Molecular assessment of the viability of osteochondral allografts; investigation of storage temperature, duration and donor pools

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master of Philosophy

by

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Declaration

I declare that this thesis has been prepared by myself, and has not been accepted in full or part, in any previous application for a higher degree. The work described in this thesis is a record of my own work; any collaborative work has been specifically acknowledged, as have all sources of information.

Mark James Eagle

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Abstract - Molecular assessment of the viability of osteochondral allografts; investigation of storage temperature, duration and donor pools - Mark James Eagle

Damage to articular cartilage in a joint caused by trauma or disease is a clinical indication, which orthopaedic surgeons in the United Kingdom are keen to treat with osteochondral allografts, avoiding or delaying the need for total knee replacement. Currently donated allograft tissue is imported and the preferred storage regimen is at 4ºC (hypothermic). However, the cartilage can only be stored for 28 days, because the success of a living osteochondral allograft is largely determined by chondrocyte viability, with 70% viability being considered as a minimum value for transplantation. Chondrocyte viability decreases with time such that by 28-days viability can be below 70%. Storage at higher temperatures such as 25ºC and 37ºC has been reported with some success at increasing storage time, but there are currently no published studies reporting the outcomes of storage at physiological resting knee temperature ("normothermic storage" at 33ºC). The aim of this work was to determine if chondrocyte function and cartilage matrix integrity can be maintained during storage at normothermic temperature for up to 12 weeks. In addition, methods of non-invasive assessment of allograft quality was investigated.

Osteochondral allografts from four human donor cohorts: normal (<45 years old), over-age (>45 years old), osteoarthritis and hypothermically stored, were retrieved from femoral condyles and stored in a bespoke culture medium- based storage solution in blood bags at 33ºC. Hemi-condyles were stored for up to 12 weeks. Storage medium was changed at weekly intervals and the conditioned medium analysed for glucose and lactate content using a test meter, and chondroitin sulphate using the dimethylmethylene blue assay. Chondrocyte viability was determined using live/dead histology and metabolic activity using the alamarBlue® assay. Presence of lubricin (PRG4) was detected by immunohistochemistry and retention of proteoglycans and glycosaminoglycans via safranin O histochemistry.

Analysis showed that glucose consumption and lactate secretion significantly decreased during 8 weeks of storage with significant difference between normothermic and hypothermic stored cohorts. It was found that chondroitin sulphate release into the storage medium was significantly higher in normal donors compared to all other cohorts, with hypothermic stored tissue releasing the least amount. Data also showed that chondrocyte viability was maintained above a mean of 70% at 12 weeks in the normal and over-age cohorts, but not in osteoarthritic or hypothermic tissue. Metabolic activity was significantly increased during the storage period in both normal and over-age donor cohorts, however in the hypothermic stored tissue it was

found to significantly decrease. Immunohistochemistry suggested that lubricin was present in the cartilage at the beginning and end of the storage period. Safranin O staining showed that there was a significant difference between normal, over-age and osteoarthritic tissue at the end of the storage period, with normal tissue retaining more proteoglycans. As a result, it is proposed that osteochondral allografts can be stored at normothermic temperature for 12 weeks with no apparent loss of integrity. Also, a combination of the non-invasive assays could potentially be used to measure tissue quality during clinical storage.

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List of abbreviations

AA	Ascorbic acid
Ab	Antibody
ACI	Autologous chondrocyte implantation
ACL	Anterior cruciate ligament
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin motifs
AFP488	Alexa Fluor® Plus 488
ALB	Anterolateral bundle
aMFL	Anterior meniscofemoral ligament
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CHI3L1	Chitinase 3-like-1
Co	Collagen
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
CT	Computed tomography
DAB	3,3'Diaminobenzidine
DAPI	4'6,-dimadino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue
DPBS	Dulbecco's Phosphate Buffered Saline
DS	Dermatan sulphate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESSKA	European Society of Sports Traumatology, Knee Surgery & Arthroscopy
FACIT	Fibril-associated collagens with interrupted triple helices
FBS	Foetal bovine serum
FCL	Fibular collateral ligament
FDR	False discovery rate
FGF-2	Fibroblast growth factor 2
FGFR	FGF receptor
GAG	Glycosaminoglycan
GH	Glucosamine hydrochloride

Glc	Glucose
H	Hypothermic
HA	Hyaluronic acid
HIF-2α	Hypoxia-inducible transcription factor 2α
HRP	Horseradish peroxidase
HS	Heparan sulphate
HTA	Human Tissue Authority
ICRS	International Cartilage Repair Society
IGF1	Insulin-like growth factor 1
IL1	Interleukin-1
IL-1α	Interleukin-1α
KS	Keratan sulphate
Lac	Lactate
MEM	Minimum Essential Medium
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
MSZ	Most superficial zone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normal
NHSBT	National Health Service Blood and Transplant
NSAID	Nonsteroidal anti-inflammatory drug
O	Over-age
OA	Osteoarthritis
OAT	Osteochondral autograft
OCA	Osteochondral allograft
OCD	Osteochondritis dissecans
ON	Osteonecrosis
PCL	Posterior cruciate ligament
PCM	Pericellular matrix
PRG4	Proteoglycan 4
PGE_2	Prostaglandin E_2
PVP-I	Poly(vinylpyrrolidone)-iodine
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SD	Standard deviation

STZ	Superficial tangential zone
TNF-α	Tumour necrosis factor α
UW	University of Wisconsin solution
WST	Water soluble tetrazolium

Chapter 1

General Introduction

1.1 - Articular cartilage structure and function within the knee joint

Human knee physiology

The human knee is a complex modified hinge joint (Fig 1.1.1), also known as a diarthrodial joint. This is a joint that consists of a layer of articular cartilage covering both ends of articulating bones (Shapiro *et al* 2012). Surrounding the joint is a fibrous joint capsule whose inner surface is lined with a synovial membrane, which secretes synovial fluid into the joint space (Shapiro *et al* 2012). A diarthrodial joint typically has greatest movement in flexion and extension about the sagittal plane (Abdulhasan and Grey 2017). The function of the knee is to maintain stability and control of the legs and therefore upper body during a number of different loading scenarios, such as during standing, walking or running. The two major bones of the knee are the distal femur and proximal tibia (part of the femur and tibia respectively) and the articulation between these supports most of the body weight (Abdulhasan and Grey 2017). The secondary articulation between the patella and femur gives rise to a frictionless transfer of forces over the knee that originate from contraction of the quadriceps muscle. The location of the knee joint between the two longest levers of the body (femur and tibia) and its roles in weight bearing makes it vulnerable to injuries (Abdulhasan and Grey 2017).

The knee is primarily stabilised by ligaments, with muscles around the knee providing secondary stabilisation. Both work synergistically to enable the knee to function. The joint space between the ends of the long bones is enclosed by a fibrous joint capsule, which contains synovial fluid. Ligaments connect bones to one another, provide joint support and comprise of bands of fibrous tissue. Reinforcement of the knee is provided by two collateral ligaments on the medial and lateral sides, along with two stronger cruciate ligaments (Fig. 1.1.1). The anterior cruciate ligament (ACL) is viewed as the main stabiliser in the knee joint, providing approximately 85% of the stabilisation and facilitating smooth, stable flexion as well as rotation of the joint (Ellison and Berg 1985). However, it is the posterior cruciate ligament (PCL) that is the strongest and comprises of the anterolateral bundle (ALB) and the posteromedial bundle (PMB) (Fig. 1.1.1) (Amis *et al* 2006). The primary function of the ACL is to resist anterior and rotational displacement of the tibia relative to the femur, whereas the PCL resists posterior displacement (Abdulhasan and Grey 2017). The medial collateral ligament provides stability to the medial side of the knee while the lateral collateral ligament (labelled as FCL in Fig. 1.1.1) stabilises the lateral side (Abdulhasan and Grey 2017).

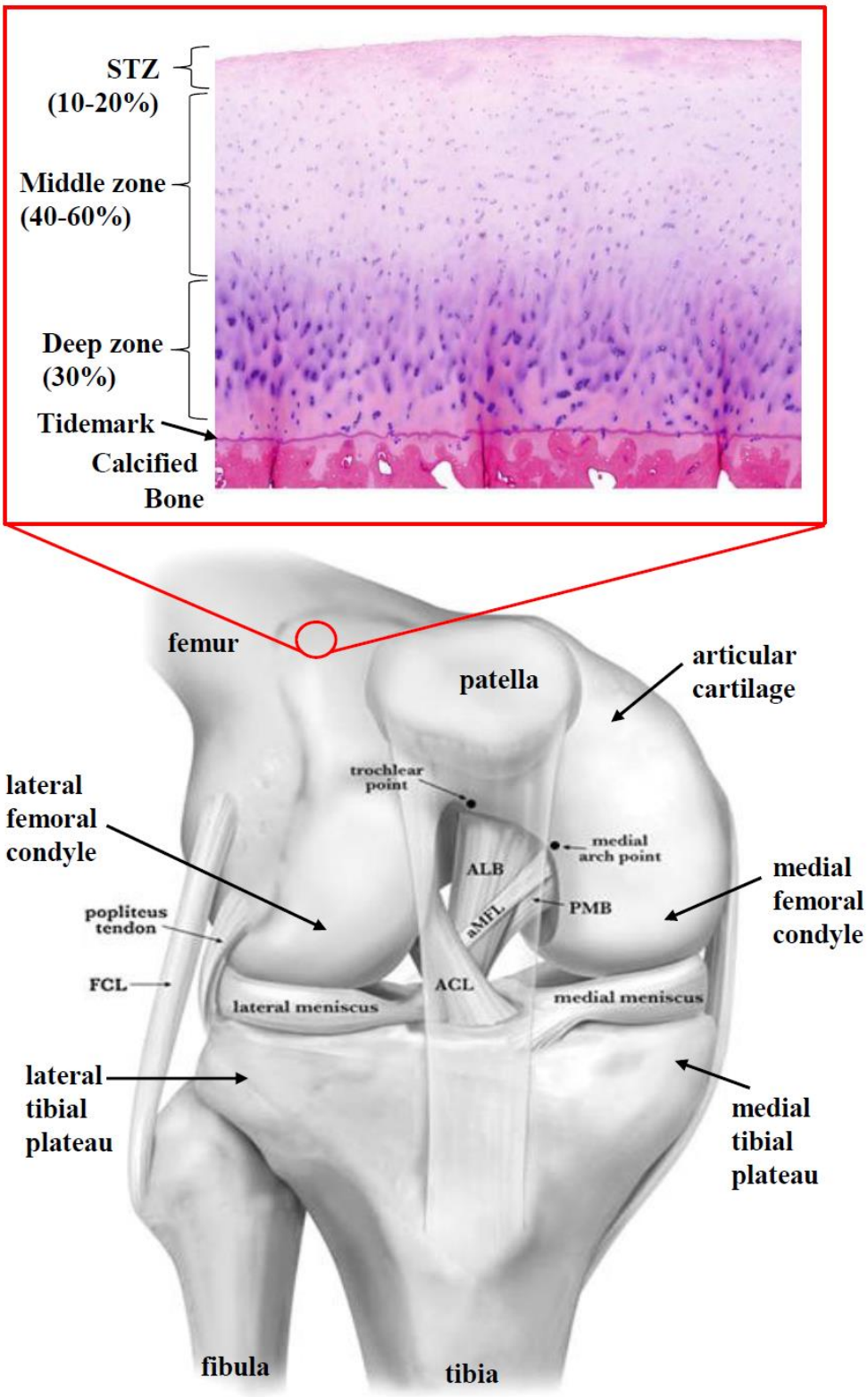

Figure 1.1.1. Anatomy of the right human knee showing major structures. Inset image of H&E stained section of articular cartilage. Knee diagram adapted from LaPrade *et al* (2015), inset image adapted from Shetty *et al* (2014). ACL = anterior cruciate ligament, ALB = anterolateral bundle, PMB = posteromedial bundle, aMFL = anterior meniscofemoral ligament, FCL = fibular collateral ligament, STZ = superficial tangential zone

Located on top of each tibial plateau is the meniscus which is composed of fibrocartilage. Structurally the meniscus comprises of two 'C' shaped pieces which originate and terminate in the bone between the two tibial condyles. The medial meniscus is positioned on the medial side of the knee, between the medial femoral condyle and medial tibial plateau, while the lateral meniscus is positioned on the lateral side between lateral femoral condyle and tibial plateau (Fig. 1.1.1). The function of the meniscus is to adapt to the movement between the articular cartilage of the distal femur and proximal tibia, but also provide a 'seat' on the tibial condyles in which the corresponding femoral condyles can locate (Abdulhasan and Grey 2017). It has been reported that the lateral menisci have a greater range of motion than the medial menisci, and as a result of this there are a higher rate of medial side meniscal injuries (Hirschmann and Muller 2015).

Cartilage found at the end of the long bones of the knee, and the posterior surface of the patella bone, is a connective tissue of which there are 3 types in the human body, elastic, hyaline and fibrous (fibrocartilage). Each type serves different functions within the body. Elastic is found in structures such as the nose and ear lobes, hyaline at the end of bones in the joints such as the knee which is the focus of this study, and the support rings of the upper airway, with fibrocartilage located in meniscus (previously mentioned) and intervertebral discs.

Chondrocytes

The function of articular cartilage tissue as a facilitator of joint articulation and dissipation of biomechanical loading is dependent upon the molecular architecture and structural integrity of the extracellular matrix (ECM) which is synthesised and assembled by one cell type, the chondrocyte (Williams *et al* 2004). Articular cartilage is an avascular, aneural, and alymphatic tissue comprising of both a fluid and solid phase. Chondrocyte cell death occurring from trauma or pathology results in the dysregulation of ECM homeostasis and the gradual breakdown of the matrix surrounding the cell, leading to exposure of subchondral bone. Further to this, chondrocytes surrounding the area of damage have a limited capacity to repair defects in the cartilage matrix due to their limited ability to replicate and inability to migrate within its collagenous structure (Fox *et al* 2009). Articulating joints exhibit intrinsically differential repair responses, for example the cartilage found in the ankle has a higher regenerative potential compared to that in the knee (Schreiner *et al* 2017).

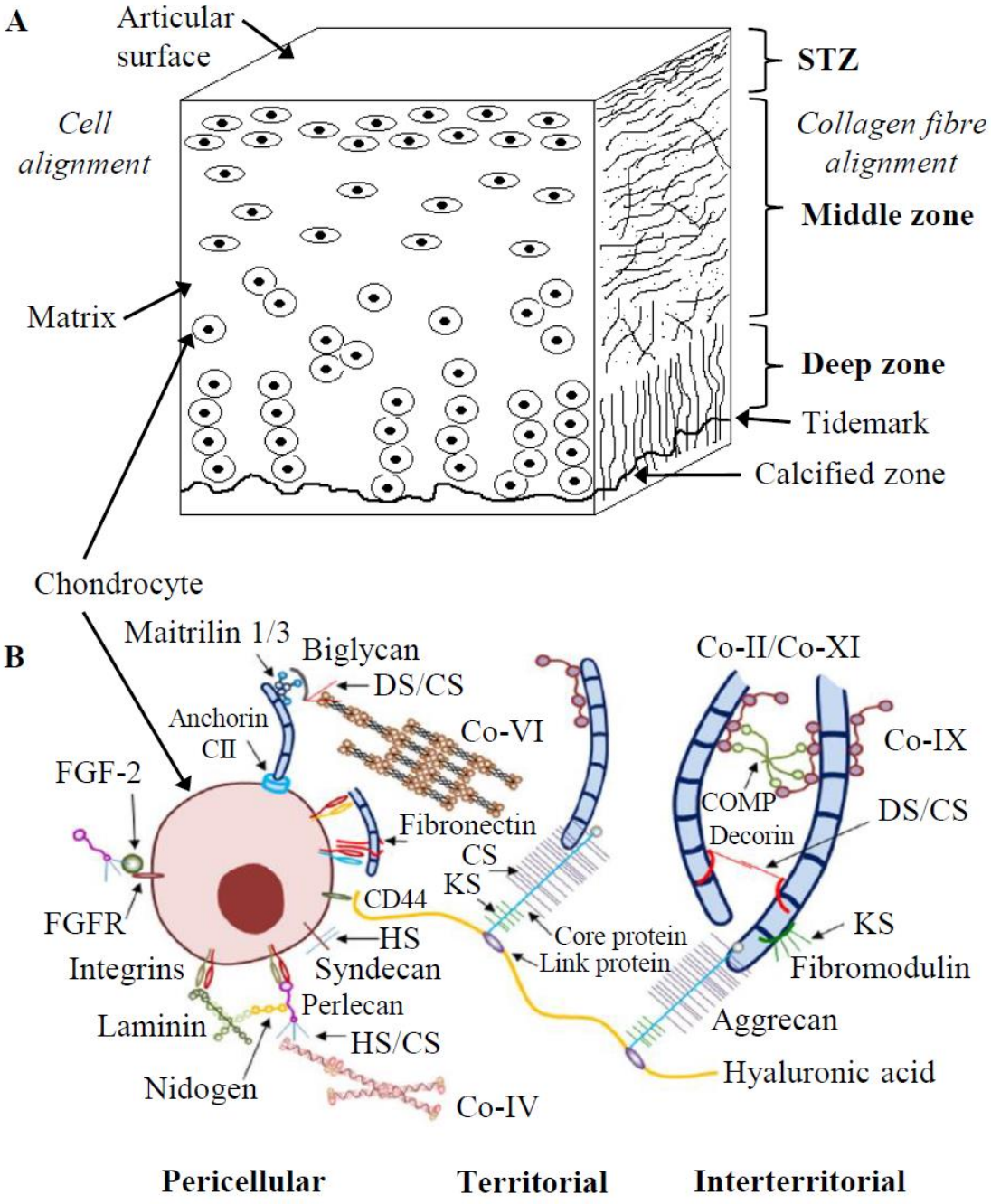

Figure 1.1.2. Cellular and molecular schematic diagrams of normal articular cartilage. A) Organisation of cells (front aspect) and collagen fibres (side aspect) within the cartilage matrix, showing different zonal organisation. Adapted from Fox *et al* (2009), STZ = superficial tangential zone. B) Molecular structures surrounding chondrocytes which are organised into different regions, adapted from Bottini *et al* (2016), CS = chondroitin sulphate, Co = collagen, COMP = cartilage oligomeric matrix protein, DS = dermatan sulphate, FGF-2 = fibroblast growth factor-2, FGFR = FGF receptor, HS = heparan sulphate, KS = keratan sulphate

Chondrocytes are organised in distinct zones within the cartilage matrix (Fig. 1.1.2A). The zone just below the articular surface is known as the superficial tangential zone (STZ), below this is the middle or transitional zone and then below this is the deep or radial zone (Fox *et al* 2009). The tidemark indicates the border between the deep zone and calcified cartilage. Within the matrix chondrocytes are located inside discrete structures called lacunae (Manafi *et al* 2017). Below the articular surface there is an acellular superficial zone, termed the most superficial zone (MSZ), which is further subdivided into 3 layers (Fujioka *et al* 2013). The first of these, which is in contact with the synovial fluid, comprises of lipids, proteins (such as lubricin), proteoglycans, and fibronectin. The second has undefined small fibrils; and the third layer is comprised of type I, II and III collagen fibrils (Fujioka *et al* 2013). The tangential zone itself contains densely packed collagen type II fibrils which are connected with proteoglycans (Fig. 1.1.2B). Chondrocytes found in this zone are fibroblast-like as they appear elongated and flattened. Both cells and fibrils are aligned parallel to the cartilage surface. In the middle zone collagen fibrils are more randomly organised (Fig. 1.1.2A) and the cells are more rounded. There is also the highest concentration of glycoproteins.

Fibrils in the deep zone are of larger diameter and become orientated at right angles to the cartilage surface, with cells organised into columns. The concentration of proteoglycan decreases as the matrix depth increases in the deep zone (Bottini *et al* 2016). The deep zone provides the greatest resistance to compressive forces, has the highest proteoglycan content and the lowest water concentration (Fox *et al* 2009, Yin *et al* 2013). At the base of the matrix is the calcified zone which contains inert chondrocytes that are trapped in the mineralised matrix (Bottini *et al* 2016).

The chondrocytes are themselves surrounded by three distinct regions within the matrix (Fig. 1.1.2B). A pericellular region is located proximally to the cell surface and facilitates the transmission of mechanical as well as biomechanical signals from the matrix to each chondrocyte (Bottini *et al* 2016). The territorial region supports the cells against mechanical load through its hydrodynamic properties, while the outer-most inter-territorial region contributes the greatest to mechanical properties of the cartilage matrix, and is the largest of the three regions (Fox *et al* 2009). This region has an abundance of proteoglycans and collagen fibrils that are organised differently depending on depth in the cartilage matrix (Fig. 1.1.2A).

Collagens

The most abundant macromolecule in cartilage matrix is collagen, the types of which are divided into fibrillar and non-fibrillar (Bottini *et al* 2016). Currently 28 types of collagen have

been identified in vertebrates, and in humans they contribute to a third of total protein content (Shoulders and Raines 2009). The structure of collagen consists of a triple helix of parallel polypeptide strands (Shoulders and Raines 2009). The types of collagen found in cartilage perform various functions and are summarised in Table 1.1.1.

Table 1.1.1. Collagens associated with cartilage.[a]

Type	Class	Composition	Function
I	Fibrillar	$\alpha1[I]_2\alpha2[I]$	Basic cartilage architecture
II	Fibrillar	$\alpha1[II]_3$	Basic cartilage architecture
III	Fibrillar	$\alpha1[III]_3$	Supports collagen II that has been weakened by damage
IV	Network	$\alpha1[IV]_2\alpha2[IV]$ $\alpha3[IV]\alpha4[IV]\alpha5[IV]$ $\alpha5[IV]_2\alpha6[IV]$	May maintain chondrocyte phenotype and viability
VI	Network	$\alpha1[VI]\alpha2[VI]\alpha3[VI]$ $\alpha1[VI]\alpha2[VI]\alpha4[VI]$	Anchors chondrocyte to PCM Mediate cell-matrix and intermolecular interaction Filter or transducer for signals from ECM
IX	FACIT	$\alpha1[IX]\alpha2[IX]\alpha3[IX]$	Formation of stable collagen network Maintenance of cartilage organisation and integrity
X	Network	$\alpha1[X]_3$	Modify cartilage matrix for calcification Maintain tissue stiffness
XI	Fibrillar	$\alpha1[XI]\alpha2[XI]\alpha3[XI]$	May regulate cartilage formation as it is the first cartilage collagen deposited by chondrogenic MSCs
XII	FACIT	$\alpha1[XII]_3$	Mediate interaction between fibrils and other ECM macromolecules/cells Protects tissue integrity
XIV	FACIT	$\alpha1[XIV]_3$	Fibrillogenesis Maintain integrity and mechanical properties
XVI	FACIT	$[\alpha1(XVI)]_3$	Organise ECM by stabilising collagen fibrils Mediate intracellular signalling (cell adhesion, proliferation, and invasiveness)
XXII	FACIT	$[\alpha1(XXII)]_3$	Contributes to stability of myotendinous junctions
XXVII	Fibrillar	$[\alpha1(XXVII)]_3$	Transform cartilage to bone Structural role in the PCM of the growth plate Organisation of the proliferation zone

[a]Table adapted from Luo *et al* (2017) and Shoulders and Raines (2009), ECM = extracellular matrix, FACIT = fibril-associated collagens with interrupted triple helices, PCM= pericellular matrix, MSC = mesenchymal stem cell

Approximately 90% of collagen in cartilage is collagen type II, which forms banded fibrils associated with collagens type IX and XI. Cross-linking of these collagens occurs with components of small leucine rich proteoglycans and cartilage oligomeric matrix protein (COMP). Collagen type II fibrils are of larger diameter and contain more banding the further their distal location is from chondrocytes (Bottini *et al* 2016) (Fig. 1.1.2B). Type III collagen has been reported as a suggested covalent modifier which may add cohesion to a collagen type II network that has been damaged by trauma, in a chondrocyte-led healing response (Wu *et al* 2010). The collagens from Type IV onwards have been termed minor collagens due to their less frequent occurrence in cartilage (Luo *et al* 2017). Type VI collagen is predominantly found in the pericellular region of the ECM and is arranged around one or more chondrocytes in microfibril form organised in elastic fibres. Type IV and X collagen which are non-fibrillar are found in lower amounts compared to type II collagen within articular cartilage. Type IV is predominantly located in the tangential zone while collagen X can be found in both the calcified zone and the non-calcified matrix immediately adjacent to it (Bottini *et al* 2016).
Even though the minor collagens are less abundant, it can be appreciated from Table 1.1.1 that they perform important roles within the cartilage, and are implicated in a number of joint diseases (Luo *et al* 2017).

Proteoglycan

Proteoglycans are a special type of glycoprotein containing at least one glycosaminoglycan (GAG) sidechain (Bottini *et al* 2016). Examples of GAGs such as chondroitin sulphate (CS) and hyaluronic acid (HA) are linear heteropolysaccharides that consist of recurring disaccharide units that are often formed by sulphated and carboxylated monosaccharides. This propensity for sulphuric and carboxylic groups results in proteoglycans having an overall negative charge. This property of proteoglycans is important for the hydration of cartilage, its stiffness and chondrocyte metabolism (Bottini *et al* 2016).
Aggrecan is the largest and most common “aggregating” proteoglycan in cartilage matrix and aggregates with HA chains by interaction with link proteins (Fig. 1.1.2B). The combined macromolecular network provides compressive stiffness, water permeability and osmotic pressure. Chondrocyte membrane proteoglycans include CD44 which binds HA, and parts of the syndecan family (Bottini *et al* 2016).

Glycoprotein

Chondrocyte membranes also have glycoproteins such as anchorin II and B1 integrins,

fibronectin and laminin attached to them (Fig. 1.1.2B). These proteins anchor chondrocytes to the pericellular matrix and allow the cells to detect mechanical and biomechanical dynamics within the matrix surrounding each cell. Superficial layers of articular cartilage produce lubricin also known as proteoglycan 4 (PRG4), which coats the cartilage surface (Szychlinska *et al* 2016), allowing the two opposing joint surfaces to freely move against each other by reducing friction. Lubricin has been found to co-localise with human cartilage glycoprotein chitinase 3-like-1 (CHI3L1) in articular cartilage (Szychlinska *et al* 2016). The over-expression of CHI3L1 (a mediator of inflammation) along with decrease in expression of lubricin is thought to be potentially associated with the development of osteoarthritis (Szychlinska *et al* 2016).

Water

In healthy cartilage matrix up to 80% of its wet weight comprises of water, with 30% of this linked to the intrafibrillar space located in the collagen network (Fox *et al* 2009). A small percent is located within the intracellular space. The remaining water content is located in the pore space of the matrix. Relative water concentration decreases from 80% at the superficial zone to 65% in the deep (Fox *et al* 2009). This concentration gradient along with compressive forces encourages water to dissipate through the cartilage tissue, and provides the means by which chondrocytes receive hydration and nutrition from the synovial fluid, as well as providing lubrication to the cartilage. Much of the water found in the interfibrillar space appears to be in the form of a gel, and it is mostly moved through the cartilage matrix by pressure applied across it or by compression of the solid matrix (Fox *et al* 2009). Resistance to water flow in the form of friction, and therefore pressurisation of the water forms the two hydrodynamic methods by which cartilage is able to withstand significant load, particularly in the human knee (Fox *et al* 2009).

Matrix turnover

The cartilage matrix restricts the movement of molecules through according to their size, charge, and structural configuration. It has been estimated that the average pore size within the ECM is ~6.0 nm (Fox *et al* 2009). As a result of this, the chondrocytes rely primarily on anaerobic metabolism. Chondrocytes use a group of degradative enzymes for maintenance of the ECM which also encompasses development and repair (Fox *et al* 2009). They synthesize macromolecules that are the building blocks of the matrix, such as the proteins and GAGs previously mentioned. The metabolic activity of chondrocytes can potentially be changed by

a number of chemical and mechanical cues in their surrounding environment. Proinflammatory cytokines for example interleukin-1 (IL1) and tumour necrosis factor-α (TNF-α) have catabolic and anabolic functions, which are involved in the degradation and synthesis of matrix macromolecules (Buckwalter and Mankin 1997). Chondrocytes synthesise, maintain and secrete proteoglycans into the ECM, and this process is thought to be regulated by growth factors and peptides such as insulin-like growth factors, transforming growth factor-β, IL1 and TNF-α. Little is known how these growth factors and peptides regulate proteoglycan synthesis.

The primary proteinases that are responsible for matrix turnover include metalloproteinases, including collagenase, gelatinase, stromelysin and cathepsins B and D (Fox *et al* 2009). Of significance in ECM turnover, and in particular degradation, are the aggrecanases. These are enzymes that degrade aggrecan (a major component of the ECM, as previously mentioned) by cleavage of the molecule between its two N-terminal globular domains (Nagase and Kashiwagi 2003). They are part of a group of enzymes called A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS) (Prasadam *et al* 2012). In this family ADAMTS4 and ADAMTS5 are expressed in articular cartilage and are implicated in the progression of OA (Prasadam *et al* 2012), along with matrix metalloproteinase-13 (MMP)-13, an enzyme that degrades collagen. This occurs due to overexpression of the enzymes by chondrocytes. It has also been suggested that crosstalk can occur between chondrocytes and the cells present in subchondral bone, contributing to abnormal levels of ADAMTS and MMP in OA cartilage (Prasadam *et al* 2012).

1.2 - Articular cartilage damage and disease

The drivers for the need to repair articular cartilage in a joint such as the knee come from physical trauma to the knee beyond the normal daily activities such as walking, or disease that affects the normal cartilage function and/or morphology.

Physical trauma

Sports injuries are a source of damage to otherwise normal articular cartilage, particularly high impact sports such as rugby. In the knee the meniscus acts as a load dissipator between the femoral condyle and tibial plateau (Fig. 1.1.1). Any damage to this as well as excessive force occurring between the two areas of cartilage can create lesions in the cartilage matrix. Because the chondrocytes are fixed in position they cannot migrate to the damaged area and repair the surrounding matrix: meaning that any endogenous repair which does take place is very slow

(Fox *et al* 2009). In the meantime, further damage can take place exacerbating the injury further. Once the cartilage has been worn away, damage can then extend to the subchondral bone, potentially weakening the remaining cartilage. With obesity, extra weight in the lower limb joints such as knee or hips can increase the load placed on the cartilage (Lima *et al* 2013). A study looking at the correlation between obesity and forces measured in the knee found a 24% increase in force measured between overweight (27 - 29.9 kg/m^2) and class 2+ obese (35 – 41.3 kg/m^2) participants (Messier *et al* 2014). As with sport injuries, failure of the meniscus can add to the burden taken by the articular cartilage (Abdulhasan and Grey 2017). The extent of the damage to cartilage can be classified into three types of mechanical injury (Buckwalter 1992):

1) Disruption or alteration of the macromolecular framework, loss of matrix macromolecules, or cell injury without visible tissue disruption.
2) Disruption of articular cartilage alone (chondral fractures)
3) Mechanical disruption of articular cartilage and subchondral bone (osteochondral fractures)

Normal physiologic levels of joint loading, for example during walking, can be between two to three times body weight. As a result, the net effect of each extra kilogram of body weight is multiplied by two to three (Lima *et al* 2013). Comparable to how bone and muscle require loading to maintain healthy structure, cartilage also requires periodic loading and movement, with a study suggesting that moderate exercise may be a good treatment for improving joint symptoms but also improve GAG content of articular cartilage (Roos and Dahlberg 2005). Loeser (2006) reported increasing evidence to suggest that the degradation of cartilage in osteoarthritis (OA) is as a result of inflammation at the molecular level in the matrix. It is thought that abnormal mechanical force can stimulate chondrocytes from normal low to higher metabolic activity, and as a result cause them to secrete inflammatory mediators, many of which are usually produced by macrophages in response to injury or infection (Loeser 2006). As a result of their release, these mediators instigate catabolic activity in the chondrocyte, causing it to release proteolytic enzymes which go on to degrade the cartilage matrix. It is thought that cartilage destruction in OA, instigated by an insult to the articular cartilage, may be partly due to chondrocytes over-compensating for the damage and removing material from the matrix quicker than it can be replaced (Loeser 2006). Normally cell signalling and feedback systems will keep this process of matrix remodelling, which is a constant feature of matrix turnover in check, as it is in bone, but it appears that in an OA joint there is a breakdown in this regulation resulting in an imbalance between anabolic and catabolic synergy. It has been

suggested that ageing plays an important role in the progression from damage to OA (Loeser 2006).

Osteoarthritis

OA is a type of joint disease, which results in articular cartilage degeneration, inflammation of the synovium and changes to bone to which the cartilage is attached. It is thought that OA is a disease of the entire joint and the tissues within it (Yuan *et al* 2014). As the disease progresses key features can be observed in the joint as shown in Fig. 1.2.1.

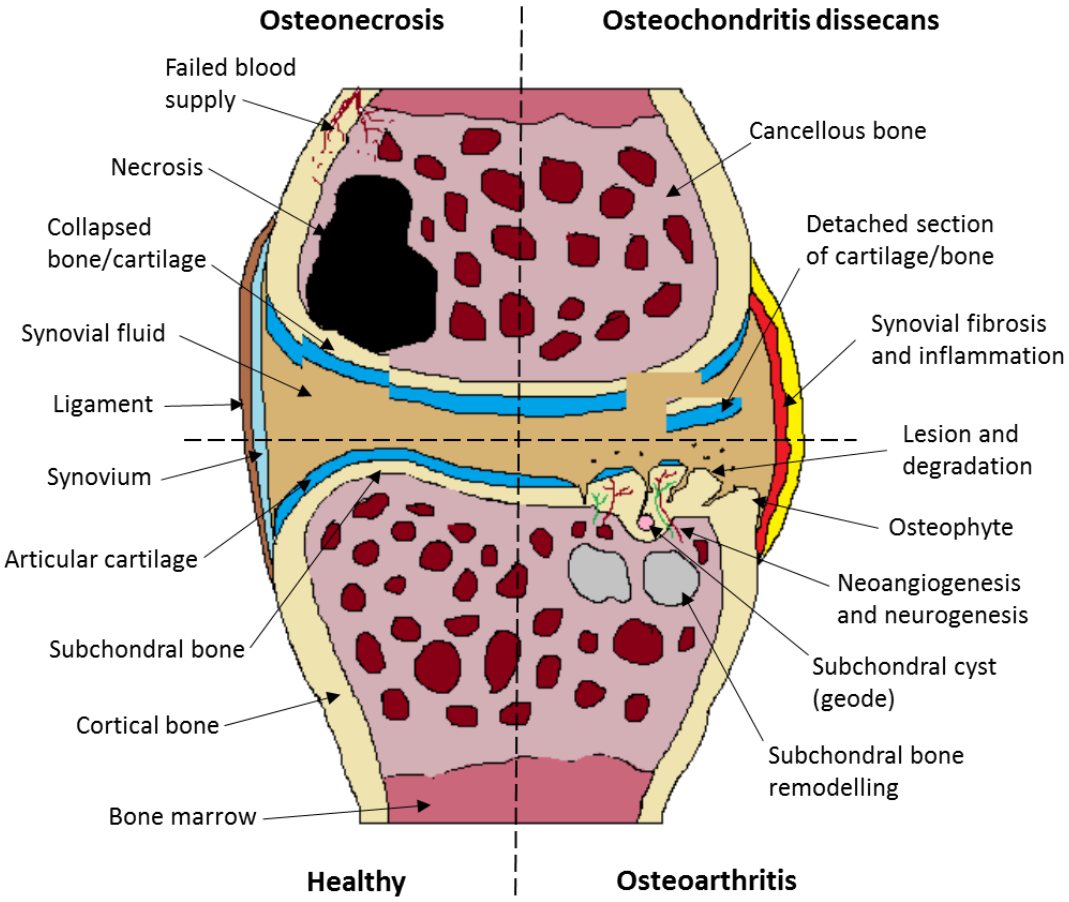

Figure 1.2.1. Diagram of a typical diarthrodial joint in saggital plane cross-section, demonstrating joint diseases that could be treated with an osteochondral allograft, alongside representation of healthy tissue. Though not shown, physical trauma can lead to damaged cartilage and/or inflammation which can then lead to osteoarthritis, or bone tumours that require removal along with a large portion of the articular cartilage surface. Adapted from Yuan *et al* (2014)

The changes observed in subchondral bone as a result of cartilage lesions, allows so called

crosstalk between the previously impenetrable subchondral bone and the cancellous bone beneath (Yuan *et al* 2014). Microcracks and fissures along with increased blood vessel formation could act as a means for molecular transport (Yuan *et al* 2014). Pressurised synovial fluid can also invade these new channels and enter the cancellous bone, forcing it to be remodelled by osteocytes in response to dynamic forces acting on it. This can thicken the cancellous and subchondral bone as well as cause the formation of fluid and or soft tissue filled cysts (Yuan *et al* 2014). Increased bone remodelling and stimulation of neoangiogenesis are believed to be crucial for development from early to late stage OA (Yuan *et al* 2014). Osteophyte formation (overgrowth of bone) is particularly prevalent in progression of the disease and is one of its visual hallmarks. These osteophytes in particular can inhibit free movement of the joint and as a result initiate pain within it.

Chondrocytes within diseased cartilage increase the synthesis of matrix molecules but also synthesise cytokines such as IL1 and enzymes such as MMP. There is chondrocyte proliferation due to vascular endothelial growth factor, runt-related transcription factor 2 and MMP-13 induced differentiation (Yuan *et al* 2014). The increased activity in the chondrocytes results in a rough surface to the articular cartilage, along with fibrillation and reduced thickness, often seen in histological sections of affected tissue (Fig. 1.2.2).

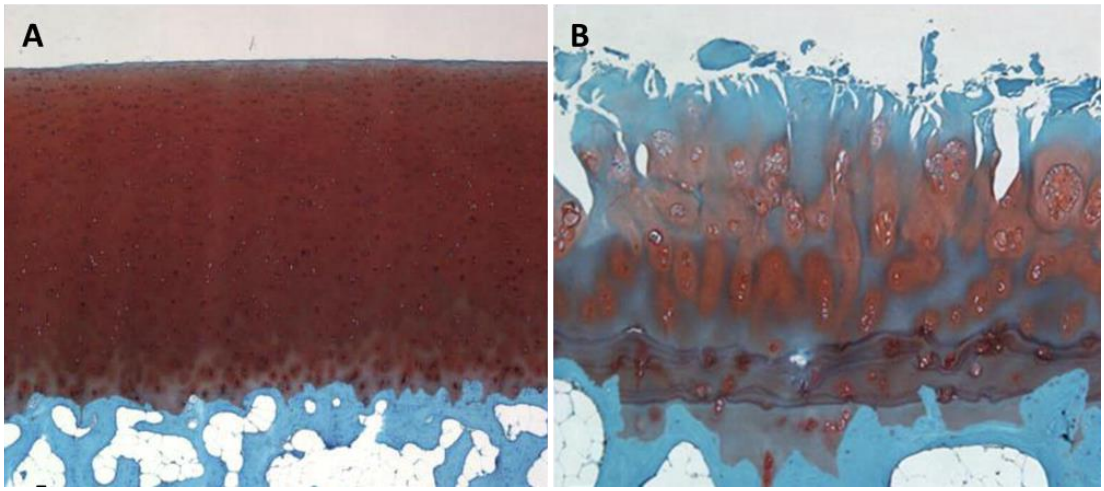

Figure 1.2.2. Safranin O / fast green histological sections of articular cartilage. A) Healthy cartilage, Mankin score 1. B) Osteoarthritic cartilage showing clefts to the deep zone, Mankin score 4. Images captured with x4 objective, and adapted from Pauli *et al* (2012)

Osteochondritis dissecans

Osteochondritis dissecans (OCD) is a condition which has been reported clinically for

centuries (Tarabella *et al* 2016). The term was introduced by Konig in 1887 and describes a process by which subchondral bone delaminates with or without involvement of articular cartilage. The condition mostly affects young patients (10-20 years of age) with reports of its occurrence increasing (Pareek *et al* 2017). OCD lesions have a scale of severity from asymptomatic to mild pain to then advanced stages of joint instability and locking (Wood and Carter 2018). The lesions themselves can develop from stable to fragmentation of cartilage above the lesion with formation of a loose body within the joint space (Fig. 1.2.1). Progression of the disease can also lead to OA. OCD can be divided into two cohorts, juvenile OCD or adult OCD. A cause for the condition has so far not been elucidated, though a number of theories have been postulated.

According to Andriolo *et al* (2018) the etiology of OCD can be attributed to either biological or mechanical factors. The first of the biological factors are genetic, with a number of studies finding OCD occurring within families, with one group even identifying a possible gene candidate (ACAN) which codes for the proteoglycan aggrecan (Stattin *et al* 2010). The second is ossification deficit, where defects in the ossification centres of the distal femur form during early growth (Ellerman *et al* 2017). Another hypothesis involves the endocrine system where hormonal changes could induce bone remodelling and then cause OCD lesions (Hussain *et al* 2011).

For the mechanical factors, injury and overuse are postulated with a suggestion that repetitive microtrauma or chronic loading might play a role in OCD progression (Andriolo *et al* 2018). The next hypothesis is tibial spine impingement where abnormal shear forces during movement of the tibia relative to the femur occur as a result of the impingement (Cavaignac *et al* 2017). However, the theory is specific to a certain area of the knee joint. Another theory is linked to discoid meniscus, where predominantly the lateral meniscus becomes thickened and shaped like a half-moon as compared to the normal crescent shape (Deie *et al* 2006). The abnormal shape leads to a change in forces acting on the cartilage in the immediate vicinity of the meniscus. Finally, biomechanical alteration could have chronic effect on load distribution over the cartilage surface, with subsequent influence on joint homeostasis (Grimm *et al* 2013). Conditions that can instigate this include meniscectomy, joint instability and condylar flattening (Crawford and Safran 2006).

Other pathogenic mechanisms for causing OCD have been suggested. The first of these is ischaemia, with the initial change being necrosis in the subchondral area, ultimately leading to fracture (Green and Banks 1953). One suggestion for this being reduced blood supply during developmental growth phases. The second is fractures either through trauma alone or underlying issues with subchondral bone with macrotrauma causing the fracture (Shea *et al*

2013).

Osteonecrosis

According to a paper by Cusano *et al* (2018), osteonecrosis (ON) is caused by a decline in the microperfusion of bone, ultimately resulting in joint collapse (Fig. 1.2.1). ON occurs most commonly after fractures or other non-traumatic means, such as prolonged glucocorticoid use (Early *et al* 2018), alcohol consumption, pancreatitis, antileukaemia treatment (Kuhlen *et al* 2017), or lipid disorders. The last of these conditions is thought to lead to accumulation of fat emboli in small blood vessels or bone marrow, inhibiting blood flow, resulting in cell death within surrounding tissue. ON more often affects the hip (femoral head) but can also present in the knee joint. Other joints that can be affected include the shoulder, ankle, jaw and spine (Cusano *et al* 2018). As with most lesions of the knee the last surgical option is total joint arthroplasty, however in the case reported by Cusano *et al* 2018, young patients are generally contraindicated from having this type of surgery. Therefore, a fresh hemi-condyle allograft transplant was used, though according to the authors this procedure is rare and to their knowledge this was the only one carried out with the size of allograft used.

1.3 - Clinical assessment

The first step in clinical assessment is examination of the patient medical history along with a physical examination (Berta *et al* 2015). During the patient interview it should be determined when any injury occurred and what symptoms are present. Previous treatment including any history of knee surgery should be investigated as some can affect outcome of resurfacing (Minas *et al* 2009). During physical examination, knee swelling may be evident with a chondral lesion, and localisation may be determined via tenderness around the joint line. Also, if the lesion is deep, or scar tissue has formed then a grinding sensation of crepitus, or catching in the joint can be felt (Moyad 2011). Patella or trochlea lesions often present symptoms as a results of shear stress, along with signs of apprehension or pain in the patient. This can be uncovered with a patella grind test and knee flexion (Moyad 2011). Medical history will often reveal the presence of a limp after certain activities (high-impact) or prolonged weightbearing, that may not be obvious on examination. Next a set of radiographs should be obtained, both locally of the affected knee, as well as full length from hip to ankle

There are a number of other methods available for clinicians to assess damage of cartilage such as Magnetic Resonance Imaging (MRI) or computed tomography (CT) scans. CT is used in some centres to determine integrity of the cartilage, and enable diagnosis of patellar femoral

maltracking (Moyad 2011). There is a risk however of exposure to ionising radiation, so the benefit of CT scanning should be evaluated for each patient. MRI is frequently used to validate the presence of chondral defects, but also to investigate related pathology such as meniscal and cruciate ligament tears. Even with advanced technology, the most reliable is seen as arthroscopic assessment by a clinician which provides the most accurate diagnosis (Berta *et al* 2015). This is however associated with some risk, due to it being a surgical procedure. Ultimately it is when there is loss of tissue or a change in structure that prevents the free movement of the joint that surgical intervention becomes necessary, however not all patients become candidates for cartilage resurfacing surgery (Moyad 2011).

A number of factors have to be assessed before surgery can take place (Moyad 2011):

- Limb alignment
- Pre-existing concomitant injury or knee arthritis
- Patient expectation and rehabilitation potential
- Instability and ligament damage
- Previous total or subtotal meniscectomy
- Age of the patient
- Size, number and location of defect(s)
- BMI

In some cases, non-operative treatments may be better for a patient (Table 1.4.1). Also, the ability of a patient to comply with post-operative rehabilitation guidelines needs to be considered (Berta *et al* 2015), with most resurfacing techniques requiring 6 to 12 weeks of protected weight bearing. Some require extended rehabilitation and restriction beyond 1 year after surgery (Moyad 2011).

1.4 - Surgical intervention

There are a number of techniques available to orthopaedic surgeons for repair of cartilage defects, not all of which require the use of donor tissue. Table 1.4.1 adapted from Moyad (2011) summarises all the techniques available as well as the lesion size suitable for treatment. Small defects <2 cm^2 can be repaired with marrow stimulation techniques, the two most common are microfracture and subchondral drilling. Microfracture has a theoretical advantage as there is no issue with generation of heat from the drilling that occurs in the other procedure which can potentially lead to bone necrosis (Chen *et al* 2008).

Table 1.4.1. Cartilage repair (●), restoration (●), and non-operative (●) treatments.[a]

Cartilage lesion treatment			
Non-operative	**Lesion size <2 cm²**	**Lesion size 2-6 cm²**	**Lesion size >6 cm²**
Activity modification Weight reduction Physiotherapy Bracing NSAIDs Hyaluronic acids	Microfracture Subchondral drilling Chondroplasty	Microfracture Subchondral drilling Chondroplasty	Durability issues?
	OAT	OAT? (morbidity) ACI OCA	ACI OCA

[a]Adapted from Moyad (2011). NSAIDs = nonsteroidal anti-inflammatory drugs, OAT = osteochondral autograft, ACI = autologous chondrocyte implantation, OCA = osteochondral allograft

Prior to both procedures, it is important that devitalised and/or damaged cartilage has been removed, resulting in a clean bed directly above an intact subchondral plate. Multipotent progenitor cells can then adhere and generate fibrocartilage. The aim is to have a stable vertical wall of cartilage surrounding the defect (Mirza *et al* 2015). Small holes are then created through the subchondral plate to allow bone marrow to coat the exposed bone. The advantage of both techniques is their simplicity and low cost, as a result they are often used as a primary procedure for cartilage defects. However, the lower durability of resultant fibrocartilage compared to the physiological hyaline cartilage, coupled with the defect not being completely filled presents major disadvantages (Mirza *et al* 2015). With the use of chondroplasty for small defects (<2 cm^2), degenerative cartilage is simply excised from the articular surface as well as removing unstable flaps of cartilage tissue. It is carried out via arthroscopy and is reported to be the most common arthroscopic procedure in the knee (Ward and Lubowitz 2013). The main disadvantage is that any indentations in the cartilage surface remain untreated.

Osteochondral autograft (OAT) is also used for small lesions (<2 cm^2) in a single stage procedure using an OAT harvester (Moyad 2011). The cartilage lesion is prepared and then the diameter accurately measured. Dowels (plugs) of autograft cartilage, with 10-15 mm of underlying bone are harvested from limited weight bearing areas within the knee (Bobic 1996). These osteochondral plugs are normally harvested from the medial or lateral trochlea ridge, or sometimes from the intercondylar notch. Once the grafts have been harvested they are then impacted into the prepared defect with minimal force, the key is to tightly pack the plugs into the defect to give secure fixation and reproduce the normal radius of curvature of the cartilage surface (Huang *et al* 2004). This can be the most challenging aspect of the operation. The other disadvantage is donor site morbidity of the harvested cartilage and bone plugs.

For larger sizes of cartilage lesion (2-6 cm^2) the same procedures that are used for small defects can also be used, however with OAT the limited scope for harvesting enough autograft material can contraindicate this (Moyad 2011). In addition, surgeons can use autologous chondrocyte implantation (ACI), which is carried out using a two-stage procedure. First a diagnostic arthroscopy is carried out to retrieve a full-thickness biopsy of cartilage. Biopsy sites utilised are similar to the ones used for the OAT procedure (Niethammer *et al* 2014). Chondrocytes are then extracted from the cartilage biopsies and expanded in cell culture. In approximately 4 to 6 weeks the cells are ready for re-implantation. During the second stage an arthrotomy is carried out and the cartilage defect debrided, resulting in a stable rim of cartilage around the defect. A periosteal patch or newer collagen membrane substitutes can be sutured over the defect area to cover the cells. The integrity of the patch is tested before the cells are injected into the defect area, fibrin glue is used between the border of the patch and healthy cartilage (Moyad 2011). Technically difficult aspects of the procedure include sizing and creation of the patch, and obtaining complete cessation of bleeding prior to injection of the cells. It can take 1 to 2 years for chondrocyte maturation to fully occur (Niethammer *et al* 2014).

The other possible surgical treatment for defects between 2-6 cm^2 is the use of stored allograft tissue, retrieved from suitable consented donors. They continue to be frequently used to repair lesions or where procedures such as debridement, microfracture, or subchondral drilling (marrow stimulation) have failed, or where lesions affect a larger area (>2 cm^2) of the cartilage surface. For lesions larger than 6 cm^2 both ACI and OCA are predominantly used (Moyad 2011). A consensus paper on the management of symptomatic cartilage lesions (Biant *et al* 2015) outlined the surgical techniques which are available to UK clinicians. It was felt that there are four groups available: bone marrow stimulation, osteochondral grafting, osteochondral scaffolds, and cell therapy (autologous chondrocyte implantation (ACI)). The paper also outlined seven consensus statements regarding the suitability of patients and lesion size for each of the treatments. One of the main limiting factors however was cost of treatments and availability of them, particularly for allograft tissue in the UK. Overall it was felt these techniques should only be used to treat symptomatic lesions.

Cartilage trauma can manifest as partial lesions affecting only the hyaline/articular cartilage tissue or full thickness which penetrates down to the cortical and/or subchondral bone underneath. Whole pieces of the cartilage and underlying bone can become detached and end up in the joint space as a loose body which can cause further damage to surrounding tissues as well as impeding articulation of the joint (Wood and Carter 2018). Chronic pathology can also cause overgrowth or inflamed cartilage providing a focal point for the forces acting on the

joint. This can either be as a result of physical trauma or genetic pre-disposition to diseases such as OA or rheumatoid arthritis (RA).

A study by Torrie *et al* (2015) stated that surgical use of the dowel technique has increased (time-frame not stated) with common defects affecting central weight bearing areas of the femoral condyle. Shell grafts are occasionally needed in areas such as the posterior femoral condyles, tibia or edges of the condyles, these are where a section of tissue with large contour changes are required. A matching shape to the area of tissue removed from the patient is created in the donor tissue, and fixed in place with screws and pins (Demange and Gomoll 2012). Contour matching of the OCA to the patient's own cartilage surface and careful manipulation contribute to the success of an operation (Torrie *et al* 2015), as well as precise fitment (Bugbee *et al* 2016). A study carried out by McCormick *et al* (2014) summarised knee cartilage specific procedures recorded on a private-payer database through a period from 2004 to 2011 in the United States. A summary graph of data pertaining to numbers of restorative procedures using autograft cells or autograft/allograft tissues from that study is shown in Fig. 1.4.1. The authors noted that far greater palliative procedures (chondroplasty) were taking place, closely followed by marrow-stimulation procedures compared to restorative ones. The authors deduced that this was likely due to cost of each graft, but also perhaps clinicians being more comfortable with well-established techniques. Ironically the palliative approach has been shown by microfracture, abrasion chondroplasty, or subchondral drilling techniques to result in formation of fibrocartilage, a less biomechanically resilient form of cartilage and a short-term fix that will likely lead to further operations. Due to the findings it was suggested that there is a need for education and policy that includes repair and restorative procedures in cartilage lesion management processes. Limitations of the study suggested by the authors included lack of information in the database for lesion size or grade, and demographic or epidemiological factors. These could have had a great influence over the type of procedure chosen.

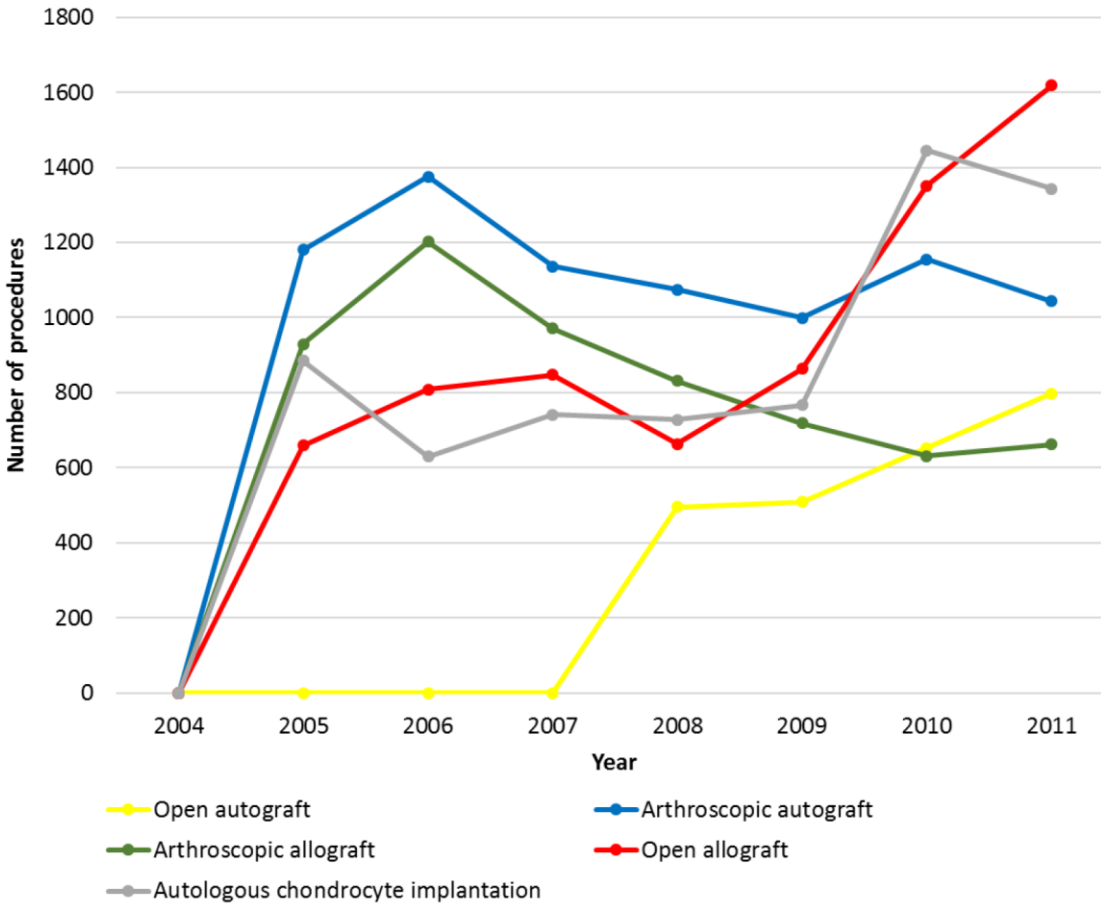

Figure 1.4.1. Restorative articular cartilage procedures of the knee carried out in the United States between 2004 and 2011 according to the PearlDiver database, data obtained from McCormick *et al* (2014). For comparison, an average 186588.8 chondroplasty's (palliative) and 76080.3 microfracture/drilling (repair) procedures were carried out over the same period

1.5 - Osteochondral allografts

The use of osteochondral allograft (OCA) as a treatment for defects to joint surfaces emerged in the 1990's (Okeagu *et al* 2017), and a study published by McCormick *et al* 2014 reporting on a private-payer medical record database (PearlDiver) in the United States, showed increasing use of cartilage allograft tissue (Fig. 1.4.1). From no use in 2004 to 1590 out of 257114 total cartilage operations in 2005, procedures increased to 2281 out of 281273 in 2011, an increase of 0.2%. It was concluded that the number of procedures of the knee involving the cartilage surface increased annually by 5%. Storage and surgical use of the stored tissue form part of a therapeutic pipeline, which starts with the tissue donor progressing through to post-op follow-up, as shown in Fig. 1.5.1. Part of this includes a preliminary International Cartilage Repair Society (ICRS) Cartilage Injury Evaluation Package (Brittburg *et al* 2000), for the

patient and surgeon to assess general health, pain, mental wellbeing, joint function, previous surgery, grading and examination criteria of the osteochondral damage, prior to deciding on what course of surgery to undertake.

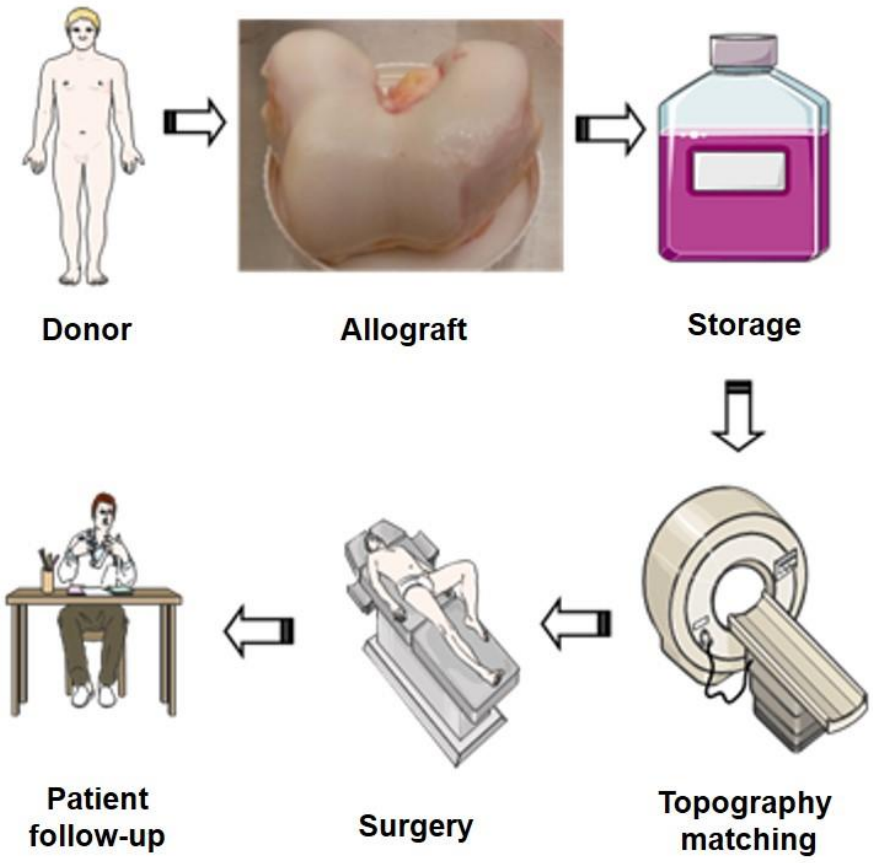

Figure 1.5.1. Therapeutic pipeline for stored osteochondral allograft repair of articular cartilage defects. Magnetic Resonance Imaging (MRI) can be used to match the topography of the donor and recipient cartilage, amongst other techniques

It is well known that viable chondrocytes are required for articular cartilage to function as it should, and that is also true for allograft cartilage. According to a paper by Cook *et al* (2016), 70% viability of chondrocytes has been proposed as the minimum required to ensure success of the allograft transplant. Most of the work published in journals relates to 4°C storage of cartilage allografts (Table 1.5.1). This is probably due to it currently being the preferred method of storage in tissue banks, which supply articular cartilage to clinicians. However, the data generally supports storage for up to 28 days only (Williams *et al* 2005, Hunter *et al* 2006, Ball *et al* 2002, Linn *et al* 2011). As the viability at cold storage tends to decrease after 28 days, this is the timescale invariably chosen for maximum storage, and to provide as large a

window of opportunity as possible for OCA suitability to be determined. It is known that prolonged storage at 4°C gives rise to hypothermic ischaemic cell damage (Belzer and Southard 1988, McAnulty 2010). This is either due to effects of reduced cell metabolism (Ishikawa *et al* 2015, Katayama *et al* 2014), impaired transport of intracellular calcium (Katayama *et al* 2014, Guthrie *et al* 2011, Boutilier 2001) or inhibition of adenosine triphosphate (ATP) driven sodium/potassium cell membrane pumps, which upsets the balance between sodium influx and potassium efflux (Boutilier 2001), in favour of sodium influx. The resultant accumulation of intracellular sodium is further exacerbated by hypothermic induced activation of the sodium/hydrogen exchanger. This net increase in cytoplasmic sodium content leads to cell swelling due to osmosis, and ultimately necrosis. This is why chondrocyte viability appears to have a 28-day upper limit at hypothermic storage. There are exceptions to this in the literature, with some reporting worse results of 52% viability after 3 weeks (Teng *et al* 2008) or ~40% after 2 weeks (Yamada *et al* 2015), and <30% after 3 weeks (Brockbank *et al* 2012). Some report better figures of 64% after 45 days (Williams *et al* 2004) and ~67% after 34 days (Pearsall *et al* 2004). More recently a study showed that 4°C storage yielded ~79% viability after 35 days (Qi *et al* 2016). This variability might suggest the limitation of storage at 4°C, and/or the different methods used to detect viability, however this appears to be the current preferred method of storage by the majority of tissue banks, who converted to the use of culture medium (Capeci *et al* 2013). There are however some papers reporting results of studies using much higher temperatures such as 37°C (Pallente *et al* 2009, Garrity *et al* 2012) or 25°C (Cook *et al* 2014, 2016). The highlighted studies have produced good data with high residual viability, in the case of Pallente's work ~80% after 28 days storage and 70% after 56 days in Garrity's results. In the 2014 Cook paper 90% viability was observed after 60 days storage. The Cook studies in particular show data that favours the use of temperatures higher than 4°C.

Table 1.5.1. Summary of published studies in storage of articular cartilage.[a]

Year published	Journal	Storage temp(s)	Storage period	Storage medium	Species	Viability assay
2002 Csonge L	Cell Tissue Bank	4°C, < -100°C	60 days	X-VIVO™ 10	Human	Live/dead, MTT
2003 Williams S	J Bone Joint Surg	4°C	28 days	MEM	Human	Live/dead
2004 Ball S	Clin Orthop Relat Res	4°C	28 days	Lactated Ringers, DMEM + IGF1	Human	Live/dead
2004 Pearsall A	Am J Sports Med	4°C	17-44 days	Bespoke, Incell	Human	Flow cytometry
2004 Williams R	Am J Sports Med	4°C	60 days	DMEM	Ovine	Live/dead
2005 Allen G	Am J Sports Med	4°C	20 days	MEM	Human	Live/dead
2005 Drobnic M	Folia Biol	4, 35°C	9 days	Saline	Human	Live/dead
2005 Williams J	J Ortho Res	4°C	28 days	MEM	Canine	Live/dead
2006 Pennock A	J Knee Surg	4°C	28 days	MEM	Human	Live/dead
2007 McCulloch	Am. J Sports Med	4°C	15-43 days	Ringer's lactate	Human	NA, follow-up study
2008 Ranawat	Clinic Ortho Rel Res	4°C	42 days	Low glucose DMEM	Ovine	Live/dead
2008 Teng BS	Clinic Ortho Rel Res	4°C	3 weeks	Lactated Ringers, DMEM + IGF1	Bovine	Live/dead
2009 LaPrade R	J Bone Joint Surg	4°C	15-25 days	Culture medium serum free	Human	NA, follow-up study
2009 Pallente A	Am. J Sports Med	4, 37°C	28 days	MEM	Hircine	Live/dead
2011 Brockbank	Transfusion Med & Hemo	4°C	28 days	DMEM	Porcine	Resazurin, chondrocyte isolate
2011 Linn MS	Am J Sports Med	4°C	28 days	DMEM + etancercept	Hircine	Live/dead

2012 Garrity JT	Am. J Sports Med	4, 37ºC	56 days	DMEM	Canine	Live/dead
2012 Onuma K	Clinic Ortho Rel Res	4ºC	21 days	UW	Murine	WST
2014 Cook JL	Clinic Ortho Rel Res	4, 25ºC	28 & 60 days	Missouri Storage medium	Canine	Live/dead, resazurin
2014 Wright J	Cells Tissues Organs	4ºC	28 days	DMEM, Unisol, SPS1	Porcine	Resazurin
2015 Mickevicius	BMC Musculoskel Disord	4, 37, -70ºC	28 days	Low glucose DMEM	Hircine	Live/dead
2015 Yamada T	Scientific World J	4ºC	14 days	UW + HA	Murine	WST
2016 Qi J	Cell Tissue Bank	4, 37ºC	7, 21, 35 days	MEM	Hircine	Live/dead
2018 Cinats D	Cartilage	4ºC	7-21 days	X-VIVO™ 10	Human	Live/dead
2019 Han Y	Cell Tissue Bank	4ºC	28 days	DMEM + hydrogen	Porcine	Live/dead

[a]HA = hyaluronic acid, IGF1 = insulin-like growth factor 1, UW = University of Wisconsin solution, DMEM = Dulbecco's Modified Eagle's Medium, MEM = minimum essential medium, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, WST = water soluble tetrazolium

A summary of viability data from published work is shown in figure 1.5.2**.** This shows that only a small number of the papers published achieve the 70% viability threshold, with tissue culture medium providing the superior support for cell survival. There are however studies which contradict the higher storage temperatures such as the work reported by Drobnic *et al* (2005), where it was found that storage at 35ºC resulted in a lower viability (mean of 5% at 9 days) than tissue stored at 4ºC (mean of 55% at 9 days). The tissue used was of human origin, however the donor was 60 years old (much higher than the upper age limit of 45 as recommended by American tissue banks, and the normal donor cohort used in this study) and only one donor was sampled. Of particular note was that the group was using saline as the storage solution, which might be appropriate for hypothermic storage but does not supply the nutrients required for the chondrocytes to function at their near physiologic metabolic rate. The tissue itself was also 6 mm diameter osteochondral dowels isolated from femoral condyles.

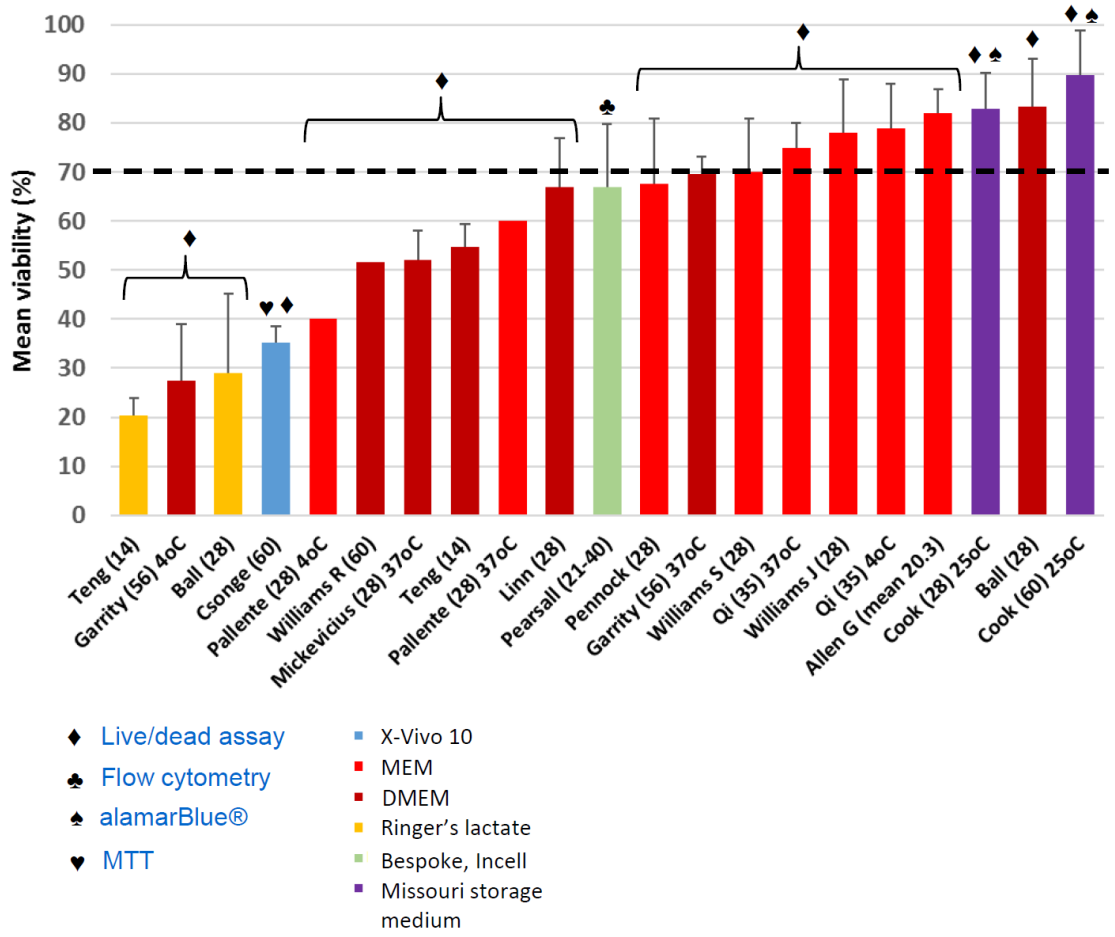

Figure 1.5.2. Summary of viability data from referenced papers shown in table 1.4.1, some publications omitted due to exact figures not stated, numbers in parentheses on x-axis labels show number of days tissue stored for. All values are means +/- standard deviation, except for Williams S and Garrity which shows standard error of the mean. Where error bars are not shown, exact data could not be determined. DMEM = Dulbecco's Modified Eagle's Medium, MEM = minimum essential medium, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Another paper by Mikevicius *et al* (2015) reported improved data at 4°C compared to 37°C, however the viability was only significantly different on the 14 day sampling point ($p = 0.003$), with no significant difference observed after 28 days storage ($p > 0.05$). For this study animal tissue was used as isolated biopsies of 5 mm diameter. While it appears that storage at higher temperatures is of benefit, the main disadvantage is the increased potential for the proliferation of microbial contaminants, which if present at 4°C are kept in a state of quiescence. All of the studies either take biopsies of the tissue, or store small diameter (<6 mm) dowels of OCA tissue, however it has been shown that cutting into the cartilage surface like this will influence cell viability (Tew *et al* 2000), with trephine cuts causing increased cell death compared to

those made with a scalpel (Archer *et al* 2006). Most of the studies shown in table 1.5.1 were carried out with animal tissues (64%) rather than human (36%) (excluding follow-up studies). A lot of scientific research is done using human tissue, but when trying to extrapolate the results to determine how OCAs might behave when transplanted between humans, it can be risky when the tissue involved is from joints. These will always be retrieved under optimal conditions from healthy young animals free of disease. Of course, being quadrupeds the knee joints will be subjected to different force distribution as would be experienced in bipeds. This is why it is important to carry out analysis on stored human cartilage to determine whether this data can be replicated, or indeed extrapolated to a different species. There are a small number of papers that compare cartilage thickness between species, one large study was carried out to investigate differences between the femoral condyles of a large cohort of species (Malda *et al* 2013), a summary of the measured thickness from those species is shown in Fig. 1.5.3.

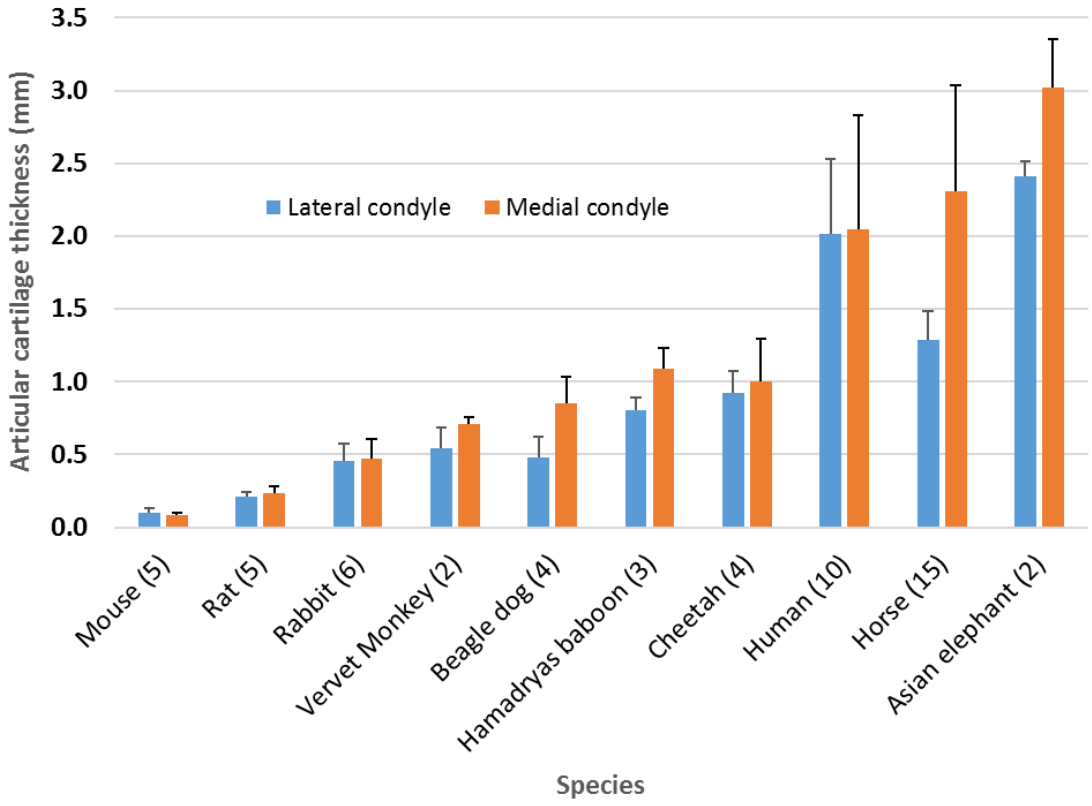

Figure 1.5.3. Species variation of mean cartilage thickness (mm) of the lateral or medial femoral condyle, values in parentheses represent number of biological replicates, with error bars showing standard deviation (data from Malda *et al* (2013))

The study found that cartilage thickness demonstrates a negative allometric relationship to body mass, with the larger animals not having as thick cartilage relative to their size as expected. It was thought that this is due to diffusional constraints of nutrients to chondrocytes in the cartilage deep zone. The authors also found greater cell density in the cartilage of smaller animal species compared to the larger species, again this was thought to be due to diffusion having to take place in the ECM of thicker cartilage in the larger animals, restricting cell numbers. This cell density trend was also reported by Moran *et al* (2016).

A study that had been carried out due to the same concerns as us over animal tissue use in cartilage storage studies (Taylor *et al* 2012), showed that human articular cartilage was significantly different ($p < 0.05$) than porcine, bovine and ovine samples, with ovine significantly different than all other species ($p < 0.05$). Although this cartilage isn't from the knee, it can be seen that with the human tissue, femoral head cartilage thickness is comparable with that from the distal femur (see study by Devrimsel *et al* (2016)). The study also showed significant differences in the mechanical behaviour and properties of human and animal tissues and underlines the limitations of using animal tissue in experiments to determine the suitability of stored human cartilage. The article does however have limited sample numbers for each species, and there wasn't any demographic data for the human tissue.

Devrimsel *et al* (2016), and Yildizgoren *et al* (2016) reported that human femoral condyle cartilage thickness ranged from 1.83 to 2.26 mm in healthy individuals (Table 1.5.2).

Table 1.5.2. Summary of human femoral condyle cartilage thickness.[a]

	Thickness (mm)		
Condyle	**Devrimsel (n = 30)**	**Condyle**	**Yildizgoren (n = 60)**
Right lateral	1.88 ± 0.08	Lateral	2.18 ± 0.3
Right intercondylar	1.92 ± 0.09	Intercondylar	2.26 ± 0.4
Right medial	1.83 ± 0.08	Medial	2.21 ± 0.3
Left lateral	1.91 ± 0.08		
Left intercondylar	1.93 ± 0.10		
Left medial	1.83 ± 0.08		

[a]Cartilage measured ultrasonically in two studies (Devrimsel *et al* (2016) and Yildizgoren *et al* (2016)). Data shows mean ± standard deviation

Cartilage thickness between species has been investigated by Frisbie *et al* (2006), they found that mean femoral condyle thickness for humans was 2.2-2.5 mm, 0.3 mm for lapine, 0.4 - 0.5

mm for ovine, 0.6 - 1.3 mm for canine, 0.7 - 1.5 mm for caprine and 1.5 – 2 mm for equine, concluding that equine tissue was a close match for human. Another study suggested that caprine tissue was more appropriate with similar anatomy and biomechanics to human tissue (Moran *et al* 2016). The authors compiled a table comparing species, summarising their advantages and disadvantages as animal models (Table 1.5.3).

Table 1.5.3. Animal models in common use for cartilage repair, advantages and disadvantages.[a]

Species	Advantage	Disadvantage
Murine (mouse)	Low cost, manageable, easily available, with transgenic and athymic strains	Very small joints-*in situ* examination impossible
Murine (rat)	Low cost, easily available, athymic strains, maintain in-house	Permanently open growth plates accelerate healing, cell density
Lapine	Low cost, maintain in-house	Increased intrinsic healing, load characteristics, defects difficult
Canine	Naturally occurring disease state, co-operate with rehabilitation	Thin cartilage, small critical size defect (4 mm), complex ethical approval
Porcine	Biochemistry similar to humans, bone apposition rate/trabecular thickness similar to human, partial thickness defects possible	Expensive, difficult to obtain at skeletal maturity, specialised habitat, temperament
Caprine	Anatomy and biomechanics similar to human, partial thickness defects possible, easily available, low maintenance	Subchondral cyst formation
Ovine	Anatomy similar to humans, partial thickness defects possible, easily available, low maintenance	Subchondral cyst formation
Equine	Large defects similar to humans, partial thickness large diameter defects possible, naturally occurring defects, similar biomechanics in trochlea groove, second look arthroscopy possible	Expensive to acquire and maintain – specialised centre required, unable to avoid weight bearing, very dense subchondral bone, MRI/CT not possible

[a]Contents of table adapted from Moran *et al* (2016), MRI = magnetic resonance imaging, CT = computer tomography

Other investigators have also voiced concern over animal tissue use in pre-clinical studies (McIlwraith *et al* (2011), Malda *et al* (2012), Chevrier *et al* (2014)) of osteochondral repair, and as a result looked at equine or lapine models of human cartilage repair. Another study by Temple *et al* (2016) looked at the viscoelastic properties of bovine and human articular cartilage from femoral heads. They found a statistical difference ($p < 0.05$) in the storage and loss modulus between bovine and human samples, with the overall conclusion that bovine tissue is "stiffer" than human. This was however a very small study with bovine tissue isolated from 3 animals, likewise the human tissue was retrieved from 3 donors, all female over the age of 69. The authors stated that the bovine tissue came from healthy animals, but there was no mention of disease status for the human donors. Proteoglycan distribution has also been found to be statistically different ($p < 0.05$) in the patella articular cartilage between human, bovine and porcine tissue samples Rieppo *et al* (2003). Another study highlighted the variation in relationship between cartilage thickness, chondrocyte density and proteoglycan content of three animal species, commonly used as models of OA disease (murine, lapine, and hircine). As a result, the authors recommended caution should be exercised when data is interpreted and used in translational research (Kamisan *et al* 2013).

Conversely data obtained in animal studies appears to have less variability, whereas the data from human tissue in this study, and other studies also using human tissue shows greater variability within and between each donor. This will most likely be due to differences in donor physiology, age and any unrecorded diseases that they might have been suffering from prior to death. Animals used in scientific studies also tend to be in-bred which minimises genetic variation, have a strict diet and are relatively sedentary in their laboratory-based surroundings as well as reaching their end point prior to disease onset.

It is due to the evidence supporting the potential for longer storage periods at elevated temperature that this was chosen as the parameter we would use for cartilage used in the current study. However, we chose the temperature of 33ºC which is the normal physiological temperature in the human knee joint (32.6 ± 0.9ºC, Ammer (2012)). A study by Becher *et al* (2008) found resting intra-articular temperature to be at a median of 31.4ºC with min/max range of 29.7ºC to 34.3ºC. During jogging however, the temperature rose by 6.1ºC after 60 minutes to give a median 37.5ºC with min/max range of 36.1ºC to 38.8ºC. Even though the temperature of the knee has been shown to increase during physical activity, storing the allografts at this elevated temperature for 12 weeks may be detrimental to chondrocyte function. There are however no current published papers we are aware of that use 33ºC as a storage temperature for OCAs.

1.6 - Outcome of cartilage repair with osteochondral allografts

A successful outcome of OCA transplantation not only comes down to the quality of the donor tissue, but also any underlying preoperative issues with the patient receiving the OCA. A retrospective study by Nuelle *et al* (2017) suggested that a number of factors could contribute to a successful outcome. Whether the patient had a body mass index (BMI) below 35 had a significant impact on clinical success, another paper suggests this might be due to improper loading on the implant site (Everhart *et al* 2017). Whilst age however was not found to be a significant factor (Nuelle *et al* 2017), another study by Levy *et al* (2013), found that age was a significant factor, with patients over the age of 30 at the time of surgery experiencing allograft failure. Another article also states that not every patient is suitable for surgery to resurface their cartilage (Moyad 2011). The following factors can influence this: limb alignment, pre-existing concomitant injury or arthritis within the knee, patient expectations and rehabilitation potential, instability and ligament damage, previous total or subtotal meniscectomy, age, defect (size, location and number), BMI. However, no data was included as to how allografts were stored or analysed. The study by Everhart *et al* (2017) also suggested that psychological factors such as patient self-motivation and efficacy, optimism, non-compliance or poor pain tolerance can lead to poor outcomes.

With OCD of the knee, which is characterised by subchondral bone fragmentation, a group have published data showing that allograft transplantation is a successful treatment for this condition (Sadr *et al* 2016). In the study 135 patients underwent surgery, with a minimum follow-up of 2 years. The study reported a 95% satisfaction rate and a low rate of graft failure. A follow-up study with a mean of 22 years by Raz *et al* (2014) looking at patients treated with allografts for OCD and posttraumatic defects had successful results. From 58 patients there was graft survival rates of 91 to 59% from 10 to 25 years. Those surviving grafts had good function with a mean modified Hospital for Special Surgery (HSS) score of 86.

According to a study by Briggs *et al* (2015), allograft transplantation is perceived as a salvage procedure where previously attempted repair strategies have failed. They argued however that the use of allografts should be considered as a primary option for cartilage repair. In their cohort of 55 patients (30 male, 25 female), graft survival was at 89.5% after 5 years and 74.7% at 10 years. Ten patients required procedures to repair graft failure. Graft size used ranged from 3.2-34.8 cm^2, with the mean being 9.6 cm^2. Normally allograft tissue is used to repair larger lesions, however this study supports primary use in a broad range of defect sizes.

A summary paper published by Bugbee *et al* (2016) of his group's work since they started investigating cartilage allograft transplant, showed good outcomes from osteochondral allograft treatment of a number of knee related conditions. The reported data for this is shown in Table 1.6.1. The paper also reported follow-up data showing that after 10-12 years, repairs of traumatic injury with allograft had the most successful survival rate (98%), compared to OA which had the worst (41%). OCD was second (85%), followed by fracture (80%), avascular necrosis (74%), and degenerative chondral lesion (68%).

Table 1.6.1. Summary of osteochondral allograft (OCA) outcomes.[a]

Diagnosis	**Location**	**Knees/ Patients N/N**	**Age in years** Mean (range)	**Follow-Up in years** Mean (range)	**Outcome**
OCD	Femoral condyle	66/64	29 (15-54)	7.7 (2-22)	72% good - excellent
ON	Femoral condyle	28/22	24 (26-44)	5.6 (2-20)	89% survived
Focal traumatic or degenerative	Femoral condyle	129/122	33 (15-68)	13.5 (2-28)	79% good - excellent
Various	Patello-femoral	20/18	42 (19-64)	7.8	75% successful
Various - adolescent	Various	43/39	16 (11-18)	8.4 (2-27)	88% good - excellent
Various – no previous surgery	Various	55/61	33 (15-67)	7.6 (2-23)	86% extremely or satisfied
OCA revision	Various	33/33	37 (17-65)	10.6 (17-65)	63% good - excellent

[a]Data from a clinical study performed by Bugbee *et al* (2016). OCD = osteochondritis dissecans, ON = osteonecrosis

A small study published by a Brazilian group (Tirico *et al* 2017) reported favourable results with the use of femoral condyles to treat 8 patients, with follow-up of at least 2 years. Patients had lesions that were bigger than 4cm^2, they were also between the ages of 15 to 45. The lesions were traumatic or acquired in origin and had failed previous attempts at repair (no

detail given). Two surgical techniques were used with the allografts, dowel and shell type. The authors attributed the success of the allografts to the relatively short time (maximum 16 days) between retrieval, storage and implantation. However, they did admit that the small number of patients treated, the lack of control group and combined evaluation of the surgical techniques were limiting factors of the study. Another small study by Bardos *et al* (2015) followed the use of chondral allografts from storage, to implantation and then follow-up. Unusually these were carefully cut from the subchondral bone and multiply incised prior to surgery, then sutured in place where damaged cartilage had been removed in the patient. Seven patients were treated, ages 14-44 with focal lesions. At 2 year's post-op, 6 out of 7 patients had good results, with delamination occurring in the failed knee.

Another successful clinical outcome was reported by Maury *et al* (2007) who removed a previously implanted cartilage allograft 25 years after it was first inserted. The bony portion of the implant was securely integrated into the recipient, with chondrocytes visible in all layers of the cartilage matrix. The use of the allograft tisssue had taken place after the removal of a giant cell bone tumour. Published in the same year, another clinical report paper by Jamali *et al* (2007) showed how donor cells were detected in a previously implanted osteochondral allograft, 29 years after surgery. The tissue was from a female donor, but implanted into a male donor, however the chondrocytes in the allograft still retained their female genotype.

A recent paper showed the successful use of a large osteochondral allograft to repair a defect in a patient's medial femoral condyle, caused by alcohol induced ON (Cusano *et al* 2018). The surgery only had a 1-year follow-up however with evidence of excellent clinical outcome, and post-operative MRI investigation demonstrating excellent allograft incorporation. Another study reporting on follow-up of 25 patients who had undergone osteochondral allograft transplant for ON showed allograft survival was 90% at 5 years, and 82% at 10 years (Early *et al* 2018). It was also found that 73% of the patients avoided further surgical intervention.

A number of factors have been reported in the literature that contribute to either the success or failure of osteochondral allografts. Pearsall *et al* (2008) reported that lesion size <2 cm2 had improved outcomes. Latt *et al* (2011) reported detrimental effect of allograft of improper sizing and positioning, with Cook *et al* (2014) reporting on importance of storage and viability. Finally Hunt *et al* (2014) reported that grafts larger than 10 cm^2 were 36 times more likely to induce an immune response. According to a paper by Bastian *et al* (2011) the osteocytes located in the subchondral and cancellous bone of an OCA do not survive well in tissue culture conditions at 37ºC, and so storing the allografts in this manner may help reduce the immunogenicity of the tissue.

Review of the literature has found that there is a large amount of published evidence for the continued success of osteochondral allograft storage, and its subsequent use in patients. The need however to retrieve suitable tissue from healthy donors within a certain age group does present great challenge to a tissue bank. When this is competing with other tissues retrieved from a joint, the combination can result in an inadequate supply for clinicians to use. Not only this but there is often the need for the graft to be matched, so that once it is implanted the topography of the donor tissue is synchronous with the patients. While the cartilage itself is immuno-privileged, the underlying cancellous bone is not, and this is where graft failure occurs most often when the tissue does not integrate with the recipient (Okeagu *et al* 2017). The range of measures used to maintain chondrocyte viability above a threshold, and the surrounding transplant pipeline, will hopefully continue to prevent the need for revision osteochondral/allograft surgery, or the final resort of total joint replacement with prostheses. Much of the supporting evidence however comes from animal studies to verify the need for tissue with viable chondrocytes then being linked to successful osteochondral transplants in the clinic. While data from the literature supports allograft use, it would be ideal to have a large study of osteochondral allograft tissue which has had its viability and structural integrity checked, implanted and then patient follow-up carried out. The recent study by Cinats *et al* (2018) in part satisfies this need, though the variable storage time used prevents suggestion of an approved maximum storage time.

1.7 - Aims of the project

The aims of the project were as follows:

- To determine whether articular cartilage can be stored for up to 12 weeks using normothermic temperature, and maintain the required level of chondrocyte viability.

- Whether the cartilage matrix can be preserved during the storage period.

- Establish whether any differences between donor age groups, damage due to OA, or different storage conditions can be detected via functional assays used in the study.

- To determine if a profile of good articular cartilage allograft function can be determined and routinely measured using non-invasive methods.

Hypothesis statements

Glucose/lactate

Metabolic function of articular cartilage can be measured in a culture system by measuring the glucose and lactate levels in conditioned and control storage media samples using an Accutrend® Plus meter (GCTL, Roche, Mannheim, Germany). As a result, changes in glucose consumption and lactate secretion will be observed under different conditions. This meter uses test strips to detect the amount of either glucose or lactate in a small sample of biofluid, normally within plasma or blood samples.

Dimethylmethylene blue

Integrity of cartilage matrix in osteochondral allografts stored in culture medium at normothermic temperature is preserved. As determined by measurement of chondroitin sulphate (CS) released into the culture medium.

Live/dead

Viability of the chondrocytes in cartilage of osteochondral allografts is maintained above a 70% threshold during normothermic storage in culture medium, as determined by the live/dead assay.

AlamarBlue®

Metabolic activity of the chondrocytes in cartilage of osteochondral allografts is maintained above day 0 level during normothermic storage in culture medium, as determined by the alamarBlue® assay.

Lubricin (PRG4) immunohistochemistry

Storage of articular cartilage at normothermic temperature in a culture system results in retention of viable lubricin secreting chondrocytes in the superficial layer of cartilage matrix.

Safranin O histology

Storage of articular cartilage osteochondral allografts at normothermic temperature in a culture system results in retention of proteoglycan and glycosaminoglycans within the cartilage matrix, as determined by safranin O staining of histological sections.

Chapter 2

General Materials and Methods

2.1 - Collection: Donor information, age range, inclusion criteria

Deceased donor tissue

Whole knees were retrieved from donors (Table 2.1.1) whose next of kin had given full ethical consent (consent form shown in Appendix 1) to the NHS Blood and Transplant National Referral Centre for the removal of tissues for the purposes of research and development. This was in accordance with the Human Tissue Authority (HTA) Codes of Practice. The research study was performed under the auspices of HTA licenses 11018 and 12608. The tissue was removed from the body either in a mortuary or at the dedicated retrieval suite located at NHS Blood and Transplant Tissue and Eye Services tissue bank in Speke, Liverpool. Retrieval was carried out by a dedicated retrieval team of trained technicians.

Table 2.1.1. Summary of donor information for the cohorts of cartilage tissue used in the study.[a]

Cohort	Age range	Gender	Comments
Normal	26 - 27	F (1), M (2)	One donor had liver failure
Over-age	57 - 77	F (2), M (2)	Small lesion
Osteoarthritic	45 - 70	F (1), M (2)	Living donors
Hypothermic	27 – 61	F (2), M (1)	
Bone only	70 - 77	F (1), M (1)	
Negative control	62	M (1)	Freeze thawed tissue

[a]Numbers in parentheses represent the number of donors used in each cohort

To remove the whole knee from deceased donors, the donor was prepared by wiping down the skin with chlorhexidine (Hydrex™, Ecolab, Northwich, UK) which was then rinsed off with sterile water for irrigation (UKF7114, Baxter, UK). The area surrounding the knee was covered in sterile drapes. Working in sterile gowns with face mask and sterile gloves, an incision was made through the skin longitudinally with a disposable PM40 knife (Swann Morton®, Sheffield, UK) over the knee to enable careful separation of the subdermal fat layer, taking care not to pierce the joint capsule, muscle tissue surrounding the distal femur was cut, so that the muscle was parted. This allowed access to the bone with a Stryker battery powered oscillating saw (System 5 Precision, Stryker, Newbury, UK). At the proximal tibia the bone was cut without a requirement to dissect muscle as none is located anteriorly to the bone. Once separated from remaining muscle and connective tissue the knees were removed and placed in

an inner and outer bag and closed by heat-sealing. The tissue was then placed on wet ice inside an insulated box before being transferred to the Tissue Bank and the R&D laboratory.

Living donor tissue

For living donor OA tissue, samples of damaged cartilage and subchondral bone were retrieved from patients undergoing total knee replacement surgery at University Hospital Aintree by Mr Michael McNicholas, with full ethical consent having been obtained from the patients (consent form and patient information form shown in Appendix 1). These samples were submersed in sterile saline inside an inner and outer sterile resealable polybag (XX1005, Helapet, Houghton Regis, UK) and sealed before being placed in a refrigerator. The samples were collected on the day of surgery and transferred to the National Health Service Blood and Transplant (NHSBT) Tissue and Eye Services R&D laboratory.

2.2 - Dissection of whole knees

Double bagged whole knees were transferred into a grade B cleanroom along with equipment and consumables required for dissection and processing of the knees. The bags containing one of the knees were opened inside a class II safety cabinet and transferred into a sterile 800 ml polypropylene kidney dish (RML140-005, Rocialle, Mountain Ash, UK). Skeletal muscle and connective tissues were carefully dissected away until the joint capsule was exposed. The joint capsule was then cut into from above the patella tendon at the distal femur side of the knee, such that the patella tendon could be reflected anteriorly, with it still attached to the proximal tibia. Connective tissue was removed from the sides of the distal femur and proximal tibia. Cruciate ligaments were exposed by flexing the knee joint so that they could be separated in two. Remaining connective tissue at the back of the knee that was still holding the knee together was removed so that the distal femur and proximal tibia were no longer attached.
Once separated excess bone was cut away from the distal femur using a mains powered deSoutter oscillating autopsy saw (CleanCut^{TM} CNS3, deSoutter, Aston Clinton, UK) the cut was made at the margin of the posterior and anterior cartilage and bone. A sample of the cartilage at the periphery of the trochlea groove was taken using a 5 mm diameter biopsy punch (Meditech, Shaftsbury, UK), with a sterile disposable scalpel (Swann Morton®, Sheffield, UK) used to separate it from the subchondral bone. Using the same scalpel, a small rectangular piece of cartilage (~3 x 5 mm) was cut close to where the biopsy punch was taken in the trochlea groove. Both pieces of cartilage were placed in a 7 ml polypropylene bijou (192A, ThermoFisher Scientific, Runcorn, UK) filled with Dulbecco's phosphate buffered saline

(DPBS) (D8537, Sigma, Gillingham, UK). The femoral condyle was then bisected along the trochlea groove (Fig. 2.2.1) using the deSoutter saw. Dissection and sampling was then completed on the second knee. From a pair of donor knees up to four hemi-condyles were prepared.

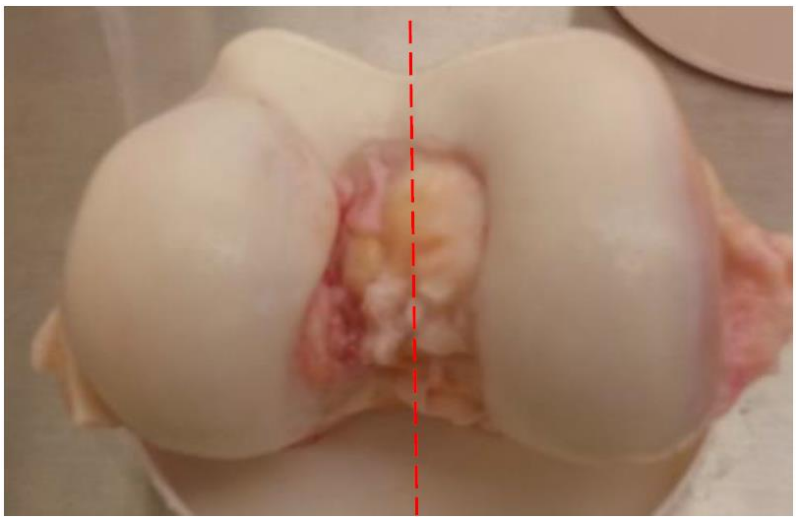

Figure 2.2.1. Image of right distal femur showing medial condyle on the right and lateral condyle on the left. Dotted line shows where the cut with a deSoutter saw was made to separate the condyle into hemi-condyles

2.3 - Hemi-condyle/OA cartilage decontamination

Each hemi-condyle or donation of OA cartilage samples were placed in individual 300 ml polypropylene jars (HJC0300E, Medfor, Aldershot, UK). Poly(vinylpyrrolidone)-iodine (PVP-I) was prepared by adding the dry powder (PVP1, Sigma, Gillingham, UK) to sterile water for irrigation (UKF7114, Baxter, UK) at a concentration of 5 mg/ml which was then filter sterilised. To each hemi-condyle, or OA donation, 100 ml of PVP-I solution was added, and then left for 2 minutes at room temperature to decontaminate the tissue. Periodically each container was agitated by hand. After 2 minutes the PVP-I was removed (Fig. 2.3.1) and replaced with 100 ml of sodium thiosulphate pentahydrate (S6672, Sigma, Gillingham, UK) (0.3% wt / vol in DPBS, filter-sterilised) and left for another 2 minutes to inactivate the PVP-I. Periodically each container was agitated. The sodium thiosulphate was removed after 2 minutes and replaced with 100 ml of DPBS, which was left for a further 2 minutes. Periodically each container was agitated. Each hemi-condyle was then placed in individual 1000 ml blood bags (R4R2022, Fenwal, London, UK) into which the storage solution was also placed.

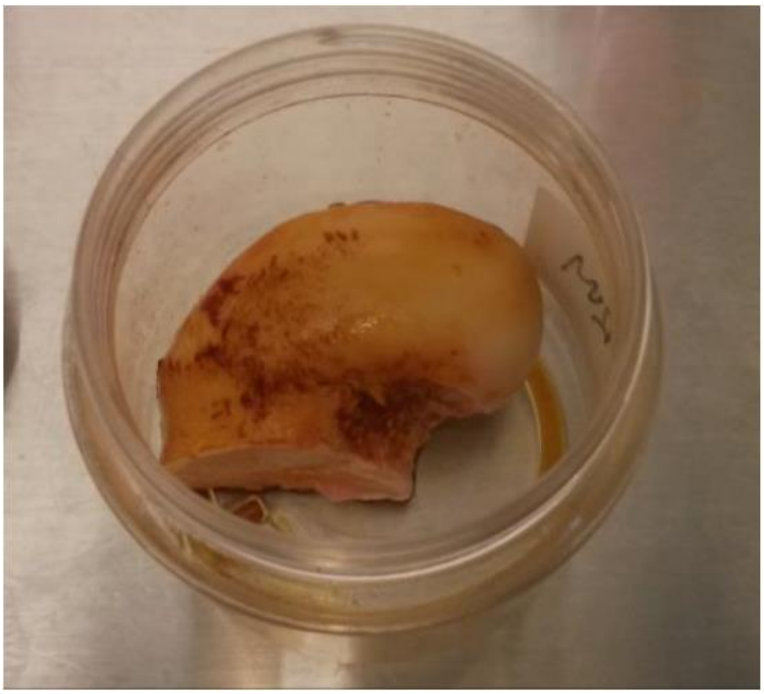

Figure 2.3.1. Hemi-condyle after treatment with poly(vinylpyrrolidone)-iodine (PVP-I) inside a 300 ml polypropylene container prior to inactivation of the PVP-I with sodium thiosulphate pentahydrate

2.4 - Culture, controls, storage, transport

Cartilage hemi-condyles and living donor OA samples were stored at 33ºC in blood bags (Fig. 2.4.1) containing filter sterilised Dulbecco's Modified Eagle's Medium (DMEM) / Ham's F-12 nutrient mixture (12-719F, Lonza, Basel, Switzerland), 10% (vol / vol) foetal bovine serum (FBS; FB-1001/500, Biosera, Labtech, UK), 1% (vol / vol) penicillin/streptomycin (P4333, Sigma, Gillingham, UK), 1% (vol / vol) sodium pyruvate (S8636, Sigma, Gillingham, UK), 0.05% (wt / vol) L-ascorbic acid (A4544, Sigma, Gillingham, UK). Hemi-condyles were stored in volumes of 100 ml for deceased donors, and 80 ml volumes for living donors due to smaller volume of cartilage pieces. Once the hemi-condyles had been placed in the opened bags, they were heat-sealed closed using a double-sided heat-sealer (Stand Up Pouch / Tube Sealer, Hulme Martin, Woking, UK). An un-conditioned control sample of medium in a blood bag without cartilage allograft was also stored under the same conditions. Media was removed at weekly intervals by cutting the connection tube of the blood bag and emptying a small volume (~5 ml) into a 7 ml polypropylene bijou (ThermoFisher Scientific, Runcorn, UK). The media was centrifuged at 35,500 x g for 5 minutes

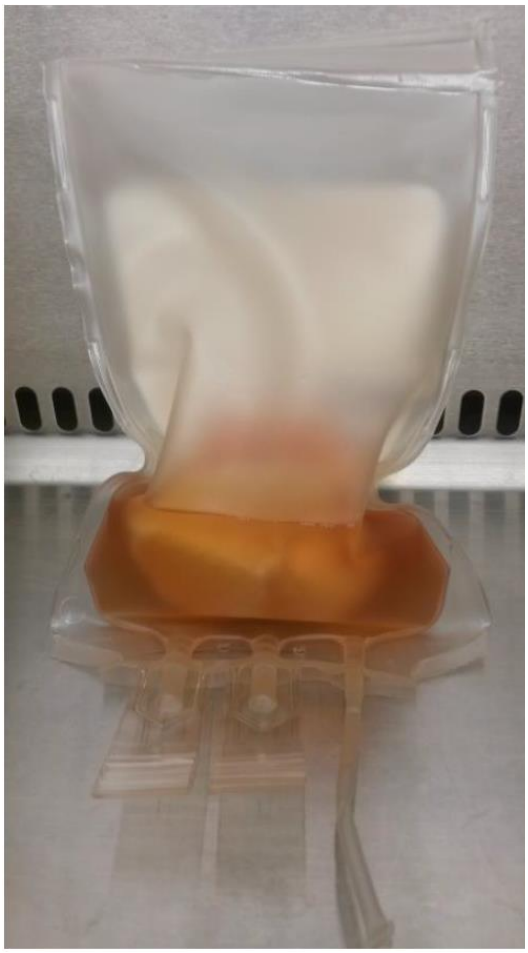

Figure 2.4.1. Hemi-condyle sealed inside a 1000 ml blood storage bag (R4R2022, Fenwal, London, UK) with tissue immersed in 100 ml Dulbecco's modified Eagle's Medium F12 Ham plus additions culture medium

at 4°C and the supernatant transferred between a new 7 ml bijou, and 2 ml cryovial (431386, Corning, Amsterdam). The supernatent was snap frozen by being immersed in liquid nitrogen and stored at -80°C. The remaining spent media was removed before fresh pre-warmed medium was then added to the bag using a 50 ml syringe (SS+50L1, Terumo, Surrey, UK) and hypodermic needle (BD Microlance 3, Co Louth, Ireland) through the bag's connection tube, which was then heat-sealed using a tube sealer (AC-155 Teruflex, Terumo, Surrey, UK). Hemi-condyles were kept in storage for up to 84 days, living donor OA samples were stored for 56 days.

2.5 - Glucose/lactate methodology

In order to infer the metabolic function of the cartilage allografts based on consumption of glucose and secretion of lactate, measurements of glucose and lactate concentrations in

conditioned and control un-conditioned storage media collected from donors shown in Table 2.5.1 were performed.

Table 2.5.1. Summary of cartilage donor cohorts tested with the glucose/lactate meter.[a]

	Number of donors (n)	Living/deceased
Normal	3	Deceased
Over-age	4	Deceased
Hypothermic	3	Deceased
Negative	1	Deceased
Bone only	2	Deceased
Osteoarthitis	3	Living

[a]Tissue from normal (<45 years of age), over-age (>45 years of age), negative, bone only and living donor osteoarthritis stored at normothermic (33ºC) temperature. Hypothermic tissue was stored at 4ºC. Tissue was retrieved from either living or deceased donors

Glucose/lactate assay mode of action

The glucose test strip is based on the following reaction:
Quinonediimine oxide + glucose ⟶ hydroxylamine derivative (reduced)
Hydroxylamine derivative (reduced) ⟶ quinonediimine
(Quinonediimine + glucose ⟶ phenylendiamine) reduction catalysed by glucose oxidase
Phosphomolybdic acid oxidiser + phenylendiamine ⟶ quinonediimine + phosphomolybdic acid (reduced)
Phosphomolybdic acid (reduced) ⟶ molybdenum blue
Therefore, the colour change from yellow to blue/green was measured by the test meter.

The lactate test strip is based on the following reaction:
L-lactate + mediator (form1) ⟶ mediator (reduced)
Mediator (reduced) + 2,18-phosphomolybdate ⟶ molybdenum blue + mediator (form2)
Therefore, the colour change from yellow to blue/green was measured by the test meter.

Glucose assay culture media samples and metabolite standards

Conditioned and un-conditioned media samples from donor cohorts shown in Table 2.5.1 were

taken from storage at -80°C and thawed at room temperature for one hour with sampling and measurements carried out.

A glucose standard (PHR1000, Sigma, Gillingham, UK) was prepared from a stock concentration of 20 mmol/L in Dulbecco's phosphate buffered saline (DPBS) (D8537, Sigma, Gillingham, UK), serially diluted to 10 and 5 mmol/L with DPBS. This diluent was used to mimic the osmotic pressure of the culture medium. Culture medium couldn't be used as it already contains glucose.

For each sample and standard tested, a glucose test strip (11447475, Roche, Mannheim, Germany) was inserted into an Accutrend® Plus meter (GCTL, Roche, Mannheim, Germany). The lid was then opened and 15 µl of the sample pipetted onto the sampling area of the test strip, after which the lid of the meter was closed and the concentration recorded.

The measurement range of the test meter for glucose was 1.1 - 33.3 mmol/L (imprecision >4.2 mmol/L CV: ≤6%). If the concentration measured by the meter was higher than the upper limit of the meter's measurement range, then a sample of the solution was diluted by half and re-measured, the measured value was then corrected by the dilution factor.

According to the manufacturer; ascorbic acid (AA) may affect results showing higher than normal glucose. As the culture medium contained AA, samples of the medium with or without AA were tested in the meter. Data showed that there was no significant difference ($p = 0.37$) observed between media samples either with or without AA (Table 2.5.2)

Table 2.5.2. Concentration of glucose measured with or without ascorbic acid (AA).[a]

Mean glucose concentration (mmol/L) media with AA	Mean glucose concentration (mmol/L) media without AA	*P* value
15.95 ± 0.41	15.65 ± 0.47	0.37

[a]Values show mean ± standard deviation, n = 4 technical replicates with samples of medium tested on the Accutrend® Plus glucose/lactate meter, *p* value was FDR adjusted

Lactate assay culture media samples and metabolite standards

A lactic acid standard (PHR1215, Sigma, Gillingham, UK) was prepared to concentrations of 20, 10, and 5 mmol/L in DPBS. A standard curve was not generated for lactate as the meter accurately measured lactate content in the samples. When the lactate standard was prepared at

a concentration of 20 mmol/L the mean reading from triplicate samples was 19.67 ± 0.61 (SD). At 10 mmol/L the mean was 9.63 ± 0.15 and at 5 mmol/L the mean was 4.57 ± 0.058.

To measure lactate concentration in samples and the standard, a lactate test strip (03012654, Roche, Mannheim, Germany) was inserted in the Accutrend® Plus meter. As before the lid was then opened and 15 µl of medium sample or standard pipetted onto the sampling area of the test strip. The lid was closed and the concentration recorded.

The measurement range of the test meter for lactate was 0.7 - 26 mmol/L (imprecision CV: 5.5% in normal range, 5% in higher range). If the concentration measured by the meter was higher than the upper limit of the meter's measurement range, a sample of the solution was diluted by half and re-measured, the measured value was then corrected by a dilution factor.

Data analysis

Data was transferred to Excel where experimental cohorts were grouped. For the glucose data, a standard curve was generated from the concentration values recorded for the known glucose concentrations (Fig. 2.5.2). The concentrations of experimental media samples were corrected with a formula generated from the trend line of the standard curve. The formula was rearranged to bring the unknown term 'x' to the left side of the equation. Using this equation actual glucose concentrations were calculated from those measured by the meter. Matched un-conditioned media control sample data was then subtracted from conditioned with both glucose and lactate results.

(1) $$\text{Metabolite turnover} = \frac{\text{Metabolite concentration (µmol/L) x volume (L)}}{\frac{\text{area (cm}^2\text{)}}{\text{time (day)}}}$$

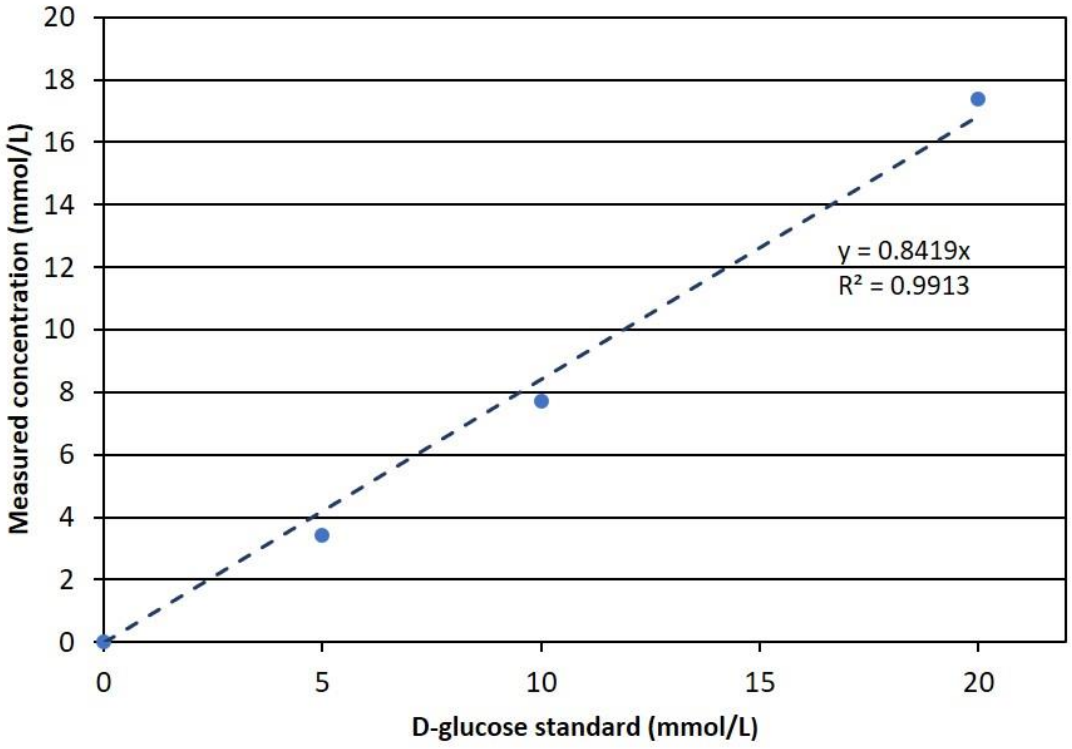

Figure 2.5.2. Standard curve for glucose as measured using the Accutrend® meter. The glucose standard was prepared at a concentration of 20 mmol/L in Dulbecco's phosphate buffered saline and serially diluted, 15 µl of each sample was added to individual test strips that were analysed by the meter

Measurement of cartilage surface area

Normalisation was carried out using the surface area of each hemi-condyle. This was measured by capturing a digital image of each hemi-condyle with a measurement scale visible next to the tissue. Each image was opened in the ImageJ software (Fiji). The line draw tool was then used to draw a line along the measurement scale that was included in the image. Set Scale was selected in the Go to Analyze options on the menu bar. In the subsequent window the known distance was typed in, and then unit of length, before Ok was selected. The freehand draw tool was then selected, and the mouse used to draw round the edge of the cartilage. Analyze was selected again and then Measure. In the next window the area of the cartilage was shown in the chosen unit squared, based on the outline drawing that was done.

2.6 - Dimethylmethylene blue methodology

To determine the turnover of cartilage matrix within stored osteochondral allografts, conditioned culture medium collected from the tissue storage system was tested for chondroitin sulphate C content using the dimethyl methylene blue (DMMB) assay.
Conditioned and control media samples from donor cohorts shown in Table 2.6.1 that had been collected during weekly media changes were taken from storage at -80^{o}C and thawed at room temperature for one hour.

Table 2.6.1. Summary of cartilage donor cohorts tested with the dimethylmethylene blue assay.[a]

	Number of donors (n)	Living/deceased
Normal	3	Deceased
Over-age	4	Deceased
Hypothermic	3	Deceased
Negative	1	Deceased
Bone only	2	Deceased
Osteoarthitis	3	Living

[a]Tissue from normal (<45 years of age), over-age (>45 years of age), negative, bone only and living donor osteoarthritis stored at normothermic (33^{o}C) temperature. Hypothermic tissue was stored at 4^{o}C. Tissue was retrieved from either living or deceased donors

The chondroitin sulphate C standard (C4834, Sigma, Gillingham, UK) was prepared at a concentration of 5 mg/ml in DMEM F12 Ham's with additions as previously described. Subsequent dilutions were also made with DMEM F12 Ham's to give a range of chondroitin sulphate C standards (10 – 50 µg/ml (vol / vol)). Normally standard curves are prepared in basic solutions, however a research paper (Zheng and Levenston 2015) reported that components of culture media can affect the outcome of the chondroitin sulphate C standard curve.

Chondroitin sulphate measurement

The DMMB assay reagent was prepared with 0.16% (wt / vol) 1,9-dimethylmethylene blue (341088, Sigma, Gillingham, UK), 0.2% (vol / vol) formic acid (0961, VWR, Lutterworth,

UK) and 30 mM sodium formate (456020, Sigma, Gillingham, UK) in distilled water (UK F7114, Baxter, Northampton, UK). The pH of the reagent was adjusted to 3.5 using 1N sodium hydroxide (S2770, Sigma, Gillingham, UK).

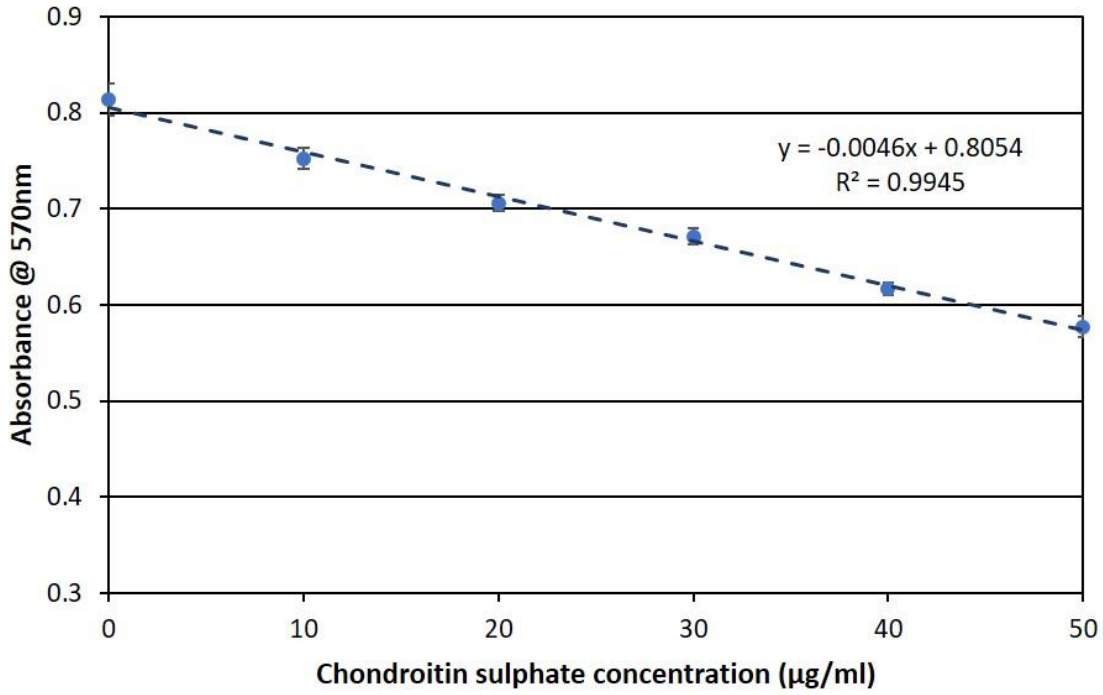

Figure 2.6.1. Standard curve for chondroitin sulphate C prepared from stock 5 mg/ml in complete culture medium (Dulbecco's Modified Eagle's Medium F12 Ham's plus additions), 40 µl of each sample was added in triplicate to wells of a microplate and then 250 µl of dimethylmethylene blue reagent added before samples were read on an absorbance plate reader at 570 nm, error bars show standard deviation (n = 3 technical replicates)

For each sample and standard tested 40 µl was pipetted in triplicate into wells of a 96 well microplate (655180, Cellstar, Greiner, Sigma, Gillingham, UK) and 250 µl of the DMMB assay reagent was added to each well. The microplate was then immediately read on an absorbance plate reader (ELx808iu, Biotek, UK) at 570 nm. If sample values were outside the range of the standard curve, these were diluted using complete media and re-measured.

Data analysis

Data was transferred to Excel where experimental cohorts were grouped. For the chondroitin sulphate data, a standard curve was generated from the concentration values measured for the

known chondroitin sulphate C concentrations (Fig. 2.6.1). The concentrations of experimental media samples were corrected with a formula generated from the trend line of the standard curve. The formula was rearranged to bring the unknown term x to the left side of the equation. Using this equation actual chondroitin sulphate C concentrations were calculated from those measured by the plate reader. Values for concentration were then placed in equation 2 to calculate the normalized amount of chondroitin sulphate C released into the culture medium

$$(2) \qquad \text{Chondroitin sulphate} = \frac{\text{CS concentration } (\mu g/ml) \times \text{volume } (ml)}{\frac{\text{area } (cm^2)}{\text{time (day)}}}$$

Measurement of cartilage surface area

Normalisation was carried out using the surface area of each hemi-condyle. This achieved following the method described in section 2.5.

2.7 - Live/dead staining methodology

To determine the viability of chondrocytes in the articular cartilage of stored osteochondral allografts, measurement of cell viability at the beginning of week 1 and final day of storage (week 8 (OA) or 12) of cartilage from the donor cohorts shown in Table 2.7.1 was performed.

Table 2.7.1. Summary of cartilage donor cohorts tested with the live/dead assay.[a]

	Number of donors (n)	Living/deceased
Normal	3	Deceased
Over-age	4	Deceased
Hypothermic	3	Deceased
Negative	1	Deceased
Osteoarthitis	3	Living

[a]Tissue from normal (<45 years of age), over-age (>45 years of age), negative, and living donor osteoarthritis stored at normothermic (33°C) temperature. Hypothermic tissue was stored at 4°C. Tissue was retrieved from either living or deceased donors

Live/dead assay mode of action

One of the assay reagents known as calcein acetoxymethyl is hydrophobic and permeates intact cells where it is hydrolysed in the cell cytoplasm by esterases to produce the hydrophilic calcein that fluoresces green. The other reagent ethidium homodimer-1 is unable to penetrate intact cell membranes, unless the cell is dead. Once inside a dead cell it binds to nucleic acids where it undergoes a 40-fold increase in red fluorescence.

Cartilage sampling and sectioning

Rectangular cartilage biopsies that had been taken at the beginning of week 1 or final week of storage (week 8 for living donor osteoarthritic (OA) tissue, and week 12 for deceased tissue) were mounted on a vibratome specimen disc using a small amount of cyanoacrylate glue. The cartilage was orientated so that the 5 mm side projected vertically from the specimen disc. The specimen disc was then secured in the vibratome (VT1000S, Leica, Milton Keynes, UK) with the cartilage piece at right angle to the vibratome knife (Fig. 2.7.1). The buffer tray in which the specimen disc and cartilage had been secured was then filled with DPBS so that the cartilage was totally immersed. After adjustment to align the vibratome knife, 50 µm thick sections were cut from the tissue with the cutting frequency set to 100 Hz and speed set to 0.25 mm/s. Once each section had been cut it was transferred from the DPBS in the buffer tray to a 7 ml polypropylene bijou (129A, ThermoFisher, Runcorn, UK) containing DPBS.

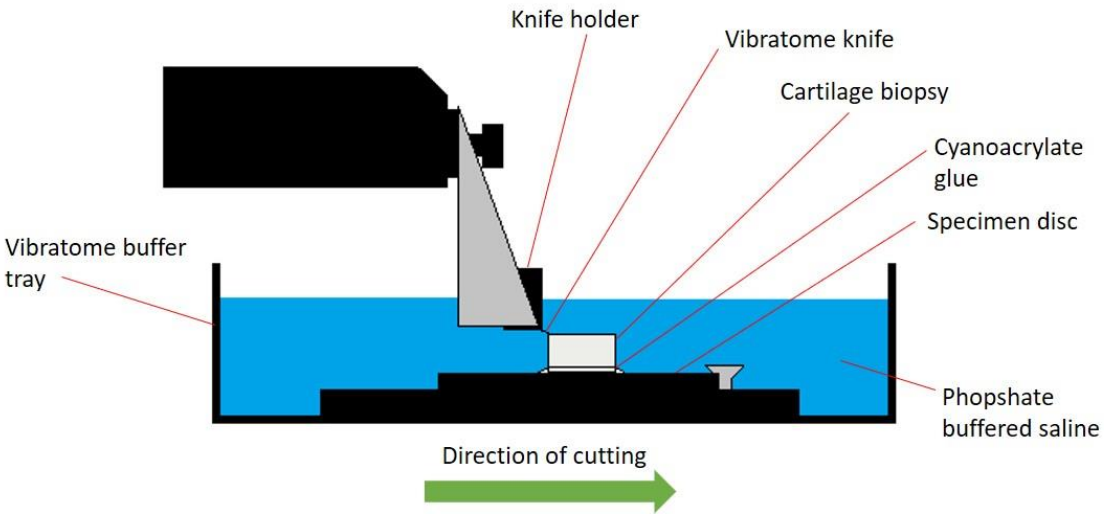

Figure 2.7.1. Sagittal plane section diagram of cartilage biopsy mounted on the specimen disc in the buffer tray, with the vibratome knife orientated at the leading edge of the cartilage, prior to section cutting taking place. Cartilage immersed in Dulbecco's phosphate buffered saline

Live/dead assay

After all sections had been cut a 5 µl aliquot of calcein acetoxymethyl (4 mM) and 10 µl aliquot of ethidium homodimer-1 (2 mM) from a live/dead assay kit (L3224, Invitrogen, ThermoFisher, UK), were added to 20 ml of DPBS in a 30 ml universal tube (201172, Sterilin, UK) and well mixed. This gave a working concentration of 1 µM for both components of the assay kit. DPBS was carefully removed from the 7 ml bijou, leaving the cartilage sections behind, after which 5 ml of the working assay reagent was added. The bijou was then wrapped in aluminium foil before it was incubated at room temperature for 30 minutes. After this time the reagent was removed from the bijou leaving the cartilage sections inside before 5 ml of DPBS was added. After 5 minutes of incubation at room temperature, the DPBS was removed before it was replaced with 5ml of DPBS. Again, this was incubated at room temperature for 5 minutes. Each section was then transferred onto a Polysine microscope slide (631-0107, VWR, Lutterworth, UK) along with a few drops of DPBS before a glass coverslip was placed on top of the sections. Any visible air bubbles were removed. Treated tissue sections were imaged on a fluorescence microscope (Leica DM2000 LED, Milton Keynes, UK) with LED fluorescence source (CoolLED pE-300^{white}, Leica, Milton Keynes, UK) with images captured using a digital camera (Leica DFC450 C, Milton Keynes, UK) linked to a desktop PC running LAS v4.9 (Leica, Milton Keynes, UK). Images were captured through red/green filters (Leica, Milton Keynes, UK) and saved as TIFF files.

Data analysis

Saved images were opened in ImageJ v1.50i (Fiji) running in a Windows 10 operating system. The images were then processed using the software according to the method shown in Fig. 2.7.2. With adjustment of noise tolerance, a number between 20 and 60 was selected depending whether point selection was highlighting points in the image that were actually background noise rather than the cells themselves.

The number count obtained from the green channel represented living cells within the tissue while the count from red represented dead cells. For each image the number of green stained cells was added to red to give a total cell count. The numbers were then placed in equation 3 to determine percent viability in the cartilage tissue

$$(3)\quad \text{Viability (\%)} = \frac{\text{Green cell count}}{\text{Total cell count}} \times 100$$

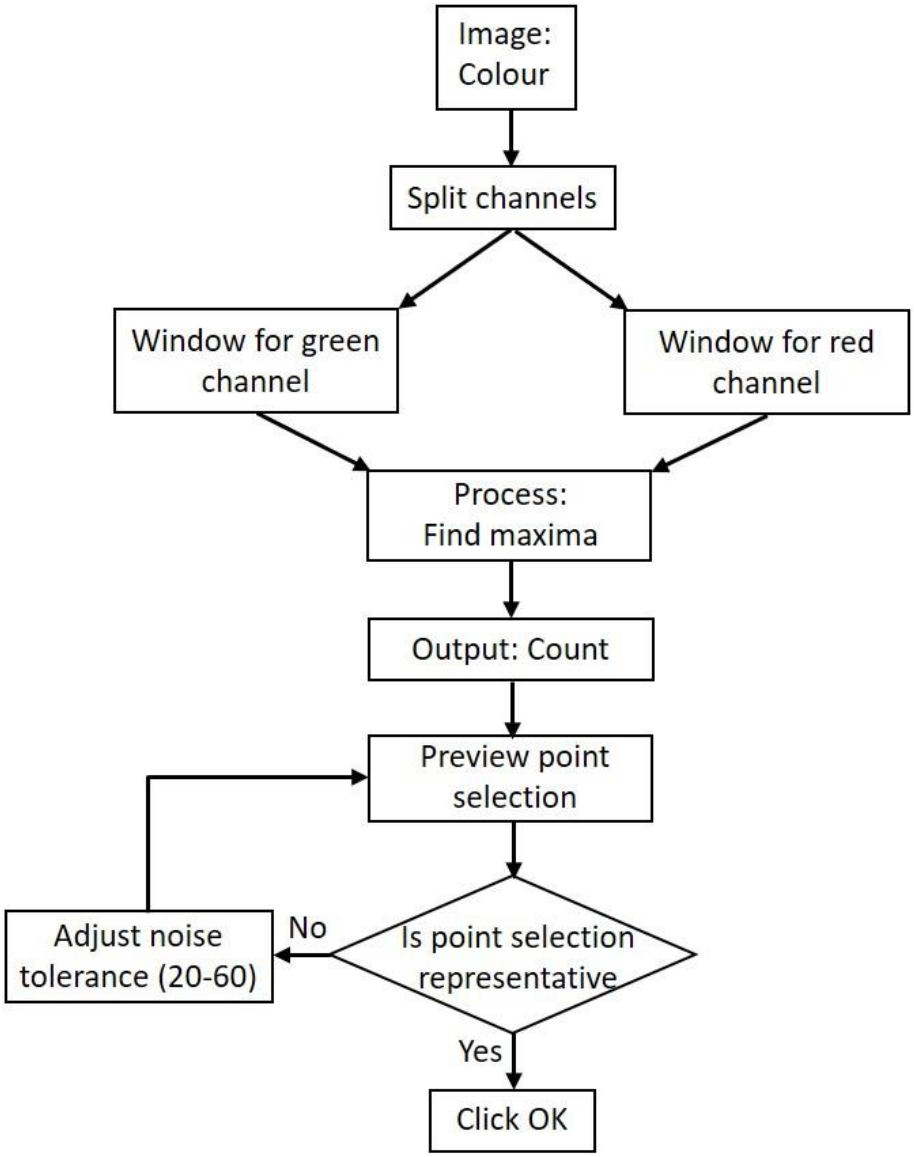

Figure 2.7.2. Flow diagram of the method used to process and analyse live/dead TIFF images using the ImageJ (Fiji) analysis software

2.8 - AlamarBlue® methodology

Metabolism in the chondrocytes of cartilage was measured using the alamarBlue® assay. Metabolic activity in stored osteochondral allografts from donor cohorts shown in Table 2.8.1, was determined via this assay in weeks 1, 4, 8, and 12.

Table 2.8.1. Summary of cartilage donor cohorts tested with the alamarBlue® assay.[a]

	Number of donors (n)	Living/deceased
Normal	3	Deceased
Over-age	4	Deceased
Hypothermic	3	Deceased
Negative	1	Deceased
Bone only	2	Deceased

[a]Tissue from normal (<45 years of age), over-age (>45 years of age), negative, and bone only stored at normothermic (33°C) temperature. Hypothermic tissue was stored at 4°C. Tissue was retrieved from deceased donors

AlamarBlue® assay

At the beginning of week 1 after each hemi-condyle had been rinsed with Dulbecco's phosphate buffered saline (DPBS) (D8537, Sigma, Gillingham, UK) and then sealed inside individual blood bags (R4R2022, Fenwal, London, UK), 40ml of the alamarBlue® assay reagent (TOX8, Sigma, Gillingham, UK) at 5% concentration in DPBS was added. This had been pre-heated to 37°C. Each hemi-condyle was orientated so that only the articular cartilage surface was immersed in the reagent. The blood bags were positioned upright inside an incubator at 37°C for 2 hours, along with a control sample of the reagent which was also stored in a blood bag. After 2 hours the metabolised reagent was removed from each blood bag and stored in individual 60 ml plastic containers. Standards of resorufin (4247455, Sigma, Gillingham, UK) were prepared from a stock solution of 10 μM by mixing the dry powder in DPBS. To a black-bottomed 96 well microplate (267342, Nunc, Thermo Scientific, Denmark) 50 μl samples of the standards were added in triplicate. To the remainder of the plate 50 μl samples of the controls and metabolized alamarBlue® reagent were added in quintuplicate. The plate was then read on a fluorescence microplate reader (FLx800, Biotek, UK) at 530 nm excitation and 590 nm emission, with gain set at 53.

At each subsequent testing time point the medium was removed from the storage bags and

replaced with 37ºC pre-heated 5% alamarBlue® assay reagent. The tissue was then incubated as previously described and after 2 hours, samples of the metabolised alamarBlue® reagent added to wells of a microplate with blanks and measured on the fluorescence plate reader.

Data analysis

All data were transferred to Excel where a standard curve was generated (Fig. 2.8.1). The formula was rearranged to bring the unknown term 'x' to the left side of the equation. Using this equation resorufin concentrations were calculated from fluorescence values measured by the microplate reader. Data was then normalised by applying equation 4 to it.

(4) $$\text{Metabolic activity} = \frac{\text{Resorufin concentration (µM)}}{\text{Cartilage area (dm}^2\text{)}}$$

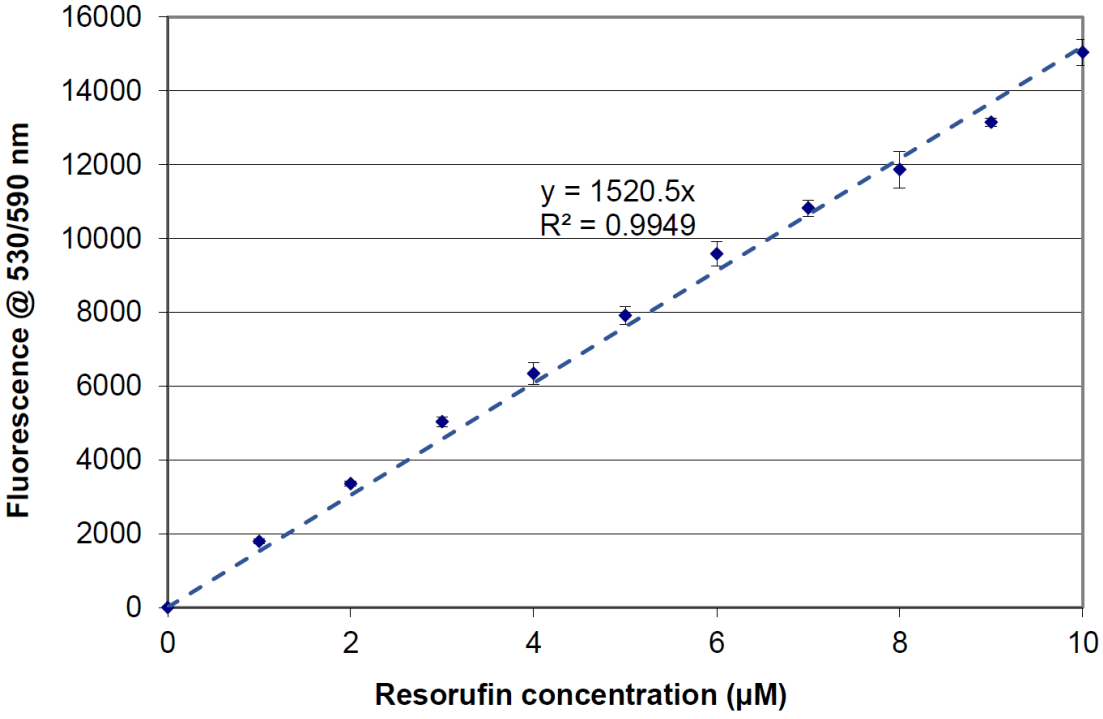

Figure 2.8.1. Standard curve for resorufin prepared from 10 µM stock prepared in Dulbecco's phosphate buffered saline. 50 µl of each dilution in triplicate to wells of a black bottomed 96 well microplate before the plate was read on a fluorescence plate reader at 530/590 nm excitation and emission, error bars show standard deviation (n = 3 technical replicates)

Measurement of cartilage surface area

Normalisation was carried out using the surface area of each hemi-condyle. This was achieved by following the method described in section 2.5.

2.9 - Lubricin (PRG4) immunohistochemistry methodology

Full thickness 5 mm diameter biopsy samples which had been taken at the beginning of week 1 and week 12 (or 8 for living donor OA tissue) of storage of cartilage tissue from donor cohorts are shown in Table 2.9.1. The biopsies were placed in 10% (vol / vol) neutral buffered formalin (NBF) (361367L, VWR, Lutterworth, UK) for up to 7 days at room temperature. Once fixed the biopsies were taken through the automated protocol shown in table 2.9.2, carried out in a tissue processor (Citadel 2000, Thermo Scientific, Runcorn, UK).

Table 2.9.1. Summary of cartilage donor cohorts tested with the lubricin (PRG4) assay.[a]

	Number of donors (n)	Living/deceased
Normal	3	Deceased
Over-age	4	Deceased
Hypothermic	3	Deceased
Negative	1	Deceased
Osteoarthitis	3	Living

[a]Tissue from normal (<45 years of age), over-age (>45 years of age), osteoarthritis, and negative, stored at normothermic (33^{o}C) temperature. Hypothermic tissue was stored at 4^{o}C

Table 2.9.2. Protocol for fixing, dehydrating and infusion of paraffin wax in cartilage biopsies.[a]

Chemical	Details	Dwell time (minutes)
10% (vol / vol) neutral buffered formalin	361367L, VWR, Lutterworth, UK	30
70% (vol / vol) ethanol	302444E, VWR, Lutterworth, UK	90
90% (vol / vol) ethanol		120
100% ethanol		90
100% ethanol		90
100% ethanol		90
100% ethanol		90
100% xylene	28975.360, VWR, Lutterworth, UK	120
100% xylene		120
100% xylene		120
100% paraffin wax	6774060, Thermo Scientific, Runcorn, UK	180
100% paraffin wax		180

[a]Protocol carried out in an automated tissue processor (Citadel 2000, Thermo Scientific, Runcorn, UK)

Preparation of sections

The biopsies were then paraffin wax embedded (HistoStar, Thermo Scientific, Runcorn, UK) in moulds with the articular surface perpendicular to the base of the mould.
Transverse sections of fixed articular cartilage were cut on a microtome (Finesse 325, Thermo Shandon, Runcorn, UK) at 5 µm in thickness, and transferred onto Polysine (631-0107, VWR, Lutterworth, UK) microscope slides (4 sections per slide) in a tissue flotation bath set at 45°C (3120059, R.A. Lamb, Thermo Shandon, Eastbourne, UK). The slides were then dried overnight in an incubator at 37°C. Finally, the slides were heat-fixed for 10 minutes on a slide hot plate set at 45°C (3120061, R.A. Lamb, Thermo Shandon, Eastbourne, UK).

Dewaxing and rehydration

Slides were dewaxed and rehydrated using the following process:
100% xylene - 5 minutes
100% xylene - 5 minutes

100% ethanol - 5 minutes
90% ethanol - 5 minutes

Lubricin (PRG4) immunohistochemistry

Chromogenic staining with horseradish peroxidase

Slides were briefly rinsed in water before being dabbed dry. Each section was drawn round with a Daco pen. Endogenase activity was blocked on the slides via addition of 0.3% (vol / vol) hydrogen peroxide in methanol for 5 minutes. The slides were incubated with chondroitinase ABC (C3667, Sigma, Gillingham, UK) (0.1 U/ml in buffer (10% (vol / vol) goat serum, 10% (wt / vol) bovine serum albumin (BSA) in DPBS)) for 1 hour at 37ºC, all incubations were carried out inside a humid chamber. Block buffer (10% (vol / vol) goat serum, 10% (wt / vol) BSA in DPBS) was then added and incubated for 1 hour at room temperature. Sections were then incubated with rabbit polyclonal anti-human lubricin antibody (ab28484, abcam, Cambridge, UK) diluted to 10 µg/ml ((1:100 from the stock) in blocking buffer) overnight at 4ºC.
Sections were rinsed extensively with PBS prior to incubation with the secondary antibody for 1 hour at room temperature. Sections were incubated with horseradish peroxidase (HRP) - conjugated goat anti-rabbit IgG ((R&D Systems, Abingdon, UK) 2 µg/ml in blocking buffer). Amplification of the HRP signal was achieved by incubation with the Vectastain ABC kit (Vector Laboratories, Peterborough, UK) for 30 minutes. After this the 3,3'Diaminobenzidine (DAB) substrate (ab64238, abcam, Cambridge, UK) was added until the characteristic brown stain was observed. Sections were then counterstained by immersion in haematoxylin for 5 minutes.
Sections were then rinsed in DPBS before dehydration using the following protocol:
70% ethanol – 30 seconds
90% ethanol – 30 seconds
100% ethanol – 30 seconds
100% xylene – 1 minute
100% xylene – 1 minute
The slides then had DPX mountant added before a coverslip was placed on each slide. Slides were then left in a cold room at 4ºC, inside a humid chamber and protected from light.

Control reactions were performed in parallel with sections either incubated with a rabbit IgG isotype control antibody ((ab28484, abcam, Cambridge, UK) 10 µg/ml diluted 1:100 in blocking buffer), or no secondary antibody.

All slides were left overnight in a cold room at 4ºC, inside a humid chamber and protected from light.

Sections were images using a light microscope (Leica DM2000 LED, Milton Keynes, UK) with images captured using a digital camera (Leica DFC450 C, Milton Keynes, UK) linked to a desktop PC running LAS v4.9 (Leica, Milton Keynes, UK). Images were captured through red/green filters (Leica, Milton Keynes, UK) and saved as TIFF files.

Fluorescence staining with Alexa Fluor® Plus 488

Slides were briefly rinsed in water before being dabbed dry. Each section was drawn round with a Daco pen. Endogenase activity was blocked on the slides via addition of 0.3% (vol / vol) hydrogen peroxide in methanol for 5 minutes. Slides were incubated with chondroitinase ABC (C3667, Sigma, Gillingham, UK) (0.1 U/ml in buffer (10% (vol / vol) goat serum, 10% (wt / vol) BSA in DPBS)) for 1 hour at 37ºC, all incubations were carried out inside a humid chamber. Block buffer (10% (vol / vol) goat serum, 10% (wt / vol) BSA in DPBS) was then added and incubated for 1 hour at room temperature. Sections were then incubated with dilute rabbit polyclonal anti-human lubricin antibody (ab28484, abcam, Cambridge, UK) diluted to 10 µg/ml ((1:100 from the stock) in blocking buffer) overnight at 4ºC.
Sections were rinsed extensively with PBS prior to incubation with the secondary antibody for 1 hour at room temperature. Sections were incubated with goat anti-rabbit IgG Alexa Fluor® Plus 488 (AFP488) ((A32731, ThermoFisher, UK) 4 µg/ml in blocking buffer). All sections were rinsed with DPBS.
The slides then had 4'6,-dimadino-2-phenylindole (DAPI) Vectorshield (Vector Laboratories, Peterborough, UK) added to each section and coverslips placed on the slides.

Control reactions were performed in parallel with sections either incubated with a rabbit IgG isotype control antibody ((ab28484, abcam, Cambridge, UK) 10 µg/ml diluted 1:100 in blocking buffer), or no secondary antibody.
All slides were left overnight in a cold room at 4ºC, inside a humid chamber and protected from light.

Immunofluorescence treated tissue sections were imaged on a fluorescence microscope (Leica DM2000 LED, Milton Keynes, UK) with LED fluorescence source (CoolLED pE-300^{white}, Leica, Milton Keynes, UK) with images captured using the same camera as used for the HRP chromogen stained images.

Lubricin (PRG4) image scoring

Images were scored via a double-blind scoring method with three people examining images prepared in a 4 x 8 table.

Scoring method

Slides were scored using the following scale with results recorded on a scorecard (see Appendix 2).

1 = No stain

2 = Minimal stain

3 = Intermediate stain

4 = Submaximal stain

5 = Maximal stain

Examples of sections at the upper and lower ends of the scale and controls for imaging with HRP are shown in Fig. 2.9.1.

Examples of sections at the upper and lower ends of the scale and controls for imaging with AFP488 are shown in Fig. 2.9.2.

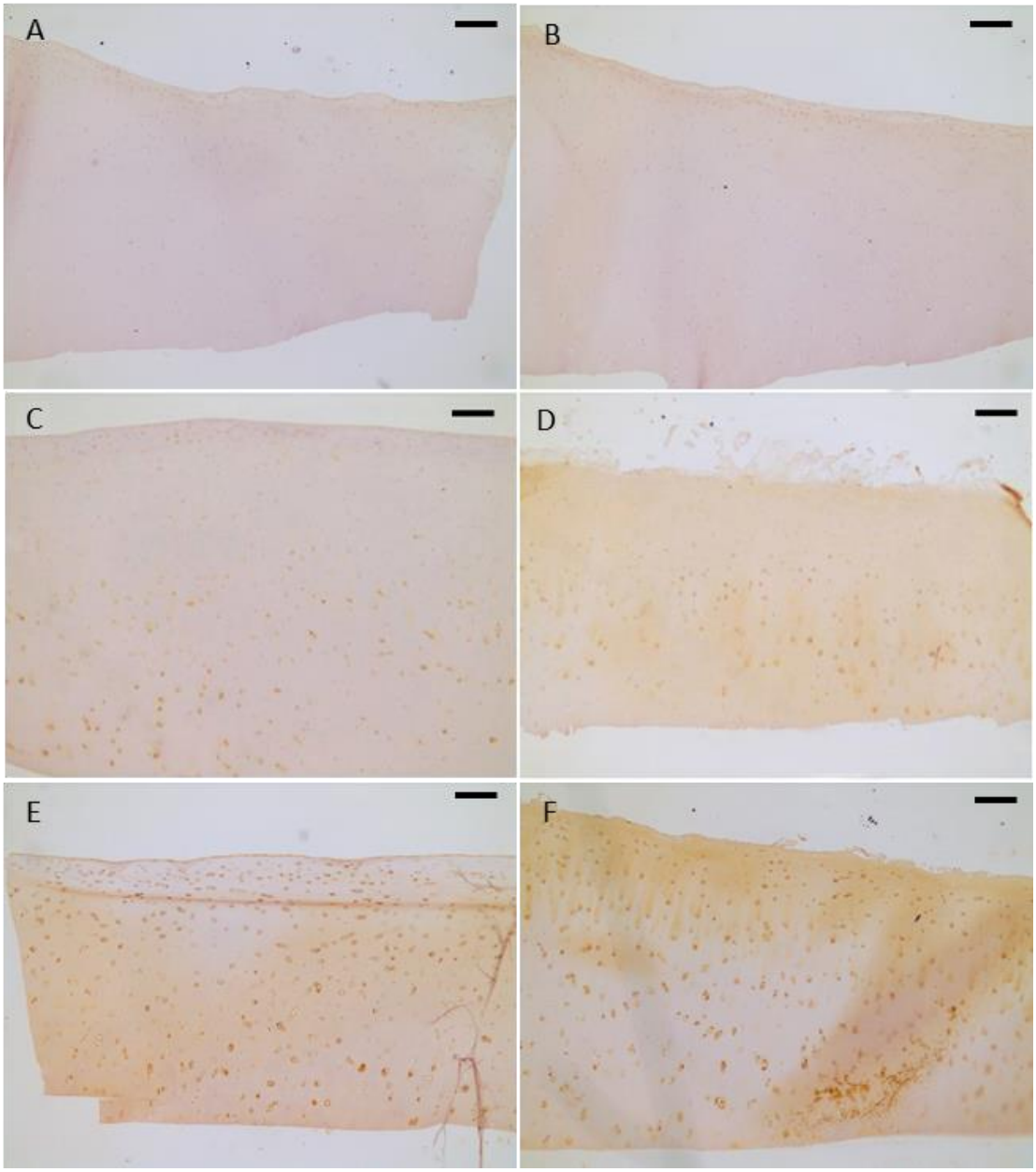

Figure 2.9.1. Sections of cartilage tissue stained via horseradish peroxidase immunohistochemistry demonstrating assigned scores. A) Image of no secondary antibody control, demonstrating an assigned score level of 1. B) Image of isotype control, demonstrating an assigned score level of 1. C) Image from normal donor cohort (<45 year old), demonstrating an assigned score level of 2. D) Image from osteoarthritic living donor cohort, demonstrating an assigned score level of 3. E) Image from over-age donor cohort (>45 year old), demonstrating an assigned score level of 4. F) Image from positive control, demonstrating an assigned score level of 5. Magnification (x50) scale bars 100 μm.

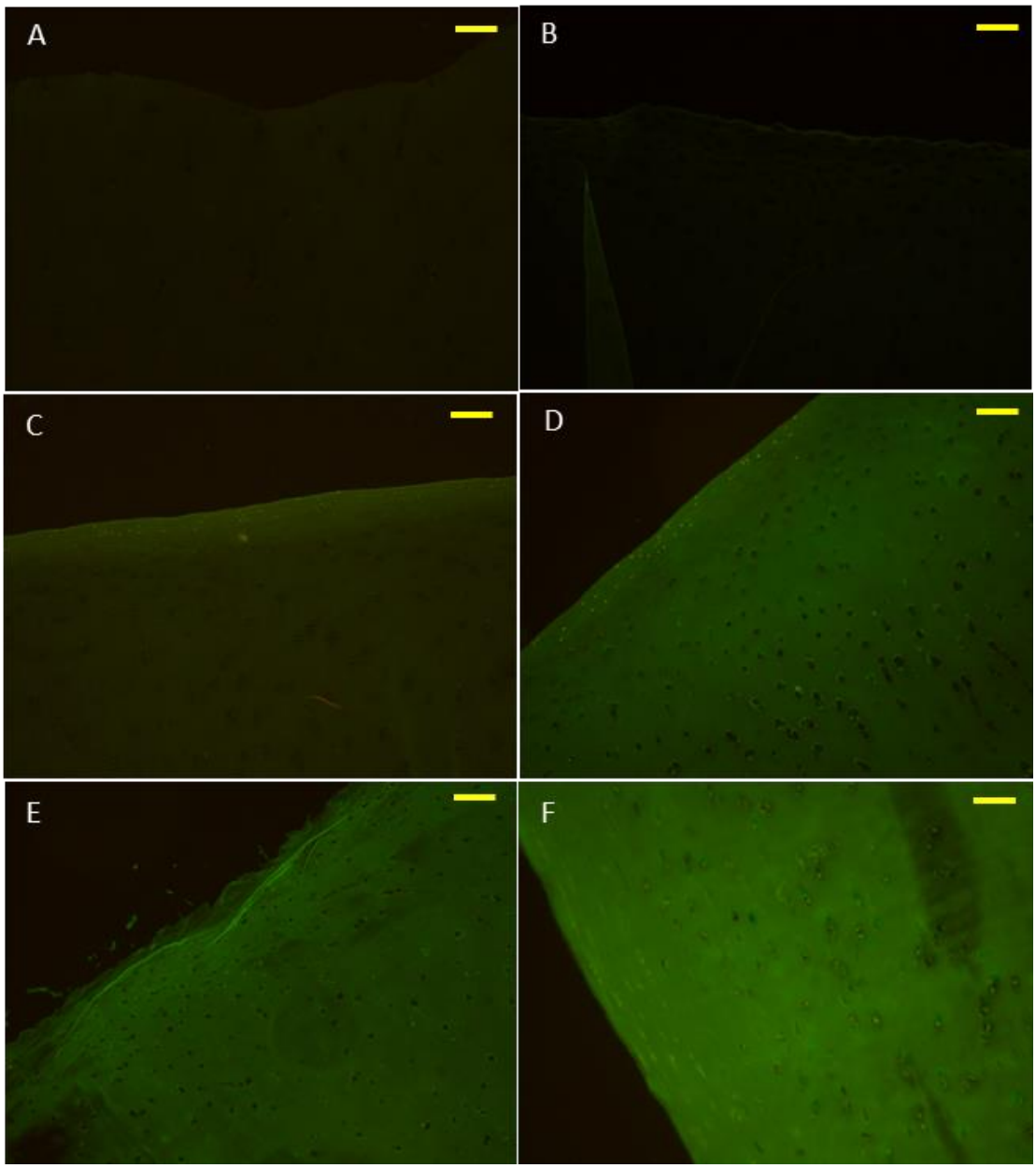

Figure 2.9.2. Sections of cartilage tissue stained via Alexa Fluor Plus® 488 fluorescence demonstrating assigned scores. A) Image of no secondary antibody control, demonstrating an assigned score level of 1. B) Image of isotype control, demonstrating an assigned score level of 1. C) Image from normal donor cohort (<45 year old), demonstrating an assigned score level of 2. D) Image from normal donor cohort, demonstrating an assigned score level of 3. E) Image from over-age donor cohort (>45 year old), demonstrating an assigned score level of 4. F) Image from positive control, demonstrating an assigned score level of 5. Magnification (x100) scale bars 200 µm

2.10 - Safranin O histology methodology

Safranin O staining, with fast green counterstaining was performed to identify the presence of proteoglycans and glycosaminoglycans within the matrix of the articular cartilage.

Safranin O assay mechanism of action

Safranin O binds to the negatively-charged glycosaminoglycans on proteoglycans in cartilage with strong affinity, if these aren't present, or in low amounts the counter-stain fast green is more prevalent in the sections. Fast green stains cytoplasmic proteins.

Fixing and embedding of biopsies

The protocol described in section 2.9 for the same donor cohorts as shown in Table 2.9.2 was carried out on biopsies of cartilage tissue.

Preparation of sections

Cartilage biopsies were further processed and cut into sections as described in section 2.9, before staining was carried out according to the method shown in Table 2.10.1.

Safranin O staining

Once the sections of cartilage tissue had been fixed onto the microscope slides, they were taken through the staining process shown in Table 2.10.2.
After following the protocol shown in Table 2.10.2 the slides were placed on a level surface before DPX (LAMB/DPX, Thermo Scientific, Runcorn, UK) was placed on top of the stained sections and a glass coverslip gently lowered onto the slides. The slides were then allowed to air dry.

Image capture

All slides were imaged on a light microscope (Leica DM2000 LED, Milton Keynes, UK) with images captured using a digital camera (Leica DFC450 C, Milton Keynes, UK) linked to a desktop PC running LAS v4.9 (Leica, Milton Keynes, UK).

Table 2.10.1. Protocol for preparing, and staining cartilage sections with safranin O and fast green.[a]

Chemical	Details	Dwell time (mins)
100% xylene	28975.360, VWR, Lutterworth, UK	5.0
100% ethanol	302444E, VWR, Lutterworth, UK	5.0
90% (vol / vol) ethanol		5.0
70% (vol / vol) ethanol		5.0
Sterile water for irrigation	UK F7114, Baxter, Northampton, UK	2.0
Harris hematoxylin	6765004, Thermo Scientific, Runcorn, UK	10.0
Tap water		1.0
0.1% Fast green FCF (wt / vol)	F7258, Sigma, Gillingham, UK	5.0
1% Glacial acetic acid (vol / vol)	A6283, Sigma, Gillingham, UK	0.5
0.1% Safranin O (wt / vol)	S8884, Sigma, Gillingham, UK	15.0
Sterile water for irrigation	UK F7114, Baxter, Northampton, UK	0.1
90% (vol / vol) ethanol	302444E, VWR, Lutterworth, UK	0.5
100% ethanol		0.5
100% ethanol		1.0
100% xylene	28975.360, VWR, Lutterworth, UK	10.0
100% xylene		10.0

[a]Safranin O prepared in sterile water for irrigation, fast green prepared in 1% glacial acetic acid with cartilage sections placed on Polysine microscope slides

Analysis of percent coverage of safranin O in sections

Each image was opened in ImageJ v1.50i (Fiji) running in a Windows 10 operating system. Images were then processed using the software according to the method shown in Fig. 2.10.1. Once measurements of pixel area had been made they were copied from the results window of ImageJ into Excel. Percent area of safranin O coverage in each section was then calculated using equation 5.

$$(5) \quad \text{Safranin O (\%)} = \frac{\text{Pixel area of safranin O}}{\text{Total pixel area of safranin O and fast green}} \times 100$$

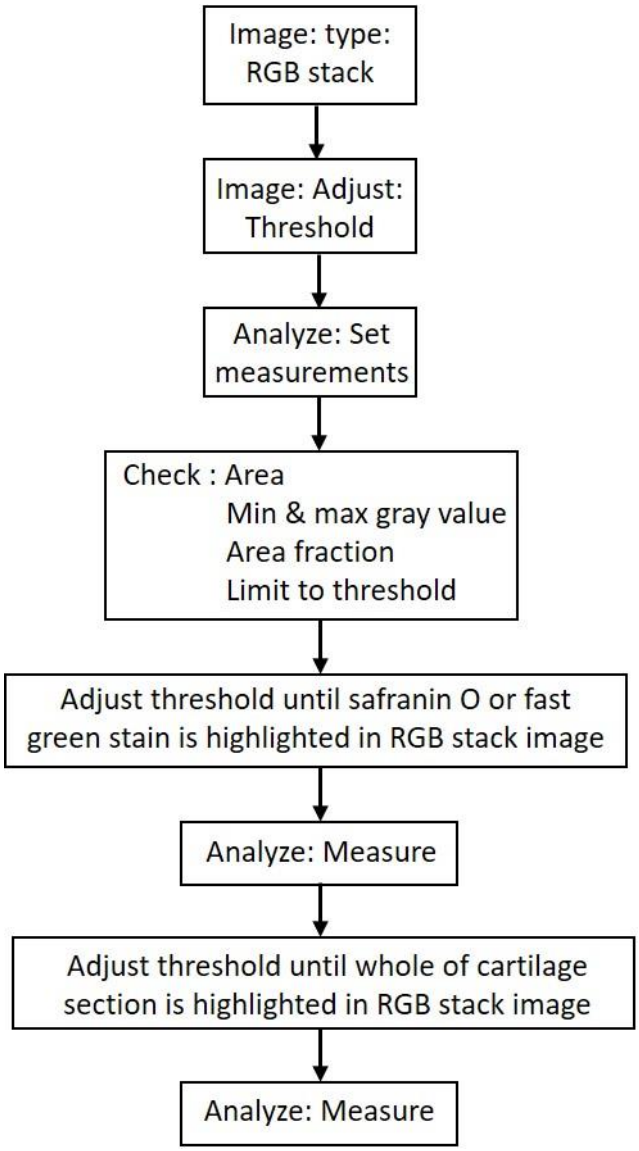

Figure 2.10.1. Flow diagram of the method used to process and analyse safranin O jpeg images using the ImageJ (Fiji) analysis software

2.11 - Statistical analysis

Data was arranged and grouped in Excel according to experimental cohorts.

Tables were created to arrange the data in a format suitable for use in MetaboAnalyst (www.metaboanalyst.ca) as shown in Tables 2.11.1 and 2.11.2. To compare data between cohorts it was arranged in the format shown in Table 2.11.1, for comparison between storage days the data was arranged as shown in Table 2.11.2.

Table 2.11.1. Example of Excel table layout for data to be analysed in MetaboAnalyst.[a]

name	group	Lactate level week 1	Glucose level week 1	Lactate level week 2
normal1	normal			
normal2	normal			
normal3	normal			
overage1	overage			
overage2	overage			
overage3	overage			
OA1	OA			
OA2	OA			
OA3	OA			

[a]Table organised to compare data between donor cohorts, name and group columns contain cohort groups that will be compared, normal (<45 years of age), over-age (>45 years of age), and OA was living donor osteoarthritic. Glucose and lactate week columns show metabolite turnover during corresponding week (μmol/cm^2/day)

Table 2.11.2. Example setup of Excel table for data to be analysed in MetaboAnalyst.[a]

	Label	Lactate level	Glucose level
Normal1.1	Week 1		
Normal2.1	Week 1		
Normal3.1	Week 1		
Normal1.2	Week 2		
Normal2.2	Week 2		
Normal3.2	Week 2		
Normal1.3	Week 3		
Normal2.3	Week 3		

[a]Table organised to compare data between weeks. Glucose and lactate columns show metabolite turnover (μmol/cm^2/day)

The procedure shown in Fig. 2.12.1 was used to enter data arranged in either Table 2.12.1 or 2.12.2 into MetaboAnalyst. Data integrity check was skipped as this uses a missing value algorithm which isn't required and no sample normalisation was carried out using the analysis package. The spreadsheet icon was selected to view statistical analysis. *Post-hoc* analysis remained as Fisher's LSD. Download was selected in the left-hand options pane and from the results window the Download.zip, data_normalized.csv, anove_posthoc.csv and

data_processed.csv files were saved. From this data, any significant differences ($p < 0.05$) between cohorts or weeks was determined.

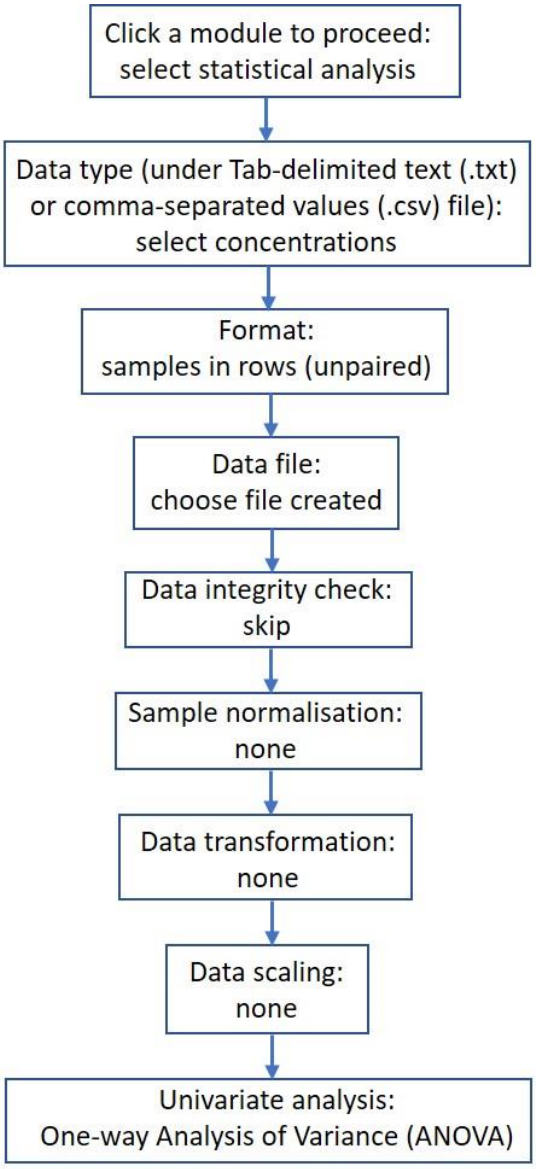

Figure 2.12.1. Flow diagram showing process of data analysis used in MetaboAnalyst, a web based statistical analysis tool, on data generated from one of the assays used in this study

Chapter 3

Cartilage Assay Results

3.1 - Glucose/lactate assay results

Glucose

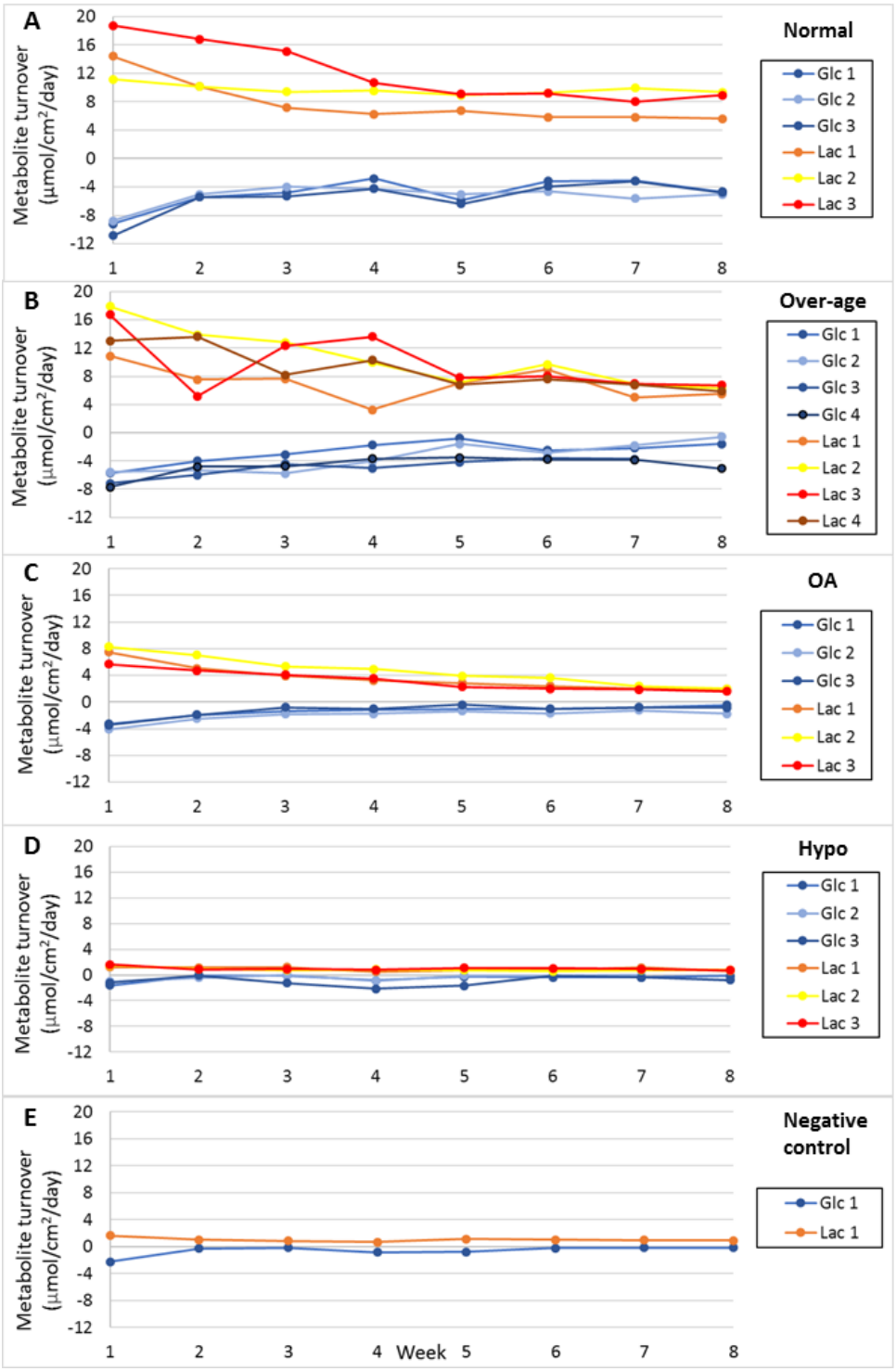

Figure 3.1.1. Scatter charts of data obtained using glucose/lactate meter. A) Normal donor cartilage (<45 years old). B) Over-age donor cartilage (>45 years old). C) Living donor osteoarthritic cartilage (OA). D) Hypothermic (Hypo, 4ºC) stored cartilage. E) Negative control. Glc = glucose, Lac = lactate

Variance within cohorts

Glucose consumption in week 1 for the normal donor samples (n = 3) was higher than at any point throughout the storage period, with a range of -8.8 µmol/cm^2/day to -11.6 µmol/cm^2/day (Fig. 3.1A). Consumption then decreased at week 2 to a range of -5.0 µmol/cm^2/day to -5.4 µmol/cm^2/day. Consumption then decreased until week 4 where a range of -2.8 µmol/cm^2/day to -4.3 µmol/cm^2/day was observed. After consumption briefly increased at week 5 (range -5.0 µmol/cm^2/day to -6.4 µmol/cm^2/day) Glucose consumption stabilised between weeks 6 to 8 with the range at week 8 of -4.7 µmol/cm^2/day to -5.0 µmol/cm^2/day.

In over-age donor samples (n = 4) week 1 glucose consumption was also greatest with range of -5.6 µmol/cm^2/day to -7.7 µmol/cm^2/day (Fig. 3.1.1B). At week 2 consumption had decreased to a range of -4.0 µmol/cm^2/day to -6.2 µmol/cm^2/day. Thereafter there was a week on week decrease until week 5 (range -0.8 µmol/cm^2/day to -4.5 µmol/cm^2/day). Consumption then stabilised at week 6 with a range of -2.5 µmol/cm^2/day to -3.8 µmol/cm^2/day with no significant differences identified beyond this. Beyond this the range of consumption increased by week 8 to -0.5 µmol/cm^2/day to -5.1 µmol/cm^2/day.

With the OA donor samples (n = 3) glucose consumption was again highest at week 1 with a range of -3.3 µmol/cm^2/day to -4.1 µmol/cm^2/day (Fig. 3.1.1C). Consumption then decreased until week 3 with a range of -0.8 µmol/cm^2/day to -1.8 µmol/cm^2/day. From week 4 onwards consumption appeared to be relatively stable with range at week 4 of -1.0 µmol/cm^2/day to -1.8 µmol/cm^2/day, and then at week 8 range of -0.4 µmol/cm^2/day to -1.8 µmol/cm^2/day.

In hypothermic stored tissue (n = 3) glucose consumption was limited and different donor samples behaved differently with week 4 the highest consumption of -2.7 µmol/cm^2/day was observed (Fig. 3.1.1D). Week 1 (range -1.1 µmol/cm^2/day to -1.5 µmol/cm^2/day) however did have higher consumption compared to week 2 (range -0.1 µmol/cm^2/day to -0.4 µmol/cm^2/day). Overall glucose consumption remained below -1.0 µmol/cm^2/day for the remainder of the 8 weeks shown. At week 8 the range of consumption was -0.1 µmol/cm^2/day to -0.8 µmol/cm^2/day.

The single negative control donor showed a peak glucose consumption of -2.3 µmol/cm^2/day at week 1 (Fig. 3.1.1E). Thereafter consumption remained below -1.0 µmol/cm^2/day for the rest of the 8-week storage period.

Variance between cohorts

Mean data showed that in the normal donor samples, glucose consumption was consistently higher than that in the over-age donor samples, starting at week 1 where normal was -9.6 µmol/cm^2/day and over-age was -6.6 µmol/cm^2/day (Table 3.1.1 and Fig. 3.1.2, *p*-value for normal vs over-age = 2.0×10^{-5}). For the next 3 weeks the difference between these cohorts was smaller with a maximum of -0.3 µmol/cm^2/day difference that occurred on week 2 (*p*-value for normal vs over-age = 1.9×10^{-5}). From week 5 onwards however, the glucose consumption in over-age donor samples decreased compared to normal donors' samples, so that by week 8 over-age glucose consumption was -2.5 µmol/cm^2/day, while normal was -4.8 µmol/cm^2/day (*p*-value for normal vs over-age = 0.0080).

Table 3.1.1. Summary of data for glucose consumption from storage medium.[a]

Concentration (glucose)	**Mean metabolite turnover (µmol/cm^2/day ± standard deviation)**								**ANOVA**
	Wk1	**Wk2**	**Wk3**	**Wk4**	**Wk5**	**Wk6**	**Wk7**	**Wk8**	***P***
Normal	-9.6 ± 1.1	-5.3 ± 0.2	-4.7 ± 0.7	-3.8 ± 0.8	-5.8 ± 0.7	-3.9 ± 0.7	-4.0 ± 1.4	-4.8 ± 0.2	8.4×10^{-6}
Over-age	-6.6 ± 1.1	-5.0 ± 0.8	-4.5 ± 1.1	-3.6 ± 1.4	-2.5 ± 1.6	-3.2 ± 0.6	-2.9 ± 1.1	-2.5 ± 2.0	0.0025
Hypothermic	-1.3 ± 0.3	-0.2 ± 0.2	-0.4 ± 0.7	-1.3 ± 0.7	-0.7 ± 0.9	-0.2 ± 0.1	-0.2 ± 0.2	-0.5 ± 0.3	0.076
Osteoarthitis	-3.6 ± 0.4	-2.1 ± 0.3	-1.3 ± 0.5	-1.3 ± 0.4	-0.9 ± 0.5	-1.3 ± 0.4	-0.9 ± 0.3	-1.0 ± 0.7	2.2×10^{-5}

[a]Data shows means with standard deviation from normal (<45 years of age), over-age (>45 years old), hypothermic (4ºC) and osteoarthritic (living donors) where at least 3 donor samples were obtained (over-age donor samples n = 4), negative control not included due to lack of biological replicates

When mean data of glucose consumption from normal donor samples was compared with living donor OA the difference was even greater than that with over-age, with week 1 normal at -9.6 µmol/cm^2/day and OA at -3.6 µmol/cm^2/day (Table 3.1.1 and Fig. 3.1.2 (*p*-value for normal vs living donor OA = 2.0×10^{-5})). Consumption was consistently higher in the normal donor samples compared to OA throughout the 8 week storage period shown with consumption in the OA cohort having dropped to as little as -0.9 µmol/cm^2/day on weeks 5 and 7. At week

8 mean consumption was at -4.8 µmol/cm^2/day in normal donors, whereas in the OA donors consumption was only -1.0 µmol/cm^2/day (*p*-value for normal vs living donor OA = 0.0080).

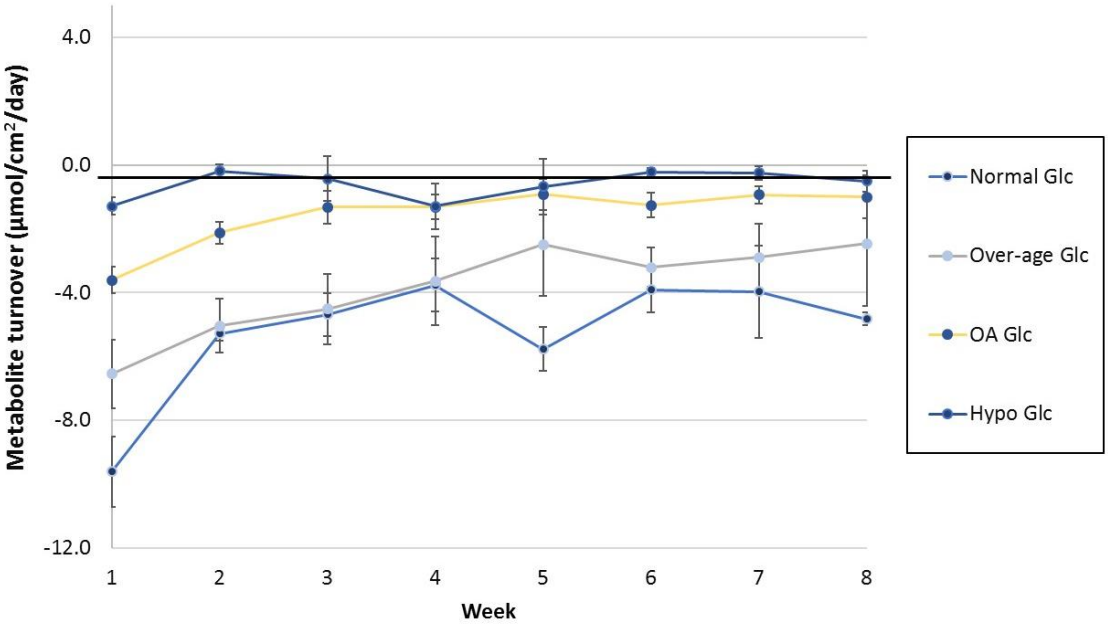

Figure 3.1.2. Scatter chart of mean data obtained using glucose/lactate meter of glucose data from normal donor cartilage (<45 years old), over-age donor cartilage (>45 years old), living donor osteoarthritic cartilage (OA) and hypothermic (Hypo, 4ºC) stored. Glc = glucose, n = 3 biological replicates except for over-age where n = 4 with error bars showing standard deviation and black bar showing baseline calculated from mean negative control values, negative control and bone only not compared due to lack of biological replicates

Comparison of mean data between normal and hypothermic donor cohorts showed that glucose consumption was consistently higher in the normal donor samples compared to hypothermic, with week 1 normal at -9.6 µmol/cm^2/day and hypothermic at -1.3 µmol/cm^2/day (Table 3.1.1 and Fig. 3.1.2 (*p*-value for normal vs hypothermic = 2.0×10^{-5}). By week 8 the consumption was -4.8 µmol/cm^2/day for normal donor samples compared to -0.5 µmol/cm^2/day for hypothermic (*p*-value for normal vs hypothermic = 0.0080).

For over-age donor samples the mean data compared with OA showed that the over-age cohort was also consistently consuming more glucose, with week 1 at -6.6 µmol/cm^2/day compared to -3.6 µmol/cm^2/day (Table 3.1.1 and Fig. 3.1.2 (*p*-value for over-age vs OA = 2.0×10^{-5})). This trend continued throughout the storage period in both cohorts, so that by week 8 the mean data for over-age was -2.5 µmol/cm^2/day and -1.0 µmol/cm^2/day for OA (*p*-value for over-age vs OA = 0.28).

Data from over-age donor samples showed that this cohort also had consistent higher mean glucose consumption compared to hypothermic with week 1 at -6.6 µmol/cm^2/day for over-age, with -1.3 µmol/cm^2/day for hypothermic (Table 3.1.1 and Fig. 3.1.2 (*p*-value for over-age vs hypothermic = 2.0×10^{-5})). By week 8 over-age was at -2.5 µmol/cm^2/day compared to -0.5 µmol/cm^2/day for hypothermic (*p*-value for over-age vs hypothermic = 0.15).

In contrast to all other comparisons made with mean data, OA and hypothermic cohorts mean glucose consumption was much closer to each other. Week 1 data showed that OA was at -3.6 µmol/cm^2/day and hypothermic -1.3 µmol/cm^2/day (Table 3.1.1 and Fig. 3.1.2 (*p*-value for OA vs hypothermic = 2.0×10^{-5})). At week 4 the mean consumption was the same for both cohorts at -1.3 µmol/cm^2/day. By week 8 mean consumption was at -1.0 µmol/cm^2/day for the OA cohort and -0.5 for hypothermic (*p*-value for OA vs hypothermic = 0.36).

Table 3.1.2. Statistical analysis of glucose consumption by osteochondral allografts from culture medium.[a]

Week	Glucose *P*-Value	Glucose Fisher's *post-hoc* (cohorts)
1	2.0×10^{-5}	H-N, H-OA, H-O, OA-N, O-N, OA-O
2	1.9×10^{-5}	H-N, H-OA, H-O, OA-N, OA-O
3	0.00034	H-N, H-OA, OA-N, OA-O
4	0.012	H-N, H-OA, OA-N, OA-O
5	0.0016	H-N, OA-N, O-N
6	9.1×10^{-5}	H-N, H-OA, H-O, OA-N, OA-O
7	0.0034	H-N, H-OA, OA-N, OA-O
8	0.0080	H-N, OA-N, O-N

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4ºC) donor cohorts. H = hypothermic, N = normal, OA = osteoarthritis, O = over-age from weeks 1 to 8 of storage. *Post-hoc* result shows which cohorts had significant difference (FDR adjusted) with each other, negative control not compared due to lack of replicates

Statistical analysis showed that at week 1 there was a significant difference (FDR adjusted $p = 2.0 \times 10^{-5}$) in glucose consumption between all cohorts (Table 3.1.2). At week 2 however there was no longer a significant difference ($p > 0.05$) between normal and over-age donor cohorts, with significant difference still having occurred between all other cohorts (FDR adjusted $p = 1.9 \times 10^{-5}$) (Table 3.1.2).

At weeks 3 and 4 there was no longer a significant difference ($p > 0.05$) between hypothermic and over-age donor cohorts, as well as normal and over-age (Table 3.1.2). A significant difference was still observed between the remaining cohorts with FDR adjusted $p = 0.00034$ at week 3 and FDR adjusted $p = 0.012$ at week 4.

Week 5 had the joint fewest cohorts, with three demonstrating significant difference (FDR adjusted $p = 0.0016$) between each other, with normal and hypothermic, normal and over-age and normal and OA having demonstrated this difference (Table 3.1.2).

Week 6 had the same pattern of significant difference (FDR adjusted $p = 9.1 \times 10^{-5}$) as that which had occurred in week 2, with no significant difference observed between normal and over-age donor cohorts ($p > 0.05$) (Table 3.1.2). Week 7 shared the same pattern of significant difference (FDR adjusted $p = 0.0034$) as that observed in weeks 3 and 4 (Table 3.1.2), and week 8 the significant difference (FDR adjusted $p = 0.0080$) occurred between the same cohorts as in week 5 (Table 3.1.2).

Table 3.1.3. Statistical analysis of glucose consumption by osteochondral allografts from culture medium.[a]

Cohort	Glucose *P*-Value	Glucose Fisher's *post-hoc* (weeks)
Normal	8.4×10^{-6}	1-(2-8), 4-2, 4-5, 6-5, 7-5
Over-age	0.0025	1-(3-8), 5-2, 7-2, 8-2, 5-3, 8-3
Osteoarthritis	2.2×10^{-5}	1-(2-8), 2-(3-8)
Hypothermic	0.076	N/A

[a]Analysis of variance compares weeks 1 to 8 of storage, numbers in parentheses represent the range of weeks significantly different from the week number outside of parentheses, for example 1-(2-8) means week 1 is significantly different from weeks 2 to 8. Normal cohort were donors <45 years old, over-age were <45 years old, osteoarthritis were living donors and hypothermic was tissue stored at 4°C. *Post-hoc* result shows which cohorts had significant difference (FDR adjusted) with each other, negative control not compared due to lack of biological replicates

In normal donors, glucose consumption at week 1 was shown to be significantly different (FDR adjusted $p = 8.4 \times 10^{-6}$) from that at any other week. ANOVA results also showed significant difference between weeks 4 and 2, 4 and 5, 6 and 5 as well as 7 and 5 (Table 3.1.3 and Fig. 3.1.3A).

With over-age donor samples there was a significant difference (FDR adjusted $p = 0.0025$) in glucose consumption between weeks 1 and 3 to 8. There was also significant difference between weeks 5 and 2, 7 and 2, 8 and 2, then 5 and 3 as well as 8 and 3 (Table 3.1.3 and Fig. 3.1.3B).

For OA tissue a significant difference (FDR adjusted $p = 2.2 \times 10^{-5}$) was seen between week 1 and all other weeks, as well as week 2 with weeks 3 to 8 (Table 3.1.3 and Fig. 3.1.3C). Finally, in hypothermic stored tissue, no significant difference was observed (FDR adjusted $p = 0.076$) between all weeks (Table 3.1.3 and Fig. 3.1.3D).

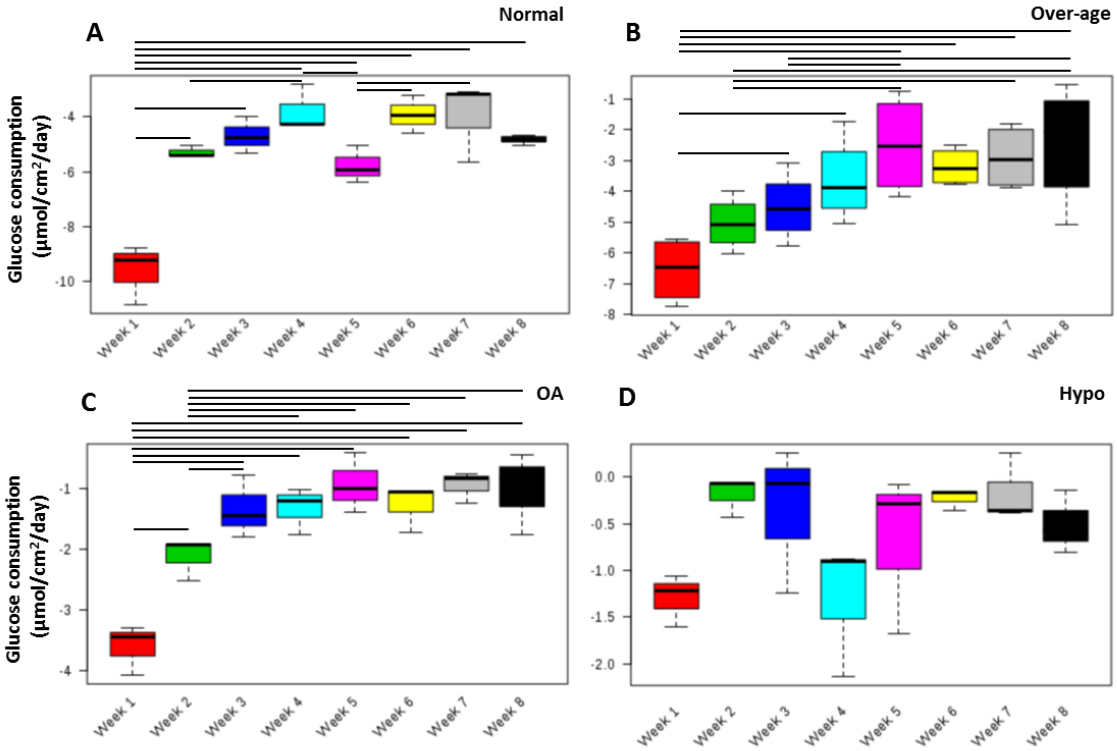

Figure 3.1.3. Box plots showing results from analysis of variance carried out on weeks 1 to 8 comparing weekly glucose consumption. A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) Osteoarthritic (OA). D) Hypothermic (Hypo, 4°C). Significance bars (FDR adjusted) show $p < 0.01$, negative control not compared due to lack of biological replicates

In summary for glucose consumption the data suggested that at the end of week 1 the normal donor cohort had a significantly higher consumption than all other cohorts, with over-age having the second highest, OA the third highest with hypothermic having the lowest consumption. Thereafter the normal and over-age donor cohorts only had significant difference from each other in weeks 5 and 8, therefore following a similar trend through the 8 weeks

analysed. Tissue from OA and hypothermic cohorts demonstrate a similar trend to each other, although it was only in weeks 5 and 8 where there was no significant difference between these cohorts. It was only the hypothermic cohort that didn't show a significant difference between week 1 and subsequent weeks of storage.

Lactate

Variance within cohorts

For normal donor samples, lactate secretion at week 1 was at its highest throughout the 8-week storage period with a range of 11.2 µmol/cm^2/day to 18.7 µmol/cm^2/day (Fig. 3.1.1A). At week 2 this had dropped to a range of 10.2 µmol/cm^2/day to 16.8 µmol/cm^2/day. By week 5 the decline in lactate secretion had stabilised to a range of 6.7 µmol/cm^2/day to 9.1 µmol/cm^2/day. At week 8 the range had increased to 5.6 µmol/cm^2/day to 9.3 µmol/cm^2/day.

With over-age donor samples, lactate secretion was more erratic in some of the donor samples compared to other cohorts, at week 1 the range was 10.9 µmol/cm^2/day to 17.9 µmol/cm^2/day (Fig. 3.1.1B). By week 2 the range of secretion was 5.2 µmol/cm^2/day to 13.9 µmol/cm^2/day. With increase and decrease occurring within individual donor samples it was not until week 5 that lactate secretion became more stable, with a range of 6.8 µmol/cm^2/day to 7.1 µmol/cm^2/day. By week 8 the range was similar to week 5 with values from 5.5 µmol/cm^2/day to 6.7 µmol/cm^2/day.

For OA tissue the lactate secretion at week 1 was higher than at any point during the remainder of the 8-week storage period with a range of 5.7 µmol/cm^2/day to 8.3 µmol/cm^2/day (Fig. 3.1.1C). At week 2 lactate secretion had dropped to a range of 4.7 µmol/cm^2/day to 7.0 µmol/cm^2/day. Secretion progressively dropped at each week so that at week 8 it had dropped to a range of 1.6 µmol/cm^2/day.

In the same way as glucose, lactate secretion in hypothermic stored tissue was greatly reduced compared to normal, over-age and OA cohorts. At week 1 the range of lactate secretion was 1.2 µmol/cm^2/day to 1.6 µmol/cm^2/day (Fig. 3.1.1D). As the secretion level for lactate was relatively low in the hypothermic stored tissue, there was not much scope for a progressive decline during the 8-week storage period, by week 8 the range was 0.6 µmol/cm^2/day to 0.8 µmol/cm^2/day.

A similar pattern to hypothermic tissue was observed in freeze thawed negative control tissue with the amount of lactate secretion at week 1 at 1.6 µmol/cm^2/day (Fig. 3.1.1E). By week 8 this had dropped to just 0.9 µmol/cm^2/day.

Variance between cohorts

At weeks 1 to 8 of storage significant differences in lactate secretion were observed between different cohorts as shown in Table 3.1.4. The cohorts that had the most significant differences between each other occurred in weeks 1 (p = 0.00062), 5 (p = 1.9 x 10^{-5}) and 8 (p = 7.7 x 10^{-5}) of storage, with the fewest significant differences between donor samples (or cohorts) occurring in weeks 2 (p = 0.012) and 4 (p = 0.011).

Table 3.1.4. Summary of data for lactate secretion into storage medium.[a]

Concentration (lactate)	**Mean metabolite turnover (µmol/cm^2/day ± standard deviation)**								**ANOVA**
	Wk1	**Wk2**	**Wk3**	**Wk4**	**Wk5**	**Wk6**	**Wk7**	**Wk8**	***P***
Normal	14.8 ± 3.8	12.4 ± 4.6	10.6 ± 4.1	8.8 ± 2.3	8.3 ± 1.3	8.1 ± 2.0	7.9 ± 2.1	8.0 ± 2.0	0.072
Over-age	14.6 ± 3.3	10.1 ± 4.4	10.3 ± 3.8	9.3 ± 4.3	7.2 ± 0.4	8.6 ± 0.9	6.4 ± 0.9	6.1 ± 0.5	0.0041
Hypothermic	1.4 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	0.011
Osteoarthitis	7.2 ± 1.4	5.6 ± 1.2	4.5 ± 0.8	3.9 ± 0.9	3.0 ± 0.8	2.7 ± 0.8	2.1 ± 0.3	1.8 ± 0.2	2.2 x 10^{-5}

[a]Data shows mean with standard deviation from normal (<45 years of age), over-age (>45 years old), hypothermic (4ºC) and osteoarthritic (living donors) where at least three donor samples were obtained (over-age donors n = 4), negative control not included due to lack of biological replicates

Mean data showed that between normal and over-age cohorts, lactate secretion was closely related, with week 1 values at 14.8 µmol/cm^2/day for normal and 14.6 µmol/cm^2/day for over-age (Table 3.1.4 and Fig. 3.1.4) (p-value for normal vs over-age = 0.96). At week 2 the difference had increased with normal at 12.4 µmol/cm^2/day and over-age at 10.1 µmol/cm^2/day (p-value for normal vs over-age = 0.76). Thereafter the values were similar again until week 8 when normal was 8.0 µmol/cm^2/day and over-age 6.1 µmol/cm^2/day (p-value for normal vs over-age = 0.49).

When mean lactate data from the normal cohort was compared with the living OA cohort, it was consistently higher during the storage period. At week 1 lactate secretion from normal donor samples was double that seen in OA, with 14.8 µmol/cm^2/day for normal, compared to 7.2 µmol/cm^2/day for OA (Table 3.1.4 and Fig. 3.1.4 (*p*-value for normal vs OA = 0.00062). This doubling factor continued until week 4, after that normal lactate secretion remained relatively stable and OA decreased to the point where at week 8 normal secretion was 8.0 µmol/cm^2/day and OA was 1.8 µmol/cm^2/day (*p*-value for normal vs OA = 7.7 x 10^{-5}).

For the comparison between normal and hypothermic donor cohorts it was found that mean lactate secretion was consistently higher in the normal donor samples, with week 1 values at 14.8 µmol/cm^2/day for normal and 1.4 µmol/cm^2/day for hypothermic (Table 3.1.4 and Fig. 3.1.4 (*p*-value for normal vs hypothermic = 0.00062). By week 8 normal had decreased to 8.0 µmol/cm^2/day while hypothermic had decreased to 0.7 µmol/cm^2/day (*p*-value for normal vs hypothermic = 7.7 x 10^{-5}).

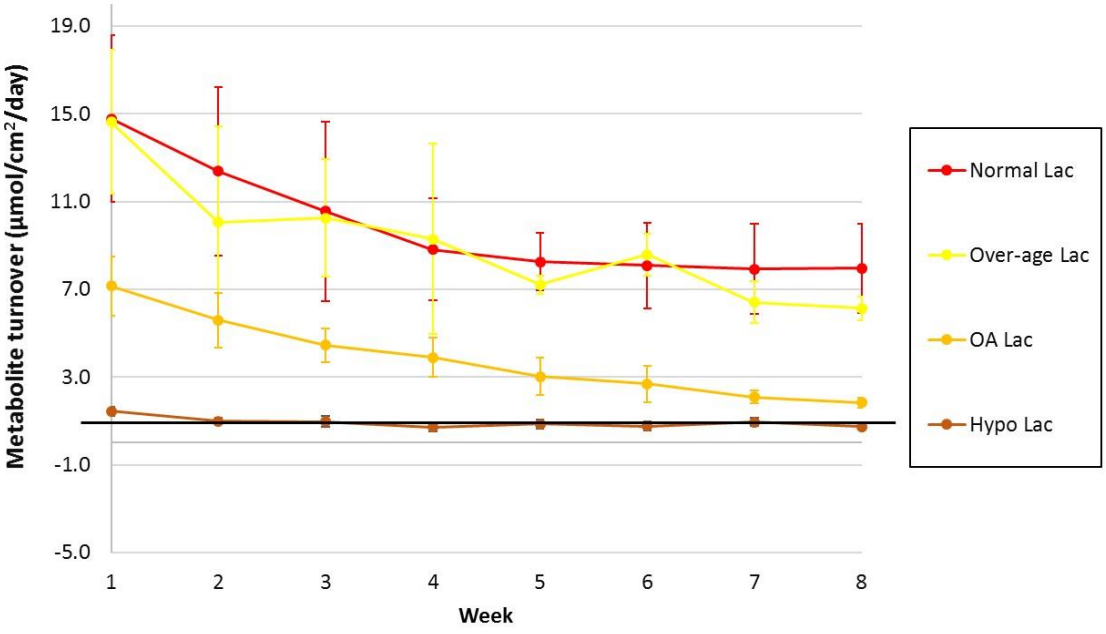

Figure 3.1.4. Scatter chart of mean data obtained using glucose/lactate meter for lactate secretion from osteochondrals into culture medium. Normal donor (<45 years old). Over-age donor (>45 years old), living donor osteoarthritic (OA), and hypothermic (Hypo, 4ºC) stored, Lac = lactate, n = 3 except for over-age where n = 4 with error bars showing standard deviation and black line showing baseline calculated from mean negative control values, negative control and bone only samples not compared due to lack of biological replicates

With over-age and living donor OA cohorts, it was found that mean lactate secretion from OA was approximately half that of over-age tissue. At week 1, secretion from over-age tissue was 14.6 μmol/cm^2/day, while OA was 7.2 μmol/cm^2/day (Table 3.1.4 and Fig. 3.1.4 (*p*-value for over-age vs OA = 0.00062). By week 2 mean secretion had decreased to a mean of 10.1 μmol/cm^2/day in the over-age cohort while in the OA cohort secretion had decreased to 5.6 μmol/cm^2/day. At week 5 onwards secretion from OA tissue was decreasing while in the over-age cohort secretion was relatively stable, so that by week 8 over-age was at 6.1 μmol/cm^2/day compared to 1.8 μmol/cm^2/day for OA (*p*-value for over-age vs OA = 7.7 x 10^{-5}).

Mean data from both over-age and living donor OA cohorts was consistently higher than the hypothermic cohort. At week 1 over-age and OA lactate secretion were at 14.6 μmol/cm^2/day and 7.2 μmol/cm^2/day respectively compared to 1.4 μmol/cm^2/day for hypothermic (Table 3.1.4 and Fig. 3.1.4 (*p*-value for over-age vs hypothermic = 0.00062)). By week 8 over-age and OA lactate secretion had decreased to 6.1 μmol/cm^2/day and 1.8 μmol/cm^2/day respectively, while secretion from hypothermic tissue was 0.7 μmol/cm^2/day (*p*-value for over-age vs hypothermic = 7.7 x 10^{-5}).

Table 3.1.5. Statistical analysis of lactate secretion from osteochondral allografts in to culture medium.[a]

Week	Lactate *P*-Value	Lactate Fisher's *post-hoc* (cohorts)
1	0.00062	N-H, OA-H, O-H, N-OA, O-OA
2	0.012	N-H, O-H, N-OA
3	0.0032	N-H, O-H, N-OA, O-OA
4	0.011	N-H, O-H, N-OA
5	1.9 x 10^{-5}	N-H, OA-H, O-H, N-OA, O-OA
6	7.7 x 10^{-5}	N-H, O-H, N-OA, O-OA
7	0.00018	N-H, O-H, N-OA, O-OA
8	7.7 x 10^{-5}	N-H, O-H, N-OA, N-O, O-OA

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4^{o}C) donor cohorts. H = hypothermic, N = normal, OA = osteoarthritis, O = over-age. *Post-hoc* result shows which cohorts were significantly different (FDR adjusted) to each other during each storage week, negative control not compared due to lack of biological replicates

Statistical analysis showed that in week 1 there was significant difference (FDR adjusted p = 0.00062) between all cohorts except normal and over-age (FDR adjusted $p > 0.05$) (Table 3.1.5). By week 2 there was significant difference (FDR adjusted $p = 0.012$) between three sets of cohorts: normal and hypothermic, over-age and hypothermic, and normal and OA (Table 3.1.5).

At week 3 there was a significant difference (FDR adjusted $p = 0.0032$) between four sets of cohorts: normal and hypothermic, over-age and hypothermic, normal and OA, and over-age and OA (Table 3.1.5). Week 4 had significant difference (FDR adjusted $p = 0.011$) between normal and hypothermic, over-age and hypothermic, and normal and OA cohorts (Table 3.1.5). In weeks 6 and 7 a significant difference (FDR adjusted $p = 7.7 \times 10^{-5}$ and $p = 0.00018$ respectively) was seen between normal, hypothermic and OA cohorts, then over-age, hypothermic and OA cohorts (Table 3.1.5).

At week 8 a significant difference (FDR adjusted $p = 7.7 \times 10^{-5}$) was observed between normal and all other cohorts, and then over-age, hypothermic and OA (Table 3.1.5).

Statistical analysis of data from normal donor samples showed no significant difference (FDR adjusted $p = 0.072$) in lactate levels between all weeks of storage (Table 3.1.6 and Fig. 3.1.5A). In contrast with over-age donor samples a significant difference was seen (FDR adjusted p = 0.0041) between weeks 1 and all other time points, there was also a significant difference between weeks 3 and 8 (Table 3.1.6 and Fig. 3.1.5B).

Table 3.1.6. Statistical analysis of lactate secretion from osteochondral allografts into storage medium.[a]

Cohort	Lactate *P*-Value	Lactate Fisher's *post-hoc* (weeks)
Normal	0.072	N/A
Over-age	0.0041	1-(2-8), 3-8
Osteoarthritis	2.2×10^{-5}	1-(2-8), 2-(4-8), 3-(6-8), 4-7, 4-8
Hypothermic	0.011	1-(2-8)

[a]Analysis of variance compares weeks 1 to 8 of storage, numbers in parentheses represent the range of weeks significantly different from the week number outside of parentheses, for example 1-(2-8) means week 1 is significantly different from weeks 2 to 8. Normal cohort were donors <45 years old, over-age were <45 years old, osteoarthritis were living donors and hypothermic was tissue stored at 4ºC. *Post-hoc* result shows which weeks (1 to 8) had significant difference (FDR adjusted) with each other, negative control not included due to lack of replicates

In OA donor samples a significant difference was observed (FDR adjusted $p = 2.2 \times 10^{-5}$) in lactate levels between week 1 and weeks 2 to 8. There was also a significant difference between week 2 and weeks 4 to 8, week 3 and 6 to 8, week 4 and 7 as well as 8 (Table 3.1.6 and Fig. 3.1.5C).

With hypothermic stored tissue a significant difference (FDR adjusted $p = 0.011$) was seen between week 1 and the remaining weeks of storage (Table 3.1.6 and Fig. 3.1.5D).

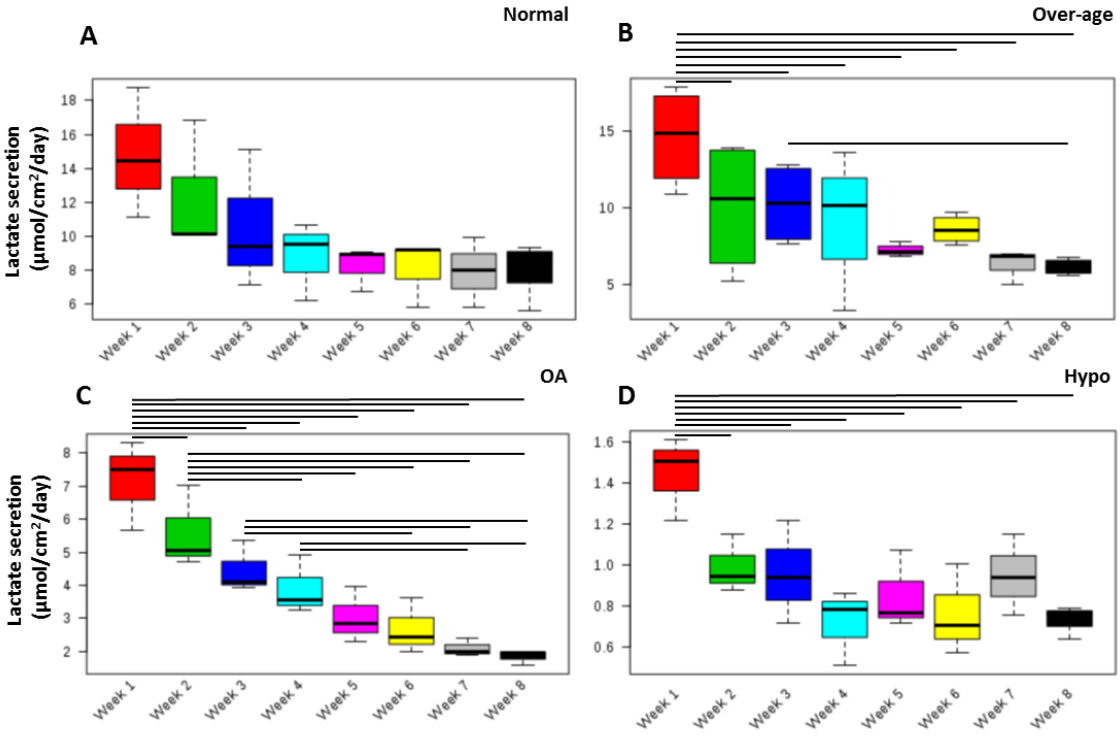

Figure 3.1.5. Box plots showing results from analysis of variance carried out on weeks 1 to 8 comparing weekly lactate secretion. A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) Osteoarthritic (OA). D) Hypothermic (Hypo, 4ºC). Significance bars show $p < 0.001$, negative control not included due to lack of biological replicates

In summary for lactate secretion the data suggested there was no significant difference between the normal and over-age cohorts throughout the storage period analysed, indicating a similar trend in secretion. There was a significant difference however between the normal and OA cohorts throughout the 8-week storage period analysed, and the hypothermic cohort was significantly lower compared to all others. Only the normal donor cohort had no significant change in lactate secretion between week 1 and the remaining storage weeks analysed, though in the normal and OA cohorts the

data suggested that the amount of lactate secreted from the allografts progressively decreased from week 1 to 8. In the over-age and hypothermic cohorts during the second half of the storage period analysed the amount of lactate secreted fluctuated.

3.2 - Dimethylmethylene blue assay results

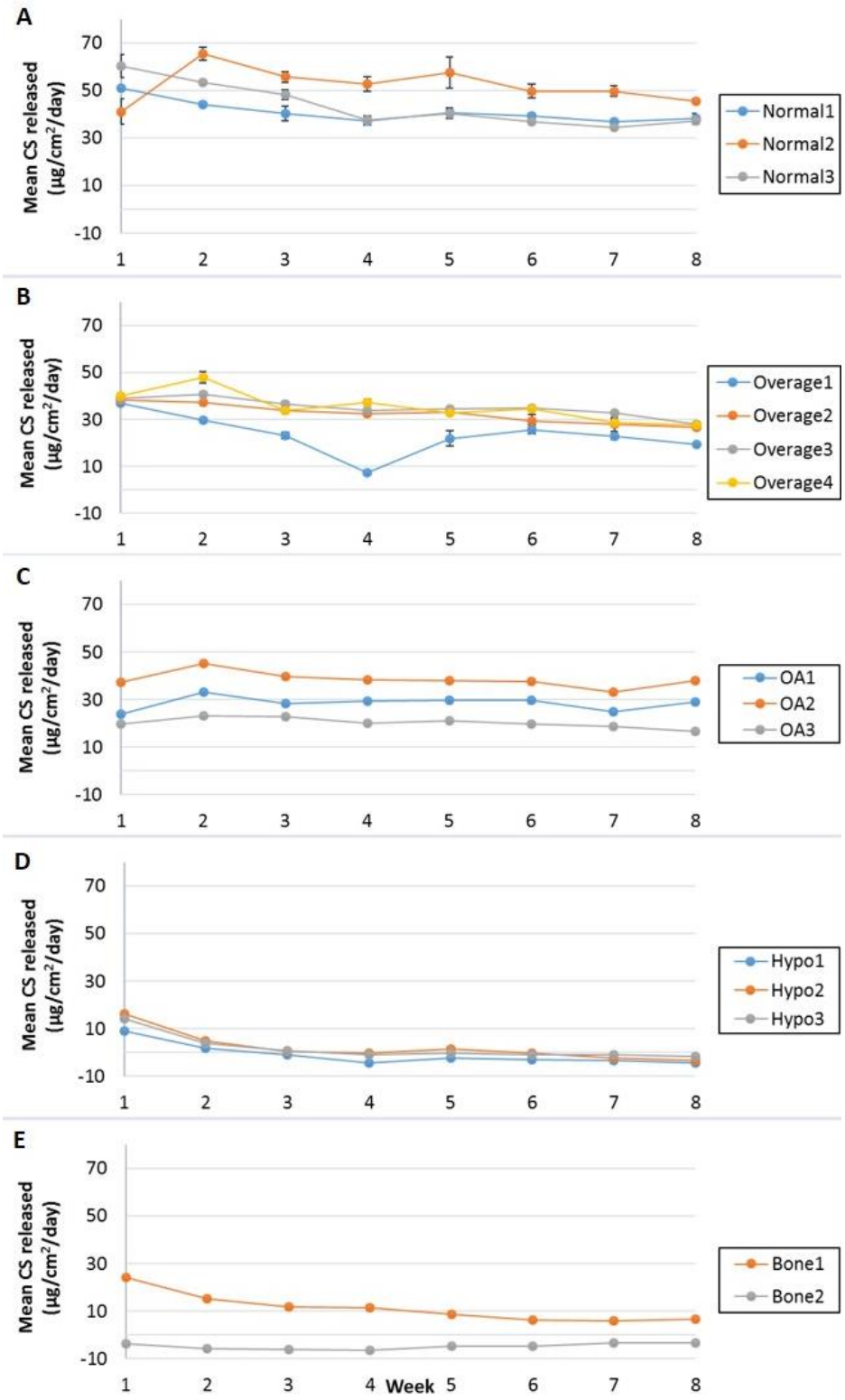

Figure 3.2.1. Scatter charts of mean ± standard deviation chondroitin sulphate (CS) detected in culture medium. A) Normal donor (<45 years of age) cartilage. B) Over-age donor (>45 years of age) cartilage. C) Living donor osteoarthritic (OA) cartilage. D) Hypothermic (Hypo, 4°C) stored cartilage. E) Bone only, n = 3 technical replicates for each data point

It was found that standards prepared in complete media reduced the slope of the curve (from -0.00902 to -0.00464) when compared with standards prepared in distilled water. Therefore, standard curves from which concentrations of chondroitin sulphate (CS) were calculated, was prepared in culture medium.

Variance within cohorts

In the normal donor samples cohort (n = 3) the amount of CS released into the culture medium was shown to be relatively stable with a range of 41.2 µg/cm^2/day to 60.3 µg/cm^2/day on week 1 to a range of 40.5 to 55.7 at week 3 (Fig. 3.2.1A). The range then slightly decreases on week 4 from 37.4 µg/cm^2/day to 52.8 µg/cm^2/day. After an increase on week 4 from 40.4 µg/cm^2/day to 57.5 µg/cm^2/day, CS release decreased to a range of 37.3 µg/cm^2/day to 45.7 µg/cm^2/day at week 8.

In the over-age donor cohort (n = 4) the amount of CS released increased from a range of 37.0 µg/cm^2/day to 40.2 µg/cm^2/day at week 1 to 29.7 µg/cm^2/day to 48 µg/cm^2/day in week 2, (Fig. 3.2.1B). CS released on week 3 then dropped to a range of 23.2 µg/cm^2/day to 36.5 µg/cm^2/day. At week 4 the data was skewed by one donor where the CS release decreased to 7.3 µg/cm^2/day, resulting in a range from 7.3 to 37.3 µg/cm^2/day. Thereafter CS release stabilised to a range of 21.9 µg/cm^2/day to 34.7 on week 5 and then 26.5 µg/cm^2/day to 34.8 µg/cm^2/day on week 6 before declining to a range of 19.2 µg/cm^2/day to 28.1 µg/cm^2/day on week 8.

Within the living donor osteoarthritic tissue cohort (n = 3), CS released at week 1 ranged from 19.6 µg/cm^2/day to 37.4 µg/cm^2/day which then increased to a range of 23.3 µg/cm^2/day to 45.4 µg/cm^2/day on week 2 (Fig. 3.2.1C). Thereafter the amount of CS released was shown to stabilise between weeks 3 to 6 with a range of 23.0 µg/cm^2/day to 39.6 on week 3 and then 19.7 to 37.6 at week 6. After a slight decrease in range from 18.7 µg/cm^2/day to 33.2 µg/cm^2/day at week 7 the amount of CS released at week 8 had returned to a comparable range seen at week 6 (16.7 to 38.0 µg/cm^2/day).

The hypothermic stored tissue cohort (n = 3) had a higher value for release of CS at week 1 (range of 9.2 µg/cm^2/day to 16.1 µg/cm^2/day) compared to all remaining weeks (Fig. 3.2.1D). The amount of CS released from the tissue decreased to a range of 1.7 µg/cm^2/day to 5.0 µg/cm^2/day at week 2, and thereafter to below 0 µg/cm^2/day up until week 8.

With bone only samples, only one of the donor samples had positive values for the amount of CS released, and this gradually declined throughout the storage period from an initial value of 24.1 µg/cm^2/day in week 1 to 6.6 µg/cm^2/day in week 8 (Fig. 3.2.1E).

Overall it was found that the OA cohort had a consistently greater variance in CS release compared to other cohorts with a difference of 17.7 µg/cm^2/day between lowest and highest amount of CS release in week 1 (*p*-value for OA vs other cohorts at week 1 = 0.00040), which increased to 18.3 µg/cm^2/day in week 4 (*p*-value for OA vs hypothermic at week 4 = 0.0027) and then 21.3 µg/cm^2/day in week 8 (*p*-value for OA vs other cohorts at week 8 = 0.00014) (Fig. 3.2.1C).
Other cohorts did have peak variance of similar magnitude, for example in the normal cohort the difference between lowest and highest amount of CS release in week 1 was 19.6 µg/cm^2/day, however by week 8 this had decreased to 8.4 µg/cm^2/day (*p*-value for normal week 1 vs week 8 = 0.39) (Fig. 3.2.1A). In the over-age cohort the difference between lowest and highest CS release in week 2 was 18.3 µg/cm^2/day, which increased to 30.0 µg/cm^2/day in week 4 (*p*-value for over-age week 2 vs week 4 = 0.082) before decreasing to 8.4 µg/cm^2/day in week 8 (*p*-value for over-age week 4 vs week 8 = 0.082).

Variance between cohorts

Overall mean data showed that CS release from normal tissue was consistently higher than that from over-age during all 8 weeks, with week 1 normal at 50.9 µg/cm^2/day compared to over-age 38.6 µg/cm^2/day (Table 3.2.1 and Fig. 3.2.2 (*p*-value for normal v over-age = 0.00040). By week 8 normal was at 40.4 µg/cm^2/day and over-age 25.3 µg/cm^2/day (*p*-value for normal vs over-age = 0.00014). With the normal donor cohort this related to a 20.1% decrease from week 1 to week 8 (*p*-value for week 1 vs week 8 normal = 0.39), whereas in the over-age cohort this related to a 34.5% decrease (*p*-value for week 1 vs week 8 over-age = 0.082).

When overall mean data from normal donors was compared with living donor OA tissue it was again found to be consistently higher throughout all 8 weeks analysed. At week 1 normal tissue CS release was a mean of 50.9 µg/cm^2/day, whereas OA released 27.0 µg/cm^2/day (Table 3.2.1 and Fig. 3.2.2 (*p*-value for normal vs OA = 0.00040)). By week 8 normal released 40.4 µg/cm^2/day compared to 27.9 µg/cm^2/day for OA (*p*-value for normal vs OA = 0.00014). With the normal donor cohort this related to a 20.1% decrease from week 1 to week 8, whereas in the living donor OA tissue this related to a 3.2% increase (*p*-value for week 1 vs week 8 OA =

0.54).

Mean data from normal donor cartilage was also consistently higher than hypothermic stored tissue, the largest amount of CS released was 13.2 µg/cm^2/day in week 1 compared to 50.9 µg/cm^2/day for normal (Table 3.2.1, Fig. 3.2.2 (*p*-value for normal vs hypothermic = 0.00040)), while at week 8 CS released from hypothermic tissue was -3.0 µg/cm^2/day compared to 40.4 µg/cm^2/day for normal tissue (*p*-value for normal vs hypothermic = 0.00014).

Table 3.2.1. Summary of data for release of chondroitin sulphate (CS) into storage medium.[a]

Concentration (CS)	**Mean released (µg/cm^2/day ± standard deviation)**								**ANOVA**
	Wk1	**Wk2**	**Wk3**	**Wk4**	**Wk5**	**Wk6**	**Wk7**	**Wk8**	***P***
Normal	50.9 ± 9.6	54.4 ± 10.7	48.2 ± 7.6	42.6 ± 8.9	46.1 ± 9.8	42.0 ± 6.8	40.3 ± 8.2	40.4 ± 4.6	0.39
Over-age	38.6 ± 1.3	39.0 ± 7.6	31.8 ± 5.9	27.7 ± 13.8	30.6 ± 5.9	31.2 ± 4.4	28.0 ± 4.1	25.3 ± 4.1	0.082
Hypothermic	13.2 ± 3.6	3.5 ± 1.7	-0.09 ± 0.9	-1.8 ± 2.3	-0.3 ± 2.0	-1.4 ± 1.6	-2.3 ± 1.2	-3.0 ± 1.4	5.1 x 10^{-7}
Osteoarthitis	27.0 ± 9.3	33.9 ± 11.1	30.3 ± 8.5	29.3 ± 9.2	29.6 ± 8.5	29.0 ± 9.0	25.6 ± 7.3	27.9 ± 10.7	0.54

[a]Data shows mean with standard deviation from each donor cohort where at least three donor samples were obtained (n = 4 for over-age donors). Normal donor (<45 years old) cartilage, over-age (>45 years old), living donor osteoarthritic and hypothermic (4^{o}C). Each donor had n = 3 technical replicates, bone only not included due to lack of technical replicates

Over-age and living donor OA tissue had similar mean amounts of CS released throughout the 8-week storage period, compared to all other cohorts. Over-age at week 1 was higher than OA with a mean of 38.6 µg/cm^2/day compared to 27.0 µg/cm^2/day (Table 3.2.1, Fig. 3.2.2 (*p*-value for over-age vs OA = 0.00040)), but by week 8 OA had a higher amount of CS released with a mean of 27.9 µg/cm^2/day compared to 25.3 µg/cm^2/day (*p*-value for over-age vs OA = 0.87). With the over-age cohort this related to a 34.5% decrease (*p*-value for over-age week 1 vs week 8 = 0.082), while for OA this was a 3.2% increase (*p*-value for OA week 1 vs week 8 = 0.54).

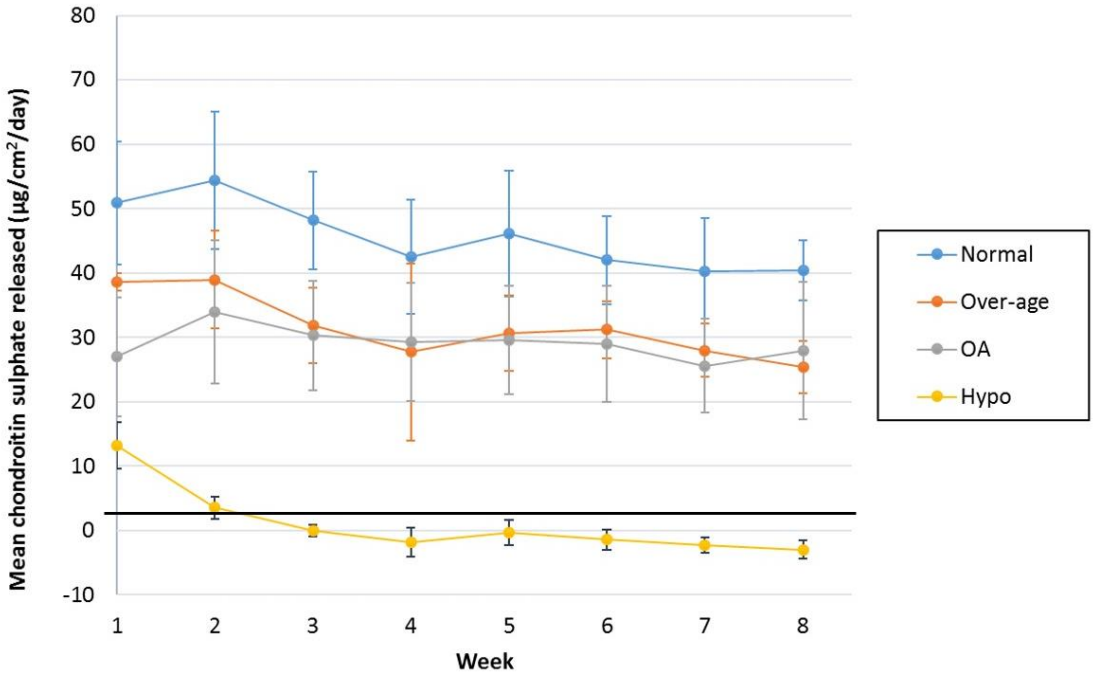

Figure 3.2.2. Scatter chart of mean chondroitin sulphate detected in culture medium. Normal donor (<45 year old) cartilage. Over-age donor (>45 year old) cartilage. Living donor osteoarthritic (OA) cartilage. Hypothermic cartilage (Hypo) was stored at 4°C, n = 3 except for over-age where n = 4 biological replicates, for all donors a technical replicate of n = 3 was performed, error bars show standard deviation, black line shows baseline calculated from mean bone only values, negative control and bone only not included due to lack of biological replicates

Both over-age and OA donor cohorts were consistently higher than hypothermic stored tissue throughout all 8 weeks. At week 1 over-age mean value was 38.6 µg/cm^2/day compared to 13.2 µg/cm^2/day for hypothermic (Table 3.2.1, Fig. 3.2.2 (*p*-value for over-age vs hypothermic = 0.00040)). OA mean value was at 27.0 µg/cm^2/day compared to 13.2 µg/cm^2/day for hypothermic (*p*-value for OA vs hypothermic = 0.00040). By week 8 over-age was at a mean of 25.3 µg/cm^2/day compared to -3.0 µg/cm^2/day for hypothermic (*p*-value for over-age v hypothermic = 0.00014). OA tissues were at a mean of 27.9 µg/cm^2/day compared to hypothermic at -3.0 µg/cm^2/day (*p*-value for OA vs hypothermic = 0.00014).

With comparison between cohorts at each week, the statistical analysis showed that at week 1 there was a significant difference between all donor cohorts with FDR adjusted $p = 0.00040$ (Table 3.2.2). By week 2 however there was no significant difference ($p > 0.05$) between over-

age and OA cohorts, with significant difference still apparent between all other cohorts (FDR adjusted $p = 0.00040$).

At week 3 there was a significant difference (FDR adjusted $p = 0.00014$) between all cohorts except over-age and OA (Table 3.2.2). At week 4 there was only significant difference (FDR adjusted $p = 0.0027$) between normal and hypothermic, OA and hypothermic and then over-age and hypothermic. By week 5 there was a significant difference (FDR adjusted $p = 0.00024$) between all cohorts except over-age and OA (Table 3.2.2). This trend continued for the remaining weeks of storage with significant difference observed in week 6 (FDR adjusted $p = 0.00014$), week 7 (FDR adjusted $p = 0.00014$), and week 8 (FDR adjusted $p = 0.00014$).

Table 3.2.2. Statistical analysis of chondroitin sulphate release into storage medium.[a]

Week	Chondroitin sulphate *P*-Value	Chondroitin sulphate Fisher's *post-hoc* (cohorts)
1	0.00040	N-H, OA-H, O-H, N-OA, N-O, O-OA
2	0.00040	N-H, OA-H, O-H, N-OA, N-O
3	0.00014	N-H, OA-H, O-H, N-OA, N-O
4	0.0027	N-H, OA-H, O-H
5	0.00024	N-H, OA-H, O-H, N-OA, N-O
6	0.00014	N-H, OA-H, O-H, N-OA, N-O
7	0.00014	N-H, OA-H, O-H, N-OA, N-O
8	0.00014	N-H, OA-H, O-H, N-OA, N-O

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts, at each week of storage. H = hypothermic, N = normal, OA = osteoarthritis, O = over-age. *Post-hoc* result shows which cohorts had significant difference with each other, bone only not compared due to lack of biological replicates

In normal donors, statistical analysis using analysis of variance showed that the small changes in CS release were not significantly different (FDR adjusted $p = 0.00086$) between early and mid to late storage weeks (Table 3.2.3, Fig. 3.2.3A). This lack of significant difference between storage weeks suggested release of CS was relatively stable.

In over-age donors there was no significant difference (FDR adjusted $p = 0.082$) between early, mid and late storage weeks (Table 3.2.3, Fig. 3.2.3B), this suggested that CS release from this cohort of tissue was also relatively stable during the storage period.

For living donor OA tissue, statistical analysis using analysis of variance showed that there was no significant difference (FDR adjusted $p = 0.54$) between any of the storage weeks, suggesting that in living osteoarthritic tissue the amount of CS release was consistent and not likely to change in week 9 onwards (Table 3.2.3 and Fig. 3.2.3C).

Table 3.2.3. Statistical analysis of chondroitin sulphate release into storage medium.[a]

Cohort	Chondroitin sulphate *P*-Value	Chondroitin sulphate Fisher's *post-hoc* (weeks)
Normal	0.39	N/A
Over-age	0.082	N/A
Osteoarthritis	0.54	N/A
Hypothermic	5.1×10^{-7}	1-(2-8), 2-(3-8)

[a]Analysis of variance compares weeks 1 to 8 of normal donors (<45 years old), over-age (<45 years old), osteoarthritis (living donors) and hypothermic (4°C) donor cohorts. Numbers.in parentheses represent the range of weeks significantly different from the week number outside of parentheses, for example 1-(2-8) means week 1 is significantly different from weeks 2 to 8. *Post-hoc* result shows which cohorts had significant difference with each other

With hypothermic stored tissue there was significant difference (FDR adjusted $p = 5.1 \times 10^{-7}$) between week 1 and the remainder of the weeks in storage (Table 3.2.3 and Fig. 3.2.3D). This data suggests that by week 3 CS release from the tissue had stabilised and that there was no release of CS from week 9 onwards.

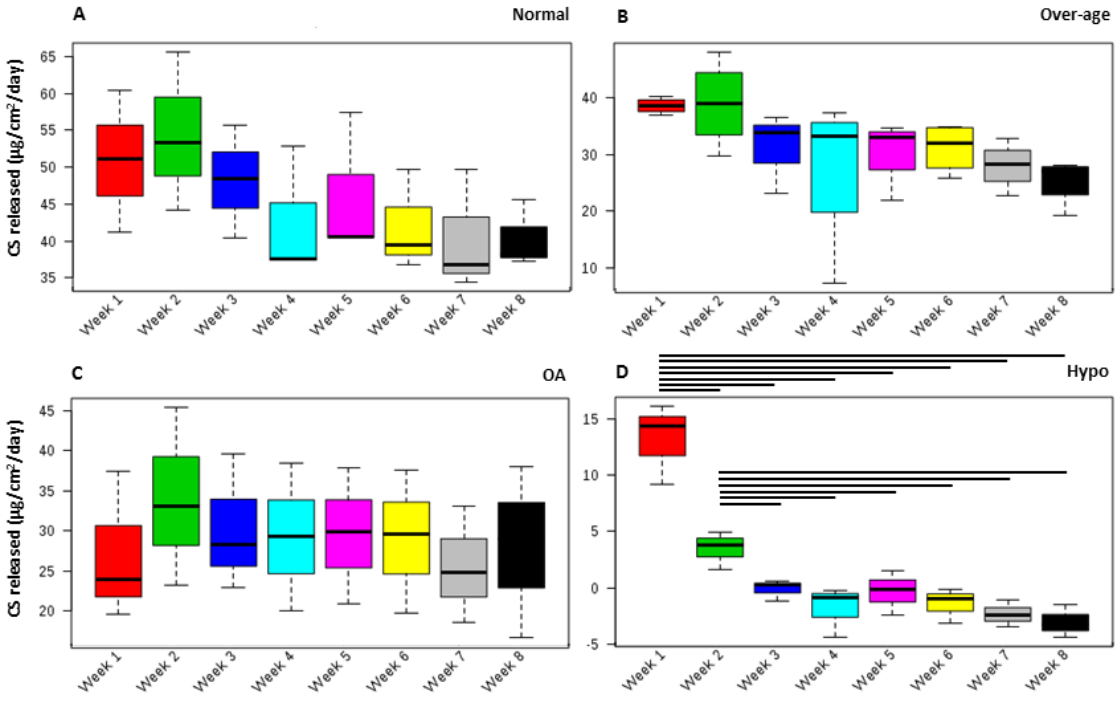

Figure 3.2.3. Box plots showing results from analysis of variance carried out on weeks 1 to 8 comparing weekly chondroitin sulphate (CS) release. A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) Osteoarthritic (OA). D) Hypothermic (Hypo, 4ºC), n = 3 except for over-age where n = 4 biological replicates, for all donor's technical replicate n = 3 was performed, significance bars show FDR adjusted $p < 0.001$

In summary the data suggested that CS release from the normal donor cohort was significantly higher than all other cohorts, except during week 4. Over-age and OA cohorts had a similar trend to each other in the amount of CS released, with only week 1 demonstrating a significant difference between these cohorts. Hypothermic stored allografts had significantly low CS release into the culture medium compared to all other cohorts. Data suggested that it was only the hypothermic stored tissue that showed a significant change in CS release when comparison was made between the storage weeks analysed.

3.3 - Live/dead assay results

Variance within cohorts

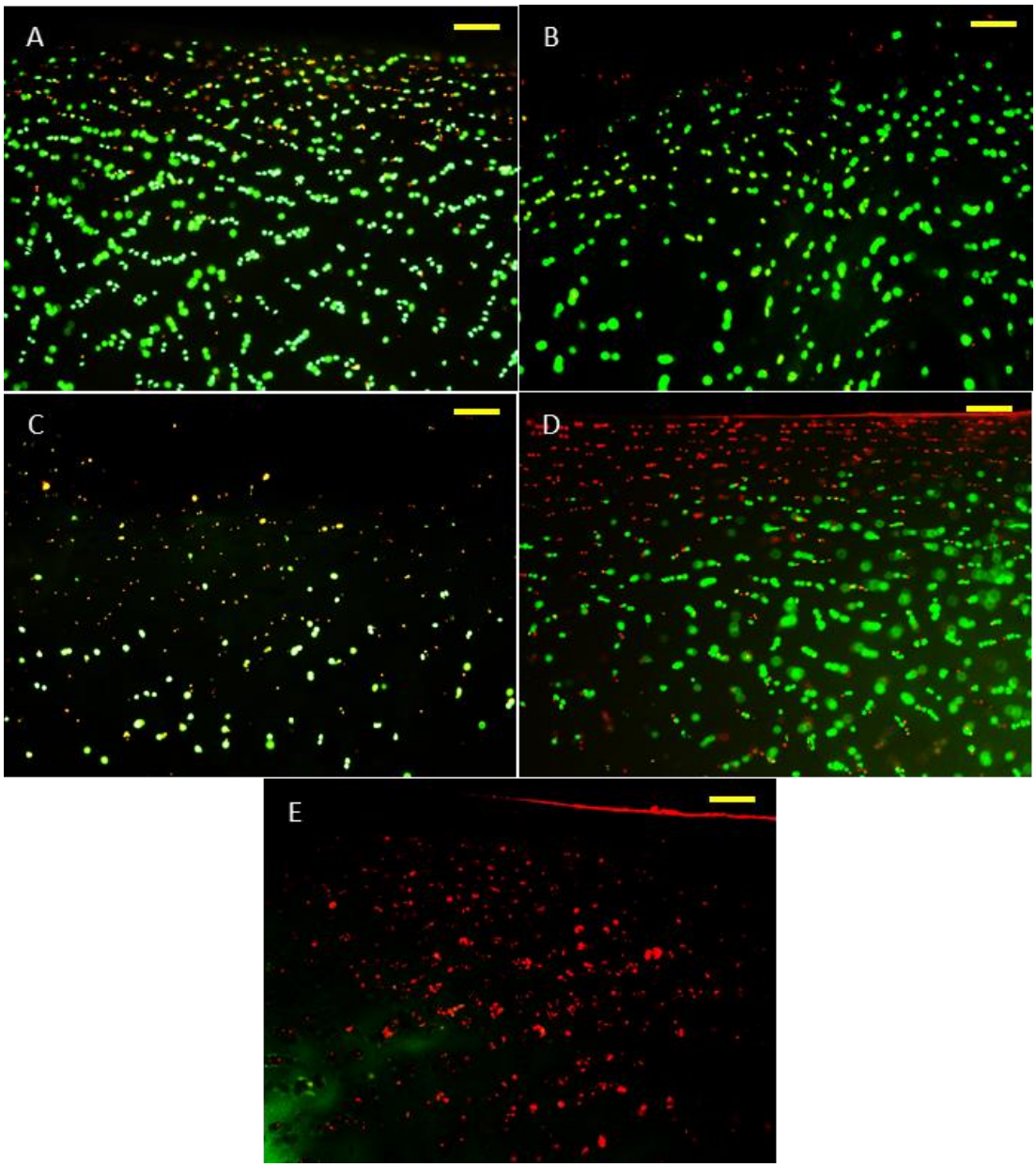

Figure 3.3.1. Representative fluorescence images of cartilage sections from each donor cohort stained with calcein acetoxymethyl to detect living chondrocytes (green) and ethidium homodimer-1 to detect dead chondrocytes (red). A) Example from normal donor (<45 years of age) at week 12, B) example from over-age donor (>45 years of age) at week 12, C) example from living donor osteoarthritic tissue at week 8 (due to final storage at week 8), D) example from hypothermic (4°C) stored tissue at week 12, E) example from negative control tissue at week 12. Images at x100 magnification, scale bars = 100 µm

Representative images from each donor cohort showing fluorescent staining of live or dead cells are shown in Fig. 3.3.1.

The results of the live/dead assay in normal donors (n = 3) showed that at the beginning of week 1 chondrocyte viability was consistently above 90%, with values ranging from a mean of 93.9% to 96.9%, and overall mean of 95.0% (Table. 3.3.1). At the end of the storage period in week 12, the remaining chondrocyte viability in the tissue was more varied (standard deviation increased from 4.3 to 16.9) with values ranging from a mean of 64.7% to 94.5%.

Table 3.3.1. Summary of chondrocyte viability data, as determined by the live/dead assay.[a]

Viability (%)	**Mean (± standard deviation)**		**t-test**
	Week 1	**Week 12 (8)**	***P***
Normal	95.0 ± 1.6	80.0 ± 14.9	0.16
Over-age	96.4 ± 2.2	85.1 ± 13.7	0.20
Osteoarthitis	81.3 ± 24.4	61.8 ± 19.0	0.34
Hypothermic	97.6 ± 1.0	42.7 ± 24.2	0.017

[a]Data shows mean with standard deviation from each donor cohort from week 1, 8 (osteoarthritic), and 12, where at least three donor samples were obtained (n = 4 for over-age donors). Each donor had n = 5 technical replicates

With over-age donor cohort (n = 4) mean viability was again above 90% at the beginning of week 1, with values ranging from a mean of 93.5% to 98.8% (Table 3.3.1). At the end of the storage period in week 12 in three of the donors the viability was relatively consistent with mean viability range from 88.8% to 95.9%, however the fourth donor had a mean viability of 66.2%.

In living donor OA donor tissue (n = 3) mean viability at the beginning of week 1 was more varied compared to that seen in either normal or over-age donors, with viability ranging from 53.1% to 96.1% (Table 3.3.1). In week 8 mean viability decreased with values ranging from 40.0% to 75.2%.

For hypothermically stored donor tissue (n = 3) mean viability was above 90% at the beginning of week 1, with a range from 96.9% to 98.8% (Table 3.3.1). In week 12 mean viability decreased to a range of 16.9% and 65%.

Even though only one donor of devitalised cartilage was used in this dataset, it showed that viability in this tissue was greatly reduced as opposed to all other donors, with mean viability at 15.0% at the beginning of week 1 as opposed to 0.7% in week 12 (Fig. 3.3.1E).

Variance between cohorts

Overall mean data from the normal donor cohort showed that at the beginning of week 1 the viability was similar to that observed in the over-age cohort with a mean of 95.0% for normal compared to 96.4% for over-age (Table 3.3.1). By week 12 however the mean viability had decreased to 80.0% for normal donor samples (p-value for week 1 vs week 12 = 0.16) whereas in the over-age group it had decreased to 85.1% (p-value for week 1 vs week 12 = 0.20).

When the overall mean viability data from normal donors was compared with living donor OA, at the beginning of week 1 normal donor viability was much greater at 95.0% compared to 81.3% for OA (Table 3.3.1 (p-value for normal vs OA = 0.33)). By week 12 (or 8 for living donors) the difference was still large with normal donor mean viability at 80.0% compared to 61.8% for OA (p-value for normal vs OA = 0.096).

With normal donor samples compared to hypothermic stored tissue it was found that data from normal donors at the beginning of week 1 was comparable to that from hypothermic, with normal donor mean viability at 95.0% compared to 97.6% for hypothermic (Table 3.3.1 (p-value for normal vs hypothermic = 0.33)). However, by week 12, while normal donor viability was at 80.0%, hypothermic viability had decreased to almost half the viability of normal donors at 42.7% (p-value for normal vs hypothermic = 0.096).

When over-age donors were compared with living donor OA it was found that at the beginning of week 1 over-age viability was higher than OA, with over-age at 96.4% compared to 81.3% for OA (Table 3.3.1 (p-value for over-age vs OA = 0.33). By week 12 (or 8 for OA) over-age had decreased to 85.1% while OA had decreased further to 61.8% (p-value for over-age vs OA = 0.096).

For comparison between over-age donors and hypothermic stored tissue it was found that at the beginning of week 1, mean data for normal was 96.4% while hypothermic was slightly higher at 97.6% (Table 3.3.1 (p-value for over-age vs hypothermic = 0.33). By week 12 the mean data for over-age had decreased to 85.1 % whereas hypothermic had decreased to 42.7% (p-value for over-age vs hypothermic = 0.096).

Finally, when data from the living donor OA cohort was compared with hypothermic samples, at the beginning of week 1 mean viability was 81.3% while for hypothermic it was 97.6% (Table 3.3.1 (*p*-value for OA vs hypothermic = 0.33). By week 12 (or 8 for OA) mean viability for OA donors had decreased to 61.8% while hypothermic had decreased further to 42.7% (*p*-value for OA vs hypothermic = 0.096).

When cohorts were compared, data showed that at the beginning of week 1, none of the cohorts were significantly different (FDR adjusted $p = 0.33$) (Table 3.3.2).

Table 3.3.2. Statistical analysis of chondrocyte viability in sections of articular cartilage, as determined by the live/dead assay.[a]

Week	Chondrocyte viability *P*-Value
1	0.33
12 (8)	0.096

[a]Analysis of variance completed on data from weeks 1, 8 (osteoarthritic) or 12 of storage, normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts. Result shows which cohorts had FDR adjusted significant difference with each other, n = 3 biological replicates except for over-age where n = 4. Each donor had n = 5 technical replicates

In week 12 (or 8 for OA tissue) there was also no significant difference (FDR adjusted $p = 0.096$) between all donor cohorts (Table 3.3.2).

With normal donor samples, statistical analysis with t-test showed that there was not a significant (FDR adjusted $p = 0.16$) decrease in viability between week 1 and 12 (Table 3.3.3 and Fig. 3.3.2A).

Table 3.3.3. Statistical analysis of chondrocyte viability in sections of articular cartilage, as determined by the live/dead assay.[a]

Cohort	Chondrocyte viability *P*-Value
Normal	0.16
Over-age	0.20
Osteoarthritis	0.34
Hypothermic	0.017

[a]Analysis via t-test completed on each cohort comparing weeks 1, 8 (osteoarthritic), or 12. Normal cohort were donors <45 years old, over-age were >45 years old, osteoarthritis were living donors and hypothermic was tissue stored at 4°C, n = 3 biological replicates except for over-age where n = 4. Each donor had n = 5 technical replicates

For over-age donor samples the decrease in viability between the beginning of week 1 to week 12 was also found to not be a significant change (FDR adjusted $p = 0.20$) (Table 3.3.3 and Fig. 3.3.2B).

It was found that in the living donor OA cohort the decrease in viability between the beginning of week 1 to week 8 was also not significant (FDR adjusted $p = 0.34$) (Table 3.3.3 and Fig. 3.3.2C).

Finally, with hypothermic stored tissue it was found that the decrease in viability from the beginning of week 1 to week 12 was found to be a significant change (FDR adjusted $p = 0.017$) (Table 3.3.3 and Fig. 3.3.2D).

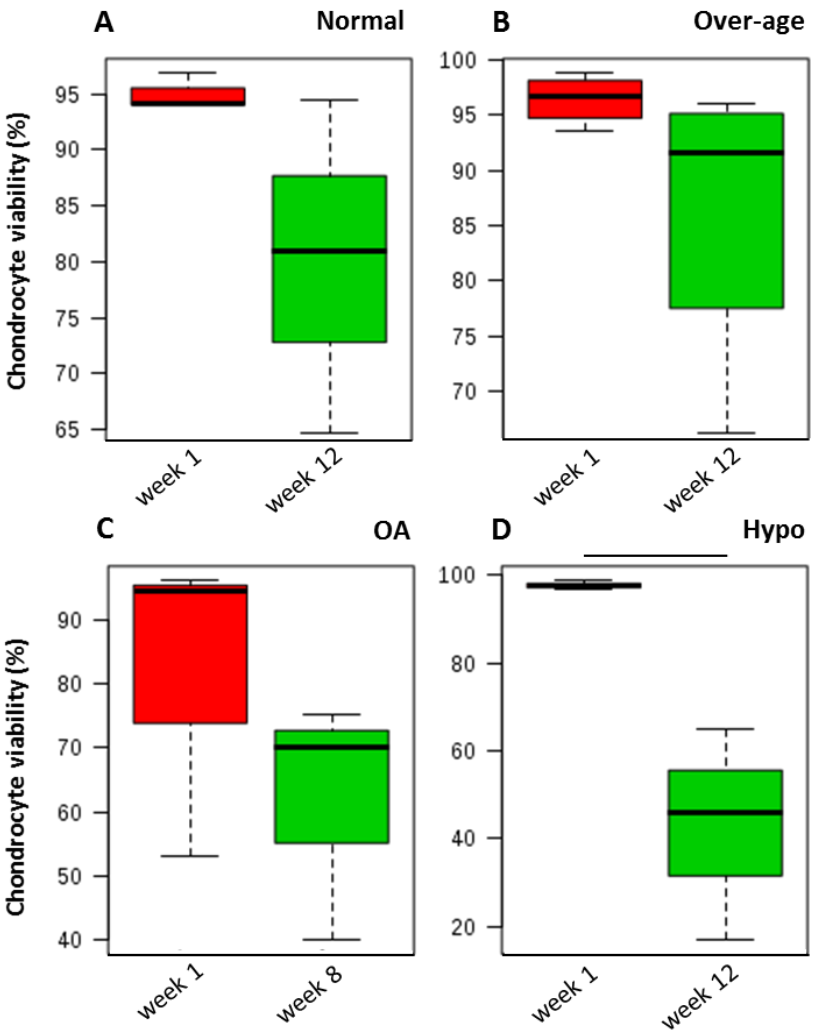

Figure 3.3.2. Box plots showing results from t-test carried out on chondrocyte viability, determined by live/dead assay, at the beginning of week 1, and week 12 (week 8 with osteoarthritic (OA) tissue), as determined by the live/dead assay. A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) OA. D) Hypothermic (Hypo, 4ºC), n = 3 biological replicates except for over-age where n = 4. Each donor had n = 5 technical replicates, significance bars: FDR adjusted $p < 0.05$

In summary for the live/dead assay the data suggested that there was no significant difference between all cohorts at the beginning of week 1 storage, while it might have been expected to find a significant difference with the OA cohort. This trend was repeated at the end of the storage period for each cohort, where it might have been expected to find a significant decrease between hypothermic or OA and all other cohorts. A significant difference was only found in the hypothermic cohort when the beginning and end of the storage periods were compared within cohorts. Again, it might have been expected to find a significant difference in the OA cohort.

3.4 - AlamarBlue® assay results

Variance within cohorts

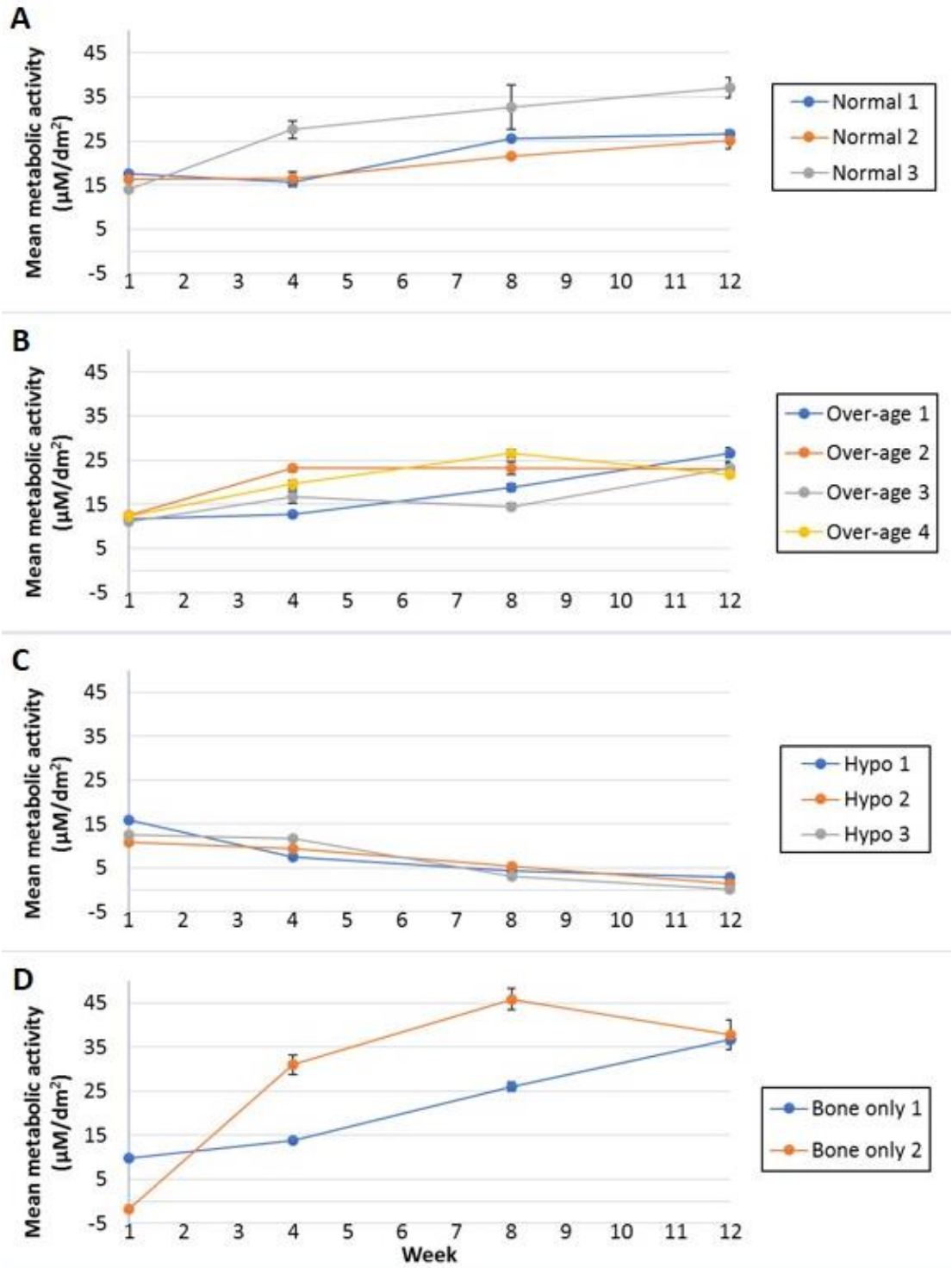

Figure 3.4.1. Scatter charts of mean chondrocyte metabolic activity, as determined by the alamarBlue® assay. A) Normal donor (<45 year old) cartilage. B) Over-age (>45 year old) donor cartilage. C) Hypothermic (Hypo, 4ºC) stored cartilage. D) Bone only, n = 5 technical replicates, error bars show standard deviation

In the normal donor cohort (n = 3), metabolic activity as measured by the alamarBlue® assay showed that at the beginning of week 1 the range of mean data was from 14.0 μM/dm^2 to 16.4 (Fig. 3.4.1A). By week 4 the range had increased to 15.7 μM/dm^2 to 27.6 μM/dm^2, with the third donor higher than donors 1 and 2. At week 8 metabolic activity had increased again, with a range of 21.6 μM/dm^2 to 32.7 μM/dm^2, again with the third donor offset from the first two. In the final storage week 12, data showed that metabolic activity had increased again, with a range of 25.1 μM/dm^2 to 37.1 μM/dm^2, again with the third donor offset from the first two.

For the over-age donor cohort (n = 4) mean metabolic activity at the beginning of week 1 ranged from 11.2 μM/dm^2 to 12.6 μM/dm^2 (Fig. 3.4.1B). At week 4 the metabolic activity had increased to a range of 12.7 μM/dm^2 to 23.1 μM/dm^2, indicating an increase in the variance of activity. By week 8 the metabolic activity had slightly increased again, with a range of data from 14.5 μM/dm^2 to 26.6 μM/dm^2. At week 12 the metabolic activity had stabilised to a range of 21.8 μM/dm^2 to 26.7 μM/dm^2, indicating that the variance had decreased.

With hypothermic stored tissue (n = 3), the mean metabolic activity at the beginning of week 1 ranged from 12.6 μM/dm^2 to 16.0 μM/dm^2 (Fig. 3.4.1C). By week 4 the metabolic activity had decreased to a range of 9.4 μM/dm^2 to 11.7 μM/dm^2. At week 8 the mean metabolic activity had decreased still further with a range from 3.0 μM/dm^2 to 5.5 μM/dm^2. Finally, in week 12 mean metabolic activity had decreased to its lowest level with a range from 0.13 μM/dm^2 to 1.44 μM/dm^2.

For bone only, there was only two donors used, however at the beginning of week 1 the range of mean metabolic activity was -1.8 μM/dm^2 to 9.8 μM/dm^2 (Fig. 3.4.1D). By week 4 this activity had increased to a range of 13.9 μM/dm^2 to 31.0 μM/dm^2, therefore this gave a greater variance. At week 8 metabolic activity had increased to a range of 26.0 μM/dm^2 to 45.9 μM/dm^2. Finally, on week 12 variance had decreased with the range in metabolic activity from 36.7 μM/dm^2 to 37.8 μM/dm^2.

Looking at the data for the devitalised (negative control) tissue, of which there was only one donor, metabolic activity at the beginning of week 1 was 2.0 μM/dm^2. At the end of the storage period on week 12 the mean activity had decreased to -0.4 μM/dm^2.

Metabolic activity in the cartilage of normal donors showed a progressive increase from an overall mean of 16.0 μM/dm^2 on week 1 to 20.0 μM/dm^2 on week 4 (*p*-value for week 1 vs week 4 = 0.37), 26.6 μM/dm^2 on week 8 (*p*-value for week 1 vs week 8 = 0.038), and then 29.6 μM/dm^2 on week 12 (*p*-value for week 1 vs week 12 = 0.038), equating to a 1.85-fold increase compared to week 1 (Table 3.4.1, and Fig. 3.4.2). When this cohort was compared to the over-age cohort it was found that metabolic activity in the normal donors was consistently higher throughout all weeks that activity was measured.

At the beginning of week 1 activity from normal donors was 16.0 μM/dm^2, compared to 12.0 μM/dm^2 for over-age (Table 3.4.1 and Fig. 3.4.2 (*p*-value for normal vs over-age = 0.057)). By week 12 activity in normal donors was 29.6 μM/dm^2 compared to 23.7 μM/dm^2 for over-age (*p*-value for normal vs over-age = 0.00038).

When data from normal donors was compared with hypothermic, it was found that again the mean metabolic activity was consistently higher in normal donors. At the beginning of week 1, normal metabolic activity was 16.0 μM/dm^2 with hypothermic at 13.2 μM/dm^2 (Table 3.4.1 and Fig. 3.4.2 (*p*-value for normal vs hypothermic = 0.057), by week 12 normal was 29.6 μM/dm^2 compared to 1.5 μM/dm^2 for hypothermic (*p*-value for normal vs hypothermic = 0.00038).

Table 3.4.1. Summary of metabolic activity data from chondrocytes in cartilage allografts as determined by the alamarBlue® assay.[a]

Metabolic activity	**Mean metabolic activity (μM/dm^2 ± standard deviation)**				**ANOVA**
	Week 1	**Week 4**	**Week 8**	**Week 12**	***P***
Normal	16.0 ± 1.8	20.0 ± 6.6	26.6 ± 5.6	29.6 ± 6.6	0.038
Over-age	12.0 ± 0.7	18.1 ± 4.4	20.8 ± 5.2	23.7 ± 2.1	0.0038
Hypothermic	13.2 ± 2.7	9.5 ± 2.1	4.3 ± 1.3	1.5 ± 1.4	0.00032

[a]Data shows mean with standard deviation from each donor cohort from week 1, 4, 8, and 12, where at least three donor samples were obtained (n = 4 for over-age donors). Each donor had n = 5 technical replicates

For over-age donor cohorts the metabolic activity also increased from an overall mean of 12.0 μM/dm^2 at the beginning of week 1 to 18.1 μM/dm^2 on week 4 (*p*-value for week 1 vs week 4 = 0.0038), then 20.8 μM/dm^2 on week 8 (*p*-value for week 1 vs week 8 = 0.0038) before finally increasing to 23.7 μM/dm^2 on week 12 (*p*-value for week 1 vs week 12 = 0.0038, a 1.98-fold increase compared to week 1 (Table 3.4.1, and Fig. 3.4.2).

Comparison with hypothermic tissue showed that with the exception of week 1, over-age tissue had consistently higher metabolic activity on those weeks when it was measured. On week 4 over-age mean metabolic activity was 18.1 µM/dm^2, while hypothermic was 9.5 µM/dm^2 (*p*-value for over-age vs hypothermic = 0.060), by week 8 over-age had increased to 20.8 µM/dm^2 while hypothermic had decreased to 4.3 µM/dm^2 (*p*-value for over-age vs hypothermic = 0.0028). At week 12 over-age was at 23.7 µM/dm^2 compared to 1.5 µM/dm^2 for hypothermic (Table 3.4.1, and Fig. 3.4.2 (*p*-value for over-age vs hypothermic = 0.00038).

Looking at hypothermically stored tissue, metabolic activity progressively decreased from a mean value of 13.2 µM/dm^2 at the beginning of week 1 to 9.5 µM/dm^2 on week 4 (*p*-value for week 1 vs week 4 = 0.14), then 4.3 µM/dm^2 on week 8 (*p*-value for week 1 vs week 8 = 0.00032) to a final value of 1.5 µM/dm^2 on week 12 (*p*-value for week 1 vs week 12 = 0.00032), an 8.8-fold decrease compared to week 1 (Table 3.4.1, and Fig. 3.4.2).

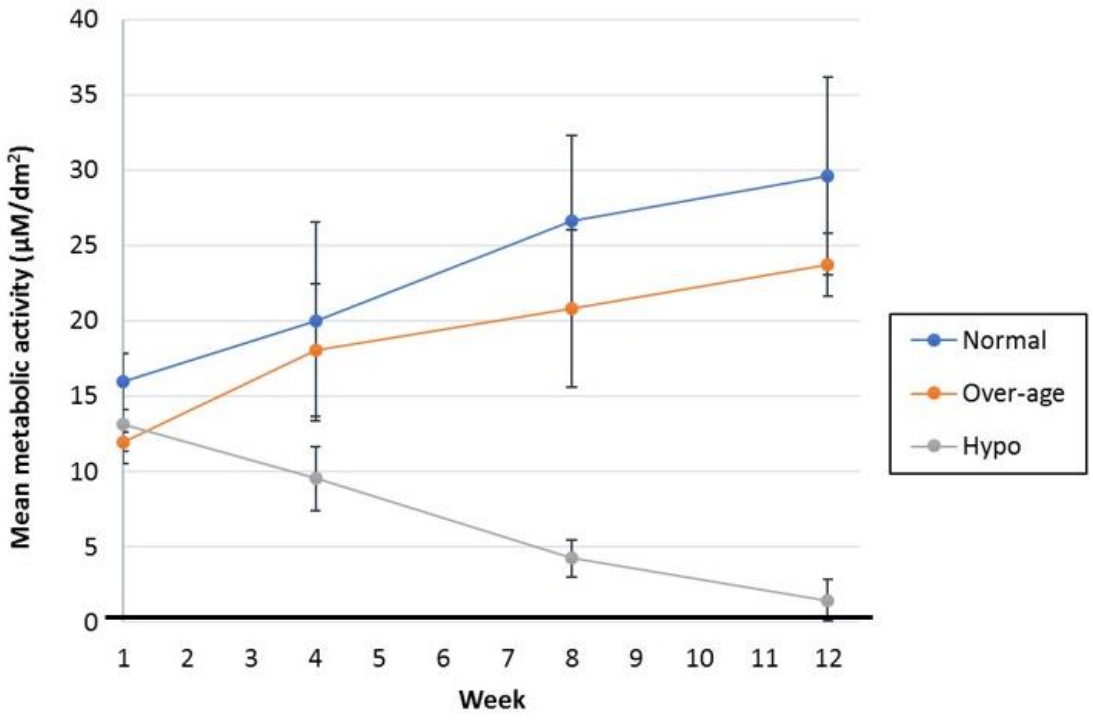

Figure 3.4.2. Scatter charts of mean ± standard deviation of chondrocyte metabolic activity as determined by alamarBlue® assay. Each donor cohort had at least an n of 3 (n = 4 for over-age donors), each donor had technical replicate samples (n = 5). Normal donor (<45 year old) cartilage. Over-age donor (>45 year old) cartilage. Hypothermic (Hypo) cartilage stored at 4^{o}C, black bar shows baseline calculated from mean negative control values. Negative control and bone only cohorts not compared due to lack of biological replicates

Variance between cohorts

Activity went up over time for normal and over-age groups, but down in the hypothermic stored group. The rate of increase was lower after week 4 in the over-age cohort, while the rate of increase remained consistent in the normal cohort (Fig. 3.4.2).

When comparisons were made between cohorts at each test week, statistical analysis showed that there was no significant difference between all cohorts in week 1 (FDR adjusted p = 0.057), or in week 4 (FDR adjusted p = 0.060) (Table 3.4.2). It was only in weeks 8 and 12 where a significant difference (FDR adjusted p = 0.0028, and p = 0.00038 respectively) was seen between normal and hypothermic and then over-age and hypothermic cohorts (Table 3.4.2).

Table 3.4.2. Statistical analysis of chondrocyte metabolic activity, determined by the alamarBlue® assay.[a]

Week	Metabolic activity *P*-Value	Metabolic activity Fisher's *post-hoc* (cohorts)
1	0.057	N/A
4	0.060	N/A
8	0.0028	N-H, O-H
12	0.00038	N-H, O-H

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), and hypothermic (4°C) donor cohorts from week 1, 4, 8, and 12. H = hypothermic, N = normal, O = over-age. *Post-hoc* result shows which cohorts had FDR adjusted significant difference with each other

Variance between weeks

With normal donors, analysis of variance (ANOVA) showed that the increase in metabolic activity was significantly different (FDR adjusted p = 0.038) when comparing weeks 8, and 12 with week 1 (Table 3.4.3 and Fig. 3.4.3A).

For over-age donor samples ANOVA showed that the increase in metabolic activity was significantly different (FDR adjusted p = 0.0038) when comparing weeks 4, 8, and 12 with week 1, and then week 12 with week 4 (Table 3.4.3 and Fig. 3.4.3B).

Table 3.4.3. Statistical analysis of chondrocyte metabolic activity as determined by the alamarBlue® assay.[a]

Cohort	Metabolic activity *P*-Value	Metabolic activity Fisher's *post-hoc* (weeks)
Normal	0.038	8-1, 12-1
Over-age	0.0038	4-1, 8-1, 12-1, 12-4
Hypothermic	0.00032	1-8, 1-12, 4-8, 4-12

[a]Analysis of variance compares normal (<45 years old), over-age (<45 years old), and hypothermic (4°C) donor cohorts in week 1, 4, 8, and 12. *Post-hoc* result shows which cohorts had FDR adjusted significant difference with each other

In the hypothermic donor cohort, ANOVA showed that the decrease in metabolic activity in each week was significantly different (FDR adjusted $p = 0.00032$) from one another when comparing weeks 8 and 12 with week 1, and then weeks 8 and 12 with week 4 (Table 3.4.3 and Fig. 3.4.3C).

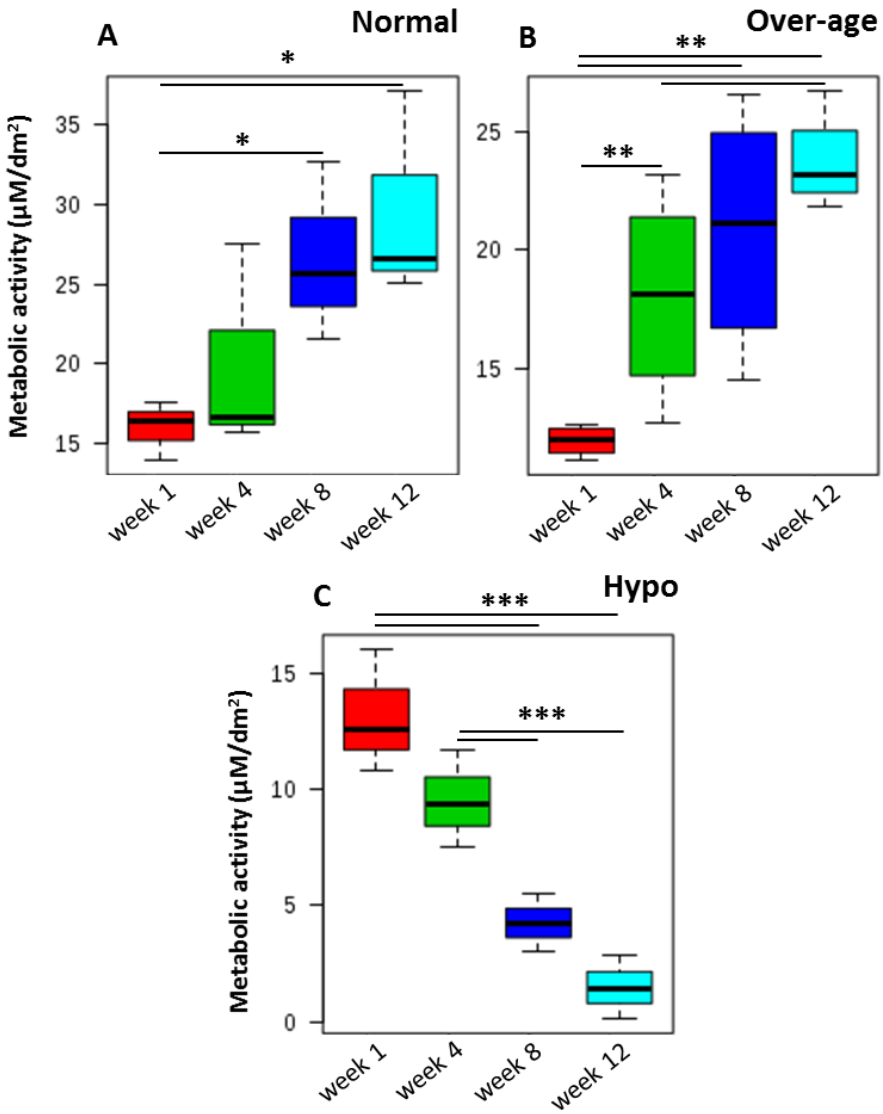

Figure 3.4.3. Box plots showing results from analysis of variance carried out on week 1, 4, 8, and 12 comparing chondrocyte metabolic activity as determined by the alamarBlue® assay. A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old), C) Hypothermic (Hypo, 4°C). Each donor cohort had at least an n of 3 biological replicates (n = 4 for over-age donors), each donor had technical replicate samples (n = 5). Significance bars: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, FDR adjusted

In summary the data suggested that metabolic activity in the cartilage was only significantly different between normal and hypothermic cohorts, and then over-age and hypothermic cohorts in weeks 8 and 12 of storage, while it might have been expected to also find a significant difference between hypothermic and the other donor cohorts in week 4. Data suggested that there was a significant increase in metabolic activity in the normal donor cohort between week 1 and 8 and then week 1 and 12. With the over-age cohort there was a significant increase in metabolic activity between week 1 and all subsequent storage weeks, and then

between week 4 and 12. With the hypothermic donor cohort there was a significant decrease in metabolic activity between week 1 and 8 and then week 12, and then also between week 4 and 8 and then week 12.

3.5 - Lubricin (PRG4) assay results

Immunohistochemistry - HRP

Variance within cohorts

Representative images from each donor cohort at the end of their respective storage periods that were stained for the presence of lubricin (PRG4) via HRP are shown in Fig. 3.5.1.

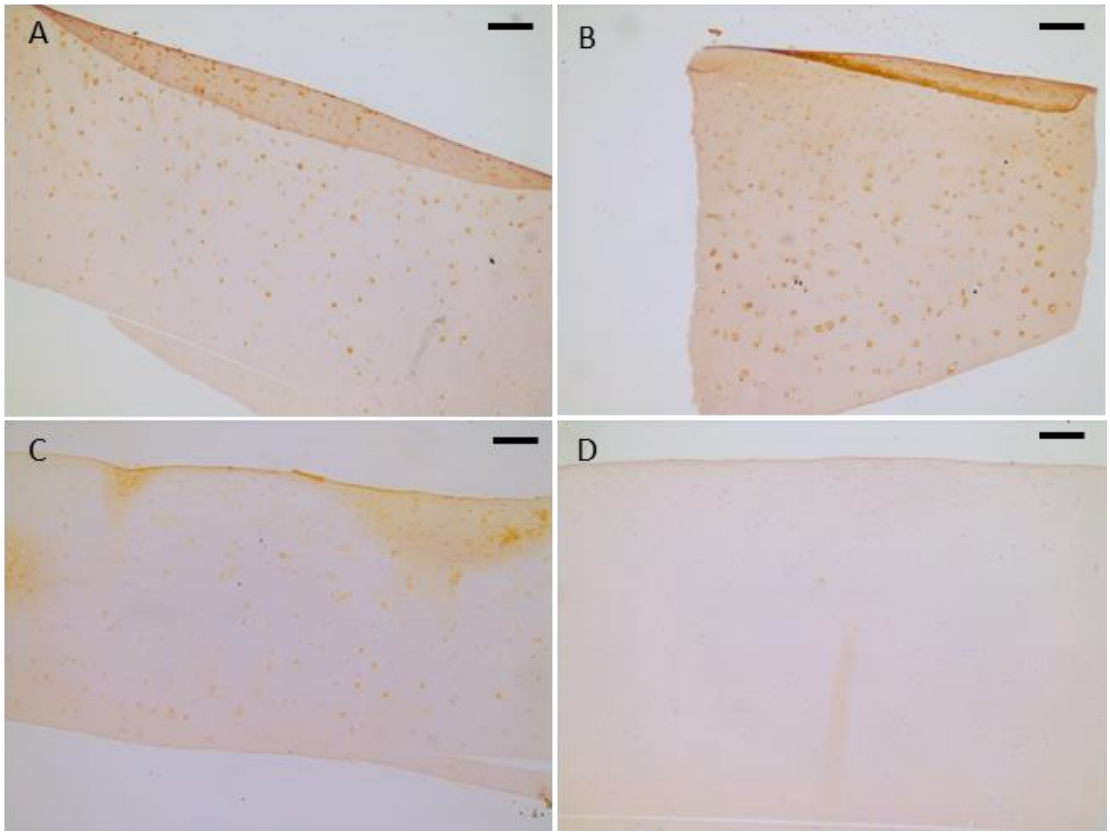

Figure 3.5.1. Immunohistochemistry sections of articular cartilage stained for the presence of lubricin (PRG4) via horseradish peroxidase. A) Normal donor (<45 year old) at the end of week 12. B) Over-age donor (>45 year old) at the end of week 12. C) Osteoarthritic living donor at the end of week 8. D) Hypothermic (4°C) donor at the end of week 12. Images at x50 magnification, scale bars = 200 µm

Results of the HRP staining of lubricin in normal donor samples (n = 3) showed that the beginning of week 1 mean scoring ranged from 1.67 to 4.00, with an overall mean of 2.78 (Table 3.5.1). At the end of the storage period in week 12 the scoring in most cases had increased to a mean range of 2.00 to 3.67 with only the third donor having a decrease in score

from 4.00 to 3.00 (Table 3.5.1). This resulted in an overall mean in week 12 of 2.89 (Table 3.5.1), representing an increase in scoring from week 1 to 12.

Table 3.5.1. Summary of double blind scoring of cartilage section stained for the presence of lubricin.[a]

Scores (HRP)	**Wk 1**				**Mean**	**SD**	**Wk 12 (8)**				**Mean**	**SD**	***P***
	Rep 1	Rep 2	Rep 3	Rep 4			Rep 1	Rep 2	Rep 3	Rep 4			
Normal	3, 1, 1	1, 5, 2	3, 4, 5		2.78	1.2	2, 3, 1	2, 5, 4	3, 2, 4		2.89	0.8	0.90
Over-age	5, 5, 5	3, 1, 3	2, 1, 1	4, 5, 3	3.17	1.6	4, 4, 4	3, 4, 2	4, 5, 5	1, 4, 2	3.50	1.0	0.74
Hypo	5, 5, 5	3, 4, 5	2, 1, 1		3.44	1.9	5, 4, 5	1, 1, 1	2, 2, 2		2.56	1.9	0.60
OA	4, 4, 3	1, 3, 1	3, 3.5, 2		2.72	1.0	1, 2, 1	3, 3, 2	4.5, 5, 3		2.72	1.4	1.00
Negative	5, 5, 4						1, 1, 2						
Isotype Control	1, 3, 1	2, 2, 1											
Control no Ab	1, 3, 1	1, 1, 2											

[a]Immunohistochemistry images assessed using a scoring scale ranging from 1 (no stain) to 5 (maximal stain) of tissue stored at week 1 or week 12 (8 for osteoarthritic), previously stained via horseradish peroxidase (HRP) chromogen. OA = osteoarthritic, hypo = hypothermic, Wk = week, Ab = antibody, *P* = *p* value obtained via t-test

With the over-age donor cohort (n = 4), scoring of the HRP staining showed that at the beginning of week 1 the mean score ranged from 1.33 to 4.00, with an overall mean of 3.17 (Table 3.5.1). By the end of the storage period on week 12 half of the donors had an increase in scoring while the other half had a decrease, this resulted in a mean range from 2.33 to 4.67, with an overall mean of 3.50 (Table 3.5.1). Therefore, the scoring had increased on week 12 compared to week 1 in the over-age cohort (*p*-value for week 1 vs week 12 = 0.90).

In the living donor OA cohort (n = 3), scoring of the HRP staining showed that at the beginning of week 1 the mean score ranged from 1.67 to 3.67, with an overall mean of 2.72 (Table 3.5.1). At the end of the storage period on week 8 the range of mean scores increased from 1.33 to

4.17, as a result the overall mean score was 2.72. This showed that there was no change in apparent staining visible in the OA cartilage sections (*p*-value for week 1 vs week 8 = 1.00).

For hypothermic stored tissue (n = 3), scoring of the HRP staining showed that at the beginning of week 1 the mean score ranged from 1.33 to 5, with an overall mean of 3.44 (Table 3.5.1). By the end of the storage period on week 12 two of the donors had a decrease in mean scoring, with the other donor having a slight increase, this resulted in a range of mean scores from 1.00 to 4.67, which gave an overall mean of 2.56 (Table 3.5.1). This indicated an overall mean decrease in scoring from week 1 to 12 (*p*-value for week 1 vs week 12 = 0.60).

Although there was only one donor of devitalised tissue used in this dataset, it showed that the mean HRP scoring at the beginning of week 1 was 4.67, but by week 12 this had decreased to 1.33 (Table 3.5.1, *p*-value for week 1 vs week 12 = 0.0021).

Variance between cohorts

Overall mean data from the normal donor cohort showed that the scoring was below that seen for the over-age cohort, with a mean score of 2.78 ± 1.2 compared to 3.17 ± 1.6 (Table 3.5.1 (*p*-value for normal vs over-age = 0.92). In week 12 the score for the normal donor cohort had increased to 2.89 ± 0.8, while the over-age cohort had increased to 3.50 ± 1.0 (*p*-value for normal vs over-age = 0.92). The over-age cohort therefore had consistently higher mean scoring over both cartilage sampling time points. However, the standard deviation showed large variance in both sets of data.

When the normal donor cohort was compared with the living donor OA cohort it was found that the mean scoring for the normal donors was slightly higher at the beginning of week 1, with a mean of 2.78 ± 1.2 for normal compared to 2.72 ± 1.0 for OA (*p*-value for normal vs OA = 0.92) (Table 3.5.1). By week 12 (week 8 for OA) normal donor mean scoring had increased to 2.89 ± 0.8 (*p*-value for normal week 1 vs week 12 = 0.90) whereas OA remained the same with a mean of 2.72 ± 1.4 (*p*-value for OA week 1 vs week 8 = 1.00). Normal donor scoring was therefore higher on both storage weeks, but again standard deviation demonstrated large variance in both cohorts.

With comparison between the normal donor cohort and hypothermic stored tissue it was found that the mean scoring at the beginning of week 1 was higher in hypothermic tissue compared to normal with a mean score of 2.78 ± 1.2 for normal compared to 3.44 ± 1.9 for hypothermic (Table 3.5.1 (*p*-value for normal vs hypothermic = 0.92)). By week 12 mean scoring for

hypothermic tissue had decreased to 2.56 ± 1.9 which was lower than the scored given to the normal cohort at 2.89 ± 0.8 (*p*-value for normal vs hypothermic = 0.92). As before, standard deviation showed large variance in both cohorts.

With overall mean data comparison between the over-age cohort and living donor OA it was found that at the beginning of week 1 the over-age scoring was higher with the over-age score at 3.17 ± 1.6 compared to 2.72 ± 1.0 for OA (Table 3.5.1 (*p*-value for over-age vs OA = 0.92)). By week 12 (week 8 for OA donors), while the mean score had remained the same for OA tissue at 2.72 ± 1.4, in the over-age the mean score had increased to 3.50 ± 1.0 (*p*-value for over-age vs OA = 0.92).

For comparison between the mean data from over-age and hypothermic donor cohorts it was found that at the beginning of week 1 the over-age scoring was lower than that for hypothermic, 3.17 ± 1.6 compared to 3.44 ± 1.9 (Table 3.5.1 (*p*-value for over-age vs hypothermic = 0.92)). By week 12 hypothermic scoring had decreased to a mean of 2.56 ± 1.9 while over-age had increased to 3.50 ± 1.0 (*p*-value for over-age vs hypothermic = 0.92). The standard deviation again demonstrated the large variance in the cohorts.

Finally, when overall mean data from the living donor OA cohort was compared with hypothermic the results showed that at the beginning of week 1 the scoring for OA was lower with a value of 2.72 ± 1.0 compared to 3.44 ± 1.9 (Table 3.5.1 (*p*-value for OA vs hypothermic = 0.92)). By week 12 (week 8 for OA donors) the mean score for OA had remained the same at 2.72 ± 1.4 while hypothermic had decreased to a lower value of 2.56 ± 1.9 (*p*-value for OA vs hypothermic = 0.92). Standard deviation showed large variance in this data.

Table 3.5.2. Statistical analysis of cartilage sections scored for the presence of lubricin.[a]

Week	Lubricin score *P*-Value
1	0.92
12 (8)	0.92

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts, sections stained via horseradish peroxidase (HRP) immunohistochemistry in sections from week 1, 8 (osteoarthritic), or 12

When all the cohorts were compared by one-way analysis of variance, no significant difference ($p > 0.05$) was observed between all cohorts at any time point, week 1 (FDR adjusted $p = 0.92$), week 12 (week 8 for OA cohort) (FDR adjusted $p = 0.43$) (Table 3.5.2).

With statistical comparison using t-test between week 1 and 12 (or 8 for OA donors) in each cohort it was found that there was no significant difference ($p > 0.05$) between either week within each cohort (normal FDR adjusted $p = 0.90$, over-age FDR adjusted $p = 0.74$, OA FDR adjusted $p = 1.00$, hypothermic FDR adjusted $p = 0.60$) (Table 3.5.3 and Fig. 3.5.2).

Table 3.5.3. Statistical analysis of cartilage sections scored for the presence of lubricin.[a]

Cohort	Lubricin *P*-Value
Normal	0.90
Over-age	0.74
Osteoarthritis	1.00
Hypothermic	0.60

[a]T-test analysis compares normal cohort were donors <45 years old, over-age were <45 years old, osteoarthritis were living donors and hypothermic was tissue stored at 4°C, sections stained via horseradish peroxidase (HRP) immunohistochemistry in sections from week 1, 8 (osteoarthritic), or 12

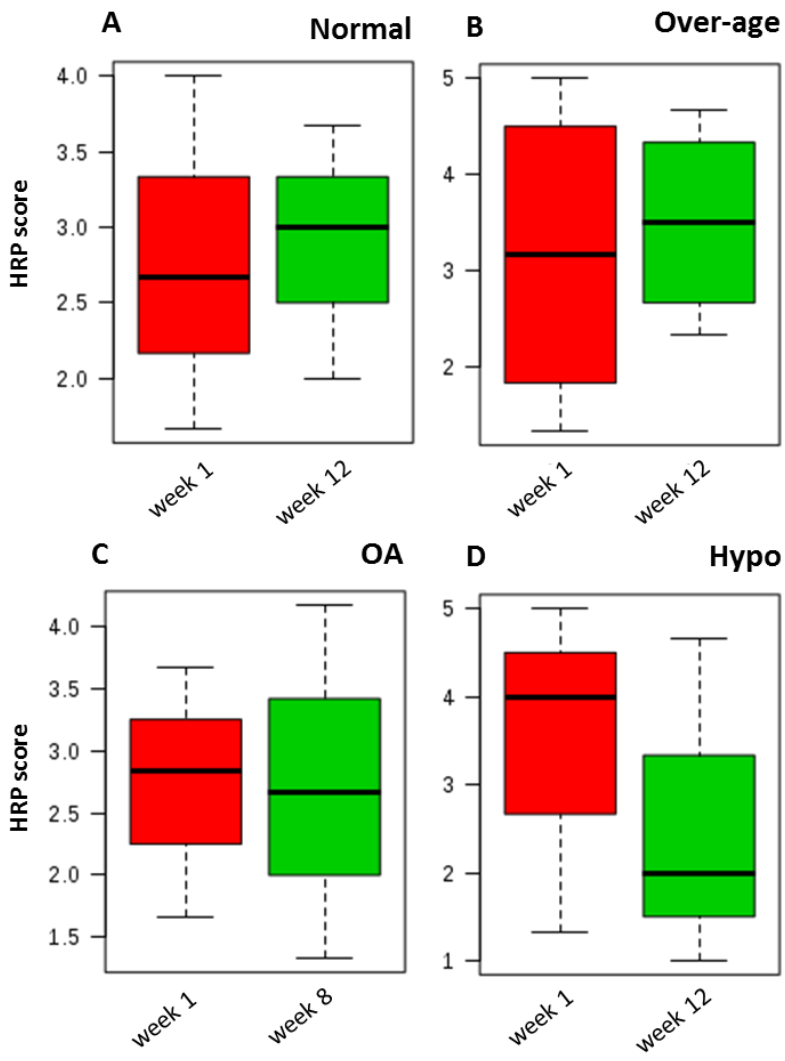

Figure 3.5.2. Box plots showing results from t-test carried out on horseradish peroxidase (HRP) immunohistochemistry scoring to detect lubricin in cartilage sections in week 1, and week 12 (Week 8 with osteoarthritic (OA) tissue). A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) OA. D) Hypothermic (Hypo, 4°C), n = 3, except for over-age where n = 4, n = 3 technical replicates were performed. Negative control tissue was not compared due to lack of biological replicates

In summary for the HRP stained cartilage sections the data suggested no significant difference was observed between cohorts either at the beginning or end of the storage periods. It could be suggested that perhaps it might have been expected to find a significant difference in OA tissue compared to other cohorts at the beginning of week 1, and then at the end of the storage period as well as between hypothermic and all other cohorts. There was also no significant difference observed within cohorts at the beginning and end of the storage periods, when it might be expected to find a significant difference within the OA and hypothermic cohorts.

Immunofluorescence – Alexa Fluor® Plus 488

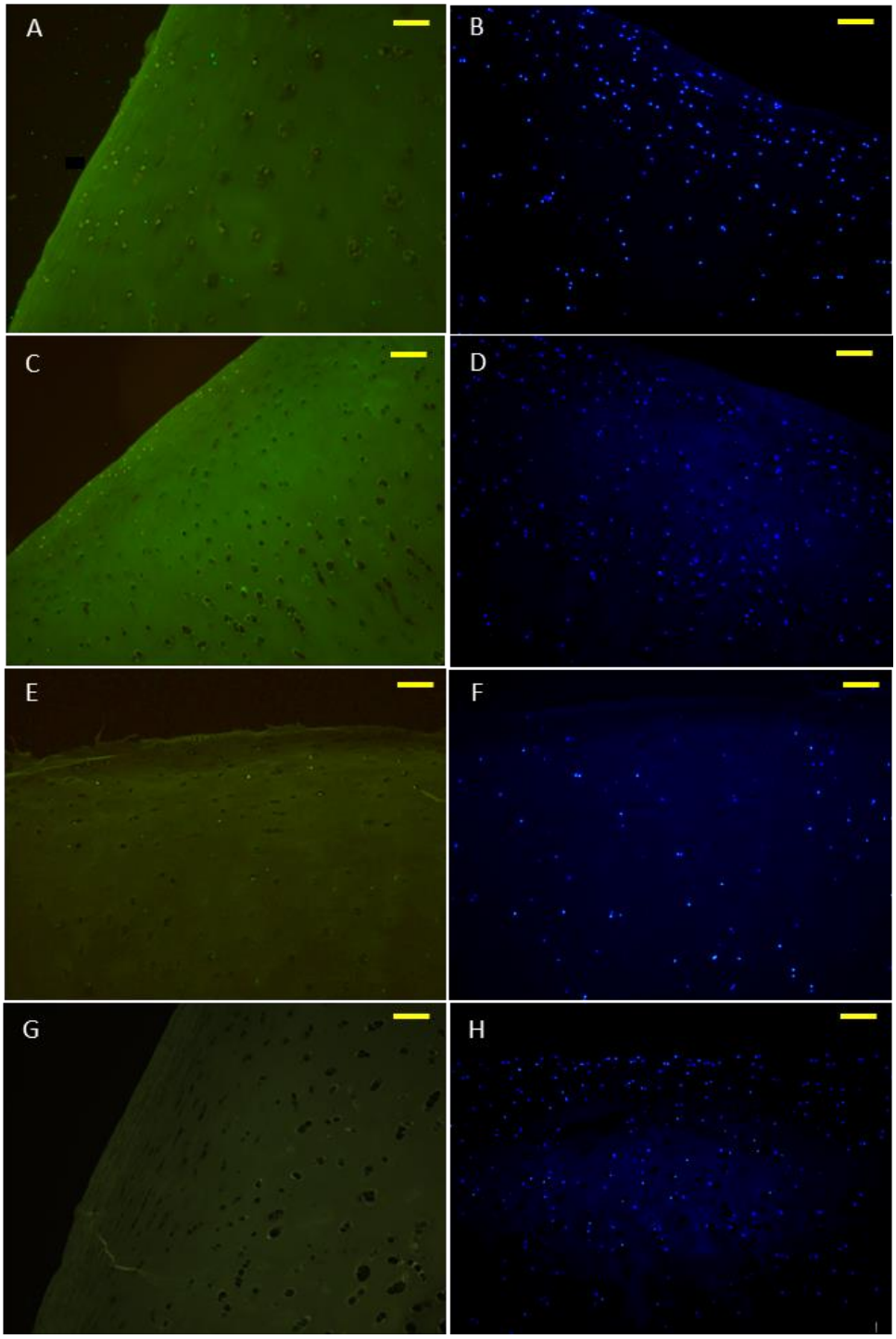

Figure 3.5.3. Immunohistochemistry sections of articular cartilage stained for the presence of lubricin (PRG4) via Alexa Fluor® Plus 488, alongside corresponding 4′,6-diamidino-2-phenylindole (DAPI) image to demonstrate presence of cells. A and B) Normal donor (<45 year old) at the end of week 12. C and D) Over-age donor (>45 year old) at the end of week 12. E and F) Osteoarthritic living donor at end of week 8. G and H) Hypothermic (4°C) donor at end of week 12. Images at x100 magnification, scale bars = 100 µm

Variance within cohorts

Representative images from each donor cohort at the end of their respective storage periods that were stained for the presence of lubricin (PRG4) via Alexa Fluor® Plus 488 are shown in Fig. Fig. 3.5.3. Alongside each image are images of DAPI stain from the same sections of cartilage, that show evidence of cells via staining of cellular DNA.

Results of the immunofluorescence staining of normal donor cartilage sections showed that at the beginning of week 1 the mean scoring ranged from 1.00 to 4.67, with an overall mean of 2.89 (Table 3.5.4). By week 12 the range had reduced from 1.33 to 3.67, with an overall mean of 2.78. Therefore, a decrease in staining for lubricin was observed at the end of the storage period for this cohort (*p*-value for week 1 vs week 12 = 0.94).

In the over-age donor cohort results showed that at the beginning of week 1 the mean scoring of sections ranged from 1.67 to 4.33, with an overall mean value of 2.75 (Table 3.5.4). At the end of the storage period on week 12 the variance of data had decreased with the scoring range from 2.33 to 3.33, with an overall mean of 2.75. This suggests there was no change in the presence of lubricin in the cartilage sections from this cohort from week 1 to week 12 (*p*-value for week 1 vs week 12 = 1.00).

With the living donor OA cohort, results showed that at the beginning of week 12 the mean scoring of sections was consistently at 2 which therefore gave an overall mean score of 2 (Table 3.5.4). The results were not as consistent on week 8 however with a score range of 1.33 to 2, which resulted in an overall mean score of 1.56. The result suggests there was a decrease of lubricin in sections from this cohort (*p*-value for week 1 vs week 8 = 0.12).

For hypothermic stored tissue, results showed that at the beginning of week 1 the mean score ranged from 1.00 to 2.67, with an overall mean score of 1.78 (Table 3.5.4). By week 12 the range had increased from 1.67 to 4, with an overall mean score of 2.89. This suggests there was an increase in staining for lubricin at the end of the storage period in this cohort (*p*-value for week 1 vs week 12 = 0.25).

Although there was only one donor of devitalised tissue used in this dataset, it showed that the mean immunofluorescence scoring at the beginning of week 1 was 3.67, by week 12 this had decreased to a mean of 1. This suggested a decrease in lubricin staining in this tissue.

Table 3.5.4. Summary of double blind scoring of cartilage section stained for the presence of lubricin.[a]

Scores (AFP488)	**Wk 1**				**Mean**	**SD**	**Wk 12 (8)**				**Mean**	**SD**	***P***
	Rep 1	Rep 2	Rep 3	Rep 4			Rep 1	Rep 2	Rep 3	Rep 4			
Normal	5, 5, 4	2, 3, 4	1, 1, 1		2.89	1.8	2, 1, 1	4, 1, 5	3, 5, 3		2.78	1.3	0.94
Over-age	2, 2, 1	4, 4, 5	3, 2, 3	1, 4, 2	2.75	1.1	2, 2, 3	4, 1, 4	4, 3, 3	3, 2, 2	2.75	0.5	1.00
Hypo	2, 2, 1	1, 1, 1	3, 2, 3		1.78	0.8	5, 2, 5	2, 1, 2	3, 2, 4		2.89	1.2	0.25
OA	1, 2, 3	1, 1, 4	1, 1, 4		2.00	0.0	1, 1, 2	3, 2, 1	2, 1, 1		1.56	0.4	0.12
Negative	4, 5, 2						1, 1, 1						
Isotype Cont.	1, 2, 1	1, 1, 1											
Control no Ab	1, 1, 1	3, 1, 1											

[a]Immunohistochemistry images assessed using a scoring scale ranging from 1 (no stain) to 5 (maximal stain) of tissue stored at week 1 or week 12 (8 for osteoarthritic), previously stained via Alexa Fluor® Plus 488 (AFP488). OA = osteoarthritic, Wk = week, *P* = *p* value obtained via t-test

Variance between cohorts

When the overall mean data of scores from AFP488 stained sections of normal donors was compared with the data from over-age at the beginning of week 1, it was found that the score from normal donors 2.89 ± 1.8 was higher than that for over-age 2.75 ± 1.1 (Table 3.5.4 and Fig. 3.5.4 (*p*-value for normal vs over-age = 0.57)). At week 12 the data showed that the scoring for normal donors was still slightly higher than that for over-age donors with a value of 2.78 ± 1.3 for normal compared to 2.75 ± 0.5 for over-age (*p*-value for normal vs over-age = 0.53). The standard deviation demonstrated a large variance in the scoring.

For comparison of overall mean data in normal donor samples with living donor OA at the beginning of week 1 it was found that the score for the normal cohort was higher with a value of 2.89 ± 1.8 compared to 2.00 ± 0.0 (Table 3.5.4 and Fig. 3.5.4 (*p*-value for normal vs OA =

0.57)). By week 12 (or week 8 for living donor OA) while the score for normal donors had decreased slightly to 2.78 ± 1.3 (p-value for week 1 vs week 12 = 0.94), the score for OA tissue had decreased further to 1.56 ± 0.4 (p-value for week 1 vas week 8 = 0.12). Again, the standard deviation highlighted the large variance in the scoring.

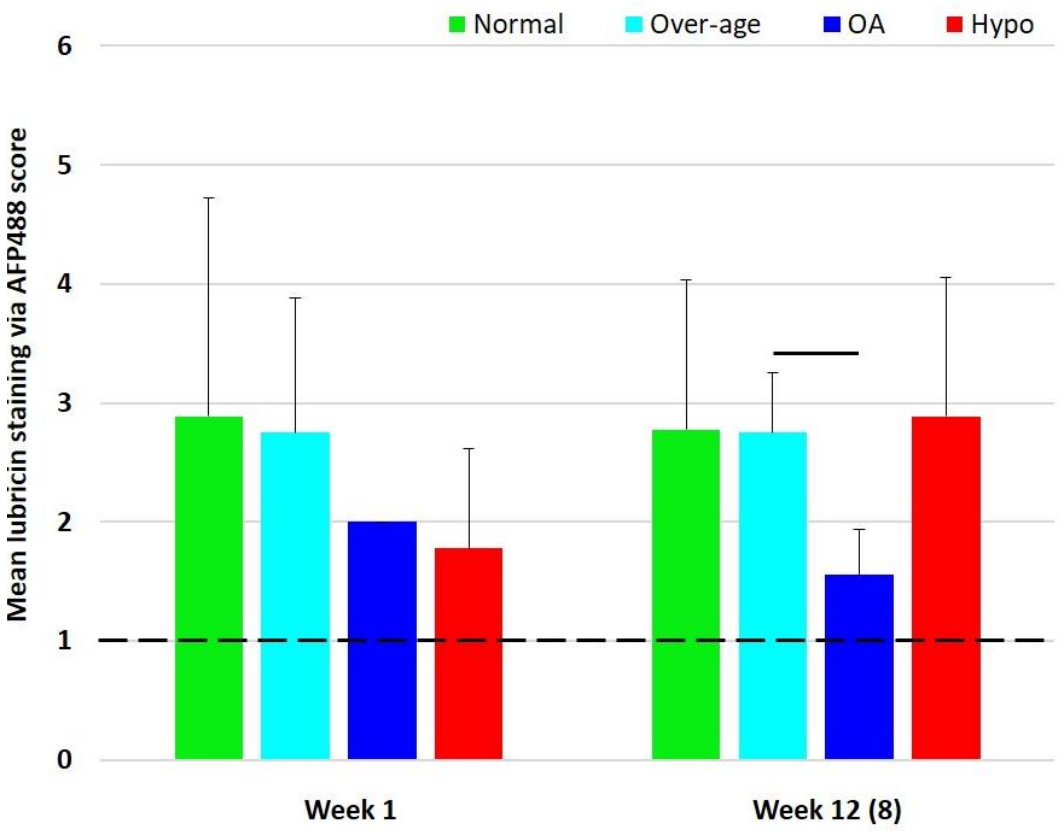

Figure 3.5.4. Bar chart of mean Alexa Fluor® Plus 488 scoring for the presence of lubricin in cartilage sections. Normal donor cartilage (<45 years of age). Over-age donor cartilage (>45 years of age). Living donor osteoarthritic cartilage (OA). Hypothermic stored (Hypo, 4°C) cartilage, n = 3, except for over-age where n = 4, n = 3 technical replicates were performed, with dashed black line showing baseline calculated from mean negative control scores at end of the storage period, negative control not compared due to lack of biological replicates, significance bar shows FDR adjusted $p < 0.05$

With normal compared to hypothermic stored tissue at the beginning of week 1 it was found that the overall mean scoring for normal cartilage 2.89 ± 1.8 was higher compared to hypothermic 1.78 ± 0.8 (Table 3.5.4 and Fig. 3.5.4 (p-value for normal vs hypothermic = 0.57). In week 12 the score for normal cartilage had decreased slightly to 2.78 ± 1.3 (p-value for week 1 vs week 12 = 0.94) whereas in hypothermic stored tissue the score had increased to 2.89 ± 1.2 (p-value for week 1 vs week 12 = 0.25), therefore making it higher. However, the standard deviation showed large variance present in both cohorts.

When the overall mean data for the over-age cohort was compared with living donor OA at the beginning of week 1, the over-age cohort had a higher score of 2.75 ± 1.1 compared to 2.00 ± 0.0 (Table 3.5.4 and Fig. 3.5.4 (p-value for over-age vs OA = 0.57). At the end of the storage period in week 12 (week 8 for OA donors) the score for over-age was still higher at 2.75 ± 0.5 compared to 1.56 ± 0.4 for OA (p-value for over-age vs OA = 0.038), which had decreased from week 1. There was a large variance in the scoring however, as demonstrated by standard deviation.

Comparison of overall mean scores between over-age and hypothermic cohorts showed that at the beginning of week 1 over-age score was higher at 2.75 ± 1.1 compared to 1.78 ± 0.8 (Table 3.5.4 and Fig. 3.5.4 (p-value for over-age vs hypothermic = 0.57). However, in week 12 the mean score for hypothermic had increased to 2.89 ± 1.2 while over-age remained at 2.75 ± 0.5 (p-value for over-age vs hypothermic = 0.53). Standard deviation showed that the scoring again showed large variance in the cohorts compared.

Finally, when overall mean scores were compared between the living donor OA cohort and hypothermic at the beginning of week 1 it was found that the OA score was slightly higher at 2.00 ± 0.0 compared to 1.78 ± 0.8 (Table 3.5.4 and Fig. 3.5.4 (p-value for OA vs hypothermic = 0.57). However, by week 12 (week 8 for OA donors) while OA decreased to 1.56 ± 0.4 (p-value for week 1 vs week 8 = 0.12), hypothermic score had increased to 2.89 ± 1.2 (p-value for week 1 vs week 12 = 0.25). As with previous comparisons it was found that the variance in the scoring was large, as demonstrated by the standard deviation.

When all the cohorts were compared by one-way analysis of variance, no significant difference was observed between all cohorts at the beginning of week 1 (p = 0.24), but by week 12 (week 8 for OA) a significant difference was observed between over-age and OA cohorts (p = 0.038) (Table 3.5.5 and Fig 3.5.4).

Table 3.5.5. Statistical analysis of cartilage sections scored for the presence of lubricin.[a]

Week	Lubricin score *P*-Value	Fisher's *post-hoc* (cohorts)
1	0.57	N/A
12 (8)	0.038	O-OA

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts. O = over-age, OA = osteoarthritic, sections from week 1, 8 (osteoarthritic), or 12, stained via Alexa Fluor® Plus 488, n = 3 except for over-age where n = 4, n = 3 technical replicates were performed

With statistical comparison using t-test between weeks 1 and 12 (week 8 for OA donors) in each cohort it was found that there was no significant difference between either week within each cohort, normal ($p = 0.94$), over-age ($p = 1.00$), OA ($p = 0.12$) and hypothermic ($p = 0.25$) (Table 3.5.6 and Fig. 3.5.5).

Table 3.5.6. Statistical analysis of cartilage sections scored for the presence of lubricin.[a]

Cohort	Lubricin *P*-Value
Normal	0.94
Over-age	1.00
Osteoarthritis	0.12
Hypothermic	0.25

[a]T-test analysis compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts, sections from week 1, 8 (osteoarthritic), or 12, stained via Alexa Fluor Plus® 488, n = 3 except for over-age where n = 4, n = 3 technical replicates were performed

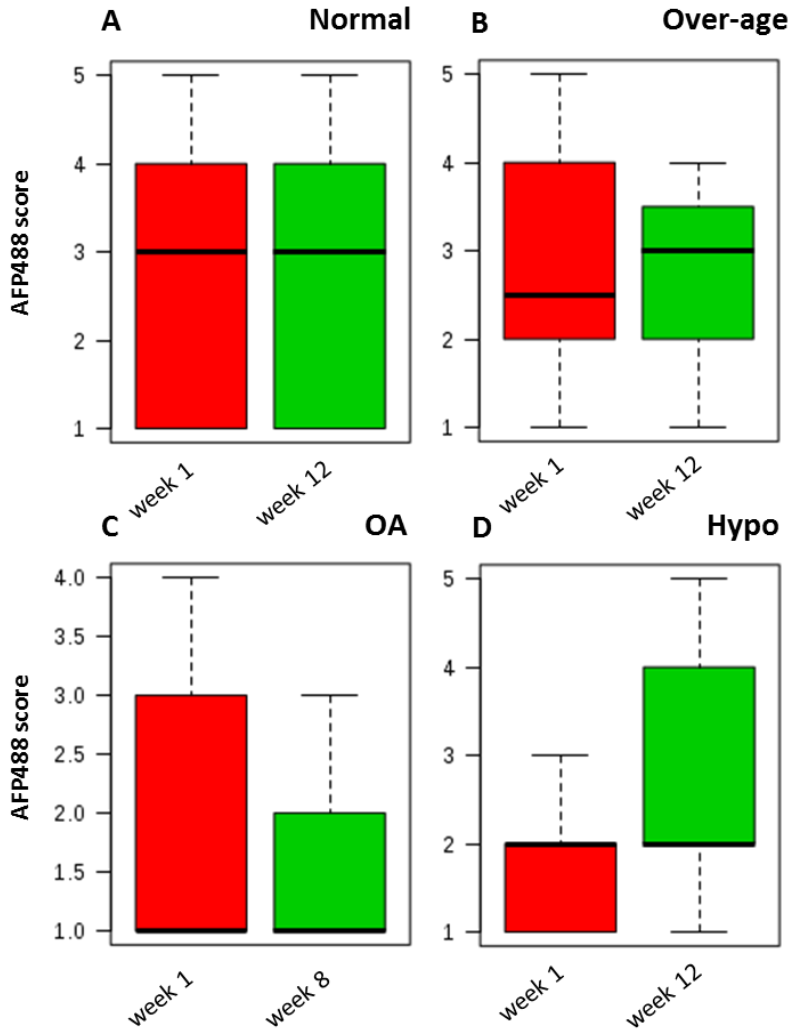

Figure 3.5.5. Box plots showing results from t-test carried out on Alexa Fluor® Plus 488 (AFP488) stained and scored sections to detect lubricin in cartilage at the beginning of week 1, and week 12 (Week 8 with osteoarthritic (OA) tissue). A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) OA. D) Hypothermic (Hypo, 4ºC), n = 3 except for over-age where n = 4, n = 3 technical replicates were performed

In summary for the immunofluorescence stained sections of cartilage, the data suggested that the only significant change in scoring for the presence of lubricin was found between over-age and OA cartilage at the end of the storage periods. It might have been expected to also find a significant change between hypothermic and all other cohorts, and between OA compared to other cohorts at the beginning of week 1. When storage weeks were compared within cohorts the data suggested that there was no significant difference, when perhaps it might have been expected to find significant change between the beginning of week 1 and the end of storage periods between hypothermic and OA donor cohorts.

3.6 - Safranin O histology results

Variance within cohorts

Representative images from each donor cohort that show the safranin O and fast green staining of cartilage sections are shown in Fig. 3.6.1.

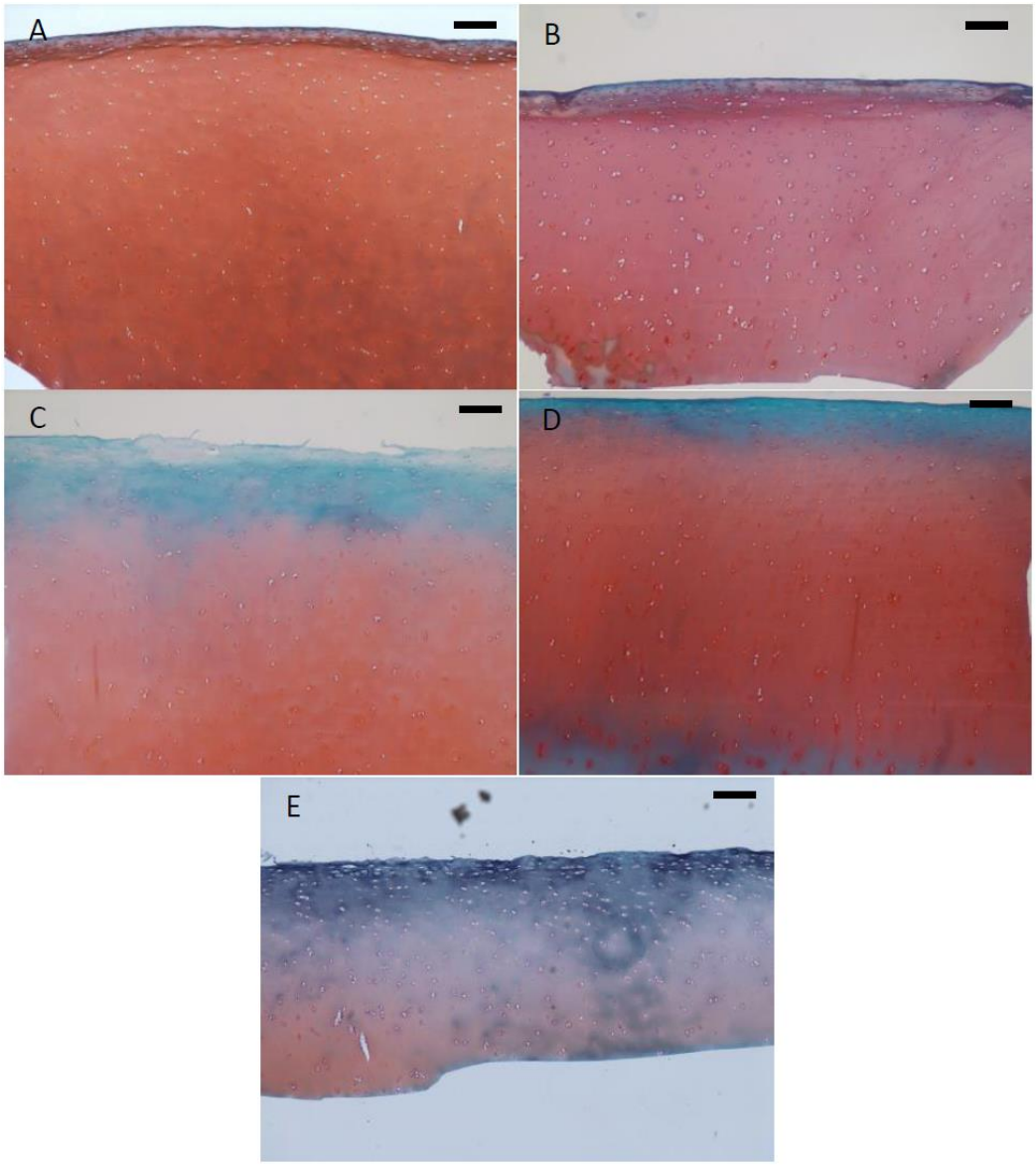

Figure 3.6.1. Representative histology images of cartilage sections from each donor cohort stained with safranin O to detect proteoglycan and glycosaminoglycans (GAGs) (red) and counter-stained with fast green to determine absence of proteoglycans and GAGs (blue). A) Example from normal donor (<45 years of age) at week 12, B) example from over-age donor (>45 years of age) at week 12, C) example from living donor osteoarthritic tissue at week 8, D) example from hypothermic (4ºC) stored tissue at week 12, E) example from negative control tissue at week 12. Images at x50 magnification, scale bars = 200 µm

Results of the histology staining in the normal donor (n = 3) cohort showed that the percent coverage of proteoglycan and glycosaminoglycan (GAG) remaining increased from a range of 46.2% to 90.9% at the beginning of week 1 to a range of 93.6% to 97.0% in week 12. This resulted in a mean value of 70.8% ± 22.7 in week 1 compared to 95.6% ± 1.8 in week 12 (FDR adjusted $p = 0.13$) (Table 3.6.1 and Fig. 3.6.2).

With the over-age donor cohort (n = 4), data showed that at the beginning of week 1, proteoglycan and GAG remaining in the sections ranged from 62.0% to 89.3%. By week 12 the range had improved to 85.9% to 91.1%. As a result, there was a mean of 81.2% ± 3.2 at the beginning of week 1 and 88.0 ± 2.3 in week 12 (FDR adjusted $p = 0.34$) (Table 3.6.1 and Fig. 3.6.2).

In the living donor OA cohort (n = 3), proteoglycan and GAG remaining in the sections ranged from 83.3% to 90.0% at the beginning of week 1, however by week 8 the range had decreased to 70.2% and then 84.2%. The outcome of this was a mean value of 86.1% ± 3.5 at the beginning of week 1 which decreased to 76.7% ± 7.1 in week 8 (FDR adjusted $p = 0.11$) (Table 3.6.1 and Fig. 3.6.2).

Table 3.6.1. Summary of data for coverage of safranin O (%) in histological sections of cartilage.[a]

Safranin O (%)	**Mean (± standard deviation)**		**t-test**
	Week 1	**Week 12 (8)**	***P***
Normal	70.8 ± 22.7	95.6 ± 1.8	0.13
Over-age	81.2 ± 3.2	88.0 ± 2.3	0.34
Hypothermic	88.7 ± 2.6	77.8 ± 17.1	0.11
Osteoarthitis	86.1 ± 3.5	76.7 ± 7.1	0.34

[a]Data shows means with standard deviation from normal (<45 years of age), over-age (>45 years old), hypothermic (4°C) and osteoarthritic (living donors) where at least 3 donor samples were obtained (over-age donor samples n = 4) in weeks 1, 8 (osteoarthritic), and 12 of storage

For hypothermic stored tissue (n =3), proteoglycan and GAG remaining in the sections of cartilage ranged from 85.8% to 90.9% at the beginning of week 1, in week 12 the coverage of staining in one of the donors had greatly decreased so that the range was then 58.3% to 90.1%. Mean data showed that a value of 88.7% ± 2.6 was determined at the beginning of week 1, and then a mean of 77.8% ± 17.1 in week 12 (Table 3.6.1 and Fig. 3.6.2 (p-value for week 1 vs week 12 = 0.11).

There was only one donation available for negative control results, however this showed that the amount of proteoglycan and GAG in the cartilage sections had decreased from 85.0% at the beginning of week 1 to 60.1% in week 12.

Variance between cohorts

Mean data showed that when staining of normal cartilage was compared with over-age, at the beginning of week 1 the over-age cohort had a higher percentage coverage of safranin O (81.2% ± 3.2 for over-age compared to 70.8% ± 22.7 for normal) (p = 0.41) and therefore amount of remaining proteoglycan and GAG (Table 3.6.1 and Fig. 3.6.2). By week 12 however the percent coverage for the normal donors had increased to a higher amount compared to over-age (95.6% ± 1.8 for normal with 88.0% ± 2.3 for over-age) ($p = 4.47 \times 10^{-6}$) (Table 3.6.1 and Fig. 3.6.2).

When the mean data from safranin O staining of normal cartilage was compared with living donor OA tissue, at the beginning of week 1 the OA cohort had a higher percent coverage of safranin O (86.1% ± 3.5 for OA compared to 70.8% ± 22.7 for normal) (p = 0.41) (Table 3.6.1 and Fig. 3.6.2). By week 12 however (week 8 for OA tissue) normal donors had increased to a percent coverage of 95.6% ± 1.8 while OA had decreased to 76.7% ± 7.1 ($p = 4.47 \times 10^{-6}$) (Table 3.6.1 and Fig. 3.6.2).

For comparison between normal and hypothermic tissue, mean data showed that at the beginning of week 1 hypothermic tissue had a higher percent coverage of safranin O at 88.7% ± 2.6 compared to 70.8% ± 22.7 for normal (p = 0.41) (Table 3.6.1 and Fig. 3.6.2). However, by week 12 safranin O percent coverage had decreased to 77.8% ± 17.1 in the hypothermic cohort, whereas in the normal donor's coverage had increased to 95.6% ± 1.8 ($p > 0.05$) (Table 3.6.1 and Fig. 3.6.2).

Comparison of mean data between over-age and OA cohorts showed that mean safranin O percent coverage at the beginning of week 1 was relatively close with over-age at 81.2% ± 3.2 compared to 86.1% ± 3.5 (p = 0.41) for OA tissue (Table 3.6.1 and Fig. 3.6.2). At week 12 (week 8 for OA tissue) over-age had increased to 88.0% ± 2.3 compared to 76.7% ± 7.1 for OA ($p = 4.47 \times 10^{-6}$) (Table 3.6.1 and Fig. 3.6.2).

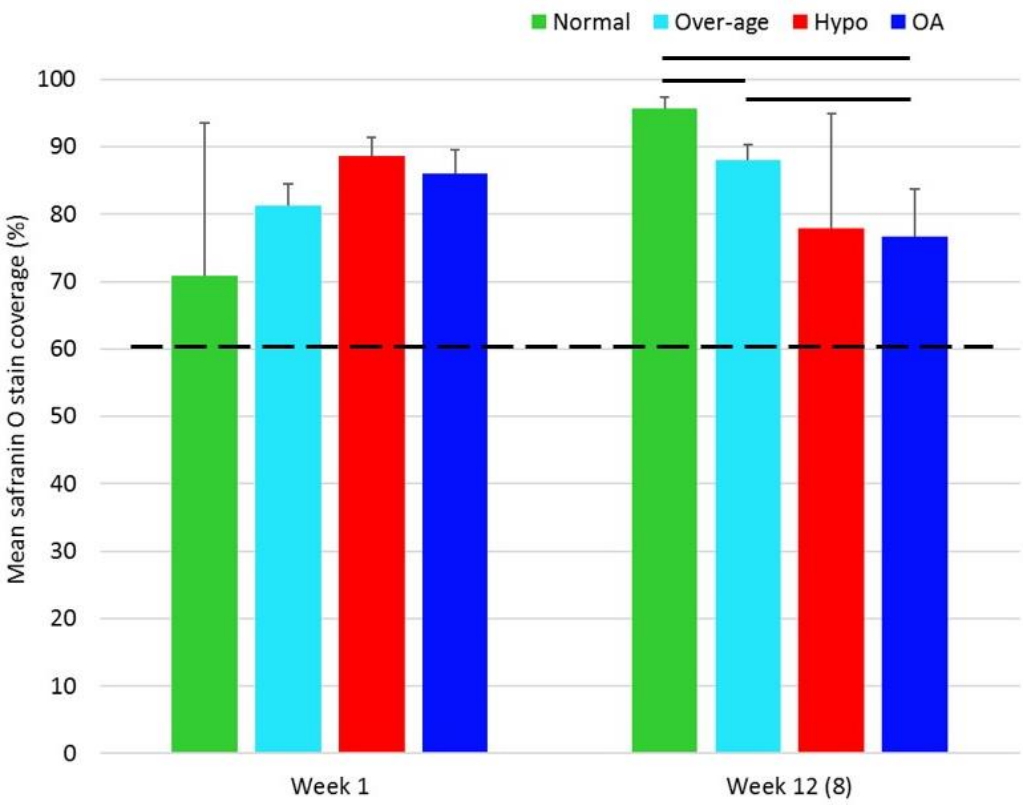

Figure 3.6.2. Bar chart of mean safranin O stain coverage (%) ± standard deviation, of cartilage sections, at weeks 1, 8 (osteoarthritic (OA)), and 12. Normal donor (<45 years of age) cartilage. Over-age donor (>45 years of age) cartilage. Living donor OA cartilage. Hypothermic (Hypo) stored (4ºC) cartilage, n = 3 biological replicates except for over-age where n = 4, with dashed line showing baseline of negative control value at the end of the storage period, negative control not compared due to lack of biological replicates, significance bars show $p < 0.01$, which were FDR-corrected

For comparison between over-age and hypothermic cohorts, data showed that mean safranin O percent coverage at the beginning of week 1 was higher in hypothermic with a mean value of 88.7% ± 2.6 compared to 81.2% ± 3.2 for over-age ($p = 0.41$) (Table 3.6.1 and Fig. 3.6.2). By week 12 the mean value for over-age had increased to 88.0% ± 2.3, whereas the mean value for hypothermic had decreased to 77.8% ± 17.1 ($p > 0.05$) (Table 3.6.1 and Fig. 3.6.2).

When the mean data from OA was compared with that from hypothermic stored tissue it was found that at the beginning of week 1 the percent coverage was relatively close with a mean value of 86.1% ± 3.5 for OA compared to 88.7% ± 2.6 for hypothermic ($p = 0.41$) (Table 3.6.1 and Fig. 3.6.2). At the end of storage in week 12 (week 8 for OA tissue) it was found that percent coverage of safranin O remained relatively close between the two cohorts with a mean

value of 76.7% ± 7.1 for OA and 77.8% ± 17.1 for hypothermic ($p > 0.05$) (Table 3.6.1 and Fig. 3.6.2).

Variance over time

When all the cohorts were compared by one-way analysis of variance, no significant difference was observed between all cohorts at the beginning of week 1 ($p = 0.41$), but by week 12 (week 8 for OA) a significant difference was observed between normal and OA, normal and over-age, then over-age and OA cohorts ($p = 4.47 \times 10^{-6}$) (Table 3.6.2).

Table 3.6.2. Statistical analysis of variance of the coverage of safranin O (%) in histological sections of cartilage.[a]

Week	Safranin O *P*-Value	Coverage (%) Fisher's *post-hoc* (cohorts)
1	0.41	N/A
12 (8)	4.47×10^{-6}	N-OA, N-O, O-OA

[a]Analysis of variance compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts stored for weeks 1, 8 (osteoarthritic) or 12. N = normal, OA = osteoarthritis, O = over-age. *Post-hoc* result shows which cohorts had significant difference with each other

Table 3.6.3. Statistical analysis of the coverage of safranin O (%) in histological sections of cartilage.[a]

Cohort	Safranin O *P*-Value
Normal	0.13
Over-age	0.34
Osteoarthritis	0.11
Hypothermic	0.34

[a]T-test Analysis compares normal (<45 years old), over-age (>45 years old), osteoarthritic (living donor) and hypothermic (4°C) donor cohorts stored for weeks 1, 8 (osteoarthritic) or 12

With statistical comparison using t-test between weeks 1 and 12 (week 8 for OA donors) in each cohort it was found that there was no significant difference between either week within

each cohort, normal ($p = 0.13$), over-age ($p = 0.34$), OA ($p = 0.11$) and hypothermic ($p = 0.34$) (Table 3.6.3 and Fig. 3.6.3).

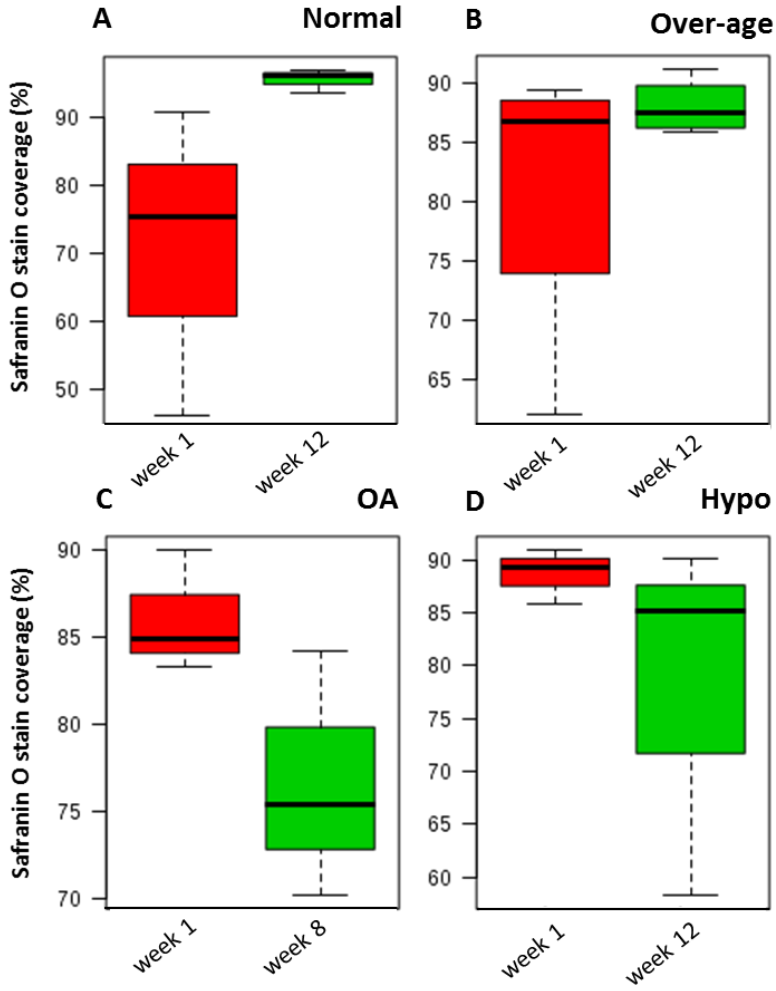

Figure 3.6.3. Box plots showing results from t-test carried out on safranin O stained sections of cartilage on week 1, and week 12 (week 8 with osteoarthritic (OA) tissue). A) Normal donor (<45 years old) cartilage. B) Over-age (>45 years old). C) OA. D) Hypothermic (Hypo, 4°C), n = 3 biological replicates except for over-age where n = 4

In summary, the results of the analysis for staining of cartilage sections with safranin O suggested that in all donor cohorts, residual proteoglycan and GAG had significantly decreased at the end of the storage period between normal and over-age, and then between normal and OA. There was also a significant decrease between over-age and OA. When storage weeks were compared within cohorts, data suggested that there was no significant change in all of the cohorts, when perhaps it might have been expected to observe a significant change in the hypothermic and OA cohorts.

Chapter 4
General discussion

4.1 - Glucose/lactate, metabolite turnover

Glucose consumption by osteochondral cells

Normal donors demonstrated higher glucose consumption than over-age, OA and hypothermic at the end of storage week 1. One possible interpretation for the initial elevated glucose consumption could be nutrient starved chondrocytes being transferred to a nutrient rich environment. However, it could be that the high glucose content of the storage medium (17.5 mM measured) is initially over-stimulating the chondrocytes. A paper published by Laiguillon *et al* (2015) showed that under high glucose (25 mM compared to 5.5 mM) culture conditions, isolated murine chondrocytes showed markedly increased glucose uptake. The authors stated that increased glucose causes the glycolytic pathway to become saturated resulting in activation of the secondary polyol metabolic pathway. The authors also suggested that in a pro-inflammatory and high-glucose environment the mitochondrial respiratory chain could be hyperactive, resulting in increased mitochondrial reactive oxygen species (ROS) production. Activation of the polyol pathway (mediated by aldose reductase) also leads to intracellular accumulation of sorbitol which can cause osmotic stress within the cell (Tang *et al* 2012).

The chondrocytes appear to then regulate turnover of glucose during the remainder of the storage period analysed, hence why observed significant differences in metabolites are more often seen between early and late storage weeks. How the chondrocytes in stored allografts then appear to modulate glucose consumption is unclear.

Storage at hypothermic temperature shows that chondrocytes enter a state of reduced activity as the test meter is able to determine the lower consumption in the culture medium. In a recent paper by Bugbee *et al* (2016) it was stated that in this "quiescent state" viable cartilage remains suitable for transplant for only 2-4 weeks. It is difficult to determine whether the quiescent state continues for the whole 8 weeks of the donors used in this study, as data in the literature (Bugbee *et al* 2016), and our own work suggests that the chondrocytes aren't well maintained beyond 4 weeks of storage. Live/dead data presented in section 3.3 showed that by week 12, chondrocyte viability had dropped from a mean of 97.6% ± 1.0 (SD) at the beginning of week 1 to 42.7% ± 24.2 (SD) at the end of week 12. Samples of the cartilage were not taken at weeks 4 or 8 to be able to determine when this decline significantly changed. But the reduction in viable chondrocytes could possibly account for the relatively low glucose consumption in the latter half of the storage period. Other researchers have reported that hypothermic storage causes a gradual decline in viable chondrocytes (Williams *et al* 2003), and that the cell death is likely due to apoptosis (Robertson *et al* 2006), though it is more likely due to necrosis as apoptosis requires energy from ATP (Boutilier 2001). While week 1 shows significant difference in glucose consumption between the most groups, if this is still shown when the

number of donors is increased then it might be interpreted that high glucose media as used in this study could be over-stimulating cells in the osteochondral allografts as previously discussed, and switching to a low glucose medium may be beneficial.

The significant difference seen between normal and OA tissue in glucose consumption and at all weeks suggests that severely damaged tissue consumes less of this metabolite and could perhaps be used to determine good metabolic function. However, the previously cited paper by Laiguillon *et al* (2015) suggested that chondrocytes from patients with OA would demonstrate higher glucose uptake in a high-glucose environment. But they are extrapolating data from their murine work due to not having access to normal human cartilage. Data shown in the live/dead viability results section showed that chondrocyte viability was not as well maintained with mean data for the OA cohort recorded as 81.3% ± 24.4 (SD) at the beginning of week 1 compared to 61.8% ± 19.0 (SD) at the end of week 8, so the reduced glucose consumption could be as a result of reduced viability compared to normal or over-age donor cohorts. However, the live/dead data for OA tissue was taken at week 8 compared to week 12 for all other cohorts, therefore the 12-week viability could have been lower for OA at the end of week 12.

The relatively small variation seen in glucose levels of OA donors during each week could be due to close proximity in time when all 3 donors were stored and therefore batches of media used. There is also the possibility that this tissue was taken from living donors and therefore not exposed to an increasingly hypoxic environment as would be experienced in a deceased donor knee up to 48 hours after death. Or perhaps that initial storage of whole knee at hypothermic temperature is enough to destabilise normal chondrocyte function in deceased donor tissue. It is known that chondrocytes are adapted to existence in a hypoxic environment (Lafont 2008) and that cells use hypoxia-inducible transcription factor 2α (HIF-2α) to detect oxygen concentration surrounding them. The paper also reported a study that suggested HIF-2α has a positive role in maintaining cartilage integrity. A study by Henderson *et al* (2010) showed that lapine chondrocytes incubated up to 21 days in 5% O_2 produced aggregates with more GAG content than those incubated at 21% O_2. While this was demonstrated in an animal model, the work suggested that chondrocytes are able to maintain normal function for extended periods in low oxygen concentration. It would be useful to obtain healthy cartilage from a living human donor to compare with healthy deceased, but this was not possible.

The effect of temperature on reaction rate in a biological process can be modelled using the temperature coefficient Q_{10} (Ito *et al* 2015). This coefficient is the factor by which a reaction rate increases for every ten degrees that the temperature is raised, and is calculated using the van't Hoff equation (equation 6). Where R_1 is the reaction rate at temperature T_1, and R_2 is the reaction rate at temperature T_2.

(6) $Q_{10} = (R_2/R_1)^{(10/T2-T1)}$

This can infer the energy consumption used by cells in a system, with more temperature-dependant processes having higher Q_{10} values. If a reaction has a Q_{10} of 1 then it can be explained by diffusion of ions, if it is higher than 2 then the process is thought to use large-scale protein conformational changes (Ito *et al* 2015). With glucose consumption by the OCAs in the normal cohort a Q_{10} value of 2.0 was calculated at the end of week 1. By the end of week 2 this had increased to 3.1. At the end of week 3 this had decreased to 2.3 and by the end of week 8 the factor was 2.2. This shows that at the early stage of storage the OCAs are in a high state of energy consumption. As this is based on measurement of a metabolite consumed from the storage medium, this could be a function of both the cartilage and bone in the allograft. This could be an indicator of tissue or cell repair taking place.

Lactate secretion from osteochondral cells

Although normal donors had the largest glucose consumption in week 1 compared to other cohorts, they had a similar level of lactate secretion to over-age donors in week 1. Otherwise, throughout the storage period analysed the normal and over-age cohorts followed a similar trend in lactate release, similar to the trend in how glucose was consumed. This should be expected as lactate secretion from cells is linked to the amount of glucose consumed (Shakhawath Hossein *et al* 2015), with metabolism of one glucose molecule yielding two lactate molecules. Though the mean trend was for lactate secretion to decline during 8 weeks, this was not found to be significant. Greater variance in this cohort during the first 4 weeks compared to the latter 4 weeks could account for this occurrence.

The OA cohort had lower lactate secretion than normal which would be expected as the glucose consumption was less than that when the two cohorts were compared. Similarly, the hypothermic stored tissue consistently had the lowest lactate secretion, though based on the glucose data the levels secreted during the storage period analysed should have been closer to that measured in the OA cohort

Data presented in this study supports the hypothesis that the metabolites glucose and lactate can be measured in the culture medium in which osteochondral allografts have been immersed. Not only this, it is possible to see in the data trends in glucose consumption and lactate secretion during the first 8 weeks of storage

Limitations of the glucose/lactate assays include the small number of donors in each cohort, so the work will need to be repeated in more donors. In particular, further work is needed with

bone only samples and freeze/thawed negative controls. The Accutrend® meter isn't necessarily designed for measuring metabolite concentration in culture media, as it is normally used to measure these in plasma or blood samples. There are benchtop machines available which have greater measurement range and precision (Glucose 0.5 – 50.0 mmol/L, lactate 0.5 - 40.0 mmol/L, imprecision: CV ≤1.5% at 12 mmol/L) (Biosen C-line, EKF Diagnostics, Cardiff, UK), but these are expensive with limited sensor lifespan and again are setup for measuring metabolites in plasma or blood samples, rather than culture media. However, papers have been published using these machines to measure glucose in culture medium (Barker *et al* 2013).

Measurement of turnover of these metabolites was not just limited to the cartilage in each allograft as there was also subchondral bone attached to it with associated bone and bone marrow cells, and small pieces of soft tissue that weren't fully removed (for example bone periosteum). Throughout the storage period bone marrow and lipids found within the subchondral bone were progressively removed when the culture medium was changed, potentially altering the number of different cell populations contributing to metabolite kinetics. It has been shown in section 3.4 (alamarBlue®) that during storage, bone begins to show signs of metabolic activity. It hasn't been determined how much of this activity is contributing to overall glucose and lactate kinetics in the culture medium.

4.2 - Dimethylmethylene blue, chondroitin sulphate release from the ECM

The release of CS into the culture medium from normal donors during the storage period was found to be significantly higher in almost all timepoints analysed. This could mean that normal cartilage that contains healthy cells has a constant higher turnover of the cartilage matrix taking place compared to all other cohorts. Sub-optimal tissue that is either damaged (OA) or in slow decline (over-age) could also perhaps contain cells that are either unable to turn over the matrix as efficiently, or their metabolism is lower. The data from hypothermic stored tissue suggests and partly supports this as the chondrocytes enter "quiescence" to eventual cell death (apoptosis) after 28 days as discussed in the glucose/lactate section of this discussion chapter. The reduction in activity of these cells resulted in less CS getting released into the culture medium. Over-age and OA tissue had similar CS release into the medium, both at levels below that observed in normal tissue, perhaps suggesting that damaged tissue/cells were not turning over the cartilage matrix to the same degree. Normalisation of CS release from the cartilage was determined by surface area, but the OA tissue had many lesions around which the cartilage thickness would have decreased, therefore reducing the effective volume of cartilage from which CS could be released. It is interesting to note that in the normothermic stored cohorts,

the over-age donors had the greatest percent reduction in rate of CS release from week 1 to week 8, with a 34.5% reduction compared to 20.1% in normal donors.
However, there is reported work on CS release from bovine cartilage explants in culture medium (Bascoul-Colombo *et al* 2016). In this study interleukin-1α (IL-1α) was added to the medium in which test explants were being incubated, to induce degradation in them. After 3 days incubation, results of the DMMB assay showed that presence of IL-1α significantly ($p < 0.05$) increased CS release into the medium compared to control. The authors also found that adding glucosamine hydrochloride (GH) significantly ($p < 0.05$) reduced CS release into the medium. It was also found that a combination of CS and GH helped reduce the synthesis of Prostaglandin E_2 (PGE_2) and nitrous oxide, both mediators of inflammation. It might be useful therefore to look for these in the culture medium in the same way Bascoul-Colombo *et al* did in their medium samples, particularly because they report PGE_2 as one of the most characteristic prostaglandins found in OA joints. Another study (Xu *et al* 2016) reported on the use of cartilage from human femoral heads to investigate CS release into culture medium. This was to investigate the effect of inhibiting the fibroblast growth factor receptor 1 (FGFR1) with compound G141. The authors found that loss of GAG into the culture medium as measured by the DMMB assay was reduced in the presence of the inhibitor.

Though the literature suggests elevated CS release from cartilage is an indication of degradation, other assays carried out on the tissue used in this project suggested that the cartilage is healthy, with the safranin O histology assay having demonstrated retention of proteoglycans and GAG in all but the superficial zone of normal cartilage. Live/dead assay also showed good remaining chondrocyte viability within the tissue compared to control.
Future work could involve taking biopsies of the cartilage at the beginning of week 1 and in week 12 and then using a decellularization method along with tissue digestion prior to analysis with the DMMB assay, as reported in work by Sutherland *et al* (2015). As this would give a quantitative measure of the amount of CS retained in the cartilage matrix. It was noted that CS was released from freeze-thawed negative control samples, even though most of the chondrocytes were dead (as evidenced by the live/dead assay). This could suggest that at normothermic temperature there is breakdown of the ECM releasing CS, this could be investigated by storing freeze-thawed allografts at hypothermic temperature.

Although there were not enough donors to compare bone only with other cohort groups, data shown in Fig. 3.2.1E showed that in the donor that contained just bone, and not the sample that consisted of a hemi-condyle with cartilage dissected off, no CS was detected in the culture medium. This suggests that in all other donor cohorts, the CS detected was being released from the cartilage alone, with none coming from the underlying subchondral/cancellous bone. It has

been reported (Pecchi *et al* 2012) however that CS is present within bone and is actively synthesised by osteoblasts as well as being localized in osteoblast membranes and extracellular matrix. Work in this project using the alamarBlue® assay has suggested that osteocytes were still functioning in isolated bone samples incubated in culture medium, therefore it is interesting to apparently find no CS release into the culture medium. One explanation could be that the CS is locked into the cell and tissue and is not actively released as a result of cellular activity. Further work with isolated bone samples will be required to confirm this initial finding.

This work would need to be repeated with further donors to improve statistical analysis. The issue with assay sensitivity at 0 µg/ml chondroitin sulphate concentration also needs to be investigated further.

4.3 - Live/dead, chondrocyte viability

Chondrocyte viability as determined by the live/dead assay demonstrated that all tissue had similar live/dead ratios at the beginning of week 1. While this might be an unusual finding in the OA cohort, a study by Ganguly *et al* (2010) found that in cartilage samples taken from patients undergoing total knee replacement, also retained high chondrocyte viability as determined by live/dead assay. However, the authors quoted no actual percent values, nor quoted any statistics values when stating there was no significant difference. There was however large variance in the OA cohort which could have resulted in the lack of significant difference.

Not a lot of differences were observed between cohorts and time points, and this may well be due to the variance observed in the data. In turn this could be due to differences between tissue samples within each cohort, for example in the normal cohort it was known that one donor died from liver failure as a consequence of alcohol abuse. Each cohort also had a small sample size.

It was not unexpected to find that hypothermic stored cartilage would show a significant decrease ($p = 0.017$) in chondrocyte viability from the beginning of week 1 to week 12 as there are many studies showing how viability significantly decreases after 28 days storage at 4ºC (Stoker *et al* 2018, Bugbee *et al* 2016, LaPrade *et al* 2009). The latest of these studies by Stoker *et al* using the Missouri storage system at 4ºC showed a decrease in mean viability to 52.4% on day 28 and then to 8.8% on day 70. However, in one of the donors used in this study, mean viability was >60% which was higher than expected compared to other results in this study, and what has been reported in the literature. This may indicate that the storage medium works better than expected at low temperature.

Viability data presented in this study supports the hypothesis that chondrocytes can be maintained above the 70% threshold, that has been suggested as the minimum viability required to ensure osteochondral allograft survival in the recipient (Cook *et al* 2016). This was suggested as osteochondral tissue with viability less than 70% did not have successful outcomes after implantation, whereas those above 70% did. For a potential osteochondral storage service, donor tissue would come from the normal group of donors and though the overall mean viability was 80.0% one of the donors had a mean value of 64.7% viability, which unfortunately meant this donor tissue wouldn't be suitable for transplant, based on the minimum viability required. However, the lower viability compared to the other two donors could be attributed to the fact this donor suffered from, and ultimately succumbed, to liver failure as a result of alcohol induced cirrhosis. A recent publication by Stoker *et al* (2018) demonstrated mean residual viability of 73.9% at day 70 in human femoral condyle cartilage stored in the Missouri storage system at 25^{o}C. Unfortunately, clinical details of the donors were not reported, but the tissue was obtained from a tissue bank and reported to be for clinical use, so it could be presumed that the tissue was from donors less than 45 years of age. The authors also related their live/dead cell viability to tissue section area.

Perhaps unexpectedly, the over-age donors had better overall mean viability compared to normal donors (85.1% compared to 80.0%) and this cohort also had a donor whose viability was lower than all others in its group. What this could suggest is if the cartilage has good structural integrity, as determined visually and microscopically, the age restriction for donation as advised by American Tissue Banks could be increased above 45. Further work would be required to determine what the upper threshold could be increased to. Results obtained from living donor OA tissue demonstrates that severely damaged cartilage culminates in greater variability of the chondrocyte viability. This cohort of tissue was only stored for 8 weeks so it is difficult to make conclusions on chondrocyte viability between OA and other cohorts on their final storage days. OA is a complex disease, but studies have shown that increasing age is the most prominent risk factor (Xi *et al* 2013) for its establishment and progression in an individual's joint(s). With regards to chondrocytes, age can bring about changes in these cells, such as telomere shortening and increased senescence-associated β-galactosidase activity (Xi *et al* 2013). As a result of this, the function of chondrocytes to maintain the cartilage matrix is impaired. Also, in aged chondrocytes their synthetic activity is reduced, and proteoglycans are smaller and more irregular (Xi *et al* 2013). A recent study by Zhang *et al* (2016) suggests that chondrocyte cell death may not initially lead to degradation of the cartilage ECM, and that the ECM is intact up to 8 months after cell death in a mouse model. The authors proposed that post-injury and prior to cell death, the chondrocytes enter an ADAMTS, MMP-13 mediated state of catabolic activity that contributes to ECM degradation. However, this study used a murine model and it is known that mice have thinner articular cartilage, with a denser

population of chondrocytes compared to humans (Moran *et al* 2016), that could have differential responses to injury as a result. As well as murine cartilage experiencing reduced mechanical forces.

Data from devitalised tissue (negative control) showed that the assay worked as expected and was able to detect dead cells alongside live, though increasing the number of tissue samples used to test this is required to confirm the observation.

The live/dead assay is widely used in published studies investigating storage of osteochondral allografts for implantation, so the positive data shown in this study suggests that the normothermic storage regimen used will give allografts the best chance of surviving in a recipient joint. To date there has been no work published with allografts stored at normothermic temperature, so there is currently no other data from research groups with which to compare. To improve the statistical analysis, further work will need to be done with more donors, particularly in the normal donor cohort but also negative control. Though not ethically possible, it would have been advantageous to have had undamaged cartilage retrieved from the OA patients to compare with the damaged cartilage that was retrieved during surgery.

4.4 - AlamarBlue®, chondrocyte metabolic activity

Chondrocyte metabolic activity as determined by the alamarBlue assay showed that significant difference was only observed in week 8 and 12 when comparing both normal and over-age cohorts with hypothermic. It might have been expected to find a significant difference in week 4, however variance in the data and the small number of donors in all cohorts could potentially account for this. As expected the metabolic activity in chondrocytes that were stored hypothermically significantly declined during storage. In published cartilage storage studies, metabolic based assays (Brockbank *et al* 2011, Cook *et al* 2014, Wright *et al* 2014, and Yamada *et al* 2015) were not as frequently used as other methods, such as live/dead. Of these published studies, only the Cook *et al* (2014) paper looked at storage temperature higher than 4ºC, and while this study used the resazurin based assay, it was only briefly mentioned in the results section in which the data obtained was correlated with that obtained from the live/dead assay. The authors found a significant ($p < 0.00001$) positive correlation with viability results obtained using a live/dead assay. They found that activity counts greater than 1000 indicated grafts maintained chondrocyte viability above 70%. This suggests that metabolic activity in articular cartilage is linked to the number of living chondrocytes, rather than an increase or decrease in each chondrocyte's metabolic activity. The authors were also using a canine model in their research. Of those studies cited it was only the Cook *et al* (2014) paper that used both a metabolic and cell viability assay, and it was only the Yamada *et al* (2015) paper that didn't use a resazurin based metabolic assay.

Data presented in this study shows that in the normal and over-age cohorts, metabolic activity in the cartilage cells is significantly increasing during the 12-week storage period, with no apparent sign of stabilising. This is unusual considering other data obtained in this study where glucose and lactate turnover appeared to stabilise after week 8. This could be a function of the alamarBlue® assay where the cells are subjected to an increased temperature, although briefly for 2 hours. The manufacturer recommends incubation at 37ºC, however due to the normal working temperature range of the cells, perhaps the hemi-condyles should have been incubated in the alamarBlue® reagent at normothermic temperature. Currently there is no published data available looking at normothermic stored cartilage that investigate metabolic function using the alamarBlue® assay. The hypothermic stored tissue in this study demonstrated a continual loss in metabolic function during the storage period. This trend was demonstrated in the Brockbank (2011) paper where resazurin data showed an assessment value <30% of fresh control at 28 days. It has been discussed in the glucose/lactate report how chondrocytes enter "quiescence" and then eventual cell death which will ultimately result in less chondrocytes being able to contribute to the measured metabolic activity of the cartilage.

OA tissue from living donors was not exposed to this assay reagent, due to the fact that the tissue comprised of small pieces of cartilage and bone, which would mean exposure of bone to the assay reagent could not be minimised. Exposure of the bone has shown in this study that osteocytes survive during storage and can therefore contribute to the overall cell metabolic function measured in the osteochondral allograft. This was thought to not be the case as a paper by Bastian *et al* (2011) suggested that osteoblasts were not as successful as chondrocytes in surviving during tissue culture storage at 37ºC. However, that study only looked at cells during a 24-hour period. Future work could look at whole hemi-condyles from deceased donors who had suffered from osteoarthritis.

The rationale for using the hemi-condyle immersion technique was to investigate whether this non-invasive assay could be used to determine if chondrocytes are still functioning after 12 weeks storage at normothermic temperature. The data suggests this approach has likely worked with no apparent decline in chondrocyte function after repeated exposure to the working reagent. A study by Pace and Leadbetter Burg (2015) presented data that supported the manufacturers claim that the assay is non-destructive when used at a maximum concentration of 10% for up to 3 hours exposure. Traditionally when metabolic assays were used in cartilage studies, biopsies of the cartilage were taken and immersed in the assay reagent for the required time, but it is known that the act of cutting into the cartilage can cause cell death and proliferation (Archer *et al* 2006, Tew *et al* 2000), potentially affecting the data obtained.

This study of metabolic activity supports the hypothesis that during normothermic storage the metabolic function of chondrocytes in the cartilage remains above the levels measured at the beginning of week 1. To improve confidence of statistical results, further work using this assay

will need to be done in more donors.

4.5 - Lubricin (PRG4), secretion from chondrocytes

HRP – chromogen immunohistochemistry

Analysis of lubricin (PRG4) secreting chondrocytes via HRP chromogen immunohistochemistry demonstrated no significant differences between all cohorts at either time point analysed. This might be unexpected at the beginning of the first week of storage as there could be significant difference between OA and all other cohorts. This could also be true at the end of the storage period, even accounting for OA samples taken at 8 weeks compared to 12. A published study showed that the absence of lubricin expression in cartilage tissue is partially implicated in the onset of OA (Ruan *et al* 2013). However, in the same paper the authors suggested that lubricin was up-regulated in chondrocytes from OA patients, and they proposed that this occurred as a repair response mechanism. Therefore, it might be expected to see continued expression of lubricin in the tissue sections from OA donors, and perhaps even an increase as was shown in the AFP488 fluorophore stained sections. The authors also went on to say that this normal response in humans may be insufficient to prevent progression of the disease.

It might have been expected to find a significant difference in lubricin staining between the beginning of week 1 and the final storage week in hypothermic stored tissue, however none was found. A study published on the storage of hircine cartilage found that tissue which had been stored frozen at -70^{o}C for 10 days had a significant ($p < 0.05$) reduction in lubricin secretion compared to fresh tissue stored at 4^{o}C for 3 days (Pallente-Kichura *et al* 2013). The authors went on to say that their results demonstrated that storage conditions which affect chondrocyte viability also affect subsequent secretion of lubricin. They found that frozen-stored cartilage was absent of surface chondrocytes and as a result secreted lubricin at a low rate. As no significant differences were found between storage periods and cohorts in the live/dead assay, this could suggest why no significant differences were found in lubricin staining as measured by HRP immunohistochemistry.

Scoring from the devitalised tissue (negative control) showed that at the beginning of week 1 there was lubricin present in the cartilage, suggesting the presence of chondrocytes that could secrete the molecule, however by the end of the storage period there was no staining visible suggesting that as would be expected, no lubricin secreting chondrocytes had survived. The lack of HRP immunohistochemistry staining in the isotype and no secondary antibody controls suggests that no non-specific binding of the proteoglycan antibody, or the secondary antibody used in these studies took place.

As discussed, the unexpected observation of no significant differences could be due to small sample size in each cohort, heterogeneity in some of the cohorts (for example over-age) and subjectivity of the double-blind scoring carried out by observers.
Results of scoring for the assessment of lubricin (PRG4) secreting chondrocytes appears to support the hypothesis that storage of cartilage allografts at normothermic temperature leads to their retention in the tissue.

AFP488 – fluorophore immunohistochemistry

Analysis of lubricin secreting chondrocytes via AFP488 fluorophore immunohistochemistry demonstrated significant decrease in lubricin secretion in the OA cohort compared to over-age at the end of their respective storage periods. This was an expected outcome as the over-age cohort represent a healthier group of tissue compared to OA.
No significant differences were observed between hypothermic and normal, hypothermic and over-age or hypothermic and OA. For the same reasons as described in the discussion for HRP, this was unexpected as hypothermic tissue would have chondrocyte cell death in the superficial zone of the ECM.
In the same way as the HRP chromogen method, unexpected observations could be due to small sample size, heterogeneity, and subjectivity in the scoring applied to the images.

There are ELISA based assays that can measure the presence of lubricin in solutions, therefore these could be used in future work to develop a non-invasive assay for detection of this molecule in the storage medium. The study by Pallente-Kichura *et al* (2013) which used an ELISA based assay to determine lubricin content in conditioned medium, also suggested this non-invasive method, utilising lubricin as a biomarker for superficial chondrocyte health and therefore as a potentially useful assay for determining OCA integrity.

4.6 - Safranin O, proteoglycan histology

Analysis of proteoglycan and GAGs content in the articular cartilage as determined by the safranin O assay, showed that there was significantly less of these molecules present in the ECM of tissue from the over-age cohort compared to normal, at the end of the storage period. It has been reported in a study that proteoglycan content was lost when old normal (76 year old) donor tissue was compared to young normal (40 year old) tissue, with reduced safranin O staining in the superficial zone (Lotz and Loeser 2012).
Analysis also showed that there was a significant decrease in proteoglycan and GAG content at the final week of storage between normal and OA and also over-age and OA. It was also

demonstrated by Lotz and Loeser (2012) that OA tissue had further reduced staining when compared to normal old donors. A study by Mastbergen *et al* (2006) stated that OA cartilage typically has low proteoglycan synthesis, and high proteoglycan release before going on to show that nonsteroidal anti-inflammatory drugs (NSAIDs) can influence its turnover in OA cartilage tissue obtained from human patients undergoing knee replacement. With the drug aceclofenac causing improved proteoglycan synthesis, retention of newly formed proteoglycan and then a reduction in total proteoglycan release. Another study using a canine model of OA showed that proteoglycan concentration in articular cartilage significantly decreased 12 weeks ($p = 0.018$) and 2 years ($p < 0.001$) after transection of the ACL, when compared to controls (Yin and Xia 2014).

Surprisingly there was no significant decline shown with the hypothermic cohort, though one of the donors in this group demonstrated the lowest percent coverage of safranin O staining compared to all other donors. A study by Qi *et al* (2016) using animal articular cartilage reported a significant decrease (*p*-value not stated) in proteoglycan content after 21 days storage at 4^{o}C, also determined via the safranin O assay.

The possible reason for not finding a significant difference in the data presented in this study is likely due to the large variance observed in the data, along with the small number of donors used. In a study comparing the Mankin and Osteoarthritis Cartilage Histopathology Assessment System scoring systems that use safranin O stained sections of cartilage, the authors stated that safranin O staining intensity as a parameter in a grading system may lead to false results (Pauli *et al* 2012). They also suggested that safranin O stained sections are not quantitative indicators of proteoglycan depletion, and that they may to some extent be reversible. Their suggested solution was automated image analysis, based on the Mankin system.

The results could suggest that sub-optimal or diseased tissue was not replacing proteoglycan in the cartilage ECM during the time they were lost throughout the storage period. Combining this data with that from the DMMB assay also suggests that even though more CS is detected in the conditioned culture medium from normal donors compared to all other cohorts, healthy chondrocytes are able to replace the CS quicker than or as quickly as the CS is released from the matrix. This could be confirmed by repeating the work using the ^{35}sulphate incorporation assay, which measures the rate of proteoglycan synthesis.

Data shown in this study supports the hypothesis that proteoglycans linked to GAGs are retained in the cartilage matrix during storage at normothermic temperature.

4.7 - Normothermic storage for 12 weeks

The results of work presented in this thesis suggest that OCAs can be stored for up to 12 weeks and maintain the minimum required 70% viability. The assays that lead to this conclusion were live/dead, and safranin O. Live/dead is an important assay as this has been reported on more than any other in any of the published papers referenced in this thesis and appears to be used by tissue banks as a standard method to determine OCA viability. Therefore, the minimum required viability of 70% determined by this assay demonstrates that the storage regimen reported here works. Clinically the OCAs from the "normal" cohort would be used in a tissue bank, however data obtained from the over-age cohort showed that these also met the minimum requirement. Success of the storage was then supported by data from the alamarBlue® assay which showed that metabolic activity in the allografts remained higher after 12 weeks of storage compared to the beginning of week 1, however it is difficult with the relatively small number of biological replicates to determine how the metabolic activity can be related to chondrocyte viability. This in turn was supported by data from the glucose/lactate assay which demonstrated the continuing metabolic activity of cells within the OCAs. The safranin O histology was able to demonstrate retention of proteoglycan within the ECM during 12 week storage and the lubricin immunohistochemistry suggested that there were still lubricin secreting chondrocytes present within the OCAs at the end of 12 weeks. Though the non-invasive assays glucose/lactate and DMMB only had samples analysed up to 8 weeks storage, results from the other assays suggest these could also have provided evidence to support satisfactory OCA storage after 12 weeks.

4.8 - Test comparisons

The most informative tests carried out would be live/dead assay as this has been used in many studies to assess the most important aspect of articular cartilage transplant using OCAs, and that is living chondrocytes with a minimum viability. Even though for scientific interest there was not a lot of differences observed, this could suggest that the storage regimen is working, and the cartilage is sufficiently viable compared to when it was initially placed in storage. Next could be safranin O as this demonstrated that viable chondrocytes were maintaining the ECM as determined by retention of proteoglycan. AlamarBlue® was also informative as this showed continued metabolic activity of the chondrocytes, and could potentially be used as a non-invasive assay, it was able to show significant changes between cohorts.

It could be argued that the glucose/lactate assay was one of the least informative, mainly because it measured indirectly the metabolism of cells in the whole of the OCA, rather than articular cartilage alone. So, while it could have been a useful non-invasive assay its reduced

specificity means that without more data it isn't able to reliably infer the health of the articular cartilage. Similarly, the DMMB assay measured CS release from the whole OCA, though initial results from bone only samples suggested the CS release was originating from the articular cartilage, and not from the bone as well. Finally, the lubricin immunohistochemistry was perhaps the least informative as even though lubricin secreting chondrocytes were detected, no clear correlations were found over time and between cohorts.

4.9 - Test integration

This study has utilised six different assays to assess the health of the articular cartilage tissue at the beginning, during and end of the storage period. This was because there are two main factors involved with cartilage allograft quality: chondrocyte viability and ECM integrity. Therefore, assays were chosen to assess chondrocyte viability (live/dead, glucose/lactate, alamarBlue®, and lubricin) and ECM integrity (safranin O, DMMB, and lubricin). Use of multiple tests to look at both requirements for OCA suitability is also important to corroborate findings within each assay, for example alamarBlue® data can support the data found in the live/dead assay.

4.10 - Limitations of the study

Beyond the requirement to increase donor numbers within each cohort, as well as devitalised cartilage and bone only controls there are other limitations to this study. Relating to donors themselves, information regarding BMI was not recorded and could not be retrospectively retrieved. This data would have been useful for removing possible effects of increased weight on trends seen in the results. Extensive medical histories were also not available to examine which potential drugs the donors may have been taking prior to their death, this could be important, particularly if the donors had been taking NSAIDs, as these can affect proteoglycan turnover in the cartilage (discussed in section 4.6). Whether the donors were diabetic could have had an influence on the OCA as diabetes mellitus has been associated with cartilage degradation and OA (Chanchek *et al* 2018).

Formal scoring of the macroscopic cartilage structure by an expert according to a recognised system such as Noyes and Stabler was not carried out after dissection of whole knee's, this would have been useful to verify assignment of donor samples into the correct experimental cohorts.

Normalisation of data in the assays measuring molecules in the culture medium via surface area, wouldn't have taken into account variation in cartilage thickness, particularly in the living donor OA samples, however fissures in the OA cartilage would have increased the

surface area in those sections in which they were present. It was apparent in some live/dead assay treated sections that there was a variation in cell density, for example OA verses normal, therefore it would have been informative to measure cell density in the cartilage.

4.11 - Future research questions

Future work could investigate the use of the more sensitive benchtop glucose/lactate machine (Biosen) previously described in section 4.1, as this would more accurately measure the amount of glucose and lactate present in the culture medium, perhaps giving greater clarity between cohorts.

Further potential work regarding glucose could be the reported effect of high glucose content in culture medium on chondrocytes (Laiguillon *et al* 2015). As a result, it would be necessary to investigate reducing glucose content of the culture medium. Concurrently the concentration of FBS could also be investigated to examine the potential for reducing it from the current 10% concentration. This would be useful as tissue banks are currently required to use FBS sourced from countries not affected by bovine spongiform encephalopathy, which increases its cost per 500 ml bottle. The change of metabolite use by the allografts during storage could be further investigated, to determine whether it is a permanent or transient process.

A technique that examines the integrity of cartilage ECM via electrochemical measurement (Mikevicius *et al* 2015) could be looked at as a further non-invasive means of determining the structural properties of the stored articular cartilage. Another technique that would give further understanding of how the ECM is being regulated during storage, in particular synthesis of molecules by chondrocytes, could be investigated by a ^{35}sulphate incorporation assay as this will allow us to trace synthesis of proteoglycan in the cartilage ECM and potentially give more insight into the results seen with the DMMB and safranin O assays.

The live/dead assay can only highlight cells that are either living or dead, therefore it would be useful to investigate how many cells are potentially undergoing apoptosis using the TUNEL assay as this would allow us to stratify different cell fates.

Due to the effect oxygen level, and pH can have on cell homeostasis **(**Shakhawath Hossain *et al* 2015) it could be useful to measure oxygen and pH level in the culture medium, particularly as these can be non-invasive assays. For pH, samples of conditioned and un-conditioned medium could be measured using a standard electrode pH meter. Dissolved oxygen could be measured in the same samples using a probe-based system (NeoFox-GT).

To further assess the chondrocytes at the end of the storage period, samples of the articular cartilage could be taken, digested and expand under cell culture conditions to further prove functionality after storage.

As an overall assessment of OCA suitability, after research ethics committee approval, samples of stored cartilage could be implanted in an animal model and assessed 6 or 12 months post-implantation. This would show whether the OCAs are able to perform as expected when transplanted after the storage regimen. Alternatively, once an agreement has been setup between clinicians and the NHSBT, a clinical study could be carried out where samples are used in articular cartilage regenerative procedures of the knee, again with follow-up studies.
Our own work and that of others has shown that after 28 days of storage at hypothermic temperature, chondrocytes begin to undergo cell death. It would be interesting to see if this process is reversible by changing storage temperature to normothermic, either after 3 or 4 weeks at hypothermic temperature. This would also potentially show what happens to the chondrocytes when transplanted into the patient after hypothermic storage alone.

4.12 - ICRS congress app

An abstract reporting on part of the work done in this study was submitted to the International Cartilage Repair Society congress which took place in Macau, China in 2018, and was accepted for oral presentation. As part of the congress the abstract was included in the congress app which was available for download on devices running either the Android or iOS operating systems.
The presentation was given by one of the authors, Mr Michael McNicholas. A question was raised by Dr William Bugbee regarding possible softening of cancellous bone within the osteochondral allografts during storage for 12 weeks at normothermic temperature. As a result of this question, a further study investigating strength of knee cancellous bone under these storage conditions is being undertaken.

4.13 - Conclusion

Supply of OCAs to orthopaedic surgeons for the repair of damage to articular cartilage as a result of trauma or joint related disease is vitally important, as one of the many treatment options available. Results presented here suggest that storage at normothermic temperature is capable of maintaining the required cartilage viability and ECM integrity for up to 12 weeks, giving a greater window of opportunity for obtaining necessary donor information and matching allograft to patient. Data from the over-age cohort suggests that donors above the clinical age range of 45 could have tissue retrieved from them and stored with the required minimal viability and ECM integrity. Also, that the bag storage system allows sterile transfer of fresh medium on a weekly basis, outside of a sterile environment. While the non-invasive techniques of glucose/lactate measurement, alamarBlue®, and DMMB assays show potential

for monitoring allograft integrity during storage at normothermic temperature; relating these to chondrocyte viability has been difficult and requires further investigation. The addition of other non-invasive techniques could also assist with validating an OCAs suitability.

References

Abdulhasan J.F., Grey M. J. (2017) Anatomy and Physiology of Knee Stability. J Funct Morphol Kinesiol, 2(34): doi:10.3390/jfmk2040034

Allen R.T., Robertson C.M., Pennock A.T., Bugbee W.D., Harwood F.L., Wong V.W., Chen A.C., Sah R.L. Amiel D. (2005) Analysis of Stored Osteochondral Allografts at the Time of Surgical Implantation. Am J Sports Med, 33(10): 1479-1484

Amis A.A., Gupte C.M., Bull A.M.J. Edwards A. (2006) Anatomy of the posterior cruciate ligament and the meniscofemoral ligaments. Knee Surg Sports Traumatol Arthrosc, 14(3): 257-63

Andriolo L., Crawford D.C., Reale D., Zaffagnini S., Candrian C., Cavicchili A., Filardo G. (2018) Osteochondritis Dissecans of the Knee: Etiology and Pathogenetic Mechanisms. A Systematic Review. Cartilage, doi: 10.1177/1947603518786557. Epub ahead of print

Archer C.W., Redman S., Khan I., Bishop J., Richardson K. (2006) Enhancing tissue integration in cartilage repair procedures. J Anat, 209(4): 481-493

Ball S.T., Amiel D., Williams S.K., Tontz W., Chen A.C., Sah R.L., Bugbee W.D. (2004) The Effects of Storage on Fresh Human Osteochondral Allografts. Clin Orthop Relat Res, 418: 246-252

Bardos T., Vancsodi1 J., Farkas B., Fazekas A., Nagy S.A., Bogner P., Vermes C., Than P. (2015) Pilot Study of Cartilage Repair in the Knee Joint with Multiply Incised Chondral Allograft. Cartilage, 6(2): 73-81

Barker B.M., Kroll K., Vödisch M., Mazurie A., Kniemeyer O., Cramer R.A. (2012) Transcriptomic and proteomic analyses of the Aspergillus fumigatus hypoxia response using an oxygen-controlled fermenter. BMC Genomics, 13:62 doi: 10.1186/1471-2164-13-62

Bascoul-Colombo C., Garaiova I, Plummer S.F., Harwood J.L., Caterson B., Hughes C.E. (2016) Glucosamine Hydrochloride but Not Chondroitin Sulfate Prevents Cartilage Degradation and Inflammation Induced by Interleukin-1α in Bovine Cartilage Explants. Cartilage, 7(1): 70-81

Bastian J.D., Egli R.J., Ganz R., Hofstetter W., Leunig M. (2011) Chondrocytes within Osteochondral Grafts Are More Resistant than Osteoblasts to Tissue Culture at 37ºC. J Investig Surg, 24: 28-34

Becher C., Springer J., Feil S., Cerulli G., Paessler H.H. (2008) Intra-articular temperatures of the knee in sports – An in-vivo study of jogging and alpine skiing. BMC Musculoskelet Disord, 9:46

Belzer F.O., Southard J.H. (1988) Principles of solid-organ preservation by cold storage. Transplantation, 45(4): 673-6

Berta Á., Duska Z., Tóth F., Hangody L. (2015) Clinical experiences with cartilage repair techniques: outcomes, indications, contraindications and rehabilitation. Eklem Hastalik Cerrahisi, 26(2): 84-96

Biant L.C., McNicholas M.J., Sprowson A.P., Spalding T. (2015) The surgical management of symptomatic articular cartilage defects of the knee: Consensus statements from United Kingdom knee surgeons. Knee, 22(5): 446-9

Bobic V. (1996) Arthroscopic osteochondral autograft transplantation in anterior cruciate ligament reconstruction: a preliminary clinical study. Knee Surg Sports Traumatol Arthroscopy, 3: 262-264

Bottini M., Bhattacharya K., Fadeel B., Mangrini A., Bottini N., Rosato N. (2016) Nanodrugs to target articular cartilage: An emerging platform for osteoarthritis therapy. Nanomedicine, 12(6): 255-268

Boutilier R.G. (2001) Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol, 204(Pt 18): 3171-3181

Briggs D.T., Sadr K.N., Pulido P.A., Bugbee W.D. (2015) The Use of Osteochondral Allograft Transplantation for Primary Treatment of Cartilage Lesions in the Knee. Cartilage, 6(4): 203-

Brittberg M., Aglietti P., Gambardella R., Hangody L., Hauselmann H.J., Jakob R.P., Levine D., Lohmander S., Mandelbaum B.R., Peterson L., Staubli H-U. (2000) ICRS Cartilage Injury Evaluation Package

Brockbank K.G.M., Rahn E., Wright G.J., Chen Zhenzhen, Yao H. (2011) Impact of Hypothermia upon Chondrocyte Viability and Cartilage Matrix Permeability after 1 Month of Refrigerated Storage. Transfus Med Hemother, 38: 387-392

Brockbank K.G.M., Rahn E., Wright G.J., Chen Zhenzhen, Yao H. (2011) Impact of Hypothermia upon Chondrocyte Viability and Cartilage Matrix Permeability after 1 Month of Refrigerated Storage. Transfus Med Hemother, 38: 387-392

Buckwalter J.A. (1992) Mechanical Injuries of Articular Cartilage. Iowa Orthop J, 12: 50-57

Buckwalter J.A., Mankin H.J. (1997) Articular cartilage, part I: tissue design and chondrocyte-matrix interaction. J Bone Joint Surg Am, 79: 600-611

Bugbee W.D., Pallente-Kichura A.L., Gortz S., Amiel D., Sah R. (2016) Osteochondral Allograft Transplantation in Cartilage Repair: Graft Storage Paradigm, Translational Models, and Clinical Applications. J Orthop Res, 34(1): 31-38

Capeci C.M., Turchiano M., Strauss E.J., Youm T. (2013) Osteochondral allografts: applications in treating articular cartilage defects in the knee. Bull Hosp Jt Dis, 71(1): 60-7

Cavaignac E., Perroncel G., Thepaut M., Vial J., Accadbled F., De Gauzy J.S. (2017) Relationship between tibial spine size and the occurrence of osteochondritis dissecans: an argument in favour of the impingement theory. Knee Surg Sports Traumatol Arthrosc, 25(8): 2442-6

Chen H., Sun J, Hoemann C.D., Lascau-Coman V., Ouyang W., McKee M.D., Shive M.S., Buschmann M.D. (2009) Drilling and microfracture lead to different bone structure and necrosis during bone-marrow stimulation for cartilage repair. J Orthop Res, 27(11): 1432-8. doi: 10.1002/jor.20905

Chanchek N., Gersing A.S., Schwaiger B.J., Nevitt M.C., Neumann J., Joseph G.B., Lane N.E., Zarnowski J., Hofmann F.C., Heilmeier U., McCulloch C.E., Link T. M. (2018) Association of Diabetes Mellitus and Biochemical Knee Cartilage Composition Assessed by T2 Relaxation Time Measurements: Data From the Osteoarthritis Initiative. J Magn Reson Imaging, 47(2): 380–390

Cinats D., Miller S., Abusara Z., Heard S.M., Hutchison C., Schachar N., Timmermann S. (2018) Evolution of a Novel Tissue Preservation Protocol to Optimize Osteochondral Transplantation Outcomes. Cartilage, doi:10.1177/1947603518812557. Epub

Cole B.J., Malek M.M. (2004) Articular Cartilage Lesions: A Practical Guide to Assessment and Treatment, Chapter 2: Patient Assessment. Springer, 13-22

Cook J.L., Stannard J.P., Stoker A.M., Bozynski C., Kuroki K., Cook C.R., Pfieffer F.M., (2016) Importance of Donor Chondrocyte Viability for Osteochondral Allografts. Am J Sports Med, 44(5): 1260-1268

Cook J.L., Stoker A.M., Stannard J.P., Kuroki K., Cook C.R., Pfieffer F.M., Bozynski C., Hung C.T. (2014) A Novel System Improves Preservation of Osteochondral Allografts. Clin Orthop Relat Res, 472: 3403-3414

Crawford D.C., Safran M.R. (2006) Osteochondritis dissecans of the knee. J Am Acad Orthop Surg, 14(2): 90-100

Csonge L., Bravo D., Newman-Gage H., Rigley T., U.Conrad E., Bakay A., Strong M., Pellet S. (2002) Banking of osteochondral allografts. Part I. Viability assays adapted for osteochondral and cartilage studies. Cell Tissue Bank, 3: 151-159

Cusano J., Curry E.J., Murakami A.M., Li X. (2018) Fresh Femoral Condyle Allograft Transplant for Knee Osteonecrosis in a Young, Active Patient. Orthop J Sports Med, 6(9): 2325967118798355

Deie M., Ochi M., Sumen Y., Kawasaki K., Adachi N., Yasunaga Y. (2006) Relationship between osteochondritis dissecans of the lateral femoral condyle and lateral menisci types. J Pediatr Orthop, 26(1): 79-82

Demange M., Gomoll A.H. (2012) The use of osteochondral allografts in the management of cartilage defects. Curr Rev Musculoskeletal Med, 5(3): 229-35

Devrimsel G., Beyazal M.S., Turkyilmaz A.K., Sahin S.B. (2016) Ultrasonographic evaluation of the femoral cartilage thickness in patients with hypothyroidism. J Phys Ther Sci, 28: 2249-2252

Drobnic M., Mars T., Alibeqovic A., Bole V., Balazic J., Grubic Z., Breceli J. (2005) Viability of human chondrocytes in an ex vivo model in relation to temperature and cartilage depth. Folia Biol, 51(4): 103-8

Early S., Tírico L.E.P., Pulido P.A., McCauley J.C., Bugbee W.D. (2018) Long-Term Retrospective Follow-Up of Fresh Osteochondral Allograft Transplantation for Steroid-Associated Osteonecrosis of the Femoral Condyles. Cartilage, https://doi.org/10.1177/1947603518809399

Ellermann J., Johnson C.P., Wang L., Macalena J.A., Nelson B.J., LaPrade R.F. (2017) Insights into the epiphyseal cartilage origin and subsequent osseous manifestation of juvenile osteochondritis dissecans with a modified clinical MR imaging protocol: a pilot study. Radiology, 282(3): 798-80

Ellison A., Berg E. (1985) Embryology, anatomy, and function of the anterior cruciate ligament. Orthop Clin North Am, 16(1): 3-14

Everhart J.S., DiBartola A.C., Early N.A., Flanigan D.C. (2017) Outcomes and clinical role of osteochondral allograft transplantation. Annals of Joint. Editorial,
doi: 10.21037/aoj.2017.04.02

Fox A.J.S., Bedi A., Rodeo S.A. (2009) The Basic Science of Articular Cartilage: Structure, Composition, and Function. Sports Health, 1(6): 461-468

Frisbie D.D., Cross M.W., McIlwraith C.W. (2006) A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. Vet Comp Orthop Traumatol, 19(3): 142-6

Fujioka R., Aoyama T., Takakuwa T. (2013) The layered structure of the articular cartilage. Osteoarthritis Cartilage, 21(8): 1092-8

Ganguly K., McRury I.D., Goodwin P.M., Morgan R.E., Augé, II W.K. (2010) Native Chondrocyte Viability during Cartilage Lesion Progression Normal to Surface Fibrillation. Cartilage, 1(4): 306-311

Garrity J.T., Stoker A.M., Sims H.J., Cook J.L. (2012) Improved osteochondral allograft preservation using serum-free media at body temperature. Am J Sports Med, 40(11): 2542-8

Green W.T., Banks H.H. (1953) Osteochondritis dissecans in children. J Bone Joint Surg Am, 35-A(1): 26-47

Grimm N.L., Tisano B., Carey J.L. (2013) Three osteochondritis dissecans lesions in one knee: a case report. Clin Orthop Relat Res, 471(4): 1186-90

Guthrie H.D., Welch G.R., Theisen D.D., Woods L.C. 3rd (2011) Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and plasma membrane integrity in striped bass (Morone saxatilis) sperm. Theriogenology, doi:10.1016/j.theriogenology.2010.10.037. Epub

Han Y., Qu P., Zhang K., Bi Y., Zhou L., Xie D., Song H., Dong J., Qi J. (2019) Storage solution containing hydrogen improves the preservation effect of osteochondral allograft. Cell Tissue Bank, 20(2): 201-208

Henderson J.H., Ginley N.M., Caplan A.I., Niyibizi C., Dennis J.E. (2010) Low Oxygen Tension During Incubation Periods of Chondrocyte Expansion Is Sufficient to Enhance Postexpansion Chondrogenesis. Tissue Eng Part A, 16(5): 1585-1593

Hirschmann M., Muller W. (2015) Complex function of the knee joint: The current understanding of the knee. Knee Surg Sports Traumatol Arthrosc, 23: 2780-2788

Huang F.S., Simonian P.T., Norman A.G., Clark J.M. (2004) Effects of small incongruities in a sheep model of osteochondral autografting. Am J Sports Med, 32(8): 1842-8

Hunt H.E., Sadr K., DeYoung A.J., Gortz S., Bugbee W.D. (2014) The role of immunologic response in fresh osteochondral allo -grafting of the knee. Am J Sports Med, 42(4): 886-891

Hunter S., Timmerman S., Schachar N., Muldrew K. (2006) The Effects of Hypothermic Storage on Chondrocyte Survival and Apoptosis in Human Articular Cartilage. Cell Preserv

Technol, 4(2): 82-90

Hussain W.M., Hussain H.M., Hussain M.S., Ho S.S. (2011) Human growth hormone and the development of osteochondritis dissecans lesions. Knee Surg Sports Traumatol Arthrosc, 19(12): 2108-10

Ishikawa J., Oshima M., Iwasaki F., Suzuki R., Park J., Nakao K., Matsuzawa-Adachi Y., Mizutsuki T., Kobayashi A., Abe Y., Kobayashi E., Tezuka K., Tsuji T. (2015) Hypothermic temperature effects on organ survival and restoration. Sci Rep, doi: 10.1038/srep09563

Ito E., Ikemoto Y., Yoshioka T. (2015) Thermodynamic implications of high Q_{10} of thermo-TRP channels in living cells. Biophysics (Naqoya-shi), 11: 33-38

Jamali A.A., Hatcher S.L., You Z. (2007) Donor cell survival in a fresh osteochondral allograft at twenty-nine years. A case report. J Bone Joint Surg Am, 89(1): 166-9

Kamisan N., Naveen S.V., Ahmad R.E., Tunku K. (2013) Chondrocyte density, proteoglycan content and gene expressions from native cartilage are species specific and not dependent on cartilage thickness: a comparative analysis between rat, rabbit and goat. BMC Vet Res, 9: 62

Katayama Y., Inaba T., Nito C., Ueda M. (2014) Neuroprotective effects of erythromycin on ischemic injury following permanent focal cerebral ischemia in rats. Neurol Res, 38(3): 275-84

Kuhlen M., Kunstreich M., Krull K., Meisel R., Borkhardt A. (2017) Osteonecrosis in children and adolescents with acute lymphoblastic leukemia: a therapeutic challenge. Blood Advances, 1: 981-984

Lafont J.E., Talma S., Hopfgarten C., Murphy C.L. (2008) Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. J. Biol. Chem, 283: 4778–4786

Laiguillon M.C., Courties A., Houard X., Auclair M., Sautet A., Capeau J., Eeve B., Berenbaum F., Sellam J. (2015) Characterization of diabetic osteoarthritic cartilage and role of high glucose environment on chondrocyte activation: toward pathophysiological delineation of diabetes mellitus-related osteoarthritis. Osteoarthritis Cartilage, 23(9): 1513-1522

LaPrade R., Botker J., Herzog M., Agel J. (2009) Refrigerated Osteoarticular Allografts to Treat Articular Cartilage Defects of the Femoral Condyles. J Bone Joint Surg, 91: 805-11

LaPrade R.F., Moulton S.G., Nitri M., Mueller W., Engebretsen L. (2015) Clinically relevant anatomy and what anatomic reconstruction means. Knee Surg Sport Traumatol Arthrosc, 23: 2950-2959

Latt L.D., Glisson R.R., Montijo H.E., Usuelli F.G., Easley M.E. (2011) Effect of graft height mismatch on contact pressures with osteochondral grafting of the talus. Am J Sports Med, 39(12): 2662-2669

Levy Y.D., Gortz S., Pulido P.A., McCauley J.C., Bugbee W.D. (2013) Do Fresh Osteochondral Allografts Successfully Treat Femoral Condyle Lesions? Clin Orthop Relat Res, 471(1): 231-237

Li Y., Wei X., Zhou J., Wei l. (2013) The age-related changes in cartilage and osteoarthritis. Biomed Res Int, doi: 10.1155/2013/916530. Epub

Lima D.D., Fregly B.J., Patil S., Steklov N., Colwell Jr C.W. (2013) Knee joint forces: prediction, measurement, and significance. Proc Inst Mech Eng H, 226(2): 95-102

Linn M.S., Chase D.C., Healey R.M., Harwood F.L., Bugbee W.D., Amiel D. (2011) Etanercept Enhances Preservation of Osteochondral Allograft Viability. Am J Sports Med, 39(7): 1494-1499

Loeser R.F. (2006) Molecular Mechanisms of Cartilage Destruction: Mechanics, Inflammatory Mediators, and Aging Collide. Arthritis Rheum, 54(5): 1357-1360

Lotz M., Loeser R.F. (2012) Effects of aging on articular cartilage homeostasis. Bone, 51(2): 241-248

Luo Y., Sinkeviciute D., He Y., Karsdal M., Henrotin Y., Mobasheri A., Önnerfjord P., Bay-Jensen A. (2017) The minor collagens in articular cartilage. Protein Cell, 8(8): 560-572

Malda J., Benders K.E., Klein T.J., de Grauw J.C., Kik M.J., Hutmacher D.W., Saris D.B., van Weeren P.R., Dhert W.J. (2012) Comparative study of depth-dependent characteristics of

equine and human osteochondral tissue from the medial and lateral femoral condyles. Osteoarthritis Cartilage, 20(10): 1147-51

Malda J., de Grauw J.C., Benders K.E.M., Kik M.J.L., van de Lest C.H.A., Creemers L.B., Dhert W.J.A., van Weeren P.R. (2013) Of Mice, Men and Elephants: The Relation between Articular Cartilage Thickness and Body Mass. PLoS ONE, 8(2): e57683. doi: 10.1371/journal.pone.0057683

Manafi A., Kaviani A., Hamedi Z.S., Rajabiani A., Manafi N. (2017) Evidence-Based Efficacy of Autologous Grated Cartilage in Primary and Secondary Rhinoplasty. World J Plast Surg, 6(2): 137-143

Mastbergen S.C., Jansen N.W.D, Bijlsma J.W.J., Lafeber F.P.J.G. (2006) Differential direct effects of cyclo-oxygenase-1/2 inhibition on proteoglycan turnover of human osteoarthritic cartilage: an in vitro study. Arthritis Res Ther, 8(1): R2

Maury A., Safir O., Heras F., Pritzker K., Gross A. (2007) Twenty-five-year chondrocyte viability in fresh osteochondral allograft. A case report. J Bone Joint Surg, 89(1): 159-65

McAnulty J.F. (2010) Hypothermic organ preservation by static storage methods: Current status and a view to the future. Cryobiology, doi: 10.1016/j.cryobiol.2009.06.004. Epub

McCormick F., Harris J.D., Abrams G.D., Frank R., Gupta A., Hussey K., Wilson H., Bach B.R. Jr, Cole B. (2014) Trends in the surgical treatment of articular cartilage lesions in the United States: an analysis of a large private-payer database over a period of 8 years. Arthroscopy, 30(2): 222-6

McIlwraith C., Fortier L., Frisbie D., Nixon A. (2011) Equine Models of Articular Cartilage Repair. Cartilage, 2(4): 317-326

Messier S.P., Pater M., Beavers D.P., Legault C., Loeser R.F., Hunter D.J., DeVita P. (2014) Influences of Alignment and Obesity on Knee Joint Loading in Osteoarthritic Gait. Osteoarthritis Cartilage, 22(7): 912-917

Mickevicius T., Pockevicius A., Kucinskas A., Gudas R., Maciulaitis J., Noreikaite A., Usas A., (2015) Impact of storage conditions on electromechanical, histological and histochemical properties of osteochondral allografts. BMC Musculoskelet Discord, 16(1): 314

Minas T., Gomoll A.H., Rosenberger R., Royce R.O., Bryant T. (2009) Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques. Am J Sports Med, 37: 902-8

Mirza M.Z., Swenson R.D., Lynch S.A. (2015) Knee cartilage defect: marrow stimulating techniques. Curr Rev Musculoskeletal Med, 8(4): 451–456, doi: 10.1007/s12178-015-9303-x

Moran C.J., Ramesh A., Brama P.A.J., O'Byrne J.M., O'Brien F.J., Levingstone T.J. (2016) The benefits and limitations of animal models for translational research in cartilage repair. J Exp Orthop, 3:1. doi: 10.1186/s40634-015-0037-x

Moyad T.F. (2011) Cartilage Injuries in the Adult Knee: Evaluation and Management. Cartilage, 2(3): 226-236

Nagase H., Kashiwagi M. (2003) Aggrecanases and cartilage matrix degradation. Arthritis Res Ther, 5(2): 94-103

Niethammer T.R., Safi E., Ficklscherer A. (2014) Graft Maturation of Autologous Chondrocyte Implantation: Magnetic Resonance Investigation With T2 Mapping. Am J Sports Med, 42(9):2199-204. doi: 10.1177/0363546514538756

Nuelle C.W., Nuelle J.A., Cook J.L., Stannard J.P. (2017) Patient Factors, Donor Age, and Graft Storage Duration Affect Osteochondral Allograft Outcomes in Knees with or without Comorbidities. J Knee Surg, 30(2): 179-184. doi: 10.1055/s-0036-1584183

Okeagu C., Baker E., Barreras N., Vaupel Z., Fortin P., Baker K. (2017) Review of Mechanical, Processing, and Immunologic Factors Associated With Outcomes of Fresh Osteochondral Allograft Transplantation of the Talus. Foot Ankle Int, 38(7): 808-819

Onuma K., Urabe K., Naruse K., Park H.J., Uchida K., Itoman M. (2009) Cold preservation of rat osteochondral tissues in two types of solid organ preservation solution, culture medium and saline. Cell Tissue Bank, 10: 1-9

Pace R.T., Leadbetter Burg K.J. (2015) Toxic effects of resazurin on cell cultures. Cytotechnology, 67(1): 13-7

Pallente A.L., Bae W.C., Chen A.C., Gortz S., Bugbee W.D. Sah R.L. (2009) Chondrocyte Viability Is Higher After Prolonged Storage at 37°C Than at 4°C for Osteochondral Grafts. Am J Sports Med, 37(Suppl 1): 24S-32S

Pallente A.L., Chen A.C., Temple-Wong M.M.., Bugbee W.D. Sah R.L. (2013) In Vivo Efficacy of Fresh vs. Frozen Osteochondral Allografts in the Goat at 6 Months is Associated with PRG4 Secretion. J Orthop Res, 31(6): 880–886

Pareek A., Sanders T.L., Wu I.T., Larson D.R., Saris D.B.F., Krych A.J. (2017) Incidence of symptomatic osteochondritis dissecans lesions of the knee: a population-based study in Olmsted County. Osteoarthritis Cartilage, 25(10): 1663-1671

Pauli C., Whiteside R., Hera F.L., Nesic D., Koziol J., Grogan S.P., Matyas J., Pritzker K.P.H., D'Lima D.D., Lotz M.K. (2012) Comparison of cartilage histopathology assessment systems on human knee joints at all stages of osteoarthritis development. Osteoarthritis Cartilage, 6(20): 476-485

Pearsall A.W., Tucker J.A., Hester R.B., Heitman R.J. (2004) Chondrocyte Viability in Refrigerated Osteochondral Allografts Used for Transplantation Within the Knee. Am J Sports Med, 32(1): 125-131

Pearsall A.W., Madanagopal S.G., Hughey J.T. (2008) Osteoarticular autograft and allograft transplantation of the knee: 3 year follow-up. Orthopedics, 31(1): 73

Pecchi E., Priam S., Mladenovic Z., Gosset M., Saurel A.-S., Aguilar L., Berenbaum F., Jacques C. (2012) A potential role of chondroitin sulfate on bone in osteoarthritis: inhibition of prostaglandin E2 and matrix metalloproteinases synthesis in interleukin-1β- stimulated osteoblasts. Osteoarthritis and Cartilage, 20(2): 127-135

Pennock A., Wagner F., Robertson C., Harwood F., Bugbee W., Amiel D. (2006) Prolonged storage of osteochondral allografts: does the addition of fetal bovine serum improve chondrocyte viability? J Knee Surg, 19(4): 265-72

Prasadam I., Crawford R., Xiao Y. (2012) Aggravation of ADAMTS and matrix metalloproteinase production and role of ERK1/2 pathway in the interaction of osteoarthritic subchondral bone osteoblasts and articular cartilage chondrocytes -- possible pathogenic role in osteoarthritis. J Rheumatol, 39(3): 621-34. doi: 10.3899/jrheum.110777

Qi J., Hu Z., Song H., Chen B., Xie D., Zhou L., Zhang Y. (2016) Cartilage storage at 4°C with regular culture medium replacement benefits chondrocyte viability of osteochondral grafts in vitro. Cell Tissue Bank, 17: 473-479

Raz G., Safir O., Backstein D., Lee P., Gross A. (2014) Distal Femoral Fresh Osteochondral Allografts Follow-up at a Mean of Twenty-two Years. J Bone Joint Surg, 96: 1101-7

Rieppo J., Halmesmäki E., Siitonen U., Laasanen M. S., Töyräs J., Kiviranta I., Hyttinen M.M., Jurvelin J., Helminen H.J. (2003) Histological differences of human, bovine and porcine cartilage. Orthopaedic Research Society. Transactions of the Annual Meeting; No. 28

Robertson C.M., Allen R.T., Pennock A.T., Bugbee W.D., Amiel D. (2006) Upregulation of apoptotic and matrix-related gene expression during fresh osteochondral allograft storage. Clin Orthop Relat Res, 442: 260–6

Roos E.M., Dahlberg L. (2005) Positive effects of moderate exercise on glycosaminoglycan content in knee cartilage: a four-month, randomized, controlled trial in patients at risk of osteoarthritis. Arthritis Rheum, 52(1): 3507-14

Ruan M.Z., Erez A., Guse K., Dawson B., Bertin T., Chen Y., Jiang M-M., Yustein J., Gannon F., Lee B.H.L. (2013) Proteoglycan 4 Expression Protects Against The Development Of Osteoarthritis. Sci Transl Med, 5(176): 176ra34

Sadr K.N., Pulido P.A., McCauley J.C., Bugbee W.D. (2016) Osteochondral Allograft Transplantation in Patients With Osteochondritis Dissecans of the Knee. Am J Sports Med, 44(11): 2870-2875

Schreiner MM, Mlynarik V, Zbyn S, Szomolanyi P, Apprich S, Windhager R, Trattnig S. (2017) New Technology in Imaging Cartilage of the Ankle. Cartilage, 8(1): 31-41

Shakhawath Hossain Md, Bergstrom D.J., Chen X.B. (2015) Modelling and simulation of the chondrocyte cell growth, glucose consumption and lactate production within a porous tissue scaffold inside a perfusion bioreactor. Biotechnol Rep (Amst), 5: 55–62

Shapiro I.M., Vresilovic E.J., Risbud M.V. (2012) IS THE SPINAL MOTION SEGMENT A DIARTHRODIAL POLYAXIAL JOINT: WHAT A NICE NUCLEUS LIKE YOU DOING IN

A JOINT LIKE THIS? Bone, 50(3): 771-776

Shea K.G., Jacobs J.C.Jr, Grimm N.L., Pfeiffer R.P. (2013) Osteochondritis dissecans development after bone contusion of the knee in the skeletally immature: a case series. Knee Surg Sports Traumatol Arthrosc, 21(2): 403-7

Shetty A.A., Kim S.-K., Nakamura N., Brittberg M. (2014) Techniques in Cartilage Repair Surgery. Springer (eBook)

Shoulders M.D., Raines R.T. (2009) COLLAGEN STRUCTURE AND STABILITY. Annu Rev Biochem, 78: 929-958

Stattin E.L., Wiklund F., Lindblom K., Önnerfjord P., Jonsson B.A., Tegner Y., Sasaki T., Struglics A., Lohmander S., Dahl N., Heinegård D., Aspberg A. A Missense Mutation in the Aggrecan C-type Lectin Domain Disrupts Extracellular Matrix Interactions and Causes Dominant Familial Osteochondritis Dissecans. Am J Hum Genet, 86(2): 126-137

Stoker A.M., Stannard J.P., Kuroki K., Bozynski C.C., Pfeiffer F.M. Cook J.L. (2018) Validation of the Missouri Osteochondral Allograft Preservation System for the Maintenance of Osteochondral Allograft Quality During Prolonged Storage. Am J Sports Med, 46(1): 58-65

Sutherland A.J., Beck E.C., Dennis S.C., Converse G.L., Hopkins A., Berkland C.J., Detamore M.S. (2015) Decellularized Cartilage May Be a Chondroinductive Material for Osteochondral Tissue Engineering. Tissue Eng PLoS, 10(5): e0121966. doi:10.1371/journal.pone.0121966

Szychlinska M.A., Trovato F.M., Di Rosa M., Malaguarnera L., Puzzo L., Leonardi R., Castrogiovanni P., Musumeci G. (2016) Co-Expression and Co-Localization of Cartilage Glycoproteins CHI3L1 and Lubricin in Osteoarthritic Cartilage: Morphological, Immunohistochemical and Gene Expression Profiles. Int J Mol Sci, 17(3): 359; doi:10.3390/ijms17030359

Tang W.H., Martin K.A., Hwa J. (2012) Aldose Reductase, Oxidative Stress, and Diabetic Mellitus. Front Pharmacol, 3: 87; doi: 10.3389/fphar.2012.00087

Tarabella V., Filardo G., Di Matteo B., Andriolo L., Tomba P., Vigano A., Marcacci M. (2016) From loose body to osteochondritis dissecans: a historical account of disease definition. Joints, 4(3): 165-170

Taylor S.D., Siridis E.T., Ingham E., Jin Z., Fisher J., Williams S. (2012) Comparison of human and animal femoral head chondral properties and geometries. Proc Inst Mech Eng H, 226(1): 55-62

Temple D., Cederlund A., Lawless B., Aspden R., Espino D. (2016) Viscoelastic properties of human and bovine articular cartilage: a comparison of frequency-dependent trends. BMC musculoskelet disorders, 17(1): 419

Teng M.S., Yuen A.S., Kim H.T. (2008) Enhancing Osteochondral Allograft Viability. Clin Orthop Relat Res, 466: 1804-1809

Tew S.R., Kwan A.L.P., Hann A., Thomson B.M., Archer C.W. (2000) The Reactions of Articular Cartilage to Experimental Wounding. Arthritis Rheum, 43(1): 215-25

Tirico L., Demange M., Santos L., Pécora J., Croci A., Camanho G. (2017). Fresh osteochondral knee allografts in Brazil with a minimum two-year follow-up Rev Bras Ortop, 52(1): 75-81

Torrie A.M., Kesler W.W., Elkin J., Gallo R.A. (2015) Osteochondral allograft. Curr Rev Musculoskelet Med, 8(4): 413-422

Ward B.D., Lubowitz J.H. (2013) Basic Knee Arthroscopy Part 4: Chondroplasty, Meniscectomy, and Cruciate Ligament Evaluation. Arthrosc Tech, 2(4): e507-e508

Williams J.M., Virdi A.S., Pylawka T.K., Edwards R.B. 3rd, Markel M.D., Cole B.J. (2005) Prolonged-fresh preservation of intact whole canine femoral condyles for the potential use as osteochondral allografts. J Orthop Res, 23(4): 831-7

Williams III R.J., Dreese J.C., Chen C.T. (2004) Chondrocyte survival and material properties of hypothermically stored cartilage: an evaluation of tissue used for osteochondral allograft transplantation. Am J Sports Med, 32(1): 132-9

Williams S.K., Amiel D., Ball S.T., Allen R.T., Wong V.W., Chen A.C., Sah R.L., Bugbee W.D.

(2003) Prolonged storage effects on the articular cartilage of fresh human osteochondral allografts. J Bone Joint Surg Am, 85-A: 2111–20

Wood D., Carter K.R. (2018) Osteochondritis Dissecans. StatPearls https://www.ncbi.nlm.nih.gov/books/NBK526091/. Accessed 28/11/18

Wright J., Brockbank K.G.M., Rahn,E. Halwani D.O., Chen Z., Yao H. (2014) Impact of Storage Solution Formulation during Refrigerated Storage upon Chondrocyte Viability and Cartilage Matrix. Cells Tissues Organs, 199: 51-58

Wu J.J., Weis M.A., Kim L.S., Eyre D.R. (2010) Type III collagen, a fibril network modifier in articular cartilage. J Biol Chem, 285(24): 18537-44

Xu W., Xie Y., Wang Q., Wang X., Luo F., Zhou S., Wang Z., Huang J., Tan Q., Jin M., Qi H., Tang J., Chen L., Du X., Zhao C., Liang G., Lin C. (2016) A novel fibroblast growth factor receptor 1 inhibitor protects against cartilage degradation in a murine model of osteoarthritis. Scientific Reports volume 6, Article number: 24042

Yamada T., Uchida K., Onuma K., Inoue G., Alkawa J., Takano S., Sekiguchi H., Fujimaki H., Miyagi M., Takaso M. (2015) Hyaluronic Acid (800 kDa) Supplementation of University of Wisconsin Solution Improves Viability of Osteochondral Grafts and Reduces Matrix Metalloproteinasse Expression during Cold Preservation. ScientificWorld Journal, [Epub ahead of print]

Yildizgoren M.T., Helvaci M.R., Ustun N., Osmanolgu K., Turhanoglu A.D. (2016) Ultrasonographic Assessment of the Distal Femoral Cartilage Thickness in Patients with Homozygous Sickle Cell Disease. Cartilage, 7(3): 217-221

Yin J., Xia Y., Lu M. (2013) Concentration Profiles of Collagen and Proteoglycan in Articular Cartilage by Fourier Transform Infrared Imaging and Principal Component Regression. Spectrochim Acta A Mol Biomol Spectrosc, 88: 90-96

Yin J., Xia Y. (2014) Proteoglycan Concentrations in Healthy and Diseased Articular Cartilage by Fourier Transform Infrared Imaging and Principal Component Regression. Spectrochim Acta A Mol Biomol Spectrosc, 133: 825-830

Yuan X.L., Meng H.Y., Wang Y.C., Peng J., Guo Q.Y., Wang A.Y., Lu S.B. (2014) Bone–cartilage interface crosstalk in osteoarthritis: potential pathways and future therapeutic strategies. Osteoarthritis and Cartilage, 22(8): 1077-1089

Zhang M., Mani S.B., He Y., Hall A.M., Xu L., Li Y., Zurakowski D., Jay G.D., Warman M.L. (2016) Induced superficial chondrocyte death reduces catabolic cartilage damage in murine posttraumatic osteoarthritis. J Clin Invest, 126(8): 2893-902. doi: 10.1172/JCI83676

Zheng C., Levenston M.E. (2015) Fact versus artifact: Avoiding erroneous estimates of sulfated glycosaminoglycan content using the dimethylmethylene blue colorimetric assay for tissue-engineered constructs. Eur Cell Mater, (29): 224-236

Appendices

Appendix 1 - Consent forms

Consent form for the retrieval of tissues from donors by NHS Blood and Transplant.

FORM FRM4281/3.2 Effective: 12/05/14

Consent in accordance with the Human Tissue Act (2004)

NHS Blood and Transplant

Unique Tissue Number K | | | | | | | | | ODT Donor number | | | | | | |

Consent - Solid Organ and Tissue Donation

Directions for Completion:

1. This form must be completed in accordance with the Human Tissue Act 2004.
2. This six page form should be completed by the Specialist Nurse-Organ Donation (SN-OD)/ Nurse Practitioner – Tissue Services in **black or dark blue ink**, and signed and dated as appropriate.
3. Where an option box requires completion, it **must** be initialled by the SN-OD/Nurse Practitioner – Tissue Services/ Tissue Donor Co-ordinator.
4. The original should be retained by the SN-OD/ Nurse Practitioner – Tissue Services/ Tissue Donor Co-ordinator for the donor file.
5. A copy should be made for the patient's medical records.
6. Another copy should be offered to the patient's family.
7. A copy should be sent to relevent tissue banks.

NOTE: The term 'patient' is used throughout the form to refer to the potential donor.

Page 1 of 6

FORM FRM4281/3.2 Effective: 12/05/14

NHS Blood and Transplant

Consent in accordance with the Human Tissue Act (2004)

Unique Tissue Number K | | | | | | | | | ODT Donor number | | | | | | |

PATIENT DETAILS — Section 1

Name

Address

Postcode

NHS number

Hospital number

Date of birth

Age (If under 3 years record years and months) years months

CONSENT FOR ORGANS AND TISSUE — Section 2

Complete **Box A** if the patient is/has giving/given first person consent
OR
Complete **Box B** if consent is given by the person ranking highest in the qualifying relationship

Box A

Name of patient

gave/gives* first person consent for the donation of the following organs/tissue for transplantation via the Organ Donor Register/donor card/expressed wish/will*
*(*Delete as appropriate)*

Box B

I, (Name) the (Relationship to the patient) of (Patient's name)

give my consent for the donation of organs and tissue as detailed below.

NOTE:
Proceed to Section 3 for Telephone Consent for Organ and/or Tissue Donation
Proceed to Section 4 for Organ and Tissue Donation
Proceed to Section 5 for Tissue Only Donation

OBTAINING CONSENT VIA TELEPHONE — Section 3

To obtain consent via telephone is in accordance with the Human Tissue Act 2004 and the Human Tissue Authority Codes of Practice 2009 – it is not a legal requirement for relatives to sign a consent form. However, the interviewer **must** ask the following and **initial** the appropriate boxes:

	Yes	No
Do you agree to the conversation about donation between (name of HCP) of NHS Blood and Transplant and you being voice recorded? The recording will be stored as proof of the information that I give to you and of the consent and information that you give to me.		
For the purpose of the recording can you tell me again your full name and relationship to (name of the patient)		
May we use the recording and case details for training purposes?		

NOTE:
Proceed to Section 4 for Organ and Tissue Donation
Proceed to Section 5 for Tissue Only Donation

Consent in accordance with the Human Tissue Act (2004)

NHS Blood and Transplant

Unique Tissue Number K | | | | | | | | |

ODT Donor number | | | | | | |

CONSENT FOR ORGAN DONATION — Section 4

Organ Group

	Yes	No
All Abdominal Organs		
All Cardiothoracic Organs		

Organ

	Yes	No	Coroner Objection	Outside Criteria
Kidneys				
Liver				
Liver hepatocytes				
Pancreas				
Pancreas Islet cells				
Bowel				
Other (consider multivisceral donation where appropriate) If YES	Please specify			
Heart				
Lungs				
Blood Vessels				

CONSENT FOR TISSUE DONATION — Section 5

All Tissue

Yes	No	Coroner Objection

Tissue

	Yes	No	Coroner Objection	Outside Criteria
Eyes				
Whole heart for heart valve donation				
Skin				
Bone				
Tendons				
Meniscus				
Spleen (tissue only donation)				
Blood Vessels				
Other				

Specific Tissue

	Yes	No	Coroner Objection	Outside Criteria
Corneas				
Sclera				
Whole heart for heart valve donation				
Pericardium				
Femur(s)				
Iliac crest(s)				
Achilles tendons				
Semi tendinosus tendons				
Patella tendons				
Anterior tibialis tendons				
Meniscus				

Please specify

Please specify

Consent in accordance with the Human Tissue Act (2004)

NHS Blood and Transplant

Unique Tissue Number K | | | | | | | | |

ODT Donor number | | | | | | |

REQUIRED INFORMATION TO SUPPORT ORGAN AND TISSUE DONATION — Section 6

IF APPLICABLE (Organ and Tissue Donation)

Do you consent to the patient being transferred from their place of death to the dedicated donation facility for the donation procedure to be undertaken? **Yes** ☐ **No** ☐

I have been informed of and am aware of the following:

That blood and tissue samples will be obtained from the patient (and the patient's mother where the patient is under 18 months old **and/or** the patient has been breast fed in the last 12 months) for testing, including tissue typing, HIV, Hepatitis, HTLV and Syphilis. These samples may be subsequently stored for future testing as necessary. In the event of any screening results that may have implications for the family, relevant individuals may be contacted if their health could be affected.

That blood and tissue samples e.g lymph node and spleen that have been obtained for screening will be subsequently biopsied, analysed and stored for future testing as necessary.

Blood vessels will be retrieved and stored to support surgical procedures and if not used within 14 days will be disposed of in accordance with the hospital/tissue establishment policy. The tissue donated for transplantation will be stored for extended periods in tissue establishments whilst it is prepared for transplantation.

The patient's medical records will be accessed by relevant healthcare professionals to obtain a past medical/behavioural history. This information may be passed on a need-to-know basis to other healthcare professionals in support of the transplantation process. This information may also be retained by the Organ Donation Teams/Tissue Establishments.

Organs and / or tissue which you have agreed to donate may be used in research prior to transplantation to improve transplant outcomes for the recipient. The organs and / or tissue will still be transplanted. Do you consent to this? **Yes** ☐ **No** ☐

CONSENT FOR SCHEDULED PURPOSES — Section 7

Organs and/or tissue can also be used for the scheduled purposes listed below.

Scheduled Purposes Include:
Research
Education or Training related to Human Health
Clinical audit
Quality Assurance
Performance Assessment

Organs and/or tissue may at times be found to be unsuitable for transplantation once removed.
Do you consent to organs and/or tissue being used for a scheduled purpose listed above if they are removed for transplant and subsequently found unsuitable? **Yes** ☐ **No** ☐

Other organs and/or tissue unsuitable for transplantation may also be donated to aid future health care.
Do you consent to organs and/or tissue being removed solely for a scheduled purpose as listed above (please detail in notes on page 6)? **Yes** ☐ **No** ☐

NOTE:
Organs and/or tissue will be used and stored for a scheduled purpose in accordance with The Human Tissue Act (2004)

Organs and/or tissue that are not used for a scheduled purpose, or following the completion of a scheduled purpose, will be disposed of as per local establishment policy in accordance with the Human Tissue Act (2004).

Consent in accordance with the Human Tissue Act (2004)

NHS Blood and Transplant

Unique Tissue Number K | | | | | | | | | ODT Donor number | | | | | | |

CONFIRMATION OF CONSENT — Section 8

I have read and understood the above and I have had the opportunity to ask and have had my questions answered

Patient/Relationship to patient

Name (Please print) | Signed

Date | | | | 2 0 | | | Time *(24 hr)* at | | : | |

Address of person giving consent

Telephone number

Mobile

Co-signatory Name *(Where applicable)* (Please print) | Signed

Healthcare Professional Details (Witness)

Designation

Name (Please print) | Signed

Date | | | | 2 0 | | | Time *(24 hr)* at | | : | |

Specialist Nurse – Organ Donation/Nurse Practitioner – Tissue Services/ Tissue Donor Co-ordinator

Designation

Name (Please print) | Signed

Date | | | | 2 0 | | | Time *(24 hr)* at | | : | |

	Yes	No
Did the patient give first person consent for the donation via the Organ Donor Register/donor card/expressed wish/will?		
Did the patient have a nominated representative?		
Was consent obtained from the person ranking highest in the qualifying relationship?		

Persons are ranked in the following descending order:

A) Spouse or partner (including civil or same sex partner)
B) Parent or child
C) Brother or sister
D) Grandparent or grandchild
E) Niece or nephew
F) Stepfather or stepmother
G) Half-brother or half-sister
H) Friend of longstanding

If no, please give details below:

Information leaflets given to family? | OR | Information leaflets to be sent to the family?

Consent in accordance with the Human Tissue Act (2004)

NHS Blood and Transplant

Unique Tissue Number K | | | | | | | | |

ODT Donor number | | | | | | |

NOTES

Aintree University Hospital NHS
NHS Foundation Trust

Version 1 06 January 2017
IRAS Project ID: **221550**
Centre Number: 1
Study Number: 1
Randomised Patient Identification Number:

Investigating the Viability of Cartilage Tissue during Storage - Version 1

CONSENT FORM

1. I confirm that I have read and understand the information sheet dated…….. to the above study. I have had the opportunity to consider the information, ask questions and that my questions answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities of from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that the sample will be transferred off-site to a secure research laboratory at the University of Liverpool or the research department of NHS Blood and Tissue Transplant Services, Speke, Liverpool. At the end of the research study any remaining tissue will be disposed of in accordance with the Human Tissue Act. ☐

5. I agree to take part in the above study. ☐

_____	_____	_____	
Name of Patient	Date	Signature	
_____	_____	_____	_____
Name of Person taking consent	Date	Signature	Position

1 copy for patient; 1 to be kept with hospital notes; 1 copy for site file

Aintree University Hospital NHS
NHS Foundation Trust

Version 1 06 January 2017
IRAS Project ID: 221550

Patient Information Sheet

Study title

A study Investigating the Viability of Cartilage Tissue during Storage

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. (Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

The purpose of this study is to look into how cartilage tissue can be stored so that it can be used to treat patients with damaged knees. Cartilage tissue can be damaged easily and this can be repaired by transplanting new tissue into the knee joint. We want to understand how we can keep alive the cartilage tissue that is used to treat patients for longer so that we are able to treat as many people as possible and they have a better chance for a successful outcome.

Why have I been invited?
You have been given this information booklet as your treating doctors have assessed your knee condition and have concluded that you require a total knee replacement. This is a common injury which is seen on a daily basis in hospitals. All patients presenting with a similar condition at Aintree University Hospital will be invited to take part in this study.

Do I have to take part?
This is solely your decision. The study design will be outlined to you and you will be given adequate time to read the information sheet and any concerns will be answered. Should you wish to participate then you will be asked to sign a consent form. **You are free to withdraw at any time, without giving a reason. This would IN NO WAY influence the standard of care you receive.**

What will happen to me if I take part?

Firstly, the project will be discussed again with you once you have read this booklet. Common questions are:

1. How long will I need to be involved in the research?

   If you agree to take part in the study you will only need to be involved during the time which the surgery is performed on your knee. You will not have to attend any additional hospital appointments for the study.

2. What will happen?

   The doctor will perform the surgery on your knee joint. As part of this procedure the ends of the bones within the knee joint will be removed. We are asking you to agree for this tissue that would otherwise be discarded to be used for our research study.

3. Is this procedure normally carried out?

   Yes, this procedure would take place routinely to correct the condition that you have. The study permission is required for us to store and use the waste tissue that would normally be discarded.

4. What will happen next?

   You will be given the same clinical care whether or not you are in the study.

What will I have to do?

Once we have taken the tissue there is nothing else you need to do.

Are there any risks?

The risk is no higher than if you were undergoing the knee surgery without being involved in this study. All appropriate measures will be taken including sanitising the skin and formal draping so as to avoid contamination.

What are the benefits of taking part?

The potential benefits are a greater understanding of how cartilage tissue transplants can be improved. We cannot promise this study will help you, but the information we get from this study will help improve the treatment of people with knee injuries in the future.

What if there is a problem?

Any complaint about the way you have been treated during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes, we will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in part 2.

PART 2

What if relevant new information becomes available?

Sometimes we get new information about the condition being studied. If this happens, your research doctor will tell you and discuss whether you should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study he may ask you to sign an updated consent form.

If this happens, your research doctor might consider you should withdraw from the study. He/she will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, we will tell you and arrange your continuing care.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the clinician responsible for your care. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

This is part of a study which is led by Aintree University Hospital NHS Foundation Trust. In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence, you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will be available to you.

Aintree University Hospital has a dedicated Patient Advice and Complaints Team (PACT) who deal with comments, concerns and complaints. The team will listen to you, respond in a timely manner and learn from you to help improve our services.

Contacting the PACT team

1) Visit the help desk: The PACT team have a helpdesk, located on the ground floor of the main hospital building near to main reception. The helpdesk, open Monday to Friday between 9am and 4pm, is staffed by volunteers and members of staff who will be happy to help you deal with any queries or provide advice or support.

2) Telephone: 0151 529 3287/2400

3) Fax: 0151 529 2019

4) Write to us: Concerns and complaints can be sent for the attention of the PACT team or the Chief Executive at:

Aintree University Hospital NHS Foundation Trust
Lower Lane
Liverpool
L9 7AL

5) E-mail us: complaints@aintree.nhs.uk

6) If you require support in raising a concern or making a complaint, you can contact the Independent Complaints Advisory Service (ICAS) at: The Gateway Conference Centre 71 London Road Liverpool L3 8HY

Will my taking part in this study be kept confidential?

All information collected about you during the course of the research will be kept strictly confidential and any information about you which leaves the hospital/surgery will have your name and address **removed** so that you cannot be recognised by anyone including members of the research team.

Data which is generated during the course of the study will be published in scientific journals in order to increase the knowledge of people working on similar research. Because the research team has no access to your personal information you will not be able to be identified.

Who will have access to my personal information?

Research studies which involve the participation of patients are strictly controlled. In some circumstances authorised people from the NHS may access your notes to check that your participation within the study is being managed according to National and European law.

It is also possible that authorised people from regulatory bodies request access to your notes to check that your participation within the study is being managed according to National and European law.

What will happen to any samples that I give?

Common questions are:

1. Will my samples be stored in a secure area and how?

Once the sample is taken it will be sent off site to a secure research laboratory either at NHS Blood and Tissue Transplant Services (NHSBT) or the University of Liverpool.

2. Who will have access to the samples?

Only the study team directly involved in the study.

3. How will my samples be used?

Researchers at NHSBT and the University of Liverpool. They will carry out a series of experiments to understand how the cartilage tissue behaves when it is used for transplanting into people with damaged knees.

4. Will my samples be anonymous?

All samples will be fully anonymised, meaning that research staff at the laboratory will not have any of your personal details.

5. What happens to my samples at the end of the study?

Once the study is complete all samples will be destroyed in accordance with the Human Tissue Act.

6. Will samples be transferred outside the UK?

No samples will be sent outside the UK

What will happen with the results of the study?

Results will be presented at a national and international level at conferences

and in scientific journals. All data will be anonymous.

Who is funding this study?

The study is funded by the Medical Research Council, the Wellcome Trust and the University of Liverpool.

Who has reviewed the study?

A research ethics committee, which has responsibility for scrutinising all proposals for research on humans, has examined the proposal and has raised no objections from the point of view of research ethics. It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from the University of Liverpool and Aintree University Hospital NHS Foundation Trust, whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

Who do I speak to if I have further questions?

You can contact Professor Michael McNicholas to discuss any matter related to the research via contacting Professor McNicholas' secretary at Aintree University Hospital. Tel 0151 529 2548

Appendix 2 - Lubricin (PRG4) imaging score card

Scores (HRP)	Wk1				mean	SD	Wk12				mean	SD
	ID1	ID2	ID3	...			ID1	ID2	ID3	...		
Normal												
Over-age												
Hypo												
OA												
Negative												
Isotype control												
Control no Ab												
Scores (AFP488)	Wk1				mean	SD	Wk12				mean	SD
	ID1	ID2	ID3	...			ID1	ID2	ID3	...		
Normal												
Over-age												
Hypo												
OA												
Negative												
Isotype control												
Control no Ab												

PROTOCOL FOR THE USE OF THE GLUCOSE/LACTATE METER

Purpose: To describe the use of the Accutrend® Plus glucose/lactate meter for measuring glucose or lactate concentration in solutions.

Scope: This protocol applies to the preparation of the meter and equipment used.

Equipment Required:

- Accutrend Plus glucose/lactate meter (Roche)
- Balance
- Micropipette

Consumables required:

- Glucose test strips (Roche, 11447475)
- Lactate test strips (Roche, 03012654)
- Pipette tips
- 7ml Bijou's

Chemicals required:

- Glucose standard (Dextrose) (Sigma, PHR1000)
- Lactic acid standard (Sigma, PHR1215)
- Dulbecco's phosphate buffered saline (DPBS)

Preparation of standards

Glucose

Prepare a 20mmol/L stock by dissolving supplied standard in DPBS, molecular weight of Dextrose = 180.16g/mol

Lactic acid

Prepare a 20mmol/L stock by diluting supplied standard in DPBS, molecular weight of lactate = 90.1g/mol. Standard supplied as a 12N solution from Sigma.

Protocol:

1. Dilute stock glucose and lactate standards in DPBS to give the following concentrations: 10mmol/L and 5mmol/L.
2. If a new batch of test strips are being used, insert the supplied batch information barcode strip into the meter and then withdraw immediately, leaving the measurement chamber closed.
3. Insert either a glucose or lactate test strip into the meter until two audible beeps are heard.
4. Open the test chamber lid and pipette 15ul of sample to be tested onto the sample application area of the test strip.
5. Close the test chamber lid.
6. Wait for the meter to measure the sample, this takes 12 seconds for glucose test strips and 60 seconds for lactate.
7. Once measurement has been completed, open the test chamber lid and remove the test strip.
8. Repeat steps 3 to 7 for all standards and test samples.
9. If the concentration of a sample is outside the range of the meter, take an aliquot from the sample and dilute it before testing in the meter.
10. Transfer glucose standards measurements into Excel to create a standard curve, with glucose standard (mmol/L) on x-axis and measured concentration (mmol/L) on y-axis.
11. Add a trend line to the curve and use the equation from this trend line to correct glucose measurements from test solutions, re-arrange the equation so that the x-axis value is calculated (ie move unknown term ‘x’ to the left side of the equation).

DMMB ASSAY PROTOCOL

Purpose: To determine the amount of chondroitin sulphate in biological solutions.

Scope: This protocol applies to the preparation of reagents, running of the assays and calculation of results.

Equipment required

- Absorbance microplate reader.
- 20-200 and 200-1,000μl capacity micropipettes
- Pipette
- pH meter

Consumables required

- Disposable 96 well microplate
- Yellow and blue micropipette tips
- ~1ml Eppendorf tubes

Chemicals required

- Distilled water (Baxter)
- Chondroitin sulphate C (Sigma C4834)
- 1,9 dimethyl methylene blue (Sigma, 341088)
- Formic acid (VWR, 0961)
- Sodium formate (Sigma, 456020)
- Sodium hydroxide (Sigma)
- DMEM F12 Ham (Lonza)
- Foetal bovine serum (Biosera)
- Penicillin/streptomycin (Sigma)
- L-ascorbic acid (Sigma)
- Sodium pyruvate (Sigma)

Preparation of reagents and solutions

Chondroitin sulphate

Dissolve 5mg/ml of the chondroitin sulphate in standard culture medium.

DMMB assay solution

0.16% (w/v) 1,9 dimethyl methylene blue – 16mg / 1L water
0.2 % (v/v) formic acid – 2ml / 1L water
30mM sodium formate – 2g / 1L water

pH 3.5 with 1M NaOH

Standard culture medium

DMEM F12 Ham solution plus:
10% (v/v) FCS
1% (v/v) penicillin/streptomycin
1% (v/v) sodium pyruvate
0.048mg/ml L-ascorbic acid

Assay protocol

1. Equilibrate all reagents to room temperature.
2. Dilute standard in culture medium as follows:

Standard Conc. (µg/ml)	Volume of stock added (µl)	Volume medium (µL)
0	0	1000
10	2	998
20	4	996
30	6	994
40	8	992
50	10	990
60	12	988
70	14	986

3. Dilute conditioned medium samples at ½, 1/5, 1/10, and 1/20 dilutions, using standard culture medium as the diluent.
4. Transfer 40µL standards and media samples into separate wells of a 96 well microplate in triplicate.
5. Add 250µl DMMB reagent to each well.
6. Immediately read the plate on the plate reader at 570nm.
7. Construct a standard curve from the absorbance values.
8. Using the formula generated by the curve trendline, calculate the concentration of chondroitin sulphate in the media samples.
9. If sample values fall outside of standard curve then these samples will need to be diluted and re-measured.

PROTOCOL FOR THE STAINING OF FRESH SECTIONS OF CARTILAGE WITH LIVE/DEAD ASSAY SOLUTIONS

Purpose: To stain sections from cartilage tissue samples that have been cut on a vibratome, stained with the combined live/dead assay solutions before being placed on microscope slides prior to observation under a microscope, to visualise living and dead cells present in the cartilage via fluorescence.

Scope: This protocol applies to the preparation of the tissue and reagents used.

Equipment Required:

- 7ml plastic bijous
- 30ml universal tube
- Microscope slide coverslips
- Microscope with fluorescence filters and UV source with camera
- Pasteur pipette
- Microscope slide
- Aluminium foil
- Scalpel
- Forceps
- Vibratome (Leica VT1000S)
- Razor blades
- Eppendorf tubes
- Red/green fluorescence test microscope slide
- Fiji image analysis software on a PC.

Chemicals required

- Dulbecco's PBS (Sigma)
- Live/dead assay kit (component A (4mM calcein acetoxymethyl), component B (2mM ethidium homodimer-1))
- Cyanoacrylate glue

Protocol:

1. Aliquot the live/dead assay kit reagents into volumes required in small Eppendorf tubes and store at -20ºC protected from light and moisture. The final concentration of both reagents in the working solution should be 1µM, therefore for a 20ml solution 10µl EthD-1 (component B), and 5µl calcein acetoxymethyl (component A) are required.
2. Cut a rectangular section of cartilage from tissue to be tested, full depth to the subchondral bone, ~3mm wide and 5mm long.
3. Mount the piece of tissue on a vibratome cutting plate using a small blob of cyanoacrylate glue, such that the 5mm side projects vertically from the plate.
4. Secure the plate in the vibratome and orientate the tissue such that the flat profile is at right angles to the blade holder.
5. Fill the trough in which the plate is located with Dulbecco's PBS, until the tissue is fully covered.
6. Switch on the vibratome and press the V-max button.
7. Make sure a blade is securely fitted in the blade holder.
8. Move the cutting blade holder forward until it is a few mm away from the tissue, using the forward/reverse toggle switch.
9. Press the button with opposing arrows.
10. Move the blade past the tissue and a few mm beyond.
11. Press the button with opposing arrows again, this sets the cutting window.
12. Press the start button to deactivate V-max, the vibratome will now start cutting.
13. Move the tissue up or down until the blade is very close to the top of the tissue.
14. Ensure the cut depth is set to 50µm, with frequency set to 100Hz and speed of 0.25mm/s.
15. Make sure the cutting is set to continuous before pressing start.
16. Wait until the blade starts cutting sections before using forceps to carefully lift the sections out of the PBS and place them in a 7ml bijou full of PBS.
17. Allow the vibratome to cut as many sections as required, placing them all in the same bijou of PBS.
18. Thaw out a vial of each working reagent (component A and B).
19. Add them both to 20ml of PBS in a 30ml universal tube and mix.
20. Carefully pour the PBS out of the 7ml bijou containing the cut sections of tissue, making sure the tissue stays in the bottom.
21. Fill the bijou with the working live/dead reagent and leave at room temperature for 30 minutes. Cover the tube in aluminium foil to protect it from light.
22. After 30 minutes remove the foil and empty the working reagent, again make sure the tissue stays inside.
23. Fill the bijou with fresh PBS and leave at room temperature for 5 minutes with the tube covered in the foil.
24. Remove foil and empty the bijou, leaving tissue inside.
25. Repeat step 23.
26. Take the sections of cartilage out of the bijou with forceps and carefully place them on a microscope slide.
27. Place a few drops of PBS on the slide using a Pasteur pipette.
28. Place a microscope coverslip over the cartilage sections and remove any air bubbles.
29. Switch on the Leica microscope, associated computer, and control box for the UV light source. Make sure the white light source is off.
30. Move the filter wheel at the front of the microscope to position 2.
31. Make sure all the levers at the bottom right of the microscope are in the forward position.
32. Put the stop lever at the back of the microscope into the down position.
33. Start the Leica image capture software on the computer.

34. Check the red/green fluorescence is picked up on the digital camera using the test microscope slide located in the dark room.
35. Place the microscope slide with the stained sections on the stage of the microscope.
36. Select the x10 objective on the microscope.
37. Move the stage so that one of the sections is visible, made sure the superficial layer of the cartilage is visible in the image captured by the camera.
38. Focus the image if necessary and adjust the gamma, exposure, saturation and colour settings on the image capture software to give a near black background with easily visible red/green fluorescence.
39. Acquire the image once satisfied and save it as a TIFF file.
40. Collect at least 5 images from each biopsy sampled.
41. Transfer images to a PC with Fiji image analysis software installed.
42. Load an image into the Fiji software.
43. Select process and then find maxima in the drop-down menu.
44. Select count in the output type box.
45. Select preview point selection in its check box.
46. Review the crosses overlaid in the image, if the program has put too many crosses in an area of cells, increase the noise tolerance figure, adjust until satisfied, recommended value between 20-60.
47. Press OK, results of total cell count will be added to a new window.
48. If required a selection box can be drawn around an area of interest.
49. Select Image > Colour > Split channels, this will separate the image into red, blue and green colours.
50. Select green colour window and repeat steps 43-47.
51. Divide the number of green cells by the total cells counted and multiply by 100 to give percent viability.

PROTOCOL FOR THE ROUTINE FIXING AND WAX EMBEDDING OF HUMAN TISSUE

Purpose: To fix fresh tissue samples and embed them in paraffin wax prior to wax block preparation.

Scope: This protocol applies to the preparation of the tissue and equipment used.

Equipment Required:

- Citadel 2000 tissue processor
- Histostar tissue embedder
- 5ml Universal tubes
- Plastic tissue cassettes
- Wax block moulds

Chemicals required:

- 10% neutral buffered formalin (VWR, 361367L)
- Ethanol (VWR, 302444E)
- Xylene (VWR, 28975.360)
- Paraffin wax (Thermo Fisher Scientific - Histoplast 6774060)

Protocol:

1. A section of fresh tissue that is to be fixed is placed in a 5ml universal tube filled with 10% neutral buffered formalin.
2. This is then left for up to 7 days in a fume hood depending on the tissue being fixed.
3. After this time the tissue is taken out of the formalin and placed in the trough of a plastic tissue embedding cassette.
4. The dipping buckets of the Citadel 2000 tissue processor has the following chemicals placed inside: bucket 1 contains 10% neutral buffered formalin.
5. Bucket 2 contains 70% ethanol.
6. Bucket 3 contains 90% ethanol.
7. Buckets 4-7 contain 100% alcohol.
8. The 3 metal buckets contain 100% xylene.
9. The final 2 heated buckets contain paraffin wax.
10. All prepared tissue cassettes are then placed in the dipping basket of the processor and the loading door closed.
11. The dipping basket is set at position 1 on the processor and started on program A.
12. Once the program has finished remove the tissue cassettes from the processor and place them in the wax bath of the Histostar tissue embedder.
13. Enough molten paraffin wax is then dispensed into a wax block mould to fill the recessed mould which is then placed on the cold plate of the Histostar.
14. A tissue cassette is taken from the wax tank and opened up, using the heated forceps the tissue is taken out and carefully orientated in the base of the wax block mould.
15. The tissue cassette is then placed on the recess of the wax block mould and more paraffin wax dispensed onto the cassette until the cassette recess has been filled.
16. The cassette and wax block mould are then left on the cold plate until the wax has solidified.
17. Once hardened, the wax block and cassette are removed from the mould and excess wax trimmed from the cassette and block.

PROTOCOL FOR THE CUTTING OF SECTIONS FROM WAX BLOCKS

Purpose: To cut sections from tissue samples embedded in wax blocks prior to staining for observation under a microscope.

Scope: This protocol applies to the preparation of the tissue and equipment used.

Equipment Required:

- Rotary microtome (Finesse 325)
- Tissue flotation bath (RA Lamb)
- Microscope slides
- Slide drying rack
- Small paintbrush
- Forceps
- Mounted needle

Chemicals required:

- Distilled water (Baxter)

Protocol:

1. A previously prepared wax block is placed in the chuck of the rotary microtome.
2. Using a worn blade in the microtome the wax is sectioned from the block by turning the right handle to oscillate the wax block over the blade. The smaller handle on the left of the microtome is used to move the chuck closer or further away from the blade.
3. Once the edge of the tissue is exposed, the blade is changed for a new one.
4. Serial sections are cut from the block with as many sections cut as required.
5. Holding the leading edge of the cut sections, they are separated from the blade by passing a small paintbrush underneath the ribbon of sections.
6. The ribbon of sections are then laid out on the surface of the water in the flotation bath. The flotation bath temperature is set at 45^{o}C.
7. Using the mounted needle the sections are separated into groups depending how many sections will fit onto a slide joined together. This is done by repeatedly making holes along the join between sections. Or teasing them apart with a pair of forceps.
8. Once separated the sections are collected onto microscope slides by inserting the slides vertically into the water close to the sections. The slide is then pulled out of the water at a 45 degree angle from underneath the sections, so that they are dragged onto the slide.
9. Slides are then dried by placing them in a drying rack, and then in an incubator at 37^{o}C.

PROTOCOL FOR THE IMMUNOHISTOCHEMISTRY OF LUBRICIN IN SECTIONS OF CARTILAGE

Purpose: To visualise the presence of lubricin secreting chondrocytes in sections of articular cartilage using both chomogenic or fluorescence based protocols.

Scope: This protocol applies to the preparation of the tissue and reagents used.

Equipment Required:

- Microscope slide heater
- Glass staining troughs
- Metal staining rack
- Microscope slide coverslips
- Microscope
- Micropipette
- Pipette
- Stripettes
- Fume hood
- Humidity chamber
- Incubator
- Cold room
- Daco pen

Consumables required:

- Tissue paper
- Pipette tips
- Aluminium foil

Chemicals required:

- Xylene
- Ethanol
- Bovine serum albumin (BSA)
- Goat serum
- Haematoxylin
- Chondroitinase ABC
- Rabbit polyclonal anti-human lubricin antibody
- Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG
- 3,3’Diaminobenzidine (DAB) substrate
- Rabbit IgG isotype control antibody
- Alexa Fluor® Plus 488
- DAPI Vectorshield
- Distilled water
- Dulbecco’s phosphate buffered saline (DPBS)
- DPX mountant

Preparation of reagents:

Block buffer
10% goat serum and 10% BSA in DPBS

Chondroitinase ABC
0.1U/ml in block buffer

HRP chromogen protocol:

Primary antibody
Rabbit polyclonal anti-human lubricin antibody diluted to 10µg/ml (1:100) from stock using blocking buffer as diluent

Secondary antibody
HRP-conjugated goat anti-rabbit IgG 2µg/ml in blocking buffer

Isotype control antibody
Rabbit IgG isotype control antibody 10 µg/ml (1:100) from stock using blocking buffer as diluent

1. Place slides on a microscope slide heater and heat fix/melt wax for 5-10 minutes.
2. Place the slides in a metal staining rack and place in a trough of 100% xylene for 5 minutes.
3. Transfer the staining rack to another trough of 100% xylene and leave for a further 5 minutes.
4. Transfer the staining rack to a trough of 100% ethanol for 5 minutes.
5. Transfer the staining rack to a trough of 90% ethanol for 5 minutes.
6. Briefly rinse the slides in water before dabbing dry with tissue paper.
7. Draw round each section on the slide with a Daco pen.
8. Place each microscope slide on the ledges inside the base of a humidity chamber, with wet tissue paper placed between the ledges, underneath the slides
9. Pipette hydrogen peroxide in methanol (0.3% (vol/vol)) onto each section, so that each section is totally immersed, leave for 5 minutes.
10. Pipette chondroitinase ABC onto each section, so that each section is immersed and then incubate for 1 hour at 37ºC, with the humidity chamber wrapped in aluminium foil.
11. Pipette block buffer onto each section and incubate for 1 hour at room temperature with humidity chamber wrapped in aluminium foil.
12. Rinse slides with DPBS.
13. Pipette primary antibody onto each section and incubate at 4ºC overnight inside humid chamber and protect from light.
14. Rinse slides with DPBS.
15. Pipette secondary antibody onto each section and incubate for 1 hour at room temperature, protect from light.
16. Rinse slides with DPBS.
17. Pipette Vectastain ABC reagent onto each section and incubate for 30 minutes at room temperature, protect from light.
18. Rinse slides with DPBS.
19. Pipette DAB substrate onto each section and wait for brown stain to appear, check staining under a microscope.
20. Rinse slides with DPBS.
21. Immerse slides in haemtoxylin and leave for 5 minutes.
22. Rinse slides with DPBS.
23. Place slides in a metal staining rack and then immerse in 70% ethanol for 30

seconds.
24. Immerse slides in 90% ethanol for 30 seconds.
25. Immerse slides in 100% ethanol for 30 seconds.
26. Immerse slides in 100% xylene for 1 minute.
27. Immerse slides in 100% xylene for 1 minute.
28. Add DPX mountant to each section before placing a glass coverslip on each slide.

29. Incubate slides in a humid chamber overnight at 4°C and protect from light.

Controls

Control sections were processed in parallel either with an isotype control substitute for the primary antibody, or with no secondary antibody.

Alexa Fluor® Plus 488 fluorescence protocol:

Secondary antibody
Goat anti-rabbit IgG Alexa Fluor Plus 488 4µg/ml in block buffer.

30. Repeat steps 1 – 15 on a separate set of cartilage sections.
31. Pipette secondary antibody onto each section and incubate for 1 hour at room temperature.
32. Rinse slides with DPBS.
33. Add drops of DAPI Vectorshield to each section before placing a coverslip on each slide.
34. Incubate slides in a humid chamber overnight at 4°C and protect from light.

Controls

Control sections were processed in parallel either with an isotype control substitute for the primary antibody, or with no secondary antibody.

PROTOCOL FOR THE STAINING OF SECTIONS WITH SAFRANIN O

Purpose: To stain with Safranin O sections from tissue samples that have been cut and put on microscope slides prior to observation under a microscope.

Scope: This protocol applies to the preparation of the tissue and reagents used.

Equipment Required:

- Microscope slide heater
- Glass staining troughs
- Plastic staining rack
- Microscope slide coverslips
- Microscope
- Fume hood

Chemicals required:

- Xylene
- Ethanol
- Safranin O
- Harris haematoxylin
- Fast green FCF
- Glacial acetic acid
- Distilled water
- DPX mountant

Preparation of reagents:

Safranin O
Safranin O is used at a concentration of 0.1% using 0.1g Safranin O in 100ml of distilled water.

FCF
Fast green FCF is used at a concentration of 0.1% using 0.1g fast green in 100ml of 1% acetic acid (1ml glacial acetic acid to 99ml Water)

Protocol:

1. Place slides on a microscope slide heater and heat fix/melt wax for 5-10 minutes.

   The following steps are carried out inside a fume hood

2. Place slides in plastic staining rack and dip in glass trough of xylene for 5 minutes.
3. Dip in a trough of absolute ethanol (100%) for 5 minutes.
4. Slides are then dipped in a trough of 90% ethanol for 5 minutes.
5. Next the slides are dipped in another trough containing 70% ethanol for 5 minutes.
6. Dip slides in distilled water for 2 minutes
7. Incubate slides in Harris haematoxylin at room temperature for 10 minutes.
8. Slides are dipped in tap water
9. Slides are then counterstained in 0.1% Fast Green FCF for 5 minutes
10. Slides are then cleared using 1% glacial acetic acid for 30 seconds.
11. Incubate slides at room temperature in 0.1% Safranin O for 15-20 minutes.
12. Following incubation slides are washed briefly in distilled water.
13. The slides are then dipped into 90% ethanol for 30 seconds.
14. Next the slides are dipped in a trough of fresh 100% ethanol for 30 seconds.
15. Slides are then dipped into a second trough of 100% ethanol for 1 minute.
16. The slides are then dipped in a trough of xylene for 10 minutes.
17. Slides are then dipped in a second trough of xylene for another 10 minutes.
18. A drop of DPX mountant is then placed onto the fixed and stained sample and a coverslip carefully lowered onto it.
19. The slide is left on a level surface and the mountant allowed to fill out under the coverslip. Any bubbles present are forced out from under the coverslip with gentle pressure.
20. Allow the slides to dry out on a level surface.
21. Once dry, the slides are viewed and imaged using a microscope.