

Molecular and Cellular Features of Anteromedial Gonarthrosis

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by

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For Dad

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Abstract

Anteromedial Gonarthrosis – a molecular and cellular study

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Anteromedial Gonarthrosis (AMG) is a distinct phenotype of knee osteoarthritis (OA), with a specific pattern of disease on the tibia. There is full thickness cartilage loss anteromedially, progressing to an area of damaged cartilage, and then to an area of macroscopically and histologically normal cartilage posteriorly. This reproducible pattern of disease can be considered to be a spatial model of OA progression and provides an alternative and less biologically varied set of specimens than the commonly used multiple joint compartments in which to quantify disease-related changes. The aim of this thesis was to explore in detail spatial and quantitative differences in cell, matrix and molecular features between areas of cartilage in AMG.

A *real time PCR* study was undertaken comparing damaged and undamaged cartilage in AMG. Previous work from our research group had shown increased type I collagen content of undamaged cartilage in AMG. This gene expression study corroborated this finding by demonstrating increased COL1A1 expression in undamaged cartilage, compared to damaged cartilage. MMP1, MMP3, MMP13 and ADAMTS4 were also shown to have increased expression in undamaged cartilage. Since these changes arise in tissue before any macroscopic damage is apparent, these may indicate early changes of the cartilage matrix in AMG.

In order to confirm that these changes are directly related to the damage process and not only to normal regional variations, *Above Knee Amputations* were collected from patients with peripheral vascular disease but no overt OA and a template of the AMG joint surface created to allow for matched regional sampling. Studies into their *histology, immunoassays and qPCR* were performed in order to compare results with those from AMG specimens. Histology demonstrated low scores throughout but allowed for a division into lower (macroscopically and histologically normal) and higher grade (surface wear/possible early signs of OA) groups. Immunoassays showed elevated type I collagen in high grade but not low grade groups in the posterior cartilage. No differences in mRNA expression were detected using qPCR suggesting that changes seen in AMG specimens were specific to the OA disease process.

Because the causes of cell death in OA are not fully understood an *immunohistochemical study into apoptosis* was performed. TUNEL staining demonstrated higher levels of apoptosis, the more damaged the cartilage. The presence of Active Caspase 3, Cytochrome C, Active Bax and Bim with the same distributions demonstrated apoptosis occurring via the intrinsic or mitochondrial pathway. The high levels of 3-Nitrotyrosine in more damaged cartilage implicated reactive oxygen species in apoptotic mechanisms.

A *microarray* study was conducted comparing regions of damaged and undamaged cartilage in AMG. 389 genes were found to be significantly differentially expressed between regions. Several pathways rich in gene expression changes were highlighted including cellular development, inflammatory response and skeletal disorders. Results suggest complex changes in the signaling microenvironment of AMG and identify areas for future study.

In summary, this thesis has highlighted several quantitative molecular and cellular differences between regions of cartilage in AMG, demonstrating its usefulness as a tight disease model. Gene expression differences corroborate changes previously seen by immunohistochemistry; microarray has shown the wider picture of gene expression changes. Apoptosis has been shown to occur via the intrinsic pathway and involve reactive oxygen species, highlighting this damage pathway as an important driver of cell loss. Most importantly this thesis has identified the apparently normal region in AMG specimens as a region undergoing considerable molecular changes and as a potential early disease model. The dysregulation of collagen I distribution seen in AMG and slightly worn AKA specimens is very interesting and suggests a possible early response to loading alterations caused by joint wear and opening the way for future experiments. The identification of wear patterns in AKA specimens specifically mapping to the zone of maximal damage in AMG confirms the site of initial injury and progression over time proposed in this model. These AKA specimens with no overt OA should therefore be used in future studies to assess emerging biomarkers of disease progression.

Chapter One

1 GENERAL INTRODUCTION

1.1 Osteoarthritis

Osteoarthritis (OA) is the most common musculoskeletal condition affecting joints. It is primarily considered to be a degenerative joint disease, and it is the major cause of chronic musculoskeletal pain and disability in elderly populations worldwide [1].

1.1.1 Definition

There have been many iterations of a definition for OA, each aiming to include the multiple factors affecting the many constituents of a joint and the resulting pathological features.

A consensus definition of osteoarthritis (OA) was defined in 1994:

“OA diseases are a result of both mechanical and biologic events that destabilise the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix and subchondral bone. Although they may be initiated by multiple factors, including genetic, developmental, metabolic, and traumatic, OA diseases involve all of the tissues of the diarthrodial joint. Ultimately, OA diseases are manifested by morphologic, biochemical, molecular, and biomechanical changes of both cells and matrix which lead to a softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes, and subchondral cysts. When clinically evident, OA diseases are characterised by joint pain,

tenderness, limitation of movement, crepitus, occasion effusion, and variable degrees of inflammation without systemic effects.” [2, 3]

1.1.2 Epidemiology

In the UK, OA affects approximately 8.5 million people [4]. More recently there has been a summary of its epidemiology in the USA that estimates its prevalence based on large cohort studies [5]. In particular, with reference to knee OA, radiological prevalence ranged from 14.1% (aged >26) to 31.2% (aged >60). The clinical and radiological prevalence ranged from 4.6% (aged >26) to 13.5% (aged >45) [6-8]. It appears to be more prevalent in females than males [9, 10].

The incidence of OA is increasing, and Kurtz et al, in 2007 [11] used the numbers of hip and knee replacements, census data, and population predictions to produce somewhat alarming figures. His paper predicts hip replacement surgery to grow by 174% and knee replacement surgery by 673% by the year 2030. Similarly, the number of revision surgeries is expected to increase by 137% and 601% for hips and knees respectively, in the same time frame, adding considerable stresses to the healthcare system. In 1986, in the UK, the direct costs of OA were estimated at £1 billion [12], and since then has increased with the growing number of joint replacements being performed.

1.1.3 Aetiology

The aetiology of OA has traditionally been categorised as being either primary (or idiopathic) or secondary. There are many theories about the factors that predispose a person to ‘primary’ OA, and these include an individual’s genetic pre-disposition,

biological, anatomical and mechanical factors (Figure 1-1). However, their interaction and relationships to the initiation and progression of OA are poorly understood. There are more identifiable links between certain conditions/events and OA, and these are often grouped together as ‘secondary’ OA (Table 1-1).

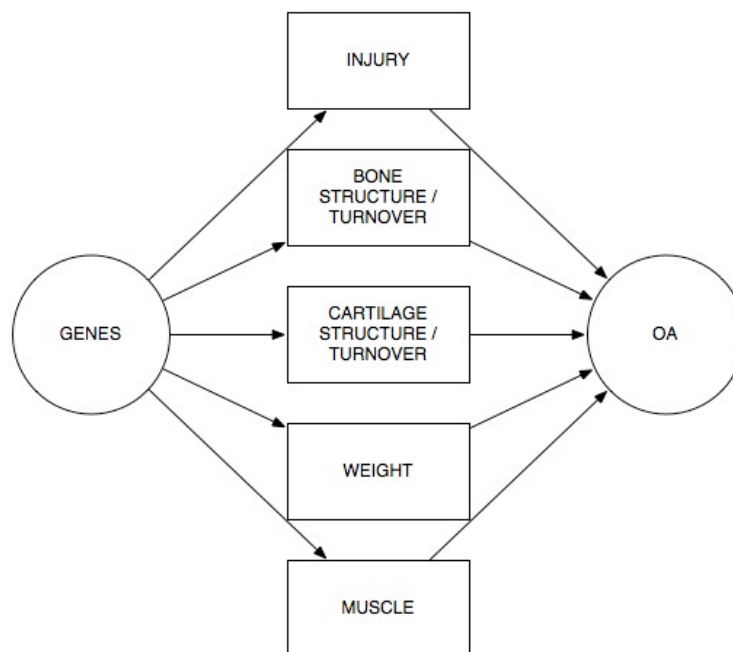


Figure 1-1 - A suggested relationship between genes and OA (adapted from Spector and MacGregor [13]).

Given the polyfactorial nature of OA, the current philosophy proposes that all OA is secondary, and that the historical divide of primary and secondary OA is not a useful one [14, 15].

Secondary OA
Trauma <i>Acute (accident)</i> <i>Chronic (occupational, obesity)</i> Underlying joint disorder <i>Fracture</i> <i>Infection</i> <i>Rheumatoid arthritis</i> <i>Hypermobility syndrome</i> Systemic metabolic or endocrine disorder <i>Wilson disease</i> <i>Haemochromatosis</i> <i>Hyperparathyroidism</i> <i>Acromegaly</i> Neuropathic disease <i>Diabetes mellitus</i> <i>Overuse of intra-articular steroids</i> Crystal deposition disease <i>Calcium pyrophosphate dehydrate (pseudogout)</i> <i>Monosodiumurate monohydrate (gout)</i> Miscellaneous <i>Bone dysplasia</i> <i>(multiple epiphyseal dysplasia, achondrodysplasia)</i> <i>Frostbite</i>

Table 1-1 A categorised list of some of the conditions that have a known association with OA, and have therefore previously been classed ‘secondary’. Adapted from Moskowitz 2007 [16].

1.1.3.1 Genetic Factors

There appears to be a genetic predisposition to developing OA [17]. This relationship was noted as early as the 1940s, with hand OA and the presence of Heberden’s nodes [18]. Further large cohort studies of twins and siblings have demonstrated a higher risk of developing OA. This has been demonstrated for hip OA [19], knee OA (tricompartmental) [19-22], and hand OA [23]. More recently there has been shown to be similar evidence for a genetic risk for developing Anteromedial Gonarthrosis

(AMG). The relative risk for AMG in siblings was 3.2 [24]. Some of the earlier studies took joint replacement for OA as their end point [19]; more recent work has used radiological progression and investigated other morphological/biological factors [25]. A review by Spector et al in 2004 [13] included the factors shown in Figure 1-1. Genome-wide association studies have also identified potential candidate genes (for a recent review see [26]). However, given that our understanding of the initiation and progression of OA is limited, it is difficult to study and draw conclusions on the relationship between genes and OA. Furthermore, it is important to note that OA is polyfactorial in its nature, and thus the environmental and other factors discussed below contribute greatly.

1.1.3.2 Environmental Factors

A number of cohort studies have collected large numbers of variables attempting to correlate environmental factors with OA. For example, the following have been identified as having a relationship with OA: occupation, obesity, physical activity, joint injury and trauma [27-30] (trauma is discussed in more detail in section 1.1.3.3). Occupational risk factors include working in a kneeling position, squatting and heavy lifting [28, 31-33]. Obesity has also shown an association to knee OA [34-36].

1.1.3.3 Traumatic Factors

Previous injury has been linked to OA. The relationship, in humans, between meniscectomy and OA was described by Fairbank in the 1940s [37]. Since the advent of arthroscopic surgery, this procedure is now rarely performed, with surgeons aiming to debride meniscal tears to a stable rim, or to repair them. However, even with meniscus-preserving surgery, there is progressive degenerative change [38-41]. Anterior cruciate ligament (ACL) tears are also common in the sporting population. Gait studies show evidence of altered loading in the ACL deficient knee [42], and

there is an increased risk of mensical damage and OA change in the ACL deficient knee [43]. The Pond-Nuki animal model (canine ACL transection) of OA is well described in the literature [44]. Other joint trauma, such as intra-articular fractures and chondral lesions also lead to OA changes, as demonstrated in the knee and ankle joints [45, 46].

1.1.3.4 Mechanical and Morphological Factors

Abnormalities of gait have been shown in patients that have AMG [47-49]. More recently, researchers have considered the effect of changes of gait on normal cartilage, and conclude that such abnormalities may predispose a joint to OA [42, 50]. The normal mechanical axis of the lower limb is aligned through the medial compartment of the knee, just medial to the tibial spine [51]. The anatomical alignment of the distal femur and proximal tibia is between 5 and 7 degrees of valgus. Changes in either of these alters the load-bearing areas of the knee and predisposes an individual to OA as demonstrated by resection specimens from the time of arthroplasty [52-54] and, more recently, by MRI studies [55-57].

Morphological features have long been associated with hip OA, where conditions such as developmental dysplasia of the hip, Perthes' disease and SUFE have been shown to accelerate the progression of OA. In the young adult, the study of hip morphology has given rise to the term Femoro-Acetabular Impingement (FAI) [58]. Relationships have been noted with patients that develop OA, and the presence of FAI in their siblings [59], however longitudinal studies are yet to be conducted considering the outcome for joints exhibiting such morphological changes. Similarly, in AMG, one group's findings suggest that the morphology of the tibial slope and the

extension facet angle of the tibial plateau may be features of patients that develop AMG [25]. The reasons why individuals exhibit such features are not understood.

1.1.3.5 Pathogenesis and Biological Factors

The initiation and progression of OA is poorly understood. There are a combination of factors that contribute to the pathogenesis of OA, and despite being described as a disease of all tissues of a synovial joint, the literature suggests uncertainty as to whether it is a primary disease of bone or cartilage. There is certainly growing interest in the relationship between cartilage and subchondral bone [15, 60], however, this thesis concentrates on the changes of cartilage in OA

Cartilage degeneration leads to fibrillation, fissuring, and ultimately complete cartilage loss, exposing the subchondral bone plate. Osteophytes are formed, and their formation may precede cartilage damage [61]. The remodeling of normal tissue is absent in OA, and repair mechanisms falter. Chondrocytes are responsible for the generation and maintenance of tissue homeostasis [62] and in OA, their failure is a result of an imbalance in the physiochemical resisting properties, together with an altered biological mechanical stress environment on cartilage. At some point the biosynthetic anabolic activity is unable to keep pace with the degradative catabolic activity and tissue degeneration results [63].

1.1.4 Clinical Presentation / Phenotype

The clinical presentation of a patient with OA is typically of pain and loss of function. A clinician's role is to assess the severity of the pain and the degree to which a patient's activities are impaired. To this extent, the use of Patient Reported Outcome Measures (PROMs), such as the Oxford Knee Score [64] and WOMAC score [65] are

becoming much more commonplace in clinical practice. Clinical examination will reveal findings including: an antalgic gait, deformity, effusions, muscle wasting, tenderness, fixed flexion deformity and a decreased range of movement which may be painful.

For many years, OA has been considered to be a homogeneous disease of joint degeneration [15]. There has been much recent interest in patterns, or phenotypes, of disease and epidemiological studies have highlighted population differences between races and ethnicities [66, 67]. However further studies are required to emphasize potential explanatory factors such as genetic, environmental, anatomical and biomechanical features [68]. Similarly, when considering the basic science research into OA, it is important to pursue phenotype-specific study as is the case in this thesis.

1.1.5 Investigation

The workhorse of clinical investigation remains the plain radiograph. In knee OA, weight bearing, skyline (patellofemoral joint) and stress view Xrays are common additional views taken. MRI is also an adjunct, however its role in diagnosis and management is not well defined. It is more often used in the assessment of early OA. MRI is a useful research tool, and in large cohort studies such as the Osteoarthritis Initiative, it is used to image patients at intervals to assess progression [57]. Advances in MRI imaging include dGEMERIC scanning (GAG specific), and T2 mapping (Collagen network specific) though these currently are predominantly used in the research setting [69].

1.1.6 Staging

The clinical severity of the disease and its staging is a very topical subject in terms of; how one measures it, how a pain score or a PROM score should be interpreted, and how it should affect treatment. Radiographic disease staging has a number of scoring systems based around osteophytes, subchondral cysts, subchondral sclerosis and joint space narrowing. The most common of these is the Kellgren-Lawrence grade [70] (Table 1-2).

<u>Kellgren Lawrence Grade</u>	<u>Features</u>
I	Doubtful narrowing of joint space and possible osteophytic lipping
II	Definite osteophytes, definite narrowing of joint space
III	Moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone contour
IV	Large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour

Table 1-2 – The Kellgren-Lawrence grading scale for the assessment of radiographic osteoarthritis.

Other radiographic scoring systems, include those of Ahlback [71] and Altman [72].

Newer MRI scoring systems have been developed such as the Boston Leeds Osteoarthritis Score [73] and the Knee Osteoarthritis Scoring System [74] however they are not yet common place in clinical practice and Xray remains the gold standard.

1.1.7 Treatment

The Osteoarthritis Research Society International (OARSI) produced a systematic review of 23 guidelines that exist for the treatment of OA, and produced their own consensus guideline documents in 2007-10 [75-77]. They reviewed treatment options available and analyse the evidence base.

In summary, they recommended a combination of non-pharmacological and pharmacological modalities which included:

1. Physiotherapy, and undertaking regular exercise
2. Analgesia
3. Steroid / hyaluronic acid injection
4. Osteotomy
5. Joint replacement surgery

Perhaps the most important factor in the progression of treatments for OA is that there are no available disease modifying treatments. Therefore most subjects that present with symptoms of OA will progress, at varying rates, toward joint replacement.

1.1.8 Current Treatment of Anteromedial Gonarthrosis

The treatment for Anteromedial Gonarthrosis (AMG) follows the same principle as highlighted in section 1.1.7. However the ‘end-stage’ surgical treatment of AMG remains an area of some debate. Options include high tibial osteotomy, unicompartmental (UKA) and total knee arthroplasty (TKA) (for systematic review see [78]).

Interestingly, despite the figures described in section 1.1.2, which gives an approximation of the prevalence of each of the knee OA phenotypes, joint registry

data demonstrates far more total knee replacements being conducted (7% of knee replacements were UKA in 2012 NJR data) [79], implying that a large number of patients with AMG receive TKA as opposed to UKA. This apparent dichotomy has a number of potential causes which include surgeon training, surgeon preference, and lack of high quality evidence either supporting or refuting their use [78].

1.1.9 Unicompartmental Knee Arthroplasty

The study of AMG in this thesis was made possible by obtaining resection samples from patients undergoing UKA. In brief, UKA was first used in the 1950s [80]. It is used to treat medial or lateral compartmental OA, with the underlying principle being preservation of normal structures. It has been shown to be successful and have shorter rehabilitation and shorter inpatient hospital stays than TKA [81].

All patients studied in this thesis underwent medial UKA with the Oxford Knee. This arthroplasty, designed by Goodfellow and O'Connor [82], employs a spherical femoral component and a flat tibial component with a fully congruent mobile bearing [83]. The minimally invasive surgical approach [84] now used to implant the Oxford Knee does not resect the femoral articulation with saw cuts. Instead, it uses a bone mill to reshape the femur in situ. Unfortunately this means that the femoral cartilage is not available for similar biological studies as were conducted on the tibial plateau.

1.1.10 Study of Osteoarthritis using Animal Models

Animal models have widely been used in the study of OA. These vary widely from genetically modified small animals to rabbit or dog 'mechanical' models. However, their transferability to human OA is debatable, with onset of disease and rate of

progression being the key factors that make their relevance difficult to ascertain [85, 86].

1.2 Knee Osteoarthritis

1.2.1 The Knee Joint Anatomy and Function

The knee is the largest synovial joint in the human body, and is formed between the femur, tibia and patella [87] (Figure 1-2). It has two articulations; tibiofemoral and patellofemoral. The tibiofemoral articulation is comprised of two compartments, the medial and lateral compartments. Stability is conferred to the knee by two collateral ligaments, the medial and lateral collaterals, and by two cruciate ligaments, the anterior and posterior cruciates. The collateral ligaments provide stability against valgus and varus forces and the cruciates against anterior-posterior translation of the tibia relative to the femur.

In both the medial and lateral tibiofemoral compartments there are ‘C’ shaped fibrocartilaginous structures called menisci (Figure 1-2). Their role is to spread load bearing in the joint. They perform this by improving congruity and are able to conform slightly throughout the range of flexion [88].

As a synovial joint there are three other important characteristic features of the knee; a joint capsule which encompasses distal femur and proximal tibia, synovium (a soft tissue lining of the joint, containing synoviocytes), and synovial fluid (a fluid secreted from the synovial membrane responsible for lubrication and nutrition to the articular cartilage). As cartilage is an avascular and aneural tissue synovium must also

metabolise cartilage waste products [89]. Figure 1-3 demonstrates pictorially some of the joint changes that occur in OA.

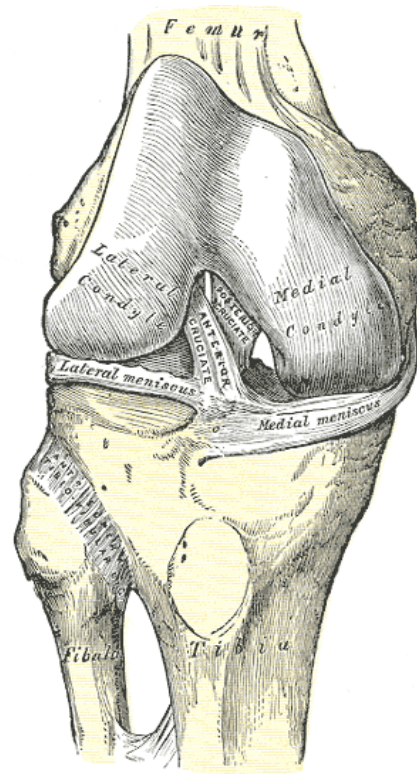


Figure 1-2 Diagram illustrating the major structures of the knee. Taken from Gray's Anatomy [90].

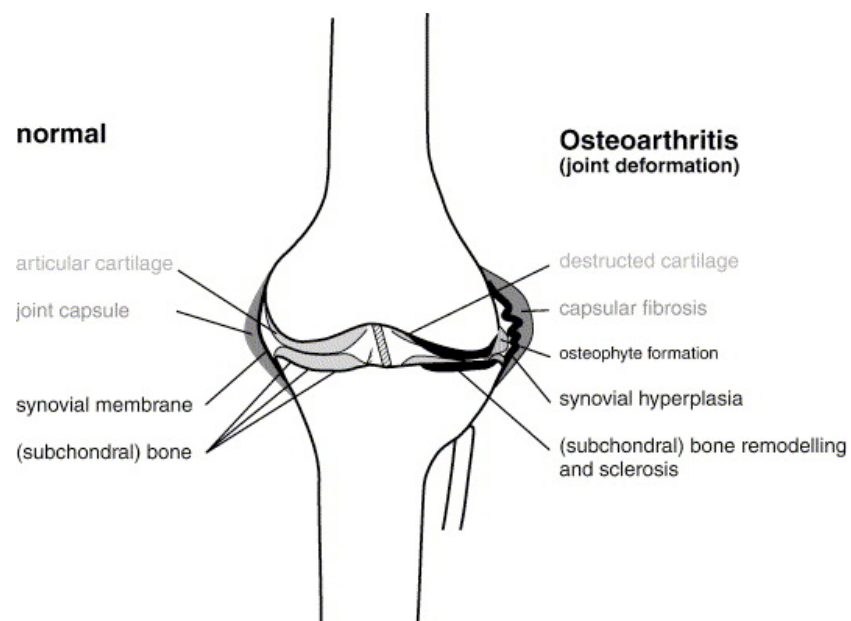


Figure 1-3 A schematic demonstrating joint changes in osteoarthritis [89].

1.2.2 Phenotypes of Knee Osteoarthritis

As described in section 1.2.1, the knee joint has three compartments; the medial tibiofemoral compartment, the lateral tibiofemoral compartment, and the patellofemoral joint. OA can affect each of these in isolation, or in varying combinations. Anteromedial Gonarthrosis presents an opportunity to study the changes in a specific phenotype of OA.

1.2.3 Anteromedial Gonarthrosis

AMG was first described by White et al. in 1971 [91]. They described a repeatable pattern of macroscopic disease; an area of full thickness cartilage loss in the anterior third of medial tibial plateau, an area of damaged cartilage in the middle third, and posteriorly, an area of full thickness, macroscopically-normal cartilage. Thus, their description described the lesion as anteromedial on the entire tibial plateau. Our group has shown, by examination and analysis of specimens, that the lesion is in the anteromedial quadrant of the medial tibial plateau (Figure 1-4). It does grow in size with deterioration of the ACL (Rout et al, in press; 2013) and when the ACL is no longer functioning, tricompartmental OA ensues [43, 53, 92]. Histological analysis of AMG corroborates the macroscopic findings, with progressively less damaged cartilage from anterior to posterior [93]. It could, therefore, be considered to be a spatial model of OA progression. Intraoperative studies have shown a matching ‘kissing’ lesion on the femoral side of the joint [94].

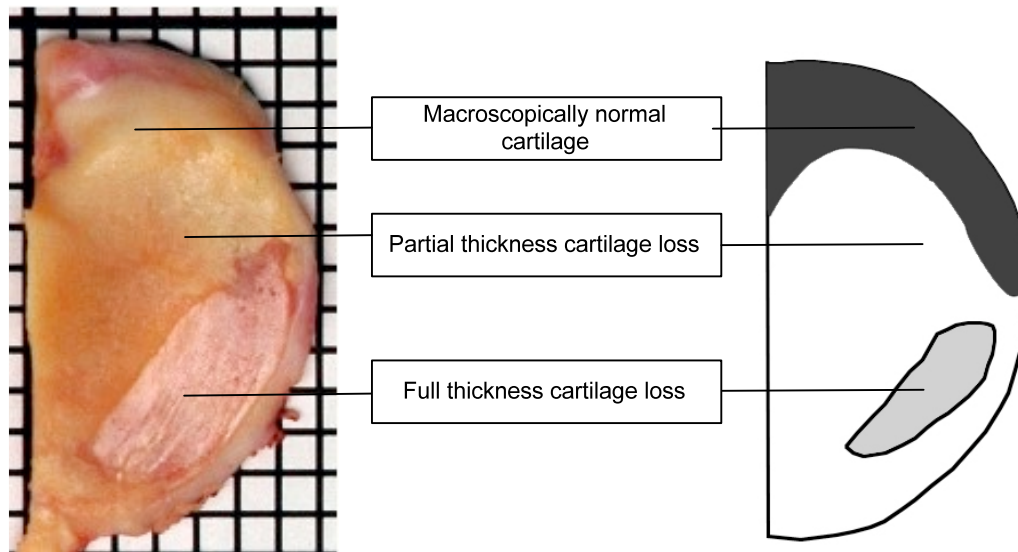


Figure 1-4 A medial tibial plateau resection specimen from a patient having UKA for AMG. The schematic on the right helps better demonstrate the areas of full thickness cartilage loss, partial thickness cartilage loss and macroscopically normal cartilage.

1.3 Cartilage

Articular cartilage is a highly specialised tissue. Its main role is the distribution of load within synovial joints, over its smooth and low friction surface. It is avascular, aneural and alymphatic. It consists of an extracellular matrix (ECM) sparsely populated with chondrocytes. The ECM is made up of primarily water, proteoglycans and collagens.

1.3.1 Structure

Articular cartilage can be divided into different zones based on depth and the arrangement of chondrocytes and matrix components (Figure 1-5). The main differences are cell shape and volume, collagen orientation and diameter, proteoglycan and water content. It can be divided into four zones; superficial, middle and deep zones, and a zone of calcified cartilage (a boundary between cartilage and subchondral bone). The superficial zone contains chondrocytes that are flattened and

elongated, and both they and the collagen fibres are arranged parallel to the articular surface. In the middle zone, chondrocytes are more rounded in shape and the collagen fibres have a larger diameter and appear more randomly arranged. The middle zone is the thickest of the zones. The deep zone has chondrocytes and collagen fibres which are arranged perpendicular to the articular surface in a columnar fashion. The zone of calcified cartilage is visible beneath a tidemark and, in this layer, collagen is arranged similar to the deep zone and appears to 'anchor' cartilage to subchondral bone with collagen fibres crossing zones. The cell population is more scarce and chondrocytes are hypertrophic.

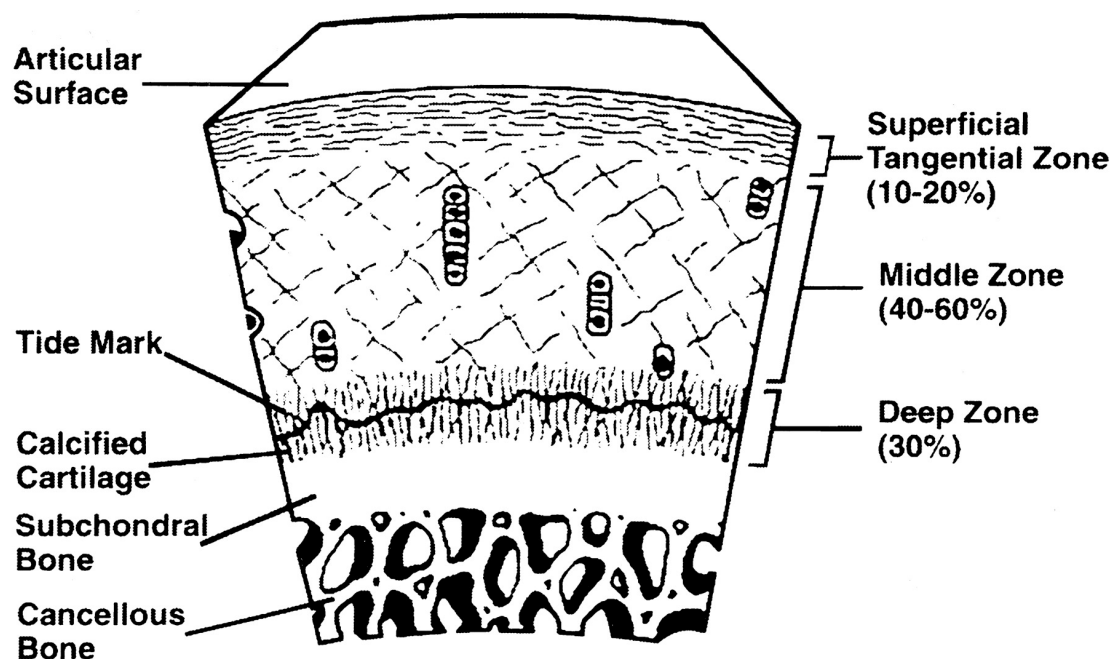


Figure 1-5 Schematic demonstrating the layers of normal human cartilage [95].

In addition, the ECM can be divided into regions characterised by the proximity to chondrocytes. The pericellular region is the region immediately surrounding the chondrocyte; the territorial region matrix lies outside the pericellular region and lies between the large regions of interterritorial matrix which encompasses the general tissue-wide matrix [96].

1.3.1.1 Chondrocytes

Chondrocytes are the resident cells of articular cartilage and they originate from the mesenchymal stem cell lineage [97]. They occupy approximately 5% of the matrix volume and are responsible for the maintenance of the ECM. Cartilage is a post-mitotic tissue and chondrocytes demonstrate extremely low levels of proliferation [98-100]. They are metabolically active and respond to a number of environmental stimuli including mechanical load. The biological stimuli and communication between chondrocytes occurs through soluble mediators which include cytokines, and growth factors.

Chondrocytes secrete the matrix components of cartilage, and through a balance of anabolic and catabolic activity regulate homeostasis – an imbalance in these functions leads to some of the changes observed in disease. Changes also occur with age, with some evidence for senescence, which may contribute to OA initiation and progression [101, 102]. A decreased responsiveness to biological signals may contribute to ECM degradation by an inability to increase anabolic activity [103].

1.3.1.2 The Extracellular Matrix

Water is the main constituent of the ECM and normal cartilage contains between 65-80%. The highest concentration is found near the cartilage surface, and decreases gradually with increasing depth. Most of the water is extracellular and contributes to the lubrication system. In addition it contains nutrients from synovial fluid.

The other two major components of the ECM are collagens and proteoglycans. Collagens vary in diameter from 20 nm in the superficial zone to 70-120 nm in the deep zone. Type II collagen is the primary collagen in cartilage accounting for 90-

98% of total collagens. The main property that collagens confer to cartilage is the tensile stiffness and strength. Proteoglycans are the next most abundant molecules with aggrecan being the commonest. They are complex macromolecules consisting of a central hyaluronic acid filament (HA) to which multiple monomers are covalently attached. Proteoglycans confer the osmotic properties necessary for cartilage to resist compressive load.

There are a number of other collagens and proteoglycans, discussed below, which even though present in smaller quantities may contribute to cartilage structure and function in both normal and diseased states.

Cartilage Constituent	Percentage
Water	65-80
Collagen	10-20
Proteoglycans	10-20

Table 1-3 - The basic constituents of cartilage as percentage of wet weight.

1.3.1.3 Collagens

Collagens make up 50-80% of dry weight of cartilage and the major collagen of articular cartilage is type II collagen [104]. It is a fibrillar protein in which the basic monomer is made up of three identical $\alpha 1$ polypeptides orientated in a left-handed helix. These polypeptides have a triple repeat amino acid sequence (Glycine-X-Y), where X and Y are frequently proline and hydroxyproline. These polypeptides associate at the C-terminal ends and condense forming a nucleus. Nucleation of the C-terminals facilitates chain alignment and formation of a right-handed triple helical structure (superhelix) that is stabilised by hydrogen bonding along the backbones of the chains. The triple helical structure is highly resistant to most proteinases. N- and C-terminal propeptides are cleaved enabling fibril formation. Processed triple helix monomers cross-link at the N- and C-terminals (non-helical regions) to form the

collagen fibrils. This covalent cross-linking occurs usually between two lysine or hydroxylysine residues and is catalysed by lysyl oxidase.

The other collagens of cartilage include types III, VI, IX, X, XI, XII, and XIV [105]. The most detail is known about type IX and XI collagens which form a heteropolymer with type II collagen, demonstrated using electron microscopy to exist as subunits of the same fibril network [106]. Type IX collagen molecules decorate the surface of type II collagen fibrils [107], and seven cross-linking sites have been identified in the type IX collagen molecule. Type XI collagen cross-link to each other and are believed to form a template that constrains the lateral growth of the type II collagen fibril [108, 109].

Type I collagen has been demonstrated in normal and osteoarthritic cartilage [110, 111]. It has been seen to colocalise with type II collagen and in OA cartilage is seen predominantly in the superficial zone in a similar fashion to that of wound healing in type I collagen based tissues [105]. The precise function of type VI collagen (distributed sparsely in the matrix) type XII and XIV collagens (found to be bound to collagen fibril surfaces) is unknown.

The fibres and network of collagen provides the high tensile strength that is resistant to deformation and important for resisting the swelling capacity of aggrecan upon hydration [112].

Interestingly in review papers of cartilage collagens [105, 113], type I collagen is not described as being a constituent of normal cartilage. Comment is passed about it being present in the normal articular cartilage of birds, where it predominates at the

articular surface and decreases with depth in an interchanging gradient with type II collagen [114]. It is the main collagen of tendon, skin and bone and is also found in fibrocartilaginous repair tissue. There is some debate, covered in more detail in the next chapter, about its presence in OA cartilage.

Collagen Type	Function	% of total collagen
I	Cartilage repair	
II	Main collagen of articular cartilage	75%
III	Adult articular cartilage	<10%
VI	Chondron basket	<1%
IX	Covalently fibril-associated cartilage	10% fetal 1% adult
X	Hypertrophic cartilage only	
XI	Fibril template	10% fetal 3% adult
XII / XIV	Non-covalently fibril-associated collagen	
XIII	Transmembrane	

Table 1-4 Table of collagens in articular cartilage, function and % by dry weight. Adapted from Eyre 2006 [113].

1.3.1.4 Proteoglycans

Proteoglycans are complex macromolecules consisting of a core protein to which covalently bound polysaccharide (glycosaminoglycans (GAG)) are covalently bound. Aggrecan is the main proteoglycan of cartilage. It is present as an aggregate of up to 100 aggrecan molecules, in association with a central filament of hyaluronan and link proteins. Aggrecan has three globular domains. G1, G2 and G3. G1 and G2 are separated by a short interglobular domain; G2 and G3 are separated by a long GAG attachment region. G1 contains the domain where HA and link proteins interact. The function of the G2 domain is unknown. The G3 domain is at the C- terminus and essential for aggrecan secretion [115].

The GAG molecules contain chondroitin sulphate and keratan sulphate, which are repeating polydimeric saccharide units of D-glucuronate and N-acetyl-D-galactosamine, and D-galactose and N-acetyl-D-glucosamine respectively. They have an overall negative charge which is crucial to control of the hydration of the matrix. GAGs are hydrated occupying a volume many times greater than their dry weight. When compressed, water is extruded from the molecule, but complete compression is sterically hindered by the polyanionic nature of the GAG molecule, thus it serves as an excellent impact absorber. Upon release of the compressive force and subsequent return of water, the inflexible collagen component resists the swelling pressure and prevents over-hydration of the proteoglycan [116].

In addition, this movement of water in and out of the cartilage and inevitable interaction with the synovial fluid is crucial for delivering nutrients and removal of waste products to and from the chondrocytes that reside in this avascular environment [117]. This interaction of aggrecan and collagen confers the ability of cartilage to maintain its integral shape and resistance to deformity (Figure 1-6).

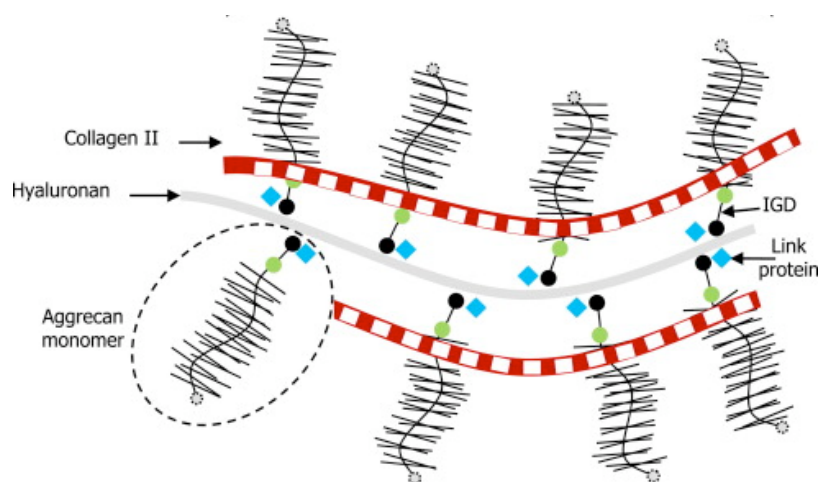


Figure 1-6 Schematic demonstrating the arrangement of aggrecan, hyaluronan and collagen in cartilage [118].

1.3.2 Cartilage in Osteoarthritis

As the definition above in section 1.1.1 implies, OA is a joint-wide phenomenon affecting all of the tissues of a synovial joint. The order in which these occur is undetermined and, as outlined previously, so is the question of where the pathology begins. One of the predominating visible features of end-stage osteoarthritis is cartilage loss, hence the description of OA being ‘wear and tear’. It is understandable that much of the study of OA has centered around cartilage degeneration and the factors driving this change. As techniques have evolved it has become possible to delve deeper into the molecular and cellular mechanisms underpinning cartilage change in OA, and some of these techniques are described in section 1.4.

1.3.2.1 Macroscopic Features

The non-invasive (imaging) features of OA cartilage are described in section 1.1.5. The next ‘level’ of descriptive features are those observed macroscopically, either at arthroscopy, or at the time of joint arthroplasty. Normal cartilage has a smooth shiny surface, and changes such as flaking or softening, fissuring and cartilage loss form the basis of two commonly used macroscopic grading systems of Collins [119] and Outerbridge [120] (See Table 1-5 and Table 1-6). Interestingly, in the description of his grade Collins wrote that, *“This grading system is, of course, arbitrary and in no way represents fixed stages in the progress of the disease.”* [119].

Collins grade	Features
Grade 0	Normal healthy joint with smooth cartilage
Grade I	Superficial flaking of cartilage in areas of pressure and movement
Grade II	More extensive destruction of cartilage not denuding bone
Grade III	Total loss of cartilage in one or more pressure areas and obvious margins
Grade IV	Complete loss of cartilage from large areas with eburnation of bone; prominent osteophytes

Table 1-5 – Collins grade of macroscopic damage to cartilage. Taken from Collins (1949) [119].

Outerbridge grade	Features
Grade 0	Normal cartilage
Grade I	Cartilage with softening and swelling
Grade II	A partial-thickness defect with fissures on the surface that do not reach subchondral bone or exceed 1.5 cm in diameter
Grade III	Fissuring to the level of subchondral bone in an area with a diameter more than 1.5 cm.
Grade IV	Exposed subchondral bone

Table 1-6 – The Outerbridge grade. Taken from Outerbridge (1961) [120].

These are relatively crude assessments of cartilage damage, though Acebes et al. showed that there is a moderate correlation between arthroscopic and histological grading systems [121]. It is also interesting to note that in macroscopic descriptions of OA, osteophyte formation can develop prior to joint space narrowing [61].

1.3.2.2 Microscopic Features

The traditional microscopic features of OA cartilage are defined by histological analysis. These include surface fibrillation and fissuring, cloning and hypercellularity, and decreased GAG staining. They are discussed in more detail in section 1.4.1 together with grading systems.

1.3.2.3 Cellular features

The cellular responses in OA cartilage include; proliferation and apoptosis, changes in synthetic activity, changes in degradation, and phenotypic modulation of chondrocytes [63]. Chondrocytes have low proliferative activity in osteoarthritic cartilage [122]. The reported levels of apoptosis are variable, ranging from 20 to 50% [123-125]. A more detailed consideration of apoptosis is presented in Chapter 5.

OA cartilage has been shown to have some metabolic activity and synthesis of ECM components [126-128], under the influence of anabolic growth factors such as transforming growth factors beta, bone morphogenic proteins, insulin-like growth factors and fibroblast growth factors. However there is an imbalance in their breakdown with the increased synthesis of two main families of enzymes, the matrix metalloproteinases (MMPs) and the Disintegrin-like and metalloproteinase-like domain with thrombospondin motifs (ADAMTS). Chapter 2 describes the roles of these enzymes in more detail.

1.4 Experimental techniques

The earliest studies of cartilage in OA involved macroscopic assessment alone [119]. There has been a rapid evolution in biological techniques and this thesis uses a number of molecular and cellular techniques to examine cartilage. An overview of their principles, development and historical use is given below.

1.4.1 Histology

Histology remains the gold standard, both clinically and in research, for the study and grading of damaged cartilage in OA. Mankin et al. in 1971 [129], produced

pioneering work on a pathological grading system for OA cartilage. The Mankin grade of Histological and Histochemical Grading System (HHGS), as it is also known, examines the following pathological features; the structure (in terms of damage to cartilage), cellularity, safranin-O staining, and tidemark integrity. Each is scored as shown in Table 1-7) with the range being 0-14.

Category	Subcategory	Score
Structure	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to transitional zone	3
	Clefts to radial zone	4
	Clefts to calcified zone	5
	Complete disorganisation	6
Cells	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity	Intact	0
	Crossed by blood vessels	1
Total		0–14

Table 1-7 – The Histological and Histochemical Grading system for osteoarthritic articular cartilage [129].

There have been several modifications to the Mankin grade. For example that of Armstrong et al [130] takes into account chondrocyte cloning. More recently, due to concerns about the validity and reproducibility of this scoring system [131, 132] OARSI produced a new grade, stage and scoring system [133] subsequently named the Osteoarthritis Cartilage Histopathology Assessment System (OOCHAS) [134]. The grade is driven by the key feature of the degree of cartilage damage, with

subtypes defined by associated criteria as demonstrated in Figure 1-7. The stage is dependent upon the horizontal extent of the damage across a joint's cartilage surface. Thus for experimental models analysing regional areas of cartilage, the stage is redundant [93].

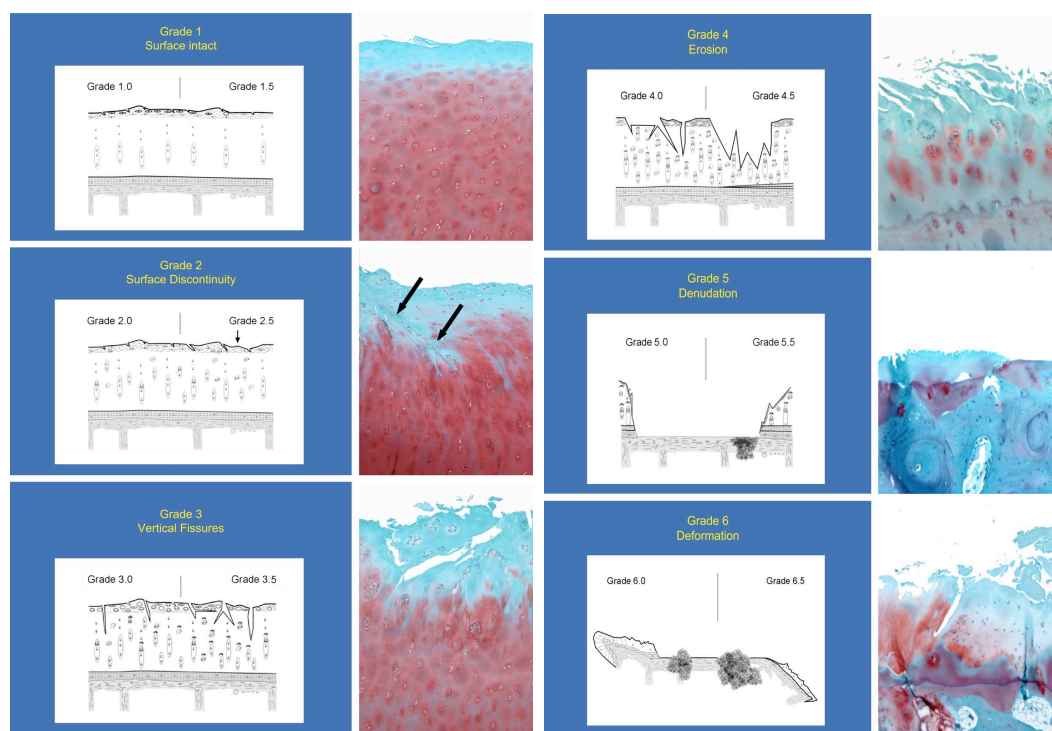


Figure 1-7 The Osteoarthritis Cartilage Histopathology Assessment System (OOCCHAS). This scoring system grades sections based on the surface phenomena of cartilage as demonstrated in the schematic representations and histological sections provided in the original paper [133].

1.4.2 Light Microscopy Techniques

Immunohistochemistry was first developed in the 1940s [135] to allow for the detection of and localisation of molecules in cells and tissues. It does this through an antigen-antibody interaction that is visualised using an enzyme, dye or radioactive element. It remains a useful tool but only allows for semi-quantitative analyses, for example the number of cells in which a protein is found, but not the absolute amount of that protein.

Fluorescence *in situ* hybridization (FISH) is a technique developed in the 1980s [136] to detect and localise specific DNA sequences. It can be used to detect mRNA in tissue, thus helping define the spatial pattern of gene expression. Specific probe sequences are created and tagged with fluorophores. The amount of fluorescence detected can be used in a comparative fashion. It has previously been used as a semi-quantitative technique, though with increased specificity of probes and improved microscopy, it can be used quantitatively.

1.4.3 Other Microscopy Techniques

The advances in microscopy, such as those below, can produce some elegant images including those of fluorescent markers and co-localisation and orientation of molecules being investigated. Confocal microscopy produces clearer images by excluding light from outside of the focal plane, using pin holes. Thus it allows for imaging of greater depth of a section, with the ability to reconstruct a three dimensional picture. Such analysis can provide interesting data on chondrocytes at different depths of a section and the 3D morphology of cells [137, 138].

Multiphoton microscopy uses non-linear optical effect, amplified at very tight focus, to produce images (fluorescence) of sub micron resolution. Due to the near infra-red pump beams, the depth of penetration is improved relative to visible light. The use of high intensity pulsed lasers also lends itself to second harmonic generation microscopy. Molecules, such as collagen that lack centro-symmetry (are piezo electric) generate second harmonic signals that can be related to spatial organization and orientation [139, 140].

Electron microscopy uses a beam of electrons, instead of light, to image a specimen. Its main advantage over light microscopy is its ability to produce image resolution on the nanometer scale. However, it is limited by its expense and the extensive preparation of tissue required. It is not possible to image live tissues/cells.

1.4.4 Immunoassay

Enzyme Linked Immuno Sorbent Assay (ELISA) allows quantitative measurement of the levels of protein in cartilage sample. It uses antigen-antibody interactions in a similar manner to immunohistochemistry, and can determine the amount of a protein present in a sample by measurement of level of the optical signal of enzymatic substrate. Their use in the study of cartilage has particularly been driven by the need to assess the quality of engineered cartilage [141-143].

1.4.5 Polymerase Chain Reaction

Conventional Polymerase Chain Reaction (PCR) is a technique that was developed in the 1980s to amplify DNA strands. Primers that are specific to a strand of DNA are used in conjunction with an enzyme, DNA polymerase, to create complimentary strands. The principle involves three cyclical steps: denaturation of double stranded DNA, annealing of primers, and primer extension [144]. RNA sequences are first reverse transcribed into cDNA and then undergo the same process. The presence of amplified products is detected using gel electrophoresis at the end of the procedure. This does not allow for accurate quantification, though band thickness can be used to crudely approximate or compare levels of gene expression [145].

Real Time quantitative PCR (qPCR) was developed in 1996 [146] as a method of DNA quantification. It monitors the progress of a PCR reaction in real time, allowing for quantification of even small amount of PCR product. This is possible due to the detection of fluorescence produced by a reporter molecule. These can be either dyes that bind to DNA or sequence specific probes such as TaqMan probes [147]. In each cycle of PCR, if the target sequence is present, the fluorophore attached to the probe is cleaved thus increasing the reporter dye signal. The detection of this signal can then be quantified absolutely, or relative to a control specimen or target ‘housekeeping’ gene.

1.4.6 Microarray

Microarray technology was first described in the 1990s [148]. It allows the simultaneous study of gene expression levels across large numbers of genes in parallel. A glass chip is coated with selected probe “spots” and DNA added. Hybridization of the probe occurs to the complementary DNA which is fluorescently tagged. The plate can then be scanned and the intensity of signal from each spot calculated. A vast amount of data is generated. This can undergo complex bioinformatic analyses in conjunction with gene ontology data to demonstrate clusters of genes that are up or down regulated, together with pathway analyses.

1.5 Previous Findings in the study of Anteromedial Gonarthrosis

Over the last six years, our group has focused on the specific OA subtype of AMG. This is most common pattern of disease in patients presenting for knee replacement, and the specific pattern of lesion development lends itself to mechanical and biological study. Previous studies have focused on macroscopic and histological

features. This thesis considers, in more detail, regional cellular and molecular variations.

1.5.1 Macroscopic

The macroscopic features of AMG were first described by White et al. in 1971 [91] and are summarized in section 1.2.3. Further, more focused work by our group on the size of the area of full thickness cartilage loss shows that with increasing damage of the ACL, the lesion size increases (Rout et al, in press; JOR 2013) and the macroscopically normal area of cartilage posteriorly remains. At the point at which the ACL is no longer functional, tricompartmental OA ensues, with a loss of the retained posterior cartilage on the medial tibial plateau, and extension of disease into the lateral compartment.

1.5.2 Histological

Previous work by our group has characterised in detail the histological features of AMG [93]. Samples were sectioned through regions of full thickness cartilage loss, damaged cartilage and undamaged cartilage, as demonstrated by the schematic below (Figure 1-8). The sections then underwent staining using Haematoxylin and Eosin and Safranin-O stains, and were graded using both Modified Mankin and OOCAS scoring systems (Figure 1-9). A significant decrease in histological grade was demonstrated from anterior to posterior ($p < 0.001$) with the posterior region classified as being within a normal range (Figure 1-10). This study showed that there was a good correlation between Modified Mankin and OOCAS grades ($\rho = 0.886$; $p = 0.001$), with both demonstrating similar inter- and intra- observer error, suggesting that they can both grading systems can be used for the purposes of such studies.

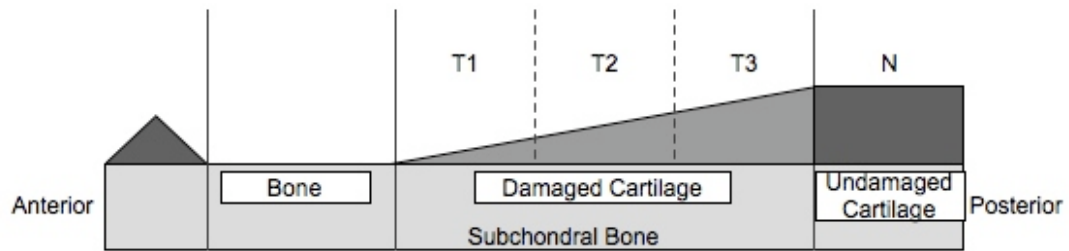


Figure 1-8 - Schematic of a cross section of AMG. It demonstrates the regions of full thickness cartilage loss (Bone), damaged and undamaged cartilage. The damaged cartilage was divided into equal thirds T1, T2 and T3. N is the undamaged region of cartilage posteriorly.

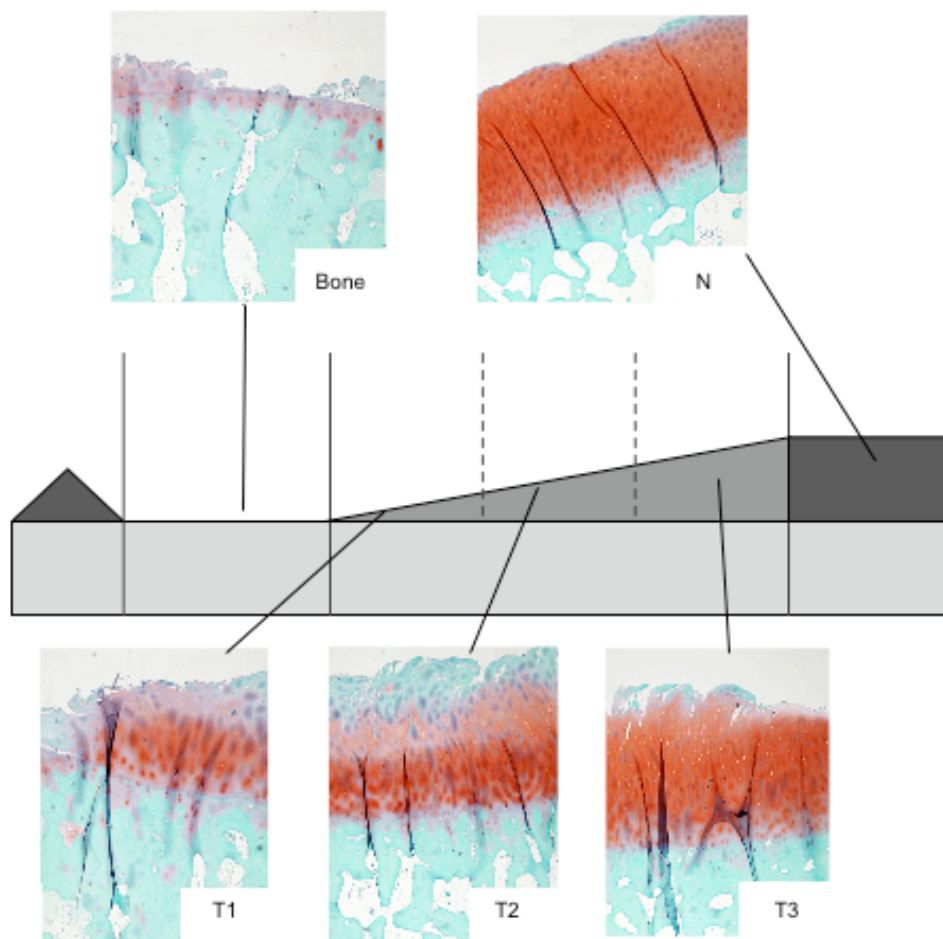


Figure 1-9 Schematic of regions with the histological Safranin-O staining of each region: T1, T2, T3 (damaged areas) and N (undamaged area).

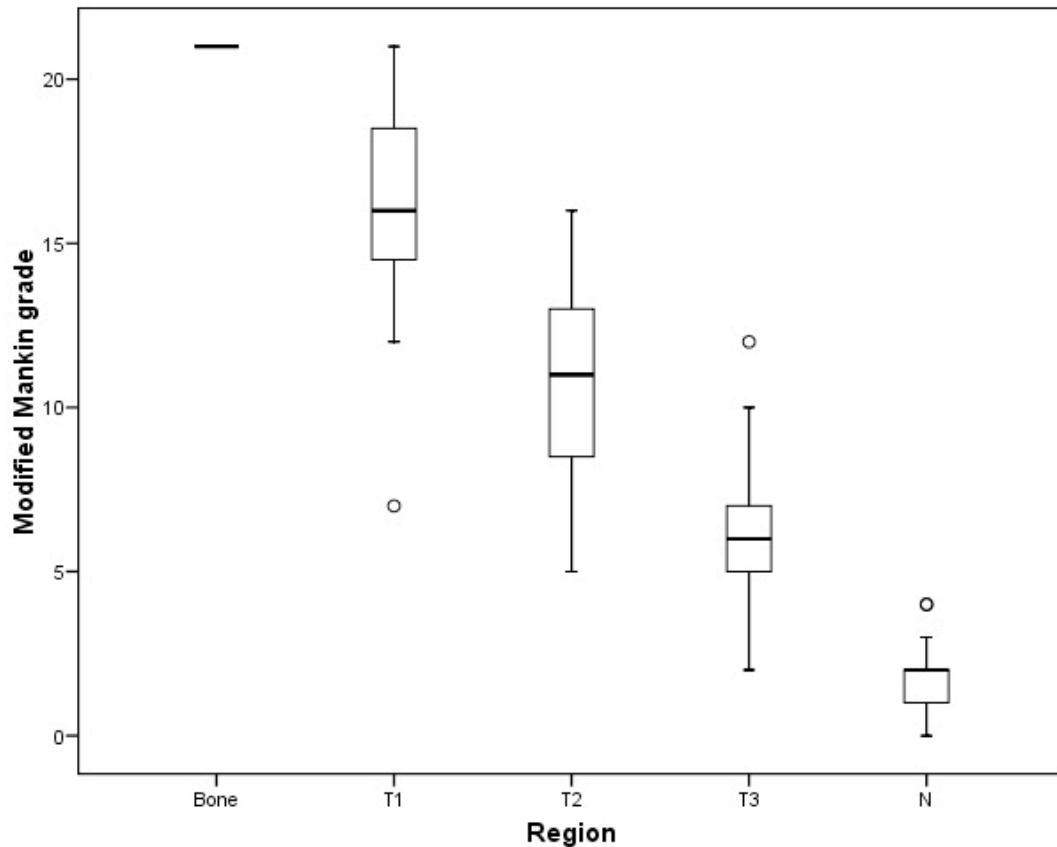


Figure 1-10 - The modified Mankin grade of AMG by region. A statistical difference was shown between regions ($p < 0.001$).

Immunohistochemical analyses revealed low levels of cell proliferation across all regions of the specimen, as one might expect, given the low turnover of cartilage in both normal and diseased states. Staining was undertaken for Types I and II collagens. Type II collagen appeared evenly distributed in all regions of cartilage. However, Type I collagen staining was shown in an inter-territorial distribution in undamaged cartilage only (Figure 1-11). This interesting finding led to further investigation with immunoassays, and is considered in more detail in Chapter 2.

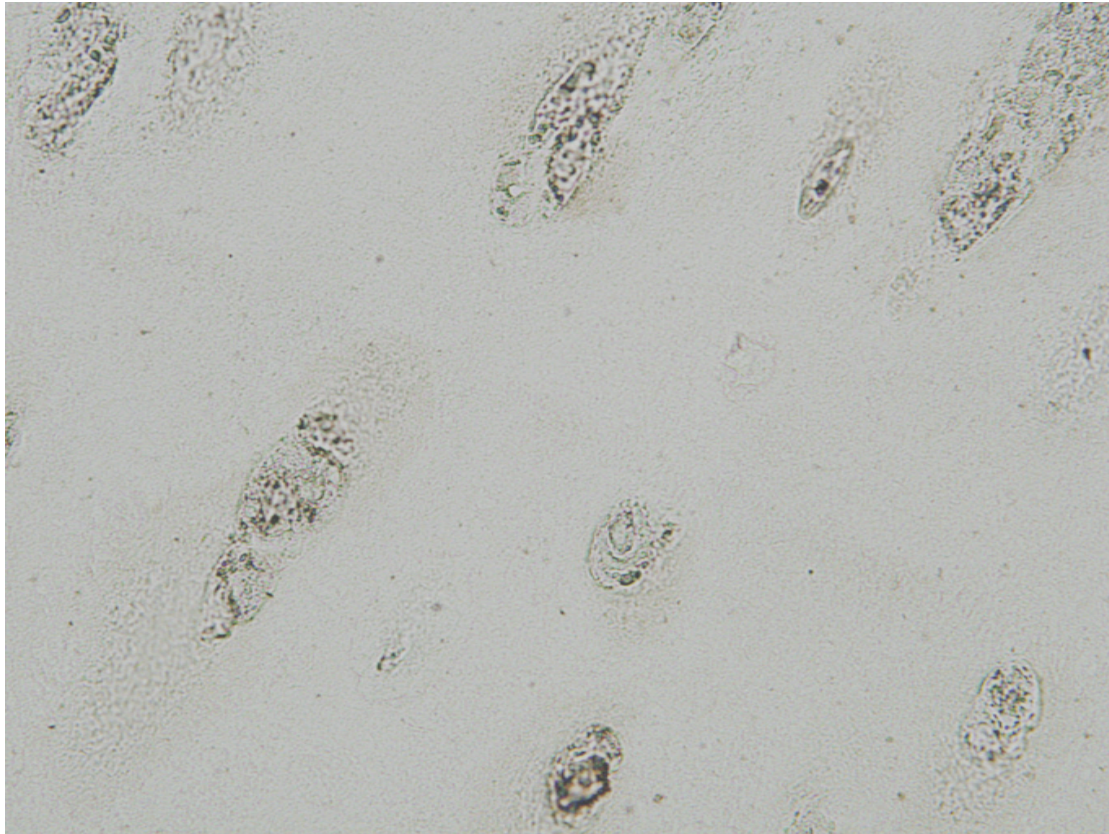


Figure 1-11 Type I Collagen immunohistochemistry. Positive staining was found in some chondrocytes and the interterritorial regions of the matrix.

1.5.3 Immunoassays

Immunoassays were undertaken for matrix components GAG, Type II collagen and Type I Collagen. The regions used were those defined in the study of histology of AMG [93]. T1-T3 were equal thirds of damaged cartilage, with T1 being the most damaged, T3 being the least damaged, and N, undamaged cartilage. There was a significant decrease in GAG levels with more damaged cartilage ($p < 0.0001$) (Figure 1-12) and no difference in type II collagen levels ($p = 0.78$) (Figure 1-13). However, the Type I collagen assay revealed an unexpected finding, with increased levels in the macroscopically and histologically normal cartilage (Figure 1-14).

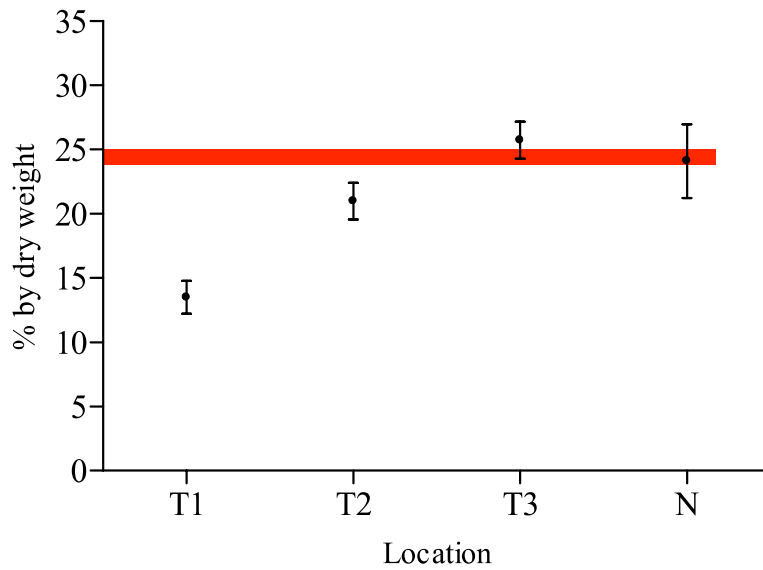


Figure 1-12 – Graph of GAG % dry weight by region (reproduced from S.McDonnell thesis, The Phenotype of Anteromedial gonarthrosis, 2008). T1-T3 represent damaged cartilage (most damaged to least damaged). N is undamaged cartilage. The red line represents lab control data (bovine). There was a significant difference ($p < 0.0001$; ANOVA) between regions.

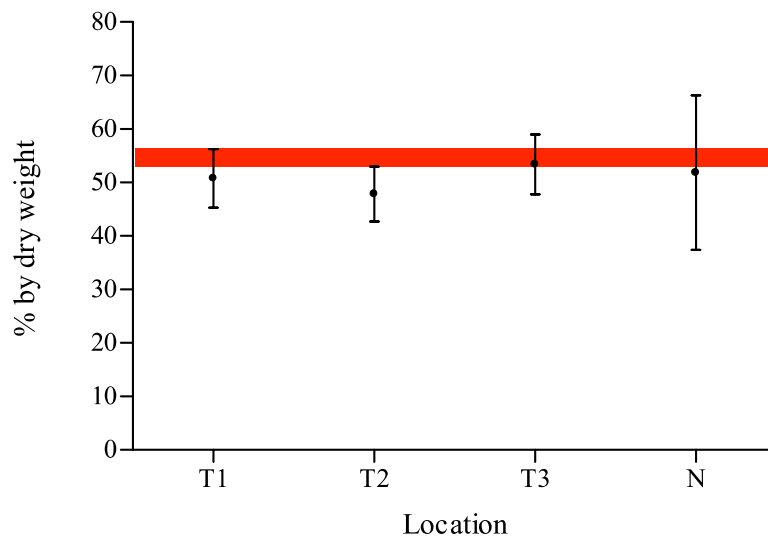


Figure 1-13 – Graph of Type II Collagen % dry weight by region (reproduced from S.McDonnell thesis, The Phenotype of Anteromedial gonarthrosis, 2008). There was no significant difference ($p = 0.78$) between regions.

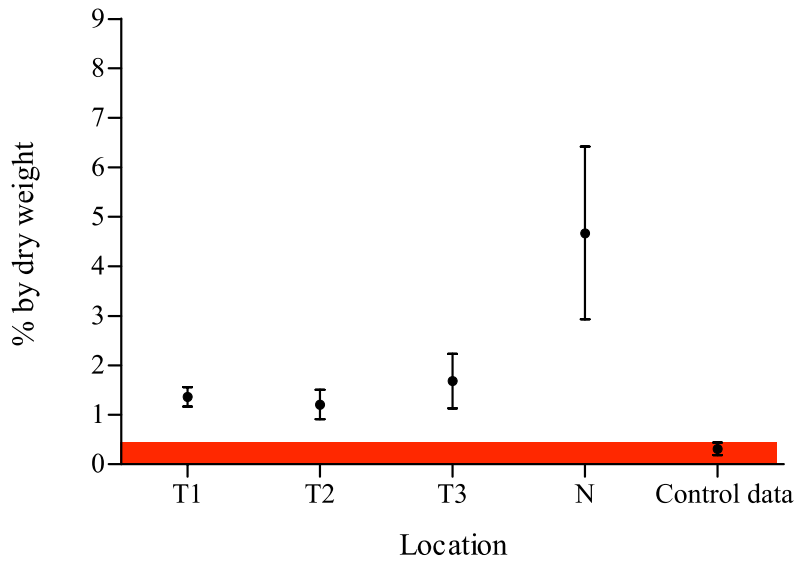


Figure 1-14 – Graph of Type I Collagen % dry weight by region (reproduced from S.McDonnell thesis, *The Phenotype of Anteromedial gonarthrosis*, 2009). There was a significant difference ($p<0.0001$) between groups.

1.5.4 Conclusions of Previous Studies and Motivation for this Work

The work above forms the basis of the model of AMG, this thesis, and subsequent work. It is suggestive that within AMG, there are well-defined histological regions with varying degrees of cartilage damage in close proximity. Therefore one could consider them to be at different stages of OA disease with the undamaged posterior region exemplifying early pre-OA and the anterior lesion, end-stage disease. As previously discussed, there are no human models of OA disease progression, and though obtaining tissue for temporal studies will always remain a challenge, AMG provides a platform for the study of a spatial model of OA.

The expected results were demonstrated for levels of GAG, with decreased amounts in the more damaged tissue. However the unexpected finding of increased Type I collagen, demonstrated qualitatively with immunohistochemistry, and quantitatively with immunoassays led to the hypothesis that this may be a pre-histological (early)

change of OA, worthy of investigation, and formed the basis for further molecular and cellular studies in AMG.

Even the earliest presentation of OA to the clinician is often that of end-stage OA. Clinical imaging shows changes of cartilage erosion, and treatments are limited to those mentioned previously in sections 1.1.7 and 1.1.8. One could consider the clinical presentation of OA as being synonymous with ‘a failing joint’.

There are currently no disease modifying treatments for OA. We have a very limited understanding of the changes that occur prior to visible histological damage. AMG provides a potential model for studying and gaining a better understanding of ‘earlier’ changes of osteoarthritic cartilage; one would hope that it might be possible to identify markers of early disease, and develop targets for earlier intervention.

1.6 The Scope of this Thesis

The scope of this thesis is to move beyond standard pathological descriptions of AMG cartilage, and to begin to explore altered cellular responses, signalling pathways and pathogenic mechanisms in a well-defined phenotype of OA.

In addition to the evident changes in damaged cartilage, the data presented above (section 1.5.4) provides evidence for previously undocumented changes occurring within the undamaged cartilage of AMG. These are interesting because they may represent pre-OA changes. A major technical aim of this thesis is to collect knee cartilage from age-matched patients without OA and to develop a sampling technique

to isolate precisely matched regions for comparison with AMG specimens, relying on our understanding of lesion topography from previous studies.

1.7 Aims of this thesis

This thesis aims to study cartilage biology in AMG, a specific phenotype of disease. AMG presents an opportunity to study regions of damaged and undamaged cartilage that are in close proximity and exhibit progressive histological features of disease. I aim to demonstrate regional differences in the content and gene expression of these areas. By investigating the molecular and cellular changes that occur in these regions of cartilage, I hope to identify new potential markers of disease and provide new insight into the mechanisms underlying these changes.

A series of cellular and molecular techniques are used both on AMG samples and control tissue to address the following questions:

1. Are there gene expression differences between undamaged and damaged cartilage in AMG?
2. Do these gene expression studies corroborate our previous findings from immunoassays?
3. Does healthy control tissue confirm these findings as changes due to OA?
4. What regulates apoptotic cell death in cartilage degeneration in AMG?
5. In a whole genome microarray is it possible to identify further genes and pathways involved in cartilage degeneration in AMG?

Chapter 2 will explore regional gene expression differences in AMG. The following ‘matrix-relevant genes will be investigated using Real-Time Polymerase Chain Reactions (qPCR) comparing damaged and undamaged cartilage regions: COL1A1, COL2A1, COL3A1, ACP, MMP 1, MMP 3, MMP 13, MMP 28, ADAMTS 4, ADAMTS 5, ADAMTS 16.

Chapter 3 will describe the methods by which control tissue is obtained. In order that results for AMG can be compared, above knee amputations are used as control tissue, and a template created to allow for topographical sampling from equivalent regions of cartilage.

Chapter 4 will present a series of experiments (histology, immunoassays and qPCR) conducted on AKA samples, as have previously been undertaken on AMG specimens.

Chapter 5 explores apoptosis in AMG. Apoptosis has been shown to play a role in cartilage degeneration in OA. Pilot data from our group suggests increased levels of apoptosis in degraded cartilage. This study will use a series of immunohistochemical experiments to explore potential mechanisms of apoptosis in AMG and its pathways.

Chapter 6 uses whole genome microarray technology to consider a global picture of cellular alteration between damaged and undamaged cartilage in AMG.

A specific hypothesis for each study is presented at the start of each chapter

Chapter Two

2 STUDY 1 – REGIONAL GENE EXPRESSION DIFFERENCES IN ANTEROMEDIAL GONARTHROSIS

2.1 Introduction

Chapter 1 outlined our group's findings in Anteromedial Gonarthrosis using biochemical and immunoassays. In light of these intriguing findings, the aim of this study was to investigate relative differences in gene expression between damaged and undamaged cartilage in AMG. In particular, the finding of increased Type I collagen in the macroscopically and histologically normal cartilage of AMG has not been documented in the literature and was worthy of further investigation. In addition we noted a high loss of GAG in the damaged region and were interested to assess whether this correlated with decreased aggrecan mRNA expression and other relevant matrix degrading enzymes mRNA.

A study was therefore designed to make use of the highly uniform graded damage features in AMG specimens to map regional gene expression changes in a number of matrix-relevant genes. The ability to compare expression patterns in damaged and undamaged regions from the same specimen is a considerable advantage when compared to the widely published approach of using damaged and undamaged cartilage from different patients. The genes studied were those most relevant to the immunoassays conducted in previous studies (COL1A1, COL2A1, ACP). In addition a selection of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) were also chosen for

study. There was limited RNA available for gene expression studies, and the MMPs and ADAMTSs chosen were on the basis of either a previously well documented role in OA (MMPs 1, 3, 13; ADAMTSs 4,5), or because of emerging evidence (MMP 28 and ADAMTS 16) from a collaborating group (Dr. Ian Clark, University of East Anglia)

Chondrocytes are responsible for cartilage homeostasis. The cytokines, growth factors, and enzymes produced by chondrocytes control the anabolic and catabolic processes that occur within cartilage. The MMPs and ADAMTSs have been previously studied and shown to play a key role in cartilage extracellular matrix (ECM) degradation [149-151]. Their precise role in OA is not fully understood, in terms of their interaction with other proteinases and their inhibitors. Similarly, the triggers and predisposing factors for their up- and down- regulation in disease states has not been characterised.

The MMPs, in humans, consist of a group of 24 zinc-dependent endopeptidases (they require a zinc ion in their active site for catalytic activity). They are active at neutral pH and can therefore catalyze the normal turnover of ECM macromolecules (collagens, proteoglycans, elastins, and matrix glycoproteins). Table 2-1 shows the MMPs that were selected for study.

MMP1 was the first MMP discovered in 1962 [152], where its action was noted on resorbing tadpole tails. As the first MMP discovered, much early work in OA cartilage focused on its collagenase activity in cleaving Type II collagen [153].

It was not until MMP13 was sequenced from breast cancer tissue [154] that interest was raised as to the relative collagenase action of MMPs in OA. MMP13 activity has been shown to be 10-fold that of MMP1 activity against Type II collagen. Hence MMP13 became considered pathognomic of OA.

MMP3 is another enzyme that has been well documented as having a role in cartilage degeneration in OA [155, 156]. This is not only due to its direct action on its substrates, but because of its capacity to activate pro-MMPs -1, -8, -9 and -13.

MMP28 is the newest member of the MMP family. It was first reported in the literature in 2001[157, 158]. Kevorkian et al [159] and Davidson [160] have shown this to be amongst the most highly upregulated MMPs in OA cartilage and synovium. Its role and function have been studied in chondrosarcoma cell lines [161] however a more specific action in OA cartilage has not yet been identified.

MMP	Enzyme name	Substrates
MMP-1	Collagenase-1, interstitial collagenase	Collagens I, II, III, VII, VIII and X, gelatin, aggrecan, versican, proteoglycan link protein, casein, α 1-proteinase inhibitor, α 2-M, pregnancy zone protein, ovostatin, nidogen, MBP, proTNF, L-selectin, proMMP-2, proMMP-9
MMP-3	Stromelysin-1	Collagens III, IV, IX and X, gelatin, aggrecan, versican, perlecan, nidogen, proteoglycan link protein, fibronectin, laminin, elastin, casein, fibrinogen, antithrombin-III, α 2M, ovostatin, α 1-proteinase inhibitor, MBP, proTNF, proMMP-1, proMMP-7, proMMP-8, proMMP-9, proMMP-13
MMP-13	Collagenase-3	Collagens I, II, III and IV, gelatin, plasminogen activator inhibitor 2, aggrecan, perlecan, tenascin
MMP-28	Epilysin	Casein

Table 2-1 - Table summarising the matrix metalloproteinases (MMPs) examined and their substrates. Adapted from Murphy *et al* (2002) [162]

The ADAMTSs consist of a group of 19 enzymes, the first of which, ADAMTS1 was documented in 1997 [163], though prior to this others had been discovered but labelled proteases rather than ADAMTSs. These enzymes have a complex domain structure consisting of at least a signal peptide, pro domain, metalloproteinase domain, disintegrin domain, thrombospondin type I motif and cysteine rich domain [164]. Table 2-2 shows the ADAMTSs selected in this study.

Initial work into OA and the ADAMTSs suggested that ADAMTS5 was the major aggrecanase involved in its pathogenesis [165, 166]. However, more recent work has shown that both ADAMTS 4 and 5 contribute to the structural damage in OA cartilage [167-169]. ADAMTS16 was described more recently [164] and has also been shown to be upregulated in OA cartilage and synovium [159, 160].

ADAMTS	Enzyme name	Substrates
ADAMTS4	Aggrecanase-1, KIAA0688*	Aggrecan; brevican; versican V1; fibromodulin; decorin; carboxymethylated transferrin
ADAMTS5	Aggrecanase-2, ADAMTS-11	Aggrecan
ADAMTS16		Unknown

Table 2-2 - Table to summarise the “a disintegrin and metalloproteinase with thrombospondin motifs” (ADAMTSs) examined and their substrates. Adapted from Porter et al (2005) [164].

This study aims to assess the relative regional gene expression differences between the damaged and undamaged cartilage of AMG specimens.

2.2 Null Hypothesis

There are no gene expression differences between damaged and undamaged regions of cartilage in AMG as determined by mRNA quantification measured by quantitative real time polymerase chain reaction.

2.3 Materials and Methods

2.3.1 Sample collection and preparation

Six tibial plateau resection specimens were collected from patients undergoing UKA for AMG (Ethics Reference C01.071) (Appendix 1) at the Nuffield Orthopaedic Centre, Oxford. Preliminary work undertaken for four samples' damaged regions (n=12 regions), had shown that in order to obtain suitable amounts of RNA for gene expression analysis it was not possible to divide the regions up as described previously (T1-N), in Chapter 1, for immunoassay work. Instead, it was necessary to compare damaged versus undamaged regions *en masse*. Therefore damaged cartilage (T1-T3) was compared to undamaged cartilage (N) (Figure 2-1).

For each sample, the cartilage was identified as being either undamaged (macroscopically) or damaged. Under a bright light, the samples were examined and a line marked between the posterior crescent of undamaged cartilage and damaged cartilage. The rim of the sample (the medial and posterior borders) was discarded to avoid contamination with tissue such as osteophyte. The cartilage was lifted off the bone using a size 12 scalpel blade. It was immediately snap frozen in liquid nitrogen and transported to a -80 °C freezer, where it remained pending processing.

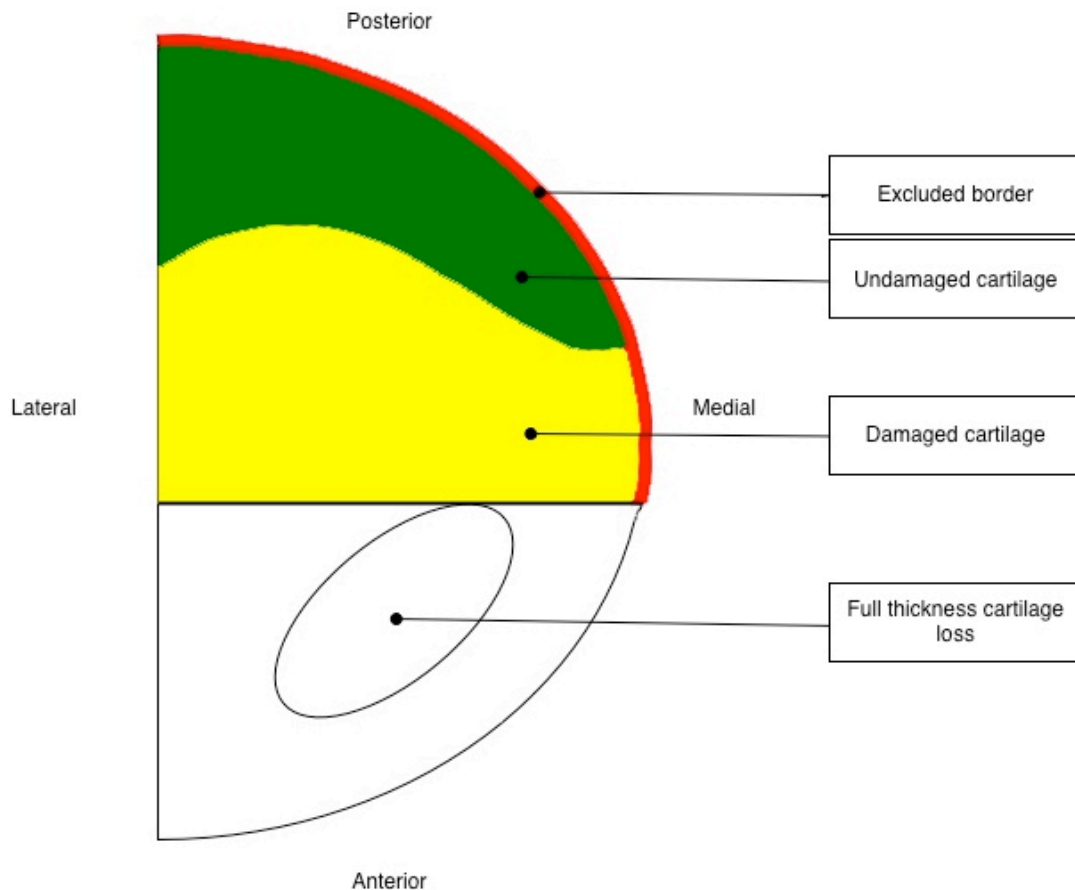


Figure 2-1 – Schematic of a medial tibial plateau demonstrating the features of AMG. The regions of cartilage sampled. The marked regions represent: the excluded border the undamaged cartilage (green), and the damaged cartilage (yellow).

2.3.2 RNA extraction

The samples were processed in a batch, and messenger RNA (mRNA) was isolated from the areas of undamaged and damaged cartilage. All surfaces, vessels, and equipment used were made RNase free through use of RNaseZap (Applied Biosystems, California, USA), or baking of relevant equipment to 180°C for 2 h.

The specimens were ground under liquid nitrogen using the Type 6750 Freezer Mill (Spex Certiprep) and transferred to an Eppendorf tube. They were further ground using a pre-chilled pestle and mortar. Following grinding they were quickly weighed before TRIzol[®] reagent (Invitrogen) was added to the Eppendorf (1ml/200mg

cartilage). After being vortexed for 15 sec, they were incubated at room temperature for 5 min.

Samples were centrifuged at 4°C and 9500g for 10 min. The cartilage formed a pellet, and the supernatant was recovered. Chloroform was added to the supernatant (600µl per 1ml of TRIzol[®]). The mixture was vortexed for 15 sec and incubated at room temperature for 10 min before being centrifuged at 4°C and 9500g for 15 min. The aqueous layer was recovered into a fresh tube. Having closely estimated the volume when pipetting off the aqueous layer, 0.5x volume of 100% ethanol was added, and the tubes shaken vigorously.

The RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used according to manufacturer's protocols. The remaining centrifuge steps were conducted at room temperature.

Samples were loaded onto the columns and centrifuged at 9,500g for 15 sec and the flow-through discarded. 700µl buffer RW1 (Qiagen) was added, centrifuged at 9,500g for 15 sec and the flow-through discarded. Columns were transferred to new collection tubes, 500µl buffer RPE (Qiagen) added, and centrifuged at 9,500g for 15 sec and the flow-through was discarded. Another 500µl buffer RPE (Qiagen) was added and centrifuged for 2mins, at 9,500g. The columns were transferred to new collection tubes.

RNA was eluted with 30µl RNase free water. After adding the water, they were left to stand for 2 min to increase the RNA yield. They were then centrifuged at 9,500g for

1min. Quantification at 260nm was undertaken using a NanoDrop[®] spectrophotometer (NanoDrop Technologies, Delaware, USA). Samples were then stored at -80°C.

2.3.3 Reverse Transcription

Complementary DNA (cDNA) was synthesized as follows; 2mM random hexamers (Amersham Biosciences, Amersham, UK) were added to 1µg RNA in a total volume of 11µl and incubated at 70°C, for 10min. The master mix containing 4µl 5x first strand buffer (Invitrogen, Paisley, UK), 2µl 0.1M DTT (Invitrogen), 5µM dNTPs (Bioline, London, UK), 200U Superscript II reverse transcriptase (Invitrogen) and 30U RNasin[®] ribonuclease inhibitor (Promega, WI, USA) in a total volume of 9µl was prepared. The master mix was added to the randomly primed RNA to give a total volume of 20µl and incubated for 1h, at 42°C followed by heat inactivation of the Superscript at 70°C, for 10 min. cDNA was stored at - 80°C.

2.3.4 Quantitative Polymerase Chain Reaction

qPCR was undertaken for the genes shown in Table 2-3.

Matrix related genes	Matrix Metalloproteinases	ADAMTSs
COL1A1	MMP 1	ADAMTS 4
COL2A1	MMP 3	ADAMTS 5
COL3A1	MMP 13	ADAMTS 16
ACP	MMP 28	

Table 2-3 - List of genes that underwent quantitative real time polymerase chain reaction (qPCR).

Gene expression data was normalized. 18S was used as an endogenous reference genes. This accounts for differences in RNA concentration between samples.

All samples achieved quality control by falling within a $1.5C_t$ range. C_t is the threshold cycle where the signal is above baseline and can be detected.

For each of the genes in Table 2-3 a standard curve was prepared to control for primer/probe efficiency. 18S cDNA standards were serially diluted as follows; 4, 2, 1, 0.5, 0.25, 0.125ng/10 μ l. All other genes were serially diluted as 20, 10, 5, 2.5, 1.25, 0.625ng/10 μ l. The results were plotted in Microsoft[™] Excel 2004 for Mac (Microsoft, Washington, USA) as log₁₀ of the C_t against concentration, the line equation calculated.

Relative quantification was determined using the standard curve and normalising to a housekeeping gene. RT-qPCR was carried out using the ABI Prism 7700 Sequence Detector in MicroAmp optical 96-well plates (Applied Biosystems). 18S assays required 1ng cDNA and all other genes required 5ng cDNA per reaction.

The following were added to each well; 2x TaqMan[®] Universal PCR Master Mix (Applied Biosystems), 100nM forward and reverse primers and 200nM probe to a total reaction volume of 25 μ l with water. Plates were sealed using optical adhesive covers (Applied Biosystems). RT-qPCR cycling conditions were; 2 min at 50°C, 10 min at 95°C followed by 15 sec at 95°C and 1 min at 60°C for 40 cycles. Data was analysed using SDS 1.9.1 (Applied Biosystems).

2.3.5 Data Analysis

2.3.5.1 Relative Quantification

Relative quantification was performed using the standard curve method [170]. This allows direct comparison across samples. The relative expression of the genes above was compared between damaged and undamaged regions of cartilage. As it is a relative comparison (without a normal reference), gene expression levels are expressed as being higher or lower, and not up- or down- regulated.

2.3.5.2 Statistical analysis

Non-parametric statistical tests were used to compare groups, given the small numbers analysed, thus making no assumption about their distribution.

Pairwise analysis, undertaken using a Wilcoxon test, was performed using PASW version 18 for Mac (Illinois, USA). Significance was set at the $p < 0.05$ level.

2.4 Results

2.4.1 Samples

Six tibial plateau were analysed. The mean age of the patients was 70.8 (range 61 - 78). There were 4 male and 2 female and 3 left and 3 right sided specimens.

2.4.2 Summary

Table 2-4 shows a summary of the genes examined, together with fold change and significance. They are presented as expression in undamaged cartilage, compared to damaged cartilage. There were five genes in total that showed statistically significant higher levels in undamaged cartilage. There was an 8.8 fold higher expression of

COL1A1 in undamaged cartilage relative to damaged cartilage ($p=0.028$, Figure 2-2).

No significant difference was noted for the other matrix genes.

Of the MMPs examined, there was significantly higher expression in undamaged cartilage compared to damaged cartilage; 1.88 fold for MMP1 ($p=0.046$, Figure 2-3), 3.68 fold for MMP3 ($p=0.028$, Figure 2-4), and 4.62 fold for MMP13 ($p=0.028$, Figure 2-5). No significant difference was seen for MMP28 (Figure 2-7).

ADAMTS 4 expression was 26.86 higher in undamaged cartilage compared to damaged cartilage ($p=0.046$, Figure 2-6). No significant difference was noted for ADAMTS 5 and 16 (Figure 2-7).

There were no genes that showed lower levels in undamaged, compared to damaged cartilage.

Gene	Fold change Undamaged compared to damaged	p value
COL1A1	8.80 (+)	0.028
COL2A1		0.116
COL3A1		0.116
ACP		0.917
MMP 1	1.88 (+)	0.046
MMP 3	3.68 (+)	0.028
MMP 13	4.62 (+)	0.028
MMP 28		0.917
ADAMTS 4	26.86 (+)	0.046
ADAMTS 5		0.173
ADAMTS 16		0.249

Table 2-4 - Changes in gene expression between undamaged and damaged cartilage in AMG. Gene expression is expressed as fold change with p values (+ indicates higher levels in undamaged cartilage relative to damaged cartilage, and red p values highlight significant changes).

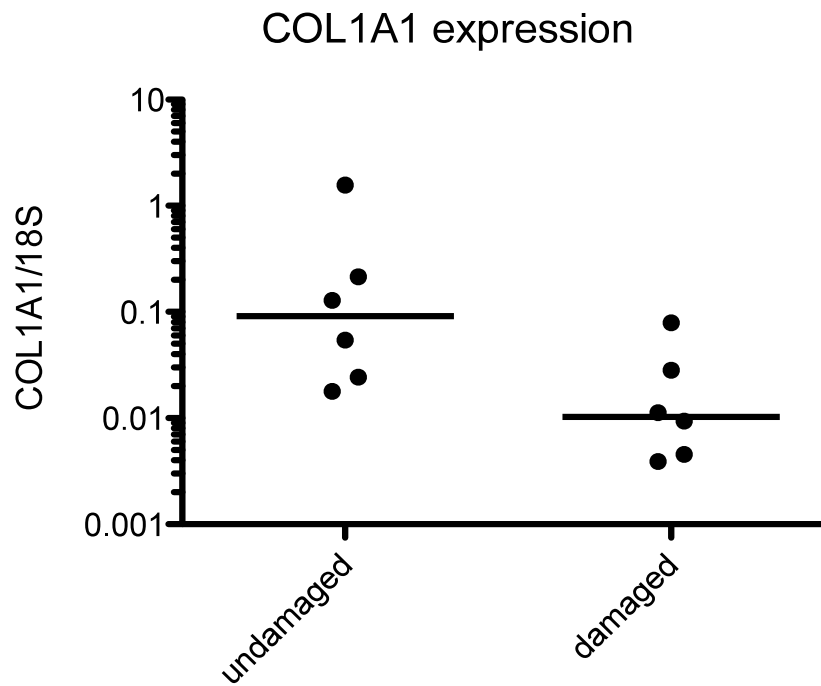


Figure 2-2 –Gene expression of COL2A1 in undamaged and damaged cartilage. There is 8.8 fold higher expression of COL2A1 in undamaged cartilage (p=0.028, Wilcoxon).

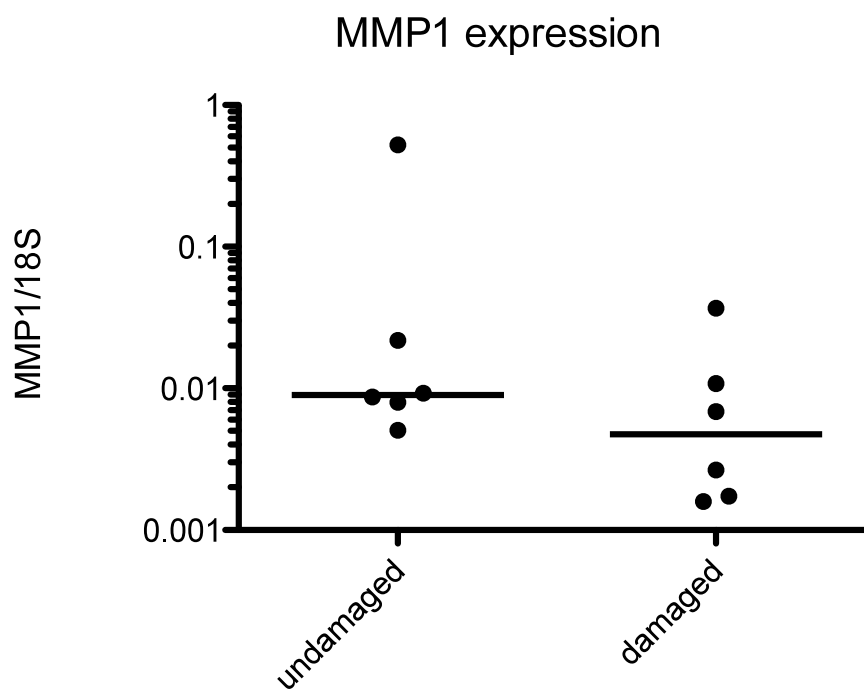


Figure 2-3 - Gene expression of MMP1 in undamaged and damaged cartilage. There is 1.88 fold higher expression of COL2A1 in undamaged cartilage (p=0.0246, Wilcoxon).

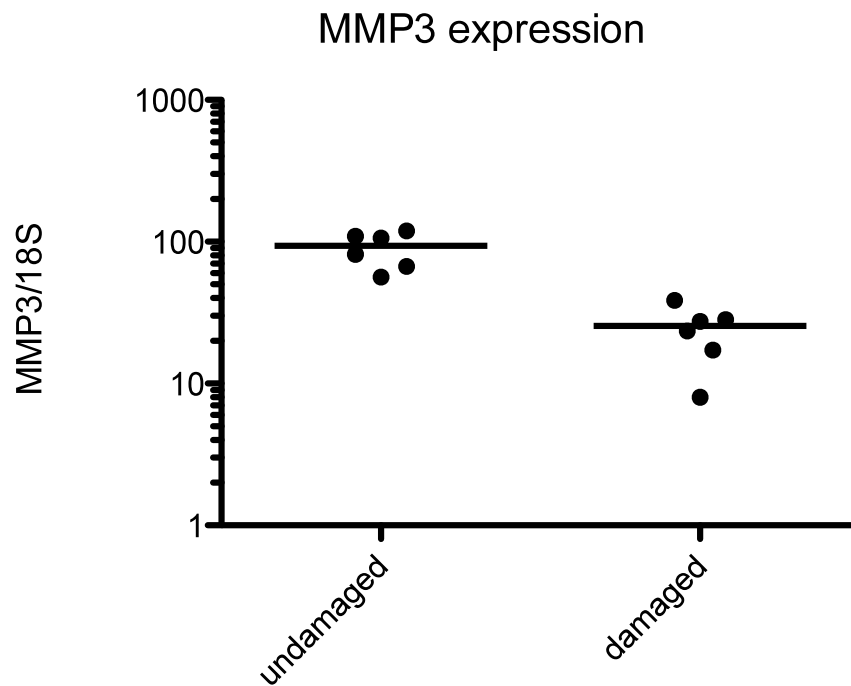


Figure 2-4 - Gene expression of MMP3 in undamaged and damaged cartilage. There is 3.68 fold higher expression of MMP3 in undamaged cartilage ($p=0.028$, Wilcoxon).

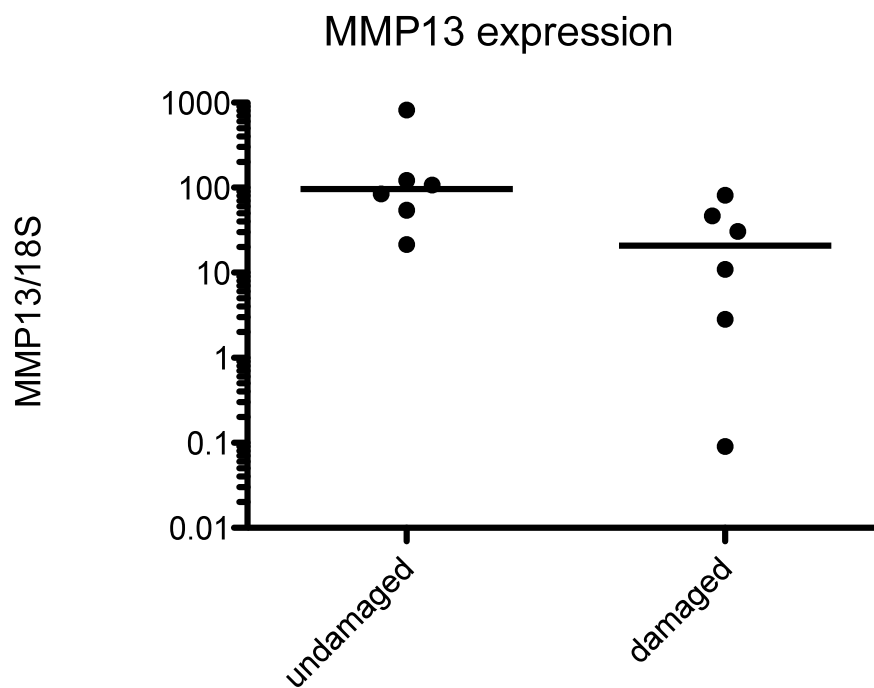


Figure 2-5 - Gene expression of MMP13 in undamaged and damaged cartilage. There is 4.62 fold higher expression of MMP13 in undamaged cartilage ($p=0.028$, Wilcoxon).

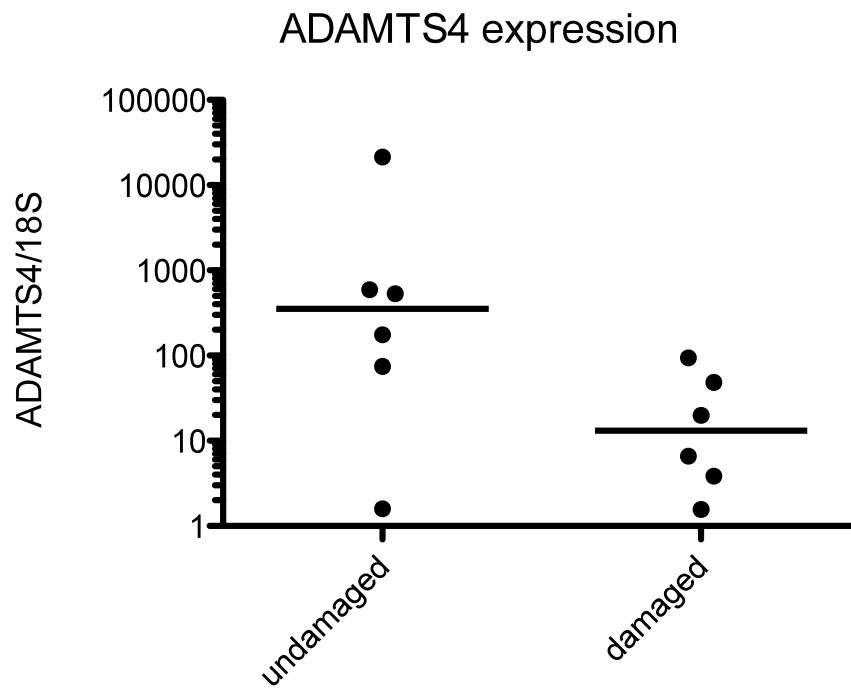


Figure 2-6 - Gene expression of ADAMTS4 in undamaged and damaged cartilage. There is 26.86 fold higher expression of MMP13 in undamaged cartilage ($p=0.046$, Wilcoxon).

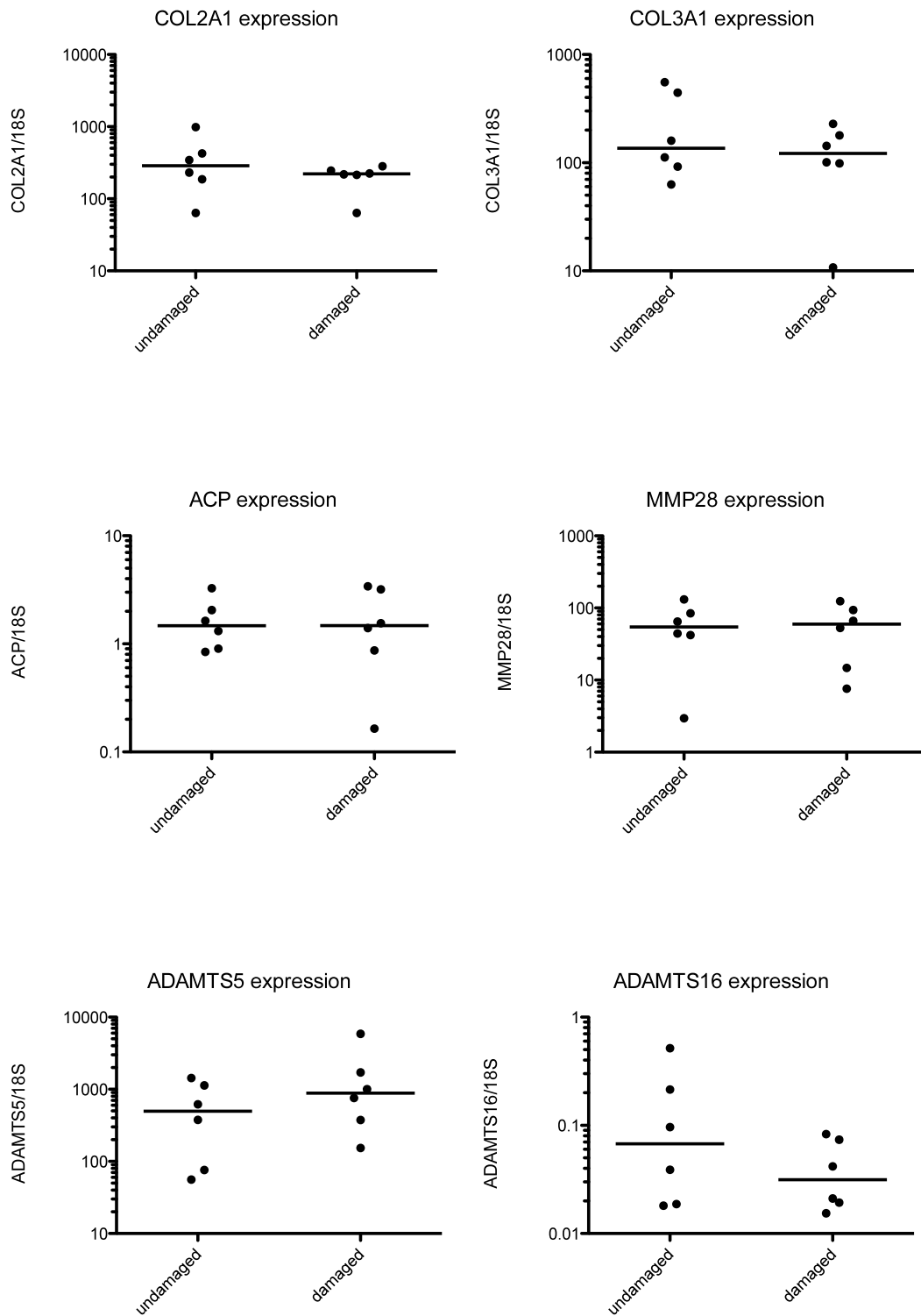


Figure 2-7 - Gene expression for further genes. These results, for COL2A1, COL3A1, ACP, MMP28, ADAMTS5 and ADAMTS16 are plotted for undamaged and damaged cartilage. There was no significant difference for any of these genes (Wilcoxon).

2.5 Discussion

The null hypothesis “*There are no gene expression differences between damaged and undamaged regions of cartilage in AMG as determined by mRNA quantification measured by quantitative real time polymerase chain reaction.*” is rejected.

The aim of this study was to gain insight into the complex matrix biology that, over time, may have contributed to the region-specific and cumulative matrix changes identified in our group’s previous work. I sought to take advantage of the tight phenotype in AMG and the highly conserved pattern of damage to make pairwise comparisons of regional gene expression differences in AMG for a number of key matrix related components and enzymes. It is somewhat disappointing not to be able to consider all of the regions (T1, T2, T3 and N) but this was an experimental limitation secondary to difficulties in obtaining good quality and quantity of RNA from small amounts of cartilage. However, the main strength of the study is that this is a statistical pairwise analysis of samples from the same patients, all of whom exhibit the same pattern of disease. In considering how to present the data, I chose to present it as level in undamaged cartilage relative to damaged cartilage. It is important to note that this data could easily be presented as the opposite of this. In the absence of normal reference value, it could be the case that both values are up-regulated, both down-regulated, or that normal expression falls between damaged and undamaged cartilage.

2.5.1 Matrix related genes

Firstly if we consider the evidence for changes in the matrix related genes group:

This experiment shows a higher level of COL1A1 gene expression in the undamaged and histologically normal cartilage of AMG, compared to the damaged cartilage; COL2A1 demonstrated no differential gene expression. This is the first study to my knowledge that has shown this relative difference between damaged and undamaged cartilage. Perhaps more interestingly, it would appear to corroborate the results of previous work on AMG, where the Type I collagen protein content was shown to be increased. Whereas the immunoassays show a cumulative change in the formation and homeostasis of cartilage over a longer time frame, the gene expression has given a ‘snapshot’ of chondrocyte metabolism at one time point.

There are a number of studies that have examined differences in collagen content and expression in osteoarthritic cartilage [111, 114, 171-176]. Early experiments used a variety of cellular and molecular techniques to examine the presence and expression of Type I collagen in normal and osteoarthritic cartilage. These included immunohistochemistry [111, 177] and *in situ* hybridisation [111, 173, 176] and semi-quantitative RT-PCR [175, 176, 178]. Results have been conflicting with some studies suggesting no change in Type I collagen and others that corroborate the findings of this study. Certainly, Type I collagen is well recognised as being present in fibrocartilage and ‘repaired’ cartilage [141, 142]. It would appear that as techniques have become more sensitive and accurate that Type I collagen has been more commonly detected in OA specimens.

Eyre [105, 174], in several review articles, makes no mention of Type I Collagen being a constituent part of normal cartilage nor of the osteoarthritis disease process. Mankin [179] described the presence of Type I Collagen, but only in repaired cartilage that was, when examined histologically, a mixture of hyaline and fibrocartilage. The same is true for osteophytic tissue.

The earlier techniques of electrophoresis, used by Goldwasser et al showed no Type I collagen in normal or osteoarthritic cartilage [171]. However he discussed the limitations of the technique, suggesting that if Type I collagen levels were less than three percent then it would be difficult to show its presence. In our groups previous work, the mean content of Type I collagen in the undamaged tissue was 4.5% by dry weight, and in damaged tissue just over 1%.

As techniques progressed, Aigner et al in 1993 studied osteoarthritic cartilage from hip and knees using *in situ* hybridisation for Type I, II and III collagen [111]. In 1997, he published further work on knee OA biopsies, examined using immunohistochemistry and *in situ* hybridisation [172]. In both of these studies, he did not find positive staining for Type I Collagen in any sample. In the second study he did show changes in Type II and III collagen, which were increased in OA. Gebhard et al, using real time PCR did show a 13-fold increase in Type I collagen in osteoarthritic cartilage [175]. However, their commentary suggested no change in chondrocyte phenotype because the Type I to Type II collagen ratio was unchanged, whereas in this study Type II collagen expression was unchanged.

A number of other studies in the 1980s and early 1990s did show the presence of type I collagen in OA using immunohistochemical techniques [177, 180, 181]. Our group's immunohistochemical studies on AMG showed the same distribution (McDonnell et al, unpublished work). This was typically located in the surface layer of damaged cartilage and around clonal clusters. However the quantitative contribution of this surface expression appears to be outweighed by overall tissue distribution when regional full depth mRNA expression is studied. The evolution of gene expression techniques and increased sensitivity of molecular techniques appears to have contributed to being able to study, in far greater detail, phenotypic alterations in the chondrocyte in OA.

Since the late 1990s a number of studies have shown increased Type I Collagen gene expression in OA, in cartilage at various stages of disease. Young et al. showed positive staining for Type I Collagen and an 11.7 fold increase in COL1A1 mRNA expression in histologically damaged regions [182]. Miosge et al showed Type I Collagen mRNA presence in the deep zones of OA chondrocytes [173], and later showed a 100 fold increased between Collins grade [128] I and III disease [176].

The immunoassay data for AMG cartilage has shown increased levels of Type I collagen in undamaged cartilage. In the absence of a normal reference for COL1A1 gene expression, one cannot comment on the level of gene expression being up- or down- regulated compared to a non-disease state. However, what is clear is that there is a relative difference in the gene expression. One possible hypothesis, taking into account the gene expression and immunoassay data, is that the presence of Type I collagen may be an early marker of OA damage or possibly indicative of altered

mechanical loading as the joint surface erodes. This is certainly worthwhile studying further.

2.5.2 MMPs and ADAMTSs

The MMPs and ADAMTSs have been widely studied in OA, however it is believed that this is the first study that has considered their differential expression between damaged and undamaged cartilage within the same joint. It has shown significantly higher levels of gene expression in undamaged cartilage of MMP1, 3 and 13, and ADAMTS4.

MMP13 has been shown to be increased in a number of studies, and is likely to be the key collagenase in OA [159, 160, 183-185]. However, in this study its expression is at a relatively higher level in undamaged cartilage.

MMP1 and 3 have been shown to be upregulated in rheumatoid arthritis, however appear to be down-regulated in OA cartilage [159, 160, 186, 187]. Other studies have shown MMP3 to be present in greater amounts following cartilage damage secondary to cyclical loading, with loss of proteoglycans [156] and OA models [155, 156, 188, 189]. MMP3 has been described as central [190] due to its pro-MMP activity, in particular on MMPs 1, 8, 9 and 13. In this study, MMP1 and 3 expression had higher levels in undamaged cartilage.

ADAMTS4 and 5 expression levels were similarly higher in the undamaged zone of AMG cartilage. Previous studies have described ADAMTS5 as being the major aggrecanase [165, 166, 191] with ablation of its activity in a mouse model, protecting

against OA. More recent work has also shown the presence of and increased gene expression for ADAMTS4 [167-169].

MMP28 and ADAMTS16 were first reported to be upregulated in OA by Kevorkian et al. [159]. With little known about their substrate and function, it is difficult to speculate on their role in OA pathogenesis.

Previous studies do not provide straightforward answers to which up- or down-regulated genes contribute to OA pathogenesis. What is clear from the literature is there is co-activation and interaction between a number of cytokines, and the underlying processes are not fully understood. Some of the findings of this study may initially be perceived as contradictory to previous work. There are a number of possible reasons why this might be the case. Tissue used for OA research is generally a heterogeneous mix of damaged and undamaged regions from resected human or animal joints. The majority of previous studies comparing OA versus normal cartilage excise OA cartilage *en masse* from joints. However within a diseased joint there may be both undamaged and damaged cartilage. Therefore if one takes both of these together their gene expression is, in effect, mathematically ‘added together’. The topographical pattern of AMG cartilage erosion enables the reproducible sampling of these separate areas, and consideration of gene expression changes of cartilage at different stages of disease.

It should be noted that in isolation one should be wary of interpreting gene expression data as it may not be indicative of protein synthesis due to post transcription / translation regulation. However there is some apparent corroboration of these results, in

particular the Type I collagen expression, with previous immunoassay data of ECM content. However, there is no disease-free comparator available in the literature or this study that allows a conclusion of whether this is a real up-regulation.

There are a number of possible hypotheses as to why there are unexpected changes in undamaged cartilage. First and foremost it should be noted that the standard for determining whether cartilage is osteoarthritic or not is histology [192]. However, given our evolving understanding of osteoarthritis, many cellular and molecular features are becoming evident that occur prior to histological damage. These are mostly from animal models, as one can appreciate the ethical issues of removing normal looking cartilage from human joints.

This rise in Type I collagen, and the changes in MMP and ADAMTS expression may be early features of OA. But other possibilities are that these are topographic feature, one that is present in normal joints, or perhaps one that is secondary to ageing.

If we consider this to be a change due to OA, there are a number of reasons why this might be the case. OA is a joint-wide phenomenon, affecting cartilage, synovial fluid, synovium, ligaments, bone, as well as cartilage [3, 15]. In AMG, cartilage erosion begins in the anteromedial quadrant of the tibial plateau [91] (Rout et al, 2013, in press). It extends posteriorly as the disease progresses, but there remains an intact crescent of macroscopically and histologically normal cartilage [93], until the ACL fails. During this process there are many chondrocytes that can be considered to be in varying stages of OA, and thus varying stages of OA phenotype. There is a complex combination of biological and mechanical changes within this OA joint, and one

could hypothesize that changes in weight bearing status and consequent signalling changes could contribute to Type I collagen gene expression / content changes.

From a biological perspective, it could be the case that the late stage of disease within regions T1-3 has caused cessation of cytokine stimulation. However, the joint-wide disease processes may be causing changes in the undamaged cartilage due to cytokine activity in synovial fluid, from chondrocytes or synoviocytes elsewhere in the joint.

Mechanical loading has been shown, using *in vitro* studies, to affect the ECM by inducing collagen and proteoglycan degradation [156, 193, 194]. It has been shown to induce MMP13 [195]. Loading of the knee, and tibio-femoral movement has been studied in great depth in the normal knee, with pivotal work by Freeman et al [196-199] demonstrating the centres of femoral condyles on the tibial plateau. Further studies using pressure-sensitive film [200] and finite element (FE) modelling [201] have shown load distribution patterns within a normal knee and knees that have undergone meniscectomy. These show the highest load is close to the middle of the medial tibial plateau with more dispersed loads peripherally. This is mainly due to the menisci whose role is load distribution [88]. MRI studies of AMG have shown antero-medial (but not posterior) meniscal extrusion to be a common finding [202]. It is logical that this change will alter the load distribution on the tibial plateau and this perhaps contributes to the initiation of AMG. Once there is cartilage erosion, the load distribution must again change, but there are no studies that investigate this directly. Unloading of skeletal tissues is strongly linked to up regulation of MMPs both in bone [203] and tendon [204] and lack of loading has also been linked to high collagen I content in fibrocartilage Autologous Chondrocyte Implantation (ACI) grafts as

chondrocytes are passaged in culture [205]. It will be important to measure loading in the conserved posterior of AMG cartilage once the joint has realigned with the anterior lesion, since relative unloading would be consistent with the molecular changes observed here.

2.5.3 Further Work

This work, together with our group's previous work, provides evidence for a change in the matrix content between regions of AMG (section 1.5.3). Furthermore, the MMP and ADAMTS data demonstrate interesting changes in the environment of the undamaged cartilage of AMG. Two further studies in this thesis were influenced by these findings:

One hypothesis of the interpretation of the immunoassay and gene expression data from AMG cartilage is that these changes are secondary to OA. In order to investigate this further, Chapter 4 examines control tissue, and uses the same series of experiments used with AMG specimens to attempt to validate these findings as changes of the disease state, and not simply regional variations.

A further exploratory experiment builds on the hypothesis of an altered signalling environment in AMG giving rise to the matrix and gene expression differences described above. Chapter 6 uses microarray technology to address the question of other differences in gene expression of tens of thousands of genes, and their interacting pathways.

Chapter Three

3 COLLECTION OF CONTROL DATA

3.1 Introduction

Chapters 1 and 2 have demonstrated that Anteromedial Gonarthrosis exhibits novel regional differences of OA that have not previously been described. These findings, of regional differences in matrix composition and gene expression, highlight the potential use of AMG as a spatial model of disease progression. Type I collagen has not been previously described in the literature in undamaged cartilage, and differences in COL1A1, MMPs and ADAMTSs gene expression, though not quantitative, provide further evidence of altered metabolism within damaged and undamaged cartilage.

In order to further our understanding of why and when these changes might be occurring, it was necessary to collect normal control tissue for further experiments.

Historically there has been poor control data in the study of OA. This is partly due to the difficulty in obtaining normal human tissue from asymptomatic individuals. Thus researchers have used a number of other sources of tissue. Animal models have been used widely in the study of OA [85] however their relevance to human disease is questionable [86, 206]. Their patterns of OA are different and the rate at which they develop OA is far shorter, making comparisons to humans difficult. There are other sources of human cartilage: for example, when studying hip OA, proximal femoral neck fracture samples are used as control data [159, 160, 207]. Similarly, with knee OA, amputations have been used as control samples in previous studies [208, 209]. In

some cases, where amputation was undertaken for lower limb tumours, neoplasia and their treatment may be confounders [210] of cellular and molecular studies.

As discussed in section 1.5.4, one of the strengths of studying cartilage in AMG is the reproducible pattern of disease. Many studies into OA cartilage have taken all remaining cartilage from OA joints with little regard to disease topography. Similarly, studies that have used control tissue often take all available cartilage for experiments. The sub-anatomical location of cartilage is an important feature of a local micro-environment where there are unique biological and mechanical factors. Studies have shown differences in ECM based on location and weight bearing status in knee joints [210].

The control tissues collected for this work were above knee amputations (AKA) from patients undergoing the procedure due to lower limb ischaemia caused by peripheral vascular disease (PVD). As described above, one typical shortcoming of OA cartilage experiments is the lack of reproducible topographical sampling. A major aim of the work presented in this Chapter was to use the predictive power conferred by the tight disease phenotype of AMG to develop an accurate method to achieve this in normal knee specimens. In order to be able to compare diseased with control specimens, a template was created to lay over harvested samples so that cartilage was taken from equivalent topographical locations of the medial tibial plateau.

3.2 Materials and Methods

3.2.1 Ethics

I obtained Ethical approval (Reference 06/Q1606 /97) to harvest knee joints from patients undergoing AKA for PVD from the Oxford Research Ethics Committee (Appendix 1). A substantial amendment was subsequently submitted to permit harvesting joint from patients who lacked capacity to consent.

3.2.2 Site Initiation, Patient Recruitment and Consent

The two hospitals recruited into providing tissue for the study were the John Radcliffe Hospital, Oxford, and Stoke Mandeville, Aylesbury, under the authority of the local vascular surgeon: Mr. Jeremy Perkins, and Mr. Andrew Northeast, respectively.

Both sites were visited at the start of the study, and intermittently during the study to ensure the vascular surgery teams, nurses and theatre staff were aware of the procedures. The doctors were advised to contact me if it was decided that a patient under their care would undergo an AKA. I would then meet the patient, give them information pertaining to the study and obtain informed consent/assent (Appendix 1).

3.2.3 Surgical Technique

Once the operating vascular surgeon had amputated the leg, it was handed out to me. I worked on a side table within a sterile field. The skin, fat and muscle were dissected away from the knee joint. The joint was opened, and the status of the cruciate ligaments and menisci were documented. They were then removed and the tibial and femoral the articular surfaces were photographed (Figure 3-1, Figure 3-2, Figure 3-3).

Specimens exhibiting macroscopic disease were discarded. Using an oscillating saw, the excess bone was removed and the distal femur, proximal tibia and patella were harvested.

The remaining tissue was placed in a limb bin and discarded according to hospital policy.

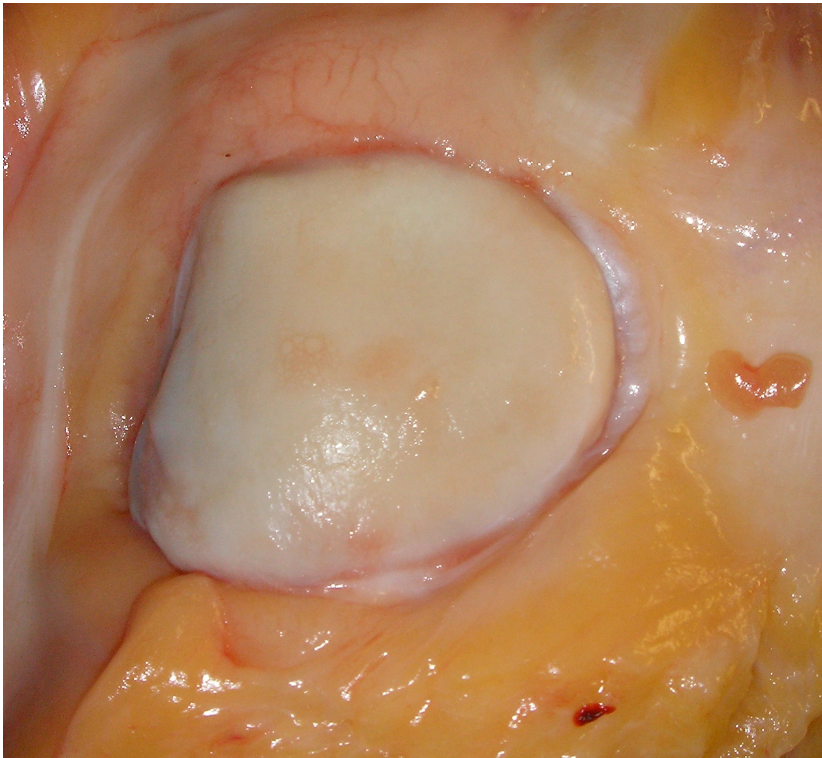


Figure 3-1 Macroscopic appearances of patella from an AKA sample collected from a patient having surgery for peripheral vascular disease.



Figure 3-2 Macroscopic appearances of femur from an AKA sample collected from patients having surgery for peripheral vascular disease.

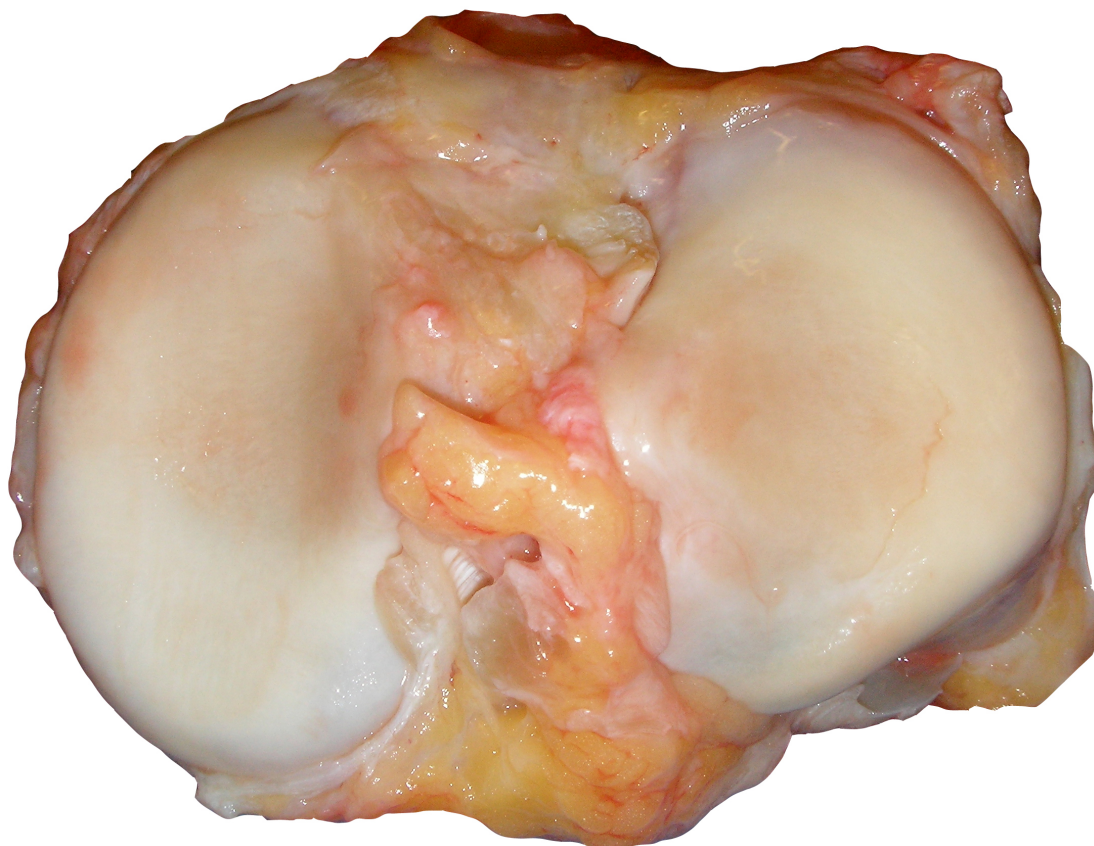


Figure 3-3 Macroscopic appearances of tibial plateau from an AKA sample collected from patients having surgery for peripheral vascular disease.

3.2.4 Transport and Storage

The samples were then transferred, on ice, to the laboratory where they were stored in a -80°C freezer, pending batch processing for experiments.

3.2.5 Creating a Template of Anteromedial Gonarthrosis

In order to generate a topographical map or template of AMG, the precise site and extent of typical lesions in patients with this phenotype of disease needed to be mapped. The varying size of tibial plateaus also needed to be taken into account, and a range of template sizes were created to account for the varying size of AKA samples that would be collected.

Ten tibial plateau resection specimens from patients undergoing UKA for AMG were collected. They all demonstrated typical lesions. Each resection specimen was placed onto a standardised grid and digitally photographed. The resulting digital image was then processed first using Photoshop (version CS2, Adobe Software, California, USA) and then with a custom routine, performing automated image analysis in Matlab (version 7, The MathWorks Inc., MA, USA). With Photoshop, the outlines of the entire medial tibial plateau, the cartilage lesion, and the region of retained full thickness cartilage, present posteriorly, were marked.

The automated image processing routine automatically determined the overall specimen characterisation parameters (Figure 3-4) in pixels.

L_T : total length of specimen

W_T : total width of specimen

Two templates were required. The first of these was to analyse the specimen cross-sectionally for histology, immunohistochemistry and immunoassays. The samples of AMG had previously been sectioned along a line joining the area of the full thickness cartilage loss and the most posterior point of damaged cartilage. Experimental assays were performed at three points of damaged cartilage (T1-T3) and then in the undamaged cartilage posteriorly (N) as shown in the schematic, Figure 1-8. This cross section template was calculated as demonstrated by the lines L_1 , L_2 and L_3 in Figure 3-4. These values were expressed as a percentages of L_4 . The distance between L_2 and L_3 was divided into three sections to represent regions T1-T3.

A second template was required for sectioning (as previously undertaken in AMG) and for sampling in preparation for qPCR experiments. Figure 2-1, in chapter 2 demonstrates the areas of damaged and undamaged cartilage that were sampled. The width (W_T) of the specimen was divided into ten, and a line extended from the posterior border of the specimen to the most posterior point of macroscopically damaged cartilage (Figure 3-5). Using the mean of these lines of the ten specimens, the morphology of macroscopically undamaged cartilage was calculated. In addition, for each lesion, the Matlab routine fitted an ellipse. Using edge detection of the lesion and the fitted ellipse, the radii (major and minor axes, D_{maj} and D_{min}) as shown in Figure 3-4 were calculated, as was the position of the centre of the lesion. The angle of orientation (θ) of the major axis, in relation to the horizontal was also calculated. This region was marked for a further region of sampling (the equivalent of the lesion in AMG). The angle of sectioning was calculated relative to vertical line (φ).

Templates were drawn using CorelDraw (Corel, Ottawa, Canada), and printed onto acetate sheets. They were printed in 1 mm increments of L_4 and L_T so that they could be matched to the size of resection specimens.

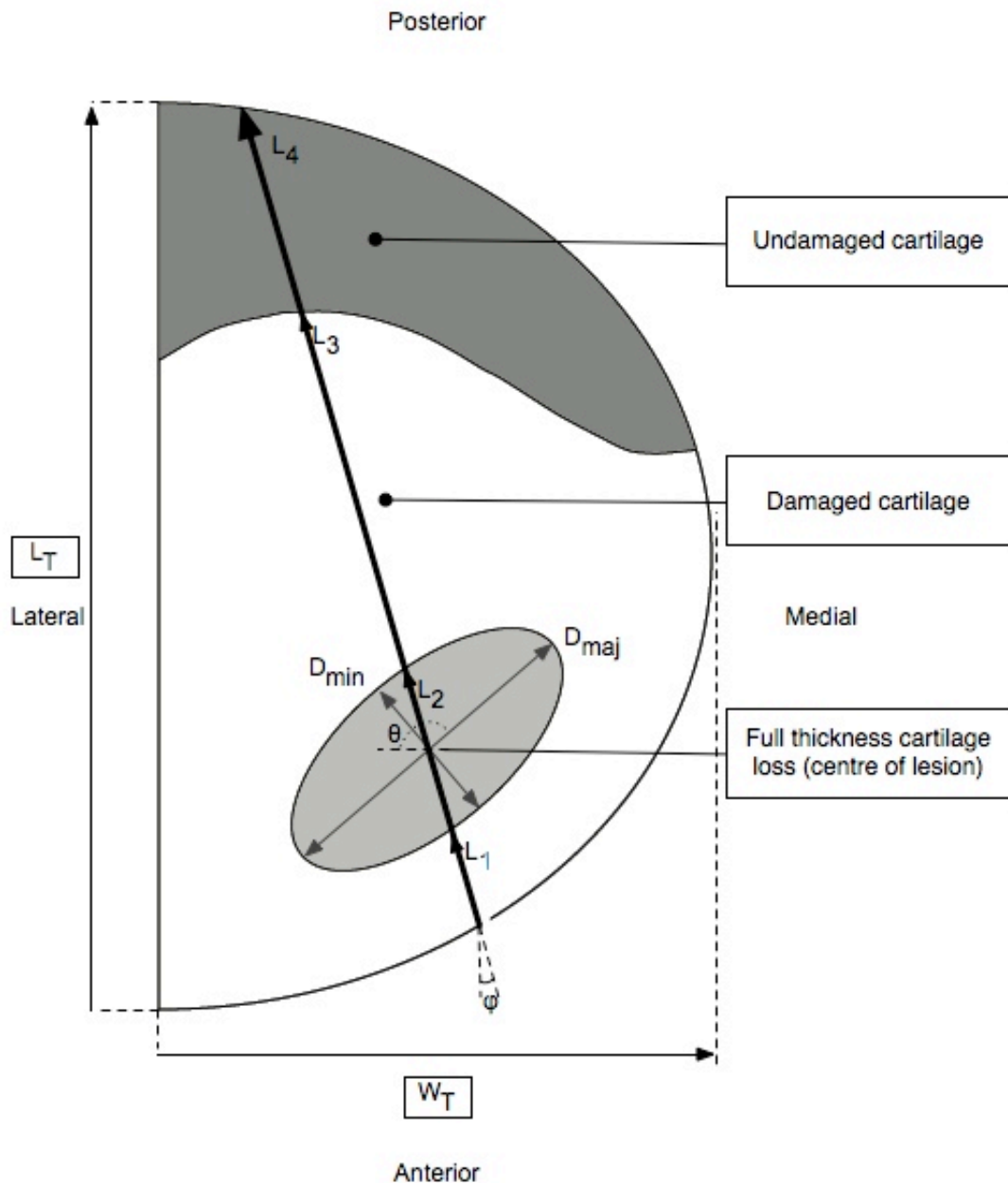


Figure 3-4 – The AMG measurements calculated. L_T and W_T are the total length and width of the specimen respectively. The line drawn through the centre of the lesion and the most posterior aspect of damaged cartilage represents the section taken through the specimen. ϕ represents the angle of this cross section. Measurements were taken from the front of the specimen to the start of the area of full thickness cartilage loss (L_1), to the end of the area of full thickness cartilage loss (L_2) to the most posterior point of damaged cartilage (L_3) and to the posterior of the section (L_4). D_{maj} and D_{min} represent the major and minor axes of the fitted ellipse of the cartilage lesion. θ represents the angle of orientation of D_{maj} relative to the horizontal.

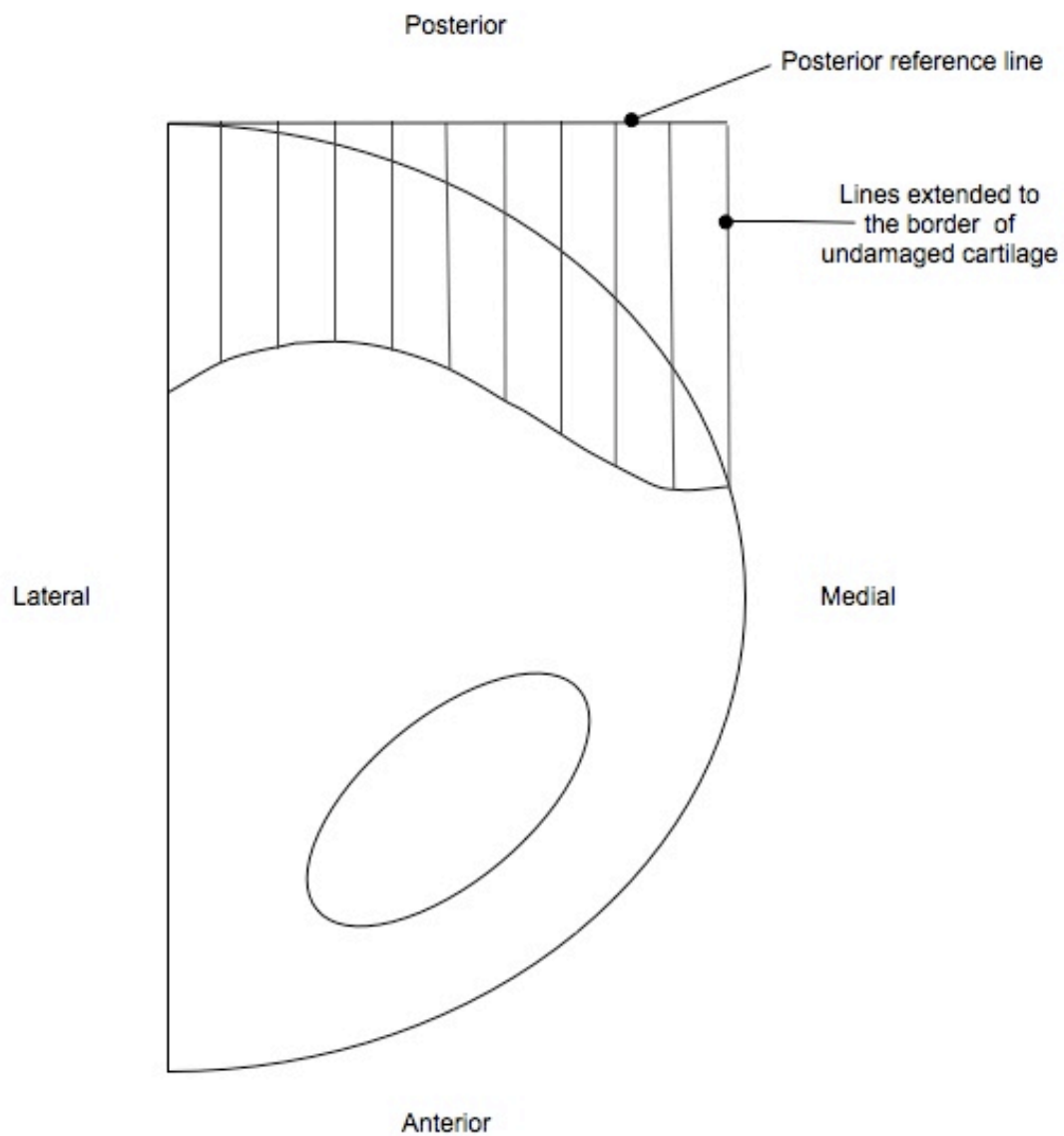


Figure 3-5 - An AMG resection demonstrating measurement of the border of undamaged cartilage. A line was drawn from the posterior reference line to the border of undamaged cartilage. The mean of each of line was calculated.

3.3 Results

3.3.1 AKA samples

Seventeen AKA samples were obtained; one was discarded as it had macroscopic OA disease of the tibiofemoral joint. Of the remaining 16 samples: there were 8 female and 8 male subjects, and 8 knees were right sided, and 8 left sided, with an average age of 77.5 years (range 37-93)(Table 3-1).

Sample number	Hospital	Gender	Date of Birth	Age	Side
1	JR	F	04/01/1934	72	R
2	SMH	M	21/10/1913	93	R
3	JR	M	16/03/1918	88	L
4	JR	M	25/11/1919	87	L
5	SMH	M	28/07/1930	76	R
6	SMH	F	14/12/1926	80	L
7	SMH	F	11/05/1929	78	L
8	SMH	F	12/09/1969	37	R
9	SMH	F	14/02/1922	85	R
10	JR	M	05/11/1933	74	L
11	SMH	M	28/02/1934	73	R
12	JR	M	13/11/1934	73	R
13	JR	F	04/08/1924	83	L
14	SMH	F	15/03/1934	73	L
15	SMH	M	19/09/1926	80	R
16	SMH	F	28/07/1934	74	R
rejected specimen	SMH	M	1/02/1915	92	R

Table 3-1 – Collection and Demographics of AKA specimens. Seventeen samples were collected. One was rejected as it demonstrated macroscopic OA. (JR – John Radcliffe Hospital, SMH – Stoke Mandeville Hospital, M – Male, F – Female, R – Right, L – left).

Of the 16 samples used, all had macroscopically normal cartilage of the tibio-femoral joint, with a Collins score [119] of 0, and they all had intact ACLs and menisci. The samples were later graded histologically as described in Chapter 4. A complete data set including standard deviations is presented in Table 3-2.

3.3.2 Template 1

Template 1 is for sampling the cross sections taken and is shown in Figure 3-6.

The mean measurements were, as percentages of L_4 : L_1 was 9.2%, L_2 was 40.8% and L_3 78.5%.



Figure 3-6 - Template 1. This template was used to sample the cross sections of AKA specimens. The lesion refers to the equivalent region to full thickness cartilage loss in AMG. T1 – T3 are equal thirds of the equivalent of damaged cartilage in AMG, and N is the equivalent of the undamaged cartilage in AMG.

3.3.3 Template 2

Template 2 is shown in Figure 3-7. The mean length and width of the AMG specimens was 54.7 and 29.9 mm respectively and the mean angle of cross sectioning was 17.2 degrees.

D_{maj} and D_{min} were 25.1 and 12.5 mm respectively, and the mean orientation of D_{maj} was 120.2 degrees.

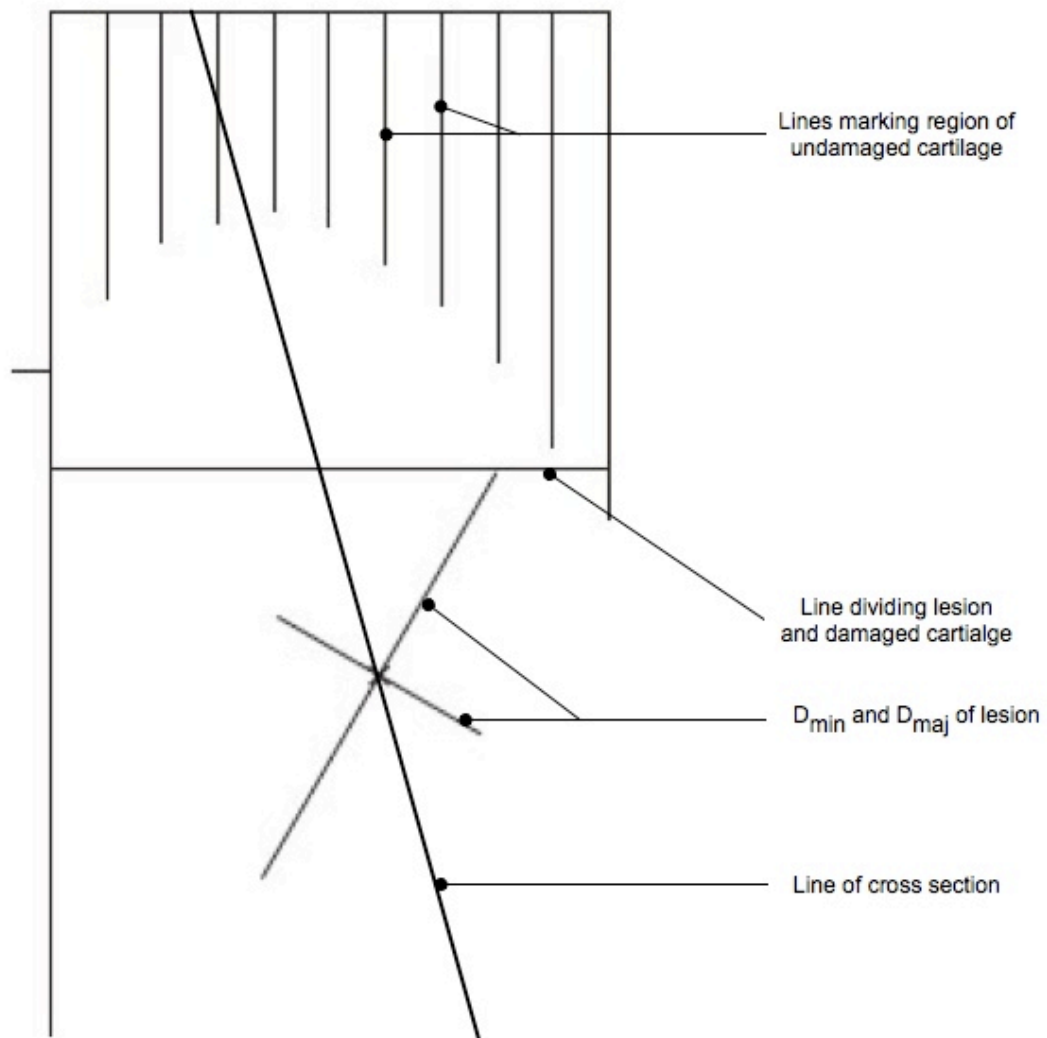


Figure 3-7 - Template 2. This template was used for cutting the cross section, and also laying over specimens to harvest cartilage for mRNA quantification experiments.

Measurement	Mean Length (mm) Angle (degrees)	Standard Deviation
L_T	54.7	3.2
L_W	29.9	3.0
L_1	4.5 (9.2% of L_4)	1.4
L_2	19.9 (40.8% of L_4)	3.9
L_3	38.3 (78.5% of L_4)	3.1
L_4	48.8	3.0
φ	17.2	5.0
D_{maj}	25.1	5.0
D_{min}	12.6	2.9
θ	120.2	12.3

Table 3-2 - Measurements taken for templating of AMG. The abbreviations are all referenced in Figure 3-4.

3.4 Discussion

This chapter describes the method developed to collect healthy control tissue from above knee amputations, and their templating for use in further experiments matched precisely with AMG specimens. The aim of collecting healthy control samples was to obtain tissue that could be used to evaluate the results from AMG specimens. All samples obtained were from patients who were undergoing AKA for PVD. AKA is the end-stage treatment for patients with PVD that have critical ischaemia.

Subjects with PVD have decreased blood flow, leading to hypoxia, which in turn can lead to irreversible tissue necrosis beginning distally. It is easy to argue that these patients may indeed not have ‘normal’ knee cartilage. However, cartilage is an avascular tissue, that therefore survives at low oxygen tensions. The superficial zones at approximately 6% O_2 and the deep zones at less than 1% O_2 [211]. Furthermore, the disease process of OA involves invasion of the tidemark with blood vessels [212] and synovial fluid penetrating into fissures, both of which increase oxygen delivery in

diseased cartilage. Therefore it is increased oxygenation, rather than hypoxia that has been associated with cartilage degeneration.

Certainly, there appears to be no evidence to suggest an increase in incidence of OA in patients with PVD. However this is biased by the fact that OA symptoms are much less likely to come to the fore and be investigated, given the other comorbidities of this group of patients. Patients with symptoms of claudication may not ambulate as much as those without such symptoms, and those with end-stage PVD will definitely have much lower activity levels. However, it seems logical that macrovascular disease ought not to have a detrimental effect on cartilage in terms of OA pathogenesis.

In order to gain access to unequivocally healthy and normal cartilage, during the course of this work I applied for additional ethics to obtain knee specimens from a cohort of brain-stem dead patients that had consented to organ donation (Appendix 1). Unfortunately no such samples became available. This would have provided an invaluable source of tissue, and such samples may become obtainable in the future.

The average age of samples obtained was 77.5 years, and this is older than the average age of someone undergoing knee arthroplasty surgery, which, in the UK National Joint Register 2009, was 64 years (NJR 6th edition, 2009). The average age of patients with AMG specimens for which we have histology and immunoassay data was 68. This is a significant difference ($p=0.02$, Mann Whitney-U), therefore the AKA samples cannot be considered to be an age-matched group. However 77.5 years is clearly an age group with a normally very high incidence of OA and one likely to display the full range of other age related changes to knee cartilage.

Collected tissue was stored at -80°C and processed in batches. There were a number of technical issues with regard to the experimental techniques and protocols (described in Chapter 4) which required a large amount of pilot work with other sources of cartilage, prior to conducting experiments on these valuable AKA specimens. In particular, it was noted that in order to maximise the number of experiments that each of these samples could be used for, precise handling and sampling would be required.

Chapter Four

4 STUDY 2 – THE REGIONAL DIFFERENCES IN AGED, UNDAMAGED CARTILAGE – A CELLULAR AND MOLECULAR STUDY

4.1 Introduction

Anteromedial Gonarthrosis has been previously studied in terms of its histology [93], its molecular matrix content, using immunohistochemistry and immunoassays (Rout et al., unpublished work) and its gene expression profile (Chapter 2). As previously discussed (Chapter 3) the control data in the field of cartilage biology is often limited by the availability of human tissue. In taking macroscopically undamaged cartilage from AKA specimens, I sought to provide data against which AMG data could be compared. Ultimately, the aim was to try and distinguish the findings in AMG as component of OA pathogenesis.

Given the age difference between AMG and AKA samples, 68 and 77.5 years respectively, comparisons of data would not serve as age-matched data. However given that the AKA samples were from subjects approximately 10 years older, a finding of no regional differences would possibly lead one to conclude that changes were not age-related.

In order to study these specimens systematically, a series of experiments were planned, akin to those previously undertaken in AMG (Section 1.5).

1. Histology – This was undertaken using Modified Mankin [129] and OOCAS [133] grades.
2. Immunohistochemistry – for Type I and II collagens
3. Immunoassays – for Type I and II collagens, total collagen, and GAG
4. Real time PCR – for the same ‘matrix-related’ genes, MMPs and ADAMTSs investigated in Chapter 2.

Topographical sampling was possible using the templates described in Chapter 3. This allows for pairwise analysis of the AKA equivalent regions of the undamaged and damaged cartilage of AMG. It also allows direct quantitative comparisons between AKA and AMG samples for the immunoassay data. However, given that qPCR was run at different times, it would only allow for pairwise analysis across either AKA or AMG samples, and not a direct comparison across samples.

In addition to the regions designated equivalent to the damaged and undamaged cartilage of AMG, there is an additional region, equivalent to the area of the full thickness cartilage loss lesion of AMG. This region is termed “X” henceforth. This is an interesting region of cartilage given that if subjects were to develop OA, in the form of AMG, this would be the region of cartilage that would be the first to degenerate.

4.2 Null Hypotheses

1. *The AKA specimens are not histologically normal.*
2. *There are no regional differences in matrix content (type I and II collagens and GAG) in AKA specimens as measured using immunoassays.*
3. *There are no regional differences in gene expression in AKA specimens as measured by real-time PCR.*

4.3 Materials and Methods

As described in the previous chapter (section 3.3.1), sixteen AKA samples were collected. The samples were collected in batches from the -80°C freezer and processed as follows:

An RNase free area was created by preparing surfaces and instruments with RNasezap (Applied Biosystems, California, USA).

Firstly, template 2, printed on acetate, was placed over the sample, and the axis of sampling marked. Using a band saw, two sections were taken along this axis. Each section was approximately 2 mm thick. The first section, for use in histology and immunohistochemical studies and was placed in 10% formalin. The second section, to be used for immunoassays, was placed on top of template 1. The lines were then used as guides, and cuts made with a scalpel at the appropriate junctions between zones. The cartilage was lifted off the bone and each piece placed into an Eppendorf tube. The remaining tibial plateau was aligned beneath template 2, and scored along lines separating zone X from the area equivalent to damaged and undamaged cartilage of AMG. Once scored, the cartilage was again lifted off using a scalpel. It was placed in RNeasy (Applied Biosystems), in Eppendorf tubes and transported back to storage in a -80°C freezer.

4.3.1 Histology

Following fixation in 10% formalin, the sections were then decalcified in nitric acid for 24h before wax embedding. Previous lab data had shown radiologically that 24h was a suitable duration for complete decalcification of a section of this width. Five micron sections were cut using a Leica RM2134 microtome (Leica Microsystems, Germany) and heat adhered onto Snowcoat X-tra™ pre-cleaned micro slides (Surgipath, Illinois, USA). They were stained using Haematoxylin and Eosin (H&E) and Safranin-O stains using standard protocols.

Once stained, both the H&E and Safranin-O slides were digitally photographed. Using an Olympus BX40 microscope (x40 magnification) and Olympus DP70 camera, images were taken and *photomerged*™ together using Adobe Photoshop CS2 (Adobe, California, USA).

By applying the values obtained from template 1, to the image it was possible to assess the equivalent regions to the cartilage of AMG. Therefore results were obtained for regions equivalent to T1, T2, T3 and N, as well as the additional region, X.

The histological characteristics of each region were assessed using the Modified Mankin score [129] and the OOCAS [133].

Rather than simply classifying the samples as controls, they were grouped on the basis of their histological score as encountered in previous studies in the literature [126, 175, 213]. Those of lower grade (< 3) were named LAKA and higher grade specimens (>3), HAKA

4.3.2 Immunohistochemistry

Further sections were cut from the wax embedded blocks, and immunohistochemistry undertaken for Type I and Type II Collagens using the protocol in Appendix 3.

4.3.3 Immunoassays

Immunoassays were undertaken for Types I and II collagen, total collagen and GAG, for regions X, T1, T2, T2 and N. These were undertaken in collaboration with Professor Anthony Hollander (University of Bristol), using his patented and validated assays [141, 142].

4.3.3.1 Sample preparation

For the assays undertaken, it was first necessary to digest and solubilise the cartilage matrix. The samples were thawed, and the wet weight was recorded. A pestle and mortar were used to grind the cartilage, and, after freeze-drying, the dry weight was recorded. Samples were digested using Trypsin (Sigma).

In brief, samples were digested with 2 mg/ml TPCK-treated bovine pancreatic trypsin containing 1mM iodoacetamide, 1mM EDTA, and 10g/ml pepstatin A (all from Sigma). An initial incubation for 15 hr at 37°C with 250 µl trypsin was followed by a further 2 hr incubation at 65°C after the addition of a further 250 µl of the freshly prepared proteinase. All samples were boiled at 100°C for 15 min at the end of incubation to denature remaining enzymes.

The published protocol [214] for this and the following summarised sections and can be found in Appendix 2.

4.3.3.2 *Type I Collagen*

The Type I collagen assay used was first described by Dickinson et al in 2005 [141] as a method for extracting and immunoassaying type I collagen from tissue engineering cartilage.

Inhibition ELISA with a rabbit anti-peptide antibody to Type I collagen was used as described in previously published work [141, 142]. Peptide SFLPQPPQ was synthesized using 9-fluorenyl-methoxycarbonyl chemistry (Fmoc) (from Dr. A. Moir, Krebs Institute, Sheffield University, UK) and was used as a standard in all of the immunoassays.

4.3.3.3 *Type II Collagen*

The α -chains of type II Collagen can be assayed using antibodies against specific amino acid sequences. This inhibition ELISA uses a mouse IgG monoclonal antibody to Col2-3/4m, raised against a synthetic peptide CB11B. Peptide CB11B (CGKVGPSGAP[OH]GEDGRP-[OH]GPP[OH]GPQY) was synthesized using standard Fmoc chemistry (from Dr. A. Moir).

4.3.3.4 *GAG*

Sulphated GAG levels, in solution, can be quantified using 1,9-Dimethylmethylen blue (DMB) [215-217]. The DMB itself and the GAG-DMB complex are known to have a degree of instability; precipitates can form, creating measurement error. Therefore rapid processing and immediate reading of samples was undertaken (within one minute).

4.3.4 Real time PCR

The methods of RNA extraction, reverse transcription and real time PCR have been described previously in section 2.3. qPCR was attempted for three regions; X, undamaged, and damaged cartilage. Unfortunately, region X yielded too little cartilage to obtain sufficient RNA for qPCR. The amount of RNA obtained from the undamaged and damaged regions also limited the number of genes that could be studied. The genes selected are highlighted in bold in Table 4-1; All of them were chosen, with the exception of COL2A1, because they had shown a significant difference in AMG (see section 2.4.2).

Matrix related genes	Matrix Metalloproteinase	ADAMTSs
COL1A1	MMP 1	ADAMTS 4
COL2A1	MMP 3	ADAMTS 5
COL3A1	MMP 13	ADAMTS 16
ACP	MMP 28	

Table 4-1 – List of genes that underwent qPCR (from Chapter 2). Those in bold were undertaken on AKA samples.

4.3.5 Statistical analysis

Graphical data are displayed as box and whisker plots with median values and error bars representing range. Statistical analyses were performed using SPSS (version 15, SPSS Inc., Chicago, USA). The histology grading was analysed using a Friedman test between regions. *Post hoc* testing was also undertaken in a pairwise manner using a Wilcoxon test. Data was also compared to AMG histology data [93] using a Mann Whitney-U test between equivalent regions. Inter- and intra- observer error was determined by calculating the Intra-Class-Coefficient (ICC) [218].

Similarly, the immunoassays were compared in the same fashion. The real time PCR results were compared using a pairwise analysis with a Wilcoxon test. The standard curve relative quantification method was used. It does not allow quantitative or relative comparison between AKA and AMG samples.

4.4 Results

4.4.1 Histology

The median modified Mankin score of all regions graded, across all samples, was 2 (range 0-6); the median OOCAS was 1.5 (range 1-3). There was a significant difference in histological grade for both Mankin and OOCAS when compared to AMG data ($p < 0.001$). Images of H&E and Safranin-O staining are shown in Figure 4-1.

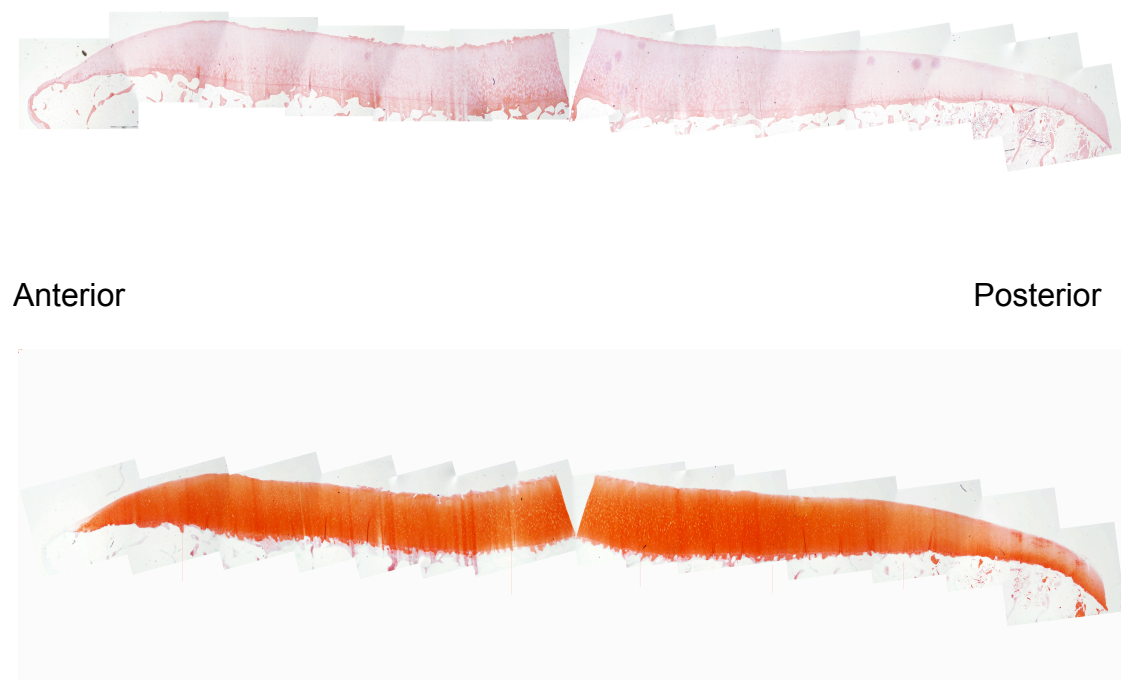


Figure 4-1 – Above knee amputation section stained with Haematoxylin and Eosin (top) and Safranin-O (bottom). Multiples images were taken under the microscope and they were merged together using Adobe Photoshop.

There was a significant difference between regions for both Mankin ($p=0.003$) and OOC HAS ($p=0.015$) scores, and post hoc testing demonstrated a significant difference, in Mankin grade between region X vs T3 ($p=0.018$) (Figure 4-2).

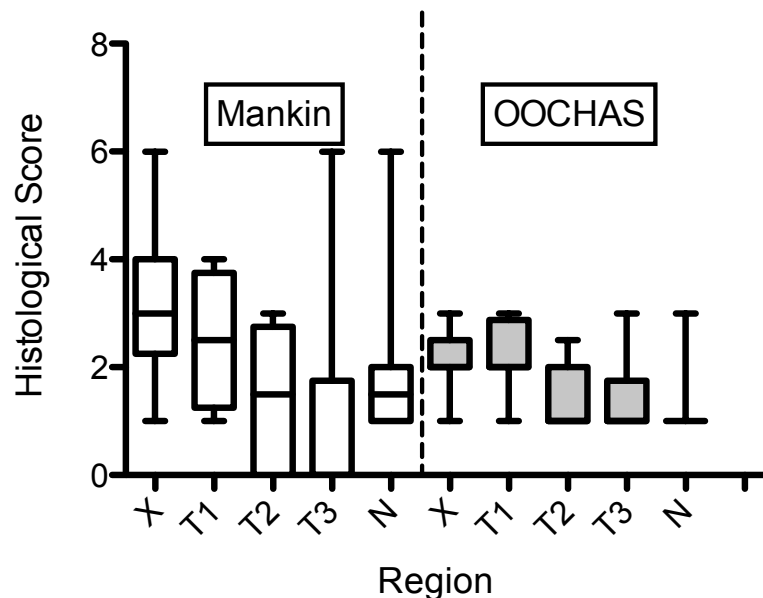


Figure 4-2 – Graph demonstrating histological grade (Mankin/OOC HAS) by region. The white boxes are for Mankin grade and a significant difference was demonstrated ($p=0.003$;Friedman). The grey boxes are for OOC HAS scoring, and a significant difference was demonstrated ($p=0.015$). Post hoc testing identified a significant difference between region X and T3 ($p=0.018$).

Using criteria that have previously been documented in the literature [126, 175, 213] for Mankin grading, there were eight samples that were classed as ‘lower grade’, and eight that were ‘higher grade’. These are referred to as LAKA and HAKA respectively. LAKA included samples with a grade of not more than 3 in any region; HAKA samples had a region that scored more than 3 (Figure 4-3). There is no such suggested value for the OOC HAS though the data has been included for reference (Figure 4-3).

The median modified Mankin grade of the samples in the LAKA group was 1 (range 0-3) and the HAKA group was 3 (range 2-6). The LAKA group did show a significant difference between regions ($p<0.001$), though *post hoc* testing did not demonstrate

this between individual regions. There was no significant difference between regions in the HAKA group.

There was a significant difference between these LAKA and HAKA groups ($p < 0.001$)

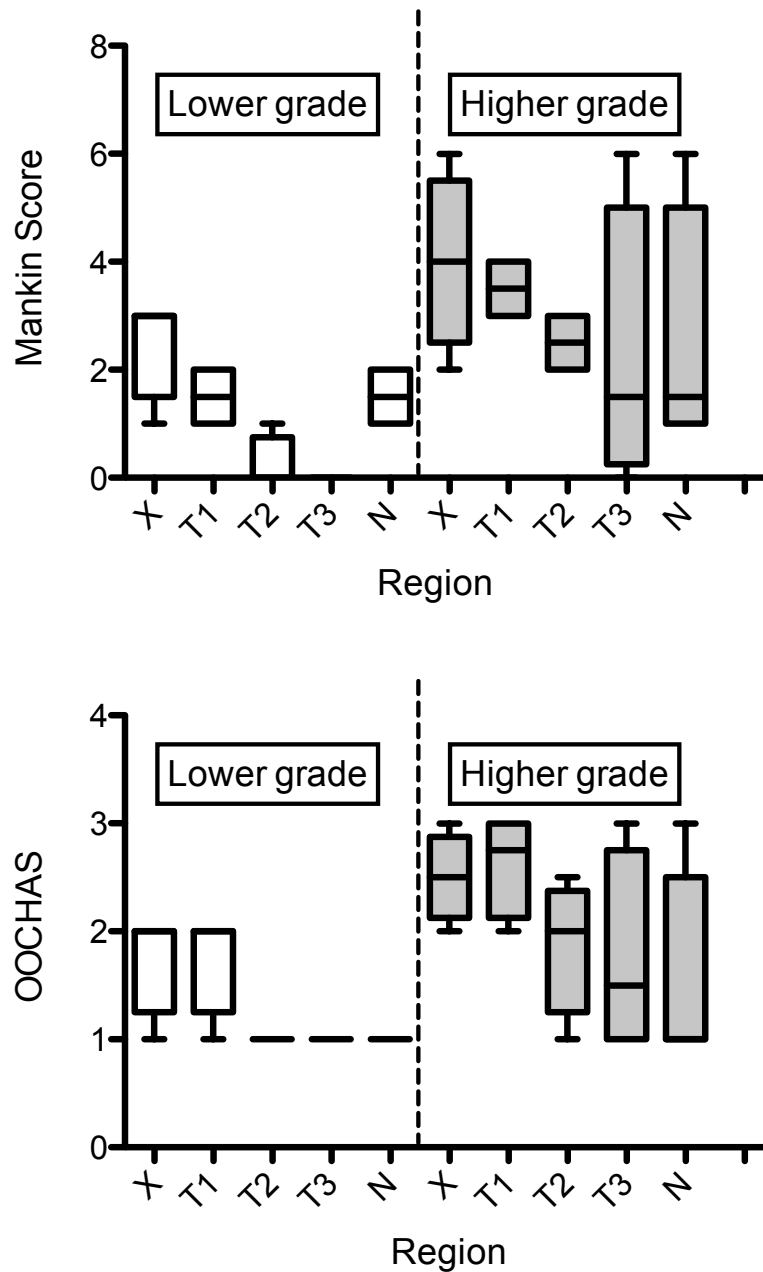


Figure 4-3 – Histology grading (Mankin (top), OOCAS (bottom)) for lower grade (LAKA; white boxes) and higher grade (HAKA; grey boxes) samples. For lower grade groups there was a statistically significant difference ($p < 0.001$) between regions. There was no significant difference for the higher grade groups. Comparing Lower grade to Higher grade demonstrated a significant difference ($p < 0.001$).

4.4.2 Immunoassays

The values presented are percentage of dry weight. For the regions T1-N there were 16 values, of which 8 were LAKA and 8 were HAKA. There were 2 samples for which there were no values for region X due to experimental errors, therefore n=14 for the analysis when X is included.

The data is firstly presented for regions T1-N. This is comparable data to that available for AMG (Section 1.5.3). The analysis of region X-N is then also presented.

4.4.2.1 Glycosaminoglycans

The median sGAG across all regions and all specimens was 16.3% (range 8.8 to 43.3%). When analysed by region, the median values ranged from 14.2 to 17.7%. There was no statistically significant difference when T1-N values (n=16) were analysed.

Comparing regions X-N (n=14), Friedman's test showed statistical significance (p=0.040) and a Wilcoxon tests revealed differences between X vs T3 (p=0.016) and X vs N (p=0.041) (Figure 4-4).

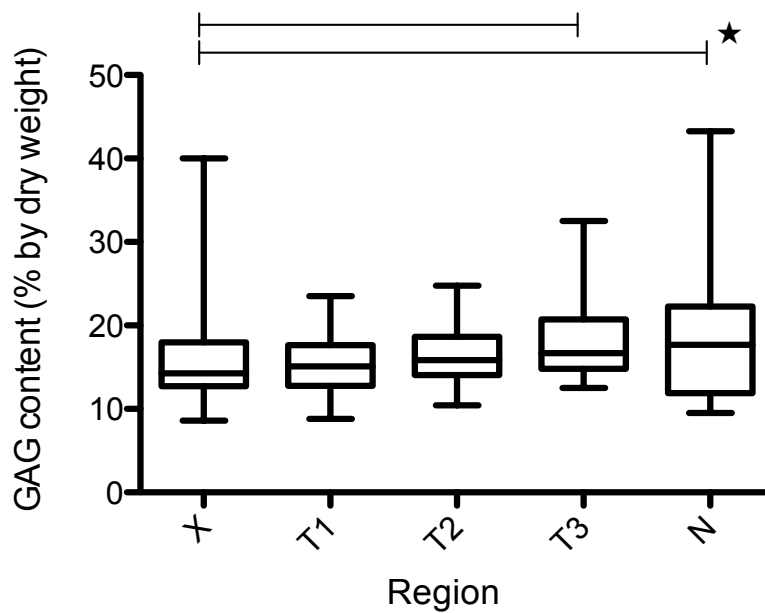


Figure 4-4 - GAG content in AKA specimens by region. n=16 for all regions and n=14 when X included. There was no statistical significance between regions T1-N. There was a statistical difference when including region X, $p=0.040$ (Friedman). Post hoc testing demonstrated statistical differences between X vs T3 ($p=0.016$) and X vs N ($p=0.041$). * Indicates significant differences.

The data was subdivided into LAKA and HAKA groups. There was no statistical difference between regions T1-N for either group ($n=16$). When region X was included there was also no significant difference between regions ($n=14$). Each region of LAKA specimens was also compared to the same region of HAKA specimen, and there was no significant difference (Figure 4-5).

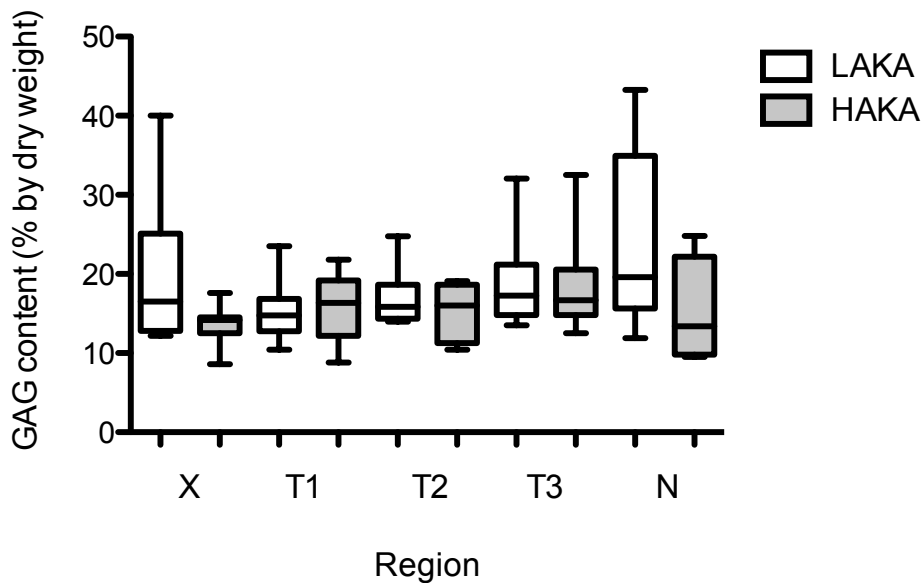


Figure 4-5 - GAG content (% by dry weight) of LAKA / HAKA samples. There was no significant difference across the regions, nor between LAKA and HAKA within each region.

4.4.2.2 Type I Collagen

The Type I collagen content median values ranged from 0.81 to 3.74% (overall range 0.06% to 7%) collagen by dry weight; values are further broken down in Table 4-2.

Overall, the AKA specimens showed a statistically significant difference between regions; $p < 0.001$ (comparing regions T1-N) and $p < 0.001$ (comparing region X-N).

Post hoc analyses showed statistically significant differences between regions X vs N, T1 vs N, and T2 vs N (Figure 4-6).

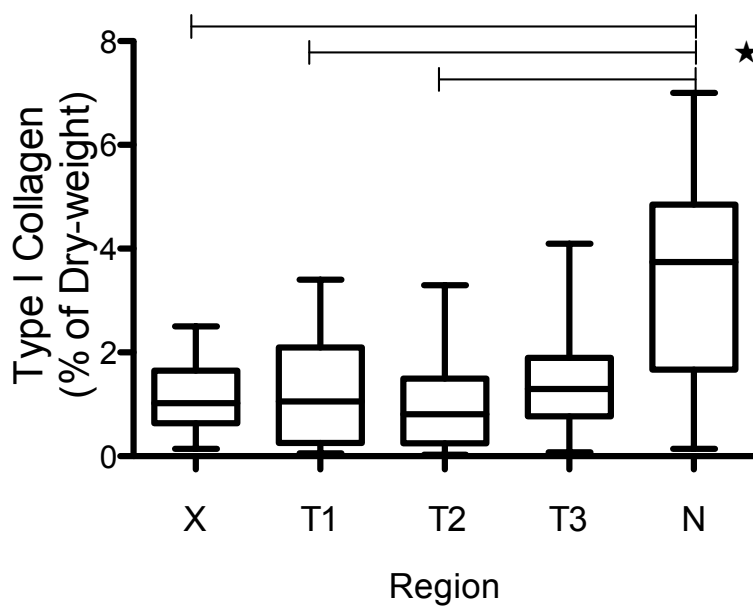


Figure 4-6 – Type I Collagen (% by dry weight) by region, including all AKA samples. There was a statistically significant difference between regions if X is excluded ($p < 0.001$) and if included ($p < 0.001$). Post hoc testing showed a significant difference between regions X vs N, T1 vs N and T2 vs N. * Indicates significant differences.

When analyzed, taking into account histological grade, the LAKA showed no significant difference between groups ($p = 0.14$ (T1-N)). However, the HAKA group did show a statistical difference ($p = 0.02$ (T1-N)). When region X was included, the significance was unchanged (LAKA, $p = 0.23$; HAKA, $p = 0.02$). *Post hoc* testing revealed statistical differences between X vs N and T2 vs N (Figure 4-7).

Sample/Region	X	T1	T2	T3	N	P value
All AKA	1.023	1.06	0.81	1.3	3.74	$p < 0.001$
LAKA	1.15	0.93	0.73	1.16	2.23	$P = 0.23$
HAKA	0.90	1.10	1.30	1.35	4.85	$p = 0.02$

Table 4-2 – Type I Collagen (% dry weight) shown by region, divided into the groups examined. LAKA – low grade AKA samples; HAKA – high grade AKA samples. Red indicates significant differences.

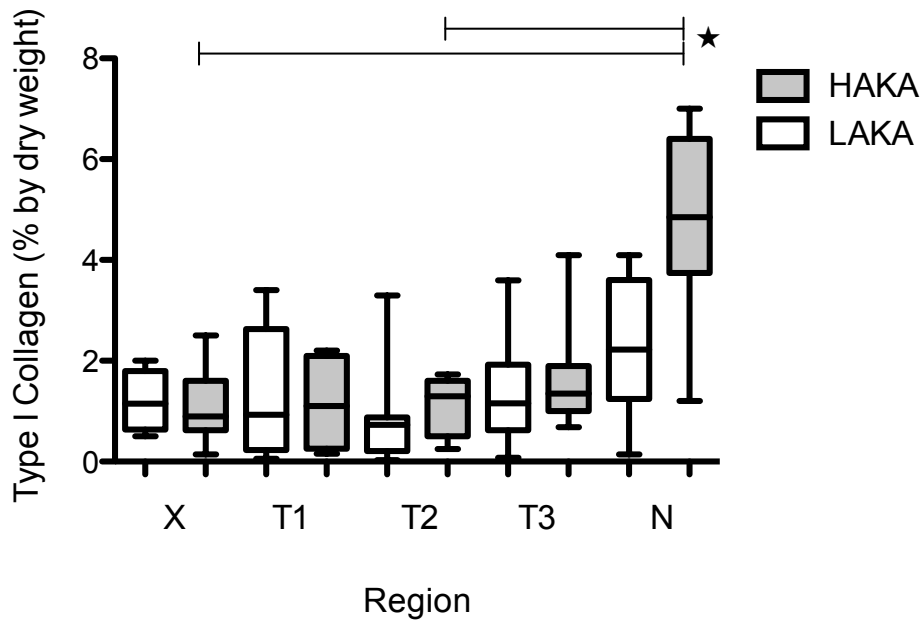


Figure 4-7 Type I Collagen (% by dry weight) in each region of HAKA/LAKA samples. For LAKA samples there was no significant difference between regions. For HAKA, there was a significant difference ($p=0.02$ when both excluding and including x) between regions (post hoc analysis showed differences between T2 vs N, and X vs N). * Indicates significant differences.

4.4.2.3 Type II Collagen

Overall, the AKA specimens showed no statistically significant differences between regions when comparing regions T1-N and regions X-N (Figure 4-8). Median values ranged from 48% to 62% by dry weight.

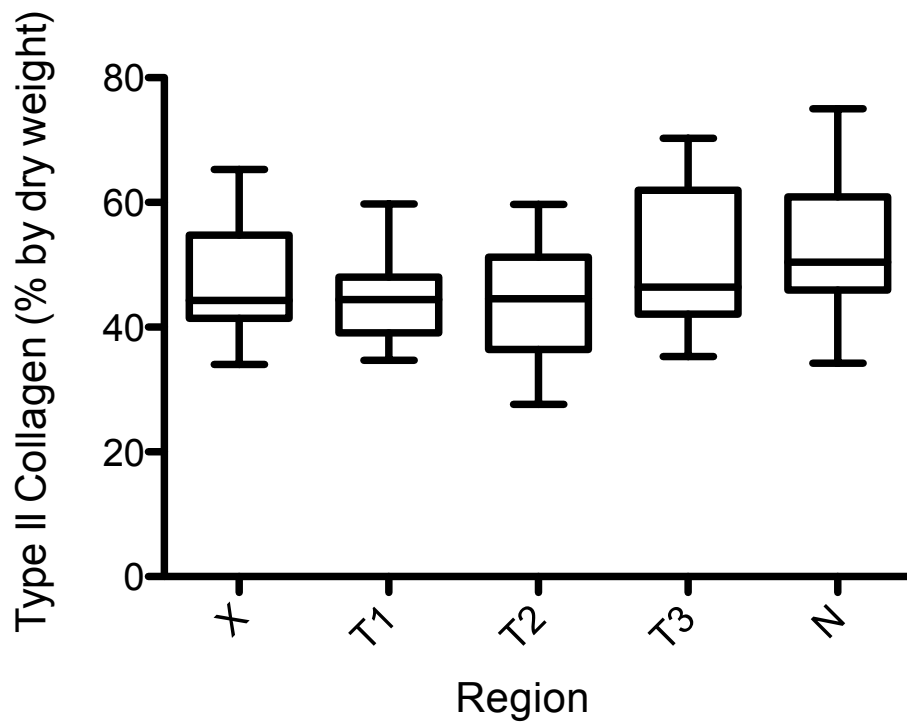


Figure 4-8 - Type II Collagen (% by dry weight) in each region, including all AKA samples. There was no statistically significant difference between regions.

Similarly, when this was analysed by histological group, there was no significant difference between regions for both LAKA ($p=0.319$) or HAKA ($p=0.062$) when X was excluded, or included ($p=0.216$ and 0.194) (Figure 4-9).

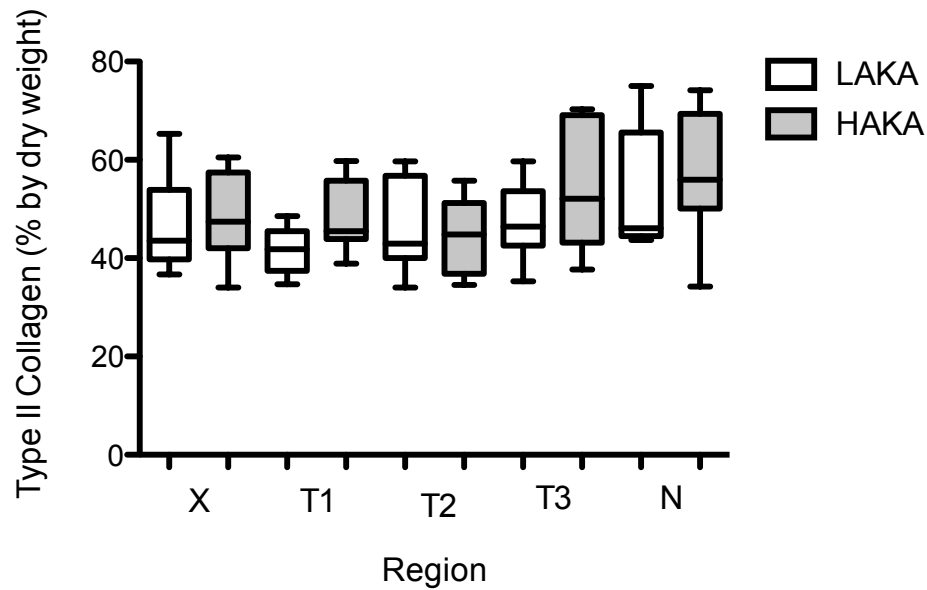
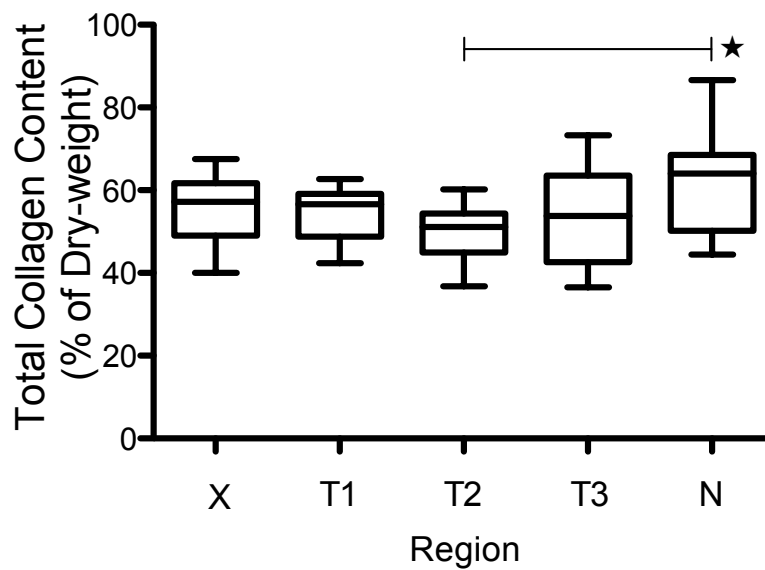


Figure 4-9 - Type II Collagen (% by dry weight) in each region of HAKA/LAKA samples. For both HAKA and LAKA samples, there was no significant difference between regions.

4.4.2.4 Total Collagen

The median total collagen levels ranged from 51-64%. There was no statistical difference between regions for total collagen levels for regions T1-N ($p=0.125$). However when X was included there was a difference ($p=0.011$). *Post hoc* testing showed the difference between regions T2 vs N (Figure 4-10)

When analysed by histological group, there was a statistical difference between regions for both LAKA ($p=0.05$), and HAKA ($p=0.019$) for regions T1-N (*post hoc* testing showed this to be between regions T2 and N). This became not significant ($p=0.178$ and 0.11) when X was included (Figure 4-11).



**Figure 4-10 - Total Collagen (% by dry weight) in all AKA samples. There was no significant difference when region X was excluded ($p=0.125$), and there was a difference with X included in the analysis ($p=0.011$). Post hoc testing showed the difference to be between regions T2 vs N.
* Indicates significant differences.**

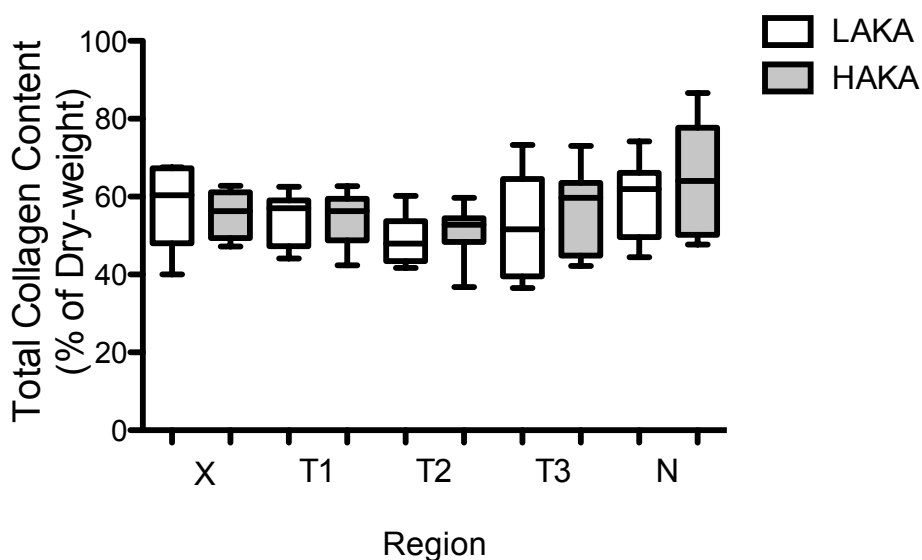


Figure 4-11 - Total Collagen (% by dry weight) in each region of HAKA/LAKA samples. For both LAKA and HAKA samples, there was a significant difference when X was excluded ($p=0.05$ and 0.019 respectively), but not when X was included.

4.4.3 Real Time PCR

The quality and yield was such that fewer samples were suitable for analysis and thus it was possible to conduct fewer experiments than planned. Furthermore, only paired data was included in the analysis. As a result of these reduced numbers, the data for HAKA and LAKA were analysed firstly as combined, and then, for those where there were larger numbers, they were analysed separately.

Table 2-4 shows a summary of the genes examined, together with fold change and significance. They are presented as expression in regions T1-3 combined compared to region N. There were no genes that showed differential expression between regions. Results are shown in Figure 4-18.

Gene	n	Fold change Damaged compared to Undamaged	p value
COL1A1	10	0.30	0.646
<i>LAKA</i>	4	2.25	0.465
<i>HAKA</i>	6	0.20	0.173
COL2A1	9	1.18	0.241
<i>LAKA</i>	4	1.31	0.465
<i>HAKA</i>	5	1.06	0.686
MMP 1	6	1.78	0.249
MMP 3	10	0.56	0.241
<i>LAKA</i>	4	0.35	0.273
<i>HAKA</i>	6	0.73	0.600
MMP 13	2	1.57	0.655
ADAMTS 4	5	1.35	0.686

Table 4-3 – AKA changes in gene expression between regions equivalent to the undamaged and damaged cartilage of AMG (values greater than 1 indicate higher expression in regions N compared to T1-3)

4.4.4 Comparison of Data to AMG

4.4.4.1 Immunoassays

In order to fully interpret these results, it is necessary to consider the data alongside the data obtained from AMG. The graphs below first demonstrate regional findings

for GAG, Type I, II and total collagen. These data are taken from results of Rout et al (unpublished data).

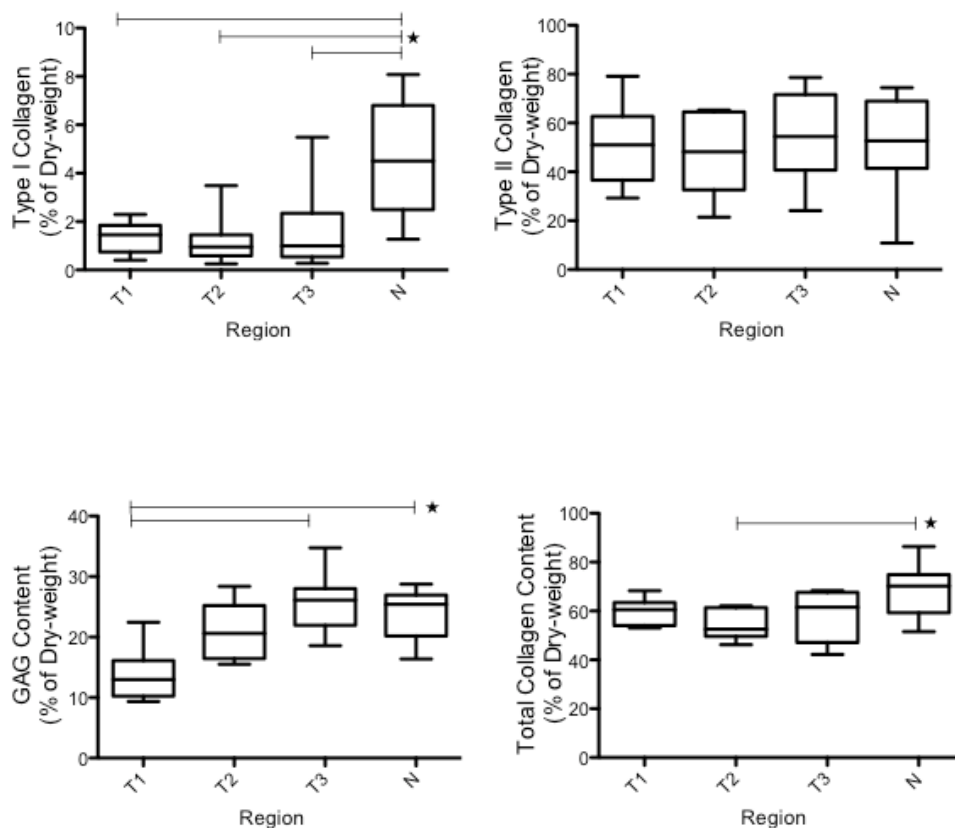


Figure 4-12 – Regional variation in AMG of Type I Collagen ($p < 0.001$) Type II Collagen ($p = 0.840$), GAG ($p < 0.001$) and Total Collagen ($p = 0.003$). Post hoc testing and significant differences are starred.

In brief, GAG follows the expected pattern of decreased amounts in more damaged cartilage and of particular interest were the changes in Type I Collagen content in the macroscopically and histologically normal cartilage of AMG (see section 1.5.4).

This data is shown plotted together, for comparison, with the result of AKA specimens in Figure 4-13 – 16.

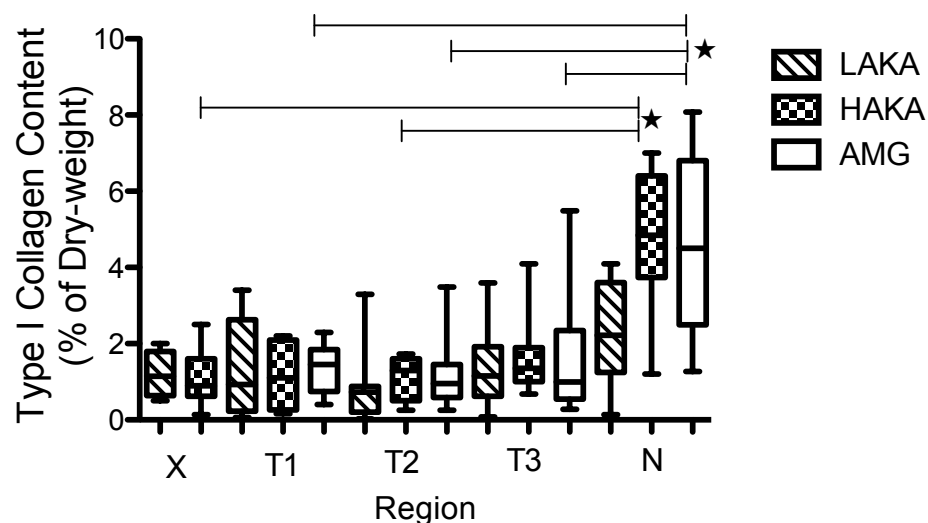


Figure 4-13 – Type I Collagen regional differences in LAKA, HAKA and AMG samples.
 * Indicates significant differences; in LAKA there were no significant differences. In HAKA, post hoc tests showed significant differences between X vs N and T2 vs N. In AMG, there were statistical differences between T1 vs N, T2 vs N and T3 vs N.

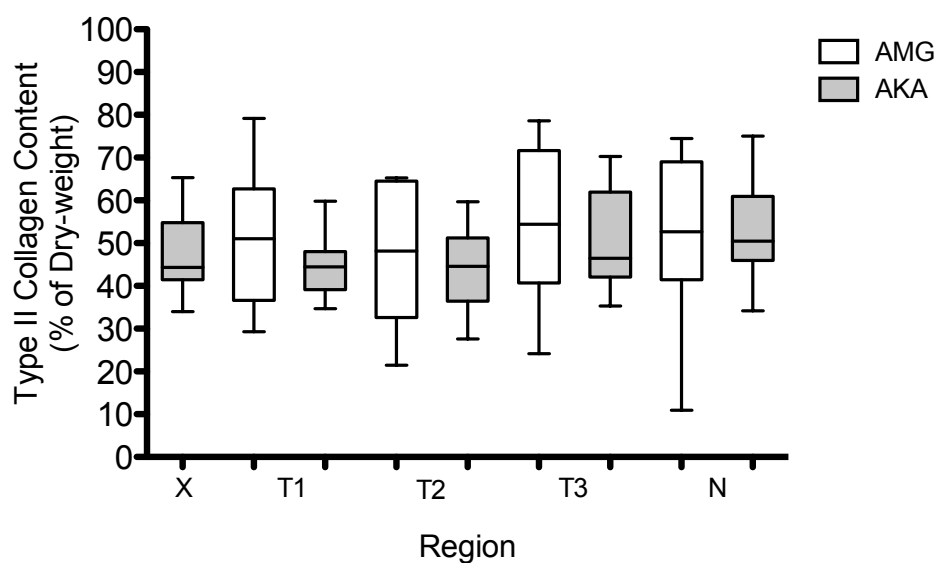


Figure 4-14 - Type II Collagen regional differences in AKA and AMG samples. There were no significant differences.

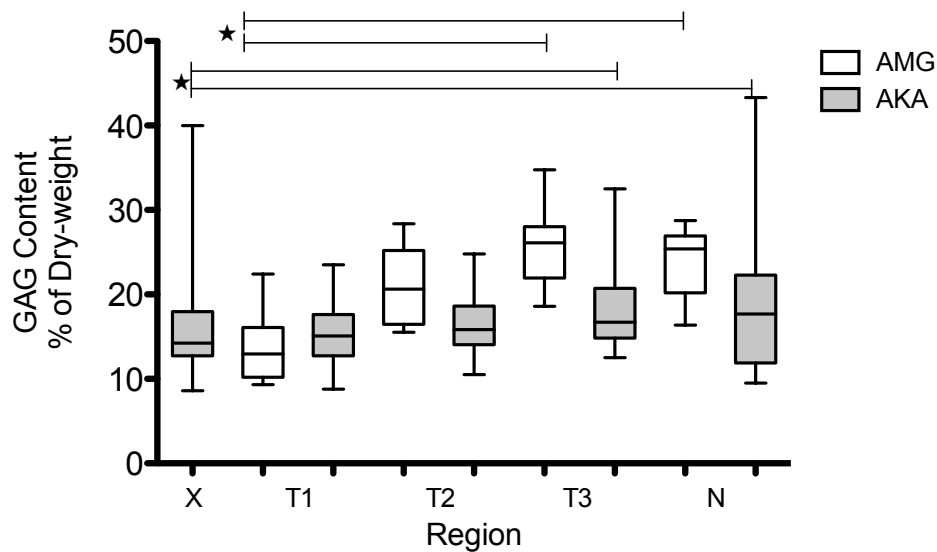


Figure 4-15 - GAG regional differences in AKA and AMG samples. * Indicates significant differences; In AKA *post hoc* testing showed significant differences were between X vs T3 and X vs N. In AMG, significant difference were shown between T1 vs T3 and T1 vs N.

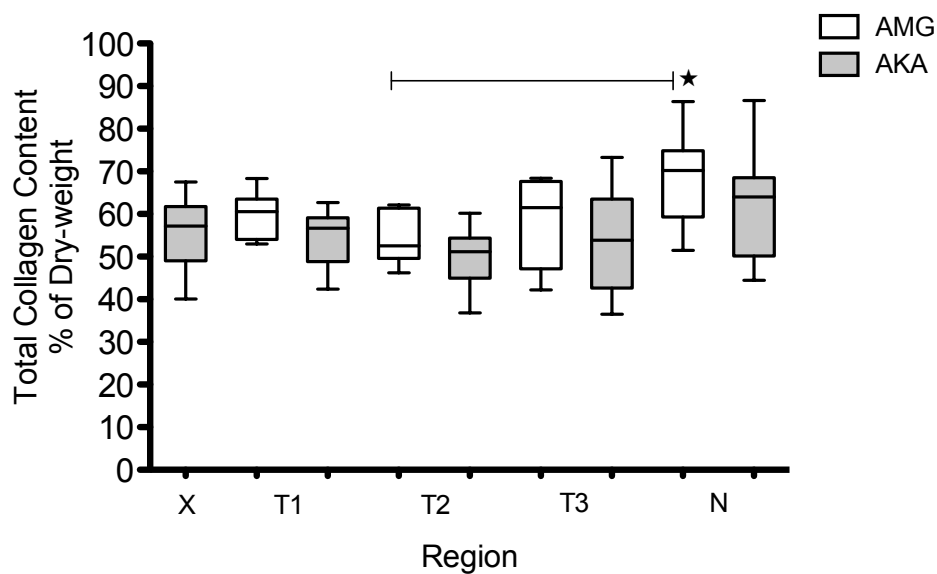


Figure 4-16 - Total Collagen regional differences in AKA and AMG samples. * Indicates significant differences; In AMG *post hoc* testing demonstrated a significant difference between T2 vs N.

4.4.4.2 Real time PCR

The results for regional differences in gene expression in AMG are presented in detail in Chapter 2. Above (4.4.3), no regional differences have been shown in any of the genes examined in AKA specimens.

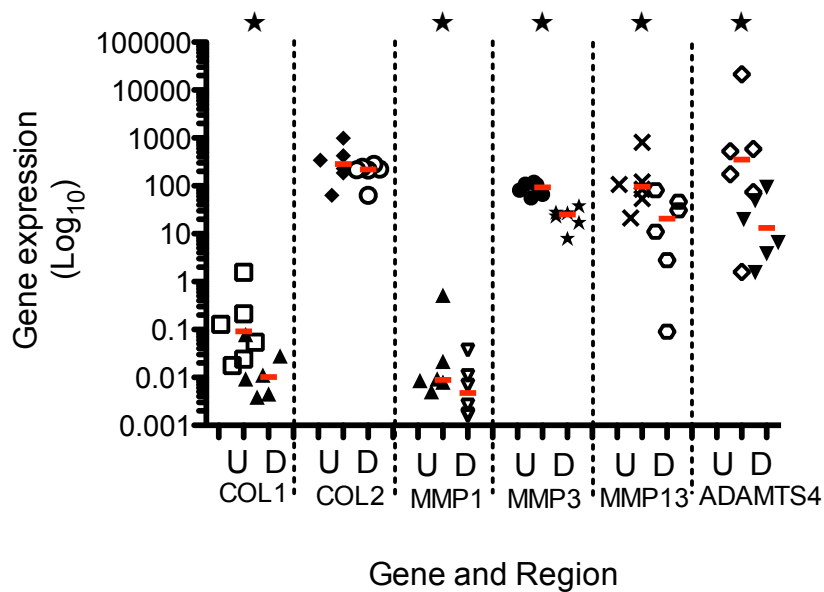


Figure 4-17 – Regional differences in Gene expression between the undamaged and damaged cartilage of AMG. * denotes significant differences (COL1A1, p=0.028; COL2A1, ns; MMP1, p=0.046; MMP3, p=0.028; MMP13, p=0.028 and ADAMTS4, p=0.046). The red line represents median values.

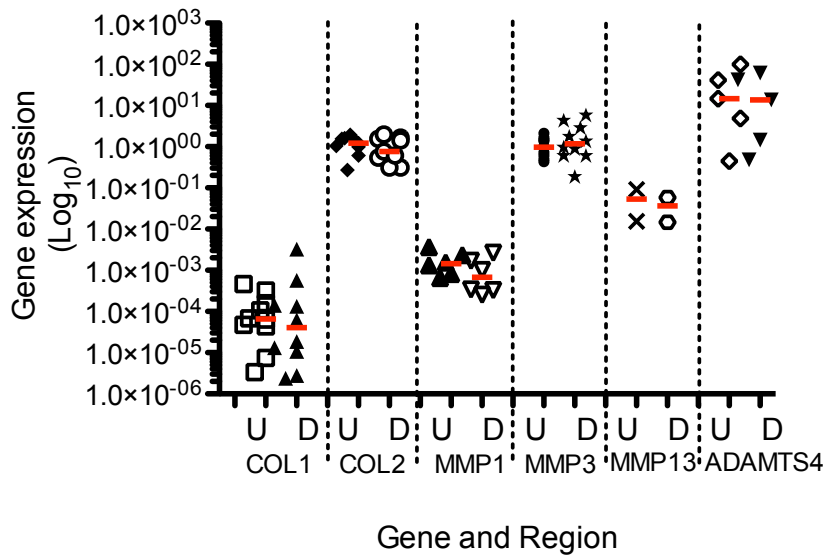


Figure 4-18 - Regional differences in Gene expression in AKA samples between the equivalent regions of undamaged and damaged cartilage of AMG. No significant differences were demonstrated. The red line represents median values.

4.4.5 Summary of main findings

There is a statistical difference in the amount of Type I Collagen in AMG and in high grade AKA samples. It is found in greater levels in the posterior area of cartilage (region N) in both cases. This regional difference is not found in low grade AKA samples.

GAG content exhibits regional variation in the disease state of AMG, with lower GAG in more damaged cartilage. In addition, it is significantly lower in region X of AKA specimens.

Total collagen is higher in the undamaged cartilage of AMG, with no variation between regions in AKA specimens. Type II collagen shows no significant variation across regions in either AMG or AKA specimens.

In AMG, regional differences in gene expression, COL1A1, MMP1, 3, 13, and ADAMTS4 have been demonstrated, however there are no regional differences in gene expression in AKA specimens for these genes.

4.5 Discussion

The aims of this study were to study regional variation in aged cartilage and also to provide data to aid comparison of AMG data in a topographical manner. The null hypotheses:

The AKA specimens are not histologically normal – is rejected.

There is no difference in matrix content (type I and II collagens and GAG) between AMG and AKA specimens as measured using immunoassays:

Type I Collagen - is rejected.

Type II Collagen – is accepted.

GAG – is rejected.

There are no regional differences in matrix content (type I and II collagens and GAG) in AKA specimens as measured using immunoassays – is rejected.

4.5.1 Histology interpretation

The study was initially conceived to validate conclusions reached in the study of AMG, namely that the matrix content and gene expression differences found are part of the OA disease process. In order to do this control tissue was sought. There are obvious difficulties in obtaining human tissue, and, as previously discussed, the most accessible source was AKA from patients undergoing this operation for peripheral vascular disease.

During the collection of this tissue, it was noted that one sample demonstrated frank macroscopic features of OA, however the 16 samples that were processed did not. However, when studied histologically, there was a range of histological scores.

Histology remains the gold standard for the confirmation of diagnosis of OA. Microscopic features, including surface fibrillation, loss of GAG, and cell clustering, are considered pathognomonic of OA. Numerous grading systems have been developed to study histology. The most popularly used remains the Mankin score [129] and its subsequent modifications, including the one used in this study [130]. More recently, the OOCAS method [133] has been introduced and is becoming increasingly more used, having been shown to be reliable and reproducible [134].

The range of histological scores presented the question of how one could classify these samples, as they did not represent true ‘control’ tissue as was initially planned. Previous studies in the literature were identified that encountered this issue [126, 175,

213] and value of 3 on a Mankin scale has previously been used to distinguish 'normal' or age-related change from OA cartilage. No such criteria have been suggested using the OOCAS, thus the Mankin grade was used to classify the samples. It is interesting to note that in most fields of medicine there are ranges assigned to normal values, be it clinical measurements, for example, temperature, or biochemical levels as in blood results. However, in certain fields such as tumours, there is a binary approach of being either positive or negative. There is no such agreed approach to histological scoring in OA.

OA is considered in the same way as the latter, and therefore any histological changes are often considered to be changes due to the disease process. One of the problems that arises with OA is that even though it is not an obligatory consequence of ageing [219], there are changes in cartilage that occur with age, and it is feasible that though they may appear, microscopically, to look like early OA, they may never progress. Cadaveric studies would suggest that in an aged population of similar age to these AKA samples, that the prevalence of OA is approximately 30-60% [220, 221].

In this study, the convention of previous work in the literature was used and therefore a distinction was made, rather than classifying the samples as controls, they were termed aged cartilage and divided into those of lower grade (< 3) or higher grade (>3), named LAKA, and HAKA respectively.

For the immunohistochemistry, it was difficult to obtain clean and interpretable staining using the immunohistochemistry protocol, despite attempts to optimise the blocking steps and antibody concentrations. Therefore this experiment was abandoned,

and the immunoassays alone used to qualitatively assess Type I and II Collagen content.

4.5.2 Immunoassay interpretation

The central finding of this study relates to Type I collagen. This was shown to be elevated in AMG in histologically and macroscopically normal tissue. Chapter 2 (section 2.5.1) discusses the presence of Type I collagen in more detail, but in summary, its presence in elevated amounts was an unexpected finding, leading to the hypothesis of it being part of the AMG disease process. When examined by histological grade, the HAKA specimens exhibited a significant difference in the same pattern as AMG. In region N, there was no significant difference between the values for Type I collagen content between AMG and HAKA specimens. In contrast, the LAKA samples had significantly less Type I Collagen in region N. Figure 4-13 demonstrates the regional variation of type I Collagen in AKA alongside the LAKA and HAKA samples. Thus, one could hypothesize that increased Type I collagen is not a finding in low grade aged cartilage, and is instead one that occurs progressively with increasing histological damage. However, its topographical location is not in the regions of cartilage damage, but instead in the posterior region of the tibial plateau. This is discussed in more detail below in section 4.5.4.

When all AKA samples were considered together, there was a significant difference in GAG content ($p=0.04$). *Post hoc* testing showed differences in GAG between regions X-T3 and X-N. This is interesting, as region X represents the topographical location of cartilage failure and bone erosion in AMG. However, when divided into LAKA and HAKA samples, these findings were no longer significant. Therefore, I

believe the conclusions drawn from this should be guarded and more samples are required in LAKA and HAKA groups to allow a more in depth analysis.

4.5.3 Real time PCR interpretation

The use of the standard curve method does not allow for comparison of gene expression across different genes, for example, MMP1 cannot be compared to MMP3. Furthermore as all AMG samples were processed together, and AKA specimens were processed on a separate occasion, the same gene cannot be compared across the two groups. The comparisons are therefore only between damaged and undamaged regions for either AMG or AKA, and have been assessed statistically in a pairwise manner.

The results from AMG and AKA data are demonstrated above (Figure 4-17 and Figure 4-18 respectively). There were several significant differences in gene expression in AMG (COL1A1, MMP1, 3, 13, and ADAMTS4), however there was no differential gene expression in AKA samples, even for those that were subdivided into LAKA and HAKA. This suggests that differential gene expression is related to the OA disease process.

4.5.4 Implications of findings

This is the first study to analyse aged cartilage in a topographical fashion, using a template of knee OA, in the form of AMG, to allow comparisons between matched areas of cartilage. The repeatable pattern of AMG allows it to be used as a spatial model of OA progression. However, the data our group has previously acquired is difficult to interpret in isolation without control data. As described in section 4.1 the

AKA samples are not true controls. They do exhibit some histological changes that are impossible to categorise into possible age-related changes versus early OA changes. Nevertheless, the AKA samples do provide some extremely useful data.

Towards the end of this study, ethical approval was given to collect samples from patients who were brain stem dead and donating organs as part of the national organ donation program. Unfortunately, there were no suitable specimens, but these samples would potentially provide true control tissue.

The presence of Type I collagen and differential gene expression in AMG is discussed in chapter 2 (section 2.5.1). This study points to these changes perhaps being related to early histological changes, but not in the area of histologically damaged cartilage. It appears to be a phenomenon of the posterior-most cartilage of the medial tibial plateau. Animal studies have shown that cartilage damage, in its early stages, causes changes in gene expression, cytokine release and inflammatory mediation. Cartilage thinning in humans has been found to be related to altered synovial fluid profiles, for example IL15, TNF-alpha, IL-6 and IL-21 [222]. Even early disease produces an altered joint wide environment, as evidenced by changes in bone [15, 60], cartilage and synovium [223, 224]. It is likely that these changes alter chondrocyte phenotype. However, it is unlikely to be due to these factors in isolation, due to the regional specificity of these changes in both AMG and HAKA specimens.

The distribution of weight, contact and stress areas in the knee have been demonstrated using film pressure sensors [200] and finite element modelling [201]. The posterior area of cartilage is sub-meniscal and thus has a more evenly spread load than more central areas of the tibial plateau.

It would be interesting to conduct two mechanical studies which might lead to a better insight into the mechanical element of OA changes in AMG as a lesion occurs. Firstly it would be interesting to understand if the cartilage in zone X becomes softer as OA ensues, and secondary to that how the loading of the tibial plateau changes. I would hypothesize that as the lesion progresses that there is increased weight bearing through this area and off-loading of the posterior undamaged cartilage. This would potentially explain some of the changes described in AMG.

Interestingly, the thickest regions of cartilage are in the primary load bearing areas [42]. It has been hypothesized that abnormal loading during gait may be a predisposing factor to initiation and progression of AMG [50, 94]. Studies have shown that gait changes with cartilage loss in AMG [48, 225], however no work has been conducted to look at change in weight-bearing areas in either early damaged cartilage or in AMG. It has been suggested that histological features alone are likely not to be the strongest associated measure of the functional properties of cartilage [226]; as an indicator of cartilage damage it is logical to assume that both biological and mechanical environments are altered. This may lead to altered nutrition to cells and altered mechanotransduction which in turn leads to increased Type I collagen synthesis. It is also important to note that in this study, care was taken to exclude peripheral cartilage when preparing the samples, and there was no subchondral bone, fibrocartilage or synovium.

It is interesting that there is no difference in Type II collagen in both AMG and AKA specimens in terms of content or differential gene expression. Its gene expression has been shown to be increased in OA [227, 228] however has not previously been

assessed in a regional manner. We have shown no differential gene expression between regions, however it could still be the case that there is a difference between AKA and AMG specimens, but as explained earlier, the methodology does not allow for this direct comparison.

GAG exhibits the pattern that one would expect in AMG; the more histologically damaged, the less the GAG content. This was shown using histological staining (Safranin-O) and DMB immunoassays (Figure 4-15). When analysing all AKA samples the statistical difference between regions X-T3 and X-N, is an extremely interesting one. X has less GAG and is the equivalent of the area that first fails in AMG. It is difficult to draw strong conclusions from this because when divided into LAKA and HAKA subgroups, there was no statistical difference. Larger numbers in each sub group may clarify this finding, but it is possible that this early loss of GAG is a phenomenon secondary to early mechanical changes prior to more advanced OA disease. Rogers et al (2006) showed increased GAG in areas of higher weight bearing (using femoral condyle cartilage) [210]. This study appears to contradict that, as region X is a higher weight bearing area, with lower GAG content. One weakness of the GAG assay is the instability of DMB. A statistical difference was shown in each region between AKA and AMG samples, with the AKA GAG content being lower. These experiments were conducted at different time points, with different stock of DMB, which is notorious for being unstable depending on factors such as duration since the stock was made, storage and light conditions [217].

This study has demonstrated differences in regions of cartilage in both AMG and AKA specimens. Whilst it is easy to accept that AKA samples are not the best

controls, they are an excellent source of aged tissue which has definitely generated some interesting data. Peripheral vascular disease, for which all AKA samples were undergoing surgery is a possible confounder. However, given that the usual conditions of cartilage are at low oxygen tensions [211], one would hope that it is less of a confounder than, for example, nearby tumour, as used in other studies [210]. Another shortcoming is that although matrix content was assessed in smaller regions (X, T1, T2, T3 and N) it was not possible to assess by depth. Furthermore, gene expression was only possible, due to constraints of the amount and quality, to be assessed in the pooled regions T1-3. Weight-bearing status of the subjects undergoing AKA was not documented. It is feasible that a lack of loading in some subjects contributes to changes in gene expression, and ultimately matrix content perhaps dependent on the duration of altered weight bearing. One analogy would be with tendon and bone, where the effects of decreased loading have been shown to cause increase in MMP expression.

AMG provides an excellent spatial model of OA progression. The gold standard in assessment of cartilage in OA would be to couple this with a temporal model and also to couple its study with parallel mechanical studies, with the key questions that still remain unanswered in the study of OA relating to its initiation and progression. An ideal set of samples would include young undamaged cartilage, early diseased cartilage, aged diseased and undiseased cartilage and AMG (Figure 4-19).

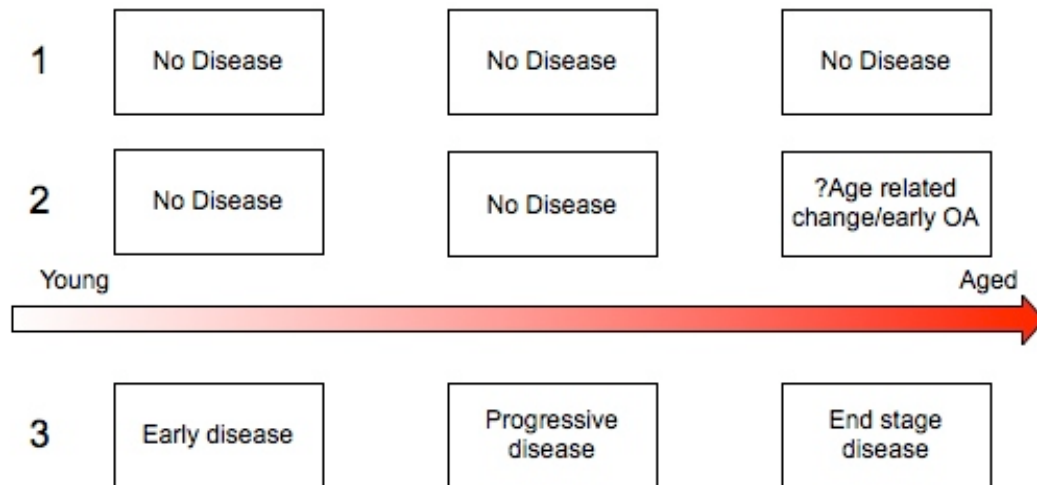


Figure 4-19 – Ideal sample collection at varying ages and stages of disease. The numbered lines 1,2, and 3 represent three groups: undiseased cartilage, aged cartilage, and progressive osteoarthritic cartilage.

It would also be possible to apply the same approach to the study of lateral, patellofemoral and tricompartmental disease, in that regional variation in disease and controls sampled in the same manner provide a persuasive argument of better understanding these changes. In addition, earlier detection techniques, for example, MRI imaging may make such early disease more amenable to study.

It is not clearly understood whether TCOA is a consequence of late AMG and the failing ACL, or whether it exists, in addition as a phenotype in itself. AKA samples, and a complete analysis of all cartilage surfaces could provide further insight into the initiation of OA. Mechanical testing of the cartilage of AKA samples could also yield interesting results especially when coupled with the findings of this study, and remains an area for further investigation.

Chapter 5

5 STUDY 3 - CELL DEATH IN ANTEROMEDIAL GONARTHROSIS

5.1 Introduction

Cartilage is an avascular, aneural and alymphatic tissue with poor regenerative potential (as discussed in Chapter 1). Chondrocytes are responsible for synthesising and maintaining the extracellular matrix of articular cartilage. They have low proliferation rates [229] thus cell death does not elicit an appropriate homeostatic mechanism of repair. Instead, once a threshold of chondrocyte death is reached, it is likely to be a pre-terminal event for the joint.

Cell death is divided into two broad categories: apoptosis and necrosis. Apoptosis, was first described by Wylie et al in 1972, as programmed cell death [230]. They described the concept of a genetically-controlled programme of nuclear and cytoplasmic changes with defined morphological and biochemical features, but without cellular constituents released into the surrounding tissues, and thus without the presence of inflammatory changes consistent with release of lysosomal enzymes [231-235]. In addition, because it involves hierarchical cascades with activation thresholds, it has been shown to be a reversible process, provided intervention occurs early enough [236].

Necrosis, on the other hand, is considered to be caused by exogenous factors, when the insult is large [237], and occurs rapidly. It would appear not to include the

complex signalling pathways of apoptosis. It is always pathological, and an inflammatory response is mounted [238]. It is a passive or energy-independent mode of death, which has been attributed to two main mechanisms; interference with the energy supply of the cell, and direct damage to cell membranes [239].

The difference between apoptosis and necrosis is an interesting and important distinction to make, and in cartilage both appear to be pathological processes [234, 240], however, necrosis is irreversible. Thus, in a study that aims to find reversible pathways as potential targets for therapeutic intervention, it is the incidence and cellular pathways of apoptosis that have been investigated.

Apoptosis has more than one pathway leading to cell death; the intrinsic (or mitochondrial) pathway, and the extrinsic (or cell surface death receptor) pathway [241] (for overview see Figure 5-1)

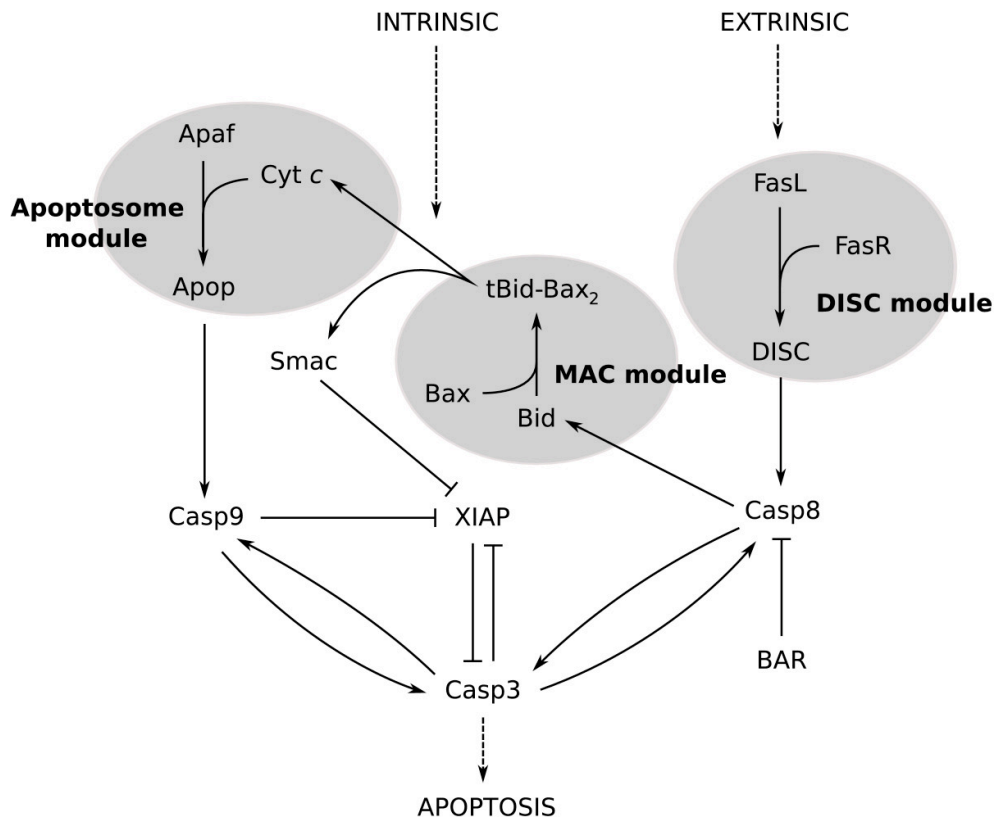


Figure 5-1 - Image demonstrating the intrinsic and extrinsic pathways of apoptosis taken from Harrington et al (2008) [242].

The extrinsic pathway involves transmembrane receptor-mediated interactions and is p53 independent. These involve extracellular death ligands that are part of the TNF receptor gene superfamily, for example FasL and TNF α . They bind with their respective receptor causing recruitment of FADD and formation of a death-inducing signalling complex (DISC), ultimately resulting in activation of caspase 8 and apoptosis execution [243].

The intrinsic pathway is initiated within the cell and there are number of known triggers which include; radiation, toxins, hyperthermia, viruses and reactive oxygen species. Such stimuli act directly within the cell, ultimately resulting in opening of mitochondrial pores and release of pro-apoptotic proteins such cytochrome c [244-246] into the cytosol [247, 248]. These proteins go on to activate Apaf-1 and pro-

caspase 9, forming an apoptosome. Thus begins the initiation and subsequent execution of the caspase cascade of proteolysis. Once activated, it has been suggested that there is an irreversible commitment towards death. However, more recently, there is some evidence that inhibition of caspase activity can prevent apoptosis [249].

A further form of programmed cell death is anoikis [250]: this is also known as “detachment-induced apoptosis “ or “integrin-mediated apoptosis”, and elements of both the intrinsic and extrinsic pathways of caspase-dependent apoptosis are implicated [251]. The loss of integrin-mediated adhesion to the extra cellular matrix results in the faltering of survival signals, including Fak, Src, and Akt, and the activation of the initiator caspase 8 via unknown mechanisms [251].

The early stages of the intrinsic pathway are highly regulated and modulation occurs with a number of proteins [252] including p53 and the Bcl-2 family Table 5-1. There are several functional classes of the Bcl-2 family. A first class inhibits apoptosis (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bcl-B). A second class forms the pores that disrupt mitochondrial membrane integrity, allowing cytochrome c release and caspase 9 activation (Bax, Bak). A third class facilitates apoptosis by binding to and decoying anti-apoptotic Bcl2 etc (Bad, Noxa and Bmf). A fourth class can bind and directly activate pore formers Bak and Bax (Bim, puma, truncated Bid) [236]. In functional terms this means that decoy-type proapoptotic members like noxa are less potent than direct activators of pore formation like Bim.

Pro-apoptotic	Anti-apoptotic	BH3-only Triggers of apoptosis
Bcl-10	Bcl-2	Bad
Bax	Bcl-x	Bik
Bak	Bcl-xl	Bid
Bok	Bcl-xs	Bim
	Bcl-w	Noxa
	BAG	Puma

Table 5-1 – The Bcl-2 family of proteins. The Bcl-2 family have both pro- and anti-apoptotic functions. There are 25 genes in total, and a selection are listed above as pro/anti apoptotic or BH3-only.

This family of proteins has also been shown to be involved with Endoplasmic Reticulum (ER) stress-induced apoptosis. The ER is an organelle responsible for the synthesis and folding of intracellular proteins. It is highly sensitive to stresses that affect cellular energy levels, the oxidation-reduction (redox) state and calcium ion concentration. The Bcl-2 family of genes has also been implicated in ER stress-induced apoptosis, with over expression of Bcl2, or Bax and Bak deficiencies being shown experimentally to confer protection against ER stresses [253, 254]. However, their regulation in ER stress is not fully understood.

The caspases have been described as the ‘executioners of cell death’ [255]. In broad terms there are three main groups of caspases; the initiators, the executioners and the inflammatory caspases (Table 5-2). Executioner caspases such as caspase 3, 6 and 7 are responsible for the activation of cytoplasmic endonucleases, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins [256].

Category	Caspases
Initiator	2, 8, 9, 10
Executioner	3, 6, 7
Inflammatory or cytokine processing	1,4, 5, 11, 12, 13, 14

Table 5-2 – Caspases listed by role. There are 14 caspases that are broadly categorised into the roles above. In particular, caspase 8 is the first activated in the extrinsic pathway, and caspase 9 in the intrinsic pathway.

Reactive oxygen species (ROS) have been implicated in a number of pathological processes. “Oxidative stress” is the term used for an imbalance in oxidants and antioxidants large enough to induce cellular and/or tissue structural or functional changes [257]. Mitochondria are an important source of ROS, they have been shown to be triggers of mitochondrial-mediated apoptosis. 3-Nitrotyrosine can be used as a surrogate marker of ROS induced damage. It is formed from the nitration of tyrosine residues in a chemical reaction caused by exposure to high levels of ROS [258]. It has been suggested that nitric oxide (NO) donors are one of the primary inducers of cell death. It appears that this process is modulated by other ROS [259]. Likely causes of excess ROS generation include excessive mechanical load [260] or proinflammatory cytokines [222], both of which are known factors in the OA disease process.

There are multiple triggers that may influence why a cell that is under stressed conditions may start down a route towards apoptosis. Similarly, there are protective mechanisms that attempt to prevent this. It is a complex, and not fully understood, balance. One family of proteins that have been implicated in the process is the Forkhead box (FOXO) family of transcription factors. The role of FOXOs is not clearly defined in the literature, showing both protective and cell death effects.

FOXO1 and 3A are key responders to oxidative stress and mediate cellular responses to ROS, including initial upregulation of protective enzymes such as SOD [261], but followed, in chronic exposure states, by upregulation of pro-apoptotic proteins such as Bim and noxa, resulting in apoptosis [262, 263]. As discussed above, while both Bim and noxa play a role in oxidative stress induced death, Bim can directly mediate cell death while noxa predisposes to it, hence presence of Bim in damaged tissue is a stronger indicator of probable apoptotic consequences. Bim was first linked to oxidative stress induced apoptosis [264] and subsequently identified as a FOXO target gene [265].

This study investigates the incidence of cell death in AMG using immunohistochemical methods, with the aim of exploring possible pathways causing apoptosis and the role they may play in cartilage degradation in AMG. Table 5-3 lists the immunohistochemical studies conducted and function of the marker.

Immunohistochemical study	Role of marker
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)	Detects DNA fragmentation resulting from apoptotic signalling cascades [266]
Active Caspase 3	Executioner caspase.
Cytochrome C	Intermediary in apoptosis; released from inner membrane of mitochondria and readily detectable only when released.
Active Bax	Bcl-2 family, found primarily in cytosol, triggers mitochondrial permeability transition in the mitochondrial membrane once conformationally active.
3-Nitrotyrosine	Indicator of nitric oxide and ROS induced damage.
Bim	Bcl-2 family, proapoptotic molecule in BH-3 only or direct activator group.
Forkhead box O3A (FOXO 3A)	Forkhead transcription factor; oxidative stress responder and mediator of apoptosis in conjunction with other proteins

Table 5-3 - Immunohistochemical studies conducted. The list above demonstrates the immunohistochemical staining undertaken, with a brief overview of the role of the marker/protein.

5.2 Null Hypotheses

- 1. Apoptosis does not correlate with degradation of cartilage in OA.*
- 2. There are no regional differences in cell death in AMG.*
- 3. Reactive oxygen species are not implicated in the process.*
- 4. There is no difference in cell death between AMG and control specimens.*

5.3 Methods

5.3.1 Sample collection

Ten tibial plateau resection specimens were collected from patients undergoing UKA for AMG (Ethics Reference C01.071) at the Nuffield Orthopaedic Centre). These were paraffin embedded and sectioned as described in previously (section 4.3.1).

Of the sixteen AKA specimens, described in the previous chapter, eight were used as control samples, having also been prepared into paraffin embedded sections.

Sample number	Gender	Date of Birth	Age	Side
1	M	02/07/1933	74	R
2	F	22/8/54	53	R
3	M	9/10/1939	68	L
4	F	2/8/1926	81	L
5	F	16/11/1945	62	R
6	F	29/2/32	75	L
7	F	26/3/1938	69	L
8	M	9/12/1936	71	R
9	M	7/12/1943	64	R
10	F	18/06/1929	78	L

Table 5-4- Demographics of AMG specimens used in this study. (M – Male, F – Female, R – Right, L – left).

5.3.2 Immunohistochemical studies

Table 5-3 shows the immunohistochemical studies that were undertaken. A protocol is included as Appendix 3.

A number of optimising steps were required to the initial basic immunohistochemistry protocol in order to gain satisfactory results, in terms of appropriate staining. These are highlighted below.

5.3.2.1 Heating sections

When difficulty has been encountered with paraffin embedded sections in the past, a number of immunohistochemical protocols describe microwaving of sections to aid with antigen retrieval [267, 268]. This was attempted to improve the quality of staining with these sections. However it was abandoned due to problems with the sections lifting off the slides. Upon discussing this with other groups, it is a common occurrence with very large cartilage sections.

5.3.2.2 *Blocking non-specific staining*

Blocking solutions reduce background staining by binding to generic protein-binding sites or highly charged molecules. The primary antibody then competitively binds to its respective antigen in the next step. Despite increasing concentrations of antibody, a number of sections were still showing only faint staining. By varying the length of the blocking step, more pronounced specific staining was achieved, with no excess of background staining; 200µl of 3% Horse serum was added per slide.

5.3.2.3 *Primary Antibody*

The data sheet information provided with each antibody often gave concentrations for immunohistochemistry conducted on cryosections. Therefore a number of pilot experiments were required to optimise the concentrations of antibody on paraffin embedded sections.

200µl of each primary antibody was added per slide. They were then covered in parafilm and incubated overnight (approximately 18 hr) at 4°C. Table 5-5 shows the concentrations that each antibody was optimised to.

5.3.2.4 *Positive and negative controls*

The following controls were used for:

TUNEL – Positive control was sections of colon, provided with the lab kit; negative control was performed with no terminal transferase (label solution only).

Caspase/cytochrome c – Positive controls for the antibody have been used widely by our lab on induced apoptosis in cultured cells. Negative controls were isotype specific controls.

FOXO / Bim – these have been used extensively by our research group to confirm specific knockdown of Bim or FOXO3A by siRNA procedures. On Western blot,

bands of the correct size are induced by expected inducers and removed by siRNA, confirming antibody specificity.

5.3.3 Assessment

TUNEL, 3-nitrotyrosine (abbreviated to acronym 3-NT although tyrosine is correctly symbolized as Y), and FOXO were assessed quantitatively. Each region (T1-3 and N) was divided into superficial, middle and deep areas as shown in Figure 5-2. Cells were counted at 200x magnification and the percentage positive cells (number of positive cells divided by total number of cells x 100) was calculated for three frames per area. For the AKA specimens, the template described previously was used (Figure 3-6)

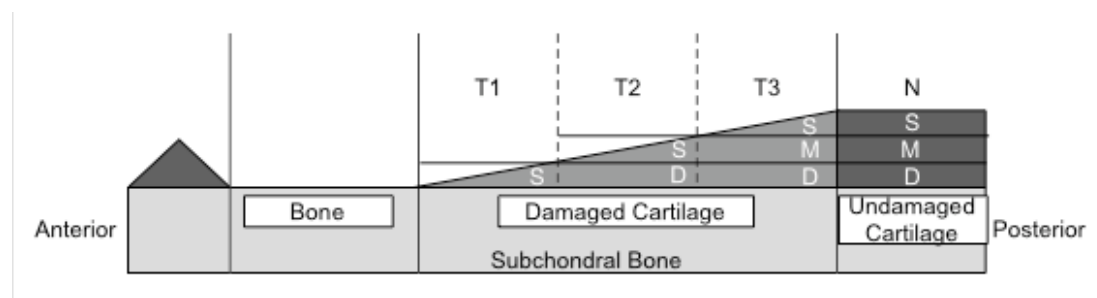


Figure 5-2 - Schematic representation of AMG. The damaged area was divided into equal thirds (T1, T2, T3). The undamaged cartilage is region N. The cartilage was divided into equal thirds, S being the most superficial, M the middle region and D being the deepest.

Active Caspase 3, Cytochrome C and Active Bax and Bim were assessed qualitatively.

Antibody to:	Manufacturer	Concentration used
Active Caspase 3	Cell signalling technologies (MA, USA)	1:100
Cytochrome C	Cell signalling technologies	1:100
Active Bax	Upstate (Virginia, USA)	1:100
3-Nitrotyrosine	Invitrogen (Paisley, UK)	1:100
Bim	Epitomics (California, USA)	1:20
FOXO3A	Cell signalling technologies	1:20

Table 5-5 - Antibodies (manufacturer, concentration). The antibodies were optimised by changing the concentrations to obtain appropriate staining.

5.3.4 Statistical Analysis

Graphical data are displayed as box and whisker plots with median values and error bars representing range. Analysis was undertaken using Friedman's tests, comparing percentage positive staining between regions in PASW version 18 (IBM, New York, USA). *Post hoc* testing was undertaken using Wilcoxon tests and Spearman's rank correlations were undertaken to assess the relationship between percentage positive staining and histological grade. Graphs were created using GraphPad Prism version 5 (Graphpad, California, USA)

5.3.5 Inter/Intra observer variability

Two random sections were analysed by a second observer, and at two different time points by one observer. Each section equates to twelve regions, and three frames were counted in each region, therefore 72 fields were analysed. Results were judged by the

intraclass correlation coefficient. The inter and intra observer variability was 0.994 and 0.995 respectively.

5.4 Results

There was a significant difference in TUNEL staining between regions ($p < 0.001$) showing progressively less staining with less damage to the cartilage (Figure 5-3 and Figure 5-4). This ranged from a median value of 26% in the most damaged cartilage (T1) to 4.4% in the undamaged cartilage (N). *Post hoc* analysis showed significance in comparison of T1 to other groups, but not within pairs T2-N.

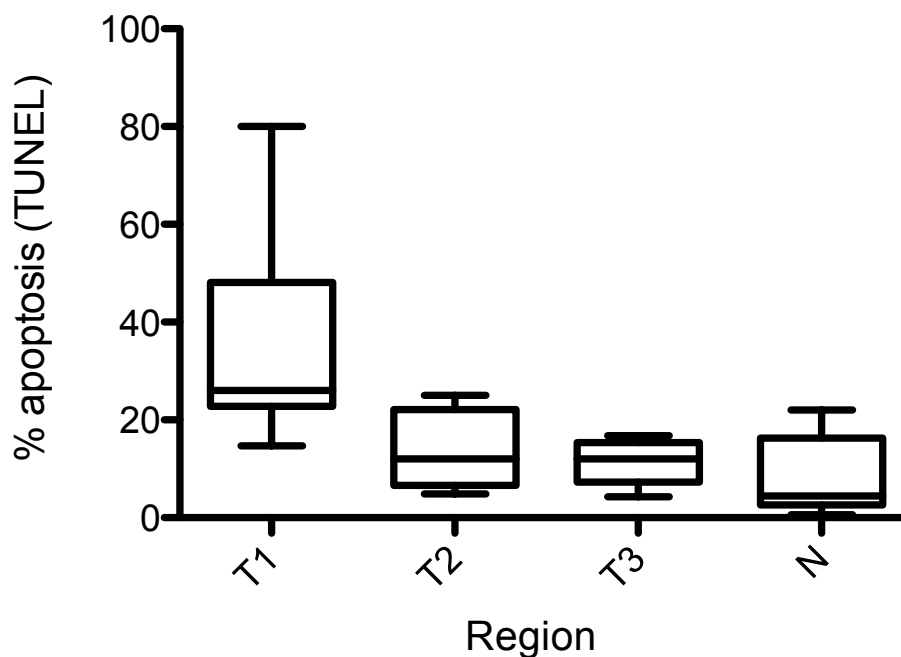


Figure 5-3 – Percentage apoptosis (as shown by TUNEL staining) by zone in AMG specimens. The cartilage sections were divided into regions from anterior to posterior (Figure 5-2). There were equal divisions of damaged cartilage (T1-T3) between the full thickness cartilage and the start of the area of undamaged cartilage (N). Percentage positive TUNEL staining (TUNEL positive cells/total number of cells) were counted in multiple frames. There was a significant decrease in levels of TUNEL staining between regions ($p < 0.001$, Friedman), from a median of 26.0% in T1 to 4.4% in N. *Post hoc* testing revealed significance between pairs of zone T1 and other zones, but not for pairs within T2-N.

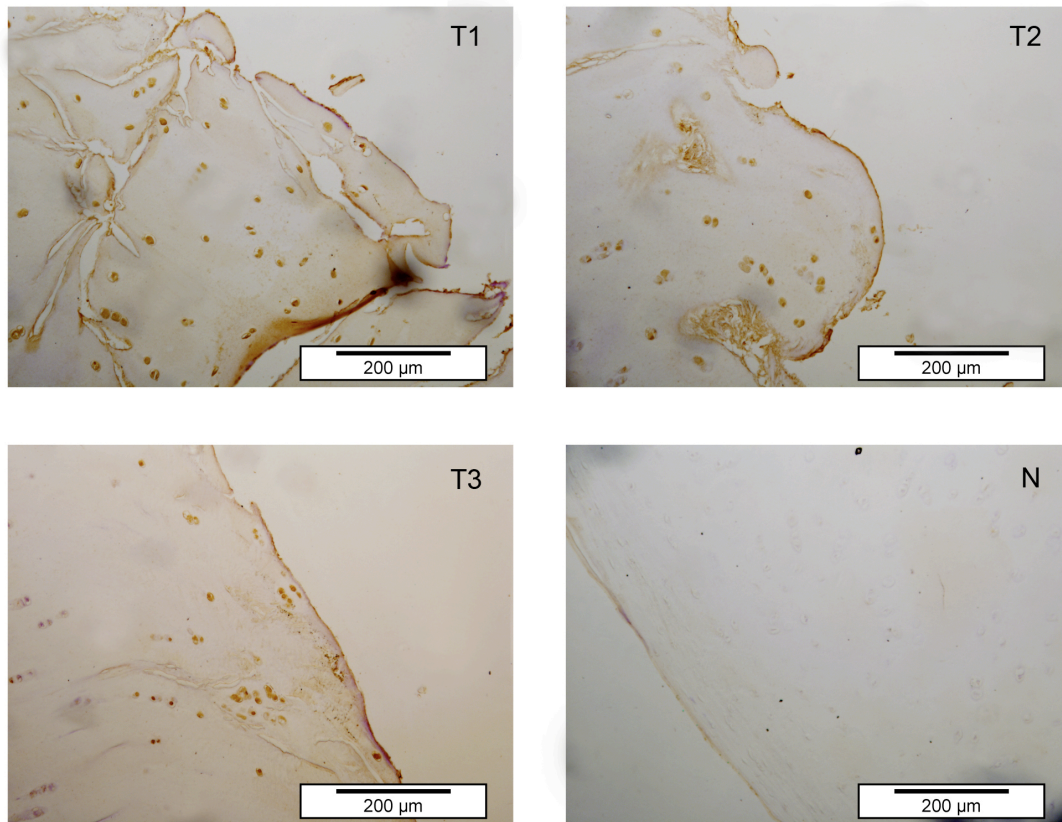


Figure 5-4 - Immunohistochemistry pictures of TUNEL staining for regions T1, T2, T3 and N. Cell counting demonstrated significantly higher percentage of positive staining in more damaged cartilage ($p<0.001$; see Figure 5-3).

Cell death, as detected by TUNEL, appeared predominantly in surface chondrocytes of damaged cartilage. The median value in the superficial layer across all zones was 22.7% compared to 0.0% (25% percentile 0.0; 75% percentile 3.4) in the deeper layers ($p<0.001$) (Figure 5-5).

An analysis of percentage of apoptosis compared to histological grade showed that there was a good correlation (Spearman's $r=0.66$, $p<0.001$), as illustrated in Figure 5-6.

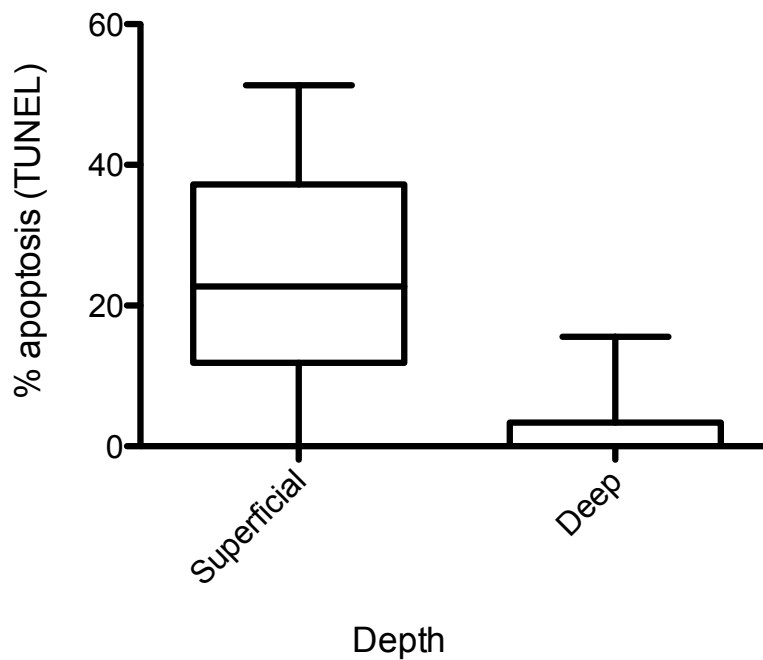


Figure 5-5 - Percentage apoptosis (as shown by TUNEL staining) by depth in AMG specimens. The cartilage sections were divided into regions by depth, of superficial (top third by depth) or other (including middle and deep) Percentage positive TUNEL staining (TUNEL positive cells/total number of cells) were counted in multiple viewing fields. There were significantly greater amounts of apoptosis in superficial cartilage when compared to deeper regions ($p < 0.001$, Friedman).

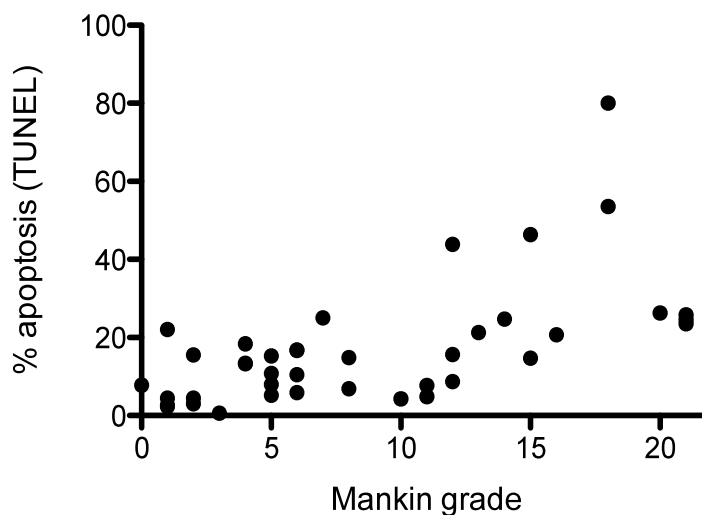


Figure 5-6 - Scatterplot showing percentage apoptosis by histological grade. Each region had percentage of TUNEL positive cells counted and was also graded histologically using a modified Mankin grade (range 0-21). The percentage of apoptosis showed a good correlation with histological damage ($r = 0.66$, $p < 0.001$, Spearman's rank correlation).

Active caspase 3, Cytochrome C, Bim and active Bax were assessed qualitatively and shared a similar distribution to TUNEL (Figure 5-7). There were highest numbers of positive cells in the surface layer of damaged cartilage. There was little staining in deeper layers or in undamaged cartilage.

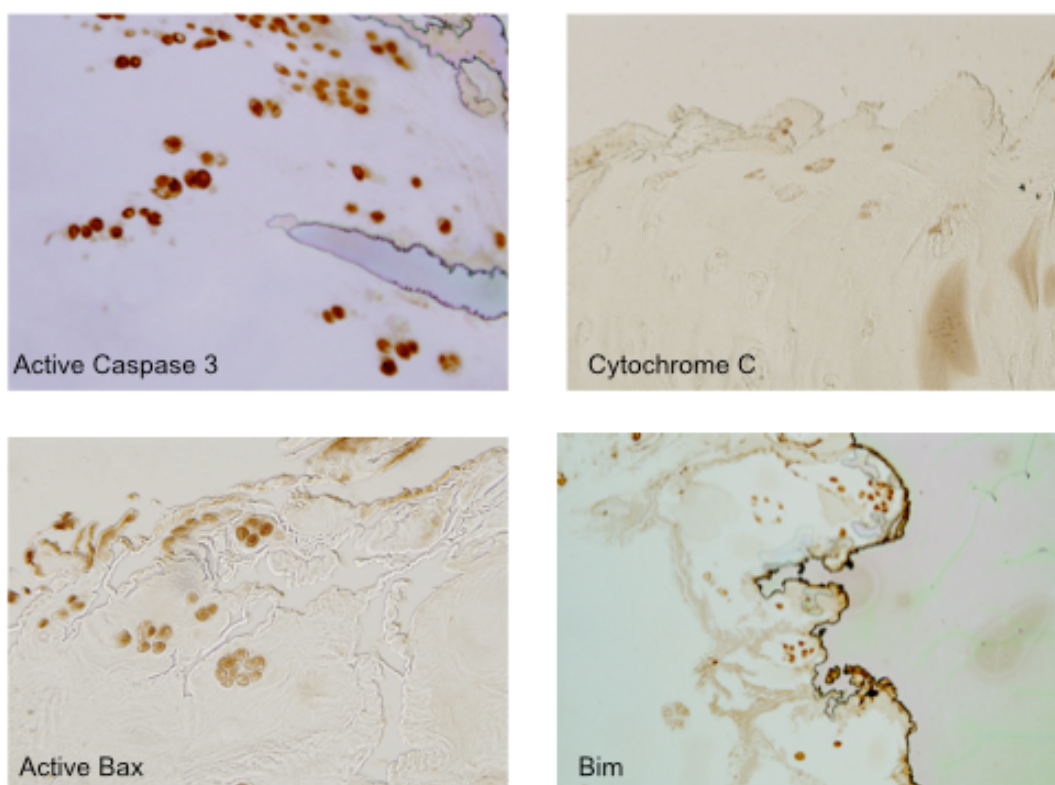


Figure 5-7 - Immunohistochemistry pictures demonstrating the presence of Active Caspase 3, Cytochrome C, Active Bax and Bim. These markers of apoptosis were seen in a similar distribution to that of TUNEL – more prevalent in more damaged cartilage (see Figure 5-3).

3-Nitrotyrosine was also shown to be present with a similar distribution to TUNEL (Figure 5-8). Quantification revealed a progressive decrease in percentage positive cells from 58.3% in T1 to 10.3% in N, and there was a significant difference between regions ($p < 0.001$) (Figure 5-9). Pairs of data (by zone) were analysed using a Wilcoxon test, and were significantly different between all pairs ($p = 0.002$) except for T3 compared to N.

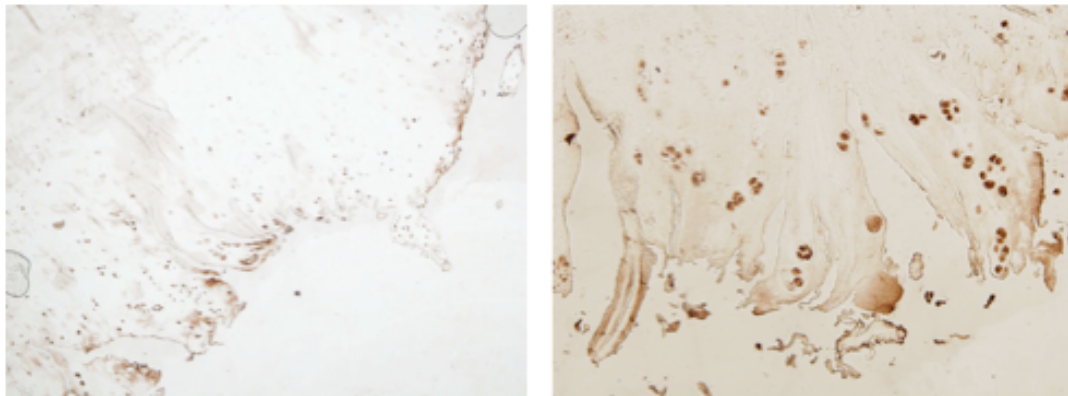


Figure 5-8 – Immunohistochemistry pictures demonstrating the presence of 3-nitrotyrosine in a similar distribution to TUNEL. Positive staining was seen predominantly in the surface layer of damaged cartilage.

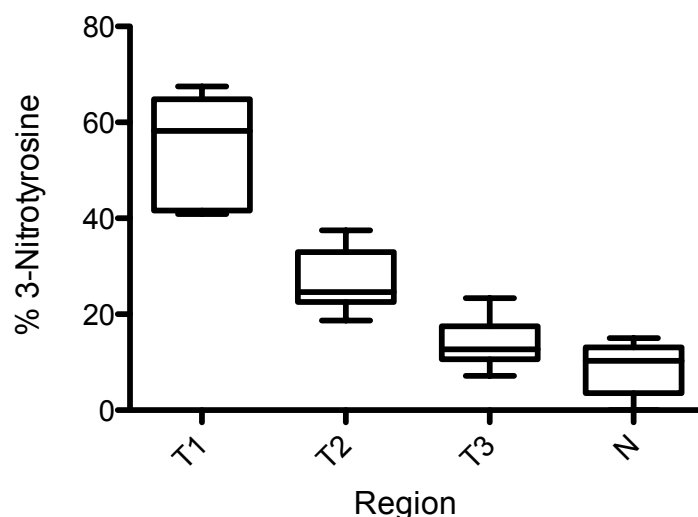


Figure 5-9 - Percentage 3-nitrotyrosine immunostaining by region. The percentage of 3-nitrotyrosine staining (positive cells/total cells) in multiple frames was shown to be significantly different ($p < 0.001$, Friedman) by region. The median values decreased from 58.3% in T1 to 10.3% in N. There was a significant difference ($p = 0.002$) between all pairs except T3 and N.

Plotted together, the relative amount of 3NT positive cells was greater than TUNEL positive cells in each zone (Figure 5-10). It was significantly higher in zones T1 and T2 ($p < 0.04$ and $p < 0.01$ respectively). This may be due to these methods having differing sensitivities, for example 3NT may be a more sensitive antibody than the TUNEL antibody kit, or 3NT may be more abundant as a marker.

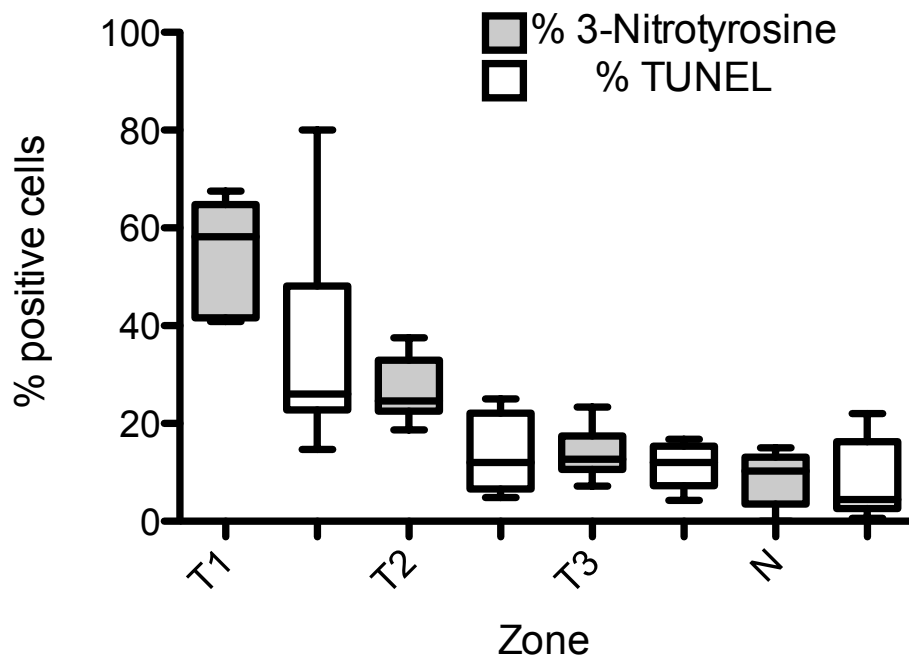


Figure 5-10 - Percentage 3-nitrotyrosine and percentage apoptosis by region. This graph represents Figure 5-9 and Figure 5-3 plotted together. In each region there appears to be a higher level of 3-NT staining than TUNEL staining. 3NT boxes are patterned, TUNEL boxes are empty. This was significant in zones T1 ($p < 0.04$) and T2 ($p < 0.01$, Wilcoxon).

No significant difference was demonstrated in levels of FOXO3A between regions ($p=0.06$) (Figure 5-11). Figure 5-12 demonstrates, for interest, levels of FOXO, 3-NT and TUNEL in regions of AMG. They are not compared statistically as each are different assays/antibodies with different sensitivities (Table 5-6).

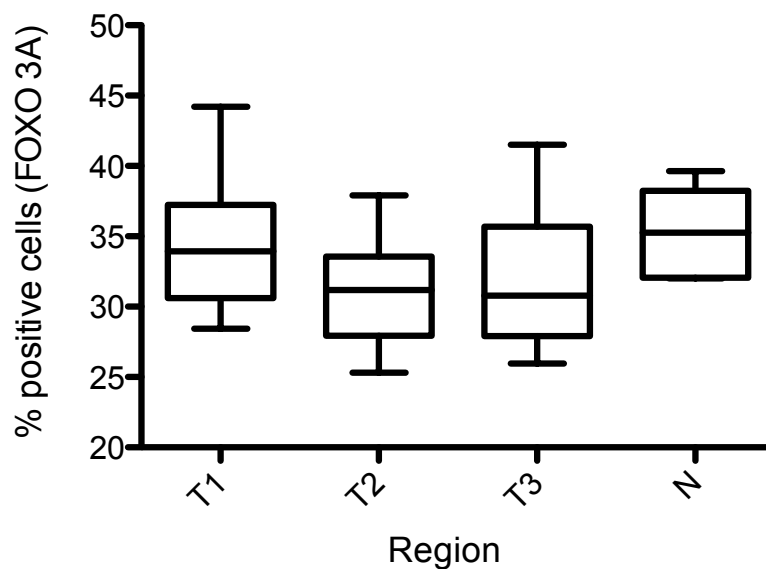


Figure 5-11 – Percentage FOXO3A by region. Median values ranged from 30.8 (T3) to 35.3 (N). There was no significant difference between regions ($p=0.06$).

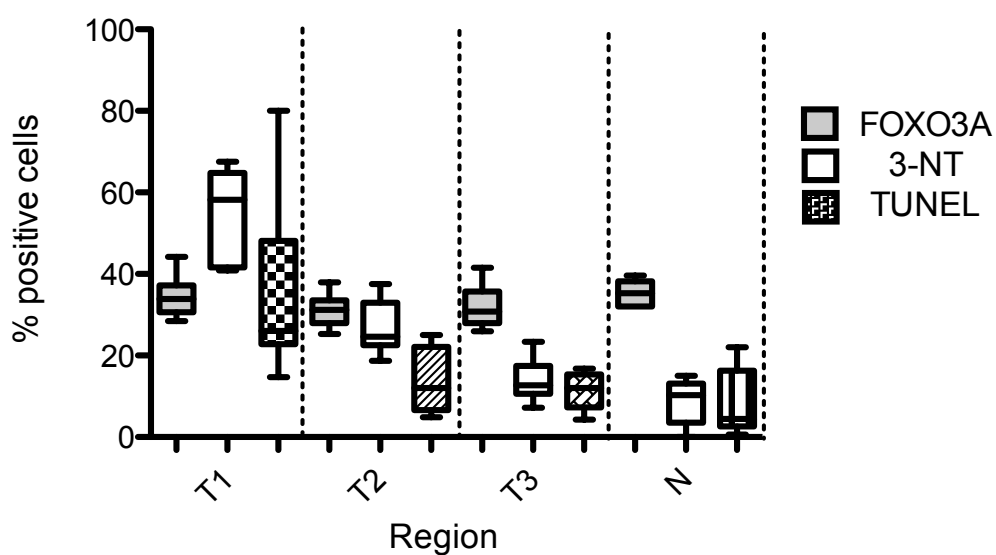


Figure 5-12 – Percentage positive cells of FOXO 3A, 3-NT and TUNEL, by region.

Assay/Region	T1	T2	T3	N
TUNEL	26.0	12.0	12.0	4.5
Range	14.7 – 80.0	4.9 – 25.0	4.3 – 16.8	0.6 – 22.0
3-NT	58.3	24.7	12.7	10.3
Range	41.0 – 67.5	18.7 – 37.5	7.2 – 23.4	0.0 – 15.0
FOXO3A	33.9	31.2	30.8	35.25
Range	28.4 – 44.2	25.3 – 37.9	25.9 – 41.5	32.1 – 39.6

Table 5-6 - Median percentage positive cells by region. Median values and ranges are shown for TUNEL, 3-NT and FOXO3A staining by region.

Comparing this to apoptosis in the age-matched but relatively undamaged AKA specimens, levels were significantly lower ($p < 0.001$) than in AMG across all regions (Figure 5-13). Median values were low throughout AKA specimens, ranging from 1.0 to 3.2%. In AKA specimens, there was a significant difference between regions ($p < 0.001$), with post hoc testing demonstrating this to be between regions X v T2, X v T3 and X v N ($p = 0.012$).

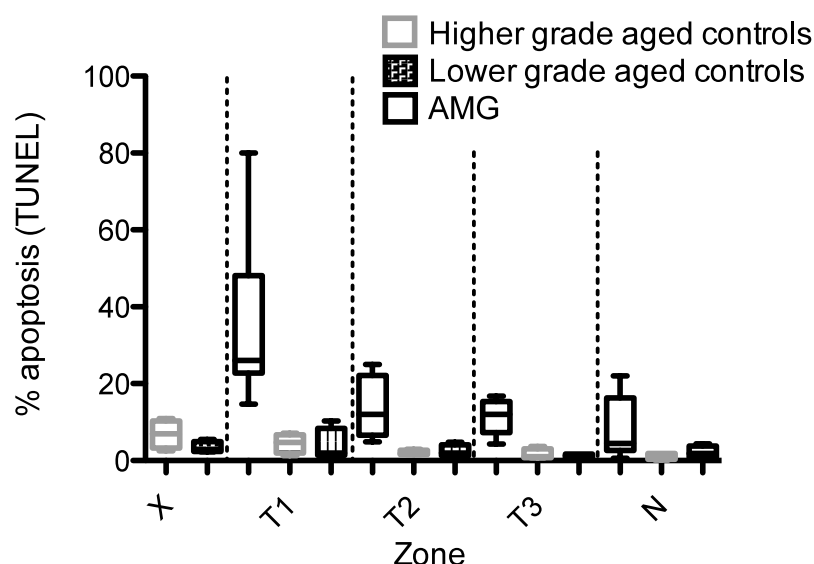


Figure 5-13 – Percentage apoptosis in aged samples, by region. Within each zone, the percentage of apoptosis is plotted for AMG specimens and for undamaged aged control samples (higher and lower histological grade). Within each zone (T1-N) there was a significantly greater percentage of apoptosis in AMG compared to undamaged control samples (T1 $p < 0.001$, T2 $p < 0.001$, T3 $p < 0.001$, N $p = 0.02$, Kruskal Wallis).

In the AKA samples, 3-NT demonstrated a significant difference ($p<0.001$) between regions (Figure 5-14). *Post hoc* testing showed that region X significantly higher numbers of cells than all other regions. There was no significant difference when higher and lower grade AKA specimens were compared.

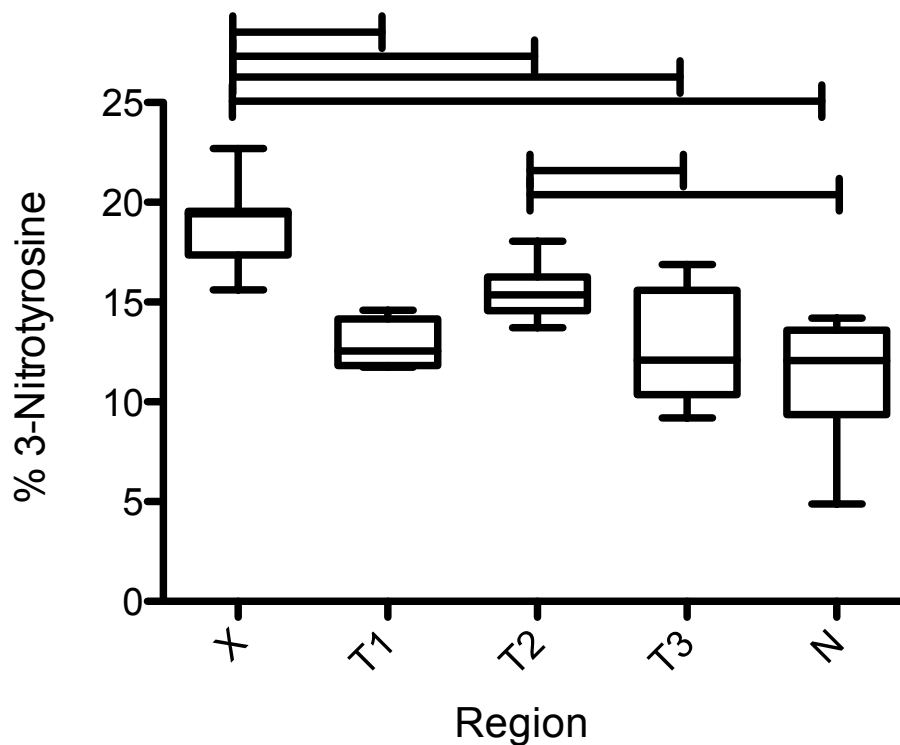


Figure 5-14 - Percentage 3 Nitrotyrosine by region. Median values ranged from 12.1 (N) to 19.41 (X). There was a significant difference between regions ($p<0.001$) demonstrated with post hoc testing to be 30.8 between X and all other regions, T2 v T3 and T2 v N).

FOXO3a staining was very consistent across all regions but there was a statistically significant difference ($p=0.04$) between regions. Post hoc testing showed this to be between regions X v T3 and T1 v T3. FOXO3A was most prevalent in region X (Figure 5-15). N displayed the least variation in staining between specimens. There was no significant difference between higher and lower grade AKA specimens.

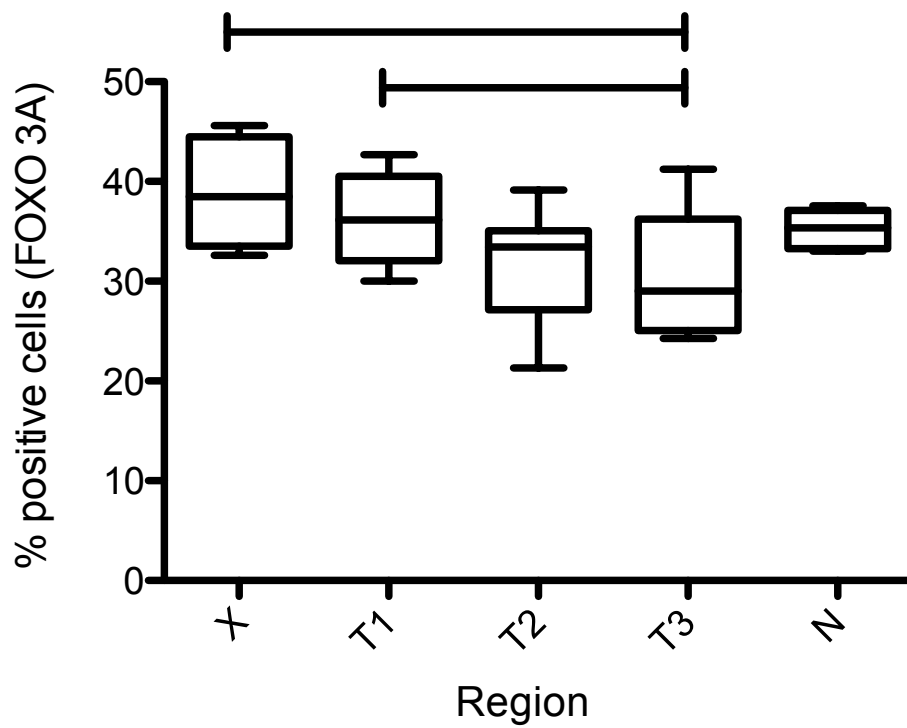


Figure 5-15 - Percentage FOXO3A by region. Median values ranged from 29.01 (T3) to 38.48 (X). There was a significant difference ($p=0.04$). Post hoc testing demonstrated statistically significant differences between X v T3 and T1 v T3.

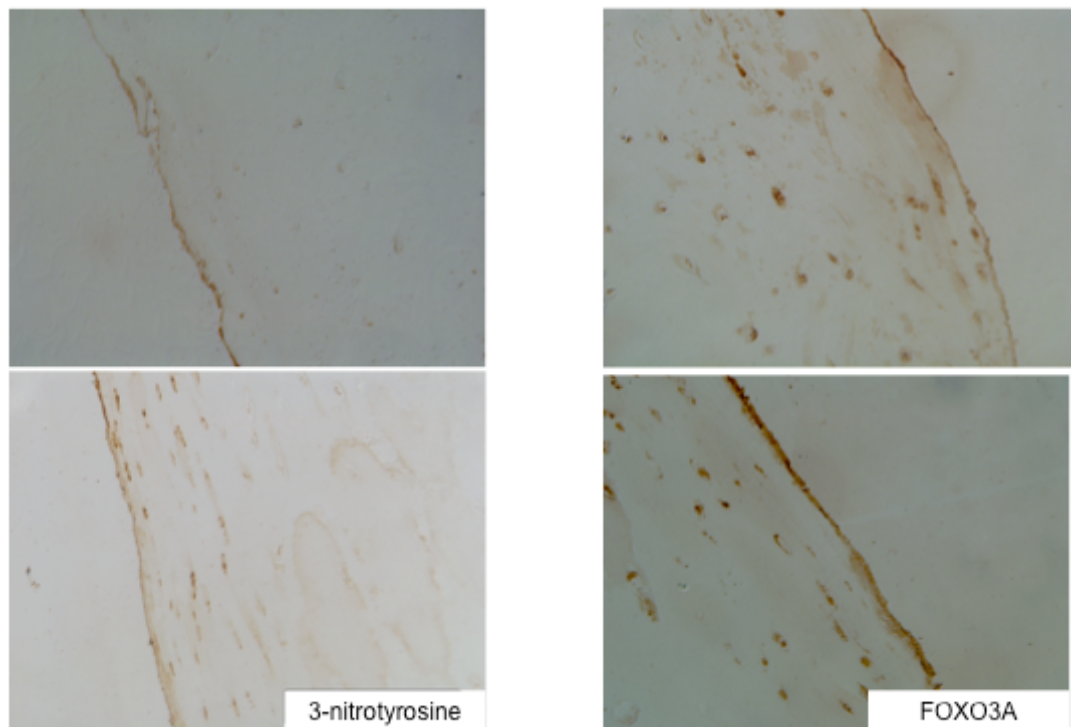


Figure 5-16 - Immunohistochemistry pictures demonstrating 3-nitrotyrosine and FOXO 3A staining in AKA samples. The smooth cartilage surface, representative of a low histological grade, can be seen together with positive staining.

When AMG levels of 3-NT and FOXO3A were compared to those from AKA samples significant differences were demonstrated in regions T1 ($p<0.001$) and T2 ($p<0.001$) for 3NT; there were no significant differences between regions for FOXO3A.

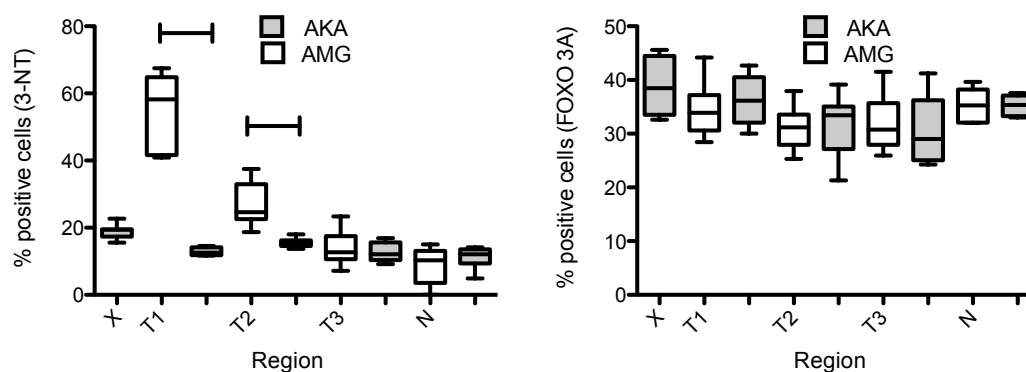


Figure 5-17 – Graphs demonstrating percentage positive cells, by region, of 3-NT and FOXO3A between AMG and AKA specimens. Significant differences were shown in regions T1 ($p<0.001$) and T2 ($p<0.001$) for 3-NT. There were no significant differences for FOXO3A.

5.5 Discussion

5.5.1 Apoptosis

The null hypotheses:

1. *Apoptosis does not correlate with degradation of cartilage in OA.*
2. *There are no regional differences in cell death in AMG.*
3. *Reactive oxygen species are not implicated in the process.*
4. *There is no difference in cell death between AMG and control specimens.*

are all rejected.

The results from this study have a number of interesting findings:

1. There is a progressive increase in the amount of positive staining per region as the cartilage becomes more damaged and there is a good correlation of cell death to histological grade.
2. Positive staining is predominantly a surface phenomenon.

3. The presence of active caspase 3 and cytochrome C release support the theory of a programmed signalling pathway leading to cell death
4. Appearance of Bim and activation of Bax and cytochrome c release suggest an “intrinsic” apoptotic mechanism that involves the mitochondria and possibly apoptosis secondary to endoplasmic reticulum stress.
5. 3NT presence demonstrates a role of ROS mediated apoptosis in cartilage

Apoptosis is an area within cartilage biology that has been controversial for over a decade. In broad terms, apoptosis has a role in several processes in the eukaryotic mammalian cell. It is required for embryogenesis and development, and in the adult, for tissue remodeling. However, it also has pathological implications, and to take a few simple examples, too much apoptosis may lead to tissue atrophy (e.g. neurodegeneration or skin thinning), and too little, to hyperplasia (e.g. cancer or atherosclerosis). In cartilage, it appears to be a pathological process and one which is poorly dealt with by the tissue. There are low rates of proliferation and little in the way of cellular turnover [122, 269] both in normal and diseased tissue. This means that an area of cartilage with depleted numbers of functioning chondrocytes has no mechanism for maintaining the ECM. This lack of a ‘regenerative’ homeostatic mechanism is one of the reasons that once OA is diagnosed that it is considered to be pre-terminal for the joint.

Aigner, in a perhaps controversially titled paper, “Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage” [122] in 2001, reported only occasional TUNEL positive cells in osteoarthritic cartilage. Aigner’s paper stresses one group’s conclusions, but there

have been both agreement and contradictory findings prior to and since its publication.

Roach et al [239] addressed this further by coining the term “chondroptosis”. Her interpretation of programmed cell death focuses on the morphological differences between classical apoptosis and features seen in chondrocytes. The presence, under electron microscopy, of TUNEL-positive clusters of particles containing nuclear material is rare. There are several reports of failure to find apoptotic bodies in OA cartilage [125, 270]. Roach et al thus use the term chondroptosis as a descriptive term for the events that they note using electron microscopy, not a sequence of biochemical events.

TUNEL staining [266] is one of the most commonly used assays for detection of apoptotic cells. It identifies DNA fragments but there is debate with regards to its accuracy and possible false positive rate [234, 239, 271], with it detecting end-stage necrotic cells that also display DNA fragmentation. This uncertainty about the specificity of TUNEL led us to use other recognised ‘end-stage’ markers of apoptosis which have also been used by other groups and for which published data exists. Active caspase 3 has been used as another marker of apoptosis in several studies [272-274] in cartilage, yielding similar results to TUNEL, however it is interesting to note that reported levels of apoptosis in OA cartilage vary widely from <1% [122] to 44% [274]. Released cytochrome c is a widely used marker of intrinsically driven apoptosis involving mitochondria [275, 276]. Despite the differences in nomenclature, it is evident from previous work into cell death in cartilage that a number of markers for a ‘programmed’ cascade are present.

There is a statistically greater percentage of positive cells in the surface layer when compared to the deeper layers. The most superficial layer of remaining cartilage was taken as the surface layer, and compared to the other layers (this was defined as all of the cartilage in T1, the most superficial half of the cartilage in T2 and the most superficial third of the cartilage in T3 and N). This result is as one might expect. In OA it is the surface layer of cartilage which histologically exhibits changes first hence grading is driven by surface phenomena [133]. In mechanical loading models *in vitro* [277], and also in animal models of OA [274, 278], it is the surface layer that exhibits changes in cellular viability or increase in staining of apoptotic markers. This has also been demonstrated in human cartilage [124].

This study demonstrates surface driven damage in a human model of OA progression, where even in cartilage with a normal histological grade, some positive staining was present with the vast majority in the superficial layer. There is a statistical difference in the percentage of positive cells when compared by region, with higher rates of cell death detected in more damaged cartilage. This is further supported statistically by a good correlation with histological grade. In the phenotype of AMG, there is a progressive change in histological grade and thus the severity of OA across specimens. These results show that in these regions of cartilage, cell death, certainly in more damaged cartilage, is a good marker of disease progression. However, one of the interesting results is in region 'N' where, though histologically classified as 'normal', there are 4% positive cells.

This again relates to a recurring theme of the lack of good human control data, but some human data [122, 239] and certainly available animal data would suggest that this is a higher percentage than one might expect. If one were to hypothesize why this might be the case, it again relates to the overall environment of the joint. In an AMG joint, the biological ‘stability’ of the joint firstly becomes compromised in the anteromedial quadrant of the tibial plateau, which is where the lesion is found [91]. As the disease progresses, the size of the lesion increases, more histological damage is evident, and there an increase in the amount of cell death. This will inevitably have sequelae, with a combination of a loss of chondrocyte survival signals [279] and a pro-apoptotic environment.

5.5.1.1 Mechanism of apoptosis

Given that TUNEL shows the variation described above in cartilage with increasing degrees of damage, it is important to try and ascertain the pathways causing cell death. Immunohistochemical staining of active caspase 3, cytochrome C, active Bax, and Bim showed their presence with a similar distribution to TUNEL staining.

Active Caspase 3 is termed an executioner caspase, in other words, its activation and effects are at the end stages of apoptosis. In particular it has interactions with caspases 8 and 9 as shown in Figure 5-1 [242]. It is activated by both the extrinsic and intrinsic pathways [280, 281]. Active caspase 3 has been used in previous studies [274] as a means of quantifying apoptosis in cartilage. The conclusions of this paper, which used equine metacarpophalangeal and proximal and distal interphalangeal joints, are analogous to the present study, showing the presence of active caspase 3 to be correlated to histological grading. This study, in assessing AMG, supports this data.

Cytochrome C is a protein normally found bound to the inner membrane of mitochondria. It is highly toxic outside the mitochondrion and upon release acts as an intermediary in the apoptosis cascade [246]. Cytochrome c release has been explained by a number of proposed theories. A significant amount begins bound to the inner mitochondrial membrane by cardiolipin [282]. Cardiolipin is oxidised, (and this can occur by ROS interactions), leading to release of cytochrome C from the membrane. Other evidence suggests that a change in the concentration of potassium ions is enough in itself to release cytochrome C from the membrane without other molecular interactions [283]. Its release into the cytosol then has downstream effects on procaspase 9, which is activated and in turn activates executioner caspase mediated cell death. While double-staining was not carried out, the data from this study supports apoptosis induction via the mitochondrial pathway, as evidenced by the presence of cytochrome C in cells from tissue regions also positive for active caspase 3 and TUNEL.

Further immunohistochemical studies examined upstream markers of intrinsic pathway apoptosis.

Bax is one of a family of apoptosis regulating molecules; the Bcl-2 superfamily [252]. Bax, in its inactive state, is present in the cytosol. Apoptotic stimuli cause its translocation into mitochondria and when it is activated, it creates pores in the outer mitochondrial membrane through which cytochrome C is released.

Bim is another one of the Bcl2 family of proteins that has been shown to have a pro-apoptotic action. This also been shown to be through interactions with a number of other Bcl-2 family members including by direct binding to and activation of Bax

[284]. Bim is also known to be upregulated by FOXOs [285, 286] (see section 5.5.2). Since the start of this work evidence for the involvement of Bim in ROS-mediated stress response has emerged [287] but it has also now been found that FOXO1 is the primary driver of Bim in apoptosis induction [287, 288], and in hindsight this would have been the better FOXO to study.

The molecules discussed are the ones primarily addressed in this study, however, there are numerous other proteins including other caspases and Bcl-2 proteins that interact in the regulation of apoptosis.

In summary, we have shown the presence of markers that are recognised as forming a part of the intrinsic pathway of apoptosis. Their prevalence is highest in the most damaged cartilage of AMG, and sequentially decrease as the cartilage becomes increasingly less damaged.

5.5.2 Reactive Oxygen Species

Oxidative stress is a term used to describe an imbalance between reactive oxygen species and the anti-oxidant capabilities of cells.

ROS are molecules and ions that contain oxygen. They are recognised as being highly reactive secondary to the presence of unpaired electrons, and examples include hydrogen peroxide (H_2O_2), the hypochlorite ion (OCl^-), the hydroxyl radical ($\cdot\text{OH}$) and superoxide anion (O_2^-) [257]. ROS have been implicated in numerous processes from ageing skin to cancers, however, more recently there is evidence pointing towards there being a balance of beneficial physiological signalling, and pathological processes [289]. Indeed, this has previously been considered in cartilage and an aptly

named review paper by Henrotin and Aigner in 2005 questioned their roles as “friends or foes” [257] with one of the defining factors appearing to be the amount of ROS.

To simplify the role of ROS, low levels are typically involved in cellular signalling, and high levels with apoptosis. ROS are created as a result of normal cellular metabolism, and are countered by the antioxidant effect of superoxide dismutases (SOD) and other cellular anti-oxidant strategies. At the point where ROS production is greater than the antioxidant effect, oxidative stress arises and this is considered to be a pathological catabolic event.

ROS and NO can react with tyrosine residues from nearby proteins to produce 3-nitrotyrosine (3-NT) [290, 291] although nitration can be reversed. 3-NT is a reasonably stable marker, remaining in tissues to serve as surrogate marker of reactions between NO and ROS. In isolation it simply provides an indicator of oxidative stress and an idea of levels of ROS mediated damage. Nitrotyrosine residues have been detected previously in OA cartilage [258]. Their presence provides evidence of ROS-induced damage, and nitric oxide dependent oxidative stress. Work by Hashimoto et al (1998) [278] demonstrated high levels of NO production in an animal model of OA. NO has previously been shown to induce apoptosis *in vitro* [123].

This study provides evidence of ROS having a potential role in cartilage degradation in AMG. Studies have shown that ROS are linked to apoptosis [123, 211, 244, 254, 257-259, 265, 292], and this study shows the percentage of positive staining follows a similar pattern to that of TUNEL staining, with the highest percentage of positive

cells in the most damaged cartilage. This percentage decreases as the cartilage becomes less damaged. Within each zone, the amount of positive staining is higher for 3-NT than TUNEL. There are a number of possible reasons why this might be the case. The antibody itself may be more sensitive than TUNEL, the tissue may be damaged but protects itself, and it is probable that 3-NT presence is a marker of a pre-apoptotic cell. In other words, the presence of 3-NT may indicate high levels of NO toxicity, and subsequent DNA or mitochondrial damage that forces a cell to begin the cascade of apoptosis. This raises the question if there is increased oxidative stress, and a resulting increase in 3-NT formation, is this reversible, and at what stage, or are these cells beyond the stage of 'recovery'?

In isolation, the presence of 3-Nitrotyrosine suggests ROS damage, however one would expect if that is the case then additional intermediate markers would be present, and we have shown evidence for the involvement of Bim. Bim expression has been shown to be regulated by oxidative stress and by redox sensitive FOXO family transcription factors [286, 293-295].

Forkhead box 3A (FOXO3a) is a transcription factor belonging to the forkhead group. They have been shown to be involved in numerous cellular processes across a wide range of physiological and pathological states. Its role in apoptosis is not fully understood, though upregulation has been shown to be associated with increased apoptosis in relation to several diseases [285, 296]. In neurons, it has been shown to induce expression of Fas ligand, triggering the death receptor pathway [297].

In musculoskeletal disease little work has been conducted regarding FOXO3a. Other papers have studied inflammatory arthropathy and found that FOXO3a was required

to prevent Fas-ligand induced neutrophil apoptosis [298, 299]. However these papers consider a neutrophil dependent inflammatory component (and Fas-mediated) of apoptosis. This is also predominant in synovium, and not in chondrocytes. In OA, studies generally have not observed Fas-induced apoptosis.

With regards to oxidative stress, FOXO3a presence has been shown to cause SOD upregulation [300], implying a protective role when under oxidative stress, however its role would appear to be much more complex than this. Other studies have shown that FOXOs upregulate Bim, which in turn can lead to intrinsic pathway apoptosis [301]. The reality is that FOXOs role differs in cell types, between diseases and probably has a changing role with ageing. In AMG, we have shown the presence of FOXO3a in cartilage of differing stages of disease. Levels were similar (no significant difference between regions T1-N) across regions potentially suggesting a complex role in both chondroprotection and apoptosis in a ROS rich environment, in conjunction with other pro and anti apoptotic signalling molecules. Its role has not clearly been defined and other family members, such as FOXO1 and FOXO4 may be equally involved. As discussed before, FOXO1 is now more clearly linked with Bim mediated apoptosis than FOXO3a but this information was not available at the time of this immunostaining study.

Figure 5-18 shows a proposed mechanism for apoptosis in AMG as suggested by the presence of markers in this study. The causative factors of OA initiation are poorly understood. In studies of 'end stage' OA, where cartilage degradation has begun, the joint is a pro-inflammatory environment [222]. There are a number of cytokines present which contribute to the disease process. In addition to the biological changes

within the joint, the mechanical properties of cartilage change [260] and weight distribution changes. These factors have shown to be present in the OA joint [302, 303], and also to stimulate over-production of ROS.

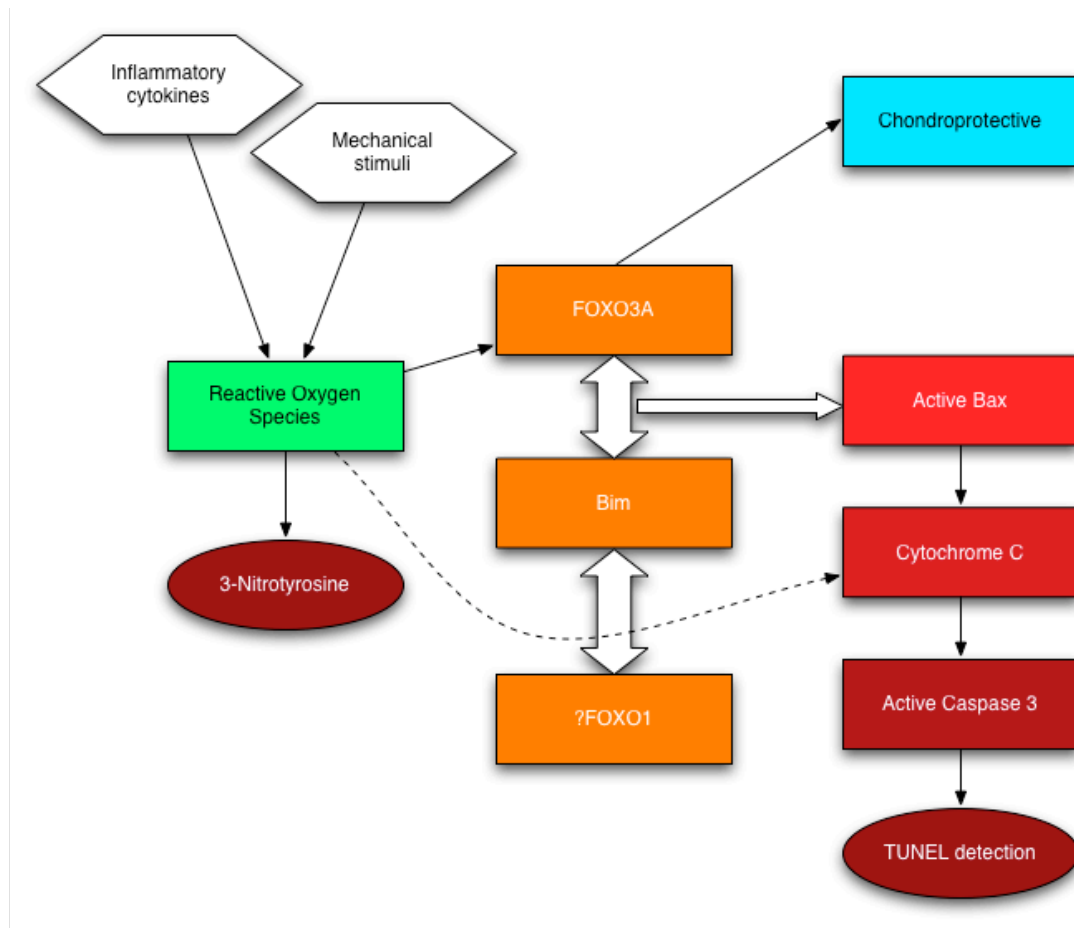


Figure 5-18 – A proposed mechanism for apoptosis in AMG of the markers identified in this study. Excess reactive oxygen species are present secondary to stimuli such as inflammatory cytokines and mechanical overload. Their presence is detected using 3-Nitrotyrosine. FOXO3a is activated though not detectably upregulated and launches an anti-oxidant protective response. ROS presence becomes excessive at some point so that the antioxidant threshold is exceeded and FOXO - probably FOXO1- causes upregulation of Bim, and subsequent activation of Bax. Bax causes permeability of the mitochondrial membrane, and Cytochrome C is released. Caspase 3 is activated and executes apoptosis, which is detected using TUNEL.

5.5.3 Diseased versus aged control samples

In comparing the AMG specimens to AKA specimens, this study sought to address the question of firstly whether an earlier stage of disease (the undamaged cartilage of

AMG) was related to apoptosis, and secondly if, and how much, ROS were detected, in the globally less damaged cartilage of AKA specimens, and its localisation.

The AKA specimens were methodically divided into the same regions of AMG (T1-N) and a further region X, corresponding to the area of the lesion in AMG. In AKA specimens, the earliest signs of damage TUNEL levels were low throughout all regions (less than 4% for regions T1-N), but at 6.9% were significantly higher in region X. The same was true for 3NT, the implication of this being that the area that corresponds to the region of AMG where cartilage damage is initiated exhibits higher levels of ROS-mediated damage. Both AMG and AKA samples demonstrated no significant difference of the presence of FOX3A across regions or between specimens with a median of 33%. There is a limited understanding of the complex interaction of ROS, FOXO3A and the factors that may induce cells on a pathway to apoptosis. In the AKA samples, the implication of similar levels of FOXO3A, but significantly less apoptosis may suggest that it has a chondroprotective role. One of the best previously studied interaction is that of Sirt1 and FOXO3A where the interaction was shown to confer cell resistance to oxidative stress and apoptosis, however in the presence of high levels of ROS, it can induce apoptosis [292]. It would appear from this study that the FOXO3A-Bim plays a role in this progression, with much lower levels of Bim found in AKA specimens when compared to AMG. One important factor to consider in comparing AKA to AMG tissue is that the AKAs were from an older population, in whom one would expect there to be higher levels of ROS, as implicated by its process in ageing. Further *in vitro* work is required to better understand the interactions of FOXO3A and pro and anti apoptotic signals in the progression of cells onto apoptosis and their role in redox systems in the initiation and progression of OA.

5.6 Summary

The presence of markers of apoptosis provides evidence for it playing a key role in cartilage degeneration. The nature of the model of AMG allows for the study of disease progression in regions of cartilage at different stages of disease. It has clearly been demonstrated that in more diseased cartilage that apoptosis level are higher, as are the markers associated with reactive oxygen species, implicating this process in the evolution of cartilage degradation. This study has shown evidence for apoptosis and the involvement of reactive oxygen species. There are little previous data in the literature available on the presence of the markers studied in this chapter in human cartilage that is not diseased. It has been demonstrated that aged, but macroscopically normal cartilage demonstrates low levels of apoptosis and ROS mediated damage, but a similar levels of FOXO3A compared to OA cartilage. Perhaps the most useful outcome of this work is the positive 3-nitrotyrosine staining in AKA patients who have normal but elderly cartilage, seen in zone X, the region of maximum future damage were they to develop AMG. This highlights the potential role of ROS in disease initiation and suggests that other useful biomarkers of very early disease/damage may be ROS scavengers such as SOD, that may already be upregulated and protecting the cartilage.

The intrinsic pathway of apoptosis has been studied in more depth in other tissues and disease processes and further work is required to define its role in chondrocytes and cartilage degradation. If, indeed, the pathways of cell death can be studied, manipulated, and even reversed, *in vitro*, then further work into the field of anti-apoptotic treatment may yield potential treatment strategies [304].

Chapter Six

6 STUDY 4 - A MICROARRAY GENE EXPRESSION STUDY OF NORMAL VERSUS DAMAGED CARTILAGE IN ANTEROMEDIAL GONARTHROSIS

6.1 Introduction

Previous chapters have demonstrated several distinct regional variations within AMG specimens. Chapter two, in particular, highlighted gene expression changes between the areas of damaged and undamaged cartilage in AMG and chapter four compares the same regions in aged AKA samples where these gene expression differences were not found.

Chondrocytes have been shown to undergo phenotypic change as part of the osteoarthritic disease process [63]. Many studies have used quantitative real time polymerase chain reaction assays (qPCR) to investigate several genes of interest at once, but with our increasing knowledge of gene expression, interactions and pathways comes the desire to study a wider range of genes simultaneously.

Microarray technology provides a powerful tool to investigate global gene expression changes, and though used widely in neoplastic disease, has been used much less in orthopaedics and human cartilage biology [186, 305-309]. It provides the opportunity to study the differential expression of tens of thousands of genes in a single experiment. Its use, to date, in cartilage biology has been in animal studies [310, 311], injured cartilage explants [305] and one study has considered damaged versus intact

human cartilage within the same knee joint of OA patients [306]. One of the reasons why microarray studies on human cartilage may be relatively limited is the difficulty in obtaining a high enough yield of RNA, of suitable quality.

AMG provides a topographically reproducible pattern of disease in which regions of damaged and undamaged cartilage can be sampled within the same joint surface and compartment. In focussing on AMG, the aims of this study were to characterize areas of cartilage at different stages of damage within this phenotype, in terms of their gene expression. Chapter 2 demonstrated differences in type I collagen expression and MMPs/ADAMTSs, and this microarray aimed to investigate collagen expression and breakdown, and also highlight a number of genes and pathways of interest for future work.

6.2 Method

6.2.1 Sample collection and preparation

Ten tibial plateau resection specimens were collected from patients undergoing UKA for AMG (Ethics Reference C01.071) at the Nuffield Orthopaedic Centre, Oxford.

Regions were defined, cartilage was harvested and RNA extraction was undertaken as described previously (Section 2.3). A further sample from each specimens' damaged and undamaged regions also underwent extraction, and was retained for later qPCR analysis and microarray validation. Samples were stored in a -80°C freezer.

Sample number	Gender	Date of Birth	Age	Side
1	M	02/03/1964	54	R
2	F	06/07/1938	70	R
3	F	26/05/1957	51	R
4	M	17/01/1944	64	R
5	F	11/04/2027	81	L
6	F	16/09/1948	60	L
7	M	10/09/1944	64	R
8	F	07/05/1946	62	R
9	M	04/05/1939	69	L
10	F	17/12/1937	71	L

Table 6-1 - Demographics of AMG specimens used in this study. (M – Male, F – Female, R – Right, L – left).

AMG samples used in previous studies [93] had remaining paraffin embedded tissue sections in storage and these were used for immunohistochemistry validation studies.

6.2.2 RNA quantification and preparation

RNA yield and quality was determined using an Agilent Bioanalyzer (Agilent Technologies, California, USA) and Nanodrop Spectrophotometer (Nanodrop Technologies, Delaware, USA).

The RNA integrity number [312, 313] is a measure of RNA quality and has been used in the study of other tissues (for example breast [314], brain [315] and blood [316]. Previous published microarray studies in cartilage appear to omit this data; for this study, it is provided below. Of note, Agilent's online database does not have data for two matrix tissues, cartilage and tendon, both recognised as being challenging sources from which to retrieve high quality RNA [317].

Due to the variable amounts of starting RNA, the amount used for each pair of samples was matched to that of the lower concentration sample in each pair. Prior to labelling, the samples were concentrated to 8.3µl in a rotary evaporator.

6.2.3 Microarray

Each pair of samples was combined and prepared as per Agilent's Two-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) protocol (version 5.7) [318]. The RNA was then fragmented at 60°C for 30 min and the reaction was stopped by the addition of 2x Agilent Hi-RPM hybridisation buffer. The samples were then loaded on to the arrays and hybridised at 65°C for 17 hr at 10 RPM. The slides were then washed and scanned as per Agilent's protocol using Agilent's stabilisation and drying solution, without using TritonX supplemented wash buffers.

6.2.4 Immunohistochemistry and qPCR

The quality of gene expression data obtained from microarray can vary greatly depending on the platforms and methods used; qPCR is commonly used as a validation tool for confirming gene expression data [319]. It is also of interest to validate gene expression data with the presence of proteins therefore, for the purposes of validation, a few proteins / genes of interest were chosen. The following genes were chosen for immunohistochemistry; CCL3, FGF18, and TNFSR11 (OPG). The following genes were chosen for qPCR and paired samples were used: TNFSR11 (OPG), SOX11, IGF1 and FGF18.

The methods used are described in Appendix 3 (immunohistochemistry) and section 2.3 (qPCR). These genes were included amongst the top hits of the microarray and chosen because of previous documented links to OA. Previous work surrounding these genes is cited in the discussion section.

6.2.5 Statistical Analysis

6.2.5.1 *Microarray*

Normalisation is the process of adjusting microarray data for effects arising from variation in the technology, in particular the variation in dye balance [320]. This was carried out using Agilent Feature Extraction 9.5.3, which applied a global Lowess normalisation to remove systematic errors resulting from the fluorescence profiles of the two dyes. The normalised fluorescence data was analysed using GeneSpring GX 10 (Agilent Technologies) to identify genes with significantly differing expression.

As all samples were paired, genes of interest were identified by applying a Wilcoxon signed rank test with the null hypothesis of there being no difference in expression in the undamaged and damaged tissue types across the samples. Significance was set at the $p < 0.01$ level.

Pathways analysis was undertaken using Ingenuity software (Ingenuity Systems, California, USA)

6.2.5.2 *Immunohistochemistry*

Immunohistochemistry was performed on a further set of AMG specimens that had been well characterised for histopathology and Mankin grading (Chapter 1). These samples had paraffin embedded blocks stored at the time of these previous experiments. These were assessed qualitatively from representative photos of immunostained damaged and undamaged regions.

6.2.5.3 *Real time PCR*

Each gene was assessed using the standard curve method [170], and normalised to 18S housekeeper as described in Chapter 2. Because the samples for each gene of

interest were two regions matched from the same individual, paired statistics were used. A Wilcoxon test was performed using PASW Statistics 18.0 (IBM, Illinois, USA). Significance was set at the $p < 0.01$ level.

Statistical advice was sought and advice given to increase the stringency of the p value and not apply a correction (see discussion, section 6.4).

6.3 Results

6.3.1 Samples

Ten AMG samples were collected between May and July 2008. The median age was 64 (range 54-81). Six were right knees, four were left; six were female, and four were male. All of the specimens demonstrated the characteristic pattern of AMG macroscopically. No power calculation was undertaken. Previous published microarray work by other groups have used a similar number of samples [305, 321, 322], with the purpose of this technical approach being to highlight potential genes/pathways for further analysis.

6.3.2 Quantity / Quality

The median RNA sample concentration was 18.06 ng/ul (range 8.14-30.11). The median 260/280 ratio was 1.9 (range 1.64-2.08). The 260/280 ratio is a spectrophotometric analysis of sample purity of nucleic acids. A complete set of values is shown in Table 6-2.

<u>Sample ID</u>	<u>Concentration (ng/ul)</u>	<u>A260</u>	<u>A280</u>	<u>260/280 ratio</u>
1d	8.14	0.203	0.108	1.89
1u	16.21	0.405	0.194	2.08
2d	17.14	0.428	0.217	1.97
2u	28.13	0.703	0.376	1.87
3d	12.35	0.309	0.188	1.64
3u	15.24	0.381	0.225	1.69
4d	9.072	0.227	0.12	1.90
4u	13.11	0.328	0.17	1.93
5d	19.72	0.493	0.249	1.98
5u	20.42	0.510	0.257	1.98
6d	20.53	0.513	0.297	1.73
6u	14.12	0.353	0.226	1.56
7d	23.4	0.585	0.313	1.87
7u	17.09	0.427	0.225	1.90
8d	22.49	0.562	0.286	1.97
8u	30.11	0.753	0.442	1.70
9d	8.04	0.201	0.104	1.93
9u	19.54	0.489	0.262	1.86
0d	25.38	0.634	0.318	1.99
0u	18.98	0.474	0.275	1.73

Table 6-2 – Table showing the samples used for the microarray analysis. Each samples' concentrations and quality data is presented. 'd' refers to damaged cartilage, 'u' to undamaged cartilage. Quantification and quality assessment (260/280 ratio) was undertaken using a Nanodrop spectrophotometer. The 260/280 ratio is a measure of nucleic acid purity.

The median RIN was 7.3 (range 6.5 – 8.8) as shown in Table 6-3.. An example of the data output from the Agilent bioanalyser is included in appendix 4.

<u>Sample ID</u>	<u>RIN</u>
1d	7.5
1u	6.5
2d	7.1
2u	6.5
3d	6.9
3u	6.9
4d	7.0
4u	6.5
5d	7.9
5u	7.0
6d	8.8
6u	7.2
7d	7.1
7u	7.1
8d	7.0
8u	7.0
9d	7.8
9u	8.1
10d	8.0
10u	8.1

Table 6-3 – Table demonstrating RNA integrity number (RIN) for each of the samples used in the microarray. The median value was 7.3 (range 6.5 – 8.8)

6.3.3 Microarray

Initial analysis showed that one sample (4u) had clear errors which were likely to be due to the low RNA yield; therefore this sample was excluded from the analysis.

Boxplots of the expression log ratios for the filtered probes show acceptable consistency across the slides used as shown in Figure 6-1.

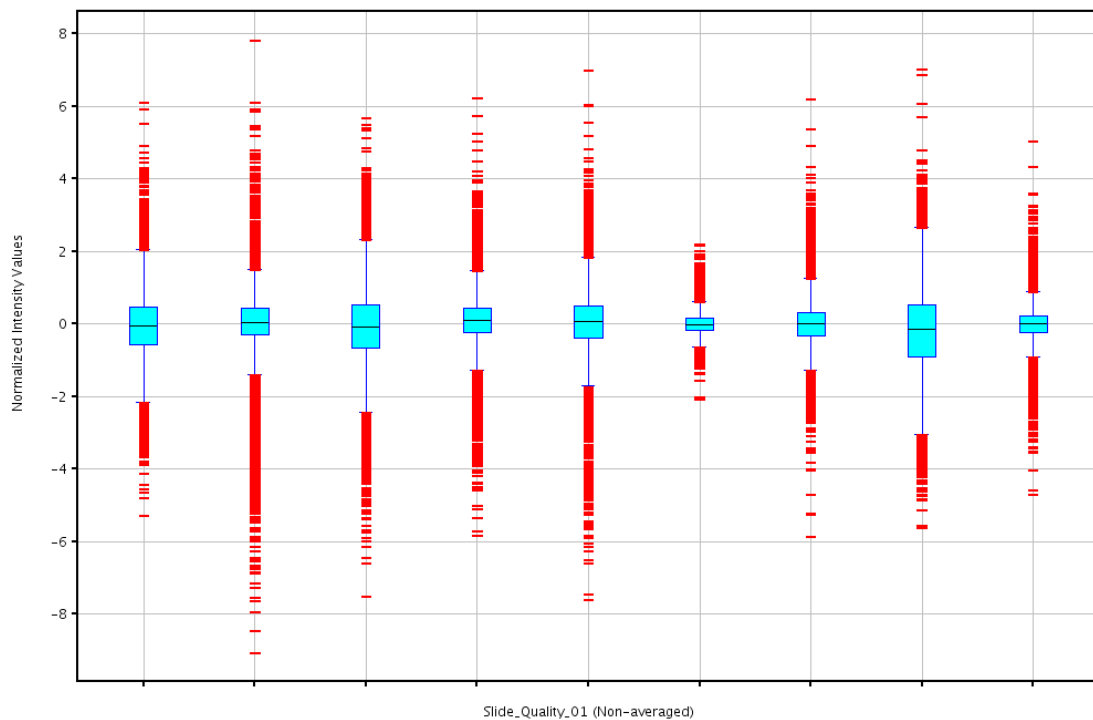


Figure 6-1 – Boxplots of expression log ratios for the filtered probes by sample. Samples are, from left to right, 101-103, 105-110.

In preparation for identifying genes of interest, control samples were removed and probes with few readings above background levels were excluded. Use of a Wilcoxon test yielded a list of 389 genes that were found to be significantly ($p < 0.01$) differentially expressed by a fold change of at least 2 between tissue types. This consists of 197 genes found to be expressed at a higher level in the damaged tissue than the normal tissue and 192 genes found to be expressed at a lower level in the damaged tissue compared to the normal tissue.

The list in Table 6-4 shows the top 50 genes listed by fold change order in damaged relative to undamaged regions. The full list of these genes is provided as supplementary material in Appendix 5.

Gene	P value	Up/Down regulation	Fold change
IL11	0.008	up	15.866
LAMB1	0.008	down	15.274
GPNMB	0.008	down	9.535
ENST00000357303	0.008	up	8.754
SOX11	0.008	up	8.662
COL14A1	0.008	down	8.283
DNER	0.008	up	8.145
C15orf48	0.008	up	7.880
PDE1A	0.008	down	7.879
MMP2	0.008	down	7.211
PCSK1	0.008	down	6.841
SOX11	0.008	up	6.683
IFI44	0.008	down	6.530
RFTN1	0.008	down	6.353
GFRA2	0.008	up	6.193
CXCL14	0.008	up	6.168
PLXDC1	0.008	down	6.016
GALNTL1	0.008	up	5.977
SERPINF1	0.008	down	5.824
BAALC	0.008	up	5.595
CDH11	0.008	down	5.505
OLFML3	0.008	down	5.471
MMP9	0.008	down	5.427
GPR124	0.008	down	5.277
HAVCR2	0.008	down	5.267

Gene	P value	Up/Down regulation	Fold change
AY358804	0.008	down	5.258
TWIST1	0.008	down	5.163
IGF1	0.008	down	5.092
CCL2	0.008	down	5.032
BST2	0.008	down	5.028
FAM20A	0.008	down	5.011
OLFML3	0.008	down	4.976
GFRA2	0.008	up	4.958
ACOX2	0.008	down	4.947
LEF1	0.008	down	4.939
IL13RA2	0.008	down	4.885
MAN1C1	0.008	down	4.783
PLCL2	0.008	down	4.704
FGF18	0.008	up	4.685
WISP2	0.008	down	4.550
CASP1	0.008	down	4.535
CCL3	0.008	down	4.497
CRISPLD1	0.008	down	4.464
S100A2	0.008	up	4.412
THC2540738	0.008	down	4.391
AYTL1	0.008	down	4.346
VIT	0.008	down	4.345
THC2668815	0.008	up	4.245
EBF3	0.008	down	4.237
CLIC3	0.008	up	4.233

Table 6-4 – Table listing the highest ranked fifty genes based on most statistically significant. All genes had significance of p=0.008, and are listed in decreasing order of up (green) / downregulation (orange) in damaged cartilage relative to undamaged cartilage.

A search was then made for Gene Ontologies (GO groups). The GO project is one which aims to describe gene products in terms of their associated biological processes, cellular components and molecular functions [323]. A search was run to find GO groups enriched within the list of genes ($p < 0.01$). This led to the identification of 67 GO groups which are shown in Appendix 5.

A number of pathways were revealed, in particular:

1. Cellular development, Skeletal and muscular system development and function, Lymphoid tissue structure development
2. Inflammatory response, cell death, haematological disease
3. Skeletal and muscular disorders, cell signalling, small molecule biochemistry

Grey shaded proteins are positive hits; white are genes that were not significantly different. The pathways are shown in Figure 6-2, Figure 6-3, and Figure 6-4 and the legend showing what each represents is presented below each diagram.

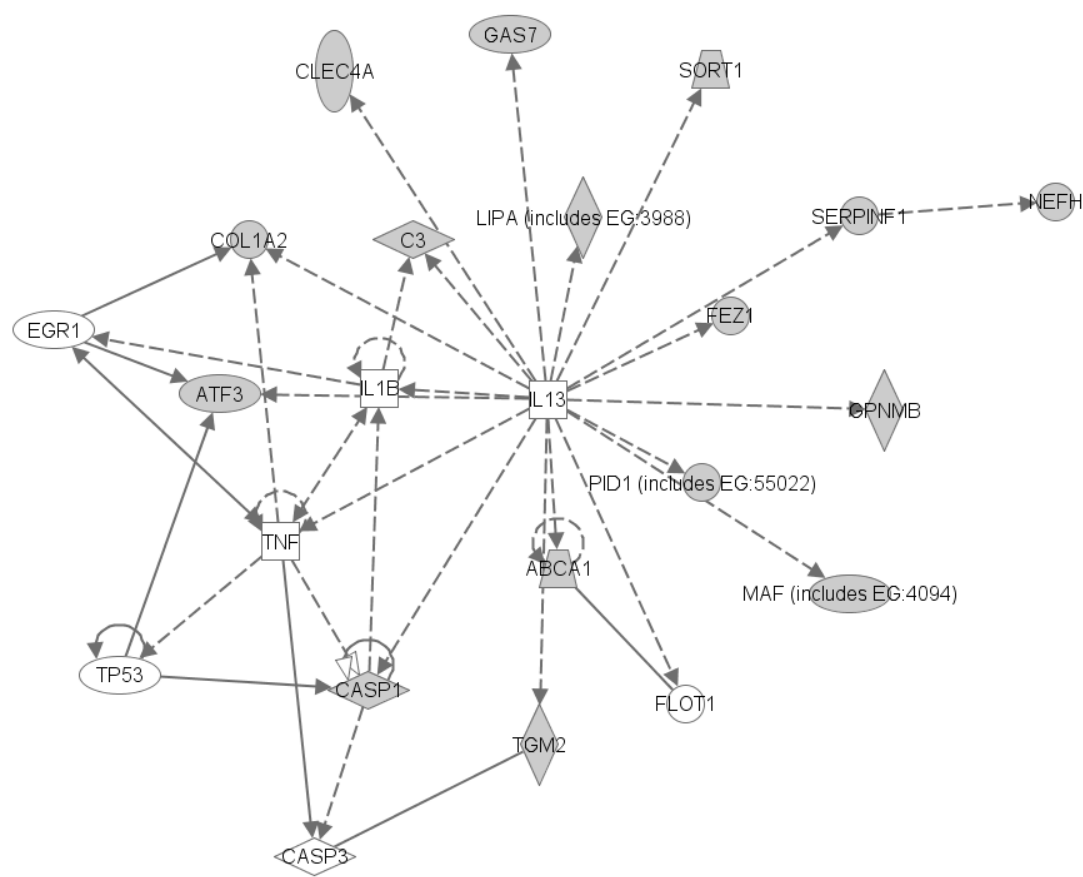
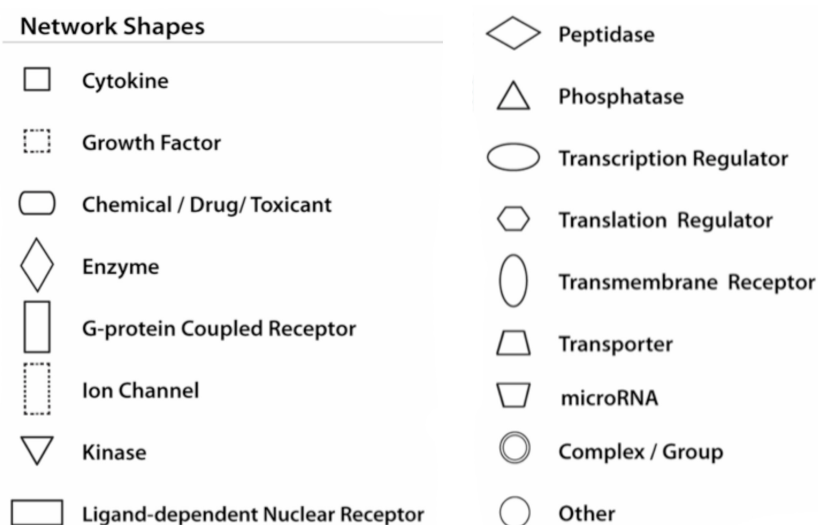


Figure 6-2 – Pathways analysis - Cellular development, Skeletal and muscular system development and function, Lymphoid tissue structure development. Grey shapes showed significant differences in gene expression. Shape legend below.



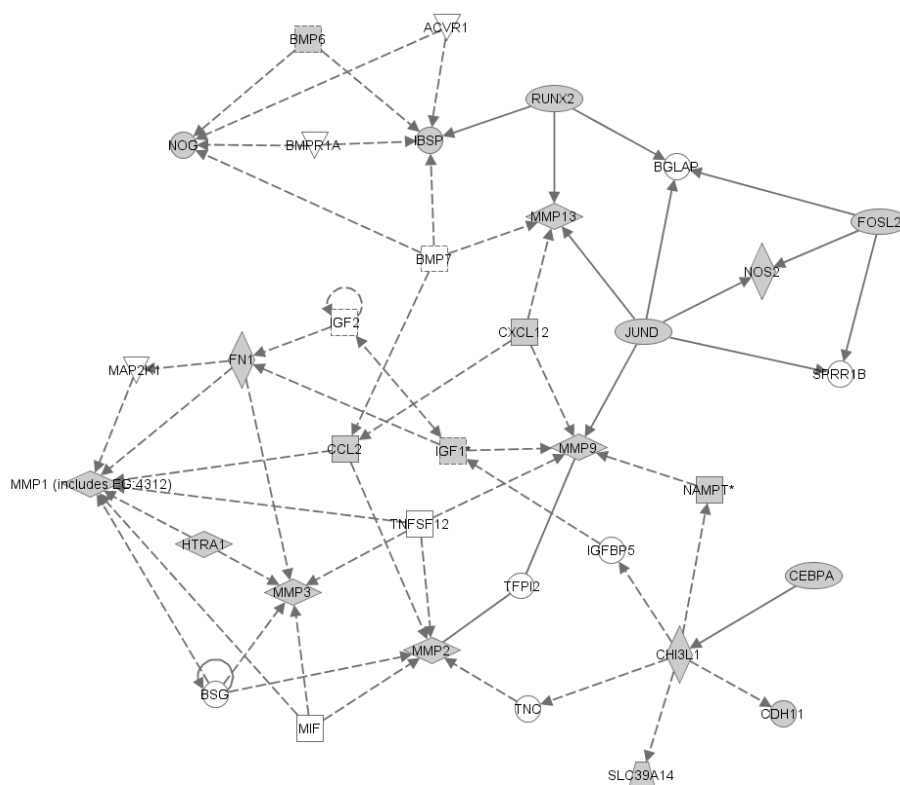
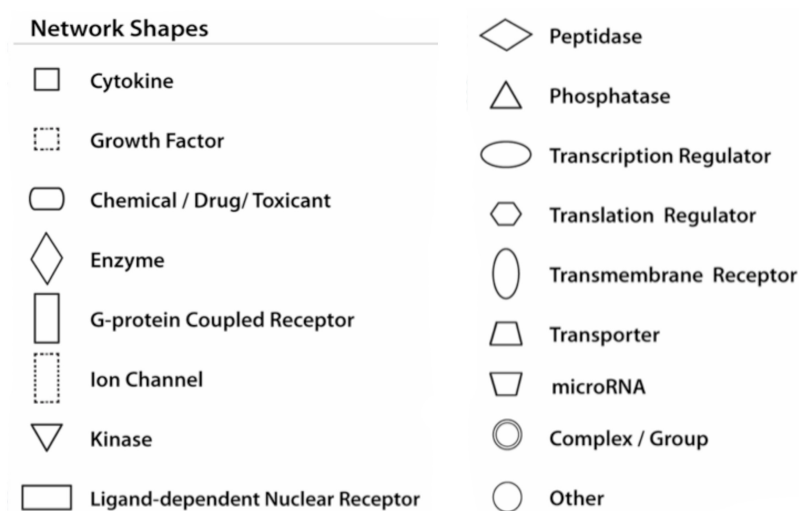


Figure 6-3 – Pathways analysis - Inflammatory response, cell death, haematological disease. Grey shapes showed significant differences in gene expression. Shape legend below



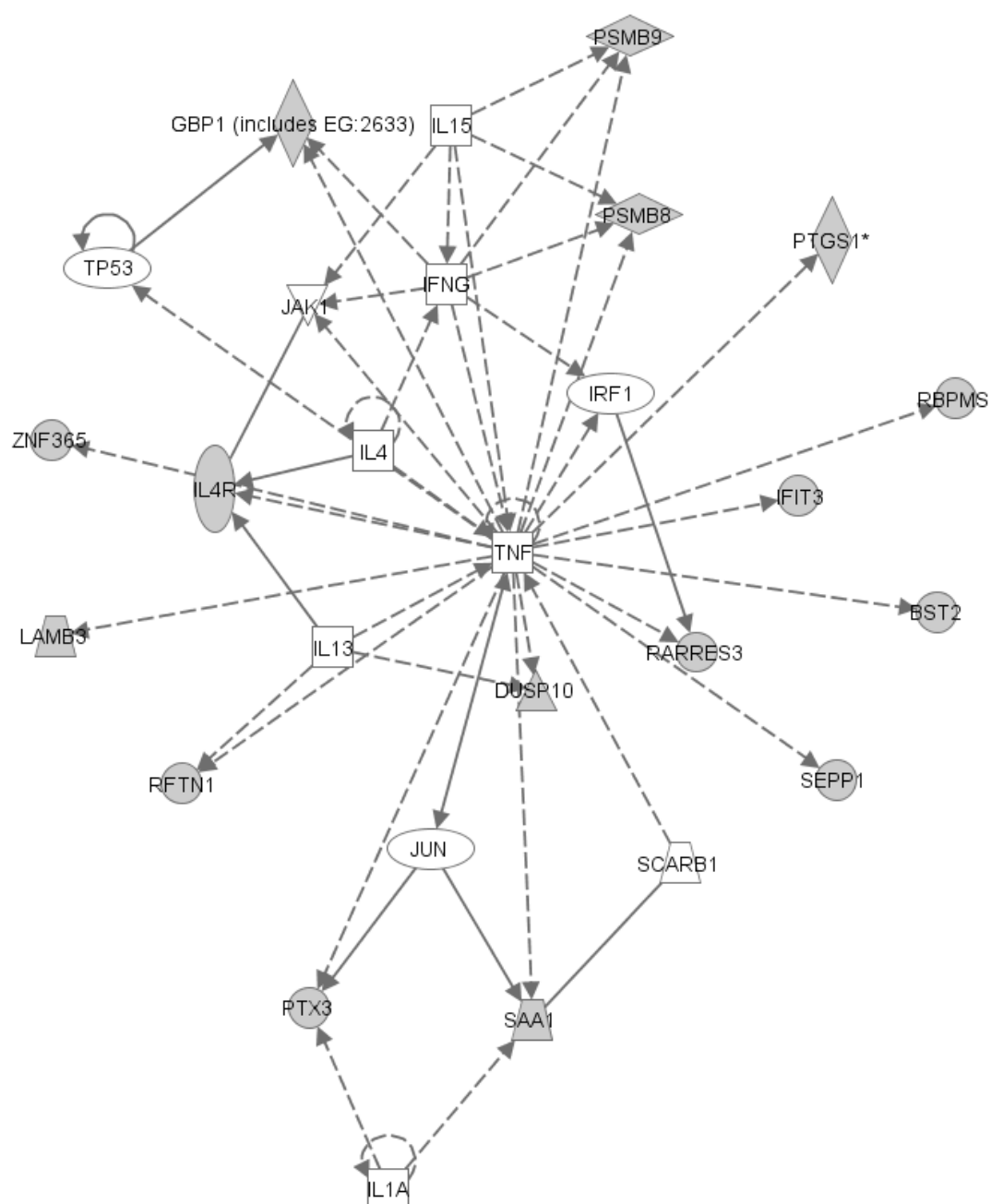
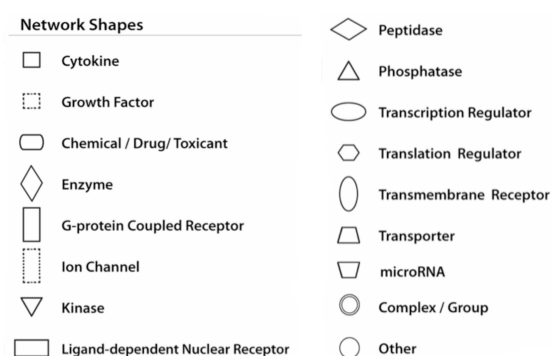


Figure 6-4 - Pathways analysis - Skeletal and muscular disorders, cell signalling, small molecule biochemistry. Grey shapes showed significant differences in gene expression. Shape legend below.



6.3.4 Immunohistochemistry

Confirming the microarray data at the protein level, the following proteins were found to be present in greater amounts in undamaged compared to damaged cartilage: FGF18, and OPG (Figure 6-5). CCL3 was present in greater levels in damaged cartilage (Figure 6-6), the opposite direction to the microarray.

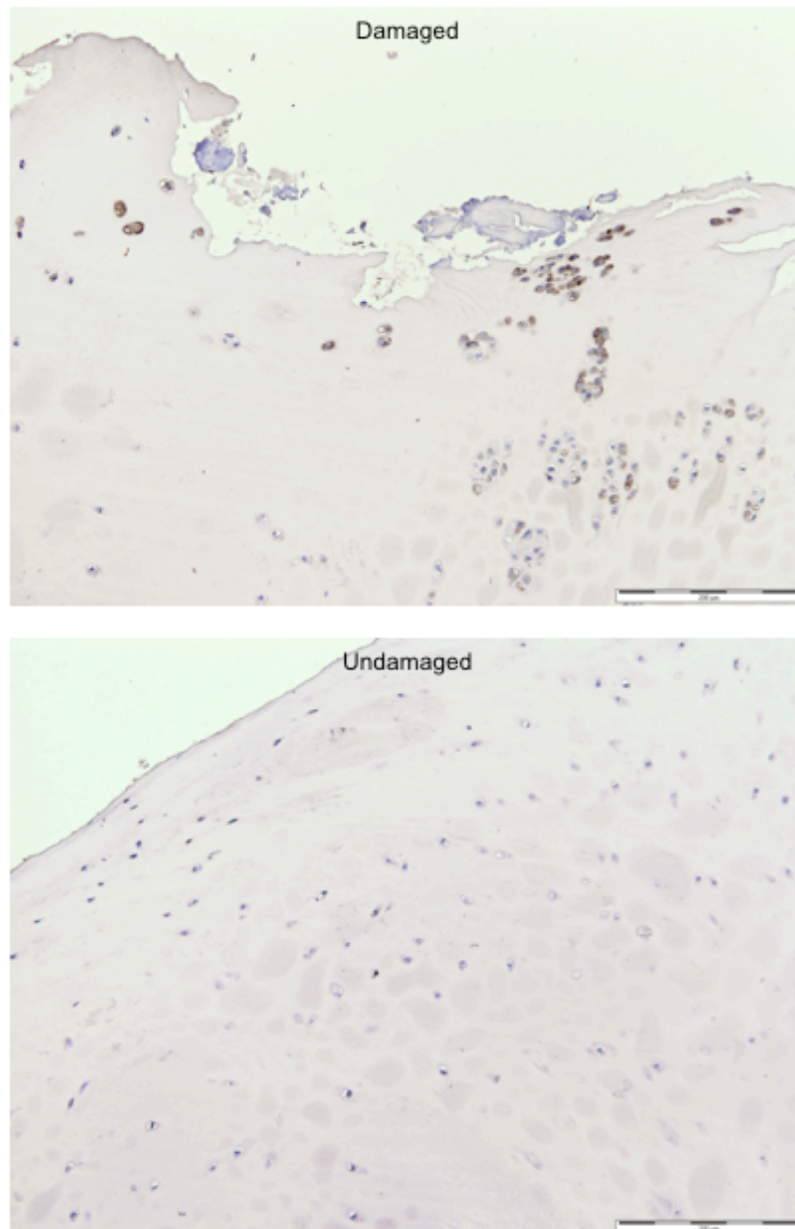


Figure 6-5 - Immunohistochemistry picture of FGF18 presence in damaged and undamaged cartilage. Positive cells were demonstrated in both regions and there was a higher percentage of positive cells present in undamaged cartilage. This matched the microarray finding of increased CCL3 gene expression in damaged cartilage.

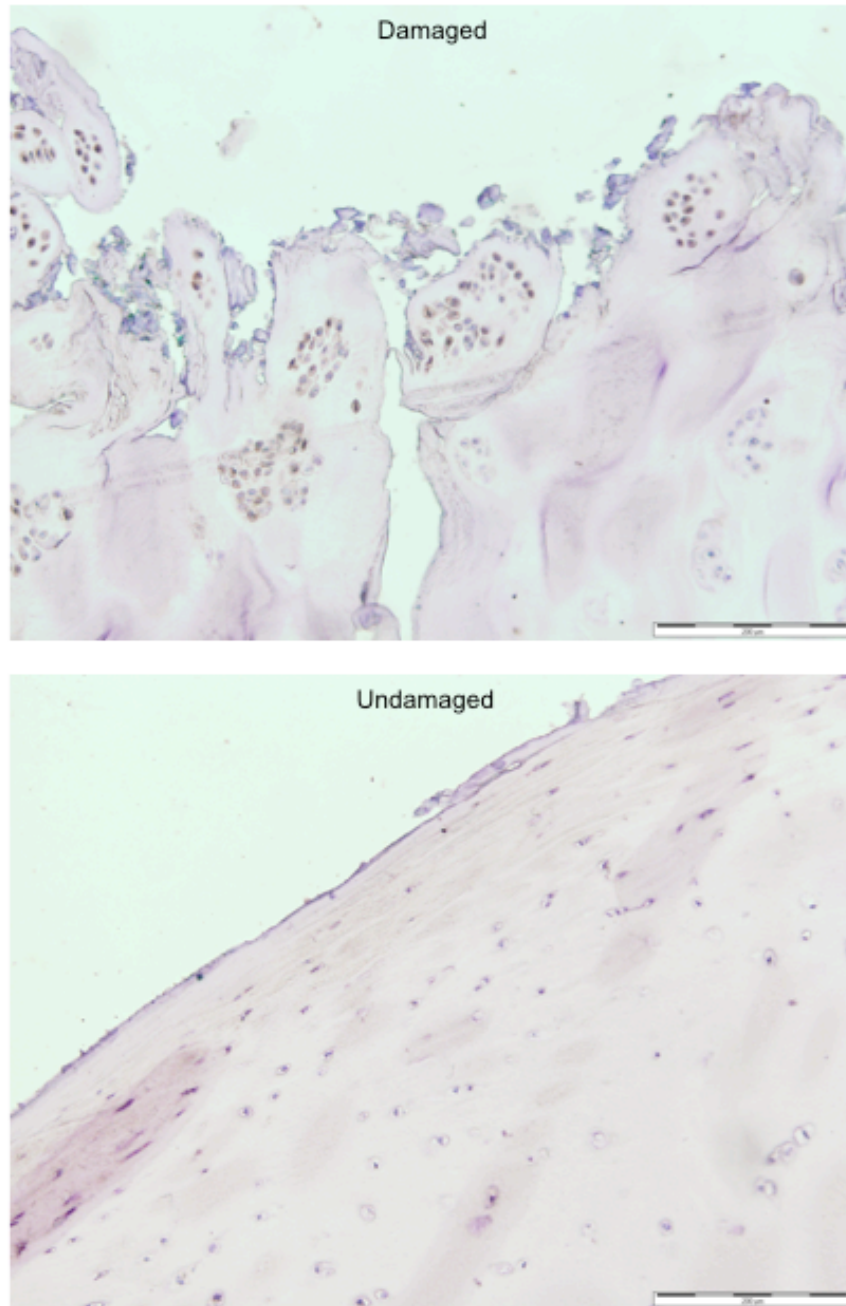


Figure 6-6 – Immunohistochemistry picture of CCL3 presence in damaged and undamaged cartilage. Positive cells were demonstrated in both regions and there was a higher percentage of positive cells present in damaged cartilage. This did not match the microarray finding of increased CCL3 gene expression in undamaged cartilage.

6.3.5 Real Time PCR validation of the Microarray results

qPCR was performed on a selected subset of genes to validate the array data, SOX11, IGF1, OPG and FGF 18. The qPCR Analysis showed significant differences in gene expression within the mirrored samples for the following genes; SOX11 (6.7 fold higher in damaged ($p=0.043$)), IGF1 (5.2 fold lower in damaged ($p=0.043$)) and OPG (6.9 fold higher in damaged ($p=0.043$)). The changes for SOX11 (Figure 6-7), IGF1 (Figure 6-8), and OPG (Figure 6-9) displayed the same pattern as the microarray. There was no significant difference for FGF18 gene expression ($p=0.08$) (Figure 6-10).

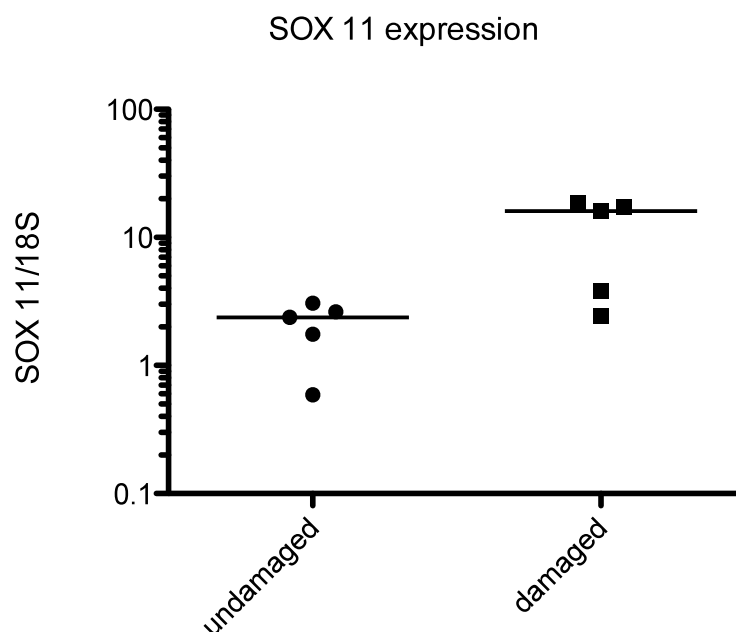


Figure 6-7 - Gene expression of SOX11 in undamaged and damaged cartilage. There is a 6.7 fold higher expression of SOX11 in damaged cartilage ($p=0.043$, Wilcoxon).

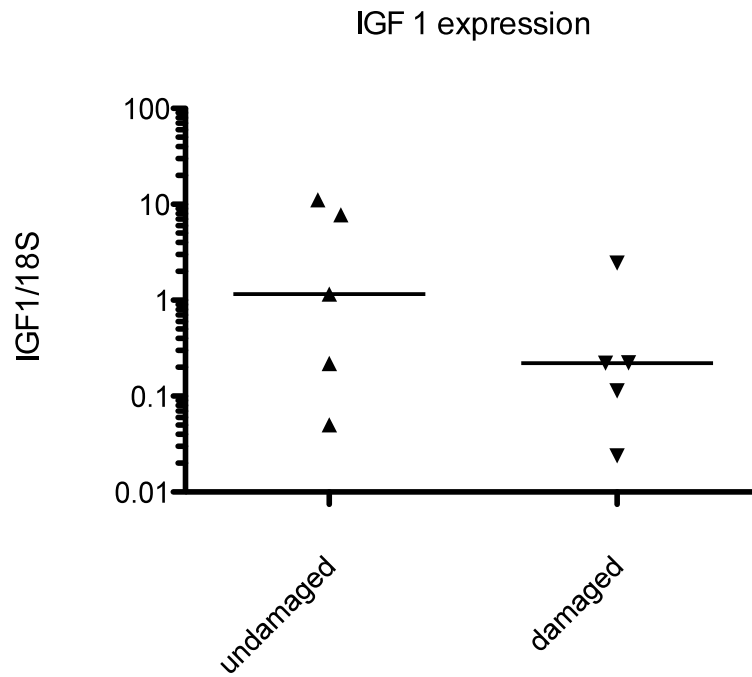


Figure 6-8 - Gene expression of IGF1 in undamaged and damaged cartilage. There is 5.2 fold lower expression of IGF1 in damaged cartilage ($p=0.043$, Wilcoxon).

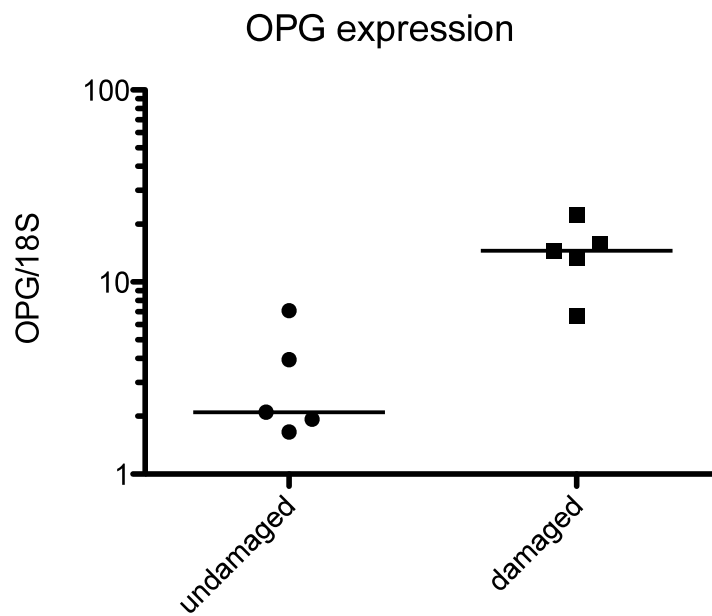


Figure 6-9 - Gene expression of OPG in undamaged and damaged cartilage. There is 6.7 fold higher expression of OPG in damaged cartilage ($p=0.043$, Wilcoxon).

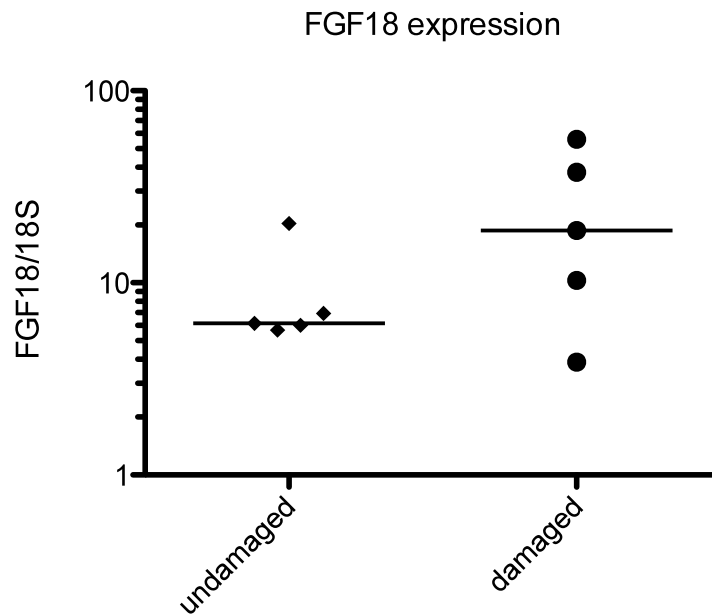


Figure 6-10 - Gene expression of FGF18 in undamaged and damaged cartilage. There is no statistical difference in expression between undamaged and damaged cartilage ($p=0.08$, Wilcoxon).

Further validation is also noted from qPCR results from Chapter 2 which used the same regions from matched AMG samples (section 2.4). These results corroborate microarray results with significant differences in expression of MMP1 - 1.88 fold change ($p=0.046$); MMP 3 - 3.68 fold change ($p=0.028$); MMP13 - 4.63 fold change ($p=0.028$) and COL1A1 - 8.8 fold change ($p=0.028$) (Figure 6-11) (increased in the undamaged cartilage of AMG in all cases).

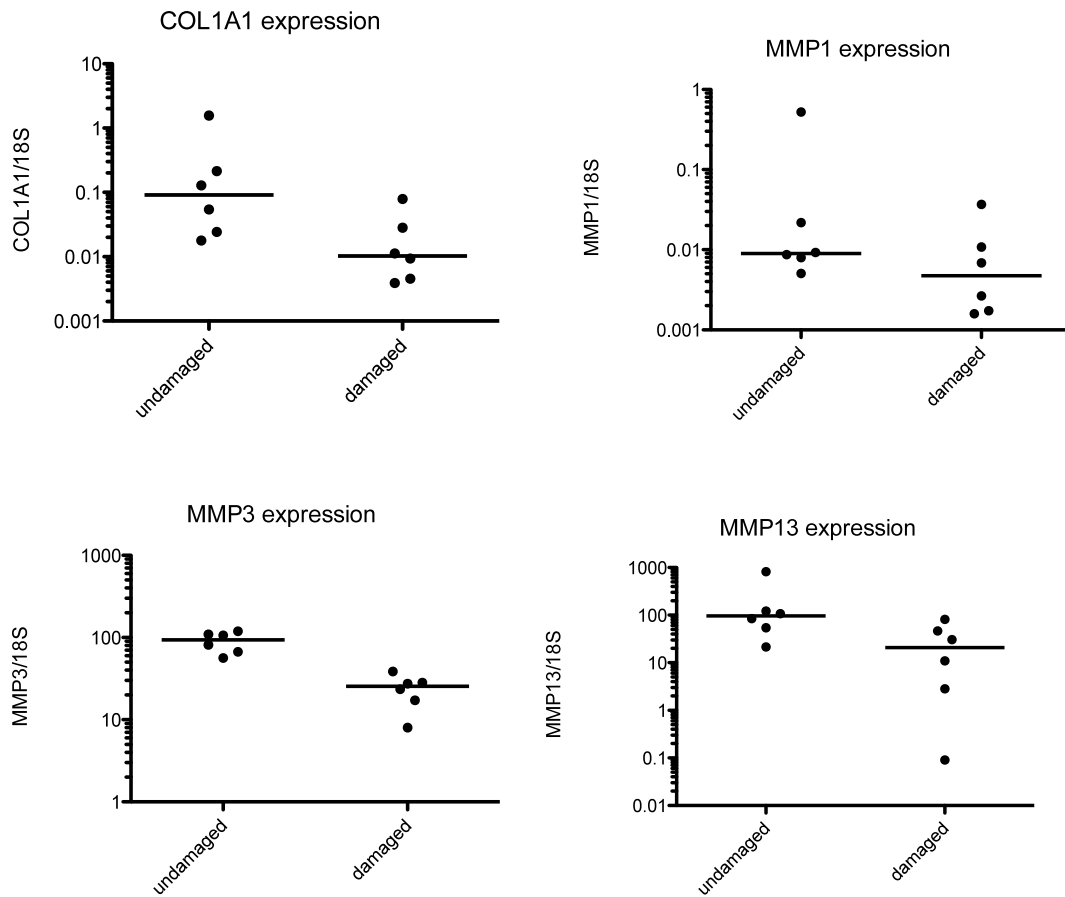


Figure 6-11 - qPCR results showing statistically significant differences for COL1 ($p=0.028$), MMP1 ($p=0.046$), MMP3 ($p=0.028$) and MMP13 ($p=0.028$) comparing expression in damaged and undamaged cartilage.

6.4 Discussion

This study is the first to describe the detailed gene expression changes between undamaged and damaged cartilage within a specific phenotype of OA and within the same specimen, in other words a direct comparison of gene expression changes with the same genetic background. It has highlighted a number of interesting differences across a genome wide microarray.

This study stemmed from previous gene expression work that highlighted differences between the undamaged and damaged cartilage of AMG (see Chapters 1 and 2). Some of these changes were unexpected and thus raised the question of what might be happening within these regions of cartilage on a wider level of gene expression. Some of the areas of particular interest raised were the collagens and collagen degrading enzymes coupled with other changes of the extracellular matrix. Other questions include the role of the bone-cartilage interface, and bone relevant factors being expressed (for example WNTs, BMPs, OPG), and pro-inflammatory cytokines, those that might alter protein expression. Ultimately, microarray studies are only partially hypothesis driven, with the overall aim being to reveal new pathways or novel targets that may lead to therapeutic interventions.

AMG is a distinct phenotype in which cartilage becomes damaged in a predictable manner. It accounts for up to 60% of individuals presenting with OA of the knee. Resection specimens have shown [91, 324] that it exhibits a repeatable pattern of disease that can be considered to be a spatial model of OA disease progression. This has also been demonstrated histologically using grading systems [93]. We highlight a number of gene expression changes between damaged and undamaged cartilage, and

these may shed light upon the mechanisms involved in cartilage homeostasis and degradation in OA. A particular strength of using AMG specimens is the ability to compare cartilage regions from the same individual, thus removing confounding factors related to drug treatments, activity, environment and genetics. Additionally the reliability with which damaged and undamaged cartilage can be distinguished allows delineation of gene expression differences that may be missed when excising cartilage *en masse* for comparison of OA and non-OA cartilage.

There are no published figures of RIN values in previous qPCR or microarray studies of cartilage. The quality of the RNA extracted from cartilage for this study was satisfactory when compared to other tissues that have been cited in the literature.

One of the difficulties in analysis of microarray results is addressing the vast amount of data generated. The complete data set of upregulated and down regulated gene expression and well as differences in GO group data is provided as appendices (Appendix 5).

In terms of assessing this data statistically, paired, non-parametric tests were used to compare undamaged and damaged regions (Wilcoxon). *Post hoc* adjustments were not made. This increases the likelihood of a Type I error (false positive), but most corrections (for example, Bonferroni [325] are too stringent, and statistically questionable [325, 326]). By not applying a correction, there is an increased risk of a Type II error (false negative), however the purpose of this study was identification of genes for further work. In genome wide association studies the threshold for reporting significance is lowered; in this study significance was lowered to $p < 0.01$ level, and other methods of reporting were also utilised: significance of GO groups of genes and

pathways analyses, as well as validation of selected results with qPCR and immunoassays.

As this is a comparative study of damaged and undamaged cartilage, the results can be interpreted as either higher or lower in each region of cartilage. For example, a decreased level of expression in damaged cartilage can also be read as an increased level of expression in undamaged cartilage. For ease of use, all the data here is referred back to the undamaged regions. However, note should be made that some proteins are over expressed in undamaged regions, for example type I collagen, suggesting that the normal regions are not necessarily stable and one cannot assume that the majority of dysregulation happens in the damaged cartilage.

Contrary to the results from other groups which have suggested higher numbers of genes displaying increased expression in OA cartilage [322], this study demonstrates a similar number of higher and lower expressed genes (197 and 192 respectively) in damaged cartilage. This can partly be accounted for by the more distinct phenotype of OA studied here as well as the specific geographical regions studied. Additionally, in the majority of other reports, OA samples have been compared to non-OA or early-OA samples from different individuals, and containing both damaged and undamaged cartilage from these joints. Thus the regions defined as “normal” in our study may well form the majority of analysed cartilage in other studies, such that an increased number of overexpressed genes is found to be present when compared to non-OA specimens.

The main aim of this study was to profile expression of a large number of genes with a view to identification of known and novel pathways and candidate genes worthy of a more in depth analysis.

6.4.1 Matrix related genes

Genes of interest include those of matrix components and enzymes indicative of matrix turnover. Several matrix related genes and pathways express higher or lower regulation in AMG cartilage regions. The impact of these changes may influence the loading environment and physical interaction between cells. In addition to the collagens and enzymes discussed in detail below, other matrix related genes showing differential expression include CLIP, NTN1, COMP and FMOD.

The collagen gene included in Table 6-4 above is COL14A1. This gene has only recently been identified in two studies as being implicated in cartilage in OA. The first study was a microarray comparing OA and normal donors by Karlsson et al [308], and the second, a study of cultured chondrocytes and the effects of GIN, a Gsk3 β inhibitor from mouse embryos by Miclea et al [327]. In the first study, COL14A1 was upregulated in OA cartilage. In the second study, where GIN was shown to induce loss of the chondrocytic phenotype, there was downregulation of COL14A1 in treated cells. This study shows lower gene expression in damaged cartilage, a finding of unknown consequence as yet.

Other collagen genes expressing significant changes (shown in Appendix 5) were; COL5A1 (FC=2.02 $\uparrow$ in damaged; p=0.008), COL12A1 (FC=3.42 $\downarrow$ in damaged, p=0.008), and COL24A1 (FC=2.93 $\downarrow$ in damaged, p=0.008). There is limited

information in the literature regarding these genes in cartilage and in osteoarthritis. COL5A1, though known to be involved in Ehlers-Danlos syndrome, and more recently with Achilles tendinopathy [328-330] has been much less researched in OA. It is a quantitatively minor fibrillar collagen that regulates fibrillogenesis [331] and is characteristically synthesized by cells with a fibrocartilagenous phenotype [332]. Aigner et al [322], showed its increased expression in moderate/severe OA in a microarray study, and when this was compared to rat ACL transection model microarray by Wei et al [311], this result was corroborated, however with the suggestion that it is a feature of early osteoarthritic cartilage. Our finding agrees with the former of these two studies, with it being a feature of end stage osteoarthritic cartilage. Other studies have shown its increased expression in OA cartilage [307, 310, 321, 333]. The mechanism by which this occurs and its implications have not been researched, but one logical conclusion is that its expression is part of an attempted reparative process [310].

COL12A1 is also sparsely studied in OA. Fukui et al [306], in a microarray study of zonal expression in osteoarthritic cartilage, identified it as having increased expression in the superficial zone compared to deep. The title of their study is “Zonal gene expression of chondrocytes in osteoarthritic cartilage”. However they do not comment on the location of such cartilage and its macroscopic or histological appearance. One assumes that this cartilage was taken from an area similar to that which we have termed undamaged cartilage, in which case the two study results would appear to concur. Type XII collagen function is unknown, but cooperation with other fibril binding proteins in cartilage, such as small proteoglycans, decorin, biglycan and fibromodulin seems probable [105].

COL24A1 is a poorly characterised fibrillar collagen; it has been shown to be expressed in the forming skeleton of the mouse embryo [334], and mouse osteoblasts [335]. In our study it has decreased expression in damaged cartilage. It has never previously been shown to demonstrate altered gene expression in osteoarthritis.

Of interest in relation to qPCR results of Chapter 2 (section 2.4) is COL1A1. This did appear in the microarray data set, showing a 2.50 fold change, with a p value of 0.015 (hence did not fit into the criteria of the results reported above). This was in the same pattern as previously described; a higher expression in undamaged cartilage. COL1A2 showed a fold change of 2.08 with a significance of $p=0.011$. These two genes are both required to produce pro- $\alpha 1$ and pro- $\alpha 2$ chains respectively, which together combine to produce type I procollagen, therefore one would expect their gene expression to be comparable when there is increased type I collagen present.

The MMPs generated several results of interest in AMG (Chapter 2). The MMPs are key to matrix turnover in cartilage. Four MMPs, MMP1, MMP2, MMP9, MMP13, were shown to demonstrate altered gene expression between damaged and undamaged cartilage in this microarray study. They all showed lower levels of expression in damaged cartilage. In Karlsson's [308] microarray study, all four of these genes were in the significantly altered group, demonstrating higher expression in OA cartilage, however in the most thorough MMP profiling studies of OA cartilage [159, 160] MMP 13 was upregulated in OA, but MMPs 1 and 3 were downregulated in OA cartilage. These studies [159, 160] were not topographical studies of areas of cartilage within an OA joint, and this highlights that different regions of cartilage in OA have different catalytic activity. The higher levels of expression in undamaged

cartilage of MMPs may be markers of impending cartilage degeneration, perhaps related to the OA disease process and altered mechanical and biological stimuli.

6.4.1.1 Glycoproteins

Laminins are a family of glycoproteins whose role is poorly understood. They have been shown in fetuses to control proliferation and differentiation of various cell types [336, 337] and Laminin-1 is present in epithelial cells and has been localised to basement membranes [338]. Laminin 1 has been shown to be present in fetal (physis) and adult cartilage (superficial layer) using immunohistochemistry [339], however there are no gene expression studies in OA or normal cartilage. This microarray study shows a highly significant 15-fold difference in LAMB1 gene expression between damaged and undamaged cartilage. It is higher in the undamaged region which does have a superficial zone, however without a direct comparison to normal cartilage, one cannot comment on its significance, and given the limited knowledge of its function, there are no previous study results upon which one can build a meaningful hypothesis.

The GPNMB gene codes for another glycoprotein, transmembrane glycoprotein NMB. In this microarray it was shown to have decreased expression in damaged cartilage; in another microarray study it was upregulated in OA cartilage [308]. GPNMB has not been studied in relation to OA, however has been shown to be related to bone formation with overexpression resulting in abnormalities of osteoblast expression and function [340].

6.4.2 Cytokines and chemokines

6.4.2.1 *IL11*

IL11 exhibited the greatest fold change (15-fold higher regulation in damaged cartilage) of all the genes tested. Previous studies have shown it to have both pro- and anti-inflammatory properties [341, 342]. It appears to stimulate production of TIMPs, [343] (there was no differential expression of TIMPs in this microarray). It has been shown to have increased expression in both RA and OA synovial fluid [344], and mouse studies showed more severe RA in IL11 injected joints, and less severe disease in those receiving IL11 antibody [345]. It clearly demonstrates a pro-inflammatory role, and more recent work showed similar concentration in RA and OA synovial fluid, but greater than that of control joints [346]. Further data on this cytokine is limited, but defining its role and its potential as a biomarker or treatment pathway is warranted.

6.4.2.2 *OPG*

TNFRSF11B is also known as Osteoprotegerin (OPG). OPG is a member of the TNF receptor family that acts as a decoy receptor for RANKL (TNFSF11) [347, 348] and subsequently has actions on bone, inhibiting osteoclast activation. It is involved in disruption of growth plates, subchondral bone and joint surface collapse in rheumatoid arthritis and Paget's disease. OPG has been shown to be expressed in normal cartilage, and previous studies describe upregulation in OA [349, 350]. Genotyping studies have also identified its association with knee OA [351]. This study demonstrates higher OPG expression in damaged cartilage, by microarray, qPCR and of protein expression, in the same pattern, by immunohistochemistry. Studies into OPG and OA have shown it to have a chondroprotective effect in an induced model of murine OA [352, 353], via prevention of chondrocyte apoptosis.

The previous chapter of this thesis addresses apoptosis in AMG, and finds increased apoptosis in damaged cartilage. One would hypothesize that the increased expression of OPG is a chondroprotective mechanism, which despite being initiated at the gene expression level is not significant to the extent that it can overcome the apoptosis signalling from the biological and mechanical stresses on the cartilage. A comparison to normal cartilage would also be interesting in order to compare it to the undamaged cartilage of AMG. It could be the case, in conjunction with other pathways, that if more highly expressed in this undamaged cartilage in an OA joint compared to normal cartilage, that it may be a contributing factor to this area of cartilage remaining undamaged.

Another factor of note is the role of OPG in bone resorption [354]. Of growing interest are the interaction between bone and cartilage in OA, with suggestions that their relationship is key [15, 60] and some studies suggest bone changes precede those of cartilage degeneration [355]. This has further been considered by Kadri et al. in relation to OPG and inhibition of cartilage degradation through an effect on trabecular bone in a murine model [356]. In his study, bone volume increased in mice treated with OPG. In AMG it was observed that the bone was sclerotic under the more damaged cartilage suggesting a possible adaptation response to the damaged cartilage above, perhaps in response to the OPG/RANK/RANKL balance or to altered mechanical load. Evidence from other studies would suggest that the earlier changes in bone structure are increased resorption and bone thinning [357, 358], however later changes in OA, such as those seen in AMG in conjunction with cartilage loss are increases in bone volume and density, and the formation of the typical subchondral sclerotic bone seen in end stage OA [359].

A recent paper by Sanchez et al [360], speculated that OPG expression is also mechanically mediated, with compression downregulating its expression in subchondral bone. The modulation of OPG, RANK (TNFRSF11A) and RANKL has only recently been studied in more details in chondrocytes [361]. Tat's study (2009) describes decreased OPG expression in OA chondrocytes, in contrast to the results of the only other study of the role of RANK signalling in cartilage, Komuro et al (2001) [349], and their suggestion for these conflicting results was cited as experimental differences (semi-quantitative versus real-time PCR). They do, however, demonstrate significant differences of ratios of OPG/RANKL and RANK/RANKL in human OA chondrocytes, and suggest these as potential targets for therapeutic intervention.

An *in vivo* cartilage injury model microarray screen [305] also identified OPG as exhibiting altered expression and implicated it in the regulation of Wnt Signalling and FRZB genes associated with cell signalling, wound healing and response to injury. OPG may therefore have beneficial effects against the progression of OA via several different mechanistic routes.

6.4.3 Inflammatory, Injury and Signalling responses

Rheumatoid Arthritis has traditionally worn the label of the 'inflammatory arthritis', however there is an increasing amount of work being undertaken into the inflammatory component of OA. This microarray highlights changes in inflammatory and NF- κ B signalling that may contribute to this component. There was decreased expression of inflammatory genes in damaged cartilage, including those of the complement system, C2, C1A and C4BPA. Complements have previously been identified in synovial fluid: C3 [362] and C5 dysregulation plays a critical role in OA

pathogenesis [363]. In addition to one of the greatest fold change genes, IL11, CCL2 and CCL3 (Figure 6-6) are expressed in higher levels in undamaged cartilage. PTSG1 (COX1) is also expressed at higher levels in undamaged cartilage. COX1 is a key enzyme in prostaglandin synthesis, and is inhibited by the NSAID pharmacological agents such as ibuprofen and diclofenac, anti-inflammatory analgesics used for symptom control in OA. Animal models have suggested possible protective effects of certain COX inhibitors on the progression of OA, however, this is an area of debate with other studies showing they were detrimental (for review see Ding 2002; [364]).

Cytokines that have been shown to stimulate chondrogenesis and chondrocyte proliferation include IGF 1 (FC 5.09 ↓ in damaged; p=0.008) and FGF 18 (FC 4.68 ↑ in damaged; p=0.008). It has been postulated that FGF18 facilitates BMP activity by reducing expression of the BMP antagonist Noggin. However in our study an increase in FGF18 in damaged cartilage occurs with an increase in Noggin expression.

6.4.4 WNT Pathway

The exact role of the Wnt pathway in OA is not fully understood, however cartilage homeostasis and development have shown under and over activation in OA mouse models [365, 366]. Of note, the Wnt antagonist, FRZB is downregulated in damaged cartilage in this study. Genome-wide association studies have shown FRZB polymorphisms associated with Caucasian populations with OA [367] and FRZB knockout mice show increased proteoglycan loss in OA models [368]. Expression of a number of Wnt-inducible genes including LEF1, WISP2 and TWIST1 were decreased in damaged cartilage in our study, as was the Wnt receptor FZD5 and

modulator DACT1. Furthermore FGF18, RUNX2, IGF1, FMOD, COL5A1, FN1 are also Wnt target genes showing altered expression, and involvement in other key dysregulated networks in this study. FZD5, downregulated in damaged cartilage in our study, has previously been shown to be upregulated in knee OA. In agreement with previous studies, this microarray highlights that there is dysregulation of the Wnt pathway, however the mechanisms underlying these changes are not known but are worth further study.

6.4.5 BMP Pathways

A number of BMP antagonists show altered regulation in AMG; Noggin, FSTL2 and BMPER. Noggin has been studied in relation to cartilage development and is highly expressed in growth plate chondrocytes, however it appears not, in the literature, to have been found to be expressed in adult cartilage [369]. BMPER (FC 2.98 ↓ in damaged; $p=0.008$) can antagonise BMP-induced chondrogenesis by ATDC5 chondroprogenitor cells, however it has also been reported to have pro-BMP activity through binding to chordin. A microarray of Kashin Beck Disease [309], an osteochondropathy seen in Asia, showed lower expression in damaged cartilage regions compared to undamaged cartilage, leading to the conclusion that this gene expression change precedes visible damage.

Up and down-regulation of these BMP antagonists in damaged regions of OA cartilage suggest the balance of BMP signalling is altered in different zones of cartilage possibly resulting in dysregulated chondrocyte phenotype in the OA joint. Our data suggests that dysregulation of the BMP pathway occurs through altered expression, activities and specificities of the regulated BMP antagonists and their modulators (e.g. FGF18).

6.4.6 Developmental/Skeletal injury networks

In this significantly altered network, regulated genes include SOX11, TNFSFR11B, CASP1, FGF18, RUNX2 and IGF1. TNFSFR11B (OPG) has been discussed in some detail above (section 6.4.2.2).

SOX11 gene expression differences (FC 8.66 ↑ in damaged; $p=0.008$) were validated by qPCR. It is a transcription factor highly expressed in the growth plate during chondrogenesis, though its function is not known, and increased SOX11 has been detected in OA cartilage in a microarray study by Sato et al [321]. SOX11 KO mice show skeletal malformations and it has been shown to play an important role in skeletal tissue remodelling [370]. CASP1 encodes caspase1, a modulator of apoptosis, previously shown to be increased in OA clonal clusters, and apoptosis is a theme investigated in more detail in the previous chapter. RUNX2 has been reported to be increased in OA and is a key transcription factor and regulator of osteoblast differentiation, essential for correct regulation of differentiation towards chondrogenic or osteoblastic lineages. It has also been found that RUNX2 expression is associated with MMP-13 expression [371], and both these genes are regulated in the same direction in this microarray.

6.4.7 Summary

This study is the first to consider gene expression differences within a specific phenotype of OA. It highlights a number of genes and pathways worthy of further study, and a better understanding of these changes may lead to potential diagnostic markers and therapeutic targets. In addition, it highlights changes of damaged and undamaged cartilage within AMG that have previously only been attributed to OA cartilage in a broader sense, but are now shown to demonstrate regional specificity.

In AMG, there is a specific, repeatable pattern of disease which allows for topographic sampling from adjacent regions of damaged and undamaged cartilage. The other studies presented in this thesis have highlighted some changes in the matrix content of these regions, and differences in gene expression, which provided justification for a broader screening approach.

It is possible that these changes may be progressive, or indeed reactive, to a joint-wide phenomenon. One shortcoming of this study is being unable to distinguish in which region of cartilage genes are being up- or down- regulated because of the lack of healthy control tissue to provide baseline quantitation. It could be the case that in end-stage disease, cell metabolism slows and gene expression is downregulated, and that the over expression of genes, traditionally linked to OA cartilage degeneration, such as MMPs etc, is occurring in the undamaged cartilage as a signalling response to surrounding damaged cells in cartilage and synovium, and circulating synovial fluid cytokines. The changes in cartilage integrity result in altered weight bearing over the joint surface, and this mechanical stimulus may also contribute to a change in chondrocyte phenotype.

It is interesting to note that some of our results corroborate those in the literature, whilst others suggest the opposite. As previously described, I hypothesize this is the case due to previous studies comparing OA versus normal cartilage where OA cartilage is excised *en masse* from both joints. However within a diseased joint there may be both undamaged and damaged cartilage. Therefore if one takes both of these together their gene expression is, in effect, mathematically ‘added together’. The topographical pattern of AMG cartilage erosion enables the reproducible sampling of

these separate areas of undamaged and damaged areas within the same joint, a process which appears not to have been undertaken previously in the literature.

Microarray is a powerful tool to assess thousands of genes simultaneously, however, in isolation its use has been questioned [372, 373]. Particular drawbacks include that results may be too complex to interpret and results are not always reproducible. Thus, even though this study has produced many results of interest, further work is required, in particular, to address some of the findings of this study using human healthy control tissue. In this way a baseline for gene expression can be identified, and one might find that even those genes which are differentially expressed are both higher or lower than that of normal cartilage.

The microarray study by Sato et al [321] does compare damaged and undamaged cartilage from the same joint, however this is taken from individuals undergoing total knee arthroplasty, which may include joints with only one or several affected compartments affected and to differing degrees, thus is less defined than AMG both as a phenotype of disease and as the geographical regions sampled. These more global studies of gene expression in OA are not without value as the dominant gene expression changes may define the overriding changes that could be targeted to increase our understanding of disease mechanism and potential therapies. The advantage of further delineation using AMG and distinct geographical regions is the potential to define specific mechanisms in a common form of OA. Therein lies the possibility of the study of AMG leading to target genes and pathways which are driving OA. The main focus of study, if AMG does represent progressive OA change, is that targets can be identified from macroscopically normal cartilage which, if altered, prevent subsequent degradation. Findings of particular interest include

pathways involving BMP and Wnt signalling, two traditionally bone related pathways. Interest has been growing for a number of years with regards to the relationship between bone and cartilage, with some groups suggesting that OA might be a primary disease of subchondral bone [14, 15, 60].

This study demonstrates the importance of studying positionally well-defined and precisely mapped regions of cartilage. Future work is required to better understand how these gene expression changes relate to normal human cartilage, and this microarray perhaps points to changes and interactions between bone and cartilage being a worthwhile path of further study. Feasible future work might involve studying the underlying bone beneath regions of damaged and undamaged cartilage in AMG.

Chapter 7

7 DISCUSSION AND FUTURE WORK

Anteromedial Gonarthrosis is a specific phenotype of Osteoarthritis. It is common, and its reproducible pattern of disease lends itself to the study of osteoarthritic cartilage at different stages of disease. The progressive nature of AMG has previously been demonstrated macroscopically and histologically. This thesis has concentrated on the molecular and cellular features of AMG and used undamaged cartilage from above knee amputations in order to validate these changes as being part of the AMG disease process.

7.1 Summary of Results and Conclusions

In brief, each of the studies findings and conclusions are summarised below:

7.1.1 Study 1 - Quantitative Real Time PCR

This chapter demonstrated differences in the gene expression between regions of damaged and undamaged cartilage. Significant differences (increased in undamaged cartilage) were demonstrated for COL1A1, ADAMTS4 and the MMPs, MMP1, MMP3, and MMP13.

We can conclude that these changes, which are previously undocumented findings, may be a result of regional variation in the AMG phenotype. They may be due to the OA disease process, changes in loading of cartilage, and a result of joint wide signaling processes. In order to further investigate this, above knee amputations, exhibiting undamaged cartilage were collected and analysed.

7.1.2 Study 2 - Assessment of Aged Undamaged Cartilage

This chapter's results showed that from 16 AKA samples collected that it was possible, using histological classification, to divide them into two groups; a lower grade and a higher grade group. The real time PCR demonstrated no significant differences between equivalent regions in contrast to clear differences between damaged and undamaged cartilage in AMG, supporting the hypothesis that the gene expression differences described above are specific to AMG. Immunoassay data demonstrated higher levels of Type I collagen in higher grade AKA specimens in posterior cartilage (as previously found in AMG), but not in lower grade specimens, suggesting that it may be a change of undamaged cartilage that occurs when there is increasing histological damage elsewhere on the medial tibial plateau or an early response to altered loading. GAG was demonstrated to be lower in region X, the equivalent region to the epicentre of the lesion in AMG. This is in keeping with evidence that this is the region where AMG commences.

7.1.3 Study 3 – Apoptosis

This study considered apoptosis in AMG and AKA specimens to seek molecular evidence of the damage pathways involved. There was progressively less apoptosis in the less damaged cartilage of AMG. The presence of active Caspase 3, cytochrome c, Bim and active Bax illustrate that apoptosis is occurring via the intrinsic pathway. Reactive Oxygen Species-mediated damage was demonstrated with the increased presence of 3-nitrotyrosine in areas of increased apoptosis.

7.1.4 Study 4 - Microarray

This complex study, that considered tens of thousands of genes' expression, revealed 389 expression differences between regions of damaged and undamaged cartilage in AMG. Highlighted gene ontology groups included cellular and skeletal development, inflammatory response, cell death and cell signalling. It corroborated several findings in the literature and suggests new genes that may be linked to OA pathogenesis in the specific phenotype of AMG.

7.2 Directions for Further Work

There are a number of interesting avenues to pursue on the basis of the results of this thesis.

1. Obtaining better control data including young, undamaged cartilage.
2. Study of further apoptotic markers, including FOXO1 and *in vitro* / *ex vivo* work considering the effects of anti-oxidants and/or inhibitors of apoptotic pathways.
3. Mechanical studies to better understand the changes of loading as disease progresses.
4. Mechanobiology studies to better understand the relationship between changes in load and cartilage biology.
5. Regional bone biology in AMG and bone-cartilage interactions
6. Studies into synovial fluid as a potential carrier for joint-wide signals.
7. Further similar work into to other phenotypes of disease, both in the knee and other joints.

This thesis has considered the regional cartilage zones affected in AMG and, where possible, compared them to the equivalent regions of cartilage from AKA specimens. The conclusions that have been drawn suggest that many of the changes seen are part of the AMG disease process. As discussed in Chapter 4, whilst the AKA samples were of great benefit, they were not ideal controls, being slightly older and at least transiently less ambulant. The collection of human control tissue represents a significant challenge in the study of cartilage biology. During the course of this thesis, ethical approval was obtained to collect knee cartilage from brain stem dead patients that were undergoing organ collection for donation. However, none of these samples became available during the time frame. In the one year that this ethics was in place, contact was made about two patients; one was under the age of 18, and was not suitable as limited by ethical approval. The second had had previous knee surgery.

The nature of the study of cartilage biology means that studies generally have small numbers. This is a particular limitation when quantifying small changes. A power calculation is not appropriate in this situation, non parametric statistics are used, but one's conclusions need to be guarded. The results form the basis for further investigation, and with the advent of a 'biobank' for tissue collection, at the Nuffield Orthopaedic Centre, it would be possible to repeat some of these experiments on greater numbers. One major limitation is assay based: a well optimised and controlled immunoassay can be run at any time and data compared with previous assays, however with the qPCR method used, samples are only comparable if undertaken on the same run. There is too much potential for variability if undertaken at different times, furthermore the RNA in cartilage, even if stored appropriately does degrade

over time. Whilst it would be feasible for one to collect AMG samples rapidly, the same is not true for AKA specimens which become available less frequently.

Apoptosis is an interesting field, relevant to most human diseases. At the time of starting this study, the FOXO family was gaining attention in ROS-mediated cell death, and FOXO3a had been identified as a potential candidate driving expression of pro-apoptotic Bim. FOXO3a was demonstrated as being present in similar levels in both AMG and AKA samples, potentially suggesting a homeostatic role, rather than one of an apoptotic trigger or signaller. As described in Chapter 5, interest has shifted to FOXO1, which directly mediates cell death via upregulation of Bim. Future studies should consider FOXO1 and its role in ROS-mediated apoptosis. Given that ROS damage has been demonstrated in cartilage where there is apoptosis, it would be interesting to conduct *in vitro* and *ex vivo* work on the modulation of apoptosis using anti-oxidants and to assess ROS generators such as mechanical load and cytokines. Further study into regional proteomics may help quantify absolute amounts of pro- and anti- apoptotic markers present in regions of AMG.

The microarray study presents several findings which stimulate ideas for further work. Some of the top hits of the array, (see section 6.4) have had limited study in the context of OA, and should be investigated further. Of growing interest, fuelled by the presence of genes traditionally associated with bone metabolism, is the interaction between bone and cartilage. A straightforward study could consider regional variations of subchondral bone in AMG, undertaken in the same manner as this study. Similarly, in AKA samples demonstrating early histological changes, it would be of great interest to study the underlying bone as many scientists hypothesize that changes are driven by the bone-cartilage interaction.

OA is a joint wide disease, and this thesis has demonstrated changes in terms of cartilage matrix content and metabolism, a wide range of gene expression changes, in that apoptosis contributes to progressive cartilage damage. The signals that trigger some of the changes described occur over a large area and it is possible that they have a mechanical basis. It is also probable that as the overall environment changes in a joint that 'long distance' signalling occurs via synovial fluid changes. Future studies should consider the synovial fluid of joints at differing stages of disease, as it may be possible to identify markers that are responsible for initiating some of the cellular and molecular features described in this thesis.

Typically, over a large number of years, the mechanics of OA progression and the biology of OA have been studied in isolation. There is growing interest in mechanotransduction and the role that mechanics play on cellular changes. Of particular interest, and previously not investigated, is how loading changes in AMG. As a lesion develops the distribution of load must change, and it is possible that that is one of the triggers for the biological changes described in this thesis. With regard to the AKA samples, changes were demonstrated in region X, the equivalent of the central lesion in AMG, and the area of cartilage that fails first. It would be interesting firstly, to mechanically test this area of cartilage in further samples. Though not possible, due to sample limitations, it would also be very interesting to consider the changes in loading with changes in the mechanical properties of this area of cartilage, something that may become possible with finite element modelling.

This thesis considers the phenotype of AMG, however the study of other phenotypes in the knee joint, and of OA of other joints, in a similar fashion would be of great interest.

Ultimately, a better understanding of the initiation and progression of early OA is sought. With investigation of the mechanical and biological factors that occur early in the disease process one can then consider which of these factors may be either reversible, or amenable to therapies to slow or halt progression.

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Appendices

Appendix 1 Ethics, Patient Information and Consent

14-JUN-2006 11:26 FROM N.D.O.S.

TO 987892249546

P.01

**Oxford
Radcliffe**
HOSPITALS

CENTRAL OXFORD RESEARCH
ETHICS COMMITTEE

THE JOHN RADCLIFFE

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CJC/imp/C01.071

22 March 2001

Dr N A Athanasou
Reader & Consultant in Orthopaedic Pathology
Department of Pathology
NUFFIELD ORTHOPAEDIC CENTRE

Dear Dr Athanasou

Re: C01.071 – The pathobiology of neoplastic and non-neoplastic disease of bone, joint and soft tissue

Thank you for your letter dated the 19 March 2001 addressing the concerns raised by the Committee at their meeting on the 2 March 2001. In accordance with the authority set out in the Terms of Reference, I am happy to confirm ethical approval and wish you every success with the study.

Please note:

- Ethical approval is valid for three years from the date of this letter. Annual updates of the progress of the research and a report of the outcome are required. (A reminder letter will be sent when these reports are due).
- No significant changes to the research protocol should be made without appropriate research ethics committee/committee's approval. Any deviations from or changes to the protocol which increase the risk to subjects, or affect the conduct of the research, or are made to eliminate hazards to the research subjects, should be made known to COREC.
- COREC should be made aware of any serious adverse events.
- Whilst the study has received approval on ethical grounds, it is necessary for you to obtain management approval from the relevant Clinical Directors and/or Chief Executive of the Trusts (or Health Boards/DHAs) in which the work will be done.

I should be very grateful if you could send me a copy of any publication which may arise from this study.

NB: Any research which will be conducted on NHS patients or staff, and which has been approved by a research ethics committee must carry the appropriate indemnity. May I remind you that COREC final approval is contingent on the appropriate indemnity being in place.

Chairman: Dr C J Chapman

ICH GCP Compliance

The Oxfordshire Research Ethics Committees are compliant with the International Committee on Harmonisation/Good Clinical Practice (ICH/GCP) Guidelines for the Conduct of Trials involving participation of human subjects as they relate to the responsibilities, composition, function, operations and records of an Independent Ethics Committee/Independent Review Board. To this end it undertakes to adhere as far as is consistent with its Constitution, to the relevant clauses of the ICH Harmonised Tripartite Guideline for Good Clinical Practice, adopted by the Certification of the European Association of Hospital

National Health Service Trust

W14 486



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19 September 2006

Mr Andrew J Price
Clinical Lecturer
Nuffield Department of Orthopaedic Surgery
NDOS, Nuffield Orthopaedic Centre
Windmill Road
Oxford
OX4 1SZ

Dear Mr Price

Full title of study: Investigation into the histological, immunohistochemical,
and molecular features of aged cartilage of the knee.
REC reference number: 06/Q1606/97

Thank you for your letter of 30 August 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application		31 July 2006
Investigator CV	Mr A Price	
Protocol	2	21 July 2006
Covering Letter		21 July 2006

An advisory committee to South Central Strategic Health Authority



National Research Ethics Service

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21 December 2007

Mr Andrew Price
Reader in Musculoskeletal Science and Consultant Orthopaedic Surgeon
Nuffield Department of Orthopaedic Surgery
Nuffield Orthopaedic Centre

Dear Mr Price

Full title of study: Investigation into Degenerative Joint Diseases: The collection of control tissue for genetic and molecular work in the fields of cartilage, tendon and bone pathologies
REC reference number: 07/H0606/156

Thank you for your letter of 10 December 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application		02 November 2007
Investigator CV	Mr A Price	01 November 2007

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England.



PATIENT INFORMATION SHEET

Investigation into the features of Aged Cartilage.

What is the purpose of the study?

The purpose of this study is to investigate changes that occur in aged cartilage in people who do not suffer from severe osteoarthritis.

Why have I been chosen to take part?

You have been chosen to take part as you are due to have an above knee amputation. Following the surgery the amputated limb is disposed of. We wish to take some tissue samples from the limb prior to this. Following this the limb will be disposed of in the normal way.

Why are these samples of interest?

We are currently investigating the causes of osteoarthritis and other bone and joint conditions. We have looked at the features of diseased cartilage in patients who have undergone joint replacements for severe osteoarthritis. In order for us to fully understand the changes that occur in disease it is important to look at the changes in tissues that have not suffered from severe osteoarthritis and are approximately the same age.

Will my vascular problems change things?

No. Cartilage has very little blood supply, so it will not have been affected by your vascular history

Do I have to take part?

You do **not** have to take part in this investigation. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive; your planned operation will proceed as normal.

What would happen to me if I take part?

If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. Your planned operation will proceed as normal.

Will taking part change anything during my operation?

No, your operation will be exactly the same

What are the risks of taking part?

There are no risks to you if you participate in the study.

What are the benefits of taking part?

There will be no direct medical benefit to you but we hope very much that this research will help increase our understanding which will help patients with osteoarthritis in the future.

Will my taking part in this study be kept confidential?

All information collected about you during this study will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Will I retain any rights to samples when they are taken?

No once the samples have been donated it will be considered a gift that is an anonymous donation.

What happens if something goes wrong?

If you are not happy with the general care and treatment you receive during this study, please talk to the study supervisor – Mr Andrew Price, who will try and solve the problem. He will also tell you about the Hospital standard complaints procedure in case you wish to take the matter further.

What will happen to the results of the research study?

The results of the study we hope would be published in scientific journals.

Who has reviewed the study?

It has been reviewed by experts in the field and has been approved by the Oxfordshire Clinical Research Ethics Committee (OREC:)

Who is organising this study?

The study is being organised by the Nuffield Department of Orthopaedic Surgery, University of Oxford.

Where can I get more information?

If you have more questions about this study, please contact:

Mr. Andrew Price - Clinical Lecturer & Honorary Consultant contact details are below.

Nuffield Orthopaedic Centre, Headington, Oxford, OX3 7LD

Email: andrew.price@ndos.ox.ac.uk or Telephone: 01865 737 543

CERES (consumers for ethics in Research) produce a leaflet about participating in research, called '*Medical Research and You*' which you may find helpful. This is available from:

Postal address: CERES, PO Box 1365, London N16 0BW

e-mail: info@ceres.org.uk

web-site: www.ceres.org.uk



RELATIVE INFORMATION SHEET

Investigation into the feature of aged cartilage

Why am I being approached?

Currently your relative is listed to have an above knee amputation for vascular disease. At the moment he is unable to give informed consent for the procedure or for participation in this research project.

What is the study?

This study investigates changes that occur in aged cartilage in people who do not suffer from severe osteoarthritis

Why are these samples of interest?

Osteoarthritic cartilage has been investigated in depth by obtaining samples from patients having joint replacement. In order for us to fully understand the changes that occur in disease, it is important to look at changes that have occurred in cartilage of people of a similar age but who have not suffered severe osteoarthritis

Do his/her vascular problems change things?

No. Cartilage has very little blood supply, so it will not have been affected by your relative's vascular problems

Does he/she have to take part?

No. You are free, as his/her relative **not** to agree to taking part in this study. You are free to withdraw your assent at any time, as is your relative, without giving a reason. Not taking part, or withdrawing from the study will **not** affect the medical care in any way; the operation will proceed as normal, and the tissue incinerated as normally happens in these cases.

Will taking part change anything during the operation?

No. The operation is exactly the same.

What are the risks of taking part?

There are no risks of participating in this study

What are the benefits of taking part?

There is no direct medical benefit to your relative in taking part. We hope that the research will increase our understanding of osteoarthritis and benefit patients in the future.

Will my relative's details remain confidential?

All information collected about your relative during this study will remain confidential. Any information that leaves the hospital will have your relative's name/address removed from it. Samples are kept anonymous.

Will I/my relative retain any rights to samples when they are taken?

No. Once samples are donated they are considered a gift that is an anonymous donation.

What happens if I/my relative are not happy with something?

If you are not happy with the general care and treatment you receive during the study, please talk to the study supervisor, Mr Andrew Price, who will try to solve the problem. He will also tell you about the hospital standard complaints procedure in case you wish to take the matter further.

What will happen to the results of research study?

We hope to publish the results in scientific journals.

Who is organising the study?

The study is organised by the Nuffield Department of Orthopaedic Surgery, part of the University of Oxford.

Who has reviewed the study?

It has been reviewed by experts in the field and approved by the Oxfordshire Clinical Research Ethics Committee (OREC)

Where can I get more information?

If you have any further questions please contact:

Mr. Andrew Price – Clinical Lecturer and Honorary consultant
Nuffield Orthopaedic Centre. Windmill Road, Oxford. OX3 0DH
Email: Andrew.price@ndos.ox.ac.uk Tel: 01865 737543

CERES (consumers for ethics in research) produce a leaflet about participating in research called '*medical research and you*' which you may find helpful. This is available from:

CERES, PO Box 1365, London. N16 0BW
Email: info@ceres.org.uk
Website: www.ceres.org.uk



CONSENT FORM

Investigation into the features of Aged Cartilage.

In patients undergoing above knee amputation.

Researchers: Mr. Andrew Price - Clinical Lecturer & Honorary Consultant
Professor Andrew Carr – Nuffield Professor of Orthopaedic Surgery
Dr Philippa Hulley - University Lecturer, University of Oxford
Mr. Stephen McDonnell - Research Fellow
Mr Rajesh Rout – Research Fellow
Vascular Surgeons – John Radcliffe Hospital, Oxford

(Please initial as yes)

1. I confirm that I have read and understood the Participant Information sheet dated 30/08/2006 (Version 3.0) for the above study. I have had the time to consider it and the opportunity to ask questions about the project. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. Have you received satisfactory answers to all your questions? ☐
4. Following my above knee amputation I give permission for tissue specimens to be taken from my limb before it is disposed of. ☐
5. I give permission for these tissue specimens to be used for scientific research. ☐
6. I understand that this study will not affect my clinical care in any way ☐
7. I agree to take part in the above study. ☐

<i>Name of Subject</i> <i>Date</i> <i>Signature</i>	<i>Please fill in write box</i>
<i>Name of Researcher</i> <i>Date</i> <i>Signature</i>	

1 for patient; 1 for researcher: 1 to be kept with hospital notes

If you have more questions about this study, please contact:

Mr. Andrew Price - Clinical Lecturer & Honorary Consultant contact details are below.

Nuffield Orthopaedic Centre, Headington, Oxford, OX3 7LD

Email: andrew.price@ndos.ox.ac.uk or Telephone: 01865 737 543



ASSENT FORM

Researchers: Mr. Andrew Price – Clinical Lecturer and Honorary consultant
 Professor Andrew Carr – Nuffield Professor of Orthopaedic Surgery
 Dr. Philippa Hulley – University Lecturer, University of Oxford
 Mr. Rajesh Rout – Research Fellow
 Vascular Surgeons – John Radcliffe Hospital, Oxford
 Buckinghamshire NHS Trust

Please initial boxes as yes

1. I confirm that I have read the information sheet dated 3rd April 2007 (version 3.1). I have had time to consider it and the opportunity to ask questions about the project. ☐
2. I am free to withdraw my assent at any point; my relative, should they become competent, may also withdraw his/her consent. I understand that participation is voluntary and that my relatives medical care and legal rights are not affected. ☐
3. I have received satisfactory answers to all my questions. ☐
4. Following the above knee amputation, I give assent for tissue specimens to be taken prior to its disposal. ☐
5. I give assent for these tissue specimens to be used for scientific research. ☐
6. I understand that it will not affect my relatives care in any way. ☐
7. I give assent for my relative to take part in this study. ☐

Name of Patient	Date
Name of relative	Signature
Relationship to Patient	

Name of Researcher	Date
	Signature

1 copy for patient; 1 for relative; 1 for researcher; 1 for hospital notes
 If you have further questions, please contact:
 Mr Andrew Price
 Nuffield Orthopaedic Centre, Oxford. OX3 7LD
 Email: Andrew.price@ndos.ox.ac.uk

DAY 2

8. Using a multi-channel pipette transfer 50 μ l from each well to the equivalent well of an AH23-coated flat-bottom plate.

NB. This must be accomplished in less than 45 sec. Do not stop to change tips between pipetting.

9. Incubate at room temperature for exactly 30 min.

10. Prepare secondary antibody (AP conjugated goat anti-rabbit) by diluting 1/1000 in 1% BSA/PBS containing Tween-20 also diluted 1/1000.

e.g. Prepare 5ml per plate consisting of 5 ml BSA/PBS
 5 μ l 2 $^{\circ}$ antibody
 5 μ l Tween-20

11. After exactly 30 min, wash plate 3x with PBS-Tween.

12. Add 50 μ l of 2° antibody to all wells. Wrap plate in parafilm and incubate at 37°C for 2 hours.

13. Wash plate 3x with PBS-Tween and 1x with distilled water to eliminate bubbles.

14. Prepare substrate by adding 1 alkaline phosphatase substrate tablet to 10ml diethanolamine buffer.

15. Add 50 μ l of substrate solution to each well. Allow plate to develop (approx 20 – 30 min) and read at 405 nm.

Type II Collagen

DAY 1

- 1) Block wells B2 to O13 with 40µl PBS-1% BSA per well at RT for 30mins.
- 2) Wash plate once with PBS-Tween.
- 3) Add 10µl Tris to rows B, C and D in triplicate (maximum binding).
- 4) Add 10µl standards (6µg/ml down to 0.5µg/ml) to rows E-K in triplicate.
- 5) Add 20µl Tris to rows L-O in triplicate (blanks).
- 6) Add 10µl sample in triplicate starting at well B5. There is space for 42 samples up to position O13.
- 7) Prepare 1st antibody diluted 1 in 600 (5µl COL2-3/4m antibody in 3ml Tris per plate) and add 10µl to all wells EXCEPT THE BLANK WELLS
- 8) Seal with a plate-seal and incubate o/n at 37°C.

	1	2	3	4	5	6	7	8	9	10	11	12	13
A													
B		MB	MB	MB	1	1	1	15	15	15	29	29	29
C		MB	MB	MB	2	2	2	16	16	16	30	30	30
D		MB	MB	MB	3	3	3	17	17	17	31	31	31
E		Std1	Std1	Std1	4	4	4	18	18	18	32	32	32
F		Std2	Std2	Std2	5	5	5	19	19	19	33	33	33
G		Std3	Std3	Std3	6	6	6	20	20	20	34	34	34
H		Std4	Std4	Std4	7	7	7	21	21	21	35	35	35
I		Std5	Std5	Std5	8	8	8	22	22	22	36	36	36
J		Std6	Std6	Std6	9	9	9	23	23	23	37	37	37
K		Std7	Std7	Std7	10	10	10	24	24	24	38	38	38
L		B	B	B	11	11	11	25	25	25	39	39	39
M		B	B	B	12	12	12	26	26	26	40	40	40
N		B	B	B	13	13	13	27	27	27	41	41	41
O		B	B	B	14	14	14	28	28	28	42	42	42
P													

DAY 2

1. Transfer 10µl from each well to equivalent well in collagen-coated plate.
2. Incubate at RT for EXACTLY 30mins and then wash 3 times with PBS-Tween.
3. Add 10µl anti-mouse second antibody, diluted 1/1000 in PBS-1%BSA-0.1% Tween (3µl antibody + 3µl Tween in 3ml PBS-BSA per plate) to all wells (reverse pipetting). Seal and incubate at 37°C for 2 hours.
4. Wash 3 times with PBS-Tween and once with dH₂O to remove bubbles.
5. Add 10µl substrate to all wells (1 tablet in 10ml diethanolamine buffer) using reverse pipetting. Allow to develop at RT or 37°C and read OD at 405nm.

GAG Assay

1. DMB Solution
16mg DMB
3.04g glycine
2.37g NaCl

Mix in 900ml water for 2 hours, covered in foil. Adjust pH to 3.0 with HCl and adjust volume to 1L. Store at RT, covered in foil.

2. Chondroitin sulphate (CS) standards

Prepare a stock at 50µg/ml in water and store at -20°C. Dilute as follows:-

Standard Number	Standard Conc (µg/ml)	Amount of stock CS (µl)	Amount of water (µl)
1	0	0	300
2	5	30	270
3	10	60	240
4	15	90	210
5	20	120	180
6	25	150	150
7	30	180	120
8	35	210	90
9	40	240	60
10	45	270	30
11	50	300	0

Method

1. Dilute samples as required in water.
2. Add 20µl water to first column as blanks (wells A1-H1).
3. Add standards, in duplicate, using 20µl / well (wells A2-F4).
4. Add samples, in duplicate, using 20µl / well (wells G4-H12).
5. Add 250µl DMB to all wells and read OD immediately at 525nm.

Appendix 3 Immunohistochemistry Protocols

Protocol for Immunohistochemistry of Paraffin Embedded Cartilage sections

1. Re-hydrate section
 - a. Xylene 2 x 5 min
 - b. 100% EtOH 2 x 2 min
 - c. 90% EtOH 2 min
2. Peroxidase block – 10 mins
3. Continue rehydration
 - a. 70% EtOH 2min
4. Wash
 - a. PBS 3 x 2min
5. Permeabilization in Triton X (0.5%) – 10 mins
6. Wash
7. Blocking stage with Horse Serum (3%) 1 hour (do not wash – drain only)
8. Primary antibody – Over night @ 4°C
9. Wash
10. Use **VECTASTAIN® ABC kit** (Vectorlab, Peterborough, UK)
11. Secondary antibody – 30 mins
12. Make up AB reagent (30 mins prior to use)
13. Wash
14. AB reagent – 30 mins
15. Wash
16. DAB – 8 mins
17. Wash
18. PI (1:500) – 10 mins
19. Wash
20. Mount with fluorosave

Solutions:

Peroxidase block = hydrogen peroxide + methanol (20 + 180µl)/slide

Triton X = Triton X + PBS (50 + 950µl) 200µl /slide

Horse Serum = Horse serum + PBS (6 + 194µl)/slide

Primary antibody = for TUNEL, cytochrome C, Active caspase-3, active Bax, Bim, FOXO3a 3-NT - 200µl /slide

Secondary antibody = II + HS + PBS (4 + 4 + 192µl)/slide

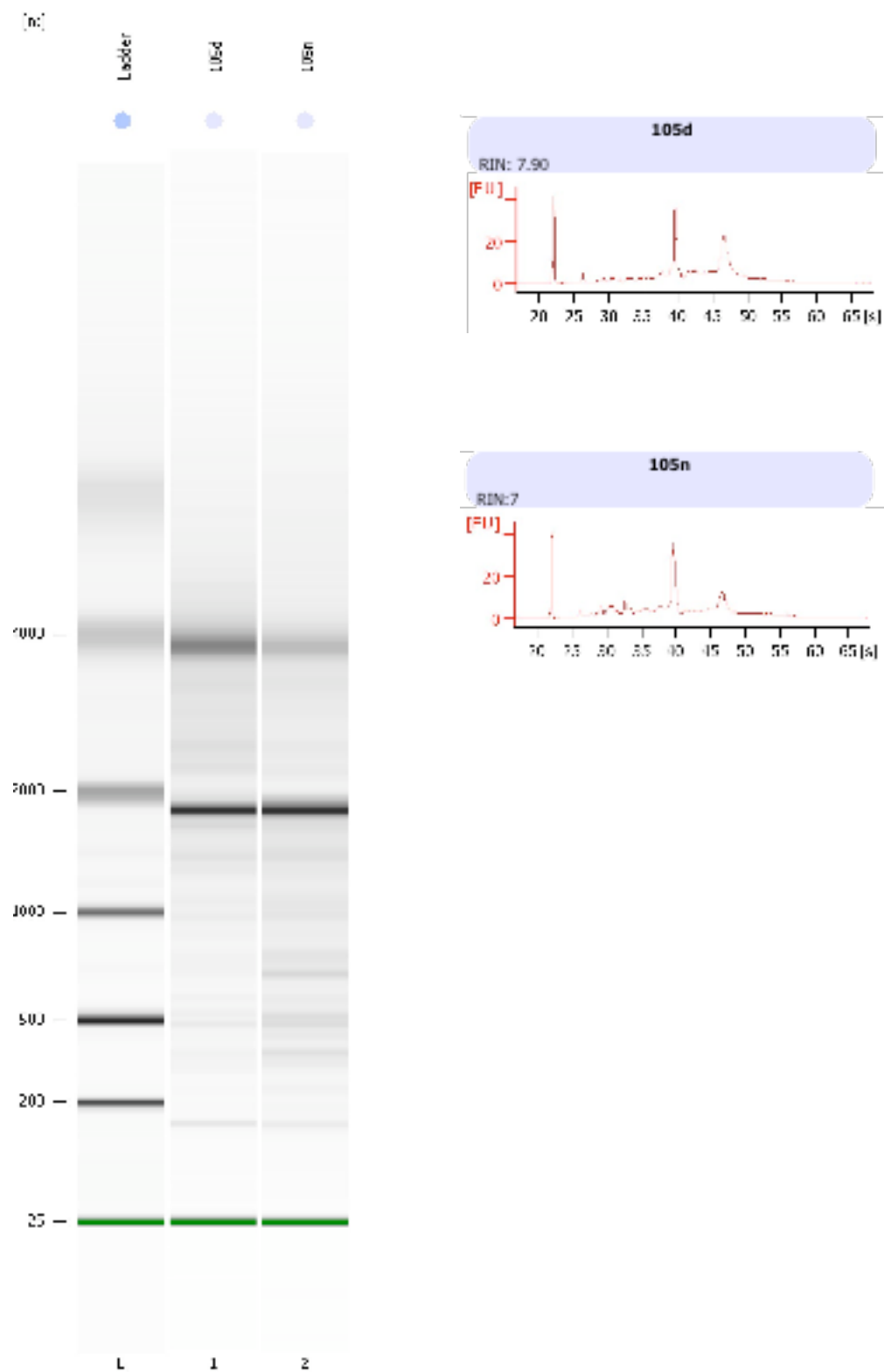
AB reagent = A + B + PBS (4 + 4 + 192µl)/slide

DAB = DAB concentrate + DAB diluent (1 drop + 1ml) 200µl /slide

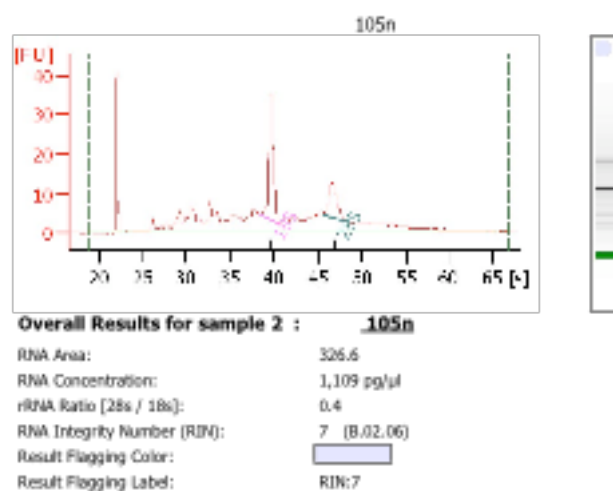
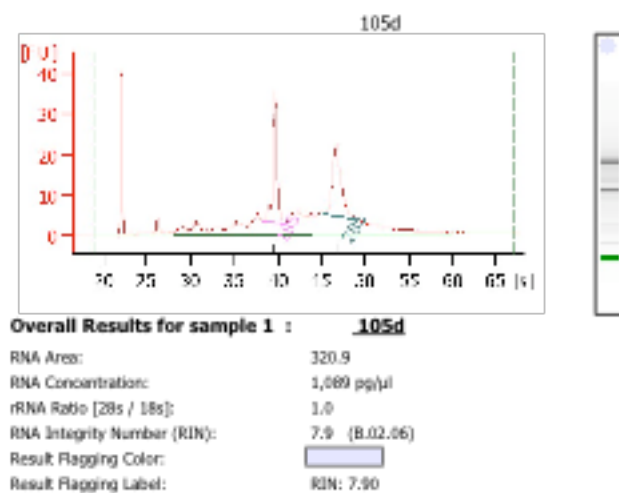
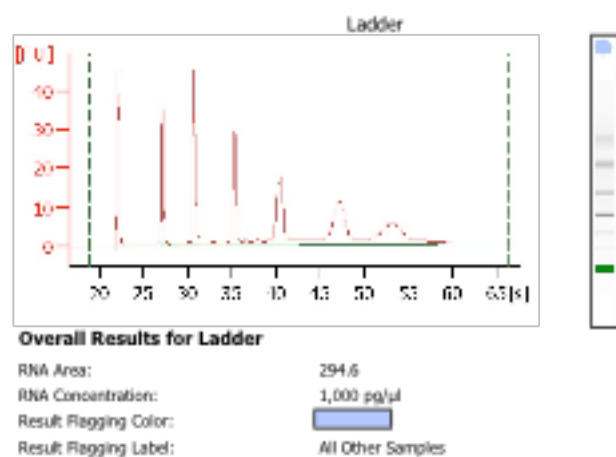
PI = PI + PBS (2 + 998µl) 200µl /slide

Appendix 4 Microarray Agilent Bioanalyser output extract

Electrophoresis File Run Summary



Electropherogram File Run Summary



Appendix 5 Microarray Results

**List of genes found to be differentially expression
between regions of damaged and undamaged cartilage
(Fold change>2; p=0.008)**

Gene Symbol	Up/Down regulation	Fold Change
IL11	up	15.866
LAMB1	down	15.274
GPNMB	down	9.536
ENST00000357303	up	8.755
SOX11	up	8.662
COL14A1	down	8.284
DNER	up	8.145
C15orf48	up	7.880
PDE1A	down	7.880
MMP2	down	7.212
PCSK1	down	6.841
IFI44	down	6.531
RFTN1	down	6.354
GFRA2	up	6.194
CXCL14	up	6.168
PLXDC1	down	6.016
GALNTL1	up	5.978

SERPINF1	down	5.824
BAALC	up	5.595
CDH11	down	5.506
OLFML3	down	5.471
MMP9	down	5.428
GPR124	down	5.277
HAVCR2	down	5.268
AY358804	down	5.258
TWIST1	down	5.164
IGF1	down	5.093
CCL2	down	5.033
BST2	down	5.029
FAM20A	down	5.012
ACOX2	down	4.947
LEF1	down	4.939
IL13RA2	down	4.885
MAN1C1	down	4.783
PLCL2	down	4.705
FGF18	up	4.685
WISP2	down	4.550
CASP1	down	4.536
CCL3	down	4.497
CRISPLD1	down	4.465
S100A2	up	4.413
THC2540738	down	4.391

Gene Symbol	Up/Down regulation	Fold Change
AYTL1	down	4.347
VIT	down	4.346
THC2668815	up	4.245
EBF3	down	4.237
CLIC3	up	4.233
TGM2	up	4.103
FLJ20035	down	4.023
THC2673862	down	4.006
KIAA1622	up	3.932
GRIA2	up	3.885
HLA-DPA1	down	3.871
COMP	up	3.864
ANGPTL4	up	3.843
MAFB	down	3.837
SEPP1	down	3.806
LAMA4	down	3.791
RNF152	up	3.774
CYFIP2	up	3.773
OR7E13P	up	3.766
IL4R	down	3.743
SVEP1	down	3.733
AK023574	down	3.704
TNFRSF11B	up	3.687
TNFSF13B	down	3.665

ARHGEF15	up	3.634
SERPINE2	up	3.616
DB381305	up	3.615
THC2667205	down	3.550
SPON1	down	3.542
DACT1	down	3.528
MEG3	down	3.523
HS3ST3A1	up	3.517
C2	down	3.515
HTRA3	up	3.479
FBLN1	down	3.463
ENST00000360879	up	3.462
PAQR9	up	3.449
LAMB3	up	3.436
LMO6	up	3.434
A_23_P170719	up	3.430
COL12A1	down	3.422
hCG_1815504	up	3.419
ADAMTSL2	up	3.400
FCGR2A	down	3.357
BTBD16	up	3.345
GLP2R	up	3.321
MARCKS	down	3.307
RUNX2	down	3.279
FSTL3	up	3.237
C4BPA	down	3.237

Gene Symbol	Up/Down regulation	Fold Change
STX1A	up	3.235
HTRA1	up	3.234
ARHGAP18	down	3.212
CADM1	down	3.204
LAMC3	up	3.197
MMP3	down	3.190
NOG	up	3.185
ATP8B4	down	3.179
TMSL3	down	3.165
C1orf54	down	3.134
AK098835	down	3.128
GABRE	down	3.119
MATN2	down	3.105
GPC4	down	3.103
CD55	up	3.095
PRUNE2	down	3.083
ABCA6	down	3.056
NLGN1	down	3.048
BNC2	down	3.046
GRASP	down	3.041
AK022044	down	3.030
USH1C	up	3.027
PCSK1N	up	3.024
CITED4	up	3.024
PSAT1	up	3.002
CHRD12	down	3.002

FLJ21963	down	2.999
PDGFRB	down	2.993
BMPER	down	2.987
S100P	up	2.982
CDH19	up	2.981
A_32_P68205	up	2.976
NKX2-5	up	2.971
BE710245	up	2.967
C19orf46	up	2.958
ZEB2	down	2.942
RASL11B	down	2.939
COL24A1	down	2.933
FLJ13236	up	2.929
SLC16A10	up	2.922
CILP	up	2.922
PDCD1	up	2.917
SLPI	down	2.912
SLC7A5	up	2.907
A_24_P511776	up	2.897
TRIM29	up	2.880
ST6GALNAC5	up	2.870
CENPP	up	2.864
STAB1	down	2.861
DKFZp761P0423	down	2.848
SPON2	down	2.813
TMSB4X	down	2.811
GBP1	down	2.792
AK026881	down	2.789

Gene Symbol	Up/Down regulation	Fold Change
ENST00000334647	down	2.770
TNFSF10	up	2.768
RHO	up	2.760
KLHL34	up	2.755
TLR4	down	2.749
KRT18P20	up	2.726
CA314451	down	2.707
SULF1	down	2.706
BC070125	down	2.705
KGFLP1	down	2.702
CAPS	up	2.698
BX427435	up	2.690
TMEM105	up	2.688
GATM	down	2.677
SLFN11	down	2.674
A_32_P121640	up	2.668
STAC2	down	2.668
C13orf33	up	2.666
ARHGAP28	down	2.654
TNNI2	up	2.648
FGF14	down	2.647
INHBA	up	2.620
MSX2	up	2.618
CDC42EP3	down	2.613
PSMB9	down	2.612
AF263545	down	2.597

LOC146439	up	2.589
AF143325	up	2.586
RAB11FIP4	up	2.585
C2orf39	up	2.582
AK094799	down	2.578
PRR16	down	2.572
BVES	up	2.567
C3orf52	up	2.563
AI801879	up	2.557
FAM80A	up	2.552
KIAA0367	down	2.551
SLITRK4	down	2.549
NT5E	up	2.546
BC037919	up	2.539
W91942	down	2.536
PTGS1	down	2.521
KCNG1	up	2.514
LY96	down	2.512
LEPREL1	up	2.502
RASSF7	up	2.502
ENST00000330640	up	2.499
CLCF1	up	2.496
CFHR3	down	2.494
FGD6	down	2.492
TPD52	up	2.491
ENST00000340381	up	2.490
ABCA1	down	2.490
COLEC12	down	2.489

Gene Symbol	Up/Down regulation	Fold Change
FN1	up	2.488
MGC20983	up	2.487
AI652176	up	2.486
TPM1	down	2.484
FMNL2	down	2.478
SAMD5	down	2.472
RPESP	up	2.462
AI754733	up	2.460
FMOD	up	2.458
SCUBE1	down	2.456
FRMD4A	down	2.449
SRPX2	up	2.448
THC2565608	up	2.445
IBRDC3	up	2.444
LOXL3	up	2.441
NRP1	down	2.436
SORT1	up	2.427
ZNF697	down	2.420
A_32_P15706	up	2.413
FRZB	down	2.412
MX1	down	2.409
IGSF3	up	2.408
LOC440731	up	2.404
CNN2	down	2.401
GADD45A	down	2.400
TMEM140	down	2.398

MYOZ3	down	2.389
CDH23	down	2.389
SDC3	down	2.384
AI916036	up	2.367
MERTK	down	2.353
hCG_1988300	up	2.350
HRASLS	up	2.348
FLJ30901	down	2.347
MSI2	up	2.342
MT1F	up	2.342
FABP5	down	2.341
RAB31	down	2.332
ENOX1	up	2.331
GLIS1	up	2.329
FGF7	down	2.327
DB518505	up	2.324
ANXA6	down	2.323
FZD5	down	2.310
C1orf113	up	2.310
ST3GAL1	up	2.307
FBXO16	up	2.306
LOC649344	up	2.306
C1orf170	up	2.305
CAMK1	down	2.305
A_24_P93111	up	2.303
ZNF365	up	2.302
TF	up	2.302
MAP4K4	down	2.297

Gene Symbol	Up/Down regulation	Fold Change
CB984746	down	2.297
ATF3	down	2.292
FLNA	down	2.291
AF161344	up	2.285
LOC648448	up	2.283
AF086329	up	2.282
B3GALNT1	down	2.281
C6orf208	up	2.279
LRRC8E	up	2.276
GALNTL2	up	2.276
SCARF2	down	2.263
TRPV2	down	2.262
A_32_P90468	down	2.262
MFI2	up	2.262
GPR162	down	2.251
SLC4A3	up	2.245
DDEF1	down	2.245
PALMD	up	2.245
SLC7A8	down	2.244
FXD2	up	2.238
KALRN	up	2.237
CFH	down	2.226
C14orf65	up	2.224
MSX2P	up	2.224
LOX	up	2.222
THC2645398	down	2.222

AL833309	up	2.222
IL18BP	down	2.221
SUV39H1	down	2.221
PRSS2	up	2.218
SEMA3B	up	2.217
PHTF2	up	2.217
PHKG1	down	2.207
SH2B3	down	2.207
OR7E156P	up	2.206
CENPP	up	2.203
GLUL	down	2.202
CDH3	up	2.201
KIAA1822	up	2.199
KIF13B	up	2.194
PLCB4	up	2.194
OR7E91P	up	2.189
ASCL5	up	2.187
A_23_P56868	up	2.187
SLC40A1	down	2.185
SPECC1	up	2.184
RUNX1T1	down	2.181
USP18	down	2.174
CPD	down	2.159
SAMD4A	up	2.157
MYLC2PL	up	2.156
AK025758	up	2.154
LOC196264	up	2.153
USP5	down	2.149

Gene Symbol	Up/Down regulation	Fold Change
SARDH	up	2.149
RPH3AL	up	2.149
DUSP10	down	2.148
SCUBE2	up	2.147
RALGPS1	up	2.143
SH3PXD2B	down	2.139
SORBS2	up	2.139
UNC13D	up	2.138
CCDC19	up	2.138
HRASLS5	up	2.136
MARVELD2	up	2.134
INPP4B	up	2.134
PIK3R1	down	2.132
C14orf139	down	2.130
PCDH12	down	2.128
A_24_P147849	up	2.126
ID2	up	2.124
FLJ14712	up	2.124
A_24_P255836	up	2.124
ULK3	down	2.120
A_32_P20613	up	2.114
ITGB8	down	2.113
C13orf18	down	2.108
TP53I11	down	2.106
PBEF1	down	2.106
FKBP5	down	2.102

ARSE	up	2.101
C9orf19	down	2.099
NTN1	up	2.097
NEFH	up	2.094
C1QTNF2	down	2.092
THC2624202	down	2.090
DSP	up	2.089
BM504117	up	2.087
RARRES3	down	2.087
GRK5	up	2.085
UBE2L6	down	2.084
CHST13	up	2.082
MFAP2	down	2.081
ENST00000370548	up	2.080
PDGFRA	down	2.075
LOC284998	up	2.073
TPCN1	down	2.072
ELL2	down	2.071
SDK2	up	2.071
GBA2	down	2.063
BEST1	down	2.051
GALNT8	up	2.051
HRASLS3	down	2.051
HGS	up	2.050
ATP1B1	up	2.046
AK055915	down	2.044
TBC1D24	up	2.042
SLC14A1	down	2.041

Gene Symbol	Up/Down regulation	Fold Change
ASNA1	down	2.039
GBP3	down	2.038
KAZALD1	up	2.035
MKS1	down	2.035
THC2683228	up	2.034
CDA	up	2.034
ZNF289	down	2.029
BTBD14B	up	2.029
C1S	down	2.027
CFHR1	down	2.024
VWA1	up	2.024
AJ420543	up	2.024
ETS1	down	2.023
TSPAN14	down	2.022

COL5A1	up	2.021
FLJ32255	down	2.021
A_23_P210297	up	2.019
THC2662587	down	2.019
PTPRE	up	2.017
GLTSCR1	up	2.016
ENST00000331096	up	2.015
RP11-262H14.4	up	2.014
ABCC8	up	2.012
C18orf1	up	2.010
BAP1	down	2.010
THBS3	up	2.008
KRT18P34	up	2.004

List of Gene Ontology groups shown to be significantly different between regions of damaged and undamaged cartilage (p<0.01)

GO Accession	GO Term	p-value
GO:0005576	extracellular region	2.09E-24
GO:0044421	extracellular region part	6.35E-18
GO:0031012	extracellular matrix	5.32E-17
GO:0005578	proteinaceous extracellular matrix	1.40E-16
GO:0006955	immune response	5.88E-15
GO:0002376	immune system process	8.39E-14
GO:0005576	extracellular region	8.39E-14
GO:0044421	extracellular region part	1.07E-10
GO:0005578	proteinaceous extracellular matrix	9.42E-10
GO:0031012	extracellular matrix	1.17E-09
GO:0002376	immune system process	1.85E-09
GO:0006955	immune response	4.62E-09
GO:0005576	extracellular region	1.10E-06
GO:0022610	biological adhesion	1.47E-06
GO:0007155	cell adhesion	1.47E-06
GO:0005615	extracellular space	2.80E-06
GO:0042613	MHC class II protein complex	3.58E-06
GO:0050896	response to stimulus	2.39E-05
GO:0022610	biological adhesion	3.76E-05
GO:0007155	cell adhesion	3.76E-05
GO:0048856	anatomical structure development	8.85E-05
GO:0042613	MHC class II protein complex	2.06E-04
GO:0005201	extracellular matrix structural constituent	2.06E-04
GO:0048731	system development	2.48E-04
GO:0007165	signal transduction	3.48E-04
GO:0005201	extracellular matrix structural constituent	4.43E-04
GO:0044259	multicellular organismal macromolecule metabolic process	5.36E-04
GO:0030574	collagen catabolic process	5.36E-04
GO:0032963	collagen metabolic process	5.36E-04
GO:0044254	multicellular organismal protein catabolic process	5.36E-04
GO:0044256	protein digestion	5.36E-04
GO:0044268	multicellular organismal protein metabolic process	5.36E-04
GO:0044266	multicellular organismal macromolecule catabolic process	5.36E-04
GO:0002252	immune effector process	6.28E-04
GO:0044236	multicellular organismal metabolic	6.95E-04

	process	
GO:0044243	multicellular organismal catabolic process	6.95E-04
GO:0042611	MHC protein complex	7.61E-04
GO:0044420	extracellular matrix part	8.16E-04
GO:0032502	developmental process	9.76E-04
GO:0044254	multicellular organismal protein catabolic process	0.001042428
GO:0044256	protein digestion	0.001042428
GO:0044259	multicellular organismal macromolecule metabolic process	0.001042428
GO:0032963	collagen metabolic process	0.001042428
GO:0044266	multicellular organismal macromolecule catabolic process	0.001042428
GO:0044268	multicellular organismal protein metabolic process	0.001042428
GO:0030574	collagen catabolic process	0.001042428
GO:0044421	extracellular region part	0.001189432
GO:0031012	extracellular matrix	0.001189432
GO:0005615	extracellular space	0.001229581
GO:0007154	cell communication	0.001264797
GO:0044243	multicellular organismal catabolic process	0.00149125
GO:0044236	multicellular organismal metabolic process	0.00149125
GO:0009611	response to wounding	0.001537514
GO:0050776	regulation of immune response	0.00162074
GO:0007275	multicellular organismal development	0.001699252
GO:0050778	positive regulation of immune response	0.001896418
GO:0009611	response to wounding	0.002584188
GO:0002253	activation of immune response	0.003176867
GO:0005578	proteinaceous extracellular matrix	0.003938509
GO:0048513	organ development	0.003996241
GO:0048583	regulation of response to stimulus	0.004877152
GO:0002253	activation of immune response	0.004974672
GO:0005509	calcium ion binding	0.005442143
GO:0050778	positive regulation of immune response	0.005442143
GO:0006954	inflammatory response	0.005805675
GO:0005044	scavenger receptor activity	0.00625266
GO:0050776	regulation of immune response	0.008448659