Investigations of the origins of altered pain perception in a commercial pig strain

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Abstract

Lameness affects over 20% of commercial pigs in the UK and is a significant threat to the sustainability of current pig production techniques worldwide, raising animal welfare, economic and food security concerns. Among the most common causes of porcine lameness are joint diseases such as osteochondrosis (OC), a skeletal disease affecting bone growth, and osteoarthritis (OA), which results from the progressive breakdown of articular cartilage. OC and OA are reported to affect over 80% of commercial pigs, however, despite this prevalence, the diseases are poorly characterised in pigs. OA also affects over 8.75 million people in the UK, so spontaneous porcine OA could provide a useful animal model of the human condition. The aims of this thesis were to characterise the pathological features of spontaneous OC and OA in a commercial pig strain, examining their relationship to pain and inflammation and identifying the molecular mechanisms involved. Spontaneous porcine OA could then be evaluated as a model for the human disease.

The presence of lameness and pain-related behaviours were investigated in female commercial pigs at a number of ages between 9 and 42 months old. Knee joint OC and OA were assessed macroscopically and the synovium was examined histologically for synovitis. Expression of matrix metalloproteinases (MMP) and their inhibitors (TIMP) and pro-inflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNFα) were determined by qPCR. Cytokine protein levels were also assessed using an immunoassay. Activation of astrocytes and microglia in the lumbar spinal cord and the phenotype of primary afferent fibres in dorsal root ganglia (DRG) were examined using immunohistochemistry.

Mild OC, consistent with *osteochondrosis latens and manifesta,* was identified in 91% of knee joints in skeletally immature pigs (9 and 15 months). OA was identified in 95% of knee joints in skeletally mature pigs (24 and 42 months) and ranged from slight cartilage erosion to end-stage disease with subchondral bone exposure. Severity of OC and OA significantly correlated with lameness, but a number of

subclinical cases of both conditions were identified. It could not be confirmed whether the pain associated with porcine OC or OA was chronic, but astrocytes and microglia were generally activated suggesting there may be a chronic pain component. Synovitis was identified in 57% of knee joints and was increased in more severe OA. However, synovitis showed no correlation to OC severity, therefore mild OC may have a weaker association with inflammation than more severe forms of the disease.

OC showed an unclear association with synovium pro-inflammatory cytokines, although slightly increased TNFα expression was observed. However, OC was associated with elevated MMP but unchanged TIMP expression. Increased MMP and limited inhibition by TIMP may lead to degradation of cartilage matrix components; this could help OC lesions spontaneously repair but may also leave cartilage vulnerable to further damage and disease progression. OA was associated with increased synovium pro-inflammatory cytokines but not in end-stage disease, suggesting the inflammatory profile changes as OA progresses. OA was also associated with elevated MMP and reduced TIMP expression. The increase in MMPs and their disinhibition could lead to further cartilage degeneration and worsen the OA pathology.

The findings of this thesis highlight the poor state of joint health in commercial pigs and provide new insight into the involvement of inflammation and the molecular changes occurring in the synovium during spontaneous OC and OA. Spontaneous porcine OA showed a number of similarities to human OA and animal models, suggesting pigs could be a good model, however, the limited availability of healthy pigs may restrict their use. The findings of this thesis will support future studies to characterise spontaneous OC and OA in pigs, enabling these conditions to be better prevented or treated, thereby improving the sustainability of the commercial pig industry worldwide.

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Abbreviations

ABC	Avidin-biotin complex
ACLT	Anterior cruciate ligament transection
ACTB	Actin Beta
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	Analysis of variance
ARUK	Arthritis Research UK
ASPA	Animals (Scientific Procedures) Act 1986
BSA	Bovine serum albumin
C2C	Carboxy-terminal telopeptide of type II collagen 3/4-length fragment
cDNA	Complementary DNA
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CPII	Carboxypropeptide of type II collagen
Ct	Cycle threshold
CT	Computerised tomography
CV	Conduction velocity
DAB	3,3'diaminobenzidine
DEFRA	Department for Environment, Food & Rural Affairs
DMM	Destabilisation of the medial meniscus
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FC	Femoral condyle
FMIA	Fluorescent microsphere immunoassay
GFAP	Glial fibrillary acidic protein
H&E	Haematoxylin and eosin
HRP	Horseradish peroxidase
IASP	International Association for the Study of Pain

IBA1	Ionized calcium binding adaptor molecule 1
ICC	Intraclass correlation coefficients
ICRS	International Cartilage Repair Society
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IMS	Industrial methylated spirit
LC	Locus coeruleus
LFC	Lateral femoral condyle
LTP	Lateral tibial plateau
MFI	Mean fluorescent intensity
MFC	Medial femoral condyle
MIA	Monosodium iodoacetate
miRNA	Micro-ribonucleic acid
MMP	Matrix metalloproteinases
MNX	Meniscal transection
mRNA	Messenger RNA
MTP	Medial tibial plateau
NFwater	Nuclease-free water
NGF	Nerve growth factor
NoRT	No reverse transcriptase
NRS	Numerical rating scale
NSAID	Non-steroidal anti-inflammatory drug
NTC	No template control
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OC	Osteochondrosis
OCD	Osteochondritis dissecans
OCT	Optimal cutting temperature mounting media
P	Patella
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde

PNS	Peripheral nervous system
PPIA	Peptidylprolyl isomerase A
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Real-time quantitative reverse transcriptase polymerase chain reaction
QST	Quantitative sensory testing
RIPA	Radio-immunoprecipitation buffer
RNA	Ribonucleic acid
rSFA	Revised Société Française D'Arthroscopie
RVM	Rostral ventromedial medulla
SEM	Standard error of the mean
SFA	Société Française D'Arthroscopie
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF	Tumour necrosis factor
TP	Tibial plateau
TRAP	Tartrate resistant acid phosphatase
TRPV1	Transient receptor potential cation channel vanilloid subfamily member 1
VAS	Visual analogue scale

Chapter 1

General Introduction

1.1 Introduction

Lameness in commercial pigs is a significant threat to the sustainability of current pig production methods worldwide (Quinn et al., 2013). The increased scale of production and selection of pigs that are faster growing has left them vulnerable to developing lameness. Despite continued attempts to improve genetic susceptibility, nutrition and housing conditions, lameness is still a major issue for pig production, particularly in female breeding stock (Crenshaw, 2006). Premature animal slaughter due to lameness has important economic impacts and the associated reduction in productivity is a food security issue (DEFRA, 2004). Lameness also indicates the presence of pain, creating major concerns in relation to animal health and welfare. Any disorder of the musculoskeletal system can result in lameness (Main et al., 2000), but the joint diseases osteochondrosis (OC) and osteoarthritis (OA) have been indicated as the most prevalent lameness-causing conditions found in growing and skeletally mature pigs, respectively (Reiland, 1977).

Since the welfare and economic consequences of these conditions are significant, increasing our knowledge of these diseases' pathologies is important. In particular, understanding the involvement of inflammation and molecular mechanisms that may alter the perception of pain, potentially leading to a chronic pain state, could lead to better treatment options. This has the potential to improve pig welfare and increase the lifespan of breeding stock, reducing the economic impact and food security concerns. Furthermore, due to the physiological similarities between pigs and humans, investigating naturally occurring OA in pigs provides a unique animal model for the human condition. This project has therefore investigated the pathological features associated with naturally occurring OC and OA in commercial pigs, examining their relationship with pain and identifying some of the molecular changes in both joint tissues and the peripheral and central nervous systems.

1.2 Lameness in commercial pigs

Lameness, as defined by Wells (1984), is an impairment in movement or a deviation from normal gait. Smith (1988) added that movement is a result of a functional integration of the nervous system, muscles, tendons, joints, ligaments and the feet.

Any disorder of this system can alter movement and researchers often describe these changes in broad terms, such as leg weakness to describe changed leg conformation (Jørgensen, 1995). Although lameness or leg weakness commonly indicates painful lesions, they can result from any functional deficit in the locomotive system, including mechanical or neurological deficits. As such, lameness has a multifactorial aetiology and is a major concern for the commercial pig industry worldwide (DEFRA, 2004).

1.2.1 Prevalence of pig lameness

The prevalence of lameness within the commercial pig industry varies in different countries due to preferences for particular housing and husbandry conditions and pig breeds (Willgert, 2011). In the USA, lameness affects an estimated 11% of all commercial pigs, but on individual farms this can range from 0 - 38% (Dewey et al., 1992, 1993). Across Europe, the prevalence of lameness is reported to vary from 8 - 49%. Lameness is estimated to affect 14% of pigs in Denmark (Bonde et al., 2004; Knage-Rasmussen et al., 2014), 8% in Finland (Heinonen et al., 2006), 13% in Belgium (Pluym et al., 2017), and 15% of gilts (female pigs that have not given birth) and 24% of castrates in Sweden (Etterlin et al., 2015). In Ireland the incidence is reported as being much higher, with one study finding lameness in 49% of pigs, which was severe in 3.6% of cases (Ryan et al., 2010). Another study in Ireland identified a 28 - 39% prevalence in finishers (pigs over 70kg and in last stage of rearing) and gilts and 48% in pregnant gilts and sows (Quinn et al., 2013).

Studies of UK pig herds have identified lameness as affecting 4 - 20% of pigs (UFAW, 2015). Kilbride et al. (2009) assessed over 4000 pigs on 549 farms and identified lameness in 19.7% of finishing pigs, 11.8% of maiden gilts (gilts yet to be mated), 17.4% of pregnant gilts and 16.9% of pregnant sows. However, another study of 2260 sows by Willgert (2011) found that only 4.3% exhibited lameness. In this study, 71% of farms had culled sows due to lameness in the previous year, with an average of 20 sows per year, which may have reduced the observed incidence. Overall, the high incidence of porcine lameness found in the UK and worldwide has significant

implications for industry, due to not only animal welfare considerations but also economic and food security implications.

1.2.2 Animal welfare implications of pig lameness

The 'Five Freedoms' of animal welfare (Farm Animal Welfare Council, 1993):

1. ***Freedom from hunger and thirst***
2. ***Freedom from discomfort***
3. ***Freedom from pain, injury and disease***
4. ***Freedom to express normal behaviour***
5. ***Freedom from fear and distress***

These 'Five Freedoms' of animal welfare, originally proposed by the UK Farm Animal Welfare Council in 1979, are generally used to determine if an animal has good welfare. Lameness usually indicates an animal is in pain or discomfort but it has the potential to indirectly affect all five freedoms, reducing animal welfare and ultimately resulting in animal slaughter to prevent further suffering (reviewed by Anil et al., 2009a).

Lameness reduces the ability of an animal to move, which can directly affect its capacity to access food and water, as well as reducing the pig's capability to compete for this food and water, thereby directly impacting the first freedom (Madec et al., 1986; Cornou et al., 2008; Anil et al., 2009b). Lame animals often show a diminished motivation to move, increased lying behaviour, and reduced weight bearing on the affected limbs, indicating pain and discomfort (DEFRA, 2004; Heinonen et al., 2006; Willgert, 2011; Ala-Kurikka et al., 2017). Lameness also alters behaviour by reducing normal social interactions and exploratory behaviours (Anil et al., 2009b). Untreated lame pigs are more passive and show reduced pen and bedding exploration than non-lame or treated lame pigs (Ala-Kurikka et al., 2017). Furthermore, the impact of lameness can increase social pressures, causing distress. Lame animals cannot move away from others as easily and studies suggest non-lame pigs show increased social interest in lame pigs (Anil et al., 2009b; Heinonen et al., 2013). Due to the impact of lameness on animal welfare and the suffering

caused, lame pigs are often removed from the herd which can have significant economic implications (Dewey et al., 1993).

1.2.3 Economic impact of pig lameness

Pig lameness has direct economic implications due to costs associated with treatment or slaughter, but it also reduces profits. Lameness most often results in removal of animals from the herd, either as a direct consequence of lameness, or indirectly due to the associated reduction in productivity (Anil et al., 2009b). Lameness-causing pathologies, such as abscesses, are estimated to result in losses of over £1.5million per year in the UK solely due to condemnation of meat at time of slaughter (DEFRA, 2004). The direct cost of vet fees, treatment and increased labour can also be significant. In the UK lameness is estimated to cost £19/pig in treatment and £13/pig in increased labour (DEFRA, 2004; Willgert, 2011). Pig slaughter is predicted to cost £39/pig but this can rise to over £160 if emergency slaughter is required (Willgert, 2011). Furthermore, the loss of production due to sow lameness is estimated at £60 per outdoor sow and £84 per indoor sow (Willgert, 2011). When these costs are combined, a single lame sow can cost anywhere from £19, if only treatment is required, to over £260.

Lameness in growing or finishing pigs and their subsequent culling results in fewer pigs reaching slaughter weight (Quinn et al., 2013). Lame finishing pigs often have reduced growth rates, which also increases the time and cost to reach slaughter weight (DEFRA, 2004; Willgert, 2011). The most common cause of sow culling is age-associated reduction in breeding productivity (Dewey et al., 1992; Jørgensen, 1995). However, lameness has been suggested as the second most common reason, with the majority of studies citing lameness as responsible for up to 15% of selective sow culling (Dagorn and Aumaitre, 1979; DEFRA, 2004; Tarrés et al., 2006; Pluym et al., 2013). This loss of productivity is a significant food security concern and reduces the sustainability of current pig production methods (Quinn et al., 2013). Due to the costs of rearing and maintaining breeding animals, it takes at least three litters of piglets before a sow becomes profitable; early slaughter can result in sow removal before this time point (Stalder et al., 2000; 2003). Lameness reduces

breeding productivity, as lame sows have smaller litters of piglets compared to healthy sows. Lame sows produce fewer live piglets per litter due to an increased incidence of mummified piglets and reduced sow agility increases piglet overlaying (DEFRA, 2004; Anil et al., 2009b; Pluym et al., 2013; Quinn et al., 2013). Therefore, an awareness of the causes and risk factors associated with lameness is vital in farm management to prevent its onset, reduce the associated economic burden and improve animal welfare.

1.3 Aetiology of lameness and leg weakness in commercial pigs

Lameness and leg weakness can result from a number of diseases, disorders, infections and injuries affecting different aspects of the locomotive system (DEFRA, 2004; Crenshaw, 2006). Disorders of the nervous system caused by a bacterial or viral infection such as meningoencephalitis, encephalomyelitis, tetanus and meningitis can disrupt the musculoskeletal system (Wells, 1984; Taylor, 2006). Porcine viral infections, although rare in the UK, commonly present with lameness. Swine vesicular disease caused by an enterovirus, and foot-and-mouth disease caused by an aphthovirus, both present with lameness, tender feet and vesicles on the feet, mouth and snout (Grist, 2007; McOrist, 2014). Although these viruses have largely been eradicated they can still be found in the Western hemisphere, particularly parts of the USA (Taylor, 2006; McOrist, 2014). Other bacterial infections include *mycoplasma hyosynovia* and *hyorihinis*, which cause mycoplasma arthritis that presents with a reluctance to move and joint swelling (Taylor, 2006).

Nerve trauma and brain abscesses can directly disrupt neural pathways and nerve impulses, thereby disrupting movement (Wells, 1984). Trauma can directly result in lameness by causing fractures but also leaves bone susceptible osteomyelitis, an infection of bone or bone marrow that creates pus-filled lesions within bone (Kirk et al., 2005; Taylor, 2006). Nutritional deficiencies affect bone growth and health, which can result in leg weakness or lameness. For example, vitamin A deficiency causes slow bone growth, and calcium and phosphorus deficiencies can result in osteoporosis or rickets that presents with sudden onset lameness (Wells, 1984).

Muscle health is also important for locomotive function, conditions such as porcine stress syndrome and muscular dystrophy can affect muscle health (Wells, 1984).

The most common causes of porcine lameness, particularly in the UK, are joint diseases and foot lesions. Foot-rot, also called brush foot, caused by infections of the claw, results in swelling and pigs walking on 'tip-toes' (Smith et al., 1990; Dewey et al., 1993; Taylor, 2006). Claw lesions, including cracks, abscesses, calluses and overgrowth also result in abnormal gait and leg weakness (Smith et al., 1990; Heinonen et al., 2006; Heinonen et al., 2013; Quinn et al., 2013). Bursitis, an inflammation of the fluid-filled sac under the skin, causes pain and swelling and presents with lameness (Taylor, 2006; Quinn et al., 2013). Laminitis, an inflammation of the soft tissue in the foot that is common in boars and pregnant sows, presents with stiffness, a reluctance to move and can cause pigs to walk on their knees (Taylor, 2006). However, the most common joint diseases presenting with lameness in pigs are OC and arthritic disease, such as OA (Reiland, 1977; Willgert, 2011). OC is a skeletal disease affecting normal bone growth in pigs under 18 months of age (Wells, 1984; Dewey et al., 1993; Kirk et al., 2005; Taylor, 2006; Quinn et al., 2013). Arthritis such as OA, characterised by the breakdown of articular cartilage in joints of mature animals, is found in over 88% of sows (Dewey et al., 1993; Kirk et al., 2005; Quinn et al., 2013). A study of 79 sows by Ryan et al. (2010) found that all pigs contained an OC or OA lesion in at least one joint.

Depending upon their age, pigs are susceptible to different conditions that present with lameness. Pre-weaned piglets are more vulnerable to splay-leg syndrome, skin abrasions and trauma (Taylor, 2006). Growing and finishing pigs are more susceptible to OC and the bacterial infections *mycoplasma hyosynovia* and *hyorihinis* (Wells, 1984; Taylor, 2006). Adult pigs, including breeding stock, suffer more from OA and foot lesions including torn claws and abscesses (Wells, 1984; Taylor, 2006). The incidence of lameness, particularly that caused by joint disease, is strongly influenced by a number of factors including genetic factors and housing conditions.

1.3.1 Risk factors influencing the incidence of lameness in commercial pigs

Many studies have suggested there is a genetic predisposition to lameness and leg conformation abnormalities in pigs (Crenshaw, 2006). Leg conformation, particularly standing under the abdomen, shows a high heritability and requires selection against when breeding (Le et al., 2015). Different pig breeds also show different lameness susceptibility, with Landrace and Large White breeds showing a higher incidence compared to Yorkshire pigs (Webb et al., 1983; Jørgensen and Andersen, 2000). Furthermore, selection of pigs with a genetic predisposition for higher growth rates has also increased lameness. Faster growth can result in bones not forming sufficiently fast to cope with an increase in muscle mass, which leaves bone more susceptible to injury (DEFRA, 2004). Although adequate feeding is required to prevent hunger, the use of restrictive feeding rather than feed available *ad libitum,* may alter the incidence of lameness. Restrictive feeding, of approximately 20% less food than consumed by pigs fed *ad libitum,* has been shown to reduce the incidence of both joint disease and leg weakness traits (Jørgensen, 1995; van Grevenhof et al., 2011). Restrictive feeding reduces growth rate, helping to maintain the balance of skeletal and muscle growth and prevents overloading of joints (van Grevenhof et al., 2011; de Koning et al., 2013).

Furthermore, correct husbandry conditions can alter the prevalence of lameness and leg weakness. Having more stockmen per pig has been shown to reduce lameness (Willgert et al., 2014). Hence it has been suggested that lameness increases in some herds during spring and summer due to crop harvesting, which results in stockmen spending less time with the herd (Knage-Rasmussen et al., 2014). A greater number of stockmen spending a greater amount of time with the animals allows for a more thorough assessment of the animal's condition, thereby enabling earlier identification of problems and earlier implementation of preventative techniques.

1.3.1.1 Housing conditions

As well as husbandry practices, housing conditions pose a large risk factor in the onset of lameness. There is some discussion as to whether indoor or outdoor reared

pigs show a difference in lameness prevalence. Some studies have identified indoor pigs as suffering more often from lameness (Kilbride et al., 2009; Willgert et al., 2014). Conversely, other studies have suggested outdoor reared pigs have a higher incidence of lameness (Pandolfi et al., 2017; 2018). It is likely this controversy is partly due to the flooring or soil type present on different farms. For outdoor pigs, the soil needs to be free-draining, as muddy conditions can soften the feet leaving them vulnerable to infection or injury. The soil also needs to be free from stones, especially flints, as they can damage the legs and feet (DEFRA, 2004). Flooring and bedding used in indoor systems are particularly important for preventing lameness (Crenshaw, 2006; Willgert et al., 2014; Wang et al., 2018). Indoor flooring needs to be soft, even and not slippery or abrasive to prevent injury and disease (DEFRA, 2004; Quinn et al., 2013). It is generally accepted that solid flooring results in significantly less lameness than slatted concrete or metal flooring (DEFRA, 2004; Heinonen et al., 2006; Kilbride et al., 2009). If slats are too far apart the pig's feet can fall through causing injury, and in addition, the rough edges which form on slats can cause heel overgrowth and foot cracks (DEFRA, 2004; Quinn et al., 2013).

The use of bedding material in indoor-reared pigs can also influence lameness and leg weakness. Deep straw bedding or roughage has been suggested as the best form, partly due to its cushioning effect and slip prevention but also due to the behavioural enrichment it provides (DEFRA, 2004; Heinonen et al., 2006; Kilbride et al., 2009). Deep litter, such as wood shaving or sawdust, can also reduce the prevalence of OC and therefore lameness (van Grevenhof et al., 2011). However, straw and litter bedding are not compatible with many modern housing systems, so it has been suggested that rubber matting can also reduce lameness effectively (Quinn et al., 2013).

The European Union (EU) Directive 2001/88/EC states that in all member states "sows and gilts shall be kept in groups during a period starting from 4 weeks after service to 1 week before the expected time of farrowing" (EU Directive, 2008). Group housing is good in terms of welfare as animals can exercise and socially interact, which reduces stress (Quinn et al., 2013). However, group housing can lead

to increased competition and aggression, which can cause injury. In group-housed pigs, stocking density is important as overstocking significantly increases lameness and reduces the quality of life. The EU directive states the minimum area per pig for gilts is 1.64m^2, sows require 2.25m^2 and boars need 6m^2 (EU Directive, 2008). Overstocking not only increases the risk of physical injury, due to increased aggression and physical contact, but can also increase the spread of bacterial and viral infections (Crenshaw, 2006; Willgert, 2011; Quinn et al., 2013; Willgert et al., 2014; Pandolfi et al., 2017; Pluym et al., 2017).

1.4 Assessing lameness and pain in pigs

Diseases and problems affecting joints in pigs are often subclinical in the early stages and it is not until they become severe that symptoms present. Lameness is commonly caused by and indicates pain and the use of analgesics can reduce lameness in pigs (Reyes et al., 2002). However, lameness does not always indicate a painful condition, so indicators of both lameness and pain are often scored together to determine the extent of suffering; this can be assessed using a number of techniques. Measuring pain or suffering in animals is complex and follows the assumption that animal pain serves the same purpose as human pain - to provide a protective mechanism against actual or potential tissue damage (Molony and Kent, 1997). Pain can change an animal's physiology and behaviour to reduce or avoid damage, to prevent its reoccurrence or improve recovery, but behavioural changes are often the first detectable sign of illness, injury or pain (Molony and Kent, 1997).

Alterations in pig motion are most commonly assessed, in both research settings and on-farm, using observational gait or conformation assessments. Leg weakness traits, or altered conformation, are commonly used to identify locomotive changes in pigs (Kirk et al., 2008). These changes include a varus malalignment, also called o-shaped or bow-legged, which indicates dysfunction in the medial joint aspect (see Figure 1.1). A valgus malalignment, also called x-shaped or cow-hocked, indicates dysfunction in the lateral joint aspect (Heijink et al., 2012). Conformational changes indicating problems in the stifle and hock (equivalent to the human knee and ankle) can include hind feet positioned under the abdomen (standing under) or behind the

stifle joint (standing back). These traits are simple to identify, particularly when severe, so little observer training is required.



Figure 1.1: Examples of pig leg weakness traits seen in the hind limbs. Drawings to illustrate conformation changes that can occur in the hind limbs of pigs. Image adapted from Van Steenbergen (1989).

Changes in gait often initially present as stiffness in the limbs or altered strides that are short and uneven (Kirk et al., 2008; Johnson, 2010; d'Eath, 2012). As lameness progresses, these changes become more apparent with features such as a swaying gait or head-bobbing. Severe lameness presents with limping due to a reduction in weight bearing on the affected limbs; if left untreated this can progress into a complete inability to move (d'Eath, 2012). Other behavioural changes associated with locomotive dysfunction include a reluctance to move, often assessed by lying-to-standing transition time, and an inability to accelerate or change direction quickly (Gregoire et al., 2013).

Alongside changes in gait and conformation, other behaviours are often assessed to indicate if there is pain or suffering associated with locomotive dysfunction. Changes in activity are often associated with lameness and pain; time spent lying rather than standing, often described as posture change, is increased in pigs with lameness or induced pain (Castel et al., 2014; Ala-Kurikka et al., 2017). Lame pigs also spend less time conducting exploratory or 'play' activities and show increased aggression (Gigliuto et al., 2014). Pain can also elicit other behaviours including vocalisation (squealing), avoidance behaviours and agitation or restlessness, all of which can be assessed, usually by observational scoring (reviewed by Landa, 2012; and Ison et al., 2016).

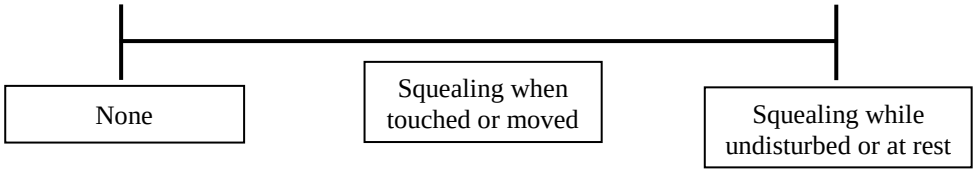
1.4.1 Porcine lameness and pain behaviour scoring systems

The severity of gait deficiencies and other behavioural changes are frequently combined into simple descriptive scoring systems to assess pig lameness and pain. These usually take the form of numeric rating scales (NRS), visual analogue scales (VAS) where a mark is placed on a 10 cm line, or simple descriptive scales (see Figure 1.2). Multiple behaviours are usually measured in conjunction and combined to give a total or global pain score (Main et al., 2000; Reyes et al., 2002; Royal et al., 2013). For example, Main et al. (2000) identified pig lameness by evaluating behaviours including the response to human presence, standing posture, gait and the behaviour of individual pigs within a group.

Numerical Rating Scale (NRS) from Reyes et al., (2002)

Score	Vocalisation
1	Low volume, occasional cries
2	Low volume, continuous cry
3	High volume, quite frequent cries
4	Screaming that lasts a long time

Visual Analogue Scale (VAS) from Royal et al., (2013)



Simple Descriptive Scale adapted from Royal et al., (2013)

Score	Vocalisation
Normal	Normal grunting/squealing when interacting
Mild	Squealing when moved or touched
Moderate	Squealing when standing or walking
Severe	Squealing when undisturbed

Figure 1.2: Different scoring systems used to assess pig vocalisations. Examples of a numerical rating scale, visual analogue scale and simple descriptive scale to assess pig vocalisations associated with pain or suffering.

These types of behaviour scoring are simple to conduct and show relatively good inter- and intra- observer agreement and reliability (Main et al., 2000). The limitations of these assessment techniques lie in the difficulty in observing pig behaviour and scoring subjectivity. Pigs have stilted locomotion and their disturbance usually results in rapid motion, not the steady walking which is required to assess gait (Main et al., 2000). The subjectivity of scoring also reduces scorer agreement particularly for mild changes in behaviour (Johnson, 2010). To limit this subjectivity, the scoring range is often reduced; on-farm scoring methods usually have up to three severities, whereas in a research setting up to 10 severities per behaviour can be included. By reducing the score range, the reliability and repeatability can be increased but this results in a failure to detect subtle differences between animals (reviewed by Nalon et al., 2013a). However, due to its simplicity and reliability, behavioural scoring forms the basis of pig welfare assessment and is the technique advised by the EU (Welfare Quality®, 2009).

1.4.2 Biomechanical assessment of gait

In research settings, kinematic analysis of gait has been conducted to quantify motion in terms of position, velocities and angular range of motion of joints and body segments. Reflective markers are placed on the skin and the pig walks down a corridor whilst being video recorded. A computer tracking programme is then used to analyse marker movements and determines features such as walk speed, stride length, leg swing time, foot height or joint angles (Gregoire et al., 2013; Stavrakakis, 2014). This analysis gives detailed information on subtle gait changes but is complex to implement, in terms of both the equipment calibration and analysis required. A simpler technique is to use accelerometers fixed to one or more limbs. The acceleration data collected can be used to identify lameness features such as steps per minute, latency to lie down after feeding or standing posture (Ringgenberg et al., 2010; Gregoire et al., 2013). However, the attachment of these devices to the animal can interfere with normal gait. Furthermore, force-plates or pressure mats have also been used to identify lameness. By walking animals along pressure sensitive runways, changes in weight-bearing or shifting of weight, both associated with lameness, can be detected (Anil et al., 2007; Karriker et al., 2013). However,

force-plates are limited as they cannot always distinguish between pressures exerted by different feet. Although both these apparatus could be used to automatically detect lameness, they can only determine weight-bearing differences and not mild lameness traits such as joint stiffness.

Footprint analysis has also been used to investigate lameness in pigs. Gregoire et al. (2013) walked pigs along a corridor covered in clay and analysed the footprint pattern, assessing features including stride length and the distance between contralateral or ipsilateral feet. Other researchers have used inking or painting of feet to identify similar gait changes (reviewed by Nalon et al., 2013a). However, the slipperiness created by painting feet or walking on clay may alter gait and might miss traits such as joint stiffness, making these techniques less reliable at detecting lameness. Researchers have also used object-tracking techniques to identify lameness of group indoor-housed animals. For example, Traulsen et al. (2016) used an ear sensor to measure the position and acceleration of group-housed sows to describe their activity patterns and identify lameness. However, these techniques are currently too expensive and require further optimisation before they can be used on-farm as an affordable automatic lameness detection system.

1.4.3 Techniques used to assess porcine pain

A number of techniques have been developed to quantify pain severity or changes in sensitivity of pigs in a research setting. Quantitative sensory testing (QST) assesses sensory nerve fibre function by measuring the amount of stimulation required to elicit a response, enabling changes in the nociceptive threshold (stimulus strength deemed painful) to be evaluated. Altered sensitivity to mechanical stimuli has been measured using hand-held probes or limb-mounted actuators (Sandercock et al., 2011; Nalon et al., 2013b; Castel et al., 2014; Mohling et al., 2014; Di Giminiani et al., 2016b). Altered thermal sensitivity has been measured using light beams and carbon dioxide lasers (Di Giminiani et al., 2013; Mohling et al., 2014). These QST techniques have proved useful at identifying changes in sensitivity of peripheral sensory neurones but do not address pain as a multidimensional experience (reviewed by Ison et al., 2016), involving both the

sensory experience and a cognitive component that elicits complex behavioural responses (Melzack and Casey, 1968; Ahles et al., 1983).

Physiological changes associated with pain can also be measured to indicate pain severity. Studies show post-operative pain in pigs alters sympathetic nervous system activity and changes in the cardiovascular system (heart rate and blood pressure), respiratory system (breathing rate, depth, and pattern) and body temperature can all be assessed (Reyes et al., 2002; Royal et al., 2013). Pain also alters neuroendocrine function, commonly measured by plasma cortisol concentrations (Hay et al., 2003; Sutherland et al., 2008). Other indicators of physiological function, including digestive function (body weight and faecal quality), plasma lactate, plasma glucose or endogenous opioid levels, have also been used to indicate pain severity in pigs (reviewed by Landa, 2012).

Finally, identification of pain facial expressions in animals have been used as non-invasive pain assessment techniques. In humans, facial expressions are considered a reliable method for evaluating pain in non-vocal patients such as those with dementia. Pain facial expressions, or grimace scales, have been created for rodents, rabbits, horses, cats, sheep and primates (Langford et al., 2010; Sotocinal et al., 2011; Keating et al., 2012; Dalla Costa et al., 2014; Holden et al., 2014; McLennan et al., 2016). In piglets, pain due to tail docking or castration resulted in orbital tightening but other facial changes were not consistent across studies (Di Giminiani et al., 2016a; Viscardi et al., 2017). In adult pigs treated with capsaicin (the active component in chilli peppers), the pain face showed lowered ears, snout wrinkling, facial muscle tension and an angled eye appearance (Göransson, 2016). However, it was noted that changes in piglets and adult pigs were very subtle and may not be applicable in a clinical setting.

Despite the range of pain and lameness assessment techniques available, behavioural assessment remains the most commonly reported method used to identify and classify pig lameness and pain, both on-farm and in a research setting, due to both the simplicity and low costs.

1.5 Structure of the knee joint

The knee joint, or stifle in animals, is a diarthrodial joint where the femur, tibia, patella and fibula meet (see Figure 1.3). The structure is maintained by ligaments and the joint capsule and synovium encapsulate the entire joint. Compared to humans, the porcine knee is anatomically almost identical, although the pig joint has a smaller range of motion and slight differences in ligament and menisci size (Proffen et al., 2012)**.** Where the femur, tibia and patella meet, the bone is protected by cartilage. The meniscus, made of fibrocartilage, disperses weight and helps protect the femur and tibia (Fairbank, 1948). Hyaline cartilage, in the form of articular cartilage, covers the end of bones, which cushions and protects the joint surfaces.

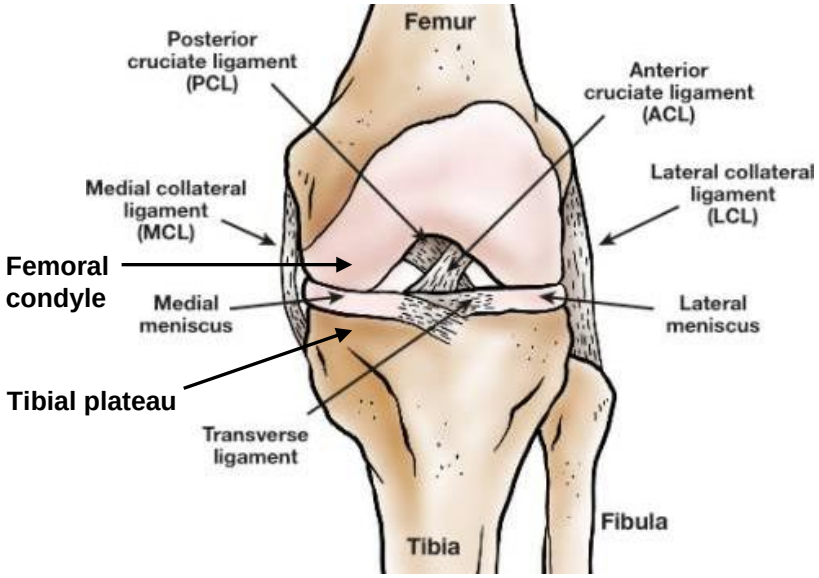


Figure 1.3: Anatomy of the knee joint. Diagram showing the anterior view of the knee joint. Image adapted from Makris et al. (2011).

1.5.1 Articular cartilage

Articular cartilage is a highly specialised tissue and its thickness varies with age, species and location within the joint. In both the healthy adult human and porcine knee, articular cartilage is 1 - 2 mm thick (Nakano et al., 1979; Shepherd and Seedhom, 1999). Cartilage provides a smooth lubricated surface for load transmission with low amounts of friction (Fox et al., 2009). Articular cartilage consists mostly of an extracellular matrix (ECM) but also contains chondrocyte cells.

In adults, the articular cartilage is devoid of blood vessels, lymphatics and nerves (Buckwalter et al., 2005).

Chondrocytes are highly specialised cells responsible for the synthesis of ECM components (Buckwalter et al., 2005). Chondrocytes perceive and respond to mechanical stress, which influences their integrity and alters ECM synthesis (Kurz et al., 2005; Harvey et al., 2014). In response to mechanical stress, chondrocytes show reduced nucleus size and altered expressions of collagen, aggrecan and ECM degrading enzymes (Liu et al., 2016). The ECM consists of tissue fluid (mostly water), collagen and proteoglycans (Buckwalter and Mankin, 1998). There are many types of collagen protein found in different distributions across the body. Collagen makes up 60% of the cartilage dry weight in mammalian hyaline cartilage, of this approximately 90% is type II collagen (Eyre, 2002). Collagen creates a network of fibres to which proteoglycans can attach, providing cartilage with its structure and tensile strength (Mayne, 1989). Aggrecan is the most abundant proteoglycan found in cartilage but others include biglycan, decorin and fibromodulin (Fox et al., 2009). Proteoglycans attach between collagen fibres, providing cartilage with its osmotic properties so that it can resist compression (Roughley and Lee, 1994). The structure of articular cartilage is divided into four distinct zones: the superficial, transitional or middle, deep and calcified zones. The cartilage components are organised differently within each layer (see Figure 1.4).

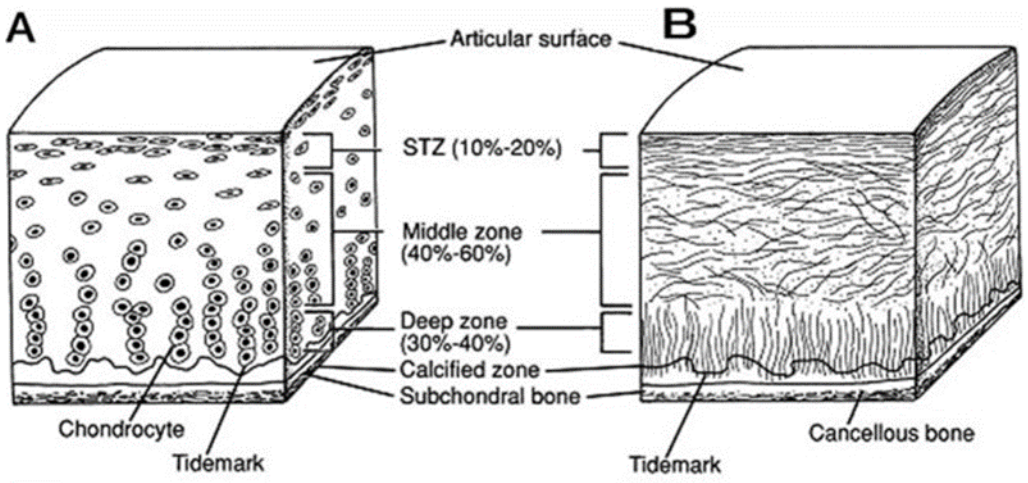


Figure 1.4: Structure of mature articular cartilage. A) Cellular organisation in zones of articular cartilage. B) Collagen arrangement in zones of articular cartilage. Image taken from Fox et al. (2009). STZ denotes superficial zone.

The superficial zone is the outermost layer and comprises 10-20% of the cartilage depth. Within this layer there are a large number of flattened chondrocytes and the collagen fibres are tightly packed parallel to the surface (Fox et al., 2009). This arrangement protects underlying structures from shear stress. The middle or transitional zone contains a high proteoglycan content, thick collagen fibres and relatively few chondrocytes (Bhosale and Richardson, 2008), providing the first line of resistance to compression. The deep zone contains collagen fibres perpendicular to the bone, a high proteoglycan content and chondrocytes are in a columnar arrangement (Fox et al., 2009). This zone provides the most resistance to compressive forces exerted across the joint. Finally, the calcified zone consists of cartilage containing calcium salts within the matrix, providing attachment between the subchondral bone and cartilage, thereby anchoring it in place (Buckwalter et al., 2005). The deep zone and calcified zone are distinguished by the tidemark that marks the interface between calcified and non-calcified cartilage.

1.5.2 Bone

The subchondral bone consists of the bone plate made of cortical bone (or compact bone) and trabecular bone (also known as cancellous or spongy) and is adapted to resist the mechanical forces exerted across the joint (Li et al., 2013). The cortical

bone creates a hard outer surface surrounding the inner trabecular bone (see Figure 1.5).

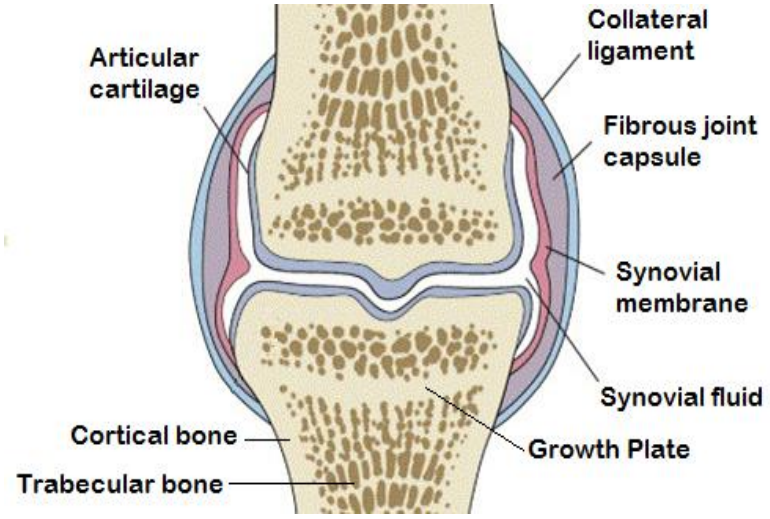


Figure 1.5: Structure of the knee joint. Schematic cross-section of the knee joint showing the articular cartilage, subchondral bone and joint capsule. Image adapted from van Weeren (2006).

The subchondral bone is highly vascularised and contains three cell types; osteoblasts, osteoclasts and osteocytes, which are derived from mesenchymal stem cells within bone marrow (Gray and Lewis, 1918). Osteoblasts are bone forming cells; they synthesise a matrix called the osteoid which is mineralised by the deposition of mineral salts that form the bone's lamellae matrix (Dodds, 1932; Grist, 2007). During this process, osteoblasts can become embedded within the matrix and mature into osteocyte cells (N.I.H., 2011). Osteocytes are thought to respond to changes in the local mechanical and chemical environment and are involved in the regulation of bone remodelling. Osteoclasts are bone resorbing cells which destroy calcified portions of the matrix including calcified bone or cartilage (Dodds, 1932). They secrete hydrogen ions and enzymes to dissolve the bone matrix, and are central to bone mass and quality. The activity of osteoblasts and osteoclasts is balanced to maintain healthy bone, but their dysregulation results in pathological conditions such as OA (Teitelbaum, 2007; Strassle et al., 2010).

1.5.3 Synovium

Synovial joints, such as the knee, are characterised by a fibrous capsule (articular capsule) that surrounds the joints and contains synovial fluid. The capsule is vital for joint function as it provides stability and maintains the fluid filled space (Edwards, 1994). The capsule is made of an outer layer of dense fibrous tissue and an inner synovial membrane or synovium (Ralphs and Benjamin, 1994). The synovium consists of two layers of cells; the outer intima layer and a subintima layer (see Figure 1.6).

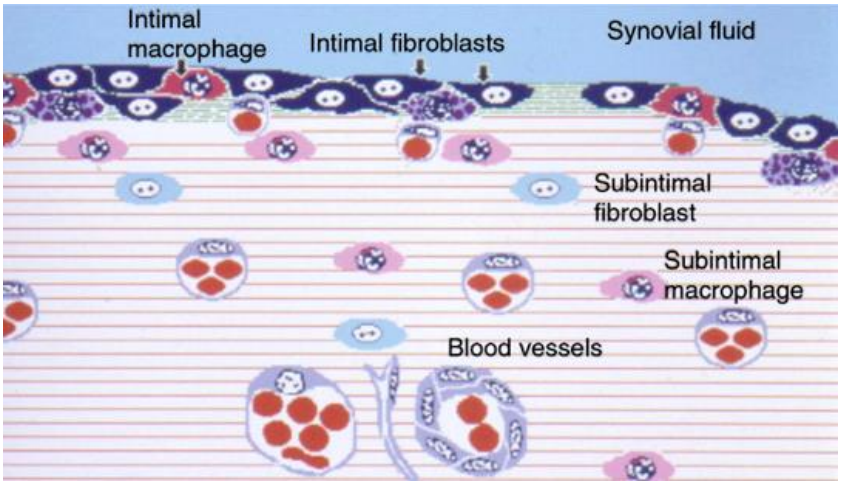


Figure 1.6: Structure of the synovium. The intima layer contains macrophage-like and fibroblast-like synoviocytes. The subintima layer contains blood vessels and sparsely distributed inflammatory cell. Image taken from Edwards (2000).

The intima layer is approximately 1-3 cells thick in healthy tissue and contains specialised synovial lining cells or synoviocytes. Two distinct types of synoviocytes have been identified: type A macrophage-like synoviocytes can phagocytose cell debris and waste products within the joint; and type B fibroblast-like synoviocytes produce matrix components (Tiwari et al., 2010). The subintima layer has a loose collagen network containing non-specialised cells, which includes fibroblasts, adipocytes, macrophages, large blood vessels and sometimes mast cells (Tiwari et al., 2010). In the healthy synovium, inflammatory cells within the subintima layer are sparsely distributed (Edwards, 1994). The synovium functions to synthesise and

secrete components of synovial fluid such as hyaluronic acid, providing synovial fluid with its viscosity to lubricate the joint (Edwards, 1994).

1.5.4 Bone growth

The process of bone growth by endochondral ossification takes place at the growth plates and the epiphyseal-articular cartilage complex. The primary centre of ossification (growth plates) appears during prenatal development in the middle of the bone shaft, then secondary centres of ossification (epiphyseal-articular cartilage complex) appear postnatally between the growth plate and articular cartilage (reviewed by Byers and Brown, 2006). Endochondral ossification at the growth plate causes longitudinal bone growth, whereas ossification at the epiphyseal-articular cartilage complex shapes the ends of long bones (Olsson, 1987; Safadi et al., 2009). During growth, new cartilage is formed and subsequently replaced by bone, allowing the bone to bear weight whilst growing (reviewed by Ytrehus et al., 2007).

During growth, the articular-epiphyseal cartilage complex can be classified into distinct zones (see Figure 1.7). The resting zone sits underneath the articular cartilage and contains irregularly scattered chondrocytes (Safadi et al., 2009). These cells may act as precursors for proliferating chondrocytes but may also produce factors which influence chondrocyte alignment or differentiation (Abad et al., 2002). The proliferating zone contains rapidly dividing chondrocytes. The columnar arrangement of these cells is responsible for the directionality of growth (Safadi et al., 2009). In the hypertrophic zone, terminally differentiated chondrocytes enlarge, pushing the cartilage complex outwards. These hypertrophic chondrocytes then secrete a specialised matrix containing osteoid that acts as a scaffold for bone formation (Ytrehus et al., 2007). In the mineralization or calcifying zone this matrix is calcified, turning into bone at the ossification front (Siegling, 1941).

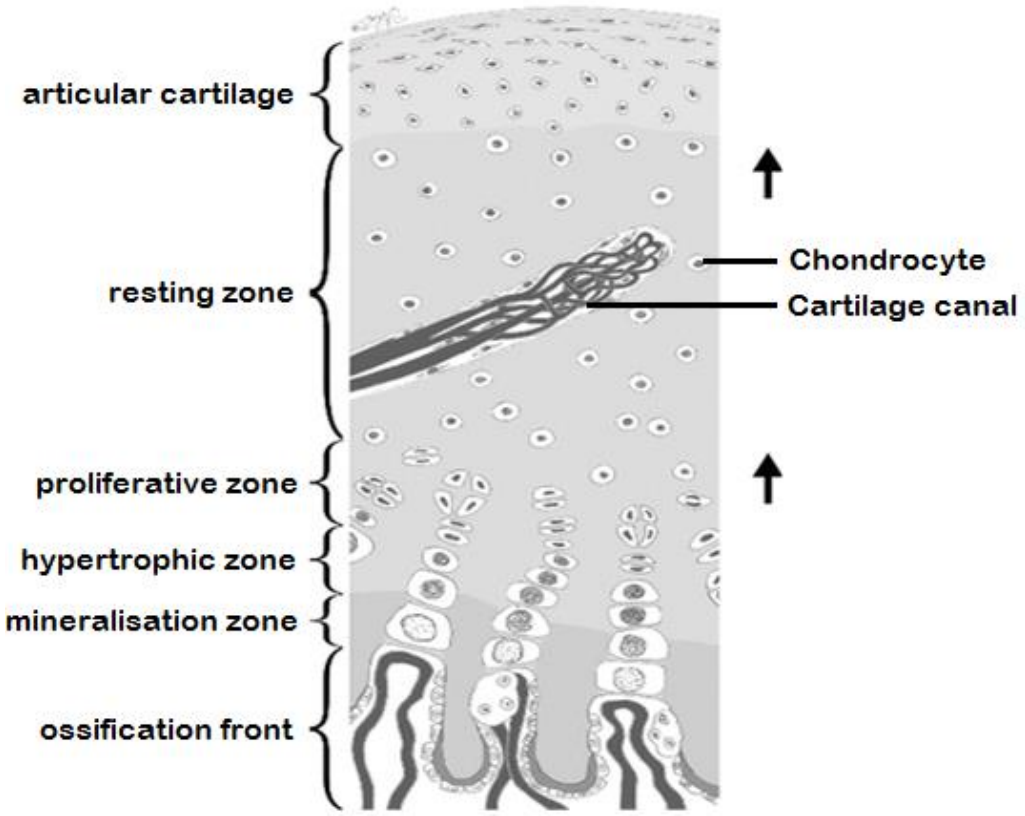


Figure 1.7: Schematic cross section of the articular-epiphyseal cartilage complex. The cartilage is arranged into distinct zones of resting, proliferating and hypertrophic chondrocytes, which causes the cartilage to grow outwards (arrows show growth direction). Hypertrophic chondrocytes secrete a matrix that is mineralised, resulting in new bone forming at the ossification front and bone growth. Image taken from Ytrehus et al. (2007).

Although articular cartilage is avascular, the epiphyseal cartilage is highly vascularised to support growth. Cartilage canals, formed by invaginations of the perichondrium (connective tissue covering non-articular cartilage in growing bones), invade the epiphyseal cartilage. These canals form a tunnel containing arterioles that branch into a network of capillaries (Blumer et al., 2005). This blood supply provides nourishment to chondrocytes deep within the cartilage, beyond the reach of synovial fluid nutrients, and is essential for normal growth. Their dysfunction results in conditions such as OC.

1.6 Osteochondrosis

1.6.1 Pathology of porcine osteochondrosis

OC is a generalised skeletal abnormality found in growing animals resulting from a non-infectious disturbance in endochondral ossification (Reiland, 1978; Nakano et al., 1984b; Crenshaw, 2006). The majority of studies, across all species, investigate late or chronic stages of the disease when clinical symptoms or macroscopic joint changes are present. However, this often results in primary changes associated with OC being missed (reviewed by Laverty and Girard, 2013). OC can occur at both the epiphyseal growth plate and the articular-epiphyseal cartilage complex (Reiland, 1978; Crenshaw, 2006), but OC affecting articular-epiphyseal cartilage is thought to be the most common and debilitating form of OC found in domestic animals (reviewed by van Weeren, 2006; and Laverty and Girard, 2013).

OC results from a focal failure in endochondral ossification and an area of epiphyseal cartilage is not calcified properly (Olsson, 1987). This focal failure is caused by a disturbance in the blood supply, due to cartilage canal alterations and necrotic blood vessels in the epiphyseal cartilage, which results in ischemic necrosis of the surrounding tissue (Woodard et al., 1987; Carlson et al., 1989; Ytrehus et al., 2004a). Initially, this results in an area of damage confined to the epiphyseal cartilage, with focal chondrocyte necrosis and disruption of the cartilage matrix (Reiland, 1978; Thorp et al., 1995). This early stage of the disease is termed *osteochondrosis latens.* A small lesion of this nature can resolve when it is reached by the advancing ossification front and the necrotic area can be mineralised into bone (see Figure 1.8).

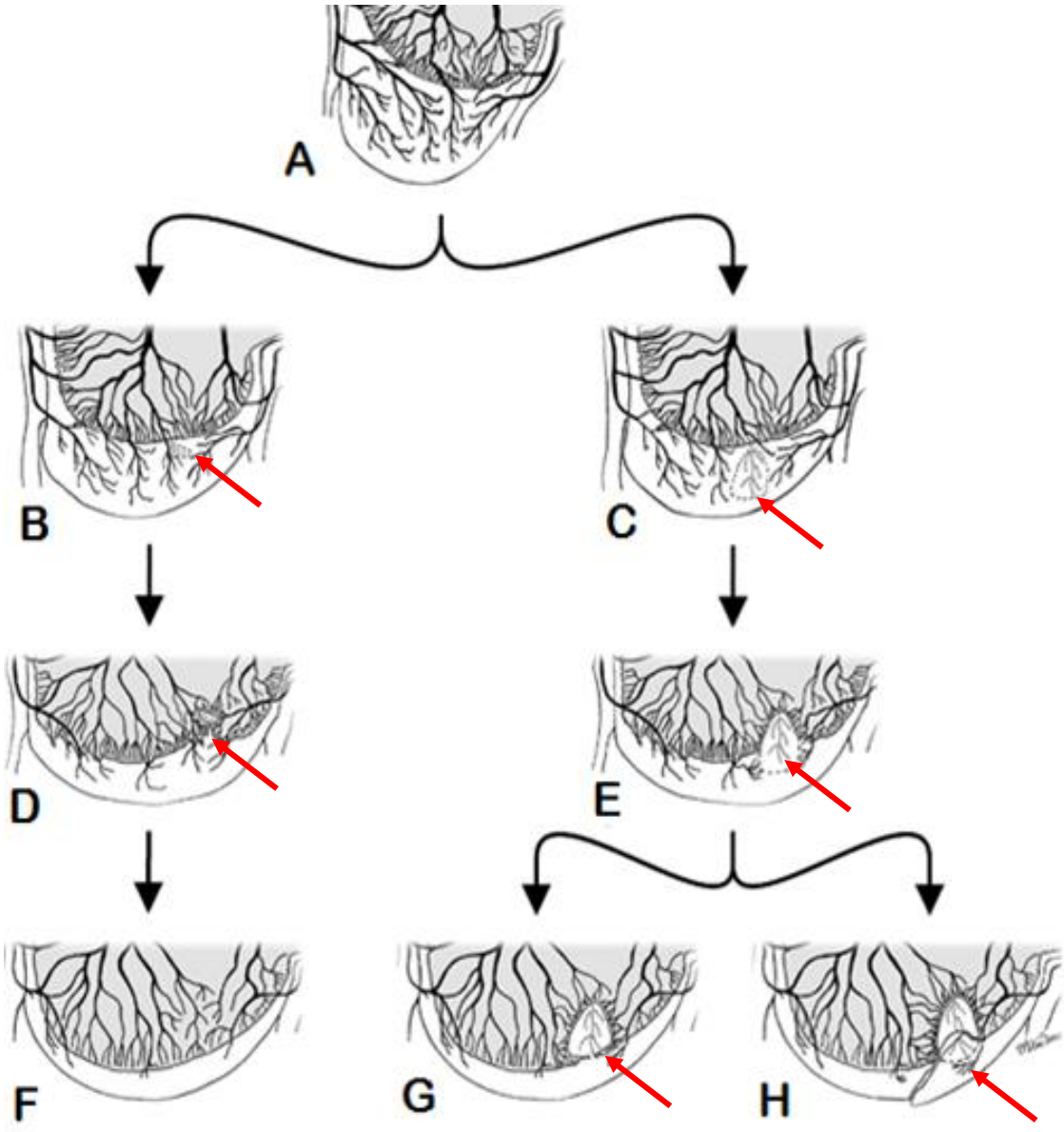


Figure 1.8: Pathogenesis of articular osteochondrosis. Healthy cartilage in young animals is highly vascularised via cartilage canals (A). Blood supply disruption causes ischemic necrosis of the surrounding cartilage (*osteochondrosis latens,* B and C). A small defect may resolve when it reaches the advancing ossification front (D and F). Larger defects are unable to resolve, resulting in a cone of necrotic cartilage, which the ossification front advances around (*osteochondrosis manifesta,* E). These lesions may be surrounded by bone as the ossification front advances (G), or the overlying cartilage can rupture or detach causing *osteochondrosis dissecans* (H). Arrows indicate lesions. Image adapted from Ytrehus et al. (2007).

Larger lesions progress into *osteochondrosis manifesta,* which is often visible radiographically and macroscopically. The necrotic area suffers from a failure in matrix mineralisation and the cartilage will not ossify correctly (Wardale and Duance, 1994; Thorp et al., 1995). A core of necrotic cartilage develops that the ossification front advances around, which creates a local thickening and retention of cartilage, causing flattening and creasing of the articular cartilage surface (Wardale and Duance, 1994; Crenshaw, 2006). In some cases the *osteochondrosis manifesta* lesion will become completely surrounded by bone and will gradually reabsorb or turn into a bone cyst (Nixon et al., 1988). However, if the lesion is too large it may not become surrounded, and this necrotic cartilage is vulnerable to rupturing, creating clefts within the cartilage termed *osteochondrosis dissecans (OCD).* This exposes the subchondral bone and flaps of cartilage or bone can become dislodged entirely, resulting in loose bodies within the joint (Omer, 1981; Crenshaw, 2006). Although many OC lesions resolve, if the ossification disruption persists it can lead to abnormal skeletal growth, altering bone shape and leaving joints susceptible to further problems, such as OA onset (Reiland, 1978). The onset of symptoms depends on the severity and location of OC, with OCD presenting with the most severe lameness (Reiland, 1978).

1.6.2 Prevalence of osteochondrosis

The disruption of normal ossification during growth and the resultant OC is a condition common in many species including pigs, horses, dogs, cattle, sheep, poultry and humans. In humans, OC is estimated to affect < 0.5% of the population but has been reported in up to 4% of males over 15 years of age (Nielsen and übersetzt, 1933; Lindén, 1976). OC is reported to affect over 20% of horses (Kane et al., 2003), with some studies identifying a higher prevalence of over 60% (van Grevenhof et al., 2009). In pigs, OC has been identified as one of the most important cause of leg weakness, with manifestations of OC seen in 80 - 100% of skeletally immature pigs (Reiland, 1978; Nakano et al., 1984b; Taylor, 2006).

The reported prevalence of porcine OC varies between studies, partly due to animal age, country and particular joint examined. The ankle, knee and elbow joints are

most commonly identified as having OC (Luther et al., 2007; van Grevenhof et al., 2011; Tóth et al., 2016). The particular scoring techniques used may also alter prevalence of OC identified in different studies. Lesions visible macroscopically have been reported in 40 - 65% of pigs, which were severe in 15% of cases (Busch and Wachmann, 2011; van Grevenhof et al., 2011; Etterlin et al., 2014). However, macroscopic changes in joint surfaces are usually not apparent in early disease stages, but radiographic examination can identify earlier and milder OC. Jørgensen and Andersen (2000) examined 2042 Landrace boars and 1946 Yorkshire boars radiographically and found radiographic OC in 84% and 56% of pigs respectively. Histological analysis is capable of detecting more subtle changes in cellular organisation, thereby detecting earlier OC lesions. OC identified using histology is consistently found in 65 - 100% of joints examined, although they are mostly mild in severity (Dewey et al., 1993; Bertholle et al., 2016; Tóth et al., 2016). As such, over 80% of commercial pigs are thought to have OC in at least one joint, but the majority of these lesions are thought to be mild and subclinical in nature (Taylor, 2006).

1.6.3 Aetiology of porcine osteochondrosis

Although many studies have identified ischemia as the cause of OC, the reason for this vascular failure is still unclear. OC is regarded as having a multifactorial aetiology with trauma, genetic predisposition, growth rate and diet all implicated in its onset (Ekman and Carlson, 1998; van Weeren, 2006; Ytrehus et al., 2007; Vidal et al., 2011). Trauma is considered a leading cause of OC and piglets dropped from a height show significantly increased lesions (Nakano and Aherne, 1988). Biomechanical factors within joints are also involved, such that in pigs medial joint aspects show increased OC compared to lateral aspects, due to their increased biomechanical stress and load bearing (Grondalen, 1974). Furthermore, housing conditions pose a significant risk factor for OC. The use of slatted flooring increases the prevalence of OC, however, solid flooring and deep bedding reduces slips and falls whilst also providing cushioning, decreasing the risk of trauma and injury, and is therefore associated with a lower incidence of OC (Kilbride et al., 2009; van Grevenhof et al., 2011; Etterlin et al., 2014; Pluym et al., 2017).

Genetic influences are also important; OC is absent or very rare in mini-pigs and wild boar but common in commercial pigs (Reiland, 1978; Etterlin et al., 2017). This is thought to result from high growth rates combined with an underlying genetic susceptibility to OC in commercial pig strains (reviewed by Ekman and Carlson, 1998). Different breeds show varying susceptibility with Landrace pigs more prone to OC than other breeds (Jørgensen and Andersen, 2000; Luther et al., 2007; Tóth et al., 2016). Heredity factors also influence the prevalence, severity and location of OC lesions (Ytrehus et al., 2004b). Genetic susceptibility to OC may be, in part, due to selection for pigs with high growth rates in order to increase production. Jørgensen and Andersen (2000) found that OC was unfavourably genetically associated with daily weight gain and restricting energy intake has been suggested to reduce OC onset (Jørgensen, 1995; van Grevenhof et al., 2011; de Koning et al., 2013; Tóth et al., 2016). Other studies have suggested there may be a critical period, between 7 and 13 weeks of age, where weight gain or growth rate has the greatest effect on OC onset (Ytrehus et al., 2004a; 2004b; de Koning et al., 2013). Outside of this period, nutrition has a limited effect on OC onset but deficiencies in vitamins and minerals can alter OC susceptibility (Nakano et al., 1984a; Sugiyama et al., 2013; Tóth et al., 2016).

1.6.4 Biochemical markers of osteochondrosis

The biological pathways and molecular mechanisms involved in the pathology of OC are still unclear. However, studies to identify candidate genes and molecular markers of OC are providing new insight into potential molecular pathways. Genome-wide association and micro-ribonucleic acid (miRNA) studies have identified a number of cellular processes that may be involved in the pathology of OC. Alterations in genes and miRNA involved in immune response, angiogenesis (formation of new blood vessels), cartilage ECM synthesis and structure, cell cycle differentiation, and metabolism have been implicated in OC (Chiaradia et al., 2012; Rangkasenee et al., 2013; Desjardin et al., 2014). It is hypothesised that alterations in these processes leaves cartilage vulnerable to injury or dysfunction, but it is unclear whether they cause the alterations in cartilage canals and resulting ischemia seen in OC, or whether they are precursive factors.

Studies of equine and porcine OC have identified altered turnover and aggregation of the proteoglycan aggrecan (Laverty et al., 2000; Kuroki et al., 2005). Changes in the cartilage collagen profile have also been identified in OC with altered amounts of type I, VI and X collagen (Wardale and Duance, 1994; Henson et al., 1997; Mirams et al., 2009). Furthermore, changes in type II collagen synthesis and degradation have been identified in OC. This has been assessed by measuring the circulating levels of carboxy-propeptide of type II collagen (CPII), a collagen precursor molecule, and carboxy-terminal telopeptide of type II collagen 3/4-length fragment (C2C), a degradation fragment formed during collagen cleavage. It has been suggested the ratio of CPII:C2C, indicating the rate of synthesis to degradation, in either serum or synovial fluid may be a good indicator of cartilage health (Laverty et al., 2000; Billinghurst et al., 2004; Frantz et al., 2010; de Grauw et al., 2011). Alterations in matrix metalloproteinases (MMPs), the enzymes responsible for degrading ECM proteins, have also been indicated in the pathology of OC. There are 25 known mammalian MMPs, and gene variants or increased expression of MMP3 and MMP13 have been identified in canine and equine OC (Kuroki et al., 2005; Mirams et al., 2009; Laenoi et al., 2012; Riddick et al., 2012).

Other molecular changes identified in both human and animal OC include reduced carbonic anhydrase, an enzyme responsible for alkalising the cartilage matrix for calcification, and changes in the growth factors insulin-like growth factor (IGF), transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF) (Laenoi et al., 2012; Riddick et al., 2012; Schmal et al., 2014). Identification of these molecular changes provides insight into the disease's pathophysiology and provides potential biomarkers for the disease. However, it is unclear whether these changes are causal or resultant of OC, and features such as the role of inflammation, cytokines and proteases are yet to be thoroughly investigated.

1.6.5 Treatment of osteochondrosis

Treatment of OC in pigs is limited and the emphasis has been placed on prevention techniques. In more valuable species, such as dogs and horses, traditionally treatment of OC is confined to exercise restriction and analgesic treatment. In some

animals palliative surgery may be conducted to remove loose cartilage bodies within the joint (Cook et al., 2008). More recently, the use of osteochondral autografting and autologous chondrocyte implantation have been investigated as a treatment of OC in dogs, horses and humans, often using pigs as a model (Cook et al., 2008; Betsch et al., 2014; Matsuo et al., 2015). However, these surgical techniques are expensive and not commercially viable for the pig industry. To treat lameness, due to suspected OC or other conditions in pigs, it is recommended that the affected animals are isolated and confined to small well-bedded pens for an extended period of up to 6 weeks (Oomah, 2008). This reduces exercise and trauma by limiting animal interactions, which may enable lesions in young animals to repair spontaneously. The systematic use of non-steroidal anti-inflammatory drugs (NSAIDs), including flunixin, ketoprofen, meloxicam or isoflupredone, is also recommended to provide analgesia (Oomah, 2008). These palliative techniques may reduce pain and allow OC lesions to heal, but more often pigs with suspected OC are culled, as it is not cost effective to treat or keep a pig with OC. Due to the subclinical nature of OC and lack of curative treatments, OC lesions often progress into more severe irreversible conditions, especially OA (Crenshaw, 2006).

1.7 Osteoarthritis

OA is often referred to as degenerative joint disease in pigs. It is a chronic joint disease characterised by degeneration of cartilage and underlying bone and usually involves joint inflammation. In commercial pigs under 18 months the incidence of OA is low, only affecting around 7% of pigs (Reiland (1975) cited in Straw (1999)). However, OA significantly increases with age and in sows over 18 months of age the incidence is estimated at over 82% (Reiland, 1975; Reiland, 1978; Dagorn and Aumaitre, 1979; Kirk et al., 2005). This high prevalence in adult pigs is thought to result partly from progression of an OC pathology (Grondalen, 1974; Reiland, 1978; Crenshaw, 2006). Despite the prevalence of OA in breeding sows, it has received little research attention. Commercial sows have a short lifespan of 2 - 4 years, beginning their reproductive life at 6 - 8 months of age (Whittemore and Kyriazakis, 2008). This short lifespan, combined with the low economic value of breeding stock, has resulted in research focusing on OC and other disorders in growing pigs, as their

prevention can provide faster economic benefits. Despite porcine OA receiving little attention, human OA has been the focus of much research, often using animal models. OA affects over 33% of humans over 45 years of age in the UK, which is approximately 8.75 million people (GBD, 2017; ARUK, 2018). OA in humans is estimated to cost the UK economy 1% of its gross national product and £250 million/year is spent on social care due to OA-related problems (NICE et al., 2008; ARUK, 2013). Therefore, understanding porcine OA has the potential to not only benefit the commercial pig industry, but also offer insight into the human condition.

1.7.1 Pathology of osteoarthritis

OA is a degenerative condition characterised by the progressive breakdown of the structure and function of articular cartilage (Collins, 1949). Features include degeneration of the cartilage ECM and cartilage thinning, which can lead to full-thickness erosion exposing subchondral bone (Buck et al., 2013; Lee et al., 2013; Siebelt et al., 2014). The subchondral bone undergoes remodelling and sclerosis (increased density and hardening) and osteophytes or bony projections form (Crenshaw, 2006; Siebelt et al., 2014; see Figure 1.9). Traditionally OA was considered a non-inflammatory 'wear-and-tear' disease; however, in the 1980s it was identified that the majority of OA patients have synovial inflammation (Goldenberg et al., 1982; Crenshaw, 2006; Berenbaum, 2013).

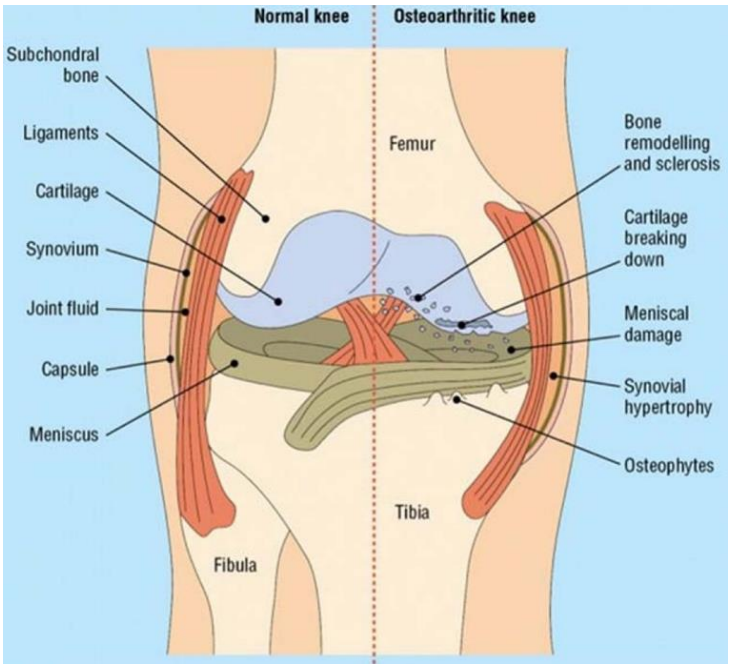


Figure 1.9: Pathophysiology of osteoarthritis. Schematic drawing of a healthy (left) and osteoarthritic (right) knee joint. Image from Shapiro et al. (2014).

Degradation of the joint can begin a long time before OA becomes symptomatic, by which point structural changes can be significant and irreversible (McAlindon et al., 1992; Wieland et al., 2005). It has been identified that an imbalance of anabolic and catabolic activities within the joint leads to irreversible tissue damage and pain in OA, but the trigger for this imbalance is still unclear (Shapiro et al., 2014). Multiple risk factors, including age, obesity, trauma and genetic predisposition, have been suggested in the development of OA (Sharma et al., 2000; GBD, 2017; ARUK, 2018).

1.7.2 Osteoarthritic changes in cartilage and subchondral bone

In healthy cartilage, chondrocytes have a low metabolic activity with little cell division or death, and they act to maintain an equilibrium of anabolic and catabolic activities to support a healthy ECM (Goldring and Goldring, 2004; Heijink et al., 2012). Cartilage is adapted to respond to load bearing in different regions of the joint, and chondrocytes can respond to biomechanical changes by upregulating synthetic activities or increasing inflammatory molecules (Vincent et al., 2012). There are thought to be two fundamental mechanisms of OA: abnormal

biomechanical loading onto healthy cartilage or normal biomechanical loading onto abnormal cartilage (Goldring and Goldring, 2007).

Normal cartilage ageing can occur without the development of OA but ageing is considered a significant risk factor for the disease. Chondrocytes are long-living cells so accumulate age-related changes including oxidative stress and decreased growth factor responsiveness (Heijink et al., 2012). This results in a change to a senescent secretory phenotype, with reduced ECM synthesis and increased cytokine and protease release (Anderson and Loeser, 2010; Heijink et al., 2012). This alters the ECM structure, particularly the content and organisation of type II collagen and proteoglycans (Goldring and Goldring, 2007). These ageing processes reduce the ability of chondrocytes to maintain healthy cartilage, which increases the risk of degeneration and can predispose OA onset (Buckwalter et al., 2005).

The features of early OA are proteoglycan loss, cartilage thinning and loss of elasticity (Mankin et al., 1971; McDevitt, 1973). These changes can increase synthetic activity of chondrocytes in an attempt to repair the cartilage matrix that leads to local cartilage thickening due to hypertrophy, but also thickening in areas of the posterior non-weight-bearing cartilage (Mankin et al., 1971; Omoumi et al., 2015). As OA progresses, articular cartilage loses its structural and functional properties due to disturbances in the matrix components (see Figure 1.10). Chondrocyte morphology is altered and they show signs of senescence, with increased oxidative deoxyribonucleic acid (DNA) damage that can result in chondrocyte apoptosis (Price et al., 2002; Chen et al., 2008). Chondrocytes cluster together in ‘clones’ and shift towards more catabolic activities, increasing inflammatory cytokine and proteolytic enzyme production and reducing anabolic signalling (Lee et al., 2013). Cytokines as small proteins, which can be pro- or anti-inflammatory, involved in interactions and communications between cells. Increases in cytokines and proteases reduces the synthesis and increases the breakdown of collagen, weakening the ECM (Eyre, 2002). Cytokines and proteases also reduce proteoglycans and increase cartilage water content, further disrupting ECM structure and function (Heijink et al., 2012; Siebelt et al., 2014; Stoppiello et

al., 2014). Loss of homeostasis within the cartilage results in fibrillation, formation of fissures and a load-bearing inability which can progress into full-thickness cartilage defects, exposing the subchondral bone (Mankin et al., 1971; Musumeci et al., 2013).

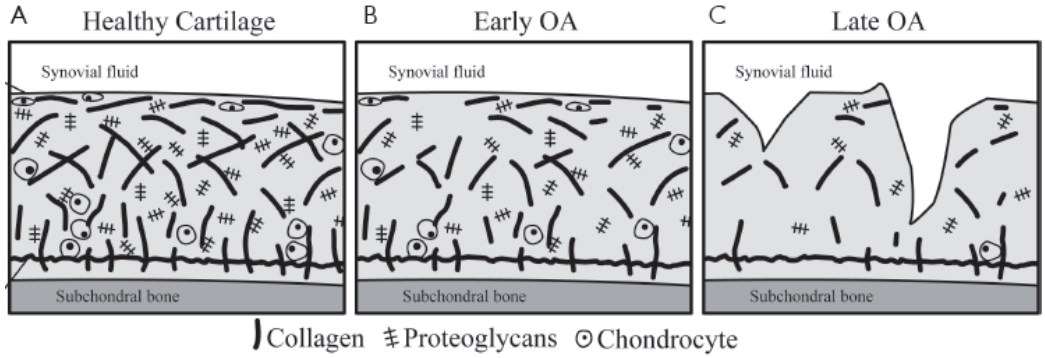


Figure 1.10: Articular cartilage composition changes in osteoarthritis. OA results in the progressive dysfunction of chondrocytes and alterations in the cartilage extracellular matrix, with the loss of collagen and proteoglycans. This compromises the cartilage's structural integrity, leading to cartilage fibrillation and fissures. Image taken from Matzat et al. (2013).

Although much of OA pathology is concerned with changes to articular cartilage, subchondral bone is also altered. It is unclear if changes to subchondral bone are causal or resultant of cartilage dysfunction but subchondral bone changes are strongly associated with symptomatic disease (Heijink et al., 2012). In early OA, bone micro-damage is apparent and increased bone remodelling occurs, resulting in thinning of the subchondral bone plate and deterioration in trabecular bone structure (Siebelt et al., 2014). As OA progresses, there is persistent remodelling, increasing bone volume and vascularity that may result in subchondral sclerosis (Mapp et al., 2008; Li et al., 2013). It has been suggested the presence or absence of bone sclerosis may represent different subtypes of OA (Steinbeck et al., 2015). Bone cysts or osteophytes on the outer joint edges can form and are associated with increased OA severity and pain (Sakao et al., 2009; Musumeci et al., 2013). The driving force behind these changes is thought to be the activity of osteoclasts.

Inhibition of osteoclasts in animal models prevents changes in bone mineralization and osteophyte formation, reducing structural changes within the joint (Strassle et al., 2010; Sagar et al., 2013). Inhibition of osteoclasts has also been shown to reduce changes in the cartilage ECM and synovial macrophage activity, suggesting osteoclast activity may drive a number of pathological changes seen in OA (Siebelt et al., 2014).

1.7.3 Osteoarthritic changes in the synovium

Although OA is traditionally not considered an inflammatory disease, due to the lack of synovial fluid neutrophils and systemic inflammation, OA is associated with signs and symptoms of inflammation including pain, swelling and synovitis (Goldenberg et al., 1982). OA causes the joint to swell due to synovial fluid effusion (excess fluid production) and thickening of the synovium because of intima layer hyperplasia and increased stromal cellularity (Berenbaum, 2013; Siebelt et al., 2014; Sieker et al., 2018). Cartilage breakdown fragments within the synovial fluid are thought to contact the synovium, resulting in an inflammatory response intended to repair and protect the joint. However, in OA the production of inflammatory mediators contributes to chondrocyte dysfunction and further cartilage degradation (Berenbaum, 2013). The inflammation results in synovial cells and chondrocytes increasing production of cytokines and proteases, including MMPs and aggrecanases. Protease release causes local proteoglycan loss and type II collagen cleavage at the cartilage surface, disrupting chondrocytes and the ECM, resulting in a loss of tensile strength (Sieker et al., 2018). The inflammation also elicits synovial angiogenesis and increased synovial macrophages (Musumeci et al., 2013; Stoppiello et al., 2014). It has been suggested that the particular pattern of synovial inflammation that develops in OA may cause distinct patterns in altered pain perception and form different pathological OA subtypes (de Lange-Brokaar et al., 2015).

1.7.4 Animal models of osteoarthritis

Much of our understanding of the pathology of OA is a result of animal model studies, using both spontaneous and induced arthritis in large and small animals.

Their use allows a greater comprehension of specific aspects of the disease whilst controlling phenotypic, genetic and environmental influences (reviewed by Bendele et al., 1999; 2001; and Dinser, 2008). There are advantages and disadvantages of using any animal model and careful selection is required, taking into account costs, ease of study, joint anatomy and biomechanics and public perceptions of the animal's use (reviewed by Gregory et al., 2012; and McCoy, 2015). These models can either be naturally occurring or require chemical or surgical induction.

1.7.4.1 Spontaneous small animal models

Naturally occurring OA models mirror the slow progressive nature of OA without the requirement for intervention. The Dunkin Hartley guinea pig has been extensively used as a spontaneous OA model as it mimics human OA with features including bilateral cartilage degeneration, osteophyte formation and subchondral bone damage (Jimenez et al., 1997). The incidence of these pathological changes increases with age and body weight, and degeneration is mostly found in medial joint aspects, similar to human OA (Bendele and Hulman, 1988). A number of mice strains also develop spontaneous OA including STR/ort, Balb/c, and Col9a1-/- transgenic mice. The use of these models has suggested roles for TGF-β1 signalling, cytokine levels and MMP expression in OA, but these models are most useful for identifying genetic predisposition factors rather than pathological features (Chen et al., 2017).

Spontaneous small animal models are useful for identifying mechanisms involved in the slow onset of OA; however, they are expensive as it can take a long time for OA to progress. For example, the Dunkin Hartley guinea pig begins to develop OA at around 3 months of age but does not reach skeletal maturity and show severe OA until around 6 months (Bendele and Hulman, 1988). Also, larger sample numbers are required as outcomes are less consistent between individuals, further increasing costs (Lampropoulou-Adamidou et al., 2014).

1.7.4.2 Induced small animal models

Induction of OA in small animals is achieved by either surgical intervention or chemical induction. Surgical models are useful for understanding the pathology of traumatic OA and usually involve disruption of the anterior cruciate ligament or menisci. Anterior cruciate ligament transection (ACLT model) causes joint instability and cartilage degeneration, and can be performed in a number of species including rats and rabbits (as well as large animals). ACTL rapidly induces severe OA, with pathological features present in rats after 2-3 weeks (Bendele et al., 1999). Surgical destabilisation of the menisci is also commonly performed to induce OA. Meniscal transection (MNX model) or destabilisation of the medial meniscus (DMM model) can also be performed. The MNX model can show weight bearing changes within 5 days of induction and cartilage lesions can be present within 7 days; however, the model shows no change in response to mechanical stimulation (Bendele, 2001). Both the MNX and DMM models mimic symptoms of meniscal injury in humans, which is a known precursor for OA development (Janusz et al., 2002; Cruz et al., 2015).

Chemical induction of OA can be performed by intra-articular injection of a number of possible compounds to induce cartilage degeneration. Injection of monosodium iodoacetate (MIA) disrupts cellular glycolysis, inducing cell death that leads to loss of chondrocytes and subsequent OA-related changes within the joint (Lampropoulou-Adamidou et al., 2014). The MIA model is well established in rats, producing proteoglycan loss and increased osteoclasts within 7 days and pain behaviours that can persist for over 60 days (Bove et al., 2006). By 21 days, extensive cartilage thinning, osteophyte formation and subchondral bone thickening occurs (Dinser, 2008). This rapid induction and change in pain sensitivity makes the MIA model useful for short-term studies and is more reproducible than surgical models. Other chemical induction models include injection of the proteolytic enzymes papain or collagenase. Papain degrades proteoglycans and can be used to study OA progression in a dose-dependent manner (Pomonis et al., 2005). Collagenase damages collagen-containing structures including cartilage, tendons and ligaments, which destabilises the joint. However, induction by

collagenase is much slower than MIA, with lesions appearing 3 weeks after injection (Lampropoulou-Adamidou et al., 2014). Carrageenan, derived from seaweed, induces inflammation in the joint and mimics acute inflammation with increases in interleukins (IL) and MMPs, and is useful for identifying inflammatory components in OA (Achari et al., 2012). These induced models are useful for identifying specific disease aspects in a short timeframe. Their use also allows for internal controls, whereby only one joint is treated, enabling direct comparison of a healthy and OA joint within the same animal. However, they do not mimic the age-related slow progressive nature of human OA.

1.7.4.3 Large animal models

There are a number of large animal models available to study OA, such as dogs, horses, pigs and monkeys (Bendele, 2001). The disadvantage of using large animals lies in the increased costs and difficulties in handling compared to small animals. Large animals such as pigs have an anatomy and physiology more similar to humans than rodents, with similar joint size and cartilage thickness (Gigliuto et al., 2014). Large animals also show widespread natural OA so are likely to have similar mechanisms of onset and progression to humans, so may predict more accurately the pathophysiology and pathogenesis of human OA. Both surgically induced and spontaneous models have been used in large animals and species studied include dogs, cats, sheep, goats, horses, pigs and non-human primates (reviewed by Mastbergen and Lafeber, 2009).

Both commercial breeds and miniature pigs have been used to study OA. The high prevalence of spontaneous OA in commercial breeds results in a large availability of abattoir specimens from adult pigs. Younger commercial pigs or miniature pigs can have OA induced, often by ACLT or MNX surgery. The similarity of the pig joint anatomy, cartilage thickness and other physiological characteristics, such as the distribution and properties of nociceptive (pain transmitting) fibres, makes them a good model for humans diseases (Gigliuto et al., 2014). However, there has been little investigation into spontaneous OA in commercial pigs, in terms of both

pathological changes occurring in joints, but also chronic pain and alterations in the nervous system that may occur.

1.8 Pain Perception

Pain is a complex phenomenon described by the International Society for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Loeser and Treede, 2008). Pain is distinct from nociception which is described by IASP as "the neural process of encoding noxious stimuli" (Loeser and Treede, 2008). Hence, pain is the physiological process by which a noxious (painful) stimulus is detected and transferred to the central nervous system (CNS); this signal becomes recognised as pain when it reaches higher centres within the brain. Nociceptive pain acts as a protective mechanism, alerting the individual to actual or potential tissue damage (Loeser and Melzack, 1999). Acute pain is transient, triggered by a noxious event and resolves following repair (Loeser and Melzack, 1999). Pathological or chronic pain, associated with many diseases, serves no physiological purpose and persists after tissue healing may have occurred (Cheng and Ji, 2008). Chronic pain can be identified by the presence of hyperalgesia (the increase in pain from a normally painful stimulus), allodynia (pain arising from a normally innocuous stimulus) and spontaneous pain (pain in the absence of an external stimulus), and represents the inability of the body to restore normal functioning of the nociceptive system (McMahon et al., 2013). The processing and perception of pain involves activation of peripheral sensory neurones that transfer the stimulus to the spinal cord and then secondary projection neurones relay this information to higher centres within the brain (see Figure 1.11).

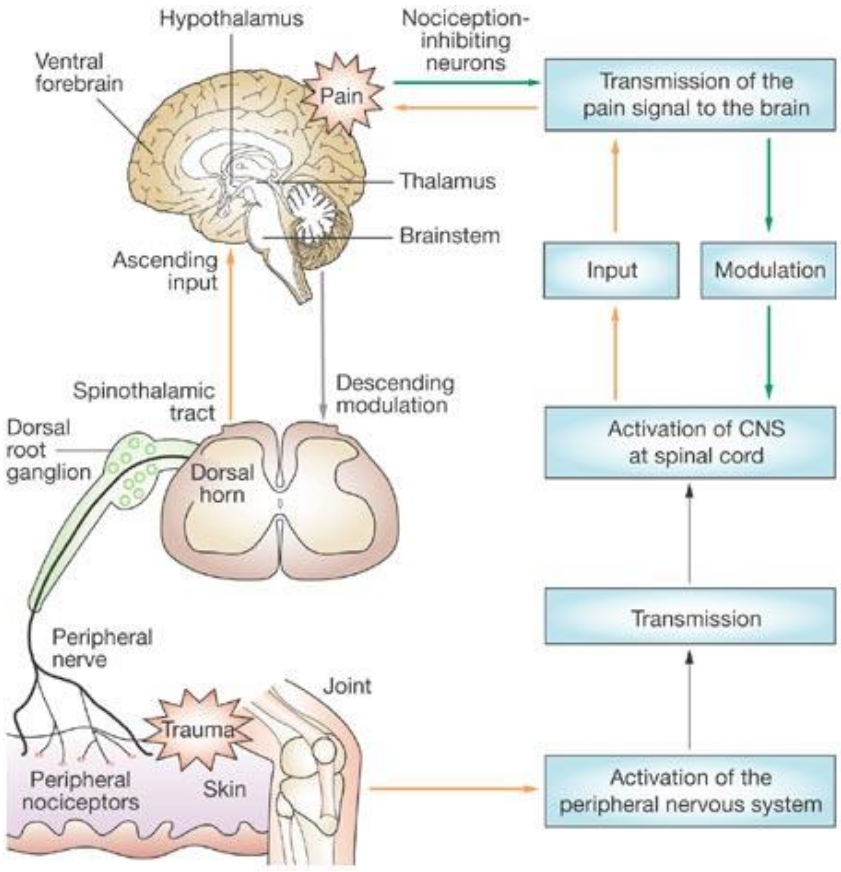


Figure 1.11: Schematic of the pain processing system. Activation of peripheral nociceptors results in signal transduction to the spinal cord dorsal horn. Signals then travel along the ascending pathways to the higher centres in the brain, which can send descending signals to modulate activity in the spinal cord. Image taken from Bingham et al. (2009).

1.8.1 Pain processing system

Primary afferent fibres are specialised pseudo-unipolar sensory neurones of the peripheral nervous system (PNS), which connect to the CNS. Hence, a single axonal process leaves the soma, situated within the dorsal root ganglia (DRG) or trigeminal root ganglia, and splits to provide terminals at both peripheral and central sites (McMahon et al., 2013; Krames, 2015; Kozłowska et al., 2017). The peripheral axon terminals of different types of primary afferents detect and relay diverse sensory information, sensing low threshold mechanosensory or thermosensory stimulation (e.g. touch, brush, cool and warm temperatures) or high threshold stimuli (Murphy et al., 2012; McMahon et al., 2013). The latter are termed nociceptors, responding

to painful (noxious) stimuli that could potentially damage tissue (Willis and Westlund, 1997).

Mammalian primary afferent fibres can be divided into three main classes based on their size, conduction velocity (CV) and morphology (Lawson, 2005). Firstly, Aα and Aβ fibres are large myelinated fibres with axon diameters of 10 - 20µm and fast CVs of 40 - 120 m/s (McGlone and Reilly, 2010). These fibres are typically classified as proprioceptors (responding to position and movement) and low threshold mechanoreceptors (Harper and Lawson, 1985a). Aα and Aβ fibres generally transmit innocuous sensory information, however, Aβ fibres have been implicated in nociception during both normal and disease states (Harper and Lawson, 1985b). Secondly, Aδ fibres are small myelinated fibres of medium diameter (2 - 5µm) with an intermediate CVs of 1 - 30 m/s (McGlone and Reilly, 2010). Aδ fibres are mostly responsible for the detection of temperature and noxious stimuli, particularly the fast, sharp 'first pain' (Magerl et al., 2001; McMahon et al., 2013). Finally, C-fibres are small unmyelinated fibres with a diameter of 1µm and have slow CVs (< 2m/s), hence they are generally responsible for the slow, dull and aching pain associated with 'second pain' (Magerl et al., 2001; McMahon et al., 2013).

C-fibres are often polymodal, meaning they respond to noxious chemical, mechanical and thermal stimuli, although a small population can also respond to tactile input such as light touch (McGlone and Reilly, 2010). This polymodal nature is partly due to the high expression of receptors from the transient receptor potential (TRP) channel family, which can respond to multiple stimulus modalities (reviewed by Patapoutian et al., 2009; and Mickle et al., 2016). For example, TRP vanilloid subfamily member 1 (TRPV1) responds to capsaicin, acidic conditions and high temperatures above 43°C, TRP vanilloid subfamily member 3 responds to warm temperatures and camphor (a chemical irritant), and TRP melastatin subfamily member 8 is activated by cold temperatures, methanol and eucalyptol (reviewed by Levine and Alessandri-Haber, 2007; and Knowlton and McKemy, 2011). C-fibres can be further subdivided as peptidergic, containing substance P, nerve growth factor (NGF) or calcitonin gene-related peptide (CGRP), or non-

peptidergic, expressing isolectin B4 and purinergic P2X3 receptors (Todd, 2010). There is also a class of C-fibres called 'silent nociceptors' which are unresponsive to acute noxious stimulation under normal conditions but become sensitized and activated during pathological conditions (Michaelis et al., 1996). C-fibres can also have an efferent function, releasing neuropeptides at the site of injury to cause the flare response, leading to the reddening of the skin by axon mediated vasodilation (Dusch et al., 2009), as well as oedema (swelling) due to plasma extravasation from blood vessels (Gee et al., 1997).

The central branches of the primary afferent fibres enter the spinal cord through the dorsal root and terminate within laminae of the dorsal horn (see Figure 1.12). There are ten distinct laminae within the spinal cord (Rexed, 1952) but Aδ and C fibres almost exclusively project to the superficial laminae I and II of the dorsal horn (Todd, 2010; McMahon et al., 2013). Activation of any of these primary afferent fibres elicits the conduction of action potentials, causing the release of neurotransmitters and neuromodulators at their central nerve terminals within the dorsal horn of the spinal cord (Schnitzler and Ploner, 2000). There is a vast array of neurotransmitters that can be released into the spinal cord, including the excitatory peptides CGRP, substance P and bradykinin, and the inhibitory molecules neuropeptide Y, somatostatin and galanin (Lawson, 2005). However, the primary excitatory neurotransmitter released into the dorsal horn by primary afferents is glutamate. These neurotransmitters bind to receptors present on spinal neurones, producing action potentials in interneurones and projection neurones within the dorsal horn eliciting a spinal response.

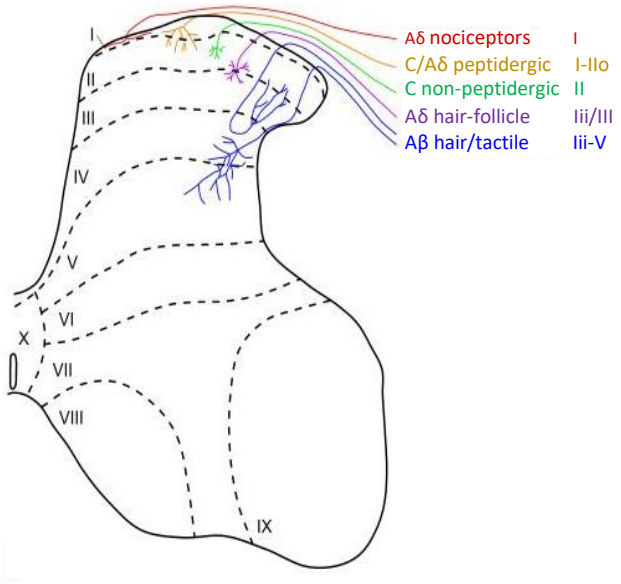


Figure 1.12: Laminar organisation of the spinal cord. Nociceptive fibres teminate mostly in laminae I and II of the spinal cord dorsal horn. Image taken from Todd (2010).

Dorsal horn neurones send axonal projections along ascending pathways to supra-spinal sites. The spinothalamic tract is the major ascending pathway but others include the spinoparabrachial and spinoreticular tracts (Gauriau and Bernard, 2002; McMahon et al., 2013). These pathways terminate in brain areas, including the thalamus and medulla, which connect with many other areas such as the amygdala, prefrontal cortex and motor cortex. This enables pain perception as both a sensory and emotional experience, eliciting complex behavioural responses (Tracey and Mantyh, 2007). As well as ascending pathways, there are also descending pain modulatory pathways. These pathways transmit inhibitory and excitatory information from the brain, mainly via the rostral ventromedial medulla (RVM) and locus coeruleus (LC), to the spinal cord (Tracey and Mantyh, 2007; McMahon et al., 2013). The RVM and LC project to spinal and medullary dorsal horns to directly and indirectly enhance or diminish nociceptive signals, modulating the pain experience (reviewed by Ossipov et al., 2010). These descending pathways release neurotransmitters, principally the monoamines serotonin (5-hydroxytrytamine) and noradrenaline. Their release alters the excitability of primary afferent nociceptors

and spinal neurones, thereby facilitating or inhibiting pain signalling (Willis and Westlund, 1997). It is thought that imbalances in these ascending and descending pain systems could affect pain processing during chronic pain conditions.

1.8.2 Persistent joint pain

The knee joint is innervated by nerves to maintain movement and proprioception but also to detect potentially damaging stimuli, such as overloading of the joint. The knee receives innervation from 2 - 3 primary articular nerves (Freeman and Wyke, 1967). The lateral articular nerve (found in large animals) projects to areas including the head of the fibula and the lateral and anterior aspects of the joint capsule. The posterior articular nerve innervates areas including the posterior joint capsule and cruciate ligaments. The medial articular nerve projects to areas such as the joint capsule, cruciate ligaments and the skin (Freeman and Wyke, 1967; Horner and Dellon, 1994).

Pain arising from the joint is often dull, aching and poorly localised. During joint diseases such as OA the pain is often initially episodic, during movement or loading, but as the disease progresses the pain can become chronic resulting in constant resting pain (Schaible, 2012). However, in conditions such as OA, the severity of pain experienced often poorly correlates with the extent of joint damage (Hannan et al., 2000), suggesting the pain experienced is determined by complex changes in the underlying neurophysiology and not solely by structural changes within the joint. As well as OA pain being chronic, it may also have a neuropathic pain (caused by a lesion to the nervous system) component. Some primary afferent fibres express activating transcription factor 3 in OA, which is a marker of nerve injury, suggesting neuropathic pain (Schaible, 2012; Thakur et al., 2014).

1.8.2.1 Peripheral sensitisation

Injury, inflammation and bone remodelling are common features of joint diseases and can elicit nociceptive pain (Nagy et al., 2006). As cartilage is aneural the pain in joint disease is thought to originate from changes in the synovium and subchondral bone. Joint pain is often associated with inflammation of the synovium, which has

been correlated with pain severity in a number of diseases (Nagy et al., 2006; Witt and Vilensky, 2014). Furthermore, subchondral bone contains free nerve endings of nociceptive fibres, which, when pathological changes in the bone occur, may elicit sensitisation and joint pain.

Following injury many chemicals are released, including prostaglandins, NGF, histamine, serotonin, tumour necrosis factor α (TNFα), CGRP, substance P and interleukins, creating an 'inflammatory soup' at the periphery that sensitises primary afferent nerve fibres (Schaible et al., 2002; Nagy et al., 2006; McMahon et al., 2013). This peripheral sensitisation results in increased neuronal firing, increasing the afferent action potential firing rate and causing hyperalgesia (Loeser and Treede, 2008; Murphy et al., 2012). Peripheral sensitisation also increases spontaneous neuronal firing, causing spontaneous pain and can recruit silent C fibres to become active nociceptors (Goldring and Goldring, 2007; Murphy et al., 2012). Joint pain results from sensitisation of both the peripheral and central pain pathways and it is thought that these changes at the periphery trigger changes in central pain processing, known as central sensitisation, which underlies and maintains chronic joint pain (Schaible et al., 2009a; McMahon et al., 2013).

1.8.2.2 Central sensitisation

The increased afferent fibre input to the spinal cord, as a result of peripheral sensitisation, causes spinal neurones to become hyperexcitable and results in their sensitisation (Schaible et al., 2002). This increases responsiveness to joint stimulation and causes the receptive field of peripheral neurones to expand (Nagy et al., 2006; Kidd, 2012). Central sensitisation also causes allodynia, increases spinal neurone firing rates and enhances spontaneous discharge (Nagy et al., 2006; Kidd, 2012). In rodent models of OA, increased cytokines and chemokines are found within the spinal cord dorsal horn, which alters neuronal activity (Im et al., 2010). Furthermore, there is evidence of neuroinflammation in articular disease with increases in the number and activity of spinal cord astrocytes and microglia (Fusco et al., 2017).

Astrocytes provide structural and functional support to the nervous system and are involved in maintaining homeostasis of the extracellular environment (Grace et al., 2014). Microglia are the resident immune cells within the nervous system and can become phagocytic (Grace et al., 2014). It is thought that reciprocal signalling between the nervous system and these immune system cells may modulate pain mechanisms and their upregulated activity in joint disease may facilitate pain (Burston et al., 2013; Grace et al., 2014). As well as changes occurring in the spinal cord, pain processing in the brain can also be altered. Chronic pain can result in atrophy of brain regions, including the thalamus, anterior cingulate cortex, amygdala and brainstem (Rodriguez-Raecke et al., 2009), but may also reduce descending inhibitory signals, further increasing pain perception (Schaible, 2012). This change in the central pain processing increases pain, resulting in joint tenderness, referred pain and helps maintain the chronic pain state (Murphy et al., 2012).

1.8.3 Porcine pain processing system

The pain system in pigs has been found to be functionally and anatomically very similar to that of humans and other mammals, making pigs a good model for studying pain and disease. The localisation of nociceptive fibres in the spinal cord differs only slightly to humans and primates (Breazile and Kitchell, 1968). Primary afferent nociceptors in pigs are also similar to those found in humans and rodents (see Table 1.1). Porcine A-fibres are mechanosensitive or nociceptive, have large receptive field sizes and CVs of 6.2 - 64 m/s, making them similar to humans and rat A-fibres (Lynn et al., 1995). One difference, however, is that porcine A-fibres have minimal heat-sensitivity in healthy tissue, which is not observed in humans (Lynn et al., 1995). Porcine C-fibres show a CV below 2m/s and are grouped based on their response to different stimulus modalities as in humans and other animals (Lynn et al., 1995). They are categorised as mechanosensitive, thermosensitive or polymodal (Gee et al., 1999). Porcine C-fibres have a similar heat threshold to humans and show similar changes in CV following activity (Obreja et al., 2010). There has also been observed in pigs a class of C-fibres which show no activity under normal

conditions, indicating the presence of fibres which are comparable to silent nociceptors found in humans and rodents (Lynn et al., 1995).

Table 1.1: Comparison of pig, human and rat primary afferent fibre properties. Data from Harper and Lawson (1985b; 1985a), Lynn et al. (1995), Gee et al. (1999), Obreja and Schmelz (2010) and Churyukanov et al. (2012).

Fibre	Property	Pig	Human	Rodent
Aα	CV	6.2 - 64 m/s	80 - 120m/s	70 - 120 m/s
	Modality		Proprioceptive	Proprioceptive
Aβ	CV	Proprioception, mechanical	33 - 75 m/s	40 – 70 m/s
	Modality		Proprioception, nociceptive	Proprioception, nociceptive
Aδ	CV	6.3 - 19.1 m/s	3 - 30 m/s	1 - 15 m/s
	Modality	Nociceptive (mechanical, thermal > 59°C)	Thermal, nociceptive (mechanical, chemical, thermal > 45°C)	Thermal, nociceptive (mechanical, chemical, thermal > 45°C)
C	CV	< 2 m/s	< 2 m/s	< 1.3 m/s
	Modality	Polymodal, thermal (33 - 55°C), nociceptive (mechanical, thermal, chemical), silent	Polymodal, thermal (> 45°C), nociceptive (mechanical, thermal, chemical), silent	Polymodal, thermal (> 45°C), nociceptive (mechanical, thermal, chemical), silent

Furthermore, the expression of many neuropeptides, enzymes and hormones have been characterised in the porcine nervous system and are similar to expressions in humans and rodents. Neuropeptide Y, which is involved in chronic pain, has a comparable spinal cord distribution in pigs and humans, with the highest expressions in the lumbar and sacral dorsal horn (Roddy et al., 1990). TRPV1 receptors that are highly involved in pain signalling are found in the spinal cord and DRG and have an 88% homology between humans and pigs (Ohta et al., 2005). Furthermore, the expression of substance P, CGRP, vasoactive intestinal peptide,

enkephalin, galanin, somatostatin and calbindin have been characterised in the DRG of healthy pigs (Zacharko-Siembida et al., 2014). It has been shown that in pigs, as in humans, substance P and CGRP are highly involved in pain transmission (Jana et al., 2012).

1.8.3.1 Porcine pain system response to inflammation and injury

The porcine pain system shows a number of characteristics in response to inflammation and damage that are found in humans and rodents. Studies have demonstrated that inflammation can elicit allodynia and hyperalgesia in pigs (Lynn et al., 1996; Sandercock et al., 2011). The flare response of axon-mediated vasodilation in pigs was also found to have similar characteristics to humans. Flare caused the activation of silent nociceptors and sensitisation of mechanical and heat sensitive nociceptors. This response indicated pig C-fibres can have an efferent as well as an afferent function, as found in humans and rodents (Lynn et al., 1996; Rukwied et al., 2008). Similarly, capsaicin, an agonist of TRPV1 receptors, produces similar thermal hyperalgesia and individual subject variations in the extent of mechanical hyperalgesia in both humans and pigs (Di Giminiani et al., 2014).

These studies have shown that the pain system in pigs and humans share a number of characteristics. Both species demonstrate peripheral and central sensitisation and inflammation is able to alter nociceptor characteristics (Rukwied et al., 2010; Obreja et al., 2011a; Obreja et al., 2011b). The differences in pain processing detected between the species could result from factors such as altered neuropeptide sensitivity or receptor expression (Dusch et al., 2009). Study of these differences may enable a better understanding of the human pain system. The porcine pain system has been well characterised in its response to flare; however, there is little work on the relationship between joint disease and pain pathways. Joint diseases in pigs often present with lameness and pain and it has been shown that lame limbs have reduced mechanical thresholds, indicating sensitisation and hyperalgesia (Spadavecchia and Ranheim, 2014). However, to what extent porcine joint diseases such as OC and OA cause chronic pain, involving peripheral or central sensitisation, has yet to be determined.

1.9 Hypothesis

Commercial pigs suffer from a high degree of joint diseases such as OC and OA, which are likely to be similar to diseases seen in humans and other animals. Alterations to the cartilage during OC and OA could trigger inflammation within the joint. This inflammation may alter gene expression within the synovium by altering inflammatory cytokines or proteases, which could influence disease progression or pain perception. The cartilage damage and inflammation is likely to cause pain that may become chronic in nature, sensitising the peripheral and central nervous systems.

1.10 Aims of this thesis

Lameness in pigs has been identified as a key problem for the commercial pig industry, creating significant economic burden and reducing animal welfare. However, the pathological features of joint diseases that cause lameness have received little research attention. The overall aim of this thesis was to characterise the pathological features of spontaneous OC and OA in a commercial pig strain. The relationship between the pathological features of spontaneous disease and pain behaviours were investigated. The role of synovial inflammation and potential molecular mechanisms involved in both porcine OC and OA were evaluated. Furthermore, changes in the cellular response of the spinal cord and the phenotype of primary afferent sensory neurones associated with disease and pain were investigated. A more detailed characterisation of spontaneous porcine OA would then allow the commercial pig to be evaluated as a spontaneous human disease model.

Chapter 2

Materials and Methods

2.1 Animals

All procedures were carried out in accordance with UK animal welfare regulations and institute guidelines. Even though the experiments did not fall under the Animals (Scientific Procedures) Act 1986 (ASPA), all experiments involving the use of animals were carried out in accordance with ASPA guidelines and were approved by the University of Nottingham Animal Welfare and Ethical Review Body (non-ASPA study number 000094).

For a summary of animals used and experiments performed see Figure 2.1. Four different age groups of clinically healthy female commercial pigs (sows or gilts) of the crossbred genotype 'Landrace x Large white x Duroc' were supplied by Yorkshire Farmers, with a total experiment population of 27 animals. Animals arrived with no clinical history, so no information was available regarding features such as previous health issues or number of parities. The crossbred genotype was selected as it consists of common commercial pig breeds found in the UK. Pigs were aged 9 months old (n = 6, weight 78 - 92 kg), 15 months old (n = 6, weight 124 - 156 kg), 24 months old (n = 8, weight 215 - 300 kg) and 3-4 years old (42 month old group, n = 7, weight 200 - 260 kg). Each age groups was studied at separate times and animals were group housed indoors, at the University of Nottingham Sutton Bonington Campus Animal Unit, with the exception of one 42 month old sow that was isolated due to ill-health. This pig appeared to be bullied by the other animals and stopped eating, so was segregated to ensure she had free access to food and water. The housing pens consisted of a solid concrete floor with deep straw bedding. Animals had access to food (Heygated Country Feeds Breeding Sow Nuts or Farmgate Pig Rearer Pellets) and water *ad libitum*. Feed bins were topped up daily and water was available from drinking spouts. Animals were habituated to the housing facility and the presence of an observer outside their enclosure for at least 2 days prior to the onset of behavioural assessment. For all age groups, behaviours were recorded at least 3 days after the animal had arrived at the facility to reduce the effect of recent transportation.

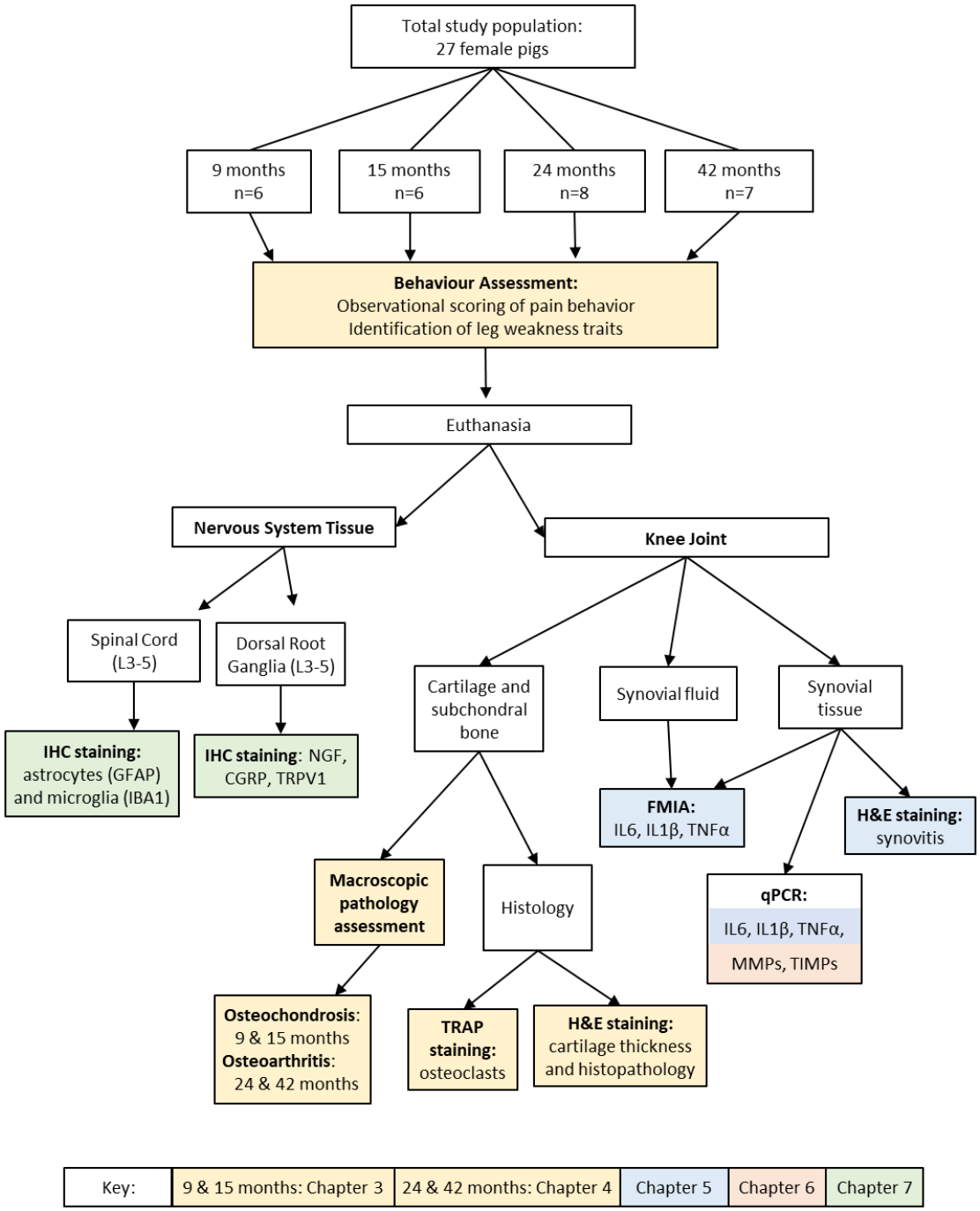


Figure 2.1: Flow diagram highlighting animals used and experiments performed on different tissue samples. Chapters containing results from each experiment are highlighted in different colours.

One 9 month old and one 15 month old pig became severely lame during the study, on the advice of the Named Veterinary Surgeon they were treated with the NSAID Metacam® (0.4ml/25kg body weight), delivered by intramuscular injection. The 9 month old pig received one dose, and the 15 month old pig received two doses of Metacam given 24 hours apart. For both animals, metacam was administered during the habituation period and at least three days prior to behaviour assessment.

2.2 Assessment of porcine pain behaviours and leg soundness

Pigs were assessed daily, over a period of 5 to 7 days, for pain behaviours and leg soundness. Animals were scored in situ from outside their enclosure. This allowed them to freely interact with one another during the observation period and minimised stress. A descriptive numerical scoring system was used to score pain behaviours. Leg soundness was assessed by recording leg weakness traits, which indicate musculoskeletal system dysfunction (Van Steenbergen, 1989). Throughout the study, observations were made by the same individual (Catherine Owles), although on some occasions observations were made by two independent scorers to assess the scoring system for inter-observer reliability and repeatability (Catherine Owles and Lara Yarwood or Mhairi Macfadyen). During the observation period, photographs and video recordings were also taken for subsequent scoring or record keeping. Whenever possible inspections were made at a similar time each day, generally 3 - 4 pm, in order to reduce any influence of circadian rhythms but also to preclude changes in behaviour caused by husbandry activities such as animal weighing, enclosure cleaning and replenishing of feed bins.

2.2.1 Observational scoring of porcine pain behaviours

Pigs were assessed visually for pain behaviours and scored using a numerical rating system. The scoring system was created by taking different behaviours from previously validated scoring systems used by Main et al. (2000), Royal et al. (2013) and Stavrakakis (2014). Royal et al. (2013) used VAS scoring to investigate post-operative pain in pigs following experimentally induced femoral fractures. They identified changes in activity on arrival, response to touch, willingness to ambulate

and vocalisations associated with pain or discomfort that were reduced by local anaesthesia. Main et al. (2000) investigated the repeatability of an NRS scoring system to assess lameness in finishing pigs, which was then simplified by Stavrakakis (2014). Characteristics including the response to human presence, standing posture and gait were assessed, and a high agreement in scores from trained individuals familiar with the animals was identified. Six of the behaviours detailed in these studies were considered good measures of lameness and pain or discomfort in pigs potentially suffering from spontaneous joint disease, which could easily be assessed, and were therefore selected for use in this study.

Seven different behaviours were assessed (see Table 2.1, behaviours A-G) and scores for A to F summated to give a total pain behaviour score. The behaviours assessed were lameness, activity on arrival, response to touch, ability or willingness to ambulate, vocalisations and response to human interaction. Behaviour G, hind leg lameness, was scored separately to indicate problems in the hind limbs only, and was not included in the total pain score to avoid double weighting of lameness behaviours. Response to touch was measured by observing the pig's interactions and recording the response to pigs touching one another.

Table 2.1: Porcine pain behaviour scoring system. Observational scoring system used to assess lameness and pain-related behaviours in commercial pigs. Adapted from Main et al. (2000) +, Royal et al. (2013) *, and Stavrakakis (2014) #.

Score	A: Lameness (whole body) (#)	B: Activity on arrival (*)	C: Response to touch (*)	D: Ability/willingness to ambulate (*)	E: Vocalisation (*)	F: Response to human interaction (+)	G: Hind leg lameness (#)
1	No evidence of lameness	Resting, calm, asleep	Minimal to no response	Walks voluntarily	None or normal noise when interacting	Inquisitive and alert: moves immediately	No lameness
2	Barely detectable: altered stride length, slight stiffness	Active but no noticeable tension or discomfort	Moderate movement	Walks with encouragement (i.e. for food or water)	Squealing when moved or touched	Bright but less responsive: remains stationary	Slight lameness barely detectable
3	Easily detectable: noticeable head nod, movements not fluent	Mild agitation, somewhat restless, may pace	Mild response: shifting, wariness, vocalisation	Reluctant to walk but can without problem	Squealing when getting up or walking	Dull: response only when motivated	Lameness in one hind leg
4	Severe: marked head nod, minimal weight bearing	Moderate agitation: shifting, pacing, moves away when approached	Strong response: vocalisation, avoidance behaviour, flinching	Difficulty standing up but can do so without assistance	Squealing while undisturbed or at rest	Unresponsive: even when motivated	Lameness in both hind legs
5	Severe: non weight bearing	Severe agitation: anxious, tense, constant shifting	-	Will not or cannot walk without assistance	-	-	Severe: one leg non weight bearing
6	Unable to move	-	-	-	-	-	Severe: both legs non weight bearing
Total	Scores summated to give a total pain score						-

2.2.2 Identification of porcine leg weakness traits

Leg soundness was investigated by recording observed leg weakness traits. These traits show exterior conformation of the limbs and have been demonstrated to associate with lameness and sow longevity (Jørgensen, 1995; de Sevilla et al., 2008; Le et al., 2016). The traits were identified using a qualitative description of the conformational abnormalities present (see Figure 2.2).

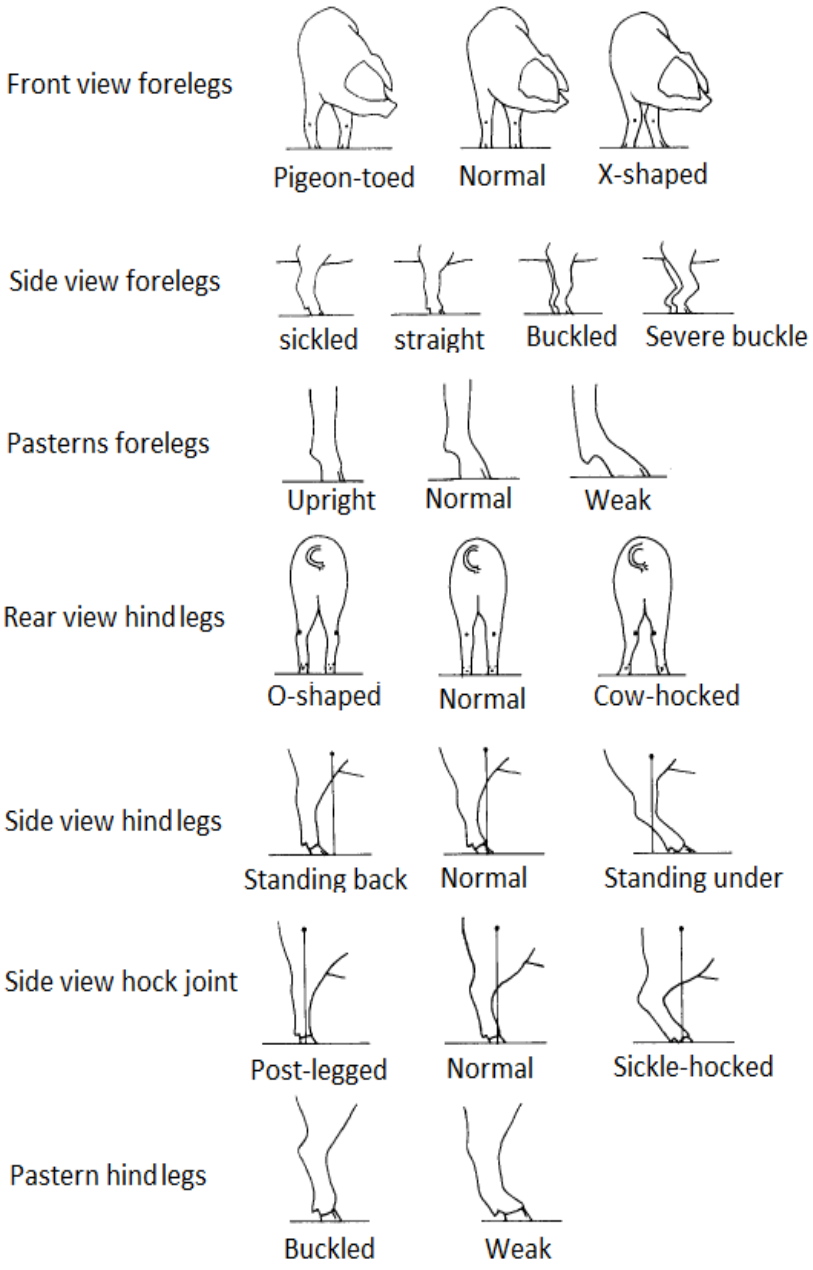


Figure 2.2: Pig leg weakness traits. Taken from Van Steenbergen (1989) and Stavrakakis (2014).

2.3 Tissue collection and processing

2.3.1 Euthanasia

At the end of each study, animals were killed by a licensed slaughterman in accordance with the recommended schedule 1 methods (appropriate methods of humane killing) of ASPA. Animals were rendered unconscious by electrical stunning before exsanguination by severance of the carotid arteries. Death was confirmed by permanent cessation of circulation.

2.3.2 Harvesting knee joint synovial fluid

Hind legs were removed by cutting through the femur above the knee joint. Removal of the entire hind limb allowed easier handling of the knee for tissue collection. The skin and muscle were dissected away until the knee joint capsule was reached. Using a 5 ml syringe and a 1.5-inch, 21-gauge needle the joint capsule was pierced at a point between the patella and femoral condyle and the synovial fluid aspirated. To increase sample collection, if required, a small section of the joint capsule was cut to reveal the joint space and contained synovial fluid, allowing for easier fluid aspiration. The synovial fluid was placed in a sterile cryovial and rapidly frozen over dry ice before storing at -80˚C until use. The average volume of synovial fluid collected per knee was 0.95 ml (range 0.08 ml to 4.5 ml).

2.3.3 Harvesting knee joint synovial tissue

Once the joint capsule was exposed, a sample of synovial membrane could be collected. Due to the thinness of the synovial membrane, a sample was taken containing synovial membrane and some underlying tissue (termed synovial tissue). Using a No. 22 scalpel blade, an incision was made in the joint capsule lateral to the patella and the patellar tendon was severed; this allowed the patella to be reflected outwards to reveal the anterior joint surfaces. Using forceps, the synovial tissue was pinched adjacent to the sampling area, between the patella and femoral condyle, and a scalpel used to dissect out the desired tissue. The tissue was then either placed in a sterile cryovial and rapidly frozen over dry ice (for protein and RNA analysis) or embedded in optimal cutting temperature (OCT) mounting media (for histology). For embedded samples, the tissue was placed in an OCT filled plastic

mould and allowed to equilibrate for a few minutes, before rapidly freezing over dry ice. Once frozen, the blocks were wrapped in Parafilm® and aluminium foil and stored at -80°C until use. For the 42 month old pigs, samples of synovial tissue were only collected into a cryovial and rapidly frozen over dry ice; for histological assessment a section of the frozen tissue was subsequently cut just prior to analysis and embedded in OCT.

2.3.4 Harvesting knee joint cartilage and bone

The tibiofemoral joint was dissected open to allow sampling of the cartilage and underlying subchondral bone. Once the patella had been removed, the femur and tibia were separated. Using a scalpel, the medial and lateral collateral ligaments were severed along with any remaining tissue present at the junction between the two bone surfaces. Flexion was applied to the joint, and using a scalpel (being careful not to damage the articular surfaces) the anterior followed by the posterior cruciate ligaments were severed. This was done superior to the menisci, leaving them attached to the tibial plateau. Applying flexion increased the accessibility of the cruciate ligaments to aid cutting whilst helping to minimise cartilage damage. If the articular surface was inadvertently damaged, this was noted and samples were taken from a different area. Once the femoral condyle was separated, the menisci were removed from the tibial plateau to expose the articular surface.

Photographs of all the articular surfaces were taken for macroscopic pathology assessment (see section 2.4). Samples from medial aspects of the femoral condyles and tibial plateaus were then collected using a hacksaw, taking a sample of approximately 1 cm x 1 cm x 1 cm in size. Samples of cartilage and underlying bone were post-fixed by immersion in 4% paraformaldehyde (PFA, Fisher Scientific) for 48 hours at 4°C. The bone was decalcified in 10% ethylenediaminetetraacetic acid (EDTA, Alfa Aesar), which is a chelating agent that works by capturing the calcium ions found in bone; their removal results in tissue softening enabling it to be thinly sectioned for histology. EDTA was used as it is a less harsh and slower decalcifying agent than other methods, such as formic acid, and has fewer effects on the ability to stain subsequent tissue sections (Sanjai et al., 2012). Decalcification occurred at

room temperature for 8 – 16 weeks (depending on sample size and animal age) whilst on a shaker to gently agitate the samples, with EDTA replaced weekly. For all pigs, a section of the medial femoral condyle and medial tibial plateau were obtained from both knee joints, with the exception of 42 month old pigs where a sample was taken from the medial femoral condyle of one knee only, and one 9 month old pig, where the tibial plateau sample from one knee was lost.

2.3.5 Spinal cord and DRG isolation

Following removal of the hind limbs, the lumbar regions L3 - L5 of the spinal cord and DRGs were isolated. Firstly, an incision was made in the musculature along the spinal column, close to the dorsal spinous processes and extending the length of the lumbar region. The start of the lumbar region was identified by the location of the last rib. The muscle and tissue surrounding the spinal processes were removed until the spinal vertebrae became clearly visible. The vertebrae then required removal to allow access to the spinal cord and DRGs. An electric saw (Bosch multi-tool) was used to cut through the thinner layer of bone between the dorsal spinous process and transverse processes of each vertebra, and then the entire dorsal aspect of the vertebra was removed, exposing the spinal cord. To remove the spinal cord and DRGs, an incision in the spinal cord was made distal to the L5 region. The cord was picked up using forceps and gently pulled away from the spinal column, and a scalpel used to detach the nerve roots distal to the DRGs. This allowed the spinal cord and DRGs to be removed as one intact piece. The tissue was then placed in a petri-dish of phosphate buffered saline (PBS, Sigma-Aldrich), the dura was removed and the DRGs were dissected free from the spinal cord. Spinal cords and DRGs were post-fixed by immersion in 4% PFA overnight at 4°C and cryoprotected in 30% sucrose solution with 0.02% sodium azide (Sigma-Aldrich). To identify the orientation of spinal cord sections, the left side was marked using a needle.

2.4 Macroscopic scoring of knee joint pathology

To assess for the presence and severity of joint disease, the knee articular surfaces were scored macroscopically for the presence of OC or OA using a photographic scoring system. Briefly, once the knee joints were dissected open (and prior to

cartilage collection), all the articular surfaces (femoral condyle, tibial plateau and patella) were photographed using a Panasonic Lumix digital camera. At the same time, a scoring sheet was annotated noting the animal identification number, joint orientation and indicating any areas of damage (see Figure 2.3). This scoring sheet then acted as an *aide-memoire* when scoring the photographs but the scorer was blinded to animal age and identification number. Images were analysed using ImageJ® to determine the presence and severity of OC or OA. Initially, all joints from every age group were assessed for both OC and OA; however, OC was only clinically relevant in 9 and 15 month old pigs and OA in 24 and 42 month old pigs.

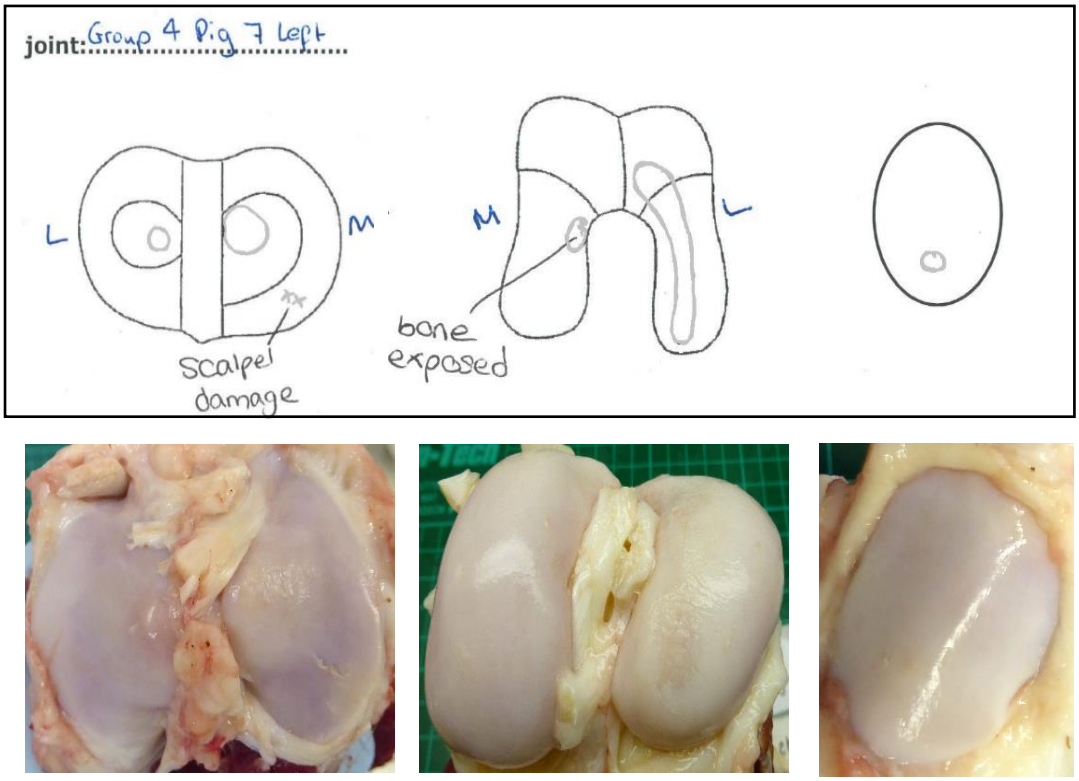


Figure 2.3: Example joint photographs and corresponding annotated scoring sheet used to assess lesions in the porcine knee. Photographs and scoring sheet from a 24 month old pig's left knee. L lateral, M medial.

2.4.1 Macroscopic scoring of osteochondrosis

The presence of OC in skeletally immature 9 and 15 month old pig knee joints was assessed using the method of van Grevenhof et al. (2011), which is adapted from the method of Van Weeren and Barneveld (1999) used to assess equine OC. Each

articular surface was graded for the extent of OC (see Table 2.2), with the highest scoring articular surface being taken as the score for the entire joint.

Table 2.2: Macroscopic scoring of osteochondrosis. Scoring system taken from Van Weeren and Barneveld (1999) and van Grevenhof et al. (2011).

Grade	Articular surface appearance
0	Normal cartilage
1	Flattening of the articular cartilage
2	Slight irregularity of the cartilage
3	Severe irregularity of the cartilage
4	Classic OCD lesion with osteochondral fragment or cyst formation

2.4.2 Macroscopic scoring of osteoarthritis

The presence of knee OA in skeletally mature 24 and 42 month old pigs was assessed using the photographic scoring system used by Walsh et al. (2009) to assess human OA. Lesions of the articular surface were graded based on the extent of changes in the cartilage surface integrity (see Table 2.3).

Table 2.3: Macroscopic scoring of osteoarthritis. Grading cartilage surface integrity, taken from Walsh et al. (2009).

Grade	Articular surface appearance
0	Normal cartilage – smooth, unbroken surface and a homogenous white to off-white colour
1	Swelling and softening of the cartilage, a little brown homogenous colouration
2	Superficial fibrillation, lightly broken surface and white to off-white/light brown in colour
3	Deep fibrillation – coarsely broken cartilage surface, dark brown, grey or red in colour
4	Subchondral bone exposure – stifled white and dark brown/red in colour

Using ImageJ®, the percentage of each articular surface area associated with each grade of surface integrity was calculated. The revised Société Française D'Arthroscopie (rSFA) scoring system was then applied to give a score for the severity of OA present (Ayral et al., 1994; Pessis et al., 2003; Walsh et al., 2009). The scoring system takes into account the type, depth and size of lesions present and calculates a score based on the percentage of each articular surface integrity grade present (see Equation 1).

$$\textit{Equation 1}: \boldsymbol{revised\ SFA\ score\ calculation\ for\ each\ articular\ surface}$$
$$\boldsymbol{score} = (\%\ \boldsymbol{Grade\ 1} \times \mathbf{0.14}) + (\%\ \boldsymbol{Grade\ 2} \times \mathbf{0.34}) + (\%\ \boldsymbol{Grade\ 3} \times \mathbf{0.65}) + (\%\ \boldsymbol{Grade\ 4})$$

The rSFA score has a possible range of 0 - 100 for each articular surface, with a higher score indicating worse disease severity. The scores for each surface (the medial and lateral femoral condyles, medial and lateral tibial plateau and patella) were summated to give a total joint score with a possible range of 0 - 500.

2.4.3 Macroscopic scoring of osteophytes

The photographs were also scored for the development of osteophytes using the Osteoarthritis Research Society International (OARSI) recommended histological assessment of OA in sheep and goats (Little et al., 2010). For osteophyte development, score 0 indicated no osteophytes present. Score 1 indicated mild osteophytes with less than 2 mm outgrowth. Score 2 indicated moderate osteophytes with 2-4 mm outgrowth. Score 3 indicated large osteophytes with more than 4 mm outgrowth.

2.5 Microscopic histopathology of knee cartilage and subchondral bone

2.5.1 Processing and sectioning of knee cartilage and bone samples

Once decalcified, the bone samples were cut using a scalpel so they fitted in plastic cassettes, then underwent a routine histological technique to embed them in paraffin wax. Briefly, the bone filled cassettes were placed in an automated tissue processor (Leica TP1020) and underwent a series of steps of dehydrating in graded

industrial methylated spirit (IMS, Fischer Scientific) and clearing in histoclear® (National Diagnostics) before impregnating with molten paraffin wax (Fischer Scientific). The final stage of paraffin impregnation was to set the tissue in blocks of paraffin by placing it in paraffin-filled metal moulds and leaving to cool until the wax had solidified. Sections of 7 µm thickness were collected onto SuperFrost™ Plus slides (Thermo Fischer Scientific) using a rotary microtome (Sigma-Aldrich Microm HM355) for histology and osteoclast staining.

2.5.2 Haematoxylin and eosin staining of cartilage and bone

Sections of cartilage and bone from the knee joints were stained with haematoxylin and eosin (H&E) to assess cartilage thickness and tissue morphology. Haematoxylin is a basic dye and binds to acidic structures within the sample, such as nucleic acid within the nucleus, staining them blue or black. Eosin is an acid dye which binds to basic structures within the sample, such as the cytoplasm, staining them shades of pink (King and King, 1986).

Tissue sections were deparaffinised in histoclear® and rehydrated in graded IMS (100%, 95% and 50%), before washing in water twice for 5 minutes each. Nuclear staining was achieved by immersing sections in haematoxylin (VWR International) for 5 minutes, before differentiating the stain by rinsing in running tap water, dipping in acid alcohol for 10 seconds (70% IMS with 0.5% hydrochloric acid) and immersing in tap water again for 3 minutes. The other tissue structures were stained by immersion in eosin (made in-house) for 5 minutes. Sections were then dehydrated in graded alcohol and cleared with histoclear® (to remove dehydrants and alcohol) before coverslipping with Omnimount® (National Diagnostics).

Images of the stained cartilage were visualised using a Leica MX16 light microscope (x1.6 objective lens) or an Olympus BHS B2 light microscope (x4 and x10 objective lens). Images were acquired with a Leica DFC320 camera and LAS v4.2 software. At least six sections per articular surface were analysed for cartilage thickness and integrity (microscopic scoring of OA and OC histopathology). The scorer was blinded to animal age, identification number and joint location.

2.5.3 Cartilage thickness

Cartilage thickness was measured using ImageJ® and the 'Distance between Polylines' plugin. This allowed a semi-automated analysis of cartilage thickness by measuring the average cartilage thickness (in μm) for a given section.

2.5.4 Microscopic assessment of the osteochondrosis histopathology

Assessment of OC at a microscopic level was carried out on H&E stained sections of cartilage and bone from 9 and 15 month old pigs. Scoring was carried out according to the method of Van Weeren and Barneveld (1999). Each section was scored on a scale of 0 - 4 (see Table 2.4) and the highest scoring section taken as the score of that particular joint surface.

Table 2.4: Microscopic scoring of cartilage and bone for osteochondrosis. Scoring system taken from Van Weeren and Barneveld (1999).

Score	Lesion characterisation
0	Normal
1	Locally thicker cartilage: indentation of ossification front
2	Slight OC indication: loss of normal columnar arrangement of chondrocytes
3	Clear OC: fissures and necrosis but articular cartilage still intact
4	Clear OC: fissures and necrosis, articular cartilage no longer intact and includes OC fragment and cysts

2.5.5 Microscopic assessment of the osteoarthritis histopathology

Assessment of OA at a microscopic level was carried out on H&E stained sections of cartilage and bone from 24 and 42 month old pigs. Scoring was carried out based on a modified version of the OARSI histopathology scoring system for the cartilage of sheep and goats (Little et al., 2010), which is a version of the Mankin scoring system (Mankin et al., 1971) for grading osteoarthritic cartilage (see Table 2.5). The scoring system assessed three factors (cartilage structure, chondrocyte density and formation of cell clones), and the scores summated to give a total OA score. The

highest scoring section of each articular surface was then taken as the score for that joint surface.

Table 2.5: Microscopic scoring of cartilage and subchondral bone for osteoarthritis. Scoring system adapted from Little et al., (2010).

Lesion characterisation	
Cartilage structure	
0	Normal
1	Slight surface irregularities (surface barely disturbed)
2	Moderate surface irregularities (surface roughened)
3	Severe surface irregularities (disruption, fissuring/fibrillation to < 10% depth)
4	Fissures to transitional zone (1/3 depth)
5	Fissures to radial zone (2/3 depth)
6	Fissures to calcified zone (full depth)
7	Erosion or severe fibrillation to mid zone (1/3 depth)
8	Erosion or severe fibrillation to deep zone (2/3 depth)
9	Erosion or severe fibrillation to calcified zone (full depth)
10	Erosion or severe fibrillation to subchondral bone
Cell cloning	
0	Normal
1	Several doublets
2	Many doublets
3	Doublets and triplets
4	Multiple cell nests or no cells in section
Chondrocyte density	
0	Normal
1	Increase or slight decrease
2	Moderate decrease
3	Severe decrease
4	No cells

2.6 Tartrate resistant acid phosphatase staining of osteoclasts

Sections of cartilage and bone from the knee were stained for tartrate resistant acid phosphatase (TRAP), a histochemical marker for osteoclasts. TRAP is an enzyme expressed by a number of cells such as osteoclasts, activated macrophages and some neuronal cells (Sells Galvin et al., 1996; Sagar et al., 2013). Commercially available TRAP test kits have been developed to detect TRAP in osteoclasts. At least 6 sections of 7 µm thick paraffin embedded bone per articular surface were stained for TRAP activity.

Sections were first deparaffinised in histoclear® twice for 5 minutes before rehydrating in graded alcohol (100% and 70% IMS, 3 minutes each) and washing in distilled water twice for 5 minutes. Sections were then incubated overnight with a recalcifying buffer containing 1 mM calcium chloride (FSA Laboratory Supplies) and 1 mM magnesium chloride (Sigma-Aldrich) in PBS, before rinsing in distilled water for 5 minutes. The TRAP stain was then applied directly onto each section after filtering with a 0.45 µm MiniSart filter (Sigma-Aldrich). Sections were incubated with the stain in a humidified chamber for 1 hour at 37^{o}C. The TRAP stain contained 2 ml acetate solution, 2 ml Naphthol AS-BI phosphoric acid, 2 ml tartrate solution and 1 capsule of Fast Garnet GBC salt, all dissolved in 44 ml of distilled water at 37°C (all from Sigma-Aldrich TRAP staining kit #387A). Following incubation, the sections were rinsed in distilled water for 5 minutes and counterstained with acid haematoxylin for 5 minutes. Sections were rinsed again in distilled water for 5 minutes before coverslipping with a 1:1 ratio of PBS and glycerol (Sigma-Aldrich). Varnish was applied around the coverslip to seal the edges. Images of the stained bone were visualised using an Olympus BHS B2 light microscope (x4, x10 and x20 objective lens). The images were acquired with a Leica DFC320 camera and LAS v4.2 software.

2.6.1 Osteoclast counting

Osteoclasts were counted at 10x magnification. The scorer was blinded to animal age, identification number and joint location. Positively stained osteoclasts were found in dark purplish stained areas and were counted if they contained 3 or more

nuclei. In the event that the stained area was too dark to identify the number of nuclei (due to haematoxylin overstaining), the size of the area was compared to an adjacent negatively stained area, and if it was twice as large as the adjacent cells, it was counted as positive. Areas of nonspecific haematoxylin staining, resulting from the extended period of decalcification, were ignored. Osteoclast count was expressed per mm^2 of tissue.

2.7 Microscopic histopathology of knee synovial tissue

2.7.1 Haematoxylin and eosin staining of synovial tissue

Serial sections of frozen unfixed synovial tissue were cut at 7 µm using a cryostat (Scilogex OTF5000-004) and every 10^{th} section collected onto SuperFrost Plus™ slides. At least 20 sections of synovial tissue per joint were routinely stained with H&E. The frozen sections were left to air dry for 30 minutes before staining to improve tissue adhesion to the slide. The sections were fixed in cold acetone (VWR International) for 10 minutes, before washing in PBS and distilled water for 5 minutes each. Sections were then stained with haematoxylin for 5 minutes, before rinsing in running tap water and staining with eosin for 1 minute, and then rinsed again in water. Sections were dehydrated in graded alcohol (70% and 100% IMS) and cleared with histoclear® before coverslipping with omnimount®. Images of the stained synovial tissue were visualised using an Olympus BHS B2 light microscope (x10 and x20 objective lens). The images were acquired with a Leica DFC320 camera and LAS v4.2 software (Leica Microsystems).

2.7.2 Assessment of synovitis

Severity of synovitis in the synovial tissue was scored according to the presence of hyperplasia - the enlargement and increased cellularity of the synovial membrane layer. Tissue sections were scored on a scale of 0 - 3 according to the methods of Mapp et al. (2008) and Cruz et al. (2015) (see Table 2.6). The scorer was blinded to animal age, identification number and joint location.

Table 2.6: Synovitis grading criteria. Scoring system taken from Mapp et al. (2008) and Cruz et al. (2015).

Score	Thickening of synovial membrane
0	Absent – lining cell layer 1 - 2 cells thick
1	Slight enlargement – lining cell layer 3 - 5 cells thick
2	Moderate enlargement – lining cell layer 6 - 8 cells thick
3	Strong enlargement – lining cell layer > 9 cells thick

2.8 Synovial tissue RNA expression

The synovial tissue messenger RNA (mRNA) expression of cytokines (IL-6, IL-1β, and TNFα), matrix metalloproteinases (MMP1, MMP3 and MMP9) and tissue inhibitors of MMPs (TIMP1 and TIMP3) were determined using SYBR green real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). RNA was extracted from homogenised samples, transcribed into complementary DNA (cDNA) and then expression determined using qPCR.

2.8.1 RNA extraction

Before use, all equipment was cleaned with RNase Zap and with ethanol between biological samples. RNA was extracted from unfixed, frozen samples of pig synovial tissue by homogenising 20 mg of tissue with 400 µl of Buffer RLT Plus (Qiagen) containing 1% β-mercaptoethanol (Sigma-Aldrich). Once homogenised, the tissue lysates were centrifuged for 3 minutes at 14000g and the supernatant collected. RNA was extracted from the supernatant using the Qiagen QIAsymphony and the 'fibrous tissue 400' protocol. The QIAsymphony allows automated RNA extraction with a silica-based RNA purification technique using magnetic separation. Tissue lysates went through a process of lysis and digestion with proteinase K followed by magnetic separation; the resulting RNA was eluted into RNase-free water ready for use. Purity of extracted RNA was determined with a Nanodrop® 1000 (Thermo Fischer Scientific) before samples were frozen at -80°C until use.

2.8.2 cDNA synthesis by reverse transcription

Synovial tissue RNA was reverse transcribed into cDNA using the Promega GoScript™ Reverse transcription system (A5000) according to the manufacturer's instructions. Briefly, 4 µl of RNA was incubated with 1 µl random primers at 70°C for 5 minutes in a heat block, before immediately chilling over ice for 5 minutes. The reverse transcription mix was then prepared; each reaction contained 4 µl GoScript reaction buffer, 2 µl magnesium chloride, 1 µl dNTP mix (deoxyribonucleotide triphosphate mix), 0.5 µl recombinant RNasin® ribonuclease inhibitor, 1 µl GoScript reverse transcriptase and 6.5 µl nuclease-free water. 15 µl of this reverse transcription mix was added to the RNA/primer mix and allowed to anneal by heating to 25°C for 5 minutes. The reaction was then incubated at 42°C for 1 hour before heating to 70°C for 15 minutes to inactivate the reverse transcriptase, terminating the reaction. The cDNA was stored at 4°C until qPCR was performed. A negative control was conducted by incubating a pooled RNA sample with the reverse transcription reaction mix without the reverse transcriptase enzyme (noRT sample).

2.8.3 Primer design and cDNA dilutions

Primer sequences for the housekeeping genes actin beta (ACTB) and Peptidylprolyl isomerase A (PPIA) were sourced from published papers (see Table 2.7). Primers for the genes of interest; IL-6, IL-1β, TNFα, MMPs and TIMPs were designed in-house. These primers were designed using Genebank® to identify the target gene sequence and Primerblast® to design the primer sequence. IDT oligoanalyser® was then used to test if the primers contained hairpins or would form self/heterodimers. All primers were purchased from Integrated DNA Technologies. The primers were tested on pooled cDNA from a number of different synovial tissue samples. A standard curve, of 2.5-fold serial dilutions, was prepared and run. The primer sequences, efficiencies, cycle threshold (Ct) range, melt temperatures and product size are found in Table 2.7.

Table 2.7: List of primers used in qPCR studies.

Target	Reference	Primer strand	Primer sequence 5'-3'	Tm	Efficiency (%)	C_t Range	Product length (bp)
ACTB	Park et al., (2015)	F R	TCT GGC ACC ACA CCT TCT TGA TCT GGG TCA TCT CAC	56 53	88.4	19-26	114
PPIA	Uddin et al., (2011)	F R	CAC AAA CGG TTC CCA GTT TT TGT CCA CAG TCA GCA ATG GT	54 57	88.2	20-26	171
IL-6	-	F R	TCT GGG TTC AAT CAG GAG ACC T AAT CTG CAC AGC CTC GAC AT	57 57	112.3	27-33	126
IL-1β	-	F R	TGA CTT TGT CTG TGA TGC CAA GCC AGC CAG TAG AGA TT	54 57	105.4	30-36	82
TNFα	-	F R	GTA GCC AAT GTC AAA GCA GA TTG TCT TTC AGC TTC ACG CC	53 56	93.3	29-36	89
MMP1	-	F R	TGA TGG ACC TGG AGG AAA CC TTG GTC CAC CTG TCA TCT TC	55 50	95.2	16-39	92
MMP3	-	F R	TAA CAC TCT GGA GGT GAT GC TGA AGT CTC CGT GTT CTC G	56 56	124.3	13-37	272
MMP9	-	F R	ATG TGA TTG ACG CCT TT ATC TCC GTG CTC CTT AAC AC	58 57	112.7	27-40*	128
TIMP1	-	F R	TTC ACC GAG ATC TAT GCT GC CCA CAA GCA GTG AGT GTCA	57 57	88.4	20-29	177
TIMP3	-	F R	ACT TTG TGG AGA GAT GGG AAG CAA GGC AGG TAG	53 53	88.6	18-29	112

Ct – cycle threshold, Tm – melting temperature, bp – base pairs, F - forward, R- reverse, 40* - No Ct value obtained for some samples

2.8.4 SYBR Green quantitative real-time qPCR

RNA expression was quantified using SYRB Green qPCR. The cDNA samples were diluted 1:10 in nuclease-free water (NFwater, Qiagen) and run in triplicate in a 96 well-plate. 5 µl of cDNA and 15 µl of qPCR master mix was pipetted into each well. Per well, the qPCR master mix contained 10 µl qPCR mastermix (Agilent Brilliant III Ultrafast SYBR Green qPCR Mastermix), 1 µl of 200 nM primer stock solution, 0.3 µl ROX reference dye (diluted 1:500 in NFwater) and 3.7 µl NFwater. The plate was spun and bubbles removed before sealing. The qPCR was run using a Mx3005P qPCR system (Aglient Technologies). The reaction was activated by heating to 95°C for 3 minutes followed by 40 cycles of heating to 95°C for 5 seconds and cooling to 60°C for 20 seconds. Results were analysed using mxPro qPCR software (Aglient Technologies). A 'no template control' (NTC) was carried out by running the qPCR master mix without the presence of cDNA to test for contamination and primer-dimer formation. A no reverse transcriptase control was run by running the noRT sample to monitor genomic DNA contamination.

2.8.5 Analysis of qPCR data

The C_t values were obtained and the relative quantity of each gene of interest (cytokines, MMPs and TIMPs) was expressed relative to the mean expression of the two housekeeping genes (ACTB and PPIA). This controlled for variations in the initial sample size and RNA starting concentration. The results were analysed using the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). This indicates the fold changes in gene expression for a given sample or for a particular group of animals, such as animals with mild OC compared to healthy animals (see Equation 2 and Equation 3). Statistical analysis was conducted using $2^{-\Delta Ct}$ values. Due to the small sample sizes, data were also assessed using $2^{-\Delta\Delta Ct}$ values (without statistical tests) to indicate mean fold change in gene expression for a given group of animals.

Equation 2: Calculating $2^{-\Delta Ct}$

$$2^{-\Delta Ct} = 2^{-(Ct\ Gene\ of\ Interest\ -mean\ Ct\ ACTB\ and\ PPIA)}$$

Equation 3: Calculating $2^{-\Delta\Delta Ct}$

$$2^{-\Delta\Delta Ct} = 2^{-(Average\ \Delta Ct\ Diseased\ -\ Average\ \Delta Ct\ healthy\ animals)}$$

2.9 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences v24 (SPSS Inc., Chicago, USA) and GraphPad Prism v7.03 (Graph Pad, San Diego, USA). Details of the specific statistical tests used to assess significance can be found in the relevant results chapters.

Chapter 3

Characterising Pain Behaviours and Pathological Features of Osteochondrosis in the Porcine Knee Joint

3.1 Introduction

Lameness is estimated to affect at least 20% of commercial pigs and is a major cause of premature culling of breeding sows (Ryan et al., 2010). This not only raises animal welfare concerns but also has economic implications (see section 1.2). The most prominent cause of lameness in growing swine is OC, which is reported to affect between 40% and 100% of pigs (Kirk et al., 2005; Crenshaw, 2006; Ryan et al., 2010; van Grevenhof et al., 2011; Tóth et al., 2016). The disease can cause joint pain, effusion, swelling and a decreased range of motion, resulting in the onset of lameness (McCoy et al., 2013). OC also affects humans and many other animal species, including horses, cows, dogs, cats and rats (Ytrehus et al., 2007). Despite the high prevalence, the disease's aetiology is still unclear, but advances have been made in understanding some of the cellular events involved (Crenshaw, 2006).

OC results from a focal disturbance in endochondral ossification (see section 1.6), the process of cartilage replacement by growing bone (Crenshaw, 2006; McCoy et al., 2013). The current consensus is that the disturbance results from a failure in blood supply but it is unclear why this disturbance occurs (Ytrehus et al., 2007). The loss of blood supply causes ischaemic necrosis, altering chondrocytes and the collagen matrix, which weakens cartilage and disrupts ossification (Wardale and Duance, 1994; Laverty and Girard, 2013). This creates a focal thickening of the cartilage and can progress into fissures in the cartilage and subchondral bone (Crenshaw, 2006).

Although lameness is a common feature of OC, particularly when severe, the disease is often subclinical. OC and other joint diseases are often identified in pigs showing no lameness, and as such, lameness is not always a good diagnostic tool for identifying joint problems (Dewey et al., 1993; Nakano and Aherne, 1993). Similarly, studies have struggled to identify clear leg weakness traits and behavioural changes associated with OC (Jørgensen, 1995; Etterlin et al., 2015). The bilateral nature of joint diseases also makes identification of lameness difficult, as it can be harder to identify gait changes when an animal is lame on more than one leg, which may mask the association between disease and lameness (Etterlin et al., 2015). This has

led to the hypothesis that pigs are required to reach a threshold of joint disease, in either severity or location, before clinical symptoms present (Jørgensen, 1995). However, the nature of this threshold has not been identified. This difficulty in identifying OC before slaughter, coupled with the OC lesions' ability to repair, makes studying the disease difficult.

A number of advances have been made using both radiographs and computerised tomography (CT) to detect OC in live animals for longitudinal disease studies. Radiographs show good correlation to lesions identified histologically but are poor at identifying early lesion formation (Jørgensen et al., 1995; Bertholle et al., 2016). CT scans are able to identify areas of ischemic cartilage that indicate vascular failure and the beginning of OC (Olstad et al., 2014; Olstad et al., 2015). However, both these techniques are expensive and require animal sedation, so are not practical for the majority of studies. Therefore, most studies use post-mortem assessment to identify the disease by histological and macroscopic assessments. Histological examination is considered by many as the gold standard of assessment, identifying changes in cartilage and the advancing ossification front, and is often used in conjunction with macroscopic assessment of joint surfaces. It has been shown that over 77% of OC lesions identified histologically also result in changes to the joint's surface, which can be identified macroscopically (Busch and Wachmann, 2011). However, despite the majority of studies using macroscopic OC assessments, there is no consensus on the most appropriate scoring method to use, with each research group using a different method (Reiland, 1978; Kirk et al., 2005; Scott et al., 2006; Ryan et al., 2010; van Grevenhof et al., 2011). These scoring systems often use different criteria to classify lesions and do not always assess for the same lesion types.

3.1.1 Aim

The aim of the work presented in this chapter was to characterise pain behaviours associated with pathological features of knee osteochondrosis in commercial pigs. Knee joints were assessed macroscopically for OC to identify the disease's prevalence and pathological features in 9 and 15 month old pigs, an age group

which has not typically been assessed for OC previously. The association between pain behaviour, macroscopic OC pathology and histological features of OC were then examined. A comparison of different macroscopic OC scoring methods was also conducted. The pain behaviour scoring system was also assessed for reproducibility by analysing the inter-observer reliability.

3.2 Methods

3.2.1 Animals

Two age cohorts of clinically healthy female commercial pigs of the crossbred genotype 'Landrace x Large White x Duroc' were used in this study. Pigs were aged 9 months (n = 6) with an average weight of 84 ± 2.21 kg and 15 months (n = 6) with an average weight of 141.2 ± 4.20 kg.

Pigs were assessed for 5-7 days prior to slaughter, for pain behaviours and lameness (see section 2.2). Both knee joints were assessed macroscopically post-mortem (see section 2.4). In pigs showing OC but normal gait, the disease was termed asymptomatic, where as in pigs showing OC and lameness, the disease was termed symptomatic. Sections of cartilage and bone from the medial femoral condyles and tibial plateaus were stained with H&E to assess cartilage thickness and microscopic histopathology (see section 2.5), or stained with TRAP to identify osteoclast (see section 2.6).

3.2.2 Macroscopic osteochondrosis scoring of porcine knee joints

Knee joints were assessed for OC, by a single observer, using four macroscopic classification systems previously reported in pig studies (methods of Scott et al., 2006, Kirk et al., 2008, Ryan et al., 2010 and Van Grevenhof et al., 2011). This allowed a comparison of different macroscopic OC scoring methods and the most appropriate method for identifying OC could be selected. Details of the Van Grevenhof method can be found in section 2.4.1.

Using the method of Scott et al. (2006), each joint surface was subjectively scored on a scale of 0 - 4. Score 0 indicated normal healthy cartilage and score 1 indicated thinning of the articular cartilage. Score 2 - 3 indicated the presence of cartilage irregularities such as fissures, ridges or clefts. Score 4 indicated extensive cartilage erosion, ulceration or the formation of cartilage flaps.

Using the method of Kirk et al. (2008), each joint surface was assessed for the presence of: i) infolding of the cartilage and ii) cartilage repair reactions. The joint

was scored as normal (score 0) when no lesion was present, moderate (score 1) when the particular lesion involved less than 20% of the articular surface area, or severe (score 2) when the lesion involved more than 20% of the articular surface area.

Using the method of Ryan et al. (2010), joint surfaces were subjectively scored on a scale of 1 - 7. Score 1 indicated normal cartilage free from abnormalities. Scores 2 - 4 indicated a slight to mild disturbed endochondral ossification with cartilage thickening and/or cartilage grooves. Scores 5 - 7 indicated moderate to severe degrees of disturbed endochondral ossification with fractures or separation of cartilage and/or subchondral bone collapse or fibrosis.

3.2.3 Statistical analysis

Data were tested for normality using the D'Agostino-Pearson normality test. Parametric data were analysed using two-way ANOVA with Sidak's or Tukey's multiple comparisons post-hoc tests. Non-parametric data were analysed with Mann-Whitney tests or Kruskal-Wallis tests with Dunn's multiple comparisons post-hoc test. Paired non-parametric data were analysed using a Wilcoxon test. Correlation between data was determined using Pearson's or Spearman's Rank Correlation. A significance level of $p < 0.05$ was used for all statistical tests.

Inter-observer and intra-observer agreements were estimated by intra-class correlation coefficients (ICC); intra-rater analysis used a one-way random single measures and inter-rater analysis used a two-way mixed model absolute agreement. Reliability was regarded as excellent if ICC > 0.9, good if ICC 0.6 – 0.9 and poor if ICC < 0.6 (Bruton et al., 2000).

3.3 Results

3.3.1 Comparison of macroscopic osteochondrosis scoring techniques

There was a positive correlation between macroscopic OC scores derived using the van Grevenhof method and the Scott method (see Table 3.1). Scores derived using the Kirk and Ryan methods did not correlate with the van Grevenhof scores. Furthermore, scores from Ryan, Kirk and Scott methods did not correlate with one another. However, the methods of Ryan, Scott and van Grevenhof did agree on the same joints being healthy and showing the most severe OC. Severity of OC identified macroscopically did not correlate with severity identified using histology for any of the macroscopic scoring techniques.

Table 3.1: Association of results produced by different methods of macroscopic osteochondrosis severity assessment. Correlation of macroscopic OC scores derived using the method of van Grevenhof et al. (2011) and Scott et al. (2006), Kirk et al. (2008) and Ryan et al. (2010). Association expressed as Spearman's Rank r^2 Correlation Coefficients (*p < 0.05, ****p < 0.0001).

	van Grevenhof
Scott	0.859****
Kirk - Infolding	0.169
Kirk - Repair	0.474*
Ryan	0.079

A positive correlation was also observed between macroscopic OC scores, derived using the van Grevenhof method, from the same observer ($r^2 = 0.9182$, $p < 0.0001$, Spearman's Rank Correlation), and inter-rater reliability analysis displayed good intra-observer reliability with an excellent ICC score (0.9130, n = 24). As the scoring method of Van Grevenhof appeared the most OC specific and sensitive method for identifying OC, and showed the strongest correlation to other methods, scores from the Van Grevenhof method were subsequently used to indicate OC severity.

3.3.2 Macroscopic osteochondrosis of porcine knee joints

Macroscopic examination of porcine knees identified evidence of OC in 22 out of the 24 knee joints (91%), with flattening of cartilage present in 9 joints. Lesions consistent with OA and osteophytes were not identified in any joints of the 9 and 15 month old pigs. Disturbances in endochondral ossification were evident in a number of joints but the cartilage surface was still intact, consistent with an *osteochondrosis latens or manifesta* pathology (see section 1.6.1). Cartilage fissures or cartilage infolding was present in 50% of joints (see Figure 3.1 for examples). However, all OC lesions were relatively mild with no OCD or loose fragments of cartilage identified.

There was no significant difference in total knee joint macroscopic OC severity, determined using the van Grevenhof et al. (2011) method, between 9 and 15 month old pigs ($p = 0.5736$, Mann-Whitney test). OC presented bilaterally in most cases, with no difference in the OC severity of left and right knee joints ($p = 0.6133$, Wilcoxon test).

However, a difference in OC severity between articular surfaces was noted in both 9 and 15 month old pigs ($p = 0.0150$ and $p < 0.0001$ respectively, Kruskal-Wallis tests). In 9 month old pigs, the patella consistently had less severe OC than medial femoral condyles and lateral tibial plateaus ($p = 0.0384$ and $p = 0.0282$ respectively, Dunn's multiple comparisons), but there was no significant difference between femoral condyles and tibial plateaus (see Figure 3.2A). In 15 month old pigs, the patella consistently had less severe OC than medial and lateral femoral condyles and lateral tibial plateaus ($p = 0.0033$, $p < 0.0001$ and $p = 0.00272$ respectively, Dunn's multiple comparisons), but there was no significant difference between femoral condyles and tibial plateaus (see Figure 3.2B).

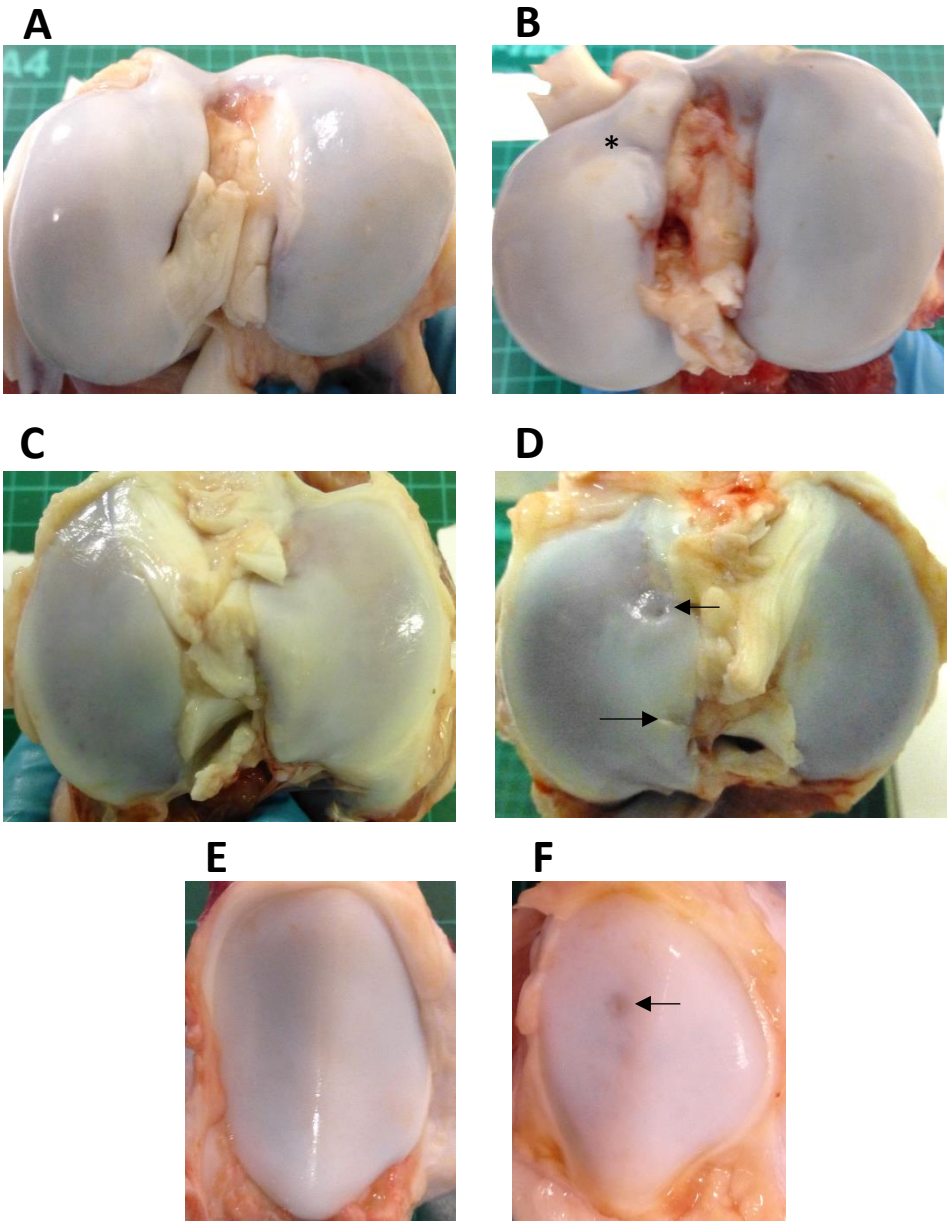


Figure 3.1: Photographs of healthy and diseased pig knee joints. Representative photographs of the femoral condyle (A & B), tibial plateau (C & D) and patella surfaces (E & F). Healthy joints (A, C & E) showed thick cartilage free from fissures or areas of necrosis. OC was identified in the majority of joints (B, D, F) with features such as disturbed ossification (*) and areas of infolded cartilage and necrotic cartilage (arrow).

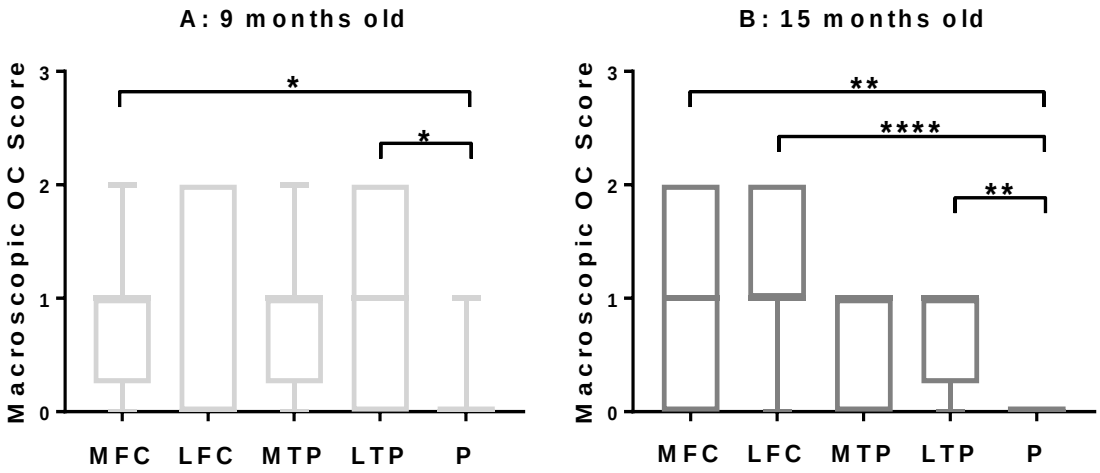


Figure 3.2: Macroscopic osteochondrosis severity of knee joint articular surfaces in two age groups of pigs. MFC medial femoral condyle, LFC lateral femoral condyle, MTP medial tibial plateau, LTP lateral tibial plateau, P patella. Macroscopic OC in 9 month old (A) and 15 month old (B) pigs. *p < 0.05, ** p < 0.001, **** p < 0.0001, Kruskal-Wallis tests with Dunn's multiple comparisons. Box plot with whiskers showing the minimum and maximum scores and a median line.

3.3.3 Histological examination of osteochondrosis in the porcine knee

Histological examination of cartilage and subchondral bone identified a number of changes in microscopic pathology consistent with OC. Indentation in the advancing ossification front and loss of the columnar arrangement of chondrocytes were seen in a number of sections (see Figure 3.3). Defects within the cartilage, indicating necrotic areas, were also present but the articular cartilage surfaces were still intact. Some joints showed an infolding in the cartilage being filled by fibrous tissue (see Figure 3.3D), consistent with an *osteochondrosis manifesta* pathology.

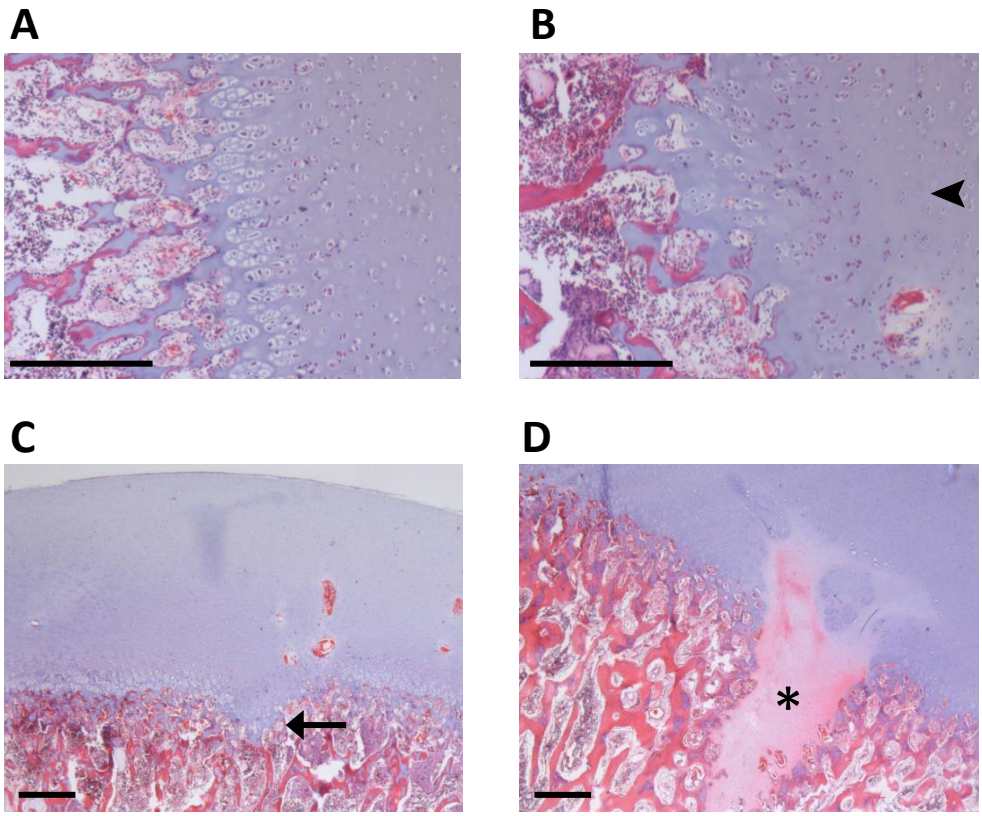


Figure 3.3: Microscopic histopathology of porcine knee joint articular cartilage. Representative images of H&E stained femoral condyle cartilage from 9 month old pigs. Healthy joints showed smooth cartilage with normal chondrocyte arrangement (A). OC caused a loss of normal chondrocyte arrangement (B, ➤), infolding of retained cartilage (C, arrow) and fibrous tissue filling of defects within the cartilage and bone (D, *). Images taken at 4x magnification (A & B) and 1.6x magnification (C & D), scale bars 500 µm.

There was no significant difference in the OC histopathology scores of different age pigs or different articular surfaces (p = 0.2022, Kruskal-Wallis test, see Figure 3.4).

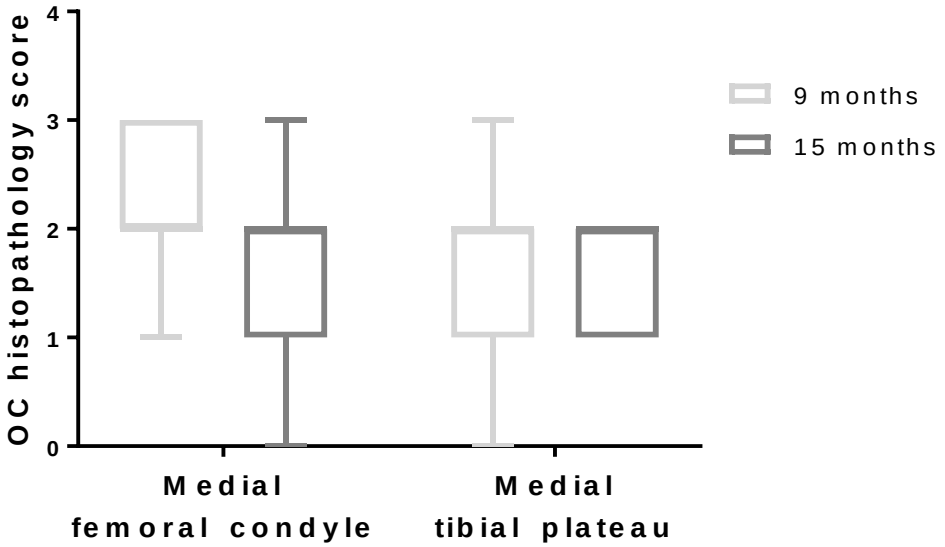


Figure 3.4: Microscopic osteochondrosis histopathology of knee joint articular surfaces in two age groups of pigs. Box plot with whiskers showing the minimum and maximum scores.

Across all pigs, the mean cartilage thickness of femoral condyles was 1679 ± 76.42 µm and tibial plateaus was 1164 ± 85.06 µm. The 9 and 15 month old pigs showed no difference in cartilage thickness of each articular surface, however, within each age group there were differences between the femoral condyles and tibial plateaus (p = 0.0617 and p < 0.0001 respectively, two-way ANOVA, see Figure 3.5). Medial femoral condyle cartilage was significantly thicker than medial tibial plateau cartilage in both 9 and 15 month old pigs (p = 0.0002 and p = 0.0174 respectively, Sidak's multiple comparisons).

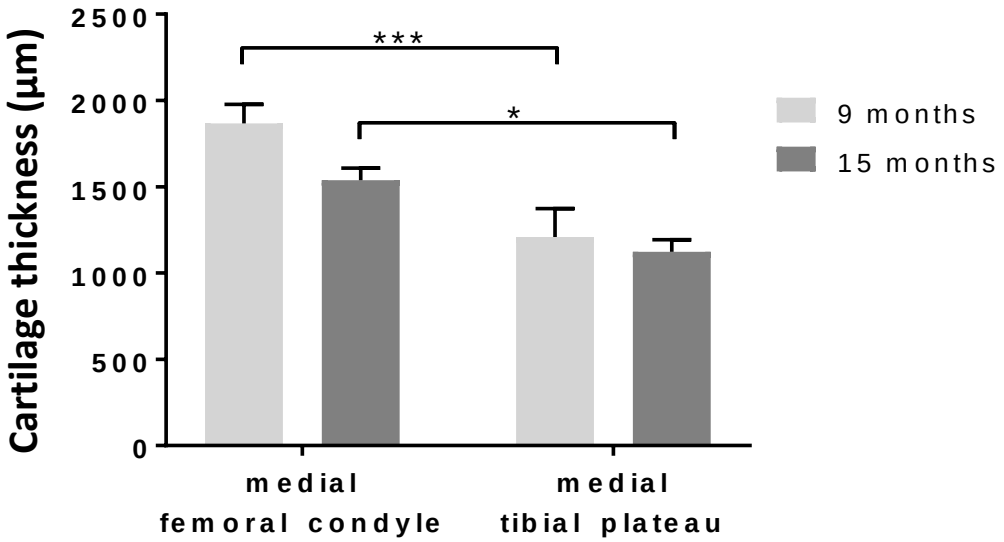


Figure 3.5: Thickness of pig knee joint articular cartilage in two age groups of pigs. Data presented as mean ± SEM. ***$p < 0.001$, *$p < 0.05$, two-way ANOVA with Sidak's multiple comparisons.

Multi-nucleated osteoclasts were visible in the medial femoral condyle and medial tibial plateau of both age groups, and differences in their density were observed between different age groups and joint surfaces ($p < 0.0001$, two-way ANOVA, see Figure 3.6). Medial femoral condyles had significantly more osteoclasts than medial tibial plateaus in both 9 and 15 month old pigs ($p < 0.0001$ and $p = 0.0109$ respectively, Tukey's multiple comparisons). There were also significantly more osteoclasts in medial femoral condyles of 9 month old pigs than 15 month old pigs, but there was no difference in these cells in medial tibial plateaus ($p < 0.0001$ and $p = 0.3217$ respectively, Tukey's multiple comparisons).

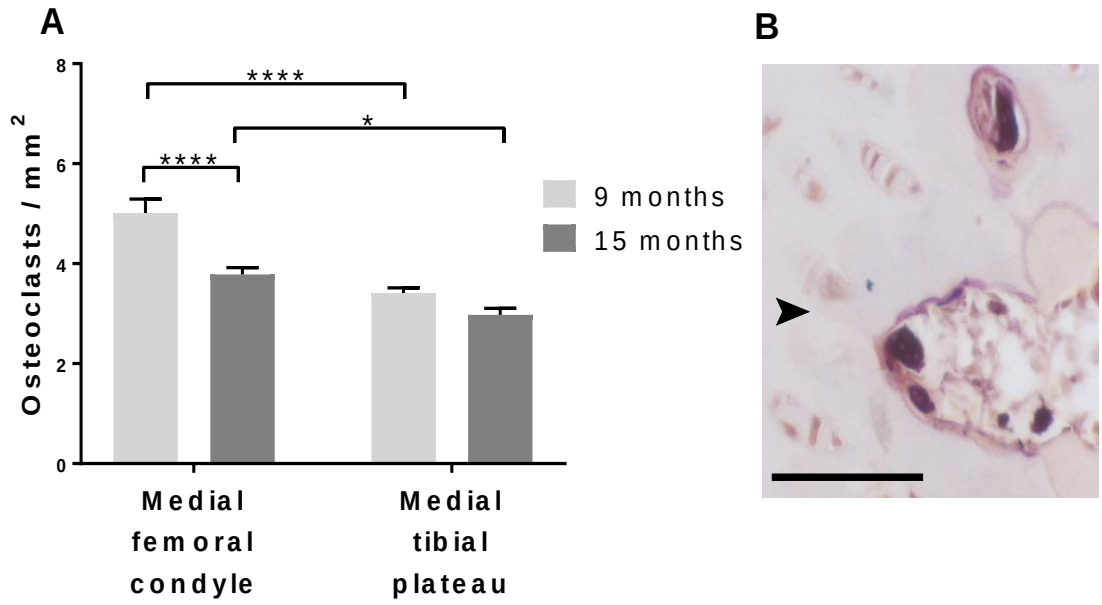


Figure 3.6: Osteoclasts in pig knee joints. A) Number of osteoclasts in 9 and 15 month old pig knee joints. Data presented as mean ± SEM. *p < 0.05, ****p < 0.0001, two-way ANOVA with Tukey's multiple comparisons. B) Representative image of TRAP stained osteoclasts in the femoral condyle of a 15 month old pig (➤), image taken at 20x magnification, scale bar 100 µm.

There was no correlation between macroscopic OC severity and microscopic histopathology scores for each joint surface (r^2 = -0.1317 and p = 0.5397, Spearman's Rank Correlation). There was, however, a positive correlation between cartilage thickness and OC histopathology (r^2 = 0.6930, p = 0.0150, Spearman's Rank Correlation) and between cartilage thickness and osteoclast number (r^2 = 0.6800, p = 0.0150 respectively, Pearson's Correlation).

3.3.4 Porcine pain behaviours

Leg weakness traits were variable across the study period and frequently changed between observations. Traits affecting the hind limbs included standing under the abdomen, cow-hocked hind legs and standing back. All pigs showed a leg weakness trait at least once during the observation period. Two 9 month old pigs and four 15 month old pigs showed leg weakness traits that were consistent across the study period.

Two independent observers scored pain behaviour of 9 and 15 month old pigs and 42 month old pigs (see chapter 4, total n = 19). Inter-observer reliability analysis showed good agreement with good to excellent ICCs across all behaviour measures (0.79-0.96), except for vocalisation scores (ICC = 0), which was due to lack of variation within the vocalisation scores.

The 9 month old pigs showed lameness severities ranging from absent to severe, and 4 out of 6 pigs showed lameness during the observation period. The 15 month old pigs also showed lameness ranging from absent to severe, and 5 out of 6 pigs showed lameness during the observation period. One pig in each age group showed severe lameness with minimal weight bearing on the affected limb (see summary in Table 3.2).

Table 3.2: Percentage of 9 and 15 month old pigs suffering from lameness and leg weakness.

	% animals	
	9 months old	**15 months old**
Lame	67 (n = 4)	83 (n = 5)
Severely lame	16 (n = 1)	16 (n = 1)
Consistent leg weakness	33 (n = 2)	67 (n = 4)

There was no difference in lameness, willingness to ambulate, response to touch, vocalisations, response to human interaction or total pain behaviour of the differently aged pigs ($p = 0.9372$, $p > 0.9999$, $p = 0.2424$, $p > 0.9999$, $p = 0.3398$ and $p = 0.4848$ respectively; all Mann-Whitney tests, see Figure 3.7). However, there was a statistical difference in the activity on arrival scores, such that 9 month old pigs were less active than 15 month old pigs ($p = 0.0043$, Mann-Whitney test). However, all pigs scored either 1 or 2 for activity on arrival for all observations.

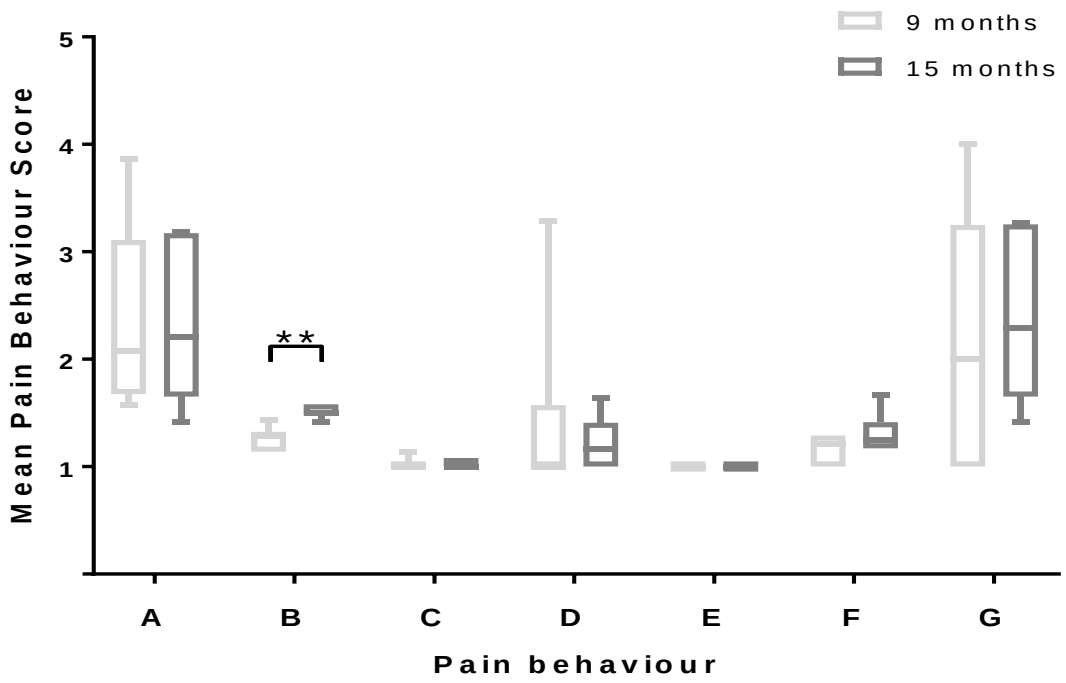


Figure 3.7: Pig pain behaviours. A: lameness, B: activity on arrival, C: response to touch, D: willingness to ambulate, E: vocalisation, F: response to human interaction, G: hind leg lameness. **p < 0.01, Mann-Whitney test. Box plot with whiskers showing the minimum and maximum scores and a median line.

There was a positive correlation between lameness and the animal's willingness to ambulate and response to touch ($r^2 = 0.702$, $p = 0.015$ and $r^2 = 0.760$, $p = 0.003$ respectively, Spearman's Rank Correlation). Willingness to ambulate also correlated with activity on arrival and response to touch ($r^2 = 0.687$, $p = 0.018$ and $r^2 = 0.847$, $p = 0.002$ respectively, Spearman's Rank Correlation).

3.3.5 Association of porcine pain behaviours and knee osteochondrosis

Although consistent leg weakness traits were identified in lame animals, no association between particular leg weakness traits and macroscopic OC severity was identified. There was also no association between OC severity of each knee joint and lameness or pain behaviours. However, there was a positive correlation between the animal's average macroscopic OC severity and hind leg lameness ($r^2 = 0.6527$, $p = 0.0220$, Spearman's Rank Correlation). Average macroscopic OC severity also positively correlated with the pig's activity on arrival, response to touch,

willingness to ambulate and response to human interaction (r^2 = 0.5999, p = 0.0354; r^2 = 0.6773, p = 0.0258; r^2 = 0.7253, p = 0.0097; and r^2 = 0.5867, p = 0.0461 respectively, Spearman's Rank Correlation).

The macroscopic severity of OC in femoral condyles, but not tibial plateaus, correlated with lameness (r^2 = 0.5137, p = 0.0102, and r^2 = 0.2015, p = 0.3565 respectively, Spearman's Rank Correlation). Furthermore, medial femoral condyle macroscopic OC severity correlated more strongly with hind leg lameness than lateral aspect OC severity (r^2 = 0.4671, p = 0.0214 and r^2 = 0.356, p = 0.0877 respectively, Spearman's Rank Correlation).

Although there was a good association between average lameness and OC severity, there was not complete agreement between the two factors and a number of pigs had asymptomatic OC. There was no difference in the severity of OC lesions seen in asymptomatic and symptomatic disease (p = 0.3153, Kruskal-Wallis test, see Figure 3.8). Of the three animals that showed no lameness, five knee joints showed OC lesions.

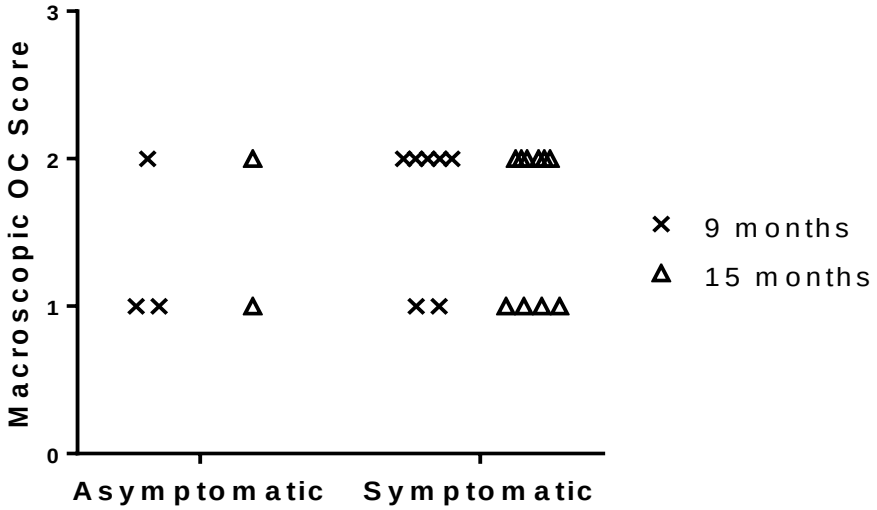


Figure 3.8: Severity of macroscopic osteochondrosis lesions seen in asymptomatic and symptomatic disease. There was no difference in lesion severity seen in asymptomatic and symptomatic OC.

3.4 Discussion

The work presented in this chapter characterised the pain behaviours and pathological features associated with knee OC in commercial pigs and compared different methods of assessing the OC pathology macroscopically. Identifying the prevalence and pathological features of spontaneous porcine knee OC at different age points, particularly in breeding age animals whom are not usually specifically assessed for OC, is an important step in characterising the disease.

3.4.1 Comparison of methods to assess osteochondrosis macroscopically

A number of different techniques to assess OC both macroscopically and histologically have been used in the pig. Many studies often assess OC using both techniques but there is little consensus on the most appropriate scoring method, particularly at a macroscopic level. In the present study, there was little agreement between the different macroscopic scoring methods investigated, with the exception of the van Grevenhof et al. (2011) and Scott et al. (2006) scoring methods, which showed a good correlation. This agreement is likely due to the similarity of their scoring criteria and scoring scale. The lack of agreement identified between the other scoring methods highlights the absence of an ideal OC scoring system that is both sensitive and specific to pig OC, taking into account lesion severity, size and type.

For example, the method of Ryan et al. (2010) scores both OC and OA on a single scale, which although useful if comparing joint diseases across a large age range, is not specific to the OC pathology and does not take into consideration lesion size. The method of Kirk et al. (2008) is designed to consider if a lesion is greater than a certain size but also investigates both OC and arthritic lesions. In the present study, the criteria selected from the scoring system were OC-specific (cartilage infolding and repair reactions) but this did not give a clear and specific indication of the overall joint disease state. The methods of Scott et al. (2006) and van Grevenhof et al. (2011) are both OC-specific, but do not discriminate between the size of lesions, making the systems lack sensitivity. These limited score parameters may be useful when a large number of joints require assessment quickly, but they lack the ability

to distinguish subtle changes in pathology. The descriptors used in these systems are also open to subjective interpretation. Inter-observer reliability of both the Scott et al. (2006) and van Grevenhof et al. (2011) methods have previously been reported as relatively poor (Gould, 2017), however in the present study, the intra-observer reliability of the van Grevenhof et al. (2011) method was good. It is important, therefore, that scoring is performed by the same individual. The comparison of different macroscopic scoring methods shows that current systems lack either sensitivity or specificity to OC, but are good at providing some guidance on the presence and severity of OC.

3.4.2 Porcine knee joint osteochondrosis

This study identified spontaneous OC in commercial pig knee joints that ranged in severity from slight flattening of articular cartilage to more severe cartilage infolding, but severe OCD was not observed. Alterations in the cartilage were consistent with OC identified in other pig studies but also in other species, including horses, cattle and dogs (Jensen et al., 1981; Jørgensen et al., 1995; Van Weeren and Barneveld, 1999; Cook et al., 2008; van Grevenhof et al., 2011; de Koning et al., 2013). Histological examination of the joints showed further features of OC, with disrupted endochondral ossification and altered chondrocyte arrangement (Etterlin et al., 2017).

The articular cartilage was approximately 1.5 mm thick, which is similar to that seen in other studies of pigs of a similar age (Nakano et al., 1979; Frisbie et al., 2006). The pigs had thicker cartilage in the femoral condyle than tibial plateau, which again is consistent with other studies (McLure et al., 2012; Kiapour et al., 2015). It might have been expected that 9 month old pigs would have thicker cartilage than 15 month old pigs, due to cartilage thinning which occurs as the animals growth rate reduces (Nakano et al., 1979). However, this was not seen and is likely due to the pigs being skeletally immature and the presence of OC, which can cause a local thickening of articular cartilage (Nakano et al., 1984b; Wardale and Duance, 1994; Jørgensen et al., 1995). The high prevalence of OC in both age groups and the

associated local cartilage thickening may have obscured any age-related differences in cartilage thickness.

The present study identified a high prevalence of spontaneous OC in commercial pigs, with 91% of knee joints showing lesions. This is supported by some studies which identified a 70-95% OC prevalence (Ytrehus et al., 2004b; Busch and Wachmann, 2011; Tóth et al., 2016); however, others have only seen a 40-52% incidence (van Grevenhof et al., 2011; Olstad et al., 2014). These previous studies did not always reference the pig's age, breed or husbandry conditions used, which could influence OC prevalence. Housing conditions, including stocking density, floor type and diet have all been shown to alter the prevalence of OC (Scott et al., 2006; Kilbride et al., 2009; van Grevenhof et al., 2011; de Koning et al., 2013; Sugiyama et al., 2013). Furthermore, the age of animals used here is not typical when studying OC. Usually younger animals are studied before they reach slaughter weight, at approximately 24 weeks old, so the present study's use of much older pigs could have influenced OC prevalence.

Higher growth rates in younger animals have been implicated as a risk for developing OC, with faster growth and weight gain linked to a higher prevalence (Nakano et al., 1984b; Busch and Wachmann, 2011; Tóth et al., 2016). A critical period has been identified in pigs, between 56 - 106 days old, when animals are most susceptible to OC development due to a reduction in cartilage vascularity that subsequently occurs (Ytrehus et al., 2007; van Grevenhof et al., 2012; Bertholle et al., 2016). Therefore, it might have been expected that the younger pigs would show increased OC. However, the present study identified no age-associated difference. The difference in growth rate of the two age groups studied here will be much smaller than would be seen with a similar age gap in younger pigs, as the animals are beginning to reach the plateau in their growth. Therefore, the age difference will have less effect on OC status. Furthermore, it might have been expected that the pigs would show reduced OC compared to studies of pre-slaughter weight pigs who have higher growth rates. The present study identified a higher prevalence than some studies of pre-slaughter weight pigs. Although the

reduced growth rate after reaching slaughter weight may leave joints less susceptible to OC onset, it also leaves them less able to repair lesions which are already present or newly formed, which may account for the high OC prevalence identified.

Examination of the knee joints identified a similar bilateral symmetry of OC as reported previously (Dewey et al., 1993; Kirk et al., 2008; van Grevenhof et al., 2009; Bertholle et al., 2016). Furthermore, no difference in the OC severity of medial and lateral aspects of the femoral condyles and tibial plateaus were identified macroscopically or histologically. It has been suggested that medial aspects show increased OC and that the femoral condyle is more susceptible than the tibial plateau due to the increased weight bearing on these articular surfaces (Grondalen, 1974; van Grevenhof et al., 2012). However, the present study identified no such difference in the OC severity of different articular surfaces, except for the patella, which was significantly less affected. This was likely due to the reduced weight bearing of the patella compared to other articular surfaces.

Previous studies have identified macroscopic assessment as being less effective than histological methods at identifying OC. However, they still show good agreements, with 79% of OC lesions identified histologically also showing visible macroscopic changes (Olstad et al., 2014; Bertholle et al., 2016). However, the present study showed no association between the severity of lesions identified macroscopically and histologically. This is likely a result of the sampling technique used, whereby bone sections were only collected from the medial aspect of the femoral condyle and tibial plateau, and not necessarily from areas containing lesions. This technique was used in part due to time constraints and tissue availability when sampling, as cartilage samples were required by other researchers, but also because a consistent sample location was required to compare different aspects of the histology across different age groups. If multiple bone sections were obtained it may have improved the agreement between OC severities identified macroscopically and histologically. This could have resulted in an underreporting of

OC severity if using histology, therefore macroscopic OC assessment was used to indicate the animals overall OC status during subsequent analysis.

Osteoclasts were identified in all pig joints using a commercially available TRAP staining kit. This kit's use has not been reported previously in porcine joint sections, only in porcine cell culture experiments. In equine OC, it has been shown there increased expression of genes involved in osteoclast differentiation (Desjardin et al., 2014), but there have been no other investigations into osteoclast involvement in OC. The present study found no association between osteoclast number and OC severity but there were significantly more osteoclasts in the femoral condyle than tibial plateau of both age groups. This is likely due to the increased bone activity of the femoral condyle compared to the tibial plateau, which can result from increased endochondral ossification (Nakano et al., 1979). There were also significantly more osteoclasts in the femoral condyle of 9 month old than 15 month old pigs. Opinion on the change in osteoclasts associated with ageing is mixed, with some studies identifying an increase with age but others identifying a decrease (Jevon et al., 2002; Chung et al., 2014; Becerikli et al., 2017). It is likely that, as with the difference seen in the femoral condyle and tibial plateau, this difference is a result of increased endochondral ossification and therefore increased bone activity occurring in this region of younger pigs.

3.4.3 Pig pain behaviours and their association to osteochondrosis

The present study identified a high prevalence of lameness and pain behaviours in 9 and 15 month old pigs, which was significantly higher than the 11-20% incidence of lameness reported in previous studies of the UK pig herd (Kilbride et al., 2009). This may partly be due to the small sample number used in this study, which may not be representative of the entire pig population. Furthermore, the high incidence of lameness could result from the animals' transport prior to the study, which could have resulted in injury and influenced lameness (Kephart et al., 2010). However, the incidence of severe lameness affecting weight bearing was 16% of pigs, which is similar to previous studies. The small number of animals in the present study also allowed a more thorough assessment of lameness, particularly of very slight

changes in gait, which may not be detected as easily when recording from large groups of animals.

There was no difference in the lameness and pain behaviours of 9 and 15 month old pigs, with the exception of their activity on arrival. This is similar to other studies which have found no difference in lameness severity between finishing pigs, gilts and sows, although the specific ages were not detailed (Heinonen et al., 2006; Kilbride et al., 2009). The 9 month old pigs did show reduced activity on arrival scores, although the range of scores for both age groups was very small. The 9 month old pigs were more often asleep on arrival, whereas the 15 month old pigs were more often awake, but neither group showed signs of agitation.

The pain scoring system was a simple and easy procedure to conduct and displayed good repeatability, with high inter-rater reliability. Although lameness in pigs has been shown to correlate to hyperalgesia, suggesting nervous system sensitisation, lameness is not necessarily always indicative of pain. Other factors such as biomechanical abnormalities can cause lameness in the absence of pain (Ison et al., 2016), so results from this type of pain behaviour scoring require caution in their interpretation. Lameness was therefore scored alongside other pain related behaviours to provide some indication as to whether the lameness was painful. The individual pain related behaviours showed a good correlation with each other, in particular lameness correlated with willingness to ambulate and response to touch, suggesting that lame animals were experiencing pain.

Leg weakness traits were seen consistently in severely lame animals but not identified in non-lame animals. However, as with other studies, there was no association between particular leg weakness traits and OC (Jørgensen, 1995; de Koning et al., 2012). There was, however, a positive correlation between pig lameness and OC severity, indicating that porcine OC was painful. This is supported by other studies that identified an association between joint lesion severity and lameness (Dewey et al., 1993; Kirk et al., 2008). It has been reported that OC in medial aspects of joints are more likely to present with lameness than lesions to

lateral aspects, which are more often subclinical (Nakano et al., 1979; Nakano et al., 1985; Nakano and Aherne, 1993; van Grevenhof et al., 2012). A similar trend was identified in the present study, whereby the severity of medial femoral condyle OC most greatly correlated with the presence and severity of lameness, with lateral aspect and tibial plateau lesions showing less association with lameness.

However, there were several occurrences of subclinical (asymptomatic) disease, where joint lesions were present but the pig showed no lameness. This has been reported previously; for example, in a study of the causes of pig lameness, Dewey et al. (1993) reported that of 6 out of 10 non-lame sows had arthrosis or OC. Many studies have suggested that this is because there is a lesion severity threshold for lameness to become apparent (Jørgensen et al., 1995; Etterlin et al., 2015). However, in the present study we identified no difference in lesion severity of asymptomatic and symptomatic disease. This highlights the complex relationship between lameness, conformation changes and joint disease (Etterlin et al., 2015). OC is often subclinical and is not identifiable until the lesions are either severe or affecting multiple joints or locations within a joint, making understanding the disease challenging as OC is not easy to identify in live animals without invasive investigation.

3.5 Conclusions

Adult female pigs of 9 and 15 months of age showed a high incidence of knee OC, with features such as loss of normal chondrocyte arrangement and infolding of the articular cartilage. OC was identified macroscopically using a number of different scoring methods, but they all had limitations, particularly their lack of sensitivity to subtle differences in disease severity. The pigs showed a high prevalence of lameness which correlated with OC severity; however, there were a number of non-lame animals who had knee OC. This highlights the difficulty in understanding diseases such as OC, as they are often subclinical and are not identifiable without invasive examinations.

Chapter 4

Characterising Pain Behaviours and Pathological Features of Osteoarthritis in the Porcine Knee Joint

4.1 Introduction

There are more than 8.75 million people over the age of 45 in the UK suffering from OA (GBD, 2017; ARUK, 2018). Consequently, extensive research is conducted to understand the disease's pathology, often by using animal models (see section 1.7.4). Pigs have been suggested as a good model for human OA and are often used to investigate bone and cartilage grafts (Teeple et al., 2013; Matsuo et al., 2015), but few investigations into the spontaneous disease have been conducted. Therefore, studying OA in commercial pigs provides a unique opportunity to address this issue as they suffer from a high prevalence of spontaneous joint disease, with over 80% of adult pigs thought to suffer from OA (Reiland, 1975). This high incidence of spontaneous OA in commercial pigs also has important implications for the industry as it may result in chronic pain, raising animal welfare concerns. Pig OA results in lameness, which has significant economic impacts as it results in early slaughter and reduces breeding productivity (see section 1.2). Characterising porcine OA is, therefore, important for improving the sustainability of the commercial pig industry, but is also the first step in validating the pig as a model of the human disease.

OA is characterised by erosion of articular cartilage and exposure of subchondral bone (see section 1.7) and a variety of methods have been developed to measure these structural changes in both humans and animals. Changes in cartilage structure and thickness, chondrocyte density and osteoclast number can be identified using histological assessment (Mankin et al., 1971). Gross changes in the joint's surface integrity, such as erosion of the cartilage and osteophyte formation, can be identified macroscopically (Walsh et al., 2009). Most macroscopic OA scoring techniques are derived from the Collin's classification system, which identifies four pathological grades of cartilage integrity ranging from healthy cartilage to complete erosion with subchondral bone exposure (Collins, 1949). This system was then built upon by the Société Français d'Arthroscopie (SFA) to take into account the location, size and depth of lesions to the articular cartilage (Dougados et al., 1994). The SFA system was then simplified to the revised SFA system (rSFA) by taking into account just the size and depth of different articular cartilage lesions (Ayral et al., 1994).

A number of scoring systems have been used to assess joint disease in pigs and many are based upon the Collin's classification system. Most methods take into consideration the size and type of lesion present but not the location. In addition, the majority try to capture multiple conditions in one scoring system, such as both OC and OA. For example, Kirk et al. (2005) scored the extent of cartilage erosion, ulceration, infolding, repair and osteophyte formation separately. Ryan et al. (2010) scored the progression of OC to OA by including factors such as disrupted endochondral ossification and subchondral bone exposure on a single scale. This may be useful when investigating joint pathology across a large animal age range, but these techniques lack sensitivity to OA. Other methods have solely scored OA, such as Hennerbichler et al. (2008), who based the macroscopic scoring on the International Cartilage Repair Society's (ICRS) scoring system, which in turn is based on the Collin's classification system. However, the method only scores on a range of 0 - 4 so may lack sensitivity to subtle differences in gross pathology. An ideal macroscopic scoring of OA needs to be simple, feasible, scalable, valid and discriminant (Nwosu, 2015), which is lacking in the current methods of porcine OA assessment. Use of the rSFA system may provide a more sensitive method of macroscopic OA assessment in the pig, detecting more subtle differences in pathology severity whilst also allowing severity to be directly compared to human OA.

4.1.1 Aim

The aim of the work presented in this chapter was to characterise pain behaviours and pathological features associated with spontaneous knee OA in commercial pigs. Porcine knee joints were assessed macroscopically for OA to identify the disease's prevalence and pathological features. The associations between pain behaviour, macroscopic OA pathology and histological measures of OA severity were then examined. A comparison of different methods of scoring macroscopic OA pathology in the knee joint was conducted to identify whether the rSFA scoring method used to assess human knee OA was suited to scoring porcine knee OA. The rSFA macroscopic OA scoring system was also assessed for reproducibility by analysing inter-observer and intra-observer reliability.

4.2 Methods

4.2.1 Animals

Two age cohorts of clinically healthy female commercial pigs of the crossbred genotype 'Landrace x Large White x Duroc' were used in this study. Pigs were aged 24 months (n = 8) with an average weight of 245.1 ± 9.00 kg and approximately 42 months (n = 7) with an average weight of 230.6 ± 8.96 kg.

Pigs were assessed for 5-7 days prior to slaughter for pain behaviours and lameness (see section 2.2). Knee joints were assessed macroscopically for OA post-mortem (see section 2.4). As for OC in Chapter 3, in pigs showing OA but a normal gait, the disease was termed asymptomatic, whereas in pigs showing OA and lameness, the disease was termed symptomatic. Sections of cartilage and bone from the femoral condyle and tibial plateau were stained with H&E to assess cartilage thickness and microscopic histopathology (see section 2.5), and stained with TRAP for osteoclast counting (see section 2.6). For the 42 month old pigs, cartilage sections were H&E and TRAP stained from the femoral condyles only.

4.2.2 Macroscopic chondropathy scoring of porcine knee joints

Macroscopic chondropathy was scored in both knees of 24 month old pigs (n = 16), but only one knee of 42 month old pigs, with the exception of one pig where both knees were assessed (n = 8), as the other hind limbs were stored for subsequent Dual-energy X-ray absorptiometry scanning by other researchers. Osteophytes were scored according to the OARSI histopathology system (see section 2.4.3).

The presence and severity of knee OA was scored macroscopically using the rSFA system described in section 2.4.2. The inter-observer reliability was determined by two independent observers (Catherine Owles and Lara Yarwood) scoring knee joint photographs (n = 32; joints from 9, 15 and 42 month old pigs). Intra-observer reliability was determined by one scorer (Catherine Owles) scoring all knee joint photographs on two separate occasions (n = 24; 24 and 42 month old pig knee joints). In order to evaluate this system's use in pigs, knee joint photographs were also scored using four other classification systems previously reported in pig studies

(methods of Hennerbichler et al., 2008; Kirk et al., 2008; Ryan et al., 2010 and the OARSI recommended assessment of OA in sheep and goats of Little et al., 2010). This enabled the use of the rSFA chondropathy scoring in pigs to be evaluated and compared to current porcine macroscopic scoring methods.

Using the method of Hennerbichler et al. (2008), each joint surface was graded 0 - 4 according to the ICRS macroscopic grading. Arthritic areas of the joint's surface were classified as grade 0 when no lesions were visible and grade 1 when superficial fissures and cracks were evident. Lesions were scored grade 2 when they extended down to less than 50% of the cartilage depth and grade 3 when lesions extended more than 50% of the cartilage depth but did not reach the subchondral bone. Grade 4 was scored when the subchondral bone was exposed.

Using the method of Kirk et al. (2008), each joint surface was assessed for the presence of: i) erosions and ii) ulcerations. The joint was scored as normal (score 0) when no lesion was present, moderate (score 1) when the particular lesion involved less than 20% of the articular surface area, or severe (score 2) when the lesion involved more than 20% of the articular surface area.

Using the method of Ryan et al. (2010), joint surfaces were subjectively scored on a scale of 1 - 7. Score 1 indicated normal cartilage free from abnormalities. Scores 2 - 4 indicated slight to mild disturbed endochondral ossification with cartilage thickening or cartilage grooves. Scores 5 - 7 indicated moderate to severe degrees of disturbed endochondral ossification with fractures or separation of cartilage and/or subchondral bone collapse or fibrosis.

The final method used was the OARSI recommended histological assessment of OA in sheep and goats of Little et al. (2010) which scored gross articular damage of each surface on a scale of 0 - 4. Joint surfaces were given a score 0 when the cartilage was normal, score 1 when cartilage roughening was present and score 2 when cartilage showed fibrillation and fissures. Score 3 indicated small erosions of cartilage to the subchondral bone that were less than 5 mm in diameter and score 4

indicated erosions to the subchondral bone that were greater than 5 mm in diameter.

4.2.3 Statistical analysis

Data were tested for normality using the D'Agostino-Pearson normality test. Parametric data were analysed using t-tests, one-way ANOVA with Tukey's multiple comparisons or two-way ANOVA with Sidak's multiple comparisons post-hoc tests. Non-parametric data were analysed with Mann-Whitney tests or Kruskal-Wallis tests with Dunn's multiple comparisons post-hoc test. Correlation between data was determined using Pearson's or Spearman's Rank Correlation. A significance of $p < 0.05$ was used for all statistical tests.

Inter-observer and intra-observer agreements were estimated by ICC; intra-rater analysis used one-way random single measures and inter-rater analysis used two-way mixed model absolute agreement. Reliability was regarded as excellent if ICC > 0.9, good if ICC 0.6 – 0.9 and poor if ICC < 0.6 (Bruton et al., 2000).

4.3 Results

4.3.1 Validity of the rSFA macroscopic scoring systems

There was a positive correlation between macroscopic chondropathy scores derived using the rSFA method and other scoring methods that were tested (see Table 4.1). The methods of Hennerbichler et al. (2008), Kirk et al. (2005), Ryan et al. (2010) and the OARSI method from Little et al. (2010) also positively correlated with each other (data not shown).

Table 4.1: Association of results produced by different methods of macroscopic osteoarthritis severity assessment. Correlation of macroscopic OA chondropathy determined using rSFA scoring and by the methods of Hennerbichler et al. (2008), Kirk et al. (2005), Ryan et al. (2010) and the OARSI method from Little et al. (2010). Association expressed as Spearman's Rank Correlation r^2 Coefficient (**p < 0.01, ****p < 0.0001).

	Revised SFA
Hennerbichler	0.7483****
Kirk - Erosion	0.5790**
Kirk - Ulceration	0.8036****
Ryan	0.7959****
OARSI cartilage	0.6291**

Positive correlations were observed between rSFA scores of both observers (see Figure 4.1A). Inter-rater reliability analysis displayed good inter-observer reliability with an excellent ICC score (0.94, n = 32). A positive correlation was also observed between rSFA scores from the same observer (see Figure 4.1B). Inter-rater reliability analysis displayed good intra-observer reliability with a good ICC score (0.86, n = 24). The rSFA scoring method showed a number of advantages over the other methods as the data produced was continuous and the system was more sensitive to subtle changes in pathology. As the rSFA system also showed good agreement with other methods previously used in pigs, the rSFA system was subsequently selected to indicate OA severity.

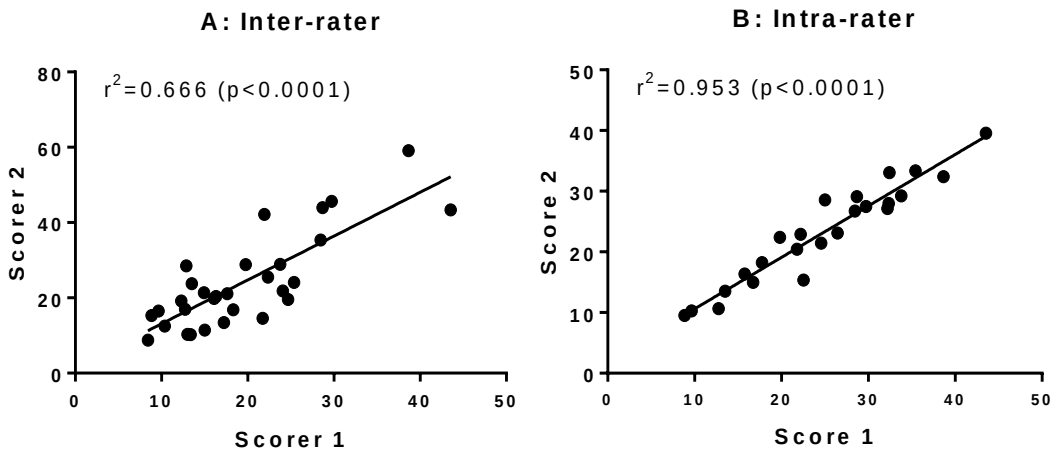


Figure 4.1: High inter- (A) and intra-rater (B) reliability of rSFA scoring. Scatter plots with linear regression line and Spearman's Rank Correlation Coefficients.

4.3.2 Macroscopic chondropathy of porcine knee joints

Macroscopic examination of porcine knees identified evidence of OA in a number of joints, with all but one knee containing areas of altered cartilage. The cartilage was often discoloured, showing signs of thinning and erosion (see Figure 4.2). Deep fibrillation of cartilage was identified in 10 out of 24 joints and 7 joints had areas of exposed subchondral bone. OC was not identified in any joints of the 24 and 42 month old pigs. The formation of osteophytes was seen in 22 joints and ranged from absent to osteophytes less than 5 mm in size.

There was no significant difference in total macroscopic OA severity between the 24 and 42 month old pigs ($p = 0.7109$, unpaired t-test). However, 42 month old pigs had more joints with subchondral bone exposure than 24 month old pigs (50% and 19% of joints, respectively). OA also presented with bilateral symmetry, with no difference in severity of left and right knees ($p = 0.3518$, paired t-test).

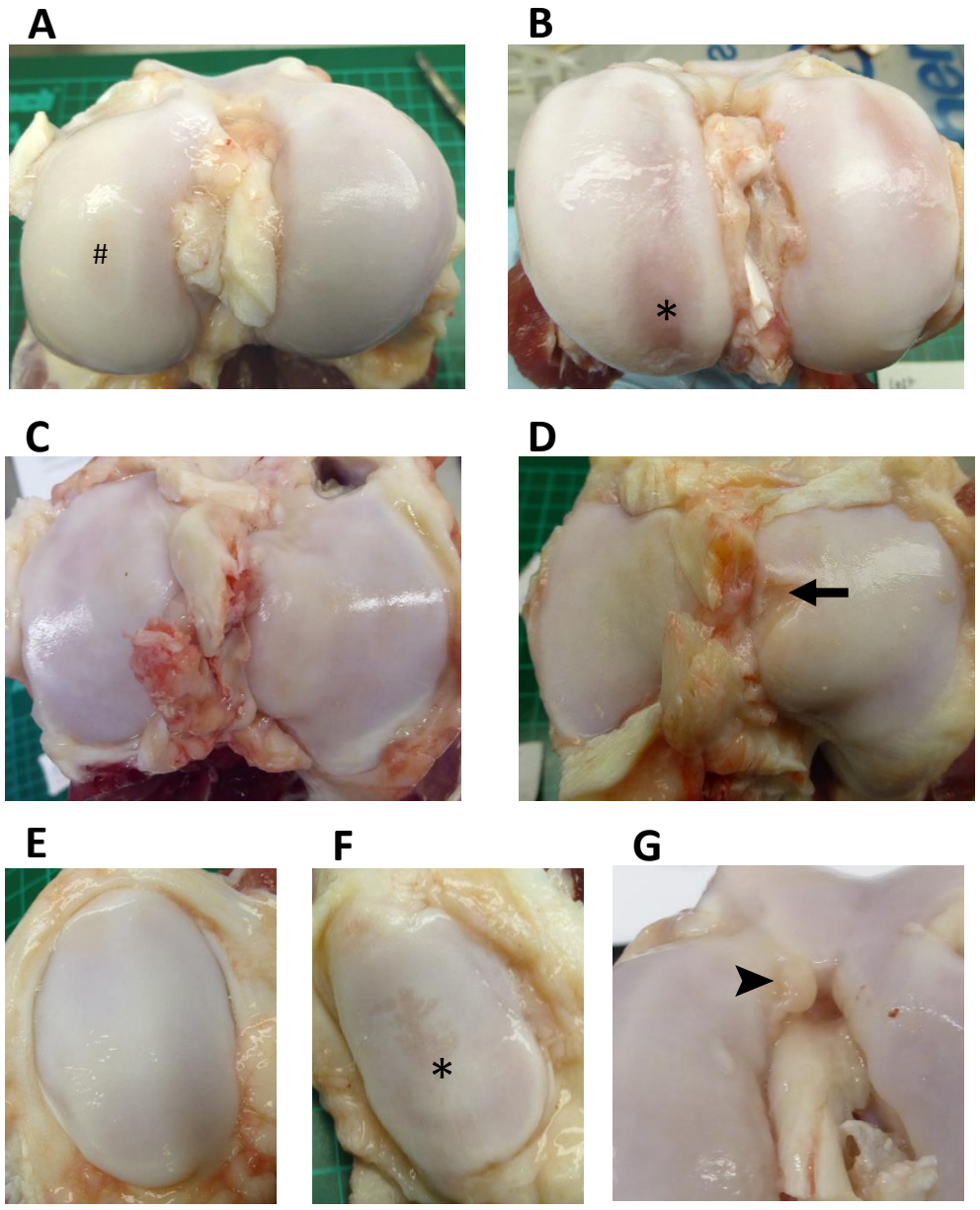


Figure 4.2: Macroscopic chondropathy of pig knee joints. Representative photographs of pig femoral condyle (A, B and G), tibial plateau (C and D) and patella surfaces (E and F). Healthy joints showed smooth and intact cartilage (A, C, E). OA caused pathological changes including erosion of the cartilage (B, *), exposure of subchondral bone (D, arrow), osteophyte formation (G, ➤), and scalpel damage (A, #).

The pigs showed no difference in OA severity between the age groups, however, within each group there were differences between the articular surfaces ($p = 0.9438$ and $p < 0.0001$ respectively, two-way ANOVA). In 24 month old pigs, the patella consistently scored less than medial femoral condyles and medial and lateral tibial plateaus ($p < 0.0001$, $p < 0.0001$ and $p = 0.0240$ respectively, Sidak's multiple comparisons, see Figure 4.3A). In 42 month old pigs the patella scored less than medial femoral condyles and medial tibial plateaus ($p = 0.0002$ and $p < 0.0001$ respectively, Sidak's multiple comparisons, see Figure 4.3B). Furthermore, medial femoral condyles and tibial plateaus showed significantly worse macroscopic OA than lateral aspects in both 24 month old pigs ($p = 0.0005$ and $p = 0.0236$ respectively, Sidak's multiple comparison) and 42 month old pigs ($p = 0.0024$ and $p = 0.0340$ respectively, Sidak's multiple comparison).

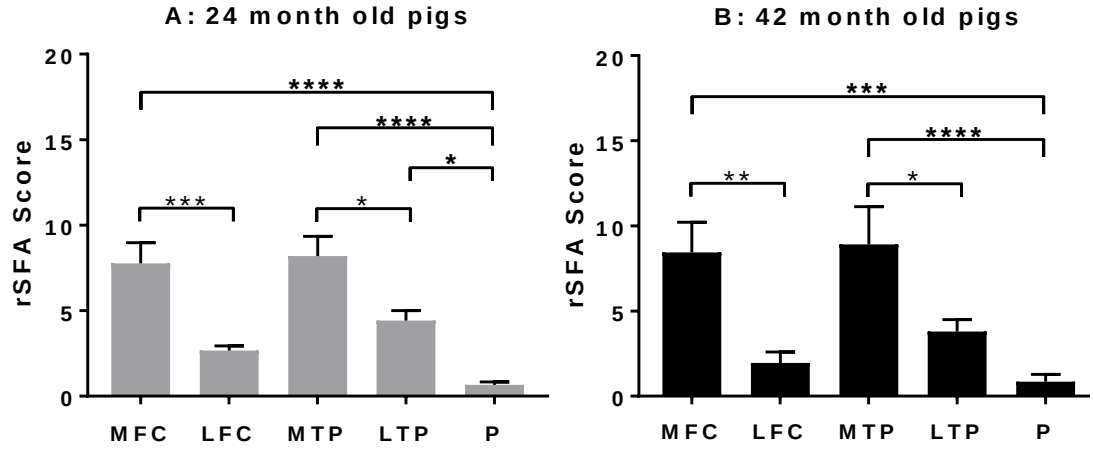


Figure 4.3: Macroscopic chondropathy of knee joint articular surfaces in two age groups of pigs. MFC medial femoral condyle, LFC lateral femoral condyle, MTP medial tibial plateau, LTP lateral tibial plateau, P patella. Macroscopic knee OA severity in 24 month old pigs (A) and 42 month old pigs (B), determined using rSFA scoring. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Sidak's multiple comparisons. Data presented as mean ± SEM.

4.3.3 Histological assessment of osteoarthritis in porcine knee

Histological examination of cartilage and subchondral bone identified a number of changes in microscopic pathology indicating OA (see Figure 4.4). Irregular cartilage and cartilage fissures consistent with OA, as well as decreased chondrocyte density and increased cell cloning, were present in a number of sections.

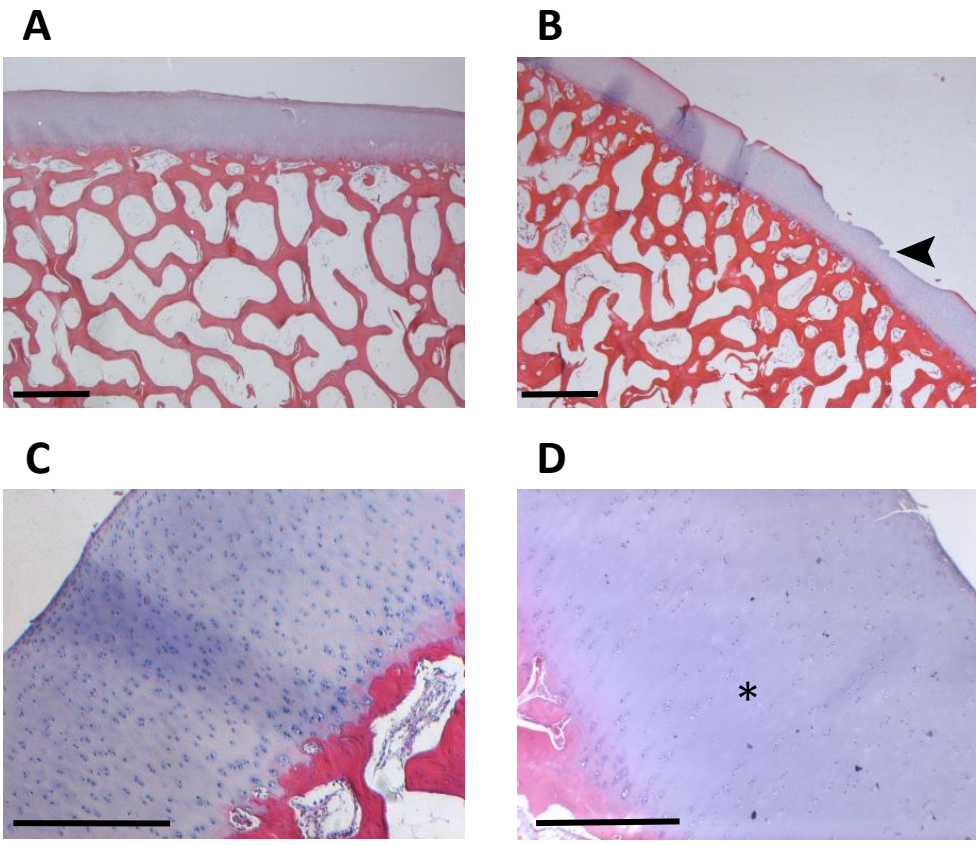


Figure 4.4 Microscopic histopathology of porcine knee joint articular cartilage. Representative images of 42 month old pig medial femoral condyle cartilage. Healthy joints showing smooth cartilage (A) with a normal chondrocyte density (C). Joints with OA showed cartilage erosion (B, ➤) and decreased chondrocyte density (D, *). Images taken at 1.6x magnification (A and B, scale bar 1000 µm) and 4x magnification (C and D, scale bar 500 µm).

There was no significant difference in the microscopic pathology scores for cartilage structure or cell cloning between age groups or articular surfaces (p = 0.9164 and p = 0.4231 respectively, Kruskal-Wallis tests, see Figure 4.5). However, there were differences in the chondrocyte density scores (p = 0.0443, Kruskal-Wallis test). In the femoral condyle, 42 month old pigs had a significantly higher chondrocyte density score, indicating fewer chondrocytes, than 24 month old pigs (p = 0.0392, Dunn's multiple comparison) but there was no difference in chondrocyte density between the medial femoral condyle and tibial plateau of 24 month old pigs (p = 0.1556, Dunn's multiple comparison).

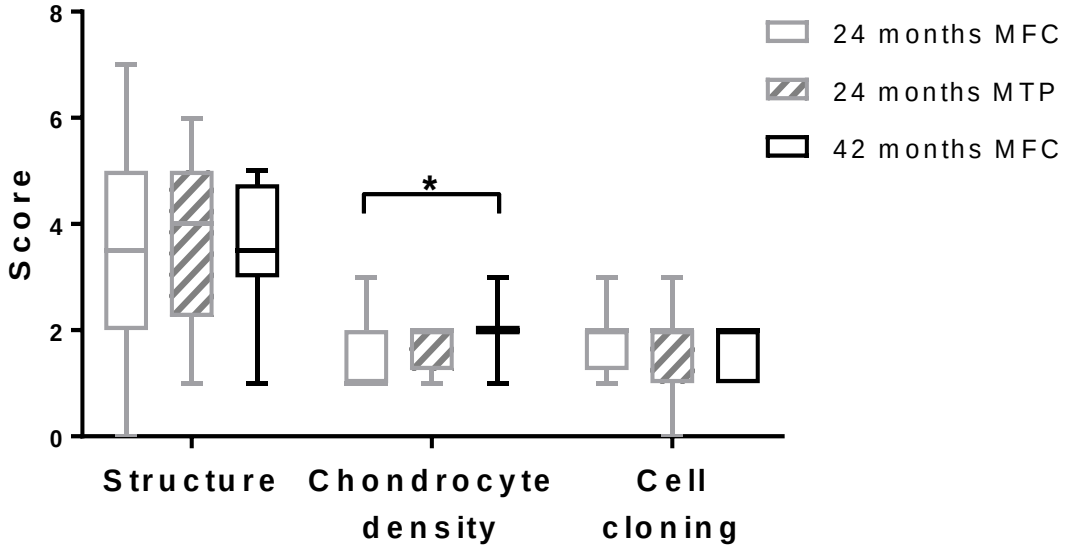


Figure 4.5 Microscopic osteoarthritis histopathology of knee joint articular surfaces in two age groups of pigs. MFC medial femoral condyle, MTP medial tibial plateau. *p < 0.05, Kruskal-Wallis test with Dunn's multiple comparison. Box plot with whiskers showing the minimum and maximum scores and a median line.

The mean femoral condyle cartilage thickness of all pigs was 1037 ± 85.08 µm and the tibial plateau cartilage was 1117 ± 89.32 µm. There was no difference in femoral condyle articular cartilage thickness between 24 month old and 42 months old pigs, or between the medial femoral condyles and medial tibial plateaus of 24 month old pigs (p = 0.8231, one-way ANOVA, see Figure 4.6).

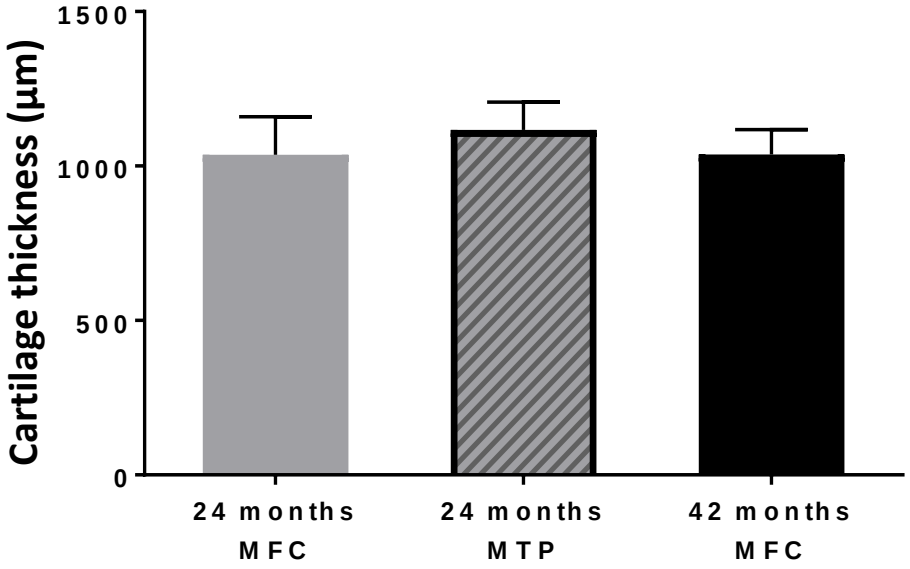


Figure 4.6: Thickness of pig knee joint articular cartilage in two age groups of pigs. MFC medial femoral condyle, MTP medial tibial plateau. Data presented as mean ± SEM.

Multi-nucleated osteoclasts were visible in the femoral condyle and tibial plateau of both age groups (see Figure 4.7B). Femoral condyle samples had an average of 1.1 ± 0.10 osteoclasts per mm^2 and the tibial plateau had an average of 0.7 ± 0.04 osteoclasts per mm^2, and a difference in osteoclasts number was observed ($p = 0.0213$, one-way ANOVA, see Figure 4.7A). There was no statistical difference in osteoclast number in femoral condyles of 24 and 42 month old pigs ($p = 0.9991$, Tukey's multiple comparison). However, in 24 month old pigs there were significantly more osteoclasts in femoral condyles than tibial plateaus ($p = 0.0282$, Tukey's multiple comparisons). The number of osteoclasts did not significantly correlate with the presence of osteophytes ($r^2 = 0.468$, $p = 0.081$, Spearman's Rank Correlation).

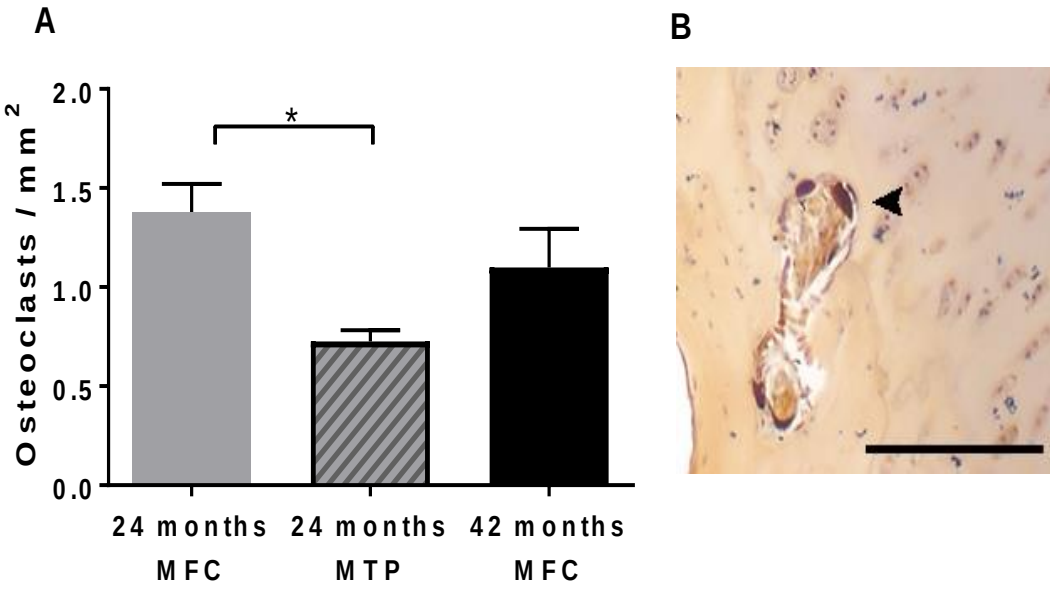


Figure 4.7: Osteoclasts in pig knee joints. MFC medial femoral condyle, MTP medial tibial plateau. A) Osteoclast count. Data presented as mean ± SEM. *p < 0.05, one-way ANOVA with Tukey's multiple comparisons. B) Representative image of TRAP stained osteoclasts from femoral condyle of a 24 month old pig (➤), image taken at 10x magnification, scale bar 200 µm.

A very slight statistically significant positive correlation was observed between macroscopic chondropathy (rSFA score) and the microscopic histopathology scores for chondrocyte density and total pathology in femoral condyles (see Table 4.2). Macroscopic chondropathy of tibial plateaus did not correlate with microscopic histopathology. A slight statistically significant negative correlation between macroscopic chondropathy and osteoclast number in the femoral condyles was also observed.

Table 4.2: Association of macroscopic and microscopic osteoarthritis severity. Correlation of revised SFA macroscopic OA scores and microscopic histopathology scores in 24 and 42 month old pigs. Association expressed as Pearson's or Spearman's Rank r^2 Correlation Coefficients (*p < 0.05, **p < 0.01).

	Revised SFA	
	Femoral condyle	**Tibial plateau**
Cartilage structure	0.2124	0.1037
Chondrocyte density	0.3748*	0.0001
Cell clones	0.1426	-0.0673
Total histopathology score	0.3612*	-0.0030
Cartilage thickness	0.1172	-0.1181
Osteoclast count	-0.5309**	-0.4131

4.3.4 Porcine pain behaviours

Within the group of 42 month old pigs, 5 out of 7 animals showed lameness, which ranged from absent to severe lameness with minimal weight bearing on the affected limb. The 24 month old pigs showed lameness ranging from absent to moderate lameness that was easily detectable, with 7 out of 8 pigs showing lameness during the observation period (for summary see Table 4.3). Leg weakness traits that affected the hind limbs, included o-shaped hind legs, sickle hocks, standing under the abdomen, cow-hocked hind legs and weak pasterns. Three 42 month old pigs and five 24 month old pigs showed leg weakness traits that were consistent across the observation period.

Table 4.3: Percentage of 24 and 42 month old pigs suffering from lameness and leg weakness.

	% animals	
	24 months old	**42 months old**
Lame	88 (n = 7)	71 (n = 5)
Severely lame	0 (n = 0)	14 (n = 1)
Consistent leg weakness	63 (n = 5)	43 (n = 3)

The 24 month old pigs were significantly more active on arrival, more responsive to touch but less willing to ambulate than 42 month old pigs (p = 0.0003, p = 0.0256 and p = 0.0067 respectively, Mann-Whitney tests, see Figure 4.8). However, all pigs scored 1 or 2 for activity on arrival, response to touch and willingness to ambulate. There was no difference in lameness, vocalisation, response to human interaction or total pain behaviour scores between the different age pigs (p = 0.7512, p = 0.4667, p = 0.0657 and p = 0.2657 respectively, Mann-Whitney tests).

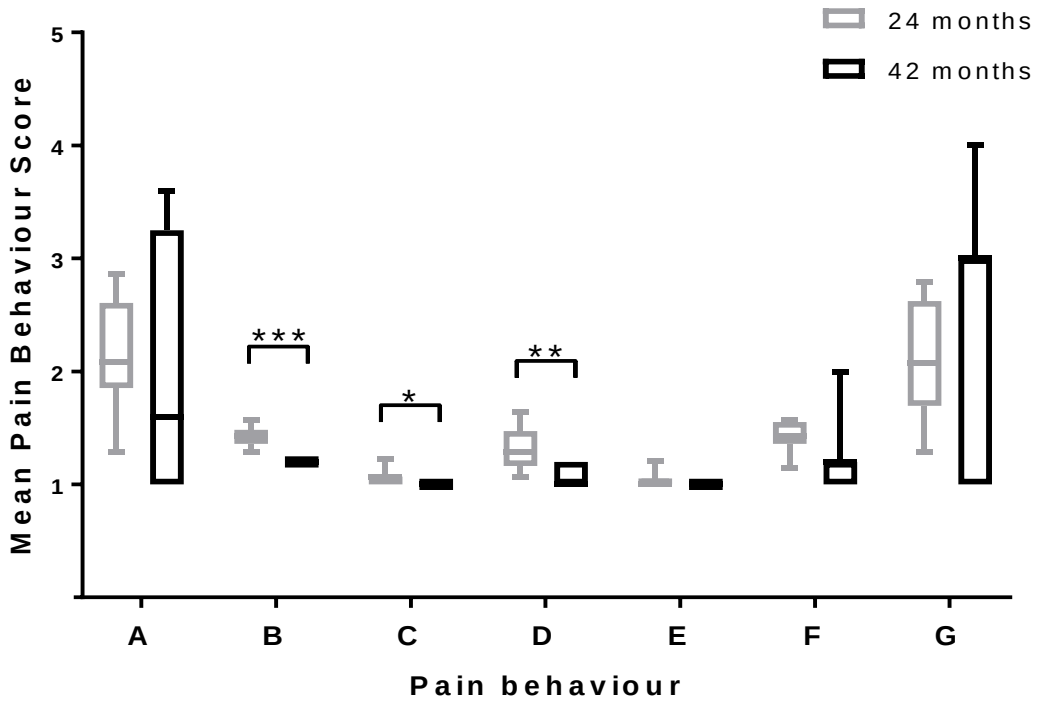


Figure 4.8: Pig pain behaviour. A: lameness, B: activity on arrival, C: response to touch, D: willingness to ambulate, E: vocalisation, F: response to human interaction, G: hind leg lameness. * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Mann-Whitney tests. Box plot with whiskers showing the minimum and maximum scores and a median line.

There was a positive correlation between lameness in 42 month old pigs and their willingness to ambulate ($r^2 = 0.8660$, p = 0.0360, Spearman's rank correlation). Lameness in 24 month old pigs positively correlated with their vocalisation scores and response to touch ($r^2 = 0.7640$, p = 0.0270, and $r^2 = 0.6260$, p = 0.0110 respectively, Spearman's Rank Correlation).

4.3.5 Association of porcine pain behaviours and knee osteoarthritis

There was a slight statistically significant positive correlation between macroscopic chondropathy and hind leg lameness of each leg across all animals ($r^2 = 0.4812$, $p = 0.0173$, Spearman's Rank Correlation). This correlation was strengthened when looking at an animal's average macroscopic chondropathy score and hind leg lameness ($r^2 = 0.6260$, $p = 0.0125$, Spearman's Rank Correlation). The chondropathy severity of the medial femoral condyle correlated most strongly with hind leg lameness ($r^2 = 0.6368$, $p = 0.0107$, Spearman's Rank Correlation). However, no association was identified between particular leg weakness traits and OA severity or different pain behaviours.

Although there was a correlation between lameness and OA severity there was not an absolute agreement between the two factors. Of the three animals identified as having a normal gait, all knees showed OA that ranged in severity from mild to severe cartilage erosion. There were also differences in chondropathy severity of asymptomatic and symptomatic OA within age groups, but no difference between age groups ($p = 0.0093$ and $p = 0.4087$ respectively, two-way ANOVA, see Figure 4.9). Chondropathy severity in symptomatic OA was significantly worse than in asymptomatic OA in 42 month old pigs, but there was no difference in 24 month old pigs ($p = 0.0003$ and $p = 0.8703$ respectively, Sidak's multiple comparisons, see Figure 4.9).

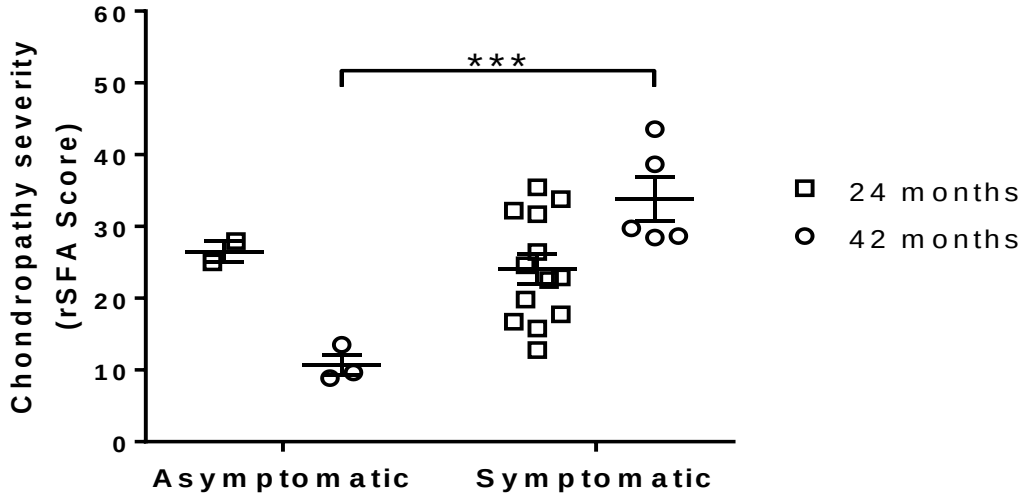


Figure 4.9: Chondropathy severity in asymptomatic and symptomatic osteoarthritis. Data presented as mean ± SEM, ***p < 0.001, two-way ANOVA with Sidak's multiple comparisons.

4.4 Discussion

The work presented in this chapter characterised pain behaviours and pathological features associated with spontaneous knee OA in skeletally mature breeding age commercial pigs and compared different methods of assessing macroscopic OA severity. Identifying the prevalence and pathological features of spontaneous porcine knee OA will allow the disease to be evaluated as a model of human disease.

4.4.1 Validation of the revised SFA scoring system's use in pigs

Macroscopic scoring of OA chondropathy in the porcine knee joint using the rSFA system displayed good repeatability between different observers and between the same observer's repeated scoring. This was consistent with the scoring system's use in humans (Walsh et al., 2009). A comparison of the rSFA and other macroscopic scoring methods previously reported in pigs was conducted to evaluate the use of rSFA scoring in pigs, and all scoring systems showed a high correlation to rSFA scoring. However, rSFA scoring displayed a number of advantages over the other methods.

Firstly, the rSFA system takes into account the exact percentage area of each lesion type. The method of Hennerbichler et al. (2008) and the OARSI scoring method only indicated whether a lesion exceeded a certain threshold percentage area or size. This makes the rSFA more sensitive to subtle differences in OA lesion type and size. The rSFA scoring system also produces continuous data, whereas other systems are scored on an ordinal scale, making them even less sensitive. Furthermore, some of the scoring methods previously used in pigs do not necessarily capture the OA pathology effectively. The method of Kirk et al. (2008) scored erosions and ulcerations to the cartilage separately, but also scored factors such as cartilage repair and infolding, which were not conducted in this study as these changes are more indicative of conditions such as OC. Similarly, the method of Ryan et al. (2010) scored animals for both OC and OA, with factors such as disturbed endochondral ossification included in the system.

Rodent studies using rSFA scoring of photographs have previously shown a poor correlation compared to scoring the joint directly (Nwosu, 2015). This partially results from the scoring system using cartilage colour to indicate cartilage surface integrity, as capturing photographs of the joint is greatly affected by lighting and camera angle, which can alter the perceived cartilage colour. To overcome this issue, at the time of photographing, a scoring sheet was annotated to indicate lesions. This also allowed recording of accidental damage made to the cartilage during the dissection process. Rodent studies have suggested that rSFA scoring is not as effective as other OA scoring methods (Nwosu, 2015). However, this is largely due to the small size of rodent joints, which is not an issue for studying pigs due to their comparative size to humans. These findings suggest that rSFA scoring is a suitable and sensitive method for assessing OA in porcine knee joints.

4.4.2 Porcine knee joint osteoarthritis

This study identified spontaneous OA in commercial pig knee joints, which ranged from mild erosion of the cartilage to severe erosion exposing the subchondral bone. This progressive breakdown in articular cartilage integrity was consistent with spontaneous OA identified in humans and a number of animal species including guinea pigs, mice, Syrian hamsters and non-human primates (Bendele, 2001). Histological examination of cartilage also identified features consistent with OA, including fibrillation and changes in chondrocyte density (van den Berg, 2011). The porcine articular cartilage was approximately 1.1 mm thick. This was thinner than seen in other studies of porcine knee joints, which measured the thickness at 1.5 - 2 mm (Hembry et al., 2001; Chiang et al., 2005; Ahern et al., 2009). This is likely due to cartilage thinning because of OA; articular cartilage has been reported to reduce by 0.2 - 0.4 mm per year in human OA (Buck et al., 2013; Eckstein et al., 2015).

The present study found a high prevalence of spontaneous OA in the knee joint of commercial pigs, with all but one joint presenting osteoarthritic changes. This OA prevalence is consistent with the study of Ryan et al. (2010), which found a 100% OA prevalence in the forelimbs of breeding sows. The current finding is, however, higher than other studies which identified an OA incidence ranging from 42% to

88% (Kirk et al., 2005; Kirk et al., 2008). These studies did not cite the age of animals used, but as they assessed the joints for signs of OC as well as OA, it suggests that these pigs may have been younger than those used in the present study. As OA is strongly linked to ageing (Anderson and Loeser, 2010), and this may explain why the prevalence identified here is higher than some studies. Due to this age-related nature of OA, it was expected that 42 month old pigs might have worse OA but there was no difference in macroscopic or microscopic pathology scores of the 24 and 42 month old pigs, with the exception of cartilage chondrocyte density. Older pigs had fewer chondrocytes within the cartilage, which is associated with more severe OA (Stoppiello et al., 2014). Furthermore, 42 month old pigs displayed more joints with subchondral bone exposure than 24 month old pigs, suggesting that more of the 42 month old pigs were reaching end-stage disease.

Examination of different knee joint surfaces identified the medial aspects of the femoral condyle and tibial plateau as showing the most severe OA. This is likely due to biomechanical factors affecting the joint as increased loading is placed on the medial surfaces. This loading is further increased when conformational changes occur, such as cow-hocked hind legs or a varus malalignment (Sharma et al., 2000; Heijink et al., 2012). This is consistent with studies of human OA and other joint problems that have identified the medial joint aspect as the most commonly affected (McAlindon et al., 1992; Curl et al., 1997; Thorp et al., 2007). The lower OA pathology found in the patella is likely due to reduced load bearing experienced by this surface, particularly its superior aspect, compared to the femoral condyle and tibial plateau (Erasmus, 2002).

Severity of OA identified using histology only correlated very slightly with severity identified macroscopically. It might have been expected that a stronger correlation would have been seen as changes at a cellular level will associate with gross pathological changes, but this did not occur. However, the correlation strength was similar to other studies of human and rodent OA that compared microscopic pathology and macroscopic chondropathy determined using photographic scoring (Walsh et al., 2009; Nwosu, 2015). Histological assessment of joint tissue allows

identification of extent and severity of cellular changes, particularly chondrocyte density and cell cloning (Janusz et al., 2002; Pritzker et al., 2006). However, histological assessment only allows for a small portion of the joint to be assessed, especially in animals with large joints like pigs. It was not feasible to section the entire joint so only a small sample was analysed, which may explain why severity of OA identified macroscopically was not entirely reflected in the histopathology.

Osteoclasts were identified and counted using a commercially available TRAP staining kit. In rodent studies, osteoclasts increase during OA and their inhibition prevents pain and cartilage loss (Strassle et al., 2010; Sagar et al., 2013). The present study contradicted these findings as osteoclast number did not associate with the histopathology and negatively correlated with macroscopic severity. It is likely that, as with the histopathology, this is a reflection of the small size of bone samples taken and the joint surface location used. Analysis of a larger number of bone samples or from areas showing more severe OA or osteophyte formation, which was not feasible in this study, is required to evaluate further the association of osteoclasts and spontaneous porcine OA.

4.4.3 Pig pain behaviour and its association to osteoarthritis

The present study identified a high incidence of lameness and pain-related behaviours that was much higher than other studies which have identified an 11-20% incidence of lameness (Kilbride et al., 2009). This could partly be due to the small number of animals which allowed a more thorough assessment of lameness to be carried out, or due to an underreporting of lameness that is thought to occur in the UK pig herd (DEFRA, 2004; UFAW, 2015). In addition, the pigs used in this study were old and reaching the end of their commercial lifespan, which may have increased the incidence of lameness compared to studies of younger breeding age pigs. Furthermore, as with the younger pigs, the animals transport prior to the study may have increased the incidence of lameness (Kephart et al., 2010).

The different pig age cohorts showed no difference in prevalence and severity of lameness but did show slight differences in other behavioural parameters. The 24

month old pigs were more active on initial observation, which was likely a result of their age as older animals appeared to spend more time sleeping and generally being inactive. Younger pigs also showed an increased responsiveness to touch and reduced willingness to ambulate, although the score range across all animals was very small. This may have resulted from a confounding factor caused by the reduced activity of older pigs. As they were less active, it was more difficult to measure their responsiveness or willingness to move, whereas for the more active younger pigs the changes in behaviour were more easily identified.

Knee joint OA causes pain and stiffness, affecting gait and increasing pain behaviours in humans and animal models. The association of pain and OA occurs due to tissue damage, and pain can become chronic due to sensitisation of the peripheral and central nervous systems (McMahon et al., 2013). Hind leg lameness, which indicates more specifically problems occurring in the knee joint than total lameness, correlated with the pig's knee joint chondropathy. Other pain behaviours did not correlate to OA pathology; this was likely due to their non-specificity to the knee joint as they indicate pain across the entire body. It is likely that the pigs had OA in other joints (such as the elbow and ankle), which would influence behaviour, but these joints were not examined. The findings of this study, therefore, indicate pigs suffering from knee OA are experiencing pain that increases lameness of the affected limb. However, there were a number of cases of subclinical or asymptomatic OA, where lesions to the joint were identified but the animals showed no lameness. This is a similar finding to other studies that have identified many pig joint diseases as subclinical in the early stages (Dewey et al., 1993; Etterlin et al., 2014).

It is hypothesised that a threshold of joint damage, in either severity or location, is required before an animal will present with lameness (Jørgensen, 1995). This was seen in 42 month old pigs in the present study, as chondropathy severity of symptomatic OA was significantly higher than asymptomatic OA, but this was not seen in younger pigs. This difference may have resulted from the chondropathy range seen in the different age groups. The 42 month old pigs had some animals

with very mild chondropathy but the rest showed severe chondropathy, whereas the 24 month old pigs had a much smaller severity range. Furthermore, changes occurring outside the knee, such as ankle OA or claw lesions, will also influence lameness but were not assessed in this study. This could have influenced the onset of lameness independently to knee joint OA severity. The findings suggest that symptomatic disease becomes more likely as chondropathy worsens and increased pain behaviours are associated with worse joint pathology. However, a number of other factors are also involved in the onset of lameness in pigs.

4.5 Conclusions

Adult female pigs suffered from a high prevalence of knee joint OA, which ranged from mild cartilage erosion to severe erosion exposing subchondral bone. Severity of OA correlated with lameness severity, indicating knee OA is a painful condition in pigs. The revised SFA system for scoring macroscopic OA was identified as a simple, reliable and sensitive measure of the gross OA pathology in pigs. Its use allowed a more sensitive OA severity assessment to be conducted than other methods previously reported in pigs, whilst also allowing porcine OA severity to be directly compared to human OA severity.

Chapter 5

Inflammation of the Synovium and its Association with Porcine Joint Disease

5.1 Introduction

The synovium is a layer of tissue lining the joint capsule that encases joints such as the knee. The synovium has an intima layer of macrophages, fibroblasts and synoviocytes, and a subintima layer containing the blood supply, lymphatics and adipose tissue (see section 1.5.3). The healthy synovium membrane layer is usually 1-2 cells thick and is central to maintaining a healthy joint by secreting components of the synovial fluid (Smith, 2011). When normal homeostasis of the joint is disrupted, the synovium can become inflamed (synovitis), which results in a number of changes that can be examined macroscopically and histologically. A macroscopic examination can identify features such as discolouration, hyper-vascularity, fibrillation or thickening of the membrane. Imaging techniques, such as magnetic resonance imaging and ultrasound, have been used to identify synovial effusion and changes in synovium volume (Sellam and Berenbaum, 2010). However, the majority of studies assess synovitis using histology (Cook et al., 2010; Sellam and Berenbaum, 2010). Inflammation results in hyperplasia of the membrane layer, increased inflammatory cell infiltration and angiogenesis (Revell et al., 1988; Ashraf et al., 2010; Stoppiello et al., 2014; Cruz et al., 2015). These histological changes are consistent across multiple species, including mice, rats, horses, dogs, sheep, pigs and humans (Cook et al., 2010; Gerwin et al., 2010; Glasson et al., 2010; Little et al., 2010; McIlwraith et al., 2010). However, despite these synovitis-associated cellular changes being well characterised, the link between synovial inflammation and porcine joint disease is unclear.

Due to the synovium's role in supporting healthy cartilage, examining synovial changes during disease may offer greater insight into disease mechanisms than, for example, changes in plasma, which indicate a more general health status (Billinghurst et al., 2004). A number of studies have tried to identify molecular changes occurring in the synovium or synovial fluid during equine OC, in order to understand underlying disease mechanisms and identify possible diagnostic or prognostic markers (McIlwraith, 2005). There has also been a lot of interest in the involvement of inflammation in OA as it has been identified as an important part of

the disease's pathogenesis (Fell and Jubb, 1977). As yet, however, the underlying molecular mechanisms of porcine OC and OA are unclear.

Inflammatory cytokines, both pro- and anti-, are commonly examined for their involvement in OA as they tend to be locally acting so reveal localised processes within a tissue (Vilček and Feldmann, 2004). Cytokines are involved in a variety of processes including inflammation, tissue degeneration and repair, and they act as immunomodulatory proteins to mediate cell-cell interactions (Goldring and Goldring, 2004). In healthy synovium, cytokines are present at low levels to maintain normal homeostasis (Smith, 2011). However, cytokines also play a central role in joint inflammation and cartilage destruction (Goldring and Goldring, 2004). During inflammation, there is increased cytokine production and release, predominantly from synovial macrophages but also fibroblasts (Bondeson et al., 2006). These cytokines can enter the synovial fluid and stimulate further cytokine and proteinase release within the joint. Although multiple mammalian cytokines have been identified, the pro-inflammatory cytokines IL-6, IL-1β and TNFα have gathered the most interest in relation to human and animal joint disease.

In its active form IL-1β consists of 153 amino acids (Mosley et al., 1987) and stimulates the release of chemokines, cytokines, inflammatory mediators and enzymes, which can induce further inflammation and tissue damage (Guerne et al., 1990; Roman-Blas and Jimenez, 2006). In addition, IL-1β suppresses the production of type II collagen and aggrecan, which are constituent components of cartilage (Stöve et al., 2000). IL-1β also causes chondrocytes to release MMPs that break down collagen, worsening cartilage damage (Monemdjou et al., 2010). TNFα is made of 157 amino acids and promotes the release of other cytokines, including IL-6 and IL-8 (Guerne et al., 1990; Wojdasiewicz et al., 2014). TNFα's actions mostly coincide with IL-1β, blocking type II collagen and proteoglycan synthesis and promoting MMPs release from chondrocytes (Saklatvala, 1986). IL-6 is a pro-inflammatory protein comprised of 184 amino acids (Hammacher et al., 1994), whose synthesis or release can be stimulated by increased IL-1β and TNFα or in response to injury (Guerne et al., 1990; Sui et al., 2009). In animal models, IL-6 has

been shown to act synergistically with IL-1β and TNFα to inhibit type II collagen production and increase MMP production (Porée et al., 2008). IL-6 also promotes osteoclast activity and stimulates cytokine release from osteoblasts, contributing to disruption in the subchondral bone (Sakao et al., 2009).

The actions of cytokines produced during inflammation may help to repair the damaged joint but could also worsen the pathology by initiating further cartilage breakdown and pain. IL-1β, IL-6, and TNFα have all been shown to be involved in the pathology of horse joint disease but limited studies of porcine joint disease have been conducted. Studies of equine joint diseases have identified increased IL-6, IL-1β and TNFα in both the synovium and synovial fluid (Bertone et al., 2001; Kamm et al., 2010). In human OA, the release of cytokines can cause different pain characteristics due to their ability to sensitise nerve endings. In this respect increased IL-6 and IL-1β are associated with movement-induced pain, whereas elevated TNFα is associated with worse total pain (Orita et al., 2011a; Orita et al., 2011b; Ashraf et al., 2013; Leung et al., 2017).

5.1.1 Aim

The aim of the work presented in this chapter was to characterise synovial inflammation in the porcine knee joint associated with joint disease. Synovial tissue was assessed for hyperplasia of the membrane layer. The amount of cytokine protein was determined using a fluorescent microsphere immunoassay (FMIA) and mRNA expression determined using qPCR. Alterations in cytokines associated with synovitis, OC and OA were assessed to determine the involvement of inflammation in porcine joint diseases.

5.2 Methods

5.2.1 Animals

The same four age cohorts of clinically healthy female commercial pigs of the crossbred genotype 'Landrace x Large White x Duroc' were used in this study. Pigs were aged 9 months (n = 6), 15 months (n = 6), 24 months (n = 8) and approximately 42 months (n = 7).

Pigs were assessed prior to slaughter for pain behaviours and lameness (see section 2.2). Both knee joints of 9 and 15 month old pigs were assessed macroscopically for OC (see section 2.4.1). Both knees of 24 month old pigs and one knee from each 42 month old pig were assessed macroscopically for OA (see section 2.4.2). In pigs showing joint disease (OC or OA) but a normal gait, the disease was termed asymptomatic. In pigs showing joint disease and lameness, the disease was termed symptomatic. Samples of synovial fluid were collected and frozen post-mortem (see section 2.3.3). Excised synovial tissue was either frozen or mounted in OCT. Synovial tissue sections were routinely stained with H&E and assessed for synovitis, indicated by hyperplasia of the membrane layer (see section 2.7). Non-inflammatory joint disease was identified when no synovitis was present in joints with OC or OA; when synovial hyperplasia was present, the disease was termed inflammatory. The expression of cytokine mRNA in synovial tissue was determined, relative to the ACTB and PPIA housekeeping genes, using qPCR (see section 2.8). The amount of cytokine protein in synovial tissue and fluid was determined using a fluorescent microsphere immunoassay (FMIA).

5.2.2 Synovial tissue and fluid cytokine protein concentration

The concentration of IL-6, IL-1β and TNFα in synovial tissue and fluid was determined using a FMIA. Synovial tissue was homogenised to extract the cytokines before conducting the FMIA with a Bio-Plex® 200 system (Bio-Rad, UK). Concentrations of cytokines were expressed relative to the sample's total protein concentration.

5.2.2.1 Synovial tissue homogenisation and cytokine extraction

Cytokines were extracted from synovial tissue by homogenisation in PBS. Approximately 200 mg of frozen tissue was added to 2 ml of sterile PBS and homogenised over ice using a hand-held homogeniser (VWR VDI12). Homogenates then underwent two freeze-thaw cycles before centrifuging at 5000g for 5 minutes. Supernatants were collected, aliquoted and tissue lysates immediately frozen at -80°C. Some samples required a second centrifugation due to a high fat content. A pooled synovial fluid sample from four pigs was created for validation experiments. The fluid was centrifuged at 1000g for 15 minutes and the supernatant collected and stored at -80°C.

5.2.2.2 Bradford protein assay

The protein concentration of synovial tissue lysates was determined using a Bradford assay. The assay was run according to the manufacturer's instructions (Bio-Rad Quick start Bradford assay). Briefly, 5 µl of synovial tissue lysate (diluted 1:5 in water) or protein standard (Bio-Rad Quick Start BSA Set) was added to 250 µl of assay dye in a 96-well plate and incubated for 5 minutes. The plate was then read using a 595 nm spectrophotometer (Multiskan^{TM} FC Microplate Photometer, Thermo Fischer Scientific) to determine total protein concentration.

5.2.2.3 Coupling of capture antibodies to magnetic microspheres

For each cytokine, the relevant capture antibodies were coupled to magnetic microspheres (beads) using the Bio-Plex® amine coupling kit (Bio-Rad) according to the manufacturer's instructions. Briefly, 100 µl of fluorescently distinct microspheres (1.25 x10^7 magnetic COOH microspheres, Bio-Rad, see Table 5.1) were separated from their supernatant using a magnetic separator and washed in 100 µl wash buffer by vortexing. The supernatant was removed by magnetic separation and beads suspended in 80 µl activation buffer. To this, 10 µl of 50 mg/ml N-hydroxysulfosuccinimide (Thermo Fischer Scientific) and 10 µl of 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Thermo Fischer Scientific), both prepared in bead activation buffer, was added, vortexed and incubated for 20 minutes at room temperature in the dark whilst shaking.

After incubation, 150 µl PBS was added, vortexed and the supernatant removed using magnetic separation. This step was repeated before suspending the beads in 100 µl PBS. Recombinant capture antibody (R&D systems porcine DuoSet ELISA capture antibodies) was added, vortexed and incubated for 2 hours in the dark whilst shaking. The supernatant was removed and the now coupled beads suspended in 500 µl PBS. The supernatant was removed again and the beads suspended in 250 µl blocking buffer, vortexed and incubated for 30 minutes in the dark whilst shaking. The supernatant was removed and the beads suspended in 500 µl storage buffer. The supernatant was removed again and beads suspended in a final volume of 150 µl storage buffer. The concentration of coupled beads was determined using an automated cell counter (Bio-Rad TC20, see Table 5.1 for concentration). The coupled beads were stored in the dark at 4°C until use.

5.2.2.4 Quantification of cytokines using a multiplex FMIA

Concentrations of IL-6, IL-1β and TNFα were measured using the Bio-Rad multiple cytokine Bio-Plex® FMIA. The optimal assay buffer was 81% distilled water, 10% reagent diluent (R&D systems) and 9% heat-inactivated foetal calf serum (Gibco). For the FMIA, 50 µl of coupled beads were added to a 96-well, black, flat-bottomed plate (Bio-Rad) and washed twice with wash buffer using a Bio-Plex® Pro II Wash Station (BioRad). To this, 50 µl of recombinant protein standards (R&D systems porcine DuoSet ELISA recombinant standard), synovial samples or controls were added. The plate was then incubated in the dark whilst shaking for 2 hours at room temperature. The plate was washed three times and 100 µl of detection antibody was added and incubated for 1 hour in the dark whilst shaking. Detection antibodies were diluted in assay buffer to 0.5 µg/ml for TNFα and IL-1β, and to 0.75 µg/ml for IL-6 (R&D systems porcine DuoSet ELISA biotinylated detection antibodies, see Table 5.1). The plate was washed three times and 50 µl streptavidin-PE (Bio-Rad) added and incubated for 30 minutes at room temperature in the dark whilst shaking. After washing three times, 125 µl assay buffer was added per well and the plate incubated for 5 minutes before reading using the Bio-Rad Bio-Plex® 200® system. Mean fluorescent intensity (MFI) for 100 microspheres, corresponding to

each individual cytokine analyte, was recorded for each well, indicating the cytokine content in each sample.

Table 5.1: Cytokines, beads and antibodies used for the multiplex fluorescent microsphere immunoassay (FMIA).

Cytokine	Bead region	Coupled bead concentration (bead/µl)	Capture antibody (µg/ml)	Detection antibody (µg/ml)	Source
IL-6	26	6000	Goat anti-porcine (360)	Biotinylated goat anti-porcine (0.75)	R&D Systems #DY686
IL-1β	29	5930	Mouse anti-porcine (144)	Biotinylated goat anti-porcine (0.50)	R&D Systems #DY681
TNFα	35	6980	Mouse anti-porcine (1440)	Biotinylated mouse anti-porcine (0.50)	R&D Systems #DY960b

5.2.2.5 Bio-Plex® FMIA validation studies

The use of the Bio-Plex® FMIA with porcine synovial tissue and fluid was validated using a number of measures. Four samples of synovial tissue lysate, representing the full range of sample protein concentrations, were selected for validation studies. The synovial tissue lysates were run at different dilutions (neat or 1:2, 1:5 and 1:10 dilutions in PBS) to determine any effect of dilution. The synovial tissue lysates were also spiked with a known amount of cytokine protein standard, of concentrations 4000 pg/ml, 640 pg/ml or 102.4 pg/ml (50 µl sample and 50 µl standard), to assess cytokine recovery. The recovery of the known amounts of cytokines had to be within 70-130% of the expected concentration to be regarded as successful (Hall et al., 2015).

For synovial fluid validation, the pooled sample was run at different dilutions (neat, 1:5 and 1:10 in PBS) to determine any dilution effects. The synovial fluid was also incubated with a blocking buffer of either bovine plasma (Biosera) or a mixture of

horse and goat serum (50% each, both Sigma). The synovial fluid was incubated (1:1) with blocking buffer for 30 minutes at room temperature before centrifuging at 1000g for 15 minutes. A sample of neat and incubated synovial fluid was then spiked with a known amount of cytokine standard to assess cytokine recovery.

The repeatability of the assay was previously determined by preparing dilutions or recombinant cytokine standards and assaying in triplicate over a three day period and expressed as intra and inter-assay variations. Intra-assay variation was evaluated by analysing multiple replicates (n = 3) of the cytokine standards of known concentration during a single assay run. Inter-assay variability was determined using a range of standard concentrations analysed in triplicate over three consecutive days (conducted by Sarah Hall and Jenny Coe, SRUC). Intra and inter-assay variations were expressed as the coefficient of variation of the repeated measurements.

5.2.3 Statistical analysis

Data were tested for normality using a D'Agostino-Pearson normality test. Parametric data were analysed using a t-test or one-way ANOVA. Non-parametric data were analysed with a Mann-Whitney test or a Kruskal-Wallis test. Paired non-parametric data were analysed using a Wilcoxon test. Correlations between data were determined using Pearson's or Spearman's Rank Correlation. A significance of $p < 0.05$ was used for all statistical tests. Statistical analysis of qPCR data was conducted using $2^{-\Delta Ct}$ values (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Where statistical analysis was not appropriate due to small sample sizes, cytokine concentrations and expressions were assessed by looking at fold changes in expression ($2^{-\Delta\Delta Ct}$ method for qPCR data) or concentration.

5.3 Results

For a discussion of the prevalence and pathological features of osteochondrosis and osteoarthritis in the porcine knee joints, see Chapters 3 and 4 respectively.

5.3.1 Inflammation of the synovial membrane

Macroscopically healthy synovial tissue was smooth and appeared white or translucent. Healthy synovial membrane was 1-2 cells thick when examined histologically (see Figure 5.1). Inflammation of synovial tissue resulted in synovitis with fibrillation or thickening of the tissue, which became yellow in colour and sometimes appeared red due to increased vascularisation that was visible macroscopically. Histologically, the inflammation resulted in hyperplasia of the membrane layer, with 3 or more cells in this layer (see Figure 5.1).

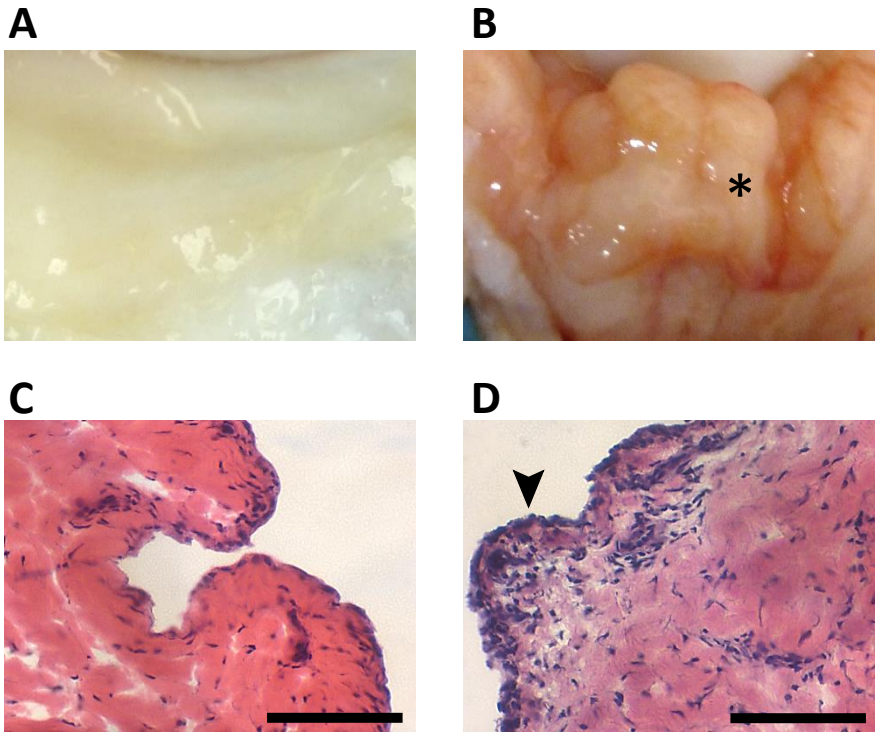


Figure 5.1: Inflammation of porcine knee synovial tissue. Representative photographs (A & B) and H&E stained sections (C & D) of pig synovial tissue. Healthy synovial tissue was smooth and translucent (A), with a membrane thickness of 1 - 2 cells (C). Inflammation caused fibrillation of the synovial tissue, which became yellow/red (*, B), and synovitis was evident by increased cellularity of the membrane layer (➤, D). Images of H&E sections taken at 20x magnification, scale bar 50 µm.

Synovitis, identified histologically, was seen in 27 of the 47 joints. Moderate synovitis, with 6-8 cells in the membrane, was seen in five joints. Severe synovitis, with more than 9 cells in the membrane layer, was seen in two joints. Synovitis presented bilaterally in many cases and there was no difference in synovitis severity of left and right knee joints in the youngest three age groups ($p = 0.9597$, Wilcoxon test). There was also no significant difference in synovitis severity between different age groups ($p = 0.3310$, Kruskal-Wallis test, see Figure 5.2). The 15, 24 and 42 month old pigs had synovitis ranging from absent to moderate synovitis (membrane thickness 6-8 cells). The 9 month old pigs showed synovitis ranging from absent to severe synovitis (membrane thickness > 9 cells) and was the only group to show severe synovitis which was present in both knee joints of one pig (see Table 5.2).

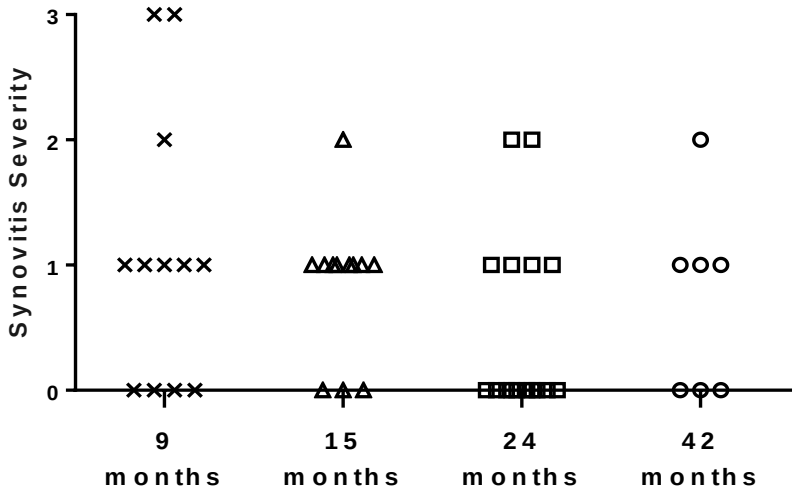


Figure 5.2: Severity of synovitis in knee joint of different age pigs. There was no difference in severity of synovitis between 9, 15, 24 and 42 month old pigs.

Table 5.2: Percentage of joints with synovitis in different age pigs.

Synovitis Score	% joints			
	9 months	15 months	24 months	42 months
0	34 (n = 4)	25 (n = 3)	63 (n = 10)	43 (n = 3)
1	42 (n = 5)	67 (n = 8)	25 (n = 4)	43 (n = 3)
2	8 (n = 1)	8 (n = 1)	12 (n = 2)	14 (n = 1)
3	16 (n = 2)	0	0	0

5.3.2 Association of synovitis with porcine joint disease and lameness

There was no correlation between severity of synovitis and OC in 9 or 15 month old pigs (r^2 = 0.4972, p = 0.1188 and r^2 = -0.2942, p = 0.4167 respectively, Spearman's Rank Correlation, see Figure 5.3A). However, synovitis was only seen in joints with OC, affecting 77% of them. There was no correlation between synovitis and OA severity in 24 month old pigs (r^2 = -0.0683, p = 0.8037, Spearman's Rank Correlation), but there was a strong positive correlation in 42 month old pigs (r^2 = 0.9258, p = 0.0143, Spearman's Rank Correlation, see Figure 5.3B). Furthermore, synovitis was only seen in joints with OA, affecting 45% of them.

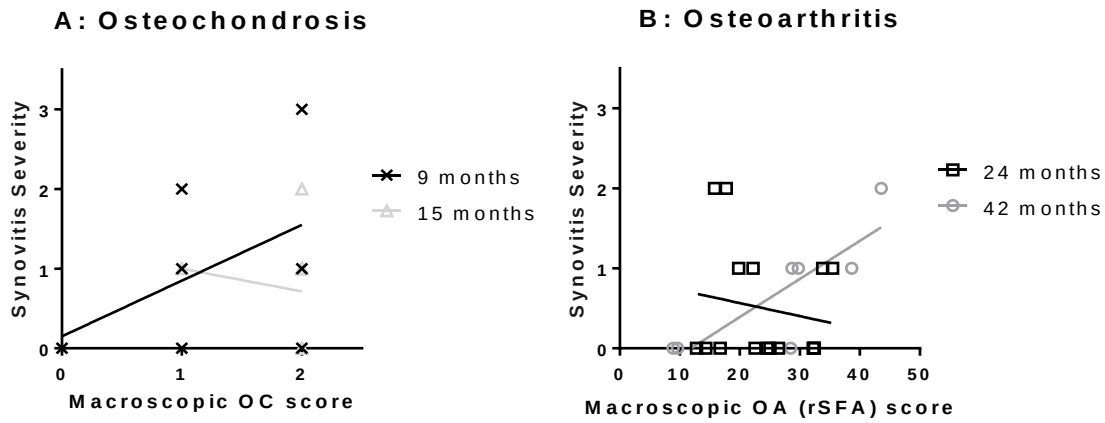


Figure 5.3: Association of synovitis with porcine joint disease. A) There was no correlation between synovitis and OC severity in 9 or 15 month old pigs. B) There was no correlation between synovitis and OA severity in 24 month old pigs but there was in 42 month old pigs (r^2 = 0.9258, p = 0.0028, Spearman's Rank Correlation). Scatter plots fitted with linear regression lines.

There was no significant correlation between hind leg lameness and synovitis across all age groups (r^2 = 0.2867, p = 0.0534, Spearman's Rank Correlation, see Figure 5.4A). Similarly, there was no significant correlation between total pain behaviour and synovitis severity (r^2 = 0.1959, p = 0.1820, Spearman's Rank Correlation). Furthermore, there was no statistical difference between synovitis seen in asymptomatic and symptomatic OC (p = 0.6439, Mann-Whitney test), but only symptomatic OC showed moderate to severe synovitis (see Figure 5.4B). There was

no statistical difference in synovitis severity of asymptomatic and symptomatic OA ($p = 0.0677$, Mann-Whitney test). However, synovitis was only seen in symptomatic OA, suggesting synovitis may be associated with a worse OA state (see Figure 5.4B). It was also noted that the pig with severe synovitis (score 3) showed the most severe total pain, which combined with the increased synovitis severity associated with symptomatic disease, suggests that worse synovitis may be associated with increased pain in pigs.

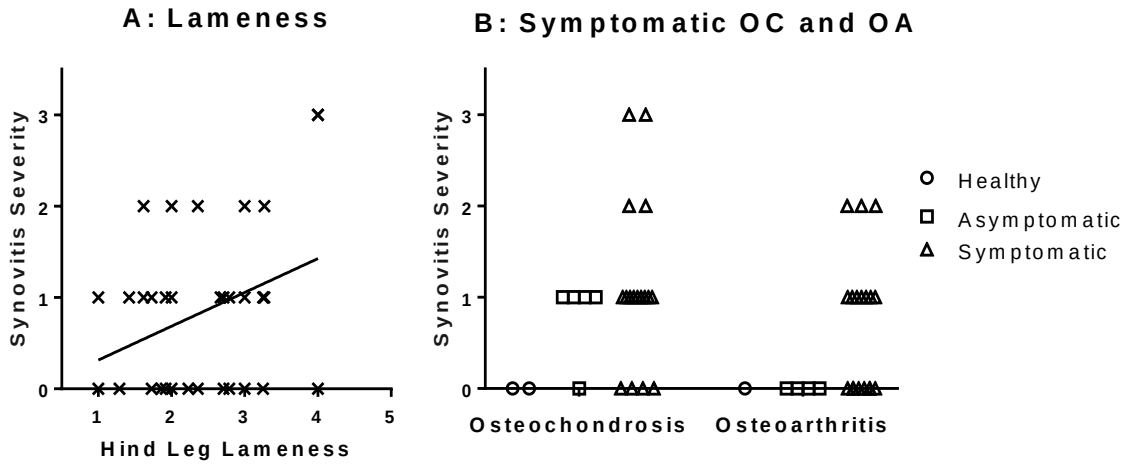


Figure 5.4: Association of synovitis with porcine lameness. A) There was no correlation between synovitis and hind leg lameness. Scatter plots fitted with linear regression line. B) There was no difference in the severity of synovitis seen in asymptomatic and symptomatic OC or OA.

5.3.3 Synovial tissue cytokine concentration determined using the FMIA

Standard curves were obtained for each recombinant cytokine standard (see Figure 5.5) and the observed concentration was within 86-120% of the expected concentration. However, when room temperature was below 16°C, the observed concentration was only 0-128% of the expected concentration, and no recombinant standard was detected below 102.4 pg/ml for IL-1β and 40.96 pg/ml for IL-6.

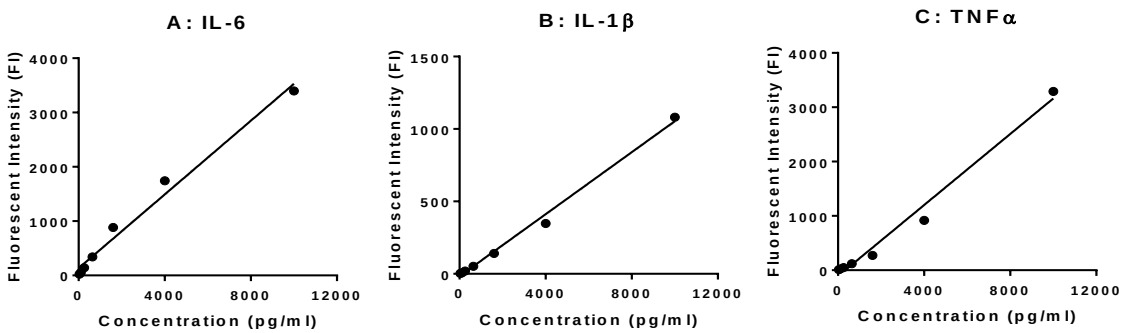


Figure 5.5: Standard curves of porcine cytokines obtained by the FMIA multiplex assay. Values are given as fluorescent intensity (FI) for serial diluted porcine recombinant cytokine standards for IL-6 (A), IL-1β (B) and TNFα (C).

IL-1β was detected at the highest concentration and was identified in all synovial tissue lysates, with a concentration range of 172.17 - 1493.95 pg/ml. IL-6 was detected in 98% of synovial tissue lysates and the concentration ranged from 0 - 515.89 pg/ml. TNFα was only detected in 17% of samples, so no further analysis on its association with disease could be conducted. In samples where the amount of TNFα was above the Bio-Plex® sensitivity threshold the concentration range was 11.74 - 176.87 pg/ml.

The average protein concentration of synovial tissue lysates determined using the Bradford assay was 1.61 mg/ml (range 0.17 - 3.4 mg/ml). There was no difference in protein content of synovial tissue lysates in different age pigs ($p = 0.2119$, one-way ANOVA). Due to this large range in protein concentrations, cytokine concentrations determined using the FMIA were expressed per mg protein to normalise for differences in initial sample weight and fat content.

5.3.3.1 Bio-Plex® FMIA validation studies

The intra-assay coefficient of variation (CV) for IL-6, IL-1β and TNFα was 3.73%, 3.72% and 4.64% respectively. The inter-assay coefficient of variation (CV) for IL-6, IL-1β and TNFα was 4.14%, 3.75% and 4.95% respectively. In Bio-Plex® multiplex FMIA assay validation studies, a dilution effect was seen in both synovial tissue lysates and pooled synovial fluid (see Figure 5.6). However, this dilution effect was

not consistent across dilutions or between samples; therefore all synovial tissue lysates were subsequently assayed undiluted.

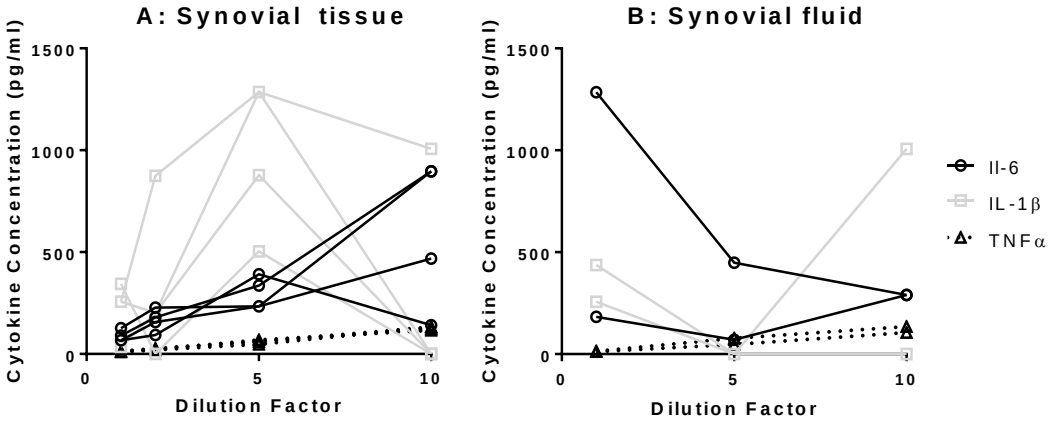


Figure 5.6: Effect of dilution on synovial tissue and fluid cytokine concentrations determined using the FMIA. Cytokine concentrations corrected for dilution factor.

Cytokine recovery in synovial tissue samples spiked with a known amount of recombinant standard was at acceptable levels for all cytokines. When room temperature was above 16°C, spike recovery of IL-6 was 93.94%, IL-1β was 98.63% and TNFα was 88.70%. Cytokine recovery in synovial fluid was much lower and not within the acceptable range of 70-130%. Incubation of synovial fluid with two different blocking buffers improved spike recovery of all cytokines (see Table 5.3), however this improvement was not sufficient to reach the acceptable cytokine recovery range.

Table 5.3: Synovial fluid percentage recovery following blocking buffer incubation. Percentage recovery of synovial fluid interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNFα).

Cytokine	% Recovery			
	Bovine plasma		Goat & horse serum	
	Untreated	Incubated	Untreated	Incubated
IL-6	13	22	4	18
IL-1β	5	60	29	64
TNFα	1	1	0	45

5.3.3.2 Synovial tissue cytokine concentrations in different age pigs

There was no difference in concentrations of IL-6, IL-1β or TNFα measured using the FMIA in different age pigs (p = 0.4491, p = 0.4357 and p = 0.8115 respectively, one-way ANOVAs, see Figure 5.7).

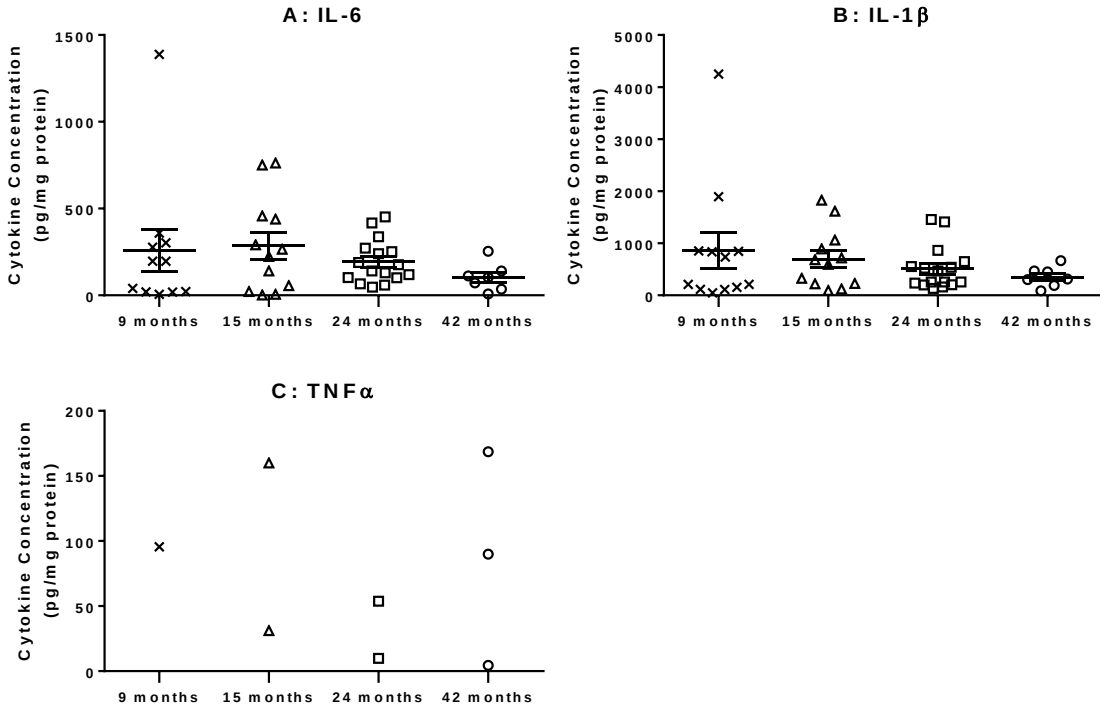


Figure 5.7: Synovial tissue cytokine concentration in different age pigs. There was no difference in synovial tissue cytokine concentration of different aged pigs for IL-6 (A), IL-1β (B) or TNFα (C), however TNFα was only detectable in 17% of samples (n = 8). Data presented as mean ± SEM.

5.3.4 Synovial tissue cytokine mRNA expression determined using qPCR

5.3.4.1 Stability of housekeeping gene expression

Starting RNA concentration ranged from 6.9 - 161 ng/ml and the RNA purity was within an acceptable range, with the ratio of absorbance at 260 nm and 280 nm above 1.8 (1.87 – 2.15). There was no amplification of the NTC and noRT controls (no C_t values obtained), indicating there was no reaction contamination, primer dimer formation or genomic DNA contamination. Expression of the housekeeping genes ACTB and PPIA varied in different samples due to initial RNA concentration

variations, but expression of ACTB and PPIA significantly correlated with one another ($r^2 = 0.975$, $p < 0.0001$, Pearson's Correlation). Expression of ACTB and PPIA also correlated with initial RNA concentration ($r^2 = 0.872$, $p < 0.0001$ and $r^2 = 0.857$, $p < 0.0001$ respectively, Pearson's Correlation), indicating they can control for variations in starting RNA concentration.

The expression of ACTB and PPIA was stable across different age groups ($p = 0.3095$ and $p = 0.1197$ respectively, one-way ANOVAs, see Figure 5.8). Expression of ACTB was stable across different disease states and was not changed by synovitis, OC or OA ($p = 0.1362$, $p = 0.5623$ and $p = 0.1362$ respectively, one-way ANOVA), as was expression of PPIA ($p = 0.0824$, $p = 0.4588$ and $p = 0.1093$ respectively, one-way ANOVA). As the expression of both genes was stable, the mean expression of ACTB and PPIA was calculated and used to determine ΔCt values for cytokine expression.

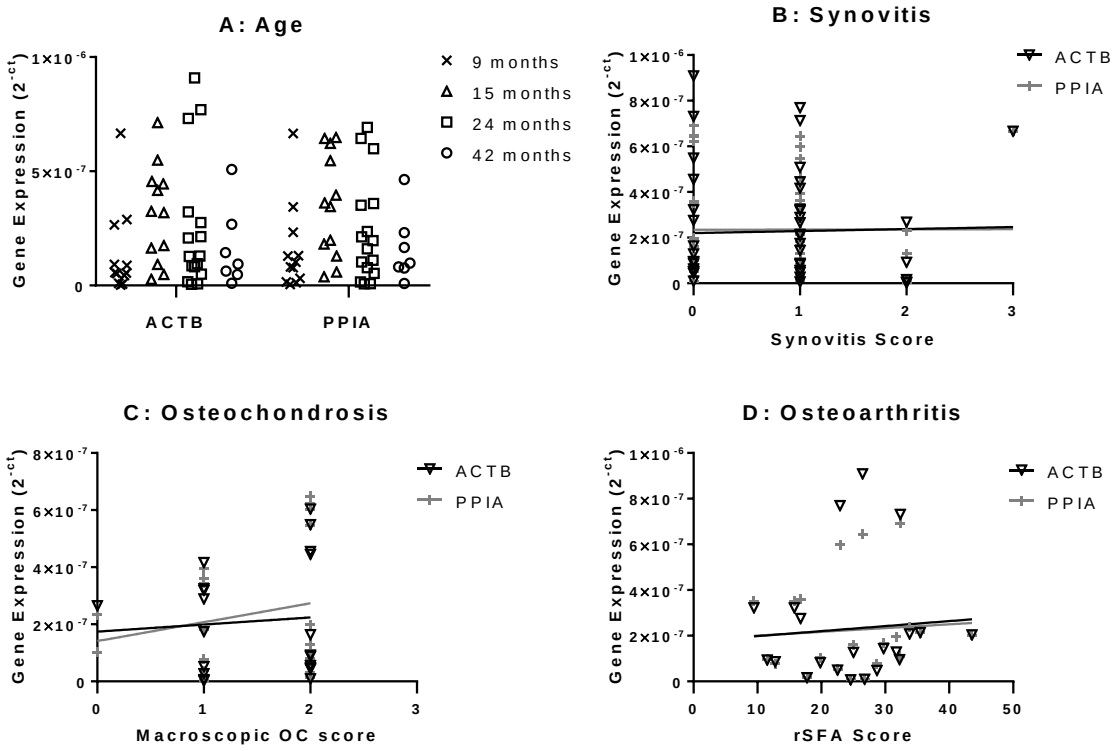


Figure 5.8: Expression of housekeeping genes ACTB and PPIA in different age pigs and different disease states. Data expressed as 2^{-Ct}. Expression of housekeeping genes was stable across different pig ages (A), synovitis severity (B), osteochondrosis severity (C) and osteoarthritis severity (D). Scatter plots fitted with linear regression lines (B, C, and D).

5.3.4.2 Synovial tissue cytokine mRNA expression in different age pigs

There was no difference in synovial tissue mRNA expression of IL-6, IL-1β or TNFα in different age pigs (p = 0.2445, p = 0.1893 and p = 0.2645 respectively, one-way ANOVA, see Figure 5.9).

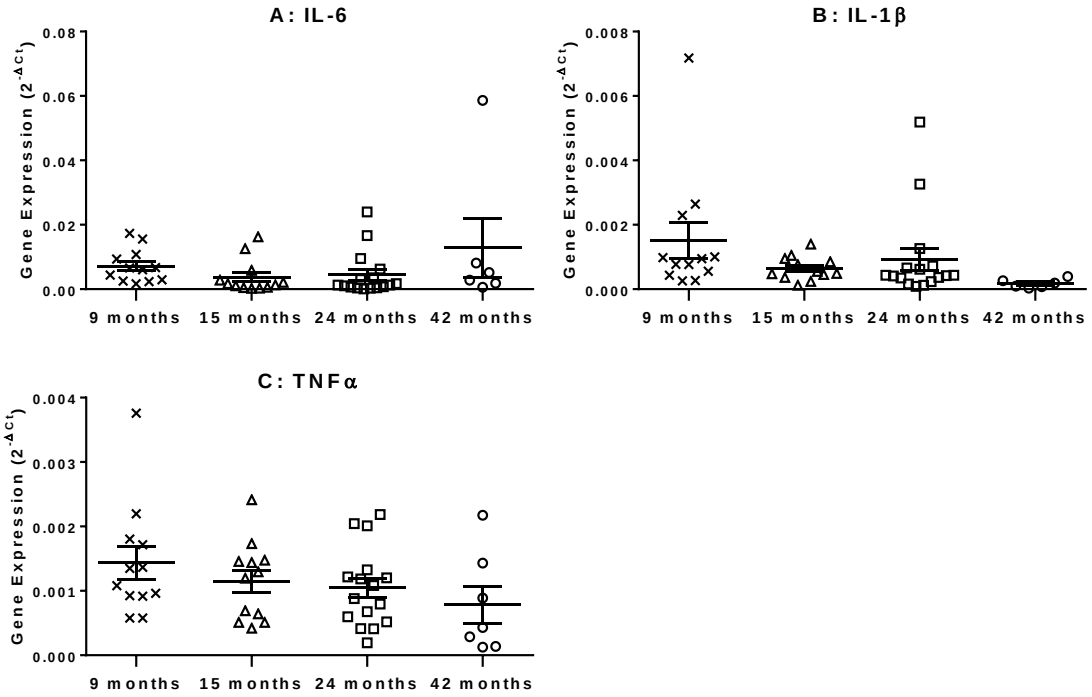


Figure 5.9: Expression of cytokines in synovial tissue of different age pigs. There was no difference in expression of IL-6 (A), IL-1β (B) or TNFα (C) between 9, 15, 24 and 42 month old pigs. Data presented as mean ± SEM.

5.3.5 Associations of synovial tissue cytokine protein concentrations and mRNA expression

There was a strong positive correlation between synovial tissue IL-6 and IL-1β protein concentrations determined using the FMIA ($r^2 = 0.954$, $p < 0.0001$, Pearson's Correlation). There was a slight positive correlation between mRNA expression of IL-1β and TNFα ($r^2 = 0.591$, $p < 0.0001$, Pearson's Correlation), but no correlations for IL-6 expression. There was no correlation between synovial tissue cytokine concentrations determined using the FMIA and mRNA expressions determined using qPCR for IL-6 or IL1β ($r^2 = 0.062$, $p = 0.686$ and $r^2 = -0.046$, $p = 0.785$ respectively, Pearson's Correlation).

5.3.6 Association of synovitis and synovial tissue cytokines

As there was no difference in cytokine concentration or expression associated with age, the data from all four age groups were pooled before analysis of cytokines associated with synovial inflammation. There was no statistically significant difference in the concentration of IL-6 and IL-1β protein in joints with synovitis (p = 033148 and p = 0.2335 respectively, t-test). However, it was noted joints with severe synovitis (score 3) there was 4 times the amount of IL-6 and 5 times the amounts of IL-1β than seen in healthy joints (see Figure 5.10).

IL-6 mRNA expression and synovitis severity had a slight but significant correlation (r^2 = 0.4157, p = 0.0041 Spearman's Rank Correlation) and IL-6 expression was significantly increased in synovitis (p = 0.0265, t-test). This equated to over a 3-fold upregulation in IL-6 expression, in severe synovitis this increased to a 9-fold upregulation compared to the healthy synovium ($2^{-\Delta\Delta Ct}$ 3.10 and 9.22, respectively). Expression of IL-1β or TNFα mRNA was also higher in joints with synovitis, but this was only just non-significant (p = 0.0642 and p = 0.0988 respectively, t-test). This equated to nearly 2-fold upregulation in IL-1β and TNFα ($2^{-\Delta\Delta Ct}$ 1.82 and 1.89, respectively).

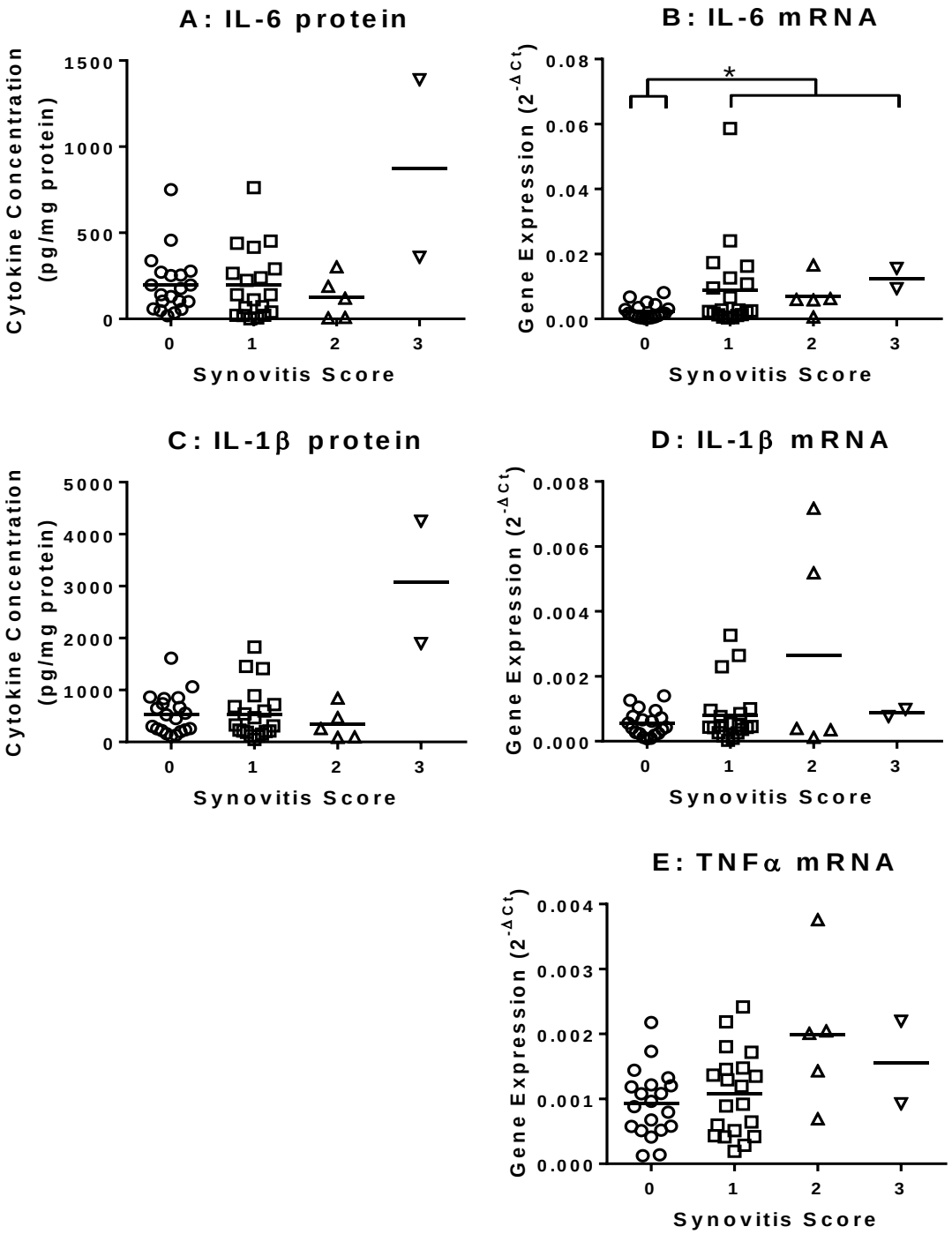


Figure 5.10: Synovial tissue cytokines associated with porcine knee synovitis. There was no difference in IL-6 (A) and IL-1β (C) protein concentrations or IL-1β (D) and TNFα (E) mRNA expressions in synovitis, but IL-6 mRNA expression was increased in synovitis (B) across all pigs studied. *$p < 0.05$, t-test. Lines represent means.

5.3.7 Association of osteochondrosis and synovial tissue cytokines

As there was no difference in cytokine concentration or expression associated with the age and no difference in OC severity of 9 and 15 month old pigs, the data from the two age groups was pooled before analysis of cytokines associated with OC. The small number of pigs without OC made it difficult to determine its effect on synovium cytokines. There was no statistical difference in cytokine protein concentrations or mRNA expressions in the different OC severities (see Figure 5.11). However, in OC the mean expression of TNFα was 2 times higher than in healthy joints ($2^{-\Delta\Delta Ct}$ 2.05), and the highest cytokine concentrations or expressions were always found in joints with OC.

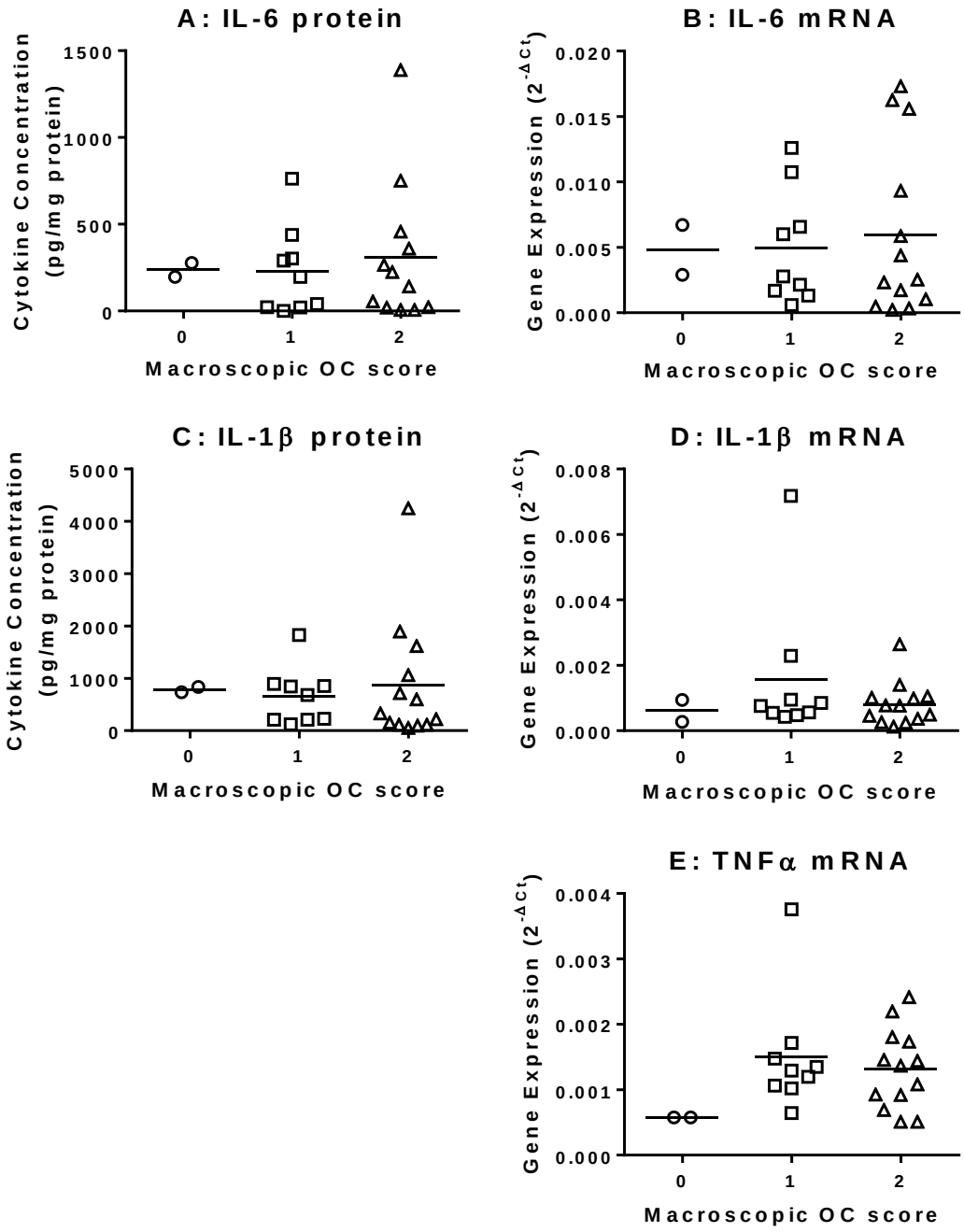


Figure 5.11: Synovial tissue cytokines in porcine knee osteochondrosis. There was no statistical difference in IL-6 (A) or IL-1β (C) protein concentration or IL-6 (B), IL-1β (D) or TNFα (E) mRNA expression in OC. Lines represent means.

Due to the small number of healthy pigs and small severity range, it was not possible to determine statistically the effect of an interaction between OC and synovial inflammation on cytokines. There was no difference in IL-6 or IL-1β protein in joints with non-inflammatory and inflammatory OC ($p = 0.4402$ and $p = 0.4959$

respectively, t-test). There was also no difference in mRNA expression of IL-1β and TNFα in non-inflammatory and inflammatory OC (p = 0.3016 and p = 0.2238 respectively, t-test), but IL-6 was significantly higher in inflammatory OC (p = 0.0405, t-test). Furthermore, the highest cytokine levels and expressions were always seen in joints with inflammatory OC (see Figure 5.12).

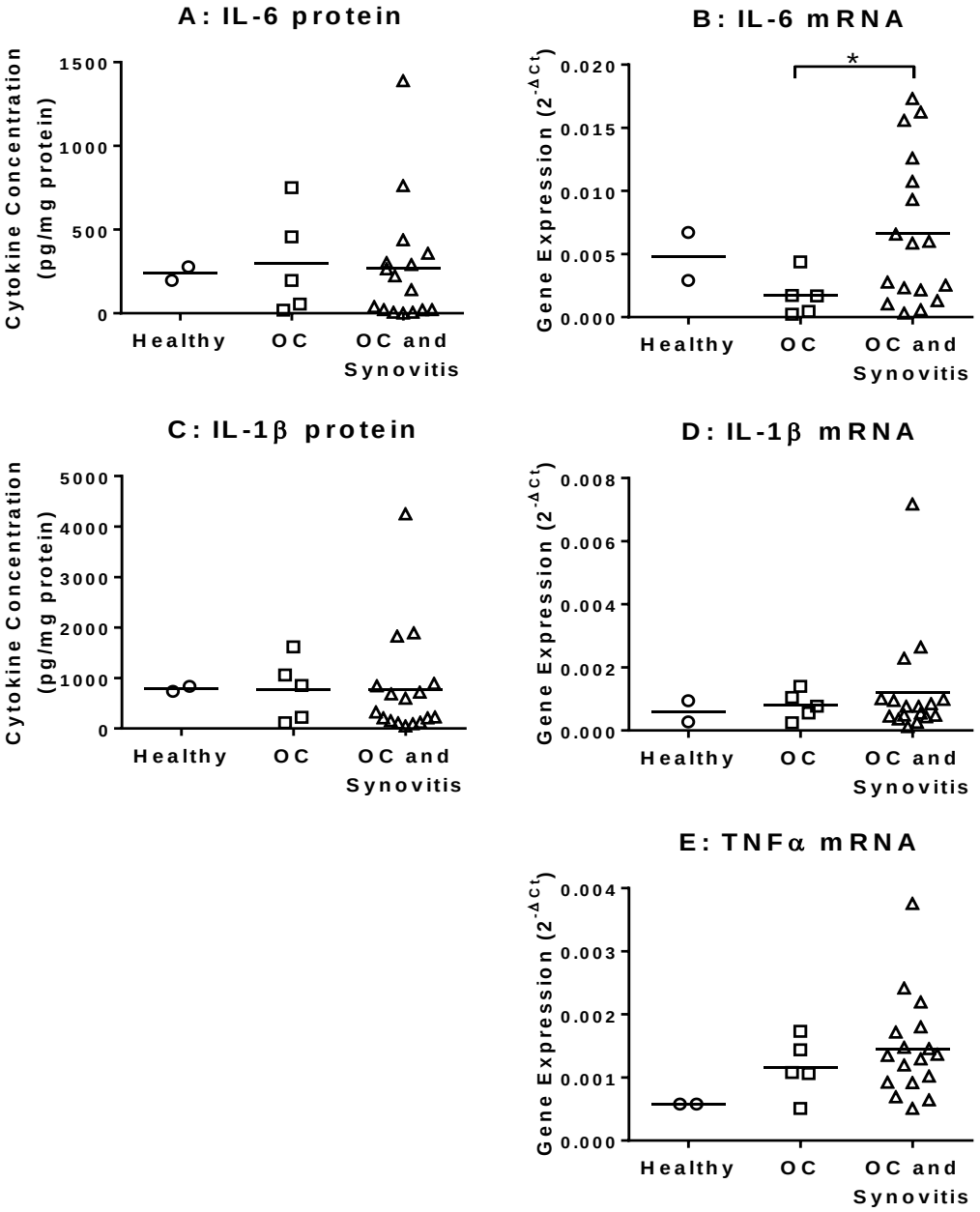


Figure 5.12: Synovial tissue cytokines in porcine osteochondrosis with or without synovitis. There was difference in IL-6 (A) or IL-1β (C) protein concentrations or IL-1β (D) or TNFα (E) mRNA expressions, but IL-6 expression was increased in OC joints with synovitis (B). *p < 0.05, t-test. Lines represent means.

5.3.8 Association of osteoarthritis and synovial tissue cytokines

As there was no difference in cytokine concentration or expression associated with age and no difference in OA severity of 24 and 42 month old pigs, the data from the two age groups was pooled before analysis of cytokines associated with OA. Due to there only being one healthy joint it was difficult to determine what effect OA had on synovial tissue cytokines. However, if the healthy joint is representative of healthy pigs, there was a 3-fold upregulation in IL-6 mRNA expression in OA ($2^{-\Delta\Delta Ct}$ 3.35), and the highest expressions of IL-1β and TNFα were seen in joints with OA. Mean protein concentrations of IL-6 and IL-1β mRNA were also 3 times higher in OA than the healthy joint (see Figure 5.13).

It was also noted that in more severe OA with subchondral bone exposure, there was reduced cytokine protein and mRNA levels compared to pigs with less severe OA, particularly IL-6 protein ($p = 0.0393$, t-test). The same trend was seen in IL-6 and IL-1β mRNA expression and IL-1β protein concentrations, although this was not statistically significant. For example, synovial IL-6 mRNA expression was over 4-fold upregulated in OA joints with unexposed subchondral bone (compared to the healthy joint), but in joints with subchondral bone exposure this upregulation was less than 2-fold ($2^{-\Delta\Delta Ct}$ 4.37 and 1.73 respectively).

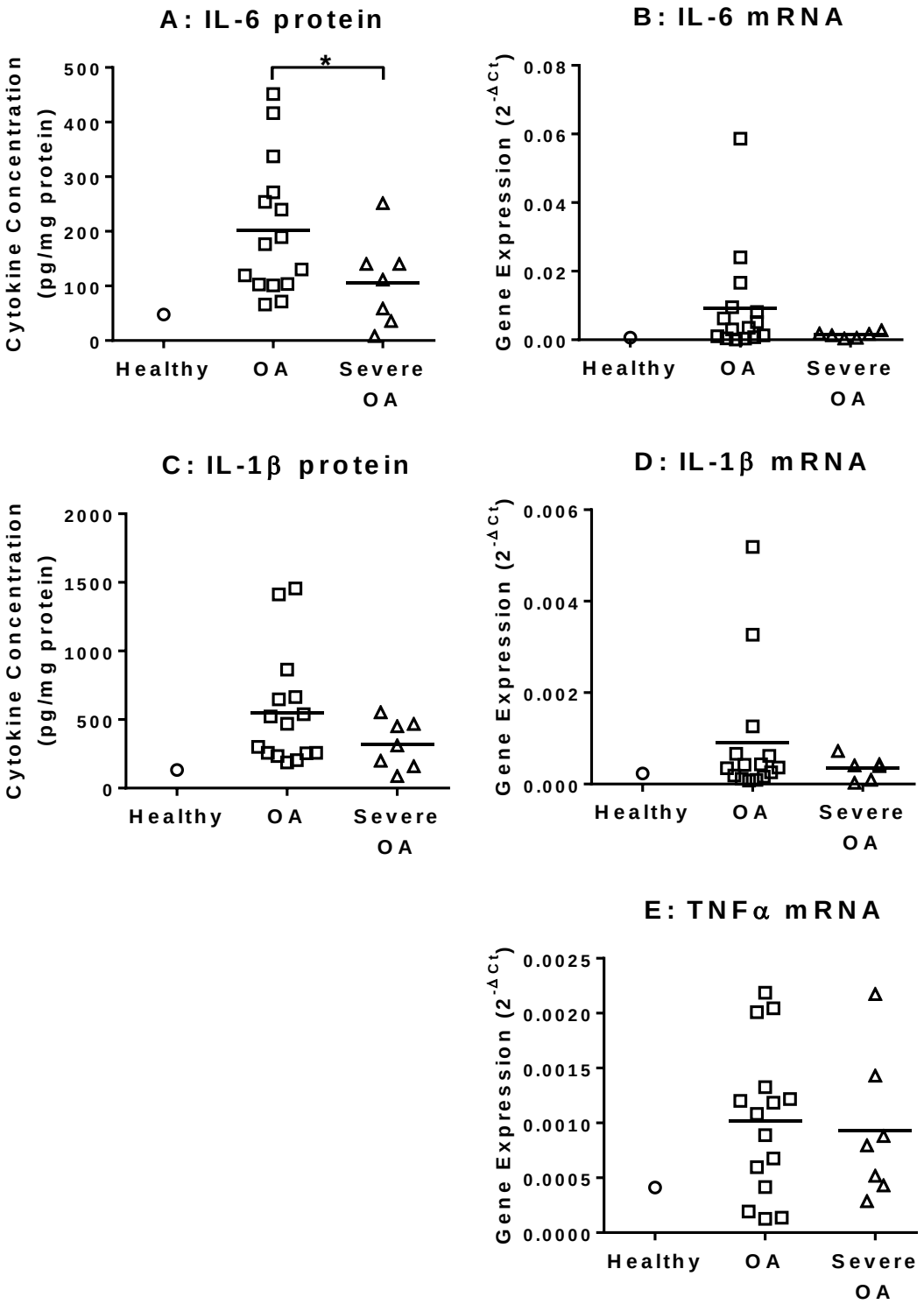


Figure 5.13: Synovial tissue cytokines in porcine knee osteoarthritis. Severe OA indicates joints with subchondral bone exposure. Higher levels of IL-6 (A) and IL-1β (C) protein and IL-6 (B), IL-1β (D) or TNFα (E) mRNA were seen in joints with OA, but this increase was smaller in joints with severe OA, which was statically significant for IL-6 protein. *$p < 0.05$, t-test. Lines represent means.

Due to the lack of healthy pigs, it was not possible to determine statistically the interaction between OA and synovial inflammation on cytokines. There was no statistical difference in mRNA expression of IL-1β and TNFα or IL-6 and IL-1β cytokine protein concentrations in pigs with non-inflammatory and inflammatory OA (see Figure 5.14). However, IL-6 mRNA expression was significantly upregulated in inflammatory OA compared to non-inflammatory OA ($p = 0.0443$, t-test). Compared to the healthy joint IL-6 was upregulated 2-fold in non-inflammatory OA but to over 6-fold in inflammatory OA ($2^{-\Delta\Delta Ct}$ 2.10 and 6.47 respectively).The same trend was also seen in IL-6 and IL-1β protein concentrations and IL-1β mRNA, with the highest levels seen in inflammatory OA.

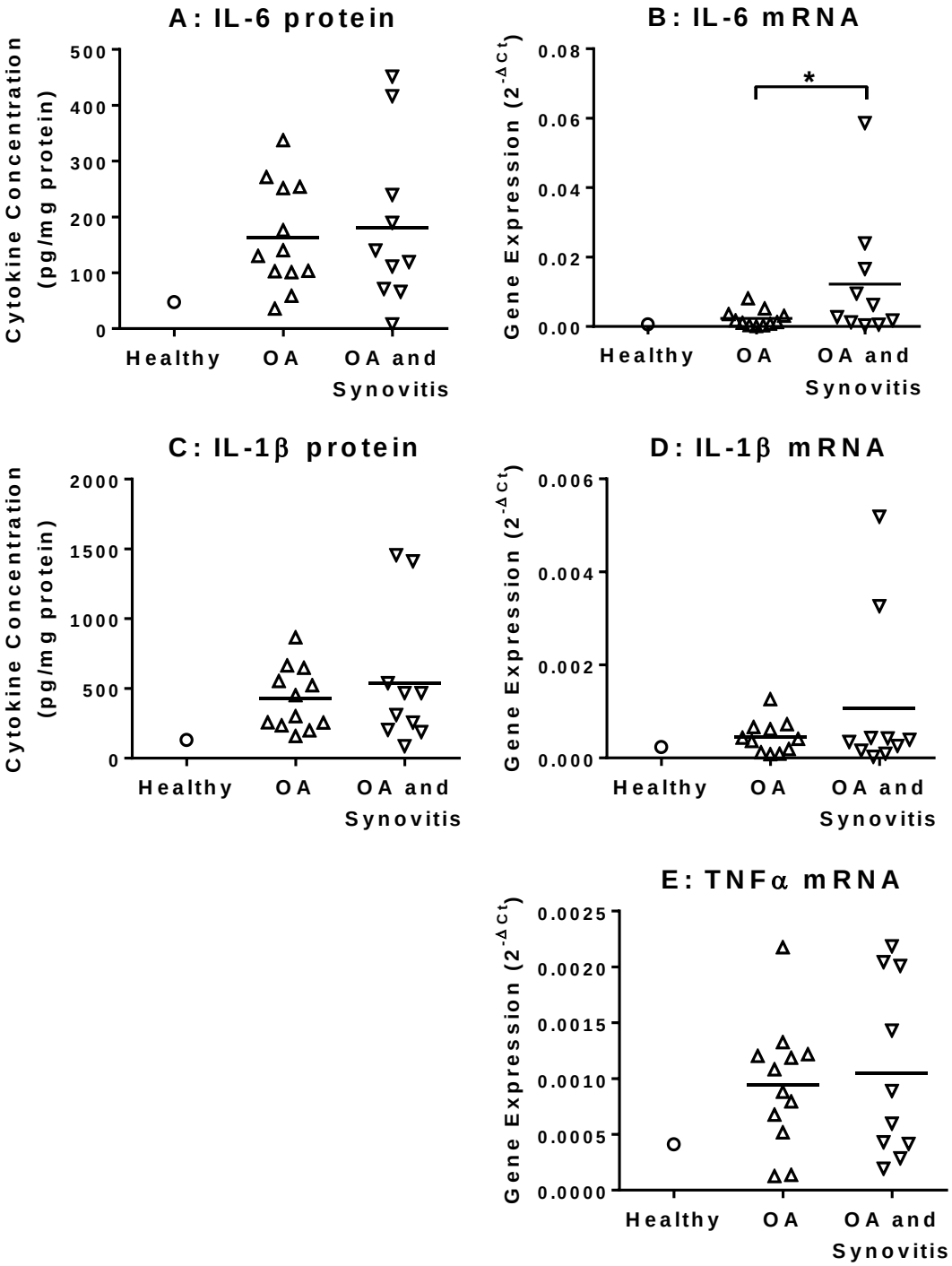


Figure 5.14: Synovial tissue cytokines in porcine osteoarthritis with or without synovitis. There was no statistical difference in cytokine protein concentrations of IL-6 (A) and IL-1β (C) or mRNA expressions of IL-1β (D) and TNFα (E) but IL-6 mRNA expression (B) was significantly increased in OA when synovitis was present. *$p < 0.05$, t-test. Lines represent means.

5.4 Discussion

The work presented in this chapter characterised prevalence and pathological features of synovitis in the porcine knee joint and the association of synovitis with joint disease and lameness. Cytokine protein and mRNA levels in synovial tissue were determined using an FMIA and qPCR. The involvement of these cytokines in OC, OA, and synovitis was then assessed.

5.4.1 Bio-Plex® FMIA validation studies

The Bio-Plex® FMIA was previously validated for a multiplex procedure for simultaneously assessing multiple cytokines in porcine and equine plasma (Bjerre et al., 2009; Wagner and Freer, 2009; Hall et al., 2015). However, this is the first time the procedure has been reported in porcine synovial tissue and fluid. The protein content of homogenates varied between samples due to sample starting weight and fat content. The thin nature of the synovial membrane meant it could not be extracted in isolation and samples had different amounts of fat underlying the membrane layer. To control for this cytokine levels were expressed per mg of protein.

A dilution effect was seen in both synovial tissue and synovial fluid; however, this was not consistent across dilutions or between samples, therefore it is important that all samples were assayed at the same dilution to control for this effect. Furthermore, when room temperature dropped below 16°C, antibody binding was affected and this prevented detection of lower cytokine concentrations and standard curves did not form correctly. To mitigate for this, the well plate should be placed in an incubator to improve antibody binding if the room temperature is low.

Homogenisation of synovial tissue was required for the FMIA and a number of different buffers have been used to extract cytokines with different efficiencies. In the optimisation of this protocol, radio-immunoprecipitation buffer (RIPA buffer) was initially tested, however only IL-1β was extracted sufficiently to be detected (data not included). Homogenisation with Bio-Rad cell lysis buffer was also trialled, however this only extracted TNFα sufficiently (study conducted by Louisa Gould

(2017)). It was discovered that detergents present in both RIPA and cell lysis buffers interacted with the Bradford protein assay so accurate tissue lysate protein concentrations could not be determined. Percentage recovery of samples spiked with a known concentration of cytokine were also low after extraction with RIPA buffer (16 - 96%) and cell lysis buffer (13% - 49%), therefore these buffers may also interfere with the FMIA. Extraction with PBS improved recovery of all three cytokines to within 70 - 130% acceptable range (Bjerre et al., 2009). As PBS contains no detergents it did not interfere with the FMIA, but this also meant freeze-thaw cycles were required to disrupt cell membranes. This resulted in successful extraction of IL-6 and IL-1β but not TNFα. However, when using PBS for homogenisation there was no proteinase inhibitor in the assay buffer. Proteinases released from cells during homogenisation could therefore cleave proteins within the lysate. This may have reduced the total protein content but also degraded the target cytokines in the synovial tissue lysates. Further work is required to optimise the homogenisation protocol to extract all cytokines effectively and to identify a suitable proteinase inhibitor for the assay buffer that does not interfere with the FMIA.

Use of the FMIA with synovial fluid samples proved more problematic and the recovery of cytokines was very low. This may be a result of pipetting errors due to the fluid's viscosity or the complex synovial fluid matrix could be interfering with the assay. The synovial fluid was not centrifuged on collection, so the cellular components of the fluid were not removed prior to freezing. Synovial fluid can contain a number of different cells including mononuclear cells and mesenchymal progenitor cells (Takasugi and Hollingsworth, 1967; Kurose et al., 2010). Formation of ice crystals during freezing could cause cells to rupture, releasing their intracellular components into the fluid matrix, and can alter the stability of proteins. This process could have caused some of the issues that occurred during synovial fluid analysis. Centrifugation of the fluid prior to freezing may overcome some of these issues by removing large cells prior to freezing, reducing assay interference. Synovial fluid can also contain a number of antibodies, rheumatoid factors, enzymes, lubricin and hyaluronic acid, all of which may interfere with the assay (de

Jager et al., 2007; Tsuchida et al., 2014). In immunoassays using human synovial fluid, a number of protocols suggest incubation with a blocking buffer reduces antibody or rheumatoid factor interference (Raza et al., 2005; de Jager et al., 2007; Wright et al., 2012; Tsuchida et al., 2014; Valentina et al., 2015). As such, synovial fluid was incubated using two different blocking buffers before conducting the FMIA; this improved cytokine recovery but not a sufficient amount. A number of other processing techniques have been trialled to improve cytokine recovery; these include hyaluronidase digestion and centrifugal concentration (Jayadev et al., 2012; Bennike et al., 2014; Moret et al., 2014; Tsuchida et al., 2014; Bhattacharjee et al., 2016; Cuéllar et al., 2016; Mariani et al., 2016; Svala et al., 2017). However, these also produced no improvement in analyte recovery in porcine synovial fluid samples (Hall et al., 2019, under review). Therefore, further investigation is required to determine if the Bio-Plex® FMIA is suitable for measuring synovial fluid cytokine concentrations.

5.4.2 Correlation of cytokine protein levels and mRNA expression

There was no correlation between cytokine mRNA expression and protein concentration. Genetic information is turned into proteins by transcription of DNA into RNA and its subsequent translation into a protein sequence (Crick, 1970). Therefore, it could be expected that RNA and protein levels would correlate. However, biological systems are more complicated than this simple two-step process. There are numerous post-translational and transcriptional modification systems and protein degradation systems which affect protein production, stability and secretion (Maier et al., 2009; Vogel and Marcotte, 2012; Kendrick, 2014; Payne, 2015). Many studies of mammalian tissue and yeast have shown there is often very little correlation between RNA and protein levels, with correlation coefficients of 0.4 or lower (Futcher et al., 1999; Gygi et al., 1999; Kendrick, 2014). It has been suggested that only 40% of variations in protein levels are explained by RNA expression, however higher RNA expressions show a stronger correlation to protein levels (Gygi et al., 1999; Schwanhäusser et al., 2011). As such, mRNA expressions in disease are useful for identifying biomarkers or predicting disease mechanisms, but it cannot necessarily be confirmed without a corresponding proteomic study. In the

present study, although there was no absolute correlation between cytokine mRNA and protein levels, they showed similar changes associated with porcine joint diseases. Therefore, it is likely that changes in cytokine protein levels are at least partly a consequence of upregulation or downregulation in gene expression.

5.4.3 Synovitis in the porcine knee joint

The macroscopic features of synovitis identified, such as yellowing and surface fibrillation, were similar to those previously seen in pigs and other species, including rodents, dogs, horses, sheep and goats (Cook et al., 2010; Little et al., 2010; McIlwraith et al., 2010; Cruz et al., 2015). In animal studies, synovitis is commonly diagnosed using histological assessment to identify membrane hyperplasia but also changes in vascularity and inflammatory cells (El-Gabalawy, 1999; McIlwraith, 2005; Wenham and Conaghan, 2010; Scanzello and Goldring, 2012). Healthy porcine synovial membrane was 1-2 cells thick but hyperplasia was seen in a number of joints. Some samples showed over 9 cells in the membrane layer, which is similar to synovitis-associated hyperplasia seen in other porcine studies (Cruz et al., 2015; Etterlin et al., 2015).

This study identified a high prevalence of synovitis, affecting 57% of joints with 14% showing moderate or severe synovitis. This prevalence was much higher than seen in other studies that identified only an 8-30% prevalence of synovitis in commercial pigs (Etterlin et al., 2014; Etterlin et al., 2015). However, previous studies only histologically examined tissue from pigs showing discolouration of synovial tissue or fluid or increased synovial fluid volume at the time of slaughter. This may have resulted in an underreported synovitis, as pigs with no macroscopic change were assumed to have no inflammation so were excluded from histological examination. In the present study, a number of synovial samples showed slight hyperplasia but there was little or no discolouration observed macroscopically. There was also no difference in synovitis severity identified in different pig age groups. It might have been expected that age would have increased prevalence of synovitis due to an accumulation of inflammation-causing factors, such as trauma or disease over time, as has been seen in humans where increased synovitis is associated with ageing

(Guermazi et al., 2012). The present data identified no association with age, which in part may be due to the small population studied, particularly of 42 month old pigs. However, the high prevalence of diseases such as OC and OA is likely to have obscured any age-associated change in synovitis and altered the prevalence of inflammation.

5.4.3.1 Cytokines associated with porcine synovitis

In healthy synovium, there are usually small amounts of cytokines present to help support normal homeostasis within the joint but this is altered during disease (Smith et al., 2003; Smith, 2011). Hyperplasia of porcine synovial membrane was associated with an upregulation of IL-6 mRNA expression, and severe hyperplasia was associated with increased IL-6 and IL-1β protein levels. These cytokines are predominately released by activated synoviocytes, both macrophages and fibroblasts, within the synovium intima layer or chondrocytes within cartilage (Bondeson et al., 2006; Sutton et al., 2009; Bondeson et al., 2010). The findings suggest activation of these synoviocytes during inflammation caused an upregulation in pro-inflammatory cytokine gene expression or increased translation into protein. This increase may elicit further cytokine production, resulting in a cycle of inflammation that has been found in joint diseases such as OA (Sutton et al., 2009; Monemdjou et al., 2010). This may also increase pain, as cytokines can sensitise peripheral neurones, thereby eliciting peripheral sensitisation that can lead to chronic pain (Schaible et al., 2002; Nagy et al., 2006).

5.4.4 Association of synovial inflammation and porcine osteochondrosis

The present study found no relationship between severity of synovitis and OC in 9 and 15 month old pigs. Synovitis was not seen in healthy joints and affected 77% of OC joints. However, joints with the same OC severity showed very different synovitis severities. Other studies examining the relationship between OC and synovitis have found stronger associations. The majority of OC lesions in pigs are thought to present with synovitis and over 88% of dogs with OC show synovitis (Etterlin et al., 2014; Wall et al., 2015). However, these studies mainly examined the more severe OCD pathology. It has been suggested that for synovitis to present with

OC, the joint is required to reach a threshold, such as a certain number of lesions or the onset of OCD (Etterlin et al., 2015). In the present study, OC was relatively mild, no OCD was identified and joints with the same OC severity showed very different inflammation severities. This suggests that in mild porcine OC, there may be a weaker association with inflammation than more severe OC, and it is not solely determined by the extent of cartilage lesions present.

Furthermore, moderate to severe synovitis was only present in symptomatic disease, suggesting synovitis may influence pain and the onset of symptoms in OC. Inflammatory molecules such as cytokines are capable of sensitising nociceptive neurones, causing pain and increasing the likelihood of symptom onset (Orita et al., 2011b; Leung et al., 2017). Synovitis can be caused by a number of factors including trauma, joint disease, and viral or bacterial infections (Vela et al., 2017). Although the synovitis identified was not purulent (pus-containing), so is unlikely to be bacterial in origin, the cause of synovitis could not be definitively identified as OC. The ability of OC lesions to repair may also have influenced the association of OC and synovial inflammation identified in this study. It could not be determined whether the OC lesions identified were forming, remaining consistent or beginning to repair; these stages may have differing involvements in inflammation and pain. Further investigation on a larger sample is therefore required to determine the involvement of inflammation in mild forms of porcine OC and its link with pain.

Although there was some upregulation in TNFα expression in joints with OC, the present study identified no clear association between other cytokine mRNA or protein levels and severity of OC in the porcine knee joint. There has been little investigation into the molecular mechanisms involved in inflammation and OC. A study of human OC identified altered IGF in the ankle but involvement of other cytokines was unclear (Schmal et al., 2014). The cytokines IL-6, IL-1β and TNFα were selected for study as they show the highest association with equine joint disease (Bertone et al., 2001). Studies of equine joint diseases identified synovial fluid IL-6 as a good predictive marker of disease and IL-1β and TNFα as good markers of acute disease (Bertone et al., 2001). In porcine OC the presence of circulating cartilage

metabolism markers has been investigated (Frantz et al., 2010), but there has been no discussion of the inflammatory markers associated with OC. The lack of association identified in the present study is perhaps unsurprising given the unclear involvement of synovitis in mild OC. Porcine inflammatory OC was associated with the highest levels of all cytokines, and the study indicated that there may be some increase in TNFα expression during OC, but there was no clear involvement of other cytokines. There may be a subset of pigs with OC that show increased synovial cytokines, but in isolation elevated cytokines may not be an indicator of the disease.

It could not be determined whether the inflammation seen in the present study was resultant of OC or an entirely separate pathology that coincided with OC. However, as synovitis was only seen in OC, these data suggest that mild porcine OC increases the likelihood of developing synovitis even if OC lesions are not necessarily the cause. Although highest cytokine levels were seen in inflammatory OC, in mild disease states there may be no consistent change in inflammatory cytokines, with only a subset of animals displaying high levels of inflammation. Therefore, the presence of synovitis and elevated cytokines in OC may represent a separate pathological state. Inflammation and elevated cytokines could elicit further pathological changes as cytokines trigger proteolytic enzyme production and reduce production of cartilage components (Saklatvala, 1986; Stöve et al., 2000; Porée et al., 2008; Monemdjou et al., 2010). This may lead to two separate pathological states in mild OC: a non-inflammatory OC pathology and an inflammatory OC pathology that should be termed osteochondritis. Osteochondritis may represent a separate disease to OC, with different outcomes and cytokine involvements that may alter disease progression and pain severity. A large population with more healthy pigs and study of a greater range of cytokines is required to evaluate this further, in order to determine the involvement of inflammation in OC, and how this may affect lesion repair or progression.

5.4.5 Association of synovial inflammation and porcine osteoarthritis

The synovitis seen in spontaneous porcine OA showed similar pathological features to synovitis seen in porcine models of surgically induced OA (Cruz et al., 2015; Reisig

et al., 2016). However, determining the association of OA and synovitis was limited by the lack of healthy joints. The 24 month old pigs showed no association but 42 month old pigs showed a strong correlation between chondropathy severity and synovitis. Traditionally OA was thought to be a 'wear and tear' disease caused by damage to joint cartilage (Mathiessen and Conaghan, 2017), but synovial inflammation has since been identified in the majority of OA patients (Goldenberg et al., 1982). It is thought that inflammation is an important part of OA pathophysiology and that inflammatory OA may be a separate distinct subtype of OA (Revell et al., 1988; Sutton et al., 2009). Studies have also suggested synovitis is involved in different aspects of OA pathology and is elevated in end-stage disease (Haywood et al., 2003; Benito et al., 2005; Bondeson et al., 2010). This may explain the differing association of synovitis and chondropathy severity in different pig age groups, as more of the 42 month old pigs had subchondral bone exposure, which is more strongly associated with inflammation.

Studies of human OA have identified increased cytokines in more severe OA pathologies (Orita et al., 2011b; Wojdasiewicz et al., 2014; Mabey and Honsawek, 2015) and studies of equine OA suggest increased IL-1β and TNFα are associated with worse articular chondropathy (Sutton et al., 2009; Kamm et al., 2010). However, there has been little investigation into the involvement of synovial inflammation in spontaneous porcine OA. A previous study of porcine post-traumatic arthritis identified increased inflammation and expression of 87 genes, including IL-6 expression (Baatartsogt et al., 2011). The present study identified no statistically significant association between severity of OA and cytokine protein or mRNA levels in the porcine knee joint, however the lack of healthy pigs was severely limiting. Despite this, IL-6 expression was upregulated in OA and the highest levels of cytokines seen in joints with OA, suggesting elevated cytokines may be associated with spontaneous porcine OA. The presence of synovitis in OA appeared to further increase synovial cytokines, which was statistically significant for IL-6 mRNA expression levels.

Other studies have identified a more complex involvement than a general increase in cytokines during OA, with cytokines changing depending on disease state and symptoms present (Vangsness et al., 2011). In human OA, increased cytokines, including IL-6 and TNFα, are related to increased symptoms but not changes in articular cartilage integrity and inflammation is an important contributing factor to the pain experienced (Stannus et al., 2010; Orita et al., 2011a; Orita et al., 2011b; Leung et al., 2017). This results in increased synovitis in symptomatic OA, whereas asymptomatic OA showed lower levels of inflammation (Stoppiello et al., 2014). This was seen in the present study as only pigs with synovitis had symptomatic OA. This indicates that, as with human OA, the presence of inflammation in OA may increase pain and worsen symptoms. Inflammation may also contribute to further cartilage degeneration, altering OA progression by increasing proteinase production, which triggers cartilage catabolism (Wittenberg et al., 1993; Sutton et al., 2009).

Furthermore, studies have identified a changing cytokine profile as OA progresses (Mabey and Honsawek, 2015). In rabbit OA, IL-1β is increased in early but not late OA (Xiaoqiang et al., 2012), and in canine OA, TNFα expression is associated with different rates of cartilage degradation (Kammermann et al., 1996). Due to the small number of pigs studied, it was not possible to conduct a thorough analysis of cytokine levels associated with different disease stages. However, this may explain why there was little correlation between OA chondropathy severity and cytokine levels. It may also explain why pigs with end-stage OA (with subchondral bone exposure) showed reduced synovial cytokines compared to pigs at earlier disease stages. In spontaneous porcine OA, the inflammatory profile may change as the disease progresses, recruiting different molecules at different times, but further study on a larger population with more healthy animals is required to evaluate this fully.

5.5 Conclusions

Adult female pigs suffered from a high prevalence of synovial inflammation, which presented with tissue discolouration and hyperplasia of the synovial membrane layer. The severity of synovial inflammation was associated with increased pro-

inflammatory cytokines, namely IL-6 and IL-1β. However, the association of synovial inflammation and porcine OC was unclear. Although OC was associated with a general increase in synovial inflammation and TNFα expression, this relationship for mild disease stages seen in the present study is still unclear. In spontaneous porcine OA, more severe cartilage degeneration was associated with increased synovitis and the onset of clinical symptoms. Spontaneous OA was also associated with a general increase in synovial cytokines but this may change as the disease progresses. Cytokines were less elevated in joints with severe cartilage degeneration and subchondral bone exposure compared to milder forms of OA.

Chapter 6

Expression of Matrix Metalloproteinases and their Inhibitors in the Synovium during Porcine Joint Disease

6.1 Introduction

The role of proteolytic enzymes and their inhibitors in joint disease gained much research interest due to the potential therapeutic targets or disease biomarkers they may provide. MMPs are a family of closely related proteinases that destroy ECM components such as cartilage collagen (Cawston, 1996; 2002). MMPs are responsible for timely ECM degradation and are important for development, morphogenesis, repair and remodelling in both normal physiology and pathological conditions (reviewed by Nagase et al., 2006), hence this family of enzymes is thought to be responsible for cartilage degeneration in a number of diseases. There are 25 known mammalian MMPs that share a number of properties, including a common amino acid sequence, a zinc active centre, secretion in an inactive form, and activity at neutral pH (reviewed by Murphy et al., 2002). Within the knee, MMPs can be produced by chondrocytes and synoviocytes (McCachren, 1991; Gepstein et al., 2002).

MMPs can be broadly categorised into four groups determined by their substrate specificity; these are collagenases, gelatinases, stromelysins and membrane-type MMPs (reviewed by Cawston, 1998; and Murphy et al., 2002). The collagenase subgroup includes MMP1 (interstitial collagenases), MMP8, MMP13 and MMP18; their main function is cleaving interstitial collagen including type I, II, III, VII, VIII and X collagen, but they also break down other ECM components, such as gelatines and aggrecan (Evanson et al., 1967). The gelatinases include MMP2 (gelatinase-A) and MMP9 (gelatinase-B). These have the highest specificity to gelatine but may also digest type I, IV, V, VII, X, and XI collagen, elastin and aggrecan (Murphy et al., 1989). The stromelysins include MMP3 (stromelysin-1), MMP10 and MMP11. Stromelysins cleave a broad range of ECM components, including aggrecan, fibronectin, proteoglycans and type I, III and IV collagen (Chin et al., 1985). Stromelysins are also thought to be highly involved in the activation of pro-collagenases. Membrane-type MMPs, including MMP14 and MMP26, are stored intracellularly and function to activate a number of pro-MMPs but they can also degrade collagen, fibronectin and gelatine (Sato et al., 1994; Pei, 1999). However, some MMPs do not fall into the four categories; for example, MMP12, MMP19 and

MMP23 can be classified into more than one group, and MMP14 is both a collagenase and a membrane-type MMP.

Cartilage's ECM contains different collagens and proteoglycans (see section 1.5.1), which can be degraded by MMP mediated proteolysis. In conditions such as OA, MMP proteolysis is partly stimulated by elevated cytokine levels, resulting in cartilage breakdown (Woessner, 1991; Cawston, 1996). The MMPs cleave cartilage proteins at different points along their amino acid sequence, forming different size degradation fragments and resulting in cartilage breakdown (see Figure 6.1). For example, collagenases such as MMP1 and MMP13 cleave type II collagen into a ¾-length N-terminal fragment and a ¼-length C-terminal fragment, thereby causing collagen to lose its helical structure (Billinghurst et al., 1997). Aggrecan can also be cleaved at different points by MMPs, creating fragments such as G1-IPEN and FFGV-G3 (Fosang and Hardingham, 1989; Fosang et al., 1995; Struglics et al., 2006). These new N and C terminals of the degraded proteins are called neo-epitopes and their creation can be used in antibody studies to indicate collagen and proteoglycan activity (Janusz et al., 2004; Fosang et al., 2010).

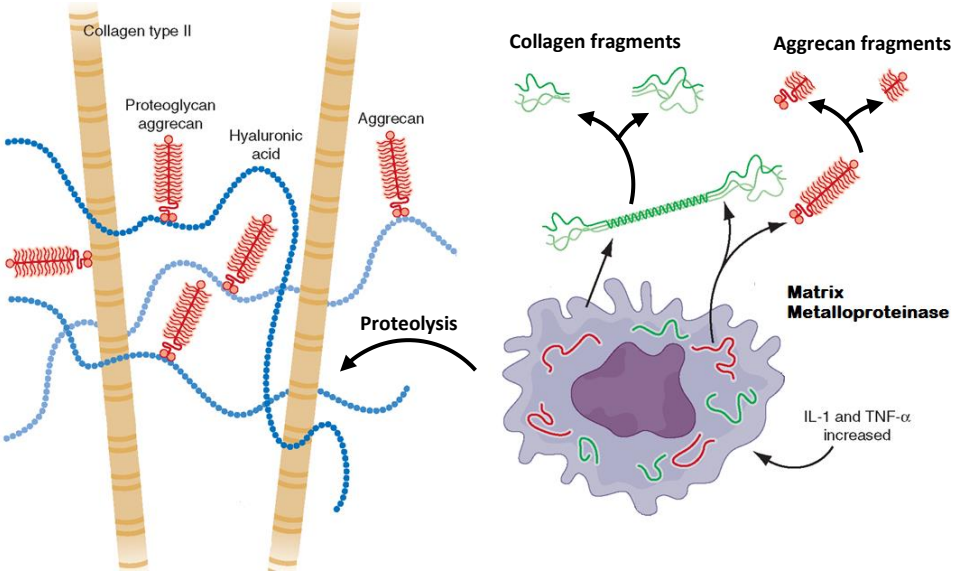


Figure 6.1: Cartilage degradation by matrix metalloproteinases. MMP activation, partly elicited by increased cytokines, results in proteolysis of collagen and aggrecan. This forms degradation fragments of different sizes and results in cartilage breakdown. Image adapted from Poole et al. (1993; 2002).

MMPs are produced in a pro-enzyme form that requires activation by removal of a 10kDa N-terminal fragment, which is an important regulatory step in MMP activity (Springman et al., 1990). Many activated MMPs can stimulate other pro-MMPs, suggesting there is an activation cascade between members of the MMP family. The enzyme plasmin (found in blood) and reactive oxygen species released during inflammation, are both capable of activating pro-MMPs (Saari et al., 1990; Murphy et al., 1992). MMPs are also inhibited by the glycoprotein α2-macroglobulin in serum and synovial fluid (Birkedal-Hansen et al., 1993), as well as tissue inhibitors of MMPs (TIMPs), which are inhibitory glycoproteins found in and synthesised by connective tissue (Nagase et al., 2006). There are 4 TIMPs with a molecular mass of approximately 21kDa (Baker et al., 2002). TIMPs bind to MMPs with a 1:1 stoichiometry, blocking the enzymes active site but also preventing activation of pro-MMPs (Gomis-Ruth et al., 1997). All TIMPs are capable of inhibiting all MMPs but with different affinities. TIMP3 has affinity for the broadest range of MMPs but can also inhibit other proteinases, including members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family which degrade proteoglycans (Porter et al., 2005). TIMP1 has the highest affinity for MMP3 and MMP7 (Brew and Nagase, 2010). The balance of MMP and TIMP activity requires tight control and any imbalance can result in excessive or insufficient ECM breakdown, leading to pathological changes (Murphy et al., 2002).

Activities of MMPs and TIMPs have been implicated in a number of diseases, including many forms of arthritis, cancer and heart disease (Murphy et al., 2002; Nuttall et al., 2003; Bonnema et al., 2007). However, it is unclear what form of MMPs (active, proenzyme or TIMP complexed) provide the best biomarker of disease. Altered expressions or imbalances in MMP and TIMP activity disturb normal ECM homeostasis, resulting in pathological changes. This disruption can be caused by a number of factors but is particularly triggered by inflammation. Cytokines and growth factors, including IL-1, IL-6, TNFα, PDGF and fibroblast growth factor 2, have all been implicated in stimulating synthesis or secretion of MMPs (reviewed by Cawston, 1998). In both OA and rheumatoid arthritis, elevations in MMPs are associated with increased cartilage degradation (Garnero et al., 2000).

MMPs have also been implicated in bone matrix turnover and may alter osteoblast and osteoclast function, which promotes the formation of osteophytes in OA (Janusz et al., 2002; Sakao et al., 2009). Furthermore, in inflamed synovial tissue during many forms of arthritis, changes in these proteolytic enzymes have been detected (Sutton et al., 2009). In the synovium of osteoarthritic joints, both macrophages and fibroblasts express altered amounts of MMPs (Bondeson et al., 2010). However, it is unclear whether changes seen in expression or activity of MMPs and TIMPs during joint diseases are species- or tissue-specific, particularly in relation to spontaneous joint diseases in pigs.

6.1.1 Aim

The aim of the work presented in this chapter was to characterise changes in expression of ECM degrading enzymes in porcine knee joint synovium. To achieve this, synovial tissue from different age pigs was assessed for changes in mRNA expression of matrix metalloproteinase 1, 3 and 9 (MMP-1, 3 and 9) and tissue inhibitors of matrix metalloproteinase 1 and 3 (TIMP-1 and 3) using qPCR. Alterations in synovium MMPs and TIMPs associated with porcine knee OC, OA and synovitis were then assessed and compared to expressions of synovium pro-inflammatory cytokines.

6.2 Methods

6.2.1 Animals

The same four age cohorts of clinically healthy female commercial pigs of the crossbred genotype 'Landrace x Large White x Duroc' were used in this study. Pigs were aged 9 months (n = 6), 15 months (n = 6), 24 months (n = 8) and approximately 42 months (n = 7).

Pigs were assessed for 5 to 7 days prior to slaughter for pain behaviours and lameness (see section 2.2). Both knee joints from 9 and 15 month old pigs were assessed macroscopically for OC (see section 2.4.1). Both knees from 24 month old and one knee from 42 month old pigs were assessed macroscopically for OA (see section 2.4.2). In pigs showing joint disease (OC or OA) but a normal gait, the disease was termed asymptomatic. In pigs showing joint disease and lameness, the disease was termed symptomatic. Excised synovial tissue was frozen for qPCR or mounted in OCT for histology (see section 2.3.3). Synovial tissue sections were routinely stained with H&E and assessed for synovitis (see section 2.7). Non-inflammatory joint disease was identified when no synovitis was present in joints with OC or OA; when synovitis was present, the disease was termed inflammatory. The synovial tissue expression of cytokine mRNA was determined using qPCR (see section 2.8) and cytokine protein levels determined using an FMIA (see section 5.2.3).

6.2.2 Expression of MMPs and TIMPs in porcine synovial tissue

mRNA expression of MMP1, MMP3, MMP9, TIMP1 and TIMP3 in synovial tissue was assessed using qPCR (see section 2.7). RNA was extracted using a Qiagen QIAsymphony instrument and reverse transcribed into cDNA before SYBR Green qPCR was performed to determine expressions of MMPs and TIMPs relative to the ACTB and PPIA housekeeping genes.

6.2.3 Statistical analysis

Data were tested for normality using the D'Agostino-Pearson normality test. Parametric data were analysed using t-tests or one-way ANOVA with Tukey's

multiple comparisons post-hoc tests. Correlations between data were determined using either Pearson's or Spearman's Rank Correlation. A significance of $p < 0.05$ was used for all statistical tests. Statistical analysis of qPCR data was conducted using $2^{-\Delta Ct}$ values (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Where statistical analysis was not appropriate due to small sample sizes, MMP and TIMP expressions were assessed by looking at fold changes ($2^{-\Delta\Delta Ct}$ method).

6.3 Results

For a discussion of the prevalence and pathological features of OC and OA in the porcine knee joints, see Chapters 3 and 4 respectively. For a discussion of the stability of the housekeeping genes (ACTB and PPIA) see section 5.3.3.1. For a discussion of synovial inflammation and cytokine protein levels and mRNA expressions in the porcine knee joints see Chapter 5.

Expression of MMP1, MMP3, TIMP1 and TIMP3 was measured in all samples, but MMP9 was only sufficiently expressed to be detected in 70% of samples. Expression of MMP1 and MMP3 significantly correlated across all animals ($r^2 = 0.9000$, $p < 0.0001$, Pearson's Correlation) as did the expressions of TIMP1 and TIMP3 ($r^2 = 0.7900$, $p < 0.0001$, Pearson's Correlation).

6.3.1 Synovial tissue MMP and TIMP expression in different age pigs

There was no difference in MMP9 expression in different age pigs ($p = 0.1960$, one-way ANOVA) but there were differences in MMP1, MMP3, TIMP1 and TIMP3 expression ($p = 0.0388$, $p = 0.0292$, $p = 0.0342$ and $p = 0.0409$ respectively, one-way ANOVAs, see Figure 6.2). MMP1 and MMP3 were significantly more expressed in 9 month old pigs than 24 month old pigs ($p = 0.0481$ and $p = 0.0373$ respectively, Tukey's multiple comparisons). TIMP1 was significantly less expressed in 9 month old pigs than 24 month old pigs ($p = 0.0496$, Tukey's multiple comparisons). TIMP3 was significantly less expressed in 9 and 15 month old pigs than 42 month old pigs ($p = 0.0179$ and $p = 0.0245$ respectively, Tukey's multiple comparisons).

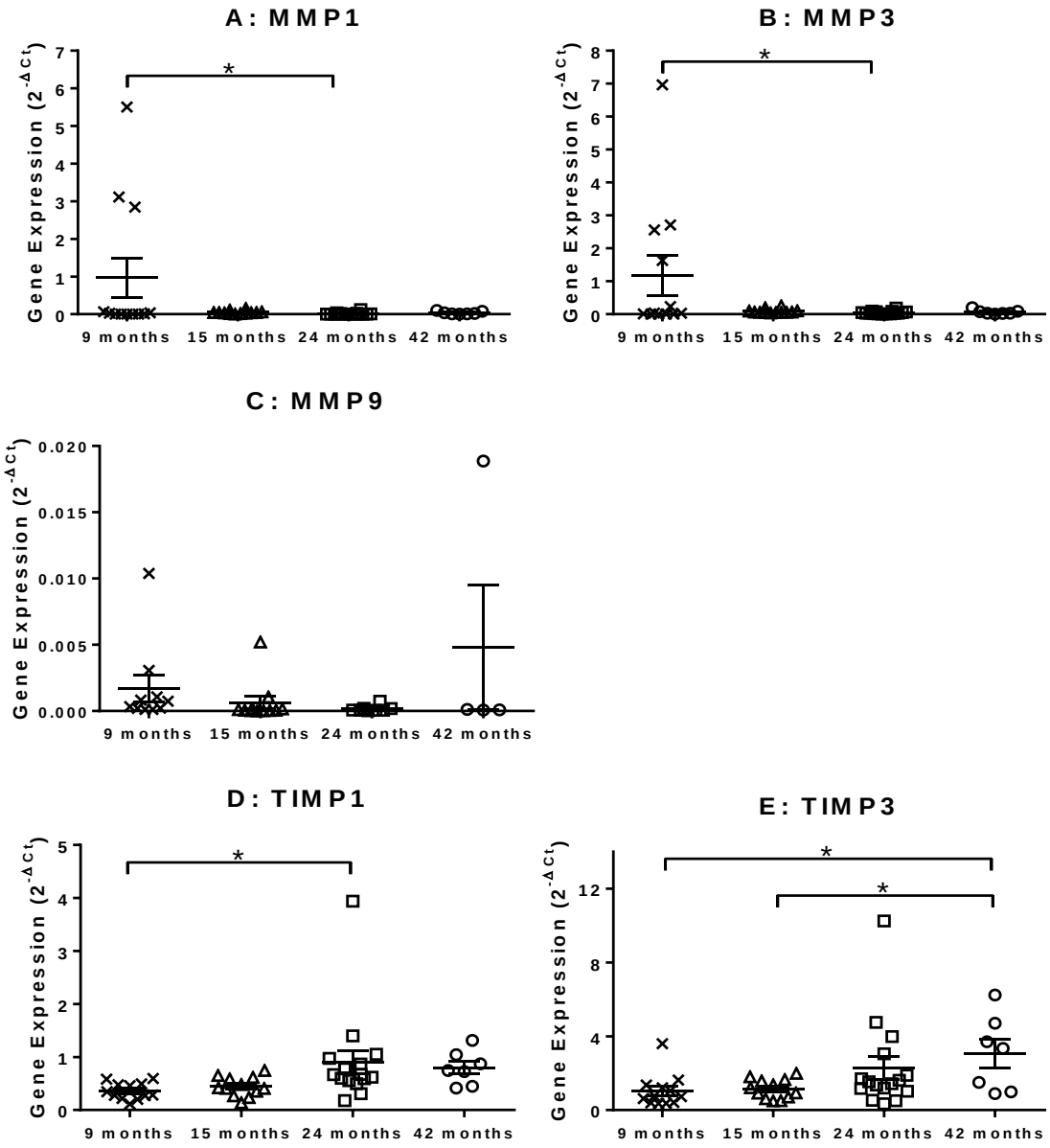


Figure 6.2: Synovial tissue MMP and TIMP expression in different age pigs. MMP1 (A) and MMP3 (B) expression was significantly higher in younger pigs. MMP9 (C) was unchanged with age. TIMP1 (D) and TIMP3 (E) expression was significantly lower in young pigs. *p < 0.05, one-way ANOVA with Tukey's multiple comparisons. Data presented as mean ± SEM.

6.3.2 Association of synovitis and synovial tissue MMP and TIMP expression

Due to the age-associated differences in MMP and TIMP, their expressions were assessed separately in young (9 and 15 months) and old (24 and 42 months) pigs. In young pigs, there was no statistical correlation between synovitis severity and expression of MMP1, MMP9, TIMP1 or TIMP3, but there was a slight but significant correlation between synovitis severity and MMP3 expression ($r^2 = 0.4499$, $p = 0.0274$, Spearman's Rank Correlation). There was no statistical difference in the expression of MMPs and TIMPs in joints with synovitis compared with healthy joints. However, MMP expression was generally increased in synovitis, particularly in severe forms (see Figure 6.3). Expression of MMP1 was upregulated over 9-fold in synovitis and over 790-fold in severe forms ($2^{-\Delta\Delta Ct}$ 9.89 and 792.14 respectively). MMP3 was upregulated over 6-fold in synovitis and over 200-fold in severe forms ($2^{-\Delta\Delta Ct}$ 6.15 and 204.68 respectively). MMP9 was also upregulated over 8-fold in severe synovitis ($2^{-\Delta\Delta Ct}$ 8.51). Furthermore, TIMP1 and TIMP3 expression was unchanged by synovitis ($2^{-\Delta\Delta Ct}$ 1.21 and 0.80 respectively).

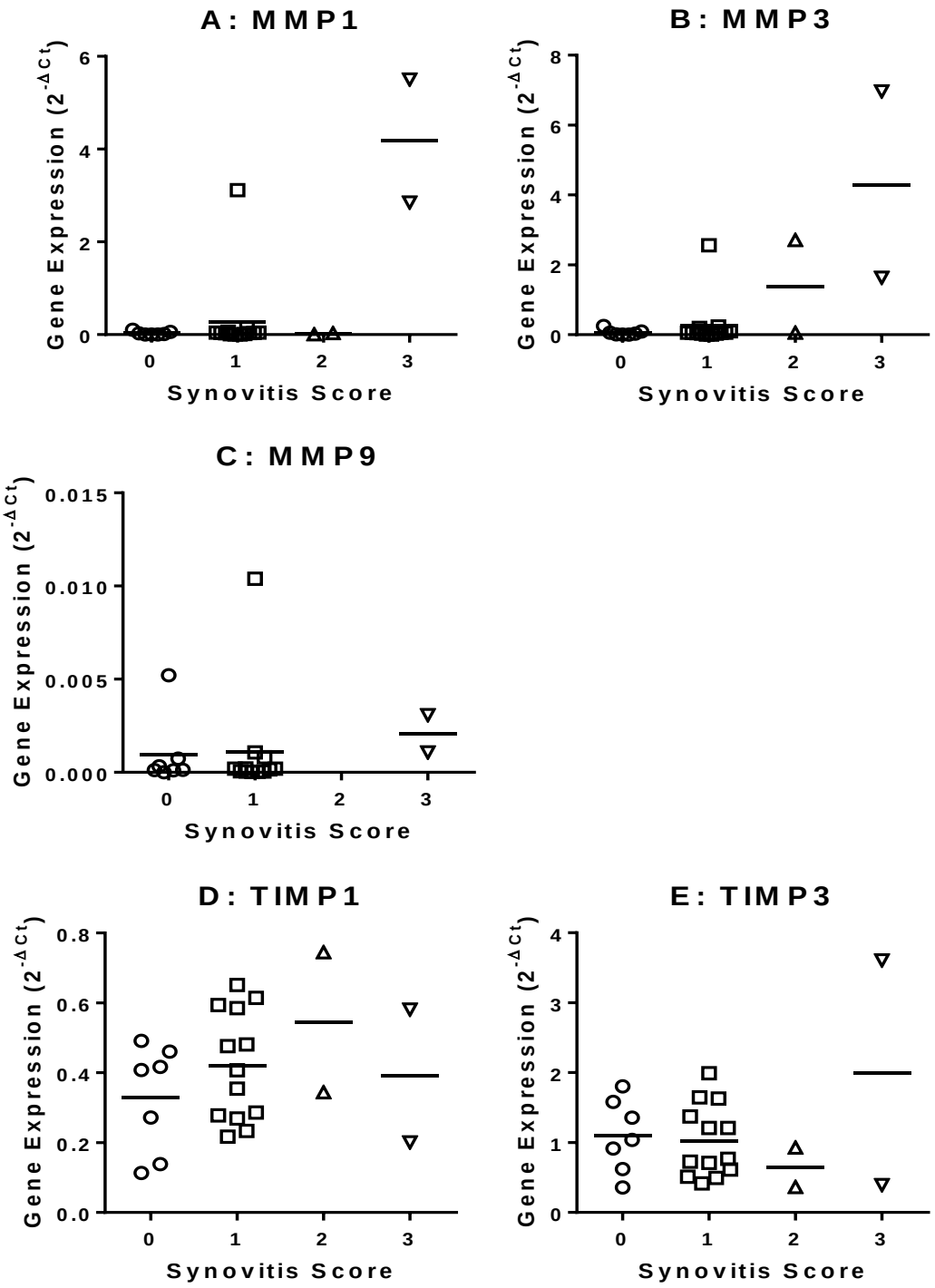


Figure 6.3: Synovial tissue MMP and TIMP expression associated with synovitis in 9 and 15 month old pigs. Expression of MMP1 (A), MMP3 (B), MMP9 (C), TIMP1 (D) and TIMP3 (E) in synovitis. Lines represent means.

In old pigs, MMP1 expression was significantly increased in synovitis ($p = 0.0049$, t-test, see Figure 6.4), which equated to a 2-fold increase in MMP1 in synovitis ($2^{-\Delta\Delta Ct}$

2.68). There was no statistically significant change in expression of MMP9 in synovitis; however, MMP9 was more expressed in the joint with moderate synovitis. This equated to a 180-fold upregulation in expression in moderate synovitis ($2^{-\Delta\Delta Ct}$ 182.74). There was no significant fold change in the expression of MMP3, TIMP1 or TIMP3 during synovitis ($2^{-\Delta\Delta Ct}$ 0.7-1).

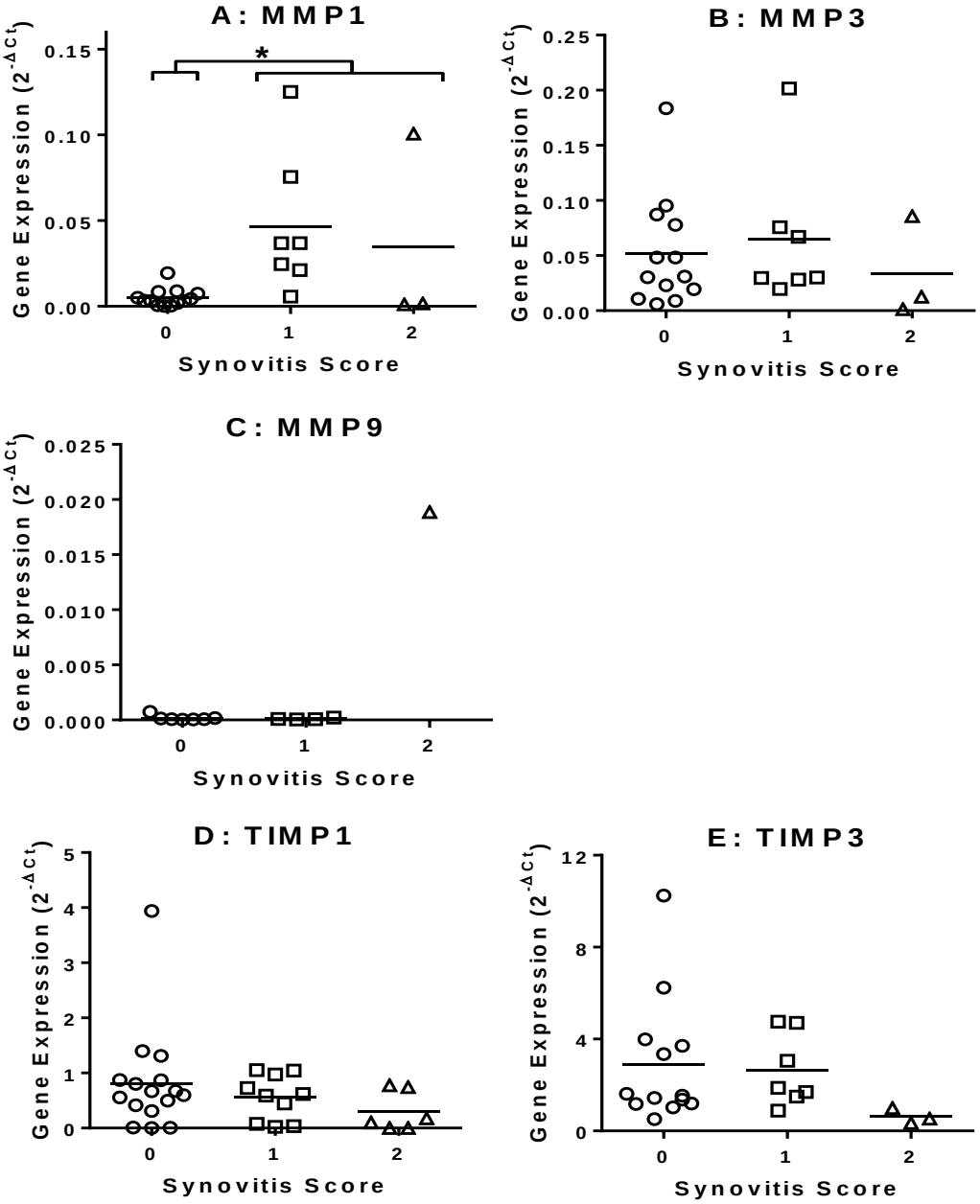


Figure 6.4: Synovial tissue MMP and TIMP expression associated with synovitis in 24 and 42 month old pigs. MMP1 (A) was significantly increased in synovitis but there was no statistical difference in MMP3 (B), MMP9 (C), TIMP1 (D) or TIMP3 (E) expression in synovitis. *$p < 0.05$, t-test. Lines represent means.

6.3.3 Association of osteochondrosis and synovial tissue MMP and TIMP expression

As there was no difference in MMP or TIMP expression associated with age in the young age groups and no difference in OC severity of 9 and 15 month old pigs, the data from the two age groups was pooled before analysis. Due to the small number of healthy pigs, statistical analysis could not be performed (see Figure 6.5). However, the mean expression of MMP1 was over 30-fold higher and MMP3 was 29-fold higher in joints with OC compared to healthy joints ($2^{-\Delta\Delta Ct}$ 33.66 and 29.10 respectively). There was no change in MMP9, TIMP1 or TIMP3 expression associated with synovitis ($2^{-\Delta\Delta Ct}$ 0.7 - 1.64). There was also no difference in MMP or TIMP expression in asymptomatic and symptomatic OC, but it was noted that higher MMP expressions were found in pigs with symptomatic OC (data not shown).

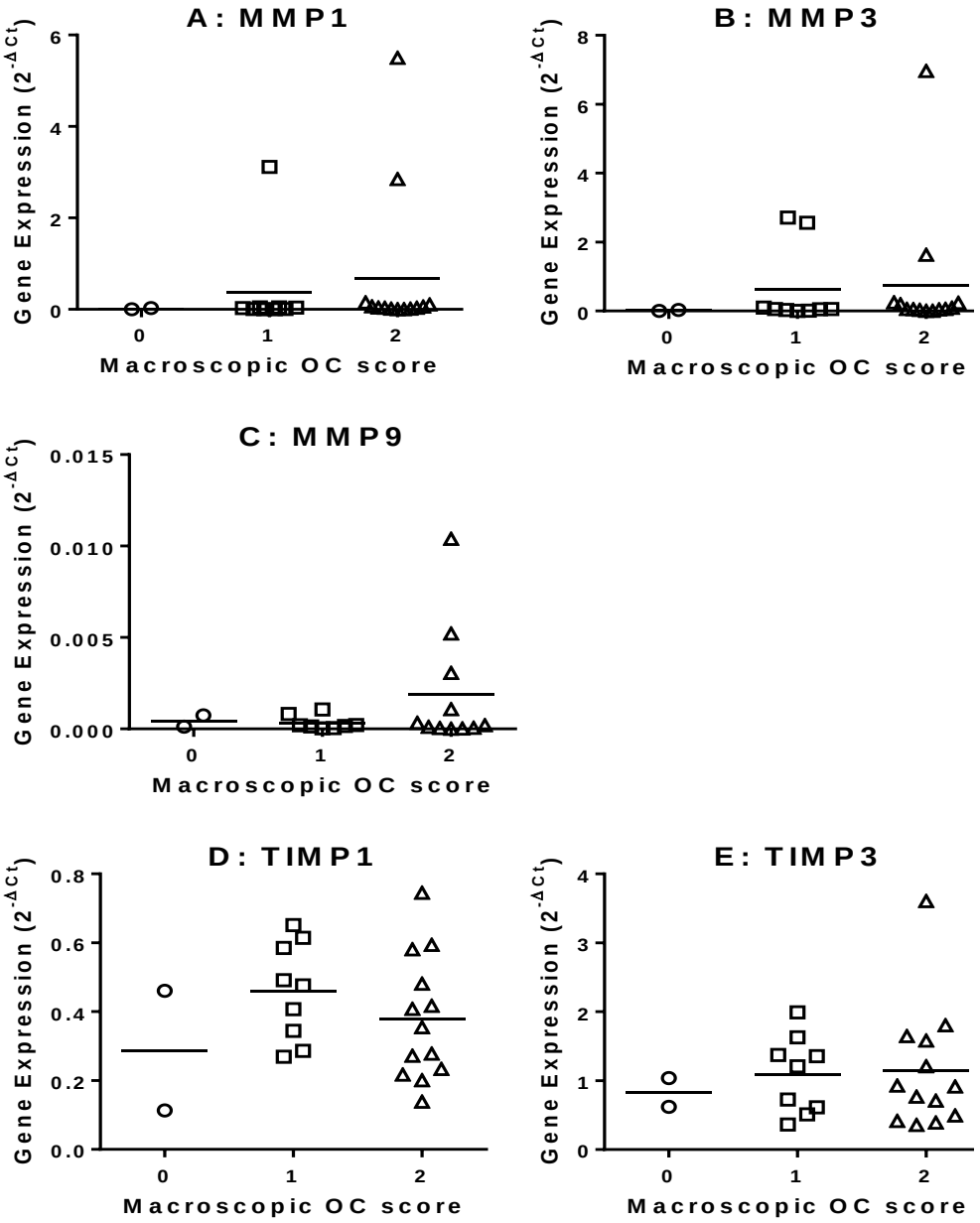


Figure 6.5: Synovial tissue MMP and TIMP expression in porcine knee osteochondrosis. There was no statistically association between OC and MMP1 (A), MMP3 (B), MMP9 (C), TIMP1 (D) or TIMP3 (E) expression. Lines represent means.

Due to the limited number of healthy pigs and severity range, it was not possible to determine statistically the effect of an interaction between OC and inflammation on MMPs and TIMPs. There was no statistical difference in MMP1, MMP3, MMP9, TIMP1 or TIMP3 expression in non-inflammatory or inflammatory OC (see Figure 6.6). However, MMP1 was upregulated 10-fold in non-inflammatory OC and 45-fold in inflammatory OC ($2^{-\Delta\Delta Ct}$ 10.70 and 45.00 respectively), and MMP3 was increased

14-fold in non-inflammatory OC and 38-fold in inflammatory OC ($2^{-\Delta\Delta Ct}$ 14.75 and 38.32 respectively). There was no fold change in MMP9, TIMP1 or TIMP3 expression in non-inflammatory or inflammatory OC ($2^{-\Delta\Delta Ct}$ 0.6 – 1.31). This suggests some MMPs may be increased in OC and further elevated when inflammation is present.

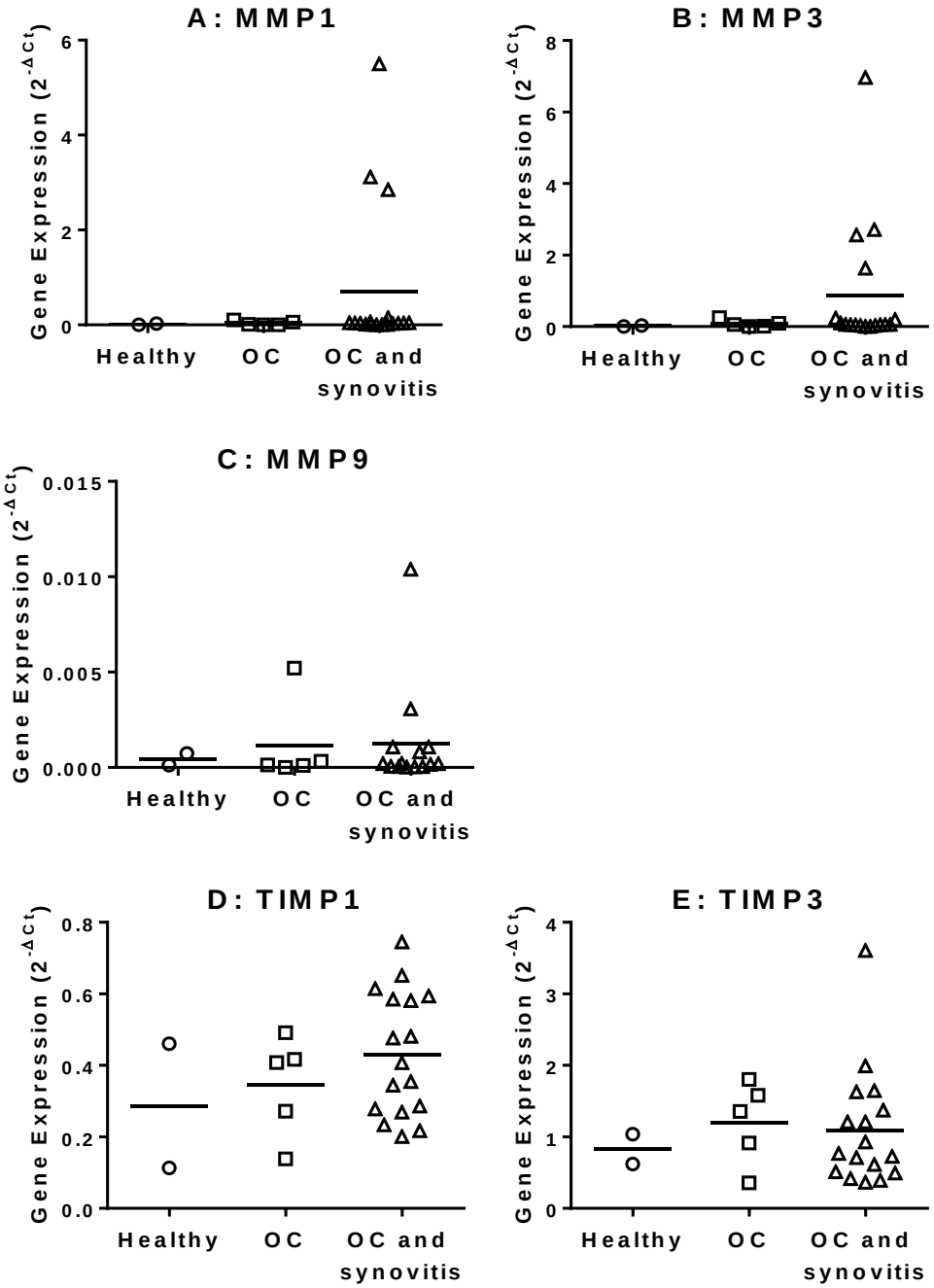


Figure 6.6: Synovial tissue MMP and TIMP expression in porcine osteochondrosis with or without synovitis. There was no difference in MMP1 (A), MMP3 (B), MMP9 (C), TIMP1 (D) or TIMP3 (E) expression in OC with our without synovitis. Lines represent means.

6.3.4 Association of osteoarthritis and synovial tissue MMP and TIMP expression

As there was no difference in MMP or TIMP expression associated with the age and no difference in OA severity of 24 and 42 month old pigs, the data from the two age groups was pooled before analysis. In 24 and 42 month old pigs, there was no significant association between different OA severities and expression of MMP3, MMP9, TIMP1 or TIMP3 (see Figure 6.7). However, there was a weak but significant correlation between MMP1 expression and macroscopic OA severity ($r^2 = 0.4491$, $p = 0.0316$, Pearson's Correlation). Due to there only being one healthy joint it was difficult to determine what effect OA had on MMPs or TIMPs. However, if the healthy joint is representative of healthy pigs, there was no fold change in the expression of MMP3 in OA ($2^{-\Delta\Delta Ct}$ 0.7), but MMP1 and MMP9 were over 2-fold upregulated in OA ($2^{-\Delta\Delta Ct}$ 2.11 and 3.77 respectively).

It was also noted that the healthy joint had significantly higher expression of TIMP1 and TIMP3 than joints with OA (see Figure 6.7). This equated to a 6-fold decrease in TIMP1 and a 10-fold decrease in TIMP3 in OA ($2^{-\Delta\Delta Ct}$ 0.17 and 0.11 respectively). There was no difference in the MMPs or TIMP1 expression in asymptomatic and symptomatic OA, however expression of TIMP3 was significantly decreased in symptomatic OA compared to asymptomatic OA ($p = 0.0228$, t-test, data not shown), which equated to over a 2-fold decrease ($2^{-\Delta\Delta Ct}$ 0.4).

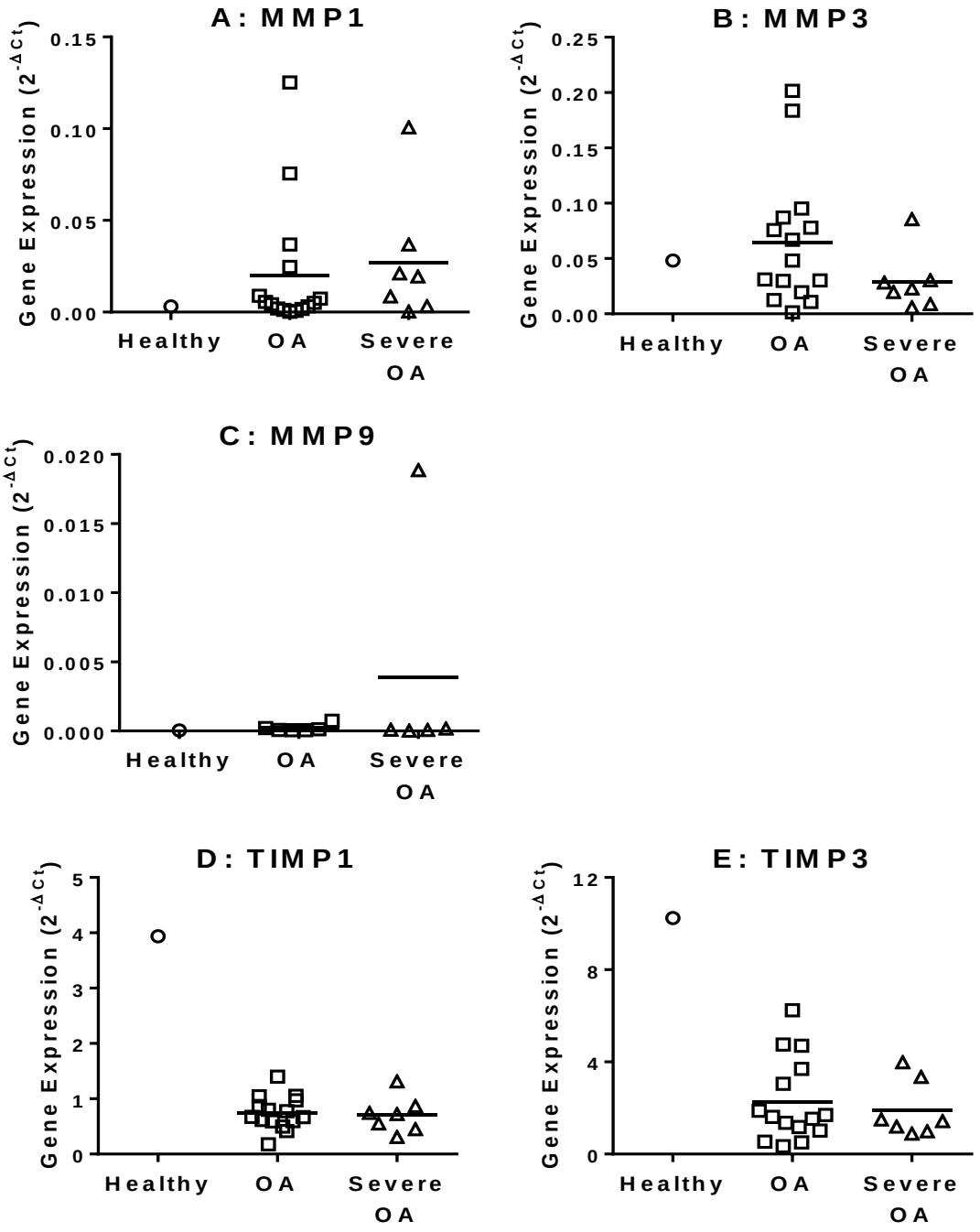


Figure 6.7: Synovial tissue MMP and TIMP expression in porcine knee osteoarthritis. There was no statistical difference in MMP1 (A) MMP3 (B), MMP9 (C), TIMP1 (D) or TIMP3 (E) expression between OA with or without subchondral bone exposure. Lines represent means.

Due to the lack of healthy pigs, it was not possible to determine statistically the interaction between OA and synovial inflammation on the expression of MMPs and

TIMPs in synovial tissue. The presence of inflammation with OA did not alter expression of MMP3, MMP9, TIMP1 or TIMP3 (see Figure 6.8). However, MMP1 expression was significantly increased ($p = 0.0071$, t-test) in inflammatory OA compared to non-inflammatory OA by more than 2-fold ($2^{-\Delta\Delta Ct}$ 3.56 and 1.36 respectively). There was also a significant correlation between MMP1 expression and non-inflammatory OA severity ($r^2 = 0.5534$, $p = 0.0310$, Pearson's Correlation). Furthermore, compared to the healthy joint, MMP9 was also over 2-fold upregulated in non-inflammatory OA and over 5-fold upregulated in inflammatory OA ($2^{-\Delta\Delta Ct}$ 2.58 and 5.92 respectively). This suggests MMP1 and MMP9 may be increased in more severe forms of OA when inflammation is present.

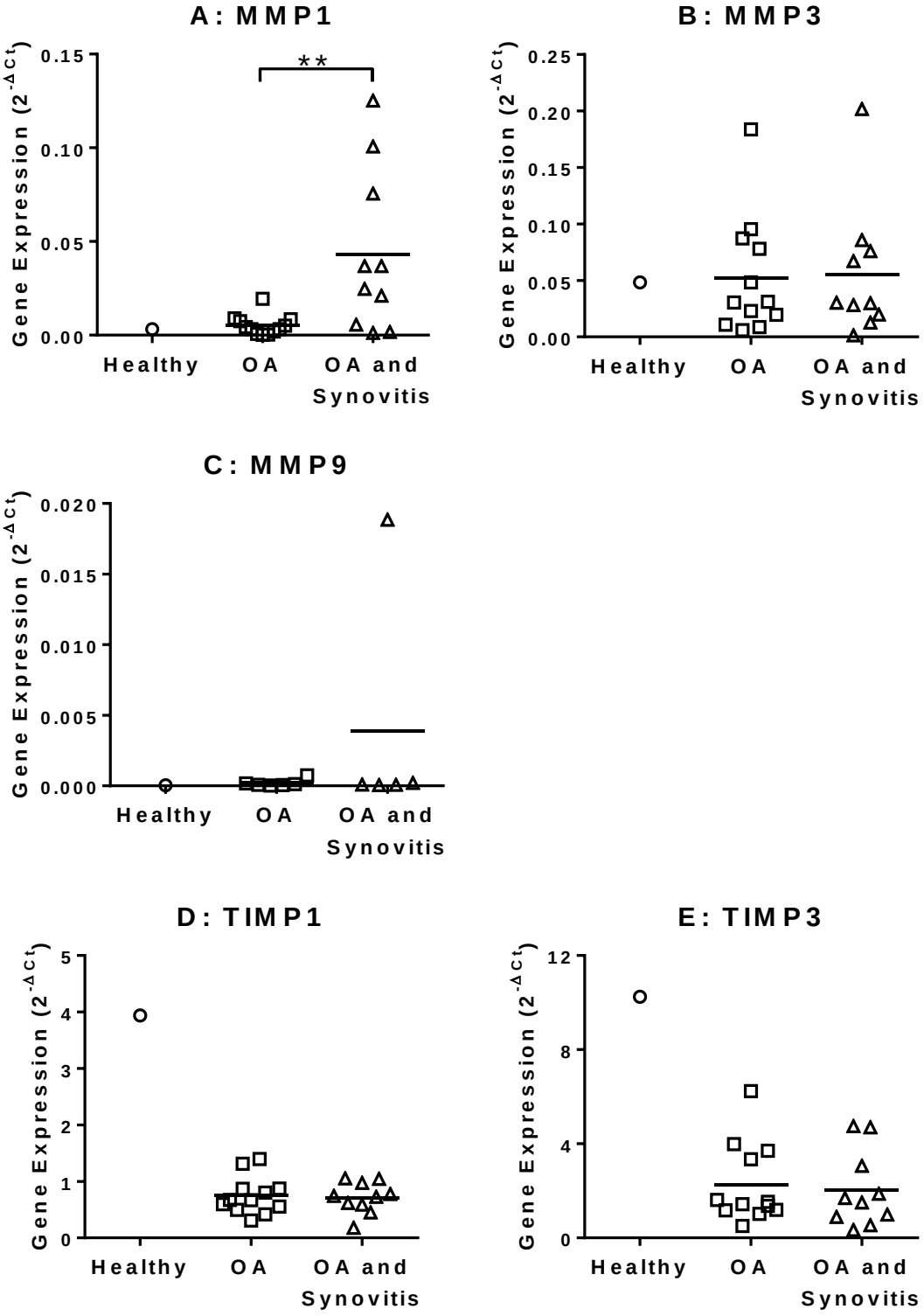


Figure 6.8: Synovial tissue MMP and TIMP expression in porcine osteoarthritis with or without synovitis. MMP1 (A) was increased in OA when synovitis was present but there was no difference in MMP3 (B), MMP9 (C), TIMP1 (D) or TIMP3 (E) expression in OA with or without synovitis. **p < 0.01, t-test. Lines represent means.

6.3.5 Association of synovial tissue cytokines with MMP and TIMP expression

Due to ability of cytokines to elicit changes in MMP expression, the correlation of cytokine (both protein and mRNA levels) and MMP expression was examined. In young pigs, synovial tissue mRNA expression of MMP1 and MMP3 positively correlated with IL-6 protein levels ($r^2 = 0.5635$, $p = 0.0051$, and $r^2 = 0.6317$, $p = 0.0012$ respectively, Pearson's Correlation, see Figure 6.9). In young pigs the expression of MMP1 and MMP3 also correlated with IL-1β protein levels ($r^2 = 0.7165$, $p < 0.0001$, and $r^2 = 0.7143$, $p < 0.0001$ respectively, Pearson's Correlation). In old pigs, mRNA expression of MMP1 and MMP3 correlated with the mRNA expression of IL-6 ($r^2 = 0.5151$, $p = 0.0142$, and $r^2 = 0.5690$, $p = 0.0057$ respectively, Pearson's Correlation).

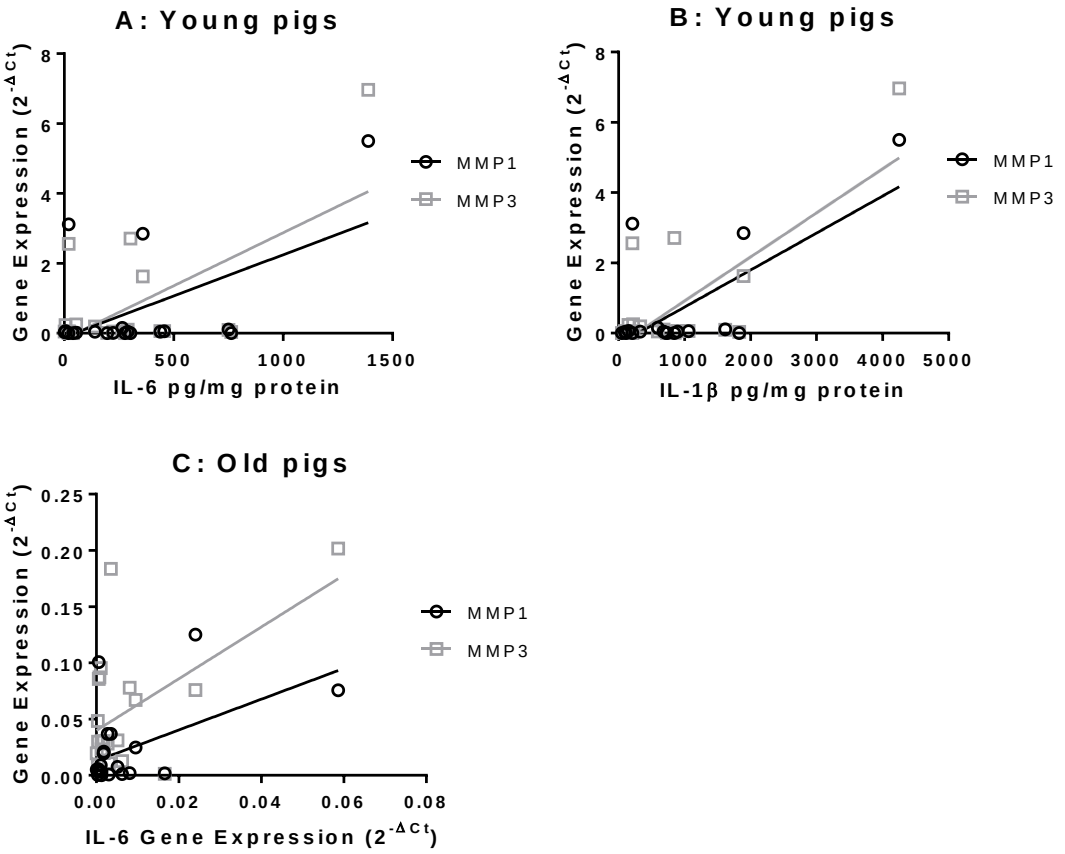


Figure 6.9: Correlation of synovial tissue MMP mRNA expression and cytokine expression or protein levels. Expression of MMP1 and MMP3 correlated with protein levels of IL-6 (A) and IL-1β (B) in young pigs (9 and 15 months old), and with the mRNA expression of IL-6 (C) in old pigs (24 and 42 months old). Scatter plots fitted with linear regression lines.

6.4 Discussion

The work presented in this chapter characterised mRNA expression of MMPs and TIMPs in porcine synovial tissue and their involvement in OC, OA and synovitis. MMPs and TIMPs were highly expressed across all samples, with the exception of MMP9 that was only detected in 70% of samples. Expression of MMP1 and MMP3 significantly correlated, as did expression of TIMP1 and TIMP3. This is likely due to their similar activation and production mechanisms and has been reported previously for MMPs in both healthy and diseased tissue (Kevorkian et al., 2004; Basaran et al., 2017).

It is important to note that mRNA expression of MMPs indicates transcription of the pro-enzyme gene. This mRNA sequence requires translation into the pro-enzyme protein and subsequent activation by removal of its N-terminal fragment (Springman et al., 1990). This is an important regulatory step in MMP activity, so although expression of MMPs gives an indication of MMP activity, it does not show if these enzymes are ultimately translated and activated. Furthermore, expression of the proteolytic enzymes was determined only in synovial tissue. Synoviocytes in the synovium and chondrocytes in the cartilage are both capable of generating MMPs and TIMPs, so regional variations in MMP synthesis across the joint are likely (Hembry 1995). Changes in synovial tissue expression of MMPs and TIMPs are likely to be reflected in the synovial fluid, which could subsequently influence cartilage degeneration. However, due to the ability of chondrocytes to also synthesise these enzymes, changes in the synovium will not solely influence cartilage degeneration but provide some indication of proteolytic activity within the joint.

6.4.1 Age-related differences in MMP and TIMP expression

A number of age-related differences in synovial tissue expression of MMPs and TIMPs were seen in this study. MMP1 and MMP3 were significantly more expressed, and TIMP1 and TIMP3 were significantly less expressed in younger pigs. Previous studies have shown that juvenile horses and mice have increased MMP activity compared to adults (Brama et al., 1998; Gepstein et al., 2002; Gepstein et al., 2003) and previous studies of TIMPs have identified elevated expression and

activity associated with the growth period (Joronen et al., 2000; Gepstein et al., 2003; Tayebjee et al., 2005). The activity MMPs and TIMPs are thought to increase from birth, reaching a peak during growth and then decrease once skeletal maturity is reached (Joronen et al., 2000). This change supports the greater ECM turnover required for growth. This is in agreement with the finding of increased MMP expression in younger animals but is contrary to reduced TIMP expression. However, studies have also identified a number of changes in MMPs and TIMPs associated with joint disease (Brama et al., 1998; Davidson et al., 2006; Bonnema et al., 2007). Due to the lack of healthy pigs across all age groups, it was not possible to determine whether the age-related differences seen were solely due to age or also influenced by disease state, which may explain the reduced TIMP expression in younger animals.

6.4.2 MMP and TIMP expression in porcine synovitis

Synovial inflammation resulted in a number of changes in MMP expression across the entire study population; however, the particular enzymes involved differed depending on the pig's age. In young pigs, synovitis was most associated with increased MMP1 and MMP3 expression. In old pigs, synovitis was more associated with increased MMP1 and MMP9 expression. These findings are in agreement with a number of studies, which identified increased MMP expression in the synovium and synovial fluid during inflammation (Hembry et al., 1995; Sutton et al., 2009).

Both macrophage-like and fibroblast-like synoviocytes can synthesise MMPs (Clegg et al., 1997; Bondeson et al., 2006). During inflammation, expression of these enzymes can be altered, partly driven by changes in inflammatory cytokines. In equine synovial fluid increased pro- and active forms of MMP2, MMP3 and MMP9 have been associated with inflammation (Clegg et al., 1997; Brama et al., 1998; Trumble et al., 2001). Furthermore, in a minipig model of OA, increased MMP1 has been identified in inflamed synovium (Haslauer et al., 2013). These studies concluded that synovitis results in increased proteolytic enzymes and an imbalance in MMP and TIMP expression (McCachren, 1991). This is in agreement with the present study that identified elevated MMP expression associated with synovitis,

but no change in TIMP expression, which could result in an imbalance of their activity and the disinhibition of MMPs. This could lead to increased catabolic activities within the joint, potentially increasing ECM degradation and resulting in cartilage disruption.

6.4.3 MMP and TIMP expression in porcine osteochondrosis

There was no statistical association between OC severity and mRNA expression of MMPs and TIMPs in the synovium of 9 and 15 month old pigs. However, this was limited by the small number of pigs and small severity range. The fold change in gene expression does suggest there may be some upregulation in synovium MMP expression during OC. There has been little investigation into the expression of these enzymes in the synovium during OC. Previous studies of synovial fluid in equine OC identified no significant change in MMP expression (Brama et al., 1998; de Grauw et al., 2011). However, studies of equine and canine cartilage have identified a more variable association of proteolytic enzymes and their inhibitors with OC. Kuroki et al. (2005) found no change in MMP1 or MMP3 but reduced TIMP3 in canine OC. Riddick et al. (2012) found no change in MMP1 but increased MMP3 in equine OC. These differences may partly result from the tissue type and species being investigated. It is expected that synovial fluid would reflect changes in metabolic and proteolytic activity occurring in both the synovium and cartilage, whereas changes in the synovium or cartilage would be more localised as these tissues contain the protein-synthesising cells (Brama et al., 1998).

The presence of inflammation in some pigs with OC may also complicate the association of MMPs and TIMPs expression. The highest MMP and TIMP expressions were seen in joints showing symptomatic OC with synovial inflammation. Furthermore, there was a general trend of upregulation in MMP1 and MMP3 expression in non-inflammatory OC, which appeared further upregulated in inflammatory OC, although not statistically significant. This indicates there may be some increases in synovium MMPs associated with osteochondritic changes in the cartilage, which is exacerbated if inflammation is present. No significant change in TIMP expression was associated with OC or inflammation, suggesting that there

may be an imbalance in synovium MMPs and TIMPs during OC and inflammation that could lead to in MMP disinhibition. A similar imbalance of MMPs and TIMPs has been reported in the cartilage during canine OC (Kuroki et al., 2005).

The reason for alterations in MMPs in the cartilage or synovium during OC is unclear. Studies suggest that this increase is not causative of initial OC lesion but a reaction to the damaged cartilage and may be involved in matrix reabsorption to repair lesions (Brama et al., 1998; Kuroki et al., 2005; Riddick et al., 2012). Increases in cartilage MMPs, as identified in other studies, may help lesions repair by removing dysfunctional cartilage components. However, it is also suggested that increased MMPs may weaken the cartilage matrix, leading to cartilage necrosis and the appearance or expansion of OC lesions (Ohata et al., 2002; Riddick et al., 2012). The disinhibition of synovium MMPs, as suggested in the present study, may increase MMPs in synovial fluid which could drive further inflammatory changes but also the degradation of cartilage, thereby reducing the ability of OC lesions to repair.

A larger study population with more healthy animals is required to evaluate fully the changes in proteolytic enzymes associated with porcine OC. Identifying changes in the synovium, synovial fluid and cartilage may help to create a more complete picture of their involvement in OC. Furthermore, assessment of expressions of other MMPs, particularly MMP13, is also required. Several studies have identified increased MMP13 in cartilage during equine and canine OC (Kuroki et al., 2005; Mirams et al., 2009; Riddick et al., 2012); however, no such study of MMP13 expression in the synovium during OC has been conducted. For the present study, a number of MMP13 primers were designed and tested for qPCR, but they were not specific enough and formed multiple peaks in the melt curve, suggesting more than one product was formed or primer dimerization occurred.

6.4.4 MMP and TIMP expression in porcine osteoarthritis

There was a slight correlation between OA chondropathy and MMP1 expression, this and the fold change in MMP1 and MMP9 expression suggest there may be

some MMP upregulation in joints with OA. Synovial cell cultures from osteoarthritic joints produce large amounts of MMPs, and in human OA the synovium shows increases in a number of different MMPs, including MMP9 (Bondeson et al., 2006; Davidson et al., 2006). Studies of induced OA in minipigs have identified elevated MMP1 and MMP13 expression in the synovium (Haslauer et al., 2013; Sieker et al., 2018). Increases in MMPs, including MMP1, -3, -13 and -14, have also been identified in the cartilage of minipigs following OA induction (Hembry et al., 2001; Cruz et al., 2015). However, other studies of human OA have identified MMP1 and MMP3 as decreased in cartilage and unchanged in the synovium, and MMP9 as elevated in both cartilage and synovium (Kevorkian et al., 2004; Davidson et al., 2006).

The results of the present study showed a similar general pattern to that found in previous human and animal studies, with either no change or an upregulation in MMP expression in the synovium during OA. Furthermore, it is suggested that inflammatory OA may represent a separate subtype of OA. The present study identified increased MMP1 associated with more severe chondropathy in non-inflammatory OA, whereas the fold change suggests MMP1 and MMP9 are both upregulated in inflammatory OA. This suggests that changes in particular MMPs expressed during spontaneous porcine OA may depend on the inflammatory state.

Although the expression of TIMPs showed no statistical correlation to OA severity, if the single healthy joint was representative of healthy tissue, there was a large reduction in TIMP1 and TIMP3 expression in OA. Furthermore, symptomatic OA, which suggests a worse disease state, was associated with a significant downregulation in TIMP3 expression. This suggests spontaneous porcine OA is associated with decreased TIMP expression. Studies of cartilage and synovial TIMP expression in human and animal OA have found reduced TIMP1 in cartilage but unchanged or elevated TIMP1 in synovium (Kevorkian et al., 2004; Davidson et al., 2006). TIMP3 expression has also been shown as increased or unaltered in cartilage and synovium of joints with OA (Su et al., 1999; Kevorkian et al., 2004; Davidson et al., 2006). However, these studies characterised only OA and non-OA joints and not

the severity or disease stage; another study of early canine OA identified decreased synovial fluid TIMP2 (Alam et al., 2011). In the present study, the reduced TIMP and increased MMP expressed by the synovium suggest that as OA progresses the balance of TIMP and MMP activity is lost. TIMPs inhibit MMPs and genetic knock-out of TIMP3 in mice increases collagen cleavage similarly to OA (Sahebjam et al., 2007), therefore imbalance of MMPs and TIMPs could drive further degeneration of the ECM and lead to further cartilage degeneration in spontaneous porcine OA.

The findings of the present study suggest there is reduced TIMP and increased MMP expression in the synovium associated with spontaneous porcine OA, and that inflammation is associated with further increases in MMPs. This suggests that as OA progresses, and inflammation increases, MMP and TIMP activity in porcine synovium becomes more imbalanced. The specific changes in particular proteolytic enzyme and inhibitor expression are contradictory to some studies of OA in humans and animal models. However, these studies agree with the main finding of the present study, that an imbalance of MMP and TIMP is present in the synovium during OA. A larger study population, with more healthy animals, is required to evaluate further the association of MMP and TIMP expression with porcine OA, in the cartilage, synovium and synovial fluid.

6.4.5 Association of synovial tissue cytokines and the expression of MMPs and TIMPs

In young pigs, synovium expression of MMP1 and MMP3 strongly correlated with protein levels of IL-6 and IL-1β. In old pigs, synovium expression of MMP1 and MMP3 strongly correlated with mRNA expression of IL-6. Furthermore, similar changes in cytokine and MMP expression in different pathological states were observed in the present study. For example, during synovial inflammation, the increase in cytokines occurred in parallel to elevated MMP expression. A number of studies have identified a role for cytokines and growth factors in stimulating synthesis or secretion of pro-MMPs (Cawston, 1998; McIlwraith, 2005). IL-1 and IL-6 have been shown to stimulate MMP and TIMP production by synovial fibroblasts (Sato et al., 1990), and synovial macrophages produce MMPs in response to IL-1β

and TNFα stimulation (Bondeson et al., 2006). The presence of catabolic factors, particularly interleukins, can also stimulate pro-MMP enzyme activation. This supports the findings of the present study, which identified an association between cytokine levels and MMP activity (Sato et al., 1990; Bondeson et al., 2006; Hyc et al., 2016). It is therefore likely that a change in joint pathology alters synovium cytokines, which in turn influences MMP expression in the synovium during porcine joint disease.

6.5 Conclusions

Synovial tissue of female pigs showed high mRNA expressions of MMPs and TIMPs, which varied depending on age and disease status. Synovial inflammation corresponded with increased MMP expression. Spontaneous porcine knee joint OC was associated with increased MMP expression, which may be exacerbated by the presence of inflammation. Similarly, increased MMP and decreased TIMP expression was associated with spontaneous porcine knee joint OA, although the particular MMPs involved may vary depending on the inflammatory state. Changes in inflammatory cytokines may partly elicit the alterations in MMPs identified during the different disease states. The findings suggest that in porcine joint disease, both OC and OA, there is increased MMPs and an imbalance of MMP and TIMP expression. This could elicit further pathological changes within the joint such as cartilage degeneration, worsening the disease state or altering disease progression.

Chapter 7

Characterising Changes in the Nervous System Associated with Porcine Joint Disease

7.1 Introduction

Pain is a predominant symptom of joint diseases and the most common reason for people with OA seeking treatment (Murphy et al., 2012). However, treatment of this pain is often ineffective, partly due to limited understanding of the underlying mechanisms. In early disease, joint pain is often episodic and induced by movement or loading. As the disease progresses, it can result in a shift towards a chronic pain state, resulting in spontaneous pain, hyperalgesia and allodynia (Nagy et al., 2006; Schaible, 2012). This chronic pain seen in OA and other joint diseases arises due to complex neurophysiological changes occurring in both the PNS and CNS (Kidd, 2012).

Injury or damage causes the release of inflammatory mediators, such as cytokines and growth factors. These mediators can directly stimulate peripheral nerves or sensitise them to mechanical, chemical or thermal stimuli, resulting in peripheral sensitisation (see section 1.8.2.1). This increases excitability and sensitivity of primary afferent fibres (McMahon et al., 2013), increasing neuronal firing rate, causing hyperalgesia and activation of silent C-fibres. During chronic pain, this sensitisation can alter the phenotype of primary afferent fibres, changing the production of chemical mediators of pain such as bradykinin, substance P, NGF, CGRP and receptors, including TRPV1. These changes can be identified in neuronal cell bodies found within DRGs.

NGF is part of the neurotrophin protein family involved in neuronal survival and differentiation, and helps regulate the functional properties of mature nerves (Lee et al., 1998). In human rheumatoid arthritis and OA, NGF is increased in synovial fluid and NGF inhibition reduces pain (Chang et al., 2016), although trials of anti-NGF therapies for OA pain were initially halted after some patients showed rapidly progressing OA which resulted in joint replacement (Hochberg, 2015). In rodent models of joint pain, increased DRG NGF expression is associated with worse pain behaviours, worse chondropathy and increased synovial inflammation (Ashraf et al., 2013; Stoppiello et al., 2014). CGRP is a neuropeptide and a potent vasodilator involved in maintaining cardiovascular system function and wound healing, but it

also has nociceptive functions (Russell et al., 2014). In human OA, CGRP is increased in neurones that innervate the synovium (Saxler et al., 2007). In the rat MIA model, CGRP is increased in plasma, synovium and peripheral neurones, and CGRP inhibition reduces mechanical hypersensitivity (Orita et al., 2011a; Ogbonna et al., 2013). TRPV1 is a nonselective cation channel and is important for polymodal nociceptor activity, and is activated by heat, acidic conditions (protons) and capsaicin (Brito et al., 2014). In human OA, synovium TRPV1 expression is increased (Kelly et al., 2015). Similarly, in rat models of pain, TRPV1 expression in DRGs is increased and TRPV1 antagonists can reduce sensitisation of peripheral neurones (Nishigami et al., 2013; Kelly et al., 2015). Therefore, overall, these studies have implicated the expression and activity of NGF, CGRP and TRPV1 receptors as important in pain perception and peripheral sensitisation in humans and rodent models of pain and OA, but their involvement in porcine disease or pain has not been investigated.

Changes in the PNS increase the probability that a stimulus will result in action potential conduction to the spinal cord. This, in turn, increases spinal cord neuronal activity and can result in central sensitisation (see section 1.8.2.2). Increased cytokine and neuropeptide release in the spinal cord contributes to hyperalgesia and allodynia seen in OA, and other diseases, by causing spinal neurones to become hyper-excitable (Schaible et al., 2009b; Orita et al., 2011b). This also activates nervous system immune cells and there is increasing evidence that neuroinflammation occurs during articular diseases (reviewed by Fusco et al., 2017). Reciprocal signalling between the CNS and immune system is emerging as a key phenomenon occurring in pathological conditions and chronic pain (Grace et al., 2014). In models of OA, spinal cord microglia and astrocytes become activated, which contributes towards pain behaviours and facilitates central sensitisation; preventing glial cell activation reduces pain behaviours (Sagar et al., 2011; Burston et al., 2013).

Astrocytes are specialised star-shaped glial cells that are essential for healthy functioning of the nervous system. They provide structural and trophic support,

contribute towards synaptic transmission, promote repair and help maintain homeostasis of the extracellular environment (Sofroniew and Vinters, 2010; Grace et al., 2014). In response to insult or injury, astrocytes are activated and undergo reactive astrogliosis, which is a graded continuum of changes ranging from reversible alterations in gene expression and cell morphology to long-lasting scar formation (see Figure 7.1A). The main features of astrogliosis include cell hypertrophy, projection thickening, increased expression of glial fibrillary acidic protein (GFAP) and astrocyte proliferation (Sofroniew and Vinters, 2010). In rodent models of OA, increased astrocyte activation correlates with allodynia and subchondral bone changes (Sagar et al., 2011; Ogbonna et al., 2013; Yu et al., 2013).

Microglia are immune cells of the CNS, acting as resident macrophages to control the CNS microenvironment. They are involved in neurogenesis, synaptic plasticity and repair but also eliminate dead cells, proteins or antigens by phagocytosis (Colonna and Butovsky, 2017). Under normal conditions microglia are in a resting state, with small-ramified cell bodies and long fine projections. Their activation results in reactive gliosis; initially microglia become hyper-ramified but then undergo hypertrophy and projections thicken (see Figure 7.1B). Activated microglia also show increased ionized calcium-binding adaptor molecule 1 (IBA1) expression, which can be used as an activation marker (Imai and Kohsaka, 2002). Persistent activation can result in microglia becoming amoeboid, indicating they are phagocytic (Grace et al., 2014). These morphological changes associated with microglia activation have been implicated in the induction and maintenance of persistent pain in rodent models of OA (Lee et al., 2011; Orita et al., 2011a; Miller et al., 2013; Ogbonna et al., 2013; Yu et al., 2013; Ogbonna et al., 2015; Tran et al., 2017). Increased microglia activation also correlates with allodynia in rodents, and inhibiting their activation reduces pain behaviours (Sagar et al., 2011; Burston et al., 2013).

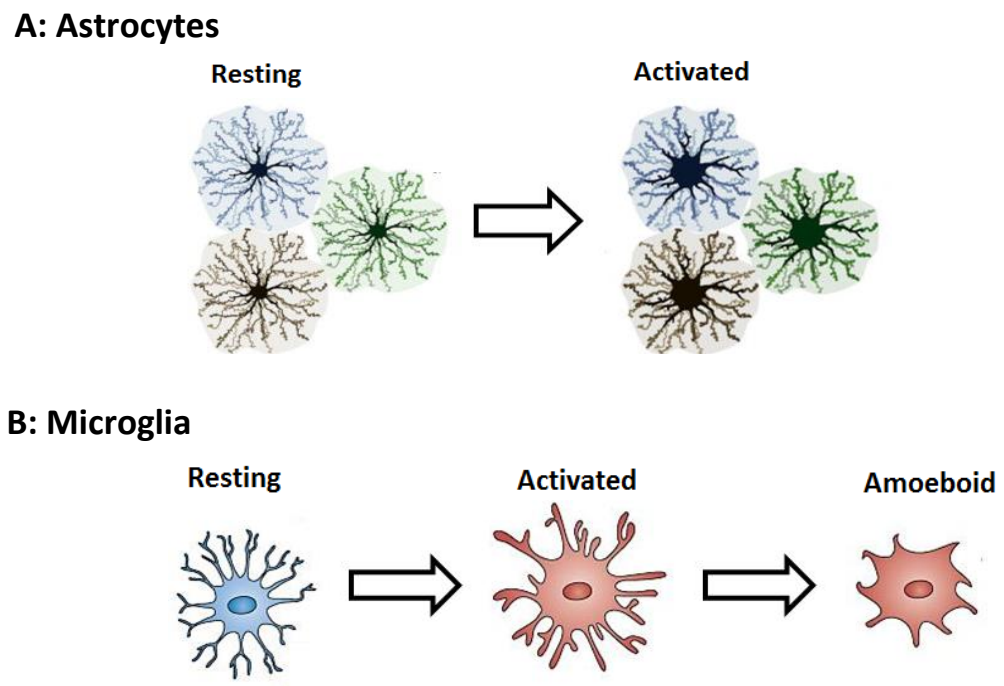


Figure 7.1: Morphology of resting and activated astrocytes and microglia undergoing reactive astrogliosis. A) Activation of astrocytes results in cell hypertrophy and projection thickening. B) Activation of microglia results in cell hypertrophy and projection thickening, with microglia eventually becoming amoeboid. Images adapted from Sofroniew (2009) and Perry et al. (2010).

Changes in the phenotype of primary afferent fibres and immune cell activation in the spinal cord are well characterised in rodent models of joint disease and chronic pain (Sagar et al., 2011; Burston et al., 2013). However, their involvement in other species is much less well defined. Furthermore, the extent of chronic pain experienced in porcine disease is poorly characterised despite the similarities between porcine, rodent and human pain processing systems. Although lame sows show increased mechanical sensitivity, indicating hyperalgesia and potential nervous system sensitisation (Nalon et al., 2013b), there has been no investigation as to whether porcine joint diseases, such as OC or OA, result in chronic pain, or whether changes in the phenotype of peripheral neurones and/or activation of spinal cord immune cells occurs.

7.1.1 Aim

The aim of the work presented in this chapter was to investigate the cellular response of the spinal cord and the phenotype of primary afferent fibres associated with porcine joint disease. Changes in cellular responses within the spinal cord dorsal horn, indicated by activation of astrocytes and microglia, were assessed using immunohistochemical staining for GFAP and IBA1, respectively. Changes in primary afferent fibre phenotype were assessed by immunohistochemical staining for NGF, CGRP and TRPV1 within DRGs.

7.2 Methods

7.2.1 Animals

The same four age cohorts of clinically healthy female commercial pigs of the crossbred genotype 'Landrace x Large White x Duroc' were used in this study. Pigs were aged 9 months (n = 6), 15 months (n = 6), 24 months (n = 8) and approximately 42 months (n = 7). Pigs were assessed for 5 to 7 days prior to slaughter for pain behaviours and lameness (see section 2.2). The lumbar regions L3-L5 of the spinal cord and DRGs were isolated (see section 2.3.5) and post-fixed by immersion in 4% PFA overnight then cryoprotected in 30% sucrose solution at 4°C. Both knee joints from 9 and 15 month old pigs were assessed macroscopically for OC (see section 2.4.1). Both knee joints from 24 month old pigs and one knee from 42 month old pigs were assessed macroscopically for OA (see section 2.4.2).

7.2.2 Processing and sectioning of porcine spinal cord and DRGs

After fixing and cryoprotecting, spinal cord segments and DRGs were embedded in OCT for sectioning. The tissue was initially placed in a mixture of 30% sucrose solution and OCT (1:1) overnight, which reduced formation of cracks and artefacts during freezing. DRGs and spinal cords were placed in plastic moulds filled with OCT and allowed to equilibrate before freezing with isopentane (Fisher Scientific) chilled over liquid nitrogen. Once frozen, the blocks were wrapped in parafilm® and aluminium foil and stored at -80ºC. DRGs and spinal cord were sectioned at 10 µm using a cryostat (Bright OFT5000-04, Bright Instruments) and collected onto SuperFrost™ Plus or Polysine™ coated slides (Fisher Scientific). Spinal cords were serially sectioned and every 15th section collected. Slides were wrapped in pairs in parafilm® and aluminium foil and stored at -20˚C until use.

7.2.3 Source of rat DRGs

Rat DRGs were stained alongside pig DRGs to help immunohistochemistry (IHC) optimisation and act as a positive control. Rat DRGs were obtained from animals used in other studies conducted within the research group. These studies were carried out in accordance with ASPA and licensed procedure carried out under project license number 40/3246, all experiments were approved by the University

of Nottingham Animal Welfare and Ethical Review Body and carried out in accordance with ARRIVE guidelines. Rats were deeply anaesthetized with sodium pentobarbital and transcardially perfused with cold PBS followed by 4% PFA. DRGs were removed and post-fixed overnight in 4% PFA, cryoprotected in 30% sucrose at 4°C and embedded in OCT filled plastic moulds using isopentane chilled over liquid nitrogen (conducted by Craig Bullock and Kenneth Paton). DRGs were sectioned at 10 - 13 µm onto SuperFrost™ Plus slides using a cryostat.

7.2.4 Immunohistochemistry

Tissue staining by IHC follows the fundamental principle that a specific antibody will bind to a specific antigen, forming an antigen-antibody complex that can be visualised directly or indirectly (see Figure 7.2). For the direct method, the antibody is conjugated to a fluorochrome or an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (Nakane and Pierce, 1966; Mason and Sammons, 1978). Using the indirect method, the primary antibody detecting the antigen is unconjugated. A conjugated secondary antibody is then added which is directed against the species the primary antibody was raised in. In this study, the indirect avidin-biotin complex (ABC) coupled to HRP (ABC-HRP) method was used. Avidin is a glycoprotein that binds with high affinity to biotin. The secondary antibody was biotin-conjugated and attached to the primary antibody (Guesdon et al., 1979; Hsu et al., 1981). ABC-HRP complex was then added, consisting of avidin bound to biotinylated HRP enzyme, which binds to the conjugated secondary antibody. A 3,3'diaminobenzidine (DAB) substrate was then added, which is oxidised by the HRP enzyme, producing a visible brown precipitate, or a black precipitate if a nickel-DAB substrate is used (Shu et al., 1988).

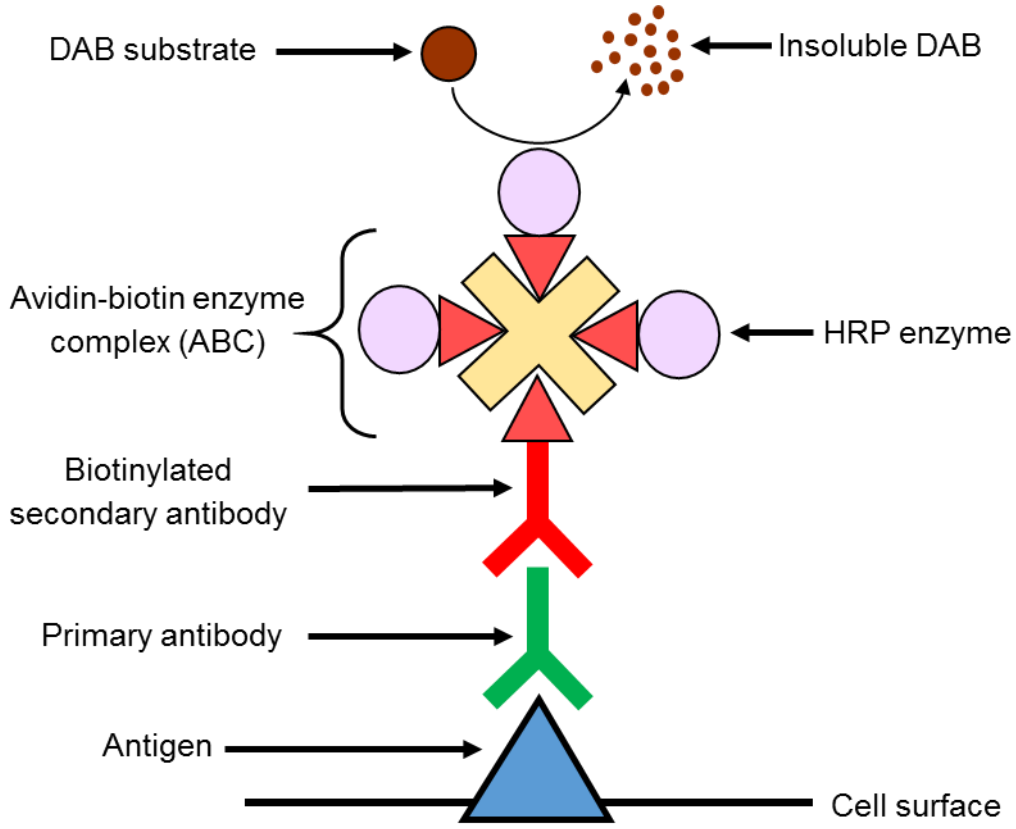


Figure 7.2: Schematic illustration of the ABC-HRP DAB IHC. A specific primary antibody binds to a specific cell surface antigen, which then binds with a biotinylated secondary antibody. The secondary antibody binds to an avidin-biotin complex coupled to a HRP enzyme. Incubation of the enzyme and a DAB substrate produces a visible precipitate.

7.2.4.1 Primary and secondary antibodies

The primary and secondary antibodies were aliquoted into single-use volumes upon arrival and stored at -20°C, with the exception of NGF primary antibody which was stored at 4°C. Details of antibodies used can be found in Table 7.1.

Table 7.1: Details of primary and secondary antibodies used for IHC.

Target	Species specificity	Host	Dilutions	Incubation time	Supplier	Cat. Number
GFAP	Human, pig, rat	Mouse	1:400	18 hours	Sigma-Aldrich	G3893
IBA1	Human, pig, rat, guinea pig, cow, dog	Goat	1:500	18 hours	Abcam	ab5076
NGF	Human, pig, rat, mouse, horse, dog, cow	Rabbit	**	**	Santa Cruz Biotechnology	sc-548
CGRP	Human, rat, pig*	Rabbit	**	**	Sigma-Aldrich	C8798
TRPV1	Human, rat, pig*	Rabbit	**	**	Merck	AB5370
Anti-mouse biotinylated secondary	Mouse	Horse	1:500	1 hour	Vector Laboratories	BA-2000
Anti-goat biotinylated secondary	Goat	Horse	1:500	1 hour	Vector Laboratories	BA-9500
Anti-rabbit biotinylated secondary	Rabbit	Horse	1:500	1 hour	Vector Laboratories	BA-1100

* - pig not listed in species specificity but its use in pigs has been published previously

** - a number of different combinations of dilutions and incubation times were tested

7.2.4.2 Spinal cord immunohistochemistry

Spinal cord sections were briefly air dried to improve slide adhesion before washing in PBS. The tissue's endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide (in PBS, Sigma-Aldrich) for 10 minutes. Sections were rinsed in PBS and blocked with a 5% blocking solution (10 ml PBS and 500 µl horse serum, Sigma-Aldrich) for 1 hour before incubating with primary antibody for 18 hours (IBA1 1:400 or GFAP, 1:500 in blocking solution). Following a PBS wash, sections were incubated with a biotinylated secondary antibody for 1 hour (horse anti-goat or anti-mouse, 1:500 in PBS).

Sections were washed again in PBS and incubated with ABC reagent for 30 minutes. ABC reagent was prepared by adding 2 drops of reagent A (avidin DH) followed by 2 drops of reagent B (biotinylated horseradish peroxidase H) to 5 ml of PBS (Vector Laboratories Vectastain Elite ABC kit #PK6100). The ABC reagent was left to stand for 30 minutes before use. Sections were then washed in distilled water and staining developed by incubation with a DAB substrate. DAB was prepared immediately before use by adding 2 drops of buffer solution, 4 drops of DAB solution and 2 drops of hydrogen peroxide solution to 5 ml of distilled water (Vector Laboratories DAB peroxidase substrate kit #SK-4100). Sections were incubated with DAB until staining was visible (approximately 6 minutes). Sections were rinsed in water to stop the reaction before dehydrating in graded alcohol (50%, 90%, and 100% IMS), clearing with histoclear® and coverslipped using VectaMount (Vector Laboratories #H-5000). All incubations were performed at room temperature and slides kept in a humidified chamber to prevent sections drying out. Images of stained spinal cord were visualised using an Olympus BHS B2 light microscope and acquired using Leica DFC320 camera and LAS v4.2 software.

Antibody specificity was determined by omission of the primary antibody (incubated with blocking serum). To reduce background staining, sections were incubated with different concentrations of blocking solutions, including 2%, 5% and 10% horse serum, 2% bovine serum albumin (BSA, Fischer Scientific) or 2% BSA with 2% horse serum, or with different concentrations of hydrogen peroxide (3-10%) and

with an avidin-biotin blocking kit (Vector Laboratories #SP-2001). An antigen retrieval step was also tested by incubating sections with 10 mM EDTA or 10 mM citrate buffer in a boiling water bath for 10 minutes prior to the hydrogen peroxide incubation step.

7.2.4.3 DRG immunohistochemistry

Pig and rat DRG sections were stained using a similar protocol to spinal cord IHC. Sections were air-dried and endogenous peroxidase activity blocked using 3% hydrogen peroxide. Sections were incubated with 5% blocking solution for 1 hour before incubation with primary antibody for either NGF, CGRP, or TRPV1. Sections were then incubated with biotinylated horse anti-rabbit secondary antibody (1:500 in PBS) for 1 hour followed by ABC reagent for 30 minutes. Staining was developed using the nickel-DAB substrate, which was prepared by adding 2 drops of Buffer solution, 4 drops of DAB solution, 2 drops of hydrogen peroxide solution and 2 drops of Nickel solution to 5 ml of distilled water. Sections were rinsed in water before being dehydrated in graded alcohol, cleared with histoclear® and coverslipped. Images of DRG sections were visualised using an Olympus BHS B2 light microscope and acquired using a Leica DFC320 camera and LAS v4.2 software.

A number of protocols using different primary antibody dilutions, incubation times and temperatures were tested to optimise staining of both pig and rat DRGs. To optimise incubation time, DRGs were incubated with primary antibody at room temperature for 1, 18, 24, 48 or 72 hours. To optimise incubation temperature, DRGs were incubated with primary antibodies for 18 hours at room temperature or at 4°C. To optimise primary antibody dilution factor, DRGs were incubated with different concentrations of NGF (1:100 - 1:10000), CGRP (1:200 - 1:10000) or TRPV1 (1:200 - 1:10000). Specificity of antibodies was tested by omission of primary antibody. To reduce background staining, sections were also incubated with different concentrations of blocking solution, hydrogen peroxide and an avidin-biotin blocking kit.

7.2.5 Assessment of astrocyte and microglia activation

Activation of microglia and astrocytes within the spinal cord was determined by the appearance of these cells within laminae I - III of the dorsal horn (see Table 7.2). This region was selected as nociceptive Aδ and C-fibres almost exclusively project to the superficial dorsal horn laminae I – II. Activated astrocytes undergo somatic hypertrophy and have an increased number and thickness of projections. Activated microglia show cell body hypertrophy, retraction and thickening of projections and can become amoeboid. Due to high background staining, only glial cells with a clear morphology were assessed. The most occurring activation (mode) score in the dorsal horn was taken as the overall score for that particular dorsal horn. The scorer was blinded to animal age and identification number.

Table 7.2: Astrocyte and microglia activation grading criteria. Scoring criteria for activation morphology of astrocytes and microglia, adapted from Colburn et al. (1997), Navarro et al. (2012) and Ali et al. (2015).

Activation score	Morphology features	
	Astrocytes	**Microglia**
0	Small with fine projections and well-spaced	Small cell body with long, fine projections and well-spaced
1	Somatic enlargement and thicker projections	Cell body enlargement and thicker projections
2	Somatic enlargement, increased number and thickness of projections	Cell body enlargement and few thicker projections
3	Rounded with few projections	Amoeboid – large round cell body with no projections

7.2.6 Statistical analysis

Data were analysed using Kruskal-Wallis tests with Dunn's multiple comparisons post-hoc tests. Correlation was assessed using Spearman's Rank correlation. A significance of $p < 0.05$ was used for all statistical tests.

7.3 Results

7.3.1 Spinal cord astrocytes and microglia

The omission of primary antibodies for IBA1 and GFAP confirmed their specificity, however, some non-specific background staining was still present. To reduce this, sections were incubated with different blocking buffer and hydrogen peroxide concentrations. This was optimised to 5% blocking buffer and 3% hydrogen peroxide but this did not completely eliminate background staining. Use of an avidin-biotin blocking kit did not alter background staining. Furthermore, antigen retrieval did not improve staining quality and resulted in a number of sections disintegrating. Therefore, in summary, despite these attempts, background staining could not be entirely eliminated but was reduced to an extent where cell morphology of some microglia and astrocytes within the dorsal horn became visible.

Inactive astrocytes had small cell bodies and fine projections. Activation resulted in somatic hypertrophy and an increase in the number and thickness of projections (see Figure 7.3). Severe astrocyte activation, resulting in rounded cells with few projections, was not seen.

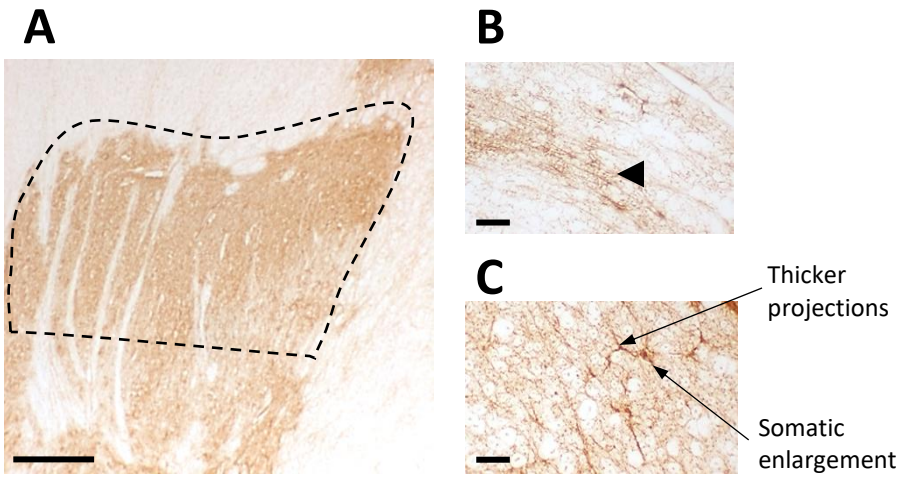


Figure 7.3: Representative images of astrocytes in porcine spinal cord dorsal horn (laminae I-III). Despite a high non-specific background stain (A), some inactive (B) and activated (C) astrocytes were identifiable (arrows). Images taken at 4x magnification (A, scale bar 250 µm, dotted line denotes scored area), 10x magnification (B, scale bar 50 µm) and 20x magnification (C, scale bar 25 µm).

Inactive, resting microglia were well ramified with many wispy projections. Activation resulted in hypertrophy of the cell body and the retraction and thickening of microglial projections (see Figure 7.4). Severe activation, with cells becoming completely amoeboid, was not seen.

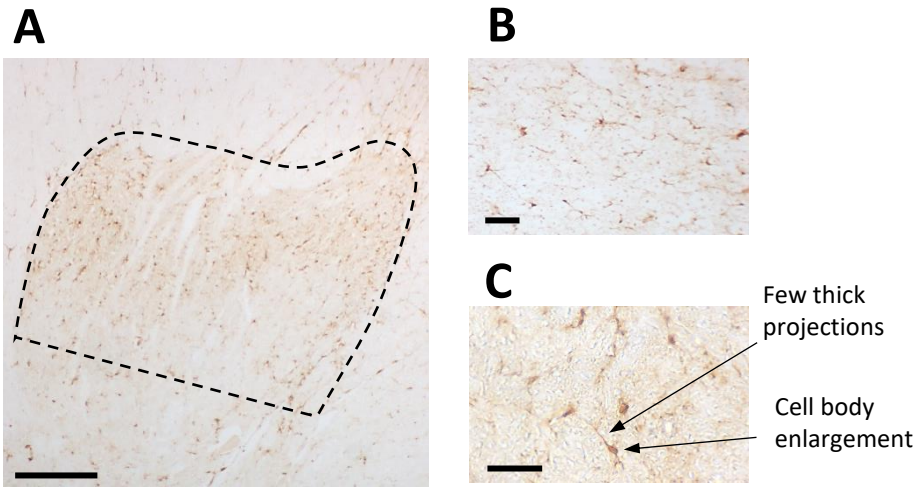


Figure 7.4: Representative images of microglia in porcine spinal cord dorsal horn (laminae I-III). Despite a high non-specific background stain (A), some inactive (B) and activated (C) microglia were identifiable (arrows). Images at 4x magnification (A, scale bar 250 µm, dotted line denotes scored area), 10x magnification (B, scale bar 50 µm) and 20x magnification (C, scale bar 25 µm).

Dorsal horn astrocyte activation ranged from score 0, indicating no activation, to score 2, indicating moderate activation with somatic enlargement and an increase in number and thickness of projections. Astrocyte activation was significantly different between the pig age groups ($p < 0.0001$, Kruskal-Wallis test). Spinal cord dorsal horn astrocytes were significantly more activated in 42 month old pigs than 9, 15 and 24 month old pigs ($p = 0.0106$, $p = 0.0005$ and $p < 0.0001$ respectively, Dunn's multiple comparisons, see Figure 7.5A). Dorsal horn microglia activation ranged from score 0, indicating no activation, to score 2, indicating moderate activation with somatic enlargement and fewer thicker projections. There was no difference in microglia activation in different age pigs ($p = 0.1202$, Kruskal-Wallis test, see Figure 7.5B).

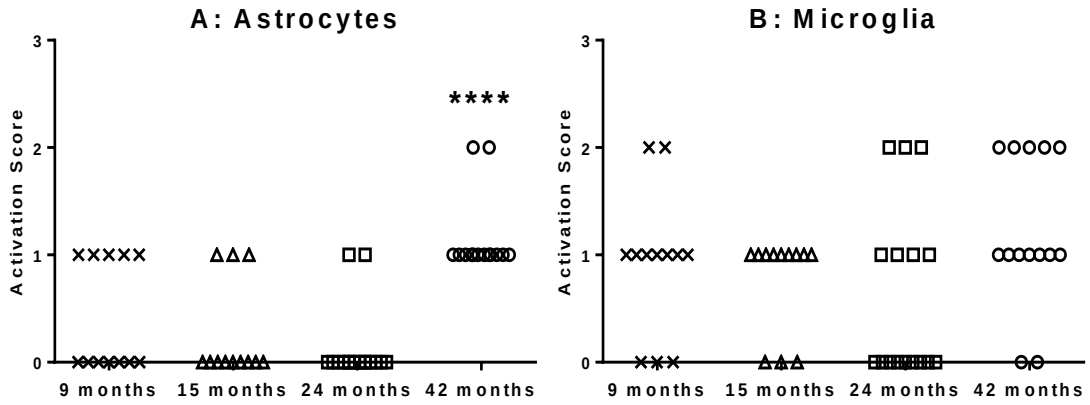


Figure 7.5: Astrocyte and microglia activation in the spinal cord dorsal horn of different age pigs. Astrocytes (A) were significantly more activated in 42 month old pigs but microglia (B) activation was unaltered in different age pigs. ****p < 0.0001, Kruskal-Wallis test.

7.3.1.1 Astrocyte and microglia activation associated with lameness

Astrocyte activation showed a very weak but significant association with lameness across all pigs ($r^2 = 0.3006$, p = 0.0379, Spearman's Rank Correlation). When investigating each age separately, there was an association between astrocyte activation and lameness in 15 month old pigs ($r^2 = 0.7325$, p = 0.0182, Spearman's Rank Correlation, see Figure 7.6), but no correlation in the other age groups.

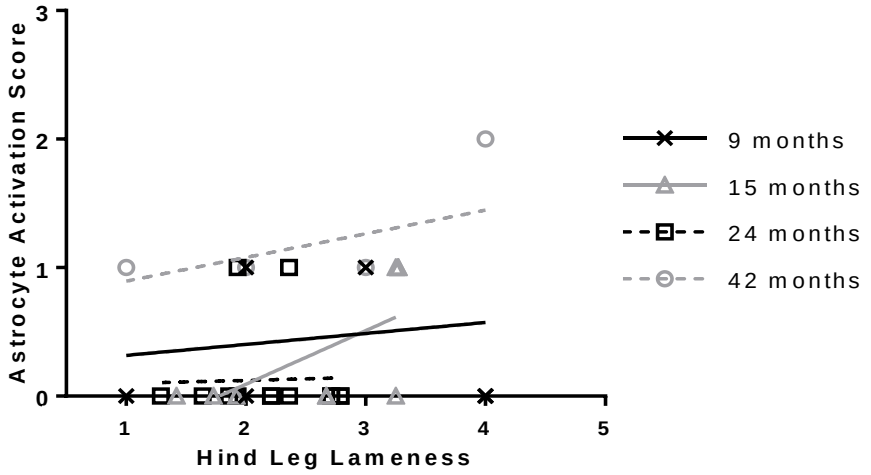


Figure 7.6: Astrocytes activation associated with hind leg lameness. There was a slight correlation between the astrocyte activation and hind leg lameness in 15 month old pigs ($r^2 = 0.7325$, p = 0.0182, Spearman's Rank Correlation). Scatter plots fitted with linear regression lines.

Microglia activation showed no association with hind leg lameness across all pigs (r^2 = 0.1562, p = 0.2891, Spearman's Rank Correlation) or in each age group (see Figure 7.7).

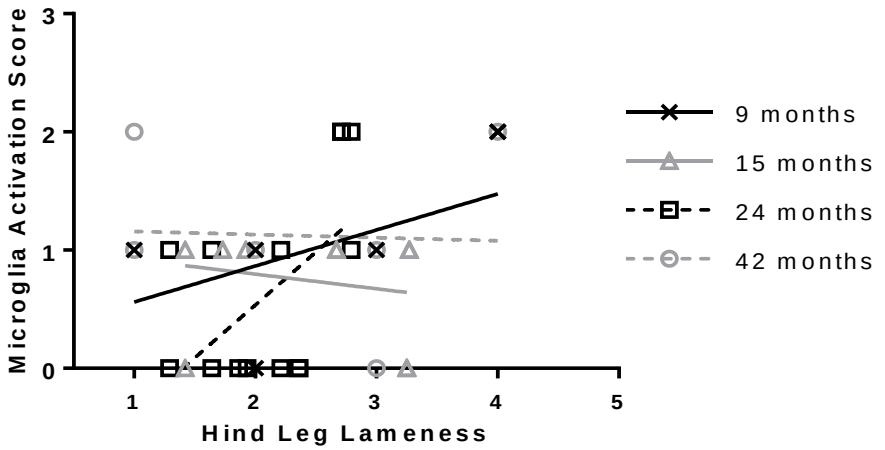


Figure 7.7: Microglia activation associated with hind leg lameness. There was no correlation between microglia activation and hind leg lameness across all pigs. Scatter plots fitted with linear regression lines.

7.3.1.2 Astrocyte and microglia activation associated with joint disease

There was no association between knee OC severity and astrocyte or microglia activity in 9 and 15 month old pigs (r^2 = -0.079, p = 0.713 and r^2 = 0.117, p = 0.587 respectively, Spearman's Rank Correlation, see Figure 7.8 A and B). There was no association between knee OA severity and astrocyte or microglia activation in 24 and 42 month old pigs (r^2 = -0.0105, p = 0.9610 and r^2 = 0.1256, p = 0.5588 respectively, Spearman's Rank Correlation, see Figure 7.8 C and D).

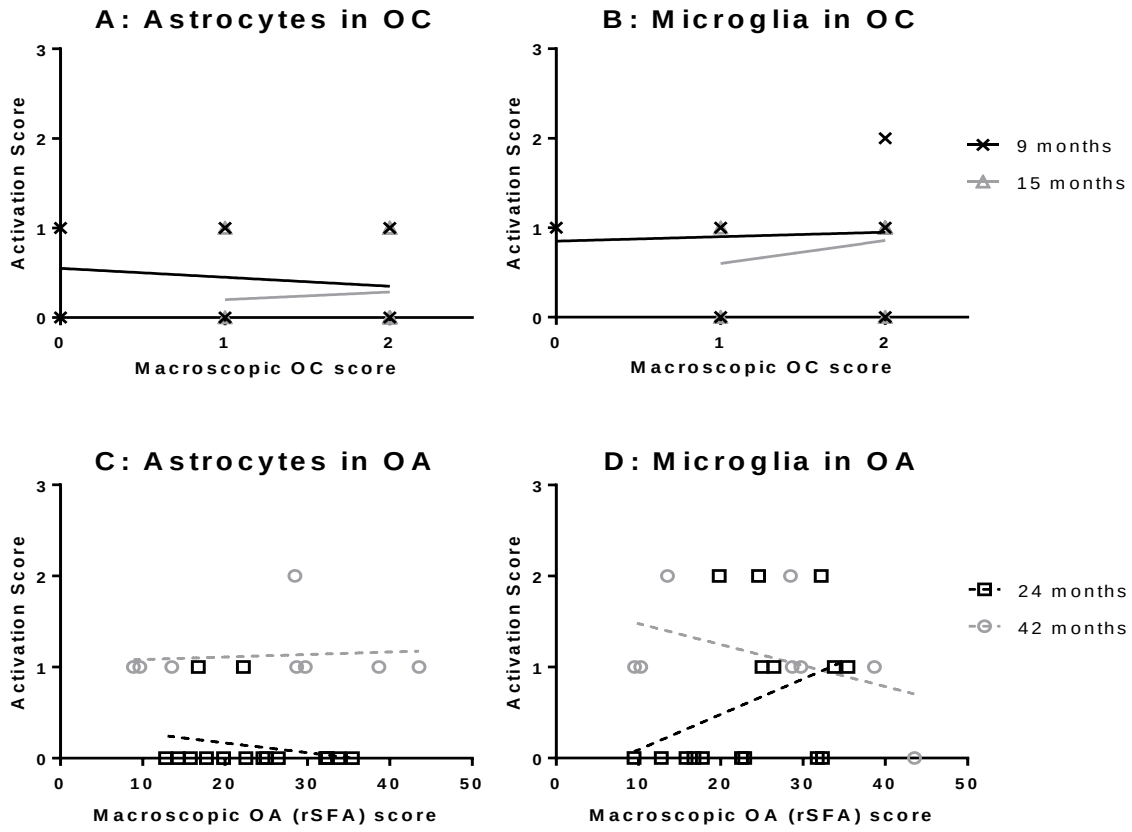


Figure 7.8: Astrocytes and microglia activation associated with porcine osteochondrosis and osteoarthritis. There was no association between OC severity and astrocytes (A) or microglia (B) activation in 9 and 15 month old pigs. There was no association between OA severity and astrocytes (C) or microglia (D) activation in 24 and 42 month old pigs (C and D). Scatter plots fitted with linear regression lines.

7.3.1.3 Astrocyte and microglia activation associated with synovial inflammation

There was no association between knee synovitis severity and activation of astrocytes in the 9, 15, 24 and 42 month old pigs (r^2 = -0.2904, p = 0.3485; r^2 = 0.5025, p = 0.2409; r^2 = 0.0476, p > 0.9999 and r^2 = -0.441, p = 0.5714 respectively, Spearman's Rank Correlation, see Figure 7.9A). Similarly, there no association between knee synovitis severity and activation of microglia in the 9, 15, 24 and 42 month old pigs (r^2 = 0.4251, p = 0.1439; r^2 = 0.1675, p = 0.8909; r^2 = -0.0267, p = -0.02669 and r^2 = 0.3443, p = 0.4857 respectively, Spearman's Rank Correlation, see Figure 7.9B).

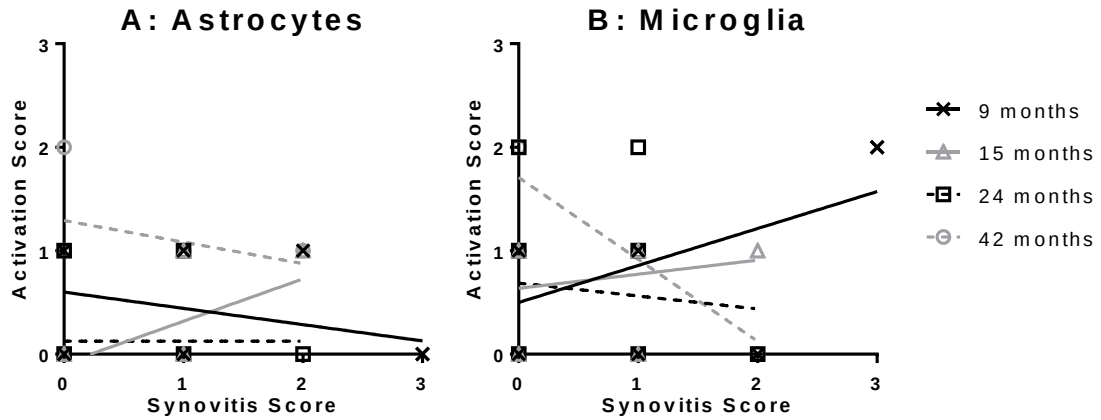


Figure 7.9: Astrocytes and microglia activation associated with porcine knee joint synovitis. There was no association between synovitis severity and activation of spinal cord astrocytes (A) or microglia (B) in different age pigs. Scatter plots fitted with linear regression lines

7.3.2 Optimising ABC-HRP DAB immunohistochemistry

The IHC protocol using the ABC-HRP method was developed to determine expression of CGRP, TRPV1 or NGF in L3-L5 DRG neuronal cell bodies. Rat DRGs were used as a positive control to aid optimisation of the staining protocol. Immunohistochemical staining for CGRP was detected in rat DRGs following incubation with a 1:1000 dilution of primary antibody for 18 hours at room temperature. Staining for TRPV1 was detected in rat DRGs following incubation with a 1:6000 dilution of primary antibody for 24 hours at room temperature. Staining for NGF was detected in rat DRGs following incubation with a 1:4000 dilution of primary antibody for 24 hours at room temperature (see Figure 7.10). Omission of primary antibodies resulted in a lack of staining in rat DRG sections, confirming specificity of the primary antibodies in rat tissue.

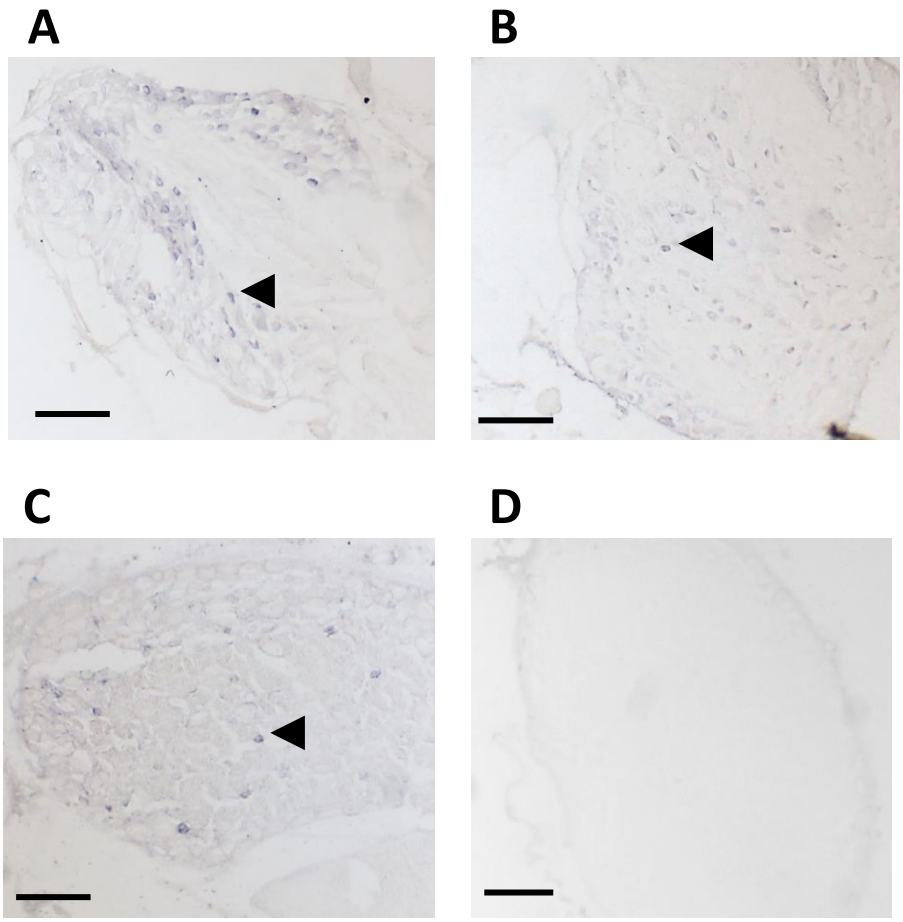


Figure 7.10: CGRP, TRPV1 and NGF immunoreactivity in rat DRGs. Representative rat IHC stained DRG sections with CGRP (A), TRPV1 (B) and NGF (C) immunoreactivity (arrowheads). Primary antibody omission resulted in no staining (D). Images taken at 4x magnification, scale bar 200 µm.

A lack of positive staining for CGRP, TRPV1 and NGF was found in pig DRGs, and only non-specific staining was identified (see Figure 7.11). The omission of primary antibodies reduced non-specific staining of pig DRGs sections slightly but did not eliminate staining. To reduce this non-specific staining, a number of optimisation protocols were performed, including incubation with different concentrations of blocking solution and hydrogen peroxide and with an avidin-biotin blocking kit. However, these steps did not alter non-specific background staining significantly.

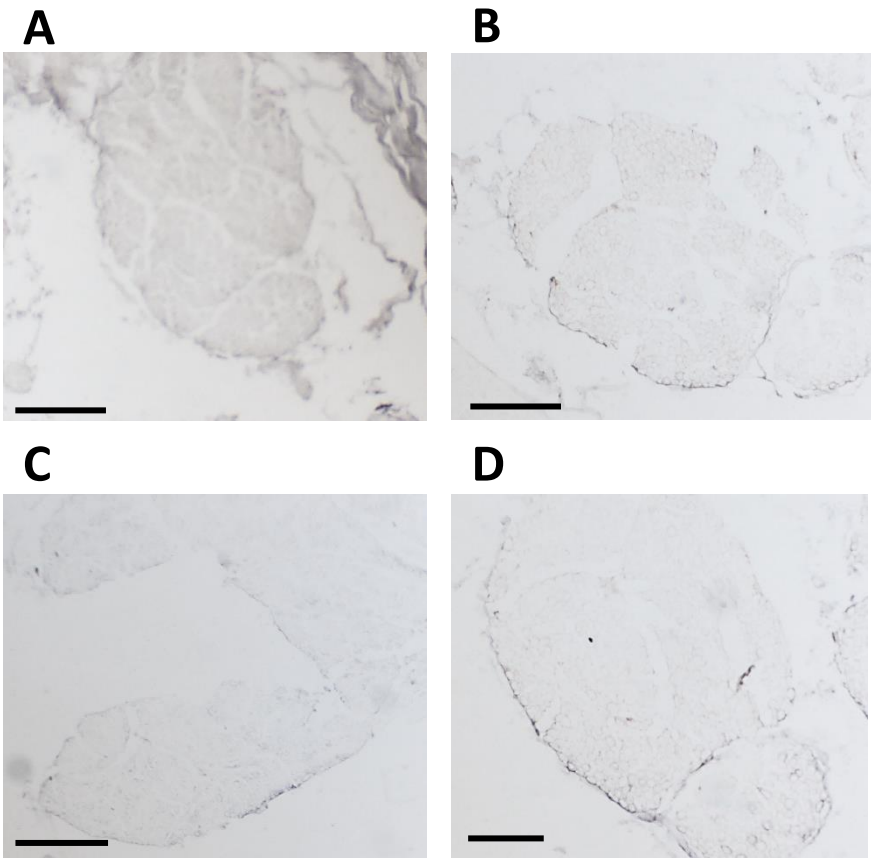


Figure 7.11: Non-specific immunoreactivity in pig DRGs. Representative pig IHC stained DRG sections. No positive staining for CGRP (A), TRPV1 (B) and NGF (C) was observed. Primary antibody omission resulted in non-specific background staining (D). Images taken at 4x magnification, scale bar 200 µm.

Furthermore, a number of protocols using different primary antibody dilutions, incubation times and temperatures were conducted to optimise porcine DRG staining for CGRP, TRPV1 and NGF, but this failed to result in specific staining. It was not possible to determine if the antibodies were specific enough to react with pig antigens, despite their use being previously reported in porcine tissue.

7.4 Discussion

The work presented in this chapter characterised the activation of astrocytes and microglia in the spinal cord dorsal horn of commercial pigs. The phenotype of primary afferent fibres in DRGs were also investigated. Determining whether these changes occur in the PNS and CNS is an important step in understanding the involvement of chronic pain in porcine joint disease.

7.4.1 Age-related differences in spinal cord astrocytes and microglia activation

The study found no difference in microglia activation between age groups but astrocytes were more activated in the oldest pigs. Age-associated changes in the phenotype of brain astrocytes and microglia have been implicated in a number of neurological diseases such as Alzheimer's (Rawji et al., 2016). However, it is unclear to what extent ageing affects microglia and astrocyte activation, particularly in the spinal cord. Some rodent studies have identified increased microglia and astrocyte immunoreactivity and altered microglia reactivity in older animals (Kullberg et al., 1998; Lee et al., 2015; Ogbonna et al., 2015). The increased astrocyte activation in 42 month old pigs may result from ageing processes but may also result from the pigs' disease state. Further work on a large sample is therefore required to determine if porcine spinal cord astrocytes or microglia are altered during ageing.

7.4.2 Nervous system changes associated with porcine joint disease

Many studies in rodent models of OA and chronic pain have identified increased activation of spinal glia that correlates with features of chronic pain such as allodynia (Lee et al., 2011; Orita et al., 2011a; Sagar et al., 2011; Burston et al., 2013; Miller et al., 2013; Ogbonna et al., 2013; Yu et al., 2013; Ogbonna et al., 2015). Joint disease or persistent painful stimulation causes peripheral sensitisation that, in turn, sensitises spinal neurones and activates glial cells. These spinal changes result in central sensitisation and the maintenance of chronic pain. The present findings identified no changes in porcine spinal cord astrocyte or microglia activation associated with knee joint OC, OA or synovitis. However, the association with porcine disease may be obscured by the poor IHC quality, which resulted in not all microglia and astrocytes being assessed as their morphology was not clear.

Furthermore, only the knee joint was assessed for pathological changes, however, the L3-L5 spinal cord receives afferent inputs from other locations including the skin. Pathological changes occurring outside the knee, which were not assessed in the present study, could have influenced glial cell activation. The association was also limited by the small sample size and severity range of OC and OA identified in this study, particularly the lack of healthy pigs. Furthermore, in rodent models there is a consistent level of joint disease between animals, which makes disease-associated changes more apparent. Generally, astrocytes and microglia were slightly activated, with few inactive glial cells observed, suggesting pigs were not in a normal healthy state but this may not be a chronic pain state. Although glial activation in rodent OA models has been characterised, glial activation in other joint diseases such as OC or in porcine diseases has not been investigated. A much larger study population with a greater range of joint disease and pain severities is required to evaluate spinal cord cellular changes, determining if central sensitisation occurs in porcine spontaneous OC or OA.

Activation of glial cells showed no clear association with hind leg lameness severity, with the exception of 15 month old pigs who showed a slight correlation between lameness and astrocyte activation. Only one study, conducted in horses, has evaluated the association of lameness and spinal glial activation. The study, conducted by Meneses et al. (2018), evaluated five horses with unilateral lameness and identified increased microglia activation but the association of astrocyte activation was less clear. In the present study, association of lameness and astrocyte activation in one age group does suggest that pain may cause some sensitisation of the porcine spinal cord. The lack of association in other age groups and with microglia activation, may result from age-associated differences in glial activation or differences in individual pig pain states. The bilateral nature of joint disease seen in the pigs may also have affected glial cell activation, making disease-associated differences less clear as there is no internal control within each animal, unlike with unilateral lameness. However, the association of astrocyte activation and lameness in one age group does suggest that pain in pigs may result in changes

in spinal cord cellular responses, but further work on a larger population is required to evaluate this.

The phenotype of primary afferent fibres in porcine DRGs could not be determined due to lack of positive immunoreactivity to NGF, CGRP and TRPV1. Joint disease or injury results in inflammatory mediator release which directly activates or sensitises peripheral neurones, resulting in hyperalgesia and increased pain perception. This sensitisation is associated with increased CGRP, NGF and TRPV1 in peripheral nerves that can be assessed by their expressions in DRGs. These targets were selected as they are often upregulated in chronic pain, particularly during OA (Fernandes et al., 2001; Saxler et al., 2007; Ashraf et al., 2013; Nishigami et al., 2013; Ogbonna et al., 2013; Zhang et al., 2013; Kelly et al., 2015; Ogbonna et al., 2015; McNearney et al., 2016; Miyamoto et al., 2017). However, positive immunoreactivity within porcine DRGs could not be identified, so their association with porcine joint disease and pain could not be evaluated.

7.4.3 Limitations in immunohistochemical staining of porcine tissue

IHC ABC-DAB staining was chosen over other staining techniques, such as immunofluorescence, in part due to limited availability of a fluorescent microscope but also because PFA fixation is known to cause auto-fluorescence. However, IHC staining of porcine tissue, both spinal cord and DRGs, produced a high level of non-specific staining and limited positive antigen-antibody reactions. For GFAP and IBA1 staining of spinal cord, this resulted in limited visualisation of cell morphology but also prevented more quantitative assessments, such as cell size or area stained, from being conducted.

A number of protocol optimisation steps were conducted to improve IHC staining. Heat-induced antigen retrieval, to remove crosslinks between the fixative and antigen epitopes (Daneshtalab et al., 2010; Stradleigh and Ishida, 2015), failed to improve staining and resulted in disintegration of a number of the fragile frozen sections. Endogenous peroxidase, avidin and biotin were also blocked using a number of protocols. However, these failed to remove non-specific staining,

suggesting they were not the source. Different blocking solutions, to prevent non-specific antibody binding, were also tested and optimised to some extent. However, this still did not completely eliminate all non-specific staining. A number of different antibody incubation concentrations, durations and temperatures were also tested but failed to produce specific staining of the DRGs.

The non-specific staining and lack of positive staining in DRGs could be caused by a number of factors. Firstly, the tissue was immersion fixed in PFA, which itself can cause some non-specific staining. Many IHC studies use perfusion-fixation, which rapidly and uniformly fixes tissue, whereas immersion fixation is a much slower process. Perfusion-fixation is relatively simple to perform in small animals, such as rodents, but is not practical for large animals such as pigs. Perfusion fixation of an adult pig would require excessively large quantities of carcinogenic fixatives, specialised equipment and training, which would increase costs and raise safety concerns. Animal slaughter by exsanguination and the subsequent extraction and immersion fixation of tissue is significantly cheaper, easier and safer but results in a delay in fixation due to the time it takes to extract nervous tissue for a large animal. This may have compromised tissue integrity, reducing IHC staining quality.

Another cause of poor staining may have been antibody specificity. There are few antibodies developed specifically for porcine tissue. Many of the antibodies used in pig studies are designed to specifically target rodents or humans, but show reactivity to other mammalian species. Although all the antibodies used in this study were either listed as showing pig reactivity or have been previously published in porcine tissue, the primary antibodies for CGRP, NGF and TRPV1 showed no porcine reactivity. Antibodies for CGRP and TRPV1 had previously been reported in porcine DRGs (Obreja et al., 2008; Botti et al., 2012; Russo et al., 2013) and the NGF antibody had been reported in porcine tissue from the ovaries, uterus and oesophagus (Jana et al., 2011; Jana and Andronowska, 2012; Samarasena et al., 2015). Staining of porcine tissue is often unsuccessful due to this lack of species-specific antibodies, and staining which works for one research group often does not work when tried by another (personal communications with Dale Sandercock,

SRUC, and Amal Mohammed, University of Nottingham). The loss of tissue integrity, caused by tissue extraction time and immersion fixation, and limited specificity of primary antibodies may have combined to result in the poor quality staining observed. A more detailed investigation using different antibodies is required to determine if this method of IHC staining is effective for assessing porcine tissue. However, it may be better to assess porcine tissues using other techniques such as gene expression analysis, which was not feasible in this study due to time restrictions, to identify if changes in the pain processing system associated with chronic pain occur during porcine joint disease.

7.5 Conclusions

Immune cell activation within the spinal cord dorsal horn was evaluated by immunohistochemical staining for GFAP reactive astrocytes and IBA1 reactive microglia. However, immunohistochemical staining to assess the phenotype of primary afferent neurones was unsuccessful. The oldest pigs showed increased astrocyte activation and lameness was associated with increased astrocyte activation in 15 month old pigs, but there was no age or disease associated change in microglia activation. This suggests that joint disease may alter pain perception at the level of the spinal cord, however, further work is required to evaluate this and changes in pain processing at the periphery that are occurring during porcine joint disease.

Chapter 8

General Discussion

8.1 Discussion of findings

The present study assessed female pigs between 9 and 42 months of age, representing the full age range of breeding stock, both before and after skeletal maturity was reached. Spontaneous OC was seen in 9 and 15 month old pigs and spontaneous OA was seen in 24 and 42 month old pigs. The findings of this thesis highlight the high prevalence of painful joint disease in commercial pigs. Knee joints also showed a high occurrence of synovial inflammation that was associated with increased inflammatory cytokines and proteolytic enzymes. OC showed an unclear association with inflammation, but some increases in cytokines and MMPs were noted. More severe OA was associated with increased synovial inflammation. Porcine OA was also associated with decreased TIMP and some increases in MMPs and cytokines, but the particular cytokines involved may change as OA progresses.

Skeletally immature 9 and 15 month old pigs showed a high prevalence of mild OC, consistent with *osteochondrosis latens* and *manifesta*, but more severe OCD was not identified. Skeletally mature 24 and 42 month old pigs showed a high prevalence of OA, ranging from slight cartilage erosion to end-stage disease with subchondral bone exposure. The present study only examined knee joints, but other studies have suggested elbows, ankles and shoulders show a similar susceptibility to developing OC and OA (Reiland, 1977; Kirk et al., 2005; Luther et al., 2007; Ryan et al., 2010; Tóth et al., 2016). Therefore, the pigs studied likely had these diseases affecting other joints, which emphasises the poor state of joint health in commercial pigs.

Severity of OC and OA was strongly associated with lameness, which was associated with other pain-related behaviours, indicating that these are painful conditions in pigs. However, it could not be determined whether this was chronic pain involving nervous system sensitisation. Peripheral sensitisation can be assessed by identifying changes in the phenotype of primary afferent neurones, such as increased CGRP, NGF and TRPV1 expression (Fernihough et al., 2005; Orita et al., 2011a; Nishigami et al., 2013). However, IHC staining for these markers in porcine DGRs was unsuccessful, thus the presence of peripheral sensitisation could not be evaluated.

Central sensitisation can be assessed by identifying reactive gliosis of spinal cord astrocytes and microglia, which alters their morphology (Sagar et al., 2011; Burston et al., 2013). Due to the poor spinal cord staining quality, it could not be conclusively determined whether OC or OA elicited central sensitisation. However, lameness in 15 month old pigs correlated with astrocyte activation, and across all the pigs studied the astrocytes and microglia were generally quite activated with few inactive cells identified. This suggests there may be some central sensitisation and therefore chronic pain in these animals, but a more detailed study is required to evaluate this further.

Although there was a strong association between lameness and joint disease severity, a number of subclinical cases of both OC and OA were identified. The subclinical nature of these conditions has been discussed previously, in animals and humans, and it is suggested that a threshold in lesion size, severity or location needs to be reached before symptoms present (Dewey et al., 1993; Jørgensen et al., 1995; Hannan et al., 2000; Etterlin et al., 2015). This is likely why the severity of medial femoral condyle OC and OA correlated most strongly with lameness. As medial joint surfaces receive a high mechanical load during weight bearing they are more susceptible to damage, which then has a greater impact on movement (Curl et al., 1997; Thorp et al., 2007). However, this lameness threshold will also be affected by the inflammatory state. Synovitis was identified in a number of joints, across all age groups, with more severe forms associated with worse pain. In human OA, pain is influenced by both synovial inflammation and pathological changes within cartilage and bone (Sakao et al., 2009; Musumeci et al., 2013; de Lange-Brokaar et al., 2015). In 24 and 42 month old pigs, synovitis was only seen in symptomatic OA, suggesting that in pig OA, as in human OA, inflammation influences pain. Therefore, the joint's inflammatory state, combined with location and severity of cartilage lesions, likely determines if porcine joint disease becomes symptomatic.

The inflamed synovium showed increased pro-inflammatory cytokines, elevated MMPs but unchanged TIMPs, although the particular MMPs involved were dependent upon pig age. This is consistent with findings in humans, rodents, horses

and minipigs that showed synovitis elevated pro-inflammatory cytokines and proteolytic enzymes (Trumble et al., 2001; Bondeson et al., 2006; Davidson et al., 2006; Sutton et al., 2009; Haslauer et al., 2013). Severity of synovial inflammation correlated with OA chondropathy but only in 42 month old pigs. This was likely due to stage of OA seen in the different age groups; more of the older pigs were reaching end-stage disease with subchondral bone exposure, which is more strongly associated with synovitis (Haywood et al., 2003; Benito et al., 2005).

Synovitis showed a more complex association with OC. Although 77% of OC joints had synovitis, there was no correlation between severities; joints with the same OC pathology showed synovitis ranging from absent to severe. Therefore, it was uncertain whether inflammation was resultant of OC or occurring independently to but alongside changes in cartilage integrity. Studies of porcine and canine OC have suggested OC is strongly associated with synovitis but these studies mainly examined more severe OCD (Etterlin et al., 2014; Wall et al., 2015). Mild OC, as seen in the present study, may have a weaker association with inflammation. Hence in mild or early OC, separate non-inflammatory osteochondrosis or inflammatory osteochondritis pathologies may develop, which could represent two different conditions with altered symptoms or abilities to repair and influence disease progression.

The association of OC and synovium biomarkers showed a complex picture, particularly due to the unclear involvement of inflammation in OC. A number of studies in horses have identified that MMP expression is upregulated in cartilage chondrocytes during OC, particular MMP3 and MMP13 (Kuroki et al., 2005; Mirams et al., 2009; Riddick et al., 2012). Furthermore, elevated synovial fluid cytokines, produced by activated synoviocytes, have been reported in several equine diseases, including OCD (Bertone et al., 2001; Trumble et al., 2001; Bondeson et al., 2006). The present study identified some elevation in synovium TNFα and MMP expression in joints with OC, but whether this was a result of OC, inflammation induced by OC, or inflammation independent to OC was difficult to determine. Although non-inflammatory OC was associated with some upregulation in synovium TNFα, the

other cytokines showed no clear alterations. As such, cytokines in general may not be altered during mild OC. MMP1 and MMP3 were upregulated during OC, and the presence of synovitis appeared to exacerbate increases in synovium cytokines and MMPs. The increase in MMP, but lack of a corresponding change in TIMP, suggests there may be an imbalanced MMP and TIMP activity, which could result in MMP disinhibition.

Increased cytokines and proteolytic enzymes produced by synoviocytes, irrespective of whether this is caused by OC, could lead to further pathological changes in the joint. If MMPs produced by synoviocytes enter synovial fluid, combined with MMPs produced by disrupted chondrocytes, this could further disrupt the cartilage matrix. Elevations in MMP may partly be involved in repairing lesions by degrading dysfunctional ECM components, such as collagen and aggrecan. However, the enzymes may also breakdown healthy cartilage components. This could worsen the disease state and limit the ability of OC to spontaneously repair as the animal grows. This could, therefore, leave cartilage vulnerable to lesion expansion or new damage, increasing the likelihood of disease progression (summarised in Figure 8.1). Altered cytokines, MMPs and any subsequent cartilage disruption may also elicit synovial inflammation, which could create a cycle of inflammation and joint damage.

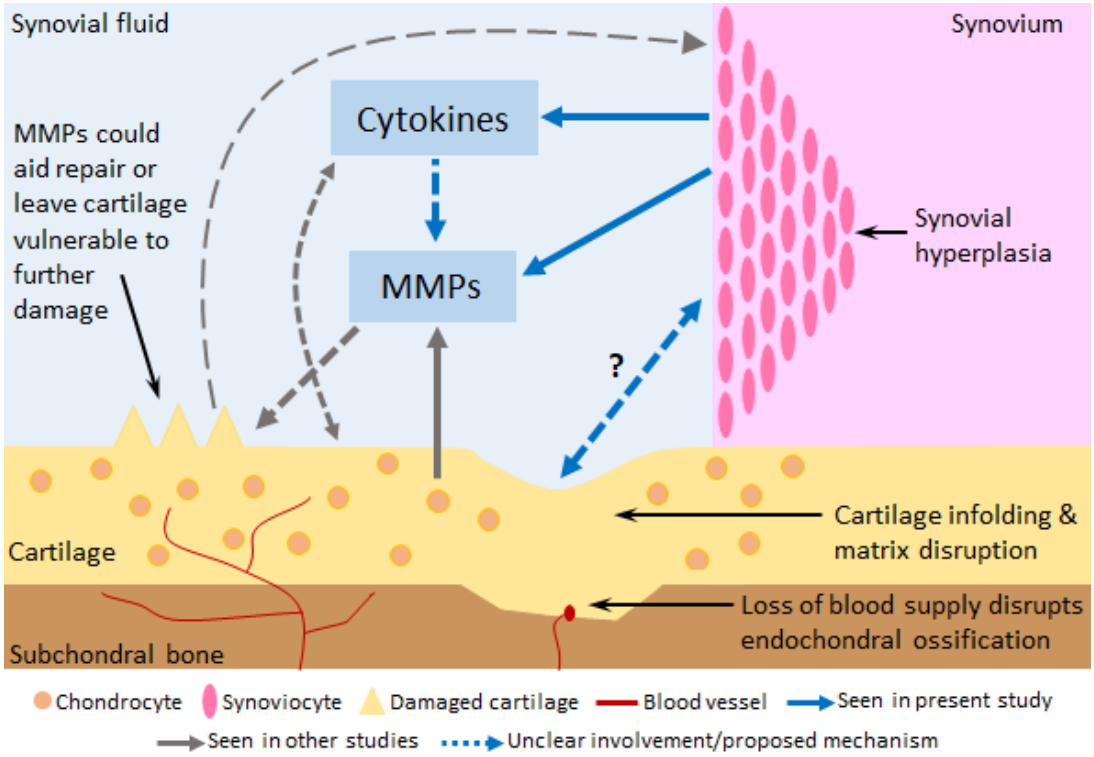


Figure 8.1: Summary of the proposed molecular mechanism involved in porcine osteochondrosis. Blood supply disruption interrupts endochondral ossification, resulting in OC with cartilage infolds, matrix disruptions and altered chondrocytes. Synovitis resulted in membrane hyperplasia and increased inflammatory cytokines and MMPs. The ability of OC, particularly mild OC, to elicit synovitis and alter synovial gene expression is still unclear, but some elevation in synovium TNFα and MMPs were identified in OC without inflammation. Increased MMPs, from cartilage chondrocytes or the synovium, could be involved in lesion repair. However, too much MMP could limit the ability to repair or damage healthy cartilage, leaving it vulnerable to disease progression into more severe OCD or OA. Elevated cytokines, MMPs and cartilage disruption may also elicit further inflammation, creating a cycle of damage.

Despite it being unclear if mild OC elicits synovial inflammation, OC and synovitis were both associated with some increases in inflammatory cytokines and MMPs. As such the inflammation, irrespective of its cause, may worsen OC and treatments to prevent or reduce inflammation may offer outcomes that are more favourable by decreasing the likelihood of OC progression. This could be especially important for

pigs reaching skeletal maturity when bone growth slows and the ability of cartilage to repair is already compromised. This leaves joints susceptible to OA, a condition that showed a number of similarities in terms of alterations in cytokines and proteolytic enzymes. These similarities may partly explain why OC strongly predisposes joints to OA, as molecular changes in the synovium associated with OA may have already begun before OA-associated changes in the cartilage develop.

Identifying the association of OA and synovial biomarkers was limited due to the lack of healthy joints. However, if the healthy joint identified was representative, there appeared to be elevated cytokines and MMP, and reduced TIMP expression, associated with OA. Several studies have identified a similar increase in synovial cytokines and synovial or cartilage MMPs associated with OA in humans and animals (Kevorkian et al., 2004; Davidson et al., 2006; Orita et al., 2011b; Haslauer et al., 2013; Wojdasiewicz et al., 2014; Sieker et al., 2018). Association of TIMPs and OA is more uncertain, with some studies identifying an elevation and others a reduction (Kevorkian et al., 2004; Davidson et al., 2006; Alam et al., 2011; Heard et al., 2012). However, the consensus is that during OA, irrespective of particular changes in TIMPs, there is an imbalance of MMP and TIMP activity, which may promote cartilage degradation. The presence of synovitis in OA further increased synovium IL-6 and MMP1. Chondropathy severity in non-inflammatory and inflammatory OA was assocaited with different MMP expressions, suggesting they may represent different pathological subtypes, which has been reported in other species (Revell et al., 1988). Furthermore, as OA progresses the particular synovial cytokines involved may change, with reduced IL-6 in end-stage disease compared to earlier OA. This suggests that in porcine OA the inflammatory profile changes as OA progresses, which has also been suggested in human and rabbit OA (Vangsness et al., 2011; Xiaoqiang et al., 2012; Mabey and Honsawek, 2015).

In a similar fashion as described in porcine OC, increased synovial cytokines and proteolytic enzymes produced by synoviocytes in OA may elicit further pathological changes within the joint. Increased expression of disinhibited MMPs, produced by synoviocytes or cartilage chondrocytes, is likely to increase the pathological

breakdown of cartilage ECM components, worsening the disease state. This inflammation and subsequent cartilage disruption may elicit further synovitis, creating a self-perpetuating cycle of inflammation and cartilage degradation (summarised in Figure 8.2).

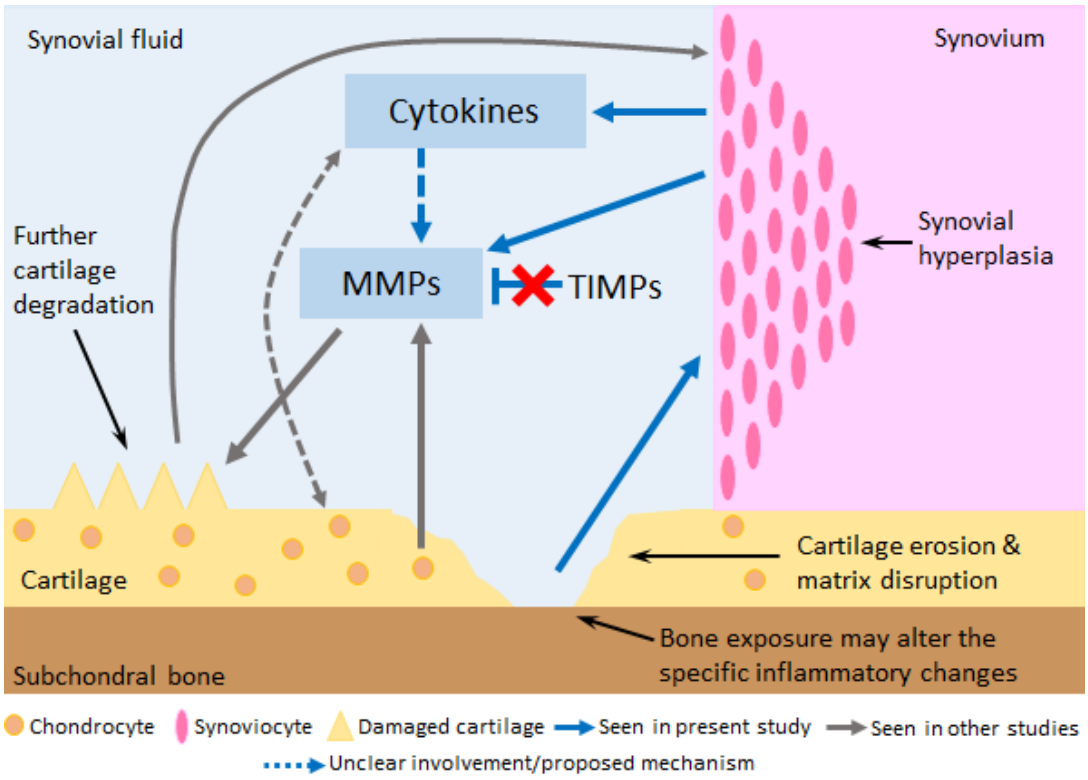


Figure 8.2: Summary of the proposed molecular mechanism involved in porcine osteoarthritis. Cartilage erosion and matrix disruption in OA elicits synovial inflammation. Synovitis results in membrane hyperplasia and increased inflammatory cytokines and MMP production, while dysfunctional chondrocytes also produce MMPs. OA resulted in reduced TIMP expression, suggesting MMPs are disinhibited. Increased uninhibited MMPs may elicit further cartilage degradation. The increased cytokines, MMPs and cartilage damage may cause further inflammation, creating a cycle of inflammation and tissue damage.

The pathological features seen in knee joints of skeletally mature female pigs showed a number of similarities to human OA. Human and porcine knees show

similar cartilage thickness and pathological changes associated with OA, such as fibrillation, subchondral bone exposure and osteophyte formation, indicating the pig is a good model of human OA. Pig OA also showed comparable alterations in cytokines and proteolytic enzymes to that seen in human OA and other animal models, which could make the pig an ideal species for bridging the gap between findings in rodent studies and the human condition. The high prevalence of spontaneous OA identified also suggests that pigs with OA could be easily acquired from the commercial population. Pigs could, therefore, be obtained and studied prior to slaughter at the end of their commercial breeding life. This would eliminate the need for breeding animals specifically for research in accordance with the 3R's (replacement, reduction and refinement), thereby making use of animals who would otherwise be destined for slaughter.

However, the high prevalence of spontaneous OA also presents a problem, as finding healthy age-matched animals may be difficult. The subclinical nature of OA makes identifying live healthy animals problematic. Porcine OA was often bilateral, which although supporting its ability to mimic the human condition, results in there being no internal control, unlike in induced animal models of OA. This makes determining changes that are resultant of OA more challenging. Even non-age-matched healthy animals may be difficult to obtain due to the high prevalence of OC in younger animals. The 9 and 15 month old pigs were initially destined to act as healthy (non-age-matched) controls, as by this age point the growth rate is beginning to slow, so they were expected to have fewer OC or OA pathologies compared to younger or older animals. However, the high prevalence of OC identified in these pigs prevented them from acting as healthy controls, suggesting finding a healthy commercial pig may be challenging.

Furthermore, the costs associated with using large animals may also limit the pig's use as a human model. Practicalities of housing, slaughter and tissue collection from such large animals reduces the feasibility of using pigs, as many research institutions will not have the facilities to conduct such studies. Despite the pig presenting as a good spontaneous model of human OA, the limitations of using a

large animal may outweigh the benefits for some studies. However, spontaneous OA is clearly a problem for the commercial pig industry in terms of reduced animal welfare and economic and food security implications. Therefore, studying this condition is important for improving sustainability of the commercial pig industry worldwide. Research into OA for this purpose may, therefore, be more important and feasible than studying it just for its human model potential, but the benefit of being able to provide insight into the human condition will still exist.

8.2 Limitations of the work presented

As with any scientific study, there were some limitations associated with the work presented in this thesis. Due to the cost of buying and housing pigs, only a small number were used. An ideal study would have larger numbers of healthy and diseased animals with different severities, allowing for changes between healthy, mild and severe disease to be evaluated. The small number of pigs made this more difficult and individual variability could not be easily accounted for. The nature of investigating spontaneous disease also meant there was no control over what disease states were seen, resulting in a near absence of healthy joints, but also a lack of severe OC. The lack of healthy animals, was particularly limiting, as it could not be determined how representative these animals were of healthy pigs, but highlights the poor state of commercial pig health. Furthermore, the need to transport the animals may have increased the presence of lameness or joint damage in the animals studied. The proximity of the study to transportation, combined with the lack of clinical history available, may have affected how representative the pigs studied were of the commercial population.

Furthermore, only female pigs were used in this study, so gender differences in porcine OC and OA were not evaluated. Many animal studies routinely use males as they have fewer hormonal fluctuations that can alter biological processes. Female pigs were used because studying the disease has more commercial relevance to adult females than males. In December 2017 the UK pig herd was approximately 4.7 million pigs, of these 490,000 were breeding females but only 12,000 were breeding males (DEFRA, 2018). Therefore, despite problems caused by hormonal changes,

such as altered behaviour during oestrus, the availability of adult females and potential impact of improving these diseases in females made them a more appropriate choice than male pigs. Furthermore, in the UK 60% of people who have sought treatment for OA are females (ARUK, 2018). Therefore, studying OA in female pigs also has more clinical relevance than studying the disease in males.

A number of difficulties were also encountered in establishing reliable protocols for tissue analysis, namely IHC staining of nervous tissue. There are relatively few antibodies designed specifically for porcine tissue, which combined with the tissue degradation that occurs due to the time required to extract nervous tissue from large animals, resulted in poor quality IHC staining. Although IHC staining was optimised in spinal cord tissue, the DRGs could not be examined for phenotypic changes. A study of different antibodies and different tissue fixation or embedding techniques may enable better IHC analysis. However, other investigative methods such as qPCR studies may be more appropriate for porcine nervous tissues, but not was possible for this study due to time constraints.

8.3 Future directions

Given the evidence presented in this thesis, further work is required on a larger sample and a greater range of disease severities to characterise fully spontaneously occurring porcine OC and OA. A more complete evaluation of porcine OA will strengthen support for its use as a spontaneous model of human OA, although the lack of healthy pigs in the commercial population may limit this to some extent. Further studies are also required to clarify the role of inflammation in OC, determining if synovitis forms as part of an OC pathology during early disease, or if synovitis forms independently to OC and subsequently interacts with the disease. Identifying the involvement of other cytokines, MMPs or TIMPs will allow a more complete profile of the molecular mechanism involved in porcine joint disease to be determined, particularly the role of MMP13 in porcine OC. Examining cytokines, proteolytic enzymes and their inhibitors in synovial fluid would also identify if changes in both synovium and cartilage gene expression occur and result in altered protein activity within the joint, which could impact cartilage integrity. Investigating

the activity of proteolytic enzymes using a fluorometric assay, instead of mRNA expression, would also identify if the enzymes are in an active form or if they are inactive pro-enzymes or TIMP-complexed. Furthermore, investigating whether porcine OC and OA result in chronic pain is required to determine the extent of pain experienced, which could lead to better treatments. This could be achieved by assessing nervous system sensitisation using nociceptive threshold testing or identifying cellular changes in the PNS and CNS.

Furthermore, to assess OC, in a manner that is comparable across studies, a new macroscopic assessment technique is required. This method needs to be reproducible across studies, sensitive to subtle changes in pathology and specific to OC and not generalised joint disease. There is currently no universally used method for assessing OC macroscopically; researchers use many different methods that show poor agreement. The revised SFA scoring provides this for porcine OA, while also allowing it to be directly comparable to the human condition, but an equivalent specific and sensitive system is required for porcine OC.

8.4 Concluding remarks

Overall, the studies presented in this thesis provide new insight into molecular changes occurring in the synovium during spontaneously occurring porcine joint disease. The studies identified an upregulation of synovium pro-inflammatory cytokines and MMPs during porcine OC and OA. This increase in inflammation and proteolytic enzymes may act to worsen the disease state, limiting the ability of lesions to repair in growing animals, thereby leaving joints vulnerable to disease progression. Although the involvement of inflammation, particularly in OC, is still unclear, changes associated with synovitis may alter the pathological state and influence the disease outcomes or symptom severity. These studies also confirmed the high prevalence of painful spontaneously occurring joint disease in pigs, which affects pigs across their entire reproductive lifespan. This current state of poor joint health is not justifiable for the commercial pig industry, and improvements are required to ensure good animal welfare, but also to increase the industry's sustainability. The outcomes of this research could help inform future studies

characterising OC and OA, to enable the development of better prevention, detection or treatments of porcine joint disease. These data also support the use of commercial pigs as a spontaneous model of human OA, which could overcome some of the issues faced when using traditional induced small animal models, such as the mechanism of OA onset and differences in joint anatomy. Commercial pigs could provide an alternative model of human OA and make valuable contributions towards the understanding of this debilitating condition.

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