Biomarker Profiling of Biologic Fluids and Tissues from Horses with Induced Carpal Osteoarthritis

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Abstract

Osteoarthritis (OA) is a progressive, irreversible disease of synovial joints associated with chronic pain, which negatively impacts on the quality of life of millions of people worldwide. Similarly, OA in horses is common and has a major financial impact on the equine industry, in treatment costs, time lost to training and animal wastage.

Diagnosis of OA generally relies on clinical examination and imaging findings. However, there is a poor correlation between them, and imaging modalities have low sensitivity and specificity, and high costs. Extensive research has been conducted to identify possible molecular biomarkers of OA in early disease, stratify the likelihood of progression, and explore new interventions. Despite significant advancements in biomarker research, the adoption and validation of candidate biomarkers within a clinical practice setting is yet to be established due to the lack of consistency, the limited practicality of the tests, and the relatively high costs when used as a tool for disease surveillance. There is a need for non-invasive, cost effective, repeatable diagnostic and screening tests with high sensitivity and specificity for early disease diagnosis. The primary objective of the body of research presented in this dissertation was to investigate alternative economical, and potentially more accurate techniques for the determination of biomarker profiles of equine OA.

An established surgical and exercise model was used in this study to induce carpal OA in nine of seventeen young female Thoroughbred horses. Weekly blood and synovial fluid samples were collected for 9 weeks after induction of OA and joint tissue samples were collected at the end of the study.

The first study (Chapter 3) assessed the use of cell-free DNA (cfDNA) as a potential biomarker of OA. The results showed that cfDNA concentrations were significantly higher in synovial fluid

from horses with OA than in the control group at 4 and 9 weeks. Conversely, cfDNA concentrations in plasma did not significantly differ between groups. It was concluded that plasma cfDNA measurement is not sufficiently sensitive for early diagnosis of OA, while measurement of this biomarker in joint fluid may be useful.

The second and third studies (Chapters 4 and 5) investigated the use of infrared (IR) spectroscopy techniques to determine the serum and synovial fluid biomarker profiles of the same group of horses with induced OA and controls. Although serum IR spectroscopy profiles were not significantly different between groups, this technique showed good accuracy when used with synovial fluid.

Raman spectroscopy (Chapter 6) was used to assess the biochemical profiles of cartilage and bone samples obtained from the same cohort of horses. This technique successfully identified clear differences between articular cartilage and bone but failed to detect significant differences between diseased and healthy joints for both cartilage and bone.

Overall, the results of these studies showed that with the described model of equine OA, the techniques and methodologies used proved more useful to identify biomarker profiles of horses with OA when used on synovial fluid compared with blood (serum or plasma). This suggests that biomarkers concentrations (cfDNA) and profiles (IR signals) are excessively low in the peripheral circulation for the detection threshold of these techniques, and that more invasive methodologies of sampling (arthrocentesis) are the preferred method for this model of OA. Raman spectroscopic analysis of tissues failed to detect significant differences in spectroscopic profiles between groups, possibly due to the nature of the OA model used, with a relatively short duration and the potential confounding effect of exercise and lameness.

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List of abbreviations

ac	Articular cartilage
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
BAP	Bone specific alkaline phosphatase
BIPEDS	Burden of disease, Investigative, Prognostic, Efficacy of intervention, Diagnostic, Safety
BK	Bradykinin
BML	Bone marrow lesions
BSP	Bone sialoprotein
CCD	Charge-coupled device
cfDNA	Cell-free DNA
cm	Centimetre
COMP	Cartilage oligomeric matrix protein
COX-2	Cyclooxygenase 2
CPII	C-propeptide of type II procollagen
CRP	C-reactive protein
CS	Chondroitin sulphate
CS-846	Epitope 846 of chondroitin sulphate
CT	Computed tomography
CTX-I	Type I collagen C-telopeptides
CTX-II	C-terminal cross-linked telopeptide of type II collagen
CV	Coefficient of variation
DJD	Degenerative joint disease
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMEA	European Agency for the Evaluation of Medicinal Products
FDA	Food and Drug Administration
FTIR	Fourier transform infrared
GAG	Glycosaminoglycan
GCMS	Gas chromatography mass spectrometry
Glc-Gal-PYD	Glucosyl-galactosyl pyridinoline

H&E	Haematoxylin and eosin
HA	Hyaluronic acid
HMGB-1	High-mobility group box-1
ICTP	Type I collagen non-helical telopeptide
IGF	Insulin-like growth factor
IGFBPs	Insulin-like growth factor binding proteins
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Infrared
IV	Intravenous
JSN	Joint space narrowing
JSW	Joint space width
kg	Kilogram
KL	Kellgren-Lawrence
L	Litre
LCMS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharide
m	Metre
mg	Milligram
MIR	Mid-infrared
miRNAs	MicroRNAs
mL	Millilitre
MMPs	Metalloproteinases
MOAKS	MRI Osteoarthritis Knee Score
MRI	Magnetic resonance imaging
MUAEC	Massey University Animal Ethics Committee
mW	Milliwatt
NIR	Near-infrared
nm	Nanometre
NMR	Nuclear magnetic spectroscopy
NO	Nitric oxide

OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OC	Osteocalcin
OOB	Out-of-bag
PCA	Principal component analysis
PET	Positron emission tomography
PG	Proteoglycans
PGE2	Prostaglandin E2
PICP	Procollagen I carboxy terminal propeptide
PIIANP	N-propeptide of type IIA procollagen
PINP	Procollagen I amino terminal propeptide
PLSDA	Partial least squares discriminant analysis
PO	Per os
RA	Rheumatoid arthritis
RS	Raman spectroscopy
SAA	Serum amyloid A
sc	Subchondral bone
sec	Second
SF	Synovial fluid
SNV	Standard normal variate
SP	Substance P
TA	Traumatic arthritis
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases
TNCC	Total nucleated cell count
TNF	Tumour necrosis factor
uCTX-II	Urinary CTX-II
US	Ultrasonography
WHO	World Health Organization
WN	Wavenumber
WORMS	Whole Organ Magnetic Resonance Imaging Score

YKL-40	Human cartilage glycoprotein-39
µm	Micrometre

Chapter 1
General Introduction

Osteoarthritis (OA) is a progressive disease of synovial joints characterised by failure of damage repair [1]. Although originally it was thought to be a degenerative condition of cartilage, it is now recognised that it has a strong inflammatory component involving a multitude of joint tissues including subchondral bone, periarticular ligaments, menisci, periarticular muscles, nerves, synovium, or a combination of the above [2-4]. The main hallmarks of OA are progressive, irreversible cartilage breakdown, along with subchondral bone sclerosis and demineralisation [5-7]. It is a painful, chronic, slowly progressive, and debilitating condition affecting a large proportion of the human population and is becoming a global issue due to the growing world population and overall increasing life expectancy. It has a significant negative impact on the physical and mental health of millions of people and has become a major social and financial burden [8-13].

Musculoskeletal injuries and lameness in horses are common and are associated with reduced performance, loss of training days and horse wastage [14, 15], and OA is responsible for the majority of lameness cases [16, 17]. OA affects millions of horses worldwide and has a major financial impact on the equine industry due to direct and indirect costs [18, 19]. It is clear that OA represents an important economic burden for the equine industry worldwide and that further conclusive research on this topic is needed.

There is currently no cure for OA and the treatments available are palliative, and these treatments may ameliorate the clinical signs or at best only slow down the progression of disease [20, 21]. Diagnosis of OA is usually based on clinical examination and imaging, with radiographic assessment still the gold standard, despite the recent development of advanced

imaging techniques [22-24]. Common clinical signs of OA are pain and joint stiffness, while radiographic features of OA include joint space narrowing, subchondral bone sclerosis and cysts, osteophyte formation, and altered epiphyseal shape [23, 25, 26]. However, imaging techniques for OA have several limitations such as poor correlation between clinical signs of pain and imaging findings [27], low sensitivity and specificity, and high costs [24, 28]. Unfortunately, OA is often diagnosed in an advanced stage of disease when the tissue changes are significantly advanced. Early diagnosis of OA is crucial for early intervention in order to slow down the progression of disease in clinical cases or to prevent further damage in subjects at risk [25]. Moreover, to stratify the severity of disease and its likelihood of progression, and to investigate new therapeutic approaches there is a need to identify scientifically robust biomarkers of OA [29-31].

In the last few decades a large body of work has been undertaken on molecular biomarkers of joint metabolism and pathology in people to identify potential markers of OA [32-36]. Similarly, significant progress in OA biomarker research has been made in horses using serum and synovial fluid [37-45]. Despite the advancements in research [46, 47], these have not translated into a wider adoption and validation in clinical practice of candidate biomarkers [35]. The reasons for this include poor practicality and high costs of the tests used, and the inconsistency of results when these are used as a screening tool for disease surveillance [48]. With the commonly available tools, making an early diagnosis of OA and monitoring its progression and response to therapy continues to remain a challenge; therefore, practical, non-invasive, cost-effective, and reliable tests and methodologies are needed [49].

The aim of this dissertation is to investigate alternative, economical, and potentially more accurate techniques for determination of biomarker profiles of equine OA. The following

chapters describe approaches and techniques for identification of OA in horses that could potentially translate into clinical use as tools for early diagnosis of OA, and ultimately as screening tools for disease prevention and moderation. These encompass the exploration of direct (e.g., synovial fluid) and indirect (e.g. blood) biomarkers, as well as giving consideration to those that may be associated with responses at the biochemical, cellular (e.g. cell-free DNA) and tissue levels (e.g. cartilage and bone). The objective of the first study (Chapter 3) was to investigate the use of cell-free DNA concentration in synovial fluid and plasma of horses as a potential early biomarker of OA. This was a novel study as there was no published literature on the concentrations cell-free DNA in normal horses (prior to intervention in our study) or horses with OA. The objectives of second and third studies (Chapters 4 and 5) were to determine the feasibility and accuracy of IR-based biomarker profiling of serum and synovial fluid respectively to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal OA from controls. Finally, in the last study (Chapter 6) the objective was to assess the use of Raman spectroscopy to differentiate the spectral profiles of subchondral bone and articular cartilage of horses with induced carpal OA from controls.

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Chapter 2
Literature Review

2.1 Joint Anatomy and Physiology

Joints are classified into three main categories based on their structure: fibrous joints, cartilaginous joints, and synovial joints. Synovial joints are characterised by the presence of a hyaline cartilage cover on the opposing bones of the epiphyses that make up the joint. These types of joints are filled with a viscous liquid called synovial fluid, which facilitates gliding of the opposing cartilaginous surfaces [1, 2]. Synovial joints are mainly located in the appendicular skeleton and are the main subject of osteoarthritis (OA) research. For the purpose of the following description joints are intended as synovial joints. Fibrous and cartilaginous joints will not be discussed as they do not share the same pathophysiology of disease as synovial joints.

The main components of joints are articular cartilage, subchondral bone, joint capsule, and synovial fluid.

2.1.1 Articular Cartilage

Hyaline cartilage has a number of characteristics that make it a unique tissue, such as no direct blood supply in the adult, no nerve endings, and no lymphatic vessels [3]. It is predominantly composed of water (70-80%) with the remainder of the dry matter being extracellular matrix (ECM). Collagen is the predominant protein in the ECM (50%), followed by proteoglycans (PG, 35%), glycoproteins (10%), minerals (3%), lipids (1%) and miscellaneous components (1%). The cellular component (chondrocytes) varies from between 1% and 12% of the volume [2].

Morphologically hyaline cartilage is divided into four zones defined as superficial, intermediate, deep and calcified. The deep zone is separated from the calcified cartilage by a tidemark. Each zone is characterised by different ratios of the main cartilage components and by variable orientation of collagen fibrils and chondrocytes [2, 3].

The main collagen in cartilage is type II and this is organised into fibrils. These fibrils are made up of three proteins (α-chains) which interweave in a triple helix configuration. The collagen triple helices are synthesised intracellularly as propeptides which are then cleaved extracellularly at the amino- and carboxy- terminals to form the tropocollagen molecule [4]. Aggregation of several tropocollagen molecules via cross-links forms collagen fibrils [2]. Collagen fibrils are arranged in three-dimensional arcades to provide mechanical strength to cartilage [5].

Proteoglycans are monomers consisting of a core protein with attached glycosaminoglycan chains, the most abundant of which are chondroitin sulphate and keratan sulphate. These are negatively charged which causes them to attract water creating an osmotic gradient, a crucial mechanism for articular cartilage function [6, 7]. The major proteoglycan is called aggrecan and is bound to hyaluronan (hyaluronic acid). Hyaluronan is a non-sulphated glycosaminoglycan and a major component of cartilage and synovial fluid.

The cellular component of articular cartilage consists of chondrocytes which are of mesenchymal origin and are considered a form of differentiated fibroblasts. They are present in small numbers within the ECM and their number appear to vary inversely proportional to the size and age of the species [8]. Their morphology also differs significantly between zones, with deeper chondrocytes being round-shaped and more metabolically active, and superficial ones having a more flattened appearance. Chondrocytes produce the ECM and are involved

in local homeostasis. They are located in lacunae within the ECM and, due to the large intercellular distance they are not connected with one another [9, 10].

2.1.2 Subchondral Bone

Subchondral bone consists of a thin layer of compact bone in direct contact with the calcified cartilage layer on one side, and trabecular bone on the opposite side. Compact bone provides stiffness and supports the overlying cartilage, while trabecular bone which is more deformable and elastic, provides some degree of shock absorption thus contributing to load distribution and adaptation to exercise [2, 6]. Subchondral bone has been shown to be ten times more deformable than cortical bone in the shaft of long bones [11, 12]. Bone is able to remodel based on the loads and stimuli that it receives according to the Wolff's law, with bone resorption occurring when loads decrease, while bone formation is associated with increased loads [13]. According to the more recent mechanostat model, bone adaptation depends on the amount of strain a bone is subjected to and its intrinsic strength, as well as other factors such as hormones, nutrition, nervous system, drugs and environment [14]. Thickening and architectural changes of the subchondral bone ensue when increased loads are applied (i.e., exercise or repetitive trauma) and this leads to changes in joint biomechanics. A stiffer subchondral plate reduces shock absorption and increases the load on the articular cartilage. Stiffening and sclerosis of subchondral bone is found in association with osteoarthritis (OA), and it is speculated that it may be an inciting cause rather than a consequence of OA as originally thought [12, 15]. It is likely that cartilage degeneration and changes in the subchondral bone are intimately related and affect one another in the early stage of disease [16].

2.1.3 Joint capsule

The function of the joint capsule is to stabilise the joint passively through its stiffness, and actively via proprioceptive and nociceptive stimuli as well as to provide a closed space around the articulating bones, synovium, and synovial fluid [17]. The outer layer of the joint capsule consists of fibrous tissue and is often in tight connection with periarticular ligaments and tendons. The inner layer, called synovium or synovial membrane, is composed of a subintimal and an intimal layer [18]. The subintima is composed of loose connective tissue and is rich in nerve endings and vessels. The intima, which is in direct contact with the joint cavity, is a thin layer of cells (synoviocytes) and is characterised by the absence of a basement membrane. This peculiarity facilitates fluid exchange between the blood and the synovial fluid. Synoviocytes are classified as type A or B. Type A synoviocytes are macrophage-like cells with predominantly phagocytic and antigen-processing functions [19]. Type B synoviocytes are fibroblast-like cells responsible for the production of hyaluronan and proteins. The existence of a third type of synoviocyte called type C has been postulated, but this appears to be a transitional form between A and B types, rather than a distinct one [2, 20].

2.1.4 Synovial Fluid

Synovial fluid is a dialysate of blood plasma with additional substances, in particular high concentrations of hyaluronic acid; this polymerises with proteins to form a complex that acts as a viscous lubricant [21]. Normal synovial fluid is a straw-coloured, clear fluid with a very low cellularity consisting mainly of mononuclear cells. As adult cartilage is not vascularised, the supply of nutrients and waste removal rely on diffusion from the synovial fluid. The exchange of molecules between the blood plasma and synovial fluid is facilitated by the absence of a basement membrane in the synovium and is dictated by hydrostatic and colloid osmotic

pressure differences between the two compartments. This is also affected by pressure changes during locomotion and by the sub-atmospheric pressure of joints [22, 23].

2.1.5 Pathophysiology of Osteoarthritis

The Osteoarthritis Research Society International (OARSI) consensus defines OA as a progressive disease of synovial joints due to failure of damage repair. This can be caused by abnormalities either in the articular cartilage, subchondral bone, periarticular ligaments, menisci, periarticular muscles, nerves, synovium, or a combination of the above. The end result is an irreversible breakdown of articular cartilage and alterations in subchondral bone [24].

Gross and histological similarities between degenerative arthritis (nowadays referred to as OA) in humans and horses were noticed in the late 1930s, with the only difference that the severity of disease seemed to be greater in humans compared to horses because the latter were generally euthanised before changes became severe [25]. From a clinical standpoint, the main difference between the species appeared to be the fact that the clinical signs of disease become manifest in horses with less advanced lesions than those observed in people.

Extrapolation from human literature has been commonly used when classifying equine OA and this has led to use the terms “primary OA” and “secondary OA”. Primary OA indicates the disease for when a specific cause is not evident, while secondary OA is used when a cause is identifiable [26, 27]. Primary OA has also been used to refer to degenerative joint disease (DJD). The distinction between the two forms though is not always clear and for a time DJD was used to indicate any type of OA [28]. Nowadays OA is favoured as the preferred terminology since DJD does not account for the inflammatory nor the regenerative components of the disease [29].

Osteoarthritis is a complex disease process that can be triggered by multiple causes including septic arthritis, osteochondrosis or trauma. Trauma often plays an important role as an inciting cause. This can occur in the form of a single event or as repetitive episodes that can lead to tissue fatigue and eventually to traumatic arthritis (TA) [30]. Synovitis, capsulitis, intra-articular sprain, intra-articular fractures, and meniscal tears are all forms of TA which may lead to OA [30]. The two main mechanisms of joint injury that have been broadly identified are either abnormal loading on normal cartilage or normal loading on abnormal cartilage [31].

The role of synovitis in the pathophysiology of OA was demonstrated using a model of synovitis in horses where cartilage degradation occurred in the absence of joint instability or trauma [32]. Capsulitis and synovitis, common findings in equine athletes, cause a release of anabolic and catabolic enzymes, inflammatory mediators and cytokines which can lead to cartilage degradation [33]. Interleukin (IL)-1 is one pro-inflammatory cytokine which plays a major role in cartilage breakdown, interferes with the activity of growth factors, and decreases the synthesis of ECM [34, 35]. Synoviocytes cultured *in vitro* and stimulated with extracts of IL-1 can release high concentrations of proteolytic enzymes, such as metalloproteinases (MMPs) [36]. The MMP activity in joints with OA has been found to be twice as high as in normal joints, due to the role of MMPs in the process of matrix destruction [37]. Within the MMP family three collagenases have been identified, specifically MMP-1, MMP-8, MMP-13, the latter of which is the main collagenase responsible for breakdown of collagen II in the articular cartilage [38, 39]. The MMPs are regulated by four tissue inhibitors of metalloproteinases (TIMPs), in particular by TIMP-1 and TIMP-2, and imbalance between MMPs and TIMPs in favour of the former was found in cartilage of human patients affected by OA [40]. High levels of TIMP-1 and their variation over a one-year period were correlated with lack of disease progression in patients with hip OA [41]. Stimulation of canine chondrocyte

cultures with IL-1 β was shown to cause downregulation of TIMP-2 [42]. Other catabolic enzymes known as aggrecanases also contribute to the process of cartilage degradation targeting aggrecan molecules, in particular the aggrecanases A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) -4 and -5 [43-45]. Another pro-inflammatory cytokine thought to play an important role in OA is IL-6, which has been found in high concentrations in serum of people with knee OA [46, 47], and in cartilage from patients undergoing knee replacement surgery [48]. Experimental studies in mice showed that IL-6 stimulation on chondrocyte cultures induced lower glycosaminoglycan (GAG) content in ECM and higher GAG release in the culture medium [49]. Interleukin-6 also upregulates MMP-3, MMP-13, ADAMTS-4 and -5, thus leading to cartilage breakdown [49].

Other cytokines that modulate MMPs and TIMPs include transforming growth factor (TGF) – β, insulin-like growth factor (IGF) – 1 and tumour necrosis factor (TNF) – α [29]. The role of growth factors in the pathophysiology of OA has not been fully elucidated. In fact, although they are keys molecules in growth and homeostasis of normal cartilage, their synthesis increases with inflammation in early stages of OA before cartilage damage occurs [50]. Repeated intra-articular injections of TGF – β have been associated with OA-like signs in both the articular cartilage (proteoglycan loss) and bone (osteophyte formation) [51, 52]. TNF – α is a pro-inflammatory cytokine with a crucial role in matrix degradation by stimulating release of proteolytic enzymes from synovial fibroblasts and chondrocytes [53]. TNF – expression has been found to be increased in synovium and cartilage from equine joints with naturally occurring OA, while IL-1 β expression was increased in cartilage, but not synovium [54]. Once the inflammatory process has been initiated, a cascade of cytokine release results in an imbalance in the MMP to TIMP ratio, with the former being predominant in OA joints [40]. The ensuing effect is an increase in cartilage breakdown. The central role of IL-1 in the development of OA

was shown by its inhibition using a model of equine OA [55]. While TNF – α is considered the main cytokine in the acute phase of OA in people, IL-1 β remains increased in all stages of disease [56]. Other molecules involved in the pathophysiology of OA are prostaglandins, in particular E_2 (PGE_2). In fact, these have been associated with inflammation and pain in OA joints and contribute to proteoglycan loss in the ECM [57, 58]. Oxygen-derived free radicals such as superoxide anion, hydroxyl radicals, and hydrogen peroxide have also been shown to cause cleavage of hyaluronic acid [59, 60] and proteoglycans [61], and degradation of collagen [61]. IL-1 β and TNF are able to induce production of free radicals [62], but also downregulate their scavengers superoxide dismutase, catalase, and glutathione peroxidase, thus potentiating the catabolic effects of free radicals on cartilage [63, 64].

The importance of synovitis and capsulitis has only recently been recognised as one of the main factors in the pathogenesis of OA, but another important component to the disease process is the role of subchondral bone [65, 66]. Sclerosis of subchondral bone is often encountered in joints affected with OA and it has been suggested that loss of the normal bone elasticity and its consequent shock-absorption properties places the articular cartilage at greater risk of stress, which may predispose to failure [12, 15, 67]. Although subchondral bone sclerosis was previously thought to be secondary to cartilage damage [50], it can occur simultaneously or even precede cartilage lesions as shown in a dog [68] and horse [67] model of OA, respectively. Although the body of literature supporting the fact that increased bone density is associated with joints affected with OA is extensive, there is also evidence that osteoporosis and decreased bone modulus increases the chances of developing OA [69]. Exercise in horses is known to cause an increase in subchondral bone density in the metacarpal condyles [67, 70], radial carpal and third carpal bones [71] in association with the presence of microfractures (microcracks) and osteocyte death [67, 70]. In equine athletes,

articular cartilage lesions in the distal row of carpal bones were almost exclusively associated with bone sclerosis, and there was good correlation between the extent of cartilage lesions and the degree of sclerosis [72]. It is therefore likely that a combination of several repetitive events, rather than a single one, is at the base of the pathophysiology of OA in this case.

2.2 Biomarkers of Osteoarthritis

The National Institutes of Health Biomarkers Definitions Working Group of the United States of America defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [73]. For OA, there are two classes of biomarkers that have been described: "dry" and "wet". Dry biomarkers refer to imaging parameters such as radiography, magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, and nuclear scintigraphy. In contrast, wet biomarkers are soluble and include DNA, RNA, carbohydrates, proteins, peptides, and metabolites and are measurable in biological fluids [74]. Biomarkers can also be classified as "direct" and "indirect". While direct biomarkers reflect known anabolic and catabolic processes, indirect biomarkers are not produced by normal tissue synthesis or breakdown (although they can influence tissue turnover), instead, they are generated by trauma or disease [75].

Biomarkers of OA have also been classified based on the tissue of origin or their potential use. The classification system based on their potential use was first proposed by Bauer *et al.* (2006) and was identified with the acronym BIPED [76]. Specifically, each letter of the acronym stands for a category: **B**urden of disease, **I**nvestigative, **P**rognostic, **E**fficacy of intervention, and **D**iagnostic.

Burden of disease markers are markers that provide information on the extent or severity of OA at a point in time, without reference to the socioeconomic impact [76]. These markers are generally assessed against a gold standard test used to evaluate OA severity and are established using cross-sectional data of affected individuals. Investigative markers include those that cannot be classified into any other categories and require further research to elucidate their potential role. Prognostic markers encompass those markers that can provide information on either the onset or the progression of OA in healthy or affected individuals, respectively. Assessment of these markers requires prospective or retrospective longitudinal studies, and their variables can be continuous, dichotomous, or categorical. Efficacy of intervention markers are markers that are indicative or predictive of treatment efficacy and are assessed using clinical trials for OA, generally the outcomes would be continuous or dichotomous (e.g., improvement of symptoms or based on imaging). Finally, diagnostic markers are those that can differentiate diseased from non-diseased individuals and are assessed against a gold standard, such as radiographs. These markers must be evaluated in studies including both healthy and affected individuals [76].

Subsequently, Kraus *et al.* (2011) added an additional category to this classification system to include **S**afety, thus renaming the system BIPEDS. Safety biomarkers provide information on toxic effects on organs and/or tissues caused by an intervention or an agent, with the goal of assessing potential adverse effects of a treatment and safe therapeutic dosages [77].

The following review of biomarkers of OA is in accordance with the classification based on the tissue of origin. The scientific literature on this topic over the last few decades is extremely vast and comprises thousands of publications encompassing *in vitro* studies, animal model studies and clinical research. For the purpose of reviewing the literature relevant to the

author's body of work in this thesis, a limited and selected portion of the literature on the topic has been included. Therefore, only the biomarkers more consistently studied and with the most supporting evidence have been included. This review is not meant to encompass all the biomarkers reported in the literature.

2.2.1 Imaging biomarkers of Osteoarthritis

Due to its widespread availability, limited cost, ease of use, short testing time, relatively low impact and high tolerance by patients, conventional radiography (X-rays) is considered the gold standard imaging technique for OA diagnosis despite the development of newer imaging technologies in the last decade [78, 79]. The main signs of OA detected by radiography are osteophytes, joint space narrowing (JSN), subchondral bone sclerosis, subchondral cysts, and altered shape of bone ends. The most widely used grading scale to assess OA severity based on these features is the Kellgren-Lawrence (KL) scale, which rates these features from 0 (normal) to 4 (severe) [79-81].

Joint space width (JSW) can be relatively well assessed by radiography and is used as a surrogate for cartilage thickness and integrity of the menisci in human knee OA, although these structures are not directly observed on radiographs and therefore cannot be measured accurately [82, 83]. However, the only structural endpoint to date approved by the U.S. Food and Drug Administration (FDA) [84] and the European Agency for the Evaluation of Medicinal Products (EMEA) to assess the efficacy of disease modifying drugs in phase-III clinical trials is JSN [85]. Also, its progression over time is used as a proxy of OA progression and the complete loss of JSW is among the indicators of the need for knee joint replacement in humans [86]. Unfortunately, radiographic imaging has several drawbacks that limit its usefulness for an early diagnosis of disease. Joint positioning in relation to the X-ray beam, for example, can

significantly influence JSN assessment [87, 88], and the diagnostic accuracy depends on the radiographic projection used as well as the number of views obtained [89, 90]. It is also well known that in some individuals, there is poor correlation between radiographic features of OA and clinical symptoms [91]. A systematic review of the association between radiographic and clinical features of OA found that an association was present in only 10%, inconsistent in 72%, and absent in 18% of 39 studies [92]. Moreover, radiography lacks sensitivity and specificity in detecting damage to articular tissues and has poor sensitivity to changes in follow-up studies [93]. Therefore, as OA is now considered a disease of the whole joint, rather than the cartilage only as previously thought, it is clear that an imaging modality such as radiology, which is unable to detect non-osseous pathology, is largely inadequate to assess the multiple tissues of joints affected by OA [94-96].

Magnetic resonance imaging (MRI) however, provides excellent soft tissue contrast and allows the visualisation of all joint tissues in a tomographic manner [78] including those not visualised by radiography such as cartilage, menisci, ligaments, synovium, joint capsule, and bone marrow [97-102]. MRI allows detection of pathological changes before any radiographic signs become apparent and can be used to identify physiological changes in tissues before morphologic changes become evident [78, 93, 103, 104]. The association of pain with bone marrow lesions (BML) [105] and synovitis [106] have also been elucidated thanks to this imaging technology. Several semiquantitative MRI scoring systems have been created and described in the literature, with the Whole Organ Magnetic Resonance Imaging Score (WORMS) being the most commonly used [107]. This system assigns scores to fourteen independent articular features such as cartilage signal and morphology, subarticular bone marrow abnormality, subarticular cysts, subarticular bone attrition, marginal osteophytes, medial and lateral meniscal integrity, anterior and posterior cruciate ligament integrity, medial and lateral

collateral ligament integrity, synovitis, loose bodies, and periarticular cysts/bursitis. More recently, an alternative scoring system, the MRI Osteoarthritis Knee Score (MOAKS), has been developed to address the limitations of WORMS and other scoring systems [108]. Although the features assessed with MOAKS are fundamentally the same as in previous systems, the differences mostly lie in the refinement of BMLs (by introducing regions and scoring across regions), cartilage (sub-regional scoring) and meniscal morphology scoring (by adding meniscal hypertrophy, partial maceration, and progressive partial maceration) [108].

Quantitative assessment with accurate measurement of cartilage and bone have been described [109]. For human knee OA the identification and validation of multiple 3D MRI biomarkers has been possible with the use of large datasets [24]. Subchondral surface area [110, 111] and parametrised shape descriptions [112, 113] have been used as efficacy of intervention biomarkers in some clinical trials [114, 115]. With modern supervised machine-learning techniques, specifically statistical shape modelling, measurements of bone area and shape obtained from MRI images allowed prediction of radiographic onset [116] and progression [117] of OA, and discrimination between OA and normal joints [118]. Contrary to radiographic examination where JSW is not a reliable marker, the use of 3D measurement of JSW with MRI has become a relatively new imaging biomarker [119]. Quantitative MRI measurements of other joint tissues has led to identification of other imaging biomarkers, for example cartilage volume and thickness [87] and areas of denuded bone [120]. Newer MRI techniques defined under the umbrella of compositional MRI (relaxometry measurements, sodium imaging, delayed gadolinium enhanced MRI of cartilage, glycosaminoglycan specific chemical exchange saturation transfer, diffusion weighted imaging, and diffusion tensor imaging) [121], currently only used for research purposes, allow identification of biochemical properties of tissues and may be able to detect early pre-morphologic changes of OA in the future [103]. Unfortunately,

MRI is not routinely used for initial assessment or follow-up of OA patients due to its high equipment cost, limited availability, long scanning times, need for skilled personnel and the lack of a validated international score [78, 93, 103, 104]. In addition, despite the high specificity of MRI, its sensitivity is below clinical diagnostic standards [122].

Computed tomography (CT) is the method of choice for imaging cortical bone and soft tissue mineralisation [93], and subchondral bone cysts and osteophytes are more readily identified than when MRI or X-rays are used [123]. Weight bearing CT imaging was successfully used for 3D JSW mapping in a group of patients with a greater accuracy compared with X-rays [124, 125]. With the intra-articular injection of a contrast medium (arthrography) CT is the method of choice for assessing superficial and focal cartilage defects [126]. Arthrography, utilised with either MRI or CT, also allows visualisation of central osteophytes (not detected by X-rays) which are associated with more severe OA than marginal osteophytes [127]. This technique though is not commonly employed due to cost, its invasive nature and the potential risks associated with intra-articular injections [103]. One advantage of CT over MRI is the lack of soft tissue distortion that can be observed in MRI images when there are inhomogeneities in the magnetic field [128]. The routine use of CT in practice is limited by the poor visualisation of soft tissues and the risks associated with use of ionising radiation [103].

Ultrasonography (US) is a low-cost, real-time imaging modality that has no known side effects as it does not employ ionising radiation and allows multiplanar imaging and visualisation of multiple tissues in a single session without the need for contrast enhancement [78, 103, 129, 130]. The main limitations are that it is an operator dependent modality, its ability to examine only tissues superficial to bone, and the tendency to over interpret the findings [78]. Compared with radiographs, US performed equally or better at identifying osteophytes in human knees and

hips [131-133]. Other features associated with OA that can be detected with US are synovial hypertrophy, joint effusion and increased soft tissue vascularity [134]. For hand OA a semiquantitative scoring system has been described [135] and for knee OA correlation was found between US-detected synovitis and advanced radiographic OA and clinical symptoms consistent with joint inflammation [136].

Nuclear medicine imaging involves the use of radioisotopes injected systemically and their detection in tissues with active metabolism. Specifically for OA nuclear medicine allows visualisation of osteophytes, sclerosis and cysts in the subchondral bone, BML and synovitis [137]. As nuclear imaging provides poor anatomical resolution, newer hybrid technologies that combine nuclear medicine imaging such as positron emission tomography (PET) with CT or MRI (PET-CT, PET-MRI) can overcome this limitation [138, 139]. Due to the need for a radiopharmaceutical with radiating activity and the potential allergic reactions nuclear medicine imaging is not routinely used in orthopaedic practice [78, 103].

Similar to human medicine, the main imaging modalities currently used for horses are radiography, ultrasonography, nuclear medicine imaging, CT, and MRI [140]. As most of the imaging equipment is designed for use in humans, its main limitation is the inability to accommodate certain body parts such as the upper limb or trunk in adult horses, and the need for general anaesthesia for some of the imaging modalities. The use of standing low-field MRI and CT is becoming more popular in horses as it avoids the need for general anaesthesia. Standing MRI has been used in the distal limb of racehorses to identify bone abnormalities given their association with the risk of catastrophic injuries [141, 142], but due to the low-quality resolution the assessment of cartilage is very limited. Although imaging grading systems for horses have been described for radiography, CT, and MRI, these are not as developed as in

human medicine [143-145]. Despite the anatomical differences between people and horses the features of OA that are generally assessed are fundamentally the same such as osteophytosis, synovial effusion, subchondral bone lysis and sclerosis, joint fragments, BML, and collateral desmopathy [145].

Since OA is a disease with a slow onset, unfortunately by the time clinical signs occur and imaging is performed, significant pathology is often already present. In addition, most of the imaging modalities commonly available do not provide any information on the tissue molecular changes associated with OA, hence the need to develop reliable and specific biomarkers that will allow early diagnosis of disease and timely treatment [85, 140, 146, 147].

2.2.2 Direct Biomarkers of Cartilage Metabolism in Osteoarthritis

2.2.2.1 Biomarkers of anabolic processes

Type II collagen is the most abundant type of collagen in articular cartilage (90-95%) [148, 149], and is synthesised as a procollagen molecule intracellularly followed by secretion into the ECM. The procollagen molecule is cleaved extracellularly at the carboxy- (C-) and amino- (N-) propeptides by proteinases to form tropocollagen molecules which aggregate to form fibrils [4, 150, 151]. The C-propeptide of type II procollagen (CPII) can be detected either *in vitro* via immunohistochemistry or *in vivo* with an immunoassay and can be used as potential marker of type II procollagen synthesis [152-154]. Increased synthesis of collagen has been observed in early OA [155] and is believed to be representative of the biologic responses to catabolic processes associated with the disease. The CPII content in cartilage affected by OA is significantly increased and directly associated with total type II collagen content. The release of CPII from OA cartilage results in increased synovial fluid (SF) CPII concentrations, which directly correlate with CPII cartilage content, but interestingly serum CPII concentrations are

reduced [156]. Two human studies found that SF concentration of CPII was increased in patients with early radiographic signs of OA, but that this decreased in advanced stages of disease [157, 158], while another found that CPII concentration in SF correlated well with radiographic progression of knee OA [159]. In an equine osteochondral fragmentation model of OA both SF and serum concentrations of CPII were consistently increased in affected horses compared with controls [160]. In horses with clinical OA CPII concentrations were increased in serum, but not in SF [161]. Several equine studies have shown the potential of CPII as an efficacy of intervention marker following treatment with either intra-articular corticosteroids [162, 163] or oral non-steroidal anti-inflammatory drugs [164, 165].

Chondroitin sulphate (CS) makes up over 80% of the sulphated glycosaminoglycans (GAGs) that make up the large protein aggrecan found in articular cartilage, and together they are responsible for the functional and structural characteristics of cartilage [166]. Epitope 846 of CS (CS-846) has been identified in abundant concentrations in fetal cartilage. The concentration progressively decreases with age and is almost absent in adults [167, 168]. In OA cartilage however CS-846 is found in high concentrations associated with recently formed aggrecan molecules, suggesting this may be a marker of aggrecan synthesis rather than degradation [168]. In fact, in order for the epitope to be detected cleavage by the ECM is necessary after its synthesis, so CS-846 may be indicative of an anabolic state rather than being an anabolic marker itself [75]. Concentrations of CS-846 were shown to be significantly increased in SF [169, 170] of OA joints and serum [170] of patients affected by OA with it being 38-times higher in SF compared with serum [170]. In horses CS-846 is found in high concentrations of both SF and serum of horses with experimentally induced traumatic OA [160] as well as in horses with naturally occurring disease [161].

2.2.2.2 Biomarkers of catabolic processes

The cartilage-associated proteins that have been more extensively studied in biomarker research are type II collagen and aggrecan. Their breakdown products in particular have shown the greatest potential in the diagnosis of OA [171, 172]. Degradation of type II collagen can be measured by identifying denatured and/or cleaved type II collagen fragments using specific antibodies. These collagen fragments expose regions that are only accessible after degradation of the original collagen molecules [148].

C-terminal cross-linked telopeptide of type II collagen (CTX-II) is released from the collagen triple helix as a degradation by-product and has been used as a marker of OA [173]. *In vitro* CTX-II was released in bovine cartilage culture supernatants upon stimulation with IL-α, and this was revoked by addition of MMP inhibitors [174, 175]. Using different models of arthritis in rats it has been shown that CTX-II concentrations are significantly increased in affected animals in serum, synovial fluid, and urine, and that these are positively correlated with the histological scores of cartilage destruction [174, 176, 177]. Urinary CTX-II (uCTX-II) is one of the most studied markers of type II collagen degradation, with multiple studies confirming its potential as a biomarker of OA [178-182]. The biggest advantage of uCTX-II is the ease and lack of invasiveness of sample collection, which makes it a very appealing biomarker. In patients with hip and knee OA uCTX-II concentrations are higher than in controls and associated with increased risk of rapid OA progression [183-185]. A correlation has been found between radiographic scores of different joints with OA and increased concentrations of uCTX-II [186]. Similarly, uCTX-II concentrations correlated well with bone marrow abnormalities detected with MRI in knees with OA [187]. Contrasting results have been reported in regard to the effect of age and gender on uCTX-II concentration: some authors reported no effect of age and gender [183] while others found differences in uCTX-II concentrations between men and women and also between

different age categories [188], with some authors also reporting an effect of diurnal variation [189]. It has been suggested that the clinical usefulness of high concentrations of uCTX-II may be best when used together with N-propeptide of type IIA procollagen (PIIANP), a marker of collagen which was found to have low concentrations in patients with knee OA [190]. In Thoroughbred racehorses with naturally occurring traumatic arthritis (osteochondral fragmentation) CTX-II concentrations in SF are significantly higher than in the uninjured controls, but not in serum. Serum CTX-II concentrations were positively correlated with radiographic scores, but not with arthroscopic scores [191]. Another study in racehorses confirmed that CTX-II concentrations increase in SF but not serum of horses with traumatic arthritis, leading to an increase in the SF:serum CTX-II ratio [192].

Other products of degradation of type II collagen have been identified and their use investigated in OA. Specifically, the peptides COL2-1 and its nitrated form COL2-1 NO_2, as well as C2C and C1,2C, also known as COL2-3/4C long and COL2-3/4C short, respectively. COL2-1 and COL2-1 NO_2 are released upon degradation or unwinding of the type II collagen triple helix following oxidative stress. High concentrations in urine and serum have been found in patients with knee OA [193, 194]. When serum COL2-1 NO_2 was used in conjunction with serum CS-846, cartilage oligomeric matrix protein (COMP) and uCTX-II, an association between their concentrations and severity of OA was identified [195]. A correlation was found between increased urinary concentrations of COL2-1 and COL2-1 NO_2, and the MRI-based OA scoring system Whole-Organ Magnetic Resonance Imaging Score (WORMS), and these markers have been deemed helpful in identifying patients at risk of OA progression [196]. An advantageous characteristic of serum COL2-1, when compared with other markers such as uCTX-II, is that it is not affected by sampling conditions, diurnal variation, seasonality, or physical activity [197-199]. This makes COL2-1 sufficiently robust to support its use in OA clinical trials, together with its

nitrated form, as a biomarker of type II collagen degradation [199]. While C1,2C derives from both type I and II collagen, C2C is specific to type II collagen [200]. Increased concentrations of C2C have been found in SF of canine knees with induced OA [201] as well as in SF, serum, and urine of people with clinical OA, in which a correlation was found with progression of disease [202-206]. In racehorses with naturally occurring traumatic arthritis, C2C concentrations are increased in both SF and serum and are positively correlated with radiographic and arthroscopic scores [207]. The COL2-3/4C short (C1,2C) immunoassay, originally developed for human cartilage, has also been used in studies on equine cartilage, particularly to monitor collagenase-induced collagen degradation [208]. More recently an equine-specific assay has been developed using the antibody 234CEQ [209]. Combined anabolic and catabolic biomarkers (CPII, COL2-3/4C short, and 234CEQ) were found to be correlated with the severity of osteochondrosis in foals [210].

Proteoglycans are composed of glycosaminoglycans attached to a core protein, and, as such glycosaminoglycans (GAGs) may be detected when there is degradation of proteoglycans, which are the main components of the cartilage ECM [211]. The main GAGs reflective of cartilage breakdown are chondroitin sulphate, keratan sulphate and dermatan sulphate, all of which were found in increased concentrations in SF from knee joints of people with OA [212, 213]. A correlation between increased GAGs concentrations in SF of patients with knee OA and radiographic grades of OA has been described; interestingly a decrease in GAGs was associated with the highest radiographic grade suggesting near total cartilage loss in these cases [214]. The measurement of serum concentrations of only keratan sulphate has yielded equivocal results with some studies showing an association with OA, but not others [215, 216]. Increased GAGs concentrations were found in synovial fluid of horses affected by osteochondritis dissecans and OA [217] but varied depending on joint effusion and cartilage OA

status. Therefore, GAGs in SF were deemed more useful if serial samples were taken, rather than a single sample. However, this limitation makes their use impractical in clinical practice. Conversely, serum GAGs concentrations have been useful in differentiating exercised control horses from exercised horses with induced OA, with higher GAGs concentrations in the latter [160].

Another biomarker indicative of active catabolic processes is cartilage oligomeric matrix protein (COMP), which is a non-collagenous protein found in large amounts in normal cartilage [218]. Increased COMP concentrations were found in SF and serum of people with OA [219-223] and have been positively correlated with radiographic grades of OA [223], radiographic progression [224], and nuclear scintigraphy [225]. In horses with naturally occurring OA, increased COMP concentrations were found both in SF [226] and urine [227]. However, as COMP is also synthesised by osteoblasts, synoviocytes and tendon fibroblasts, and not only chondrocytes as originally thought, its utility as a biomarker of OA remains questionable [228, 229].

2.2.3 Direct Biomarkers of Bone Metabolism in Osteoarthritis

2.2.3.1 Biomarkers of anabolic processes

Type I collagen is the predominant protein in bone matrix and during its synthesis cleavage of the procollagen I carboxy- (C-) and amino (N-) terminal propeptides (PICP and PINP) occurs. Such cleaved fragments are detectable in the circulation and their concentrations reflect synthesis of type I collagen, as such they are used as markers of bone formation [230]. Although type I collagen is also present in smaller amounts in skin, teeth, cornea, vessels, and tendons [231], these tissues generally have a much slower turnover compared with bone and have a marginal effect on circulating concentrations of PINP [230]. In humans with knee OA, serum concentrations of PINP are correlated with radiographic grading and OA progression,

especially in the early stages of OA, thus showing prognostic and diagnostic value [232]. In dogs with induced OA by transection of the cranial cruciate ligament increased PINP concentrations are found both in SF and serum [233]. Synovial fluid concentrations of PICP in people with knee OA show no correlation with the severity of the disease as assessed intra-operatively [234]. In horses, these two markers have not been extensively studied, but it was observed that serum PICP concentrations decrease with age [235] and increase with exercise [236, 237].

Osteocalcin (OC) is a non-collagenous protein associated with bone turnover. In people increased concentrations of synovial fluid and serum OC were found in OA patients compared with controls and are positively correlated with other markers of bone metabolism [238] and findings of nuclear scintigraphic examination [225]. It has also been observed that synovial fluid OC concentrations are lower than those found in serum, and it was concluded that synovial fluid OC is derived from the circulation [238]. Contrasting findings though have been reported in hand OA with some authors reporting lower serum concentrations of intact OC in patients with OA compared with non-affected people [239], while others found higher serum intact OC concentrations in affected individuals and a positive correlation with severity of radiographic grading, joint space narrowing and osteophyte formation scales [240]. Similarly, contrasting results have also been found for knee OA [178, 241]. As serum OC concentration reflects bone metabolism of the whole skeletal system and not specifically subchondral abnormalities related to OA, other factors such as age, menopausal status or other bone diseases can influence systemic OC concentrations [200]. Moreover, OC also plays a role in glucose [242] and lipid [243] metabolism, and its concentrations have significant circadian variations [244]. In horses, serum OC concentration decreases with exercise, and it has been postulated that this may reflect decreased bone formation [236, 245]. However, other researchers found increased serum and SF OC concentrations following exercise [160]. The same authors also found that induced

carpal OA caused an increase in SF and serum OC concentrations [160]. Osteocalcin has also been used in multiple experimental studies in horses as a marker of efficacy of intervention [246, 247].

Bone specific alkaline phosphatase (BAP) is highly expressed on the surface of osteoblasts and is associated with bone formation and mineralisation [75, 248, 249]. The use of BAP as a marker of OA has yielded variable results. Synovial fluid BAP activity was increased in the early stages of disease in patients with hip OA [250]. Kenanidis *et al.* described the use of serum BAP activity and other bone-turnover markers in patients with hip and knee OA against radiographic assessment and did not find a correlation between BAP serum activity and radiographic grading of OA, thus suggesting BAP cannot be used as surrogate for radiographic imaging [251]. Other authors report no statistically significant difference in serum or plasma BAP activities between patients with symptomatic knee OA and controls [252]. In the same study a positive correlation was found between serum BAP activity and knee stiffness scores, but no correlation between BAP and pain or physical function. In racehorses with traumatic OA, increased BAP activity was positively correlated with arthroscopic scores of cartilage damage in carpal or metacarpophalangeal joints, thus suggesting the presence of a link between bone-turnover and cartilage damage in OA [253]. Another study on racehorses undergoing arthroscopic surgery for removal of exercise-induced osteochondral fragments from carpal or metacarpophalangeal joints found that SF BAP activity was significantly higher in joints with osteochondral fragments than in normal joints [254]. In addition, BAP activity in serum, SF and SF:serum BAP ratio were predictive of osteochondral injury. In the same study radiographic and arthroscopic scores correlated with serum BAP activity, while SF:serum BAP ratio correlated with arthroscopic scores. In another study SF BAP activity from the small tarsal joints of horses with OA was significantly higher compared to controls and positively correlated with overall radiographic scores [255]. Exercise in horses does not affect serum BAP

activity, as no difference has been detected between a group of exercised horses and controls [245].

2.2.3.2 Biomarkers of catabolic processes

Type I collagen non-helical telopeptide (ICTP) is a breakdown product of type I collagen and in human patients with rheumatoid arthritis has been suggested to be an indicator of bone resorption [256, 257]. Its usefulness in detecting pathology in horses has not been positively demonstrated, but lower concentrations seem to be associated with exercise in different studies [236, 237, 245].

Type I collagen C-telopeptides (CTX-I), another product of type I collagen breakdown, has been used as a marker of bone resorption in the serum of people with rheumatoid arthritis [257]. Urine concentrations of CTX-I were greater in patients with progressive knee OA compared with patients that had non-progressive OA or controls [258]. In dogs with induced OA following transection of the cranial cruciate ligament, both serum and SF concentrations of CTX-I were increased, although in SF this was not statistically significant [233]. In horses, serum concentrations of CTX-I were significantly correlated with age and training [259, 260], but it has not proven to be a good indicator of disease [261].

Bone sialoprotein (BSP) is a protein rich in sialic acid forming closed bonds to hydroxyapatite and is found in newly synthesised osteoid [262, 263]. It is particularly abundant at the cartilage-bone interface in the rat [264] and pig [265]. BSP concentrations are increased in serum of human patients with clinical signs of knee OA and with positive scintigraphic findings, as well as in SF of people with rheumatoid arthritis [266-268]. Although BSP has been identified in the cartilage-bone interface of the proximal aspect of the third carpal bone of horses with OA, there is no published information on synovial fluid or serum concentrations of BSP in horses [269].

2.2.4 Biomarkers of Synovium Metabolism in Osteoarthritis

Hyaluronic acid (HA) is a molecule produced by synoviocytes which plays an important role in articular cartilage lubrication and homeostasis [270]. Elevated serum concentrations of HA have been identified in multiple human studies of patients affected by OA in one or more joints including knee, hip, spine, wrist, and finger [271]. It was also observed that higher basal serum HA concentrations were associated with rapid radiographic progression of knee OA [272] and were predictive of radiographic progression in patients with erosive hand OA [273]. As HA is a molecule not unique to joint tissues but is found in multiple other tissues in the body, serum concentrations can be influenced by multiple other diseases such as liver disease among others, thus making the use of this molecule difficult as a diagnostic biomarker of OA [274]. In horses an early study (1984) reported significantly reduced SF concentrations of HA in cases with traumatic OA compared to normal joints [275], but no further studies have been published on HA as a potential biomarker of OA in this species, although its usefulness as a therapeutic molecule in equine OA has been established [276-280].

Glucosyl-galactosyl pyridinoline (Glc-Gal-PYD), a glycosylated analogue of pyridinoline, is a degradation product from the synovium and is not released from bone and cartilage [281]. Increased urinary concentrations of Glc-Gal-PYD were detected with high pressure liquid chromatography in people with knee OA and were correlated with pain and physical dysfunction scores [178]. Similarly, a strong association between presence of knee OA and urinary Glc-Gal-PYD was found between urinary Glc-Gal-PYD and the severity of radiographic grades, joint space narrowing, and osteophyte scores [282].

The human cartilage glycoprotein-39 (YKL-40), secreted by chondrocytes and synoviocytes and other tissues such brain, kidney, and placenta [274], has been found in increased

concentrations in serum and SF of patients with severe knee OA, where a correlation was also identified between serum and SF concentrations, with 10-fold higher values in the latter [283]. In patients with hip OA, serum YKL-40 concentrations were increased and correlated with serum concentrations of inflammatory marker C-reactive protein (CRP) [284]. Moreover, correlations were found between SF concentrations of YKL-40 and MMP-1, MMP-3, interleukin (IL)-6 and IL-17 [285]. No work has been done on this molecule in horses.

2.2.5 Indirect Biomarkers of Joint Metabolism

Some researchers have suggested that limiting joint health assessment only to cartilage and bone biomarkers may not provide a full understanding of the complex interactions between the different joint tissues, and that other markers of joint homeostasis should also be considered; in particular inflammatory markers [286]. The central role of synovial inflammation in the pathogenesis of OA is well known [32]. Inflammatory markers have been shown to be among the best markers for assessing the categories of burden of disease, prognostic, and efficacy of intervention [286]. There is currently no consensus on the intrinsic nature of OA, given the simultaneous presence of degenerative and inflammatory events in joints affected by OA. However, some speculate that low grade inflammation drives or accompanies OA and therefore OA should be considered an inflammatory disease [286].

2.2.5.1 Mediators of inflammation and pain

As adult cartilage has no vascularisation, most of the inflammation in disease states occurs in the synovial membrane, which produces several inflammatory mediators. These can activate nociceptors and stimulate peripheral neurons to release neuropeptides which contribute to progression of OA [287].

Kinins, for example bradykinin (BK), are a family of peptides with pro-inflammatory functions and are implicated in pain pathways [288]. They selectively activate kinin B_2 receptors which induce release of inflammatory mediators to sensitise nociceptive fibres [288, 289]. The B_2 receptors have been found in synoviocytes, fibroblasts and endothelial cells of synovium from individuals with OA [290]. In people with knee OA the presence of BK in synovial fluid correlates positively with markers of cartilage degradation (GAGs) and inflammation (IL-6), thus supporting a role of BK in OA [291]. In rats, intra-articular injection of knee joints with BK causes proteoglycan loss from articular cartilage as confirmed with histopathological staining [292]. Similarly in a rat model of synovitis intra-articular administration of BK induced endothelial cell proliferation without synovial proliferation [293]. In horses, contrasting results have been reported. In horses with fetlock joint pain or clinical OA, SF BK concentration was not increased [294], while BK surges were found in a model of synovitis 24h post-induction, which were reduced by meloxicam and morphine [164, 295, 296].

Serum amyloid A (SAA) is a an acute-phase protein synthesised by the liver in the event of systemic or severe local inflammation [297, 298]. Although in horses SAA expression has been identified in synovium [299], most of the SF SAA derives from blood ultrafiltration [300, 301]. In people, SAA has proven to be a useful marker, mostly of rheumatoid arthritis (RA) [302-304], but some evidence also supports its role in the pathophysiology of OA [300, 305, 306]. In horses, a significant increase in serum and SF concentrations of SAA were reported in association with induced inflammatory arthritis [307, 308] , septic arthritis and tenosynovitis, but not OA [309].

Substance P (SP) is a neurotransmitter associated with pain which is synthesised in the spinal cord and reaches the peripheral nerve ending through the axons [310]. Upon stimulation of the articular primary afferent neurons SP release has been associated with radiographic signs of

joint degradation after experimentally induced arthritis [311]. Substance P is capable of stimulating release of inflammatory mediators such as IL-1, TNF-α , PGE_2 [312-314], proteases [313, 315], and oxygen free radicals [316]. In SF of patients with rheumatoid arthritis increased concentrations of SP were found [317, 318], and SP production was demonstrated in the synovium from people with RA or OA [319]. Similarly, significant increases of SP concentrations in SF were observed in an equine synovitis model [164, 295, 296], and in horses with OA [320].

Prostaglandins are inflammatory lipid mediators which have been found in SF and synovium of people with OA [321]. Their role in joint metabolism is not fully understood as they appear to have catabolic effects on ECM [322, 323] , chondrogenic activity [324, 325], and inhibit MMPs expression by synovial fibroblasts [326]. Prostaglandin E_2 (PGE_2) is one of the key players involved in the process of cartilage degeneration and OA progression [327-329]. A study by Li *et al.* (2009) using SF and chondrocyte cultures from people with OA demonstrated that PGE_2 inhibited synthesis of aggrecan and subsequently proteoglycan accumulation in cartilage mainly through the EP2 receptor, with only marginal stimulation of MMPs and ADAMTS [330]. They also found a decrease in type II to type I collagen ratio, resulting in an ECM poorer in chondrocytes and richer in fibroblasts, a finding that has been described previously in tissues during progression of OA [331]. Similarly to humans, an increase in SF concentrations of PGE_2 in horses has been shown in association with joint inflammation in experimentally induced synovitis[164, 295, 296] or OA [320], and its decrease has been used to assess efficacy of treatment [164, 279, 296].

2.2.5.2 Growth factors and cytokines

Insulin-like growth factor (IGF) is a peptide structurally similar to proinsulin and is present in two isoforms (IGF-1 and IGF-2) [332, 333]. An important function of IGF-1 is stimulation of proteoglycan synthesis, counteracting the catabolic activity of IL-1 in cartilage [334-337].It also

plays a major role in cartilage repair [338]. The effects of IGFs are modulated by six binding proteins (IGFBPs), each with specific functions themselves [339]. Increased SF concentrations of IGF-1 have been found in human [340, 341] and equine joints with OA [342]. In dogs with OA, similar findings were reported in association with high concentrations of IGFBP -3 and -4; this led to speculation that there may be a limited anabolic effect of IGF-1 in OA joints due to its reduced bioavailability associated with its binding of IGFBPs [343]. This is in agreement with other studies reporting a lack of response to IGF of chondrocytes from joints with OA in the presence of high IGFBPs concentrations [344-348]. Therefore, cartilage catabolism in OA may be in part a consequence of binding of free IGF to IGFBPs in SF [349]. Due to the complexity of interactions of IGF with IGFBPs it has limited usefulness as a marker of OA, but it has proven useful to monitor response to therapy in horses [350].

Transforming growth factor β (TGF – β) plays a vital role in homeostasis and injury response of several tissues [351]. Specifically, in cartilage it has been shown both *in vitro* and *in vivo* that TGF – β stimulates synthesis of collagen type II and proteoglycans [52, 352, 353]. In both spontaneous OA and an induced OA murine model, TGF – β signalling was found to be significantly reduced [354], as was also the case in genetically OA-prone aged mice [355]. Experimentally induced loss of responsiveness to TGF – β led to terminal chondrocyte differentiation and OA in mice [356]. In addition, it has been demonstrated that TGF – β is able to counteract IL-1-mediated upregulation of MMPs and reduce TNF-α-mediated collagen breakdown [357]. Although TGF – β appears to have anabolic and protective effects on cartilage, repeated joint exposure to TGF – β in a murine model induced synovial fibrosis and osteophyte formation, two traits that are commonly observed in OA [52, 358].

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine identified as playing an important role in the pathogenesis of OA [359, 360], contributing to cartilage degradation and reduced ECM synthesis [34, 35]. Despite its role in inflammation and catabolism, its use as a marker of OA has yielded poor results. In fact, in multiple studies the SF concentration of IL-1 β in patients with OA has been found to be low [291, 361-363]. Therefore, IL-1 tends to be mostly used in experimental OA as a marker to assess the response to therapy rather than a marker of OA itself [364, 365].

Interleukin-6 (IL-6) is a pro-inflammatory cytokine which is produced by multiple cells including synovial fibroblasts [366, 367], chondrocytes [368] and osteoblasts [369]. Mice chondrocyte cultures stimulated with IL-6 had reduced GAGs content in the ECM and increased GAGs release in the culture medium, indicating cartilage degradation, as well as increased production of MMP-3 and -13 and aggrecanases ADAMTS-4 and -5 [49]. Increased IL-6 concentrations were found in serum [363] and the SF of people with OA [291, 363, 370], and its concentration was even greater in cartilage [370]. In people with OA, IL-6 concentrations in serum [371, 372] and SF [373] are correlated with pain and joint function scores. An association between high circulating levels of IL-6 and radiographic severity of knee OA was also found [46]. Another study reported an association between serum IL-6 concentrations and knee BML in patients with symptomatic OA, as well as an increase in BML score as assessed by MRI [374]. In a model of equine synovitis, the SF concentrations of IL-6 markedly increased after intra-articular injection of lipopolysaccharide (LPS) [375]. Synovial fluid IL-6 concentrations were also increased in horses with naturally occurring OA and IL-6 proved to be an excellent predictor of joint disease [376-378]. These findings suggest that IL-6 is a good indirect marker of OA in both human and horses, although the effects of gender and age on IL-6 concentration in equine studies, which have had a limited number of subjects, should be taken into consideration [379].

Tumor necrosis factor-α (TNF-α) is an important cytokine involved in the pathophysiology of OA and is found in high concentrations in SF, synovium, cartilage, and subchondral bone of patients with OA [380]. It binds to two receptors, one of which (TNFRI or p55) has been found to be overexpressed in OA chondrocytes and synovial fibroblasts [381]. The detrimental impact of TNF-α on chondrocytes has been demonstrated by it causing a reduction of PG and collagen type II synthesis [382, 383]. In addition, its catabolic activity is exerted through stimulation of chondrocytes to release proteases such as MMP-1, -3 and -13 [384-386], and ADAMTS-4 [387], as well as upregulation of ADAMTS-4 expression from synovium of patients with OA [388]. Tumor necrosis factor-α is also capable of inducing the release of other pro-inflammatory mediators such as IL-6 [389], IL-8 [390] and PGE_2, and upregulates expression of genes encoding inducible nitric oxide synthase (iNOS), soluble phospholipase A_2, cyclooxygenase 2 (COX-2) and microsomal prostaglandin E synthase 1 [380]. Moreover, TNF-α stimulates the production of nitric oxide (NO) and other reactive oxygen species, which in turn produce free radicals that participate in the process of cartilage breakdown [62], as well as downregulating expression of antioxidant enzymes, further perpetuating the catabolic effects on cartilage [63, 64]. Contrasting results have been found in horses with regards the impact of TNF- α. In a model of equine lipopolysaccharide induced synovitis, some authors report increased SF TNF-α concentrations [391], while others have failed to show an increase [392]. Similarly, in horses with clinical OA, TNF-α concentration in SF was deemed to be a good predictor of joint disease in one study [377], while another found low concentrations and no association with any joint lesions [376]. Kamm *et al.* (2010) showed that expression of TNF-α was upregulated in both synovium and cartilage from horses with OA, but that SF concentrations were increased only in cases with severe OA [54]. Given the lack of consistent results in the equine species, the use of TNF-α as a biomarker of OA is controversial at this stage.

High-mobility group box-1 (HMGB-1) is a nuclear protein which also acts as a cytokine [393]. Its release from the nucleus occurs following cell necrosis or hypoxia, and its production can be stimulated by TNF-α [394]. The presence of HMGB-1 extracellularly elicits inflammation and is thought to be a major contributor to the pathogenesis of chronic inflammatory diseases like OA [395]. HMGB-1 is able to induce production of NO, MMP-3, MMP-13, and ADAMTS-5 by chondrocytes, thus showing a similar pro-inflammatory activity to TNF- α and IL-1β [396]. Overexpression of HMGB-1 was identified in human OA cartilage [397, 398] and higher concentrations than controls were reported in synovium and SF of knees with OA which were associated with the severity of synovitis and pain [399]. In SF, but not serum, of knees with OA, HMGB-1 concentrations were significantly higher than controls and were positively associated with the severity of radiographic scores [400]. The local nature of HMGB-1 activity was also shown in horses, where an increase in its concentrations was observed in SF, but not in serum [392]. The presence of HMGB-1 was found via immunohistochemistry both in synovium and osteochondral fragments of horses with traumatic arthritis undergoing arthroscopic fragment removal [401]. In racehorses with naturally occurring traumatic arthritis of the metacarpo/tarso-phalangeal and carpal joints, increased HMGB-1 concentrations were found in synovial fluid of affected joints compared to controls, but these were not correlated with radiographic and arthroscopic scores [402].

2.2.5.3 Enzymatic activity

Matrix metalloproteinases (MMPs) were first discovered in 1962 [403] and approximately twenty of them are now known in people, among which the most important ones are: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), and matrilysins (MMP-7, MMP-26) [404]. In human OA [405] and mouse models of OA [406] MMP-13 appears to be the main MMP involved in type II collagen destruction, and together

with MMP-3 [407], ADAMTS-4 and -5 [408, 409], it is one of the most abundant proteases secreted by OA-affected cartilage [208, 386]. Higher concentrations of MMP-3 were found in SF and blood samples from people with knee and hip OA [157, 410, 411], compared to those of MMP-1 in patients with knee OA [157, 411]. However, in other studies high concentrations of MMP-3 or MMP-1 in serum of patient with OA were not found [412, 413]. An association was identified in people with knee OA between plasma MMP-3 concentrations and radiographic joint space narrowing, thus suggesting the potential of MMP-3 as a surrogate for radiography in clinical trials [414]. Imbalance between MMPs and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), in favour of MMPs has been identified in cartilage of people with clinical OA [40]. As many of these enzymes are normally present in SF bound to their inhibitors, measuring their concentration does not provide a true representation of their destructive potential, and their actual activity (conversion of a substrate) should be used instead [415]. In horses, in a model of joint inflammation (intra-articular amphotericin B) increased activity of MMP-2 and MMP-9 in SF was observed, with a sustained prolonged increase especially of MMP-2, to a degree consistent with the potential to cause cartilage degradation [416]. In horses with clinical OA, MMP-2 but not MMP-9 SF activities were increased using both a zymographic analytical method and immunocapture assays when compared with controls [417]. The zymographic method only detected the latent forms of MMPs, but not the active ones. Moreover, this technique is subject to gel-to-gel variability. On the contrary, the immunocapture technique was able to detect the active forms, which better reflect the process of degradation [417]. General MMP activity in horses has been used in an acute synovitis model as a marker of efficacy of intervention after administration of anti-inflammatory drugs [164, 165].

2.2.6 Advances in Biomarkers Research

2.2.6.1 Genetic markers

In the last few years several studies have explored markers other than tissue-specific and inflammatory biomarkers, as potential markers for OA diagnosis and prognosis, in particular microRNAs (miRNAs). These are non-coding RNA molecules that regulate gene expression of catabolic (MMPs, aggrecanases) and inflammatory (IL-1β, TNF-α) mediators and are believed to play a role in OA pathogenesis [418-423]. A study in which expression levels of 380 miRNAs were measured in the plasma of patients with knee OA found that 12 (miR-16, miR-20b, miR-19c, miR-30b, miR-93, miR-126, miR-146a, miR-184, miR-186, miR-195, miR-345 and miR-885-5p) were overexpressed compared with healthy controls [424]. Similarly, three other miRNAs (let-7e, miR-454 and miR-885-5p) were correlated with knee and hip OA by other authors [425]. Other studies confirmed that the exploration of serum miRNAs as markers of OA holds potential and have identified other miRNAs that are either overexpressed or underexpressed in patients with OA [426-429]. Evidence has been presented that miRNAs are present in SF fluid in patients with OA, but their concentrations are significantly lower than in plasma, and there is no correlation between plasma and SF miRNAs. In the same study plasma miR-132 measurements were used successfully to differentiate healthy individuals from patients with OA or rheumatoid arthritis (RA), while SF miRNAs could only differentiate RA from OA patients, but not OA from healthy individuals [430]. Another study in patients with knee OA identified 7 out of 752 SF miRNAs tested as significantly differentially expressed in early-stage versus late-stage disease [431]. The potential use of miRNAs in horses has been explored by a group of researchers who found that SF miR-92a was downregulated in those with severe OA of the distal interphalangeal joint compared with horses with mild disease. Cartilage explants from the same horses subsequently cultured *in* vitro and transfected with a miR-92a mimic showed

increased expression of chondrogenic and hypertrophic markers, suggesting a role for this microRNA in equine OA [432]. Another study identified 13 miRNAs differentially expressed in SF of metacarpophalangeal joints from horses with early OA compared with controls [433].

2.2.6.2 Vibrational spectroscopy

Vibrational spectroscopy includes different modalities such as infrared (IR) and Raman. Infrared spectroscopy is divided into mid-infrared (MIR) with a frequency range of 4,000–400 cm^{-1} and near-infrared (NIR) with a frequency range of 12,500–4000 cm^{-1} [434]. Mid-infrared spectroscopy is commonly referred to as Fourier transform infrared (FTIR) spectroscopy, as modern technology includes an interferometer which converts the data to the frequency domain using a Fourier transform function. Vibrational spectroscopy is based on the specific interaction of the chemical bonds of a sample with the incident radiation of a light source, which, depending on the detector used, creates an absorption or reflectance spectrum characteristic of the sample, commonly called signature fingerprint, and provides information on the chemical composition of the sample [435]. Vibrational spectroscopy techniques have been used for diagnostic purposes on various biological fluids including serum, tears, saliva, urine, amniotic fluid, whole blood, synovial fluid, semen, and vaginal secretions in both medical and forensic settings [436-439]. The advantages of vibrational spectroscopy are its accuracy, low cost, small sample size requirements, speed, and low invasiveness [440].

Raman spectroscopy has been used successfully to classify SF from patients with clinical and radiographic evidence of knee OA [441]. Several Raman bands associated with protein secondary structure and content (protein backbone and amide linkages) were increased. Calculation of band intensity ratios has allowed identification of changes in the chemical composition of SF of patients with knee OA and these correlated with radiographic scoring of OA [441].

An early study using FTIR on SF from patients with different arthropathies, including rheumatoid arthritis, OA and spondyloarthropathy, reported an accuracy of 89%-96% in differentiating these conditions [442]. Another study described the use of NIR to differentiate SF collected from patients with the same arthropathies as above from controls with a sensitivity of 97%, 92% and 100% respectively [443]. More recently, attenuated total reflection FTIR allowed discrimination of serum from patients with knee OA from controls with 74% accuracy [444]. The use of IR spectroscopy on serum of rabbits with induced OA by transection of the cranial cruciate ligament allowed discrimination of affected animals from controls [445]. Infrared spectroscopy was successfully used to discriminate dogs with naturally occurring OA associated with rupture of the cranial cruciate ligament both in SF and serum with an accuracy of 98% and 92%, respectively [446, 447]. In horses, IR spectroscopy of SF allowed classification of diseased joints with an accuracy of 89% for traumatic arthritis [448] and 77% for osteochondrosis [449]. An unvalidated study evaluating IR spectroscopy on serum of racehorses with naturally occurring OA and controls reported a sensitivity and specificity of 100% [450]. These findings suggest that IR spectroscopy has utility in the diagnosis of OA.

2.2.6.3 Proteomics and metabolomics

Proteomics and metabolomics belong to the "omics" family of sciences, which also includes genomics and transcriptomics [451]. Genomics studies the genome or DNA of organisms, while transcriptomics studies the mRNA, which is the template for protein synthesis and more closely reflects actual gene expression [452]. Proteomics study the structure and function of proteins, which carry out the gene functions [452, 453]. Metabolomics is the study of metabolites such as carbohydrates, amino acids, oligopeptides, organic acids, nucleotides or lipids and exogenous molecules (drugs, food, toxins) [454]. Proteomics and metabolomics aim to detect proteins or metabolites in biological samples in an untargeted manner [452], an approach in a

way similar to that of vibrational spectroscopy. Proteomics have the advantage over genomics and transcriptomics of providing a true representation of the status of a tissue, given that the latter two approaches do not account for post-translation protein modifications, or alternative transcriptional and translational steps [453]. The advantage of metabolomics over transcriptomics and proteomics is that metabolites are the final products which reflect in an amplified manner all changes that have occurred upstream [455]. Despite the smaller number of metabolites (~5,000) compared to proteins (>100,000) or genes (~35,000), the former are more diverse and biologically more complex compared to the other "omics" [452]. Conversely, proteomics has the advantage that proteins are more stable than metabolites [456]. Some of the technologies most commonly used in metabolomics include nuclear magnetic spectroscopy (NMR) [457], liquid chromatography mass spectrometry (LCMS) [458], and gas chromatography mass spectrometry (GCMS) [459]. These techniques have relatively low operating cost, can be used in different types of samples, and can identify metabolites with high efficiency [460]. In particular, NMR spectroscopy has the advantage of requiring minimal sample preparation and being fast, reliable, non-discriminating (known and unknown compounds), and non-destructive [461]. The use of metabolomics in the biomedical field has developed rapidly in recent years, with applications in cardiovascular disease [462], cancer [463] and OA [464].

Several metabolites have been identified for OA in a multitude of tissues such as bone, cartilage, and synovium [457, 465], or bodily fluids including urine, SF, plasma, and serum [464, 466-468]. A 2023 systematic review of articles on the use of metabolomic approaches to identify potential biomarkers of OA found over 40 articles, of which 6 were on urine, 13 on plasma, 11 SF samples and 11 on serum [469]. Of particular importance from the above studies was the identification of common metabolic pathways including tricarboxylic acid cycle, fatty acid metabolism, amino acid metabolism (among which the branched-chain amino acids

metabolism and tryptophan metabolism), nucleotide metabolism, urea cycle, cartilage matrix components, and phospholipid metabolism. However, given the large scale of altered metabolism identified, a single metabolite or combination thereof that could be used for early diagnosis of OA has not determined at this stage [469].

In horses, quantitative proteomics have been used in an *in vitro* cartilage inflammation model to identify three COMP neoepitopes [470]. For one of these a new ELISA test was subsequently developed and high concentrations of this neoepitope were found in SF of horses with early OA. Another study used a proteomic approach on SF to describe a comprehensive proteome of 764 proteins, with 10 of them differentially expressed in racehorses affected by OA compared with healthy horses, which could be used in the future as biomarkers [471]. A proteomics study on horses with either OA or osteochondrosis identified a set of dysregulated proteins related to the inflammatory cascade, coagulation pathway, oxidative stress, and matrix damage, consistent with alterations in articular homeostasis, plasma-SF exchange, joint nutritional status, and vessel permeability [472]. Metabolomic approaches used on equine cartilage explants stimulated with TNF-α and interleukin-1β led to the discovery of nine novel neopeptides with a potential for use as OA biomarkers [473]. Another study using metabolomics on SF collected from metacarpophalangeal joints of horses identified 10 metabolites (lactate, alanine, acetate, N-acetylglucosamine, pyruvate, citrate, creatine/creatinine, glycerol, HDL choline, and α-glucose) which were significantly increased in OA joints compared with healthy joints [474]. Another study comparing metabolites abundance in SF between normal joints and joints affected by palmar osteochondral disease (a manifestation of OA in horses) found no significant differences, although the most influential metabolites were glucose and lactate [475]. Metabolomics were also used on a small number of placebo horses (n=4) in a separate pharmacokinetics study to investigate possible correlations in metabolite intensities between

SF (middle carpal joint) and serum [476]. A total 1850 metabolites were co-detected in SF and serum and for 93 of them there were significant correlations (65 positive and 28 negative) between SF and serum. These findings suggest that quantification of the intensity of a metabolite in the circulation may allow prediction of its intensity within the SF, thus potentially leading to identification of useful serum biomarkers which may avoid the need for invasive sample collection methods such as arthrocentesis.

2.3 Models of Osteoarthritis

A significant body of knowledge on OA has been obtained from clinical studies and preclinical models. Clinical studies in humans pose numerous limitations and challenges due to the variability between the onset of symptoms and disease, the slow progression of naturally occurring OA as well its unpredictable nature [477, 478]. Also, often clinical symptoms occur late in the disease process and do not accurately correlate with biochemical or structural changes in the joint [479, 480]. Tissue samples from humans are generally collected for research at the time of joint replacement (advanced stage of disease), making it very difficult to study the early stages of OA [481]. As such preclinical models have been developed to overcome these limitations and advance the understanding of the pathogenesis of OA and efficacy of therapeutic interventions [482, 483].

The main advantages of animal models in general, are knowledge of the onset and cause of disease, control of environmental factors, consistent phenotype, possibility of developing genetic models, and ability to collect samples at multiple stages of OA [482]. Nonetheless, they have several limitations when compared to humans, including anatomical and biomechanical differences, different cartilage thicknesses, variable progression of disease, difficulties in recreating lesions similar to all stages of human OA, and multiple sources of variability in age,

species, sex, and method of induction of disease across studies which makes comparison between studies challenging [482]. Because of these limitations, there is no ”gold standard” animal model of OA, as no single model allows researchers to study all features of the disease, and their translatability to understanding and managing human OA varies [481, 484-486]. Therefore, several models have been developed in small and large animal species.

Small animal models include mouse, rat, rabbit, and guinea pig, while large animal models include dog, sheep, goat, and horse [487, 488]. Of published reports utilising models of OA, small animal models have been used in 77%, with large animal models featuring in the remaining 23% [481]. In general, small animal models are easier to manage, quicker in disease progression and cheaper compared with large animal ones and are suited to the study of the pathogenesis and pathophysiology of OA [487, 489]. Their main disadvantages are the dissimilarities with people in tissue structure, joint size, and biomechanics [490, 491]. Conversely, large animal models have the advantage of greater anatomical similarity to humans, allow multiple tissue or fluid sampling, have a longer lifespan with the progression of disease more similar to people, and are amenable to arthroscopy and diagnostic imaging [489, 492]. Despite the fact that small animal models are more commonly used, large animal models produce more clinically relevant data and are a necessary requirement for regulatory approval of therapeutics [492, 493]. On the other hand, large animal models are expensive, time consuming, require trained personnel for safety, and have greater ethical considerations associated with public perception [489, 494]. Non-human primates would be an ideal species for OA research for their close biological similarity to humans, but they present multiple challenges associated with high costs of housing and management, their long life span with slow OA progression, but they can experience depression and post-traumatic stress disorder, thus posing significant ethical concerns [487, 495, 496].

Animal models of OA can be divided into primary, secondary, and tertiary. Primary models consist of those which do not require external intervention to induce disease and include naturally occurring disease and genetically engineered animals that develop spontaneous disease [497]. Examples of naturally occurring disease are the commercial porcine model, where OA develops in 3-4 years [498], and the Hartley guinea pig which reaches skeletal maturity in 6 months [487]. Genetically modified lineages of knockout mice have been used to reproduce OA and study its pathophysiology [499], for example the mice strains *Fgf2*^{LMWKO}and *Fgf2*^{HMWK} in which different isoforms of fibroblast growth factor 2 were ablated [500]. Secondary models are those that utilise an external stimulus to induce OA and include dynamic, synthetic, and surgical models. Dynamic models utilise non-invasive methods and are carried out with machine loading or impacting joints to induce for example tibial plateau fractures [501] or rupture of the anterior cruciate ligament in mice [502]. Synthetic models generally employ injection of chemicals into the joint space to cause cartilage destruction such as monoiodoacetate, collagenase, quinolones, papain, and lipopolysaccharide [503]. Surgical models are invasive methods to induce a joint defect, joint instability or systemic endocrine alteration leading to OA. The most commonly used surgical methods include anterior cruciate ligament transection in rabbits [504], rodents [505] and dogs [506], meniscectomy [507], osteochondral defects, orchidectomy, and ovariectomy in multiple species [487, 503]. Each of these models have specific advantages and disadvantages which have been well described in the literature [482]. Finally, tertiary models utilise a combination of a surgical method and exercise to induce OA and have been described in multiple species including the rat[508], rabbit [509], sheep [510], goat [511] and horse [160, 512, 513].

Equine models of OA represent an attractive option for translational research. In fact, horses have close similarities to humans in joint size and thickness of cartilage and subchondral bone

[514, 515]. They allow serial sample collection and outcome measurements, use of diagnostic imaging, and arthroscopic assessment [55, 513, 515]. Compared with humans, articular cartilage is half the thickness in dogs, ten times thinner in rabbits [490, 516], and seventy times thinner in mice [481, 487]. Cartilage thickness of the medial femoral condyle in particular has been reported to vary between 1.6-2.6 mm [8, 517] in people and 1.5-2.3 mm in horses [8, 518]. This allows the creation of cartilage defects in horses that have translational utility [519]. Both naturally occurring and post-traumatic OA has been described in horses [520, 521], and equine models of OA have been extensively used in the past decades for investigation of the pathogenesis of OA as well as efficacy of intervention trials [32, 160, 161, 280, 522-525]. The main disadvantages of using equine models are the difficulties related to housing and managing horses and the costs associated with it, as well as general ethical considerations associated with use of large animal species in research [495].

Several equine models have been described in the literature involving different joints. In one study, desmotomy of the lateral collateral and lateral collateral sesamoidean ligaments was performed on the metacarpophalangeal joint to induce instability that led to OA similar to naturally occurring OA in horses, resulting in lameness, osteophyte formation, cartilage erosions and score lines [526]. Another model has utilised an impactor device to create contusive trauma to the medial femoral condyle of the femur, similar to trauma-induced lesions observed clinically, resulting in decreased SF GAG concentrations, micro- and macroscopic cartilage lesions, and mild lameness, however radiographic abnormalities were not detected [527]. Another model employed a similar technique, but on the medial trochlear ridge of the talus [528]. Non-terminal surgically created osteochondral fragmentation on the dorsal aspect of the first phalanx in the metacarpophalangeal joint has also been described, resulting in clinical, imaging, and histological changes in cartilage and synovium typical of early post-

traumatic OA [529]. In more recent years two variations of the metacarpophalangeal osteochondral fragmentation model have been reported. In one model the fracture bed was enlarged with a curette to create instability of the fragment and the subchondral bone was exposed [513], while in the other one a horizontal full-thickness cartilage defect on the distal dorsomedial aspect of the third metacarpal bone was also made opposite to the osteochondral fragment [512].

The OA model that has been used more extensively in horses is the carpal osteochondral fragmentation model [32, 55, 160, 514]. This involves creating an 8 mm osteochondral fragment on the dorsal distal aspect of the radial carpal bone in the middle carpal joint and leaving it hinged at the capsular reflection. A motorised burr is then used to enlarge the parent bone to a 15 mm-wide defect to prevent premature healing of the fragment and create inflammation in the joint. After a 2-week recovery period a treadmill exercise program is implemented 5 days a week until day 70. This model mimics many of the characteristics of naturally occurring post-traumatic OA of racehorses, who often experience similar osteochondral fragmentation during their racing career [55]. This model, which produces clinical signs, biomarker alterations, and histological changes consistent with OA, has been used in several studies to investigate biomarkers profile changes as well as the efficacy of a large number of potential treatments of OA [280, 522-525]. This is the OA model the author has used in the current series of studies in this dissertation.

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Chapter 3
Plasma and Synovial Fluid Cell-Free DNA Concentrations Following Induction of Osteoarthritis in Horses

3.1 Abstract

Objective: to compare the concentrations of synovial fluid (SF) and plasma cell-free DNA (cfDNA) over time in control horses with those with induced carpal osteoarthritis (OA).

Methods: following an established model, unilateral carpal OA was induced in 9 of 17 healthy Thoroughbred fillies, while the remainder were sham-operated controls. Synovial fluid and plasma samples were obtained before induction of OA (Day 0) and weekly thereafter until Day 63, and cfDNA concentrations were determined using fluorometry.

Results: SF cfDNA concentrations were significantly higher for OA joints than for sham-operated joints on Days 28 (median 1430 µg/L and 631 µg/L, respectively, $p = 0.017$) and 63 (median 1537 µg/L and 606 µg/L, respectively, $p = 0.021$). There were no significant differences in plasma cfDNA between the OA and the sham groups after induction of OA.

Conclusions: plasma cfDNA measurement is not sufficiently sensitive for diagnostic purposes in this induced model of OA. Synovial fluid cfDNA measurement may be used as a biomarker to monitor early disease progression in horses with OA.

3.2 Introduction

Musculoskeletal injuries resulting in lameness are a leading cause of wastage in racing Thoroughbreds [1-4] and are associated with the highest number of training days lost [5]. Most lameness cases in the general horse population are associated with osteoarthritis (OA) [6-8]. The

diagnosis of equine OA is commonly based on clinical evaluation supported by imaging ("dry biomarkers") [9]. Unfortunately, imaging techniques have significant limitations in the early prediction and characterization of many musculoskeletal injuries and providing sufficient data to inform prognostication. The primary constraints are a modest correlation between pain and imaging findings [10], low sensitivity [11] and specificity [12], and high cost for higher resolution modalities [13].

Extensive research on molecular biomarkers ("wet biomarkers") for human OA during the last two decades [14, 15] has paralleled progress in the characterization of equine orthopaedic disease biomarkers [16, 17]. Synovial fluid biomarkers have been used for horses in experimental settings to evaluate the health of cartilage and synovium using ELISA-based techniques [18-22]. However, these techniques have not translated into clinical use due to the tests' lack of consistency, cost, and practicality of the tests [23]. Although serum biomarkers can identify horses with musculoskeletal injuries [24], the high cost of multiple ELISA techniques and limited sensitivity may prevent using these as a screening tool for OA in a clinical setting. There is a need for non-invasive, repeatable diagnostic and screening tests with high sensitivity and specificity for early disease diagnosis, stratification of orthopaedic disease, and the development and evaluation of new therapeutic approaches [9, 25-27].

Cell-free DNA (cfDNA) consists of short sequences of double-stranded DNA that circulate in plasma in an unbound form [28]. The presence of unbound DNA in human serum has been known for over 70 years [29]. It has been found in increased concentrations in patients affected by myocardial infarction [30], lupus erythematosus [31], lymphoma, and other neoplastic conditions [32]. In the case of neoplasia, it is thought to derive from cell apoptosis and necrosis and has been associated with neutrophils in disease states [33]. Canine cfDNA has been

measured and evaluated as a prognostic indicator of lymphoid neoplasia [34], for genomic aberration detection in mammary carcinomas [35], and for assessing the severity of acute pulmonary thromboembolism [36]. Equine fetal cfDNA in the maternal circulation has been used for fetal sexing [37, 38]. Plasma cfDNA has been explored as a prognostic marker for septic and non-septic illness in foals, but significant differences from healthy foals were not found [39]. In healthy subjects, circulating cfDNA originates predominantly from blood cells, endothelial cells, and hepatocytes, but in disease, the affected tissues also contribute to the circulating cfDNA [40, 41]. Increased release of cfDNA into bodily fluids or decreased clearance may result in elevations or differences associated with disease, age or sex [42, 43]. Circulating cfDNA comprises sources from totally normal tissue sources, diseased tissues and circulating mitochondrial DNA [44].

There is a growing interest in the role of cfDNA release from orthopaedic tissues and responses in biofluid concentrations to disease and exercise [45-47]. An early report comparing human cfDNA values in synovial fluid and serum from patients with rheumatoid arthritis (RA), OA, and other orthopaedic conditions found the highest concentrations in patients with RA compared to low levels in patients with OA or traumatic arthritis [48]. Further work exploring cfDNA in cases of RA has found that not only are elevations in the blood and synovial fluid indicators of disease, but they are also associated with disease progression [48]. Concentrations can also be monitored for other manifestations of immune-mediated joint disease [49]. Peer-reviewed publications investigating the diagnostic potential of cfDNA in equine orthopaedic disease are lacking. Human plasma cfDNA concentration also increases with intense exercise [50-55]. For example, strenuous intermittent exercise increases plasma cfDNA in athletes [56, 57]. Comparable published data for the horse are currently unavailable.

The objectives of this study were to report the baseline concentrations of synovial fluid (SF) and plasma cfDNA in a group of young, healthy Thoroughbreds and to compare the concentrations of SF and plasma cfDNA over time in control horses with those with traumatically induced carpal osteoarthritis undergoing a standardised exercise regimen.

3.3 Materials and Methods

The study was approved by Massey University Animal Ethics Committee (protocol MUAEC 14/18). Fifteen 2-year-old and two 3-year-old New Zealand-bred Thoroughbred fillies (*n* = 17) that had not been used or trained for athletic activity were selected for the study. Inclusion criteria were lack of clinical abnormalities based on a daily general physical examination, lameness at the walk and trot, and absence of radiographic abnormalities of the carpi. Before the study, all horses were kept at pasture as per normal New Zealand husbandry practice [58]. Before the trial and assignment to control and treatment groups, cfDNA was measured in plasma as described below from 16 of these horses during the same month (one (1/17) horse was not available until closer to the time of the experimental phase of the study). Based on the mean ± s.d. of these pre-trial values (632 ± 91 μg/L), $\alpha = 0.05$, and $\beta = 0.20$, a sample size for each group in the trial was estimated as $n = 8$. After blocking horses for sire (ensuring animals with the same sire were not within the same group) and age, each horse was assigned to one of two groups. For nine horses, an 8 mm osteochondral fragment was created arthroscopically in the distal dorsal aspect of the radial carpal bone of one randomly selected limb to induce OA using an established equine model [59, 60]. Per the published protocol, the radial carpal bone fragment was left attached to the dorsal joint capsule reflection. The parent bone was debrided with a motorized burr to make a ~15 mm-wide defect (including the fragment's width), and the debris was left in the joint as previously described. These horses

were identified as the OA horse group, their operated middle carpal joints as OA joints, and the unoperated contralateral middle carpal joint as OA-control joints. The other eight horses (sham horse group) underwent arthroscopic exploration only of one randomly selected middle carpal joint (sham joint), and the unoperated contralateral middle carpal joint served as a sham-control joint. All horses were administered procaine penicillin (22 mg/kg, IM) once before surgery and phenylbutazone immediately after completion of the procedure (4.4 mg/kg, IV) and for the following 4 days (4.4 mg/kg, PO, q 24 h). During the trial, horses were housed in stalls with limited free exercise (30 min/day in a 6 m × 6 m yard) throughout the study. After a 14-day post-surgical recovery period, all horses started a 7-week-long treadmill exercise protocol (5 consecutive days, Monday through Friday each week, followed by 2 days of rest). On each treadmill day, horses were exercised for 2 min at a trot (4–5 m/s), followed by 2 min at a gallop (8–9 m/s), and then a further 2 min at a trot (4–5 m/s). The model has been reported to mimic naturally occurring equine traumatic OA [61]. Horses were assessed and scored for lameness at the walk and trot, joint effusion, and response to carpal flexion preoperatively and weekly thereafter by the same board-certified surgeon (Appendix A, Tables 1-3). Lameness scores were assigned according to the American Association of Equine Practitioners lameness scale [62].

Radiographs of both carpi were obtained for all horses before entering the study and at its completion. For these images, scores were assigned by two board-certified radiologists blinded to group allocation based on lysis and osteophyte formation (Appendix A, Table 4). These clinical and radiographic scores confirming the establishment of OA in this study have been published [63].

Blood (~10 mL) was collected by venipuncture and SF (~3-4 mL) via bilateral middle carpal arthrocentesis from all horses starting on Day 0 immediately before induction of OA or sham surgery and weekly each Monday before the commencement of exercise until Day 63. Blood samples were chilled on ice, and approximately 4–5 mL of plasma was obtained after centrifugation within 60 min of collection. The plasma obtained was divided into 1 mL aliquots and stored at −80 °C for later analysis. An aliquot (~0.5 mL) of SF was placed in ethylenediaminetetraacetic acid (EDTA) tubes and processed to determine the total nucleated cell count (TNCC) and nucleated cell differential count. A refractometer determined the total protein concentration on fresh SF immediately after collection. The remainder of the SF was divided into ~1 mL aliquots and stored at −80°C until later analysis. At the time of cfDNA analysis, plasma and SF samples were thawed at room temperature and divided into aliquots of 20 µL. cfDNA concentrations were measured by fluorometry (Qubit dsDNA High Sensitivity Assay kit, Life Technologies, Carlsbad, CA, USA) in triplicate using a fluorometer (Qubit 2.0 fluorometer, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's specifications [64]. Calibration was performed with the standards provided by the manufacturer before each run. The fluorescence detected by the fluorometer is proportional to the concentration of double-strand DNA in the sample. For each plasma sample, cfDNA replicate values were averaged.

Lameness, flexion tests, effusion, radiographic scores, and TNCC were analysed using Freidman's two-way ANOVA. Where differences were significant, post hoc analysis using Mann–Whitney U tests was performed. Similarly, Freidman's two-way ANOVA and Wilcoxon matched-pairs test were used for total protein concentrations. Significance was set at $p < 0.05$. Triplicate fluorimetry plasma samples were averaged . Plasma cfDNA data were not normally distributed and were analysed with Freidman's two-way ANOVA to account for repeated

measures (Statistica 11, StatSoft, Tulsa, OK, USA). When significant differences were observed, post hoc analysis was performed using Wilcoxon matched-pairs test to detect the sources of temporal differences within OA and control groups. Synovial fluid data were not normally distributed, and outliers were eliminated (excluded if a value outside of the range of [Q1 − 1.5 x IQR, Q3 + 1.5 x IQR]) at each time point. For each SF sample, cfDNA replicate values were averaged. A paired comparison of SF cfDNA between left and right joints on Day 0 was performed using Kruskal–Wallis ANOVA. Synovial fluid from OA joints was compared with SF from OA-control joints and SF from sham joints over time using Freidman's two-way ANOVA. Where differences were significant, post hoc analysis using Wilcoxon matched-pairs test and Mann–Whitney U tests was performed to detect the sources of temporal differences within groups. The precision of the assay in SF and plasma was determined for these samples. The coefficient of variation (CV) was calculated using standard deviation (s.d.) and mean (μ) of the replicates for each sample (CV = s.d./μ) [65].

3.4 Results

On Day 0, the baseline median SF cfDNA concentration was 504 μg/L (IQR = 236), and there was no significant difference between groups and between carpi within horses. After the creation of the osteochondral fragment, cfDNA concentrations in SF were significantly higher for OA joints than for sham-operated joints on Days 28 (median 1430 and 631, respectively, p = 0.017) and 63 (median 1537 and 606, respectively, p = 0.021); SF concentrations were trending towards significance for OA joints on Days 35 (p = 0.059) and 56 (p = 0.070). Within OA horses, there was a significant difference in concentrations over time in the OA joints (p = 0.036) but not in the OA controls (p = 0.12). SF cfDNA concentrations for OA joints were

significantly higher at all time points (*p* range: 0.010– 0.043) compared to Day 0, except for Day 49 (Figure 3.1).

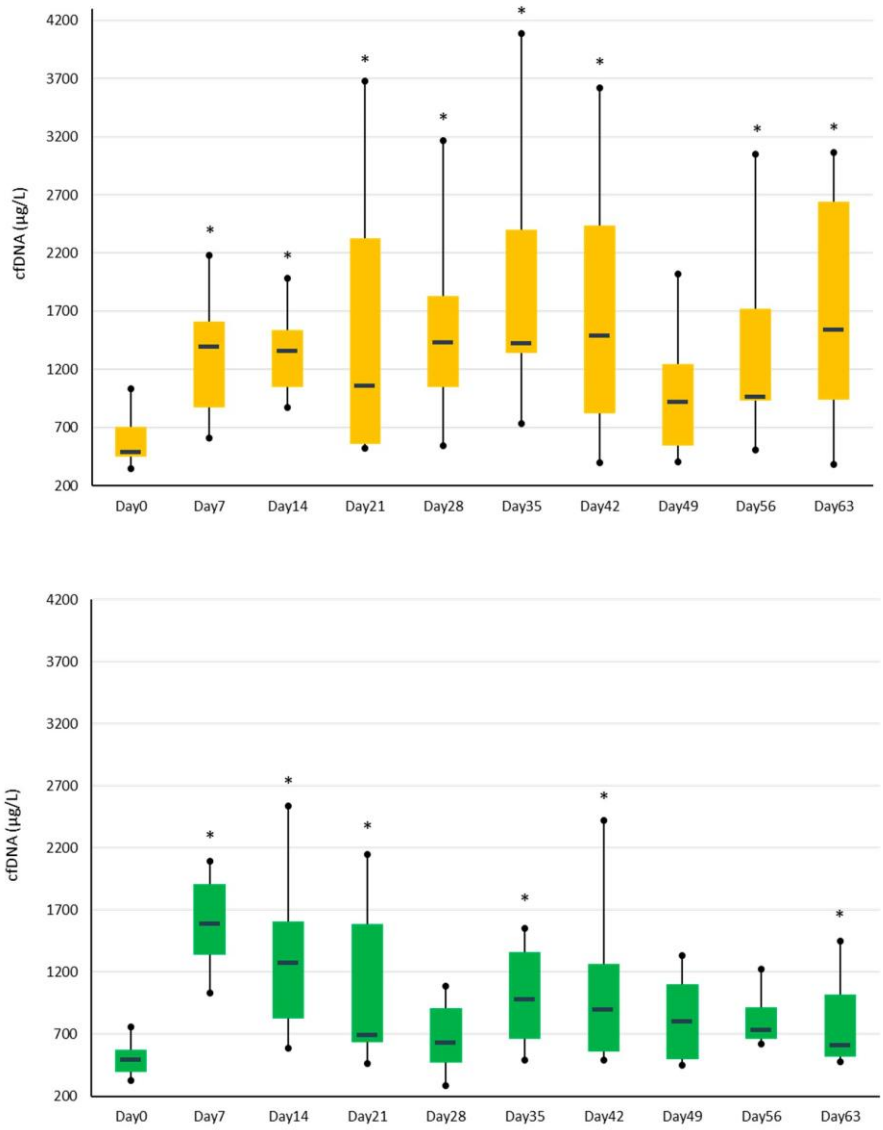

Figure 3.1 Synovial fluid cell-free DNA concentrations in the OA (top graph) and sham (bottom graph) joints over time. The * indicates significant differences ($p < 0.05$) from the respective Day 0 values. The bars represent the interquartile range, and the horizontal lines within the bars are the median values. Whiskers indicate the minimum and maximum values.

The baseline median plasma cfDNA concentration on Day 0 was 626 µg/L (IQR 117), and there was no significant difference between groups. No statistically significant effect of the OA model on plasma cfDNA concentrations was detected. However, significant differences in

plasma cfDNA concentrations over time were identified. This was true for pooled data (all horses), where Day 0 values significantly differed from those at Days 14, 21, 28, 35, and 42. This was also confirmed within groups: In the OA group, Day 0 concentrations were significantly higher than those for Days 28, 35, and 42 ($p < 0.05$); in the sham group, Day 0 values were significantly higher than those for Days 14, 21, 28, and 42 ($p < 0.05$) as shown in Figure 3.2.

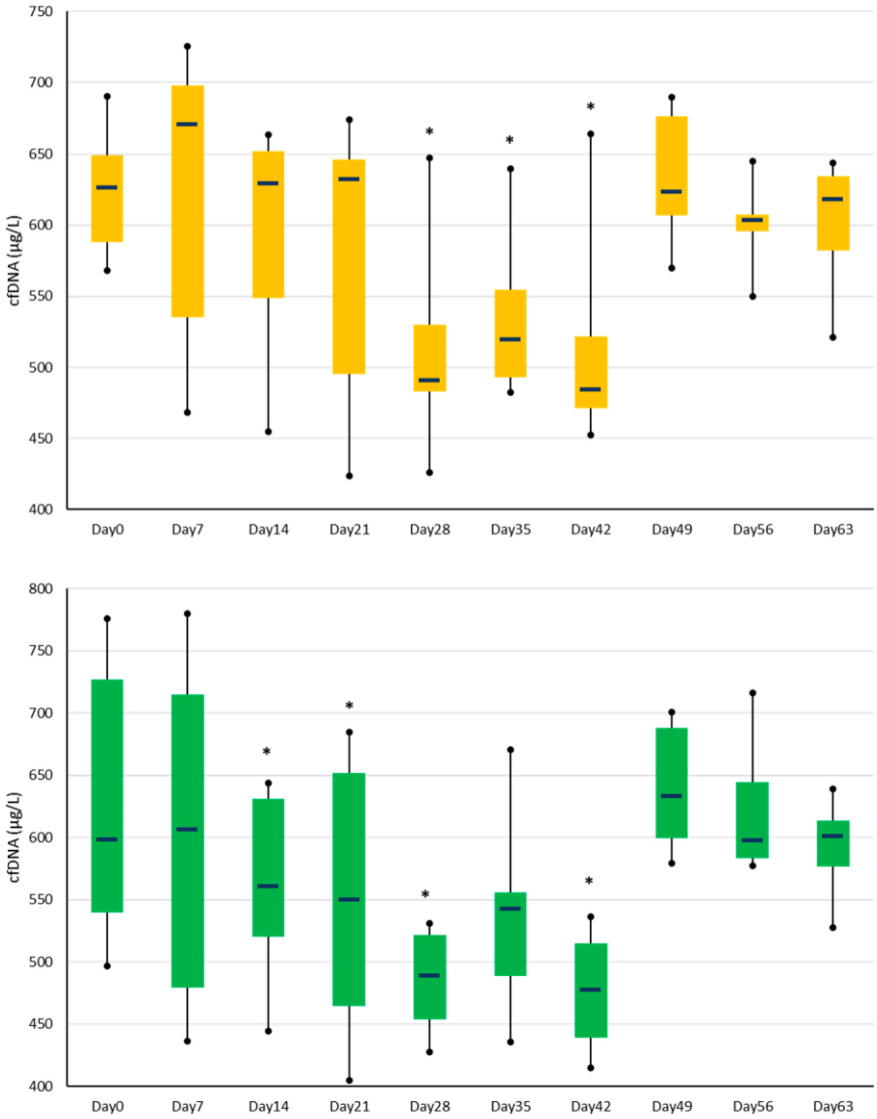

Figure 3.2 Plasma cell-free DNA concentrations in the OA (top graph) and sham (bottom chart) groups over time. The * indicates significant differences ($p < 0.05$) from the respective group Day 0 values. The bars represent the interquartile range, and the horizontal lines within the bars are the median values. Whiskers indicate the minimum and maximum values.

Synovial fluid TNCCs did not differ significantly between groups on Day 0 and over time within groups. There were no significant TNCC differences between OA and sham joints, OA and OA-control joints, sham and sham-control joints, and OA-control and sham-control joints. Total protein concentrations in SF were not significantly different between operated sham joints and OA joints at Day 0 or any other time throughout the study, except on Day 14 (p = 0.01). No significant differences were observed between sham-control and OA-control joints.

The precision of the assay for cfDNA was high. The mean plasma coefficient of variation (CV) was 2.70% (95% CI 2.45–2.96), and almost all CV values fell within 5% of the mean CV. The synovial fluid mean CV was 0.95 % (95% CI 0.87–1.03), and almost all CV values fell within 3% of the mean CV.

3.5 Discussion

To the authors' knowledge, this study is the first to report cfDNA concentrations in SF and plasma of healthy young racehorses. An osteochondral fragment model of carpal OA was used to investigate the use of cfDNA as a potential biomarker for OA. Our results show that, although the concentration of cfDNA in plasma was not useful for differentiating horses affected by induced carpal OA from controls, its use in the synovial fluid has potential value for monitoring change over time.

The SF of both OA and sham joints contained significantly increased cfDNA concentrations compared to the respective Day 0 values. The increase in sham joints may be due to the effect of multiple arthrocentesis during the study. However, although multiple arthrocenteses have been shown to induce an increase in OA biomarkers in SF of exercised horses, this effect is minimized when weekly intervals between arthrocentesis are used, as was completed in our

study [66]. The early increase in cfDNA concentration in sham joints may be attributed to the traumatic effect of the sham surgery. Similar observations have been made in sham joints with significant increases in the anti-inflammatory mediator interleukin-1 receptor antagonist and the pro-inflammatory cytokine tumour necrosis factor alpha after arthroscopy [67].

Compared with sham joints, the significant change over time of SF cfDNA concentrations in OA joints suggests that the effects of induced OA were sustained, and that exercise was not responsible for the observed differences. This is consistent with previous research where the effects of exercise on synovial fluid biomarkers differed from those caused by the induction of OA [16]. Although the half-life of cfDNA in horses has not been documented, it is likely to be within the range reported in people and dogs (1.5 h and 5.6 h, respectively) [68, 69]. In the current study, synovial fluid samples were taken weekly approximately 72 h after cessation of the previous week's exercise. The authors, therefore, suggest that any increase in the concentration of cfDNA is unlikely to be associated with exercise but rather with the ongoing contributions from the diseased tissues.

To the authors' knowledge, there is no peer-reviewed published literature on cfDNA in SF in horses. The significance of the current findings concerning naturally occurring OA remains unknown at this stage. When compared with sham joints, the significantly higher concentrations of SF cfDNA at Days 28 and 63 in OA joints confirm that SF cfDNA measurement can identify OA-affected joints in a research setting. However, validation in cases with naturally occurring disease is necessary before making recommendations for its clinical use.

The current study did not show a significant difference between plasma cfDNA concentrations of horses with induced OA of the middle carpal joint and horses of the sham group over 2 months. Median plasma cfDNA concentrations in the OA group were higher than in the sham

group at most time points. However, because significant differences in this biomarker between OA and sham horses were not found for plasma cfDNA, the authors conclude that it is not a sensitive predictor of experimental OA for this biofluid. An ideal property of plasma biomarkers is a high level of sensitivity in detecting disease. This means that the molecules being detected, in this case, cfDNA, must be circulating in sufficient concentrations associated with the burden of disease to be detected. Studies exploring the role of cfDNA in people and dogs have investigated more severe conditions (cancer, severe trauma, sepsis, rheumatoid arthritis, and acute pulmonary thromboembolism), with significant systemic responses and a high burden of disease [36, 45-47]. Frisbie et al. [16] reported significant changes in serum biomarkers of cartilage degradation using the same OA model. However, as observed for other species with different medical conditions, a more substantial or naturally occurring disease burden may be required to generate significant increases of OA-associated cfDNA concentrations in the peripheral circulation [36, 45-47]. Even though, a similar lack of diagnostic sensitivity was demonstrated in a recent study in foals that showed no significant differences in cfDNA concentrations between critically ill and healthy foals [39], suggesting that even with significant systemic illness, plasma cfDNA concentration may not be a useful marker in young horses. In contrast, cfDNA is significantly higher in emergency patients $\geq$ 2 years old with colic than in control horses and ponies [70]. In the current OA model, plasma cfDNA measurement was not sufficiently sensitive for diagnostic purposes.

Nevertheless, temporal changes in plasma cfDNA concentrations were observed in both groups at Days 14 and 28 after surgery in the sham and OA groups, respectively. Thereafter, a decline in cfDNA concentrations was seen until 4 weeks after the start of training, after which cfDNA concentrations returned to baselines. The reason for this is unknown, as there is limited information on the effects in vivo of physical loading on the integrity and metabolism of

synovium [71]. In humans, plasma cfDNA concentrations increase with exercise but rapidly decrease within 30 min of cessation of exercise [53], returning to baseline values within 2 h [52]. Similarly, in working dogs, exercise is associated with a transient increase in cfDNA concentrations 30 min after the end of the exercise, returning to baseline levels 90 min thereafter [72]. Details of the effects of the training regime on cfDNA concentrations in the horses in the current study are unknown, but further investigation of the half-life cfDNA in equids and the optimal time points for sampling after exercise or disease induction is recommended.

Based on the current results, plasma cfDNA concentrations alone do not provide significant information for focal diseases such as that evaluated in this study. In the future, it may be helpful to investigate whether specific sequences within the cfDNA rather than total cfDNA concentrations, as described for specific human cancers [73, 74], better identify horses affected by OA.

The small CV found for the cfDNA assay in horses is consistent with the mean CV (2%) using the same analytical method reported in dogs [75] and a report that used the assay in horses admitted as emergency patients with colic and a control group [70]. The precision of the assay meets the USA Food and Drug Administration's requirements for bioanalytical method validation (Food and Drug Administration, 2018). The precision of the assay supports further use of this fluorometric method for future studies of equine conditions with a high burden of disease.

The TNCC in SF did not change significantly within and between groups over time. Synoviocentesis causes an increase in SF TNCC [76], although transient (1-2 days), which may be a confounding factor when utilizing TNCC as an outcome measure. Since both groups were

sampled during the same timeframe and with equal frequency, small differences in TNCC may have been skewed by the response to synoviocentesis. However, the effects of repeated arthrocentesis on TNCC are generally short-lived and not likely to be a factor in our study, given the 7-day interval between samplings. Total protein concentrations in SF did not significantly change over time and among groups. The lack of significant increases in SF TNCC and total protein concentration in our study is consistent with mild to no increase generally observed in joints affected by OA [77] but in contrast with other studies in which this same carpal OA model was used [16]. The reasons for the differences between the work of Frisbie et al. [16] and the current study are unclear as the technique used for OA model was under the direction of an author common to both studies, and both reports used 2-year-old horses. To reduce interhorse variation as a random source of error, the current study used a cohort of the same breed and gender, bred, and raised at pasture in the same country. These signalment details were not described in the report by Frisbie et al. [16]. Such differences, if present, may have contributed to SF TNCC findings in the current study that were not consistent with those from the previously published work.

The lack of an age-matched unexercised control group limited the interpretation of the role of exercise on cfDNA concentrations. However, based on the short half-life of cfDNA reported in other species, the timing of sampling 72 h after treadmill exercise is likely to have prevented the detection of confounding exercise-related effects in the current study. Determination of the half-life of this marker in horses may facilitate the identification of the optimal time for sampling.

To reduce variability in results attributed to age or gender, differences due to these factors were not explored in the current study. Plasma cfDNA concentrations are higher in older (20

months) rats compared to younger ones (3 months) [78]. In humans, individuals over 60 [79] and 90 years of age [80] have higher concentrations of cfDNA. Gender has not been associated with differences in human cfDNA concentration except in women over 60 who had higher concentrations [79]. A larger equine population with different age categories, inclusive of intact and castrated males, may be more representative for establishing baseline concentration ranges for cfDNA in the wider population of horses.

This study reports baseline plasma and SF cfDNA concentrations in horses and examines the effects of focal orthopaedic trauma on this marker. The OA induced using a well-established model in the middle carpal joint in horses did not affect plasma cfDNA concentrations over 2 months suggesting this biomarker is not a valuable tool for early detection using this biofluid. Conversely, SF cfDNA concentrations have the potential to detect OA in an experimental setting and with further evaluation may be suitable for monitoring disease progression. Further study and clinical validation are necessary before recommending its use in the field for equine practice.

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Foreword to Chapter 4

In Chapter 3 an established equine model of osteoarthritis was used in a group of 17 Thoroughbred fillies and cfDNA was investigated as a potential biomarker of OA. The results indicated that cfDNA concentrations in plasma are not a good marker of OA in the early stages of disease, but in synovial fluid these showed potential to detect OA in a research setting. The next step of this research in Chapter 4 utilises a different approach to detect OA in the same group of horses, where instead of targeting a single biomarker, a “biochemical fingerprint” of all infrared absorptive molecules in serum is evaluated with the use of infrared spectroscopy.

Chapter 4
Infrared Spectroscopy of Serum Fails to Identify Early Biomarker Changes in an Equine Model of Traumatic Osteoarthritis

4.1 Abstract

Objective: to determine the accuracy of infrared (IR)-based serum biomarker profiling to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal osteoarthritis (OA) from controls.

Methods: unilateral carpal OA was induced in 9 of 17 healthy Thoroughbred fillies, while the remainder served as sham operated controls. Serum samples were obtained before induction of OA (Day 0) and weekly thereafter until Day 63 from both groups. Films of dried serum were created, and IR absorbance spectra acquired. Following pre-processing, partial least squares discriminant analysis (PLSDA) and principal component analysis (PCA) were used to assess group and time differences and generate predictive models for wavenumber ranges 1300-1800 cm^{-1} and 2600-3700 cm^{-1}.

Results: the overall correct classification rate when classifying samples by group (OA or Sham) was 52.7% (s.d. = 12.8%), while it was 94.0% (s.d. = 1.4%) by sampling Day. The correct classification results by group-sampling Day combinations with pre-intervention serum (Day 0) was 50.5% (s.d. = 21.7%).

Conclusions: with the current approach IR spectroscopic analysis could not differentiate serum of horses with induced carpal OA from that of controls. The high classification rate obtained by Day of sampling may reflect the effect of exercise on the biomarker profile. A longer study

period (advanced disease) or naturally occurring disease may provide further information on the suitability of this technique in horses.

4.2 Introduction

Osteoarthritis (OA) is the leading cause of joint disease in humans and is responsible for significant morbidity, with clinical signs such as pain and disability leading to marked economic losses worldwide [1-4]. It is estimated that 100 million Europeans suffer from OA [5], with knee OA estimated to affect between 18-40% of 60–79-year-old people [6, 7]. In the U.S. over 91 million adults (37%) were affected by OA in 2015 [8]. Similarly, joint disease represents a significant clinical condition in horses. Approximately 50 ± 3.2% of U.S. multi-horse operations experience one or more lameness cases annually, with approximately 50% limb or joint related [9]. In 2007, 4 million of the 7.3 million horses in the U.S. were estimated to be affected by OA, with a negative impact on 4.6 million people (owners, service providers, and businesses) associated with the equine industry producing goods and services of US$38.3 billion [10].

Compared with advances in human orthopaedic research [11], there is a lack of a validated and repeatable approach to staging clinical osteoarthritis in the horse and case definitions referable to OA in specific joints. This has led to reliance upon well-validated models of traumatic OA in the horse as a proxy for naturally occurring disease, particularly in its early stages [12-16]. The need for inexpensive and reliable tests for early detection remains; it is currently difficult to obtain an early diagnosis of OA and objectively monitor disease progression or response to therapy in clinical cases [17].

Many different soluble biomarkers associated with joint metabolism and pathology have been investigated as potential markers of OA [18-20]. In parallel with medical advances, a significant body of research in horses has been published, and progress has been made in identifying

potentially clinically useful markers of OA [12, 16, 21]. Despite advancements in biomarker research [22-25] and limited application to equine clinical cases [26], the wider adoption and validation within a clinical practice setting of candidate biomarkers is yet to be established [19]. The main limitations of the techniques evaluated are the lack of consistency, the limited practicality of the tests in a clinical setting, and the relatively high costs when used as a tool for disease surveillance [27].

Fourier-transform infrared (IR) spectroscopy has been used to identify a "biochemical fingerprint" of all molecules in biofluids, rather than separating single molecules or biomarkers associated with a disease or physiological states [28]. It provides a means for detecting quantitative and qualitative changes in the molecular profile of a biofluid, accounting for known and unknown markers of disease. This aspect is particularly important since it is recognized that OA may be initiated by trauma and inflammation in any or all of the multiple joint tissues, including the synovial membrane, fibrous joint capsule, subchondral bone, periarticular ligaments, or articular cartilage [29]. The advantages of IR spectroscopy as a diagnostic tool are its high sensitivity and specificity in other diagnostic applications [30, 31], the low cost (no reagents are required), and the low-invasive nature of the sample collection (serum) for testing. This technique has been investigated as a potential diagnostic and screening tool for joint disease in human synovial fluid [32-34] and serum [35], focusing on rheumatoid arthritis. However, these investigations have been in clinical cases with advanced disease and not applied to early detection of OA. In a rabbit model of knee osteoarthritis, IR spectroscopy of serum proved to be a sensitive approach to differentiate between rabbits with OA and controls [36]. This model produces severe acute joint instability, rather than focal osteochondral trauma often seen in young human and equine athletes [37, 38]. Serum-based IR spectroscopy is capable of differentiating dogs with naturally occurring knee OA from

unmatched controls [39]. In the horse, synovial fluid IR spectroscopy has been used in the diagnosis of clinical osteochondrosis and traumatic osteoarthritis [38, 40]. This method has been applied to the diagnosis of clinical OA based on spectra of equine serum, but the comparison group used in this study was not verified as free of OA [41].

The objective of this study is to determine the feasibility and accuracy of IR-based serum biomarker profiling to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal OA from controls. Our hypothesis is that IR biomarker profiling of serum will be able to differentiate horses with induced carpal OA from controls.

4.3 Materials and Methods

This randomized prospective experimental study was approved by the Massey University Animal Ethics Committee (MUAEC 14/18). The sample size for treatment and control groups was estimated based upon previous publications using the same model for biomarker and treatment trials [12-15].

4.3.1 Animals

As part of a larger biomarker study, fifteen 2-year-old and two 3-year-old female Thoroughbred horses (n=17) not previously used or trained for any athletic activity were selected for the study. The inclusion criteria were lack of clinical abnormalities on physical examination by two equine surgical specialists, absence of lameness at the walk and trot before and after limb flexion tests, and no abnormalities on radiographs of the carpi immediately before the study. All horses were kept on grass pasture before the study. Nine horses were randomly assigned to the OA group, and eight to the Sham horse group, after

blocking horses for sire and age. The order of procedures, Sham operation or surgical OA induction, was randomized. At the end of the trial the horses in the OA group were euthanised for tissue collection as part of another study (unpublished data), while Sham operated horses were rehomed.

OA group: Procaine penicillin at 22 mg/kg IM (Phoenix Pharmacillin 300, 300mg/ml, Phoenix Pharm, New Zealand) was administered to all horses once before surgical intervention. All horses then were subjected to general anesthesia for arthroscopic surgery. Anesthesia was induced with 2.5 mg/kg of ketamine (Ceva Ketamine injection; Ceva Animal Health Pty Ltd. Australia) and 0.01 mg/kg of Diazepam (ilium Diazepam Injection USP; Troy Laboratories, Australia) intravenously. Following orotracheal intubation, anesthesia was maintained with isoflurane (Isoflurane; Bayer New Zealand Ltd, New Zealand) in 5 L/minute of 100% oxygen. Following general anaesthetic induction and aseptic preparation of a randomly chosen carpus, an 8 mm osteochondral fragment was arthroscopically created in the middle carpal joint using a bone gouge in the distal dorsal aspect of the radial carpal bone to induce traumatic OA as previously described [14, 15]. The osteochondral fragment remained attached to the dorsal joint capsule reflection. The parent bone from where the fragment had been separated was debrided with a motorized bone burr to create a ~15 mm-wide defect (including the width of the fragment). The articular debris was left in the joint. These horses were identified as the OA horse group. All horses were administered phenylbutazone immediately after completion of the procedure at 4.4 mg/kg IV (Nabudone P, 200 mg/ml, Troy Laboratories, Australia) and for the following four days at 4.4 mg/kg PO, every 24h (Equine Bute Paste, 200mg/ml, Randlab, Australia). Postoperatively horses were examined twice daily to evaluate their comfort and well-being.

Sham control group: Sham horses underwent arthroscopic exploration only of one randomly selected middle carpal joint. The perioperative pharmaceutical treatment protocol and monitoring plan was identical to that of the OA group.

4.3.2 Post-operative exercise and clinical assessment

After a 14-day recovery period in horse stalls with 30 minutes of daily turnout in a 6 m x 6 m yard, a 7-week-long treadmill exercise protocol (5 days a week) was initiated. Horses were exercised daily for 2 minutes at a trot (4-5 m/sec), then 2 minutes at a gallop (8-9 m/sec), and then 2 minutes at a trot (4-5 m/sec). The model mimics naturally occurring equine traumatic OA [12, 13]. Scores were assigned on a weekly basis for lameness, joint effusion, and response to carpal flexion and at the end of the study for radiographic changes (Appendix A, Tables 1-4) as confirmation of the establishment of OA [12-16].

4.3.3 Serum sample collection

Blood (~10 mL) was collected by left jugular venipuncture immediately before induction of OA (or Sham surgery) (Day 0), and then weekly from all horses until Day 63. After collection, blood samples were allowed to clot in plain tubes, and 4 to 5 mL of serum obtained after centrifugation at 5000 rpm (3400 g) for 5 minutes within 60 minutes of sample collection. The serum was divided into 1 mL aliquots and stored at -80°C until later analysis.

4.3.4 Infrared spectroscopy

Serum samples were thawed at room temperature and replicate (x 6) dry films of 8 µL made for each sample on a silicon 96-well microplate [17, 38, 39]. The microplate was mounted on a multi-sampler accessory (XY Microtiter Plate Accessory, PIKE Technologies, Madison, WI, USA) interfaced with an IR spectrometer (Tensor 27, Bruker Optics, Preston, Victoria, Australia). Infrared absorbance spectra in the range of 400 to 4,000 cm^{-1} were generated and recorded

in transmission mode with proprietary software (OPUS software, version 6.5, Bruker Optics, Ettlingen, Germany). For each acquisition, 512 interferograms were averaged, and Fourier transformed to obtain a spectrum with a resolution of 4 cm^{-1}.

4.3.5 Analyses and model development for classification of spectral data

Spectral files were imported into proprietary spectral manipulation software (The Unscrambler Xv10.5.1, Camo Software, Oslo, Norway) and converted into delimited csv files for further analyses. All subsequent analyses were performed in R (V3.6.3, R Core Team, Auckland, New Zealand; ChemoSpec and MixOmix packages V 4.3.34 and V6.8.5 respectively).

Spectral pre-processing: Savitzky-Golay filtering was applied to all spectra with a 1st-order derivative of the signal, a 1st order polynomials function, and a smoothing window of width 5; parameters were tuned to maximize spectral separation by Day. An analysis of sensitivity was performed, and similar performances were also retrieved for other combinations of filter parameters. Then, standard normal variate (SNV) transformation was used to normalize spectra and remove baseline effects, reducing within-class variance [42]. Spectra values in the ”fingerprint” regions between 1300-1800 cm^{-1} and 2600-3700 cm^{-1} were selected for further analyses. We did not remove any outlier samples as their number did not exceed the threshold of extreme PCA scores expected by chance (5%), and further inspection did not reveal any anomaly with the samples.

Classification model development: a series of predictive models were built to predict the horse group (task i; OA vs Sham), the sampling Day (task ii; 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63) and to assign samples a day-group class label, except Day 0 (i.e. prior to interventions) for which OA and Sham groups were pooled (task iii; n= 19 classes). Partial least squares discriminant analysis (PLSDA) [43] was initially used in multilevel mode to account for repeated

measures. Predictions were made using the first ten (10) principal components (PC) of the PLSDA as no significant performance improvement was observed with > 10 PCs and more than 50% of the variance of the design matrix was accounted for in all explored scenarios. More specifically, scores along each PC was computed for each new sample. Then new samples were assigned to the class with the closest centroid, relying on the Mahalanobis metric, as it was observed to lead to the most accurate class prediction [44]. The classification rates were computed based on the classification outcome of the spectra from each horse. Each horse was used in turn as a test horse (”leave-one-horse-out”), while all other horses (16) were used for model training. For task i, we denoted TP1 as the count (among all the leave one horse out repeats) of the true OA spectra correctly classified as OA, FP1 as the count of true OA spectra wrongly classified as Sham spectra, TP2 as the count of true Sham spectra correctly classified as Sham, and FP2 as the count of true Sham spectra wrongly classified as OA spectra. Then, the OA classification rate was defined as TP1/(TP1+FP1), the Sham classification rate was defined as TP2/(TP2+FP2), and the overall classification rate was defined as (TP1+TP2)/(TP1+TP2+FP1+FP2). We defined the overall and class-specific classification rates similarly for tasks ii and iii.

Logistic and multinomial regression with L1-regularization, random forests, Support Vector Machines, and convolutional neural networks were also explored as classification methods to check that the PLSDA underlying assumptions were not limiting the performance. The performance of these different models offered no significant advantage over, and was similar to, that obtained with PLSDA. Moreover, PLSDA offered a visualisation of the samples by plotting their scores in its principal component space. Therefore, only the results obtained with PLSDA are reported.

4.4 Results

4.4.1 Spectral pre-processing

The normalized spectra of serum from OA and Sham groups are shown in Fig. 4.1 (left). There was no visually apparent difference in the pattern between the two groups. The greater peaks representing infrared absorption are associated with proteins: bands centred at 1,650 cm^{-1}(amide I) and 1,545 cm^{-1} (amide II) correspond to stretching and bending vibrations on the amide C=O and N-H groups, respectively; the broadband at 3,300 cm^{-1} corresponds to the N-H group as well, but is a stretching vibration called the amide A mode [45]. The image of the pre-processed spectra is shown in Fig. 4.1 (right).

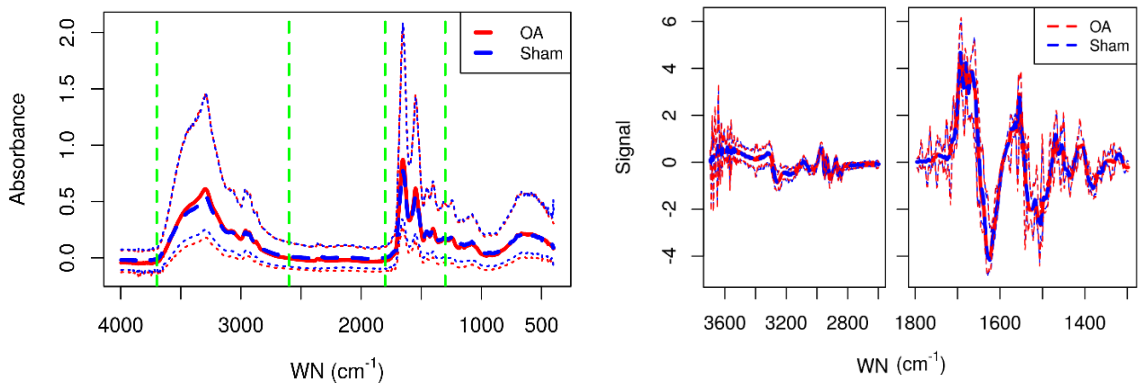

Figure 4.1 Spectra are shown (left). Savitzky-Golay filtered and smoothed spectra in fingerprint regions (3700-2600 cm^{-1} and 1800-1300 cm^{-1}) are shown (right). The median (thick line) and 2.5%- and 97.5%-quantiles (thin lines) for both groups are shown: osteoarthritis (OA) in red, Sham in blue.

4.4.2 Classification of IR spectra from OA versus Sham horses

The overall correct classification rate when classifying sample spectra according to their treatment group (OA or Sham) was 52.7% (standard deviation = 12.8%) using 10 PC, with 50.2% of the total variance explained. Fig. 4.2 shows the lack of clear separation for the first

2 components in the first sample space. The classification rates obtained overall and for each group (OA or Sham) for a number of PC comprised between 1 and 10 is shown in Appendix A, Fig. 1. Although the total variance associated with the model increases with the number of PCs, the classification accuracy is not improved by using more PCs.

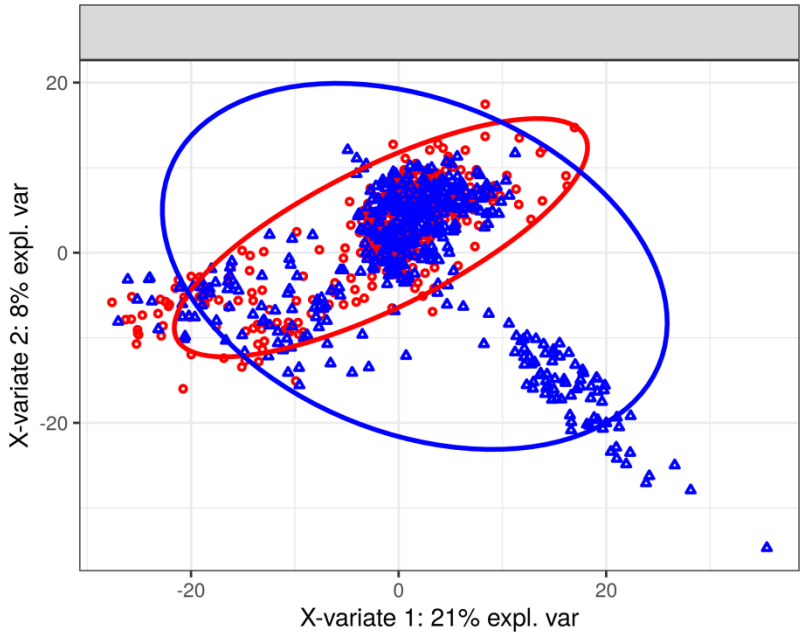

Figure 4.2 Sample score plots by group for the first 2 components of partial least squares discriminant analysis (PLSDA) (task i). Osteoarthritis (OA) horses in red circles, Sham in blue triangles.

4.4.3 Classification of IR spectra at different sampling times (Days)

The overall correct classification rate for samples according to their sampling Day (Days 0 to 63) was 94.0% (s.d. = 1.4%) when using 10 PC, with 53.2% of the total variance explained. The classification rates obtained overall and for each sampling Day for a number of PC comprised between 1 and 10 are reported in Appendix A, Fig. 2. In Fig. 4.3 are plots of all samples in the first 3 PC defined by the PLSDA performed with sampling Day as the outcome (not applying the leave-one-horse-out scheme).

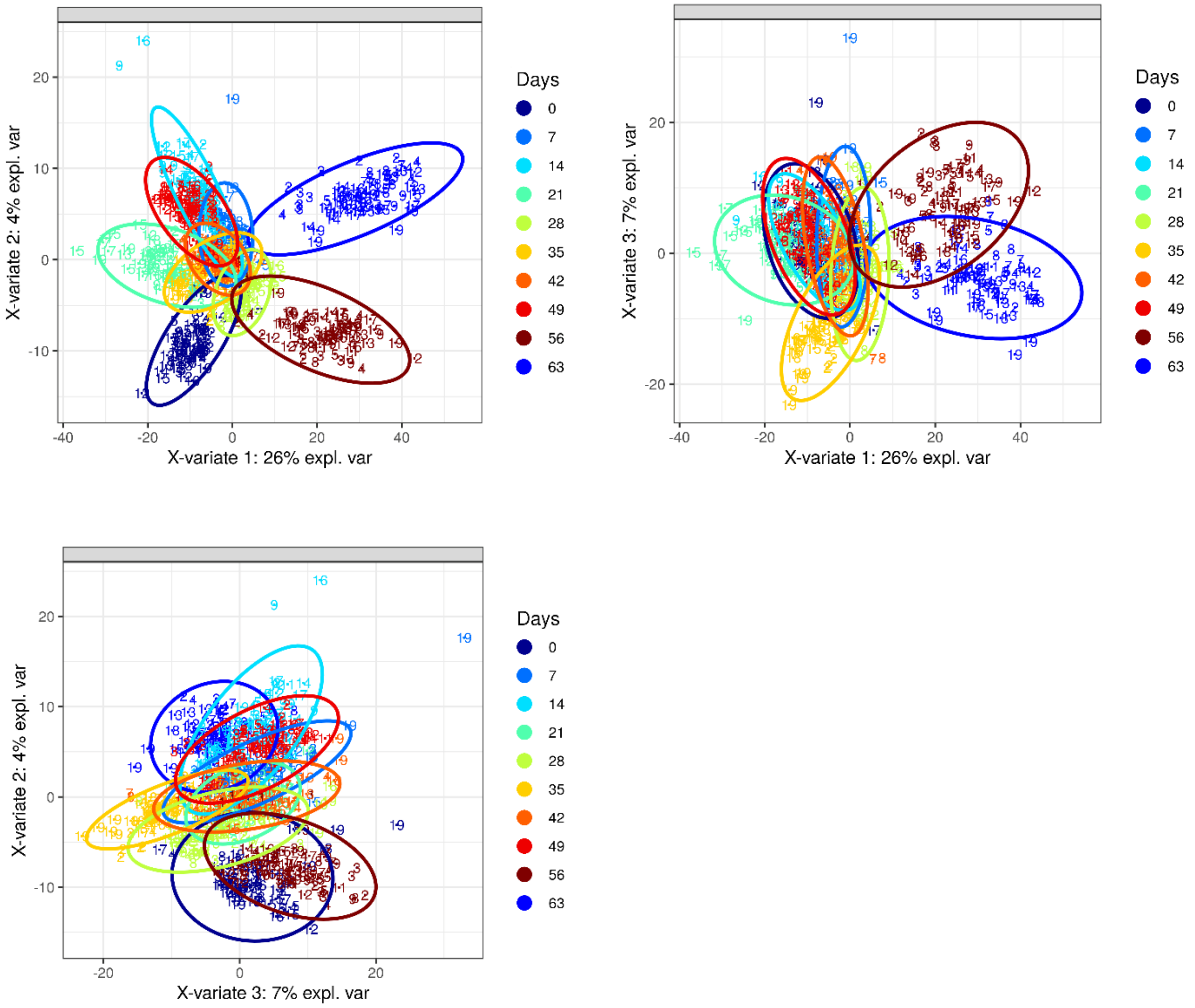

Figure 4.3 Sample plots of the partial least squares discriminant analysis (PLSDA) for sample day identification (task ii) in the first three principal components (PC) (PC2 vs PC1, top left plot; PC3 vs PC1, top right plot; and PC2 vs PC 3, bottom plot).

4.4.4 Classification of group-sampling day combinations with serum from Day 0

For task iii, the overall classification rate is 50.5% (s.d. = 21.7%) using 10 PC, with 53.2% of the total variance explained. All class-specific classification rates for this task are reported in Appendix A, Table 5, and Fig. 3.

The specificity for a class (group x Day) identification varies. Sampling Day identification was still performed accurately, with classification rates similar to those shown in Appendix A, Fig. 2. However, within sampling Days, the correct identification of groups (OA vs Sham) was limited, with classification rates ranging from 94.1% for samples collected prior to interventions (Day 0) to 85.0% (Day 35, Sham) and 20.0% (Day 42, OA). Lastly, Fig. 4.4 shows all samples for the first three PC of the PLSDA for this task (not applying the leave-one-horse-out scheme) and confirms the lack of separation by group. Incidentally, the separation of spectra by days is observed for a few samples.

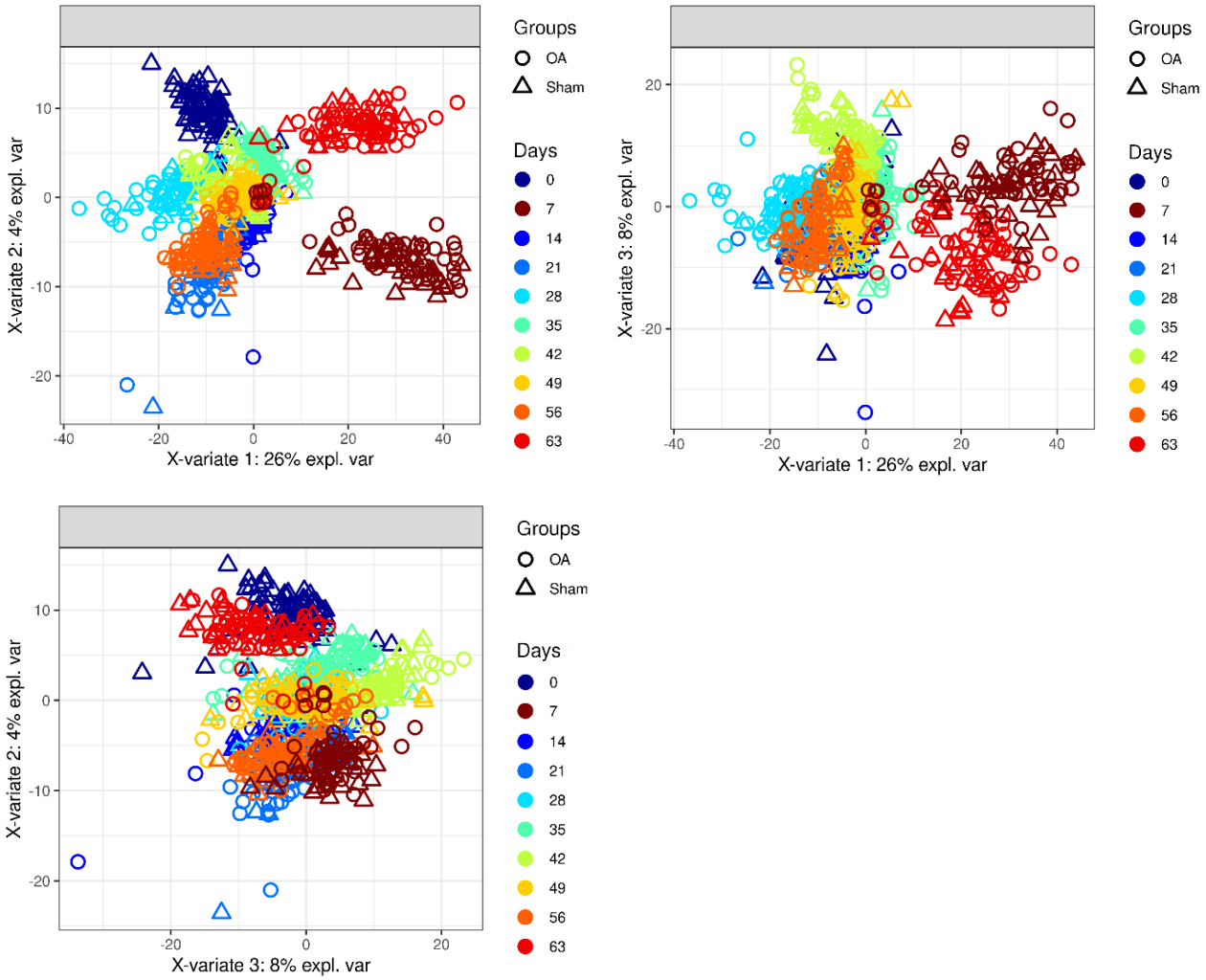

Figure 4.4 Individual sample partial least squares discriminant analysis (PLSDA) score plots in the first three planes for task iii. Samples are presented by Day (colour-coded) and groups: circles represent osteoarthritis (OA) samples, while triangles represent Sham samples.

4.5 Discussion

This study is the first to attempt evaluation of IR spectroscopy and spectral analyses to discriminate between serum from horses with traumatically induced OA and strictly characterized controls. However, our results showed that the approach described here failed to differentiate serum spectra of horses with early traumatically induced osteoarthritic changes (OA) from those of controls (Sham). Significant differences in the spectroscopic profile of serum were detected at different time points during a postoperative exercise program in both groups.

In a previous experimental study in rabbits using a model of knee OA (cranial cruciate ligament transection inducing marked instability) IR spectroscopy on serum allowed differentiation of affected animals from controls at biweekly intervals between 2 and 12 weeks post injury [36]. The joint instability created with this model results in early onset of OA, with a severe and debilitating burden of disease of all articular structures within 2 weeks, sufficient to contribute to detectable changes in the IR spectrum of rabbit serum [46]. Similarly, IR spectroscopy was able to identify serum of dogs with naturally occurring knee OA associated with cranial cruciate ligament rupture from controls, with high sensitivity and specificity [39]. In this prospective controlled study, the dogs recruited had clinically evident naturally occurring OA due to joint instability that was sufficiently advanced to warrant surgical intervention. The equine model used in the current study does not create marked joint instability. In agreement with other reports using the model, clinical signs attributed to the osteochondral fragments were minimal [12, 14, 15]. This model has been sufficient to demonstrate biochemical differences based on ELISA biomarkers [12]. However, a more severe equine model of joint injury that

includes instability, or prolonged follow-up period, may be required to exploit the discriminatory potential of IR spectroscopy of serum.

In contrast with our study, others utilizing the same equine model and one of naturally occurring OA in similarly aged horses of the same breed have demonstrated significant differences in specific ELISA based biomarkers (e.g. epitope CS846 and type II collagen carboxy-propeptide) between OA and control groups [12, 26]. Although an IR spectroscopy-based approach provides for a broad-based "fingerprint" of known and unknown biomarkers, few IR based assays of biofluids rely upon the detection of a single molecular species. Where such assays are successful, they are typically for biomolecules in high concentrations (e.g. immunoglobulins) that overshadow contributions to serum IR spectra of solutes in lower concentrations [30, 31, 47]. In the case of previously established ELISA-based soluble biomarkers for this equine model, concentrations in the serum are 25 to 1000 fold lower than in synovial fluid, and these levels approach or are below the detection threshold for a single molecular species with mid-IR transmission spectroscopy [12, 26, 47]. In contrast, IR spectroscopy is effective at identifying synovial fluid from horses affected by osteochondrosis and traumatic arthritis from controls, presumably because disease-related IR active biomolecules are in sufficient concentrations for IR based detection and discrimination [38, 40]. To improve the quantification of proteins with low serum concentrations (e.g. immunoglobulin A) an ultrafiltration method has been described to separate molecules based on their molecular size [48]. In future studies, pre-processing of serum samples with techniques such as ultrafiltration may enhance the relative contribution of specific proteins in the IR spectral profile but is unlikely to separate specific peptides or glycosaminoglycan-based biomarkers.

A recent study utilizing IR spectroscopy of serum claimed success in discriminating between horses with naturally occurring OA from controls [41]. The study compared 15 horses with OA and 48 designated controls. Serum from horses affected with metacarpophalangeal, metatarsophalangeal, or carpal joints were included. However, conventional, and IR-based comparisons of equine synovial fluid from different normal joints differ significantly biochemically [49, 50]. Therefore, in our study a single joint was evaluated to eliminate possible variability arising from different anatomic locations. The control group in the report by Paraskevaidi *et al.* (2020) included 12 different breeds, ranging in age from 1 day to 26 years, and the clinical OA group consisted only of Thoroughbred racehorses ranging in age from 3 to 10 years. Age has been recognized to play an effect on clinical disease and on the performance of IR-based spectroscopy techniques in horses affected by osteochondrosis and dogs with cranial cruciate ligament rupture associated OA [39, 40]. Therefore, in agreement with the approach taken by others [12, 26], we report the narrow inclusion criteria of unraced 2 to 3-year-old female Thoroughbreds to minimize the possible confounding effects of age or gender on biomarker profiles. Nevertheless, the narrow selection criteria included in the current study did not improve the ability of IR spectroscopy to detect changes associated with induced OA.

Excellent classification rates by sampling Day were obtained when using 10 PC of the PLSDA. There was clear separation of classes for samples collected at Day 0 and 7. Lameness, flexion test, and effusion scores were significantly different between these days for the OA group (Appendix A, Tables 1-3) providing clinical evidence of inflammation. For the Sham group only lameness and effusion scores were significantly different, suggesting perhaps a less severe inflammatory response. However, differences in these scores between OA and Sham groups were not significant. This may be attributed to the clinical response to the use of a non-

steroidal anti-inflammatory drug for five days following arthroscopy in both groups (as dictated by the animal ethics protocol).

The inability to reliably classify the treatment group (OA or Sham) at the remaining sampling times may reflect the effect of exercise on the biomarker profile contained within the serum spectra rather than disease [12, 51]. This finding is in agreement with previous works using the same equine model that found significant changes in the concentrations of specific soluble biomarkers (epitope CS846, epitope CPII, glycosaminoglycans, osteocalcin, type 1 and 2 collagen degradation fragments, and bone specific type I collagen) in serum in response to the same exercise protocol [12]. Adaptations to exercise are complex and encompass the musculoskeletal, cardiovascular, respiratory, and other systems. The IR-active biomolecular contributions to each spectrum reflect both physiologic (i.e. exercise induced or natural temporal variations) and disease (OA) contributions. In the current study, the exercise-related changes in the spectra may have obscured the detection of those associated with early disease-related responses in the equine OA model.

The comparison of group-sampling day combination with serum from Day 0 was performed in an attempt to further explore the poor results observed for the OA-Sham analysis. However, the results were highly variable with performance best at Day 35. Although the precise reasons for these results are unknown, the limitations discussed above are likely to apply similarly.

A limitation of our study was the lack of an age-matched unexercised control group, which did not allow the determination of the role of exercise on IR spectra. Moreover, although the selection criteria of the animals in our study (young age, female gender and Thoroughbred breed) were chosen to limit variability, differences may exist in serum spectra of animals of

different age, gender or breed, which were not explored in this study. In addition, the IR spectra obtained in horses with induced OA may differ significantly from those in horses with naturally occurring OA.

In conclusion, this is the first study to investigate the use of IR spectroscopy on serum from horses with traumatically induced OA. This technique did not facilitate the early discrimination of horses affected by OA from controls. A prospective study using horses affected by naturally occurring OA with precise case definitions and appropriately matched controls may provide more useful information on the suitability of this technique in horses.

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Foreword to Chapter 5

In Chapter 4 the accuracy of infrared-based serum biomarker profiling to differentiate horses with early OA from controls was assessed. The results showed that this technique failed to identify horses with OA. A reason for this could be that the serum concentrations of potential biomarkers of OA could be below the detection threshold of infrared spectroscopy. The next step of this research in Chapter 5 was to determine if a similar infrared-based approach in the same research horses would yield better results when used on synovial fluid.

Chapter 5
Infrared Spectroscopy of Synovial Fluid Shows Good Accuracy as an Early Biomarker in an Equine Model of Traumatic Osteoarthritis

5.1 Abstract

Objective: to determine the accuracy of infrared (IR)-based synovial fluid biomarker profiling to differentiate horses with early inflammatory changes associated with a traumatically induced model of equine carpal osteoarthritis (OA) from controls.

Methods: unilateral carpal OA was induced in 9 of 17 healthy Thoroughbred fillies, while the remainder served as sham operated controls. Synovial fluid (SF) samples were obtained before induction of OA (Day 0) and weekly thereafter until Day 63. Films of dried synovial fluid were created, and IR absorbance spectra acquired. Following pre-processing of the spectra, a series of predictive models using random forests were used to assess the ability to characterise samples according to different classification tasks.

Results: the estimated classification accuracy by sampling Day was 87%. The accuracies of classification by joint were 75% and 70% for OA vs OA Control and OA vs Sham, respectively. The classification accuracy by joint among all horses was 53%, while the accuracy for Horse group (OA vs Sham) was 68%.

Conclusions: the current analytical approach of IR spectroscopy was able to discriminate SF from joints with induced OA from controls with good accuracy. As the results of this study do not compare as favourably with studies performed with naturally occurring disease, a longer study period or a more invasive equine model of joint injury may be required to explore the full research potential of SF IR spectroscopy.

5.2 Introduction

Osteoarthritis (OA) is a common, painful, slowly progressive, and debilitating disease. [1, 2] It places a significant economic burden on society, leading to physical disability and impaired mental health. [3] The disease is estimated to affect 100 million people in the European Union [4] and 54 million [5, 6] to 66 million [7] people in the U.S.A. The annual direct medical costs associated with OA in the U.S.A. in the period 2008-2014 was $11,502 per patient, and the annual total direct costs were estimated at $373.2 billion; as life expectancy and medical expenses increase, these costs are likely to rise [8]. Osteoarthritis is also a significant cause of lameness and joint disease in horses, with 50 +/- 3.2% U.S. multi-horse operations reported as experiencing one or more lameness cases yearly, of which half were limb or joint related [9]. The majority of lameness cases in the equine population is associated with osteoarthritis OA [10-12]. According to a 2017 report of the American Horse Council Foundation the estimated total U.S. equine population was 7.2 million horses, and the equine industry generated an impact of $122 billion on the U.S. economy [13]. The direct costs associated with the care of a horse affected by OA may vary from $3,000/year to as high as $15,000/year if indirect costs are also considered [14].

To identify early disease, stratify the likelihood of progression, and explore new interventions, there is a need to identify biomarkers of OA [15-17]. The term biomarker has been defined as a collection of medical signs that can be measured with accuracy in a reproducible manner [18]. In a joint venture on chemical safety led by the World Health Organization (WHO) with the collaboration of the United Nations and the International Labor Organization, a biomarker has been defined as “any substance, structure, or process that can be measured in the body or its products and influences or predicts the incidence or outcome of disease” [19]. Several soluble

biomarkers of joint metabolism and disease have been explored for their use as potential markers of OA in synovial fluid, blood, and urine [20-23]. Although important progress has been made in biomarker research [24-30] and there is evidence of potential clinical application in horses in serum [31] and synovial fluid [32, 33], validation and widespread clinical use of biomarkers has not become common practice [21]. Multiple factors contribute to this lack of uptake, including high costs, low practicality, and inconsistency of results for the purpose of disease surveillance [34]. Obtaining an early diagnosis of OA remains a challenge and it is difficult to assess progression of disease clinically and response of disease to therapeutic interventions; hence cost effective and reliable testing methodologies are needed, especially in the early stages of OA [35]. Unlike in humans [36], clinical staging of disease and case definitions of OA for specific joints has not been established in animals, but a significant body of knowledge has been gained from models including dogs [35] and horses especially for the early stages of disease [24, 37-40]. Proteomic approaches have been developed in recent years using blood, synovial fluid, and other body fluids with the aim to investigate their use for diagnostic, prognostic, and therapeutic purposes in OA [41]. These techniques rely on the separation of a large number of proteins and their identification by mass spectrometry. However, an alternative approach is the identification of a biochemical profile of multiple molecules in biological fluids using Fourier-transform infrared (IR) spectroscopy. This technique does not require the separation of single molecular species associated with disease, but instead provides a complex IR signal produced by an array of molecules [42]. Since OA is a complex disease that can be triggered by trauma or inflammation in any one of multiple joint tissues [12] with each of them possibly contributing to joint homeostasis, the use of IR spectroscopy has been investigated. In human SF from joints affected by rheumatoid arthritis, IR spectroscopy has shown potential as a diagnostic and screening tool [43-45]. Similarly, the use of IR spectroscopy on SF has been shown

to identify osteochondrosis and naturally occurring traumatic arthritis (TA) in equine clinical cases [32, 33]. In dogs with naturally occurring OA associated with cranial cruciate ligament rupture IR spectroscopy on synovial fluid allowed differentiation between affected and control joints with high sensitivity (97.6%), specificity (99.7%) and overall accuracy (98.6%) [46]. The advantages of IR spectroscopy are its accuracy, low cost, and low invasiveness [47]. Although this technique has performed well in SF from horses with naturally occurring disease, to date there are no studies assessing its use for the early detection of OA in a controlled research setting using a homogenous cohort of horses. The objective of this study was to determine the feasibility and accuracy of IR-based SF biomarker profiling to differentiate joints with early inflammatory changes associated with a traumatically induced model of equine carpal osteoarthritis (OA) from controls. The authors hypothesised that IR biomarker profiling using IR spectral analyses of SF can differentiate joints with induced carpal OA from controls.

5.3 Materials and Methods

This study was approved by Massey University Animal Ethics Committee (MUAEC 14/18). The sample size for treatment and sham control groups was estimated based on previous work using the same OA model for other biomarker studies and treatments trials [37-40].

5.3.1 Animals

Seventeen female Thoroughbred horses including fifteen 2-year-olds and two 3-year-olds were recruited for the IR-based biomarker study. These horses had not previously been trained or used for any athletic activity. To determine eligibility for enrolment in the study the animals were checked for physical abnormalities or illness, lameness at walk and trot as assessed by two specialist equine surgeons, carpal flexion tests and radiographic examination of the carpi; all findings were negative. Procaine penicillin at 22 mg/kg IM (Phoenix

Pharmacillin 300, 300mg/ml, Phoenix Pharm, New Zealand) was given to all horses once before surgical intervention. The assignment of horses for the Sham operation (control) or the surgical induction of traumatic OA was randomized.

Nine horses were randomly assigned to the OA group. General anaesthesia was induced with 2.5 mg/kg of ketamine HCl (Ceva Ketamine injection; Ceva Animal Health Pty Ltd. Australia) and 0.01 mg/kg of diazepam (ilium Diazepam Injection USP; Troy Laboratories, Australia) intravenously. General anaesthesia was maintained with isoflurane (Isoflurane; Bayer New Zealand Ltd, New Zealand) in 5 L/minute of 100% oxygen. After aseptic preparation of the carpus, an 8 mm osteochondral fragment was arthroscopically created in the middle carpal joint using a bone gouge in the distal dorsal aspect of the radial carpal bone to induce traumatic OA as previously described [39, 40]. The osteochondral fragment was left in place, hinged at the dorsal joint capsule reflection. The parent bone from which the fragment originated was debrided with a motorized bone burr to create a ~15 mm-wide defect (including the width of the fragment) and the associated debris left in the joint. These horses were identified as members of the OA horse group, their operated middle carpal joints as OA joints, and the unoperated contralateral middle carpal joint as OA Control joints.

The other eight horses (Sham horse group) underwent arthroscopic exploration only using the same general anaesthetic protocol, without creation of an osteochondral defect of one randomly selected middle carpal joint (Sham joint). In these horses the unoperated contralateral middle carpal joint served as a Sham Control joint. All horses were administered phenylbutazone immediately after completion of the procedure at 4.4 mg/kg IV (Nabudone P, 200 mg/ml, Troy Laboratories, Australia) and for the following four days at 4.4 mg/kg PO, every 24h (Equine Bute Paste, 200mg/ml, Randlab, Australia). Postoperatively, horses underwent clinical examination twice daily to evaluate their comfort and well-being.

5.3.2 Post-operative exercise and clinical assessment

Horses were allowed a 14-day recovery period in box stalls with 30 minutes of daily turnout, after which a 7-week-long treadmill exercise protocol (5 days/week) was initiated. For the treadmill exercise protocol all horses were exercised once daily for two minutes at a trot (4-5 m/sec), then two minutes at a gallop (8-9 m/sec), and then two minutes at a trot (4-5 m/sec). The model has been used previously by other researchers to mimic naturally occurring equine traumatic OA [37, 38]. Each horse was assessed pre-intervention and once weekly thereafter to assign scores for lameness [48], joint effusion and response to carpal flexion. At the end of the study radiographs were taken and scores given for radiographic changes to confirm the establishment of OA. Results of the lameness, flexion tests, effusion and radiographic scores for this study are presented in other sections in this thesis (Chapter 4, and Appendix A Tables 1-4).

5.3.3 Synovial fluid sample collection

Approximately 3 mL of synovial fluid (SF) was collected from both carpi of each horse before induction of OA (or Sham surgery) on Day 0, and then weekly from all horses until Day 63. SF aliquots of ~1 mL were stored at -80°C for later batch analysis.

5.3.4 Infrared spectroscopy

Synovial fluid samples were thawed at room temperature and replicate (x 6) dry films of 8 µL made for each sample on a silicon 96-well microplate [33, 35, 49]. The microplate was mounted on a multi-sampler accessory (XY Microtiter Plate Accessory, PIKE Technologies, Madison, WI, USA) interfaced with an IR spectrometer (Tensor 27, Bruker Optics, Preston, Victoria, Australia). Infrared absorbance spectra in the wave number (WN) range of 400 to 4,000 cm^{-1} were generated and recorded in transmission mode with proprietary software (OPUS

software, version 6.5, Bruker Optics, Ettlingen, Germany). For each acquisition, 512 IR interferograms were averaged, and Fourier transformed to obtain a spectrum with a resolution of 4 cm^{-1}.

5.3.5 Analyses of synovial fluid spectral data

Spectral files were imported into proprietary spectral manipulation software (The Unscrambler Xv10.5.1, Camo Software, Oslo, Norway) and converted into delimited csv files for further analyses. All subsequent analyses were performed in R (V4.2.2, R Core Team, Auckland, New Zealand).

Spectral pre-processing: The prospectr R package V 0.2.4 was used for spectral pre-processing and analyses. Savitzky-Golay filtering was applied to all spectra with a 2nd-order derivative of the signal, and a 2nd order polynomial function and a smoothing window of width 15; parameters were tuned to maximize spectral separation by Day. We performed an analysis of sensitivity to filtering parameters, and similar performances were also retrieved for other combinations of filter parameters. Then, standard normal variate (SNV) transformation was used to normalize spectra and remove baseline effects, reducing within-class variance [50]. Spectra values in the regions between 1300-1800 cm^{-1} and 2600-3700 cm^{-1} were selected for further analyses. Outlier samples were not removed as their number did not exceed the threshold of extreme PCA scores expected by chance (5%), and further inspection did not reveal any anomaly with the samples.

Classification model development: A series of predictive models were built to predict the sampling Day (task 1; Days 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63; 10 classes); the joint sampled in OA horses (task 2; OA joint vs OA Control; 2 classes); the joint sampled in Sham horses (task 3; Sham joint vs Sham Control, 2 classes); the intervention joint sampled between horse

groups (task 4: OA joint vs Sham joint, 2 classes); all joints sampled in both horse groups (task 5a: OA joint, OA Control, Sham Joint vs Sham Control; 4 classes); the OA joint sample vs any other (task 5b; 2 classes); the horse group (task 6; OA versus Sham; 2 classes); the samples classified Day x joint group except for Day 0 (i.e. prior to interventions) for which OA and Sham groups were pooled (task 7a; 0, 7 x OA joint, 7 x OA Control, 7 x Sham joint, 7 x Sham Control joint, 14 x OA joint,…, 63 x Sham Control; 37 classes); and similarly comparing the Day x OA joint sampled vs any other (task 7b ; 19 classes), and the variation among horses (task 8, horse label 1 to 17; 17 classes).

Sparse partial least squares discriminant analysis [51], logistic and multinomial regression with L1-regularization [52], random forests [53], Support Vector Machines [54], and convolutional neural networks [55] were explored as classification methods to make sure that the model/algorithm assumptions were not limiting the performance.

Overall, random forests allowed efficient detection of the best classification performance and were easy to implement, without requiring complex tuning of the method. The random Forest v. 4.7-1[56] and ranger v 0.15.1[57] R packages with default parameter settings were used. The number of trees to ensure convergence were explored by monitoring the global accuracy, and it was found that 1000 trees were enough for all considered tasks. We tried doubling and dividing by a factor of two the default number of splits (√(number of WN)) and this did not change the performance. We re-weighted the samples in unbalanced classification tasks. For example, task 5a included 25% of OA joints versus 75% of other joints.

Predictions were made for each tree in the forest using out-of-bag (OOB) samples [58]. A confusion matrix was then obtained by comparing the predicted class to the actual class of the OOB samples for all the trees in the forest. The authors report the overall classification

rate (computed as the ratio of the sum of the diagonal elements of the confusion matrix to that of the sum of all its elements) for each task, and when relevant (i.e., when imbalanced performance was observed) the classification rate per class (proportion of sample of one class correctly identified as such). Notice that the overall classification rate is commonly referred to as accuracy. A random classification for a balanced problem would lead to a performance rate of 1/(number of classes), so 50% for a 2-class problem, 25% for a 4-class problem, etc. The accuracy of the predictive model was defined as very good (>90%), good (70%-90%), acceptable (60-69%) and poor (<60%).

5.4 Results

5.4.1 Spectral pre-processing

The raw spectra of SF from all joints by class are shown in Fig. 5.1 (top). There was no obvious visually appreciable difference in the spectral pattern between joints. The higher peaks of the spectra are associated with intermolecular bond vibrations with proteins; the absorption bands centred at 1650 cm^{-1} (amide I) and 1545 cm^{-1} (amide II) are associated with stretching and bending vibrations of the amide C=O and N–H groups, respectively. Absorption at 3300 cm^{-1} is also associated with the N–H group but is a stretching vibration (amide A mode) [59]. The image of the pre-processed spectra is shown in Fig. 5.1 (bottom).

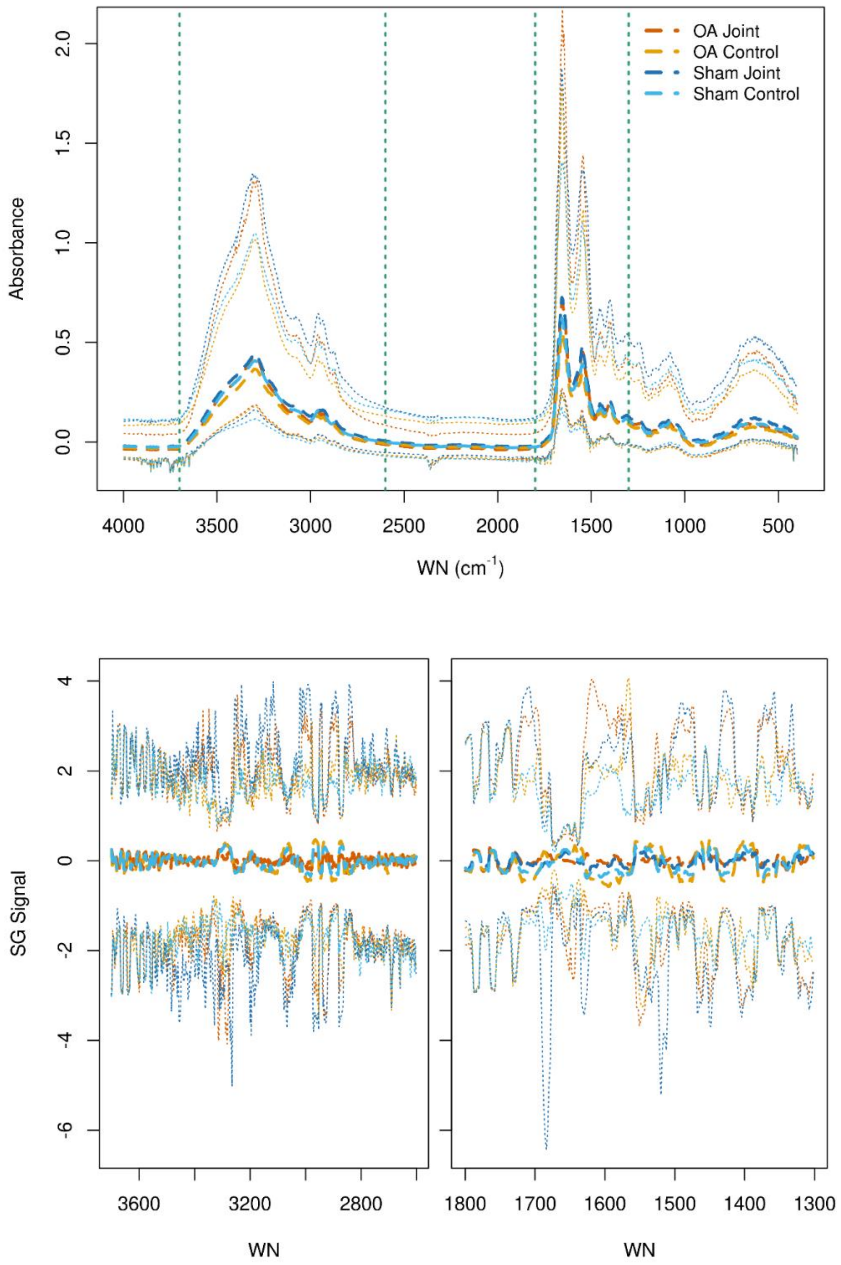

Figure 5.1 Raw (top) spectra of synovial fluid from all joints. The fingerprint regions 3700-2600 cm^{-1} and 1800-1300 cm^{-1} are indicated by the vertical dashed lines. Savitzky-Golay transformed (bottom) spectra of synovial fluid from all joints in the selected fingerprint regions. The median (thick dashed lines) and 2.5%- and 97.5%-quantiles (thin dotted lines) are shown. The osteoarthritis (OA) and OA Control joints spectra are represented in dark and light orange, respectively. The Sham and Sham Control joints are represented in dark and light blue, respectively. WN = wavenumber.

5.4.2 Classification of IR spectra

For task 1 the accuracy in all horses was 87.0%, with a very good per class correct classification rate (over 88.0%), except for Day 56 (51.5%) and Day 63 (52.9%), which the model could not distinguish. The accuracy within the OA horse group (task 2) was 75.0%, which contrasts with a lower accuracy (61.0%) within the Sham horse group (task 3). When prediction was attempted on the operated joints (OA joints Vs Sham joints) in task 4 the accuracy was good (70.0%). The accuracy by joint group in all horses (task 5) was 53.0% with OA joints which more precisely classified than Sham joints (66.4% and 64.2% for OA Control joints and OA joints, respectively Vs 37.2% and 40.3% for Sham Control joints and Sham joints, respectively). For task 5b the accuracy in identifying OA joint vs any other in all horses was 80.0%, but the per class classification rate was 21.5% for OA joints (sensitivity) and 99.1% for the other class (specificity). When we investigated the possible effect of class imbalance on the model performance by re-weighting the observations with the inverse of their frequency, the sensitivity to detect OA joint was increased (61.1%), at the expense of specificity (81.9%), while the accuracy remained virtually unchanged (79.0%). The accuracies for all the classification tasks considered are reported in Table 5.1.

Table 5.1 - Accuracy of the prediction models by task.

Task	Number of classes	Accuracy in prediction
1 – Day	10	0.87
2 – OA vs OA Control joints	2	0.75
3 – Sham vs Sham Control joints	2	0.61
4 – OA joint vs Sham joint	2	0.70
5a – Joint group (OA, OA Control, Sham, Sham Control)	4	0.53
5b – OA joint vs any other joint	2	0.80
6 – Horse group (OA vs Sham)	2	0.68
7a – Day x joint group	37	0.38
7b – Day x OA joint	9	0.67
8 – Horse sampled among all horses	17	0.46

5.5 Discussion

This study is the first to assess the utility of IR spectroscopy and spectral analyses to differentiate synovial fluid of horses with induced OA from controls. With the approach described here we obtained good accuracy in discriminating synovial fluid of joints with induced OA from sham operated joints and OA joints from contralateral unoperated joints. This suggests that the response to the surgical creation of the osteochondral fragment was a key variable responsible for alterations in the spectral patterns of synovial fluid. Further supporting this is the lower accuracy obtained in differentiating Sham from Sham Control joints, indicating that the Sham operation does not cause marked changes in joint metabolism and homeostasis compared to contralateral unoperated controls for IR-based techniques. The classification rate by day of sampling was good. Overall, differentiation between horses with and without OA by IR spectroscopy was greater for SF than for serum in the same cohort of

horses [60]. This finding is consistent with those reported for previous IR studies in dogs [46, 49]. Synovial fluid is likely to more closely reflect joint metabolism and disease compared with serum due to higher concentration of joint biomarkers in SF [37, 61, 62].

The accuracy of the model in the current experimental study is similar to the one previously reported using IR techniques on synovial fluid to differentiate horses with osteochondrosis from controls (77%) [32], but lower than for horses affected by naturally occurring TA (89-97%) [33]. The difference in accuracy between the two studies previously published could be attributable to the nature of the two conditions or different characteristics of the populations studied. A developmental disease such as osteochondrosis may potentially have a lower burden and shorter duration of disease, compared with TA. In the TA study the authors speculated that misclassification of some diseased joints as controls could have been due to the variation in duration and degree of inflammation, with joints with mild arthritis possibly not differing significantly from controls. The osteochondral fragment model used in our study may not have caused sufficient changes in joint metabolism to affect the IR spectral profiles of SF to the same degree as in the ones associated with naturally occurring TA. The clinical signs associated with the current carpal osteochondral model were relatively mild in accordance with other studies using the same model [37, 39, 40]. In dogs with naturally occurring OA associated with cranial cruciate ligament rupture, the use of IR spectroscopy on SF showed significantly higher accuracy in differentiating diseased joint from controls compared with our study [46]. The main difference between the two studies, apart from the species difference, consists in the naturally occurring disease in dogs which contrasts with the induced disease in horses. Cranial cruciate ligament rupture in dogs is believed to be most commonly caused by prior ligament degeneration [63, 64] and to have immune mediated components to it [65, 66], which could significantly contribute to the IR profiles. Also, the dogs recruited in that study had

sufficiently advanced clinical OA and marked joint instability to warrant surgical correction [46]. The short duration of our study may also have had a significant effect on the SF IR spectral profiles when compared with profiles from clinically diseased horses, where OA may be more advanced or chronic.

The use of ELISA techniques on SF has proven successful in horses in being able to differentiate joints with induced OA using the same carpal osteochondral fragment model we used in our study [37]. More recently, another study reported excellent accuracy in discriminating SF of joints with induced OA from controls using ELISA techniques targeting specific markers (BAP, C2C, C12C, CPII, CS846, CTXII), although the authors used a different equine osteochondral fragment model of OA in which the metacarpophalangeal joints were used [67]. The difference in accuracy between our study and those studies could be due to the laboratory techniques chosen (ELISA Vs IR). In fact, said studies targeted the detection and quantification of specific markers, while our study investigated changes in an array of molecules, in which the contribution to the spectral signal of known molecules could be obscured or diluted by a large number of other unknown molecules. Another difference of note is that the study from of Malek *et al.* (2023) utilised the metacarpophalangeal joints as opposed the middle carpal joint in our study and it has been previously reported that SF biochemical composition varies between joints within the limb [68, 69].

In conclusion, this is the first study to evaluate the use of IR spectroscopy on synovial fluid from horses with experimental traumatically induced OA. Our results showed that this approach can differentiate affected joints from controls with a good degree of accuracy in a research setting, although it does not compare as favourably as in previous studies with naturally occurring joint disease. A longer study period, or a more invasive equine model of

joint injury that more closely resembles naturally occurring disease, may be required to explore the full potential of IR spectroscopy.

5.6 References

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Foreword to Chapter 6

In Chapters 3, 4 and 5 potential biomarker profiles of early OA were assessed in a group of horses with induced carpal OA. The evidence suggested that synovial fluid is a better biological fluid than plasma or serum when utilised with the described techniques and methodologies. In the next section (Chapter 6) joint tissues collected from a subset of horses (9) with induced OA are subjected to histopathological analysis and investigation using Raman spectroscopy with the aim of determining the useability of this spectroscopic technique to differentiate the spectral profiles of OA affected tissues from controls.

Chapter 6
Raman Spectroscopy of Carpal Bone and Cartilage from Young Horses with Induced Osteoarthritis

6.1 Abstract

Objective: To assess the useability of Raman spectroscopy to differentiate the spectral profiles of subchondral bone and articular cartilage of carpal bones from horses with induced osteoarthritis (OA) from controls.

Methods: Synovium and slices of carpal bones including the overlying cartilage, from 9 horses subjected to an osteochondral fragment and exercise model of OA, were collected *ex vivo* at the end of the trail and stored at -80°C until analysis. Histopathological examination was performed on all tissues, and scores assigned. Raman spectroscopy (RS) was used on the articular cartilage (ac) and subchondral bone (sc) sections to determine spectral profile differences between joints with OA and contralateral controls using PCA and other methods.

Results: Histopathological scores were significantly higher in affected joints compared with controls. PCA of Raman spectra only allowed clear separation of ac from sc tissues but could not classify samples by joint (OA vs control), location within joints, horses, or combinations thereof. Further data classification with random forest did not improve classification rates compared with PCA separation. Bayesian logistic regression identified significant relationships between peak areas and the probability of a sample being classified as OA for some specific peaks in the Raman range.

Conclusions: The equine surgical model of OA used induced histopathological changes consistent with OA. Raman spectroscopy detected differences in spectral profiles between OA

and control joints in some subchondral bone locations, but clear separation could not be proven between groups. The use of a Bayesian logistic regression models provided insights into the probability of correctly identifying affected tissues in selected peaks of the Raman spectra for some specific carpal joint locations.

6.2 Introduction

Osteoarthritis (OA) is a chronic, slowly progressive, and debilitating disease associated with pain and physical disability that affects millions of people worldwide [1-5] and estimated direct costs of over $370 billion annually in the United States [6]. There are significant psychological consequences related to reduced mobility and lower quality of life associated with having OA, including distress, loneliness, and decreased self-esteem [7]. In horses, half of lameness cases are limb or joint related [8], and the majority of the latter are due to OA [9-11]. The cost of managing OA ranges from 3,000 USD (direct) to 15,000 USD (indirect) annually per horse [12].

Osteoarthritis is characterised by progressive cartilage degradation with depletion of glycosaminoglycans (GAGs) and loss of organisation and alignment of collagen fibres [13-15]. Subchondral bone changes also occur with OA, in particular sclerosis and demineralisation of the subchondral bone plate with significant remodeling involving cortical and trabecular bone [16-19]. During the progression of OA all of the joint tissues develop changes to their viscoelastic properties and chemical composition [20].

Vibrational spectroscopy is a non-destructive technique for the characterisation of materials and tissues which encompasses infrared (IR) and Raman spectroscopy (RS). The approach is based on the interaction of light with molecular chemical bonds which periodically stretch and bend [21]. The chemical bonds of different molecules vibrate with different frequencies and have characteristic interactions with incident light, which, when measured, create unique spectral

fingerprints. Analysis of the resultant spectra provides information on molecular composition and properties of materials or biological samples. While IR spectroscopy is based on the absorbance or reflectance of electromagnetic radiation by the sample examined, RS is instead based on the inelastic scattering originating from the interaction of an incident laser source with the molecular bonds. As also observed in several vibrational spectroscopy techniques, Raman spectra are unique to the molecules being examined [21]. In a microscopy context, the main advantages of RS over IR spectroscopy are the high spatial resolution and the limited interference from water [22, 23]. The latter characteristic allows minimal sample preparation (e.g., no dehydration is required) and the potential for *in vivo* examination without the need for external labelling [23-26].

Vibrational spectroscopy has been used in a wide range of scientific and industrial settings, including pharmacology [27], archaeology [28], forensic [29], food [30], agriculture [31] and medicine [32] among others. In people, spectroscopy has been used as a diagnostic or monitoring tool for several conditions such as diabetes mellitus [33], kidney disease [34], Alzheimer's disease [35], and cardiovascular disease [36]. The number of publications on the use of vibrational spectroscopy to investigate cancers and infections alone has grown enormously in the last two decades, with 14 and 416 publications using RS in the years 2000 and 2020, respectively [37]. Use of RS on bone dates back to 1970 [38] but in the following two decades only a handful of studies were published on this subject [39-41]. With the commercialisation of Raman instrumentation, the number of publications on RS applied to interrogate mineralised tissues markedly increased in the period 2000-2021, with over 630 resulting publications [42]. The use of RS to study molecular changes associated with OA has initially focused on subchondral bone given the strong signal originating from some of the bone associated molecules [22, 43, 44]. As subchondral bone becomes exposed only in advanced stages of OA, significant interest has been placed in

the investigation of cartilage. Raman spectroscopy has shown potential for the detection of changes in cartilage proteoglycans [45, 46] with high sensitivity and specificity, and has allowed for the classification of different OA stages in human samples [25]. The biomolecular changes in affected cartilage can be detected by RS before histological alterations become visible [45]. This has sparked interest in exploring the clinical application of RS such as its integration within fiber-optic probes for intra-operative use [47-49].

Despite the growing body of literature on the use of RS on cartilage and bone, limited information is available on its use in horses. Its use has been reported in studies investigating the properties of equine bone [50-53], the effect of exercise on bone mineral density and the discrimination of horses with proximal sesamoid bone fractures from controls [54]. Other studies used RS to describe the chemical composition of equine bone affected by bone oedema as identified by use of MRI [55], and the proteomic profile of cartilage from horses affected by osteochondrosis [56]. To the best of the authors' knowledge there are no strictly controlled studies on the use of RS on horses affected by OA.

The objective of this study was to assess the use of RS to differentiate the spectral profiles of subchondral bone and articular cartilage of horses with induced carpal OA from controls.

6.3 Materials and Methods

This study was approved by Massey University Animal Ethics Committee (MUAEC 14/18).

6.3.1 Animals

Fifteen 2-year-old and two 3-year-old Thoroughbred fillies bred and raised in New Zealand were recruited. These horses had not undertaken any athletic training activity prior to entering the study. Exclusion criteria included: physical abnormalities or illness, lameness at

walk and trot evaluated by two specialist equine surgeons, positive carpal flexion tests, and abnormalities identified on standing radiographic examination of the carpi. Nine horses (eight 2-year-old and one 3-year-old) were randomly assigned to the surgical induction of traumatic OA in one randomly selected carpus, while the remaining eight horses underwent a sham operation in one randomly selected carpus as part of a larger study on biomarkers of OA. Only joints from the nine horses with induced traumatic OA were included in the current study.

Prophylactic antimicrobial therapy consisted of procaine penicillin at 22 mg/kg IM (Phoenix Pharmacillin 300, 300 mg/mL, Phoenix Pharm, New Zealand) administered once before surgical intervention. General anaesthesia was induced with 2.5 mg/kg of ketamine HCl (Ceva Ketamine injection; Ceva Animal Health Pty Ltd. Australia) and 0.01 mg/kg of diazepam (ilium Diazepam Injection USP; Troy Laboratories, Australia) intravenously and maintained with isoflurane (Isoflurane; Bayer New Zealand Ltd, New Zealand) in 5 L/minute of 100% oxygen. One carpus on each of the nine horses was clipped and aseptically prepared for surgery. Via arthroscopy of the middle carpal joint an 8 mm osteochondral fragment was created using a bone gouge in the distal dorsal aspect of the radial carpal bone to induce traumatic OA, as previously described [57-59]. The operated middle carpal joints were identified as OA joints (n=9), while the unoperated contralateral middle carpal joints were considered control joints (n=9).

Phenylbutazone was given to all horses shortly after completion of the procedure at 4.4 mg/kg IV (Nabudone P, 200 mg/ml, Troy Laboratories, Australia) and for four days at 4.4 mg/kg PO, every 24 h (Equine Bute Paste, 200mg/ml, Randlab, Australia). Clinical examinations were performed twice daily for the first seven days to ensure the horses' well-being after surgery.

6.3.2 Post-operative exercise and clinical assessment

The post-operative recovery period consisted of a 14-day confinement in box stalls with 30 minutes of daily turnout in small yards. After the recovery period a 7-week- (4 horses) or 8-week-long (5 horses) treadmill exercise protocol (5 days/week) was initiated. All horses were exercised once daily for two minutes at a trot (4-5 m/sec), then two minutes at a gallop (8-9 m/sec), and then two minutes at a trot (4-5 m/sec). Exercise is an important part of this equine model of OA to mimic naturally occurring equine traumatic OA [60, 61]. Each horse was assessed pre-intervention and once weekly thereafter to assign scores for lameness [62], joint effusion and response to carpal flexion. At the end of the study radiographs were taken and scores given for radiographic changes to confirm the establishment of OA. Results of the lameness, flexion tests, effusion and radiographic scores have been presented in a previous section in this thesis (Chapter 4 and Appendix A, Tables 1-4). Horses were euthanised 63 (n = 4 horses) and 70 (n = 5 horses) days after induction of OA respectively, with an overdose of sodium pentobarbitone (Pentobarb 500, 500mg/mL, Provet NZ Pty Limited, New Zealand).

6.3.3 Histopathological examination

Samples of synovial membrane, adjacent to the osteochondral fragment on the radial carpal bone (Syn-CR) and adjacent to the 4th carpal bone (Syn-C4), were collected into 10% neutral buffered formalin. Bone/cartilage sections (approximately 3 mm thick) of osteochondral fragment on the radial carpal bone (R2), radial carpal bone adjacent to the osteochondral fragment (R3), 3rd carpal bone (C3, opposed the osteochondral fragment) and the 4th carpal bone (C4) were collected using a orthopaedic handsaw and placed into 10% neutral buffered formalin. These sections were demineralised in a commercial 10% hydrochloric acid product (Decalcifier Hydrochloric acid, Amber Scientific Ltd, Midvale, Australia). Demineralised

sections and synovial membrane samples were placed into a cassette, embedded in paraffin wax and sections (3 mm) cut and stained with haematoxylin and eosin (H&E) for microscopic examination. Sections of bone, cartilage and synovium were also obtained in the equivalent locations in the contralateral (control) carpus of each horse and processed using the same methodology. Bone, cartilage, and synovial sections were evaluated by a single evaluator blinded to group assignments. Bone and cartilage sections were assessed for cartilage fibrillation, chondrocyte necrosis, presence of chondrones, and focal cell loss, and graded 0-4 based on severity of the change (0= no abnormalities detected, 1= slight changes present, 2= mild changes present, 3= moderate changes present, 4= severe changes present) as previously described [61]. Synovial membrane sections were evaluated for cellular infiltration, intimal hyperplasia, subintimal oedema, subintimal fibrosis and subintimal vascularity, and graded 0-4 based on the severity of changes as above.

6.3.4 Raman spectroscopy

At the time of bone and cartilage sample collection for histological examination, 3 mm sections were also collected from the same locations (R2, R3, C3, C4) and stored at -80°C for later Raman spectroscopic analysis. The Raman microspectroscopy system used was custom-made and consisted of a 785 nm, 50 mW single-mode, 50 µm diameter fibre-coupled diode laser (Warsash Scientific, Sydney, Australia) which served as the near-infrared monochromatic excitation source, to reduce the inherent fluorescence of biological samples. An Olympus IX-70 inverted fluorescence microscope body was fitted with a 10× magnification objective (NA 0.25, Edmund Optics, Singapore) and a 785 nm filter (Iridian Technologies, Ottawa, Ontario, Canada). The scattered light was directed through a series of Volume Bragg Grating interference filters (OptiGrate, Oviedo, Florida) into a LS 785 spectrograph (Princeton Instruments, Trenton, New Jersey). A PIXIS 400 liquid nitrogen-cooled charge-coupled device

(CCD) detector was used (Princeton Instruments, Trenton, New Jersey), with a slit width set to 50 µm. LightField software (version 6.0.4.1611, Princeton Instruments, Trenton, New Jersey) was used to obtain the Raman measurements. The bone and cartilage sections were allowed to thaw at room temperature before beginning Raman spectra collection. Sample collection points were identified either as articular cartilage (ac) or subchondral bone (sc) by preview examination of spectra with LightField software based on the presence of a collagenous or mineralised component for the ac and sc, respectively. A Raman shift range of -500 – 2000 cm^{-1} was used for spectra collection, with a resolution of approximately 5 cm^{-1}. Spectral pre-processing and statistical analysis focused on the wavelength range 300 – 2000 cm^{-1}. Dark field correction, cosmic ray removal, and CCD hardware orientation were applied to all spectra before file were exported in .csv format. From each carpal bone section two spectra from the ac and two spectra from the sc were obtained. To reduce the potential burning effect on the samples from the laser, a low power was used (~3-7.5 mW at the sample). Total collection time was 120 seconds (6 x 20 sec).

6.3.4.1 Spectral data processing

Spectral data was analysed by bone location (R2, R3, C3, C4) using Python™ 3.9.× code (www.python.org) written in Jupyter notebooks (https:// jupyter.org), or as scripts from the command line. For each individual spectrum a baseline correction (using a modified asymmetric least squares baseline algorithm) was applied [63]. Background removal was achieved with an asymmetric least square algorithm modified for improved performance with broad overlapping bands with long tail as found in our data sets. An average spectrum over the two replicates was calculated and used for further analysis. To identify potential artifacts introduced by baseline subtraction a visual inspection of the baseline functions was performed and, if necessary, the baseline parameters were adjusted to limit the introduction

of artifacts for the entire dataset of spectra. Asymmetric least squares were used with λ (offset) of 120000 and P (sensitivity) of $5x10^{-4}$.

6.3.4.2 Peak-fitting of spectral data

A plot was generated of a random spectrum from each sample type (ac or sc) and carpal bone location. Working spectral ranges were selected to include all identifiable peaks based on visual inspection which were consistent across all horses sampled. A pseudoVoigt function was used as the component function in the fit. A component function was used for each selected peak in the working spectral range and was fitted for position, amplitude, and width manually. Components were only added for the most significant spectral features. An example of peak-fitting of a random spectrum is shown in Appendix B, Figure 1. Unfitted components were accounted for by the tails of the added components, potentially leading to an over-estimation of the spectral width and amplitude. After a reasonable fit was obtained, a non-linear least squares optimization using the scipy.optimize.curve_fit package (https://docs.scipy.org/doc/scipy/reference/generated/scipy.optimize.curve_fit.html) was used to find the optimal parameter values. For each location, peak parameters (including peak location, amplitude, and width) were obtained and averaged. The peak areas of characteristic bands were calculated from the fitted peak parameters. Corrections for spectrometer sensitivity and differential band-pass were not made as these were kept constant.

6.3.5 Statistical analysis

For bone/cartilage sections, composite histological scores were summed. These were tested for normality using a Shapiro-Wilk test (Statistica 11, StatSoft, Tulsa, OK, USA) and were not normally distributed. The OA and control values for each sampling site were considered independent of each other (i.e., from different horse groups). Therefore, scores for samples

from the OA and control joints were compared using the Mann-Whitney U test (Statistica 11, StatSoft, Tulsa, OK, USA). Differences were considered significant for values of $p < 0.05$. The same approach was taken for synovial membrane scores. All the analyses of Raman spectroscopy were conducted in R Statistical Software (v4.3.2, R Core Team, Auckland, New Zealand, 2023). Raw spectra peaks characteristics (centre in wavenumber [WN], height in relative intensity and width in WN) for 12 and 11 peaks from the full Raman spectra were obtained for cartilage and bone sections, respectively. The area of each spectrum was computed as area = c*height*width, with c = π (pseudoVoigt). The peaks in each spectrum were labelled consecutively as peak 1 to peak 12. Only peaks that were present in all samples and that could be determined unambiguously from their centres were used, therefore peak 4 and peak 12 were excluded accordingly from further analysis. For each peak, areas corresponding to the different conditions (location x tissue x joint) were visualised using boxplots. Due to varied ranges, the 10 peak areas across the different samples and replicates were standardised by removing the mean and dividing by the standard deviation. Pairwise correlation structure in the data was explored by plotting scatterplots of each peak area as a function of other peak areas. Principal component analysis (PCA) from the mixOmics package v6.24.0 [64] was then used to explore the structure in the spectral data. Next, random forests were applied [65] to predict the three factors in the data: joint (OA/control), location (C3/C4/R2/R3) and tissue (ac/sc) or combinations thereof: joint x tissue, tissue x location, and joint x tissue x location. The randomForest R package v4.7-1.1 [66] was used to access the so-called importance of each peak in the correct classification performance. Lastly, the joint status was modelled with a Bayesian logistic regression using Stan v2.26.1 and Rstan v2.32.3 (Stan Development Team, 2023). More specifically, the most general model considered can be written as:

Model:

$$Joint \sim Binomial(p)$$

$$logit(p) = \alpha_0[Tissue, Location] + \sum_i \alpha_i\,[Tissue, Location] \times peakArea_i$$

Prior:

$$\alpha_0 \sim Normal(mean = 0, sd = 0.5)$$

$$\alpha_i \sim Normal(mean = 0, sd = 2), i = 1,2,3,5,6,7,8,9,10,11$$

where p is the probability for a sample to be classified OA (vs Control). α_0 is the intercept in the model and the other α_*i*'s are the effect of peak *i* area in the logit(p). All parameters are tissue x location specific. An increase of +1 unit in peak *i* area induces an average increase of α_i on the log-odds of p. The most general model contains 88 (=11 parameters α x 4 locations x 2 tissues) parameters. Despite efforts to regularise these parameters, the available data did not allow the authors to obtain a reliable estimation of the model. The authors only show the results for the dependency towards either tissue or location on each peak effect in the models, with 22 and 44 parameters, respectively.

6.4 Results

6.4.1 Histopathological examination

Synovial membrane pathology scores were significantly higher in the OA joints compared with control joints at both locations (Syn-CR and Syn-C4). For bone/cartilage sections, the scores were significantly higher in OA joints than in control joints only at the two locations closest to

the area of the surgically created osteochondral fragment (R2 and C3). Descriptive statistics are shown in Table 6.1.

Table 6.1 Descriptive statistics of the histopathological scores for control and osteoarthritis (OA) joints.

	Median		Minimum		Maximum		Range		Quartile Range	
	Control	OA	Control	OA	Control	OA	Control	OA	Control	OA
R2	0[a]	8[b]	0	4	1	13	1	9	0	4
R3	0	0	0	0	1	2	1	2	0	1
C3	0[a]	2[b]	0	1	0	10	0	9	0	2
C4	0	0	0	0	0	2	0	2	0	1
Syn-CR	2[a]	8[b]	0	6	5	13	5	7	3	1
Syn-C4	1[a]	5[b]	0	2	6	11	6	9	2	2

R2 = osteochondral fragment on the radial carpal bone; R3 = radial carpal bone adjacent to the osteochondral fragment; C3 = 3rd carpal bone; C4 = 4th carpal bone; Syn-CR = synovium adjacent to the osteochondral fragment; Syn-C4 = synovium adjacent to the 4th carpal bone. In the median column different superscripts within the same row indicate $p < 0.05$.

An example of typical histological synovial changes in OA joints is shown in Figure 6.1. Examples of osteochondral changes identified histologically are shown in Figures 6.2 and 6.3.

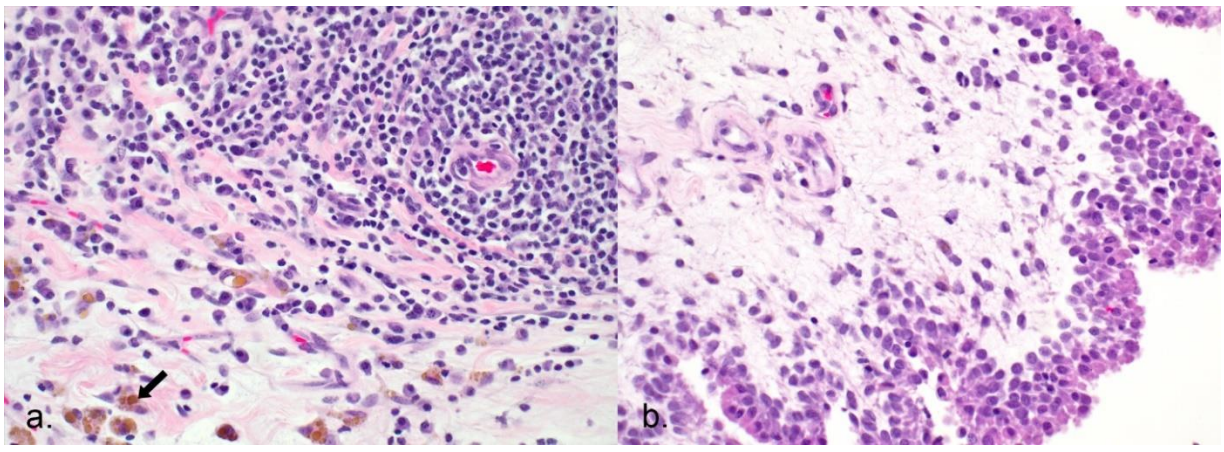

Figure 6.1 Histological sections of synovium from OA joint showing haemosiderin laden macrophages (arrow) and perivascular lymphoplasmacytic infiltrate (a), and mild synovial hyperplasia (b). H&E

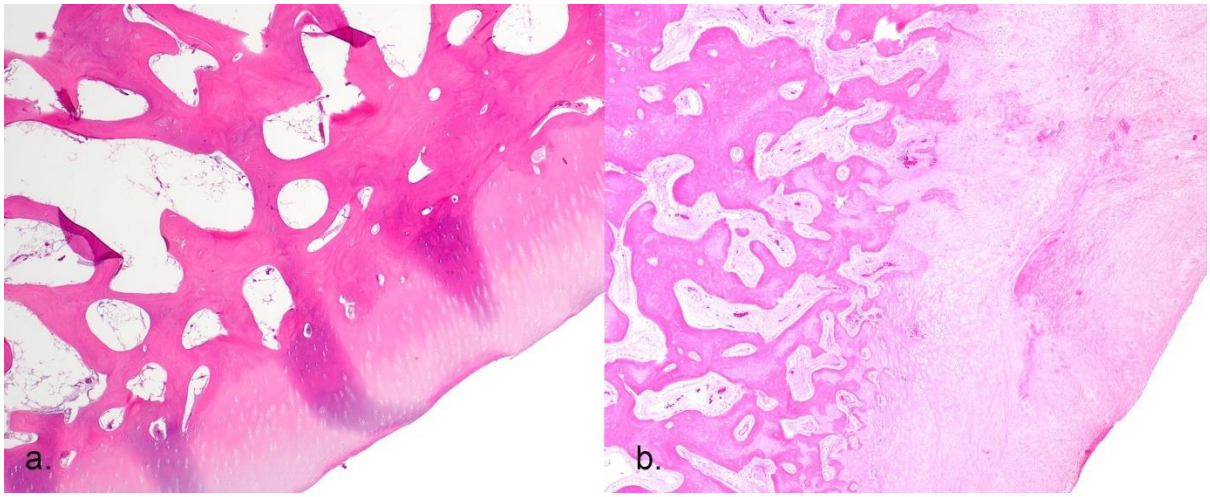

Figure 6.2 Histological section from C3 location of a control joint (a). Note smooth cartilage surface and organised chondrocytes. Histological section from R2 location of an OA joint (b). Cartilage is unorganised and replaced by fibrocartilage. H&E

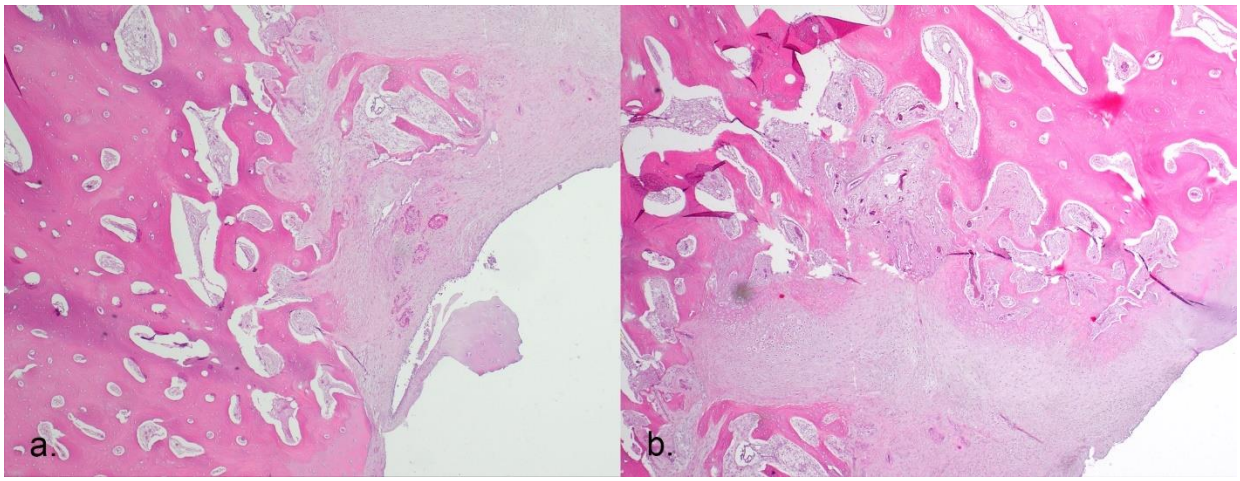

Figure 6.3 Histological section from R2 location of an OA joint. Large cartilage defect filled with fibrous tissue (a) and fibrocartilage and new woven bone formation (b). H&E.

6.4.2 Raman Spectroscopy

The spectral ranges within which each peak centre was identified are shown in Table 6.2. The area and Raman shift of the 10 peaks in the data set used for the analysis for each tissue type is shown in Figure 6.4.

Table 6.2 Spectral ranges within which each peak was identified.

Peak number	**Spectral range (Raman shift cm^{-1})**
1 ($\upsilon_2PO_4^{3-}$, phosphate)	380-439
2 ($\upsilon_4PO_4^{3-}$, phosphate)	500-622
3 (υ(C-S), cysteine)	637-766
5 (υ(C-C), collagen proline)	820-952
6 (bone mineral phase)	925-961
7 ($\upsilon_3PO_4^{3-}$, phosphate)	1021-1083
8 (Amide III)	1210-1288
9 (Amide III)	1302-1400
10 ($\delta(CH_2)$, protein deformation)	1445-1491
11 (Amide I)	1651-1677

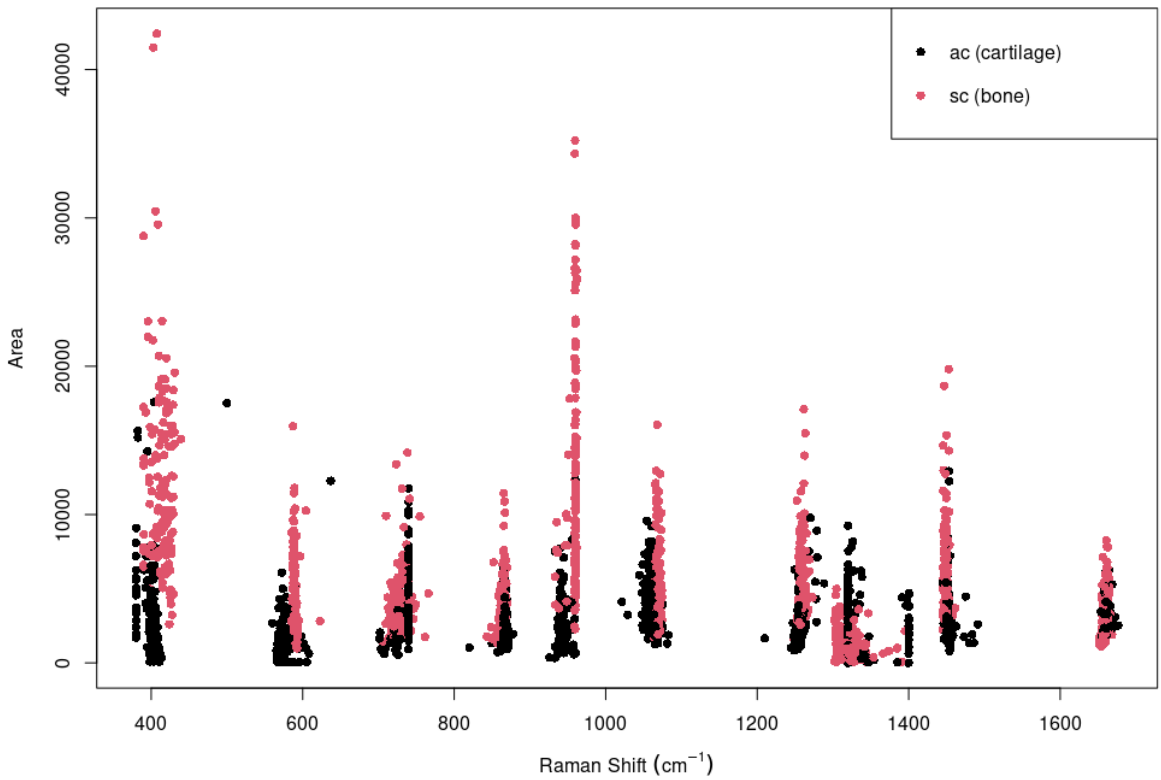

Figure 6.4 Area (pre-standardisation) versus Raman shift for all analysed peaks in the data set by sample tissue (articular cartilage in black, subchondral bone in red).

Exploration of the data was conducted by examining the relationship between area and joint (OA vs control), collected tissue (ac/sc), and the sample location (C3, C4, R2 and R3); these are shown as boxplots of standardised areas in Figures 6.5-6.7, and Appendix B, Figures 2-8.

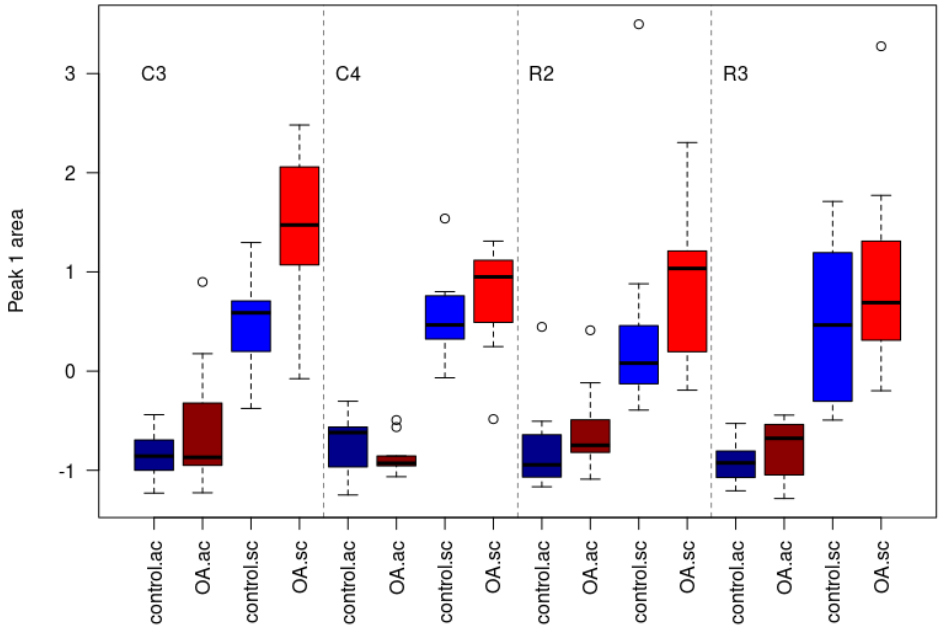

Figure 6.5 Peak 1 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

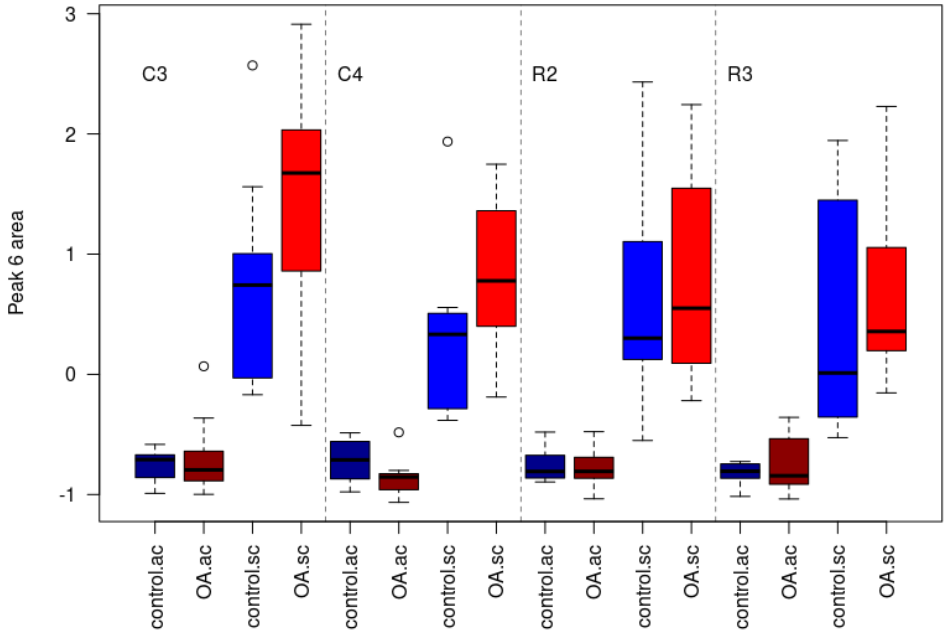

Figure 6.6 Peak 6 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

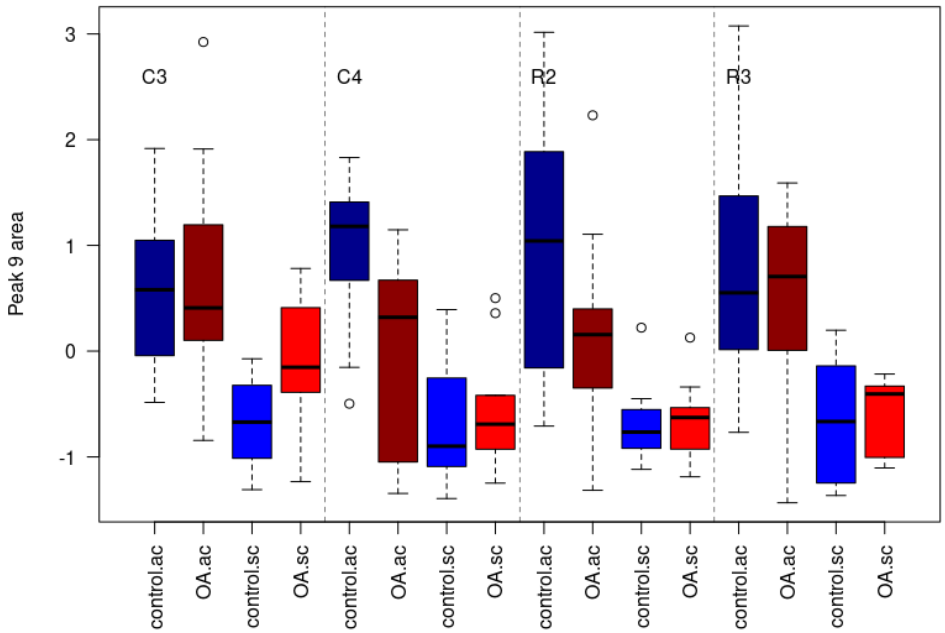

Figure 6.7 Peak 9 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

The two tissues (ac and sc) show different area distribution across the 10 spectral peaks. The joint factor appears to affect the area of some peaks with different effects according to the location and tissue. For example, peak 1 area is increased in the sc tissue for OA joints in locations C3, C4 and R2, but stays constant in all ac locations and for sc in location R3. For some peaks the joint factor affects differentially the area in the two tissues in some locations. As an example, the peak 9 area decreases in C4/ac samples between control and OA, while it is constant in C4/sc samples. In contrast, the area under peak 9 increases in C3/sc samples between control and OA but remains constant for C3/ac samples. This suggests that the area may be useful to classify the joint (OA or control), but the direction of the relationship might depend on the tissue and/or on the location of the sample. The authors standardised the peak

areas so that only their relative patterns among samples could be compared directly. Therefore, among all samples, the average of each peak area was 0 and its standard deviation was 1.

The first six components of the PCA applied to the standardised peak areas included 68%, 14%, 8%, 3%, 2% and 2% of the total variation, respectively. Among all the factors in these data, only a clear separation of ac and sc samples could be observed, using a combination of PC1 and PC2 (Figure 6.8). No separation by joint, sample location, horse, or combination thereof with tissue type was observed. Performing two separate PCAs on standardised peak areas of the ac and sc samples, respectively, was tested, and this also led to no separation by joint, sample location, horse. The first six components of these PCAs resulted in 61%, 13%, 10%, 6%, 3% and 3%, and in 80%, 7%, 4%, 3%, 2% and 1% of explained variance for the ac and sc sample analyses, respectively.

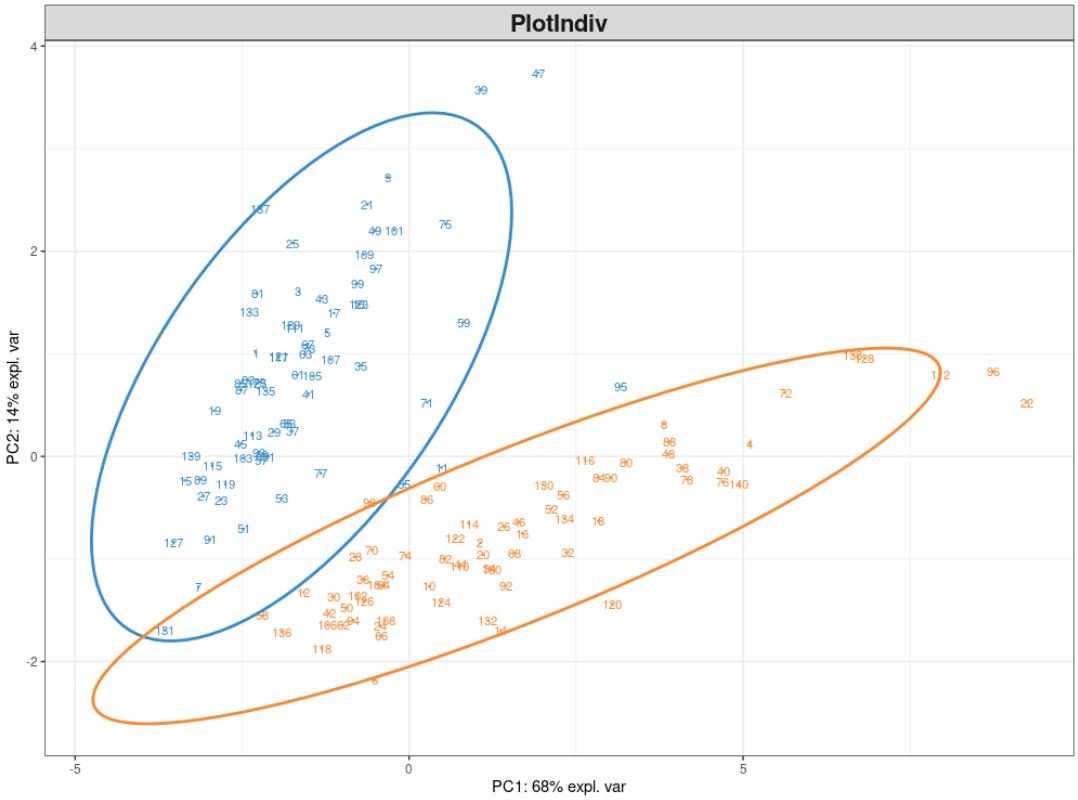

Figure 6.8 PCA sample plot for component 1 (x-axis) and component 2 (y-axis) for ac (blue) and sc (orange) samples.

When random forest was used on the different classification tasks the accuracy obtained was: 97.9% by tissue (2 classes) with peaks 1 and 6 being the main driving factors (followed by peaks 2, 9 and10); 47.9% by joint (2 classes); 27.1% by location (4 classes); 46.4% by joint x tissue combination (4 classes), 27.1% by tissue x location (8 classes); 8.6% by joint x tissue x location combination (16 classes); and 10.7% by horse (9 classes). For a random classification an accuracy of $1/k$ is expected, where k is the number of classes to differentiate. For example, with 2 classes, 1/2=50%, and for 8 classes, 1/8=12.5%.

Last, the authors present the result of the Bayesian logistic regression. The effect of a few peak areas on the probability of a sample to be predicted OA in different tissues or in different locations is visualised. We first fitted a model (model 1) that expresses the probability of OA

as a function of all 10 standardised peak areas; model 1 has 11 parameters. The model depicted in Fig. 6.9 and 6.10 (model 2) expresses the probability of OA as a function of all 10 standardised peak areas, whose effects are tissue specific. Model 2 has 22 parameters. For example, Figure 6.9 shows the probability of a joint to be OA according to peak 11 standardised area, when all other peak areas are kept constant. Increasing peak 11 area tends to identify OA (as opposed to control) joints in either ac (black) or sc (red) samples. The figure also shows the corresponding 90% predicted credible intervals. Another example is shown in Figure 6.10 which shows the probability of a joint to be OA according to peak 8 standardised area for ac and sc samples, while other peak areas are kept constant. Increasing peak 8 area tends to identify OA (as opposed to control) joints in ac tissue (black) but has the opposite effect of classifying samples as control in sc tissue (red). Note that simplifying assumptions were made as peak areas were correlated (Figure 6.11).

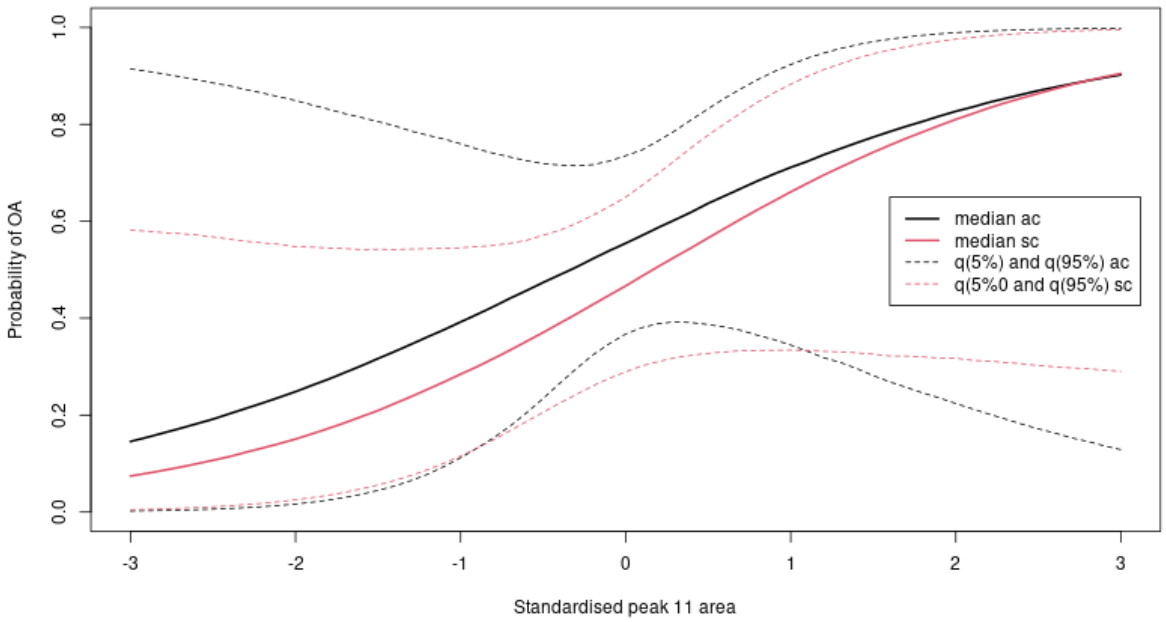

Figure 6.9 Predictive probabilities of sample to be classified as OA as a function of the peak 11 standardised area; median (thick line) with 90% credible bands (thin dashed lines) for ac (black) and sc (red) samples.

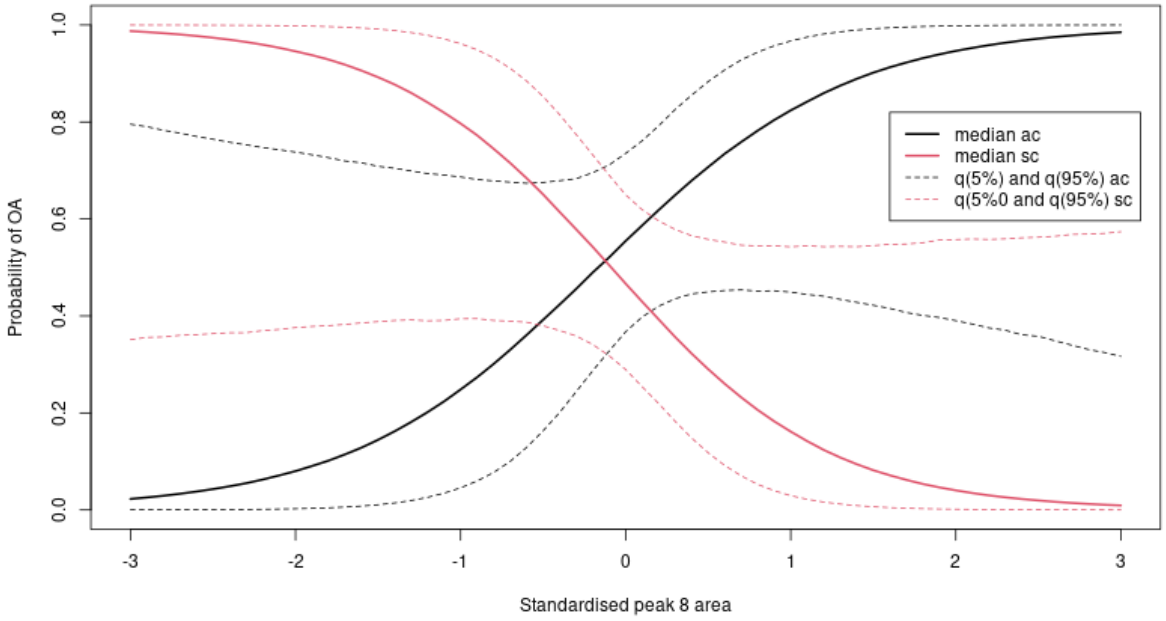

Figure 6.10 Predictive probabilities of a sample to be classified as OA as a function of the peak 8 standardised area; median (thick line) with 90% credible bands (thin dashed lines) for ac (black) and sc (red) samples.

The authors also modelled the probability of OA as a function of all 10 standardised peak areas, with location-specific effects (model 3). Model 3 has 44 parameters and large uncertainty in effect coefficient estimation. The effect of peak1 area on the probability of a joint to be OA for C3, C4, R2 and R3 locations is shown in Figure 6.12. The C3 or R2 peak 1 area effect is different from the peak1 area effect for C4 or R3, which is positive versus not impactful, respectively. A final model (model 4) expressed the probability of OA as a function of all 10 standardised peak areas, with an interaction of location and tissue for each peak effect. This model was less reliable as it contained 88 parameters (model not shown). The results for the effect of the peak area on the prediction of AO Vs control for models 1-3 are reported in Appendix B, Table 1.

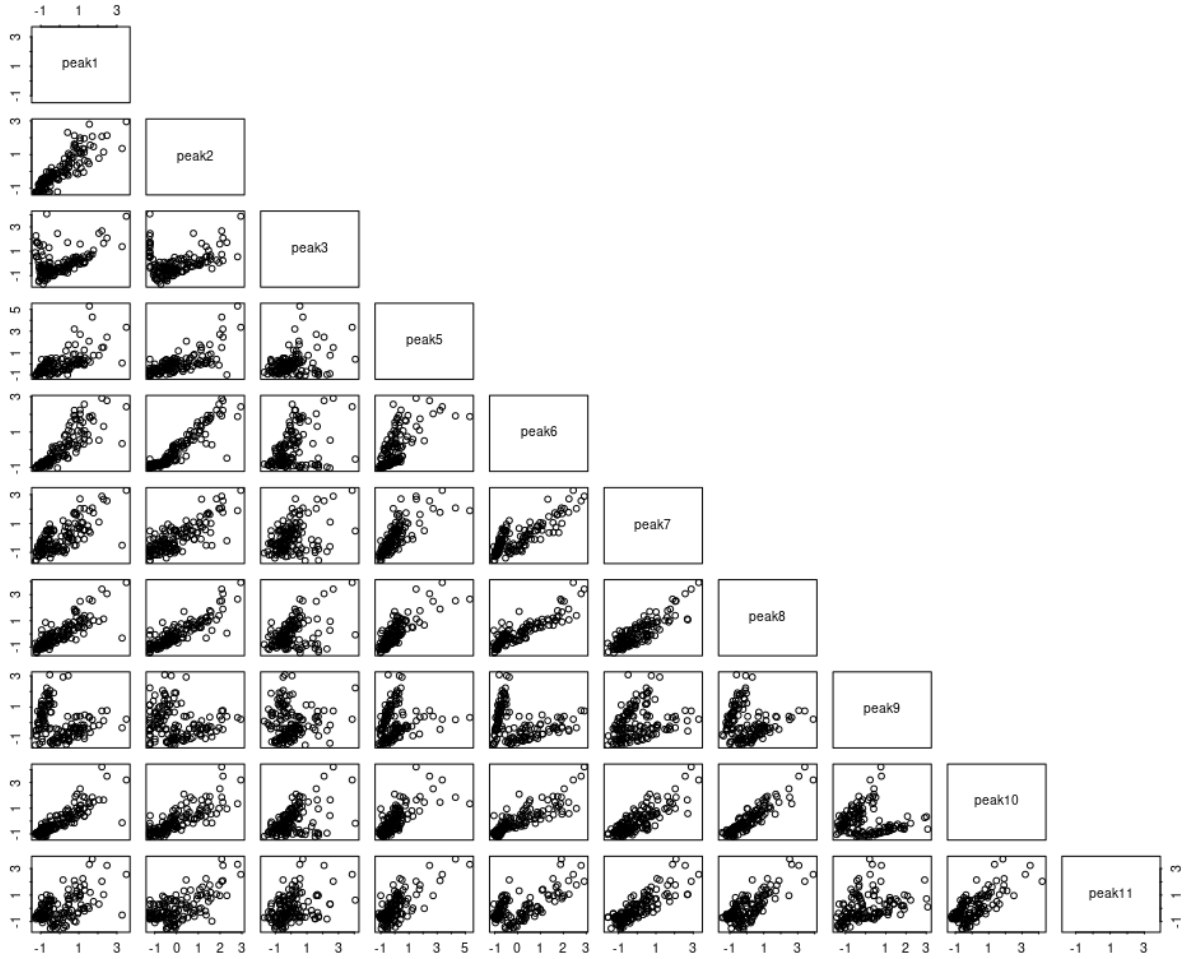

Figure 6.11 Pair scatterplots of each peak area as a function of other peak areas. Plot in row *i* column *j* is the scatterplot of the standardised peak *i* aera as a function of the standardised peak *j* area.

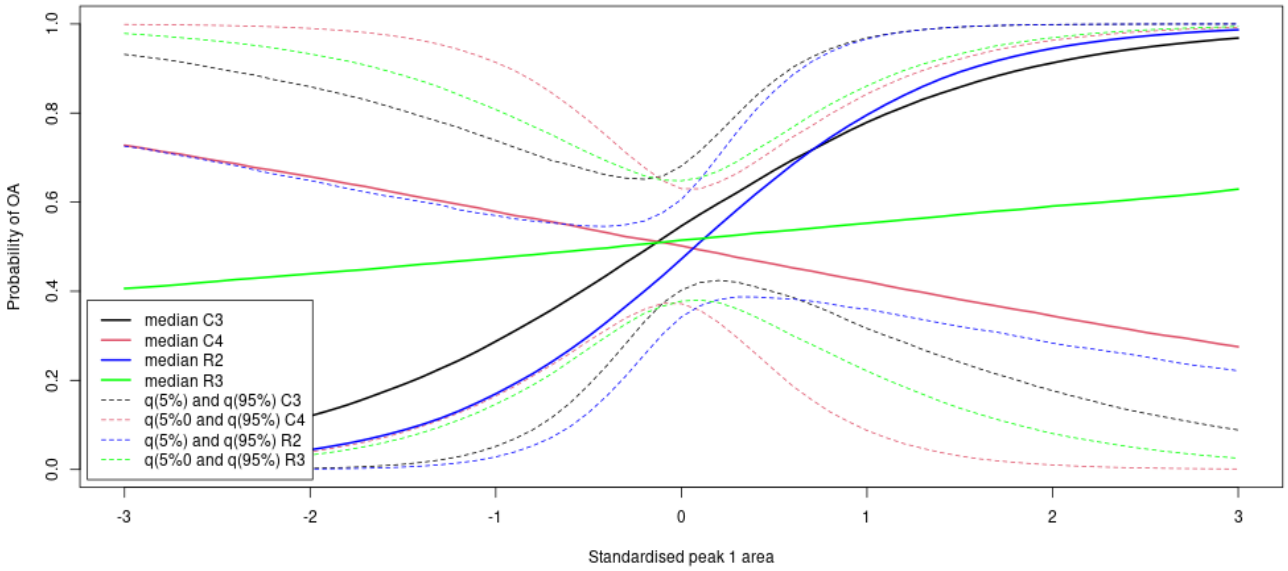

Figure 6.12 Predictive probabilities of sample to be classified as OA as a function of the peak 1 standardised area; median (thick line) with 90% credible bands (thin dashed lines) for C3 (black), C4 (red), R2 (blue) and R3 (green) samples.

In summary, most peaks exhibited an increased area in the subchondral bone of the third carpal bone (location C3, tissue sc) in OA joints compared with controls. Other specific relationships exist. As another example, in R2/sc, peak 1 area for the OA joints is larger than that of the corresponding control joints. This effect is also seen to a lesser extent in peak 3.

6.5 Discussion

This study described the use of RS on cartilage and bone from joints in an attempt to distinguish those with surgically induced OA from contralateral controls. The results show that clear separation could be observed with PCA between ac and sc samples, but not between joints (OA Vs control), locations within joints, horses, or combinations thereof. Statistical modelling allowed estimations of the probability of classifying samples as OA by location and tissue type and identified some peaks of interest.

On histopathological examination the synovial tissue features of OA joints were consistent with synovitis, and the scores were significantly higher in the OA joints compared with controls, regardless of the location. Histological scores were also increased in areas that were not in close proximity to the surgically induced osteochondral fragmentation, such as for Syn-C4 samples, which suggests that inflammation affected all of the synovium of OA joints rather than just synovium local to the osteochondral defect. Conversely, the bone/cartilage scores of the OA joints were significantly higher than controls only in the sections obtained at the level of the osteochondral fragment itself (R2) and the area directly opposite (distal) to it in the lower row of carpal bones (C3), thus suggesting that significant bone/cartilage changes only occurred focally, in proximity of the fragment.

Raman spectroscopy of bone has traditionally relied on the analysis of specific scattering bands considered characteristic, in particular the ones associated with υ^2 phosphate, carbonate type B, υ^1 phosphate and amide III [67, 68]. Using these bands, intensity ratios are then calculated to obtain the mineral-to-matrix ratio, carbonate-to-phosphate ratio, and crystallinity and these are compared between groups [42, 69]. A different approach was used in the current study as the authors considered all peaks which were clearly identifiable or present in the spectra. The authors did not calculate the ratios but used standardised peak areas for modelling to classify the samples and Bayesian logistic regression to estimate the probability of a sample to belong to OA or control joint groups. The advantage of this approach consists in using all the information present in the spectra rather than a small number of arbitrarily selected peaks with the standard approaches. Moreover, we relied on the models to inform which peaks would provide the best predictions.

On a preliminary data exploration with box plots using peak areas by location and tissue, a clear difference was observed between OA and control joints for sc tissue, specifically in the third carpal bone (C3) in all peaks and in correspondence to the osteochondral fragment (R2) in some peaks (peaks 1 and 3). These findings are particularly noteworthy as they mirror the histopathological scores obtained in the same locations, closest to the osteochondral fragment. This suggests, that with the equine OA model used, RS was able to detect differences in peak areas only in locations within the joints where direct damage was induced (fragment) or secondary damage ("kissing lesion" on C3). The use of PCA though did not prove useful to detect changes induced by this OA model, as it allowed us to only separate tissue types (ac Vs sc) regardless of the origin of the sample (OA vs control, location, or horse). Further modelling confirmed these findings as poor classification rates were obtained for all factors, except for tissue type. When models were fitted using Bayesian logistic regression some insights were gained on the probability of classifying samples as OA or control joints. These depended on the individual peaks and tissues as exemplified in Fig. 6.9 and Fig. 6.10. Interestingly, for peak 1 (Fig. 6.12) the probability of classifying samples as OA increases as the peak area increases for locations R2 and C3, while the probability remains virtually unchanged for R3 and C4. Once again, R2 and C3 correspond to the areas where most tissue changes were expected based on the proximity to the osteochondral fragment, and as confirmed with histopathology and suggested by visual assessment of the box plots of the peak areas.

The effect of exercise on the spectral data in our study cannot be determined due to the lack of a group of unexercised horses that could be used as negative controls. The effect of exercise (13 weeks in duration) on bone mineral density in horses has been described in the literature for the third carpal bone, with increased density in exercised horses when compared with controls [70]. The degree of the changes varied though depending on the location within the

bone. In the same study exercise was also associated with an increase in cartilage thickness. Another study that used a treadmill exercise protocol similar the one described in the current study, but with a much longer duration (6 months), found an increase in bone density in the metacarpal condyles of exercised horses, but not in their third carpal bone and radial carpal bone [71]. Comparison between studies is difficult due to the different type and duration of exercise and other variables. Although in the current study the authors did not measure the bone mineral density, it is unknown if the Raman spectra were influenced by the effect of exercise. The extent of this effect is not quantifiable from the current study, as a negative control group of unexercised horses was unavailable. Moreover, as the horses with induced OA had a variable degree of lameness on the affected (OA) limb (Appendix A, Table 1), the opposite limb would have been subjected to higher loading during exercise. This may have influenced the chemical composition of bone and cartilage of the control joints, potentially even obscuring to some the degree the true extent of the effect of osteochondral fragmentation on the Raman spectra of OA joints. Finally, it should be noted that changes in bone density in equine limbs does not necessarily correlate with chemical changes identifiable with RS, as has been shown in horses with bone marrow oedema-like abnormalities associated with increased bone density, but no detectable changes in the chemical composition of bone using RS [55].

The current study presents some limitations, and the results need to be interpreted taking these into consideration. Specifically, the limited number of study subjects prevented further statistical modelling to account for correlations between peaks, and a location-specific effect (C3/C4/R2/R3) in interaction with tissue type. Furthermore, the statistical models used were based on a linear effect of peak areas on the logit of probability of OA Vs control. Additionally, it was not possible to reproducibly standardise the region of spectra collection from cartilage

and bone sections, likely introducing variation into the data. Finally, for logistical reasons beyond the authors' control, four horses were euthanised at 63 days while five at 70 days after surgical induction of OA. Although this introduced some source of variability in the study, histopathology revealed that synovial inflammation, bone, and cartilage changes were well established by this time point, as such was likely to have minimal impact on the Raman spectra.

In conclusion, the model of OA used induced diffuse synovial, and focal cartilage and bone histopathological changes. Using Raman spectroscopy clear identification of OA joints was difficult, although for sc tissue type the use of peak areas may be useful to identify affected joints. The use of Bayesian logistic regression models provided insights on the probability of correctly identifying affected tissues in some locations using some peaks of interest in the spectrum. To the author's knowledge this is the first study applying RS techniques to a group of horses with induced OA using a carpal osteochondral fragmentation model.

6.6 References

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Chapter 7
General Discussion and Concluding Remarks

7.1 General Discussion

Osteoarthritic disease dates back to the prehistoric era, with evidence found in dinosaurs [1]. Bioarchaeological studies report that OA was by far the most common joint disease found in hominid fossils from Neolithic sites and Egyptian mummies [2-4]. Osteoarthritic changes in the distal tarsal joints and proximal interphalangeal joints have also been identified from paleopathological studies of cattle and horses from ancient Roman times [5]. Nowadays, OA is a leading cause of joint disease in people worldwide, with estimates of 100 million people [6] and 91 million adults [7] affected in Europe and the U.S.A., respectively. The economic impact of OA is substantial with total annual direct medical costs estimated in excess of US$370 billion in the U.S.A., with an average of over US$11,000 per patient [8]. With the growing age of the population, obesity and sedentary lifestyle, the prevalence of OA is expected to rise in the future [9, 10]. The burden of OA goes far beyond its economic impact, representing a psychological burden on society due to reduced quality of life associated with physical disability, chronic pain, loneliness, and low self-esteem [11].

The prevalence of lameness in horses is high and, in most cases, is caused by OA [12-14]. The estimated direct and indirect annual costs to manage a horse with OA in the U.S.A. are US$3,000 and US$15,000 , respectively [15]. Despite extensive research in the past several decades, there is currently no cure for OA in horses or people, and available treatments are symptomatic or only partially modify the course of the disease or its progression [16, 17].

Diagnosis of OA mostly relies on history, clinical signs, and imaging (“dry biomarkers”). The latter most commonly in the form of radiography, despite more advanced techniques

becoming progressively more available [18-21]. Unfortunately, there is poor correlation between clinical signs and radiographic findings [22], and imaging techniques have low sensitivity [23] and specificity [24]. Additionally the use of advanced imaging modalities is hampered by the high cost [25]. Early diagnosis is paramount to prevent further joint damage and slow disease progression [26]. There is a need to investigate and validate biomarkers of OA that allow clinicians to stratify disease, identify risk of progression and test alternative therapeutic approaches [21, 27, 28].

A large body of research in people has been published in the last decades on potential markers of joint metabolism and OA in synovial fluid, blood, and urine [29-33]. Likewise in horses, multiple studies have investigated the use of biomarkers of OA [22, 34-39]. Despite encouraging results in some studies [36-39] and the potential for clinical application to equine serum [40] and synovial fluid [41, 42], adoption of these biomarkers in daily practice has not been established [30]. This is due to the limitations of these techniques which are associated with high costs, impracticality, and lack consistency of results to be able to implement them for disease surveillance [43]. Making an early diagnosis of OA and monitoring progression and response to therapy continues to remain a challenge, so there is a need to develop alternative, affordable tests with higher reliability and practicality [44].

This thesis presents a series of studies which aimed at investigating alternative biomarkers or biomarker profiles of early OA in horses with the potential for clinical application. Although there are several models of equine OA in the literature [45-50], the one that has been most widely used is the carpal osteochondral fragment and exercise model [51-54]; this was used in my study. This model aims to closely reproduce the characteristics of naturally occurring post-traumatic OA in racehorses by surgically creating an osteochondral fragment in the radial carpal bone, a

common site of injury of racehorses [52]. The model also exploits the effects of treadmill exercise to generate clinical signs, biomarker, and histological changes consistent with OA in a relatively short period of time and has been used in multiple drug efficacy studies [55-59]. The cohort of animals used in our study was carefully selected with the intent of limiting the sources of variation and reducing confounding factors associated with breed, sex and age. In fact, unlike in previous studies I used untrained horses of a single breed, of the same gender and of very similar age (fifteen 2-year-old and two 3-year-old horses).

In **Chapter 3** I investigated the use of cfDNA as a potential marker of OA in horses. The presence of cfDNA in the peripheral circulation has been known to be present in people since 1948 [60], but its significance was not further investigated until more recent years when its presence was associated with a plethora of human medical conditions, such as myocardial infarction, immune-mediated and neoplastic diseases [61-63]. Although in veterinary medicine cfDNA has been described in some diseases in dogs [64, 65], there is very limited published literature in horses. The results of the current study demonstrated that cfDNA in plasma is not a useful marker of OA in the model utilised. Similar findings were reported in foals where cfDNA failed to differentiate critically ill from healthy foals [66], although in adults with colic cfDNA concentrations were significantly increased compared with controls in another study [67]. When cfDNA concentrations were used for synovial fluid in my study, significant differences were detected at two sampling times, and at two additional times these approached statistical significance (i.e., a trend). The latter results on synovial fluid are encouraging, but at this stage cfDNA concentrations cannot be recommended broadly for use in equine research, given the lack of consistency over our studied time period. As this was the first study on cfDNA in horses with focal orthopaedic disease, the author believes that further assessment is necessary before accepting this marker for use in synovial fluid of horses with experimental OA. The

results of this study also do not provide sufficient evidence for the clinical use of cfDNA concentrations, but clinical research in horses with naturally occurring OA may provide further insights. Of particular interest in our study was the ease of use of the fluorometric method and the precision of the assay consistent with other studies [67, 68], which supports its further development.

While Chapter 3 investigated a potentially novel biomarker for OA in horses, the fundamental approach is similar to the traditional methodologies commonly employed in OA research. These aim at identifying a marker or a combination of markers that allow differentiation of affected from unaffected individuals or joints, for diagnostic purposes, monitoring progression of disease or assessment of efficacy of intervention. **Chapter 4** and **Chapter 5** used an alternative approach to identify horses or joints with induced carpal OA. Rather than separating or detecting a single molecule, IR spectroscopy produces a profile of all IR absorbing molecules contained within the fluid being examined [69]. This characteristic is particularly attractive for a complex disease such as OA, where multiple joint tissues play a vital role in the disease, anabolic and catabolic states alternate, and inflammation and degeneration generate complex interactions and signalling pathways [14, 70]. Moreover, IR spectroscopy has shown high sensitivity and specificity in other diagnostic applications [71, 72], and is a low-cost technique that requires no reagents. Infrared spectroscopy was successfully used on serum of rabbits with induced OA by transection of the cranial cruciate ligament [73] and serum of dogs [74] with naturally occurring OA associated with cranial cruciate ligament rupture. In both the rabbits and the dogs, subjects with OA were identified with high sensitivity and specificity. Conversely, in my study IR spectroscopy on serum failed to identify with good accuracy the horses with OA. However, there are significant differences between these studies and a direct comparison cannot be made. In the rabbit and the dog studies two

factors could have contributed to generate a stronger IR signal, specifically the joint inflammation from transection or rupture of the intra-articular cranial cruciate ligament, as well the significant joint instability associated with it, which was not present in the model I used. In addition, in the dog study the subjects were clinical cases, and it is likely that the degree of OA was significantly more chronic and advanced than in my horses. An equine study on the use of IR spectroscopy on serum showed excellent accuracy in separating horses affected by OA from controls [75]. Some differences with our study though could contribute to the markedly different results, specifically the selection of study subjects, joints, the nature of disease and a more scientifically robust method of spectral analyses. In that study multiple breeds were used, and the age range was very wide, but importantly horses affected by OA had naturally occurring disease and in some of them multiple joints were affected. It is reasonable to assume that these horses had more advanced and chronic disease compared with our subjects and that having more than one affected joint in some subjects could provide a stronger IR signal in serum. Although IR spectroscopy did not prove useful when used on serum in our group of horses to differentiate affected horses from controls, its use on synovial fluid yielded good results. In fact, I was able to show excellent accuracy in differentiating OA joints from all other joints (80%) and good accuracy in differentiating OA joints from OA-control (75%) or Sham joints (70%). These findings are consistent with previously published literature in dogs with naturally occurring knee OA, where IR spectroscopy showed a greater accuracy when used on synovial fluid rather than serum [74, 76]. In horses, IR spectroscopy techniques used on synovial fluid to differentiate joints affected by osteochondrosis from normal joints showed comparable accuracy (77%) to my study [41]. In another equine study, IR spectroscopy showed excellent accuracy (89-97%) [42] to separate joints affected by traumatic arthritis of clinical cases from controls. The intrinsic nature of the model of OA I used is

possibly the main reason for the lower performance of IR in our study. The degree of joint injury we induced is unlikely to precisely mirror the degree of injury experienced by joints with naturally occurring disease. Also, the duration of our study was relatively short compared to the likely longer duration of disease of horses presented for surgical intervention in the previous studies. Overall, the results of our studies in Chapters 4 and 5 demonstrate that IR spectroscopy holds value in a research setting using this model of equine OA, but only when applied on synovial fluid. It has been shown that some biomarkers of joint metabolism are present in concentrations 25-1000 times lower in serum than synovial fluid, and that these are below the detection threshold of the IR spectroscopy techniques used [40, 51, 77]. It is likely that synovial fluid better reflects the metabolic and pathologic state of joints compared to serum due to significantly higher concentrations of some joint biomarkers [51, 78, 79].

In **Chapter 6** I investigated the use of Raman spectroscopy (RS) on cartilage and bone from my group of horses with the aim of assessing the accuracy of this technique to discriminate osteoarthritic cartilage and bone from controls. Similarly to IR, RS provides a chemical fingerprint unique to the material being examined [80]. While IR is based on the absorbance or reflectance of electromagnetic radiation by the sample material, RS utilises the inelastic scattering generated when an incident laser source interacts with molecular bonds [80]. Although RS techniques have been used in a wide range of scientific and industrial settings, limited published literature is available on horses. Thus far research using RS has focused on the general properties of equine bone [81-84], bone oedema [85], sesamoid bone fractures [86], and osteochondrotic cartilage [87], but to the author's knowledge there are no reports on the application of RS techniques to cartilage and bone of horses with OA. In the current study, the histopathological findings and scores of synovial samples obtained from OA joints were consistent with diffuse synovitis. Conversely, the histopathological findings and scores of the

osteochondral sections in OA joints were only limited to areas in direct proximity to the surgically induced fragmentation in the radial carpal bone (R2) and the third carpal bone (C3), consistent with focal OA. With these types of tissue changes, inflammatory mediators and catabolites are released into the joint space [53, 88, 89]. Therefore, it is not surprising that in my previous studies on cfDNA and IR spectroscopy synovial fluid proved to be a better biological sample for OA investigation compared with plasma or serum, respectively. The histopathological findings were also mirrored in the analysis of the Raman spectra. In fact, for all peaks used in the analysis the standardised peak area of subchondral bone (sc) in OA joints was greater than in the control joints at the level of C3, and for some peaks at the level of R2 as well, both corresponding to areas close to the osteochondral fragment. Using PCA of Raman spectra, it was possible to clearly separate only articular cartilage (ac) from subchondral bone (sc), but samples could not be classified by joint (OA vs control), location within joints, horses, or combinations thereof. Further data classification with random forest yielded high error rates for all classification tasks except for tissue type, thus confirming the results of PCA. Bayesian logistic regression allowed identification of significant relationships between peak areas and the probability of a sample being classified as OA for some specific peaks in the Raman range. Noteworthy is the fact that for peak 1 the probability of classifying samples as OA increased as the peak area increased for the locations C3 and R2, areas in close proximity to the osteochondral fragmentation. In conclusion, Raman spectroscopy detected differences in spectral profiles between OA and control joints in some subchondral bone locations, but clear separation could not be obtained between groups. Bayesian logistic regression models provided insights into the probability of correctly identifying affected tissues in selected peaks of the Raman spectra for some of carpal joint locations.

7.2 Limitations

A potential limitation in this series of studies is the relatively low number of horses. Although a larger population may have strengthened the probability that our results were not affected by low statistical power, ethical considerations are of concern when using a large animal species like horses when compared with laboratory animals, especially if the endpoint of the study involves the sacrifice of a group animals. Previous biomarker studies [51, 52, 90] which used eight horses per group have been able to demonstrate significant differences between groups, hence our decision to use similar numbers. In addition to ethical considerations, a significant challenge that limited the number of horses we were able to use was the high cost of such a large-scale project.

Another limitation is the lack of an age-matched unexercised control group. This limited our ability to assess the effect of exercise on the concentration of cfDNA and on the IR spectral profiles of plasma, serum, and synovial fluid as well as on the RS fingerprint of cartilage and bone. Exercise is known to cause rapid release of cfDNA into the circulation, but this rapidly returns to baseline values in 30-120 minutes in other species. Our plasma and synovial fluid samples were obtained over 72 hours after exercise, thus reducing the possible confounding effects of exercise, although admittedly the half-life of cfDNA in horses is unknown. The effect of exercise on serum and synovial fluid IR spectral profiles is currently unknown, therefore we cannot exclude that this may have influenced the biochemical composition of our biological samples and reduced our ability to detect differences between groups. Although exercise has been shown to alter the bone mineral density in horses, thus potentially having the ability to change the bone RS profile, this was not the case in carpal bones [23]. As our horses developed lameness in the OA affected limbs, the contralateral limb was subjected to higher load during

treadmill exercise, a factor that could have potentially obscured changes in the Raman spectra in OA joints.

Age, breed, and sex of our animals were chosen to reduce variability in this study, but generalisation of the findings should be made cautiously as differences may exist in different breeds, horses of different ages and gender.

Another limitation is that only horses with induced OA were sacrificed at the end of the study for ethical reasons, therefore results from the Sham operated horses could not be included in Chapter 6. Given the results of Chapter 6 though, where OA joints and control joints did not show significant differences in RS profiles, it is unlikely that including the Sham and Sham control joints would have altered the significance of the results of that study.

The results of these studies can only apply to this model of OA, as significant differences exist between models. Moreover, this model of OA may not necessarily closely reflect all the changes associated with naturally occurring OA in horses, therefore clinical validation of these techniques is necessary before recommendations can be made for use in practice.

7.3 Future Work

The work undertaken and described in this dissertation has generated novel information in the field of equine OA. Considering the results obtained and the limitations described, there are some areas that would deserve further consideration or development.

The origin and significance of cfDNA in the literature is still not conclusively understood, but its use as a marker has proven useful in several medical conditions in other species [61, 65]. In my study, baseline concentrations of cfDNA are only providing a “snapshot” in time and cannot be used as reference of normal values for horses in general. To do so, collection of samples

from a larger population, with a broader range of age, breed and gender would be needed. This would be valuable information to guide future studies utilising cfDNA in horses. Moreover, I only considered the total concentration of cfDNA in the samples, but I did not investigate specific sequences of DNA as has been done in some studies [91, 92]. Further research in this field could consider whether specific sequences of cfDNA can be associated with OA, rather than total cfDNA concentrations. Unfortunately though, this would inevitably involve the use of more sophisticated laboratory techniques and increase costs, with the risk of potentially making cfDNA a less desirable biomarker.

The results of the two IR studies suggested that the degree of changes associated with OA achieved with this model may be too subtle to be detected in serum with the IR technique we described. Although the length of the study period was similar to other studies in which ELISA techniques were used in conjunction with the same OA model [51, 52], it is possible that the study period may have been too short for OA to develop to a degree suitable for the detection threshold of IR techniques in serum. It could be warranted to extend the duration of the study period beyond 63 days, if serum is to be further investigated. In addition, the significance of the two IR studies is unknown from a clinical standpoint as the results obtained from a model do not always necessarily translate to clinical disease. Naturally occurring disease is likely associated with more advanced and/or chronic tissue changes and may be able to generate IR spectra with significant differences from controls. A prospective clinical trial with well-defined inclusion criteria for study subjects affected by naturally occurring OA may be able to explore the full potential of IR techniques. Similarly, there is no information on the use of cfDNA in horses with clinical OA, therefore this would be an area to explore further to gain a deeper understanding on the potential of cfDNA in horses.

The results of the RS study do not support the use of this technique under the same study conditions in the future, but similar considerations could be given as above in regard to the length of the study period and the degree of OA. I used RS *ex vivo* with the aim of assessing this technique for tissues affected by OA. Unfortunately *ex vivo* studies are intrinsically associated with terminal procedures as they involve collection of cadaveric tissues. There are reports in the literature on the use RS coupled with fiberoptics technology that have the potential for *in vivo* assessment via arthroscopy [93, 94]. This particular feature would allow cartilage assessment in clinical cases undergoing arthroscopy in young untrained horses with osteochondrosis, for example. The data collected could be assessed against future career performance to build a database for prognostication purposes. Raman spectroscopy has also been described in combination with needle arthroscopy [95], a minimally invasive technique that could have wide application in general practice. Currently there are no reports of this technology used *in vivo* in humans or horses, therefore this is an area that deserves consideration for future research. Other studies in humans and rats have reported the use of spatially offset Raman spectroscopic techniques on bone tissue that allow transcutaneous applications *in vivo* [96-99]. The non-invasive nature of these techniques would suit well the investigation of bone in horses for research purposes or as screening and diagnostic tools in a clinical setting.

7.4 Conclusions

The biomarkers/biomarkers profiles investigated in our study did not perform satisfactorily with the OA model utilised when used in serum/plasma. Synovial fluid is a better suited biological sample for the techniques and markers described in this dissertation and could be useful in a research setting for future studies. The significance of these results for use in cases

with naturally occurring disease is unknown and further validation is needed. Raman spectroscopy could not detect significant spectral differences between joints under the conditions examined in this study.

7.5 References

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Appendices

Appendix A

Figures

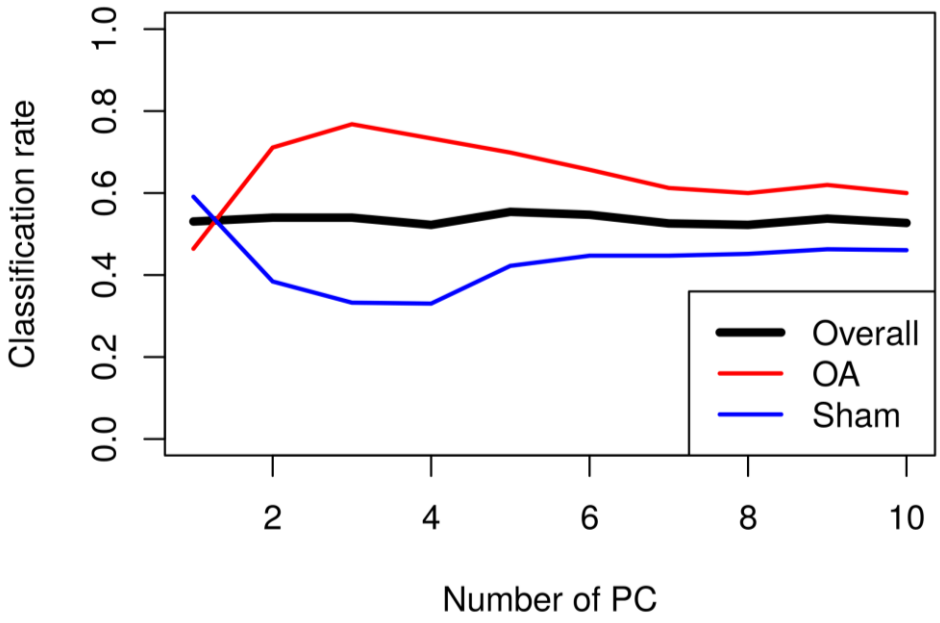

Figure 1 Correct classification rates overall (thick black line) and by horse group (two thin lines, red for osteoarthritis (OA), blue for Sham) as a function of the number of principal components (PC) used in the partial least squares discriminant analysis (PLSDA) with group as the output (task i). Note the lack of classification performance improvement with a higher number of PC.

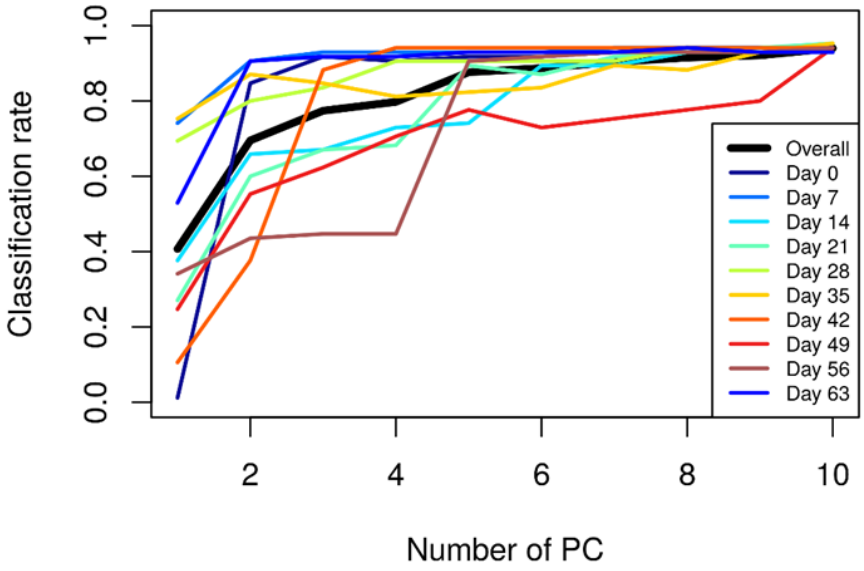

Figure 2 Correct classification rates overall (thick black line) and by sample Day (thin coloured lines) as a function of the number of principal components (PC) used in the partial least squares discriminant analysis (PLSDA) with sampling Day as the output (task ii).

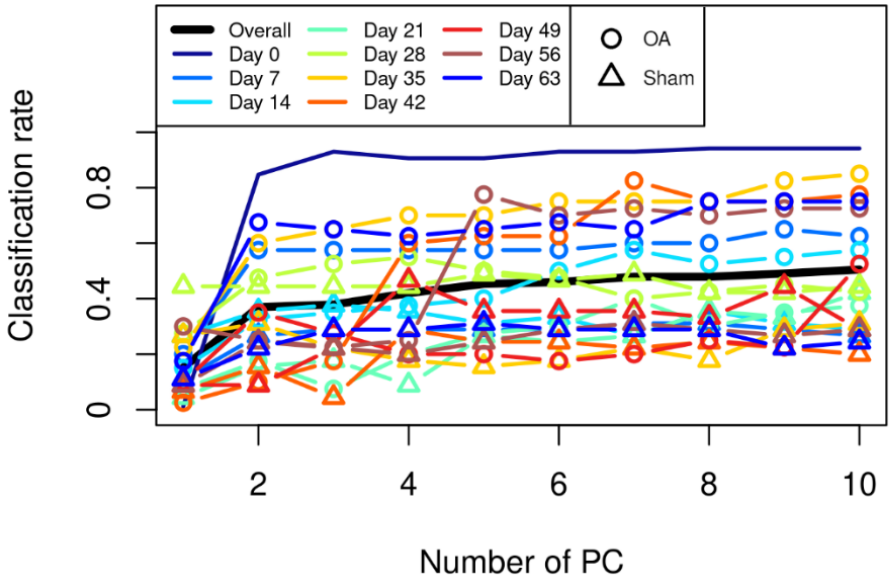

Figure 3 Correct classification rates overall (thick black line) and for all Days x horse combinations (thin lines, coloured by day, with circles for osteoarthritis (OA), and triangles for Sham) as a function of the number of principal components (PC) used in the partial least squares discriminant analysis (PLSDA) for task iii.

Tables

Table 1 Lameness scores of the osteoarthritis (OA) and Sham groups over time (0 = sound; 5 = non weight bearing lameness).

	OA	**Sham**	
Days	**Median**	**Median**	***P***
0	0^{a} (0)	0^{a} (0)	1.000
7	1^{b} (2)	1^{b} (1)	0.564
14	1^{b} (2)	0^{a} (0)	0.001
21	1^{b} (2)	0^{a} (0)	0.001
28	2^{b} (3)	0^{a} (1)	0.008
35	1^{b} (2)	0^{a} (1)	0.075
42	1^{b} (2)	0^{a} (0)	0.024
49	1^{b} (2)	0^{a} (1)	0.014
56	2^{b} (2)	0^{a} (1)	0.004
63	2^{b} (2)	0.5^{b} (1)	0.014

Values in brackets indicate the range. Different superscripts within columns indicate $P<0.05$

Table 2 Flexion test scores of the osteoarthritis (OA) and Sham groups over time (0 = normal; 4 = severe).

	OA	Sham	
Days	**Median**	**Median**	***P***
0	0^{a} (0)	0^{a} (0)	1.000
7	1^{b} (4)	0^{a} (2)	0.102
14	3^{b} (3)	0^{a} (1)	0.001
21	1^{b} (3)	0^{a} (0)	0.001
28	2^{b} (3)	0^{a} (0)	0.001
35	2^{b} (3)	0^{a} (0)	0.001
42	1^{b} (3)	0^{a} (0)	0.008
49	2^{b} (2)	0^{a} (0)	0.001
56	2^{b} (3)	0^{a} (0)	0.002
63	2^{b} (3)	0^{a} (0)	0.014

Values in brackets indicate the range. Different superscripts within columns indicate $P<0.05$

Table 3 Effusion scores of the osteoarthritis (OA) and Sham groups over time (0 = normal; 4 = severe).

	OA	Sham	
Days	**Median**	**Median**	***P***
0	0[a] (0)	0[a] (0)	1.000
7	2[b] (3)	1[b] (2)	0.163
14	2[b] (2)	1[b] (1)	0.061
21	3[b] (3)	2[b] (1)	0.007
28	3[b] (2)	1[b] (1)	0.002
35	3[b] (2)	1[b] (1)	0.001
42	3[b] (2)	1[b] (2)	0.005
49	3[b] (3)	1.5[b] (2)	0.075
56	2[b] (2)	0.5[b] (2)	0.011
63	2[b] (3)	1[b] (2)	0.007

Values in brackets indicate the range. Different superscripts within columns indicate P<0.05

Table 4 Median radiographic scores of the osteoarthritis (OA) and Sham groups for osteophytosis (0 = normal; 3 = severe) and lysis (0 = normal; 3 = diffuse/deep) before intervention and at the end of the study period.

	Osteophytosis			Lysis		
	OA	**Sham**	***P***	**OA**	**Sham**	***P***
Pre-intervention	0 (1)	0 (1)	0.501	0 (0)	0 (1)	0.700
End of study	2 (3)	0 (0)	0.002	3 (2)	0 (2)	0.003

Values in brackets indicate the range.

Table 5 Correct classification rates for classification task iii between Day 0 and all group-Day combinations (osteoarthritis (OA) top and Sham bottom).

	Correct classification rate	
Sampling Day	**OA**	**Sham**
0	0.941	-
7	0.267	0.625
14	0.289	0.575
21	0.422	0.375
28	0.444	0.425
35	0.311	0.850
42	0.200	0.775
49	0.289	0.525
56	0.489	0.725
63	0.244	0.750

Appendix B

Figures

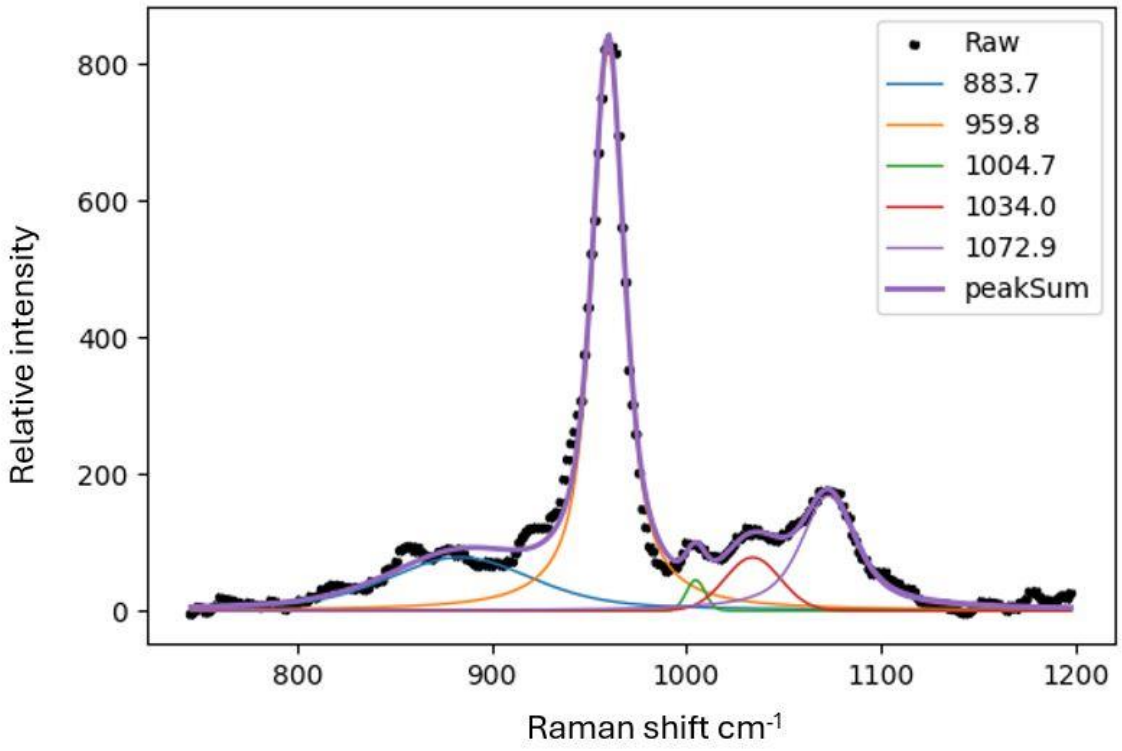

Figure 1 Example of peak-fitting of a select portion of a random spectrum from an sc sample (peak positions are not optimised).

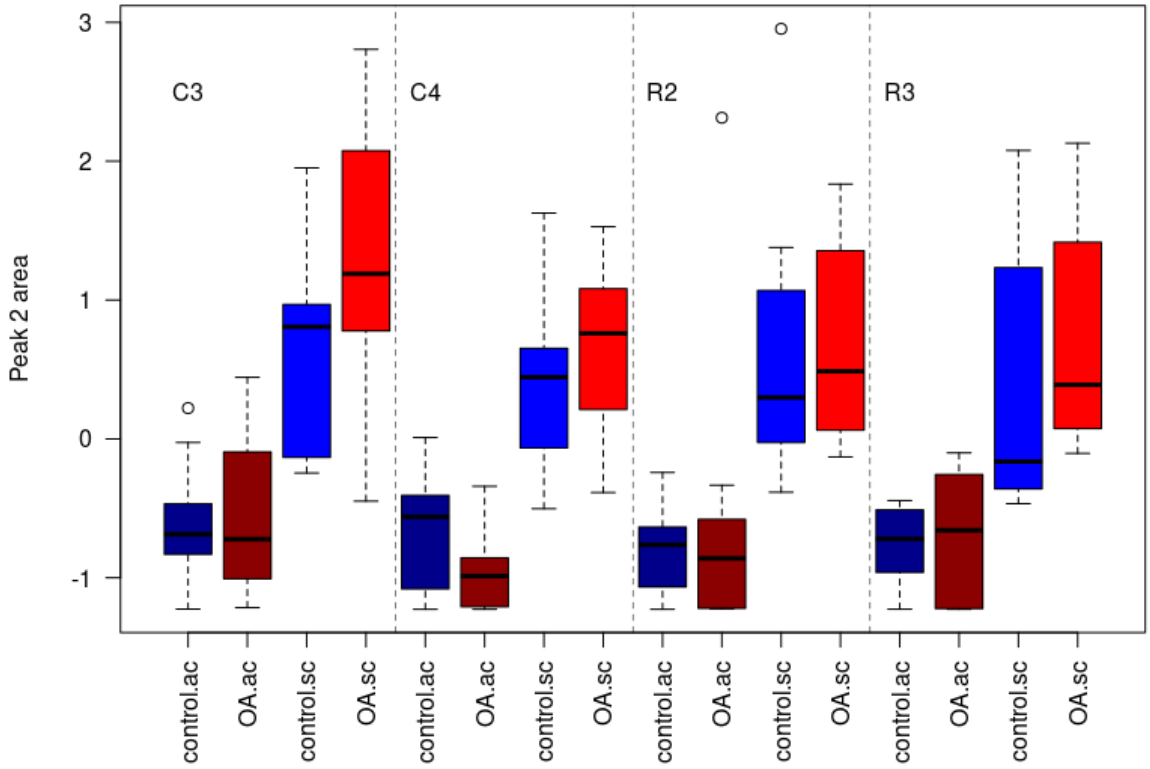

Figure 2 Peak 2 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

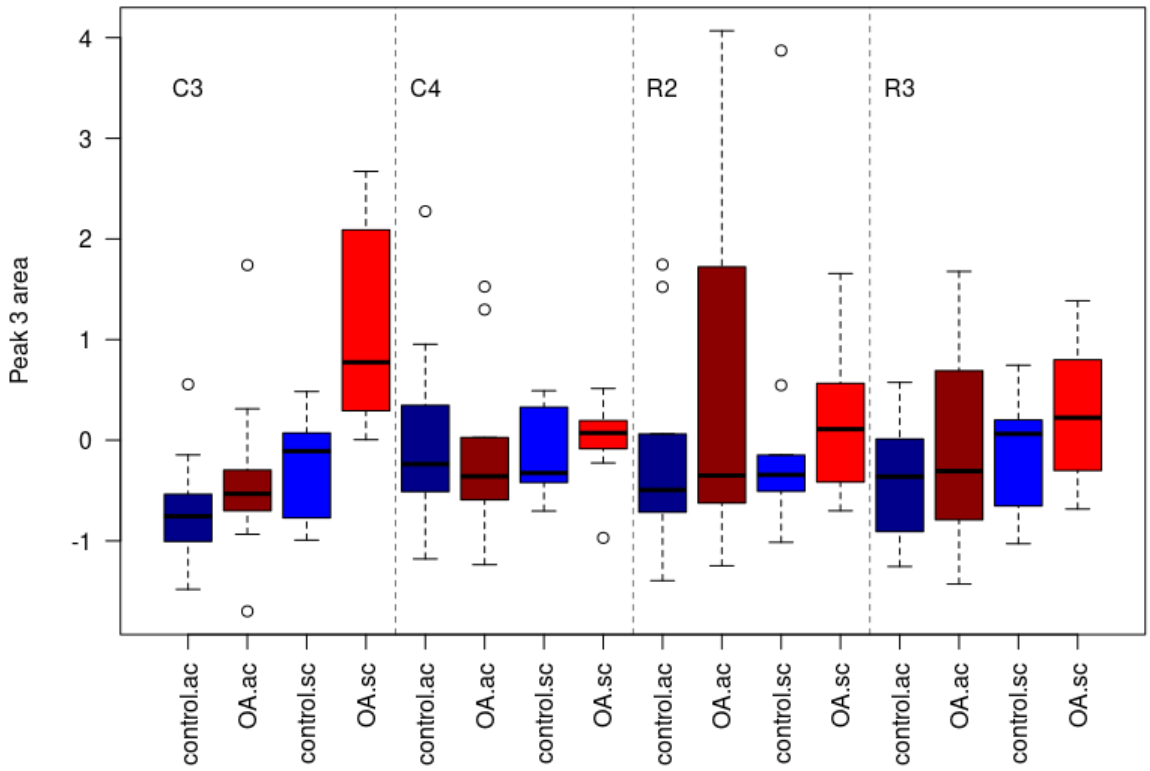

Figure 3 Peak 3 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

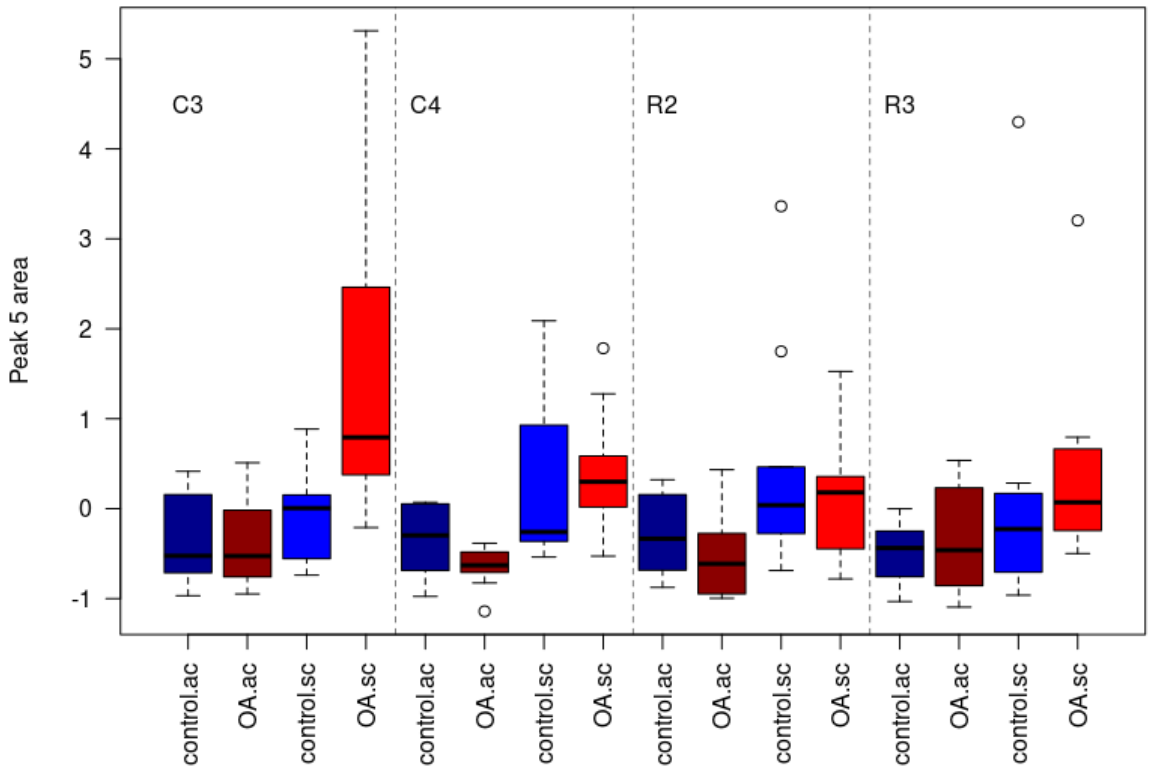

Figure 4 Peak 5 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

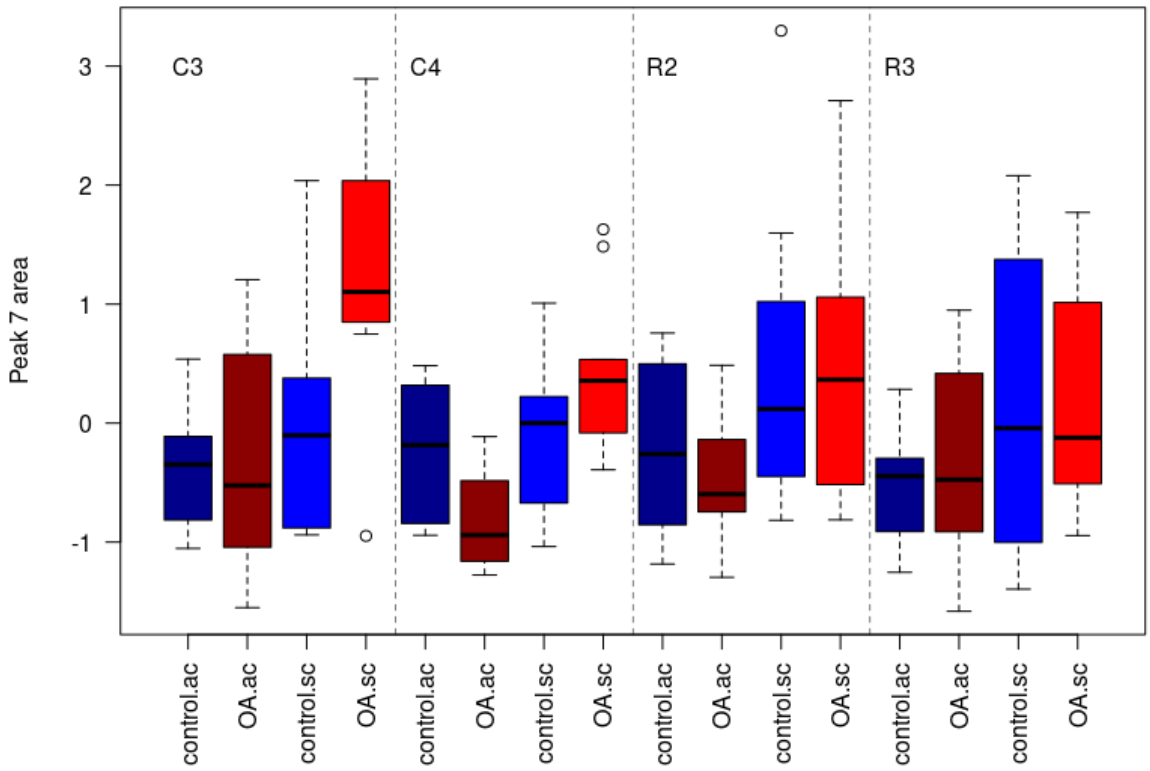

Figure 5 Peak 7 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

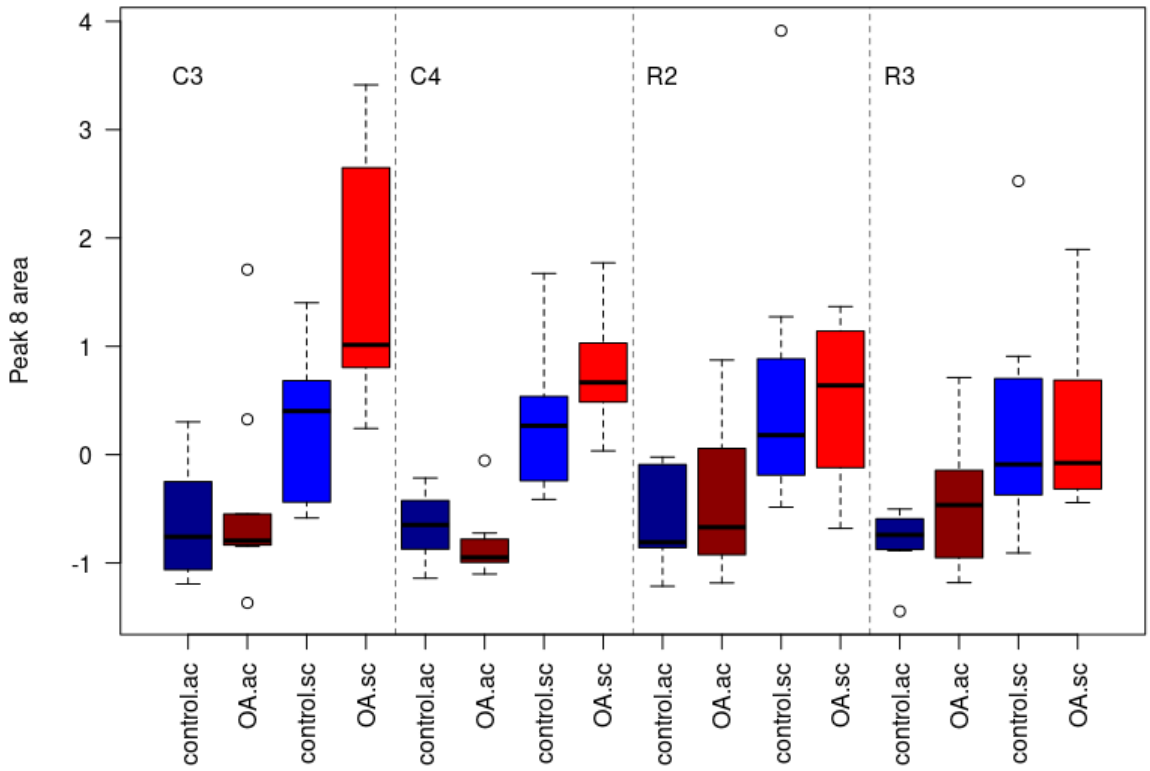

Figure 6 Peak 8 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

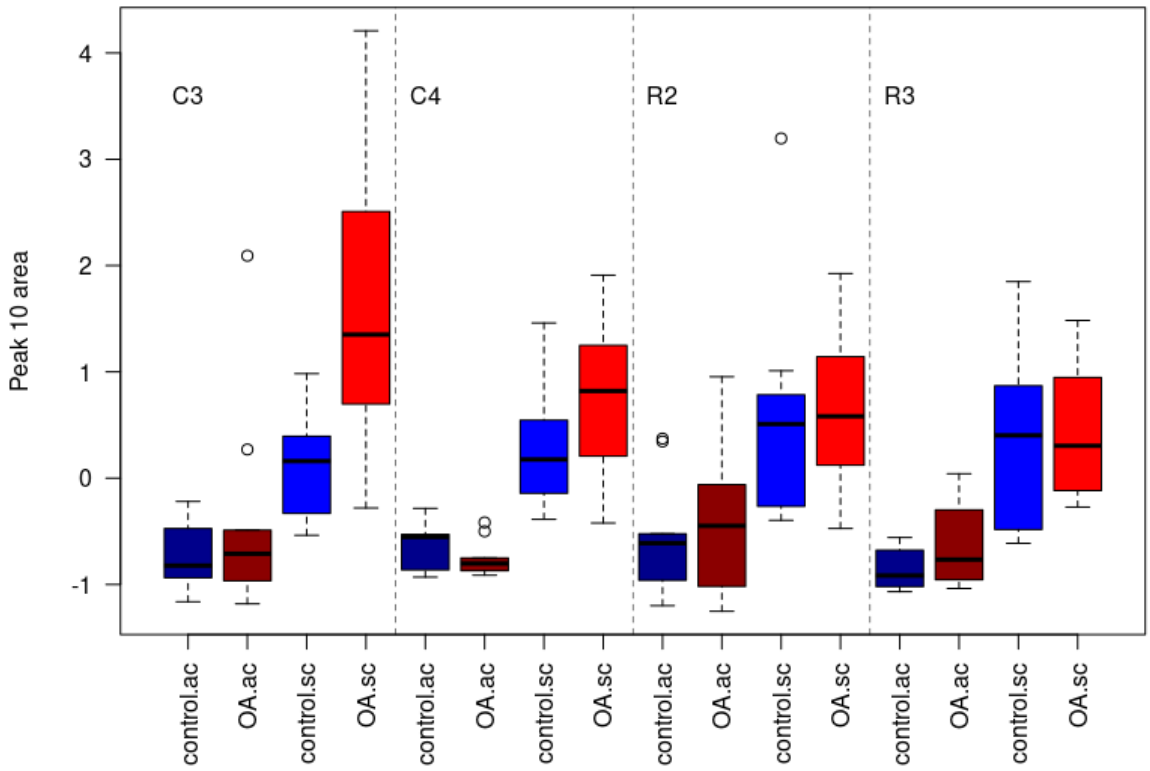

Figure 7 Peak 10 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

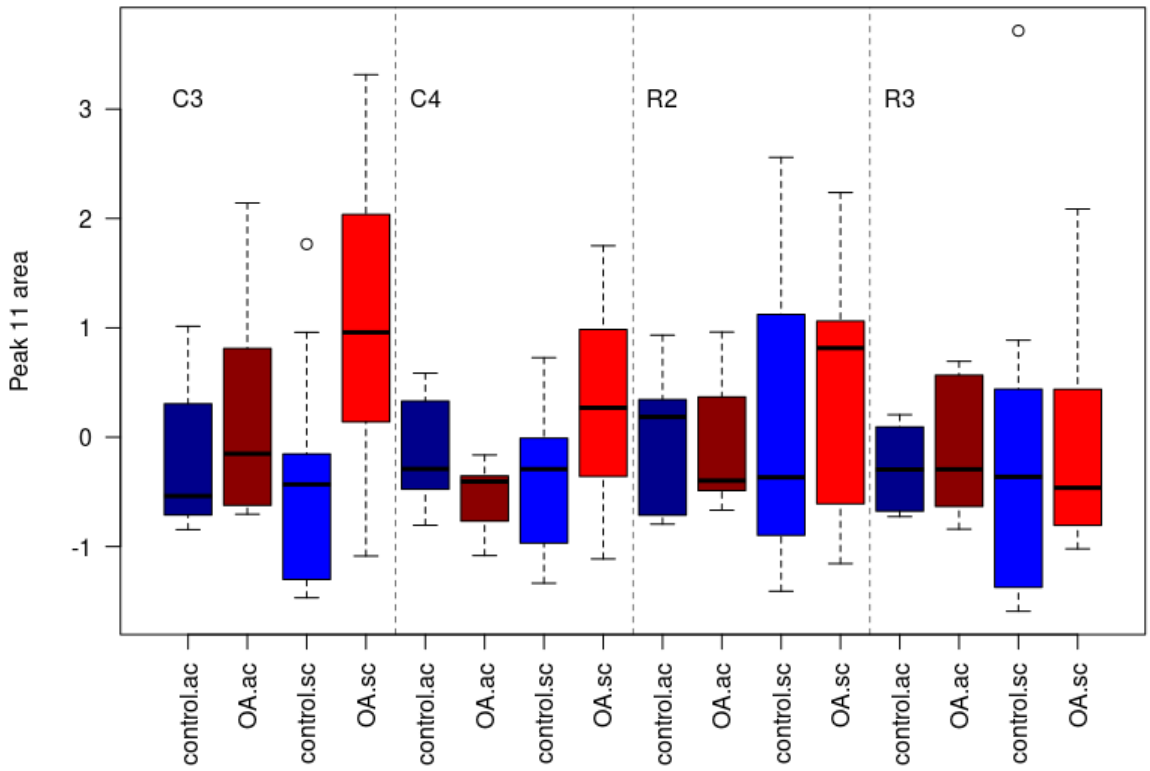

Figure 8 Peak11 standardised area as a function of joint (OA/control), tissue (ac/sc) and location (C3/C4/R2/R3). The vertical dashed lines separate the four locations. Dark blue: control/ac; brown: OA/ac; blue: control/sc; red: OA/sc. The bars represent the interquartile ranges, the thick horizontal line within the bars is the median, the whiskers show values 1.5 times above or below the median.

Tables

Table 1 Summary of each peak effect in models 1, 2 and 3 fitted to predict the probability of the inspected joint to be OA.

Peak	Effect in model 1	Effect in model 2	Effect in model 3
1	N	Y, s+ (ac), N (sc)	Y, s+ (C3, 2), N (C4, R3)
2	N	N	Y, s- (C3, C4), N (R2), Y w+ (R3)
3	N	N (ac), Y, s+ (sc)	Y, s+ (C3, R3), N (C4), Y, w+ (R2)
5	N	Y, s- (ac), N (sc)	Y, m+ (C3), N (C4), Y, s- (R2), w+ (R3)
6	Y, s-	Y, s- (ac), N (sc)	Y, s- (C3, R2, R3), s+ (C4)
7	N	Y, s- (ac), N (sc)	Y, w- (C3), N (C4, R2, R3)
8	N	Y, s+ (ac), s- (sc)	N (C3, C4), Y, m- (R2), m+ (R3)
9	Y, w-	Y, m- (ac, sc)	N (C3, R3), Y, s- (C4), w- (R2)
10	Y, w+	N (ac), Y, m+ (sc)	Y, m+ (C3), w+ (C4), N (R2, R3)
11	Y, s+	Y, s+ (ac, sc)	Y, s+ (C3, C4, R2), m- (R3)

Y/N = effect/no effect; +/- = increasing the peak area increases/decreases the probability of OA, respectively; w/m/s = the effect is “weak”/“medium”/”strong”, i.e. 0.2-0.3, 0.3-0.4 or more than 0.4 variation in probability (OA) when the peak area varies between mean - 1 s.d. to mean + 1 s.d.; and in relevant models: per tissue (model 2: ac or sc) or per location (model 3: C3, C4, R2 or R3)