The Role of Chondrocyte Senescence in the Pathogenesis of Canine Osteoarthritis

Kristina Pollock **MVB CertSAS MRCVS**

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

School of Veterinary Medicine, College of Medicine, Veterinary and Life Sciences, University of Glasgow

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Abstract

The aims of this study were to (1) evaluate cellular senescence in chondrocytes from osteoarthritic articular cartilage, (2) investigate the hypothesis that oxidative stress is a feature of canine OA chondrocytes and that oxidative stress contributes to cellular senescence in canine chondrocytes, (3) investigate the hypothesis that osteoarthritic chondrocytes alter the gene expression of adjacent normal chondrocytes in OA joints leading to modulation of genes known to play a role in the pathogenesis of OA and (4) evaluate the presentation of dogs undergoing femoral head excision in veterinary referral practice in the UK as a treatment for osteoarthritis of the coxofemoral joint, and to categorise the distribution and severity of associated pathological lesions.

Chondrocytes from osteoarthritic and normal cartilage were examined for levels of senescence. Initially chondrocytes were cultured using an alginate bead culture system, thought to mimic the extracellular matrix of articular cartilage. However, these chondrocytes showed almost no growth as compared to monolayer culture where they grew rapidly. OA chondrocytes entered the senescent state after 1.5 to 4.9 population doublings in monolayer culture, while normal chondrocytes underwent 4.8 to 14.6 population doublings before entering the senescent state. Osteoarthritic chondrocytes had increased levels of markers of cellular senescence (senescence associated beta-galactosidase accumulation and p16 protein accumulation) as compared to normal chondrocytes, suggesting that chondrocyte senescence is a feature of canine osteoarthritis.

An experimental model for the induction of oxidative stress in chondrocyte cell culture was developed using tert-Butyl hydroperoxide and total cellular glutathione was measured as an indicator of cellular oxidative stress levels. Experimental induction of oxidative stress in both normal and osteoarthritic chondrocytes in cell culture resulted in increased amounts of cellular senescence, shown by an increase in levels of senescence associated beta-galactosidase accumulation and decreased replicative capacity. Experimental induction of oxidative stress also resulted in altered gene expression of three genes important to the degradation of the extracellular matrix; MMP-13, MMP-3 and Col-3A1, measured by RT-PCR, in normal canine

chondrocytes in monolayer cell culture. MMP-3 showed the greatest relative expression change, with a fold-change of between 1.43 and 4.78. MMP-13 had a fold change of 1.16 to 1.38. Col-3A1 was down regulated, with a fold-change of between 0.21 and 0.31. These data demonstrate that experimentally induced oxidative stress in chondrocytes in monolayer culture increases levels of cellular senescence and alters the expression of genes relevant to the pathogenesis of canine OA.

Coculture of osteoarthritic chondrocytes with normal canine chondrocytes resulted in gene modulation in the normal chondrocytes. Altered gene expression of ten genes known to play a role in the pathogenesis of osteoarthritis was detected in the normal chondrocytes (fold change shown in brackets); TNF-alpha (11.95), MMP-13 (5.93), MMP-3 (5.48), IL-4 (7.03), IL-6 (5.3), IL-8 (4.92), IL-F3 (4.22), COL-3A1 (4.12), ADAMTS-4 (3.78) and ADAMTS-5 (4.27). In total, 594 genes were significantly modulated suggesting that osteoarthritic chondrocytes contribute to the disease propagation by altering the gene expression of adjacent normal chondrocytes, thus recruiting them into the disease process. Gene expression changes were measured by microarray analysis and validated by RT-PCR and Western blot analysis.

An epidemiological study of femoral heads collected from dogs undergoing total hip replacement surgery as a treatment for osteoarthritis of the coxofemoral joint secondary to canine hip dysplasia revealed that there was no characteristic pattern of cartilage lesion for canine hip dysplasia. Severe pathology of the femoral head with cartilage erosion occurred in 63.9% of cases and exposure of subchondral bone in 31.3% of cases.

The work presented in this thesis has demonstrated that cellular senescence is a feature of chondrocytes from canine osteoarthritic cartilage and suggests that cellular senescence and oxidative stress play an important role in the pathogenesis of osteoarthritis in dogs.

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Presentations

1. Senescence as a feature of canine osteoarthritis
At British Small Animal Veterinary Association Congress,
Birmingham, 9th April 2010.

2. The role of senescence in canine osteoarthritis
At American College of Veterinary Surgeons Annual Congress,
Chicago, 5th November 2011.

Declaration

I hereby declare that the work carried out in this thesis is original and was carried out by either myself or with due acknowledgement. All additional sources of information have likewise been acknowledged. This work has not been presented for the award of a degree at any other university.

Signed: 

Date: 28th December, 2014

List of Abbreviations

ADAMTS- A disintergrin and metalloproteinase with thombospondin motifs

ARF-alternative reading frame

ATM-Ataxia telangiectasia mutated

ATP- adenosine triphosphate

ATR- ataxia telangiectasia and Rad3-related

CDK-cyclin dependent kinase

cDNA- complimentary DNA

CHD- canine hip dysplasia

COX- cyclooxygenases

cRNA-complimentary RNA

CT- computed tomography

DAB- diaminobenzidine

DDR- DNA damage response

DEPC- diethylpyrocarbonate

DHA- docosahexaenoic acid

DMEM-Dulbecco's Modified Eagle's Medium

DMOADs- Disease modifying osteoarthritic drugs

DNA- deoxyribonucleic acid

ECM- extracellular matrix

ED- elbow dysplasia

EDTA- ethylenediamenetetraacetic acid

ERK- extracellular signal-regulated kinase

FBS- foetal bovine serum

FC- fold change

FGF-fibroblast growth factor

GAG- glycosaminoglycan

GSH- total cellular glutathione

HPF- high powered fields

HRPO- horseradish peroxidase stept-avidin

iGA-iterative group analysis

IGF- insulin-like growth factor

IL- interleukin

INF-interferon

iNO-inducible nitric oxide

IRAP-Interleukin 1 receptor antagonist protein

JNK- c-Jun NH2-terminal kinase

LIF-leukemia inhibitory factory

LOX- lipoxygenses

MAPK- mitogen activated protein kinase

MDA- malondialdehyde

MMP- matrix metalloproteinase

MRI- magnetic resonance imaging

mRNA- messenger RNA

NO- nitric oxide

NSAIDS- non-steroidal anti-inflammatory drugs

OA- osteoarthritis

OPG-osteoprotegrin

PAGE- poly acrylamide gel electrophoresis

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PGE-prostaglandin E

QPCR- quantitative PCR

QTL- quantitative trait loci

RANK-receptor activator of nuclear kappa B

RB-retinoblastoma protein

RNA- ribonucleic acid

RNA-seq- RNA sequencing technologies

RNS- reactive nitrogen species

ROS- reactive oxygen species

RP-rank product

PSGAGs- Polysulphated glycosaminoglycans

RT-PCR- reverse transcription PCR

PUFA- poly-unsaturated fatty acid

SA-beta-gal- senescence associated beta-galactosidase

SAHF- senescence associated heterochromatin foci

SASP- senescence-associated secretory phenotype

SIPS- stress-induced premature senescence

SOD- superoxide dimutase

tBhP- *tert*-Butyl hydroperoxide

TBST-Tris Buffer saline with tween

TGF- transforming growth factor

TIMP- tissue inhibitors of metalloproteinase

TNF- tumor necrosis factor

TNFR-tumor necrosis factor receptor

TRF- terminal restriction fragment

UHTDSTs- ultra-high-throughput DNA sequencing technologies

U.K.- United Kingdom

U.S.A.- United States of America

Chapter I

Introduction to Canine Osteoarthritis and Review of the Literature

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1.1 Introduction to Canine Osteoarthritis

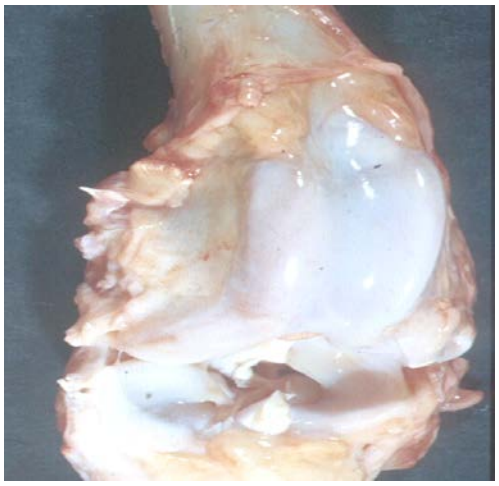
Osteoarthritis (OA) is the most common disease of the dog (Bennett and May, 1995). Prevalence is estimated at 20% for adult dogs in both the UK (Moore *et al*., 2001) and the United States (Johnston, 1997). OA is also the most common disease of the musculoskeletal system in human beings (Pelletier *et al.*, 2006), affecting 80% of people aged over 55 years in the U.S.A. (Control, 2001). OA has been identified in dinosaur fossils and on examination of the remains of prehistoric humans (D'Anastasio and Capasso, 2004). It is a major cause of pain and suffering in dogs and a common reason for euthanasia due to deteriorating quality of life, which occurs with advanced disease. Although OA can be initiated early in life, it is predominantly in later years that the disease causes pain and suffering. This is generally explained by the fact that the disease is gradual in onset and slowly progressive. It has been suggested that OA in humans is a disease of gradual onset due to the ageing of the cartilage cells (senescence) enhanced by oxidative stress (Parinello *et al.*, 2003). Thus reversing or delaying chondrocyte senescence could provide a novel and exciting approach to the treatment of OA in veterinary medicine if the same hypothesis can be shown to be true in canine OA.

OA is a degenerative disease affecting movable joints. It is characterised by degeneration of articular cartilage with new bone formation at the articular surface and changes to the synovium and adjacent soft tissues (Bennett and May, 1995; Brandt *et al.*, 2006), (Fig 1-1B and 1-1C). There are two forms of osteoarthritis; primary (or idiopathic) and secondary. Primary OA is a process in which articular degeneration occurs without an obvious underlying abnormality (Samson *et al.*, 2007). Secondary osteoarthritis occurs as a result of another underlying condition (Bennett and May, 1995), such as occurs following a traumatic injury to the cartilage surface. Secondary OA in dogs can commonly occur when abnormal loads act on the joint surface of a normal joint (e.g. repetitive trauma, fracture, luxation or cartilage injury) or during normal loading of a dysplastic joint (e.g. canine hip dysplasia and elbow dysplasia), (Bennett and May, 1995; Innes, 2005). OA is a complex disease process and is considered to be a final common pathway of joint failure resulting from many diverse biological pathways including cellular senescence, inflammation and oxidative stress.

1.2 Normal joint structure

The mammalian skeleton has two types of joints; diarthrotic and synarthrotic. Synarthrotic joints are characterised by a low range of movement and are tightly bound by either connecting cartilage (synchondroses) or by a bony connection (synostoses). Examples in the dog include the intervertebral joints of the spinal column, which are synchrondroses, and the skull bones, which are synostoses. Diarthrotic joints are characterised by a high range of motion and are the most common joints in both dogs and humans. These joints predominantly consist of two long bones connected by a joint capsule with a joint space between them containing synovial fluid. The joint capsule encloses the joint and the synovial fluid acts as a lubricating fluid, providing nutrition to the articular cartilage. The joint capsule consists of two components: an inner synovium and an outer fibrous capsule. Articular cartilage covers the ends of the articulating bones and provides a shock-absorbing surface for the concussive forces applied to bones during movement (Fig 1-1A). There are four cartilage zones: the articular zone, proliferative zone, fibrocartilaginous zone and the calcified cartilage zone (Figure 1-3). In the superficial articular zone the chondrocytes and matrix are aligned parallel to the joint surface, while in the proliferative and fibrocartiaginous zones the components are aligned perpendicularly. The stiffer calcified cartilage layer, which attaches the articular cartilage to the subchondral bone, acts to diffuse biomechanical forces limiting their impact on the underlying bone (Oegema *et al.*, 1997). The calcified cartilage gradually transitions into subchondral bone with the intrusion of blood vessels and nerves from the underlying bone (Clark, 1990).

Figure 1-1.A



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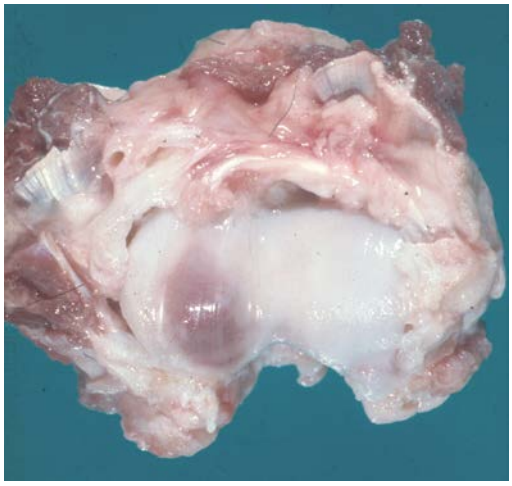

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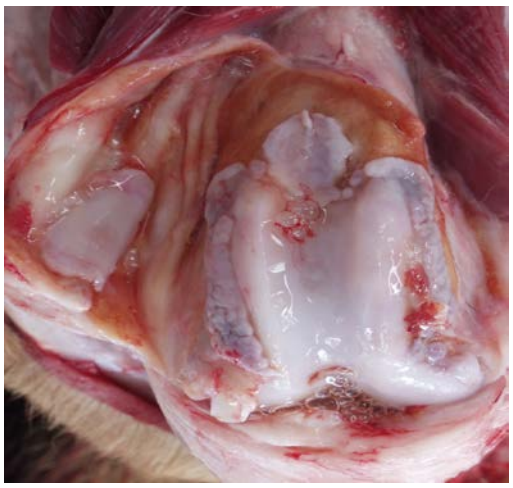


Figure 1-1. A. Normal canine stifle joint showing smooth articular cartilage on the femoral trochlea. **B.** Canine elbow joint with OA, with loss of articular cartilage from the humeral condyle. **C**. Canine stifle joint showing cartilage erosion and fibrillation of the trochlea groove with osteophyte formation along the trochlear margin.

1.2.1 The structure and function of normal articular cartilage

Articular cartilage is a specialised connective tissue consisting of hyaline cartilage. It is avascular, aneural and has a low cell density and as such consists predominantly of an extracellular matrix. It is characterised by a high tensile strength and shock-absorbing capacity. The only cell type found in articular cartilage is the chondrocyte, constituting up to 5% of cartilage by volume, depending on age and disease state (Stockwell, 1967).

The extracellular matrix is a dynamic system in a constant state of flux controlled by anabolic and catabolic factors (Dijkgraaf *et al.,* 1995). It consists of approximately 60-80% water and 20-40% Type II collagen, proteoglycan and hyaluronic acid aggregates, which are secreted by chondrocytes (Goldring, 2000; Maroudas *et al.,* 1980), (Figure 1-2). Small amounts of lipid and inorganic compounds are also present. The gross structure is a tightly bound collagen structural mesh bathed in a proteoglycan-water gelatinous fluid. The type II collagen framework consists of bundles and sheets of collagen fibrils that are protein bound by chondronectin and fibronectin and cross-linked by type IX collagen (Dean, 1991; Howell *et al.,* 1992). Type XI collagen is present in small quantities, providing a supportive framework for the chondrocyte exoskeleton (Seyer and Kang, 1989). The collagen network confers tensile strength to the cartilage and limits the water absorbing capacity of the proteoglycan aggregates (Cremer *et al.,* 1998).

Proteoglycans are large negatively charged, hydrophilic aggregate molecules consisting of a core protein molecule to which bind glycosaminoglycan (GAG) chains, bound to hyaluronic acid via a link protein. The main proteoglycan is aggrecan. The GAG chains consist predominantly (90%) of chondroitin 6-sulphate and keratan sulphate, with chondroitin 4-sulphate accounting for 5% (Howell, 1989). These hydrophilic aggregates trap and hold water in between the strands of collagen in the extracellular matrix (May, 1994; Muir, 1995), (Figure 1-2). It is the combination of proteoglycan aggregates and water that give cartilage its extraordinary shock absorbing capacity. Upon compression, articular cartilage deforms and in the presence of high loads the hydrostatic pressure caused by loading exceeds the osmotic pull of the cartilage and water is squeezed from the matrix into the synovial fluid, contributing to joint lubrication (Dijkgraaf *et al.,* 1995). This water can then be resorbed after the compressive force has ceased, restoring the normal cartilage structure. Thus cartilage allows compressive forces to be absorbed without injury to the underlying bone (Buckwalter and Mankin, 1998). Lower forces are distributed by

the boundary lubricating glycoproteins (Mankin and Radin, 1989). These lubricin glycoproteins form a monolayer that is adsorbed to the articular cartilage surface and deforms upon loading to reduce friction. The remainder of the extracellular matrix is made up of structural glycoproteins (primarily fibronectin and laminin), which regulate chondrocyte adhesion, migration, proliferation and differentiation (Trelstad, 1989).

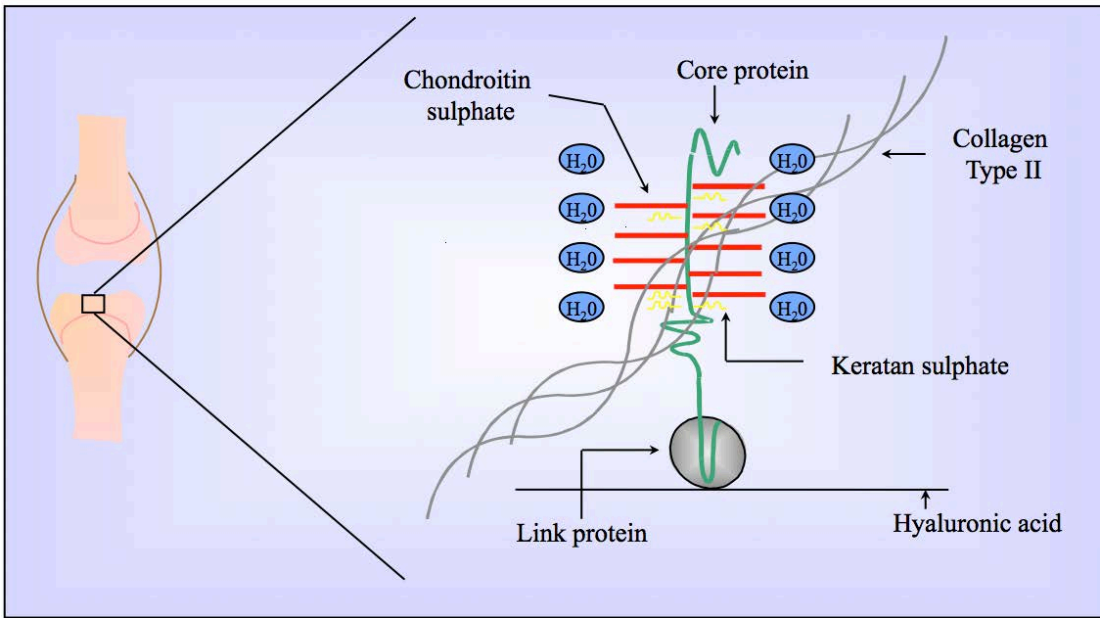


Figure 1-2. Schematic diagram of the molecular structure of cartilage.
Cartilage consists of 60-80% water and 20-40% Type II collagen, proteoglycan and hyaluronic acid aggregates.

Cartilage is in a constant state of flux (Clark, 1991) and chondrocytes maintain the fine balance between synthesis and destruction of both collagen and glycosaminoglycans in articular cartilage (Buckwalter and Mankin, 1998). This occurs in response to stimuli such as joint loading (Lee *et al.*, 2002), levels of matrix breakdown and the presence of growth factors and cytokines (Guilak *et al.*, 2006; Rowan and Young, 2007).

Chondrocytes survive in an avascular and alymphatic tissue relying on diffusion of nutrition and waste products to and from the synovial fluid under the stimulation of normal joint movement and loading (Howell, 1989). They have low rates of metabolic activity and can survive in a low oxygen tension environment. The chondrocytes secrete all the components of the extracellular matrix, and also regulate the catabolism of the matrix, influenced by growth factors and cytokines. Extracellular matrix turnover is regulated by the secretion of proteases and protease inhibitors from the articular chondrocytes. Articular cartilage allows motion between bones by providing a hardwearing, shock absorbing and

load-transferring surface capable of withstanding repetitive cyclical loading. These functions of articular cartilage are closely tied to and dependent upon its unique structure and biomechanics.

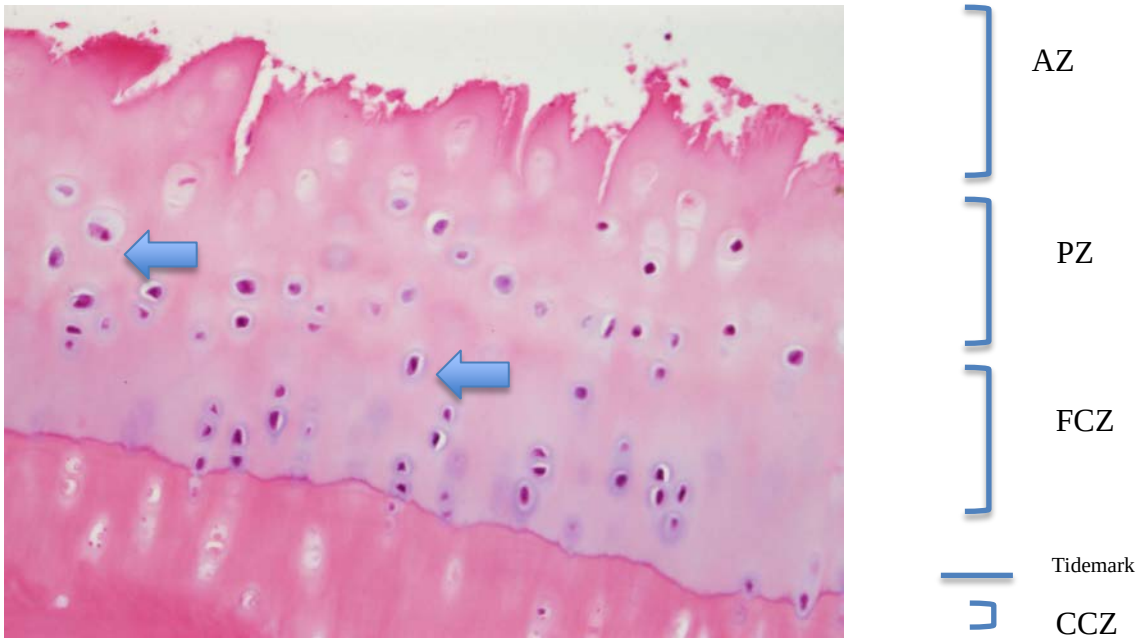


Figure 1-3. Histopathology showing structure of articular cartilage

Histopathology of articular cartilage showing the four layers: articular zone (AZ), proliferative zone (PZ), fibrocartilaginous (FCZ) and the calcified cartilage zone (CCZ). The blue arrows show chondrocytes. H&E stain at 200x magnification. This cartilage section shows vertical fissures in the articular cartilage surface consistent with OA. The articular zone demonstrates the earliest pathological changes when OA develops. These changes include fissures of the cartilage surface and proliferation of chondrocytes visible as clusters of cells.

1.3 The pathogenesis of osteoarthritis

OA usually occurs secondary to underlying musculoskeletal disease in dogs (Henrotin *et al.*, 2005). Underlying disorders may be developmental or acquired. Developmental disorders include canine hip dysplasia (CHD), elbow dysplasia, non-traumatic patellar luxation and osteochondrosis dissecans (Corley, 1992; Guthrie, 1989; Lust, 1977; Remedios and Fries, 1995). These developmental disorders lead to abnormal joint development and thus abnormal joint loading which in turn leads to OA in response to normal physical activity.

Acquired disorders are usually traumatic and include articular fractures, joint luxation, tendon and ligament injury and excessive joint loading (both isolated and repetitive), all of which result in direct cartilage trauma (Marcellin-Little *et al.,* 1994; Martinez and Coronado, 1997; Mclaughlin, 1995). Whether the initiating insult is a developmental or an acquired abnormality of the joint, disruption occurs to the delicate balance between anabolism and catabolism of the cartilage matrix leading to alterations in the structure of the extracellular matrix and ultimately to degradation of the extracellular matrix (Aigner *et al.,* 2001; Buckwalter and Mankin, 1997b; Fay *et al.,* 2006). As a result articular cartilage looses its unique elastic properties and is less able to endure normal physiological forces, which results in further degradation.

In the early stages of OA, chondrocytes undergo hypertrophy (Braunstein *et al.,* 1990) and division forming cell clusters with increased production of Type II collagen and aggrecan as they attempt to repair cartilage damage (Aigner *et al.,* 2001; Drissi *et al.,* 2005; Pullig *et al.,* 2000). New collagens are also secreted including type I, IIA, III and X (Schmid *et al.,* 1990; Yasuda and Poole, 2002). During this phase cellular metabolism increases, resulting in lactate secretion into the synovial fluid causing a drop in pH (Schmid *et al.,* 1990). At the same time, destruction of cartilage is mediated by decreased secretion of tissue inhibitors of metalloproteinases (TIMPs) with increased secretion of matrix metalloproteinases (MMPs), interleukins and aggrecanases (Kamekura *et al.,* 2005; Wang *et al.,* 2008) by the chondrocytes, which degrade proteoglycans and collagens. There is a decrease in TGF-beta receptor expression resulting in impaired TGF-beta signalling (Ballock *et al.,* 1993; Grimsrud *et al.,* 2001). This signal pathway regulates chondrocyte hypertrophy, division, chondrocyte matrix secretion and TIMP secretion (Kupcsik *et al.,* 2010; Qureshi *et al.,* 2008). As proteoglycans are degraded, they are replaced by new smaller proteoglycans with decreased water binding capacity. At the same time the degradation of the collagen fibrils further limits the water holding capacity of the articular cartilage. There is a limited regenerative capacity of the chondrocytes with replacement of the extracellular matrix over time but the articular cartilage is very vulnerable to damage during this phase as it has lost its resistance to large and repetitive mechanical loading (Loeser *et al.,* 2002; Yudoh *et al.,* 2005). The resulting imbalance between the levels of these catabolic enzymes and enzyme inhibitors (Charni *et al.,* 2005; Christgau *et al.,* 2001) results in destruction of proteoglycans and type II collagen. This occurs initially in the articular zone, with spread to the deeper cartilage layers and subchondral bone as the disease progresses (Wu *et al.,* 2002).

Damage to subchondral bone also plays a role in the pathogenesis of OA. It has been suggested that subchondral bone functions as an additional force-absorbing structure within the joint. Traumatic injury to subchondral bone results in haemorrhage and oedema and in severe cases necrosis (Radin and Rose, 1986). Bone cysts and osteophyte formation are common changes seen in OA (Altman *et al.*, 1986; Buckwalter *et al.*, 2000), along with sclerosis of subchondral bone. Bone cysts, lined with fibrous connective tissue, may communicate with the joint space where articular cartilage has been completely eroded (Pouders *et al.*, 2008). Damage to the subchondral bone disrupts the vascular channels travelling from the subchondral bone to the basal articular cartilage. This leads to failure of diffusion of nutrients and oxygen to cartilage, disruption of cartilage matrix synthesis (Imhof *et al.*, 1997) and thinning of the calcified cartilage layer. This is evidenced by disruption of the tidemark (the junction of calcified and non-calcified cartilage), which can be visualised during histopathological examination (Hwang *et al.*, 2008). It has been suggested that an increase in bone density that occurs following subchondral bone sclerosis results in a decrease in the force-absorbing capacity of the bony tissue and this may exacerbate damage to articular cartilage in OA (Radin and Rose, 1986). The exact order of progression of these changes has yet to be demonstrated (Burr, 2004).

The synovial lining of the joint capsule also plays a role the pathogenesis of OA. While OA is not considered to be an inflammatory arthropathy, inflammation of the synovium is a feature of the disease. Indeed joint swelling and effusion are classic clinical features during examination of dogs with OA. Synovial cell hyperplasia and hypertrophy occur along with infiltration of inflammatory cells, mainly lymphocytes and macrophages. The release of cartilage degradation products into the synovial fluid results in the production of catabolic cytokines by the synovium (Smith *et al.*, 1997). Synovial TNF-alpha and IL-1 are secreted resulting in MMP production (Goldring, 2000), which further propagates cartilage damage.

1.3.1 Enzymatic destruction of articular cartilage

Degradation of articular cartilage by proteolytic enzymes is a critical process in the pathogenesis of OA.

1.3.1.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are produced by both chondrocytes and synovial cells in OA and are associated with extracellular matrix destruction (Martel-Pelletier, 1999). Type II collagen and aggrecan are both degraded by MMPs. The MMP family has 25 members that regulate communication between chondrocytes and between chondrocytes and the extracellular matrix, modulating important cell functions such as proliferation and migration (Baker *et al.,* 2002). The MMP family is capable of degrading the entire cartilage matrix (Murphy *et al.,* 2002; Nagase and Woessner, 2000). The MMPs include collagenases, stromelysins, gelatinases, metrilysins and membrane type MMPs. These molecules are secreted by cells as inactive precursors, which become activated extracellularly by proteolytic cleavage (Knäuper *et al.,* 1996). Enzymes capable of activating the MMPs are varied, thus there is a wide variety of molecules regulating their activity. Such enzymes include cathespin B, stromelysin-1, collagenase-3 and gelatinase A (Martel-Pelletier, 1999). In healthy cartilage the MMPs regulate cell turnover and matrix homeostasis in response to stress or injury (Fitzgerald *et al.,* 2004; Goldring and Goldring, 2007; Kurz *et al.,* 2005).

The MMP family includes three collagenases; MMP-1 (which is also known as collagenase 1), MMP-8 (known as collagenase 2) and MMP-13 (known as collagenase 3). Collagenases “unwind” the triple helical structure of Type II collagen, which is formed from the three alpha-chains (Murphy *et al.,* 2002; Nagase and Fushimi, 2008). Once the fibrils are unwound they are cleaved into shorter fragments (Mitchell *et al.,* 1996).

1.3.1.2 ADAMTS Proteinases

The ‘A disintergrin and metalloproteinase with thombospondin motifs’ (ADAMTS) molecules are a second family of metalloproteinases which degrade articular cartilage. These molecules include a group of aggrecanases, which degrade aggrecan in the extracellular matrix. ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9 and ADAMTS-15 are all aggrecanases (Glasson *et al.,* 2005; Stanton *et al.,* 2005).

1.3.1.3 TIMP inhibition of metalloproteinases

The activity of MMPs and ADAMTS proteinases are regulated mainly by inhibition by the tissue inhibitors of metalloproteinases (TIMPs). TIMPs are a small family of four molecules that regulate the activation of the MMPs and ADAMTS proteinases by directly

binding to them. The activity of TIMPs in normal cartilage is to control the rate of extracellular matrix turnover and the TIMPs are similarly regulated by other cytokines and growth factors (Borden and Heller, 1997) including IL-1, PGE-2 and TGF-beta. All MMPs and ADAMTS-1, ADAMTS-4 and ADAMTS-5 can all be inhibited by binding to TIMPs (Baker *et al.*, 2002; Kashiwagi *et al.*, 2001; Rodriguez-Manzaneque *et al.*, 2002), though some aggrecanases are unaffected by TIMP inhibition of matrix degradation.

Cartilage destruction occurs as a result of an imbalance between proteinases and proteinase inhibitors (Figure 1-4). This balance is very complex and influenced by a wide variety of cytokines and pathways (Figure 1-5). The precise role of the various pathways and molecules is not yet fully understood, however some cytokines have been identified as being particularly important to the pathogenesis of OA.

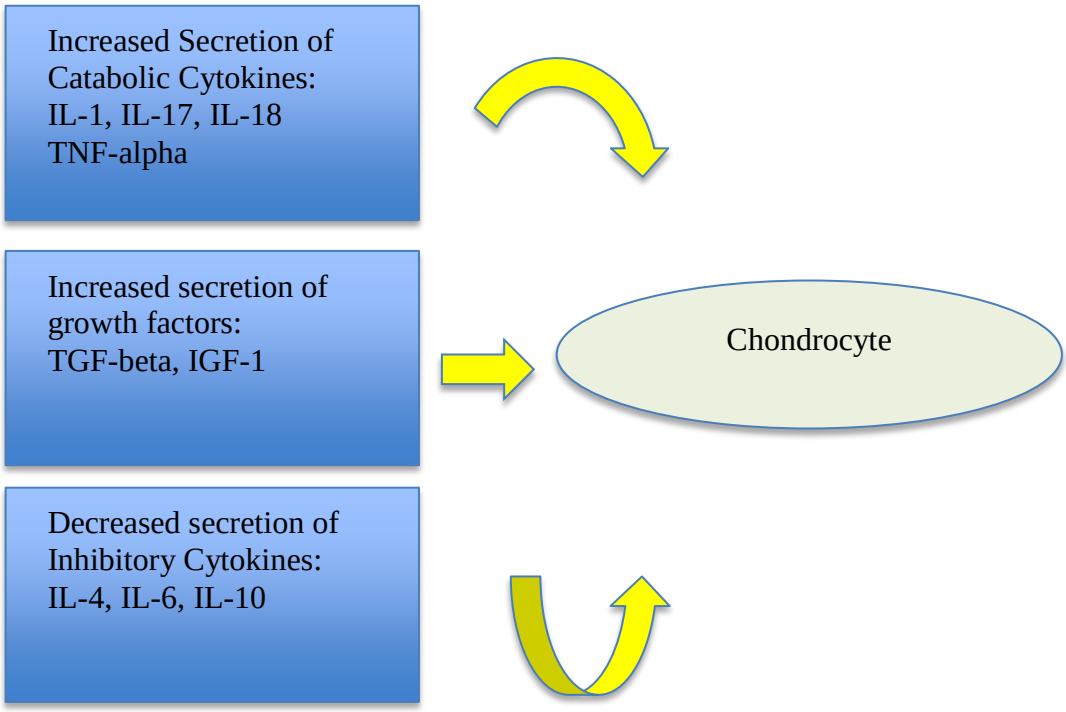


Figure 1-4. The balance of anabolic growth factors with destructive and inhibitory cytokines acting on articular chondrocytes in the pathogenesis of canine OA. Degradation of articular cartilage occurs via increased secretion of MMPs, interleukins and aggrecanases (mediated by IL-1 and TNF-alpha) alongside decreased secretion of TIMPs and TGF-beta receptor expression, which impairs important chondrocyte pathways of hypertrophy, division and matrix secretion. Secretion of crucial regulatory cytokines, such as IL-4 and IL-10, which influence levels of TNF-alpha and IL-1 acting on chondrocytes, is decreased. Ultimately there is destruction of proteoglycans and type II collagen resulting in loss of the extracellular matrix.

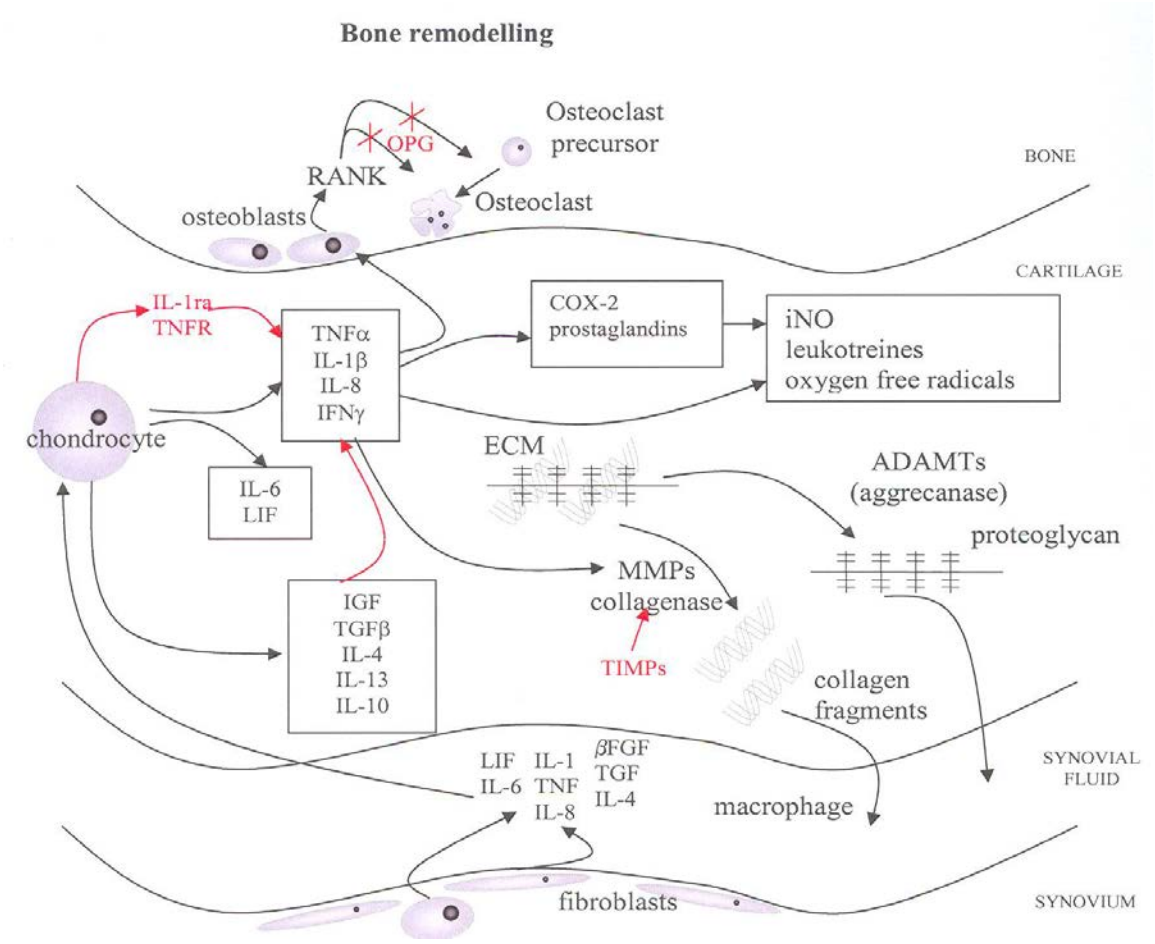


Figure 1-5. The molecular pathogenesis of OA is cytokine driven. Cytokines mediate pathological changes throughout the joint in OA, acting on synovial fibroblasts, macrophages in the synovial fluid, chondrocytes in the ECM and osteoblasts and osteoclasts in the subchondral bone. The release of cytokines is initially stimulated by the release of collagen and proteoglycan fragments into the synovial fluid when articular cartilage is damaged. This causes synovial macrophages and fibroblasts to release LIF, IL-6, IL-1, TNF-alpha, IL-8, beta-FGF, TGF and IL-4 into the synovial fluid. These cytokines diffuse through the ECM and subsequently stimulate a cascade of both anabolic and catabolic cytokine release from chondrocytes including IL-1RA, TNF-R, IL-6, LIF, IGF, TGF-beta, IL-8, INF-gamma. This results in an increase in inflammatory molecules such as nitric oxide, oxygen free radicals, leukotrienes, prostaglandins and COX-2, which further damage the ECM. Increases in TNF-alpha, IL-1beta, IL-8 and INF-gamma stimulate the release of a pro-osteoclastic factor, receptor activator of nuclear factor kappa B (RANK), from osteoblasts inhibiting OPG resulting in osteoclastogenesis, important for bone remodelling. Red arrows show inhibitory pathways and black arrows show promoting pathways.

When cartilage is damaged, components of the ECM are released into the synovial fluid. These fragments stimulate cytokine production from synovial cells and activation of MMPs, which then increase cartilage matrix destruction. Further ECM components are released resulting in a positive feedback cycle of MMP and cytokine production, including

IL-1 and TNF-alpha. IL-1 and TNF-alpha are known to cause extracellular matrix degradation (Poole, 2005). An increase in chondrocyte IL-1 receptors (Martell-Pelletier *et al.*, 1992) and TNF-alpha receptors (Shlopov *et al.*, 2000; Webb *et al.*, 1997) is known to occur. IL-1 triggers production of cyclooxygenase-2 (COX-2) in chondrocytes which inhibits synthesis of type II collagen and stimulates type I and III collagen production, contributing to fibrosis (Goldring and Goldring, 2004). It has been demonstrated that inhibition of IL-1 or TNF-alpha can supress matrix degradation and increase aggrecan production *in vitro* (Nagase and Kashiwagi, 2003). Experimental injury of cartilage explants has been shown to increase levels of MMPs and aggrecanases (Lee *et al.*, 2005; Patwari *et al.*, 2005). Little demonstrated that MMP-13 deficient mice had less cartilage erosion in experimentally induced OA due to decreased collagen loss, despite aggrecan loss (Little *et al.*, 2009). Up-regulation of collagenases occurs in response to increases in IL-1, IL-17 and TNF-alpha secretion by articular chondrocytes (Mitchell *et al.*, 1996). In addition, synovially secreted cytokines, including IL-1, IL-6, IL-17, IL-18, and TNF-alpha, dysregulate proteoglycan synthesis leading to enzyme-mediated cartilage destruction. (May, 1994; Miossec, 2004; Yim *et al.*, 2007).

As cartilage is degraded, there is a decrease in proteoglycan, hyaluronic acid and collagen content, decreasing the water absorbing capacity of the cartilage and causing a corresponding decrease in shock-absorbing capacity with localized areas of fibrillation and softening (Bennett and May 1995; Clark, 1991). Following further normal physiological loading, cartilage fissures, flaking and fractures occur with loss of the previously smooth articular surface and secretion of extracellular matrix components into the joint fluid as discussed above and further loss of normal load-bearing capacity (Bennett and May, 1995). Type II collagen and proteoglycans from degraded extracellular matrix induce inflammation of the synovium. This causes secretion of IL-1, TNF-alpha and nitric oxide (NO), (Goldring and Goldring, 2004). As these lesions progress, the cartilage is eroded and subchondral bone may eventually become exposed. Subchondral bone has a rich nerve supply and marked pain may be experienced by dogs once the articular cartilage has been completely eroded and the subchondral bone exposed. Inflammation of the synovial lining and increases in joint fluid volume are also associated with increased nociception. Increased hyaluronic acid catabolism and impaired synthesis result in a decrease in the shock absorbing and lubricating quality of the synovial fluid, increasing the risk of cartilage trauma in response to load bearing.

Anti-inflammatory molecules work to counteract cartilage degeneration caused by pro-inflammatory mediators (Goldring and Goldring, 2004). TGF-beta is a potent anti-inflammatory molecule with many protective actions. It acts to repair matrix damage by stimulating synthesis of proteoglycans and inhibiting further matrix destruction by reducing collagenase activity mediated by TNF-alpha (Shlopov *et al.*, 2000). It directly inhibits MMP release and induces the synthesis of protease inhibitors (Roberts and Sporn, 1990). TGF-beta can, however, increase levels of IL-1 thus contributing to cartilage damage as well as having a protective effect (Wahl *et al.*, 1993). Insulin-like growth factor-1 (IGF-1) increases the synthesis of prostaglandin and proteoglycan in OA joints, though it has also been associated with the development of pathological bony changes in OA (Martel-Pelletier *et al.*, 1999). IL-1RA competes with IL-1 for IL-1 receptors, thus down-regulating IL-1 activity (Arend, 2002; Caron *et al.*, 1996). TNF activity is modulated by direct binding and inactivation by TNF inhibitor molecules (Mageed *et al.*, 1998). IL-4 directly counteracts the activity of IL-1 mediated collagen damage and IL-13 increases IL-1RA synthesis and inhibits synthesis of MMPs.

The action of inflammatory and anti-inflammatory molecules, and indeed their interaction and feedback on each other, is a highly complex process involving many biological pathways which are still under investigation.

1.4 The role of senescence in age-related disease

Cellular ageing is associated with loss of tissue homeostasis and function resulting in organ and biological system impairment and an increase in the incidence of disease. This age related decline in cell function has been attributed to the onset of cellular senescence (van Deursen, 2014). It is thought that age-related cellular senescence occurs as a final end point of multiple senescence pathways, with accumulation of damage over time. Cellular senescence is a state of cell quiescence where replication has ceased but cells are still alive and exhibit ongoing secretory and metabolic functions with dramatic alterations in phenotype. Senescence is a critically important biological state, which protects against uncontrolled cellular replication as occurs in neoplasia (Bodnar *et al.*, 1998; Hayflick and Moorhead, 1961; Serrano *et al.*, 1997). Investigation of the causes of cellular senescence is an active area of current research. Four main senescence triggers have been identified; (1) telomere shortening, (2) activation of DNA damage responses, (3) stress-induced premature senescence (SIPS) and (4) activation of oncogenes. In cell culture, the onset of

experimentally induced senescence usually occurs over a period of weeks with cell viability lasting for several months (DeCecco *et al.*, 2013).

Senescence occurs with the onset of cell-cycle arrest via the p21-p53 and p16-pRB pathways (Figure 1-6). In acute senescence, the senescent cells are cleared and no further cycle arrest occurs. In chronic senescence, lamin B1 is down regulated resulting in the formation of senescence-associated heterchromatin foci as a result of altered methylation of chomatin (Freund *et al.*, 2012; Shah *et al.*, 2013). These foci "trap" cell-cycle genes promoting the state of replicative arrest and altered gene expression rates (Shah *et al.*, 2013; Zhang *et al.*, 2003). The cell adopts a senescence-associated secretory phenotype, which is characterised by up-regulation of pro-inflammatory cytokines and chemokines (Coppe *et al.*, 2008; Rodier *et al.*, 2009). This senescence-associated secretory phenotype is characteristic of almost all senescent cells regardless of the inducing cause of senescence (Coppe *et al.*, 2008).

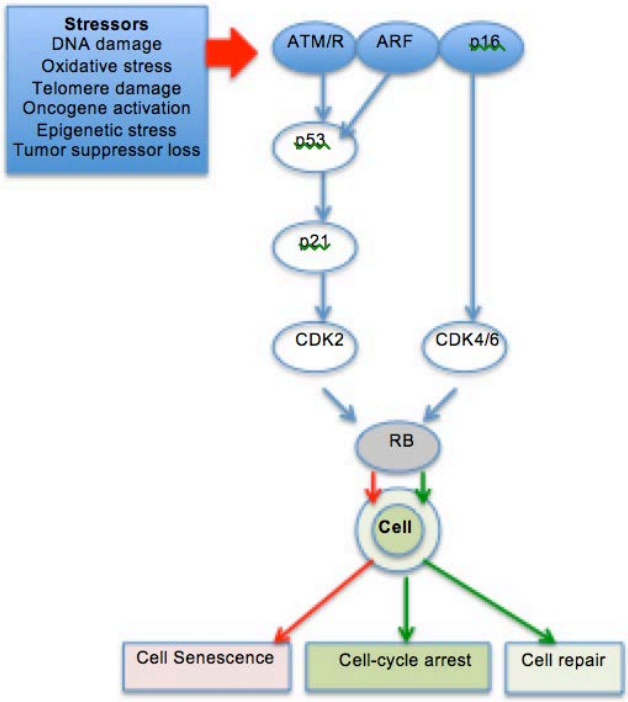


Figure 1-6. Stimuli of senescence and the main senescence pathways. "Stressors" activate two main senescence pathways; p53, p16 or both. The DNA damage response (including oxidative damage by ROS) activates p53, which in turn activates p21 causing cell cycle arrest via cyclin E-Cdk2 inhibition. The p16 pathways cause cell cycle arrest by inhibition of cyclin D-Cdk4 and cyclin D-Cdk6. Inhibition of Cdk prevents inactivation of Rb causing suppression of E2F target genes thus preventing the onset of S-phase in the cell replication cycle. Red arrows indicate senescence promoting and green arrows indicate senescence inhibiting activity.

Two distinct senescence processes have been identified: acute and chronic (discussed in Chapter IV, Introduction). It is thought that acute senescence is closely regulated, cell specific and has important homeostatic, developmental and reparative functions. The senescent cells are eliminated by the immune system once they have fulfilled their purpose (Jun and Lau, 2010; Krizhanovsky *et al.*, 2008; Storer *et al.*, 2013). Two examples of acute senescence include senescence of myofibroblasts in skin wound healing, and remodelling of tissues during embryogenesis (Storer *et al.*, 2013). In wound healing, myofibroblasts become senescent secreting cytokines and chemokines limiting excessive fibrosis of the healing skin (Jun and Lau, 2010). A similar process also occurs in acute liver injury, preventing fibrosis. Chronic senescence is related to ageing and the age-related decline in tissue function. This persistent growth arrest is a cumulative unscheduled event, with failure of clearance of these cells by an ageing immune system (Nikolich-Zugich, 2008; Wang *et al.*, 2011). The precise mechanism by which the immune system clears senescent cells is not yet understood.

Cellular senescence is a key factor in age-related disease (Baker *et al.*, 2012; Campisi, 2013; Hayflick and Moorhead, 1961), and has been associated with Alzheimer's disease, atherosclerosis, pulmonary fibrosis and OA (Naylor *et al.*, 2013). It has been previously demonstrated that senescent cells accumulate in both aged tissues (Herbig *et al.*, 2006; Lawless *et al.*, 2010; Wang *et al.*, 2011) and also in tissues undergoing trauma or remodelling (Jun and Lau, 2010; Krizhanovsky *et al.*, 2008; Storer *et al.*, 2013). Cellular senescence leads to age-related disease by cessation of cellular replication in tissues, by chronic tissue inflammation, by tissue fibrosis (Laberge *et al.*, 2012; Parinello *et al.*, 2005) and by increased incidence of cell death (Freund *et al.*, 2010). Senescent cells affect normal paracrine signalling leading to impaired tissue healing and repair (Jun and Lau, 2010). The senescent cell phenotype can also spread from cell to cell via IL-1 and TGF-beta secretion (Acosta *et al.*, 2013; Nelson *et al.*, 2012) leading to further tissue impairment as increasing numbers of cells are affected. While the senescent cell state is thought to protect against neoplasia, it has been demonstrated that some secreted cytokines together with MMPs can promote proliferation and spread of cancer cells (Campisi, 2011; Campisi, 2013) which may explain the age-related increase in rates of cancer noted in humans.

1.5 The role of senescence in Osteoarthritis

An established link exists between the development of OA and increasing age (Buckwalter, 1995; Buckwalter, 1997b; Buckwalter and Lappin, 2000; Buckwalter *et al.*, 2000; Buckwalter *et al.*, 2001). However, age-related changes to articular cartilage and the development of OA are two distinct events and one does not necessarily precede the other. The changes that occur with age mean that chondrocytes have less reparative and restorative capacity (Buckwalter and Mankin, 1997b; Buckwalter *et al.*, 2000), predisposing them to injury.

The normal functions of chondrocytes are impaired with age (Buckwalter *et al.*, 1993a; Buckwalter *et al.*, 1993b; Buckwalter *et al.*, 1994; Buckwalter and Lane, 1996; Buckwalter *et al.*, 2000; Bullough *et al.*, 1993; Mow *et al.*, 1995; Roth *et al.*, 1980; Verzijl *et al.*, 2000). Fibrillation of articular cartilage occurs with advancing age in humans; however there is no loss of function associated with this change (Buckwalter *et al.*, 2000; Koepp *et al.*, 1999). There are significant alterations to the structure of the extracellular matrix, with changes in the structure of both aggrecan and proteoglycan. (Buckwalter and Rosenberg, 1983; Buckwalter *et al.*, 1985). It has been shown that the size of aggrecan and chondroitin sulphate molecules decrease in an age-dependant manner (Buckwalter *et al.*, 1985), with link proteins undergoing structural changes also (Buckwalter *et al.*, 1986; Buckwalter and Rosenberg, 1988; Tang *et al.*, 1996). These changes result in significant loss of normal extracellular matrix structure, a decrease in water binding capacity and an overall loss in tensile strength and stiffness leading to altered load-bearing capacity (Buckwalter *et al.*, 1993a, Buckwalter *et al.*, 1993b; DeGroot *et al.*, 1999; Verzijl *et al.*, 2000). In addition to significant structural changes to cartilage, loss of secretory activity (Martin and Buckwalter, 2001) and loss of responsiveness of chondrocytes to anabolic growth factors and cytokines also occur with age (Loeser *et al.*, 2000; Martin and Buckwalter, 2000). The ultimate outcome of these changes is an aged tissue with a decreased ability to secrete normal extracellular matrix, an abnormal matrix structure and a decreased ability to respond to traumatic injury (Poole, 1997).

Similar to aged chondrocytes, chondrocytes affected by OA have been shown to have increased levels of senescence. This has been established by measurement of telomere lengths in OA chondrocytes. Telomeres are DNA sequences that cap the ends of linear

chromosomes. As cells replicate, telomeres shorten with each cell cycle until they reach a critical length beyond which replication ceases and cellular senescence occurs (Blackburn, 1991; Campisi, 1999). Thus each cell type can undergo a finite number of population doublings before growth arrest occurs. This is known as the Hayflick limit, a function which exists to protect cells and tissues from uncontrolled replication (Hayflick, 1965; Hayflick, 1996; Hayflick and Moorhead 1961). Chondrocytes can only undergo a limited number of cell divisions *in vitro*, with a Hayflick limit for human chondrocytes of approximately 35 population doublings (Evans and Georgescu, 1983). Cartilage is a post-mitotic tissue, with cellular proliferation occurring predominantly in juvenile tissue. Telomere erosion occurs progressively with age in chondrocytes leading to senescence (Martin and Buckwalter, 2001). Additionally, rates of chondrocyte replication have been shown to increase in response to trauma (as occurs in the development of OA) thus accelerating the rate of telomere erosion and increasing the time to onset of senescence (Buckwalter *et al.*, 2001). Telomere lengths have been shown to be decreased in human OA chondrocytes compared to normal human chondrocytes (Price *et al.*, 2002). Similarly, increased accumulation of senescence-associated beta galactosidase (SA-beta-gal), a marker of cell senescence, has been shown to occur in human OA chondrocytes compared to normal chondrocytes (Price *et al.*, 2002). Oxidative stress has been shown to erode chondrocyte telomeres resulting in the onset of premature senescence (Yudoh *et al.*, 2005). Chondrocyte cells have limited mitotic activity compared to other mammalian cell types, making them vulnerable to cumulative damage over time. Senescent chondrocytes have no replicative capacity and as such are unable to multiply in response to anabolic growth factors (Guerne *et al.*, 1995). Mechanical injury and senescence may be inter-related in the pathogenesis of OA. One study demonstrated that shear stress applied to cartilage explants increased levels of reactive oxygen species (ROS) and also stress induced premature senescence of chondrocytes (Martin *et al.*, 2004).

1.6 The role of oxidative stress in cartilage senescence

Oxidative stress is thought to play a role in chondrocyte senescence and OA pathogenesis (Dumont *et al.*, 2000; Toussaint *et al.*, 2000). It occurs as a result of an imbalance between the production of ROS and ROS scavenging enzymes (Figure 1-7). Oxidative stress levels increase with age *in vivo* and induce cellular senescence *in vitro* (Muller, 2009) by damaging cell signalling, DNA, lipids and proteins (Finkel and Holbrook, 2000). Mitochondrial DNA damage occurs as a cumulative effect of chronic oxidative stress and

is a feature of chondrocyte senescence (Martin and Buckwalter, 2002; Martin and Buckwalter, 2003). Mitochondrial DNA degradation leads to impairment of the electron transport chain and increased production of free radicals (Arnheim and Cortopassi, 1992; Kang *et al.*, 1998). It has also been suggested that increased ROS-mediated DNA damage leads to premature telomere shortening (Kurz *et al.*, 2005). An imbalance in the redox state has been associated with many disease processes (as discussed in Chapter VI). This imbalance has been linked to the pathogenesis of OA (Shah *et al.*, 2005), however the precise mechanism is still unclear. Oxidative stress is a highly dynamic process that undergoes rapid change. In the acute phase of oxidative stress, there is an increase in the level of antioxidant scavenging in response to increased oxidative stress, followed by a later phase characterised by impaired antioxidant protection and cell damage. The later stage of oxidative stress is associated with a reduction in antioxidant capacity in OA joints (Regan *et al.*, 2005; Yudoh *et al.*, 2005). Oxidative stress was increased in both a model of canine OA (Goranov, 2007) and a model of human OA (Pinto *et al.*, 2008; Rubyk *et al.*, 1988; Suprapaneni and Venkataramana, 2007), and has been shown to play a role in extracellular matrix breakdown, reduction in type II collagen and elevated levels of MMP-13 (Tiku *et al.*, 2000). *In vitro* loading of cartilage explants increases cartilage metabolism and free radical production (Heiner *et al.*, 2001).

Chondrocytes are particularly susceptible to cellular senescence. Once the cell population of articular cartilage is established with the cessation of skeletal development, there is no replenishment of chondrocytes (Buckwalter and Mankin, 1997a). Chondrocytes are metabolically quiescent and can exist in an environment of low oxygen tension (Grimshaw and Mason, 2000; Grimshaw and Mason, 2001; Murphy and Sambanis, 2001). When injury occurs the chondrocytes respond to repair the extracellular matrix, increasing synthetic activity and thus metabolic rate (Buckwalter and Mankin, 1997) resulting in the synthesis of ROS by chondrocyte mitochondria (Henrotin *et al.*, 1993; Mazetti *et al.*, 2001). Additionally, injury exposes the chondrocytes to external ROS, which over time can result in the onset of the senescent phenotype. When many cells are affected the extracellular matrix of articular cartilage can no longer be maintained and repaired (Martin and Buckwalter, 2001; Martin and Buckwalter, 2003). Research into the precise interactions between chondrocyte senescence, oxidative stress and OA pathogenesis is nascent but presents an important opportunity in devising new targets for disease prevention.

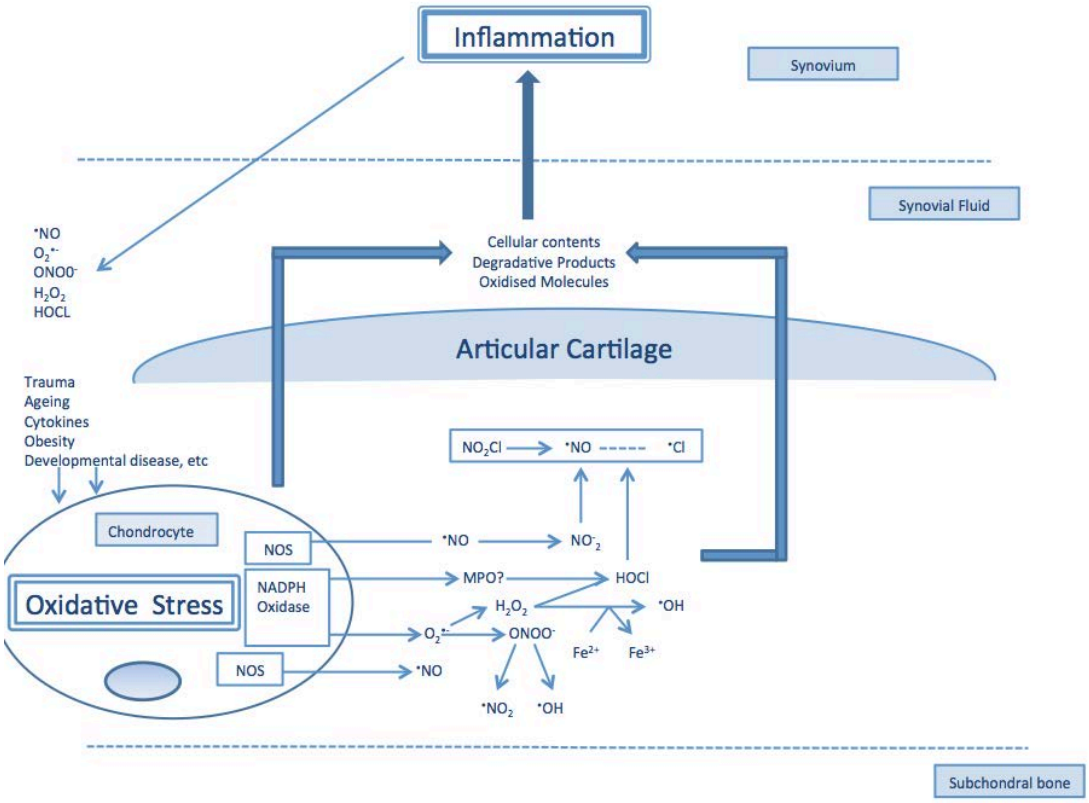


Figure 1-7. Sources of ROS and RNS in OA joints. ROS are primarily produced by chondrocyte mitochondria in response to increased metabolic rate induced in response to damage to the extracellular matrix, excessive cartilage loading or shear stress. IL-1 and TNF-alpha released in response to extracellular matrix damage induce nitric oxide (NO) formation by synovial cells via up-regulation of inducible nitric oxide synthase (iNOS) in inflamed joints. NO has a positive feedback effect, increasing IL-1 and TNF-alpha secretion by synovial fibroblasts, further increasing NO and iNOS production by chondrocytes and thus oxidative stress levels within the joint.

1.7 Epidemiology and clinical presentation

Primary canine OA occurs as a result of defective cartilage structure. It is very rare and has strong breed associations, affecting Chows, Dalmatians and Samoyed dogs (May, 1994). Causes of secondary OA are outlined above and secondary OA is the most common form of OA in domestic dogs. Canine hip dysplasia (CHD), elbow dysplasia and cranial cruciate ligament failure are the most common underlying causes of canine OA (Innes, 2005). Two age cohorts commonly present with clinical signs of OA; young dogs with joint pain and lameness related to an underlying cause of OA, and older dogs with established OA. In humans, age is also a risk factor for the development of OA (Hunter and Felson, 2006). This is discussed further below. Excessive body weight has also been identified as a risk factor in both canines and man (Felson *et al.*, 1988, 1992; Kealy *et al.*, 2000; Smith *et al.*,

2006). Marked reductions in lameness and pain have been achieved in dogs suffering from OA using weight reduction alone (Impellizeri *et al.,* 2000).

Dogs with established OA present clinically with lameness, decreased range of motion, loss of function and pain on joint manipulation (Todhunter and Johnston, 2003). These are the same signs observed in humans (Hunter and Felson, 2006; Moskowitz, 2007). The most common presenting sign in dogs is joint pain (Budsberg, 2004). As the disease progresses, crepitus on joint manipulation, periarticular fibrosis and muscle atrophy of the affected limb can often be detected during physical examination. Owners commonly describe an intermittent lameness that is worse after a period of rest. Chronic pain can be difficult to detect and associated signs noted by the owner may be the first suggestion of joint disease such as decreased exercise tolerance, reluctance to go up and down stairs or not wanting to jump in and out of the car.

1.8 Diagnosis

A tentative diagnosis of OA can be made based upon the physical examination findings and the history provided by the owner. Diagnostic imaging remains the primary method of confirming a diagnosis of OA in small animal veterinary patients (Carrig, 1997). A number of more advanced techniques are often employed in human medicine. To date these have largely been confined to research use in veterinary medicine, however advanced imaging modalities are likely to be increasingly used in veterinary medicine in the future. It should be remembered that radiographic osteoarthritic changes are a feature of end-stage OA and occur relatively late in the disease process. Radiographic features include joint effusion, osteophyte formation, the presence of bone cysts and increased radio-opacity of subchondral bone (Allan, 1998).

1.8.1 Diagnostic imaging

The main uses of radiography in canine OA are to confirm diagnosis and to identify dogs at risk of developing OA due to developmental joint disease (for example as part of a breed scoring scheme for CHD or elbow dysplasia). The original radiographic scoring system of OA lesions in humans was developed in 1957 by Kellgren and Lawrence (Kellgren and Lawrence, 1957). Many other scoring systems have been developed since, including

canine specific scoring systems for breed screening schemes (discussed in more detail in Chapter III). Radiographic assessment is limited and does not detect the early degenerative changes that occur in OA, with significant limitations incurred due to the two-dimensional nature of images obtained (Figure 1-8A&B). Computed tomography (CT) provides a three-dimensional radiographic image of joints and is very useful in diagnosing common canine arthropathies such as elbow dysplasia and CHD, providing more detailed information regarding joint congruity and bony changes compared to plain radiography alone. As with conventional radiographs, imaging of soft-tissue joint structures is poor. There is a significant cost differential between CT and plain radiography, with CT not as widely available, and most first opinion veterinary practices being equipped with an x-ray facility. Magnetic resonance imaging (MRI) is a superior imaging modality to both radiography and CT, providing high-resolution images of all joint structures including cross-sectional views in dogs (Hunter, 2008). Changes to articular cartilage, subchondral bone, periarticular soft-tissue structures, the synovial joint capsule and synovial fluid can be examined (Eckstein *et al.,* 2009; Teichtahl *et al.,* 2008). Bone marrow oedema was first identified as a feature of canine OA using MRI in 1988 (Wilson *et al.,* 1988). While MRI provides the best images for the diagnosis of OA, the cost and availability is prohibitive for routine use in domestic pets.

Figure 1-8A.

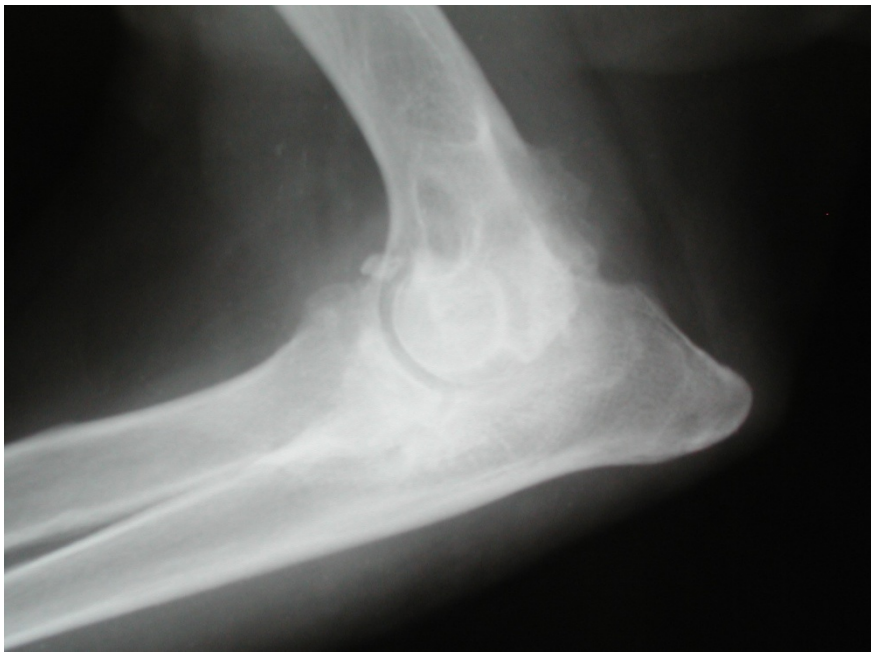


Figure 1-8B.

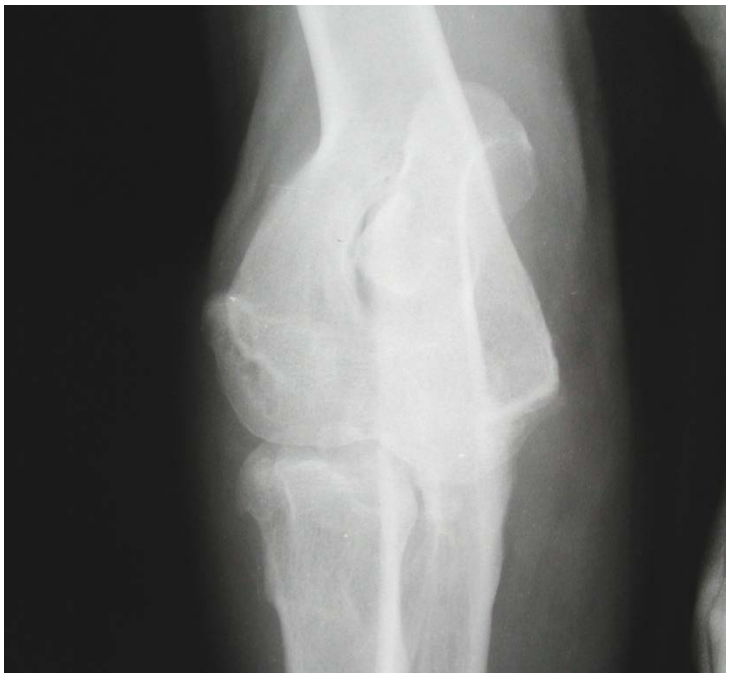


Figure 1-8. A mediolateral (1-8A) and a craniocaudal (1-8B) radiograph of a canine elbow joint showing marked OA changes including increased radio-opacity of subchondral bone and periarticular new bone formation.

1.8.2 Histological assessment

Histological assessment of affected cartilage provides a definitive diagnosis of OA. However, histopathology is rarely, if ever, used in the clinical diagnosis of canine OA. In both humans and dogs with suspected OA, collection of tissue samples for histopathological examination is particularly challenging and usually confined to the examination of excised tissues collected during joint replacement surgery or during *post-mortem* examination. Histopathological assessment is used for diagnostic purposes to assess the extent of pathological changes in human patients undergoing knee and hip replacement surgery. Several scoring systems are commonly used to grade human OA lesions including; the modified MANKIN-score (Mankin *et al.*, 1971), the Modified O'Driscoll Scale (O'Driscoll, 2001) and the International Cartilage Repair Society (ICRS) Visual Histological Assessment Scale (Mainil-Varlet, 2003).

1.8.3 Osteoarthritis biomarkers

A biomarker is "a substance, physiological characteristic, gene, etc. that indicates, or may indicate, the presence of disease, a physiological abnormality or a psychological condition"

(Collins English Dictionary, 2003). In relation to OA, biomarkers are measurable molecules that are released into body fluids as a result of cartilage, synovial and subchondral bone tissue damage, which serve to indicate the presence of OA (Lohmander, 2004). Currently there are no specific markers or combination of markers that can provide a definite diagnosis of OA (Rousseau and Delmas, 2007). However biomarkers can aid in the diagnosis of OA when evaluated in combination with other diagnostic evaluation, such as physical examination and diagnostic imaging. Research in this field is ongoing and it is hoped that a diagnostic panel of biomarkers will be discovered to enable early detection of the onset of OA, at which time there is an opportunity for delaying disease progression and the onset of clinical signs. Biomarkers used in human OA diagnosis are categorised into five types (Bauer *et al.,* 2006). These are diagnostic markers, burden of disease markers, prognostic markers, efficacy of intervention markers and investigative markers. Biomarkers currently used in both research and in the diagnosis of human OA include markers of cartilage metabolism. Type II collagen degradation products can be detected in serum and urine (Elsaid and Chichester, 2006; Rousseau and Delmas, 2007). MMPs and TIMPs can be detected in synovial fluid by immunoassay (Muddasani *et al.,* 2007), with increased levels of MMPs and decreased levels of TIMPs in patients with OA. The presence of urinary glycosyl-galactosyl-pyridinoline can be measured by immunoassay and is associated with synovial membrane damage in OA patients (Garnero *et al.,* 2001; Rousseau and Delmas, 2007). Biomarkers are not currently routinely used in the diagnosis of canine OA, but this represents a potentially useful tool in future early detection of disease.

1.9 Treatment options

There is currently no cure for OA and as such current treatment options focus on symptomatic management and amelioration of clinical signs. Treatment options can be divided into medical and surgical treatments (Kapatkin *et al.,* 2002).

1.9.1 Medical management of canine OA

Multimodal therapy is used in conservative medical management of canine OA. This approach involves achieving and maintaining a lean body weight, controlled regular exercise and pain reduction using pharmaceuticals (Lascelles and Main, 2002; Millis and

Levine, 2002; Pascoe, 2002; Fransen *et al.,* 2003). Pain reduction is an important factor in maintaining exercise levels and thus prolonging joint function whilst retaining muscle mass to support affected joints and sustaining a functional range of joint motion. The use of analgesic and anti-inflammatory therapeutic drugs has been the keystone of veterinary treatment of OA in dogs. Exercise is controlled to prevent further traumatic injury to compromised cartilage and subchondral bone.

1.9.1.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDS) are traditionally the mainstay of therapeutic drug treatment of canine OA (Johnston and Budsberg, 1997). These drugs are carboxylic and enolic acids with anti-inflammatory and analgesic effects. NSAIDS exert their action by the inhibition of cyclooxygenases (COX) and lipoxygenases (LOX), (Doherty, 1989). It has been demonstrated that there are increased levels of prostaglandins produced from both synovial cells and chondrocytes in OA joints, compared to normal joints (Knott *et al.,* 1994). In OA joints synovial membrane inflammation results in prostaglandin and leukotriene production via arachadonic acid metabolism by COX and LOX when cell membrane phospholipids are released (Knott *et al.,* 1994). Prostaglandins and leukotrienes result in the secretion of inflammatory mediators. NSAIDS act by blocking this inflammatory cascade. Most NSAIDS block both COX-1 and COX-2 enzymes (Fox and Johnston, 1997). COX-1 is present in most cells of the body. It inhibits the synthesis of prostaglandin E2 and has many important homeostatic roles, such as blood clotting and renal perfusion. Accordingly, COX-1 inhibition can have adverse effects. NSAID inhibition of COX-1 enzymes has been associated with renal papillary necrosis and GI ulceration (MacPhail *et al.,* 1998; McDonald and Langston, 1995). The therapeutic effects of NSAIDS result predominantly from COX-2 inhibition. COX-2 is induced at sites of inflammation and produces the inflammatory mediator prostaglandin E2. However, it also has important homeostatic functions in some tissues and long term use has been associated with impaired wound healing, renal impairment and cardiac disease (Simon *et al.,* 2002).

More recently COX and LOX dual inhibitors have been introduced for treatment of OA in dogs (including firocoxib, rofecoxib and licofelone), blocking both prostaglandin and leukotriene synthesis. However, the dual inhibition has been shown to be transient in dogs, with marked COX-1 inhibition. For canine OA, carprofen, meloxicam and robenacoxib are

the NSAIDs mostly commonly used in the U.K. Due to their potential side-effects, these drugs do have limitations to their usage and research to find new therapeutics with less potential adverse effects is ongoing (Pendleton *et al.,* 2000). The NSAID mavacoxib (Trocoxcil™), like robenacoxib, is a COX-2 specific inhibitor and has the advantage of having a long plasma half-life, requiring less frequent dosing than other NSAIDS (Reymond *et al.,* 2012).

1.9.1.2 Disease modifying osteoarthritic agents

Disease modifying osteoarthritic agents are commonly used in both canine and human medicine. The therapeutic target of these agents is to increase the intra-articular levels of extracellular matrix components. Nutraceutical supplementation with glucosamine sulphate, chondroitin sulphate (Black *et al.,* 2009) and hyaluronic acid (Brzusek and Petron, 2008) has been reported to improve clinical signs of OA in man, however this evidence has been widely challenged. Chan *et al.,* (2006) showed that glucosamine and chondroitin sulphate inhibit the expression of MMPs and ADAMTSs and increase the expression of one of their natural inhibitors, TIMP-3, *in vitro*. In veterinary medicine, glucosamine hydrochloride, chondroitin sulphate and essential fatty acids are commonly used as oral supplements. All three have been shown to have beneficial effects in treating OA in dogs (Clegg *et al.,* 2006; McCarthy *et al.,* 2007; Roush *et al.,* 2010). The use of these agents in the treatment of OA remains controversial with reports describing conflicting efficacy. It was demonstrated that glucosamine reduced signs of OA in dogs in two studies (Johnson *et al.,* 2001; McCarthy *et al.,* 2007), while another study reported no improvement (Moreau *et al.,* 2003). A 2003 human study compared the effects of NSAIDS and glucosamine sulphate and demonstrated superior effects using glucosamine (Towheed *et al.,* 2003). Chondroitin sulphate has been shown to cause a reduction of OA symptoms in man (Clegg *et al.,* 2006; Pipitone *et al.,* 1992; Morreale *et al.,* 1996; Uebelhart *et al.,* 1998; Verbruggen *et al.,* 1998).

Polysulphated glycosaminoglycans (PSGAGs) are derived from bovine trachea and pulmonary tissue. They have an inhibitory effect on inflammatory mediators and destructive enzymes (MacPhail, 2000). Pentosan polysulphate is a semi-synthetic polysulphated polysaccharide derived from xylan in plants. These matrix supplements are supplied as commercial preparations for dogs with OA and are usually administered orally. Their mode of action is thought to involve increasing proteoglycan synthesis by

chondrocytes, increasing hyaluronic acid synthesis by synovial fibroblasts and increasing synovial and subchondral blood flow (Francis and Read, 1993; Read *et al.,* 1996; Rogachefsky *et al.,* 1994).

Dietary supplementation of Omega-3 essential fatty acids and changes in the ratio of dietary Omega-3 and Omega-6 essential fatty acids have been shown to decrease inflammation associated with OA in some studies (Campbell, 1993; Miller *et al.,* 1992; Vaughn *et al.,* 1994) and a beneficial effect in dogs with OA has been demonstrated (Miller *et al.,* 1992). Their mode of action is by alterations in the types of prostaglandin and leukotriene produced within the diseased joint by altering the composition of cell membrane phospholipids. Recently a further mode of action was identified with the discovery of a new class of anti-inflammatory Omega-3 poly-unsaturated-fatty-acid-derived lipid mediators called resolvins and protectins (Kohli and Levy, 2009; Weylandt *et al.,* 2012). These compounds are hydroxylated derivatives of the parent Omega-3 poly-unsaturated-fatty-acid eicosapentaenoic acid for the E-resolvins, and docosahexaenoic acid for the D-resolvins and protectin D1 (Weylandt *et al.,* 2012). It has been demonstrated in dogs with chronic osteoarthritis receiving carprofen as an analgesic, that the use of Omega-3 supplementation allowed for a reduction in the carprofen dosage required to reduce clinical signs of OA (Roush *et al.,* 2010). The use of green-lipped mussel extract has also been shown to have an anti-inflammatory effect in canine OA (Croft, 1995; Korthauer and De-La-Torre, 1992), with improvement in pain and lameness (Croft, 1995). It has also been suggested that this extract can have gastrointestinal protective effects against the development of ulceration in dogs receiving concurrent NSAID therapy (Croft, 1995).

1.9.2 Surgical treatment options

Surgical management of canine OA is generally employed as a salvage procedure. Early surgical intervention in cases of severe joint deformity (such as hip dysplasia or elbow dysplasia) has been recommended to prevent the onset of severe cartilage degeneration. Surgery can effectively treat articular fractures, cruciate ligament rupture, osteochondrosis, incongruity, hip dysplasia and elbow dysplasia. The goal of surgical treatment is to prevent or delay the progression of OA. Surgical treatment can also be used as an end-stage salvage procedure to manage severe pain and joint dysfunction, which is refractory to medical management. Osteotomy removes the affected joint surfaces to alleviate the clinical signs of OA, for example the use of femoral head and neck excisional arthroplasty

to treat hip OA. Partial and total joint arthroplasty has been successful in the treatment of end stage OA (Kurtz *et al.*, 2007) and is used in the treatment of OA of the hip, elbow and stifle joints of dogs. Complications can be significant, and implants have a finite lifespan due to time dependent loosening (Wylde *et al.*, 2007). Regeneration of damaged cartilage is currently the focus of new treatment modalities and research. Techniques without tissue and cell transplantation involve debridement of OA cartilage lesions and the exposure of the underlying bone marrow (for example by drilling holes or creating microfractures) to provide a source of progenitor cells for repairing the damaged cartilage (Falah *et al.*, 2010).

1.9.3 Adjunctive therapies

Adjunctive therapies include physical therapy and acupuncture, a full discussion of which is beyond the scope of this chapter, though physiotherapy may be helpful in assisting to maintain muscle mass and range of joint motion.

1.9.4 Cell based therapies

Multiple cell based therapies are undergoing development for the treatment of OA. The goal of these therapies is to facilitate tissue repair by regeneration of functional articular cartilage. There have been two main areas of research; the use of directly-injected mesenchymal stem cells and the use of stem cell scaffold constructs. A tissue engineering approach to articular cartilage regeneration was first described in 1994 (Brittberg *et al.*, 1994) and involved injection of expanded autologous chondrocytes below the periosteum of the joint undergoing treatment. This technique had limited success as OA lesions continued to increase in size and regeneration of new articular cartilage took a long time (Davies-Tuck *et al.*, 2008; Peterson *et al.*, 2000). There are currently a wide variety of tissue engineering treatments available for the treatment of OA in both humans and dogs. Tissue transplantation methods include transplantation of autologous or allogenic osteochondral plugs (such as Osteochondral Autograft Transfer System which is an autograft of ostechondral cylinders) and periosteal transplantation (Attmanspacher *et al.*, 1999).

The next phase of cell therapy development involved the use of mesenchymal stem cells. Mesenchymal stem cells harvested from the patient were stimulated to induce

chondrogenic differentiation and were either injected directly into OA joints (Murphy *et al.,* 2002; Noth *et al.,* 2008) or implanted on bioscaffolds. It is not known exactly how mesenchymal stem cells migrate to sites of cartilage damage to facilitate repair following direct injection into the joint. It has been shown that mesenchymal stem cells can differentiate into chondrocytes and that they secrete bioactive factors which support stem cell replication and inhibit apoptosis (Chen *et al*., 2006; Kan *et al*., 2007; Uccelli *et al*., 2007). Allogeneic mesenchymal stem cell transplants have also been used in a clinical trial (Uth *et al.,* 2014). The stem cells were harvested from donated human umbilical cord tissue and then combined with with hyaluronan before injection into OA-affected joints. Mesenchymal stem cells have also be cultured on a bioscaffold which was then implanted at the site of cartilage injury (Noth *et al.,* 2008), resulting in the production of new articular cartilage following scaffold implantation (Hollander *et al.,* 2006; Moretti *et al.,* 2005). Both synthetic and native (Kuo *et al*., 2006; Nesic *et al*., 2006) materials have been used for bioscaffold production. Collagen type I hydrogels containing mesenchymal stem cells were successfully used in a clinical trial in humans, resulting in the repair of full thickness articular cartilage lesions with a five year follow-up (Wakitani *et al*., 2004). One important challenge in the development of cell- bioscaffold constructs is the development of scaffold materials that can resist weight-bearing forces over a period of many years (Hollander, 2010; Noth, 2008). These cell based therapies are still undergoing clinical trials and evidence based clinical treatment protocols have not yet been developed.

Intra-articular administration of platelet rich plasma is another form of cell therapy, which has undergone clinical trial in dogs (Fahie *et al.,* 2013). With this treatment, blood is collected from the dog and treated to obtain a platelet cell concentrate which is then injected into the articular joint undergoing treatment for OA. The mechanism of action of platelet cell injection is currently unknown, but is thought to involve stem cell chemotaxis (Lee *et al.,* 2012) and growth factor release (Nguyen *et al.,* 2011). In clinical trial in dogs with OA, the use of a single intra-articular injection of platelet-rich plasma resulted in a significant improvement of pain and lameness at twelve weeks (Lee *et al.,* 2012).

1.9.5 Gene therapy treatment of OA

It has been hypothesised that very early identification of the initiation of OA may present a treatment window where the disease process can be reversed or stopped (Stoker *et al.,* 2006). Molecular targets for the treatment of OA are currently being intensively

researched. Positive results have been found in initial studies looking at gene therapy (Gelse *et al.*, 2005). Transplantation of genetically modified mesenchymal stem cells and fibroblasts (by transfection of cells with both viral and non-viral vectors) is a promising area of research and these techniques have been used successfully to treat rheumatoid arthritis (Robbins *et al.*, 2003). Interleukin-1 Receptor Antagonist Protein (IRAP) gene therapy was first used to treat rheumatoid arthritis in humans in 2005 (Evans *et al.*, 2005) and has undergone evaluation in pre-clinical trials as treatment for rheumatoid arthritis in humans (Wehling, 2009). Inhibition of IL-1 has been shown to inhibit articular cartilage degradation (Pelletier *et al.*, 2000). Deletion of ADAMTS-5 has been shown to prevent OA in mice *in vitro* (Glasson *et al.*, 2005). Etanercept is a licenced gene therapy viral vector TNF antagonist used to treat rheumatoid arthritis in humans. It is administered as bi-weekly subcutaneous injections, though limited efficacy in reduction of clinical signs has been reported (Kerensky *et al.*, 2011). An injectable IFN-beta gene therapy has been under development as an anti-inflammatory treatment for rheumatoid arthritis (Vervoordeldonk *et al.*, 2009), though early clinical outcomes were also disappointing (van Holten *et al.*, 2005). It is likely that as future studies unravel the complex pathogenesis of OA, other potential therapeutic gene targets will emerge. The present study has been concerned with the role of chondrocyte senescence and the role of oxidative stress in the pathogenesis of canine OA, areas which are amenable to therapeutic intervention in the future.

1.10 General Aims and objectives

The overall aim of this thesis was to investigate the role of cellular senescence in the pathogenesis of canine osteoarthritis. It has long been known that damage to articular cartilage causes inflammation within a joint. More recently it has been shown that degeneration of articular cartilage mediates increased production of reactive oxygen species by chondrocytes causing oxidative stress in articular cartilage. In studies investigating osteoarthritis as an age related disease in humans, it was discovered that human chondrocytes had increased levels of cellular senescence and other studies have determined oxidative stress to be a cause of cellular senescence. In seeking to further our understanding of the pathogenesis of canine osteoarthritis, a hypothesis was formulated linking cellular senescence and oxidative stress to the disease process. The hypothesis was firstly, that cellular senescence is a feature of OA chondrocytes and secondly, that oxidative stress causes senescence of canine articular chondrocytes. Considering recent findings that senescent articular chondrocytes have impaired biological functions contributing to the pathogenesis of OA, we hypothesised that senescent OA chondrocytes contribute to the pathogenesis of OA by altering the gene expression of adjacent normal chondrocytes. We also hypothesised that oxidative stress can alter the expression of genes key to OA pathogenesis in normal canine chondrocytes.

Specifically, the aims and objectives were to:

1. evaluate the presentation of dogs undergoing femoral head excision in veterinary referral practice in the UK as a treatment for osteoarthritis of the coxofemoral joint and to study the distribution and severity of associated pathological lesions.
2. evaluate cellular senescence in chondrocytes from osteoarthritic articular cartilage.
3. investigate the hypothesis that oxidative stress is a feature of canine OA chondrocytes and that oxidative stress contributes to cellular senescence in canine chondrocytes.
4. investigate the hypothesis that osteoarthritic chondrocytes alter the gene expression of adjacent normal chondrocytes in OA joints leading to modulation of genes known to play a role in the pathogenesis of OA in normal chondrocytes.

Chapter II

General Materials and Methods

The following General Materials and Methods detail the equipment and methodology used in the experiments described in this thesis. Where additional materials were utilised or the methodology differed from that described below, a description will follow in the subsequent chapters.

2.1 Materials

2.1.1 Plasticware

-Syringe top cell strainer filters (0.2mm pore size), were supplied by Nalgene (NY, U.S.A.).

-Ependorf tubes: screw top 1.5ml tubes were from Camlab (Cambridgeshire, U.K.), and 0.5ml and 1.5ml flip top tubes were supplied by Anachem (Luton, U.K.).

-Pipette tips, universal fit yellow and blue tips, were supplied by Greiner Bio-one (Gloucestershire, U.K.).

-Filter tip pipette tips; 10µl from Greiner Bio-one (Gloucestershire, U.K.); 100µl, 200µl and 1000µl were supplied by Starlab (Milton Keynes, U.K.).

-Petri dishes were supplied by Sterilin (Staffordshire, U.K.).

-ThinCert 6-well plate tissue culture inserts were supplied by Greiner Bio-one (Gloucestershire, U.K.).

-Scalpel blades were supplied by Swan-Morton (Sheffield, U.K.).

-Parafilm was supplied by Sigma Aldrich (Dorset, U.K.).

-Tissue culture flasks and Falcon conical centrifuge tubes (15ml and 50ml) were supplied by Greiner Bio-one (Gloucestershire, U.K.).

-6-well plates and pipettes (5ml, 10ml, 25ml, 50ml) and cell filters were supplied by Corning (supplied by Fisher Scientific in West Sussex, U.K.).

-20ml syringes and 22 gauge needles were made by Terumo (supplied by Henry Schein in Glasgow, U.K.).

-Gene Chip: Gene Chip canine genome microarray 2.0 from Affymetrix (California, U.S.A.).

2.1.2. Common reagents, solutions and media

All solutions and media for cell culture were supplied by Invitrogen (U.K.), unless otherwise stated.

2.1.2.1 Cell Culture Media

-Complete growth medium: DMEM supplemented with 10% foetal calf serum, 100 U/ml Penicillin Streptomycin and 1.25 μg/ml Fungizone.
-Freeze medium for cryopreservation of chondrocytes for long-term storage in liquid nitrogen: 9ml of complete growth medium with 9ml foetal calf serum and 2ml DMSO solution.
-Dimethylsulphoxide (DMSO) solution: (45% medium/ 45% foetal calf serum/ 10% DMSO). DMSO supplied by Invitrogen, (U.K).
-Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-1 with sodium pyruvate, glucose and pyridoxine (Invitrogen, U.K.).
-Foetal Bovine Serum (FBS); (Invitrogen, UK): heat inactivated at 56°C for 30 minutes, then stored in 50ml aliquots at -20°C.
-Penicillin/streptomycin (Sigma, U.K.): supplied as a 100x stock solution of 10,000 units penicillin and 10,000 units streptomycin per ml and stored in 5 ml aliquots at -20°C.
-Ciproxin (Sigma, U.K.): supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.
-Fungizone (Sigma, U.K.): supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.
-Trypsin-EDTA: supplied as a 100x stock solution and stored in 5 ml aliquots at -20°C.
-Collagenase from Sigma Aldrich (Dorset, U.K.): Made up in serum free DMEM at a concentration of 0.25g dry collagenase in 50ml DMEM (final concentration of 5mg/ml solution).
-Alginate: 1.2% sodium-alginate from Fluka (Deisenhofen, Germany).

2.1.2.2. Buffers and solutions

-Lysis Buffer: 25mM Tris HCL pH 8.0 with glacial acetic acid and made up to 2L volume

-Lysis Buffer for Western Blotting: 0.5% NP40, 150mM NaCl, 50mM TrisHCL adjusted to pH 8.0, protease inhibitor tablet (Roche, U.K.), 1 tablet was added per 10ml of lysis buffer mix.

-1M Tris HCL: 121g Tris base, 800ml distilled H_2O. Adjusted to the desired pH with concentrated HCL and made up to 1L.

-TE Buffer: 10mM Tris-HCL (pH 8.0), 1mM EDTA.

-1 x PBS: 140mM NaCl, 2.7mM KCL, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3).

-0.2M (2x) Phosphate Buffered Saline (PBS): 0.2M Na2HPO4, 0.2M NaH_2PO_4, 1.8% w/v NaCl, pH 7.4

-10x DNA Gel Loading Buffer: 30% w/v glycerol, 0.25% bromophenol blue, 70% TE buffer. Stored at room temperature and used at a 1:10 dilution.

-Ethidium bromide: made to a working dilution of 10mg/ml with distilled H_2O in a fume cupboard. Stored away from light.

-DEPC water: 0.5ml of Diethylpyrocarbonate in 500ml water, overnight at room temperature in the fume cupboard and autoclaved.

-Lysis mix: 0.32M sucrose, 10mM Tris, 5mM MgCl, 1% Triton X.

-Nuclei lysis mix: 10mM Tris, 0.4 NaCl, 2mM EDTA.

-TRIS-EDTA: (10/1 pH 7.4).

-TRIS-EDTA: (10/0.1 pH 7.5).

-NU-PAGE Transfer Buffer: supplied by Invitrogen and diluted 1:20.

-NU-PAGE Running Buffer: supplied by Invitrogen and diluted 1:20.

-10x Ponceau's Red Stain: 2g Ponceau S, 30g Trichloroacetic acid, 30g Sulphopalicylic acid. Made up to 100 ml by addition of dH2O.

-TBS-Tween: 150 mM NaCl (8.77 g), 50 mM Tris (6.06 g) and 0.05% Tween (0.5 ml). Made up to 1L by addition of distilled H_2O.

-Blocking Buffer: 1 g non-fat skimmed milk powder in 20 ml TBS-Tween.

-Antibody diluent: 0.2 g non-fat skimmed milk powder in 20 ml TBS-Tween.

-Bio-Rad Reagent: supplied Bio-Rad Laboratories (Hemmel Hempstead, U.K.).

-Bovine Serum Albumin (BSA): supplied by Sigma Fraction V, (U.K.).

-Superscript III reaction mix for first strand cDNA synthesis: 20µl 10x RT buffer (10x), 4µl 25mM MgCl2, 2ul 0.1M DTT and 1ul 40U/µl RNase OUT.

-Tert- Butyl hydroperoxide from Sigma Aldrich (Dorset, U.K.).

2.1.3. Antibodies

Antibodies used in Western blot experiments are listed below, along with dilution and supplier.

Table 2-1. List of all antibodies used in the experiments presented in this thesis.

Antibody	Species	Dilution	Supplier
p16	rabbit	1:50	Santa Cruz Biotechnology
P38 MAPK pP38 MAPK	rabbit rabbit	1:1000 1:1000	Cell Signalling Technologies
TNF-alpha	rabbit	1:200	Santa Cruz Biotechnology
MMP-13	rabbit	1:200	Santa Cruz Biotechnology
Tubulin	rabbit	1:200	Santa Cruz Biotechnology

2.1.4 Complete kits

-SA-beta-gal kit: Senescence cells histochemical staining kit, Sigma-Aldrich (Dorset, U.K.).

-Glutathione assay kit: Glutathione Assay kit, Sigma-Aldrich (Dorset, U.K.).

-Live/Dead staining kit: LIVE/DEAD Viability/Cytotoxicity Kit, Life Technologies (Paisley, U.K.).

-ECL-PLUS Western Botting Detection Reagents, GE Healthcare (Buckinghamshire, U.K.).

-SuperScript III First-Strand Synthesis System for RT-PCR Invitrogen (Paisley, U.K.).

-RNeasy Mini Kit from Qiagen (Crawley, U.K.).

-Oligonucleotide primers and probes: Oligonucleotides for use in polymerase chain reactions (PCR) and cycle sequencing were synthesised by Integrated DNA Technologies, (U.K.). Primers and probes were Poly Acrylamide Gel Electrophoresis (PAGE) purified and supplied as lyophilised DNA.

2.1.5 Cell lines

Table 2-2. OA samples used in this thesis.

Case no.	Age in months	Breed	Sex
OA1	13	Rottweiler	FE
OA 2	13	Labrador	MN
OA 3	58	Labrador	MN
OA 4	11	Crossbreed	ME
OA 5	17	Crossbreed	FN
OA 6	12	Golden Retriever	MN
OA 7	84	Crossbreed	FE
OA 8	109	German Shepherd Dog	FN
OA 9	10	Springer Spaniel	ME
OA10	8	Crossbreed	FN
OA 11	11	Labrador	ME
OA 12	7	Border Collie	ME
OA 13	95	Weimeraner	FN
OA 14	27	Bearded Collie	FE
OA 15	120	Border Collie	FN
OA 16	31	Crossbreed	MN
OA 17	42	Border Collie	MN
OA 18	111	Crossbreed	FN
OA 19	13	Old English Sheepdog	MN
OA 20	18	Labrador	MN
OA 21	72	German Shepherd Dog	FN
OA 22	84	German Shepherd Dog	FE
OA 23	12	Crossbreed	FN
OA 24	36	Siberian Husky	FN
OA 25	60	Labrador	FN
OA 26	84	Labrador	MN
OA 27	60	Border Collie	MN
OA 28	94	Labrador	FE
OA 29	6	Cavalier King Charles Spaniel	FE
OA 30	60	Labrador	MN
OA 31	72	Border Collie	MN
OA 32	48	Rough Collie	ME
OA 33	72	Labrador	FN
OA 34	84	Crossbreed	MN
OA 35	72	German Shepherd Dog	FN
OA 36	18	Cocker Spaniel	MN
OA 37	156	Golden Retriever	FN
OA 38	60	Rough Collie	ME
OA 39	9	Golden Retriever	FE
OA 40	14	Labrador	ME
OA 41	96	Springer Spaniel	ME
OA 42	13	Leonberger	ME
OA 43	36	German Shepherd Dog	FN
OA 44	54	Rough Collie	MN
OA 45	7	Labrador	FE
OA 46	30	Labrador	ME
OA 47	90	German Shepherd Dog	FE
OA 48	72	Labrador	ME
OA 49	14	Jack Russell Terrier	ME
OA 50	31	Labradoodle	MN
OA 51	48	Crossbreed	FN
OA 52	100	German Shepherd Dog	FN

Table 2-2 (continued). OA samples used in this thesis.

Case no	Age in months	Breed	Sex
OA 53	13	German Shepherd Dog	FE
OA 54	96	German Shepherd Dog	ME
OA 55	13	Labrador	ME
OA 56	32	Rottweiler	MN
OA 57	110	German Shepherd Dog	FN
OA 58	72	Springer Spaniel	ME
OA 59	18	Dogue de Bordeaux	ME
OA 60	26	German Shepherd Dog	MN
OA 61	10	Leonberger	ME
OA 62	69	Labradoodle	MN
OA 63	48	Labrador	FN
OA 64	132	Golden Retriever	FN
OA 65	28	German Shepherd Dog	ME
OA 66	72	Crossbreed	FN
OA 67	84	Labrador	FN
OA 68	82	Labrador	FN
OA 69	29	Springer Spaniel	FN
OA 70	84	West Highland White Terrier	FE
OA 71	17	Labrador	FE
OA 72	73	Border Collie	ME
OA 73	73	Irish Red Setter	MN
OA 74	10	Labrador	ME
OA 75	8	German Shepherd Dog	ME
OA 76	11	Border Collie	FE
OA 77	11	Border Collie	FE
OA 78	96	Border Collie	FN
OA 79	72	German Shepherd Dog	FE
OA 80	26	Labrador	ME
OA 81	39	German Shepherd Dog	FE
OA 82	84	Crossbreed	MN
OA 83	72	German Shepherd Dog	FN

Table 2-3. Normal cartilage samples used in this thesis.

Case no	Age in months	Breed	Sex
N1	0	Rottweiler	FE
N2	0	Shih Tzu	ME
N3	0	Cavalier King Charles Spaniel	ME
N4	132	Crossbreed	ME
N5	110	Border Collie	FE
N6	72	Staffordshire Terrier	ME
N7	36	Staffordshire Terrier	MN

2.1.6 Major Equipment

-Benchtop centrifuge: CPR Centrifuge (Beckman)
-Microcentrifuges: Centrifuge 5402, 5415R and Minispin (Eppendorf)
-Water baths: Sub 36, and W6 (Grant)
-Spectrophotometer: GeneQuant pro RNA/DNA calculator
-Automatic Sequencing Apparatus: ABI 3010
-Pipettes: Finnipipette Techpette (0.5-10, 5-40, 40-200, 200-1000 μl)
-Ultraviolet trans-illuminator: T2201 (Sigma Chemical Company)
-Gel documentation system: Uvi tec (Thistle Scientific)
-Horizontal orbital shaker: 4628-1CE Labline Instruments inc (U.K.)
-Incubator: B5042 (Heraeus)
-Gel systems: Hoefer HE 33 Mini Horizontal Submarine Unit
-Balance: Precisa 100A-300M (Precisa Balances Ltd, Bucks, U.K.)
-Stirrer: Magnetic Stirrer Hotplate (Stuart Scientific, U.K.)
-PCR Machines: GeneAmp PCR System 2400, 2700 and DNA Thermal Cycler 480 (Perkin Elmer)
-Leica DMIL LED light microscope

2.2 Methods

Commonly used methods in this thesis are described in this chapter and specific techniques are described in the relevant chapters.

2.2.1 Collection of femoral heads

Tissue samples were collected from the femoral heads of dogs at The Small Animal Hospital, School of Veterinary Medicine, University of Glasgow, from the SSPCA Cardonald Centre and from specialist referral centres in the U.K. with informed owner consent. The OA chondrocyte group included dogs undergoing a total hip replacement for osteoarthritis and/or hip dysplasia. The normal chondrocyte group consisted of dogs of various breeds that were euthanatised for reasons unrelated to musculoskeletal disease. In addition three foetal samples were obtained from still born puppies, and one sample was obtained from an eight-week old puppy undergoing *post-mortem* examination (included in

the normal chondrocyte group). The following information was recorded for each femoral head sample; patient/case number, age of donor animal, breed, sex, the date that the sample was collected and from which veterinary practice the sample was received. Femoral heads samples were collected into a sterile pot containing PBS Glycerol. Samples from veterinary referral hospitals were transported using a next day delivery service. The samples were photographed and the OA lesions were scored for gross pathology (as described in Chapter III) upon receipt. Cartilage samples were collected from the femoral head by careful dissection using sterile technique. Excised cartilage was placed in a 15ml conical tube filled with DMEM complete culture medium. The tubes were spun at 1000 rpm for 5 minutes and culture medium was removed. To digest chondrocytes from the cartilage samples 4.5ml plain DMEM and 0.5ml trypsin was added to the spun sample. This was then placed on an orbital shaker in a room heated to 37°C for 1 hour and the digest solution was then removed. Complete DMEM (4.5ml), 5µl ciproxin and 0.5ml collagenase was added and the sample tube was placed on an orbital shaker in room heated to 37°C overnight. Following digestion, the solution was filtered through a cell strainer into a 50ml falcon tube and spun at 1000rpm for 5 mins. The liquid was removed leaving a chondrocyte cell pellet. Complete DMEM cell culture medium was added (10ml) and the cell pellet was resuspended. A small volume of cell suspension (20µl) was removed for cell counting. Ciproxin was added (10µl) and the cells were transferred to a $25cm^2$ flask for culture.

2.2.2 Tissue Culture

Cell culture was carried out in a laminar flow hood using aseptic technique. Chondrocytes for cell culture were processed within two hours of surgical removal of donor femoral heads. Femoral heads collected from distant veterinary practices that were transported in PBS Glycerol were not utilised for cell culture.

Monolayer cell culture

Chondrocytes were cultured as an adherent monolayer in $25cm^2$ culture flasks in complete culture media and grown in an incubator at 37°C with 5% CO_2. Cells were passaged by removal of culture medium, washing in sterile PBS and incubation with trypsin-EDTA in the incubator. Once detached cells were resuspended in 10ml DMEM with FBS and spun for 5 minutes at 1000rpm to deactivate the trypsin and wash the cells. Cell pellets were

resuspended in complete culture media at the required density following cell counting using a haemocytometer.

Coculture system

For the coculture system ThinCert tissue culture inserts and 6 well Multiwell Plates (Greiner bio-one, U.K.) were used. This two-chamber culture system allows co-culture of two cell populations without direct cell to cell contact but with exchange of secreted factors. Cells were seeded in both the 6 well plates and in the tissue culture well inserts at a density of 1.6 x 10^5 cells. Complete culture medium was added at a volume of 2.5ml to each well. Medium was changed daily. Cells were harvested after three days of culture.

Culture in Alginate beads

After culture of harvested chondrocytes from femoral heads to P1 in monolayer, the cells were trypsinised. They were washed twice in PBS and then suspended in alginate solution, consisting of sterile 1.2% Sodium alginate in 150mM sodium chloride. Cells were suspended at a density of 3 x 10^6 cells per ml of alginate solution. Alginate beads of approximately 2mm diameter were constructed using a 20ml sterile syringe and a 22 gauge sterile needle. The alginate cell suspension mix was dropped from the needle into a 102mM calcium chloride solution to form small gelatinous alginate beads. The alginate cell suspension beads were washed twice in complete DMEM. These beads were then cultured in 6 well plates using ThinCert well inserts, in complete media as described for monolayer culture. Culture medium was changed daily. For cell counting the beads were dissolved in a dissolving buffer solution (0.55 M Sodium Citrate, 1.5 M Sodium Chloride and 0.5 M EDTA). The cells were then centrifuged at 400g for 10 minutes and the pellet treated with collagenase (9.18 kU/ml in DMEM) for 1 h.

Cryopreserved chondrocyte stocks

Harvested chondrocytes from femoral heads were cultured in monolayer to confluence. They were then harvested and counted before re-suspension at a density of 2 x10^6 cells in DMSO solution for storage in 1ml aliquots in cryovials (Nalgene). Cells were cooled to minus 80^0C overnight and then stored long term in liquid nitrogen. When required for use, frozen cells were rapidly thawed in a water bath heated to 37^0C and then washed twice in PBS before culture as described above.

Culture to senescence

Cells were cultured in monolayer as described above. Once the cells reached confluence they were trypsinised and counted as described above. The cells were then reseeded and the process repeated. Cells were considered to have ceased replication following a failure of cells to double in number over a period of seven days. The number of population doublings was calculated using the formula:

n = 3.32 (log UCY - log l) + X, where n = the final population doubling number at end of a given subculture, UCY = the cell yield at that point, l = the cell number used as inoculum to begin that subculture, and X = the doubling level of the inoculum used to initiate the subculture being quantitated. Growth curves were obtained by graphing the number of population doublings on the x-axis against the number of days in culture on the y-axis.

2.2.3 Western Blotting

2.2.3.1 Extraction of total cellular protein

Chondrocytes in monolayer culture were harvested, washed and counted. A cell volume of 2x 10^5 cells were suspended in 60 µl of lysis buffer incubated on ice for 30 minutes and then centrifuged at 13, 000 rpm for 30 minutes at 4 °C.

2.2.3.2 Protein Quantification

Protein quantification was determined using the Bradford Assay (Bradford, 1976). In summary, 10µl of the extracted protein solution was mixed with 1x Bradford reagent (Bio-rad, Hertfordshire, U.K.). Protein standards (BSA from Sigma-Aldrich, Dorset, U.K.) at doubling concentrations of 0.1 - 2 mg/ml were used to create a standard curve by measurement of absorbance at 595 nm and a standard curve was drawn. Protein concentrations in unknown samples were extrapolated from the linear part of this curve.

2.2.3.3 Sample preparation

Extracted protein at a concentration of 20 µg was added to 1x NuPAGE sample reducing agent and 1x NuPAGE protein loading dye (Invitrogen, U.K.). The total sample volume

was made up to 25μL using deionised water. The solution was heated to 70°C for 10 minutes to denature any secondary structures in the sample.

2.2.3.4. SDS-PAGE Gel Electrophresis

Samples were resolved using gel electrophoresis on NuPAGE Novex 4-12% gradient Bis-Tris polyacrylamide gel (Invitrogen, U.K.). This pre-cast gel was formulated with an increasing gradient of polyacrylamide (4% - 12%), which causes separation of proteins between 1-200 kDa in size. The gel was placed in the tank (Xcell SureLock Mini-Cell supplied by Invitrogen, U.K.) and loaded with 25μL of protein sample and 10 μL of Seeblue marker per well (Seeblue Plus 2 pre-stained standard; Invitrogen, U.K.). Running buffer (1 x MES), (Invitrogen, U.K.) was added to the tank along with antioxidant agent to maintain protein reduction. Electrophoresis was carried out at 200V for 60 minutes.

2.2.3.5 Membrane Transfer and Antibody staining

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Invitrogen iBlot; Invitrogen, U.K.). The membrane was blocked using 5% dried milk (Marvel Dried Milk Powder by Premier Foods, U.K.) in PBS-T for 1 hour at room temperature and then incubated with rotation with the relevant primary antibody overnight at 4°C. Detection of the primary antibody was carried out using a secondary antibody. Secondary antibodies were conjugated to hydrogen peroxidase resulting in a colour change in the presence of the primary antibody. The colour change was visualised using light microscopy. In all experiments tubulin was used as a loading control.

Following incubation overnight, the membrane was washed in 0.1% PBS-T with four washes at five minutes per cycle. The membrane was then incubated with the relevant secondary antibody for 2 hours at room temperature. Following secondary antibody incubation, the membrane was washed as above followed by protein detection by chemiluminescence. A 1ml volume of detection reagent (ECL-Plus supplied by Amersham, U.K.) was dispersed across the membrane and incubated at room temperature for 5 minutes. Proteins were detected by radiography on Kodak 18x24 Medical Radiographic Film on a Xograph Compact X4 processor.

2.2.4 Real-time relative quantitative PCR (RQ-PCR)

2.2.4.1 Extraction and Purification of RNA

RNA extraction from cultured cells was carried out using the Qiagen RNeasy Mini Kit according to the kit instructions. The RNA samples were treated using DNA-free (Ambion, U.K.) to remove any remaining DNA. Digestion was carried out for 1 hour at 37°C and then the reaction was stopped by incubation with DNase Inactivation Reagent with intermittent agitation over 2 minutes at room temperature. The RNA was separated by centrifugation of the sample for 90 seconds at 10,000g and removal of the RNA supernatant to a fresh Epindorf tube. First strand cDNA was synthesised by reverse transcription of the collected RNA using the Superscript III kit (Invitrogen, U.K.) according to the manufacturer's instructions. A volume of 1-5µg of RNA sample was added to 1µl of Oligo dT primer and 1µl of 10mM dNTP mix, made up to a final volume of 10µl using DEPC water and then incubated for 5 minutes at 65°C to remove any secondary structures before placing on ice. Reaction mix was added and the reaction was incubated at 50°C for 50 minutes with incubation at 85 °C for 5 minutes to end the reaction. The samples were placed on ice and 1 µl RNase H was added before incubation at 37°C for 20 minutes to remove the original RNA strands. A Perkin-Elmer thermal cycler 480 was used for the reaction.

2.2.4.2 PCR

PCR was carried out using 0.1 µg of cDNA sample, using the required primer and probe set (Table 2-4) for the gene under examination in a particular experiment. Primer and probe sets were designed using Primer Express software and are listed in Table 2-4.
PCR reaction mixes were made up for each cDNA sample using 0.1 µg of cDNA sample in 25 µl of 2x PCR UDG SuperMix, 1 µl ROX Reference Dye, 0.2 µM forward and reverse primers, 0.1 µM probe and deionised water to a total reaction volume of 50 µl. Reactions were carried out in triplicate for each sample. GAPDH was used as an internal control gene. The PCR reactions were performed on an Applied Biosystems 7500 Real Time PCR machine. The 2-ΔΔCt method was used to show the relative fold change in gene expression levels.

PCR reactions consisted of an initial 50°C for 2 minutes Platinum Taq DNA polymerase activation step. This was followed by a 5 minute 95°C denaturing step and then 45 cycles for 15 seconds at 95°C and 45 seconds at 60°C. Fluorescence measurements (6-carboxyfluorescein, ROX, and 6-carboxytetramethylrhodamine) were collected for every cycle at 60°C.

Table 2-4. Primer and probe sets used in RQ-PCR experiments.

Target Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	Probe
TNF	TGAGCCGACGTGCCAAT	TGACGGCACTATCAGCTGGTT	TCCTGGCCAACGGCGTGGA
IL-6	AGGG CCAGACTGTCTA TTGTACTTC	CTGTGTATTTTCCAGGAGGGAAA	TTTCGCTGCTGGTCTCAGGGAGCTC
MMP-13	CGGCCACTCCTTAGGTCTTG	CGTGTAGGTGTAGATGGGAAACAT	CACTCCAAGGACCCGGGAGCACT
COL-3A1	AGCTCAGGCCACGATCTCA	AATCCCAAGCAGCCTCCAT	CCGGGACCTCAAATTCTGCCATCC
IL-8	AGCTAGGCCCCAT GCACAATGA	AATCACCCCACCCCCAAACCTTCG	AACTCGGATGTTTCCTCTTC
MMP-3	AAACCAACACTGTCGCCCTATAA	CTATGACGTCTTAGTGCCTTGCT	AGTCGGGCTCCAGAGAGTGCATC
IL-F3	AGGCCCCATGCACAATGA	CTCGGATGTTCCCTCCTCTTC	CCCCCACCCCCAAACCTTCG
ADAMTS-5	GGGTTCCTGGGTGGCTTAG	AGGGCTGGGTCTCAGG ATTC	AGTG TTTGACTCTTGGTTTCAGCTCAGGCC
IL-4	CAATACGACGAGAAC A CATGGATAG	CAGCTTGTAGCCCAACTTCTCA	CTGAAGCTGCGGAACTATGTGGTAGA

2.2.5. Senescence associated beta-galactosidase (SA-beta-gal) staining

Levels of cellular senescence were assessed using the senescence associated beta-galactosidase (SA-beta-gal) staining kit (Cell Signalling Technology, U.K.). Following removal of culture medium, cells were washed twice in 2ml of PBS. Cells were then fixed for 15 minutes at room temperature in 1ml fixative solution. Cells were again washed twice in PBS before staining with staining solution (comprising a mix of 930µl staining solution, 10µl staining supplement A, 10µl staining supplement B, 50µl X-Gal per well). Cells were incubated with staining solution overnight in the 37°C room. Staining was assessed by light microscopy (Leica DMIL LED light microscope).

Chapter III

Osteoarthritis of the Coxofemoral Joint in Dogs

3.1 Introduction

Secondary OA is the most common form of OA in dogs. Causes of secondary OA include developmental abnormalities, the presence of osteochondritis disescans, joint luxation or subluxation, joint laxity, traumatic injury, obesity leading to abnormal loading and wear of articular cartilage and prolonged steroid therapy and diabetes which can lead to increased joint laxity (Mele, 2007). Secondary OA constitutes 70% of cases of articular disease presenting for veterinary treatment (Mele, 2007). Canine Hip Dysplasia (CHD) is the most common form of developmental orthopaedic disease in dogs (Smith *et al.,* 2012) and the most common cause of OA of the coxofemoral joint with many pedigree breeds as well as crossbreed dogs affected (Todhunter *et al.,* 2003).

In order to study the pathogenesis of canine OA, femoral heads were collected from dogs with OA of the coxofemoral joint. The hip joint was chosen since the femoral head is removed when dogs undergo surgical treatment of coxofemoral OA, thus providing a readily available and an experimentally ethical source of degenerate articular cartilage for study. Hip OA is a major cause of pain, lameness and loss of mobility in domestic and working dogs (Flückiger *et al.,* 1995). An overall species prevalence, irrespective of breed, of 42% for hip dysplasia was reported for the years 1970 and 1994 (Flückiger *et al.,* 1995). Corley (Corley, 2000) estimated an overall prevalence for hip dysplasia of 12% in 1994 while a study by Rettenmaier (Rettenmaier *et al.,* 2002), which included both pedigree and crossbreed dogs reported a prevalence of 18%.

CHD is a common developmental disease with hereditary and environmental components, first described in 1935 (Schnelle, 1935). It most commonly presents bilaterally, but can also be unilateral (Citi *et al.,* 2005; Keller and Corley, 1989; Lust *et al.,* 1973; Olsewski *et al.,* 1983; Todhunter *et al.,* 1997). OA of the coxofemoral joint occurring as a result of CHD is a progressive disease (Kealy *et al.,* 1997; Kealy *et al.,* 2000; Smith *et al.,* 2006) and can lead to euthanasia due to severe pain and suffering (Bonnett *et al.,* 1997; Malm *et al.,* 2010; Proschowsky *et al.,* 2003). Affected puppies have abnormal coxofemoral joint laxity (originally described by Henricson *et al.,* 1966), which results in conformational abnormalities as the dog grows (Brass *et al.,* 1989; Morgan and Stephens, 1985; Prieur, 1978). The conformational abnormality results in incongruity of the developing joint with deformation of the femoral head and acetabulum (Olsson, 1980; Prieur, 1978) resulting in subluxation or luxation of the coxofemoral joint (Hedhammar *et al.,* 1979; Olsewski, 1982;

Riser 1973). This results in abnormal wear of the articular cartilage causing fibrillation, erosion, subchondral bone thickening and osteophyte formation (Morgan, 1987; Morgan and Stephens, 1985; Prieur, 1980). Hip dysplasia is also a common cause of OA in humans (developmental dysplasia of the hip) and in cats but it is reported less commonly in pigs, cows, rabbits and horses (Haakenstad, 1953; Johnston, 1966; Keller *et al.*, 1999; Olsson, 1978; Owiny *et al.*, 2001; Punto and Puranen, 1978; Roser, 1979).

3.1.1 Aetiology of CHD

The aetiopathogenesis of CHD is complex and incompletely understood. It is thought to result from a combination of genetic and environmental factors (Distl *et al.*, 1985; Distl *et al.*, 1991; Hedhammar *et al.*, 1979; Hutt, 1969; Jessen and Spurell, 1972; Lust and Farrell, 1977; Todhunter *et al.*, 1999; Willis, 1977). CHD is a continuous genetic trait with multiple genes affecting its inheritance (Todhunter *et al.*, 2003), however, it has been shown to be an inherited disease in Labradors, German Shepherd Dogs and Rottweilers (Flückiger *et al.*, 1998; Kealy *et al.*, 1997; Kealy *et al.*, 2000; Popovitch *et al.*, 1995; Runge *et al.*, 2010; Smith *et al.*, 2001; Smith *et al.*, 2006).

3.1.1.1 Environmental factors

Many environmental factors have been identified in the development of CHD including nutrition, body weight and biomechanical trauma. While environmental factors alone cannot cause hip dysplasia, they increase the risk of disease and the severity of disease in genetically predisposed dogs. Rapid growth rate has also been identified as a risk factor (Fries, 1995; Kealy *et al.*, 1992; Todhunter and Lust, 2003) in dogs with a genetic predisposition to CHD and as such nutrition is thought to play a key role in the development of this disease. Excessive calorie consumption during growth was shown to cause acceleration in growth rate and an increased severity of disease in a population of Labradors (Kealy *et al.*, 1992). Over supplementation of dietary calcium is thought to lead to abnormal bone formation due to delayed endochondral ossification (Hazewinkel, 2004) thus contributing to the development of disease. Increasing body weight has also been associated with an increased prevalence of CHD (Roberts and McGreevy, 2010).

3.1.1.2 Genetic Factors

The exact mode of inheritance of CHD is poorly understood. Grounds *et al.* (1955) and Schales (1957) first suspected that CHD was an inherited disorder. Heritability was proven in Swedish Army Dogs in 1966 (Henricson *et al.,* 1972). Heritability in various breeds has been extensively studied and is reported to vary between 0.1 and 0.6 (Corley, 1992; Flückiger *et al.,* 1995; Hedhammer *et al.,* 1979; Henricson *et al.,* 1966; Janutta *et al.,* 2005; Janutta and Distl, 2006; Leighton, 1997; Leppänen *et al.,* 1999; Rettenmaier, 2002; Zhang *et al.,* 2009; Zhu *et al.,* 2009). Quantitative trait loci (QTL) are the DNA regions controlling the expression of continuous (quantitative) genetic traits (Geldermann *et al.,* 1985). Major genes for CHD and OA of the coxofemoral joint have been identified in multiple breeds, including Labradors, German Shepherd dogs, Golden Retrievers and Rottweilers (Chase *et al.,* 2005; Janutta and Distl 2006; Leighton, 1997; Maki *et al.*, 2004; Phavaphutanon *et al.,* 2009; Todhunter *et al.,* 2005). The mode of heritability of CHD is thought to involve a complex interaction of multiple genes, similar to the interaction of environmental factors. Environmental impact on variance is reported to be less than 10% (Hamann *et al.,* 2003; Leppänen *et al.*, 2000; Maki *et al.,* 2004). Research to identify specific candidate genes causing CHD is ongoing (Janutta and Distl, 2006; Leighton, 1997; Maki *et al.,* 2002; Maki *et al.,* 2004; Todhunter *et al.,* 1999).

Figure 3-1A. **Figure 3-1B.**

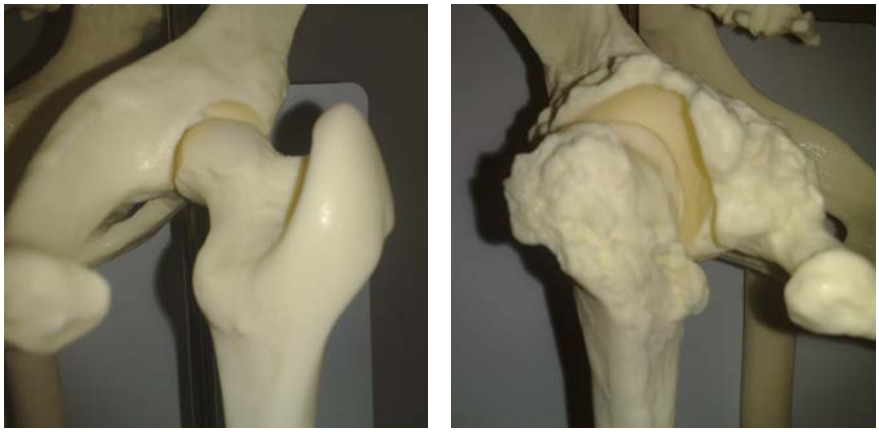


Figure 3-1A&B. Morphological differences between a normal and OA canine hip joint.
The Canine Pelvis Hip model (Access Equipment Corporation, Syalacuaga, Alabama, U.S.A.) below illustrates the differences between a normal canine hip joint (3-1A), and a canine hip joint with extensive remodeling and degeneration of the femur and acetabulum (3-1B). Figure 3-1B shows deformation of the acetabulum, deformation of the femoral head and neck, subluxation of the joint and extensive new bone formation on the acetabulum and femoral head.

3.1.2 Normal anatomy of the canine coxofemoral joint

The coxofemoral joint is a diarthrodial (synovial) joint comprising the spherical femoral head articulating with the concave acetabular fossa. The acetabulum is formed by the ilium craniolaterally, the ischium caudolaterally and the pubis medially. At its central point the round (teres) ligament of the femoral head inserts onto the acetabulum. The joint is stabilized by the round ligament, the dorsal rim of the acetabulum and the joint capsule (Trostel *et al.,* 2000).

3.1.3 Development of pathological changes leading to osteoarthritis

CHD occurs as a result of two principal mechanisms; abnormal joint laxity and abnormal progression of endochondral ossification (Lust *et al.,* 1993; Norberg, 1961; Smith *et al.,* 1990; Smith *et al.,* 1993; Todhunter *et al.,* 2003). Abnormal joint laxity results in incongruity of the femoral head and acetabulum. All affected animals are normal at birth (Gustafsson *et al.,* 1975; Riser, 1975). Once there is joint laxity, abnormal biomechanics occur as the femur fails to articulate normally with the acetabulum. This results in synovitis, joint effusion, fibroplasia and oedema of the round ligament (Fries and Remedios, 1995; Henry, 1992). Inflammation and oedema of the round ligament occurs and secondary stretching further exacerbates joint laxity and facilitates further lateral displacement of the femoral head. Abnormal loading during weight bearing leads to microfractures of the acetabulum, flattening of the head of the femur and hypertrophy of the femoral neck (Brass, 1989; Henry, 1992). Changes to the biomechanics of the coxofemoral joint whilst the dog is growing lead to a delay in the ossification of the acetabulum and of the proximal femur (Madsen *et al.,* 1991; Todhunter *et al.,* 1997). Ultimately, subluxation and luxation of the femoral head occur in some cases. Abnormal wear and loading of the articular surface results in fibrillation and erosion of articular cartilage and heralds the onset of OA by the pathways detailed in Chapter I. The end sequelae for affected dogs is pain and varying degrees of loss of joint function (Alexander, 1992; Todhunter and Lust, 2003). The model shown in Figure 3-1A and 3-1B illustrates the gross morphological differences between a normal coxofemoral joint and a dysplastic one. The dysplastic joint (Figure 3-1B) shows deformation of the acetabulum, deformation of the femoral head and neck, subluxation of the joint and extensive new bone formation on the acetabulum and femoral head. A radiograph of a pelvis including both coxofemoral joints with OA is shown in Figure 3-2, illustrating radiographic changes observed with CHD. Both coxofemoral joints are affected by coxofemoral OA on this radiograph and

marked deformation of the acetabulum, deformation of the femoral head and neck, subluxation of the coxofemoral joints, osteophyte formation and increased bone radio-opacity are visible bilaterally.

3.1.4 Clinical findings and diagnosis

Dogs affected by OA of the coxofemoral joint present with clinical signs of lameness. There is commonly a history of intermittent mild lameness with difficulty rising after a rest period, which then progresses over time to severe lameness and reluctance to exercise (Dassler, 2003; Fry and Clark, 1992). Physical examination findings include pain on manipulation of the coxofemoral joint, decreased range of joint motion, atrophy of quadriceps and gluteal musculature and increased weight bearing on the forelimbs (facilitated by tipping the bodyweight forwards and tucking the hind limbs further under the abdomen), (Dassler, 2003; Fry and Clark, 1991).

The population of dogs presented for veterinary treatment for CHD has a bimodal age distribution (Dassler, 2003). The first population consists of dogs aged between three and 12 months of age and the second population is made up of skeletally mature dogs, generally between two and 12 years of age. The two cohorts present with different joint pathology. In the younger group, pain and lameness occur following the development of acetabular microfractures secondary to luxation or subluxation of the femoral head. The older group of dogs present with pain and lameness secondary to the development of OA changes within the joint (Dassler, 2003). During orthopaedic examination of dogs in the younger group, an increase in hip joint laxity can sometimes be detected using the Barlow, Barden or Ortolani tests. These tests are subjective assessments and to date the diagnostic or prognostic value of these tests in dogs with CHD have not been evaluated quantitatively (Puerto *et al.,* 1999). Increased laxity is usually specific to young dogs in the early stages of disease, because OA and secondary DJD in advanced disease and in older dogs results in changes to the joint such that laxity is no longer palpable during physical examination.

A definitive diagnosis of both CHD and OA of the coxofemoral joint is made using radiography. Ventrodorsal radiographs are taken under sedation or general anaesthesia to assess joint congruency and to detect OA changes (Dassler, 2003; Fry and Clark, 1992; Ginja *et al.,* 2010). Early investigation of CHD in skeletally immature dogs (between the age of four and eight months) can be carried out using stressed radiographic views of the

joint to reveal hip laxity (Adams *et al.,* 2000; Fischer *et al.,* 2010; Ginja *et al.,* 2008a), though a definitive diagnosis is not usually made until the skeleton has matured (Adams *et al.,* 2000; Fischer *et al.,* 2010; Ginja *et al.,* 2008a; Ginja *et al.,* 2008b; Lopez *et al.,* 2006; Todhunter *et al.,* 2003), with the exception of the PennHIP scoring system which can be used from sixteen weeks of age. It is important to note that, as in many diseases of the joint, the severity of clinical signs does not necessarily correlate with the severity of radiographic changes (Corley, 1983; Dassler, 2003; Fry and Clark, 1992; Prieur, 1990). The time to onset of radiographic changes and clinical signs of OA is associated with the severity of underlying CHD and the lifestyle of the animal (Runge *et al.,* 2010; Smith *et al.,* 2006).

Correct positioning is critical for the ventrodorsal radiograph. The pelvis must be symmetrical with both femora fully extended and internally rotated. The patellae should sit over the mid-sagittal plane of the corresponding femur (Brass, 1993) such that a straight ventrodorsal view is obtained. Radiographic screening for hip dysplasia is used in a number of countries, with some variation in the scoring systems used for each particular country. These hip scoring programs aim to decrease the incidence of disease by implementing recommendations whereby affected dogs are not used for breeding. Animals are assessed after 12 months of age, and a ventrodorsal radiograph as described above is used to determine the severity of disease, if present. In the U.K. the hip score is the sum of the scores awarded for each of nine features of CHD relating to the Norberg angle, the degree of subluxation, deformation of the acetabulum and femoral head and neck and osteophyte formation (Gibbs, 1997). The minimum score is 0 and the maximum is 106, with 53 points being allocated for each joint. Lower scores correlate with less severe CHD and each breed has a mean score. Individuals used for breeding should score below the mean breed score. An alternative system is the PennHIP system, which measures hip joint laxity using three separate radiographs: the distraction view, the compression view and the hip-extended view (Smith, 2002). Despite the use of scoring schemes in many countries, there has been no associated decrease in the incidence of CHD (Coopman *et al.,* 2008; Flückiger *et al.,* 1995; Ginja *et al.,* 2009) and as such the efficacy of these programs is questionable. These schemes do not apply to crossbreeds and generally only dogs from established breeding lines are screened. As major genes and single nucleotide polymorphisms predisposing to CHD are identified, genetic testing may replace radiography as a more accurate indicator of breeding values in the future (Guo *et al.,* 2011).

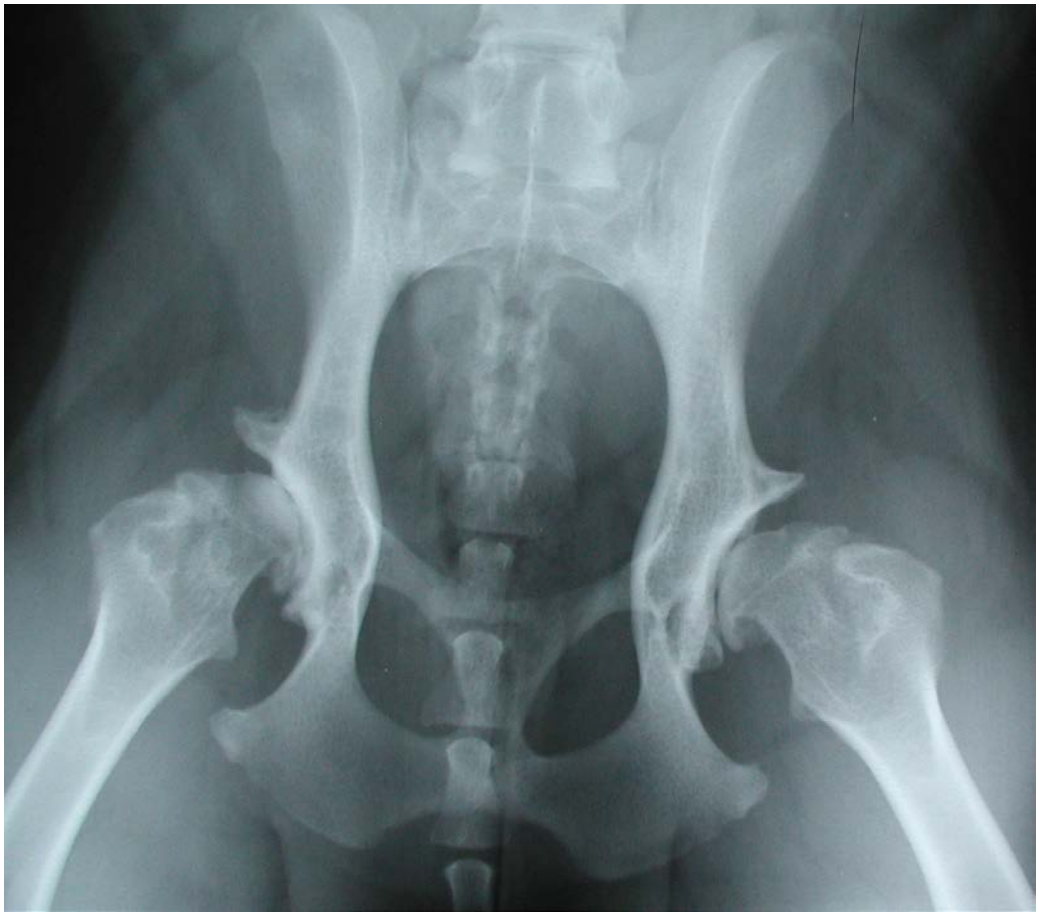


Figure 3-2. Radiograph of a dog with CHD and OA.
Marked deformation of the acetabulum, deformation of the femoral head and neck, subluxation of the joint, osteophyte formation and increased bone radio-opacity of the acetabular rim are visible.

3.1.5 Treatment of CHD

Treatment of CHD falls into three categories; (i) animals that are diagnosed at less than one year of age and may be treated by surgical correction of joint incongruity, (ii) animals showing early signs of OA which are often managed conservatively and (iii) animals with end stage OA which are candidates for salvage surgery. In all affected dogs treatment is focused on minimising the development and progression of OA secondary to CHD. Controlling the impact of environmental risk factors is important for dogs showing early signs of disease and calorie intake is often regulated to control skeletal growth rate.

In dogs affected by OA, treatment is focused on analgesia and maintaining joint function. Management of pain and joint inflammation is achieved using anti-inflammatory medications such as non-steroidal anti-inflammatory drugs. A careful exercise programme

can help to maintain muscle-mass and comfortable range of joint motion whilst potentially reducing microtrauma from excessive concussive exercise. Conservative treatment of OA in dogs is discussed in detail in Chapter I. In the case of CHD, the use of conservative treatment without surgical intervention before 18 months of age has a reported success rate of 72%, with a favourable outcome defined as resolution of pain and good joint function (Barr *et al.*, 1987).

Some dogs with hip dysplasia present whilst still skeletally immature (four to eight months of age) with pain, lameness and joint subluxation may be treated by corrective osteotomy of the coxofemoral joint. These procedures include intertrochanteric osteotomy (though rarely used now), pubic symphysiodesis and double or triple pelvic osteotomy (Braden, 1994; Slocum and Devine, 1986; Slocum and Devine, 1990). These procedures aim to improve joint incongruity and laxity thus preventing or delaying the development of OA of the coxofemoral joint. Other surgical techniques have been described for the treatment of CHD, including pectineal myectomy (Wallace, 1992), biocompatible osteoconductive polymer acetabular shelf arthroplasty and lengthening of the femoral neck (Devine and Slocum, 1995) but are seldom used nowadays. Salvage surgeries are indicated in dogs with refractory pain, loss of joint function, lameness and severe OA. Salvage surgeries include femoral head and neck excisional arthroplasty and total hip replacement (Jensen and Sertl, 1992; Olmstead, *et al.*, 1981; Olmstead *et al.*, 1983). The selection of treatment options for CHD is complex and depends upon the severity of disease, age at presentation, level of pain and joint function, concurrent medical disease, response to medication, financial constraints and availability of surgical treatment. As such there is no exact protocol for treatment of this disease.

This chapter reports on the age, breed and sex of 82 dogs presenting for surgical treatment of OA of the coxofemoral joint secondary to CHD in veterinary practice in the U.K. and describes the pathological changes affecting the excised femoral heads.

3.2 Aims and Objectives

The aims of this study were to evaluate the presentation of dogs undergoing femoral head excision in veterinary referral practice in the U.K. and to study the pathological lesions that occur on the femoral heads of dogs with osteoarthritis of the hip joint. Specifically, to evaluate the sex and neuter status, age and breed of dogs undergoing total hip replacement surgery for the treatment of CHD and OA and to identify the severity and classify the morphological distribution of OA lesions of excised femoral heads.

3.3 Materials and Methods

3.3.1 Sample collection and categorisation

Femoral heads for the study were obtained from the University of Glasgow, Small Animal Hospital and from veterinary referral centers across the U.K. (detailed in the general materials and methods). Information on age, sex and neuter status, breed, side from which the femoral head was collected (left or right), whether a radiographic diagnosis of OA was made prior to surgical treatment, the date of surgery and the name of the submitting veterinary practice were all recorded. Samples were transported in sterile PBS glycerol as described in Chapter 2, General Materials and Methods, and were examined within 72 hours of collection.

For analyses of sex and neuter status four categories applied: female entire, female neutered, male entire and male neutered. For analyses of age four categories were used; two months to one year (12 months), one year (13 months) to 4 years (48 months), four years (49 months) to seven years (84 months) and older than seven years (85 months). For analyses involving breed, recognised British Kennel Club breeds were used to categorise dogs with the additional inclusion of Labradoodles (strictly speaking a crossbreed) due to the prevalence of this crossbreed in the U.K. All other dogs were analysed as crossbreed dogs. Prevalence was calculated by dividing the number of dogs in a particular category of interest (e.g. a specific age or breed group) undergoing femoral head excision by the total number of dogs undergoing femoral head excision in this study.

3.3.2 Lesion scoring system

There is no one scoring system used to grade OA lesions in humans or animals, and authors of research studies often define a unique scoring system specific to their study (Burton-Wurster, 1999; Chase *et al.*, 2004; Meachim, 1972; Pelletier and Martel-Pelletier, 1989). One of the first scoring systems utilised in human medicine was the Collins grade (Collins, 1949). This system graded gross cartilage lesions identified at *post-mortem* examination using India ink to highlight areas of cartilage erosion and fibrillation. Application of India ink to the articular cartilage was not possible for the present study as the effects of India ink on live chondrocytes are unknown and these cells were required for

subsequent culture and analysis, so the Collins grading system could not be utilised. Lust defined a scoring system based on the size of cartilage lesions in mm in a colony of Greyhounds, Labradors and Greyhound-Labrador crossbreed dogs (Lust *et al.,* 2001). In that study the femoral heads were very similar in size and measuring the extent of lesions was thus relevant. However in the present study there was marked variation in the size of femoral heads corresponding to the sizes of the dog breeds and thus the size of lesions could not be used as an assessment of lesion severity. Farrell and colleagues (Farrell *et al.,* 2014) developed a scoring system for elbow OA in dogs based on gross cartilage lesions identified arthroscopically, however arthroscopy only allows a limited examination of the articular cartilage within a joint and in contrast to the elbow joint, arthroscopy is not used routinely to evaluate hip OA in clinical diagnosis. As such a new scoring system was developed, based primarily upon the Collins grade. This new scoring system is referred to as the modified Collins score.

Lesions were classified based on location and type. Each lesion type was given a score as outlined in Table 3-1. There are three OA lesion types: cartilage fibrillation, cartilage erosion and exposed subchondral bone. A score of 0 indicated no lesion in that area. Lesions were identified in three distinct locations: the primary area (perifoveal), the secondary area and the distant area, as previously described in dogs by Burton–Wurster (Burton-Wurster *et al.,* 1999). These are identified in Figure 3-3. Each femoral head was given a score for each of the three areas, such that the overall score was a three-digit number indicating the pathology recorded on each area of the femoral head. For example, a score of 4-2-3 indicted cartilage erosion of the primary area (4), cartilage fibrillation of the secondary area (2) and cartilage fibrillation of the distant area (3). Femoral heads were graded upon arrival at the laboratory (within 72 hours of collection from the patient) and the lesions were classified using a dissection microscope.

3.3.3 Statistical analysis

For sample size calculations using logistic regression, standard parameters of power and confidence were set (a power of 80% and confidence of 95%). Evaluation of hypotheses was carried out using Pearson Chi-Squared tests and Likelihood Ratio Chi-Squared tests, using SPSS software.

Figure 3-3. Schematic representation of the three femoral head areas.
1A= primary (perifoveal) area, 2A=secondary area, DA= distant area.

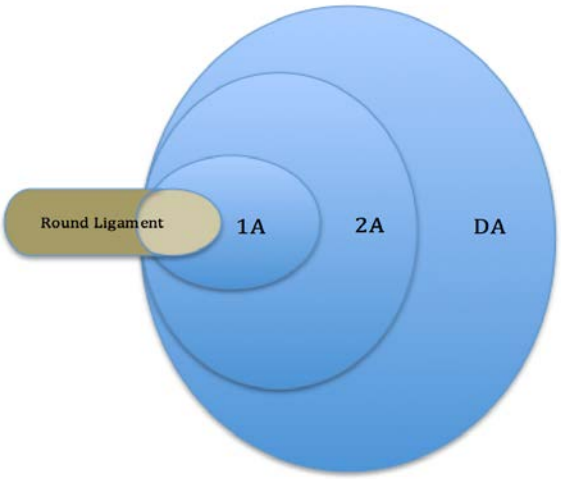


Table 3-1. Description of lesion scores.
This table shows the modified Collins scoring system of pathological lesions of the femoral head according to location and type or lesion used in this study.

Lesion score	Description
1	Cartilage fibrillation (including fissuring, flaking or pitting) of the primary area
2	Cartilage fibrillation of the secondary area
3	Cartilage fibrillation of the distant area
4	Cartilage erosion of the primary area
5	Cartilage erosion of the secondary area
6	Cartilage erosion of the distant area
7	Exposed subchondral bone on the primary area
8	Exposed subchondral bone on the secondary area
9	Exposed subchondral bone on the distant area

3.4 Results

3.4.1. Assessment of collected femoral heads from dogs undergoing total hip replacement surgery revealed cartilage erosion to be the dominant lesion observed. No characteristic pattern of femoral head pathology was observed in dogs with CHD.

Eighty-two femoral heads with OA were included in this study (Table 3-2). Forty four femoral heads were from the left coxofemoral joint, 38 from the right. Of the samples, 30.5% were collected from female neutered dogs, 26.8% from male entire dogs, 24.4% from male neutered dogs and 18.3% from female entire dogs (Table 3-3). Dogs aged between one and four years of age were most commonly presented for femoral head excision (36.6%), followed by dogs aged four to seven years (29.3%). Dogs aged between two and 12 months represented 18.3% of collected samples, and dogs aged greater than seven years represented the smallest sample group at 15.8% (Table 3-4). The most commonly represented breeds were Labradors (24.4%), German Shepherd Dogs (19.5%), Border Collies (11.1%), Crossbreed dogs (13.4%), Golden retrievers (4.9%) and Springer spaniels (4.9%), (Table 3-5). Examples of the morphological lesions seen are illustrated in Figures 3-8 to 3-10. Cartilage fibrillation, cartilage erosion and exposed subchondral bone lesions are visible in Figures 3-9 and 3-10). The OA lesion scores recorded on the 82 femoral heads are shown in Table 3-6.

The most severe OA lesion recorded on each femoral head sample was analysed by age group (Figure 3-4) and breed (Figure 3-5), with exposure of subchondral bone being the most severe lesion followed by erosion of articular cartilage. Cartilage fibrillation was the mildest lesion grade. In dogs in the three age groups; 2-12 months, 1-4 years and older than seven years, erosion of articular cartilage was recorded as the most common lesion (53.7%; 89.7%; 57.1% respectively). In dogs aged 4-7 years, exposure of subchondral bone was the most frequent lesion observed, with cartilage erosion accounting for 44% of lesions in this age cohort. In the 1-4 years of age cohort, cartilage erosion was the dominant lesion. A total of 89.7% of the femoral head samples collected in this age group had cartilage erosion as the most severe lesion and 6.9% of the femoral head samples collected in this age group had cartilage fibrillation as the only lesion observed. This was distinct from the three other age cohorts, where there were similar percentages of cartilage erosion and subchondral bone exposure recorded. Fibrillation was the most severe lesion

recorded in 4.8% of cases overall, with little variation between age cohorts (6.7% of 2-12 months, 3.4% of 1 to 4 years, 4% of 4-7 years and 7.1% of older than seven years). This shows that cartilage fibrillation alone is not a common reason for surgical treatment of OA of the hip and dogs presenting for such surgery usually have severe cartilage pathology with either cartilage erosion alone (63.9% of cases overall) or with exposure of subchondral bone (31.3% of cases overall).

Osteophytes were observed on 26.5% of collected femoral heads. Most samples had the round ligament transected at the level of the femoral head at the time of surgery and as such assessment of hypertrophy was not possible. Some femoral heads may have had no round ligament attached at the time of surgical excision because the round ligament may rupture and be resorbed due to chronic synovitis. In samples where the round ligament was still attached and intact (19 samples), oedema of the round ligament was evident.

When distributed according to the top six presenting breeds, cartilage erosion was the most dominant lesion of the femoral head for each breed affecting 50% of Labradors, 55.5% of Border Collies, 72.7% of crossbreeds, 75% of German Shepherd Dogs, 75% of Springer spaniels and 50% of Golden retrievers. Labradors and Border Collies had similar levels of exposed subchondral bone and cartilage erosion recorded, whereas the other commonly affected breeds had a greater relative proportion of cartilage erosion lesions than exposed bone lesions (approximately twice as many for German Shepherd Dogs, Crossbreeds, Golden retrievers and Springer spaniels). The Labradors were the only breed presenting with cartilage fibrillation as the most severe lesion observed (15% of Labrador femoral heads).

No one single pattern of lesions was observed (Table 3-6). Thirty-five of the 82 femoral heads collected showed a lesion distribution where the most severe lesion occurred adjacent to the fovea, with less severe pathology as the distance from the central lesion increased. However an equal number of femoral heads showed a lesion distribution where the most severe lesion occurred adjacent to the fovea, followed by the least severe changes and then a final band of more severe pathology at the margin of the joint. These two patterns of cartilage damage constituted a total of 84.3% of the specimens examined.

Table 3-2. FH OA in dogs undergoing FHEA/THR in veterinary practices in the U.K.

This table shows the age, breed, sex and side affected for each of the eighty two femoral heads examined in this study.

Case no.	Age in months	Breed	Sex
OA1	13	Rottweiler	FE
OA 2	13	Labrador	MN
OA 3	58	Labrador	MN
OA 4	11	Crossbreed	ME
OA 5	17	Crossbreed	FN
OA 6	12	Golden Retriever	MN
OA 7	84	Crossbreed	FE
OA 8	109	German Shepherd Dog	FN
OA 9	10	Springer Spaniel	ME
OA10	8	Crossbreed	FN
OA 11	11	Labrador	ME
OA 12	7	Border Collie	ME
OA 13	95	Weimeraner	FN
OA 14	27	Bearded Collie	FE
OA 15	120	Border Collie	FN
OA 16	31	Crossbreed	MN
OA 17	42	Border Collie	MN
OA 18	111	Crossbreed	FN
OA 19	13	Old English Sheepdog	MN
OA 20	18	Labrador	MN
OA 21	72	German Shepherd Dog	FN
OA 22	84	German Shepherd Dog	FE
OA 23	12	Crossbreed	FN
OA 24	36	Siberian Husky	FN
OA 25	60	Labrador	FN
OA 26	84	Labrador	MN
OA 27	60	Border Collie	MN
OA 28	94	Labrador	FE
OA 29	6	Cavalier King Charles Spaniel	FE
OA 30	60	Labrador	MN
OA 31	72	Border Collie	MN
OA 32	48	Rough Collie	ME
OA 33	72	Labrador	FN
OA 34	84	Crossbreed	MN
OA 35	72	German Shepherd Dog	FN
OA 36	18	Cocker Spaniel	MN
OA 37	156	Golden Retriever	FN
OA 38	60	Rough Collie	ME
OA 39	9	Golden Retriever	FE
OA 40	14	Labrador	ME
OA 41	96	Springer Spaniel	ME
OA 42	13	Leonberger	ME
OA 43	36	German Shepherd Dog	FN
OA 44	54	Rough Collie	MN
OA 45	7	Labrador	FE
OA 46	30	Labrador	ME
OA 47	90	German Shepherd Dog	FE
OA 48	72	Labrador	ME
OA 49	14	Jack Russell Terrier	ME
OA 50	31	Labradoodle	MN
OA 51	48	Crossbreed	FN
OA 52	100	German Shepherd Dog	FN

Table 3-2 (continued). FH OA in dogs undergoing FHEA/THR in veterinary practices in the U.K.

Case no	Age in months	Breed	Sex
OA 53	13	German Shepherd Dog	FE
OA 54	96	German Shepherd Dog	ME
OA 55	13	Labrador	ME
OA 56	32	Rottweiler	MN
OA 57	110	German Shepherd Dog	FN
OA 58	72	Springer Spaniel	ME
OA 59	18	Dogue de Bordeaux	ME
OA 60	26	German Shepherd Dog	MN
OA 61	10	Leonberger	ME
OA 62	69	Labradoodle	MN
OA 63	48	Labrador	FN
OA 64	132	Golden Retriever	FN
OA 65	28	German Shepherd Dog	ME
OA 66	72	Crossbreed	FN
OA 67	84	Labrador	FN
OA 68	82	Labrador	FN
OA 69	29	Springer Spaniel	FN
OA 70	17	Labrador	FE
OA 71	73	Border Collie	ME
OA 72	73	Irish Red Setter	MN
OA 73	10	Labrador	ME
OA 74	8	German Shepherd Dog	ME
OA 75	11	Border Collie	FE
OA 76	11	Border Collie	FE
OA 77	96	Border Collie	FN
OA 78	72	German Shepherd Dog	FE
OA 79	26	Labrador	ME
OA 80	39	German Shepherd Dog	FE
OA 81	84	Crossbreed	MN
OA 82	72	German Shepherd Dog	FN

Table 3-3. Prevalence of FH OA in dogs, grouped on the basis of sex and neuter status, treated by FHEA/THR in veterinary practices in the U.K.

30.5% of the samples were collected from female neutered dogs, 26.8% from male entire dogs, 24.4% from male neutered dogs and 18.3% from female entire dogs.

Group	Number of dogs	% of total dogs
Female entire	15	18.3
Female neutered	25	30.5
Male entire	22	26.8
Male neutered	20	24.4

Table 3-4. Prevalence of FH OA in dogs, grouped on the basis of age, treated by FHEA/THR in veterinary practices in the U.K.

Dogs aged between one and four years of age were most commonly presented for femoral head excision (36.6%), followed by dogs aged four to seven years (29.3%). Dogs aged between two and 12 months represented 18.3% of collected samples, and dogs aged greater than seven years represented the smallest sample group at 15.8%.

Group	Number of dogs	% of total dogs
2months-12months	15	18.3
1-4 years	30	36.6
4-7 years	24	29.3
>7 years	13	15.8

Table 3-5. Prevalence of FH OA in dogs, grouped on the basis of breed, treated by FHEA/THR in veterinary practices in the UK.

The most commonly represented breeds were Labradors (24.4%), German Shepherds (19.5%), Border Collies (11.1%), Crossbreed dogs (13.4%), Golden retrievers (4.9%) and Springer spaniels (4.9%).

Breed	Number of dogs	% of total
Labrador	20	24.4
German Shepherd Dog	16	19.5
Crossbreed	11	13.4
Border Collie	9	11
Golden Retriever	4	4.9
Springer Spaniel	4	4.9
Leonberger	2	2.5
Rough Collie	3	3.6
Labradoodle	2	2.5
Rottweiler	2	2.5
Dogue de Bordeaux	1	1.2
Cavalier King Spaniel	1	1.2
Jack Russell Terrier	1	1.2
Siberian Husky	1	1.2
Irish Setter	1	1.2
Cocker Spaniel	1	1.2
Weimeraner	1	1.2
Bearded Collie	1	1.2
Old English Sheepdog	1	1.2

Table 3-6. Pathological lesions of the femoral heads of 82 dogs graded using a modified Collins scoring system.

HRL refers to hypertrophy of the round ligament and OP refers to the presence of osteophytes on the femoral head. Lesions were classified based on location and type. Each lesion type was given a score as outlined in Table 3-1. There are three OA lesion types: cartilage fibrillation, cartilage erosion and exposed subchondral bone. A score of 0 indicated no lesion in that area. Lesions were identified in three distinct locations: the primary area, the secondary area and the distant area, identified in Figure 3-3. Each femoral head was given a score for each of the three areas, such that the overall score was a three-digit number indicating the pathology recorded on each area of the femoral head. For example, a score of 4-2-9 indicates cartilage erosion of the primary area (4), cartilage fibrillation of the secondary area (2) and exposed subchondral bone of the distant area (9).

Case number	Lesion Score	HRL	OP
1	4-2-3	y	y
2	4-2-0	y	y
3	4-2-0	y	y
4	4-2-3	y	y
5	4-2-3	n	n
6	7-5-3	y	y
7	7-8-6	y	n
8	9-8-7	y	n
9	4-2-0	n	y
10	9-8-7	n	n
11	9-8-7	y	n
12	4-2-3	n	n
13	9-8-7	y	y
14	4-2-3	y	y
15	6-2-3	n	n
16	6-2-3	n	n
17	5-3-0	n	n
18	4-2-3	y	y
19	4-2-3	y	y
20	4-2-3	n	y
21	7-5-3	n	n
22	1-5-6	n	n
23	4-2-3	y	y
24	4-2-3	y	y
25	6-1-2	n	y
26	9-8-7	n	y
27	7-5-3	n	n
28	1-2-0	n	n
29	9-8-7	n	n
30	4-2-9	n	n
31	7-2-6	n	y
32	6-5-4	n	n

Table 3-6 (continued). Pathological lesions of the femoral heads of 82 dogs.

Case number	Lesion Score	HRL	OP
33	7-8-3	n	n
34	1-5-6	n	n
35	7-5-3	n	n
36	6-2-3	n	n
37	7-5-3	y	n
38	6-2-3	n	n
39	4-2-0	n	n
40	7-5-9	n	n
41	9-8-7	n	n
42	4-2-0	n	n
43	6-2-3	n	n
44	4-2-0	n	n
45	1-2-3	n	n
46	6-2-3	n	n
47	6-5-4	n	n
48	7-5-9	n	n
49	1-2-3	n	n
50	6-5-4	n	n
51	6-5-4	n	n
52	6-2-3	n	n
53	4-2-0	n	n
54	1-5-6	n	n
55	6-1-2	n	n
56	6-5-4	n	n
57	4-2-3	n	n
58	4-2-3	n	n
59	7-5-3	y	n
60	6-2-3	n	n
61	4-2-3	n	n
62	6-5-4	n	y
63	6-1-2	n	n
64	1-5-6	y	n
65	1-5-6	y	n
66	9-8-7	n	y
67	1-2-0	y	n
68	7-5-9	n	y
69	4-2-3	n	n
70	4-5-3	n	n
71	7-5-3	n	n
72	7-5-3	n	n
73	7-2-3	n	n
74	6-5-4	n	n
75	6-5-4	n	n
76	7-5-9	n	y
77	6-2-3	n	n
78	9-8-7	n	y
79	4-2-3	n	n
80	1-5-6	n	n
81	6-5-4	n	n
82	6-2-3	n	n

Figure 3-4. Score of pathological lesions according to age of dog.
In dogs in age groups 2-12 months, 1-4 years and older than seven years, erosion of articular cartilage was recorded as the most common lesion (53.7%; 89.7%; 57.1% respectively). In dogs aged 4-7 years, exposure of subchondral bone was the most frequent lesion observed (52%), with cartilage erosion accounting for 44% of lesions in this age cohort. In the 1-4 year cohort, cartilage erosion was the dominant lesion, affecting 89.7% of the femoral heads of this age group and cartilage fibrillation as the only lesion affecting 6.9%. This was distinct from the three other age cohorts, where there were similar percentages of cartilage erosion and subchondral bone exposure recorded. Fibrillation was the most severe lesion recorded in 4.8% of cases overall, with little variation between age cohorts (6.7% of 2-12 months, 3.4% of 1 to 4 years, 4% of 4-7 years and 7.1% of older than seven years).

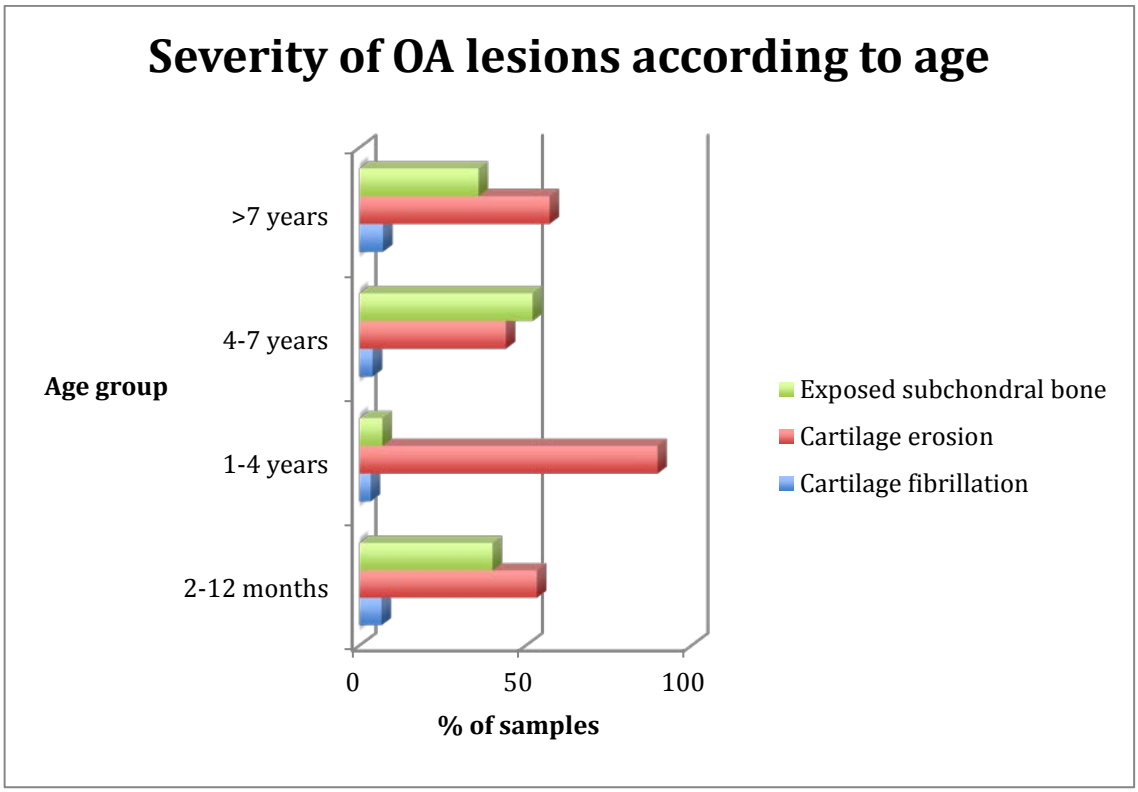


Figure 3-5. Distribution of the most severe pathological lesion according to breed of dog.

When distributed according to the top six presenting breeds, cartilage erosion was the most dominant lesion of the femoral head for each breed affecting 50% of Labradors, 55.5% of Border Collies, 72.7% of crossbreeds, 75% of German shepherds, 75% of Springer spaniels and 50% of Golden retrievers. Labradors and Border Collies had similar levels of exposed subchondral bone and cartilage erosion recorded, whereas the other commonly affected breeds had a greater relative proportion of cartilage erosion lesions than exposed bone lesions (approximately twice as many for German shepherds, Crossbreeds, Golden retrievers and Springer spaniels). The Labradors were the only breed presenting with cartilage fibrillation as the most severe lesion observed (15% of Labrador femoral heads).

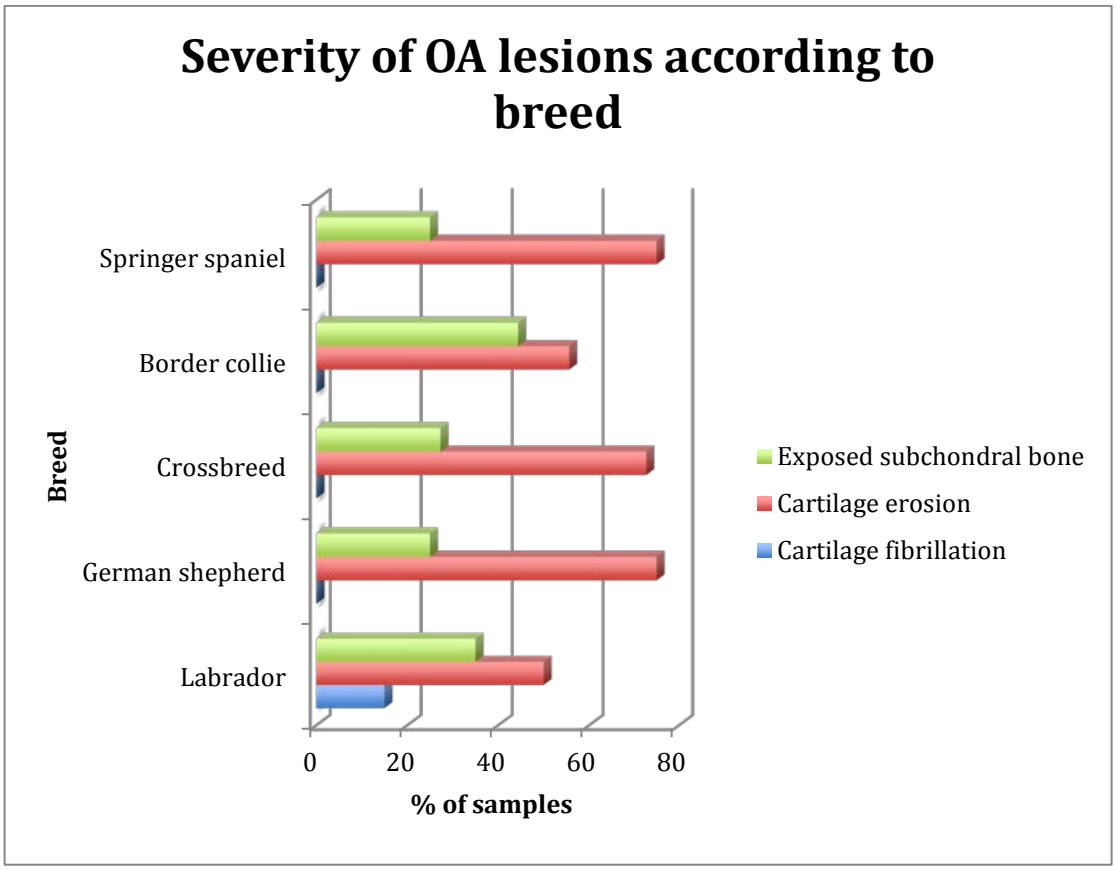


Figure 3-6. Distribution of the most severe pathological lesion according to age of dog.

The most severe lesion on each femoral head was perifoveal in the majority of cases (between 75% and 89.4% of cases for the four age groups), with no statistically significant difference between the four age cohorts. Thus damage to perifoveal cartilage is a hallmark of coxofemoral OA secondary to CHD irrespective of age. The percentage of total lesions located at the primary area (blue bars) secondary area (red bars) and tertiary area (green bars) is shown on the graph below for each age cohort.

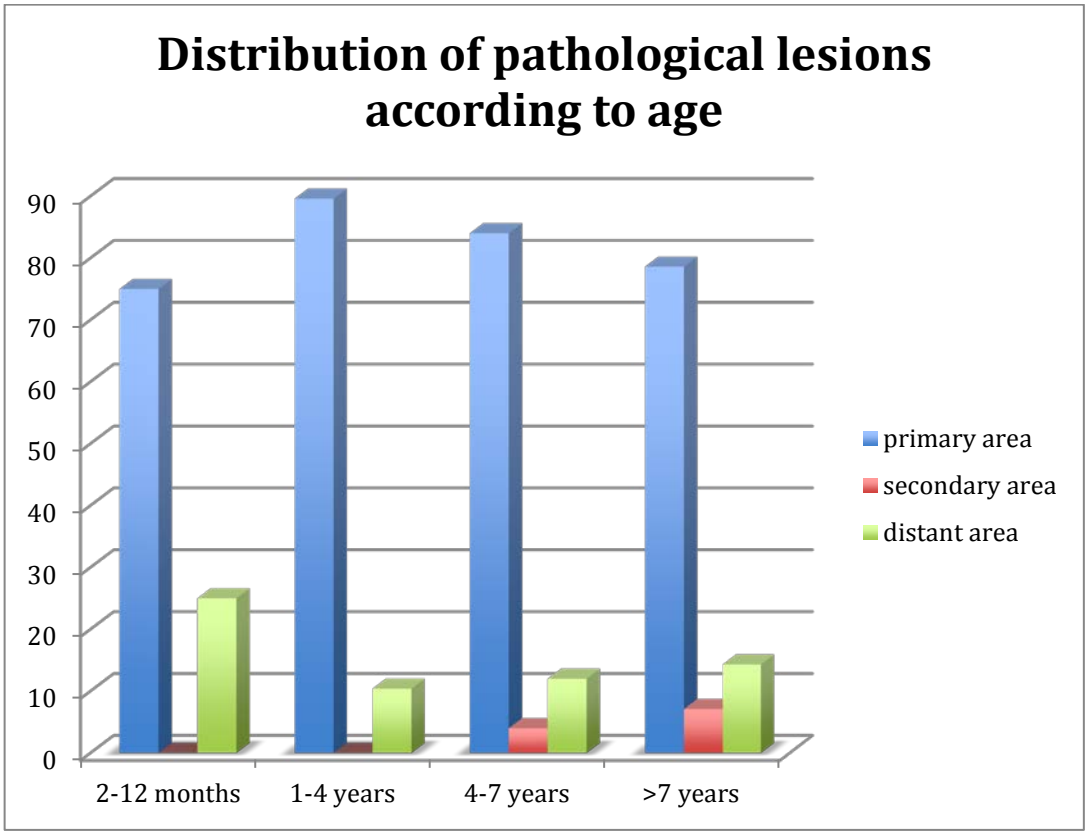
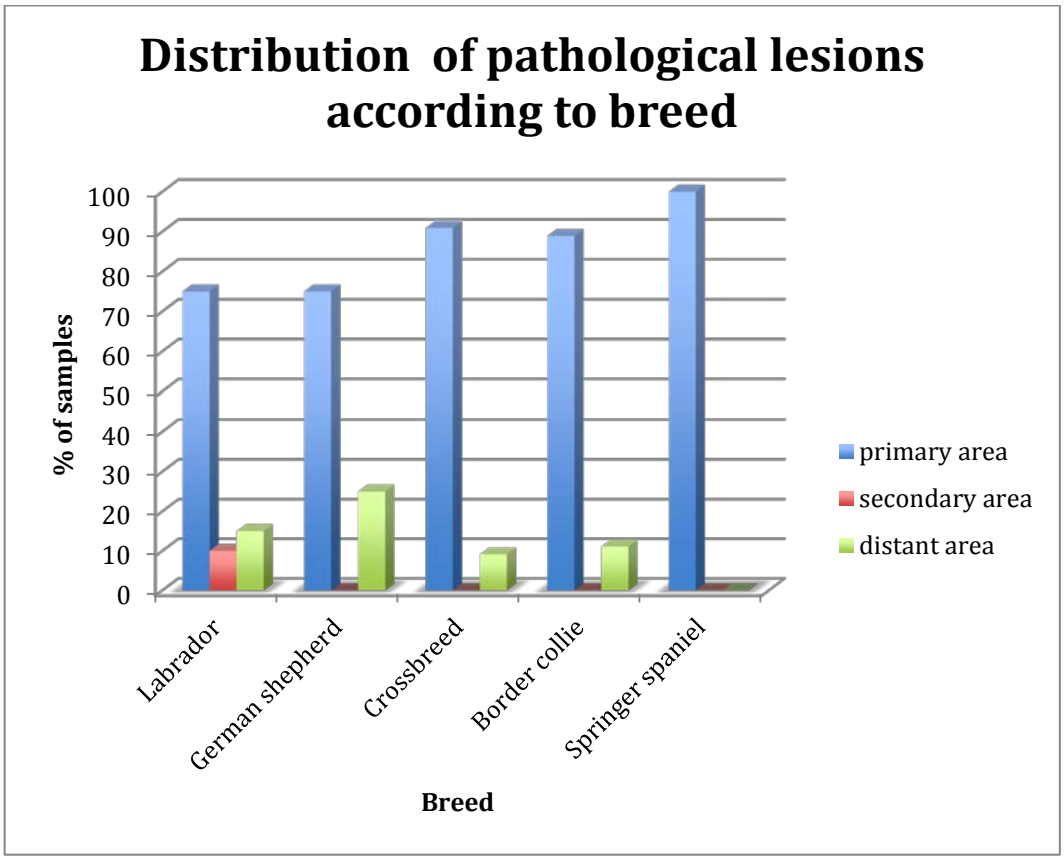


Figure 3-7. Distribution of pathological lesions within different breeds of dog.

When variation in pathological lesion location was examined according to breed it was found that for the top five breeds recorded in the study, the most severe cartilage damage occurred in the perifoveal primary area irrespective of breed occurring in 75% of Labrador samples, 75% of German Shepherd samples, 90.9% of crossbreed samples, 88.9% of Border Collie samples and 100% of springer spaniel samples. Thus taken in conjunction with the results in Figure 3-6, breed and age did not affect the distribution of pathological changes to the femoral head in dogs with OA secondary to CHD.



Figures 3-8 to 3-15. Samples of femoral heads collected showing some of the commonly observed lesions.

Figure 3-8 shows a normal femoral head collected from a dog with no OA. The femoral head is of round morphology, with no erosion or fibrillation of the articular cartilage and no remodelling. Figure 3-9 shows exposed subchondral bone, cartilage erosion and cartilage fibrillation of the primary area, secondary area and distant area respectively. Figure 3-10 shows eroded subchondral bone, cartilage erosion and cartilage fibrillation of the primary area, secondary area and distant area respectively. Osteophytes at the margin of the distant area adjacent to the site of joint capsule attachment are visible (Figure 3-10).

Figure 3-8.

Figure 3-9.

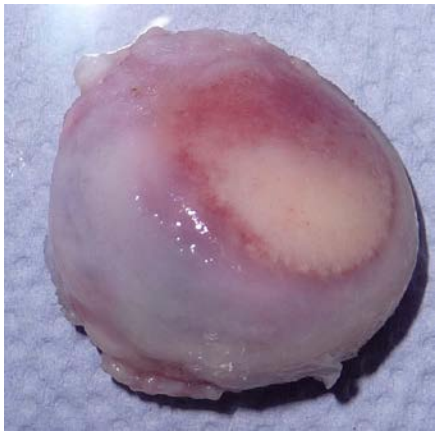
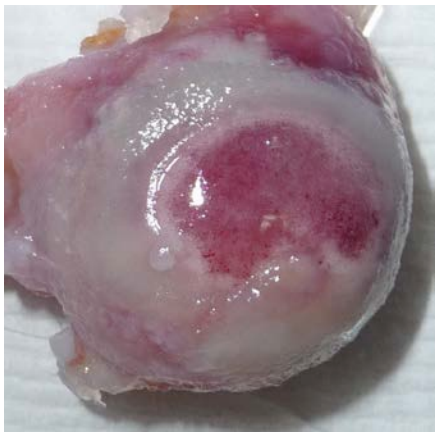


Figure 3-10.



3.4.2. Evaluation of multiple hypotheses using chi squared testing showed that dogs older than 4 years had a greater incidence of exposed subchondral bone than dogs aged less than 4 years.

Chi squared tests were used to evaluate if a link existed between the presence of exposed subchondral bone with either age or sex of animal and also if a link existed between cartilage erosion and age of animal. In total, four hypotheses were investigated. A significant link was established for one hypothesis (p=0.003) and established that in the present study dogs older than 4 years of age had a greater incidence of exposed subchondral bone than dogs aged less than 4 years (Table 3-7). A significant link was not established for the remaining hypotheses; that dogs aged less than 4 years were more likely to have cartilage erosion as the most severe OA lesion than dogs older than 4 years of age, p>0.1 (Table 3-8), that neutered dogs were more likely to have exposed subchondral bone than entire dogs, p=0.89 (Table 3-8) and that female dogs were more likely to have exposed subchondral bone than male dogs, p=0.24 (Table 3-10).

Table 3-7. Chi squared analysis of the link between dog age the incidence of exposed subchondral bone on the femoral head.

Dog age	SCB exposed	SCB not exposed
Age <4yrs	8	37
Age >4yrs	18	19

Hypothesis: dogs older than 4 years had a greater incidence of exposed subchondral bone (SCB) than dogs aged less than 4 years.
Pearson Chi-Square = 8.936, P-Value = 0.003
Likelihood Ratio Chi-Square = 9.055, P-Value = 0.003

Table 3-8. Chi squared analysis of the link between dog age the incidence of exposed subchondral bone on the femoral head.

Dog age	Cartilage erosion most severe lesion	Cartilage erosion not most severe lesion
Age <4yrs	31	14
Age >4yrs	19	18

Hypothesis: dogs aged less than 4 years were more likely to have cartilage erosion as the most severe OA lesion than dogs older than 4 years of age.
Pearson Chi-Square = 2.624, P-Value = 0.105
Likelihood Ratio Chi-Square = 2.56, P-Value = 0.247

Table 3-9. Chi squared analysis of the link between dog age the incidence of exposed subchondral bone on the femoral head.

Dog age	SCB exposed	SCB not exposed
neuter	14	31
entire	11	26

Hypothesis: neutered dogs were more likely to have exposed subchondral bone (SCB) than entire dogs.
Pearson Chi-Square = 0.018, P-Value = 0.892
Likelihood Ratio Chi-Square = 0.018, P-Value = 0.892

Table 3-10. Chi squared analysis of the link between dog age the incidence of exposed subchondral bone on the femoral head.

Dog age	SCB exposed	SCB not exposed
Female	12	28
Male	8	34

Hypothesis: female dogs were more likely to have exposed subchondral bone (SCB) than male dogs.
Pearson Chi-Square = 1.333, P-Value = 0.248
Likelihood Ratio Chi-Square = 1.338, P-Value = 0.247

3.5 Discussion

OA of the coxofemoral joint secondary to CHD occurs widely and affects dogs of all breeds, ages and sex/neuter status. It is a significant cause of pain and loss of mobility in dogs in the U.K. Whilst studies into the pathogenesis of this disease are many in number, the precise impact of causative factors remains unknown and exact preventative measures and non-surgical treatment options remain elusive. The majority of studies focus on radiographical evaluation for diagnosis and little data has been presented based on gross pathology (Leppänen and Saloniemi, 1999; Maki, 2000; Paster *et al.,* 2005). In this study, age at presention, sex/neuter status and breed of dogs undergoing surgical treatment of coxofemoral OA in the U.K. was evaluated. Additionally the types and severity of cartilage lesions on the femoral heads were evaluated providing a new insight into the pattern and distribution of pathology that occurs with CHD and further elucidating the pathogenesis of this common and debilitating disease.

3.5.1. Evaluation of the presentation of dogs undergoing femoral head excision in veterinary referral practice in the U.K. as a treatment for osteoarthritis of the coxofemoral joint showed no sexual dimorphism associated with CHD and that large breeds were most commonly affected along with dogs aged one to four years.

There has been some debate regarding a sex predisposition to CHD due to conflicting studies (Distl *et al.,* 1991; Morgan *et al.,* 1999). More recently it has been determined that there is no sexual dimorphism associated with CHD (Reed, 2000; Rettenmaier *et al.,* 2002). Previously an increased incidence in males was recorded (Distl *et al.,* 1991) followed by reports of an increased incidence in females (Morgan *et al.,* 1999; Swenson *et al.,* 1997; Wood *et al.,* 2002;). Other studies showed an increased prevalence in neutered males (Spain *et al.,* 2004; Witsberger *et al.,* 2008). Our findings showed no statistically significant sex prevalence for surgical treatment of CHD, with 30.5% of the samples from spayed female dogs, 26.8% from entire male, 24.4% from neutered male dogs and 18.3% from entire female dogs. It would be interesting to determine if these results proportionally represent the overall population of dogs kept in the U.K.; however, as in the case of breed, these data have never been recorded and collated for the entire population of dogs in the U.K.

Forty-four of the collected femoral heads were from the left coxofemoral joint, and 38 were from the right. In the present study, we did not record whether the disease was unilateral or bilateral, and as such no direct conclusions regarding prevalence of OA in right and left hip joints can be drawn. In a study into CHD in Portuguese water dogs, two QTL were identified as regulating the variation of the Norberg angle, with one locus affecting the right hip and one affecting the left (Chase *et al.*, 2004).

CHD occurs in all breeds with large breed dogs most commonly affected (Fries, 1995). This may be a result of genetic predisposition rather than as a result of body mass as some studies have demonstrated a negligible effect of body weight in CHD pathogenesis (Flückiger 1995; Morgan and Stephens 1985), and some large breeds such as Greyhounds are unaffected. Medium and small sized breeds are reported to have a lower prevalence of CHD (Coopman *et al.,* 2008; Flückiger *et al.,* 1995; Genevois *et. al.,* 2008; Leppanen and Saloniemi, 1999). Dog breeds with well developed hindlimb and pelvic musculature have been shown to be at decreased risk of CHD in comparison to breeds with poor muscle mass and a more straight angled limb stance (Flückiger *et al.,* 1998; Smith *et al.*, 2001).

A wide range of breeds of all sizes were represented by the samples collected here, from a Jack Russell Terrier to a Leonberger. A genetic component of CHD has been identified in some breeds including Labradors, German Shepherd Dogs, Golden Retrievers and Rottweilers (Chase *et al.,* 2005; Leighton, 1997; Maki *et al.,* 2004; Smith *et al.,* 2006). The most commonly represented breed in this study was the Labrador, constituting 24.4% of the collected samples, followed closely by German Shepherd dogs (19.5%), Border Collies (11.1%), Crossbreed dogs (13.4%), Golden Retrievers (4.9%) and Springer Spaniels (4.9%). It should be considered that the ten most popular dog breeds by numbers of registration with the British Kennel Club in 2006 were Labrador Retriever (45,700), Cocker Spaniel (20,459), Springer Spaniel (15,133), German Shepherd Dogs (12,857), Staffordshire Bull Terriers (12,759) Cavalier King Charles Spaniel (11,411), Golden Retriever (9,373) and West Highland White Terrier (9,300). Rottweiler, Yorkshire terrier, Weimeraner, Dogue de Bordeaux, Border Collie and Siberian Husky all featured in the top 30 breeds and were represented in this study. Thus the high prevalence of samples collected from Labradors may represent not only a genetic predisposition to this disease, but also their popularity as pets in this country. Breeds not featuring in the top 30 registered breeds but included in the current study were Rough Collie, Bearded Collie, Old English Sheep Dog, Jack Russell Terrier, Irish Setter and Leonberger. Breed specific prevalence for CHD has been reported to be between 11-32% for Labradors, 17-46% for

German Shepherds, 17-41% for Golden Retrievers, 11-25% for Border Collies, 13-20% for Leonbergers, 5-25% for Old English Sheepdogs, 10-45% for Rottweilers, 6-24% for Bearded Collies and 9-40% for Irish Setters (Leppänen and Saloniemi, 1999; Maki *et al.*, 2000; Morgan *et al.*, 1999; Swenson *et al.*, 1997; Wood *et al.*, 2002). The species prevalence of CHD, as estimated by the Orthopaedic Foundation for Animals, is between 10 and 48% depending on breed. Studies based on radiographic screening schemes for CHD are likely to under-report actual population prevalence given that radiographs are submitted voluntarily for a negative diagnosis predominantly in breeding animals (Todhunter *et al.*, 2003). Breed heritability of CHD has been reported as 0.34 for Labradors (Wood *et al.*, 2000), 0.4 for German Shepherd Dogs (Hedhammar *et al.*, 1979) and 0.58 for Rottweilers (Maki *et al.*, 2000).

There is little data available regarding the age prevalence of coxofemoral OA in canines. OA in the general canine population has been reported to represent 70% of veterinary hospital visits for disease of the appendicular skeleton, with 22% of cases occurring in dogs aged less than one year (Richardson and Toll, 1997). A bimodal age distribution has been observed (Smith *et al.*, 2012; Witsberger *et al.*, 2008) with the first group being aged four months to three-four years, and a second group being aged older than seven years. Data collated in the study by Witsberger were obtained from the North American Veterinary Medical Database, which consisted of a collection of medical record information supplied by 27 veterinary medical teaching hospitals in North America and included dogs with a diagnosis of CHD or hip OA. Thus data from this study should be representative of clinically affected dogs. There is significant bias in data collected from radiographic studies of dogs undergoing screening for CHD; in one study 71·4% of dogs were less than two years old (Coopman *et al.*, 2008) reflecting the age at which most animals are screened radiographically as part of CHD breeding schemes. Additionally, different radiographic scoring systems exist to evaluate the canine coxofemoral joint so it is difficult to draw comparisons between studies (Coopman *et al.*, 2008). In the present study, the bimodal age distribution reported in the literature was not observed. Dogs aged between one and four years of age were most commonly presented for femoral head excision (36.6%), followed by dogs aged four to seven years (29.3%). Dogs aged between two and 12 months represented 18.3% of collected samples and dogs aged greater than seven years represented the smallest sample group at 15.8%. These data were unexpected as it is generally accepted that the prevalence of OA in dogs increases with age (Vaughan-Scott and Taylor, 2007), as is the case with humans (discussed in Chapter I, Introduction). In a study of over 15,000 pedigree dogs, increasing age was shown to predispose to the

development of coxofemoral OA (Smith *et al.,* 2001) and accordingly one would expect to see increasing numbers of dogs presenting for surgical treatment with advancing age. There is a different pattern in ages of dogs presenting for surgical treatment to the age pattern observed in canine OA. Similarly the severity of lesions affecting different ages of dog seen in the present study was different to reports in the literature, with some young animals showing severe pathological changes and some old dogs presenting with only cartilage fibrillation. Clearly the clinical decision to treat a case surgically will be influenced by the attending clinician and this was a factor which could not be standardised in the present study and this is a possible explanation as to why the severity of lesions affecting different ages of dog seen in the present study did not follow the expected pattern. Another possible factor is the increased availability of total hip replacement surgery as a treatment option with more surgery referral practices emerging over time and with the development of better surgical implants. These data show that OA secondary to CHD is an important differential in the investigation of hindlimb lameness of young dogs aged less than four years and that the prevalence of this condition does not necessarily increase with age as previously thought. It is thus important to move away from current thinking of hip OA as an age-related disease of dogs in clinical practice.

3.5.2. Assessment of collected femoral heads from dogs undergoing total hip replacement surgery revealed cartilage erosion to be the dominant lesion observed. No characteristic pattern of femoral head pathology was observed in dogs with CHD.

While the etiology of canine hip dysplasia has been extensively studied and is well understood, the precise explanation of biomechanical events leading to morphological changes of the femoral head in dogs with HD relies upon theoretical mechanics to explain the observed gross pathological changes. It has been demonstrated that joint laxity associated with CHD changes load distribution in the hip joint so that the cartilage and the underlying bone are damaged (Burton-Wurster *et al.,* 1999). The initial site of cartilage injury occurs adjacent to the fovea of the femoral head (perifoveal at the insertion point of the round ligament) and is centrally located (Burton-Wurster *et al.,* 1999). Subsequently, secondary changes occur adjacent to this area moving in a lateral direction across the femoral head (Burton-Wurster *et al.,* 1999). The site of initiation of cartilage lesions in CHD was shown to be the dorsal femoral head immediately adjacent to the fovea in a study by Inerot (Inerot *et al.,* 1991) using a surgical model of CHD. It has been proposed

that this lesion distribution occurs as a result of subluxation of the hip during the swing phase of locomotion with subsequent reduction of the hip upon weight bearing due to gravitational forces and the co-contraction of the muscles of the hip (which act to form a resolved force of the gravitational force and the muscle force thus reducing the hip medially). It has been suggested that the medial reduction of subluxation which theoretically occurs as the foot contacts the ground and weight-bears leads to a central dorsomedial location of cartilage erosion on the femoral head adjacent to the fovea and fibrillation of the dorsolateral cartilage adjacent to this region (Smith *et al.*, 2012). The current explanation of lesion development remains theoretical, and as such we sought to examine the location and types of femoral head lesions seen in dogs with CHD.

In examining the distribution of cartilage lesions over the surface of the femoral head no one pattern of lesion distribution was associated with CHD, in contrast to the hypothetical lesion distribution outlined above. In humans with OA of the knee, people will modify their gait such that mechanical loading of the affected joint is altered resulting in a slower progression of OA and an alteration in the morphological changes in different regions of cartilage within the joint which are loaded during motion (Miyazaki *et al.*, 2002; Prodromos *et al.*, 1985; Wang *et al.*, 1990). We know from radiographical studies that there is considerable variation in the degree of coxofemoral joint laxity measured in canines affected by CHD (Fluckiger *et al.*, 1999; Smith *et al.*, 1990; Smith *et al.*, 1993; Todhunter *et al.*, 2003) and it follows that the contact area of the femoral head, which is loaded during motion, may change in association with the degree of laxity. Similarly in humans it has been demonstrated that variations in joint laxity and congruency alter cartilage contact stress (Ateshian *et al.*, 1991; Cicuttini *et al.*, 2002; Cohen *et al.*, 1999). Individual variation may explain the wide variety of lesion distribution seen in the femoral heads collected. Pre-operative force plate gait analysis in dogs undergoing femoral head excision could potentially answer this question, as gait could potentially be correlated with femoral head lesions observed, by investigating if there is an association between gait abnormality and gross pathological lesions recorded on the excised femoral head for each dog. The lack of a consistent pattern of lesion distribution suggests that the current biomechanical theories of lesion pathogenesis are incorrect and as such the exact mechanism by which lesions progress across the femoral head from the initial perifoveal location are not yet known, but could potentially be explained by individual variation as occurs with human hip OA.

Hypertrophy of the round ligament has been reported to be an early OA change in joints affected by CHD (Lust and Summers, 1981). In all samples in the present study where the round ligament was present, oedema of the round ligament was grossly visible, however histopathological examination was not carried out in order to confirm if hypertrophy of the round ligament was present. Dogs aged 2-12 months represented not only 18.3% of the total samples collected, but also included femoral heads with severe pathological changes. In the 1-4 year age group, cartilage erosion was the primary lesion observed, recorded in 89.7% of the femoral heads. The milder pathology of cartilage fibrillation represented 6.9% of lesions and at the other end of the spectrum, samples with exposed subchondral bone represented 3.4% of lesions. In all other age cohorts there were similar percentages of cartilage erosion and subchondral bone exposure recorded.

Dogs with moderate or severe coxofemoral OA, which is unresponsive to conservative management, are candidates for surgical treatment (Jensen and Sertl, 1992; Olmstead *et al.,* 1981; Olmstead *et al.,* 1983) and the severity of cartilage changes observed here was consistent with moderate to severe OA. Cartilage fibrillation alone was not a common finding. Most specimens had severe pathology of the femoral head with either cartilage erosion (63.9% of cases overall) or exposure of subchondral bone present (31.3% of cases overall). The Labrador was the only breed presenting with cartilage fibrillation as the most severe lesion observed (15% of Labrador femoral heads). It is unclear why this one breed should be selected for surgical treatment of OA with only fibrillation of the articular cartilage of the femoral head present.

It is estimated that 20% of dogs have chronic pain associated with OA (Sharkey, 2013). Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (International Veterinary Academy of Pain Management). Pain is clearly an important feature of OA and a key component of treatment involves management of chronic pain associated with this disease. In the current study, it would have been interesting to attempt to correlate the severity of OA lesions observed on excised femoral heads with chronic pain levels in dogs undergoing hip replacement surgery. Measuring chronic pain in dogs is made challenging by an absence of patient communication, an important feature of human pain assessment techniques. Currently a number of subjective and objective assessment tools are in use in companion animal veterinary medicine. Subjective pain assessment techniques include owner questionnaires such as the Helsinki Chronic Pain Index (Hielm-Bjorkman and Tulamo, 2009), Liverpool Osteoarthritis Clinical Metrology Instrument (Hercock *et al.*, 2009) and GUVQuest

(Wiseman-Orr *et al.*, 2004). Objective techniques measuring limb motion have also been utilised as a measure of pain assessment in dogs with OA, primarily to measure treatment outcomes. These techniques include force plate analysis and omnidirectional accelerometers (Wernham *et al.*, 2011). To date these techniques have not been correlated with subjective owner questionnaires and clinical examination findings and they are also subject to influencing factors other than pain, such as loss of mechanical limb function associated with disease. Pain is often masked in dogs as part of their innate survival instinct (Sharkey, 2013), and this can influence the reliability of assessments based on altered behavior (owner questionnaires for example). Behaviour can also be altered due to factors other than pain, such as breed, age and concurrent disease. Measurement of chronic pain was not included as part of this study due to the factors outlined above and also because participating clinicians would not have time to complete pain assessments. Additionally, controlling for inter-clinician variation in pain assessment scoring would be difficult.

The data described here make an important contribution to our understanding of OA of the coxofemoral joints of dogs via two novel findings. Firstly the previously observed bimodal age distribution of dogs with OA secondary to CHD was not repeated in our data. We found that dogs between one and seven years were most commonly presented. It is generally accepted that the prevalence of OA in canines increases with age and as such these data are unexpected and also have important clinical implications in that OA should be considered to be a common cause of hindlimb lameness in young dogs and not limited to aged canines. Secondly, a previously undescribed pattern of lesion distribution was discovered on examination of the excised femoral heads. Prior to this study very little data has been published regarding gross pathological changes of the femoral head due to OA as a result of CHD and our understanding of OA pathogenesis has been based on theoretical mechanical models of CHD. Our data did not show any one consistent pattern of lesion distribution. This would suggest that the biomechanical events leading to femoral head lesion development are not fully understood, and that the current theory of subluxation of the joint during the swing phase of locomotion is not a complete explanation of how these lesions develop. Thus the precise pathogenesis of OA of the femoral head from the initial site of perifoveal trauma is not yet known. It could potentially be explained by individual variation as occurs with human hip OA or by pathological events at cellular and molecular levels, which are investigated by other studies in this thesis (in Chapter III, Chapter IV and Chapter V).

3.5.3 Future studies

It would be very interesting to continue this study in order to increase sample numbers and perhaps further our overall understanding of the clinical aspects of CHD. It would be interesting to see if one particular pattern of lesion distribution did emerge as sample size increased or if the random pattern of lesion distribution increased suggesting that individual variation plays an important role in lesion and disease progression as is the case with humans with coxofemoral OA. It would also be of interest to see if the current pattern of age distribution continued with an increase in sample size as this would impact upon the current emphasis on age as a factor in disease progression of CHD and may influence treatment of this disease in younger dogs. If such a study were undertaken additional information could be obtained on sample collection, including body mass, if the dog had unilateral or bilateral hip OA, if the dog had OA of other joints, or concurrent illness which could contribute to joint disease such as diabetes mellitus or hypothyroidism, drug treatment history and levels of physical activity. Submission of digital radiographic images of affected hip joints along with femoral head samples would enable comparison of radiographic changes with grossly observed lesions and would facilitate assessment of acetabular pathology. In the current study, only the femoral head was examined as it is excised at the time of surgery. With CHD there is pathology the acetabular rim also, which was not examined as part of this study.

More complex analyses or statistical modeling of data with categorical outcomes as is the case in this work is difficult. However, it may be possible in future work to dichotomise the categorical outcome to either investigate the likelihood of cartilage erosion or of subchondral bone exposure in different age, gender and breeds of dog. Although this would result in some loss of information with respect to the severity of OA lesions observed, it would enable logistic regression models to be built. For example, one could build a model to investigate if age, gender or breed (or indeed other potential risk factors) were associated with exposure of the subchondral bone as the outcome variable. Likewise, a similar model could be produced with erosion of the cartilage as the outcome variable. In order to do this type of statistical modeling it is important to conduct sample size calculations prior to the commencement of data collection. In these scenarios, given 80% power, 95% confidence and expected incidence of the outcome in the unexposed group of 10% one would require 394 dogs to identify a relative risk indicating a two-fold difference in the likelihood of the outcome or 118 dogs to identify a 3-fold relative risk.

In conclusion, this study has provided new insights into the pathogenesis of OA of the coxofemoral joint of dogs with CHD. These data provide important new information on the gross pathological changes that occur in canine coxofemoral OA, supplementing the current understanding of this disease which is primarily derived from radiographic changes studied as part of CHD screening schemes rather than on the gross pathological findings of clinically affected dogs.

Chapter IV

Senescence of canine articular chondrocytes- a comparison of osteoarthritic and normal cells

4.1 Introduction

As cells age their replicative potential decreases and they enter a senescent phenotype. Ageing is a major risk factor for the development of osteoarthritis in humans and dogs (as discussed in Chapter I). Chondrocytes from osteoarthritic joints in humans show greater evidence of senescence when compared to normal chondrocytes (Price *et al.*, 2002). In this chapter the hypothesis that senescence of articular chondrocytes is a feature of canine OA was investigated.

4.1.1 Cellular senescence

Replicative senescence occurs when normal somatic cells stop dividing after a finite number of divisions. It was first described in 1965 by Hayflick as a process that limits the growth of normal human cells in culture: the so-called Hayflick phenomenon. Twenty-five years later it was discovered that telomeres progressively decrease in length as human primary fibroblasts are cultured towards senescence (Harley *et al.*, 1990). The Hayflick limit refers to the number of times a cell type can replicate in vitro before it undergoes senescence. Human chondrocytes have a short replicative lifespan when compared to other mammalian cell types; the estimated Hayflick limit is approximately 35 population doublings for chondrocytes grown in monolayer culture (Martin and Buckwalter, 2001). Senescent cells remain viable and metabolically active but undergo phenotypic changes associated with an alteration in gene expression (Bodnar *et al.,* 1988; Campisi, 1999). Hayflick (1997) identified changes in cell saturation density, an increase in cell surface volume and changes in multiple biochemical and physiological parameters. Senescent cells produce high levels of reactive oxygen species (ROS), (Chen et al., 1995), which have been shown to play a role in multiple disease processes including cardiovascular disease, neurological disease, neoplastic transformation of cells and indeed OA (Brieger *et al.,* 2012).

Cellular senescence occurs when normal cells are exposed to a variety of “stressors”. It prevents uncontrolled cell replication and as such is a powerful tumour suppressive mechanism (Campisi, 2005; Serrano *et al.,* 1997). However, it may also have the effect of contributing to age-related pathologies, such as osteoarthritis, because many cellular functions are altered or impaired in senescent cells (Kuilman *et al.,* 2010; Price *et al.,* 2002). Different stressors may activate one or multiple senescence pathways. The exact

mechanisms by which different stressors activate these pathways is unknown at present. In addition, the molecular pathways that lead to cellular senescence have not been demonstrated in detail and research to elucidate these pathways is ongoing.

4.1.2 Mechanisms of cellular senescence

As described above, cellular senescence functions to prevent further cellular replication. It is activated in response to physiologic stress (Ben-Porath and Weinberg, 2004). It parallels the cellular program of apoptosis-programmed cell death, though senescent cells remain viable in a quiescent state. There are four main activators of cellular senescence and within a population of cells there may be subsets of cells activating different pathways to senescence (described in Chapter I). Replicative senescence mediated by telomere shortening represents the primary method of cellular senescence in mammalian tissues; however cellular senescence can also occur by DNA damage from exogenous and endogenous sources and due to inappropriate activation of mitogenic pathways, by activation of oncogenes or as a result of "stress". As such there are four main triggers; (i) telomere shortening, (ii) activation of DNA damage responses, (iii) stress-induced premature senescence (SIPS) and (iv) activation of oncogenes.

4.1.2.1 Telomere shortening

Telomeres are DNA sequences that cap the ends of linear chromosomes and are necessary for chromosomal replication and stability (Zakian, 1995). In dogs, they are made up of tandem repeats of the base sequence TTAGGG. Replicative senescence occurs primarily as a result of progressive telomere shortening (30-200bp in human somatic cells) with each cell cycle. Telomeres shorten with each cell cycle as DNA polymerases are unidirectional and cannot prime a new DNA strand. Shortened telomeres maintain the senescent state by generation of a persistent DNA damage response (DDR), (Rodier and Campisi, 2011). Erosion of telomeres beyond a critical minimum length triggers genetic pathways that result in cell cycle arrest and cellular senescence. In dogs, telomeres span approximately 3-23 Kbp (Yazawa *et al.,* 2001) and it has previously been shown that canine telomeres undergo attrition with both *in vivo* and *in vitro* ageing (Rodier and Campisi, 2011).

4.1.2.2 Activation of DNA damage responses (DDR)

The DDR can be induced by direct DNA damage, for example DNA double strand breaks (Di Leonardo *et al.*, 1994). In culture the DDR can be induced by irradiation and exposure to DNA damaging agents (Wahl and Carr, 2001). It has yet to be determined what the deciding factors are in determining the cellular outcome of the DDR, with some cells undergoing apoptosis and other cells entering the senescent state (Ben Porath, 2005). DNA damage has been shown to induce the p53 cellular pathway. It has been suggested that a post-translational modification of p53 occurs and that it is this post-translational modification that determines whether a cell undergoes apoptosis or senescence (Webley *et al.*, 2000). It has also been suggested that the post-translational modification of p53 is determined by the initiating stimulus. The DDR activates the protein kinase ataxia-telangiectasia mutated (ATM) and p53 thus inducing senescence via the p53 pathway (Herbig *et al.*, 2004).

4.1.2.3. Stress-induced premature senescence (SIPS)

SIPS has been demonstrated *in vitro* by exposing cells to sub-lethal stresses including oxidative stress (Toussaint *et al.*, 2000), radiation (Toussaint *et al.*, 2000) and DNA-damaging drugs (Ithahana *et al.*, 2001; Wei *et al.*, 2001). SIPS has been shown to be mediated by activation of p53 and p21 (Toussaint *et al.*, 2000).

While four distinct triggers of cellular senescence have been identified, there is some overlap between these mechanisms. The DDR can be activated by eroded telomeres (d'Adda di Fagagna *et al.*, 2003; Rodier *et al.*, 2009; Rodier and Campisi, 2001). Similarly, activation of oncogenes can induce the DDR (Dimri *et al.*, 2000; Serrano *et al.*, 1997; Zhu *et al.*, 1998) and when fibrblasts are subjected to both chronic low-grade hyperoxia and acute oxidative stress there is marked acceleration of the rate of telomere loss (Von Zglinicki *et al.*, 1995; Von Zglinicki *et al.*, 2000). Von Zglinicki and other researchers suggested the possibility of viewing cellular senescence as one common type of cellular stress response, comparable to apoptosis (Ben-Porath and Weinberg, 2004; Itahana *et al.*, 2004).

4.1.3 Senescence and chondrocytes

Senescent cells accumulate with age (Dimri *et al.*, 1995; Martin and Buckwalter, 2003; Campisi, 2007). They have also been shown to accumulate at sites of age-related pathology, for example OA lesions in cartilage (Price *et al.*, 2002). Comparison of telomere lengths from young and old donors shows a correlation between telomere length and donor age for many cell types, including cartilage, across a number of species (Lindsey *et al.*, 1991). Age-independent accumulation of senescent cells has been shown to occur in many diseases. Senescence has been identified as a feature of atherosclerosis (Foreman and Tang, 2003; Minamino *et al.*, 2002), end-stage renal disease (Melk, 2003), cardiac disease secondary to hypertension (Westhoff *et al.*, 2008), idiopathic pulmonary fibrosis (Minagawa *et al*., 2011) and Alzheimers disease (McShea *et al.*, 1997).

The role of cellular senescence in the pathogenesis of OA is discussed in Chapter I, Introduction. In summary, the cornerstone of OA pathogenesis is an imbalance between articular cartilage destruction and its synthesis leading to progressive loss of articular cartilage. Cartilage has many functions which are critical to maintaining joint health, including absorption of mechanical forces, provision of a protective articular surface and maintenance of joint fluid (Poole, 2005). These functions are provided by the extracellular matrix, which is synthesised and maintained by chondrocytes. Short-term DNA labeling studies have shown that chondrocyte mitoses are present but relatively rare in normal cartilage and mature articular cartilage is regarded as a post-mitotic tissue with little cellular turnover (Martin and Buckwalter, 2001). However, mitotic activity increases several-fold following cartilage injury and this could significantly accelerate telomere erosion. It has previously been demonstrated that there is an increase in the degree of chondrocyte senescence with age (Lotz, 2012), with an age related increase in SA-beta-gal activity and a decline in both telomere length and mitotic activity of chondrocytes (Martin and Buckwalter, 2002). Martin and Buckwalter (Martin and Buckwalter, 2001) also showed that cell turnover in cartilage is dependent upon mechanical stress exposure (Chapter I).

OA is considered to be an age related disease as the incidence of disease increases with increasing age, however ageing alone does not cause OA. Ageing simply increases the risk of developing OA in response to trauma or disease because aged chondrocytes have decreased ability to maintain and restore articular cartilage following insult (Lotz, 2012). Chondrocytes from human OA joints have been shown to have alterations in the

expression of collagenases MMP-1, MMP-8 and MMP-13 compared to normal controls. This results in increased destruction of type II collagen. Additionally TIMP-1 expression has been shown to be decreased in OA affected chondrocytes, resulting in decreased inhibition of enzymatic degradation of collagen and compounding cartilage matrix destruction (Price *et al.*, 2002). Senescent chondrocytes synthesise smaller aggrecan molecules and less functional link proteins, leading to the formation of dysfunctional proteoglycan aggregates (Poole, 2005). This affects the ability of articular cartilage to withstand compressive loads and it is known that aged articular cartilage has decreased tensile stiffness and strength (Buckwalter *et al.*, 1994; Martin *et al.*, 1997). These alterations to normal cellular function and gene expression of senescent chondrocytes impair the vital functions of articular chondrocytes. Senescence of articular chondrocytes thus represents an important aspect of the molecular pathogenesis of OA. The present study investigated the extent to which canine OA chondrocytes were senescent in order to determine if senescence of chondrocytes is a feature of this disease in dogs.

4.1.4. Markers of cellular senescence in mammalian cells

Cellular senescence can be identified by a number of biomarkers enabling the identification of senescent cells both *in vivo* and *in vitro*. These biomarkers include cell cycle arrest, changes in cell morphology, SA-beta-gal accumulation, the presence of senescence associated heterochromatin foci, activation of tumor suppressor pathways (for example p53 and p16-RB cascades) and cell cycle inhibitors (p16, p21 and p53), (Campisi and Fagagna, 2007; Dimri *et al.*, 1995; Kang *et al.*, 2005; Krishnamurthy *et al.*, 2006). Currently there is no one marker which can identify the presence of cellular senescence in isolation and as such a combination of markers are routinely used. For the purpose of this study the replicative lifespan of cells, activity of SA-beta-gal and the presence of p16 were assessed in order to identify the senescent state. Although a marker of apoptosis rather than senescence, the presence of the phosphorylated form of p38MAPK was determined, as this protein has been linked to OA changes in chondrocytes in both humans and mice.

(a) Replicative lifespan of cells

Replicative senescence in primary cell culture is marked by termination of cellular replication. Three phases of cellular proliferation were identified in monolayer cell culture by Hayflick and Moorhead in 1961. Phase I refers to the initial proliferation of cells which is characteristically slow, prior to establishment of the first passage. Phase II is

characterised by rapid cell proliferation and precedes Phase III, during which replication ceases and the cells enter the senescent state. The number of population doublings of cells in tissue culture prior to the onset of senescence can be measured in order to determine the replicative lifespan of cells.

(b) Morphological changes of cells

Changes in cell morphology are also a feature of senescence and are used as a marker of senescence in association with other quantifiable biomarkers, such as p16 and SA-beta-gal. These alterations in morphology were first noted by Hayflick in 1965. Senescent chondrocytes have been shown to develop a flattened morphology with gross cell enlargement (Schnabel *et al*., 2002).

(c) SA-beta-gal activity

SA-beta-gal is a commonly used biomarker for both in vivo and in vitro senescence. Its origin and function remain unknown. Its use was first described by Dimri in 1995 (Dimri et al., 1995) as a histochemically detectable marker which specifically identifies senescent cells, but not pre-senescent, quiescent, terminally differentiated or immortal cells. Cartilage was successfully stained for SA-beta-gal for the first time by Price in 2002 (Price *et al.*, 2002).

(d) p38 MAPK

P38 is a member of the mitogen activated protein kinase (MAPK) family, which is comprised of three main pathways; extracellular signal-regulated kinase (ERK); c-Jun NH2-terminal kinase (JNK) and p38 MAPK. These are major signaling pathways involved in cellular differentiation, proliferation and apoptosis (Chang and Karin, 2001). Activation of p38 (referred to as P38) to its phosphorylated from (referred to as pP38) occurs as a stress response and can be brought about by exposure to reactive nitrogen species (RNS) (such as nitric oxide), IL-1 and TNF-alpha (Freshney *et al.*, 1994; Kyrjakis *et al*., 1994; Raingeaud *et al.*, 1995). RNS, ROS, IL-1 and TNF-alpha all play an important role in the pathogenesis of canine OA (Chernajovsky *et al.*, 2002; Ziskoven *et al.*, 2010), and the expression of pP38 is increased in both human (Takebe *et al*., 2010) and rabbit (Wang *et al.*, 2007) OA chondrocytes.

(e) p16 expression

P16 is a cell-cycle inhibitor, which is known to play an important role in regulating cell growth and the p16 signal transduction cascade is a common mediator of cellular

senescence (Campisi and Fagagna, 2007). Cellular senescence brought about as a result of environmental and intrinsic stress is primarily associated with an up-regulated expression of p16 (Campisi and Fagagna, 2007) and the protein has been shown to accumulate within ageing articular chondrocytes (Zhou, 2004). P16 prevents phosphorylation of the pRb proteins that control G1 exit, by competing with activated D-type cyclins for association with CDK4 or CDK6. P16 has been shown to have a tumor suppressive function in both humans (Sharpless *et al*., 1999) and mice (Krimpenfort *et al*., 2001; Sharpless *et al.*, 2001). It has been widely used as a marker of senescence and has also been shown to be a feature of senescent chondrocytes in human OA cartilage (Zhou *et al*., 2004).

4.2 Aims and Objectives

In the work presented in this chapter, the hypothesis that cellular senescence plays a role in the pathogenesis of canine osteoarthritis was tested. More specifically, the aims and objectives were to:

(i) Establish primary chondrocyte cultures from canine OA and normal cartilage.

(ii) Compare the *in vitro* lifespan of OA and normal canine chondrocytes in culture.

(iii) Compare SA-beta-gal activity between OA and normal canine chondrocytes in culture.

(iv) Compare the expression of p38 MAPK and phosphorylated p38 MAPK between OA and normal chondrocytes in culture.

(v) Compare p16 immunoreactivity between OA and normal chondrocytes in formalin fixed tissue sections.

4.3 Materials and Methods

4.3.1 Sample collection

OA cartilage samples were collected from the femoral heads of dogs. These femoral heads came from The Small Animal Hospital, University of Glasgow Veterinary School and from specialist referral centers in the U.K. The OA sample group was obtained from dogs undergoing a total hip replacement surgery for OA and/or hip dysplasia. The most commonly represented breeds were German Shepherd Dog, Border Collie and Labrador. The control group consisted of dogs of various breeds that were euthanatised for reasons unrelated to the musculoskeletal system (normal chondrocyte samples). In addition three femoral heads were obtained from canine foetuses as normal chondrocyte control samples.

4.3.1.1 Collection of cells for primary cultures

Chondrocytes were harvested from normal and OA femoral head cartilage and counted as described earlier (Chapter II, Materials and Methods).

4.3.1.2 Culture of chondrocytes in alginate beads

Following sequential digestion of cartilage in collagenase and trypsin, chondrocyte cells were isolated from the harvested cartilage samples. Chondrocytes were grown in monolayer culture to P2 and frozen for later use (as described in Chapter II). *In vitro* replicative senescence is defined as a failure of cells in culture to double in number over a period of four weeks. In order to culture chondrocytes to senescence the cells were transferred from monolayer culture to 3D alginate culture. This was because chondrocytes grown in monolayer undergo fibroblastic change after 5-8 passages and also alter their gene expression. Culture of chondrocytes in alginate beads is thought to mimic the *in vivo* tissue matrix of articular chondrocytes more accurately than monolayer culture. After seven days alginate beads were dissolved and the cells were counted using a coulter counter. Cell number was calculated and the cells were then re-suspended in alginate and the process repeated a further three times (Chapter II). Once placed in alginate beads the chondrocytes showed almost no growth, as compared to monolayer culture where they grow rapidly (see results below). As such it was not possible to grow chondrocytes to

senescence in alginate and thus the cells were cultured to senescence in monolayer culture using routine culture techniques previously described (Chapter II).
The following three cell lines were used in the alginate culture study:
Cell line N1: Rottweiler foetus 0 days old normal cartilage
Cell line N7: Staffordshire Terrier 3 years old normal cartilage
Cell line OA78: Border Collie 8 years old OA cartilage

4.3.1.3 Culture of chondrocytes in monolayer to establish growth curves

Four OA primary cells lines (OA65, OA74, OA26, OA78) and four normal primary cell lines (N1, N6, N4, N5) were cultured in monolayer for the growth curve study. The collected data was used to calculate population doublings for each cell line.
Cell lines utilised in this experiment were as follows:
Four OA cell lines:
OA65: German Shepherd Dog, male entire, 28 months
OA74: Labrador, male entire, 10 months
OA26: Labrador, male neuter, 84 months
OA78: Border Collie, female neuter, 96 months
Four Normal cell lines used:
N1: Foetus, Rottweiler, female entire, 0 days (at birth)
N6: Staffordshire terrier, male neuter 3 months
N4: Cross breed, male entire, 132 months
N5: Border collie, female entire, 132 months

Cells were brought up from frozen stocks and seeded into 60mm culture dishes at 2x 10^5 cells per dish, in triplicate, with 5ml complete media. When cells reached confluence cells were trypsinised, counted and reseeded again in triplicate at $2x10^5$ cells in 5ml complete media per 60mm dish. The collected data was used to calculate growth curves and life span of the OA and normal chondrocytes.

4.3.2 Assessment of senescence

Replicative senescence of cell lines was confirmed using a number of additional markers.

4.3.2.1 Senescence associated beta-galactosidase activity

SA-beta-gal activity was determined by immunocytochemistry. Immunocytochemistry allows identification of cellular antigens by detection of specific antibody-antigen binding. Measurement of SA-beta-gal activity was described by Dimri (Dimri *et al.* 1995) and is carried out using a commercial kit (Senescence Associated Beta-Galactosidase Staining Kit, Cell Signaling). Cells were grown on cover slips contained in 6 well plates and the cells were stained on the cover slips. Growth medium was removed from the cells and the cells were washed once with 1X PBS. Cells were then fixed with 1X Fixative Solution for 10–15 minutes at room temperature before washing twice with 1X PBS. Staining solution was added and the cover slips were incubated overnight at 37^{o}C. Positive staining for SA-beta-gal was evident as the development of a perinuclear blue precipitate. Evaluation of slides: three high power fields per slide were examined by light microscopy at 40X magnification and the percentage of positive staining cells per high power field was recorded. Two slides were examined for each chondrocyte cell sample. An average of the percentage of positive staining cells for SA-beta-gal per high power field for each chondrocyte sample over the two slides was obtained. Staining was recorded by digital photography.

4.3.2.2 p16 expression

In order to demonstrate that OA chondrocytes show disease-related senescence, immunohistochemistry was used to show the distribution of p16 in normal and arthritic cartilage. Initially immunocytochemistry of cells grown on cover slips (as described for SA-beta-gal above) was attempted. Immunoperoxidase immunostaining is a type of immunocytochemistry that utilizes a peroxidase (horseradish peroxidise) to catalyse the antibody-antigen reaction to produce a coloured product at the site of the primary antibody, which can then be used to detect the presence of the antigen by microscopy. This technique has been described to detect p16 in cells. Following several attempts at optimisation, no staining was detected and the technique was deemed unsuccessful. As such immunohistochemistry was used as an alternative method of p16 detection. In these experiments immunohistochemistry allowed identification of cellular antigens using a dual antibody system. The unlabeled primary p16 antibody was bound to a second biotinylated antibody that was then conjugated with horseradish peroxidase stept-avidin (HRPO). This provided signal amplification at the primary antibody site. HRPO then reacted with

diaminobenzidine (DAB, Sigma Aldrich) resulting in a brown stain formation in the presence of p16 allowing identification of the protein. Cartilage was removed from collected femoral heads under sterile conditions and placed in formalin for immunohistochemical staining with p16 antibody (Santa Cruz Biotechnology H-156 rabbit polyclonal antibody raised against a recombinant protein corresponding to full length human p16). The antibody concentration and cartilage fixing procedure was optimised. This involved titration of the primary antibody over a range of dilutions to determine optimum staining with minimum background. Canine lymphatic tissue was used as a positive control for optimisation and concentrations of 1/50, 1/100, 1/200 and 1/300 were tested. Staining was optimal for 1/50 antibody dilution.

4.3.2.3 Immunohistochemical staining procedure

Cartilage tissue sections were fixed in acetone for 5 minutes and allowed to air dry for 20 minutes. The sections were then cut and mounted on histogrip coated slides (Zymed Laboratories Inc), then dried at 56°C for 15 minutes. This did not result in stable fixing of cartilage samples to the slides, and with subsequent steps the cartilage slices slid off the slides. As such additional fixing of the samples to the slides by incubating at 80°C overnight was required. This resulted in obliteration of the extracellular matrix, though chondrocyte cells were well preserved and successfully fixed to the slide. Endogenous peroxidase activity was blocked by immersion of slides in peroxidase block solution (Dako Real Peroxidase-blocking solution) for 10 minutes. After washing for 5 minutes with Tris Buffer saline with Tween 20 (TBST), sections were incubated with primary antibody against p16 (rabbit monoclonal anti-p16, H-156; sc-759, Santa Cruz Biotechnology), for 60 minutes at room temperature. The dilution used was 1:50 (using Dako Real Antibody Diluent). Sections were then washed for 5 minutes in TBST buffer. Sections were then incubated with primary antibody against p16 (rabbit monoclonal anti-p16, H-156; sc-759, Santa Cruz Biotechnology), for 60 minutes at room temperature. The dilution used was 1:50 (using Dako Real Antibody Diluent). Sections were then washed again for 5 minutes in TBST buffer. Sections were incubated with secondary antibody (Secondary HRP anti-rabbit, Dako EnVision+ System HRP labeled polymer Anti-Rabbit), for 40 minutes at room temperature. Slides were wiped to remove excess buffer, and incubated with peroxidase labeled polymer complex (HRPO conjugated streptavidin complex, Dako) for 35 minutes. Slides were washed with 1X TBST buffer and then with distilled water. Sections were developed with Dako K5007 DAB (3,4,3’,4’-tetra aminobiphenyl

hydrochloride), incubated twice for 5 minutes at 37°C, washed thoroughly with distilled water and counterstained with Gill's Haematoxylin for 27 seconds. DPX mountant was used to permanently fix the slides with cover slips. Positive staining for p16 was identified by examination of the slides by light microscopy. Cartilage sections were examined for the presence of brown cytoplasmic staining. The proportion of positive cells per high power field was calculated for each cartilage sample. This was repeated for 3 separate high-powered fields (HPF) per slide, and each cartilage sample was run in duplicate. For each cartilage sample, the resulting percentages of positively staining cells per HPF were averaged to give a final positive stain percentage. Staining was recorded by digital photography. For negative controls, duplicate slides were incubated with a non-related serum rather than p16 primary antibody. Positive controls for p16 protein consisted of lymphatic tissue sections.

4.3.2.4 P38 kinase and phosphorylated p38 protein expression.

In order to evaluate the expression of phosphorylated p38 MAPK in OA and normal chondrocytes, cell pellets were collected from eight cell lines at passage one and once the cells had ceased replication. P38 kinase and phosphorylated p38 kinase protein expression were examined by Western blot using anti-p38 monoclonal antibody (p38 MAP Kinase Thr180/Tyr182 and Phospho-p38 MAP Kinase Thr180/Tyr182 antibody, Cell Signalling Technology) and a HRP-conjugated anti-rabbit IgG antibody (Cell Signalling Technology) as described in Chapter II. Protein expression can also be assessed using immunohistochemistry in formalin fixed tissue samples and cultured cells. However P38 and pP38 antibodies for immunohistochemistry were not available. As such western blotting was used to assess the presence of these proteins in this experiment.

4.3.3 Statistical analysis

For statistical analysis of the significance of differences in growth rates of chondrocytes in monolayer, the Students paired t-test was used. Evaluation of statistically significant differences in staining levels of SA-beta-gal and p16 were calculated using Wilcoxin rank –sum nonparametric tests, using SPSS software. To calculate staining levels the proportion of positive cells per high power field (HPF) was calculated for each cartilage sample over 3 HPF per slide with each cartilage sample measured in duplicate. The percentages of positively staining cells per HPF were averaged to give a final positive

stain percentage for SA-beta-gal and p16. P-values of <0.05 were considered statistically significant.

4.4 Results

OA canine chondrocytes showed increased levels of cellular senescence compared to normal canine chondrocytes. OA chondrocytes had less replicative capacity than normal cells, they had increased levels of SA-beta-gal accumulation and they had higher levels of p16 positivity than normal chondrocytes. Both OA and normal chondrocytes were positive for the presence of phosphorylated p38 at the onset of cellular senescence, however both OA and normal chondrocytes were negative for the presence of this protein at the time of initial culture.

4.4.1 Assessment of the replicative lifespan of chondrocytes showed that OA chondrocytes undergo replicative senescence earlier than normal chondrocytes.

In order to determine the replicative life span of chondrocytes, cells were cultured to senescence. Initially alginate beads were used as chondrocytes grown in monolayer culture can undergo fibroblastic change after 5-8 passages and also alter their gene expression. Culture of chondrocytes in alginate beads is thought to mimic the *in vivo* tissue matrix of articular chondrocytes more accurately than monolayer culture. Once normal and OA chondrocytes were placed in this culture system, all three cell lines showed almost no growth (Figure 4-1), with less than one population doubling across all cell lines over the 28 day culture period. As such the cost of culturing chondrocytes to senescence using this method would have been excessive both in time and culture materials. In all subsequent experiments, chondrocytes were grown in monolayer cultures (as described in Chapter II). Using monolayer cell culture, chondrocytes grew rapidly (Table 4-1) compared to the alginate bead culture system.

Changes in cell morphology were observed once chondrocytes became senescent. Both OA and normal chondrocytes grown in monolayer culture to replicative senescence showed a change in cell morphology. The late passage chondrocytes became shorter and flatter in shape compared to early passage chondrocytes, which had an elongated morphology. The senescent population also displayed diverse morphotypes with decreased cellular density due to increased cell-to-cell contact inhibition. Figure 4-2 shows early passage chondrocytes growing in monolayer while Figure 4-3 shows late passage chondrocytes growing in monolayer, illustrating the change in morphology of senescent chondrocytes. To compare the *in vitro* life span of OA and normal chondrocytes, four OA primary

chondrocyte cells lines (OA65, OA26, OA74, OA78) and four normal primary chondrocyte cell lines (N1, N6, N4, N5) were cultured to senescence in monolayer culture and growth curves were generated. Growth curves for OA and normal chondrocytes are shown in Figures 4-4 and 4-5 respectively. The OA cell lines underwent replicative senescence between day 13 and day 25 of culture and the normal chondrocyte cell lines underwent replicative senescence between day 14 and day 32 of culture. Table 4-1 shows the number of population doublings to onset of cellular senescence for each cell line. The OA cell lines (OA65, OA74, OA26, OA78) ceased growth between 1.15 and 4.93 population doublings. In contrast, normal chondrocyte cell lines (N1, N6, N4, N5) reached population doublings of between 5.11 and 14.68 before cessation of cellular replication. The chondrocytes derived from foetal cartilage showed a much higher number of population doublings to senescence (14.68) compared to the other cell lines. These data show that OA chondrocytes cease replicating after fewer population doublings than normal chondrocytes ($p=0.01$).

Figure 4-1. Growth rates of chondrocytes in alginate beads.

Chondrocytes were initially grown in 3D alginate culture as culture of chondrocytes in alginate beads is thought to mimic the *in vivo* tissue matrix of articular chondrocytes more accurately than monolayer culture. After seven days of culture alginate beads were dissolved and the cell number was calculated. The cells were then re-suspended in alginate and the process repeated 3 further times in order to calculate the cell growth rate and generate growth curves for each cell line. Once placed in alginate beads the chondrocytes showed almost no growth, as compared to monolayer culture where they grew rapidly. There was less than one population doubling for both normal and OA chondrocytes cultured over the 28-day period, as shown in the graph below which illustrates the total cell numbers for each of the 7-day growth periods (weeks 1, 2, 3 and 4) for the three cell lines (N1, N7 and OA78). As such it was not possible to grow chondrocytes to senescence in alginate due to the time limitations of the study and the cells were cultured to senescence in monolayer culture.

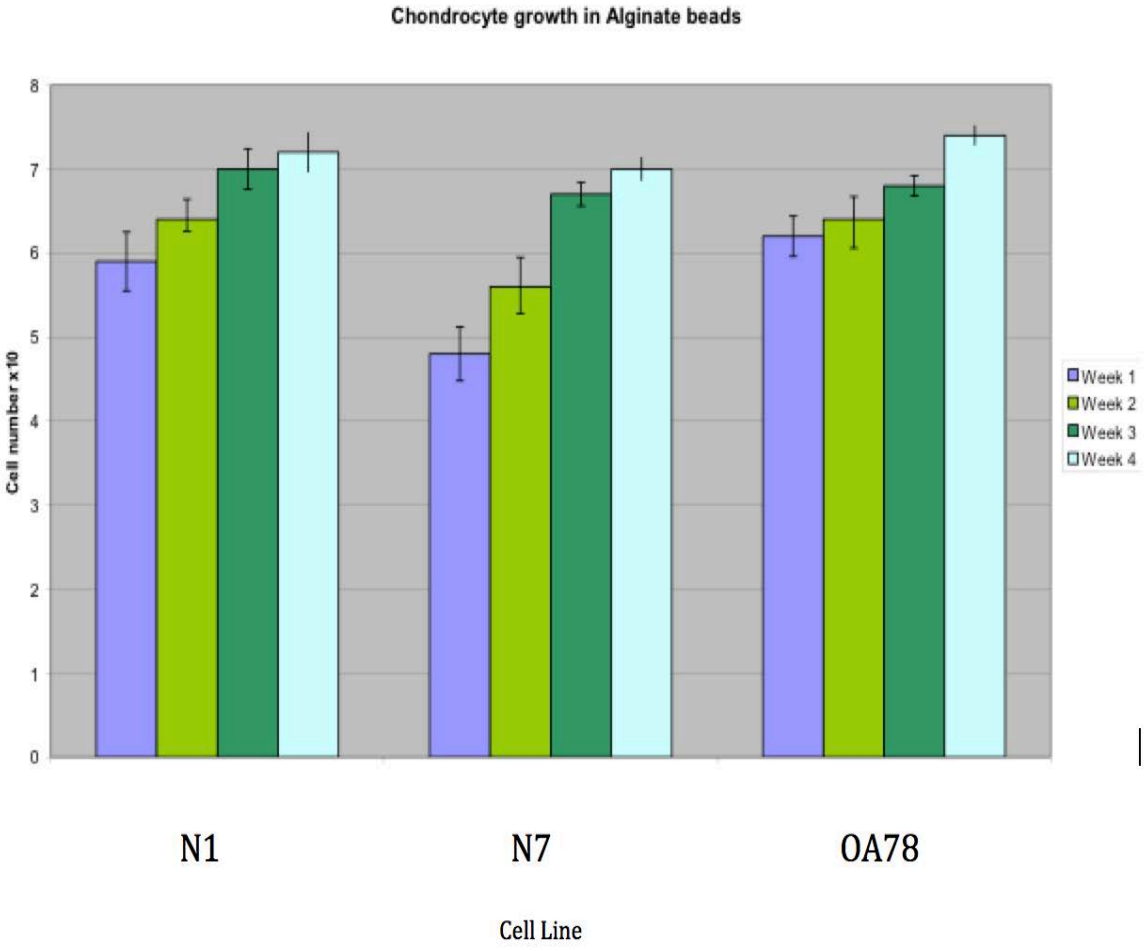


Table 4-1. Life span of articular chondrocytes in monolayer culture.
Chondrocyte cell lines grew rapidly in monolayer culture as compared to alginate culture. The cells were cultured to senescence, growth curves were generated and the number of population doublings to senescence (the replicative capacity) was calculated for each cell line. Population doublings to senescence for the eight cell lines cultured are shown in the table below. Four OA chondrocyte cell lines and four normal chondrocyte cell lines were utilised for this study. The OA cell lines (OA65, OA74, OA26, OA78) ceased growth between 1.15 to 4.93 population doublings. In contrast, normal chondrocyte cell lines (N1, N6, N4, N5) reached population doublings between 5.11 and 14.68 before cessation of cellular replication. The cells derived from a foetal sample showed a much higher population doubling to senescence of 14.68 compared to the other cell lines.
These data show that OA chondrocytes have lower replicative capacity than normal chondrocytes as they cease replicating after fewer population doublings compared to normal chondrocytes (p=0.01).

Cell line	*Population doublings to senescence*
OA26	1.51
OA74	1.15
OA78	1.99
OA65	4.93
N3	5.11
N4	4.81
N5	3.17
N1	14.68

Figure 4-2A and 4-2B. The cellular morphology of early passage chondrocytes grown in monolayer culture.

The images below illustrate the morphological features of chondrocytes grown in monolayer culture. Images 4-2A and 4-2B show normal chondrocyte morphology.
A: OA65 chondrocytes at first passage. X 400 Magnification.
B: N7 cells at second passage. X 400 Magnification.

A B

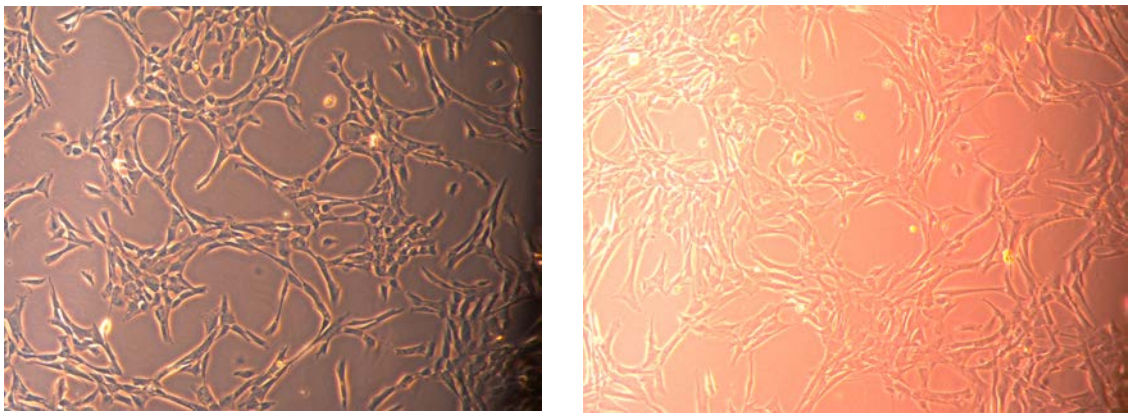


Figure 4-3A and 4-3B. The cellular morphology of late passage senescent OA and normal chondrocytes grown in monolayer culture.

The images below illustrate the morphological features of senescent chondrocytes grown in monolayer culture. Images 4-3A and 4-3B show a more rounded morphology compared to normal chondrocytes. The presence of these morphological changes suggests the onset of replicative senescence in cell culture.

A: N1 chondrocytes at senescence. X 200 Magnification.
D: OA65 chondrocytes at senescence. X 200 Magnification.

A. B.

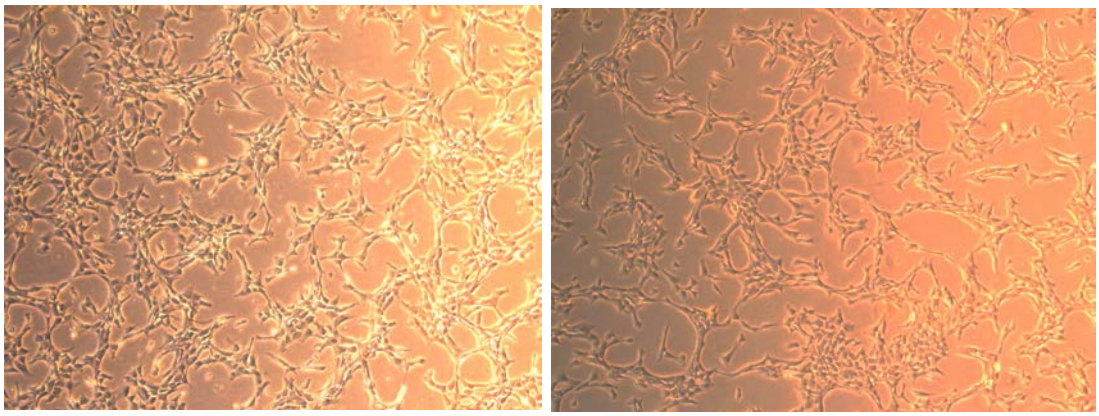


Figure 4-4. Growth Curves of OA chondrocytes cultured to senescence.
The growth curves of four OA chondrocyte cell lines cultured to senescence are shown below. The OA cell lines underwent replicative senescence between day 13 and day 25 of culture, after a total of between 1.15 and 4.93 population doublings. OA chondrocyte cell lines used were: OA65 German Shepherd Dog, male entire; OA74 Labrador, male entire; OA26 Labrador, male neuter; OA78 Border Collie, female neuter.

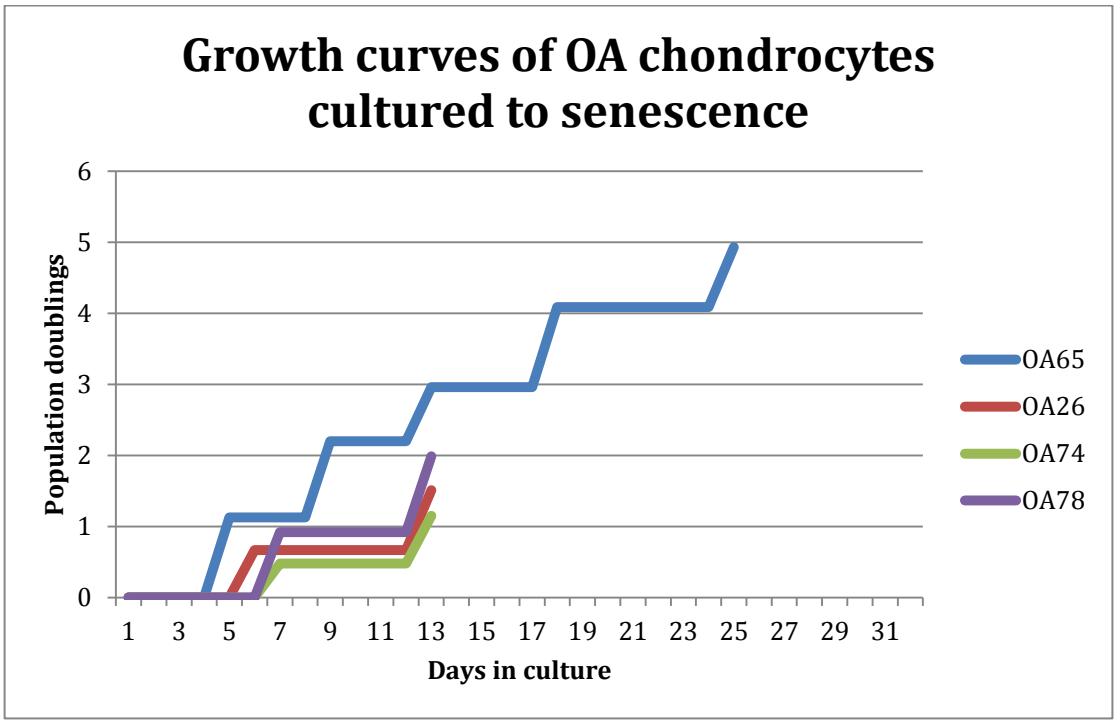


Figure 4-5. Growth Curve of normal chondrocytes cultured to senescence. The growth curves of four normal chondrocyte cell lines cultured to senescence are shown below. The normal chondrocyte cell lines underwent replicative senescence between day 14 and day 32 of culture, after a total of between 3.17 and 14.68 population doublings. OA chondrocytes had shorter replicative capacity than the normal chondrocytes (p=0.01) based on number of population doublings to senescence. Normal chondrocyte cell lines used were: N1, Rottweiler, female entire; N6 Staffordshire terrier, male neuter; N4 Cross breed, male entire; N5 Border Collie, female entire.

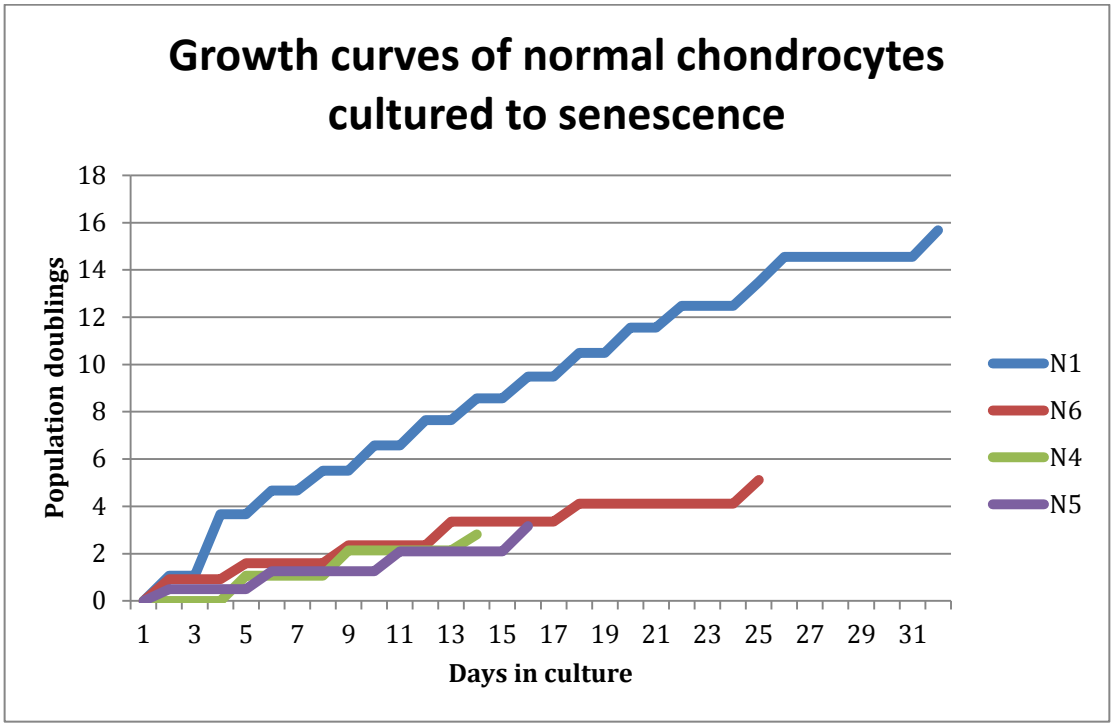


Figure 4-6 and 4-7. Growth curves of normal chondrocytes and OA chondrocytes from young dogs and growth curves of normal chondrocytes and OA chondrocytes from old dogs.

To determine if the animal's age impacted upon the chondrocyte lifespan, the growth curves and population doublings to senescence were compared between young (less than four years of age) and old (greater than eight years of age) dogs. The OA cell lines from old dogs underwent replicative senescence after fewer population doublings than normal cell lines from old dogs (p=0.01). Similarly, OA cell lines from young dogs underwent replicative senescence after fewer population doublings than normal cell lines from young dogs (p=0.01): Figure 4.6A shows the growth curves of OA and normal chondrocytes from young dogs and Figure 6B shows growth curves of OA and normal chondrocytes from old dogs.

4-6.

OA chondrocyte cell lines used were: OA65 German Shepherd Dog, male entire; OA74 Labrador, male entire. Normal chondrocyte cell lines used were N1 Rottweiler, female entire; N6 Staffordshire terrier, male neuter.

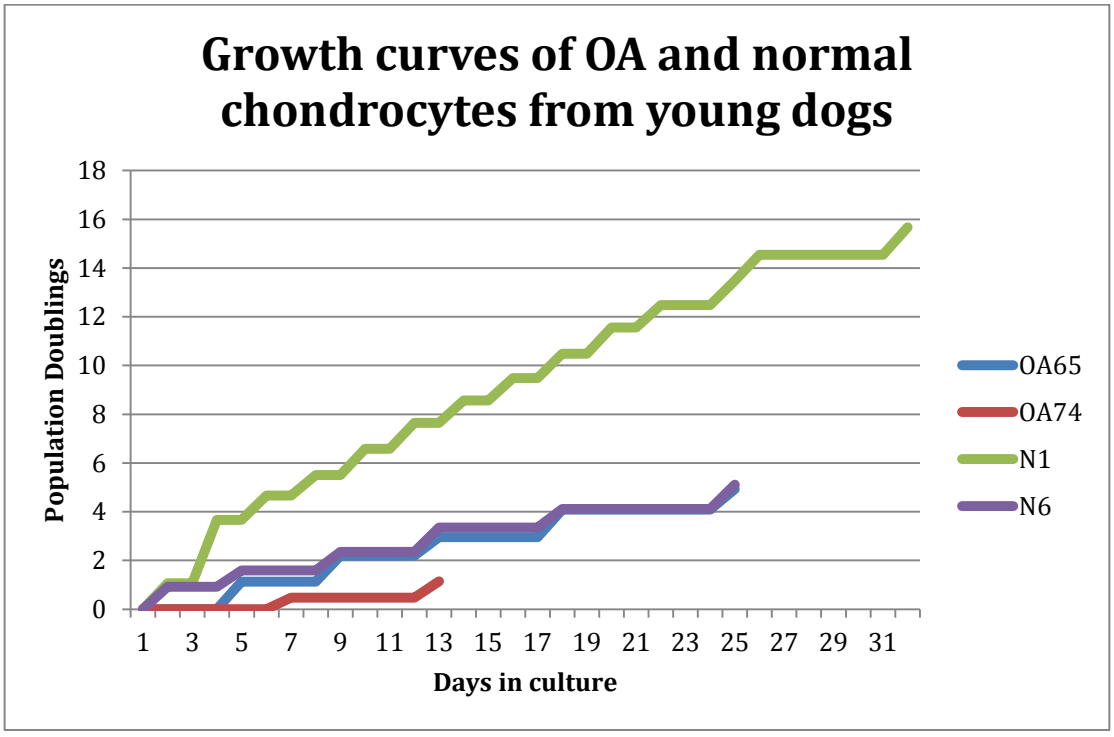
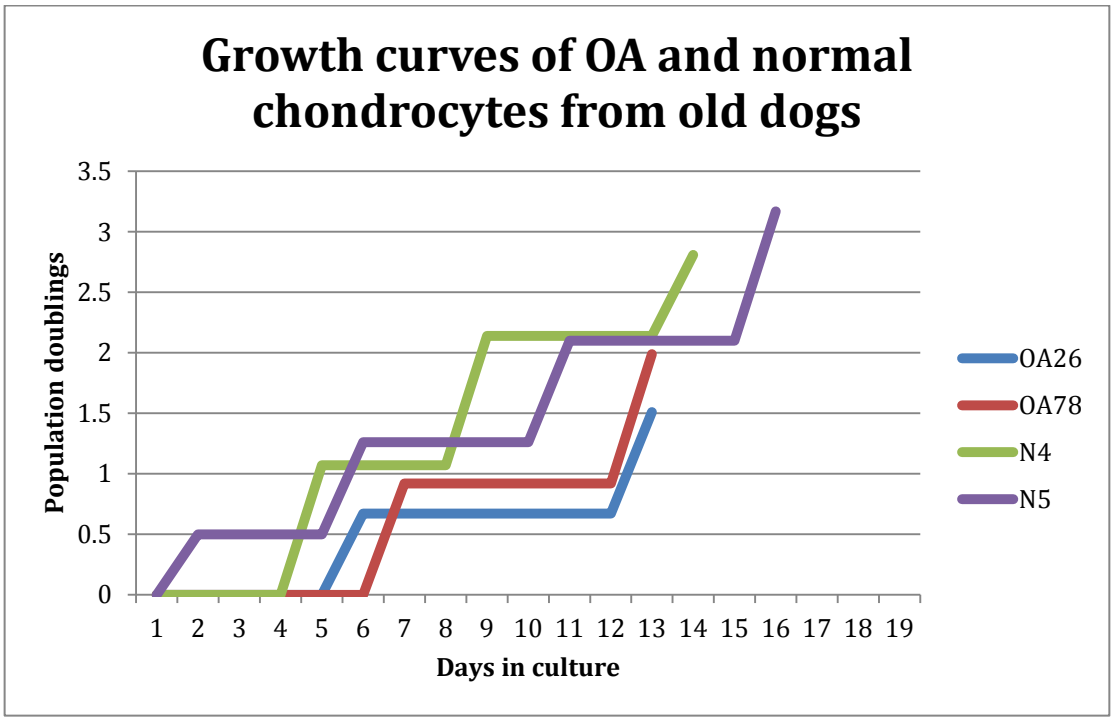


Figure 4-6 and 4-7. Growth curves of normal chondrocytes and OA chondrocytes from young dogs and growth curves of normal chondrocytes and OA chondrocytes from old dogs.

4-7.

OA chondrocyte cell lines used were: OA26 Labrador, male neuter; OA78 Border Collie, female neuter. Normal chondrocyte cell lines used were N4 Cross breed, male entire; N5 Border Collie, female entire.

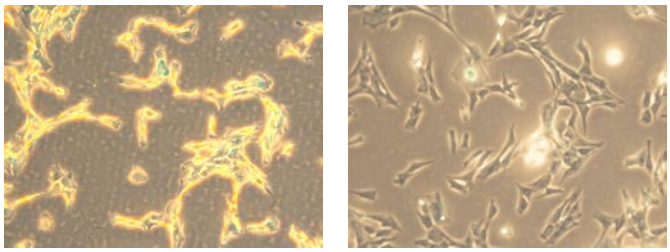


4.4.2 OA chondrocytes had increased levels of SA-beta-gal activity compared to normal chondrocytes indicating higher levels of cellular senescence in OA chondrocytes than normal chondrocytes.

The senescent cell state was established by visual identification of a perinuclear blue dye indicating the presence of SA-beta-gal within the chondrocyte. Figures 4-8 and 4-9 show SA-beta-gal stained cartilage cells for cell line N4 and cell line OA26 respectively. Levels of senescence within a cell population were calculated as a percentage of positively staining cells for SA-beta-gal. Figures 4-10A, B, C and D show the percentage of SA-beta-gal positive staining cells at day 0, 7, 14 and 21 of culture in the OA chondrocyte cell lines and the normal chondrocyte cell lines. At 24 hours after seeding in monolayer (day 0) the normal cell lines showed between 0% and 10% positive SA-beta-gal staining. The OA cells at the same time point showed between 9% and 51% positive staining (Figure 4-10A). At day seven of culture in monolayer the normal cell lines showed between 0% and 10% positive staining and the OA cell lines showed from 57% to 64% positive staining (Figure 4-10B). By day 14 the percentage of positively staining cells had increased in the normal chondrocytes to between 0% and 41%, with an even higher level of positive staining seen in the OA cell lines of 85% to 100% Sa-beta-gal positive cells (Figure 4-10C). At day 21 of monolayer culture 97% to 100% of OA cells stained positive for SA-beta-gal and 0% to 63% of normal chondrocytes stained positive (Figure 4-10D). All four OA cells showed significantly higher levels ($p<0.001$) of SA-beta-gal staining than non-OA cells at the four time points examined.

Figure 4-8. SA-beta-gal activity in N4 chondrocytes.
SA-beta-gal activity in N4 chondrocytes at (A) day 0, (B) day 7 and (C) day 14 of monolayer culture. Cells were stained for SA-beta-gal activity and examined under a light microscope. Perinuclear blue staining is indicative of SA-beta-gal accumulation. At day zero 10% of the cells were senescent. At day seven 12 % of cells were senescent. This increased to 41% at day 14.

A. B.



C.

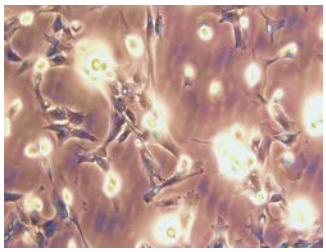
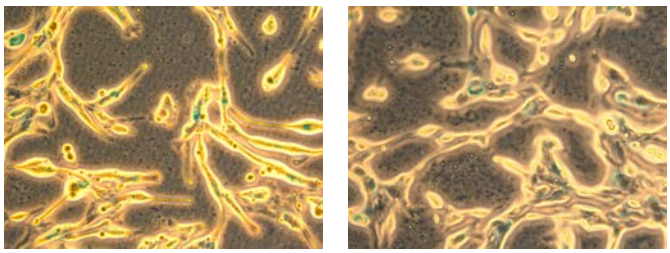


Figure 4-9. SA-beta-gal activity in OA26 chondrocytes
SA-beta-gal activity in OA26 chondrocytes at day 0 of culture (A), day 7 (B) and day 14 (C). At initial culture 24% of the cells were SA-beta-gal positive. At day seven this had increased to 64% positive staining and at day 14 all of the cells were senescent, staining positively for SA-beta-gal.

A. B.



C.

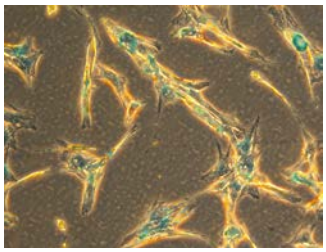
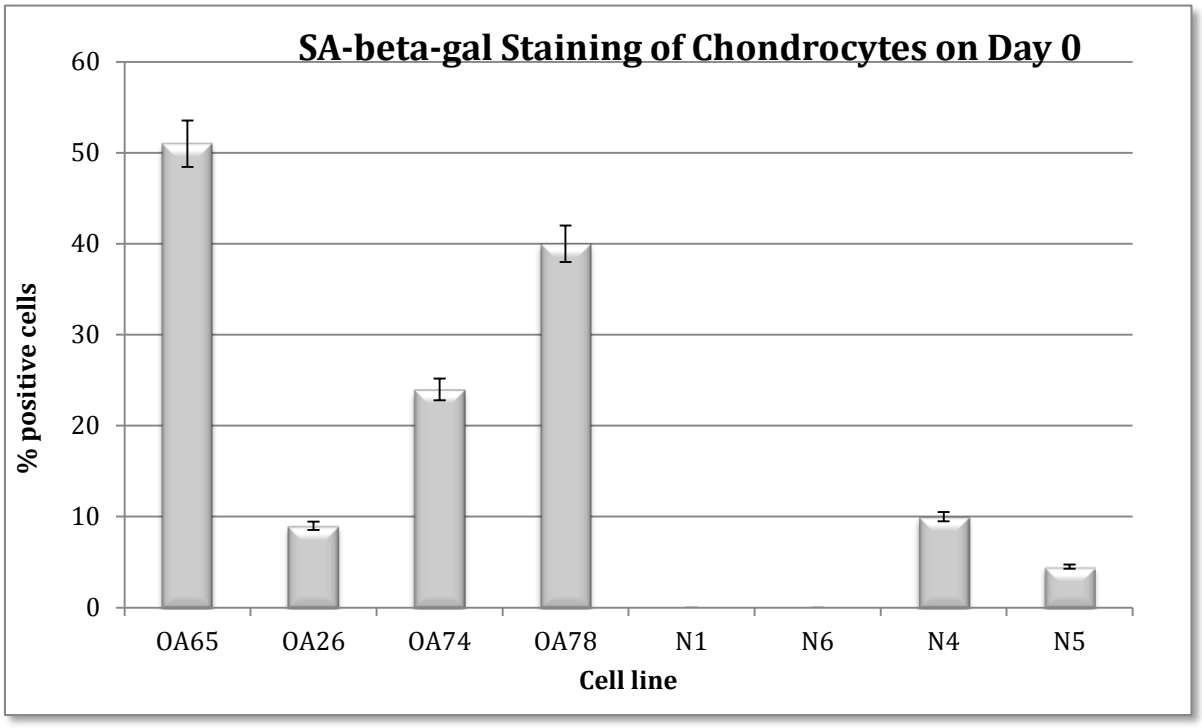


Figure 4-10A, 4-10B, 4-10C and 4-10D. SA-beta-gal activity in OA and normal chondrocytes over 28 days of monolayer cell culture.

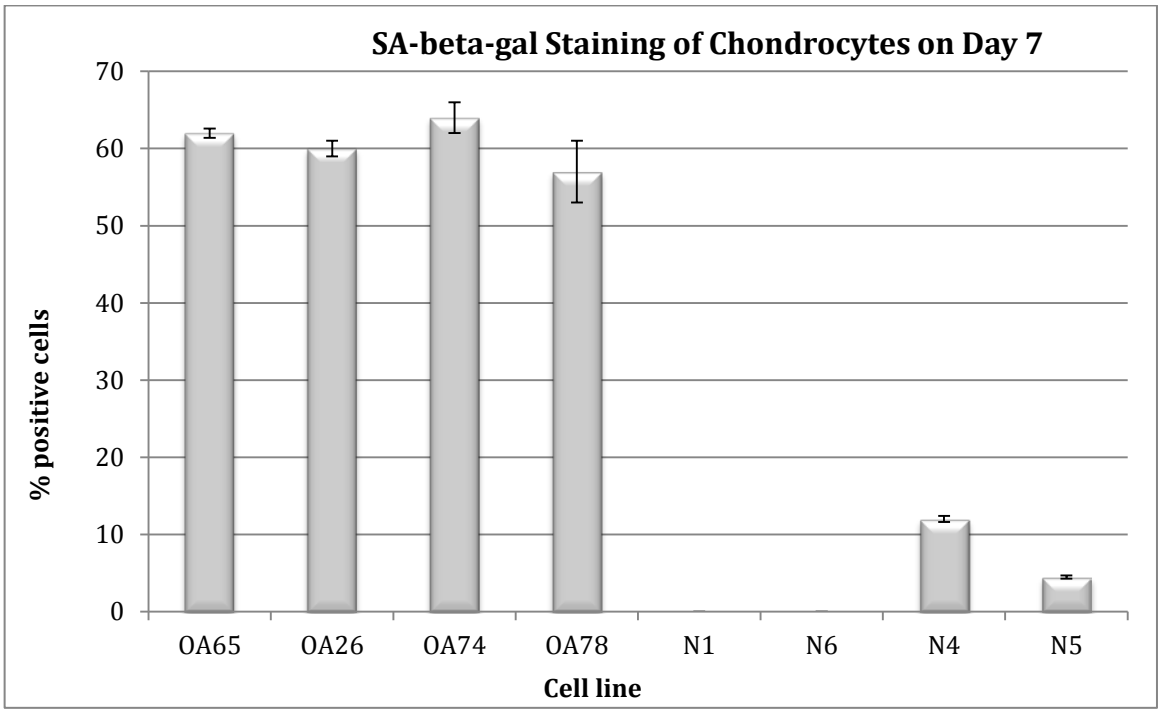
The graphs below show the results of SA-beta-gal staining of chondrocyte cells cultured in monolayer over a 28 day period for four OA (OA) and four normal (N) chondrocyte cell lines. Chondrocytes were stained at day 0, day 7, day 14 and day 21 of culture. All four OA cell lines showed significantly higher levels ($p<0.001$) of SA-beta-gal staining than non-OA cells at the four time points examined. Graphs illustrate the percentage of SA-beta-gal positive staining cells for each cell line.

OA chondrocyte cell lines used were: OA65 German Shepherd Dog, male entire; OA74 Labrador, male entire; OA26 Labrador, male neuter; OA78 Border Collie, female neuter. Normal chondrocyte cell lines used were: N1, Rottweiler, female entire; N6 Staffordshire terrier, male neuter; N4 Cross breed, male entire; N5 Border Collie, female entire.

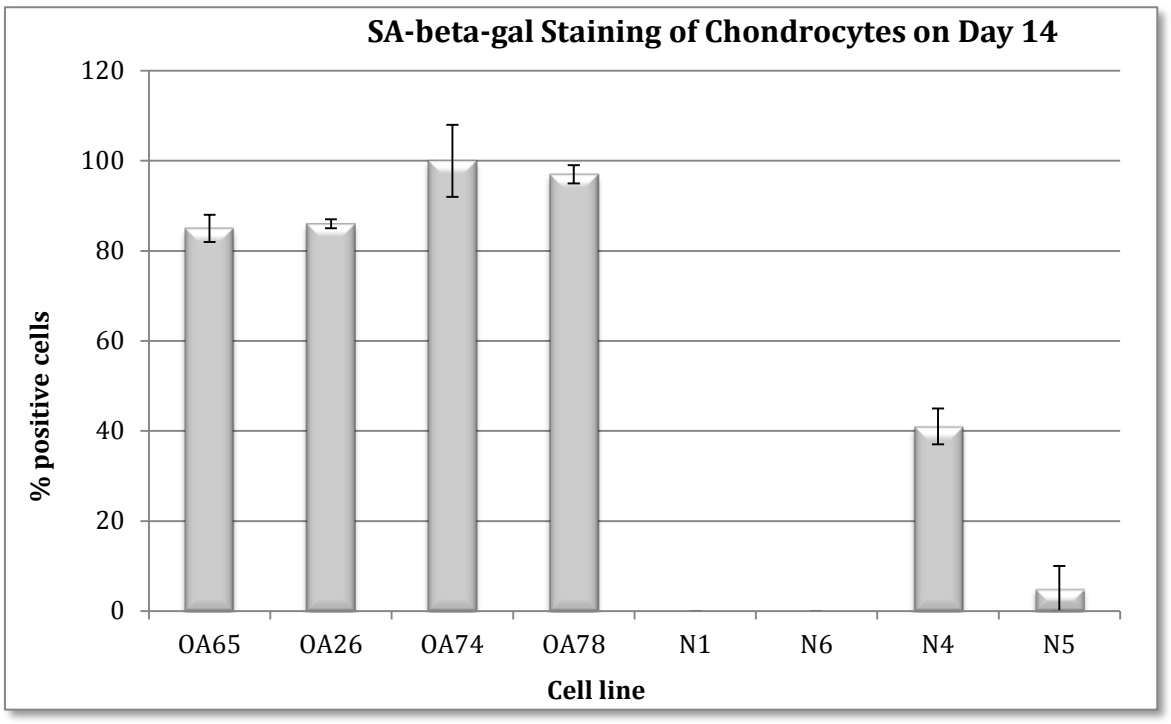
4-10A. At 24 hours after seeding in monolayer (day 0) the normal cell lines (N1, N6, N4, N5) showed between 0 and 10% positive Sa-beta-gal staining. The OA cells (OA65, OA26, OA74, OA78) at the same time point showed between 9 and 51% positive staining.



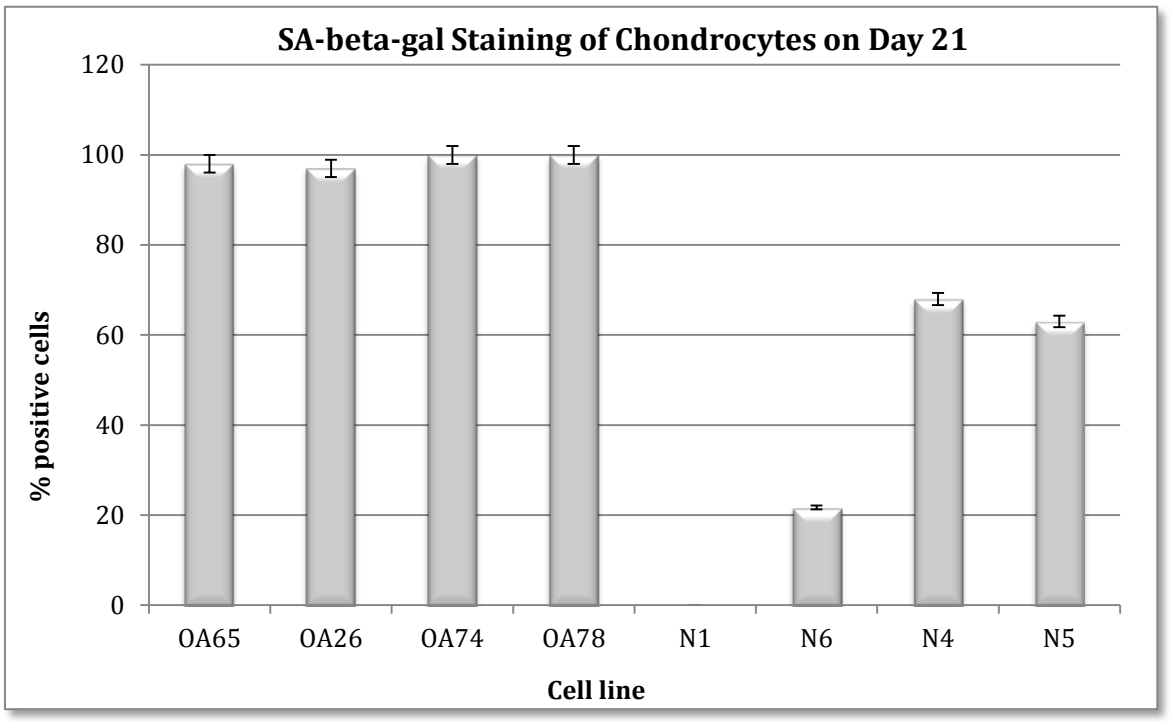
4-10B. At day seven of culture in monolayer the normal cell lines (N1, N6, N4, N5) showed between 0 and 10% positive staining and the OA cell lines (OA65, OA26, OA74, OA78) showed from 57 to 64% positive staining.



4-10C. By day 14 the percentage of positively staining cells had increased in the normal chondrocytes (N1, N6, N4, N5) to between 0 and 41%, with an even higher level of positive staining seen in the OA cell lines (OA65, OA26, OA74, OA78) of 85 to 100% Sa-beta-gal positive cells.



4-10D. At day 21 of monolayer culture 97-100% of OA cells (OA65, OA26, OA74, OA78) stained positive for Sa-beta-gal and 0-63% of normal chondrocytes (N1, N6, N4, N5) stained positive.

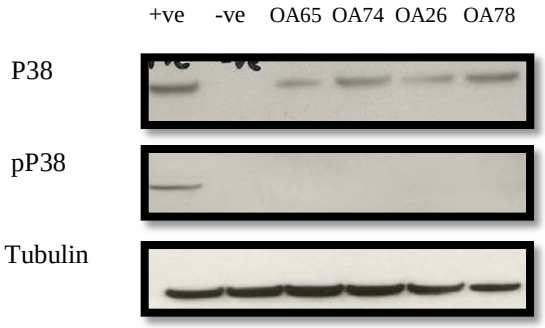


4.4.3 P38 MAPK and phosphorylated P38 MAPK were activated in both normal and OA canine chondrocytes.

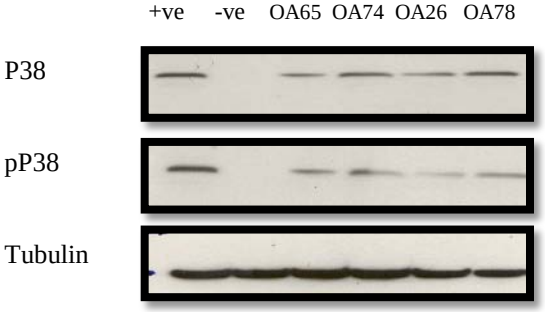
To investigate the hypothesis that OA chondrocytes have increased levels of cellular senescence compared to normal chondrocytes, western blot analysis for the presence of p38 and phosphorylated p38 protein was carried out for both OA and normal cells cultured in monolayer. Western blot analysis at initial culture and at replicative senescence for four OA and four normal chondrocyte cells lines is shown in Figure 4-11. Once protein bands had been transferred from the western blot to the paper membrane for probing with antibody, the paper membrane was divided at the level of the 45kDa marker. The upper portion of the membrane was probed with P38 and then stripped and re-probed with pP38 antibody. The lower portion of the membrane was probed with tubulin antibody. Western blot analysis demonstrated the presence of the p38 protein (referred to as P38) in all OA and normal cells (eight cell lines in total) at passage one of monolayer culture and once the cells had reached replicative senescence (Figure 4-11). Probing with the phosphorylated p38 (pP38) antibody showed that OA65, OA74, OA26 and OA78 cells contained the phosphorylated active form of p38 at the time of senescence but not at passage one of monolayer culture (Figure 4-11A and 4-11B). Similarly, normal chondrocytes (N1, N6, N4, N5) did not contain the phosphorylated active form of p38 at initial culture (Figure 4-11C) but were positive for pP38 at replicative senescence (Figure 4-11D). As such pP38 is a useful marker to confirm cellular senescence but not as an indicator of replicative capacity as it was only present in chondrocytes after replicative senescence had been reached.

Figure 4-11. Western Blot analysis for p38/pP38 in canine chondrocytes.
Western blot analysis for the presence of p38 and phosphorylated p38 protein was carried out for four OA and four normal chondrocyte cell lines cultured in monolayer at the time of initial culture and again once replicative senescence had been reached. Probing with the phosphorylated p38 (pP38) antibody showed that OA65, OA74, OA26 and OA78 cells contained the phosphorylated active form of p38 at the time of senescence but not at passage one of monolayer culture (Figure 4-11A and 4-11B). Similarly, normal chondrocytes (N1, N6, N4, N5) did not contain the phosphorylated active form of p38 at initial culture (Figure 4-11C) but were positive for pP38 at replicative senescence (Figure 4-11D).
4-11A: OA cells at day 0 of culture. 4-11B: OA cells at senescence. 4-11C: Normal cells at day 0 of culture. 4-11D: Normal cells at senescence.

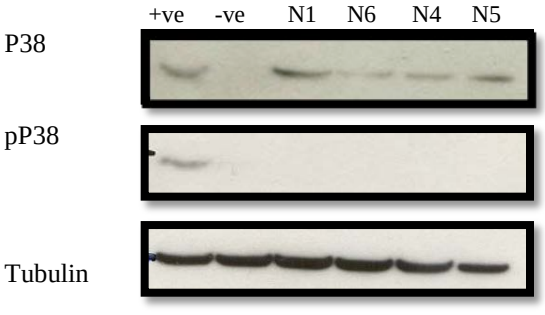
4-11A. OA chondrocytes did not contain pP38 at initial monolayer culture.



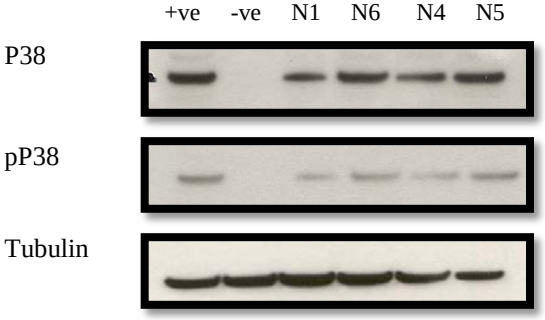
4-11B. OA chondrocytes were positive for pP38 once they reached cellular senescence.



4-11C. Normal chondrocytes did not contain pP38 at initial monolayer culture.



4-11D. Normal chondrocytes were positive for pP38 once they reached cellular senescence

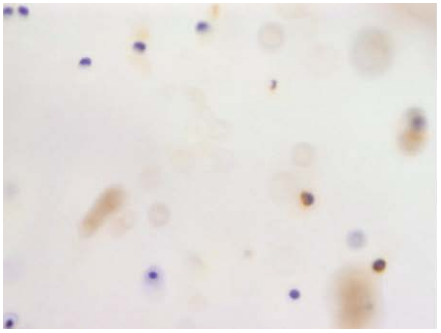


4.4.4 OA cartilage had significantly higher levels of p16 expression compared to normal cartilage samples.

To investigate the hypothesis that OA chondrocytes have higher levels of cellular senescence than normal chondrocytes, levels of p16 were measured in OA and normal articular cartilage samples as a marker of cellular senescence levels. Nineteen OA and six normal cartilage samples were analysed using immunohistochemical staining of fixed cartilage samples that had been stained with p16 antibody. P16 positive staining was recorded as a brown cytoplasmic stain in chondrocytes, visualised by light microscopy. For each cartilage sample two slides of fixed cartilage were examined, with analysis of three high power fields per slide. The percentage of positive staining slides was calculated for each high power field and the results were averaged to obtain a final value for p16 positivity for each cartilage sample. Figure 4-12 shows positive p16 staining observed in OA cartilage samples OA20 and OA24. The percentage positive staining for p16 of the 19 OA and 6 normal articular cartilage samples is illustrated in Figure 4-13. The 25 samples analysed are detailed in Table 4-2 below. The OA cartilage samples ranged from 65 to 97.2% p16 positive staining. In normal cartilage samples the level of p16 positive staining varied from no staining in one sample, to another sample at 58% p16 positivity and a further sample at 65% p16 positivity. The latter two samples were obtained from 11 year old dogs suggesting levels of p16 increase with advancing age in articular cartilage, though further study is required to investigate this relationship. In the OA cartilage samples the average p16 positivity was 78.52%, compared to an average p16 positivity of 31.5% in the normal chondrocyte samples. Thus OA cells showed higher levels of p16 expression than normal cells ($p<0.01$).

Figure 4-12. P16 expression was detected by immunohistochemistry.
Staining for p16 expression was carried out on 25 articular cartilage samples collected from OA and normal canine femoral heads. Following tissue fixation and immunohistochemical staining with p16 antibody, samples were examined for p16 levels by light microscopy. P16 staining was visible as brown cytoplasmic staining. The proportion of positive cells per high power field was calculated for each cartilage sample. This was repeated for 3 separate high power fields per slide, and each cartilage sample was run in duplicate slides. For each cartilage sample, the resulting percentages of positively staining cells per high powered field for each replicate were averaged to give a final positive p16 stain percentage. Images A. and B. below show examples of cytoplasmic brown staining, indicative of p16 positivity.
A. OA20 (400X magnification); B. OA24 (X200 magnification).

A.



B.

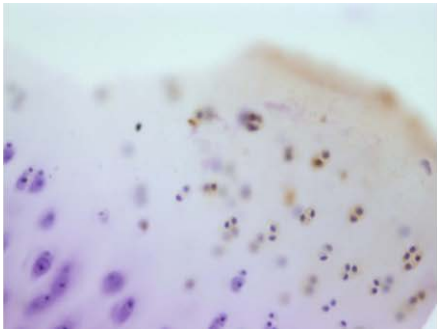


Figure 4-13. Percentage positivity for p16 staining in canine chondrocytes. The percentage positive staining for p16 for each of the 25 cartilage samples is shown below. 19 OA (blue bars below) and six normal (red bars below) cartilage samples were examined. The average p16 positivity was 31.5% in the normal chondrocyte samples and 78.52% in the OA chondrocyte samples.

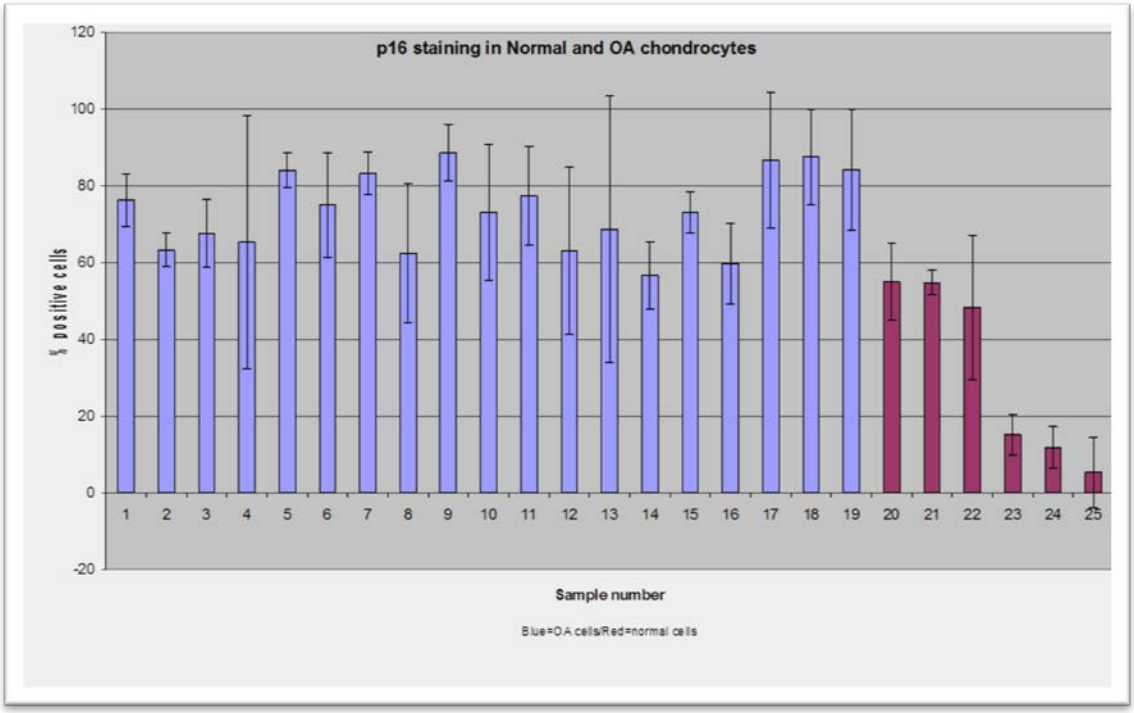


Table 4-2. Canine cartilage samples stained for p16 expression.
Sample details along with average percentage of p16 positively staining chondrocytes are detailed in the table below. OA cartilage samples ranged from 65 to 97.2% p16 positive staining. In normal cartilage samples the level of p16 positive staining varied from 0% staining to 65% positive staining. In OA cartilage samples the average p16 positivity was 78.52% compared to an average of 31.5% in the normal chondrocyte samples.
GSD= German Shepherd Dog, GRT=Golden Retriever, ST= Staffordshire Bull Terrier.

Sample no./cell line	Age/months	Breed	Average % of positive cells	staining cells/total cell number
1/OA45	7	Labrador	81.5	29/38
2/ OA46	30	Labrador	65	38/60
3/OA47	90	Labrador	76.4	23/34
4/OA20	18	Labrador	69.2	22/26
5/OA21	72	GSD	79.5	37/44
6/OA22	84	GSD	90.6	24/32
7/OA23	12	Crossbreed	77.7	15/18
8/OA24	36	Husky	83.3	15/24
9/OA25	60	Labrador	80.6	55/62
10/OA39	9	GRT	66	26/30
11/OA40	14	Labrador	90.3	24/31
12/OA10	8	Border Collie	82.1	23/28
13/OA43	36	GSD	93.7	33/48
14/OA44	54	Border Collie	66.6	17/30
15/OA27	60	Border Collie	79.4	25/34
16/OA28	92	Labrador	68.9	26/29
17/OA37	174	Labrador	93.3	26/30
18/OA38	84	Newfoundland	87.5	28/32
19/OA8	139	GSD	97.2	35/36
20/N7	36	Border Collie	65	13/20
21/N4	132	Crossbreed	58	17/31
22/N5	132	SBT	37.5	11/24
23/N1	0	Rottweiler	12.1	5/33
24/N3	0	Crossbreed	10.7	3/28
25/N2	2	Shih tzu	4.6	1/30

4.5 Discussion

It has long been established that both human and canine mitotic cells undergo senescence as a consequence of ageing and during a number of disease processes. Researchers have been investigating levels of cellular senescence in human OA cartilage since shortened telomeres were first identified as a feature of OA chondrocytes (Martin and Buckwalter, 2001). The overarching aim of this piece of research was to investigate levels of senescence in canine OA articular cartilage compared to normal articular cartilage in order to establish the role of cellular senescence in the pathogenesis of osteoarthritis in dogs.

4.5.1 Canine chondrocytes grew more rapidly in monolayer cell culture than in alginate bead culture.

When human articular chondrocytes are cultured in monolayer they develop a fibroblastic morphology over time, with alteration to collagen and proteoglycan synthesis and development of an unstable phenotype (Benya and Schaffer, 1982; Hauselmann *et al*., 1994). Suspension cultures facilitate expression of a normal chondrocyte phenotype, maintenance of normal cell morphology, synthesis of normal cartilage matrix molecules (Hauselmann *et al*., 1996; Kolettas *et al*., 1995) and collagen type II and aggregan synthesis (Rai *et al*., 2009). Initially suspensions over agarose were used as an alternative culture system to monolayer chondrocyte culture (Benya and Shaffer, 1982). Agarose gel culture maintained chondrocyte cell morphology and matrix synthesis. In 1989, Guo developed the alginate bead culture system (Guo *et al*., 1989) and it was subsequently demonstrated that human chondrocytes cultured in alginate form a matrix similar to both native bovine and human articular cartilage (Hauselmann *et al*., 1994; Hauselmann *et al*., 1996). The alginate bead system was found to be superior to other 3D cell culture constructs, because the alginate beads are readily dissolved with the use of chelating agents allowing rapid recovery and analysis for quantitation of chondrocytes in this culture system (Guo, 1989). For these reasons, attempts were made to culture chondrocytes to senescence using the alginate bead culture system. In alginate beads the canine chondrocytes showed almost no growth, as compared to monolayer culture where they grew rapidly. This may be because the 3D structure of alginate culture mimics the conditions for growth within the joint, and *in vivo* articular chondrocytes show very little proliferation under normal conditions. As such, despite being a potentially superior culture system as compared to monolayer culture, it was not possible to culture chondrocytes to senescence using alginate beads. Previous studies using human chondroctyes have reported

a threefold increase in cell number per bead over 51 days in culture (Hauselmann *et al*., 1996) using 14- to 66-year old human donors. Guo reported linear growth of foetal chick chondrocytes in alginate beads cultured over 30 days, with just 20% the growth rate observed in monolayer culture (Guo *et al.*, 1989). Both of these reported growth rates far exceed those obtained for canine chondrocytes over 28 days in this study. While linear growth did occur, there was less than one population doubling for all cell lines (using both normal and OA chondrocytes) cultured over a 28-day period.

4.5.2. Comparison of the *in vitro* lifespan of OA and normal canine chondrocytes showed that OA chondrocytes were more senescent than normal chondrocytes.

Assessment of the replicative lifespan of chondrocytes demonstrated that OA chondrocytes entered the senescent state after 1.5 and 4.9 population doublings, while normal chondrocytes underwent 4.8 to 14.6 population doublings before entering the senescent state. Normal human chondrocytes undergo between 24 and 40 population doublings before entering senescence (Evans and Georgescu, 1983; Kolettas *et al*., 1995) and normal rabbit chondrocytes undergo between 24 and 40 population doublings before becoming senescent (Adolphe *et al*., 1983). Human OA chondrocytes undergo senescence between three and 20 population doublings (Piera-Velazquez *et al*., 2002). The results of the present study also showed that OA chondrocytes from young dogs underwent senescence after fewer population doublings than normal chondrocytes from young dogs. Similarly OA chondrocytes from aged dogs underwent senescence after less population doublings than normal chondrocytes from aged dogs. However the numbers of cell lines cultured for this experiment was very small, and the experiment should be repeated with a larger sample size to establish if these results are repeatable. Senescent chondrocytes changed morphology, which has been associated with the onset of cellular senescence (Benya and Shaffer, 1982; Watt and Dudhia, 1988).

4.5.3 Comparison of SA-beta-gal activity between OA and normal canine chondrocytes showed higher levels of SA-beta-gal accumulation in OA chondrocytes than in normal chondrocytes.

The presence of SA-beta-gal was used to assess the level of cellular senescence in OA and normal chondrocytes at first passage in monolayer culture. SA-beta-gal staining was further utilised to confirm the senescent state of chondrocytes that were cultured to

replicative quiescence in experiments comparing the replicative potential of OA and normal chondrocytes in monolayer culture. OA cells showed statistically significant higher levels of SA-beta-gal staining than normal cells at all four time points in the study described here ($p<0.001$). This showed that canine OA chondrocytes had increased levels of senescence compared to normal chondrocytes. For the eight cell lines cultured, between 95% and 100% of the cells stained positive for SA-beta-gal once replicative senescence was reached in monolayer culture. In this study, OA canine chondrocytes stained at first passage in monolayer culture showed a range of 8% to 52% positive staining for SA-beta-gal. Similarly, human OA chondrocytes stained at first passage in monolayer culture showed a range of 23% to 52% positive staining for SA-beta-gal (Price *et al*., 2002). The levels of SA-beta-gal staining in this study correlate closely with levels obtained in human studies (Martin and Buckwalter, 2001; Price *et al.,* 2002). It has previously been shown that SA-beta-gal staining chondrocytes accumulate with age in humans at a rate of 4% per decade (Martin and Buckwalter, 2001). This suggests that senescent chondrocytes accumulate in articular cartilage with age coinciding with a dramatic increase in the incidence of OA in humans with every passing decade (Zhang and Jordan, 2010).

4.5.4 Comparison of the expression levels of p38MAPK between OA and normal chondrocytes showed no difference between OA and normal chondrocytes.

Oncogenic RAS has been shown to induce premature senescence in normal primary cell culture (Serrano *et al*., 1997) by activation of the p38 MAPK pathway in response to pro-inflammatory cytokines and environmental stress (Nebreda and Porass, 2000). Investigation of normal and OA articular canine chondrocytes showed that both normal and OA cells contained the P38 MAPK protein at the time of initial monolayer culture. Neither normal nor OA cells contained the activated phosphorylated from of the protein (pP38) at initial culture. Once the cells had ceased replication in monolayer culture, both normal and OA chondrocyte cell lines were found to contain the activated form of P38 known as phosphorylated P38 (pP38). These data are consistent with previous studies using rabbit articular chondrocytes where pre-senescent normal chondrocytes were positive for P38 and negative for pP38 by Western blot analysis, however following monolayer culture to senescence, the chondrocytes were found to be positive for pP38 (Kang *et al*., 2005). As such, this study suggests that the presence of pP38 can be used to identify the senescent state in canine chondrocytes and also that activation of P38 is not a marker for the presence of osteoarthritic chondrocytes *in vivo*. P38 inhibition has been identified as a

potential therapeutic target for the treatment of inflammatory conditions in humans (Goldstein and Gabriel, 2005). This treatment could potentially represent an option for canine osteoarthritis if successfully developed for humans. An orally administered p38 MAPK inhibitor, Pamapimod, has undergone clinical trials for use in humans (Hill *et al.*, 2008) and is now in phase two clinical studies. Further studies examining the precise role of the p38 MAPK pathway in the role of OA pathogenesis are required to understand potential therapeutic benefits of P38 inhibition.

4.5.5 P16 staining was increased in OA chondrocytes compared to normal chondrocytes.

To investigate the hypothesis that OA chondrocytes have higher levels of cellular senescence than normal chondrocytes, levels of p16 were measured in OA and normal articular cartilage samples as a marker of cellular senescence levels. Nineteen OA and six normal cartilage samples were analysed using immunohistochemical staining of fixed cartilage samples that had been stained with p16 antibody. Avoiding expansion of the cartilage cells in culture and staining chondrocytes in fixed tissue samples facilitated evaluation of cellular senescence *in vivo.* The results showed that there was a higher percentage of p16 positive cells in OA cartilage samples than in normal cartilage samples ($p=0.01$) indicating that OA chondrocytes show increased levels of cellular senescence compared to normal chondrocytes. In OA cartilage samples there was a mean of 78.5% p16 positively stained chondrocytes compared with a mean of 31.5% positively stained chondrocytes in the normal chondrocyte samples. It is important to note that there was a relatively small number of normal samples ($n=6$) compared to OA samples ($n=19$). The OA and normal chondrocyte samples were not age-matched, due to a difficulty in obtaining normal articular cartilage samples. In this study, the normal chondrocyte samples came from a younger population of animals than the OA samples. It is interesting to note that p16 staining was present in all of the cartilage samples. This suggests that senescence is not an unusual feature of articular cartilage in general. This study should be repeated with a larger number of normal chondrocyte samples, age-matched to the OA samples (to give an even number of normal and OA samples) so that the results do not report p16 positive staining caused by age rather then OA. In a study of age-matched normal and OA human chondrocyte samples, Zhou demonstrated that p16 is more highly expressed in OA chondrocytes compared to normal chondrocytes (Zhou *et al.*, 2004). In the study by Zhou, it was also demonstrated that silencing p16 decreases the response of OA chondrocytes to catabolic cytokines and increases the response to anabolic growth factor, with an increase

in DNA synthesis and cellular growth rate, essentially altering the response of chondrocytes to pathogenic pathways essential to the development of OA. As such if it were possible to block p16 expression *in vivo* the rate of OA progression in OA joints could potentially be slowed in both humans and dogs.

There is a lack of age-matched samples for all of the experiments outlined here, due to the lack of availability of normal canine cartilage samples. A lack of age-matched samples could potentially falsely report cellular senescence as OA related, when in fact the cellular senescence could be age-related. The lack of age-matched controls was addressed in the current study by using SA-beta-gal activity as a marker of cellular senescence. SA-beta-gal activity has been shown to correlate with levels of cellular senescence but not with the chronological age of the donor (Dimri *et al.*, 1995). As such, levels of chondrocyte senescence measured using SA-beta-gal activity are representative of replicative senescence independent of donor age. Currently the reason for this distinction is unknown, as the precise function of SA-beta-gal within the cell remains to be elucidated.

4.5.6 Conclusions

This study is the first to report that senescence is a feature of canine OA cartilage. These data closely correlate with the findings in human studies. There is strong evidence, based on human data, that cellular senescence of OA chondrocytes causes pathological alteration to normal chondrocyte functions. The data in the present study support a potential molecular pathway, p16, that could be developed as a therapeutic target to delay the onset of the senescent state in chondrocytes. The biological role of cellular senescence in canine OA warrants further investigation.

4.5.7. Future Studies

In order to verify these data, several experiments should be repeated using larger sample numbers, as highlighted in the discussion above. In particular, the lack of age-matched samples should be addressed in any future studies. Cellular senescence occurs as sequellae to both ageing and disease (such as atherosclerosis, hypertensive cardiac disease, pulmonary fibrosis and OA). Accordingly, it is important to distinguish between cellular senescence of chondrocytes as a result of ageing and cellular senescence of chondrocytes as a result of osteoarthritis. This could be achieved by collecting chondrocytes from an OA

joint and chondrocytes from a normal joint within the same donor animal. In order to achieve collection of such samples, patients with OA would need to be either euthanatised or subjected to biopsies of normal articular cartilage in healthy joints, both of which raise ethical issues. Alternative methods of removing the confounding issue of age would be to use age-matched samples from different donor animals, or to assess leukocyte telomere lengths in the donor animals as an internal control of age-related cellular senescence. For the present study, the cartilage samples were not accompanied by blood samples and as such leukocyte telomere lengths could not be used as an internal control for age-related levels of cellular senescence. Telomere lengths of chondrocytes have been shown to decrease as a result of OA (Aigner *et al*., 2001; Martin *et al.,* 2001) and as such telomere length could not be used as an internal control for age of donor sample. Future studies could involve the collection of a blood sample at the time of cartilage sample collection to facilitate the measurement of leukocyte telomere lengths, measured by QPCR (Cawthon, 2009).

As there is no one marker for cellular senescence, multiple markers are used to confirm the senescent cell state. These data could be further verified by use of additional markers of senescence to further prove increased levels of senescence in OA chondrocytes compared to normal chondrocytes. Such studies could compare telomere lengths in OA versus normal chondrocytes as an indicator of senescence as outlined above. Further studies could also investigate the accumulation of p21 (a cyclin-dependant kinase inhibitor), or senescence associated heterochromatic foci (SAHF) as additional markers of cellular senescence by western blot analysis and by immunohistochemical staining.

Extended studies would be beneficial to confirm senescence as a feature of canine OA chondrocytes and the role of senescence in the pathogenesis of canine OA. This could be achieved by *in vitro* modification of senescence in OA chondrocytes to investigate if reversing senescence results in recovery of crucial biological functions known to be relevant to the pathogenesis of OA. The present study showed that OA chondrocytes have increased levels of p16 compared to normal chondrocytes *in vivo.* Treatment of the OA chondrocytes with p16-*si*-RNA could reverse senescence in these cells, in accordance with previous studies (Bond *et al.,* 2004). Reversal of senescence could be confirmed by subsequent assessment of cellular senescence in p16-*si*-RNA-treated OA chondrocytes using the markers described in our experiments (growth curves to assess replicative capacity, SA-beta-gal activity and p16 positivity). The principal function of chondrocytes is secretion of the cartilage matrix and this function is lost in OA chondrocytes (Clark *et*

al., 1993; Ishiguro *et al.*, 1999). Following treatment of OA chondrocytes with p16-*si*-RNA, mRNA expression levels of key cartilage component molecules, type II collagen and aggrecan, could be measured by RT-PCR. Type II collagen and aggrecan mRNA levels could then be compared in the p16-*si*-RNA-treated OA chondrocytes and the untreated OA chondrocytes. This would demonstrate if reversing senescence in OA chondrocytes results in recovery of the key biological process of extracellular matrix production, thus establishing a key role of cellular senescence in the pathogenesis of canine OA.

Chapter V

Investigation of the propagation of OA within the joint using a cell coculture model

5.1 Introduction

OA is the most prevalent disease in dogs and is a major cause of pain and deterioration of quality of life (Bennett and May 1995; Moore *et al.*, 2001). To investigate the mechanisms by which OA is propagated within a joint, gene expression profiling was used to examine the effect of secreted factors from OA cells on adjacent normal chondrocytes. Currently little is known about the role of OA chondrocytes in the progression of disease to adjacent normal cells (Sokolove and Lepus, 2013). The experiments described in this chapter demonstrate that small populations of OA chondrocytes can alter the gene expression of neighbouring chondrocytes and also that dysregulation of genes associated with OA pathogenesis occurs when normal chondrocytes are exposed to secreted factors from OA chondrocytes. The principal aim of the work presented in this Chapter was to study the molecular mechanisms of OA disease propagation as may occur in synovial joints. One goal of OA research is to identify treatment targets and therefore investigating whether injury to individual chondrocytes can impact upon gene expression of adjacent normal chondrocytes is an important study.

5.1.1 Pathogenesis of OA at a cellular level within the joint

Whilst many factors are associated with a susceptibility to OA resulting in initiation of the pathological process, it is generally accepted that mechanical loading of the articular surfaces within a joint play a primary role (Loeser *et al.*, 2002; Yudoh *et al.*, 2005).

Articular cartilage is composed of the extracellular matrix and chondrocytes (as described in Chapter I). The chondrocyte is responsible for production and maintenance of articular cartilage and thus plays a pivotal role in the maintenance of its tensile strength and stiffness. Biomechanical stress results in chondrocyte injury and initiation of OA (Goldring, 2000) as injured chondrocytes secrete inflammatory cytokines, inflammatory mediators and proteases (Bau *et al.*, 2002; Dessau *et al.*, 1981; Duerr *et al.*, 2004; Glasson *et al.*, 2005; Hughes *et al.*, 1998; James *et al.*, 2005; Malfait *et al.*, 2002). When cartilage is subjected to abnormal mechanical stress cartilage breakdown products are released into the synovial fluid. These products stimulate the synovial fibroblasts and macrophages to secrete several pro-inflammatory cytokines, which include IL-1, IL-6 and TNF-alpha. Following an initial phase of cellular proliferation and Type II collagen and aggrecan synthesis, injured chondrocytes secrete aggrecanases, MMPs, TIMPs, plasminogen

activator and NOS, which degrade the ECM and erode the structure of the articular cartilage (Clark *et al.*, 1993; Hembry *et al.*, 1995; Ishiguro *et al.*, 1999; Okada *et al.*, 1992).

Articular cartilage is an avascular tissue and as such chondrocytes receive nutrition from synovial fluid, an ultra-filtrate of blood which is formed within the synovial vasculature. Aside from nutrients and oxygen, synovial fluid also contains other important molecules such as hyaluronate, and it also acts as a transporter for chemokines and cytokines. Molecules of up to 6.8nm can diffuse through the cartilage matrix to reach chondrocytes (Moskowitz *et al.,* 1992) and it has been demonstrated that cytokines reach chondrocytes via the synovial fluid (Tsuchida *et al.*, 2014). It has also been demonstrated that ROS production can be induced *in vitro* by exposing chondrocytes to IL-1 and TNF-alpha and furthermore, that ROS act as second messengers which can alter chondrocyte gene expression (Lo *et al.,* 1996).

5.1.2 Genes associated with the pathogenesis of OA

OA is a highly dynamic disease. Cytokines and chemokines play a key role in the pathogenesis of OA and much recent research into OA in both humans and animals has focused on gene expression in this disease (Aigner *et al.,* 2006; de Lange Brokaar *et al.*, 2012; Martin *et al.*, 2001,). There are two main sources of inflammatory mediators; synovial cells and chondrocytes. Gene expression changes involved in the early pathogenesis of OA include up-regulation of collagen genes resulting in additional extracellular matrix collagen deposition during the initial proliferative phase (Aigner *et al.*, 1992; Aigner *et al.,* 1993; Aigner *et al.,* 2006). During the subsequent catabolic phase cartilage degradation is the central event and IL-1, TNF-alpha, metalloproteinases (MMPs), aggrecanases, plasminogen activators, cathepsins and elastase genes are up-regulated (Aigner *et al.*, 2006; de Lange Brokaar *et al.*, 2012).

5.1.3 The coculture model

The coculture model is a very useful cell culture system as it facilitates the study of cell-to-cell interactions. There are various types of coculture models (Acharya *et al.*, 2010; Domm *et al.,* 2002; Malfait *et al.*, 1994). In some studies direct cell-to-cell contact interaction is desirable. However, articular cartilage is a relatively acellular tissue with little or no direct contact between chondrocytes and the primary mechanism of interaction between cells is via soluble diffusible factors. As such, a non-contact coculture system was utilised for this

study. Two chamber dishes with a porous membrane between each chamber allow diffusion of soluble factors through a membrane with a pore size of 8nm (molecules of up to 6.8nm can diffuse through the cartilage matrix to reach chondrocytes), (Data sheet, ThinCert™ Cell Culture Inserts for Multiwell Plates, Greiner Bio One). This prevents direct contact between the two cartilage cell populations but facilitates diffusion of cytokines and chemokines. This model represents a very useful tool for studying interactions between chondrocyte populations mediated by secreted soluble factors. Furthermore, it distinguishes between these interactions and those that occur as result of direct contact between inflammatory cells and chondrocytes. Coculture models have previously been used to demonstrate cytokine-mediated interactions between inflammatory cells and chondrocytes (Malfait *et al.*, 1994) and to examine the impact of senescent cells on the development of oncogenic mutations in epithelial cells (Krtolica *et al.*, 2001).

5.1.4 DNA microarray technology

DNA microarray technology was first developed in the 1990s as a result of DNA sequencing (Schena *et al.*, 1995). DNA microarrays are made up of thousands of DNA probes spotted onto a solid surface in a high-density array consisting of rows and columns. This configuration facilitates parallel measurement of labeled target nucleic acids. There are many different microarray platforms available (Hardiman, 2004). Affymetrix GeneChip oligonucleotide arrays have been widely used from the early days of microarray research (Hardiman, 2004; Nau *et al.*, 2000).

More recently ultra-high-throughput DNA sequencing technologies (UHTDSTs) have been developed (Fox *et al.*, 2009; Primig, 2012). This method obtains a short sequence to assign the site of origin in the genome for each read rather than trying to determine the entire sequence of each read. A short sequence read is determined for millions of nucleic acid molecules from a sample. This shorter sequence of 25–35 base pairs enables identification of the location of each fragment in the reference genome using informatics. Once the sequences are mapped their distribution is mapped throughout the target genome. The end product is hundreds of thousands of individual reads, each of which corresponds to a different molecule in the sample of origin. This overcomes some of the challenges of hybridisation microarray, including the requirement to synthesize millions of DNA probes, and avoids cross-hybridization. Another recent advance in microarray analysis is the use of RNA sequencing technologies (also referred to as RNA-seq). Using this technique, RNA is converted cDNA fragments which then undergo ultra high- throughput sequencing. This

obtains short-sequence reads of 30-400 base pairs, which are then correlated to the reference genome using informatics. While DNA microarray is associated with a small dynamic range due to poor sensitivity in detecting genes with very low or very high levels of expression, RNA-seq is associated with a high dynamic range with no upper limit for quantification. Additionally, smaller quantities of RNA are required for this analysis as there is no requirement for cloning or amplification of samples (Wang *et al.* 2009).

Despite the evolution of microarray technology, hybridization-based microarray approaches still offer a number of advantages in gene profiling studies (Fox *et al.*, 2009). They are widely available and at a significantly lower cost than the newer sequencing technologies. In addition, microarray technology is more accessible in terms of bioinformatics and hardware availability and cost. The initial sample preparation techniques are the same for hybridization and sequencing-based microarrays.

5.1.5 Affymetrix Canine GeneChip 2.0 Microarray

The GeneChip Canine Genome 2.0 Microarray enables interrogation of 18,000 canine mRNA transcripts and over 20,000 non-redundant predicted genes (datasheet, GeneChip Canine Genome 2.0 Microarray, Affymetrix). It consists of high-density oligonucleotide probe sets imprinted upon a glass slide. In a microarray experiment the first step is isolation of RNA from the samples to be analysed. The RNA is then reverse transcribed to cDNA, which is more stable than RNA. Biotin labeled cRNA is then produced followed by *in vitro* hybridization to the array surface. Finally, staining with phycoerythrin fluorophore enables detection. The samples undergo laser excitation and the generated fluorescence signal images are measured and recorded. Software analysis of obtained images measures the abundance of each transcript on the array. Each detection is evaluated for statistical significance, indicated by a p-value. Control hybridisations run simultaneously allowing assessment of experiment validity, by measuring sample integrity and assay efficiency. Software analysis also removes contributions from stray hybridisation signals in the data analysis.

5.1.6 Applications of DNA microarray technology in the study of disease

A principal use of DNA microarray technology has been for the analysis of mRNA expression from thousands of genes in one experiment. Advances in microarray gene

expression analysis have facilitated detailed studies of disease pathogenesis at a molecular level. Using this technology the gene expression patterns of a wide range of diseases can be studied. Altered gene expression profiles have been identified in colon cancer (Notterman *et al.*, 2001) pulmonary tumors (Beer *et al.*, 2002), leukaemia (Golub *et al.*, 1999) and osteosarcoma (Pang *et al.*, 2014). This technology is also being used in pharmacology to facilitate the development of therapeutics (Brachat, 2002). In addition to the study of gene expression profiling, DNA microarray can also be used to measure DNA methylation (Yan *et al.*, 2001), alternative RNA splicing (Relogio *et. al.*, 2005), detection of single nucleotide polymorphims (Cutler *et al.*, 2001) and alterations in gene copy number (Pollack *et al.*, 1999).

5.1.7 Applications of DNA microarray technology in the study of OA

DNA microarray studies have lead to profound advances in our understanding of OA disease mechanisms. In a comparison of human OA cartilage versus normal cartilage using DNA microarray, Aigner and others (Aigner *et al.*, 2001) showed an increased gene expression of type II collagen, type IV collagen, fibronectin, MMP-2, MMP-3 and MMP-13. A study on altered gene expression in canine articular cartilage following mechanical injury (Burton-Wurster *et al.*, 2005) showed increased expression of 16 genes significant in the pathogenesis of OA, including TNF-alpha, MMP-3, MMP-13, TIMP-1 and TIMP-2. An analysis of gene expression in early experimental OA (Appleton *et al.*, 2007) in dogs showed increased expression levels of proteases including ADAMTS-5, MMP-2, MMP-13, Prostaglandin E synthase and TGF-β2. In addition the study reported herein demonstrated altered production of collagens, for example COL-1A1, which is not expressed at high levels in non-OA cartilage.

Inflammatory mediators secreted by chondrocytes are of particular interest, not just in furthering our understanding of the pathogenesis of OA, but because they present a possible therapeutic target for the treatment of OA. Examples include the use of cytokine receptor agonists or direct cytokine antagonists and proteinase inhibitors that could halt the inflammatory cascade and disrupt disease progression (Harris and Mant, 2013; Kim *et al.*, 2013; Saava and Rodger, 2013). The general dysregulation of chondrocyte genes in OA has been examined by various research groups (Aigner *et al.*, 2006; de Lange Brokaar *et al.*, 2012; Martin *et al.*, 2001). In this chapter we investigate the effect of OA chondrocytes

on adjacent normal chondrocytes in order to provide insight into the molecular mechanisms involved in disease progression at a cellular level using Affymetrix gene expression analysis.

5.2 Aims and objectives

The aim of this study was to investigate the hypothesis that the presence of osteoarthritic chondrocytes within a joint influences the gene expression of adjacent normal chondrocytes, leading to up-regulation of OA genes in these chondrocytes.

More specifically the aims and objectives were to;

(i) evaluate the biological effects of secreted factors from OA chondrocytes on normal canine chondrocytes using a coculture system and canine gene microarray analysis.

(ii) identify genes whose expression is associated with the pathogenesis of OA.

(iii) verify gene modulation by expression analysis using RT-PCR and Western blotting for relevant proteins.

5.3 Materials and methods

5.3.1 Study design

In order to evaluate the biological effects of secreted soluble factors from canine OA articular chondrocytes on adjacent normal chondrocytes, canine gene microarray analysis was used. OA chondrocytes were cultured in the presence of early passage foetal chondrocytes (normal chondrocytes) using a coculture system as described in Chapter II, General Materials and Methods. Briefly, cells were cultured in two chamber culture dishes separated by a porous membrane, which prevented direct contact between the two cell populations but allowed the transfer of soluble secreted factors. Cells were cocultured for three days using standard chondrocyte culture media and conditions. Normal foetal chondrocytes (Cell line N1) were cocultured with normal foetal chondrocytes as a control sample. Normal foetal chondrocytes were then cocultured along with chondrocytes from four different osteoarthritic femoral heads from Labradors (these were the exposed foetal chondrocyte samples).

<u>The OA samples were:</u>

(a) Sample OA74: 10 month old male entire Labrador retriever

(b) Sample OA71: 1year 5 month old female entire Labrador retriever

(c) Sample OA26: 8year 3 month old male neutered Labrador retrieve

(d) Sample OA68: 6year 10 month old female neutered Labrador retriever

<u>Samples for microarray analysis were:</u>

(a) Sample 1- foetal chondrocytes cocultured with foetal chondrocytes (internal control sample)

(b) Sample 2- foetal chondrocytes cocultured with OA74 chondrocytes

(c) Sample 3- foetal chondrocytes cocultured with OA71 chondrocytes

(d) Sample 4- foetal chondrocytes cocultured with OA26 chondrocytes

(e) Sample 5- foetal chondrocytes cocultured with OA68 chondrocytes

After three days, total RNA was isolated from the foetal chondrocytes grown in coculture with osteoarthritic cells and from the normal control cells. Isolated RNA was subjected to canine microarray analyses to examine variation in gene expression of the cocultured cells, using the Affymetrix Canine Genechip 2.0. Changes in the level of gene transcription were measured between normal chondrocytes cocultured with normal chondrocytes (the control

cells) and normal chondrocytes cocultured with osteoarthritic chondrocytes for each of the four OA samples. Cut-off values for significant altered gene expression were defined as a level of differential expression of p </= 0.05 and fold change greater than 2.

5.3.2 Cell cultures

Four different cell lines of OA chondrocytes were cocultured in the presence of early passage chondrocytes from a stillborn neonate (a control population of normal chondrocytes) in ThinCert tissue culture inserts and Multiwell Plates (Greiner bio-one, U.K.). This two-chamber culture system allowed coculture of two cell lines with separation by a porous membrane. This prevented contact between different cell types but allowed the transfer of soluble secreted factors. The tissue culture inserts had a pore size of 8.0 µm and a pore density of 0.15 x 106 /cm ². OA cells were cultured in the upper suspended inserts, with normal chondrocytes cultured in the six well plate. A control sample was generated by coculture of normal chondrocytes with normal chondrocytes. Cells were cocultured for 3 days using standard chondrocyte culture media and conditions (Chapter II, General Materials and Methods).

5.3.3 RNA isolation, microarray analysis and statistical analysis

RNA was isolated from the foetal cocultured chondrocytes using RNeasy Mini kit (Qiagen, Crawley, United Kingdom) in accordance with the manufacturer's instructions as described in Chapter II, Materials and methods. Double-stranded cDNA was synthesized from RNA using SuperScript III first-strand synthesis kit (Invitrogen, United Kingdom). Double-stranded cDNA was transcribed overnight using biotinylated nucleotides to produce biotinylated cRNA. The cRNA was then purified using a spin column and quantified using spectrophotometry. RNA quality was determined by Experion RNA StdSens analysis kit using Experion automated electrophoresis (Bio-Rad Laboratories, Hercules, CA). The cRNA was fragmented using a buffer solution (GeneChip *in vitro* transcription labeling kit) and fragmentation of cDNA was confirmed using gel electrophoresis. The Affymetrix Canine 2.0 GeneChip was loaded with the fragmented and labeled cRNA. Hybridization of the chip took place overnight at 45^0C and 60rpm. The array was then washed and stained on a Fluidics Station 400 apparatus (Affymetrix) using the appropriate fluidics scripts (Protocol EukGE-vs4.v2). The array was scanned using an Affymetrix GeneChip Scanner 3000 and the data was collected and processed using

GeneChip operating software (Affymetrix, GCOS v1.2.1). Genes were annotated using the Gene Ontology database (Ashburner, *et al.*, 2000).

Statistical analysis of microarray data was carried out using the rank product technique where for each gene *g* in *k* replicates *i*, each examining *n i* genes, one can calculate the corresponding combined probability as a rank product RP *g* up=$\prod$ *i*=1 *k* (*r i,g* up/*n i*), where *r i,g* up is the position of gene *g* in the list of genes in the *i*th replicate sorted by decreasing FC, i.e. *r* up=1 for the most strongly upregulated gene. Analogously, RPdown *g* is calculated from the list of genes sorted by increasing FC, i.e. *r* down=1 for the most strongly downregulated gene (Breitling *et al.*, 2004). Iterative Group Analysis (iGA) identified modulation of functional gene classes using iterative calculation of p-values (Breitling *et al.*, 2004).

5.3.4 RT-PCR Analysis of Gene Expression

The gene expression levels of nine genes selected for microarray validation were quantified using RT-PCR as described in Chapter II. Primer and probe sets were designed using Primer Express software (Applied Biosystems, United Kingdom). Primer and probe sequences are presented in Chapter Two, General Materials and Methods. Briefly, total RNA from cells was isolated using a RNeasy Mini kit (Qiagen, Crawley, United Kingdom). First-strand cDNA was synthesised using SuperScript III first-strand synthesis kit (Invitrogen, Paisley, United Kingdom). Quantitative gene expression analysis was performed with 100 ng of cDNA. PCR reactions consisted of 0.2 μM forward and reverse primers, 0.1 μM probe, 25 μl of 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen, UK), 1 μl ROX Reference Dye and water to a final volume of 50 μl. Following a 2 min activation at 50°C of Platinum Taq DNA polymerase and a 5 min denaturing step at 95°C, 45 cycles of 15 s at 95°C and 45 s at 60°C were run. Fluorescence measurements were made for every cycle at 60°C. Each OA coculture cell sample was analysed in triplicate. Mean fold changes in gene expression were calculated using 7500 Real Time PCR System software, relative to the expression of the coculture control cells using the 2-ΔΔCT method.

5.3.5 Western Blot

In order to determine if alteration in cellular mRNA in the exposed OA chondrocytes was

consistent with altered protein expression, western blot analysis of two proteins was performed. The western blotting protocol used in these experiments is outlined in Chapter II, General Materials and Methods. Two proteins significant to OA pathogenesis were chosen; TNF-alpha and MMP-13. Specific antibodies to canine proteins were not readily available, and therefore cross-reacting human antibodies were utilised in these experiments. Tubulin, which has a molecular weight of 55kDa, was used as a loading control. TNF-alpha has a molecular weight of 17kDa in its soluble form and 20kDa in its insoluble form. MMP-13 has a molecular weight of 54kDa and therefore the membrane had to be stripped and re-probed between detection of tubulin and MMP-13 proteins. This was not necessary when probing for tubulin and TNF-alpha because there is a significant difference in the molecular weights of these two proteins such that the membrane can be split and incubated with two different antibodies.

5.5 Results

5.5.1. Coculture of normal chondrocytes with OA chondrocytes lead to modulation of genes central to the pathogenesis of OA.

In order to explore the impact of OA chondrocytes on the pathogenesis of OA, normal chondrocytes were cocultured with OA chondrocytes. Microarray analysis was utilised to identify whether OA chondrocytes influenced the gene expression of adjacent normal chondrocytes. Gene expression profiling of normal canine chondrocytes cocultured with OA canine chondrocytes was performed using an Affymetrix GeneChip Canine 2.0 microarray. A total of 594 genes were found to be significantly modulated in the cocultured chondrocytes ($p=0.05$); 568 genes were up-regulated and 26 genes were down-regulated. The top 100 up-regulated genes and the 26 down-regulated genes are listed in a table attached in Appendix 1 and Appendix 2 respectively. Table 5-2 shows the top ten up-regulated genes and Table 5-3 shows the top ten down-regulated genes. Ten modulated genes highly associated with the pathogenesis of OA in cartilage showed significant fold changes in up or down regulation as a result of coculture demonstrating that OA chondrocytes play a role in the pathogenesis of OA by modulating the gene expression of adjacent normal chondrocytes in a coculture model.

The genes known to play a role in the pathogenesis of OA along with their specific role in OA are shown in Table 5-1; TNF-alpha, IL-4, IL-6, MMP-13, MMP-3, ADAMTS-5, COL-3A1, IL-8, IL-F3 and ADAMTS-4. The role of each of these molecules in OA is outlined in detail in the discussion. Whilst all of the genes above result in pathological change to articular cartilage leading to OA, notable genes are TNF-alpha (11.95-fold increased expression), MMP-13 (5.93 -fold increased expression), MMP-3 (5.48-fold increased expression) and ADAMTS-5 (4.27-fold increased expression), which directly result in cartilage matrix degradation. These results demonstrate that coculture of normal chondrocytes with OA chondrocytes alters the gene expression of the normal chondrocytes such that they develop an OA genotype, increasing the expression of genes known to play a role in OA pathogenesis.

While the top ten up-regulated (Table 5-2) and down-regulated genes (Table 5-3) do not include genes known to play a key role in OA, they do include genes of interest for potential further study of OA pathogenesis. SON DNA binding protein, which is thought to

play a role in maintaining genome stability and hematopoietic stem cell differentiation (Ahn *et al.,* 2011; Ahn *et al.,* 2013), showed a 252-fold increase in gene expression. Similarly interferon-beta 1 and interferon-induced protein were up-regulated 121-fold and 107-fold respectively. These genes are associated with host defense functions in apoptosis and modulating immune function (Chawla-Sarkar, 2003). These data show activation of pathological pathways in the normal chondrocytes following exposure to OA chondrocytes. Another modulated gene of particular interest is secreted phosphoprotein 1, which showed a seven-fold decrease in gene expression. Secreted phosphoprotein 1 acts as a cytokine and up-regulates expression of interferon gamma and IL-12 (both proinflammatory cytokines), (Weber and Cantor, 1996; Sodek *et al.,* 2000). These genes have not been previously associated with the pathogenesis of OA.

To investigate if genes differentially expressed as a result of coculture with OA cells belonged to biological pathways significant to the pathogenesis of OA, ingenuity pathway analysis was used. The ingenuity pathway analysis confirmed up-regulation of biological functions known to play a role in the pathogenesis of canine OA (Figure 5-2A and 5-2B). These biological functions were cytokine activity, chemokine activity, chemotaxis, regulation of cell proliferation, NOS activity, superoxide metabolic processes, IL-8 receptor binding, IL-10 receptor activity, collagen metabolic process, prostaglandin metabolic process, defense response pathways and inflammatory response pathways (Figure 5-2A). Between 10% and 100% of the molecules in each of these groups were modulated (Figure 5-2 B). Of particular significance to OA pathogenesis are the superoxide metabolic process and NOS activity as oxidative stress is an early event in the destruction of articular cartilage. Additionally up-regulation of IL-8 and IL-10 receptor pathways is of particular significance. IL-8 is a pro-inflammatory cytokine shown to have a key role in the induction of the OA process (Takahashi *et al.,* 2015). IL-10 is an anti-inflammatory cytokine that inhibits MMPs thus inhibiting degradation of the cartilage matrix producing a protective effect in early OA (Wang and Lou, 2001). Up-regulation of collagen metabolic process is also highly significant as degradation of the collagen matrix is a key event in the destruction of articular cartilage that occurs with OA. These data show that inflammation, oxidative stress and collagen destruction pathways are up-regulated in normal chondrocytes that are exposed to OA chondrocytes and thus confirm that OA chondrocytes play a role in altering the behaviour of normal chondrocytes in OA. These data also demonstrate that normal chondrocytes that have been cocultured with OA chondrocytes have significant up-regulation of collagen metabolic processes compared to

non-exposed normal chondrocytes such that these normal cocultured chondrocytes may contribute to collagen degradation and articular cartilage destruction.

The individual up-regulated genes in five of the up-regulated biological pathways are shown in Figure 5.3. Some of the genes encompassed multiple biological groups. Chemokine ligands and TNF-alpha are represented in the inflammatory, defense and chemokine activity pathways. Chemokine ligands function in chemotaxis, recruiting other immune cells as part of the inflammation that occurs with OA. The importance of TNF-alpha to OA pathogenesis has been discussed above.

These data illustrate that OA chondrocytes play a role in altering the behaviour of normal chondrocytes in OA. The gene expression patterns of ADAMTS, aggrecanase, TNF, collagen and interleukin genes are shown according to fold change along with the role of these molecules in the pathogenesis of OA in Table 5.4. These gene families play key roles in OA pathogenesis by degradation of articular cartilage via destruction or modulation of the structure of the extracellular matrix. The up-regulation of multiple genes within each family shows a shift in overall gene expression of normal chondrocytes to an OA genotype as a result of coculture with OA cells.

Table 5-1. Coculture of normal chondrocytes with OA chondrocytes leads to dysregulation of ten genes critical to the pathogenesis of canine OA in the normal chondrocytes.

TNF-alpha, MMP-13 and MMP-3 play a key role in the destruction of articular cartilage and showed 11.9-fold, 5.9-fold and 5.4-fold up-regulated gene expression. Further genes well established as key to OA pathogenesis were also significantly modulated, including COL-3A1, ADAM-TS4 and ADAM-TS5. Overall there was significant up-regulation of genes playing a role in degradation of the cartilage matrix and inflammation. This shows that normal chondrocytes develop an OA genotype as a result of exposure to OA chondrocytes and suggests that OA chondrocytes propagate OA within joints by altering the behaviour of normal cells. FDR refers to the false discovery rate (FDR < 5.0 =p value of <0.05).

Gene		FDR	Fold Change	Role in OA pathogenesis
1	TNF-alpha	4.54	11.95	Cartilage degradation Antagonism of aggrecan synthesis Antagonism of type II collagen synthesis Induction of increased production of MMPs by synoviocytes
2	IL-4	3.28	7.03	Anti-inflammatory role in OA via inhibition of TNF-alpha
3	IL-6	2.76	5.30	Cartilage proliferation
4	MMP-13	2.96	5.93	Cartilage degradation via cleavage of collagen types I, II and III
5	MMP-3	2.82	5.48	Cartilage degradation via cleavage of laminin and fibronectin
6	ADAMTS-5	2.42	4.27	Cartilage destruction via aggrecan degradation
7	COL-3A1	2.37	4.12	Collagen synthesis and cartilage tensile strength and stiffness
8	IL-8	2.64	4.92	Cartilage degradation by chemotaxis of neutrophils Promotion of MMP-3 activity Cartilage hypertrophy
9	ILF-3	-2.40	-4.22	Associated with joint inflammation Required for T-cell expression of IL-2
10	ADAMTS-4	-2.25	3.78	Cartilage destruction via aggrecan degradation

Table 5-2. Rank product differential expression of top 10 up-regulated genes in normal chondrocytes following coculture with OA chondrocytes. The table shows the most upregulated genes in normal chondrocytes following coculture of normal chondrocytes with OA chondrocytes. SON DNA binding protein, which is thought to play a role in maintaining genome stability and haematopoetic cell differentiation showed a 252-fold increase in gene expression. Similarly Interferon beta 1 and interferon-induced protein were upregulated 121-fold and 107-fold respectively.

Gene Name	Gene Symbol	Accession number	Fold Change	Function
SON DNA binding protein	SON	478406	252.8	Genome stability Hematopoietic stem cell differentiation
Unidentified gene	---	---	185.65	Currently unknown
chemokine (C-C motif) ligand 8	CCL8	448792	130.15	Chemotaxis of inflammatory cells
interferon, beta 1	IFNB1	481558	121.67	Apoptosis and immune function
guanylate binding protein 1, interferon-inducible,	GBP1	490172	119.16	haemostasis
interferon-induced protein with tetratricopeptide repeats 1	IFIT1	488947	107.04	Apoptosis and immune function
myeloid cell nuclear differentiation antigen	MNDA	488622	99.25	Interaction with interferons

Table 5-2 (continued). Rank product differential expression of top 10 up-regulated genes in normal chondrocytes following coculture with OA chondrocytes.

Gene Name	Gene Symbol	Accession number	Fold Change	Function
serglycin	SRGN	609421	81.91	Inflammation and apoptosis
indoleamine 2,3-dioxygenase 1	IDO1	475574	82.07	Immunoregulation and antioxidant activity
similar to interferon gamma inducible protein 47	LOC481471	481471	92.12	

Table 5-3. Top ten down-regulated genes in normal chondrocytes cocultured with OA chondrocytes.

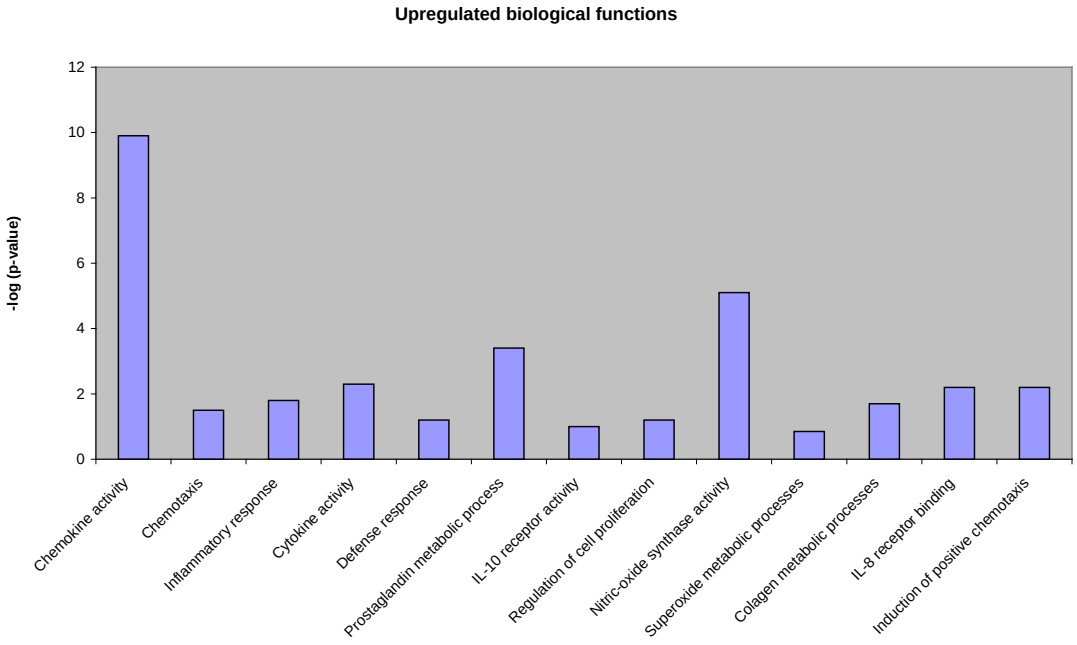
The table shows the most down-regulated genes in normal chondrocytes following coculture of normal chondrocytes with OA chondrocytes. Similar to the top ten most up-regulated genes, the down-regulated genes also do not encompass genes previously linked specifically to OA pathogenesis. One modulated gene of particular interest is secreted phosphoprotein 1, which showed seven-fold decrease in gene expression and up-regulates expression of proinflammatory cytokines and plays a role in the degradation of the extracellular matrix.

Gene Name	Gene Symbol	Accession number	Fold change	Function
Unidentified gene	---	---	-11.95	Currently unknown
secreted phosphoprotein 1	SPP1	478471	-7.03	Proinflammatory cytokine
leucine rich repeat neuronal 1	LRRN1	484682	-5.3	transmembrane protein
Unidentified gene	---	---	-5.93	Currently unknown
protocadherin 8	PCDH8	485469	-5.48	integral membrane protein that is thought to function in cell adhesion
NAD(P)H dehydrogenase, quinone 1	NQO1	610935	-4.27	cellular metabolism
similar to plasticity-related protein 3	LOC479934	479934	-4.12	cell membrance signal transduction and phospholipid metabolism
---	---	---	-4.92	currently unknown
RAB3A interacting protein (rabin3)	RAB3IP	481159	-4.22	cellular metabolism
NAD(P)H dehydrogenase, quinone 1	NQO1	610935	-3.78	cellular metabolism

Figure 5-1A and 5-1B. Gene enrichment analysis of cocultured normal chondrocytes with OA chondrocytes highlighted biological functions central to OA pathogenesis.

The microarray results showed that the inflammatory response and oxidative stress (NOS activity and superoxide metabolic processes) pathways were up=regulated in normal chondrocytes that had been cocultured with OA chondrocytes. Other up-regulated biological functions of importance to the pathogenesis of OA include cytokine and chemokine activity. Up-reguation of IL-8 and IL-10 receptor pathways are of particular significance. IL-8 is a pro-inflammatory cytokine shown to have a key role in the induction of the OA process. IL-10 is an anti-inflammatory cytokine that inhibits MMPs. Up-regulation of The collagen metabolic process biological function was also upregulated.

5-2A. Up-regulated biological functions



5-2B. Percentage change within each biological function group.

Biologial function	Number of molecules	Percent changed
Cytokine activity	9	17.65
Chemokine activity	7	36.84
Chemotaxis	8	35.0
Regulation of cell proliferation	4	18.18
NOS activity	2	33.33
IL-8 receptor binding	3	25.0
Induction of positive chemotaxis	3	75.0
Collagen metabolic process	2	28.57
IL-10 receptor activity	4	18.18
Prostaglandin metabolic process	3	100.0
Defense response	7	33.33
Cytokine activity	9	17.65
Inflammatory response	9	33.33
Superoxide metabolic processes	9	11.69

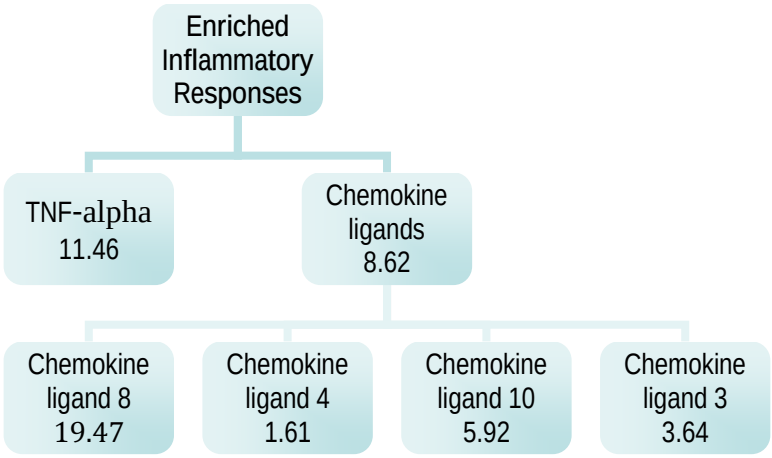
Table 5.4 Coculture of normal chondrocytes with OA chondrocytes lead to dysregulation of molecular groups central to the pathogenesis of canine OA in the normal chondrocytes.

The gene expression patterns of ADAMTS, aggrecanase, TNF, collagen and interleukin genes are shown according to fold change along with the role of these molecules in the pathogenesis of OA. These up-regulated molecular groups function in the degradation of articular cartilage by destruction or modulation of the structure of the extracellular matrix.

Table 5.4			
Molecular Group	**Gene**	**Fold change**	**Role in OA pathogenesis**
ADAMTS	ADAMTS-2 ADAMTS-4 ADAMTS-5 ADAMTS-6	1.92 2.38 2.10 1.7	-degrade articular cartilage
Aggrecanase	MMP-1 MMP-2 MMP -3 MMP -13 MMP -14	1.51 1.6 3.25 5.88 1.81	-degrade proteoglycans and collagens -degrade articular cartilage
TNF	TNF SF -10 TNF SF -13B TNF SF -15 TNF SF -18 TNF AIP -2 TNF AIP -3 TNF AIP -6	3.92 1.82 9.28 2.76 3.92 2.05 9.21	-ECM degradation - dysregulate proteoglycan synthesis
Collagen	Col-3A1 Col-6A3 Col -4A1 Col-15A1	2.0 1.44 1.37 1.33	-type II collagen replaced with type I and III collagen causing fibrosis
Interleukin	IL-23A IL-4I1 IL-27 IL-6 IL-10RB IL-15RA IL-15 IL-8 IL-18BP IL-1RA IL-12A IL-1R2	9.62 9.87 7.75 7.0 4.57 3.64 3.32 2.73 2.15 1.93 1.74 1.53	-upregulate MMP expression -inhibit chondrocyte repair of damaged ECM -degrade articular cartilage

Figure 5-2. Functional gene classes of differentially expressed genes showed enrichment of inflammatory responses, chemokine activity, defense responses and collagen catabolic processes.
Five enriched biological gene families of importance to OA disease progression were upregulated: A. inflammatory responses, B. chemokine activity, C. defense responses, D. collagen catabolic processes and E. regulation of cell proliferation. Individual genes within each family showing increased expression are illustrated. Biologically significant gene modulation is defined as a two-fold increase in gene expression with $p<0.05$ corresponding to a false discovery rate of 5%. Chemokine ligands and TNF-alpha were represented in the inflammatory, defense and chemokine activity pathways.

5-2A.



5-2B.

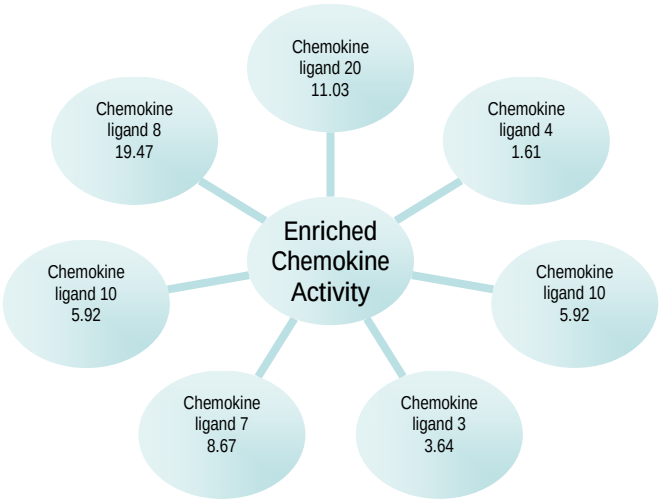
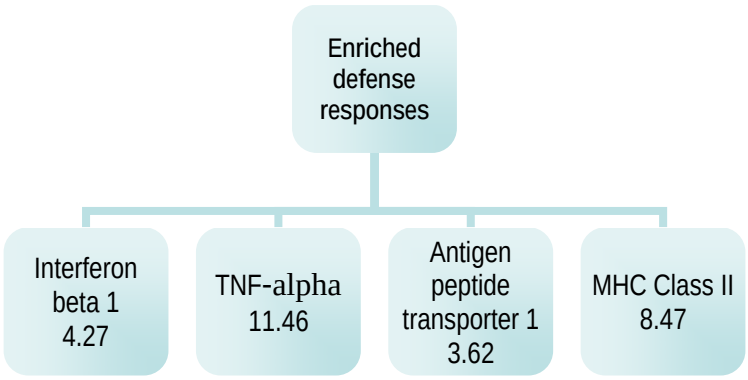
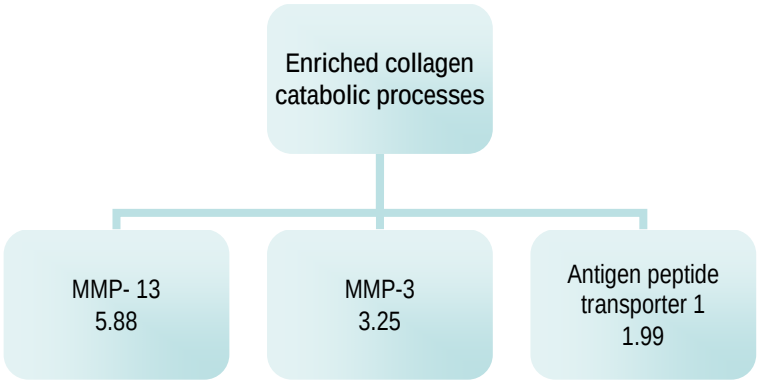


Figure 5-2 Functional gene classes of differentially expressed genes show enrichment of inflammatory responses, chemokine activity, defense responses and collagen catabolic processes.

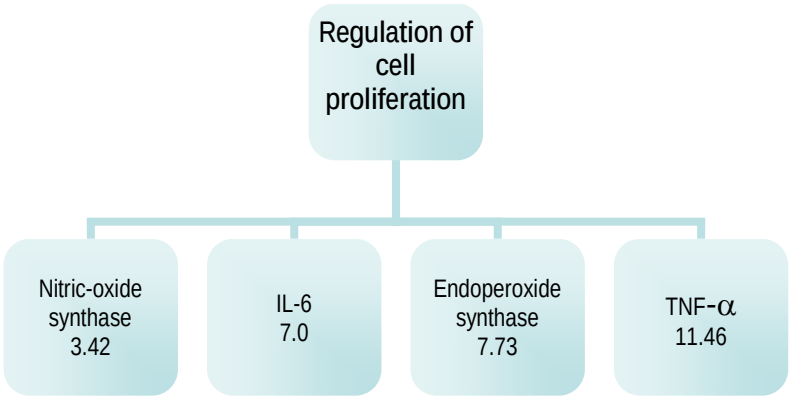
5-2C.



5-2D.



5-2E.



5.5.2 RT-PCR analysis validated the microarray data.

Validation of the differential gene expression profiles obtained by microarray analyses of nine genes with fold-changes of two or higher using RT-PCR showed similar changes in fold changes and expression directions (Figure 5-4). The nine genes were chosen for their role in the pathogenesis of OA. Validations were performed using the original cell lines under the same coculture conditions with which the microarray analyses were performed. Up- regulation of MMP-3 (Figure 5-4D), MMP-13 (Figure 5-4E), COL-3A1 (Figure 5-4F), IL-6 (Figure 5-4G), ADAMTS-5 (Figure 5-4H), TNF-alpha (Figure 5-4I), and down regulation of IL-F3 (Figure 5-4A) was confirmed (all $p<0.05$). Expression levels for IL-4 (Figure 5-4B) and IL-8 (Figure 5-4C) did not correlate with the microarray results (both $p>0.05$). Thus the microarray expression pattern of seven of the nine genes assessed in these experiments corresponded with gene expression levels. These data show that overall gene expression profiles obtained by the microarray analysis were validated by RT-PCR.

Figure 5-3. RT-PCR experiments validated the microarray data.
Figures 5-4A, 5-4B, 5-4C, 5-4D, 5-4E, 5-4F, 5-4G, 5-4H and 5-4I show the results of RT-PCR analysis of gene expression levels of ILF-3, IL-4, IL-8, MMP-3, MMP-13, Col-3A1, IL-6, ADAMTS-5 and TNF-alpha, respectively. The data are presented as several fold changes relative to control cells (normal chondrocyte with normal chondrocyte cocultures), the RQ or relative quantification value. The GAPDH housekeeping gene served as an internal control. Data represent the mean of the results obtained from three replicate experiments; error bars indicate standard deviations from the mean.
Graph legends:
Sample 1- normal chondrocytes cocultured with foetal chondrocytes (internal control sample).
Sample 2- normal chondrocytes cocultured with OA74 chondrocytes
Sample 3- normal chondrocytes cocultured with OA71 chondrocytes
Sample 4- normal chondrocytes cocultured with OA26 chondrocytes
Sample 5- normal chondrocytes cocultured with OA68 chondrocytes

Figure 5-3A. ILF-3 relative gene expression
RT-PCR analysis was consistent with the microarray findings, with an average RQ value of 0.57 across the four cell sample replicates. This shows down-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes.

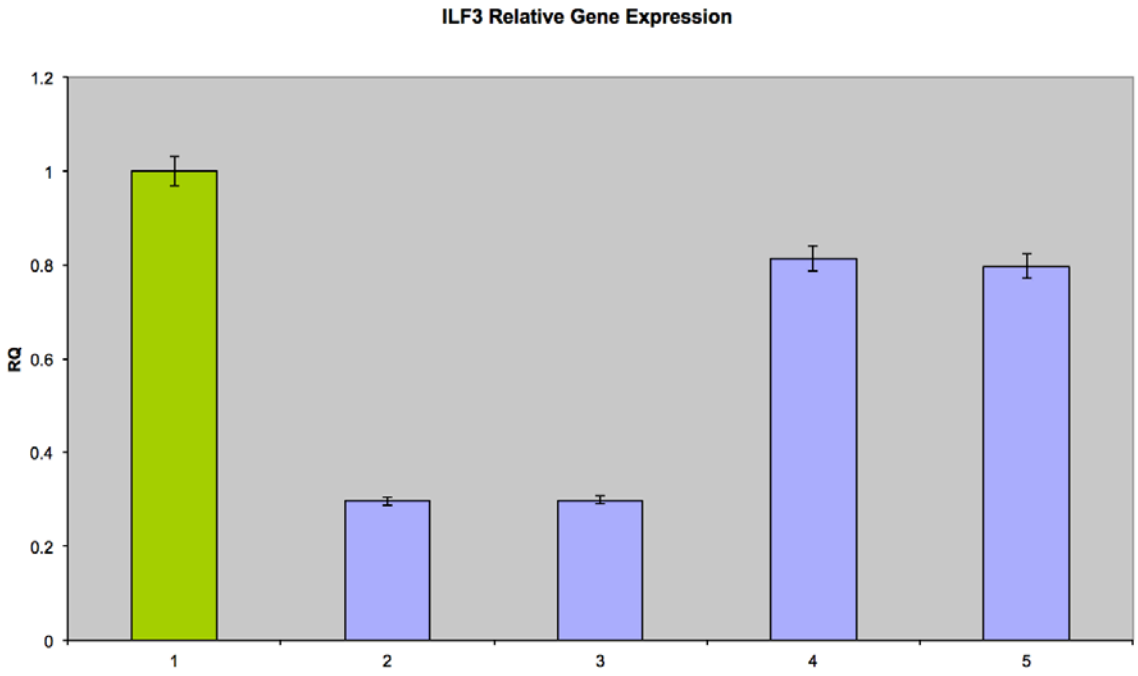


Figure 5-4B. IL-4 relative gene expression
RT-PCR analysis was inconsistent with the microarray findings, with an average RQ value of 0.9 showing down-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes, while the microarray results showed up-regulation of IL-4 in normal chondrocytes following coculture with OA chondrocytes.

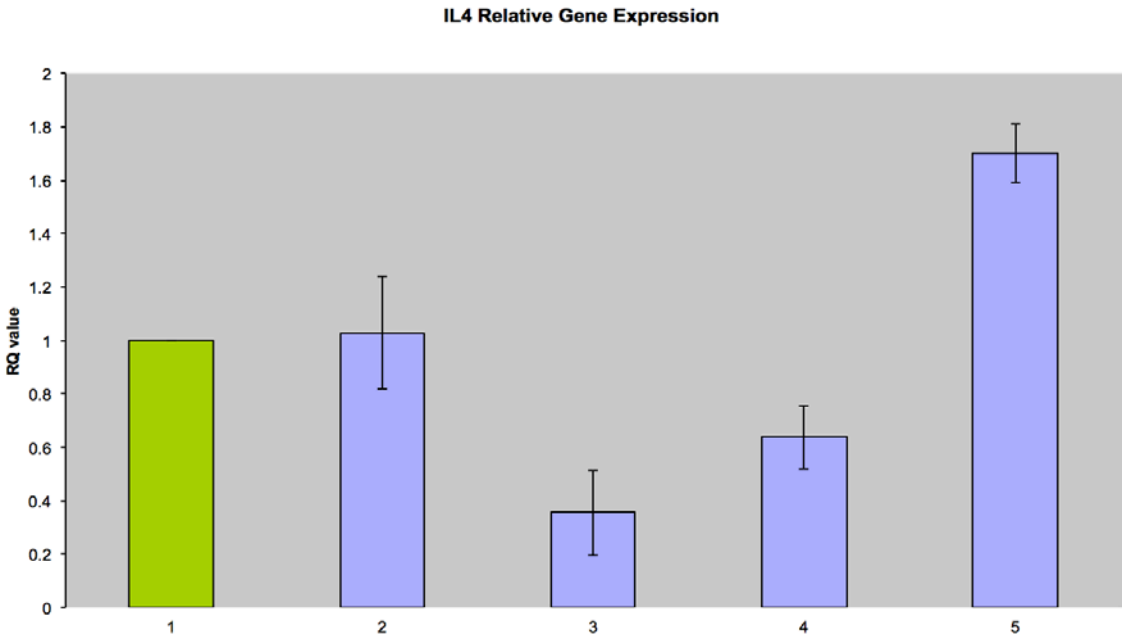


Figure 5-4C. IL-8 relative gene expression
RT-PCR analysis was also inconsistent with the microarray findings, with an average RQ value of 0.26 showing down-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes, while microarray analysis results showed an increase in relative gene expression.

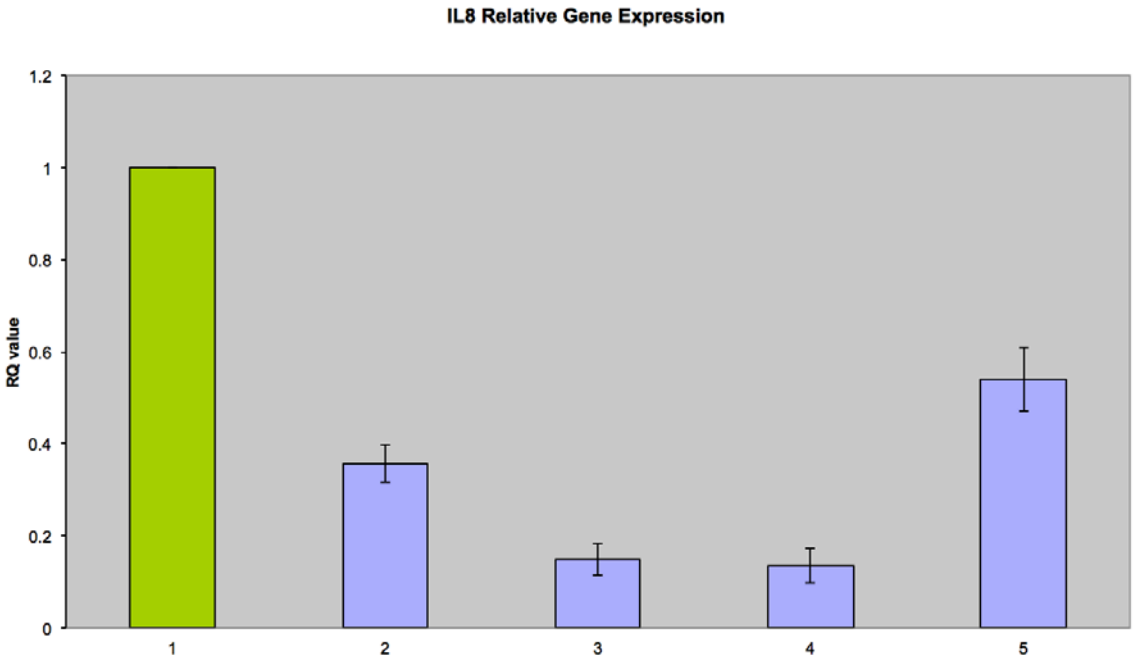


Figure 5-4D. MMP-3 relative gene expression
RT-PCR analysis was consistent with the microarray findings for gene expression levels of MMP-3, with an average RQ value of 1.2 showing up-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes.

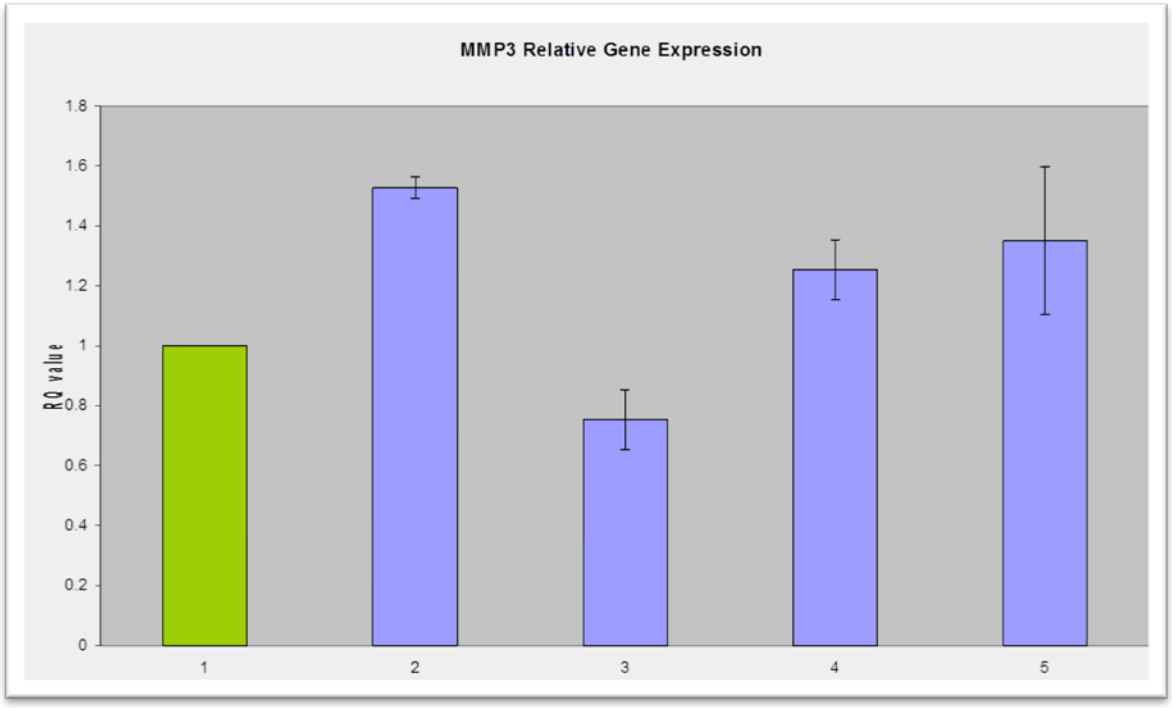


Figure 5-4E. MMP-13 relative gene expression
RT-PCR analysis validated the microarray findings, with an average RQ value of 7.9 showing increased expression of this gene in normal chondrocytes following coculture with OA chondrocytes.

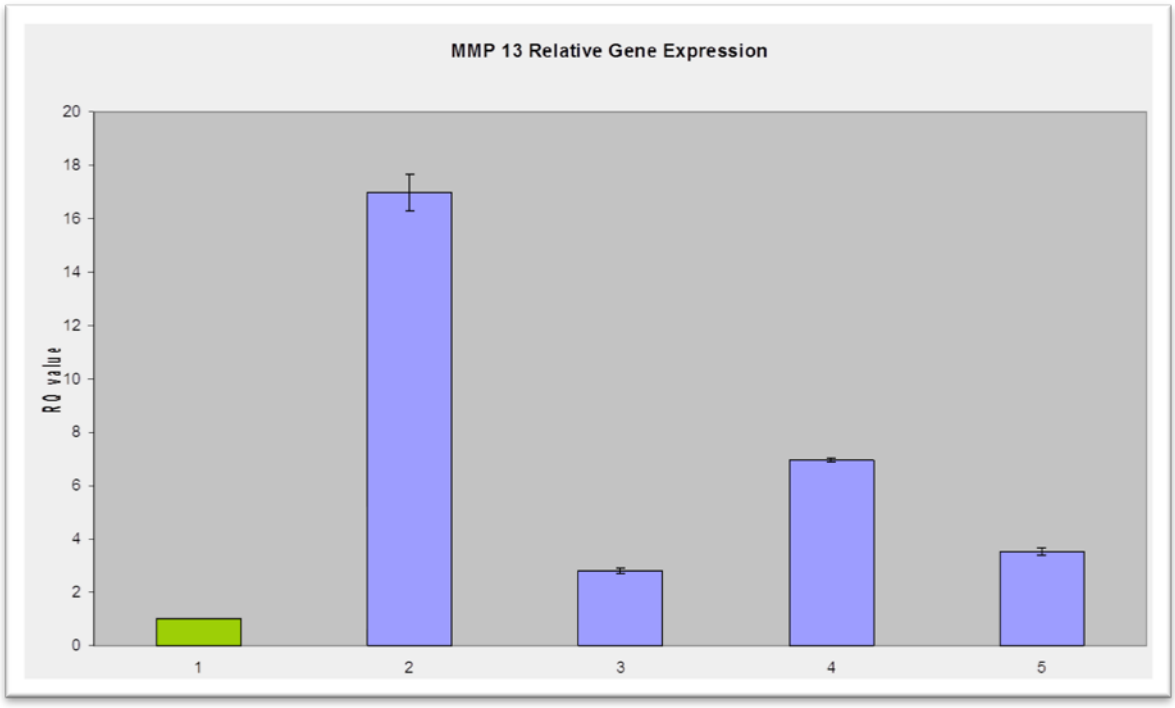


Figure 5-4F. Col-3A1 relative gene expression
RT-PCR analysis was consistent with the microarray findings for gene expression levels of Col-3A1, with an average RQ value of 3.8 showing up-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes.

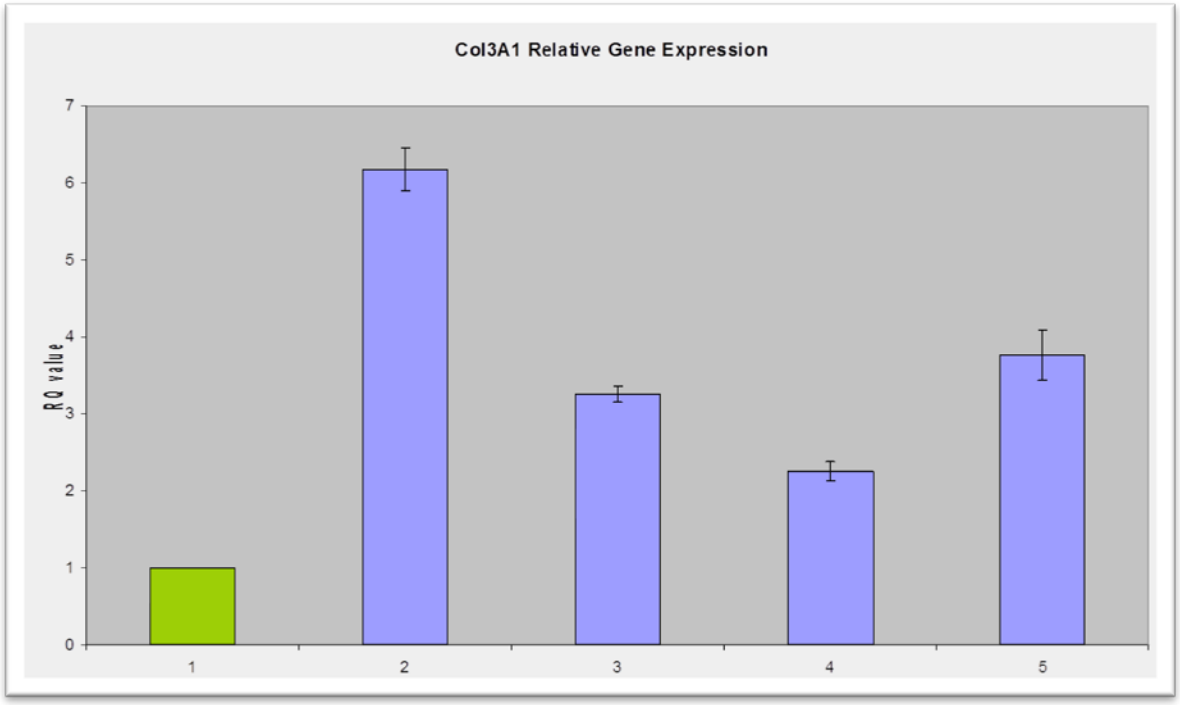


Figure 5-4G. **IL-6 relative gene expression**
RT-PCR analysis validated the microarray findings, with an average RQ value of 2.2 showing increased expression of this gene in normal chondrocytes following coculture with OA chondrocytes.

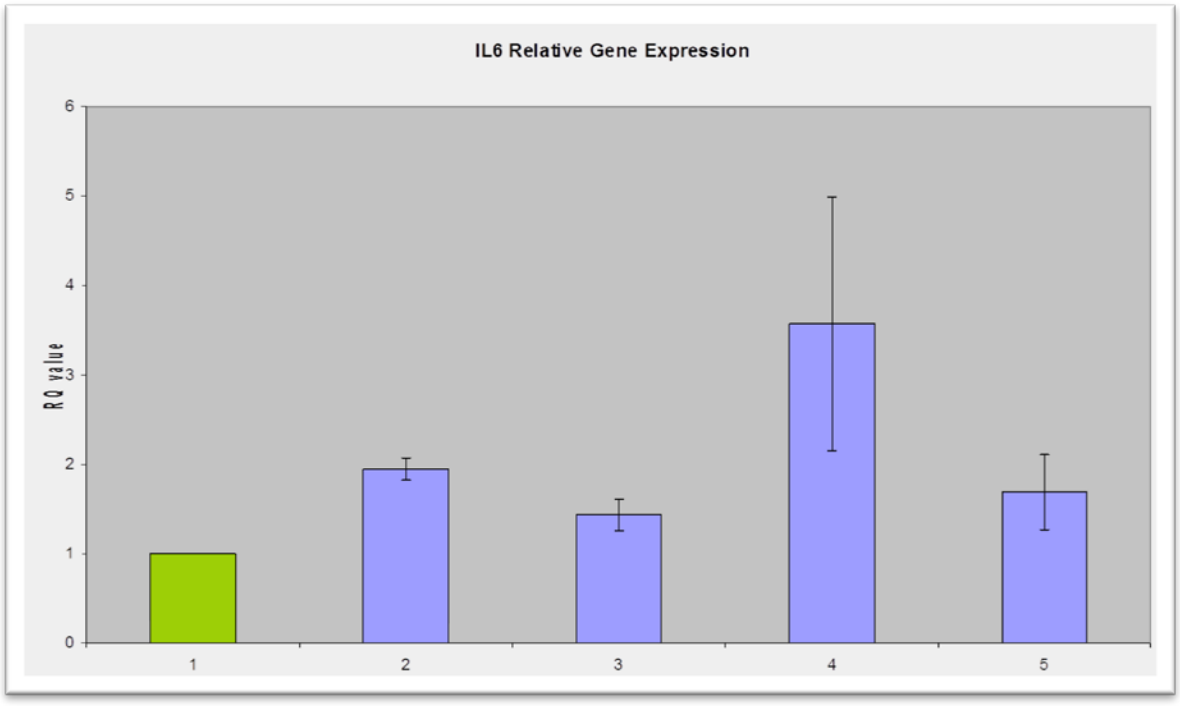


Figure 5-4H. ADAMTS-5 relative gene expression
RT-PCR analysis validated the microarray findings, with an average RQ value of 1.5 showing increased expression of this gene in normal chondrocytes following coculture with OA chondrocytes.

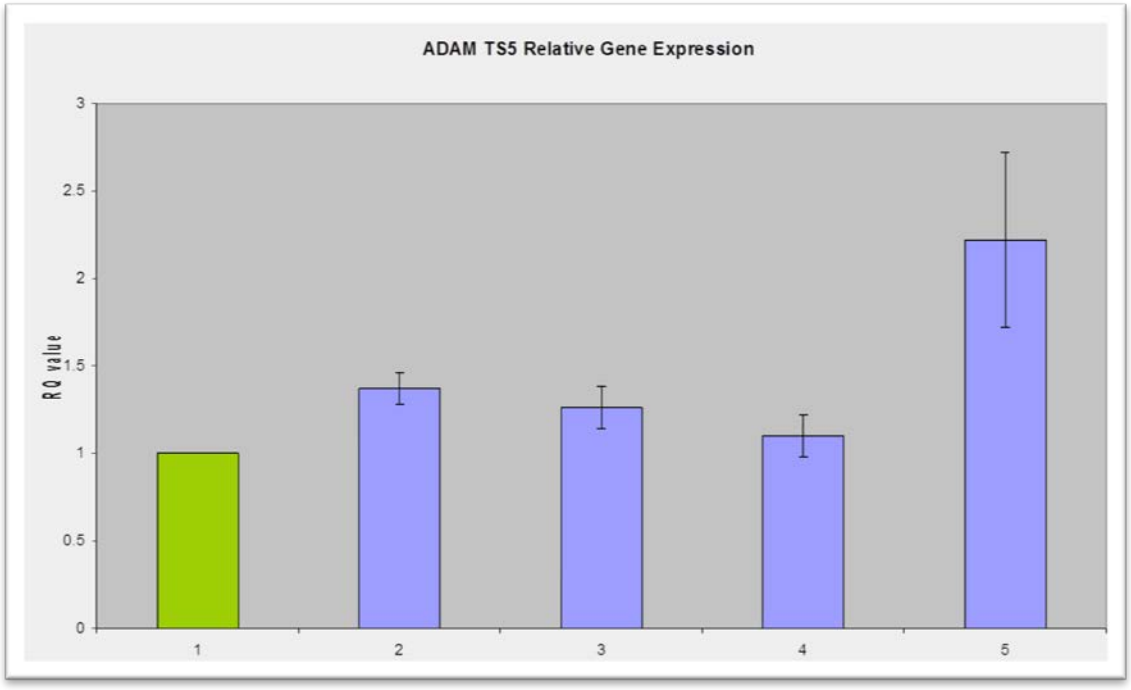


Figure 5-4I. TNF-alpha relative gene expression
RT-PCR analysis was consistent with the microarray findings, with an average RQ value of 0.57 across the four cell sample replicates. This shows up-regulation of this gene in normal chondrocytes following coculture with OA chondrocytes

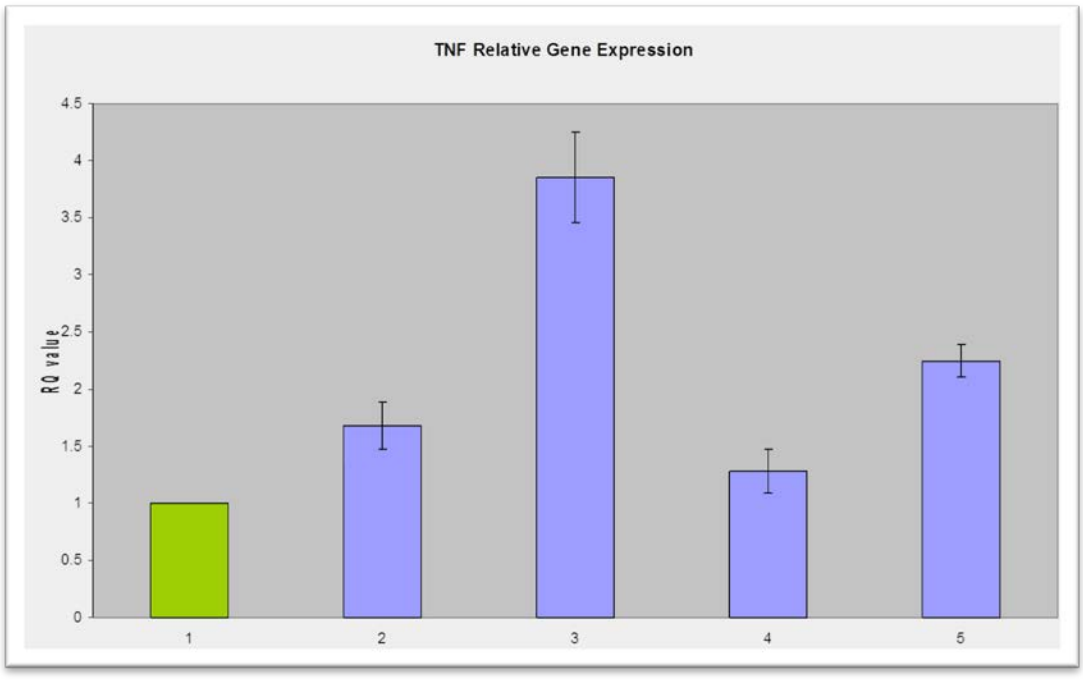
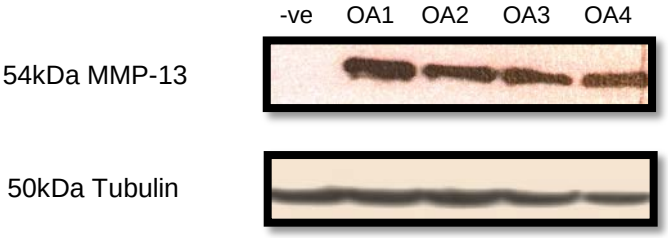
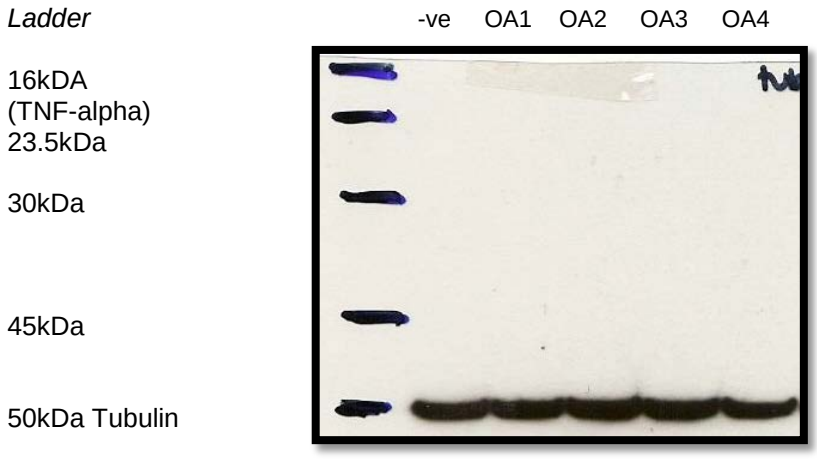


Figure 5-5A and 5.5B. Western Blot analysis showed expression of MMP-13, a protein which plays a key role in the pathogenesis of OA.
Western blot analysis of MMP-13 (Figure 5-5A) showed that this protein was present in the normal-with-OA cocultured chondrocytes but not in normal-with-normal cocultured control chondrocyte cells. Western blot analysis of TNF-alpha (Figure 5-5B) was unsuccessful.

5-5A. Western blot analysis for MMP-13.
This blot was stripped and re-probed as MMP-13 is 54kDa in size and tubulin is 50kDa in size, hence both bands were not detectable simultaneously on the Western blot.



5-5B. Western blot analysis for TNF-alpha.
Western blot analysis for TNF-alpha was unsuccessful. TNF-alpha is a 17kDa monomer, no bands of this size were visible on the blot as shown below.



5.6 Discussion

A cornerstone of OA research is elucidating the pathogenesis of this disease on a molecular level in order to identify potential therapeutic targets. Extensive research into understanding and elucidating the complex molecular pathways involved in establishing and maintaining the OA disease state is ongoing, and complex biological pathways and molecular interactions are being identified. Much OA research has focused on the role of chemokines and cytokines originating from the synovial membrane, fibroblasts or chondrocytes within the joint (Appleton *et al.,* 2007).

The mechanisms by which trauma to individual chondrocytes leads to joint inflammation and OA development are still poorly understood, though certain facets are emerging (Bau *et al.,* 2002; Dessau *et al.,* 1981; Duerr *et al.,* 2004; Glasson *et al.,* 2005; Hughes *et al.,* 1998; James *et al.,* 2005; Malfait *et al.,* 2002). The impact of individual OA chondrocytes on adjacent normal chondrocytes and the role of this cellular interaction in the pathogenesis of canine OA remains to be explained. To advance our understanding of the role of OA chondrocyte cells in disease propagation to adjacent normal chondrocyte cells this study investigated global changes in gene expression in canine chondrocytes in response to coculture with OA canine chondrocytes using microarray technology. By evaluating the biological effects of OA chondrocyte secreted factors on normal canine chondrocytes using canine gene microarray analyses, ten genes whose expression is key to the pathogenesis of OA, were identified. Gene modulation was subsequently verified by expression analysis using RT-PCR and by Western blotting for MMP-13. Additionally, a large number of OA biological processes were found to be up-regulated.

5.6.1 Coculture of OA chondrocytes with normal chondrocytes resulted in modulation of OA genes in normal chondrocytes.

The coculture experiments demonstrated that OA chondrocytes can affect the gene expression of normal chondrocytes, with up-regulation of genes crucial to the pathogenesis of OA. Coculture of these two cell populations resulted in a total of 594 significantly altered genes (i.e. with a fold change in expression level greater than two and a false discovery rate of less than 5%). Of these, a wide range of genes previously determined to be key to the development of OA, were shown to be dysregulated. This proves the hypothesis that OA chondrocytes can play an important role in the progression of canine OA. Of 594 genes identified with a change in mRNA levels greater than two fold, a total of

489 genes had greater than a four-fold increase in mRNA, deemed to be highly significant. It should be noted that there can be disparity between a mathematical change in level of gene expression and the biological significance of that change. It is possible that very small fold changes in gene expression can be extremely significant depending on the role of the genes in question to a particular disease process. Conventionally, a fold-change of two is considered significant and a fold-change of four is considered highly significant (DeRisi *et al.,* 1996; Schena *et al.,* 1995). Combining fold-change with a p-value of less than 0.05 (corresponding to a false discovery rate of five percent) increases correlation of fold change to biological significance (Huggins *et al.,* 2008). These criteria were applied in the current study. One should always bear in mind that the biological significance of a gene fold-change will always be related to the function and thus biological impact of that gene. Some of the highly dysregulated genes have not previously been identified as playing a role in OA pathogenesis, or their presence has not previously been reported in cartilage (Table 5-2, Table 5-3).

5.6.2 Genes associated with OA pathogenesis showed dysregulation.

Microarray analysis of exposed normal chondrocytes revealed modulation of ten genes that have been identified as playing a key role in the pathogenesis of OA. Two of the most significant results in this group were TNF-alpha and IL-1. TNF-alpha and IL-1 are key to OA pathogenesis, playing a primary role in the degradation of articular cartilage (as detailed in Chapter I, Introduction). Together they tip the delicate balance between catabolism and anabolism that exists in maintaining a healthy ECM, towards catabolism. Production is disrupted via the antagonism of aggrecan and type II collagen synthesis (Arner and Pratter, 1989; Klamfeldt *et al.,* 1986; Verbruggen *et al.,* 1991), while destruction is increased by inducing chondrocytes and synoviocytes to increase production of MMPs (Campbell *et al.,* 1986; Pratta *et al.,* 1989). It has previously been demonstrated that TNF-alpha expression is increased in canine OA synovial cells (Burton-Wurster *et al.,* 2005; Fujita *et al.,* 2005). To the author's knowledge, up-regulation of TNF-alpha in canine chondrocytes in association with OA has not previously been demonstrated. Interleukins are cytokines, which are primarily associated with inflammation and immunity. Interleukins which have been shown to be increased specifically in OA include: IL-1 (Loughlin *et al.,* 2002; Tyler and Saklatvala, 1985; Verbruggen *et al.,* 1991; Wilbrink *et al.,* 1991); IL-4 (Mahr *et al.,* 2003), IL-12 (Sakkas *et al.,* 1998); IL-6 (Ainger *et al.,*

2001; Maccoux *et al.,* 2007; Wilbrink *et al.,* 1991); and IL-10 (Maccoux *et al.,* 2007). Of these molecules IL-4, IL-6 and IL-8 were significantly upregulated in this study. The finding of an increase in IL-8 gene expression is of particular interest. IL-8 activity in OA chondrocytes has been shown to promote MMP-3 activity (Borzi *et al.,* 2004) and also to induce hypertrophy of cartilage with pathological changes to normal cartilage functionality (Merz *et al.,* 2003). IL-8 also results in cartilage degradation by chemotaxis of neutrophils. As such IL-8 plays an important role in the pathological changes of OA and this change in gene expression in the normal adjacent chondrocytes is highly significant. Further studies examining the levels of transcribed protein resulting from this up-regulation would be of interest in helping our understanding of OA development within a joint. An additional ten members of the interleukin group were also up-regulated.

MMP-13 and MMP-3 both showed marked increases in gene expression (5.88 and 3.25 fold respectively). These molecules are enzymes capable of degrading the ECM. They are part of the normal anabolic-catabolic homeostatic cycle in articular cartilage. Increased production of MMPs results in cartilage matrix destruction (Billinghurst *et al.,* 1997; Karran *et al.,* 1995; Sondergaard *et al.,* 2009). MMP-3 is also known as Stromelysin-1. It cleaves the ECM proteins laminin and fibronectin and induces activation of other MMPs. MMP-13 is a collagenase which cleaves Type I, II and III collagen. Increased levels of MMP-3 have been identified in human (Lohmander *et al,* 1993) and canine (Panua *et al.,* 1998) OA cartilage and synovial fluid. Increased levels of MMP-13 have been identified in OA cartilage samples (Shlopov *et al.,* 1997). Culture of normal canine chondrocytes with IL-1β has been shown to increase levels of MMP-3 (Cook *et al.,* 2000) and MMP-13 (Kuroki *et al.,* 2005), illustrating the complex interactions that exist between cytokines.

Changes of gene expression levels of ADAMTS molecules are also of particular interest. The ADAMTS group is a family of proteases associated with cell adhesion and migration, inflammation, angiogenesis and coagulation. It has been shown that inhibition of ADAMTS-4 and ADAMTS-5 prevents aggrecan degradation in OA cartilage (Malfait *et al.,* 2002). Thus upregulation of aggrecanases seems to be a significant response to intercellular communication within the OA joint. The data presented here shows increased expression of both ADAMTS-4 and ADAMTS-5 and MMP-13, another potent aggrecanase.

One gene associated with OA pathogenesis showed down-regulation, IL-F3 (interleukin enhancer binding factor 3), a transcription factor required for T-cell expression of IL-2,

which has been shown to be increased in chronic inflammatory joint diseases such as rheumatoid arthritis (Firestein *et al.,* 1988). It is interesting that only 26 genes with a fold change greater than two were down-regulated in this study and only one of these has previously been identified as significant to the pathogenesis of OA. One possible explanation for this is that upregulation of genes occurs before there is any inhibition of expression. Another study showed that stimulation of chondrocytes with IL-1β increases the expression of cytokines, chemokines and MMPs before there is any downregulation of matrix genes (Sandell *et al.,* 2008). This suggests that the cytokine response occurs more rapidly than other molecular events and may explain why there is a much greater proportion of up-regulated genes in the present study. At any one time within an OA joint, there is an ongoing dynamic complex interaction of cytokines and chemokines between surrounding chondrocytes and synoviocytes (Borzi *et al.,* 2004; Kuroki *et al.,* 2005; Villinger *et al.,* 1992). This interaction is responsible for altering the balance between repair and degradation of the extracellular matrix (Fernandes, 2002). IL-1β increases the expression of IL-8 (Martel-Pelletier et al., 1999), and IL-1 in the presence of TNF has been shown to cause both release and increased production of IL-8 in the ECM (Pulsatelli *et al.,* 1999). IL-1β and TNF-alpha also activate MMPs (Cook *et al.,* 2000; Pulsatelli *et al.,* 1999; Sandell *et al.,* 2008).

5.6.3 Biological processes associated with OA pathogenesis show dysregulation.

Data mining for biologically relevant processes identified that up-regulated genes in cocultured chondrocytes are associated with inflammation, chemotaxis, chemokine activity, cytokine activity, defense responses, regulation of cell proliferation, superoxide metabolic processes and collagen metabolic processes. The overall trend of altered biological processes is consistent with those processes known to be involved in the pathogenesis of OA and this further strengthens the evidence of a causal relationship between intercellular communication and the pathogenesis of OA. Proteins are the major effector molecules in biological systems. It has been well established that the amounts of protein transcribed do not directly correlate with levels of mRNA within a cell (Gygi *et al.,* 1999). It is therefore important to confirm the significance of results obtained by microarray studies. The data in this study were validated using RT-PCR analyses for nine known OA genes. Up-regulation of TNF-alpha, IL-6, MMP-13, COL-3A1, ADAMTS-5, MMP-3 and down regulation of IL-F3 was confirmed ($p < 0.05$). RT-PCR for IL-8 and IL-

4 was not consistent with the microarray results ($p > 0.05$) thus seven of the nine microarray results were validated. The data demonstrated that the overall results of the RT-PCR experiments were consistent with those of the microarray analysis. Similarly, Western blot analysis verified that changes in cellular mRNAs in the cocultured normal chondrocytes corresponded to changes in MMP-13 protein expression.

5.6.4 Novel genes previously unassociated with OA pathogenesis showed dysregulation.

As in other gene expression studies profiling OA genes, the present data provides an additional source of candidate genes for continued study and analysis (Aigner *et al.*, 2001; Burton-Wurster *et al.*, 2005; James *et al.*, 2005; Clements *et al.*, 2006). In this study four potential genes of interest for further study were identified. These genes have not been previously identified as playing a role in the pathogenesis of OA but showed high levels of modulation. The four genes of interest identified were SON DNA binding protein which showed a 252-fold increase in gene expression, interferon beta-1 which had a 121-fold increase, interferon-induced protein with a 107-fold increase and secreted phosphoprotein 1, which showed a seven-fold decrease in gene expression. These genes are associated with maintaining genome stability, hematopoietic stem cell differentiation, host defense functions in apoptosis, modulation of immune function and cytokine activity (as outlined in the results section). These pathological processes may play a previously unidentified role in OA and further work examining a potential function of these genes in OA pathogenesis could prove fruitful. Unfortunately, due to time constraints these findings were not investigated further as part of this research.

5.6.5 Conclusions

In conclusion these data show for the first time that coculture of OA chondrocytes with normal chondrocytes results in altered gene expression of the normal cells. Following culture with OA chondrocytes, the normal chondrocytes show up-regulation of genes playing a key role in OA pathogenesis. The study confirmed the crucial role that chondrocytes play in disease pathogenesis by impacting upon the regulation of OA associated genes. These data fit with current hypotheses on disease propagation from sites of minor trauma to the joint-wide OA disease state. For instance, these findings may begin to explain why seemingly minor trauma can instigate a cascade of events, which over time

lead to catastrophic changes within joints with progression to OA. Individually damaged chondrocytes will alter the gene expression of adjacent normal chondrocytes, thus facilitating the spread of OA from cell to cell, without ongoing mechanical trauma. The present study has shown that normal chondrocytes will show up-regulation of genes key to OA pathogenesis (TNF-alpha, IL-4, IL-6, MMP-13, MMP-3, ADAMTS-5, COL-3A1, IL-8, IL-F3 and ADAMTS-4) as a result of coculture with OA chondrocytes in vitro. The identification of differentially expressed genes in this experimental model furthers our knowledge of the biological mechanisms involved in the propagation of OA within the canine joint.

It is hoped that a comprehensive understanding of the genes involved in the pathogenesis of OA will establish new targets for therapeutic interventions and new candidates for the development of more efficient biomarkers of early disease. The identification of patients in a pre-clinical phase could facilitate early interventions and halt or slow disease progression. Identifying the very earliest events in disease propagation is key to achieving this goal. Therapeutic options can potentially be approached using this preliminary data. Interleukins are currently used as therapeutic targets in cancer treatment in humans, for example the use of synthetic IL-2 (Aldesleukin) in the treatment of renal cell carcinoma (Proleukin, Novartis). Targeting one single cytokine as a therapeutic approach has been shown to be of limited success (Hansbro *et al.*, 2011) in many diseases due to the complex interactions between these molecules (as outlined above in the case of OA) and further understanding of these biological pathways and cascades of interaction will likely be required to understand potential targets and side-effects in using these molecules as treatment targets. Research into the use of cytokines and chemokines as treatment targets is a rapidly developing area of research, with asthma (Hansbro *et al.*, 2011), IBD (MacDonald, 2011) and gout (Neogi *et al.*, 2010) being current fields of investigation. The use of gene therapy and cell therapies for the treatment of OA also rheumatoid arthritis is discussed in Chapter 1, Introduction.

5.6.5 Future Studies

It would be of interest to repeat this study using a larger sample size, and also utilising different cohorts of OA cell donors such as aged versus young animals The use of newer technologies such as UHTDSTs and RNAseq could facilitate rapid study of greater sample numbers.

Some questions still remain unanswered in these experiments, particularly relating to the assessment of protein levels corresponding to up-regulated gene expression. The present experiments demonstrated the presence of MMP-13 protein associated with increased MMP-13 gene expression, however Western blot analysis did not demonstrate the presence of TNF-alpha protein in concurrent experiments. It was not possible to pursue these experiments further due to time constraints, however it would be interesting to generate positive control samples for a number of proteins (using transfection with siRNA) in order to determine if there is cross-reactivity between commercially available antibodies and the canine proteins corresponding to the up-regulated genes in the RT-PCR experiments. Other protein detection techniques, such as immunofluorescence and flow cytometry could also be utilised as not all antibodies work well in Western Blot experiments.

Having established that OA chondrocytes impact upon the gene expression of adjacent normal chondrocytes in vitro, the next step would be to assess the soluble factors secreted by OA chondrocytes in order to determine the molecular pathways by which gene expression is altered in adjacent normal cells. With this information it may be possible to identify targets by which these chemical messages could be disrupted, preserving the normal function of non-OA cells in injured joints. The coculture cell inserts have a pore size of 8nm, allowing the diffusion of soluble factors up to 120 kDa in size from chondrocytes. Cytokines and chemokines are 5-20 kDa in size and therefore can diffuse easily through the coculture system. Proteomics could be used investigate which soluble factors are present in the cell culture fluid following coculture, using enzyme-linked immunosorbent assay for specific molecules (such as MMPs and TNF-alpha) and using mass spectrometry-based techniques to identify unknown proteins.

Chapter VI

The Role of Oxidative Stress in the Pathogenesis of Canine Osteoarthritis.

6.1 Introduction

Oxidative stress is a pathological condition that occurs when excess reactive oxygen species (ROS) damage cells. ROS are free radicals containing oxygen. Free radical molecules are highly chemically reactive due to the presence of either unpaired electrons or an open external orbital. As such they act as oxidants, seeking to gain an electron from another molecule. Electron gain stabilises the free radical but alters the structure of the oxidised molecule, causing the molecule itself to become a free radical and causing a chain reaction within cells. These molecules have the potential to damage amino acids, proteins, cellular DNA, mitochondrial DNA and intracellular lipids (Beckman and Ames, 1999; Golden and Melov, 2001;). Oxidative stress results when there is an imbalance between the production of ROS and active scavenging by cellular mechanisms, as occurs in brain ageing, Parkinsons disease, atherosclerosis and OA (Finkel, 2003; Ikebe *et al.*, 1990). The consequence of damage caused by ROS is alteration of structure and function in both intra- and extracellular molecules. ROS are continuously produced during the process of cellular energy production. ROS can also be produced via NADPH oxidase, xanthine oxidase and cytochrome P450.

Aerobic respiration by mitochondria is the main mechanism of energy production in cells and accounts for 90% of cellular ROS production (Figure 6.1), (Balaban *et al.*, 2005). Mitochondria are cellular organelles that produce energy by oxidative phosphorylation, generating fifteen times more ATP than glycolysis. Mitochondria regulate both ROS production and scavenging (Landolfi *et al.*, 1998; Newmeyer *et al.*, 1994; Turrens and Boveris, 1980). Within mitochondria, superoxide anion ($O_2^{\bullet -}$) is produced by the single electron reduction of O_2. $O_2^{\bullet -}$ is then re-organised to hydrogen peroxide (H_2O_2), catalysed by superoxide dismutases (SODs). H_2O_2 is scavenged by catalase or glutathione peroxidase to produce water and oxygen. In the absence or dysfunction of free radical scavenging ROS accumulate, including H_2O_2, $O_2^{\bullet -}$ and hydroxyl free radical ($OH^{\bullet -}$). A further free radical, peroxynitrite ($ONOO^{\bullet -}$) may form when nitric oxide reacts with H_2O_2. Peroxynitrite is considered to be both a reactive nitrogen species (RNS) and a ROS.

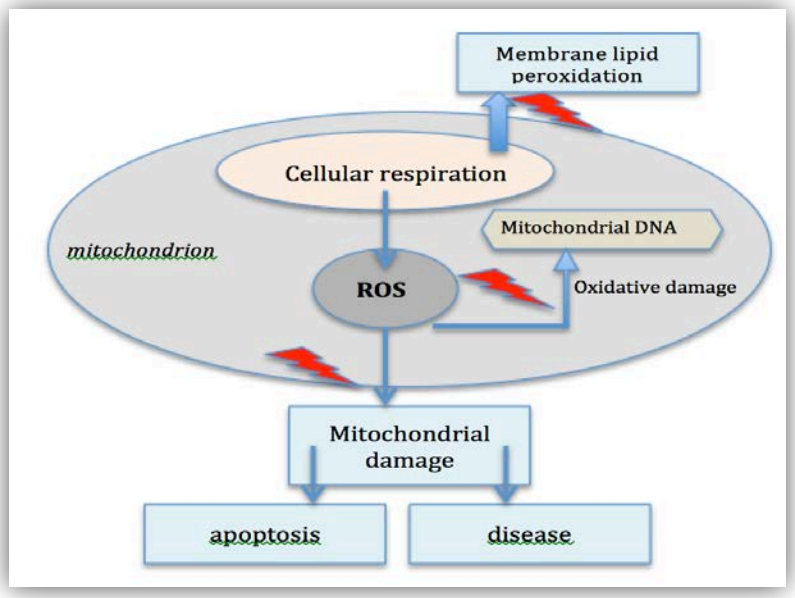


Figure 6-1. Production of reactive oxygen species (ROS) by mitochondria. ROS are produced within mitochondria as a product of cellular respiration. In the absence of efficient scavenging of ROS, lipid peroxidation and mitochondrial damage occur resulting in cellular apoptosis. This process plays a role in many disease states, including OA.

In the presence of ROS, lipid peroxidation can occur producing highly damaging lipid peroxides such as malondialdehyde (Porter, 2013). These molecules alter chemical, osmotic and electrical gradients within cells by damaging lysosomal and mitochondrial membranes. Malondialdehyde causes DNA damage resulting in alterations to DNA structure and function (Marnett, 1999).

ROS also have homeostatic functions, acting as intracellular messengers in a number of important pathways and undertaking signaling functions in cell activation, proliferation and cytokine control (Clancy *et al.,* 2004). These include MAPK signaling (pP38 upregulation), interleukin release (IL-6, IL-1β and IL-18), transcription of IL-6 and caspase activation (Bulua *et al.,* 2011; Nakahira *et al.,* 2011; Zhou *et al.,* 2011). Cytokines, including interleukins (IL-1β, IL-3, IL-6), TNF-alpha and growth factors (TGF-β) increase cellular levels of ROS (Thannickal and Fanburg, 2000). Consequently, it is when ROS levels exceed cellular scavenging capacity, that they become pathological rather than homeostatic molecules. As free radicals can oxidise other molecules leading to altered function and cell damage, continuous scavenging of ROS is required to protect cells from oxidative stress. Multiple ROS scavenging systems exist, however, the most prevalent systems are glutathione (Luschak, 2012), catalase (Linares *et al.,* 2010) and SOD (Johnson and Guilivi, 2005). Glutathione peroxidase is a mitochondrial hydrogen peroxide

scavenger whilst catalase is a cytosolic hydrogen peroxide scavenger (Linares et al., 2010; Luschak, 2012). Glutathione is also a substrate for glutathione peroxidase scavenging of hydroperoxides (Luschak, 2012; Sinha *et al.,* 1993). SOD exists in three forms (cytosolic SOD known as SOD1, mitochondrial SOD known as SOD2 and secreted extracellular SOD referred to as SOD3). SOD3 catalyses the regeneration of reduced glutathione from oxidized glutathione by glutathione reductase, at the expense of NADPH (Gadoth, 2010; Deahl *et al.,* 1992). Scavenging of ROS also occurs via antioxidants, such as vitamins C and E (Gilgun-Sherki *et al.,* 2002).

6.1.1 The role of oxidative stress in disease

Oxidative stress plays a pathological role in cellular aging, senescence and apoptosis. The free radical theory of aging hypothesises that damage caused by ROS is associated with ageing of cells (Carlo and Loeser, 2003; Finkel and Holbrook, 2000). Increased levels of ROS and RNS have been detected in many disease states (Mates *et al.,* 1999), such as atherosclerosis (Harrison *et al.,* 2003) and OA (Bae *et al.,* 2003; Mrowicka *et al.,* 2008). The exact mechanisms by which free radicals cause disease have yet to be elucidated. ROS damage to mitochondria is thought to play a significant part in the pathogenesis of oxidative damage to tissues. Within mitochondria, oxidative stress damages proteins, mitochondrial DNA (causing base modifications, single and double strand breaks and cross-linking) and cell membranes and impairs the process of oxidative phosphorylation (Arheim and Cortopassi, 1992; Beckman and Ames, 1999; Golden and Melov, 2001) as illustrated in Figure 6-1.

6.1.2 Reactive oxygen species in healthy articular cartilage

Nutrition of articular cartilage occurs by diffusion of nutrients from the synovial fluid (Levick, 1995), as articular cartilage is an avascular tissue. Similarly, oxygen and metabolic products diffuse back to the synovial fluid from chondrocytes, giving cartilage a low oxygen tension (Zhou et al., 2004). Oxygen tension in cartilage is 1% in the deep zone, 8% in the superficial zone and 7-8% in synovial fluid creating a diffusion gradient (Brighton and Heppenstall, 1971). ATP in chondrocytes is predominantly produced by substrate level phosphorylation (which is oxygen independent), however chondrocyte mitochondria use some of this ATP in aerobic respiration in regions of higher oxygen tension- principally at the articular surface (Lee and Urban, 1997). Chondrocytes have

10% lower density of mitochondria as compared to other cells in the body (Brighton *et al.*, 1984). The density of mitochondria is higher in cells of the superficial cartilage zones than in the deeper cartilage layers where the oxygen tension is lower (Clancy *et al.*, 2004; Stockwell, 1991) and superficially located chondrocytes have five times less catalase than chondrocytes in the deeper cartilage layer (Fragonas *et al.*, 1998). Articular chondrocytes have little tolerance for free radical buffering and they are uniquely adapted for this by existing in a low oxygen environment. Chondrocytes are also metabolically inert compared to other cells, showing low levels of oxygen consumption, low levels of cellular replication and limited anabolic/catabolic activity (Henrotin *et al.*, 2005). As in other biological tissues, ROS act as chemical messengers in articular cartilage (Gibson *et al.*, 2008; Lo *et al.*, 1998; Clancy *et al.*, 2004). Cellular pathways of importance to chondrocytes (including mitogen-activated protein kinase signaling, HIF-1 signaling pathways, NF-κB signaling pathways and AP-1 signaling pathways) are mediated by ROS as signaling molecules (Dröge, 2002; Valko *et al.*, 2007). ROS have also been shown to down regulate pro-inflammatory chondrocyte genes (Ziskoven, 2010), having an anti-inflammatory function in cartilage homeostasis.

6.1.3. The role of oxidative stress in the pathogenesis of OA

Free radical production by chondrocytes is low in healthy articular cartilage as discussed above. With joint inflammation there is a pathological rise in tissue oxygen tension and this promotes the production of free radicals. IL-1 induces nitric oxide formation by up-regulation of iNOS in inflamed joints (Pelletier, 1998; Vuolteenaho *et al.*, 2007). Nitric oxide is produced by synovial cells as a result of inflammation (Pelletier, 1998). Excessive mechanical loading of articular cartilage has also been shown to increase the production of ROS (Tomiyama *et al.*, 2007) and RNS (Healy *et al.*, 2005) in chondrocytes, causing oxidative stress. Similarly, excessive shearing forces applied to articular cartilage increase chondrocyte mitochondrial ROS production (Green *et al.*, 2006). Sources of ROS/RNS within the joint in oxidative stress are shown in Figure 1-9, Chapter I. Due to the poor capacity of chondrocytes for free radical scavenging, increased ROS production caused by inflammation, can lead to oxidative stress in articular cartilage. It has been suggested that the capacity of chondrocytes for free radical scavenging may be impaired in OA. Oxidative stress in articular cartilage has been shown to cause degradation of the extracellular matrix, to impair extracellular matrix synthesis, to induce chondrocyte senescence and apoptosis, to cause DNA damage, to alter gene expression and to cause breakdown of intracellular

and extracellular proteins (Henrotin *et al.,* 2003; Lo *et al.,* 1998; Tiku *et al.,* 1999; Tiku *et al.,* 2003).

6.1.3.1. Cartilage matrix degradation

Health of articular cartilage depends upon the maintenance of the extracellular matrix structure, which confers tensile stiffness and strength. The structure of the extracellular matrix exists as a fine balance of anabolic and catabolic processes. This makes articular cartilage particularly vulnerable to alterations of normal homeostatic mechanisms, as occurs with oxidative stress. ROS damage the extracellular matrix by direct degradation of component molecules. Degradation of component molecules occurs by direct oxidation, by disruption of cell signaling pathways, by damage to chondrocyte mitochondrial DNA and by reduction of the number of functional (i.e. extracellular matrix-secreting) chondrocytes caused by the induction of senescence and apoptosis (Fragonas *et al.,* 1998; Henrotin *et al.,* 2003; Henrotin *et al.,* 2005). Degradation of component molecules is further mediated by increased levels of MMPs. MMPs are produced by both chondrocytes and synovial cells in response to inflammatory cytokines (TNF-alpha and IL-1), produced following cartilage damage (Goldring, 2000). MMPs are capable of degrading all components of the extracellular matrix of articular cartilage (Martel-Pelletier, 1999). They play a key role in the pathogenesis of OA. MMP-3 and MMP-13 are increased in both human and canine OA (Lohmander *et al.,* 1993; Panula *et al.,* 1998; Shlopov *et al.,* 1997). MMP-3 is also known as Stromelysin-1, it cleaves the extracellular matrix proteins laminin and fibronectin and induces activation of other MMPs. MMP-13 is a collagenase which cleaves Type I, Type II and Type III collagen. Concentrations of MMPs have been shown to be increased by oxidative stress in OA (Murrell *et al.,* 1995). In the early stages of OA, chondrocytes undergo hypertrophy (Braunstein *et al.,* 1990), division and form cell clusters with increased production of Type II collagen and aggrecan as they attempt to repair cartilage damage (Aigner *et al.,* 2001; Drissi *et al.,* 2005; Pullig *et al.,* 2000). New collagens are also secreted including Type I, IIA, III and X (Schmid *et al.,* 1991; Yasuda and Poole, 2002). The gene expression of several matrix collagens has been shown to be up-regulated in OA, including Col-3A1 gene expression (Aigner *et al.,* 2006). ROS inhibit matrix synthesis by inhibiting the production of extracellular matrix components including collagen (Shah *et al.,* 2005), proteoglycan (Henrotin *et al.,* 2005; Tschan *et al.,* 1990; Wang *et al.,* 2002) and PSGAG's (Yudoh *et al.,* 2005).

6.1.3.2. Mitochondrial damage

As cartilage is a post-mitotic tissue, chondrocytes are less dependent on mitochondrial energy production than other biological tissues and it is thought that they are more resistant to the effects of oxidative stress mediated by mitochondrial dysfunction. However, once a critical threshold of oxidative stress has been exceeded, oxidative damage to chondrocyte mitochondria is an important pathway of cartilage damage causing decreased production and increased degradation of the cartilage extracellular matrix (Johnson *et al.,* 2004; Maneiro *et al.,* 2003). Nitric oxide production by chondrocytes and synovial cells increases in OA (Pelletier, 1998; Vuolteenaho *et al.,* 2007). Nitric oxide is thought to alter mitochondrial function by inhibiting the electron transport chain during respiration. Oxidatively damaged mitochondria can lead to cell growth arrest and replicative senescence (Toussaint *et al.,* 2000).

6.1.3.3. Senescence and apoptosis

The role of stress-induced premature senescence (SIPS) in the pathogenesis of OA has been described in Chapter IV Introduction, and it is known that as chondrocytes age their telomeres shorten and the amount of cellular senescence increases. Several studies have shown that ROS play a role in the onset of senescence in articular chondrocytes. Premature senescence and chondrocyte apoptosis can result from increases in ROS and nitric oxide production. It has been demonstrated that mechanical stress applied to articular cartilage induces ROS production and that these ROS can induce premature senescence (Martin *et al.,* 2004). Increases in RNS have been shown to induce telomere erosion in chondrocytes (Yudoh *et al.,* 2005), an effect that was inhibited by pre-treating cells with an antioxidant. Chondrocyte generated ROS have also been shown to induce apoptosis (Aigner *et al.,* 2001; Blanco *et al.,* 1998; Del Carlo and Loeser, 2002; Hashimoto *et al.,* 1998), though the importance of apoptosis in OA remains controversial.

6.1.4 Measurement of oxidative stress in tissues

The measurement of ROS in tissues is very difficult, largely due to the dynamic flux of these molecules in cells and extracellular fluid. Instead, direct measurement of end products of oxidative stress, as well as indirect measurement of pathway enzymes, are frequently used to assess levels of oxidative stress in cells or tissues (Niki, 2008). Various

molecules are produced during oxidative damage and many can be detected in biological samples. Commercial kits are available to measure lipid peroxidation in plasma, serum, urine, tissue homogenates and cell lysates. Urinary hydrogen peroxide can be quantified using a hydrogen peroxide assay. Eight-isoprostane and isoprostane-iPF2α-VI levels (produced by oxidation of tissue phospholipid) can be detected in plasma, serum, saliva and cell lysates. Eight-hydroxy-2-deoxy-guanosine, a product of oxidative damage of DNA by ROS and RNS, can be quantified in cell culture and plasma. Free radical generation has been detected *in vivo* in animals by measuring electron spin resonance (Sonta *et al.,* 2004), however this technique requires miniaturised electron spin resonance spectrometers (Micro-ESR), which are not routinely available in medical laboratories. Glutathione can be measured in plasma, cells and urine. Glutathione is a thiol tripeptide synthesised in mammals from amino acids. It is the most abundant antioxidant present in mammalian cells and plays a key role in maintaining ROS homeostasis by free radical scavenging (Meister and Anderson, 1983). Low levels of GSH reflect increased susceptibility to oxidative stress (Ballatori et al., 2009). Measurement of cellular glutathione has been used an indicator of the antioxidant capacity of chondrocytes (Carlo and Loeser, 2003).

6.1.5 Current treatment options for oxidative stress

Antioxidant therapy represents a potential treatment option for diseases in which oxidative stress plays a pathological role. The use of naturally occurring antioxidants in the treatment of OA has been evaluated for glutathione and catalase, as well as SOD, peroxidases, and vitamins A, C and E. It has been shown that N-acetyl cysteine may protect articular chondrocytes from oxidative stress in vivo (Nakagawa *et al.,* 2010; Ross *et al.,* 2004; Ueno *et al.,* 2011). Other studies have demonstrated that apoptosis induced by mechanical trauma to cartilage in human and equine experimental models can be inhibited by the use of caspase inhibitors (D'Lima *et al.,* 2001; Huser *et al.,* 2006). A high dietary content of antioxidants (Vitamin C, Vitamin E and beta-carotene) has been associated with extracellular matrix destruction in MRI studies of articular cartilage (Henrotin *et al.,* 2005), thought to be caused by antioxidant breakdown of collagen and hyaluronan (Gao *et al.,* 2008; Petersen *et al.,* 2004). Therefore, there may be adverse effects associated with dietary antioxidant supplementation as a treatment for oxidative stress. It should also be considered that ROS play an important role in intracellular signaling, and it is only when there is an imbalance in normal ROS homeostasis that oxidative stress occurs. The

scavenging efficacy of commonly used antioxidant supplements, including N-acetyl cysteine, is currently unknown, as is the impact of these supplements on the clinical syndrome of OA. More research is required to fully clarify the role of supplementary antioxidants in oxidative stress processes.

6.2 Aims and Objectives

Oxidative stress has been shown to play a role in the pathogenesis of OA in humans and horses. To date, little data has been published regarding the role of oxidative stress in the pathogenesis of canine OA. The experiments described in Chapter IV suggested that OA canine chondrocytes have greater amounts of cellular senescence compared to normal canine chondrocytes. It has been shown that damage to articular cartilage causes inflammation leading to oxidative stress and also that oxidative stress is a cause of cellular senescence. This lead to our hypothesis that oxidative stress is a feature of canine OA chondrocytes and that this oxidative stress is a cause of senescence in canine OA chondrocytes. To extend this idea we hypothesised that oxidative stress alters the secretory function of articular chondrocytes by altering the expression of genes responsible for maintaining the extracellular matrix of articular cartilage (MMP-3, MMP-13 and Col-3A1), thus establishing a pathological link between oxidative stress, senescence and OA. The specific aims and objectives of the work presented in this chapter were:

1. To optimise the use of *tert*-Butyl hydroperoxide (tBhP) as an inducer of oxidative stress in canine chondrocytes, by treating monolayer chondrocyte cultures with varying concentrations of tBhP and performing Live/Dead cell assays for cell viability following treatment.
2. To establish oxidative stress as a cause of cellular senescence in normal canine chondrocytes by measuring SA-beta-gal activity and replicative capacity to show cellular senescence in tBhP-treated chondrocytes.
3. To compare the amount of oxidative stress in OA and normal canine chondrocytes by measuring cellular levels of total glutathione, to establish that oxidative stress is a feature of OA chondrocytes.
4. To examine the impact of experimentally induced oxidative stress on MMP-3, MMP-13 and Col-3A1 gene expression in normal canine chondrocytes by RT-PCR to demonstrate that oxidative stress alters the expression of genes key to the maintenance of the extracellular matrix of articular cartilage.

6.3 Materials and methods

6.3.1 Sample collection

OA cartilage samples were collected from the femoral heads of dogs, as previously described. These femoral heads came from The Small Animal Hospital, School of Veterinary Medicine, University of Glasgow and from specialist referral centers in the UK. The OA sample group was obtained from dogs undergoing total hip replacement surgery for OA and/or hip dysplasia. The normal control group consisted of dogs of various breeds that were euthanatised for reasons unrelated to the musculoskeletal system including three samples obtained from canine foetuses. Chondrocytes were harvested from normal and OA femoral head cartilage (Chapter II, Materials and Methods).

6.3.2 Culture of chondrocytes in monolayer

Culture of chondrocyte cells in monolayer was carried out as described in Chapter II, General Materials and Methods.

6.3.3 Induction of oxidative stress in cultured chondrocytes

In order to induce oxidative stress in normal canine chondrocytes, cells were treated with tert-Butyl hydroperoxide (tBhP). Three normal chondrocyte cell lines were cultured in monolayer in 6 well plates at a density of $1x10^6$ cells/ml, with a well volume of 2ml, in DMEM supplemented with 10% FBS and 10μl ciproxin. After 5-7 days the cells reached confluence and were transferred to serum-free DMEM overnight prior to treatment with tBhP. Varying concentrations of tBhP were added to the monolayer chondrocyte cultures in order to determine the optimal concentration of tBHP required to induce oxidative stress without inducing cell death. The concentrations of tBhP used were 5 μM, 10μM, 25μM, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM and 500 μM tBhP. Cells were incubated for one, four, eight and sixteen hours at each concentration of tBhP. These concentrations and incubation times were selected based on previously reported doses of tBhP used to induce oxidative stress in cultured chondrocytes (Kurz *et al.*, 2004; Yin *et al.*, 2009).
Three cell lines of normal canine chondrocytes were cultured:
Cell line F1=Rottweiler, foetus at birth, female entire

Cell line F2= Shih tzu, foetus at birth, male entire

Cell line F3= Cavalier King Charles spaniel, foetus at birth, male entire

6.3.4 Culture of OA and normal tBhP treated chondrocytes to senescence

Cells were cultured to senescence in monolayer culture using routine culture techniques described previously (Chapter II, General Materials and Methods). Four OA primary cells lines (OA65, OA74, OA26, OA78) and four normal primary cell lines (N1, N6, N4, N5) were cultured, to investigate the effect of oxidative stress induction on replicative capacity of normal and OA chondrocytes.

Samples utilised in this study were:

OA cell lines:

Cell line OA65: German Shepherd Dog, male entire, 28 months

Cell line OA74: Labrador, male entire, 10 months

Cell line OA26: Labrador, male neuter, 84 months

Cell line OA78: Border Collie, female neuter, 96 months

Normal cell lines used:

Cell line N1: Foetus, Rottweiler, female entire, 0 days (at birth)

Cell line N6: Staffordshire Terrier, male neuter 3 months

Cell line N4: Cross breed, male entire, 132 months

Cell line N5: Border Collie, female entire, 132 months

In order to examine the effect of oxidative stress on cell survival in OA and normal chondrocytes, cell lines N1, N6, N4, N5, OA65, OA74, OA26 and OA78 were cultured in monolayer in 6 well plates at a density of $1x10^6$ cells/ml (in triplicate), with a well volume of 2ml, in DMEM supplemented with 10% FBS and 10µl ciproxin. Once the cells reached confluence; they were then maintained in serum-free DMEM overnight prior to treatment with tBhP.

6.3.5 Measurement of cell viability using Live/Dead cell staining

Viability of normal chondrocytes following induction of oxidative stress by treatment with tBhP was assessed using Live/Dead cell staining. Cell survival was determined by a total cell count with the Live/Dead Viability Cytotoxicity Kit (Life Technologies, U.K.). Two fluorescent dyes are utilised for the viability assay, one of which stains for live cells and

the other of which stains for dead cells. Ethidium bromide homodimer-1 stains nuclear DNA when there is compromise of the cell membrane (dead cell staining). It will not stain the nuclei of cells with an intact cell membrane as it is cell impermeable. Calcein AM produces a fluorescent stain in the presence of live cells when it is cleaved from its non-fluorescent state by intracellular esterases of live cells.

Chondrocytes were cultured on sterile glass coverslips as confluent monolayers in 6 well plates for two days, as described in Chapter II, General Materials and Methods. Following treatment with tBHP, culture medium was removed and cells were washed twice with sterile PBS. LIVE/DEAD kit assay reagents were allowed to thaw at room temperature and cells were stained by adding LIVE/DEAD cell stain mix. This was prepared by adding 20mL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 ml of sterile, tissue culture–grade D-PBS to prepare a 4 mM EthD-1 solution. 5mL of the supplied 4mM calcein AM stock solution (Component A) was then added to the 10 mL EthD-1 solution. 100ml of the cell stain mix was added to coverslips in the 6 well plates. The plates were covered and the cells were incubated for 30 minutes at room temperature. For assessment of cell viability, 10ml of cell stain mix was added to a microscope slide before inversion of the stained glass cover slip onto the slide. Stained slides were sealed with clear nail polish. The labeled chondrocytes were assessed by fluorescence microscopy. Cell viability was calculated by counting the percentage of live and dead cells per high power field at 40X magnification using a fluorescence microscope as described in Chapter II, General Materials and Methods. The number of positive live and positive dead staining cells over three high power fields (with a minimum of 100 cells per field) was calculated and a percentage positive live stain and positive dead stain was calculated. A mean value of live/dead staining across three high power fields per slide was obtained for each sample.

6.3.5 Assessment of cellular levels of total glutathione

To assess the amount of oxidative stress within chondrocytes, the concentration of total cellular glutathione was measured in cell culture supernatant using spectrophotometry as first described by Tietze (Tietze ,1969) and subsequently modified by Griffith (Griffith, 1980), using a glutathione assay kit (Sigma-Aldrich, U.K.). Six cell lines consisting of three tBhP-treated chondrocyte cell lines and three tBhP untreated control chondrocyte cell lines (F1, F2 and F3 as described above) were grown in six-well plates until confluent after seeding at a density of 1x 10^6 cells (according to the method described in Chapter II,

General Materials and Methods). Once confluent, cells were harvested and de-proteinised using a 2.5% solution of 5-sulfosalicylic acid, lysed by freezing and thawing and then centrifuged to remove precipitated protein. 15ml of working reaction mixture, consisting of 8ml 100mM potassium phosphate at pH 7.0, 228 µl of glutathione reductase and 228µl DNTB stock solution made up to 15ml using DEPC-treated water, was added to a volume (up to 10µl) of the 5-sulfosalicylic acid cell supernatant and mixed. Following incubation at room temperature for 5 minutes, 50µl of diluted NADPH was added. A standard curve using serial dilution of a known stock solution (provided with the kit) was used for calibration. A plate reader was used to measure the absorbance in each well at a wavelength of 412nm. Total cellular glutathione was calculated in nM per 10^8 cells by dividing the reaction rate of the unknown sample by the reaction rate of the known control sample.

6.3.6 Assessment of senescence levels

Replicative senescence of cell lines was assessed by measurement of Sa-beta-gal staining and measurement of cellular replicative capacity.

6.3.6.1 Senescence associated beta-galactosidase activity

Described in Chapter IV, Materials and Methods section (4.3.2.1).

6.3.6.2 Cellular replicative capacity

Described in Chapter IV, Materials and Methods section (4.3.1.3).

6.3.7 RT-PCR analysis of gene expression

The gene expression levels of three genes, MMP-13, MMP-3 and Col-3A1 were quantified using RT-PCR as described in Chapter II, General Materials and Methods. These genes were chosen as their expression levels change in the pathogenesis of early OA following articular cartilage damage. Primer and probe sets were designed using Primer Express software (Applied Biosystems, U.K.). Primer and probe sequences are presented in Chapter II, General Materials and Methods. Briefly, total RNA from cells was isolated

using a RNeasy Mini kit (Qiagen, U.K.). First-strand cDNA was synthesised using SuperScript III first-strand synthesis kit (Invitrogen, U.K.). Quantitative gene expression analysis was performed with 100 ng of cDNA. PCR reactions consisted of 0.2 μM forward and reverse primers, 0.1 μM probe, 25 μl of 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen, UK), 1 μl ROX Reference Dye and water to a final volume of 50 μl. Following a 2 min activation at 50°C of Platinum Taq DNA polymerase and a 5 min denaturing step at 95°C, 45 cycles of 15 s at 95°C and 45 s at 60°C were run. Fluorescence measurements were made for every cycle at 60°C. Each cell sample was analysed in triplicate. Mean fold changes in gene expression were calculated using 7500 Real Time PCR System software, relative to the expression of the untreated control cells using the 2-ΔΔCT method.

6.3.8 Statistical analysis

For statistical analysis of the significance of differences in concentration of total cellular glutathione and replicative capacity of chondrocytes in monolayer, the Students paired t-test was used. Evaluation of statistically significant differences in amount of staining of SA-beta-gal and Live/Dead cell staining were calculated using Wilcoxin rank–sum nonparametric tests, using SPSS software. P-values of <0.05 were considered statistically significant.

6.4 Results

6.4.1. Treatment of monolayer cultured canine chondrocytes with 25μM *tert*-Butyl hydroperoxide for four hours induced oxidative stress without inducing cell death.

To optimise the use of *tert*-Butyl hydroperoxide (tBhP) as an inducer of oxidative stress in canine chondrocytes, monolayer chondrocyte cultures were treated with varying concentrations of tBhP. A Live/Dead cell assay was used to assess cell viability following treatment. Three normal canine chondrocyte cell lines (F1, F2 and F3) were cultured to confluence in monolayer in six well plates (as described in the Materials and Methods) and incubated with increasing concentrations (5μM, 10μM, 25μM, 50μM, 100μM, 200μM, 250μM, 500μM) of tBhP. Cells were incubated for one, four, eight and sixteen hours for each concentration of tBhP. Time and dose increments were in accordance with previously published data on the use of tBhP for induction of oxidative stress in cell culture. Total glutathione concentration is a cellular marker of oxidative stress. Total glutathione concentration in tBhP treated chondrocytes and tBhP untreated chondrocytes (negative control cells) was measured by spectrophotometric assay to determine the optimum concentration of tBHP required to induce oxidative stress. Experimental induction of oxidative stress *in vitro* is defined as a greater than four-fold decrease in total cellular glutathione concentration. Multiple tBhP dosages and incubation times resulted in oxidative stress in the treated chondrocytes, highlighted in bold in Table 6-1 below.

In order to determine the optimal tBhP dose and incubation time required to induce oxidative stress, Live/Dead cell assay was used. High levels of oxidative stress are cytotoxic and induce cell death. Accordingly, following treatment with tBhP, each sample was analysed for cell viability by Live/Dead cell staining (Figure 6-2). The lowest concentration of tBhP over the shortest incubation period that resulted in a four-fold decrease in total glutathione whilst maintaining a live cell rate of greater than 95% was chosen to induce oxidative stress in subsequent experiments. Incubation of chondrocytes with 5μM and 10μM of tBhP had no impact on total cellular glutathione concentration, irrespective of the length of incubation period. Incubation of chondrocytes for four hours at a concentration of tBhP greater than 50μM resulted in a four-fold decrease in total cellular glutathione concentration, but treatment of chondrocytes with tBhP at these concentrations resulted in cell death of 14%, as shown in Figure 6-3 below. Incubation of chondrocytes

with 25μM of tBhP for four hours resulted in a 4.5 fold decrease in total cellular glutathione concentration whilst maintaining cell viability at 97%.

Table 6-1. Effect of tBhP treatment on total cellular glutathione levels (GSH) in normal canine chondrocytes in monolayer culture. Chondrocytes were incubated with increasing concentrations of tBhP; 5μM, 10μM, 25μM, 50μM, 100μM, 200μM, 250μM and 500μM. For each treatment volume of tBhP, one cell sample was incubated for each of the four incubation durations studied; one hour, four hours, eight hours and sixteen hours. Following treatment, total cellular glutathione was measured for each cell sample using spectrophotometry. Incubation with 5μM and 10μM had no impact on total cellular glutathione concentration regardless of the length of incubation. Incubation for four hours at a concentration greater than 50μM resulted in a four-fold decrease in total cellular glutathione concentration, however there was an increased level of cell death at these concentrations. Incubation with 25μM for four hours resulted in a 4.5 fold decrease in total cellular glutathione concentration whilst maintaining cell viability. Bold text indicates a four-fold or greater decrease in total cellular glutathione concentration and successful induction of oxidative stress *in vitro* whilst maintaining cell viability.

Volume of tBhP (μM)	**Total GSH nMx10^8 cells**			
	1 hour	**4 hours**	**8 hours**	**16 hours**
5	32.41	32.23	31.79	31.92
10	32.70	32.25	28.85	30.43
25	30.81	**6.78**	**5.72**	3.38
50	32.80	**2.68**	**2.61**	0.87
100	32.42	**0.83**	**0.48**	0.44
200	33.53	**0.74**	**0.81**	0.78
250	32.47	**0.52**	**0.39**	0.86
500	0.64	**0.61**	**0.66**	0.59

Figure 6-2. Live/Dead assay of canine chondrocytes identified levels of cell viability following treatment with tBhP.

Green fluorescent staining of the nucleus indicated live cell staining. Red fluorescent staining of the nucleus indicated dead cell staining.

A and **B**. F1 cells, photographed under light microscopic examination at 40X magnification after treatment with 25μM tBhP for four hours, showed A. 97% live and B. 3% dead cell staining.

C and **D.** F1 cells, photographed under light microscopy examination at 40X magnification after treatment with 100μM tBhP for four hours, showed C. 24% live and D. 76% dead cell staining.

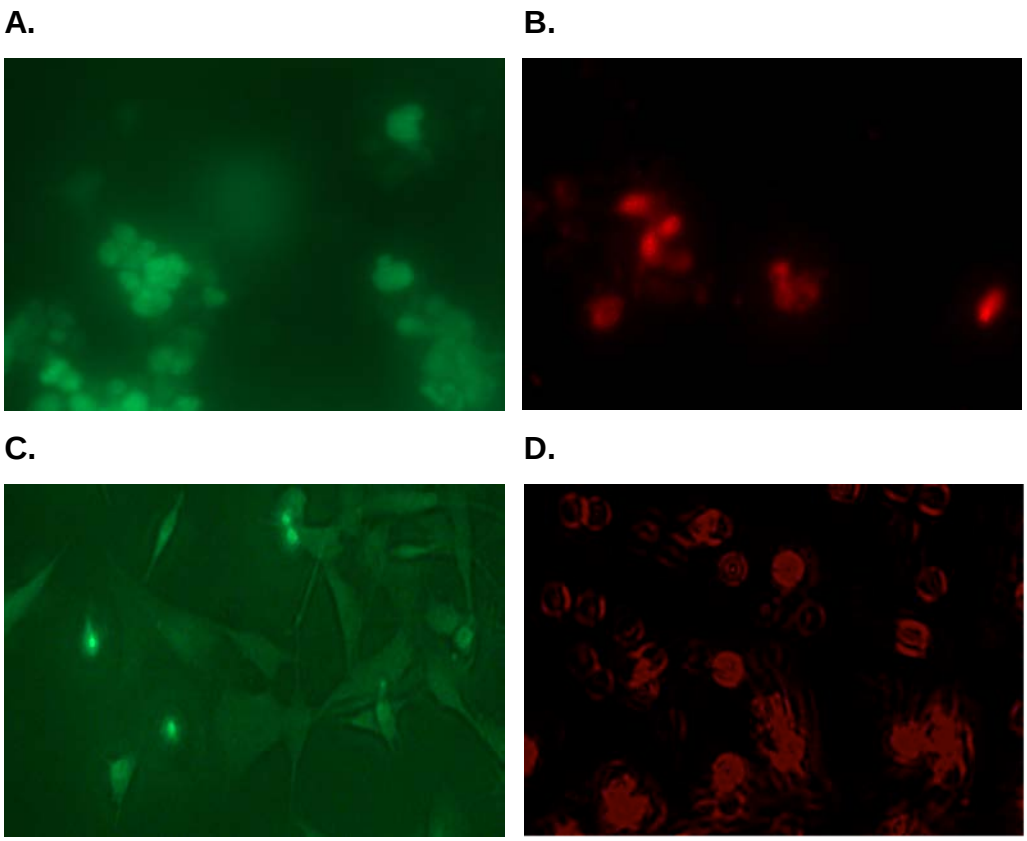
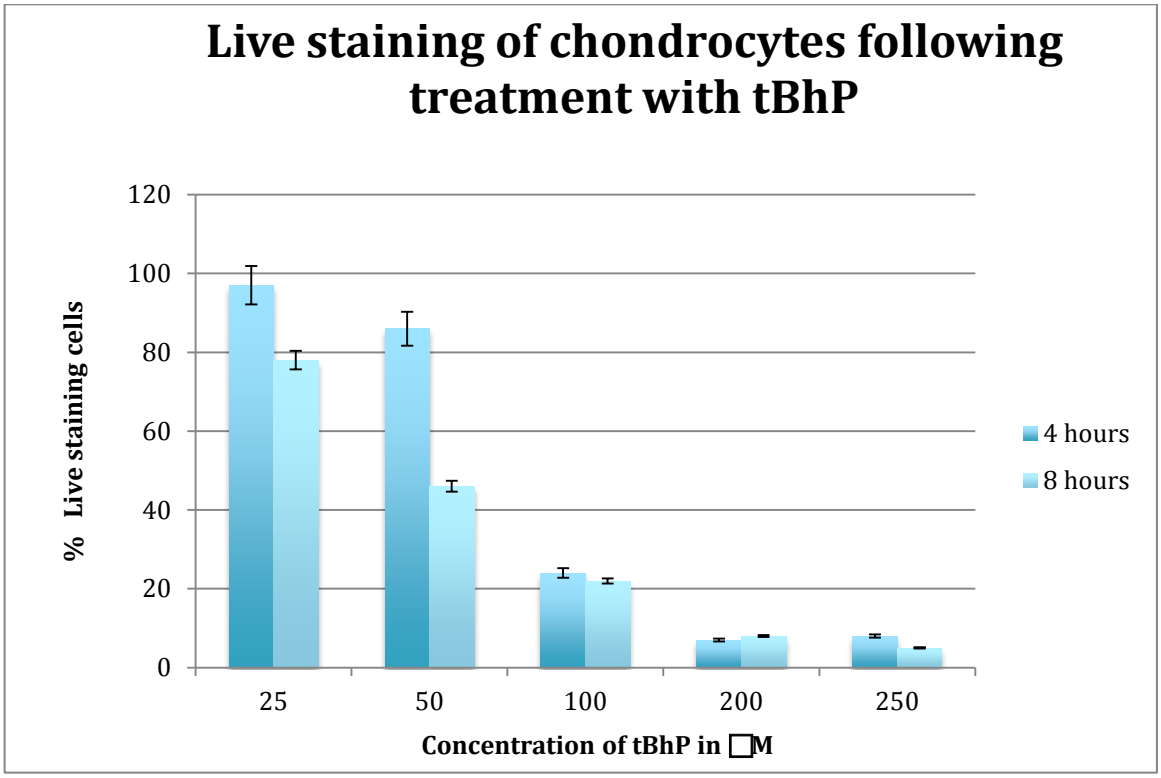


Figure 6-3. Effect of tBhP treatment on viability of chondrocytes in monolayer culture measured by Live/Dead assay.

Treatment of chondrocytes with 25μM of tBhP for a period of four hours resulted in 97% live cell staining. Treatment of chondrocytes with 25μM of tBhP for eight hours decreased the percentage of live cell staining to 78%. Treatment of chondrocytes with 50μM for a period of four hours resulted in 86% live cell staining. Treatment of chondrocytes with greater than 100μM of tBhP for a period of four hours or greater resulted in high levels of cell death, with live cell staining of 24% or less. Treatment with 25μM of tBhP for a period of four hours preserved 97% live cells. Dark blue bars show the percentage of live staining after 4 hours of incubation and light blue bars show the percentage of live staining after eight hours of incubation for each concentration of tBhP (25μM, 50μM, 100μM, 200μM and 250μM).



6.4.2. Induction of oxidative stress in normal chondrocytes by treatment with *tert*-Butyl hydroperoxide increased levels of cellular senescence in treated chondrocytes.

To investigate the hypothesis that oxidative stress results in cellular senescence in canine chondrocytes, oxidative stress was induced in normal canine chondrocyte cell lines by treatment of cells with 25μM of tBhP for a period of four hours. This resulted in a four-fold decrease in total cellular glutathione concentration and live cell staining of 97%, indicating that oxidative stress had been induced and that the chondrocytes were still viable following treatment. Levels of cellular senescence were measured in chondrocytes following induction of oxidative stress using tBhP by measurement of SA-beta-gal positivity and replicative capacity. Untreated matched chondrocyte samples were used as a control. Following induction of oxidative stress in normal chondrocytes using tBhP, the amount of SA-beta-gal activity increased from between 0-4% prior to tBhP treatment, to greater than 76% positive staining for SA-beta-gal in all three cell lines (Table 6-2). Assessment of the replicative lifespan of normal canine chondrocytes following treatment with tBhP showed that chondrocytes with experimentally induced oxidative stress underwent replicative senescence significantly earlier than untreated chondrocytes ($p=0.01$). The number of population doublings in monolayer culture to the onset of replicative senescence and the number of days in monolayer culture to the onset of replicative senescence were measured to assess replicative lifespan. The tBhP treated chondrocytes underwent replicative senescence significantly earlier than untreated chondrocytes, after between 1.89 and 2.4 population doublings ($p=0.01$), shown in Table 6-2. The tBhP treated chondrocytes also underwent replicative senescence, significantly earlier than the untreated chondrocytes, after 13 to 15 days in culture, shown in Figure 6-4.

6.4.2.1. Normal chondrocyte cell lines consistently showed significantly higher amounts of SA-beta-gal activity following treatment with tBhP compared to untreated chondrocytes.

Induction of oxidative stress in normal chondrocytes by treatment with 25μM of tBhP for a period of four hours resulted in significantly increased amounts of SA-beta-gal activity in tBhP treated chondrocytes compared to untreated normal chondrocytes ($p=0.01$). Chondrocytes were grown on glass coverslips in monolayer culture and stained for SA-beta-Gal (as described in Materials and Methods) and the senescent cell state was established by visual identification of a perinuclear blue dye by light microscopy at 40X

magnification. SA-beta-gal levels for each sample were calculated as a mean percentage of positively staining cells per high power field, by analysis of three high power fields per slide. Two slides were examined for each chondrocyte sample. Prior to treatment with tBhP, three foetal chondrocyte cell lines (F1, F2 and F3) were stained for Sa-beta-gal. Cell lines F1 and F2 had no positive Sa-beta-gal staining prior to tBhP treatment and cell line F3 had six percent positively stained cells for Sa-beta-gal. Following incubation with 25μM tBhP for four hours, all three cell lines had greater than 76% positive staining, with 76%, 86% and 84% Sa-beta-gal positive staining for cell lines F1, F2 and F3 respectively. These data show that treatment of normal canine chondrocytes with tBhP resulted in significantly increased amounts of SA-beta-gal positivity. It can therefore be concluded that experimental induction of oxidative stress in monolayer cultured normal canine chondrocytes resulted in an increased amount of cellular senescence.

Table 6-2. Effect of tBhP treatment on senescence associated beta-galactosidase staining in chondrocytes in monolayer cell culture.
The percentage of SA-beta-gal positive staining cells, before and after treatment with tBhP, is shown. Monolayer cultures of treated and untreated chondrocytes were stained for SA-beta-gal using a commercial kit. The mean SA-beta-gal positive staining for each cell sample is shown. Prior to treatment with tBhP, there were low levels of SA-beta-gal positivity, with no positive staining in both F1 and F2 chondrocytes, and 6% positive staining in F3 chondrocytes. Following treatment with tBhP there was a significant increase in SA-beta-gal positive staining (p=0.01). 76% of tBhP treated F1 cells, 86% of tBhP treated F2 cells and 84% of tBhP treated F3 cells showed SA-beta-gal positive staining.

Cell line	% of SA-beta-gal positivity before treatment	% of SA-beta-gal positivity after tBhP treatment
F1	0	76
F2	0	86
F3	6	84

6.4.2.2 Normal chondrocyte cell lines showed significantly decreased replicative potential following treatment with tBhP compared to untreated chondrocytes.

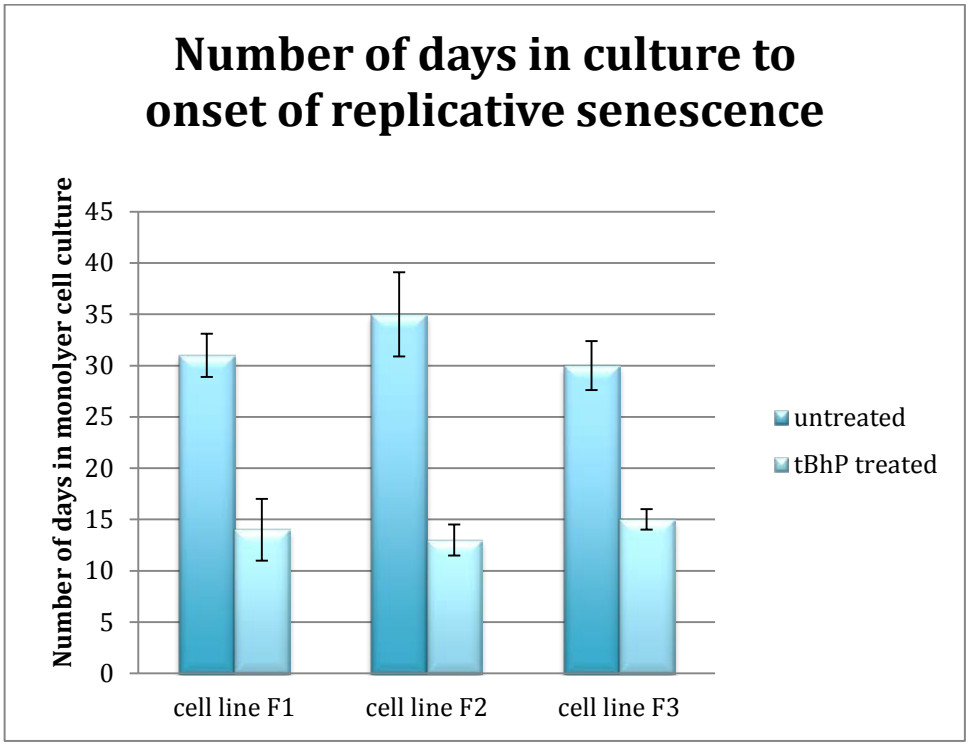
Assessment of the replicative lifespan of normal canine chondrocytes following treatment with tBhP showed that chondrocytes with experimentally induced oxidative stress underwent replicative senescence significantly earlier than untreated chondrocytes. Oxidative stress was induced in normal canine chondrocyte cell lines by treatment of three foetal cell lines with 25μM of tBhP for a period of four hours. In order to compare the in vitro life span of tBhP-treated normal chondrocytes with untreated normal chondrocytes (F1, F2 and F3), three untreated cell lines and three tBhP treated cell lines were cultured to senescence in monolayer cell culture as previously described (Chapter IV, Materials and Methods). Population doublings to senescence were calculated for each of the cell lines; untreated F1, F2, and F3 cells and tBhp treated F1, F2 and F3 cells, as shown in Table 6-3. The untreated F1, F2, and F3 chondrocytes underwent replicative senescence after 14.1, 15.68 and 18.6 population doublings respectively. The tBhP treated F1, F2, and F3 chondrocytes underwent replicative senescence after 1.89, 2.14 and 2.4 population doublings, showing a significant decline in replicative capacity in canine chondrocytes following treatment with tBhP ($p=0.01$). Similarly, untreated chondrocytes underwent replicative senescence after 30 to 35 days in culture, shown in Figure 6.4. The tBhP treated chondrocytes underwent replicative senescence after 13 to 15 days in culture, showing a significant decline in replicative capacity in canine chondrocytes following treatment with tBhP ($p=0.01$). By extension, it can be concluded that experimental induction of oxidative stress in monolayer cultured normal canine chondrocytes resulted in an increased amount of cellular senescence evidenced as decreased replicative capacity in this experiment.

Table 6-3. Effect of tBhP treatment on replicative life span of chondrocytes in monolayer cell culture.

Untreated chondrocytes and tBhP treated chondrocytes were cultured to replicative senescence in monolayer culture. The number of population doublings to failure of cellular replication for the three untreated and the three tBhP treated cell lines is shown. Three untreated (F1, F2 and F3) chondrocyte cell lines underwent replicative senescence after between 14.1 to 18.6 population doublings. The tBhP treated (F1, F2 and F3) chondrocytes underwent replicative senescence after between 1.89 and 2.4 population doublings, showing a significant decline in replicative capacity ($p=0.01$) of chondrocytes following treatment with tBhP.

Cell line	Population Doublings to senescence	Population doublings to senescence after tBhP treatment
F1	15.68	1.89
F2	18.6	2.14
F3	14.1	2.4

Figure 6-4. Effect of tBhP treatment on number of days in culture to onset of replicative senescence of chondrocytes in monolayer cell culture.
The number of days in monolayer cell culture to onset of cellular senescence of three foetal chondrocyte cell lines before and after treatment with tBhP is shown. Untreated cell lines (F1, F2 and F3 dark blue bars on graph) underwent replicative senescence after 31, 35 and 30 days respectively. The tBhP treated cell lines (F1, F2 and F3 light blue bars on graph) underwent replicative senescence after 14, 13 and 15 days respectively, showing a significant decline in replicative capacity ($p=0.01$) of chondrocytes following treatment with tBhP across the three cell lines.



6.4.3. OA chondrocytes did not have significantly decreased levels of cellular glutathione compared to normal chondrocytes.

To investigate the hypothesis that oxidative stress is a feature of canine OA chondrocytes, the concentration of total cellular glutathione was measured in OA and normal canine chondrocytes. These data established that oxidative stress is a feature of both OA and normal chondrocytes. Initial concentrations of total cellular glutathione were not significantly different for OA (OA65, OA74, OA26 and OA78) and normal (N1, N6, N4 and N5) canine chondrocytes, ranging from 13.95nM to 29.8nM, as shown in Table 6-4.

Table 6-4. Total cellular glutathione levels in normal and OA canine chondrocytes detected by spectrophotometry.
Total cellular glutathione was measured in OA and normal chondrocytes grown in monolayer culture using a commercial kit and spectrophotometry. OA chondrocytes had similar concentrations of total cellular glutathione compared to normal chondrocytes.

Cell Line	Total cellular glutathione (nM per 10^8 cells)
N1	28.42
N6	19.14
N4	22.93
N5	23.66
OA65	13.95
OA74	29.80
OA26	27.38
OA78	10.80

6.4.4 OA chondrocytes had decreased antioxidant capacity compared to normal chondrocytes.

In order to investigate the hypothesis that OA chondrocytes have increased levels of oxidative stress compared to normal chondrocytes, four OA cell lines and 4 normal cell lines were treated with tBhP to induce oxidative stress. Oxidative stress occurs when the antioxidative capacity of cells is depleted. In accordance with our hypothesis, OA cells would thus have depleted antioxidative capacity predisposing them to increased levels of oxidative stress and increased levels of cellular senescence following treatment with tBhP. The impact of experimentally induced oxidative stress (by treatment with 25μM of tBhP for a period of four hours) on total cellular glutathione concentration, cell viability and the amount of cellular senescence in chondrocytes collected from normal and OA canine chondrocytes was investigated using a glutathione assay, Live/Dead cell staining, SA-beta-gal staining, and measurement of replicative capacity (as described in Materials and Methods).

Total cellular glutathione concentration was significantly reduced in cell lines following treatment of cells with tBhP. There was a 2.68 (or greater)-fold decrease in total cellular glutathione concentration for all cell lines. Interestingly, initial concentrations of glutathione (prior to tBhP treatment) were similar for both OA and normal chondrocytes (from 13.95μM to 29.8μM), as shown in Table 6-5. However, OA chondrocytes showed a significantly greater decrease in total cellular glutathione concentration following tBhP treatment, in comparison to normal chondrocytes ($p<0.01$). The mean fold decrease in total cellular glutathione concentration over the four normal chondrocyte cell lines was 4.51, while the mean fold decrease over the four OA chondrocyte cell lines was 33.05. These data demonstrate a clear difference in the response of OA and normal chondrocytes to oxidative stress.

Measurement of cell viability by Live/Dead cell staining (as per methodology described in Materials and Methods) of normal and OA chondrocytes following tBhP treatment showed decreased cell viability in OA chondrocytes compared to normal chondrocytes. The percentage of positive staining live and dead cells was calculated for each chondrocyte sample following tBhP treatment, as shown in Figure 6-5. The percentage of live staining cells varied from 74% to 94% in normal chondrocytes following oxidative stress induction. OA chondrocytes showed lower percentages of live cell staining of between 38% to 56%.

These results showed that normal chondrocytes have higher numbers of viable cells following induction of oxidative stress by treatment with tBHP compared to OA chondrocytes ($p<0.01$).

OA chondrocytes were associated with similar increases in the amount of of SA-beta-gal activity following oxidative stress to normal chondrocytes, with no significant difference between the increase in SA-beta-gal staining between normal and OA chondrocytes following treatment with tBhP (Table 6-6). Prior to treatment with tBhP the normal chondrocytes showed between 0% and 10 % SA-beta-gal positivity. The OA cells showed between 9% and 51 % positivity. Following tBhP incubation the amount of SA-beta-gal activity increased for all cells lines, with between 92% and 100% of cells staining positive for SA-beta-gal. All four normal and four OA cell lines showed significantly greater amounts ($p<0.01$) of SA-beta-gal positivity indicating an increased amount of senescence across all cell lines following treatment with tBhP.

OA chondrocytes showed significantly decreased replicative capacity following treatment with tBhP, in comparison to normal chondrocytes. This was evidenced as a lower number of population doublings and fewer days in monolayer culture to onset of cellular senescence. Population doublings to senescence were calculated for each of the OA (OA65, OA74, OA26 and OA78) and normal (N1, N6, N4 and N5) cell lines as shown in Table 6-7. The normal N1, N6, N4 and N5 chondrocytes underwent replicative senescence after 15.68, 5.11. 2.81 and 3.17 population doublings prior to tBhP treatment and 4.22, 1.9, 1.73 and 2.81 population doublings after tBhP treatment, respectively. The OA chondrocytes, OA65, OA74, OA26 and OA78, underwent replicative senescence after 4.93, 1.51, 1.15 and 1.99 population doublings prior to tBhP treatment and 2.4, 1.26, 1.02 and 0.57 population doublings after tBhP treatment, respectively. These data show a significant decline in replicative capacity in both OA and normal canine chondrocyte cell lines following treatment with tBhP ($p<0.01$). Similarly, both normal and OA chondrocytes underwent replicative senescence fewer days in culture following tBhP treatment, shown in Figure 6-6.

These data suggest that OA cells have a greater amount of pre-existing oxidative stress than normal cells as they have less antioxidant capacity, suggested by an increased rate of cell death after treatment with tBhP and an increased amount of cellular senescence after treatment with tBhP when compared to normal chondrocytes.

Table 6-5. Effect of tBhP treatment on total cellular glutathione levels in normal and OA canine chondrocytes in monolayer cell culture.

OA (OA65, OA74, OA26 and OA78) and normal (N1, N6, N4 and N5) chondrocytes were incubated with 25μM tBhP for four hours to induce oxidative stress and total cellular glutathione concentration was measured for each cell sample using a glutathione assay kit and spectrophotometry. The quantity of total cellular glutathione was significantly reduced following treatment of cells with tBhP for all cell lines ($p<0.01$). The mean fold decrease in total cellular glutathione concentration over the four normal chondrocyte cell lines was 4.51, while the mean fold decrease over the four OA chondrocyte cell lines was 33.05.

Cell Line	Total cellular glutathione (nM per 10^8 cells) before tBhP treatment	Total cellular glutathione (nM per 10^8 cells) following tBhP treatment	Fold decrease in total cellular glutathione (nM per 10^8 cells) following treatment with tBhP
N1	28.4	4.22	6.73
N6	19.09	5.38	3.55
N4	22.91	8.55	2.68
N5	23.66	4.64	5.10
OA65	13.95	0.48	29.06
OA74	29.80	0.55	54.18
OA26	27.38	0.84	32.59
OA78	10.80	0.66	16.36

Figure 6-5. Effect of tBhP treatment on Live/Dead staining of normal and OA canine chondrocytes in monolayer cell culture.

OA (OA65, OA74, OA26 and OA78) and normal (N1, N6, N4 and N5) chondrocytes were assayed for cell viability following treatment with tBhP to induce oxidative stress by Live/Dead cell staining. Live/Dead cell staining was calculated as a percentage of positively staining cells. Before treatment with tBhP 99% to 100% live cell staining was confirmed for all cell lines. Following treatment with tBhP the percentage of live staining cells varied from 74% to 94% in normal chondrocytes and from 38% to 56% in OA chondrocytes.

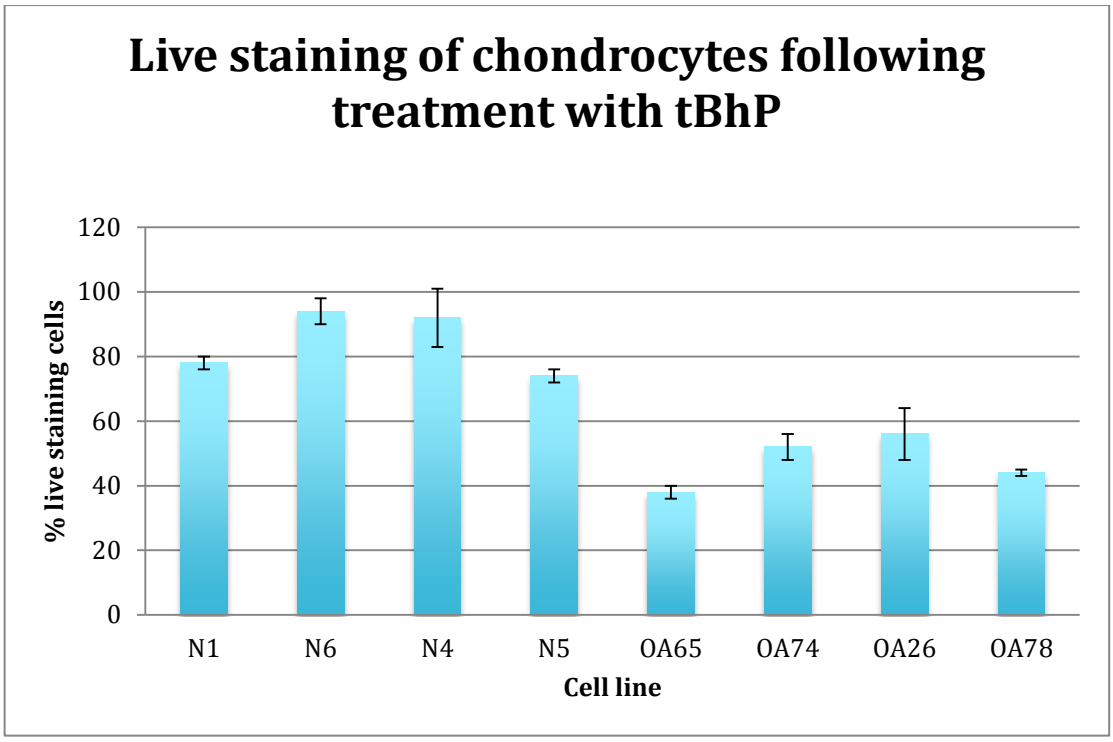


Table 6-6. Effect of tBhP treatment on SA-beta-gal positivity of normal and OA canine chondrocytes in monolayer cell culture.

The senescent cell state was established by staining of monolayer chondrocyte cultures fixed on a microscope slide using a commercial kit. The percentage of SA-beta-gal positivity in the four OA (OA65, OA74 OA26 and OA78) and four normal (N1, N6, N4 and N5) chondrocyte cell lines before and after exposure to tBhP is shown below. Prior to treatment with tBhP the normal chondrocytes showed between 0% and 10 % SA-beta-gal positivity. The OA cells showed between 9% and 51 % positivity. Following tBhP incubation the amount of SA-beta-gal activity increased for all cells lines, with between 92% and 100% of cells staining positive for SA-beta-gal. All four normal and four OA cell lines showed significantly greater amounts (p=0.01) of SA-beta-gal positivity following tBhP treatment indicating an increased amount of senescence across all cell lines following treatment with tBhP.

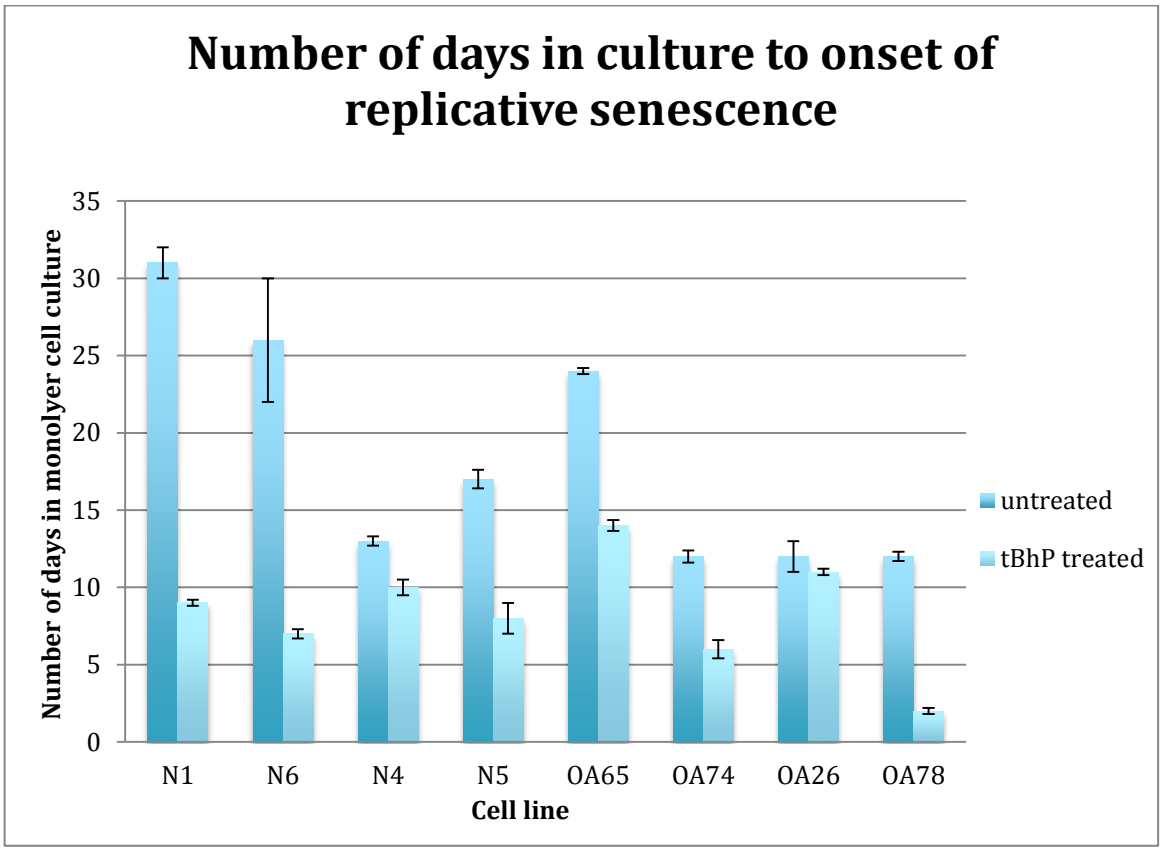
Cell line	% SA-beta-gal positivity before treatment	% SA-beta-gal positivity after tBhP treatment
N1	0	92
N6	0	72
N4	10	83
N5	4	79
OA65	51	100
OA74	9	75
OA26	24	87
OA78	40	72

Table 6-7. Effect of tBhP treatment on number of population doublings to onset of replicative senescence of normal and OA canine chondrocytes in monolayer cell culture.

Four OA (OA65, OA74 OA26 and OA78) and four normal (N1, N6, N4 and N5) chondrocyte cell lines were treated with tBhP and then cultured to replicative senescence in monolayer culture. The number of population doublings to failure of cellular replication for all cell lines is shown. The OA chondrocyte cell lines, OA65, OA74 OA26 and OA78, underwent replicative senescence after between 0.57 and 2.4 population doublings following induction of oxidative stress with tBhP. The normal chondrocyte cell lines, N1, N6, N4 and N5, underwent replicative senescence after between 1.73 and 4.24 population doublings following induction of oxidative stress with tBhP. Normal cell lines underwent a greater reduction in replication rate following induction of oxidative stress with tBhP compared to OA chondrocytes.

Cell line	Population Doublings to senescence	Population doublings to senescence after tBhP treatment
N1	15.68	4.24
N6	5.11	1.9
N4	2.81	1.73
N5	3.17	2.81
OA65	4.93	2.4
OA74	1.51	1.26
OA26	1.15	1.02
OA78	1.99	0.57

Figure 6-6. Effect of tBhP treatment on number of days in monolayer culture to onset of cellular senescence of normal and OA canine chondrocytes.
The number of days in monolayer cell culture to onset of cellular senescence of four OA (OA65, OA74 OA26 and OA78) and four normal (N1, N6, N4 and N5) chondrocyte cell lines before and after treatment with tBhP is shown. Untreated OA cell lines underwent replicative senescence after between 12 and 24 days while tBhP treated OA cells underwent replicative senescence after between 2 and 14 days. Untreated normal cell lines underwent replicative senescence after between 13 and 31 days while tBhP treated normal cells underwent replicative senescence after between 7 and 10 days. All cell lines showed a significant decline in replicative capacity following treatment with tBhP ($p<0.01$).



6.4.5 Induction of oxidative stress in normal canine chondrocytes using tBhP caused altered expression of MMP-3, MMP-13 and Col-3A1 genes.

In order to investigate the effect of oxidative stress on the pathogenesis of canine OA, the differential gene expression profiles of three genes specific to the pathogenesis of OA were measured in normal canine chondrocytes following experimental induction of oxidative stress. More specifically, the hypothesis that oxidative stress alters the extracellular matrix secretory function of canine articular chondrocytes by altering the gene expression profile of MMP-3, MMP-13 and Col-3A1 genes was investigated using RT-PCR. Oxidative stress was induced in three normal chondrocyte cell lines in monolayer culture by incubation with 25μM tBhP for four hours. An untreated normal chondrocyte sample was used as a relative control. MMP-13 (Figure 6-7A) and MMP-3 (Figure 6-7B) genes were up-regulated in normal canine chondrocytes treated with tBhP ($p < 0.05$). Col-3A1 gene expression was down-regulated (Figure 6-7C) in normal canine chondrocytes treated with tBhP ($p < 0.05$). MMP-3 showed the greatest relative expression change following experimental induction of oxidative stress using tBhP, with a fold-change of between 1.43 and 4.78. MMP-13 had a fold change of 1.16 to 1.38. Interestingly, Col-3A1 was down regulated, with a fold-change of between 0.21 and 0.31. These data demonstrate that experimentally induced oxidative stress in chondrocytes in monolayer culture alters the expression of genes relevant to the pathogenesis of canine OA.

Figure 6-7. Effect of tBhP treatment on the relative gene expression of MMP-13, MMP-3 and Col-3A1 genes in normal canine chondrocytes.
The graphs below show the results of RT-PCR analysis of gene expression levels of A. MMP-13, B. MMP-3 and C. Col-3A1 genes in normal canine chondrocytes with experimentally induced oxidative stress. The data are presented as several fold changes, the RQ or relative quantification value, of gene expression relative to the control cells. GAPDH served as the internal control. Data represent the mean of the results obtained from three replicate experiments, error bars indicate standard deviations from the mean. Figures 6.A and B show a relative increase in gene expression of MMP-13 and MMP-3 respectively. Figure 6.C shows a relative decrease in expression of Col-3A1 relative to the control population.

Figure 6-7A. MMP-13 relative gene expression in tBhP treated chondrocytes.
MMP-13 had a fold change in gene expression of between 1.16 and 1.38.

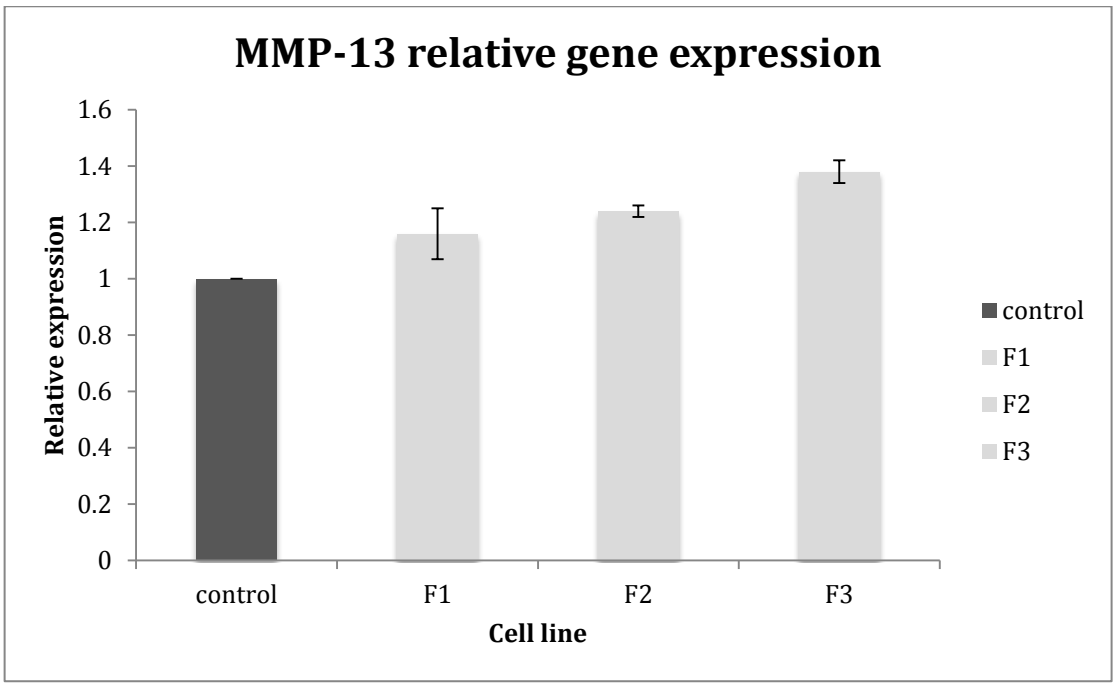


Figure 6-7B. MMP-3 relative gene expression in tBhP treated chondrocytes. MMP-3 showed the greatest relative expression change in chondrocytes treated with tBhp, with a fold-change of between 1.43 and 4.78.

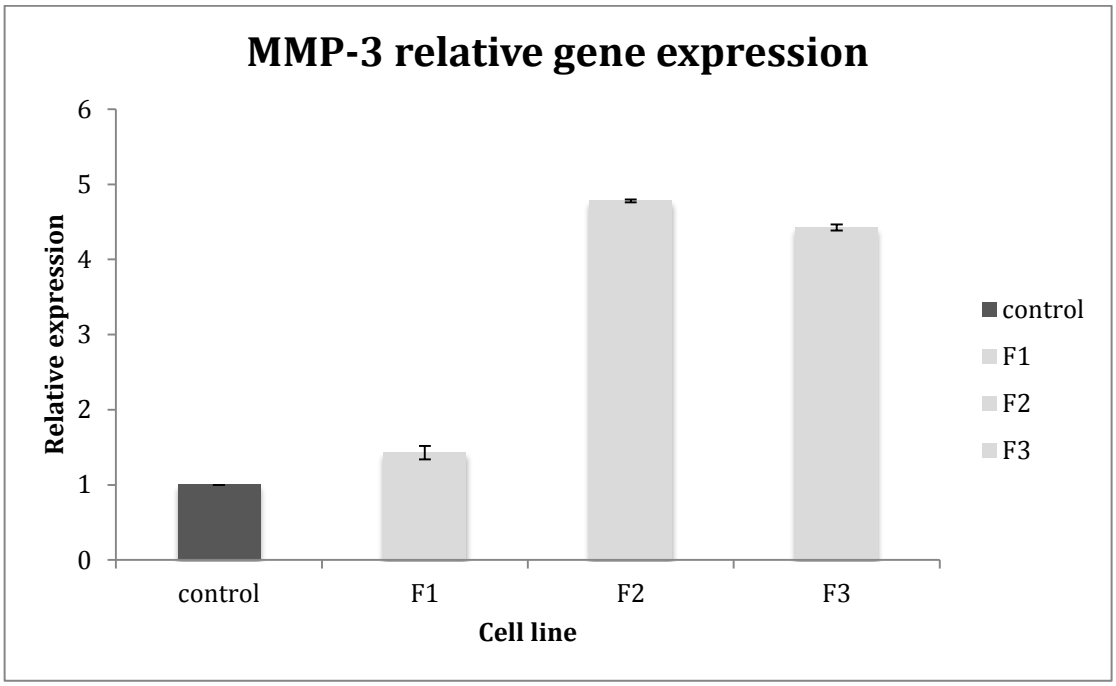
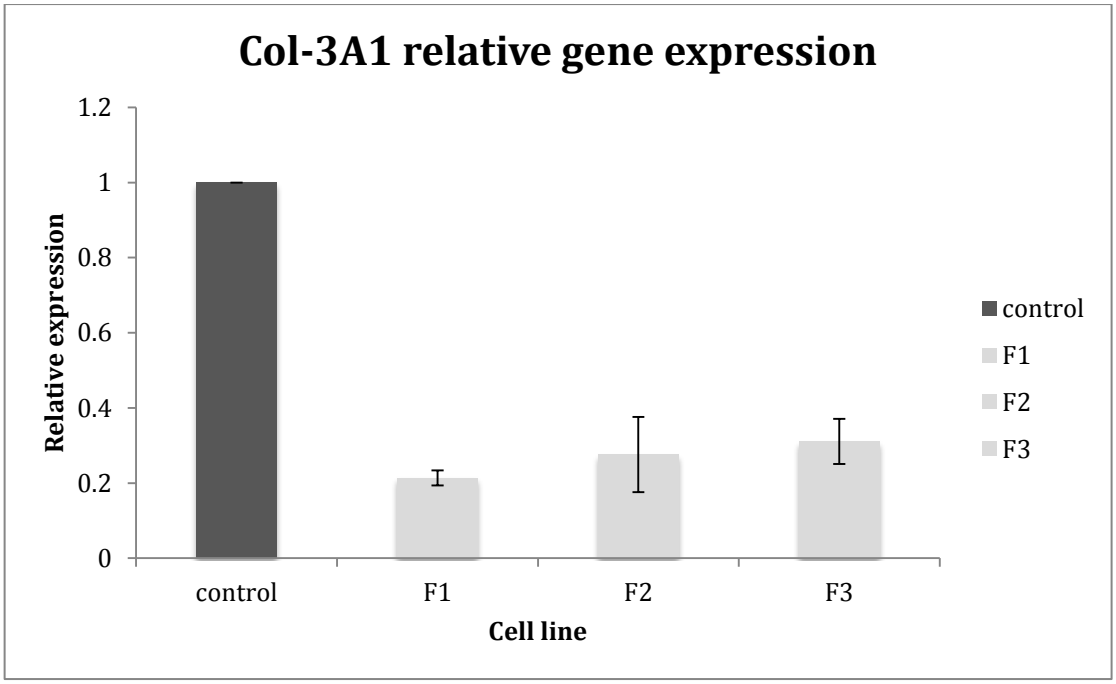


Figure 6-7C. Col-3A1 relative gene expression in tBhP treated chondrocytes. Col-3A1 gene expression was down regulated in chondrocytes following treatment with tBhp, with a fold-change of between 0.21 and 0.31.



6.5 Discussion

There is extensive evidence that oxidative stress plays a role in the pathogenesis of OA in humans (Carlo and Loeser, 2003; Henrotin et al., 2003; Henrotin et al., 2005; Studer et al., 1999; Vuolteenaho et al., 2007; Ziskowen, 2010), however little is known about the role of oxidative stress in the pathogenesis of canine OA. It has been shown that dogs with experimentally induced OA had systemic elevation of MDA, a peroxidated lipid (Goranov, 2007). The aim of this chapter was to investigate the role of oxidative stress in the pathogenesis of canine OA. It is known that inflammation leads to oxidative stress in many disease processes, including OA. Having established that cellular senescence is a feature of canine OA chondrocytes in Chapter IV, our hypothesis was that inflammation that occurs in OA causes oxidative stress which in turn causes senescence of canine chondrocytes. Our hypothesis was investigated by determining if experimentally induced oxidative stress resulted in an increased amount of senescence in normal canine chondrocytes in vitro. Additionally, we investigated if OA chondrocytes had greater amounts of cellular senescence compared to normal chondrocytes and if OA chondrocytes had less antioxidant capacity compared to normal chondrocytes. Finally, we examined the impact of experimentally induced oxidative stress on the expression level of three genes key to early OA pathogenesis in normal cultured canine chondrocytes.

6.5.1 Treatment of canine chondrocytes with tBhP caused oxidative stress and induced cellular senescence.

Experiments to establish an oxidative stress model for in vitro cell culture using tBhP showed a concentration of 25μM tBhP applied for four hours to monolayer cultured chondrocytes was required to induce oxidative stress while maintaining cell viability. This treatment protocol resulted in oxidative stress, defined as a four-fold decrease in intracellular glutathione concentration with a survival rate of 97% for treated chondrocytes. Various protocols have been reported in the literature for oxidative stress induction in chondrocytes *in vitro* using tBhP at a wide range of concentrations, including the concentration shown to be optimal for oxidative stress induction in canine chondrocytes with maintenance of cell viability in these experiments (Kurz *et al.*, 2004; Yammani and Loeser, 2014; Yin *et al.*, 2009). To investigate if oxidative stress can induce cellular senescence in canine chondrocytes, the amount of cellular senescence present in three normal chondrocyte cell lines was measured, following the induction of oxidative

stress with tBhP. Experimental induction of oxidative stress resulted in the onset of premature senescence of the normal canine chondrocytes in vitro. This was shown by a significant increase in SA-beta-gal staining from 0%-6% prior to tBhP treatment, to 72% to 84% positive staining after tBhP treatment ($p<0.01$). Assessment of the replicative capacity of chondrocytes, following induction of oxidative stress with tBhP showed a significant decrease in the number of population doublings to onset of replicative senescence from between 14.1 and 18.6 population doublings in the untreated chondrocytes to between 1.89 and 2.4 population doublings in the tBhP treated chondrocytes. These results showed a decrease in replicative capacity and increase in SA-beta-gal positivity of normal chondrocytes following experimental induction of oxidative stress, proving our hypothesis that oxidative stress plays a role in inducing cellular senescence in normal canine chondrocytes in vitro.

6.5.2 OA chondrocytes did not have a significantly greater amount of total cellular glutathione when compared to normal chondrocytes but had decreased antioxidant capacity compared to normal chondrocytes.

In order to investigate the hypothesis that OA chondrocytes have increased amounts of oxidative stress compared to normal chondrocytes, the concentration of total cellular glutathione in normal and OA chondrocytes collected from articular cartilage of coxofemoral joints was measured. OA chondrocytes did not have a significantly higher concentration of total cellular glutathione compared to normal chondrocytes. This finding was in contrast to the results of multiple studies investigating the amount of oxidative stress in human chondrocytes. In humans, it has been demonstrated that OA chondrocytes contain increased concentrations of ROS compared to normal chondrocytes (Ruiz- Romero *et al.,* 2009). Oxidative stress in OA chondrocytes is thought to occur due to increased aerobic cellular respiration secondary to inflammation (Le Grand *et al.,* 2001), leading to an increase in the amount of ROS production by chondrocyte mitochondria. Increased concentrations of lipid peroxidation products have also been detected in human OA cartilage, indicating an increased amount of oxidative stress (Bae *et al.,* 2003; Loeser *et al.,* 2002). Lipid peroxidation products are thought to play a role in OA via extracellular matrix breakdown (Morquette *et al.,* 2006; Pinto *et al.,* 2008; Rubyk *et al.,* 1988; Shah *et al.,* 2005; Surapaneni and Venkataramana, 2007; Tiku *et al.,* 2000;) and circulating products of lipid peroxidation such as malondialdehyde and acylhydroperoxide (Pinto, 2008; Surapaneni and Venkataramana, 2007) and other ROS products were found to be increased

in joints and biological fluid samples from humans with OA (Otte, 1991; Pufe *et al.,* 2004; Safran, 2003). A further study showed that the concentration of hydrogen peroxide is increased in humans with OA (Wang *et al.,* 2002). The increased amount of ROS found in OA chondrocytes in man, also occurs in the synovium of joints affected by OA. Inflammation of the synovium has been associated with an increase in synovial fluid oxygen tension and a pathological increase in aerobic metabolism in chondrocytes (Muir, 1995). Synovitis mediated by NF-κ-B, resulted in NO secretion and an increased concentration of NO in human synovial fluid (Carlo and Loeser, 2003). Increased amounts of ROS and RNS have also been shown to occur in articular cartilage as a result of excessive mechanical loading and shearing forces (Healy *et al.,* 2005; Tomiyama *et al.,* 2007). It is possible that the amount of oxidative stress is not increased in canine OA chondrocytes compared to normal chondrocytes, as was suggested in the current study. However, only eight cell lines were examined in these experiments. For conclusive evidence that this is in fact the case, a much greater number of OA and normal chondrocyte samples should be examined. Another possible explanation for this result is the highly dynamic state of ROS in biological systems. Some human studies investigating the amount of oxidative stress in OA chondrocytes have shown conflicting results as to whether levels of ROS are increased or decreased. It was demonstrated that OA joints had decreased concentrations of antioxidants in one study (Yudoh *et al.,* 2005), while another showed an increase in antioxidants in OA (Regan *et al.,* 2005). One study found that SOD was increased in human OA chondrocytes (Mazzetti *et al.,* 2001) while reduced concentrations of SOD were demonstrated in a subsequent study (Scott *et al.,* 2010).

These findings are potentially explained by the fact that both ROS and antioxidants are in a state of flux, with constant up and down regulation of intracellular ROS scavengers. Concentrations of ROS and antioxidants vary in disease states and also temporally, which can lead to conflicting or equivocal study results. This may explain the disparity between our findings for canine OA chondrocytes and those reported for human OA chondrocytes. In order to investigate the hypothesis that OA chondrocytes have decreased antioxidant capacity compared to normal chondrocytes, four OA cell lines and four normal cell lines were treated with tBhP to induce oxidative stress according to the protocol outlined previously. The effect of oxidative stress on total cellular glutathione levels in normal canine chondrocytes and OA canine chondrocytes was compared following tBhP treatment and the resultant drop in total cellular glutathione concentration in each cell line was then measured. The replicative capacity and SA-beta-gal positivity of cells following antioxidant treatment was also examined. Our findings showed that OA chondrocytes had

a significantly greater decrease in total cellular glutathione concentration as compared to normal chondrocytes following induction of oxidative stress by treatment with tBhP. Additionally, OA chondrocytes had a significantly greater amount of cell death compared to normal chondrocytes following experimental induction of oxidative stress. Both OA and normal canine chondrocytes showed a significant decrease in replicative capacity following treatment with tBhP, with a decrease in the number of population doublings to the onset of replicative senescence and a decrease in the number of days in monolayer culture to onset of replicative senescence. The decrease in replicative capacity following treatment with tBhP was more pronounced in the normal chondrocytes than the OA chondrocytes. Experimental induction of oxidative stress by treatment of monolayer chondrocyte cultures with tBhP also resulted in an increased amount of cellular senescence in both normal chondrocytes and OA chondrocytes. Both normal and OA chondrocytes had a significantly increased amount of SA-beta-gal activity, with a mean of 81.5% SA-beta-gal positivity in the normal chondrocytes and 83.5% SA-beta-gal positivity in the OA chondrocytes. There was no clear relationship between SA-beta-gal levels before and after treatment of both OA and normal cells with tBhP. Findings for canine chondrocytes with experimentally induced oxidative stress are similar to those reported for human chondrocytes with experimentally induced oxidative stress. Human OA chondrocytes have been shown to have decreased antioxidant capacity when compared to normal chondrocytes, with decreased concentrations of antioxidant enzymes (Ruiz- Romero *et al.*, 2009; Shah *et al.*, 2005; Yudoh *et al.*, 2005). It is likely that the impact of oxidative stress upon OA chondrocyte replicative capacity is less pronounced when compared to normal chondrocytes as seen in the experiments outlined here, because OA chondrocytes have pre-existing oxidative stress and cellular senescence (as discussed in detail in Chapter IV) prior to tBhP treatment.

As discussed in the introduction, measurement of oxidative stress is difficult as the redox state of a cell or body tissue is in a constant state of flux. While total cellular glutathione has been utilised as a measure of oxidative stress in cells, it should be noted that levels of total cellular gluathione are influenced by a multitude of factors and homeostasis of glutathione involves a complex metabolic pathway (Meister and Anderson, 1983). The cellular glutathione level occurs as a balance between glutathione synthesis as well as consumption (as occurs in the scavenging of free radicals in oxidative stress). Synthesis of cellular glutathione is affected by physiological and nutritional state, so levels of total cellular glutathione may not be truly representative of oxidative stress levels (Ballatori *et al.*, 2009). Additionally, the impact of cell culture techniques upon cellular glutathione

levels is unknown. While these confounding factors should be considered, currently direct measurement of oxidative stress levels relies is not possible and so direct measurement of end products of oxidative stress, as well as indirect measurement of pathway enzymes, are used to assess levels of oxidative stress in cells and tissues (Niki, 2008).

6.5.3 Oxidative stress caused altered expression of MMP-3 MMP-13 and Col-3A1 genes in normal canine chondrocytes.

IL-1 is a catabolic cytokine secreted in response to articular cartilage damage. It has been shown that IL-1 causes NO formation in synovial cells in OA, resulting in increased concentrations of ROS within the joint leading to oxidative stress. IL-1 also causes MMP production by chondrocytes in OA. Up-regulation of collagen production is an early event in OA pathogenesis (discussed in Chapter 1, Pathogenesis of Osteoarthritis), with increased production of Type 3 collagen. Having established that oxidative stress is a feature of OA cartilage in dogs, a study was designed to explore the hypothesis that experimental induction of oxidative stress in normal canine chondrocytes would cause increased expression of MMP and collagen genes. Gene expression levels of MMP-3, MMP-13 and Col-3A1 were measured, following experimental induction of oxidative stress in normal canine chondrocytes, using RT-PCR. There is extensive evidence that MMP-13 and MMP-3 are key players in the pathogenesis of canine OA. MMP-13 (Collagenase 3) degrades Type II collagen and MMP-3 (Stromelysin-1) degrades the laminin and fibronectin components of the extracellular matrix of articular cartilage. In a comparison of human OA cartilage versus normal cartilage using DNA microarray, Aigner showed increased gene expression levels of MMP-3 and MMP-13 (Aigner *et al.*, 2001). A study on altered gene expression in canine articular cartilage following mechanical injury (Burton-Wurster *et al.*, 2005) showed increased expression of 16 genes significant in the pathogenesis of OA, including MMP-3 and MMP-13, while an analysis of gene expression in early experimental OA (Appleton *et al.*, 2007) in dogs showed increased expression levels of MMP-13, with altered production of extracellular matrix collagens. Our data showed that experimental induction of oxidative stress in normal canine chondrocytes resulted in up-regulated gene expression of MMP-13 and MMP-3 genes in normal canine chondrocytes. The collagen gene Col-3A1 was down regulated following experimental induction of oxidative stress in normal canine chondrocytes. Col-3A1 is widely reported to be up-regulated in OA cartilage as part of increased metabolic activity that occurs with osteoarthritis (Aigner *et al.*, 2001; Fukui *et al.*, 2008). Accordingly, an increase in the gene

expression level of Col-3A1 had been anticipated. However, it has been demonstrated that experimental induction of oxidative stress in human trabecular meshwork cells resulted in decreased expression of several extracellular matrix genes, including Col-3A1, (Luna *et al.*, 2009). Oxidative stress has also been shown to regulate collagen synthesis and MMP activity in myocardial cells, with down-regulation of Col-3A1 (Siwik *et al.*, 2001). As such, it is possible that oxidative stress reduces collagen synthesis in multiple disease processes, including OA. It is also possible that expression levels of Col-3A1 vary depending on whether oxidative stress occurs as part of acute or chronic OA. Further study to investigate this question could compare Col-3A1 gene expression levels in canine chondrocytes that had been exposed to acute oxidative stress (as in the current oxidative stress model) and chronic oxidative stress (with development of a chronic oxidative stress cell culture model) to see if the expression levels of Col-3A1 vary with ongoing oxidative stress. Other studies have shown altered gene expression in chondrocytes in response to oxidative stress, demonstrating increased collagen II mRNA and Sox9 mRNA in cultured chondrocytes in response to ROS (Yin *et al.*, 2009).

The overall impact of oxidative stress on the pathogenesis of OA is yet to be fully elucidated. Some reports have suggested a deleterious effect on articular cartilage, while other studies have reported a protective effect. It has been shown that increases in ROS result in increased amounts of chondrocyte cell death and also altered cell function via TGF-β1 suppression and impaired extracellular matrix synthesis (Nakagawa *et al.*, 2010; Studer *et al.*, 1999). Other studies have reported a protective role of ROS species with an associated increase in TGF-beta-1 production and an anti-apoptotic effect (Abramson, 2008; Kuhn *et al.*, 2003). Chondrocyte cell death negatively impacts extracellular matrix production as the number of matrix secreting cells in articular cartilage is reduced. The gene expression studies reported here showed that oxidative stress can alter the expression of genes known to contribute to OA pathogenesis in normal canine chondrocytes in vitro, suggesting that oxidative stress contributes to disease progression. Our finding, that MMPs were up-regulated in canine chondrocytes in response to experimental induction of oxidative stress has been previously reported in the literature for human chondrocytes. ROS have been shown to contribute to cartilage degradation by up-regulation of MMP genes and increased MMP synthesis in human articular chondrocytes (Burkhardt *et al.*, 1986; Sasaki *et al.*, 2011) with amelioration of this effect by inhibition of mitochondrial aerobic respiration (Del Carlo *et al.*, 2002). Elevation of lipid peroxidation products has been associated with collagen oxidation, decreased levels of type II collagen (Tiku *et al.*, 2000) and increased MMP-13 in synovial fluid (Morquette *et. al.*, 2006) of human OA

patients. We can conclude that experimental induction of oxidative stress in normal chondrocytes in cell culture leads to alterations to expression levels of chondrocyte gene that contribute to OA pathogenesis in both dogs and man.

It should be noted that, for all experiments comparing normal and OA chondrocytes, the samples were not age matched. As discussed in Chapter IV Discussion, we know that the amount of cellular senescence of chondrocytes increases with age and also that the amount of oxidative stress of chondrocytes is thought to increase with age. In the present study, a comparison between young and aged chondrocytes was not possible due to the sample size of normal and OA chondrocyte cell lines utilised in the experiments. A study evaluating the relationship between donor age and oxidative stress in normal human articular chondrocytes (Carlo and Loeser, 2003) demonstrated that aged normal chondrocytes had similar concentrations of total cellular glutathione to young normal chondrocytes, but that aged cells had a greater decrease in concentration of total cellular glutathione and underwent an increased amount of cell death following induction of oxidative stress compared to chondrocytes from younger donors. These findings from Carlo and Loeser suggest that aged chondrocytes have increased amounts of oxidative stress resulting in decreased antioxidant capacity. It is thought that this age-related decrease in antioxidant capacity occurs due to age related increases in oxidative stress via dysregulation of the glutathione antioxidant system. As the OA and normal cells compared in this study were not age-matched, it is possible that age related changes contributed to some of the comparisons made, specifically the concentrations of total cellular glutathione in normal and OA chondrocytes. Accordingly, these experiments should be repeated with age-matched controls.

6.5.4 Conclusions

The precise role of oxidative stress in the pathogenesis of osteoarthritis remains unclear, with conflicting reports of both damaging and protective effects. The highly dynamic state of ROS and antioxidants makes these molecules difficult to assess. It has been established that oxidative stress plays a role in OA pathogenesis in humans. The data presented here suggest that this is also true for domestic dogs. Clearly, oxidative stress is a potential therapeutic target for OA treatment in both man and dogs. The use of antioxidant therapy as a treatment for human OA has been investigated with varied results. ROS have been shown to directly degrade the cartilage extracellular matrix by release of aggrecan (Tiku *et al.,* 1999) and collagen (Tiku *et al.,* 2000) from the matrix, while enzyme inhibition and

antioxidant treatment was shown to decrease these effects. Oxidation of collagen by malondialdehyde predisposed it to degradation (Tiku *et. al.*, 2003), while inhibition of ROS by antioxidants decreased collagen degradation (Tiku *et. al.,* 2000). Studies utilising anti-TNF-alpha and IL-1β therapy to reduce ROS in joint fluid in OA were unsuccessful (Calich *et al.,* 2010). It has been demonstrated, in an experimental model of OA in dogs, that NOS inhibition prevents cartilage degradation (Pelletier, 1989). It seems unlikely that complete inhibition of ROS represents the best treatment target for OA given the complex interactions of ROS with other biological pathways and the role of ROS in normal cell homeostasis. More precise knowledge of the role of specific oxidative species in OA pathogenesis would facilitate the development of more targeted therapeutics, as opposed to a blanket blockade of all oxidative molecules. Our understanding of specific oxidative stress molecules and pathways in disease pathogenesis is in its infancy but presents a key area of future research.

6.5.5 Future studies

It would be useful to repeat these experiments with age-matched samples to confirm the results and similarly, the number of cell lines examined for all experiments would ideally be increased, for the reasons outlined above. It would be of interest to unravel the link between oxidative stress and cellular senescence in canine OA to further our understanding of molecular pathways contributing to pathogenesis of OA. In the current study, we examined SA-beta-gal and replicative potential as markers of cellular senescence. However these markers are not pathway specific and do not provide any information on which pathways to cellular senescence are activated in response to oxidative stress. Future studies could investigate the impact of oxidative stress on specific senescence pathways in chondrocytes using immunohistochemistry or western blot analysis to detect the presence of p53 and p16 proteins. Telomere length analysis by RT-PCR or TRF analysis would identify the impact of oxidative stress on telomere lengths in chondrocytes. It would also be interesting to investigate the impact of oxidative stress on the gene expression levels of MMP3, MMP13 and Col3A1 in immortalised canine chondrocytes, in order to identify if alterations in the gene expression levels shown here are mediated by cellular senescence or occur solely due to oxidative stress.

Chapter VII

General Discussion

7.1 General perspectives

There is emerging evidence that cellular senescence plays a role in the pathogenesis of a number of major diseases. There has been significant research into the role of cellular senescence in the pathogenesis of human OA in an attempt to unravel the molecular biology of OA and also as part of the search for new therapeutic targets. Following recent research linking senescence and OA in humans, the aim of this thesis was to investigate the role of cellular senescence in the pathogenesis of canine OA. Extensive evidence has shown that chondrocyte senescence plays a role in the development and progression of human OA. Chondrocyte senescence had not previously been demonstrated to be a feature of canine OA. In the hunt for new therapeutic targets for this high morbidity and high mortality disease, it was deemed valuable to investigate if senescence plays a role in the pathogenesis of canine OA.

Examination of the excised femoral heads of dogs with CHD revealed novel findings regarding the presentation of dogs for surgical treatment of this common disease. Dogs aged between one and four years of age were most commonly presented for total hip replacement surgery. This was surprising as it is generally accepted that older dogs most commonly present for end stage surgical treatment of CHD and previously reported data suggest that the prevalence of OA in dogs increases with age (Vaughan, 1990). In the current study it was found that young dogs (aged between two and twelve months) often presented with severe end stage articular cartilage pathology despite their young age. Additionally, 15% of the excised femoral heads obtained from Labradors undergoing total hip replacement surgery had cartilage fibrillation as the most severe pathological lesion observed, considered to be a very mild degenerative change and not a common reason for joint excision. The data provided novel findings on the gross pathological changes to the femoral heads of dogs presenting for surgical treatment of CHD. To date, studies on CHD have largely been based upon radiographic findings obtained as part of breed screening schemes. Additional data collection would have provided further insight but was omitted as part of the original study, including; animal weight, if the dog had unilateral or bilateral hip OA, if the animal had OA of other joints, if there was concurrent illness which could have contributed to the development of joint disease, lameness scoring prior to surgery and a record of levels of physical activity for each patient. It would have been interesting to continue this study in order to increase the sample size and collect the additional data outlined above. Additionally, submission of digital radiographic images of affected hip

joints along with femoral head samples would enable comparison of radiographic changes with grossly observed lesions. In this study, no characteristic distribution of cartilage lesions over the surface of the femoral head was identified for CHD. It has been demonstrated in humans with OA of the coxofemoral joint, that variations in joint laxity alter the cartilage contact stress thus altering the site of cartilage injury on the femoral head (Ateshian *et al.,* 1991; Cicuttini *et al.,* 2002; Cohen *et al.,* 1999). To date a correlation between the site of articular cartilage contact stress and gait has not been demonstrated in humans or canines. In future studies, force plate analysis of the gait of dogs just prior to hip replacement surgery could be used to determine if gait can be correlated with the distribution of lesions observed on the femoral head. Such a study may help to explain the distribution of lesions reported here.

Previous studies have shown that human chondrocytes from joints affected by OA have higher amounts of senescence than chondrocytes from normal joints. OA chondrocytes were shown to have less replicative potential than normal chondrocytes (Piera-Velazquez *et al*., 2002), p16 (a cell cycle inhibitor and mediator of cellular senescence) was shown to be more highly expressed in OA chondrocytes compared to normal chondrocytes (Zhou *et al.,* 2004) and senescent chondrocytes were shown to accumulate with age (Martin and Buckwalter, 2001; Price *et al.,* 2002) in humans. The work presented in this thesis demonstrated for the first time that chondrocyte senescence is a feature of canine OA. Our findings demonstrated that OA chondrocytes had higher amounts of cellular senescence than normal chondrocytes. A growth curve study showed that canine OA chondrocytes had less replicative potential than normal chondrocytes. Similarly OA chondrocytes had increased levels of SA-beta-gal staining (a commonly used marker of cellular senescence *in vitro*) than normal chondrocytes and OA cartilage samples were shown to have increased levels of p16 staining compared to normal chondrocytes. The presence of phosphorylated p38 was not found to be a distinguishing feature of senescence in canine OA chondrocytes. While p38 MAPK inhibition has been identified as an anti-inflammatory therapeutic target and has been undergoing clinical trials for human use (Hill *et. al.,* 2008), from the data collected here it seems unlikely that p38 MAPK inhibition would be effective in moderating cellular senescence in canine chondrocytes. Based on our finding that canine OA chondrocytes having significantly higher levels of p16 positivity than normal chondrocytes, p16 silencing presents a potential therapeutic target for canine OA. It has been previously demonstrated that *in vitro* inhibition of p16 resulted in a decreased response of OA chondrocytes to catabolic cytokines and an increased response of OA chondrocytes to anabolic growth factors causing an increase in cellular growth rate

and inhibiting OA pathogenic pathways (Zhou *et al.*, 2004). Further study on the role of p16 in OA pathogenesis in canines is required to expand our initial findings, ideally repeating experiments with a much larger sample size and age-matched normal and OA samples to confirm the results presented here and to eliminate the age bias that was present in this study. Both the growth curve study and the SA-beta-gal staining experiments were conducted using monolayer cultured chondrocytes. As discussed in Chapter IV, three-dimensional culture of chondrocytes is preferable to monolayer culture as chondrocytes cultured in monolayer can develop a fibroblastic morphology over time, with alteration to collagen and proteoglycan synthesis and development of an unstable phenotype (Benya and Schaffer, 1982; Hauselmann *et al*., 1994). Three-dimensional suspension cultures facilitate expression of a normal chondrocyte phenotype, maintenance of normal cell morphology and synthesis of normal cartilage matrix molecules (Hauselmann *et al*., 1996) including type II collagen and aggregan expression (Rai *et al*., 2009). Standard methodology for growth curve analysis of chondrocytes utilises monolayer cell culture and as such our data can be compared to human studies using this technique. Repeating the growth curve studies and SA-beta-gal staining experiments using chondrocytes grown in three-dimensional suspension culture would likely provide a more accurate reflection of how chondrocytes behave within articular cartilage *in vivo,* however, such studies could take many years. When in suspension culture, chondrocytes seem to enter a quiescent state (as observed in the alginate bead culture study), much like within the joint itself, and as such culture to senescence could be prolonged.

A major finding presented in this thesis was the impact of OA chondrocytes on the gene expression of adjacent normal chondrocytes in coculture, providing evidence that OA chondrocytes contribute to the propagation of OA within a joint by modulationg the expression of genes associated with OA pathogenesis. This may help to explain why microtrauma affecting relatively small areas of cartilage can be the initiating event that ultimately results in diffuse OA changes to articular cartilage throughout a joint. Using a coculture system and microarray analysis, it was shown that OA chondrocytes modulated the gene expression of normal canine chondrocytes, resulting in significantly altered expression of 594 genes. Ten genes known to play a key role in the pathogenesis of OA were modulated (TNF-alpha, IL-1, IL-4, IL-6, IL-8, MMP-13, MMP-3, ADAMTS-4, ADAMTS-5 and IL-F3). Ingenuity pathway analysis showed up-regulation of biological processes that feature in OA pathogenesis, including inflammation, chemotaxis, chemokine activity, cytokine activity, defense responses, regulation of cell proliferation, superoxide metabolic processes and collagen metabolic processes. A limitation of this

study was the use of a small number of replicates due to the costs of microarray data analysis. In the time since these experiments were conducted, new sequencing technologies have become available enabling examination of much higher sample numbers over a shorter time period, though costs are still significant. It would be interesting to repeat these experiments using OA chondrocytes from donors of various ages to determine if altering the age of the OA chondrocyte donor used for the coculture experiments would alter the differential gene expression in normal chondrocytes, given that cellular senescence occurs not only as a feature of disease but also as a feature of increasing age.

Having established that cellular senescence is a feature of canine OA chondrocytes, the impact of oxidative stress on levels of cellular senescence in normal and OA canine chondrocytes was investigated. ROS have important homeostatic functions, acting as intracellular messengers in MAPK signaling (pP38 upregulation), interleukin release (IL-6, IL-1β and IL-18), transcription of IL-6 and caspase activation (Bulua *et. al.,* 2011; Nakahira *et al.,* 2011; Zhou *et al.,* 2011). ROS also have signaling functions in cell activation, proliferation and cytokine control (Clancy *et al.,* 2004). Experiments outlined in chapter VI present evidence that the experimental induction of oxidative stress in normal canine chondrocytes *in vitro* induced cellular senescence. Normal chondrocytes with experimentally induced oxidative stress underwent replicative senescence much earlier than untreated normal chondrocytes. Experimental induction of oxidative stress also resulted in higher amounts of cellular senescence in both normal and OA chondrocytes, with increased SA-beta-gal staining and decreased replicative capacity. OA cells had higher amounts of oxidative stress and decreased antioxidant capacity compared to normal chondrocytes. Furthermore, normal chondrocytes had significantly improved survival rates following experimental induction of oxidative stress then OA chondrocytes. Finally, it was shown that experimentally induced oxidative stress altered the expression of extracellular matrix genes key to the pathogenesis of OA in dogs. Up regulation of MMP-13 and MMP-3 occurred as a result of oxidative stress induction and Col-3A1 was down regulated. There is extensive evidence that MMP-13 and MMP-3 are key players in the pathogenesis of OA and are up-regulated as a result of cartilage damage (Aigner *et al.,* 2001; Burton-Wurster *et al.,* 2005). Col-3A1 is widely reported to be up-regulated in OA cartilage as a result of increased metabolic activity that occurs with osteoarthritis (Aigner *et al.,* 2001; Fukui *et al.,* 2008). Accordingly, we had anticipated an increase in the gene expression level of Col-3A1 in this experiment. However, oxidative stress in human trabecular meshwork cells (Luna *et al.,* 2009) and myocardial cells (Siwik *et al.,* 2001) has been shown to down-

regulate Col-3A1 gene expression. It is possible that oxidative stress reduces collagen synthesis in multiple disease processes, including OA, or that expression levels of Col-3A1 vary depending on whether oxidative stress is acute or chronic. This question merits further investigation to examine the impact of oxidative stress on the extracellular matrix structure in OA. While it was shown that initial levels of total cellular glutathione were similar for both OA and normal chondrocytes, OA chondrocytes had a higher fold-decrease in total cellular glutathione levels in comparison to the normal chondrocytes following induction of oxidative stress, showing decreased antioxidant capacity in OA chondrocytes as compared to normal chondrocytes. The findings presented here are comparable with the findings of human oxidative stress studies. Similar to our finding in canine chondrocytes, it has been previously demonstrated that human OA chondrocytes have increased levels of oxidative stress compared to normal chondrocytes (Ruiz- Romero *et al.,* 2009) and that human OA joints have decreased levels of antioxidants (Yudoh *et al.,* 2005). When evaluating data related to both ROS and antioxidants, it should be remembered that oxidative stress is a highly dynamic state and levels of ROS and antioxidants are in a constant state of flux. Alterations in cellular levels of these molecules occur, not only in disease states, but also in normal tissue homeostasis, which may explain conflicting and equivocal study results. Levels of oxidative stress may also vary with chronicity of disease. Many molecular pathways identified as playing a role in the pathogenesis of OA have become potential targets for new therapeutics, including oxidative stress. The use of antioxidants as therapeutic drugs has been associated with many adverse effects including extracellular matrix destruction (Henrotin *et al.,* 2005), by breakdown of the matrix molecules collagen and hyaluronan (Gao *et al.,* 2008; Petersen *et al.,* 2004). The scavenging efficacy and the broader cellular impact of commonly used antioxidant supplements is currently unknown. More research is required to fully clarify the role of supplementary antioxidants in oxidative stress processes. Perhaps the goal of OA treatment should be maintaining a healthy chondrocyte phenotype to protect the endogenous antioxidant mechanisms of articular cartilage, rather than on inhibiting oxidative stress or supplementing antioxidants. It would seem likely that oxidative damage occurs as a combination of both increased levels of stress combined with impaired scavenging mechanisms as chondrocyte function is impaired in OA, and may even be caused by cellular senescence in OA chondrocytes.

7.2 Future directions

7.2.1 Telomere length analysis

The study of senescence in canine chondrocytes could be expanded in future studies, as telomere length analysis has not been investigated as part of this thesis. Initially it had been hoped to measure telomere lengths of normal and OA canine chondrocytes in order to determine if telomere shortening was a feature of canine OA chondrocytes. However, there were significant technical difficulties encountered in measuring telomere lengths using RT-PCR, with failure of the assay to work on canine samples in an initial pilot study. The RT-PCR protocol used had been previously developed to measure telomere lengths in avian blood cells and was adapted from the human technique as described by Cawthon (2002). Given that this assay had never been used in canines and that the author had no previous experience of using this technique, a finite amount of time was allocated to optimising the protocol for canine use. The precise reasons for assay failure were not determined and telomere length analysis was not used as a measure of cellular senescence in further experiments. Prior to the use of RT-PCR to measure telomere lengths, telomere lengths were measured using an alternative technique called terminal restriction fragment measurement (TRF), a type of southern blot. This technique is very time consuming and requires a large amount of DNA for each sample measurement. It was not possible to use TRF telomere length measurement as the quantity of DNA obtained from the canine cartilage samples was insufficient for this assay. Telomere length analysis would enable evaluation of the contribution of telomere shortening to the onset of senescence in canine chondrocytes and it is hoped that the samples collected as part of this study may be analysed in the future. It would also be of interest to evaluate the impact of oxidative stress on telomere lengths, to further understand the interaction between canine OA, oxidative stress and cellular senescence. This could be achieved by inducing oxidative stress in normal canine chondrocytes and measuring the resultant change in telomere lengths. Increases in RNS have been shown to induce telomere erosion in human chondrocytes (Yudoh *et al.*, 2005), an effect which was inhibited by pre-treating cells with antioxidant.

7.2.2 Gene expression studies

It would be interesting to investigate if experimental induction of oxidative stress in normal canine chondrocytes resulted in altered expression of OA genes, as was observed

when OA canine chondrocytes were cocultured with normal canine chondrocytes. This could be examined by microarray analysis of gene expression in normal canine chondrocytes with experimentally induced oxidative stress. Perhaps the most interesting finding of this research was that OA chondrocytes alter the gene expression of adjacent normal chondrocytes with modulation of genes highly important to the pathogenesis of OA. The number of samples studied in the gene expression experiment was small. Future studies could determine if the gene modulation reported here occurs across large sample numbers and could also examine the impact of OA chondrocyte donor age on the relative gene expression of OA genes in cocultured normal chondrocytes.

7.2.3 Cellular senescence as a therapeutic target

Having confirmed that cellular senescence is a feature of canine OA, it seems likely that future development of therapeutics for human OA that mediate cellular senescence will also be relevant to dogs. Pelletier suggested that in time a cure for OA may be found as a result of new molecular therapies for OA (Pelletier *et al.,* 2006). Traditional treatment has been symptom based. Current research into new therapeutic targets is investigating specific microscopic events in disease pathogenesis, with the goal of therapy being prevention of progression from the early stages of disease to end stage OA. There is a vast field of research investigating experimental therapeutics to prevent the onset of cellular senescence and to remove senescent cells from the body. It has been hypothesised that the immune system could be harnessed to induce clearance of senescent cells from tissues, though the mechanism by which the immune system naturally clears senescent cells from the body is not yet known (van Deursen, 2014). Given that articular cartilage is an avascular tissue it is not yet known if immune clearance of senescent cells would affect chondrocytes in articular cartilage.

7.3 Conclusions

The findings outlined in this thesis achieved our aim of demonstrating that cellular senescence plays a role in the pathogenesis of canine OA.

The main findings of this PhD thesis were that:

1. Cellular senescence is a feature of canine chondrocytes from OA cartilage.
2. Experimentally induced oxidative stress increases levels of cellular senescence in normal canine chondrocytes and OA chondrocytes have increased amounts of oxidative stress compared to normal chondrocytes.
3. Osteoarthritic chondrocytes alter the expression of genes key to OA pathogenesis in normal chondrocytes in cell coculture.
4. Examination of the femoral heads of dogs presenting for surgical treatment of canine hip dysplasia did not reveal a characteristic pathology of the femoral head, with wide variation in the types and distribution of cartilage lesions observed.
5. Questions regarding the molecular mechanisms by which senescence and oxidative stress contribute to the pathogenesis of OA in dogs remain unanswered. While it was not aim of this thesis to answer these questions, our findings outline important areas for future studies in canine OA.

Chapter VIII

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Appendix 1. Rank product differential expression of top 100 up-regulated genes.

0	Probe-set ID	RPscore	FC_rma	FC_nom	"Gene ID"	"EntrezGene"	Gene Symbol	Title
1	CfaAffx.1703.1.S1_x_at	1.19	29.18	252.80	pmrna1603	478406	SON	SON DNA binding protein
2	CfaAffx.1703.1.S1_at	2.91	24.17	185.65	pmrna1603	---	---	---
3	CfaAffx.28084.1.S1_s_at	3.52	19.47	130.15	pmrna27984	448792	CCL8	chemokine (C-C motif) ligand 8
4	CfaAffx.3389.1.S1_at	5.90	18.68	121.67	pmrna3289	481558	IFNB1	interferon, beta 1, fibroblast
5	CfaAffx.30940.1.S1_s_at	7.27	18.45	119.16	pmrna30840	490172	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa
6	CfaAffx.15121.1.S1_at	7.61	17.28	107.04	pmrna15021	488947	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
7	CfaAffx.18191.1.S1_s_at	9.64	16.50	99.25	pmrna18091	488622	MNDA	myeloid cell nuclear differentiation antigen
8	Cfa.20785.1.S1_s_at	11.19	14.68	81.91	DN271015	609421	SRGN	serglycin
9	CfaAffx.9573.1.S1_at	12.26	14.70	82.07	pmrna9473	475574	IDO1	indoleamine 2,3-dioxygenase 1
10	CfaAffx.1705.1.S1_at	13.85	15.77	92.12	pmrna1605	481471	LOC481471	similar to interferon gamma inducible protein 47
11	Cfa.9758.1.A1_at	15.29	15.12	86.02	CO590687	---	---	---
12	Cfa.9955.1.A1_at	17.54	14.68	81.94	CO586666	---	---	---
13	Cfa.20785.1.S1_at	19.75	13.37	70.27	DN271015	609421	SRGN	serglycin
14	Cfa.5944.1.A1_at	20.29	12.89	66.15	CO694999	474853	CFB	complement factor B
15	Cfa.3619.1.S1_at	20.45	12.84	65.79	NM_001003	403756	CD38	CD38 molecule

16	Cfa.3619.1.S1_s_at	20.63	13.10	67.96	NM_001003143	403756	CD38	CD38 molecule
17	Cfa.18819.1.S1_at	21.16	12.41	62.16	DN270978	490172	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa
18	Cfa.19015.1.S1_s_at	21.86	11.72	56.59	DN268276	490172	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa
19	Cfa.20456.1.S1_at	23.44	12.56	63.47	CN002479	478170	LOC478170	similar to interferon induced 6-16 protein isoform a
20	Cfa.6458.1.A1_s_at	25.06	11.97	58.65	CO654346	474853	CFB	complement factor B
21	Cfa.3609.1.S1_s_at	25.59	11.89	57.99	NM_001003133	403744	MX2	myxovirus (influenza virus) resistance 2 (mouse)
22	Cfa.54.1.S1_s_at	27.05	11.46	54.55	NM_001003244	403922	TNF A	tumor necrosis factor (TNF superfamily, member 2)
23	CfaAffx.15377.1.S1_at	28.62	11.30	53.31	pmrna15277	478474	HERC5	hect domain and RLD 5
24	Cfa.4589.1.A1_s_at	29.58	11.96	58.57	CO707981	477707	C1R	complement component 1, r subcomponent
25	Cfa.18826.1.S1_at	30.70	11.12	51.96	DN270575	488585	RGS1	regulator of G-protein signaling 1
26	CfaAffx.16422.1.S1_s_at	30.75	11.03	51.27	pmrna16322	448790	CCL20	chemokine (C-C motif) ligand 20
27	CfaAffx.27804.1.S1_s_at	30.75	10.47	47.10	pmrna27704	---	---	---
28	CfaAffx.16440.1.S1_at	31.59	11.13	52.01	pmrna16340	477518	OASL	2'-5'-oligoadenylate synthetase-like
29	Cfa.20723.1.S1_at	31.65	10.97	50.82	DN382966	---	---	---
30	Cfa.11073.1.A1_at	31.77	11.12	51.97	CO594083	476931	EPSTI1	epithelial stromal interaction 1 (breast)
31	CfaAffx.1156.1.S1_at	34.22	9.62	40.97	pmrna1056	481110	IL23A	interleukin 23, alpha subunit p19
32	Cfa.180.1.S1_s_at	35.65	10.50	47.29	NM_001002	403469	CD40	CD40 molecule, TNF receptor superfamily member 5

33	CfaAffx.17917.1.S1_at	38.47	10.32	45.94	pmrna17817	488729	ISG20	interferon stimulated exonuclease gene 20kDa
34	CfaAffx.6110.1.S1_at	40.00	9.87	42.72	pmrna6010	484371	IL4I1	interleukin 4 induced 1
35	CfaAffx.16575.1.S1_s_at	41.13	9.88	42.79	pmrna16475	488585	RGS1	regulator of G-protein signaling 1
36	Cfa.15795.1.A1_s_at	41.60	9.13	37.63	CO676693	448786	CCL4	chemokine (C-C motif) ligand 4
37	CfaAffx.5863.1.S1_at	41.66	8.95	36.39	pmrna5763	609005	RSAD2	radical S-adenosyl methionine domain containing 2
38	Cfa.16593.1.S1_at	41.89	10.15	44.76	DN383058	---	---	---
39	CfaAffx.18427.1.S1_at	42.46	10.16	44.82	pmrna18327	488634	IGSF9	immunoglobulin superfamily, member 9
40	Cfa.12298.1.A1_a_at	43.31	10.04	43.93	CO660248	474865	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
41	CfaAffx.18567.1.S1_s_at	44.17	9.73	41.75	pmrna18467	608673	LOC608673	similar to TCDD-inducible poly(ADP-ribose) polymerase
42	CfaAffx.30585.1.S1_s_at	44.41	9.55	40.48	pmrna30485	480698	PTGES	prostaglandin E synthase
43	CfaAffx.5975.1.S1_at	45.91	9.28	38.59	pmrna5875	481688	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
44	Cfa.19154.1.S1_a_at	46.78	9.00	36.76	DN355397	608996	LOC608996	similar to thymidylate kinase family LPS-inducible member
45	Cfa.12477.1.A1_at	46.95	9.53	40.36	CO625727	---	---	---
46	CfaAffx.18567.1.S1_at	47.09	9.33	38.96	pmrna18467	608673	LOC608673	similar to TCDD-inducible poly(ADP-ribose) polymerase
47	CfaAffx.19610.1.S1_at	48.04	9.07	37.20	pmrna19510	606875	LOC606875	similar to immunity-related GTPase family, cinema 1
48	Cfa.15622.1.A1_s_at	50.34	9.34	39.00	CO683291	477623	RND1	Rho family GTPase 1

49	CfaAffx.28491.1.S1_at	52.45	8.21	31.58	pmrna28391	403822	NOS2	nitric oxide synthase 2, inducible
50	CfaAffx.22221.1.S1_s_at	52.79	9.10	37.37	pmrna22121	486714	C1S	complement component 1, s subcomponent
51	Cfa.10757.1.S1_at	53.12	9.00	36.70	DN272024	479575	ISG15	ISG15 ubiquitin-like modifier
52	CfaAffx.2164.1.S1_at	53.15	8.96	36.46	pmrna2064	474865	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
53	CfaAffx.670.1.S1_s_at	54.85	8.76	35.15	pmrna570	608996	LOC608996	similar to thymidylate kinase family LPS-inducible member
54	CfaAffx.30585.1.S1_at	56.76	8.70	34.76	pmrna30485	480698	PTGES	prostaglandin E synthase
55	Cfa.12190.1.A1_at	57.17	8.72	34.88	CO657202	480698	PTGES	prostaglandin E synthase
56	Cfa.21168.1.S1_s_at	57.44	8.79	35.34	DN267433	477707	C1R	complement component 1, r subcomponent
57	CfaAffx.30944.1.S1_at	60.52	8.56	33.84	pmrna30844	490170	GBP5	guanylate binding protein 5
58	Cfa.12151.1.A1_at	60.61	8.22	31.68	CO598370	---	---	---
59	CfaAffx.13934.1.S1_at	63.55	8.10	30.92	pmrna13834	477623	RND1	Rho family GTPase 1
60	Cfa.16472.2.S1_s_at	65.47	8.28	32.04	DN380906	486714	C1S	complement component 1, s subcomponent
61	Cfa.10821.1.A1_s_at	65.88	8.17	31.34	CO702013	486714	C1S	complement component 1, s subcomponent
62	CfaAffx.1699.1.S1_at	67.34	7.87	29.45	pmrna1599	---	---	---
63	Cfa.12240.1.A1_at	67.56	8.21	31.60	CO707940	476728	C3	complement component 3
64	CfaAffx.26832.1.S1_s_at	71.72	7.81	29.12	pmrna26732	---	---	---
65	Cfa.20456.1.S1_s_at	71.88	7.74	28.69	CN002479	478170	LOC478170	similar to interferon induced 6-16 protein isoform a
66	Cfa.8846.1.A1_s_at	72.15	7.81	29.12	CO701403	474853	CFB	complement factor B

67	CfaAffx.3613.1.S1_at	72.19	7.42	26.74	pmrna3513	---	---	---
68	CfaAffx.23214.1.S1_at	72.80	7.67	28.25	pmrna23114	483772	RARRES3	retinoic acid receptor responder (tazarotene induced) 3
69	CfaAffx.26206.1.S1_at	73.16	7.75	28.76	pmrna26106	607880	IL27	interleukin 27
70	CfaAffx.5498.1.S1_at	74.89	7.75	28.75	pmrna5398	482196	PPBP	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)
71	Cfa.3660.1.A1_s_at	75.94	7.30	26.07	NM_001003186	403822	NOS2	nitric oxide synthase 2, inducible
72	CfaAffx.16068.1.S1_at	76.52	7.15	25.20	pmrna15968	490222	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
73	CfaAffx.5973.1.S1_s_at	78.52	7.28	25.92	pmrna5873	481688	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
74	Cfa.1661.1.S1_at	79.45	7.24	25.72	NM_001002938	403400	CTSS	cathepsin S
75	Cfa.16590.1.S1_s_at	79.65	7.08	24.78	NM_001010949	478432	CXCL10	chemokine (C-X-C motif) ligand 10
76	Cfa.15810.1.S1_at	80.81	7.49	27.15	NM_001005255	448792	CCL8	chemokine (C-C motif) ligand 8
77	Cfa.14352.1.A1_at	82.45	7.22	25.58	CO678929	448787	CCL3	chemokine (C-C motif) ligand 3
78	Cfa.16440.1.S1_at	83.60	7.24	25.71	DN271207	490954	IFI35	interferon-induced protein 35
79	Cfa.14528.1.A1_at	84.14	7.31	26.09	CO674938	483594	DLA-79	MHC class Ib
80	Cfa.3550.2.A1_at	85.64	6.49	21.46	CO703931	477683	MGST1	microsomal glutathione S-transferase 1
81	Cfa.3528.1.S1_s_at	86.07	7.00	24.31	NM_001003301	403985	IL6	interleukin 6 (interferon, beta 2)
82	CfaAffx.9471.1.S1_s_at	86.11	6.95	24.04	pmrna9371	483355	RNF213	ring finger protein 213

83	Cfa.16337.1.S1_s_at	89.38	7.08	24.78	NM_001010960	491148	CCL7	chemokine (C-C motif) ligand 7
84	Cfa.15815.1.S1_s_at	89.61	6.92	23.84	NM_001005251	448787	CCL3	chemokine (C-C motif) ligand 3
85	CfaAffx.19613.1.S1_at	89.78	6.99	24.27	pmrna19513	606863	IRGM	immunity-related GTPase family, M
86	Cfa.8798.1.A1_at	89.90	6.86	23.50	CO595599	---	---	---
87	CfaAffx.14389.1.S1_at	90.75	6.85	23.50	pmrna14289	478406	SON	SON DNA binding protein
88	Cfa.3868.1.S1_at	90.80	6.21	19.97	NM_001003310	403999	SELE	selectin E
89	CfaAffx.16456.1.S1_s_at	93.61	6.85	23.45	pmrna16356	477518	OASL	2'-5'-oligoadenylate synthetase-like
90	Cfa.12214.1.A1_at	94.50	6.59	22.05	CO660813	490222	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
91	Cfa.1333.1.S1_at	94.67	6.92	23.88	DN270854	---	---	---
92	Cfa.170.1.S1_at	97.23	6.53	21.71	NM_001002975	403461	BDNF	brain-derived neurotrophic factor
93	CfaAffx.14116.1.S1_at	97.35	6.59	22.01	pmrna14016	---	---	---
94	CfaAffx.31150.1.S1_at	98.30	6.64	22.32	pmrna31050	479980	IFI44L	interferon-induced protein 44-like
95	CfaAffx.10779.1.S1_at	98.80	6.71	22.68	pmrna10679	483406	LOC483406	similar to Interferon regulatory factor 7 (IRF-7)
96	Cfa.5163.1.A1_at	99.14	6.58	21.97	CO707032	485549	RAB20	RAB20, member RAS oncogene family
97	Cfa.18892.1.S1_s_at	99.40	6.46	21.29	DN267574	479980	IFI44L	interferon-induced protein 44-like
98	Cfa.18376.1.S1_at	99.99	6.25	20.19	DN366676	489433	BIRC3	baculoviral IAP repeat-containing 3

99	CfaAffx.3929.1.S1_at	103.10	6.48	21.40	pmrna3829	482305	SAMD9L	sterile alpha motif domain containing 9-like
00	Cfa.6456.1.S1_at	103.29	6.35	20.74	NM_001011723	---	---	---

Appendix 2. Rank product differential expression of down-regulated genes

0	Probe-set ID	RPscore	FC_rma	FC_nom	"Gene ID"	"EntrezGene"	Gene Symbol
1	Cfa.4779.1.A1_at	3.64	-4.54	-11.95	CO710123	---	---
2	CfaAffx.15042.1.S1_s_at	7.09	-3.28	-7.03	pmrna14942	478471	SPP1
3	Cfa.2662.1.A1_at	18.17	-2.76	-5.30	CF409522	484682	LRRN1
4	Cfa.10516.1.A1_at	18.31	-2.96	-5.93	BU749417	---	---
5	CfaAffx.8206.1.S1_s_at	19.68	-2.82	-5.48	pmrna8106	485469	PCDH8
6	Cfa.16827.1.A1_at	31.65	-2.42	-4.27	DN427842	610935	NQO1
7	CfaAffx.30692.1.S1_s_at	35.92	-2.37	-4.12	pmrna30592	479934	LOC479934
8	Cfa.9602.1.A1_at	40.61	-2.64	-4.92	CO633047	---	---
9	Cfa.1634.2.A1_a_at	45.86	-2.40	-4.22	BU747116	481159	RAB3IP
10	CfaAffx.31023.1.S1_at	46.19	-2.25	-3.78	pmrna30923	610935	NQO1
11	Cfa.14158.1.S1_at	55.63	-2.14	-3.48	CO703555	486666	ARHGDIB
12	Cfa.299.1.A1_s_at	56.09	-2.10	-3.38	BU745341	478156	FABP3
13	Cfa.9613.1.A1_at	71.16	-2.16	-3.54	CO593111	---	---
14	CfaAffx.3090.1.S1_s_at	73.35	-2.17	-3.56	pmrna2990	481262	GCAT
15	Cfa.9240.1.S1_at	73.47	-2.10	-3.37	CO584391	478471	SPP1
16	Cfa.12793.1.A1_at	76.31	-2.21	-3.68	CO716482	---	---
17	Cfa.299.1.A1_at	76.97	-1.94	-2.97	BU745341	478156	FABP3
18	CfaAffx.11454.1.S1_at	80.66	-2.00	-3.10	pmrna11354	483056	MALL
19	Cfa.363.2.S1_at	85.22	-2.02	-3.17	CF413486	479934	LOC479934

20	Cfa.6471.1.A1_at	88.90	-1.94	-2.97	CO679849	608809	AKR1CL2
21	CfaAffx.19964.1.S1_s_at	90.05	-1.99	-3.09	pmrna19864	486666	ARHGDIB
22	CfaAffx.15408.1.S1_at	91.08	-2.10	-3.37	pmrna15308	486605	PDZRN4
23	Cfa.13413.1.A1_at	91.17	-2.07	-3.30	CO698532	---	---
24	CfaAffx.5497.1.S1_s_at	98.32	-2.16	-3.53	pmrna5397	607717	MEST
25	CfaAffx.7333.1.S1_at	102.79	-2.51	-4.54	pmrna7233	---	---
26	CfaAffx.12221.1.S1_s_at	106.75	-2.32	-3.98	pmrna12121	486804	LOC486804