



**Do the intrinsic properties of the chondrocytes  
account for the difference in prevalence of OA in  
the Knee and Ankle?**

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## SUMMARY OF THESIS: POSTGRADUATE RESEARCH DEGREES

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<b>Full Title of Thesis</b>	Do the intrinsic properties of the chondrocytes account for the difference in prevalence of OA in the Knee and Ankle?

## Abstract

**Introduction** The lifetime prevalence of symptomatic osteoarthritis (OA) in the knee is < 44% compared to 3.4-4.4% in the ankle. This has led to the theory that ankle articular cartilage must have some degree of inherent resistance to osteoarthritis (OA), although it is unknown whether this is attributed to biomechanical and/or biochemical differences. It has been previously hypothesised that biochemical differences in ankle cartilage extracellular matrix composition confers this resilience. Interestingly, previous studies have demonstrated that the ankle is also more resistant to pro-inflammatory cytokine mediated degeneration. Therefore, this thesis aimed to further characterise the response of human ankle and knee cartilage to pro-inflammatory cytokines, commonly found in OA, to elucidate whether this may account for the inherent difference in OA prevalence between these two different joints of origin.

**Methods** Talar domes and femoral condyles donated by patients following amputation were harvested and full-depth articular cartilage explants cultured in the absence or presence of a combination of physiological 'low' and pathological 'high' concentrations of cytokines, namely Interleukin-1 alpha (IL-1 $\alpha$ ), Oncostatin M (OSM) and Tumour Necrosis Factor alpha (TNF $\alpha$ ) over a short-term (7 days) or long-term (28 days) culture period. Media was assessed for sulphated glycosaminoglycan loss (sGAG), lactate dehydrogenase, synthesis of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and synthesis and/or activation of the Matrix Metalloproteinases MMP-2 and 9.

**Results** A significantly higher proportion of sGAG loss, increased production of PGE<sub>2</sub> and NO, in addition to induction and activation of pro MMP-9 was observed for knee cartilage explants only; furthermore, significant differences between joints was independently observed following culture in 'high' concentration TNF $\alpha$ . Consistent patient specific heterogeneity was observed across all outcome measurements confirming the hypothesis that there is an 'inflammatory osteoarthritic phenotype'.

**Conclusion** Novel findings have identified that in the presence of cytokines, ankle cartilage chondrocytes do not synthesise significant levels of NO, PGE<sub>2</sub> or MMP-9, unlike that observed in the knee. In contrast, knee cartilage was most responsive to TNF $\alpha$  stimulation in inducing potential degenerative effects, therefore targeting of the TNF $\alpha$  pathway may aid production of bespoke biological treatments to prevent inflammatory knee OA. Further characterisation is necessary to elucidate the mechanism(s) that protect the ankle cartilage from cytokine insult; utilisation of this knowledge will undoubtedly inform on therapeutic approaches for consideration in treatment strategies for primary knee OA.

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To all the patients that have donated samples towards this project, your gracious gifts have not been squandered.

Lastly, to Alexa and my girls  
this, like everything I do, is for you.

## Abbreviations

COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
Da	Dalton
DMEM	Dulbecco's Modified Eagles Medium
DMMB	Dimethylmethylene Blue
DS	Dermatan sulphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACIT	Fibril associated collagens with interrupted triple helices
FBS	Foetal Bovine Serum
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
HA	Hyaluronan
HPR	Hydroxyproline
HS	Heparan sulphate
IL	Interleukin
kDa	Kilo Dalton
KS	Keratan sulphate
MMP	Matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
OA	Osteoarthritis
OSM	Oncostatin M
PBS	Phosphate buffered saline
PG	Proteoglycan
RNA	Ribonucleic acid
sGAG	Sulphated Glycosaminoglycan
SLRPs	Small leucine-rich proteoglycans
TGF- $\beta$	Transforming Growth Factor $\beta$
TNF- $\alpha$	Tumour necrosis factor $\alpha$

## Grants, publications, presentations and awards associated with this study

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### Publications

Miller A., Hodgson P., Blain E. Differential gene expression in ankle cartilage chondrocytes compared with knee: Might this explain the difference in prevalence of osteoarthritis in these joints and identify a potential treatment target? Bone and Joint Journal: Orthopaedic Proceedings 2017 Volume 99-B, Issue SUP 21

### Presentations

Date	Title	Meeting
May-2018	The difference between gene expression in Ankle and Knee Chondrocytes may have provided a target for the prevention of OA	Welsh Orthopaedic Society meeting
Nov-2017	Differential gene expression in ankle cartilage chondrocytes compared to knee: might this explain the difference in prevalence of osteoarthritis in these joints and identify a potential treatment target?	British Orthopaedic Foot and Ankle Society Meeting
Sep-2017	Ankle cartilage is more resilient to cytokine-induced catabolism than knee cartilage: A potential target for prevention of knee arthritis?	British Orthopaedic Association
Oct-2016	Ankle cartilage is more resilient to cytokine-induced catabolism than knee cartilage: A potential target for prevention of knee arthritis?	World Congress of Bone, Muscle and Joint Disease

### Awards

May-2018	Presidents Gold Medal Presentation	Welsh Orthopaedic Society
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Oct-2016	Young Scientist of the Year Award	World Congress Bone, Muscle and Joint Disease

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# Chapter 1

## Introduction

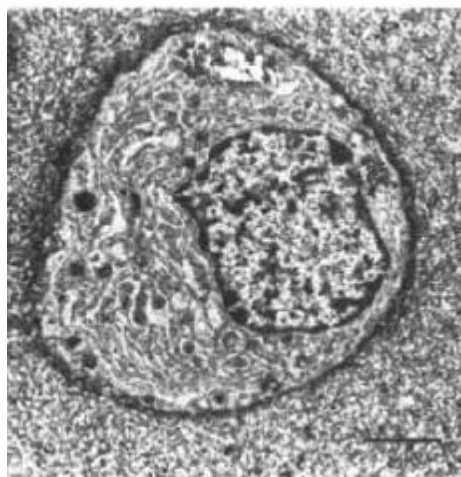
# 1 Introduction

## 1.1 Composition of Articular Cartilage

Hyaline cartilage or “articular” cartilage is a specialised tissue that facilitates low friction movement between joint surfaces in synovial joints. Cartilage is an avascular, alymphatic and aneural tissue that comprises predominantly of extracellular matrix (ECM) with chondrocytes sparsely spread throughout. The tissues’ biological composition confers biomechanical functionality in providing: a low-friction gliding surface, acting as a shock absorber and minimising peak pressures on the subchondral bone (Bhosale and Richardson, 2008); these mechanical properties are imparted mainly by the collagen and proteoglycans that make up the ECM.

## 1.2 Chondrocytes

Cartilage is mesenchymal in origin and is first seen *in utero* at 5 weeks of gestation. Chondroblasts in the blastema start to produce ECM, separating the cells. Once totally separated by primitive matrix, the cells become chondrocytes. In healthy adult articular cartilage the cellular volume comprises between 5-10% of the total cartilage volume (Lin et al., 2006).



**Figure 1.1:** Metacarpal phalangeal chondrocyte taken with a transmission electron microscope (>13 500x magnification); Modified from Muir, 2005.

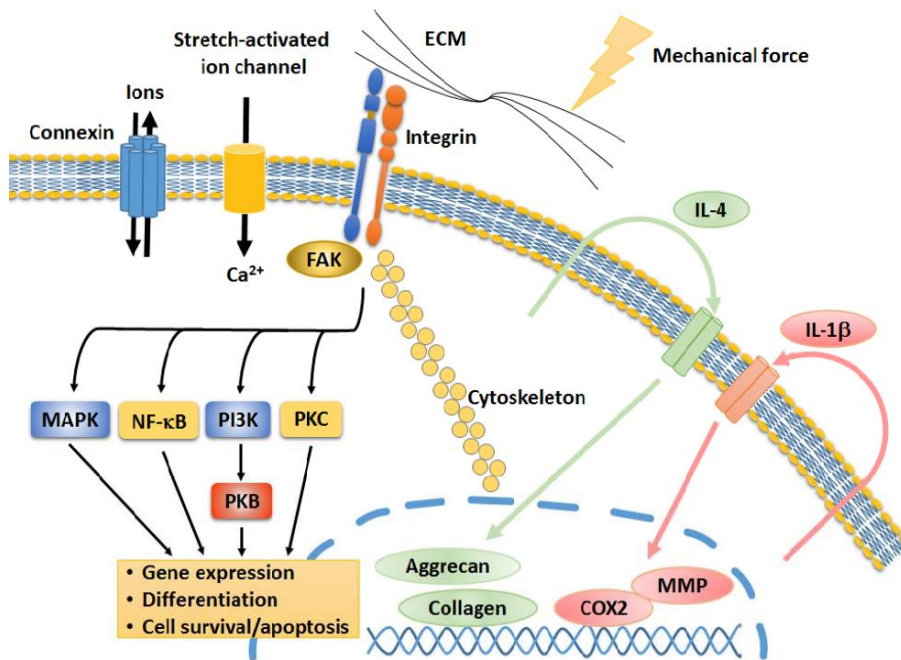
In the absence of mechanical load, chondrocytes are spherical with a diameter of around 13µm and are isolated in lacunae. The cells have a single nucleus in humans and well developed golgi apparatus (Figure 1.1) which become unusually large during matrix synthesis (Muir, 1995). The chondrocytes' role is to maintain homeostasis of the ECM by "*mediating synthesis, assembly and degeneration of the matrix proteins*" (Lin et al., 2006), the main two components of which are type II collagen and aggrecan. Furthermore, cell-matrix interaction is a reciprocal process. The chondrocyte is responsible for production and homeostasis of the ECM whilst the matrix components themselves influence cell migration, adhesion, differentiation and survival.

The metabolism of the chondrocyte is predominantly anaerobic as would be expected given that the deep zone of cartilage has oxygen tensions as low as 1%. Interestingly however, the cells will preferentially continue to use glycolysis to produce lactate even in the presence of an aerobic environment (Muir, 1995). These resilient chondrocytes are capable of living as long as their occupant and retain the ability to divide at any stage in response to disruption of the local collagen network.

Single or small groups of chondrocytes are encapsulated in a network of fibrils containing type VI, II and IX collagens called chondrons. These chondrons appear to be compression-resistant fluid-filled bladders that dampen mechanical, osmotic and physico-chemical changes induced by dynamic loading (Muir, 1995). Chondrons are consistently orientated parallel to compressive forces, but the number of cells within each chondron is variable between joints (Kuettner and Cole, 2005).

### 1.3 Cell-matrix Interactions

Chondrocytes have an important mechanotransductive function with physiological dynamic load promoting matrix production and excessive, reduced or static compressive load resulting in matrix degeneration. Mechanical load is perceived by the pericellular matrix and mechano-signals transmitted to the cell via mechanoreceptors including integrins, connexins, stretch activated ion channels and the primary cilia.



**Figure 1.2:** The major mechano-signalling pathways identified in chondrocytes. Modified from Lee and Salter (2015).

These mechanoreceptors are stimulated by the mechanical forces transduced via the ECM. One of the most well characterised is the integral membrane complex proteins or “integrins” which are transmembrane receptors that bind ligands such as collagen and fibronectin fragments, the critical mechanoreceptor being  $\alpha 5 \beta 1$  integrin in chondrocytes (Lee and Salter, 2015). They transmit mechanical signals directly to the cytoskeleton and ligand-integrin complexes increase intracellular calcium and tyrosine phosphorylation.  $\beta 1$  integrins, found predominantly in the chondrocyte membrane, have been strongly implicated in the disease process associated with osteoarthritis (OA). Fibronectin-integrin and collagen-integrin complexes reduce chondrocyte apoptosis and are down regulated in OA. TGF-integrin reduces cellular adhesion and prevents retention at cartilage defects, while IL-1 binds to integrins and down regulates ECM production (Gao et al., 2014). Downstream signal transduction pathways involve the cytoskeleton and signalling molecules, including FAK, PKC, PI3K, PKB, NF- $\kappa$ B, and MAPK, which act to regulate gene expression, cell function and survival/apoptosis (Lee and Salter, 2015).

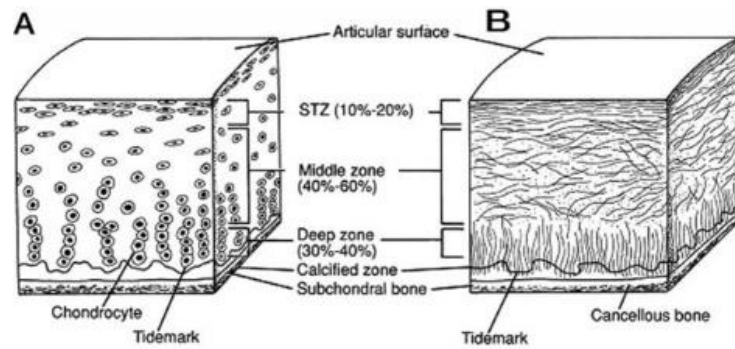
Chondrocytes are also influenced by autocrine, paracrine and endocrine systems (although the endocrine system is relatively ineffective due to the avascular nature of cartilage and requirement for any hormone to diffuse through the dense ECM before reaching the cells). The majority of these autocrine and paracrine factors are integrin mediated or released in response to cell membrane deformity via stretch-activated channels. Growth factors and anabolic cytokines have been shown to increase matrix production and antagonise their catabolic counterparts. Prostaglandins, particularly PGE<sub>2</sub>, and nitric oxide (NO) production are inhibited in response to beneficial cyclical load and increased with detrimental static load or offload. Interleukins can be both beneficial and destructive; for example, IL-4 is considered beneficial as it can upregulate aggrecan and MMP3 mRNA (Lee and Salter, 2015). However, IL-1 $\beta$  has been shown to be detrimental as it increases the release of sulphated glycosaminoglycans from bovine cartilage tissue (Stabellini et al., 2003) and in human cartilage, inhibits sGAG production, increasing matrix metalloproteinase (MMP) activation and suppressing type II collagen production (van der Kraan and van den Berg, 2000). Catabolic cytokines such as IL-1 may also have a secondary effect of producing NO, which leads to chondrocyte apoptosis. TNF $\alpha$  and IL-1 are both known to activate aggrecanases – the principal enzymes involved in aggrecan degradation, however it seems that Oncostatin M (OSM), one of the IL-6 family, acts synergistically to increase aggrecan and hyaluronic acid degeneration (Durigova et al., 2008) and reduce collagen synthesis (Cawston et al., 1998).

#### 1..4 Articular Cartilage Morphological Zones

Articular cartilage, comprising chondrocytes dispersed within an extensive ECM, receives nutrition via diffusion from the adjacent synovial fluid. Within mammalian cartilage there are distinct morphological zones: superficial, middle and the deep zone, before the cartilage is calcified and becomes subchondral bone (Figure 1.3). Each of these zones contains: a pericellular region around the chondrocyte, a territorial region surrounding this with a basket like weave of collagen fibrils and an interterritorial



region containing the majority of the proteoglycans with well organised collagen fibrils. Each zone varies in its ECM composition, collagen orientation, chondrocyte shape and distribution. These zone specific adaptations are thought to confer zone specific biomechanical properties through the tissue depth (Sophia Fox et al., 2009).



**Figure 1.3:** Diagram A – Chondrocyte orientation and shape in zones of articular cartilage, Diagram B – Collagen orientation in different zones of articular cartilage. Modified from (Buckwalter et al., 1994).

### 1..5 The Superficial “tangential” Zone

The superficial zone has relatively low proteoglycan content and the predominantly type II and IX collagen is orientated parallel to the joint surface. The disc shaped chondrocyte are orientated in chondrons that contain multiple cells in the ankle but only single cells in the knee and all secrete superficial zone protein (SZP). This protein is made only by chondrocytes in this superficial zone and does not incorporate into the ECM (Schumacher et al., 1994). It is believed that SZP is “a multifunctional protein with potential growth-promoting, cytoprotective and lubricating properties” (Flannery et al., 1999). The Superficial zone normally comprises only 10-20% of the total depth of the cartilage but protects the deeper layers from the majority of the “sheer and tensile forces” (Sophia Fox et al., 2009).

### **1..6 The Middle “transitional” Zone**

The middle zone makes up between 40% and 60% of the total thickness of the cartilage depending on whether the tissue is isolated from the ankle or knee (Oegema et al., 2003). The type II collagen is obliquely orientated and rotates through 90 degrees from a parallel to perpendicular orientation. The spherical chondrocytes are more sparsely orientated and the ECM has a much higher concentration of proteoglycans (Sophia Fox et al., 2009).

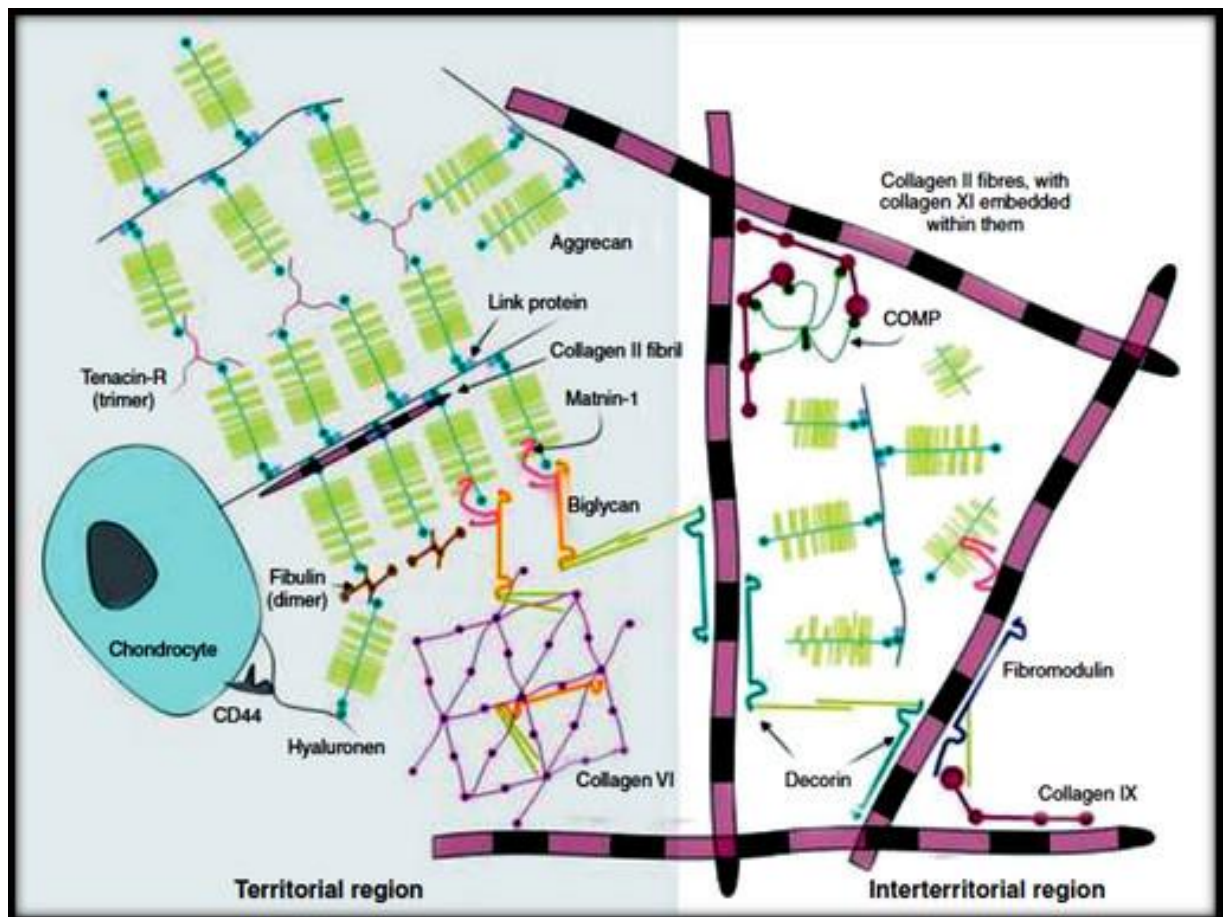
### **1..7 The Deep “radial” Zone**

The deep zone comprises about 30% of the total cartilage thickness. It has the highest concentration of proteoglycans, lowest water content and large diameter perpendicularly orientated collagen fibrils. This zone is responsible for resisting the majority of the compressive force imparted upon it during axial loading.

### **1..8 The Zone of Calcified Cartilage**

The main function of this zone is to anchor the cartilage to the bone and is found deep to the tidemark with chondrocytes becoming hypertrophic.

## 1.9 Extracellular Matrix



**Figure 1.4:** Schematic diagram of cartilage components and their interactions. Its major components are: proteoglycans (e.g. aggrecan), smaller proteoglycans (e.g. biglycan, decorin, fibromodulin) collagens (mainly Type II) and non-proteoglycans (e.g. cartilage oligomeric matrix protein (COMP) and link proteins). The territorial region surrounding the pericellular region of the chondrocyte is proteoglycan-rich compared to the interterritorial region which is located between the territorial matrices. (Dudhia, 2005)

The extracellular matrix comprises roughly 80% of the weight of cartilage with the vast majority of that weight being water retained by the strong negative charge of the proteoglycans. The remaining organic contents are made of proteoglycans such as aggrecan, collagen fibrils, particularly type II collagen but also Type IX and XI, small proteoglycans such as biglycan and fibronectin and non-proteoglycan molecules, such as Cartilage Oligomeric Matrix Protein (COMP).

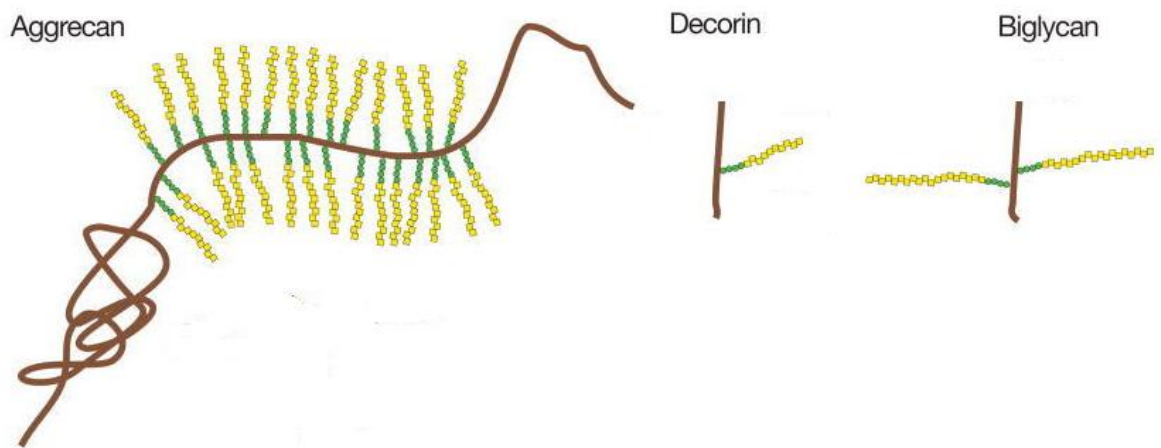
## 1.10 Proteoglycans

### 1.11 Glycosaminoglycans (GAG)

Initially named mucopolysaccharides, glycosaminoglycans have been studied for around 50 years. As our understanding of their involvement in specific disease processes improves more attention has been paid to congenital “glycosylation” disorders, biosynthesis and degeneration of the extracellular matrix. The main role of these highly negatively charged structures is to attract and bind water to produce cartilages’ compressive and lubricating properties.

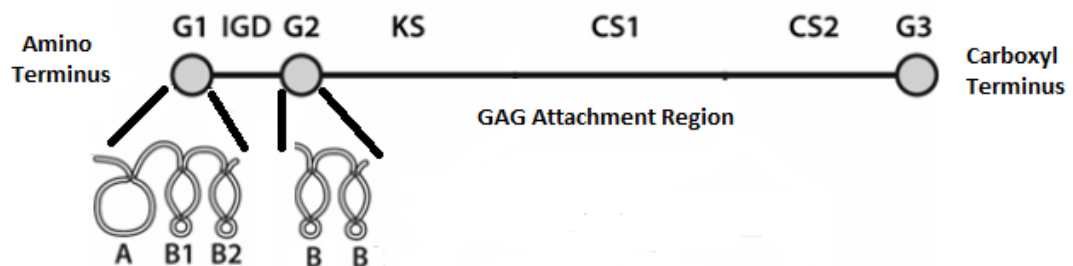
More recently, improved scientific procedures, such as density-gradient ultracentrifugation, has enabled the structure of hyaluronan and the sulphated glycosaminoglycans (Keratin Sulphate, Chondroitin Sulphate, Dermatan Sulphate and Heparan Sulphate) to be identified (Esko JD, 2009). These polysaccharide chains are made of a repeated disaccharide unit containing a uronic sugar or galactose in addition to an amino sugar (N-acetylglucosamine or N-acetylgalactosamine). Huge heterogeneity is seen between glycosaminoglycans as they are enzymatically modified, not produced by templating like proteins. However, it is still possible to classify the molecules according to gross disaccharide structure and sulphation. Chondroitin sulphate (CS) is the most abundant, heparan sulphate (HS) closely resembles heparin, the most negatively charged molecule known to man, keratan sulphate (KS) is fucosylated and doesn’t contain an amino sugar, while hyaluronic acid (HA) is not sulphated.

Glycosaminoglycans covalently bind to core proteins to produce proteoglycans. There can be anywhere between one GAG bound to the core protein, as is the case with decorin, or over a hundred GAGs bound to a single core protein, as is the case with aggrecan (Figure 1.5). The function of proteoglycans is determined by the number and type of these highly charged GAG chains, the structure of the core protein (Roughley, 2006) and the subsequent aggregates that are formed.



**Figure 1.5:** Proteoglycans consist of a core protein (Brown) covalently bound to glycosaminoglycan chains (Yellow). Proteoglycans can have as few as one side chain such as Decorin or more than 100 such as Aggrecan (Modified from Esko JD, 2009).

### 1.12 Proteoglycan core protein



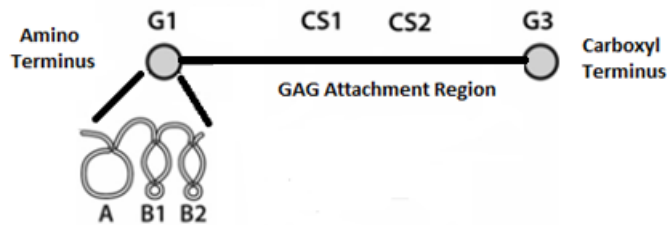
**Figure 1.6:** The core protein of aggrecan. This core protein contains 3 Globular zones (G1, G2, G3), an interglobular domain (IGD) and GAG Attachment Region that is subdivided into Keratin Sulphate (KS), Chondroitin Sulphate 1 (CS1) and Chondroitin Sulphate 2 (CS2) binding sites (Modified from Roughley 2006).

The structure of the proteoglycan core protein is highly variable but relatively well preserved across mammalian species. Figure 6 depicts the basic structure of the aggrecan core protein. It contains 3 Globular zones (G1, G2, G3) made of cysteine residues that permit disulphate binding (Sandy et al., 1990). Each globular zone

contains specific subdomain regions that allow aggregation and further binding. Globular zone 1 is located at the amino terminus (NH<sub>2</sub>) and has 3 subdomain regions: region A allows bonding with link proteins (LP), B1 and B2 are responsible for Hyaluronic acid binding. Between G1 and G2 lies the interglobular domain a 150 residue section that is one of the five metalloproteinases cleavage sites (Tortorella et al., 1998). The second globular zone contains B subdomain regions but these are not known to bind hyaluronic acid or link proteins. This globular zone is unique to aggrecan and is strongly conserved during degradation (Aspberg, 2012) The GAG attachment region between G2 and G3 is subdivided into keratan sulphate binding (KS) and two distinct chondroitin sulphate (CS1 and CS2) binding zones. The CS2 region contains four matrix metalloproteinases cleavage sites and the length and density of GAG in this section is hypothesised to influence an individual's risk of developing osteoarthritis (Roughley and White, 1980). The third globular zone is at the carboxyl terminus and has four subunits: 2 Epidermal-like Growth Factors (EGF), a C type domain and a Complement Regulating Protein repeat (CRP). The C type domain binds carbohydrate, including GAGs, in the extracellular matrix and is responsible for facilitating GAG chain attachment and enhancing secretion. The roles of the remaining domains are unclear (Kiani et al., 2002).

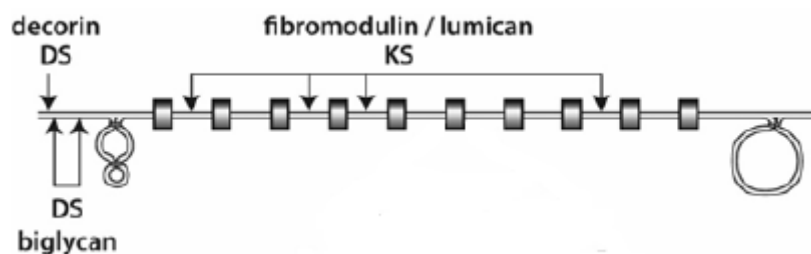
The core protein of versican (Figure 1.7) is very similar to that of aggrecan but does not contain the G2 domain. It also has significantly less GAG binding sites with between 12-15 chondroitin sulphate molecules attached to each core protein depending on which isoform is examined. Both Aggrecan and Versican are "Hyalectins" as the G1 attachment stabilised by the link protein binds hyaluronic acid allowing large aggregates to form. The much smaller GAG binding zone can be single or split into two but contains far fewer GAGs than aggrecan. While the G3 or carboxyl end of the core protein is almost identical to that of aggrecan with 2 Epidermal-like Growth Factors (EGF), a C type domain and a Complement Regulating Protein repeat (CRP) it seems to have additional properties that enhance cell proliferation and inhibit differentiation. Experimental deletion of the EGF regions at the G3 domain in extra-articular Versican has been shown to inhibit cell proliferation in astrocytes (Wu et al., 2001) and the

carboxyl end has been shown to modulate cellular apoptosis by binding to beta-1-integrin in glial cells (Wu et al., 2002).



**Figure 1.7:** The core protein of versican. This core protein contains 2 Globular zones (G1, G3) and a GAG Attachment Region for between 12 and 15 Chondroitin Sulphate GAGs. The GAG attachment region can be single, split into two or (Modified from Roughley 2006).

The small leucine-rich repeat proteoglycans share a common core protein structure which is very different to the larger aggrecan and versican core proteins (Figure 1.8). Between each of the ten leucine rich repeats is a cysteine cluster that forms a disulphide bonding domain capable of attaching to GAGs. There is a type B subdomain located near the  $\text{NH}_3^-$  terminus and a type A subdomain at the carboxyl end. The dermatan sulphate proteoglycans, decorin and biglycan, have one or two DS GAGs attached between the amino terminus and the type B subdomain. Keratan sulphate proteoglycans, fibromodulin and lumican, have between one and four KS GAGs attached to the disulphide bond domains between the leucine rich repeats.



**Figure 1.8:** Common core protein of the small leucine-rich repeat proteoglycans (decorin, biglycan, fibromodulin and lumican). Square boxes depict leucine-rich repeats between cysteine clusters that form disulphide bonding domains for GAGs (Modified from Roughley 2006).

### 1.13 Hyaluronic Acid

Hyaluronic acid is a non-sulphanated glycosaminoglycan made by the hyaluronan synthase enzyme in the cell membrane (Weigel et al., 1997). The long polysaccharide structure consists of glucuronic acid and N-acetyl Glucosamine disaccharide repeats made from cytoplasmic monosaccharides. The hyaluronic acid molecule exits the cell via pores in the cell membrane and forms large aggregates with the hyalectans, aggrecan and versican, in the pericellular region. It remains unclear, however, how this large aggregate moves to the territorial and interterritorial zones.

### 1.14 Proteoglycans of Articular Cartilage

Proteoglycans of articular cartilage can be classified as large aggregating proteoglycans such as aggrecan and versican, which bind to hyaluronic acid via a link protein, and non-aggregating proteoglycans. Non-aggregating proteoglycans can be either small leucine rich repeat proteoglycans (SLRP) or other non-specific proteoglycans, such as perlecan or lubricin (Table 1.1).

Aggregating	Non Aggregating	
	Small Leucine-rich repeat	Other
Aggrecan	Biglycan	Perlecan
Versican	Decorin	Surface Zone Protein or Lubricin
	Epiphygan*	
	Fibromodulin	
	Lumican	

**Table 1.1:** Classification of Proteoglycans



### 1.15 Aggregating Proteoglycans

#### 1.16 Aggrecan

Aggrecan is the most abundant proteoglycan in articular cartilage comprising almost 10% of the overall weight of the tissue. Aggrecan forms huge aggregates with hyaluronic acid by attaching via non-covalent bonds, and is stabilised by link proteins at the N terminus (Kiani et al., 2002). This large aggregating proteoglycan produces the typical viscoelastic properties of cartilage by creating an osmotic potential and through its interactions with collagen. The osmotic action occurs due to the high concentration of negatively charged glycosaminoglycans in the GAG attachment region. This negative charge attracts positively charged sodium ions and thus water follows, causing aggrecan to swell and expand. The hydrated aggregate produces the biomechanical resistance to compression and resilience to deformation that is typical of healthy cartilage. The tensile strength of cartilage is a result of the close association of these aggregating proteoglycans to collagen. It was initially believed that there was no direct bond between aggrecan and collagen itself but it is now felt that the junction of the G2 region and the Keratan sulphate section of the proteoglycan creates multiple weak molecular bonds directly with type II collagen (Hedlund et al., 1999).

#### 1.17 Versican

Versican is present in a large variety of different connective tissues and is far less abundant in cartilage than aggrecan. Versican is a member of the hyalactin family, binding to hyaluronic acid via the G1 region and stabilised by a link protein in a similar manner to aggrecan. In addition to attracting water and producing cartilage resistant to compressive load, versican has a role in cell adhesion, proliferation and ECM assembly. Versican is believed to modulate the inflammatory process by binding directly to the CD44 cell surface receptor of inflammatory leukocytes via the chondroitin sulphate chains and link protein on the protein core. It may also indirectly influence proliferation of chondrocytes by creating a more rigid environment immediately around the cells, thus maintaining a proliferative phenotype (Wight, 2002).

### 1.18 Non-aggregating Proteoglycans

Non-aggregating proteoglycans can be classified as either SLRPs or other non-aggregating proteoglycans depending on the structure of their core proteins. The SLRPs share a common ten leucine repeat core protein, while the other non-aggregating proteoglycans do not have a common core protein structure. Although these non-aggregating molecules do not exert the same osmotic forces as that of aggrecan and versican they remain essential to the normal development of connective tissue and bone.

### 1.19 Small Leucine Rich Repeat Proteoglycans

The SLRPs can be divided into dermatan sulphate or keratan sulphate proteoglycans. Dermatan sulphate proteoglycans such as decorin and biglycan contain either one (decorin) or two (biglycan) dermatan sulphate glycosaminoglycans near the amino terminus. Keratan sulphate proteoglycans such as lumican and fibromodulin have between one and four keratan sulphate glycosaminoglycans attached to the disulphide bonds between leucine rich repeats. Epiphykan is a third class of SLRP that has a core protein containing between 6 and 8 LRRs and are glycosylated with chondroitin or dermatan sulphate. Epiphykan is however unique to epiphyseal cartilage and present only in immature cartilage. The SLRPs play a key role in collagen fibrillogenesis and are believed to regulate collagen fibril diameter (Kalamajski and Oldberg, 2010). They are also able to block access of collagenases to specific collagen cleavage site preventing proteolysis, and store growth factors such as EGF, TGF $\beta$  and TNF before releasing them to chondrocytes (Ni et al.). SLRPs are essential to the development of normal connective tissue and bony development. Decorin and lumican knockout mice exhibit skin laxity secondary to irregular or excessively large collagen fibrils (Chakravarti et al., 1998) biglycan knockouts display an osteoporotic phenotype (Xu et al., 1998) and fibromodulin knockouts produce abnormal tendon collagens (Chakravarti, 2002)

### 1.20 Others

Perlecan is a non-aggregating proteoglycan with a core protein that does not contain multiple leucine rich repeats. The core protein contains 5 major domains and allows binding of 3 glycosaminoglycan chains of predominantly heparin sulphate near the amino terminus. Perlecan is seen in greatest concentration in the pericellular ECM and is believed to act as a reservoir regulating the exposure of chondrocytes to growth factors (R. Gomes, 2002) and is essential to the normal development of cartilage and the hypertrophic zone of the physis. Proteoglycan 4, previously known as Surface Zone Protein (SZP) or Lubricin, is produced by superficial articular side chondrocytes and synovial tissue. It helps to maintain boundary lubrication of articulating joints (Reesink et al., 2016) and has a role in synovial fluid homeostasis. Controversially however, proteoglycan 4 may not in fact be a true proteoglycan as not all isoforms are substituted with a glycosaminoglycan chain (Lord et al., 2012).

### 1.21 Collagens

The word “collagen” is derived from two Greek words meaning “Glue Producing” after the practice of boiling down animal bones to make glue. Collagen when denatured will produce a gelatinous substance but forms a fibrillar and microfibrillar scaffold in native form. The ECM is built around this network of fibrils and is the most abundant protein in all connective tissues.

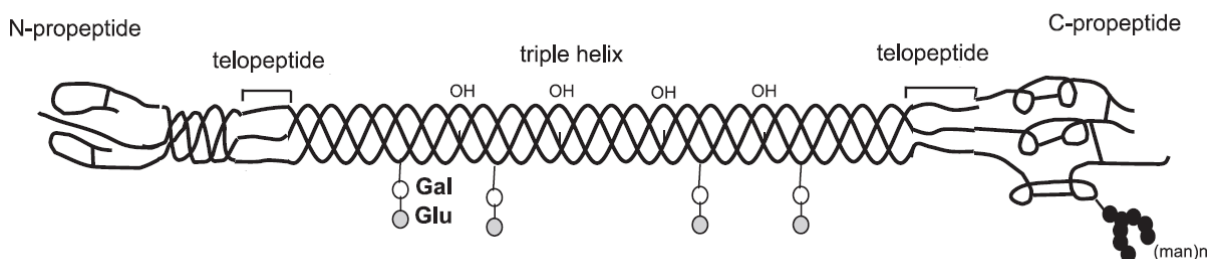
The biomechanical properties of connective tissues is determined by the “arrangement of fibrillar element, microfibrillar network as well as soluble proteins [and] glycoproteins” (Gelse et al., 2003) . The components of the ECM are determined by the “resident” cell expression and synthesis. Reciprocally, extracellular matrix feeds back to the cell via a number of different mechanisms. Collagen fibrils bind to cell-surface receptors and mediate cellular attachment, migration, differentiation and gene expression. The amount and orientation of collagen fibrils in the pericellular matrix also plays a role in protecting or exposing the cell to mechanical stimuli which alters cellular phenotype and subsequent function. Certain pericellular collagens are also known to act as a reservoir for growth factors which, when released, alters cellular “morphogenesis and metabolism” (Gelse et al., 2003).

Collagen is a heterogenous group of proteins, but all members form a triple helix structure of proline rich tripeptides (Gly-X-Y). Each super-molecule contains three alpha chains which can be either identical (homotrimers) such as type II collagen, or different (heterotrimers) such a type I collagen. The three chains form a right-handed helix that passes through 360 degrees every 18 amino acids. The small glycine residue is central within the spiral, with the larger residues, such as lysine and proline, arranged peripherally to allow close packing of the spirals. These peripheral proline residues confer additional stability to the helical structure as they form interstrand hydrogen bonds after hydroxylation.

So far 26 different collagens have been identified and grouped according to structure and organisation. The main groups are:

- Fibril-forming (Type I, II, III, IV and XI)
- Fibril-associated collagens (Type IX, XII, XIV)
- Microfibrillar (Type VI)
- Short Chain (Type VIII, X)
- Basement membrane (Type IV)

Fibril forming collagen is by far the most abundant and represents around 90% of all collagens. The fibrillar structure of type 1 collagen is shown (Figure 1.9).



**Figure 1.9:** Fibrillar structure of type 1 collagen. Triple helix central with subdomains bound to this. Non-helical telopeptides followed by terminal propeptides. procollagenases cleavage sites are at the terminal end of the telopeptides. (Modified from Gelse et al., 2003)

## 1..22 Synthesis

Collagen synthesis is a complex process involving intracellular post-transcriptional processing and extracellular enzymatic modifications. Following transcription of one of the 34 known “COL” genes, mRNA enters the cell cytoplasm from the nucleus. Ribosomal subunits link to the mRNA strand producing a peptide called the signal sequence at the amino terminus of the molecule. This specific sequence on the newly formed pre-pro-peptide correlates with a signal recognition particle within the endoplasmic reticulum. The pre-pro-collagen then undergoes three modifications to become pro-collagen. First, the signal sequence is removed to form a pro-peptide, then vitamin C-mediated enzymatic hydroxylation of lysine and proline occurs producing cross-linking of alpha peptides. The hydroxylysine molecules are then glycosylated with either glucose or galactose monomers. The pro-peptides then twist together in a left-handed spiral to form pro-collagen. The final post translational modification then occurs in the golgi apparatus when oligosaccharides are added to the pro-collagen before they are secreted out of the cell. Once in the extracellular space, membrane bound collagen peptidases remove the unwound ends of the pro-collagen producing tropocollagen. Collagen fibrils are produced when the tropocollagens are covalently bound to each other as the lysine and hydroxylysine residues are enzymatically oxidised to aldehyde groups.

## 1..23 Collagens of Articular Cartilage

Collagen comprises 2/3 of the dry weight of articular cartilage with the predominant type being type II collagen. The tensile strength produced by the collagen-rich tissue is related to the degree of crosslinking between collagen chains and the orientation of the collagen fibres. In developing cartilage, type II collagen is found in close proximity to type IX and XI collagen in a heteropolymer at a ratio of 80:10:10, but as the tissue matures the relative quantities of type IX and XI reduce as more type II is produced. It has also been noted that finer fibres closer to the chondron of the chondrocyte have higher proportions of Type IX and XI while courser fibres found in the territorial and

interterritorial regions have less type IX and XI collagen (Eyre, 2002) . It is hypothesised that this microstructure of collagens traps proteoglycans, which produce an osmotic potential drawing more water into the cartilage and altering the biomechanical properties of the tissue.

Type III collagen is co-localised to type II collagen in the superficial and middle zones and may be produced by chondrocytes as a response to injury. There is some evidence to support the theory that it may act as a “covalent modifier that may add cohesion to a weakened existing collagen” (Wu et al., 2010). Other much less abundant collagens such as short chain type X collagen are found predominantly at the tidemark as it plays a role in calcifying cartilage. The function of microfibrillar type VI and fibril-associated type XII and XIV collagens remain to be determined.

#### 1..24 Collagen degeneration

Very little collagen synthesis occurs in mature articular cartilage, with the estimated turnover time of femoral head cartilage being over 400 years (Maroudas, 1979). The majority of collagen turnover occurs in the pericellular region, while the collagen fibres in the territorial and interterritorial region undergo almost no remodelling under normal conditions. The limited ability of collagen to be remodelled following acute injury and chronic degeneration is one of the factors contributing to the development of osteoarthritis.

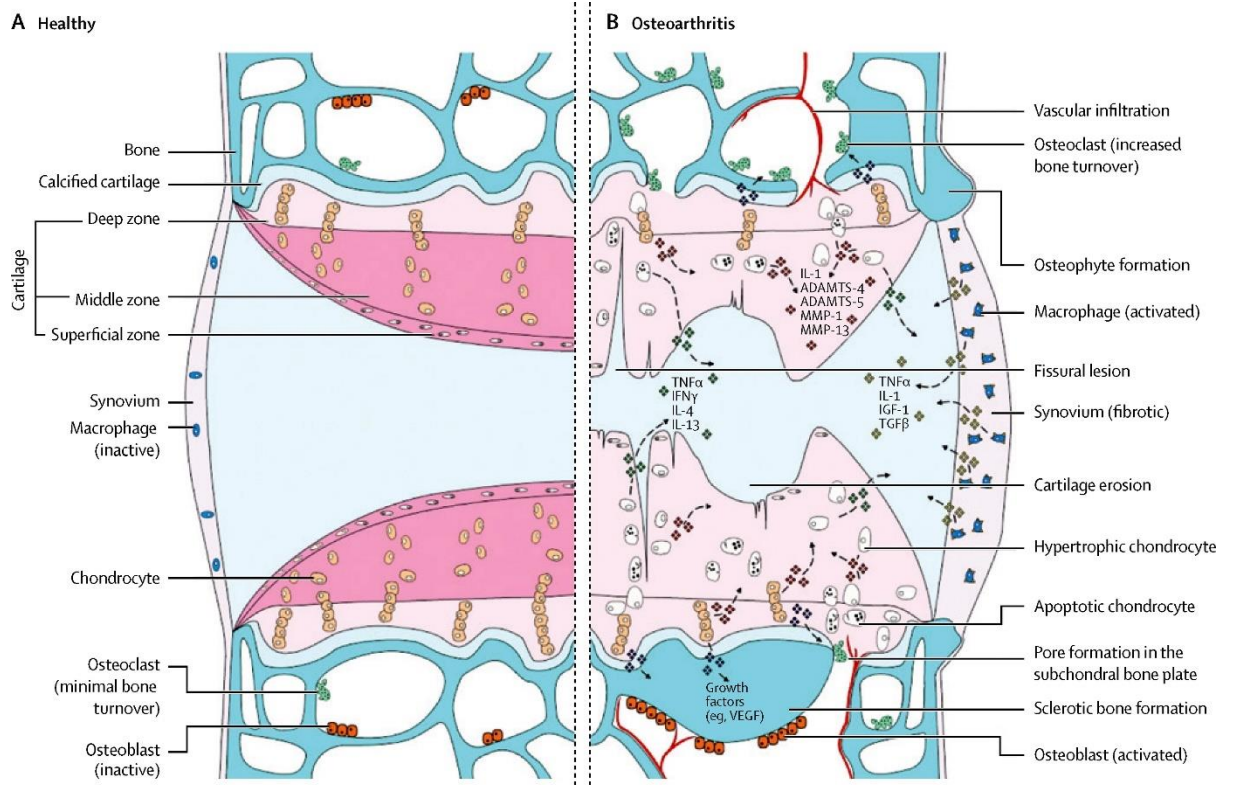
#### 1..25 Osteoarthritis

Osteoarthritis (OA) is a chronic musculoskeletal disorder that affects all parts of diarthrodial joints including the synovial membrane, ligaments, tendons, cartilage and bone. Contrary to the initial theory that this is a disorder of “wear and tear”, initial manifestation are in fact secondary to abnormal joint tissue metabolism which results in anatomical and physiological changes including cartilage degradation, subchondral bone remodelling, osteophyte formation and joint inflammation (Kraus et al., 2015). It typically presents with insidious onset of joint pain, swelling, stiffness and progressive

loss of function. Primary osteoarthritis clearly has a multifactorial aetiology with a combination of genetic predisposition and lifestyle factors such as obesity, manual work and sporting participation (Hoaglund and Steinbach, 2001). Secondary osteoarthritis occurs due to anatomic abnormalities, osteonecrosis, trauma, sepsis, rheumatoid arthritis or any other specific causative insult. Osteoarthritis has a huge health and financial burden to society with 8.5 million people suffering with osteoarthritis at a cost to the economy of £3 Billion a year and over 36 million working days lost annually in the UK (NICE, 2015).

### 1.26 Pathophysiology of OA

The pathophysiology of osteoarthritis is complex and multifactorial but is now generally accepted to be an inflammatory and biomechanical process. Abnormal joint anatomies, such as congenital dysplasia, are well known to predispose to early degenerative changes in the hip. Malalignment around the knee, whether post traumatic or constitutional, result in point loading on cartilage with predictable patterns of wear. Some of the strongest evidence for biomechanical predisposition can be seen when cartilage regenerates following offloading osteotomies or distraction bracing. Measurable increases in cartilage depth and collagen synthesis have been shown following corrective periarticular osteotomies in patients with symptomatic knee osteoarthritis (Wiegant et al., 2013). Inflammation can be either locally produced by macrophages in the synovium, osteoblasts or chondrocytes themselves, or secondary to a systemic inflammatory state. Obesity and metabolic syndrome may produce a double hit, predisposing patients to osteoarthritis by increasing the joint reaction forces and by altering the inflammatory environment. Adipokines such as adiponectin, resistin and leptin produce systemic and intra-articular release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF $\alpha$  (Urban and Little, 2018). This adipose tissue-mediated inflammation may go some way in explaining why obesity is an independent risk factor for osteoarthritis in both weight bearing and non-weight bearing joints (Yusuf et al., 2010).



**Figure 1.10.** Healthy versus Osteoarthritic joint demonstrating sites of cytokine action and effect on target tissues (Glyn-Jones et al., 2015)

The theory that OA is an inflammatory process has gained popularity since it was first proposed over 40 years ago (Ehrlich, 1975). Certain subgroups of patients, such as post-menopausal women with hand OA, were noted to share very similar characteristics with rheumatoid patients. Recent studies, however, have not linked systemic inflammation as measured with C reactive protein with progression of a osteoarthritic joint degeneration but it does seem to correlate with patient reported pain scores (Jin et al., 2015). Intra-articular inflammation is however strongly associated with disease progression (Berenbaum, 2013).

The process by which intra-articular inflammation leads to the cardinal macroscopic osteoarthritic features of cartilage fissuring, joint space narrowing, subchondral cysts, sclerosis and osteophyte formation is complex. Synovial tissue, subchondral bone and cartilage are sites of both pro-inflammatory cytokine production and pathological damage. Osteoblasts and chondrocytes respond to a hostile mechanical environment such as excessive load or prolonged offload by producing proinflammatory cytokines, nitric oxide and prostaglandin E2. Cytokines such as Interleuken-1 $\beta$ , TNF and IL-6 are felt



to upregulate cartilage degrading enzymes; matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTs). Unlike rheumatoid arthritis, the synovium is not the primary driver of inflammation but becomes inflamed secondary to cartilage breakdown fragments in a macrophage-mediated reaction similar to a foreign body reaction. Cytokine-mediated cartilage breakdown continues which produces more matrix fragments and the inflammatory-breakdown cycle continues (Kapoor et al., 2010).

Inflammation occurs even during the very early stages of osteoarthritis in the tibiofemoral joint. The degree of intra-articular inflammation has also been directly linked to disease progression and pain levels. The pro-inflammatory cytokines that have attracted most interest from researchers include TNF $\alpha$  and the IL-1 and IL-6 families. IL-1, both alpha and beta, are directly linked with cartilage degeneration with IL-1 $\alpha$  elevated in early disease and IL-1 $\beta$  levels increasing later as disease become more clinically apparent (McNulty et al., 2013). TNF $\alpha$  is a promoter of the inflammatory cascade, although it is likely that it acts synergistically with IL-1, as injection of both cytokines in an animal knee model results in an exponential increase in cartilage degeneration. Both TNF $\alpha$  and IL-1 inhibit anabolic activity of the chondrocyte resulting in reduced synthesis of type 2 collagen, proteoglycans and link proteins. The cytokines also upregulate cartilage degenerative enzymes; MMP-1, 3, 13 and ADAMTS-4 and stimulate other inflammatory mediators via the inducible nitric oxide synthase (iNOS) and soluble phospholipase A2 enzymes. This results in an increase in Prostaglandin E2 (PGE2) and Nitric Oxide (NO) production which further activates matrix metalloproteases, inhibits matrix component synthesis, down regulates IL-1 receptor antagonists and ultimately promotes chondrocyte apoptosis (Kapoor et al., 2010). The pathways by which TNF $\alpha$  and IL-1 upregulate catabolic and anti-anabolic pathways seem to be predominantly via the nuclear factor  $\kappa$ B (NF- $\kappa$ B) signalling pathway with some involvement of the c-jun n-terminal kinase (jnk) and the p38 mitogen activated pathway. IL-1 $\beta$ -mediated catabolism is further amplified by the wnt- $\beta$ -catenin signalling pathway, producing further cartilage degeneration.

IL-6 is produced in response to the activation of the NF- $\kappa$ B intracellular pathway by either IL-1 or TNF $\alpha$ . Although present at low levels under normal physiological load, IL-6 has been shown to significantly increase in concentration via a PGE2 mediated response following application of shear stress to cartilage. The role of IL-6 in the development of osteoarthritis is less straightforward than that of IL-1 or TNF $\alpha$ . In order to produce upregulation of IL-6- specific genes, the IL-6 receptor requires additional soluble receptors (sIL-6R) to be present in the cytoplasm. It would also seem that IL-6 is not an initiator of inflammation, but part of the inflammatory cascade downstream from IL-1, TNF $\alpha$  and oncostatin. Once the IL-6R-sIL-6R complex forms, there is an increase in MMP-1 and 13 activity and decrease in type 2 collagen production, however the effect on extracellular proteoglycans is unclear, with IL-6R knockout mice continuing to show a decrease in proteoglycan production (Kapoor et al., 2010).

Oncostatin M (OSM) belongs to the IL-6 superfamily of cytokines and has a function and structure very similar to leukaemia Inhibitory factor (LIF). OSM is so similar to LIF that it is able to activate both gp130/LIFR receptors (type 1 OSM receptors) and gp130/OSMR (type 2 OSM receptors) allowing it to act on a “wide variety of cells and elicit diverse overlapping biological responses” (Tanaka M., 2003). OSM is able to initiate both an anti-inflammatory cascade via the activation of the type 1 OSM receptor, or a pro-inflammatory reaction via the type 2 receptor (Zarling et al., 1986). Both receptors have been identified in high levels in osteoarthritic cartilage and have been heavily implicated in the pro-inflammatory pathogenesis of osteoarthritis. LIFR is seen throughout both healthy and osteoarthritic cartilage, while OSMR is seen throughout osteoarthritic cartilage, but only in the superficial zone in healthy individuals. Directly blocking OSM activity with activated inhibitory antibodies has been shown to have a protective and anabolic effect on extracellular GAG. However, adding recombinant OSM at 5 and 50pg/ml to harvested explants did not inhibit GAG production, suggesting “factors present in synovial fluid act in concert with OSM” (Beekhuizen et al., 2013) to produce a catabolic environment. Although blocking OSM seems to improve the chondrocytes reparative efficiency in some explants, it is important to remember that this cytokine is only detectable in 30% of osteoarthritic synovial samples and therefore may not be an ideal therapeutic target (Beekhuizen et al., 2013).

Individual cytokines rarely work in isolation and are often part of a general pro or anti-inflammatory environment. The combination of OSM and IL-1 has been shown to synergistically increase upregulation of degenerative enzymes such as MMP 1, 8 and 13 in addition to ADAMTs 4 and other cytokines such as IL-8 found further down the inflammatory cascade. The combination of 20pg/ml of IL-1 and 10ng/ml OSM to monolayers of chondrocytes from 4 different osteoarthritis patients produced a synergistic upregulation of the MMP1 gene. This response consistently exceeded the same individuals response to IL-1 alone by between 1.85 to 3.55 times (Barksby et al., 2006). The authors did, however, note a heterogenous response of individual patients to pro-inflammatory cytokines with some patients having minimal MMP1 gene upregulation following stimulation with either cytokine alone or in combination. It would seem that cytokine-mediated inflammation and subsequent cartilage degeneration is bespoke to the individual patient but the relative gene upregulation between IL-1 in isolation and IL-1 with OSM is more consistent.

### **1..27 Ankle vs Knee**

### **1..28 Prevalence of symptomatic osteoarthritis in the knee and ankle**

During 2017 the UK National Joint registry recorded 734 total ankle replacements compared to in excess of 100,000 knee arthroplasty procedures (Report, 2018). The dramatically different number of procedures is in part down to the relative infancy of total ankle replacement compared to total knee replacement. Ankle arthrodesis is an acceptable option for many patients while knee arthrodesis is now performed almost solely as a salvage procedure following failed arthroplasty. Despite this, there remains a significant difference in the prevalence of symptomatic OA of the knee and ankle with one study suggesting that this may be as high as 41% in the knee and only 4.4% in the ankle (Cushnaghan and Dieppe, 1991). More conservative estimates based on UK symptomatic populations quote prevalence of knee OA as 11-19% (Peat et al., 2001) and ankle osteoarthritis as 3.4% (Murray et al., 2018) Cadaveric studies have also shown the presence of joint degeneration in 66% of knees compared to only 21% of ankles

(Muehleman et al., 1997). Knee arthritis is therefore at least 3 times more prevalent than ankle arthritis but it is unclear why this is the case. Further research by the same group compared paired knee and ankle cartilage in 545 organ donors in a bid to investigate the relationship between knee and ankle OA. They found that of the donors in their 6<sup>th</sup> decade or older, only 4% of knee joints showed no evidence of arthritis, while 50% of ankle joints demonstrated no cartilage degeneration (Muehleman et al., 2010). Interestingly, knee arthritis was present in all patients with ankle OA and the severity exceeded that of the ankle 98.9% of the time. Severe ankle degeneration (3 and 4 modified Collins scale) never occurred in the absence of knee OA, suggesting ankle arthritis occurs only when preceded by abnormal knee biomechanics or alignment. It is important to note, however, that this study did exclude patients who had previously undergone arthroplasty or arthrodesis procedures and could not identify symptomatic joints.

The underlying aetiology of ankle and knee osteoarthritis is also very different with the vast majority of ankle arthritis being post traumatic (Saltzman et al., 2005) whilst knee OA is most often primary in nature with no known underlying cause. One study of over 400 ankles with end stage OA identified a traumatic cause in 78% of patients and primary OA was felt to be the cause in only 9% of cases (Valderrabano et al., 2009). Of the 78% of post-traumatic OA cases, 62% occurred following fractures and 16% following ligamentous injuries making ankle fractures the leading cause of symptomatic ankle OA. As would be expected, any fracture extending into the joint can result in chondral damage (Thomas and Daniels, 2003) and lead to OA but malalignment from extra-articular tibial fractures can also lead to degenerate changes as ankle biomechanics is altered. Fracture personality has a significant correlation with the likelihood of developing post traumatic OA, with comminution fractures, fracture-dislocations and malunions having the greatest association with long term degenerate changes (Horisberger et al., 2009). Although less common, multi-ligament instability around the ankle can also cause talar shift, reducing joint contact area and increasing peak stress on the ankle cartilage (Harrington, 1979). This acute alteration in biomechanics, or subsequent chronic instability, has been shown to lead to predictable patterns of wear in the ankle. Chronic lateral ligament injuries have been shown to develop anteromedial

osteoarthritis as a consequence of ongoing anterior subluxation around an intact deltoid ligament (Taga et al., 1993). Although ankle OA is relatively rare it still remains a significant physical, emotional and functional problem and pain scores may exceed that of patients with primary OA of the knee (Brown et al., 2006, Saltzman et al., 2006).

Primary osteoarthritis is far more common than secondary arthritis in the knee. Recent data from the National Joint registry in 2017 reported that 98% of the 103, 983 knee arthroplasty procedures were performed for idiopathic arthritis (Report, 2018). This high proportion of primary osteoarthritics may represent a degree of surgical over-reporting, however large epidemiological studies of the same patient group confirmed idiopathic arthritis accounts for around 82% of symptomatic knee arthritis in the UK (Peat et al., 2001). Primary or idiopathic arthritis is multifactorial in origin, with factors such as genetic predisposition, obesity, occupation and sex influencing its development.

What remains unclear is why the prevalence of ankle osteoarthritis is significantly less than that of knee arthritis, and why the two have such different underlying aetiologies.

### 1.29 Joint Biomechanics

Although weight bearing activities can increase joint reaction forces to ten times patient weight (Ratcliffe and van Mow, 1996) normal repetitive physiological loading of a joint is considered beneficial. Repetitive physiological load is transmitted through the chondrocytes cytoskeleton and creates physicochemical signals that maintain extracellular homeostasis. Repetitive dynamic compression within physiological tolerances has been shown to increase type 2 collagen and proteoglycan production (Grodzinsky et al., 2000). The converse is also true as constant offload or sustained compression may adversely effect biosynthesis of extra cellular components (Palmoski and Brandt, 1984). An adverse biomechanical environment undoubtedly influences contact stresses contributing to cartilage degeneration (Rao et al., 2010).

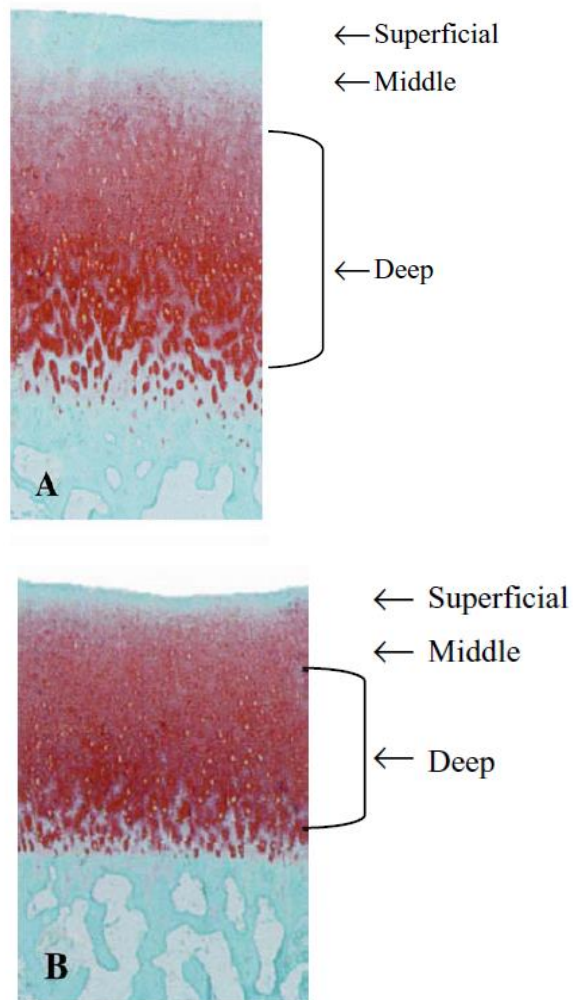
The ankle is biomechanically more complex than a simple hinge joint. The ankle or tibiocrural joint is comprised of the tibiotalar, the fibulotalar and inferior tibiofibular or

ankle syndesmosis (Leardini et al., 2014). The ankle mortise opens between two- and three-millimetres during dorsiflexion as the talar body is wider anteriorly than it is posteriorly, which requires a degree of play within the ankle syndesmosis. The talus itself is similar to a frustrum of a cone with a smaller radius of curvature medially than laterally that is orientated 24 degrees (+/- 6 degrees) from the coronal plane. The tibiotalar joint remains highly congruent during all phases of gait and even at high loads. It has a large surface contact area of between 11 and 13cm<sup>2</sup> which allows reduced point contact stressed in comparison the knee (Mauffrey, 2009). Minor alterations in axis or alignment do lead to a significant alteration in biomechanical environment. One sentinel study suggested that 1mm of talar shift within the mortise results in a reduction in joint contact area by 42% resulting in a significant increase in joint contact stress (Ramsey and Hamilton, 1976).

In contrast to the ankle, the knee is comprised of the medial and lateral tibiofemoral compartments and the patellofemoral joint. The knee is a more unstable hinge which is much less constraint, with six degrees of motion (including a rotational screw home mechanism) that places significant sheer stresses on the tibiofemoral compartments. Femoral roll back occurs to prevent posterior impingement of the femur on the tibia in deep flexion. As knee flexion progresses past 100 degrees, the mismatch in radius of curvature between the medial and lateral femoral condyle produces relative internal rotation of the tibia as the lateral condyle rolls off the back of the tibia. The fibrocartilaginous menisci have developed as compensatory mechanisms to improve stability and dissipate load within the tibiofemoral joint. Further restraints to excessive translation or rotation include the cruciate ligaments, collaterals and capsular ligamentous structures (Ramachandran, 2007). The knee has a significantly larger articular contact when compared to the ankle, however due to the semi-constraint nature of the joint, a degree of point loading does occur as not all chondral surfaces remain in contact at all times during the gait cycle. MRI based studies assessing healthy individuals have estimated the total knee articular area as 102cm<sup>2</sup> (+/- 13.6) and the distal femur as 64cm<sup>2</sup> (+/- 8.7); ten times and six times larger than the total contact area of the ankle. In fact the medial tibial surface, which is in constant contact with the femur, has a surface area roughly equal to that of a normal ankle joint (Hohe et al., 2002). Given

that the same weight passes through the knee and ankle and the ankle has a significantly smaller contact area, the load per unit (stress) on the tibiocrural joint is expected to be higher. The higher peak stresses at the articular cartilage create a detrimental biomechanical local environment, altering hydrostatic lubrication, increasing the shear stress, damaging the proteoglycan–collagen structure and promoting matrix failure (Ateshian et al., 1994). Logically, therefore, the ankle should be *more* susceptible to osteoarthritis, yet this is not the case. One explanation for this is that the nature of the cartilage or the chondrocyte within the two joints must be intrinsically different.

### 1.30 Histological differences



**Figure 1.11:** Histological sections stained with with Safranin O and fast green femur (A) and talus (B) at 4x magnification (Kuettnner and Cole, 2005)

Knee cartilage is, on average, twice the thickness of ankle cartilage (Juras et al., 2016) with ankle cartilage uniformly around 1 to 1.5mm throughout the joint while the depth of knee cartilage varies from 6mm in the trochlear to 2mm on the tibial plateau. Tissue from both joint shares the same general features of avascularity, lack of nerve and lymphatic supply. They are both histologically divided into superficial, middle and deep zones. The superficial zone contains horizontally orientated type 2 collagen fibres and flattened chondrocytes that produce lubricin. Clusters of chondrocytes in the ankle are



contained in chondrons while the knee chondrons contain only single chondrocytes. The middle zone is relatively large in the ankle compared to the knee and both tissues contain similar quantities of rounded chondrocytes with more vertically orientated collagen. The deep zones are again microscopically similar but occupy a larger proportion of the overall collagen in the knee than the ankle.

### 1.31 Biochemical differences

Although histologically very similar the two cartilages have very different proportions of extracellular matrix components. Several studies have demonstrated lower water content and higher sulphated-glycosaminoglycan content in ankle cartilage (Treppo et al., 2000, Huch, 2001, Eger et al., 2002). This change in proportion of one of the most crucial components of cartilage leads to a higher Young's Modulus and stiffer, more resilient cartilage. In order to achieve 65% strain of both cartilages, significantly more stress was required (11GPA vs 16 GPA) in the ankle tissue and this only produced macroscopic signs of degeneration in 17% of ankle samples compared to 81% of knee samples. This increase in stiffness may account for the finding that "knee cartilage degeneration leads to the development of OA with clinical symptoms, whereas the ankle cartilage develops fissures that do not appear to progress to later stages of OA" (Huch, 2001).

Interestingly, chondrocytes derived from ankle cartilage are metabolically different, producing significantly more glycoaminoglycans and type 2 collagen than knee chondrocytes (Kuettner and Cole, 2005). The ankle chondrocytes are metabolically more active, reducing the half life of proteoglycans and producing twice as much sGAGs mRNA as knee chondrocytes. The ankle chondrocyte also seems to be hardier, being resistant to both a hostile mechanical and a pro-inflammatory environment (Cole and Kuettner, 2002). Detrimental static compression of ankle cartilage produces a reduction in collagen, GAG and protein synthesis of 15% compared to 25-50% in knee cartilage. Ankle tissue is also less susceptible to the catabolic effects of pro-inflammatory cytokines and matrix fragments. In order to reduce the synthesis of proteoglycans by half, the presence

of significantly more IL-1 $\beta$  is required (IC<sub>50</sub> 6 pg/ml vs 35 pg/ml) and the same is true for fibronectin fragments (Cole and Kuettner, 2002, Joseph, 2009). The ankle cells also less readily express mRNA for key degenerative enzymes like matrix metalloproteinase-8 (MMP-8) (Chubinskaya et al., 1999) and return to normal function much sooner following removal of noxious stimuli. The ankle chondrocytes resistance to IL-1 is maintained when it is removed from its native matrix, suggesting the chondrocyte itself must play an important role in preventing inflammatory-mediated cartilage degeneration.

Differences in the response of knee and ankle cartilage to pro-inflammatory mediators commonly found in OA may help to identify therapeutic targets for the prevention of joint degeneration in the future.

### 1.32 Aims

The aim of the following thesis is to

1. Assess the differential response in proteoglycan loss and cell viability of ankle and knee articular cartilage following stimulation with pro-inflammatory cytokine
2. Assess if this differential cartilage degeneration observed between the ankle and the knee due to differences in the expression and/or activity of molecules involved in catabolism?

# Chapter 2

## Methods

## **2 General Methods and Materials**

All reagents were of analytical grade or above and obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

### **2.1 Sample Collection**

### **2.2 Ethical Approval**

Ethical approval was granted to collect articular cartilage from the knee and ankle joints of patients undergoing lower limb amputations due to vascular complications (Newcastle & North Tyneside 1 Research Ethics Committee Reference: 15/NE/0337 IRAS project ID: 184005). Patients were recruited through the Department of Vascular and General Surgery in collaboration with Mr Ian Williams (Consultant Vascular Surgeon, University Hospital of Wales, Heath Park, Cardiff).

### **2.3 Patient Recruitment**

Patients who presented to the University Hospital of Wales with any pathology or injury that required non-emergent lower limb amputation were provided with a "permission to determine suitability" form (Appendix 7.1). A database of patient details including hospital numbers, dates of birth and names was retained on a password protected computer within the Trauma and Orthopaedic Department of the University Hospital of Wales in order to maintain patient confidentiality and meet data protection requirements.

Patient suitability for this study was assessed according to the following criteria:

#### **Inclusion Criteria**

- Patients attending appropriate Foot and Ankle, Knee or Hip orthopaedic clinics
- Patients attending Vascular Clinic
- Inpatients awaiting non-emergent lower limb amputations

#### **Exclusion Criteria**

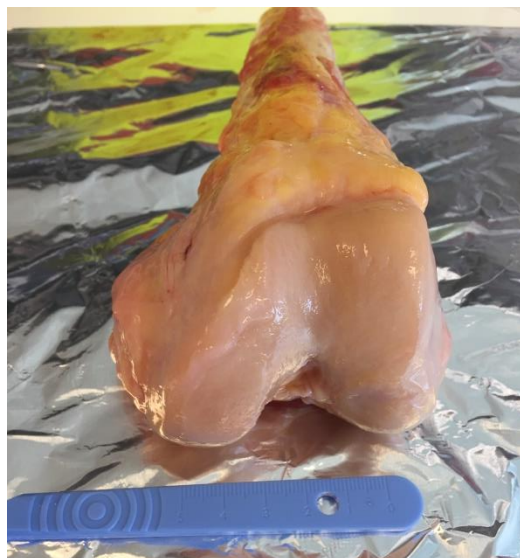
- Inability to provide written informed consent

- Patients that have any previous problem to the joint under investigation that may affect the results such as a septic joint
- Certain medication that may affect the research results such as disease modifying rheumatoid drugs like anti-TNF
- Emergency Procedures where the patient may not have adequate time to consider their inclusion in the study

A total of 10 ankles and 5 knees were collected for this study as indicated (Table 3.1).

#### 2.4 Tissue preparation, transport and storage

Following the surgical procedure, the limb was removed under sterile conditions and the distal femur (Figure 2.2) and/or talus (Figure 2.3) dissected out using separate sterile instruments and surgical table within the same theatre but outside the lamina flow ventilation system.



**Figure 2.2:** Surgical excision of distal femur (K1). Post amputation image with medial to the left side and lateral towards the right.



**Figure 2.3:** Surgical excision of talus (A1). Left image – Talar dome. Right Image – Lateral view

Samples were transferred from the University Hospital of Wales to the Cardiff University Biosciences department by the responsible principle investigator in a sealed specimen pot containing Hanks' Balanced Salt Solution (Thermo Fisher, Loughborough, UK) at room temperature.

Full thickness cartilage explants (3mm or 4mm diameter) were taken from the weight bearing surface of the talar dome, and the medial and lateral femoral condyles. Explants were produced by pushing a dermal punch (Miltex, York, PA, USA) through the articular cartilage and detaching from the subchondral bone with a scalpel blade.

### 2.5 Tissue Culture

Explants were immediately placed in 50ml Hanks' Balanced Salt Solution supplemented with 400U/ml Streptomycin and 400µg/ml Penicillin (Thermo Fisher, Loughborough, UK) and subjected to three washes, with a change of high strength antibiotic media every ten minutes for thirty minutes, to reduce the likelihood of infection during the culture period.

Following extraction and washes, explants were placed in single wells of a 48 well culture plate (Corning, Wisbaden, Germany) and cultured in 500µl of Dulbeccos Modified Eagle's Medium/Hams F12-glutamax (DMEM/F12-glutamax (1:1) (Thermo Fisher, Loughborough, UK) supplemented with 100U/ml Streptomycin and 100µl/ml Penicillin, 50µg/ml Ascorbate–2–Phosphate and 1x Insulin-transferrin-sodium selenite (Thermo Fisher, Loughborough, UK); herein referred to as culture media unless indicated otherwise. Ascorbic acid acts as cofactor of collagen prolyl hydroxylases in the endoplasmic reticulum maintaining normal collagen homeostasis (D'Aniello et al., 2017). Insulin-transferrin-sodium selenite aids the culture of chondrocytes in multiple way; the insulin enables glucose and amino acid uptake, transferrin acts as an iron carrier and helps to reduce free radicles and the selenite is a co-factor for glutathione peroxidase which also acts as an anti-oxidant (Mesalam et al., 2019). Explants were rested overnight in an incubator set at 37°C and 5% CO<sub>2</sub>. Media was removed and then discarded the following day before the explants were subjected to cytokine treatment.

## 2.6 Cytokine Treatment

To assess the effect of cytokine treatment on cartilage metabolism, explants were stimulated with either high or low concentrations of pro-inflammatory cytokines (Table 1) by adding the appropriate cytokines to 500µl of culture media per well; untreated explants served as controls. Explants were either stimulated with cytokines for 7 (short-term) or 28 days (long-term). Media was removed and replenished at day 3 for the short-term culture (knee n=1, ankle n=3) or collected and replenished at day 3, 7, 10, 14, 17, 21 and 24 for long-term culture (knee n=3, ankle n=4); resulting media was stored at -20°C prior to analysis. Cultures were subsequently terminated at day 7 or 28 with media

collected and stored at -20°C and explants blotted dry and weighed to provide a wet weight (mg). Explants were then immediately frozen in liquid nitrogen and stored at -80°C.



<b>Treatment concentrations</b>			
<b>Cytokines</b>	<b>IL-1<math>\alpha</math></b>	<b>TNF<math>\alpha</math></b>	<b>Oncostatin M</b>
<b>Untreated</b>	n/a	n/a	n/a
<b>TNF<math>\alpha</math> Low</b>	n/a	2ng/ml	n/a
<b>TNF<math>\alpha</math> High</b>	n/a	100ng/ml	n/a
<b>IL-1<math>\alpha</math> &amp; OSM Low</b>	100pg/ml	n/a	200pg/ml
<b>IL-1<math>\alpha</math> &amp; OSM High</b>	5ng/ml	n/a	10ng/ml
<b>IL-1<math>\alpha</math> &amp; TNF<math>\alpha</math> Low</b>	100pg/ml	2ng/ml	n/a
<b>IL-1<math>\alpha</math> &amp; TNF<math>\alpha</math> High</b>	5ng/ml	100ng/ml	n/a

**Table 2.1:** Experimental cytokines treatments and control used (Recombinant Human TNF $\alpha$ , Recombinant Human Oncostatin M and Recombinant Human IL-1 $\alpha$  from *E.Coli* sourced from PeproTech, USA).

## 2.7 Biochemical Assays

### 2.8 Dimethylmethylene Blue Assay

Concentrations of sulphated glycosaminoglycans (sGAG) were calculated using a 1,9 - dimethylmethylene blue assay as previously described (Little et al., 1990). Sulphated glycosaminoglycan content in the experimental media and explants was determined by comparison to a standard curve that was created by preparing solutions containing 0, 10, 20, 30, 40 and 50µg/ml of chondroitin-4-sulphate from purified shark cartilage. Forty microliters of each media sample and standards were applied to a 96 well flat bottom (Corning, Wisbaden, Germany), followed by 200µl of 1,9 dimethylmethylene blue reagent (32mg 1,9 DMMB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98% formic acid and made up to 2L with water). Absorbance measurements were measured immediately at a wavelength of 525nm using the Labsystem Multiscan MS Spectrophotometer (Optima, Japan). Where results did not fall within the standard curve, samples were appropriately diluted and re-analysed. Standards prepared for the assay were used to produce a calibration curve and sGAG amounts released into the media were calculated and then normalised to the wet weight of each explant.

### 2.9 Papain Digest of explants

The concentration of sGAG within the explant was measured in order to determine the percentage loss of sGAG from the tissue into the media. Explants were weighed, placed in 200µl papain buffer (0.05M sodium-acetate, 25mM EDTA pH 5.6, 5mM cysteine) and digested in 1mg/ml papain enzyme (papaya latex extract) for 18 hours at 60°C. Upon digestion of the tissue, supernatants were diluted appropriately and analysed using the DMMB assay as above. Results were normalised to wet weight of the explant section.

Percentage loss of sGAG from the explant to the media was then calculated using the formula:

$$\text{Percentage loss sGAG} = \frac{\text{Media Concentration}}{(\text{Papain concentration} + \text{Media Concentration})}$$

### 2..10 Griess Nitrite Assay – Nitrite

Indirect measurement of NO content within the experimental media was calculated using a Griess Nitrite Assay (Promega, Southampton, UK) as previously described (Chae et al., 2004). Nitrite content was determined based on a standard reference curve created by a serial twofold dilution of a NaNO<sub>2</sub> standard (from 100µM to 1.56µM). Fifty microliters of the culture medium and standards were applied to a 96 well flat bottom plate (Corning, Wisbaden, Germany) before being incubated with 50µl of 1% (w/v) sulfanilamide (in 5% (w/v) phosphoric acid) for 10 minutes at room temperature while protected from light. This process was then repeated with 50µl of 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride before absorbances were measured at a wavelength of 540 nm using the Labsystem Multiscan MS Spectrophotometer (Optima, Japan). Standards prepared for the assay were used to produce a calibration curve, and nitrite concentrations determined prior to normalisation to the wet weight of each explant.

### 2..11 CytoTox 96® Non-Radioactive Cytotoxicity Assay- Lactate dehydrogenase

Measurement of lactate dehydrogenase (LDH) release into the culture medium, which occurs as a result of cell death (Koh and Choi, 1987), was used as a marker of cell lysis.

The CytoTox 96® non-radioactive cytotoxicity assay (Promega, Southampton, UK ) as previously described (Riss et al., 2004) was used to determine relative LDH release across cytokine treatments. Fifty microliters of culture media was added to wells with 50µl of the CytoTox 96® Reagent in a 96 well flat bottom plate (Corning, Wisbaden, Germany); samples were incubated at room temperature and protected from light for 30 minutes. Fifty microliters of stop solution was then added, absorbance measured at a wavelength of 490nm using the Labsystem Multiscan MS Spectrophotometer (Optima, Japan) and relative LDH levels compared to untreated explants that had not been stimulated with cytokines.

#### **2..12 Prostaglandin E<sub>2</sub> ELISA**

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) released into the media from explants stimulated with cytokines was measured using a Prostaglandin E<sub>2</sub> high sensitivity ELISA (Enzo Life Sciences, Switzerland). Following a test analysis, all media samples derived from explants treated with cytokines were diluted with distilled water (1:2) to ensure an appropriate fit on the standard curve; media derived from untreated explants was left undiluted. Following manufacturer instructions (PGE<sub>2</sub> high sensitivity ELISA kit), a PGE<sub>2</sub> standard curve was produced to generate standards ranging from 1000pg/ml to 7.81pg/ml. Plate wells were filled according to manufacturer's instructions (Enzolifesciences, 2017) providing both positive and negative controls for the assay. Standards (100µl) and experimental samples (100µl) were pipetted into the remaining wells, followed by 50µl of blue conjugate and 50µl of yellow antibody pipetted into the respective wells. The plate was then sealed and incubated overnight at 4°C before each well was washed 3 times with 400µl of wash solution. pNpp solution (200µl) was added to all wells prior to the plate

being covered and incubated at 37°C for a further hour. Fifty microliters of stop solution was added to all wells and the plate was read at a wavelength of 405nm using the Labsystem Multiscan MS Spectrophotometer (Optima, Japan). Results were calculated by first producing a standard curve of percentage bound versus concentration of PGE<sub>2</sub> and unknowns determined by linear interpolation (Microsoft Excel, Microsoft, USA). Mean net Optical Density (OD) was calculated by subtracting mean non-specific binding OD (NSB) from mean bound OD. Binding of each pair of standards is calculated as a percentage of the maximum binding wells (B0).

$$\text{Mean Net OD} = \text{Mean Bound OD} - \text{Mean NSB OD}$$

$$\text{Percentage Bound} = (\text{Net OD}/\text{Net B0 OD}) \times 100$$

Dilutions were then accounted for and results normalised by dividing through by the wet weight of the corresponding explant.

### 2.13 Gelatin Zymography

Gelatin zymography was used to determine the amount of pro- to active- enzyme and the ratio of matrix metalloproteinase 9 and 2 released into the culture media at various time points post cytokine stimulation (Woessner, 1995). Experimental media samples (50µl) were denatured in 2x sample buffer (0.125M tris pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS and 10mg Bromophenol Blue) at 60°C for 30 minutes. Equivalent amounts of samples (normalised to explant wet weight) and 5µl MMP-2/-9 standard (used for identification and gel to gel comparison) were resolved on 7.5% SDS

polyacrylamide gels containing 1mg/ml gelatin (Table 2.2) using 1x Laemmli electrophoresis buffer (10x Laemmli buffer contained 30.0g Tris base (pH 8.3), 1440.0g glycine, 10.0g SDS dissolved in 1000ml of water)(Laemmli, 1970). Proteins were separated at 150V for 45 minutes or until the dye front reached the bottom of the gel.

**Table 2.2:** Composition and Reagents used to produce gels for zymography

Reagents	Resolving Gel (7.5%)	Stacking Gel (4%)
40% Bis/Acrylamide	2.72ml	575µl
1M tris/HCl pH 8.8	3.63ml	---
1M tris/HCl pH 6.8	---	1.3ml
10% (w/v) SDS	100µl	50µl
10% (w/v) APS	75µl	37µl
dH <sub>2</sub> O	6.16ml	4.075ml
TEMED	15µl	7.5µl
Gelatin (15mg/ml)	1ml	---

Following electrophoresis, gels were washed three times in 2.5% (v/v) Triton X-100 on a rotary shaker in order to renature the MMPs followed by incubation overnight at 37°C in MMP Buffer (50mM tris (pH 7.8), 50mM CaCl<sub>2</sub>, 0.5M NaCl). Gels were then rinsed with distilled water and placed in stain for 60 minutes (45% (v/v) methanol, 20% (v/v) acetic acid and 5g brilliant blue made up to 2 litres) followed by destain (10% (v/v) glacial acetic acid, 20% (v/v) Methanol) until lysis bands appeared. Gels were scanned for presentation.

#### 2.14 Statistical Analysis

All statistical analysis was undertaken using statistical software Prism 7 (GraphPad Software, California, USA). All data is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation. Prior to analysis, data was checked for Gaussian distribution using D'Agostino and Pearson's normality test. Where two groups were compared; normal data was analysed using students t-test and non-parametric data was analysed using Wilcoxon matched-pairs signed rank test (paired) or Mann-Whitney test (unpaired). Where multiple groups were compared, ANOVA was used for all parametric data and Kruskal-Wallis used for non-parametric data. All multiple comparisons are displayed with an adjusted p value where appropriate, and results considered statistically significant at  $p < 0.05$ .

# Chapter 3

**Differential response of ankle and knee  
articular cartilage to pro-  
inflammatory cytokine stimulation:  
effect on proteoglycan loss.**



### 3 Introduction

Intra-articular inflammation has been proven to be part of the pathogenesis of osteoarthritis (OA) and is present even before the onset of macroscopic degeneration (Ehrlich, 1975). As previously described (Section 1.7.1) the incidence of primary ankle OA is much lower compared to knee OA, and studies have previously been conducted to ascertain whether this is primarily due to biomechanical differences or whether there are inherent differences in matrix composition and/or chondrocyte behaviour. It is known that ankle cartilage has higher GAG content and lower water content making it stiffer and more resilient to wear than knee cartilage. Interestingly, ankle chondrocytes have been shown to be more resistant to IL-1 and fibronectin fragment mediated catabolism than knee explants (Kuettner and Cole, 2005). Eger et al (2002) investigated the rate of [<sup>35</sup>S]-sulphate incorporation into the glycosaminoglycans within ankle and knee cartilage at ascending IL-1 levels from 1-250pg/ml and found a significant difference between the joints between 5-250pg/ml. The concentration at which sGAG production (as measured by radioactive counts per minute of DNA) was reduced by 50% (IC<sub>50</sub>) in the knee was 11.8pg/ml compared to 56.1pg/ml in the ankle. This suggests that the ankle chondrocytes required significantly higher concentrations of IL-1 to halve production of sGAGs. These results were however performed on *ex vivo* chondrocytes in alginate not in their native extracellular matrix making direct correlation with concentrations found in the native arthritic joints difficult (Eger et al., 2002).

Pro-inflammatory cytokines can have an autocrine effect when produced by the chondrocyte themselves or a paracrine effect when they are produced by the joint synovium and act on the chondrocytes. In OA, both autocrine and paracrine effects occur and pro-inflammatory cytokines such as TNF $\alpha$ , IL-1 and OSM inhibit anabolic activity of the chondrocyte resulting in reduced synthesis of type II collagen, proteoglycans and link protein (Kapoor et al., 2010). These cytokines also upregulate proteolytic enzyme synthesis and activation (MMPs), stimulate prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO) production as an inflammatory response culminating in the

eventual degradation of the cartilage itself if the cytokine insult is a chronic feature. This inflammatory environment causes the breakdown of essential extracellular matrix components into the synovial fluid and cartilage degeneration occurs. To date, no study has examined the differential effect of TNF $\alpha$  or IL-1 in conjunction with OSM on the metabolic behaviours of ankle and knee cartilage.

### 3.1 Aim

This chapter aimed to quantify the effect of combination treatments of pro-inflammatory cytokines, i.e. IL-1, TNF $\alpha$  and OSM at physiological and pathophysiological concentrations on cell viability in ankle and knee articular cartilage stimulated for 28 days. Furthermore, the loss of proteoglycan from the cartilage tissue was measured as an indicator of cartilage breakdown to evidence whether there is a differential response to cytokine stimulation in these anatomically distinct cartilage tissues.

1. Donor patient demographics, co-morbidities and joint degeneration scores were collected in order to identify any baseline variables between the ankle and knee patients that may affect results.
2. Lactate dehydrogenase (LDH) levels were measured in order to determine if there was a difference in cell viability between joints in response to different cytokine treatments.
3. The percentage loss of sGAGs from cartilage explants into the media was used to determine if there was a difference in ECM proteoglycan breakdown between joints in response to different cytokine treatments.

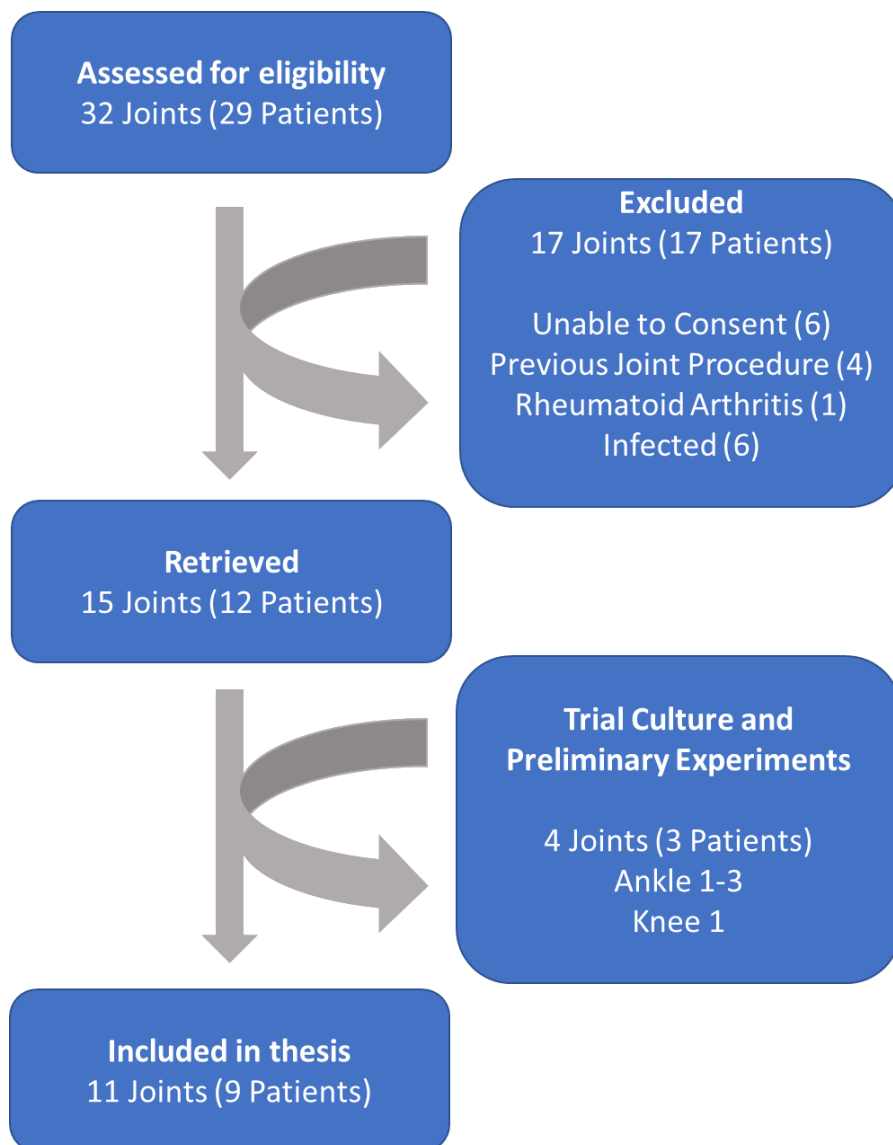
## 3.2 Results

### 3.3 Donor population patient consent

Twenty-two patients who presented to the University Hospital of Wales, Cardiff requiring non-emergent lower limb amputation were provided with a "permission to determine suitability" form (Appendix 2). Patient suitability for this study who met inclusion criteria (Section 2.1.2) were provided with a patient information sheet (PIS version 1.0 September 2016) and asked to donate surgical waste and to complete a consent form (Consent form Version 1.0 September 2016). Seventeen patients were excluded prior to sample retrieval of which: six patients were unable to consent for the study because of lack of capacity (4) or insufficient time to consider donation (2), four had previous surgical procedures involving the joints which included a total knee replacement (1), ankle fusion (2) and a previous open ankle fracture (1). One patient had rheumatoid arthritis and was on disease modifying rheumatoid drugs (1) and a significant proportion were found to be infected (6). Of the retrieved samples, 11 joints from 9 patients (Ankle 4-10 and Knee 2-5) were included for study in this thesis (Figure 3.1).

### 3.4 Classification of joint cartilage gross morphology

Following retrieval, all joints were washed and articular cartilage visually assessed to produce a 'joint score' using the Modified Collins classification for knees (Collins, 1949) and modified ankle degeneration score (Muehleman et al., 1997). All joints included scores between 0 - 1 and were not damaged during retrieval. Of the 10 ankles, 7 were grade 0 and 3 were grade 1; of the 5 knees, 2 were found to be grade 0 and 3 found to be grade 1 (Table 3.1). Chi Squared statistical test did not find any significant difference between the knee and ankle 'joint cartilage morphology score' at baseline ( $p=0.2635$ ).



**Figure 3.1** Donor patient inclusion flow chart demonstrating number of patients identified as eligible, reason for exclusion, final numbers retrieved and patients providing donor tissue used for experimental work in thesis.

Patient Code	Joint Code	Age	Sex	Co-Morbidities	Indication for Amputation	Outerbridge Classification (0-4)	Culture Period (Days)
Patient A	Ankle 1	70	M	Type 2 Diabetes, Angina	Critical Ischaemia	0	7
Patient B	Ankle 2			Type 2 Diabetes, Stroke, COPD	Critical Ischaemia	0	7
Patient B	Knee 1	67	M		Critical Ischaemia	1	7
Patient C	Ankle 3	68	M	Type 2 Diabetic, Heart Failure, Renal Failure, Asthma	Critical Ischaemia	0	7
Patient D	Ankle 4	62	M	Type 2 Diabetic, Asthma, Renal Failure	Critical Ischaemia	1	7
Patient E	Ankle 5	78	M	Type 2 Diabetic, Renal Failure, Diabetic Retinopathy	Critical Ischaemia	1	7
Patient F	Ankle 6	57	M	Type 2 Diabetes, Previous Common femoral artery stent	Critical Ischaemia	0	7
Patient G	Knees 2	60	F	Type 2 Diabetic, Myocardial Infarction, Below knee amputation	Critical Ischaemia	0	7
Patient H	Ankle 7	92	M	Type 2 Diabetic, Myocardial Infarction, heart failure, stroke	Critical Ischaemia	1	28
Patient I	Ankle 8	66	F	Type 1 Diabetes, Stroke, Myocardial Infarction	Critical Ischaemia	0	28
Patient J	Ankle 9			Type 2 Diabetes, Heart failure, Contralateral Below Knee amputation	Critical Ischaemia	0	28
Patient J	Knee 3	84	F		Critical Ischaemia	1	28
Patient K	Ankle 10			Type 2 diabetes, Myocardial Infarction	Critical Ischaemia	0	28
Patient K	Knee 4	73	M		Critical Ischaemia	1	28
Patient L	Knee 5	79	M	Myocardial Infarction, Malignant Myosarcoma	Malignancy	0	28

**Table 3.1** Donor population demographics, co-morbidities, indication for amputation, visual joint degeneration score using the Modified Collins classification for knees (Collins, 1949) and modified ankle degeneration score (Muehleman et al., 1997) and duration of culture for each sample.

### 3.5 Donor patient demographics and co-morbidities

Inpatient notes were reviewed for patient demographics and all co-morbidities listed (Table 3.1). As would be expected for a study of non-emergent amputations, the majority of patients suffered with diabetic induced peripheral vascular disease; critical ischaemia was the indication for all amputations except one (Knee 5) and the majority of diabetics were type 2. The single non-vasculopath was a patient having an above knee amputation for a myosarcoma of the calf in which the unaffected knee sample was retrieved (knee 5).

Baseline demographics such as age and proportion of male/female patients was assessed statistically using the non-parametric Mann-Whitney test for patient age (data was not normally distributed) and chi squared test for proportion of male/female. Median age in the ankle group was 69 (mean 71.7) compared to median age of 73 (mean 72.6) in the knee group, however this was not found to be statistically significant ( $p=0.7909$ ). Two patients in each group were female and again this had no significant impact ( $p=0.4090$ ).

### 3.6 Differential effect of pro-inflammatory cytokines on chondrocyte survival in the ankle and knee

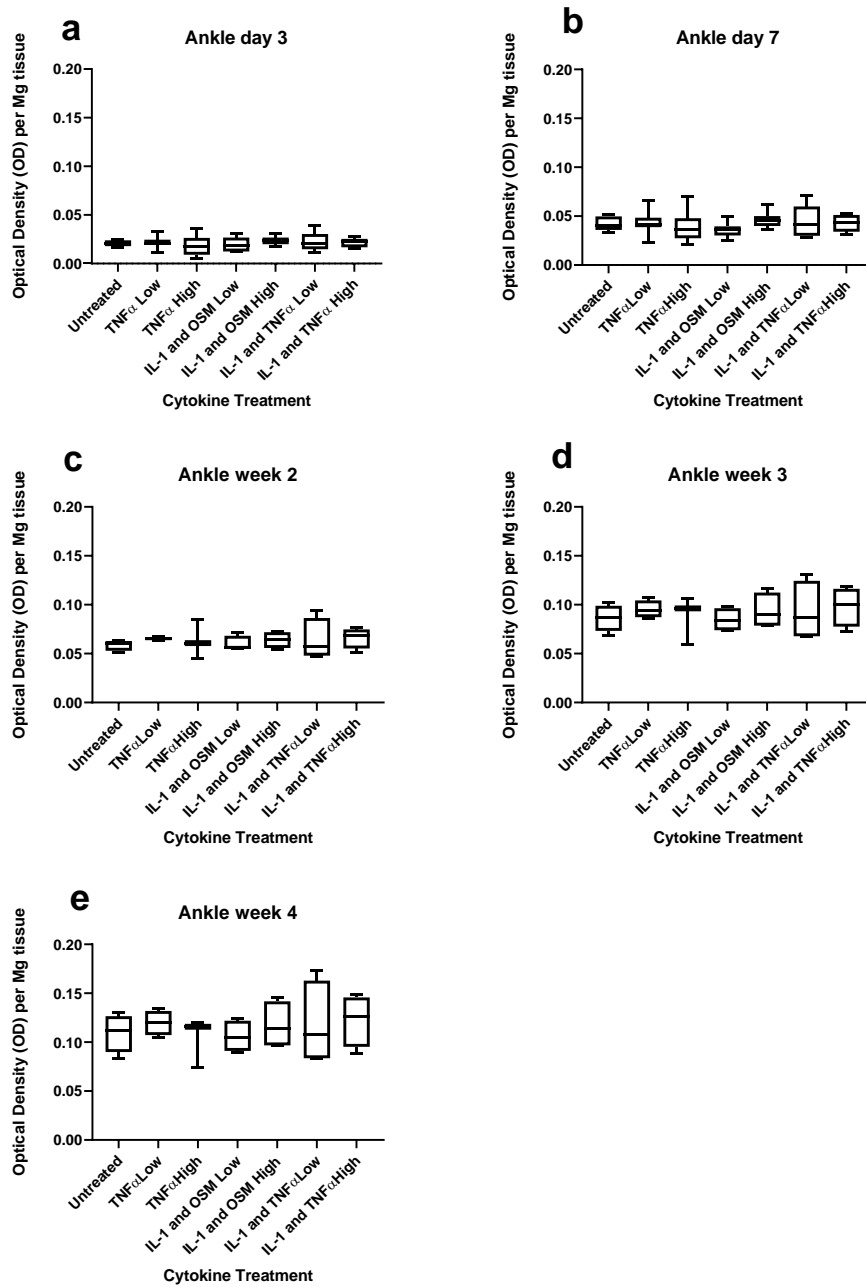
Following sample retrieval, multiple explants were taken from the tali or femoral condyles of recruited patients (Section 2.1.3); each donor had at least 21 explants removed with three explants cultured in either physiological, denoted 'low' or pathological cytokine concentrations, denoted 'high' or left untreated as a control (Section 2.2). Short-term culture was performed on 4 joints (knee  $n=1$ , ankle  $n=3$ ) with removal of media and replenishment at 3 days and termination at 7 days. Long-term culture was performed on 7 joints (knee  $n=3$ , ankle  $n=4$ ) with removal and replenishment of media containing cytokines at day 3, 7, 10, 14, 17, 21 and 24 and experiments terminated at day 28.

### 3.7 Lactate Dehydrogenase levels

Lactate dehydrogenase (LDH) was measured for all time points, up to and including day 28, to assess chondrocyte viability in order to investigate differences in chondrotoxicity of different cytokines and to ascertain if there was any difference in cell death observed in the ankle compared to the knee. A CytoTox 96® Non-Radioactive Cytotoxicity Assay (Section 2.3.4) was used to measure LDH released into the media as a marker of cell lysis (Koh and Choi, 1987), as LDH is an intracellular enzyme and would only be detected in the media due to cell death. LDH levels are presented as absorbance units normalised to tissue explant wet weight.

### 3.8 Ankle cartilage chondrocyte viability was not significantly affected by cytokine treatment

Analysis of LDH release from ankle cartilage, assessed using a one way ANOVA, demonstrated no significant difference in mean cumulative LDH levels between cytokine treatments (day 3  $p=0.881$ , week 1  $p=0.769$ , week 2  $p=0.971$ , week 3  $p=0.949$ , week 4  $p=0.926$ ; Figure 3.1a-e). Tukey multiple comparator testing using adjusted  $p$  values was performed at all time points in order to compare LDH release from untreated cartilage against tissue treated with physiological and pathological cytokine concentrations. No significant difference was observed at any time point between either the untreated or cytokine stimulated ankle cartilage suggesting that there was little cytotoxicity in response to treatment ( $p \geq 0.915$ ).

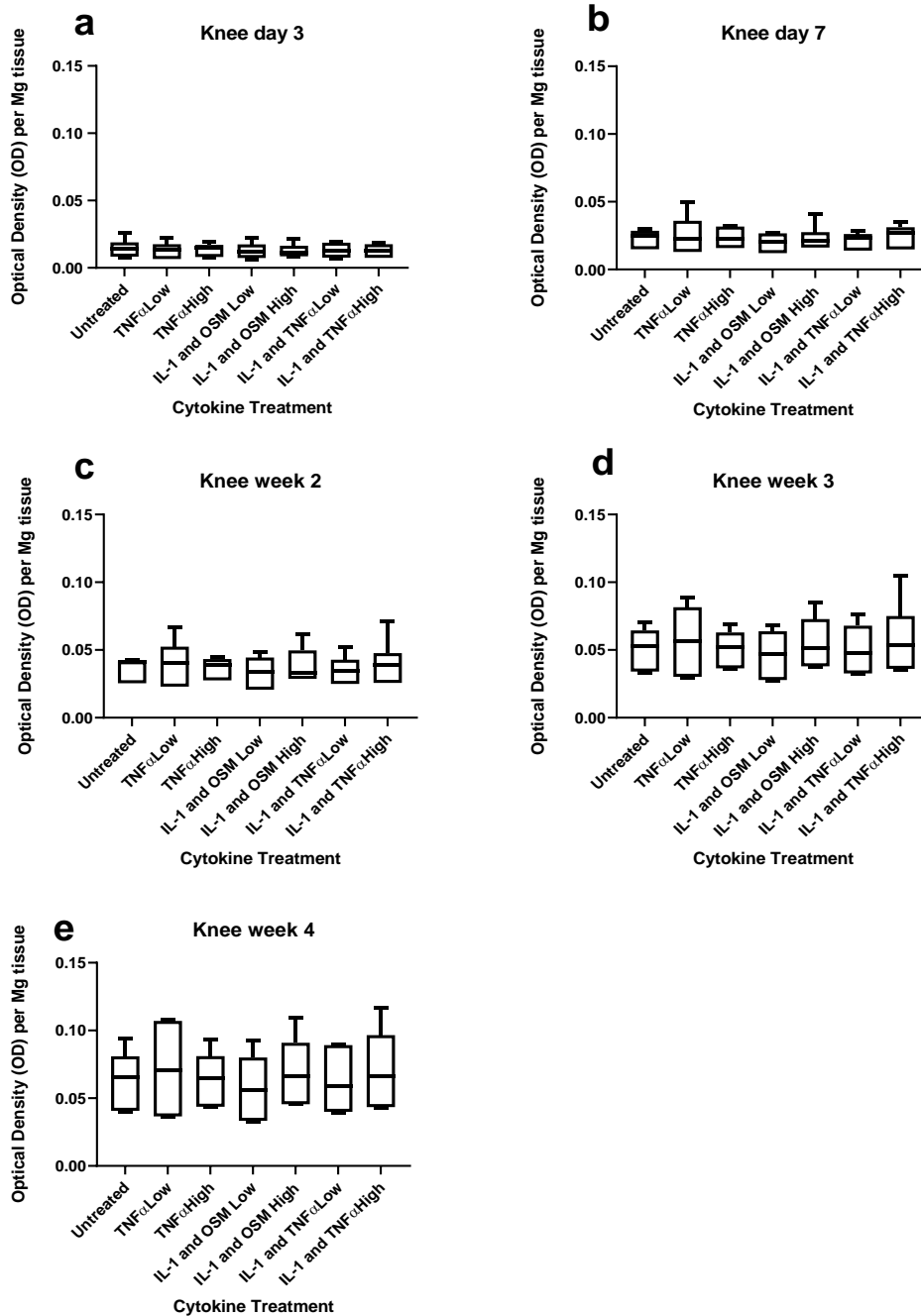


**Figure 3.2** Levels of lactate dehydrogenase released from ankle cartilage into the media following cytokine stimulation, assessed as a marker of cell viability. Ankle cartilage was either cultured ‘short term’ for 7 days (n = 7) or ‘long term’ extending out to 28 days (n = 4) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); LDH levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as the mean cumulative absorbance units per mg of wet weight tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).



### 3.9 Knee cartilage chondrocyte viability was not significantly affected by cytokine treatments

Analysis of LDH from knee cartilage, assessed using a one way ANOVA (Figure 3.3a-e) demonstrated no significant differences in mean cumulative LDH levels between cytokine treatments (day 3  $p=0.985$ , week 1  $p=0.939$ , week 2  $p=0.959$ , week 3  $p=0.949$ , week 4  $p=0.963$ ). Tukey multiple comparator testing using adjusted p values was again performed at all time points in order to compare LDH release from untreated knee cartilage against tissue treated with physiological and pathological cytokine concentrations. No significant difference was observed at any time point between either the untreated or cytokine stimulated knee cartilage suggesting that there was little cytotoxicity in response to treatment ( $p \geq 0.969$ ).

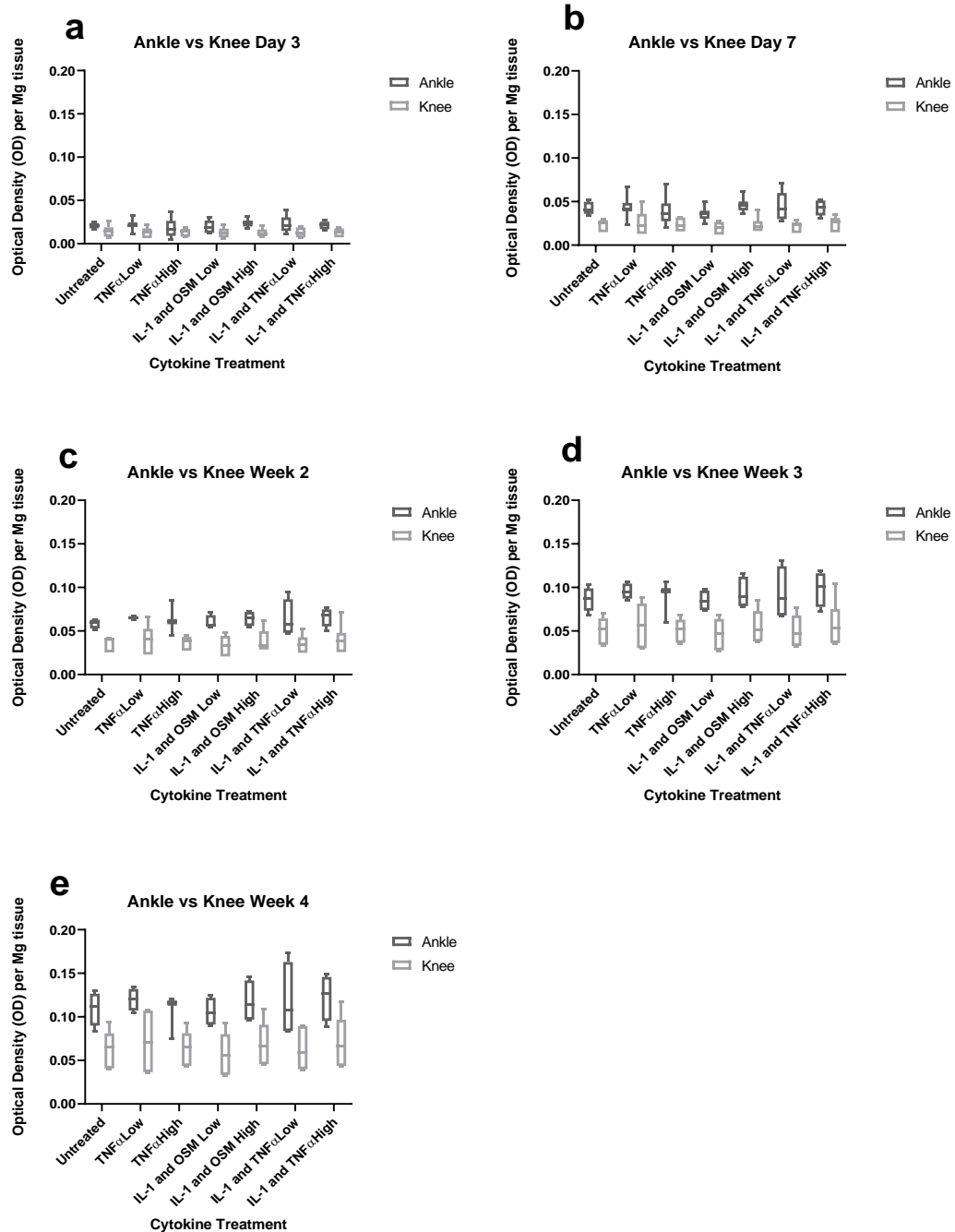


**Figure 3.3** Levels of lactate dehydrogenase released from knee cartilage into the media following cytokine stimulation, assessed as a marker of cell viability. Knee cartilage was either cultured ‘short term’ for 7 days (n = 4) or ‘long term’ extending out to 28 days (n=3) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); LDH levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as the mean cumulative optical density per mg of wet tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

### 3.10 Significantly more LDH was produced by the ankle than the knee at all timepoints

Mean cumulative LDH release from ankle and knee cartilage explants were compared at each time point using a Two-way ANOVA (Figure 3.4). This statistical test allowed for assessment of the percentage variance and significance that the joint of origin and cytokine treatment had on cell viability. Multiple comparison analysis with an adjusted p value was also undertaken using the Sidak's multiple comparator method to identify any trends between different joints exposed to the same cytokine treatment (Table 3.2).

As observed during analysis of individual joint tissues, overall the mean LDH levels were not affected by cytokine treatments at any time point (day 3  $p=0.955$ , week 1  $p=0.702$ , week 2  $p=0.888$ , week 3  $p<0.812$ , week 4  $p=0.819$ ; Figure 3.4)). However, mean LDH levels were significantly higher in the ankle compared to the knee at all time points with ankle tissue producing almost twice as much LDH compared to the same wet weight of knee cartilage (day 3: 0.021 vs 0.013  $p<0.0001$  (Figure 3.4a); week 1: 0.042 vs 0.023  $p<0.0001$  (Figure 3.4b); week 2: 0.063 vs 0.037  $p<0.0001$  (Figure 3.4c); week 3: 0.091 vs 0.052  $p<0.0001$  (Figure 3.4d); week 4: 0.114 vs 0.066  $p<0.0001$  (Figure 3.4e). Multiple comparison analysis demonstrated significant differences in LDH production between the two untreated joint cartilage tissues and for almost all cytokine treatments from 1 week onwards (Table 3.2), suggesting that the joint of origin, as opposed to the cytokine treatment, influenced LDH levels.



**Figure 3.4** Levels of lactate dehydrogenase released from ankle and knee cartilage into the media following cytokine stimulation, assessed as a marker of cell viability. Ankle cartilage (n=4) and knee cartilage (n=3) was either cultured ‘short term’ for 7 days or ‘long term’ extending out to 28 days (ankle n=3, knee n=4) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); LDH levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as the mean cumulative optical density per mg of wet tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

<b>Day 3</b>	<b>Ankle</b>	<b>Knee</b>	<b>Adjusted P Value</b>	<b>Significant</b>
Untreated	0.0205	0.0148	0.4205	No
TNF $\alpha$ Low	0.0216	0.0134	0.1008	No
TNF $\alpha$ High	0.0182	0.0134	0.6753	No
IL-1 and OSM Low	0.0198	0.0127	0.1796	No
IL-1 and OSM High	0.0235	0.0124	0.0057	Yes
IL-1 and TNF $\alpha$ Low	0.0220	0.0131	0.0418	Yes
IL-1 and TNF $\alpha$ High	0.0217	0.0134	0.0945	No
<b>Day 7</b>	<b>Ankle</b>	<b>Knee</b>	<b>Adjusted P Value</b>	<b>Significant</b>
Untreated	0.0417	0.0231	0.0128	Yes
TNF $\alpha$ Low	0.0432	0.0255	0.0198	Yes
TNF $\alpha$ High	0.0389	0.0234	0.0768	No
IL-1 and OSM Low	0.0362	0.0198	0.0384	Yes
IL-1 and OSM High	0.0460	0.0232	0.0012	Yes
IL-1 and TNF $\alpha$ Low	0.0436	0.0215	0.0018	Yes
IL-1 and TNF $\alpha$ High	0.0429	0.0248	0.0166	Yes
<b>Day 14</b>	<b>Ankle</b>	<b>Knee</b>	<b>Adjusted P Value</b>	<b>Significant</b>
Untreated	0.0587	0.0361	0.0505	No
TNF $\alpha$ Low	0.0654	0.0401	0.0203	Yes
TNF $\alpha$ High	0.0637	0.0366	0.0249	Yes
IL-1 and OSM Low	0.0599	0.0333	0.0129	Yes
IL-1 and OSM High	0.0641	0.0385	0.0181	Yes
IL-1 and TNF $\alpha$ Low	0.0640	0.0351	0.0053	Yes
IL-1 and TNF $\alpha$ High	0.0662	0.0400	0.0145	Yes
<b>Day 21</b>	<b>Ankle</b>	<b>Knee</b>	<b>Adjusted P Value</b>	<b>Significant</b>
Untreated	0.0865	0.0510	0.0482	Yes
TNF $\alpha$ Low	0.0953	0.0569	0.0259	Yes
TNF $\alpha$ High	0.0875	0.0511	0.0761	No
IL-1 and OSM Low	0.0848	0.0466	0.0270	Yes
IL-1 and OSM High	0.0937	0.0553	0.0262	Yes
IL-1 and TNF $\alpha$ Low	0.0931	0.0504	0.0096	Yes
IL-1 and TNF $\alpha$ High	0.0981	0.0583	0.0187	Yes
<b>Day 28</b>	<b>Ankle</b>	<b>Knee</b>	<b>Adjusted P Value</b>	<b>Significant</b>
Untreated	0.1096	0.06373	0.0501	No
TNF $\alpha$ Low	0.1199	0.07143	0.0332	Yes
TNF $\alpha$ High	0.1035	0.0646	0.2236	No
IL-1 and OSM Low	0.1059	0.05792	0.0355	Yes
IL-1 and OSM High	0.1175	0.06958	0.0359	Yes
IL-1 and TNF $\alpha$ Low	0.1182	0.06262	0.0095	Yes
IL-1 and TNF $\alpha$ High	0.1225	0.07102	0.0198	Yes

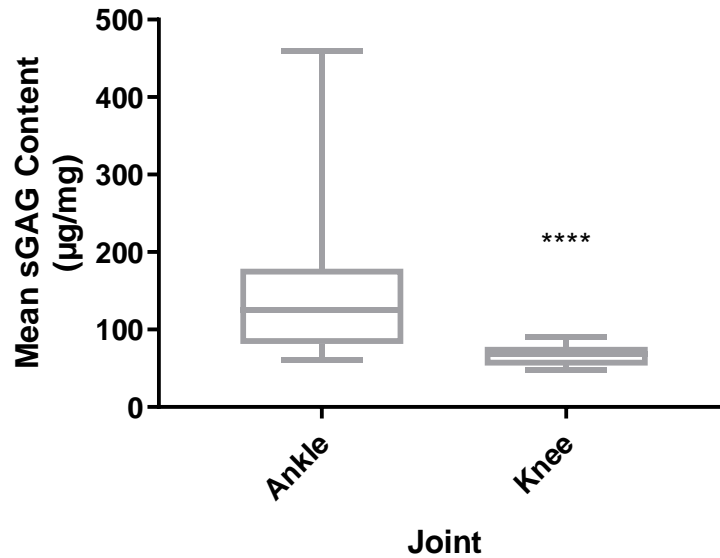
**Table 3.2:** Sudak's Multiple comparator test results with adjusted p values comparing LDH release from the different joints after 'short term' culture for 7 days (n = 7) or 'long term' culture extending out to 28 days (n = 4) in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ).

### **3..11 Differential effect of pro-inflammatory cytokines on proteoglycan loss in the ankle and knee**

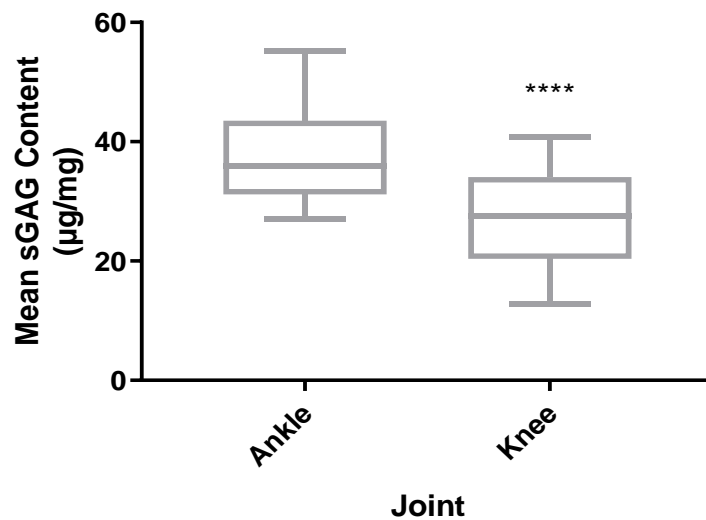
Percentage loss of sGAG from the explant into the media was measured and used as a marker to quantify cartilage proteoglycan degeneration. All media was analysed for sGAG content using the 1,9 -dimethylmethylene blue assay as previously described (Little et al., 1990). The remaining explant was then digested with papain to allow the remaining sGAG in the tissue to be measured. All results were normalised to original explant wet weight. sGAG amounts are presented below as normalised to wet weight and percentage of the total sGAG lost from explants.

### **3..12 Total sulphated glycosaminoglycan content was significantly higher in the ankle than the knee cartilage explants**

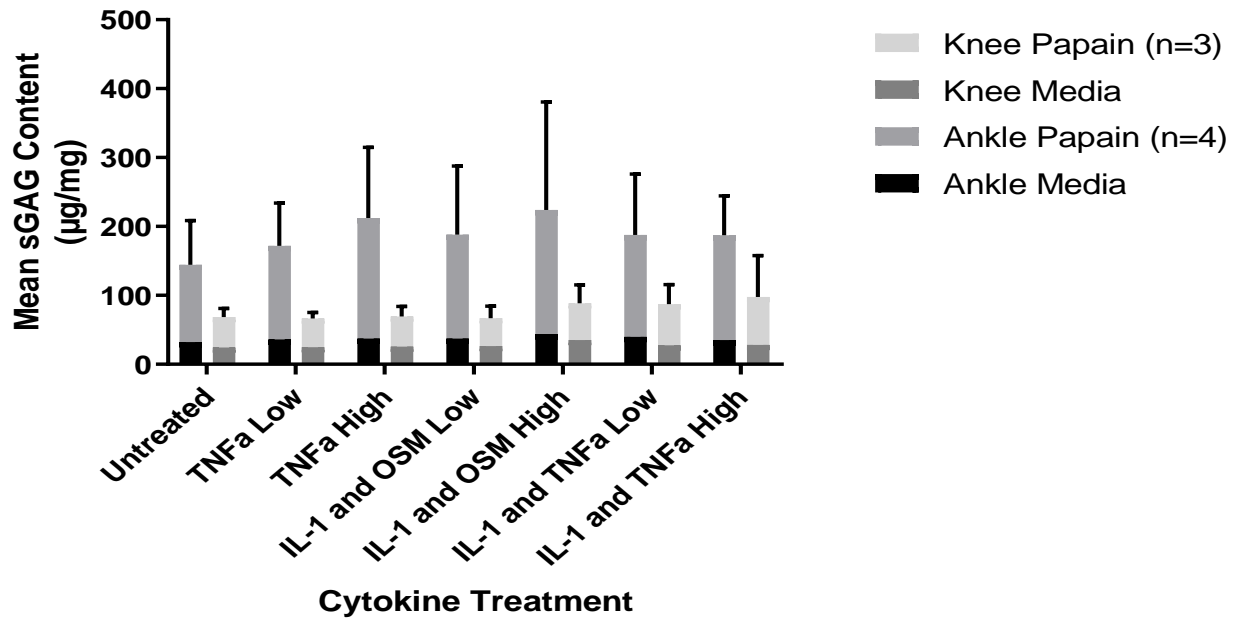
Following papain digestion, significantly higher amounts of sGAG were detected within the untreated ankle cartilage explants ( $148 \pm 75\mu\text{g}/\text{mg}$ ) compared to the knee ( $74 \pm 18\mu\text{g}/\text{mg}$ ;  $p < 0.001$ ; Figure 3.5). Significantly more sGAG was released, into the media, from the untreated ankle ( $37 \pm 7.4\mu\text{g}/\text{mg}$ ) compared to the untreated knee cartilage explants ( $27 \pm 7.6\mu\text{g}/\text{mg}$ ) over the 28 days culture period ( $p < 0.0001$ ; Figure 3.6). Hence, total sGAG levels (determined as the summation of amount released into media plus amount retained in the tissue) in the ankle cartilage explants exceeded that of the knee explants, irrespective of cytokine treatment ( $p < 0.0001$ ; Figure 3.7). However, cytokine treatment did not significantly affect total sGAG content when comparing response of ankle and knee ( $p = 0.8897$ ).



**Figure 3.5:** Total sulphated glycosaminoglycan (sGAG) content in untreated ankle (n=7) and knee articular cartilage (n=6), as assessed using the Dimethylmethylene Blue assay. Total sGAG is equal to sGAG released into the media and sGAG released from the tissue following papain digest. Data is presented as mean sGAG content per mg of wet weight tissue and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box). An unpaired t-test was performed to determine statistical significance ( $p < 0.0001$ ).



**Figure 3.6:** Mean total sulphated glycosaminoglycan (sGAG) lost to media in untreated ankle (n=7) and knee articular cartilage (n=6), as assessed using the Dimethylmethylene Blue assay. Data is presented as mean sGAG content per mg of wet weight tissue and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box). An unpaired t-test was performed to determine statistical significance ( $p < 0.0001$ ).



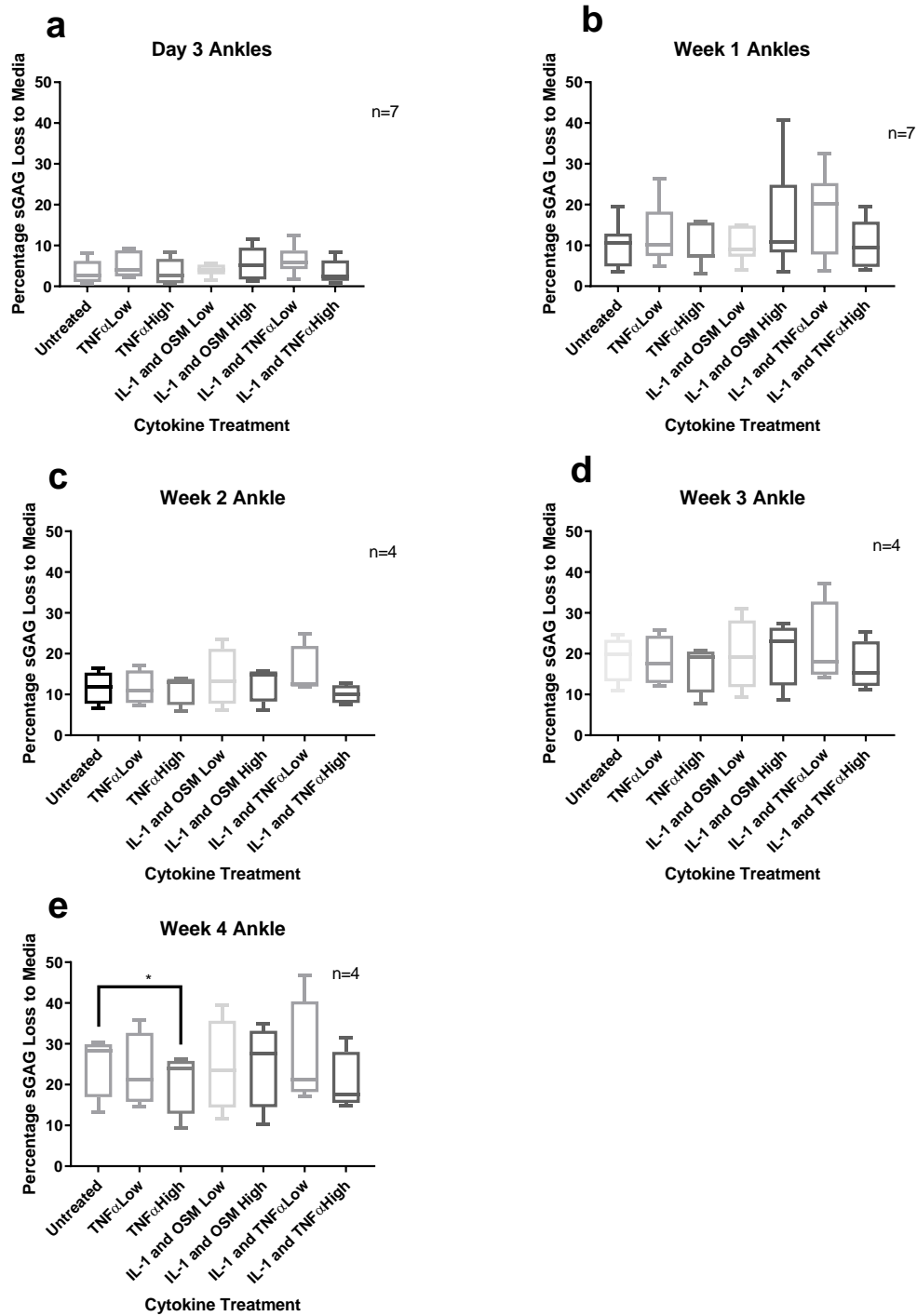
**Figure 3.7:** Total amount of sulphated glycosaminoglycans (sGAG) in ankle (n=4) and knee articular cartilage (n=3), as assessed using the Dimethylmethylene Blue assay. Total content is the summation of sGAG lost to the media plus sGAG content in the explants following papain digestion. Cartilage tissue was cultured in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ) for 28 days. Data is presented as mean sGAG content per mg of wet weight tissue  $\pm$  standard deviation. A two-way ANOVA was performed to compare differences between cytokine treatment (p=0.8897) and joint of origin (p<0.0001).



### 3.13 Significantly less proteoglycan degeneration occurred in ankle cartilage explants treated with 100ng/ml TNF $\alpha$ compared to the untreated samples at 28 days

The effect of cytokine treatments on sGAG loss from ankle cartilage was quantified as a mean cumulative percentage sGAG loss as a proportion of total sGAG content over the culture duration. Data from matched samples was analysed using a one-way ANOVA at each specific time point (Figure 3.8); surprisingly, there was no significant difference between cytokine treatments in the cumulative percentage loss of sGAG to media (day 3:  $p=0.097$  Figure 3.8a; week 1:  $p=0.073$  Figure 3.8b; week 2:  $p=0.307$  Figure 3.8c; week 3:  $p=0.467$  Figure 3.8d; week 4:  $p=0.459$  Figure 3.8e).

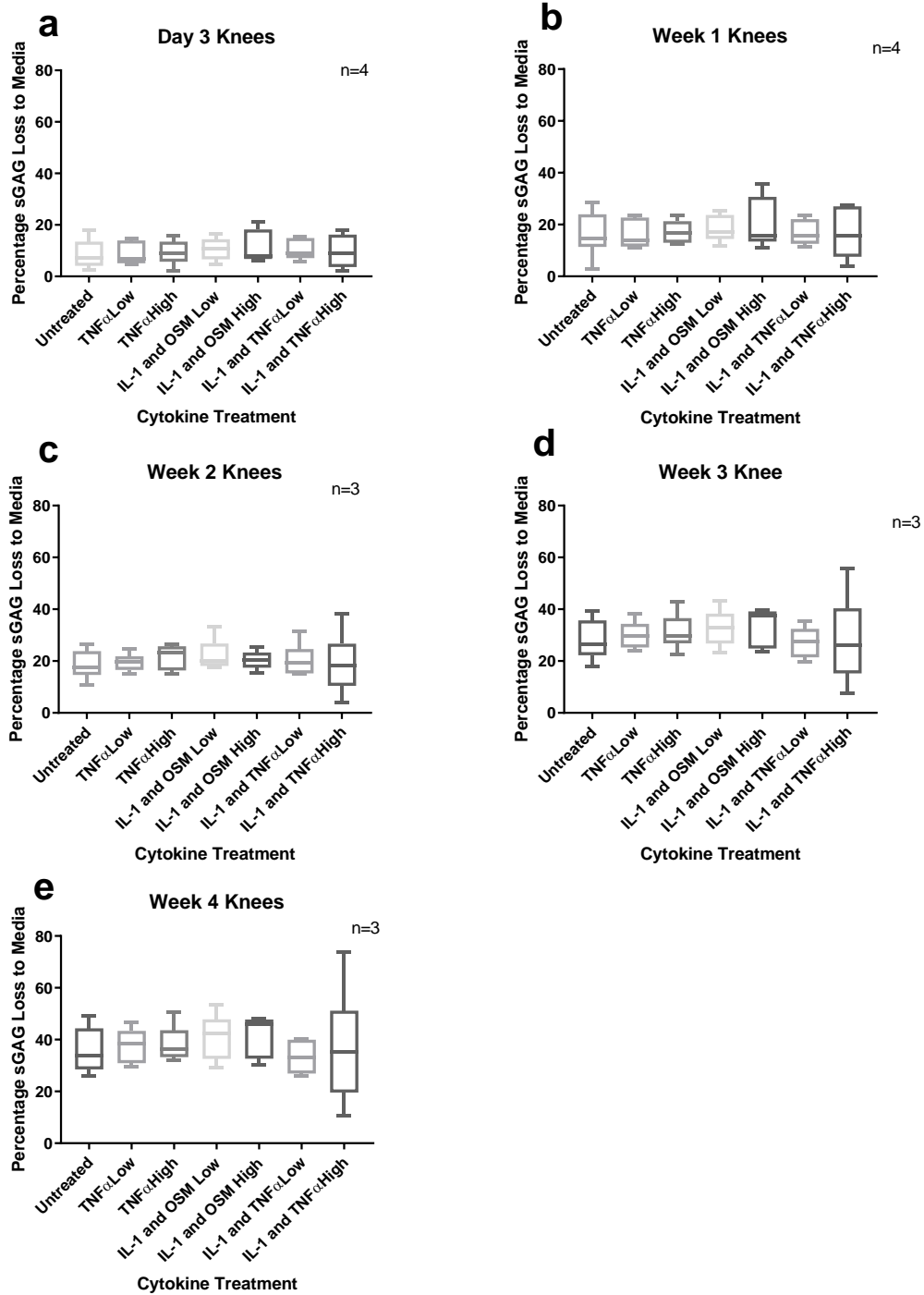
However, there was a significant difference between patients receiving the same treatments over the culture duration suggesting heterogeneity between patients (day 3  $p<0.001$ , week 1  $p<0.001$ , week 2  $p=0.002$ , week 3  $p<0.001$ , week 4  $p<0.001$ ). Tukey multiple comparator testing using adjusted  $p$  values was performed at all time points in order to compare untreated samples against cytokine treated samples and to compare high concentration treatments against low concentration treatments. Interestingly, following 4 weeks of culture in high dose TNF $\alpha$ , a significantly lower percentage sGAG loss was seen compared to the untreated samples (20.88% vs 25.03%,  $p=0.036$ ) suggesting the ankle cartilage was not responsive to this cytokine treatment. There was no evidence of increased percentage loss of sGAG to media following high concentration stimulation compared to low concentration stimulation at any time point for any cytokine concentration ( $p= 0.123$  to  $p=0.999$ ).



**Figure 3.8:** Mean cumulative percentage loss of sulphated glycosaminoglycans (sGAG) from ankle cartilage cultured in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ) for 28 days; sGAG levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as mean percentage sGAG loss to media for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box) (n = 4 – 7 donor explants) [\* p < 0.05].

### 3.14 Proteoglycan loss was not significantly affected by cytokine treatments in knee cartilage.

The effect of cytokine treatments on sGAG loss from knee cartilage (combined medial and lateral femoral condyle tissue) was quantified as a mean cumulative percentage sGAG loss as a proportion of total sGAG content over the culture duration. Data from matched samples was analysed using a one-way ANOVA at each specific time point (Figure 3.9); however, there were no significant differences in the cumulative percentage sGAG loss to media in response to the different combinations of pro-inflammatory cytokines (day 3:  $p=0.424$  Figure 3.9a; week 1:  $p=0.555$  Figure 3.9b; week 2:  $p=0.744$  Figure 3.9c; week 3:  $p=0.632$  Figure 3.9d; week 4:  $p=0.625$  Figure 3.9e). Tukey multiple comparator testing using adjusted p values was performed at all time points in order to compare untreated cartilage against tissue exposed to the various physiological and pathological concentrations of cytokine combinations. Surprisingly, cytokine treatment did not significantly influence sGAG loss at any time point relative to untreated tissue ( $p \geq 0.423$ ). Furthermore, there was also no evidence of increased percentage sGAG loss following stimulation with pathological cytokine 'high' concentration compared to physiological cytokine 'low' concentration ( $p \geq 0.593$ ).

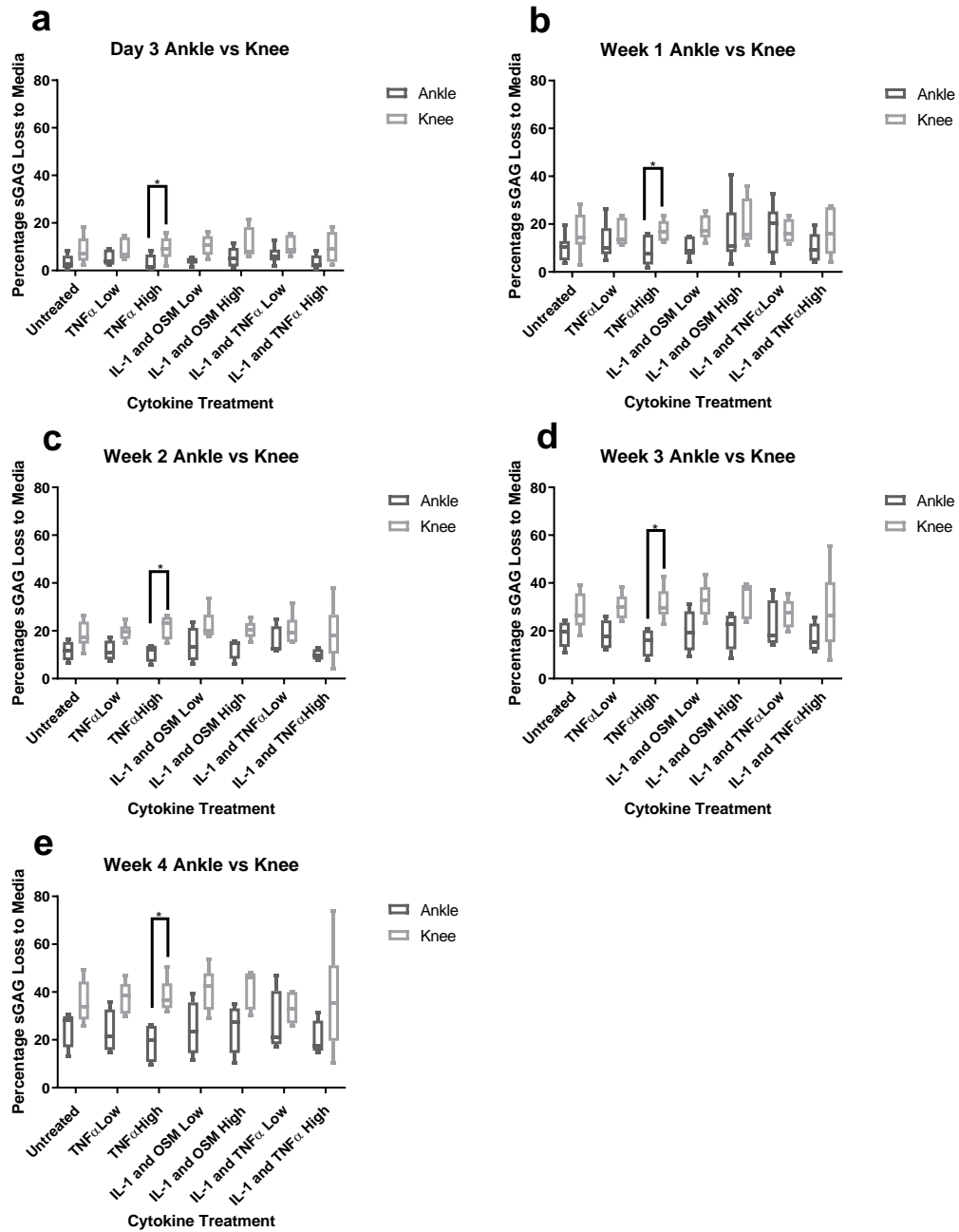


**Figure 3.9:** Mean cumulative percentage loss of sulphated glycosaminoglycans (sGAG) from knee cartilage cultured in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ) for 28 days; sGAG levels were measured at [a] day 3, [b] day 7, [c] 14, [d] day 21 and [e] day 28. Data is calculated as mean percentage sGAG loss to media for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box) (n = 3 – 4 donor explants).

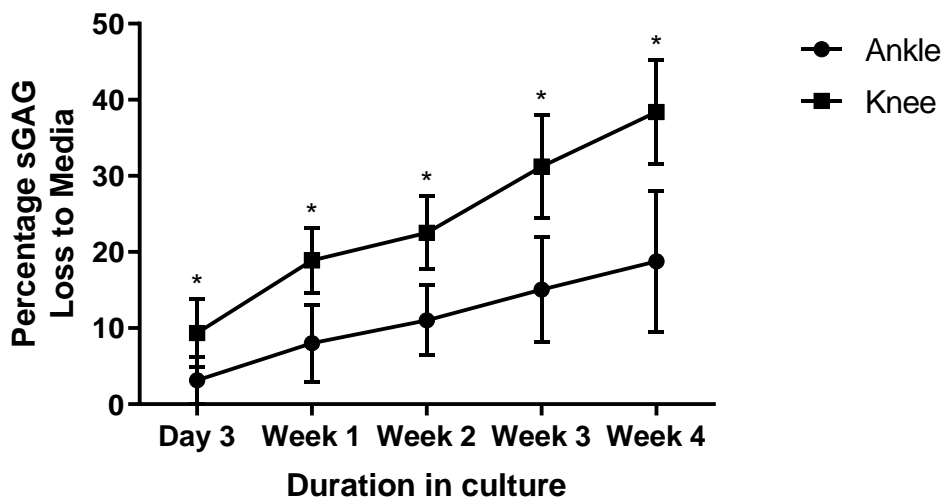
### 3.15 Significantly more proteoglycan degeneration occurred in knee cartilage compared to ankle cartilage following treatment with 100ng/ml TNF $\alpha$

Mean percentage sGAG loss (cumulative) from ankle and knee cartilage explants exposed to differing cytokine combinations/concentrations was then compared at each time point using a Two-way ANOVA (Figure 3.10). This statistical analysis allowed assessment of the percentage variance and significance that the joint of origin, cytokine treatment and interaction between these two factors had on sGAG loss during the culture period. Multiple comparison analysis with an adjusted p value was also undertaken using the Sidak's multiple comparator method to identify any trends between different joints exposed to the same cytokine treatment.

Overall variance between groups was significantly associated with the joint of origin at all time points analysed (day 3:  $p < 0.0001$  Figure 3.10a; week 1:  $p = 0.0007$  Figure 3.10b; week 2:  $p = 0.001$  Figure 3.10c; week 3:  $p < 0.0001$  Figure 3.10d; week 4:  $p < 0.0001$  Figure 3.10e). Surprisingly, when analysed together, cytokine treatments were not found to be a significant cause of variation of the mean between groups at any time point (day 3 3.7%  $p = 0.549$ , week 1 6.4%  $p = 0.300$ , week 2 3.6%  $p = 0.766$ , week 3 2.5%  $p = 0.886$  and week 4 2.0%  $p = 0.929$ ). Multiple comparison analysis did however demonstrate a significant increase in percentage sGAG loss from knee cartilage explants treated with high dose TNF $\alpha$  compared to ankle tissue (Figure 3.9). This significance was consistently observed at all time points analysed including day 3 (3.1% vs 9.3%  $p = 0.043$ , Figure 3.10a), week 1 (8.0% vs 18.9%  $p = 0.042$ , Figure 3.10b), week 2 (11.0% vs 22.5%  $p = 0.023$ , Figure 3.10c), week 3 (15.0% vs 31.2%  $p = 0.031$ , Figure 3.10d) and week 4 (18.8% vs 38.4%  $p = 0.039$ , Figure 3.10e). Over time, it was evident that pathological 'high' TNF $\alpha$  concentration significantly induced sGAG loss from the knee cartilage relative to levels measured in the ankle tissue (Figure 3.11).



**Figure 3.10** Mean cumulative percentage sulphated glycosaminoglycans (sGAG) loss from ankle and knee cartilage into the media following cytokine stimulation. Ankle cartilage (n=4) and knee cartilage (n=3) was either cultured ‘short term’ for 7 days or ‘long term’ extending out to 28 days (ankle n=3, knee n=4) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); sGAG levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as the mean cumulative percentage of total sGAG for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box) [\* p < 0.05].



**Figure 3.11:** Effect of 100ng/ml TNF $\alpha$ , considered a pathological concentration, on percentage cumulative sulphated glycosaminoglycan loss from ankle (n = 4) and knee cartilage explants (n = 3) over a 28-day period. Data is presented as mean +/- standard deviation and significance assessed using a Two-way ANOVA [\* p < 0.05].

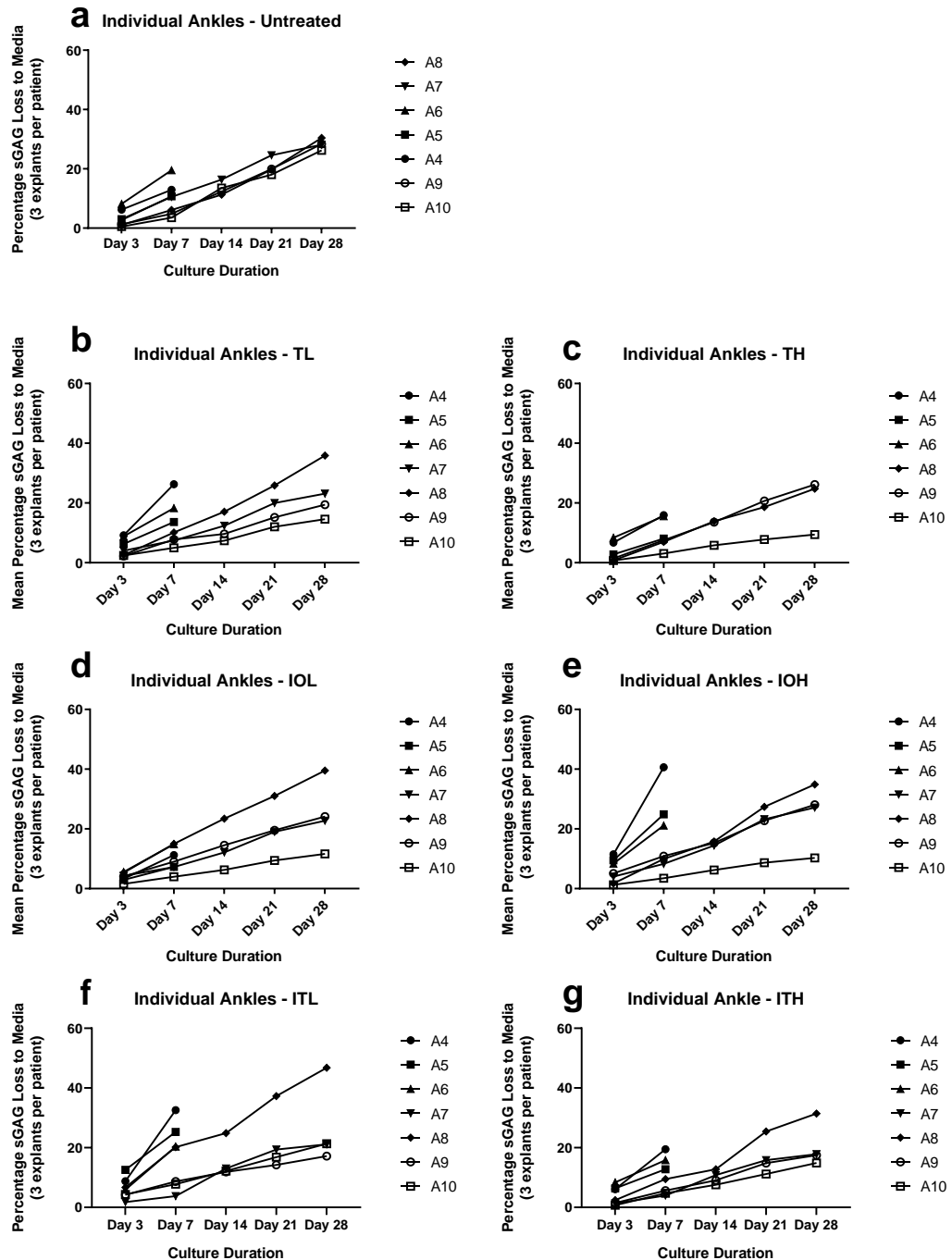
### 3.16 Importance of heterogeneity in articular cartilage response to cytokines: proteoglycan loss relative to individual patient analysis

Due to the heterogenous response of patient tissue to cytokine treatments, data was further analysed as individual responses to determine whether a trend could be identified. Individual ankle explants and individual knee explants cultured in each different combination of cytokine are displayed at all time points (Figure 3.12 – 3.13) to elicit whether there was a consistent individual patient response. In order to demonstrate individual patient susceptibility to cytokine mediated degeneration, data from matched pairs of ankle and knee tissue from the same patient donor was further analysed (Figure 3.14).

### 3.17 Response of donor-specific ankle cartilage to cytokine treatment

Very little variation was observed between patients following culture of ankle cartilage explants in the absence of cytokines i.e. untreated (Figure 3.12a), while a consistent pattern was identified for all cytokine treatments (Figure 3.12b-g). Irrespective of the cytokine treatment, donor ankle 10 released the least sGAG to media during the 28 days of culture. Furthermore, donor ankle 10, 9 and 7 consistently released the least sGAG across all treatments, whilst sGAG loss was most evident in donor ankles 4, 5 and 8, indicating enhanced degradation of the cartilage matrix. The untreated cartilage explants demonstrated very little difference (4.22%) in sGAG loss between the different donor tissues after 28 days of culture (A10 - 26.20% and A8 - 30.42%; Figure 3.12a). In contrast, there was a greater variation in sGAG loss in response to specific cytokine treatments; for example, a difference of 16.58% was observed in ankle tissue subjected to pathophysiological '*high*' concentration of IL-1 $\alpha$  in combination with TNF $\alpha$  (ITH: A10 - 14.85% and A8 - 31.43%; Figure 3.12g). In addition, a difference of 29.57% sGAG loss was detected in donor-specific ankle cartilage tissue in response to physiological '*low*' concentrations of IL-1 $\alpha$  in combination with TNF $\alpha$  (ITL: A10 - 17.2% and A8 - 46.77%; Figure 3.12e). Even greater differences between patient tissues may have been observed if longer-term culture i.e. extending the period to 28 days had been implemented for donor ankle A4, A5 and A6, as at day 7 there was already evidence of 20% sGAG loss i.e. degradation, in response to the majority of cytokine treatments. For example, donor ankle 4 had already reached 40.65% loss of GAG when cultured in a pathophysiological '*high*' concentration of IL-1 $\alpha$  in combination with OSM (Figure 3.12e), clearly demonstrating heterogeneity in the behaviour of the tissue towards cytokine stimulation.

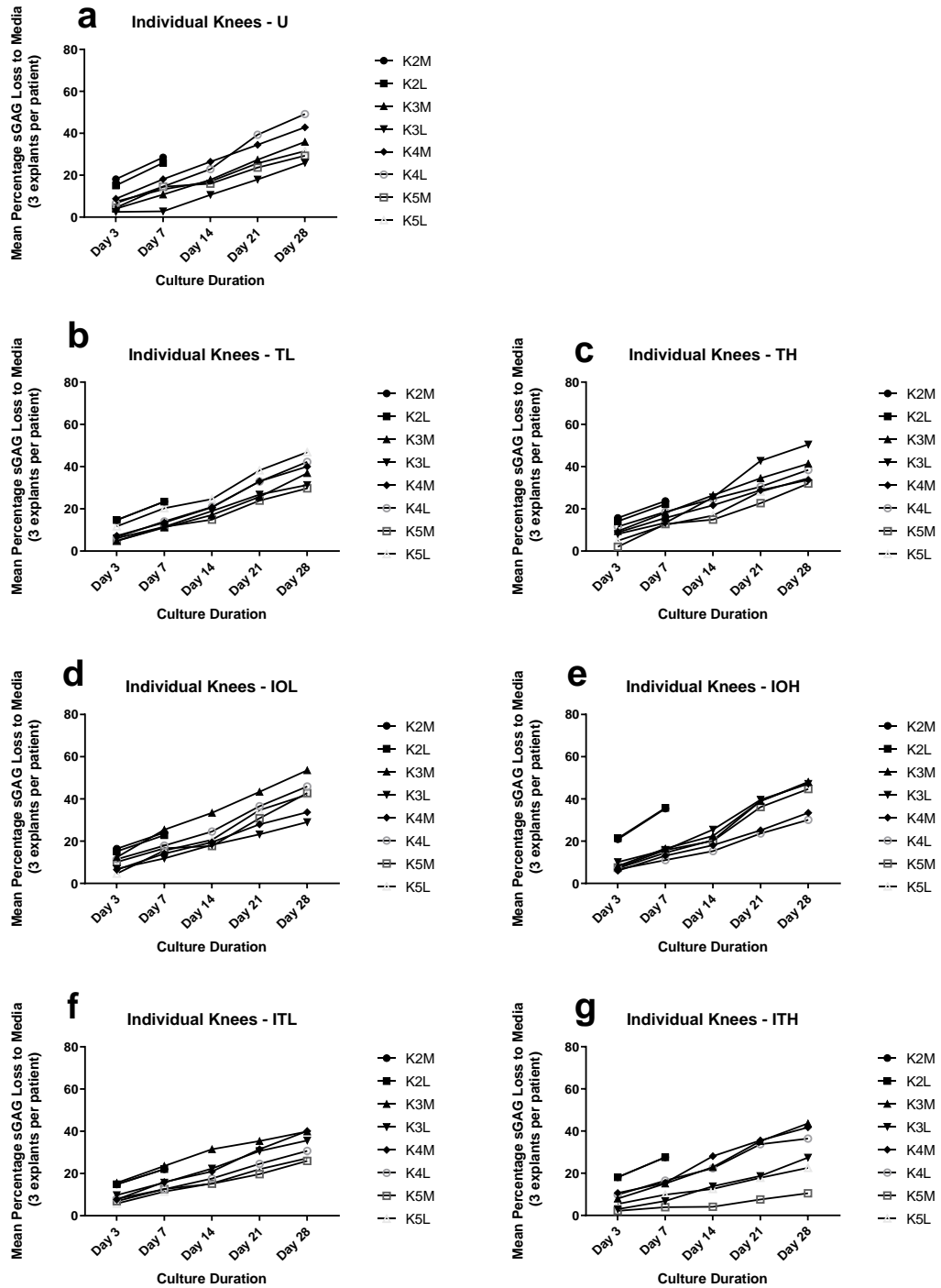




**Figure 3.12:** Mean cumulative percentage loss of sulphated glycosaminoglycans (sGAG) from individual donor-specific ankle cartilage explants cultured in the [a] absence (untreated) or presence of [b] 2ng/ml TNF $\alpha$  (TL), [c] 100ng/ml TNF $\alpha$  (TH), [d] 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM (IOL), [e] 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM (IOH), [f] 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$  (ITL), and [g] 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (ITH) for < 28 days. sGAG levels were measured at day 3, day 7, day 14, day 21 and day 28. Data is calculated as mean percentage sGAG loss to media for each treatment group. Data is representative of the mean of 3 explants for each donor.

### 3.18 Response of donor-specific knee cartilage to cytokine treatment

Less patient heterogeneity was observed across the individual knee samples, however, there remained little variation in mean sGAG loss for untreated explants (Figure 3.13a). Following 28 days of culture in the absence of cytokines the difference between the most catabolic specimen and least was only 14.9% (K3M 44.2% and K5M 29.3%). Once again, a consistent but less pronounced pattern was identified for all cytokine treatments (Figure 3.13b-g). For all cytokine treatments, Knee sample 4 and 5 released the lowest percentage sGAG to media during the 28 days of culture while Knee samples 2 and 3 released the most. This pattern was most noticeable for explants cultured in high concentration of IL-1 $\alpha$  and TNF $\alpha$ . Following 28 days of culture the difference in percentage sGAG loss between the least and most catabolic sample was 43.1% (K5M 10.5% and K4M 53.6%).



**Figure 3.13:** Mean cumulative percentage loss of sulphated glycosaminoglycans (sGAG) from individual donor-specific knee cartilage explants cultured in the [a] absence (untreated) or presence of [b] 2ng/ml TNF $\alpha$  (TL), [c] 100ng/ml TNF $\alpha$  (TH), [d] 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM (IOL), [e] 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM (IOH), [f] 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$  (ITL), and [g] 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (ITH) for < 28 days. sGAG levels were measured at day 3, day 7, day 14, day 21 and day 28. Data is calculated as mean percentage sGAG loss to media for each treatment group. Data is representative of the mean of 3 explants for each donor.

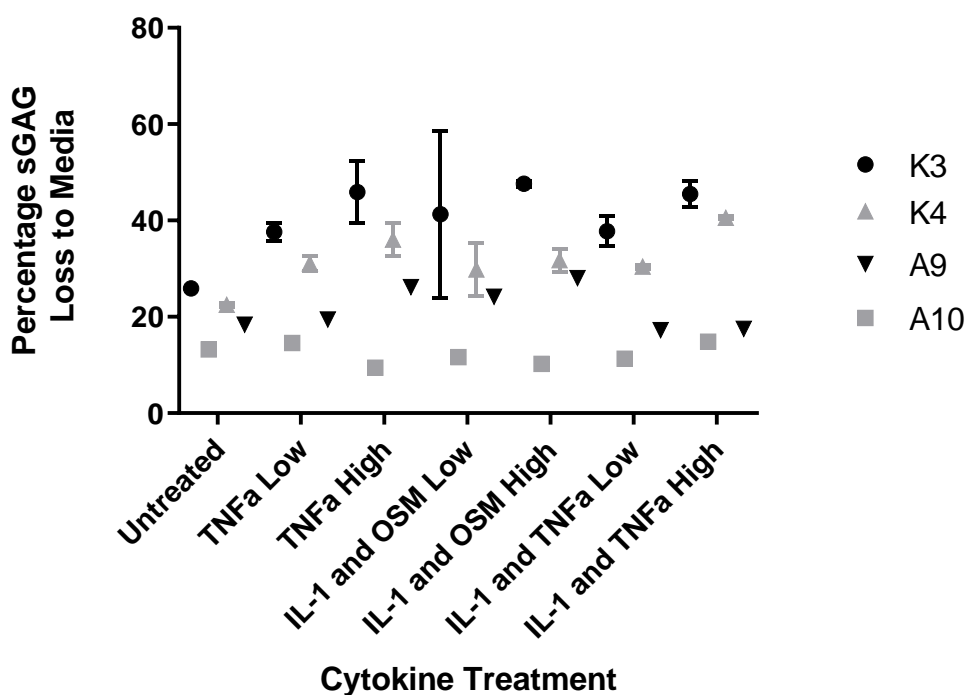
### 3..19 Comparison of cytokine-mediated response of donor-specific matched ankle and knee cartilage

Matched paired ankle and knee cartilage were collected from 3 donors: Patient B (Ankle 2 and Knee 1), Patient J (Ankle 9 and Knee 3), Patient K (Ankle 10 and Knee 4). Cartilage explants from Patient B were cultured for 7 days and explants from Patient J and Patient K underwent extended culture to 28 days. A Two-way ANOVA was used to identify any significant difference between the total percentage sGAG loss at 28 days from knee and ankle tissue from patients J and K (Figure 3.14).

A Two-way ANOVA of the ankle samples (A9 and A10) demonstrated an overall significant difference in percentage loss of sGAG to media between donors (21.55% vs 12.17%;  $p=0.027$ ) indicative of patient heterogeneity but this difference is not significantly affected by cytokine treatments ( $p=0.931$ ). Identical comparison performed on the knee cartilage explants (K4 and K5) also demonstrated a significance between donors (40.24% vs 31.75%  $p=0.001$ ) and in this case also between cytokine treatment ( $p=0.005$ ). In the knee, significant differences in proteoglycan loss are seen between the untreated tissue and the pathological 'high' dose cytokine treatments (Table 3.3). Hence, what can clearly be observed is that more sGAG is lost from knee compared with ankle in response to increasing cytokine concentration and relative to untreated explants (untreated < physiological 'low' < pathological 'high'.) Also, there is clear heterogeneity in donor cartilage behaviour with more overall sGAG lost from the explants by patient J for all cytokine treatments suggesting patient J is significantly more susceptible to the catabolic effect of pro-inflammatory cytokines (Figure 3.14).

Sidak's multiple comparisons test	Untreated (%)	Cytokine (%)	Adjusted P Value	Significant?
Untreated vs. TNF $\alpha$ Low	24.19	34.37	0.1095	No
Untreated vs. TNF $\alpha$ High	24.19	40.99	0.0038	Yes
Untreated vs. IL-1 $\alpha$ and OSM Low	24.19	35.55	0.0610	No
Untreated vs. IL-1 $\alpha$ and OSM High	24.19	39.71	0.0073	Yes
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	24.19	34.1	0.1244	No
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	24.19	43.06	0.0014	Yes

**Table 3.3:** Sidak's Multiple comparator test results with adjusted p values comparing percentage sGAG loss from the different joints after 'short term' culture for 7 days (n = 7) or 'long term' culture extending out to 28 days (n = 4) in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ).



**Figure 3.14:** Total mean cumulative percentage loss of sulphated glycosaminoglycans (sGAG) from donor-specific matched paired ankle and knee cartilage explants cultured in the absence (untreated) or presence of 2ng/ml TNF $\alpha$  (TNF $\alpha$  low), 100ng/ml TNF $\alpha$  (TNF $\alpha$  High), 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM (IL-1 and OSM Low), 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM (IL-1 and OSM High), 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$  (IL-1 and TNF $\alpha$  Low), and 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (IL-1 and TNF $\alpha$  High) for 28 days. sGAG levels were measured at day 3, day 7, day 14, day 21 and day 28, and data is calculated as a total mean percentage sGAG loss to media for each treatment group over the 28 days of culture. Data is representative of the mean of 3 explants for each donor [Samples A9 and K3 were donated by patient J (black) and samples A10 and K4 were donated by patient K (grey)].

### 3..20 Discussion

The viscoelastic properties i.e. biomechanical functionality of articular cartilage is determined by the sGAG, type II collagen and water content. Ankle cartilage demonstrates improved wear characteristics due to the high level of sGAG and relatively low water content. The data presented in this Chapter confirms the conclusions of previous work by Keuttner et al (2005) that ankle cartilage has significantly higher sGAG levels when compared to the knee. The presented findings also demonstrated that in untreated cartilage i.e. in the absence of pro-inflammatory cytokines, that more sGAG was lost from the ankle tissue into the culture media than in the knee respectively; however, as a percentage sGAG loss to represent degree of degeneration, the loss of sGAG from the cartilage into media was greater in the knee than ankle. Interestingly, when LDH levels were analysed, almost twice as much LDH was produced by the ankle than the knee explants, although overall, levels were low indicating cell viability over the culture period.

Surprisingly, when individual joints (either ankle or knee) were investigated independently, cytokine treatment did not significantly affect the percentage sGAG loss from the explants to the media relative to the untreated tissue. Furthermore, exposure to physiological 'low' or pathological 'high' concentrations of the respective cytokines also did not affect overall sGAG loss, compared to untreated tissue. This cannot have been attributed to cell death as LDH levels in ankle and knee explants showed no significant difference between the untreated and cytokine treated explants, or in response to increasing cytokine concentration over the culture period. This lack of perceived effect may be due to the sizeable loss of sGAG into the media of untreated cartilage which was unexpected; this result could be attributed to the fact that it is not experiencing any load over the 28-day culture period, as it is well reported that articular cartilage relies on a mechanical stimulus to maintain tissue homeostasis (Sophia Fox et al., 2009) . However, what is evident is the differential response according to joint of origin in how the tissue behaves.

Previous studies have reported that pro-inflammatory cytokines increase tissue degradation i.e. via breakdown of proteoglycan extracellular matrix (Fernandes et al., 2002); however, in this study, when the data is averaged biological effects are lost due to donor heterogeneity. However, what is interesting is when knee and ankle cartilage explants are directly compared following pathological 'high' TNF $\alpha$  treatment there seems to be a degree of 'chondroprotection' in the ankle, in that the tissue does not respond to the cytokine stimulus, while there is increased sGAG loss from the knee tissue. This differential response to TNF $\alpha$  results in a significant difference in proteoglycan loss/matrix degeneration between the ankle and knee cartilage which remains significant at all individual time points analysed hence diverging over time. It may be that the set point at which ankle and knee cartilage produces a catabolic response to TNF $\alpha$  is very different and therefore at the concentrations assessed in this thesis, the ankle is less responsive i.e. limited proteoglycan degradation and is thus resistant to this pro-inflammatory insult. This would confirm the study of Keuttner and Cole (2005) who demonstrated that there was a differential response between these joint tissues in how they react to pro-inflammatory IL-1 and demonstrated tissues can shift between anabolic and catabolic states depending on the cytokine concentration they are exposed to.

As previously mentioned, there was a high degree of heterogeneity in the catabolic response observed between patients, hence data was presented as individual donors to more finely analyse the extent of sGAG loss. The extent of sGAG lost to media from the untreated ankle cartilage was very similar with only an approximate 4% difference between the range of responses. Much greater differences were observed for the cytokine treated explants with an almost 30% difference in sGAG loss across the donor responses. There was a consistent pattern whereby several donors were less responsive to cytokines, with others releasing more sGAG loss over the culture period. A similar but slightly less pronounced pattern of heterogeneity was observed across the knee cartilage with a differential degree of sGAG loss i.e. proteoglycan degradation. This donor-specific inflammatory response was further demonstrated when examining the matched pairs of knee and ankle tissue that had been collected from the same patient donors (above knee amputation). For both these patients the

overall percentage loss of sGAG was very similar but there was a differential susceptibility to inflammation which was found to be significant for all cytokine treatments, as well as a joint dependent susceptibility i.e. knee cartilage released consistently more sGAG than its matched ankle.

The findings presented in this Chapter would concur with previous studies demonstrating a resistance to pro-inflammatory stimulus in the ankle compared to the knee (Eger et al., 2002). Future studies would require increasing the sample size and ideally acquiring more matched knee and ankle specimens to more fully investigate inherent differences in cartilage response to cytokines. Furthermore, the data demonstrated a considerable variability in the extent to which the cartilage reacted to an inflammatory insult. It is possible that differences observed between patient responses is related to their underlying medical conditions; however, significant patient specific proteoglycan loss was seen between patient J and K both of which were vasculopathies undergoing amputations for critical ischaemia, with similar comorbidities, of similar age with identical joint degeneration scores (Table 3.1). If the patient's medical condition was the main driver of resistance or susceptibility to cytokine induced degeneration, then one would have expected a non-vasculopathy (patient L) to have been relatively cytokine naïve and undergone the most inflammation driven degeneration which was not observed. When individual joint degeneration was plotted for each cytokine treatment, explants from the lateral and medial side of the knee donated by patient L (Knee 5) underwent the least cytokine mediated degeneration. Both these findings would support the hypothesis that cytokine mediated degeneration is due to innate patient specific differences and not attributable to their underlying medical conditions.

Patient Innate susceptibility to inflammation has previously been investigated in the context of polytrauma. Systemic Inflammatory Response Syndrome (SIRS) is an immune response following trauma or severe infections that results in excessive vascular permeability, extracellular fluid shift and in some patients acute lung and vital organ failure (Bone et al., 1992). SIRS is also associated with persistently elevated levels of serum cytokines including TNF $\alpha$ , IL-1, IL-6 and IL-8 (Jaffer et al., 2010). It is unclear why some patients develop life threatening complications from an



exaggerated and elongated inflammatory phase while others with the same injury burden or infectious load fully recover. It is theorised that individual patients may have an innate susceptibility or immunity to cytokine-mediated inflammation and tissue death. Differential regulation of proinflammatory genes such as TNF $\alpha$  and inflammatory pro-resolution genes encoding lipoxin and secretory leukocyte protease inhibitor (SLPI) have been heavily implicated (Barton, 2008). It is also felt that “genetic polymorphisms may influence the natural history of SIRS” (Jaffer et al., 2010) in particular those involving anti-inflammatory cytokines. The heterogeneous patient response to cytokines, that were identified in this Chapter - particularly for knee articular cartilage, has also been noted in clinical trials of disease modifying osteoarthritis drugs such as anti-TNF *adalimumab*. Although preclinical work would suggest blocking TNF $\alpha$  in hand OA would prevent cartilage degeneration and pain this has not been demonstrated in large clinical trials, however subgroups within the patient cohort have had an excellent response (Verbruggen et al., 2012).

In conclusion, it was found that both LDH and absolute amount of sGAG lost from the tissue were significantly elevated in the ankle compared to the knee. This is not surprising given it has previously been demonstrated that ankle proteoglycans (sGAGs) undergoes 2.1 times more turnover than knee tissue and ankle cartilage is significantly more metabolically active than knee cartilage (Kuettnner and Cole, 2005). To confirm this higher level of sGAG synthesis and more accurately assess metabolic status, [<sup>35</sup>S]-sulphate radiolabelling could be performed to measure both synthesis and degradation of sGAGs in these joint cartilages. As a percentage of total sGAG, the ankle lost less and therefore could be described as undergoing less inflammation-induced degeneration.

In the *in vitro* model of cytokine-induced cartilage degeneration, the loss of sGAG and LDH levels were all found to be cytokine independent with the notable exception of pathological ‘high’ concentration TNF $\alpha$  which induced significantly more sGAG loss in the knee compared to the ankle; this is suggestive of an innate ‘chondroprotection’ in the ankle, in contrast to the observed catabolic effect in the knee. Further investigation of pro-inflammatory mediators (nitric oxide and prostaglandin E<sub>2</sub>) within the cartilage may help to explain this behaviour further. Chapter findings have also

demonstrated a significant patient-specific susceptibility to inflammation. This heterogeneity may explain why cytokine-dependent degeneration was not conclusively demonstrated. It may also explain the surprisingly poor outcomes reported in clinical studies of anti-cytokine medications that may in fact be related to patient selection as opposed to a failure of the trial drug (Verbruggen et al., 2012).

### 3..21 Summary of Chapter Findings

- Ankle cartilage has significantly higher sGAG levels when compared to the knee; furthermore, in the absence of pro-inflammatory cytokines, more sGAG was lost from the ankle tissue than in the knee respectively
- As a percentage sGAG loss to represent degree of proteoglycan degeneration, more sGAG was lost from the knee relative to ankle cartilage
- Following pathological '*high*' TNF $\alpha$  treatment, there was increased sGAG loss from knee cartilage whereas ankle cartilage was less responsive, suggestive of a degree of '*chondroprotection*'
- Findings would support the hypothesis that cytokine mediated degeneration is due to innate patient specific differences and not attributable to their underlying medical conditions

# Chapter 4

**Is the differential cartilage degeneration observed between the ankle and the knee due to differences in the expression and/or activity of molecules involved in catabolism?**

## 4 Introduction

The data presented in Chapter 3 demonstrated that there is significantly more cartilage degeneration (determined by percentage sGAG loss) in the knee when compared to the ankle, despite a comparatively higher rate of cell death in the ankle tissue (raised LDH levels). This degeneration was observed to be cytokine independent apart from treatment with pathological 'high' TNF $\alpha$  (100ng/ml). Therefore, it has been hypothesised that the differential response of knee and ankle tissue to this high dose TNF $\alpha$  stimulation may be related to subtle, but inherent, chondroprotective mechanisms in the ankle and a more pronounced catabolic response in the knee. What remains to be determined is the mechanism by which this differential response could occur. It is known that cytokines induce ECM degeneration through a plethora of signalling pathways including stimulation of prostaglandin E2 (PGE<sub>2</sub>) and nitric oxide (NO) production, and upregulation of degenerative enzymes including the MMPs and aggrecanases (Kapoor et al., 2010).

Nitric oxide (NO) has a complex role in the catabolic processes following an inflammatory insult(s) that leads to OA development and progression. NO upregulates the production of pro-inflammatory cytokines and is itself induced by IL-1 and TNF $\alpha$  via induction of the inducible nitric oxide synthase (iNOS) isoform in chondrocytes (Abramson, 2008). NO inhibits collagen type II synthesis, proteoglycan production, activates matrix metalloproteinases (MMPs) and induces chondrocyte apoptosis (Abramson, 2008). Chondrocytes in the superficial zone of articular cartilage have been shown to upregulate iNOS expression in response to pro-inflammatory cytokines such as IL-1 and TNF $\alpha$ ; furthermore, iNOS knockout mice have been shown to be relatively resistant to OA (Melchiorri et al., 1998). Interestingly, NO in conjunction with elevated levels of IL-1 or TNF $\alpha$  acts synergistically to induce MMP-9 synthesis and activation in a dose dependant manner up to concentrations of 1mM NO. However, at higher concentrations, the combination of cytokine and NO inhibits MMP-9 activation via a cGMP dependent process. Following chondral damage ECM repair is mediated by NO produced by macrophages in a "biphasic and flux-dependent manner" (Ridnour et al., 2007). It would make sense that as macrophages accumulate and release NO, inflammation occurs and ECM enzymatic debridement can occur however there must

be some limit to this to allow cell proliferation and ECM regeneration. Once a critical number of macrophages arrive NO exceeds a set cellular limit, in this case 1mM. This inhibits the proteolytic enzymes and allows ECM production and cellular proliferation to occur. NO is also known to modulate PGE<sub>2</sub> mediated inhibition of proteoglycan synthesis in cartilage chondrocytes, adding further complexity to this inflammatory cascade (Abramson, 2008). There is however, increasing evidence that NO itself may be a less potent driver of degeneration than first believed. It may, be that NO is beneficial in the very early stages of OA (Hsu. et al., 2017) and that the balance between its degenerative and chondroprotective downstream redox derivatives are far more important (Clancy et al., 2004).

PGE<sub>2</sub> is a product of the breakdown of arachidonic acid via the cyclooxygenase 2 pathway (COX-2) (Kojima et al., 2004). Normal human chondrocytes express COX-2 and produce PGE<sub>2</sub> at low levels, however in the presence of IL-1 or TNF $\alpha$  this is significantly upregulated (Masuko-Hongo et al., 2004). PGE<sub>2</sub> (0.1 - 10 $\mu$ M) stimulation of OA cartilage dose-dependently inhibited proteoglycan production and promoted collagen degeneration (Attur et al., 2008). This response was mediated via PGE<sub>2</sub> induced activation of MMP-13 and ADAMTS-5, whilst MMP-1 expression was inhibited in a dose-dependent manner. Therefore, PGE<sub>2</sub>, like NO, is able to exert either a catabolic or anabolic response on cartilage chondrocytes depending on its concentration (Attur et al., 2008).

An important downstream target of these pro-inflammatory cascades is the MMPs, zinc dependant endopeptidases that digest the cartilage ECM. They are involved in homeostatic remodelling of articular cartilage, but, have also been heavily implicated in the pathogenesis of OA. Particular interest has been paid to MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-13 (collagenase 3) which have all been extensively implicated in degeneration of articular cartilage and onset/progression of OA pathology (Rose and Kooyman, 2016). MMP-13 is critical in initiating the degradation of cartilage collagen fibrils (Dahlberg et al., 2000) Following MMP-13 mediated cleavage of type II collagen, the gelatinases, namely MMP-2 and MMP-9 denature the collagen, contributing to its degradation and loss from the tissue. Interestingly, MMP-2 and MMP-9 cleave other targets, including growth factors, chemokines and cytokines, which results in the release of these ligands, which then have

the potential to activate other major signalling pathways, for example, those involved in inflammation and angiogenesis (Bauvois, 2012). A recent meta-analysis has confirmed that MMP-9 is significantly upregulated compared to MMP-2 in Caucasian patients with OA (Zeng et al., 2015), therefore cartilage with the highest ratio of MMP-9 to MMP-2 synthesis/activation are likely to be undergoing the most degeneration.

#### 4.1 Chapter Aims

To address whether differences in the production of cytokine-induced pro-inflammatory molecules may, in part, explain the relative resistance of ankle cartilage to degeneration, this chapter aimed to investigate whether there was differential *de novo* synthesis of:

- Downstream pro-inflammatory molecules including prostaglandin E<sub>2</sub> and nitric oxide
- Specific catabolic enzymes, namely, MMP-2 and MMP-9 expression and/or activation

in human ankle and knee explants after stimulation in the presence or absence of physiological 'low' or pathological 'high' concentrations of IL-1, OSM and TNF $\alpha$ .

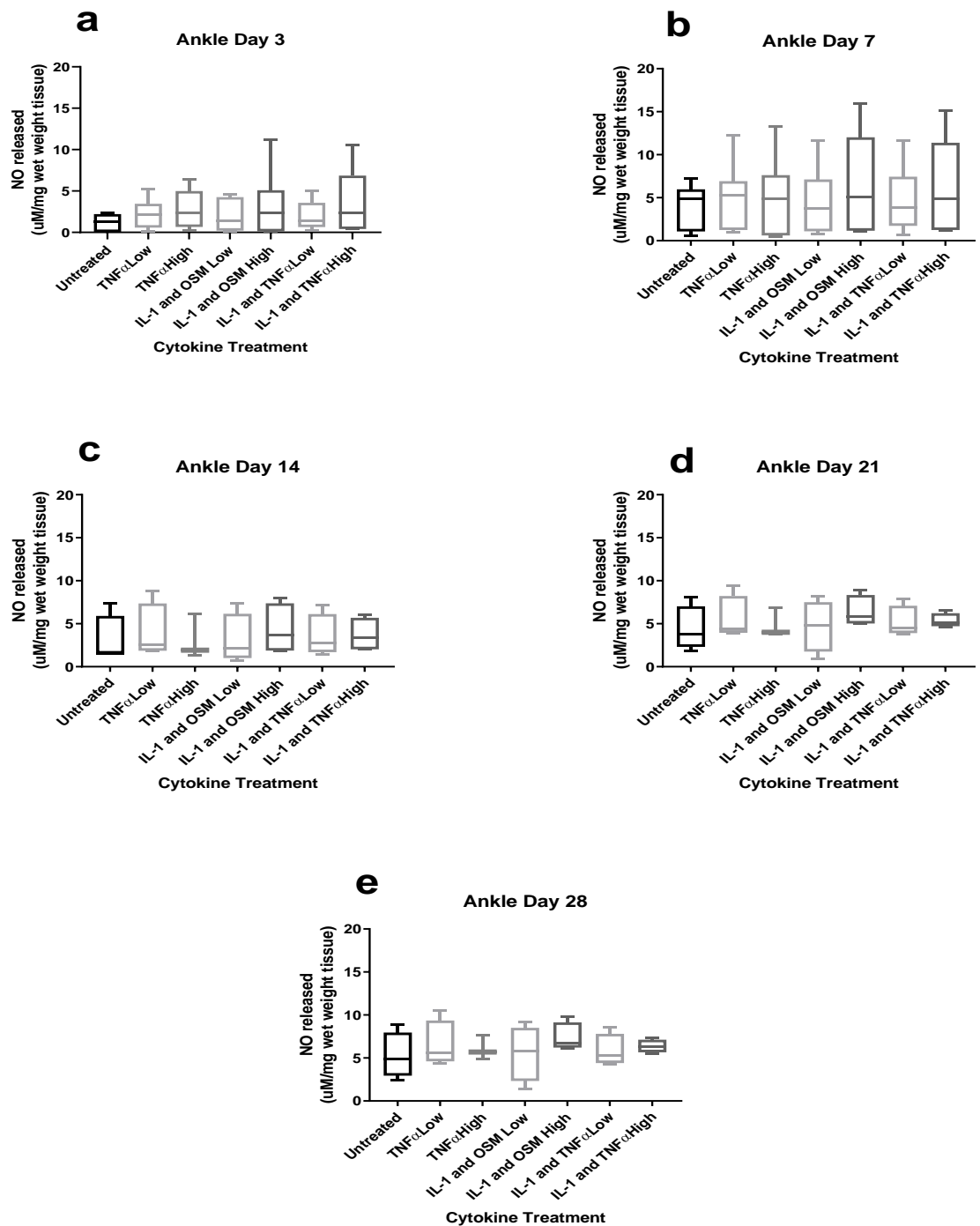
## 4.2 Results

### 4.3 What effect do pro-inflammatory cytokines have on chondrocyte nitric oxide production and are these effects different in the ankle and knee?

Levels of NO released from cytokine-treated cartilage explants into the media was measured using the Griess Assay, which indirectly measures NO as the stable end product nitrite; NO levels were compared between ankle and knee tissue at all time points during explant culture in order to investigate if there was a difference in the production of inflammatory mediators downstream from cytokine stimulation. Short-term culture was performed on 4 joints (knee n=1, ankle n=3) with media extraction and replenishment at 3 days and termination at 7 days. Long-term culture was performed on 7 joints (knee n=3, ankle n=4) with removal and replenishment of treatments at day 3, 7, 10, 14, 17, 21, 24 and termination at 28 days. All results were normalised to explant wet weight and NO levels are presented as  $\mu\text{M}/\text{mg}$  wet weight tissue.

### 4.4 Nitric Oxide production was not significantly induced by cytokine treatment in ankle

The effect of cytokine treatment on NO production and release from ankle cartilage was analysed using a One-way ANOVA at each individual time point (Figure 4.1 a-e). Minimal amounts of NO were released by the ankle cartilage over the culture period. Approximately 1 – 2.5 $\mu\text{M}$  NO was detected in the media during the early phase at day 3 (Figure 4.1a) increasing to approximately 5 - 7 $\mu\text{M}$  by day 28 (Figure 4.1e); however, there was no significant difference between cytokine treatment groups in cumulative mean NO production from the ankle cartilage (day 3 p=0.626, week 1 p=0.902, week 2 p=0.993, week 3 p=0.898, week 4 p=0.877). Tukey multiple comparator testing using adjusted p values was performed at all time points in order to compare untreated against cytokine treated ankle tissue, and to also compare high concentration against low concentration cytokine treatments. However, cytokine treatment, irrespective of concentration, had no significant effect on NO production at any of the time points measured ( $p \geq 0.678$ ).

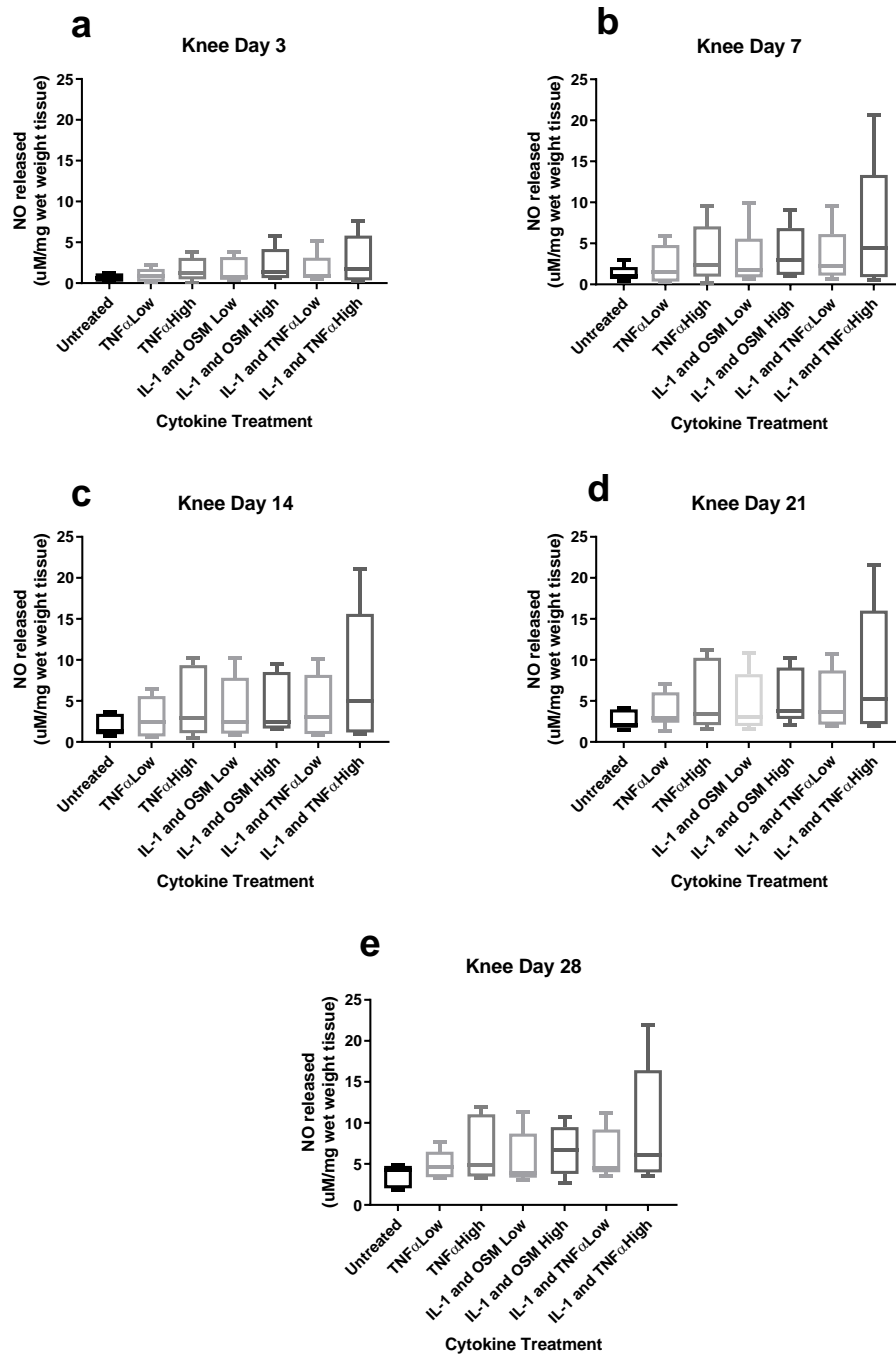


**Figure 4.1.** Levels of Nitric Oxide released from ankle cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Ankle cartilage was either cultured ‘short term’ for 7 days ( $n = 7$ ) or ‘long term’ extending out to 28 days ( $n = 4$ ) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); NO levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as mean cumulative NO ( $\mu$ M) per mg of wet weight tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).



#### 4.5 Nitric Oxide production was not significantly induced by cytokine treatment in knee cartilage

Comparable analyses were performed to measure NO release from knee cartilage using a One-way ANOVA to determine the effect of cytokine treatment at each specific time point (Figure 4.2a-e). As observed for the ankle (Figure 4.1), minimal amounts of NO were measured during the culture period, however, unlike the ankle, there was generally a much greater level of variability in NO produced by chondrocytes exposed to cytokine stimulation (Figure 4.2a-e). Due to this greater variability, no significant differences were observed between cytokine treatment groups in cumulative mean NO levels (day 3  $p=0.317$ , week 1  $p=0.202$ , week 2  $p=0.402$ , week 3  $p=0.386$ , week 4  $p=0.302$ ). Tukey multiple comparator testing using adjusted  $p$  values was again performed at each time point in order to compare untreated against cytokine treated knee tissue and to compare high against low cytokine concentrations. However, cytokine treatment, irrespective of concentration, had no significant effect on NO production at any of the time points measured ( $p=0.256$  to  $>0.999$ ).

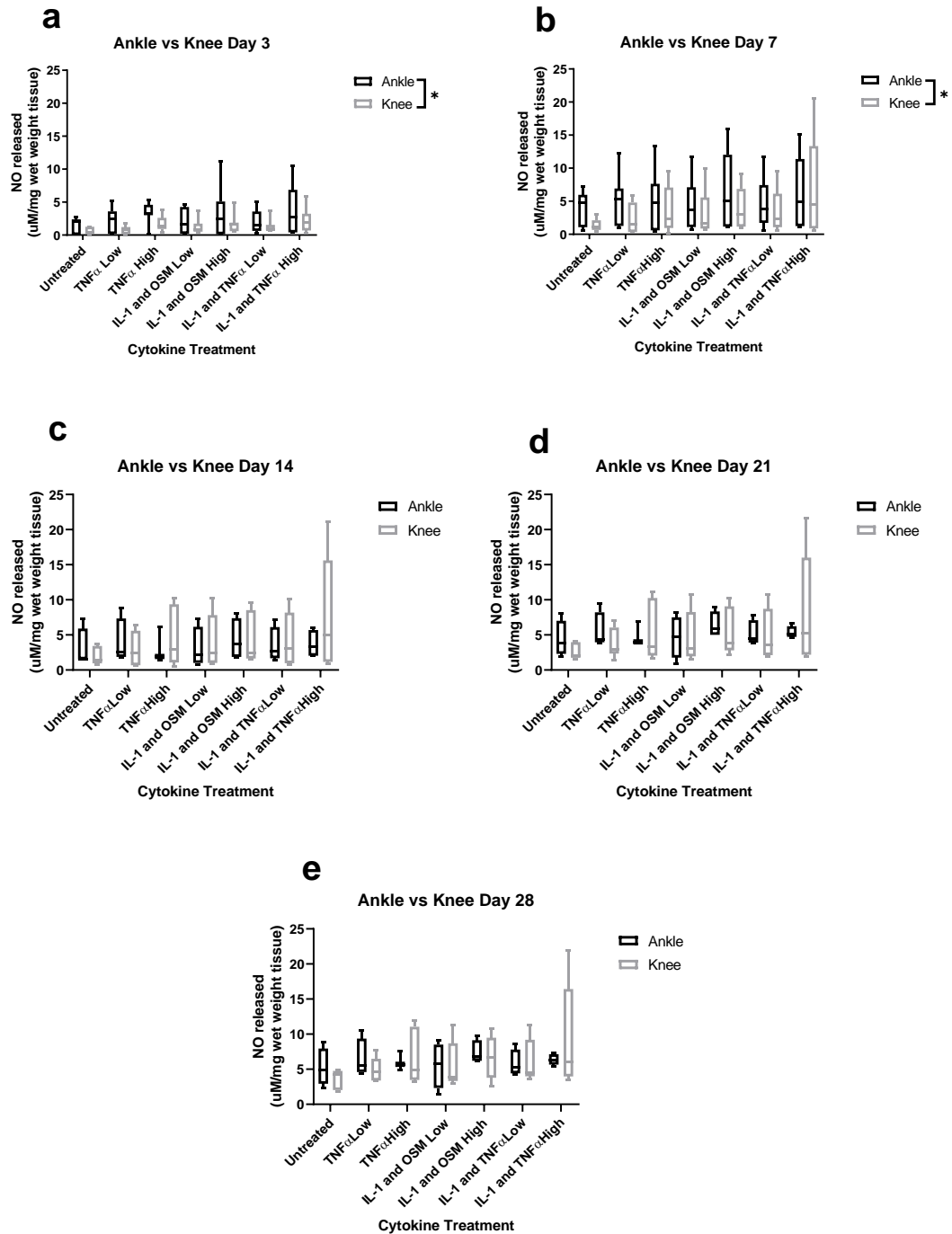


**Figure 4.2** Levels of Nitric Oxide released from knee cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Knee cartilage was either cultured ‘short term’ for 7 days (n = 4) or ‘long term’ extending out to 28 days (n = 3) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); NO levels were measured at **[a]** day 3, **[b]** day 7, **[c]** 14, **[d]** day 21 and **[e]** day 28. Data is calculated as NO released uM/mg wet weight tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

#### 4.6 Nitric Oxide production was significantly higher in the ankle compared to the knee during the first week of culture

Mean cumulative NO produced and released from the ankle and knee explants were compared at each specific time point using a Two-way ANOVA (Figure 4.3). This statistical test allowed assessment of the effect that the joint of origin and cytokine treatment had on NO levels produced by the respective tissues. Multiple comparison analysis with an adjusted p value was also undertaken using the Sidak's multiple comparator method to see if individual cytokine treatments, particularly 100ng/ml TNF $\alpha$ , had affected the concentration of NO produced.

Overall mean NO levels were not affected by cytokine treatments at any time point (day 3 p=0.105, week 1 p=0.232, week 2 p=0.686, week 3 p=0.549, week 4 p=0.427). However, mean NO levels for all samples were significantly higher in the ankle (2.61 $\mu$ M/mg wet weight tissue) than the knee (1.40 $\mu$ M/mg wet weight tissue) at day 3 (Figure 4.3a; p=0.003) and again at day 7 (Figure 4.3b: ankle - 5.12 $\mu$ M/mg wet weight tissue and 3.48 $\mu$ M/mg wet weight tissue; p=0.0447); after this point, no significant differences were seen during the remaining culture period (week 2 p=0.441, week 3 p=0.820, week 4 p=0.979). Multiple comparison analysis failed to identify any significant differences in NO production between joints for any cytokine treatment (Day 3 p=0.938, Day 7 p=0.993, Week 2 p>0.999, Week 3 p>0.999, Week 4 p>0.999). Furthermore, NO production was not significantly altered in response to 100ng/ml TNF $\alpha$  across the different joint cartilages.



**Figure 4.3** Levels of NO released from ankle and knee cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Ankle cartilage (n=4) and knee cartilage (n=3) was either cultured ‘short term’ for 7 days or ‘long term’ extending out to 28 days (ankle n=3, knee n=4) in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); NO levels were measured at [a] day 3, [b] day 7, [c] 14, [d] day 21 and [e] day 28. Data is calculated as the mean NO released  $\mu$ M/mg wet weight tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

#### 4.7 Individual patient analysis

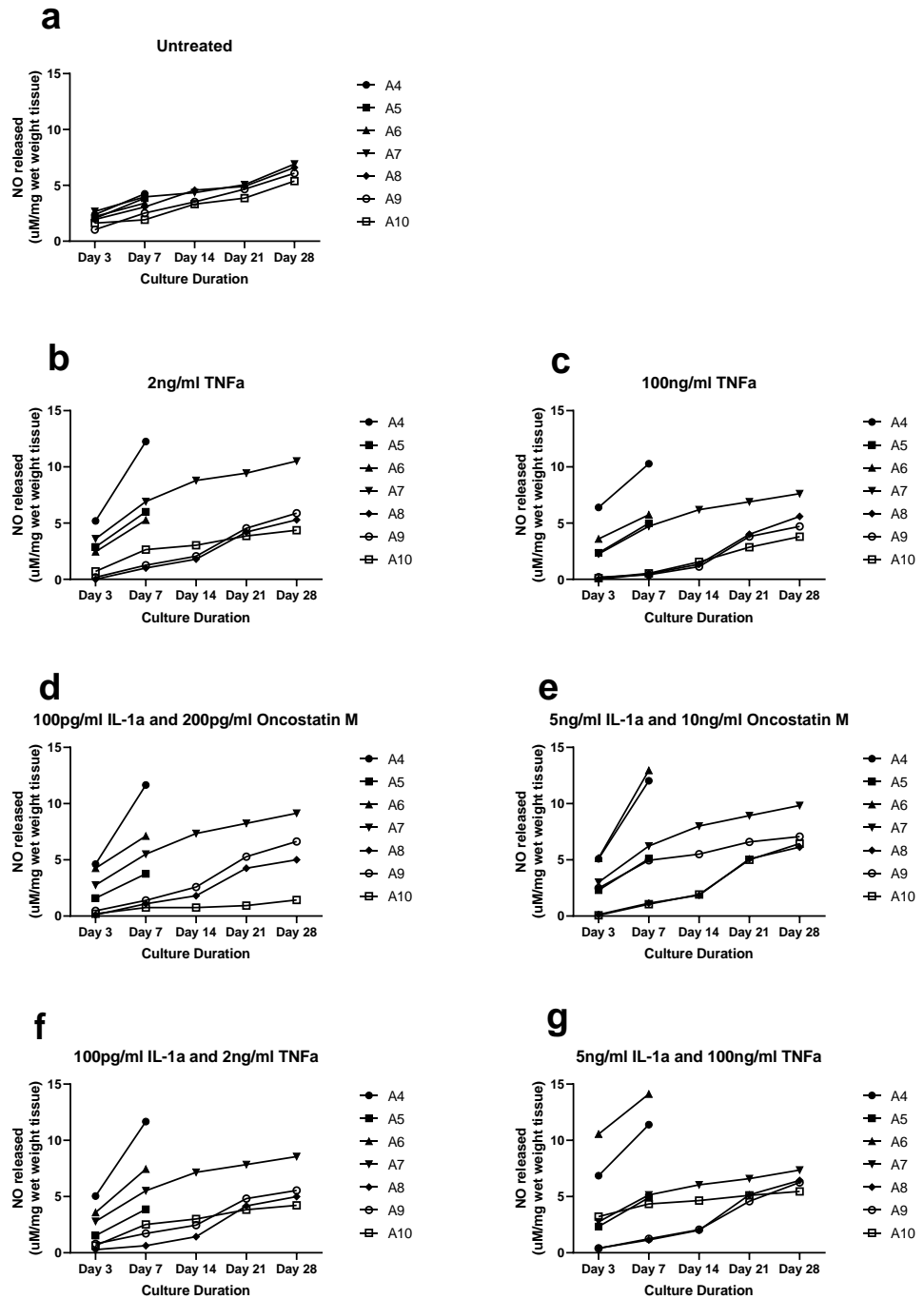
Due to the not unsurprising heterogeneous responses noted between different patients, which had previously been observed in Chapter 3, data was displayed as individual patient responses to see if a pattern could be identified (Figure 4.4 – 4.5). In order to ascertain individual patient susceptibility to cytokine induced NO production, data from matched donor pairs of ankle and knee tissue were also further analysed (Figure 4.6).

#### 4.8 Individual analysis demonstrates a consistent pattern of patient specific Nitric Oxide production following cytokine stimulation in ankle tissue

Very little variation in mean NO levels was observed between donors following culture in the absence of cytokines with approximately  $5\mu\text{M}/\text{mg}$  wet weight tissue released by day 28 (Figure 4.4a). In contrast, the ankle tissue did produce more NO in response to cytokine treatment; typically,  $8 - 10\mu\text{M}$  NO/mg wet weight tissue was observed after cytokine stimulation for 28 days. However, heterogeneity was again observed between donor tissues (Figure 4.4b-g) although a consistent pattern could be identified.

Irrespective of cytokine treatment, donor ankle 10 produced the least NO during the 28 days of culture; furthermore, donor ankles 9 and 8 also consistently produced the least NO per mg wet weight tissue reacting minimally to the cytokine insult. Interestingly, those donor ankles (ankles 7 and 4) that were only exposed to short-term culture released substantial amounts of NO in response to all cytokine treatments, typically increasing 4 – 5-fold by day 7; it would have been interesting to observe whether these increases were further perpetuated or would have plateaued over time. Donor ankle 6 also consistently increased NO production increasing by approximately 2.5-fold by day 28 in response to cytokine stimulus, although the concentration of cytokine did not appear to influence response. Overall, there was clear heterogeneity in the ankle tissue response to cytokines; for example,  $100\text{ng}/\text{ml}$  TNF $\alpha$  elicited only  $3.8\mu\text{M}$  NO/mg wet weight tissue from ankle 10 whilst inducing  $10.28\mu\text{M}$  NO/mg wet weight tissue from ankle 4 (Figure 4.4c), and  $5.45\mu\text{M}$  NO/mg wet weight tissue from ankle 10 compared

with 14.2 $\mu$ M NO/mg wet weight tissue in ankle 6 after stimulation with 5ng/ml IL-1 $\alpha$  and 100ng/ml TNF $\alpha$  (Figure 4.4g). It is also apparent that the speed in which the ankle cartilage responds to the cytokines also differs between donor patients; for example, several react within 7 days by producing increased levels of NO, whereas others, which are less receptive, only appear to elicit a sizeable response 14 days after stimulation (particularly evident in Figure 4.4b - c).

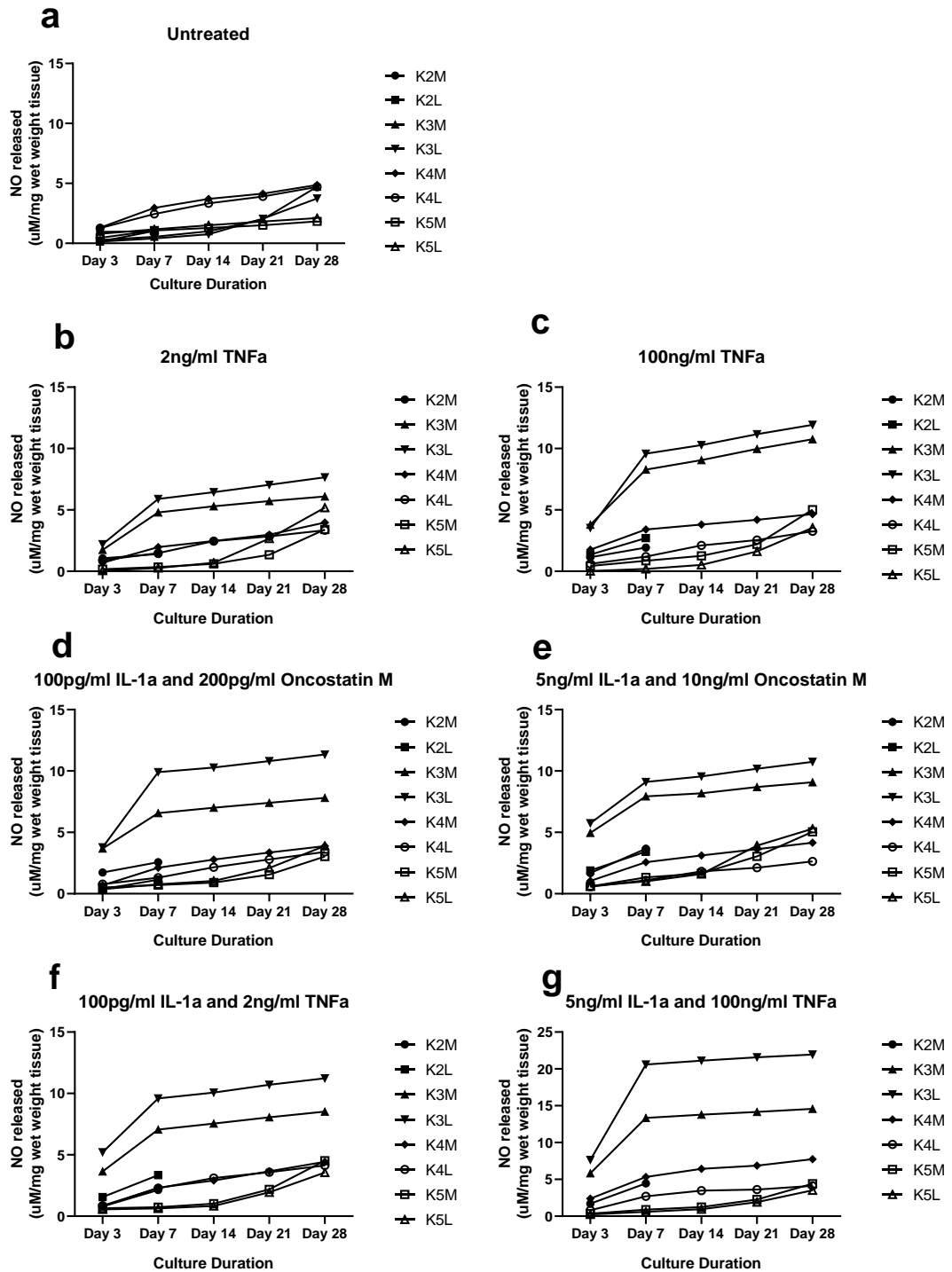


**Figure 4.4:** Mean cumulative NO production from individual donor-specific ankle cartilage explants cultured in the [a] absence (untreated) or presence of [b] 2ng/ml TNF $\alpha$ , [c] 100ng/ml TNF $\alpha$ , [d] 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM, [e] 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM, [f] 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$ , and [g] 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  for < 28 days. NO levels were measured at day 3, day 7, day 14, day 21 and day 28. Data is calculated as the mean NO released  $\mu$ M/mg wet weight tissue for each treatment group and is plotted as the mean of the 3 explants.

#### 4.9 Individual analysis demonstrates a consistent pattern of patient specific Nitric Oxide production following cytokine stimulation in knee tissue

Again, there was very little variation in NO released from untreated donor knee cartilage ranging from, on average, 1.83 to 4.87  $\mu\text{M}/\text{mg}$  wet weight tissue (Figure 4.5a). However, there were much clearer patterns of behaviour in the knee cartilage in response to specific cytokines (Figure 4.5b-g). Irrespective of cytokine treatment, donor knees 4 and 5 produced the least NO over the 28 days of culture whereas donor knees 2 and 3 released the most. Due to the low numbers of non-diseased knees that were accessible during the study, explants were harvested from both the medial and lateral components of the femoral condyle and cultured separately. Interestingly, of the major responder i.e. donor patient 3, the lateral condylar cartilage reacted more to the cytokine insult with increased NO levels detected indicating 'within joint' heterogeneity also (Figure 4.5b-g). Furthermore, of those responders, an apparent difference was observed between the physiological 'low' and pathological 'high' cytokine concentrations. For donor knee 3, an approximate 2-fold increase in NO release was observed in response to 100ng/ml TNF $\alpha$  (Figure 4.5c) compared to 2ng/ml TNF $\alpha$  (Figure 4.5b). Likewise, a concentration response was evident for donor knee 3 with a 2-fold increase in NO levels after exposure to 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (Figure 4.5g) relative to the lower concentration (Figure 4.5f). A comparison of NO levels in a non/low responder (donor 5, 3.52  $\mu\text{M}$  NO/mg wet weight tissue) relative to a strong responder (donor 3, 21.95  $\mu\text{M}$  NO/mg wet weight tissue) indicated a 6-fold difference after 28 days of exposure to 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (Figure 4.5g) demonstrating the marked heterogeneity within the population. Again, the major responders reacted quickly to cytokine insult within 7 days with levels plateauing thereafter, whereas the less responsive tissues slowly accumulated NO over a sustained period, akin to the observations for ankle cartilage (Figure 4.4). However, the magnitude of response was more pronounced in knee cartilage relative to the ankle tissue.





**Figure 4.5:** Mean cumulative NO production from individual donor-specific knee cartilage explants (M – medial and L – lateral component) cultured in the [a] absence (untreated) or presence of [b] 2ng/ml TNF $\alpha$ , [c] 100ng/ml TNF $\alpha$ , [d] 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM, [e] 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM, [f] 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$ , and [g] 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  for < 28 days. sGAG levels were measured at day 3, day 7, day 14, day 21 and day 28. Data is calculated as the mean NO released uM/mg wet weight tissue for each treatment group and is plotted as the mean of the 3 explants.

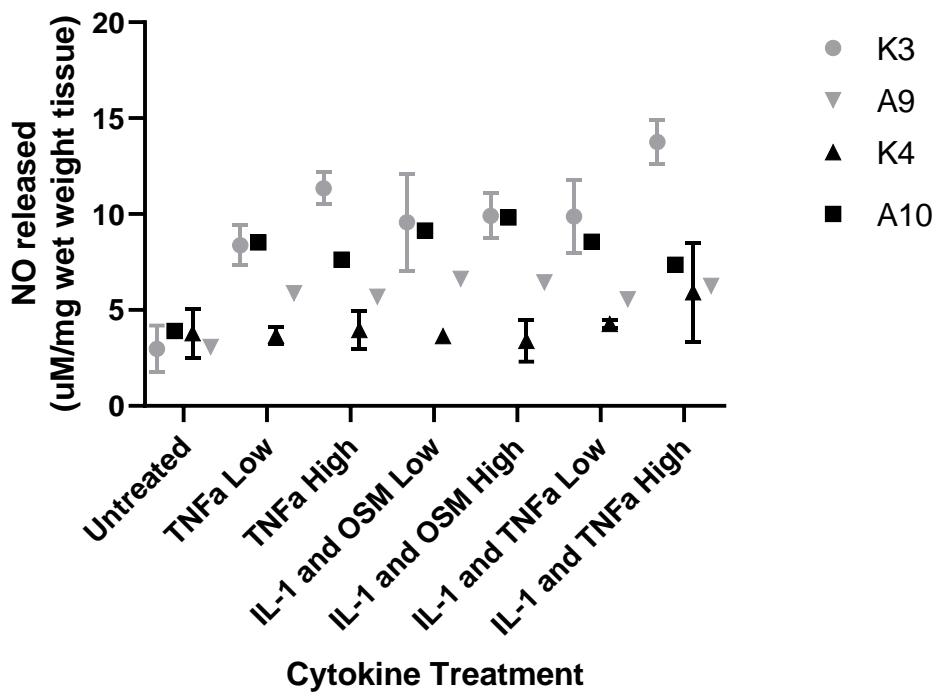
#### 4.10 Matched pairs of ankle and knee tissue demonstrate a consistent patient specific production of Nitric Oxide in response to cytokine stimulation

Matched paired ankle and knee cartilage explants were harvested from 2 donors: Patient J (Ankle 9 and Knee 3) and Patient K (Ankle 10 and Knee 4) which underwent extended culture to 28 days. A Two-way ANOVA was used to identify any significant joint of origin and/or cytokine dependent differences in the total NO concentration measured over 28 days (Figure 4.6). Surprisingly, there was a similar amount of NO released from both the untreated ankle and knee across the 2 donor tissues, however the heterogeneity was observed in response to cytokine treatment. Donor ankles (A9 and A10) demonstrated an overall significant difference in NO production between patients ( $p=0.0009$ ) as well as between cytokine treatments ( $p=0.0062$ ) (table 4.1).

Identical comparison performed on the knee cartilage (K4 and K5) also demonstrated a significance between patients ( $p<0.0001$ ) and between cytokine treatments ( $p=0.008$ ) (Table 4.1). When patient J (A9 and K3) was compared with patient K (A10 and K4) less NO was produced by patient K ( $p<0.0001$ ) for all cytokine treatments and there was also a significant difference in response to cytokines ( $p=0.0017$ ). However, multiple comparison analysis demonstrated no significant difference between high and low concentration treatments for either patient ( $p\geq 0.5393$ ). Clearly, the data confirms a significant heterogeneous response of donor cartilages to all cytokines.

Sidak's multiple comparisons test	Untreated (Mean NO uM/mg)	Cytokine (Mean NO uM/mg)	Significant?	Adjusted P Value
<b>Ankle 9 and Ankle 10</b>				
Untreated vs. TNF $\alpha$ Low	3.50	7.20	Yes	0.0328
Untreated vs. TNF $\alpha$ High	3.50	6.66	No	0.0707
Untreated vs. IL-1 $\alpha$ and OSM Low	3.50	7.89	Yes	0.0135
Untreated vs. IL-1 $\alpha$ and OSM High	3.50	8.14	Yes	0.0101
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	3.50	7.06	Yes	0.0398
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	3.50	6.81	No	0.0563
<b>Knee 3 and Knee 4</b>				
Untreated vs. TNF $\alpha$ Low	3.38	6.02	No	0.3038
Untreated vs. TNF $\alpha$ High	3.38	7.65	Yes	0.0131
Untreated vs. IL-1 $\alpha$ and OSM Low	3.38	6.61	No	0.1020
Untreated vs. IL-1 $\alpha$ and OSM High	3.38	6.65	No	0.0939
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	3.38	7.08	Yes	0.0406
Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	3.38	9.84	Yes	0.0002

**Table 4.1:** Sidak's Multiple comparator test results with adjusted p values comparing mean NO production from the Ankle 9 and 10 or from Knee 3 and 4 in the presence or absence of a combination of physiological, denoted 'low' (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ).



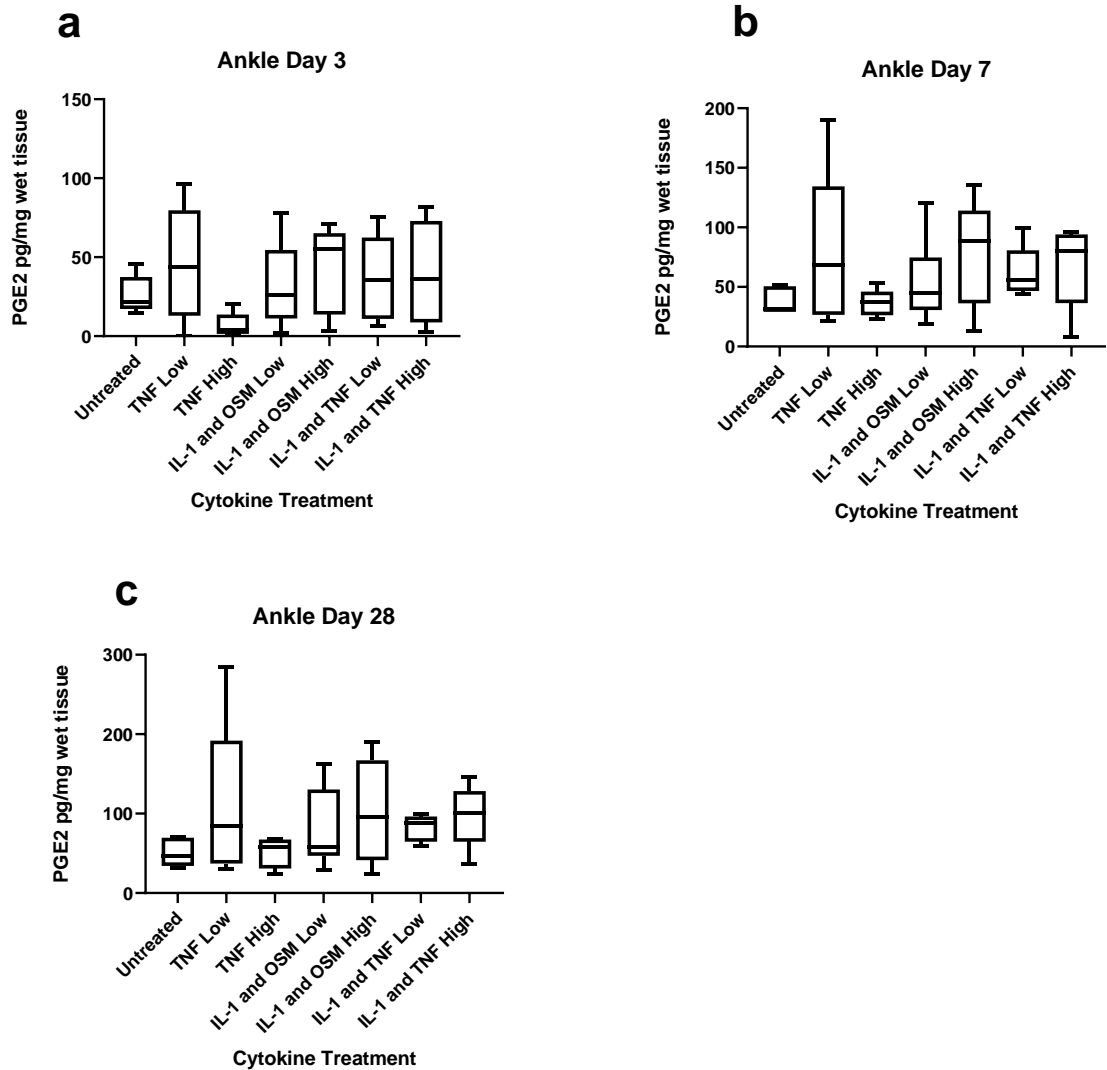
**Figure 4.6:** Total mean cumulative NO production from donor-specific matched pair ankle and knee cartilage explants cultured in the absence (untreated) or presence of 2ng/ml TNF $\alpha$  (TNF $\alpha$  low), 100ng/ml TNF $\alpha$  (TNF $\alpha$  High), 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM (IL-1 and OSM Low), 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM (IL-1 and OSM High), 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$  (IL-1 and TNF $\alpha$  Low), and 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (IL-1 and TNF $\alpha$  High) for 28 days. NO levels were measured at day 3, day 7, day 14, day 21 and day 28, for each treatment group over the 28 days of culture. Data is representative of the mean ( $\pm$ SD) of 3 explants for each donor [Samples A9 and K3 were donated by patient J (black) and samples A10 and K4 were donated by patient K (grey)].

#### **4..11 What effect do pro-inflammatory cytokines have on chondrocyte prostaglandin E<sub>2</sub> production and are these effects different in the ankle and knee?**

Levels of PGE<sub>2</sub> released from ankle and knee articular cartilage explants in the absence or presence of a combination of cytokines was measured using a PGE<sub>2</sub> high sensitivity ELISA. Mean cumulative PGE<sub>2</sub> levels were compared between the matched donor pairs of ankle and knee tissue (from patient J [ankle 9 and medial condyle from Knee 3] and patient K [ankle 10 and medial condyle Knee 4]) during the early phases of cytokine insult, namely at days 3 and day 7 and during the later stage of exposure at day 28 in order to investigate if there was a difference in the production of inflammatory mediators downstream from cytokine stimulation. All results were normalised to explant wet weight and mean cumulative PGE<sub>2</sub> levels are presented as pg/mg wet weight tissue.

#### **4..12 Prostaglandin E<sub>2</sub> production was not significantly induced by cytokine treatment in ankle cartilage**

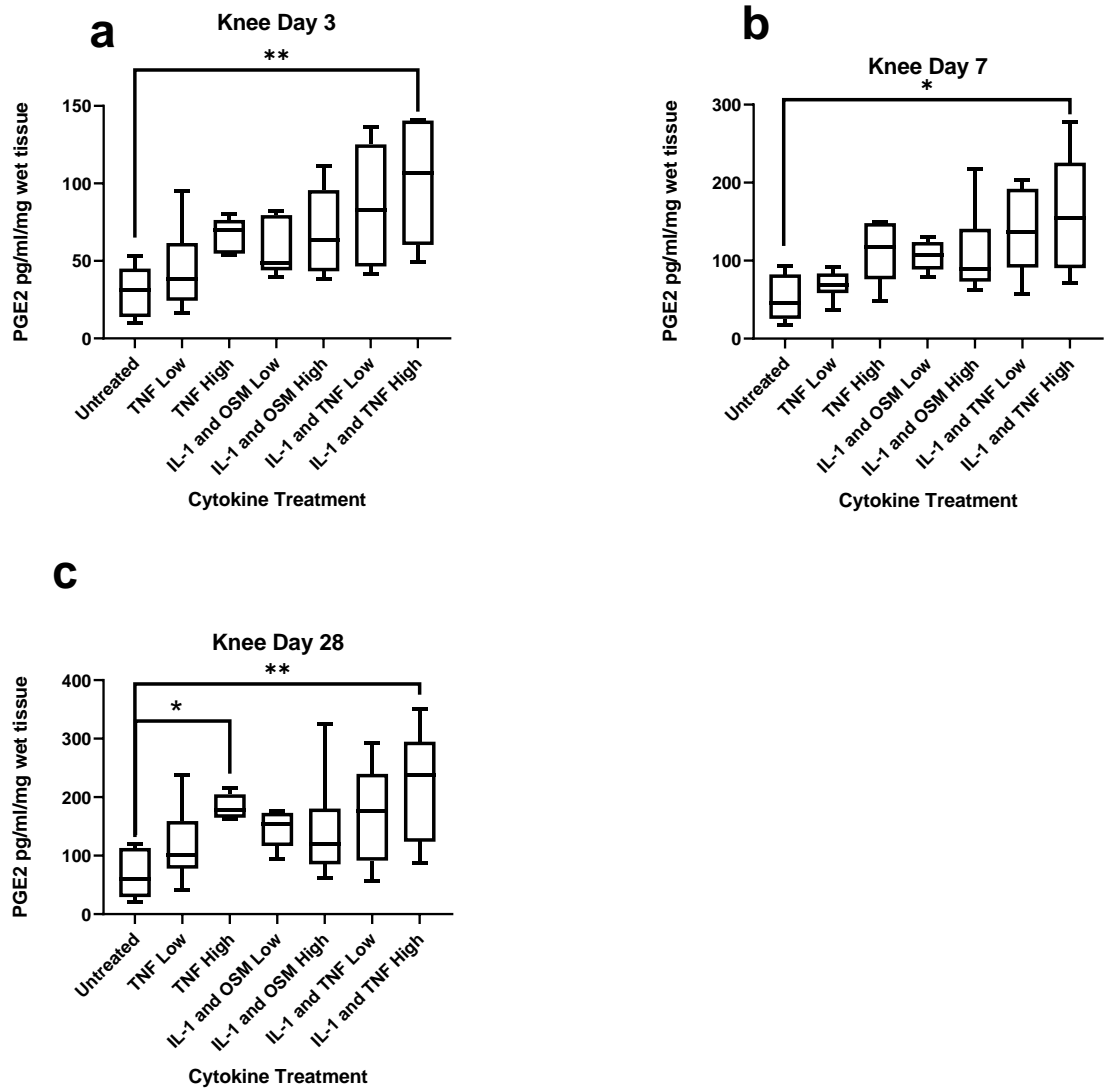
To assess the effect of cytokine treatment on PGE<sub>2</sub> release from ankle cartilage, data was analysed with a One-way ANOVA at the three specific time points (Figure 4.7a-c); however, no significant difference in cumulative mean PGE<sub>2</sub> production was observed between cytokine treatment groups at either the early (Figure 4.7a – b; day 3 p=0.250, week 1 p=0.323) or late stage (Figure 4.7c - week 4 p=0.344). Tukey multiple comparator testing using adjusted p values was performed at all three time points in order to compare response of untreated cartilage explants against cytokine treated tissue, in addition to comparing high versus low concentration treatments. No significant difference was seen at any time point between the untreated and any cytokine stimulus, or between high and low concentration treatments (p≥0.188).



**Figure 4.7** Levels of PGE<sub>2</sub> released from ankle cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Ankle (n=2) and knee cartilage (n=2) were cultured in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); PGE<sub>2</sub> levels were measured at **[a]** day 3, **[b]** day 7, and **[c]** day 28. Data is calculated as the mean cumulative PGE<sub>2</sub> pg/mg wet weight tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

#### 4.13 Prostaglandin E<sub>2</sub> production was significantly induced by cytokine treatment in knee cartilage

To assess the effect of cytokine treatment on PGE<sub>2</sub> release from the donor matched knee cartilage, data was analysed using a One-way ANOVA at the three specific time points representative of either an early or late phase of cytokine insult (Figure 4.8a-c); in contrast to the ankle (Figure 4.7), significant differences were observed in mean cumulative PGE<sub>2</sub> production between cytokine treatment groups at day 3 (Figure 4.8a,  $p=0.005$ ), day 7 (Figure 4.8b,  $p=0.013$ ) and day 28 (Figure 4.8a,  $p=0.033$ ). Tukey multiple comparator testing using adjusted  $p$  values was performed at all three time points in order to compare untreated versus cytokine treated explants, and to further compare high versus low cytokine concentrations. A cytokine dose-dependent effect on PGE<sub>2</sub> synthesis was not observed at any time point ( $p \geq 0.236$ ). However, when compared to untreated explants, significantly more PGE<sub>2</sub> was produced by knee tissue treated with pathological 'high' concentration of IL-1 $\alpha$  and TNF $\alpha$  at day 3 (29.75pg/mg vs 101.4pg/mg wet weight tissue;  $p=0.005$ ), day 7 (52.13pg/mg vs 108.7pg/mg wet weight tissue;  $p=0.0145$ ) and day 28 (68.88pg/mg vs 220.90pg/mg wet weight tissue;  $p=0.0165$ ). Interestingly, significantly more PGE<sub>2</sub> was also released after 28 days from knee cartilage treated solely with pathologically 'high' (100ng/ml) TNF $\alpha$  (68.88pg/mg vs 183.4pg/mg wet weight tissue;  $p=0.0471$ ).



**Figure 4.8** Levels of PGE<sub>2</sub> released from knee cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Knee samples (n=2) were cultured in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); PGE<sub>2</sub> levels were measured at **[a]** day 3, **[b]** day 7 and **[c]** day 28. Data is calculated as the mean cumulative PGE<sub>2</sub> pg/mg of wet tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

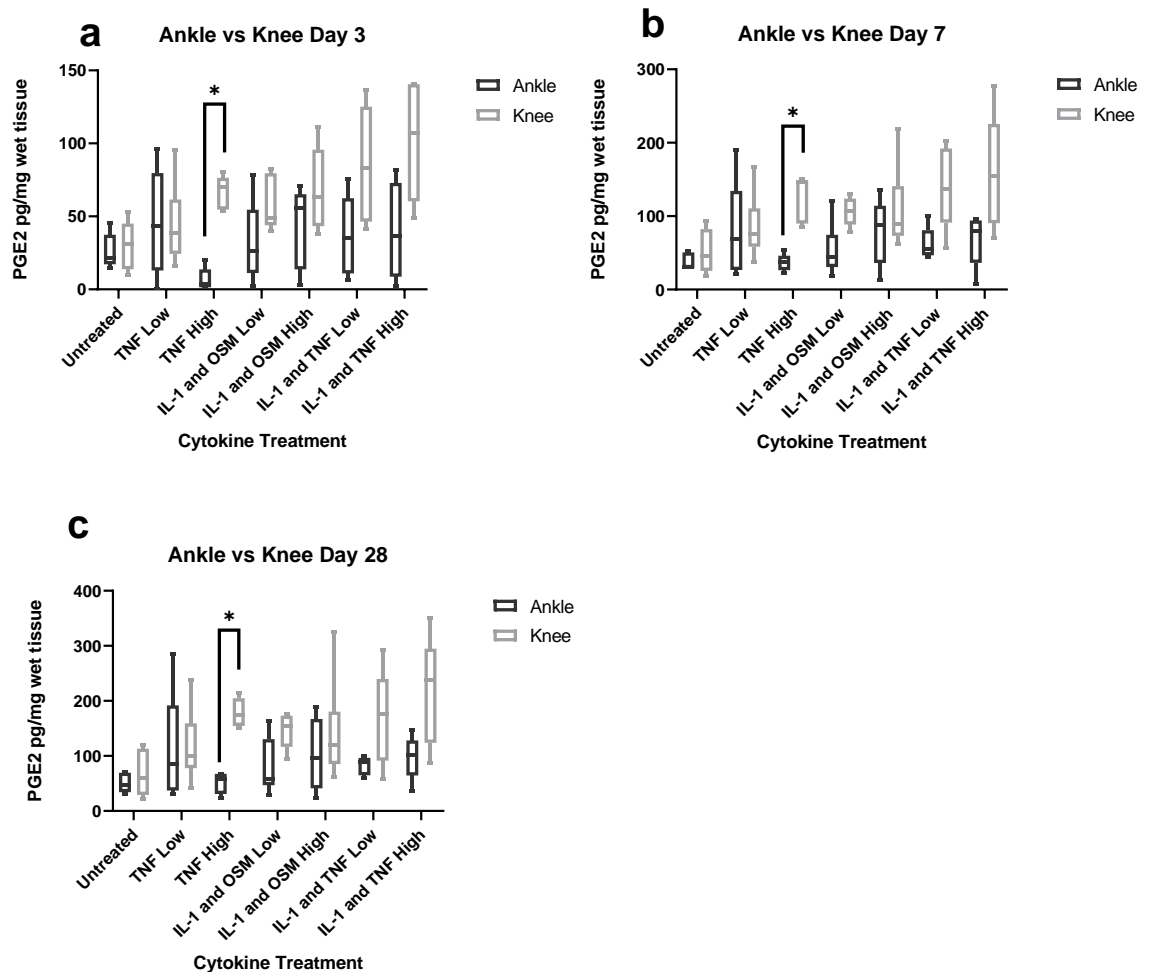


#### 4.14 Significantly more PGE<sub>2</sub> was produced by the knee than the ankle tissue following treatment with 100ng/ml TNF $\alpha$

Mean cumulative PGE<sub>2</sub> synthesised and released from the cytokine-stimulated ankle and knee cartilage explants were compared at each time point using a Two-way ANOVA, allowing assessment of the effect that the joint of origin and cytokine treatment had on cartilage-specific PGE<sub>2</sub> production (Figure 4.9). Multiple comparison analysis with an adjusted p value was also undertaken using the Sidak's multiple comparator method to see if individual cytokine treatments, particularly 100ng/ml TNF $\alpha$ , affected the concentration of PGE<sub>2</sub> produced.

What was evident was the increased amounts of PGE<sub>2</sub> produced by the knee cartilage when compared to the ankle. Significantly more PGE<sub>2</sub> was produced by the knee cartilage compared to ankle cartilage at day 3 (Figure 4.9a, 32.79pg/mg vs 64.90pg/mg wet weight tissue; p<0.0001), day 7 (Figure 4.9b, 59.91pg/mg vs 111.20pg/mg wet weight tissue; p<0.0001) and day 28 (Figure 4.9c, 82.62pg/mg vs 149.20pg/mg wet weight tissue; p<0.0001).

Overall, mean PGE<sub>2</sub> levels were significantly influenced by cytokine treatments at all time points (day 3 p=0.0207, Day 7 p=0.0391 and day 28 p=0.0461). Specifically, multiple comparison analysis demonstrated a significant difference in mean cumulative PGE<sub>2</sub> production between joints with increased synthesis in knee cartilage in response to 100ng/ml TNF $\alpha$  at day 3 (Figure 4.9a, 7.03pg/mg vs 67.38pg/mg; p=0.0387), day 7 (Figure 4.9b, 37.14pg/mg vs 127.90pg/mg; p=0.0476) and day 28 (Figure 4.9c, 51.35pg/mg vs 178.40pg/mg; p=0.0461).



**Figure 4.9** Levels of PGE<sub>2</sub> released from knee and ankle cartilage into the media following cytokine stimulation, assessed as a marker of inflammation. Ankle cartilage (n=2) and Knee samples (n=2) were cultured in the presence or absence of a combination of physiological (100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ); PGE<sub>2</sub> levels were measured at **[a]** day 3, **[b]** day 7 and **[c]** day 28. Data is calculated as the mean cumulative PGE<sub>2</sub> pg/mg of wet tissue for each treatment group and is plotted as the mean, max-min (whiskers)  $\pm$  standard deviation (box).

#### 4.15 Matched donor pairs of ankle and knee tissue demonstrate a consistent patient specific production of PGE<sub>2</sub> in response to cytokine stimulation

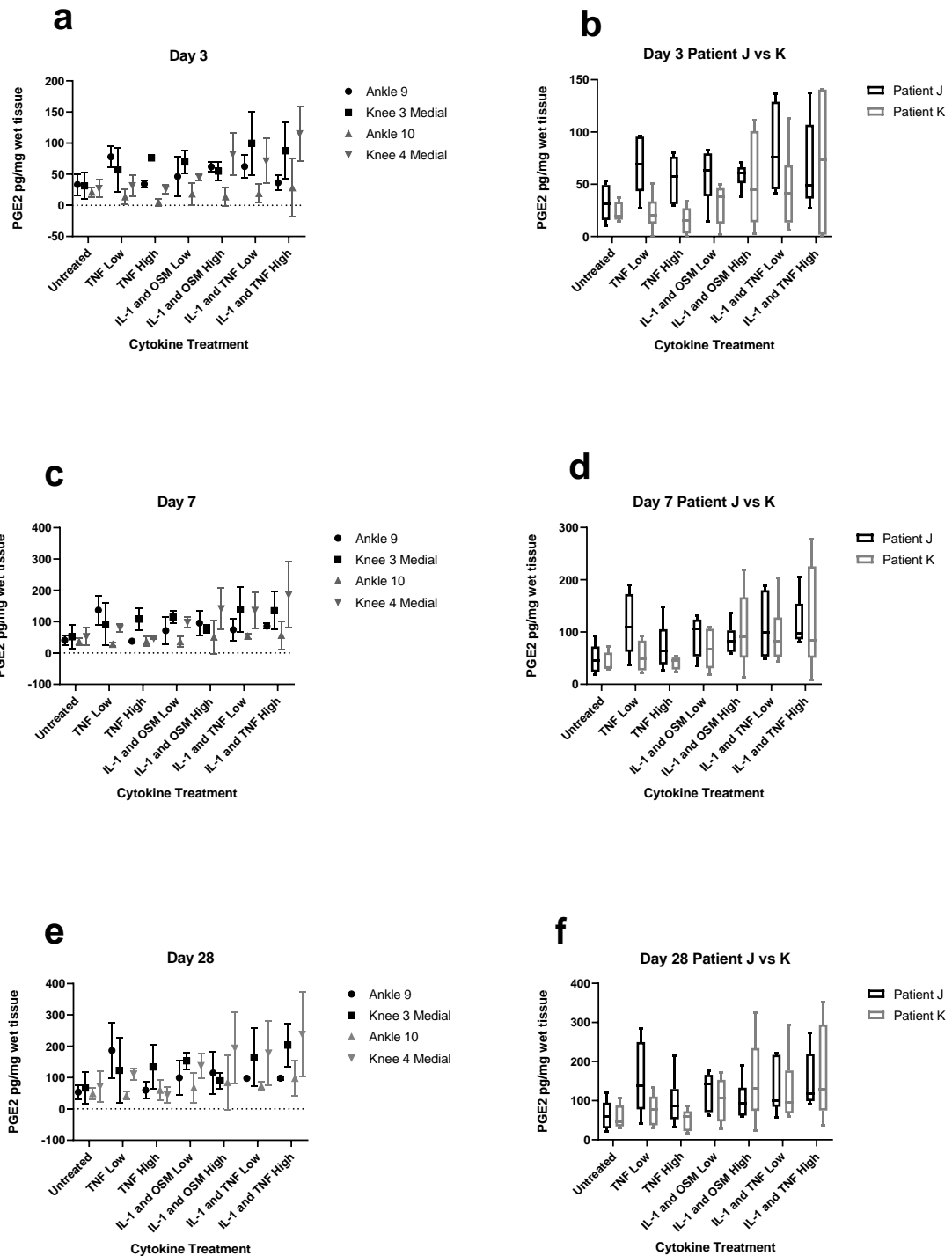
To determine whether the response of the joint of origin was comparable across donor tissues, cartilage explants harvested from patient J (A9 and K3M) were compared to explants from patient K (A10 and K4M) and PGE<sub>2</sub> levels measured (Figure 4.10a, c, e). Analysis of ankle tissue (A9 and A10) demonstrated a significant difference between patients; donor ankle 9 produced significantly more PGE<sub>2</sub> compared to donor ankle 10 at day 3 (Figure 4.10a, 50.40pg/mg vs 16.94pg/mg wet weight tissue;  $p < 0.0001$ ), day 7 (Figure 4.10c, 70.56pg/mg vs 42.54pg/mg wet weight tissue;  $p = 0.0016$ ) and day 28 (Figure 4.10e, 101.40pg/mg vs 67.44pg/mg wet weight tissue;  $p = 0.0282$ ). However, cytokine treatment did not account for the difference in PGE<sub>2</sub> production between ankle cartilages (day 3  $p = 0.401$ , day 7  $p = 0.125$  and day 28  $p = 0.286$ ).

In contrast, comparison of PGE<sub>2</sub> levels in knee cartilage between K3M and K4M demonstrated no difference between patients at day 3 (Figure 4.10a, 68.08pg/mg vs 56.98pg/mg wet weight tissue;  $p = 0.2421$ ), day 7 (Figure 4.10c, 102.30pg/mg vs 105.60 pg/mg wet weight tissue;  $p = 0.8458$ ) or day 28 (Figure 4.10e, 133.70pg/mg vs 139.50pg/mg wet weight tissue;  $p = 0.8108$ ). However, overall, there was a significant cytokine effect on PGE<sub>2</sub> synthesis at day 3 ( $p = 0.0059$ ), day 7 ( $p = 0.0395$ ) and day 28 ( $p = 0.0447$ ) (Table 4.2). These results would suggest that the production of PGE<sub>2</sub> by knee cartilage is cytokine dependent while the ankle tissue is comparatively resistant to cytokines with respect to PGE<sub>2</sub> induction.

Culture	Dunnett's multiple comparisons test	Untreated (Cumulative Mean PGE <sub>2</sub> pg/mg)	Cytokine (Cumulative Mean PGE <sub>2</sub> pg/mg)	Significant?	Adjusted P Value
Day 3	Untreated vs. TNF $\alpha$ Low	29.75	44.34	No	0.9031
	Untreated vs. TNF $\alpha$ High	29.75	67.38	No	0.1661
	Untreated vs. IL-1 $\alpha$ and OSM Low	29.75	57.28	No	0.4324
	Untreated vs. IL-1 $\alpha$ and OSM High	29.75	68.61	No	0.1453
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	29.75	85.6	Yes	0.0170
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	29.75	101.4	Yes	0.0016
Day 7	Untreated vs. TNF $\alpha$ Low	52.13	68.82	No	0.9814
	Untreated vs. TNF $\alpha$ High	52.13	111.3	No	0.2100
	Untreated vs. IL-1 $\alpha$ and OSM Low	52.13	106.1	No	0.2845
	Untreated vs. IL-1 $\alpha$ and OSM High	52.13	108.7	No	0.2455
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	52.13	137.6	Yes	0.0324
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	52.13	160.3	Yes	0.0047
Day 28	Untreated vs. TNF $\alpha$ Low	68.88	117.3	No	0.7059
	Untreated vs. TNF $\alpha$ High	68.88	183.4	Yes	0.0471
	Untreated vs. IL-1 $\alpha$ and OSM Low	68.88	145.8	No	0.2818
	Untreated vs. IL-1 $\alpha$ and OSM High	68.88	141.8	No	0.3282
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ Low	68.88	171.5	No	0.0879
	Untreated vs. IL-1 $\alpha$ and TNF $\alpha$ High	68.88	220.9	Yes	0.0052

**Table 4.2:** Dunnett's Multiple comparator test results with adjusted p values comparing cumulative mean PGE<sub>2</sub> production from Knee 3 and 4 at day 3, 7 and 28 in the presence or absence of a combination of physiological, denoted 'low' ((100pg/ml IL-1 $\alpha$ , 200pg/ml OSM, 2ng/ml TNF $\alpha$ ) or pathological concentrations of cytokines, denoted 'high' (5ng/ml IL-1 $\alpha$ , 10ng/ml OSM, 100ng/ml TNF $\alpha$ ).

PGE<sub>2</sub> production between the two patients was also compared by combining data from the ankle and knee donors (Figure 4.10b, d, f). Mean cumulative data was analysed at day 3, day 7 and day 28 using a Two-way ANOVA and multiple comparisons performed using the Sidak's multiple comparator method with adjusted p values. More PGE<sub>2</sub> was consistently produced by patient J compared to patient K demonstrating similar trends to those observed for sGAG loss (Figure 3.14) and NO production (Figure 4.6). The difference in total PGE<sub>2</sub> production was found to be significant between donors at day 3 (Figure 4.10b, 60.48pg/mg vs 37.86pg/mg; p=0.025) but failed to reach statistical significance at day 7 (Figure 4.10d, 91.70pg/mg vs 75.00pg/mg; p=0.1601) and day 28 (Figure 4.10f, 117.70pg/mg vs 104.70pg/mg; p=0.4075). Although there was a significant cytokine effect at all time points (day 3 p=0.0319, day 7 p=0.0247 and day 28 p=0.0253), following multiple comparator analysis no individual cytokine treatment produced a significant effect on PGE<sub>2</sub> synthesis between patients.



**Figure 4.10** Mean cumulative PGE<sub>2</sub> (pg/mg wet weight tissue) production by individual patients. Interleaved symbol graphs [a], [c] and [e] represent mean cumulative PGE<sub>2</sub> ± SD (whiskers) from Patient J (Black, A9 or K3M) and Patient K (Grey, A10 or K4) presented as separate joints. Box Plot graphs [b], [d] and [f] represents combined ankle and knee results and are displayed as either patient. Patient J (Black A9 combined with K3M) and Patient K (Grey A10 combined with K4M). [a] and [b] mean PGE<sub>2</sub> at Day 3, [c] and [d] mean cumulative PGE<sub>2</sub> at day 7, [e] and [f] mean cumulative PGE<sub>2</sub> at day 28. [Individual patients results were plotted with ankle and knee data separated [a,c,e] and with ankle and knee data combined [b,d,f] in order to identify trends between matched pairs or joints and between patients].

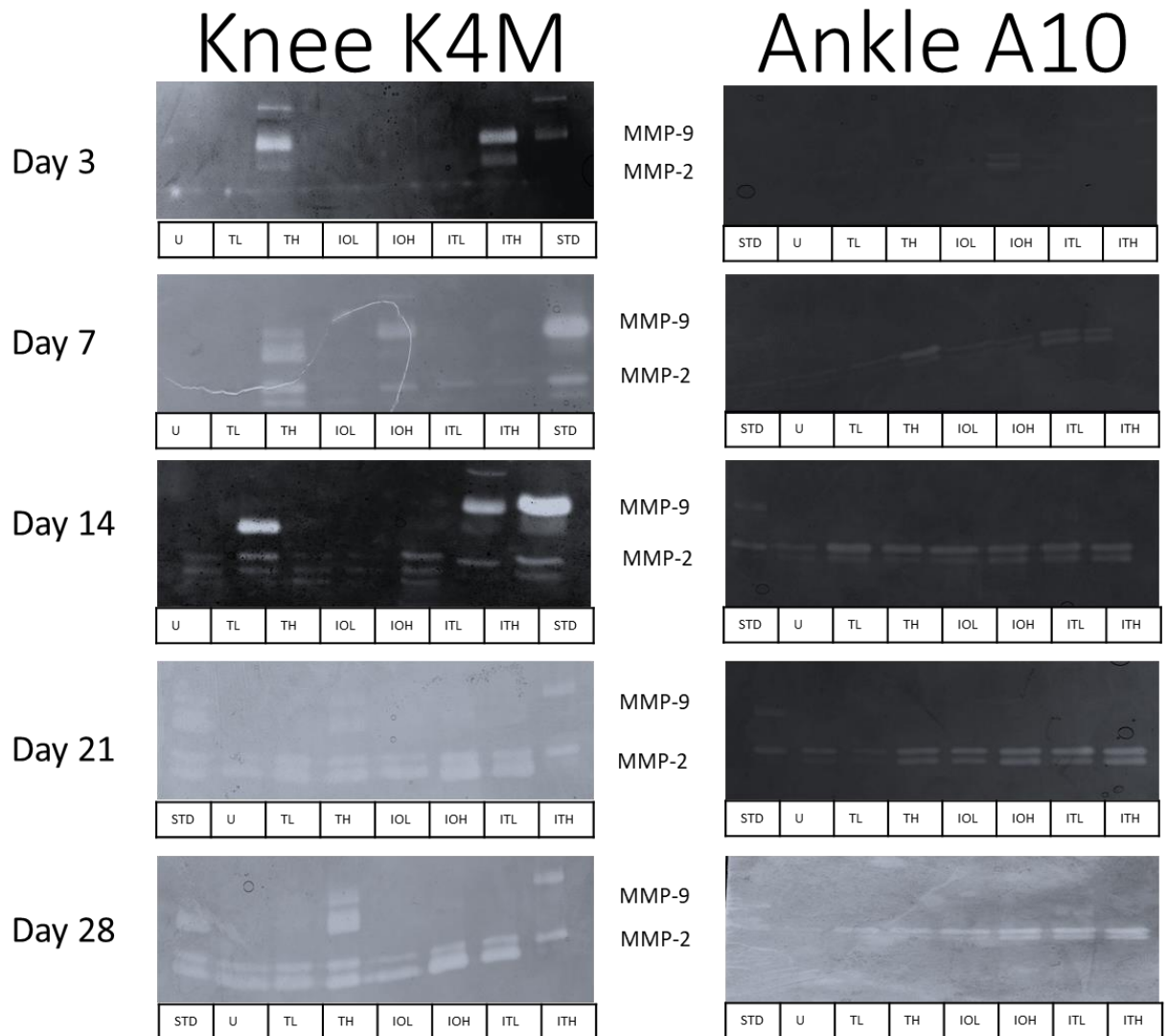
#### 4..16 **What effect do pro-inflammatory cytokines have on chondrocyte gelatinase matrix metalloproteinase (MMP-2 and 9) expression/activation and is there a differential response in the ankle and knee?**

Expression and activation status of the gelatinases, MMP-2 and MMP-9 in cytokine-treated ankle and knee cartilage explants was measured using gelatin substrate zymography (Figure 4.11). Qualitative assessment comparing ankle and knee tissue from patient K (Ankle 10 and Knee 4 medial femoral condyle) was undertaken at day 3, 7, 14, 21 and 28 of culture in order to investigate the relative ratio of MMP-9 to MMP-2, as it has previously been reported that a high ratio of MMP-9 to MMP-2 is suggestive of ongoing extracellular matrix degeneration as observed in OA (Zeng et al., 2015). Furthermore, the ratio of inactive proMMP-9 compared to the active form of MMP-9 was also assessed. MMP-9 is activated *in vivo* by MMP-3 which cleaves the pro-peptide from the heavier 92kDa pro MMP-9 molecule producing active MMP-9 enzyme with a molecular weight of 82kDa. All media samples were normalised to the explant wet weights prior to electrophoresis in order that direct visual comparison could be made between treatments.

#### 4..17 **MMP-9 is induced in knee and not ankle cartilage in response to 100ng/ml TNF $\alpha$ and 5ng/ml IL-1 $\alpha$ with 100ng/ml TNF $\alpha$**

Analysis of the zymograms containing media samples collected from cytokine exposure of the matched pair ankle and knee (patient K) illustrated that there was little to no active MMP-9 or pro-MMP-9 released from any of the ankle samples during the 28 days of culture (Figure 4.11). An MMP-9 standard loaded onto the gels was detected indicating the lack of MMP-9 in the samples reflected the tissue's activity. Although initially at very low levels, pro- and active MMP-2 was observed in the ankle samples throughout, consistent with its known constitutive expression. This is in stark comparison to the knee samples where substantially more MMP-9 in both the inactive proenzyme and active enzyme forms was detected on all gels from knee cartilage explants stimulated with either pathologically 'high' 100ng/ml TNF $\alpha$  in isolation or in conjunction with 5ng/ml IL-1 $\alpha$  (with 100ng/ml TNF $\alpha$ ). This upregulation of pro- and active MMP-9 can be seen from day 3 and continues all the way to termination of the

culture period at day 28. Interestingly, as culture time progressed the pro-MMP-9 band intensity reduced concomitant with an increased intensity in the active MMP-9 band (Figure 4.11), suggesting a potential increase in degeneration over the culture period.



**Figure 4.11** Gel zymography of matched ankle and knee tissue from patient K (A10 and K4M). Images presented for quantitative assessment of the ratio of MMP-2 to MMP-9 as a marker of enzyme induced degeneration. Talar dome and knee medial femoral condyle were cultured in the absence (U) or presence of 2ng/ml TNF $\alpha$  (TL), 100ng/ml TNF $\alpha$  (TH), 100pg/ml IL-1 $\alpha$  & 200pg/ml OSM (IOL), 5ng/ml IL-1 $\alpha$  & 10ng/ml OSM (IOH), 100pg/ml IL-1 $\alpha$  & 2ng/ml TNF $\alpha$  (ITL), and 5ng/ml IL-1 $\alpha$  & 100ng/ml TNF $\alpha$  (ITH) for 28 days with media extracted and tested at day 3, 7, 14, 21, and 28 (n = 4). All samples were compared to 5 $\mu$ l of MMP-2 and MMP-9 standard (STD) (section 2.4).



#### 4.18 Discussion

The balance of anabolic and catabolic responses in cartilage is complex, involving autocrine and paracrine responses of chondrocytes to cytokines, NO, prostaglandins and proteolytic enzymes including the MMPs and ADAMTSs. In this Chapter, the production of NO, PGE<sub>2</sub> and the gelatinases (MMP-2 and MMP-9) were investigated. When the mean cumulative data of each joint were independently examined (either ankle or knee) NO production did not appear to be significantly influenced by cytokine treatment compared to the untreated control. However, when the mean cumulative data between the ankle and the knee were compared, there was a small but significant increase in NO production in the ankle compared to the knee for the first 7 days of culture. This small difference was however lost after day 7. Ankle chondrocytes are more metabolically active with higher turnover of ECM compared to knee chondrocytes (Kuettner and Cole, 2005) which may partly explain this short term significant difference. However, research into the role of NO in cartilage degeneration has suggested that NO itself may not be an ideal marker of pro-inflammatory degeneration. Adramson (2008) and Clancy et al (2004) suggested that NO was less important than the balance of anabolic and catabolic downstream redox derivatives that it produced. NO mediates upregulation of IL-1 and TNF $\alpha$ , MMPs and iNOS via the NK- $\kappa$ B pathway. Previous evidence would suggest that NO redox derivatives may in fact antagonise one another in order to maintain cartilage homeostasis. The peroxynitrite derivative aids translocation of NK- $\kappa$ B into the nucleus of chondrocytes resulting in the upregulation of genes encoding cytokines, MMPs and pro-apoptotic molecules (Clancy et al., 2004). In contrast, S-nitrocysteine ethyl ester (SNCEE) another NO redox derivative does exactly the opposite by blocking NF- $\kappa$ B from entering the cell nucleus (Clancy et al., 2004). Therefore, to more clearly understand the reported importance of cytokine-induced NO production in cartilage degeneration, the relative levels of SNCEE and peroxynitrite would be useful to measure to determine whether the composition of these NO derivatives differ in the ankle and knee. This would provide further mechanistic insight into how the tissues might deviate in their responses. As seen with sGAG loss and LDH production, there is a consistent patient specific heterogeneity observed with NO production. The donor

ankles and knees that consistently produced the most NO in response to cytokines almost perfectly mirrored the patient specific pattern observed with sGAG loss and LDH production (Chapter 3).

Levels of cytokine-induced PGE<sub>2</sub> synthesis adds further weight to the hypothesis that differential degeneration between ankle and knee cartilage, reflecting the relative incidence of primary OA, may be mediated by pathologically '*high*' levels of TNF $\alpha$ . Analysis of ankle cartilage exposed to cytokines revealed no significant overall effect on PGE<sub>2</sub> production; however, it was interesting to note that in the 100ng/ml TNF $\alpha$  treated explants there was a trend towards less PGE<sub>2</sub> being produced by the '*high*' dose TNF $\alpha$  treatment when compared to untreated tissue. In contrast, overall production of PGE<sub>2</sub> from the knee explants was significantly increased by stimulation with pathological '*high*' levels of TNF $\alpha$ , both alone and in combination with IL-1 $\alpha$ . When PGE<sub>2</sub> production was directly compared between joints, half as much PGE<sub>2</sub> was produced by the ankle than the knee concomitant with a significant increase in knee cartilage treated with '*high*' TNF $\alpha$ . These data exactly mirror the results observed when assessing the other outcome measurements i.e. sGAG loss and NO production. In addition to this the patient specific response to inflammation reported in Chapter 3 remained consistent with patient J producing significantly more PGE<sub>2</sub> than patient K.

Lopez-Armada and colleagues investigated the effect of TNF $\alpha$  and IL-1 on human femoral head chondrocytes in monolayer (Lopez-Armada et al., 2006). They found a differential response in chondrocyte death and NF- $\kappa$ B mediated degeneration between pro-apoptotic TNF $\alpha$  and the significantly less chondrotoxic IL-1. Interestingly, these differences were accentuated in the presence of PGE<sub>2</sub> with significantly more pro-apoptotic caspase enzymes being produced following TNF $\alpha$  stimulation at 100ng/ml (Lopez-Armada et al., 2006). Both IL-1 and TNF $\alpha$  have themselves been reported to increase PGE<sub>2</sub> production (Kuhn et al., 2000), supporting the data presented in this Chapter identifying that pathologically '*high*' TNF $\alpha$  induces PGE<sub>2</sub> production in knee cartilage and that this synergistic relationship seems to result in an increase in degeneration; however, one of the novel findings in this Chapter is that the ankle cartilage is less susceptible to cytokine insult and does not respond by inducing PGE<sub>2</sub> synthesis, unlike that observed in the knee.

Matrix metalloproteinases in particular MMP 1, 2, 9 and 13 play an important role in OA by degrading cartilage extracellular matrix (Rose and Kooyman, 2016). In this Chapter, two gelatinases MMP-2 and MMP-9 were investigated to determine if ankle and knee exert differential MMP responses to pro-inflammatory cytokines, as evidence would suggest that an increased MMP-9 to MMP-2 ratio (Ben David et al., 2008) and increased activation of pro-MMP-9 are both markers of degeneration (Zeng et al., 2015). The results suggested that there is little to no MMP-9 expression in the ankle tissue exposed to pro-inflammatory cytokine insult over the 28 days culture period; furthermore, activation of the zymogen was not observed in the ankle. This is not the case for the more prolific cytokine-responding knee tissue which demonstrated substantial increases in both pro and active MMP-9 in the media of all knee explants treated with pathological 'high' levels of TNF $\alpha$ . What is also evident is that as time progressed more pro MMP-9 was converted to the active MMP-9. Previous criticisms of this technique have highlighted the change in MMP-2 and MMP-9 levels with age (Takahashi et al., 2005), however given that both the knee and ankle cartilage was harvested from the same donor patient at the same time (during above knee surgical amputation) this is unlikely to affect the results obtained. Further quantification of the MMP levels by measuring band intensity by densitometry, or analysing samples using specific MMP-9 ELISAs (which can measure pro and active enzyme levels) along with increased sample size would however undoubtedly be beneficial. Furthermore, quantification of pro and active MMP-13 levels would also be a valuable addition to better understand whether ankle cartilage is also more resistant to cytokine-induced collagenase expression. This could be conducted in parallel with measuring the extent of collagen degradation, using the hydroxyproline assay, to correlate collagen loss with MMP activity – a feature known to be critical in perpetuating OA progression (Wang et al., 2013).

What is also remarkable is the consistent patient specific responses observed across all outcome measurements investigated. The same donor tissues that have been shown to undergo the most degeneration (Chapter 3 sGAG loss) also induced the greatest PGE<sub>2</sub> synthesis making a link between these two processes highly likely, concurring with previous reports that PGE<sub>2</sub> dose-dependently inhibited proteoglycan production

in OA cartilage (Attur et al., 2008). What remains unclear is why certain donor tissues/patients possess an innate resistance or susceptibility to pro-inflammatory cytokines and whether this difference is at a transcriptome, protein or structural level.

In conclusion, this experimental chapter has validated earlier studies that indicated that the chondrocyte response to pro-inflammatory cytokines differed between knee and cartilage (Eger et al., 2002, Kuettner and Cole, 2005) by further extending the pro-inflammatory molecules being assessed. To the best of our knowledge, this is the first report demonstrating a significant differential response of knee and ankle cartilage to cytokine insult in the induction of pro-inflammatory mediators (NO and PGE<sub>2</sub>), in addition to the enhanced synthesis and activation of MMP-9 observed in knee cartilage only. This phenomenon was particularly pronounced when the cartilage was cultured with 100ng/ml TNF $\alpha$  alone or in combination with IL-1. It provides novel data on the largely resistant phenotype of the ankle cartilage in withstanding these cytokine insults, and might, in part, explain why primary ankle OA has a much lower incidence than knee OA.

#### 4..19 Summary of Chapter Findings

- This is the first report demonstrating a significant cytokine mediated induction of pro-inflammatory mediators NO and PGE<sub>2</sub> in knee relative to ankle cartilage
- Cytokine mediated induction of MMP-9 synthesis and activation was observed in knee cartilage only
- Effects were particularly pronounced when the cartilage was cultured with 100ng/ml TNF $\alpha$  alone or in combination with IL-1 $\alpha$

# Chapter 5

## General Discussion

## 5 Research Question

Osteoarthritis (OA) is a debilitating disease that causes considerable pain and suffering to patients and has huge financial and socioeconomic costs to society. The National Institute of Health Care and Economics estimated that OA cost the UK £8.5 billion per year (NICE, 2015). A disease that was once felt to be inevitable ‘wear and tear’ of the joints that occurred with age is now understood to be a disorder of chronic inflammation and mechanical malalignment with multiple genetic and environmental influences (Sokolove and Lepus, 2013). Treatment of a different musculoskeletal disorder, namely rheumatoid arthritis has been revolutionised over the last few decades with the advent of biological disease modifying anti-rheumatoid drugs (bDMARDs) such as *Anakinra* (IL-1 receptor antagonist), *Infliximab* (anti-TNF) and *Tocilizumab* (IL-6 receptor blocker). These biological DMARDs have been so effective that observing severe rheumatoid affecting large joints has become a rarity in clinical practice (Kahlenberg and Fox, 2011). Sadly, despite extensive research and multiple promising targets, the same cannot be said for disease modifying osteoarthritis drugs (DMOADs).

However, not all joints are affected by OA to the same extent; interestingly, the lifetime prevalence of knee OA is as high as 41% compared to only 4.4% in the ankle (Cushnaghan and Dieppe, 1991). Understanding why the ankle joint is largely protected from primary OA might provide mechanism(s) and/or therapeutic targets for alleviating the degeneration observed in the knee. Previous studies comparing ankle to knee articular cartilage found not only histological differences between the two joints but also differences in the biochemical composition, metabolic activity and dynamic stiffness (Eger et al., 2002, Kuettner and Cole, 2005) It is still largely unknown whether this inherent difference in biochemical composition or difference in biomechanical properties confers a level of protection to the ankle cartilage. However, what was most intriguing was the differing catabolic response of cartilage to pro-inflammatory IL-1 and fibronectin fragments. [<sup>35</sup>S]-sulphate was incorporated into sGAGs as a marker of proteoglycan synthesis following culture with IL-1 (Eger et al., 2002). At pathologically ‘high’ levels, suppression of proteoglycan production by 50% (IC<sub>50</sub>) occurred in the knee at a concentration 5 times lower than that observed in the ankle (11.8pg/ml compared to 56.1pg/ml) (Eger et al., 2002). The relative resistance of ankle tissue to pro-

inflammatory mediated degeneration was even more prominent after an artificial cartilage injury was produced (Patwari et al., 2003). Patwari and colleagues (2003) compressed human donor ankle or knee cartilage in order to produce an injurious stimulus and cultured the explants in either 1ng/ml IL-1 or 100ng/ml TNF $\alpha$ . In the knee tissue they noted that the combination of injury and cytokine significantly increased proteoglycan loss by 35% (IL-1) and 54% (TNF $\alpha$ ) compared to injury alone. When the same experiment was performed on ankle cartilage there was no increase in proteoglycan loss. These finding certainly go some way towards explaining the huge difference in prevalence between knee and ankle OA; furthermore unlike the knee, the vast majority of ankle OA only occurs after a significant injury resulting in mechanical malalignment (Saltzman et al., 2006).

Hence, in this thesis, the ankle has been utilised as a 'model of perfection' against which to compare knee cartilage proteoglycan degeneration, chondrocyte death, generation of secondary inflammatory mediators and upregulation of matrix degrading proteases following stimulation with pro-inflammatory cytokines. Characterising the differing responses between the two joints will aid identification of potential therapeutic targets to prevent knee cartilage degeneration and may identify existing biological anti-cytokine treatments that can be used to treat knee OA.

### **5.1 Biochemical implications of data findings**

The results presented in this thesis are consistent with previous findings demonstrating that the ankle contains significantly more sGAGs per milligram of tissue than knee cartilage (Kuettner and Coles, 2005). There is also higher turnover of sGAG in the ankle than the knee resulting in more sGAG being lost to media during culture. Ankle aggrecan transcript levels are reported to be expressed at twice the level of that expressed in knee cartilage and may account for the increased sGAG content detected (Kuettner and Coles, 2005). However, as a proportion of total sGAG, the ankle loses significantly less than the knee, meaning ankle cartilage undergoes less cytokine-mediated proteoglycan degeneration, in line with the observation that the ankle is more resistant to OA onset. An overall comparison suggested that sGAG loss was more pronounced in the knee i.e. more significant proteoglycan degradation, indicating that the joint of origin was central

to affecting the rate of degeneration. This lack of response in the ankle cartilage, irrespective of cytokine treatment, would suggest that the tissue has some degree of innate resistance to degeneration that is independent of cytokines; again, this finding supports the pre-existing idea that there are inherent differences between the two cartilages that confers protection in the ankle. In addition to this, there was an independent effect of pathologically 'high' TNF $\alpha$  which induced proteoglycan loss in the knee only; this is the first report of the chondroprotective behaviour of the ankle to TNF $\alpha$  insult, which has only previously been reported for IL-1 (Eger et al., 2002) and fibronectin fragments (Kang et al., 1998). The data would suggest that the ankle cartilage is much more refractory to damage than the knee cartilage. However, articular cartilage does not only comprise proteoglycans - both collagen composition and architecture are equally as critical for tissue functionality. Evidence suggests that collagen content does not alter between ankle and knee cartilage (Kuettner and Coles, 2005), hence it was not analysed in this thesis. However, the relative amounts and the types of collagen crosslinks found in these tissues would be interesting to compare, as these too can influence tissue functionality and could provide resistance to overt tissue degeneration.

Cytokine-mediated downstream pro-inflammatory molecules i.e. NO and prostaglandin E2 synthesis followed an almost identical pattern as that detected for sGAG loss from the tissue as a cumulative response over 28 days in culture. Surprisingly, in the first 7 days of stimulation, ankle cartilage produced significantly more NO than the knee, with no difference observed between joints thereafter. The evidence presented for the other outcome measurements would lead to the expectation that the ankle joint would produce less NO than the knee; however, this finding could be accounted for by the fact that NO has an important role in normal joint homeostasis and is not necessarily a marker of inflammation (Abramson, 2008). The interaction between nitric oxides' redox derivatives: anabolic S-nitrocysteine ethyl ester (SNCEE) and the catabolic peroxyxynitrite determines how NO levels affect degeneration (Clancy et al., 2004). Recent animal studies using a post meniscectomy rat model to reproduce early OA have also suggested that high NO levels may be chondroprotective in the first 2 weeks (Hsu. et al., 2017). Hsu and colleagues performed meniscectomies or sham procedures on rats and measured IL-1, TNF $\alpha$  and IL-6 in addition to NO levels for 14 days. They found significantly higher



levels of NO in the post meniscectomy rats than in the sham group, correlating with a reduction in IL-1, TNF $\alpha$  and IL-6 levels in the early OA group. They concluded that “*nitric oxide plays a protective role in OA in the early stages*” (Hsu. et al., 2017). This may therefore explain the initial observation of higher NO levels in the ankle joint, which may be mechanistically relevant in conferring resistance to cytokines. Thus, sustained elevation of NO levels, as observed in the knee, might then predispose the tissue to a more catabolic phenotype and tissue degeneration.

Both NO and PGE<sub>2</sub> production were significantly induced in knee cartilage exposed to 100ng/ml TNF $\alpha$ , both alone and in combination with 5ng/ml IL-1 at each time point over the 28 days period. These findings are consistent with that of a previous study examining the response of monolayer OA knee chondrocytes exposed to 10ng/ml IL-1, in which a significant increase in PGE<sub>2</sub> production was observed following only 24 hours of cytokine exposure (Masuko-Hongo et al., 2004). It is likely that this early increase in PGE<sub>2</sub> synthesis may be because the cells lacked an extensive ECM that may have slowed diffusion of the cytokines to the chondrocytes as observed in the *ex vivo* cartilage explants. Again, a comparison of knee versus ankle cartilage demonstrated a significant difference between the joint of origin and a TNF $\alpha$ -dependent induction of NO and PGE<sub>2</sub> synthesis – an effect that was noticeably absent in ankle cartilage. Patient-specific responses were also observed suggesting a donor cohort was more sensitive to cytokine insult and produced more downstream inflammatory mediators. Increased synthesis of these downstream molecules can then modify the catabolic and anabolic responses in chondrocytes, predisposing the tissue to degeneration, as evidenced in OA (Goldring and Berenbaum, 2004).

Up-regulation of MMP-9 synthesis and activation observed in this study is also consistent with the other cytokine-mediated pro-inflammatory responses of the knee cartilage, as described above. Cytokine-induced expression and activation of MMP-9 in knee cartilage corroborates previous studies demonstrating its increased activity in human OA cartilage (Lipari and Gerbino, 2013) Activation of *de novo* synthesised pro-MMP-9 was most markedly released from knee cartilage stimulated with pathologically ‘*high*’ dose 100ng/ml TNF $\alpha$ , alone and in combination with 5ng/ml IL-1 $\alpha$  over the culture period. TNF $\alpha$ -mediated induction of MMP-9 synthesis/activation has been previously

described as part of the mechanism that produces hypertrophic OA (extensive osteophyte formation). Subchondral bone has been shown to produce TNF $\alpha$  that acts on the chondrocyte to upregulate MMP-9 expression/activation and has a role in chondral dedifferentiation at the tidemark between bone and cartilage (Prasad et al., 2010, Chubinskaya et al., 1999), again suggesting a mechanism by which TNF $\alpha$  can elicit degenerative changes in the cartilage tissue.

In contrast, pro and/or active MMP-9 enzymes were below the limit of detection in the ankle tissue at all time points analysed, once again suggesting the ankle is more resistant to degeneration. Reported analysis of MMP transcript expression profiles indicated that MMPs 1, -2, -13 and -14 were comparable between knee and ankle cartilage, however MMP-8 was below the limit of detection in ankle cartilage (Chubinskaya et al., 1999). However, this study did not investigate the expression of MMP-9 levels in these tissues, and there are no other published reports of MMP-9 expression in ankle cartilage. Hence, induction of MMP-9 activity in knee chondrocytes can result in extensive enzymatic damage to the matrix that the cells may not be able to repair, while the weaker response of the ankle chondrocytes may allow the cells to repair their matrix damage supporting a differential incidence in OA between these two joints. Other MMPs including MMP-13 are critical for collagen degradation as observed in OA, so studies further characterising the transcriptional profile and enzyme activity status would be beneficial. To date, analysis of the ADAMTS 'aggrecanase' enzymes has not been compared between these tissues, and again this would be highly informative on elucidating potential mechanistic differences in tissue response.

Experiments to assess cell viability by testing LDH levels demonstrated no specific cytokine effect which is contrary to the results of previous studies investigating caspase induced apoptosis (Lopez-Armada et al., 2006). Although low levels were detected overall, there was more LDH produced by the ankle than the knee explants. It was expected that the knee tissue would release more LDH reflecting a more degenerative phenotype, although it is recognised that degeneration is not reliant on cell death. There is some evidence to support the use of synovial fluid LDH levels as a marker for OA changes however this is now felt to be "non-specific and insensitive in early disease" (Hurter et al., 2005). This subtle increase in LDH release may have arisen as a result of

the unloaded nature of the cartilage tissue. Healthy cartilage requires cyclical loading to maintain normal homeostasis (Grodzinsky et al., 2000) and it is speculated that the denser packed ECM of the ankle tissue inhibited the diffusion of oxygen to the centre of the explants resulting in slightly more anaerobic metabolism. LDH levels also represent quite a crude assessment of cell death. Further investigation with non-radioactive microscopic staining such as live/dead staining with ethidium homodimer 1, Thiazolyl blue tetrazolium bromide (MTT) or even LDH staining, have all been shown to be significantly more accurate and reproducible than Cytotox<sup>96</sup> assays (Stoddart et al., 2006) and may help to clarify this in future studies.

Identification of trends in the tissues' responses was difficult to discern due to the overt heterogeneity in the behaviour of the human tissue. Several patient donors were much more 'receptive' to cytokine stimulation than others; furthermore, assessment of two sets of matched pairs of ankles and knees demonstrated that this susceptibility to degeneration was patient specific and not solely caused by differences in individual samples, further adding weight to the theory that patients have an innate susceptibility to inflammation driven degeneration. Individual patient analysis allowed heterogeneity to be accounted for but reduced statistical power. Despite this, a more predictable response to cytokines, that may have been lost when averaged across the knee samples, was observed e.g. untreated < physiological 'low' concentration < pathological 'high' concentration with a synergistic effect of IL-1 in combination with either TNF $\alpha$  or Oncostatin M. These findings are consistent with those reported by Barksby et al (2006) who demonstrated that human monolayer chondrocytes stimulated with 20pg/ml IL-1 and 10ng/ml OSM increased MMP-1 mRNA expression (1.85 to 3.55 times increase) when cytokines were used in combination compared to individual cytokine treatments. Interestingly, they also noted a similar pattern of heterogeneity between their 4 osteoarthritic patients (Barksby et al., 2006). Importantly, this pattern of degeneration (untreated < physiological 'low' concentration < pathological 'high' concentration) was not observed in the ankle cartilage, which was equally resistant to all cytokines, irrespective of concentration, even when patient heterogeneity was accounted for.

## 5.2 Clinical implications of study findings

The data presented in this thesis has demonstrated that (i) ankle cartilage is significantly less prone to cytokine-mediated degenerative effects than knee cartilage and (ii) knee cartilage is significantly affected by pathologically '*high*' 100ng/ml TNF $\alpha$ . This differential response to high dose TNF $\alpha$  likely reflects the inherent chondroprotective behaviour of the ankle cartilage, whilst inducing degenerative effects in the knee. Furthermore, bimodal responses have been previously reported in other cell types following treatment with TNF $\alpha$  (van Kralingen et al., 2013) and there is also evidence to support the presence of both pro-inflammatory and anti-inflammatory cytokine receptors on the chondrocyte cell membrane (Tanaka M., 2003). Interestingly, this significant differential response following 100ng/ml TNF $\alpha$  is reflected in several of the outcome measurements: increased PGE<sub>2</sub> release concomitant with *de novo* MMP-9 synthesis and activation. It is currently unclear whether TNF $\alpha$  induced degeneration of knee cartilage may be mediated by or synergised by PGE<sub>2</sub> resulting in MMP-9 activation, and further studies are warranted to investigate this as a potential mechanistic pathway.

The findings presented in this thesis that knee cartilage is highly susceptible to pathologically '*high*' TNF $\alpha$  and that this cytokine is heavily implicated in the initiation of OA is no surprise when the proposed mechanism of novel intra-articular orthobiological treatments such as platelet rich plasma (PRP) and Mesenchymal Stem Cell (MSC) injections is considered. Interestingly, at the start of 2019 the National Institute for Health and Care Excellence have, for the first time, recommended intra-articular injections of PRP as a treatment for knee OA. The mechanism of action of this platelet rich serum is poorly understood but is speculated to combine the anti-inflammatory and anti-catabolic effect of a combination of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)- $\beta$  (Tong et al., 2017). Pre-clinical evidence has suggested that in chondrocytes primed with TNF $\alpha$ , concentrations of platelet derived growth factors in excess of 10% will actually inhibit ongoing production of TNF $\alpha$ , IL-1 and the downstream production of IL-6 (Tohidnezhad et al., 2017). It is therefore likely that the active component of PRP is PDGF, and that it acts by suppressing the autocrine effect

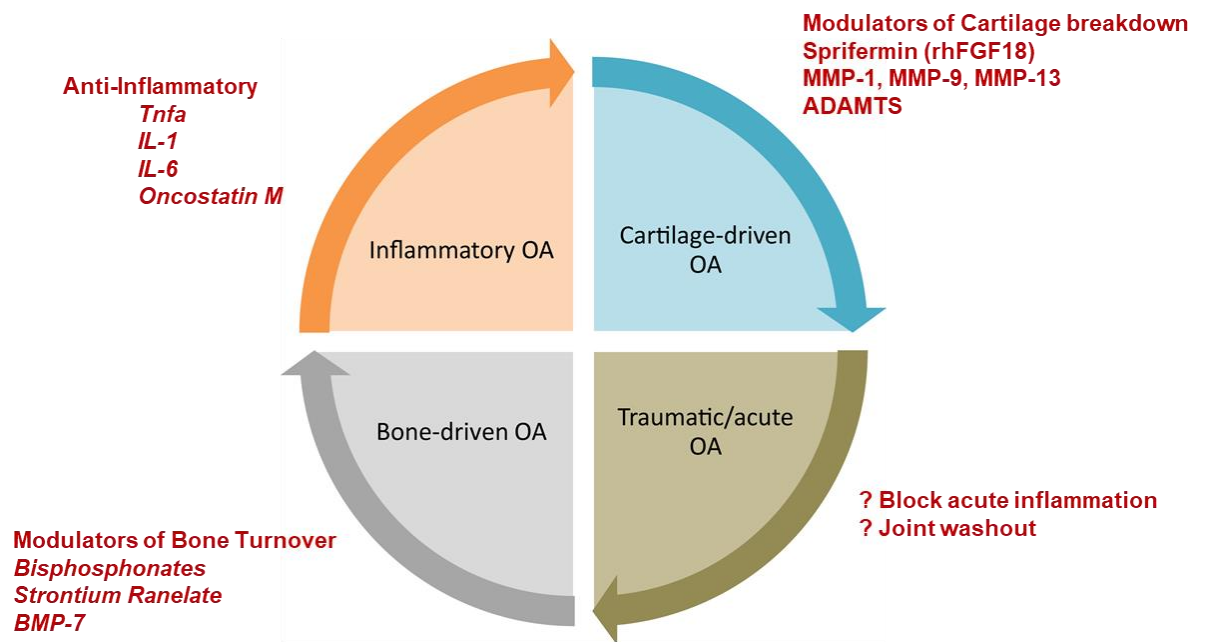
of TNF $\alpha$  on chondrocytes (Tohidnezhad et al., 2017). This is consistent with the findings presented in this thesis that TNF $\alpha$  plays a key role in proteoglycan degeneration.

Also consistent with the thesis findings presented is the relative failure of PRP to improve pain and prevent disease progression in the ankle. Importantly, a recent systematic review of injectable treatments for ankle OA failed to demonstrate any benefit of PRP over corticosteroid or hyaluronic acid (Vannabouathong et al., 2018), suggestive that the mechanism(s) involved in ankle OA cartilage differs to the knee supporting the divergence in ankle cartilage behaviour observed in this thesis. Mesenchymal stem cells (MSCs) are also attracting much clinical interest for the treatment of OA. It was initially believed that these multipotent cells would be able to 'regrow' cartilage. There is, however, limited evidence supporting MSCs' capability to regenerate hyaline cartilage but there is evidence to suggest that they are able to modulate the inflammatory environment, protecting chondrocytes and down-regulating inflammation in the synovium (van Buul et al., 2012). My hypothesis that TNF $\alpha$  is a significant driver of cartilage degeneration in the knee is supported by the study of Van Buul and colleagues (2012). The femoral canal of patients undergoing total hip replacement was aspirated to obtain bone marrow derived MSCs, while chondrocytes and synovial cells was obtained from clinical waste during total knee replacement and cultured. In the presence of TNF $\alpha$  stimulation, the MSCs showed reduced expression of IL-1, MMP-1, MMP-13 and COL2A1 transcripts while upregulating IL-1 antagonists in the cartilage and synovium. A beneficial reduction in NO and PGE<sub>2</sub> production via NF- $\kappa$ B inhibition was observed following MSC treatment, but only in the presence of TNF $\alpha$ . Mesenchymal stem cells migrate towards injured tissue in order to proliferate and aid tissue repair. They will produce anti-inflammatory effects in order to facilitate proliferation and repair but only in the presence of an inflammatory environment (Regmi et al., 2019). Currently no studies assessing the benefit of MSCs in ankle OA have been undertaken but the thesis findings would support the hypothesis that there might be limited benefit in this joint.

The thesis findings demonstrated a huge amount of heterogeneity between patients' responses to cytokines, over the 28 days culture period, where one donor patient's knee cartilage specimens underwent 40% more proteoglycan loss than another donor patient

harvested from the same joint of origin. Most interesting, however, is the fact that this relative resistance or susceptibility to degeneration (percentage sGAG loss, MMP-9 activation) and induction of downstream inflammatory mediators (NO and PGE<sub>2</sub>) was specific to that individual patient. This significant and consistent patient specific susceptibility to pro-inflammatory cytokines was observed across all experimental outcome measurements. The data presented would support the concept that there is a specific 'inflammatory OA' phenotype and that this phenotype is not directly related to the patients underlying medical conditions, age or sex.

Patient innate susceptibility to inflammatory stimuli following trauma has been heavily investigated. Systemic inflammatory response syndrome (SIRS) is a life-threatening cascade of inflammatory mediators that occurs in some trauma patients following a major injury. The difficulty for trauma surgeons is identifying patients who can safely be operated on without initiation of a 'second hit' phenomenon and those that will deteriorate post-operatively. The second physiological hit of surgery causes some patients to initiate a "cytokine storm" which results in increased endovascular permeability, pyrexia and loss of fluid into the interstitial spaces (Bone et al., 1992). Clinically, this manifests as an acute lung injury followed by multi-organ failure and death. Despite extensive investigation into biomarkers like IL-6 and appropriate physiological parameters, to date, orthopaedic surgeons are still unable to reliably predict which patients will develop this propagated inflammatory response and which will not (Jaffer et al., 2010). Certainly, the same would seem to be true in OA pathogenesis. In clinical practice, often because of financial constraints associated with the National Health Service, the majority of patients attending clinics are those with end stage OA requiring joint replacement. However, there is increasing attention being paid to early intervention, reconstruction and regeneration as a method of reducing health spending and the burden for patients. This has led clinicians to postulate that there are separate osteoarthritic phenotypes that progress towards end stage OA or "joint failure" (Figure 5.1).



**Figure 5.1** Osteoarthritic phenotypes and proposed treatments or therapeutic targets. Modified from a lecture and paper by Barr and Conaghan (Conaghan, 2013).

These OA phenotypes (Figure 5.1) are often quite apparent to clinicians on a day to day basis. Bone-driven OA tends to occur in elderly male patients who rapidly develop deformities with joints that undergo extensive osteophytosis, traditionally referred to as hypertrophic OA. Cartilage driven arthritis often manifests early with a slowly progressive varus deformity at the knee in younger male patients. On arthroscopy, wear is often confined to a single knee compartment and resembles a polished concrete surface. These patients often do well if high tibial osteotomies or uni-compartmental knee replacements are performed. Post-traumatic OA follows a significant intra-articular fracture or ligamentous injury in the knee. Inflammatory OA is often noted in female patients with raised body mass indices and is associated with extensive intra-articular synovitis and recurrent effusions. These patients initially have a good response to intra-articular corticosteroid injections but tend to progress and require total joint replacements.

Studies have confirmed that “inflammatory OA” has many similar features to rheumatoid OA, in particular extensive synovial inflammation associated with IL-17 expression and a similar pattern of inflammatory cytokines to that detected in

autoimmune disease (Pasquali Ronchetti et al., 2001). It would therefore fit that certain biological anti-rheumatoid drugs, such as anti-TNF *Adalimumab*, may be beneficial in this group of patients. To date, no clinical study has assessed anti-TNF use in knee or ankle OA however, there has been a pilot study undertaken using this biologic agent in finger joint OA (Verbruggen et al., 2012). This study however failed to show a significant difference in pain but was extremely effective at reducing swelling in a subgroup of patients with the worst inflammatory symptoms. This thesis would support the ongoing investigation of anti-TNF drugs for treatments of knee OA, however patient heterogeneity should be considered. In order to demonstrate the maximum benefit, participants should be profiled to identify patients who are most susceptible to cytokine-induced inflammation. I would agree that *“OA trials would be more useful if they targeted a specific phenotype [as] any disease-modifying ability of an intervention in subgroups of trial populations might be diluted by the lack of efficacy in others”* (Van Spil et al., 2019).

### 5.3 Study limitations

The main limitation of the study was the patient population from which the knee and ankle cartilage was harvested; most donors were vascular patients who were undergoing amputation for critical ischaemia. These patients, by the nature of their disease, will have elevated IL-1, IL-6 and TNF $\alpha$  levels (Fiotti et al., 1999). In a study looking to assess the response of cartilage to pro-inflammatory cytokines, the ideal sample population would be healthy patients with no established arthritis, however for obvious ethical reasons, this was not possible. Alternative sources were considered including use of intra-operative waste from knee and ankle replacements or arthrodesis procedures, however the cartilage samples retrieved were extremely worn and poor quality. In addition to this, ankle joint replacements are rarely performed, and arthrodesis is done arthroscopically making cartilage retrieval impossible. One knee joint was obtained from a patient undergoing an oncological amputation and the biochemical outcomes measured demonstrated that, despite being relatively cytokine naïve, this donor patient tissue was less responsive to cytokine-mediated effects. This



would fit with my hypothesis that susceptibility to inflammation driven degeneration is specific to that patient and not any underlying disease or co-morbidity. A direct comparison of vasculopathies with matched joints from the same patients was performed which should have eliminated any further unknown variables.

A further limitation of the study was the relatively low number of knee cartilage specimens (5) versus ankle samples (10) and the relative low number of samples overall. All 29 patients (32 joints) that attended the University of Wales Hospital, Cardiff for non-emergent amputations over a 2-year period were considered for enrolment in my study. The relatively low number of amputations is likely to represent the improved glycaemic control and effect of statins on peripheral vascular disease (Hsu et al., 2017). Further enrolment would have required a multicentre study or longer recruitment period which was not feasible over this project duration.

Mechanical stimulation of the explants was absent in this cytokine-induced model of cartilage degeneration which may have artificially increased rates of proteoglycan loss, as it is well established that physiological levels of cyclical compression are required for normal cartilage homeostasis (Grodzinsky et al., 2000). This may have increased the percentage sGAG loss observed in the untreated explants making it higher than would be seen *in vivo* but as samples were being directly compared, it is unlikely to have made any difference to the overall conclusions drawn in this thesis. Lack of mechanical load may also have led to artificially elevated LDH levels as chondrocytes in the centre of the explant receive less oxygen due to lack of diffusion. Future experiments would ideally stimulate the ankle and knee cartilage with pro-inflammatory cytokines in the presence of a physiological and/or pathophysiological loading regimen to recapitulate the *in vivo* environment; this would allow further interrogation of the interplay of different OA causal factors in development of knee cartilage degeneration to enable comparison against the phenotypic behaviour of ankle cartilage.

#### 5.4 Future Directions

This thesis has strongly implicated TNF $\alpha$  as a significant cause of proteoglycan loss and inducer of downstream molecules known to be involved in cartilage degeneration – a phenomena that was largely only observed in the knee compared to the ankle. Further work could focus on assessing dose dependant effects of TNF $\alpha$  in promoting knee cartilage degeneration and comparing to the response of ankle tissue to more clearly delineate differences in biochemical outcomes. This would help to confirm the hypothesised bimodal response of the knee cartilage to this cytokine, at what concentration a switch from an anabolic to catabolic effect is established, and whether this occurs in ankle cartilage. Also, a more accurate identification of a specific inflammatory OA phenotype would help to better define patient cohorts for future studies, thus establishing treatment populations for whom targeted biological agents are most likely to yield beneficial results. Elucidating differentially expressed genes in the knee and ankle of inflammatory OA patients may also unravel the pathophysiological mechanisms distinct to each joint, and new therapeutic target(s) established for the prevention of inflammatory OA.

Future work would also aim to utilise next generation sequencing (RNAseq) to investigate transcriptome profiles of ankle and knee articular cartilage (Top 50 genes expressed Appendix 7.2). This series of experiments was performed as part of my thesis, however unfortunately the sequencing data obtained was of poor quality (for reasons outside of my control and related directly to the downstream processing and running of the RNA samples), therefore the differential expression of joint-specific genes could not be identified. To the best of my knowledge, transcript profiling of ankle versus knee cartilage has still not been performed, but would enable comparison of genes that are differentially expressed in one joint of origin over the other, to inform on how the ankle cartilage is largely resistant to primary OA. This information could then be utilised to determine whether any differences might translate to possible therapeutic targets for treatment of knee OA.

### 5.5 Concluding remarks

In conclusion, the data presented in this thesis has demonstrated that there are inherent differences in how ankle articular cartilage responds to pro-inflammatory cytokine insult when compared to knee cartilage. The work has further extended original studies indicating a level of protection conferred by ankle cartilage that is not observed in knee. Novel findings have identified that in the presence of cytokines, ankle cartilage chondrocytes do not synthesise significant levels of NO, PGE<sub>2</sub> or MMP-9, unlike that observed in the knee. Further characterisation is necessary to elucidate the mechanism(s) that protect the ankle cartilage from cytokine insult; utilisation of this knowledge will undoubtedly inform on therapeutic approaches for consideration in treatment strategies for primary knee OA.

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## 7 Appendix

### 7.1 Permission to determine suitability form

Dr Helen Hodgson  
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Cardiff and Vale Orthopaedic Centre  
Llandough Hospital  
CF642XX  
02920716370



## PERMISSION TO DETERMINE SUITABILITY FORM

### Why do joint cells differ in the ankle compared to the knee?

Cardiff University and the department of Orthopaedics/Vascular at Cardiff and Vale University Health Board are working in collaboration to research into ankle tissue. The research team is looking into why the ankle joint isn't prone to some primary joint diseases (such as arthritis) compared to other joints including the knee and hip.

For this research we need patients who have suffered ankle weakness, disease, and trauma and are undergoing surgery to take part or people who are undergoing hip or knee surgery. This may involve allowing us to have the tissue removed during your surgery that would normally be disposed of after surgery so that we can undertake basic biomedical research.

We are asking you to fill in and sign this form if you are interested in taking part in our research. Filling in this form does not mean that you have to take part, and you are free to withdraw from the research at any time, and this will not affect your standard of care and you do not have to give a reason for your withdrawal from the study. Filling in this form simply gives permission for a member of your clinical team to determine whether you are suitable to take part in this study. You may also be given a patient information sheet about this study to take home to read. This sheet will give you more detailed information about the study.

If you are deemed suitable to take part in this study you will be approached again by a member of your clinical team. If you do take part in the research, we will ask you to sign a consent form.

Permission to Contact Form

If you are interested in taking part in this research study, please fill in the form below and give it to a member of your clinical team or a researcher who may be present at clinic. If you would prefer to take the form home and think about it, please send it to the Research Manager at the address above if you decide to take part in the research.

Full Name: \_\_\_\_\_

Date of Birth: \_\_\_\_\_

Hospital number (if known): \_\_\_\_\_

Address: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Telephone number: \_\_\_\_\_

Email address: \_\_\_\_\_

Patient NHS no (if known): \_\_\_\_\_

Consultant name (if known): \_\_\_\_\_

I give permission for a member of clinical team to look at my medical records to determine if I am suitable to take part in the above research study. I understand this does not mean I have to take part and that I am free to withdraw at any time.

\_\_\_\_\_

Signature

\_\_\_\_\_

Date

## 7.2 Top 50 genes expressed across both joints detected using RNA-seq

Gene Symbol	Name	Total Reads both joints
CLU	clusterin	7270785
DCN	decorin	3908191
GPX3	glutathione peroxidase 3	3147521
MMP3	matrix metalloproteinase 3	2175364
APOD	apolipoprotein D	2105799
ACAN	aggrecan	2005371
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	1948911
CHI3L1	chitinase 3 like 1	1650333
MT-CO1	cytochrome c oxidase subunit I	1643486
FN1	fibronectin 1	1568732
MGP	matrix Gla protein	1325745
TPT1	tumor protein, translationally-controlled 1	1322496
CFH	complement factor H	1263517
CYTL1	cytokine like 1	1224396
MT-CO3	cytochrome c oxidase III	1217825
MT-ND4	NADH dehydrogenase, subunit 4 (complex I)	1216283
FTH1	ferritin heavy chain 1	1214685
BEST1	bestrophin 1	1163529
COMP	cartilage oligomeric matrix protein	1161340
MT2A	metallothionein 2A	1037799
EGR1	early growth response 1	933542
FTL	ferritin light chain	930951
SERPINA3	serpin family A member 3	761244
RPL13A	ribosomal protein L13a	754566
SOD2	superoxide dismutase 2	748985
SOD2	proline and arginine rich end leucine rich repeat protein	744813
PRELP	ATP synthase F0 subunit 6	737786
MT-ATP6	Mitochondrial encoded NADH dehydrogenase 2	716412
MT-ND2	connective tissue growth factor	706839
CTGF	ribosomal protein S6	699462
RPS6	cytochrome b	699315
MT-CYB	cytochrome c oxidase subunit II	692740
MT-CO2	NADH dehydrogenase, subunit 1 (complex I)	688443
MT-ND1	vimentin	657579
VIM	ribosomal protein L4	628502
RPL4	ribosomal protein L3	619597
RPL3	ribosomal protein S27	599225
RPS27	ribosomal protein L10	586746
RPL10	ribosomal protein L13	585102
RPL13	ribosomal protein S4, X-linked	566634
RPS4X	ribosomal protein S11	547278
RPS11	chromosome 2 open reading frame 40	536251
C2orf40	ribosomal protein S18	521850
RPS18	serpin family A member 1	518622
SERPINA1	ribosomal protein lateral stalk subunit P0	503300
RPLP0	ribosomal protein L19	489013
RPL19	Mitochondrial NADH dehydrogenase, subunit 5 (complex I)	478760
MT-ND5	ribosomal protein L8	459115
RPL8	eukaryotic translation elongation factor 2	458497
EEF2	ribosomal protein S3A	455132
RPS3A		

