The investigation of metabolic profiling of human synovial fluid to provide joint disease analysis and the association with implant material wear

Thesis submitted by

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Abstract

The goal of this thesis was to establish a biobank of human synovial fluid (HSF) samples. Furthermore, the methodology, conditions, fit-for-purpose criteria were needed to ensure validity and robustness. Once those conditions were met, the relationship between HSF and the behaviour of the joint implant material was to be evaluated.

The current literature relating to the metabolism of osteoarthritis, synovial fluid, its storage and analysis was systematically reviewed.

A biobank of HSF samples was collected. Metabolite identification of HSF was achieved using a combination of published NMR studies, the Human Metabolite Database (HMDB), 1D and 2D NMR spectra and STOCSY analysis. The stability of samples to handling, collection and long-term -80ºC storage was investigated. All metabolite concentrations affected by storage were reported. This work has validated the systems and methodology to metabolically profile HSF.

A variety of protein precipitation steps to maximise the metabolic information from the samples were evaluated. An acetonitrile liquid/liquid extraction performed well with additional recovery of unknown metabolites, albeit with increased variation and diminished lipid detection.

To understand which metabolic components are important for mechanical wear, the metabolic profile of HSF and its wear model, BCS, were analysed for components of both fluids which correlate to measured wear in a bench-top testing rig. Wear analysis demonstrated variation in the HSF mechanical properties. This correlated to the presence of glycosaminoglycan (GAG) and proteoglycan molecules with binding to citrate and glucose. Furthermore, specific amino acids: lysine, glutamine, glycine, threonine, asparagine, proline, histidine and tyrosine, correlated with measured wear.

The reported unstable metabolites must be considered for any HSF study. The acetonitrile liquid/liquid extraction method is recommended to maximise metabolite detection. The small molecule components

of HSF contributing to the wear properties of implant materials has not been reported previously, is unique and opens a new field of study in implant survival.

Statement of Originality

I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

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Dedication

To my wife, Rosie who has been a model of patience and understanding throughout a challenging time for our family. For William, Arthur and Molly who have the capacity to lift my spirits and provide inspiration at any low moment. I am just sorry that I haven't been as present in their lives as I would have liked, and it doesn't reflect my adoration for them.

To my children's grandparents for their unwavering support in childcare. To my parents for their unrelenting support without reward and particularly my father for his dedication to proof reading and never giving up on improving my English.

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Finally, to Mr Pouya Akhbari for his enthusiastic collaboration and engagement with the project. His tolerance of my time keeping and receptiveness to my returned calls has been unwavering.

Glossary of abbreviations

ACR = American College of Rheumatologists, 78

adenosine triphosphate (ATP), 97

Analysis of Variance (ANOVA), 180

anterior cruciate ligaments (ACL), 88

average wear depth (AWD), 144

body mass index (BMI), 52

bovine calf serum (BCS), 70

branch chain amino acids (BCAA), 92

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-L = Kellgren & Lawrence, 78

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MCA = Macroscopic cartilage assessment, 78

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1 Chapter I: Introduction

1.1 Thesis Hypotheses

1.1.1 Hypothesis 1

Human synovial fluid (HSF) will demonstrate to be a useful analytical medium with stability and reproducibility in the detection of small molecule metabolites.

Following the establishment of the biobank and prior to investigating any joint diseases or fluid properties, it is important to understand the characteristics of any sample handling, storage, extraction and analysis and how those essential steps influence the metabolic profile.

1.1.2 Hypothesis 2

The small molecule constituents of human synovial fluid (HSF) will have a relationship with the mechanical properties of that fluid in the implanted joint.

The design and engineering features of prosthetic joint implants that contribute to increased wear have been outlined. Furthermore, the biological impacts of excessive implant wear have been discussed. Until now, the importance of HSF as the lubricating fluid has seldom been studied to date by the clinical and research community. This fluid has many constituents thought to improve the lubricating properties thereby reducing wear, but the influence of the small molecule constituents is yet to be studied.

1.2 Project aims

1.2.1 Principal aim

To ethically establish a sizeable biobank of HSF clinical samples and to demonstrate a stable and reliable methodology for metabolite detection, thereby allowing characterisation of the metabolic profile, how it relates to joint disease and the mechanical properties of the fluid.

1.2.2 Interim aims

Chapter 1 **Introduction**

The introduction of joint anatomy & physiology.

The description of joint disease, the effects and the limited treatments available.

The reporting of the links between OA and metabolism with clinical relevance.

The description of joint fluid biomechanics related to the prosthetic joint

Chapter 2 **Metabolic profiling studies of synovial fluid and osteoarthritis**

The systematic and comprehensive assessment of published synovial fluid and joint disease metabonomic research.

Chapter 3 **Metabolic Phenotyping: the methods, limitations and standards to ensure success in studying joint disease**

Outlining the methods and approach to conducting a successful and robust clinical metabolic profiling study, whilst discussing the benefits and limitations thereof.

Chapter 4 **The NMR spectroscopy evaluation of human synovial fluid**

The evaluation of sample preparation, buffer use, contaminants, NMR sequencing in the characterisation of HSF.

The metabolite assignment of HSF by NMR spectroscopy using NOESY and CPMG sequences.

Chapter 5 **The NMR spectroscopy evaluation of bovine calf serum as a potential quality control for human synovial fluid**

To evaluate the effects of pre-freezing buffer addition and the suitability of BCS as a QC sample for HSF analysis.

Chapter 6 **The metabolic stability of human synovial fluid to sample collection, sample handling and short-term storage**

To study how the metabolic profile of HSF detected by NMR spectroscopy is affected by time elapsed at varying temperatures and handling procedures prior to evaluation.

Chapter 7 **The metabolic stability of human synovial fluid to long-term low temperature storage**

To study how the metabolic profile of HSF detected by NMR spectroscopy is affected by low-temperature storage.

Chapter 8 **The evaluation of protein precipitation extraction techniques in the small molecule analysis of human synovial fluid**

To qualitatively evaluate the use of protein precipitation methods and their effect on the metabolic description of HSF by NMR methods.

Chapter 9 **Evaluating the small molecule components of prosthetic joint fluid lubricants, both model and native, and correlating these to implant material wear**

To compare the metabolic components of HSF and its wear model surrogate BCS.

To correlate the differences in metabolic components with implant material wear.

Chapter 10 **Conclusions and Summary**

1.3 Typical synovial joint

1.3.1 Joint macrostructure and the synovium

This project will evaluate the composition and mechanics of HSF to improve our understanding of joint diseases and joint biomechanics. However, in order to do so it is crucial to understand the synovial joint, the relationship and importance of the synovial fluid, its properties and its composition. Furthermore, we must appreciate the detailed structure of the cartilage components to understand the degradation products found in the synovial fluid.

Any mobile joint within the body is susceptible to destructive disease and this thesis addresses the typical synovial joints. Synovial joints have evolved to confer a large range of motion but retain stability. Furthermore, they are energy efficient with a low coefficient of friction. Synovial joints are, by definition, covered with hyaline cartilage and contain synovial fluid (see Figure 1). The joint is encapsulated in fibrous tissue which has limited elasticity. The joint capsule is rarely filled to its maximum capacity, except in disease scenarios when synovial fluid is produced in excessive amounts. At the point of joint filling, tension is applied to the fibrous capsule and hydrostatic pressure is generated (1).

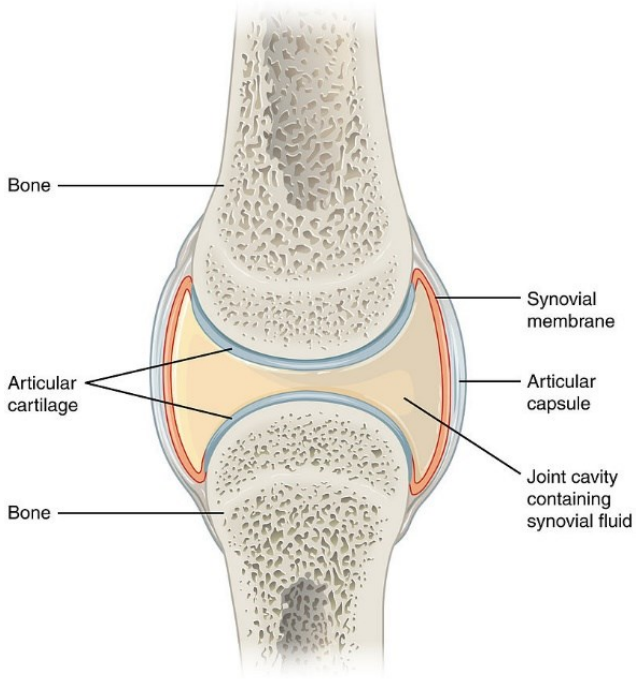


Figure 1 - Schematic representation of a Typical Synovial Joint (2)

The inner lining of the joint capsule contains synovial tissue. This tissue provides a conduit from the blood to the synovial joint fluid, produces the synovial fluid and provides an immune response in times of stress.

The bone is covered with hyaline cartilage. The interaction and behaviour of hyaline cartilage and the synovial fluid, together under load, provide the low friction environment for efficient joint movement.

In addition to the components illustrated in Fig. 1, fibrocartilaginous structures (named menisci in the knee and labra in the shoulder and hip), are encapsulated within the synovial fluid and interposed between the hyaline cartilage surfaces. When bearing weight, their function is to absorb shock and dissipate load by increasing contact area (3).

To understand how low friction is achieved requires an examination of the microstructure of both the hyaline cartilage and the synovial fluid.

1.3.2 Hyaline cartilage

The hyaline cartilage has distinct layered arrangements which are crucial in resisting shear forces and spreading the load (see Figure 2). The cartilage has cellular and extracellular constituents (see Figure 3). The cellular composition is sparse: chondrocytes are the major cell type and a small proportion of stem cells. The chondrocytes, despite being sparse, are crucial in the maintenance of the extracellular matrix (4). Extracellular matrix (ECM) is mainly composed of water, with dense type II collagen and proteoglycans (see Figure 3). The type II collagen provides tensile strength with shear resistance. The proteoglycans attract the water, providing the viscoelastic mechanical properties and resisting compression.

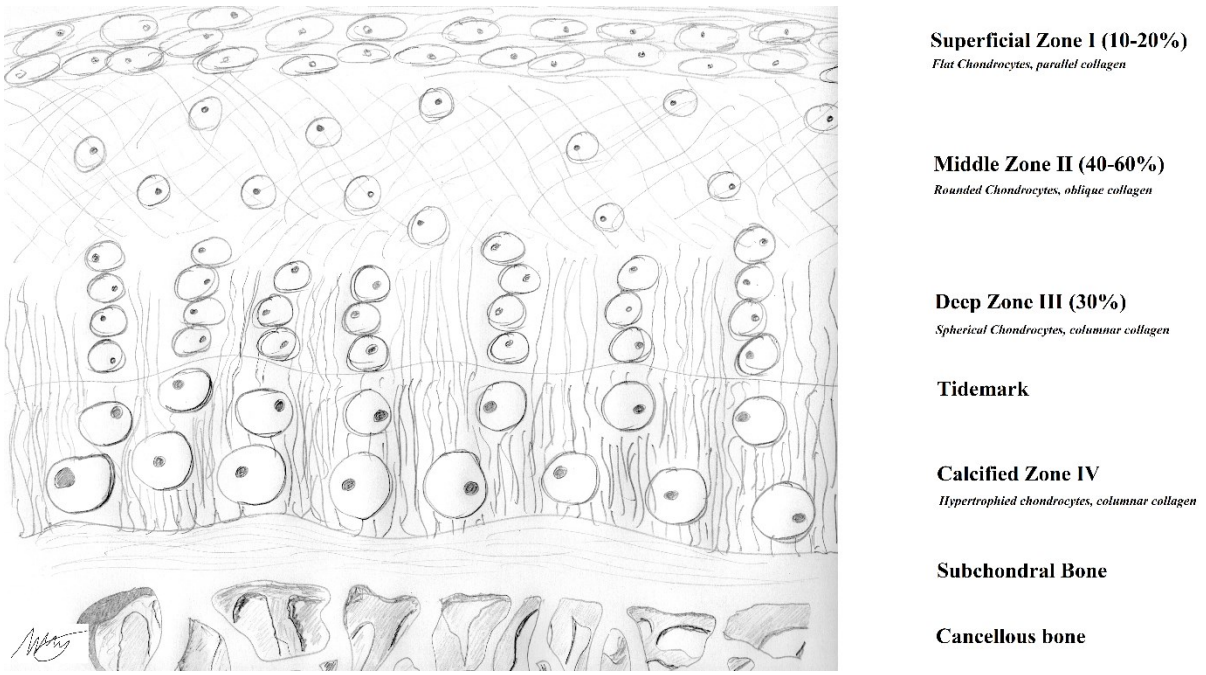


Figure 2 - A pictorial representation of the cellular and extra-cellular structure of hyaline cartilage

The relative concentrations of collagen and ECM change across the cartilage, from the superficial zone towards the bone. The superficial (or gliding) zone provides the contact surface: this is the thinnest layer and contains the lamina splendens, composed of dense collagen fibrils and flattened chondrocytes. The dense collagen fibres are arranged in flat sheets with the same orientation as the joint surface to

resist shear forces. It partially functions as a physical and immunological barrier to prevent foreign material and large molecules getting into the cartilage. This superficial layer has a high-water content despite a low proteoglycan turnover. In the early stages of joint disease, the parallel sheets of collagen fibrils degenerate and break up.

The transition or middle zone contains collagen fibrils which are arranged obliquely to the joint surface and provide a connection between the deeper layers and the superficial zone. Going from the superficial layer towards the bone, the proteoglycan concentration steadily increases. The cartilage nearest to the bone is called the radial zone: here the organisation of collagen fibres becomes tangential and hence more vertical to the joint surface. The increased concentration of proteoglycan combined with the collagen arrangement gives a viscoelastic property which resists compression forces and allows the cartilage to function like a spring and recoil when loaded.

Below the radial zone there is a transition from uncalcified to calcified cartilage. Known as the "tidemark" this layer becomes progressively acellular and migrates further towards the subchondral bone. In the calcified zone the cartilage is formed mainly of type X collagen and provides anchorage to subchondral bone. The cartilage lacks blood supply and hence above the tidemark the structure is avascular. This means that cellular and extracellular components of the cartilage are almost completely reliant upon the synovial fluid for delivery of nutrients and removal of waste products (1).

In summary, the ECM fibrils have a mechanical function which relies on their arrangement with the cartilage. Any loss of macroscopic or microscopic arrangement causes increased wear and arthritis.

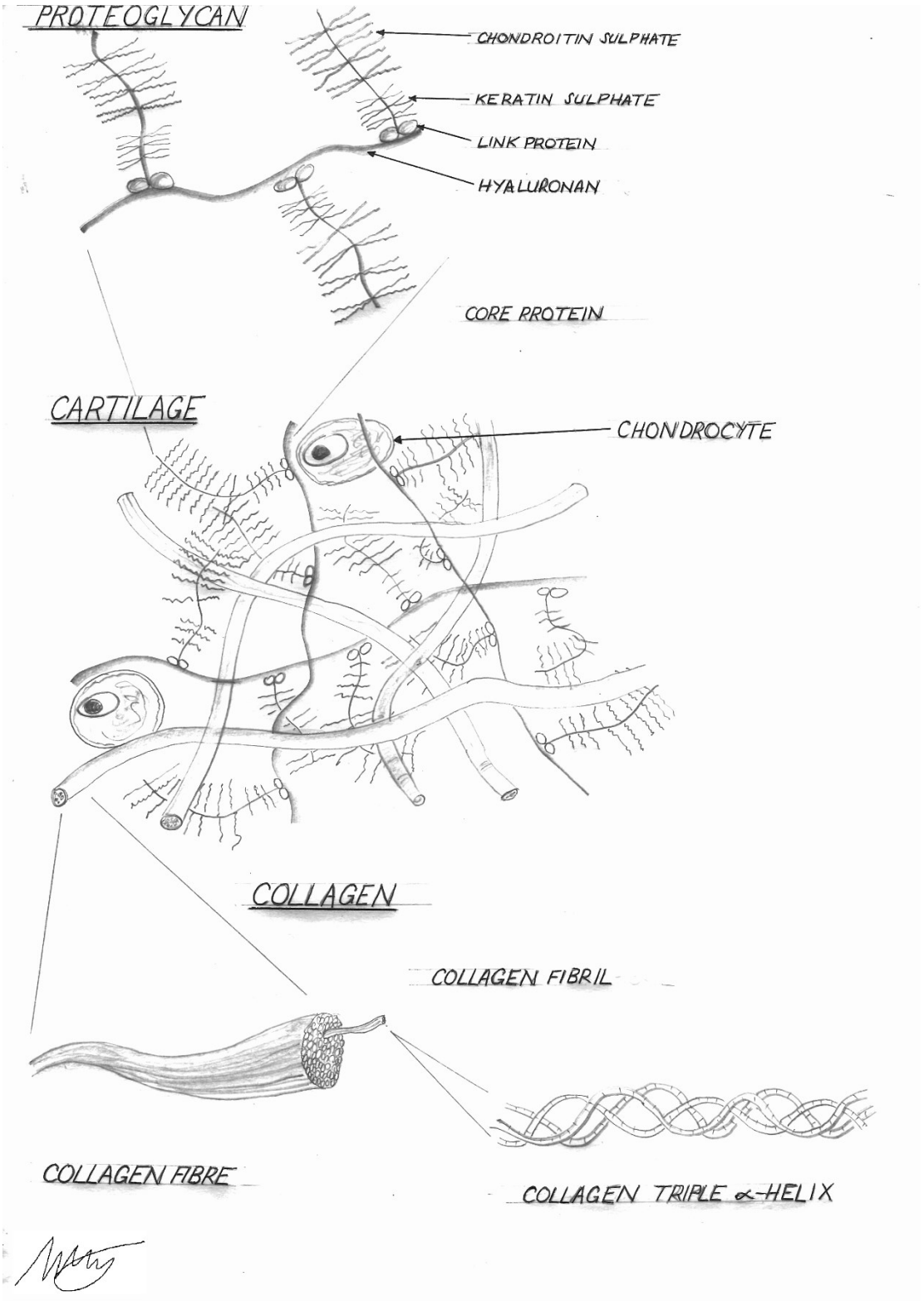


Figure 3 - Schematic representation of cartilage and its components: proteoglycan and collagen

1.3.3 Collagen

There are 28 types of collagen which have been described, although type II predominates. These serve different roles but type IX is thought to stabilise type II to limit mechanical breakdown (5).

The collagen is arranged as a mesh of fibrils layered across each other (see Figure 3). The strength of the fibrils is provided by the intertwining of the strands, like a rope. When a bundle of fibrils is loaded the force is shared by friction as the fibrils slide over each other. The collagen fibrils contain many triple α-helices. The α-helix of collagen is mostly composed of glycine, alanine, proline and hydroxyproline in a repeating pattern. Proline and its analogues allow the curvature and progression of the helix. Glycine and alanine have compact side-groups allowing tight helical turns. As well as providing strength, the mesh arrangement of the collagen matrix provides resistance to shear forces when the joint is mechanically loaded.

The strength of collagen arises from the interactions between the fibrils and the cross-linking thereof. A key process in ensuring appropriate function is the conversion to procollagen. This involves the hydroxylation of side chains amino acids lysine and proline which then allows cross-linking between fibrils. The collagen is then packaged within the cell before being excreted into the connective tissue for incorporation into the ECM.

1.3.4 Proteoglycan and glycosaminoglycans (GAG's)

Proteoglycan is integral to the properties of cartilage and synovial fluid. The proteoglycan provides a link between the collagen fibrils. This is a complex and variable molecule containing a mixture of sidechains which are anionic. The charge of the proteoglycan molecule gives a large osmotic potential to retain water. Chondroitin sulphate (CS) and keratin sulphate (KS) chains form the sidechains of the ECM structure that anchors to the hyaluronan backbone. These proteoglycans aggregate with

hyaluronic acid (HA) so that they are retained within the collagen matrix. This is achieved by aggrecan molecules (a type of proteoglycan) and the association of link proteins at binding sites. Aggrecan consists of a central protein core, KS and CS binding sites and a domain for binding HA (6, 7). Hyaluronan is a ubiquitous molecule and is a component of most animal interstitial fluids.

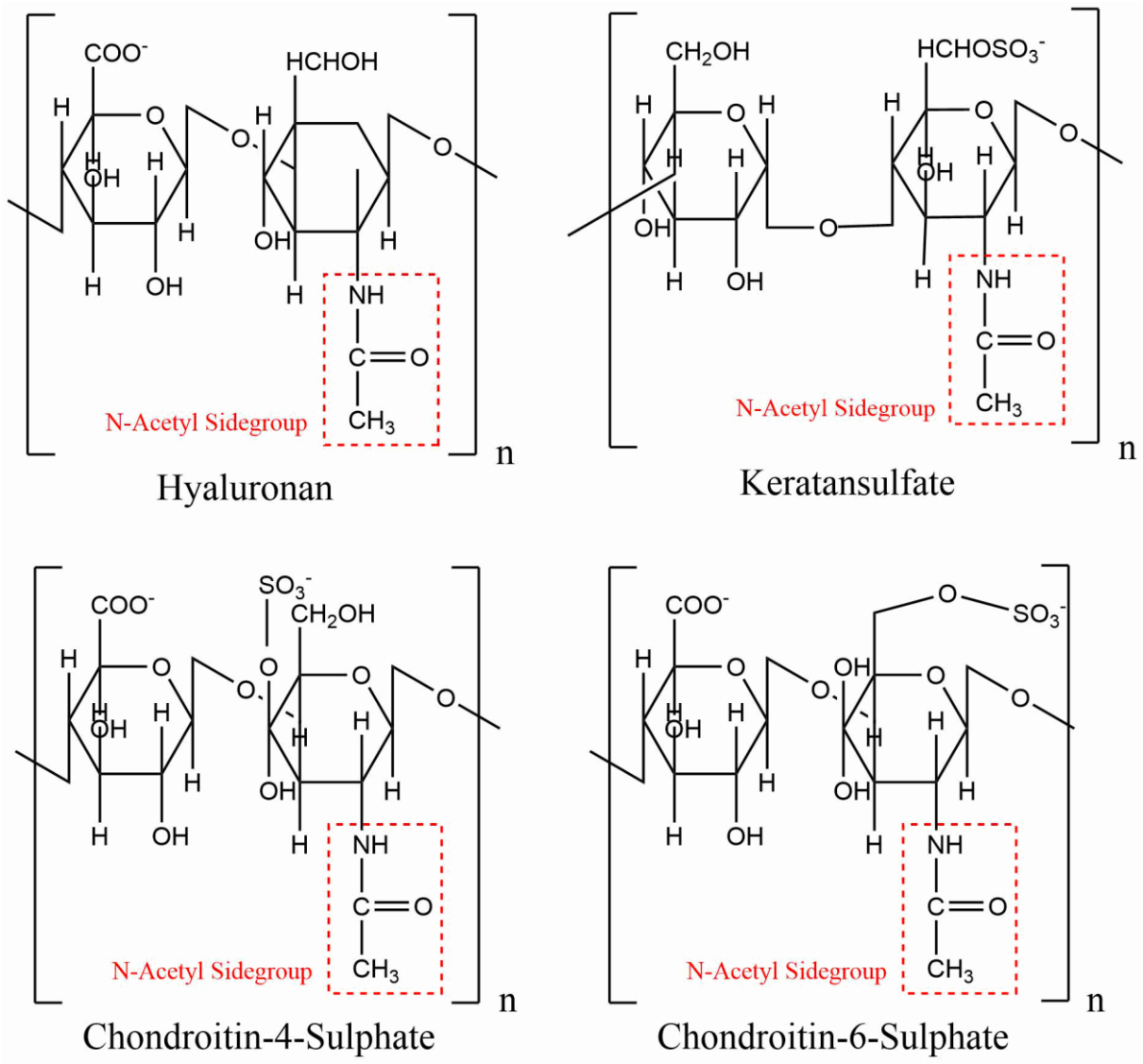


Figure 4 – Polymer structure of proteoglycan molecules

At the molecular level CS, KS and hyaluronan are commonly repeating disaccharide polymers. As repeating disaccharide monomers with an *N*-acetyl side group, they differ subtly in their monosaccharide composition (see Figure 4). Hyaluronan chains can be 10000 saccharides long and the entangling and interaction of these domains give the viscoelastic properties.

The monomer of hyaluronan is *β-D-N*-acetyl-glucosamine coupled to *β-D*-glucuronic acid. In KS *β-D*-galactose is coupled to sulphated *β-D-N*-acetyl-glucosamine. CS is also sulphated but contains sulphated *β-D-N*-acetyl-glucosamine and *β-D*-glucuronic acid (8, 9).

1.3.5 Chondrocytes

The major cell of the cartilage ECM are chondrocytes. The normal conduits (arteries, veins and lymphatics) are absent. Therefore, these rely on the diffusion of fluid through the ECM for their nutrition. The chondrocytes have a role in maintaining the proteoglycan structure and concentration. This function is inhibited by cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) (7).

1.3.6 Synovial fluid

Synovial fluid (SF) is complex and has multiple roles including immunological, mechanical, nutritional and chemotactic. Synovial fluid provides a lubrication film between two cartilage surfaces, but chemical and mechanical analysis shows it does much more. Synovial fluid is a non-Newtonian fluid and has viscoelastic properties (the viscosity varies depending on the force applied). This property is advantageous because, under load, the cartilage and synovial fluid become more viscous and therefore function as a shock absorber (10).

Since the hyaline cartilage is avascular and the movement of organic materials must rely on diffusion, any nutrients, waste products or chemotactic signals must be delivered by the synovial fluid.

1.3.7 Synovial fluid composition

Synovial fluid is produced from a dialysate of the plasma. It is formed from the capillary bed to the synovium and its composition often mirrors that of the plasma from which it was derived. Therefore, it is protein rich and has high water content. However, there are several other major components which provide HSF with its key mechanical properties. These constituents are actively secreted and added by the synovium (11).

Like the cartilage, the viscoelasticity of the synovial fluid is achieved by the proteoglycan polymer chains (particularly hyaluronan), their interactions and their ability to attract water (see 1.3.4). The ribbon structure of hyaluronan can aggregate with water to cause coiling of the ribbon to yield a sphere of 0.5μm in diameter. Under load these spheres may serve to maintain a film lubrication between surfaces. In addition to hyaluronan, HSF contains another proteoglycan: lubricin (or proteoglycan 4) which contains a large glycosylated domain and provides boundary lubrication by adhering to the joint surfaces (10).

The synovial fluid would provide a rich energy source for any infecting organism, so it must have an immunological capability. Furthermore, the normal turnover of cartilage generates polymer fragments which must be phagocytosed. Therefore, there is an abundance of polymorphonuclear leucocytes which maintain the sterility and cleanse the synovial fluid of undesirable material (12).

1.3.8 Mechanical interaction of synovial fluid and cartilage

The layered structure of the cartilage and its collagen component provides strength to resist shear forces, whilst retaining form. The outermost layer or hyaline cartilage is smooth so two joint surfaces pass easily upon one another. The proteoglycans within the ECM cause ingress of water, swelling the cartilage and providing viscoelasticity to give shock absorption and the ability to spread load. The lubrication is maintained under load due to the porosity of the cartilage, high water content, compressibility and recoil (13). When loaded, the cartilage acts like a sponge with fluid forced through

its pores towards the contact surface, ensuring constant lubrication. In addition, the binding properties of hyaluronan and lubricin fill any crevices or imperfections to give as smooth and frictionless bearing surface as possible. Lubricin is thought to bind to the collagen providing a protective boundary to any degraded area. The structure and charge of the hyaluronan molecules provide a film lubrication. This process is so effective that a near frictionless joint motion is produced (10).

1.3.9 Cartilage and synovial fluid in disease

There is ongoing chondrocyte maintenance to the proteoglycan and collagen structure within the cartilage. Synovial fluid is constantly being replenished with fresh proteoglycan by type B synovial cells located within the synovium. Polymorphonuclear leucocytes within the HSF ensure it is cleansed and free from any debris, removing the potential to cause particulate wear. However, cartilage will degrade under load if collagen breaks down, there is a loss of proteoglycan, depolymerisation of hyaluronan or the chondrocyte maintenance fails.

If the rate of degradation exceeds the rate of regeneration, then arthritis will occur. This can occur either by:

- a failure of the regeneration mechanisms in age or disease
- active degradation of the macromolecular structures due to disease
- a supramaximal application of force beyond the tolerances of the system (14).

1.4 Osteoarthritis

1.4.1 History

Osteoarthritis (OA) was described by the ancient Egyptians and has been found in mummified remains as well as in major joints of dinosaurs (15). Osteoarthritis remains one of the most common diseases in mammals.

1.4.2 Epidemiology and economic

OA is now the most disabling and most common musculoskeletal (MSK) condition in Western populations. This is due to improved treatments and prevention for other conditions like Rheumatoid Arthritis (RA), alongside an aging population and improved geriatric care. In 2002 it was estimated to affect between 1.3 and 1.75 million people in England and Wales, which given recent reports was probably an underestimate. At that time the economic burden of MSK disease accounted for 1-2.5% of gross domestic product (16). Knee osteoarthritis affects approximately 18% of the United Kingdom (UK) population over the age of 45 with a significant economic burden upon healthcare provision (17).

1.4.3 Associations of osteoarthritis

OA can be associated with environmental, genetic, nutritional or mechanical factors.

As the key component of hyaline cartilage, any disease which affects the structure and function of type II collagen will cause an early onset and increased incidence of osteoarthritis. These diseases include: Down's syndrome, achondrogenesis, spondyloepiphyseal dysplasia and Stickler disease (18).

Lifestyle factors which are associated with OA are joint injury, nutrition, exercise and smoking. It is found more commonly in women than men. The Framingham study investigated the risk of OA in older people. This confirmed an association of high body mass index (BMI), but this effect was seen preferentially in females. In part, this may explain the increased incidence in women. Non-smokers were at higher risk, although no mechanism for this reduced risk is understood (19). Those in the upper quartiles of physical activity were at greater risk (20).

Trauma or surgery to a joint increase the risk of developing OA. Weight provides a strong but not absolute association with OA. Traditionally it was assumed that this was due to excess load upon the synovial joint mechanisms. Whilst this may be the case, newer associations with metabolic disturbance

and inflammation are emerging: namely cardiovascular disease, diabetes and hyperlipidaemia (see 1.5.1).

1.4.4 Clinical features of osteoarthritis

The typical presentation is a progressive loss of function alongside an increase in pain. The symptoms are variable. In some patients, pain is the predominant symptom. Others notice a deformity or a loss of movement. Stiffness is a regular complaint. Symptoms can be particularly prevalent in the morning, after a period of immobility or at the end of a very active day. Night pain, and particularly disturbed sleep, is indicative of severe disease. Clinicians have reported cohorts of patients who suffer repeated episodes of symptoms originating from joint inflammation (or synovitis) such as: excessive swelling, pain to touch, restricted movement and even inflammation of the surrounding soft tissue. In this cohort, symptoms tend to be more periodic. Alternatively, patients will complain of activity and load-related pain (21).

1.4.5 Imaging techniques

The diagnosis of OA is made radiographically. It can be diagnosed on plain radiographs with features showing the cartilage loss (a reduction in joint space) and bone remodelling (subchondral sclerosis, osteophyte production and bone cysts). These radiographic features are only seen once the destruction of the joint is advanced. Thus, the combination of symptoms and radiographic features only emerge once disease pathology is advanced: the diagnosis is late.

Other imaging methods can be useful, but rarely yield more information than the plain radiograph. Ultrasound can determine the presence of thickened synovium or cysts around the joint. However, its role is mainly in describing the surrounding soft tissue features of joint pathologies which may be confused with OA. Computed Tomography (CT) can be useful where an isolated part of the joint may be degenerative or for planning operations. However, plain radiographs are usually satisfactory to make the diagnosis. Magnetic resonance imaging (MRI) can provide an assessment of the intra-articular

cartilaginous structures. In early OA this can provide a more detailed assessment of cartilage thickness, adhesion to bone and underlying bony changes (22).

1.4.6 Treatment

The treatment options for OA are initially confined to the control of symptoms. Sadly, unlike the other joint diseases, no supported and confirmed pharmacological disease-modifying treatment exists. Some nutritional supplements have been suggested to influence OA onset or progression including glucosamine and CS, either in isolation or in combination. The method of action is unclear. Reports on the effectiveness of these supplements are in isolation and often conflict, probably due to the high rate of positive effect from the placebo group. The recent randomised controlled Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) was inconclusive. Other trials have reported a greater response to therapies with the supplementation of glucosamine and/or chondroitin sulphate albeit with a bias in the published conclusions (23, 24). Supplementation of vitamin A, C and E are reported to be effective (25).

Non-steroidal anti-inflammatory agents are the typical treatment for pain and inflammation in OA. They are effective although there is concern about the potential for gastric mucosal degradation and renal toxicity (26).

The physical imbalance of the joint resulting from disease can cause malalignment, subluxation and functional instability. Weight loss (if applicable) can restrict symptoms in weight bearing joints. Physical therapy can be effective by the providing additional muscular support and stability (27). Patients can be advised to avoid exercise that involves repeatedly loading the joint e.g., long distance running.

Once conservative measures are unsuccessful or do not adequately control symptoms then intra-articular injections may be considered. A corticosteroid may reduce inflammatory symptoms, although this effect may be short-lived (28). The use of intra-articular HA injection can be useful in early-to-moderate OA

but the efficacy remains unclear; some studies have shown a small but statistically significant benefit, whilst others have shown no effect (29, 30). Individual reports suggest that some patients benefit, so perhaps there is a more distinct cohort of patients who respond positively to these agents.

If symptoms are derived in part from a malalignment of the joint, this can be addressed. The treatment may also prolong the effective lifetime of an arthritic joint by altering the mechanical axis and thus removing the load from an arthritic area and distributing it elsewhere. Joint realignment is not always surgical; it may be by selective and corrective bracing to a joint or the use of corrective insoles. These strategies are used to good effect with the knee (26). Surgical correction can be used to permanently realign the joint and thus shift the load bearing away from the osteoarthritic portion of the joint. This involves the use of selective osteotomy.

Secondary Mechanical symptoms such as locking of the joint from a loose body or pain originating from a degenerate meniscus can be treated with arthroscopy. In addition, there are positive effects upon the inflammatory stimuli within the joint. In most cases arthroscopy will reduce those inflammatory symptoms. The mechanism of this improvement is uncertain but is believed to be secondary to the dilution or removal of stimulating cytokines within the joint fluid (31).

Surgical options to treatment include arthrodesis or fusion of the affected joint. The advantage of this strategy is the effective removal of joint and all the associated inflammatory tissues with good symptom relief. The disadvantage is the disability which comes from removing the articulation of a joint. Therefore, the procedure is typically limited (unless absolutely necessary) to joints with little or redundant movement that can be accommodated elsewhere, for example the small joints of the foot.

Arthroplasty or prosthetic joint replacement is for the patients who experience a very significant impact on their quality of life due to the OA. Joint arthroplasty is an invasive procedure with significant risk during and after the operation. The recovery is lengthy and challenging. The prosthetic joint will not provide full function or complete pain relief. Therefore, the timing of such an intervention is crucial to

optimise the ratio of risk to reward. The outcomes of joint arthroplasty can vary greatly. Most patients will see a steady improvement in symptoms. A small proportion will endure a lingering inflammatory stimulus within the joint causing pain and stiffness and ultimately a less than desirable outcome.

All of the treatments have a variety in the symptoms and responses originating from the inflammatory mechanisms within the joint. To date these responses are unpredictable and poorly understood. The ability to understand, control and predict the inflammatory response to disease progression and/or treatment would revolutionise the available treatments.

1.4.7 The pathophysiology of osteoarthritis

OA can be thought of as the product of cartilage breakdown and the associated physical and radiographic changes. OA affects all parts of the joint including the synovium, hyaline cartilage, fibrocartilage, subchondral bone and ligaments. It affects underlying bone with remodelling, accommodation of the joint capsule and ligaments, and weakness of the muscles around the joint. Because the response to disease within the joint is integrated and affects all tissues, the joint should be considered as an organ. OA is not a typical inflammatory arthropathy because it lacks neutrophils during the acute phase (32).

The progression of the OA disease process is divided into three stages. Stage I involves a reduction in number and clustering of the chondrocytes in the ECM (their function it is to maintain the ECM). Chondrocytes are thought to respond adversely to prolonged compression, shifting towards catabolic processes in cartilage thereby causing proteoglycans to become cleaved, collagen to be damaged and a lack of protein synthesis (33). The reverse is believed to occur in cyclic loading (34). The resultant shortening of proteoglycan molecules both within the HSF and the cartilage causes a reduction in viscoelasticity and an increase in friction at the bearing surface, thereby increasing degradation of the joint. As the ECM breaks down, the structural cohesion of the cartilage is lost, fibrillation occurs by erosion and mechanical shear forces, and this constitutes stage II.

Stage III encompasses the inflammatory component by cytokine release. The synovium becomes hyperplastic (increasing the number of cells) as well as hypertrophied (increasing in cell size). OA is an inflammatory disease, and the degree of the inflammation and cytokine release directly relates to the balance of cartilage maintenance via its combination of anabolic and catabolic process. Loss of cartilage structure, either due to overwhelming forces or a lack of maintenance causes replacement with a pathological and biomechanically substandard construct. The chondrocytes are adversely affected by the products of cartilage degradation, inflammatory signals or excess mechanical stress. This further causes a shift towards the catabolic state. A host of catabolic proteins and cytokines are produced. These add to the inflammation and degrade the structure of the ECM and proteoglycan molecules. The catabolic proteins include matrix metalloproteinases (MMP's) which cause cleavage of the ECM at the level of the link proteins (Figure 3). In addition, the inflammatory cytokine producing enzymes, nitric oxide synthase (NOS)-2 and cyclooxygenase (COX)-2 are produced. Injury to the joint releases reactive oxygen species (ROS) and this indicates that free radical generation plays a part in OA pathogenesis (35).

There is contention about the validity of these three stages of disease in all patients. It is known that the inflammation associates with the destructive processes within the joint (36, 37). However, it remains unclear to what extent the inflammatory mechanisms drive the degradation of the cartilage or vice versa. A study of 422 patients with patellofemoral OA demonstrated a positive correlation of OA progression to the observation of coinciding inflammatory synovitis: implying a direct role of inflammation (38). An element of joint inflammation has been observed in early OA disease, somewhat disputing the three-stage model (39). In addition, cohorts of patients are seen with great variation in their inflammatory symptoms of OA, irrespective of the level of joint destruction. Damage to other parts of the joint (the meniscus) in the absence of OA can initiate an inflammatory response (34). Hence, whilst the three-part model may be appropriate, it should not be thought of as purely sequential and, in reality, destruction of the joint architecture and inflammation occur simultaneously.

Hyaluronan, mentioned earlier as a key constituent of synovial fluid and responsible for most physical properties, has an influence on inflammation. The presence of fragmented, high molecular weight hyaluronan inhibits phagocytosis and prostaglandin E2 (PGE2) production, whilst low molecular weight stimulates angiogenesis. Therefore, it seems that low molecular weight hyaluronan can have a deleterious inflammatory effect (14). The extent of any effect within the joint is not known.

As we become older the composition of the proteoglycan molecules changes, CS chains become shorter and fewer whilst the KS chains get longer and more numerous. Furthermore, the number of chondrocytes decreases with age (7). These factors combined with altered bone metabolism and reduced blood supply are believed to contribute to an increased risk of OA in the elderly.

1.4.8 Osteoarthritis biomarkers

A biomarker was defined in 1998 by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (40). Radiographical and clinical diagnosis has significant limitations in OA because the symptoms and/or radiographic features are often apparent only when the disease is advanced. Furthermore, radiographic markers do not give information about the degree of inflammation or the response to any treatment. Therefore, clinicians would like to use biomarkers to represent the specifics and progression of OA pathology more accurately, in addition to providing diagnosis before the disease becomes irreversible.

No biomarkers for OA are used in clinical practice at present. However, metabolic and protein biomarker candidates have been proposed experimentally. To date, the principal targets were markers related to the depolymerisation of the structural components of cartilage and/or synovial fluid. Other targets include the inflammation, protease action, ECM destruction or signalling thereof, Cartilage oligomeric matrix protein (COMP) and collagenase-cleaved type II collagen (C2C). These have been correlated radiographically by quantitative MRI imaging of joint destruction. Both had strong

correlations with radiographic features (41). Urinary C-terminal telopeptide of type II collagen can distinguish diagnostically between healthy controls and OA suffers (42).

1.5 Metabolic syndrome

It has recently emerged that the metabolic syndrome (MeS) may have a large impact upon OA pathogenesis, the severity of symptoms and possibly even treatment. MeS is defined as the clustering of cardiovascular disease risk factors and involves increased insulin resistance, a proinflammatory state, atheropathic dyslipidaemia and hypertension (43). In short, it is the clustering of metabolic diseases which are proinflammatory and carry an increased risk of atherosclerosis or lipid deposits within the vessels. These associations were discovered epidemiologically but a biochemical and molecular basis is being clarified. Various proinflammatory diseases have been identified as having associations with arthritis, including cytomegalovirus infection, Helicobacter pylori, dental infections, chronic bronchitis, systemic lupus erythematosus, diabetes mellitus, IgA nephropathy and RA (44, 45). For example, the risk of cardiovascular disease in those suffering with RA is 50% greater than the cohort without RA. Some authors have suggested that the elevated level of systemic inflammation and lipid inflammatory mediators is coupled directly to the phospholipid metabolism, thereby linking diseases and the risk of developing artheroma (46). Lactate and altered phospholipid metabolism are correlated with systemic markers of inflammation, such as C-reactive protein (CRP) (47). A variety of lipoprotein changes are known to result from the acute phase response to inflammation but Very Low-Density Lipoprotein (VLDL) and triglyceride levels are affected the most. In addition the modification of sphingolipids and hence Low Density Lipoprotein (LDL) towards more cardiovascularly damaging forms is also seen (44). This altered metabolism suggests a link between cardiovascular disease and inflammatory diseases since the same altered lipoprotein metabolism and free radical oxidation of the LDL is present in both.

1.5.1 Metabolic syndrome and osteoarthritis

An elevated risk of OA has been associated epidemiologically with the development of cardiovascular disease. Signs of radiographic OA correlate to cardiovascular risk and decreased survival in women (48). Comparisons of OA and non-OA controls have shown a larger risk of cardiovascular disease in

the OA group, although there may have been biased selection in the patient groups (49, 50). The link between OA and MeS was questioned in the past, but now a two-fold increase in the risk of developing MeS has been demonstrated in patients suffering from OA (51). This evidence has led to OA being included in the definition of MeS.

Some authors have argued for a mitochondrial role in the development of OA (52, 53). Compounding the argument is the observation that mitochondrial dysfunction also occurs with age, diabetes and obesity, all factors associated with OA and MeS (53). This strengthens the association of a metabolic component in the development of OA. Blanco et al. observed a change in mitochondrial respiratory function with a reduction in the oxidative components. They hypothesised a compensatory increase in total mitochondrial number. This finding correlates with the early synovial fluid studies suggesting an anaerobic and hypoxic environment in the pathological joint (54). Therefore, it is reasonable to conclude that the chondrocyte energy state and aerobic metabolism may provide a key role in stage I OA or the failure of the ECM structure. Furthermore, the mitochondria respond to key OA cytokines TNF-α and IL-1, with the production apoptotic nitric oxide (NO) and thus chondrocyte death. Secondary to this the production of free radicals and ROS is known to be destructive (25, 55, 56). Hence, the handling of these species depends on the oxidative processes within the mitochondria and thus could cause OA (52).

Joint destruction mechanisms may be associated with free radical production and subsequent glycoprotein macromolecule depolymerisation (55, 57-60). Therefore, antioxidants may provide some protection or even limit propagation of destructive joint disease. A study of 640 patients in the Framingham study assessed the progression of knee OA between 1983-1985 and 1992-1993. No significant associations with any dietary factor were identified. However, pain incidence and OA progression were reduced 3-fold in the high vitamin C intake group. Other anti-oxidants like beta-carotene and vitamin E had less consistent effects (25).

1.5.2 Diabetes

There is an emerging consensus of an association between non-insulin dependent diabetes mellitus (NIDDM) and OA. Whilst this may be a product of the MeS, with diabetes conferring chronic inflammation, this pathway has never been shown but is postulated to be an increased insulin resistance secondary to circulating cytokines. Therefore, it is worth considering a link between OA and diabetes. Demonstrating any link epidemiologically is statistically challenging owing to the commonality of causal factors. Despite this, the correlation of diabetes and OA is reported from a variety of sources: radiological, metabolic, epidemiological and the association of bilateral hip OA (61). Fasting glucose confers a higher risk of OA (62). Despite this, patients suffering with type II or NIDDM are more likely to suffer a bilateral OA polyarthropathy than non-diabetic patients. Studies have also suggested that the inflammatory markers and pain scores are more likely to be elevated in those suffering with OA and NIDDM compared to those suffering OA alone (62, 63). Therefore, it is proposed that NIDDM is proinflammatory in OA, driving worse symptoms and even disease progression. Hyperglycaemia increases free radial oxygen species production by chondrocytes and the potential oxidation of macromolecules within the HSF and potentially the cartilage, thereby predisposing to OA (61).

1.5.3 Altered lipid metabolism and osteoarthritis

Inflammation in the joint affects the intra-articular fatty acid metabolism and acts on the free radical pathways. The dyslipidaemia in RA is well characterised. A reduction in inflammatory activation of RA (measured by erythrocyte sedimentation rate (ESR)) correlates to increased high density lipoprotein (HDL) levels. Interestingly the concentration of systemic LDL is often low and triglycerides also correlate positively with ESR (45, 64). OA and dyslipidaemia are less extensively described but there is emerging evidence that it is a major contributing factor. A study of human OA serum found an increase in total cholesterol compared to control subjects. Elevated concentrations of HDL, LDL and VLDL were found although these were not statistically significant (64). A study of the MeS components

revealed that OA symptoms correlated with hypertension. Weight loss in people over 60 years of age decreases expression of TNF receptors and this correlates with knee symptoms of OA (65).

The value of statins in OA treatment remains unclear. Statins have an inflammatory effect and reduce the amount of circulating CRP (a known biomarker of non-specific inflammation). Animal models show statins reduce catabolic OA cytokines and preserve joints. Studies trialling treatments in humans are awaited (66). Statins can reduce IL-1, IL-6, IL-8 and upregulate NOS; all involved in the pathogenesis of OA (see 1.4.7), although it is not clear if this is their mechanism of action.

1.5.4 Obesity

Since Fletcher's report in 1938, obesity has been associated with OA, but this was always thought to be mechanical (67). However, recent hypotheses propose that this association is more than simply an increasing load on the failing joint. The first indications that biological factors may be having an impact emerged when a study of over 1000 participants in the later decades of life revealed an increased incidence of OA in the hand, i.e. a non-load bearing joint (62). Furthermore, analysis did not support the concept that OA drove an increased obesity risk, thus confounding the association (68). This finding suggests that greater body mass confers either metabolic or hormonal changes which have deleterious effects to the establishment and progression of OA.

1.5.5 Adipokines

Adipokines are proteins derived from adipose tissue and are thought to have a signalling function. Their action is widespread and systemic, stretching beyond just controlling metabolism and food intake; they also affect insulin resistance and systemic inflammation (69). Their elevated levels in OA, and more recently illustration of proinflammatory effects within the joint, have led to strong arguments for a role in disease progression.

Leptin is a polypeptide hormone known to associate with obesity and is produced from the expression of Ob gene. It has effects upon food satiety and the regulation of other metabolic controls. Leptin concentrations seem to elevate with increasing body fat, and a study of Ob knockout mice demonstrated obesity traits. Hence, the conclusion is that leptin works as a homeostatic control for weight gain (70). However, in addition to food intake, there were suggestions of effects upon other MeS diseases, including OA. Leptin stimulates a variety of joint cell types to produce catabolic cytokines which can be destructive in the presence of disease. It is thought to act upon MMP formation as well as IL-1 and subsequent NOS signalling. Leptin concentrations are elevated in OA and correlate with the severity of disease (71).

1.5.6 Osteoarthritis summary

The varied but debilitating symptoms of osteoarthritis have been outlined and this variation is largely due to the impact of inflammation on the disease progression. The identification of disease occurs towards the irreversible end-stage of pathology with few treatment options to slow disease. The mechanisms and importance of inflammation and the ROS are poorly understood. Metabolic disturbances such as diabetes, dyslipidaemia and obesity have been demonstrated to influence OA progression without a significant understanding of the mechanisms responsible.

1.6 Mechanical lubrication

1.6.1 Joint arthroplasty

More than 200000 arthroplasty operations were performed in the Uk in 2019 before the outbreak of the Covid-19 pandemic, with some 20000 being revision procedures (72). These operations are the end stage treatment for joint arthropathy (disease involving the joints). The prosthetic joints are engineering systems that introduce artificial materials within the biological environment that is the human joint. These artificial materials must operate within this environment without being toxic or

creating an inflammatory reaction. They must integrate to the underlying and supporting bony architecture. They should not react chemically or electrically with their operating environment.

The materials used in joint arthroplasty vary. Bearing surfaces are made from metal, ceramic, polyethylene (PE) or combination thereof (see Figure 5).

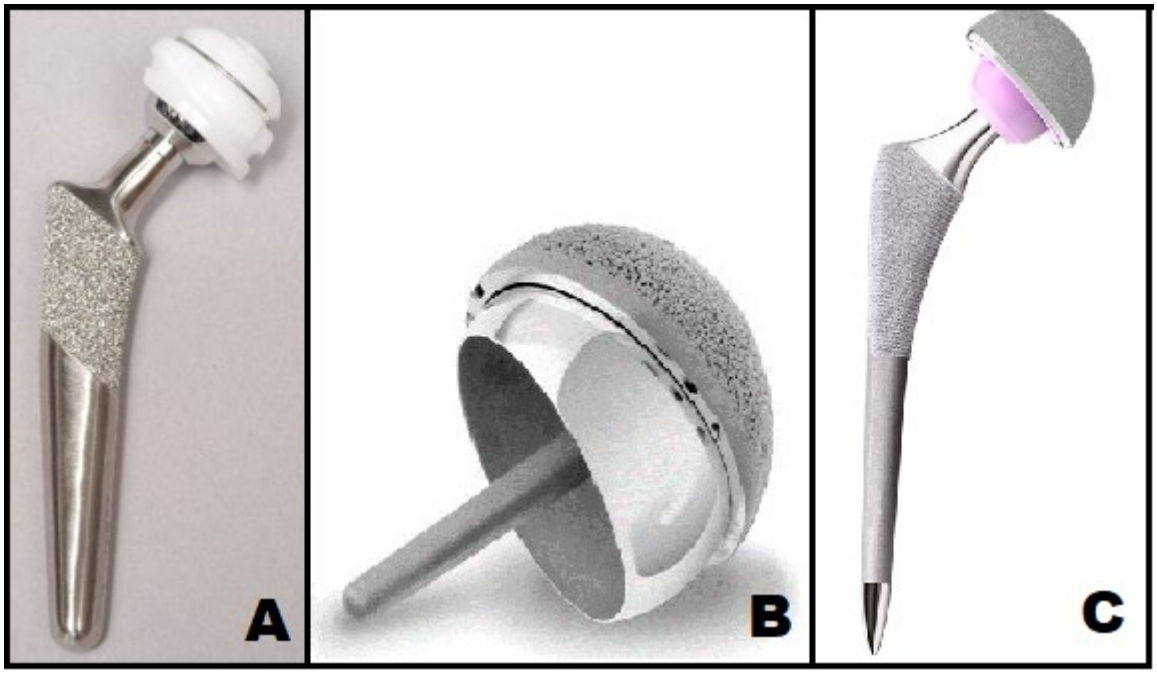


Figure 5 - Hip arthroplasty implants illustrating the variety of bearing surfaces, panel A – Cobalt Chrome (CoCrMo) on ultra-high molecular weight polyethylene (UHMW PE), panel B – Cobalt Chrome (CoCrMo) on Cobalt Chrome (CoCrMo) (73), panel C – Ceramic on ceramic (74)

The longevity of implant is directly affected by the wear of the implant surface. Furthermore, the production of wear debris causes phagocytosis and subsequent inflammatory reactions with potentially destructive effects.

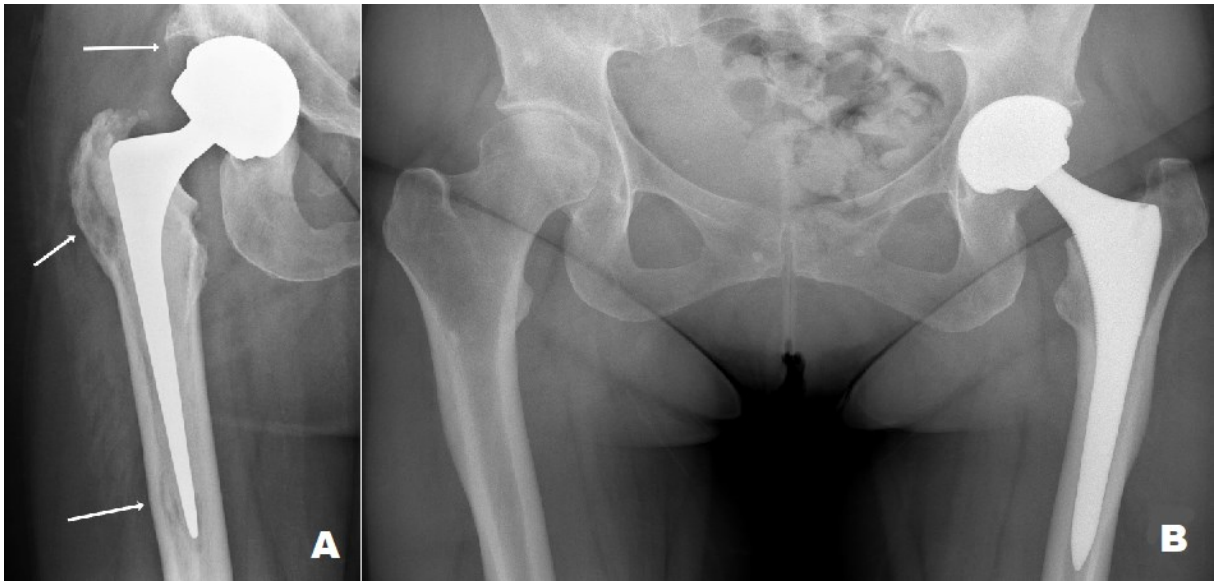


Figure 6 - Panel A - The affected joint space of a metal-on-metal hip joint in the presence of excessive wear and osteolysis (arrowed). Panel B – A normal hip joint space with a hip arthroplasty in situ

In the presence of phagocytosis, immune activation causes secondary IL-1 and IL-6 release which act upon osteoclasts cells to break down inorganic bone. Thus, in the presence of wear debris this bone destruction can cause implant loosening, instability and he need for revision surgery (see Figure 6).

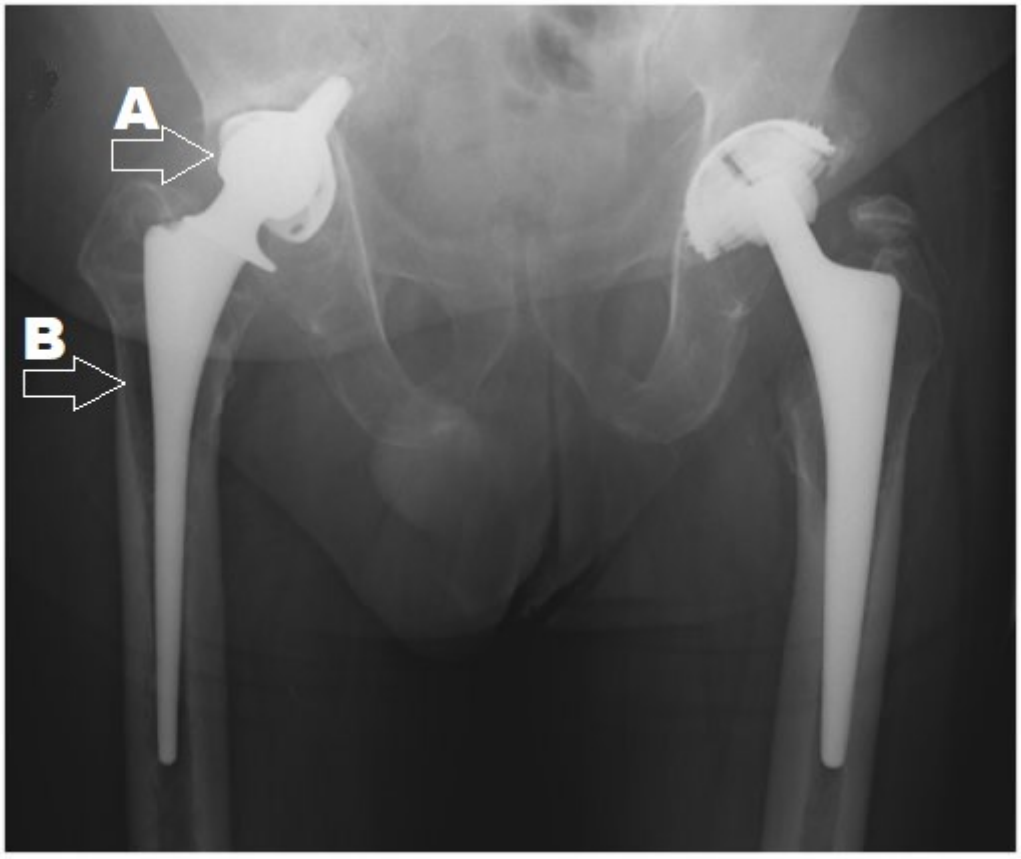


Figure 7 - A plain radiograph demonstrating eccentric positioning of the femoral head indicative of constitutional wear (arrowed A) and the subsequent bone osteolysis (arrowed B)

1.6.2 Arthroplasty implant wear

The bearing surfaces can be damaged either by mechanical wear (due to acetabular edge-loading or high friction) or corrosive wear (due to chemical reaction of the implant surface with its environment) (75).

Mechanical wear can occur at any point where the bearing surfaces contact each other, and a fluid film is absent between the surfaces. This is either by movement across each other (termed abrasive wear), or transient bonding to each other prior to movement causing a small portion of the bearing surface to be separated, termed adhesive wear (see Figure 8). Therefore, the utopian environment is one where the two bearing surfaces never touch. If this occurs (and no corrosive wear takes place) then the

bearing will never experience wear. In reality this utopia does not occur, and other factors must be considered in minimising bearing wear.

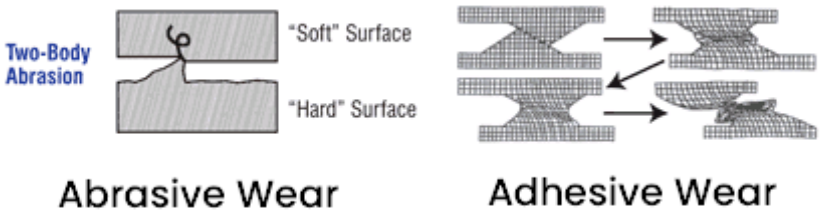


Figure 8 - Illustration of abrasive and adhesive wear (76)

Load should be evenly distributed across the bearing surface and not focused on any one area, since this will cause an increase in contact pressure displacing any fluid film. This phenomenon is known as point loading, or edge loading when it occurs at the periphery of the bearing surface. Furthermore, the lubricating surfaces should be perfectly equal in their contour and equator, separated by a thin film of fluid. This is termed perfect equatorial clearance and promotes maintenance of the fluid film (see Figure 9). The surface roughness of the bearing surfaces will result in high spots serving as focal areas of implant wear, and therefore polishing and machining is employed to remove these as much as possible prior to implantation.

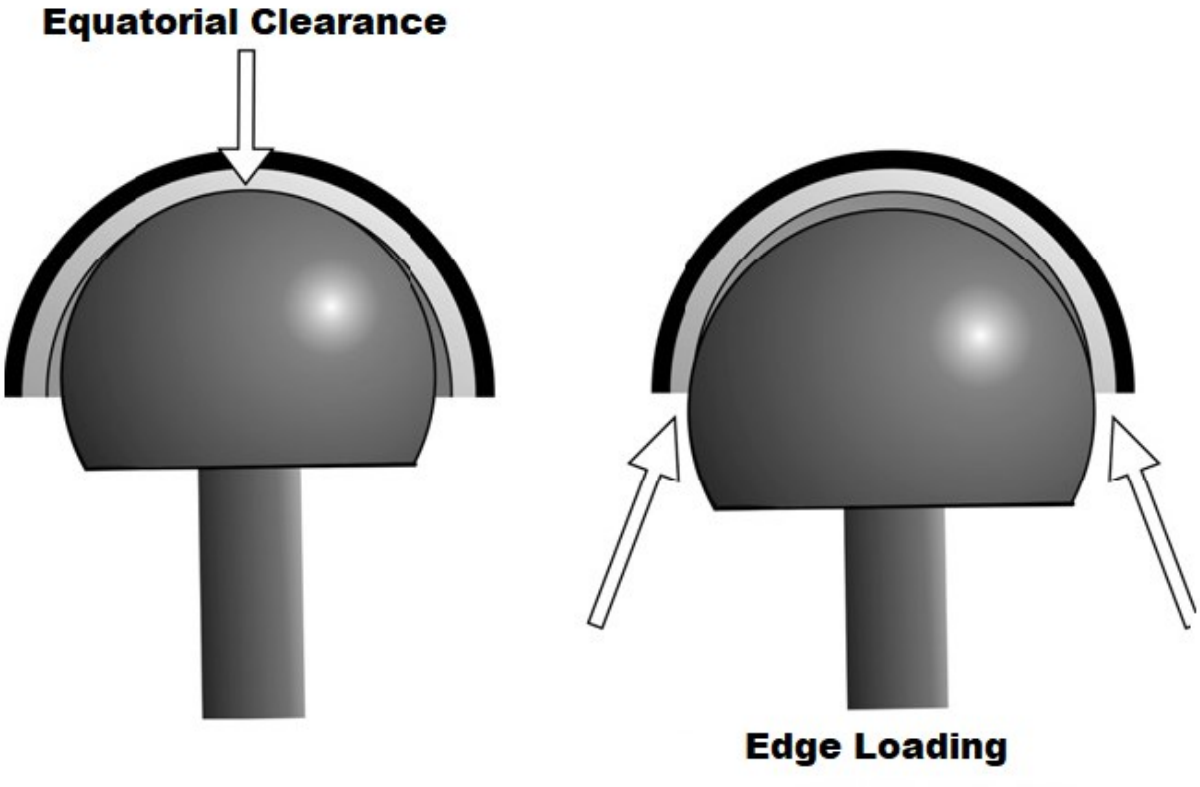


Figure 9 - Illustration of Edge Loading and equatorial clearance

1.6.3 Modes of lubrication

The prosthetic joint can be lubricated by a variety of mechanical processes. As stated (1.6.2), fluid-film lubrication maintains a separation between the surfaces with an intact viscous layer. This fails where the later becomes too thin for the surface incongruity or imperfections, or the fluid becomes unstable.

In the absence of fluid-film lubrication, boundary lubrication may occur. This occurs when a single molecular layer exists between the bearing surfaces and is typically due to chemical attraction or wettability of the surfaces. Once the boundary is lost there will be wear.

1.6.4 Lubricating fluid factors

In the metal-on-metal and the metal-on-PE prosthetic joint it is assumed that macromolecules bind to the implant surface providing boundary lubrication (10). Therefore, the composition and chemical makeup of the HSF may have a significant impact upon the wear effects of the prosthetic joint.

HSF is a viscoelastic material. As such, it has properties of strain rate dependency which define that the rate of force application will affect the internal resistance of the fluid to that force. Hence a rapidly applied, large force will produce a greater resistance to deformation. This property is important in maintaining a separation between the joint surfaces under load during the gait cycle and related to the macromolecule properties of the fluid.

Many studies have examined how the fluid components affect wear. These all involve the use of model fluids like bovine calf serum (BCS) and the supplementation of various components, concentrating solely upon the macromolecules (10, 77-81). There are no studies which examine the wear of HSF related to its composition. Furthermore, there are no studies directly comparing the small molecule content of the fluid and the subsequent effect upon wear (82).

HA is an abundant GAG which forms a component of the proteoglycan complex within synovial fluid. As such it has a role of attracting water and providing viscoelastic properties to the fluid. It has a clinical role in viscosupplementation in clinical practice, although the efficacy of this intervention is a matter of debate. Supplementation of HA has been used in simulated joints to reduce friction. It is unclear if this molecule is key to wear. Furthermore, the composition and structure of HA is not uniform, changeable and may be significant. More detailed information of structure may provide greater insight into which aspects of composition may play a role.

1.7 Metabolic profiling

Metabolic phenotyping (id metabonomics, metabolomics) has developed rapidly over the past two decades to establish itself as a resilient and well used technique for the analysis of biofluids or tissues. The concept of using excreted matter, body fluids or tissues to make inferences about systems biology or disease is not new. Many ancient civilisations including the Romans and Greeks, would assess the urine for colour and consistency, albeit making an incorrect interpretation of the information (1).

Metabonomics was conceived in the 1980's by marrying multiple simultaneous small molecule metabolite measurement using various analytical methods alongside chemometrics (multivariate statistics in chemical analysis), based mostly on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Since then, technical advances have allowed higher throughput, greater spectral resolution (the ability to distinguish between complex compounds in a biological sample), more powerful computational analysis and now machine-learning to analyse data.

The application of metabolic profiling has expanded exponentially over the last twenty years with studies in: oncology, epidemiology, gastrointestinal disease, MeS, diabetes and cardiovascular disease (83-87). Clinical practice has been influenced by the presence of metabolic biomarkers: the IMPACT study (elevated blood pressure), risk of impending mortality and chronic kidney disease treatment (88-90). There are a few reports of synovial fluid metabolism. However, there are no established or validated methodologies for the metabolic profiling of synovial fluid.

The details of metabolic profiling, its processes, methodologies and keys to success are discussed later in greater detail (see 3.2).

1.8 Summary

The human synovial joint is a complex system that provides a biologically maintained low friction environment using the interaction of cartilage and synovial fluid; this interaction is susceptible to failure.

OA is the most common joint disease and requires a huge amount of health resource. The importance of metabolism in the inflammatory mechanisms of OA has emerged in recent times. Therefore, establishing a technique and methodology which allows metabolic study of HSF may provide advances in OA understanding.

The mechanics of prosthetic joint lubrication, wear and potential failure have been outlined. The synovial fluid must play a large role in the lubrication of the prosthetic joint. The effect of synovial fluid composition upon wear of the construct has only been studied by supplementation.

2 Chapter 2: Metabolic profiling studies of synovial fluid and osteoarthritis

2.1 Why study synovial fluid?

Synovial fluid is a useful medium for the study of joint pathology. It contains representation of the systemic metabolism because it is a dialysate of blood plasma. However, despite this, the metabolic composition of the SF is nearly isolated because the joint is encapsulated as an organ. The joint is not an excretion pathway from other body systems nor is it a conduit for delivery to other organs. Therefore, the synovial fluid metabolic profile should be a good representation of the disease microenvironment including the cartilage, subchondral bone and synovial tissue.

The sparse chondrocytes which fail in OA disease rely solely on the synovial fluid for delivery of nutrients and removal of waste products. Thus, any change in chondrocyte metabolism due to disease should be measurable within the SF. In addition, the disease response of the inflammatory synovial tissue lining the joint will be reflected in the synovial fluid. Lastly, any degradation of the cartilage ECM, synovial fluid proteoglycans or cartilage collagen will be passed to the synovial fluid.

The metabolic analysis of synovial fluid predates the use of NMR spectroscopy. Early studies measured the oxygen tension of SF harvested from patients with various forms of arthroses using a Clark type microelectrode oxygen monitor. As time progressed more advanced detection techniques, such as NMR spectroscopy, were used with univariate statistical analysis used to investigate the significance of changes. Very few studies employing multi-variate statistical NMR methods have been made and none which employed a detailed description of the OA disease phenotype. The majority of recruits in this study are expected to be sourced from patients suffering with OA, due to the abundance of this condition and their presentation requiring joint surgery.

In summary, HSF is the desired analytical fluid of choice in the study of OA and joint disease due to its close relation of joint metabolism and the relative isolation from body systems. This guides our decision to study this body fluid and its favour amongst researchers.

2.2 Literature search methods and criteria: osteoarthritis

In order to comprehensively assess the current metabolic understanding of OA, a systematic review was performed according to the PRISMA guidelines (91). On 3rd June 2020 the MEDLINE and EMBASE databases were searched using the following Boolean search strings:

1. "osteoarthritis" AND ("metabolic" OR "metabonomic" OR "metabolomic" OR "metabolism")

2. ("synovial fluid" OR "cartilage" OR "synovium" OR "serum" OR "plasma" OR "urine") AND ("NMR" or "Mass spectrometry")

This search generated 15141 abstracts. These were evaluated for relevance, sample numbers, species (human or animal model), statistical power, analytical validity, quality of evidence and conclusions. A Newcastle-Ottawa score (NOWS) was used to evaluate the study design and to rate the strength of the study. Relevant metabolites were highlighted, and statistical significance was quoted where appropriate. The search generated 40 reports suitable for inclusion here (see Figure 10).

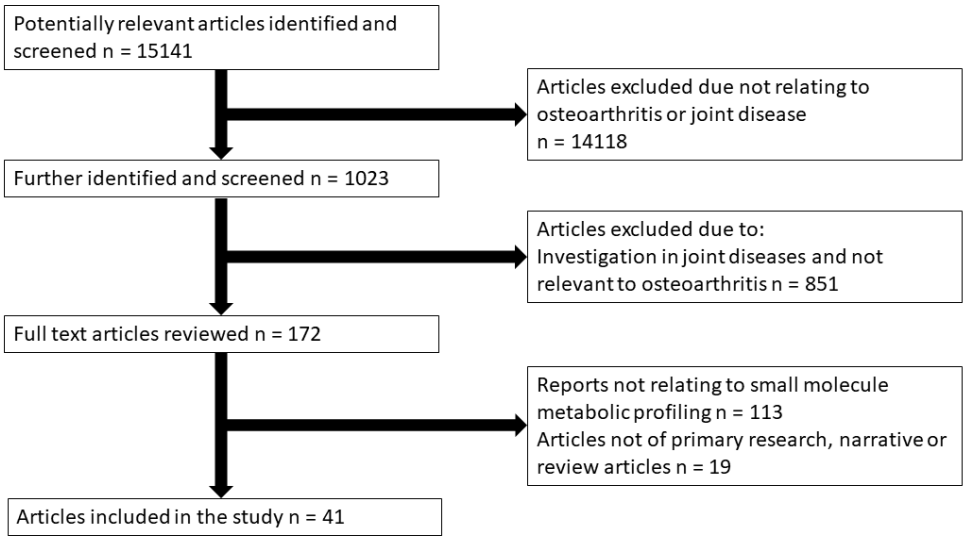


Figure 10 - Literature search strategy and outcome

The metabolic studies were classified according to the body fluid or tissue studied and where the species is not human, this is mentioned. The studies identified for meta-analysis, scrutiny and metabolic interpretation are listed (see Table 1). A collation of all the reported metabolic findings for OA is presented (see Table 2). Studies were scrutinised to evaluate: species, tissue type, number of samples, adequacy of controls, whether analysis was validated and if the statistical approach used was appropriate in the assumptions made and testing used. Furthermore, a NOWS provided a validated measure of study strength (92).

First Author & Year	Country of Origin	Species	Body fluid/Tissue	Joint	Diagnosis	Disease Staging	Controls	Sample size	Validated analysis	Controls	Statistical validity	NOWS
Adams, S.B.(93) 2011	USA	Human	Synovium	Knee	Clinical	ESP & Outerbridge grading	Mild disease (Outerbridge grade <2)	8	Not mentioned	Strong	Adequate	7
Adams, S.B.(94) 2012	USA	Human	Synovial	Ankle	Not stated	ESP	No disease (Radiologically)	20	Not mentioned	Strong	Inadequate	5
Ahn, J.K.(95) 2016	South Korea	Human	Synovium	Wrist/ Knee	RA/OA	Not stated	RA patients	3	Not mentioned	Weak	Inadequate	5
Borel, M.(96) 2009	France	Guinea pig	Cartilage	Knee	Induced OA	Outerbridge grading	No disease (Outerbridge grade 0)	20	Strong	Weak	Adequate	6
Carlson, A.K. (97) 2018	USA	Human	Synovial	Not stated	OA/RA	Not stated	Post-mortem SF	13	Not mentioned	Weak	Inadequate	6
Carlson, A.K. (98) 2019	USA	Human	Synovial	Knee	Clinical	Outerbridge grading	No disease (0 grading)	79	Not mentioned	Weak	Adequate	7
Chen, R. (99) 2018	China	Human	Serum	Knee	Clinical	K-L radiological grading	Mild disease (K-L grading)	67	Weak	Weak	Adequate	6
Damyanovich, A.Z.(100) 1999	Canada	Canine	Synovial	Knee	Induced OA	MCA	No disease (MCA)	14	Strong	Strong	Adequate	7
Damyanovich, A.Z.(101) 1999	Canada	Canine	Synovial	Knee	Induced OA	MCA	No disease (MCA)	7	Strong	Strong	Adequate	6
Deiana, Michela (102) 2019	Italy	Human	Serum	Not stated	Not stated	Not stated	Paired analysis (before and after exercise)	6	Not mentioned	Strong	Inadequate	6
Duffy, J.M.(103) 1993	Ireland	Human	Synovial	Knee	MCA	MCA	No disease (MCA)	16	Weak	None	Adequate	4
Fuchs, B. (104) 2009	Germany	Equine / Canine	Synovial	Not stated	Not stated	Not stated	Disease free animals	6 + 5	Not mentioned	Strong	Inadequate	4
Gierman, L.M.(105) 2013	Holland	Human	Infrapatellar fat pad	Knee	Clinical	ESP	No disease cadaver	13	Strong	Weak	Inadequate	4
Hugle, T(106) 2012	Switzerland	Human	Synovial	Knee	Clinical	None	Non-OA pathology	15	Not mentioned	None	Adequate	4
Jiang, Hui. (107) 2016	China	Rat	Urine	Knee	Histological	Histological	Non-OA histologically	16	Not mentioned	Strong	Adequate	5
Jiang, Miao(108) 2013	China	Human	Serum	Not stated	Clinical	Not stated	Healthy volunteers	27	Strong	Strong	Adequate	7
Kim, Sooah(109) 2016	South Korea	Human	Synovial	Knee	Clinical	K-L radiological grading	Mild disease (K-L grading)	15	Not mentioned	Weak	Inadequate	5
Kosinska, M.K. (110) 2013	Germany	Human	Synovial	Knee	Clinical	Not stated	Post-mortem	39	Strong	Weak	Adequate	7
Lacitignola, L(111) 2008	Italy	Equine	Synovial	MCPJ	Radiological	None	No radiological disease	14	Not mentioned	Strong	Adequate	7
Lamers, Robert-Jan A. N.(112) 2005	USA	Human	Urine	Knee & Hip	Radiological	K-L radiological grading	No disease (K-L grading)	45	Not mentioned	Weak	Inadequate	5

Lamers, Robert-Jan A. N.(113) 2003	Holland	Guinea pig (Hartley outbred albino model)	Urine	Not stated	None (inevitable disease)	None (inevitable disease)	Vitamin C fed animals	46	Not mentioned	Strong	Inadequate	5
Li, Xin(114) 2010	China	Human	Urine	Knee	Radiological	K-L radiological grading	Healthy volunteers	37	Not mentioned	Weak	Adequate	5
Loeser, R.F. (115)2016	USA	Human	Urine	Knee	Radiological	K-L grading & Joint space width	Disease no progression	44	Not mentioned	Strong	Inadequate	6
Maher, Anthony D.(116) 2012	Australia	Ovine	Serum	Knee	MCA	MCA	MCA	12	Not mentioned	Strong	Inadequate	5
Marshall, K.W.(117) 2000	Canada	Human	Synovial	Not stated	Clinical	Not stated	Mild disease	10	Not mentioned	Weak	Adequate	5
Maerz T. (118)2018	USA	Rat	Serum	Knee	Not stated	Not stated	ACL sacrificed	36	Strong	Strong	Adequate	7
Meshitsuka, S(119) 1999	Japan	Human	Synovial	Knee	Clinical (ACR criteria)	Not stated	RA patients	16	Not mentioned	None	Adequate	4
Mickiewicz, B.(120) 2015	Canada	Ovine	Synovial	Knee	Surgically induced	Not stated	Sham and non-surgical controls	9	Not mentioned	Strong	Adequate	5
Mickiewicz, B. (121) 2015	Canada	Human	Synovial	Knee	Clinical	Not stated	No disease cadaver	21	Strong	Weak	Adequate	6
Mustonen, A-M. (122) 2019	Finland	Human	Infrapatellar fat pad / Synovial	Knee	OA/RA	Not stated	Non-OA/RA pathology	20	Not mentioned	Weak	Inadequate	5
Rocha, Beatriz (123) 2020	Spain	Human	MSC Chondrocytes	Hip	Clinical	Not stated	Healthy donors	12	Not mentioned	Strong	Inadequate	7
Shet, Keerthi(124) 2011	USA	Human	Cartilage	Knee	ESP	Not stated	No disease cadaver	12	Weak	Weak	Adequate	5
Sitton, N.G.(125) 1986	UK	Human	Synovial	Not stated	Clinical (ACR criteria)	Not stated	No OA pathology	29	Strong	None	Adequate	4
Williamson, M.P.(126) 1989	UK	Human	Synovial	Knee	Clinical	Not stated	No OA pathology	10	Weak	None	Inadequate	4
Wu, C. (127) 2017	USA	Mouse	Serum / Synovial	Knee	Surgically induced	Histological	No OA pathology	24	Not mentioned	Strong	Inadequate	6
Zhai, Guangju(128) 2010	UK	Human	Serum	Knee	Clinical & Radiological	K-L radiological grading	No disease (KL grading	123	Strong	Strong	Adequate	7
Zhai, Guangju (129)2019	Canada	Human	Serum	Knee	Radiological	Not stated	Slow disease progression	139	Strong	None	Adequate	6
Zhang, Q.(130) 2015	China	Human	Serum	Knee	Clinical & Radiological	K-L radiological grading	Mild disease (K-L grading)	40	Not mentioned	Strong	Adequate	6
Zhang, W.(131) 2014	Canada	Human	Synovial	Knee & Hip	ESP	Not stated	Absent metabolic disease	80	Strong	None	Adequate	5

Zhang, W.(132) 2016	Canada	Human	Serum	Knee	ESP	Clinical (ACR criteria)	No disease (ACR criteria)	123	Strong	Strong	Adequate	7
Zhang, W.(133) 2016	Canada	Human	Serum	Knee	ESP	Clinical (ACR criteria)	Absent metabolic disease	144	Strong	Strong	Adequate	7
Zheng, K. (134)2017	China	Human	Synovial	Knee	Not stated	Not stated	Above knee amputees	49	Not mentioned	Strong	Inadequate	6

Table 1 - The evaluation of OA metabolic studies published to date (NOWS = Newcastle-Ottawa Score, ACR = American College of Rheumatologists, ESP = End-stage procedure, MCA = Macroscopic cartilage assessment, K-L = Kellgren & Lawrence

Metabolite	*Change*	*Species, Tissue/Fluid type and Reference*
1-arachidonoylglycerophosphoethanolamine	Increased	Human SF(94)
1-arachidonoylglycerophosphoinositol	Increased	Human SF(94)
1-monostearin	Increased	*Human SF(109)
1-oleoylglycerophosphoethanolamine	Increased	Human SF(94)
1-stearoylglycerophosphoethanolamine	Increased	Human SF(94)
1-stearoylglycerophosphoinositol	Increased	Human SF(94)
1,4-dihydroxy-2-naphthoic acid	Not stated	*Rat urine(107)
12,13S-epoxy-9Z,11,15Z-octadecatrienoic acid	Increased	*Human SF(97)
12-OPDA	Increased	*Human SF(97)
12-oxo-PDA	Increased	*Human SF(97)
13-epi-12-oxo Phytodienoic Acid	Increased	*Human SF(97)
13-keto-9Z,11E,15Z-octadecatrienoic acid	Increased	*Human SF(97)
13-Methyl-4,4-Bisnor-8,11,13-Podocarpatrien-3-One	Increased	Human SF(97)
1,5-Anhydroglucitol	Increased	*Human SF(134)
17beta-Hydroxy-2-oxa-5alpha-androstan-3-one	Increased	Human SF(97)
2-hydroxypalmitate	Increased	Human SF(94)
2-hydroxystearate	Increased	Human SF(94)
2-hydroxyvaleric acid	Decreased	*Human synovium(95)
2-ketoisocaproate	Decreased	*Human synovium(95)
2,8-dihydroxyquinoline	Not stated	*Rat urine(107)
20:4n-6 FA	Increased	*Human IFP(122)
22:6n-3 FA	Decreased	*Human SF(122)Human IFP(122)
24,24-difluoro-1Î±,25-dihydroxy-26,27-dimethyl-24a-homovitamin D3	Increased	*Human SF(97)
28-Homobrassinolide	Increased	*Human SF(97)
3-Carboxy-4-methyl-2-oxypentanoate	Increased	*Human serum(130)
3-*D*-hydroxybutyrate	Increased	*Human SF(94, 117), *canine SF(100, 101), human urine(112)
	Decreased	*Human SF(121)
3-Hydroxypropionate	Increased	*Human SF(109)
3-indoxyl-sulfate	Increased	Human SF(94)
3-Methyl histidine	Increased	Ovine serum(116)
3-Nitrotyrosine	Decreased	*Rat serum(118)
3-phenyllactate	Decreased	*Human synovium(95)
3,5-Tetradecadiencarnitine	Increased	*Rat serum(118)
4-androsten-3beta-17beta-diol-disulfate-1	Increased	Human SF(94)
4-Hydroxy-6-[2-(2-methyl-1,2,4a,5,6,7,8,8a-octahydro-1-naphthalenyl) ethyl]tetrahydro-2H-pyran-2-one	Increased	*Human SF(97)
4-hydroxy-hippurate	Increased	*Human urine(114)
4-Methyl-phenol	Decreased	*Human urine(114)
4-methyl-2-oxopentanoate	Decreased	*Human synovium(93)
4-methylene proline	Increased	*Human urine(114)
4-Oxoproline	Decreased	*Human serum(130)
4-pyridoxic acid	Not stated	*Rat urine(107)
5-aminovaleric acid	Decreased	*Rat urine(107)
5-deoxy-5-methylthioadenosine	Decreased	*Human synovium(95)
5-dodecenoate (12:1n7)	Increased	Human SF(94),
5-oxoproline	Decreased	*Human synovium(93)
7-Paradol	Increased	*Human SF(97)
8-Aminocaprylic acid	Decreased	*Human SF(134)
(-)-8-hydroxy-11E,17-octadecadien-9-ynoic acid	Increased	*Human SF(97)
8-hydroxy-9,11-octadecadiynoic acid	Increased	*Human SF(97)
8-hydroxy-10,12-octadecadiynoic acid	Increased	*Human SF(97)
(9R,13R)-12-oxo-phytodienoic acid	Increased	*Human SF(97)
9-Decenoylcarnitine	Increased	*Rat Serum(118)
9-hydroxy-10E,14Z-octadecadien-12-ynoic acid	Increased	*Human SF(97)
9-OxoOTrE	Increased	*Human SF(97)
9,10-EOT	Increased	*Human SF(97)
10-OPDA	Increased	*Human SF(97)

Acetamide	Increased	*Canine SF(100)
Acetate	Increased	*Canine SF(100, 101), *equine SF(111), *human urine(114)
Acetyl carnitine	Increased	*Human synovium(93),
	Decreased	*Human serum(99)
Aconitic Acid	Increased	*Human urine(114)
Adenosine-5-monophosphate	Decreased	*Human synovium(95)
Adipate	Increased	*Human SF(109)
	Decreased	*Human synovium(95)
Alanine	Increased	*Canine SF(100), Canine SF(101), *equine SF(111), guinea pig urine(113), *Human serum(99), *Rat Urine(102)
	Decreased	*Human joint cartilage(124)
Aleic Acid	Increased	*Human SF(109)
Alpha-licanic acid	Increased	*Human SF(97)
Alpha-tocopherol	Increased	*Human SF(109), Human SF(94)
Aminomalonic acid	Not stated	*Rat urine(107)
Androsterone sulphate	Increased	Human SF(94)
***α*–Aminobutyric acid**	Decreased	*Human serum(99)
***γ*–Aminobutyric acid**	Decreased	*Human serum(99)
Arachidic Acid	Decreased	*Human synovium(95)
Arachidonate (20:4n6)	Increased	*Human SF(109), Human SF(94)
Arginine	Increased	*Human serum(99)
	Decreased	*Human plasma,
Ascorbic acid	Increased	*Human SF(109)
Asparagine	Increased	*Human serum(108),
	Decreased	*Ovine SF(120), *human synovium(95), *Human serum(99),*Rat urine(107)
Aspartic Acid	Increased	*Human SF(135)
	Decreased	*Rat serum(118)
Aspartate (isomerized)	Decreased	*Human synovium(95)
Behenic Acid	Decreased	*Human synovium(95)
Benzamide	Increased	*Human SF(109)
	Decreased	*Human synovium(95)
Benzoate	Increased	Human SF(94), Human SF(109)
Bilirubin*E-E	Increased	*Human SF(109)
Butylcarnitine	Increased	*Rat serum(118)
C16-OH	Increased	*Rat serum(118)
C5-DC/C6-OH	Decreased	*Rat serum(118)
C5-OH/C3-DC-M	Decreased	*Rat serum(118)
Capric Acid	Increased	Human SF(94)
Carnitine	Decreased	*Human joint cartilage(124), *Rat serum(118)
Carnosine	Decreased	*Rat serum(118)
Choline	Not stated	Human SF(98)
Chondroitin Sulphate	Increased	Human SF(94)
Cis-4-decenoyl-carnitine	Increased	*Equine SF(111), Canine SF(101) *human SF(117), *human urine(114)
Citrate	Increased	*Human SF(97)*human synovium(95),
	Decreased	*Human SF(121)
Citrulline	Increased	*Human SF(97)
	Decreased	*Human serum(99)
Colnelenic acid	Increased	Human SF(94)
Cortisol	Increased	Human urine(112), *canine SF(101), *equine SF(111)
Creatine/Creatinine	Increased	Ovine serum(116)
Creatine	Increased	Ovine serum(116), *Human serum(99)
	Decreased	*Human SF(121)
Creatinine	Increased	Human SF(94)
	Decreased	*Human serum(99)
Cysteine	Increased	Human SF(94)
Cytosine	Not stated	*Rat urine(107)
***D*-(glycerol 1-phosphate)**	Not stated	*Rat urine(107)
***D*-galacturonic acid**	Not stated	*Rat urine(107)
Diglycerol	Not stated	*Rat urine(107)
Dihomo-linoleate-20-2n6	Increased	*Human serum(108)

Dihydroxyfumaric acid	Decreased	*Human serum(108)
Dimethylglycine	Decreased	*Humans serum(99)
Dimethylnonanoyl carnitine	Increased	*Human SF(109)
Ethanol	Decreased	*Human SF(121)
Ethanolamine	Increased	*Canine SF(100, 101), *human SF(126), *guinea pig joint cartilage(96)
	Decreased	*Human SF(121)
Fatty Acid CH2 NMR peak	Decreased	Human SF(126)
Fatty Acid CH2/CH NMR peak ratio (chain saturation)	Increased	*Canine SF(100), Canine SF(101)
Fatty Acid CH3 NMR peak	Decreased	Human SF(126)
Fatty Acid CH_3/CH_2 NMR peak ratio (FA chain length)	Increased	*Human SF(117)
Formate	Increased	*Human synovium(95)
	Decreased	*Rat serum(118)
Fructose	Decreased	*Human synovium(95), *Human SF(121)
Fructose-6-phosphate	Decreased	*Human synovium(95)
Fucose	Increased	*Human SF(97)
Fumaric Acid	Decreased	*Rat serum(118)
Fusidic Acid	Increased	*Human synovium(95)
Galactose	Decreased	*Human synovium(93)
Gamma-glutamylleucine	Increased	*Ovine SF(120), *equine SF(111), *human synovium(95)
Gentiobiose	Not stated	*Rat urine(107)
Gluconic lactone	Increased	*Human SF(134)
Glucose	Decreased	Canine SF(101), *human synovium(95)
	Increased	Canine SF(101), *human synovium(93), ovine serum(116), *human urine(114), *human synovium(95)
Glutamine	Increased	*Canine SF(101), *Human SF(134)
	Decreased	*Human serum(99),*Rat urine(107)
Glutamine/Glutamate	Decreased	*Human synovium(95)
Glutamate	Increased	*Human Serum(108)
	Decreased	Human serum(99)
Glyceraldehyde	Increased	*Canine SF(100, 101), *equine SF(111), *human SF(109), human urine(112)
Glycerol	Decreased	*Human joint cartilage(124), *human serum(130), *human urine(114)
α-Glycerophosphorylcholine	Increased	Human serum(99)
Glycine	Decreased	*Human synovium(95), Human serum(99)
Glycolate	Increased	*Human urine(115)2018
	Decreased	*Human synovium(95)
Guanine	Increased	*Canine SF(100), *equine SF(111)
H1	Decreased	*Rat serum(118)
HDL-Choline	Not stated	Human SF(98)
Heparin sulphate	Increased	*Human SF(109)
Heptadecanoic Acid	Decreased	*Human urine(114)
Hexanoylcarnitine	Increased	*Rat Serum(118)
	Decreased	*Human SF(121)
Hippuric Acid	Increased	*Human urine(115)2018
	Decreased	*Human urine(114)
Histamine	Increased	*Human synovium(95)
Histidine	Decreased	*Human serum(130), *human urine(114), human urine(112),
	Increased	*Human serum(130), Human serum(99), *Human urine(115)2018
Homocysteine	Increased	*Human serum(108)
Homogentisic acid	Not stated	*Rat urine(107)
Homoserine	Increased	*Human urine(114)
Homovanillic Acid	Increased	*guinea pig joint cartilage(96), *human synovium(93)
Hydroxyproline	Decreased	*Ovine SF(120)
	Increased	*Human serum(99, 130), guinea pig urine(113)
Hydroxypropionylcarnitine	Decreased	*Rat serum(118)
Hypoxanthine	Decreased	Human synovium(95)
Inosine	Increased	*Ovine SF(120)

Isobutyrate	Increased	*Human urine(114)
Isocitric Acid	Increased	*Canine SF(100), Canine SF(101) *guinea pig joint cartilage(96), *human synovium(95)
Isoleucine	Increased	*Human SF(98), *Human serum(99)
Isopropyl alcohol	Increased	*Rat serum(118)
Keratin Sulphate	Increased	Human SF(94)
***α*-ketoglutaric acid**	Not stated	*Rat urine(107)
Kynurenine	Increased	*Human SF(94, 117, 119), *Canine SF(100), Canine SF(101),*equine SF(111), *human serum(130), human urine(112), guinea pig urine(113)
Lactate, Lactate/Alanine ratio	Decreased	Human joint cartilage(124)
	Decreased	*Human synovium(95)
Lactose	Increased	*Human SF(109)
Lanosterol	Decreased	*Human synovium(95)
Lauric Acid	Increased	*Guinea pig joint cartilage(96), *human serum(128), *human plasma(132), *human synovium(95)
Leucine	Increased	*Human serum(99, 128)
Leucine/Histidine ratio	Increased	*Human SF(109)
Lignoceric Acid	Increased Decreased	*Human SF(109) *Human synovium(95)
Linoelaidyl carnitine	Decreased	*Rat serum(118)
Linolenic Acid	Decreased	*Human serum(130),
Lipoprotein associated fatty acid	Increased	*Human synovium(95)
Lysine	Increased	*Human serum(99)
	Decreased	*Human plasma(132)
Lysophosphatidylcholine	Increased	*Human SF(109, 110), guinea pig urine(113)
Lysophosphatidylcholine 18:2 / Phosphatidylcholine 44:3 ratio	Increased	*Human serum(129)
Malate	Decreased	*Human SF(121)
Malic Acid	Increased	*Human synovium(95)
Maltose	Not stated	*Rat urine(107)
Mannitol	Increased Decreased	Human SF(94) *Human synovium(95)
Mannose	Increased	*Human synovium(95)
Methionine sulfoxide	Increased	*Human serum(99, 130), Human serum(99)
	Decreased	*Human SF(121)
Methyl-hippuric acid	Decreased	human urine(112)
Methyl histidine	Increased	*Human synovium(95)
Methyl palmitoleate	Increased	*Human synovium(93)
Myo-inositol	Increased	*Human SF(109), *human synovium(95)
Myristic Acid	Increased	*Human SF(98, 103, 136), *canine SF(100, 101), *equine SF(111), *guinea pig joint cartilage(96)
***N*-acetyl glycoprotein**	Decreased	*Human joint cartilage(124)
	Increased	*Human synovium(93)
***N*-acetyl ornithine**	Increased	*Human SF(109)
***N*-acetyl-*D*-galactosamine**	Not stated	*Rat urine(107)
***N*-acetylornithine**	Decreased	*Rat serum(118)
N-carbamylaspartate	Increased	*Human synovium(95)
N-carbamylglutamate	Decreased	*Rat urine(107)
N-methylalanine	Increased	*Human synovium(95)
N-Phenylacetylglycine	Decreased	*Human SF(121)
Nicotinamide	Decreased	*Human synovium(95)
O-Acetylcarnitine	Decreased	*Human SF(121)
Octadecanol	Decreased	*Human synovium(95)
Oleic Acid	Increased	*Human synovium(95)
Oleoylcarnitine	Decreased	*Rat serum(118)
Ornithine	Decreased	*Human synovium(95), Human serum(99)
Oxalate	Increased	*Human synovium(95)
Oxoproline	Increased Decreased	*Human SF(109), *Human synovium(95)
Palmitic Acid	Increased Decreased	*Human SF(109), *Human synovium(95)
Palmitoleic Acid	Increased	Human SF(94)
Palmitoyl-sphingomyelin	Increased	*Human SF(97)
Palmitoylcarnitine	Decreased	*Rat serum(118)

Panaquinquecol 1	Increased	*Human synovium(95)
Phenylalanine	Increased	*Human SF(109)
	Decreased	*Human synovium(95), Human serum(99)
1-Phenylethylamine	Decreased	*Rat serum(118)
Phosphate	Decreased	*Human plasma(133)
Phosphatidylcholine	Increased	*Human SF(110)
Phosphatidylcholine acyl-alkyl C16:0	Decreased	*Rat serum(118)
Phosphatidylcholine acyl-alkyl C34:3	Decreased	*Human plasma(132)
Phosphatidylcholine acyl-alkyl C36:2	Decreased	*Human plasma(133)
Phosphatidylcholine acyl-alkyl C36:3	Decreased	*Human plasma(132)
Phosphatidylcholine diacyl C36:6	Decreased	*Human plasma(132)
Phosphatidylcholine acyl-alkyl C38:0	Decreased	*Human synovium(95)
Phosphatidylethanolamine	Increased	*Human SF(110)
Phosphatidylethanolamine based plasminogen	Increased	*Human SF(110)
Phosphogluconic Acid	Increased	*Human SF(109)
Pelargonic Acid	Increased	*Human SF(109)
Pentadecanoic Acid	Increased	*Human SF(109)
	Decreased	*Human synovium(95)
Phenylacetate	Decreased	*Human synovium(93)
Phenylacetylglycine	Increased	*Human SF(109), *human synovium(95)
Phenylalanine	Increased	*Human SF(97)
Phytolaccinic acid	Increased	*Human SF(97)
Pokeberrygenin	Increased	*Guinea pig joint cartilage(96)
Proline	Decreased	*Human serum(99, 130)
Propionyl-carnitine	Increased	Human SF(94)
Pseudouridine	Increased	*Human SF(109)
ω-3 PUFA	Decreased	*Human SF (122), *Human IFP (122), *Mouse serum(127)
ω-6 PUFA	Increased	*Human SF (122), Human IFP(122), *Mouse serum(127)
Putrescine	Decreased	*Human serum(130)
Pyridoxine	Increased	Canine SF(101), *equine SF(111), *human serum(130), human urine(112)
Pyruvate	Increased	*Human SF(109)
	Decreased	*Human synovium(95)
Salicylaldehyde	Increased	*Human synovium(95), Human SF(109)
	Decreased	*Ovine SF(120)
Serotonin	Decreased	*Rat serum(118)
Serine	Increased	*Human SF(98)
	Decreased	*Human serum(99)
Shikimic acid	Not stated	*Rat urine(107)
Sialic Acid Metabolism	Decreased	*Human serum(130), *human plasma(132)
Spermidine	Increased	*Rat serum(118)
Sphingomyelin	Increased	*Human SF(109)
Squalene	Increased	*Human SF(109),
	Decreased	*Human synovium(95)
Stearic Acid	Increased	*Human synovium(93), Human SF(109)
Stearoylcarnitine	Decreased	*Rat Serum(118)
Succinate	Decreased	*Canine SF(100), *human SF(117), *Rat serum(118)
Sugars, Polyols	Increased	*Human synovium(95)
Tagatose	Increased	*Human synovium(95)
Tartronic Acid	Not stated	*Rat urine(102)
Taurine	Decreased	*Human serum(99)
Threitol	Increased	*Human synovium(95)
	Decreased	*Human SF(117)
Threonine	Increased	*Human SF(109, 134)
	Decreased	Human serum(99)
Threose	Increased	Human SF(94), *human serum(130), *human synovium(95)
trans-2-Dodecenoylcarnitine	Increased	*Rat serum(118)
Trehalose	Not stated	*Rat urine(107)
Trigonelline	Decreased	*Human urine(115)
Tryptophan	Increased	Canine SF(101), *human synovium(95), *Human serum(99)
Tyramine	Decreased	*Human SF(134)

Tyrosine	Increased	Human SF(94), *human serum(99, 130), Human synovium(95)
Urate	Increased	*Human synovium(93)
Urea	Increased	*Human SF(94),
Uridine	Decreased	Ovine SF(120)
	Increased	*Human serum(128), *human synovium(95)
Urocanic acid	Decreased	*Rat urine(107)
Valine	Increased	*Human serum(99, 128)
Valine/arginine ratio	Increased	*Human serum(128)
Valine/glycine ratio	Increased	*Human serum(128)
Valine/histidine ratio	Increased	*Human serum(128)
Valine/tryptophan	Increased	*Human synovium(95)
Xanthine	Increased	*Human synovium(95)
Xylose	Not stated	*Rat urine(107)

Table 2 – A reference of identified metabolites associated with OA. Metabolites reported with a $p<0.05$ are highlighted and the relevant specimen type indicated (*)

2.3 Assessment of bias

The experimental and analytical design was assessed using the Cochrane criteria for bias (137). The similarity of the studies facilitated the use of a global statement for bias.

Patient selection was never mentioned to be randomised. Blinding is impossible at sample collection but often applied at the analysis stage. However, supervised statistical methods, by definition, require knowledge of the sample class to perform the analysis, preventing blinding of the data. Since certain analytical methodologies are common these may disproportionately report certain metabolites and ignore others, creating a reporting bias. Furthermore, more abundant metabolites will be more readily detectable and create reporting bias for the same reason. Most models have linear regression integrals to their modelling and this has the potential to overlook significant metabolites where the pattern of phenotype association is not linear (138).

Assaying several hundred metabolites may yield apparently significant ***p*** values unless a false discovery rate calculation, permutation testing or validation data sets are used (139).

2.4 Osteoarthritis synovial fluid studies

In order to compare studies, their findings and make conclusions based upon the metabolism, reports have been grouped according to which body fluid has been studied. Where possible human is mentioned as a priority and compared directly. Where the species is not human this is mentioned. The majority of studies concentrate upon synovial fluid for the reasons outlined above.

Kim et al, reported a study of SF metabolism comparing patients in early and late-stage disease using gas-chromatography mass spectrometry (GCMS) (109). Fifteen patients were studied, and t-testing was applied to confidently illustrate a significant difference in metabolic profiles between early and late-stage OA group demographics, assuming data normality. However, the late-stage group contained no males and no diabetics, causing a mismatch and potential confounding factors for both gender and diabetes. Salicylaldehyde was reported as significant and must be discounted because it is an aspirin derivative used for its analgesic and anti-inflammatory properties. The reporting of 28 significant metabolites associating with OA should be treated with caution due to the uncontrolled confounding

factors, limited numbers, lack of metabolite interpretation and the absence of any false reporting correction.

2.4.1 Anaerobic environment

Metabolic stress, lactate and pyruvate elevation and an anaerobic environment have been observed in HSF from septic arthritis and RA. RA glucose concentrations rise before dropping in later disease (125, 140). This may be the case in OA.

Different joint arthropathies were assayed by NMR spectroscopy and the results were analysed using principal component analysis (PCA): results showed no proof of an elevated anaerobic environment with OA (106). Other NMR studies showed variable lactate levels (103). However, other researchers reported a 2-6-fold elevation in lactate and a 4-fold pyruvate elevation in human OA SF using both NMR and liquid chromatography mass spectrometry (LCMS) techniques (94, 100, 117). A direct comparison of HSF from healthy, RA and OA patients revealed elevated lactate levels in diseased joints, using the lactate/alanine ratio to standardise. This effect was seen in both diseased groups but was more extreme in RA (119). The study of human post-traumatic and end-stage OA suggested elevated lactate, although the post-traumatic group also had increased oxidative metabolism with elevation in glutathione, cysteine and cysteine-glutathione (94). A comparison of human early and late stage OA showed 1.5 fold elevation of the anaerobic metabolites lactate, citrate and formate in late stage disease, but only 5 patients were involved (117). Comparing OA knee HSF to recently deceased individuals concurred with the finding of elevated citrate in OA, despite the limitations of the control group. In addition, an increase in fructose and decreases in malate, 3-hydroxybutyrate, creatine and ethanol indicated disturbed tricarboxylic acid (TCA) cycle metabolism (121). Canine OA samples had elevated pyruvate and lactate compared to controls, whilst equine OA SF only had a 3-fold elevation in lactate levels (111). However, the South Korean group reported an increase in malate, citrate, succinate and fumarate (contrary to others) in late-stage OA, arguing this demonstrates enhanced use of energy (109).

In anaerobic environments glucose levels are thought to be elevated due to increased demand. Like the observation of lactate, the glucose reports are mixed. Sitton et al. found glucose concentrations in OA SF higher than in the serum of the same patient, although the reverse was observed in RA (125).

Conversely, in advanced OA Marshall et al. noted a lower concentration of sugars and polyols, citing anaerobic metabolism as the cause. Equine studies comparing OA and normal SF showed a five-fold increase in glucose concentration in OA fluid (111). An ovine study reported significantly higher glucose levels in OA versus controls (120). Interpreting the glucose levels is difficult due to multiple influences including diet, altered glucose transport and synovial membrane permeability. In conclusion, the evidence suggests a glucose concentration rise in early disease, most likely from up-regulated transport mechanisms. In late-stage disease a decompensation of glucose consumption versus delivery could cause a drop in levels. The above conclusions are supported by the reduction of TCA cycle intermediates in late-stage OA HSF (98).

A study of OA HSF versus post-mortem "healthy" synovial fluid by LCMS analysis revealed a promotion of creatine synthesis and galactose metabolism, although the controls were imperfect (97). A better controlled study indicated gluconic lactose hypothesised to be the free radical conversion of glucose (134).

Clinically the anaerobic environment and modified glucose metabolism are not specific markers for OA but may indicate early joint disease.

2.4.2 Lipid metabolism

The clinical relevance of lipid metabolism is significant with the emerging evidence for a link with OA, statin preventative therapy, inflammatory diseases and ischaemic heart disease. Lipids may provide future biomarkers and effective pharmacological targets.

The handling of lipids is multifactorial, with contributions from both metabolic and inflammatory pathways. NMR spectroscopic analysis allows lipoprotein class information to be characterised. It is possible to distinguish between VLDL, LDL and HDL (141, 142). Individual lipid types can be measured and is validated in serum or plasma including triglycerides, cholesterol, cholesterol esters and phospholipids. Information is given about the length of the lipid chain polymer and the overall amount of mono or polyunsaturated lipid. Both these factors can be derived from the peak area ratios of CH_3, CH_2 and CH moieties within the proton NMR spectral peaks arising from the lipid molecules.

Microscopic evaluation of canine knees with transected anterior cruciate ligaments (ACL) revealed chondrocytes storing large amounts of lipid (143). Healthy human SF has very low concentrations of the VLDL, HDL and LDL lipoproteins in comparison to matched serum. In inflammatory joint disease the levels of the lipoproteins are elevated. It was suggested that this was due to inflammation and increased synovial membrane permeability (140). This finding requires cautious interpretation because controls were not age-matched and were between 25 and 42 years old.

Intra-articular phospholipase A_2 (PLA_2) is up regulated in patients with inflammatory joint disease thereby catalysing the creation of lysophospholipids from phospholipids. An LCMS characterisation of the phospholipid classes in human, equine and canine SF in each case found phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC) and sphingomyelin(104). The lysophosphatidylcholine content and lysoPC/PC ratio was correlated with RA disease, but there were no significant differences the lipid profile of OA and controls in any species (104, 144). The authors acknowledged the confounding factor of diet and the strong influence it may have in such studies and any conclusions are limited because there were only six samples (104). This was contrary to other studies measuring lipid classes in OA knee HSF. Carlson et al. revealed alteration in the glycerophospholipid metabolism and biosynthesis of glycosphingolipid(98). The total lipid content and phosphatidylcholine as the dominant lipid class was found to increase five-fold in OA. This was in agreement with the observations of the South Korean (109). LysoPC and phosphatidylethanolamine were also increased two and four-fold respectively (110).

A switch to fatty acid metabolism is supported by the report of reduced O-acetylcarnitine, hexanoylcarnitine, ethanolamine, methionine and *N*-phenylacetylglycine, all indicating the energy metabolism of lipid species (121). The ketone bodies 3-*D*-hydroxybutyrate and 3-*D*-hydroxyisobutyrate were elevated in late-stage OA in human, ovine and canine studies, again suggesting a switch to fatty acid metabolism (100, 101, 117, 120). Equine OA studies concur, with a 3-fold increase in glycerol liberation, suggesting the same (111).

Some HSF studies have compared OA and RA disease directly. OA has been reported to contain more unsaturated and shorter triglyceride chains than the other groups (122, 126). This was corroborated by

canine OA SF with increased terminal CH_3, CH_2 and CH, compared to control canine SF (100). Equine OA synovial fluid studies concurred with higher levels of unsaturated fatty acids, high density lipoprotein (HDL) and choline (111). However, not all reports are in agreement. Qualitatively RA and OA HSF reported the CH_3/CH_2 NMR peak area ratio was lower in OA HSF, indicative of longer fatty acid chains, albeit with variable results. Studies comparing OA HSF to post-mortem controls report that fatty acid chains are longer with relative unsaturation, whilst specifically decreases in ω-3 polyunsaturated fatty acid (PUFA) and increased in ω-3 PUFA. An identical finding was reported in a mouse serum model (127). Lipid precursors 22:6n-3 are also reduced (110, 122).

Zhang et al. used metabolic profiling to identify subgroups of OA which appeared to differ in fat metabolism and levels of acyl carnitine. There were also distinctions in the glycophospholipids and the sphingiomyelins (131). No correlation to clinical factors or age matching was performed so it is difficult to draw further conclusions.

2.4.3 Free amino acids

In osteoarthritic joint fluid, it is proposed that the majority of increased amino acid levels will derive from hyaline cartilage collagen and proteoglycan destruction (97). These amino acids may constitute biomarkers to early destruction of hyaline cartilage in OA.

Sitton et al. compared serum and HSF in the same patients. Patients suffered with OA or RA. Concentrations of a variety of amino acids, sugars and immunoglobulins were measured using standardised clinical biochemistry techniques. A higher histidine concentration was seen in RA SF compared to serum, an observation not seen with OA despite other studies demonstrating that histamine is produced by OA chondrocytes (125).

Samples from canine OA contained more isoleucine compared to non-diseased controls (100). Both canine and equine OA synovial fluid have higher concentrations of alanine (100, 101, 111). One human study identified lower levels of threonine in advanced compared to early OA (117). A finding disputed by a later study with a reported elevation (134). An MS study of HSF from ankle OA found elevated proline and hydroxyproline, both major constituent amino acids of collagen α-helices (94). Conversely

ovine OA samples reported a reduction in hydroxyproline levels, although samples were taken after ACL reconstruction (120). Canine OA had double the isoleucine concentration compared to non-diseased controls (100). Both canine (1.5-fold) and equine (3-fold) OA synovial fluid have higher alanine concentrations (100, 101, 111). Human OA synovial fluid versus post-mortem samples has revealed a switch to arginine biosynthesis (97). A single protein based HSF study reported isomerised aspartate a potential biomarker for arthritic joint destruction (135).

There is speculation that increased concentrations of these amino acids is due to increased synovial permeability (and thus passage from the serum). However, hyaline cartilage components, proteoglycan and type II collagen, are both rich in alanine and the amino acids discussed above. Therefore, a reasonable hypothesis is that the volume of hyaline cartilage destruction is directly related to the increases of these amino acids in the synovial fluid.

2.4.4 Hyaluronic acid (HA) metabolism

HA is a key SF target in metabolic and OA research. HA contributes to the SF viscoelastic properties by virtue of its polymer aggregates. In OA the HA depolymerises and the viscoelastic properties are lost (14).

The monomeric components of HA: *N*-acetylglucosamine and glucuronic acid, are identifiable by NMR spectroscopy. HA cleavage in SF is observed by NMR spectroscopy due to an increase in the peak area from the terminal *N*-acetyl (N-Ac) group which is a singlet at 2.044ppm, verified by treating SF with hyaluronidase enzyme, γ-radiation and hypochlorous acid (59, 60, 136). Concurrent formation of lactate, citrate and acetate, (known products of *N*-acetylglucosamine) has also been reported (145, 146). Schiller et al. noted the concurrent increase in formate with hypochlorous acid (HOCl) treatment, postulating this corresponded to the monomer carbohydrate ring breakdown (59). Unfortunately, NMR spectroscopy cannot completely distinguish between the N-Ac liberation from HA and other glycosaminoglycans, although both are probably present during joint destruction.

OA and RA HSF comparison showed *N*-Ac levels to be similar. The authors concluded the N-Ac elevation correlated with the level of inflammation, although no explanation for this observation was

provided (103). The study of *N*-Ac group liberation doubled in canine and equine OA compared to healthy controls (100, 111). This was also supported by LCMS analysis of HSF in early versus late OA disease (98).

Williamson's study of SF identified varying levels of *N*-Ac groups with RA and OA. They postulated that this was due to glycoprotein liberation and associating with disease activity but failed to associate this with HA depolymerisation. The authors observed RA and reactive arthropathy patients to show significant degrees of HA depolymerisation. This effect was observed but variable in the OA patients (126). The limitation of this study was the use of cadaveric knee aspirates as the control (no depolymerisation) and that this correlated with traumatic effusions of the knee. Animal studies with tighter controls show *N*-Ac group liberation to double in canine and increase 8-fold in equine OA (100, 111).

The link between HA depolymerisation and synovial fluid viscoelasticity is well documented (147). Thus, a pathway of inflammation, HA depolymerisation and a loss of synovial fluid mechanical properties is likely. The extent of this reaction in human OA SF might be more variable than in other inflammatory arthropathies, hence the rationale for personalised healthcare in hyaluronan therapy.

2.4.5 Vitamin metabolism

The alteration of vitamin metabolism supports the earlier conclusion of an increase in relative hypoxia during disease and the promotion of the anaerobic environment. A successful reaction to this change should promote antioxidant production. Comparing late to early OA revealed that vitamin A, E and glutathione metabolism are reduced. The B vitamins: B6, B5, B9 and B1 were also reduced. This is contrary to vitamin C (ascorbic acid) which is elevated (98). This elevation may be influenced by its role in collagen production as well as an antioxidant.

2.5 Osteoarthritis: plasma, serum and urine Studies

The majority of OA metabolic profiling has concentrated on plasma, serum and urine perturbations because the samples are easy to collect. Studies in the 1980's indicated metabolites that provided a distinction between joint arthropathies.

2.5.1 Serum and plasma studies

Maher et al. harvested ovine serum samples pre- and post-surgical ligament transection (and hence OA induction), meniscal destabilisation or a sham procedure. NMR serum analysis and multi-variate data analysis was used to identify metabolic differences between the sham surgery and surgically induced OA. At four weeks post-ligament transection they reported a reduction in the branch chain amino acids (BCAA) valine, leucine and isoleucine. In addition, 3-methylhistidine was increased. At 12 weeks, glutamine, 3-methylhistidine, creatinine and creatine concentrations increased alongside decreased BCAA. In a separate OA model, the meniscus was destabilised but none of the above features were seen except a reduction in creatine and elevation in dimethyl sulfone ($DMSO_2$). Additional metabolic changes were seen in both models but were ignored because they were also present in the sham surgery controls and were thus linked to the surgical stimulus and not necessarily to OA (116). Some of these metabolites are probably commonly altered by both OA and by the surgical stimulus, but this was not discernible due to the experimental design. Interestingly, $DMSO_2$ is a metabolite of gastrointestinal bacteria, suggesting a link between the gut microbiome and OA (148). A lower concentration of BCAA suggests a shift towards anabolic processes within the cartilage and thus OA progression. This finding might be expected in the animal models where the anabolic systems of the cartilage (and hence BCAA consumption) are presumed to be intact and OA is caused by overwhelming mechanical and supramaximal force, rather than biological failure. Proteoglycan destruction at the cleavage sites could liberate glutamine, suggesting a mechanism to cause an increase in levels. Fat metabolism is significantly influenced by OA in SF and plasma. Fatty acid metabolism increases with elevated metabolites involved in fatty acid chain oxidation. Characterising the lipids suggests triglyceride levels rise with unsaturated, longer chains contributing to higher concentrations of HDL. The analysis of the Newfoundland study data evaluated 139 OA patient's serum looking for metabolites that associated with progression of disease. They reported the significance of the Lysophosphatidylcholine 18:4 to Phosphatidylcholine 44:3 ratio as correlating strongly with OA cartilage loss (129).

The animal OA model findings of decreased BCAA and increased histidine levels are mirrored in human serum. Lamers et al. metabolically profiled albino bred guinea pigs with an inevitable early

onset OA phenotype. Urine samples were taken at various times and compared for the progressive onset of OA. They identified lactate, hypoxanthine and alanine as factors associated with OA onset. Vitamin C was found to affect the levels of these metabolites (113).

In two human studies histidine levels were lower in the serum of OA patients compared to controls: sulphydryl (SH) levels also decreased concurrently (112, 149). The source of the SH remains unclear, but it probably came from liberated cysteine.

A Chinese group made a serum and urine comparison of control subjects alongside arthropathies: RA, OA, ankylosing spondylitis and gout. The study used gas chromatography and high-performance liquid chromatography methods, combined with MS detection and multi-variate analysis to compare the groups. The authors acknowledged several age and gender differences, and these raised the possibility of confounding factors. All disease groups showed altered metabolites attributed to anaerobic metabolism: elevated lactate, aspartate, homoserine, dihydroxyfumarate and decreased 4,8-dimethylnonanoyl carnitine. This work showed a smaller change in the OA group than in the other joint diseases. They suggested it was possible to distinguish OA from other disease types when a more subtle metabolic disturbance was present (108). The findings suggest common metabolic disturbances in joint diseases, but less consistent and less marked changes in OA. Thus, the responsible metabolites are unlikely to be good biomarkers in the diagnosis or monitoring of OA. Furthermore, these findings are contrary to rat studies which reported decreases in many of the carnitine analogues and aspartic acid (118).

A further Chinese group used serum from 40 OA patients and 20 healthy controls, performing age and gender matching (130). They observed significantly less histidine and glycine as the OA disease progressed. In addition, homocysteine and L-tryptophan levels increased with progressive disease. The findings indicate chronic inflammation and biosynthesis (130). This contrasts with a rat serum study which reported a reduction in serum glycine, tryptophan and its metabolites (118).

Zhang studied serum in 72 OA patients and 76 controls, and found OA to associate with reduced arginine and selected lipid species (including PC's and sphingomyelin) (132). A separate analysis of the same dataset investigated associations between OA and diabetes. Whilst identifying those metabolic

differences were present, the researchers also concluded leucine and PCs were influenced significantly by both diseases (133). The studies were robust, with appropriate populations, patient matching and suitably powered statistical analysis. Many of the amino acid findings were correlated by an Ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-TQ-MS) study of 67 patients with varying degrees of knee OA. The targeted amino acid study supported the finding of leucine and the BCAAs to be elevated in OA. In addition, they found increased alanine, aspartate, glutamate, arginine and proline in the presence of decreased aspartate. Furthermore, they reported an elevation of gamma-aminobutyric acid (GABA) and hydroxyproline, both involved in energy metabolism and bone turnover (99).

2.5.2 Urine studies

Lamers et al. assessed 92 urinary metabolic profiles by NMR spectroscopy in patients with OA and concluded a depletion of histidine and methyl histidine, which they argued was indicative of inflammation. They found elevated hydroxybutyrate, pyruvate, creatine/creatinine ratio and glycerol which led them to hypothesise that fat metabolism may be elevated. These results were observational and without statistical significance (112).

A case-control study comparing normal and OA subjects found elevations in valine and leucine which were particularly significant when applied in a ratio to histidine (128). A human urine study comparing patients with and without inflammatory OA symptoms revealed altered histamine metabolism, an effect one might expect in the light of the inflammatory mast cell release (114).

The selected results of the IDEA (Intensive diet and Exercise for Arthritis) trial in the United States of America (USA) yielded selective participants suffering with different grades of OA. After age matching, dietary, exercise and co-morbidity selection, a subset of patients were chosen. Patients with obesity and slow progressing OA were compared to those with fast progression of disease. Progression was assessed by joint space narrowing. The authors reported significant metabolites that associated in a linear manner with joint space narrowing: there were elevations in hippurate, glycolate and histidine, alongside reductions in trigonelline and an unknown metabolite (δ=7.3ppm) (115).

Rat OA knee models evaluated the urinary profile in histological OA and non-OA animals. Decreases in amino acids: glutamine, N-carbamylglutamate, urocanic acid, 5-aminovaleric acid and asparagine were observed. This contrasted with an alanine increase, postulated to be secondary to amino acid metabolism and TCA cycle activity. The authors also postulated an inhibition of the glycolysis pathways with reduction in levels of shikimic acid, gentiobiose, trehalose and xylose (107). This is contrasts with most other reports which have findings of elevated glycolysis. Other noted findings were elevated diglycerol, 4-pyridoxic acid and cysteine, suggesting disordered fatty acid, vitamin B6 and nucleic acid metabolism respectively (107).

The blood and urine studies correlate with synovial fluid findings in many areas, potentially reflecting their close relationship and the extent to which synovial fluid is derived from serum. The BCAA have potential in clinical early diagnostics and serial monitoring. Anaerobic metabolism has been suggested as a method to distinguish between joint arthropathies. The potential for clinical diagnostics is questionable but more detailed metabolic studies may provide this capability.

2.6 Osteoarthritis: cartilage tissue and chondrocyte Studies

Cartilage tissue and cells are an attractive area of study since they relate directly to the mechanical destruction and loss of hyaline cartilage. Their metabolic disturbance may directly describe the predominant catabolism seen in osteoarthritic destruction.

2.6.1 Cartilage studies

Direct metabolic studies of cartilage tissue have been made using high resolution magic angle spinning (HRMAS) NMR spectroscopy. Early studies suggested a significant switch to fatty acid oxidation in aerobic glycolysis of cartilage, although it was the growth plate that was studied and not the joint (150). Unsurprisingly, proteoglycan depolymerisation and structural loss of the cartilage was targeted by many. Using bovine patella cartilage, the *N*-acetyl group originating from glycosaminoglycan breakdown was visualised in the same manner as for SF. An NMR signal at 3.4-4.6 ppm was measured concurrently, but not assignable to hyaluronan alone due to the significant overlap of chondroitin sulphate and collagen peaks. Many of the constituent amino acids of collagen were noted and presumed

to be free in solution within cartilage, although this may have been a misinterpretation as HRMAS studies mean amino acids in mobile polymers can also give rise to observed peaks (151). Guinea pig and bovine nasal cartilage (treated with collagenase) NMR spectra both have increased *N*-acetyl groups peaks originating from glycosaminoglycan breakdown. The collagen amino acids proline, hydroxyproline, leucine and isoleucine are elevated in guinea pig, whilst lysine, glutamate, proline, hydroxyproline and glycine are elevated in bovine-collagenase-treated cartilage (152). Alterations in lipid metabolism with increased CH_2 NMR signals suggest longer lipid chains, but the significance of this remains unclear (96). Treatment of cartilage (and hence proteoglycan) with collagenase did not liberate carbohydrates in the same way that it liberated the amino acids. This anomaly suggests that the free amino acids are retained within the collagen/proteoglycan matrix whereas the free carbohydrate moves into the synovial fluid (152). This has reverse implications when assaying SF in the presence of proteoglycan destruction.

Human OA cartilage has also been studied by HRMAS NMR spectroscopy. OA knees were sampled at arthroplasty and compared to non-OA cadaveric tissue (124). Cadaveric tissue is a poor control because it has to be assumed that metabolism after death is normal. Furthermore, samples were not age matched. OA cartilage had lower levels of *N*-acetyl, alanine, glycine, choline and lactate. The authors claimed indicative proteoglycan destruction because there was a lower NMR signal from *N*-acetyl groups. In synovial fluid the *N*-acetyl signal rises but the authors argued the reverse in cartilage due to a washout effect into the synovial fluid; no evidence was presented to support this theory. The levels of alanine and glycine depletion suggests proteoglycan depolymerisation and hence cartilage destruction.

An LCMS study of cartilage from OA knees demonstrated metabolites associating with osteophyte production. These included PC's, various amino acids and some associated products of fatty acid metabolism (153).

2.6.2 Chondrocyte studies

Chondrocytes switch to a catabolic state in the progression of OA. Dog models with structurally altered joints precipitating OA have chondrocyte hypertrophy and increased fatty acid storage, requiring a

change in metabolism (143). The ECM synthesis is dependent on adenosine triphosphate (ATP) to supply energy. This is an oxidative process, but the chondrocytes are isolated from the nutrient supply and rely upon diffusion. As detailed in *1.3.4* the production of proteoglycan requires the catalysed formation of cross-linked sidechains. To crosslink the glycosaminoglycan molecules, amino acid substrates are required, particularly glutamine. Therefore, one would predict consumption of these metabolites in the presence of an anabolic state within the joint.

Immune stimulated chrondrocytes have demonstrated a shift in anaerobic activity towards lactate production and glycolysis. This was achieved by joint injury and subsequent determination of NF-κB as the responsible immune modulator. Whilst not a metabolic study directly, an increase in lactate dehydrogenase (LDH) was measured (154). Mesenchymal Stem Cells (MSC) stimulated to differentiate to chondrocytes were harvested from OA patients and healthy donors. The differences in metabolism between the cell lines was measured yielding evidence of uridine diphosphate (UDP)-glucuronic acid synthesis and UDP-glucose accumulation in the OA group, pointing towards GAG synthesis. In addition, a reduction in *N*-acetyl groups was speculated to impair GAG production. The production of glucosamine was elevated and cell turnover suggested to be heightened by the presence of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) precursors: ribose-5-phosphate and ribose-1,5-diphosphate (123).

An Italian group evaluated MSC cells harvested from the serum of marathon runners pre and post half marathons. This was an attempt to characterise the metabolic stimuli for immature chondrocytes in their differentiation towards cartilage production. This assumes that physical activity promotes protective mechanisms against joint degeneration. They determined a significant change in the pyrimidine biosynthesis indicating that exercises do appear to promote differentiation to Chondrocytes, with an added tested finding of vitamin B6 being important (102).

An element of caution should be exercised interpreting the in vitro culture studies since these frequently use animal models and cultured chondrocytes. It is unclear to what extent these differ from the in vivo results. This was demonstrated by Batushansky et al. in the comparison of an in vivo harvest and an

equivalent in vitro harvest using a GCMS methodology which demonstrated a prevalence for nitrogen-based metabolites such as amino acids over the sugar substrates in aerobic metabolism (155).

2.7 Osteoarthritis: infrapatellar fat pad

The infrapatellar fat pad (IFP) is a specific region of inflammatory fat and synovium in the front of the knee joint. It is a source of fatty acid lipid mediators and adipocytokines, which make it a particular target for study since it is important for the inflammatory response of the knee. One study examined the oxylipid and fatty acid composition from osteoarthritic infrapatellar fat pads by LCMS using post-mortem tissue as control samples. The group suggested thromboxane A2, thromboxane B2 and lipoxin A2 distinguished between the disease and control samples. Their findings lacked significance, the controls were weak, and the fitting of the model was poor (105).

Mustonen et al. studied the IFP differences between the IFP in OA and RA patients. They found a higher content of 20:4n-6, 22:6n-3 fatty acids and increased n-3 PUFA compared to the RA group(122).

2.8 Osteoarthritis: synovium

As the epithelial tissue of the joint supplying nutrition and providing inflammatory stimuli, the synovium is a desirable target. A single study sampled synovium tissue from three RA patients undergoing wrist arthroscopy and three OA patients undergoing total knee replacement (95). Therefore, the validity of this comparison between diseases and with different joints is questionable. Furthermore, the study lacked suitable statistical validation. However, the authors cultured synoviocytes from the tissue and reported 129 metabolites, 61 of which were statistically significant between the groups using a student's T-test with an $\alpha=0.05$ (see Table 2). These included amino acids, sugars, amines and fatty acids. Most of these reports were in opposition to the reports of synovial fluid for the same metabolite.

2.9 Summary of osteoarthritis studies

In isolation, these studies are limited by the cross-species examination, differing clinical scenarios, variable strength of design and controls. Nevertheless, the studies have greater power when considered in unison, particularly when authors agree and when statistical significance has been quoted and the

experimental design is robust. Therefore, for reference purposes and to add interpretation of later results, the metabolites of significance in OA have been collated and evaluated according to authorship, species and strength of reporting (significance) (see Table 2).

Whilst the evidence to date is limited some conclusions can be made and these have generated a metabolic network pathway (see Figure 11) using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database for human OA SF in the hope of suggesting future research targets (137).

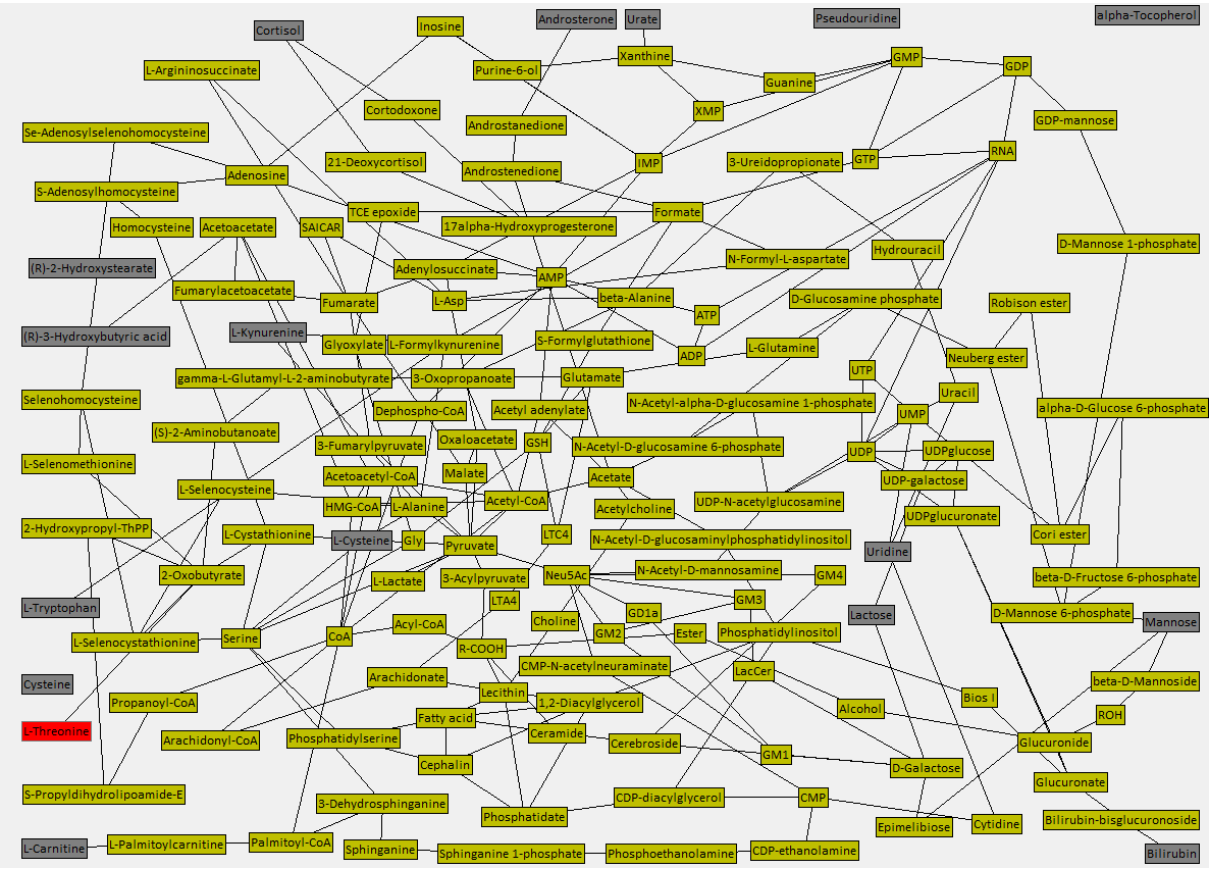


Figure 11 - Metabolic network pathway for osteoarthritic human SF generated using the KEGG database (156). Elevated metabolites in reports are displayed in grey. Those reported to be supressed are highlighted in red. Hypothesised intermediates are generated by the shortest route between reported metabolites, thereby potentially affected metabolites and pathways are highlighted in green.

It is known that LDH is released from chondrocytes in times of stress and cartilage degradation. Despite this, the reports of elevated lactate and the anaerobic environment in OA are mixed; most evidence supports an aerobic environment, particularly the animal models with robust controls. It is hypothesised

that the delivery of glucose is dependent upon the stage of disease, and therefore there are mixed reports in the literature. However, there is no direct measure to support this hypothesis.

Fat metabolism is significantly influenced by OA in synovial fluid and plasma. Characterising the lipids is challenging although evidence suggests triglyceride levels rise in OA synovial fluid with unsaturated, longer chains contributing to higher concentrations of HDL. Fatty acid metabolism increases with elevated metabolites involved in fatty acid chain oxidation. The composition of PC's and sphingomyelin is altered in OA plasma.

The study of macromolecules (including HA) in joint disease has provided clear evidence of proteoglycan chain shortening and cleavage. The described elevation of some amino acids in OA synovial fluid follows the expected pattern of collagen components and other cartilage constituents. Whether this correlates quantitatively with the volume of cartilage loss and extent of joint catabolism remains to be assessed.

OA has a variable inflammatory component, but whether this is significant in driving the disease or is a response to pathology remains unclear. No study has assessed the inflammatory status of the OA joint and correlated this to the metabolic profile, so there are no markers associated with inflammation. However, a group from Birmingham, UK have metabolically assessed serum from established RA and early arthritis patients, attempting to correlate disease to baseline inflammatory markers such a CRP (47). The limitations of their study are the lack of CRP specificity and that any measurable difference in the serum was due to joint inflammation. Their results showed a significant alteration in phospholipid metabolism with elevation of choline, LDL lipids, cholesterol and fatty acids.

Most studies have too few samples and differing degrees of suitable controls, often comparing dissimilar disease cohorts. Whilst interesting, they provide little conclusive data. Animal studies have provided valuable information with stronger controls, albeit with cross-species limitations. A large scale epidemiological metabolic phenotyping study of OA would overcome these challenges. Furthermore, the assessment of clinical outcomes, symptoms and disease progression would address the links between metabolism, the inflammatory manifestations and the clinical features of disease.

2.10 Metabolic phenotyping of synovial fluid

The above studies provide an understanding of the reported metabolites in OA. OA patients will constitute the majority of synovial fluid samples in the recruitment to this study. However, there are other metabolic phenotyping studies relating to synovial fluid. Therefore, an understanding of the metabolic changes in response to other diseases may have a bearing on a study of synovial fluid due to common metabolites, outcomes or responses. Most of these studies focus on RA, probably because the disease is common and the samples are easily available.

The detection of the endogenous metabolites is pivotal to any metabolic phenotype study. It is also essential to be able to distinguish the exogenous metabolites, or those secondary to drug administration. An examination of all the reported synovial fluid metabolic studies will assist with this information. Such metabolites may correlate well with a disease trait or condition and be erroneously identified as central to the metabolic aetiology of that condition unless they are appropriately identified. To date the exogenous metabolites of Ibuprofen, Paracetamol and 1-methyl uric acid (a known metabolite of caffeine) have been identified in synovial fluid (106).

2.11 Synovial fluid: anaerobic environment

A significant inflammatory disease within the joint causes metabolic stress. The Clark electrode analysis of HSF from patients with RA revealed a significantly lower oxygen tension. This was in combination with a more acidic HSF pH and elevated carbon dioxide concentrations, suggesting an anaerobic joint environment (157). A similar study identified elevated lactate concentrations in HSF with an associated low oxygen tension, although this was seen in a variety of arthropathies (54). Healthy HSF has elevated lactate compared to matched serum, but no direct comparison to RA HSF was mentioned (140). Comparison of RA synovium and serum in matched samples revealed an elevated synovial lactate in comparison to the corresponding serum, suggesting that the RA joint operates in an hypoxic environment with anaerobic respiration (158). Elevated lactate is found in other synovial fluid environments, such as septic arthritis. The same group concluded no significant elevation in other scenarios although they were only comparing different arthropathies in small numbers using PCA

analysis(106). A single Systemic Lupus Erythematosus (SLE) sample was found to have vastly elevated lactate concentrations (103). Another research group independently reported the same finding with paired RA synovial fluid having a significantly lower glucose concentration than RA serum (158).

The conversion of phenylalanine (Phe) to tyrosine (Tyr) requires a hydroxylation and is an oxidation reaction. Hence, in the presence of anaerobic stress this conversion is slowed. Therefore, it was hypothesised that the ratio of Phe/Tyr will differ between reactive arthropathy and spondylopathy. An NMR based study of these ratios demonstrated a significantly higher Phe/Tyr ratio in reactive arthropathy compared to RA (159).

In isolation, none of these findings conclusively demonstrate the anaerobic environment in presence of inflammatory stress, more specifically RA. However, a metabolic profiling study of RA synovial fluid against other disease types suggested metabolic perturbations consistent with an increased TCA and urea cycle turnover (160).

2.12 Synovial fluid: fat metabolism

An early NMR spectroscopic study compared paired synovial and serum samples in RA patients and demonstrated an abundance of short chain fatty acids in the RA synovial fluid compared to the serum. The observation of elevated levels of the ketone body 3-*D*-Hydroxybutyrate indicated the metabolic preference for fat metabolism as a fuel substrate (158). The authors concluded the RA joint was using fatty acids and producing ketone bodies. This was corroborated by an LCMS study examining RA versus other disease subgroups, where metabolites consistent with elevated fatty acid metabolism were found in the RA group (160).

A study sampling healthy humans found low concentrations of lipoproteins: VLDL, HDL and LDL in comparison to matched serum. Furthermore, direct SF comparison with diseased inflammatory joints showed greatly elevated concentrations of the lipoproteins. The authors suggested that this reflects the effective synovial tissue filtration in healthy individuals (140). However, they failed to address the confounding factor of age, healthy individuals were all between 25 and 42.

Giera et al, used capillary LCMS methods to identify pro-inflammatory lipid mediators in human RA samples: MaR1, RvD5, LXA_4 and 5S,12S-diHETE. However, this study had severe limitations as no correlation with any clinical variables was attempted, or even comparison to other disease subtypes. Hence there is no evidence that these lipid mediators are related to the disease (161).

2.13 Synovial fluid: amino acid Metabolism

The study of HSF in the presence of ankle fracture revealed elevations in several amino acids which the authors argued may be important in the later development of post traumatic OA due to a proinflammatory state. The key finding was elevation of tryptophan. This statement is without significant support since the metabolism of tryptophan is multifactorial, something acknowledged by the authors. They report elevations in aspartate, ornithine, cysteine, histidine, and glutamate. Some of these amino acids share pathways and elevations with OA. However, it is not clear if the commonality of these pathways is a causal link for trauma to initiate OA (162).

2.14 Synovial fluid: free radical generation

The activation of inflammatory macrophages and polymorphonucleocytes generate free radical species via destructive myeloperoxidase enzymes. Free radical release occurs in hypoxic/reperfusion insult and is a key part of the joint inflammatory reaction and a bactericidal mechanism to inhibit infection (163). These free radicals can take many forms, including reactive hydroxyl groups ($OH^{\cdot}$) and superoxide anions. Chemical agents can be used to induce them, including hydrogen peroxide and HOCl. Lymphocytes in autoimmune diseases have a reduced capacity to resist hydrogen peroxide cytotoxic killing suggesting a saturation of these free radical mechanisms (55). The effect of free radical production, whilst bactericidal, also causes diffuse reactions on protein and lipid species which results in a loss of polymerisation in glycoprotein and lipoprotein molecules.

ROS obviously need oxygen to form. This presents a conflict because the joints of diseased patients with high levels of ROS are hypoxic. The mechanism suggested to explain this phenomenon is hypoxia-reperfusion injury. The basis of this theory is that the environment starts as oxygen rich but with increased oxygen requirements due to injury or disease. A point of decompensation occurs where

oxygen delivery does not meet the demands and the environment becomes anaerobic. This could be due to increased pressure within the joint or a lack of blood flow in the synovial tissue microvasculature (14). Grootveld et al. attempted to measure the metabolic effect of this directly in RA volunteers undergoing isometric exercise followed by serial sampling during the recovery phase. They measured what were deemed to be the hyaluronan breakdown products as the joints became more hypoxic (145). The same authors also generated free radical activity observed by hyaluronan breakdown and found supplementing with *N*-Acetylcysteine or Cysteine was protective (56).

Synovial fluid samples from rheumatoid patients were analysed by NMR spectroscopy before and after treatment with γ-radiation in the presence of oxygen to assess the effect of free radical oxygen species on the synovial fluid. Peaks assigned to acetate, citrate, formate, tyrosine and lactate were increased in intensity (145, 146). Tyrosine was attributed to the hydroxylation of phenylalanine by free radical hydroxyl species. However, no comment was made of the alteration in phenylalanine concentration and little evidence is presented to support this hypothesis. Furthermore, the authors postulated that formate concentrations are elevated secondary to the degradation of carbohydrate (145). The elevations in acetate, citrate and lactate were postulated to be breakdown products of hyaluronan, although the authors suggested an alteration in the NMR peak areas of these metabolites due to an alteration in protein binding, rather than true change in metabolism (145, 146).

The study of HSF in the presence of ankle fracture has demonstrated the presence of glutathione and cysteine-glutathione disulphide suggesting its presence to mitigate the presence of ROS in this environment as a result of oxidative stress.

Free iron ions have been found within the synovial fluid in the presence of inflammation. This unbound iron increases the production of free radical species. The source of this free iron is not known but it has been postulated to arise as a result of liberation from transferrin in the acidic environments. Incubation of rheumatoid synovial fluid with deferoxamine yielded elevated levels of citrate with an altered spin/relaxation time suggesting the unbound iron chelates to citrate. Furthermore, the presence of iron reduced the intensity of the lipid-CH3 terminal chain group NMR peak, presumably via oxidation of the fatty acid chains (58).

Copper ion levels within the joint are proposed to relate to free radical generation and inflammatory disease. It is thought this is due to a chelation mechanism and saturation and defunctioning of the antioxidant species within the joint, making it more susceptible to the free radical destruction and depolymerisation. The copper ions also caused metabolic changes which were presumed to be by chelation effects, particularly with losses of detectable histidine and formate alongside reductions in detectable alanine and threonine. These complexes may contribute to the formation of free radical species (164).

Pentosidine is an oxidation product formed from the carbonyl sugar residue and amino protein residue. Its importance is unclear but elevated levels have been shown in situations of hyperglycaemia. It is believed to be linked to oxidative stress and to free radical formation. Elevated levels were seen in RA and correlated closely with the inflammatory markers CRP and ESR (165).

In summary, the inflamed joint probably has an environment abundant with oxidative free radical species. The likely targets for these species will be the complex glycoproteins and triglyceride chain fatty acids, resulting in chain shortening and cleavage of polymeric bonds. HA depolymerisation is well demonstrated (see 2.4.4).

To date, no research group has concluded that changes in triglycerides and HA in combination are due to the common susceptibility of action by free radical species, despite this phenomenon being observed in the serial synovial fluid sampling of two patients (145). This idea is supported by Schiller's demonstration that directly treating synovial fluid with HOCl caused an excess of the N-Ac breakdown alongside a strong increase of triglyceride CH_3 groups and the disappearance of CH_2 groups in the NMR spectra (60). Williamson et al. noted the change in the triglyceride CH_2/CH_3 ratio (indicative of a chain length shortening) to correlate significantly ($|r| = 0.76$) with the N-Ac levels (indicative of HA depolymerisation) and disease activity (126). Furthermore, a correlation was observed between the triglyceride and creatinine levels which would be consistent with a free radical inhibition of creatine kinase, something already shown in rabbits, and elevated creatine levels were detected in equine OA samples (111, 166).

2.15 Synovial fluid: hyaluronic acid (HA) metabolism

In vivo testing of HA breakdown products after exercise in the rheumatoid inflamed joint found the concentration of N-Ac and other breakdown products to increase, albeit transiently (145). Synovial fluid viscosity was directly reduced by the action of free radicals; the hypothesis is therefore that the viscoelastic properties of SF attributed to HA polymers are lost by free radical oxidation reaction (57).

2.16 Synovial fluid: metal iIon analysis

The presence of metal ions in SF has both an endogenous and exogenous source. The use of metal alloy joint replacements and their wear characteristics has heightened recent interest in metal ion deposition. Despite this, only one research group has examined the detection and effect of metal ions on the NMR spectra of HSF.

Silwood et al. used human SF as a matrix and supplemented it with various ion solutions, measuring the NMR spectra before and after supplementation. The addition of aluminium ions did not significantly affect the spectrum and there was no change in the fluid pH, suggesting a large buffering capacity. The authors noted some minor changes in chemical shifts of tyrosine, histidine and citrate. They concluded these changes were due to the formation of complexing species with the aluminium ions. In addition, spectra showed the presence of these complexes in their different charge forms and valences. The same study repeated using chromium (III) demonstrated alterations to the NMR spectra of threonine, alanine, glycine and citrate, with binding causing a broadening of peaks. Titanium (IV) was found to complex to Citrate with peak broadening similar to the action of chromium.

2.17 Synovial fluid: identification of metabolites from published synovial fluid NMR Spectra

Within our laboratory HSF had not been analysed before using NMR spectroscopy. It was important to gather the maximum NMR spectral information from the SF studies mentioned above. This aids characterisation of the spectra and identification of metabolites, alongside library reference databases

like the Human Metabolome Database (HMDB) (167). Published studies were scrutinised for metabolite identification and the results were collated (see Table 7).

2.18 Summary of synovial fluid studies

The switch to fat as a substrate is mirrored in other joint arthropathies. Investigation of synovial fluid in other joint conditions also found an anaerobic environment and the generation of lactate. Whether this anaerobic switch is linked to the production of free radicals is unclear. However, the effect of free radical generation upon the polymers of the joint is demonstrated and their presence is thought to catalyse a catabolism within the joint leading to loss of structure and further arthritis. Several metabolic investigations of the joint have highlighted a loss of the joint structure as described by a degradation of proteoglycans and an increase in *N*-Ac signal which is in accordance with this hypothesis.

To date, studies have concentrated on the degree of pathological destruction within the joint itself. Few studies have attempted to correlate to disease symptoms or the mode of disease presentation. This is a failing because clinicians know that the degree of pathological destruction can be independent of the symptoms. The reasons for this are not known. However, considering the metabolic disease links to OA which are reported to confer an elevation in symptoms or differing responses to treatment, I hypothesise that metabolism will be play a key role.

3 Chapter 3 - Metabolic phenotyping: the methods, limitations and standards to ensure success in studying joint disease

3.1 Introduction

In order to investigate the hypotheses, it is necessary to provide a complex metabolic description of the human joint. The methodology must be robust, reliable, reproducible, logistically simple and provide a level of quantitation. The methods form three principal components:

- the collection of HSF
- the metabolic analysis of HSF
- the analysis of the wear protection properties of HSF

Since synovial fluid is seldom studied, the description of methods relates broadly to those used in other biofluid studies. At the time of study there was no access to any ethically approved collection and tissue bank of HSF. Therefore, it was critical to gain ethical approval to establish the *Imperial College synovial fluid tissue bank subcollection.* Concurrently the *Imperial College Synovial Fluid Working Group* was formed to ensure legal, ethical and efficacious harvesting of HSF from patients, and maximise the research impact from this tissue bank (see 12.9).

3.2 General principles

Metabolic phenotyping generates and analyses complex molecular patterns to identify, represent and describe biological systems. To define formally:

- "Metabolic Profiling", "Metabonomics" or "Metabolomics" is the study of a biological system by quantitative and qualitative analysis of the small molecules present whether in the presence or absence of a disease state, genetic difference or environmental stimulus (168, 169).
- The "Metabonome" or "Metabolome" was never formally defined but is widely accepted to be the profile of small molecules observed within a biological system whether in the presence or absence of a disease state, genetic difference or environmental stimulus (170).

- The terms “metabolomics” and “metabolome” originally defined the small molecules in relation to a single cell. However, the terms are now used interchangeably (171).

Research into biological processes has evolved over the last twenty years to use systems which provide “data rich” analyses. The bioinformatic data obtained from a biosystem can provide information about the state of that biosystem at any timepoint. A study of the DNA genome (genomics) informs about the genotype but provides only partial information about the phenotype due to the influence of stimuli from electrical signals, chemotactic signals or the surrounding environment (see Figure 12). A study of the RNA (transcriptomics) gives information about gene expression and, to some extent, about external stimuli (172). The proteins (proteomics) can represent the current function or expressive state of the biosystem and thus represent one view of the phenotype (173). This last stage, metabonomics (cf metabolic phenotyping, metabolic profiling), studies the small molecules encompassing substrates, nutrients, waste products, signalling molecules and degradative products. This can provide information about the phenotype and specifically about metabolism, homeostasis, chemotaxis and environmental influences, including toxins (169).

The power of metabonomics and the other “omics” techniques lies in the complexity of data generated and the unprejudiced analysis.

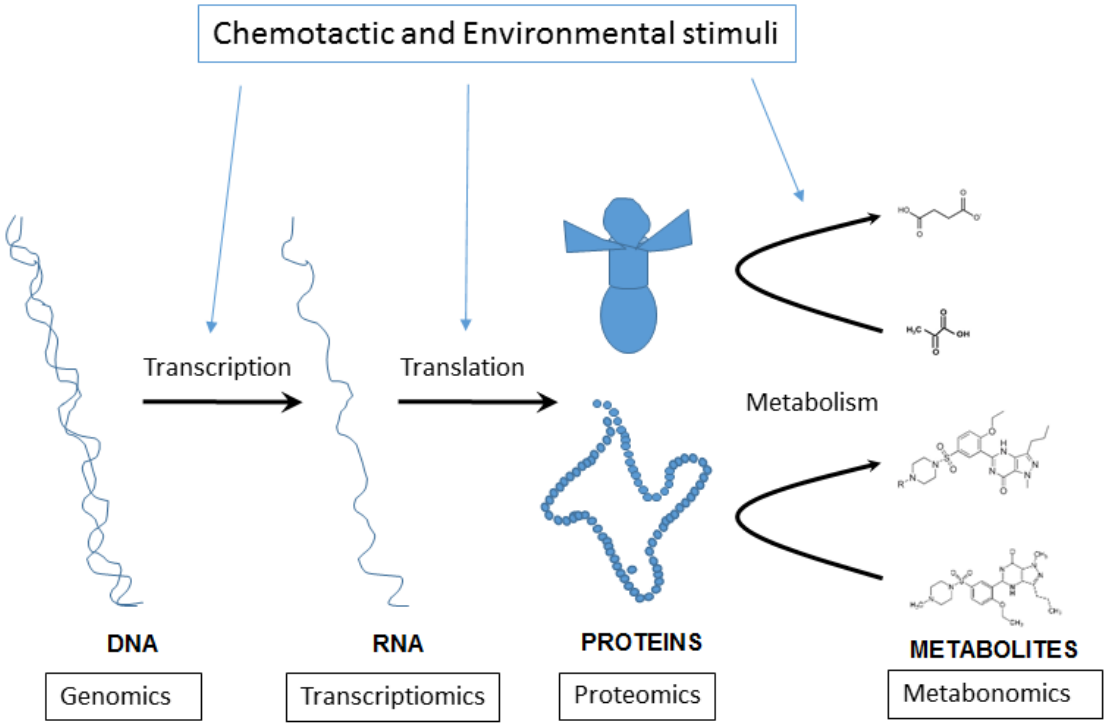


Figure 12 - Cellular expression and the "big data" methods for describing a biosystem

The response to a disease or treatment may depend on many factors including: immunosuppression, exercise, toxins, radiation, concurrent disease or diet. Studying metabolism provides a “top down” view of a biological system, with the advantage of representing the genetic disease traits but also the influence of diet and environmental pressures, thereby giving a more complete representation of the biosystem (169). The analysis of metabolites rather than genes or proteins is an attractive prospect as the numbers are smaller (typically <1000), compared to 30000 genes and up to 10 million proteins when considering alternative splicing and post translational modification.

The comprehensive representation of the phenotype means confounding factors of environment, diet and exercise can cause sample heterogeneity. In addition, there are multi-factorial influences on metabolite levels. It is rarely the presence or absence of a metabolite which is conclusive: more likely is a change in the pattern of several metabolites or even a metabolic ratio. Therefore, identifying these changes and demonstrating statistical validity is difficult.

Clinical metabolic phenotyping specifically studies the small molecules or metabolism of a clinical state or disease. Clinicians studying joint disease have typically focused on the genomic and proteomic disease descriptions, not the metabonomic.

There are many ways the metabonomic technique can provide information about a disease. Understanding how the metabolic homeostasis has been lost can provide information about the disease aetiology. A unique and specific identifier only present in the disease state or trait thereof may give rise to diagnostic biomarkers. A serial change in the metabolic homeostasis mirroring the progression of disease can demonstrate and measure disease staging (169). The prediction of metabolic phenotypes after the dosing by a pharmacological agent was originally defined as pharmaco-metabonomics (174, 175). The same phenomenon may be used in reverse to observe the metabolic disturbance and hence phenotype change in response to a pharmacological treatment; useful in the pharmaceutical industry and in drug development. In summary the technique can be used for: understanding disease mechanisms, disease staging, classification, diagnostics, prognostics, treatment monitoring, pharmacological response and the identification of novel biomarkers (169).

Despite the limitations, metabolic profiling (*id* metabolomics, metabonomics, metabolic phenotyping) is a resilient technique which has produced a wealth of useful studies in oncology, epidemiology, gastrointestinal disease, MeS, diabetes and cardiovascular disease (83-87, 176, 177). In particular the likelihood of yielding clinical translational value from metabolic profiling studies appears to be greater in diseases with a known or expected metabolic link. Traditional metrics to describe disease (i.e., symptoms, clinical signs, clinical imaging and phlebotomy) can be expanded, allowing the potential for better understanding, description, classification, monitoring, diagnosis and research. Metabolic biomarkers have influenced clinical practice in several disciplines: the IMPACT study (elevated blood pressure), risk of impending mortality and chronic kidney disease treatment (88-90). The technique is still quite new and is likely to have major positive impacts on clinical practice.

3.3 The stages of metabonomic investigation

Metabonomics can be split into distinct stages; often requiring specific locations and expertise (Figure 13). Metabonomics provides specific challenges in the study, design and interpretation of the data; published reports often lack some of these areas. There is a need for education about agreed minimum standards of methodology and reporting(178). These standards have been agreed by the Metabolomics Standards Initiative (MSI) and form the starting point but do not fully describe the minimum requirement for a clinical metabolic phenotyping study. The standards have been expanded and explained in Table 3. These steps form the model for the experimental analysis upon the tissue bank of HSF and should provide a guide to the reader of the ideal steps in establishing any such study.

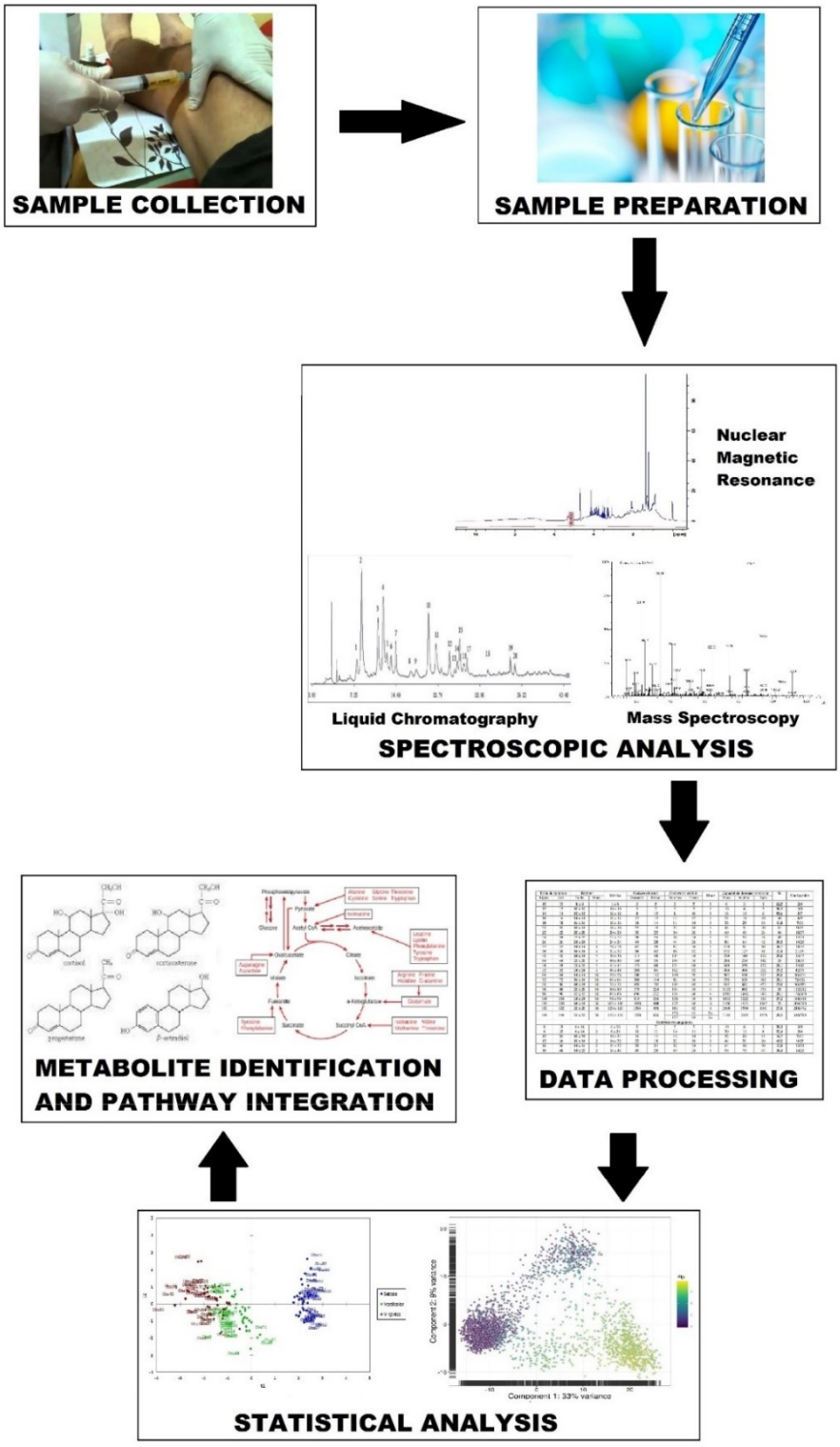


Figure 13 - Diagrammatic representation of the stepwise processes of metabolic profiling

Reporting standards		
Regulatory and Ethical Approval	Human studies	Should include details of the awarding body and the date.
	Animal models	Statement of approved licence and compliance with applicable governing guidelines
Study Design	Sample numbers	Should reflect the degree of confounding variables in the study. Pilot studies may require less numbers but describing effect by significance will require more patients
	Study type	The study approach e.g., Epidemiological/Case control/randomisation/clinical trial etc should accompany a statement of rationale.
	Controls	Controls should be precisely defined and how the clinical question is answered should be outlined.
	Inclusion/Exclusion Criteria	Should be clearly stated.
	Patient Demographics	As a minimum, age, gender, body mass index and diet should be recorded. Additional data to include: smoking status, current therapeutic treatments, co-morbidities and lifestyle.
	Sample matching	The metabolic assessment of disease requires matching to control for as many external influences as possible. Matching of the above "patient demographic" factors should be considered.
	Treatment group	The details of any treatment whether pharmacological or not should include: timings, dosage, duration and route
	Disease Classification	Patient disease should be classified as per an agreed, validated standard with diagnosis verified by at least two specialist clinicians in that field. The disease group(s) should be homogeneous – e.g., avoidance of sub-types – tumour stages, etc in one group.
	Sample type	Metabolism is tissue, body fluid and species specific. This needs to be taken into account for metabolite interpretation and the limited relevance of cross-species analysis should not be forgotten.
	Collection time-point	Collection time-points and particularly serial collection should be precisely stated in the context of disease evolution.
	Sample Amount	Volume or amount of tissue should be recorded.
	Metabolic influences	Any obvious metabolic limitations or known confounding factors should be assessed and reported.
	Sample collection	Where sample collection requires a medical or surgical intervention this should be stated as a potential source of collection bias. This is due to variation in personnel, equipment or technique. Any variation in time point of collection will also introduce bias

	Polypharmacy	All administered drugs should be recorded and considered as variables. Anaesthesia should not be overlooked when the samples are collected peri-operatively.
Analytical Methods	Sample Storage	The storage methods including temperature and timeframe to storage should be stated. Any prolonged storage should mandate demonstration of metabolic stability during storage or a statement thereof
	Sample preparation	Sample collection and preparation methodology should be validated or referenced as such. This should include a consideration of contaminants.
	Analytical quality control	A method to ensure analytical quality control and the consistency of analysis must be used. QC samples should be prepared to represent the samples (pooling is preferable). QC samples should be used to identify variation within a single batch analysis and also between analyses on different dates.
	Chemical supply	The manufacturer of all chemicals used in the preparation and analysis should be stated.
	Analytical instruments	Instrument methodology must be stated in full so that studies can be repeated. Batch analysis should be reported when used. Agreed standard operating procedures (SOP) should be used where possible.
Data transformation	Spectral Adjustment	Methods/software to apply any baseline, phase or mass correction in the spectra should be stated
	Spectral Calibration	Internal standard and the method used to calibrate should be stated
	Data alignment	Methods/software should be stated to align chromatographic/NMR data
	Data normalisation	The method of normalisation (if used) should be stated so that effects upon data quantitation can be understood.
Quantitation		The method used should be described. E.g., data binning, peak integration, peak line shape fitting.
Statistical Analysis	Cross validation	All linear regression models should report the quality and model fitting by virtue of the Q^2 and R^2 values
	Permutation testing (minimum)	A p-value should be stated
	Validation data set	The details of validation set should be outlined and compared to the original dataset
Metabolite identification	Database searching	The database used should be referenced
	Identification methods	Methods used for identification should be detailed.

	Identification rigour	The level of confidence reporting should be classed: 1. Identified (using reference standards) 2. Putatively annotated (without reference standards but based on chemical features and reference libraries) 3. Putatively characterised (compound classed according to similar chemical behaviour to known compounds) 4. Unknown (spectral data reported to allow differentiation)

Table 3 - Clinical metabolic phenotyping standards for a successful study, expanded from the metabolic standards initiative (MSI) reported by Sumner et al. (178)

3.4 Regulatory and ethical approval

Clinical metabolic phenotyping studies will, by definition, involve the handling and analysis of human tissue or biofluids. The regulatory steps required to ensure compliance are mandatory.

3.4.1 Research approval

The research approval to harvest human joint fluid from patients during the normal course of their treatment is required from the following regulatory bodies:

- Health Research Authority
- NHS R&D Offices

These applications require details of: full experimental design, scientific justification, personnel, timings, patient impact, recruitment, consent, confidentiality and probity of data, sponsorship and funding and insurance. These details were supplied using the "Integrated Research Application System" which collates the answers and submits the forms to the appropriate bodies (see 12.1, 12.3, 12.5) (179). Approval was given from both organisations and indemnity assured under the joint research partnership of Imperial College London and Imperial College NHS Trust (see 12.2, 12.5).

3.4.2 Ethical approval

In the UK ethical approval requires a submission to, and a hearing by a board of the NHS Research Ethics Committee. This application was also submitted via the "Integrated Research Application System" (see 12.6) (179). The case for the research, potential limitations and the impact upon patients

was debated in a forum. The outcome of this debate was the award of reference 15/LO/0388 for approval of research by the London – Chelsea Research Ethics Committee (see 12.7).

3.4.3 Tissue bank

The storage of human tissue or biofluids requires that all samples are stored in accordance with the Human Tissue Act 2004. Therefore, registrations were made for the samples to be stored and registered with the Imperial College Tissue Bank as a subcollection (see 12.8, 12.9). In accordance with legislation sample movements, storage locations, use and disposal were logged and traceable at all times (see 12.10).

3.4.4 Biosafety

In accordance with the Human Tissue Act 2004, samples must be collected, handled and stored as a biohazard. All risks to the researchers had to be appropriately assessed and minimised (See 12.10). The harvesting of HSF from unscreened patients has a containment level 2 (CL2) rating. Thus, samples must be transported in a closed and sealed vessel. All spillages must be treated with bleach products. All transfers of samples between vessels must take place within a negative pressure class II biosafety cabinet to prevent aerosol exposure. All samples must be incinerated to dispose. A standard operating procedure was required to ensure that all personnel handling the samples were appropriately trained, immunised, and adhered to all safety measures (see Appendices 12.12, 12.13, 12.14). These procedures were followed.

3.5 Imperial college synovial fluid working group

The collection of HSF was a new biofluid for the established tissue banks. There were several potential research targets for HSF outside our principal field of metabolic description in joint pathology. In light of the relationship of these disciplines and a common interest of understanding the influence of HSF composition and its subsequent behaviour, it was determined that maximal value would be returned from the samples by establishing a working group. This group would combine academics from a variety of disciplines and expertise to include genetics, metabonomics, clinical medicine and tribology. The group met on a quarterly basis to discuss hypotheses, critique each other's work, suggest new fields of

study, collaborate data and offer potential solutions to research barriers. It was apparent that the combination of data would provide additional insight into respective disciplines. The influences of these collaborations and meetings are present throughout this thesis.

3.6 Study design

3.6.1 Sample numbers

To ensure success a clinical hypothesis must be clearly defined with appropriately selected patient cohorts. Conversely, merely characterising populations to describe the disease types can allow a hypothesis to be generated before further evaluation. However, the studies may need to be large and on the epidemiological scale. Although metabonomics comprehensively represents the disease it is susceptible to confounding factors of diet, lifestyle, age, gender etc. Patient cohorts and controls must be matched to minimise the effect of any confounding factors. Therefore, once the recruited patients are separated into disease cohorts, they are matched to allow comparison resulting in a drastic reduction in the samples numbers for effective comparison. It is not possible to estimate the sample numbers using power calculations or number needed to treat formulae since the impact of metabolic disturbances upon the osteoarthritis is not known. Therefore, risk calculations cannot be performed prior to collection. A pilot group of samples may later provide data to allow a power calculation informing on required sample numbers for future analyses. Therefore, samples were collected from all patients irrespective of joint diagnosis, with the hope of comparing arthroses and studying concurrent metabolic diseases. The aim was a collection of 100 samples, but 152 patients were successfully recruited when laboratory work for this thesis was completed.

3.6.2 Study type

Given the aims of the study (see 1.2), long-term control of either the metabolic habits or the treatment regime of joint disease was not possible. Serial sampling of patients was unlikely due to ethical constraints, and metabolic representation of the joint disease would constitute a single timepoint, stage of disease, stage of treatment and pathological response. There was no intervention in the study and the data is purely observational. Therefore, the study was performed using an epidemiological approach

of sampling a population then evaluating which cohorts of disease are present in sufficient numbers to provide meaningful comparison.

3.6.3 Controls

Where control subjects or pathological samples are difficult to access, *in vitro* or animal models can lessen ethical considerations whilst controlling genetic variation, diet and the environment. However, metabolic interpretations may not be directly applicable to human metabolism or clinical environments. Therefore, a human study was preferred in this case.

Joint metabolism and the sampling of synovial fluid presents a particular challenge for control samples. Healthy joints contain synovial fluid in small volumes (<10ml in the human knee), making successful sampling unlikely. NMR analysis requires 700µl before losses. Disease cause excess volumes of synovial fluid making it easier to harvest from these patients. Therefore, an immediate bias exists since any joint where enough synovial fluid can be sampled by arthrocentesis or arthrotomy will be pathologically abnormal. At time of completing the analysis, HSF was not a commercially available biofluid. The harvest of HSF from disease free individuals with knowledge of their metabolic state for matching purposes would be the ideal control. However, this was not ethically or physically possible. Therefore, to evaluate the impact of traits and cohorts within the disease, comparisons must be made either between disease cohorts or between active joint diseases.

3.6.4 Inclusion / Exclusion criteria

Patients were recruited wherever arthrocentesis (synovial fluid aspirate) was required for clinical diagnosis or an arthrotomy (surgical opening of the joint) was performed during the course of treatment. Any patient known to harbour a communicable disease, be pregnant or under 16 years of age was excluded. Samples were collected from within the Trauma and Orthopaedic Department at Imperial College NHS Trust, a central London teaching hospital and major trauma centre.

3.6.5 Patient demographics and disease classification

The demographics of the patients were gathered to include diet, ethnicity, recent food intake, exercise and smoking. Medications were recorded so these could be identified within the analysis and excluded

where relevant. Medical history was included in the meta-analysis, and this was particularly relevant if metabolic diseases were present.

The symptoms, treatments, responses, macroscopic features and outcomes were sampled by questionnaire to attempt sub classing of the OA disease cohort (see 12.16).

3.6.6 Sample matching

The recording of patient demographics, comorbid disease, pharmacology and lifestyle factors was necessary to allow matching and to attempt control of confounding factors which could influence the metabolic profile.

3.6.7 Sample type

The selection of body fluid or tissue to analyse requires careful consideration (see 2.1). Physical barriers to synovial fluid sampling were overcome by sampling patients intra-operatively during arthrotomy (surgical opening of the joint capsule) or in arthrocentesis (needle insertion into the joint) when there was a clinical need to do so. Therefore, most patients could only be sampled once. On rare occasions where repeated joint surgeries were required, repeat samples were taken. The collection of intraoperative samples has the potential to influence metabolism. Patients are starved electively prior to anaesthetic. The medications and physiological stresses of anaesthesia will directly influence the metabolic pathways. The sampling of urine, blood plasma or serum may yield further metabolic information. However, this was deemed to be outside the scope of the current project due to the significant additional logistical challenges presented when sampling patients on the day of surgery. This was because patients rarely passed water on the morning of surgery due to restricted fluid intake and frequently urinated before arriving at hospital. Phlebotomy during canula insertion was possible but coordinating this with the anaesthetists and anaesthetic nurses was not successful. Furthermore, pharmacological contamination from anaesthetic agents was likely because sampling frequently occurred using the same line as anaesthetic infusion or was performed after inhaled agents were supplied to patients.

3.6.8 Sample collection & polypharmacy

Sample packs were designed to provide clinicians with all the necessary equipment and paperwork to consent, recruit and sample patients (see Figure 14, 12.13, 12.14, 12.15, 12.16). A standard operating procedure (SOP) was included to provide guidance and ensure samples were collected appropriately (see 12.13).

3.6.9 Contaminant control and system blanks

Sample pots, needles, syringes and Eppendorf tubes coming into contact with the samples were sourced at the start of the project. System blanks were analysed and run in parallel with all the samples to observe any contaminants that could be discounted at point of data interpretation. The same batch and supplier for each disposable item was used for the collection of all samples (see Table 4).

Disposable	**Manufacturer**	**Batch**
Microlance 18G needles	Becton Dickinson™	LOT 1412 05
30ml Universal sample container	Sterilin™	LOT 050528
20ml Plastipak syringe	Becton Dickinson™	LOT 1505285

Table 4 – The disposables used in the collection of HSF

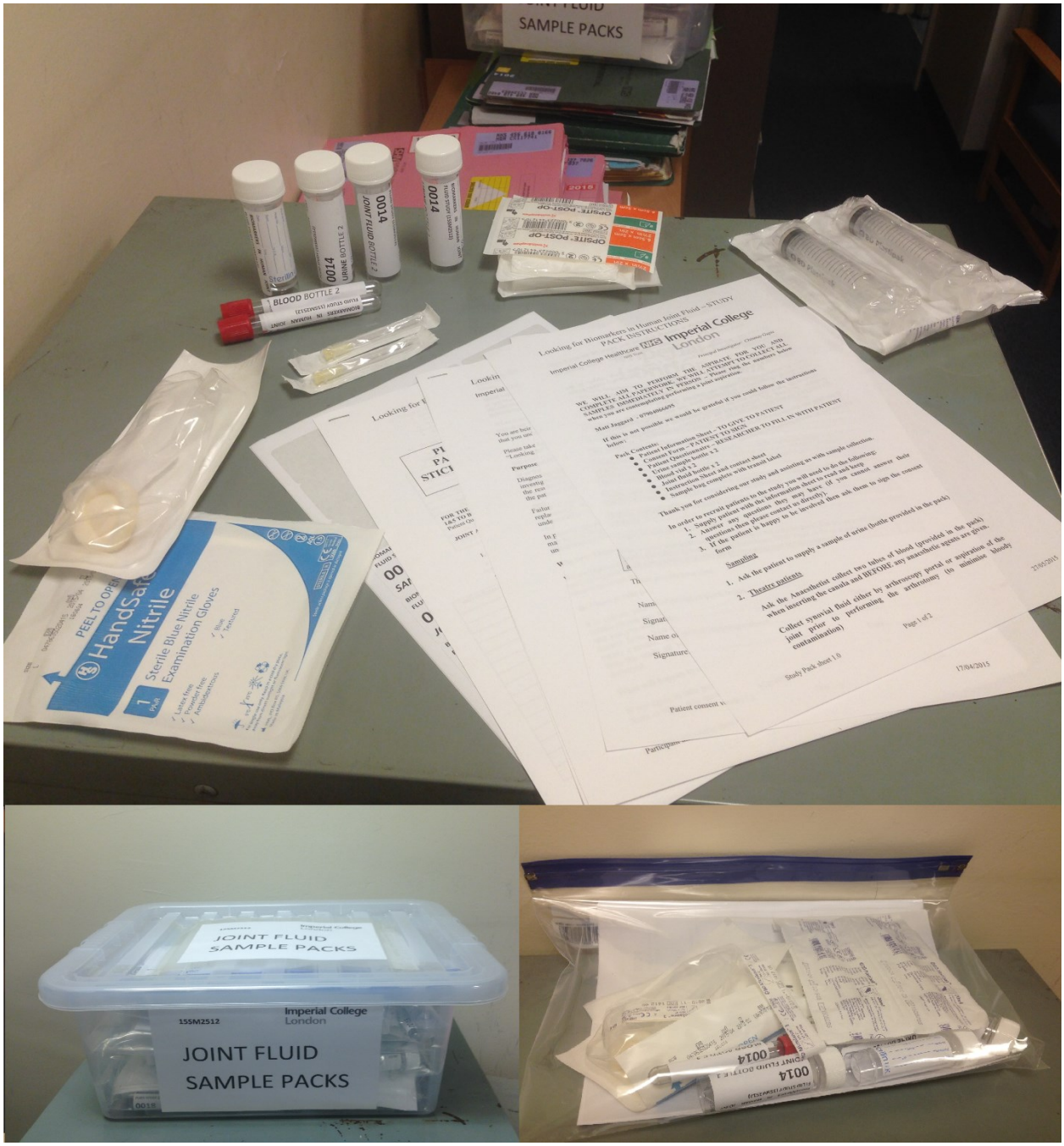


Figure 14 - Human joint fluid sample collection packs

3.7 Analytical methods

3.7.1 Sample storage

Sample collection must uniformly or not adversely alter the metabolism. It is important to halt or minimise metabolism after collection thus, providing the best representation of the disease state at the point of collection (180, 181). If not halted, metabolically active cells will exhaust the oxygen and

aerobic substrates. Thus, metabolically active cells should be removed by centrifugation or filtration, and metabolism slowed by reducing the temperature. Therefore, the time between sample collection, preparation and storage at -80ºC should be minimized. Ongoing anaerobic metabolism will be time and temperature dependant thus distorting the data. Clinicians and researchers were told to place samples at 4ºC immediately but there was an inevitable delay (<6 hours) before centrifugation and low temperature storage was achieved (see 12.13).

Variation introduced during collection and storage must be quantified and considered in the analysis (see Table 3). There is no validated method for HSF NMR metabolic profiling and therefore the impact of time and temperature from sample collection to storage upon the metabolic profile was unknown.

3.7.2 Sample preparation

Samples may need to be prepared to perform NMR spectroscopic analysis. Typically, the resolving power of NMR spectroscopy is such that the individual molecular components of complex biological samples can be distinguished. However, the background matrix of the sample may cause problems of detection or quantitation, either due to protein binding or a disturbance of the baseline. In circumstances where a particular class of metabolite is targeted or the sample type requires it, an extraction step may be used. HSF is known to be a highly proteinaceous biofluid (147).

3.7.3 Quality Control (QC) and Bioblanks

There was insufficient HSF to use a pooled sample as a bioblank:. Synovial fluid was not commercially available. Therefore, BCS was used as a bioblank since it has applications as an HSF surrogate model. It is used to mimic HSF in the biomechanical experimentation and validation of prosthetic joints. BCS is available in large batch stocks and is low cost. Preliminary analysis revealed that the NMR spectra showed HSF and BCS to share many metabolites, and the analytical methods were applicable to both biofluids (see 5.6). This meant that BCS was a logical choice for use as a QC sample. These samples were prepared alongside the HSF using the same methods and consumables. They were analysed at the start, end and every 10 samples within an analytical run. The resultant QC spectra were compared visually for reproducibility. Furthermore, a coefficient of variation was calculated using the integration

of a consistent peak within in the spectra of glucose. Therefore, the QC validation assessed any variation in both the sample preparation processes and the instrument analysis.

3.7.4 Chemical supply

All chemicals used in the preparation of samples were sourced from Sigma Aldrich UK™.

3.8 Instrument analysis

Few groups have studied synovial fluid by NMR and therefore there was little guidance to successful methodology (see 4.1.1). The background matrix of synovial fluid can vary greatly in the disease states, unlike urine or blood which remain relatively stable (14). As such, any analysis method needs to perform despite significant changes in water and protein composition.

Ideally, the analytical method would have:

- High resolution (or specificity*): the ability to see the metabolite despite the complexity of the biofluid background matrix.
- High sensitivity: the ability to detect the metabolite at low concentrations.

Analysis can be "targeted" where the analyte class e.g., lipids, is already known and a related hypothesis already exists. Alternatively, a comprehensive representation of the metabolic activity is "untargeted" analysis. The challenge of untargeted analysis is the detection and differentiation of many different and chemically diverse molecules in one assay, whilst minimising the analytical losses or failed detection. Thus, method development is a conflict between widespread detection, sensitivity, and specificity. The two most established analytical platforms used in metabolic profiling are MS and NMR spectroscopy (182, 183).

3.9 Nuclear magnetic resonance (NMR)

NMR provides a small molecule (<1kDa, or <1000Mw), analysis across a variety of metabolite classes. It is non-destructive and requires little sample preparation since even in complex biofluids the spectral resolution is highly specific. NMR analysis of intact biofluids is fast and thus cost-effective. It does not possess the sensitivity of MS but nevertheless can detect in the micromole range. It fails to

distinguish specific lipid molecules and certain glycoproteins, although spectra of biofluid extracts can achieve this. Despite these limitations it is a desirable untargeted detection method for the analysis of synovial fluid.

3.9.1 Nuclear spin

NMR is a technique which uses an intrinsic quantised property of certain positively charged atomic nuclei, designated spin, which generates a nuclear magnetic moment. The simplest nucleus with this property is that of the hydrogen atom with only one proton. The nuclear spin quantum number determines how many orientation states (and hence energy levels) the nucleus can occupy; the number of possible orientations is given by $2I+1$. Nuclei with $I > ½$ can have NMR spectra that are difficult to observe due to the complexity of the signal. Other nuclei with $I = ½$ are ^{13}C, ^{15}N and ^{31}P, all relevant in studying biochemistry, ^{1}H is most frequently studied because it is ubiquitous.

A nucleus with a spin quantum number of ½ is desirable because it can exist in two spin states ((2x½)+1); high ($I = -½$, *or* β) and low energy ($I = +½$, *or* α). In the absence of a magnetic field high spin states degenerate but as a greater magnetic field is applied, the energy between spin states (ΔE) increases proportionally (see Figure 15).

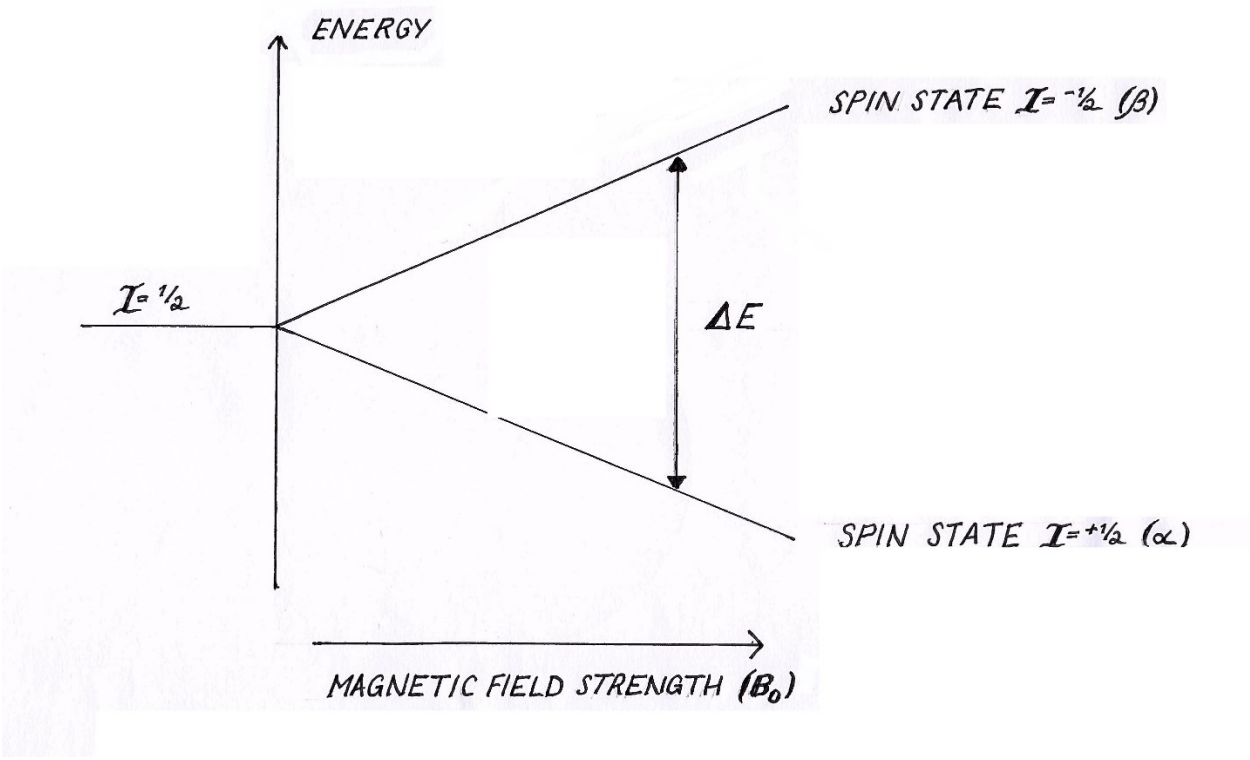


Figure 15 - The energy states for a nucleus with a spin quanta of I=½. B_0 = Magnetic field strength, α=low energy state, β=high energy state, ΔE=Energy to transition between states

The energy of transition is described by: $\Delta E = h\nu$,

where h = Plank's constant and ν = frequency. The frequency is further determined by:

$\nu = \frac{\gamma B_0}{2\pi}$, where B_0 = magnetic field strength and γ = gyromagnetic ratio of the nucleus

Therefore, the amount of energy required to shift between nuclear spin states will depend upon the frequency (ν), which in turn is dependent upon the magnetic field strength (B_0). The perfect combination of both yields resonance and a spectrum.

3.9.2 NMR nuclear behaviour

The sample is subjected to a longitudinal magnetic field *(B_0)* causing the net nuclei magnetisation (M_0) to align accordingly and a low energy state predominates, with the population difference given by the Boltzmann relationship (see Figure 16A). This difference is very small, of the order of 1 in 30,000 and this explains the relative insensitivity of the NMR phenomenon. A short radiofrequency *(RF)* magnetic

pulse across a band of frequencies is then applied via an input coil. The vector of this magnetisation is typically perpendicular to the longitudinal magnetic field *(B_0)* and in a new orientation *(B_1),* (see Figure 16B). **The frequency *(v)* of the pulse must match the resonant frequency of the nucleus, known as the Larmor frequency *(ω_0)*.** Only then is the net nuclei magnetisation (M_0) shifted away from the longitudinal magnetic field *(B_0)* to a new vector *(M_1)*, causing the nucleus to exhibit "resonance".

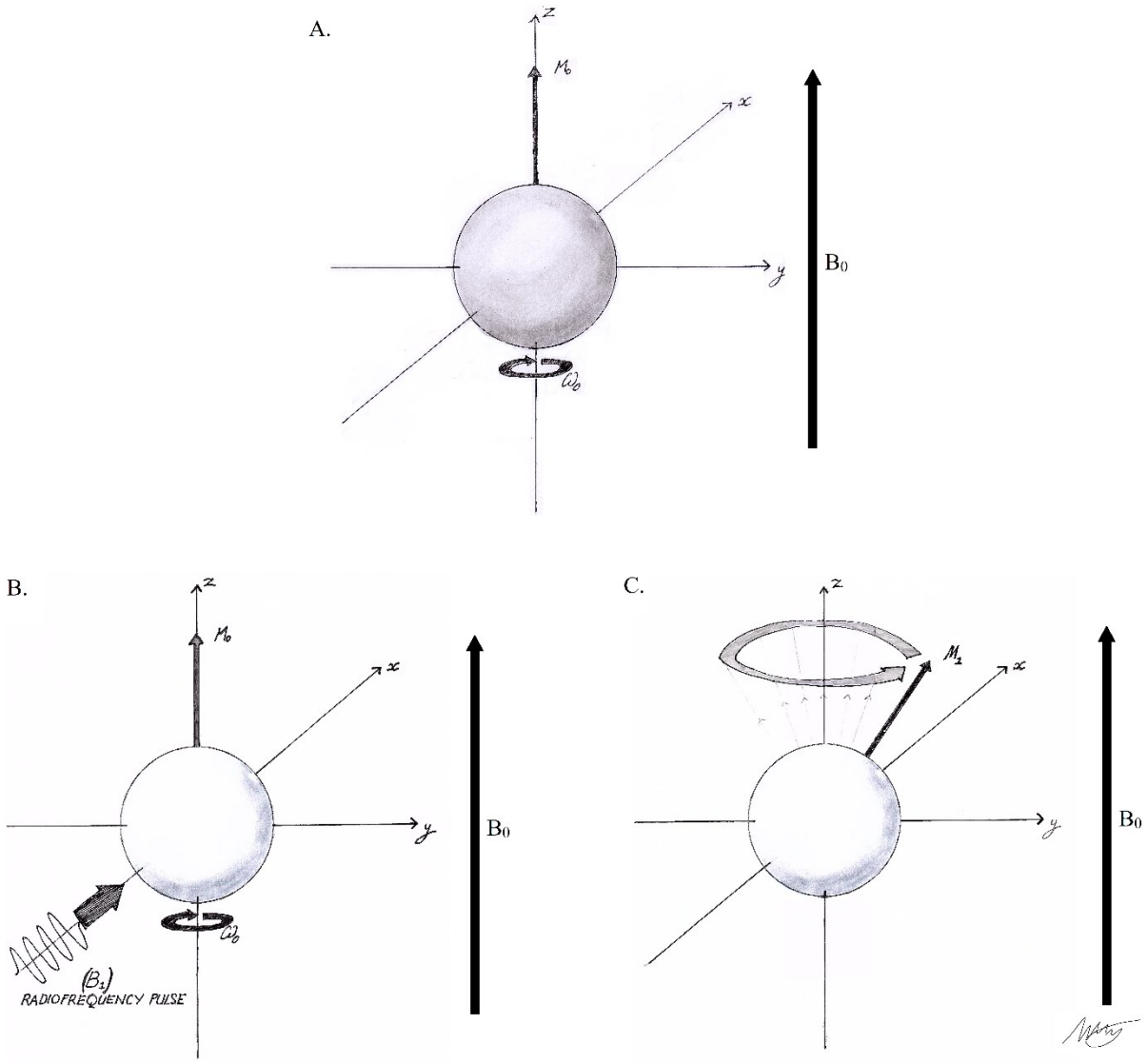


Figure 16 - Kinetic behaviour of a nucleus as it undergoes resonance due to an NMR pulse.

A illustrates the application of the longitudinal magnetic field ***(B₀)*** causing the net nuclear magnetisation ***(M₀)*** to align and the frequency of spin ***(ω₀)*** around this axis. **B** demonstrates the application of the radiofrequency pulse ***(RF)*** and a new magnetic force ***(B₁)*** causing state **C** where the net nuclear magnetisation vector has deviated to a high energy state position ***(M₁).*** As the RF pulse ceases the nucleus then relaxes with the net nuclear magnetisation vector precessing from ***M₁*** back to ***M₀*** in the motion indicated.

Once the pulse ceases, the nuclei spin but exhibit Larmor precession causing the net nuclei magnetisation *(M_1)* to move from its excited position progressively back towards the longitudinal magnetic field *(B_0)* and its previous position *(M_0),* (see Figure 16*C*). The motion of precession is such that the spin angular momentum of the particle will move around the longitudinal magnetic field in ever decreasing circles before coming to rest in the equilibrium state. While the nuclei are precessing,

pulsatile energy is released due to transition from a high energy to a low energy state. This energy is released in the form of a voltage, measured by an output radiofrequency coil. The intensity of signal will be proportional to the total number of nuclei. There are two rates that can be made from the nucleus coming to the equilibrium state, or in this case approaching the longitudinal magnetic field *(B_0)*. The half-life time required for the nuclei to achieve the equilibrium position after the RF pulse in the z-plane is called the "T_1, spin-lattice or longitudinal relaxation time". Conversely measuring the half-life of signal decay in the x-y plane gives the "T_2, spin-spin or transverse relaxation time". The oscillating time-dependent RF voltage that is picked up in a receiver coil as the nuclear magnetisation decays is termed the free induction decay (FID). FIDs can be added together to increase the signal-to-noise ratio.

3.9.3 Generating an NMR spectrum

The measured output voltage contains the sum of signals across a bandwidth of frequencies. A Fourier transformation is required to generate the spectrum consisting of signal intensity plotted against frequency (see Figure 22), (184). Each peak corresponds to a population of nuclei which are undergoing resonance at that specific frequency. The number of nuclei will be proportional to the peak area.

3.9.4 Electron shielding and the chemical shift.

The magnetic field seen by the nucleus may differ due to the surrounding chemical environment caused by the effect of nearby electrons. Electrons in motion around nuclei also have a magnetic moment which modifies the resonance frequency.

The changes in frequency due to electron shielding are small in absolute quantity. The frequency recorded will also vary depending on the strength of the instrument magnet and its precise resonance frequency. Therefore, to provide standardisation the radiofrequency scale or "chemical shift" (δ) is used. It is a relative scale and has the advantage of better representing the small absolute changes in frequency seen from electron shielding.

It is calculated as follows:

$\delta = \frac{v_{sample} - v_{ref}}{v_{spec}}$, where δ = chemical shift, v_{sample} = nuclear resonance frequency of sample, v_{ref} = nuclear resonance frequency of reference material, v_{spec} = frequency of mass spectrometer

As such it has no units and is expressed in parts per million (ppm). Since chemical shift is a relative scale with no units it requires an internal reference to provide a frequency standard point. The observed chemical shifts of protons in an NMR spectrum can provide information about different chemical moieties in a molecule (e.g., methyl groups, aromatic protons, aldehydes, etc).

3.9.5 Spin-spin coupling

Spin-spin coupling causes minor shifts in the resonance frequency of a nucleus due to the magnetic interactions from adjacent nuclei mediated via the electrons in intervening chemical bonds. The two-spin state of an adjacent nucleus causes a splitting of the energy levels of a given nucleus and this leads to a splitting of the usual NMR peak into multiplets. The magnitude of the splitting is termed a scalar coupling constant, termed J coupling (measured in Hz, and unlike the chemical shift its size is independent of the magnetic field). In proton NMR, a single adjacent nucleus will cause a splitting of the peak into a doublet of equal size due to half the population being in the I=+½ and half being in the I=-½ spin state. If two adjacent nuclei are involved, a triplet peak in the proportions of 1:2:1 is formed. The first peak represents both adjacent nuclei being in the I=-½ state, and the last peak is both nuclei in the I=+½ state. The middle peak does not shift due to a net effect of zero from one nucleus in the I=+½ state, and the other I=-½. However, since there are two combinations in which this can occur, the peak is double the area of the neighbouring split peaks. Expansion to more complex influences (transmissible up to 3 bonds away) reveals a pattern of peaks which follows Pascal's triangle. This occurs in so-called first order situations where all chemical shifts are large compared to the J values and

there is no symmetry to the spin system. The size of J couplings is affected by the type of bond, angle of bond and the nuclear properties of the coupled nuclei.

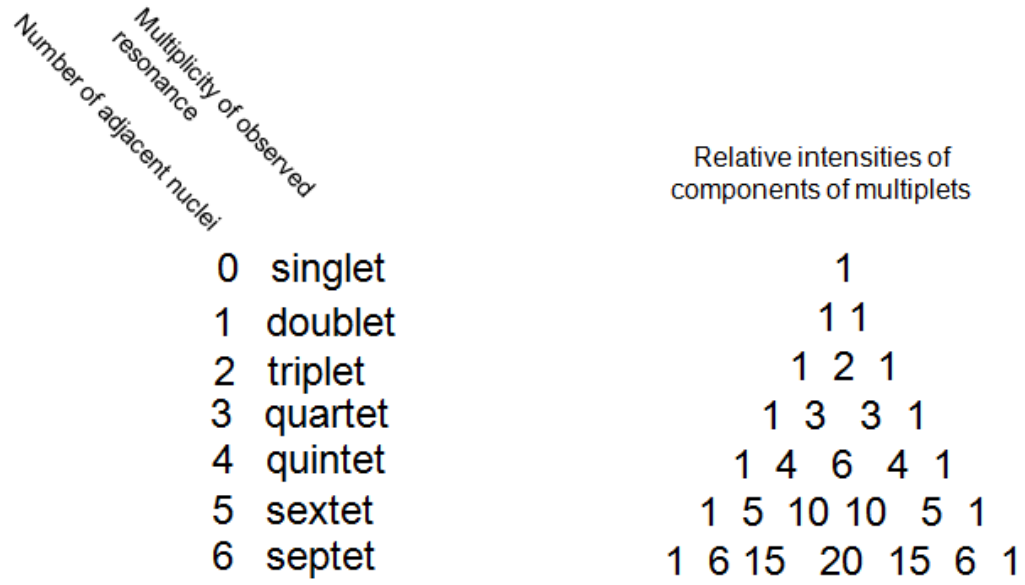


Figure 17 - NMR peak pattern seen with adjacent nuclei coupling seen with ^{1}H-NMR (or spin state I=½)

3.9.6 NMR practical considerations

NMR generates semi quantitative data, and the intensity of signal is instrument specific. The signal can have differing effects from coupled nuclei, relaxation times and spectrum optimisation which determines the peak shape. For quantitative applications, specific methodologies are usually recommended. Alternatively, calibration standards are required.

In order for nuclei to provide sharp resonances and hence to yield good spectral resolution of different species, they must be mobile. This requires the metabolites to be in solution. Therefore, a solvent is required which will exhibit its own resonance. The resultant signal will be large and undesirable. Instrument optimisation may supress the solvent peak, but it cannot be removed without deleting a section of the spectrum.

Many molecules can undergo protonation-deprotonation (acids, bases) and because the two forms are different, they will have different chemical shifts. However, they are in fast exchange on the NMR timescale and only an average set of chemical shifts is produced. Hence any variations in sample pH will shift the equilibrium and cause a variance in the chemical shift. In order to allow comparability of

chemical shift, the use of buffers is necessary. Any experimental error in the sample pH will result in difficulty comparing samples and interpreting the chemical shifts.

3.9.7 Pulse sequences

NMR experiments can yield different information about the nuclei by varying the number, timing and duration of the pulses. These are usually termed pulse sequences.

3.9.7.1 1D NMR spectroscopy

There were two main experiments used in the interpretation of the biological samples: Nuclear Overhauser Effect Spectroscopy (NOESY) and Carr-Purcell-Meiboom-Gill (CPMG). The simplest 1D-NMR experiment has the following key components:

1. A preparation, or relaxation delay phase in which the nuclei are allowed to reach their equilibrium state (see Figure 16A). In biofluids if the solvent (e.g., water) peak has to be suppressed this is often done here by an extra irradiation.
2. A radiofrequency pulse to reorientate nuclei into a higher energy spin state (see Figure 16B). If this tips the magnetisation exactly into the xy plane, this is called a 90° pulse and equalises the population to the top spin energy levels.
3. An acquisition period where acquisition of the FID signal occurs (see Figure 16C)

3.9.7.1.1 Nuclear Overhauser Effect Spectroscopy (NOESY)

In biofluids, a pulse sequence called NOESY-presat is often used as this provides better water peak suppression than simple experiment as described above. The pulse sequence provides an overall picture of all the proton-containing molecules in the sample with small molecules well resolved but macromolecules are poorly resolved and may compromise detectability of other small molecules

3.9.7.1.2 Carr-Purcell-Meiboom-Gill (CPMG)

The CPMG sequence is based on a phenomenon known as a spin-echo. This refocuses the magnetisation and detects it in such a way that components with fast T2 signals are suppressed. It

consists of a 90-degree pulse before a delay, 180-degree pulse and further delay repeated many times in a loop. The advantage of this technique is thus the suppression of macromolecular signals allowing enhancement and visualisation of the small molecules.

3.9.7.2 2D NMR spectroscopy

The advantage of the 2D-NMR experiments is several-fold when considering biological samples. The complexity of the sample means a further dimension of investigation provides additional resolution between peaks where overlap may occur. The added dimension also yields information upon the relation between peaks structurally, particularly if the peak originates from the same molecule.

There are many types of 2-D experiment and those mostly commonly used in biofluid analysis are summarised here. All comprise multiple pulses and delays often with applied magnetic field gradients as well. The sequences include an incremented time interval which together with the acquisition time provides two times for a 2-dimensional Fourier transformation.

Correlation spectroscopy (COSY) observes the spin-spin couplings between ^{1}H nuclei. Therefore, it is possible to distinguish peaks which originate from close proximity in the same molecule. The experiment consists of a relaxation delay before a 90-degree RF pulse and then an evolution time where spin relaxation occurs. A second 90-degree pulse is then applied at a fixed timepoint before acquisition of the signal. The separation of the differential effect from a second pulse is observed by varying the time between the two 90-degree pulses and repeating the experiment.

J-resolved spectroscopy uses the pulses to split the effect of J-coupling and peak chemical shifts into orthogonal dimensions. The application of the second dimension allows separation of the 1D-NMR spectrum signal into individual protons which may be responsible for that peak. Observations of the peak patterns that occur (doublet, triplet etc) can then be used to identify if peaks are related to J-coupling or originating from a separate molecule or species (185).

Heteronuclear single quantum coherence (HSQC) experiments measure the ^{1}H signal related to the adjacent bonded ^{13}C isotope. This is recorded in the first dimension. The second dimension records

the chemical shift of the ^{13}C. This experiment provides useful information to determine chemical structure (186).

3.9.8 Data transformation

Previously, univariate statistical methods would concentrate on specific metabolites. Multi-variate techniques allow the collective metabolic patterns to be studied holistically, rather than any one metabolite in isolation.

3.9.8.1 Spectral adjustment

Spectra must be transformed into data sets, considering every datum point in the spectrum as a variable during statistical analysis; a typical NMR spectrum might contain 32k data points (1k = 1024). The solvent peaks are removed (usually water) as these can cause distortion of the statistical modelling.

3.9.8.2 Data alignment

NMR spectra from different samples have to be aligned computationally using an in-house algorithm to correct for variation in peak positions due to slight pH differences, despite the use of a sample buffer. This ensures that the same region of each spectrum is compared.

3.9.8.3 Data normalisation

Variation in biofluid concentration due to dehydration means normalisation of the signal intensity is often required, particularly in urine studies. Two methods of normalisation were used in this study. Total area normalisation was used in experiments when a consistent HSF sample was analysed in a repeated manner. Median full change normalisation was used in cohort analysis due to multiple broad peaks from macromolecules presenting in HSF and making total area normalisation unsuitable.

3.9.9 Quantitation

Samples were quantified using peak integration. An isolated and well resolved peak was selected, corresponding to the metabolite of interest. Relative quantification of metabolites was achieved by non-

linear baseline adjustment followed by integration of identified and well resolved peaks using the trapezoid method.

3.10 Statistical analysis

3.10.1 Multivariate linear regression

The complex dataset must be simplified and reduced to detect the most significant variables. Two main approaches are used.

3.10.2 Principal component analysis

The first considers which data variables make the largest contribution to the total variance of the dataset (187). In this case, Principal Component Analysis (PCA) is the usual starting point to describe the dataset. PCA is an "unsupervised" method since no information about the data matrix is required in advance of the calculation. To understand the construction of a PCA model, it is important to consider each variable as an axis. Therefore, the combination of variables provides a multi-dimensional (e.g., 32k axes) representation of the data (Figure 18). Every sample then has a specific coordinate within the multi-dimensional space by virtue of the unique combination of variables. The PCA model produces a linear regression line through the multi-axial space which explains as much variance within the dataset as possible. Known as the "first principal component", the first regression line represents the variables which are contributing the largest variance in the dataset (Figure 18). The "second principal component" is a regression line which is orthogonal (or perpendicular) to the first, but incorporates as much variance not explained by the first. There are as many principal components as data points but usually only the first few describe the variation in the data and the rest merely describe the noise. These component regression lines simplify the data by disproportionately representing the metabolites contributing the most to the variance. Plotting the samples against these new component axes as a scatter graph forms the basis of the PCA scores plot (Figure 19). Samples will coalesce or separate depending upon their position on the component axes. A PCA loadings plot indicates which parts of the spectrum provide the largest contribution to the principal components, thus highlighting the

metabolic differences amongst the samples. Outlying samples can be identified, investigated and, when appropriate (e.g. an instrument error), removed from the model (187).

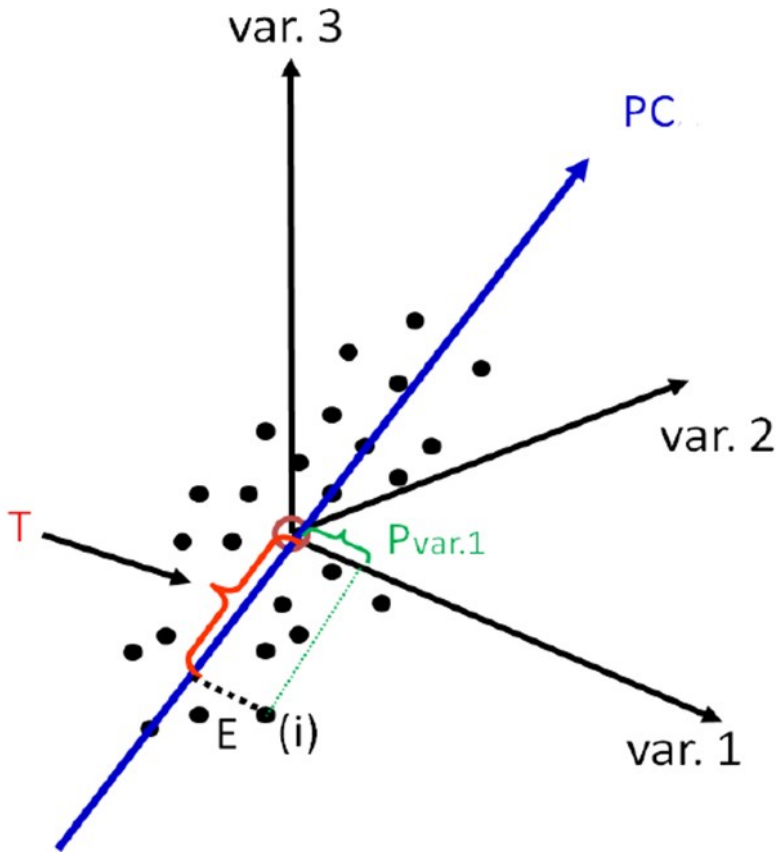


Figure 18 - Illustration of the technique used to construct the PCA plot with 3 variables, for simplicity. Each variable (var=variable) is represented by an axis. The principal component (PC) is a regression line through the dataset, explaining the variance of all three variables within the samples as best as possible. Samples represented by dots are then considered relative the new simplified axis of the principal component: In the case of sample (i), (T) is the representation according to the principle component and would be the value plotted on one axis of the PCA plot (188)

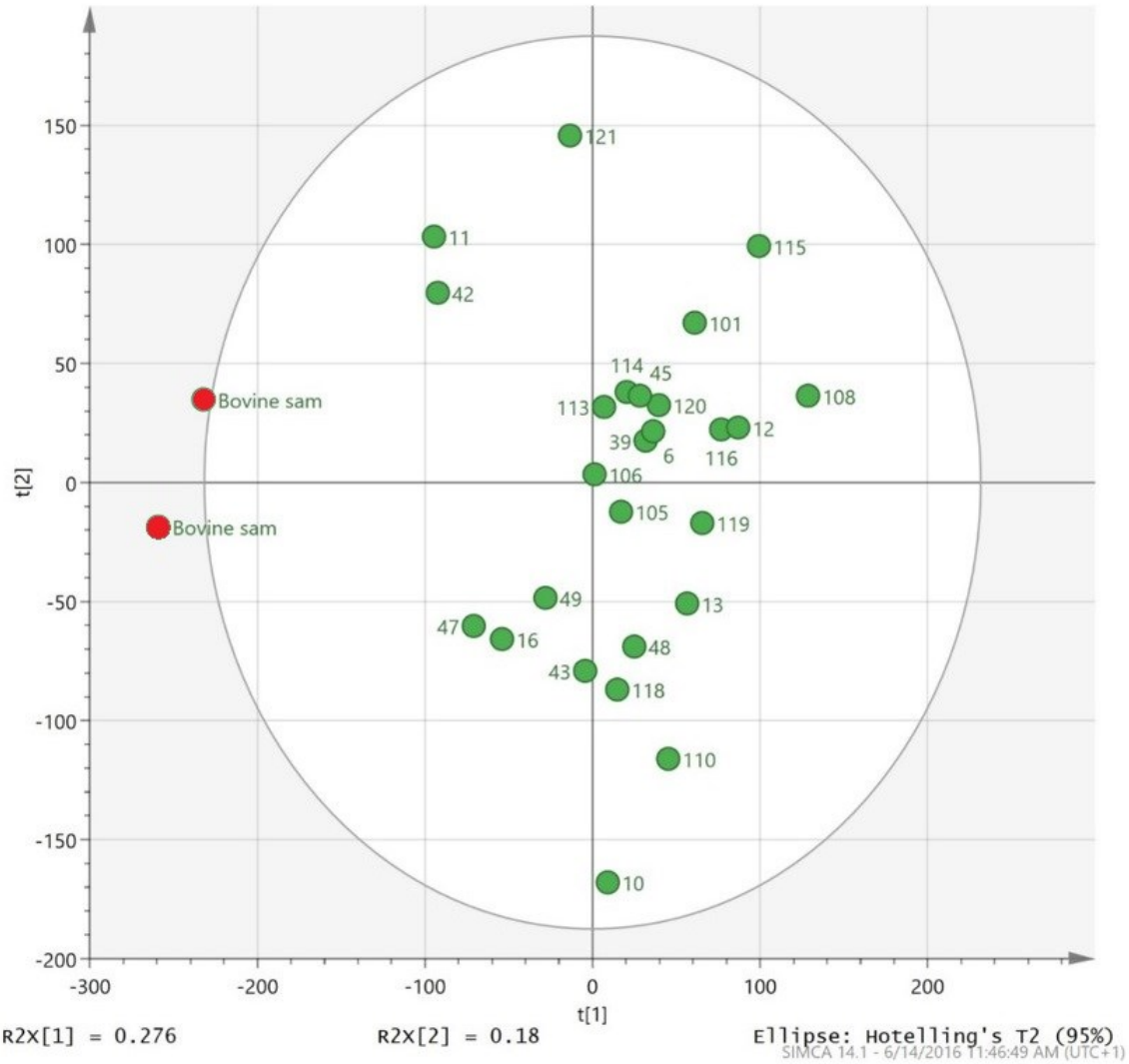


Figure 19 - An example of a PCA plot using the first and second component to represent a dataset of HSF samples. Samples from a different species (bovine - red) have been included to illustrate the identification of outliers (visible outside the T2 Hostelling representing the interval of confidence).

3.10.3 Partial least squares discriminant analysis

The metabolite variation caused by the disease is likely to be small and not part of the largest variance, which often originates from confounding factors. In this case, the next stage is the application of a "supervised" method allowing sample classification whilst control, disease or phenotype traits are incorporated before calculating the model. An example would be Partial Least Squares Discriminant Analysis (PLS-DA) which also uses linear regression modelling of component axes to represent the variables. However, the model attempts to identify the variance and thus metabolic difference between the pre-defined classes, e.g. disease and disease-free (187).

Orthogonal partial least squares (OPLS) analysis is an extension to the method in which any variance which is uncorrelated to the disease trait (i.e., orthogonal to it) is removed. This can enhance the visualisation of the metabolic differences between disease traits (189).

3.10.4 Cross validation

The validity of the multi-variate model must be evaluated because pre-defining classes in clinical scenarios can cause model over-fitting and generate false positives. There are several metrics to assess the validity of the PLS-DA model. Cross-validation statistics (such as Q^2Y) are calculated by randomly removing a section of the data before recalculating the PLS model. This is repeated until every sample has been removed at least once. The models with data removed are compared against the original model. The error between the real and the predicted score determines the Q^2Y. A positive Q^2Y, close to 1.0 means a highly predictive model. A low or negative Q^2Y means the model is not predictive and should be disregarded (190). This approach is the absolute minimum for model validation and further methods are strongly advised.

The R^2X value indicates how well the overall variance within the data (X) is represented by the regression model. When pre-defining sample classes e.g., in a PLS-DA model, the variance within the classes (Y) which is incorporated into the model is reported by the R^2Y. Therefore, all models should quote an R^2X and those with pre-defined classes should additionally quote an R^2Y value (190). A value closer to 1.0 means a stronger regression model.

3.10.5 Permutation testing

Inferring a significant difference from a model should report a p-value, calculated by correcting for false positive rates and supported by a permutation test. This randomly re-labels individual samples within the model, re-evaluates the model several times generating a series of Q^2Y values. This series of

Q^2Y values are compared to the original Q^2Y to validate the model if it is significantly different ($p<0.05$) (190).

As with any population sampling, the sample size needs to be sufficient to demonstrate a significant difference between disease classes. The overall variance can be used in a power calculation to determine how many samples are required.

Once generated, it is necessary to test the model. The receiver operating characteristic (ROC) curve is widely used when discrimination between the sample classes is seen. This effectively describes the diagnostic testing ability of the statistical model, and its useful application.

It can be desirable to revisit the data with univariate statistics to complement the models.

3.10.6 Validation data set

The most rigorous method to test a class-defining multivariate statistical model is to apply the model to a completely new set of data, known as a "validation data set", this begins to address the problem of confounding variables.

3.11 Metabolite identification

A significant but unknown metabolite may be detected. To interpret biological significance, the metabolite must be identified. Although many peaks in an NMR spectrum of a biofluid have been annotated in the literature, for unknown species, this process is not straightforward, relying upon further spectroscopic analysis, library searching and confirmation.

The information gained from NMR spectroscopy is used to help identify unknown compounds. The chemical shift value and the multiplicity of the peak informs on the chemical structure. Specific NMR methods use specially designed radiofrequency and magnetic field gradient pulse sequences that manipulate the magnetic interactions between the nuclei in the same molecule. This allows the identification of peaks originating from the same metabolite by virtue of their nuclei being coupled. Usually this is visualised as a two-dimensional (2D) NMR spectrum and the most common application

is Correlated Spectroscopy (COSY) as described above. The combined information of peak identity as well as association with other peaks from the same molecule helps metabolite identification (191).

3.11.1 Database searching

The cooperation of various research centres around the world has led to the development of metabolome databases. The Human Metabolome Project in Canada led to the HMDB containing spectroscopic data on a variety of body fluids, diseases and specific metabolites (192). The Metabolome Workbench is a National Institute for Health (NIH) collaboration to expand and improve the quality of metabolic references, data collaboration and promote metabolic profiling (193). The European Bioinformatics Institute has established "MetaboLights", a central database for metabolic profiling experiments (194). The centralisation of this data has greatly accelerated metabolite identification by expanding access to metabolite reference standards and sharing data to improve the efficacy of studies.

Two principal databases were used in the identification of metabolites in this study: HMDB for the HSF and the Livestock metabolome database when assessing BCS.

3.11.2 Identification methods & rigor

The most rigorous confirmation of metabolite identity is the spiking of a sample with a reference stock of the suspected metabolite and then measuring the spectra of the sample again under the same conditions. A quoted Level of Assignment is recommended to state confidence of identification (178).

In this study metabolites were identified with a confidence level of 2 (see Table 3)

3.12 Metabolite interpretation

Core metabolites will be influenced by the species, disease or environment being studied. However, the multiple homeostatic influences and converging pathways may cause challenges when interpreting significance. The metabolic profile of a biological system can be used in several ways. Key metabolites must be interpreted alongside what is known about disease aetiology and cellular metabolism.

Metabolite interpretation can go beyond simple concentration and relating to disease, with sampling multiple time-points giving rise to metabolite trajectories. In addition, studies using stable isotope

labels such as ^{2}H (deuterium) or ^{13}C can track changes in cellular metabolic pathways by identifying the metabolism pathway taken by that isotope.

A metabolite can be interpreted in the context of the cellular metabolic map, relating to disease aetiology and postulating a mechanism. One of the most comprehensive information pathways is the KEGG (195). This map is an interactive network of global metabolism for genes, proteins and their products. Software tools such as Metabonetworks use this information to map metabolites of interest (or biomarkers) onto the potentially disease affected metabolic pathways (156). These methods were used in the analysis of significant OA metabolites (see 2.17).

3.13 Limitations

3.13.1 Confounding factors

Like any clinical study, the influence of confounding factors: lifestyles, diet and exercise are the largest limitation. In addition, modern polypharmacy introduces metabolites and metabolism-altering drugs. These are the principal obstacles which the technique attempts to overcome by using statistics. Many previous studies have not recognised the heterogeneity in sample classes, with poor data matching between classes (e.g., by gender, BMI, ethnicity, age, smoking status, medications, etc.) and confounding variables associated with disease. Physical activity is a prime example with significant effects on metabolism which are likely to decline with any disease diagnosis, when comparing a patient with an aged-match control. Therefore, ensuring that comparative datasets are appropriately matched to eliminate these potentially confounding factors is important for study rigor and was attempted here where possible.

3.13.2 Linear association of variables

Most multi-variate statistical models use linear regression and thus any variable which has a non-linear association may not be identified; something frequently overlooked by investigators. The models are powerful, but the reporting of statistical significance is frequently over exaggerated. The PLS-DA models will generate plots showing good separation of the sample classes due to over-fitting. Subsequently, many models are not properly validated. Hence users in clinical practice tend to

misunderstand and inappropriately interpret the validity of the models. This is usually due to the failure of authors to adjust for false positives by virtue of the large number of variables or inappropriate controls.

3.13.3 Considerations for further study

The detection of metabolites depends upon technological advances, the ability to separate compounds and instrument detection limits. Any single technique will have its limits; hence it is desirable to use more than one method, combining strengths and compensating for weakness to achieve a more comprehensive metabolic analysis. In this study, constraints were the access to instrumentation and the funding available. It is partly for this reason that NMR was chosen, being cost effective and non-targeted, likely to give a broad detection of metabolite class.

Pooling data from multiple studies and centres can overcome many of the limitations and add power by numbers to a study. This can frequently occur via international collaborations. However, pooling data introduces variability from sample collection, sample preparation, sample storage, instrument detection and instrument techniques. Therefore, this is only possible when data are robustly and directly comparable between laboratories and centres. Whilst NMR studies are reproducible, variability can be seen within LCMS analyses (196-198). Therefore, this should be considered if data sharing is possible in the future.

3.14 The evaluation of lubrication upon prosthetic joint wear

3.14.1 Implant validation

Testing the performance of the implant and bearing surfaces relies upon the use of joint simulators, the implant design and the resilience of the bearing design to repetitive loads. Typically the implant will undergo a test of 5 million cycles (199, 200). The forces applied attempt to replicate the forces and joint kinematics observed during a typical gait cycle (201). The simulator gives the possibility of implant movement within the test around six degrees of freedom. The lubricant consists of 25% w/w BCS which is changed every 100000 cycles during the test.

The wear of the bearing components is measured by weight before and after the test. The consistency of the test is known to be poor and therefore conditions such as temperature are standardised as much as possible.

The advantages of the joint simulator are the versatility in testing variations of implant material and implant design. The simulator has the capacity to quickly and cheaply generate comparable wear data. Every attempt is made to utilise the mechanics of the simulator to mimic the physiological joint motion and forces. However, the in vivo joint environment remains overlooked and poorly modelled.

3.14.2 High frequency reciprocating rig (HFRR)

Bench-top testing confers the advantage of measuring wear directly whilst providing an opportunity to study small volumes of lubricant with varying compositions. The interaction between bearing surfaces and lubricants and the impact of the lubricant chemistry is lost when the fluid is constantly recycled (82). This bench-top test uses an HFRR (PCS Instruments London), and its application to HSF was developed by the Tribology Section and the Department of Mechanical Engineering, Imperial College London. That work was completed under the remit of the Imperial College Synovial Fluid Working Group and demonstrated that the fluids behave inconsistently in bench-top wear testing (202).

A bench device is required to evaluate HSF lubrication since a joint simulator requires larger volumes (202). The bench test slides a 10mm ball upon a 19.05mm contact disc. Both bearing surfaces are

composed of Cobalt Chrome (CoCrMo). The upper ball oscillates at a determined frequency and load, pressing on the static lower contact disc (see Figure 20). The test bath contains the lubricant specimen and is maintained at 37°C using a thermocouple. The atmosphere of the test is purged with N_2 for 20 minutes to reduce the potential for free radical oxidation.

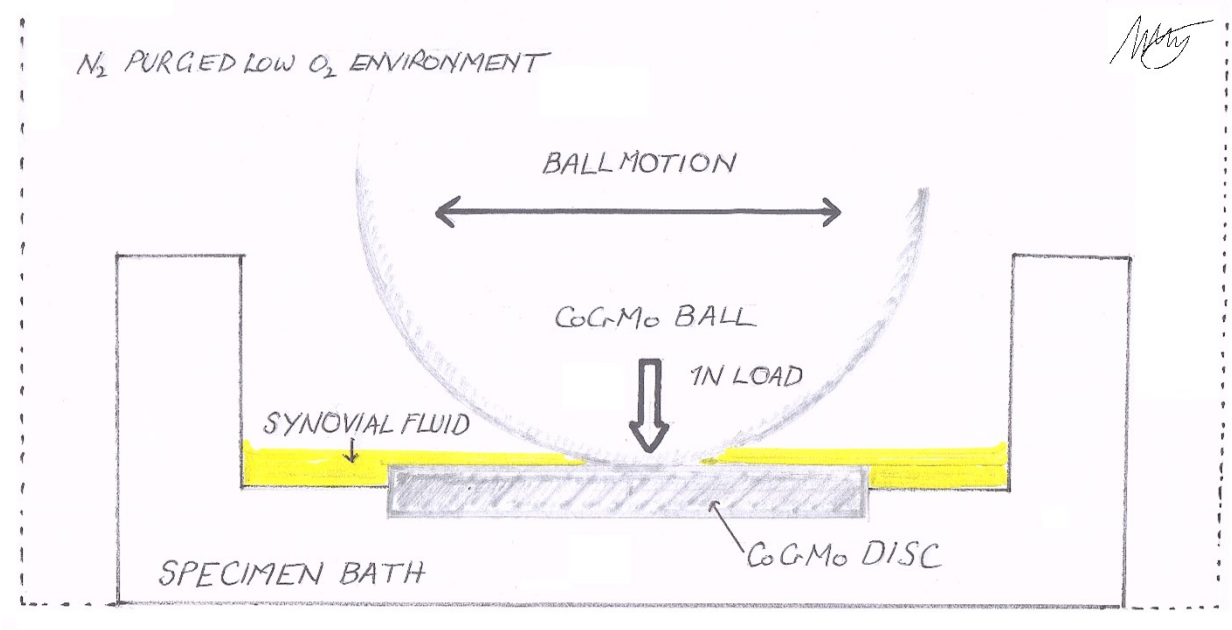


Figure 20 – Representation of the HFRR testing rig used to evaluate the wear effect from varying the biological lubricant

The testing bath is composed of nylon so as not to influence the lubricant composition during the test. Specimens were cleaned in detergent 1% v/v solution before being rinsed in laboratory grade distilled water, followed by isopropyl alcohol to ensure no cross contamination.

This test conditions replicate contact pressures, kinematics and sliding speeds (0-42mms^{-1}) experienced over the load-bearing part of the gait cycle for a weight bearing joint. A sliding frequency of 20Hz was used with a stroke length of 1mm. The resultant initial contact pressure (mean 200 MPa) is higher than quoted for the peak load during walking gait cycle but represents a "jogging" gait thereby mirroring a severe wear condition (203, 204). The test was run for 72,000 cycles or 60 minutes of testing.

The wear is measured by evaluating the resultant wear scar on the disc from the oscillatory motion of the ball. The scar or linear trough was assessed for depth and volume. Wear depth of the scar was measured at 30%, 50%, 70% along its length and averaged to produce an average wear depth (AWD).

3.15 Summary

This project has undergone significant steps to achieve legal and ethical approval for its execution and to allow a legacy of future experiments with HSF.

The measures undertaken to effectively and reliably sample HSF from a patient cohort have been outlined so the study has a predictably stable metabolic composition. The standards of the experiment were in line with the internationally agreed MSI. These standards will form the basis and process to establish a validated and reproducible metabolic profiling study of HSF.

The establishment of the Imperial College Synovial Fluid Working Group should yield maximum output from the new tissue bank of HSF samples. This collaboration with the department of Mechanical Engineering means their HFRR can be used to study HSF samples and determine if the measurable metabolic composition has any relation to the wear measured.

4 Chapter 4: The NMR spectroscopy evaluation of human synovial fluid

4.1 Introduction

Achieving regulatory approval and the establishing the mechanisms for collecting samples allowed the pilot analysis of HSF using NMR techniques.

The metabolic phenotyping of HSF has no pedigree in our department and there are very few studies in the literature with no established or validated methodology. Therefore, to metabolically evaluate HSF in disease, the first step was to confirm an analytical methodology. The method needed to be reproducible, efficient and provide a broad metabolic representation of the synovial fluid.

There have been prior descriptions of HSF by NMR, but these reports do not guarantee success for our hypotheses or samples. Neither do they guarantee a reliable and reproducible approach which can be applied to samples originating from multiple sources, at different timepoints, different pathologies and from different clinicians.

All methods follow a similar pattern to overcome the challenges outlined (see 3.2). HSF presents additional challenges in a very high protein content. This may result in an excess of macromolecules overwhelming the spectrum due to heterogeneity of the chemical moieties secondary to complex protein bindings, multiple spin interactions and unpredictable proton environments. The resulting spectrum baseline could reduce the ability to detect small molecules. This either needs to be addressed using strategies to remove the protein primarily or supress its signal and binding effects using the NMR spectrometer.

The breadth of metabolic profile from HSF should be maximised to provide the best chance of determining metabolites of significance. Therefore, all available sources of information to identify metabolites in HSF need to be researched. In addition, this research will provide information about previous methodologies.

4.1.1 Published NMR synovial fluid studies

To comprehensively assess the current studies using NMR to evaluate synovial fluid of any species, a systematic review was performed according to the PRISMA guidelines(91). On 23rd August 2018 the MEDLINE and EMBASE databases were searched using the following Boolean search strings:

1. "Synovial Fluid" AND ("nuclear magnetic resonance" OR "NMR")

This search generated 702 abstracts. These were evaluated for relevance, sample numbers, species (human or animal model), sample preparation methods, NMR methods and the identified metabolites. The search generated 26 reports suitable for inclusion here (see Figure 21). The majority have used human samples although canine, equine, guinea pig and ovine have been included (see Table 5)

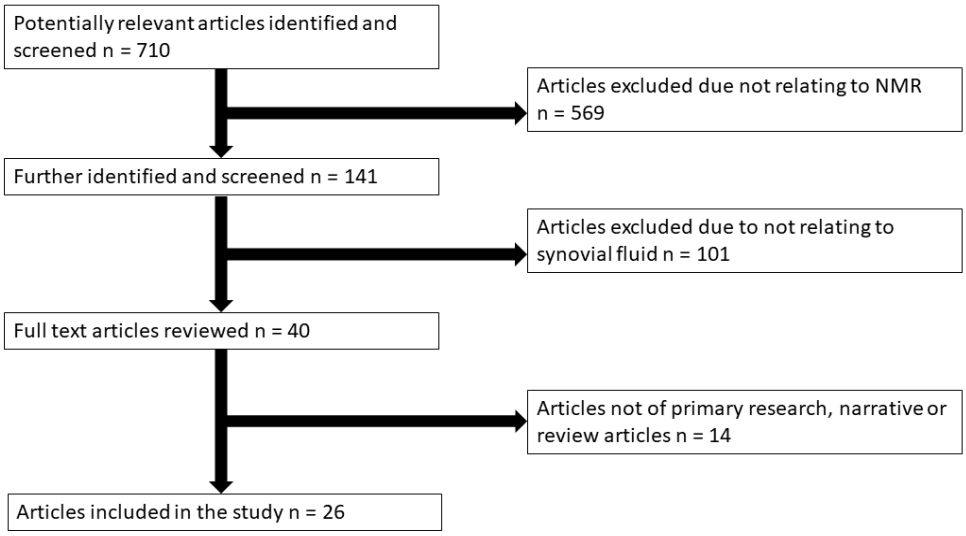


Figure 21 – Flowchart for identification of reports involving the NMR analysis of synovial fluid

4.1.2 Sample preparation (see Table 5)

In most studies the samples were prepared using a centrifugation step to precipitate debris and cells (58, 60, 100, 103, 126, 136, 140, 145). The timings and the gravity force varied (10-30min; 400 g – 17000 g). A shorter time was thought preferable to minimise the time spent at warm temperatures before analysis. In seven cases microfiltration was used as an alternative to remove macromolecules above a

specified molecular weight in a controlled manner (111, 117, 119, 140, 164). However, no clear benefits were apparent, albeit with limited data. Microfiltration required lengthy centrifugation times, and this raises the concern of uncontrolled metabolism throughout this step due to a prolonged period at room or chilled temperatures in the centrifuge. This is a factor neither mentioned, tested nor controlled by the authors in these studies. In addition, any analytes with a high affinity for the filter may cross contaminate if not eluted by washing of filters.

In two cases a protein precipitation step was included (104, 144). These experiments were both presented by the same author and targeted to the detection of phospholipids with the use of ^{31}P-NMR spectroscopy. Therefore, it was felt they were of limited value in guiding the methodology for an untargeted NMR analysis of HSF.

Reference	**Protein Precipitation**	**Microfiltration**	**Centrifugation speed**	**Centrifugation time**	**Buffer**	**Storage**
Lacitignola et al, 2008(111)	No	Yes	17000 g	15min	No	-80ºC
Fairburn et al, 1992(205)	No	No	2000 g	unstated	No	4ºC
Grootveld et al. 1991(145)	No	No	2500rpm	10min	Yes	-70ºC
Naughton et al. 1993(158)	No	No	3000rpm	15min	No	-70ºC
Hugle et al. 2012(106)	No	No	None	None	Yes	-80ºC
Damyanovich et al. 1999(101)	No	No	14000 g	15min	No	none
Damyanovich et al. 2000(206)	No	No	2000 g	30min	No	-75ºC
Parkes et al. 1991(146)	No	No	400 g	15min	No	-70ºC
Duffy et al. 1993(103)	No	No	not stated	not stated	No	-20ºC
Grootveld et al. 1999(56)	No	No	2500rpm	20min	No	4ºC
Williamson et al. 1989(126)	No	No	not stated	not stated	No	-20ºC
Schiller et al. 1996(60)	No	No	not stated	10min	No	-18ºC

Albert et al. 1993(136)	No	No	10000 g	15min	No	Liquid N_2
Naughton et al. 1995(164)	No	Yes	7000 g	15min	No	Not stated
Naughton et al. 1993(140)	No	Yes	7000 g	60min	Yes	-20ºC
Meshitsuka et al. 1999(119)	No	No	None	None	No	-80ºC
Fuchs et al. 2009(104)	Yes	No	3000 g	20min	Yes	-20ºC
Mickiewicz et al. 2015(120)	No	Yes	not stated	not stated	Yes	-80ºC
Mickiewicz et al. 2015(121)	No	Yes	3000 g	30min	Yes	-80ºC
Fuchs et al. 2005(144)	Yes	No	3000 g	20min	Yes	-20ºC
Parkes et al. 1991(146)	No	No	400 g	15min	No	-70ºC
Marshall et al. 2000(117)	No	Yes	3000 g	12-15 hours	No	-75ºC
Claxson et al. 1999(207)	No	No	2000 g	not stated	No	-70ºC
Anderson et al. 2018(208)	No	No	2000 g	5min	Yes	-20ºC
Anderson et al. 2018(162)	No	No	2540 g	5min	Yes	-80ºC
Martini et al 2017(209)	No	Yes	17000 g	15min	Yes	-80ºC

Table 5 - Synovial Fluid NMR Sample Preparation Methods

4.1.3 Use of sample buffer

Only ten reports have advocated the addition of buffer to stabilise chemical shifts across the samples (106, 145). Synovial fluid contains a significant pH buffering capacity, but despite this the pH can vary in the presence of disease (54). Multi-variate statistical analysis techniques require direct comparability of spectra. This is compromised by any instability of the chemical shift. Since the chemical environment of the molecule is directly affected by the pH, so is the chemical shift. Therefore, a stable pH is desirable in this type of analysis (106). The feasibility of buffer and any effect it may have upon the metabolic profile of the HSF model was evaluated.

4.1.4 Chemical shift reference (see Table 6)

Most authors studying synovial fluid with NMR spectroscopy analysis successfully used trimethylsilylpropanoic acid (TSP) for their chemical shift reference (56, 58, 100, 101, 103, 106, 111, 117, 119, 140, 145, 146, 158, 164, 205, 206). In one case the authors used the lactate peak to supply this reference and in one case no specific reference compound was mentioned (60, 126). A single author used phosphatidylcholine as a reference in a targeted methodology for lipid analysis (104, 144). Therefore, TSP was used in this study because it has a successful pedigree both inside and outside our department, alongside published evidence of use in synovial fluid.

Reference	Reference	Targeted/Untargeted	NMR Method	Pulse sequence	Sample stability
Lacitignola et al, 2008(111)	TSP	Untargeted	^{1}H-NMR		not stated
Fairburn et al, 1992(205)	TSP	Targeted	^{1}H-NMR	Hahn spin echo	48hrs (? tested)
Grootveld et al. 1991(145)	TSP	Targeted	^{1}H-NMR	Hahn spin echo	not stated
Naughton et al. 1993(158)	TSP	Untargeted	^{1}H-NMR	Hahn spin echo	8 days tested
Hugle et al. 2012(106)	TSP	Untargeted	^{1}H-NMR	NOESY & CPMG	12 months
Damyanovich et al. 1999(101)	TSP	Untargeted	^{1}H-NMR	CPMG	immediately analysed
Damyanovich et al. 2000(206)	TSP	Untargeted	^{1}H-NMR	CPMG	not stated
Parkes et al. 1991(58)	TSP	Untargeted	^{1}H-NMR	Hahn spin echo	not specified & tested
Duffy et al. 1993(103)	TSP	Untargeted	^{1}H-NHR		not stated
Grootveld et al. 1999(56)	TSP	Targeted	^{1}H-NMR	Hahn spin echo	18hrs (not tested)
Williamson et al. 1989(126)	None	Untargeted	^{1}H-NMR	Hahn spin echo	not stated
Schiller et al. 1996(60)	Lactate	Untargeted	^{1}H-NMR	Hahn spin echo	6 months
Albert et al. 1993(136)	TSPA	Untargeted	^{13}C-NMR		not stated
Naughton et al. 1995(164)	TSP	Targeted	^{1}H-NMR	Hahn spin echo	not stated

Naughton et al. 1993(140)	TSP	Untargeted	^{1}H-NMR	Hahn spin echo	72hrs tested
Meshitsuka et al. 1999(119)	TSP	Untargeted	^{1}H-NMR		not stated
Fuchs et al. 2009 (104)	PC	Targeted	^{31}P-NMR	NOE	within days
Mickiewicz et al. 2015(120)	not stated	Untargeted	^{1}H-NMR	NOESY	not stated
Fuchs et al. 2005(144)	PC	Targeted	^{31}P-NMR	NOE	within days
Mickiewicz et al. 2015 (120, 121)	DSS	Untargeted	^{1}H-NMR	NOESYPR1D	Not stated
Parkes et al. 1991(146)	TSP	Targeted	^{1}H-NMR	Hahn spin echo	not specified & tested
Marshall et al. 2000(117)	TSP	Untargeted	^{1}H-NMR	Hahn spin echo	not stated
Claxson et al. 1999(207)	TSP	Untargeted	^{1}H-NMR	Hahn spin echo	Not stated
Anderson et al. 2018(208)	TSP	Untargeted	^{1}H-NMR	CPMG	Not stated
Anderson et al. 2018(162)	TSP	Untargeted	^{1}H-NMR	CPMG	Not stated
Martini et al 2017(209)	DSS	Untargeted	^{1}H-NMR	CPMG, 1D ZGPR, 2D 1H J-res	Not stated

Table 6 - The reported NMR methods for the analysis of synovial fluid

4.1.5 NMR methodology

The published studies used 1D-NMR or occasionally 2D-NMR techniques. The preference was for ^{1}H-NMR for speed of analysis, ease of interpretation and ease of quantitation. Where targeted analysis was employed, some used ^{13}C-NMR or ^{31}P-NMR.

Hahn spin echo experiments were favoured in many of the traditional studies, with later investigations using NOESY or CMPG. In light of previous success with these methods in plasma analysis (with similar protein content), reported synovial fluid success and the experience of these methods in our group, NOESY and CPMG sequences were selected for first evaluation.

Metabolite	**Chemical Shift and peak pattern (ppm)**	**Reference**
Cholesterol C18	0.58(bs), 0.65(bs)	Naughton et al.(140)

Lipid CH_3 terminal groups	0.85(bs)	Schiller et al (60), Williamson et al.(126), Duffy et al.(103), Damyanovich et al.(100, 101), Parkes et al.(58, 146), Naughton et al.(140), Fairburn et al.(205)
Isoleucine	0.93(t), 1.00(d), 1.25(m), 1.46(m), 1.96(m), 3.66(d)	Damyanovich et al.(100, 101), Parkes et al.(58, 146), Naughton et al.(140), Grootveld et al.(56, 145),
Leucine	0.95(d), 0.96(d), 2.00(m)	Naughton et al.(140), Damyanovich et al.(100, 101)
Valine	0.98(d), 1.03(d), 2.26(m), 3.60(d)	Damyanovich et al.(100, 101), Williamson et al. (126), Schiller et al.(60), Duffy et al.(103), Claxson et al.(207), Parkes et al.(58, 146), Naughton et al.(140), Mickiewicz et al.(120), Meshitsuka et al.(119), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205), Duffy et al.(103)
3-Hydroxyisobutyrate	1.06(d), 2.48(m)	Damyanovich et al.(100, 101)
3-Hydroxybutyrate	1.19(d), 2.35(dd), 4.14(m)	Damyanovich et al.(100, 101), Duffy et al.(103), Claxson et al.(207), Naughton et al.(140, 158), Mickiewicz et al.(120), Grootveld et al.(56, 145)
CH3 lipid chain (L2 terminal)	1.25(s)	Damyanovich et al.(100, 101)
Lipid chain CH_2 groups	1.3(s)	Schiller et al.(60), Duffy et al.(103), Parkes et al.(58, 146), Damyanovich et al.(100, 101), Naughton et al.(140), Grootveld et al.(56, 145), Fairburn et al.(205),
alpha-Lactate	1.308, 1.34(d), 4.10(q)	Naughton et al.(140), Parkes et al.(58, 146), Mickiewicz et al.(120),Meshitsuka et al.(119), Hugle et al.(106), Fairburn et al.(205), Duffy et al.(103),
Threonine	1.31(d), 3.56(d), 4.24(dd)	Claxson et al.(207), Parkes et al.(58, 146), Naughton et al.(140), Mickiewicz et al.(120),Hugle et al.(106), Grootveld et al.(56, 145),
Alanine	1.47(d), 3.78(q)	Naughton et al., Mickiewicz et al.(120),Meshitsuka et al.(119), Lacitignola et al.(111),Grootveld et al.(56, 145), Fairburn et al.(205), Damyanovich et al.(100, 101)
Lysine	1.47(m), 1.71(m), 1.89(m), 3.03(t), 3.72(t)	Williamson et al. (126), Mickiewicz et al.(120), Hugle et al.(106)
Arginine	1.69(m), 1.90(m), 3.23(t)	Mickiewicz et al.(120), Damyanovich et al.(100, 101)
Acetate	1.91(s)	Lacitignola et al.(111), Mickiewicz et al.(120), Parkes et al.(58, 146), Naughton et al.(140, 158), Grootveld et al.(56, 145), Fairburn et al.(205)
N-acetyl CH_3 groups (GAG and Glycoproteins)	2.02(bs)	Schiller et al.(60), Williamson et al.(126), Duffy et al.(103), Lacitignola et al.(111), Claxson et al.(207), Damyanovich et al.(100, 101), Parkes et al.(58, 146), Naughton et al.(140),Meshitsuka et al.(119), Fairburn et al.(205)
N-acetyl groups	2.06(bs)	Claxson et al.(207)
Glutamate	2.06(m), 2.34(m)	Claxson et al.(207), Hugle et al.(106),
Glutamine	2.12(m), 2.45(m), 3.74(dd)	Claxson et al.(207), Parkes et al.(58, 146), Naughton et al.(140), Mickiewicz et al.(120), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205)

Proline	2.00(m), 2.08(m), 2.34(m), 3.32(dt), 3.41(m), 4.12(dd)	Mickiewicz et al.(120),
Lipid CH_2CO	2.25(s)	Naughton et al.(140, 158)
Methionine	2.14(s), 2.16(m), 2.65(t)	Mickiewicz et al.(120)
Acetone	2.22(s)	Naughton et al.(140), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205)
Acetoacetate	2.27(s), 3.44(s)	Fairburn et al.(205)
2-methyl glutamate	1.05(d), 1.57(t), 1.61(d), 1.71(m), 2.13(m)	Mickiewicz et al.(120),
N-acetyl chlorinated degradation products	2.35(s)	Schiller et al.(60)
Chloramine	2.35(s)	Schiller et al.(60)
Pyruvate	2.37(s)	Lacitignola et al.(111), Naughton et al.(140), Hugle et al.(106), Grootveld et al.(56, 145), Damyanovich et al.(100, 101)
Succinate	2.40(s)	Hugle et al.(106)
trans-4-hydroxy proline	2.41(m), 2.15(m)	Mickiewicz et al.(120),
Carnitine	2.43(s), 3.22(s), 3.419(s), 4.55(s)	Mickiewicz et al.(120), Hugle et al.(106), Grootveld et al.(56, 145)
Citrate	2.53(d), 2.67(d)	Williamson et al.(126), Lacitignola et al.(111), Damyanovich et al.(100, 101), Mickiewicz et al.(120),Naughton et al.(140), Parkes et al.(58, 146), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205)
Dimethylalanine	2.5(s)	Mickiewicz et al.(120),
Aspartate	2.67(m), 2.83(m), 3.87(m)	Mickiewicz et al.(120),
Asparagine	2.82(dd), 2.93(dd), 3.97(dd)	Mickiewicz et al.(120),
Deferoxamine	2.82 (s)	Parkes et al.(58, 146)
Creatine	3.03(s), 3.91(s)	Damyanovich et al.(100, 101), Mickiewicz et al.(120),Naughton et al.(140), Lacitignola et al.(111), Duffy et al.(103)
Creatinine	3.04(s), 4.05(s)	Williamson et al.(126), Duffy et al.(103), Lacitignola et al., Claxson et al.(207), Naughton et al.(140), Parkes et al.(58, 146), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205), Damyanovich et al.(100, 101)
Phenylalanine	3.1(d), 3.98(s), 7.37(s)	Mickiewicz et al.(120), Williamson et al.(126), Naughton et al.(140), Hugle et al.(106) , Damyanovich et al.(100, 101)
Malonate	3.12(s)	Mickiewicz et al.(120),
HDL-choline	3.21(s)	Damyonovich et al.(100, 101), Naughton et al.(140), Claxson et al.(207), Mickiewicz et al.(120),

o-phosphocholine	3.23(s)	Mickiewicz et al.(120),
alpha-Glucose	3.23(dd), 3.40 (m), 3.46 (m), 3.52 (dd), 3.73 (m), 3.82 (m), 3.88 (dd), 4.63 (d), 5.23(d)	Schiller et al.(60), Lacitignola et al.(111), Claxson et al.(207), Damyanovich et al.(100, 101), Naughton et al.(140), Mickiewicz et al.(120), Meshitsuka et al.(119), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205), Duffy et al.(103)
Taurine	3.25(t), 3.42(t)	Mickiewicz et al,(120).
Betaine	3.25(s)	Mickiewicz et al.(120), Grootveld et al.(56, 145)
Glycine	3.55(s)	Damyonovich et al.(100, 101), Claxson et al.(207), Naughton et al.(140), Mickiewicz et al.(120), Fairburn et al.(205)
HDL-choline	3.65 (m)	Lacitignola et al.(111)
Glycerol	3.55 (m), 3.64 (m), 3.77 (m)	Damyanovich et al.(100, 101), Mickiewicz et al.(120), Lacitignola et al.(111), Grootveld et al.(56, 145)
Beta-Glucose	3.40 (t), 3.89 (dd), 4.64 (d)	Schiller et al.(60), Damyanovich et al.(100, 101), Williamson et al.(126), Mickiewicz et al.(120), Meshitsuka et al.(119), Hugle et al.(106), Grootveld et al.(56, 145), Fairburn et al.(205), Duffy et al.(103)
CH=CH (fatty acyl residues, lipids)	5.27(s)	Damyonovich et al.(100, 101), Fairburn et al.(205)
Allantoin	5.38(s)	Grootveld et al.(56, 145),
Uracil	5.79(d), 7.62(d)	Hugle et al.(106)
Tyrosine	6.89(d), 7.19(d)	Damyonovich et al.(100, 101), Williamson et al.(126), Naughton et al.(140), Mickiewicz et al.(120), Hugle et al.(106),
Histidine	7.05(s), 7.77(s)	Damyonovich et al.(100, 101), Williamson et al.(126), Naughton et al.(140)
Hippuric Acid	3.96(d), 7.54(m), 7.62(m), 7.83(dd)	Hugle et al.(106)
Formate	8.45(s)	Williamson et al.(126), Claxson et al.(207), Damyanovich et al.(100, 101), Naughton et al.(140), Grootveld et al.(56, 145),
N-Acetyl Cysteine Amide Product	8.6-8.7(d)	Grootveld et al.(56, 145),

Table 7 - Reported synovial fluid metabolites from a literature search of reported studies

4.1.6 Methodology contaminants

At this stage, the evaluation of human samples mandates that the harvesting, aliquoting and processing of the HSF is screened for any contaminants that could interfere with the analysis. The use of plastic disposables, washed glassware and solvents are known to introduce contaminants at multiple stages. Typically these include phthalates from the plastic and polyethylene glycol from solvents (210).

Therefore, any contact of the sample from collection through to analysis with vessels, solvents or reagents must be screened.

4.1.7 Metabolite identification

In order to provide metabolic information on future samples it is essential that metabolites can be identified with confidence. Furthermore, the metabolite information from the samples needs to be maximised. The methods outlined will be used to achieve this (see 3.11). The literature was reviewed for all published reports of the NMR analysis of synovial fluid to aid metabolite identification.

Therefore, all 26 articles found by literature review (see 4.1.2) were searched for any reported metabolite with supportive or identifying information (see Table 7).

4.2 Hypothesis:

- The addition of buffer does not detriment the metabolic profile of synovial fluid
- Contaminants will be present in the HSF from the consumables that need to be discounted from any later metabolite identification
- The high protein content of HSF will cause a significant NMR signal. This signal may be suppressed by using CPMG sequencing.

4.3 Aims:

- The evaluation of sample preparation, buffer use, contaminants and NMR sequencing in the characterisation of HSF.
- The metabolite assignment of HSF by NMR spectroscopy using NOESY and CPMG sequences.

4.4 Materials and methods

4.4.1 Sample collection

HSF was collected according to the protocols as detailed (see 3.6.8). Nineteen patients were undergoing joint arthroplasty, five were undergoing joint arthroscopy and two patients underwent needle arthrocentesis. Patients were aged 29 to 80 with a mean age of 64. Sixteen were female and ten were male. The comorbid conditions were asthma, angina, hypertension, chronic kidney disease, diverticulitis and benign prostatic hypertrophy. Specific metabolic diseases were hyperthyroidism, obesity and diabetes. Medications were noted including two patients taking regular analgesia. Twenty patients were white, and one was mixed ethnicity white/black Caribbean, three Caribbean and two Indian.

Three blank samples were processed in tandem using HPLC grade water (5ml) to mimic HSF and collected alongside the HSF. These samples were handled using the sample collection and storage procedures at the same time and using identical disposables (see 3.6.9). These disposables were used for all future experiments to ensure consistency in the contaminants.

4.4.2 Sample processing

All samples were transferred to 2ml Eppendorf tubes before centrifugation at 10000g, room temperature for 15min. The supernatant was then decanted and stored at -80°C. Samples were then defrosted at room temperature. Sample (400µl) was combined in a 1:1 v/v mix with buffer (142mM $Na_2HPO_4x7H_2O$; 4% w/v NaN_3; TSP) before being centrifuged at 12,000g for 2 min to allow maximal transfer. Sample/buffer mix was then transferred (550µl) to a 5mm NMR tube.

4.4.3 NMR methods

Samples were analysed by NMR using a 600MHz Avance III Bruker NMR Spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.09MHz, 310°K coupled to a Samplejet 96 well rack autosampler at 4°C. Sequences were acquired (NOESYGPPR1D and CPMG) performing 32 scans, thereby generating 96K data points. The NOESYGPPR1D sequence contained magnetic field gradients

in the mixing period and was performed with a mixing time of 10ms, a delay between two 90°RF pulses of 3μs and a relaxation time between two FIDs of 4s (211). The CPMG sequence used a D - 90^{o}_{x} – τ - $(180^{o} \pm y - \tau)_{n}$, where relaxation time D = 4s, spin echo delay τ =0.3ms and the number of loops n=128 (211). A CPMG sequence leads to peak area variations based on proton T2 values and both sequences might not show fully relaxed spectra with a short T1 relaxation delay. However, parameters were typical of those used in previous HSF publications, whilst the intensity values derived will be semi-quantitative, the relative values should retain accuracy, assuming that T1 and T2 values do not differ markedly between samples.

4.4.4 Data handling

All NMR FID signals were transformed by Fourier transformation using Topspin® v3.2 software (Bruker). Spectra were manually phased, baseline corrected and calibrated to the ^{1}H proton peak signal of TSP (δ=0ppm). Data processing was performed using Matlab® software vR2014b (Mathworks®, Natwick, Massachusetts, USA). Spectral subtraction was performed to remove the water peak (δ=4.5-5.0ppm) and hence distorting variability due to water presaturation. Spectra were aligned using a published method to allow peak comparison between samples (212). Spectral intensities were normalised using median-fold change normalisation to account for any errors in dilution of the samples (213).

4.5 Results

The HSF was heterogeneous in viscosity and cellular content. The amount of sediment material upon centrifugation was significant and variable. This meant that pipetting had to be slow and deliberate to avoid error. Furthermore, a 5mm NMR tube was mandatory as despite buffer mixing, the viscosity would not allow samples to enter the tubes without an air lock.

There were contaminant peaks in the system blanks at low levels. This was in addition to solvent peaks, which were expected. Contaminant peaks were identified using library referencing and J-Resolved (JRES) spectra, thereby avoiding any interference with future HSF results and ensuring appropriate

interpretation of compounds close to the contaminant peaks. The contaminant peaks identified are listed (see Table 8).

Chemical shift	Peak type	Identity
0.097	Singlet	^{13}C satellite of TSP
1.176		
1.188	Multiplet 1:2:1	Ethanol
1.199		
1.253	Singlet	?2-methyl-2-propanolol (TSP contaminant)
1.984	Singlet	Unknown
2.235	Singlet	Acetonitrile (shifted)
3.246	Singlet	^{13}C satellite of 3.366 Methanol
3.366	Singlet	Methanol
3.429	Singlet	^{13}C satellite of 3.563 singlet
3.482	Singlet	^{13}C satellite of Methanol
3.549	Singlet	Unknown
3.563	Singlet	Unknown
3.646		
3.657	Multiplet 1:3:3:1	Ethanol
3.669		
3.681		
3.698	Singlet	^{13}C satellite of 3.563 singlet
3.846	Doublet	^{13}C satellite of 3.698 doublet
3.968	Doublet	Unknown
7.905	Singlet	Unknown
8.463	Singlet	Formate

Table 8 - Contaminants identified in the collection and storage of HSF

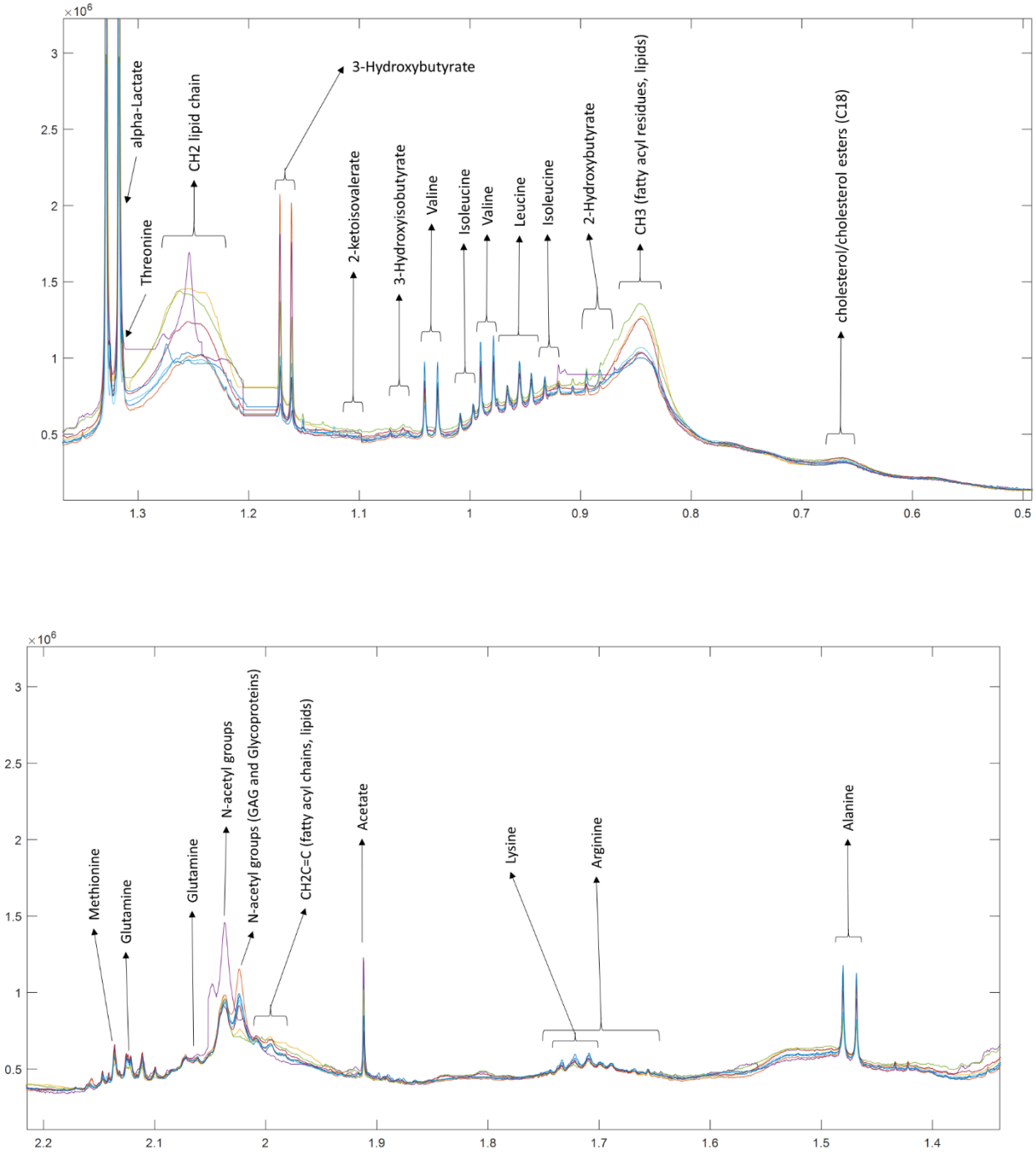
NMR spectra were of good quality, resolution and bearing a resemblance to serum. Peaks were systematically identified in the NMR spectrum for both the NOESYGPPR1D and the CPMG sequences. Metabolites were identified using a combination of published reports and experience; HMDB, JRES, TOCSY and heteronuclear single quantum clearance (HSQC) acquisitions (see 3.9.7, Table 6 and Table 9).

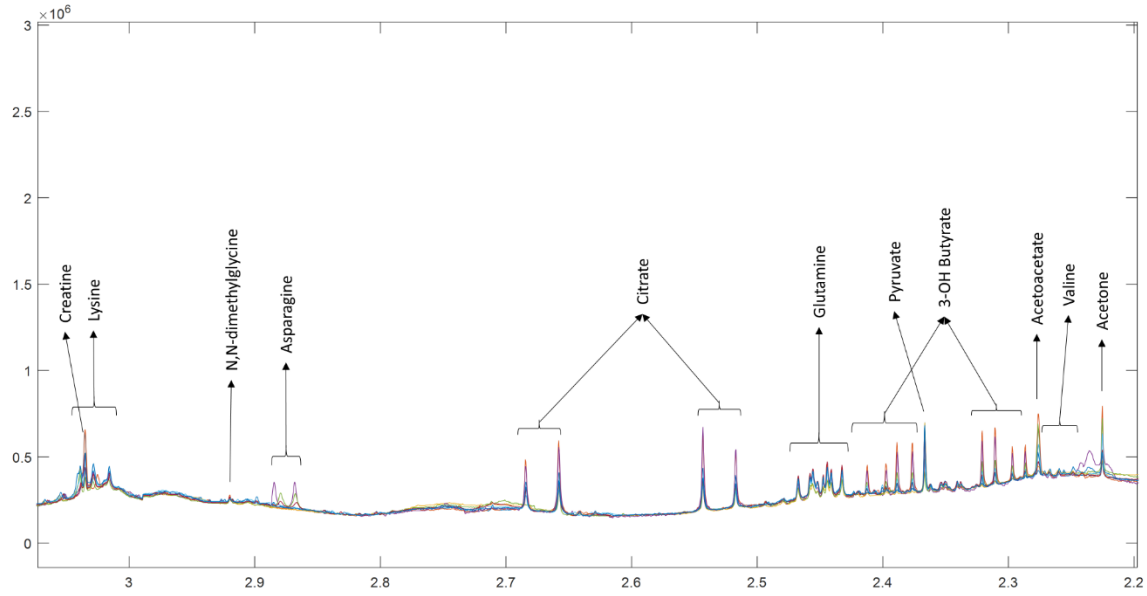
Metabolite	**Chemical Shift (ppm) & ^{1}H-NMR signature**	**HMBD Ref**	**Derived from 1D-NMR, HMDB &**
Cholesterol C18	0.58(bs), 0.65(bs)		HSQC
CH_3 (fatty acyl residues, lipids)	0.85(bs)		TOCSY, HSQC
2-Hydroxybutyrate	0.89(CH_3, t), 1.66(CH_2, m), 1.71(CH_2, m)	0000008	TOCSY, JRES
Isoleucine	0.93(CH_3, t), 1.00(CH_3, d), 1.25(CH_2, m), 1.46(CH_2, m), 1.96(CH, m), 3.66(CH, d)	0000172	TOCSY
Leucine	0.95(CH_3, d), 2.00(CH, m)	0000687	TOCSY
Valine	0.98(CH_3, d), 1.03(CH_3, d), 2.26(CH, m), 3.60(CH, d)	0000883	TOCSY
3-Hydroxyisobutyrate	1.06(CH_3, d), 2.48 (CH, m)	0000336	TOCSY, STOCSY
2-ketoisovalerate	1.11(CH_3, d), 3.00(CH, m)	0000019	TOCSY, JRES
3-Hydroxybutyrate	1.19(CH_3, d), 2.31(CH_2, dd), 2.43(CH_2, dd), 4.14(CH, m)	0000011	TOCSY
CH_2 (fatty acyl residues, lipids)	1.30 (bs)		HSQC
L-Lactate	1.31 (CH_3, d), 4.10(CH, q)	0000190	TOCSY
Threonine	1.31(CH_3, d), 3.56(CH, d), 4.24(CH, dd)	0000167	TOCSY
Alanine	1.47(CH_3, d), 3.78(CH, q)	0000161	TOCSY
Lysine	1.47(CH_2, m), 1.71(CH_2, m), 1.89(CH_2, m), 3.03(CH, t), 3.72(CH, t)	0000182	TOCSY
Arginine	1.69(CH_2, m), 1.90(CH_2, m), 3.23(CH_2, t)	0000517	TOCSY
Acetate	1.91(CH_2, s)	0000042	JRES, HSQC
CH_2C=C (fatty acyl chains, lipids)	1.99(bs)		HSQC
N-acetyl groups	2.06(bs)		HSQC
Glutamate	2.06(CH_2, m), 2.34(CH_2, m)	0000148	TOCSY, JRES
Glutamine	2.12(CH_2, m), 2.45(CH_2, m), 3.74(CH, t)	0003423	TOCSY, JRES
Methionine	2.14(CH_3, s), 2.16(CH_2, m), 2.65(CH_2, t)	0000696	TOCSY
Acetone	2.22(CH_3, s)	0001659	JRES
Acetoacetate	2.27(CH_3, s), 3.44(CH_2, s)	0000060	JRES, STOCSY
Pyruvate	2.37(CH_3, s)	0000243	JRES
Succinate	2.40(CH_2-CH_2, s)	0000254	JRES
Citrate	2.53 (CH_2, d), 2.67 (CH_2, d)	0000094	TOCSY

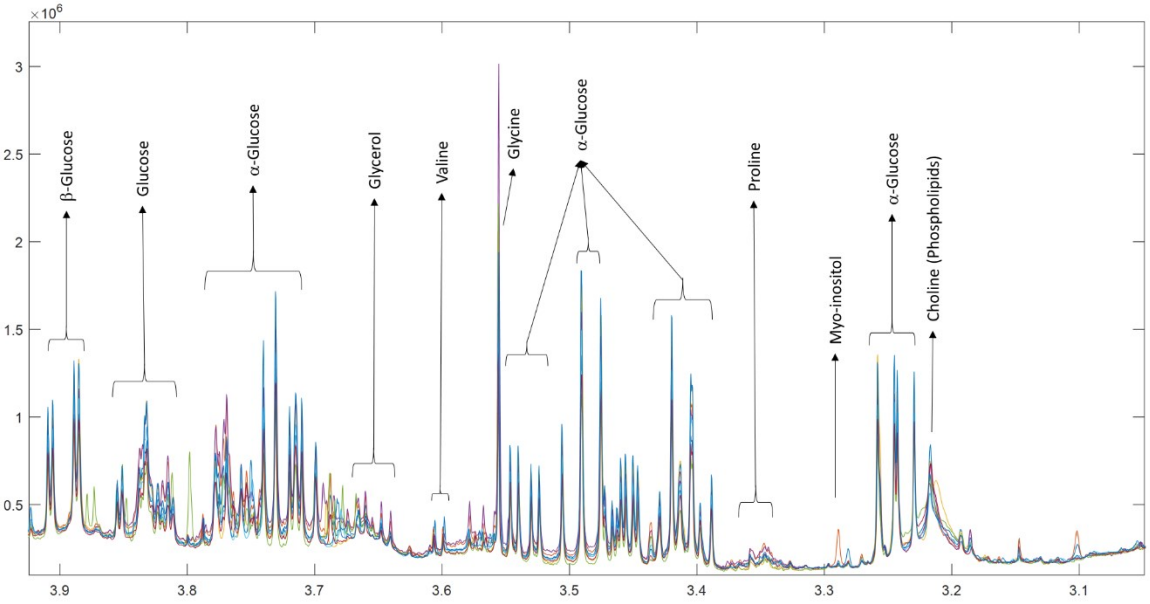
Dimethylamine	2.72(CH_3, s)	0001020	JRES
N, N-dimethylglycine	2.92(CH_2, s)	0000092	JRES
Creatine	3.03(CH_3, s), 3.91(CH_2, s)	0000064	HSQC
Creatinine	3.04(CH_3, s), 4.05(CH_2, s)	0000562	HSQC
Ornithine	1.80(CH_2, m), 1.93(CH_2, m), 3.04(NH_2, m), 3.77(CH, m)	0000214	TOCSY
Phenylalanine	3.1(CH_2, m), 3.98(CH_2, m), 7.37(COOH, s)	0000159	TOCSY
Dimethylsulfone	3.14(CH_3, s)	0004983	JRES
Choline (free)	3.21(CH_3, s)	0000097	HSQC
Choline (phospholipids)	3.23(CH_3, s)	0000097	HSQC
Glucose	3.23(CH, dd), 3.40 (CH, m), 3.46 (CH, m), 3.52 (CH, dd), 3.73 (CH, m), 3.82 (CH_2, m), 3.88 (CH, dd), 4.63 (CH, d), 5.23(CH, d)	0000122	TOCSY
Proline	2.00(CH_2, m), 2.08(CH_2, m), 2.34(CH_2, m), 3.32(CH_2, dt), 3.41(CH_2, m), 4.12(CH, dd)	0000162	TOCSY
Glycine	3.55(CH_2, s)	0000123	HSQC, JRES
Glycerol	3.55 (CH_2, m), 3.64 (CH_2, m), 3.77 (CH, m)	0000131	TOCSY, JRES
Myo-inositol	3.28(CH, t), 3.60(CH, t), 4.06(CH, t)	0000211	TOCSY
CH=CH (fatty acyl residues, lipids)	5.27(s)		BS, HSQC
Tyrosine	6.89(CH, m), 7.19(CH, m)	0000158	TOCSY
Histidine	7.05(CH, d), 7.77(CH, d)	0000177	JRES
Paracetamol glucuronide	2.16(CH_3, s), 5.09(CH, d), 7.15(CH, d), 7.36(CH, d)	0010316	TOCSY, STOCSY
GAG	2.02(s), 3.34(bs), 4.46(bs), 7.97(bs)		BS, STOCSY
Hypoxanthine	8.18(CH, s), 8.20(CH, s)	0000157	JRES
Formate	8.45(CH, s)	0000142	JRES

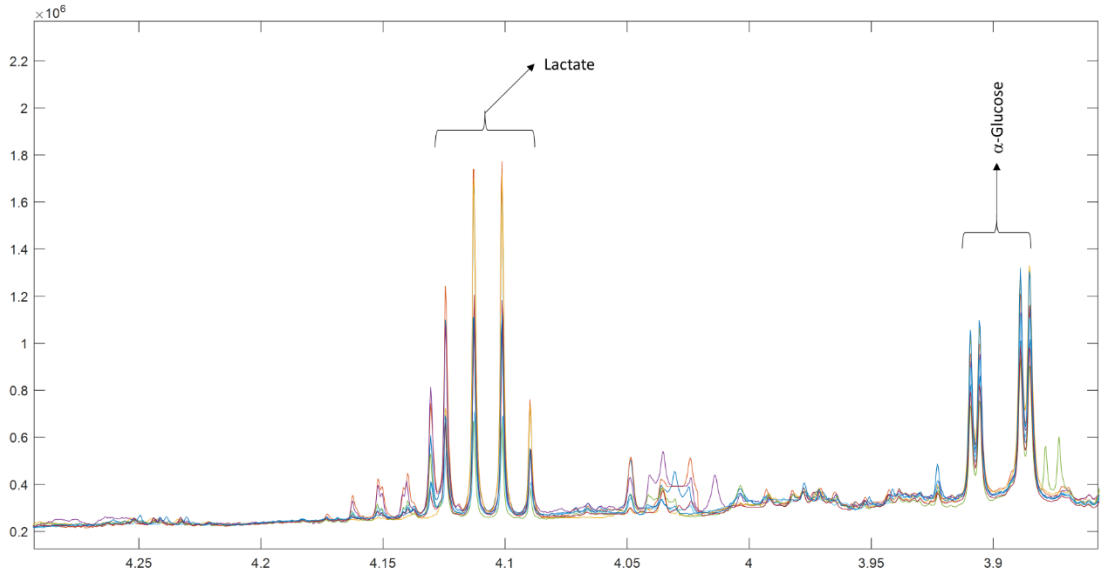
Table 9 - The metabolite assignment of noesygppr1d and CPMG NMR data using published literature and analytical techniques

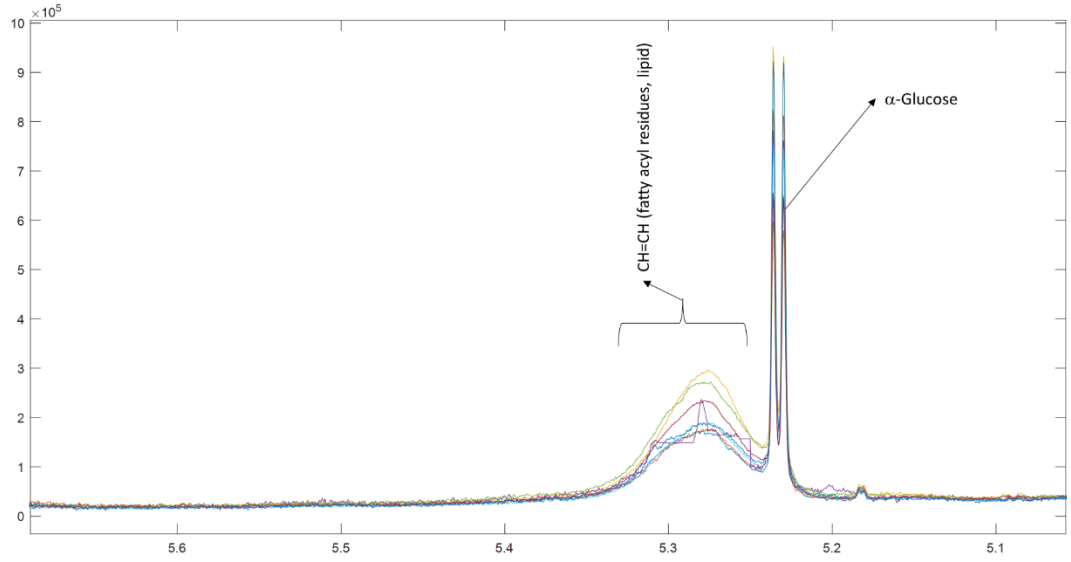
Figure 22 - The NMR NOESYGPPR1D Spectra of HSF with metabolites highlighted

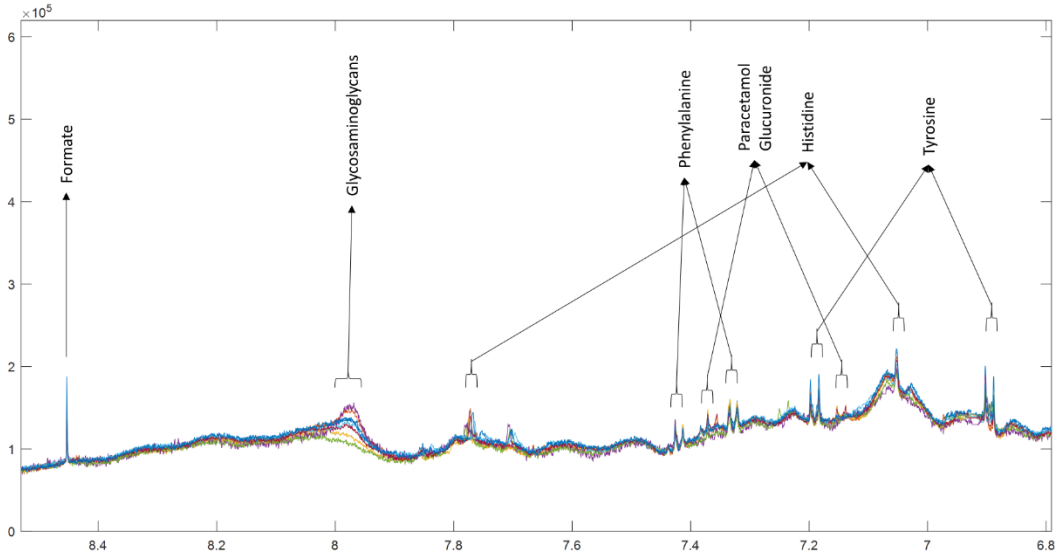



×10^6
Creatine
Lysine
N,N-dimethylglycine
Asparagine
Citrate
Glutamine
Pyruvate
3-OH Butyrate
Acetoacetate
Valine
Acetone


×10^6
β-Glucose
Glucose
α-Glucose
Glycerol
Valine
Glycine
α-Glucose
Proline
Myo-inositol
α-Glucose
Choline (Phospholipids)


×10^6
Lactate
α-Glucose
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
4.25
4.2
4.15
4.1
4.05
4
3.95
3.9


×10^5
CH=CH (fatty acyl residues, lipid)
α-Glucose
10
9
8
7
6
5
4
3
2
1
0
5.6
5.5
5.4
5.3
5.2
5.1



4.6 Discussion

The method was successful in producing quality, highly resolved spectra. The spectra were consistent and minimal spectral alignment was required to produce comparability. Therefore, the buffering of the samples appears to have been adequate despite the variable pH, as expected within pathological HSF (54). However, certain sample handling practices like pipetting, need to be done with experience and care due to the variable viscosity of the samples.

The spectral resolution was excellent and the macromolecule interactions within the HSF were manageable. The spectra represented a good breadth of metabolites for an untargeted analysis.

The contaminants identified are undesirable, but logistically difficult to remove prior to analysis. Therefore, identifying contaminants and discounting them from any future metabolite interpretation of the samples is essential.

It is concluded that the methodology is applicable for metabolic profiling an HSF cohort. However, further work is required to validate the method and establish suitable quality controls.

5 Chapter 5: The NMR spectroscopy evaluation of bovine calf serum as a potential quality control for human synovial fluid

5.1 Introduction

To ensure consistent handling and analysis of HSF, a quality control (QC) sample is required. The ideal sample would be an HSF sample which has a composition as close to the tested samples irrespective of the variation due to disease. Typically, a pooled HSF sample sourced from a variety of HSF samples, thereby representing a spectrum of pathological diseases, would achieve this. However, small sample volumes and numbers, scarce access to patients and a newly established tissue bank mean this was not possible. A synovial fluid from another species could provide a close representation of HSF, albeit without the disease representation. Commercial alternatives were prohibitively expensive and not available at the time of this study. The harvest of synovial fluid from culled farm animals was investigated but the response from local meat providers was not positive due to regulatory restrictions.

5.1.1 The proposal of bovine calf serum (BCS) as a model

Potential candidates for model synovial fluid were discussed amongst the Imperial College Synovial Fluid Working Group. A proposal to use bovine synovial fluid was made as this was cheap and available in large volumes. Fluid stocks were supplied by the working group. Whilst the metabolic profile of the model may not exactly mimic HSF as per our aspirations, the expected similarities were noted and BCS is used a synovial fluid model in many other applications including tribology, (due to be evaluated in this thesis (see 9.1.3)).

BCS remains the industry standard model fluid for HSF in the validation and testing of joint prosthesis. It is also assumed that the small molecule profile will have similarities because SF is a dialysate product of blood serum. This is in conjunction with the knowledge that BCS mirrors HSF in total protein content (see 9.1.1). Logistically BCS is commercially available at low cost and without the need for an animal testing licence. It can be batch purchased in large volumes.

5.1.2 The evaluation of bovine calf serum

The evaluated NMR methods for the analysis of HSF closely resemble those developed for the description of serum. Therefore, BCS should be a suitable QC biosample to run in parallel to HSF.

It was not clear if the addition of buffer may be possible prior to sample freezing and if this may have any detrimental effects to the measured metabolism. This has not been reported in synovial fluid or plasma. Logistically this was desirable due to removing the need for additional handling steps beyond defrosting and before NMR analysis.

5.2 Hypothesis:

The addition of pre-freezing buffer will not affect metabolic stability in BCS.

The suggested methodology will be reliable and robust for BCS analysis, indicating BCS suitability as a QC material for HSF analysis.

5.3 Aim:

To evaluate the effects of pre-freezing buffer addition and the suitability of BCS as a QC sample for HSF analysis.

5.4 Material and methods

BCS was sourced from Sigma Aldrich (Cat # 12133C; batch 11n239). BCS (400µl) was combined with 0.142M phosphate NMR buffer (400µl). Phosphate NMR buffer (pH 7.4; 1L) was prepared in D_2O by adding sodium dihydrogen phosphate (10.64mg NaH_2PO_4), sodium azide (40mg NaN_3) and 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt in deuterium oxide solution (80mg TSP). All chemicals were purchased from Sigma-Aldrich(214).

5.4.1 Sample preparation

A stock of BCS was divided and aliquoted before being mixed in equal quantities with NMR buffer or HPLC grade water. Samples were subjected to a varying number of freeze-thaw cycles with a 24hr interval. Each sample was repeated in triplicate (see Table 10).

BCS Sample	24hr Freeze thaw cycles	Buffer addition
1-3	1	Yes
4-6		No
7-9	2	Yes
10-12		No
13-15	3	Yes
16-18		No
19-21	4	Yes
22-24		No
25-27	5	Yes
28-30		No

Table 10 - Sample list for the evaluation of BCS stability to freeze-thaw cycling and the use of phosphate buffer

5.4.2 NMR analysis

The NMR methodology was identical to that used in the prior evaluation of HSF (see 4.4.3). Samples were not run in logical sequence to avoid any time dependant effect of the samples sitting on the autosampler prior to analysis.

5.4.3 Data processing

The data processing methods were identical to those used in the prior evaluation of HSF (see 4.4.4), except that spectral alignment was omitted because the samples were identical and the aim was to evaluate buffer efficacy, something which can be accommodated by spectral alignment.

5.5 Results

The BCS model fluid was consistently less viscous and easier to transfer than HSF.

5.5.1 Spectra evaluation and outliers

One sample (27) had to be discarded due to a poor spectra resolution. The cause of this instrument failure is not known. An initial spectral overlay indicated the remaining spectra were consistent and no gross differences could be observed between the cohorts (see Figure 23). TSP was found to contain some non-significant variations in peak intensity between the freeze-thawing.

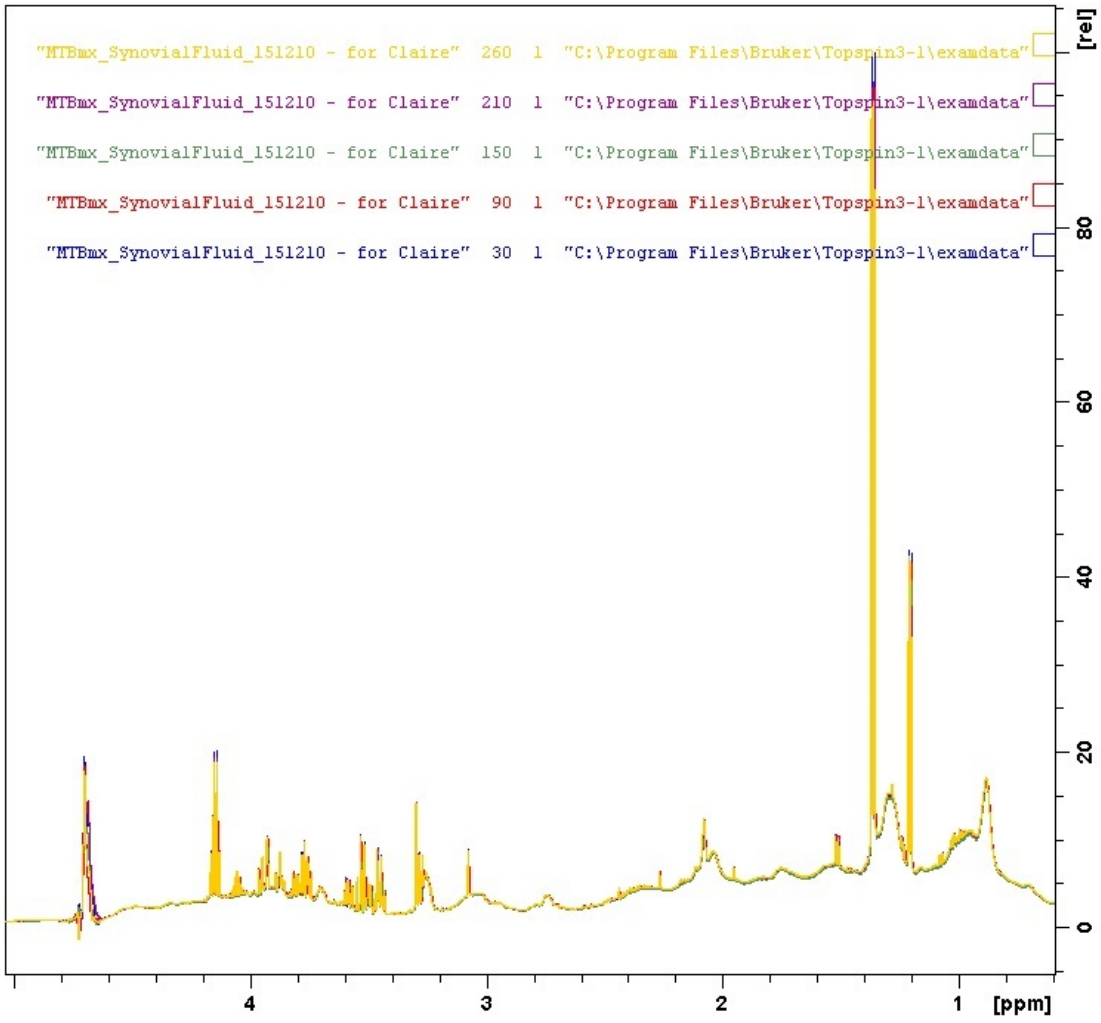


Figure 23 - NOESY Spectral overlay of BCS samples subjected to repeated freeze thaw cycling

5.5.2 Freeze-thaw effects

An initial PCA plot of all samples revealed samples 6 & 19 to be outlying in the NOESY (R^2X=0.435, Q^2=0.309) and samples 1&2 in the CPMG (R^2X=0.366, Q^2=0.228), (see Figure 24). The spectra were acceptable and not visually different when overlaid. A loadings and S plot analysis was performed to identify the spectral regions responsible for the variations. In sample 6 & 19 (NOESY) this was a general baseline effect in all regions under δ=5ppm. In the case of samples 1 & 2 (CPMG) differences corresponded to a doublet at δ=1.16ppm and a multiplet at δ=4ppm. STOCSY analysis revealed these peaks were linked and they were identified using database searches, as isopropyl alcohol. This compound was ubiquitous to the samples and not present in the blanks. Because there was no clear explanation for the outlying samples, they were not excluded from the analysis.

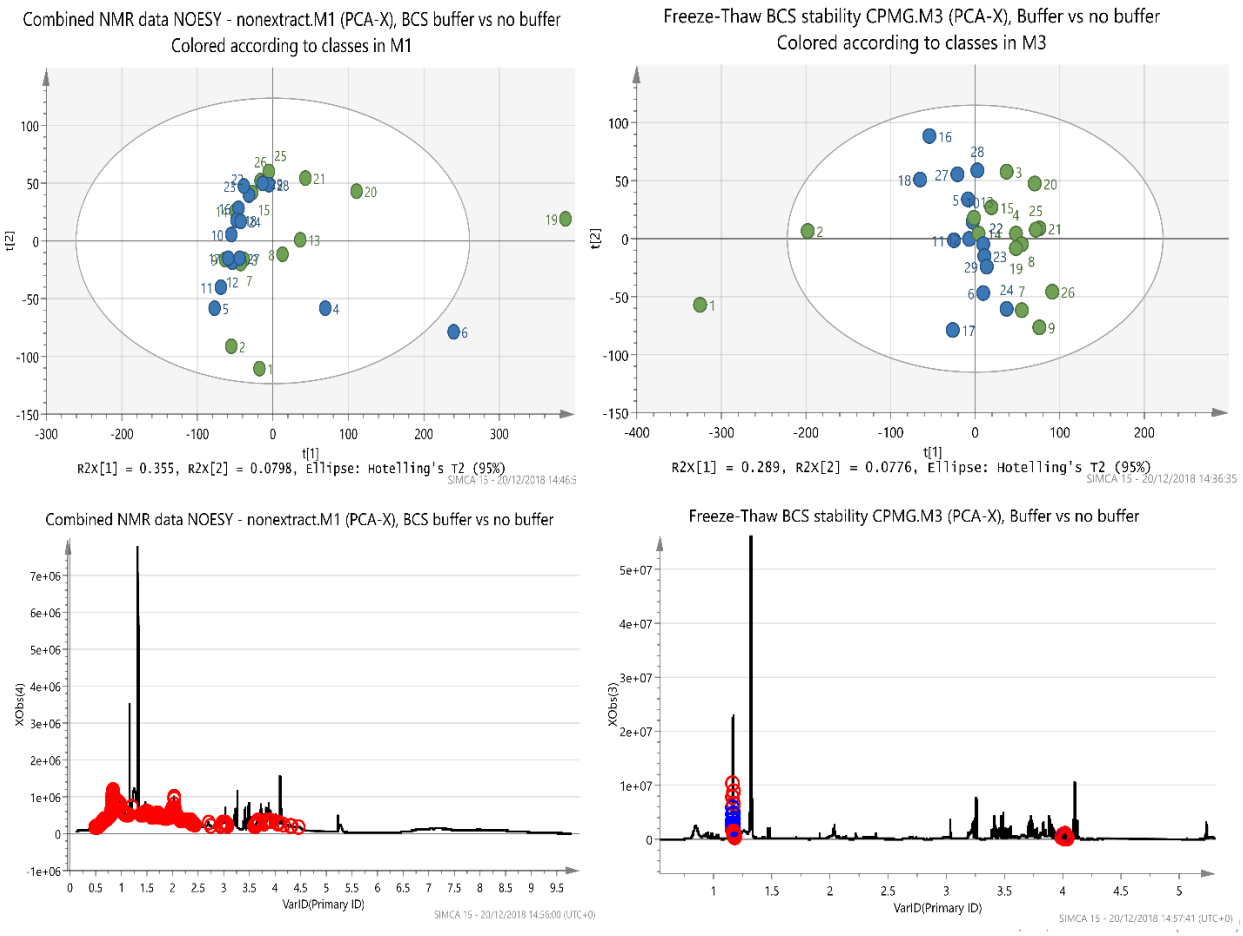


Figure 24 - PCA analysis of NOESY (left) and CPMG (right) NMR data of BCS stability to freeze-thawing and the effect of buffer use (upper panel, green = buffer, blue=no buffer). Loadings plot analysis (lower panel) to identify spectral regions responsible for outlying samples (lower panel)

The effect of freeze-thawing in the presence of buffer was evaluated using an OPLS model (NOESY R^2X=0.496, R^2Y=0.99, Q^2=0.504; CPMG R^2X=0.171, R^2Y=0.998, Q^2=0.351). A linear effect of freeze-thawing was seen in both the NOESY and CPMG spectra within the models. Examination of an S plot and using the Pearson correlation coefficients demonstrated several metabolites to be responsible including: acetone, glucose, citrate and alanine. STOCSY analysis confirmed the identity of these peaks. Evaluation of the spectra did not reveal a change in signal for these metabolites but rather a selective shift of these peaks with freeze-thawing, causing a minor peak mal-alignment (NOESY p=0.33, CPMG p=0.31). The same effect, but larger and more significant, was seen in the absence of buffer (NOESY p=0.019, CPMG p=0.055).

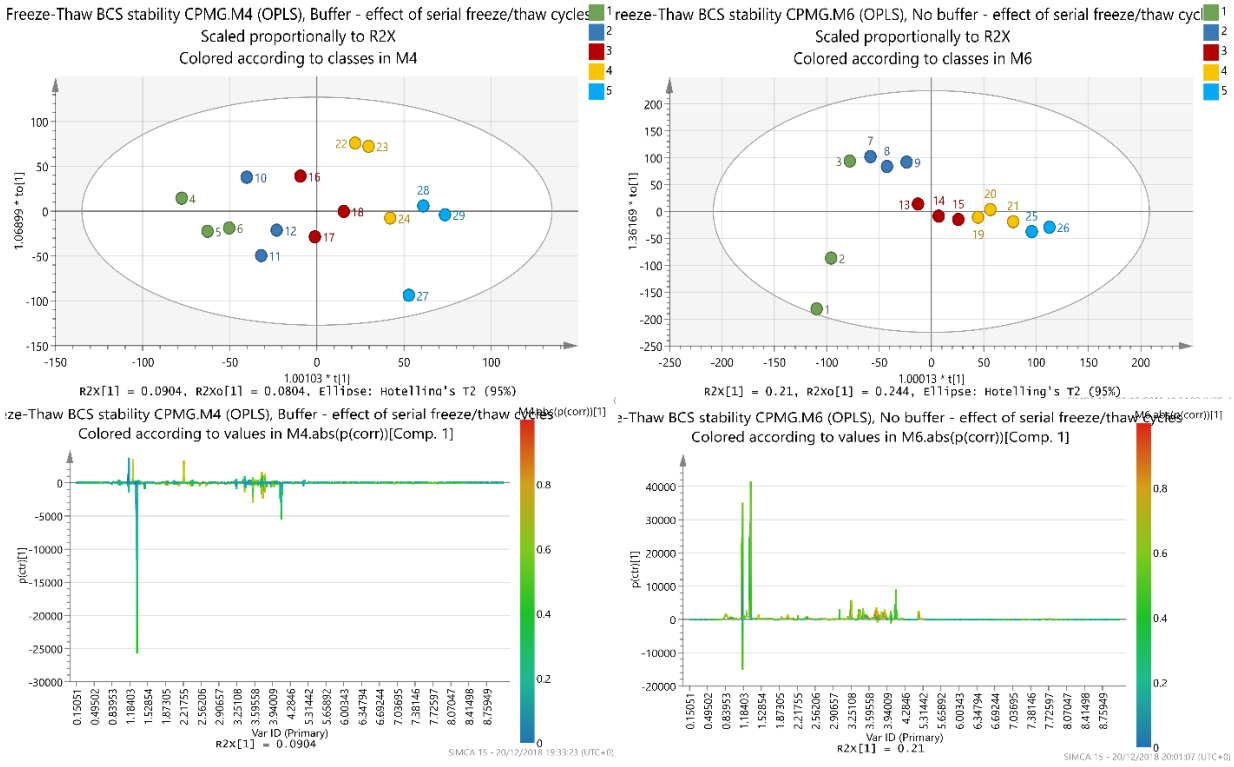


Figure 25 - OPLS analysis of CPMG NMR data of BCS stability to freeze-thawing in the presence (left panel) and absence (right panel) of phosphate buffer. OPLS plot (upper panel) and its corresponding S plot (lower panel). (Green = 1 cycle, blue = 2 cycles, red = 3 cycles, yellow = 4 cycles, magenta = 5 cycles)

5.5.3 The effect of NMR phosphate buffer

The effect of phosphate buffer addition was analysed by direct comparison of the two cohorts of samples. PCA analysis (NOESY R^2X=0.435, Q^2=0.309; CPMG R^2X=0.269, Q^2=0.117) suggested a separation between the buffered and non-buffered samples (see Figure 26). The NOESY data model did not provide a satisfactory distinction between the groups in the first two components. However, a distinction was seen when applying the second and third components. The responsible metabolites were localised to the lipids. The CPMG data concurred, identifying lipids, phospholipids, GAG and isopropyl alcohol.

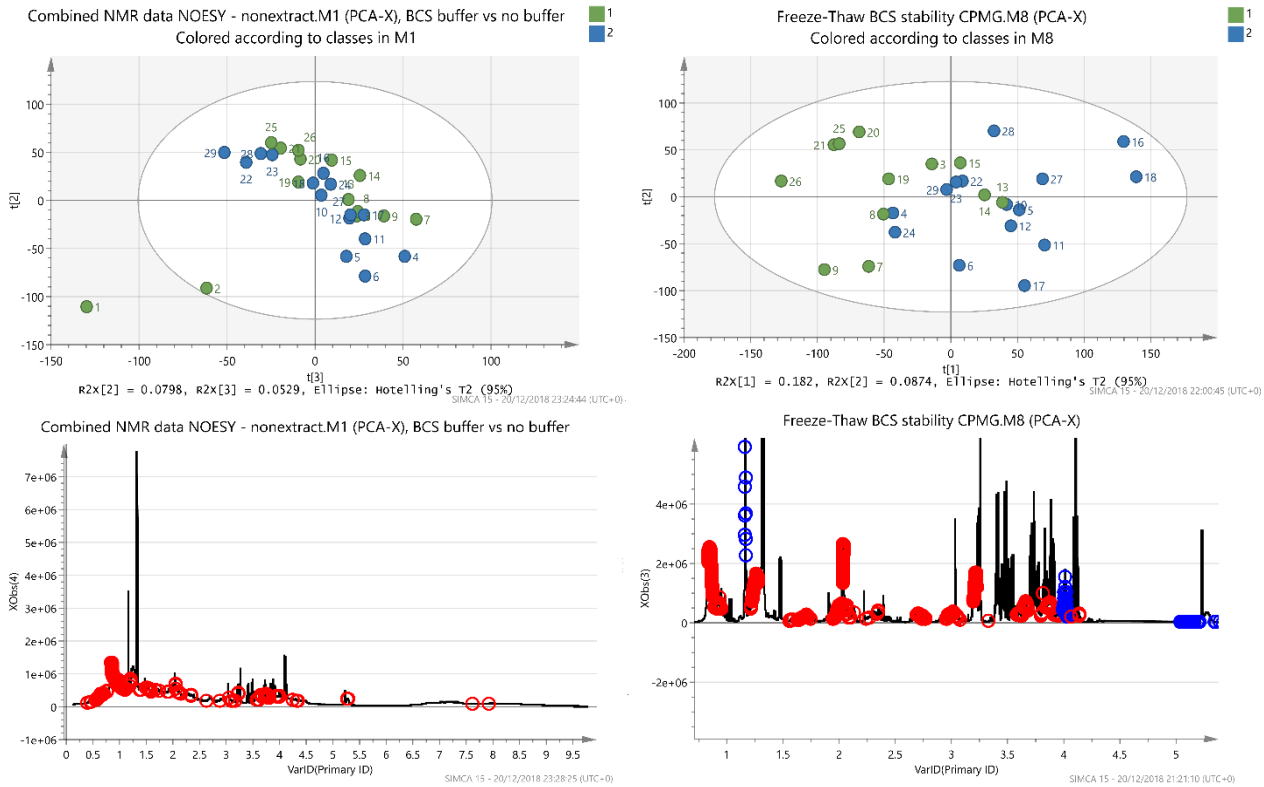


Figure 26 – PCA analysis of NOESY (left) and CPMG (right) NMR data to test the effect of phosphate buffer upon BCS metabolic stability (upper panel, green = buffer, blue=no buffer). Loadings plot analysis (lower panel) to identify spectral regions affected by presence of the phosphate buffer.

OPLS-DA models (NOESY $R^2X=0.386$, $R^2Y=0.96$, $Q^2=0.109$; CPMG $R^2X=0.234$, $R^2Y=0.957$, $Q^2=0.56$) reinforced this result with S plot analysis providing regions of high correlation to the fatty acyl lipids (NOESY p=0.57) and isopropyl alcohol (CPMG p=0.00086), (see Figure 27).

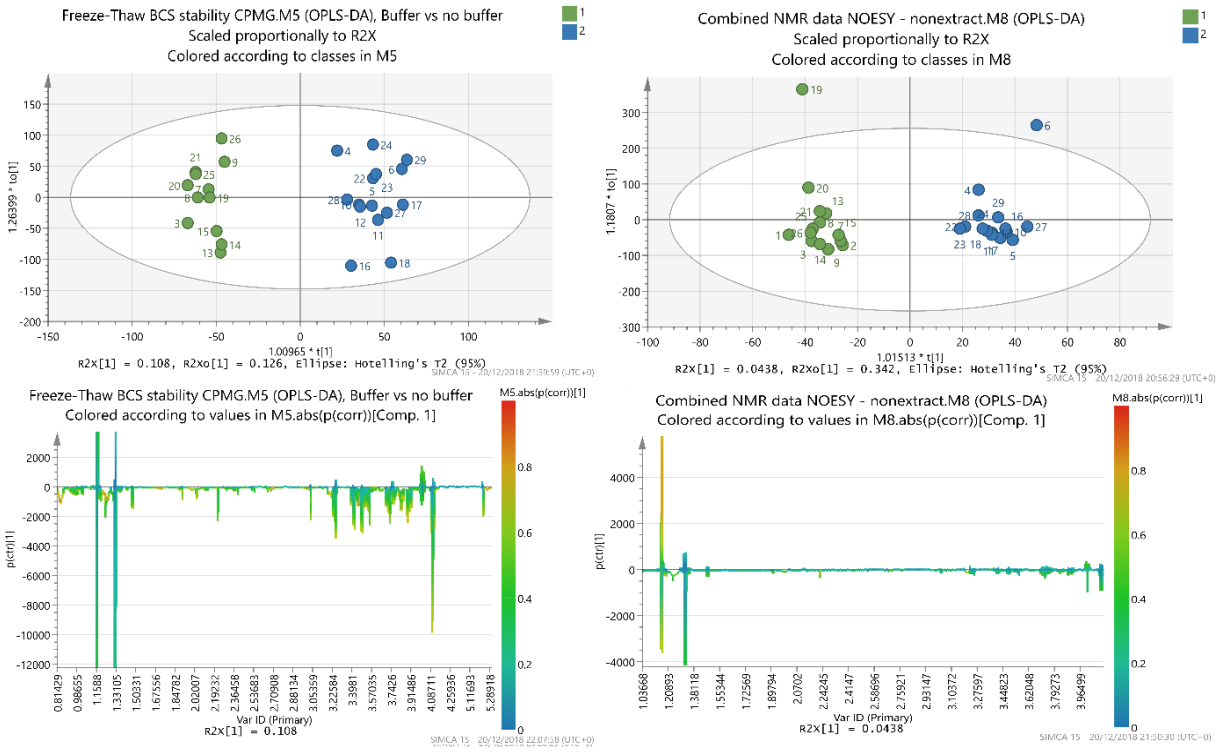


Figure 27 - OPLSDA analysis of CPMG NMR data (left panel) and NOESY NMR data (right panel) to test the effect of phosphate buffer addition. OPLS plot (upper panel) and its corresponding S plot (lower panel), (green = buffer, blue=no buffer)

5.6 Discussion

The initial observations of the handling and processing of BCS revealed significant differences in mechanical properties compared to the HSF samples.

The application of the proposed HSF methods in the BCS analysis was successful. The NMR methods were robust except for a single sample. There were no concerns with the use of TSP as a chemical shift reference. Attempts to use the TSP as an internal reference of signal intensity were unsuccessful due to the change in TSP signal magnitude with repeated freeze-thawing cycles. The reason for this is unclear. It is hypothesised that this may be secondary to a changing protein binding effect.

The BCS surrogate had a progressive change in chemistry during freeze-thaw cycles, suggesting a pH, sedimentation or macromolecule effect from repeated freeze-thawing. The effect was confined to specific metabolites (acetone, glucose, citrate and alanine). The pH effect may have caused a minor change in chemical shift. This is supported by the observation that the metabolites affected were those

reported to be readily shifted in minor pH perturbations (215). In addition, the effect was exaggerated in the absence of phosphate buffer and was statistically significant in the CPMG data. Whilst effecting the chemistry of the sample, it is unlikely that this will have a significant effect upon any metabolic profiling dataset. This is because samples undergo a spectral alignment prior to any dataset analysis and this should adjust for any minor chemical shifts between analysed samples. However, the changing chemistry of the BCS stocks with repeated freeze-thawing suggests that repeated freeze thawing should be avoided, and once defrosted, a sample should not be refrozen. Although not measured directly, it is reasonable to assume that the same effect will occur with HSF samples; repetitive freeze thawing should be avoided.

The gelatinous property of the samples resulting from their high protein content makes them difficult to pipette into the NMR tubes in the absence of buffer. It is assumed that HSF will also have these problems and to a greater extent than BCS. Minor changes were observed in the lipid peak signals in the absence of buffer.

The buffer has practical disadvantages both in efficiency and the need for water peak suppression. Despite the technical and logistical disadvantages of using buffer, the use is advocated. Perturbations of chemical shift were amplified in its absence, particularly the lipid peaks. These problems were observed in the batch BCS; variations in pH are likely to be far greater in a population of HSF samples. The use of non-homogenous human samples with disease is expected to introduce great variation into the composition, protein content and pH.

The application of buffer prior to sample freezing did raise some issues. The TSP peak intensity (present in the buffer) was affected by freeze-thawing as well as the buffering capacity of sample.

This data demonstrates that the analysis method is robust using sample buffer and TSP as a reference, when applied to BCS after defrosting. Thus, BCS is proposed as a biological QC sample in the NMR analysis of HSF, providing that all samples are defrosted simultaneously, originate from the same batch and are analysed together.

6 Chapter 6: The metabolic stability of human synovial fluid to sample collection, sample handling and short-term storage

6.1 Introduction

The metabolic evaluation of HSF requires knowledge of the impact of sample collection and handling prior to any long-term storage and subsequent analysis. Any effect on the metabolic profile of samples is likely be inconsistent and therefore produce changes which could be attributed erroneously to joint disease.

The timing of HSF sample collection is unpredictable making it impossible to control the period before they enter long-term storage. Intraoperative sampling of HSF can only take place if there is a joint effusion (meaning an excess of synovial fluid), and usually this is only discovered during the operation. Furthermore, the timings can be clinically and logistically unpredictable. Non-operative sampling (perhaps for an acutely inflamed joint) typically occurs an emergency department and thus timing cannot be anticipated.

The logistical challenges also introduce variations after HSF collection. Cross site sampling is common, often requiring inter-site transit before processing and storage. The length of time before refrigeration depends on the clinical staff and factors such as emergencies or the length of the operation. Furthermore, sample processing may only be possible at the end of the day so the refrigeration time will differ between samples harvested in the morning and the afternoon. In summary, the above factors mean that variation in the metabolic profile of HSF may be introduced due to sample collection and storage, resulting from variable timings and temperatures. This variation needs to be understood and any effect upon the measured metabolism quantified before any interpretation of metabolic perturbations can be made confidently.

6.1.1 The reported metabolic stability of human synovial fluid

The stability of other biofluids has been reported but the stability of synovial fluid has seldom been studied and is worthy of attention (see Table 11) (181, 216, 217).

A systematic literature search was performed with inclusion criteria for articles studying small molecule metabolism in synovial fluid as a primary or secondary outcome in any species analysed by MS or NMR. Studies were searched in English but review articles or narratives without original research were excluded. The MEDLINE and EMBASE databases used the following Boolean search strings (last performed 10th July 2019):

1. "metabo#omic" OR "metabolome" OR "metabolic adj profil*" OR "metabolite" OR "biomarker" OR "biologic adj marker"
2. "synovia*" OR "joint fluid" or "articular fluid" OR "synovium"

The articles were scrutinised for any investigation into the metabolic stability of the synovial fluid in the storage or analysis process. 86 abstracts were initially identified with 6 reports suitable for inclusion (see Figure 28).

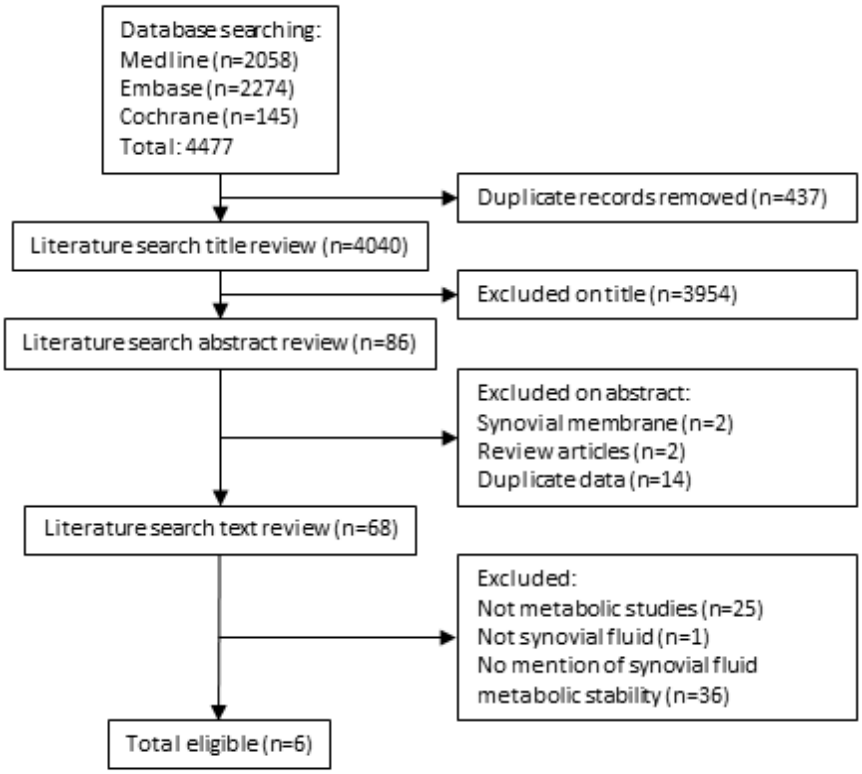


Figure 28 - Flowchart of study selection

Of the studies identified, the majority mention sample stability in a cursory statement without reference to the level of study or the methods used to validate that statement. In one case a study specifically investigated storage of HSF. The studies are summarised (see Table 11).

First Author & Year	Country of Origin	Species	Joint	Diagnosis	Controls	Sample size
Schiller et al. 1996(60)	Germany	Human	Knee	RA	Not stated	25
Parkes et al. 1991(58)	UK	Human	Knee	RA	Not stated	9
Naughton et al. 1993(140)	UK	Human	Knee	RA	Not stated	22
Naughton et al. 1993(158)	UK	Human	Knee	RA	Not stated	17
Hugle et al. 2012(106)	Switzerland	Human	Knee	Arthritis NOS	Not stated	Not stated
Damyanovich et al. 2000(206)	Canada	Human	Knee	OA	Yes	12

Table 11 - Articles reporting metabolic stability of synovial fluid

In the process of investigating the effect of hypochlorous acid on 25 synovial fluids, Schiller reported sample stability assessed by NMR spectroscopy over a period of six months at -18ºC (60). No further

details were given. The analysis of free iron ions in SF using NMR spectroscopy led to the conclusion that the sample was stable during storage. Samples were collected into heparinised tubes and stored at -70ºC with no time-period stated (58). Naughton et al. gave two reports for the metabolic features of RA and inflammation in synovial fluid, both analysed by NMR spectroscopy. They stated stability using control samples with storage at -20ºC for 72 hours and -70ºC for 8 days respectively (140, 158).

A single report studied the effect of repeat freeze-thaw cycles and long-term storage. Control samples were analysed within 24 hours of collection. There was no mention of the elapsed time to analysis or the temperature at which the control samples were kept prior to analysis. Conversely the study of repeated freeze-thawing from -75ºC to room temperature had no significant effect upon either the macromolecular or low molecular weight species. This was despite a hypothesis that the high protein content of the samples would leave them liable to denaturation, affecting the small molecule binding and content (206).

6.1.2 Study requirements

In our collection protocols it is possible (although not advised) that a sample may remain at room temperature for the duration of a surgical procedure or be refrigerated until the end of the day (see 12.13). Whilst it is likely that the sample will only be subjected to a single freeze-thaw cycle, we wished to study any effects of freeze-thawing. Therefore, a study was designed to test the effect of freeze-thawing, short-term room temperature and refrigerated storage.

The probability of detecting a storage effect on a single metabolite is greatest if the testing uses an HSF sample which represents the breadth of the metabolic variations in terms of age, sex, ethnicity, diet, metabolic disease and joint disease. Therefore, to provide breadth of representation a pooled sample was used.

6.2 Hypothesis

The metabolic profile of HSF will be susceptible in a measurable way to variations in temperature.

6.3 Aim

To study how the metabolic profile of HSF detected by NMR spectroscopy is affected by time elapsed at varying temperatures, and repetitive freeze-thawing.

6.4 Materials and methods

6.4.1 Sample collection

HSF was collected in the early morning from three patients. Patient A underwent knee joint arthroplasty for end-stage OA (female, 90 years old, white English). Patient B underwent knee arthroscopy for mechanical symptoms and had moderate osteoarthritic joint changes (female, 50 years old, black African). Patient C underwent arthrocentesis of a revision total hip arthroplasty for loosening but with no infection found (female, 67 years old, white English). The operations were coordinated so that the samples were harvested within 30 minutes of each other. Samples were immediately pooled in equal volumes to create a combined representative HSF sample.

6.4.2 Sample preparation

The pooled sample was then prepared for storage in accordance with the trialled methods (centrifugation at room temperature, 10000 ***g*** for 15min before decanting the supernatant and storing in a sealed Eppendorf container) (see 4.4, 12.12). Aliquots were then subjected to a variety of storage conditions and handling practices (see Table 12). These were designed to replicate the expected practices in the collection of clinical HSF samples: room temperature for 0, 4 or 12 hours; refrigeration for 0, 4, 8 or 12 hours, repeated freeze-thaw cycles (-80ºC to room temperature) at 24, 48, 72, 96 or 120 hours. The pooled sample was then prepared for storage in accordance with the trialled methods (centrifugation at room temperature, 10000 ***g*** for 15min before decanting the supernatant and storing in a sealed Eppendorf container) (see 4.1.2, 12.12). Each batch of pooled HSF samples was analysed in triplicate using differing research staff, preparation equipment and refrigeration equipment. As before all consumables were of the same source and batch to reduce the possibility of any contamination (see 4.4.1).

Sample number	Description
1	Immediate -80°C freezing
2	Room temperature 4 hrs, -80°C freezing
3	Room temperature 12 hrs, -80°C freezing
4	4°C for 4 hrs, -80°C freezing
5	4°C for 8 hrs, -80°C freezing
6	4°C for 12 hrs, -80°C freezing
7	Immediate -80°C freezing, rethaw at 24hrs and re-freeze -80°C
8	Immediate -80°C freezing, rethaw at 24hrs, 48hrs and re-freeze -80°C
9	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs and re-freeze -80°C
10	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80°C
11	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80°C

Table 12 - Sample storage conditions for the evaluation of HSF collection practices

6.4.3 Sample analysis

All samples were defrosted together at room temperature with no external warming applied. The samples were combined in a 1:1 v/v mix with buffer (142mM $Na_2HPO_4.x.7H_2O$; 4% w/v NaN_3; TSP) before being centrifuged at 10,000 ***g*** for 5 min at 4ºC and transferred (550µl) to a 5mm NMR tube, giving a TSP concentration of 40µgml^{-1}. Tubes were randomised in order and loaded onto the Samplejet 96 well autosampler refrigerated at 4ºC and analysed using a Bruker® Avance III 600MHz spectrometer (Bruker Biospin®, Rheinstetten, Germany). Data acquisition and processing parameters were identical to those agreed previously for HSF analysis, generating both NOESYGPPR1D and CPMG spectra (see 4.4.3). Spectra were manually phased and baseline corrected and calibrated to the ^{1}H proton peak signal of α-glucose (δ=5.23ppm). This endogenous refence was used because it was stable at the working pH and contained a non-overlapped peak suitable for referencing (218). No normalisation of the data was performed since the analysis used only one sample and this step may have the effect of supressing any measured change in metabolite levels by handling and storage.

Bioblanks of BCS were used throughout the analysis as QC samples to demonstrate no cross-contamination and the consistency of analysis.

Level 2 metabolite identification was achieved from previous assignment (see 4.1.7) (219), 2D NMR experiments (JRES, TOCSY and HSQC), the Human Metabolite Database (HMDB) and statistical total correlation spectroscopy (STOCSY).

6.4.4 Statistical analysis

Once identified, a suitable metabolite peak was chosen, and signal quantified using non-linear baseline adjustment followed by trapezoid integration. The integrals were referenced to TSP as an internal standard, thereby removing any effect of instrument variation. This peak was preferred, despite being susceptible to protein binding, a clear spectral region means peak integration is consistent. Signal intensities for an individual metabolite were then referenced to the "immediate -80ºC" sample as a control, thereby providing a ratio of change from the control sample, known hereafter as the relative ratio (RR). Replicates of analysis provided confidence intervals at each timepoint, a method used previously to report metabolite stability over time (220).

The QC samples were evaluated for five key metabolites, diverse in chemical properties (valine, alanine, glucose, lactate and isopropyl alcohol). A coefficient of variation was used to demonstrate comparability and analytical stability of samples throughout and between the analytical runs.

A one-way Analysis of Variance (ANOVA) was performed for each metabolite during each storage condition: room temperature, 4ºC refrigeration and repeated -80ºC freeze-thaw cycling. Post-hoc paired t-testing was used to examine any significance at individual timepoints.

The SIMCA™ software (Sartorius Stedim Biotech, Germany) was used to apply PCA, followed by an OPLS-DA to compare the effect of long-term low temperature storage. The models were evaluated using the R^2X and R^2Y values, thereby indicating how well the variance is represented. The cross-validation Q^2Y metric was used to assess the strength of model prediction. The significance of the metabolic differences between the models was reported using p-values of the cross-validation. A permutation test was performed 100 times. An S-line plot examined spectral regions of significance

reporting an absolute correlation of (-0.85 <pCORR> 0.85, to yield >95% confidence) (221). An S-line plot examined spectral regions of significance, reporting an absolute correlation of (-0.85 <pCORR> 0.85, to yield >95% confidence).

6.5 Results

The consistency of TSP internal standard signal in between samples was validated with a coefficient of variation consistently less than 4% (see Table 13).

Sample	TSP NOESY	TSP CPMG
Immediate -80oC freezing	96649	198035
Room temperature 4 hrs, -80oC freezing	98962	207535
Room temperature 12 hrs, -80oC freezing	103501	200548
4oC for 4 hrs, -80oC freezing	97582	204011
4oC for 8 hrs, -80oC freezing	104139	208385
4oC for 12 hrs, -80oC freezing	91340	206612
Immediate -80oC freezing, rethaw at 24hrs and re-freeze -80oC	94830	204462
Immediate -80oC freezing, rethaw at 24hrs, 48hrs and re-freeze -80oC	108336	192991
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80oC	101061	204564
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80oC	98252	205101
Immediate -80oC freezing	101198	194162
Room temperature 4 hrs, -80oC freezing	94821	197681
Room temperature 12 hrs, -80oC freezing	99812	205517
4oC for 4 hrs, -80oC freezing	97931	190107
4oC for 8 hrs, -80oC freezing	100192	199702
4oC for 12 hrs, -80oC freezing	100140	196400
Immediate -80oC freezing, rethaw at 24hrs and re-freeze -80oC	96439	190374
Immediate -80oC freezing, rethaw at 24hrs, 48hrs and re-freeze -80oC	100466	194313
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs and re-freeze -80oC	101691	200507
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80oC	96026	186661
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80oC	102337	201635
Immediate -80oC freezing	93728	209850
Room temperature 4 hrs, -80oC freezing	94045	190991
Room temperature 12 hrs, -80oC freezing	95600	193431
4oC for 4 hrs, -80oC freezing	91307	185359
4oC for 8 hrs, -80oC freezing	98162	198971
4oC for 12 hrs, -80oC freezing	95672	192352
Immediate -80oC freezing, rethaw at 24hrs and re-freeze -80oC	93293	192266
Immediate -80oC freezing, rethaw at 24hrs, 48hrs and re-freeze -80oC	95178	192450
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs and re-freeze -80oC	97006	196116
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80oC	94749	202136
Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80oC	95812	195799
Standard deviation	*3830.0*	*6542.2*
Mean	*97820.5*	*198094.5*
Coefficient of variation	***0.0392***	***0.0330***

Table 13 - TSP integrals to demonstrate analytical stability during the analysis

The signals of the selected metabolites in the QC samples demonstrated stability with a coefficient of variance (CV) consistently <1.5% (see Table 14).

	Metabolite				
Sample	*Valine*	*Alanine*	*Glucose*	*Lactate*	*Isopropyl Alcohol*
Short-term storage	2075392	5224273	8578675	28308826	9023262
Short-term storage	2084071	5307788	8613334	28961417	9140835
Short-term storage	2106275	5378402	8679144	29001017	9196154
Short-term storage	2044623	5269477	8596172	28078150	8972968
Short-term storage	2077439	5271857	8575613	28833979	9073142
Short-term storage	2070776	5295851	8603419	28436578	9143400
Standard deviation	19974	51432	37839	382081	83813
Mean	2076429	5291275	8607726	28603328	9091627
CV	0.00962	0.00972	0.00440	0.01336	0.00922

Table 14 - QC sample NMR analysis of short-term storage conditions in HSF

Close evaluation of the NMR spectra before and after storage effects revealed several differences in the metabolite signals. Furthermore, the metabolites differed between spectral acquisition methods of NOESYGPPR1D and CPMG.

Peak integral ratio change compared to the Immediate -80°C freezing and post hoc paired student T-test	Room temperature 4hrs, -80°C freezing	Room temperature 12hrs, -80°C freezing	4°C for 4 hrs, -80°C freezing	4°C for 8 hrs, -80°C freezing	4°C for 12 hrs, -80°C freezing	Immediate -80°C freezing, rethaw at 24hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs and re-freeze -80°C	Immediate -80oC freezing, rethaw at 24hrs, 48hrs,72hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80oC	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80°C
Metabolite										
Cholesterol C18	0.992+/-0.024 (0.478)	1.014+/-0.019 (0.467)	0.996+/-0.021 (0.49)	0.988+/-0.02 (0.471)	1.01+/-0.015 (0.474)	0.982+/-0.041 (0.448)	0.969+/-0.003 (0.42)	0.999+/-0.037 (0.45)	0.973+/-0.015 (0.299)	0.985+/-0.031 (0.38)
CH3 (fatty acyl residues) lipids	0.993+/-0.066 (0.491)	1.029+/-0.069 (0.47)	0.994+/-0.053 (0.492)	1.05+/-0.037 (0.443)	1.122+/-0.132 (0.392)	1.047+/-0.14 (0.446)	1.135+/-0.058 (0.357)	1.054+/-0.142 (0.384)	1.042+/-0.027 (0.243)	1.076+/-0.049 (0.192)
2-Hydroxybutyrate	0.912+/-0.077 (0.324)	0.873+/-0.095 (0.231)	0.932+/-0.028 (0.27)	0.858+/-0.088 (0.231)	0.833+/-0.085 (0.218)	0.85+/-0.092 (0.224)	0.993+/-0.067 (0.483)	0.962+/-0.035 (0.499)	0.904+/-0.114 (0.089)	0.825+/-0.09 (0.386)
Isoleucine	0.915+/-0.037 (0.271)	0.986+/-0.021 (0.452)	0.978+/-0.021 (0.419)	0.984+/-0.014 (0.426)	0.96+/-0.041 (0.385)	0.955+/-0.007 (0.322)	1.01+/-0.038 (0.468)	0.97+/-0.033 (0.464)	0.956+/-0.007 (0.344)	0.995+/-0.008 (0.208)
Leucine	0.976+/-0.033 (0.416)	0.954+/-0.028 (0.355)	1.025+/-0.017 (0.408)	0.946+/-0.009 (0.307)	0.951+/-0.063 (0.374)	0.992+/-0.02 (0.471)	1.008+/-0.019 (0.472)	1.003+/-0.064 (0.497)	0.993+/-0.036 (0.364)	0.944+/-0.012 (0.235)
Valine	1.096+/-0.005 (0.051)	1.044+/-0.034 (0.05)	1.171+/-0.028 (0.003)	1.073+/-0.019 (0.133)	1.099+/-0.033 (0.032)	1.097+/-0.057 (0.065)	1.077+/-0.062 (0.135)	1.124+/-0.026 (0.134)	1.091+/-0.055 (0.36)	1.034+/-0.022 (0.349)
3-Hydroxyisobutyrate	1.033+/-0.03 (0.138)	1.022+/-0.028 (0.324)	1.017+/-0.041 (0.425)	1.051+/-0.061 (0.337)	0.961+/-0.091 (0.399)	0.994+/-0.054 (0.48)	1.04+/-0.057 (0.355)	1.074+/-0.066 (0.146)	1.101+/-0.101 (0.135)	0.999+/-0.11 (0.455)
2-ketoisovalerate	0.859+/-0.03 (0.079)	0.78+/-0.036 (0.011)	0.962+/-0.045 (0.277)	0.966+/-0.172 (0.413)	0.89+/-0.103 (0.283)	0.785+/-0.002 (0.043)	0.935+/-0.045 (0.09)	0.825+/-0.147 (0.346)	0.802+/-0.07 (0.107)	0.948+/-0.089 (0.191)
3-Hydroxybutyrate	0.984+/-0.016 (0.476)	1.012+/-0.028 (0.483)	0.98+/-0.019 (0.47)	0.996+/-0.017 (0.494)	0.984+/-0.02 (0.476)	0.957+/-0.039 (0.44)	1.045+/-0.033 (0.438)	1.006+/-0.013 (0.388)	1.043+/-0.003 (0.318)	0.982+/-0.013 (0.441)
CH2 (fatty acyl residues) lipids	0.979+/-0.027 (0.456)	1.028+/-0.008 (0.447)	0.993+/-0.033 (0.487)	1.002+/-0.033 (0.497)	1+/-0.04 (0.5)	0.978+/-0.043 (0.453)	0.958+/-0.039 (0.403)	0.97+/-0.037 (0.424)	1.015+/-0.043 (0.277)	1.027+/-0.021 (0.323)
L-Lactate	0.95+/-0.087 (0.431)	0.954+/-0.036 (0.418)	0.868+/-0.062 (0.308)	0.998+/-0.061 (0.497)	0.884+/-0.039 (0.311)	0.855+/-0.058 (0.28)	0.926+/-0.037 (0.374)	1.077+/-0.036 (0.422)	0.932+/-0.045 (0.195)	0.87+/-0.107 (0.35)
Threonine	1.159+/-0.145 (0.276)	1.039+/-0.137 (0.44)	1.083+/-0.051 (0.312)	1.094+/-0.109 (0.343)	0.989+/-0.091 (0.461)	0.976+/-0.123 (0.459)	1.057+/-0.086 (0.373)	0.827+/-0.065 (0.385)	0.957+/-0.075 (0.298)	0.955+/-0.217 (0.32)
Alanine	0.933+/-0.054 (0.382)	0.936+/-0.04 (0.35)	0.967+/-0.03 (0.43)	0.941+/-0.011 (0.392)	0.961+/-0.04 (0.431)	0.911+/-0.042 (0.317)	0.99+/-0.03 (0.478)	1.002+/-0.012 (0.428)	1.01+/-0.019 (0.398)	0.946+/-0.042 (0.399)
Lysine	1.002+/-0.042 (0.494)	1.001+/-0.046 (0.497)	1.012+/-0.01 (0.473)	1.085+/-0.078 (0.368)	1.008+/-0.03 (0.483)	1.011+/-0.024 (0.472)	1.089+/-0.047 (0.345)	1.01+/-0.029 (0.413)	1.037+/-0.017 (0.459)	0.987+/-0.036 (0.387)
Arginine	0.993+/-0.171 (0.492)	0.971+/-0.082 (0.456)	0.983+/-0.115 (0.478)	0.849+/-0.127 (0.298)	0.966+/-0.091 (0.451)	0.96+/-0.09 (0.433)	1.127+/-0.143 (0.353)	1.095+/-0.215 (0.427)	0.947+/-0.039 (0.361)	0.978+/-0.115 (0.406)
Acetate	1.044+/-0.09 (0.334)	0.9+/-0.219 (0.31)	1.071+/-0.104 (0.342)	0.944+/-0.105 (0.423)	1.022+/-0.093 (0.429)	0.98+/-0.086 (0.471)	1.076+/-0.067 (0.333)	1.032+/-0.21 (0.374)	1.081+/-0.139 (0.223)	1.044+/-0.095 (0.306)
CH2C=C (fatty acyl chains) lipids	1.052+/-0.012 (0.36)	0.939+/-0.005 (0.347)	1.021+/-0.074 (0.413)	1.037+/-0.073 (0.433)	0.953+/-0.02 (0.391)	1.065+/-0.105 (0.393)	1.042+/-0.021 (0.388)	0.93+/-0.052 (0.489)	0.964+/-0.026 (0.442)	0.95+/-0.01 (0.227)
N-acetyl groups	1.011+/-0.009 (0.433)	1.02+/-0.007 (0.395)	1.014+/-0.014 (0.404)	1.021+/-0.012 (0.387)	1.014+/-0.012 (0.416)	1.022+/-0.011 (0.362)	0.98+/-0.009 (0.401)	1.021+/-0.009 (0.469)	0.986+/-0.009 (0.358)	1.029+/-0.004 (0.417)
Glutamate	0.961+/-0.022 (0.386)	1.035+/-0.032 (0.417)	0.962+/-0.025 (0.408)	0.947+/-0.026 (0.376)	0.974+/-0.056 (0.447)	0.992+/-0.026 (0.475)	1.009+/-0.066 (0.481)	0.976+/-0.121 (0.488)	0.968+/-0.023 (0.327)	1.013+/-0.118 (0.402)
Glutamine	0.965+/-0.021 (0.399)	0.961+/-0.032 (0.39)	0.984+/-0.013 (0.456)	0.984+/-0.023 (0.465)	0.95+/-0.031 (0.348)	0.967+/-0.017 (0.408)	0.995+/-0.02 (0.486)	0.999+/-0.013 (0.45)	1.005+/-0.014 (0.38)	0.971+/-0.006 (0.237)
Methionine	0.952+/-0.065 (0.369)	0.951+/-0.071 (0.35)	0.978+/-0.006 (0.433)	1.011+/-0.015 (0.472)	0.981+/-0.037 (0.439)	0.982+/-0.019 (0.445)	1.027+/-0.018 (0.409)	1.014+/-0.042 (0.449)	1.006+/-0.005 (0.429)	1.009+/-0.013 (0.296)
Acetone	1.075+/-0.079 (0.277)	1.023+/-0.07 (0.422)	0.84+/-0.092 (0.093)	0.839+/-0.021 (0.236)	0.969+/-0.049 (0.43)	0.976+/-0.064 (0.454)	0.972+/-0.026 (0.433)	0.919+/-0.126 (0.454)	1.002+/-0.021 (0.269)	1.101+/-0.076 (0.293)
Acetoacetate	0.928+/-0.013 (0.36)	0.904+/-0.061 (0.328)	1.02+/-0.041 (0.467)	0.981+/-0.019 (0.459)	0.908+/-0.064 (0.33)	0.963+/-0.066 (0.408)	0.977+/-0.059 (0.434)	0.949+/-0.05 (0.433)	1.042+/-0.06 (0.4)	0.942+/-0.04 (0.445)
Pyruvate	1.004+/-0.006 (0.486)	1.046+/-0.044 (0.326)	1.072+/-0.046 (0.335)	0.971+/-0.013 (0.383)	0.987+/-0.019 (0.447)	0.991+/-0.038 (0.476)	1.018+/-0.025 (0.435)	1.005+/-0.029 (0.452)	1.024+/-0.074 (0.184)	1.027+/-0.063 (0.398)
Succinate	0.895+/-0.115 (0.337)	0.867+/-0.058 (0.266)	0.906+/-0.143 (0.125)	0.971+/-0.124 (0.459)	0.885+/-0.174 (0.328)	1.025+/-0.072 (0.463)	0.987+/-0.072 (0.475)	0.937+/-0.012 (0.478)	1.017+/-0.036 (0.321)	0.921+/-0.136 (0.26)
Pyroglutamine	1.269+/-0.107 (0.264)	1.138+/-0.107 (0.364)	1.129+/-0.151 (0.197)	1.166+/-0.137 (0.347)	1.173+/-0.163 (0.356)	1.166+/-0.081 (0.328)	1.217+/-0.122 (0.307)	1.253+/-0.264 (0.299)	1.194+/-0.162 (0.279)	0.975+/-0.027 (0.376)
Citrate	0.992+/-0.083 (0.49)	0.932+/-0.055 (0.407)	1.062+/-0.081 (0.422)	0.977+/-0.103 (0.473)	0.964+/-0.086 (0.441)	0.833+/-0.063 (0.291)	1.046+/-0.062 (0.432)	0.976+/-0.127 (0.424)	1.119+/-0.023 (0.354)	0.816+/-0.041 (0.15)
Dimethylalanine	3.362+/-1.553 (0.132)	1.431+/-0.273 (0.098)	1.159+/-0.171 (0.26)	0.959+/-0.184 (0.429)	1.152+/-0.272 (0.315)	1.414+/-0.512 (0.231)	1.246+/-0.326 (0.233)	1.448+/-0.346 (0.212)	1.307+/-0.301 (0.119)	1.155+/-0.2 (0.246)
N N-dimethylglycine	1.024+/-0.235 (0.48)	1.051+/-0.168 (0.457)	0.891+/-0.042 (0.371)	0.931+/-0.15 (0.43)	0.831+/-0.118 (0.328)	0.818+/-0.155 (0.335)	0.731+/-0.145 (0.268)	0.856+/-0.082 (0.387)	0.854+/-0.332 (0.27)	0.953+/-0.204 (0.352)
Creatine	0.995+/-0.037 (0.495)	0.98+/-0.062 (0.481)	1.001+/-0.031 (0.499)	0.966+/-0.016 (0.469)	0.805+/-0.051 (0.311)	0.902+/-0.077 (0.41)	0.939+/-0.11 (0.427)	0.953+/-0.008 (0.361)	0.988+/-0.063 (0.35)	0.856+/-0.053 (0.247)
Creatinine	0.969+/-0.047 (0.439)	0.975+/-0.059 (0.454)	0.948+/-0.037 (0.397)	0.952+/-0.018 (0.413)	0.952+/-0.036 (0.4)	0.925+/-0.067 (0.337)	0.986+/-0.054 (0.477)	0.977+/-0.041 (0.47)	1.016+/-0.056 (0.331)	0.983+/-0.029 (0.248)
Ornithine	1.05+/-0.093 (0.389)	1.189+/-0.078 (0.251)	1.104+/-0.101 (0.368)	1.084+/-0.091 (0.398)	1.082+/-0.084 (0.379)	1.181+/-0.08 (0.29)	1.021+/-0.11 (0.448)	1.123+/-0.047 (0.376)	1.123+/-0.086 (0.428)	1.18+/-0.088 (0.423)
Phenylalanine	0.921+/-0.059 (0.319)	0.968+/-0.032 (0.327)	1.036+/-0.039 (0.295)	1.053+/-0.111 (0.407)	0.911+/-0.057 (0.269)	0.946+/-0.073 (0.385)	0.947+/-0.054 (0.368)	0.946+/-0.227 (0.446)	1.025+/-0.041 (0.486)	1.017+/-0.062 (0.476)
Dimethylsulfone	0.947+/-0.092 (0.398)	1.028+/-0.09 (0.466)	0.947+/-0.024 (0.427)	0.945+/-0.047 (0.434)	0.811+/-0.056 (0.244)	0.912+/-0.077 (0.366)	0.923+/-0.041 (0.38)	0.888+/-0.013 (0.475)	0.938+/-0.035 (0.477)	0.87+/-0.065 (0.161)
Choline (free)	1.094+/-0.03 (0.352)	1.078+/-0.052 (0.373)	1.054+/-0.054 (0.419)	0.995+/-0.01 (0.49)	0.959+/-0.022 (0.425)	0.97+/-0.055 (0.446)	1.012+/-0.017 (0.478)	1.008+/-0.028 (0.411)	1.05+/-0.022 (0.354)	0.996+/-0.025 (0.348)
o-phosphocholine	0.945+/-0.073 (0.419)	0.907+/-0.058 (0.344)	1.006+/-0.021 (0.489)	0.945+/-0.028 (0.408)	0.943+/-0.044 (0.409)	0.995+/-0.029 (0.491)	0.968+/-0.035 (0.444)	1.016+/-0.05 (0.448)	0.972+/-0.024 (0.458)	0.975+/-0.034 (0.356)
Glucose	0.999+/-0.085 (0.499)	1.042+/-0.092 (0.421)	1.025+/-0.014 (0.429)	0.923+/-0.037 (0.236)	0.917+/-0.061 (0.301)	0.987+/-0.078 (0.467)	1.045+/-0.094 (0.415)	1.047+/-0.134 (0.463)	1.042+/-0.087 (0.3)	0.994+/-0.024 (0.445)
Proline	0.976+/-0.035 (0.475)	0.952+/-0.034 (0.444)	0.969+/-0.041 (0.459)	0.947+/-0.019 (0.436)	0.938+/-0.045 (0.421)	0.916+/-0.056 (0.39)	0.99+/-0.044 (0.489)	0.976+/-0.047 (0.402)	0.935+/-0.055 (0.292)	0.918+/-0.056 (0.269)
Glycine	1.085+/-0.064 (0.26)	1.018+/-0.025 (0.456)	1.033+/-0.02 (0.426)	1.037+/-0.015 (0.417)	0.926+/-0.074 (0.37)	0.994+/-0.074 (0.486)	0.881+/-0.072 (0.332)	1.029+/-0.062 (0.442)	0.89+/-0.068 (0.141)	1.002+/-0.034 (0.469)
Glycerol	1.017+/-0.039 (0.459)	0.99+/-0.021 (0.475)	0.896+/-0.03 (0.296)	0.984+/-0.018 (0.466)	0.926+/-0.007 (0.344)	0.915+/-0.048 (0.261)	1.05+/-0.037 (0.371)	0.975+/-0.017 (0.47)	0.943+/-0.009 (0.452)	0.884+/-0.003 (0.129)
Myo-inositol	1.141+/-0.159 (0.398)	1.1+/-0.126 (0.419)	1.138+/-0.132 (0.397)	1.183+/-0.176 (0.376)	1.181+/-0.234 (0.381)	1.087+/-0.146 (0.434)	1.24+/-0.12 (0.324)	1.114+/-0.193 (0.354)	1.13+/-0.107 (0.377)	1.108+/-0.075 (0.452)
CH=CH (fatty acyl residues) lipids	1.089+/-0.032 (0.32)	1.043+/-0.082 (0.423)	1.056+/-0.135 (0.279)	1.029+/-0.016 (0.446)	1.079+/-0.082 (0.283)	1.153+/-0.053 (0.227)	1.033+/-0.056 (0.438)	1.062+/-0.018 (0.39)	1.059+/-0.058 (0.238)	1.049+/-0.084 (0.415)
Tyrosine	0.946+/-0.044 (0.227)	0.979+/-0.06 (0.271)	1.031+/-0.023 (0.301)	1.009+/-0.092 (0.472)	1.066+/-0.045 (0.091)	0.907+/-0.066 (0.057)	1.02+/-0.075 (0.36)	0.975+/-0.043 (0.491)	0.996+/-0.021 (0.382)	0.947+/-0.072 (0.235)
Histidine	0.849+/-0.042 (0.197)	1.105+/-0.176 (0.384)	0.963+/-0.034 (0.425)	1.009+/-0.095 (0.487)	1.185+/-0.075 (0.166)	0.999+/-0.047 (0.498)	1.066+/-0.035 (0.32)	1.184+/-0.244 (0.306)	1.153+/-0.116 (0.237)	1.022+/-0.027 (0.469)
Acetaminophen glucuronide	1.194+/-0.212 (0.183)	1.348+/-0.14 (0.217)	1.733+/-0.406 (0.169)	1.904+/-0.253 (0.097)	1.561+/-0.178 (0.138)	1.424+/-0.114 (0.145)	1.347+/-0.164 (0.026)	2.169+/-0.712 (0.169)	1.789+/-0.305 (0.149)	1.753+/-0.088 (0.075)
GAG	0.936+/-0.087 (0.336)	0.969+/-0.108 (0.429)	0.843+/-0.075 (0.144)	0.994+/-0.133 (0.488)	1.054+/-0.13 (0.391)	0.993+/-0.208 (0.49)	1.103+/-0.101 (0.435)	0.914+/-0.074 (0.276)	0.8+/-0.084 (0.017)	1.013+/-0.114 (0.442)
Formate	1.24+/-0.288 (0.286)	1.266+/-0.439 (0.319)	1.362+/-0.275 (0.191)	1.093+/-0.149 (0.353)	0.955+/-0.204 (0.435)	1.049+/-0.147 (0.422)	1.284+/-0.294 (0.249)	0.912+/-0.066 (0.404)	1.237+/-0.027 (0.034)	1.016+/-0.191 (0.468)

Table 15 - Short-term storage results for NMR noesygppr1d HSF analysis reporting the peak integral ratio change relative to the immediate freezing at -80°C result, results significant to the 0.05 confidence level are displayed in red

Peak integral ratio change compared to the Immediate -80°C freezing and post hoc paired student T-test	Room temperature 4hrs, -80°C freezing	Room temperature 12hrs, -80°C freezing	4°C for 4 hrs, -80°C freezing	4°C for 8 hrs, -80°C freezing	4°C for 12 hrs, -80°C freezing	Immediate -80°C freezing, rethaw at 24hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs and re-freeze -80°C	Immediate -80°C freezing, rethaw at 24hrs, 48hrs,72hrs, 96hrs, 120hrs and re-freeze -80°C
Metabolite										
CH3 (fatty+A5:A36 acyl residues) lipids	1.011+/-0.023 (0.482)	1.005+/-0.026 (0.493)	1.024+/-0.017 (0.462)	1.001+/-0.004 (0.498)	1.018+/-0.026 (0.472)	1.039+/-0.041 (0.44)	0.969+/-0.018 (0.412)	0.991+/-0.031 (0.38)	0.969+/-0.02 (0.448)	1.009+/-0.021 (0.486)
2-Hydroxybutyrate	0.961+/-0.049 (0.15)	1.01+/-0.082 (0.456)	1.051+/-0.008 (0.113)	0.983+/-0.057 (0.335)	1.011+/-0.022 (0.036)	0.973+/-0.044 (0.233)	0.995+/-0.079 (0.481)	0.948+/-0.004 (0.484)	0.95+/-0.049 (0.131)	0.98+/-0.057 (0.359)
Isoleucine	1.009+/-0.019 (0.452)	0.973+/-0.026 (0.374)	0.978+/-0.039 (0.4)	1.038+/-0.048 (0.364)	0.948+/-0.02 (0.26)	0.988+/-0.066 (0.461)	1.013+/-0.056 (0.457)	0.983+/-0.034 (0.437)	0.988+/-0.011 (0.428)	0.986+/-0.03 (0.434)
Leucine	0.979+/-0.016 (0.355)	1.028+/-0.019 (0.246)	0.997+/-0.025 (0.475)	1.008+/-0.003 (0.436)	0.984+/-0.023 (0.229)	0.975+/-0.018 (0.291)	1.008+/-0.019 (0.455)	1.011+/-0.024 (0.439)	1.02+/-0.011 (0.346)	0.995+/-0.018 (0.455)
Valine	1.006+/-0.021 (0.258)	1.023+/-0.012 (0.089)	0.997+/-0.012 (0.192)	0.991+/-0.013 (0.354)	1.004+/-0.004 (0.41)	0.981+/-0.018 (0.061)	1.005+/-0.004 (0.43)	1.019+/-0.002 (0.405)	0.993+/-0.007 (0.37)	1.004+/-0.021 (0.418)
3-Hydroxyisobutyrate	1.066+/-0.082 (0.401)	1.21+/-0.125 (0.267)	1.08+/-0.094 (0.363)	1.129+/-0.097 (0.331)	1.002+/-0.073 (0.496)	1.095+/-0.108 (0.377)	1.113+/-0.051 (0.359)	1.227+/-0.375 (0.304)	1.056+/-0.104 (0.422)	0.952+/-0.12 (0.439)
2-ketoisovalerate	0.964+/-0.028 (0.215)	0.77+/-0.17 (0.121)	1.028+/-0.055 (0.332)	1.117+/-0.056 (0.123)	0.869+/-0.158 (0.209)	0.854+/-0.194 (0.227)	0.783+/-0.164 (0.2)	0.84+/-0.127 (0.165)	1.113+/-0.067 (0.099)	0.85+/-0.125 (0.146)
3-Hydroxybutyrate	0.934+/-0.057 (0.391)	0.99+/-0.124 (0.486)	0.946+/-0.047 (0.407)	0.995+/-0.096 (0.492)	0.902+/-0.057 (0.351)	0.991+/-0.108 (0.489)	0.955+/-0.119 (0.457)	1.093+/-0.245 (0.437)	0.983+/-0.118 (0.478)	0.907+/-0.052 (0.342)
CH2 (fatty acyl residues) lipids	1.029+/-0.02 (0.457)	1.023+/-0.028 (0.466)	1.027+/-0.028 (0.461)	1+/-0.006 (0.5)	1.013+/-0.029 (0.48)	1.03+/-0.036 (0.457)	0.975+/-0.003 (0.411)	0.987+/-0.002 (0.378)	0.986+/-0.016 (0.478)	1.006+/-0.019 (0.491)
L-Lactate	0.989+/-0.009 (0.469)	1.003+/-0.005 (0.49)	0.985+/-0.007 (0.458)	0.993+/-0.002 (0.479)	0.987+/-0.006 (0.463)	0.986+/-0.014 (0.463)	1+/-0.006 (0.441)	0.993+/-0.003 (0.417)	1.002+/-0.003 (0.496)	0.987+/-0.007 (0.463)
Threonine	1.007+/-0.006 (0.481)	1.097+/-0.114 (0.344)	1.046+/-0.041 (0.402)	1.137+/-0.085 (0.283)	1.103+/-0.066 (0.318)	1.034+/-0.062 (0.433)	1.046+/-0.064 (0.412)	1.024+/-0.097 (0.38)	1.141+/-0.103 (0.26)	1.073+/-0.108 (0.384)
Alanine	0.983+/-0.007 (0.461)	0.992+/-0.014 (0.482)	0.965+/-0.007 (0.417)	0.982+/-0.015 (0.461)	0.966+/-0.005 (0.423)	0.98+/-0.01 (0.451)	0.992+/-0.021 (0.434)	0.983+/-0.004 (0.407)	0.998+/-0.017 (0.495)	0.962+/-0.028 (0.404)
Lysine	1.045+/-0.016 (0.293)	1.053+/-0.03 (0.286)	1.034+/-0.039 (0.364)	0.996+/-0.056 (0.469)	0.961+/-0.04 (0.238)	1.059+/-0.061 (0.338)	1.062+/-0.018 (0.361)	1.159+/-0.039 (0.306)	0.982+/-0.048 (0.207)	1.024+/-0.032 (0.335)
Arginine	1.09+/-0.079 (0.204)	1.036+/-0.147 (0.439)	1.049+/-0.091 (0.389)	0.95+/-0.117 (0.393)	1.062+/-0.081 (0.352)	1.06+/-0.053 (0.329)	1.103+/-0.066 (0.324)	0.948+/-0.094 (0.266)	1.063+/-0.06 (0.048)	1.04+/-0.132 (0.426)
Acetate	0.984+/-0.02 (0.466)	0.963+/-0.032 (0.42)	0.965+/-0.013 (0.429)	1.012+/-0.013 (0.477)	0.98+/-0.019 (0.457)	0.967+/-0.015 (0.434)	0.993+/-0.013 (0.428)	1.006+/-0.022 (0.401)	1.005+/-0.022 (0.49)	0.992+/-0.014 (0.484)
CH2C=C (fatty acyl chains) lipids	0.997+/-0.057 (0.496)	1.006+/-0.065 (0.493)	0.925+/-0.053 (0.414)	0.946+/-0.051 (0.436)	0.942+/-0.015 (0.419)	0.932+/-0.062 (0.41)	0.985+/-0.068 (0.424)	1.058+/-0.127 (0.393)	1.021+/-0.076 (0.477)	0.963+/-0.063 (0.459)
N-acetyl groups	1.011+/-0.017 (0.407)	1.012+/-0.026 (0.416)	1+/-0.02 (0.5)	1.001+/-0.007 (0.488)	0.99+/-0.033 (0.434)	1.029+/-0.036 (0.332)	0.976+/-0.006 (0.414)	0.971+/-0.001 (0.378)	0.977+/-0.01 (0.315)	1.006+/-0.015 (0.443)
Glutamate	0.965+/-0.036 (0.261)	1.042+/-0.018 (0.287)	0.913+/-0.025 (0.137)	0.992+/-0.081 (0.458)	0.922+/-0.026 (0.159)	0.988+/-0.031 (0.437)	0.916+/-0.009 (0.409)	0.94+/-0.079 (0.371)	0.88+/-0.063 (0.012)	0.978+/-0.049 (0.293)
Glutamine	0.964+/-0.007 (0.395)	0.969+/-0.027 (0.408)	0.942+/-0.049 (0.313)	0.937+/-0.047 (0.321)	0.952+/-0.019 (0.342)	0.926+/-0.04 (0.281)	0.914+/-0.074 (0.47)	0.914+/-0.042 (0.459)	0.963+/-0.014 (0.404)	0.907+/-0.076 (0.252)
Methionine	0.996+/-0.005 (0.484)	0.982+/-0.03 (0.431)	0.987+/-0.02 (0.461)	1.014+/-0.02 (0.458)	0.98+/-0.015 (0.422)	0.99+/-0.009 (0.466)	1.009+/-0.013 (0.462)	1.002+/-0.003 (0.448)	1.002+/-0.01 (0.495)	0.985+/-0.018 (0.452)
Acetone	1.023+/-0.024 (0.456)	1.103+/-0.071 (0.336)	0.928+/-0.056 (0.386)	0.92+/-0.071 (0.384)	0.997+/-0.051 (0.495)	0.953+/-0.057 (0.423)	0.989+/-0.029 (0.438)	0.96+/-0.022 (0.411)	1.02+/-0.051 (0.467)	0.997+/-0.052 (0.495)
Acetoacetate	0.968+/-0.005 (0.438)	0.917+/-0.02 (0.349)	0.979+/-0.045 (0.446)	1.001+/-0.013 (0.498)	0.977+/-0.037 (0.452)	0.97+/-0.013 (0.437)	1.006+/-0.046 (0.403)	1.016+/-0.012 (0.367)	1.032+/-0.009 (0.434)	0.965+/-0.065 (0.395)
Pyruvate	1.006+/-0.006 (0.426)	1.026+/-0.019 (0.152)	0.986+/-0.01 (0.378)	0.993+/-0.008 (0.434)	0.982+/-0.015 (0.26)	0.979+/-0.014 (0.17)	1.003+/-0.007 (0.476)	1.023+/-0.025 (0.468)	0.994+/-0.001 (0.427)	0.995+/-0.024 (0.452)
Succinate	0.865+/-0.065 (0.032)	1.014+/-0.168 (0.477)	0.79+/-0.034 (0.073)	0.848+/-0.05 (0.141)	0.767+/-0.027 (0.024)	0.866+/-0.173 (0.263)	0.887+/-0.061 (0.159)	0.889+/-0.037 (0.17)	0.983+/-0.057 (0.276)	0.89+/-0.082 (0.207)
Pyroglutamine	1.055+/-0.042 (0.297)	1.08+/-0.169 (0.312)	1.01+/-0.08 (0.398)	1.049+/-0.12 (0.28)	0.909+/-0.066 (0.000493)	0.933+/-0.188 (0.341)	0.919+/-0.112 (0.305)	1.04+/-0.121 (0.317)	1.079+/-0.079 (0.204)	0.995+/-0.17 (0.487)
Citrate	0.944+/-0.032 (0.357)	1.002+/-0.067 (0.495)	1.005+/-0.06 (0.483)	1.014+/-0.047 (0.465)	0.987+/-0.062 (0.451)	0.99+/-0.056 (0.452)	1.068+/-0.025 (0.382)	1.078+/-0.032 (0.341)	1.028+/-0.056 (0.359)	1.016+/-0.035 (0.438)
Dimethylamine	0.987+/-0.041 (0.449)	1.13+/-0.209 (0.284)	1.316+/-0.145 (0.123)	1.106+/-0.093 (0.287)	1.26+/-0.12 (0.15)	1.1+/-0.175 (0.341)	1.078+/-0.19 (0.294)	0.978+/-0.051 (0.33)	0.934+/-0.098 (0.1)	1.438+/-0.134 (0.032)
N N-dimethylglycine	1.137+/-0.139 (0.349)	1.013+/-0.016 (0.473)	1.01+/-0.1 (0.485)	1.118+/-0.109 (0.359)	1.148+/-0.17 (0.35)	1.024+/-0.059 (0.459)	0.936+/-0.02 (0.465)	0.926+/-0.077 (0.45)	1.09+/-0.129 (0.392)	1.054+/-0.084 (0.382)
Creatine	0.913+/-0.058 (0.423)	1.083+/-0.141 (0.436)	1.124+/-0.054 (0.392)	0.992+/-0.081 (0.492)	0.925+/-0.068 (0.423)	1.01+/-0.21 (0.493)	1.101+/-0.098 (0.369)	1.072+/-0.182 (0.342)	0.996+/-0.13 (0.496)	0.98+/-0.087 (0.48)
Creatinine	0.979+/-0.017 (0.458)	0.997+/-0.013 (0.494)	0.938+/-0.01 (0.373)	0.997+/-0.009 (0.495)	0.976+/-0.012 (0.45)	0.96+/-0.023 (0.415)	0.962+/-0.013 (0.469)	0.973+/-0.026 (0.462)	0.945+/-0.017 (0.398)	0.995+/-0.001 (0.489)
Ornithine	0.958+/-0.06 (0.432)	1.032+/-0.056 (0.443)	1.014+/-0.018 (0.472)	0.969+/-0.063 (0.451)	0.903+/-0.061 (0.318)	0.896+/-0.039 (0.288)	0.942+/-0.083 (0.46)	1.104+/-0.041 (0.445)	1.018+/-0.044 (0.467)	0.917+/-0.048 (0.317)
Phenylalanine	0.962+/-0.02 (0.2)	0.996+/-0.052 (0.477)	1.076+/-0.032 (0.102)	1.053+/-0.059 (0.262)	0.944+/-0.035 (0.194)	1.018+/-0.061 (0.41)	1.008+/-0.04 (0.449)	0.994+/-0.05 (0.436)	0.912+/-0.005 (0.02)	1.056+/-0.054 (0.255)
Dimethylsulfone	1.181+/-0.179 (0.374)	1.068+/-0.061 (0.437)	0.969+/-0.066 (0.468)	1.051+/-0.056 (0.455)	0.94+/-0.071 (0.442)	0.993+/-0.076 (0.494)	1.002+/-0.044 (0.386)	1.023+/-0.001 (0.347)	0.985+/-0.007 (0.485)	0.969+/-0.046 (0.464)
Choline (free)	1.047+/-0.065 (0.438)	1.126+/-0.123 (0.366)	0.964+/-0.03 (0.446)	0.976+/-0.034 (0.465)	0.971+/-0.027 (0.457)	1.001+/-0.067 (0.499)	0.944+/-0.053 (0.498)	0.932+/-0.021 (0.497)	0.993+/-0.056 (0.491)	0.988+/-0.079 (0.485)
o-phosphocholine	0.957+/-0.016 (0.253)	0.931+/-0.027 (0.156)	0.99+/-0.034 (0.455)	0.966+/-0.005 (0.307)	0.966+/-0.005 (0.318)	1.002+/-0.017 (0.492)	0.966+/-0.018 (0.334)	0.958+/-0.007 (0.304)	0.956+/-0.027 (0.314)	0.961+/-0.025 (0.346)
Glucose	0.999+/-0.009 (0.492)	1+/-0.01 (0.498)	0.989+/-0.004 (0.436)	0.998+/-0.006 (0.487)	0.99+/-0.01 (0.447)	0.997+/-0.011 (0.483)	1+/-0.007 (0.484)	0.998+/-0.017 (0.48)	0.999+/-0.007 (0.496)	0.997+/-0.002 (0.483)
Proline	0.96+/-0.041 (0.441)	0.902+/-0.031 (0.364)	0.988+/-0.035 (0.478)	0.987+/-0.041 (0.479)	0.905+/-0.042 (0.359)	0.987+/-0.066 (0.482)	0.944+/-0.045 (0.496)	1.032+/-0.08 (0.495)	0.979+/-0.018 (0.467)	0.944+/-0.037 (0.418)
Glycine	0.984+/-0.008 (0.397)	1.01+/-0.028 (0.39)	1.034+/-0.021 (0.239)	1.035+/-0.034 (0.188)	0.975+/-0.027 (0.296)	1.013+/-0.043 (0.346)	1.04+/-0.017 (0.308)	1.022+/-0.032 (0.3)	1.031+/-0.03 (0.255)	1.006+/-0.018 (0.451)
Glycerol	1+/-0.035 (0.499)	1.049+/-0.034 (0.335)	1.016+/-0.082 (0.459)	1.018+/-0.042 (0.441)	0.976+/-0.034 (0.419)	1.027+/-0.028 (0.377)	1.004+/-0.021 (0.466)	1.047+/-0.058 (0.452)	0.944+/-0.046 (0.272)	0.97+/-0.04 (0.352)
Myo-inositol	1.011+/-0.088 (0.478)	0.846+/-0.058 (0.143)	0.803+/-0.08 (0.104)	0.891+/-0.088 (0.183)	0.94+/-0.088 (0.383)	0.8+/-0.142 (0.112)	0.826+/-0.064 (0.298)	0.839+/-0.093 (0.219)	0.91+/-0.004 (0.27)	0.819+/-0.075 (0.039)
CH=CH (fatty acyl residues) lipids	1.083+/-0.026 (0.366)	1.096+/-0.023 (0.35)	1.045+/-0.021 (0.426)	1.064+/-0.068 (0.39)	1.092+/-0.021 (0.333)	1.117+/-0.014 (0.311)	1.043+/-0.04 (0.38)	1.149+/-0.053 (0.339)	1.058+/-0.038 (0.401)	1.113+/-0.047 (0.304)
Tyrosine	1.023+/-0.026 (0.273)	0.994+/-0.035 (0.427)	1.025+/-0.045 (0.292)	1.022+/-0.047 (0.359)	1.028+/-0.031 (0.289)	0.979+/-0.036 (0.255)	1.001+/-0.025 (0.485)	1.01+/-0.018 (0.483)	1.077+/-0.037 (0.095)	1.048+/-0.032 (0.19)
Histidine	1.105+/-0.019 (0.208)	1.163+/-0.061 (0.171)	1.04+/-0.041 (0.402)	1.074+/-0.047 (0.346)	1.041+/-0.019 (0.395)	1.05+/-0.034 (0.383)	1.169+/-0.016 (0.183)	1.106+/-0.106 (0.124)	1.079+/-0.016 (0.253)	1.05+/-0.073 (0.406)
Acetaminophen glucuronide	1.349+/-0.162 (0.042)	1.164+/-0.139 (0.246)	1.077+/-0.099 (0.347)	0.938+/-0.086 (0.361)	1.103+/-0.143 (0.277)	0.966+/-0.145 (0.435)	1.222+/-0.29 (0.217)	0.907+/-0.342 (0.234)	1.075+/-0.149 (0.375)	1.178+/-0.058 (0.044)
hypoxanthine	0.984+/-0.11 (0.468)	0.966+/-0.103 (0.438)	1.04+/-0.058 (0.396)	0.905+/-0.068 (0.317)	1.029+/-0.029 (0.412)	0.976+/-0.077 (0.411)	0.922+/-0.095 (0.394)	0.667+/-0.028 (0.353)	0.954+/-0.109 (0.422)	0.848+/-0.012 (0.164)
Formate	1.042+/-0.115 (0.402)	0.965+/-0.012 (0.177)	1.008+/-0.019 (0.448)	1.018+/-0.136 (0.463)	1.103+/-0.13 (0.279)	0.955+/-0.113 (0.395)	0.956+/-0.04 (0.32)	1.088+/-0.106 (0.304)	1.077+/-0.168 (0.37)	0.941+/-0.062 (0.291)

Table 16 - Short-term storage results for NMR CPMG HSF analysis reporting the peak integral ratio change relative to the immediate freezing at -80°C result, results significant to the 0.05 confidence level are displayed in red

6.5.1 Room temperature (see Table 17 & Table 18; Figure 29)

Storage at room temperature for up to 12 hours changed metabolite concentrations. The NOESYGPPR1D sequence demonstrated significant decreases for 2-ketoisovalerate ($F_{2,6}=5.679$, $p=0.041$) with post-hoc testing achieving significance only at the 12hr timepoint ($p=0.011$). Valine showed borderline significant increase by t-testing only (4hr $p=0.0509$, 12hr $p=0.0498$). In CPMG spectra only dimethylamine showed significance by ANOVA testing ($F_{2,6}=11.09$, $p=0.041$) which was not ratified by post-hoc t-testing. T-testing revealed significant decreases for succinate ($p=0.031$) and increases for acetaminophen glucuronide ($p=0.0424$) after 4hrs of exposure. Non-significant fluctuations were seen for other metabolites: pyruvate, free choline, formate, ornithine, histidine, threonine. These differences were not always linear throughout the measurement period. The exceptions were 3-hydroxyisobutyrate, CH=CH fatty acyl residues, histidine and acetaminophen glucuronide. In some cases (succinate, arginine, N-N dimethylglycine, dimethylsulfone (CPMG), dimethylamine, acetone, isoleucine and threonine (NOESYGPPR1D)), an initial change in concentration was measured at 4 hours which then normalised by 12 hours.

6.5.2 Refrigeration (see Table 17 & Table 18; Figure 30)

The NOESYGPPR1D experiment and ANOVA analysis revealed a significant increase in Valine ($F_{3,8}=5.67$, $p=0.022$), illustrated by significant post-hoc t-testing at the 4hr ($p=0.0031$) and 12hr timepoints ($p=0.034$). The CPMG experiment and ANOVA analysis showed significance for decreases in succinate ($F_{3,8}=5.29$, $p=0.024$), with post-hoc t-testing indicating the 12hr timepoint to be most responsible ($p=0.0235$). T-testing did reveal significance for the 12hr timepoint of decreases in pyroglutamine ($p=0.0005$) and increases in 2-hydroxybutyrate ($p=0.0363$). Non-significant changes were observed for increases in: CH_3 fatty acyl residues, dimethylamine, ornithine, myo-inositol, acetaminophen glucuronide and decreases in: 2-ketoisovalerate, proline, myo-inositol, acetoacetate, phenylalanine and dimethylsulfone.

6.5.3 Free-thaw cycling (see Table 17 & Table 18; Figure 31)

Repetitive freeze-thawing had little effect on most metabolites and no significance was detected by ANOVA. T-testing revealed some single time points of repetitive freeze-thawing to be significant for NOESYGPPR1D: 2-ketoisovalerate (p=0.043), GAG (p=0.0173), acetaminophen glucuronide (p=0.0257) and formate (p=0.0340). For CPMG: arginine (p=0.0483), glutamate (p=0.0123), phenylalanine (p=0.020) and paracetamol glucuronide (p=0.048). We observed non-significant elevations of CH_3 fatty acyl residues, ornithine, threonine and CH=CH fatty acyl residues. Concentrations of dimethylsulfone, 2-hydroxybutyrate, glutamine, succinate, myo-inositol and GAGs were reduced, but not significantly.

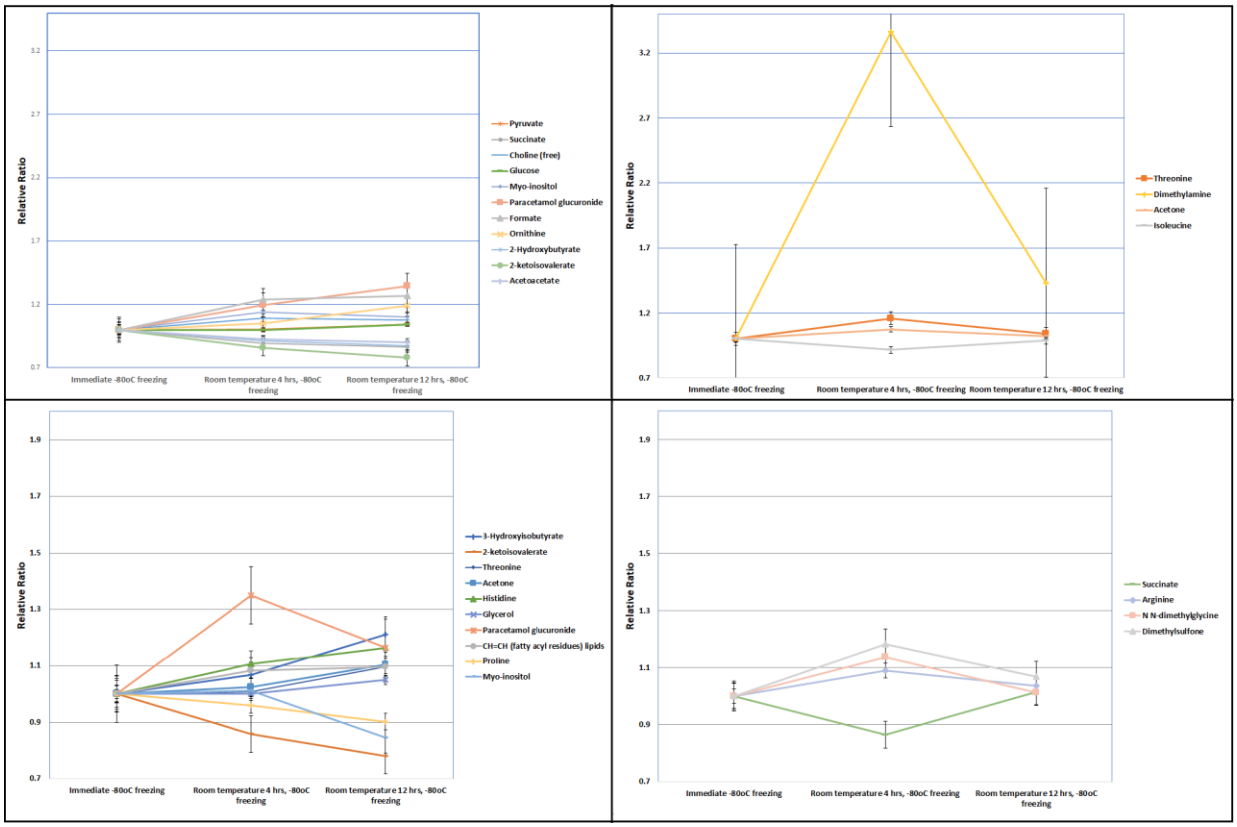


Figure 29 – The effects of room temperature upon the metabolite levels detected by NMR of HSF

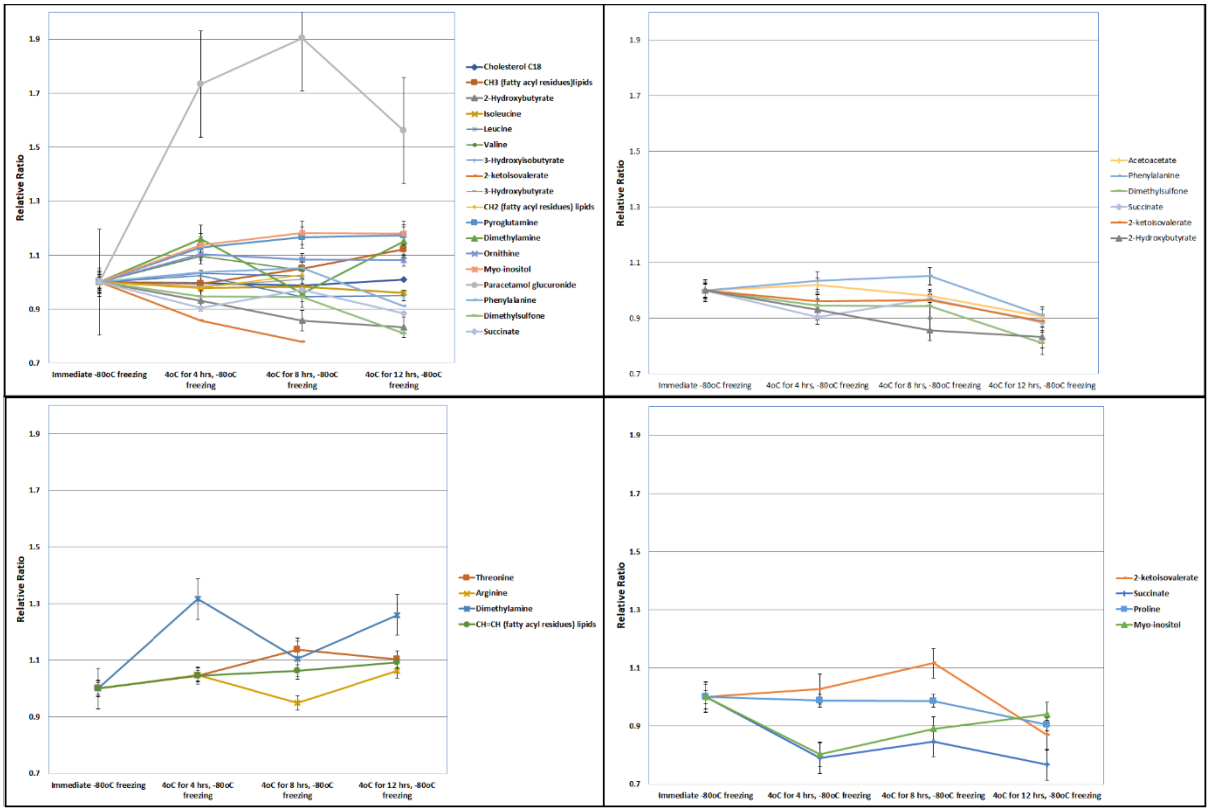


Figure 30 - The effects of refrigeration upon the metabolite levels detected by NMR of HSF

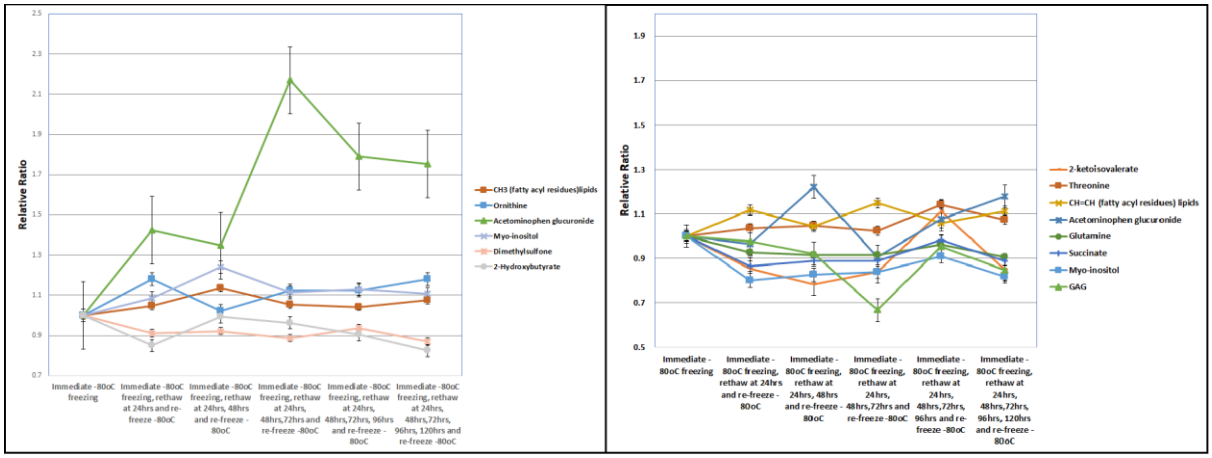


Figure 31 - The effects of freeze-thaw cycling upon metabolites levels detected by NMR in HSF

One way ANOVA	Room temperature		Fridge temperature (4ºC)		Freeze (-80ºC) thaw cycling	
Metabolite	***F(2,6)***	***p value***	***F(3,8)***	***p value***	***F(5,13)***	***p value***
Cholesterol C18	0.0205	0.9797	0.0183	0.9964	0.2471	0.9339
CH3 (fatty acyl residues) lipids	0.0126	0.9875	0.1442	0.9306	0.2891	0.9106
2-Hydroxybutyrate	0.4333	0.6671	0.7681	0.5433	0.2327	0.9413
Isoleucine	0.7678	0.5048	0.1244	0.9431	0.2088	0.9528
Leucine	0.1870	0.8341	0.5342	0.6717	0.2950	0.9072
Valine	2.7919	0.1390	5.6789	0.0221	0.6055	0.6973
3-Hydroxyisobutyrate	0.2280	0.8027	0.3541	0.7877	0.9720	0.4701
2-ketoisovalerate	5.6786	0.0413	0.1818	0.9058	0.6387	0.6745
3-Hydroxybutyrate	0.0114	0.9886	0.0072	0.9991	0.2479	0.9335
CH2 (fatty acyl residues) lipids	0.0508	0.9509	0.0014	0.9999	0.2552	0.9296
L-Lactate	0.0637	0.9389	0.5583	0.6572	0.1157	0.9867
Threonine	0.4274	0.6706	0.3760	0.7729	0.2730	0.9198
Alanine	0.1127	0.8953	0.0666	0.9761	0.1702	0.9691
Lysine	0.0002	0.9998	0.2130	0.8846	0.1482	0.9770
Arginine	0.0116	0.9885	0.3126	0.8160	0.1742	0.9676
Acetate	0.1928	0.8296	0.1858	0.9031	0.2955	0.9069
CH2C=C (fatty acyl chains) lipids	0.5624	0.5972	0.1872	0.9021	0.2244	0.9454
N-acetyl groups	0.0708	0.9324	0.0675	0.9757	0.0862	0.9932
Glutamate	0.2561	0.7821	0.1064	0.9540	0.1760	0.9668
Glutamine	0.0659	0.9369	0.0837	0.9670	0.2421	0.9365
Methionine	0.0988	0.9074	0.0606	0.9791	0.1654	0.9710
Acetone	0.1118	0.8961	0.7285	0.5632	0.3978	0.8417
Acetoacetate	0.2037	0.8211	0.2428	0.8642	0.3160	0.8946
Pyruvate	0.1631	0.8532	0.6375	0.6117	0.3056	0.9009
Succinate	0.3293	0.7317	0.1249	0.9428	0.2412	0.9370
Pyroglutamine	0.6450	0.5576	0.1995	0.8939	0.3083	0.8992
Citrate	0.0811	0.9231	0.1134	0.9498	0.7624	0.5927
Dimethylalanine	1.8981	0.2298	0.2836	0.8360	0.3974	0.8420
N N-dimethylglycine	0.0133	0.9868	0.2021	0.8921	0.4486	0.8070
Creatine	0.0020	0.9980	0.2268	0.8752	0.4584	0.8002
Creatinine	0.0224	0.9779	0.0722	0.9732	0.2856	0.9126
Ornithine	0.3857	0.6957	0.0982	0.9588	0.1947	0.9591
Phenylalanine	0.3810	0.6986	0.6444	0.6079	0.3603	0.8666
Dimethylsulfone	0.0648	0.9379	0.3932	0.7614	0.2893	0.9105
Choline (free)	0.1976	0.8259	0.1608	0.9198	0.2273	0.9440
o-phosphocholine	0.1428	0.8697	0.1173	0.9474	0.0844	0.9935
Glucose	0.0626	0.9399	0.6662	0.5960	0.2412	0.9370
Proline	0.0178	0.9824	0.0305	0.9923	0.4088	0.8343
Glycine	0.1837	0.8367	0.3091	0.8184	0.1188	0.9858
Glycerol	0.0221	0.9782	0.3730	0.7749	0.3724	0.8586
Myo-inositol	0.1054	0.9016	0.1408	0.9327	0.1827	0.9642
CH=CH (fatty acyl residues) lipids	0.1309	0.8797	0.0742	0.9722	0.2984	0.9052
Tyrosine	0.3245	0.7348	0.2765	0.8409	0.3911	0.8463
Histidine	0.9232	0.4471	1.0665	0.4159	0.7691	0.5884
Paracetamol glucuronide	0.8105	0.4880	1.9954	0.1933	1.0125	0.4490
GAG	0.1439	0.8689	0.7733	0.5408	0.9795	0.4661
Formate	0.2288	0.8022	0.9138	0.4764	0.5226	0.7552

Table 17 - One way ANOVA analysis of short-term HSF storage measured by NOESYGPPR1D NMR analysis

One way ANOVA	Room temperature		Fridge temperature (4ºC)		Freeze (-80ºC) thaw cycling	
Metabolite	***F(2,6)***	***p value***	***F(3,8)***	***p value***	***F(5,13)***	***p value***
CH3 (fatty acyl residues) lipids	0.0348	0.9661	0.0110	0.9983	0.2960	0.9052
2-Hydroxybutyrate	0.1615	0.8578	0.7911	0.5321	0.2147	0.9490
Isoleucine	0.8570	0.5077	0.8894	0.4869	0.0514	0.9979
Leucine	1.7249	0.3172	0.1408	0.9327	1.0582	0.4330
Valine	1.4833	0.3565	0.2286	0.8740	0.7724	0.5891
3-Hydroxyisobutyrate	0.2178	0.8160	0.3266	0.8064	0.6578	0.6625
2-ketoisovalerate	0.4680	0.6654	1.2848	0.3439	0.8297	0.5545
3-Hydroxybutyrate	0.3367	0.7380	0.1789	0.9078	0.0727	0.9952
CH2 (fatty acyl residues) lipids	0.0186	0.9817	0.0122	0.9980	0.2812	0.9139
L-Lactate	0.0069	0.9931	0.0120	0.9981	0.1231	0.9842
Threonine	0.1955	0.8321	0.5232	0.6784	0.5134	0.7611
Alanine	0.0279	0.9727	0.0469	0.9855	0.0730	0.9952
Lysine	0.4321	0.6841	0.3560	0.7864	0.2790	0.9151
Arginine	0.2654	0.7832	0.3158	0.8138	0.1839	0.9628
Acetate	0.0388	0.9624	0.0569	0.9809	0.3080	0.8981
CH2C=C (fatty acyl chains) lipids	0.0766	0.9280	0.0604	0.9792	0.1047	0.9890
N-acetyl groups	0.1806	0.8432	0.0430	0.9872	0.3447	0.8752
Glutamate	0.1478	0.8685	0.5761	0.6467	0.2163	0.9482
Glutamine	0.2044	0.8256	0.1695	0.9140	0.0557	0.9974
Methionine	0.4512	0.6741	0.0819	0.9680	0.1816	0.9638
Acetone	0.0895	0.9167	0.1959	0.8963	0.2258	0.9436
Acetoacetate	0.0077	0.9924	0.0193	0.9960	0.2267	0.9431
Pyruvate	0.4119	0.6949	0.1944	0.8973	0.1319	0.9817
Succinate	0.5911	0.6075	5.5288	0.0237	0.3911	0.8449
Pyroglutamine	0.1527	0.8647	0.4686	0.7124	0.3273	0.8862
Citrate	0.1572	0.8611	0.0208	0.9956	0.1177	0.9857
Dimethylamine	11.0974	0.0411	1.7042	0.2429	2.2615	0.1205
N N-dimethylglycine	0.2231	0.8122	0.2740	0.8426	0.1344	0.9809
Creatine	1.4299	0.3663	0.2087	0.8876	0.0924	0.9917
Creatinine	1.7933	0.3074	0.1061	0.9542	0.5608	0.7283
Ornithine	0.0639	0.9393	0.2740	0.8426	0.0983	0.9905
Phenylalanine	0.2873	0.7689	2.2679	0.1577	1.3325	0.3203
Dimethylsulfone	0.1378	0.8765	0.0681	0.9754	0.3092	0.8973
Choline (free)	0.2897	0.7673	0.0212	0.9955	0.1066	0.9886
o-phosphocholine	4.5309	0.1240	0.2467	0.8615	0.4522	0.8035
Glucose	0.7671	0.5382	0.0369	0.9898	0.7350	0.6124
Proline	0.5873	0.6092	0.1478	0.9282	0.2702	0.9201
Glycine	4.5319	1.5633	1.0097	0.4373	0.4396	0.8121
Glycerol	0.0033	0.9968	0.1036	0.9556	0.1899	0.9603
Myo-inositol	1.5633	0.3426	0.7623	0.5462	0.3245	0.8879
CH=CH (fatty acyl residues) lipids	0.0704	0.9335	0.1267	0.9416	0.3258	0.8871
Tyrosine	0.5445	0.6284	0.1205	0.9455	1.7170	0.2112
Histidine	0.2582	0.7880	0.2112	0.8859	0.8569	0.5386
Paracetamol glucuronide	6.6065	0.0796	0.5266	0.6762	0.6664	0.6569
Hypoxanthine	0.6975	0.5640	0.7528	0.5509	0.5837	0.7126
Formate	1.6104	0.3349	0.2453	0.8625	0.2697	0.9204

Table 18 - One way ANOVA analysis of short-term HSF storage measured by CPMG NMR analysis

6.6 Discussion

Studies using HSF as an expression of joint disease are now being reported more frequently (see 5.1.1). NMR spectroscopy remains an attractive method to provide an untargeted metabolic representation of the disease state due to the resolving power, simplicity of sample preparation breadth of description and relative quantitation. However, studies validating the analysis and sample collection procedures are rare. One study detailed concerns about sample stability to freeze-thaw cycling but no study has specifically investigated the various refrigeration practices or delays at room temperature before long-term storage.

Differences were observed between the CPMG and NOESYGPPR1D acquisition. Synovial fluid has a high proteoglycan content. These large molecules with their multiple charged species are likely to exhibit small molecular binding effects, thereby affecting their mobility in solution. This reduced mobility will directly impact the spin relaxation and hence spectral intensity observed by NMR spectroscopy. Any binding effect will be pronounced in the NOESYGPPR1D acquisition as opposed to the CPMG spin echo sequence which diminishes this effect. This may in part explain why a disparity was seen between the sequences. The concentration of the metabolite may not be affected, merely an alteration in detection by the differing NMR sequence.

The centrifugation of samples removes cellular material to halt ongoing metabolism. However, a small amount of ongoing metabolism may take place due to extracellular molecules, proteins or the inability to completely remove all cells from the samples. In circumstances where elevations or depletions are consistently seen throughout the storage, this is likely to be the cause.

Freeze thaw cycling had little effect upon metabolite concentrations. This was consistent with the findings of Damyanovich et al. (206). Furthermore, stability of the lipid peaks to room temperature and short-term refrigeration was demonstrated.

Room temperature storage had significant effects upon 2-ketoisovalerate, not known to associate with joint disease in HSF. Valine is reported to differentiate between RA and reactive arthritis or OA and

was affected by room temperature and refrigeration (222). Succinate is reported to relate to joint disease in HSF, but is significantly affected nevertheless (223).

In the light of these findings, it is recommended that samples are refrigerated immediately after harvesting. Furthermore, samples should not be refrigerated for more than 4 hours before preparation and freezing. The observations of selected metabolic changes need to be considered when interpreting the implications of any metabolic changes measured in synovial fluid.

7 Chapter 7: The metabolic stability of human synovial fluid to long-term low temperature storage

7.1 Introduction

Logistical constraints require the use of long-term low temperature storage prior to analysis of HSF samples. Batch analysis of HSF samples is required for several reasons including reducing cost, minimising inter-instrument analytical variation and maximising time efficiency. At least 50 samples are required to provide a good cohort analysis of joint disease and at best samples can be collected from only two or three patients per day. Therefore, samples must be stored for varying periods at low temperature before batch analysis. Any effect upon the metabolic profile from long-term low-temperature storage must be investigated and understood.

Only one study reports the effect of long-term storage on HSF, with an overall reduction in signal and specific reductions in metabolite detection (see Table 11) (206). Those samples were stored at -75ºC within minutes after sample preparation and centrifugation. Samples were thawed at room temperature without additional heating. Twelve samples used for long-term storage comparison were stored at -75ºC for <12 months with a single freeze thaw cycle. Sample analysis was then compared directly to the control samples. The same spectrometer and analysis method was used throughout. However, the authors failed to acknowledge the potential effect of differences in instrument performance and variability in the intervening period. Nevertheless, a reduction in intensity of low molecular weight and macromolecular species was reported. No internal standard was used to verify or calibrate this reduction. Spectra were internally referenced against TSP added to samples prior to analysis. A Student's t-test was used to test statistical significance ($p<0.05$) in: glucose, valine, leucine, isoleucine, CH_2 lipid chain and CH_3 lipid chain differences. Typically, the concentrations of these metabolites were reduced by around 40%.

7.2 Hypothesis

The metabolic profile of HSF will be altered in a measurable way by long-term low temperature storage.

7.3 Aim

To study how the metabolic profile of HSF detected by NMR spectroscopy is affected by low-temperature storage.

7.4 Materials and methods

7.4.1 Sample preparation

HSF was harvested from 27 patients. Fourteen were female and 13 were male, the mean age was 66 years. Samples were harvested from knee (n=23) and hip (n=4) joints. Ethnicities were recorded as white British/other (n=19), Indian (n=4), white/black Caribbean (n=2), Caribbean (n=1) or Pakistani (n=1). After harvesting, samples were stored at 4ºC for no longer than four hours. Samples were then prepared immediately by centrifugation (10000***g***; room temperature, 15min) before the supernatant was dispensed for analysis. The sample aliquots were then divided for early and late analysis. All samples were frozen at -80°C and treated in an identical manner. A cohort of aliquots was removed for defrosting and NMR spectroscopy analysis having spent <21 days in low temperature storage. A second cohort was defrosted for analysis having spent between 10-11 months in low temperature storage. Both cohorts underwent a single low-temperature freeze-thaw cycle. Identical samples were analysed 10 months apart and the same protocol was followed for both.

Samples were prepared using the same researcher, reagents and buffering as previously (see 4.4.2). On both occasions BCS QC samples were prepared alongside the samples. These samples were used to evaluate the consistency of instrument analysis.

7.4.2 NMR methods

The same NMR spectroscopy instrument and methods were used to prepare and analyse the samples as used previously (see 3.9.7). QC samples of BCS were prepared and analysed in line with the HSF throughout the analysis to ensure no contamination and analytical stability.

In the early analysis, assessment of the spectra quality of both the HSF samples and the BCS QC's revealed water peak interference and poor water suppression. Therefore, analysis was halted, and

samples were reanalysed after a manual readjustment of the magnet shim to improve water suppression. This finding highlighted the possibility that the water content within the HSF samples was variable and that pre-analysis optimisation of the NMR spectrometer had been performed upon an outlying sample of low water content.

7.4.3 Data processing

NMR spectroscopy FID signals were transformed by Fourier transformation using Topspin® v3.2 software (Bruker). Phase and baseline correction was performed to the ^{1}H signal of the TSP peak. The water peaks (δ=4.5-5.0ppm) and TSP (δ=-0.2-0.3ppm) were subtracted before a median fold change normalisation was applied. This method of normalisation was chosen since it would be less affected by any significant change in protein content. Spectra were aligned to accommodate any minor changes in chemical shift using the same methods as previous experiments (see 3.9.8.2).

Data was then analysed using spectral interpretation and multi-variate linear regression modelling using the same principles and methods as before (see 3.10). The spectral description of HSF (see 3.10) allowed the semi-quantitative evaluation of how individual metabolites were affected by storage. A well resolved and previously identified peak, in clear baseline (where possible) was selected. Peaks were integrated by baseline adjustment and a trapezoid method. The principal TSP integrated peak functioned as an internal standard with consistency of detection being validated.

A mean and standard deviation calculation of storage effect upon each metabolite was performed. A paired Student's t-test was used (α=0.05) to test the significance of sample storage effects upon each metabolite. A Benjamini-Hochberg correction was performed to correct for any false discovery rate (b=0.05) (224).

7.5 Results

7.5.1 QC samples

The peak integrations from the QC samples revealed a CV of <2.5% and well below any observed differences within the samples (see Table 19). Therefore, instrument analysis appeared to be stable.

	Metabolite				
Sample	*Valine*	*Alanine*	*Glucose*	*Lactate*	*Isopropyl Alcohol*
Long-term 1 month analysis	16957248	46848970	65067717	264461772	74360743
Long-term 1 month analysis	16678511	46466129	63693654	263158607	74375574
Long-term 1 month analysis	16659181	48863706	65228722	264560783	74235645
Long-term 1 month analysis	16327420	46739954	64898349	252091020	71624688
Long-term 1 month analysis	16244257	46329638	63967726	252840632	71059990
Long-term 1 month analysis	16335489	46604492	64352900	255248419	71568745
Long-term 1 month analysis	16427922	47331278	63295487	254222857	72726301
Long-term 12-month analysis	16557328	46936252	62195938	256380222	72495463
Long-term 12-month analysis	16399754	47383460	61903792	256148941	72967582
Long-term 12-month analysis	16107114	46320019	61989057	247727675	70375741
Long-term 12-month analysis	15751233	45448071	63805768	250308893	69212832
Long-term 12-month analysis	15733383	46733110	62126579	245139014	69255198
Long-term 12-month analysis	16944595	48058249	63841623	260245310	74314469
Long-term 12-month analysis	16979989	47178855	64202087	263850205	72474661
Standard deviation	385980	854319	1121390	6118772	1757066
Mean	16395860	46953324	63500129	255532506	72052838
CV	0.02354	0.01820	0.01766	0.02395	0.02439

Table 19 - QC sample NMR spectroscopy analysis results for analysis of long-term storage conditions in HSF

7.5.2 Observations (see Figure 32 to Figure 35)

A macroscopic evaluation of the spectra revealed a close resemblance despite the intervening storage period. The spectra of short-term low temperature storage were averaged. This mean spectrum was then compared directly to the mean spectrum of the long-term low temperature storage. This was performed for both the NOESYGPPR1D and the CPMG acquisitions. Gross evaluation of the mean spectra revealed subtle differences in both acquisitions. The CPMG spectra revealed a difference in both the broad lipid signals of CH_2 and CH_3 groups. In addition, there were spectral differences in the NOESYGPPR1D spectra. These were mainly focused on the baseline amongst the poorly resolved peaks, originating from the macromolecules.

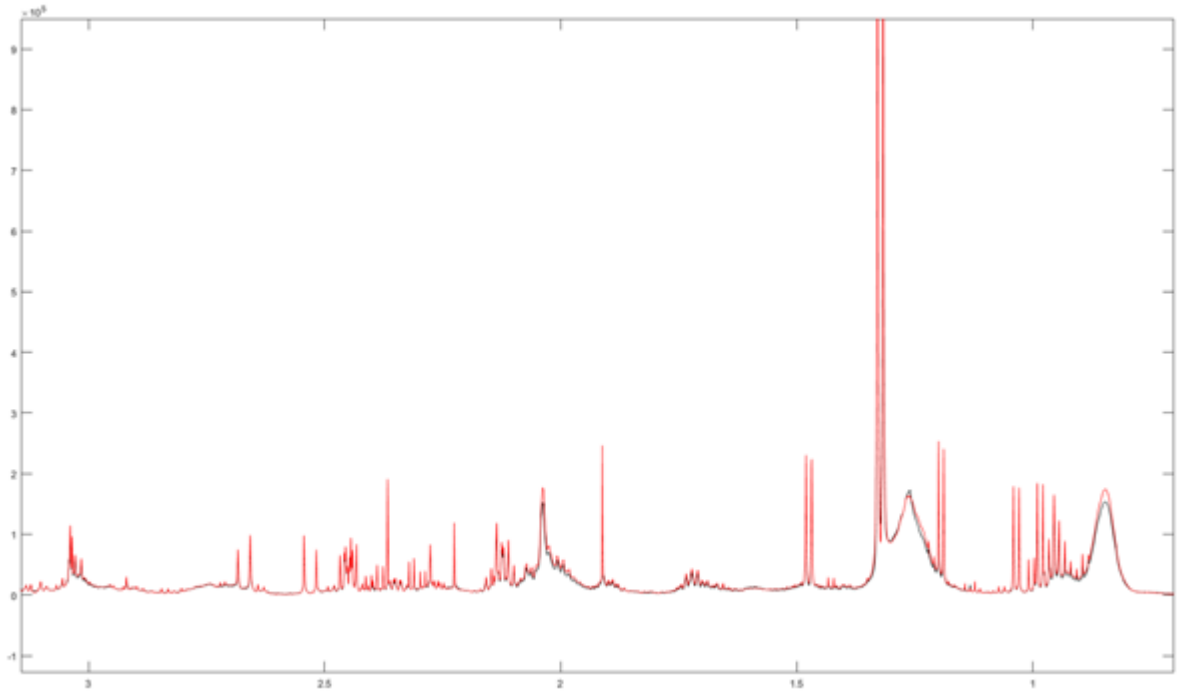


Figure 32 - CPMG HSF mean spectra comparison for the short-term (1month) and long-term (12 month) low temperature (-80ºC) storage, 0-3.0ppm

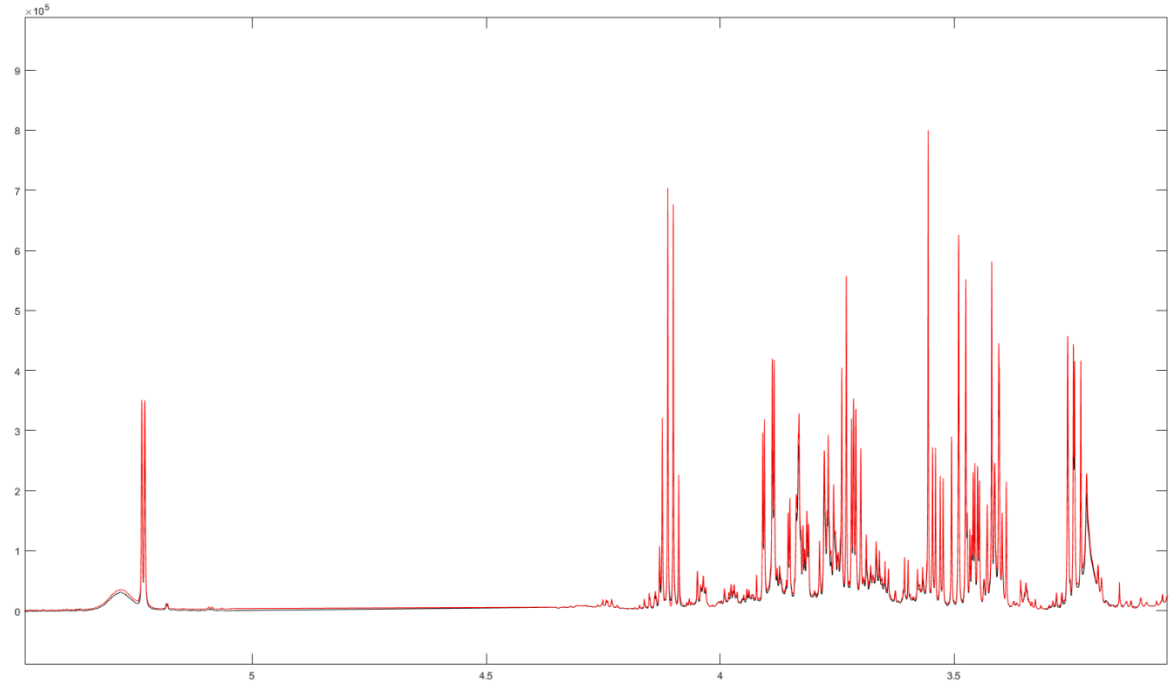


Figure 33 - CPMG HSF mean spectra comparison for the short-term (1month) and long-term (12 month) low temperature (-80ºC) storage, 3.0-5.0ppm

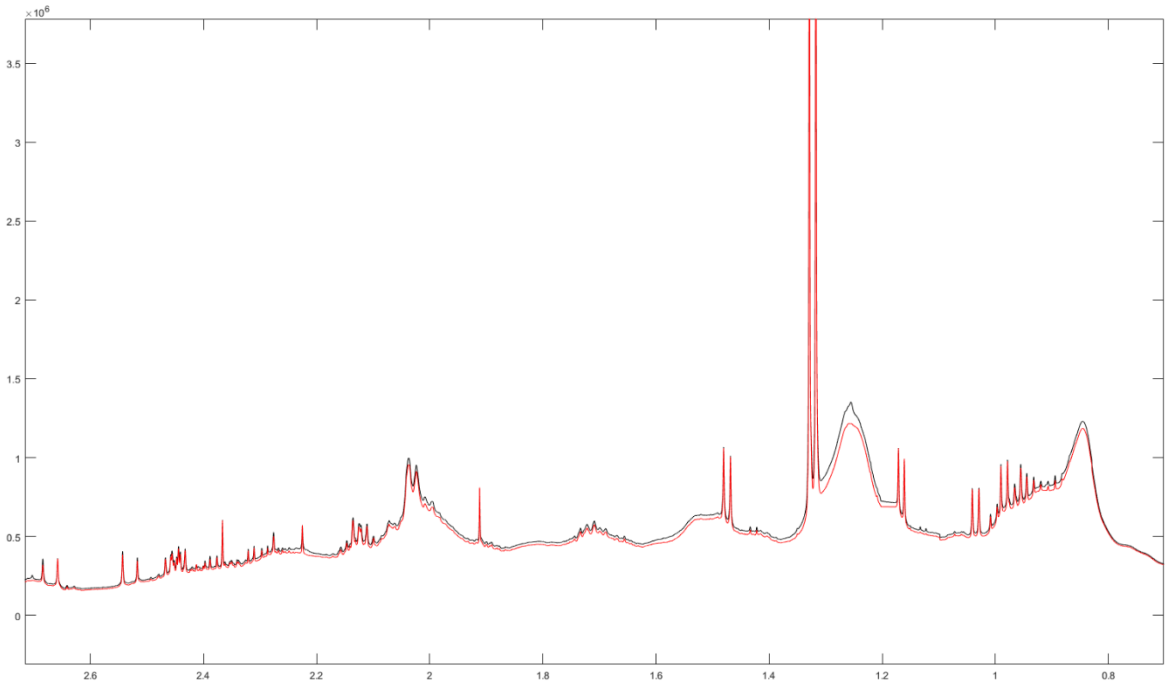


Figure 34 - NOESYGPPR1D HSF mean spectra comparison for the short-term (1month) and long-term (12 month) low temperature (-80ºC) storage, 0-3.0ppm

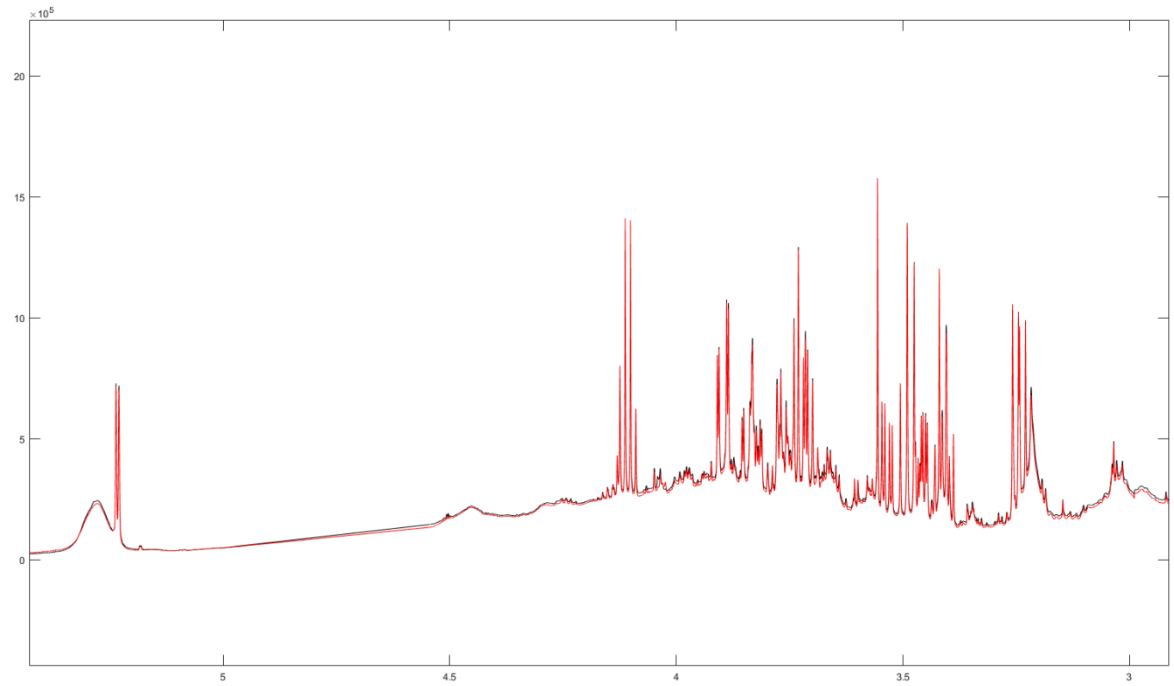


Figure 35 - NOESYGPPR1D HSF mean spectra comparison for the short-term (1month) and long-term (12 month) low temperature (-80ºC) storage, 3.0ppm 5ppm

7.5.3 Linear regression analysis – NOESYGPPR1D

Principal component analysis was used to describe the samples before and after storage. The PCA scores plot suggested a clear separation between the short and long-term storage without discrimination of the model ($R^2X=0.52$, $Q^2=0.438$), (see Figure 36).

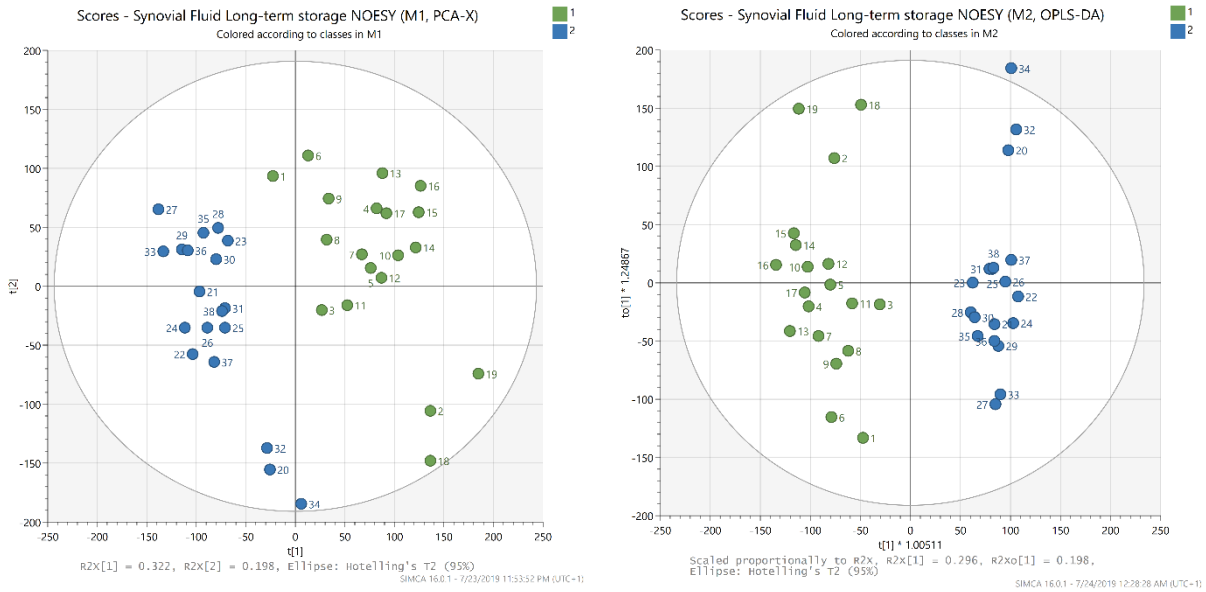


Figure 36 - PCA (left PC1 = 32.2%, PC2 = 19.8%) and OPLS-DA (right PC1 = 29.6%, PC2 = 19.8%) scores plot of low temperature HSF storage NOESYGPPR1D analysis (green=1 month, blue=12 month)

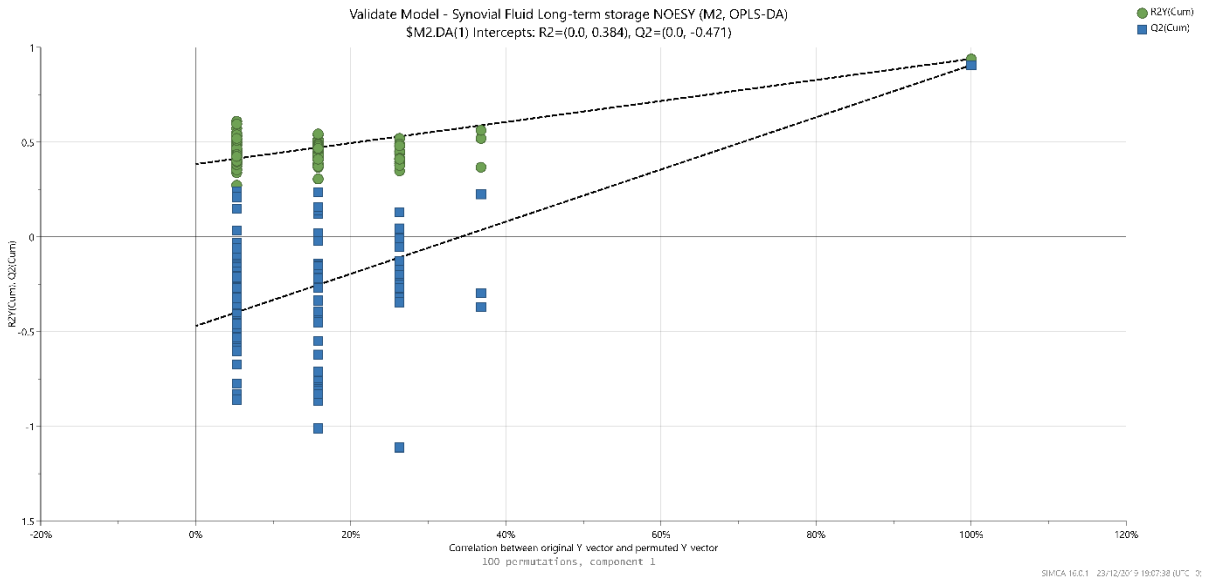


Figure 37 – OPLS-DA model permutation test for the evaluation of long-term low-temperature storage for HSF using NMR NOESYGPPR1D sequences

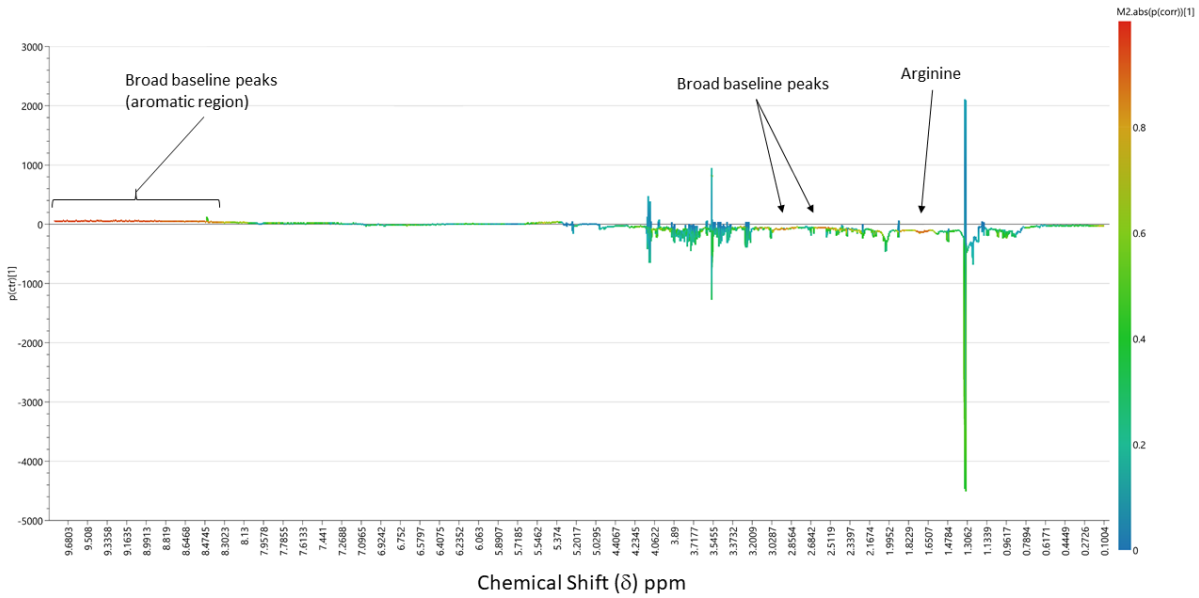


Figure 38 - S-line representation of an OPLS-DA model comparing short and long-term HSF storage after NOESYGPPR1D NMR spectroscopy analysis. The pCORR (partial correlation) is represented according to a temperature colour scale. Hotter colours represent the spectral regions with high correlation and thus are responsible for the separation between the short and long-term storage groups in the OPLS-DA model.

Application of Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to describe which spectral regions were affected by long-term low temperature storage ($R^2X=0.495$, $R^2Y=0.939$, $Q^2=0.906$, $p=1.78x10^{-16}$), (see Figure 36). The permutation test showed the model to be acceptable (see Figure 37). A resultant S-plot (see Figure 38) indicated the correlations (see Table 20). This demonstrated correlations in the upper chemical shift regions corresponding to amide groups of protein and specifically arginine.

7.5.4 Linear regression analysis - CMPG

The models were repeated for the CPMG analysis. PCA analysis revealed clear separation of the short and long-term storage data ($R^2X=0.387$, $Q^2=0.306$).

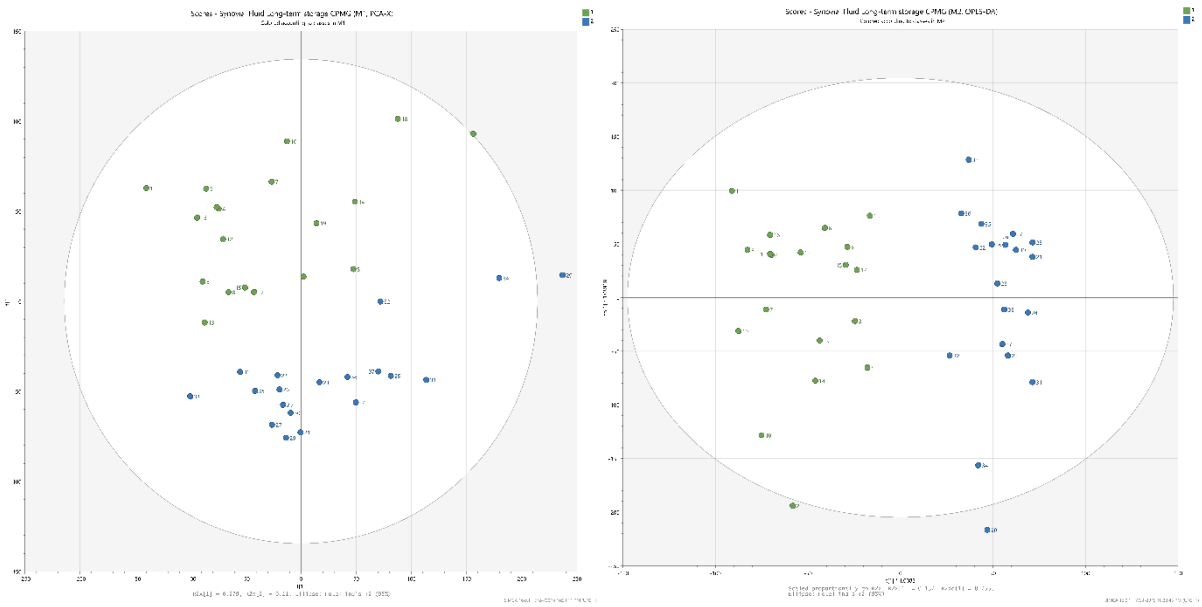


Figure 39 - PCA (left PC1 = 27.9%, PC2 = 11%) and OPLS-DA (right PC1 = 13.2%, PC2=25.5%) scores plot of low temperature HSF storage CPMG analysis (green=1 month, blue=12 month)

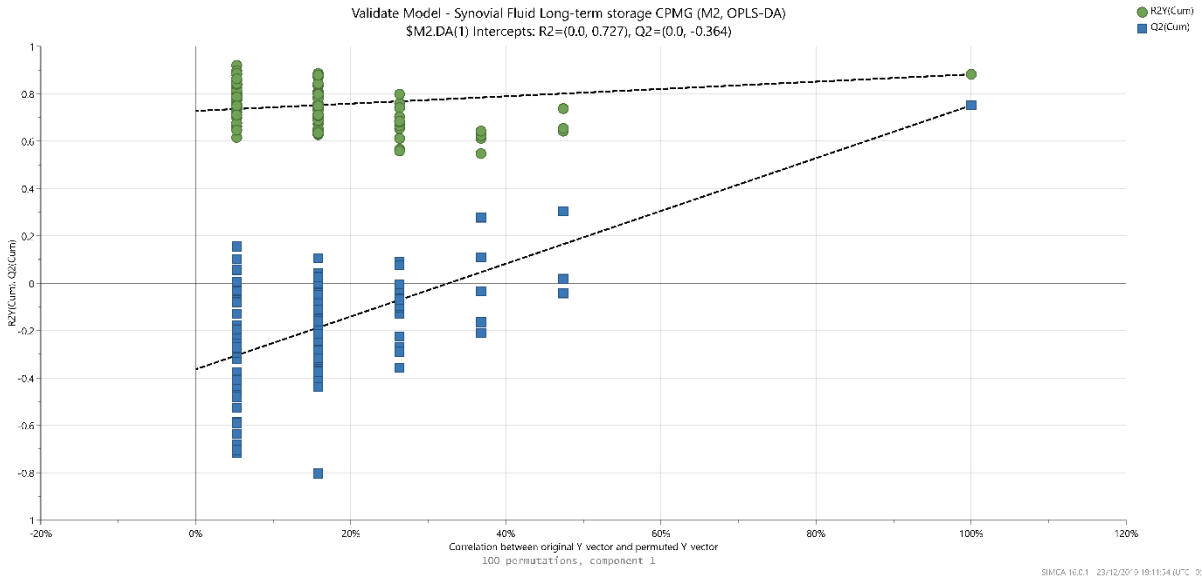


Figure 40 - OPLSDA model permutation test for the evaluation of long-term low-temperature storage for HSF using NMR CMPG sequences

An OPLS-DA model further demonstrated the significant differences between short and long-term HSF storage by CPMG analysis ($R^2X=0.387$, $R^2Y=0.882$, $Q^2=0.751$, $p=1.49x10^{-9}$; see Figure 39). The permutation testing is shown (see Figure 40). The responsible spectral region did not mirror the NOESYGPPR1D analysis and was restricted to a small spectral window of 5.04-5.16ppm, corresponding to a broad band (see Figure 41).

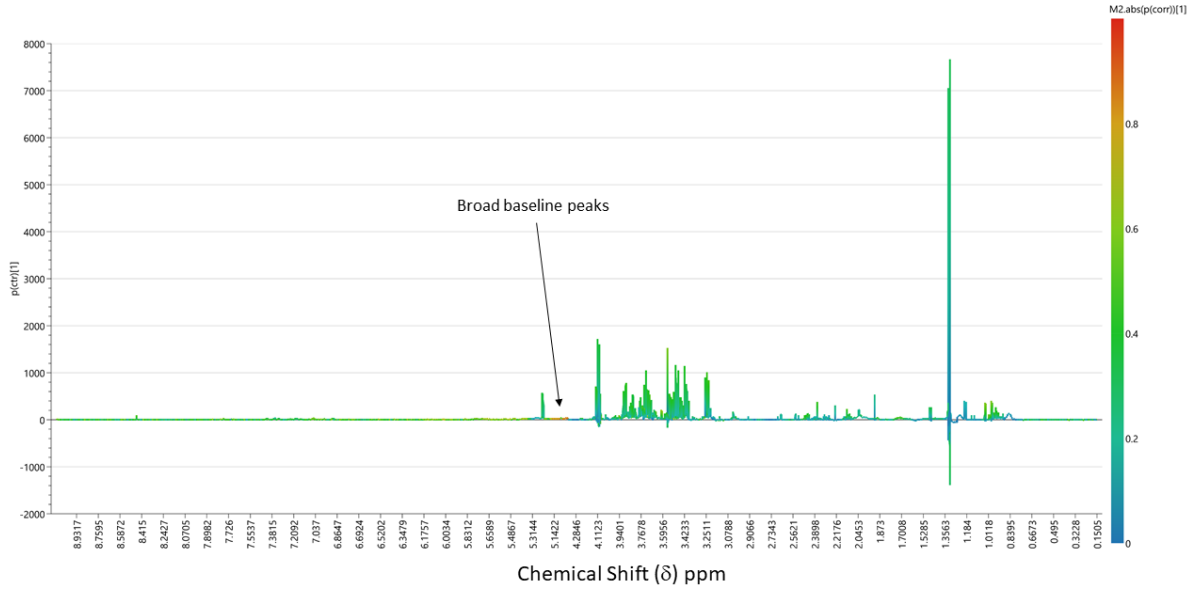


Figure 41 - S-line representation of an OPLS-DA model comparing short and long-term HSF storage after CPMG NMR spectroscopy analysis

Chemical Shift (ppm)	Metabolite	P(CORR) NOESYGPPR1D	P(CORR) CPMG
1.64-1.76	Arginine	0.92	
1.84-1.895	Unknown	0.74	
2.557-2.633	Unresolved baseline	0.86	
2.816-3.02	Unresolved baseline	0.92	
3.075-3.163	Unresolved baseline	0.90	
5.04-5.16	Unresolved baseline		0.90
8.33-9.763	Unresolved baseline (aromatic region)	0.933	

Table 20 - Spectral regions of separation between short-term (1 month) and long-term (12 months) low temperature (-80°C) from OPLSDA models of NMR spectroscopy analysis of HSF

7.5.5 Metabolite evaluation

The integrals for each metabolite correlated with the S-line plots indicating which metabolites changed during storage. As before, this is reported as a relative ratio (RR) change to the control sample. Most metabolites had a mean change centred around zero indicating no overall change. However, 95% confidence intervals demonstrate a wide variation in signal change throughout the samples. All metabolites had a 95% confidence interval which included zero (see Figure 42 & Figure 43).

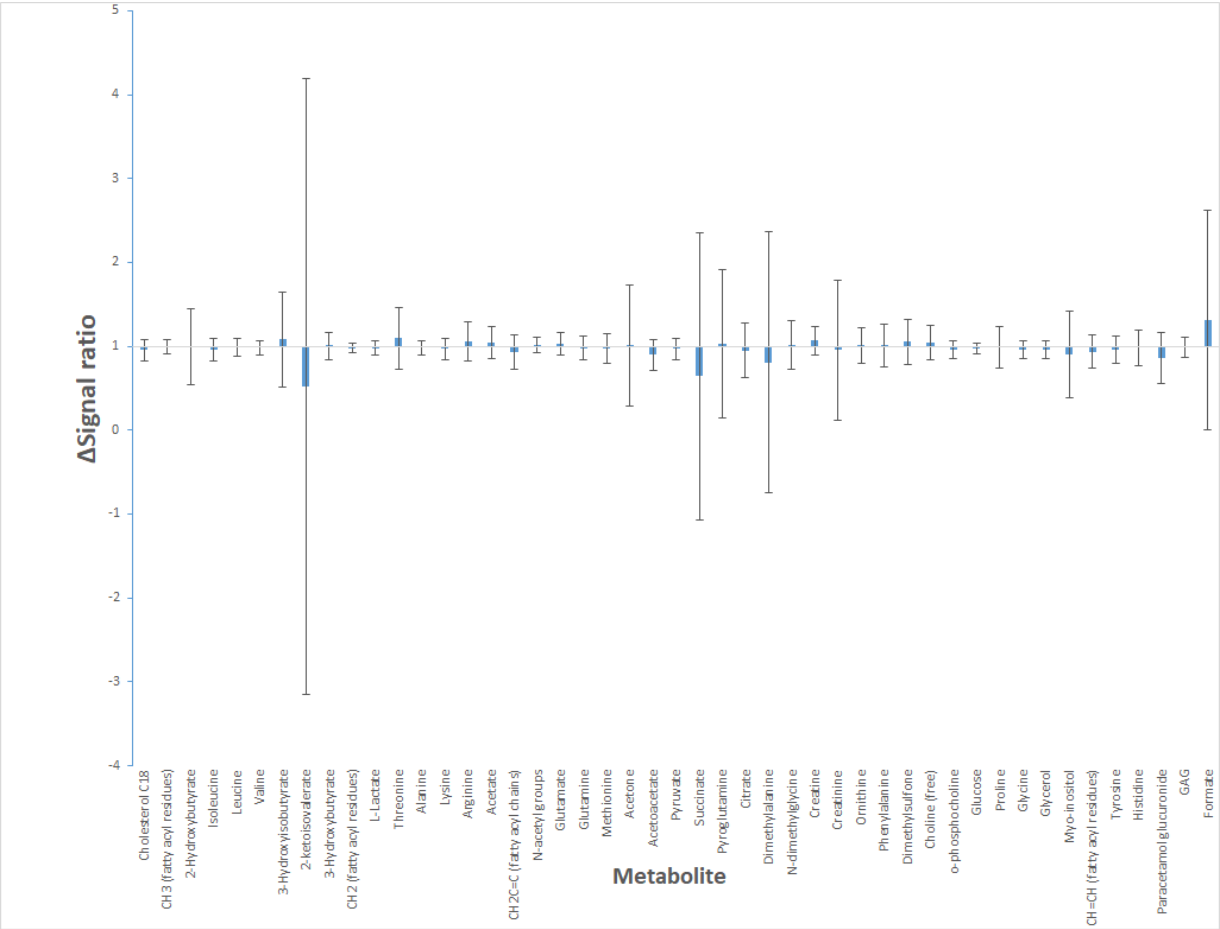


Figure 42 - Change in metabolite NOESYGPPR1D signal due to long-term low temperature storage

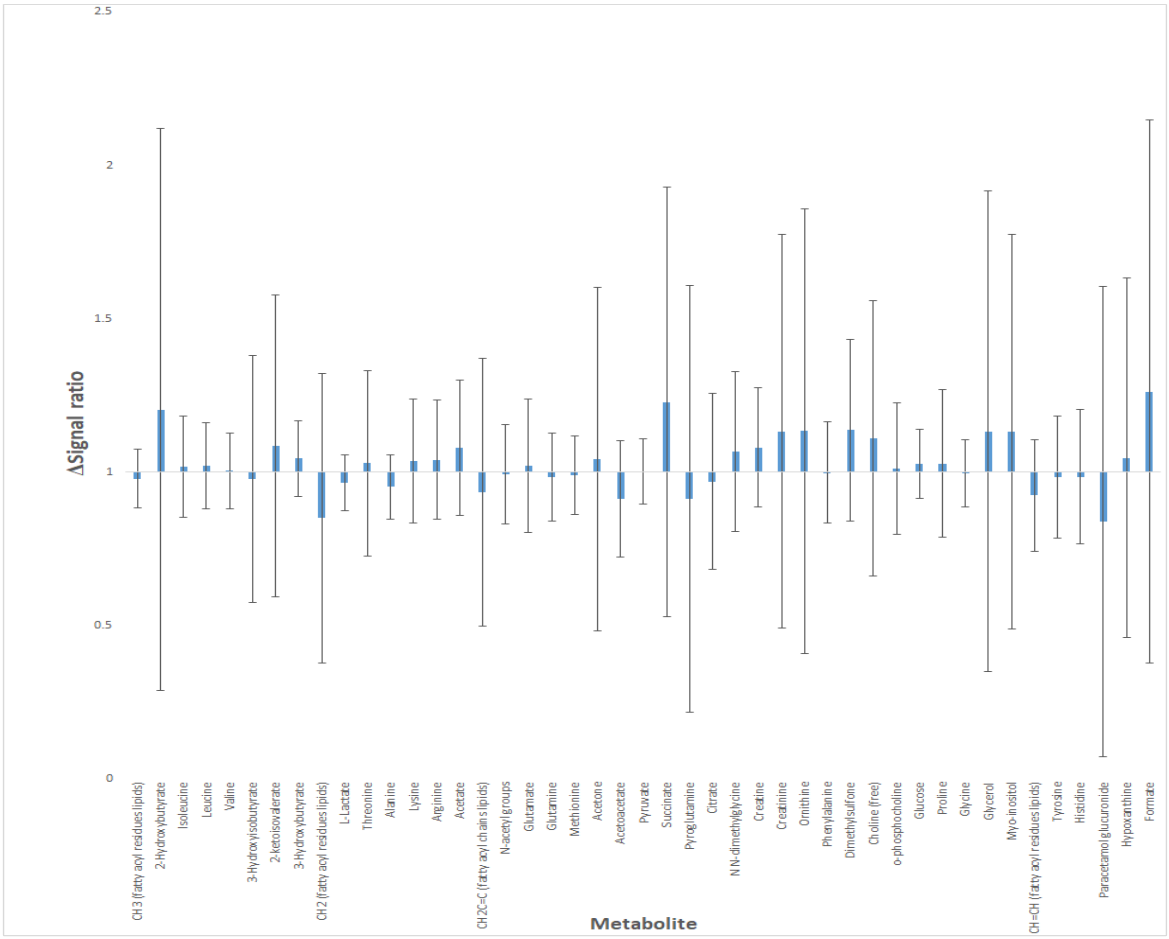


Figure 43 - Change in metabolite CPMG signal due to long-term low temperature storage

The paired t-test combined with FDR correction yielded a more sensitive detection. The NOESYGPPR1D sequences showed a significant decrease in acetoacetate (RR 0.898) and an increase of creatine (RR 1.066). CPMG sequences identified significant increases in: 3-hydroxybutyrate (RR 1.045), acetate (RR 1.08), succinate (RR 1.22), creatine (RR 1.08), dimethylsulfone (RR 1.137) and N-N dimethylglycine (RR 1.066). A significant increase was seen in acetoacetate (RR 0.898), (see Table 21 & Table 22).

Table 21 - Long-term low temperature storage impact upon HSF NOESYGPPR1D NMR metabolic profile

	NOESYGPPR1D		
Metabolite	T-test (α=0.05)	FDR correction (b=0.05)	Significant (95% confidence)
Cholesterol C18	0.054622293	0.013829787	NO
CH3 (fatty acyl residues)	0.079771957	0.015957447	NO
2-Hydroxybutyrate	0.805687848	0.044680851	NO
Isoleucine	0.21300185	0.022340426	NO
Leucine	0.421800993	0.035106383	NO
Valine	0.642269156	0.039361702	NO
3-Hydroxyisobutyrate	0.136417294	0.021276596	NO
2-ketoisovalerate	0.318099476	0.027659574	NO
3-Hydroxybutyrate	0.249117043	0.025531915	NO
CH2 (fatty acyl residues)	0.689150211	0.040425532	NO
L-Lactate	0.938846493	0.05	NO
Threonine	0.01528184	0.006382979	NO
Alanine	0.468298971	0.036170213	NO
Lysine	0.480299389	0.037234043	NO
Arginine	0.011478264	0.005319149	NO
Acetate	0.029793805	0.010638298	NO
CH2C=C (fatty acyl chains)	0.051849732	0.012765957	NO
N-acetyl groups	0.017566747	0.008510638	NO
Glutamate	0.010250457	0.004255319	NO
Glutamine	0.925393101	0.04893617	NO
Methionine	0.738240524	0.042553191	NO
Acetone	0.847186079	0.04787234	NO
Acetoacetate	**0.000362354**	**0.00212766**	**YES**
Pyruvate	0.33114324	0.029787234	NO
Succinate	0.119851096	0.020212766	NO
Pyroglutamine	0.836663838	0.046808511	NO
Citrate	0.419978343	0.034042553	NO
Dimethylalanine	0.33347302	0.030851064	NO
N-dimethylglycine	0.313802855	0.026595745	NO
Creatine	**0.000351992**	**0.00106383**	**YES**
Creatinine	0.773217173	0.043617021	NO
Ornithine	0.319016463	0.028723404	NO
Phenylalanine	0.386646408	0.032978723	NO
Dimethylsulfone	0.027244545	0.009574468	NO
Choline (free)	0.016996845	0.007446809	NO
o-phosphocholine	0.044733204	0.011702128	NO
Glucose	0.368284131	0.031914894	NO
Proline	0.724250115	0.041489362	NO
Glycine	0.091726693	0.018085106	NO
Glycerol	0.103062078	0.019148936	NO
Myo-inositol	0.234050053	0.024468085	NO
CH=CH (fatty acyl residues)	0.083389572	0.017021277	NO
Tyrosine	0.213231668	0.023404255	NO
Histidine	0.821764415	0.045744681	NO
Paracetamol glucuronide	0.005606437	0.003191489	NO
GAG	0.537926856	0.038297872	NO
Formate	0.06092296	0.014893617	NO

	CPMG		
Metabolite	T-test (α=0.05)	FDR correction (b=0.05)	Significant (95% confidence)
CH3 (fatty acyl residues lipids)	0.162557873	0.00018062	NO
2-Hydroxybutyrate	0.053299379	5.92215E-05	NO
Isoleucine	0.067117055	7.45745E-05	NO
Leucine	0.026036357	2.89293E-05	NO
Valine	0.096004235	0.000106671	NO
3-Hydroxyisobutyrate	0.883195126	0.000981328	NO
2-ketoisovalerate	0.044867396	4.98527E-05	NO
3-Hydroxybutyrate	**0.000556827**	**6.18696E-07**	**YES**
CH2 (fatty acyl residues lipids)	0.035760128	3.97335E-05	NO
L-Lactate	0.9219799	0.001024422	NO
Threonine	0.168703532	0.000187448	NO
Alanine	0.586481905	0.000651647	NO
Lysine	0.042826464	4.7585E-05	NO
Arginine	0.022034854	2.44832E-05	NO
Acetate	**0.002976777**	**3.30753E-06**	**YES**
CH2C=C (fatty acyl chains lipids)	0.456064992	0.000506739	NO
N-acetyl groups	0.135638755	0.00015071	NO
Glutamate	0.098869762	0.000109855	NO
Glutamine	0.466178169	0.000517976	NO
Methionine	0.285985501	0.000317762	NO
Acetone	0.333059118	0.000370066	NO
Acetoacetate	**0.006337261**	**7.0414E-06**	**YES**
Pyruvate	0.062072273	6.89692E-05	NO
Succinate	**0.007081239**	**7.86804E-06**	**YES**
Pyroglutamine	0.526587765	0.000585098	NO
Citrate	0.980198891	0.00108911	NO
N N-dimethylglycine	**0.00515659**	**5.72954E-06**	**YES**
Creatine	**0.000183812**	**2.04235E-07**	**YES**
Creatinine	0.04180069	4.64452E-05	NO
Ornithine	0.076123969	8.45822E-05	NO
Phenylalanine	0.159030599	0.000176701	NO
Dimethylsulfone	**0.000259529**	**2.88365E-07**	**YES**
Choline (free)	0.01849152	2.05461E-05	NO
o-phosphocholine	0.090328332	0.000100365	NO
Glucose	0.016066774	1.7852E-05	NO
Proline	0.069947129	7.7719E-05	NO
Glycine	0.101818449	0.000113132	NO
Glycerol	0.096304804	0.000107005	NO
Myo-inositol	0.057350761	6.37231E-05	NO
CH=CH (fatty acyl residues lipids)	0.067720863	7.52454E-05	NO
Tyrosine	0.374701972	0.000416336	NO
Histidine	0.528040129	0.000586711	NO
Paracetamol glucuronide	0.149286297	0.000165874	NO
Hypoxanthine	0.272414259	0.000302683	NO
Formate	0.012623108	1.40257E-05	NO

Table 22 - Long-term low temperature storage impact upon HSF CPMG NMR metabolic profile

7.6 Discussion

Does prolonged low-temperature storage exert a metabolic effect upon the synovial fluid? It is important to investigate this question prior to any clinical study of HSF. Studies validating or justifying this method of storage are rare or lack detail. These results rigorously detail instabilities essential to validate methodology for any clinical study of HSF.

7.6.1 Lipid stability

The mean spectra plots highlighted areas of the spectrum subject to change during long-term storage. The broad unresolved lipid peaks were highlighted. The PCA and OPLSDA models corroborated this finding. This agrees with the findings of Damyanovich et al (206). Lipid peaks showed significance with univariate testing, but not after FDR correction. The difference may originate from an unresolved baseline and as such would be identified by the OPLSDA models since they evaluate a single datapoint within the spectrum. However, univariate analysis evaluates a total peak integral, which dilutes the influence of peak shape. More variation was present in the detection of these lipids both in the NOESYGPPR1D and CPMG sequence acquisitions after long-term storage (see Figure 42 & Figure 43). Therefore, the effect of long-term storage on lipid metabolism should be considering when interpreting data from an HSF cohort.

7.6.2 Macromolecule stability

Significant segregation was seen on the OPLSDA model, distinguishing between short and long-term storage. The responsible spectral regions with Pcorr>0.8 were mostly due to unresolved baseline and in the broad spectral envelope, particularly in the aromatic region. Macromolecular or protein binding probably caused these unresolved baseline changes due to binding effects across the spectrum.

7.6.3 Small molecule stability

Univariate analysis showed that acetate and 2-hydroxybutyrate were significantly decreased. In canine and equine synovial fluid OA causes acetate concentrations to increase (100, 101, 111). 3-hydroxybutyrate increases in synovial fluid metabolism of OA and other joint diseases (94, 100, 101,

111, 117). The CPMG sequence identified 3-hydroxybutyrate to significantly decrease during storage. The diminishing detection of these metabolites during long-term storage decreases the likelihood of a clinically significant increase being detected. Furthermore, these methods may even falsely report a depletion of these metabolites.

A significant increase in dimethylsulfone ($DMSO_2$) was identified upon t-testing after FDR correction in the CPMG analysis. Previous work has concluded increasing dimethylsulfone in OA ovine models (116). A decrease in creatine was significant in both the NOESYGPPR1D and the CPMG sequences. Creatine has been linked to free radical processing in joint fluid. Also, it seems to be increased by OA in both ovine and equine animal models (111, 116). If long-term storage was involved in these studies, then the increase may have been exaggerated.

Decreases in acetoacetate and increases in N, N-dimethylglycine and succinate were also observed with long-term storage of synovial fluid. Any future work identifying these metabolites as significant in synovial fluid needs to consider the findings described here.

This study was not able to corroborate the findings of Damyanovich et al. that glucose, valine, leucine and isoleucine were significantly altered by long-term synovial fluid storage (206). Significance was demonstrated for glucose and leucine in the CPMG analysis ($p<0.05$). However, this significance was lost upon FDR correction. The other metabolites were not significantly altered.

Changes in pH occur with long-term storage and such variations affect NMR spectra. Previous authors have raised concerns about salt precipitation and sediment formation within the samples as a result of pH changes. Any pH effect is probably mediated by protein binding, although this remains to be clarified. We did not observe any sedimentation or precipitation. Buffering prior to freezing was one proposal to counteract this problem. However, previous experiments have shown the detrimental effects of doing so (see 5.5).

There is a disparity between CPMG and NOESYGPPR1D results. Certain metabolites e.g., myoinositol, show an increased NOESYGPPR1D signal but a reduced signal in CPMG. This correlates with other work performed upon HSF. One hypothesis is that this difference originates from abundant

charged and ionic macromolecules causing binding effects which are subsequently masked by the CPMG sequence. However, this requires clarifying with further experiments using titrated exogenous addition of these metabolites.

7.6.4 Limitations

The key limitation of this study is variability due to two analyses conducted 12 months apart. Where possible, this was controlled within the study design by using the same technician, instrument, reagent batches etc. However, inevitably the study may have failed to identify some metabolites affected by storage.

8 Chapter 8: The evaluation of protein precipitation extraction techniques in the small molecule analysis of human synovial fluid.

8.1 The qualitative evaluation of liquid/liquid protein precipitation techniques in the NMR analysis of human synovial fluid

8.1.1 Introduction

HSF is a complex biofluid with a high concentration of macromolecules including proteoglycans. The metabolic profiling of small molecule metabolites using NMR is directly affected by the quality of spectral resolution. The excess of macromolecules may overwhelm the Nuclear Magnetic Resonance (NMR) spectrum due to chemical complexity caused by complex protein bindings, multiple spin interactions and unpredictable proton environments. This can diminish or amplify the NMR signal observed for a given metabolite. The use of spin echo NMR sequences such as CPMG has the benefit of suppressing but not removing these effects by a short T_2 relaxation time. It is hypothesised that use of an extraction step may recover additional metabolites and improve detection compared to previous analyses.

In an untargeted study of HSF, the greater the breadth of metabolite detection, the greater the possibility of detecting a metabolite of significance. Hence, the provision of another method of analysing HSF by NMR spectroscopy improves the breadth of analysis and maximises the information gained from a single sample.

The macromolecular composition of HSF varies with joint disease (225). Proteoglycans increase in concentration during disease while the water content can increase with OA but decrease with other joint diseases. Therefore, among subjects recruited to a study, there will be significant variation of proteoglycan and water content in the HSF. Some metabolites can have an elevated signal while others are supressed (226). As a result of macromolecule interference, the absolute quantification of

metabolite concentrations between samples may not be reliable as the distorting effect of the macromolecules may vary between samples (227).

Metabolic profiling frequently uses multiple detection methods to provide a more comprehensive representation of the biosample. Inherently, MS does not have the resolving power to analyse complex biofluids like HSF without a preparatory extraction step. Future analyses of HSF may use MS analysis techniques in targeted and untargeted studies. A derived extraction method could be applied to both MS and NMR and provide advantages in future analyses. Absolute rather than relative quantification may be required in future to investigate specific metabolite classes. The use of protein extraction may provide the methods to achieve this.

Various methods can provide a protein precipitation. These include ultrafiltration, liquid/liquid solvent extraction, strong acid precipitation (e.g., trifluoroacetic acid, TFA) or the addition of concentrated salt (e.g., ammonium sulphate). Whilst ultrafiltration has been used successfully in serum previously, in HSF the range of macromolecule size in the presence of disease may make it inappropriate (228). Concentrated salts exert their effect by saturating the solution and causing ionic proteins to crystallise (229). In HSF this would target the proteoglycan molecules (see 1.3.4). However, the hydrophobic molecules would be unaffected. This leaves two methods to be evaluated: liquid/liquid extraction and the addition of strong acids.

The use of a solvent liquid/liquid extraction will necessitate the use of drying step to remove the solvent before reconstitution in NMR buffer prior to analysis. This stabilises the sample pH and adds the deuterated reference material required for analysis. Evaporation of the solvent under blown nitrogen or under a vacuum is efficient. However, it has the potential to cause the loss of volatiles (230). Perhaps the need for a drying stage can be overcome by reconstitution in deuterated solvent. This provides a suitable deuterated reference to stabilize the magnetic field strength of the NMR and negates the need for subtraction of the water peak (previously the predominant solvent). In addition, it might provide information about the losses suffered due to drying under nitrogen.

To date no study has evaluated the use of a protein precipitation step in the analysis of HSF by NMR and it is not known if any previously supressed metabolites will be made visible by these methods.

8.1.2 Hypotheses

- The removal of protein from HSF samples will provide analytical gains in the metabolic profiling.
- The use of deuterated solvent as an extractant will remove the need for a drying stage with the potential recovery of volatiles.

8.1.3 Aim

To qualitatively evaluate the use of protein precipitation methods and their effect on the metabolic description of HSF by NMR methods.

8.1.4 Materials and methods

8.1.4.1 Sample preparation

Three sample types were analysed with all the extraction methods: high performance liquid chromatography (HPLC) grade water (system blank), BCS (bioblank) and HSF (biosample).

System blanks were prepared using HPLC grade water to ensure no contamination from solvents, reagents or glassware during the sample preparation process. A bioblank (BCS - Sigma Aldrich Cat # 12133C; batch 11n239) was used to verify the effect of any biological sample to the elution of contaminants within it. This occurs because the NMR spectrum of this sample is known. The sample was prepared using the same disposables as the HSF samples. Therefore, any additional peaks would be due to biological elution of contaminants from disposables used in the preparation of the samples. The BCS samples were also used to as QC samples in line with previous methods (see 5.5).

At the time of analysis, the shortage of HSF samples meant that the preliminary evaluation of protein precipitation methods was to be performed upon aliquots of a single HSF sample. One large volume sample from a typical OA patient was selected: 72-year-old male, end stage OA, BMI 31.4, white English ethnicity, and a history of ischaemic heart disease, vitamin D deficiency and type II diabetes mellitus. Immediately after collection all samples were prepared by centrifugation (10000g; 15min,

room temperature) before the supernatant was dispensed into aliquots for analysis and frozen at -80ºC for less than three months.

An internal standard was used to provide accurate calculations of recovery by the extraction methods. It was important to select material that would not react with other molecules in the sample, particularly proteins. Also, the internal standard should not present endogenously in the pooled HSF sample. A literature search revealed that caffeine had no reported cross reactivity or deleterious effect upon the NMR spectrum of other studied biofluids. Each sample was fortified with caffeine (10µg).

Phosphate NMR buffer (0.142M, pH 7.4; 1L) was prepared by adding sodium dihydrogen phosphate (10.64mg NaH_2PO_4), sodium azide (40mg NaN_3) and 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt in deuterium oxide solution (80mg TSP).

Acetonitrile, methanol, deuterated methanol, trichloroacetic acid (TCAA), deuterated water, TSP and caffeine were obtained from Sigma Aldrich.

8.1.4.2 Protein precipitation

Five protein precipitation and extraction methods were studied:

- no extraction
- liquid/liquid extraction methanol
- liquid/liquid extraction acetonitrile
- liquid/liquid extraction deuterated methanol
- TCAA

Extraction methods were configured to have the same final volume and TSP concentration.

8.1.4.2.1 No extraction

Samples (400µl) were mixed with buffer (400µl) before being transferred to an NMR tube.

8.1.4.2.2 Methanol and acetonitrile

Samples (400µl) were mixed with methanol (400µl) or acetonitrile (400µl). These liquids were vortexed to mix and incubated at -20ºC for 20 minutes. The mixtures were then centrifuged at 10000rpm for 30 minutes at room temperature to pellet the protein. Supernatant was removed directly and dried under nitrogen. Samples were reconstituted using D_2O (400µl) and phosphate buffer (400µl).

8.1.4.2.3 Trichloroacetic acid (TCAA)

Sample (400µl) was cooled in an ice bath and mixed with TCAA (10µl, 10%) by vortexing and then left for 10min. It was then centrifuged at 10000rpm for 30min at room temperature to pellet proteins before removing supernatant. The dry pellet was then washed twice using deionised water before evaporation to dryness on both occasions. All dried samples were reconstituted in deuterated water (400µl), before having buffer added (400µl) and transferred to an NMR tube.

8.1.4.2.4 Deuterated methanol

Sample (400µl) was mixed with deuterated methanol (400µl). The resultant mixture was vortexed to mix and incubated at -20ºC for 20 minutes. The mixture was then centrifuged at 10000rpm for 30 minutes at room temperature to pellet the protein. Supernatant was removed directly before having TSP (0.64mg) added.

8.1.4.3 NMR analysis

The established NMR methodology from previous analyses was applied (see 4.4.3). The use of deuterated solvent resulted in an alternate section of the spectrum subtracted for solvent removal.

8.1.4.4 Data processing

All NMR FID signals were transformed by Fourier transformation using Topspin® v3.2 software (Bruker). Spectra were manually phased and baseline corrected and calibrated to the ^1H proton peak signal of TSP (δ=0ppm). Data processing was performed using Matlab® software vR2014b (Mathworks®, Natwick, Massachusetts, USA).

Spectral subtraction was performed to remove the water peak (δ=4.5-5.0ppm), except for those samples reconstituted in deuterated methanol.

8.1.5 Results

8.1.5.1 Contaminants

Evaluation of the system blanks identified the solvent peaks using the 1d spectra. There were minor additional peaks which appeared throughout the analysis at low signal intensity. Some may represent satellite peaks due to the ^{13}C isotope of the solvent peak; others could not be identified with 1d analysis alone.

8.1.5.2 Experimental observations

The addition of methanol caused a solidification of the sample. This occurred to a varying degree throughout the experiment. It did not preclude the analysis but will have compromised the recovery and quality of the spectra obtained. This is because harvesting of the supernatant after solvent addition was unreliable. One sample extracted with deuterated methanol was not useable as a result.

Spectra were evaluated for the internal standard using known HMDB libraries. The caffeine peak was not visible within the samples or the system blanks. Thus, it is likely that the spiking level was not sufficient to provide adequate detection due to binding or suppression.

8.1.5.3 Spectral evaluation NOESY

A comparison of the NOESY sequence revealed that all methods: acetonitrile, methanol and TCAA removed protein from the samples. This was shown by the consistency of the baseline in all regions of the spectra. Most notable was removal of the unresolved peaks around the *N*-acetyl region (δ=2-2.05 ppm) and removal of the lipoproteins (δ=0.82-0.87;1.18-1.302; 5.25-5.35). In addition, there was an improved resolution of the choline peak (δ=3.2-3.24) and a flattening of the baseline in the aromatic region (δ=6.6-9.0).

The TCAA was the most effective at removing the macromolecules, but this compromised the sensitivity because the signal intensity of metabolites was also reduced. The methanol was less effective at removing macromolecule signals. However, it did not cause signal reduction. Acetonitrile seemed to provide the best compromise with a removal of macromolecules whilst retaining signal (see Figure 44).

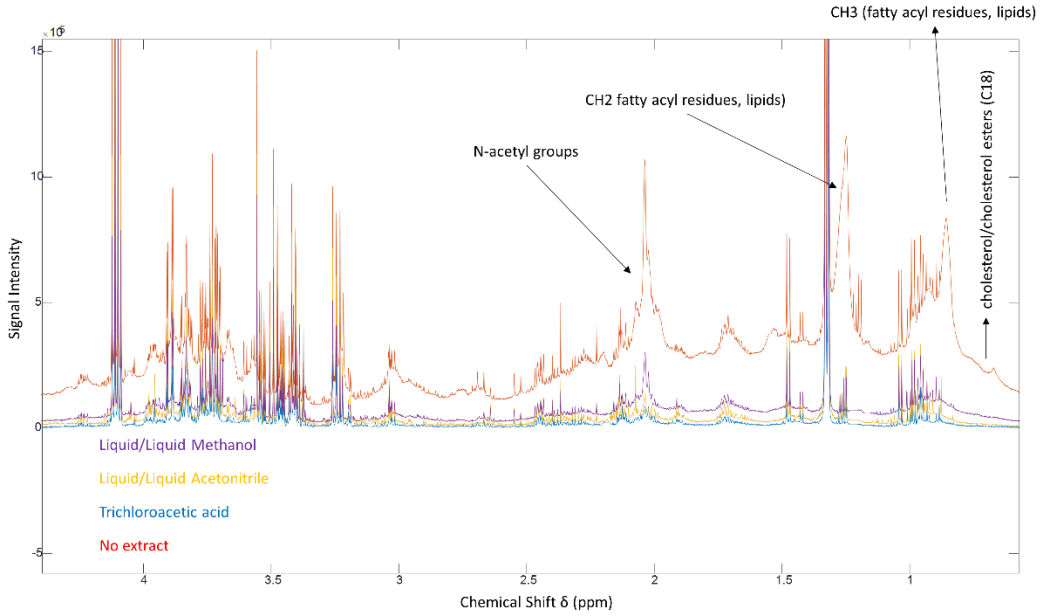


Figure 44 - NOESY NMR evaluation of HSF spectra using a variety of protein precipitation techniques

The extracted spectra were evaluated for any analytical gains compared to an unextracted sample. Four singlets not previously visible and absent from the system blanks, were present after extraction. These were not identifiable and remain unknown: δ=3.64, 3.957 & 4.049 all gained after acetonitrile extraction. A singlet at δ=1.913 was an additional recovery after both the methanol and the acetonitrile extractions.

8.1.5.4 Spectral evaluation CPMG

The comparison of extracted to non-extracted CPMG sequences is valid because this is the current and preferred method to remove macromolecule effects. Unsurprisingly, all extractions had an improvement in the macromolecular interference, mirroring the results of the NOESY spectra. As with the NOESY data, TCAA had the poorest signal intensity and thus a loss in sensitivity.

The liquid/liquid extractions of methanol and acetonitrile both provided improvements in baseline signal, over and above the macromolecular suppressive effect of the CPMG acquisition. Methanol and acetonitrile both gave enhanced small metabolite signals, although this effect was greater in the

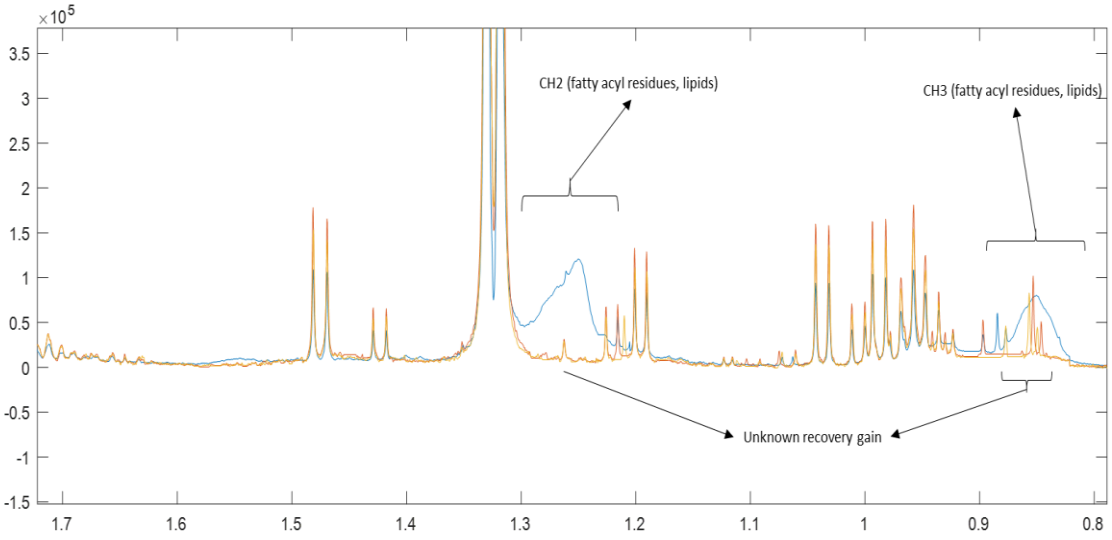


Figure 45 - NMR CPMG Spectra overlay of non-extract (blue), acetonitrile (amber) and methanol (red) extract of HSF, δ=0.8-1.8

acetonitrile phase. This was notable in the δ=2.2-3.2 ppm range which includes glutamine, pyruvate, citrate and creatine (see Figure 46).

Both methods demonstrated a loss of 2-hydroxybutyrate (δ=0.897 ppm) but with additional detectable peaks underneath the lipid signals of δ=0.844, 0.851, 0.864, 1.263, 1.291 ppm (see Figure 45). It is unclear if some of these gains are the lost 2-hydroxybutyrate which has suffered a chemical shift.

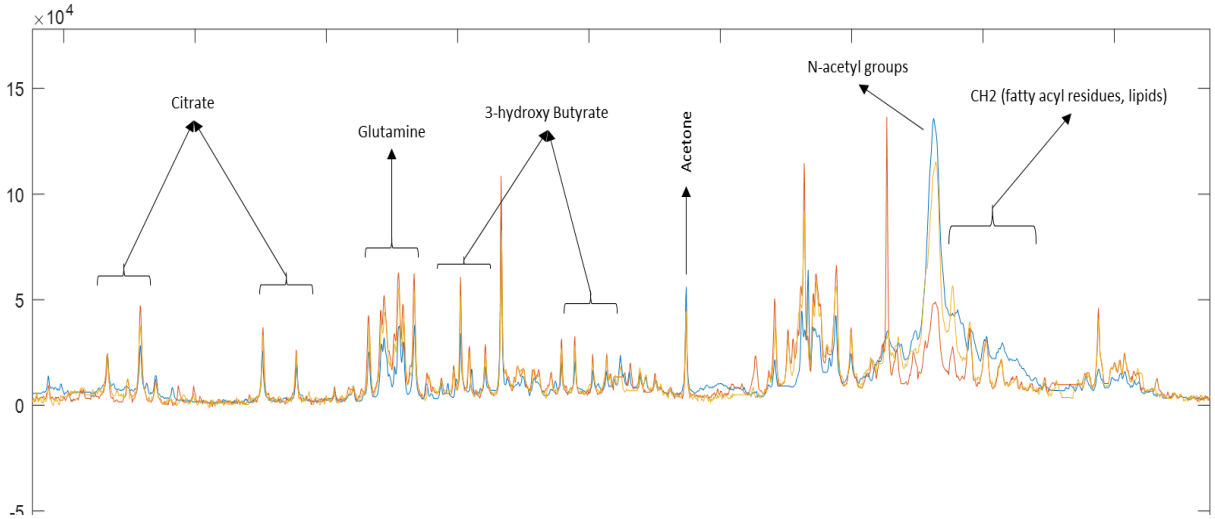


Figure 46 - NMR CPMG Spectra overlay of non-extract (blue), acetonitrile (amber) and methanol (red) extract of HSF, δ=1.8-2.7

The denaturing problems experienced with the addition of methanol to HSF resulted in only a single extract being available for analysis. Hence, it was not possible to make conclusive statements of any

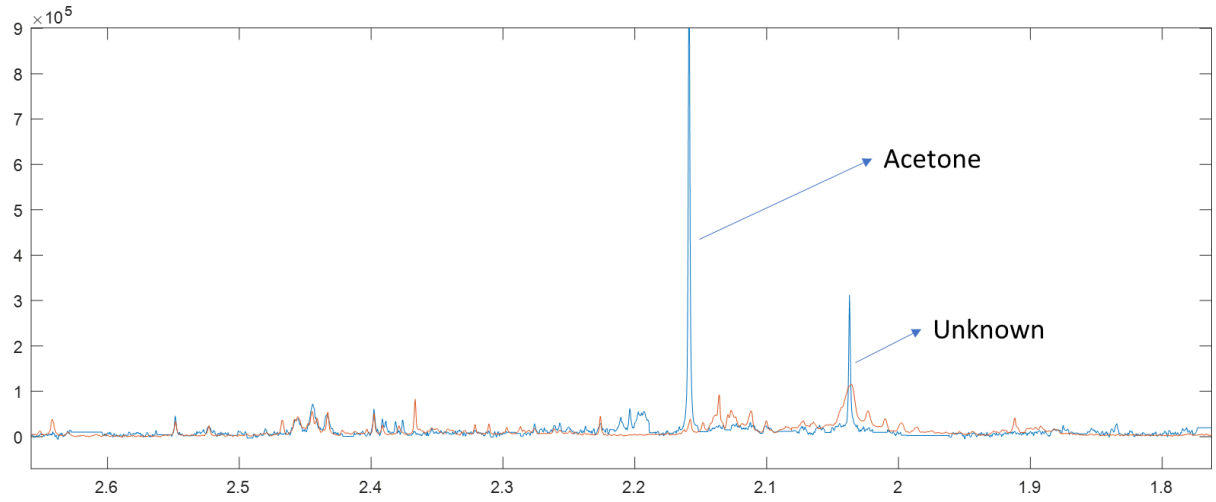


Figure 47 - NMR CPMG Spectra overlay comparing deuterated methanol (blue) and methanol liquid/liquid extraction (red)

metabolic loss due to solvent drying and volatility. As expected, there was a chemical shift seen due to the lack of pH buffer in the deuterated solvent. However, there was a clear distinction from singlet peaks at δ=2.158 & 2.037 ppm, present in the deuterated methanol but lost in the methanol liquid/liquid extraction. The peak at δ=2.158 corresponds to acetone and would be an expected volatile loss with a drying step (see Figure 47).

8.1.6 Discussion

The removal of protein in this sample type provided analytical gains in both known and unknown metabolites. The unknown metabolites at δ=1.913, 3.64, 3.957 & 4.049 were all gained after acetonitrile extraction and undescribed in this biofluid (231).

The presence of minor unidentified contaminant peaks in the system blanks needs further investigation and identification. Some of these peaks are attributable to ^{13}C isotope satellite peaks of adjacent high signal solvent peaks. However, this requires clarification and thus a further 2D NMR analysis is proposed.

The use of an internal standard was desired, and we trialled caffeine. It has limitations of not necessarily representing the entire scope of metabolites within the sample. However, it should demonstrate the recovery and analytical stability of the methods. Sadly, it appeared that the concentration of caffeine was insufficient, and this should be adjusted prior to any future analysis.

The denaturation of proteins after methanol addition was unexpected and is not yet explicable. This invariably compromised the reproducibility of the analysis due to difficulties in sample handling. It is unclear whether this effect was specific to the HSF sample analysed or which proteins were affected. The methanol method only gave one singlet gain (not previously seen) at δ=1.9.

Using TCAA to precipitate protein had more steps than the other methods. Whilst extraction of protein was efficient, it always affected analytical sensitivity, the signal was reduced by between 2% to 20%. There were no analytical benefits in the use of TCAA above the other liquid/liquid extractions and therefore its use is not recommended.

The deuterated solvent did identify potential losses in the use of liquid/liquid extraction and drying steps. However, more detailed conclusions cannot be drawn because there was only one sample and comparisons could not be made. Further work will be required to identify and quantify which metabolites are affected. Deuterated methanol has the advantage over the other methods that the water peak contributes far less interference to the spectra. Whilst this may give analytical gains in the δ=4.5-5.0 region, this is in contrast to the presence of a methanol solvent peak at δ=3.4 ppm which is likely to detract from detection of proline, glucose and other monosaccharides.

The potential drawbacks of introducing an extraction step should be considered. Additional sample handling introduces greater potential error and variation in the sample preparation. In addition to being time intensive, this also increases the time spent at warm temperatures and on the bench, thereby affecting metabolic composition. However, the results have shown promise in the untargeted study of HSF. The liquid/liquid extraction has produced the most consistent spectra and least metabolite loss. The acetonitrile was found to have minor gains above the methanol, and both were superior to the TCAA. The methanol extraction method may be impractical because of protein denaturing. The

seriousness of the protein denaturing will only become apparent when a larger stock of HSF samples is available for trialling. Therefore, a more quantitative evaluation of the methanol and acetonitrile is recommended.

8.2 Metabolite identification and the extraction recoveries of liquid/liquid extraction prior to human synovial fluid NMR analysis

8.2.1 Introduction

Preliminary analysis has suggested that a liquid/liquid extraction of HSF may provide some analytical gains in the untargeted analysis of this biofluid. However, the presence of contaminant peaks needs to be clarified and the efficiency of extraction recovery quantified to demonstrate where losses and gains are being made. Qualitative evaluations suggest acetonitrile liquid/liquid extraction has shown the most promise. However, the use of deuterated solvents still requires further evaluation. Since the initial evaluation showed the most promise with acetonitrile, it is proposed to evaluate acetonitrile compared to its deuterated form. The analytical losses due to the additional drying step required with non-deuterated solvents can then be quantified, thereby evaluating the use of deuterated solvent as an adjunct to these methods.

The shortage of HSF samples and volume limited the initial qualitative evaluation. The establishment of the HSF biobank now permits a more representative pooled sample to provide sufficient volume for replicate analysis and direct comparison.

8.2.2 Aims

- The identification of contaminants in the metabolic profiling of HSF using a solvent liquid/liquid extraction
- The quantitation of volatile losses during blown nitrogen solvent drying
- The quantitation of reproducibility and recovery for solvent liquid/liquid extractions in HSF

8.2.3 Materials and methods

HPLC grade water (Sigma Aldrich) was used as a system blank and to identify contaminants in each extraction and instrument analysis. A pooled HSF sample was created using HSF samples encompassing a spectrum of ages, ethnicities and disease presentations (n=10): age 29-93, ethnicity (White British n=6, Caribbean n=2, Irish n=2), female (n=6), male (n=4). The samples were harvested

from a variety of clinical scenarios: acute synovitis (n=2), OA of the knee (n=6), acute knee injury (n=1) and crystal arthropathy (n=1). Samples were prepared at the time of collection by centrifugation (10000g; 15min, room temperature) before the supernatant was dispensed into aliquots for analysis and frozen at -80ºC for less than three months. Aliquots were defrosted and a pooled sample made by combining equal volumes of each sample before refreezing at -80ºC.

8.2.3.1 Sample preparation

Pooled samples were prepared using the same methods as previously evaluated (see Protein precipitation). The use of deuterated acetonitrile was evaluated with direct comparison to the acetonitrile extraction.

All methods were tested using the system blank and pooled HSF sample in triplicate. Each triplicate was repeated on three occasions being prepared by different researchers. All samples were then analysed together and contained the same final volume and concentration of Trimethylsilylpropanoic acid (TSP).

8.2.3.2 Deuterated acetonitrile

Pooled sample (400µl) was mixed with Deuterated acetonitrile (400µl). The resultant mixture was vortexed to mix and incubated at -20ºC for 20 minutes. The mixture was then centrifuged at 10000rpm for 30 minutes at room temperature to pellet the protein. Supernatant was removed directly before adding TSP (0.64mg). The resultant liquid was transferred to an NMR tube.

8.2.3.3 Quality control

A single HSF pooled sample aliquot was analysed at repeated intervals throughout the sample run. Evaluation of the consistency and reproducibility of these runs serves as quality control. A CV was used to demonstrate consistent analysis of 11 metabolites across 14 peaks (see Table 24).

8.2.3.4 NMR analysis

NMR instrument protocol was identical to those used previously (see 6.4.4). In addition, a JRES acquisition protocol was used to provide 2D NMR data and assist with metabolite and contaminant identification.

8.2.3.5 Data processing

Data was processed in an identical manner to the previous analysis (see 6.4.4). For each metabolite and extraction method, the mean, standard deviation, coefficient of variation and recovery were reported. The recovery for each metabolite is referenced to the "no extract sample", thus providing a ratio of peak integral change or a recovery expressed as a percentage (216). A univariate student t-test was applied to determine if the extraction technique caused a significant difference in metabolite recovery. A Benjamini-Hochberg correction was performed to correct for any false discovery rate (b=0.05) (224).

Whilst the full relaxation might not be achieved with shorter T_1 intervals, methods were selected to be representative of commonly used HSF analysis protocols and assuming $T_{1/2}$ values vary little between samples. Semi-quantification of metabolites was achieved by non-linear baseline adjustment followed by trapezoid integration of identified and well resolved peaks (see Supplementary Table 3). The integrals were referenced to TSP as an internal standard.

8.2.4 Results

8.2.4.1 Contaminants

System blanks were used to determine the quantity of contaminants for every extraction method. The use of 2D ^{1}H-NMR served to clarify the relation of the peaks. Identifications were made in combination with the HMDB database. There were several ^{13}C isotope satellite peaks derived from adjacent large signals, with the isotope peak visible in the presence of a noiseless baseline. All identifications were derived from solvents. Six singlets, all uncoupled, were present at low levels and were not identifiable by these methods (see Table 23). All contaminants were recorded for consideration in future analyses.

		Extraction method				
Chemical shift	Peak type	None (HPLC grade water)	Methanol	Acetonitrile	Deuterated Acetonitrile	Identity
0.097	Singlet	Y	Y	Y	y	13C satellite of TSP
1.176 1.188 1.199	Multiplet 1:2:1	Y			y	Ethanol
1.253	Singlet	Y	Y	Y	y	2-methyl-2-propanol (TSP solution)
1.836 1.84 1.846 1.849 1.853	Multiplet 1:2:3:2:1				y	13C satellite of 1.96 multiplet
1.91	Singlet		Y			Unknown
1.951 1.956 1.96 1.964 1.968	Multiplet 1:2:3:2:1				y	Acetonitrile
1.984	Singlet	Y		Y	y	Acetonitrile
2.063 2.066 2.071 2.075 2.079	Multiplet 1:2:3:2:1				y	C13 satellite of 1.96 multiplet
2.235	Singlet	Y	Y	Y		Unknown
3.246	Singlet	Y	Y	Y	y	13C satellite of 3.366 Singlet
3.366	Singlet	Y	Y	Y	y	Methanol
3.429	Singlet	Y	Y	Y	y	13C satellite of 3.563 singlet
3.482	Singlet	Y	Y	Y	y	13C satellite of 3.366 Singlet
3.549	Singlet	Y	Y	Y	y	Unknown
3.563	Singlet	Y	Y	Y	y	Unknown
3.665	Singlet		Y	Y		?CH3-ester
3.646 3.657 3.669 3.681	Multiplet 1:3:3:1	Y			y	Ethanol
3.698	Singlet	Y	Y	Y		13C satellite of 3.563 singlet
3.846	Doublet	Y	Y		y	13C satellite of 3.968 doublet
3.873	Singlet				y	Unknown
3.968	Doublet	Y	Y	Y	y	Unknown
4.08	Doublet					13C satellite of 3.968 doublet
4.25	Singlet				y	Unknown
7.905	Singlet	Y	Y	Y	y	Unknown
8.463	Singlet	Y	Y	Y	y	Formate

Table 23 - Contaminant peaks identified from system blank analysis

8.2.4.2 QC samples

The repeated analysis of a single pooled HSF extraction revealed a CV that was <1.5% (see Table 24). Therefore, consistent detection could be concluded throughout the NMR analytical run.

	Pooled HSF aliquot replicate analysis								
Metabolite (ppm)	*1*	*2*	*3*	*4*	*5*	*6*	**Mean**	**SD**	**CV**
CH3-LDL (0.78-0.89)	29354805	28795487	29395724	29019564	28965497	28769390	29050077.89	246311.76	**0.848**
Valine (1.04-1.047)	1337029	1327308	1338226	1331968	1327114	1323893	1330922.95	5302.88	**0.398**
3-hydroxybutyrate (1.197-1.204)	2297187	2241950	2272053	2251795	2234976	2246545	2257417.64	21168.07	**0.938**
Lactate (1.309-1.324)	30062023	29949615	30134059	30378620	30405615	30435426	30227559.65	187585.48	**0.621**
Alanine (1.464-1.475)	1844914	1795392	1825825	1834139	1817246	1802752	1820044.91	17152.24	**0.942**
Acetate (1.907-1.915)	1362853	1357924	1371429	1388258	1384771	1365190	1371737.44	11221.27	**0.818**
N-acetyl (2.029-2.05)	8040645	7818455	7898007	7959375	7862103	7902936	7913586.83	71060.02	**0.898**
Valine (2.285-2.289)	371925	369133	373831	367419	367414	369173	369815.83	2343.90	**0.634**
3-hydroxybutyrate (2.308-2.315)	471765	458657	465003	459557	458110	460696	462298.00	4792.65	**1.037**
Citrate (2.544-2.553)	442265	439280	446365	447785	441716	436819	442371.67	3787.76	**0.856**
Lysine (3.032-3.036)	1721678	1699821	1703293	1722889	1730135	1736274	1719015.00	13286.70	**0.773**
Glucose (3.501-3.511)	2640768	2581083	2609163	2619314	2589365	2604547	2607373.28	19512.57	**0.748**
Lactate (4.084-4.093)	5399729	5497632	5565634	5519349	5531223	5506605	5503362.00	51141.58	**0.929**
Tyrosine (6.895-6.906)	321338	309960	317897	316609	321554	318587	317657.50	3873.00	**1.219**

Table 24 - Selected metabolite integration for quality control analysis using a pooled HSF sample aliquot

8.2.4.3 Observations

The addition of methanol and the production of a gelatinous sample again made it difficult to consistently decant the sample. However, this happened to a lesser extent in the pooled HSF than was observed in the specific HSF sample (see 8.1.5.2).

Samples extracted using deuterated acetonitrile had significantly more peak shift than other methods. This was probably the result of a shift in pH (this method does not have a buffer). It was not possible to align the spectra of deuterated acetonitrile to the other samples with any confidence. Therefore, data using this extraction method had to be transformed separately.

The TCAA protein precipitation again performed poorly with loss of metabolites across the spectrum in all analyses. Therefore, this method was not taken to quantitation.

One deuterated acetonitrile CPMG acquisition had to be rejected because the baseline was poor. The cause of this instrument failure is unknown.

8.2.4.4 Metabolite identification and quantitation

Metabolites were identified within the extracted spectra using metabolites collated from literature search (see 4.1.1), the human metabolome database (HMBD), unextracted NMR spectra and 2D NMR data. A peak originating from each metabolite was selected for quantitation. This peak had to be visible in all extraction methods and have clear shoulders to minimise integration errors.

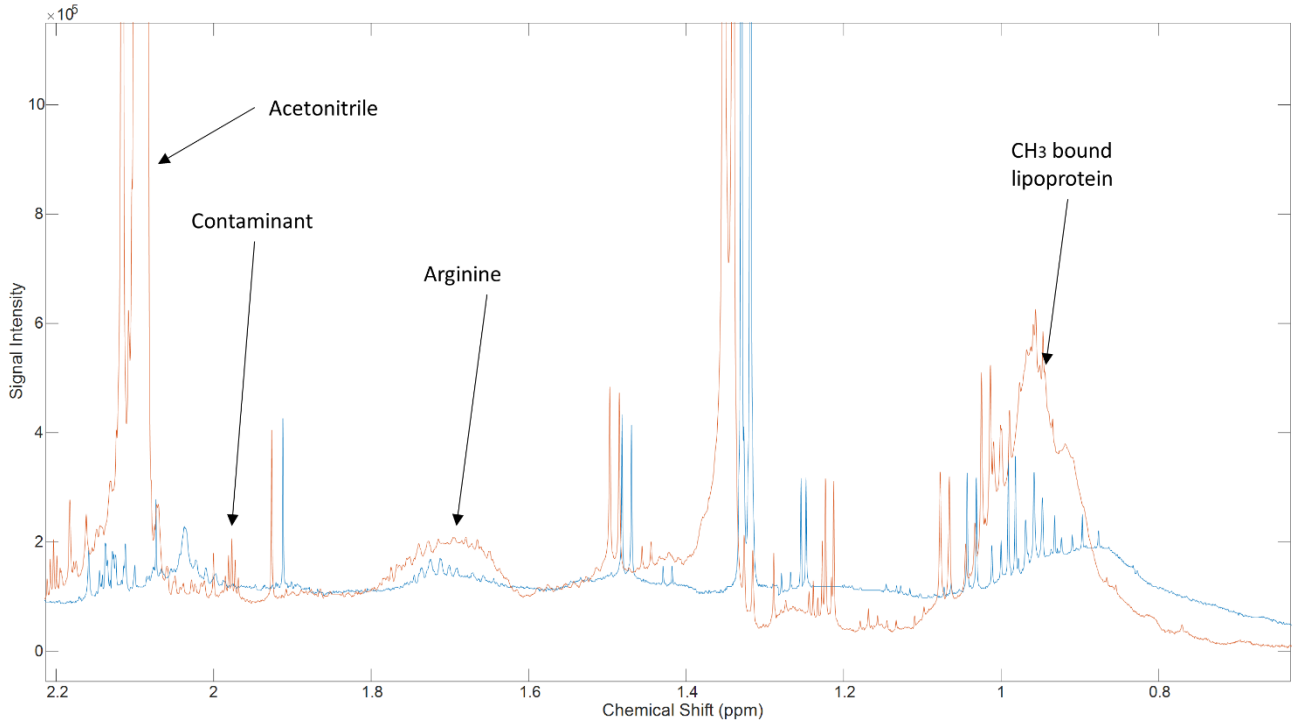


Figure 48 - NOESY NMR Spectra of HSF extracted using deuterated acetonitrile (red) and acetonitrile with a buffer reconstitute (blue)

8.2.4.5 Spectral evaluation

The previously evaluated extractions (liquid/liquid methanol liquid/liquid acetonitrile and trichloroacetic acid) provided no additional information.

A liquid/liquid deuterated acetonitrile extraction allowed an evaluation of evaporation losses seen with a non-deuterated acetonitrile liquid/liquid extraction. This was complicated by a lack of buffer in the deuterated acetonitrile method and variable shift in the peaks (see Figure 48). Therefore, to interpret

and make comparisons between methods, the spectral components were identified and compared manually without mass alignment (see Table 27).

The NOESY and CPMG spectra showed the deuterated acetonitrile extraction had a greater protein content than an acetonitrile liquid/liquid extraction with a drying step and reconstitution in buffer. This increased the baseline of the various lipid peaks (due to lipoprotein association) and arginine, presumably due to the presence of an arginine-rich protein (see Figure 49).

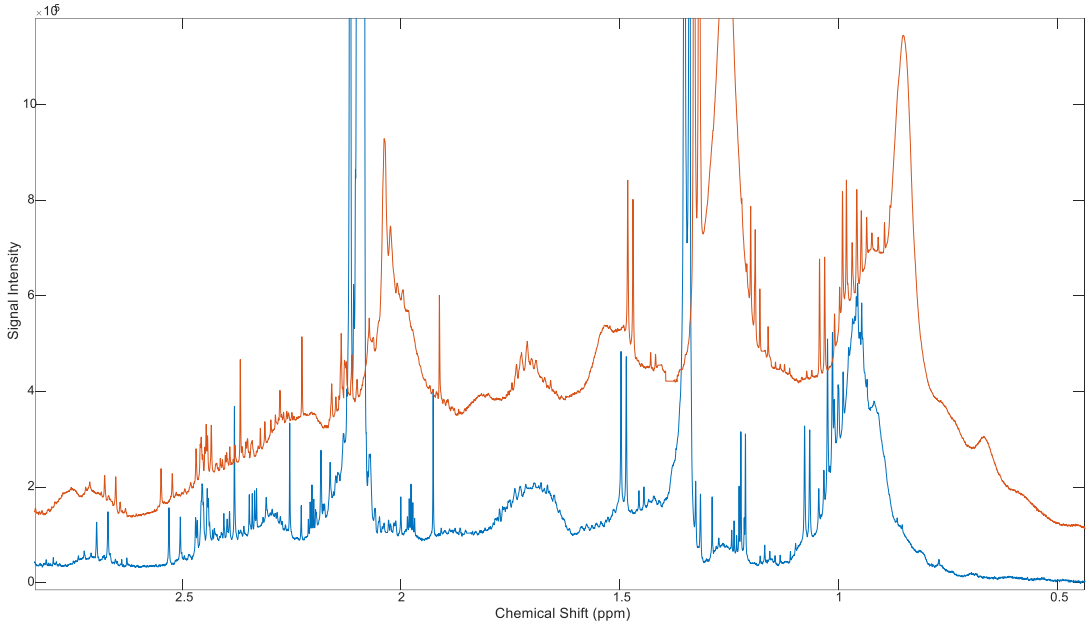


Figure 49 - NOESY NMR Spectra of HSF unextracted (red) and liquid/liquid extraction with Deuterated Acetonitrile (blue). The lack of spectral alignment and change is chemical shift is evident in addition to a baseline reduction due to protein removal.

8.2.4.6 Quantitative assessment

Forty-six metabolites were identified in the non-extracted, liquid/liquid methanol and liquid/liquid acetonitrile extracts. Twenty-five metabolites were identified in the deuterated acetonitrile where a lack of buffering and peak shifts made identification difficult. 2D-NMR data was used to assist identification (Table 27). Using a single comparable pooled HSF sample prohibited the use of any STOCSY analysis to aid identification. This is due to the lack of variation between the samples which this method relies

upon. In the presence of a liquid/liquid extraction, the macromolecular suppression of the CPMG sequence did not provide any better detection than the NOESYGPPR1D sequence.

Universally, the introduction of an extraction step caused increased variability in the detection of metabolites, as expected. The lipid detection was consistently low for all methods, which reflects the effective protein precipitation and removal of lipoprotein complexes. This was also mirrored by the loss of choline and phosphocholine in the methanol extraction. The acetonitrile detection of lipid was consistently higher than methanol (Cholesterol C18 12.5% vs 9.2%, CH_3 fatty acyl residues 5.8% vs 3.4%, CH_2 fatty acyl residues 5.3% vs 2.6%). Choline was broadly lost in the methanol but retained in acetonitrile (methanol 18.2%, ACN 87.3%). A statistically significant loss of 3-hydroxybutyrate was noted in all extractions, but without noticeable impact upon sensitivity with good resolution of the peaks. Glycerol had poor detection in the methanol extraction (9%) but well visualised in the acetonitrile (39.8%).

The hydrophobic amino acids had low detection by the methanol extraction (isoleucine 22.9%, leucine 14.9%, methionine 14.3%, alanine 13.6%, valine 18.6%), except for glycine (60.7%). By comparison the ACN detections were consistently 3-4 times larger.

The hydrophilic amino acids were also recovered poorly by the methanol: lysine (16.7%), arginine (15.3%), tyrosine (36%), glutamine (19.1%), glutamate (13.7%), threonine (26.2%) and histidine (27.4%) all had detections which were a magnitude of 3-5 less than the acetonitrile extraction.

Recovery of the energy substrates also demonstrated the same pattern of detection as the acetonitrile, consistently recovering more metabolite (glucose 13.6% vs 82.1%, citrate 27.6% vs 126%, acetoacetate 10.1% vs 27.9%, pyruvate 11.1% vs 44.4%, succinate 35.5% vs 189%, acetoacetate 10.1% vs 27.9% and lactate 15.1% vs 86.3%).

The removal of proteoglycans was effective in both liquid/liquid methods. However, the methanol outperformed the acetonitrile (*N*-acetyl groups 9% vs 26.2%).

Isolated metabolite concentrations increased following the application of an extraction, thereby providing a theoretical recovery in excess of 100%. This was recorded for: GAG, tyrosine, paracetamol,

citrate, succinate, acetone, acetate, lysine, 2-hydroxybutyrate, 2-ketoisovalerate and lactate (see Table 25 & Table 26, Figure 50 - Figure 57). This phenomenon was seen in the analysis of other body fluids.

A comparison of the acetonitrile and its deuterated counterpart did not identify any losses due to the drying step or detect any additional metabolites. Losses were seen with paracetamol glucuronide, lysine, pyruvate, 2-ketoisovalerate and acetone.

The acetonitrile method provided superior reproducibility. A CV was <20% for 36 of the 44 reported metabolites and <15% in 27 metabolites in the NOESY data. The CV was higher for the methanol data with 7 of the 44 metabolites having a CV <20%, and no metabolites having a CV of <15%.

	Mean recovery +/- SD (CV %)					
	Methanol	T-test	Acetonitrile	T-test	Deuterated Acetonitrile	T-test
Cholesterol C18	0.092 ± 0.015 (16.69)	4.2E-13	0.125 ± 0.012 (9.64)	7E-13		
CH3 (fatty acyl residues lipids)	0.034 ± 0.01 (27.89)	4.4E-12	0.058 ± 0.009 (15.5)	9E-12		
2-Hydroxybutyrate	0.269 ± 0.102 (38.)	6.6E-08	1.012 ± 0.056 (5.53)	0.218	2.101 ± 0.682 (32.47)	0.0009
Isoleucine	0.229 ± 0.051 (22.15)	2.4E-10	0.884 ± 0.092 (10.39)	0.0029	0.819 ± 0.186 (22.72)	0.0261
Leucine	0.149 ± 0.046 (30.6)	2.4E-11	0.818 ± 0.082 (9.98)	0.0001	0.506 ± 0.133 (26.21)	3E-06
Valine	0.186 ± 0.052 (28.16)	1.9E-11	0.871 ± 0.077 (8.81)	0.0005	0.82 ± 0.188 (22.93)	0.014
3-Hydroxyisobutyrate	0.451 ± 0.104 (23.18)	7.2E-08	0.986 ± 0.122 (12.4)	0.3261	1.043 ± 0.294 (28.24)	0.3559
2-ketoisovalerate	0.441 ± 0.144 (32.6)	1.8E-06	0.78 ± 0.126 (16.17)	0.0032	5.751 ± 1.784 (31.02)	3E-05
3-Hydroxybutyrate	0.086 ± 0.018 (20.46)	9.6E-11	0.38 ± 1.017 (267.93)	0.0575	0.596 ± 0.138 (23.15)	0.1343
CH2 (fatty acyl residues lipids)	0.026 ± 0.01 (38.32)	7.2E-12	0.053 ± 0.012 (22.16)	1E-11		
***L*-Lactate**	0.151 ± 0.054 (35.78)	6.8E-11	0.863 ± 0.08 (9.27)	0.0006	1.511 ± 0.119 (7.85)	5E-07
Threonine	0.262 ± 0.059 (22.73)	1.6E-08	0.572 ± 0.039 (6.84)	3E-07		
Alanine	0.136 ± 0.041 (30.21)	5.4E-12	0.717 ± 0.075 (10.49)	4E-06	0.951 ± 0.163 (17.13)	0.2145
Lysine	0.167 ± 0.059 (35.64)	9.6E-11	0.872 ± 0.083 (9.56)	0.001	1.535 ± 0.349 (22.72)	0.0012
Arginine	0.153 ± 0.046 (30.04)	1.2E-10	0.807 ± 0.111 (13.73)	0.0004	0.319 ± 0.098 (30.89)	2E-08
Acetate	0.37 ± 0.09 (24.35)	2.4E-08	1.071 ± 0.055 (5.18)	0.0041	1.329 ± 0.091 (6.88)	2E-06
CH2C=C (fatty acyl chains lipids)	0.168 ± 0.046 (27.6)	1.9E-10	0.393 ± 0.059 (15.12)	6E-09		
***N*-acetyl groups**	0.09 ± 0.037 (40.65)	4.5E-12	0.262 ± 0.032 (12.14)	8E-11		
Glutamate	0.137 ± 0.039 (28.71)	1.1E-08	0.811 ± 0.131 (16.18)	0.006		
Glutamine	0.191 ± 0.052 (27.42)	4.9E-10	1.016 ± 0.099 (9.7)	0.3547		
Methionine	0.143 ± 0.04 (27.96)	2.9E-11	0.689 ± 0.055 (7.97)	6E-07		
Acetone	0.239 ± 0.048 (20.26)	2.3E-10	0.451 ± 0.116 (25.69)	9E-07	1.287 ± 0.141 (10.97)	0.0005
Acetoacetate	0.101 ± 0.035 (34.07)	2.2E-11	0.279 ± 0.058 (20.86)	5E-09	0.64 ± 0.18 (28.18)	0.0004
Pyruvate	0.111 ± 0.035 (31.73)	4.5E-11	0.444 ± 0.061 (13.7)	2E-08	1.288 ± 0.249 (19.36)	0.0038
Succinate	0.355 ± 0.128 (36.06)	6.5E-07	1.899 ± 0.367 (19.32)	7E-05		
Pyroglutamine	0.259 ± 0.128 (49.56)	5E-08	0.622 ± 0.113 (18.14)	9E-06		
Citrate	0.276 ± 0.088 (31.99)	4.2E-08	1.265 ± 0.125 (9.87)	5E-05	1.472 ± 0.223 (15.14)	0.0001
Dimethylalanine	0.372 ± 0.063 (17.02)	5.4E-07	0.614 ± 0.155 (25.31)	1E-05	0.566 ± 0.153 (27.03)	3E-05
N N-dimethylglycine	0.131 ± 0.053 (40.74)	1.9E-10	0.706 ± 0.107 (15.12)	0.0003		
Creatine	0.044 ± 0.019 (43.13)	6.4E-09	0.065 ± 0.018 (27.6)	1E-08		
Creatinine	0.21 ± 0.059 (28.)	5.9E-10	0.812 ± 0.071 (8.73)	0.0001		
Ornithine	0.187 ± 0.051 (27.22)	5.5E-10	0.575 ± 0.104 (18.09)	1E-06		
Phenylalanine	0.337 ± 0.079 (23.54)	4.4E-08	0.767 ± 0.07 (9.08)	5E-05	0.929 ± 0.178 (19.18)	0.1509
Dimethylsulfone	0.206 ± 0.046 (22.3)	5.1E-10	0.881 ± 0.074 (8.41)	0.0066		
Choline (free)	0.182 ± 0.083 (45.63)	1.2E-08	0.873 ± 0.218 (25.02)	0.1091	0.808 ± 0.259 (32.11)	0.0626
Choline (phospholipids)	0.109 ± 0.029 (26.9)	6.1E-13	0.578 ± 0.044 (7.54)	4E-09	0.838 ± 0.186 (22.22)	0.0403
Glucose	0.136 ± 0.05 (36.65)	3.1E-11	0.821 ± 0.085 (10.38)	0.0002	0.936 ± 0.19 (20.3)	0.1751
Glycine	0.607 ± 0.022 (3.61)	2.7E-12	0.895 ± 0.027 (3.)	4E-06		
Glycerol	0.09 ± 0.017 (18.75)	6.8E-11	0.398 ± 0.095 (23.75)	5E-07		
Myo-inositol	0.369 ± 0.078 (21.09)	0.00019	0.745 ± 0.169 (22.71)	0.0352		
CH=CH (fatty acyl residues lipids)	0.061 ± 0.022 (36.46)	8E-12	0.049 ± 0.004 (9.05)	2E-11		
Tyrosine	0.36 ± 0.071 (19.8)	6.3E-10	0.872 ± 0.111 (12.7)	0.01	1.488 ± 0.177 (11.88)	1E-05
Histidine	0.274 ± 0.091 (33.08)	3.3E-08	0.665 ± 0.083 (12.44)	8E-06	1.086 ± 0.139 (12.76)	0.0768
Paracetamol glucuronide	0.355 ± 0.09 (25.4)	2.4E-08	0.895 ± 0.12 (13.36)	0.0543	2.941 ± 0.257 (8.73)	3E-08
GAG	0.44 ± 0.068 (15.44)	1.8E-08	1.274 ± 0.144 (11.26)	0.0001		
Formate	0.708 ± 0.138 (19.55)	0.00153	1.086 ± 0.106 (9.78)	0.0282	0.709 ± 0.119 (16.76)	1E-04

Table 25 - The mean recovery, standard deviation and (coefficient of variation %) with tested significance for the metabolite detection using liquid/liquid extraction by NOESYGPPR1D NMR

	Mean recovery +/- SD (CV %)					
	Methanol	T-test	Acetonitrile	T-test	Deuterated Acetonitrile	T-test
CH3 (fatty acyl residues	0.05 ± 0.015 (30.35)	7.35E-12	0.057 ± 0.006 (10.12)	1.4E-11		
2-Hydroxybutyrate	0.082 ± 0.092 (111.51)	5.19E-08	0.398 ± 0.215 (53.96)	9.3E-06		
Isoleucine	0.258 ± 0.052 (20.09)	7.92E-10	0.837 ± 0.086 (10.32)	0.00023	1.017 ± 0.296 (29.07)	0.4266
Leucine	0.157 ± 0.051 (32.69)	8.13E-11	0.822 ± 0.067 (8.21)	9E-05	0.37 ± 0.16 (43.23)	7E-06
Valine	0.186 ± 0.055 (29.77)	1.39E-10	0.856 ± 0.091 (10.68)	0.00116	0.855 ± 0.158 (18.5)	0.0216
3-Hydroxyisobutyrate	0.507 ± 0.122 (24.05)	7.36E-06	1.11 ± 0.141 (12.69)	0.01134	0.834 ± 0.156 (18.65)	0.0225
2-ketoisovalerate	0.52 ± 0.147 (28.3)	0.000131	0.713 ± 0.152 (21.32)	0.00131	1.383 ± 0.225 (16.3)	0.0015
3-Hydroxybutyrate	0.077 ± 0.03 (39.02)	1.91E-09	0.07 ± 0.025 (35.69)	7.5E-10	0.138 ± 0.021 (14.98)	1E-08
CH2 (fatty acyl residues	0.156 ± 0.095 (61.07)	3.43E-08	0.351 ± 0.082 (23.37)	5.5E-08	1.476 ± 0.663 (44.89)	0.0522
L-Lactate	0.166 ± 0.057 (34.22)	1.29E-10	0.911 ± 0.085 (9.31)	0.0099	1.573 ± 0.081 (5.16)	6E-08
Threonine	0.3 ± 0.086 (28.69)	3.39E-08	0.762 ± 0.104 (13.61)	0.00012		
Alanine	0.14 ± 0.039 (27.67)	9.68E-12	0.706 ± 0.07 (9.86)	2E-06	0.595 ± 0.107 (17.95)	7E-06
Lysine	0.191 ± 0.07 (36.76)	1.72E-09	0.934 ± 0.075 (8.06)	0.00484	1.41 ± 0.348 (24.7)	0.0077
Arginine	0.158 ± 0.047 (29.66)	3.47E-10	0.864 ± 0.1 (11.58)	0.00422	0.316 ± 0.074 (23.47)	1E-07
Acetate	0.352 ± 0.084 (24.)	1.69E-08	1.149 ± 0.1 (8.7)	0.00284	1.153 ± 0.126 (10.95)	0.0045
CH2C=C (fatty acyl chains	0.324 ± 0.065 (20.06)	1.89E-08	0.747 ± 0.074 (9.96)	7.5E-06		
N-acetyl groups	0.092 ± 0.03 (32.4)	6.12E-12	0.268 ± 0.035 (12.97)	1E-10		
Glutamate	0.186 ± 0.043 (23.15)	2.36E-08	0.951 ± 0.129 (13.6)	0.23121		
Glutamine	0.195 ± 0.077 (39.22)	2.22E-09	1.077 ± 0.13 (12.05)	0.05638		
Methionine	0.148 ± 0.055 (37.39)	3.93E-10	0.723 ± 0.047 (6.52)	1.9E-07		
Acetone	0.235 ± 0.056 (23.61)	2.53E-09	0.436 ± 0.114 (26.21)	1.4E-06	1.285 ± 0.097 (7.58)	0.0005
Acetoacetate	0.119 ± 0.025 (20.94)	1.37E-09	0.253 ± 0.045 (17.97)	1.7E-09	0.342 ± 0.121 (35.35)	2E-06
Pyruvate	0.116 ± 0.039 (33.71)	4.82E-11	0.448 ± 0.049 (10.96)	7.5E-10	0.145 ± 0.064 (44.47)	8E-09
Succinate	0.253 ± 0.058 (23.06)	9.38E-10	0.876 ± 0.058 (6.59)	0.00199		
Pyroglutamine	0.268 ± 0.101 (37.63)	4E-08	0.585 ± 0.086 (14.69)	1.2E-05		
Citrate	0.362 ± 0.076 (20.9)	5.86E-08	1.486 ± 0.16 (10.76)	1.9E-05	0.225 ± 0.034 (14.89)	3E-08
Dimethylalanine	0.5 ± 0.149 (29.89)	1.33E-05	0.845 ± 0.054 (6.43)	0.00335	0.416 ± 0.089 (21.38)	2E-06
N-dimethylglycine	0.233 ± 0.064 (27.44)	1.08E-08	0.767 ± 0.118 (15.42)	0.00136		
Creatine	0.045 ± 0.048 (105.74)	1.55E-08	0.077 ± 0.025 (32.29)	1.6E-09		
Creatinine	0.202 ± 0.041 (20.49)	4.49E-09	0.838 ± 0.067 (8.)	0.0003		
Ornithine	0.276 ± 0.068 (24.84)	1.12E-08	0.703 ± 0.128 (18.16)	0.00019		
Phenylalanine	0.423 ± 0.099 (23.44)	3.77E-07	0.82 ± 0.107 (13.05)	0.00199	0.773 ± 0.141 (18.24)	0.0006
Dimethylsulfone	0.259 ± 0.07 (27.07)	7.83E-09	0.839 ± 0.107 (12.77)	0.00093		
Choline (free)	0.201 ± 0.079 (39.51)	1.54E-11	0.918 ± 0.101 (10.99)	0.05723	0.723 ± 0.214 (29.56)	0.0097
Choline (phospholipids)	0.107 ± 0.027 (25.57)	1.36E-08	0.574 ± 0.054 (9.42)	1.1E-05	0.678 ± 0.188 (27.74)	0.0063
Glucose	0.142 ± 0.046 (32.09)	3.36E-11	0.814 ± 0.087 (10.68)	0.00018	0.776 ± 0.168 (21.6)	0.0037
Proline	0.267 ± 0.062 (23.33)	1.18E-07	0.321 ± 0.101 (31.46)	1.1E-06		
Glycine	0.628 ± 0.019 (2.96)	2E-12	0.935 ± 0.045 (4.79)	0.00172		
Glycerol	0.104 ± 0.021 (19.86)	2.38E-11	0.226 ± 0.037 (16.38)	8.9E-07	0.68 ± 0.222 (32.67)	0.0027
Myo-inositol	0.501 ± 0.132 (26.3)	2.4E-05	0.851 ± 0.139 (16.38)	0.01771		
CH=CH (fatty acyl residues	0.176 ± 0.045 (25.34)	2.92E-09	0.188 ± 0.019 (10.15)	2.7E-10		
Tyrosine	0.353 ± 0.058 (16.3)	2.16E-09	0.79 ± 0.054 (6.81)	2.4E-06	1.005 ± 0.096 (9.54)	0.4393
Histidine	0.319 ± 0.039 (12.2)	2.52E-10	0.703 ± 0.062 (8.8)	8.8E-06	0.977 ± 0.112 (11.44)	0.2723
Paracetamol glucuronide	0.473 ± 0.049 (10.43)	1.68E-07	0.953 ± 0.075 (7.9)	0.05079	0.903 ± 0.126 (13.91)	0.0296
GAG	0.627 ± 0.061 (9.78)	1.75E-06	1.215 ± 0.164 (13.49)	0.00293		
Formate	0.724 ± 0.08 (11.03)	0.000135	1.225 ± 0.066 (5.42)	0.00052	1.024 ± 0.126 (12.32)	0.4099

Table 26 - The mean recovery, standard deviation and (coefficient of variation %) with tested significance for the metabolite detection using liquid/liquid extraction by CPMG NMR

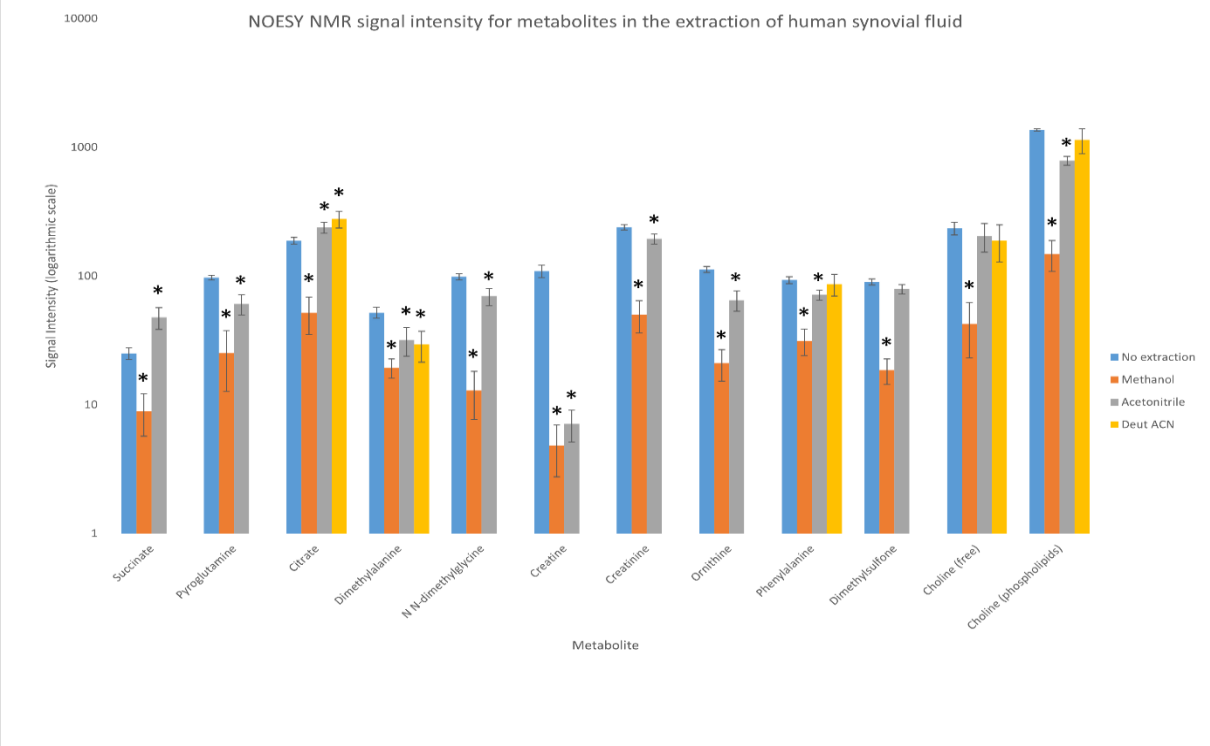


Figure 50 - Selected metabolite recoveries in HSF by various extraction methods and using NOESYGPPR1D detection. (*) denotes significance compared to the unextracted control sample

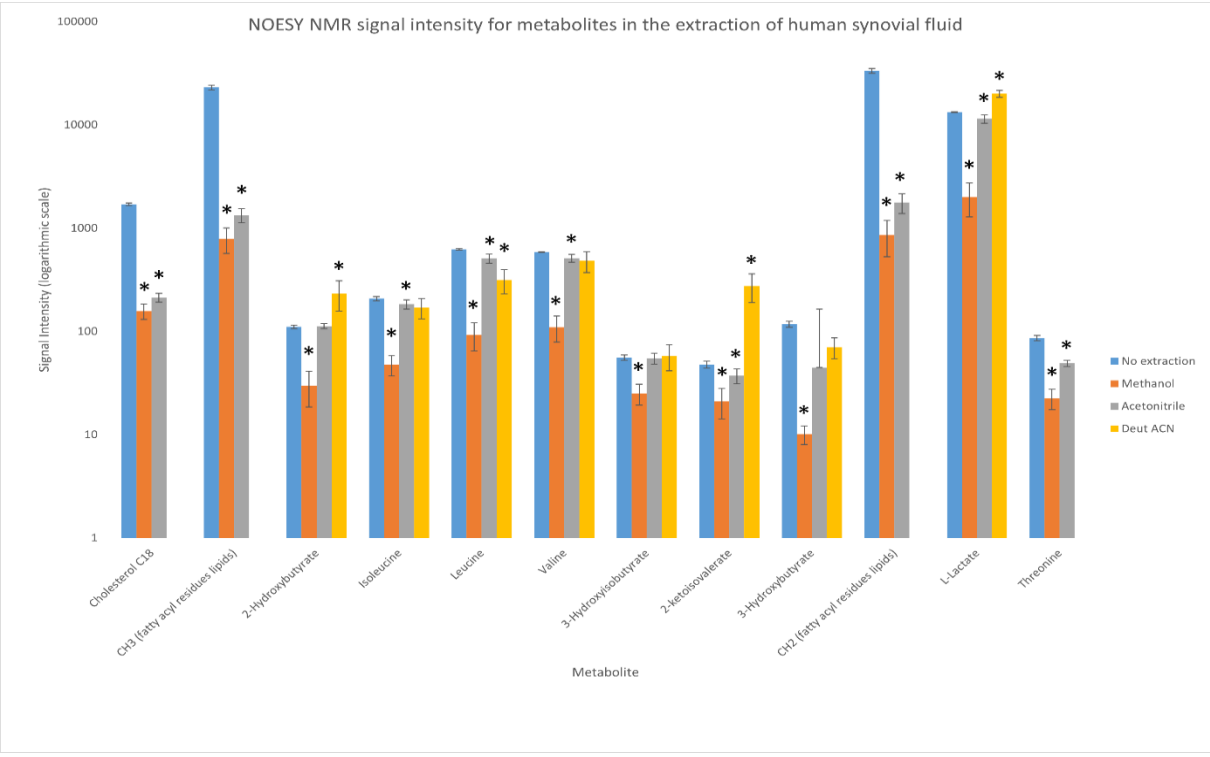


Figure 51 - Selected metabolite recoveries in HSF by various extraction methods and using NOESYGPPR1D detection. (*) denotes significance compared to the unextracted control sample

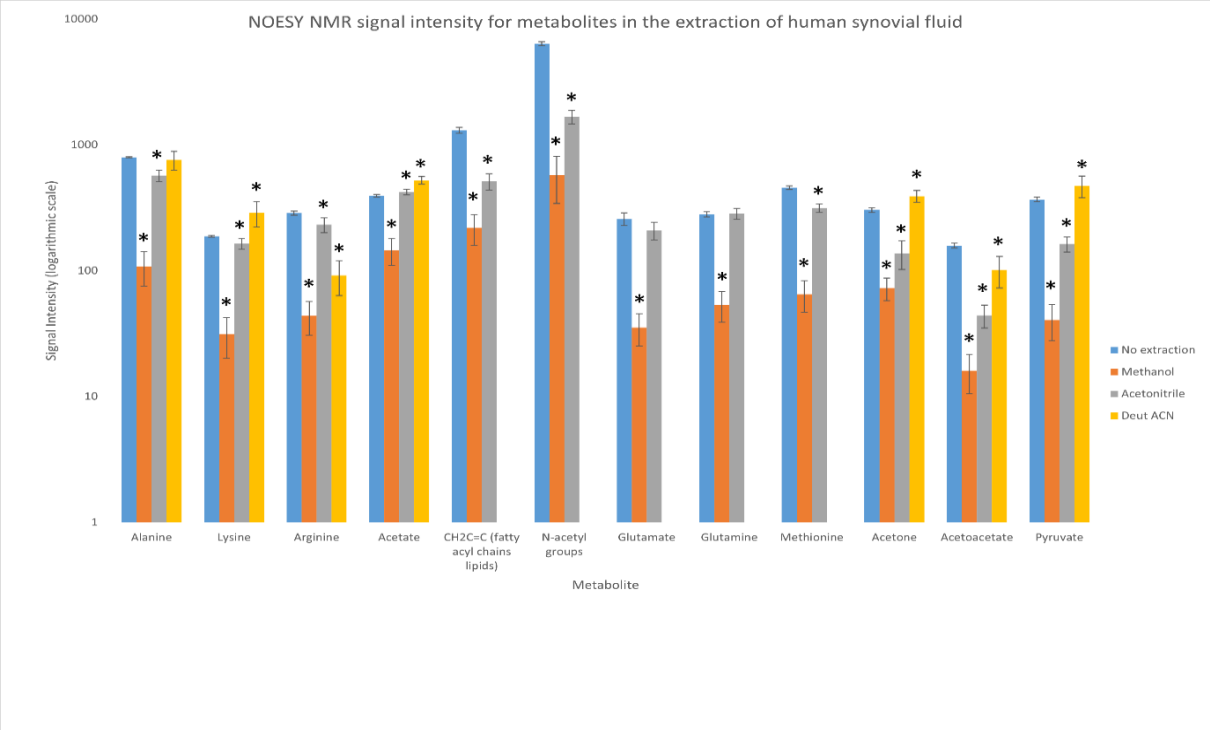


Figure 52 - Selected metabolite recoveries in HSF by various extraction methods and using NOESYGPPR1D detection. (*) denotes significance compared to the unextracted control sample

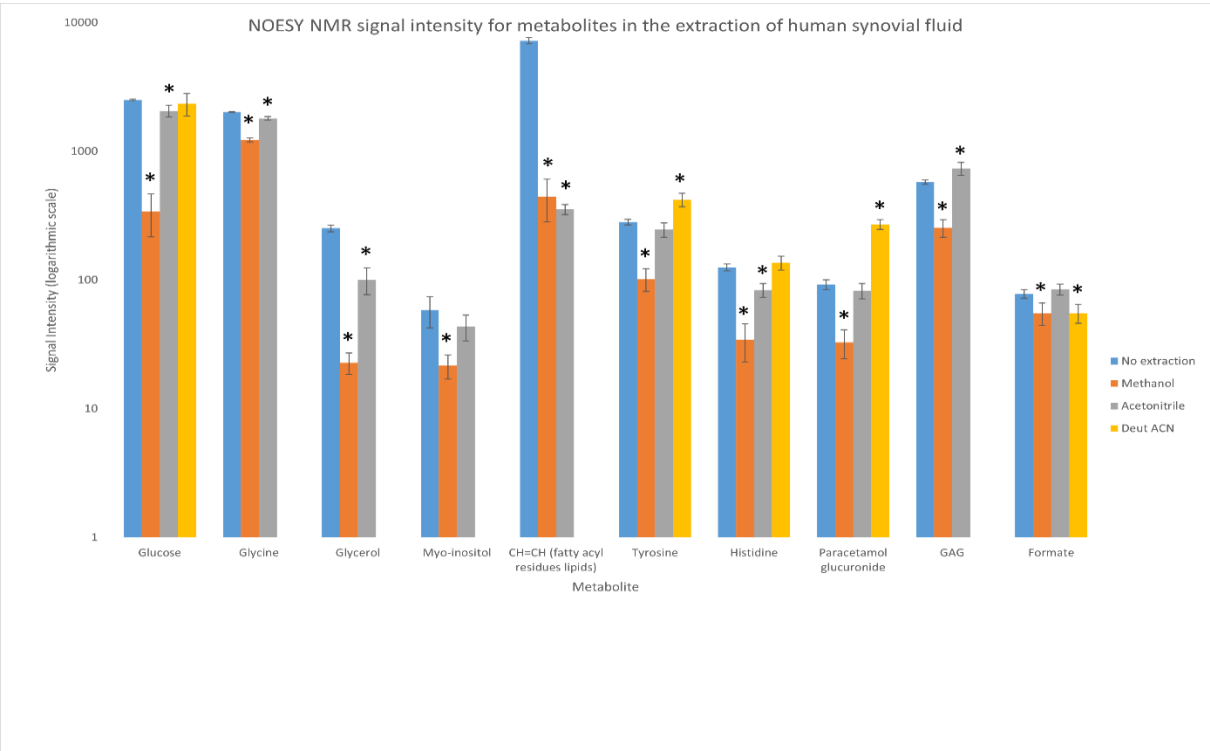


Figure 53 - Selected metabolite recoveries in HSF by various extraction methods and using NOESYGPPR1D detection. (*) denotes significance compared to the unextracted control sample

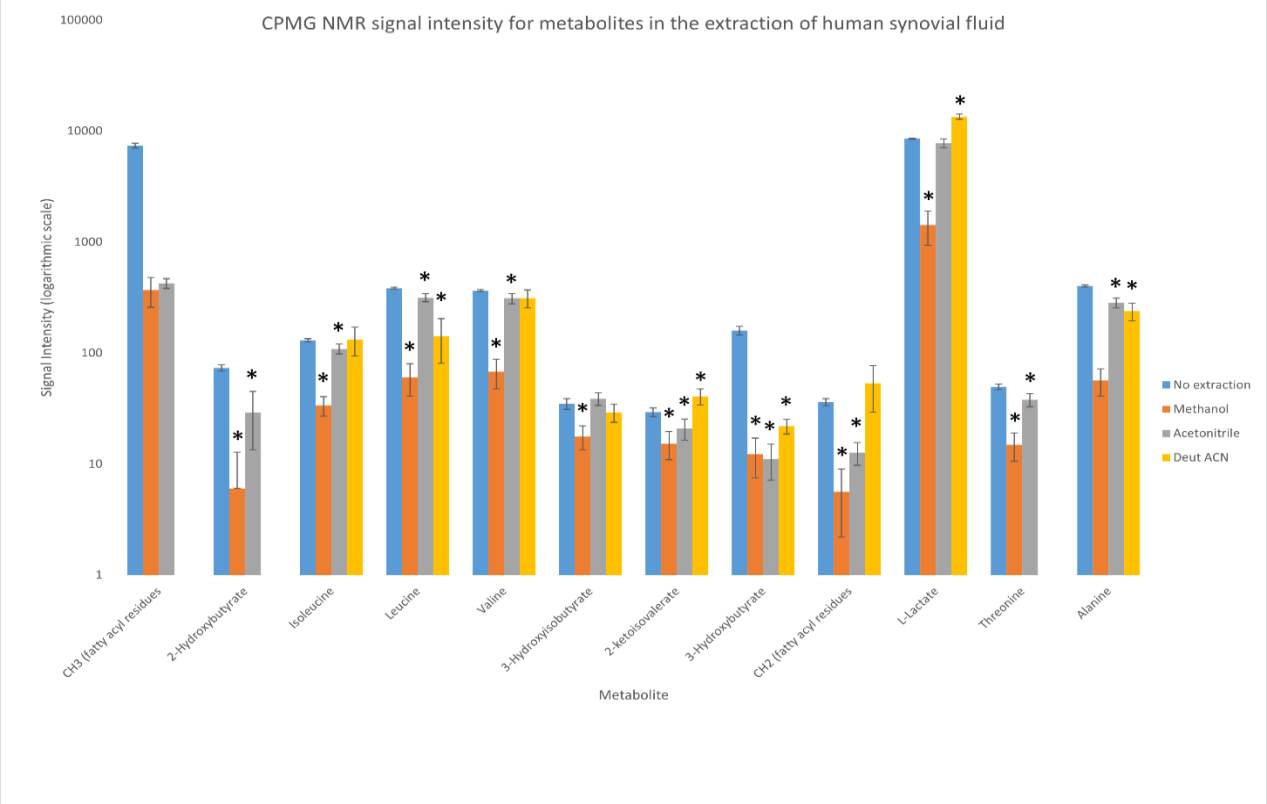


Figure 54 - Selected metabolite recoveries in HSF by various extraction methods and using CPMG detection. (*) denotes significance compared to the unextracted control sample

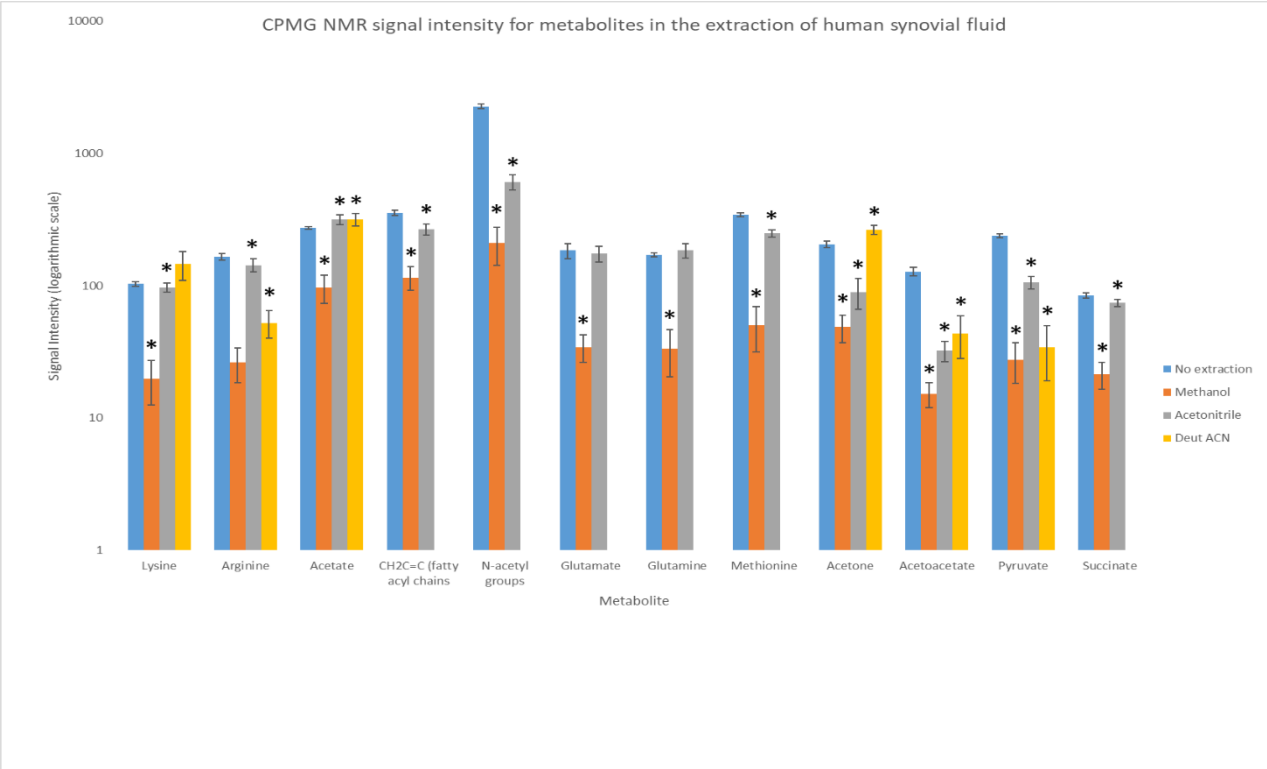


Figure 55 - Selected metabolite recoveries in HSF by various extraction methods and using CPMG detection. (*) denotes significance compared to the unextracted control sample

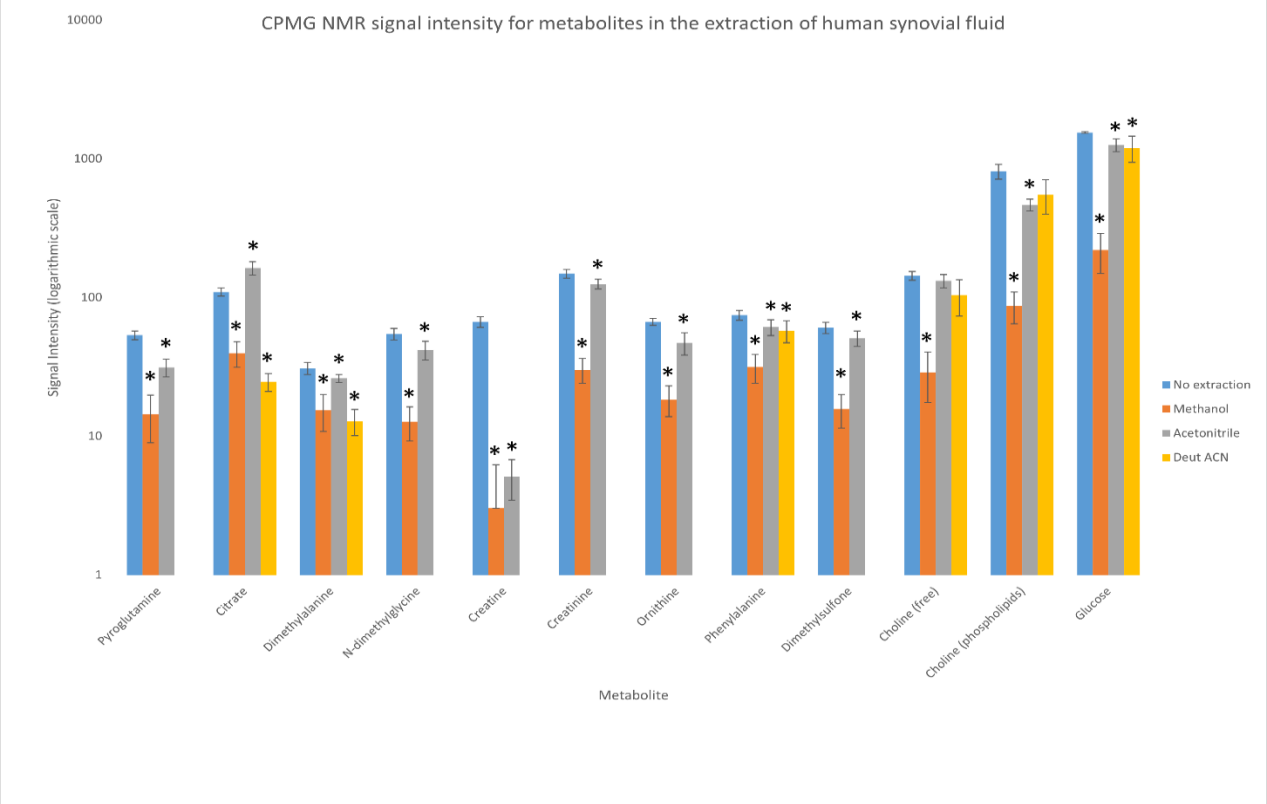


Figure 56 - Selected metabolite recoveries in HSF by various extraction methods and using CPMG detection. (*) denotes significance compared to the unextracted control sample

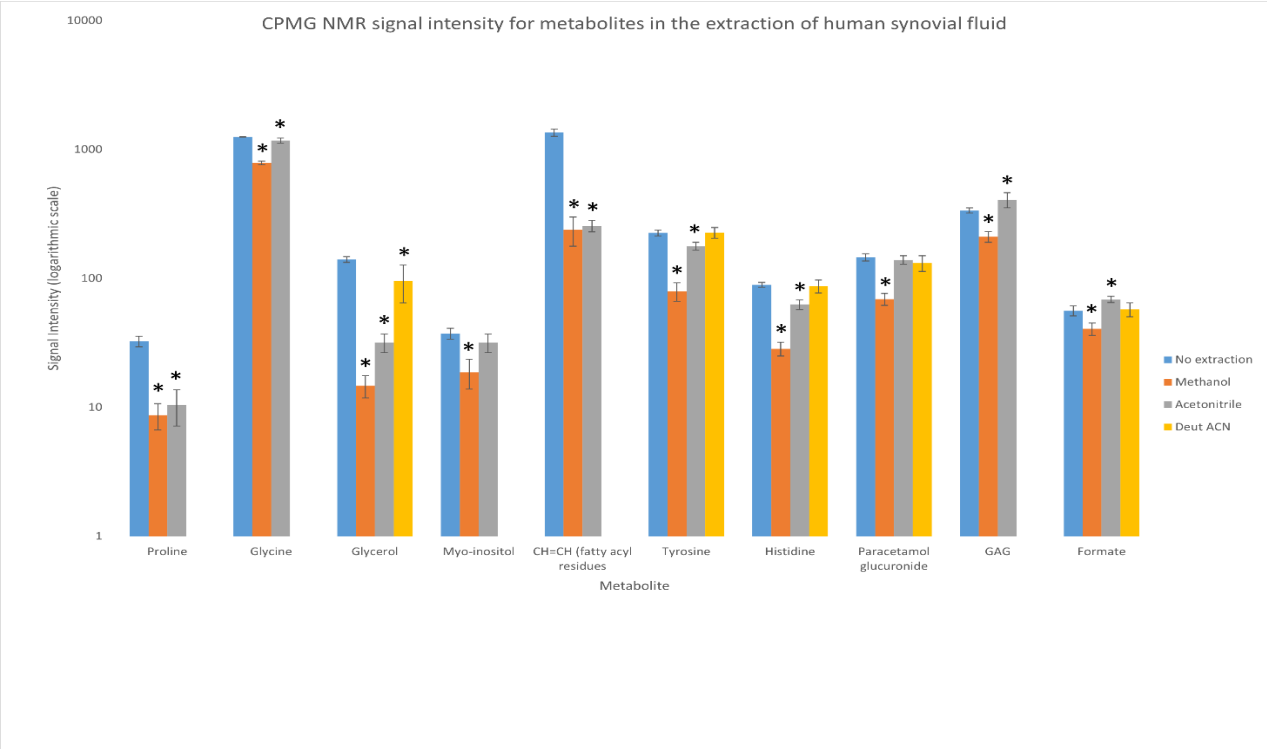


Figure 57 - Selected metabolite recoveries in HSF by various extraction methods and using CPMG detection. (*) denotes significance compared to the unextracted control sample

8.2.5 Discussion

The evaluation of extraction methods has confirmed the superior performance of acetonitrile above other protein precipitation techniques. Acetonitrile precipitation had better selectivity of metabolite recovery and the analyses were easier to perform. This was contrary to previous reports where human serum was used (228).

Poor detection (<5%), was noted for the lipid peaks for both the methanol and acetonitrile extractions. The acetonitrile extraction had non-lipid recoveries between 28% for acetoacetate and 109% for formate. Consistently detections were in excess of 80% for acetonitrile with 25 of the 44 metabolites achieving this. The exception was creatine, only achieving 6.5%. This is contrasted to the methanol extraction where none of the metabolites had a recovery over 80%. Acetonitrile achieved baseline reduction but with the retention of peaks attributable to GAG and the *N*-acetyl peak. Both these peaks have important diagnostic value in joint disease (93, 109, 124). Furthermore, there were the analytical gains already mentioned (see 8.1.6).

TCAA protein precipitation was efficient but at the expense of analytical sensitivity. The concentrations of metabolites were consistently poor with recoveries <10%, and with no additional metabolites detected. Therefore, this method was discounted as there were no analytical benefits of TCAA above the other liquid/liquid extractions.

Methanol extraction was difficult in the sample preparation stage, with the unpredictable formation of a gelatinous sample and the loss of small volumes after centrifugation; one entire sample was lost. This was consistent with the previous individual HSF sample and demonstrated that it was not a sample specific effect, strengthening the conclusion not to use the methanol extraction. This effect is probably due to the hydrophilic carbohydrate groups from the proteoglycan molecules. It is suggested that this variable solidification is responsible, at least in part, for the much higher CV with that method. The methanol liquid/liquid extraction is not recommended for HSF protein precipitation prior to small molecule analysis.

These methods restrict the detection of lipids. This should be remembered in any analysis of disease which may be linked to lipid metabolism (231). Furthermore, the application of these extraction methods in an untargeted analysis evaluating joint disease will probably require a follow-up, lipid-targeted analysis.

Comparison of deuterated acetonitrile with the acetonitrile liquid/liquid extraction demonstrated losses due to the final stage of drying before reconstitution (see Table 25 & Table 26, Figure 50 - Figure 57) but in no case was a metabolite completely lost. Except for acetone, all the metabolites with losses during drying have boiling points in excess of 130°C. Therefore, volatility alone cannot be responsible. Alternatively, the reconstitution in NMR buffer after drying may not be dissolving all the dry residue, leaving analytes on the tube. Deuterated solvent has the advantage over the other methods that the water peak contributes far less interference to the spectra, with analytical gains in the δ=4.5-5.0 region. However, this is contrasted by the lack of buffering and the acetonitrile solvent peak at δ=3.5-4.2 ppm which is likely to detract from detection of proline, glucose and other monosaccharides. The use of deuterated solvent does not confer an analytical advantage either in the sensitivity of detection or the recovery of volatile substances, as proposed originally.

Unsurprisingly the coefficient of variation was increased from the unextracted to the extracted samples. Whilst the measurement of certain metabolites may be improved by removal of protein and quantitation becomes more absolute, this is at the expense of consistency (227). Furthermore, preparation time spent at room temperatures may affect metabolic composition (232).

The enhanced recovery for some metabolites (>100%) could be due to two factors (see Table 25 & Table 26, Figure 50 - Figure 57). The first is that the removal of interfering baseline signal allowed a greater integral for the metabolite peak. Secondly, metabolite may be liberated from protein binding or protein associations when the extraction takes place. The short T2 relaxation results in a smaller NMR signal so, where the protein is removed by precipitation, the NMR signal is elevated. The larger signal could improve the metabolite description of the biofluid. If the later were true, then one would expect

all the peaks originating from that metabolite to be enhanced and this was not consistently the case. The larger signal could improve the metabolite description of the biofluid.

The CPMG sequence suppresses the protein macromolecular signals by virtue of their short T2 relaxation times. This remains the accepted method of controlling these signals in NMR detection (142). However, there are limitations to this approach. Small molecules binding to HSF proteins may be invisible or enhanced by this approach. This has been described for phenylalanine, tyrosine and histidine (226). Therefore, using this sequence to provide quantitative NMR data can be inaccurate, depending on the extent of protein binding. This effect may be less in HSF than in serum because the serum contains a wealth of transport and sequestration proteins specifically for complexing to small molecules such as albumin. However, HSF does contain a large amount of charged protein and thus some binding is expected. Therefore, any close quantitation of small metabolite levels using NMR should use an acetonitrile liquid/liquid extraction.

The generation of a large biobank of samples encourages the application of multiple analyses and detection methods to gain as much information as possible from those samples. The volume of each sample is likely to be small and limiting. Therefore, it is attractive to use a single preparation method that can be automated and allows multi-platform analyses like HPLC-MS and NMR. An acetonitrile liquid/liquid extraction would permit HPLC-MS analysis, is applicable to NMR and should be considered when the scope of analysis increases and the biobank numbers grow to match.

It is surprising that the performance of the methanol and acetonitrile methods was consistent across the spectrum of metabolites, without one method favouring one class of metabolite. This included hydrophilic, hydrophobic, lipids and proteoglycans. Our hypothesis was that these methods would be more selective for a particular class of metabolite. The relative retention of lipid in the acetonitrile will be beneficial as it permits the method to give some (albeit limited) information of lipid handling prior to any targeted lipid analysis. As already mentioned, the lipid may be a source of metabolic disturbance in many joint diseases.

		Unextracted				Methanol/Acetonitrile/TCA				DeutACN			
		NOESY		CPMG		NOESY		CPMG		NOESY		CPMG	
Metabolite	**Chemical Shift (ppm)**	**PPM start**	**PPM end**	**PPM start**	**PPM end**	**PPM start**	**PPM end**	**PPM start**	**PPM end**	**PPM start**	**PPM end**	**PPM start**	**PPM end**
Cholesterol C18	0.58(bs), 0.65(bs)	0.634	0.705	N/A		0.629	0.7	N/A		N/A		N/A	
CH3 (fatty acyl residues, lipids)	0.85(bs)	0.788	0.878	0.776	0.8792	0.775	0.892	0.7658	0.893	N/A		N/A	
2-Hydroxybutyrate	0.89(t), 1.66(m), 1.71(m)	0.8907	0.8991	0.891	0.8977	0.893	0.901	0.8924	0.9014	0.9415	0.9532	N/A	
Isoleucine	0.93(t), 1.00(d), 1.25(m), 1.46(m), 1.96(m), 3.66(d)	1.004	1.013	1.002	1.017	1.004	1.019	1.006	1.022	1.038	1.055	1.022	1.031
Leucine	0.95(d), 0.96(d), 2.00(m)	0.9499	0.9606	0.961	0.9721	0.9529	0.9627	0.9529	0.9633	0.9943	1.004	0.9532	0.9643
Valine	0.98(d), 1.03(d), 2.26(m), 3.60(d)	1.035	1.047	1.035	1.048	1.036	1.053	1.037	1.053	1.057	1.072	1.072	1.091
3-Hydroxyisobutyrate	1.06(d), 2.48(m)	1.067	1.079	1.052	1.067	1.054	1.066	1.055	1.068	1.103	1.119	1.092	1.105
2-ketoisovalerate	1.11(d), 3.00(m)	1.119	1.128	1.106	1.116	1.106	1.118	1.106	1.119	1.115	1.164	1.162	1.174
3-Hydroxybutyrate	1.19(d), 2.35(dd), 4.14(m)	1.155	1.166	2.307	2.315	1.168	1.174	1.165	1.176	1.163	1.174	1.187	1.203
CH2 (fatty acyl residues, lipids)	1.3(s)	1.207	1.306	1.207	1.305	1.206	1.31	1.206	1.213	N/A		1.225	1.229
L-Lactate	1.308, 1.34(d), 4.10(q)	1.323	1.338	1.323	1.351	1.324	1.344	1.324	1.349	1.346	1.392	1.346	1.408
Threonine	1.31(d), 3.56(d), 4.24(dd)	4.238	4.248	4.236	4.248	4.239	4.25	4.238	4.248	N/A		N/A	
Alanine	1.47(d), 3.78(q)	1.474	1.489	1.462	1.475	1.463	1.475	1.463	1.475	1.491	1.517	1.476	1.493
Lysine	1.47(m), 1.71(m), 1.89(m), 3.03(t), 3.72(t)	3.024	3.032	3.012	3.02	3.024	3.032	3.024	3.032	3.031	3.046	3.032	3.045
Arginine	1.69(m), 1.90(m), 3.23(t)	1.66	1.676	1.716	1.731	1.719	1.734	1.718	1.732	1.722	1.735	1.719	1.732
Acetate	1.91(s)	1.908	1.917	1.903	1.923	1.906	1.915	1.905	1.915	1.915	1.942	1.917	1.94
CH2C=C (fatty acyl chains, lipids)	1.99(bs)	1.987	2.002	1.959	2.019	1.949	2.011	1.949	2.011	N/A		N/A	
N-acetyl groups	2.02(bs)	2.012	2.057	2.023	2.051	2.011	2.06	2.011	2.058	N/A		N/A	
Glutamate	2.06(m), 2.34(m)	2.065	2.082	2.068	2.08	2.068	2.078	2.068	2.082	N/A		N/A	
Glutamine	2.12(m), 2.45(m), 3.74(dd)	2.118	2.129	2.105	2.117	2.105	2.118	2.106	2.119	N/A		N/A	
Methionine	2.14(s), 2.16(m), 2.65(t)	2.129	2.14	2.131	2.14	2.131	2.142	2.13	2.144	N/A		N/A	
Acetone	2.22(s)	2.221	2.229	2.218	2.234	2.221	2.134	2.219	2.234	2.244	2.263	2.245	2.266
Acetoacetate	2.27(s), 3.44(s)	2.272	2.28	2.273	2.28	2.272	2.281	2.271	2.282	2.302	2.313	2.307	2.317
Pyruvate	2.37(s)	2.364	2.371	2.363	2.37	2.364	2.372	2.364	2.372	2.371	2.386	2.361	2.374
Succinate	2.40(s)	2.403	2.41	2.394	2.399	2.396	2.4	2.392	2.404	N/A		N/A	
Pyroglutamine		2.416	2.427	2.417	2.424	2.415	2.429	2.415	2.427	N/A		N/A	
Citrate	2.53(d), 2.67(d)	2.535	2.553	2.537	2.555	2.543	2.553	2.542	2.556	2.522	2.543	2.54	2.56
Dimethylalanine	2.5(s)	2.704	2.716	2.707	2.179	2.707	2.717	2.705	2.718	2.731	2.742	2.732	2.741
N,N-dimethylglycine	2.92(s)	2.913	2.922	2.913	2.924	2.913	2.924	2.912	2.923	N/A		N/A	
Creatine	3.03(s), 3.91(s)	3.032	3.037	3.032	3.037	3.032	3.036	3.032	3.036	N/A		N/A	
Creatinine	3.04(s), 4.05(s)	4.043	4.056	4.045	4.054	4.044	4.055	4.044	4.056	N/A		N/A	
Ornithine	1.80(m), 1.93(m), 3.04(m), 3.77(m)	3.047	3.061	3.052	3.06	3.052	3.062	3.052	3.064	N/A		3.064	3.083
Phenylalanine	3.1(d), 3.98(s), 7.37(s)	7.408	7.418	7.31	7.342	7.407	7.421	7.401	7.424	7.468	7.484	7.467	7.484
Dimethylsulfone	3.14(s)	3.141	3.151	3.143	3.151	3.145	3.154	3.144	3.154	N/A		N/A	
Choline (free)	3.21(s)	3.19	3.196	3.189	3.196	3.189	3.199	3.189	3.199	3.209	3.223	3.211	3.222
Choline (phospholipids)	3.23(s)	3.199	3.226	3.212	3.224	3.199	3.227	3.199	3.227	3.254	3.269	3.221	3.234
Glucose	3.23(dd), 3.40 (m), 3.46 (m), 3.52 (dd), 3.73 (m), 3.82 (m), 3.88 (dd), 4.63 (d), 5.23(d)	3.236	3.251	3.238	3.251	3.238	3.251	3.236	3.252	3.238	3.252	3.232	3.253
Proline	2.00(m), 2.08(m), 2.34(m), 3.32(dt), 3.41(m), 4.12(dd)	3.341	3.352	3.341	3.352	N/A		3.339	3.347	N/A		N/A	
Glycine	3.55(s)	3.55	3.562	3.551	3.563	3.551	3.564	3.55	3.564	N/A		N/A	
Glycerol	3.55 (m), 3.64 (m), 3.77 (m)	3.636	3.645	3.644	3.652	3.643	3.653	3.643	3.653	N/A		3.671	3.68
Myo-inositol	3.28(t), 3.60(t), 4.06(t)	4.057	4.074	4.064	4.069	4.067	4.078	4.063	4.078	N/A		N/A	
CH=CH (fatty acyl residues, lipids)	5.27(s)	5.244	5.36	5.242	5.35	5.242	5.365	5.245	5.384	N/A		N/A	
Tyrosine	6.89(d), 7.19(d)	6.895	6.907	6.875	6.911	6.875	6.911	6.875	6.917	6.877	6.92	6.882	6.922
Histidine	7.05(s), 7.77(s)	7.046	7.059	7.044	7.062	7.044	7.06	7.046	7.065	7.037	7.06	7.038	7.064
Paracetamol glucuronide	2.16(s), 5.09(d), 7.15(d), 7.36(d)	7.13	7.146	7.133	7.146	7.135	7.151	7.13	7.169	7.153	7.193	7.155	7.19
GAG	7.97(s)	7.933	8.032	N/A		7.872	7.919	7.858	7.938	N/A		N/A	
Hypoxanthine	8.18(s), 8.20(s)	N/A		8.162	8.209	N/A		N/A		7.906	7.939	7.89	7.958
Formate	8.45(s)	8.447	8.458	8.446	8.463	8.451	8.465	8.445	8.464	8.488	8.504	8.484	8.511

Table 27 - Identified metabolites in the NMR analysis of HSF using a variety of extraction methods

9 Chapter 9: Evaluating the small molecule components of prosthetic joint fluid lubricants, both model and native, and correlating these to implant material wear

9.1 Introduction

9.1.1 The clinical relevance of comparing bovine calf serum (BCS) and human synovial fluid (HSF)

Of the 200000 arthroplasty operations are performed annually in the UK, 10% are revision procedures performed because of (72):

- Aseptic loosening
- Instability, subluxation or dislocation
- Pain
- Implant wear
- Malalignment
- Periprosthetic fracture
- Stiffness
- Progressive arthritis (where only a partial joint replacement has been performed)
- Infection .

All artificial joints will have a limited lifespan before failure. The modes of implant failure due to wear and underlying causes have been outlined (see 1.6.2). However, underpinning all of these will be lubrication but the importance of the composition of the lubricant has been largely overlooked to date.

Since BCS (in vitro) and HSF (in vivo) are both used as lubricants for prosthetic joints, it is valid to compare and contrast the composition of the two. It is assumed that BCS and HSF have equivalent mechanical and chemical effects on the implant. However, if this assumption is wrong then the BCS will be a poor model for HSF and for the *in vivo* implanted joint. There is no detailed study of the chemical differences between HSF and BCS.

9.1.2 The lubricating fluid chemistry and the impact on wear

Both HSF and BCS contain macromolecules and small molecule metabolites. The macromolecular composition has been studied and key differences exist between BCS and HSF (Table 28) (233). In HSF proteoglycan is the main protein, whereas in BCS it is albumin (81). Hence there is more glycoprotein debris in HSF. The two fluids both contain water, inflammatory proteins and albumin. BCS is far more cellular than healthy HSF (but note cells are removed from both biofluids before storage) (147). Lipid concentrations are similar, but the composition may be very different. The species and organisation of lipids could influence boundary film thickness and thus affect wear (see 1.6.3) (234).

It is implied that macromolecules bind to the implant surface providing boundary lubrication (see 1.6.3) (10). The small molecules can bind to the implant surface, either causing corrosion or providing protection. This will affect wear and, implant longevity.

Table 28 - Protein composition comparison of HSF and BCS

	Albumin (mg/ml)	Globulin (mg/ml)	Approx. Total protein (mg/ml)	pH
HSF	7-18 (235)	0.5 -2.4 (235)	7-20	7.3 -7.4
OA HSF	17.8-18.5 (236)	12.9-13.4 (236)	30-34	7.4 -8.1 (237)
100 wt.%BCS	35.00 (238)	22.00 (238)	57	7.0 -8.1 (238)
25 wt. %BCS	8.75 (238)	5.5 (238)	14	7.0 -8.1 (238)

The composition of HSF changes significantly in disease states (202). In HSF proteoglycan concentrations change with increased hyaluronan and chondroitin sulphate (225). Furthermore, the composition varies between individuals. This may explain those differences in implant longevity

between individuals, that cannot be attributed to any other factor (239). This was seen in metal on metal (CoCrMo/CoCrMo) prosthetic hip implants, where some studies report excellent outcomes whilst other cohorts (e.g. women) had inexplicably high failure rates (240).

Some studies have evaluated how specific fluid components affect the wear. These all involve using model fluids (like BCS) supplemented with the tested component, concentrating solely on the macromolecules (10, 77-81). There are no studies applying this method to HSF. There are no studies evaluating the small molecule content of the fluid (either HSF or BCS) and their influence on wear (82).

9.1.3 The testing and validation of a prosthetic joint

Joint simulators are engineered rigs which apply forces across a prosthetic joint. They attempt to recreate the motion and variable forces within a natural joint cycle (201). Lubricant is provided by constant replenishment of 25% BCS. This lubricant is a surrogate for HSF, having a similar total protein content and non-Newtonian rheological properties (10). The prosthetic joint is subjected to a million cycles, equivalent to a year of implantation, before wear is measured (199, 200).

The constant replacement of fluid means that large volumes (>300ml) are required to perform the test. Therefore, any local concentration increases or temperature changes at the bearing surface are diluted and not modelled.

Prosthetic joint testing evaluates the material properties of the implant when measuring wear, but it fails to account for the influence of the chemistry of the lubricating fluid.

9.2 Hypothesis

- The small molecule analysis of HSF and BCS will highlight areas of significant difference with potential for developing more accurate HSF models.
- The small molecule composition of the lubricating fluid will have a relationship with implant wear.

9.3 Aims

Since the established prosthesis validation tests solely use BCS, it is desirable to evaluate how closely BCS models HSF chemistry. Furthermore, the investigation of which small molecule components are key in implant wear may provide insight into premature implant failure or strategies to combat its onset. The Imperial College synovial fluid tissue bank subcollection is now large enough to facilitate the collaboration of the Imperial College Synovial Fluid Working Group. Therefore, it was possible to analyse the mechanical and chemical properties of the fluids in tandem. The aims are:

- To compare the metabolic components of HSF and its model surrogate BCS.
- To correlate the differences in metabolic components with implant material wear.

9.4 Materials and methods

The methods used in this study are summarised in a flow chart (see Figure 58).

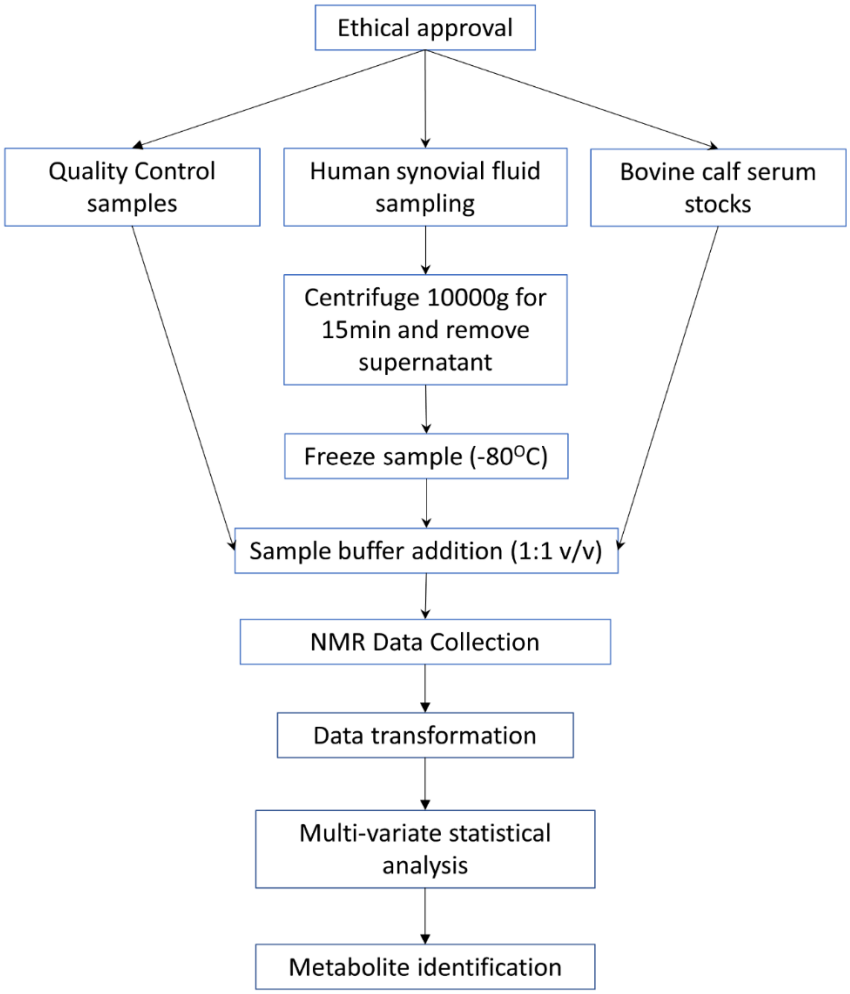


Figure 58 - Experimental process flow for the metabolic comparison of BCS and HSF

9.4.1 Sample collection

BCS was sourced from Sigma Aldrich (Cat # 12133C) and two separate batches were used in the analysis (11n239/13J360). HSF was harvested from patients with an inclusion criterion of undergoing a primary total knee replacement for end-stage OA (n=14). There were 6 men and 8 women, average age 73 years (95% confidence interval: 67.28-79.14). The average BMI was 30.22. None of the patients were smokers or vegetarians. Four patients suffered with type II diabetes mellitus. The patient ethnicities were as follows: white Caucasian (n=7), Asian (n=2), black Caribbean (n=2), black African (n=1) and ethnicity not recorded (n=2).

9.4.2 Sample preparation and NMR analysis

Pre-filtered BCS was stored at -20°C until required for analysis, when it was defrosted at room temperature.

All HSF samples were harvested perioperatively during the arthrotomy. Samples were collected and stored according to the study protocols (see 6.6). Samples were prepared using the methods evaluated previously (see 4.4.2). All samples were then stored at -80°C for a maximum of 6 months before analysis.

System blank samples containing only buffer and no biological material were handled and processed in tandem with the biological samples to determine any contaminants. The BCS samples were repeated throughout the analytical run to act as bioblanks and QC samples to ensure reproducibility.

9.4.3 NMR methods.

NMR data was acquired using the methods tested previously to generate NOESYGPPR1D and CPMG sequences (see 4.4.3).

9.4.4 Data handling

The data was manipulated using the same approach as tested previously (see Ch 4), except that spectra were referenced for chemical shifts to the anomeric ^{1}H peak of α-glucose (δ=5.23ppm) for reasons previously outlined (see 5.4.3).

9.4.5 Metabolite identification

Metabolites were identified by matching the NMR chemical shifts and peak multiplicities to information held in the literature and metabolite databases: Human Metabolite Database (HMDB) and the Livestock Metabolome Database (LMDB) (167, 241). Statistical total correlation spectroscopy (STOCSY) was used to aid metabolite identification by highlighting peaks which correlate statistically, thereby implying a relation (242).

9.4.6 Statistical analysis.

Multi-variate linear regression modelling was performed using the SIMCA® software v14.1 (MKS Umetrics™, Umeå, Sweden). The dataset was investigated using an unsupervised principal component analysis (PCA) model, where no class information about the data was used to build the model. This was followed by a supervised orthogonal partial least squares discriminant analysis (OPLS-DA) (see 3.10.3). The R^2X and R^2Y values represented the variance. The cross-validation Q^2Y metric assessed the strength of model prediction. The significance of metabolic differences was reported using cross-validation p-values. A permutation test was performed 100 times.

Regions of the spectrum that distinguish between BCS and HSF were demonstrated using an S-line plot. The partial correlation (pCORR) of peaks reported the strength of distinction between BCS or HSF. A Spearman correlation was used to illustrate spectral regions correlating with measured wear. Spectral regions with a pCORR (>0.8) or Spearman correlation (>0.9) were investigated further. This watershed was chosen to give 95% confidence or better (221, 243).

9.4.7 Wear measurement

HSF samples with adequate sample volume (>3ml, n=8) and both BCS stocks provided the lubricant for testing. The High Frequency Reciprocating Rig (HFRR, PCS Instruments London) was used to provide the data using CoCrMo surfaces (full methods, see 3.14.2). The wear scar resulting from the reciprocating ball across the disc was measured at 30%, 50% and 70% along its length using a White Light Interferometer (Wyko NT9100). The mean of these readings provided the average wear scar depth (AWD). Where the fluid volume was sufficient, the test was repeated, and the mean AWD was taken for statistical analysis against the metabolic profiling dataset. Sample volumes permitted three replicates in one case and two replicates in a further two.

9.5 Results

9.5.1 Observations

A macroscopic observation of the samples revealed large differences between the physical properties of the BCS and HSF. The cohort of osteoarthritic HSF was more viscous with a large variation in the consistency.

9.5.2 Quality control

Contaminant peaks present in the system blanks were discarded from any interpretation (see Table 29).

Chemical shift	Peak type	Identity
0.097	Singlet	^{13}C Satellite of TSP
1.176		
1.188	Multiplet 1:2:1	Ethanol
1.199		
1.253	Singlet	Unknown
1.984	Singlet	Unknown
2.235	Singlet	Acetonitrile (shifted)
3.246	Singlet	^{13}C Satellite of 3.366 Singlet
3.366	Singlet	Methanol
3.429	Singlet	^{13}C Satellite of 3.563 singlet
3.482	Singlet	^{13}C Satellite of 3.366 Singlet
3.549	Singlet	Unknown
3.563	Singlet	Unknown
3.646		
3.657	Multiplet 1:3:3:1	Ethanol
3.669		
3.681		
3.698	Singlet	^{13}C Satellite of 3.563 singlet
3.846	Doublet	^{13}C Satellite of 3.968 doublet
3.968	Doublet	CH3O-
7.905	Singlet	Unknown
8.463	Singlet	Formate

Table 29 - System blank contaminant peaks discarded from interpretation

The reproducibility of the BCS QC samples was checked and analytical reproducibility was within acceptable limits with coefficients of variation consistently less than 2%.

	Valine	*Alanine*	*Glucose*	*Lactate*	*Isopropyl Alcohol*
Peak Integral	16352177.35	46305486.03	63722192.34	252289355.9	71689926.18
	16188526.05	46127543.7	64861202.34	251956303.6	71297656.58
	16957248.96	47048970.26	65067717.91	264461772.4	74360743.09
	16678511.86	47466129.33	63693654.71	263158607.2	74375574.16
	16659181.51	48863706.4	65228722.43	254560784	74235645.77
	16327420.31	46739954.27	64898349.07	252091020.8	71624688.95
	16244257.52	46329638.69	63967726.68	252840632.4	71059990.01
	16335489.02	46604492.55	64352900.05	255248419.7	71568745.44
	16427922.08	47331278.49	63295487.28	254222857	72726301.89
	16557328.85	46936252.29	62195938.5	256380223	72495463.81
	16399754.65	47383460.69	61903792.4	256148941.7	72967582.71
	16107114.48	45320019.83	61989057.71	247727675.1	70375741.75
Standard Deviation	239604.5754	878780.3169	1206791.55	4697162.515	1363798.301
Mean	16436244.39	46871411.04	63764728.45	255090549.4	72398171.7
Coefficient of Variation	1.457781776	1.874874891	1.892569104	1.841370653	1.88374688

Table 30 - Variation observed from selected metabolites in QC samples to demonstrate analytical consistency

9.5.3 NMR evaluation of HSF vs BCS

The NMR spectra of the two biofluids were qualitatively assessed. The broad background peaks of the NOESYGPPR1D spectra (arising from large molecules) suggested differences in the polysaccharide-complexed protein seen. The NOESYGPPR1D mean spectrum of the BCS cohort was compared to a mean HSF spectrum with a 95% confidence interval. Any peaks or regions where the mean BCS spectrum fell outside this interval were noted and evaluated (see Figure 59, Table 31).

NOESY				
Chemical Shift (ppm)	**Peak type**	**Elevated**	**Peak ID (HMDB)**	**STOCSY**
0.358-0.401	Baseline	BCS	Not assignable	
0.46-0.56	Baseline	BCS	Not assignable	
0.575-0.76	Baseline	BCS	Not assignable	
0.76	Baseline	BCS	Not assignable	
0.975	Doublet	HSF	Valine	
1.03	Doublet	HSF	Valine	
1.16	Doublet	BCS	Isopropyl alcohol	
1.325	Doublet	BCS	Lactate	
1.46	Doublet	HSF	Alanine	
1.49-1.54	Baseline	HSF		
1.72	Triplet	HSF	Lysine	
1.91	Singlet	HSF	Acetic Acid	
1.996	Singlet	BCS	Lipid species (CH_2-C=C)	
2.039	Singlet	HSF	*N*-acetyl glycoproteins	
2.12	Multiplet	HSF	Glutamine	
2.365	Singlet	HSF	Pyruvate	
2.45	Multiplet	HSF	Glutamine	
2.51	Doublet	HSF	Citrate	
2.65	Singlet	HSF	Unknown	related to triplet of doublet (2.338ppm)
2.78-2.96	Baseline	HSF	Not assignable	
3.03	Singlet	BCS	Creatine	
3.21	Singlet	HSF	Choline	
3.23	Doublet of doublets	HSF	Glucose	
3.35	Doublet	HSF	Unknown	related to Citrate
3.76	Multiplet	HSF	Glucose	
3.92	Multiplet	BCS	Unknown	
4.01	Multiplet	BCS	Isopropyl alcohol	
5.1-5.14	Baseline	BCS	Not assignable	
5.33-5.7	Baseline	BCS	Not assignable	
6.09-6.15	Baseline	BCS	Not assignable	
7.05	Baseline	HSF	Not assignable	
7.4-7.78	Baseline	HSF	Not assignable	
7.74	Singlet	HSF	Unknown	
8.38-8.64	Baseline	BCS	Not assignable	
8.86-End	Baseline	BCS	Not assignable	

Table 31 – NOESYGPPR1D NMR spectral regions to distinguish between the HSF and BCS fluid model with high correlation

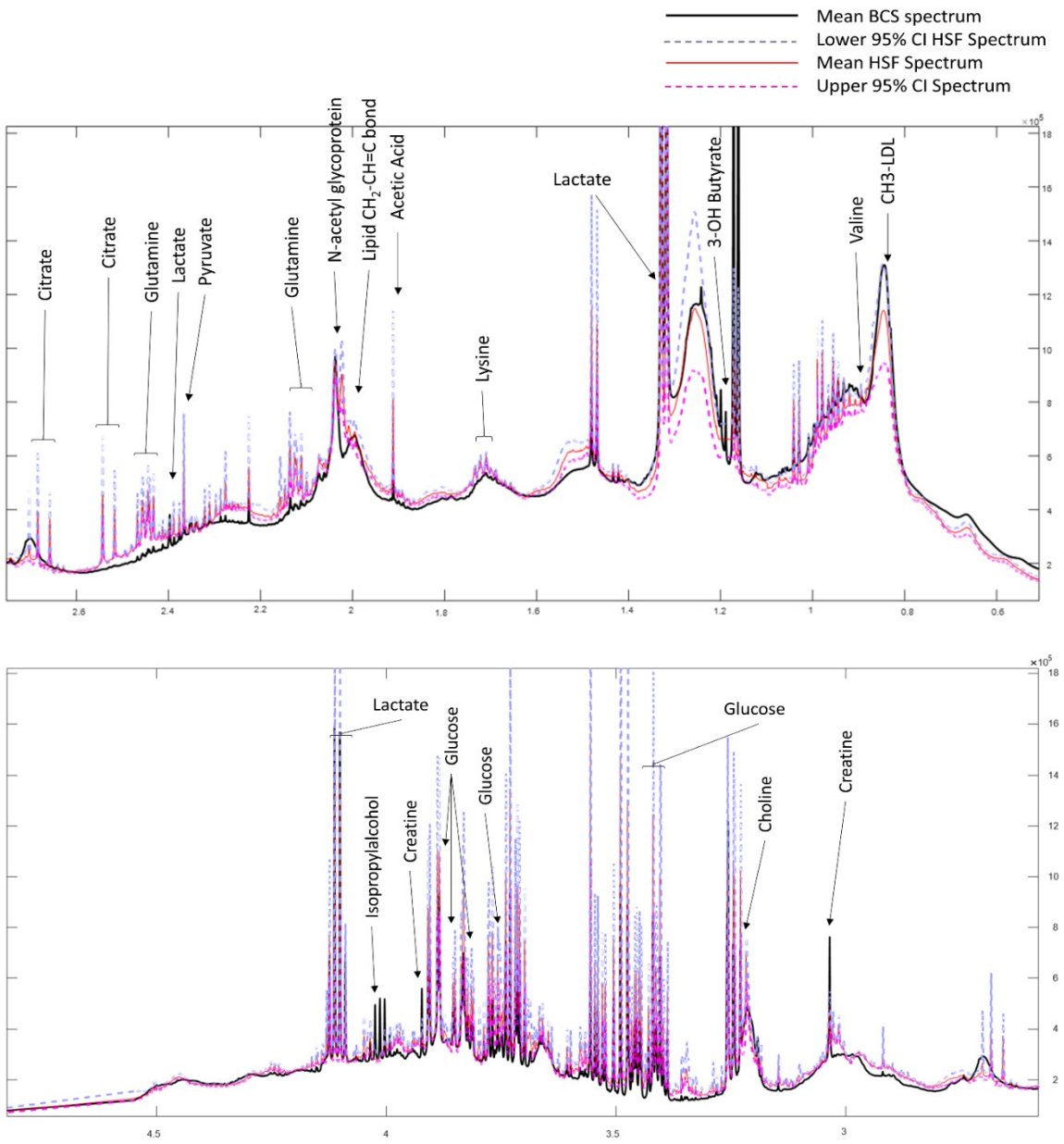


Figure 59 - Annotated mean NOESYGPPR1D spectra comparison for HSF and BCS (upper 0-2.8ppm, lower 2.5-5ppm)

The overall profiles of the lipid peaks are similar. However, the broad bands near 1.5 ppm, and 2.7 ppm differ. The latter peak (higher in the BCS samples) arises from poly-unsaturated fatty acid moieties that are probably incorporated into complex lipids as fatty acyl esters (e.g., phospholipids, gangliosides, etc). The peak at 1.5 ppm was elevated in HSF and reflects CH_2 groups adjacent to a double bond in fatty acyl esters, thereby reflecting the degree of fatty acid saturation. HSF lipoproteins are thought to derive from serum and increase with inflammation, but they provide a negligible contribution to the overall lipid profile (244). The broad peaks between 3.0 and 4.5 ppm are similar in both sample types

and are probably due to the complex carbohydrates. However, the *N*-acetyl peak of glycoproteins (δ=2.04) had noticeable differences.

CPMG NMR data was then analysed using multivariate statistical methods to compare the small molecule differences between BCS and HSF and to provide a measure of significance of the differences. PCA was also used to help identify outlier variation in the dataset resulting from collection, handling, storage or data processing. There were no outlying samples. The PCA scores plot revealed marked separation using the first component between BCS and HSF, with an R^2X of 0.521 (see Figure 60). Therefore, the main variation in the dataset was due to the differences between BCS and HSF. The differences were due to a mixture of small and macromolecules (despite CPMG sequences supressing macromolecule signals). The PCA suggested that HSF samples were separating into two groups. However, no factors could be identified as the cause of this separation.

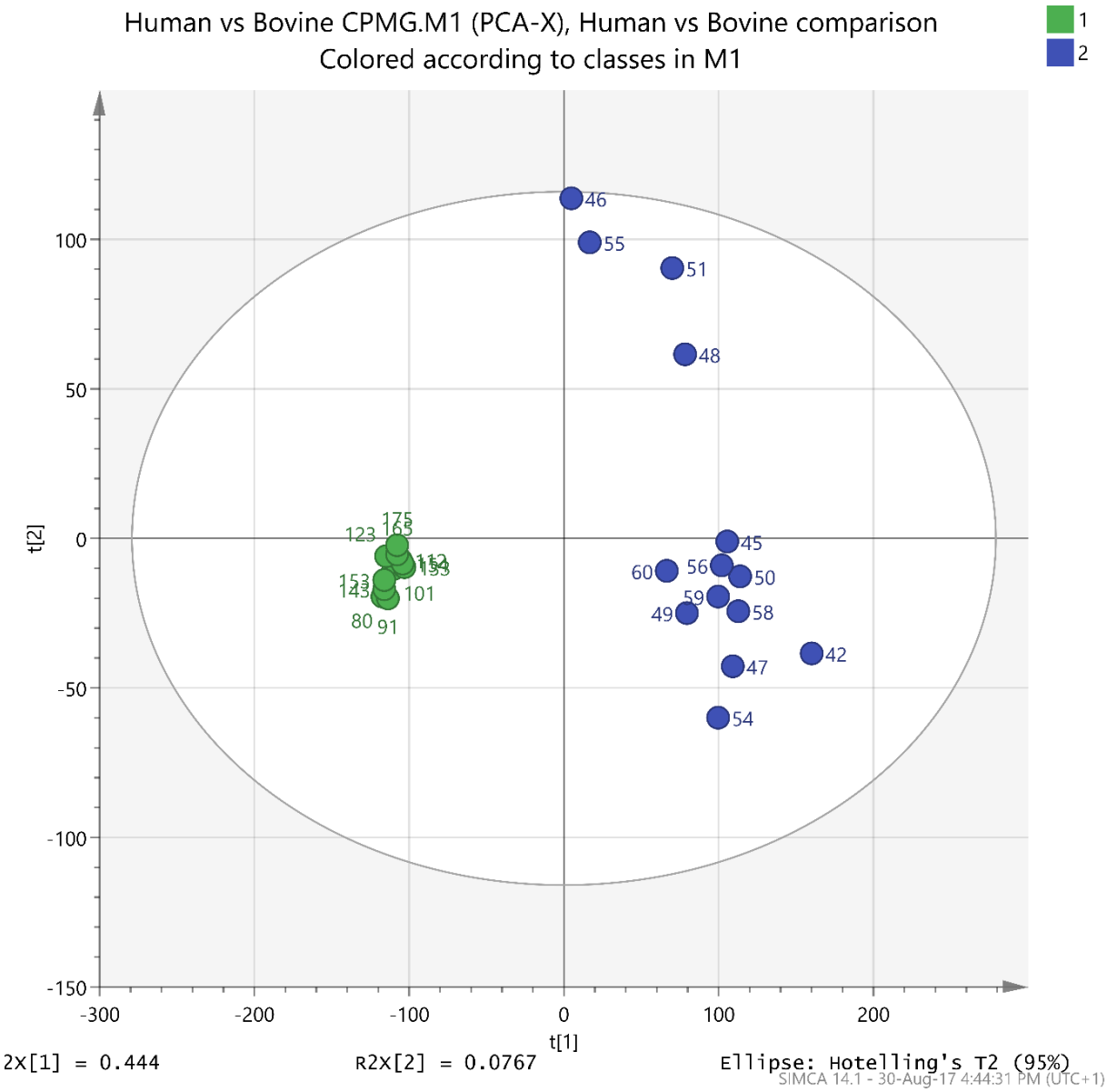


Figure 60 - PCA model comparing NMR CMPG spectra of BCS model (green) and HSF samples (blue).

The OPLS-DA further investigated the differences between BCS and HSF (1 predictive component, 1 orthogonal component, $R^2X = 0.56$, $R^2Y = 0.992$, $Q^2Y = 0.959$, $p=1.28x10^{-13}$), (see Figure 62) with an acceptable permutation test (see Figure 61).

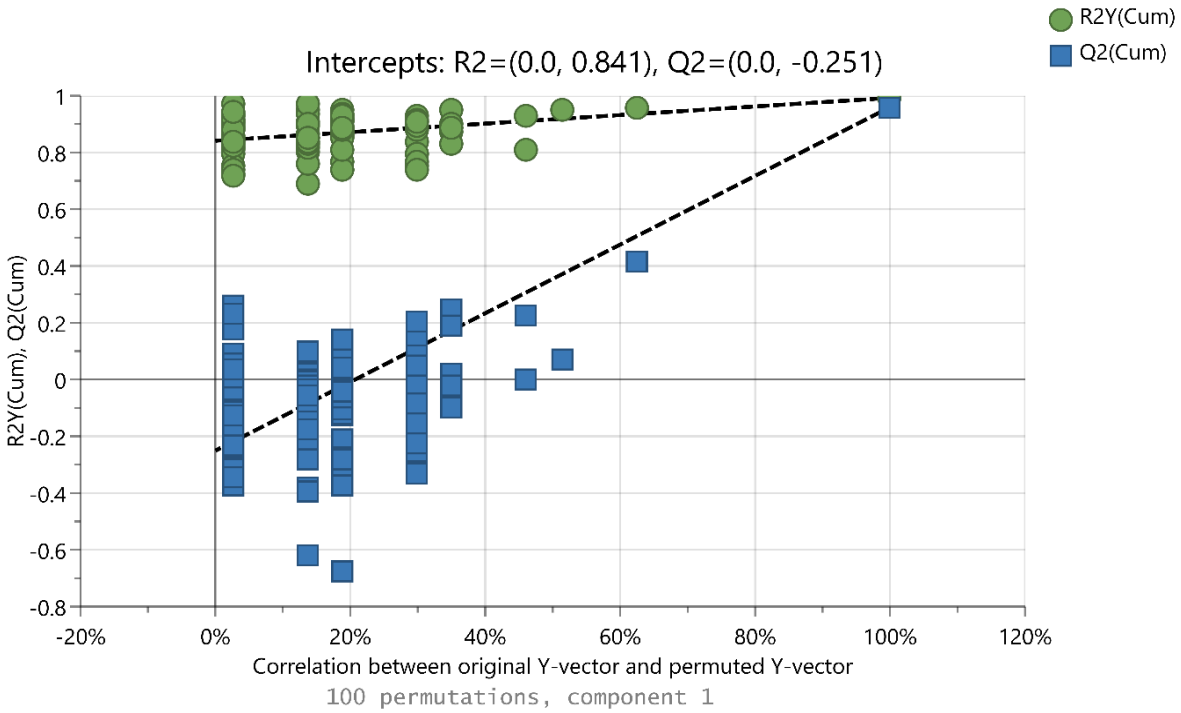


Figure 61 - The permutation test for the OPLS-DA model evaluating NMR CPMG data of HSF and BCS

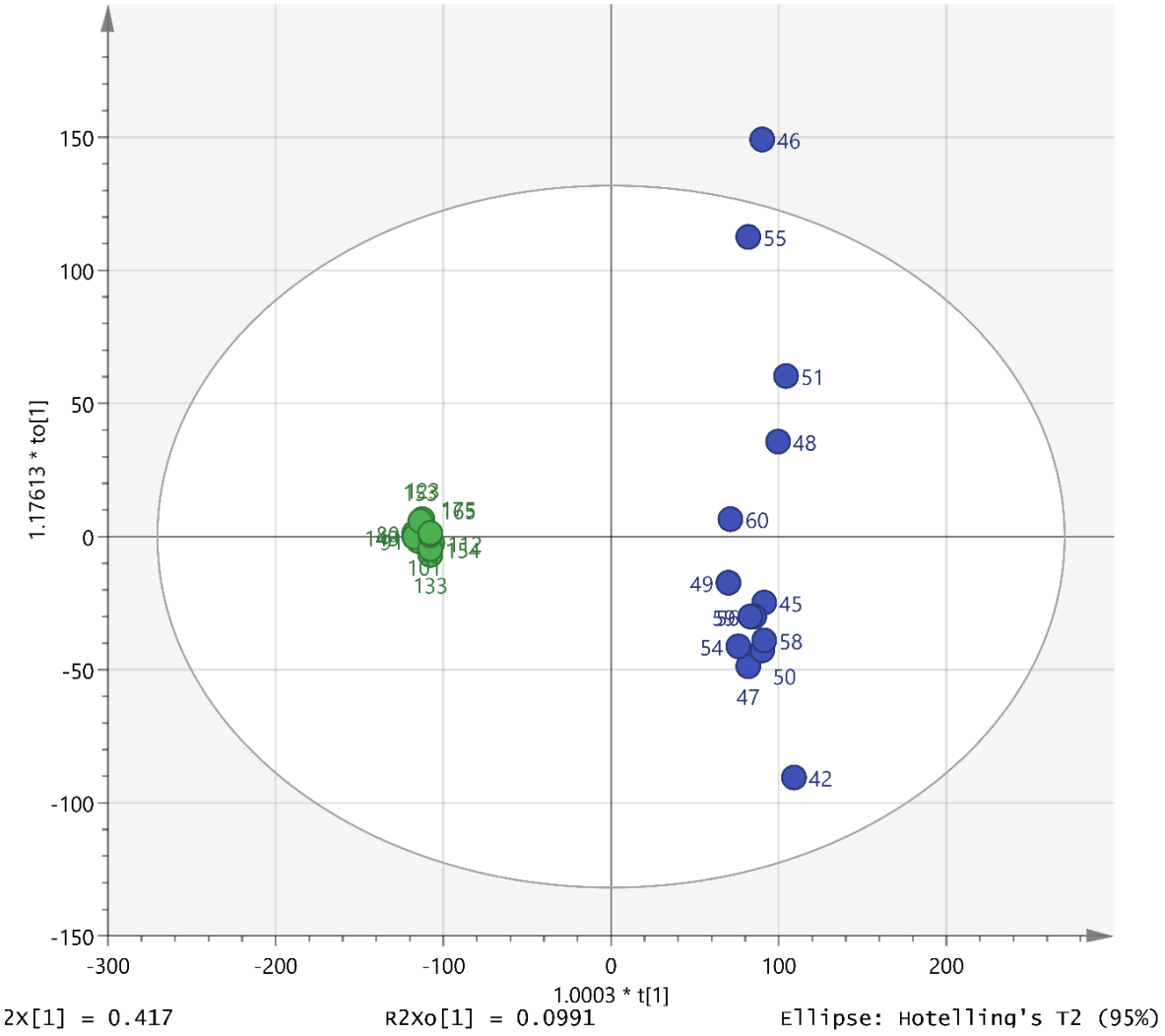


Figure 62 – OPLS-DA model comparing NMR CMPG spectra of BCS model (green) and HSF samples (blue).

The S-line plot highlighted the spectral regions contributing to separation between the BCS and HSF classes (see Figure 63).

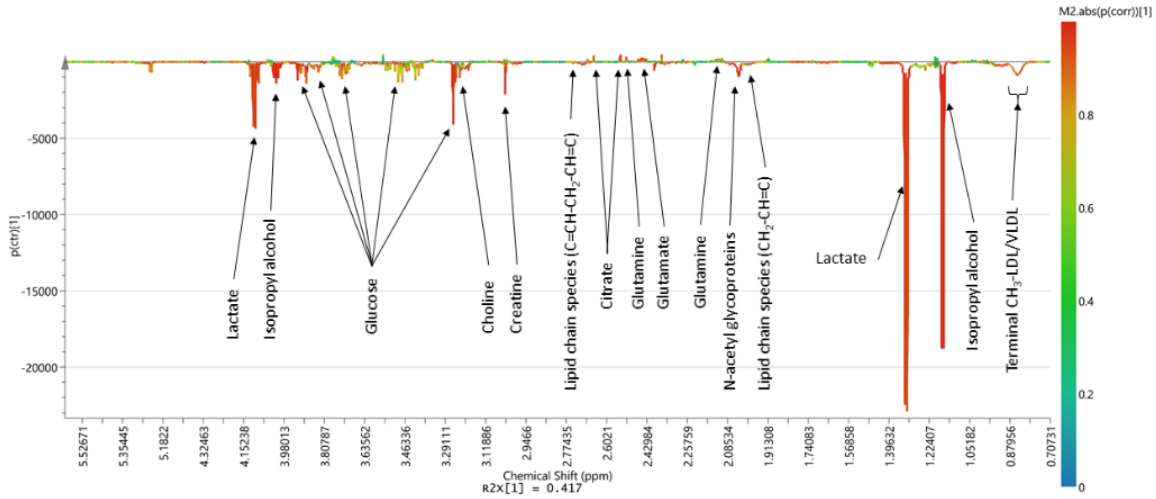


Figure 63 - S-line plot for NMR CPMG spectral sequences after applying an OPLS-DA model to compare HSF and BCS groups. The pCORR (partial correlation) is represented according to a temperature colour scale. Hotter colours represent the spectral regions with high correlation and thus are responsible for the separation between the HSF and BCS groups in the OPLS-DA model.

The peaks or background envelope within the spectra yielding a pCORR of ≥0.8, were reported (see Table 32).

Chemical shift (ppm)	Peak Type	Elevated	Peak ID (HMDB)	P(CORR)
0.85	Broad	BCS	Terminal CH_3-LDL/VLDL	0.986
1.16	Doublet	BCS	Isopropyl alcohol	0.995
1.329	Doublet	BCS	Lactate	0.988
1.995	Singlet	BCS	Lipid chain species (CH_2-CH=C)	0.978
2.039	Singlet	HSF	*N*-acetyl glycoproteins	0.983
2.11	Multiplet	HSF	Glutamine	0.940
2.338	Multiplet	BCS	Unknown	0.968
2.365	Doublet	HSF	Pyruvate	0.949
2.397	Singlet	BCS	Unknown	0.987
2.445	Quadruplet	HSF	Glutamine	0.940
2.518	Doublet	HSF	Citrate	0.930
2.655	Doublet	HSF	Citrate	0.938
2.742	Broad	BCS	Lipid chain species (C=CH-CH_2-CH=C) bond)	0.959
3.035	Singlet	BCS	Creatine	0.838
3.192	Singlet	BCS	Choline	0.976
3.23	Doublet of doublets	HSF	Glucose	0.972
3.45	Quartet	HSF	Glucose	0.938
3.74	Singlet	HSF	Glucose	0.989
3.83	Singlet	HSF	Glucose	0.995
3.852	Doublet	HSF	Glucose	0.994
3.885	Doublet	HSF	Glucose	0.985
3.922	Singlet	BCS	Creatine	0.987
3.99	Singlet	BCS	Unknown	0.991
4.003	Doublet	BCS	Unknown	0.992
4.01	Multiplet	BCS	Isopropyl alcohol	0.993
4.11	Quartet	BCS	Lactate	0.989
5.43	Singlet	BCS	Unknown	0.995

Table 32 – CPMG NMR spectral regions that distinguish between HSF and BCS samples from an OPLS-DA model

The BCS samples showed higher lactate concentrations than HSF and lower concentrations of pyruvate, glucose and creatine. The terminal CH_3 signal of fatty acid chains from LDL and VLDL, choline and 3-hydroxybutyrate were elevated. The HSF cohort had higher concentrations of the amino acids: alanine, glutamine and the branch chain amino acids of leucine and valine. Citrate, acetate and the *N*-acetyl peak were also increased.

BCS samples contained isopropyl alcohol which was not found as a contaminant in the system blanks.

9.5.4 Wear analysis

The AWD was successfully measured in each sample.

Sample ID	AWD R1	AWD R2	AWD R3	Mean AWD
041	0.456	0.7		0.578
Bioblank 1	0.65			0.65
Bioblank 2	0.65			0.65
077	0.8	0.81		0.805
110	1			1
113	0.59			0.59
135	0.78	0.97	0.91	0.89
145	0.93			0.93
156	0.9			0.9
164	1.03			1.03

Table 33 - Average Wear Scar Depth (AWD) for tested HSF and BCS samples

The STOCSY analysis of the CPMG NMR spectra showed no significant correlations within the spectra to the AWD (see Figure 64).

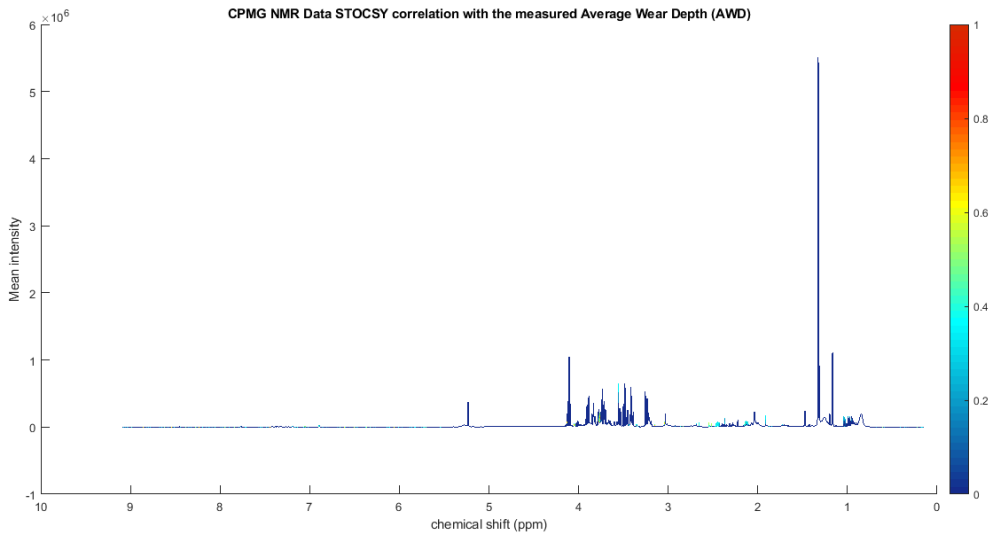


Figure 64 - A mean CPMG spectra plot depicting areas of correlation by colour. The pCORR (partial correlation) is represented according to a temperature colour scale. Hotter colours represent the spectral regions with high correlation and correlation to the AWD.

However, the same analysis applied to the NOESYGPPR1D NMR spectra yielded several spectral regions of high correlation to AWD (see Figure 65). In addition, the S-line plot revealed whether these significant metabolites were positively or negatively correlated with the simulated implant wear: all were positive (see Figure 66).

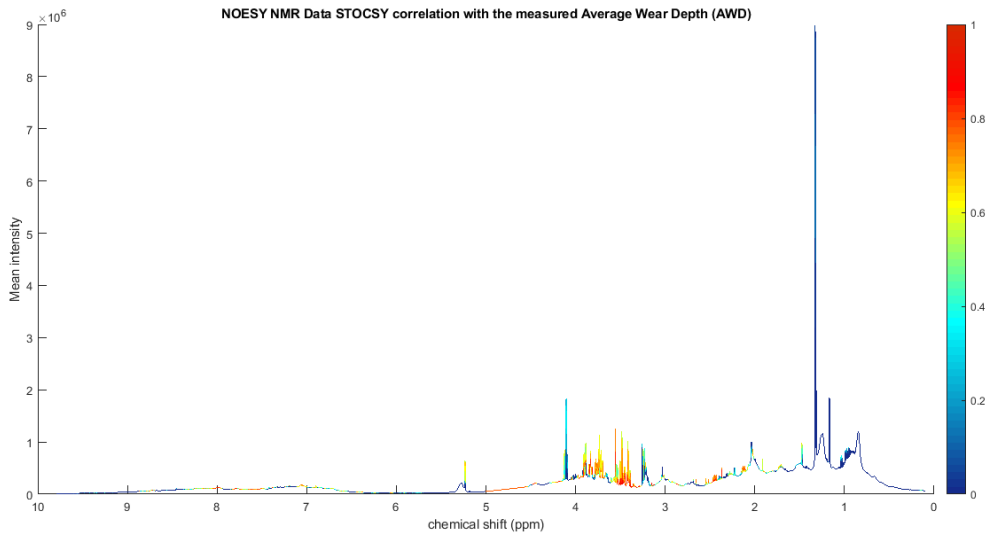


Figure 65 - A mean NOESYGPPR1D spectra plot depicting areas of correlation by colour. The Spearman correlation is represented according to a temperature colour scale. Hotter colours represent the spectral regions with high correlation and correlation to the AWD.

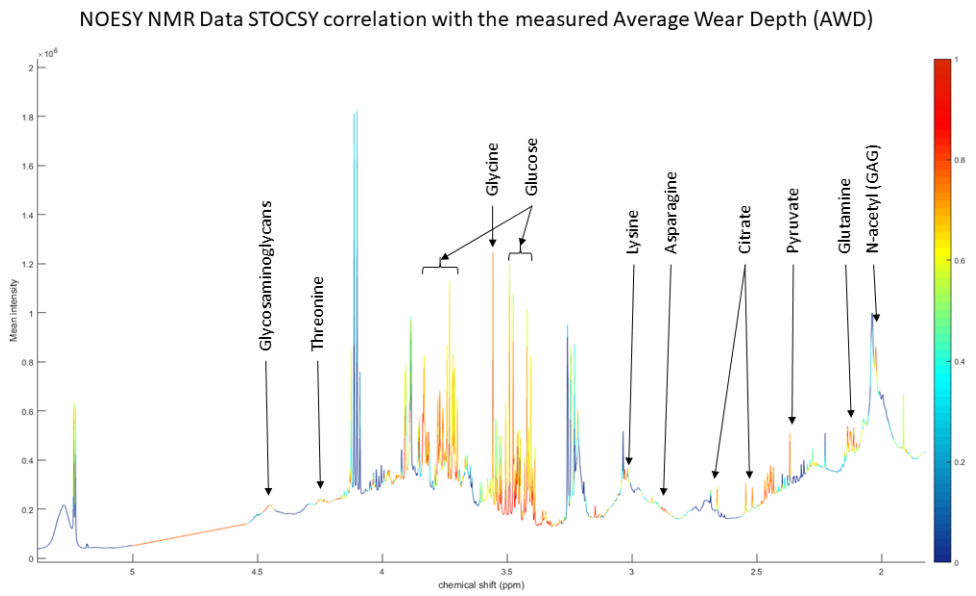


Figure 66 - An S-line plot depicting the correlation between NOESYGPPR1D NMR Spectra and AWD

Metabolite ID	Chemical Shift (ppm)	
Alanine	1.46 (d)	
Lysine	1.71 (m)	3.028 (m)
N-Acetyl (Glycosaminoglycans - GAG)	2.02 (s)	
Glutamine	2.12 (m)	2.44 (m)
Pyruvate	2.367 (s)	
Citrate	2.517 (dd)	
Asparagine	2.879 (m)	
N, N-dimethylglycine	2.92 (s)	
Dimethylsulfone	3.147 (m)	
Glucose	3.4 (m)	
Glycine	3.56 (s)	
Unknown	3.938 (m)	
Unknown	3.975 (m)	
Threonine	4.29 (m)	
Glycosaminoglycans (GAG)	4.45 (s)	
Glycosaminoglycans (GAG)	5.907 (m)	
Proteins -NH	Baseline 6.07	
Proteins -NH	Baseline 6.229-6.254	
Proteins -NH	Baseline 6.377-6.454	
Proteins -NH	Baseline 6.568-6.662	
Proteins -NH	Baseline 6.785-6.804	
Tyrosine	6.889 (d)	
Histidine	7.052 (s)	7.795 (d)
Unknown	7.067 (s) poor defined	
Proteins -NH	Baseline 7.473-7.647	
Glycosaminoglycans (GAG)	7.981 (s)	

Table 34 – Metabolites of HSF identified by NOESY NMR correlating with a Spearman correlation >0.9 with the AWD

Free amino acids were identified to correlate strongly with the average wear depth (AWD). These included: alanine, lysine, glutamine, glycine, threonine, asparagine, histidine and tyrosine. The amino acid derivative N,N-dimethylglycine was also significant. The baseline signature at >6ppm is non-specific and attributed to the amide group of protein.

The plant-based sulphur compound dimethyl sulfone, and energy substrates glucose and citrate were elevated with AWD.

There were multiplet peaks at δ=3.938ppm and 3.975ppm, and a singlet peak at δ=7.067ppm showing a strong correlation. However, their identities remain unknown.

The GAG signature in several areas of the NOESY spectrum correlated strongly with AWD. The *N*-acetyl peak which resulted from GAG within joint fluid also correlated strongly with AWD. This

correlation of the broad peak was not universal, and it corresponded specifically to the shoulder of the peak at δ=2.02ppm.

9.6 Discussion

9.6.1 Metabolic comparison of HSF and BCS

The direct comparison of HSF and the model fluid BCS, revealed a large variation in osteoarthritic HSF small molecule composition in keeping with other studies (219). This was illustrated with the multivariate linear regression analysis and the mean spectra plots. The spectra suggest variation in the glycoproteins and lipids, something also observed with other methods (245).

Synovial fluid is formed as an endocapillary dialysate of the blood plasma. Therefore, HSF and BCS are expected to have similarities but also specific differences. Any differences between the fluids could be due to active transport between the serum and SF, selective permeability of the capillary wall, and/or metabolic processes occurring within the joint itself (11). Active joint inflammation would predictably increase endocapillary wall permeability. Joint inflammation is known to reduce the oxygen tension and result in an anaerobic state in other diseases (54, 157). Animal studies of OA synovial fluid compared to healthy controls found increased anaerobic metabolism, a switch to fatty acid chain oxidation and proteoglycan degradation (100, 101, 111). The oxygen content of bovine and human arterial blood is similar (246, 247). Joints rely on the delivery of oxygen from the synovial fluid, via the synovium and ultimately the arterial blood. Therefore, due to consumption, the oxygen content of the SF is expected to be consistently lower than the concentration in the arterial blood. Therefore, it was predicted that OA HSF samples would present a more anaerobic environment than a non-diseased BCS sample. Despite this, the BCS samples had consistently higher levels of lactate. Interestingly, pyruvate concentration was lower in BCS. This may be due to a reduced production from glycolysis and consumption to lactate, supporting the hypothesis of anaerobic lactate production in the BCS samples. Additionally, an inactive Cori cycle could reduce the effective systemic lactate clearance (248). The reason for this unexpected result is not clear. It is possible that a delay between harvesting BCS and filtering large cellular material (>0.2μm) causes ongoing cellular activity and thus increasing

anaerobic metabolites. However, most noteworthy is that the biofluids of two distinct species were investigated and this factor drives the differences in composition.

Glucose levels are both elevated and depleted in OA compared to controls (111, 120, 125). The first theory is up-regulated transport, whilst the other in greater energy consumption. The HSF samples contained higher concentrations of glucose and creatine. Furthermore, the lipid peaks in BCS provided stronger NMR peaks from the $(CH_2)_n$ type, suggesting more saturation (101). Higher levels of choline and 3-hydroxyisobutyrate in the BCS samples suggest a destruction of complex lipid species and fatty acid metabolism (140). Alternatively, it could indicate a high level of gluconeogenesis in the BCS samples.

The presence of proteoglycan in the HSF is supported, although not confirmed, by the liberation of the *N*-acetyl moiety (2.03ppm) in the HSF cohort. A larger *N*-acetyl signal has previously indicated proteoglycan destruction, including hyaluronan (100, 111). Free amino acids alanine, glutamine, leucine and valine were elevated in the HSF compared to the BCS model. Changes in the levels of valine have been reported in synovial fluid after prolonged low temperature storage. It is unclear if this was the cause in our samples. No studies have referred to synovial fluid valine levels due to disease, although serum studies indicate both elevation and depletion in OA (116, 128). Leucine is elevated in canine OA synovial fluid compared to non-diseased controls (100).

Isopropyl alcohol was found in the BCS samples. This might be from skin swabbing when venepuncture was performed or from the filtration / storage processes. The effect of this alcohol contamination is not clear, although it is likely to affect the organised lipids and may disrupt any fluid-film lubrication they may provide.

The BCS model fluid contains more acidic anaerobic metabolites: lactate, pyruvate and 3-hydroxybutyrate than HSF. These molecules will be partially buffered by the solution and proteins of the BCS. Macromolecule surface binding of lipid and hyaluronan is effective in reducing implant wear (249, 250). However, the acids could affect the surface binding of charged species, thereby limiting boundary lubrication (251). In addition, acids could theoretically propagate crevice corrosion on the

implant (252). In summary, the liberation of acid is likely to limit lubrication and promote corrosion of the implant. This is more relevant in the *in vitro* scenario where any buffering effect may be limited.

9.6.2 Wear analysis

The direct correlation of HSF metabolic components with implant material wear is a significant finding. Several macromolecules have been postulated to be significant to native joint biomechanics. However, the composition of HSF has never been directly studied against wear in the *in vivo* or simulated scenario. Studies to date have shown that the degradation of hyaluronan affects sample viscosity (57). A lower molecular weight hyaluronan within the lubricating fluid increases wear (253)..

The NOESYGPPR1D NMR analysis identified several small and large molecules which were significant. However, the CPMG sequence universally showed no significance. The macromolecule signal is expected to be supressed when using the CPMG sequences (see 3.9.7.1.2). However, the loss of significance of the small molecules in this sequence is a notable finding. This implies that the wear-related small molecules seen in the NOESYGPPR1D sequence should be more visible in the CPMG sequence. Their loss suggests those small molecules are associated or complexed with macromolecules that are being supressed in the CPMG sequence.

The presence of significant *N*-acetyl and GAG peaks indicates that GAG plays a role in lubrication of the prosthetic joint. The decomposition of GAG (specifically hyaluronan) both *in vivo* and *in vitro* of HSF causes an increase in the *N*-acetyl ^{1}H-NMR signal (145, 146). HSF contains several GAG molecules including chondroitin sulphate (CS), keratan sulphate (KS) and hyaluronan (HA) (see 1.3.4). These molecules might lubricate the native joint; HA joint supplementation by injection being an accepted OA treatment(14, 29, 30) (see 1.4.6). It is not possible to specifically identify which GAG species relate to prosthetic wear in this experiment. However, since one shoulder of the *N*-acetyl peak (δ=2.02ppm) was significant then a specific GAG is involved.

The significance of glucose, citrate and pyruvate may be related to metabolic stress and changes in energy use within the samples. The glucose concentration both increases and decreases in joint disease (111, 117, 125). In addition, citrate and pyruvate consistently increase with joint disease and this change

was attributed to anaerobic metabolism (94, 100, 111, 117). Whilst these metabolites alter in joint disease and relate directly to metabolic stress, it is unclear what the correlation with prosthetic wear is attributable to. Perhaps these metabolites confound with another lubricating component of HSF which increases with metabolic stress and advanced joint disease. Alternatively, the anaerobic environment of the joint may have a direct relationship with the lubrication properties of the HSF due to alteration of the pH or interaction with other molecules in the HSF. The latter seems unlikely since no other anaerobic metabolites such as lactate and formate were concurrently significant (see 2.4.1).

An alternative hypothesis is that the glucose and citrate may originate from or be bound to a significant lubricating macromolecule within the HSF. Glucose is a constituent of the GAG backbone. Furthermore, the enzymatic destruction of GAG within HSF has specifically demonstrated the liberation of glucose and citrate (145, 146). Finally, in support of this hypothesis is the finding that supressing the macromolecule NMR signature in the CPMG sequence caused a loss of significance for both glucose and citrate.

The remaining amino acids that correlated with prosthetic wear have varying changes in joint disease. Alanine, glycine, asparagine and tyrosine are reported to increase with OA. There are no reports of changing levels of lysine or histidine with joint disease. The significance of these metabolites to lubrication is unclear. However, the absence of significance in the CPMG spectra again suggests a macromolecule relation. Cartilage NMR studies have suggested alteration of glycine and alanine levels correlating with proteoglycan and collagen destruction (124). Another report suggests that alanine levels can rise due to destruction of the proteoglycan complex and aggrecan unbinding (254).

9.6.3 Limitations

This study was limited by the stocks of BCS available. Only two batches were studied (albeit in duplicate) due to logistical and cost restraints. This is partially negated by commercial BCS being a pooled product. The HSF samples were collected from one central London research hospital and thus may suffer from selection bias.

10 Chapter 10 – Conclusions and summary

The principal purpose of this work was to establish a biobank of HSF that could robustly, informatively and safely answer multiple research questions. This and future projects would study the metabolic components of a variety of joint diseases, clinical subtypes, treatments and responses.

10.1 What is known about human synovial fluid?

To understand the scope of studies made with Human Synovial Fluid (HSF), it was important to characterise it and understand its properties. There are two distinct roles of human synovial: mechanical and biochemical (see 1.3.6). Therefore, the aims, strategy and organisation throughout this thesis mirrors these roles.

10.1.1 Mechanical properties of human synovial fluid

The understanding of the mechanical role of HSF was restricted to the macroscopic properties. Furthermore, understanding of the interaction between HSF and prosthetic implants *in vivo* was non-existent. This is serious because 200000 prosthetic joints are implanted annually in the UK alone (16, 26), and it is known that there is significant variation in macroscopic properties between individuals (10, 225). This variation might be present at the small molecule level as well. This, combined with the knowledge that so many implanted joints fail for unexplained reasons means that individual biology must be a factor (225). Little is understood about how HSF behaves within the prosthetic joint and nothing is known about the small molecule variations, so considerable value could come from an investigation of the small molecule composition of HSF and their contribution to the mechanical behaviour of joint surfaces.

10.1.2 Biochemical properties of human synovial fluid

Joint disease represents a significant health, economic and social burden to modern society. However, the treatment of osteoarthritis remains largely surgical and only addresses the mechanical features of the disease without considering the biochemical abnormality, inflammation and subsequent macromolecular degeneration which leads to a breakdown of the joint microanatomy. Furthermore,

there is a belief that the term osteoarthritis is an overarching description which simplifies the problem. It merely describes the endpoint of joint destruction, with many pathological routes and subgroups in the process. Considering the strong and recently identified links between metabolic disturbance, the progression of joint destruction and the experience of symptoms, it was felt that a metabonomic study of the OA subgroup would be a good target for future research of the HSF, in the expectation of identifying OA associations and metabolic subgroups. The comprehensive review of the literature surrounding OA and HSF separately, demonstrated use of lipid metabolism, a switch to anaerobic metabolism and the involvement of the BCAA (see 2.9).

10.2 Challenges

This project was proposed as a pilot study to evaluate the viability for metabolic phenotyping in HSF. Many proposals were submitted to secure funds for the sample collection and analysis. Sadly, these were unsuccessful, probably due to a lack of pedigree in this field. Eventually funding for sample collection was provided by Imperial College NHS Trust as a key component of their ongoing orthopaedic research program. Funding for the analysis was kindly provided by the Imperial College Phenome Centre and Metabometrix Ltd to add knowledge about the metabolic profiling of HSF to their portfolio of studied biofluids.

The preparation of the research, ethics and sponsorship proposals provided significant logistical, political, and bureaucratic challenges. Ethical approval required coordination of the local research committee, local sponsors, clinicians, and the ethics committee (see Appendix 1 - 7). Samples constituted a biohazard and sample handling protocols had to be drafted after suitable risk assessments had been completed (see Appendix 11 & 17). A full proposal was prepared; it included all documents related to the collection and storage of HSF (see Appendix 12 – 13). This was reviewed and approved before final submission to the research and ethical committees (see Appendix 6 – 7).

10.3 Key components to success

The creation of this project and the collection of an HSF biobank would permit many fields of study. The samples were harvested in a clinical setting and those volunteers should be rewarded with clinical

outputs furthering the treatment of joint diseases. It was felt that the principal method of studying the HSF should be metabolic since it provided a comprehensive representation of the phenotype (see 3.2). It was hoped that this project would facilitate many future collaborations, thereby combining data from multiple analysis methods to provide a near complete description of the phenotype. Metabolic phenotyping was deemed a suitable and relevant starting point.

Sample collection represented a large investment of time. To maximise the output of this investment the Imperial College Synovial Fluid Working Group was established. This forum, which met quarterly, had several benefits. Work was propelled forward by regular meetings and critical review. The combination of expertise from different research fields provided alternative perspectives, solutions and proposals for future work. Furthermore, the data produced from the samples could symbiotically assist all groups.

10.4 The metabolic analysis of human synovial fluid

At the start of this project HSF had seldom been studied and this remains the case although some reports have been published in the meantime (223). Other fluids: amniotic, serum, urine, saliva and cerebrospinal fluid have received much more attention (87, 220, 255-257). Therefore, it was imperative to systematically gather and collate all possible information relating to the stability, analysis methods and metabolites of HSF.

10.4.1 What is known about metabolism in synovial fluid?

To address this question, a joint disease was evaluated. Osteoarthritis was chosen as the most relevant since it was most likely to be prevalent in the harvested samples. Therefore, knowledge about the metabolism of OA in synovial fluid and other tissues was researched systematically (219). This, the first comprehensive analysis, provided a list of which compounds were potentially important, reported as statistically significant and the weight of evidence. The significance of this collated information was then analysed to provide insight into the metabolic networks affected by OA. This had not been done comprehensively before (see 2.9). The lipid pathways were highly represented. Anabolic and naturally occurring steroids were also represented for the first time. The presence of many amino acids, typically

those which are constituents of macromolecules, is explainable in view of the macromolecular decomposition seen with OA.

Interestingly glucosamine was also highlighted. This commonly available supplement for the treatment of OA has many effects. It is anti-inflammatory, reducing TNF-α and IL-1 and IL-6 (258). In addition, glucosamine has antioxidant properties and reduces oxidative stress in chondrocytes (259). However, this is the first time glucosamine has been highlighted from metabolic studies.

Besides OA, other joint diseases were researched. However, the studies typically compared diseases directly to OA but in the absence of any control subjects (126, 136, 158).

10.5 Study design

The process of collecting and storing a biobank of HSF would only be valid if the samples are not compromised, contaminated or degraded by the process. Furthermore, the small volumes of sample and limited analysis resource mean that repeated analysis is unlikely, thereby making it imperative that the study design, analysis and interpretation are robust and appropriate.

To ensure that the metabolic studies were effective, the literature was reviewed to determine the requirements for metabolic profiling in the context of relevance to clinical studies (see 3.3). These components formed the basis for the study design and informed the hypotheses and the structure of the thesis (see 1.2). This framework is used as a template and guide for any future member of the Imperial College Synovial Fluid Working Group to conduct their research and future studies, thereby ensuring the highest impact and maximum quality of data.

10.5.1 NMR methods

A comprehensive review of the synovial fluid NMR methods revealed that untargeted analysis was possible for HSF without the need for any prior extraction steps or removal of the macromolecules (see 4.1.2). The search showed a heavy reliance on buffer to stabilise the pH, which varied greatly amongst the synovial fluid samples. This was tested and the need for buffer was confirmed, without detriment to the NMR spectra.

Previous authors found that straightforward 1D ^{1}H-NMR techniques provided valuable information about the metabolic profile (101, 103, 111, 126, 146, 208). However, to facilitate metabolite identification and greater detail, the analysis was augmented with 2D-NMR. There was a concern that the significant macromolecule content of HSF would detract from the production of reliable and consistent NMR spectra. With limited samples this was tested, and the application of a CPMG sequence provided good macromolecule suppression and enhanced the small molecule detection.

10.5.2 Contaminant identification

To ensure validity of this and future projects it was essential to demonstrate and document any contaminants in the collection, storage or analysis. Since contaminants can be specific to a particular batch of consumables, meticulous care was taken to ensure that a single stock was used throughout all analyses, thereby making the contaminants predictable.

Contaminants were identified or partially identified (see Table 8). Precise identification was not always possible because since these chemicals were only present in trace amounts. ^{13}C satellite peaks originating from naturally occurring ^{13}C isotope from abundant constituents, such as solvent, were seen and catalogued for exclusion. The presence of 2-methyl-2-propanolol could not be explained. This compound is present in some water purification systems and so may have originated from the aqueous buffer (260).

This contaminant identification process had to be repeated for the evaluation of protein precipitation techniques (see Table 23). The number of contaminants increase due to increased steps, chemicals and materials. Solvent peaks alongside their ^{13}C isotope peaks were identified. However, this left four peaks (δ = 3.549, 3.563, 3.873, 4.25 and 7.905) which could not be identified. The presence of formate as a contaminant across all methods could not be explained. However, formate contamination has been described previously as a result of atmosphere absorption in aqueous samples and amongst NMR solvents (261).

10.5.3 Validation of sample collection

Samples could not be processed immediately after collection and therefore had to spend a short time refrigerated or at room temperatures. Prior to large scale sample collection, the magnitude of any metabolic effect as a result of this delay had to be characterised and quantified. A systematic literature review provided no support for the hypothesis that the samples would be stable.

Immediate energy release and phosphorylated energy systems would quickly and predictably deplete during sample collection at room temperatures. It was expected that the metabolic profile would show this. However, this was not always found. Succinate alone, of all the TCA cycle intermediates, was significantly reduced. A significant reduction of 2-ketoisovalerate may indicate energy consumption since pyruvate (the end product of glycolysis) is a precursor. However, the primary metabolic function of 2-ketoisovalerate is linked to branch chain amino acid (BCAA) formation (231). These are important in anabolic processes and macromolecular production. Valine (one of the BCAA) was seen to rise in concentration. It is pertinent that all of these metabolites have been reported in previous metabolic studies of joint disease, but only this study has demonstrated their instability (128, 162). Whilst these authors may have varied in their sample collection methods, it is likely that these findings will have unknowingly influenced results.

10.5.4 Validation of sample storage

Any future batch analysis and population-based studies will have to use stored samples. Therefore, it is imperative that metabolic effects of time spent in low temperature storage (-80°C) are known.

The results showed that many areas of the spectrum had changed after long-term storage. This included the macromolecules, aromatic compounds and lipids. However, these effects were not proven to be significant upon univariate testing.

The TCA cycle contributors: acetate, acetoacetate and succinate, were significantly reduced in concentration after long-term storage. The creatine concentration was significantly increased suggesting use of the phosphocreatine pathway. The significantly lower concentration of 3-hydroxybutyrate suggests ketone body production via the fatty acid pathway. In combination, these

findings suggest ongoing energy production and use during storage. These concentration changes were small. However, these metabolites all had similar and significant changes in magnitude in joint disease studies, including osteoarthritis (109, 117, 120, 121). These results raise the possibility that studies making conclusions of significance for these metabolites may have done so erroneously if long-term storage was used.

10.5.5 Maximising the benefit from the samples

Effective big data biological studies are not confined to only one analysis method. Maximal value is derived from the combination of the genome, transcriptome, proteome and the metabolome (see 3.2). Therefore, the future direction is a multi-platform high-throughput analysis. This will need sample preparation with extraction and protein precipitation. Potential sample extraction methods for HSF were evaluated for reproducibility, contamination, protein removal, metabolite recovery and any effects upon the NMR spectra.

10.5.6 Protein precipitation in human synovial fluid

A clear preference was reached for an acetonitrile liquid/liquid extraction in the protein precipitation of HSF; the method was reproducible and easy to use. It was most applicable to NMR analysis and provided additional detection of four singlets not previously described. Lastly it demonstrated good recovery universally compared to the other methods whilst still being effective in precipitating the protein and flattening the baseline. The principal disadvantage of this extraction is the loss of lipid, pertinent since the lipid profile is probably involved in inflammatory joint diseases. However, in many studies a separate targeted lipid method has been required and this would overcome this disadvantage (110, 127, 244). For this reason, it was felt that the detection gain of other metabolites outweighed the lipid loss.

The alternative methods tested were unsuitable due to analyte loss (TCAA wash) and protein denaturation (methanol). The use of deuterated solvent was tried because it negated the need for drying steps and a reconstitution phase. However, the lack of buffering and challenges to spectra interpretation meant the reward did not justify the effort needed to develop this method. Furthermore, it was hoped

that the reduction of the water and lack of need for water subtraction (δ=4.5-5.0) would give analytical benefits. No additional peaks were uncovered by removing water from the sample.

The additional metabolites detected by acetonitrile extraction have not been described by any other HSF study and require further work to identify them (223). These were singlets at δ=1.913, 3.64, 3.957 & 4.049. They had no significant relations elsewhere in the spectra, making it difficult to identify them by NMR methods alone.

If future analyses of HSF use HPLC then the analytical benefits of acetonitrile extraction will need to be re-evaluated, but its use would still be recommended. The extraction would present the sample in a solution that can be injected into an HPLC mobile phase.

10.5.7 The mechanical properties of human synovial fluid and metabolic profiling.

BCS is the surrogate biofluid of HSF in implant testing. Therefore, it is reasonable to compare HSF and BCS using metabolic profiling methods. Furthermore, in the event of a relationship between wear, mechanical properties and biochemical makeup of either BCS or HSF, a comparison could provide an insight into how the model may differ from reality. Any differences would assist in the development of more accurate HSF models and ultimately give a better representation of *in vivo* joint wear. A comparison challenges the assumption amongst tribology researchers that the fluids are chemically similar and will interact at the joint surface in a similar manner.

This thesis reports the first study to directly correlate the mechanical properties of HSF to its small molecule chemical composition. The importance of the glycosaminoglycan macromolecules was predicted and confirmed (see 9.6.2). Specifically, these macromolecules were strongly associated to various small molecules including amino acids, glucose and citrate.

Metabolic stress (i.e., predominance of the anaerobic and energy consumption pathways) is related to the wear properties of the HSF and this was demonstrated. Furthermore, these changes are present in pathological joints (see 2.11). This poses the question of whether the mechanical properties result from the metabolic changes or causing them. The suggestion is that joints with inflammation, and thus metabolic stress, with have detrimental lubrication. It has been shown that inflammation causes

degradation of the joint structure, the macromolecular composition of the HSF and the cartilage (4, 14, 25, 47). However, this has never been linked directly to any mechanical measurement. This study has shown that metabolic stress correlates with decreased lubrication, presumably from disease, and inflammation provides further evidence of this.

10.6 The future

10.6.1 What are the likely future advancements in metabolic phenotyping?

Metabolic profiling constantly moves forward with enhancements in instrument sensitivity. Furthermore, the advancements in engineering result in ever greater instrument resolution, yielding a higher specificity. Any future continuation of this work must ensure that researchers remain current and incorporate these advances into their methods.

The increase in computational power and software developments will allow more complex and involved statistical analysis. In NMR spectroscopy the development of higher frequency instruments and super-cooled detectors (cryoprobes) improve the signal-to-noise ratio and provide greater resolution of spectra. MS instruments are improving their mass measurement and allowing easier identification of metabolites, particularly the introduction of Fourier transformation ion cyclotron resonance mass spectrometry (FTICR-MS) (262). MS detectors are evolving in sensitivity, with better low-level detection. The re-engineering of mass analysers to provide multi-fragmentation (MS^n) techniques advances the elucidation of chemical structures. Combined, these instrument advances will yield more metabolites that can be identified with greater confidence (182).

10.6.2 Personalised medicine

Personalised medicine is a concept widely held to be a viable product of the techniques outlined in this thesis. Patients differ in responses to both disease and treatment due to genetics, diet and the environment. The detailed description of the phenotype, and hence disease, allows a greater understanding of response to and personalisation of treatment. The appreciation of the influence of diet and the environment on disease has evolved in the last 20 years, especially when considering the influence of the gut microbiome. Metabolic profiling has the potential to deliver personalised medicine

in diseases with an inflammatory and/or metabolic component (such as OA). NMR analysis of the lipoprotein subclasses is now clinically used in the evaluation of atherosclerosis and the risk of developing cardiovascular disease (263).

In addition to advanced diagnostics, a patient's treatment may be tailored from the metabolic profile. Pharmacological agents may be titrated, specific diet or lifestyle modifications made or surgery recommended with a greater confidence of success (174). The treatment response can be accurately evaluated by advanced monitoring. The risk of developing a disease can be stratified prior to any symptoms, allowing preventative medicine. The outcome of clinical trials can benefit from precise and accurate data about the effects from novel drug therapies.

The tailoring of healthcare delivery for the population is the concept of stratified medicine. This is the application of phenome data from a population to better understand the healthcare needs. This allows a targeted healthcare strategy: identifying individuals at risk , identifying diseases more prevalent in the population, strategic delivery and maximising the value of health spending. This is particularly relevant in joint disease like OA, where it consumes a sizeable proportion of the healthcare budget.

10.6.3 Metabolite identification

This study identified metabolites and contaminants. The identification of contaminants is unlikely to have a bearing on the future metabolic profiles because their concentrations are small. However, profiling contaminants at this stage is beneficial since any change in consumables in the future may cause significant contamination. Therefore, it is preferable to make confident identification of contaminants, so they do not corrupt the results.

The unknown metabolites discovered using the acetonitrile extraction will benefit future HSF phenotyping studies. At this stage they could not be identified. If these unknown metabolites are found to be important, then time and effort should be invested to provide a level 1 metabolite identification.

Several unknown metabolites differed between the HSF and the BCS samples but were not correlated to the wear properties. Whilst it was not important to provide identifications for these metabolites at this stage, any future attempt to design a better HSF surrogate would benefit from this information.

10.6.4 Understanding the metabolism of implant wear

The relationship between the presence of a metabolite and the wear of a joint prosthesis requires further investigation. Specific small components related to GAG species that significantly affected wear were demonstrated. This requires identification and clarification. The results illustrate that (contrary to previous opinion) the synovial fluid may play a role in implant longevity due to its composition, and that specific protein classes are important. Furthermore, metabolic stressors may cause or be related to a reduction in lubrication.

The joint simulator tested metal-on-metal surfaces. Further work should investigate the impact on lubrication of the other bearing surfaces e.g., metal-on-polyethylene, ceramic-on-ceramic or ceramic-on-polyethylene, and if they are affected by the same metabolic components. It is feasible that a different chemistry may be favoured with another bearing surface and this needs to be evaluated.

The effect of HSF chemistry on implant wear in prosthetic joints suggests that targeting joint metabolism or chemistry may reduce implant wear. A hypothesised personalised medicine approach could target either the composition of the fluid for the individual or even influence the selection of the implant. Diet supplements or metabolism modifying drugs are beginning to emerge as strategic targets for joint health. Therefore, it is logical that similar factors may reduce implant wear within the joint. Indeed, some of the compounds identified (N, N-dimethylglycine and derivatives of dimethylsulfone) are sold for promoting joint health. In summary, it may be possible to assess the metabolic profile and tailor a diet, medication or operation regimen to that patient (66, 179)..

These results have shown the wear effects of diseased HSF. However, the presence of an implant may itself significantly affect the metabolic profile (225). Future studies from our sample cohort should concentrate on recruiting patients with prosthetic joints *in situ* to firstly study the metabolic impact thereof and secondly correlate this impact to wear. This may provide insight into how the different modes of failure impact the metabolism.

Whilst these are long-term goals, the description of metabolic profiling having an impact upon material wear is the first step in this journey.

10.6.5 Can the human synovial fluid provide more information?

The HSF samples are collected from a spectrum of diseases, clinical cohorts, treatments and outcomes; thus, the samples have value to answer many clinical questions. A biobank of 200 samples will provide only a few samples once specific cohorts of disease are selected. Nevertheless, this biobank should provide enough statistical power to make confident metabolite distinctions between disease cohorts.

10.6.5.1 Septic arthritis

Septic arthritis remains a significant diagnostic challenge. The condition is a surgical emergency since the presence of intra-articular colonisation, particularly with gram positive organisms, causes production of lytic enzymes. These enzymes are destructive to the macromolecular structures of both the synovial fluid and the cartilage. As a result, the joint rapidly and irreversibly degrades causing early onset arthritis. The treatment is urgent surgical washout and antibiotics to prevent sepsis and preserve the native structure of the joint. However, it is a difficult clinical decision since the diagnostic tools available in clinical practice are limited both in accuracy and immediacy. Therefore, the clinician must commit a patient to emergency surgery and risk making the joint worse if the diagnosis is uncertain (264). The available clinical signs, diagnostic tests and imaging do not distinguish between infection and inflammatory arthropathy (265). Confirmatory testing requires invasive arthrocentesis and then culturing of microbes from the joint fluid, which takes 24-48 hours. Any delay in treatment can be catastrophic, so it is often prudent to proceed with surgery despite the uncertainty. This means some patients will have unnecessary surgery while, for others a delayed diagnosis will mean irrevocable damage to the joint. Diagnostic delay can also result in additional bed occupancy and thus expense, waiting in hospital for surgery to be performed or test results to be returned (266). The need for better diagnosis, prediction and monitoring of septic arthritis is clear. Metabolic profiling could differentiate between infected and non-infected HSF. Furthermore, a metabolic profile may provide an insight into specific organisms, their subtypes and yield a faster diagnosis.

The preliminary analysis comparing an infected and a non-infected HSF cohort showed significant metabolic differences. However, the sample numbers were small and this limited the significance of

the results (267). Therefore, there is a desire to expand the sample numbers. In addition, extending the analysis to include the microbiome profile will complement the metabonome by providing microbe specific information.

10.6.5.2 Osteoarthritis

Osteoarthritis remains a significant health challenge being common, disabling and expensive to treat (there are few options but arthroplasty in the late stages). The increased awareness of the importance of metabolism in this inflammatory disease is providing new targets for both treatment and diagnosis (268). The details of the link between metabolism (MeS specifically) and osteoarthritis are yet to be discovered. This remains an obvious target for the work of Imperial College Synovial Fluid Working Group, given the large number of samples harvested from OA patients. It is hoped that once the biobank is suitably established there will be cohort groups that are large enough to determine how metabolism plays a role in the different OA subgroups. These would include the following:

- Patients who respond to anti-inflammatory and steroid therapy
- Metabolic differences in patients with post-traumatic OA and non-traumatic OA
- Inflammatory and non-inflammatory OA, comparing the metabolic differences
- Rapid onset and destructive OA versus slow progressing OA
- Early versus late OA and how metabolism changes with progression

The suggested links between lipid metabolism and OA mean that it would be desirable to evaluate further with a targeted lipid profiling study to investigate the association between clinical factors. This might provide insight into OA lipid metabolism and augment many of the recent studies (43).

10.6.5.3 Expanding the scope of phenotyping

Expansion of the Imperial College Synovial Fluid Working Group to include microbiome and proteome researchers should allow a more complete description of the phenotype in the samples (see 3.2). The combination of these data streams will provide the most powerful and informative studies. Thus, going forward all samples should undergo a three-tiered analysis: metabonome, microbiome and proteome,

with the increased output that is expected to provide. This strategy succeeds to an umbrella analysis with the expectation that the combination of analyses will provide more power than the sum of their parts.

This work has been the genesis of the synovial fluid biobank and collated the research skills to utilise it. It is hoped that the outputs from, and related to this thesis will allow expansion thereof with future funding, ongoing sample collection and high-quality published research.

10.7 Publications originating from this thesis

Jaggard MKJ, Boulange CL, Graca 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.' Journal of Pharmaceutical and Biomedical Analysis 2021; 197: 113942

Jaggard MKJ, Boulange CL, Akhbari P, Vaghela U, Stevenson H, Bhattacharya R, Williams HRT, Lindon JC, Cann P, Gupte CM 'Evaluation of the relevance of bovine calf serum as a model for human synovial fluid in implant testing and validation from small-molecule differences using metabolic profiling' Submitted to BMC Musculoskeletal Disorders

Akhbari P, Karamchandani U, Jaggard MKJ, Graca 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):108–119.

Jaggard MKJ, Boulange CL, Akhbari P, Vaghela U, Bhattacharya R, Lindon JC, Williams HRT, Gupte CM 'Can metabolic profiling provide a new description of osteoarthritis and enable a personalised medicine approach?' – Clinical Rheumatology 2020 39(12):3875-3882

Jaggard MKJ, Boulange CL, Akhbari P, Vaghela U, Bhattacharya R, Lindon JC, Williams HRT, Gupte CM 'A systematic review of the small molecule studies of osteoarthritis using nuclear magnetic resonance and mass spectroscopy' – Osteoarthritis and Cartilage, 2019 27th Apr (4):560-570

Jaggard MKJ, Boulange CL, Graca G, Akhbari P, Vaghela U, Bhattacharya R, Williams HRT, Lindon JC, Gupte CM ‘The effect of liquid-liquid extraction on metabolite detection and analysis using NMR in Human Synovial Fluid’. Submitted to Metabolomics

10.8 Presentations originating from this thesis

Jaggard M, Stevenson H, Boulange C, Graca G, Akhbari P, Vaghela U, Bhattacharya R, Williams H, Lindon J Cann P, Gupte C

The Metabolic Components of Human Synovial Fluid and Bovine Calf Serum That Correlate with Cobalt Chrome (CoCrMo) Wear, 22nd EFORT Congress, VEC 2021 [p]

Jaggard M, Akhbari P, Vaghela U, Boulange C, Stevenson H, Cann P, Lindon J, Williams H, Bhattacharya R, Gupte C 'Can We Distinguish Between Infected and Non-Infected Joint Fluid Using Metabolic Profiling? A Prospective Metabonomics/Nuclear Magnetic Resonance Study'

Orthopaedic Research Society, New Orleans, March 2018 [p]

Jaggard MKJ, Akhbari P, Vaghela U, Boulange C, Stevenson H, Cann P, Lindon J, Williams H, Bhattacharya R, Gupte C

Differences in the metabolic profile of human synovial fluid in various osteoarthritic subgroups

British Orthopaedic Research Society, London 2017 [p]

Jaggard M, Akhbari P, Vaghela U, Boulange C, Stevenson H, Cann P, Lindon J, Williams H, Bhattacharya R, Gupte C

Differences in the metabolic profile of human versus pooled bovine synovial fluid

19th EFORT Congress, Barcelona 2018 [p]

British Orthopaedic Research Society, London 2017 [p]

Metabolic profiling of osteoarthritic joint fluid with correlation to metabolic disease, symptoms and functional outcome

Jaggard MKJ, Akhbari P, Vaghela U, Boulange C, Lindon J, Williams H, Bhattacharya R, Gupte CM

European Knee Society (EKS), London 2017 [p]

The correlation between synovial fluid metabolic profile and implant wear

Stevenson H, Jaggard M, Boulange C, Akhbari P, Vaghela U, Lindon J, Williams H, Bhattacharya R, Gupte C, Cann P

European Knee Society (EKS), London 2017 [p]

10.9 Publications originating from the Imperial College Synovial Fluid Working Group

Stevenson H, Parkes M, Austin L, Jaggard M, Akhbari P, Vaghela U, Williams H, Gupte C, Cann PM. 'The development of a small-scale wear test for CoCrMo specimens with human synovial fluid.' Biotribology 2018 14:1-10

Akhbari P, Jaggard M, Vaghela U, Boulange C, Gomes Da Graca G, Lindon J, Williams H, Bhattacharya R, Gupte C 'Differences in the composition of hip and knee synovial fluid in osteoarthritis: a metabolic phenotyping study' –Osteoarthritis and Cartilage 2019, 3rd Sept

Akhbari P, Jaggard MKJ, Boulange C, Vaghela U, Graca G, Bhattacharya R, Lindon JC, Williams HRT, Gupte C. 'Differences between infected and non-infected synovial fluid: an observational study using metabolic phenotyping (or "metabonomics")' Bone and Joint Research 2021;10(1):85-95

Stevenson H., Jaggard M., Akhbari P., Vaghela U., Gupte C., Cann P. 'The role of denatured synovial fluid proteins in the lubrication of artificial joints' Biotribology 2019 17:49-63

10.10 Presentations originating from the Imperial College Synovial Fluid Working Group

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

Differences Between Infected and Non-Infected Synovial Fluid: A Nuclear Magnetic Resonance (NMR) Spectroscopy Study of Metabolic Profiles, 21st EFORT Congress, Vienna Austria 2020 PODIUM

P Akhbari, MKJ Jaggard, U Vaghela, C Boulange, G. Graca, J Lindon, H Williams, R Bhattacharya, C Gupte

Differences Between Infected and Non-Infected Synovial Fluid: A Metabolic Phenotyping Study

20th EFFORT Congress, Lisbon, Portugal 2019 [p]

P Akhbari, MKJ Jaggard, U Vaghela, C Boulange, J Lindon, H Williams, R Bhattacharya, C Gupte

Differences In the Synovial Fluid of Hips and Knees: Metabolic Analysis Using Nuclear Magnetic Resonance (NMR) Spectroscopy

19th EFORT Congress, Barcelona 2018

Orthopaedic Research Society, New Orleans, March 2018 [p]

Stevenson, H., Jaggard, M., Boulange, C., Akhbari, P., Vaghela, U., Lindon, J., Williams, H., Gupte, C., Cann, P.

Synovial fluid chemical composition and wear of hip implant materials

British Orthopaedic Research Society, London 2017 [p]

The Effect of Synovial Fluid Composition on Cobalt Chrome Implant Material Wear

Stevenson, H., Jaggard, M., Boulange, C., Akhbari, P., Vaghela, U., Lindon, J., Williams, H., Gupte, C., Cann, P.

Summer Biomechanics, Bioengineering and Biotransport Conference 2017

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12 Appendices

12.1 Appendix 1 – NHS R&D form

NHS R&D Form IRAS Version 5.12

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Looking for Biomarkers in Human Joint Fluid

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(●) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

DRAFT

12.2 Appendix 2 – NHS R&D approval letter

Jo int Researc h Compliance Office
Academic Health Science Centre
Imperial College London and
Imperial College Healthcare NHS Trust
Room 5L1OD, 5th Floor, Lab Block
Gharing Cross Hospital
Fulham Palace Road
London W6 BRF
Tel: +44 (0)20 3311 0212
Fax : +44 (0)20 3311 0203
r.nicho lson@imper ial.ac .uk
www .ic.ac.uklc lini calresearc hgove rnance office

Ruth Nicholson
Research Governance Manager

Dear Mr Gupte

RE: JRCO Study Approval

Project Title: An investigation of human body fluids for the identification of biomarkers relating to joint disease

Joint Research Compliance Office Reference number: 15SM2512

Ethics reference number: 15/LO/0388

Principal Investigator: Mr C Gupte

I confirm that this project has now been approved by the Joint Research Compliance Office. The project may now start at Imperial College Healthcare NHS Trust sites. Please note that the start date of the project is the date of this letter and the duration is the same as that provided in your application form.
The list of documents reviewed and approved by the Joint Research Compliance Office under requirements of the Research Governance Framework are as follows:

Document	Version	Date
REC Aooroval letter		12May2015
Patholoav Approval		21 April 2015
Tissue Bank Approval		20 May 2015
Divisional Aooroval		30 March 2015
SSI form		21 May 2015
REC Form		05 March 2015
Non-validated questionnaire	1.2	05 February 2015
Guidelines for Joint resamolina	1.0	08 Januarv 2015
Resamplinq patient letter	1.0	09 February 2015
Chimnay Gupte CV		05 February 2015
Matthew Jaqqard CV		11 February 2015
Consent Form	1.3	17 April 2015
Information Sheet	1.5	17 April 2015
Protocol	1.3	08 January 2015

Before you commence your research, please note that you must be aware of your obligations to comply with the minimum requirements for compliance with the Research Governance indicators 17 (Data Protection); 25 (Health and Safety) and 22 (Financial Probity). Details of the requirements to be
i met can be found in the Research Governance Framework available on www.dh.gov.uk.
Under the Research Governance regulations, Serious Adverse Event Reports, Adverse Reactions and amendments to the protocol or

12.3 Appendix 3 - NHS SSI form

NHS SSI IRAS Version 4.0.0

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Looking for Biomarkers in Human Joint Fluid

1. Is your project research?

(•) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product

( ) Clinical investigation or other study of a medical device

( ) Combined trial of an investigational medicinal product and an investigational medical device

( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice

( ) Basic science study involving procedures with human participants

( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology

( ) Study involving qualitative methods only

(•) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)

( ) Study limited to working with data (specific project only)

( ) Research tissue bank

( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(•) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(•) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(•) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(•) Yes	( ) No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

12.4 Appendix 4 – Sponsorship & insurance registration form

Imperial College London

Imperial College Healthcare NHS Trust

Joint Research Compliance Office
Academic Health Science Centre
Imperial College London and
Imperial College Healthcare NHS Trust
Room 5L1DG, 5th Floor, Lab Block
Charing Cross Hospital
Fulham Palace Road
London W6 8RF
Tel. +44 (0)20 3311 0212
Fax. +44 (0)20 3311 0203
r.r.nicholson@imperial.ac.uk
www.ich.ac.uk/ichltd/researchgovernance/rec/rec

Ruth Nicholson
Research Governance Manager

05/02/2015

Mr Chinmay Gupte
Imperial College London
St Mary's Hospital
Praed Street
W2 1NY

Dear Mr Gupte

RE: An investigation of human body fluids for the identification of biomarkers relating to joint disease.

Joint Research Compliance Office Reference number: 15SM2512

This is to confirm that the above named research project utilises human participants, their organs, tissue and/or data as defined under the sponsorship requirements of the Research Governance Framework for Health and Social Care 2005, incorporating the Medicines for Human Use (Clinical Trials) Regulations 2004.

On behalf of Imperial College of Science, Technology and Medicine, we undertake to act as the identified Research Sponsor for this project.

This letter confirms:

- The research proposal has been discussed, assessed and registered with the Joint Research Compliance Office, Imperial College Academic Health Science Centre, Imperial College London and provisional sponsor approval granted.
- The Chief Investigator has undergone a process of scientific critique commensurate with the scale of the project
- Indemnity and insurance arrangements have been put in place to cover the project.
- Resources and support are available to the research team to aid delivery of the research as proposed.
- Management, monitoring and reporting responsibilities for the research have been approved.
- Imperial College will undertake and enforce those sponsor duties set out in the NHS Research Governance Framework for Health and Social Care.

Imperial College Sponsorship is conditional on the project receiving applicable ethical and regulatory approval for all research related aspects of its conduct. It is also conditional on successful contract and agreement negotiations and sign off via the Joint Research Office, where relevant, and before the study commences.

A copy of the ethics approval letter **must** be sent to the Research Governance Manager prior to the study commencing. Sponsorship is dependant on obtaining R&D Office approval for all NHS sites where the research is being conducted.

12.5 Appendix 5 – Insurance certificate

Arthur J. Gallagher International

21st July 2014

TO WHOM IT MAY CONCERN

We, the undersigned Insurance Brokers hereby certify that we have place the following Insurance:

VERIFICATION OF INSURANCE

Unique Market Reference:	B1262FIC114814	
Type:	Clinical Trials Insurance	
Insured:	Imperial College London	
Period:	From: 01st August 2014 To: 31st July 2015	Both days inclusive at Local Standard Time.
Interest:	This Policy will indemnify/cover the Insured in respect of their Legal Liabilities arising out of the Insured's activities and as more fully disclosed within the Policy Wording	
Limit of Indemnity:	GBP 12,500,000Any One Claim and GBP 12,500,000 in the Aggregate, including costs and expenses	
Excess:	GBP 2,500 Each and Every Claim, including costs and expenses	
Underwriter:	100.0000%	Newline Syndicate 1218

This document is for information only and does not make the person or organisation to whom it is issued an additional Insured, nor does it modify in any manner the Contract of Insurance between the Insured and the Insurers. Any amendment, change or extension to such Contract can only be affected by specific endorsement attached thereto.

Should the above mentioned Contract of Insurance be cancelled, assigned or changed during the above policy period in such manner as to affect this document, no obligation to inform the holder of this document is accepted by the undersigned or by the Insurers. The information provided is correct at the date of signature.

Authorised Signatory
Gallagher London.

12.6 Appendix 6 – Research ethics committee application form

NHS REC Form | Reference: 15/LO/0388 | IRAS Version 4.0.0

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please complete the questions in order. If you change the response to a question, please select 'Save' and review all the questions as your change may have affected subsequent questions.

Please enter a short title for this project (maximum 70 characters)
Looking for Biomarkers in Human Joint Fluid

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(●) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

DRAFT

12.7 Appendix 7 – Research ethics committee favourable opinion letter

NHS
Health Research Authority

NRES Committee London - Chelsea
Research Ethics Committee (REC) Bristol Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 01173421330

12 May 2015

Mr Matthew Jaggard
Clinical Research Fellow
Imperial College NHS Trust
St Mary's Hospital
Praed Street
London
W2 1NY

Dear Mr Jaggard

Study title:	**An investigation of human body fluids for the identification of biomarkers relating to joint disease**
REC reference:	**15/LO/0388**
IRAS project ID:	**167807**

Thank you for your letter of , responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Miss Gemma Oakes, nrescommittee.london-chelsea@nhs.net . Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation

12.8 Appendix 8 – tissue bank application form

Imperial College Healthcare
Tissue Bank



IMPERIAL COLLEGE HEALTHCARE NHS TRUST

TISSUE BANK (ICHTB)

APPLICATION FOR REGISTRATION OF A CLINICAL TRIAL

- This application form is for the purposes of registering a Clinical Trial with the ICHTB to facilitate the release of tissue or samples for said Trial

    - Registration is required for all Collections or Trials to allow for the release of material

    - The NRES reference and details must be supplied to the Tissue Bank.

    - Before samples from a clinical trial can be used for research, researchers must either provide the approved trial protocol, or must make an application for use of this material through the tissue bank mechanism to be provided with "deemed ethics approval".

Please email the completed application to: tissuebank.@imperial.ac.uk, or post to:

ICHTB Secretariat
Tissue Bank Laboratories
11th Floor, Laboratory Block
Charing Cross Hospital
Fulham Palace Road
London W6 8RF

Application to register a clinical trial with ICHTB

A: General information

Trial name , PI and sites	
Name of PI for the trial	Mr Chimnay Gupte
Contact details for the PI	0207 483 5160
Email	c.gupte00@imperial.ac.uk
Sites at which trial will take place *(Delete as required)*	St Mary's Hospital, Charing Cross Hospital, South Kensington Campus Mechanical Engineering, South Kensington Metabonomics
Local lead(s) for trial site(s)	***(Indicate for which site, add further lines if more than one site)***
Name	Charing Cross – Chimnay Gupte
Contact details	0207 483 5160
Email	c.gupte00@imperial.ac.uk
Name	St Marys – Matt Jaggard
Contact details	07904066695
Email	m.jaggard@nhs.net
Name	South Kensington Mechanical Engineering – Philipa Cann
Contact details	020 7594 7027
Email	p.cann@imperial.ac.uk
Name	South Kensington Computational and Systems Medicine – John Lindon
Contact details	+44 (0)20 7594 3194
Email	j.lindon@imperial.ac.uk
Trial information	
Who is the sponsor for the trial?	Imperial College
Who is funding the trial?	Imperial College
Is this an NCRN adopted trial?	No
R&D Number (if known):	15SM2512
EUdract Number (if applic):	N/A
Name and contact details of trial coordinating centre (CTU):	N/A
This form completed by	
Name	Matthew Jaggard
Contact details	07904066695
Email	m.jaggard@nhs.net

ICHTB contact	***(Leave blank – this will be allocated by ICHTB)***
Name	Sarah Chillcot-Burns
Contact details	0203-311-7342
Email	s.chilcott-burns@imperial.ac.uk

B: Consent and Ethics

NRES approval number	**15/LO/0388**
Date of NRES approval	12/05/2015
Date of expiry *(from REC applic or subsequent amendments)*	
Please supply the following: - Consent Form (sample) - REC application form - REC approval letter - Approved trial protocol.	*(These can be embedded here or sent separately)*

C: Biological Samples

Are biological samples being collected as part of the trial	**YES**
If yes, which of the following does the trial require?	
Tissue:	
Fresh or snap frozen material from the operative specimen	**NO**
Formalin fixed paraffin embedded material	**NO**
Other samples types (ie not FFPE block or fresh tissue)	**YES**

Please supply enough information to help ICHTB plan the work required.
Specify the types of samples needed, when and how they will be (or have been) taken from the patient. Indicate what will happen to the samples.

Synovial Fluid, Blood and Urine to be submitted for metabonomic analysis. Synovial fluid to be taken for Microbiology and Tribology analysis

Examples and additional information
"Extra breast biopsy taken at diagnosis. Needs sending off to the CTU"
"Block containing tumour material, if available, from original diagnostic specimen"
"Fresh tissue from surgery, to be kept at 4 degrees, placed in RNALater etc. Pots to be supplied by trial office"

Interaction with Histopathology
ICHTB have the facility to cut / stain additional slides (where these are required and agreed), but if any additional reporting is required, this must be agreed with Rob Goldin (Head of Histopathology) and you must make arrangements with the appropriate pathologist.

Note - please keep in your own office any trial materials, such as sample collection pots, jiffy bags for dispatch, CRFs etc that may have been provided by the CTU, unless you have discussed and agreed specific arrangements with your ICHTB contact.

How often do you expect to make request for samples? Once *(Consider how many patient accruals are expected)*	Supplying samples only
Fluids:	
Bodily fluid (blood/serum/urine/bile etc)	**YES**
Please specify type(s) of fluid taken	Blood (serum), urine and synovial fluid

Please provide further information about any fluid samples taken for trial purposes *Indicate by whom, where in patient treatment pathway and what you intend to do with the samples (eg to spin and freeze prior to dispatch). Please indicate if you are requesting any Tissue Bank assistance* *Samples taken by clinical staff and researchers in the clinical setting of outpatient clinics, theatres and the emergency department. No samples taken above those required in clinical practice*

D: Information on storage after release from the Tissue Bank

Will these samples be stored at any stage on Imperial College Trust premises?	**NO**
If Yes, give the location in which these samples are stored:	
Hospital (e.g. CXH/SMH/HH):	Room number: 7
Freezer/cabinet:	Container labelled:
If No please give details of how long after collection these samples will be shipped to their destination and give the address of the place to which they will be shipped: **Daily transfer to the above location:** **Room** **Imperial College London** **Room 7L34** **Musculoskeletal Research Laboratory** **7th Floor East Wing** **Charing Cross Hospital** **Fulham Palace Road** **London**	
Return of samples **Please indicate what has been stated by the CTU in terms of returning samples** *(note if the CTU has stated samples will only be returned on request, ICHTB are happy to make such requests)* *No arrangement made*	

E: Reimbursement available for bio sample collection.

For each bio sample type listed for collection for the trial please give the following information:	
Amount available for reimbursement by the CTU:	
Sample type*	
Details of reimbursement available: *(ICHTB can raise invoices independently of*	

R&D and links with the Contracts department in order to avoid duplication)	

**** Add additional rows for multiple sample types***

ICHTB Use:

Applications for use of materials from clinical trial sub-collection submitted to ICHTB

Application No	Date approved	PI

12.9 Appendix 9 - Tissue Bank Sub Collection Registration Form

IMPERIAL COLLEGE HEALTHCARE NHS TRUST TISSUE BANK
APPLICATION FOR REGISTRATION AS A SUB-COLLECTION UNDER THE HTA LICENCE

- This application is for the purposes of registering a collection of samples of human tissue or fluids that contain cells for storage under the HTA licence.
- Registration is required for all collections of material that consent patients generically for future use of their material for research.
- Such collections, where they involve **ONLY** collection of material from patients at the same time as similar diagnostic samples are being taken, may utilize the approved Trust consent forms. Consent forms and patient information sheets are available from the ICHTB Tissue Bank manager.
- Separate patient information sheets and consent forms are available when extra samples (e.g. extra blood sample or biopsy specimen) are taken specifically for research.
- Where projects seek to obtain samples from patients when a similar diagnostic sample **WOULD NOT** be taken or where patients are asked to complete questionnaires must obtain project specific NRES approval.
- Where generic consent is also sought for use of material, these projects **MUST** be registered under the HTA licence.
- Before a sub-collection can start accruing samples, the following SOPs must be lodged with the Tissue Bank using the Q-Pulse document management system (to obtain an account please contact the Tissue Bank Manager)
  - procedure for obtaining and documenting donor consent and receipt of samples
  - procedure for disposal of samples
  - procedure for transport of samples (into storage and from storage)
  - procedure for cleaning and decontamination
  - procedure for managing abnormal changes in storage temperatures
  - procedure for recording and reporting adverse events to the DI[1]

  In some instances these may be the same as the SOPs used by the main tissue bank – copies of these can be obtained from the Tissue Bank Manager.
- Before samples from a subcollection can be used for research, researchers must either provide evidence of project specific NRES approval for the use of the material from a specific subcollection, or must make an application for use of this material through the

[1] An adverse event is defined as an event resulting in specimen loss, missing or incorrect documentation, a security breach, abnormalities in storage temperature readings, issues from transport of specimens, inappropriate disposal of a sample

tissue bank mechanism to be provided with “deemed ethics approval”.

Please email the completed application for registration as a sub-collection form to tissuebank.@imperial.ac.uk to:

ICHTB Secretariat
Department of Histopathology
6th Floor, Laboratory Block
Charing Cross Hospital
Fulham Palace Road
London W6 8RF

Subcollection Application

Name of PI for the project MR Chinmay Gupte

Contact details for the PI Musculoskeletal Laboratory
7th Floor East Block
Charing Cross Hospital
Hammersmith
London

Email c.gupte00@imperial.ac.uk

Name of Sub-collection Looking for Biomarkers in Human Joint Fluid

Consent and Ethics

Which of the following applies to your project?

A Generic consent is obtained from patients using the NHS consent for procedure (please specify which consent form 1,2 or 3)

B Generic consent for samples extra to those required for diagnosis (e.g. biopsy/blood) is obtained using the NHS consent form for extra samples

C This project has its own NRES approved consent form
If C, please state the date on which NRES approval was obtained, the date on which this will expire, and give the appropriate NRES approval number. Please also supply a copy of the Consent Form and the NRES approved project/trial protocol.

C - 15/LO/0388 – Favourable opinion given 12/05/2015 – no expiry date mentioned

Samples and data collected

A Samples:

Which of the following does your project collect?

Tissue:

Fresh or snap frozen material from the operative specimen

taken directly from surgery Yes

obtained from the histopathology department No

Formalin fixed paraffin embedded material

Samples extra to that obtained for diagnosis N/A

Samples supplied from the diagnostic record N/A

Extra tissue samples taken at the same time as samples taken for diagnosis

taken preoperatively Yes

Taken at other time point (specify) No

Please specify the type of sample taken (e.g. extra breast biopsy taken at diagnosis)

_____

Fluid:

Bodily fluid (blood/serum/urine/bile etc) Yes – Synovial Fluid

Please specify type(s) of fluid taken
Are these samples taken at the same time as other similar samples are taken for diagnosis? Yes (but not always)

Please give details of the number of extra samples taken and at what stage of the patient's treatment pathway.

Details of extra samples taken and where in patient treatment pathway:

Extra sample may be taken if patient synovial fluid being sampled for diagnostic purposes

Scenarios would be a patient presenting with a swollen joint when the diagnosis is uncertain. A fluid tap would be performed and part of this sample may be retained for research.

How many samples do you expect to collect per patient per week? Five

You will be required to provide information on a regular basis on the number of samples collected – this is a requirement of using the HTA Research Licence to store samples. The predicted number of samples collected each week will determine the frequency of reporting of this data.

Give the location in which these samples are stored (Campus/room number/freezer etc)

Hospital . CXH Room number 7L34

Freezer/cabinet -80ºC freezer Container labeled: Joint Fluid Study, 2nd shelf

Freezer Microbiology TWO, Microbiology lab 1005, 10th Floor QEQM, SMH

Room LG134, Freezer CSM 808, Alexander Fleming Building, South Kensington Campus

Freezer ME470/01, Room 470, Tribology Group, Mechanical Engineering, South Kensington Campus

Electronic log of samples stored via the following link:
https://docs.google.com/spreadsheets/d/1HkCys9J7t1SMJ5LLTMj4gDg6qnKNctbLc6frZNBJwGs/edit?usp=sharing

Please provide a time-line for sample collection for each patient (see attached exemplar at the end of this document)

B Data

Do you maintain your own project specific database? Yes
Do you collect further clinical data (e.g. on outcome or treatment) Yes
If yes, is this on every case/only when requested? When requested

C Access to material/data by researchers

Is access restricted only to those working within your research group/Institution? Yes
Please note – projects using material collected using the Trust consent forms for generic consent from a sub-collection must either have project specific ethics approval for use of material from NRES or have received "deemed" ethics either via the ICHTB approval mechanism. (see http://www1.imperial.ac.uk/tissuebank/usingtissue/accessingmaterials/

If No:

How do researchers gain access to the samples in your sub-collection? A

A Approval via the Tissue Management Committee of the ICHTB
B Approval via another mechanism – please specify

Do you release the following?

Tissue samples only Yes

Processed material (e.g. to RNA and DNA, tissue sections) No

D SOP information

For each of the below, please indicate whether you will use the Tissue Bank SOPs or will supply a bespoke version for your sub collection

SOP for obtaining and documenting donor consent and receipt of samples Bespoke – Joint Fluid Study – Theatre Instructions v1.0, Joint Fluid Study – Ward Instructions v1.0

SOP for disposal of samples Bespoke - (Code of Practice lab 307 containment level 2, Joint Fluid Study –Sample Storage v1.0)

SOP for transport of samples (into storage and from storage) Bespoke – (Guidelines for transport of Infectious Substances - Category B (Guidance Note: 045), Joint Fluid Study –Sample Storage v1.0)

SOP for cleaning and decontamination Bespoke - (Code of Practice lab 307 containment level 2)

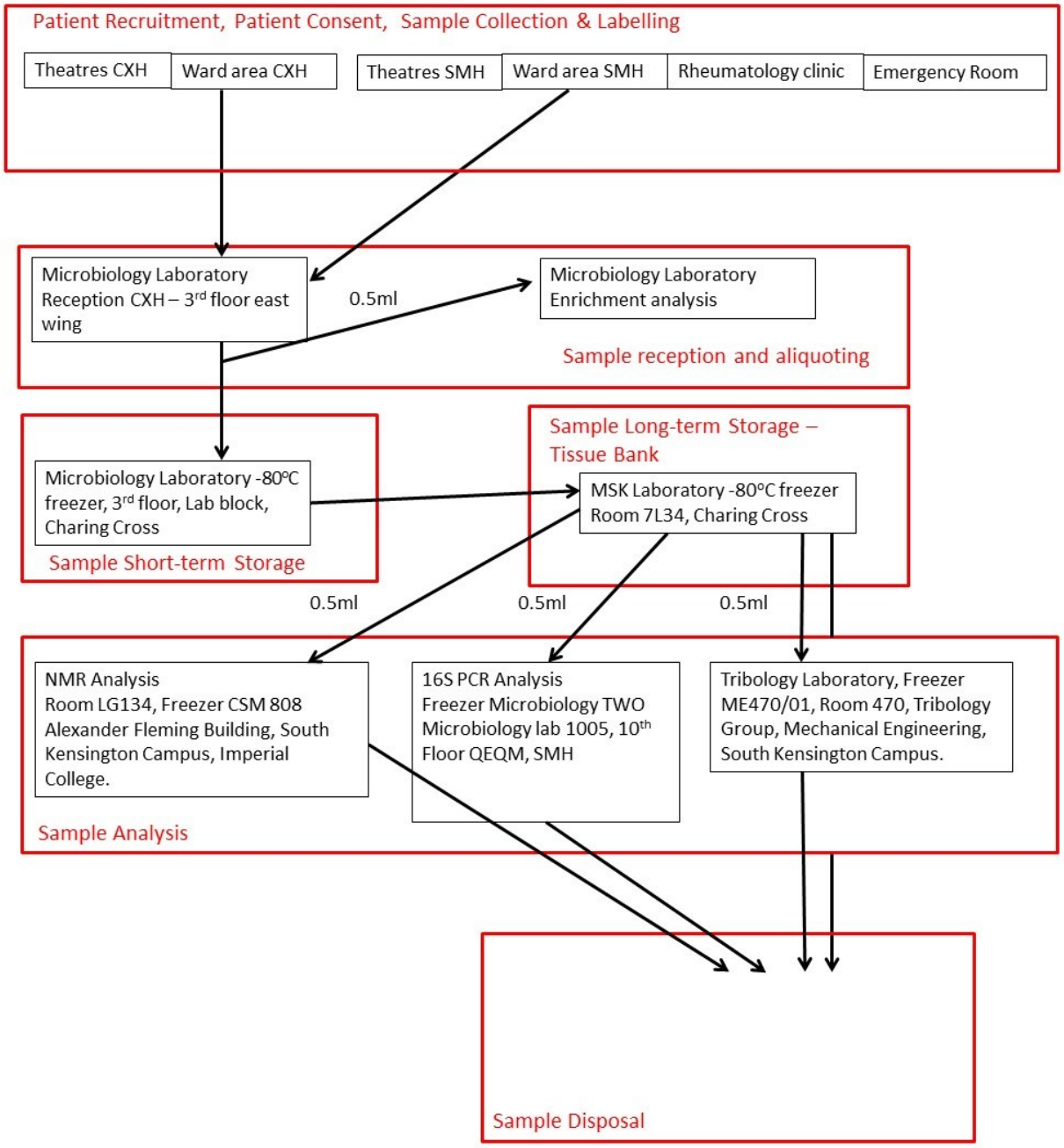
SOP for managing abnormal changes in storage temperatures Tissue Bank

SOP for recording and reporting adverse events to the DI Tissue Bank

If you elect to use the Tissue Bank SOPs, you must provide a note which should be uploaded to the files tab of your sub-collection stating the title and version of the SOP you are using.

Office Note:
Applications for use of materials from sub-collection submitted to ICHTB

Application No	Date approved	PI


Patient Recruitment, Patient Consent, Sample Collection & Labelling
Theatres CXH
Ward area CXH
Theatres SMH
Ward area SMH
Rheumatology clinic
Emergency Room
Microbiology Laboratory Reception CXH – 3rd floor east wing
0.5ml
Microbiology Laboratory Enrichment analysis
Sample reception and aliquoting
Microbiology Laboratory -80°C freezer, 3rd floor, Lab block, Charing Cross
Sample Short-term Storage
Sample Long-term Storage – Tissue Bank
MSK Laboratory -80°C freezer Room 7L34, Charing Cross
0.5ml
0.5ml
0.5ml
NMR Analysis Room LG134, Freezer CSM 808 Alexander Fleming Building, South Kensington Campus, Imperial College.
16S PCR Analysis Freezer Microbiology TWO Microbiology lab 1005, 10th Floor QEQM, SMH
Tribology Laboratory, Freezer ME470/01, Room 470, Tribology Group, Mechanical Engineering, South Kensington Campus.
Sample Analysis
Sample Disposal

12.10 Appendix 10 – Tissue bank sample storage details

Storage details of samples for OTIS disclaimer

LOCATION OF AUTHORISED COPIES	1. Q-Pulse 2. ICHTB secretariat, 11th floor lab block CXH

Related documents to this SOP :

- TB-SOP-036 – Entry of sample details for sub-collections onto OTIS

Background.

Some sub-collections have been registered with the tissue bank after the collection of many samples initially, and as such entry of each and every sample retrospectively into the OTIS database would prove difficult. Some collections also store their data in individual, separate databases, and entry into two separate systems would not be efficient.

The tissue bank and the HTA require all storage addresses of samples to be logged onto the OTIS database, and mass sample locations, existing databases and paper

12.11 Appendix 11 – Bio1 biosafety form

Imperial College London

For Safety Dept use only	Activity I.D.: v	If updated, date of first consent:	
	Consent date for this version:	CL with derogation ☐	GM Class

BIO1 FORM

PROJECT REGISTRATION AND RISK ASSESSMENT FOR WORK INVOLVING BIOLOGICAL MATERIAL



PLEASE READ THIS CAREFULLY

This form acts to register projects involving the use of Biological Agents and / or Genetically Modified Micro-Organisms, or of materials (such as cells and tissues) that may be contaminated with these agents. It also allows assessment of the hazards and risks associated with the project as well as in identifying those at risk and the measures necessary for preventing, or controlling these risks. Please ensure that sufficient detail is provided when completing this form and that the relevant written protocols are referenced where required. Note that the written protocols (e.g. Code of Practice (CoP) or Standard Operating Procedures (SOPs)) containing details of the working procedures must accompany these risk assessments at submission for work requiring Containment Level 2 or above. Once completed and approved, all risk assessments plus the protocols must be held together and supplied to all those working within this project, or who may otherwise be at risk from exposure.

Any queries during the preparation of these documents should be directed to your Safety Officer or directly to the BioSafety Team before submission. This form plus protocols should be reviewed in the first instance by your FSM/CSM and then must be submitted to biosafety@imperial.ac.uk

The work described within this Project Registration must not commence until the Principal Investigator has received a completed Biological Project Certificate.

Any changes to the work, or the persons involved, must be notified via the above email address. All changes requested must be incorporated within an amended version of this form as well as within the Code of Practice plus details of what has been changed recorded within the risk assessment change control form (Form C).

Principal Investigator	
Name: Mr Chimnay Gupte	CID: 00328445 Other employee no:
Department / Section: Department of Surgery and Cancer, Musculoskeletal Research	
Faculty: Medicine	

Details of person conducting the risk assessment	
☒ Same as PI Name:	CID: Other employee no:
Department / Section: Surgery and Cancer	
Faculty: Medicine	

The activity
Title: Investigation of the Metabolome and Microbiome of Human Joint fluid, blood and urine

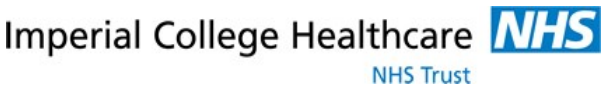
Change history *(list all previous versions of this risk assessment)*	
Date	Risk Assessment ID and version no.

The following declaration must be completed by the PI responsible for this project

- ☒ All information contained in this form is accurate and comprehensive.
- ☒ All workers involved will be instructed that their work must remain within the boundaries of this Project Registration.
- ☒ It is understood that this Registration does not comprise operating protocols or all risk assessment required for specific tasks. Such written protocols and risk assessments of individual tasks is still required. Where these must be submitted with this form is indicated within the relevant sections.
- ☒ All changes to the work covered by this form will be reassessed and the changes submitted to biosafety@imperial.ac.uk before those changes are made to the work.
- ☒ All workers have been given, or will be given before they become involved, this risk assessment, adequate training and where necessary, their competency assessed and recorded.
- ☒ All workers have, or will be before their involvement begins, enrolled with Occupational Health for health clearance where necessary.
- ☒ It is understood that this risk assessment shall not be transferred to a third party without the PI named in this form either taking responsibility for the new activities, or ensuring that a new proposal is submitted to Safety Department.

12.12 Appendix 12 – Joint fluid sample storage SOP

Looking for Biomarkers in Human Joint Fluid – SAMPLE STORAGE SOP





Principal Investigator: Chimnay Gupte

Please contact us on ……. if there are any concerns or problems

Collection:

Samples to be collected in accordance with the ethical approval and verified paperwork with the consent of the patient and treating clinician. Samples should not sacrifice any material required for clinical analysis. SOP's for the collection of samples are available:

Joint Fluid Study Ward Instructions v1.1

Joint Fluid Study Theatre Instructions v1.1

Sample Preparation:

The sample will arrive in a sterile container. All safety measures and precautions for the handling of CL2 samples should be followed *(see Code of Practice – Handling of Synovial Fluid Samples v1.1)*. All

tubes should be labelled with the 3 digit code corresponding to that research sample. Any NHS patient identifying label should on samples should be removed.

If <1.5ml sample

Taken entire sample and transfer to a 2ml Eppendorf tube

Centrifuge at 10000g for 15min

Decant sample into clean 2ml Eppendorf tubes leaving sediment pellet behind, ensuring a volume of 660µl in each tube where possible.

Store samples at -80oC

If 1.5ml>5ml sample

Decant sample from sterile container under aseptic conditions ensuring that at least 1.5ml remains in the sterile container and store at -80oC

Transfer the decanted sample to a 2ml Eppendorf tube

Centrifuge at 10000g for 15min

Decant sample into clean 2ml Eppendorf tubes leaving sediment pellet behind, ensuring a volume of 660µl in each tube where possible.

Store samples at -80oC

If >5ml sample

Decant sample from sterile container under aseptic conditions ensuring that at least 1.5ml remains in the sterile container and store at -80oC

Transfer the decanted sample to 2ml Eppendorf tubes

Centrifuge at 10000g for 15min

Decant sample into clean 2ml Eppendorf tubes leaving sedimented pellet behind, ensuring a volume of 660µl in each tube where possible.

Fill 4 x 2ml Eppendorf tubes with 660µl of sample

Fill 2 x 2ml Eppendorf tubes with 1ml of sample marking with a "T" on the lid

Any remaining volume should be placed in Eppendorf tubes in 660µl aliquots

Sample Storage:

Store all samples at -80oC immediately. Long term storage at -80oC is located on the 2nd shelf of the freezer in room 7L34 at Charing Cross Hospital Lab Block. All samples should be stored in accordance with the tissue bank sub collection guidelines.

Paperwork:

Consent forms and patient questionnaire should be filed in the joint fluid study draw,7 East Doctors office, Charing Cross.

A record of the samples should be continually updated when adding to, removing or disposing. This record is stored on the study googledrive: login jointfluidstudy@gmail.com, tissuebanksamplelog.xls

An update is required on a monthly basis to the documas system to document recruitment of patients

12.13 Appendix 13 – Joint fluid collection SOP

Looking for Biomarkers in Human Joint Fluid – STUDY PACK INSTRUCTIONS FOR THEATRE

Imperial College Healthcare NHS Trust

Imperial College London

Principal Investigator: Chimnay Gupte

Please contact us on 07904066695 if there are any concerns or problems

If this is not possible we would be grateful if you could follow the instructions below:

Pack Contents:

Patient Information Sheet – *TO GIVE TO PATIENT*

Consent Form – *PATIENT TO SIGN*

Patient Questionnaire – *OPERATING SURGEON / ASSISTANT TO PLACE PATIENT STICKER ON FRONT AND COMPLETE SECTIONS 6-8*

Instruction Sheet

Sample bag complete with transit label

Thank you for considering our study and assisting us with sample collection.

If a patient is due for theatre and you considering them for the study please do the following:

BEFORE THEATRE

Supply patient with the information sheet to read and keep

Answer any questions they may have (if you cannot answer their questions then please contact us directly).

If the patient is happy to be involved then ask them to sign the consent form (provided)

Place the pack within the notes to go to theatre with the patient

IN THEATRE

Collect synovial fluid either by arthroscopy portal or aspiration of the joint prior to performing the arthrotomy (to minimise contamination).

Place the sample into a sterile dry sample pot

Pass sample to the theatre nurses who should label it with a research sticker (contained in the pack) and place it in the specimen bag.

AFTER THEATRE

The operating or the assistant surgeon should complete the relevant information on the patient questionnaire sections 6-8 (where applicable) – this is not expected to take longer than 30 seconds

IMPORTANT: The sample pack (complete with sample) should be immediately taken to the specimen room fridge (next to theatre 10) by the theatre nursing staff. It will then be collected.

Inform researcher of patient recruitment into the study - 07904066695

If you have any queries please do not hesitate to contact us:

Or email us at: JointfluidStudy@gmail.com

12.14 Appendix 14 - Patient information sheet

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: Chimnay 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

12.15 Appendix 15 – Patient consent form

Imperial College London

Principle Investigator: Chinmay Gupte

Sponsor: Imperial College London

Looking for biomarkers in Human Joint Fluid

	Please initial
I have read the patient information sheet version 1.5 (17/04/2015)	
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……..

12.16 Appendix 16 – Patient questionnaire

Looking for Biomarkers in Human Joint Fluid

PLACE PATIENT

Imperial College London

PLACE RESEARCH SAMPLE LABEL HERE

Principle 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

"Are you known to be?" Pre-menopause

Experiencing menopause

Post menopause

"Are you Vegetarian?" Yes

No

“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)		

“What was your last meal?” ……..

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

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

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

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

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

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

Section 2 – Past Medical History

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

Section 3 – Drug History

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

"Which of the following do you suffer with?"

Osteoarthritis Yes / No

Rheumatoid Arthritis Yes / No

Psoariatic Arthritis Yes / No

Septic Arthritis Yes / No

Gout Yes / No

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

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

"Do you find great variation in the symptoms they feel on a daily basis?"

Yes / No

Is your joint ever so painful that you cannot walk/get out of bed/use the joint at all?

Yes / No

"Have you injured the joint recently?" Yes

No **go to section 5**

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

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

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

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

"Did the steroid injection relieve any symptoms and if so how long for?".....weeks

go to question 24

"Was the injection an artificial lubricant (e.g. Duralane)?" Yes

No **go to section 6**

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

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

Did the patient receive an injection today? Yes / No

Please give the drug and dosage injected today ……

Was a large joint effusion present? Yes / No

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

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

Is the patient suspected to have a septic joint? Yes

No **go to section 7**

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

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

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

What are the bearing surfaces?

Metal / Metal Metal / Poly Ceramic / Poly Ceramic / Ceramic

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

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

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

What was the reason for revision? Loosening Wear Dislocation Fracture

Was bone cement used? Yes / No

Is a hybrid fixation used? Yes

No **go to section 8**

What is the uncemented interface? Grit blasted / Hydroxyapatite

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

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

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

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)

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

12.17 Appendix 17 – Code of practice for synovial fluid handling v1.1

Code of Practice – Handling of Synovial Fluid Samples v1.1

Introduction

Synovial fluid is only to be handled by those who have received adequate training and registration as laid out in this document. Section only for handling human samples, infected with or potentially infected with hazard group 2 and 3 pathogens.

Pathogens

Human samples potentially infected with TB, Hep C, Hep B and HIV.

Personal Protective Equipment

Theatre attire

It is compulsory to wear theatre attire when collecting samples from the surgical theatres. Theatre clothes should be submitted for laundry immediate after sample collection.

Lab Coats

Wearing a Howie style lab coat is compulsory in the laboratory or when collecting samples outside the surgical theatres. Lab coats should regularly be sent for washing, in a clear autoclave bag loosely sealed with autoclave tape. Each person is provided with three laboratory coats.

Gloves

Nitrile gloves will be used to prevent skin exposure and are stored at the entrance of the lab. Disposable nitrile gloves must be changed regularly (at least every hour) and as soon as possible after a potential exposure to any hazardous agent (biological, radiological or chemical). In surgical theatres protective gloves must be worn when handling samples or any other biological material.

Safety glasses

Safety glasses or protective eye wear must be worn at all times when handling samples are not contained by a sealed container. This includes sample collection and laboratory handling.

Hand washing

Hands should be washed after removing protective clothing and before leaving the laboratory or surgical theatre.

Transportation within/between laboratories

All samples must be double contained when moving around the lab or theatre. When transporting samples between areas or to/from the freezers, samples must be carried in closed sealed at least double containers, preventing the need to wear gloves or lab coats in non-lab and non-theatre areas. See below for response to spillage during transportation.

Decontamination and Disinfection

Contaminated material to be decontaminated with Virkon (2% final concentration) for 30 minutes in the Safepak containers provided, either in the cabinet or in a designated place on the bench with closed lid, before disposal via sink or waste. Surfaces will be routinely cleaned with 70% ethanol after use.

Waste Treatment and Disposal

Liquid waste will be treated with Virkon to a final concentration of 2% and left for 30 minutes in the cabinet or in closed lid safepak containers near sink area and can be disposed through the sink after.
Contaminated solids for disposal such as pipette tips will be transferred into an autoclave or clinical waste bag (within a white bin). Material which can be closed with a cap etc. such as culture flasks can be disposed directly into the autoclave or clinical waste bag**.** White bins if 2/3 full will be closed and transported to the basement autoclave facility in SAF, where new bins are provided.
Samples in NMR tubes will be treated as sharps waste. Sharp bins will be autoclaved in yellow bins before disposal. Lids will be closed loosely with white adhesive tape and transported to autoclave facility in the SAF basement. Please ensure the exterior of the sharps bin is uncontaminated.

Spillages and Incidents

Use tissue (blue roll, Kim wipes) to take up spilled liquid. All tissues are then collected in autoclave bags. Use 70% ethanol or 1% Virkon and tissues to disinfect area. (10 minutes contact time).
After spillage in centrifuge (e.g. breakage of centrifuge tube followed by out-of-balance vibration of the centrifuge), the rotor should be left unopened for 30 mins to allow the aerosol mist to settle within the rotor. Excess sample will be taken up with tissue. Then the samples will be carefully removed, and the rotor will be taken out. Centrifuge and rotor will be cleaned with 70% ethanol. Waste tissue will be collected in autoclave bags. The same procedure applies to the bead beater.
Samples are transported in secondary containment and hence protected. If tubes break or open inside this containment, the secondary containment can only be opened within the safety cabinet and the spillage dealt with as described for spillages within the safety cabinet (see above)
Wash out with water (if eye or mouth) or soapy water (if wound or on skin). Encourage bleeding of puncture wound. Cover with sterile dressing afterwards. See Occupational Health immediately.
All incidents on South Kensington campus must be reported to the Campus Safety Manager and Ms Steffi Klier. All incidents at Charing Cross must be reported to the safety officer at the MSK lab – 7 east. An incident report form must be filled out for all unplanned events.
http://www3.imperial.ac.uk/safety/subjects/reportingaccidents (via SALUS)
Emergency contact for spillages (South Kensington): Mr Neil Chapman (mob. 07771-738927)

Storage of samples

Samples should be fully labelled with all appropriate information, and stored in a locked -80°C freezer, only accessible by a swipe card/key. Samples in freezers will be included into a sample database to track sample origin, and any potential hazards associated with it using the CSM LIMS. Freezers are audited and should any freezers fail, there is emergency freezer space.

Summary of Hazards for work as described in Bio1

Use of sharps (glass tubes for NMR and MS analysis) - Sharps bins need to be sealed when ¾ full and a pre-printed tag attached. The label must be completed by the person closing it. Potential spillages in centrifuge.

Health Surveillance

All workers handling synovial fluid must be registered with and cleared by the Occupational Health Unit.

Medical Risk Assessment

CMV: Potential risk of infection from percutaneous exposure to urine from individuals with past history of CMV infection. Risk of damaging congenital infection of fetus should mother contract primary CMV infection during pregnancy. Risk of severe disease in immune-impaired adults. Minimum infectious dose of CMV unknown but likelihood of infection being transmitted very low as transmission via blood exposure only ever reported after transfusion.

Blood borne viruses: Potential risk from percutaneous exposure.
TB: The risk of becoming infected with TB from TB-positive serum is low, as the number of circulating bacteria in a patient's bloodstream is very low. Experimental testing of serum from patients with active TB, and from patients co-infected with HIV (with high levels of active TB), demonstrated TB could not be cultured from serum when grown in Middlebrook 7H11 agar at 37°C for >3 weeks. The likelihood of bacteria being present in our serum samples is further reduced by the method of serum collection as the bacteria will sediment with the red blood cells during centrifugation when retrieving the serum. Individuals working with these samples will be required to be vaccinated using BCG.

HIV: HIV is the cause of AIDS and substantial immunosuppression can result in life threatening opportunistic infections. All individuals have been screened for HIV infection in this study and only samples originating from HIV negative individuals are to be supplied. Further reducing the risk are the low endemic rates (~2%) of this infection in the cohort studied.
Hepatitis B and C: These viruses can cause infections which affect the liver causing inflammation/liver damage and spread via infected blood and body fluid.
All individuals involved in sample preparation and analysis are required to be vaccinated against Hepatitis B. Hepatitis B risk should be low due the worker being immunised. There is no current vaccination against Hepatitis C, Hepatitis C prevalence in the UK is low but transmission requires only a small volume of inoculation and therefore, all precautions should be taken. Good laboratory practices, prohibiting the use of needles, the use of a Microbiological Safety Cabinet for sample handling, wearing gloves and lab coat and effective spillage and disposal procedures can reduce risks to the laboratory staff.

Responsible PIs

Mr Chinmay Gupte
Musculoskeletal Laboratory
Charing Cross Hospital
c.gupte00@imperial.ac.uk

Details on training, supervision and competence assessment.

All work performed with synovial fluid will have to follow approved work practices, decontamination and emergency protocols listed in the relevant Bio1.
All users need to have attended the Biosafety Foundation training, Month One Safety Training (MOST), and received a laboratory induction by Ms Steffi Klier (South Kensington) and the safety officer MSK lab (Charing Cross).
A template providing minimum requirements for training at containment level 2 is included in below. Each individual being trained by an authorized trainer needs to illustrate that they are competent in the various techniques and control measures before they are considered to be competent to work at containment level 2 unsupervised.

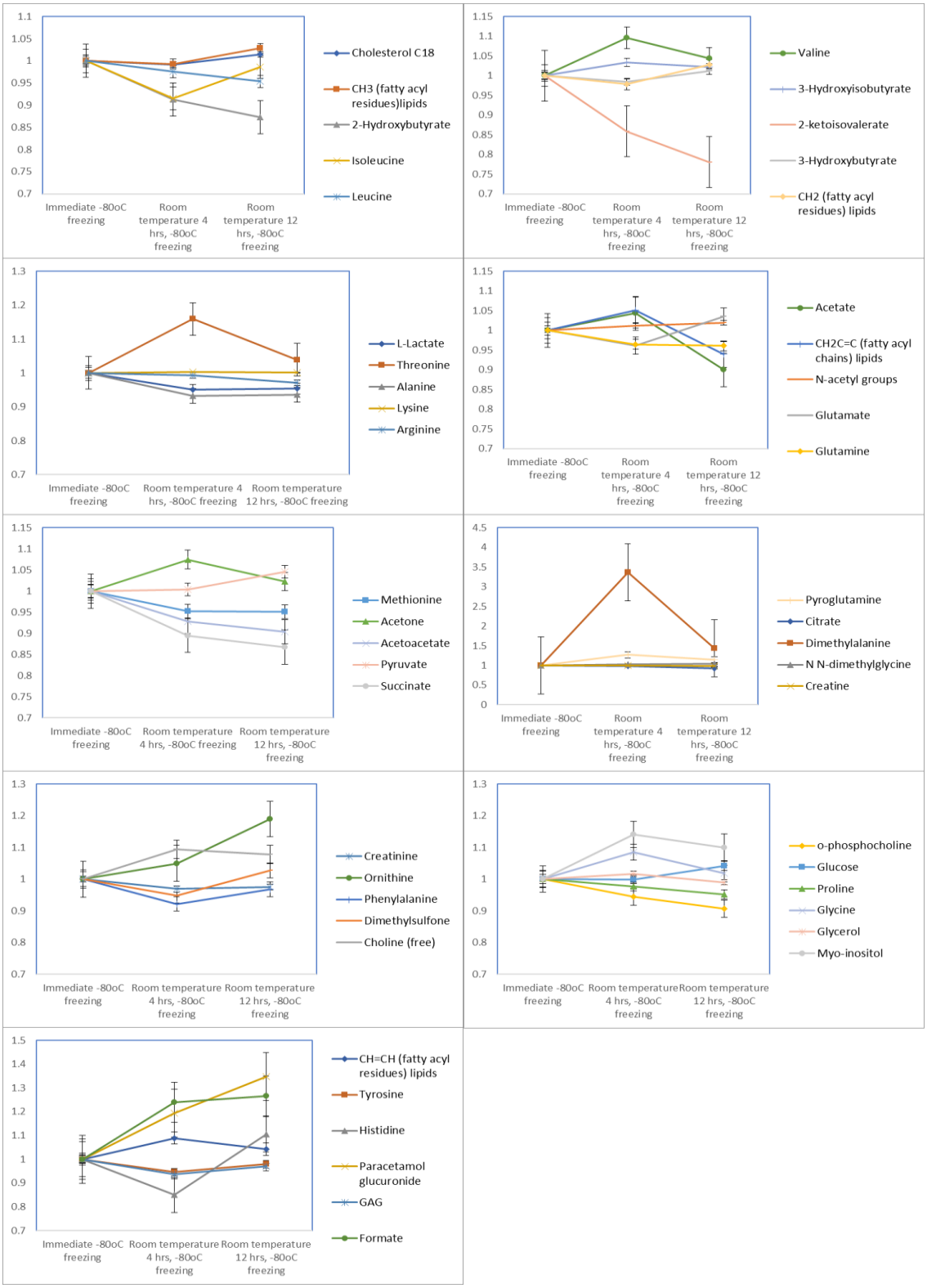
Competence requirements to work unsupervised at containment level 2

Health issues – explanation of the following to be given by the trainer, followed by description of the requirements by the trainee back to the trainer:
Issues that would preclude working with hazardous biological material (e.g. undergoing a course of antibiotic treatment, pregnancy, illness, immune deficiencies etc) Signs and symptoms of disease caused by the hazardous biological agents being used and handled Route of exposure associated with hazardous biological agents being used and handled (e.g. inhalation, ingestion, through or on the skin, splashes in the eyes etc)
Laboratory techniques – practical supervision to be given by the trainer followed by demonstration by the individual to ensure individual has appropriate level of competence and understands the control measures associated with the techniques and hazardous material:
All laboratory members are expected to observe the local safety requirements and to respect the cleanliness and maintenance of laboratory and office equipment. A minimum requirement is the maintenance of your personal safety folder to include your shadowing (laboratory training) record, a copy of the CMMI5 local rules as well as risk assessments and MSDS data specific to your research. These records must be initialled and dated as and when you have read them. Your safety folder must be available for inspection whenever required by local or Institutional safety personnel. Shadowing: This is an essential component of laboratory training. No laboratory member is authorised to perform any procedure unsupervised until such time as that procedure has been demonstrated by a named trainer. All laboratory members will keep a record of procedures they have received training in. this record will be signed by both the trainee and the trainer.
Emergency procedures - describe and then ask the trainee to explain back to the authorized trainer emergency procedures to be followed to minimise exposure to themselves and others in the event of an accident:
What to do in event of an exposure What to do in event of small spillage (< 5mls) inside and outside equipment What to do in event of large spill (>5mls) inside and outside of equipment Decontamination procedures Specific first aid procedures Reporting accidents and near misses
Disposal procedures – describe the disinfectants in use, their preparation, required contact times and routes for disposal of solid waste (autoclaves etc.). Ask trainee to demonstrate preparation of disinfectants and use of waste routes

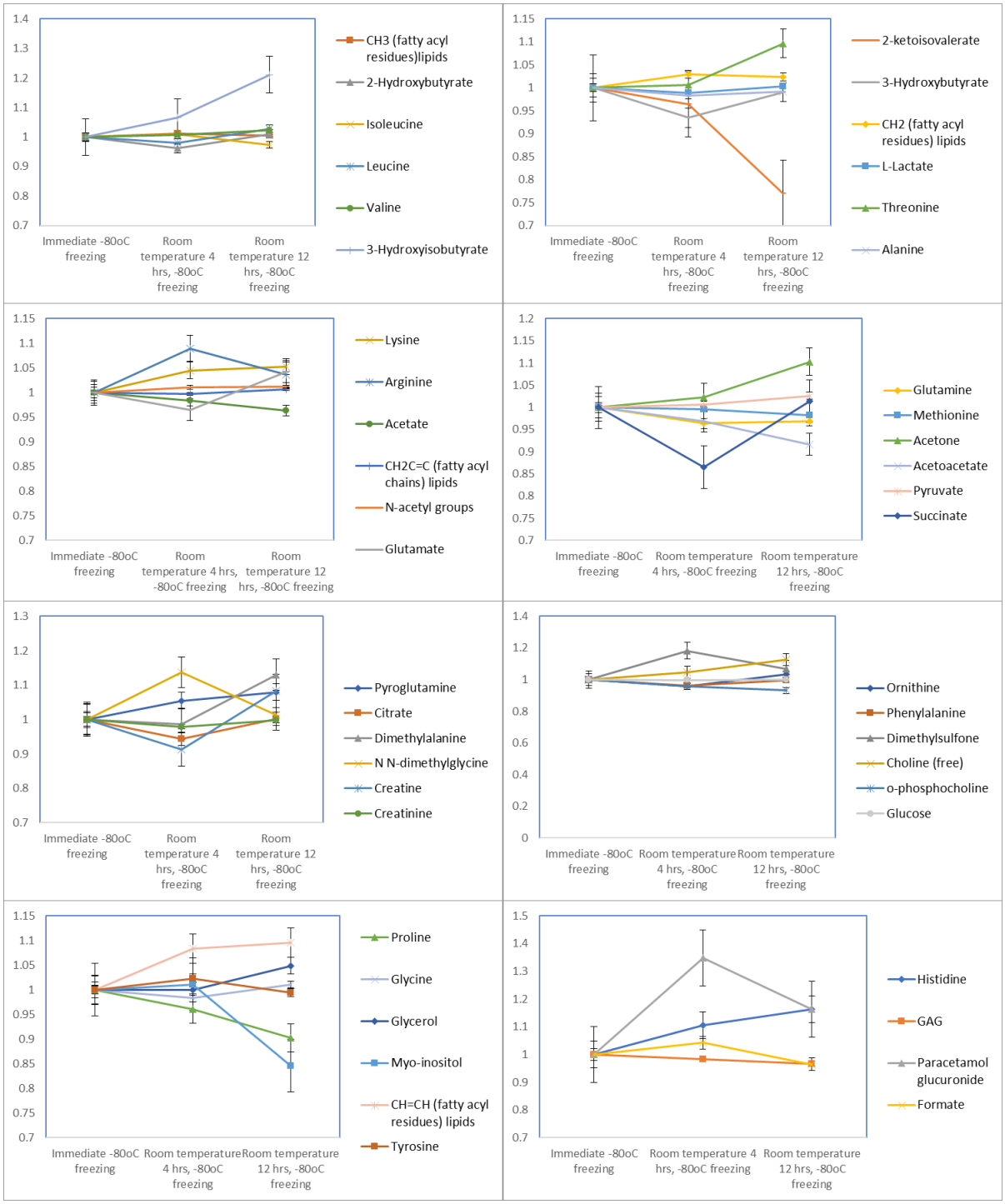
Validated disinfectants Disposal of solid waste Disposal of liquid waste

M. Jaggard March 2016

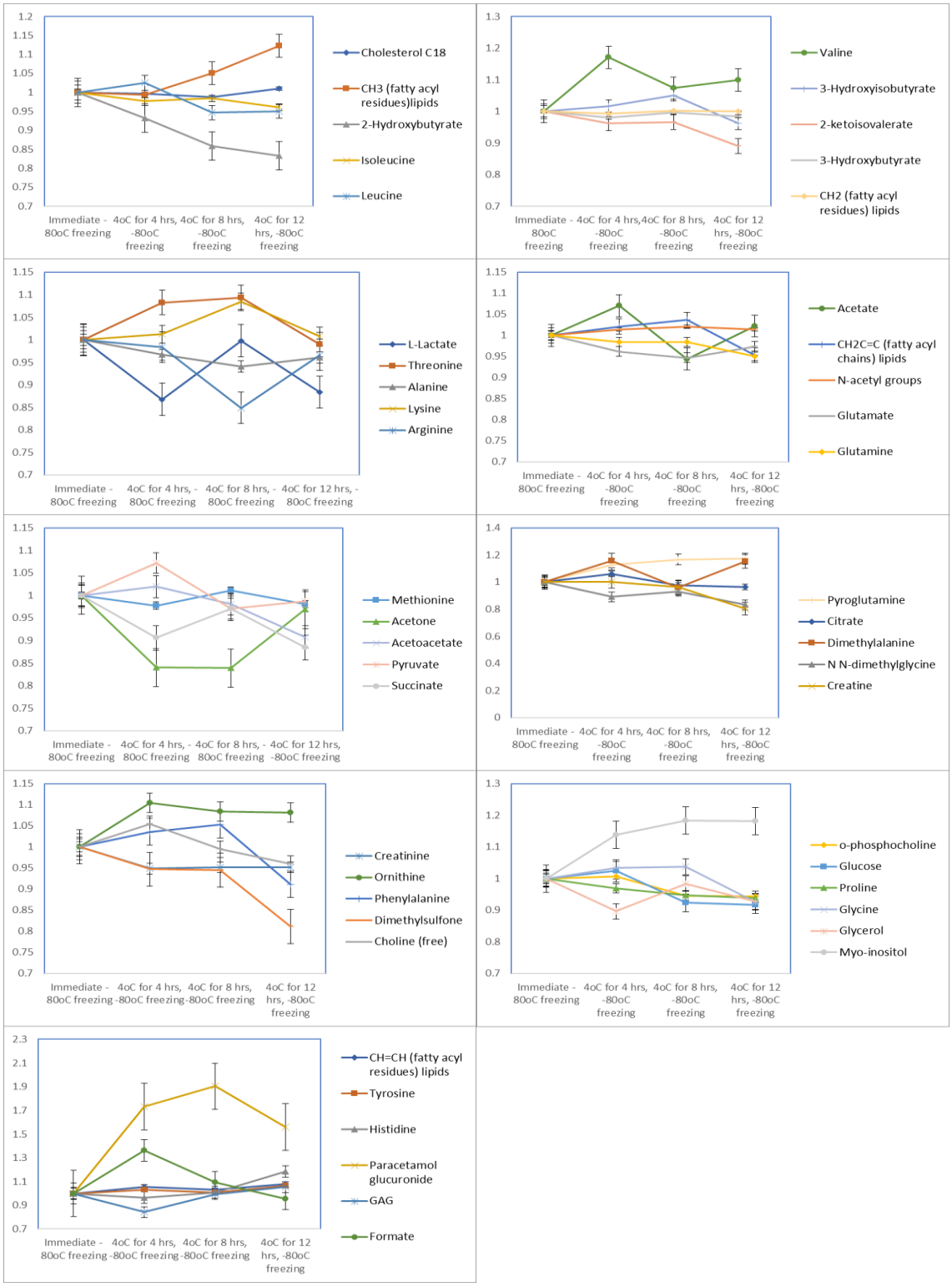
12.18 Appendix 18 – Metabolite detection in human synovial fluid by NMR methods to evaluate short-term sample storage



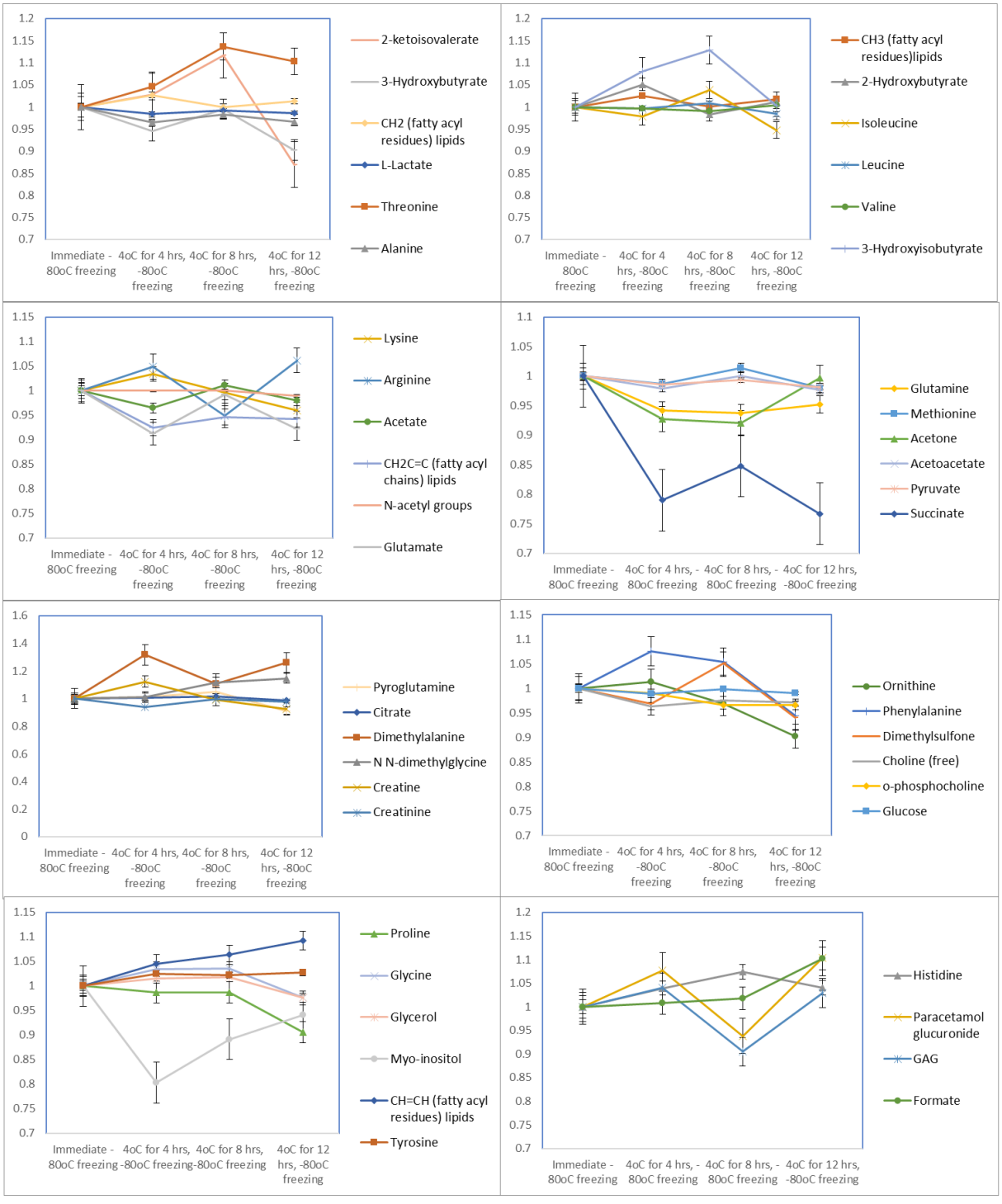
Appendix 18-1 - The change in metabolite detection within HSF by NMR noesygppr1d methods at room temperature



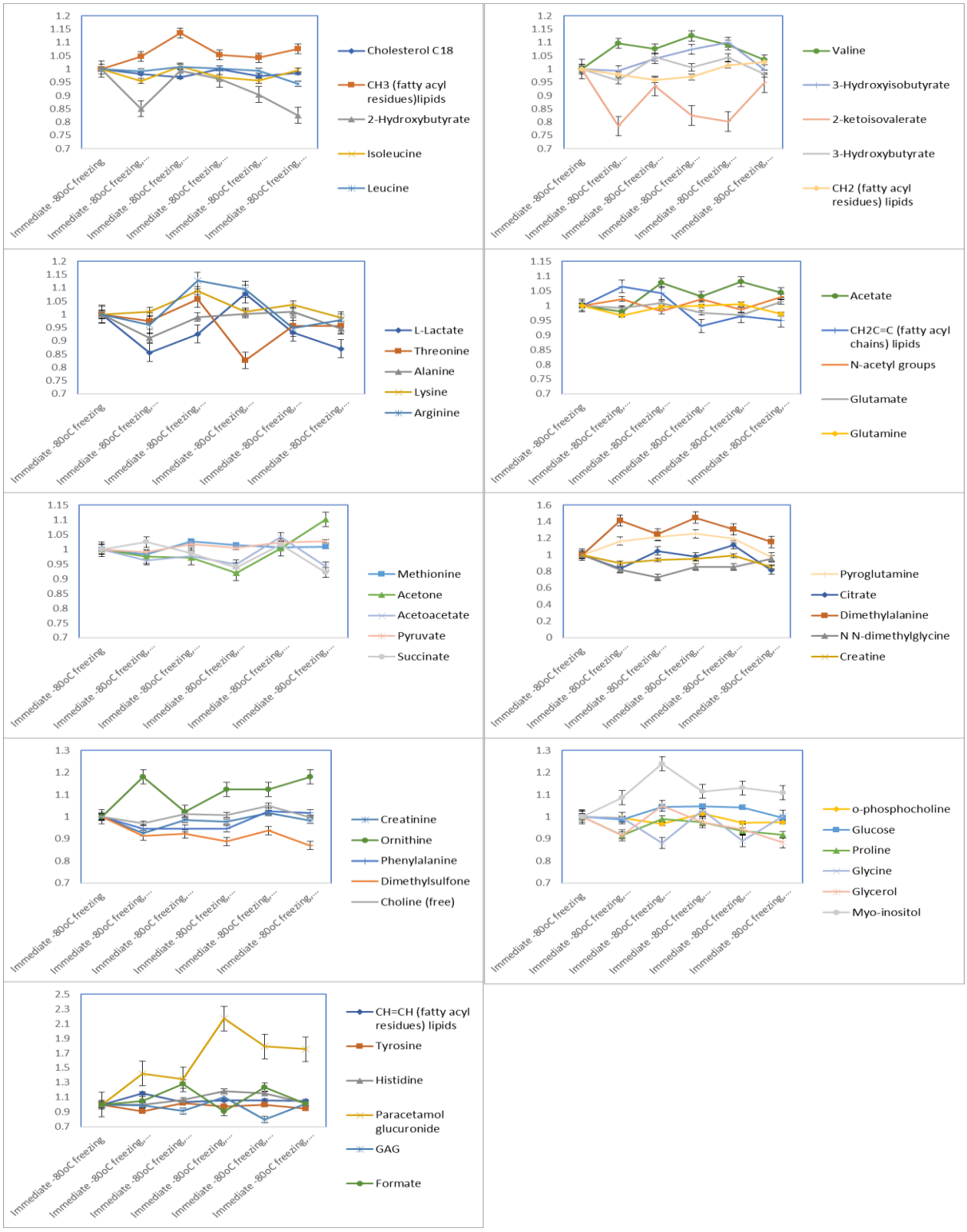
Appendix 18-2 - The change in metabolite detection within HSF by NMR cpmg methods at room temperature



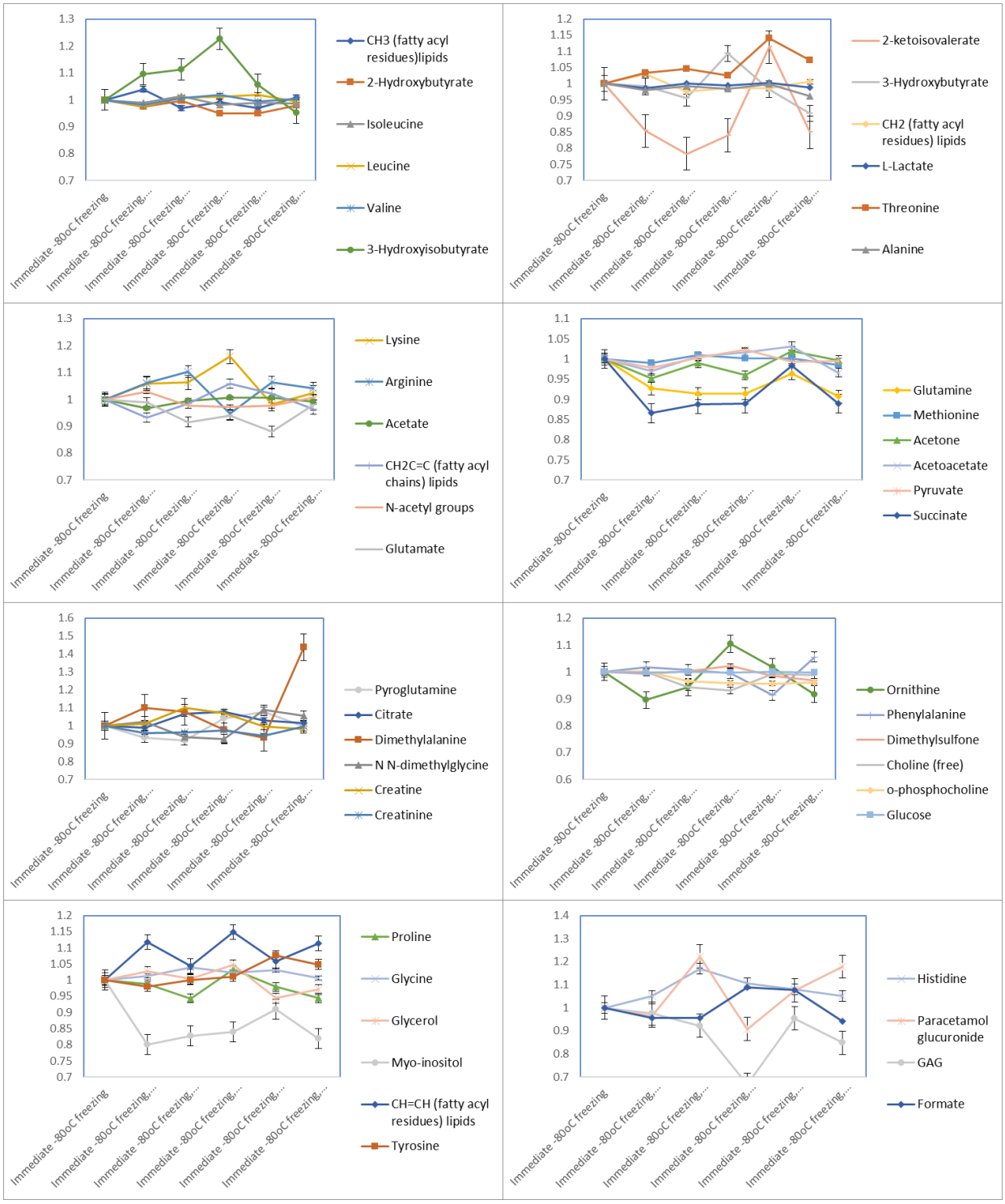
Appendix 18-3 - The change in metabolite detection within HSF by NMR noesygppr1d methods at 4oC



Appendix 18-4 - The change in metabolite detection within HSF by NMR cpmg methods at 4oC



Appendix 18-5 - The change in metabolite detection within HSF by NMR noesygppr1d methods during freeze-thaw cycling



Appendix 18-6 - The change in metabolite detection within HSF by NMR cpmg methods during freeze-thaw cycling