# CHARACTERISATION OF THE COMMERCIAL PIG AS A MODEL OF MUSCULOSKELETAL AGEING AND OSTEOARTHRITIS

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*“I would rather have questions that can't be answered than answers that can't be questioned.”*

Richard Feynman

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'Science may never come up with a better office communication system than the coffee break.'

Earl Wilson

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# ABSTRACT

Animal models of musculoskeletal ageing are required for the investigation of a number of conditions which may be difficult or unethical to investigate in humans. For this purpose, rodent models have been widely utilised, however, there are a number of differences between rodents and humans which may limit their usefulness as a model of human disease. Osteoarthritis (OA) is a musculoskeletal joint disorder characterised by degenerative loss of the articular cartilage and bone remodelling. In attempting to better understand the processes that mediate cartilage degeneration animal models have been utilised, however, OA in these models is typically induced either chemically or surgically and poorly mimics the pathological features of human OA. Spontaneous models also exist, one such model is the Dunkin Hartley guinea pig, but questions have been raised about the utility of these models due to issues with translatability of research into meaningful treatments in humans. Therefore, in order to better understand the key drivers of musculoskeletal decline and to identify and develop new therapeutics there is a high unmet need to develop more translatable animal models of musculoskeletal ageing.

Importantly, the pig shares a high degree of physiological and anatomical similarity with that of humans and as a result, the pig is considered more closely related to humans than any other non-primate mammalian species. The high prevalence of degenerative joint conditions including osteochondrosis and osteoarthritis in commercial pig breeds suggests that these animals may represent an improved model. Therefore, in this study we assessed whether the commercial pig might present an alternative model of musculoskeletal ageing and early OA.

For this purpose, this study investigated commercial pigs at a number of ages up to 4yrs paying particular attention to body composition changes, muscle changes and knee OA development within the age range. The utility of porcine cartilage and bone for *ex-vivo* studies was also examined.

The investigation of body composition changes in these pigs did not reveal significant age-related deterioration, likewise, in muscle, no indication of early sarcopenic changes were identified. Together these findings suggest that the pigs used in this study were too young to be undergoing age-related muscle decline. Examination of OA in porcine knee joints revealed development of OA-like lesions and proteoglycan loss suggesting that these commercial pigs spontaneously develop OA at a relatively young age. Similarities between porcine and human cartilage and bone were also revealed with loss of chondrocyte phenotype in 2D culture and the discovery of an altered osteoblast phenotype in cells obtained from older, damaged knee joints; this osteoblast phenotype was similar to that found in sclerotic human bone.

In summary, this work has shown that commercial pigs within this age range are too young to exhibit early indications of age-linked muscle decline, such as sarcopenia. However, pigs within this age range exhibit early OA changes in the knee joint and both porcine cartilage and bone may be of use in *ex-vivo* studies investigating OA. Together this suggests that pig could represent an appropriate animal model for the investigation of early OA.

# 1.0 INTRODUCTION

## 1.1 MUSCULOSKELETAL AGEING

As science continues to pursue life prolonging research there is growing interest in not only increasing human lifespan but also in increasing the quality of life and independence of the elderly. Central to this is the need to remain mobile and therefore the maintenance of a healthy and functioning musculoskeletal system is critical. This introduction will describe the effect of age on the different tissues of the musculoskeletal system (namely skeletal muscle, bone and cartilage), some of the purported key mechanisms behind age-related deterioration, and the consequences for health and quality of life. It will also discuss why the pig may represent a promising model of musculoskeletal ageing.

### 1.1.1 Muscle structure and function

Skeletal muscle is a highly organized tissue consisting of different muscle fibre types. The major function of this tissue is to convert chemical energy into kinetic energy, allowing movement. Muscle also has an important impact on metabolic processes within the body; contributing to basal energy metabolism and making amino acids available for other tissues as an energy source during periods of starvation or catabolic disease (1,2).

Muscle cell contractile units, known as sarcomeres, consist mainly of actin and myosin myofilaments which interact to allow muscle contraction. Sarcomeres are repeated along the length of the myofibrils within each muscle fibre. Single muscle fibres are surrounded by a membrane known as the sarcolemma and

groups of muscle fibres form bundles known as fascicles, which are surrounded by connective tissue known as the perimysium, as shown in Figure 1.1. (1,3)

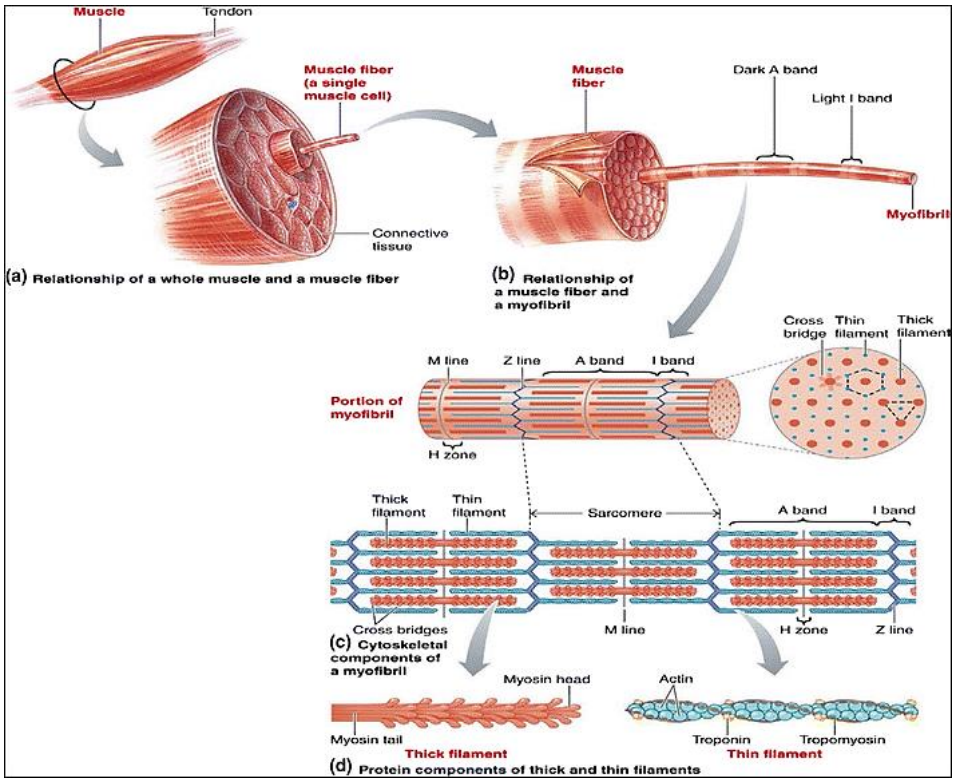


**Figure 1.1: Muscle structure** (Diagram from Frontera et al (1)) Diagram shows muscle fibre arrangement within whole muscle (parts a. and b.) and cytoskeletal components of the contractile units (part c.).

Muscle contraction is dependent on two major processes occurring within the muscle. The first is the successful transmission of nerve stimulation and the release of calcium ions (1). Calcium binds to troponin C on actin and this leads to the displacement of tropomyosin from the actin active site, and allows the binding of myosin (2). The second is the interaction of the myofilaments, actin and myosin (thin and thick filaments, respectively, shown in Figure 1.1). Cross bridges form between myosin heads and actin (1). A combination of ATP and ATPases within the myosin head facilitate the release of cross bridges and the formation of new ones to slide actin over myosin causing muscle contraction (2). The series of events linking these processes is known as excitation-contraction

coupling (1,2). More detail regarding muscle contraction and excitation-contraction coupling can be found in a number of reviews (4–6).

### 1.1.2 Muscle fibre types

There are two main fibre types in mammalian skeletal muscle: type I or slow-twitch fibres and type II or fast twitch fibres. Type II fibres are further divided into subgroups: type IIa, IIb and IIx (2). Muscle fibre type is determined by which myosin heavy chain (MyHC) is expressed in the fibre. Humans, and possibly other large mammals, are thought to have lost the expression of the fastest and least energy efficient fibre type, type IIb, in the interest of energy conservation (7). However, the pig has been shown to express MyHC IIb in the longissimus muscle (8). Muscle fibre types vary in their metabolic and contractile properties and these properties can be determined by examining their myosin heavy chain expression, as shown in Figure 1.2.

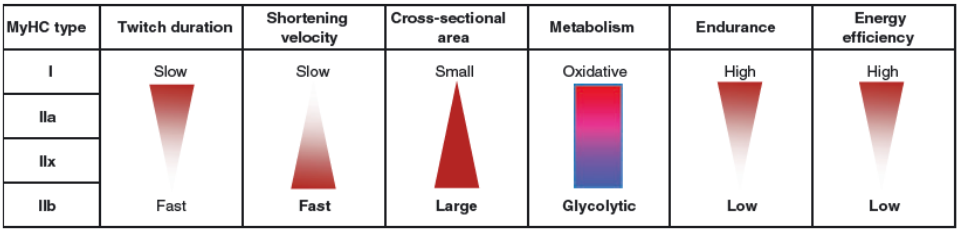


**Figure 1.2: Physiological characteristics associated with muscle fibres expression of each MyHC isoform** (Figure from Gunderson (9))

Slower twitch fibres are associated with smaller cross-sectional areas, oxidative metabolism and increase endurance and efficiency (9). Fast twitch fibres, on the other hand, are associated with large cross sectional areas, glycolitic metabolism, high fatigability and low efficiency (9). MyHC type IIb are the fastest fibres and therefore the least efficient: this is thought to be why some larger mammals have lost type IIb expression (8).

During ageing and muscle wasting, fibre specific changes are thought to occur. Original research suggested that there was loss of type II fibres in ageing muscle (10,11), however these results have been difficult to replicate (12,13). Sato et al (14) suggested that ageing resulted in a reduced number of both type I and II fibres, but a reduction in the size of type II fibres specifically, resulting in an increased proportion of muscle cross sectional area that consists of type I fibres without any changes in the proportions of each fibre type (14). These results are supported by a number of more recent papers (15,16).

There are three muscles that are of importance to this study: *tibialis anterior*, *gastrocnemius* and *soleus*. These muscles are essential for ankle movement and balance control. The *tibialis anterior* (TA) muscle is the main foot dorsiflexion muscle, meaning that it is responsible for lifting the toes (17). The TA muscle consists of predominantly type II or fast twitch muscle fibres (18–20). *Gastrocnemius* and *soleus* muscle are 2 of the plantarflexion muscles, meaning that they point the toes (21). The *gastrocnemius* muscle has a mixed fibre type with differences across the muscle making the fibre composition somewhat variable and the *soleus* muscle consists of predominantly type I or slow twitch muscle fibres. Strength in these muscles, particularly in the plantarflexion muscles, is known to decrease with age in over 40s and this is associated with an increased risk of trips and falls in elderly individuals (21).

### 1.1.3 Muscle protein synthesis

Following resistance exercise skeletal muscle undergoes hypertrophy, a process characterised by increased muscle protein synthesis and muscle fibre size (22). Latres et al (22) established that some of the genes known to change during

muscle hypertrophy are inversely regulated during muscle atrophy. Muscle hypertrophy activates Akt through the PI3K/Akt pathway and this has been found to inhibit the induction of atrophy-linked E3 ubiquitin ligases, MuRF1 (Muscle RING-finger protein-1) and MAFbx (muscle atrophy F-box)through the phosphorylation of FOXO family transcription factors. This phosphorylation prevents the translocation of the FOXO family members to the nucleus, inhibiting their ability to induce atrophy (22,23). Together these mechanisms support the hypothesis that muscle mass and protein synthesis rates are regulated in a coordinated manner in response to anabolic and catabolic stimuli.

The mTOR (mammalian target of rapamycin) signalling pathway is essential for the increased levels of muscle protein synthesis seen in response to anabolic stimuli, such as exercise (24). mTOR is a conserved protein kinase which responds to amino acids, growth factors and a number of other intracellular signals to regulate protein metabolism in skeletal muscle (25).This pathway is responsible for the control of translation initiation through the phosphorylation of 4EBP1 (4E binding protein 1), a translation repressor found downstream of mTOR (24). In the hypophosphorylated state, 4EBP1 binds eIF4E (eukaryotic translation initiation factor 4E) preventing its interaction with eIF4G and the formation of the translation initiation complex (24). mTORC1 (mTOR complex 1) activation in response to anabolic stimuli leads to hyperphosphorylation of 4EBP1. This releases eIF4E and allows the initiation of cap-dependent translation (26). 4EBP1 phosphorylation is reduced during resistance exercise suggesting that muscle contraction induces MPS independently of 4EBP1 (27). The control elicited over translational responses by 4EBP1 is therefore part of a much wider network of protein synthesis regulation but appears to be an important component of the regulatory network controlling the activity of the mTOR pathway.

### 1.1.4 Impact of muscle ageing

Geriatric care is now a major area of concern for the NHS; the age-related loss of muscle mass and strength increases the risk of trips and falls in the elderly putting pressure on this service and due to a lack of mobility in affected individuals, increasing the demand for homecare and assisted housing. In 2000, 1.5% of total healthcare spending in the US could be attributed to sarcopenia; this equates to $18.5bn, making it a huge drain on the healthcare system (28).

### 1.1.5 The age-related decline in musculoskeletal health

#### 1.1.5.1 Sarcopenia

The age-related deterioration in skeletal muscle strength (dynapedia) and decline in skeletal muscle mass (sarcopenia) can negatively impact quality of life and mobility in elderly individuals (29,30). Loss of muscle mass can start as early as 40 years of age and occurs alongside a reduction of the quality of the remaining muscle (31,32). A study by Auyeung et al (33) found that over a 4 year period elderly men lost 1.59% of appendicular skeletal muscle mass and elderly women lost 2.02%. Grip strength was also investigated; women lost 10% grip strength over 2 years whereas men lost 3.85% over the same time period, suggesting that the decline in muscle strength occurs at a faster rate than loss of muscle mass, particularly in the women within the population studied (33). Grip strength is potentially a useful predictor of mortality with a number of studies linking a decline in grip strength to an increase in all-cause mortality (34,35).

The observed decreases in muscle mass, strength and endurance lead to reductions in physical activity, and therefore reduced energy expenditure. It is

possible that this in turn leads to the accumulation of abdominal fat and an increase in the likelihood of obesity and obesity-related problems; such as insulin resistance, type-2 diabetes and hypertension; in those genetically susceptible to such conditions (30). Loss of muscle mass itself can also contribute to the development of insulin resistance as muscle is a target for insulin and is therefore important for the regulation of blood glucose levels; thus a reduction in muscle mass could contribute to the development of insulin resistance (36). In individuals with sarcopenia an increased amount of intramuscular fat is observed and this may contribute to both localised inflammation in the muscle through the production of adipose-tissue associated cytokines, known as adipokines, and the functional decline in the musculoskeletal system of the elderly (37).

#### 1.1.5.2 Cachexia

Cachexia is a condition typically associated with severe illnesses such as cancer. It is characterised by loss of body weight, muscle wasting, increased inflammation and leads to increased frailty; it also correlates strongly with morbidity (38). Elevated interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα) concentrations in blood and muscle tissue stimulate muscle proteolysis and myocyte apoptosis (39) in both cachexia and sarcopenia and are thought to interfere with cellular signalling processes associated with skeletal muscle anabolism (40).

#### 1.1.5.3 Disuse atrophy

Disuse atrophy occurs in older individuals that have been hospitalised or confined to bed for a period of time (41). Inactivity also contributes to sarcopenia; this is discussed in more detail in Section 1.1.5.1. Disuse atrophy can also occur in young individuals during the treatment of and recovery from injury.

Jones et al (42) showed that following 2 weeks of immobilisation there was a reduction in quadriceps mass by 4.7 ± 0.9% and a reduction in isometric strength by 27 ± 3%. The major difference between young and old individuals is the speed at which muscle mass and strength recovers following remobilisation. Young individuals return to full muscle mass and strength much more quickly, whilst elderly individuals recover slowly and may never regain full strength, particularly if periods of hospitalisation or bed rest become more frequent (43).

### 1.1.6 Proposed mechanisms driving age-related decline in skeletal muscle

#### 1.1.6.1 Age-related decline in muscle mass and quality

The cause of the observed reductions in muscle mass and strength with age appears multifaceted, but decreased postprandial rates of muscle protein synthesis (MPS) have been implicated, even when factors such as activity levels and diet have been taken into consideration (44).

This age-induced anabolic resistance could be due to the reduction in the ability of the body to successfully carry out the processes necessary to provide an appropriate protein synthetic response to stimuli such as contraction or following amino acid provision (25). Observed decreases in protein synthesis due to anabolic resistance have been attributed to diminished mTOR signalling, discussed in Section 1.1.3 (25).

Resistance exercise training in the elderly improves muscle mass by increasing the rate of muscle protein synthesis, as such there are many studies detailing the effects of resistance exercise in elderly populations (44,45). Fragala et al (46) showed that resistance training in the elderly can improve Muscle Quality Index

(MQI) significantly (46). MQI is calculated using an equation devised by Takai et al (47) whereby particular factors such as body mass, time taken to stand from a sitting position and leg length are taken into consideration when working out a muscle power score measured in Watts (47). Fragala et al's resistance exercise intervention produced changes in MQI from 203.4±64.31W to 244.3±82.92W thus showing a marked improvement in power output in the elderly (46). This degree of improvement could have a meaningful bearing on the quality of life experienced by elderly citizens.

#### 1.1.6.2 Reduced physical activity in the elderly

Inactivity results in the loss of muscle mass and strength in both young and aged individuals due, in part, to reduced muscle protein synthesis, although the underlying mechanisms for this may differ between the age groups (48). In adults, physical activity decreases with increased age therefore some of the age-related changes seen in muscle could be caused by or, more likely, accelerated by inactivity. Active older people still exhibit sarcopenic changes in skeletal muscle but these may be less severe and the onset of the condition may be delayed (49,50).

### 1.1.7 Modelling of muscle ageing

Animal models of muscle ageing are required for the investigation of a number of musculoskeletal conditions which may be difficult or unethical to investigate in humans. They are also required for initial testing of treatments and interventions before they are tested in a human cohort. For this purpose, rodent models have been widely utilised, however, there are a number of differences between

rodents and humans which may limit their usefulness as a model of human disease.

The pattern of growth and decline typical of rodents differs from that of humans. Rodents are often used as models of ageing but these animals do not follow the same ageing profile as humans; instead these animals grow for almost their entire life span (Figure 1.3). Near the end of the rat's lifespan the animal enters a short period of decline whereby they lose condition, whereas human growth plateaus at around 40 years of age and the long slow period of decline that follows can last 30 years or more (Figure 1.4). Aged Sprague Dawley rats have been used as a model of sarcopenia in the past due to evidence showing that aged rats exhibit reduced muscle mass compared to young adults (51). These animals do not develop joint problems, such as osteoarthritis, and although they have been used as models of osteoporosis, this condition is typically induced via immobilisation or ovariectomisation (52). Although Figure 1.4 only shows the growth data for females the growth data for males follows the same pattern with the main difference being that males reach a higher maximum weight.

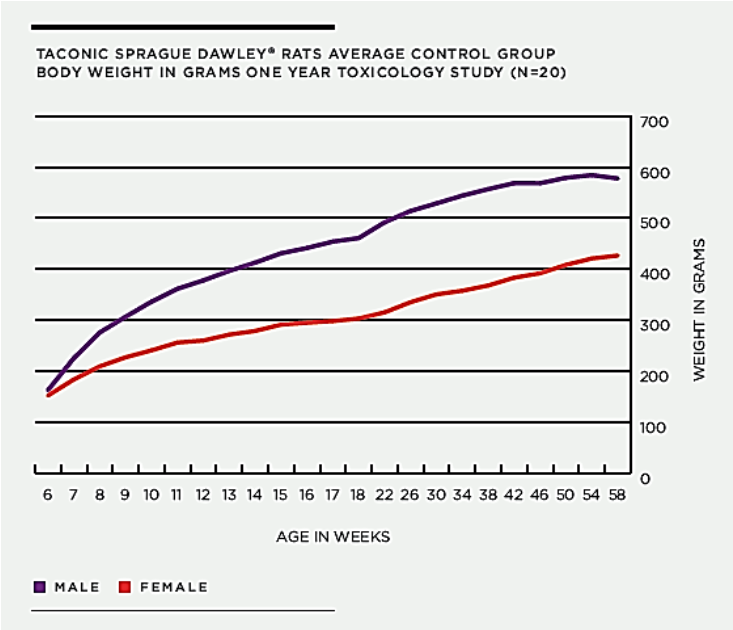


**Figure 1.3: Sprague Dawley rate growth curve.** (Figure from Taconic Sprague Dawley Rat Datasheet (53))Male rat growth curve shown in purple; female rat growth curve shown in red. Maximum age shown is 58 weeks.

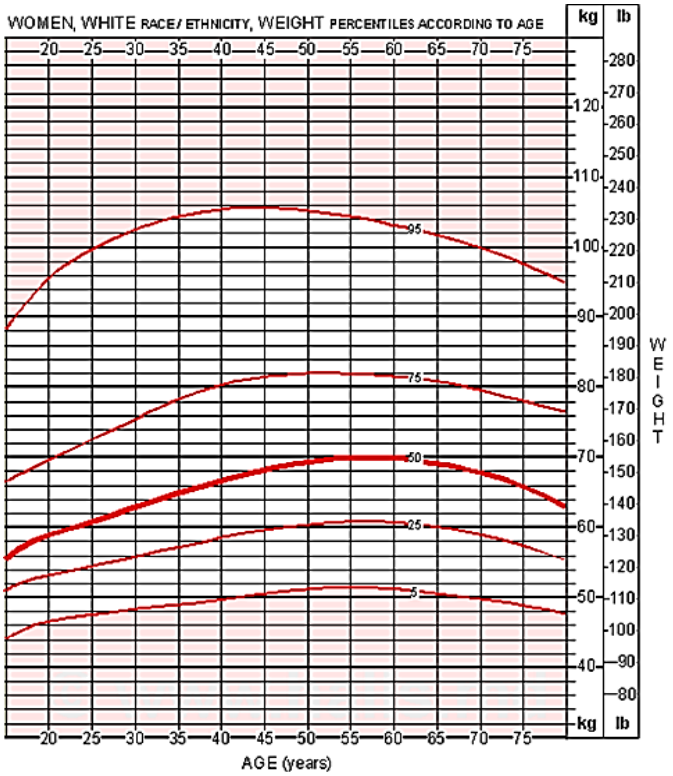


**Figure 1.4: Women's average weight chart and percentile distribution.** (Figure from Halls, S. B. & Hanson, J., (2000). Data originally from NHANES III 1988-1994) (54) The uppermost red line shows the 95$^{th}$ percentile whereby 95% of women are lighter than the weight achieved. Each subsequent line shown represents a different percentile, as labelled on the graph.

Inactivity is a common feature of human ageing thus the effect of inactivity on the muscle of any animal model should reflect human muscle changes. Rodents are often used to model ageing and responses to immobilisation. However, the response of rodents to immobilisation differs from that of humans. Kimball et al (55) determined that muscle mass decreases with age in Sprague-Dawley rats and that this is accompanied by an increase in muscle protein synthesis, suggesting that muscle protein breakdown must exhibit a similar significant increase. Therefore, muscle atrophy as a result of immobilisation in rodents is mainly the result of enhanced proteolysis rather than suppressed protein synthesis, as seen in humans (55–57). In rodent models immobilisation results in rapid and substantial muscle atrophy, losing 1.5-3% of muscle mass per day. Disuse muscle atrophy in humans occurs much more slowly than in rodents, with humans losing 0.5-0.75% of muscle mass per day. This difference is potentially due to the increased rate of protein turnover in rodents compared to humans (56,57). Additionally, many rodent studies investigating disuse atrophy use animals during their growth phase which makes relating results from these models to disuse atrophy in elderly humans difficult (58).

There are metabolic differences between many of the animals models currently used for musculoskeletal ageing research and humans, for example, the basal metabolic rate (BMR) per gram body weight of mice is 7x that of humans (59). Animals with high BMR per gram body weight also tend to have increased levels of reactive oxygen species production and are less able to maintain cellular homeostasis (59). These metabolic differences could mean that although some rodents may exhibit a similar ageing phenotype to humans the underlying mechanisms may differ.

## 1.2 INCREASED SYSTEMIC INFLAMMATION IN AGEING

Immune system function declines with age, this leads to dysregulation of inflammatory processes and increased systemic inflammation. This increased inflammation with age is referred to as inflammaging and has been implicated in a number of age-related, inflammatory conditions (60). Inflammaging is identified by an increase in the concentration of inflammatory mediators, such as cytokines, and an exaggerated acute phase response to immune challenges (60). Elevated levels of these cytokines can cause a reduction in muscle protein synthesis and muscle mass (61–63); and as mentioned in Section 1.1.5, plasma levels of IL-6 and TNFα are elevated in patients with cachexia (39,62,64) and in sarcopenia (38,65). Therefore, it is likely that inflammaging plays a key role in age-related musculoskeletal decline.

### 1.2.1 Cytokines

Cytokines are small proteins which regulate the immune system and are responsible for the initiation and termination of immune responses and inflammation (66,67). Cytokines are produced by a vast range of cell types and through interaction with their receptors, cytokines allow cell communication and signal induction. There are a number of cytokine families: these include the interferons, colony-stimulating factors, tumour necrosis factor (TNF) family and the interleukins (67). Cytokines can have either pro-inflammatory or anti-inflammatory effects, for example, IL-1 is a highly pro-inflammatory cytokine which is capable of activating various leukocytes to carry out their function as part of the immune response through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factor, a master

regulator of the immune response, and the induction of the production of other pro-inflammatory cytokines (67). IL-10 on the other hand is an anti-inflammatory cytokine which inhibits the production of pro-inflammatory cytokines and contributes to the termination of the immune response (67).

Cytokines can be produced by a number of different tissues and can be referred to by their tissue-specific names, for example, muscle derived cytokines are known as myokines and lymphocyte derived cytokines are known as lymphokines (66,67).

Of particular importance to this study are IL-1β and IL-6, these are 2 of the classical inflammatory cytokines. IL-1β has been found to be elevated in cartilage, subchondral bone, synovial fluid and the synovial membrane of osteoarthritic joints (68). Stimulation of chondrocytes with this cytokine inhibited the production of ECM components and reduced the repair capacity of cartilage. It also induced the production of cartilage degrading MMPs and ADAMTS and increased the production of pro-inflammatory cytokines such as IL-6 (68). Therefore, IL-1β is thought to be the principal cytokine responsible for the symptoms of inflammation associated with osteoarthritis (OA) (69).

As such, neutralisation of this cytokine has been an aim for a considerable amount of research, with a number of methods being investigated. One such method is to employ competitive agonists such as IL-1Ra; these receptors can inhibit the action of IL-1β by acting as a competitor to the cytokine receptor, therefore reducing cytokine receptor binding and cell signal induction (70). Alternatively, research into caspase-1 inhibitors has revealed that it could be possible to prevent or limit caspase-1 mediated cleavage of pro-IL-1β to the active form of the cytokine, IL-1β (70).

IL-6 is often used as a measure of inflammation and has been found to be elevated in the muscle of older individuals and in the synovial fluid of osteoarthritic joints (39,65,71,72). IL-6 was found to reduce type II collagen expression in cartilage, and increased IL-6 is associated with higher numbers of osteophytes and increased pain in early OA (73). It is therefore considered to have potential as a biomarker of early OA (72,73).

Another important class of cytokine are the adipose tissue derived cytokines, known as adipokines. The increased level of adiposity that often occurs during human ageing results in increased circulating levels of these adipokines. Three members of this family are of importance to this research, and in particular osteoarthritis (OA), and will be discussed in Section 1.4.2.6.

### 1.2.2 Chemokines

Chemokines are chemoattractant cytokines. This means that they drive the recruitment of specific immune cells to specific areas or tissues through a process known as chemotaxis (67). There are several classes of chemokine, based on the arrangement of cysteines in the protein: CC chemokines have adjacent cysteine residues, CXC chemokines have a single amino acid separating the cysteine residues and CXXXC chemokines have three amino acids separating the cysteine residues (66). CXCL8, also known as IL-8, is elevated in OA osteoblasts (74,75). The ability of immune cells to respond appropriately to chemokine signalling is reduced with age (76,77). For example, the migration of human innate immune cells known as neutrophils in response to IL-8 is less accurate in older individuals (76).

## 1.3 STRUCTURE OF THE KNEE JOINT

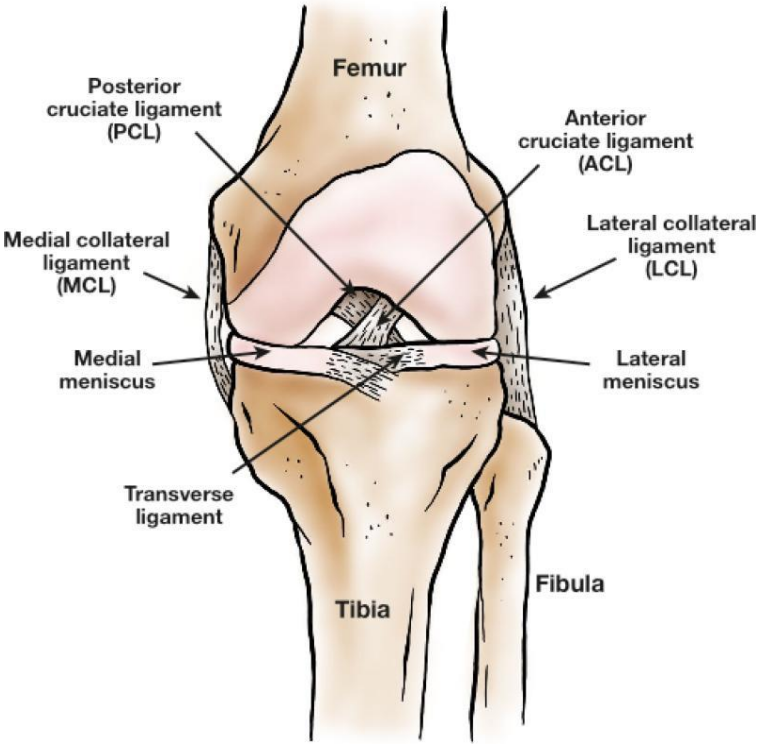


**Figure 1.5: Structure of the knee joint** (Diagram from Makris et al (78)) Diagram identifies various knee joint ligaments and bones.

The knee joint is a hinged synovial joint where the femur, patella, tibia and fibula bones all meet. This complex structure is held together by a network of ligaments and further stabilised by the lateral and medial menisci, as shown in Figure 1.5. Cartilage cushions and protects the femoral condyles, tibial plateau and patella at contact points allowing shock absorption and smooth movement of the joint (79,80).

### 1.3.1 Cartilage

Articular cartilage found in joints is also known as hyaline cartilage and is distinct from the elastic and fibro-cartilage found in ears and ligaments, respectively.

Hyaline cartilage does not have a direct blood supply and is avascular. It consists of an extracellular matrix of collagen and aggrecan, populated with a relatively small number of specialised cartilage maintaining cells, known as chondrocytes (79). Hyaline cartilage can be identified by its collagen components; there are several different types of collagen that contribute to the extracellular matrix of hyaline cartilage: types II, III, VI, IX, X, XI, XII, XIV are all present in the mature matrix. The primary component of articular cartilage is collagen type II with mature articular cartilage containing approximately 1% collagen type IX, 3% collagen type XI and over 90% collagen type II (81). Cartilage strength and loading resistance is dependent on these collagens forming cross-linked copolymers, therefore, although collagen type II is the main collagen component of this cartilage, there are a number of important collagens that although expressed at lower levels are nevertheless important.

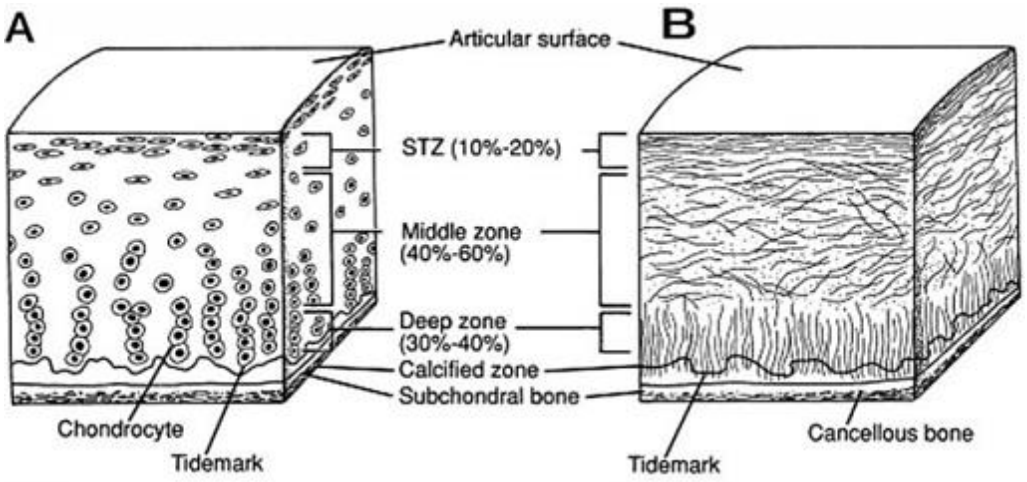


**Figure 1.6: Schematic of healthy articular cartilage. A**: Zones of articular cartilage; **B**: collagen fibre architecture. (Diagram from Buckwalter et al (82))

Cartilage is not a uniform structure and is split into zones – the superficial zone, mid zone, deep zone and calcified zone (shown in Figure 1.6). In mammalian articular cartilage the principal collagen components (types II, IX, XI) do not change between zones, however, the orientation of collagen fibrils does vary

between these regions of cartilage (81). The superficial zone is at the cartilage surface and is in contact with synovial fluid. Collagen fibres here are tightly packed and parallel to the articular surface. This zone is responsible for the ability of cartilage to resist the tensile and compressive forces of the joint (79,80). The middle zone of cartilage represents 40-60% of cartilage volume with obliquely organised collagen fibres and a low density of chondrocytes; it aids in the resistance of compressive forces (79,80). In the deep zone, collagen is arranged perpendicular to the joint surface with columnar chondrocyte orientation, parallel to the collagen fibres. Underneath the deep zone of cartilage there is a visible line known as the tide mark; this identifies the boundary between non-calcified and calcified cartilage which connects to subchondral bone (79,80). In healthy mammalian cartilage, collagen type X is only expressed in this calcified region of cartilage (81).

The collagen network of cartilage differs between the different types of cartilage: hyaline, fibrocartilage and elastic. Collagen type I is either present in small amounts or completely absent from hyaline cartilage (83). This type of collagen is more abundant in fibrocartilage such as in the meniscus or intervertebral discs. Whilst collagen type II can be found in significant amounts in both hyaline and fibrocartilage; with Roberts et al (83) reporting that the type II collagen represented 96% of the collagen in articular cartilage and 80% of the collagen in intervertebral discs; various joint conditions and treatments can influence the collagen make up of cartilage. These collagen-modifying conditions include joint degenerative diseases, such as osteoarthritis, and tissue-engineering methods for cartilage repair that produce cartilage closer to fibrocartilage than hyaline (81,83,84).

### 1.3.2 Bone

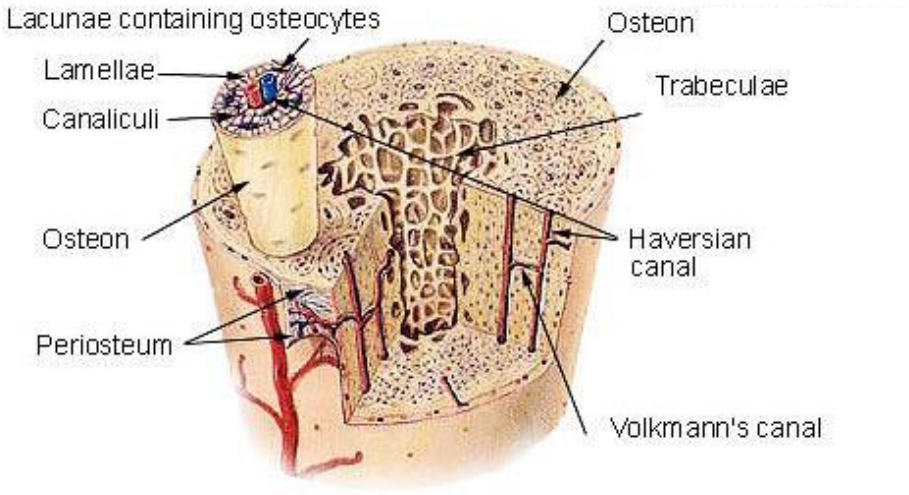


**Figure 1.7: Structure of Cortical and Cancellous Bone**. (Figure reproduced from US National Institute of Health (85)) Diagram shows the structural components of both the outer cortical bone and the interior cancellous bone.

There are 2 major types of bone: cortical and cancellous. The structure of these bone types is shown in Figure 1.7. Cortical bone forms the hard outer surface of the bone and consists of osteons. Osteons are cylindrical structures made of layers, known as lamellae, surrounding a channel containing nerves and vessels; a Haversian canal (85,86). Haversian canals can be connected through perpendicular channels known as Volkmann's canals (85). Bone resident cells, known as osteocytes (discussed later in this section), which have become trapped by the matrix that they have produced, are found in lacunae; small spaces between lamellae and are connected to lacunae by channels known as canaliculi (85,86). Long bones, such as the femur, are covered in a dense layer of connective tissue called the periosteum, except where covered with articular cartilage, such as at the knee joint (shown in Figure 1.7). Cancellous bone is found within the

cortical bone and is less dense. This bone is formed of a network known as trabeculae which is surrounded by red bone marrow (85,86).

Bone is populated by 3 main types of cell that are derived from mesenchymal stem cells (MSCs). These cell types are: osteoblasts, which are bone forming cells; osteoclasts, which are bone resorption cells; and osteocytes, which are osteoblasts that have become embedded in the mineralised matrix that they secrete and these cells are responsible for the regulation of osteoblasts and osteoclasts (87).

Osteoblast differentiation requires the expression of osteogenic transcription factors such as Runt Related Transcription Factor 2 (RUNX2) and osterix (Osx). RUNX2 expression upregulates osteoblastic genes including collagen, type I, alpha 1 (col1A1), alkaline phosphatase (ALP) and osteocalcin (OCN) (88). Bone formation by osteoblasts involves the initial laying down of organic matrix consisting of type I collagen, proteoglycan and various non-collagen proteins including osteocalcin, osteopontin and osteonectin; followed by mineralisation of the matrix. Osteoblasts can become osteocytes, these cells make up the majority of bone cells and are resident in lacunae (small cavities in the bone) surrounded by mineralised bone matrix (88).

Osteoclasts are bone resorption cells, the formation of which is promoted by factors produced by osteoblasts, osteocytes and stromal cells (88). One such factor is receptor activator of nuclear factor kappa-B ligand (RANKL). The binding of RANKL to its receptor, RANK, on osteoclast precursors promotes osteoclastogenesis, the process by which osteoclasts are formed. This process can be regulated by osteoprotegerin (OPG), which is also produced by osteoblasts. The binding of OPG to RANKL inhibits osteoclastogenesis by preventing RANKL binding to RANK (88,89). Increased osteoclast generation with age is thought to

be linked to alterations in the RANKL/OPG balance and contributes to the age-related loss of bone mass and increased incidence of osteoporosis with age (90).

Bone mass and skeletal integrity is maintained through the coupling of osteoclast: osteoblast activity and the balancing of bone resorption and formation (91). Dysregulation of this process has been linked to age-related bone disease. For example, excessive osteoclast activity is observed in osteoporosis (91). In OA, the regulation of bone remodelling by these cells is abnormal resulting in the formation of sclerotic bone; this is discussed in more detail in Section 1.4.1.

## 1.4 OSTEOARTHRITIS (OA)

OA is common in a number of joints in elderly humans (92). Knee OA is the most prevalent, followed closely by hip OA, shown in Figure 1.8 (92,93). Maintaining musculoskeletal health and functionality of both hip and knee joints is required to ensure continued independence and mobility into old age. The majority of animal models of the disease investigate knee OA (94). Therefore, this study investigated the development of spontaneous knee OA in the commercial pig.

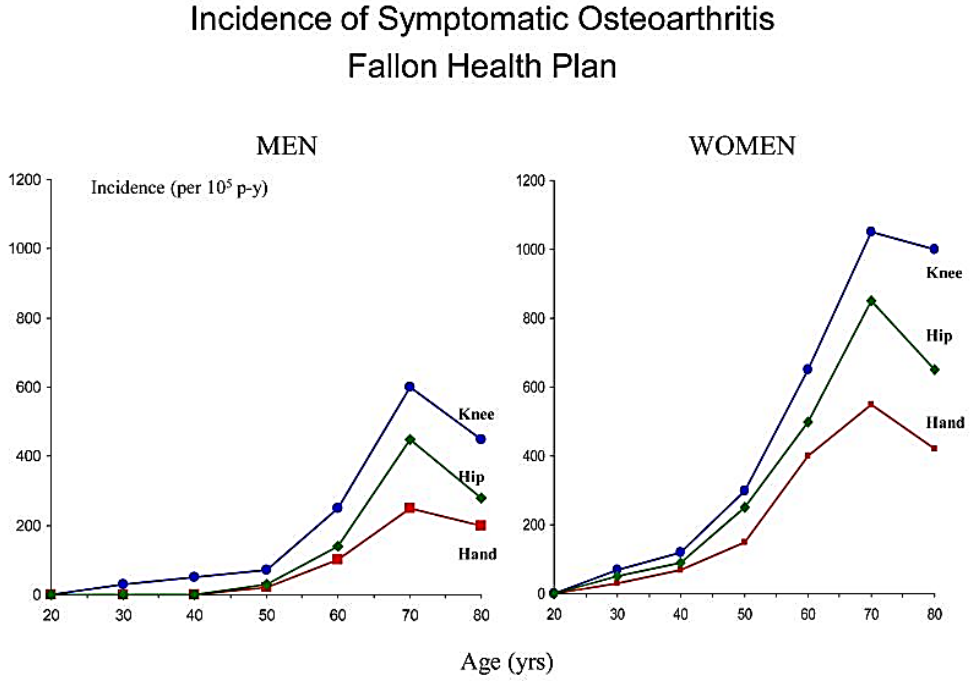


**Figure 1.8: Incidence of OA in men & women up to 80yrs of age.** (Figure from Litwic et al (92).) Incidence of knee (blue), hip (green) and hand (red) OA in men and women between the ages of 20 – 80 years.

Osteoarthritis (OA) is an age-related condition and the most common degenerative joint disorder in the World (95,96). This debilitating joint disorder is characterised by changes in the subchondral bone and degeneration of articular cartilage resulting in detrimental joint changes (95,96). Joint changes in osteoarthritis sufferers can limit mobility by reducing the functionality of the

joint and causing increased levels of pain (96). This is now a major health concern, particularly in countries with an ageing population, as it limits independence and can contribute to the social isolation of the elderly (97).

Although cartilage and bone are the most commonly investigated tissues, OA is a condition that affects the whole joint including synovium, meniscus and surrounding tissues such as muscle, particularly the quadriceps muscle which exhibits loss of strength in patients with OA (98–101).

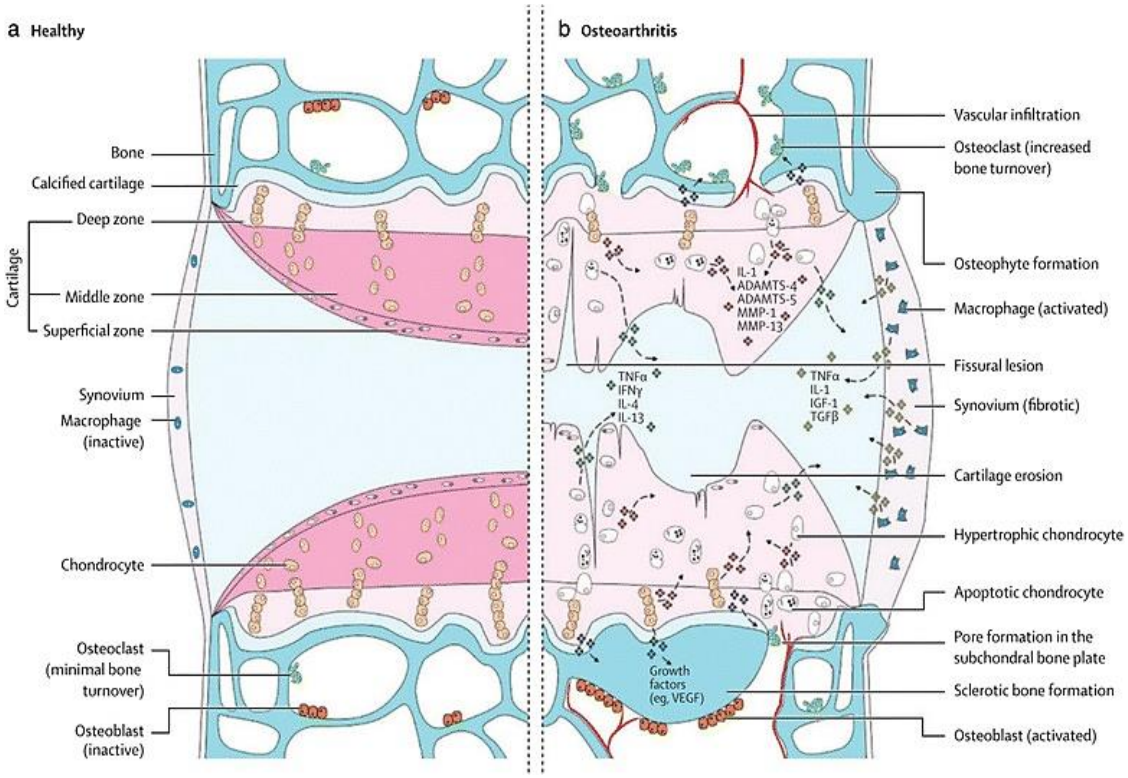


**Figure 1.9: Osteoarthritic knee joint pathology** (Diagram from Kuyinu et al (96)) (a) Healthy knee joint with anatomical labelling; (b) Osteoarthritic knee joint with anatomical changes and contributory growth factors, cytokines and proteinases.

### 1.4.1 Osteoarthritic changes in cartilage and bone

The degradation of the cartilage ECM is a consequence of osteoarthritis and is a major contributor to cartilage loss, shown in Figure 1.9. The ECM is made of type

II collagen and proteoglycans, including aggrecan which consists of a central protein with a number of glycosaminoglycan (GAG) branches. Degradation of articular cartilage is a result of an imbalance between the level of proteinases and the level of their inhibitors or control mechanisms in the joint (102). Matrix metalloproteinases (MMPs) and aggrecanases are responsible for the degradation of ECM components and can be produced by chondrocytes. These include MMP 1, 8 and 13 which have all been implicated in the degradation of the type II collagen; and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) 1,4 and 5 which have been identified as aggrecanases (103,104). There may also be other proteinases that are involved in cartilage degradation and ECM loss, including MMP 2 & 9 and cathepsins B, L & D (103). Cartilage has a very limited ability to self-repair therefore cartilage loss in conditions such as osteoarthritis can quickly become irreversible (79).

Additionally, OA chondrocytes become hypertrophic and exhibit higher levels of apoptosis. This hypertrophic phenotype is adopted by chondrocytes in healthy tissue during endochondral ossification whereby cartilage become calcified and is converted to bone during growth (105). Collagen type X is often used as a marker of hypertrophy and is expressed in the calcified regions of healthy hyaline cartilage and in other cartilage zones during OA. Collagen type X is also elevated in the serum of human OA patients when compared to healthy controls (105,106). The expression levels of other cartilage collagens are also altered in OA. The expression of collagen type VI, for example, is higher in OA cartilage, particularly in the chondrocyte cell clusters of severely damaged cartilage and this is thought to contribute to the altered metabolism in OA chondrocytes (107).

OA bone becomes sclerotic: this bone is dense but under-mineralised and exhibits increased bone turnover and aberrant remodelling leading to the

formation of osteophytes within the joint (108). Human osteoblasts from OA bone have been found to have an altered phenotype compared to osteoblasts from non-OA controls (74). This altered phenotype exhibits reduced mineralisation capacity, elevated alkaline phosphatase (ALP) activity, increased protein synthesis of osteocalcin, osteopontin, insulin-like growth factor (IGF-1), IL-6, IL-8 and transforming growth factor β1(TGFβ1), and increased gene expression of COL1A1 and collagen type 1, alpha 2 (COL1A2), MMP13, progressive ankylosis protein homolog (ANKH), ALP and vascular endothelial growth factor (VEGF) (74,75). Together these phenotypic changes promote a pro-inflammatory joint environment and could contribute to the degradation of the overlying articular cartilage. It is not clear whether bone changes occur before the cartilage damage occurs or what the driving force for these changes is.

Osteophytes; a common feature of OA; are bony outgrowths covered in fibrocartilage (109). Osteophytes occur in addition to joint space narrowing and subchondral bone sclerosis and can form early in OA development. However, it is not clear whether osteophyte formation is a direct result of OA pathology or as a consequence of the joint destabilisation secondary to OA joint damage (109). Hsia et al (110) suggested that osteophytes may form as a response to joint instability. This study investigated osteophyte formation in response to ACL rupture in mice; initial injury resulted in increased joint laxity and range of motion. Two weeks after the injury, the joint had been restabilised and this was accompanied by the formation of chondrophytes (precursor of osteophytes), and by 8 weeks, joint laxity had returned to that of uninjured controls. However, the range of motion 8 weeks post-injury was much lower than that of uninjured controls due to considerable osteophyte formation (110).

An additional consequence of osteoarthritis is the activation of immune cells, such as macrophages, which become activated by pro-inflammatory cytokines in the osteoarthritic joint (96). This further exacerbates the pro-inflammatory environment of the joint by inducing pro-inflammatory cytokine production in the macrophages (96). These numerous joint changes are summarised in Figure 1.9.

## 1.4.2 Proposed mechanisms driving osteoarthritic damage

### 1.4.2.1 Metabolism and growth factors

Numerous growth factors have been investigated as potential targets for the development of treatments for OA. These include: transforming growth factor β (TGF β) superfamily, fibroblast growth factor (FGF) family, insulin growth factor-1 (IGF-1) and platelet derived growth factor (PDGF) (111). In this thesis, IGF-1 is of importance due to its influence on the ageing process. IGF-1 has been linked to longevity and ageing in invertebrates with more recent research in mice suggesting that it may also play a role in ageing in mammals (112,113). IGF-1 is also important for adult muscle hypertrophy and the age-associated decline in muscle derived IGF-1 levels could therefore play a role in the development and progression of musculoskeletal ageing conditions such as sarcopenia (114). In healthy cartilage IGF-1 reduced matrix catabolism, however, this effect was lost in aged or osteoarthritic cartilage (111). It also stimulates the production of cartilage matrix components and has been found to be elevated in the synovial fluid of OA patients (115). There has been some suggestion that OA may arise following age-related reductions in the responsiveness of chondrocytes to growth factors, such as IGF-1, leading to reduced levels of repair following initial damage to the cartilage (111,115,116).

#### 1.4.2.2 Oxidative stress in OA

A feature of oxidative stress is the increase in production of reactive oxygen species (ROS) by mitochondria which can contribute to cartilage degradation, bone resorption and DNA damage (117,118). ROS are a by-product of mitochondrial respiration which can cause oxidative damage to the mitochondria and are thought to contribute to ageing (119). The accumulation of oxidative damage to mitochondria and mitochondrial DNA leads to mitochondrial dysfunction (119). Oxidative stress is a characteristic of OA that can drive a number of the pathological features of the disease and increases mitochondrial DNA damage within chondrocytes (120). Fang et al (121) established that different mitochondrial haplotypes can influence the risk of developing osteoarthritis, highlighting the importance of mitochondrial function in disease pathology (121). Oxidative stress in chondrocytes can induce mitochondrial dysfunction and cellular senescence, further reducing the capacity of cartilage to heal following damage (118). In bone, this increase in ROS favours differentiation of stem cells towards adipogenesis rather than osteogenesis (122), and promotes the development of cellular senescence and death through mitochondrial DNA damage in much the same way as in cartilage (118). Therefore, oxidative stress could play a significant role in the development and progression of OA pathology.

OA cartilage expresses a number of senescence associated markers suggesting that cellular senescence in chondrocytes could be playing a role in OA pathology. Chondrocyte senescence in OA cartilage could be induced by oxidative stress and could be contributing to the inflammatory environment in the joint due to the senescence-associated secretory phenotype (SASP), which consists of a number of pro-inflammatory cytokines and growth factors (123).

#### 1.4.2.3 Inflammation

It has been observed that pro-inflammatory cytokines, namely IL-1β and TNFα, are present at higher levels in the synovial fluid of OA patients. This occurs without the infiltration of classical inflammatory cells, such as neutrophils and macrophages, as seen in rheumatoid arthritis (RA) suggesting that there is some other source of inflammatory factors controlling the joint environment (103). There are a number of potential sources of cytokines, including the chondrocytes themselves, as well as the synovial fibroblasts or macrophage-like cells in the synovium of the joint, which respond to damage or inflammatory stimuli in the joint. Previous studies in preclinical animal models and *ex-vivo* human cartilage explants (124) show that cytokine stimulation of cartilage mimics a number of the pathological aspects of the OA joint, such as the induction of matrix and collagen degradation and the induction of aggrecanases, that degrade the proteoglycan, aggrecan (103).

IL-1β stimulation of chondrocytes induces the expression of a number of pro-inflammatory cytokines, chemokines and matrix modifying proteinases (125,126). It also inhibits the expression of genes associated with the components of the extracellular matrix, such as COL2A1 and aggrecan (125–127). Therefore, IL-1β induces catabolism of cartilage and reduces the ability of chondrocytes to repair cartilage damage by limiting the synthesis of matrix components.

IL-6 and IL-8 are also both elevated in the synovial fluid of OA sufferers and these pro-inflammatory cytokines are elevated further in obese OA patients (71,72). Production of IL-6 and IL-8 can be induced in chondrocytes by stimulation with potent pro-inflammatory factors like IL-1β and TNFα (68,72). Chondrocyte produced factors can have a direct impact on the inflammatory phenotype of

other joint tissue cell types, such as synovial fibroblasts which exhibit increased IL-6 secretion when cultured in chondrocyte conditioned media (71).

Whilst OA is a chronic low grade inflammatory condition it has previously been referred to as non-inflammatory arthritis. This is due to comparisons with RA which is a highly inflammatory joint condition characterised by joint degeneration and immune cell infiltration (71). OA exhibits much lower levels of immune cell infiltration and pro-inflammatory cytokine release but inflammation is still elevated beyond that of healthy joints(71). These two types of arthritis therefore share some characteristics but are also distinct. Pearson et al suggested that OA in obese individuals could be closer in some respects to RA due to the inflammatory effects of increased adiposity and the contribution of adipokines to OA pathology (71).

#### 1.4.2.4 Biomechanical factors in osteoarthritis

Abnormal biomechanics of the joint is often observed in osteoarthritic joints but it is unclear whether this is a symptom of the condition or a contributing factor. A number of methods of OA induction utilise joint destabilising surgeries resulting in biomechanical abnormalities and the development OA-like symptoms (96,128,129). Traumatic OA can develop in humans following injury; this is likely linked to the resulting biomechanical defect, however, it is not clear whether the biomechanical changes seen in non-traumatic, age-related OA occur prior to, or following, initial OA damage (128,130). Regardless, this may influence the development and progression of OA. Two of the most commonly used surgical methods of OA induction are the anterior cruciate ligament tear (ACLT) method and the destabilised medial meniscus (DMM) method, highlighting the importance of these ligaments and the menisci in maintaining the stability and health of the knee joint. One of the major drawbacks of such models is that they

cannot be used to test treatments as the biomechanical damage used to induce OA cannot be repaired. Models like these are perhaps better mimics of traumatic OA, where the condition is initiated following a joint injury, than the age-related, non-traumatic form of the condition in humans (128,130).

#### 1.4.2.5 Epigenetics

Age-related changes to epigenetic processes have been implicated in a number of ageing linked conditions (131). Currently, the sirtuin family of deacetylases are under investigation as factors affecting musculoskeletal ageing (114). Reduced SIRT1 expression or activity resulted in the development of an ageing phenotype in the skin of and reduced the longevity of transgenic mice, whereas increased SIRT1 expression is linked to increased longevity in skeletal muscle (114,132). SIRT1 expression is reduced in OA tissue suggesting a possible role in OA (133–135). Reduced mitochondrial SIRTs have also been implicated in the development of cellular senescence, although reduced expression of these proteins could be an indicator of mitochondrial dysfunction and it is this dysfunction that leads to the senescence (136,137).

#### 1.4.2.6 Obesity and osteoarthritis

Age-related changes in body composition occur, including an accumulation of abdominal fat in the elderly, coupled with reduced levels of physical activity and increased sedentary time (138). This results in an increase in the average BMI in later life and an increased likelihood of individuals becoming overweight or obese. In turn, obesity increases the risk of developing osteoarthritis in addition to other conditions, such as cardiovascular disease and diabetes (139,140). Previously, this correlation had been attributed to biomechanical factors associated with increased weight, where any minor biomechanical issues may be

exacerbated by the increased weight and stresses put on the joint. However, this does not fully explain the increased risk of the development of OA in non-weight-bearing joints, such as those in the hand (140). Therefore, adipose tissue was investigated as a source of potential mediators of joint damage. Adipokines are adipose tissue derived cytokines and are elevated in obese individuals, as mentioned in Section 1.2.1 (139,140). Adipokines, produced by white adipose tissue, were therefore identified as a potential non-biomechanical link between increased body fat and osteoarthritis (139,140). There is also some evidence to suggest that tissues such as the meniscus, which is essential in maintaining the stability of the knee joint, are more susceptible to the effects of adipokines than the likes of cartilage (141). So although much of the current body of research on adipokines and osteoarthritis has centred on the responses of cartilage, there may be further effects on the joint overall due to the impact of these adipokines on other tissue types and on the interaction between these tissues (71,142).

The adipokines of interest in this study are leptin, visfatin and resistin. Details of these adipokines are discussed in the following sections.

***Leptin***

Leptin is considered to be a pleiotropic hormone and has similar properties to that of the IL-6 family of cytokines (143). As an adipokine, it is thought to regulate food intake and energy expenditure at a hypothalamic level whilst also playing a role in other physiological processes such as bone formation and inflammation (125,144–146); more recent studies have suggested that leptin may also be involved in the regulation of chondrocyte metabolism.

Expression of leptin and its receptor are both increased in advanced OA cartilage with chondrocytes exhibiting BMI-dependent responses to the protein; high levels of leptin increased the expression of genes, such as MMP-13, more in those

with a higher BMI (147,148). Leptin, like several other adipokines, is elevated in the synovial fluid of OA patients and has a detrimental effect on chondrocyte proliferation whilst inducing IL-1β and MMPs 9 and 13. Therefore, leptin induces cartilage catabolism and promotes inflammation in the joint (147).

Pro-inflammatory stimulation of chondrocytes results in increased expression of leptin, which in turn induces the production of pro-inflammatory cytokines such as TNFα and IL-6. This inflammatory loop highlights the capacity of this adipokine to promote sustained inflammation as seen in conditions like OA (149). Together the increased expression of leptin observed in individuals with higher levels of adiposity and the elevated levels observed in the synovial fluid of OA joints indicate that leptin could, in part, explain the link between obesity and OA of non-weight bearing joints such as the hand.

Leptin has a number of effects on bone. It may act on bone marrow stromal cells to enhance their differentiation to osteoblasts whilst inhibiting adipocyte formation. However, leptin resistance is common in obese individuals which would result in increased adipocyte differentiation (144,150). Studies have shown that the ratio of leptin to adiponectin may be associated with the bone formation biomarkers; osteocalcin, a protein produced by osteoblasts; and procollagen type I N-terminal propeptide (PINP), a protein produced by osteoblasts during type I collagen synthesis (146). It has been suggested that increased leptin may drive the bone remodelling process seen in osteoarthritic joints. This hypothesis is supported by the increased expression of leptin in the osteophytes of OA joints, and links between leptin and the increased density of bone, which is observed in OA patients (145,146,151). Mutabaruka et al (152) suggested that the altered osteoblast phenotype observed in OA bone could be linked to abnormal leptin production and/ or responses, with leptin inhibition

treatment of OA osteoblasts resulting in reduced expression of ALP, osteocalcin, type I collagen and TGFβ1, all of which are elevated in OA (152). Therefore, leptin may play an important role in the development and progression of osteoarthritis (125).

***Visfatin***

Visfatin is produced by visceral adipose tissue and by a number of other tissues including OA synovial cells, chondrocytes and osteoblasts (153). It is found at increased levels in individuals with abdominal obesity (154) and is also elevated in the synovial fluid of patients with osteoarthritis (155).

This protein is known by three names: visfatin, pre-B cell colony enhancing factor 1 (PBEF1) and Nicotinamide phosphoribosyltransferase (NAMPT), and has a number of different functions. Visfatin has been shown to induce pro-inflammatory responses in chondrocytes and osteoblasts and in osteoarthritis it is mainly produced by the synovium (153). Visfatin is expressed by lymphocytes and has been investigated as an activator of B cell expansion, which is one of several immune and inflammatory functions of this protein (156). Stimulation of joint tissues with visfatin has been found to induce the production of a number of pro-inflammatory cytokines, including IL-1β, TNFα and IL-6, and chemokines such as monocyte chemoattractant protein 1 (MCP-1) and IL-8 in a dose-dependent manner, as well as inducing the production of cartilage degrading MMPs 3 and 13, and ADAMTS 4 and 5 (153,154,156,157), suggesting that elevation of this adipokine in the synovial fluid of OA patients could be further promoting the pro-inflammatory environment of the joint and driving cartilage catabolism (155,156). Visfatin also functions as the NAMPT enzyme and is important in the NAD+ biogenesis pathway suggesting that visfatin also plays an important role in cell metabolism and NAD+ dependent processes, such as sirtuin

activity (156). Fukuhara et al (158) suggested that visfatin was an insulin mimic, but this paper has since been retracted due to the irreproducibility of the results. In a more recent paper Revollo et al (159) have shown that extracellular NAMPT does not act as an insulin mimic but can regulate the secretion of insulin in pancreatic β cells through the NAMPT-dependent NAD+ biosynthetic pathway (158,159).

***Resistin***

Resistin, like other adipokines, is elevated in the synovial fluid of OA patients (142). It has been found to have pro-inflammatory effects on chondrocytes, inducing a similar pro-inflammatory phenotype when compared to IL-1β (126,160,161). Stimulation of chondrocytes with resistin increases the expression of a number of pro-inflammatory cytokines and chemokines, MMPs 1 and 13, and ADAMTS4 through the activation of the NFκB transcription factor, a master regulator of inflammation (160,161). It also reduces the expression of genes responsible for the production of matrix components, COL2A1 and aggrecan, limiting the repair capacity of cartilage (160). Resistin has also been found to promote an abnormal type I collagen phenotype in the osteoarthritic subchondral bone of obese individuals whereby the ratio of type I/II collagen is increased, further supporting the link between adipokines and OA (162). Therefore, this adipokine can promote inflammation in the joint and reduce the functional and repair capacity of both cartilage and bone.

## 1.5 MODELS OF OSTEOARTHRITIS

As with muscle ageing research, animal models are used to study OA and small animals are the most common choice of model. Owing to the relatively short lifespan (~2 years) of small animals, the development of spontaneous joint problems generally does not occur, although there are some exceptions which will be discussed in Section 1.5.2. As a result many of the animal models used have OA surgically or chemically induced; some of these models will be discussed in Section 1.5.1.

### 1.5.1 Induced small animal models

#### 1.5.1.1 Anterior Cruciate Ligament Tear (ACLT) model

The ACLT model of OA involves surgically damaging the anterior cruciate ligament of the knee and this model has been used in a number of different species including the rat, the rabbit and some large animal models such as the dog (129,163–165). Typically this method rapidly induces a form of OA that is more severe than that observed in humans (165), and the severity of OA as a result of this surgery differs between animal model species. This method of surgical OA induction, like many surgical models, can also vary in severity between the researchers carrying out the surgery due to differences in surgical skill and is not recommended for use in the mouse due to the high level of proficiency required (163,166). Culley et al (130) suggested that surgical models may better reflect traumatic OA in humans rather than the idiopathic OA associated with ageing alone (130). The rapid progression of damage following ACLT surgery makes this model helpful for short term studies which may be comparable to OA in humans following ACL injury (96).

#### 1.5.1.2 Destabilised Medial Meniscus (DMM) model

The DMM model produces a milder form of OA that progresses slowly and is considered to be more clinically relevant (165). In this model the meniscus is destabilised by the transection of the medial meniscotibial ligment. Due to simpler surgical procedures this type of surgical induction is more reproducible than ACLT, the severity of which varies considerably when the surgery is performed by different people (167,168). For this reason, Glasson et al (166) concluded that DMM is the better model for use in mice but also acknowledged that there were differences in the severity of OA induced between different mouse strains using this method (166). The slower progression of damage in this model allows the investigation of disease pathology and the study of the changes occurring in joint tissues (166). As discussed in the ACLT section, surgical methods of OA induction are considered to more closely mimic traumatically induced OA. The DMM model mimics the clinical symptoms of meniscus injury in humans and whilst this is a known predisposing factor for the development of OA it may not reflect the changes occurring in idiopathic age-related human OA (130).

#### 1.5.1.3 Mono-iodoacetate induced osteoarthritis model

Mono-iodoacetate is a metabolic poison which induces acute cartilage degradation and joint pain (167,169). Naveen et al (170) compared this model to ACLT in rats and human OA and came to the conclusion that this model caused similar levels of cartilage degradation to ACLT and that this was comparable to human OA (170). However, opinions on this model vary; Lorenz et al (167) have suggested that this method may be useful as a model of joint pain but that it is poorly reflective of human OA as it induces extensive chondrocyte apoptosis

(167,169,171). Therefore, as a method of testing pain treatments for OA, MIA models can be extremely useful and this method is considerably less invasive than surgical induction methods (171).

Whilst induced methods may not closely reflect age-related OA they can be useful to investigate OA as a result of traumatic injury to the joint or joint ligaments. Induced methods also allow for a known time point of initiation and therefore may be helpful in elucidating some of the early joint changes involved in OA development. Animals may also have an internal control if only one knee undergoes surgery allowing direct comparison to a healthy joint in the same animal.

## 1.5.2 Spontaneous small animal models

#### 1.5.2.1 Dunkin Hartley Guinea Pig

The Dunkin Hartley guinea pig has been used extensively in OA research as a spontaneous model of the condition. However, the significant influence of body mass and obesity on these animals complicates their use as an OA model (169). Guinea pigs are predominantly sedentary animals with a tendency to become obese unless dietary restriction is used, however, this can result in reduced incidence and severity of OA (129,172). In contrast to humans, male guinea pigs exhibit more severe, consistent pathology than the females, which is thought to be due to their increased size and growth rate (129). These animals also develop OA during maturation, as opposed to old age as is the case for humans and a number of the large animal models of spontaneous OA (128). This animal has been used due to its consistent and reliable development of OA pathology,

however, this reliability presents a problem in that there is no healthy control for comparison purposes.

#### 1.5.2.2 Mice

There are a number of mouse strains which develop spontaneous osteoarthritis: C57/BL6, Balb/c and STR/ort mice are examples of these strains. As with most spontaneous models there is a degree of variability in OA pathology (165). Like guinea pigs, OA is more severe in males with the degree of protection observed in females correlating with increased expression of cytokines, TGFβ1 and IL-4 which are thought to positively modulate cartilage metabolism (167). Transgenic mice are also of use in OA research but more as a research tool to investigate the contribution of particular genes to OA pathology and to establish any genetic predispositions associated with the pathology rather than models of the condition itself (169).

### 1.5.3 Large animal models

There are a number of large animal models used in OA research. All large animal models present difficulties such as increased cost compared to small animals, increased housing requirements and handling difficulties. However, these animals are more anatomically similar to humans and the larger joints increase the ease of use and reproducibility of surgical induction techniques, making them more easily comparable to the human condition. Here, three large animals will be discussed: sheep, horses and dogs.

Sheep and goats have been used in OA research and are one of the models discussed in papers published as part of The OARSI histopathology initiative (173). These animals typically do not develop spontaneous OA, with various forms of ligament tears and meniscus destabilisation being the preferred

methods of surgical induction (173,174). The sheep knee or stifle is more anatomically similar to human knees than that of small animals, as is the case for a number of other large animal models (173,175). One potentially important difference is the loading of the knee joint, meaning that ovine knee joints are loaded in flexion rather than in extension as is the case for human knee joints (174).

Burger et al (174) investigated a meniscal tear model of OA in sheep finding that this method induced similar cartilage degeneration as meniscal injuries in humans. However, it was also noted that the ACLT method of OA induction does not induce significant cartilage damage in goats (174). These animals have also been utilised in the investigation of cartilage repair following injury, for example Power et al (176) created cartilage lesions in sheep knee joints to determine the effect of rhFGF-18 on chondral repair (176). Recently the sheep has also been used to model joint repair using osteochondral plugs (177). Therefore, although the sheep may not be an appropriate animal model to investigate age-related OA pathology due to the lack of naturally-occurring disease, it has proved to be useful for research into methods of osteochondral defect repair to treat the joint damage caused by OA.

The horse develops spontaneous OA in old age and therefore has been used as a spontaneous model of OA, however, the development of the condition takes a number of years in these longer lived animals (178). As a result, induced models have also been used. This is not limited to joint instability models with the osteochondral fragment and exercise models being the most commonly published (179–181). The osteochondral fragmentation model involves the surgical creation of an osteochondral defect within the joint and the resulting joint changes have been shown to exhibit similarities with human OA (182).

Although this method induced clinically relevant symptoms and joint changes it can be difficult to separate adaptive responses to the surgery and development of the OA pathology due to the severity of the fragmentation (180). The ability of osteochondral fragmentation to induce OA in the horse is important when considering the impact of other joint pathologies on spontaneous models of disease: osteochondrosis, which is discussed further in Section 1.6, can result in the development of osteochondral fragments within the joint and can therefore lead to the development of secondary OA (183).

The dog also develops spontaneous OA, but the length of time required for these animals to develop OA and the resulting variability in pathology in individual animals means that induced models have also been used. Joint destabilisation models, such as ACLT, have been used and the 'groove' model was developed without the need to induce joint instability. In dogs, ACLT induction causes damage by creating joint instability and can lead to a form of OA in which a large number of osteophytes are formed (129,165). The groove model induces OA-like damage by cutting grooves into the cartilage of the joint and has been used in the dog only. This option resulted in damage similar to the ACLT model (184,185). Marijnissen et al (185) compared the joint condition of dogs with OA induced by these two methods and found that the groove model exhibited OA with less synovial inflammation and MMP activity than the ACLT model (185). This one time trauma method is likely to be more useful in investigating the effect of treatments over time because, unlike methods like ACLT, there is no ongoing joint instability driving joint degeneration (185).

## 1.6 THE COMMERCIAL PIG AS A MODEL OF MUSCULOSKELETAL AGEING

Every animal model has both advantages and disadvantages. The majority of issues surrounding use of the pig centre around the large size of these animals but it is hoped that the data in this thesis will show that the benefits of such a model might outweigh the challenges involved in its use and that this model may overcome some of the issues with use of rodents and other animal models. Table 1.1 shows some of the major points of consideration for the pig model.

**Table 1.1: Advantages and disadvantages of the pig model**

| Advantages | Disadvantages |
|---|---|
| Similarities in joint size and structure to humans **(186)** | Large animals present handling difficulties/challenges |
| Metabolic similarities with humans | Increased cost of housing and feeding animals |
| Spontaneously develop osteoarthritis | Longer life spans make ageing research more difficult |
| Voluntary physical activity levels similar to humans **(187)** | If drugs are used as part of a trial, larger animals require larger doses |
| Possible to take serial biopsies of muscle, for example. | |
| Large amounts of tissue available | |

### 1.6.1 Advantages of the commercial pig model

One major advantage of using porcine cartilage is that the different zones that exist in articular cartilage can be easily identified. Figure 1.10 shows porcine cartilage stained with safranin-O (method detailed in Section 2.8.5) and the cartilage zones, discussed in Section 1.3.1, have been identified.

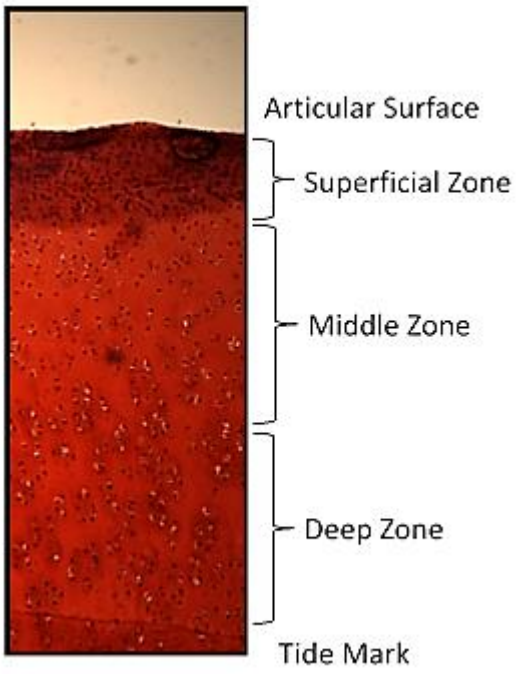


**Figure 1.10: Zones of porcine articular cartilage**

**Joint Size and tissue structure**

The knee joint or stifle of the pig is similar in terms of joint size and structure to the human knee. There are also a number of papers discussing the similarities in cartilage thickness, with one particular advantage being that partial thickness lesions can be created in the pig, and other larger animal models, which are difficult to recreate in smaller animals which have much thinner cartilage. An additional benefit of use of the pig, and of other large animals, is that it is possible to take multiple muscle biopsies over time to investigate the effect of treatments or interventions without loss of the animal.

**Metabolism**

Metabolically, the pig is very similar to humans and is used as a model of metabolic disease and diabetes. Metabolic changes in elderly humans may contribute to a number of degenerative changes in the musculoskeletal system therefore when considering the models available for the study of musculoskeletal ageing it is important to consider whether these age-related metabolic changes are likely to occur in that animal. It is likely, although not confirmed, that the pig would exhibit a degree of metabolic dysfunction similar to elderly humans in their old age.

**Behavioural Similarities**

Studies investigating physical activity in the pig have revealed a number of similarities between pigs and humans. Pigs, much like humans, tend to eat in meals as opposed to grazing and exhibit similar levels of voluntary physical activity (187).

### 1.6.2 Disadvantages of the commercial pig model

**Housing and handling**

Like all large animals, using the pig would result in higher housing and feeding costs per animal. These animals can be aggressive, therefore, there are handling precautions which must be taken into account when determining if this is the correct animal for use in any study. For example, it is recommended that there be three people present when pigs are being handled to ensure that if someone were to be injured or were to take ill when in with the pigs that there is someone to

assist them and another person to ensure the pigs are kept away from the injured party.

**Potentially unsuitable for some types of research**

If drugs are to be used in any trial involving pigs it is worth noting that the amount of the drug required per animal would be considerably higher than in smaller animals. Therefore, costs of running such a trial with pigs would be much higher.

Being long lived animals it is likely unfeasible to age pigs for the years that are required to reach old age. Therefore, the pigs available for ageing research are limited to those available from the commercial industry, these pigs tend to be equivalent to middle aged humans and are therefore likely to be suitable for the investigation of early age-related decline but not for conditions that specifically effect elderly humans, such as osteoporosis.

### 1.6.3 Porcine musculoskeletal health

There are a number of factors that can affect the health of porcine joints. Increased rearing intensity, where pigs are brought up to slaughter weight more quickly, can have a negative impact on leg health, increasing the incidence of lameness (188). Likewise, the condition of pen flooring on which the pigs are kept can have an effect on lameness incidence. This is thought to be linked to biomechanical stresses put on the joints as a result of poor or uneven surfaces (189,190).

Pigs have been known to develop a number of joint conditions and the commercial breeds, in particular, have a high incidence of lameness. These

conditions include osteochondrosis and osteoarthritis. Pigs and many other fast growing mammals, including humans, can suffer from osteochondrosis (OC). This is a condition where there is a lack of blood supply to the endochondral ossification front, resulting in cartilage being retained in the bone of joints. This presents as cartilage protrusions into the subchondral bone where ossification has failed, which can result in lameness and, if left untreated, can lead to fragmentation of the joint and the development of secondary OA (183). It is also possible for these OC lesions to spontaneously resolve. Endochondral ossification can progress past these lesions resulting in subchondral bone cysts of necrotic cartilage, this cartilage can be cleared by phagocytosis and replaced with new bone through intramembranous ossification (191). This condition is common in younger, fast growing animals therefore it is important when considering animals such as the pig as a model of OA that skeletally mature animals be used where possible rather than immature animals which may exhibit OC rather than OA and have a higher capacity for joint healing (192). The occurrence of OC in commercial pigs may complicate their use as a model of spontaneous OA due to similarities in the resulting pathology.

## 1.7 SUMMARY AND THESIS OBJECTIVES

The ageing population of the western world means that the number of older individuals requiring treatment for musculoskeletal ageing related conditions such as sarcopenia and osteoarthritis is likely to increase. The majority of research in this area utilises rodent models of these conditions, however, these models have a number of issues which limit the translatability of research carried out using these models into meaningful treatments for humans. Therefore, there is an unmet need for a more representative animal model. Due to similarities in joint structure, high incidence of joint problems and similarities in muscle metabolism it is hypothesised that the pig will develop spontaneous knee OA at an early age and will display early indications of age-related muscle decline and therefore could represent an improved model of musculoskeletal ageing,

In this thesis the utility of the commercial pig as a model of musculoskeletal ageing and osteoarthritis will be investigated.

**Chapter 3 - Analysis of porcine body composition and changes across the age range**

This chapter will determine whether the body composition changes that typically occur during human ageing also occur in the pig within the age range used in this study.

**Chapter 4 - Assessment of age-related changes in porcine muscle**

This chapter will investigate changes in porcine muscle across the age range with special consideration given to aspects of muscle that would be expected to be altered in early sarcopenia in humans.

**Chapter 5 - The pig as a model of spontaneous osteoarthritis**

This chapter will investigate osteoarthritis in the pig. It will determine if these pigs develop spontaneous osteoarthritis of the knee and at what age, and will go on to characterize some of the changes occurring in the knee joints of these animals.

**Chapter 6 - Utility of porcine cartilage for ex-vivo studies and osteoarthritis research**

This chapter will assess the utility of porcine cartilage and chondrocytes for osteoarthritis research. This will include the assessment of chondrocyte phenotype in culture and the effect of pro-inflammatory stimuli on cartilage tissue.

**Chapter 7 - Utility of the pig as a source of bone for ex-vivo studies and osteoarthritis research**

This chapter will assess the utility of porcine bone as a tool to study osteoarthritis. It will determine the functional phenotype of porcine osteoblasts and seek to establish if these changes are age-related or linked to the damage status of the knee.

## 2.0 GENERAL METHODS

### 2.1 ANIMALS

The animals used in this study were female Camb12 pigs (Large white x Landrace x Duroc). These pigs are a large commercial breed used primarily for meat production. In order to establish an ageing profile for this line of commercial pig, a number of age groups were investigated during the course of this study. The age groups were as follows: 6-10 wks (n=8), 17 wks (n=6), 40 wks (n=6), 80 wks (n=8), and 3-4 yrs (n=7). The 6-10 wks, 17 wks, 40 wks, and 80 wks groups were gilts (female pigs that have not been used for breeding). The pigs aged 3-4 yrs were breeding females, known as sows. In addition, a group of six 45 week old female pigs were kept in house for a period of 35 weeks to investigate the early development of lameness in the pig, therefore there was an additional group of 80 wk old pig (n=6) that were kept within University of Nottingham for a period of time. Ethical permission for the study was granted by the Nottingham University Animal Welfare Ethical Review Body (AWERB).

The age at which pigs reach skeletal maturity is debated in the literature but it has been suggested that maturity is reached around 2-2.5 years of age. Therefore, the 3-4 year old group of pigs would be skeletally mature and therefore more suitable for OA research. Despite widespread use it is not appropriate to use immature animals in musculoskeletal ageing research as they are still in their growth phase and therefore not likely to show true evidence of age-related decline. The age groups chosen for this study were selected following consultation of Jorgensen and Sorensen's paper on different rearing intensities of gilts (188). With the 17wk group being supposedly healthy with little risk of early slaughter due to leg disorders (see Figure 2.1), the 40wk group represent pigs at the first drop in likelihood of survival. The 80 week age group was selected to

allow the investigation of development of lameness/ leg disorders while the oldest group of 3-4 year old pigs allowed the investigation of a group of skeletally mature pigs at a stage where a number of pigs are lost due to leg problems. Together these groups would allow the generation of a musculoskeletal ageing profile for the commercial pig up to 3-4yrs and the oldest group is likely to represent the most viable for use as a model of spontaneous OA.

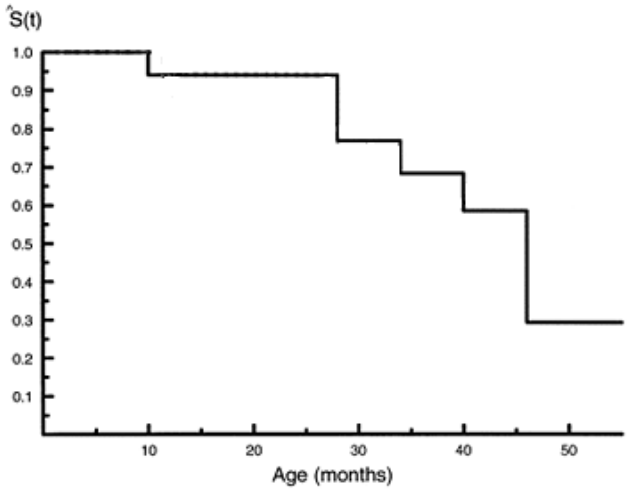


**Figure 2.1: The effect of rearing intensity on the probability of not being culled due to leg disorders.** (Figure modified from Jorgensen et al (188))

## 2.2 WEIGHT MONITORING

All pigs were weighed on arrival at the University of Nottingham Animal Unit and again prior to slaughter. Pigs kept for longer than 2 weeks were weighed weekly to establish growth rate and monitor health.

## 2.3 SAMPLE COLLECTION

Pigs were slaughtered by stunning followed by exsanguination. Blood samples were collected at slaughter and processed to provide both serum (no anticoagulant added) and plasma (Ethylenediaminetetraacetic acid disodium salt

dehydrate (EDTA) added as anticoagulant) samples, which were frozen for subsequent analysis. Following slaughter, samples of *tibialis anterior*, *gastrocnemius* and *soleus* muscles were dissected from one of the rear legs of each pig and either snap-frozen immediately in liquid nitrogen or frozen in liquid nitrogen cooled isopentane to preserve the muscle structure for histological analysis. These samples were stored at -80°C for processing at a later date.

The rear leg was then transferred to a class II safety cabinet for further dissection to reveal the articular cartilage surfaces of the joint. These internal cartilage surface of the joint were photographed to allow chondropathy scoring to be carried out at a later date and joint tissue was collected as detailed in the Tissue Culture section.

## 2.4 TISSUE CULTURE

Cartilage explant discs, primary chondrocytes and primary osteoblasts were isolated from the knee joints of pigs. The methods involved in obtaining this tissue and isolating and culturing these cells are detailed in the following sections.

### 2.4.1 Preparation of porcine cartilage explants

Using a cork borer, small cartilage discs were cut from knee articular cartilage taken from the femoral condyles of pigs at the time of slaughter. Cartilage discs were placed into the wells of a 96 well culture plate and 100µl of chondrocyte media added to each well. The plate was then incubated at 37°C, 5% $CO_2$. Media was refreshed after 24 hrs and experimental media (defined in results chapters) was added after 48 hrs.

Chondrocyte media consisted of Dulbecco's modified eagle medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% Fetal bovine serum (FBS; Sigma Aldrich, Poole, UK), 2mM L-glutamine (Sigma Aldrich, Poole, UK), 1% non-essential amino acids (GE Healthcare Life Sciences, Pittsburgh, US), 1% penicillin/streptomycin (Sigma Aldrich, Poole, UK), 0.1% gentamycin (Sigma Aldrich, Poole, UK) and 1% amphotericin-B (Sigma Aldrich, Poole, UK).

### 2.4.2 Primary porcine chondrocyte isolation

Pig femoral condyle cartilage was digested for 4 hrs in chondrocyte media containing sterile-filtered collagenase (Sigma Aldrich, Poole, UK) at a concentration of 2mg/ml. The digested cartilage was then filtered through a sterile 40 μm cell strainer, placed in sterile T75 culture flasks with 13ml chondrocyte media and incubated at 37°C, 5% $CO_2$. Media was refreshed after 48hrs to remove any dead cells and the remaining viable cells were cultured as described below.

### 2.4.3 Primary chondrocyte culture

Primary chondrocytes isolated from porcine cartilage were cultured in chondrocyte media at 37°C, 5% $CO_2$; media was refreshed every 3-4 days. Cells were monitored frequently during culture to identify any morphological changes occurring with increased passage number or increased time in culture. Isolated primary porcine chondrocytes were passaged on reaching 75-80% confluence.

### 2.4.4 Primary osteoblasts culture

Bone chips were obtained from the femoral condyles of the pig at the time of slaughter using bone cutters and were incubated in T75 culture flasks with osteoblast media at 37°C, 5% $CO_2$. Osteoblast media consisted of Dulbecco's

modified eagle medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, 1% non-essential amino acids, 1% penicillin/streptomycin, 2mM β-glycerophosphate (Merck Millipore, Darmstadt, Germany), 50μg/ml L-ascorbic acid (Sigma Aldrich, Poole, UK), 10nM dexamethasone (Alfa Aesar, Massachusetts, US) and 1% amphotericin-B. After 7-20 days of culture osteoblast outgrowth was observed. Cells were monitored frequently during culture to identify any morphological changes occurring with increased passage number or increased time in culture.

### 2.4.5 Passaging cells

Chondrocytes were passaged at 75-80% confluence in order to expand the cell population in culture. Osteoblasts were passaged at 95% confluence to prevent bone nodule formation and allow best yield and continued growth in the new flasks. For both cell types, media was removed from the flask and the cells washed briefly with sterile PBS (Phosphate-buffered saline), 0.25% Trypsin-EDTA was then added to the flask (2ml per T75 flask) and incubated at 37°C for 3-5 mins or until cells could be seen floating free of the flask surface, provided this time did not exceed 10 mins. The trypsin-EDTA was then neutralised with the addition of 8ml of chondrocyte or osteoblast media depending on the cell type being used. The cell suspension was transferred to a 25ml universal and centrifuged (200rcf for chondrocytes; 300rcf for osteoblasts) for 5 mins to obtain a cell pellet. The supernatant was discarded and the pellet re-suspended in the appropriate media and split between the desired number of plates or flasks.

### 2.4.6 Counting cells

Cells in suspension were counted using a haemocytometer. The following equation allows the estimation of cell number per ml suspension and therefore

allows consistency in seeding density or calculation of the overall cell number for normalisation purposes.

**Equation:**

**Cell number/ml = Average cell count per haemocytometer square x 10^4**

### 2.4.7 Cryopreservation and thawing of primary cells

#### Cryopreservation

To cryopreserve adherent cell cultures, cells were detached using 0.25% Trypsin-EDTA solution, diluted in chondrocyte or osteoblast media depending on the cell type being used and pelleted as described in Section 2.6.5. Cell pellets were then re-suspended in freezing media (FBS containing 10% DMSO) and immediately transferred to a cryovial and placed in a Nalgene Mr Frosty™ freezing container at -80°C for 24 hrs before being transferred to liquid nitrogen for long term storage.

#### Thawing cells

To prevent damage to the cells upon thawing, cryovials were rapidly thawed in a 37°C water bath for 4 mins or until contents were thawed. Cryovial contents were transferred to a universal tube containing 9ml of chondrocyte or osteoblast media depending on the cell type being used. The cell sample was then centrifuged at 140rcf for 4 mins, and the resulting pellet re-suspended in 10ml of chondrocyte or osteoblast media depending on the cell type being used and transferred to a T75 flask. Media was refreshed after 24 hr culture at 37°C, 5% $CO_2$ to replenish nutrients and to remove any dead cells from the culture.

## 2.5 PROTEIN EXTRACTION

### 2.5.1 Protein extraction from cells

Cells were lysed using cell lysis buffer (Appendix A) containing protease and phosphatase inhibitors (protease inhibitor cocktail; phosphatase inhibitor cocktail 2; Sigma Aldrich, Poole, UK) at 2µl/ml & 10µl/ml respectively. 50µl of lysis buffer was used per well of a 24 well plate and resultant total protein lysate stored at -40°C for subsequent analysis.

### 2.5.2 Protein concentration determination

The protein concentration of the lysed cell extract was determined using the Bradford protein assay (193). Standards ranging from 0-29µg/µl were prepared from stock solution of 1.45mg/ml bovine serum albumin**.** Samples were diluted 1:400 using distilled water to a volume of 400µl and 100µl of Bradford reagent (Bio-Rad Laboratories Ltd, Hertfordshire, UK) was added to each microcentrifuge tube. The solution was mixed by inversion and loaded onto a 96 well plate in duplicate. Absorbance was read at 550nm (Bio-Rad 680 XR microplate reader). A standard curve of A550 against the known protein content of the standards was produced and the protein concentration of the unknown samples estimated. This allowed for any results investigating the concentration or activity of a particular protein to be normalised to the overall protein content of the sample for more accurate comparison across the samples.

## 2.6 STATISTICAL ANALYSIS

Details of the specific statistical tests used to assess significance can be found in each of the results chapters.

# 3.0 ANALYSIS OF PIG BODY COMPOSITION AND CHANGES ACROSS THE AGE RANGE

## 3.1 INTRODUCTION

The typical human growth curve is characterised by initial fast growth throughout childhood and adolescence (particularly in the first 5 years of life and during puberty) whereby weight, bone mineral density and the functional capacity of the body's organs and systems are increased (194–196). Human growth plateaus at around 50 yrs of age followed by a period of long slow decline (196–198). Sarcopenic symptoms start to develop during this period of decline with loss of lean muscle mass and alteration of lipid distribution both featuring. Muscle strength is gradually lost and systemic inflammation in the muscle increases with age. Studies investigating this phenomenon have found that the decreased fat free muscle mass seen in sarcopenic patients is often accompanied by increased abdominal fat and reduced physical activity although current research has been unable to clarify the cause and effect relationship between these factors (199). It is possible that this in turn leads to an increase in the likelihood of obesity and obesity-related problems such as cardiovascular disease, type-2 diabetes and hypertension in those genetically susceptible to such conditions (30,200).

During later life the production of sex hormones, such as oestrogen, is reduced in both males and females with the drop in oestrogen levels being more pronounced in post-menopausal women. Oestrogen is a promotor of bone mineralisation and with reduced levels of this hormone studies have found a reduction in bone mineral density and increased bone fragility (201–203). Testosterone levels are reduced with age and this has been linked to the progressive loss of muscle mass

with age (204). Therefore, interventions involving testosterone supplementation to treat ageing characteristics such as sarcopenia have been investigated (205).

Geriatric care is now a major area of concern for healthcare systems globally. The combination of these body composition changes can contribute to increased physical frailty and increased risk of trips and falls in the elderly putting pressure on healthcare resources and, due to a lack of mobility in affected individuals, increasing the demand for homecare and assisted housing.

The purpose of this chapter is to determine if any early changes associated with ageing characteristics observed in humans, such as muscle loss, fat accumulation and increased systemic inflammation, occur in commercial pigs under 4 years of age.

### 3.1.1 AIMS

The main aims of this chapter are:

- Establish a growth curve for pigs over their commercial lifespan.
- Determine if body composition changes, such as increased lipid deposition and reduced lean muscle mass, seen in humans with age can be observed in the pigs
- Establish if there is an increase in systemic inflammation with age by investigating plasma IL-6 and visfatin concentrations by ELISA.

## 3.2 METHODS

The following methods were utilised in this chapter; further methods can be found in Chapter 2: General Methods.

### 3.2.1 Growth curve of weight gain

The mean weight for each age group was calculated in order to establish a growth curve up to 3-4 yrs of age for pigs raised in an industrial setting. Weekly weighing of the pigs while housed in the university's animal unit allowed the production of a growth curve as the pigs approached maturity. Further details on the animals used can be found in Section 2.1.

### 3.2.2 Body composition of commercial pigs as determined by DXA

One rear leg from each pig was scanned using a DXA (Dual energy X-ray absorptiometry) scanner 2-4 days post-slaughter; this enabled the measurement of leg mass to be separated into bone, lean and fat constitutive components. Prior to scanning, the legs were stored at 4°C. The DXA scanner (Norland XR-800 Densitometer) was calibrated using a 77-point standard QA calibration before 'whole body' scans were performed on the rear legs.

### 3.2.3 Analysis of dual-energy X-ray absorptiometry (DXA) scan data

Using the data provided by the DXA 'whole body' scan report it was possible to analyse the difference in the percentage fat, bone mineral density (BMD) and lean: fat ratio in the rear legs of pigs from 8 wk, 80 wk and 3-4 yr age groups. It was not possible to perform scans on whole pigs as the weight of the animals exceeded the maximum weight supported by the DXA scanner therefore hind legs were selected for investigation.

### 3.2.4 Plasma IL-6 and visfatin concentration

Porcine duo-set IL-6 ELISA kits (R&D systems) were used to assess IL-6 content and human duo-set visfatin ELISA kits (R&D systems) were used to assess visfatin content of blood plasma taken from the pigs during slaughter. Details of slaughter and blood collection can be found in Section 2.5.

**Plate preparation**

Capture antibodies were diluted in PBS to a working concentration of 0.8µg/ml for IL-6 and 1µg/ml for visfatin, 100µl of which was added to each well of a 96 well plate and incubated at room temperature overnight. The following morning the plate was washed several times with wash buffer and blotted to remove excess liquid. Plates were then blocked with 300µl of reagent diluent for one hour at room temperature. Following a final wash, the plate was ready for the addition of samples.

**Assay procedure**

100µl of sample or standard were added to each well and incubated for 2 hrs at room temperature. Standard concentrations ranged from 125-8000pg/ml for IL-6 and from 0.5-32ng/ml for visfatin. Following incubation, plates were washed thoroughly as before. 100µl of detection antibody was then added to each well at a working concentration of 100ng/ml for IL-6 and 500ng/ml for visfatin in reagent diluent and plates incubated for 2 hr at room temperature.

**Plate development**

Plates were washed and 100µl of a working dilution of horseradish peroxidase-conjugated streptavidin (streptavidin-HRP) was added to each well for a 20 min incubation in the dark, followed by another wash step and a 20 min incubation with 100µl of substrate solution per well in the dark. 50µl of stop solution was

then added to each well and absorbance was measured at 450nm using a FLUOstar plate reader (BMG Labtech Ltd) for both IL-6 and visfatin ELISAs.

### 3.2.5 Statistical analysis

Statistical analysis was carried out using Graphpad Prism software version 7: one-way ANOVAs were used to assess statistically significant differences between age groups for percentage fat, BMD and for lean: fat ratios. All ELISA results were analysed using four parameter logistic curve online data analysis tool, MyAssays Ltd and statistical analysis by one-way ANOVA was carried out.

3.3 RESULTS

### 3.3.1 Growth curve

The mean weight of the youngest age group (8 wk) was 37.4 ± 1.4 kg, by 17 wks of age this had more than doubled to 84.0 ± 2.2kg, the 40 wk age group mean was 141.2 ± 4.2kg. The highest mean weight was achieved by the 80 wk age group (245.1 ± 9.0kg) and a slightly lower mean was achieved by the 3-4 yr age group (230.6 ± 9.0kg). These data suggest that this genotype of pig reaches a plateau in weight gain between 80 wks and 3 yrs of age.

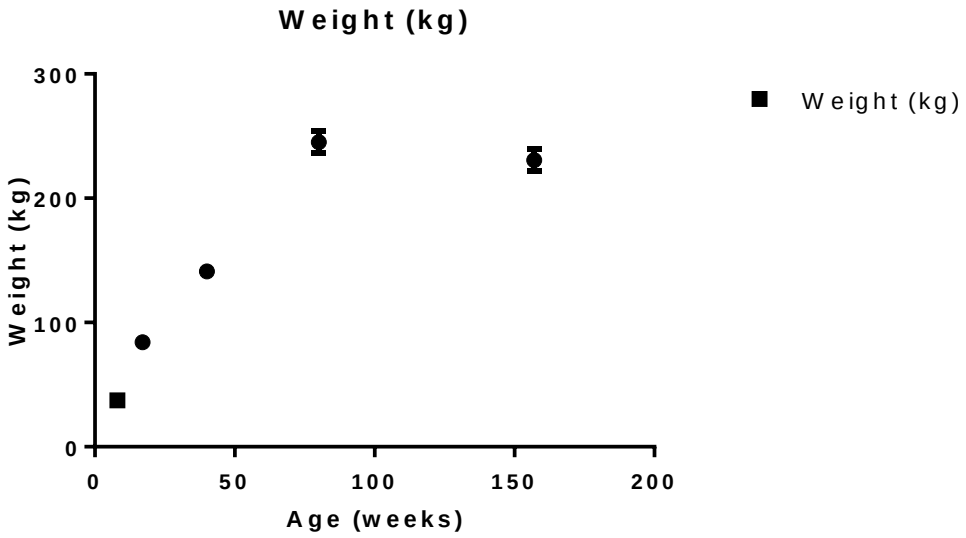


**Figure 3.1: Growth curve of pigs.** Values represent mean Weight (kg) ± SEM of pigs from each age group investigated. (8 wk n=7; 17 wk n=6; 40 wk n=6; 80 wk n=8; 3-4 yr n=7).

A group of 45 week old gilts (n=6) were monitored for behavioural changes relating to pain and deterioration in joint condition for a period of 35 weeks. During this time, weight was monitored weekly both for the establishment of a growth curve and as a measure of health. By the end of this period of monitoring

the mean weight of these pigs had increased from 149.2 ± 2.2kg at 45wks of age to 292.3 ± 9.3kg at 80 wks of age. Figure 3.2 shows the mean weight ± SEM of this group of pigs during the period of monitoring.

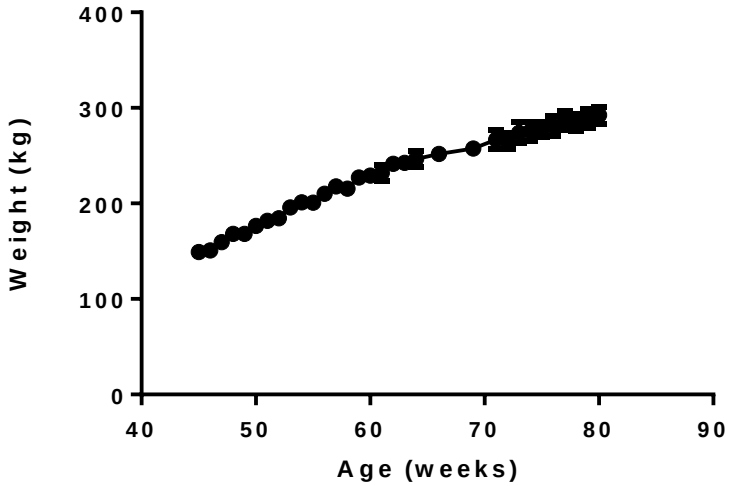


**Figure 3.2: Porcine growth curve.** Values represent mean weight (kg) ± SEM of pigs kept over a period of 35 weeks (n =6).

The Department for Environmental, Food and Rural Affairs (DEFRA) are the regulating body of the meat production industry in the UK. They implement a number of welfare and quality assurance guidelines including guidelines for the breeding of livestock (206). These guidelines suggest that sows to be used for breeding must be of a particular body condition. DEFRA's condition scoring was established to ensure that breeding pigs have adequate nutrition and this is thought to minimise the risks of pregnancy and improve welfare (206). Therefore, the lower weight of 80 wk pigs obtained directly from industry when compared to the pigs kept for a period of 35 weeks within the University of Nottingham animal facility could be linked to these pigs being prepared for breeding by ensuring that they meet the DEFRA body score requirements.

### 3.3.2 DXA scans

The rear legs of pigs from the 8 wk, 80 wk and 3-4 yr age groups were scanned using a Dual-energy X-ray absorptiometry (DXA) scanner to examine age-related change in tissue composition. Figure 3.3 shows representative images for each of the main tissue groups. From reports generated from these DXA scans bone mineral density, rear leg percentage fat and lean: fat tissue ratios could be determined.

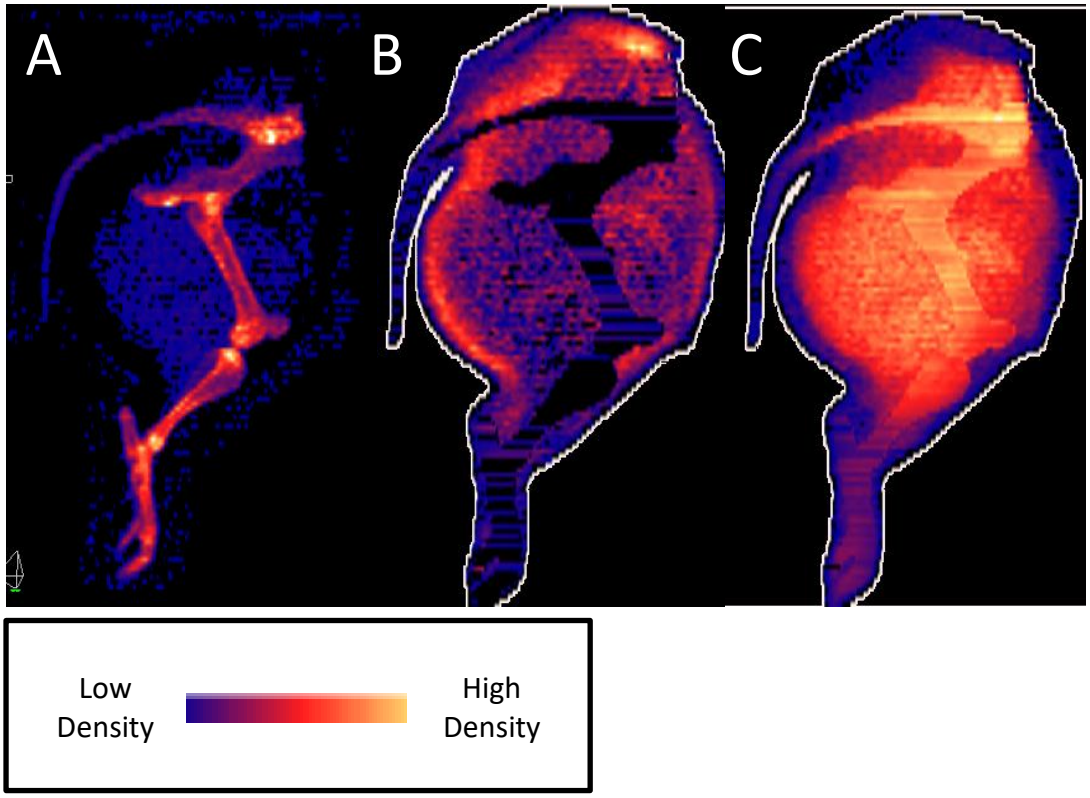


**Figure 3.3: Representative DXA scan images.** A: Bone density image, B: Fat image, C: Composite image showing lean tissue.

Examination of bone mineral density (BMD) data produced by DXA scans revealed a significant increase in BMD between the 8 wk and 80 wk age groups but no significant difference in BMD between the 80 wk and 3-4 yr age groups, as shown in Figure 3.4.

DXA scans also revealed that the percentage fat in the rear legs of the 80 wk group was significantly higher than that of the 8 wk and 3-4 yr groups with a mean fat content of 38.8% ± 1.9 compared to 14.2% ± 1.4 in the 8 wk group and 21.7% ± 2.4 in the 3-4 yr group, as shown in Figure 3.5.

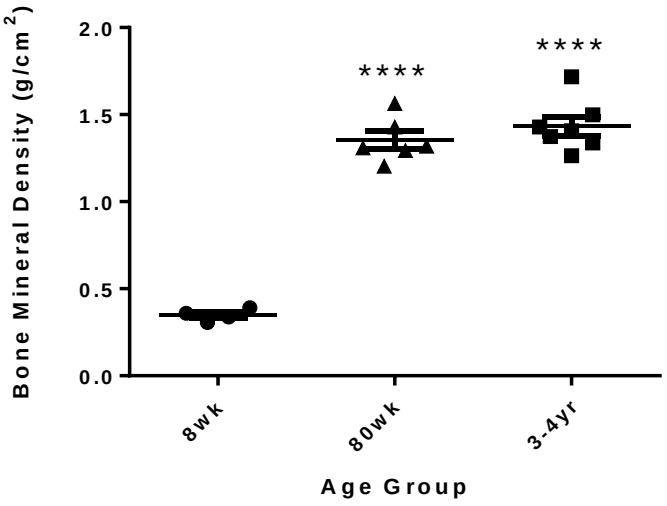


**Figure 3.4: Bone mineral density.** Individual results and mean ± SEM. Significant effect of age on BMD (p <0.0001) determined by one way ANOVA, tukey's post hoc test revealed **** p<0.0001 significantly higher BMD when compared to 8 wk pig group (8 wk n=4; 80 wk n=6; 3-4 yr n=7).

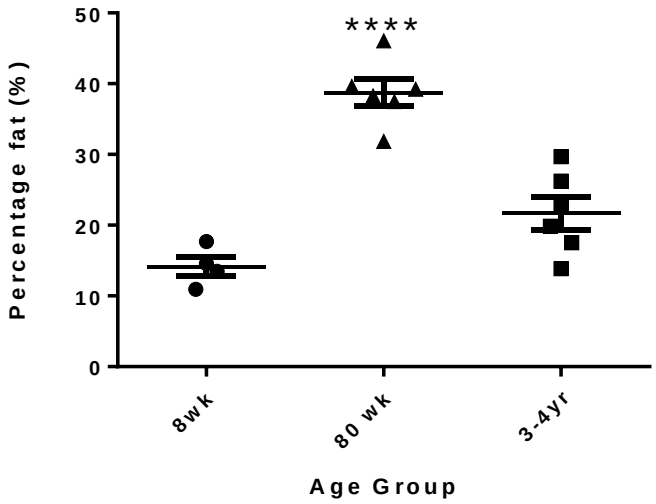


**Figure 3.5: Rear leg percentage fat.** Significant effect of age on percentage fat ($p<0.0001$) determined by one way ANOVA. Tukey's post hoc test revealed **** $p<0.0001$, significantly higher percentage fat in the rear legs of 80 wk when compared to 8 wk pigs (8 wk n=4; 80 wk n=6; 3-4 yr n=7).

Lean: fat ratios for the rear legs were used to establish the relationship between fat and muscle in the different age groups. The examination of lean: fat tissue mass in the hind legs of these pigs revealed that there was no significant difference in lean tissue mass between the 80 wk and 3-4 yr age groups. Therefore, the composition differences between 80 wk and 3-4 yr groups were in rear leg fat content only (Figure 3.6).

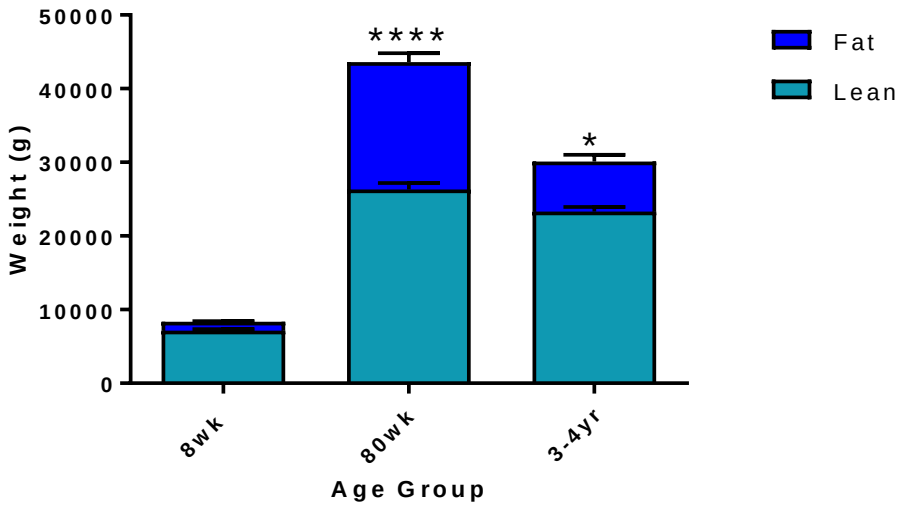


**Figure 3.6: Lean: fat tissue ratio.** Results shown as mean ± SEM for both lean and fat tissue. Significant effect of age on lean: fat ratio ($P < 0.0001$) determined by one way ANOVA. Dunnett's post hoc test revealed **** $P<0.0001$, * $P = 0.0116$ significantly higher ratio of lean: fat tissue when compared to 8 wk old pig groups.

### 3.3.3 Plasma cytokines

As discussed in Section 1.2, increased systemic inflammation is a consequence of ageing and increased circulating pro-inflammatory cytokines are associated with a number of age-linked conditions, such as sarcopenia and osteoarthritis. The investigation of plasma IL-6 levels in the pig revealed that there were no significant differences between the age groups and no relationship was observed between plasma IL-6 levels and pig age (shown in Figure 3.7A). There was also a high level of variability between individuals in all age groups with the exception of the 3-4 yr age group.

Increased risk of osteoarthritis is observed in obese individuals, with adipokines such as visfatin implicated as potential non-biomechanical mediators of the condition (207,208). Figure 3.7B shows that the mean visfatin concentration was

slightly higher in the 3-4 yr age group when compared to other age groups but there is a high level of variability within this age group and there were no significant differences in the plasma visfatin level between any of the various age groups.

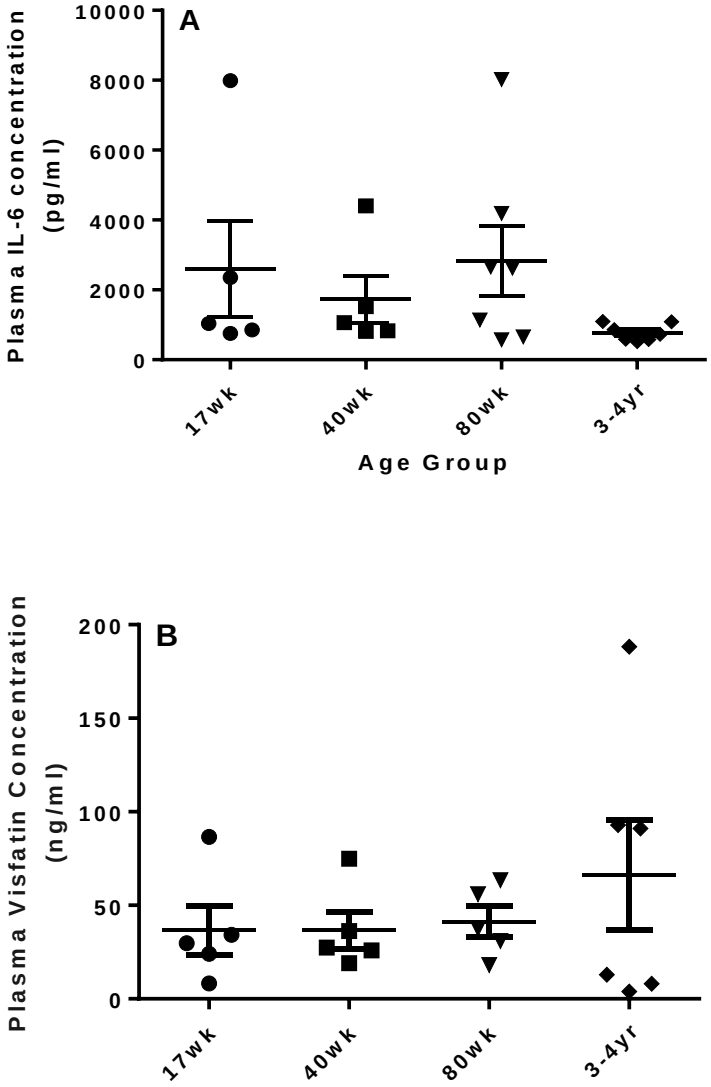


**Figure 3.7: Plasma IL-6 and visfatin concentrations in the pig. A:** Values represent mean IL-6 concentration (pg/ml) ± SEM for each age group. **B:** Values represent mean plasma visfatin concentration (ng/ml) ± SEM for each age group. No significant effect of age on IL-6 (p=0.2786) or visfatin (p=0.2861) concentrations in plasma as determined by one way ANOVA.

## 3.4 DISCUSSION

The detrimental age-associated changes in body composition start to develop in middle aged humans (53,54). This is a long slow process which is absent in a number of animal models commonly used to investigate ageing, discussed in more detail in Section 1.1.7. Many of the age-linked conditions in humans only exhibit symptoms in the elderly, such conditions include osteoporosis and sarcopenia; however, these conditions begin to develop much earlier and take many years to become symptomatic (31,32). Animal models are available to investigate some of these specific conditions affecting the elderly; for example, the bone of aged BALB/c mice requires a reduced level of energy-to-fracture suggesting that bone fragility increases with age and that these mice could be used to investigate osteoporosis (209). Yet there is an unmet need for an animal model to investigate the initial changes leading to the development of these musculoskeletal conditions. For this purpose, the pig may present an alternative model that exhibits ageing characteristics that reflect human ageing more closely and could be used as a model of early musculoskeletal change.

The age groups, discussed in Section 2.1, were used to allow the investigation of the porcine musculoskeletal system up to an age expected to be equivalent to middle aged humans. At this age, humans begin to show signs of musculoskeletal decline with early OA and initial muscle strength and mass reductions, associated with sarcopenia. The results outlined in this chapter have shown that commercial pigs within the age range investigated as part of this study reach adult weight and skeletal maturity but are not old enough to exhibit initial indications of age-related decline, as discussed in Chapter 1.0.

**The growth pattern of commercial pigs**

The investigation of the musculoskeletal ageing process in pigs is vital in determining whether these animals could prove to be a suitable alternative to more commonly used models. To this end the weight of all pigs used in the study was monitored for the duration of their stay within the university facility. The resulting growth curve revealed that the commercial pigs grew rapidly in early life, reaching maximum weight and plateauing between 80 wks and 3 yrs of age. It is difficult to discern whether the oldest pigs investigated have entered the anticipated period of decline from this information alone. The age at which pigs reach skeletal maturity is debated in the literature but it has been suggested that both skeletal growth and weight gain plateau at around 2-2.5 yrs of age (210,211). This would support the growth data obtained in this study and suggest that the 3-4 year age group have reached a growth plateau. Human growth reaches a plateau at around 40-50 years of age, followed by a long slow period of decline (54). This study have revealed that the oldest pigs in this study (3-4 yrs) have reached a growth plateau confirming that this age group is likely to be equivalent to middle aged humans.

Despite the mean weight of the 3-4 yr age group being slightly lower than that of the 80 wk age group it is unlikely that these pigs have entered a period of decline as there was no difference in lean tissue weight. A consequence of ageing in humans is the reduction in lean muscle mass and an increase in fat accumulation (29–32). No observable change in lean tissue mass between the 80 wk and 3-4 yr age groups supports the assertion that a growth plateau has been reached but that the older age group are not showing musculoskeletal decline.

The most commonly used animal models of musculoskeletal ageing are rodents, such as the Sprague Dawley rats, which grow for almost their entire lifespan with

only a short period of decline before death (53). Therefore, the growth pattern of commercial pigs mimics that of humans more closely than that of rat however further investigation in pigs older than 4 years of age is required to confirm the occurrence of the expected period of decline.

This study also revealed that 80 wk old pigs from industry had a lower mean weight than 80 wk old pigs aged with the University of Nottingham Animal Facility. The Department for Environmental, Food and Rural Affairs (DEFRA) are the regulating body of the meat production industry in the UK. They implement a number of welfare and quality assurance guidelines including guidelines for the breeding of livestock (206). These strict breeding guidelines dictate that pigs that are to be bred must be within a certain range on the DEFRA body condition scoring system to maintain the health of the sow during and after pregnancy. DEFRA's condition scoring was established to ensure that breeding pigs have adequate nutrition and this is thought to minimise the risks of pregnancy and improve welfare (206). As a result, commercial pigs in an industrial setting may undergo a degree of food restriction to maintain or achieve the required body condition prior to breeding. This type of regulation was not implemented in the 80 wk group of gilts during their time in the University Animal House and no food restriction was carried out with the aim of maintaining a particular body score. Therefore, the lower weight of 80 wk pigs obtained directly from industry could be linked to these pigs being prepared for breeding by ensuring that they meet the DEFRA body score requirements. Adherence to DEFRA guidelines for breeding sows could also explain the lower percentage fat seen in the rear legs of the 3-4 yr age group when compared to the 80 wk group. It is also possible that these sows carried less fat than expected due to the impact of pregnancy and lactation on their overall weight and body condition (206).

Therefore, the impact of DEFRA guidelines and pregnancy are important factors to take into consideration when determining if the pig is an appropriate model for each study, particularly when investigations require the use of adult female animals.

**Bone Mineral Density**

Bone mineral density (BMD) changes with age and is regulated by a number of factors. BMD increases with body mass and resistance exercise, this response is thought to be linked to increased muscle mass rather than mechanical loading caused by overall weight since weight loss does not result in reduced BMD (212–216). It is also influenced by hormones such as oestrogen, which was mentioned briefly in Section 3.1. Ageing bone exhibits reduced bone mineral density leading to the development of bone conditions, such as osteoporosis, and resulting in increased bone fragility and risk of fracture (209). These aspects of human ageing appear to be influenced by events in earlier life. A study conducted by Tveit et al (217) investigated the effect of exercise during youth on the loss of bone density and condition with age and found that exercise early in life can improve bone health in old age (217).

Examination of porcine BMD revealed a significant increase in the BMD of the rear leg between the 8 wk and 80 wk age groups, these changes in BMD are consistent with weight gain in these pigs and was to be expected in rapidly growing pigs (212). There is no significant difference in BMD between the 80 wk and 3-4 yr age groups, despite a difference of almost 62kg between the mean weights of these groups. Despite the difference in overall weight of these age groups there is no difference in lean tissue mass. This supports the theory that BMD is linked to lean muscle mass and not to total body weight in the pig, and this pattern is also observed in humans (212–216).

Reduced BMD is a symptom of osteoporosis in elderly humans. By 70 years of age humans have typically lost 30-40% of their bone mass and this loss of mass is accompanied by a loss of bone strength (218). Osteoporosis is particularly common in post-menopausal women with work by Khosla et al (219) suggesting that the rate of BMD loss increases following menopause in women (219). The percentage of women effected by osteoporosis varies between different populations with estimates ranging from 25-52% of women over 80 years of age developing osteoporosis-related bone fractures (220–222). However, the oldest age group of pigs investigated in this study were equivalent to middle-aged humans: only 2-4% of women over 50 years of age exhibit osteoporosis and the associated bone fractures (218,220–222). The 3-4 yr age group were still reproductively active and therefore any reduction in oestrogen production, as occurs in postmenopausal women and is linked to lower BMD, is unlikely. This age group were the oldest animals available from commercial breeders at the time of study; however, the estimated life expectancy of pigs is roughly 8-10 yrs (223). Therefore, it is unsurprising that the pigs have no exhibited any significant age-related reduction in BMD in the rear leg. Should older pigs become available these animal may exhibit osteoporotic symptoms.

**Fat Accumulation**

There is an increased incidence of obesity in the elderly population (224,225). Such body composition changes contribute to the development of a number of conditions effecting the elderly population such as cardio-metabolic disorders and as a result of, what has been termed, the obesity epidemic these obesity-linked conditions are becoming increasingly prevalent in younger individuals (224). Sarcopenic obesity is of particular concern in elderly humans. This is the

combined presence of both sarcopenia and obesity. Sarcopenia promotes fat accumulation through reducing muscle strength and physical activity; this in turn promotes the production of adipose-tissue derived inflammatory factors which increases sarcopenic muscle loss (226). Whilst this problem is only associated with elderly humans, changes occur first in middle aged individuals. Identifying initial changes could aid in the treatment or prevention of such age-related conditions therefore we investigated fat accumulation in pigs up to 4 years of age.

Investigation of percentage fat in the rear legs of pigs showed increased fat in the 80 wk group only, this was most likely due to the lack of food restriction and body condition controls as are in place in the DEFRA regulated UK commercial environment (206) and was not evident in the oldest age group. There was no significant difference between the lean tissue mass in these age groups. DXA imaging also revealed that the fat accumulating in the rear legs of these pigs was not able to infiltrate muscle to a degree that could be determined using this scanning method: intramuscular fat accumulation was further investigated as detailed in Section 4.3.5. Therefore, the 3-4 yr age group did not exhibit altered lipid deposition, increased fat accumulation or any initial reduction in lean muscle mass as would be expected in aged humans.

For the body composition studies only the rear legs of pigs were examined. This was due to the large size of the animals and the weight restriction on the DXA scanner. It is noted that the results obtained from the rear leg may not be representative of the whole animal and that age-related changes to the musculoskeletal system may not occur uniformly across all anatomical locations.

**Inflammation**

Increased levels of systemic inflammation is a characteristic of human ageing (227). IL-6 is a key pro-inflammatory mediator produced mainly by immune cells, vascular endothelial cells and adipocytes (228). Zhao et al (229) showed that serum levels of interleukin-6 (IL-6) correlate positively with age. IL-6 has been found to be further elevated in a number of ageing-linked conditions, including osteoarthritis and sarcopenia (72,230) and in obesity (231,232). This interleukin is involved in the activation of the acute phase response and the switching of this acute inflammatory response to chronic inflammatory conditions and has been associated with the loss of muscle strength in older adults (230,233). Evaluation of porcine IL-6 levels in plasma has not shown a direct link between IL-6 and age, however, this could reflect the relatively young age of the pigs used in this study. Elevated IL-6 is associated with the loss of muscle mass and strength (72,230). The pigs in this study do not exhibit increased IL-6 or reduced muscle mass suggesting that the age groups use are too young to be undergoing such body composition changes.

Obesity has been linked to an increased risk of the development of inflammatory conditions such as OA (72). Adipocytes within fat produce a number of adipokines, these proteins have a wide variety of different functions ranging from immune system modulation to appetite regulation (234,235). Visfatin is one such adipokine, produced by white adipose tissue and a number of other cells and tissues including chondrocytes and osteoblasts. It has been found to have pro-inflammatory effects and is elevated in the synovial fluid of osteoarthritic joints and in the blood plasma of osteoarthritis patients (155). The investigation of plasma visfatin across the age range of pigs did not show any significant relationship between plasma visfatin concentration and age but the average

visfatin level in the 3-4 yr old pigs was slightly higher and was considerably more variable than in other age groups. This suggests that the stage of disease progression and ageing is not sufficient for significant changes in the plasma cytokine profile in these pigs, if these changes occur at all.

Further discussion of inflammation and OA can be found in Chapter 5.0. Knee OA correlates with loss of muscle to some degree as observed in previous studies (98,236) and osteoarthritis has also been shown to correlate with increased inflammatory biomarkers in serum, these biomarkers include IL-6 and visfatin (229,237).

**Conclusion**

These commercial pigs do not exhibit early changes in body composition that would be considered typical during the initial ageing process in humans. However, early biological changes involved in the development of musculoskeletal ageing characteristics and the associated body composition changes could be evident and this is the basis of subsequent chapters. The lack of age-related body composition changes could reflect the relatively young age of the pigs being examined as part of this study, however, these age groups were chosen to give a snapshot of the body composition and condition of pigs across the commercial lifespan with the eldest group being breeding sows. If older pigs were required it is unlikely that these pigs would be available from commercial suppliers so they may have to be aged in a university setting, replicating as closely as possible the conditions found in the commercial meat production industry.

# 4.0 ASSESSMENT OF AGE-RELATED CHANGES IN PORCINE MUSCLE

## 4.1 INTRODUCTION

Sarcopenia, discussed further in Section 1.1.5, negatively impacts the quality of life and independence in the elderly (29,30). Primary symptoms of this condition include the loss of muscle mass and strength and the accumulation of intramuscular fat with advancing age (31,32,37). Research to better understand musculoskeletal ageing has commonly used rodent models. However, these rodent models of musculoskeletal ageing have vastly different ageing profiles when compared to humans. For example, the basal metabolic rate per gram body weight is 7x greater in mice than in humans (59). Rodent models have short life spans lacking the long slow period of decline that is typical of human ageing (54) and do not spontaneously develop many of the age-related conditions, such as osteoarthritis, that are common in the elderly, with only a few exceptions. The response of rodent models to immobilisation or inactivity, mimicking the effect of hospitalisation in elderly humans, differs significantly from that of humans with the rapid muscle atrophy in rodents being a result of enhanced proteolysis rather than suppressed muscle protein synthesis as is characteristic of the slow loss of muscle in humans (59).

### 4.1.1 AIMS

Main aim of the chapter:

- To investigate changes to the pathology of skeletal muscle in the pig during ageing, in order to establish if common sarcopenic and age-related changes typical of human ageing occur.

- To determine if porcine muscle shows early changes typical of human ageing within the age range available from the commercial meat production industry; accomplished by investigating changes in muscle fibre type composition, fibre cross sectional area and intramuscular lipid accumulation.

4.2 METHODS

### 4.2.1 Animals

Details of the animals used in this study can be found in Section 2.1.

## 4.2.2 Histological methods

A number of histological methods were employed to investigate structural changes in porcine muscle across a range of ages up to 4 years old. This section details the samples preparation and the staining protocols used.

#### 4.2.2.1 Muscle sample collection and processing for histological analysis

Muscle samples were obtained from 3 lower leg muscles at slaughter: namely the *tibialis anterior, soleus* and *gastrocnemius*. Muscle samples were frozen in liquid nitrogen-cooled isopentane and stored at -80°C until processing.

Samples were processed using a refrigerated Bright Instruments Ltd Cryostat Microtome at a temperature of -23°C. Muscle samples were mounted onto blocks using OCT (Optimal Cutting Temperature) compound and orientated so that sections were taken perpendicular to the muscle fibres. Sections were cut at a thickness of 8μm and transferred onto slides for staining. Muscle sections were stained using 2 methods: ATPase staining for fibre type analysis and Oil Red O staining for lipid content analysis.

Imaging was carried out using a Zeiss Axioskop HBO50 microscope, QIClick camera and Q-capture version 7 software. Further analysis of images was carried out using Image J software as detailed in the Chapter 4.

#### 4.2.2.2 ATPase staining of muscle cryosections

To determine fibre type and fibre cross sectional area cryosections were stained using ATPase staining as follows.

Slides were exposed to an alkaline stock consisting of 0.282% glycine, 0.3% calcium chloride, 0.225% sodium chloride, 0.14% sodium hydroxide in distilled water. To this stock 1.6mg/ml ATP was added and the resulting substrate was adjusted to pH 9.4 at a temperature of 37°C. Slides were exposed to this substrate at 37°C for 10 mins before undergoing two 3 min exposures to 1% calcium chloride at room temperature, a 2 min exposure to 2% cobalt chloride after which the slides were washed well with distilled water prior to a final exposure of 1 min in 1% ammonium sulphide. The ATP consumption of myosin ATPase catalyses the production of cobalt sulphide, a black substance in this staining protocol. The alkaline incubation inhibits myosin ATPase in slow-twitch type I fibres only, therefore less cobalt sulphide is produced by the type I fibres leading to pale staining of type I fibres and darker staining of type II fibres (238).

Cross-sectional area of muscle fibres was determined in ATPase stained muscle cryosections using image J software. Whereby a number of type I and type II fibres were selected using the freehand selections tool and the area of that fibre measured using the inbuilt Image J analysis options.

For both fibre type composition and cross-sectional area determination a minimum of 3 fibres per fibre type per image, selected randomly, were measured and a minimum of 3 different muscle images were used for each pig in each age group, excluding any sections exhibiting freezing artifacts. Fibre type composition and ratios were established by counting differently stained muscle fibres in a $1mm^2$ area of muscle cryosections.

#### 4.2.2.3 Oil Red O staining of muscle cryosections

Oil Red O is a lipid soluble stain that allows the visualisation of lipid in frozen sections of tissue such as skeletal muscle. A stock solution of 0.5% Oil Red O in isopropanol was diluted to a working concentration with water in a 3:2 ratio of stock to water and incubated for an hour at room temperature before filtering through filter paper. Muscle cryosections were fixed for 1 hr in 3.7% formaldehyde, washed thoroughly in distilled water and stained for 30 mins in the working solution of Oil Red O, washed again and mounted using omnimount mounting media (National Diagnostics) (239). Images were taken using Q-Capture software and further analysis carried out using Image J threshold analysis tools.

The percentage of total area positively stained with Oil Red O was calculated using the threshold function in the image J software whereby the amount of red staining over a given threshold is calculated. This threshold was kept constant for all images.

### 4.2.3 Statistical analysis

Fibre type ratio was analysed using one-way ANOVA followed by Tukey's multiple comparison post-hoc test and Oil red O staining results were analysed using two-way ANOVA with muscle type and age as variables. Significance was accepted at the $P < 0.05$ level. All statistics were produced using Graphpad Prism 7 software.

### 4.3.1 Muscle histology - ATPase staining

ATPase staining was carried out on muscle cryosections from *tibialis anterior*, *gastrocnemius* and *soleus* muscles from a number of age groups of pigs. This staining allowed the clear visualisation of individual muscle fibres and identification of type I and II fibres, but this method is not sensitive enough to determine which class of type II fibres are present in these muscles. Figures 4.1-4.3 show representative images from all age groups and muscles investigated.

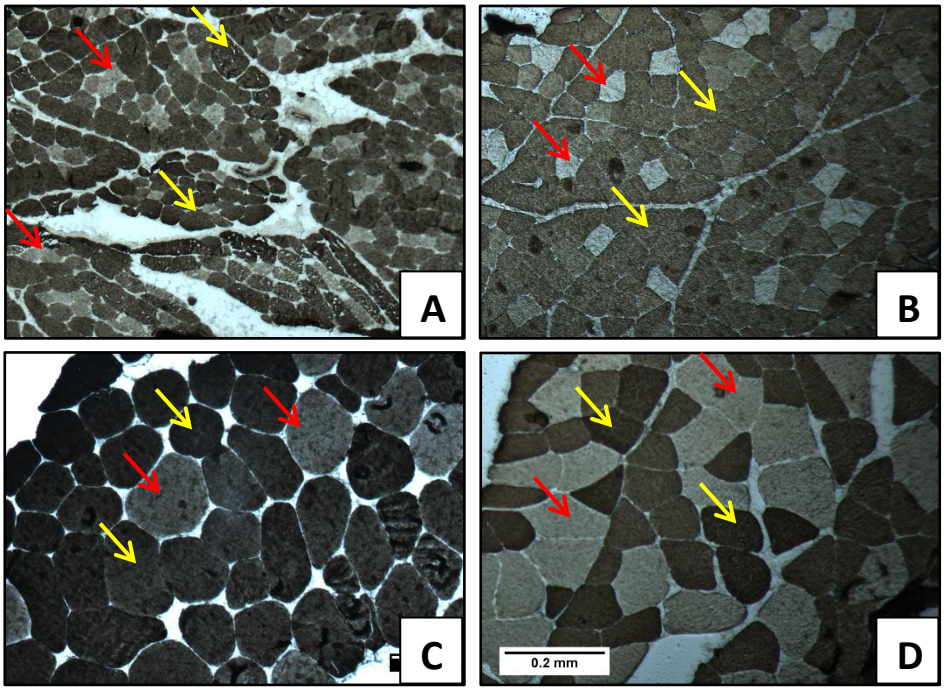


**Figure 4.1: Representative light microscope images of ATPase staining of *tibialis anterior* muscle.** 10x magnification. A: 8wk, B: 17wk, C: 80wk & D: 3yr. Pale staining is indicative of type I fibres (examples are highlighted using red arrows) and darker staining of type II fibres (examples are highlighted using yellow arrows).

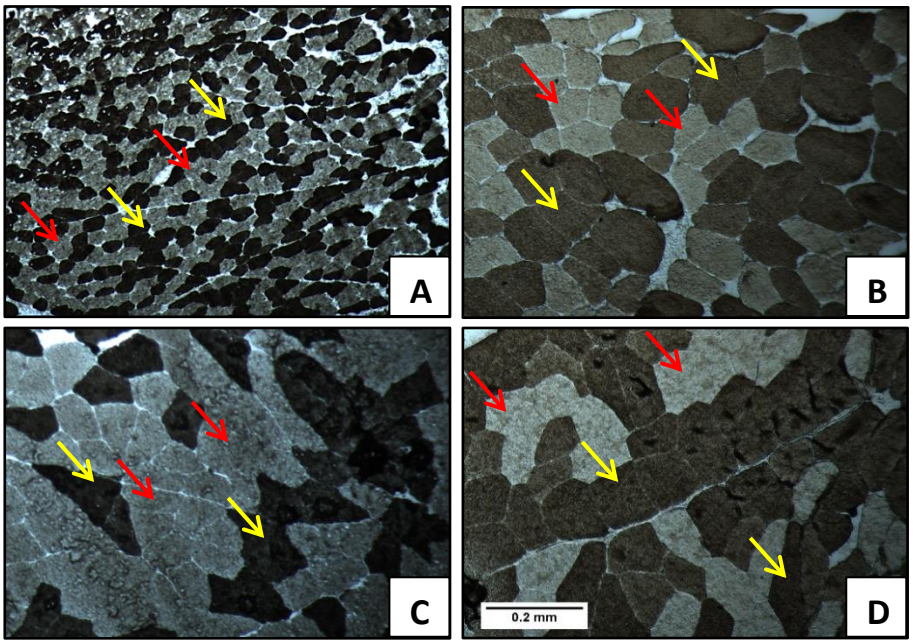


**Figure 4.2: Representative light microscope images of ATPase staining of *gastrocnemius* Muscle.** 10x magnification. A: 8wk, B: 17wk, C: 80wk & D: 3yr. Pale staining is indicative of type I fibres (examples are highlighted using red arrows) and darker staining of type II fibres (examples are highlighted using yellow arrows).

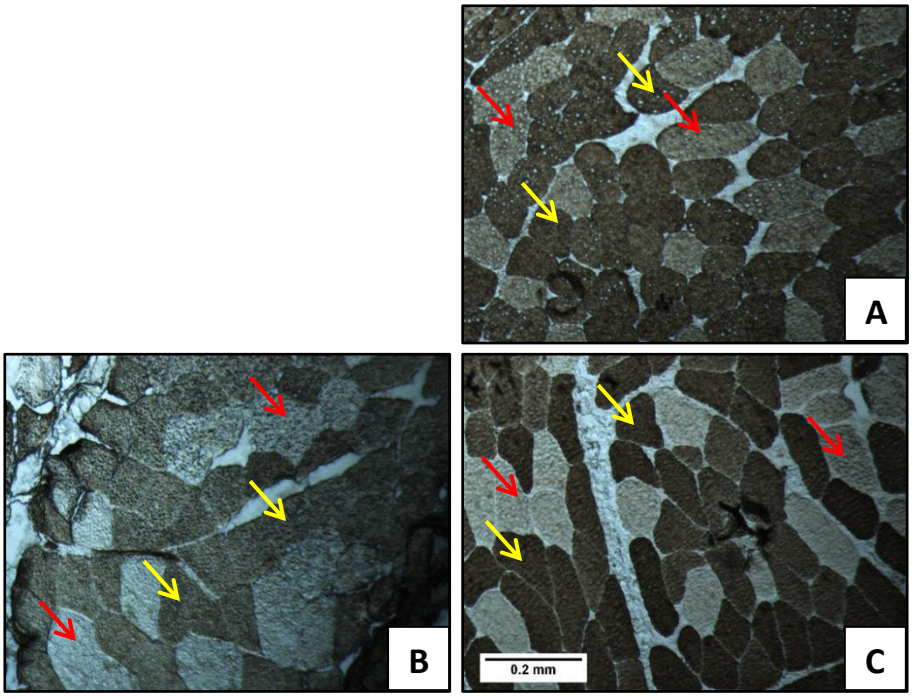


**Figure 4.3: Representative light microscope images of ATPase staining of *soleus* muscle.** 10x magnification. A: 17wk, B: 80wk & C: 3yr. *Soleus* muscle from 8 wk old pigs was not available for investigation. Pale staining is indicative of type I fibres (examples are highlighted using red arrows) and darker staining of type II fibres (examples are highlighted using yellow arrows).

### 4.3.2 Fibre type determination in *tibialis anterior, gastrocnemius and soleus* muscles

ATPase staining allowed the determination of fibre type in muscle cryosections. There was no significant difference in the muscle fibre composition of the *tibialis anterior* muscle across the age groups examined. There was no significant difference in muscle fibre composition in the *gastrocnemius* muscle across the age groups examined. However, there was some degree of variation, most likely resulting from known fibre type variation across the *gastrocnemius* muscle and difficulties in identifying the same area of this large muscle when dissecting the rear legs of pigs of such different sizes. The proportion of type I and type II muscle fibres in the *gastrocnemius* muscle did not show any pattern of change across the age range and was quite variable across the groups of pigs.

There was no significant change in muscle fibre composition across the age groups examined. 8 wk *soleus* samples were not available for examination: at the time of sample collection for this age group of pigs the *soleus* had not yet been selected as a muscle of interest.

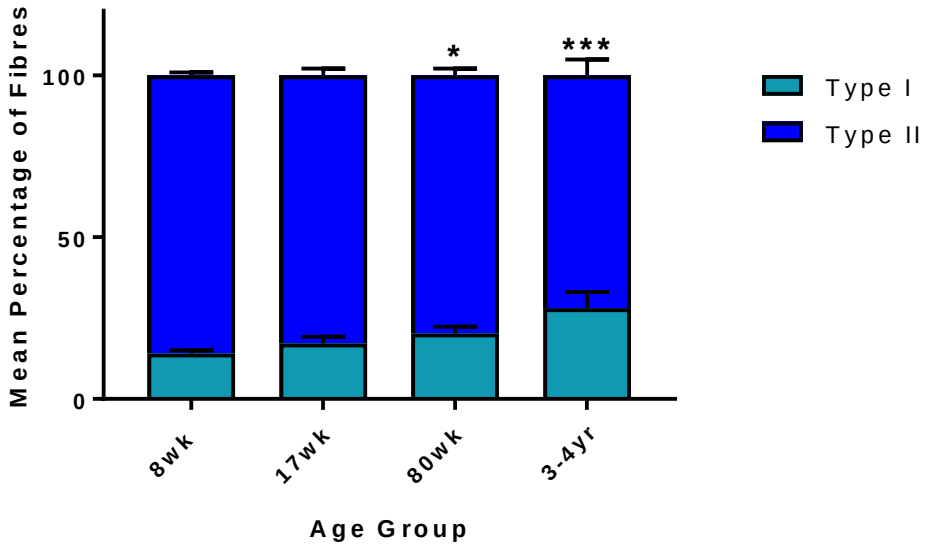


**Figure 4.4: Percentage type I & II fibres in *tibialis anterior* muscle.** Values represent mean ± SEM. Significant effect of age on percentage of type I fibres (p=0.0018) determined by one way ANOVA. Dunnett's multiple comparisons revealed significantly higher percentage type I fibres between age groups *p=0.0360, ***p=0.0008 when compared to the 8 wk age group.

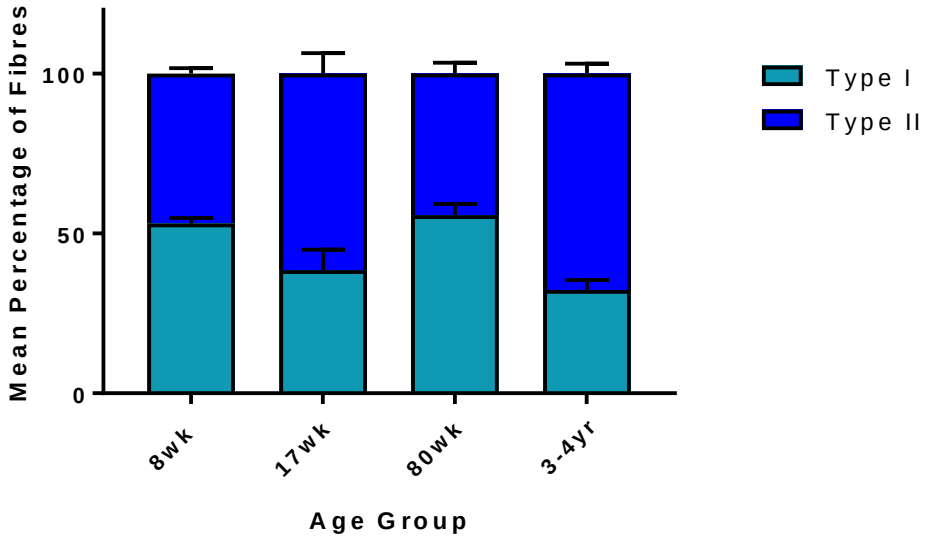


**Figure 4.5: Percentage type I & II fibres in *gastrocnemius* muscle.** Values represent mean ± SEM. Significant effect of age on percentage of type I fibres (p=0.0009) determined by one way ANOVA. Tukey's multiple comparisons revealed significantly higher percentage type I fibres between 8 wk and 3-4 yr age groups (*p=0.0145) and between 80wk and 3-4 yr age groups (**p=0.0016).

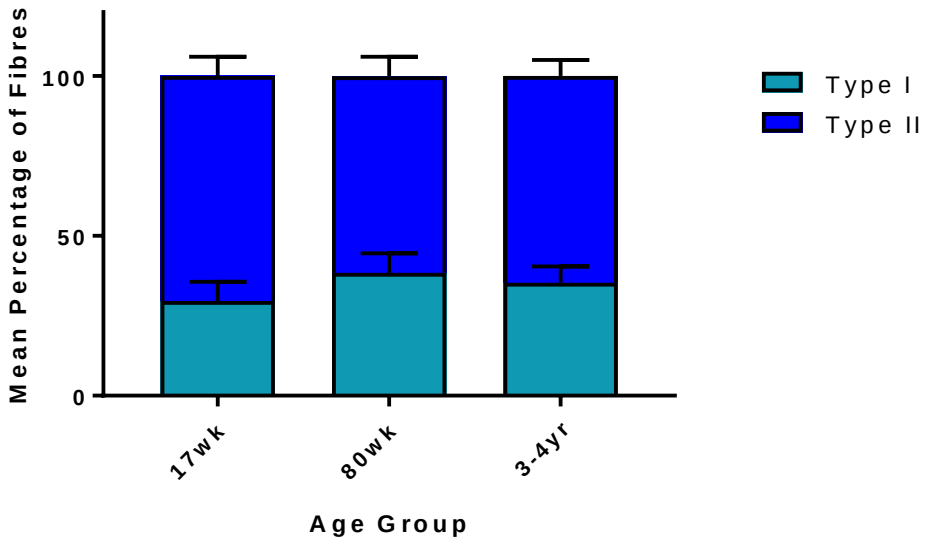


**Figure 4.6: Percentage type I & II fibres in *soleus* muscle.** Values represent mean ± SEM. No significant effect of age on percentage of type I fibres (p=0.5672) determined by one way ANOVA.

### 4.3.3 Fibre type ratios of *tibialis anterior, gastrocnemius* and *soleus* muscles

Investigation of the ratio of type I: II fibres was used to establish if there was any pattern of change in fibre type composition of the muscles across the age range. The data for *tibialis anterior*, shown in Figure 4.7A, revealed that there was an significant effect of age on the ratio of type I: II fibres (p=0.0011) and that there was a significant linear increase in the ratio of type I: II fibres (p=0.0001). There was no effect of age on this measure for the *gastrocnemius* (Figure 4.7B; p=0.0536) or *soleus* (Figure 4.7C; p=0.6798).

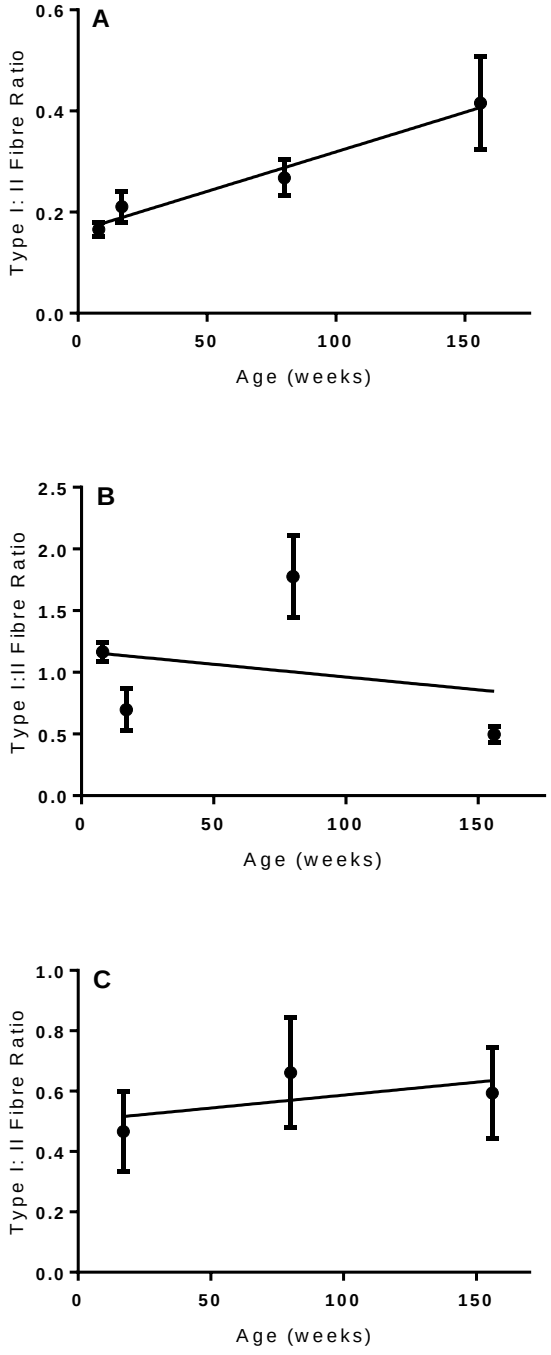


**Figure 4.7: Type I: II fibre ratios.** Values represent mean ± SEM. **A:** *Tibialis Anterior*. Significant effect of age on type I: II ratio (p=0.0011) determined by one way ANOVA. *** p=0.0001 significant linear trend towards increasing type I: II fibre ratio as determined by post-test for linear trend, linear regression line, Y = 0.001566*X + 0.1627, $r^2$=0.9712. (n≥5 for each age group)**B:** *Gastrocnemius.* No significant difference in type I: II fibre ratio as determined by one way ANOVA, p= 0.0536. Y = -0.002071*X + 1.169, $r^2$= 0.06216. (n≥5 for each age group) **C:** *Soleus.* No significant difference in type I: II fibre ratio as determined by one way ANOVA, p= 0.6798. Y = 0.0008555*X + 0.5014, $r^2$= 0.3609. (n≥3 for each age group)

### 4.3.4 Cross-sectional area of *tibialis anterior, gastrocnemius* and *soleus* muscle fibres

Cross sectional area (CSA) of muscle fibres is reduced in individuals suffering from sarcopenia, an age-dependent deterioration in muscle mass and function. This study investigated porcine muscle CSA in order to determine if the age groups of pigs being investigated showed early changes synonymous with these ageing events.

Significant increases in the CSA of type I fibres of the *tibialis anterior* muscle were observed between 8 and 17 wk groups ($p=0.0382$), 17 and 80 wk groups ($p=0.0018$), and 80 wk and 3-4 yr groups ($p=0.0003$). Likewise significant increase in the CSA of type II fibres of the *tibialis anterior* muscle were observed between 8 and 17 wk groups ($p=0.0032$), 17 and 80 wk groups ($p<0.0001$), and 80 wk and 3-4 yr groups ($p=0.0005$). Therefore, for the *tibialis anterior* muscle there was a significant effect of age on the CSA of both fibre types, as shown in Figure 4.8 ($p<0.0001$).

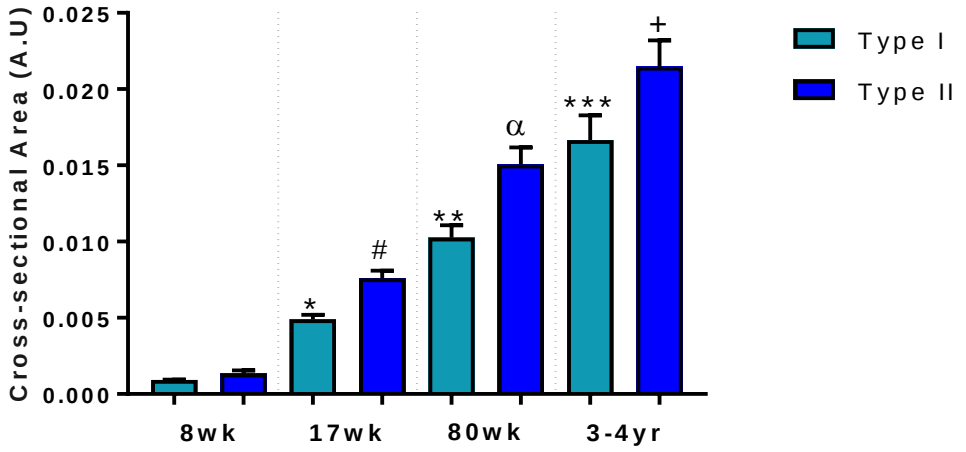


**Figure 4.8: Cross-sectional area of muscle fibres by fibre type across age groups in *tibialis anterior* muscle.** Values represent mean CSA ± SEM in arbitrary units (A.U). Significant effect of age on both type I ($p<0.0001$) and type II ($p<0.0001$) CSA determined by one way ANOVA. Holm-Sidak's multiple comparisons revealed significantly larger CSA when compared to the type I fibre CSA of the next youngest age group (*p=0.0382; **p=0.0018; ***p=0.0003) and significantly larger CSA when compared to the type II fibre CSA of the next youngest age group(#p=0.0032; αp=0.0005; +p<0.0001).

Evaluation of ATPase staining muscle cryosections revealed a significant effect of age on the CSA of both type I and II muscle fibres of the *gastrocnemius* muscle ($P<0.0001$), as shown in Figure 4.9. Significant increases in the CSA of type I fibres of the *gastrocnemius* muscle were observed between 8 and 17 wk groups ($p<0.0001$), 17 and 80 wk groups ($p=0.0069$), and 80 wk and 3-4 yr groups ($p<0.0001$). Likewise significant increase in the CSA of type II fibres of the *gastrocnemius* muscle were observed between 8 and 17 wk groups ($p<0.0001$), and 80 wk and 3-4 yr groups ($p<0.0001$) but not between the 17 and 80 wk groups ($p>0.9$).

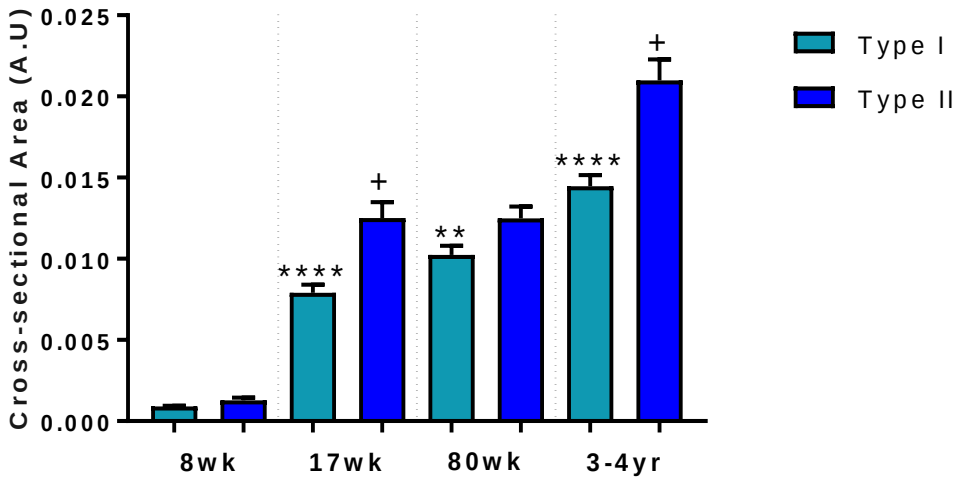


**Figure 4.9: Cross-sectional area of muscle fibres by fibre type across age groups in *gastrocnemius* muscle.** Values represent mean CSA ± SEM in arbitrary units (A.U). Significant effect of age on both type I ($p<0.0001$) and type II ($p<0.0001$) CSA determined by one way ANOVA. Holm-Sidak's multiple comparisons revealed significantly larger CSA when compared to the type I fibre CSA of the next youngest age group (****$p<0.0001$; **$p=0.0069$) and significantly larger CSA when compared to the type II fibre CSA of the next youngest age group (+$p<0.0001$).

A significant effect of age on the CSA of *soleus* type I and II fibres was discovered ($p<0.0001$). Muscle CSA of both type I and type II muscle fibres increased significantly between 80 wk and 3-4 yr age groups ($p<0.0001$) but no significant CSA changes were observed between 17 wks and 80 wks of age in either type I ($p=0.4457$) or type II ($p=0.1258$) fibres, as shown in Figure 4.10.

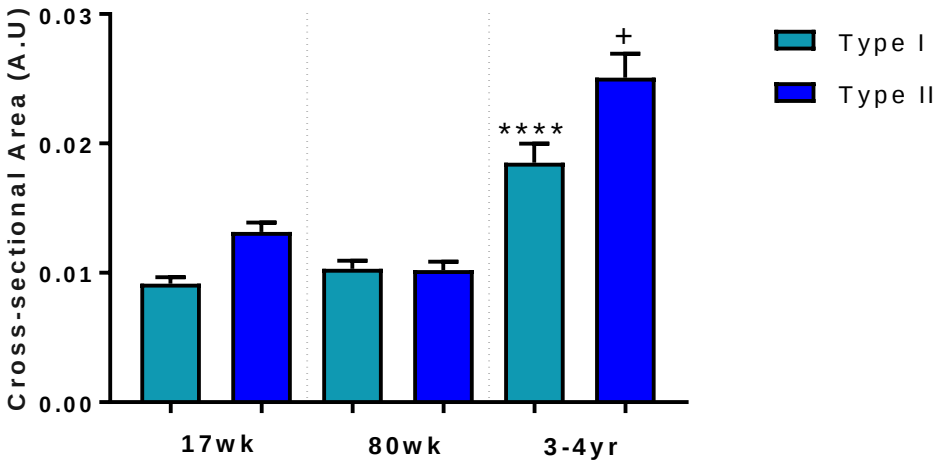


**Figure 4.10: Cross-sectional area of muscle fibres by fibre type across age groups in *soleus* muscle.** Values represent mean CSA ± SEM in arbitrary units (A.U). Significant effect of age on both type I ($p<0.0001$) and type II ($p<0.0001$) CSA determined by one way ANOVA. Holm-Sidak's multiple comparisons revealed significantly larger CSA when compared to the type I fibre CSA of the next youngest age group (****$p<0.0001$) and significantly larger CSA when compared to the type II fibre CSA of the next youngest age group (+$p<0.0001$).

### 4.3.5 Oil red O staining of muscle cryosections

Intramuscular lipid accumulation is a characteristic of human muscle age and occurs alongside loss of muscle mass. Oil red O staining was used to stain lipid within the pig muscle cryosections across three different age groups – 17 wk, 80 wk and 3-4 yr. The results, displayed in Figure 4.11 and 4.12, showed a great deal of variability between animals, and did not show any clear signs of lipid accumulation with age in any of the 3 muscles.

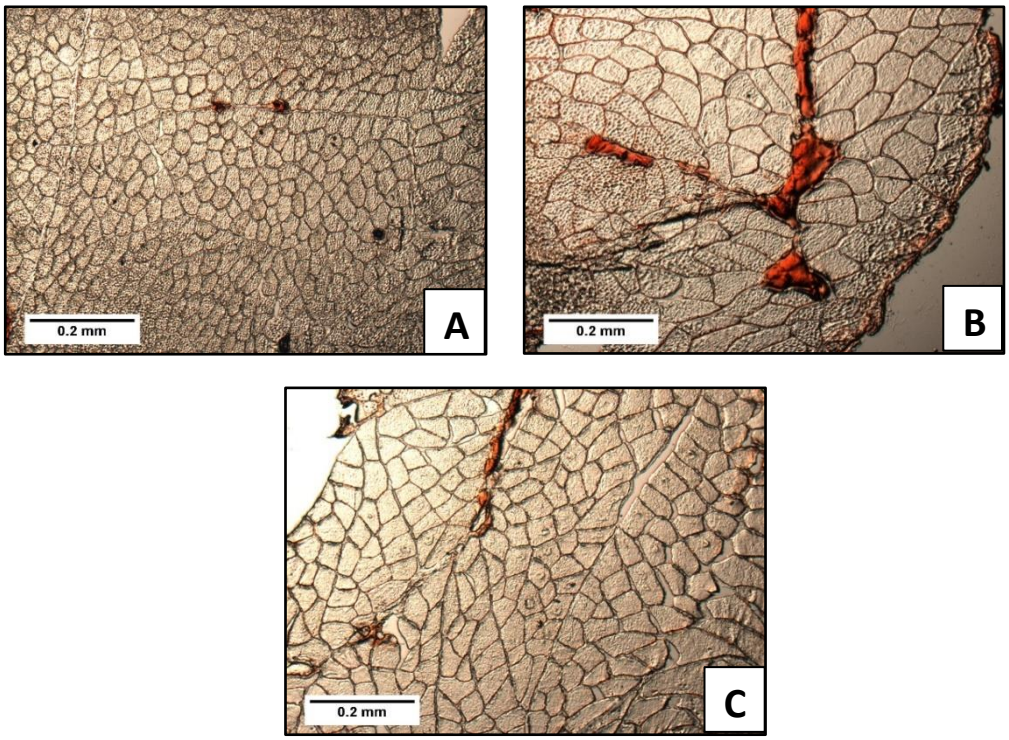


**Figure 4.11: Representative light microscope images of oil red O staining in porcine *soleus* muscle cryosections.** 10x magnification. A: 17wk; B: 80wk; C: 3yr.

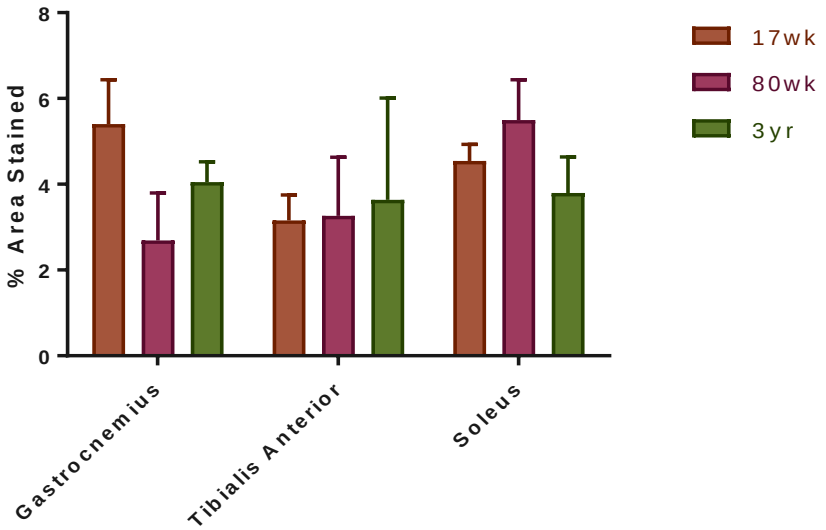


**Figure 4.12: Percentage of muscle cryosections stained positively with oil red O stain indicating presence of lipid within the muscle.** Three different age groups and three different muscles were examined for this purpose. Values represent mean ± SEM. Analysis by two-way ANOVA did not show any significant interaction between age and muscle type. There were no significant differences between muscle types or age groups.

## 4.4 DISCUSSION

This study investigated whether the commercial pig represents an appropriate model of early age-related musculoskeletal change. In order to determine the utility of the pig as a model of musculoskeletal ageing, histological methods were used to examine skeletal muscle for evidence of early changes associated with the age-related deterioration.

### Muscle fibres

Investigation of the fibre type composition and cross sectional areas of muscle fibres in 3 lower leg muscles across a selection of age groups of pigs was carried out utilising ATPase staining methods to identify type I and II fibres in muscle cryosections. The muscles selected for study were the *tibialis anterior*, *gastrocnemius* and *soleus* muscles. These lower leg muscles were identified as being of importance to lower leg strength and stability in humans, therefore maintaining strength in these muscles is essential in preventing or reducing the risk of trips and falls in the elderly (98,236).

Of these three muscles the tibialis anterior was the only muscle to show a linear relationship between increasing age and increasing type I: II muscle fibre ratios. This is likely a feature of muscle maturation as there is no deviation from this pattern in the oldest group examined (3-4 years). In contrast, the *gastrocnemius* and *soleus* muscles did not show any significant patterns in fibre type ratios.

This study has revealed that the percentage of fibres that are type I in the *tibialis anterior* muscle of the pig ranges from 8-25% at 8 wks of age, and from 11-41% in the 3-4 yr age group. The 3-4 yr group achieved a mean type I composition of 28% compared to the previously reported 39.8% type I fibres in human *tibialis anterior* muscle (240). The composition of this muscle in rats and rabbits is

slightly different; the *tibialis anterior* in these animals is predominantly fast twitch and has a lower type I content, reported to be <5% in rats (241) and approximately 4% in rabbits (242). The fibre type composition results for the *gastrocnemius* muscle were somewhat variable between age groups with a range of 32-56% type I fibres and an overall average of 45% type I fibres for these pigs; compared to the profile of human *gastrocnemius* muscle of 57% type I, reported by Gollnick et al and 48% type I, reported by Yang et al (240,243,244). *Soleus* muscle samples from the 8 wk group were not available for investigation in this study and the fibre profile observed in porcine *soleus* was predominantly type II with between 30-38% of fibres being type I contrasting with the predominantly type I muscle fibres found in human *soleus* muscle, reported to be between 62-80% type I (240,243,244). This could be a result of the selective breeding of commercial pigs to increase lean meat production (245).

Muscle fibre CSA is decreased in sarcopenia, however this is a condition that effects the elderly. This study investigated the CSA of muscle fibres in the lower leg muscles of pigs to determine if there were any indications that sarcopenic changes were beginning to occur within the age range. The CSA of *tibialis anterior* muscle fibres increased significantly with age for both fibre types. This suggests that the *tibialis anterior* is a continuously adapting muscle and that this muscle is exhibiting no signs to indicate a slowing of growth or reduction in fibre size that could be associated with early signs of musculoskeletal decline. In the *Gastrocnemius* and *soleus* muscles, there was also a significant effect of age on CSA of muscle fibres despite an apparent pause in growth in *Gastrocnemius* type II fibres and *soleus* type I and II fibres between the 17 week and 80 week groups.

As mentioned in Section 1.1.2, original research suggested that there may be a loss of type II fibres in ageing human muscle (10,11) but these results have been difficult to replicate (12,13). More recent research has suggested that, rather than a reduction in the number of type II fibres, both type I and II fibres are lost and there is a greater loss of CSA in type II fibres (14). None of the porcine muscles examined exhibited a reduction in CSA of muscle fibres, which suggests that these animals are not undergoing muscle decline within the age range investigated in this study. Only the *tibialis anterior* muscle exhibited a change in the proportion of fibre types with a reduction in the percentage of fibres that are type II fibre. However, this change was consistent across the age range and is therefore unlikely to be an indicator of age-related muscular decline. Therefore, these results are consistent with growth and maturation of the pigs and do not reflect detrimental age-related muscle changes as would be expected to occur early in ageing human muscle.

Together, this information suggests that the composition of the *tibialis anterior* and *gastrocnemius* in the pig is similar to that of humans but that the soleus muscle appears to be predominantly fast twitch in these commercial pigs, rather than the slow twitch expected in human *soleus* muscle. This study has found no evidence to suggest that commercial pigs under 4 years of age have entered a period of musculoskeletal decline and the changes that occur with age, detailed in this chapter, are a consequence of growth and maturation rather than ageing.

**Intramuscular lipid accumulation**

During ageing humans accumulate both abdominal and intramuscular fat; this is thought to contribute to the loss of functional capacity in the muscle and can increase systemic inflammation in the muscle (246,247). In this study, oil red O

staining was utilised to investigate intramuscular lipid accumulation in muscle cryosections. The large white x landrace x duroc cross pigs have been selectively bred to develop lean muscle tissue; this has resulted in reduced levels of intramuscular fat deposition (245). These pigs do not exhibit increased fat accumulation within the muscle with advancing age across the age range examined in this study. This could be explained by a combination of factors. One such factor is genetics; these pigs may not develop this characteristic of muscle ageing, possibly due to the drive for increased lean meat production in pigs. Another factor is age: the pigs used in this study may be too young for this type of age-related change in lipid deposition to occur.

### 4.4.1 Overall Conclusions

This study has not found any indications of early muscle deterioration within the age range examined, however, this work cannot rule out the possibility that these changes occur at a later age in commercial pigs.

# 5.0 THE PIG AS A MODEL OF SPONTANEOUS OSTEOARTHRITIS (OA)

## 5.1 INTRODUCTION

Osteoarthritis (OA) is a musculoskeletal joint disorder characterised by degenerative loss of the articular cartilage, joint space narrowing and bone remodelling. It is a leading cause of pain and disability and there are currently no pharmacological therapeutics that can slow or reverse disease progression (248). In attempting to better understand the pathways and processes that mediate cartilage degeneration rodent models have been utilised. However, OA in these models is typically induced either chemically or surgically and poorly mimics the pathological features of human OA (see Section 1.5). Commercial pigs develop spontaneous joint pathologies with a number of pigs being slaughtered early due to lameness (188). Therefore, the commercial pig was investigated as a possible model of early spontaneously occurring osteoarthritis.

### AIMS

The main aims of this chapter are:

- Establish the occurrence of osteoarthritis in the pig by analysing joint condition of pigs across the age range investigated.
- Determine if poor joint condition and observational joint scoring are linked to cartilage loss as is expected in osteoarthritic joints.
- Determine if there is a relationship between joint chondropathy and circulating concentrations of the OA-linked inflammatory markers, IL-6 and visfatin.

- Investigate the utility of behavioural assessment as a measure of lameness and determine if this is feasible for use in the pig.

### 5.2.1 Behavioural assessment of pigs

A 45 week old group were monitored for a period of 35 weeks prior to slaughter in order to observe the pigs for indications of the onset of lameness and the development of discomfort as they approached adult weight. During this period, weight gain was monitored weekly and pain behaviour was assessed using an adapted Visual Analogue Scale (VAS) scoring system, based on the scale used by Royal et al (249). Work by Royal et al (249) used VAS scoring to investigate post-operative pain in a group of pigs with experimentally induced femoral fracture. Similar behaviours have also been used to score chronic pain as a result of OA in dogs (250). Therefore, the behaviours detailed in these papers were considered to be appropriate to determine discomfort-related behavioural change, potentially relating to spontaneous OA, in the pigs used in this study.

Five aspects of pig behaviour were selected as potentially useful indicators of joint discomfort. The scoring system was used to assign scores to each pig in this group and was subsequently used to determine lameness in 6 wk and 3-4 yr age groups of pigs. Behavioural assessment of pigs was carried out weekly, assessment sessions typically lasted about an hour. On arrival at the facility, pigs were allowed to acclimatise to the presence of the scorer before behavioural scoring was carried out. Observation of lameness and response to touch were carried out during weighing of the animals, this allowed individual pigs' ability and willingness to move to be scored. All other scoring was carried out prior to the arrival of the animal technicians to help weigh the animals.

Pigs were observed at rest and whilst active within the pig house, the descriptions in Table 5.1 were used to score each pig. Upon entry of the scorer(s), pigs were given 5-10 minutes to settle before scoring was carried out. Scoring sessions typically lasted roughly 45mins and were immediately followed by weighing of the pigs. All scoring was carried out by one scorer with a second scorer brought in occasionally to ensure similar scores were obtained independently. Following a 4 week period of analysis each behavioural measure was assessed and any that were found to be inappropriate for use in this study were discarded. See results for further details. The modified VAS scoring used in this study was based of that used by Royal et al (249).

**Table 5.1: Observational scoring system**

| Score Assigned | Lameness | Observation before handling | Response to touch | Ability or willingness to ambulate | Vocalisation |
|---|---|---|---|---|---|
| **1** | No evidence of lameness | Resting, calm, asleep | Minimal to no response | Walks voluntarily | None or grunting/squealing when interacting with other pigs |
| **2** | Lameness barely detectable | Active but no noticeable tension or discomfort | Moderate movement | Walks with encouragement | Squealing when moved or touched |
| **3** | Lameness easily detectable, noticeable head nod | Somewhat restless, mild agitation. | Shifting, wariness, possibly some vocalisation | Reluctant to walk but no problems with doing so | Squealing when getting up or walking |
| **4** | Severe lameness, characterised by marked head nod but affected limb is still weight bearing | Shifting, anxious, moderate agitation | Strong response, vocalisation, avoidance behaviour, flinching | Difficulty in standing up but can do so without assistance | Squealing while undisturbed or at rest |
| **5** | Severe lameness, characterised by non-weight bearing on affected limb | Severe agitation. Anxious, tense, constant shifting | | Will not or cannot walk without assistance | |

### 5.2.2 Chondropathy scoring of knee joint

Images of the femoral condyles and tibial plateaus of pig knee joints were used for chondropathy scoring. Both sections of the knee were divided into medial and lateral sides and the percentage area assigned to each cartilage condition grade, from 0 to IV (see Table 5.2), determined. These percentage determinants were used to independently score each of the 4 joint areas using either Collin's grading, which has been used in the pig previously (251), or revised Système Française D'Arthroscopie (SFA) methods, which is a more informative measure, typically used in humans (see below for details). The overall chondropathy score for each joint was a sum of the scores obtained for each of the 4 joint areas. Scores

obtained from the commercial pigs were compared to photographs of human joints obtained following knee replacement surgery, provided by the University of Birmingham.

**Table 5.2: Grading of cartilage based on observed condition**

| Grade | Condition of articular cartilage surface |
|---|---|
| **0** | Normal, unbroken surface |
| **I** | Swelling/softening of cartilage |
| **II** | Superficial fibrillation |
| **III** | Deep fibrillation |
| **IV** | Subchondral bone exposure |

#### 5.2.1.1 Collin's grading

Collin's grading has been used in the pig previously and utilised the following flow chart to determine the score of each joint section (252). The maximum overall joint score for this chondropathy scoring is 16. Due to the use of this flow chart method for scoring there can be considerable variation in joints obtaining the same Collin's grade score therefore an additional scoring method was used.

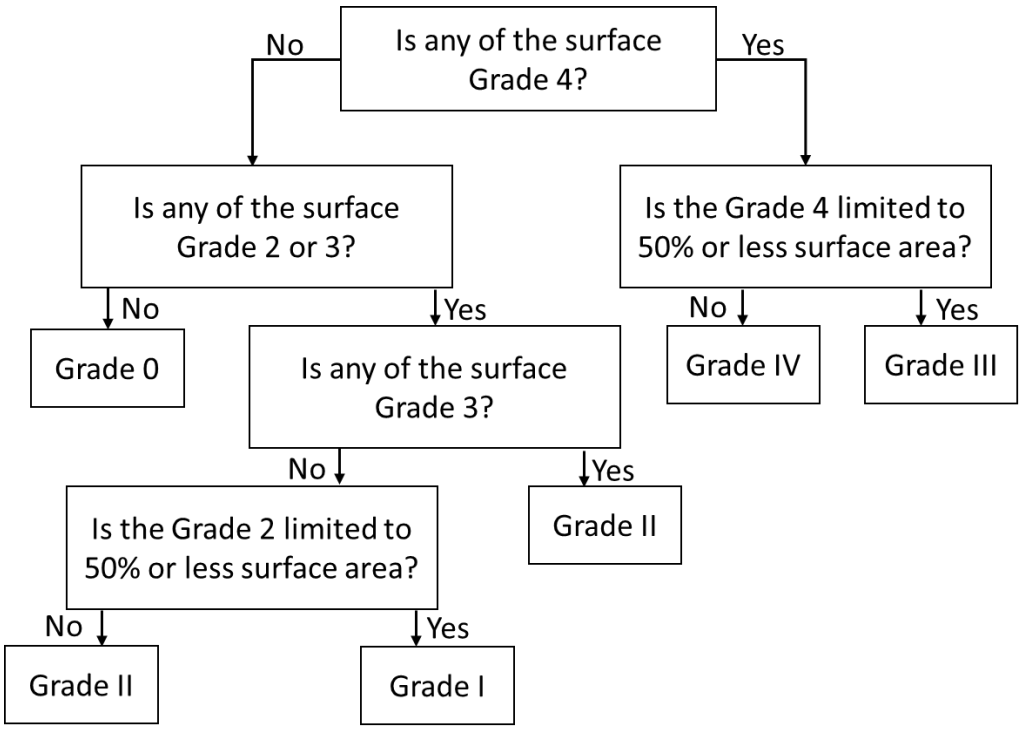


**Figure 5.1: Collin's grading flow chart.** Adapted from Walsh et al (2009) (253)

#### 5.2.1.2 Revised Système Française D'Arthroscopie (SFA)

Revised SFA uses the percentage estimates and the following equation to obtain a value for each joint section out of 100. The maximum total joint score is therefore out of 400 (253). This method allows a more detailed comparison between joints obtaining the same collin's grade and is therefore more informative.

Score = (Grade I x 0.14) + (Grade II x 0.34) + (Grade III x 0.65) + (Grade IV x 1)

### 5.2.3 Safranin-O staining of femoral condyle cryosections

8μm cryosections of these samples were cut and transferred to slides to facilitate subsequent safranin-O staining. Safranin-O is a cationic dye that stains proteoglycans and glycosaminoglycans (GAGs) in cartilage sections. Fixation

techniques can result in the loss of proteoglycan from samples, therefore this protocol was performed on cryosections without a fixation step (254).

Cryosections of femoral condyles were transferred to room temperature slides, and incubated at room temperature for 10mins before staining. Cryosections were stained for 8 mins in haematoxylin, rinsed in tap water and stained for 5 mins in 0.001% fast green, the slides were rinsed in tap water again and stained for 5 mins with 0.1% safranin-O. Images of slides were taken immediately following completion of the staining protocol. Finally, slides were rinsed with tap water and mounted using omnimount mounting media. Imaging was carried out using a Zeiss Axioskop HBO50 microscope, QIClick camera and Q-capture version 7 software.

### 5.2.4 1,9-dimethylmethylene blue (DMMB) proteoglycan release assay

This assay was used to determine the amount of sulphated glycosaminoglycan (GAG) present in the cell culture supernatant as a quantitative measure of cartilage degradation occurring in cartilage explants. A standard curve was run on each plate. For this purpose, 100μg/ml chondroitin stock solution was diluted to a range of concentrations between 5-50μg/ml. 20μl of each standard or sample was added to each well of a 96 well plate and 200μl of DMMB reagent (Appendix A) was added to each well. Absorbance was measured immediately at 550nm using a Bio-Rad 680XR microplate reader.

#### 5.2.4.1 Non-damaged vs Damaged cartilage explants

Visibly non-damaged and damaged cartilage explants were cut from the distal femoral condyle articular cartilage of 7 pigs. They were allowed to rest in

chondrocyte media for 2-3 days. The chondrocyte media (detailed in Section 2.4.3) on the explants was replaced with fresh chondrocyte media and incubated for 48hrs after which time supernatant was collected and frozen for later analysis using the DMMB proteoglycan release assay. This was carried out to confirm that visibly damaged cartilage was undergoing degradation at a faster rate than the visibly healthy cartilage, confirming the utility of the chondropathy scoring methods in the pigs.

Cartilage explants were determined to be damaged if they showed fibrillation – this was equivalent to a Collin's grade score of greater than 2 or a revised SFA score of more than 20. The fold difference in sGAG release between visibly healthy and visibly damaged cartilage explants taken from each pig was calculated.

5.3 RESULTS

### 5.3.1 Behavioural assessment

Specific pain-associated behaviours, selected based on their use in pain assessment in other animals (mainly rodents), were incorporated into a modified Visual Analogue Scale (VAS) scoring system whereby particular behavioural aspects were assigned a score from 1-5. Of the aspects originally identified as useful indicators in identifying pain perception in the pig, only the lameness scoring was selected for continued study following a period of protocol adaption and testing, whereby the practicalities of the protocol was assessed and the protocol adjusted accordingly.

**Observation before handling and Willingness to ambulate**

Neither of these observations showed any pattern, with individual pigs varying between scoring sessions.

**Response to touch**

There was limited handling of these pigs, except during weighing and none of the pigs responded to touch in a manner that would be associated with pain or discomfort.

**Vocalisation**

It became apparent that vocalisation in these pigs was not related to any pain or discomfort as might be expected of other animals, such as the rat for which VAS scoring is more commonly used.

**Lameness**

Observational scoring of lameness indicators revealed no indications of lameness in the youngest age group (6 weeks of age). However, a trend towards increasing lameness with age in the pigs as they approached 80 wks of age was identified. Overall lameness in the oldest age group investigated (3-4 yrs) was slightly lower than that of the 80 wk group but still tended to increase slightly over the 2-3 weeks that these pigs were kept at the University of Nottingham animal facility.

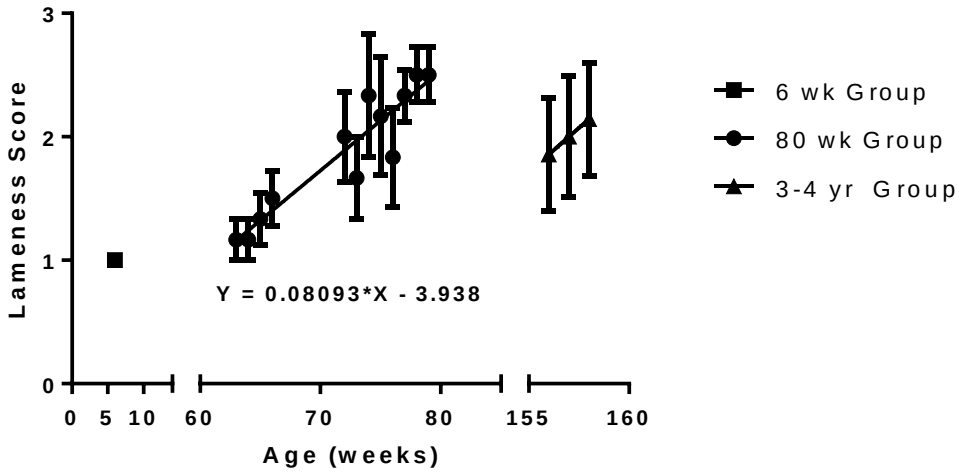


**Figure 5.2: Observational scoring of lameness.** Displayed as mean lameness score ± SEM for each assessment. Linear regression analysis of the 80 wk group revealed that the slope deviated significantly from zero( $p<0.0001$) (6 wk n=6; 80 wk n=6; 3-4 yr n=7)

### 5.3.2 Joint condition

Knee joints obtained from female commercial pigs (n=6 for 17 wk and 40 wk groups; n=8 for 80 wk group; n=7 for 3-4 yr group) and human patients undergoing knee replacement (n=4) were examined and assessed for the presence and severity of osteoarthritic lesions. Photographs of these knee joints

were taken to facilitate joint condition scoring. Figure 5.2 shows representative images of healthy and damaged porcine knee joints.

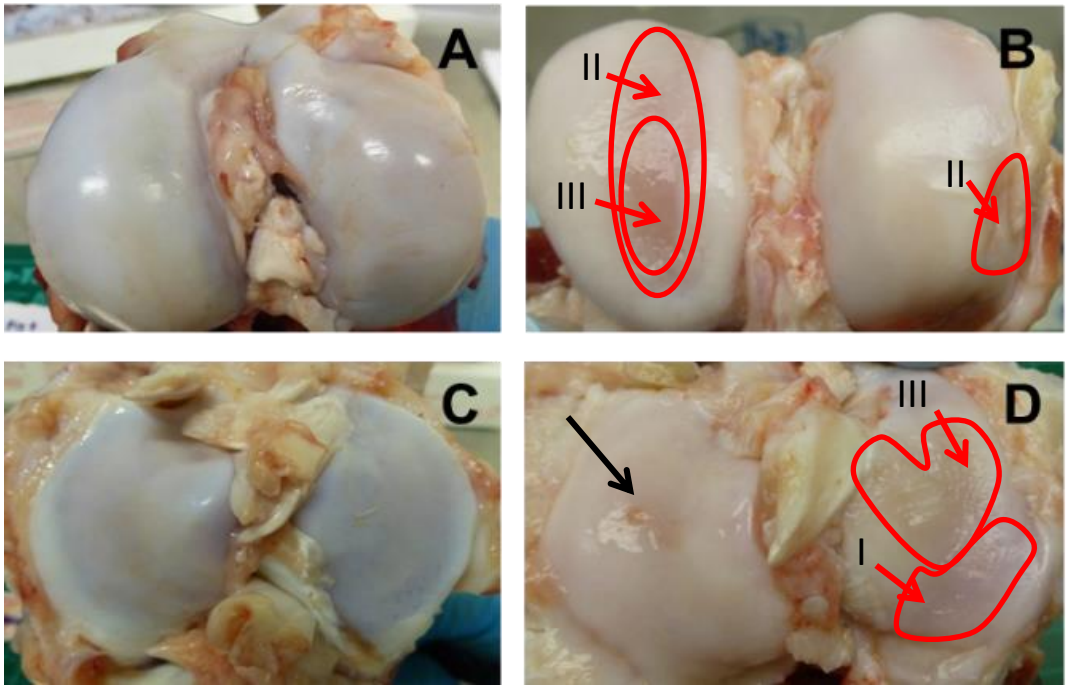


**Figure 5.3: Representative porcine joint images.** A & C: representative images of a healthy knee joint from a 40 wk old pig. B & D: representative images of a damaged joint from a 3-4 yr old pig. A: femoral condyles exhibiting no cartilage lesions. B: femoral condyles exhibiting cartilage lesions of grades II and III (circled in red). C: Tibial plateau exhibiting no cartilage lesions. D: Tibial plateau exhibiting cartilage lesions of grade I and III (circled in red) and a possible cartilage indentation indicative of osteochondrosis (denoted by a black arrow).

Figure 5.4 highlights osteophyte formation in porcine knee joints. This is indicative of bone remodelling, a process seen in osteoarthritic human joints, and was evident in pigs as young as 80 wks old.

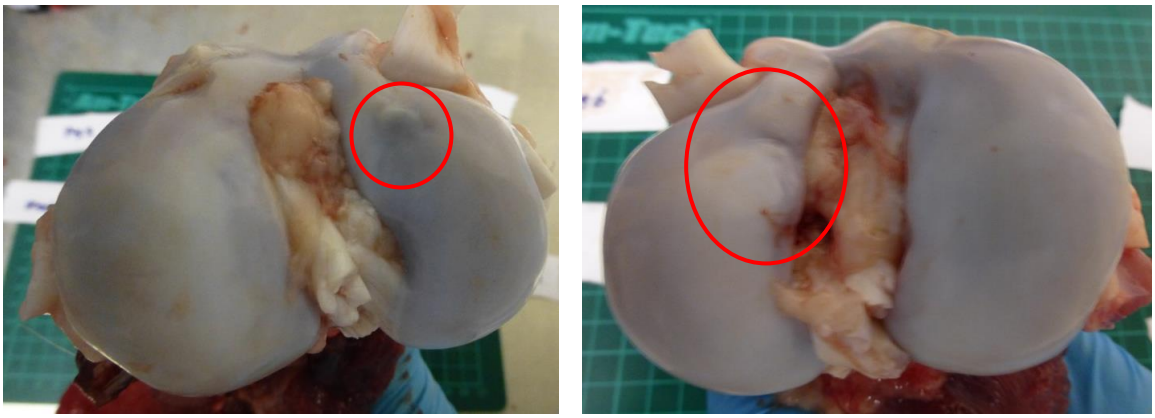

**Figure 5.4: Osteophyte formation in femoral condyles taken from 80 wk old pigs. Red circles highlight osteophytes in porcine femoral condyles from 80 wk old pigs.**

Table 5.3 shows the mean chondropathy scores obtained for each age group and for the human joints that were scored for comparison purposes. This indicated that there was an increase in joint damage with increasing age in these pigs, even at the relatively young ages used in this study; the maximum scores obtained from commercial pigs were lower than the scores obtained from human joints. However, the variation in chondropathy scoring achieved by individual pigs within each age group also increases with age for both Collin's grading (Figure 5.5A) and revised SFA scoring (Figure 5.5B) methods.

**Table 5.3: Chondropathy scoring shown as mean ± SEM.** Collin's grading and Revised SFA methods used.

| Group | Collin's Grading | Revised SFA |
|---|---|---|
| **17 weeks** | 0.7 ± 0.4 | 9.3 ± 1.5 |
| **40 weeks** | 1.8 ± 0.5 | 21.2 ± 2.3 |
| **80 weeks** | 3.9 ± 0.7 | 35.2 ± 5.1 |
| **3-4 years** | 5.3 ± 1.1 | 52.0 ± 10.8 |
| **Human (OA)** | 10.0 ± 1.1 | 106.4 ± 10.8 |

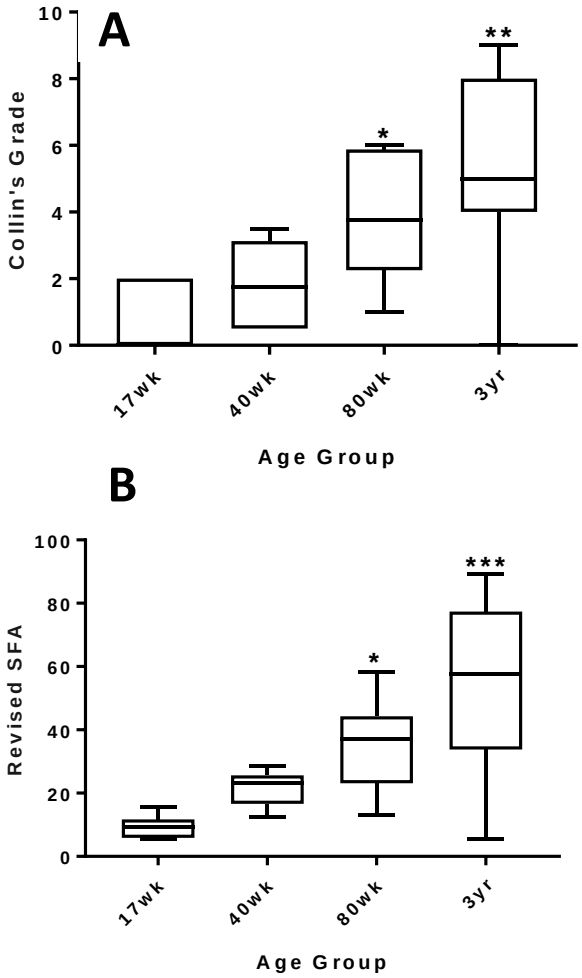


**Figure 5.5: Age group variation in chondropathy scores.** Box plot with whiskers showing the minimum and maximum scores and a median line. **A:** Collin's grading. Significant effect of age on joint chondropathy score determined by one-way ANOVA (p=0.0015). Chondropathy scores were significantly higher in the 80 wk (*p=0.03) and 3-4 yr (**p=0.0018)groups when compared to 17 wk group **B:** Revised SFA. Significant effect of age on joint chondropathy score determined by one-way ANOVA (p=0.0010). Chondropathy scores were significantly higher in the 80 wk (*p=0.0441) and 3-4 yr (**p=0.0008)groups when compared to 17 wk group.

There was a significant relationship (p=0.0074) between the Collin's grading method of chondropathy scoring and cartilage degradation, with higher chondropathy scores being linked with increased sGAG release from explants as shown in Figure 5.6A. There was a significant relationship (p=0.014) between the revised SFA method of chondropathy scoring and cartilage degradation, with

higher revised SFA scores being linked to increased sGAG release from explants as shown in Figure 5.6B.

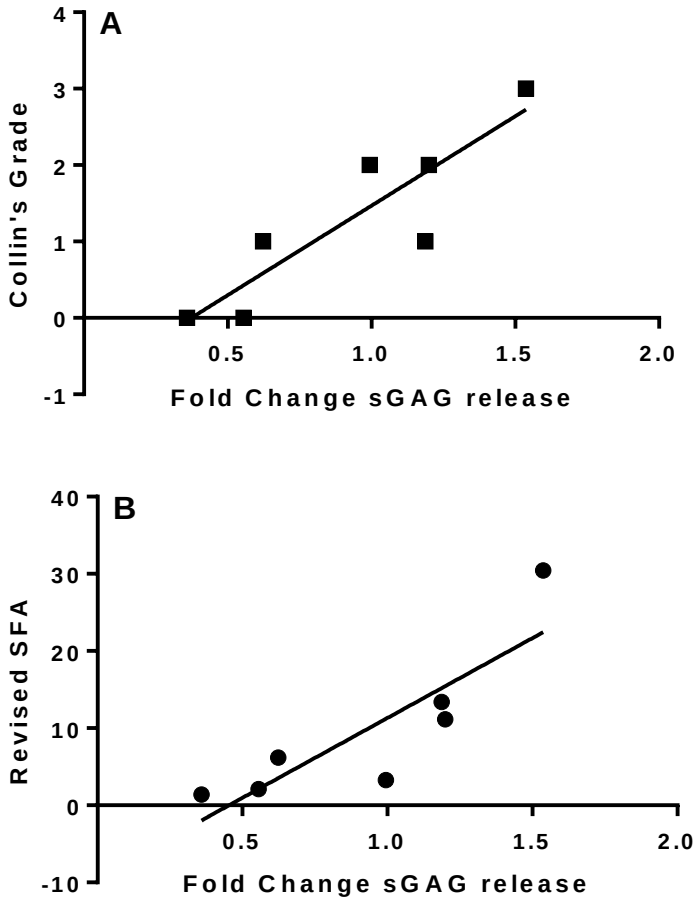


**Figure 5.6: A: Collin's grade chondropathy scoring and cartilage degradation correlation.** Pearsons r correlation coefficient. $r^2$=0.791, Correlation was significant, p=0.0074.**B: Revised SFA chondropathy scoring and cartilage degradation correlation.** Pearsons r correlation coefficient. $r^2$=0.7326, Correlation was significant, p=0.014.

Safranin-O staining was used to visualise proteoglycan and collagen expression in cartilage cryosections taken from the femoral condyles of 80 wk pigs. This staining was carried out in combination with haematoxylin staining, allowing the chondrocytes in the cartilage to be observed. Safranin-O staining of femoral condyles from a non-damaged knee joint with a score of 7.6 using the revised SFA

method showed strong proteoglycan staining, shown in red whereas the same staining carried out in cryosections obtained from a damaged knee joint, with a score of 35.4 using the revised SFA method showed much weaker proteoglycan staining, shown by increased visibility of collagen staining by fast green, which appears blue in these images. This difference in cartilage staining in the more damaged joint is suggestive of a more fibrocartilage-like composition at the articular surface of cartilage (Figure 5.7B).

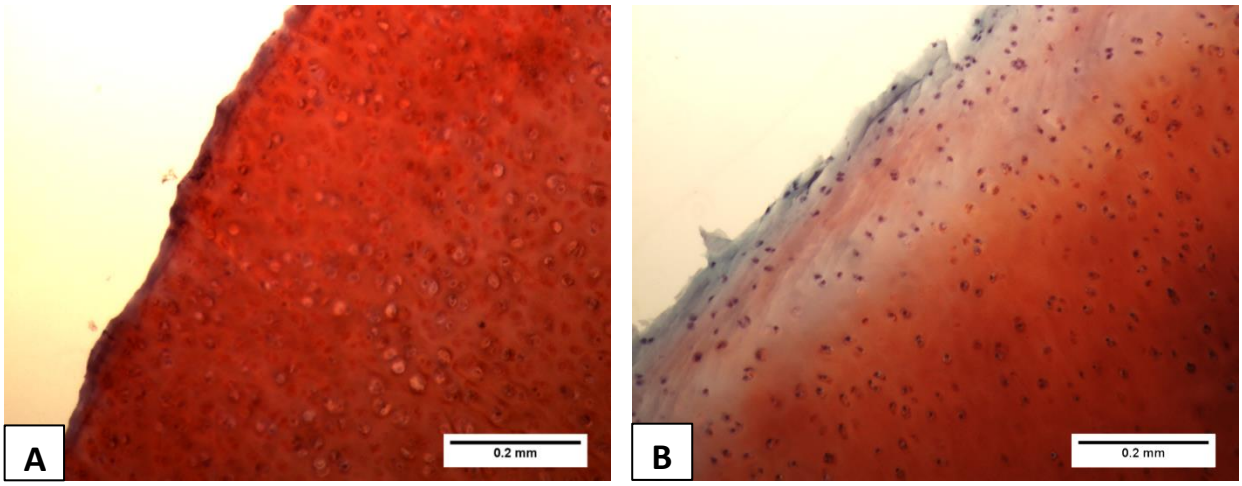


**Figure 5.7: Safranin-O staining of proteoglycan in cartilage cryosections.** Femoral condyle cartilage cryosections obtained from 80 wk old pigs. **A:** non-damaged knee joint with revised SFA score of 7.6; **B:** damaged knee joint with revised SFA score of 35.4. 10x magnification.

### 5.3.3 Plasma cytokines and chondropathy scoring

The plasma concentrations of the cytokines, IL-6 and visfatin, was determined by ELISA and discussed in Chapter 3 in relation to the age of pig groups. This data was also compared to the condition of joints as determined by the revised SFA method of chondropathy scoring. There was no correlation between joint condition and plasma IL-6 concentrations (Figure 5.7 A) or plasma visfatin concentrations (Figure 5.7 B).

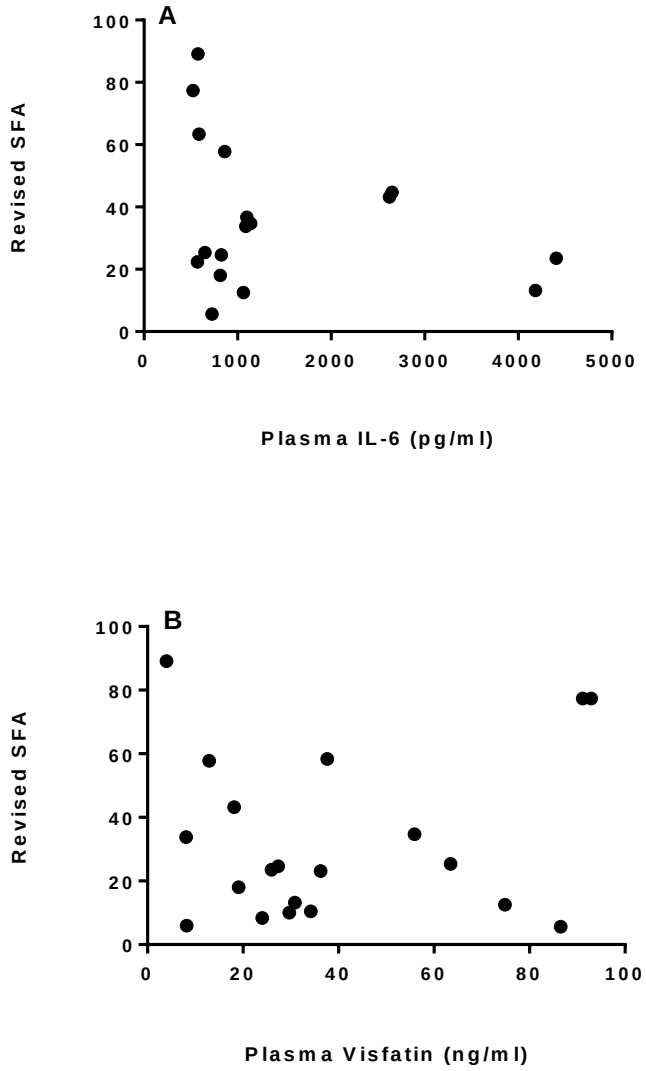


**Figure 5.8: Correlation of joint chondropathy with plasma IL-6 and visfatin concentrations. A:** Individual plasma IL-6 concentrations plotted against revised SFA. Pearsons r correlation coefficient, no significant relationship (p= 0.2797). **B:** Individual plasma visfatin concentrations plotted against revised SFA. Pearsons r correlation coefficient, no significant relationship (p=0.2351)

## 5.4 DISCUSSION

This chapter investigated spontaneous joint chondropathy and osteoarthritis in the commercial pig. The data suggested that these commercial pigs suffer from joint degeneration at an early age and may present an improved model of spontaneous osteoarthritis.

### Joint Photography and Chondropathy

Following dissection, porcine knee joints were photographed and subsequently scored using two chondropathy scoring methods: Collin's grading and revised SFA. Joint scoring methods can utilise photographs, radiographs, magnetic resonance imaging (MRI) and histological methods to score OA severity. The Kellgren and Lawrence (KL) system has been widely utilised; this method uses radiographs of the joint to score OA progression based on the presence of osteophytes, joint space narrowing and articular surface changes (255). This method assumes that OA damage in the joint progresses in a linear manner starting with osteophyte formation, progressing to joint space narrowing and articular surface deformation; therefore, it cannot score joint space narrowing when osteophytes are not present (255). MRI methods include the Whole Organ MRI Score (WORMS), Boston-Leeds OA Knee Score (BLOKS) and MR OA knee score MOAKS (256–259). BLOKS has been shown to correlate with other measures such as the KL system, however, these semi-quantitative scoring methods have limited ability to detect early OA changes and are more effective in scoring more advanced disease (256,260). Alizai et al developed a Cartilage Lesion Score (CaLS) method which was able to measure the 3D volume of cartilage defects and was recommended for the longitudinal follow up of mild cartilage defects in MRI (256). Histological methods have been used previously,

these methods were originally devised by Mankin et al (261,262). The Mankin method examines the condition of joints by evaluating decalcified sections of joint tissue stained by safranin-O with a fast green counterstain: histological methods can be prone to problems with reproducibility (261).

Although several different joint scoring techniques have previously been used, in this study the Collin's grading and revised SFA scoring were chosen based on their simplicity and robustness (253,263,264). Both of these scoring methods are based on macroscopic observations of the joint itself or of photographs of it. Collin's grading has been used to score joint damage in the pig previously (252). However, joints obtaining the same Collin's grade can exhibit a considerable level of variation; therefore, the revised SFA method was selected as an additional, more informative measure of joint chondropathy (253). Safranin-O staining was also performed on a small sample of porcine joints as part of this study; histological scoring was not carried out due to the small number of samples available and the irreproducibility of results obtained with this method.

Examination of porcine knee joints in this manner revealed that commercial pigs develop OA-like lesions at an early age and that lesions develop on both the medial and lateral articular surfaces of the femoral condyles and tibial plateaus of knee joints. There appears to be a trend towards increasing chondropathy score with age. Human knee joints were also scored to allow a direct comparison between pig and human chondropathy. These human joints were obtained from patients undergoing knee replacement surgery; therefore, the joints obtained were in the late stages of OA, showing severe damage. This level of joint deterioration would not be expected in pigs within the age range investigated in this study. The age groups used were selected to determine the utility of the pig as a model of early OA changes and disease progression. Photography of these

joints also suggested that a high degree of subchondral bone changes, such as osteophyte formation, was occurring. These changes reflect those occurring in human knee joints during the development and progression of knee osteoarthritis and are indicative of bone remodelling processes occurring in these joints (96). Additionally, the fold difference in sGAG release between non-damaged and damaged cartilage explants obtained from pigs was found to positively correlate to the revised SFA score for the joint from which that explant was taken. Therefore, increased cartilage degradation was observed in explants obtained from joints achieving a higher revised SFA score. This lends support to the revised SFA method of scoring in porcine knee joints. Knee joints scoring higher revised SFA scores also exhibited weaker safranin-O staining, further demonstrating proteoglycan loss in these damaged joints.

As discussed in Section 1.6, pigs can develop osteochondrosis (OC) which results in macroscopic joint changes similar to that occurring in OA (183,191). The methods used to investigate joint chondropathy in this study do not differentiate between the joint damage seen in OA and OC. The presence of OC lesions in the joint can lead to the development of secondary OA (183). This complicates the use of these measures of chondropathy in the pig and may complicate their use as an animal model of OA.

**Chondropathy scoring and plasma cytokines**

Plasma levels of the pro-inflammatory cytokines, IL-6 and visfatin, in the different age groups of pigs were presented and discussed in Chapter 3.0. In this chapter, this data was also compared to joint condition as measured by revised SFA. IL-6 is elevated in osteoarthritis (72) and in obesity (231,232). As

mentioned in Chapter 3.0, this interleukin is involved in the switching of acute inflammatory responses to chronic inflammatory conditions (230,233).

Obesity has been linked to an increased risk of the development of inflammatory conditions such as OA (72). Visfatin has been found to have pro-inflammatory effects and is elevated obese individuals, and in the synovial fluid of osteoarthritic joints, and in the blood plasma of osteoarthritis patients (155). As the circulating levels of both of these cytokines are increased in OA patients, it was important to determine how the plasma concentrations of these cytokines compared to the joint deterioration or OA status of the pigs. No correlation was found between plasma IL-6 or visfatin levels and revised SFA, possibly due to the complex nature of inflammatory regulation and the wide variety of factors that could be contributing to circulating cytokine levels. The level of these cytokines in the synovial fluid of the joints could also be more reflective of the damage status of that joint.

**Behavioural Assessment**

Visual Analogue Scale (VAS) scoring systems are used to measure pain or pain behaviours in animals (249,250). For the purpose of this study, a number of pain related behaviours were identified, from similar behavioural studies, as being of interest in the pig (249). Using these behaviours the VAS scoring system was modified to produce a system whereby behavioural aspects could be scored between 1-5, depending on severity of response or symptom. Initially the observational scoring protocol for the pig had 5 areas of interest: observation at rest, willingness to ambulate, response to touch, vocalisation and lameness (249,265). The scores obtained for each pig varied from one scoring session to the next with no discernible pattern for both “observation at rest” and

"willingness to ambulate", therefore, these scores were discontinued. The "response to touch" measure was determined to be unsuitable due to the lack of handling: pigs were only handled briefly during weighing and "vocalisation" in the pig is a feature of social interaction, therefore, the purpose or cause of vocalisation was not easily discernible and could not be attributed solely to pain. This is in contrast to animals in which VAS scoring is more commonly used, e.g. rats, for which vocalisation within the audible range of humans is associated with an expression of pain and is not a common feature of social vocalisation, which is mostly ultrasonic instead (266,267). Therefore, this measure was also removed from the protocol. This left "lameness" as the only measure found to be suitable for use in the pig. Our observational scoring of lameness and associated pain indicators revealed a trend towards increasing lameness with age in these pigs. The oldest age group (3-4 yr old pigs) investigated in this study achieved slightly lower lameness scores than the much younger 80 wk group. This could be due to the effect of individual variation within each group or it could be linked to the higher average weight in the 80 wk group increasing the stresses put on their joints compared to the lighter 3-4 yr group (Body weight and composition were discussed in Chapter 3.0).

This measure of lameness cannot be directly linked to knee joint damage and these behaviours could be associated with a number of other ailments, injuries or damage to other joints, such as the hip, not investigated as part of this study. However, this measure is still an important factor in determining pain perception in the pig. If used in conjunction with other indicators of pain, such as the pig grimace scale currently being developed at Newcastle University (268) and gait analysis similar to that used by Hans von Wachenfelt et al (269), this could form a strong basis for the investigation of pain during OA development and treatment in the pig.

## Conclusion

In conclusion, these results have established that commercial pigs develop osteoarthritic lesions on both the medial and lateral articular cartilage surfaces of the femoral condyles and tibial plateaus of the knee joint in pigs as young as 80 weeks, and that these lesions worsen with age. The revised SFA scoring method was found to be a simple and robust method of scoring joint damage in pigs and correlated well with cartilage degradation and proteoglycan loss, as measured by sGAG release and safranin-O staining, respectively.

Plasma levels of the pro-inflammatory cytokines, IL-6 and visfatin, did not correlate with joint chondropathy, future studies should investigate the relationship between joint chondropathy and the level of these cytokines in synovial fluid of individual joints. Finally, observational measures of lameness have revealed increasing lameness with age. Although this cannot be linked directly to knee joint deterioration, it could prove useful if used in conjunction with other measures being developed elsewhere, such as gait analysis.

# 6.0 UTILITY OF PORCINE CARTILAGE FOR EX-VIVO STUDIES IN OA RESEARCH

## 6.1 INTRODUCTION

Porcine cartilage and chondrocytes have been employed in research previously (252,270–274). For the most part, the research areas utilising porcine cartilage have centred around the testing of potential chondro-protective substances (270,273–275) or tissue engineering methods of osteochondral defect repair (276–279). However, as a source of both healthy and damaged cartilage, the pig has been underutilised.

Pro-inflammatory cytokines such as IL-1β have been suggested to be key drivers of OA cartilage pathology (280). However, confirming the role of these cytokines in mediating "early OA" pathology and the key signalling pathways activated is difficult to achieve. This is largely due to the fact that the human OA cartilage used for *in vitro* and *ex vivo* studies is most often obtained following joint replacement surgery for "end-stage" disease, where the cartilage is highly degraded (281). In addition, the quantity of cartilage obtained from rodent models is insufficient for many requirements, particularly *ex vivo* cartilage explant studies. Therefore, establishing relevant OA phenotypic assays in the pig could represent a valuable resource for the study of the signalling pathways that mediate early OA pathology.

In order to evaluate the utility of the pig as a model of early OA it is necessary to determine the effect of pro-inflammatory cytokine stimulation on primary porcine chondrocytes and cartilage explants.

### 6.1.1 AIMS

The purpose of this chapter is to determine if porcine cartilage explants and primary chondrocytes would be useful for *ex vivo* studies investigating aspects of osteoarthritis.

Main aims addressed in the chapter:

- To investigate the response of cartilage explants to acute pro-inflammatory stimuli
- To determine the response of primary chondrocytes to acute pro-inflammatory stimuli

## 6.2 METHODS

Osteoarthritis is characterised by the loss of articular cartilage therefore the level of cartilage degradation and the responses of cartilage and cartilage maintaining cells, chondrocytes, to pro-inflammatory cytokines are of importance in the validation of the commercial pig as a model of osteoarthritis.

### 6.2.1 Cartilage explants

Cartilage explants were obtained from the femoral condyles of porcine knee joints and cultured as described in the Section 2.6.1. The following experiments were carried out using porcine cartilage explants. The supernatants from these experiments underwent further analysis to investigate IL-6 production by ELISA, described in Section 3.2.4 and cartilage degradation by DMMB proteoglycan release assay, as described in Section 5.2.4.

### 6.2.2 Acute stimulation of explants with Interleukin 1 Beta (IL-1β)

Porcine cartilage explant disks were stimulated for 24 hours with 150µl chondrocyte media containing the pro-inflammatory cytokine, IL-1β. Details of the components of chondrocyte media were discussed in Section 2.4.3. Supernatant was collected from all of the following experiments and frozen for subsequent analysis. Explants were also frozen.

#### Experiment 1: High dose human IL-1β stimulation of porcine explants

Explants were exposed to media containing 10ng/ml or 30ng/ml human recombinant Interleukin 1 Beta (Sigma Aldrich, Poole, UK) in chondrocyte media for 24 hours. Supernatant from unstimulated explants was used as the control sample.

**Experiment 2: High dose porcine IL-1β stimulation of porcine explants**

Explants were exposed to media containing 10ng/ml or 30ng/ml porcine recombinant Interleukin 1 Beta (R&D systems) in chondrocyte media for 24 hours. Supernatant from unstimulated explants was used as the control sample.

**Experiment 3: Dose response to porcine IL-1β stimulation of porcine explants**

Explants were exposed to media containing increasing doses (0.1, 0.3, 1, 3 or 10ng/ml) of porcine recombinant Interleukin 1 Beta (R&D systems) in chondrocyte media for 24 hours. Supernatant from unstimulated explants was used as the control sample.

### 6.2.3 Acute stimulation of explants with adipokines

Porcine cartilage explant disks were stimulated for 24 hours with media containing the pro-inflammatory adipokines; leptin, resistin or visfatin at the concentrations detailed below in the individual experiments. Supernatant was collected from all of the following experiments and frozen for subsequent analysis. Explants were also frozen.

**Experiment 1: Human adipokine stimulation of porcine explants (leptin & resistin)**

Explants were exposed to media containing 100 or 300ng/ml human recombinant leptin, 30 or 100ng/ml human recombinant resistin (cell guidance systems) in chondrocyte media for 24 hours. Supernatant from unstimulated explants was used as the control sample.

**Experiment 2: Human visfatin stimulation of porcine explants**

Explants were exposed to media containing 30, 100 or 500ng/ml human recombinant visfatin in chondrocyte media for 24 hours. Supernatant from unstimulated explants was used as the control sample.

### 6.2.4 Primary porcine chondrocytes

The following experiments were carried out in primary porcine chondrocytes isolated from knee cartilage as described in the Section 2.4.3. The supernatant from these experiments underwent further analysis to investigate IL-6 production by ELISA, as described in Section 3.2.4.

### 6.2.5 Growth rates of primary porcine chondrocytes

Proliferation rates of porcine chondrocytes were determined by seeding cells on 24 well plates at two known densities: a lower density of 5000 cells per well and a higher density of 10000 cells per well.. Cells were trypsinised and counted using a haemocytometer at intervals over a 14 day time course. This experiment was used to determine if there were significant differences in the growth rates of cells obtained from the different age groups examined.

### 6.2.6 Acute stimulation with IL-1β

Primary porcine chondrocytes were stimulated for 24 hours with media containing the pro-inflammatory cytokine, IL-1β. Supernatant was collected from all of the following experiments and frozen for subsequent analysis.

#### Experiment 1: High dose human IL-1β stimulation of porcine chondrocytes

Chondrocytes were exposed to media containing 10ng/ml or 30ng/ml human recombinant Interleukin 1 Beta (Sigma Aldrich) in chondrocyte media for 24 hours. Chondrocytes exposed to media only were used as unstimulated control samples for comparison purposes.

#### Experiment 2: Dose response to porcine IL-1β stimulation of porcine chondrocytes

Chondrocytes were exposed to media containing 0.1, 0.3, 1 or 3ng/ml porcine recombinant Interleukin 1 Beta (R&D systems) in chondrocyte media for 24 hours. Chondrocytes exposed to media only were used as unstimulated control samples for comparison purposes.

### 6.2.7 Acute stimulation with adipokines

Primary porcine chondrocytes were stimulated for 24 hours with media containing the pro-inflammatory adipokine; leptin, resistin or visfatin. Supernatant was collected from all of the following experiments and frozen for subsequent analysis.

**Experiment 1: Human adipokine stimulation of porcine chondrocytes (leptin & resistin)**

Chondrocytes were exposed to media containing 100 or 300ng/ml human recombinant leptin, or, 30 or 100ng/ml human recombinant resistin in chondrocyte media for 24 hours. Chondrocytes exposed to media only were used as unstimulated control samples for comparison purposes.

**Experiment 2: Human visfatin stimulation of porcine chondrocytes**

Chondrocytes were exposed to media containing 500ng/ml human recombinant visfatin in chondrocyte media for 24 hours. Chondrocytes exposed to media only were used as unstimulated control samples for comparison purposes.

The doses of IL-1β selected for initial experiments used in this study are supra-physiological concentrations to ensure responsiveness to IL-1β. The concentrations of IL-1β used to investigate dose response were physiologically relevant and have been used previously (282–284). All adipokine concentrations used were also supra-physiological and the following concentrations have been used previously: leptin, up to 100ng/ml (141); resistin, up to 500ng/ml (162) and visfatin, up to 800ng/ml (162,285,286).

### 6.2.8 RNA extraction from cultured chondrocytes using Roche High Pure total RNA kit

Total RNA was extracted from cultured chondrocytes using an RNA isolation kit (Roche High Pure Isolation Kit for RNA isolation). 400µl of lysis-binding solution was added directly to cells frozen in sterile PBS and allowed to thaw, and then vortexed for 15 seconds. The 600µl sample was then centrifuged in a high pure spin column at 8000xg for 15 seconds. The flow through was discarded after each centrifugation step. RNA was treated with 10µl DNase I and 90µl of DNase-incubation buffer for 15 minutes at room temperature. 500µl of wash solutions I was added to the spin column and centrifuged at 8000xg for 15 seconds, followed by 500µl of wash buffer solution II and centrifugation at 8000xg for 15 seconds. An additional 200µl of wash buffer II was passed through the column with 2mins of centrifugation at maximum speed. The RNA was then eluted in 50µl RNase-free water into a fresh 1.5ml microcentrifuge tube by centrifugation at 8000xg for 1 minute. Eluted RNA was quantified using a Thermoscientific Nanodrop and stored at -80°C until use.

### 6.2.9 RNA extraction from cartilage using Qiagen fibrous tissue kit

300µl of buffer RLT was added to 100mg of frozen cartilage tissue in a Roche Green Beads tube and the tissue was disrupted using a MagnaLyser at 6500rpm for 60 seconds. Supernatant was then removed and added to 590µl RNase-free water. 10µl proteinase K solution was added and mixed thoroughly with the sample by pipetting. Samples were then incubated at 55°C for 10mins followed by centrifugation at 10000xg for 3mins. The samples were then transferred to clean 1.5ml microcentrifuge tubes with 450µl of 100% ethanol and mixed thoroughly by pipetting. 700µl of the sample (approx. 900µl) was then transferred to an RNeasy mini spin column and centrifuged at 8000xg for 15

seconds. Supernatant was discarded after all following centrifugation steps, except the elution step. The remaining sample (approx. 200µl) was added to the column and the previous centrifugation step repeated. 350µl of buffer RW1 was added to the spin column and centrifuged at 8000xg for 15 seconds. DNase digestion was carried out on the column by adding 80µl DNase I incubation mix to the column and incubating for 15mins (10µl of DNase I stock and 70µl buffer RDD). 350µl buffer RW1 was then added to the column and centrifuged at 8000xg for 15 seconds. 500µl buffer RPE was added to the column and centrifuged at 8000xg for 15 seconds, followed by the addition of another 500µl buffer RPE and centrifugation at 8000xg for 2 mins. RNA was eluted by the addition of 30µl RNase-free water and centrifugation at 8000xg for 1 min. All centrifugation steps were carried out at room temperature. Eluted RNA was quantified using a Thermoscientific Nanodrop and stored at -80°C until use.

### 6.2.10 cDNA synthesis using Thermo Scientific RevertAid RT kit

cDNA was generated from RNA samples using the following method, all steps were carried out on ice unless otherwise stated. 100ng total RNA was added to a sterile, nuclease free tube with 1µl random hexamer primer and nuclease free water was added to a total volume of 12µl. 4µl reaction buffer, 1µl RiboLock RNase inhibitor, 2µl 10mM dNTP mix and 1µl RevertAid RT was added in that order, bringing the total reaction volume to 20µl. The reaction tube was mixed and centrifuged briefly, incubated at room temperature for 5 mins followed by a further incubation at 42°C for 1hr. The reaction was terminated by heating to 70°C for 5 mins. cDNA was stored at -80°C until use.

### 6.2.11 Real Time Quantitative Polymerase Chain Reaction (RTqPCR)

Relative mRNA expression was determined by real time PCR using a Roche Lightcycler 480® (Roche, Burgess Hill, UK). mRNA levels were analysed by SYBR green florescence. The primer sequences used were as follows: COL1A1 Forward: AGAAGAAGACATCCCACCAGTCA, Reverse: CGTCATCGCACAACACATTG; COL2A1 Forward: GGCAACAGCAGGTTCACGTA, Reverse: CAATCATAGTCTGGCCCCACTT. Reactions were carried out in triplicate on 384 well plates. 5µl of cDNA was added to each well along with the following reagents: 7.5µl SYBR green master mix (Roche, Burgess Hill, UK), 0.45µl forward and reverse primers (10µM each) and 1.6µl RNase-free $H_2O$, resulting in a total reaction volume of 15µl per well. Samples were incubated at 95°C for 5 minutes followed by 45 PCR amplification cycles (de-naturation: 95°C for 10 seconds; annealing: 60°C for 15 seconds; elongation: 72°C for 15seconds). A standard curve was produced using serial dilutions of a pool of cDNA made from culture chondrocyte samples to check the linearity and efficiency of the PCR reactions. Transcript abundance was normalised to the total first strand cDNA concentration following reverse transcription using OliGreen.

### 6.2.12 Total cDNA quantification using Oligreen

Reactions were carried out in triplicate on 384 well plates using a Roche Lightcycler 480® (Roche, Burgess Hill, UK). 5µl of Oligreen (Invitrogen, Paisley, UK) working solution was added to 5µl cDNA. Fluorescence at 80°C was plotted against a standard curve produced using serial dilutions of a pool of cDNA obtained from cultured chondrocyte samples. This was used to normalise RTq-PCR to total cDNA content.

### 6.2.13 Statistical analysis

Analysis was carried out using Graphpad Prism software version 7.0. T-tests were used for experiments involving visfatin stimulation of chondrocytes. One-way ANOVAs were utilised for the remaining cytokine and adipokine stimulations followed by Dunnetts post hoc test with the exception of results investigating the effect of high dose porcine IL-1β stimulation of cartilage explants on sGAG release, where Tukeys post hoc test was more appropriate. Dunnets post hoc test compared the mean of each treatment to the mean of the control group. Tukeys post hoc test allows the comparison of the mean of each treatment to each other treatment and the mean of the control group. Tukeys was used to determine if the different treatments were producing significantly different results. Linear regression was carried out on proliferation rate data.

6.3 RESULTS

### 6.3.1 Response of porcine cartilage explants to IL-1β stimulation

#### 6.3.1.1 Cartilage degradation

Stimulation of porcine cartilage explants with recombinant human IL-1β for a 24 hour period did not induce cartilage proteoglycan degradation, as measured by sGAG release into the explant culture media (Figure 6.1), and was also unable to induce IL-6 production above the minimum detectable concentration of the ELISA kit used.

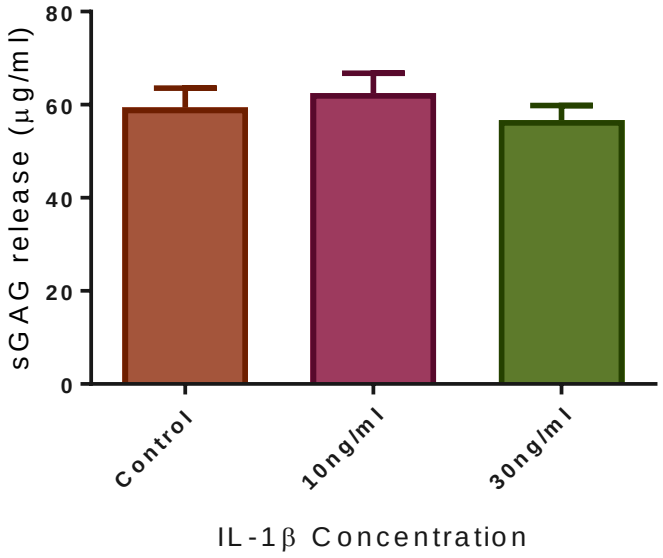


**Figure 6.1: Cartilage degradation in response to 24 hour human IL-1β stimulation of porcine cartilage explants.** Results shown as mean ± SEM. No significant effect of IL-1β stimulation on sGAG release determined by one way ANOVA. (n=7 per stimulation)

In contrast, recombinant porcine IL-1β induced cartilage degradation in porcine cartilage explant disks, as determined by the significant increase in sGAGs detected in the supernatants as shown in Figure 6.2. Hence, recombinant porcine IL-1β was used for all subsequent experiments.

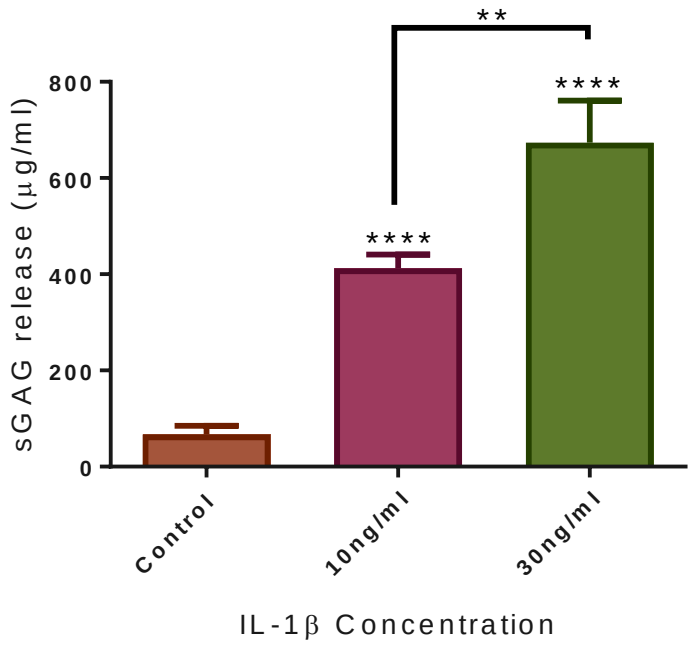


**Figure 6.2: Cartilage degradation in response to 24 hour porcine IL-1β stimulation of porcine cartilage explants.** Results shown as mean ± SEM. Significant effect of porcine IL-1β concentration on sGAG release determined by one way ANOVA ($p<0.0001$). Holm-Sidak's multiple comparisons revealed that IL-1β significantly increased sGAG release at both 10ng/ml and 30ng/ml concentrations (**** $p<0.0001$) and that there was a significant increase in sGAG release between 10ng/ml and 30ng/ml stimulations (*** $p=0.0008$). (n=26 per stimulation)

The dose response experiments detailed in this chapter were designed to gauge the response of cartilage to physiologically relevant concentrations of IL-1β. For this purpose an IL-1β dose response experiment was performed with concentrations ranging from 0.1ng/ml to 10ng/ml. There was a significant relationship between increased IL-1β concentration and higher levels of sGAG release. sGAG release from cartilage explants was significantly higher than the unstimulated control when stimulated with as little as 0.1ng/ml IL-1β (Figure 6.3).

The basal sGAG release differs between groups of pigs and this should be taken into consideration when comparing results obtained from different groups of

pigs, or from experiments carried out at different times. The previous experiments in this study exhibited basal sGAG concentration of roughly 60µg/ml after 24 hours; this dose response experiment exhibited an average basal sGAG concentration of around 200µg/ml.

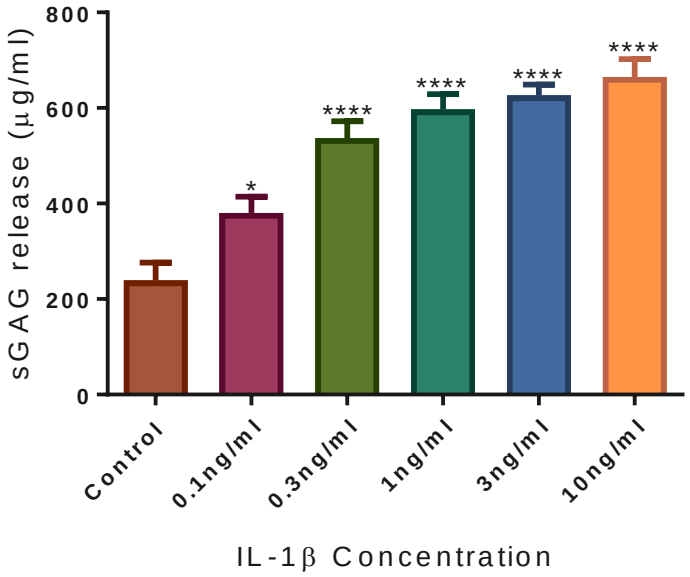


**Figure 6.3: Cartilage degradation in response to 24 hour stimulation with increasing IL-1β concentrations.** Results shown as mean ± SEM. Significant effect of IL-1β concentration on sGAG release determined by one-way ANOVA ($p<0.0001$). Dunnett's multiple comparisons revealed significant differences between treatment groups (* $P=0.0157$; **** $P<0.0001$). (n=18 per stimulation)

#### 6.3.1.2 IL-6 production

Recombinant human IL-1β was unable to induce IL-6 production above the minimum detection threshold of the ELISA kit used. Stimulation of porcine cartilage explant disks with recombinant porcine IL-1β at a concentration of 10ng/ml significantly increased IL-6 production ($P<0.0001$) when compared to that of unstimulated control explants. There was no significant difference between IL-6 production in response to 10ng/ml and 30ng/ml IL-1β (Figure 6.4).

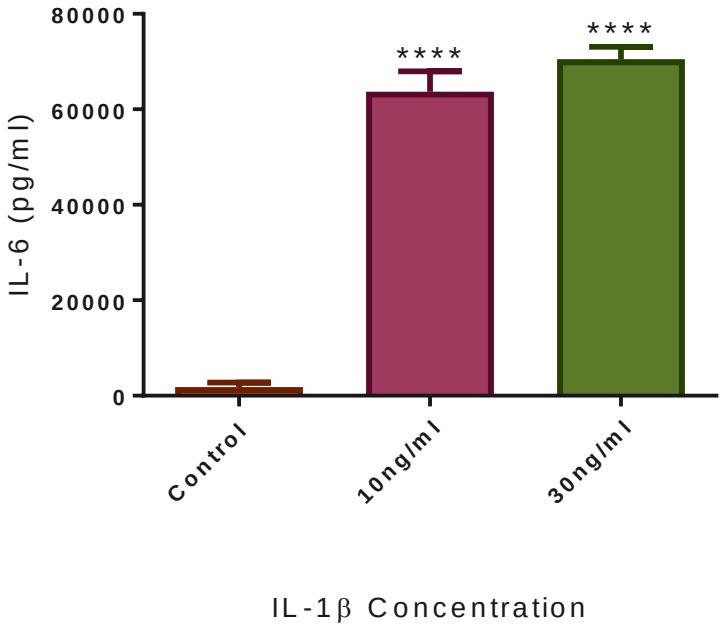


**Figure 6.4: IL-6 production in response to 24 hour stimulation with porcine IL-1β.** Results shown as mean ± SEM. Significant effect of porcine IL-1β concentration on IL-6 production determined by one way ANOVA ($p<0.0001$). Holm-Sidak's multiple comparisons revealed that IL-1β significantly increased IL-6 production at both 10ng/ml and 30ng/ml concentrations (**** $p<0.0001$) and that there was no significant increase in IL-6 production between 10ng/ml and 30ng/ml stimulations ($p=0.1365$). (Control: n=18; 10ng/ml: n=31; 30ng/ml: n=31)

Figure 6.5 shows that there was a significant relationship between increased IL-1β concentration and higher levels of IL-6 production ($P<0.0001$). IL-6 production in explants stimulated with 10ng/ml IL-1β was slightly lower than that of the 3ng/ml stimulated explants, however this difference was not statistically significant.

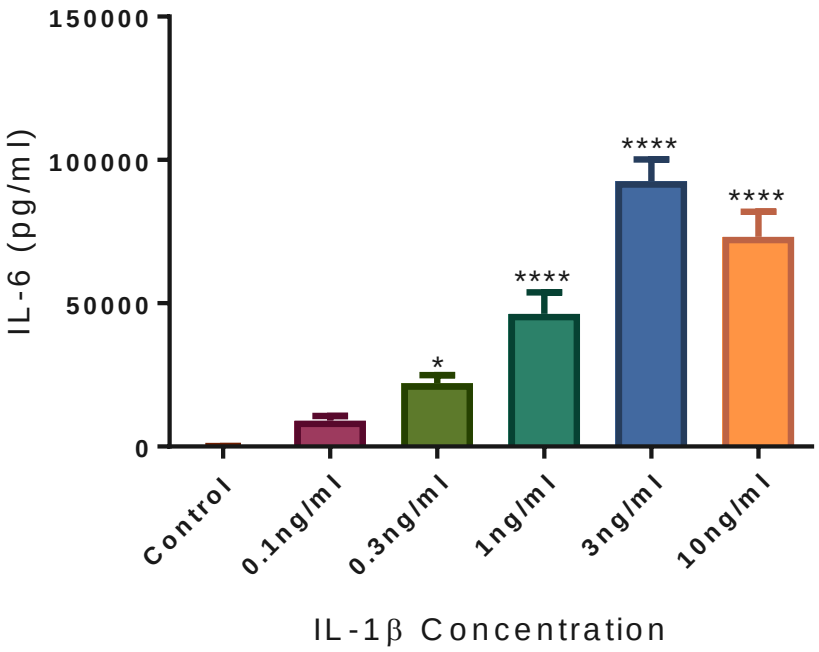


**Figure 6.5: IL-6 production in response to IL-1β stimulation.** Results shown as mean ± SEM. Significant effect of IL-1β concentration on IL-6 production determined by one-way ANOVA ($p<0.0001$). Dunnett's multiple comparisons revealed significant differences between treatment groups (* P=0.0475; **** P<0.0001). (Control: n=16; all other stimulations: n=20)

### 6.3.2 Response of porcine cartilage explants to adipokine stimulation

#### 6.3.2.1 Cartilage degradation

Neither recombinant human resistin or recombinant human leptin, at the concentrations used, induced any change in the concentration of GAG released into culture media over a 24 hour period (Figure 6.6). Human resistin has 77% homology with porcine resistin and human leptin has 85% homology with porcine leptin; porcine adipokines were not available for this study.

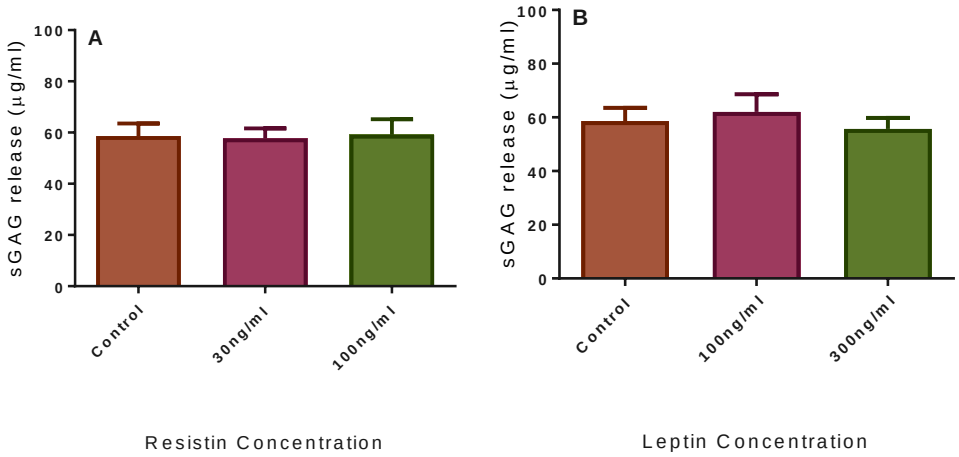


**Figure 6.6: Cartilage degradation in response to 24 hour adipokine stimulation. A: Human recombinant resistin stimulation. B: Human recombinant leptin stimulation.** Results shown as mean ± SEM. No significant effect of resistin stimulation (p=0.9784) and no significant effect of leptin stimulation (p=0.7051) on sGAG release determined by one way ANOVA. (n=11 per stimulation)

Acute stimulation of porcine cartilage explants with human recombinant visfatin, which has 97% homology with porcine visfatin, resulted in no significant change at lower concentrations (30ng/ml and 100ng/ml), but a significant increase in GAG release was observed at 500ng/ml visfatin stimulation (Figure 6.7). The basal sGAG concentration of the control group is slightly higher than that of the 30ng/ml and 100ng/ml groups, this could be explained by the use of explants of a different age. This experiment was carried out using explants from 80 wk old pigs (30ng/ml and 100ng/ml groups) and 3-4 yr old pigs (control and 500ng/ml groups). The 80 wk group exhibited slightly lower basal GAG release when compared to that of the 3-4 yr group but this difference was not significant.

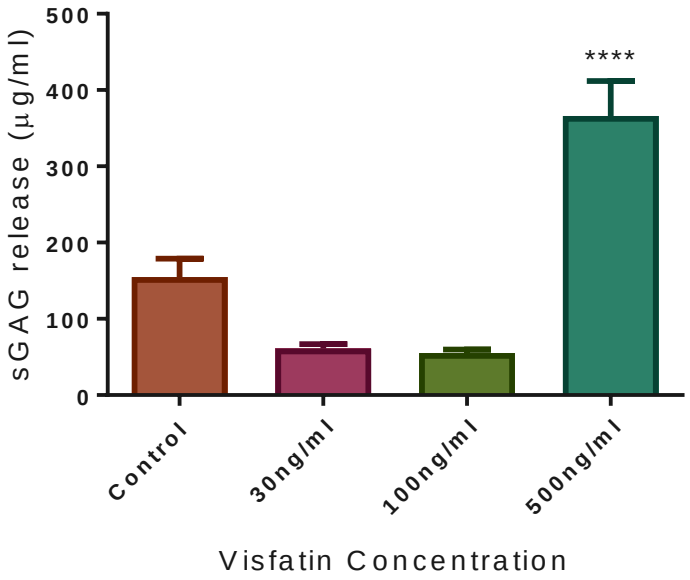


**Figure 6.7: Cartilage degradation in response to 24 hour stimulation with various concentrations of visfatin.** Results shown as mean ± SEM. Significant effect of visfatin stimulation on sGAG release determined by one way ANOVA ($p<0.0001$). Tukey's multiple comparisons revealed that there was a significant increase in sGAG release at 500ng/ml visfatin stimulation ($p<0.0001$) and no significant difference between control and either 30ng/ml or 100ng/ml visfatin sitmulations ($p=0.1918$ and $p=0.1702$, respectively). (Control: n=28; 30ng/ml: n=13; 100ng/ml: n=12; 500ng/ml; n=21)

#### 6.3.2.2 IL-6 production

24 hour stimulations of porcine cartilage explants with recombinant human leptin or resistin at the concentrations used in this study were unable to induce IL-6 production higher than the minimum detection level for the ELISA kit used. Acute stimulation of porcine cartilage explants with 500ng/ml human recombinant visfatin resulted in a significant increase in IL-6 production over 24 hours (Figure 6.8).

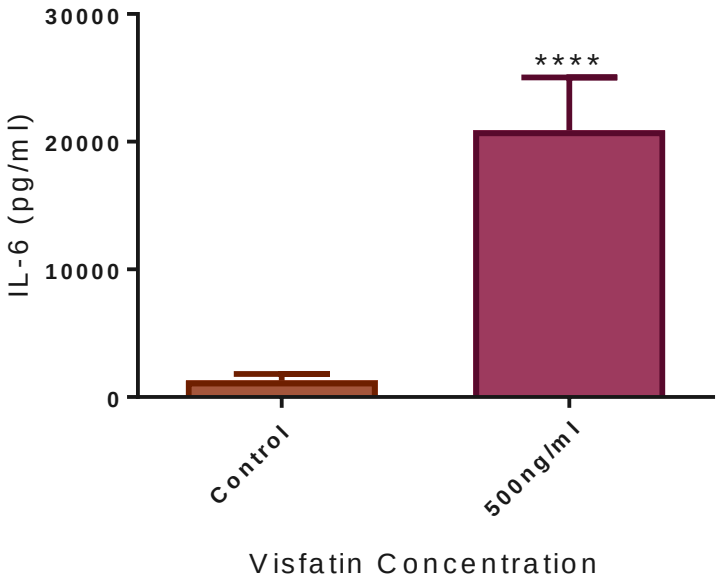


**Figure 6.8: IL-6 production in response to 24 hour visfatin stimulation.** Results shown as mean ± SEM. Significant increase in IL-6 production with visfatin stimulation at 500ng/ml determined by students t test, **** $p<0.0001$. (Control: n=21; 500ng/ml: n=20)

### 6.3.3 Characterisation of primary porcine chondrocytes isolated from knee articular cartilage

Morphological examination of primary porcine chondrocytes isolated from porcine knee joints revealed a fibroblast-like morphology similar to that of human primary chondrocytes when grown on plastic in 2D culture, shown in Figure 6.9 (287).

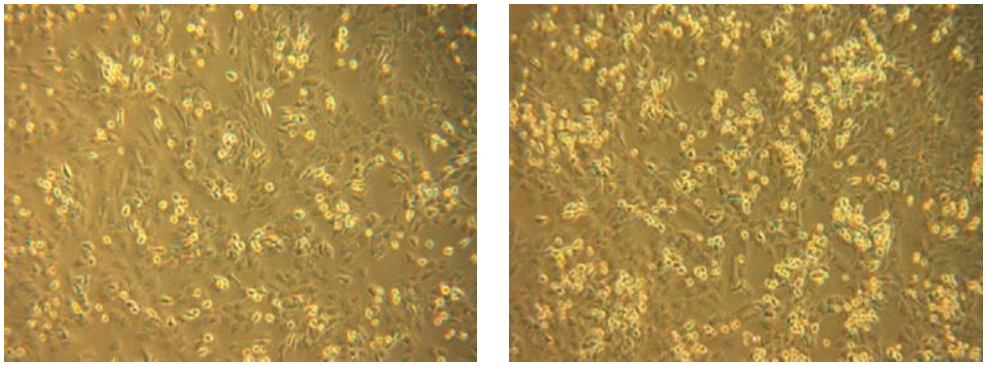

**Figure 6.9: Representative light microscope images of primary porcine chondrocytes.** (6.3x magnification)

Images of primary porcine chondrocytes were taken periodically during culture. Figure 6.10 shows that there were morphological changes in the chondrocytes with increasing passage number. Over time chondrocytes in culture change in shape from the round morphology exhibited at isolation and during early culture, to the flattened, fibroblast-like morphology observed in longer term cultures.

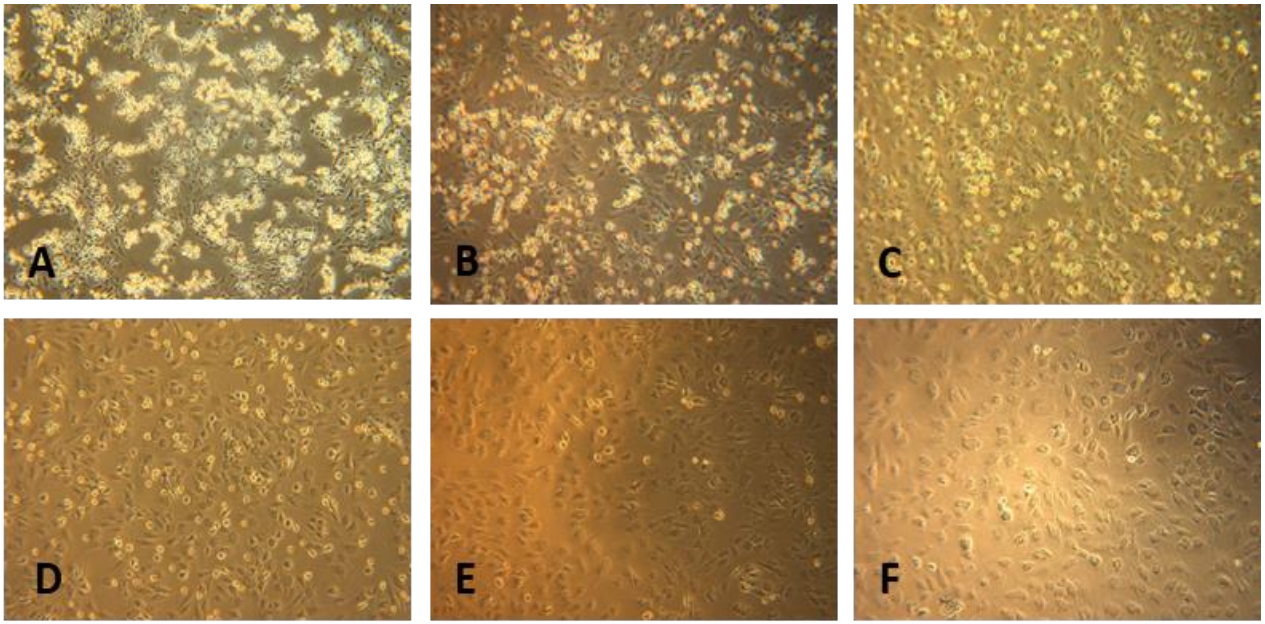


**Figure 6.10: Representative light microscope images of morphological change in primary porcine chondrocyte culture following passaging** (6.3x magnification) Chondrocytes were obtained from an 80 wk old pig. **A:** Passage 0; **B:** Passage 1; **C:** Passage 2; **D:** Passage 3; **E:** Passage 4 and **F:** Passage 5.

Previous research has suggested that the collagen expression on chondrocytes is altered in 2D culture (288,289). In this study, primary porcine chondrocytes exhibit significantly elevated collagen type I mRNA expression (Figure 6.11 A) and significantly reduced levels of collagen type II mRNA expression (Figure 6.11 B) in culture when compared to cartilage. Any potential age differences were not investigated in this study.

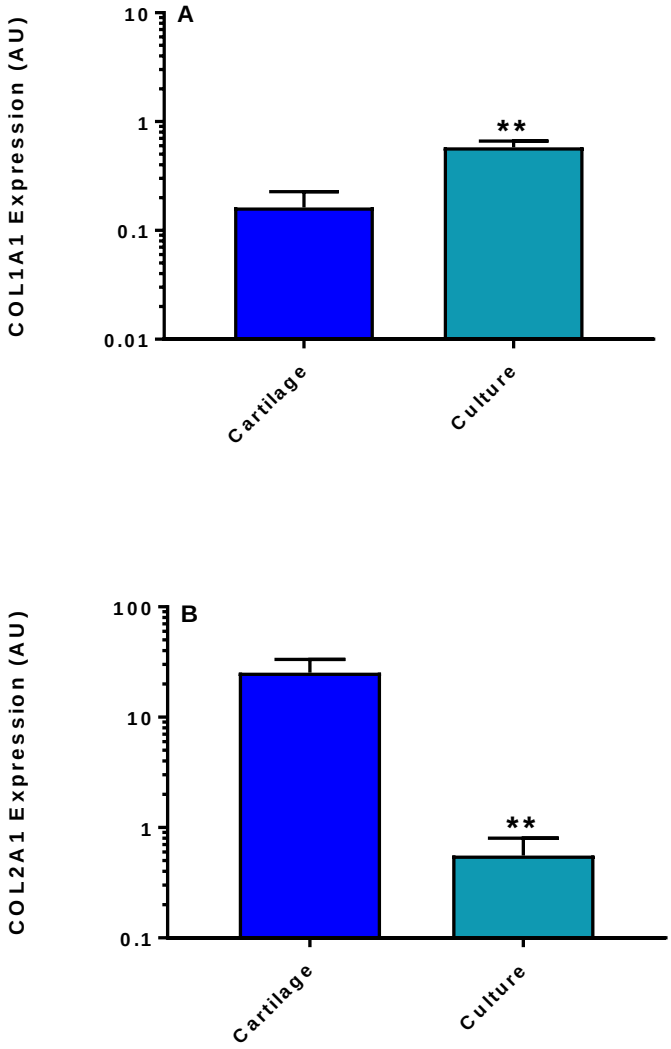


**Figure 6.11: Collagen mRNA expression in chondrocytes in culture and chondrocytes in cartilage. A:** COL1A1 expression significantly higher in cultured chondrocytes when compared to those in cartilage. Significance was determined by students t test (p=0.0035)**; B:** COL2A1 expression significantly lower in cultured chondrocytes when compared to those in cartilage. Signficance was determined by students t test (p=0.0082). (Cartilage: n=5; Culture: n=6)

PCR revealed a significantly lower ratio of COL2A1/COL1A1 expression in cultured chondrocytes compared to chondrocytes in cartilage (Figure 6.12).

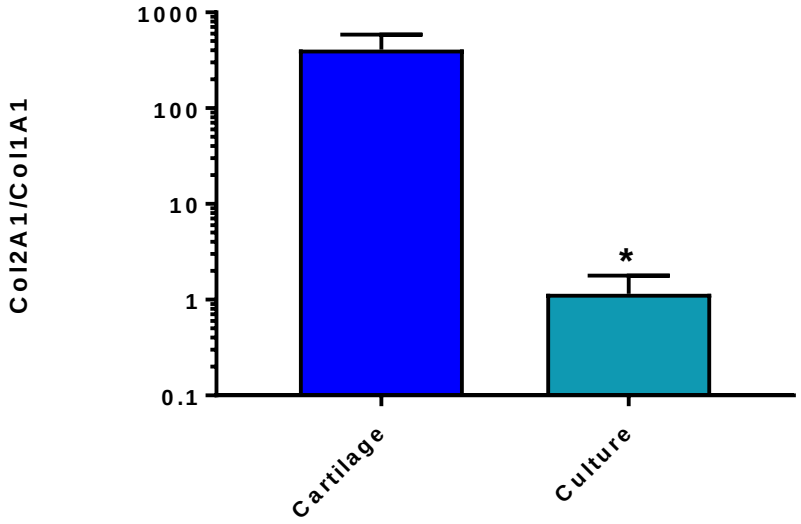


**Figure 6.12: Ratio of COL2A1/COL1A1 mRNA expression.** Ratio of COL2A1/COL1A1 significantly lower in cultured chondrocytes when compared to those in cartilage. Significance was determined by students t test (p=0.0305). (Cartilage: n=5; Culture: n=6)

The growth rate of chondrocytes isolated from 10 week old or 3-4 year old pigs was monitored over a period of 14 days. These data show that there was no change in the proliferation rate between cells seeded at the two densities investigated and that there was no difference in chondrocyte proliferation rate between the cells obtained from 10 week old pigs and those obtained from 3-4 year old pigs (Figure 6.13).

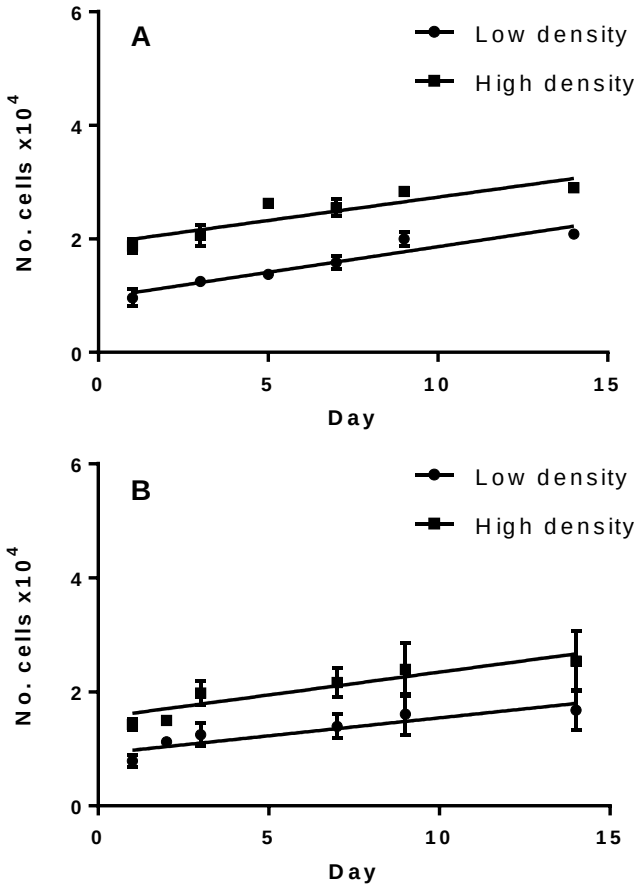


**Figure 6.13: Chondrocyte proliferation over a 14 day period when seeded at different densities.** Individual points represent the mean cell number ± SEM. Low density wells were seeded with 5000 cells per well on Day 0; high density wells were seeded with 10000 cells per well on Day 0. Chondrocytes obtained from **A:** 10 week old pigs (n=3); **B:** 3-4 year old pigs (n=7).

### 6.3.4 Response of porcine chondrocytes to IL-1β and visfatin stimulation

Stimulation of primary porcine chondrocytes with porcine IL-1β at a range of concentrations resulted in the response curve shown in Figure 6.14. 3ng/ml IL-1β stimulation resulted in significantly increased IL-6 produced by chondrocytes in culture (p=0.0008).

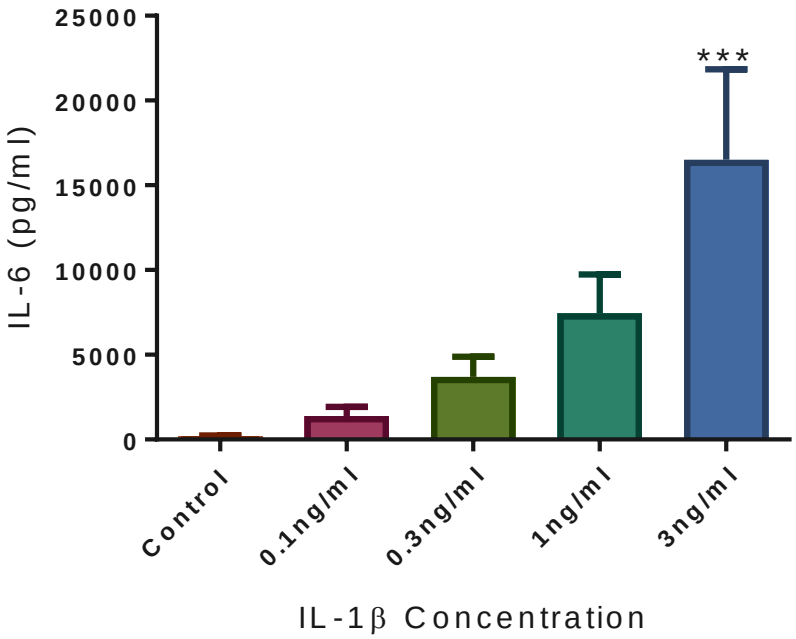


**Figure 6.14: IL-6 production by chondrocytes in response to 24 hour IL-1β stimulation.** Results shown as mean ± SEM. Significantly higher IL-6 production in response to IL-1β stimulation determined by one-way ANOVA (p=0.0015). Dunnett's multiple comparisons revealed significantly higher IL-6 production in response to 3ng/ml IL-1β stimulation (*** p=0.0008). (n=6 per stimulation)

Acute stimulation of chondrocytes with 500ng/ml of the pro-inflammatory adipokine, visfatin, significantly increased (p=0.0116) the production of IL-6 by chondrocytes in culture (Figure 6.15).

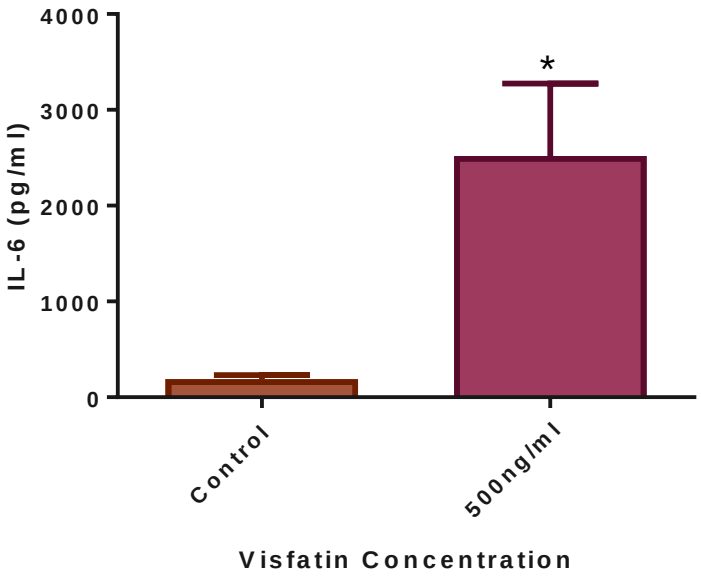


**Figure 6.15: IL-6 production in response to 24 hour visfatin stimulation.** Results shown as mean ± SEM. Significantly higher IL-6 production with visfatin stimulation determined by students t test (*p=0.0116). (n=6 per stimulation)

## 6.4 DISCUSSION

### Cartilage Explants

*Ex-vivo* studies using cartilage explant tissue presents a method of investigating the responses of cartilage to particular stimulants or conditions in a controlled setting. The pig presents a source of full thickness, healthy cartilage, as well as cartilage at an early stage of osteoarthritis. Porcine cartilage explants have been used extensively in research investigating cartilage repair and responses to drugs (271,273,274,290,291). Rodents and other small animals are generally considered to be unsuitable for explant work due to their small size but have been used in a number of studies despite the lack of tissue (292,293). Alternatively, a number of studies have used porcine cartilage explants or chondrocytes prior to *in vivo* testing in small animals, such as the mouse (271,272). However, as a source of osteoarthritic cartilage in the early stages of disease the pig is underutilised.

Human cartilage explants have been used in several studies (281,294–296).The vast majority of cartilage available for human research is obtained from patients undergoing elective joint replacement surgery; therefore the cartilage obtained from these joints is at the "end-stage" of osteoarthritis and is highly degraded. Therefore, this human cartilage may not respond as cartilage from a healthy joint would and this leaves researchers without a healthy human control. Bovine cartilage explants are also commonly employed to investigate cartilage damage and repair methods (297–299). Dairy cattle have been proposed as a model of OA, with work by Hargrave-Thomas and colleagues (300) suggesting that the bovine patella may be an appropriate model of early OA (300). However, subsequent work has suggested that the spontaneous OA in these animals

exhibits distinct differences when compared to human OA; for example, a number of OA-associated biomarkers that are elevated in human OA synovial fluid are not elevated in damaged bovine joints (301).

Hence, the data presented in this chapter would suggest that porcine cartilage mimics some of the responses that would be expected of human cartilage; therefore use of porcine cartilage may allow the elucidation of early cartilage changes in osteoarthritis and contribute to the understanding of the mechanisms underlying the development of osteoarthritis.

**Chondrocytes**

Primary chondrocytes can provide numerous insights into the mechanisms underlying key processes in joint repair and age-related deterioration. Chondrocyte isolation and experimentation has been carried out in a number of species: mice (302), rats (271,303), rabbits (288,304,305), humans (162,306), pigs (272,273,275) and cattle (307,308). This list is not exhaustive and chondrocyte growth in culture is possible for all the main musculoskeletal and osteoarthritis animal model species. Chondrocytes have been used in a vast range of different research areas; for example, they have been used in tissue engineering and xenotransplantation research, investigation of joint pathology such as OA and initial testing of potential treatment to treat cartilage damage (270,273,277,278). Porcine chondrocytes have been used to investigate OA pathology using methods of OA induction. Induction methods include LPS stimulation, TNFα stimulation, and hydrogen peroxide and mechanical stimulation; however the commercial pig may also present a readily available source of healthy and spontaneously damaged cells at a range of disease stages of OA for experimentation.

Dedifferentiation of mammalian chondrocytes in 2D culture is well established. Dedifferentiation reflects a reduction in collagen type II expression and an increase in collagen type I expression (288,289). This is accompanied by changes in cell shape, with newly isolated chondrocytes showing round morphology which flattens and elongates to a more fibroblast-like morphology (289). Morphological examination of porcine chondrocytes revealed that, immediately following isolation, the cells are rounded but over time in 2D culture they develop a fibroblast-like morphology similar to that of human primary chondrocytes.

Human chondrocytes are known to exhibit reduced collagen type II expression in culture (309,310). This is indicative of a loss of chondrocyte phenotype as the cells become more fibroblast-like. This study investigated the difference in collagen type I and II mRNA expression between porcine chondrocytes in culture and in cartilage and found that there was a significant loss of collagen type II expression in cultured chondrocytes, indicating that porcine chondrocytes also undergo a phenotype shift towards a more fibroblast-like state in culture. This is further reinforced by the observed morphological changes in chondrocytes with increased passage number. Therefore, all experiments utilising primary porcine chondrocytes in this study were carried out at passage 0-1.

Schlichting and colleagues (303) have investigated the suitability of porcine chondrocytes for micromass culture and found that when in micromass cultures porcine chondrocytes exhibit similar properties when compared to human cartilage (303). Culturing porcine chondrocytes in 3D or micromass cultures helps retain the chondrogenic phenotype in these cells and may, therefore, be more useful as an in vitro model of cartilage (311).

**Response to interleukin 1 beta stimulation**

IL-1β has been implicated as a driver of inflammation and damage in conditions such as OA and rheumatoid arthritis (68,312), therefore the response of porcine cartilage and chondrocytes to IL-1β stimulation was of importance in determining the usefulness of this tissue in OA research.

Initial experiments on cartilage explants were carried out using recombinant human IL-1β. Nietfeld et al (313) had used human IL-1 at a much lower concentration of 100pg/ml on porcine cartilage explants previously and found that this stimulation was able to reduce the proteoglycan content in human and porcine cartilage without inducing proteoglycan release (313). High doses of human IL-1β were used in this study and would have been expected to induce significant increases in cartilage degradation and pro-inflammatory cytokine production in human cartilage (314). However, we have shown that human IL-1β does not induce the expected response in porcine cartilage and that high concentrations of this human cytokine were not able to induce cartilage degradation or pro-inflammatory cytokine release in porcine cartilage explants, as would be expected in human cartilage (315). In addition, a 17-plex human cytokine luminex was carried out to identify cytokine production in the conditioned media from porcine cartilage explants. This luminex failed to identify cytokines produced by porcine tissue indicating that a number of important cytokines are poorly conserved between the two species. For IL-1β, the sequence homology between the human and porcine proteins is only 58%; this could explain why the human protein is unable to induce the pro-inflammatory response that is associated with this cytokine. A previous study investigating the effect of human cytokines on porcine endothelial cells found that, of the 4 cytokines tested, only TNFα was found to cross-react (316). Together this data

suggests that cytokines are poorly conserved between pigs and humans, therefore, human IL-1β is not suitable for use in experiments with porcine tissue and porcine recombinant IL-1β was sourced for further experimentation.

Recombinant porcine IL-1β induced significant increases in both sGAG release and IL-6 production in a dose dependent manner. IL-6 production appeared to be slightly reduced with the maximum IL-1β concentration tested (10ng/ml). Whilst this difference was not significant, it may indicate that 10ng/ml IL-1β is potentially detrimental to cell health and may be inducing apoptosis in these cells (317). Therefore, a slightly lower maximum IL-1β stimulation of 3ng/ml was used for future experiments using porcine chondrocytes. This lower maximum concentration was sufficient to induce a significant increase in IL-6 production in porcine chondrocytes over a 24 hour period. Results from a number of animal models suggest that IL-1β plays a significant role in OA pathology: IL-1β blockade was able to reduce OA pathology in Dunkin Hartley guinea pigs (318); transferal of the interleukin-1 receptor agonist (IL-1Ra) gene prevented OA progression and reduced clinical symptoms in dogs, rabbits and horses induced OA models (181,319,320); and the intra-articular injection of human IL-1Ra has been found to reduce osteophyte and cartilage lesion formation in dogs, at part through reductions in collagenase-1 expression (321). However, this is not the case for all animal models, with IL-1β -/- knockout mice showing no protection against OA development following meniscectomy (322). Numerous treatments for human OA targeting IL-1 have been developed but the results of such treatments have been disappointing; more detailed discussion of some of these treatments can be found in Jotanovic and colleagues' 2012 review on the Role of IL-1 in OA (69).

Together the results from this study suggest that porcine IL-1β is capable of inducing a strong pro-inflammatory response in both porcine cartilage and

chondrocytes, as well as increased degradation in porcine cartilage. McNulty et al (252) have investigated the levels of both alpha and beta isoforms of IL-1 in the synovial fluid of pigs exhibiting OA joint damage and found that IL-1β concentration positively correlates with meniscus degeneration. Further research is required to clarify the role of IL-1β in spontaneous porcine OA pathology.

**Response to adipokine stimulation**

Adipokines have been identified as potential biomarkers of osteoarthritis in numerous studies (140,323,324). They have also been implicated as a possible non-biomechanical mechanism linking obesity and OA development (140,325). Adipokine concentrations are elevated in the synovial fluid of OA patients and are now being investigated as potential therapeutic targets for OA treatment. Leptin has been shown to be capable of regulating cartilage and bone metabolism (326). Synovial fluid levels of visfatin correlate with cartilage damage in rheumatic disease (326). Resistin concentration in synovial fluid is elevated in OA patients and has been shown to promote an abnormal Type I collagen phenotype in the subchondral bone of obese OA patients (162). Recent research has also suggested that adipokines, such as visfatin, may act synergistically with other cytokines, such as IL-1, to further promote joint degeneration and inflammation (141).

Nishimuta et al (142) suggested that the meniscus was more susceptible to the degradative effects of adipokines than cartilage; however, this study used juvenile bovine tissue (142). The maturation and ageing process can alter the metabolism and response to inflammatory stimuli, such as IL-1, in cartilage; therefore the responses of immature cartilage may not be reflective of the responses of mature cartilage (327). Hence, cartilage from animals that are not

yet skeletally mature is considered unsuitable for the investigation of the processes involved in OA development and progression.

In this study, visfatin stimulation results in a pro-inflammatory response in cartilage, whereby cartilage degradation is increased and pro-inflammatory cytokines are released. This response is similar to that observed in human tissue (295) suggesting that visfatin activates similar signalling networks in pigs and humans, which would potentially make porcine cartilage a good source of tissue for investigation into the mechanisms by which visfatin induces these responses.

The response of porcine tissue to recombinant human resistin and leptin was also investigated. Neither of these proteins induced any change in cartilage degradation and both were unable to induce IL-6 production above the minimum detectable concentration of the ELISA kit used. The differences in sequence similarities between pig and human could explain the lack of response observed, with sequence homology for resistin at 77% and leptin at 85%. Visfatin on the other hand is highly conserved with sequence homology between human and pig being 97%. Unfortunately, porcine recombinant proteins were not available for this study.

A previous study by McNulty et al (141) used porcine joint tissue obtained from an abattoir to examine the effects of adipokines over a 3 day stimulation period. This study revealed that up to 100ng/ml porcine leptin did not induce significant changes in sGAG release, MMP activity or NO release in either cartilage or meniscus tissue, suggesting that if porcine leptin had been available for this study the results for acute stimulation of cartilage explants would be no different (141). Human visfatin, up to 100ng/ml, was found to increase sGAG release without increasing MMP activity and also synergised with IL-1β to further increase sGAG release (141). Together with the results obtained in this study these results

support the hypothesis that visfatin may play an important role in cartilage degradation and the progression of rheumatic disease.

It is unclear whether there are significant differences between breeds of pig. Within the available literature there are a variety of pig breeds used but very little investigating the differences between these animals. The Tochigi pig project (328) investigates the use of the pig as a model of research and investigates a number of different pig breeds. In vivo work tends to be done in minipigs rather than the much larger commercial pigs due to the handling issues associated with such large animals and the additional housing and feeding costs. However, when looking at musculoskeletal issues the commercial pig has a wealth of documented evidence of spontaneously occurring joint degeneration and lameness whereas this is somewhat lacking in the minipig breeds. The research that is carried out on commercial pigs tends to use tissue obtained directly from abattoirs but does not identify the specific breed make-up of the animals used. Therefore, how reproducible this research is with other breeds and whether each commercial line is sufficiently similar to human cartilage to produce reflective results is unknown. There is also great variety in the age of these animals with some papers using pigs as young as 6 months to study the development of OA models and others using 2-3yr old pigs, that are stated to be skeletally mature and are therefore more appropriate for use in musculoskeletal ageing research (271,303).

Therefore, there is a requirement for enhanced clarity in the research utilising porcine tissue and, whilst there is a considerable body of research using tissue from these animals, there is very little research investigating spontaneous OA in these animals.

## Conclusion

Together these results suggest that porcine cartilage explants and isolated chondrocytes respond to the pro-inflammatory cytokines, IL-1β and visfatin, in a similar manner to human cartilage. Previous research has found that synovial fluid concentrations of IL-6 in knee osteoarthritis ranges from 0.1-4495.3pg/ml (329). IL-1β stimulation of chondrocyte cultures up to a concentration of 0.3ng/ml and visfatin stimulation of chondrocyte cultures at 500ng/ml induce the production of IL-6 within this physiologically relevant range. Therefore, the pig could provide a source of both healthy and damaged cartilage for *ex-vivo* studies to investigate and elucidate the mechanisms underlying the development and progression of osteoarthritis.

# 7.0 UTILITY OF THE PIG AS A SOURCE OF BONE FOR EX-VIVO STUDIES IN OA RESEARCH

## 7.1 INTRODUCTION

The majority of human tissue available for research into understanding the pathogenesis of osteoarthritis is obtained following elective joint replacement surgery and therefore represents late-stage disease. Healthy human joints for comparison purposes are only available from cadavers or patients undergoing joint replacement following hip fracture and there is no guarantee that these joints will not be damaged. Therefore, primary human osteoblasts are often obtained from end-stage OA knee joints and could be exhibiting a different phenotype in culture when compared to those obtained from healthy joints, or from joints with signs of early OA (296). Therefore, the pig could present an alternative source of both healthy and damaged joints to obtain primary osteoblasts from for studies of osteoblast functionality and responses. Further details of osteoarthritic bone were discussed in Section 1.4.1.

Previous studies (153,330) have used primary murine osteoblasts but due to the size of the animals the amount of usable tissue is limited and if osteoarthritic symptoms have been induced as is commonplace, it is possible that the bone changes and remodelling occurring in the joint do not reflect the processes occurring in spontaneous human osteoarthritis. Induced models of OA also tend to result in a severe form of the condition with extensive damage to the joint making the study of OA development and early processes difficult in these models (130).

The MC3T3-E1 cell line from C57BL/6 calvaria mice are a fibroblast-like pre-osteoblast cell line which have been used as an alternative to primary osteoblasts (331). Studies investigating the phenotypic differences between these cells and

human primary osteoblasts have concluded that the cells are valuable for initial *in vitro* study of osteoblast function but they cannot replace primary cells due to significant differences in gene expression when compared to human osteoblasts (331). Primary porcine osteoblasts could potentially overcome some of the issues associated with current primary and cell line osteoblasts.

### 7.1.1 AIMS

The purpose of this chapter is to investigate the phenotypical and functional capacity of primary porcine osteoblasts and the responses of these osteoblasts to stimulation with pro-inflammatory adipokines, discussed in Chapter 6.

Main aims of this chapter:

- Establish if porcine osteoblasts mineralise in the culture conditions used.
- Investigate the functional responses of osteoblasts to pro-inflammatory adipokine stimulation.
- Determine if there are functional or metabolic differences between osteoblasts obtained from normal, healthy joints and those obtained from joints exhibiting osteoarthritic damage.

## 7.2 METHODS

Bone remodelling occurs in the joint, narrowing joint spaces and altering the biomechanics of the joint (96). These detrimental changes were investigated through the following methods by looking at the specific changes in the growth and activity of osteoblasts isolated from the femoral condyles taken from both normal and damaged joints.

### 7.2.1 Primary porcine osteoblasts

The following experiments were carried out using primary porcine osteoblasts isolated from bone chips taken from porcine knee joints. The cell lysate from these experiments underwent further analysis to investigate ALP activity, which is described later in this methods section.

### 7.2.2 Growth rates of primary porcine osteoblasts

Once primary porcine osteoblasts reached 85% confluence, flasks were split and the proliferation rates of the cells were determined by seeding cells on 24 well plates at two known densities. Low density wells were seeded with 5000 cells per well on Day 0; high density wells were seeded with 10000 cells per well on Day 0. At regular intervals over a 14 day time course, cells were trypsinised and counted using a haemocytometer, as described in Section 2.6.6.

This experiment was used to determine if there were significant differences in the growth rates of cells obtained from two different age groups (80 week and 3-4 year age groups).

### 7.2.3 Alizarin red mineralisation assay

In order to determine the mineralisation ability of osteoblasts, cells were grown for 3 weeks after reaching confluence on 24 well plates before being stained with sterile filtered 0.5% alizarin red, adjusted to pH 4.0, for 10 mins. The wells were then washed and photographed before being frozen at -20°C for stain extraction

and quantification at a later date. 80 week and 3-4 year age groups of pigs were investigated using this measure.

Alizarin red staining was quantified by thawing the stained plates, adding 200µl of 10% w/v cetyl pyridinium chloride per well of the 24 well plate and incubating the plate for 10 mins at room temperature. The supernatant was then collected and diluted 1:10 with PBS, loaded onto a 96 well plate in triplicate and the absorbance measured at 550nm (Bio-Rad 680XR microplate reader) (332).

### 7.2.4 Alkaline phosphatase (ALP) assay

A stock solution of 100 units/ml was made using human alkaline phosphatase (ALP) (from human placenta) and diluted with 1mM magnesium chloride to produce standards ranging from 0.005 to 0.3 units/ml. Osteoblast cell lysate was diluted as required using 1mM magnesium chloride and 10µl of the standard or sample was added to each well of a 96 well plate. 100µl of alkaline phosphatase substrate was then added to all wells and the plate was incubated at 37°C for 15 mins before being stopped by the addition of 20µl 0.1M sodium hydroxide to each well. Absorbance was measured at 405nm using a Bio-rad 680XR microplate reader. The absorbance readings of the standards allowed the production of a standard curve which was used, along with the absorbance reading of samples, to determine the concentration of ALP in the samples.

### 7.2.5 Mitochondrial ATP production assay

#### 7.2.5.1 Osteoblast sample preparation

Osteoblast cells obtained from bone (detailed in Section 2.6.4) suspended in 2ml Buffer A (detailed in Appendix A) were transferred into an ice cold 5ml homogenisation vessel and homogenised at 150xg for 4 mins with slow vertical plunger movements. The sample was then transferred to 1.5ml microcentrifuge tubes that had been chilled in ice and centrifuged at a temperature of 3-4°C and

720xg for 1 min to pellet any cellular debris. The resultant supernatant was transferred to clean pre-cooled microcentrifuge tubes and centrifuged at 10000xg for 5 mins to pellet the mitochondria. The supernatant was carefully removed and the pellet resuspended using 400µl Buffer B (225mM sucrose, 44mM potassium phosphate monobasic, 12.5mM magnesium acetate, 6mM EDTA).

#### 7.2.5.2 Bioluminescence protocol for ATP production measurement

A 96 well plate protocol was used for the bioluminescence measurement. Each well contained 25µl of the sample in buffer B, 110µl Tris-EDTA buffer, 25µl ADP and 40µl ATP reagent SL (Biothema ATP Reagent SL Kit). Samples were run in triplicate with a negative control where the sample was replaced with 25µl Tris-EDTA buffer, also run in triplicate. Measurements were run one well at a time using a FLUOstar plate reader on luminescent setting.

### 7.2.6 Citrate synthase assay

Citrate synthase activity is proportional to mitochondrial number, therefore this measure was used to normalise maximum mitochondrial ATP production to mitochondrial number. Prior to processing, osteoblasts suspended in PBS were frozen and stored at -80°C (333).

In this assay, citrate synthase produces thionitrobenzoic acid (TNB) in a 2 step reaction (shown below). This is a spectrophotometric assay whereby TNB production is measured based on absorption at 415nm. Increased TNB production results in increased absorption readings.

**Reactions:**


Experimental procedure:

A reaction mix was prepared as shown in Table 7.1, with the oxaloacetate being added immediately before measuring absorbance on a Bio-rad 680XR microplate reader.

**Table 7.1: Citrate synthase assay reaction components**

| Component | Samples (µl) | Positive control (µl) [Citrate Synthase (CS)] | Negative Control (µl) [Blank] |
|---|---|---|---|
| Triton X-100 | 6.25 | 6.25 | 6.25 |
| Acetyl Co-A | 6.25 | 6.25 | 6.25 |
| DTNB | 25 | 25 | 25 |
| Cell Suspension | 25 | 2 (CS standard) | 0 |
| Distilled Water | 175 | 198 | 200 |
| Oxaloacetate | 12.5 | 12.5 | 12.5 |

A kinetic protocol was used, reading one well of a 96 well plate at a time, immediately following the addition of the oxaloacetate component to that well. This protocol measured absorption at 415nm every 10 seconds over a 120 second period.

The maximum velocity of the TNB producing reaction was determined and used as a measure of citrate synthase activity in the osteoblast cell sample. Reaction components were tested using a citrate synthase standard as a positive control; a negative control sample (blank) was also run using water as a sample substitute to ensure the values obtained from the samples were a true measure of citrate synthase levels.

The citrate synthase assay was carried out and used to normalise maximum mitochondrial ATP production data for mitochondria number.

### 7.2.7 Acute stimulation with adipokines

Primary porcine osteoblasts were stimulated for 24 hours with media containing the pro-inflammatory adipokines; leptin, resistin or visfatin. Following this stimulation the osteoblasts were lysed using cell lysis buffer as detail in Section 2.5 and the lysate ALP activity was assessed (Section 7.2.4).

**Experiment 1: Human adipokine stimulation of porcine osteoblasts**

Osteoblasts were exposed to media containing 100 or 300ng/ml human recombinant leptin (141,152,334), 30 or 100ng/ml human recombinant resistin (335) or, 30 or 100ng/ml human recombinant visfatin (141) in osteoblast media, for 24 hours. Osteoblasts exposed to media only were used as unstimulated control samples for comparison purposes.

### 7.2.8 Chronic stimulation with visfatin

Primary porcine osteoblasts were stimulated for 14 days with media containing recombinant human visfatin. Following this stimulation the osteoblasts were lysed as detailed in Section 2.7 and the lysate ALP activity assessed (Section 7.2.3). RNA was also obtained from cells through harvesting cells in Trizol reagent.

**Experiment 1: Human adipokine stimulation of porcine osteoblasts**

Osteoblasts were exposed to media containing 100, 300 or 500ng/ml (152,334) recombinant human leptin; 30, 100 or 300ng/ml recombinant human resistin (162,335) or 30, 100 or 300ng/ml human recombinant visfatin (141) in osteoblast media for 14 days. Osteoblasts exposed to media only were used as unstimulated control samples for comparison purposes.

**Experiment 2: Human visfatin stimulation of porcine osteoblasts from damaged and non-damaged knee joints**

Osteoblasts were exposed to media containing 30, 100, 300 or 500ng/ml (141,162,295) human recombinant visfatin in osteoblast media for 14 days. Osteoblasts exposed to media only were used as unstimulated control samples for comparison purposes.

### 7.2.9 RNA extraction from osteoblasts using Trizol method

Cells harvested in Trizol were stored in sterile microcentrifuge tubes at -80°C until processing. 60µl of chloroform was added to each 300µl sample in trizol, microcentrifgure tubes were sealed and shaken vigorously for 15 seconds. After a 3 min room temperature incubation the samples were centrifuged at maximum speed for 15 mins at 4°C. This process separates the samples into three phases. The upper most phase was a clear aqueous layer containing the RNA and this phase was carefully removed to a new sterile microcentrifuge tube. 150µl isopropanol was added to the aqueous phase and the samples were incubated at room temperature for 10 mins and then on ice for a further hour. Samples were then centrifuged at maximum speed for 15 mins at 4°C. Supernatant was removed, leaving a small pellet of RNA. This pellet underwent 2 ethanol washes whereby 300µl 80% ethanol was added and centrifuged at maximum speed for 10 mins after a 10-15 min on ice incubation. Supernatant was removed and the ethanol wash repeated once more.

Following the second wash the resultant RNA pellet was air-dried for 10 mins, resuspended in 20µl RNase free water and incubated at 42°C for 5 mins prior to concentration measurement using a Thermoscientific Nanodrop.

### 7.2.10 Statistical analysis

Statistical analysis was carried out using Graphpad Prism software version 7.0. T-tests were used for mitochondrial ATP production and citrate synthase activity experiments. Linear regression was carried out on proliferation rate data. One-way ANOVAs were utilised for the remaining experiments investigating the response of osteoblasts to adipokine stimulation and osteoblast proliferation rates followed by Tukeys post hoc test and/or post hoc test for linear trend. Significance accepted at the $P<0.05$ level.

7.3 RESULTS

### 7.3.1 Culture of porcine osteoblasts

Porcine osteoblasts were isolated and cultured from bone chips obtained from the femoral condyle of porcine knee joints as described in Section 2.4.4. Figure 7.1 shows representative images of primary porcine osteoblasts during the initial outgrowth of cells from bone chips. The time taken for initial outgrowth from bone chips to occur varied from 7-23 days between individual pigs. Following outgrowth from bone chips, osteoblasts adopt a flattened and dendritic morphology with reaching processes.

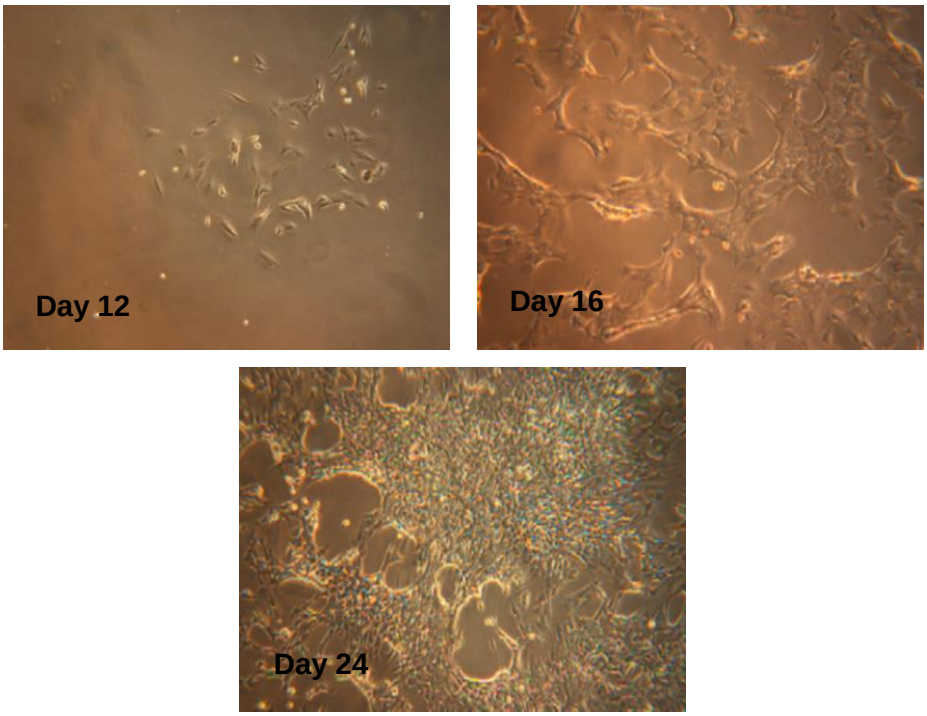


**Figure 7.1: Representative light microscope images of osteoblasts at different stages of growth approaching confluence.** (6.3x magnification) Images show growth of osteoblasts from a single flask between 12-24 days of bone chip culture. Osteoblasts were obtained from 80 wk old pig knee joint.

### 7.3.2 Proliferation rate and mineralisation capacity of porcine osteoblasts

In osteoblasts from 80wk old non-damaged knee joints a higher seeding density resulted in a significantly faster growth rate ($p=0.0091$) when compared to osteoblasts seeded at a lower density (Figure 7.2A). However, osteoblasts from 3-4 year old damaged knee joints did not exhibit this increased growth rate when seeded at the higher density (Figure 7.2B). In these osteoblasts the proliferation rates of cells at high and low density seeding levels were similar. Therefore, the response to increased seeding density in culture was lost in osteoblasts from 3-4 year old damaged joints. Joint condition was determined prior to isolation of cells by chondropathy scoring, as discussed in Section 5.3.2. Knee joints were considered to be damaged when the revised SFA score was greater than or equal to 35, joints obtaining scores higher than this level exhibited extensive minor cartilage fibrillation, some major fibrillation of cartilage, or a degree of subchondral bone exposure. Joints with revised SFA scores lower than 35 can exhibit some damage but this is restricted to cartilage discolouration and swelling or a small amount of minor cartilage fibrillation.

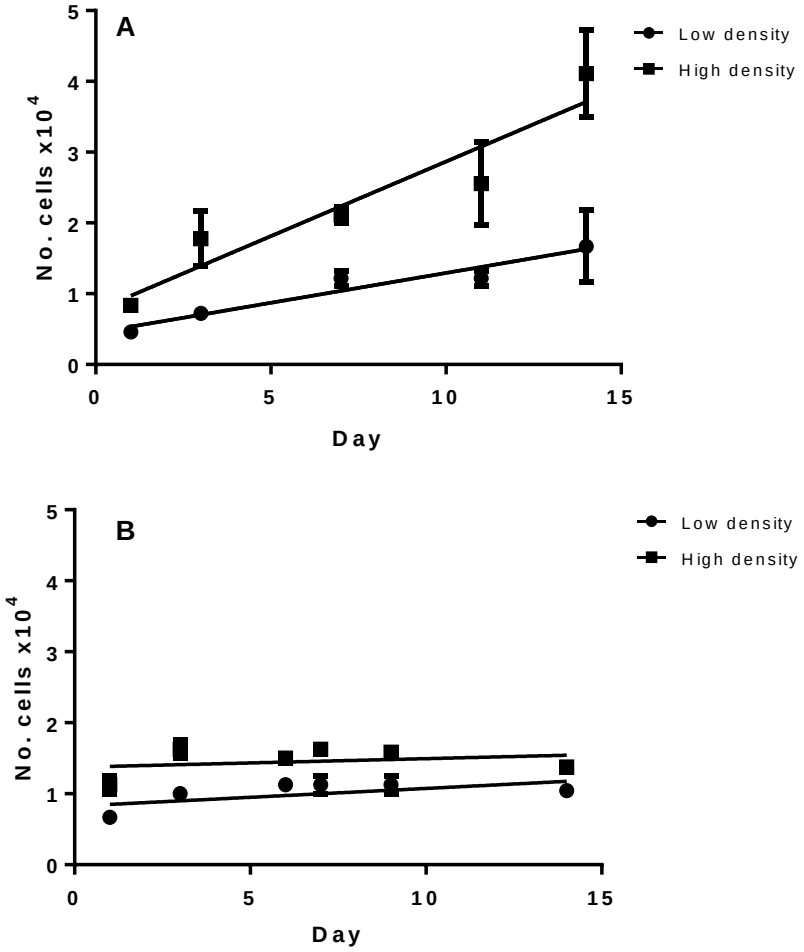


**Figure 7.2: Proliferation rate of primary porcine osteoblasts isolated from non-damaged knee joints from 80 wk old pigs and damaged knee joints from 3-4 yr old pigs.** Individual points represent the mean cell number ± SEM. **A:** Osteoblasts obtained from non-damaged joints from 80 wk old pigs **B:** Osteoblasts obtained from damaged joints from 3-4 yr old pigs.

Primary osteoblasts have been shown to form mineralised bone nodules in culture. Alizarin red staining has previously been used to visualise and quantify these mineralised deposits in osteoblast cultures. Figure 7.3 shows that primary porcine osteoblasts obtained from healthy 80wk old pig knee joints develop mineralised deposits in culture.

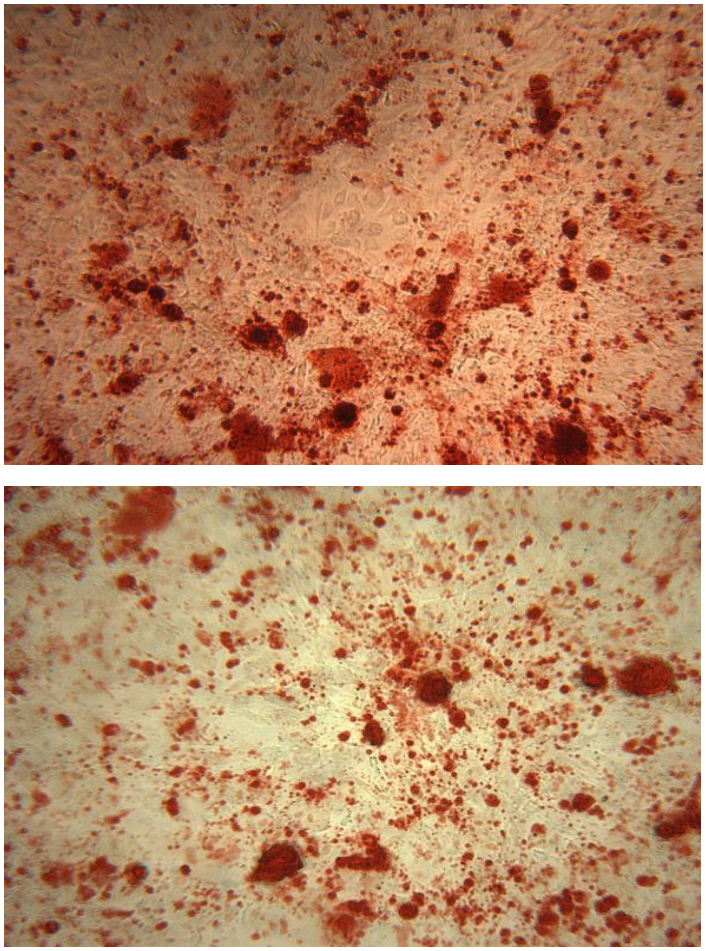

**Figure 7.3: Mineralisation in porcine osteoblast culture.** Initial alizarin red staining carried out using osteoblasts from non-damaged 80 week old pig knee joints (6.3x magnification).

Porcine osteoblasts were grown in culture for 10-12 wks after reaching confluence to allow the formation of mineralised bone nodules. Osteoblasts obtained from 80 wk old non-damaged joints were able to form mineralised bone nodules as shown in Figure 7.4A, however, osteoblasts obtained from 3-4 yr old

damaged knee joints exhibited reduced proliferation capacity (Figure 7.2B) and a lack of mineralisation as shown in Figure 7.4B, despite an additional 2 weeks of culture.

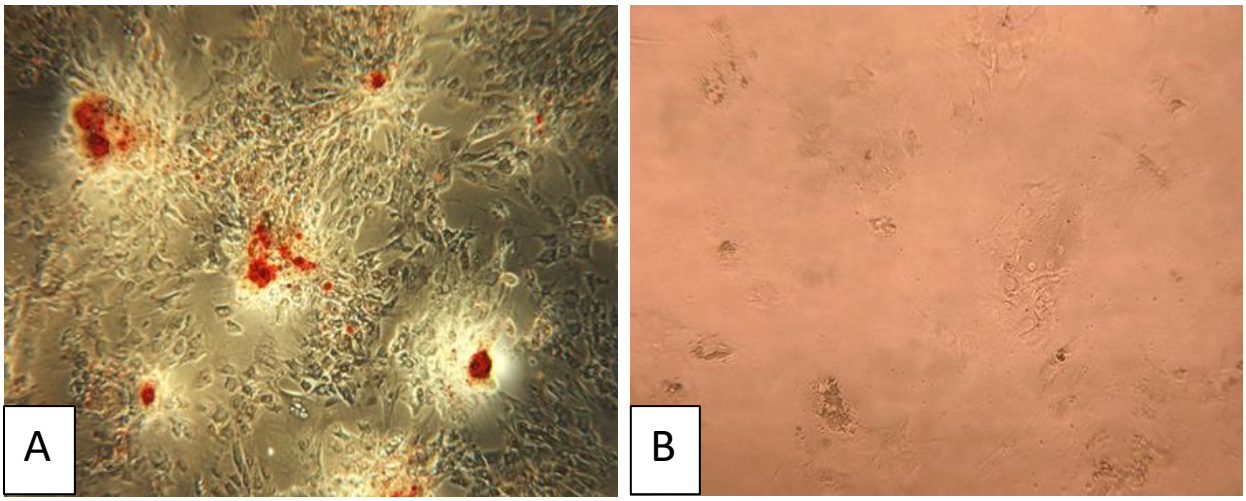


**Figure 7.4: Representative light microscope images of mineralisation in osteoblasts isolated from non-damaged and damaged joints A:** Osteoblasts from non-damaged knee joints from an 80 wk old pig 10 weeks post-seeding, stained with Alizarin red, **B:** Osteoblasts from damaged knee joints from a 3-4 yr old pig 12 weeks post-seeding (6.3x magnification).

### 7.3.3 Metabolic assessment of porcine osteoblasts

Citrate synthase activity was assessed as a measure of metabolic function in osteoblasts and to allow the normalisation of ATP production results for mitochondrial number. There was no significant difference in citrate synthase activity between 40 wk and 3-4 yr old pig groups (Figure 7.5A). However, osteoblasts obtained from damaged knee joints exhibited significantly reduced maximum velocity of a citrate synthase driven reaction and therefore reduced citrate synthase activity (Figure 7.5C; $p=0.0009$).

Maximal mitochondrial ATP production was assessed as a metabolic measure in these osteoblasts. Osteoblasts from the 3-4 year age group of pigs did not exhibit any significant difference in maximal mitochondrial ATP production when compared to the 40 week age group (Figure 7.5B). However, osteoblasts obtained from damaged knee joints exhibited significantly lower mitochondrial ATP production when compared to those obtained from non-damaged joints (Figure 7.5D; p=0.002).

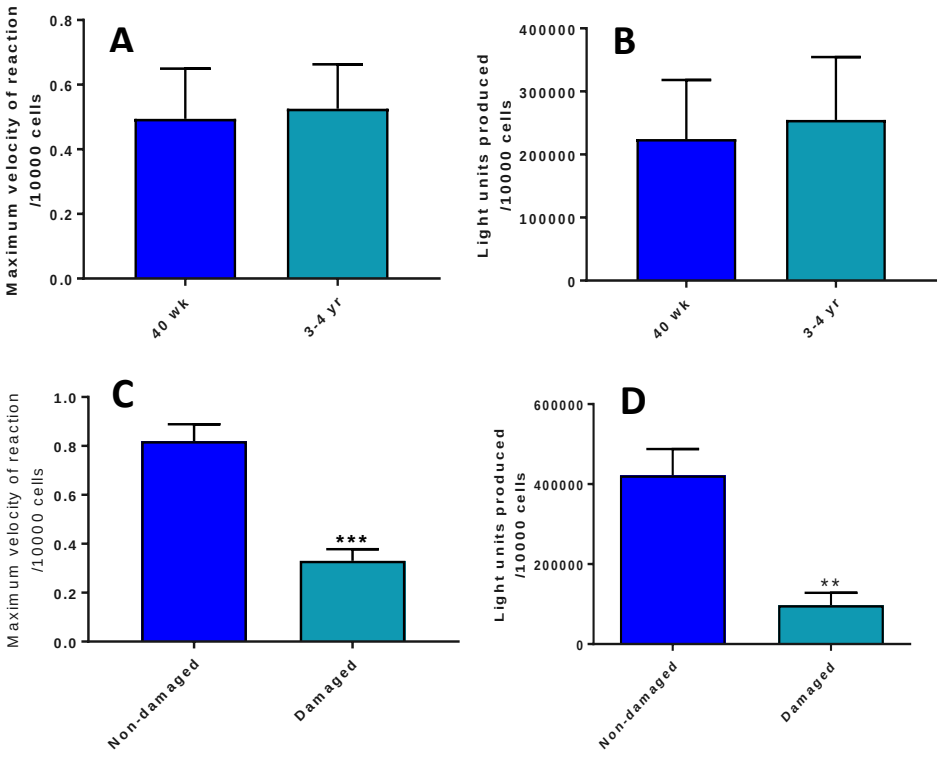


**Figure 7.5: Metabolic assessment in osteoblasts. A & C:** Citrate synthase activity. Results represent the mean maximum velocity of a citrate synthase driven reaction ± SEM. Comparison of osteoblasts from **A:** 40 wk and 3-4 yr age groups. No significant difference between the age groups determined by t test (p=0.8874); **C:** non-damaged and damaged knee joints. Significantly lower maximum velocity exhibited by osteoblasts from damaged joints determined by t-test (***p=0.0009). **B & D:** Maximal mitochondrial ATP production. Results represent mean light units produced ± SEM. Comparison of osteoblasts from **B:** 40 wk and 3-4 yr age groups. No significant difference between age groups determined by t test (p=0.8343); **D:** non-damaged and damaged knee joints. Significantly lower maximal mitochondrial ATP production exhibited by osteoblasts from damaged joints determined by t-test (** p = 0.0020)

Normalisation of maximal mitochondrial ATP production to citrate synthase activity controls for mitochondrial abundance. When normalised to citrate synthase activity there is no significant difference in ATP production in osteoblasts from the 40 week and 3-4 year age groups (Figure 7.6A) or from non-damaged and damaged knee joints (Figure 7.6B), suggesting that the lower metabolic activity in osteoblasts from damaged joints is due to reduced mitochondrial abundance.

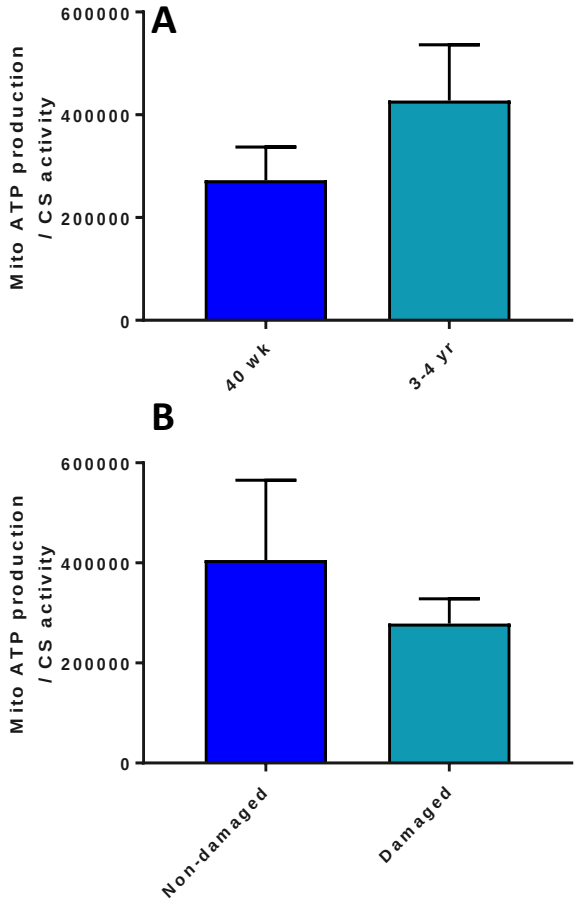


**Figure 7.6: Maximal mitochondrial ATP production normalised to citrate synthase activity.** Comparison of osteoblasts from **A:** 40 wk and 3-4 yr age groups. No significant difference between age groups determined by t test (p=0.3458); **B:** non-damaged and damaged knee joint. No significant difference between damaged and non-damaged knee joints determined by t test (p=0.4291).

### 7.3.4 Acute adipokine stimulation of primary porcine osteoblasts

Elevated adipokine concentrations in the synovial fluid of osteoarthritic joints have been implicated as a link between obesity and increased risk of osteoarthritis development. Therefore, the response of primary osteoblasts to adipokine stimulation was of interest. These experiments were initially designed to establish the acute response of osteoblasts to 24 hour stimulation with a given concentration of an adipokine. Alkaline phosphatase activity was used as a measure of osteoblast functionality (336). Stimulation of osteoblasts with recombinant human leptin (Figure 7.7A) produced a slight but non-significant reduction in ALP activity with increasing leptin concentration. Neither recombinant human resistin (Figure 7.7B) nor recombinant human visfatin (Figure 7.7C) induced any significant changes or trends in ALP activity of osteoblasts at the concentrations used.

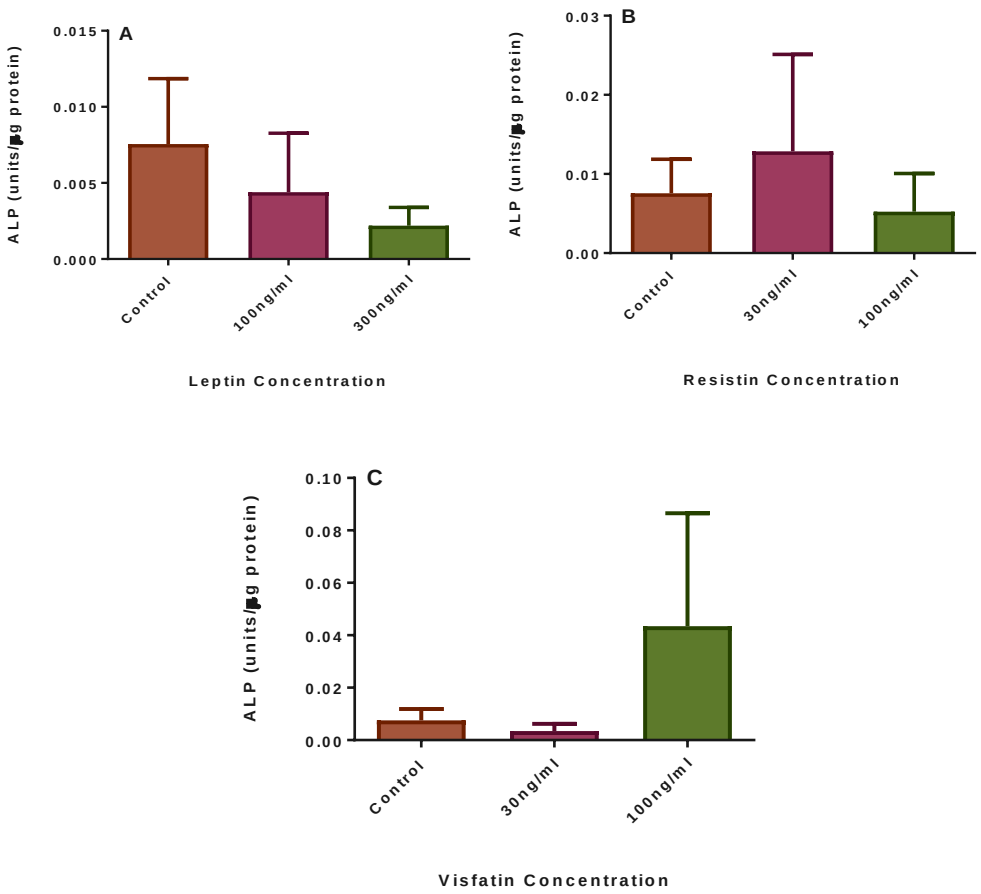


**Figure 7.7: Effect of acute adipokine stimulation on ALP activity of osteoblasts isolated from non-damaged knee joints of 80wk old pigs.** Results shown as mean ± SEM. **A:** Leptin stimulation; no significant effect of leptin stimulation on ALP activity determined by one way ANOVA (p=0.6652) **B:** Resistin stimulation; no significant effect of resistin stimulation on ALP activity determined by one way ANOVA (p=0.7726) **C:** Visfatin stimulation; no significant effect of visfatin stimulation on ALP activity determined by one way ANOVA (p=0.3423).

### 7.3.5 Chronic adipokine stimulation of primary porcine osteoblasts

Given the chronic nature of both osteoarthritis and obesity, longer term adipokine stimulations were also investigated. Stimulations were carried out over a period of 14 days and included a higher concentration of the adipokines than had previously been tested. These were preliminary experiments using cells

from 2 different pigs from the 3-4 yr age group; one pig exhibited joint damage, the other did not. These experiments would inform future work.

Chronic stimulation of osteoblasts with various concentrations of recombinant human leptin (Figure 7.8A) produced variable results with no significant changes or trends in ALP activity. Similarly, chronic stimulation of osteoblasts with various concentrations of recombinant human resistin (Figure 7.8B) produced no significant changes or trends in ALP activity. In contrast, chronic stimulation of osteoblasts with visfatin showed a significant trend towards reduced ALP activity with increased recombinant human visfatin concentration. (Figure 7.8C; *p=0.0233)

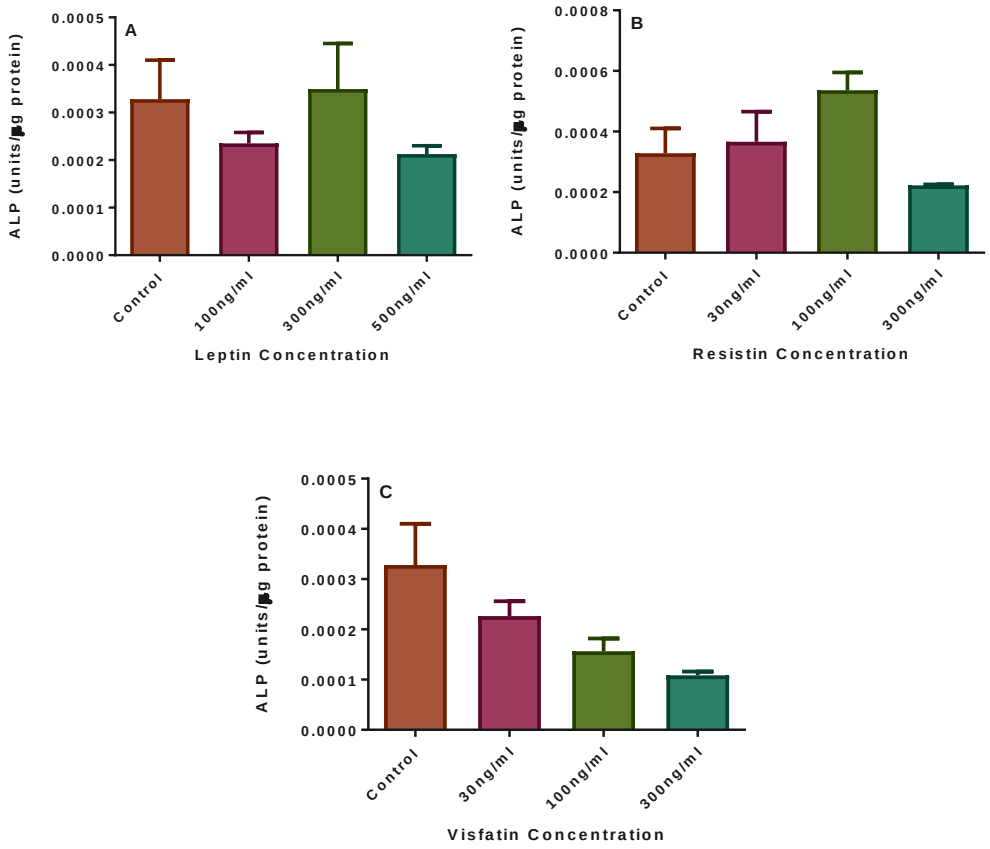


**Figure 7.8: Preliminary investigation of the effect of chronic adipokine stimulation (14 days) on ALP activity of osteoblasts isolated from the knee joints of 3-4 year old pigs.** Results shown as mean ± SEM. **A:** Stimulation with human recombinant leptin. No significant effect of leptin determined by one way ANOVA (p=0.4475); **B:** Stimulation with human recombinant resistin. No significant effect of resistin determined by one way ANOVA (p=0.1342); **C:** Stimulation with human recombinant visfatin. No significant effect of visfatin determined by one way ANOVA (p=0.0939): post-test for linear trend revealed a significant trend across the visfatin stimulations (p=0.0233). (n=2 for all stimulations)

A further set of experiments using chronic visfatin stimulation were designed and analysis of results revealed an apparent difference in the response of cells obtained from 3-4 years old damaged and 40 week old non-damaged joints. In non-damaged joints a low level of visfatin stimulation (30ng/ml) appeared to have a slightly beneficial effect on ALP activity, however, this effect was lost with increasing concentration. In damaged joints even a low visfatin concentration was detrimental to ALP activity and therefore may reflect a reduction in

osteoblast cell function with a significant trend towards decreased ALP activity with increasing concentration of visfatin.

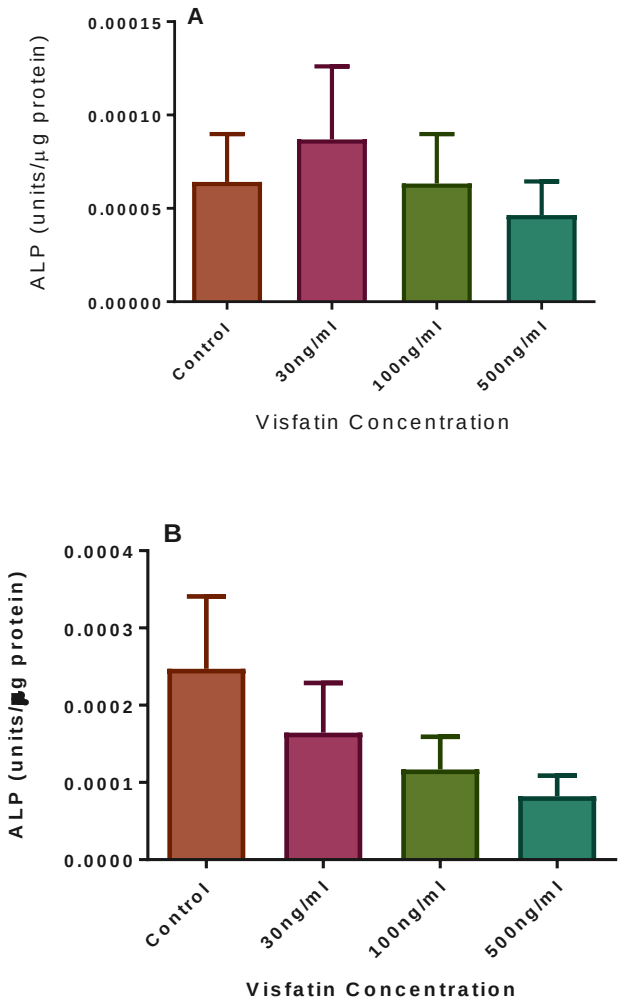


**Figure 7.9: Effect of chronic human visfatin stimulation on ALP activity of osteoblasts isolated from 40 wk non-damaged and 3-4 yr old damaged knee joints. A:** Osteoblasts from 40 wk old non-damaged knee joints; no significant linear trend towards reduced ALP activity in osteoblasts from non-damaged 40 wk old knee joints (p=0.1322). **B:** Osteoblasts from 3-4 yr old damaged knee joints; significant trend towards reduced ALP activity in osteoblasts from damaged 3-4 yr knee joints as determined by post-hoc test for trend (p=0.0159).

Investigation of ALP activity in osteoblasts from 3-4 year old damaged and 40 week old non-damaged joints revealed that the ALP activity of unstimulated cells from 3-4 year old damaged joints was 5.5 times greater than that of osteoblasts from 40 week old non-damaged joints on average (Figure 7.10). However, this

difference was not statistically significant due to high individual variability in the ALP activity in osteoblasts from 3-4 year old damaged knee joints.

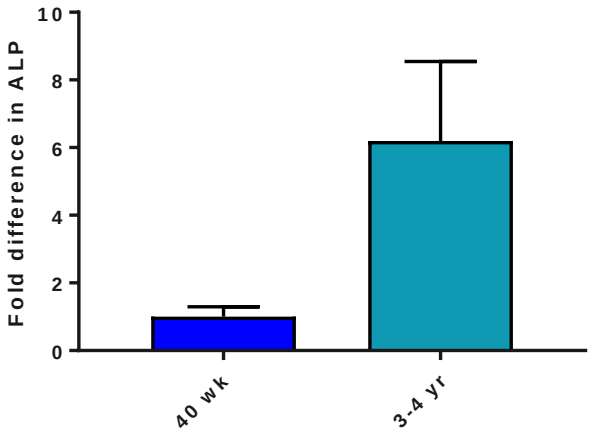


**Figure 7.10: Basal ALP activity in osteoblasts isolated from 40 wk old non-damaged knee joints and 3-4 yr old damaged knee joints.** Values represent fold change from non-damaged ALP activity ± SEM. No significant difference ALP activity in osteoblasts from non-damaged 40 wk old and damaged 3-4 yr old knee joints. Statistical significance determined by students t test (p=0.0932).

## 7.4 DISCUSSION

### Primary porcine osteoblasts as a research tool

This study has demonstrated that primary porcine osteoblasts can proliferate and mineralise in culture conditions previously used for human osteoblast research(162). This research has also confirmed that assays used for human tissue and cells; namely the ALP assay, alizarin red staining and metabolic assays, all work well in the pig. Although ALP activity has been used as a measure of osteoblast function, it is not advisable to use this as the only readout due to the apparent disconnection between ALP activity and alizarin red staining in porcine damaged joint osteoblasts and human sclerotic bone osteoblasts (74,75). Alizarin red staining provided a clear indication of osteoblast bone nodule formation and this could be quantified in osteoblasts from non-damaged joints, this measure should be used in conjunction with ALP activity measures of osteoblast functionality.

### Functional and metabolic differences between osteoblasts from damaged and non-damaged knee joints

This study has shown that the proliferation and mineralisation capacity in osteoblasts obtained from 3-4 yr old damaged knee joints was impaired compared to that of osteoblasts obtained from 80 wk old non-damaged joints. In contrast, the mean ALP activity in these 3-4 yr old damaged joint osteoblasts was slightly greater than that of the 80 wk non-damaged joint osteoblasts. An altered phenotype of OA osteoblasts has been reported previously. In a 1998 paper, Hilal et al (74) investigated the altered phenotype of OA bone looking at a number of factors in OA bone explants and primary OA osteoblasts. Of particular interest to

this research, they showed that ALP activity and osteocalcin release were both elevated in OA osteoblasts when compared to normal osteoblasts (74). Osteocalcin KO mice exhibit increased bone formation and improved functional capacity of bone (337), therefore, elevation of both ALP and osteocalcin could contribute to increased bone density observed in the dense, under-mineralized bone found in OA joints. Couchourel et al (338) proposed that this elevation in ALP coupled with reduced mineralisation could suggest that OA osteoblasts are undergoing incomplete differentiation (338). Hilal's work also investigated IGF-1 release from OA explants and showed it was increased in OA osteoblasts, but IGF-1 release was not investigated here in the pig (74).

Sanchez et al (75) expanded on this field of work and compared osteoblasts from sclerotic and non-sclerotic bone in OA patients, although a limitation of this research is that it lacked a healthy control. This approach is likely linked to the difficulties in obtaining healthy human joints for research, since non-OA joints can only be obtained from cadavers or from hip fracture patients where a joint replacement has been implemented as treatment. This does not guarantee that joints will be in good condition. In fact, hip fracture patients are most often elderly and their joints exhibit, if not osteoarthritis, then other age-related deterioration, such as osteoporosis. Use of the pig as a model of spontaneous OA would allow a readily available source of both healthy and damaged joints which exhibit many of the same characteristics as human OA joints. Sanchez et al (75) characterised the phenotype of sclerotic bone osteoblasts from OA joints finding that sclerotic osteoblasts exhibited increased ALP activity, but reduced mineralization as measured by alizarin red staining (75). These results agree with those obtained by Hilal et al and support those obtained in this study in pigs (74). A number of other aspects were also found to vary between sclerotic and non-sclerotic bone osteoblasts and although these measures have not been

investigated in porcine osteoblasts as part of this study, they would be of interest in future work using this model. These factors include increased protein synthesis of osteopontin, IL-6, IL-8 and TGF-β1 and upregulated gene expression of MMP-13, COL1A1 and COL1A2, osteopontin, alkaline phosphatase, osteocalcin, vascular endothelial growth factor and ANKH (74,75,339). Of particular interest is ANKH, which mediates transmembrane trafficking of PPi, a substrate of ALP (75,340). Whilst PPi is a substrate of ALP, high levels of PPi can also inhibit the formation of hydroxyapatite crystals during the bone mineralisation process (340). Therefore high expression of ANKH could inhibit bone mineralisation and the uncoupling of ANKH and ALP could explain the reduced mineralisation in sclerotic or OA osteoblasts despite elevated ALP activity (340). This altered phenotype may be having a detrimental impact on the overlying chondrocytes and has been shown, in a co-culture model, to reduce aggrecan production while increasing the expression of MMP-3 and MMP-13 and shifting chondrocyte phenotype towards hypertrophic differentiation, which is exhibited in OA chondrocytes (339,340).

This study has revealed that metabolic dysfunction was observed in the osteoblasts isolated from damaged pig joints and that this dysfunction did not appear to be linked to the age of the pig from which the osteoblasts were obtained. This could be contributing to the osteoblast functional issues. Both citrate synthase activity and maximal mitochondrial ATP production were significantly reduced in osteoblasts from damaged compared to non-damaged knee joints. Maximal mitochondrial ATP production was normalised to citrate synthase activity to control for mitochondrial number. The differences in mitochondrial ATP production observed between osteoblasts obtained from damaged and non-damaged joints are no longer evident once normalised to citrate synthase activity suggesting that the apparent functional differences could

reflect fewer mitochondria in the osteoblasts from damaged joints and this could, in part, explain the reduction in proliferation and mineralisation despite slightly increased ALP activity.

Work investigating mitochondrial function in osteoarthritic osteoblasts is limited; however, mitochondrial dysfunction has been identified as an important factor in osteoarthritic chondrocytes (341–344) and in osteoblasts in the pathogenesis of osteoporosis (345). Research carried out by Maneiro et al (341) and Blanco et al (342) suggested that osteoarthritic chondrocytes exhibited reduced mitochondrial complex II and II activity and that this difference was due to cartilage damage and was not associated with age (341). Similarly, Terkeltaub et al (343) proposed that perturbed ATP production mechanisms in chondrocytes could be playing a role in the development and progression of OA; this is supported by work by Johnson et al (344) that concluded that reduced chondrocyte respiration could induce or promote cartilage matrix loss. Mitochondrial dysfunction has also been implicated in pathologies involving osteoblasts, such as osteoporosis; oxidative stress in osteoporotic osteoblasts results in oxidative damage to mitochondrial function (345).

SIRT1 mRNA and protein expression has been shown to be suppressed in OA subchondral bone (346) and reduced SIRT1 activity results in diminished proliferation, mineralisation and osteoblastic differentiation of stem cells (347). Therefore, the functional phenotype observed in porcine osteoblasts from damaged joints could be linked to reduced expression of SIRT1, although this was not investigated. SIRT1 is a member of the sirtuin family of deacetylases and can contribute to the regulation of metabolic and epigenetic processes within the cell. SIRT1 has been implicated in mitochondrial biogenesis through interaction with peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) (348–350)

and in mitochondrial turnover by mitophagy (350,351). Mitochondrial dysfunction has been found to contribute to the development of cellular senescence and work by Wiley et al (136) also implicated mitochondrial SIRTs such as SIRT3 as suppressors of senescence suggesting that this family of proteins could influence the mitochondrial metabolic and functional capacity of cells (136). Sirtuin activity has been shown to reduce with age and NAD+ decline has been suggested as a driver of this decline (352). Therefore, the expression of SIRTs in OA osteoblasts and the ability of these proteins to regulate osteoblast phenotype and mitochondrial function would be of great interest for future research.

The persistence of osteoblast functional differences in culture would suggest that these cells may have undergone lasting detrimental changes within the damaged joint. One mechanism by which cells could be influenced by the damaged joint environment is through epigenetic modification. Changes to epigenetic processes have been implicated in a number of late-onset or age-related human diseases (131). In OA the majority of studies have investigated epigenetic changes in chondrocytes (131,133) although it has been shown that epigenetic processes can also affect certain processes in subchondral bone (134). Of particular interest to this research are the sirtuin deacetylases (SIRTs). As mentioned earlier, SIRT1 expression is reduced in osteoblasts, but it is also reduced in osteoarthritic cartilage and this is associated with elevated levels of chondrocyte apoptosis. SIRT1 has been shown to have a role in the termination of inflammatory signalling in chondrocytes through the blocking of the binding of the transcription factor, NFκB (133,353,354), and is also associated with a reduction in NFκB signalling in osteoblasts (134), likely through the same process. It also promotes osteoblastogenesis over osteoclastogenesis through the promotion of RUNX2 activation (134). Together this suggests that a reduction in SIRT1

expression in OA cartilage and bone might result in increased chondrocyte apoptosis and inflammation, reduced osteoblast proliferation, mineralisation and differentiation, and promotion of osteoclastogenesis in addition to the mitochondrial dysfunction and cellular senescence discussed earlier. Whilst the investigation of SIRT1 expression has not been part of this study, a reduction in SIRT1 expression could explain the functional and metabolic changes observed in osteoblasts obtained from damaged porcine joints.

Importantly, any phenotypic changes occurring in the older damaged joint osteoblasts are retained in culture, as shown by the persistence of functional and metabolic differences between osteoblast populations from damaged and non-damaged joints in long term primary cell cultures. This would support the theory that these porcine cells could provide a valuable source of damaged and non-damaged joint osteoblasts for *ex-vivo/ in vitro* studies.

**Impact of adipokine stimulation on porcine osteoblasts**

Initial experiments investigating osteoblast responses to human adipokines used acute 24 hour stimulations. These experiments showed no significant changes or trends in ALP activity with increasing concentrations of the chosen adipokines. Due to the chronic nature of both OA and obesity, longer term stimulation would be more appropriate. Hence, 14 day stimulations were used in preliminary experiments for all 3 adipokines: leptin, resistin and visfatin. The results from this preliminary work suggested that only visfatin had any response in osteoblasts, which was also evident in the cartilage-based experiments discussed in Chapter 6. This could be linked to the use of human adipokines on porcine cells and tissue. The sequence homologies between human and pig for these adipokines are as follows: 85% for leptin; 77% for resistin; and 97% for visfatin.

Therefore the decision was made to discontinue further studies with leptin and resistin.

Chronic 14 day stimulations of osteoblasts with varying concentrations of visfatin revealed a difference in response between the osteoblasts obtained from damaged and non-damaged knee joints. 30ng/ml visfatin stimulation of non-damaged cells appeared to induce a slight increase ALP activity, whereas higher visfatin concentrations reduced ALP activity slightly; none of these effects were statistically significant. In contrast, all concentrations of visfatin reduced ALP activity in damaged joint osteoblasts with increasing concentrations resulting in a significant linear reduction in ALP activity. It is important to note that the ALP activity in unstimulated osteoblasts from damaged joints was slightly higher than that of osteoblasts from non-damaged joints. Hence, although chronic stimulation of osteoblasts with visfatin reduced ALP activity in these cells, even the highest concentration of visfatin used did not return ALP activity to that of non-damaged joint osteoblasts. It is also of note that although ALP is typically used as a bone formation biomarker, in OA osteoblasts there appears to be an uncoupling of ALP activity and mineralisation, therefore additional investigation into the mineralisation capacity of osteoblasts in response to visfatin stimulation would be useful in determining whether visfatin is having a beneficial or detrimental effect on osteoblast cell function.

Visfatin is a pro-inflammatory adipokine that can be produced by a number of cell types. It is also known as pre B cell enhancing factor 1 or PBEF1, due to its role in the priming of B cells in the adaptive immune response, as well as nicotinamide phosphoribosyltransferase (NAMPT). These three different names reflect the different functions of this protein and suggest that the protein may be involved in a number of processes and pathologies. Increased synovial fluid

levels of visfatin in OA patients could be influencing cell behaviour and disease progression through both inflammatory and metabolic mechanisms (155). Visfatin is involved in the regulation of NAD/SIRT1 activity and therefore may have a role in the regulation of joint tissues both in healthy ageing and disease (355,356). It has been suggested that chronic inflammation and oxidative stress can lead to a reduction in NAMPT-dependent NAD biosynthesis and therefore reduced SIRT1 activity, linking inflammation and visfatin to the processes that may be involved in the development of the altered osteoblast phenotype in OA (355).

The slight detrimental effect of visfatin decreasing osteoblast ALP activity needs to be investigated further. Given the disconnection between ALP activity levels and mineralisation as measured by alizarin red in damaged joint osteoblasts, ALP activity results should be confirmed by the examination of the effect of visfatin on mineralisation capacity. Whether visfatin is having an impact on inflammatory pathways or NAD-dependent sirtuin activity in osteoblasts is still unknown, as is the receptor through which extracellular visfatin produces these effects. Addressing these unknowns should form the basis of future work to elucidate the role of visfatin in bone and joint health.

This project was not without challenges. Porcine osteoblasts were harvested in Trizol reagent and RNA isolated from these cells, but the total RNA yield was insufficient for further work. This is most likely due to the limited number of cells available for this work and the reduced proliferation capacity shown in Figure 7.2. However, had yields from these cells been adequate the expression of 96 genes identified as being altered in OA tissue were to be investigated using a fluidic card format. Optimisation of tissue collection and cell culture procedures could help alleviate some of these issues by improving the initial yield of

osteoblasts for experimentation. This is of particular importance in the osteoblasts from damaged joints which exhibit limited proliferative capacity.

**Conclusion**

The pig could represent a source of both healthy non-damaged joint tissue and tissue from joints showing visible signs of early-OA like damage. Functional and metabolic assessment of porcine osteoblasts has revealed significant differences between osteoblasts from damaged and non-damaged joints suggesting that osteoblasts retain their phenotype in culture, possibly through epigenetic changes occurring in cells from damaged joint tissue. This is of great importance when considering the utility of human primary osteoblasts. With no readily available healthy comparison, we are unable to investigate these features and therefore it will be difficult to determine early changes in OA bone using human primary osteoblasts as a research tool. Therefore, porcine primary osteoblasts may be a readily available resource to investigate these initial bone changes in early osteoarthritis.

The effect of chronic visfatin stimulation on osteoblast ALP activity also lends credence to the theory linking visfatin, obesity and osteoarthritis. There is further research to be done to establish the pig as a useful model for OA research but these initial results are promising and reflective of results in other joint tissues.

# 8.0 GENERAL DISCUSSION

## 8.1 INTRODUCTION AND AIMS

Rodent animal models are routinely used in musculoskeletal ageing research. Some of the drawbacks of such models were discussed in Sections 1.1.7 and 1.5. These drawbacks mean that research carried out in these models does not always reflect the physiological and pathological processes and responses seen in humans. A number of species are currently being utilised as OA models and several of these models require OA induction either surgically or chemically to present an OA phenotype (further details can be found in Section 1.5). Due to issues with translatability of research carried out in these induced models into meaningful treatments for human OA patients, the utility of such models has been questioned (128,129). The pig is of interest as a model of the condition due to joint size and structural similarities with humans, and the relatively high prevalence of joint conditions and leg weakness disorders. The aim of this work was to establish if the pig could present an improved model of musculoskeletal ageing and OA.

## 8.2 SUMMARY OF FINDINGS

### 8.2.1 Pig body composition changes up to 4yrs of age

Commercial pigs grew rapidly in early life and body weight gain reached a plateau at 18months. Percentage fat in the rear legs (as determined by DXA) changed very little across the age groups, with the exception of the 80 week group that was housed within the department for a number of weeks. These pigs had a much higher percentage hind-limb fat and slightly elevated plasma visfatin concentrations compared to the other groups. The older pigs (3-4 years) also

exhibited slightly elevated visfatin. Due to the 80 week and 3-4 year age groups exceeding the maximum weight supported by the DXA scanner, only the rear legs of the pigs were assessed. However, full body scans may have revealed an increased proportion of fat in these older pigs that is not distributed evenly across the body. Visually there appeared to be increased abdominal fat in these older groups but this was not measured as part of this study.

### 8.2.2 Lower leg muscle changes in pigs up to 4yrs of age

Lower leg muscle strength is important for balance and reducing the number of trips and falls in the elderly, therefore porcine lower leg muscles were investigated. There were no significant changes in muscle fibre composition, although the method used to assess this was restricted to type I and II fibres and could not distinguish between the various subtypes of type II muscle fibres. In future, determination of expression of the myosin heavy chain isoforms might provide a more detailed assessment of muscle fibre composition. Investigation of these muscles at older age points and possibly of other muscles such as the quadriceps muscle might also be of use. Quadriceps strength is reduced in knee OA and could contribute to mobility issues; however, this muscle was not investigated as part of this study.

### 8.2.3 Osteoarthritis in commercial pigs

Pigs developed OA lesions of the knee at a relatively young age. Some pigs as young as 17wks showed minor chondropathy, although it is possible that this could be caused by other conditions such as osteochondrosis, which the photographic chondropathy scoring (PCS) used does not fully examine. Average joint condition worsened with age; this was accompanied by an increase in the

amount of variation in joint condition within each age group. PCS correlated strongly with cartilage degradation in explant tissue and was therefore reflective of active joint deterioration. Plasma levels of IL-6 and visfatin were measured as markers of systemic inflammation, but were found not to correlate with PCS of the knee joints. In humans, some, but not all, studies have reported increased systemic concentrations of IL-6 and visfatin in patients with OA (140,357–359). Furthermore, systemic concentrations may not be representative of local concentrations within the joint, and thus the level of these cytokines in synovial fluid might be a more appropriate measure for determining levels of joint inflammation and correlation with macroscopic damage.

### 8.2.4 Utilisation of porcine cartilage and chondrocytes for ex vivo work

Porcine cartilage is already in use, with studies investigating cartilage repair and OA research using healthy cartilage explant tissue (192,360). Porcine joints could present a valuable source of damaged cartilage tissue and chondrocytes for OA research. This research demonstrated that porcine chondrocytes grew well in culture; however, much like other monolayer cultures of chondrocytes, these cells lose their phenotype and become more fibroblast-like over time (361). It is therefore advisable to use cartilage explants, 3D culture methods, such as scaffold cultures, or micromass cultures or to use chondrocytes as soon as possible following isolation from cartilage (311). Investigation of the effects of IL-1β stimulation was carried out to examine the induction of an OA-like phenotype in healthy *ex vivo* cartilage explants. Pro-inflammatory factors are commonly used for this purpose; these include IL-1β and TNFα (280,314). This work showed that recombinant human IL-1β protein is too dissimilar from porcine IL-1β to produce the classic pro-inflammatory response in pig cells, but that even low

concentrations of recombinant porcine IL-1β could induce a response within 24 hours in both cartilage explants and chondrocytes.

### 8.2.5 Investigation of the functional phenotype of osteoblasts obtained from damaged knee joints

One of the major findings of this study iss that there was a phenotypic difference between osteoblasts obtained from damaged knee joints of 3-4 year old pigs and non-damaged pig knee joints from 40-80 week old pigs and that this is reflective of results obtained from research on human osteoblasts. This is particularly relevant when considering the difficulties associated with sourcing healthy human joint tissue for comparison purposes and testing of safety for new treatments. This is where the pig could provide a valuable resource as a source of both healthy and damaged joints.

### 8.2.6 Effect of adipokines on porcine joint tissue

Adipokines have been implicated as a potential non-biomechanical link between obesity and OA (139,140,207,208). Therefore the effect of such proteins on joint tissue was of importance. There were issues with the availability of porcine proteins for research purposes. However, an increase in utilisation of the pig as a model of a number of conditions is starting to drive an increase in the availability of pig specific products such as ELISA kits and recombinant proteins, but at the time of this study, no recombinant porcine adipokine proteins were commercially available. Human visfatin however, has 97% sequence homology with the porcine protein and elicited a strong pro-inflammatory response in cartilage explants and chondrocytes when used at a similar level to that found to elicit a response in human tissue. It also reduced ALP activity in osteoblasts, however, given the

disconnection between this measure of osteoblast functionality and mineralisation seen in damaged joint osteoblasts, this data should be confirmed by alizarin red staining.

Further investigation into the effect of small elevations in visfatin over longer periods of time is also required. Obesity being a chronic condition, it is unlikely that sudden significant increases in the exposure of joint tissues to visfatin results in the same changes that we see in obesity and obesity-linked OA.

## 8.3 LIMITATIONS OF THE STUDY

As with all scientific studies, there are a number of limitations associated with this work. Musculoskeletal ageing and OA are age-linked conditions; here we have investigated such conditions in pigs up to the age of 4 years. Pigs of this age are equivalent to middle-aged humans; they are skeletally mature and may be entering a period of musculoskeletal decline and condition loss. Due to reduced condition and productivity of pigs older than 4 years of age, the commercial pig industry tends not to maintain pigs into their old age. Therefore, it is inherently difficult to obtain older pigs that may present with more severe OA or may be developing sarcopenia, without purposefully ageing the animals in a research environment.

Spontaneous models of OA can be very variable and environmental factors should be taken into consideration when using commercial pigs. Factors such as flooring type and condition or whether the pigs are housed indoors can all impact on the prevalence of joint problems (189,190). This could mean that pigs of the same breed from different suppliers could have different incidences and severity of OA.

Due to the cost of housing pigs, relatively small numbers were used in this study and this limits the ability to fully characterise OA in these animals, due to the variability in joint condition discussed in Section 5.3.2. Ideally this study, had it been designed to look at OA alone, would have used a larger group of 3-4 year old sows and split the group into healthy, mild OA and moderate OA for comparison purposes.

## 8.4 MAIN FINDINGS

The data reported in this thesis suggests that the porcine OA model could prove to be a valuable resource in the drive to produce translatable research and meaningful treatments for human OA patients. However, as a model of muscle ageing, the pig did not show any indications of early sarcopenic muscle changes and is therefore not suitable for this purpose within the age range examined in this study.

## 8.5 FUTURE WORK

Further characterisation of OA changes in the joint and surrounding tissue would strengthen the support for this model. In particular, closer examination of the damaged joint osteoblast phenotype including gene expression analysis investigating OA related genes and in depth investigation of the metabolic dysfunction observed in these osteoblasts from damaged joints would be useful. Future work could also involve the examination of osteoblast function in sclerotic and non-sclerotic bone regions within the joint, rather than simply from damaged or non-damaged joints.

Body composition and muscle changes begin to occur in early middle aged humans. The oldest age group examined in the pigs was 3-4 years of age; which is

equivalent to early middle aged humans. However, no detrimental changes were observed across the age groups examined. The age groups used may be too young to be developing detrimental age-related changes in skeletal muscle, but sarcopenic changes may occur in older animals. Therefore, similar investigations in older pigs may be of use, although these are likely to have to be purposefully aged in research facilities.

Future work on this model could also include examination of the quadriceps muscle; since atrophy in this muscle has been suggested to be involved in the progression of OA.

## 8.6 UTILISATION OF THE PIG MODEL

The age groups investigated in this study were chosen to cover the "commercial lifespan" of these animals. Commercial life span is the life span of a female pig within the commercial industry, breeding sows are typically sent to slaughter once their breeding productivity begins to fall. Reproductive capacity begins to fall at around 3 yrs of age (223,362). For OA research, pigs older than 2 years of age should be used to ensure skeletal maturity has been reached. Skeletally immature animals may exhibit higher levels of spontaneous cartilage repair than adults. The increased capacity of young cartilage to heal could influence results, therefore it is recommended that all animals used in OA research are skeletally mature. Young pigs may also be exhibiting other conditions such as osteochondrosis. Osteochondrosis can lead to osteoarthritic joint changes later in life, but is a disease of rapidly growing animals caused by disruption of the blood supply to the endochondral ossification front and is therefore active in younger, skeletally immature animals.

Given the high level of variability in chondropathy found in the knee joints of the pigs from the 3-4 year age group, it is possible that a larger group of sows of this age could provide a variety of joint conditions ranging from healthy joints to severely damaged joints. Therefore, this could provide tissue that could be categorised as non-OA, mild/moderate OA and severe OA. The majority of pigs from this age group exhibited mild/moderate OA changes, further details can be found in Chapter 5.0.

As the rate of loss of bone density and the risk of OA development are both higher in postmenopausal women it may be of interest to investigate this condition in an older group of sows. All age groups up to and including the 80 week old group were gilts: young, non-breeding females. The 3-4 year age group were reproductively active sows that had been used for breeding therefore none of the pigs used in this study were exhibiting significant reproductive decline. Carmichael et al suggested that the litter size produced by sows increases with age until the sow reaches 3 years of age and then begins to decline (223). Therefore at 3-4 years of age, commercial pigs were not undergoing significant reductions in fertility as would be associated with human menopause. Older pigs exhibit reduced reproductive capacity and would therefore be more comparable to older, postmenopausal women.

## 8.7 CLOSING REMARKS

Musculoskeletal ageing research has used a number of rodent models to investigate the pathological features of age-related musculoskeletal conditions such as sarcopenia. These models poorly reflect human ageing (52,53,56,57,59,129). The commercial pig was investigated as an alternative animal model of musculoskeletal ageing as part of this study. The data presented

in this thesis suggested that the pigs used were too young to be exhibiting early signs of age-related muscle deterioration. It is possible that pigs older than 4 years of age could be an appropriate model for the investigation of early sarcopenic muscle changes and the initiation of musculoskeletal decline however pigs of that age were not available for this study. Therefore, commercial pigs within the age range examined (8 weeks – 4 years old) do not represent a suitable model of early musculoskeletal ageing.

OA is a leading cause of pain and disability and there are currently no pharmacological therapeutics that can slow or reverse disease progression (248). In attempting to better understand the pathways and processes that mediate OA joint degeneration, rodent models where OA is induced surgically have been used extensively. However, the predictive utility of surgically induced rodent models has been questioned due to the failure of preclinical efficacy of therapeutics to translate in the clinic. Therefore alternative models are being sought. The data presented in this thesis supports the use of the commercial pig as a spontaneous model of early OA without many of the problems associated with other OA animal models. It is proposed that commercial pigs could present an alternative to commonly used models of OA to investigate disease pathology and would make a valuable addition to the research tools already available in this research area.

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# APPENDIX A – BUFFERS

## Citrate Synthase Protocol

Tris-HCl buffer (1.0M, pH 8.1): 2.4228g Tris was added to 20ml distilled water and adjust to pH 8.1 with 37% HCl.

Tris–HCl buffer (0.1M, pH 7.0): 2ml 1M Tris-HCl buffer, pH 8.1 added to 15ml distilled water and adjusted to pH 7 with concentrated HCl. Solution was then made up to a volume of 20ml using distilled water.

Triethanolamine-HCl buffer (0.5M, pH 8.0) + EDTA (Ethylenediaminetetraacetic acid disodium salt dehydrate) (5mM): 8.06g triethanolamine was added to 100ml distilled water and adjusted to pH 8 with 37% HCl. 186.1mg EDTA was then added.

Triton X-100 (10% solution): 10ml triton X-100 added to 90ml distilled water.

12.2mM acetyl-CoA: 25mg acetyl CoA added to 2.5ml distilled water. Aliquots of 250µl stored at -20°C until required.

Triethanolamine-HCl-buffer (0.1M, pH 8.0): 1ml of 0.5M triethanolamine-HCl-buffer, pH 8.0 added to 4ml distilled water.

Oxalacetate (10mM, pH 8.0): 6.6mg oxalacetate added to 5ml of 0.1M triethanolamine-HCl-buffer, pH 8.0.

DTNB (1.01mM, pH 8.1): 2mg DTNB added to 5ml of 1M Tris-HCl-buffer, pH 8.1.

## Mitochondrial ATP production protocol

Buffer A: 100mM potassium chloride, 50mM Tris, 5mM Magnesium Chloride, 1.8mM adenosine triphosphate (ATP), 1mM EDTA. pH 7.2.

Buffer B: 225mM sucrose, 44mM potassium phosphate monobasic, 12.5mM magnesium acetate, 6mM EDTA.

Tris acetate buffer: 20mM tris acetate (trizma acetate), 0.2mM magnesium acetate.

Tris-EDTA buffer: 0.1 mol/L Tris (hydroxymethyl) aminomethane, 2 mmol/L EDTA and adjusted to pH 7.75

## Protein Extraction

Cell lysis buffer: 150mM Sodium Chloride, 1% triton x-100, 50mM Tris, pH 8.0. Before use mixed protease and phosphatase inhibitors were added at a concentration of 2µl/ml and 10µl/ml respectively.

## Proteoglycan Assay

DMMB reagent (1000ml): 2.4g NaCl, 3g Glycine, 8ml 1M HCl, 16mg DMMB.