Advancing cartilage cell therapy through chondrocyte culture and patient reported outcome measures (PROMs)

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Abstract

Autologous chondrocyte implantation (ACI) is the only UK approved cell therapy for knee cartilage defects which might otherwise progress to osteoarthritis. For ACI, chondrocytes isolated from a healthy cartilage biopsy are expanded in number prior to their re-implantation into the damaged joint. ACI is cost-effective compared to alternative surgical interventions but it comes with a 50% failure rate at 10 years. Making ACI truly successful requires optimisation. Possible enhancements include improvement of the chondrogenic capacity of chondrocytes and better monitoring of the clinical outcomes.

Herein, we histologically, metabolically and biochemically compared chondrocytes sourced from the ankle and knee. We found that chondrocytes sourced from ankle cartilage exhibited higher chondrogenic potential metabolically and histologically, irrespective of external stimuli.

In separate experiments, we characterised the extracellular matrix (ECM) of chondrocytes cultured in Stemulate™ to determine whether it could be used to improve upon the existing ACI protocol. While Stemulate™ was effective at increasing chondrocyte monolayer proliferation, it impacted upon the re-differentiation potential.

To assess clinical outcome of ankle surgery and ACI we developed a brand new patient-reported outcome measure and performed validation analyses to screen a cohort of 206 participants. It demonstrated proven reliability, responsiveness and good correlation with existing scores. In clinic, our foot and ankle clinicians are using it to monitor clinical outcomes.

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Abbreviations

$2^{-\Delta CT}$	Comparative $_{CT}$ method
ACAN	Aggrecan gene
ACI	Autologous chondrocyte implantation
ALK1	Activin A receptor type II-like 1
CI	Confidence interval
COL1A1	Collagen type I, alpha 1
COL2A1	Collagen type II, alpha 1
COL10A1	Collagen type X, alpha 1
COSMIN	Consensus-based standards for the selection of health measurement instruments
C_T	Cycle threshold
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	1,9-dimethylmethylene blue
ECM	Extracellular matrix
FBS	Foetal bovine serum
GAGs	Glycosaminoglycans
H&E	Haematoxylin and Eosin
HPL	Human platelet lysate
ICC	Intraclass correlation coefficient
MACI	Matrix induced autologous chondrocyte implantation
MCS	Mental component score
MOxFQ	Manchester Oxford Foot Questionnaire
MSCs	Mesenchymal Stem Cells
OsAS	Oswestry Ankle Score
PBS	Phosphate buffered saline
PCS	Physical component score
PDs	Absolute population doublings
PDT	Population doubling time
PROMs	Patient-reported outcome measures
RJAH	The Robert Jones & Agnes Hunt Orthopaedic Hospital
SEM	Standard error of mean
sGAGs	Sulphated glycosaminoglycans
SOX9	SRY-related gene
SPSS	Statistical Package for the Social Sciences
TGF-β1	Transforming growth factor-β1
VR-12	Veteran Rand 12-Questions

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- Turbo talk presentation, postgraduate symposium. Keele, UK. May 2017
- Research day. The Robert Jones & Agnes Hunt Hospital, Oswestry, UK. April 2017

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- Osteoarthritis Research Society International. Liverpool, UK. April 2018
- Postgraduate Symposium. Keele, UK. May 2017
- Research day. The Robert Jones & Agnes Hunt Hospital, Oswestry, UK. April 2017
- Postgraduate Symposium. Keele, UK. May 2016

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- Future Investigators of Regenerative Medicine. Girona, Spain. September 2018
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Chapter 1: General introduction

Cartilage is a tough and smooth elastic tissue which has many roles within the body. Essentially, it protects the ends of long bones within synovial joints to provide frictionless, pain-free movement (Anderson, 1962). It is also a major component within several structures including the knee joint, the ear, the nose, the bronchial tubes and the intervertebral discs. There are three different types of cartilage in the human body: elastic cartilage, fibrocartilage and hyaline cartilage (Wheeless, 2016), as shown in Figure 1.

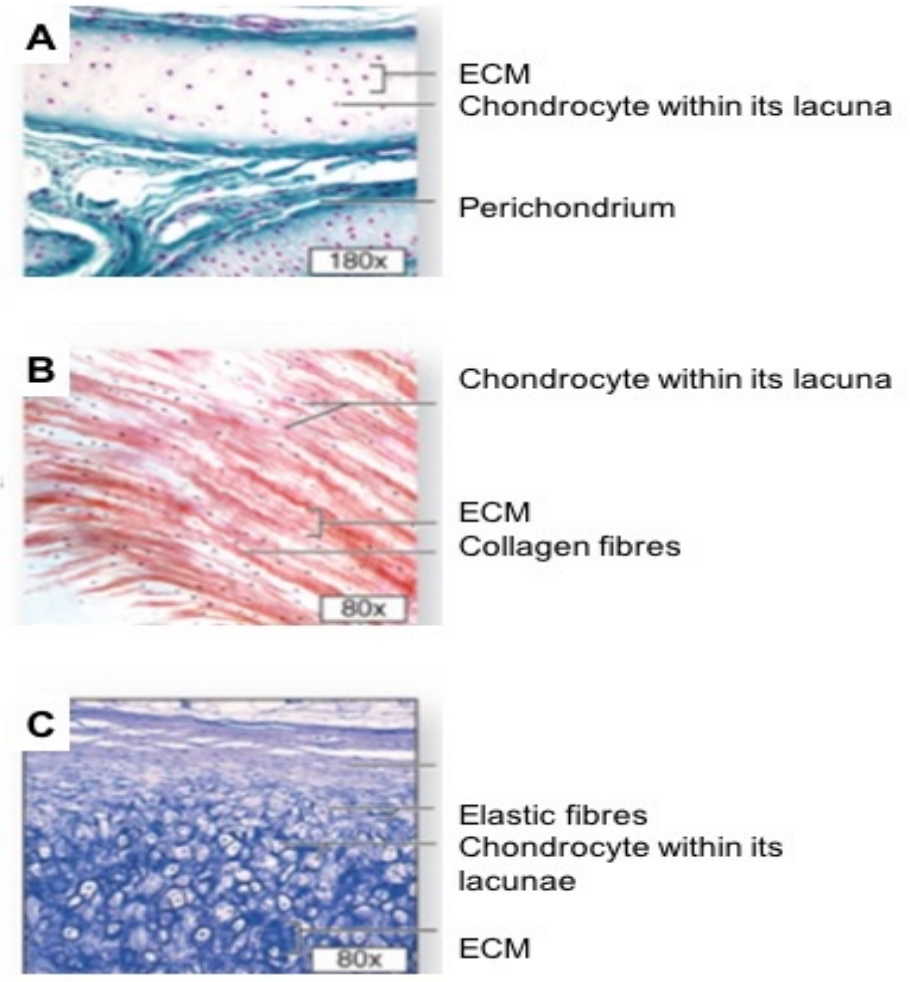


Figure 1: Histology images illustrating the three types of cartilage present in human tissue, showing (A) hyaline cartilage, (B) fibrocartilage and (C) elastic cartilage. Images taken from Junqueira's Basic Histology; Mescher, 2018.

All three types of cartilage consist of extracellular matrix (ECM), water and specialised cells called chondrocytes. Chondrocytes synthesise and organise collagens, proteoglycans, glycosaminoglycans (GAGs) and non-collagenous proteins to form the ECM. Within each cartilage type, these components differ in their amount and arrangement whilst the chondrocytes display different morphology, behaviour and metabolic activities. Together, these variations endow each type of cartilage with its unique biological and mechanical properties.

1.1. The function, composition and organisation of articular cartilage

Within synovial joints, there is a specialised form of hyaline cartilage, called hyaline articular cartilage. It functions as a shock absorber and reduces contact stress on the underlying subchondral bone by distributing load and enabling pain free movement (Buckwalter and Mankin, 1998). At its surface, healthy articular cartilage is smooth whilst through the depth of the cartilage it is avascular, aneural and alymphatic (James and Uhl, 2001)**.** Despite being aneural, sensations of pain can still occur following damage to the underlying cartilage. However, cartilage damage can still occur without pain sensations. With increasing depth from the articular surface, there are at least four architectural zones with striking variations in their chondrocyte arrangement, mechanical properties and surrounding ECM (Poole et al., 2001; Wilusz et al., 2014).

1.1.1 The extracellular matrix (ECM) within articular cartilage

Water and electrolytes lubricate the cartilage tissue and transport nutrients to the chondrocytes. The interaction between the water and the macromolecules within the ECM provide the tissue with its rigidity and resilience (Woo and Buckwalter, 1988). Despite the volume of water remaining constant in cartilage, it differs throughout the tissue and decreases from the articular surface to the subchondral bone based on the interaction with the macromolecules present in the ECM (Mow et al., 1992; Mankin et al., 2000; Buckwalter et al., 2005).

1.1.1.1 Collagens

Collagens provide the cartilage tissue with its tensile strength. There are 28 different types of collagen present in all vertebrates (Gelse et al., 2003; Luo et al., 2017; Responte et al., 2007). All collagen types contain three polypeptide strands in a left-handed helical arrangement, which bind together to form a triple helix structure stabilised through hydrogen bonds (Gelse et al., 2003). Collagens typically contain a high hydroxyproline content, of approximately 100 residues per 1,000 amino acid residues (Brown et al., 2001; Dickson and Bagga, 1985). Collagens can either be fibrillar or non-fibrillar depending on their repeat amino acid structure. Fibrillar collagens have non-interrupted repeat amino acid structures, whereas non-fibrillar collagens have interrupted repeat amino acid structures. Fibrillar collagens are present in almost all animals, including humans, and consist of types: I, II, III, V, XI, XXIV and XXVII, all of which are made up of 997-1,020 residues (Boot-Handford and Tuckwell, 2003; Exposito et al., 2002; Kadler et al., 2007). Whereas, non-fibrillar collagens consist of types: IV, VI, VII,

VIII, IX, X, XII, XIII and XIV (Luo et al., 2017; von der Mark et al., 1992; Responte et al., 2007; Van Der Rest and Mayne, 1988).

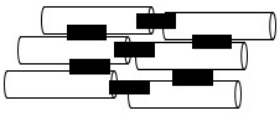
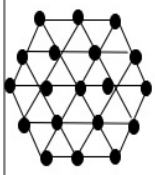
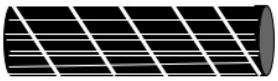
Collagen type	Structure / Diagram	Location	Function
Collagen II		Throughout articular cartilage	Form cross-banded fibrils to create fibril meshwork Maintains tissue rigidity and tensile strength Traps proteoglycans to increase water retention
Collagen VI		Pericellular matrix (PCM)	Anchors chondrocyte to PCM Mediates cell-matrix interactions
Collagen IX		Growth plate cartilage Adult articular cartilage	Maintains chondrocyte integrity Forms collagen network
Collagen X		Hypertrophic zone of the growth plate and basal calcified zone of articular cartilage	Hallmark of hypertrophy Facilitates normal distribution of proteoglycans Regulates chondrocyte metabolism
Collagen XI		Within/on macrofibrils	Regulates cartilage formation

Figure 2: The main types of collagen in articular cartilage, illustrating the structure, location and function. Figure was created using information derived from. Plumb et al., 2011.

Articular cartilage contains collagen types II, VI, IX, X and XI, as demonstrated in Figure 2. Each collagen type plays a specific role through the depth of the tissue (Luo et al., 2017; Mankin et al., 2000; Responte et al., 2007).

Collagen I is the main component of bone tissue but is found in articular cartilage. It has a role in structure and support, but has been shown to be present in fibrosis (Caron et al., 2012). There are two key genes of collagen type I, which are collagen type 1 alpha 1 and collagen type 1 alpha 2. Type one

produces pro-alpha-I chains, whereas type two produces pro-alpha-II chains. Two pro-alpha-I chains combine with one pro-alpha-II chain to create a type I procollagen molecule which combine to produce collagen I fibrils (Henriksen and Karsdal, 2016). Type one is the more abundant gene in collagen I and is used as a key gene marker for assessing chondrocyte de-differentiation; the process in which chondrocytes alter their phenotype to a more fibroblastic state following exposure to tissue culture plastic during monolayer expansion and is characterised by a reduced ECM quality with higher expression of collagen type I and a lower expression of collagen type II (Lin et al., 2008; Schulze-Tanzil, 2009; Watt, 1988). Hence, why collagen type 1 alpha 1 is used as a key gene marker to assess chondrocyte dedifferentiation, particularly in qRT-PCR.

Collagen II is the most abundant collagen type in articular cartilage and it is distributed throughout the depth. It has only one gene, namely collagen type II alpha 1, which is considered a key marker of chondrocyte phenotype. This gene produces pro-alpha-II chains and three of these chain combine to create a type II procollagen molecule (Gudman and Karsdal, 2016). The main role of collagen II is to form cross-banded fibrils to create the fibril meshwork. This meshwork provides the tissue with rigidity and high tensile strength, and entraps water binding-proteoglycans to increase water retention in order to hydrate and lubricate the tissue (Rosenburg, *et al.,* 1986; Van Der Rest, 1991)**.** Collagen II fibrils are reported to be thinner closer to the articular surface. They increase in their diameter, from 20 nm to 50-100 nm, as the cartilage tissue matures from foetal to adult cartilage tissue (Lane & Weiss, 1975).

Collagen VI is found exclusively in the pericellular matrix within articular cartilage, where it forms microfibrils in the pericellular matrix that surrounds the chondrocyte to help it attach to the ECM. It is unique in that it undergoes supramolecular assembly to form a beaded microfilament network in the ECM. Collagen VI consists of three alpha chains (α1, α2 and α3) in a 1:1:1 ratio to form a triple helix monomer (Colombatti et al., 1995; Engel et al., 1985). The associated genes are collagen type VI alpha 1, collagen type VI alpha 2, collagen type VI alpha 3 and collagen type VI alpha 5. The overall function of collagen VI is to create the three-dimensional architecture of tissues including: skeletal muscle, tendons, bone and cartilage; yet it does also have associated cytoprotective roles, including: inhibition of apoptosis (Grumati et al., 2010; Irwin et al., 2003), promotion of tumour growth (Park and Scherer, 2012) and maintenance of the cells ability to differentiate (Urciuolo et al., 2013). Collagen VI has also been reported to have a role in the regulation of cell differentiation, further supported in studies performed by Smeriglio in which supplemented soluble collagen VI was found to increase cell proliferation in monolayer culture (Smeriglio et al., 2015; Smeriglio et al., 2017). However, the main function specifically in cartilage is to aid the attachment and integrity of chondrocytes (Cescon et al., 2015). Knockout studies have also demonstrated that collagen VI has associated roles with the transmission of mechanical and osmotic stresses from the ECM to the pericellular matrix, found through decreased pericellular matrix stiffness (Alexopoulos et al., 2009), increased extent of chondrocyte swelling and increased extent of osmosis induced signalling (Zelenski et al., 2015).

Collagen IX is a fibril-associated collagen with interrupted triple helix. fibril-associated collagen with interrupted triple helix collagens do not form fibrils, but rather they decorate the surface of the fibrillar collagens (Luo et al., 2017). Collagen IX is usually found in tissues containing collagen II, including growth plate cartilage and adult articular cartilage (Eyre and Wu, 1987). The collagen IX associated genes are collagen type IX alpha 1, collagen type IX alpha 2 and collagen type IX alpha 3 (He and Karsdal, 2016). Structurally, collagen IX is composed of three helical domains: COL1, COL2 and COL3. COL2 has been identified as containing cross-linked sites where collagen II covalently attaches, to create the collagen fibril meshwork (Van Der Rest & Mayne, 1988). This meshwork is thought to be organised by this collagen type projecting into the ECM and surrounding the surface of collagen II fibrils (Buckwalter et al., 2005; Eyre, 2004; Van Der Rest and Mayne, 1988), and has the role of trapping proteoglycans and resisting their swelling pressure. Functionally collagen IX is speculated to play a role in development and maturation, as studies have shown the collagen quantity decreases from 10% in foetal cartilage tissue to 1% in adult cartilage tissue (Eyre, *et al.,* 1991; Van Der Rest, 1991). It is also speculated to provide mechanical stability and increase the tensile strength of the tissue (Blumbach et al., 2009; Posey et al., 2008).

Collagen XI is a fibril-forming collagen found within and on macrofibrils. It is usually retained at the chondrocyte surface. It has two associated genes: collagen type XI alpha 1 and collagen type XI alpha 2 (Luo and Karsdal, 2016). Structurally, collagen type XI is found primarily cross-linked to each other and is widely distributed in articular cartilage (Mio et al., 2007). This

collagen type accounts for 10% of foetal cartilage but decreases to 3% total collagen in adult articular cartilage (Eyre, 2004). Functionally, it is thought to be associated with stabilising the collagen fibrils that create the fibrillar meshwork. The meshwork increases the tensile strength and therefore accommodates the general 'wear and tear' nature of the tissue (Eyre et al., 1991; Van Der Rest, 1991).

Collagen X is a fibrillar collagen usually only found within the pericellular latticework in the calcified zone of articular cartilage. It is secreted by hypertrophic chondrocytes and found exclusively in hypertrophic cartilage and the calcified zone of articular cartilage (Gannon et al., 1991). Therefore, collagen X expression is increased during periods of hypertrophy and as it has only one associated gene, collagen type X alpha 1, this is frequently used as a hallmark of hypertrophic expression in research studies (Eyre et al., 1991; Gannon et al., 1991; Kwan et al., 1991). Structurally, it is a homotrimeric collagen consisting of three α1 chains. It constitutes approximately 1% of total collagen in adult articular cartilage (Eyre et al., 1991). Functionally, this structure is thought to assist with the role of modifying ECM for subsequent bone formation during ossification (Kwan et al., 1991). In regards to function, several studies have associated this collagen type with cartilage mineralisation and structural support (Poole, *et al.,* 1992; Eyre, 2004; Schmid & Linsenmayer, 1985). Collagen X has also been associated with maintaining tissue stiffness, regulating chondrocyte metabolism (Luckman et al., 2003) and facilitating the normal distribution of proteoglycans within the growth plate (Shen, 2005).

1.1.1.2 Glycosaminoglycans (GAGs)

GAGs are long chains of repeating disaccharides that are negatively charged and variably sulphated (Figure 3; page 10). They are usually associated with a protein core to form a proteoglycan. The variations in GAG chain length, composition and sulphation give proteoglycans their diversity. An ECM without GAGs would be drastically compromised since their biological functions include tissue hydration (Pomin and Mulloy, 2018), resistance to compressive load (Hardingham et al., 1991), intracellular signalling (Hardingham et al., 1991) and protein up-take (Fraser et al., 1993).

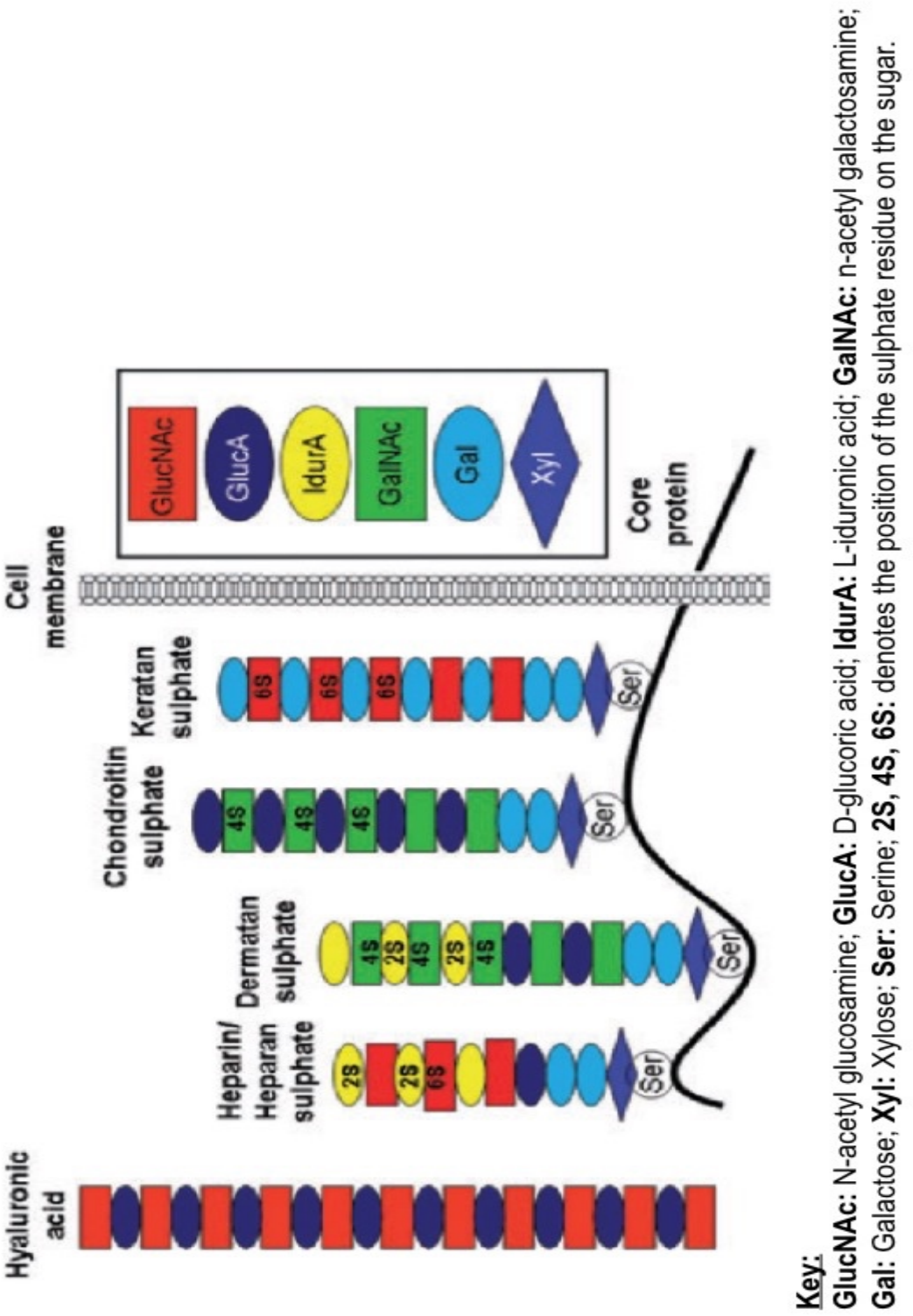


Figure 3: The molecular structure of the five main glycosaminoglycans found in human articular cartilage: hyaluronan, heparan sulphate, dermatan sulphate, chondroitin sulphate and keratan sulphate. Image adapted from Papakonstantinou and Karakiulakis, 2009.

As shown in Figure 3 (page 10), all GAGs are synthesised and covalently bound to core proteins to form proteoglycans, except for hyaluronan. All three types have a similar structure but differ in the sugar that they contain and the way the structure is linked to the core protein (Gandhi and Mancera, 2008).

In articular cartilage, chondroitin sulphate is the predominant GAG and accounts for approximately 90% of the total GAG content. It contains galactosamine as the hexosamine sugar, has a molecular weight of 5-50 KDa and is sulphated (Hardingham & Fosang, 1992; Mankin, *et al.,* 2000).

Keratan sulphate is the most heterogeneous GAG present in articular cartilage. Keratan sulphate also contains galactosamine as the hexane sugar and is sulphated. However, keratan sulphate has a smaller molecular weight of 4-19 KDa. Keratan sulphate chains contain the disaccharide: -3Galβ1-4GlcNAcβ1- and is approximately 8-9 disaccharide units in length (Caterson and Melrose, 2018). There are two types of keratan sulphate found in the human body: type I found in the cornea and type II found in cartilage.

Hyaluronan is un-sulphated and contains the hexane sugar glucosamine. It is found throughout the human body, including in the synovial fluid and loose connective tissue. Hyaluronan differs in size, ranging in molecular weight from 4,000-8,000 KDa (Hardingham and Fosang, 1992)**,** depending on the isoform of the associated hyaluronan synthases (HAS) gene. There are three hyaluronan synthases genes: hyaluronan synthases 1, hyaluronan synthases 2 and hyaluronan synthases 3, each varies in stability and the rate of HA synthesis (Itano et al., 1999). Hyaluronan synthases1 is expressed in early development and has been reported to synthesise the hyaluronan polymers of $2x10^5 – 2x10^6$

Da. Whereas the hyaluronan synthases 2 gene has associated expression with all stages of embryogenesis and synthesises the largest hyaluronan polymers of $2x10^6$ Da. Moreover, the hyaluronan synthases 3 gene is associated with late development and has been shown to synthesise the shortest hyaluronan polymers of $1x10^5$ – $1x10^6$ Da (Cyphert et al., 2015; Tien and Spicer, 2005).

Dermatan sulphate is sulphated and contains the hexane sugar galactosamine. It has associated roles in binding to proteoglycans, assisting the function of growth factors, and modifies cofactors to influence proliferation. It has also been observed that dermatan sulphate is found to circulate in the blood until needed. It is found mainly in the skin but is also found within bone, cartilage and blood vessels (Pomin and Mulloy, 2018).

Heparan sulphate is a sulphated GAG present on almost every cell within the human body. It contains the hexane sugar galactosamine and is usually a molecular weight of $0.005\text{-}0.07x10^6$ Da. It has associated roles in regulating the activity of chemokines, growth factors and enzymes. A further role it has is in assisting with cell matrix interactions involving connecting to ECM proteins such as fibronectin, laminin and various collagens, including: collagens I, II, IV and VI (Gandhi and Mancera, 2008; Papakonstantinou and Karakiulakis, 2009; Pomin and Mulloy, 2018).

1.1.1.3 Proteoglycans

Proteoglycans are defined as complex carbohydrates and are present in many tissues, including the brain, (Schwartz and Domowicz, 2004) cartilage (Roughley, 2006) and skin (Smith and Melrose, 2015). In articular cartilage, proteoglycans

are synthesised by chondrocytes. They function to retain water, facilitated by Donnan equilibrium, to allow the tissue to withstand mechanical stresses that occur to the joint (Maroudas, 1973; Maroudas, 1976; Overbeek, 1956). Proteoglycans have a fixed charge density to promotes osmosis. The osmotic pressure within the cell increases, relative to outside the cells, which encourages water to flood into the cell and results in the intracellular matrix swelling and becoming pressurised to balance the Donnan equilibrium and osmotic pressure. The collagen meshwork resists the swelling, which allows the proteoglycan complex in the ECM to remain a highly concentrated gel.

Aggrecan

The proteoglycan which is specific to articular cartilage is aggrecan (Figure 4; page 14). It accounts for approximately 90% of the total mass in articular cartilage. It has a 'bottle-brush' like structure made up of a combination of approximately 150 chondroitin sulphate and keratan sulphate chains, usually in a 2:1 ratio. The main function of aggrecan is to bind to hyaluronan using the small glycoprotein, link protein, to create large proteoglycan aggregates (Huber, *et al.,* 2000; Horkay, *et al.,* 2012). Aggregation allows the proteoglycan to increase in size and the aggregated GAG chains allow the proteoglycan to exhibit a high anionic charge, both of which are essential for normal function of both proteoglycan aggregates and articular cartilage.

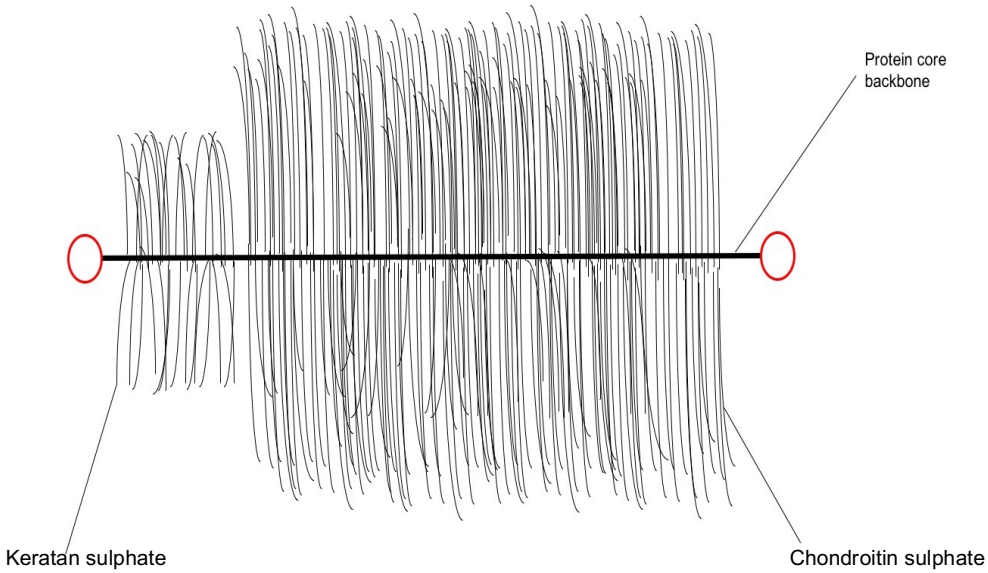


Figure 4: Schematic of the bottlebrush-like structure of aggrecan, demonstrating the central protein core, chondroitin sulphate domain made up of many chondroitin sulphate chains and the keratan sulphate domain made up of many keratan sulphate chains.

The chain ratio and size of aggrecan has been shown to vary with age, disease and during development (Hardingham & Fosang, 1992). The content and composition of aggrecan is largely related to the state of the articular cartilage, since synthetic and degradative changes occur during ageing and disease. These changes are a consequence of the long resident time of aggrecan within the ECM, but has also been speculated to be due to an altered anabolic response of chondrocytes (Bayliss et al., 1999; Maroudas et al., 1998). Degradation causes cleavage of all components of proteoglycan aggregates, leading to loss of proteoglycan aggregate function and ultimately loss of cartilage function.

Perlecan

Perlecan is a large sized proteoglycan, consisting of chondroitin sulphate and heparan sulphate chains (Farach-Carson et al., 2014). It consists of a core protein of 4,000 amino acid residues and heparan sulphate side chains (Farach-Carson and Carson, 2007). The chondroitin sulphate chains specific to perlecan, have been reported to accelerate and catalyse collagen fibre formation (Kvist et al., 2006). It has a major role in development, as shown in mouse models with a deletion of perlecan gene expression demonstrating severe skeletal defects specifically with abnormal growth plates (Farach-Carson and Carson, 2007; Melrose et al., 2004).

Small leucine-rich proteoglycans

Small leucine-rich proteoglycans account for approximately 3% of the total proteoglycan mass in articular cartilage and have the role of further stabilising the ECM. The Small leucine-rich proteoglycans in cartilage tissue are decorin and biglycan (Huber et al., 2000).

Decorin is a stromal small leucine-rich proteoglycan of 90-140 KDa. Structurally, it consists of a protein core with 10 leucine-rich repeat sequences and a GAG chain consisting of either chondroitin sulphate or dermatan sulphate (Boskey and Robey, 2013). Decorin is considered a cellular or pericellular proteoglycan where it's roles include influencing fibrillogenesis, binding to collagen type I fibres (Bock et al., 2001), cell adhesion and cell proliferation (Iozzo, 1999; Neill et al., 2012; Scott, 1991). Furthermore, studies have shown pivotal involvement in inhibiting angiogenesis, to maintain the avascular nature

of the tissue retaining the biomechanical proteins of the cartilage tissue (Mohan et al., 2011; Neill et al., 2012).

Biglycan is comprised of a protein core, leucine-rich repeat regions and two GAG chains, consisting of either chondroitin sulphate or dermatan sulphate which are attached at amino acids 5 and 10 in humans (Bock et al., 2001). It is expressed on either the cell surface, pericellularly or within the ECM of various tissues. One associated role of biglycan is in mineralisation of bone, with knockout studies in mice demonstrating an osteoporotic-like phenotype (Chen et al., 2002). Biglycan also exhibits a strong affinity to collagen type II (Bock et al., 2001). More recent studies have proven its role in the regulation of inflammation (Barreto et al., 2015) and as a modulator of morphogenesis and differentiation (Hunziker, 1992).

1.1.1.4 Non-collagenous proteins

There are many non-collagenous proteins found within articular cartilage, all of which are highly variable in structure and function with some only being present in specific cartilage zones, or during periods of development or ageing. Despite the high variability, many have been shown to hold a structural role in articular cartilage (Heinegard and Oldberg, 1989; Roughley, 2001). Several non-collagenous proteins have undergone high levels of research and have been described in more detail regarding their function and structure within articular cartilage.

Link protein is a non-collagenous glycoprotein that binds the proteoglycan molecules to hyaluronate chains. There are two main link proteins

found in human articular cartilage, link 1 and link 2. In humans, these usually consist in a 1:1 ratio, both in normal and osteoarthritic human cartilage. Link 1 has an approximate molecular weight of 45-54,000 kDA whereas link 2 has an approximate molecular weight of 40-48,000 kDA (Junnoseke et al., 1982). The associated role of link protein is to increase the strength of proteoglycan binding and thus increase stability.

Fibronectin is one of the most abundant non-collagenous proteins within the ECM. It is involved in the ECM assembly process, specifically in directing the organisation of the ECM, with key roles such as mediating cell attachment, cell aggregation (To and Midwood, 2011), and stimulating cell migration during embryonic development (Hsiao et al., 2017; Moretti et al., 2007).

Laminin has two isoforms associated with the ECM; laminin-III is secreted by chondrocytes and found in articular cartilage, whereas laminin-332 is found in embryonic cartilage (Kruegel and Miosge, 2010). There is increased expression of laminin-332 in new-born cartilage compared to mature cartilage, but it has a pericellular distribution in mature cartilage (Durr et al., 1996; Foldager et al., 2016). Laminin has also been reported to have a role in promoting chondrogenesis (Schminke et al., 2016). Studies have shown that laminin forms part of the pericellular matrix in native cartilage, but is absent in repair or degenerated tissue or in fibrocartilage (Foldager et al., 2014; Foldager et al., 2016; Kruegel and Miosge, 2010), which supports the suggestion of laminin having a role in promoting chondrogenesis (Schminke et al., 2016). Expression of laminin is also depleted in osteoarthritis, which suggests that it is associated with the homeostasis of normal articular cartilage.

Tenascin-C is a cell-interaction protein known for its adhesive and anti-adhesive interactions. Tenascin-C is not normally expressed in healthy adult tissue but has upregulated expression during inflammation and is highly expressed during wound healing, where it can promote cell migration (Chiquet-Ehrismann and Chiquet, 2003; Roughley, 2001; Yamada, 1991).

Cartilage oligomeric matrix protein is a member of the thrombospondin family and is found in all human cartilage tissue and is therefore speculated to have a major role in cartilage development and turnover (Bornstein et al., 1993). Cartilage oligomeric matrix protein is reported to cause degradation, producing degraded fragments which circulate within the synovial fluid. Higher levels of these circulating fragments are associated with early stages of primary osteoarthritis (Lohmander et al., 1994).

1.1.2. Arrangement of the ECM

1.1.2.1 The chondrocyte

Chondrocytes are the only cells present in cartilage tissue and are in sparse content, accounting for approximately 3% of the total tissue volume (James and Uhl, 2001). The average chondrocyte is spherical in shape and approximately 13 µm in size (Kempson, 1979) as shown in Figure 5 (page 19). This low cellularity of articular cartilage contributes to the limited capability of cell-to-cell communication and self-repair which will be introduced later.

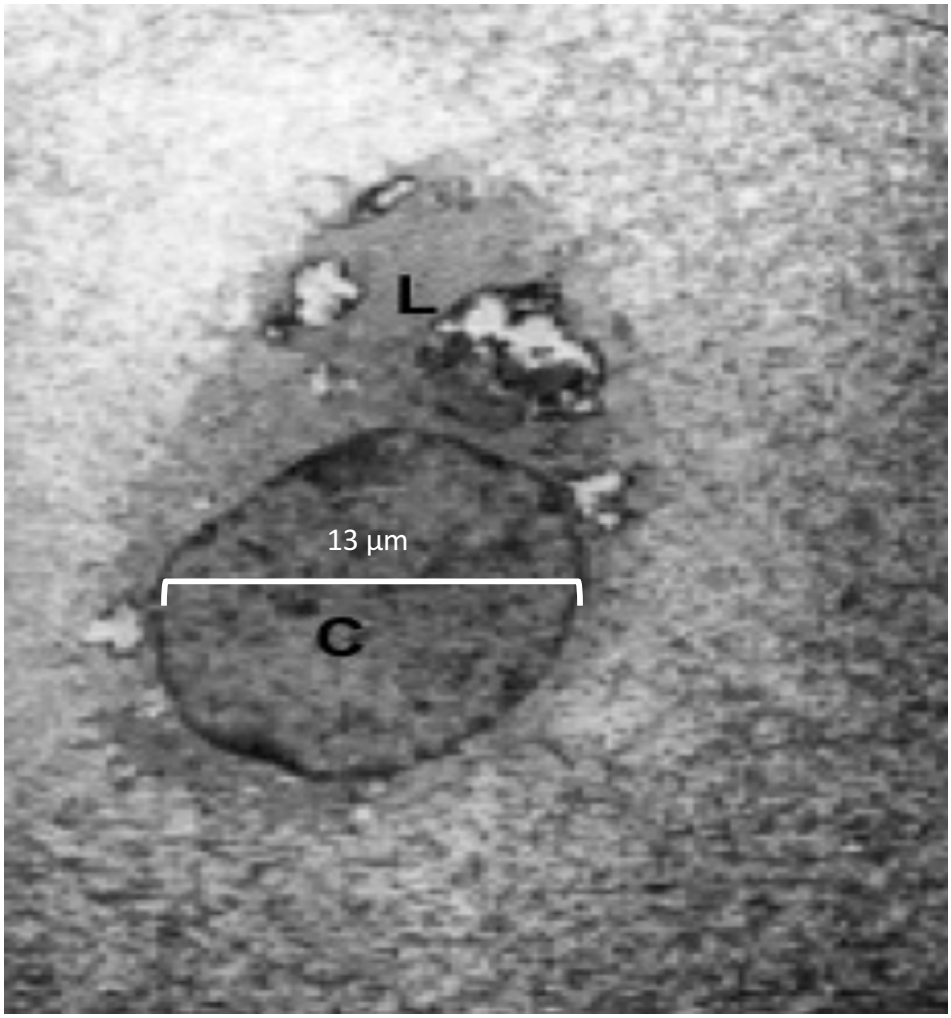


Figure 5: Electron microscopy image of a chondrocyte (C) within its lacunae (L), in articular cartilage. Image taken from Patwari et al., 2004.

1.1.2.2 Regional organisation of the ECM

There is a further hierarchical organisation within articular cartilage associated with the ECM (Figure 6; page 21), which consists of three distinct matrix regions: the pericellular matrix, the territorial matrix and the inter-territorial matrix.

A chondrocyte surrounded a pericellular matrix is termed a 'chondron', and was first described in 1925 (Benninghoff, 1925). The chondron is considered the structural, functional and metabolic unit of human articular cartilage (Poole, et al., 1992; Zhang, 2015). The pericellular matrix region is a thin

layer of approximately 1-3 µm that immediately surrounds the chondrocyte (Zhang 2015; Wilusz, *et al.,* 2014). The pericellular matrix is rich is non-sulphated GAGs, proteoglycans and collagens (Wang et al., 2008). It contains fibronectin, aggrecan, collagens type II, IX and XI and is the exclusive location of collagen VI (Wang et al., 2008; Wotton et al., 1991). It provides a microenvironment to allow the chondrocytes to anchor and attach to the ECM, as well as mediating communication between the chondrocytes and ECM. Another function of the pericellular matrix is to provide a boundary for components to penetrate before reaching the cell membrane, offering extra protection to the chondrocytes (Zhang, 2015; Wilusz, *et al.*, 2014). It is also speculated that the pericellular matrix has a further role of initiating signal transduction within cartilage for load bearing (Eggli *et al.*, 1985).

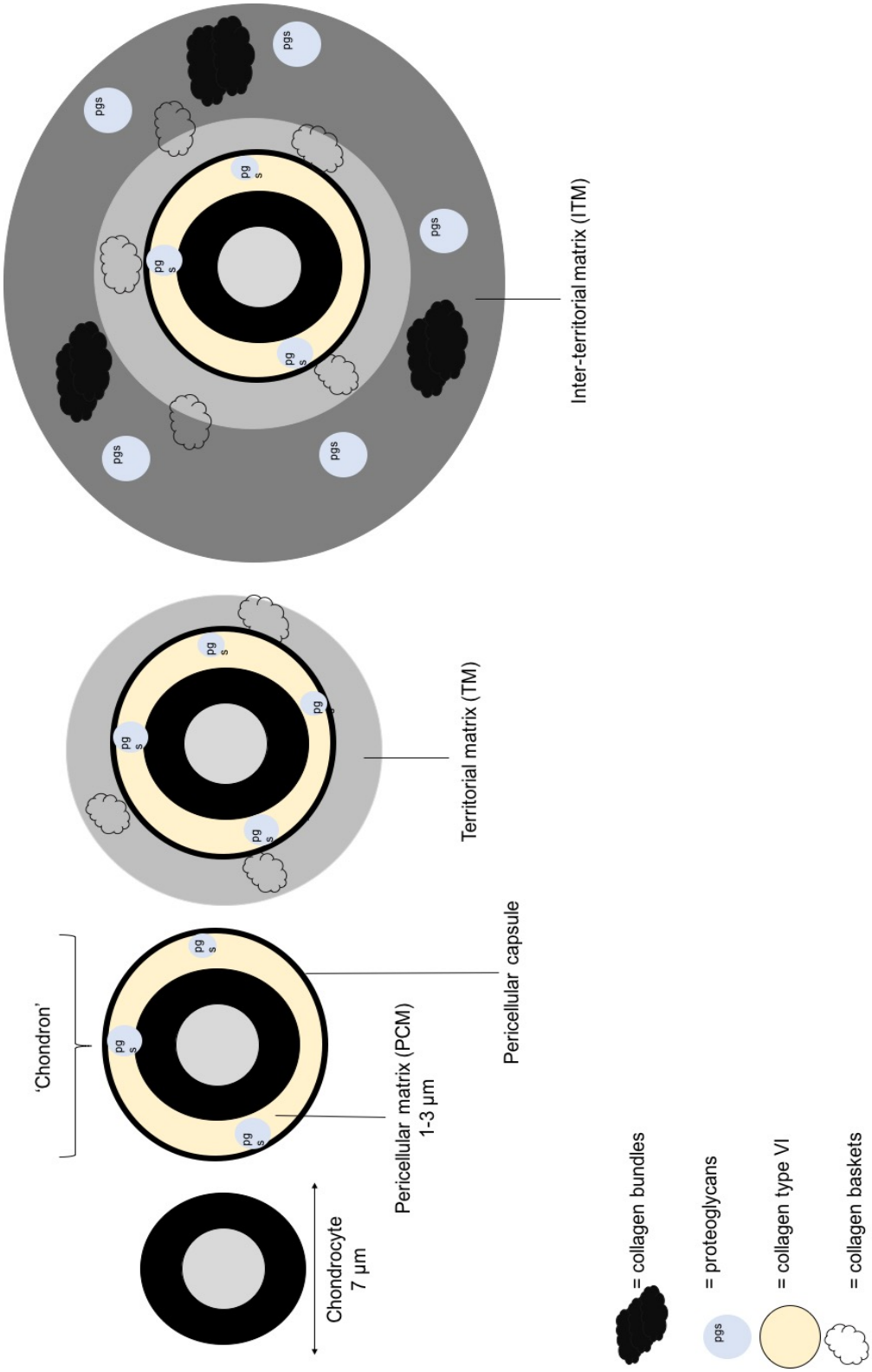


Figure 6: Schematic illustrating the three distinct regions in the organisation of the chondrocyte: the pericellular matrix, the territorial matrix and the inter-territorial matrix.

Beyond the pericellular matrix there is the territorial matrix, which surrounds individual, and isogenous chondrocytes. This region is thicker than the pericellular matrix region and has the primary role of protecting chondrocytes from mechanical stress (Poole, 1997). The territorial matrix is distinctive due to its hallmark fine collagen type IX and XI fibrils that organise themselves into 'basket-like' networks. However, some collagen fibrils do protrude into the **inter-territorial matrix**, connecting the two regions together for further structural support (Poole, 1997).

The inter-territorial matrix is situated furthest from the chondrocyte. Collagen type IX and XI fibrils within the inter-territorial matrix are organised into random bundles orientated differently depending on their location within this region (Eyre et al., 2006; Mankin et al., 2000; Poole et al., 2001). They are parallel to the surface of the superficial zone, oblique in the middle zone and perpendicular to the articular joint surface in the deep zone (Buckwalter *et al.*, 2005; Poole, 1997). The inter-territorial matrix region contributes highly to the biomechanical properties of the cartilage tissue, possibly through the abundant content of proteoglycans (Mow and Guo, 2002).

1.1.2.3 Zonal arrangement of the ECM

Articular cartilage is composed of four basic biological zones, which from the articular surface to the underlying subchondral bone comprise: the superficial, middle, deep and calcified zones (Huber, *et al.,* 2000). Each zone contributes to the biochemical properties of the cartilage tissue, including its high tensile strength and durability. The chondrocytes and their surrounding ECM differ within the architectural zones (Figure 7) (Huber, *et al.,* 2000).

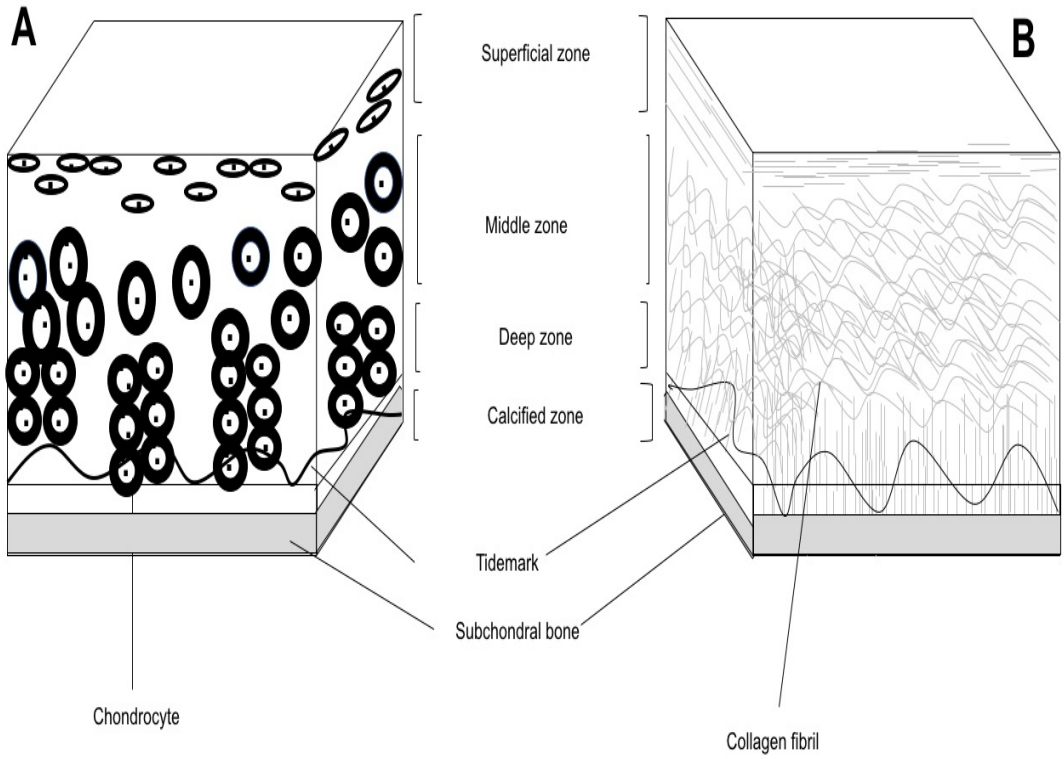


Figure 7: Representative diagram demonstrating (A) the organisation of chondrocytes and (B) the collagen fibril arrangement throughout the four zones in human articular cartilage. Diagram adapted from Fox et al., 2009.

Closest to the articular surface is the superficial zone, which accounts for 10-20% of the tissue thickness. It is the thinnest of the four zones. The function of the superficial zone is to protect the deeper zones from sheer stress

and forces, and to aid this there is a high content of collagen type II (Temenoff & Mikos, 2000; Buckwalter *et al.*, 2005). Collagens types II and IX are organised into a tight acellular sheet arranged parallel to the articular surface, and due to this arrangement, the chondrocytes are flattened in shape. There is less aggrecan content, with the most abundant SLRP in this zone being decorin (Temenoff & Mikos, 2000; Buckwalter *et al.*, 2005).

The middle zone occupies the most volume, accounting for approximately 40-60% of the total tissue thickness. The function of this zone is to protect the deeper zones from compressive forces. To enable this, the collagen type II fibrils are present at a low density but are thicker in size when compared to those found in the other zones and they are obliquely organised (Buckwalter, *et al.,* 2005; Wheeless, 2016). This zone has spherical chondrocytes, a low water content and high aggrecan content (Wheeless, C.R., 2012; Temenoff & Mikos, 2000; Buckwalter *et al.*, 2005).

The deep zone is the largest zone and accounts for 30% of the total tissue volume. The primary function of this zone is to provide the greatest resistance to compressive forces, protecting the underlying subchondral bone (Buckwalter et al., 2005; Temenoff and Mikos, 2000). The collagen fibrils here have the largest diameter and are orientated parallel to the articular surface. The fibres can extend into the tidemark, which is a basophilic line of unknown composition and function that separates the deep and calcified zones (Temenoff and Mikos, 2000; Wheeless, 2016). Here, the chondrocytes have high synthetic activity, a rounded morphology and are stacked in columns perpendicular to the articular surface and parallel to the collagen fibrils. The deep zone contains the lowest water

content, yet highest proteoglycan content, which is particularly high in aggrecan and perlecan (Buckwalter et al., 2005; Temenoff and Mikos, 2000).

The calcified zone is situated closest to the subchondral bone and provides the transition between cartilage and bone tissue. The function of the calcified zone is to secure the cartilage to the subchondral bone with collagen type X fibrils radiating through the tidemark up into the deep zone. The chondrocytes in the calcified zone are sparse in density, smaller in diameter and are hypertrophic in nature, meaning the cells become larger and thus increase the size of the tissue (Buckwalter and Mankin, 1998; Wheeless, 2016). Chondrocytes are encased in a calcified ECM that enables very little movement or cellular metabolic activity (Buckwalter & Mankin, 1998).

1.2 Osteoarthritis

Damaged articular cartilage has a limited potential for healing and untreated cartilage defects often progress to osteoarthritis (Bhosale and Richardson, 2008). Osteoarthritis is a degenerative joint disease characterised by the loss of articular cartilage and changes within the subchondral bone (Gardiner et al., 2015; Sharma et al., 2013).

Within the UK, osteoarthritis is a major healthcare concern with approximately 8.75 million people aged 45 years or older requiring treatment (Arthritis Research UK, 2013), and it has been reported that 1 million adults consult their GP with symptoms of osteoarthritis every year (Chen et al., 2012; Warner and Valdes, 2016).

There are several risk factors found to be associated with osteoarthritis, including: age, previous injury, gender, body mass index and genetics. Ageing is the most prominent risk factor and studies have shown that osteoarthritis prevalence significantly increases with age, with 40% of adults over the age of 65 years having some form of osteoarthritis (Hügle et al., 2012; Shane, Anderson and Loeser, 2010). Injury to the joint results in joint instability, altered joint biomechanics and altered joint loading, all of which can progress to osteoarthritis. Obesity is defined as a body mass index of 30 kg/m^2 or greater (King et al., 2013). The relationship between osteoarthritis and body mass index is mainly caused by the mechanical overloading of the joint. This risk factor can be decreased through reducing weight by means of a healthy diet and exercise. However, body mass index can also increase following exercise due to muscle mass, as can joint loading. Both are considered risk factors for osteoarthritis but exercise is considered a preventative measure. Females have been proven to be more at risk of developing osteoarthritis than males (Felson, 1990). This is speculated to be due to females having a higher average body fat content compared to males. Furthermore, there is a reported genetic association with osteoarthritis, in particular with nodular osteoarthritis (Fernández-Moreno et al., 2008).

1.2.1 Cartilage injury and natural healing

As mentioned previously, cartilage has a limited capability for self-repair, in part due to its aneural and avascular nature. It has long been accepted that 'cartilage injury is a troublesome thing and once injured is seldom repaired' (Hunter,

1742). Despite cartilage being aneural, sensations of pain can still occur following damage. These pain sensations mainly occur from the underlying bone interactions following the 'wear-and-tear' of cartilage through injury and aging.

There are many types of cartilage injury, including: general wear and tear and physical trauma, both of which may lead to cartilage defects. Cartilage defects can either be of full thickness or partial thickness. Full thickness defects are characterised by progressing through the cartilage tissue and penetrating the underlying subchondral bone, where access to cells in the bone marrow space allows for an immediate repair response (Shapiro et al., 1993). However, the tissue generated tends to be fibrocartilaginous in nature which does not possess the same biomechanical properties of hyaline articular cartilage (Mankin, 1982; Redman et al., 2005). In contrast, partial thickness defects are defined as existing only within the cartilage tissue above the calcified zone and not penetrating into the subchondral bone and thus do not possess the capability of healing spontaneously (Mankin, 1982; Redman et al., 2005). Any repair response that does occur is stimulated by injury to the adjacent tissue and has been shown to promote cell proliferation, chondrocyte cluster formation and ECM synthesis. However, the repair response is often short-lived and the defect fails to repair (Mankin, 1982).

Cartilage repair or regeneration is notoriously difficult but there are several surgical approaches with differing degrees of clinical success which are discussed in the next section.

1.2.1.1 Cartilage repair surgery

Repair strategies use the principle of smoothing the cartilage surface to reduce friction and pain. Regeneration strategies use the principle that 'stimulated bleeding leads to healing'. Whereas, replacement strategies either replaces the whole joint when necessary or replaces cartilage tissue where applicable, such as when filling a defect. These strategies can often be used in combination to generate a more stable repair and more successful clinical outcome.

Cartilage repair surgery was previously reserved for older patients or more severe cases of cartilage injury, such as with larger defect sizes. This was due to the belief that cartilage cannot repair itself (Hunter, 1742) and that attempted repair would not generate articular cartilage tissue. Therefore, surgical repair was avoided where possible. Since then, a plethora of studies have recorded repair tissue in a three-dimensional microenvironment maintaining the articular cartilage phenotype (Dell'Accio et al., 2001; Dell'Accio et al., 2003; Schulze-Tanzil et al., 2002; Wu et al., 2009). More current techniques aim to prevent further degeneration stemming from increasing knowledge and emerging technology. These more recent techniques seem to follow a 'regenerate and repair over replace' ideology with many progressing away from techniques of marrow stimulation or whole joint replacement. Each cartilage surgical repair technique is explained in more detail within Table 1.

Table 1: Repair techniques used to repair cartilage defects.

Technique:	**Defect size:**	**Defect thickness:**	**Process:**
Microfracture	< 15 mm	Partial thickness	Multiple holes in the surface of the subchondral bone to generate a blood supply and the migration of cells, to stimulate a healing response
Osteotomy	N/A	Partial thickness	Reshape the bone to relieve pressure on the affected joint
Soft tissue grafts	< 4 mm	Partial thickness	Implant grafts of perichondrium and periosteum into the defect area
Osteochondral transplant	Autograft < 1cm Allograft > 2cm	Full thickness	Implant of non-weight bearing cartilage and subchondral bone into the defect site
Cell therapy	> 2cm	Full thickness	Transplant cultured chondrocytes into the cartilage defect, to fill the defect area
Total joint replacement	N/A	Full thickness	Replace the defected joint by an artificial joint

Microfracture uses the process of creating multiple holes, approximately 2-3 mm apart, in the surface of the subchondral bone to create a new blood supply that subsequently causes a migration of cells and stimulates a healing response (Redman *et al.*, 2005; American Academy of Orthopaedic Surgeons, 2009). One key factor in the success of microfracture is the rehabilitation programme associated with the technique, which insists on early mobilisation of the area and continuous passive motions with reduced weight bearing for an extended period of time (Smith *et al.*, 2005). The technique has been particularly successful in professional athletes, with one study showing that 76% of American football players returned to the sport the next season following microfracture of

the knee, which may not have been possible with an alternative procedure (Steadman *et al.*, 2003). Furthermore, a study of 105 patients with ankle defects treated by microfracture showed a high success rate in lesions smaller than 15 mm (Chuckpaiwong *et al.,* 2008). The technique is usually reserved for partial thickness defects of less than 15 mm in size.

Osteotomy has the aim to cut and reshape the bone to relieve pressure from the damaged joint, subsequently relieving pain and improving joint function (American Academy of Orthopaedic Surgeons, 2011). This strategy is used to transfer weight and loading from the damaged area to a non-damaged area, correct poor alignment and extend the lifespan of the natural joint, thus prolonging the need for total joint replacement surgery. The ideal candidate for an osteotomy is active, between 40-60 years of age and experiences pain on only one side of their joint (American Academy of Orthopaedic Surgeons, 2011). Currently, osteotomy surgery is less commonly used due to the improvements associated with total joint replacement surgery and the lifespan of artificial joints. Therefore, osteotomy surgery is more commonly reserved for early asymmetric osteoarthritis (Smith *et al.*, 2005). However, osteotomy has shown great success in decreasing pain, as reported in a study of 35 patients with ankle osteoarthritis. This study demonstrated an overall average decrease in pain score from seven prior to surgery to three post-surgery, with a further ten patients presenting as pain free at their follow up appointment (Pagenstert *et al.,* 2007). Furthermore, a study of 95 patients with osteoarthritis in the knee demonstrated that osteotomy produced better coverage of the degenerative cartilage by a

thicker layer of repair tissue, when compared to debridement or bone drilling (Schultz and Gobel, 1999).

Soft tissue grafts, using the perichondrium and periosteum, involve creating a full thickness defect of 4 mm or larger, removing 1-2 mm of subchondral bone, and implanting the graft into the defect and securing into place with fibrin glue (Carranza-Bencano *et al.*, 1999). There has been no reported significant difference in the clinical outcome between the two graft sites (Carranza-Bencano, A. et al., 1999). Hyaline morphology has been reported in histological studies of the graft in the knee, but with only six of the 22 patients having a higher than 50% presence of hyaline morphology (Bouwmeester *et al.*, 1999; Smith *et al.*, 2005). Most published studies have investigated soft tissue grafts in the knee, with very limited literature reporting grafts performed in the ankle. One limitation associated with the periosteal graft procedure is the risk of calcification of the graft long-term (Redman *et al*., 2005).

Osteochondral harvest and transplantation involves harvesting non-weight bearing cartilage as a cylindrical plug and subchondral bone that is matched in size and surface area to the defect site. The plug is then implanted into the defected area. Multiple plugs can be taken, which is known as mosaicplasty. Osteochondral transplantation is usually reserved for smaller defects due to the limited area of the harvest site. The technique can be either autograft or allograft. Osteochondral autograft transplantation involves using the harvest from the individual and is often performed arthroscopically on defect areas smaller than 1 cm. Whereas, osteochondral allograft transplantation involves taking the harvest from a cadaver donor and sterilising it prior to

transplantation. It is often reserved for defects larger than 2 cm in size and is performed using open surgery. Studies performed in the ankle joint have been successful, with one study showing 80% of patients with ankle osteochondral lesions resulting in normal or nearly normal cartilage repair at 18 months post-surgery (Versier *et al.*, 2005). This is further supported by a comprehensive examination of 243 patients over nine separate studies, which showed comparable results with a 87% success rate in ankle treatment (Zengerink *et al.*, 2010).

1.2.1.2 Cartilage cell therapy

Tissue engineering strategies are another relative new approach for repairing cartilage defects, having only arisen approximately four decades ago, therefore some tissue engineering strategies are still in development or in the optimisation stages. Tissue engineering is defined as the use of a combination of cells, engineering materials and/or suitable biochemical factors, such as growth factors, to improve or replace biological functions in an effect to improve clinical procedures (Langer and Vacanti, 1993). It is mainly used to repair or regenerate damaged tissues and/or organs.

From a research perspective, successful tissue engineering strategies would provide medical and research screening models on bioengineered tissues and organs, which consequently could speed up research and drug discoveries. The successful generation of tissue engineered organs could lead to readily available 'off-the-shelf' organs for transplantation, eradicating lengthy waiting lists for transplantation. Furthermore, the use of successfully engineered tissues

and organs, would reduce the number of animal models for research, for example transplantations of heart valves are usually currently obtained from porcine origin. From a healthcare prospective, tissue engineering and cell therapies can facilitate a personalised medicine approach to medical treatments. For example, autologous cell therapies are a personalised treatment approach, but also have the added advantage of no tissue rejection possibility. There are two major issues associated with tissue engineering strategies: cell death following implantation and the large scale-up for routine clinical use, both of which limit the wide-range applicability and hinders clinical implementation.

A recent search on clinicatrials.gov website, a registry for all registered worldwide clinical trials, using the key words 'cell therapy' and 'tissue engineering' showed that 31,480 clinical trials are ongoing in cell therapy, and a further 79 clinical trials are ongoing in tissue engineering, as stated in November 2018 worldwide (National Institute of Health: US national library of medicine, 2018a; National Institute of Health: US national library of medicine, 2018b). Some examples of these clinical trials are listed in Table 2.

More specific an example of tissue engineering successfully being used to repair cartilage is autologous chondrocyte implantation (ACI), which briefly involves transplanting cells into cartilage defects to repair osteochondral full thickness defects. Several modifications to ACI have occurred over the last twenty years, and they will be outlined in the next section.

Table 2: Examples of cell therapy clinical trials conducted worldwide in 2018. Information obtained from National Institute of Health: US national library of medicine, 2018a.

Cell Type:	**Disease/Condition:**	**Location:**
T cell therapy	Liver transplants	London, UK
Dendritic cells	Non-Hodgkin lymphoma	Minnesota, USA
Autologous stem cells Autologous chondrocytes	Non-union fractures Knee cartilage defects	The Robert Jones & Agnes Hunt Orthopaedic Hospital (RJAH), Oswestry, UK
Tissue engineered corneal matrix	Corneal ulcers	Beijing, China
Mesenchymal stem cells (MSCs)	Spinal fusion treatment Cleft lip and cleft palate Cranial reconstruction Laryngeal implants Parkinson's disease	Barcelona, Spain Sao Paolo, Brazil Perth, Australia London, UK Milan, Italy

Autologous chondrocyte implantation (ACI)

ACI was developed by Brittberg and his team in 1994 by adapting a technique originally used to treat cartilage defects in rabbits

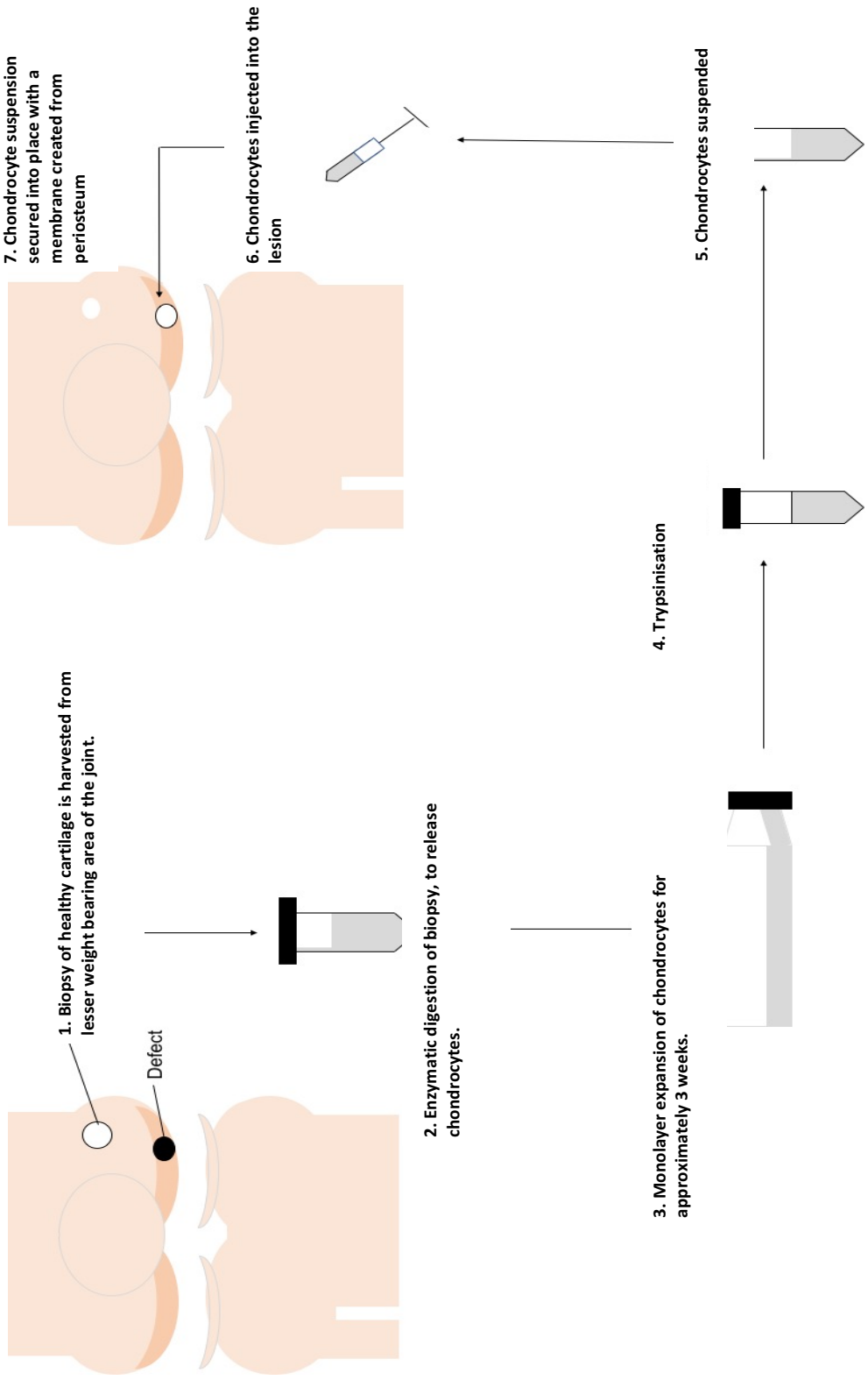


Figure 8: Schematic illustrating the procedure of ACI, using methodology from Brittberg et al., 1994.

(Brittberg, *et al.,* 1994; Grande, *et al.,* 1989). In brief, autologous chondrocytes are harvested from a lesser weight bearing area of the affected joint, expanded in monolayer and re-implanted into the damaged area under a natural or synthetic membrane via open joint surgery, as illustrated by Figure 8 (page 35).

Our centre, and others report a high percentage of good to excellent clinical results for ACI (Bhosale et al., 2009; Henderson et al., 2003). Although there is limited long-term follow-up data, positive clinical results are beginning to emerge and as a consequence, ACI has recently been approved by the National Institute for Health and Care Excellence to be available on the NHS as a first line treatment for knee cartilage defects over 2 cm^2 in size (National Institute of Health and Care Excellence, 2017).

A limitation associated with ACI is the expensive treatment cost per patient. The highest cost is associated with the expansion of chondrocytes, which can cost up to £16,000 per patient. However our centre currently cultures chondrocytes for ACI at a cost of £4,125 per patient in the specialised Good Medical Practice OsCell laboratory (National Institute for Health and Care Excellence, 2015). The expense of the technique limits the application of the technique and the availability for it to be used routinely as a first-line NHS treatment for other joint sites despite the promising results. Therefore, more research into refining the technique, and possibly reducing culture costs consequently, would be extremely beneficial. Optimisations into reducing the exposure to tissue culture plastic during the expansion process, which in turn would reduce the risk of cell de-differentiation, would be highly beneficial and

would in turn aid the potential for large scale routine use on the NHS and expansion of the treatment for other joint sites, including the ankle.

The standard ACI technique (known as first generation ACI) has since evolved using adaptations with new technology and research, streamlining the technique by eliminating any initial issues and ultimately improving clinical outcome.

Evolution of ACI

Many of the initial issues associated with first generation ACI were with the native periosteal tissue harvest and stitching. These issues related to time and quality of the harvest. A periosteum harvest is relatively time-consuming in regard to the full surgical procedure, keeping the patient under anaesthetic and exposing the open wound for longer periods of time, both of which increased the risk of possible complications and infection. A further issue is that the transplanted 'flap' regularly undergoes hypertrophy or ossification (Brittberg et al., 1996; Kuroda et al., 2011), which is considered a major reason for an inhomogeneous outcome of first generation ACI (Haddo et al., 2004).

Second generation ACI aimed to eliminate the periosteal tissue harvest issues by using a synthetic collagen membrane from porcine origin as an alternative. These were found to have superior clinical long-term outcomes compared with first generation ACI (Gooding et al., 2006; Zeifang et al., 2010). However, by using animal derived membranes this posed its own risk of possible cross-species contamination.

Third generation ACI, also known as matrix-induced ACI (MACI), optimised the technique further to utilise hydrogel scaffold constructs. An example of which is Hyalofast or CaRes, both of which are made from type I collagen gel (Kuroda et al., 2011) The scaffold is used for culturing the cells in a three-dimensional environment. This optimisation reduces the time of cells in monolayer culture and should therefore reduce the risk of de-differentiation, ensuring cells maintain their chondrogenic phenotype up until re-implantation (Schnabel et al., 2002; Schulze-Tanzil, 2009). MACI has the added advantage of requiring no patch to seal the cells into the defect and scaffolds can be trimmed to be an exact fit to the size of the defect site. When compared with first and second generation ACI, MACI has the advantages of being surgically simplistic and being able to be performed using an arthroscopic approach (Marcacci et al., 2005; Minas and Nehrer, 1997; Ossendorf et al., 2007; Peterson, 1996). Despite the three generations of ACI all having undergone optimisation, there are still some associated limitations with ACI.

A recent health-economic study commissioned by the UK National Institute for Health and Care Excellence found ACI to be cost-effective compared to alternative surgical interventions, but it also found that ACI comes with a 50% failure rate at 10 years. We need to consider the limitations of ACI to be able to optimise the technique and improve upon the current failure rate. Technical steps at the point of cell isolation and expansion, re-implantation, monitoring of clinical outcomes and the establishment and longevity of the graft itself, all require further attention. Furthermore, we need to address how we can transfer ACI to other joint types.

At the point of cell isolation and expansion, chondrocytes begin to de-differentiate once exposed to tissue culture plastic during expansion. De-differentiation results in the chondrocytes exhibiting an altered phenotype, one more characteristic of fibroblast cells (Lin et al., 2008; Schulze-Tanzil, 2009). This altered phenotype impacts upon tissue biomechanics and function. Until recently, ACI has been restricted to assess the efficacy, cost-effectiveness and efficiency compared to other treatment options that are readily available on the NHS, such as microfracture. The majority of this research has been conducted on the knee (Bartlett et al., 2005; Bentley et al., 2003; Fu et al., 2005; McCarthy et al., 2015; Steinwachs and Kreuz, 2007), with little work carried out on assessing ACI treatment into other joints, including the ankle (Giannini et al., 2014; McCarthy et al., 2015; Thermann et al., 2014; Whittaker et al., 2005; Zengerink et al., 2010). However, the use of ACI in the ankle has increased over time but still requires further work to be carried out to allow for much needed optimisation to adapt to treating a wide variety of joints, including the ankle (National Institute of Health and Care Excellence, 2017; National Institute of Health and Care Excellence, 2015).

1.2.1.3 Knee, foot and ankle cartilage

Due to the high prevalence of knee osteoarthritis, occurring in approximately 6% of the population, there are lots of surgical treatments currently available to repair cartilage damage in this joint (page 29; Felson et al., 1987). Furthermore, high levels of research are being conducted into new or improved treatments.

Ankle cartilage damage is usually treated by total ankle replacement, ankle fusion or osteotomy. Little research has been conducted into cartilage repair of the ankle, especially when compared to the plethora of research conducted into the other joints of the lower extremity (Bartlett et al., 2005; Fu et al., 2005; McCarthy et al., 2015). This is surprising, as the ankle has a higher injury rate. However, this may be explained by the ankle having a lower prevalence of osteoarthritis at just 1% of the population, compared to the 6% prevalence of osteoarthritis in the knee (Aurich et al., 2014; Felson, 1990), and ankle cartilage defects tend to be of a lesser International Cartilage Repair Society grade (Figure 9; page 41) and are less likely to require surgical intervention. Combined, these findings possibly indicate that the ankle has a protective mechanism preventing cartilage injuries either progressing to osteoarthritis or progressing in osteoarthritis severity (Aurich et al., 2014). Osteoarthritis in the ankle is less likely to develop sporadically but is most likely to be a result of trauma, termed post-traumatic osteoarthritis.

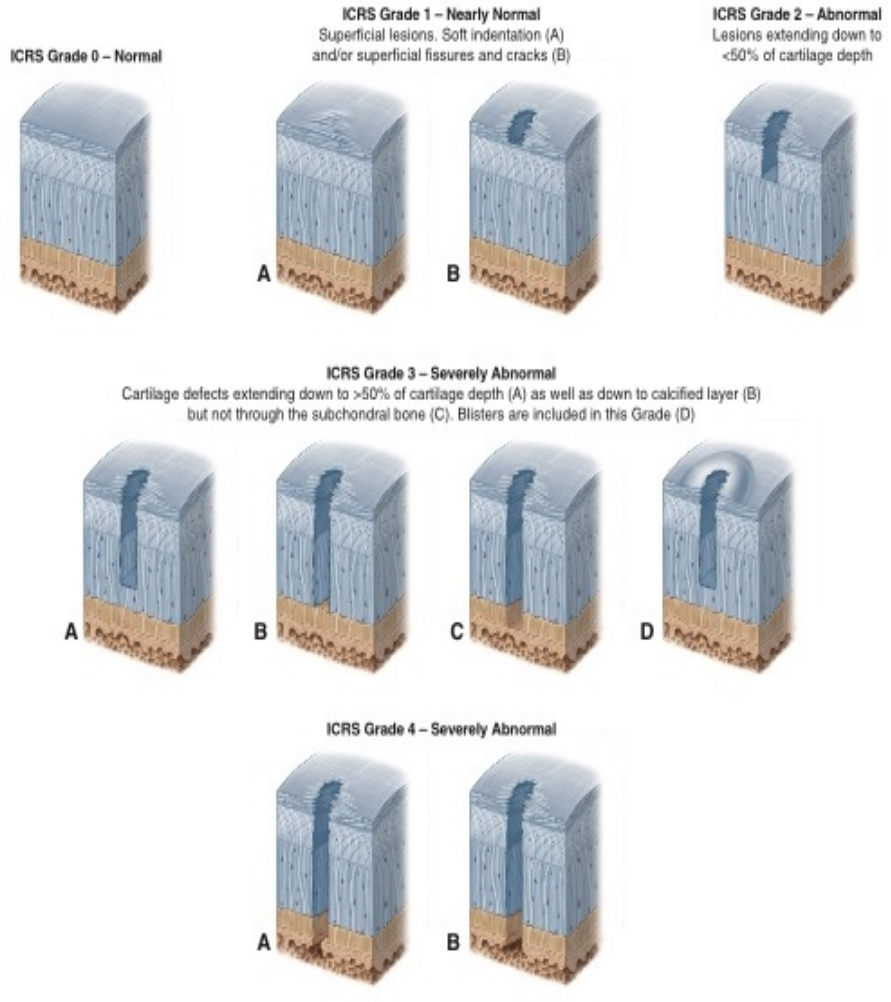


Figure 9: International cartilage repair society grading of cartilage lesions, listing key characteristics of severity. Severity ranges from normal (grade 0) to severely abnormal (grades 3-4). Image taken from Brittberg and Peterson, 1998.

There are notable anatomical differences between the ankle and knee joints. The knee is comprised of four main bones: the patella, the fibula, the tibia and the femur (Figure 10), whereas the foot is a small joint comprised of 26 bones: five metatarsal bones, 14 phalangeal bones and seven talar bones (Figure 10). The ankle joint is a relatively unstable complex joint consisting of three bones: the tibia, fibula and talus, and three main joints: the subtalar joint, talocrural joint and talocanconavicular joint (Brockett and Chapman, 2016).

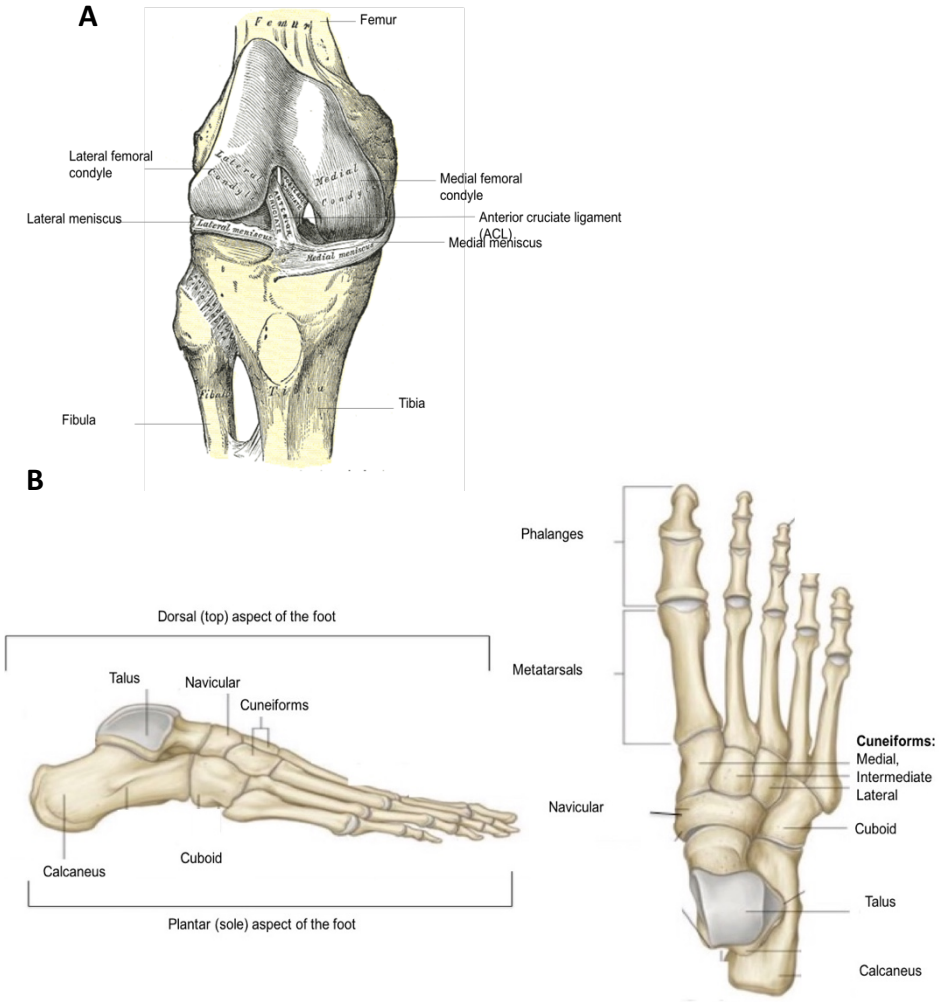


Figure 10: Basic anatomy of the (A) knee and (B) the ankle. Image adapted from Gray's Anatomy; Drake et al., 2015.

Ankle cartilage is 1-1.7 mm thick on the talus, much thinner when compared to knee cartilage which can range up to 6 mm in thickness (Ateshian et al., 1991; Schenck and Athanasiou, 1993). Simply, both joints are considered synovial hinge joints, however the ankle is a more complex joint comprised of two joints and has three planes of movement: the sagittal plane, the transverse plane and the frontal plane, whereas the knee is comprised of one joint and has only one plane of movement (Brockett and Chapman, 2016).

1.2.1.4 ACI for knee and ankle defects

A handful of studies over the last 15 years have demonstrated the recent success of translating ACI to the ankle joint to treat osteochondral defects (Giannini et al., 2014; Giza et al., 2010; Nam et al., 2009; Schneider and Karaikudi, 2009; Thermann et al., 2014; Whittaker et al., 2005).

From a clinical perspective, MRI demonstrated improvements in outcome and repair quality. This was reflected by increased Western Ontario and McMaster Universities Arthritis Index scores post-operatively, fully filled defects, a good integration observed with no lamination of matrix, and a decreased bone marrow oedema observed in 88.9% of patients (Imhoff et al., 2011; Nam et al., 2009; Thermann et al., 2014). Histological findings further support the results seen radiologically and in outcome measures. One study compared three successful ACI cases to three failed ACI cases and found that the quality of cartilage formed may be paramount to the success of ACI within the ankle. Histologically, the three successful cases demonstrated hyaline-like cartilage, which contrasted to the three failed cases that demonstrated

fibrocartilage tissue with altered cell organisation and lower concentrations of GAGs (Giannini et al., 2014). This correlates with a study performed at our centre, which evaluated six biopsies of successful ACI within the talus and found mainly hyaline cartilage, indicating good quality cartilage tissue formation (Whittaker et al., 2005).

The high levels of clinical success in ACI surgery have been partly credited to the extensive rehabilitation process associated with the technique. ACI rehabilitation therapy is considered more intense than standard rehabilitation therapies assigned with other surgical therapies.

Furthermore, high levels of patient satisfaction have been recorded following ACI from our centre and others. A study performed at RJAH reported 90% of patients being pleased with the outcome of ACI performed in their ankle (Whittaker et al., 2005), and another study reporting 100% of patients felt that they had improved following ankle MACI (Giza et al., 2010). There was no placebo effect assessed, as these studies compared one treatment to another directly.

1.3 Assessment of the outcomes for foot and ankle surgical treatments

Patient-reported outcome measures (PROMs) are defined by the US Food and Drug Administration as 'any report of the status of a patient's health condition that comes directly from the patient, without interpretation of patient response by a clinician or anyone else' (U.S. Department of Health and Human Services Food and Drug Administration, 2009). They are designed to assess the impact of the disease on the patient and provide a clear view of the treatment from the

patient perspective, which can provide invaluable information that technology and observers cannot provide (Black, 2013).

PROMs are primarily used to assess the success of surgical intervention on patients. Studies have shown that patient-reported healthcare, including the use of PROMs, results in enhanced treatment adherence and improved treatment outcomes (Pinsker et al., 2013). There are two methods used in PROMs analysis; through direct answers, for example pain severity, or through the change from a previously completed score (U.S. Department of Health and Human Services Food and Drug Administration, 2009).

As PROMs are solely patient-reported with no clinician input, this can cause barriers with wide application and can cause difficulty with administration and data input. Some scores can be specific to certain conditions or diseases and this can limit their suitability and consequently their application. Despite this, usually several PROMs are administrated to thoroughly assess the outcome of the condition or treatment. As there is currently no gold standard PROM, and not all PROMs are validated, selecting an appropriate outcome measure can be difficult and therefore PROMs are usually selected based on routine use or preference of the clinician or centre (Nelson & Berwick, 1989; McHorney & Tarlov, 1995; Martin, *et al.,* 1997; Binkley, *et al.,* 1999).

Therefore, there is high demand for a newly developed validated PROM for assessing all the relevant areas for the outcome of cell therapy, including ACI.

1.3.1 Validation of patient-reported outcome measures (PROMs)

Once developed, it is important that any PROM is validated and undergoes analyses to ensure that the score is fit for purpose, especially as the purpose is to measure clinical outcome. Only once a score is validated should it then be used in clinic.

Validity is defined as the extent to which a score actually measures what it purports to. This can be assessed using three areas, construct validity, content validity and criterion validity, all of which are further defined and summarised in Table 3. Other aspects to consider during validation are: the type of score and whether it is suitable, the characteristics of the subjects and the reliability and responsiveness of the outcome measure being assessed.

All outcome measures must be responsive, specifically it must be sensitive to changes in clinical outcome. Therefore, it must be able to accurately detect whether a patient is improving or declining in clinical outcome. The content must capture what the score aims to capture and must be considered important from the patient perspective as a PROM is designed to be solely patient-reported. The score must also be reliable. More specifically, if no change has occurred then the PROM should remain relatively consistent. Finally, the score must also be able to correlate with existing validated outcome scores.

Table 3: A summary of validation criteria for outcome measures and a summary of aspects to consider when designing, selecting or validating an outcome measure.

Aspects:	**Details:**
Content validity	Defined as the degree to which the content of the outcome measure is an adequate reflection of the construct to be measured.
Construct validity	Defined as the degree to which the scores of the outcome measure are consistent with the hypotheses.
Criterion validity	Defined as the degree to which the outcome measure is an adequate reflection of a 'gold standard'.
Type of score	There are eight types of score: generic, disease-specific, region-specific, patient-specific, population-specific, dimension-specific, summary items and utility measures.
Subjects	To ensure it addresses the needs and characteristics of the subjects.
Reliability	Defined as the degree to which an outcome measure is free form measurement error. For example, scores from patients who have not changed in outcome should remain consistent.
Responsiveness	Defined as the ability of a score to detect change over time in the construct, for example following intervention.

Aims of PhD project

This PhD project has the overall aim of advancing cartilage repair of the ankle by increasing the understanding of ankle defects and treatment, and improving upon existing cell therapies. This will be addressed through the following projects:

(1) To advance cartilage cell therapy by improving the expansion and chondrogenic capacity of cultured human chondrocytes.

(2) To better understand why the ankle is less prone to osteoarthritis than the knee by characterising the differences in the metabolism of chondrocytes isolated from knee and ankle cartilage.

(3) To assess the outcomes for ankle surgical treatments, including cartilage cell therapy, by designing a new PROM and then comparing it with existing validated scores.

Chapter 2: Methods and materials

2.1 Laboratory methods

2.1.1 Media and solution compositions

Working solution

Dulbecco's Modified Eagles Medium (DMEM):F12 containing 0.03 mg/ml deoxyribonuclease I (Sigma, Poole, UK) and 0.83 mg/ml type XI collagenase (Sigma, Poole, UK). Working solution was used for digesting cartilage samples to obtain chondrocyte cells.

Ascorbic acid solution

10 mg of ascorbic acid (Sigma, Poole, UK) was dissolved in 125 ml of sterile water for injection (Fannin, Dublin Ireland). Ascorbic acid solution was added to all media solutions used in cell culture, listed below.

Serum-free media

DMEM:F12 (Gibco - Thermo fisher Scientific, Massachusetts, USA) supplemented with 0.5% (w/v) penicillin-streptomycin-amphotericin (Lonza, Basel, Switzerland) and 0.5% (w/v) ascorbic acid solution. Serum-free media was used for re-suspending cells prior to cell counts during passaging.

Complete culture media

DMEM:F12 supplemented with 0.5% (w/v) penicillin-streptomycin-amphotericin, 0.5% (w/v) ascorbic acid solution and 10% (v/v) foetal bovine serum (FBS; Gibco – Thermo Fisher Scientific, Massachusetts, USA). Complete culture media was

used for cell culture in all experiments, unless otherwise stated, for the culture of all cells used in Chapter 4, and the FBS culture of samples in Chapter 3.

Stemulate™ culture media

DMEM:F12 supplemented with 0.5% (w/v) penicillin-streptomycin-amphotericin, 0.5% (w/v) ascorbic acid solution and 10% (v/v) Stemulate™ (Cook Regentec, Indianapolis, USA). Stemulate™ culture media was used for 'Stemulate™' defined samples in Chapter 3.

Chondrogenic media

DMEM:F12 supplemented with: 1% (v/v) FBS, 1% (v/v) non-essential amino acids (Gibco - Thermo fisher Scientific, Massachusetts, USA), 1% (v/v) L-glutamine (Lonza, Basel, Switzerland), 0.5% (v/v) penicillin-streptomycin-amphotericin, 0.5% (w/v) ascorbate, 1% (v/v) insulin-transferrin-selenium (Gibco - Thermo fisher Scientific, Massachusetts, USA), 200 ng/ml transforming growth factor-β1 (TGF-β1; Peprotech, New Jersey, USA), 10 nM dexamethasone (Sigma, Poole, UK) and 3.2 µg/ml L-proline (Sigma, Poole, UK). Chondrogenic media was used throughout Chapter 3 and Chapter 4 for all chondrogenic pellet culture.

Blocking agent for flow cytometry

1 ml of blocking agent was prepared by diluting 10% (v/v) human IgG in 2% (w/v) bovine serum albumin (w/v). Blocking agent was used to incubate cell suspensions prior to flow cytometry in Chapter 4.

Proteinase K solution

Proteinase K (Life Technologies, California, USA) was diluted in 100 mM of ammonium acetate (Sigma, Poole, UK) in a final concentration of 1 mg/ml. Proteinase K solution was used to digest three-dimensional chondrogenic pellets for biochemical analysis in Chapter 3 and Chapter 4.

1,9-dimethylmethylene blue (DMMB) dye solution

The dye was prepared by diluting 4 mg DMMB (Sigma, Poole, UK) in 225 ml of distilled water containing 0.76 g glycine (Sigma, Poole, UK) and 0.595 g sodium chloride. The final solution was adjusted to pH 3.0 using 2 M hydrochloric acid and the final volume was adjusted to 250 ml using distilled water. The solution was protected from light, kept at room temperature and filtered prior to use using Whatman filter paper. DMMB dye solution was used in Chapter 3 and Chapter 4 for GAG analysis experiments of chondrogenic pellets.

Citrate/acetate buffer

It was prepared by dissolving 34 g sodium hydroxide (Sigma, Poole, UK), 34 g citric acid monohydrate (Sigma, Poole, UK) and 120 g sodium acetate trihydrate (Sigma, Poole, UK) in 250 ml of distilled water. The final solution was adjusted to pH 6.0 and to a final volume of 1 L using distilled water. Citrate/acetate buffer was used to further prepare oxidation buffer, outlined below, for hydroxyproline assays to determine total collagen content of chondrogenic pellets in Chapter 3 and Chapter 4.

Oxidisation buffer

Oxidisation buffer was prepared by mixing 600 ml isopropanol, 330 ml distilled water and 390 ml citrate/acetate buffer. Oxidation buffer was used as a standard control, and to further prepare oxidation solution, outlined below, for hydroxyproline assays to determine total collagen content of chondrogenic pellets in Chapter 3 and Chapter 4.

Oxidisation solution

Oxidation solution was prepared in a dark reservoir by dissolving 300 mg of chloramine T (Sigma, Poole, UK) in 50 ml oxidation buffer. Oxidation solution was used for hydroxyproline assays to determine total collagen content of chondrogenic pellets in Chapter 3 and Chapter 4.

Ehrlich's reagent

Ehrlich's reagent was prepared by dissolving 6 g of p-dimethylaminobenzaldehyde (Sigma, Poole, UK) in a mixture of 52 ml of isopropanol and 16 ml of 50% perchloric acid (Sigma, Poole, UK). Ehrlich's reagent was used for hydroxyproline assays to determine total collagen content of chondrogenic pellets in Chapter 3 and Chapter 4.

Reaction mix for complementary DNA (cDNA) conversion

It was prepared by mixing 5 µl of reverse transcriptase (RT) buffer, 5 µl of random primers, 2.5 µl of multiscribe RT enzyme, 2 µl of deoxynucleotide mix

and 10.5 µl of RNAse-free water. Final volume of solution was 25 µl. Reaction mix was used to convert mRNA to cDNA for further qRT-PCR analysis for gene expression profiling.

Reaction mix for qRT-PCR

The reaction mix consisted of 10 µl of SYBR green reaction mix, 2 µl of primers for targeted genes and 6.4 µl of RNAse free water. The reaction mix (18.4 µl) was added to the wells containing cDNA to give a 20 µl reaction. Reaction mix of each gene of interest was used during qRT-PCR for gene expression profiling.

2.1.2 Obtaining patient consent

Fully informed consent was taken from individuals undergoing total joint replacement surgery at our centre, RJAH, for either the ankle or knee with ethical approval (11/NW/0875; Appendix I, page 225). All patient consents were obtained by research assistants Dr Sharon Owen and Mrs Annie Kerr. Demographics for each experiment are shown in the relevant experimental plans outlined in each chapter.

2.1.3 Chondrocyte isolation

Each tissue sample was photographed and full depth cartilage was removed from macroscopically healthy areas of the femoral condyle of knee samples and the talus of ankle samples using a sterile scalpel. Cartilage extraction was performed within 24 hours of receiving the tissue. Chondrocytes were isolated following established protocols (Harrison et al., 2000; Nguyen et al., 2010; Sykes et al.,

2018; Wang et al., 2008). Briefly, cartilage tissue (100-300 mg) was placed into 10 ml working solution in an upright T25 tissue culture flask at 37 °C with 5% CO_2 for 16 hours. After 16 hours, the digest was filtered through a 0.2 µm cell strainer and chondrocytes were pelleted at 750 *g* for 10 minutes. Trypan blue (Sigma, Poole, UK) was used to determine cell viability.

2.1.4 Chondrocyte culture

Chondrocytes were seeded into appropriately sized tissue culture flasks at a density of $5x10^4$ cells/cm^2 in serum-free media and supplemented specifically for each experiment, as listed in the experimental design of each chapter. Chondrocytes were incubated at 37 °C with 5% CO_2 for 4 days to allow for adherence, after which media were replenished three times weekly. This was considered passage zero. Once 80% confluency had been reached, chondrocytes were passaged.

2.1.5 Chondrocyte passaging to increase cell number

Once approximately 80% confluent, chondrocytes were washed with phosphate buffered saline (PBS; Gibco - Thermo fisher Scientific, Massachusetts, USA) to weaken cell attachments and 5 ml or 10 ml trypsin/EDTA (Gibco - Thermo fisher Scientific, Massachusetts, USA) was added to T75 or T175 culture flasks respectively and incubated at 37 °C with 5% CO_2 for 5 minutes. Chondrocytes were detached by gentle agitation. An equal volume of culture media was mixed with the trypsin and pelleted at 750 ×*g* for 10 minutes. Supernatant was discarded and the cell pellet was re-suspended in 1 ml of serum-free media. A

cell count was performed and cell viability was determined using trypan blue (Sigma, Poole, UK). Chondrocytes were then seeded into relevantly sized tissue culture flasks at a density of $5x10^4$ in the relevant culture media. Cells were cultured in monolayer until passage two.

2.1.6 Growth kinetics during monolayer expansion

Throughout each passage, cell counts were performed and used to determine growth kinetics of each sample. Still-images using brightfield microscopy were taken throughout monolayer culture using a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan), the Hamamatsu C4742-95 camera (Phototonics, Japan) and IP Lab imaging software (BD Biosciences, California, USA, version 3.6). Growth kinetics were assessed throughout monolayer expansion, by absolute population doublings (PDs) and population doubling times (PDTs). PDs and PDTs were recorded at each passage and calculated from cell counts using the following formula (McAteer and Davis, 2002):

$$PD = \text{time in culture (days)} \div PDT$$

$$PDT = 1 \div \text{multiplication rate}$$

$$\text{Multiplication rate} = \frac{3.32 * [\log(\text{final cell number}) - \log(\text{initial cell number})]}{\text{time difference}}$$

2.1.7 Detection of chondrocyte cell surface markers by flow cytometry analysis

Flow cytometry uses light scatter produced by light refraction to determine the count and profiles of the cells within a heterogeneous mixture. Forward scatter

is produced from light being refracted by the cells into the same path and is usually used to identify cell size, whereas side scatter is produced from light being refracted by the cells into another path and is used to identify the complexity of the cells. A dot plot of forward scatter and side scatter (Figure 11) can be used to identify 'probable' cell types. However, to determine definitive cell types using flow cytometry labelling with cell type-specific markers is required.

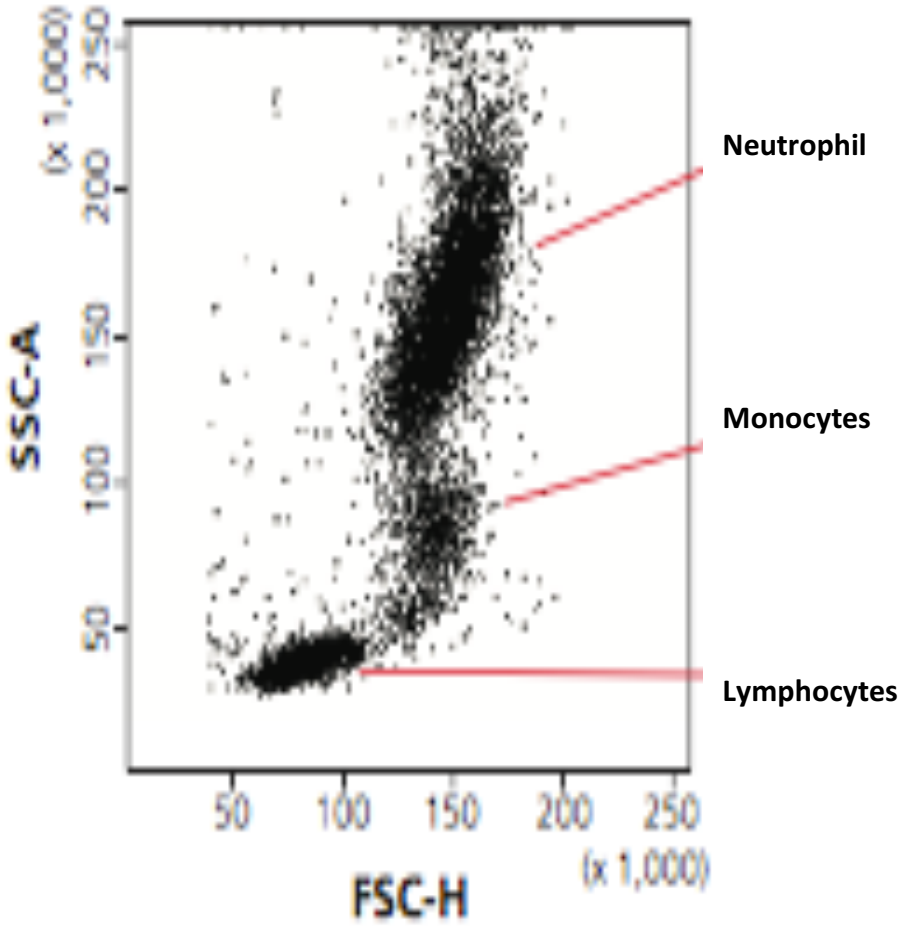


Figure 11: Examples of flow cytometry analysis profiles used to determine the profile of cells based on forward scatter and side scatter. This example was used to determine blood cells in a homogenous population. Image taken from BD Biosciences, 2008.

A profile of cell surface markers was selected to determine chondrogenic potency of chondrocytes and based upon published work from our centre (Garcia et al., 2016a). These consisted of: CD39, CD44, CD49c and CD166. Details of clones and isotype controls are listed in Table 4.

Briefly, cells were harvested from monolayer culture at passage three. $4x10^4$ cells were harvested and re-suspended in 1 ml blocking agent and incubated protected from the light for 1 hour at 4 °C. The samples were centrifuged at 350 *g* for 8 minutes and re-suspended in 200 µl of 2% (w/v) bovine serum albumin and aliquoted equally into two flow cytometry tubes. Fluorochrome conjugated antibodies against CD39, CD44, CD49c and CD166 were added in the volumes specified in Table 4 and incubated for 30 minutes at 4 °C protected from the light. The samples were then washed in 1 ml of 2% (w/v) bovine serum albumin and centrifuged at 350 ×*g* for 8 minutes. Supernatant was discarded and the pellets were re-suspended in 300 µl 2% (w/v) bovine serum albumin, before being analysed in the FACS CantoII cytometer using the FACSCanto II flow cytometry machine (BD biosciences) and FACSDiva software (BD biosciences, version 7.0).

Table 4: Cell-specific markers used to determine chondrogenic potency of the cultured chondrocytes, listed with the volumes, clone, isotype control and fluorochrome used to analyse the flow profile.

Marker:	Volume:	Clone:	Isotype control:	Fluorochrome:
CD39	5 µl	TU66	IgG2b	Allophycocyanin
CD44	5 µl	G44-26	IgG2b	Peridinin-Chlorophyll-Protein-Cyanine 5.5
CD49c	80 µl	C3 IL1	IgG1	Phycoerythrin
CD166	1 µl	3A6	IgG1	Brilliant Violet 421

2.1.8 Three-dimensional pellet culture to re-differentiate chondrocytes

Re-differentiation of chondrocytes is the process in which chondrocytes regain their original chondrogenic phenotype following de-differentiation caused by prolonged exposure to plastic during monolayer culture (Caron et al., 2012; Wu et al., 2009). Re-differentiation results in chondrocytes re-expressing the cartilage marker molecules, regaining expression of GAGs, ACAN (gene for aggrecan) and COL2A1, and having a reduced expression of COL1A1 (Elima and Vuorio, 1989; Tallheden et al., 2004). Re-differentiation is usually achieved using three-dimensional cell culture conditions (Lin et al., 2006; Lin et al., 2008).

Once 80% confluent at passage 2, chondrocytes were passaged and seeded at $2.5x10^5$ cells into 1.5 ml Eppendorf tubes, cultured in 500 µl chondrogenic media and centrifuged at 750 ×*g* for 7 minutes to create spherical chondrogenic pellets. After 24 hours, the Eppendorf tubes were inverted to

ensure the pellets did not adhere to the tubes but remained suspended in the chondrogenic media. Chondrogenic media was replenished twice weekly (Johnstone et al., 1998).

2.1.9 Chondrogenic pellet processing for biochemical analyses

Chondrogenic pellets were digested in 250 µl of proteinase K solution for 2 hours at 60 ^{o}C to release GAG and DNA content. The proteinase K was inactivated for 5 minutes at 100 °C. These samples were stored at -20 °C. Standard stock solutions for the biochemical assays are described in Table 5 (page 61).

2.1.10 Total cell number

Cell number within the chondrogenic pellets was determined using the PicoGreen® fluorescent DNA quantification assay, following the manufacturer's instructions for the PicoGreen® fluorescent DNA quantification kit (Thermo fisher Scientific, Massachusetts, USA). All reagents were included in the kit. This assay uses the PicoGreen® double stranded DNA quantification reagent, which binds to the double-stranded DNA present in the samples and creates an increase in the fluorescence proportional to the amount of DNA present (Dragan, *et al.,* 2010).

Stock standard solution and standard concentrations were prepared as listed in Table 5 (page 61). Tris-Edta buffer (10 mM Tris-HCl, 1 mM EDTA) was used as a control. PicoGreen® dye was diluted (1:200) in Tris-Edta buffer and protected from light. Experimental samples were diluted (1:20) in Tris-Edta buffer.

Standards were diluted with an equal volume (500 µl) of the diluted PicoGreen® dye solution. Experimental standards (25 µl) were mixed with 200 µl Tris-Edta buffer and 225 µl of the diluted PicoGreen® dye. The standards and experimental samples (200 µl) were transferred in duplicate to a 96-well flat-bottomed plate. Fluorescence was read at excitation 480 nm and emission 520 nm on a FluorStar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The fluorescence value for the control was subtracted from each sample and the DNA concentration was determined from the standard curve. Cell number was calculated using the widely reported value of 7.7 pg of DNA per chondrocyte (Kim et al., 1988).

The cell number obtained from the PicoGreen® assay was used to normalise the sGAG production, total collagen production and gene expression profiles to determine results per cell number and to eradicate any slight variations in pellet size.

Table 5: Details of stock solutions and standard concentrations for biochemical analyses.

Assay:	**Standard stock solution:**	**Standard concentrations:**
Total cell number determined by PicoGreen® assay	Lambda DNA (1:50 dilution) diluted 1:40 in 10 mM Tris-Edta buffer. Final stock concentration of 2 µg/ml	100 ng/ml, 200 ng/ml, 400 ng/ml, 600 ng/ml, 800 ng/ml, 1 µg/ml. Diluted in Tris-Edta buffer
Total sGAG content assessed by DMMB assay	Chondroitin sulphate A from bovine trachea, diluted in PBS. Final stock concentration of 1 mg/ml	2 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml. Diluted in distilled water
Total collagen content assessed by hydroxyproline assay	Hydroxyproline diluted in 10 mM hydrochloric acid. Final stock concentration of 1 mg/ml	0.1 µg/ml, 0.2 µg/ml, 0.4 µg/ml, 0.6 µg/ml, 0.8 µg/ml, 1.0 µg/ml. Diluted in 10 mM hydrochloric acid

2.1.11 Total sulphated glycosaminoglycan (sGAG) content

The total sGAG content was quantified using a 1,9-DMMB assay (Farndale et al., 1986). This assay uses DMMB dye (page 50), which presents a change in absorbance due to the induction of metachromasia when the dye is bound to sGAGs within cartilage tissue.

Stock DMMB solution and standard concentrations were prepared as listed (page 61; Table 5). Duplicate samples and standards (50 µl) were transferred to a 96-well flat-bottomed plate and mixed with 200 µl of the DMMB dye. Absorbance was read immediately at 530 nm on a FluorStar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The absorbance value for the control was subtracted from each sample and the sGAG concentration was determined from the standard curve. The sGAG content was normalised to cell number using the cell number data obtained from the PicoGreen® assay.

2.1.12 Total collagen content

The total collagen content was determined using a hydroxyproline assay (Brown et al., 2001). This assay measures hydroxyproline which relies on the oxidation of hydroxyproline to a pyrrole derivative that reacts with p-dimethylaminobenzaldehyde to produce a coloured product. This can be used to estimate collagen fibril content based on 100 hydroxyproline residues are present per 1,000 amino acid residues in collagen (Altman and Gerber, 1983). Stock solution and standard concentrations were prepared as listed (Table 5; page 61).

Experimental samples (100 µl) were transferred to screw cap polypropylene tubes and hydrolysed in 100 µl of hydrochloric acid (specific gravity 1.16, approximately 10 M; Sigma, Poole, UK) for 16 hours at 108 °C in a dry-heat block. On cooling, samples were partially neutralised with 800 µl of 1 M sodium hydroxide (Sigma, Poole, UK). Duplicate samples and standards (50 µl) were transferred to a 96-well flat-bottomed plate. Oxidation buffer (100 µl) was used as a negative control. Oxidation solution (100 µl) was added to all samples and standards. The plate was gently shaken and then incubated at room temperature for 5 minutes. Ehrlich's reagent (100 µl) was added to all wells and mixed well. The plate was sealed with adhesive plate seal and incubated in a 60 °C water bath for 45 minutes. The absorbance was read at 570 nm on a microplate reader and the total collagen content was calculated using the widely reported multiplication factor of 7.2 (Sims et al., 2000).

2.1.13 Gene expression analyses

2.1.13.1 Sample preparation for gene expression analysis

Chondrogenic pellets ($2.5x10^5$) were prepared for RNA extraction by homogenisation, using a 21g needle and syringe, in 350 µl RLT lysis buffer (Qiagen, Hildner, Germany) containing 1% (v/v) β-mercaptoethanol (Sigma, Poole, UK) and then stored at -80 °C in 1.5 ml Eppendorf tubes. All products for messenger RNA (mRNA) extraction, cDNA conversion and qRT-PCR analysis, including primers, were procured from Qiagen (Hildner, Germany) unless otherwise stated.

2.1.13.2 Optimisation of reference genes

Gene expression was determined relative to two reference genes according to published MIQE guidelines (Bustin et al., 2009). Five commonly used reference genes were used to determine co-efficiency of variance across chondrocyte culture conditions and were as follows: peptidylprolyl, β-Actin, glyceraldehyde 3-phosphate dehydrogenase, hypoxanthine-guanine phosphoribosyltransfrase-1 and TATA box-binding protein. The two human reference genes hypoxanthine-guanine phosphoribosyltransfrase-1 (Foldager et al., 2009; Rushton et al., 2014) and TATA box-binding protein (Foldager et al., 2009; Pombo-Suarez et al., 2008) were selected for all experiments based on the lowest co-efficiency of variance values (Figure 12). Lowest co-efficiency of variance indicates the most stable reference genes across chondrocytes cultured in both FBS and Stemulate™ sera supplements. A co-efficiency of variance of 7 or less is considered good.

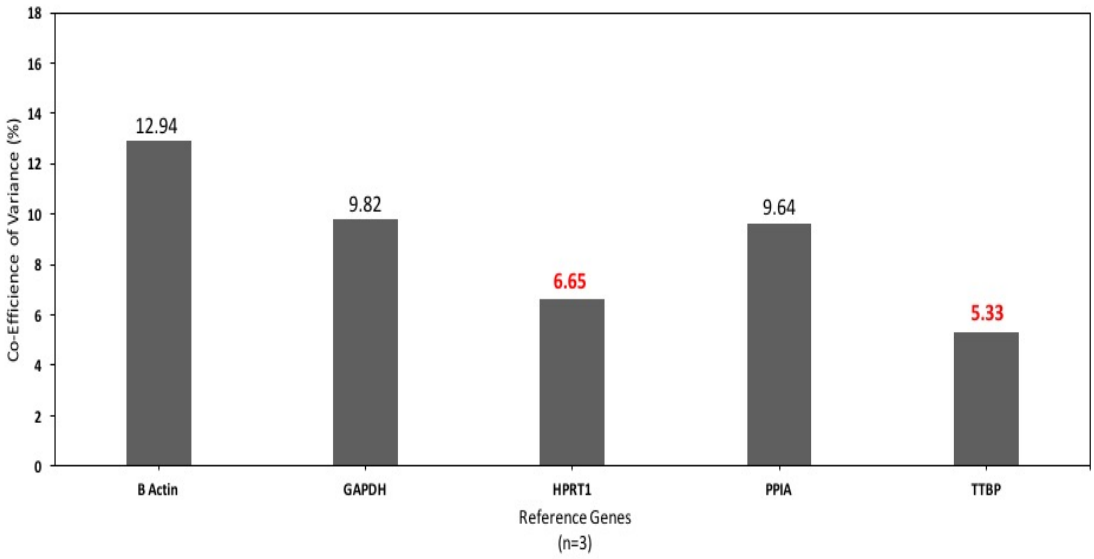


Figure 12: Preliminary experiment to determine the most suitable two reference genes from a selection of five commonly used reference genes for qRT-PCR

analysis of chondrogenic pellets, selected based on the lowest co-efficiency of variance.

2.1.13.3 **Qualitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

Amplification of targeted DNA molecules was assessed using qRT-PCR, allowing for assessment of fold changes in gene expression to quantify gene expression profiles within samples. qRT-PCR assesses the amplification of targeted DNA molecules and occurs in real time and therefore allows for the recording and analysis of fold changes in gene expression to be reported as they occur, as opposed to just the end-product analysis. mRNA was first extracted and then converted to cDNA to be able to perform qRT-PCR.

mRNA was extracted from the chondrogenic pellets using the Qiagen RNeasy mini kit as per manufacturers' instructions. All required reagents were included in the kit. In brief, cells were thawed and re-suspended in 350 µl of 70% molecular grade ethanol (Sigma, Poole, UK). The full volume was then transferred to a spin column placed inside a 2 ml collection tube and centrifuged at 8,000 ×*g* for 1.5 minutes. RPE buffer (500 µl) was used to perform two washes of the mRNA and centrifuged at 8,000 ×*g* for 1.5 minutes. RNAse-free water (50 µl) was added and centrifuged at 8,000 ×*g* for 1.5 minutes to elute the mRNA. The final volume was collected in sterile 0.5 ml Eppendorf tubes and stored at -80 °C.

mRNA was then converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, California, USA) in a Progene thermocycler (Techne, Minnesota, USA) following the manufacturers'

instructions. All reagents, apart from RNAse free water, were provided in the kit and all steps were performed on ice. mRNA (25 µl) was transferred to sterile tubes containing reaction mix, as previously described. The tubes were transferred to the thermocycler, which was pre-programmed to 25 °C for 10 minutes, 37 °C for 2 hours and a final holding step of 4 °C. Samples were stored at -20 °C.

For each patient, qRT-PCR was performed using SYBR green (Applied Biosystems, California, USA) on three pellet replicates from days 0, 21 and 28. Genes (Table 6; page 67) were chosen to determine the chondrogenic phenotype after re-differentiation. All genes were human derived. Gene expression was expressed relative to the two reference genes and normalised to cell number obtained from the PicoGreen® assay.

All steps were performed on ice. Briefly, 1.6 µl of cDNA from three pellet replicates from each patient was added to the 96-well flat-bottomed plate. A reaction mix for each gene of interest was prepared as explained previously. The plate was then sealed using optical adhesive film (Applied Biosystems, California, USA) and transferred to the Quant Studio3 Real-Time PCR machine (Applied Biosystems, California, USA) programmed to run the protocol listed in Table 7 (page 68).

Table 6: Genes selected for qRT-PCR analysis to determine the chondrogenic phenotype of the cells and to determine if the chondrocytes had de-differentiated to a hypertrophic state.

Gene:	**Official name:**	**Amplicon length:**	**Entrez ID:**
Reference genes			
HPRT1	Hypoxanthine phosphoribosyl transferase 1	- 130 (NM_000194)	3251
TBBP	TATA box-binding protein	- 132 (NM_001172085) - 132 (NM_003194)	6908
Chondrogenic markers			
ACAN	Aggrecan	- 62 (NM_001135) - 62 (NM_013227) - 62 (XM_001131727) - 62 (XM_001131734) - 62 (XM_00670419)	176
COL2A1	Collagen type 2 alpha 1	- 95 (NM_001844) - 95 (XM_006719242)	1280
SOX9	SRY (sex determining region Y)-box 9	- 112 (NM_000346)	6662
De-*differentiation markers*			
COL1A1	Collagen type 1 alpha 1	- 118 (NM_000088) - 118 (NM_003257058) - 118 (XM_005257059) - 118 (XM_006721703)	1277
Hypertrophic markers			
ALK1	Activin A receptor type II-like 1	- 74 (NM_000020)	94
COL10A1	Collagen type 10 alpha 1	- 91 (NM_000493)	1300

Table 7: Programme used during qRT-PCR.

Process:	**Stage:**	**Temperature (°C):**	**Time (mins):**	**Cycles:**
Activation of SYBR Green	Activation	95	10:00	1
Separation of DNA strands and building of cDNA	Denaturation	94	00:10	40
	Annealing	55	00:30	
	Extending	72	00:34	
SYBR green analysis of target genes	Dissociation	94	00:10	1
		55	00:30	
		94	00:10	

The Quant Studio Design and Analysis™ Software (Applied Biosystems, California, USA) was used to determine cycle threshold (CT) Values and the baseline of which cycles had no or little signal. The comparative C_T method ($2^{-\Delta CT}$) was used to determine the relative expression profiles of each gene of interest (Schmittgen and Livak, 2008). Data was expressed relative to the two reference genes as per the following equation:

$2^{-\Delta CT}$ $\Delta CT = CT_{\text{GENE OF INTEREST}} - CT_{\text{COMBINED REFERENCE GENES}}$

2.1.14 Histological analysis of chondrogenic pellets

2.1.14.1 Sample preparation for histological analyses

Pellets were placed onto 1 cm^2 pieces of Whatman filter paper (Whatman, Maidstone, UK) and snap frozen in liquid nitrogen cooled n-hexane and stored at -80 °C. All solutions and products used for sectioning were obtained from Cell Path Ltd, Newtown, USA.

Frozen pellets were mounted onto pre-cooled chucks with optimal cutting temperature compound and secured with cryospray. Sections of 7 µm

thickness were cut using the cryostat (Bright, Luton, UK; Model: OTF) and collected onto poly-L-lysine pre-coated slides. Three sections were collected per slide. Sections were stained with Haematoxylin and Eosin (H&E) or Toluidine Blue according to our established methods (Roberts and Menage, 2004). All histological stains were procured from VWR International, Pennsylvania, USA.

2.1.14.2 Haematoxylin and Eosin (H&E) staining

H&E stain is used to evaluate the general morphology of tissue or pellet samples. As haematoxylin is positively charged it reacts with negatively charged components and stains these blue-black, such as nucleic acids in the nucleus. Eosin is negatively charged and therefore reacts with positively charged components and stains these pink, such as the cytoplasm and ECM (Roberts and Menage, 2004).

To perform the H&E stains, slides of sectioned pellets were flooded in Mayer's Haematoxylin Gurr for 1-2 minutes. Excess dye was removed and slides were washed in a running flow of tap water. Slides were transferred into a coplin jar of tap water for 5 minutes to allow tissue sections to 'blue'. Slides were flooded in 1% Eosin for 1 minute and then washed in tap water. Slides were dehydrated through increasing concentrations of isopropanol from: 70%, 90%, 100% in which the slides were left for 2 minutes, after which the slides were placed into a fresh jar of 100% isopropanol for a further 2 minutes then cleared in xylene for 5 minutes and then transferred into a fresh jar of xylene for a further 5 minutes. Slides were mounted with a coverslip using pertex (Histolab Products, Othenburg, Sweden) and left to air-dry for at least 1 hour.

2.1.14.3 Toluidine blue staining

Toluidine blue is a thiazine metachromatic dye used to visualise proteoglycans in cartilage tissue. The dye changes from a blue to a purple colour when attached to GAGs within the tissue ECM (Horobin, 2010).

To perform the toluidine blue stains, slides of sectioned pellets were flooded in 1% aqueous toluidine blue for 30 seconds. Excess dye was removed and slides were washed in a running flow of tap water. Excess water was removed and slides were left to air-dry for at least 30 minutes. Slides were mounted with a coverslip using pertex and left to air-dry for at least 1 hour (Roberts and Menage, 2004).

Once dry, the mounted tissue sections were viewed using the Leitz Diaplan 307-148-001 light microscopy (Leitz, Stuttgart, Germany) to assess the cell and tissue morphology of the stained structures. Images were taken using ×6.3, ×25, ×40 and ×63 objective lenses. Images were captured using the Nikon DS-Fi1 camera (Nikon, Tokyo, Japan) and analysed with NIS-Elements BR imaging software (Nikon, Tokyo, Japan; version 3.2). Images were white balanced using Photoshop Elements (Adobe, California, USA; version 15).

2.1.14.4 Semi-quantification of toluidine blue staining intensity

To semi-quantify the toluidine blue staining intensity, four central images of each pellet were randomly selected and the integrated intensity was determined using ImageJ Software (National Institute of Health, Washington, USA; version: 24) according to published methodology (Jensen, 2013; Owida et al., 2017; Prasad and Prabhu, 2012). Using ImageJ software an area of stained ECM was

selected and measured for integrated density. An area of slide background containing neither cells nor ECM was selected and measured for integrated density. The total staining intensity was then calculated accounting for the background using the following formula:

Total staining intensity = Integrated density $_{ECM}$ – integrated density $_{BACKGROUND}$

2.1.14.5 Histological grading

The BERN score was used to grade the histological quality of the chondrogenic pellets (Table 8; page 72). This score accounts for uniformity and intensity of ECM staining, cell density/extent of the ECM produced and cellular morphologies. There are three set criteria, each given equal weight in the BERN scoring system. The minimum score is 0 indicating the poorest histological quality and the maximum score is 9 indicating the highest histological quality. There is a reported relationship between the BERN score and the GAG/cell content within pellets, which generally indicates the lower the BERN score, the lower the GAG/cell score (Grogan, *et al;* 2006).

Table 8: The BERN score used to grade histological quality of the chondrogenic pellets.

Scoring category:	Assessment:	Score:
A – uniformity and darkness of stain Observed using a ×10 objective lens	No stain	0
	Weak staining of poorly formed ECM	1
	Moderately even staining	2
	Even dark staining	3
B – distance between cells/amount of ECM accumulated Observed using a ×20 objective lens	High cell densities with no ECM in between	0
	High cell densities with little ECM in between	1
	Moderate cell density with ECM	2
	Low cell density with moderate distance between cells and an extensive ECM	3
C – cell morphologies represented Observed using a ×40 objective lens	Condensed/necrotic/pycnotic bodies	0
	Spindle/fibrous	1
	Mixed spindle/fibrous with rounded chondrogenic morphology	2
	Majority rounded/chondrogenic	3
Total:		/9

2.1.15 Statistical methods for biochemical and gene expression tests

All independent outcomes (PDTs, cell number, sGAG, total collagen, q-RTPCR and staining intensity), patient variability and differences between conditions over the experimental time courses were analysed using multilevel modelling in Statistical Package for the Social Sciences (SPSS) statistical software package (IBM, New York, USA; version 24). In this model, condition (either supplement type or cartilage source) and time points were considered fixed variables. Patient

was considered a random intercept variable to accommodate for natural patient variability. All qRT-PCR data was log-transformed before any statistical analysis to make the data fit a normal distribution. Log-transformed data was used for reporting the qRT-PCR data.

Cell number, sGAG and total collagen data were expressed as mean ± standard error of the mean (SEM). Gene expression data was shown as a box plot with the box representing the second and third quartiles, the horizontal line inside the box representing the median and the whiskers either side of the box representing the lower and upper quartiles. All statistical analyses were performed with SPSS statistical software and for all analyses $p \leq 0.05$ was deemed to denote significance.

Chapter 3: Investigating the impact of Stemulate™, a type of human platelet lysate, on the expansion and chondrogenic capacity of cultured human chondrocytes for cartilage cell therapy.

3.1 Introduction

To obtain sufficient cell numbers from small cartilage biopsies for use in cell therapy, chondrocytes need to undergo *in vitro* expansion, which usually means the chondrocytes undergo 2-3 passages. De-differentiation is a key limitation in ACI, as chondrocytes lose their chondrogenic phenotype (Benya and Shaffer, 1982; Schnabel et al., 2002). Consequently, de-differentiation decreases the capacity of re-implanted chondrocytes to regenerate functional cartilage (Schulze-Tanzil, 2009). To reduce the risk of de-differentiation, RJAH culture the majority of freshly isolated chondrocytes up to passage 2 to achieve sufficient cell numbers for clinical cell therapy, with a small percentage of approximately 15% of patients having their cells cultured up to passage 3 (Harrison et al., 2000). Complete de-differentiation is known to occur in as few as three passages (Kang et al., 2007). Nevertheless, further minimising of conditions which promote chondrocyte de-differentiation may improve ACI and this includes reducing the number of passages the chondrocytes undergo.

Current standardised protocols for chondrocyte expansion involve culture in serum-free media, or growth medium supplemented with FBS or human autologous serum. Sera is commonly used as it is known to support cell growth and proliferation, in addition to stabilising detoxifying factors, including pH and

aiding transport of protein carrying hormones. FBS is traditionally used in research for *in vitro* expansion of many different cell types including chondrocytes.

However, there is a high demand to find alternatives to the use of animal sera, such as FBS, in cell research. FBS is an ill-defined cocktail of growth factors, cytokines and adhesion molecules used to support the basic cell functions, including proliferation, growth and cell adhesion. However, there have been several issues raised regarding the use of FBS in cell culture. One issue is there is a reported high batch-to-batch variation, meaning that batch testing is required prior to conducting research and only the appropriate batch can be used throughout that research project in an attempt to reduce variability (Jayme et al., 1988; Jochems et al., 2002). An increase in demand, from typically 500,000 L per year, and limited stocks are consistently increasing the current cost price. Another issue is the possibility of cross-species contamination and the Good Manufacturing Practice guidelines for treatments stating any animal product must be replaced with a human alternative whenever possible (European Medicines Agency, 2013). These Good Manufacturing Practice guidelines compliment the guidelines set by the National Centre of the Replacement, Refinement and Reduction of Animals in Research (National Centre of the Three Rs, 2011). A final issue raised is concerning the ethics regarding the animal welfare during the production and collection of animal sera. FBS is produced from a cardiac puncture of bovine foetuses collected from pregnant cows at the slaughter house. The cardiac puncture is performed without anaesthetic to minimise risk of serum contamination, but it is thought that the foetuses begin

to feel once exposed to oxygen. A bovine foetus produces approximately 150-550 ml of raw FBS, which increases as the foetus ages (Jochems et al., 2002). Therefore, a human alternative is in high demand. A natural choice for an alternative would be human autologous serum, however this is not always feasible due to the volumes of sera required for long-term cell expansion.

Human platelet lysate (HPL) is one alternative currently available for long-term cell expansion and has been used since the 1980's (Choi et al., 1980). Much of the work conducted into the use of HPL as an alternative to FBS in cell expansion has been studied using Mesenchymal Stem Cells (MSCs) and studies have shown that HPL increases the proliferative rates without comprising the MSC phenotype (Bieback et al., 2009; Juhl et al., 2016). Very little work has been conducted into the effect of HPL on human chondrocytes (Doucet et al., 2005; Hildner et al., 2015; Juhl et al., 2016; Kim et al., 2015).

Stemulate™ is a commercially available source of HPL provided by Cook Regentec (Indianapolis, USA). It contains a cocktail of all essential ingredients to support the requirements of the cell, including: growth, proliferation and cell adhesion. It is prepared in accordance with Good Manufacturing Practice guidelines using large donor pools from accredited blood centres that screen for routine human viruses, including HIV. Stemulate™ has been used to expand a range of different cell types, including adipose tissue-derived MSCs and bone marrow-derived MSCs (Badowski et al., 2017; Juhl et al., 2016; Mangum et al., 2017; Riis et al., 2016; Søndergaard et al., 2017). Despite little research conducted into the effect of Stemulate™ on chondrocytes, it could be used as an alternative supplement to increase chondrocyte proliferation for cartilage cell

therapy. If Stemulate™ did indeed increase the proliferation of articular human chondrocytes, it could reduce time in monolayer culture and reduce exposure to tissue culture plastic, which in turn could decrease the possibility of de-differentiation, and by extension reduce associated cell culture cost.

3.2 Experimental design

Figure 13 (page 78) illustrates the experimental design for this project.

Chondrocytes were isolated following enzymatic digestion and split equally into two sera supplement types: FBS and Stemulate™ and expanded in monolayer up to passage 2. Once approximately 80% confluent at passage 2, chondrocytes were pelleted (2.5 x10^5 cells/pellet) in CM and retained in culture for up to 28 days. The chondrogenic capacity was investigated at 7-day intervals over a 28-day period (day 0, 7, 14, 21, 28) assessed by histological analysis, biochemical analysis (determining cell number, sGAG synthesis and total collagen synthesis) and through gene expression profiles specifically for markers of chondrogenesis, de-differentiation and hypertrophy.

Five knee samples were received from patients undergoing total knee replacement surgery at RJAH. Demographics were recorded for each patient, including: the age and gender of the patient and surgery performed (Table 9; page 79). All cartilage was taken from macroscopically healthy areas of the femoral condyle region of the knee joint.

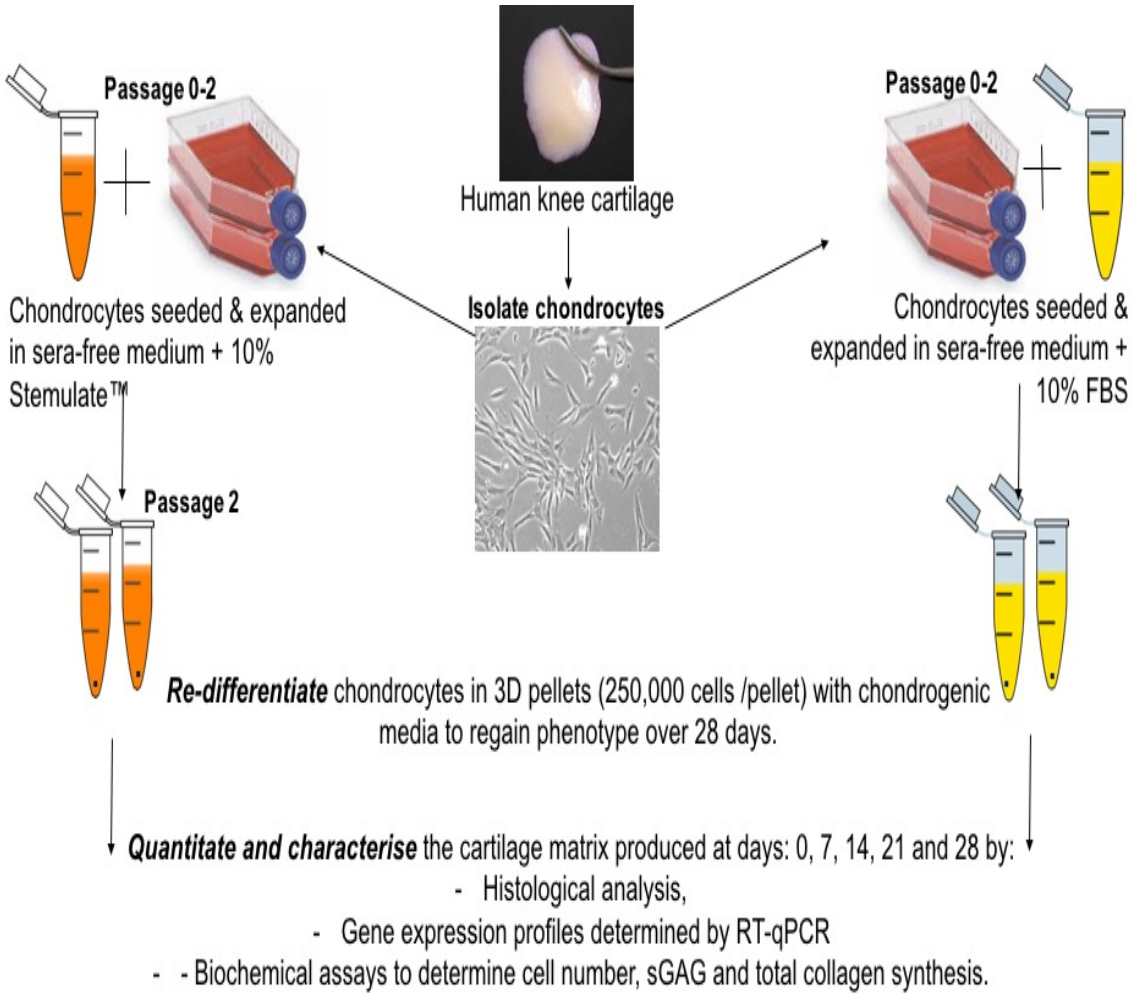


Figure 13: Schematic illustrating the experimental design of the project comparing the chondrogenic capacity of chondrocytes cultured in FBS and Stemulate™ sera supplements.

Table 9: Demographics of the human knee tissue sample donors from which the chondrocytes were obtained.

Gender:	**Age:**	**Procedure:**
Male	55 years	Total knee replacement
Female	62 years	Total knee replacement
Female	74 years	Total knee replacement
Male	70 years	Total knee replacement
Male	76 years	Total knee replacement
Total: 2 females 3 males	Mean age: 67.4 years Age range: 55-76 years	

Isolated chondrocytes (section 2.1.3, page 53) extracted from macroscopically healthy cartilage tissue on the femoral condyle of the knee joint and was split equally into two culture conditions: FBS and Stemulate™. Samples were then cultured in monolayer until passage 2 (section 2.1.4 page 54). Representative images were taken of the knee samples received and are displayed in Figure 14 (page 80).

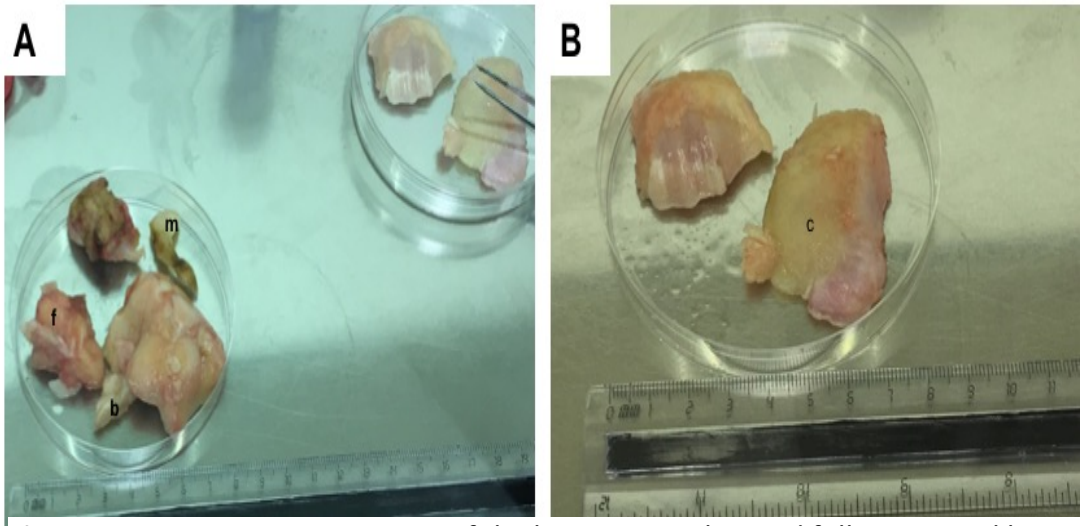


Figure 14: Representative images of the knee tissue obtained following total knee replacement surgery.

(A) All tissue received that was not macroscopically healthy cartilage, such as meniscus (m), bone (b) and fat (f) was discarded. Macroscopically healthy cartilage tissue (c) was extracted using a sterile scalpel and digested to obtain chondrocytes, avoiding any obvious cartilage lesions or artefact damage.

Once confluent at passage 2, chondrocytes were passaged and seeded into three-dimensional pellets ($2.5x10^5$) in chondrogenic media, using methods outlined in section 2.1.8 (page 58). Pellets were maintained in culture for up to 28 days for three patients and 21 days for two patients, with assessments being carried out at days 0, 7, 14, 21 and 28 as outlined in Figure 13 (page 78).

Chondrogenic pellets (n>3) were snap frozen at days 7, 21 and 28. Frozen pellets were sectioned and stained with toluidine blue (section 2.1.14.3, page 70) and H&E (section 2.1.14.2, page 69).

3.3 Results

3.3.1 Stemulate™ positively influences proliferation of chondrocytes in monolayer culture

For all five patients, the chondrocytes expanded in Stemulate™ (13.10 days ± 2.57 SEM mean PDT) proliferated at a significantly quicker rate than the chondrocytes expanded in FBS (25.07 days ± 6.98 SEM mean PDT, p=0.050). Patient variability was considered not significant (p=0.48) in affecting the PDT. Cell number obtained during monolayer expansion (Figure 15) indicated cell number increased over time for both sera supplement types, as expected (p<0.001) but was consistently higher in Stemulate™-expanded chondrocytes (p=0.014) across all passages supported by the increased PDTs.

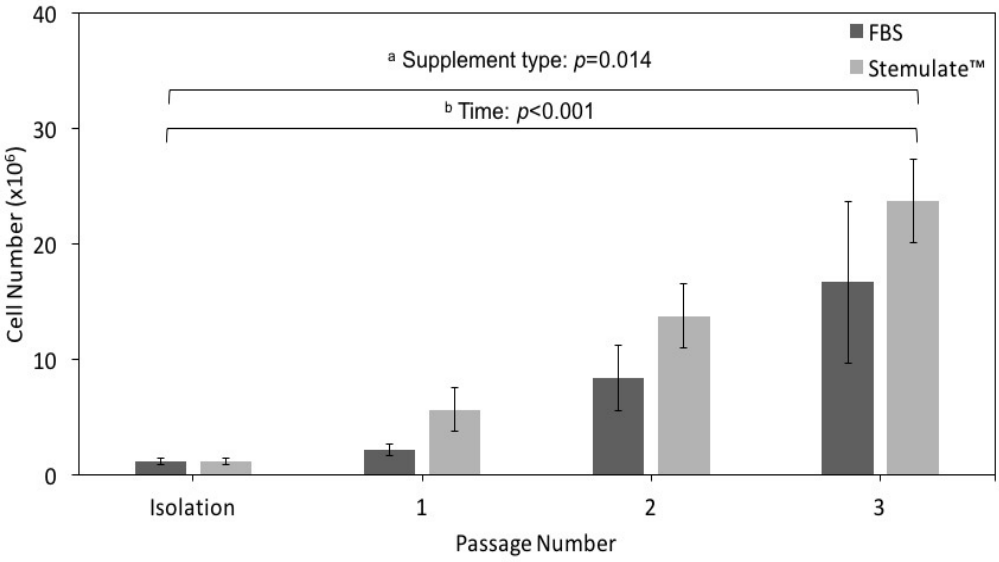


Figure 15: Cell number during monolayer expansion of chondrocytes expanded in FBS and Stemulate™ sera supplements. Cell number was calculated from cell counts during chondrocyte isolation, were seeded at an equal cell density, and across three passages. Data is presented as mean ± SEM and was obtained from five patients in total.

Despite proliferation rates being higher in Stemulate™-expanded chondrocytes, Figure 16 shows both sets of chondrocytes underwent less than 1.5 PDs with no significant difference seen between sera supplement type.

Figure 17 (page 83) shows the morphology of the human chondrocytes, obtained using still images taken by light microscopy at day 4 of each passage (passage 0, passage 1 and passage 2) in the presence of either Stemulate™ or FBS. For Stemulate™-expanded chondrocytes, there were markedly more chondrocytes that appeared to be more clustered together at all passages. There appeared to be little difference in cell shape between the two supplement types, as the chondrocytes appeared elongated and flattened in both sera supplement conditions.

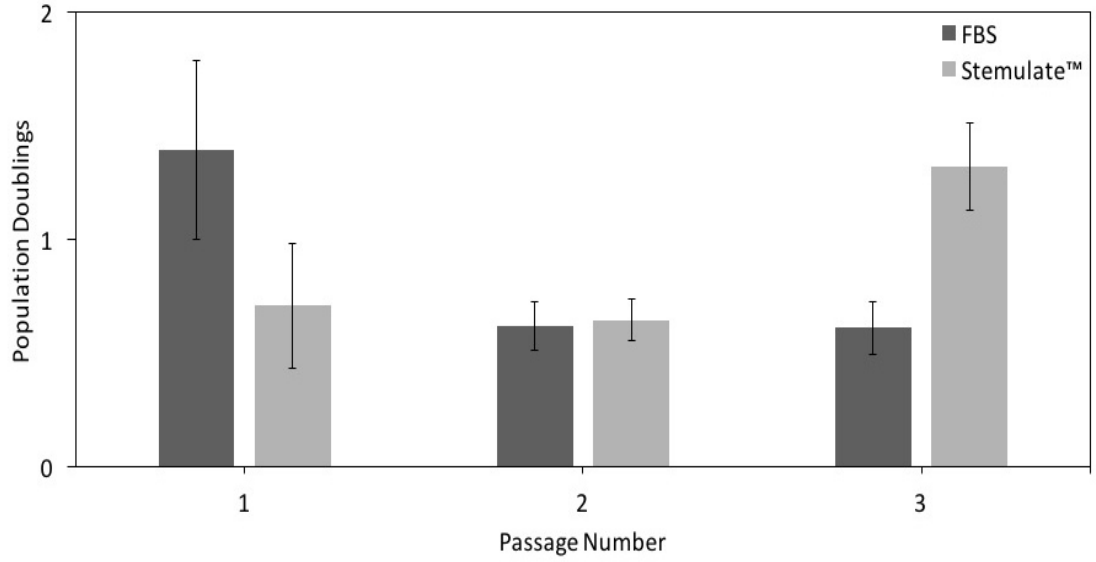


Figure 16: Average absolute population doublings of chondrocytes expanded in FBS and Stemulate™ at each of the three passages. Data is presented as mean ± SEM and was obtained from five patients in total.

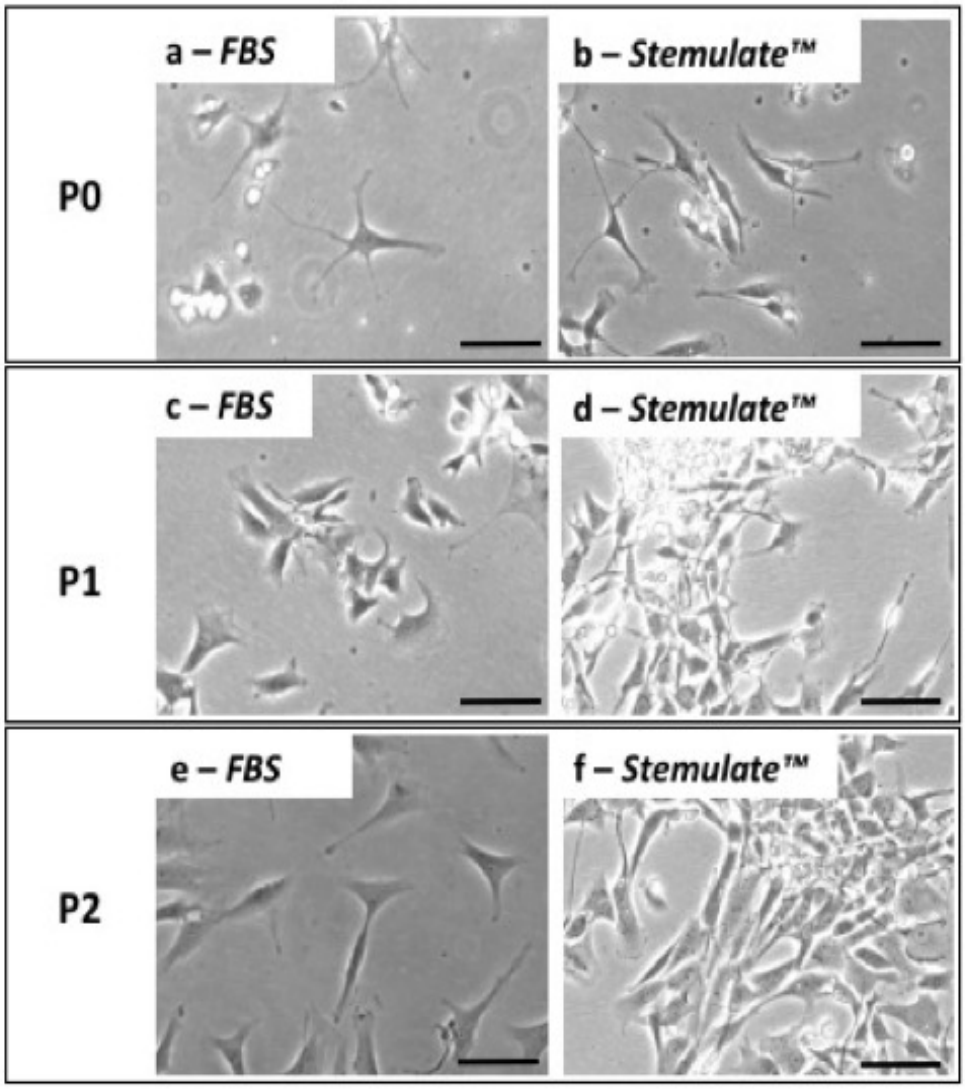


Figure 17: Representative images of chondrocytes expanded in culture media supplemented with 10% (A) FBS and (B) Stemulate™ at each passage. Images were taken at day 4 of each passage to allow the chondrocytes to adhere. All images were taken using a Nikon TS100 light microscope, Hamamatsu C4742-95 camera and ×20 magnification lens. Images were acquired using IPLab 3.2.6 software. All scale bars represent 10 μm.

3.3.2 Stemulate™ negatively influences the re-differentiation potential of chondrocytes in three-dimensional pellet culture, compared to standard FBS.

To investigate the impact of the sera supplements on the re-differentiation potential of the chondrocytes at the end of monolayer expansion, both sera supplements were removed and chondrocytes were cultured into three-dimensional chondrogenic pellets in chondrogenic media. The pellets were assessed for cell number (Figure 18), total sGAG (Figure 19; page 86), total collagen (Figure 20; page 87), and gene expression by qRT-PCR (Figure 21; page 88).

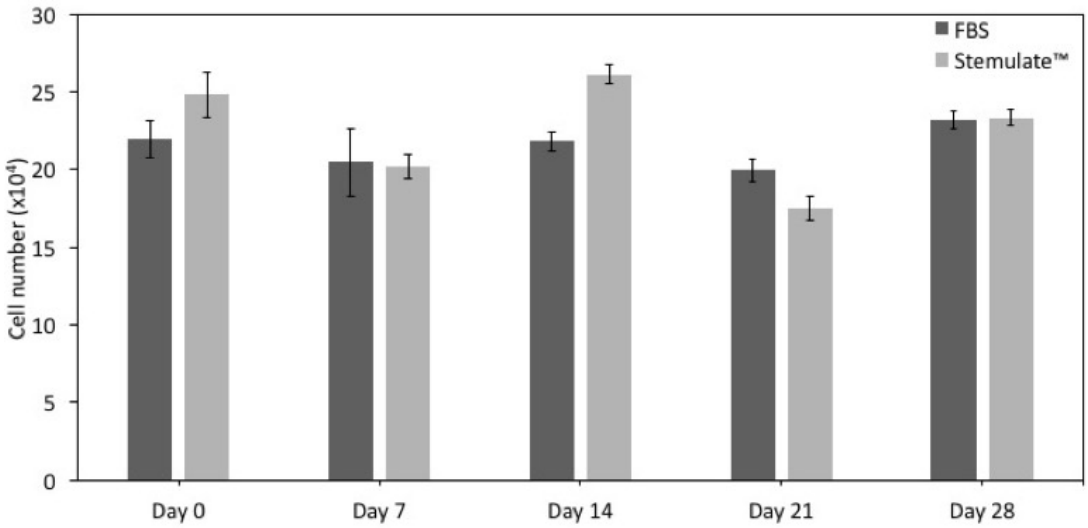


Figure 18: Cell number in chondrogenic pellets created from chondrocytes expanded in FBS and Stemulate™ sera supplement over a 28-day time course. Data is presented as the mean ± SEM and was obtained from five patients in total for day 0 – day 21 and three patients up to day 28.

Cell number was assessed by the PicoGreen® assay throughout the time course (Figure 18). This was to determine if the chondrocytes could recover their

morphology and prevent further proliferation once in chondrogenic pellet structures. For both supplement types, cell number remained consistent throughout the time course, at approximately the number of cells that were originally pelleted (2.5 x10^6*; p*=0.84). Cell number was used to normalise the total sGAG and total collagen production.

Chondrocytes expanded in both supplement types demonstrated a steady and significant increase in sGAG production within the three-dimensional pellet (Figure 19; page 86) across all time points (p<0.001). sGAG concentration was higher in FBS compared to Stemulate™ across all time points (p=0.009), as determined by multi-level modelling. There was a steady increase between days 0 and 7, a significant sharp increase in sGAG concentration between days 7 and 14 (p<0.001) and then appeared to slightly level off at day 28 for FBS-expanded chondrocytes. Whereas, there was a steady increase between days 0 and 21, and then a sharp increase in sGAG concentration between days 21 and 28 in Stemulate™-expanded chondrocytes. The trend for Stemulate™-expanded chondrocytes appeared to lag behind that of FBS-expanded chondrocytes. There was no evidence of variability between individual patients at any time point

(*p*=0.60).

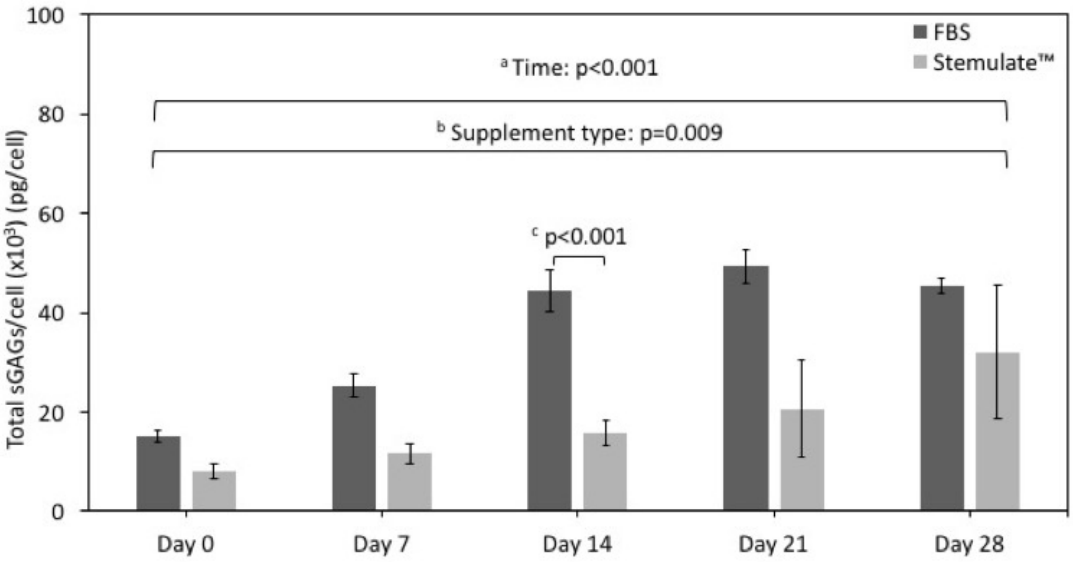


Figure 19: sGAG production per cell in chondrogenic pellets from chondrocytes expanded in FBS and Stemulate™ sera supplement over 28-days. Data is presented as the mean ± SEM and was obtained from five patients in total for days 0–21 and three patients up to day 28. [a, b, c] denotes statistical significance of time, supplement type and at the specific time point of day 14 respectively.

Total collagen production (Figure 20; page 87) demonstrated a similar trend in both sera supplement types. The production levels remained consistent until an increase between day 21 and day 28. Total collagen production demonstrated a significant interaction between treatment and time (*p*=0.001). Total collagen levels were significantly higher in FBS compared to Stemulate™ (*p*=0.001) with FBS-expanded chondrocytes having a sharper increase between day 21 and day 28 than Stemulate™-expanded chondrocytes. There was no evidence of variability between individual patients at any time point (*p*=0.26).

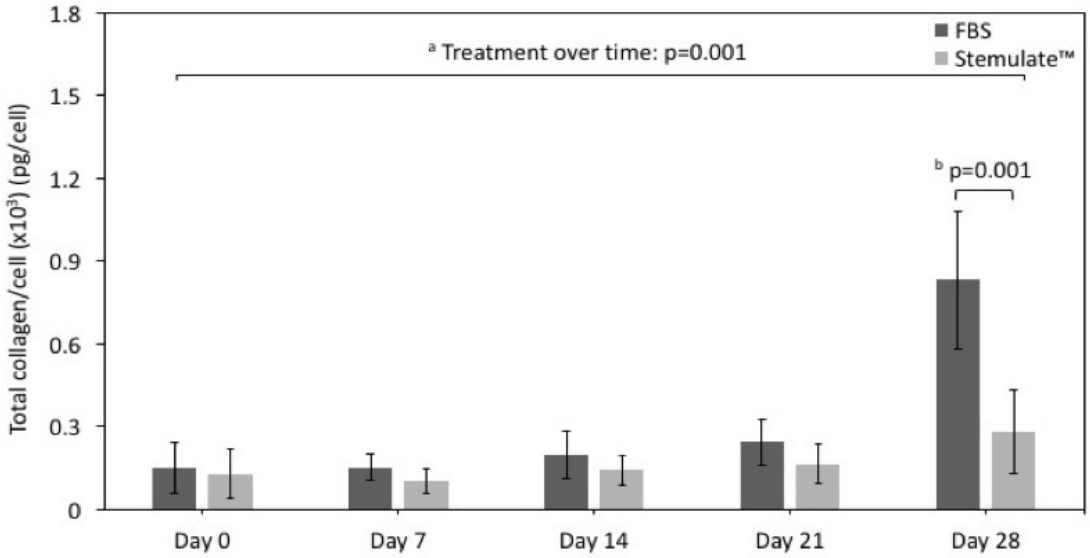


Figure 20: Total collagen production per cell in chondrogenic pellets created from chondrocytes expanded in FBS and Stemulate™ sera supplement over a 28-day time course. Data is presented as the mean ± SEM and was obtained from five patients in total for days 0-21 and three patients up to day 28. [a] and [b] denote statistical significance of time and at the specific time point of day 28

Gene expression profiles were investigated using qRT-PCR to determine the chondrogenic capacity of the pellets and establish whether the pellets were in a hypertrophic or de-differentiated state (Figure 21; page 88).

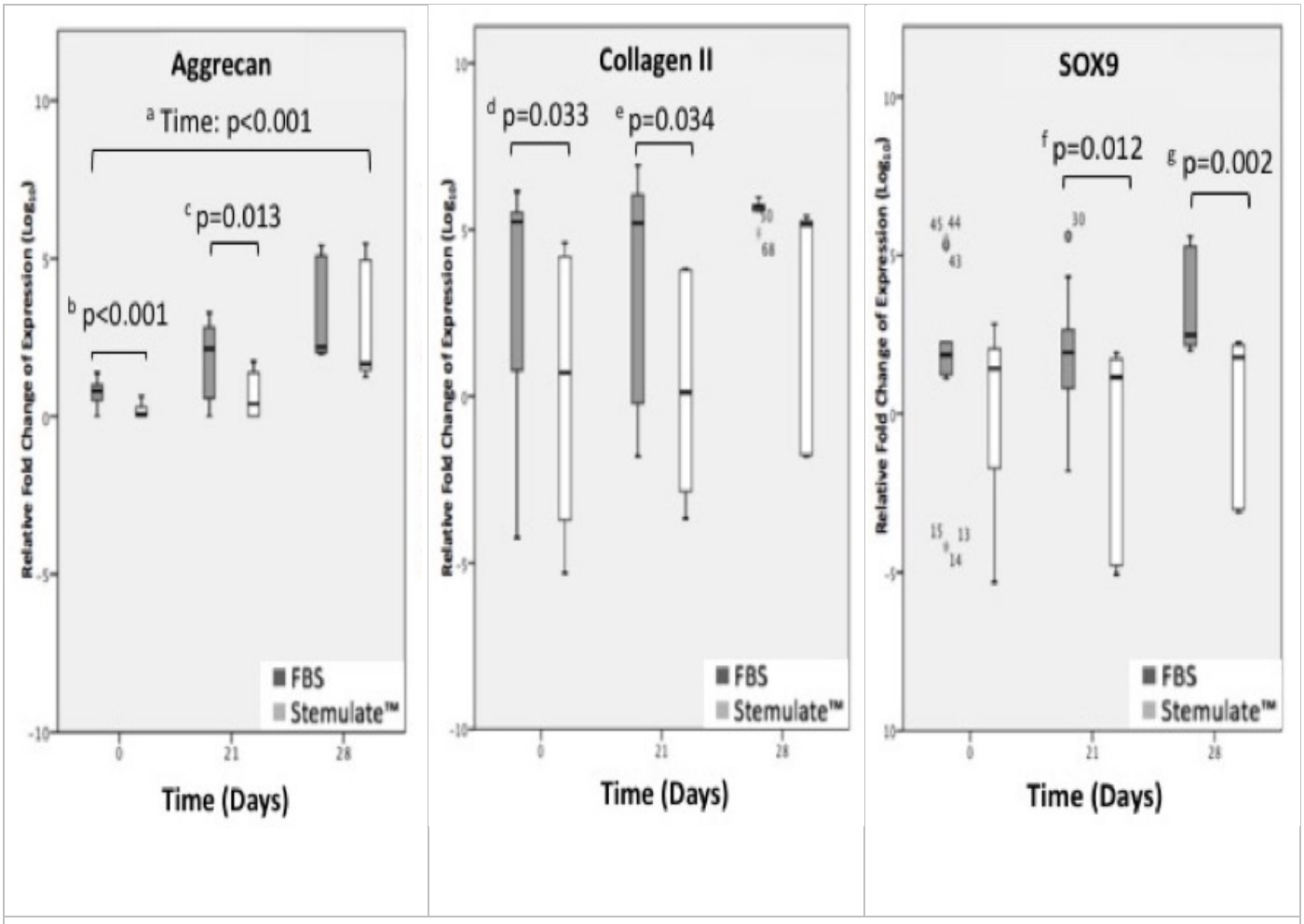


Figure 21: Box and whisker plots of the gene expression profiles of the chondrogenic pellets created from chondrocytes expanded in FBS and Stemulate™. Data is expressed relative to two reference genes, normalised to cell number and was obtained from five patients using three experimental pellet replicates per patient. [a-g] denotes significance of time, significance of ACAN expression at day 0, significance of ACAN expression between supplement types at day 21, significance of COL2A1 expression between supplement type at day 0, significance of COL2A1 expression between supplement types at day 21, significance of SOX9 expression between supplement types at day 21 and significance of SOX9 expression between supplement type at day 28 respectively.

Gene expression profiles was determined by the comparative C_T method (Schmittgen and Livak, 2008) at days 0, 21 and 28. Data was expressed relative to two reference genes and normalised to cell number. No gene expression of COL1A1, COL10A1 or ALK1 were observed in either supplement type. There were striking differences between gene expression profiles for each serum supplement types, for the key chondrogenic markers: ACAN, COL2A1 and SOX9.

ACAN expression increased over the time course in both supplements (p<0.001 for time) but was significantly higher in FBS-expanded pellets than Stemulate™-expanded pellets at day 0 (p<0.001) and day 21 (p=0.013), with day 28 being equal. There was no evidence for a general increase in COL2A1 expression over time (p=0.20 for time). However, COL2A1 expression was significantly higher in FBS cultured cells than that seen in Stemulate™-expanded pellets at day 0 (p=0.033) and day 21 (p=0.034), with day 28 again being equal. Similar to COL2A1, there was no evidence for a general change over time in SOX9 expression (p=0.15 for time). SOX9 expression was similar at day 0 in both sera supplements, then FBS-expanded pellets demonstrated a significantly higher expression of SOX9 at day 21 (p=0.012) and day 28 (p=0.002) when compared with Stemulate™-expanded pellets. As the samples obtained were from humans, significance of patient variability was determined using mixed model analysis. There was no evidence of variability between individual patients for any of three key chondrogenic markers: ACAN expression (p=0.48), COL2A1 expression (p=0.33), or SOX9 expression (p=0.203)

3.3.3 FBS improves the quality of the ECM produced in three-dimensional pellet culture

Frozen chondrogenic pellets at day 7, day 21 and day 28 were sectioned at a thickness of 7 µm using a Bright cryostat (Bright, Luton, UK; Model: OTF). Central sections were stained with H&E to show general morphology or toluidine blue to show proteoglycan distribution (Figure 22; page 92). At day 7, chondrocytes expanded in Stemulate™ appeared to form less stable and more fragile pellets compared with chondrocytes expanded in FBS. Morphological differences were apparent in the chondrocytes occupying the periphery of the pellets. These cells appeared to more fibroblastic in nature in pellets created from the Stemulate™-expanded chondrocytes compared to those created from FBS-expanded chondrocytes.

Histological grading of the toluidine blue stained chondrogenic pellets were assessed at day 7, day 21 and day 28 using the BERN scoring system. FBS-expanded chondrocytes formed chondrogenic pellets of a higher mean histological grading across all three time points (Table 10; page 91). The range of scores obtained from several pellet replicates demonstrated that the chondrogenic pellets formed by the Stemulate-expanded chondrocytes had consistently lower scores than the chondrogenic pellets formed by the FBS-expanded chondrocytes, indicating lesser histological quality.

Table 10: Histological grading of the FBS-expanded and Stemulate™-expanded chondrogenic pellets at day 7, day 21 and day 28.

Time point:	**FBS grading:**	**Stemulate™ grading:**
Day 7 Day 21	7 9	6 7
Day 7 Day 21	7 9	0 7
Day 7 Day 21 Day 28	8 7 9	6 6 6
Day 7 Day 21 Day 28	8 8 8	7 6 6
Day 7 Day 21 Day 28	7 9 8	6 6 5
Mean grades Day 7 Day 21 Day 28	 7.4 8.4 8.3	 5 6.4 5.6
Range of grades: *Day 7* *Day 21* *Day 28*	7-8 7-9 8-9	0-6 6-7 5-6

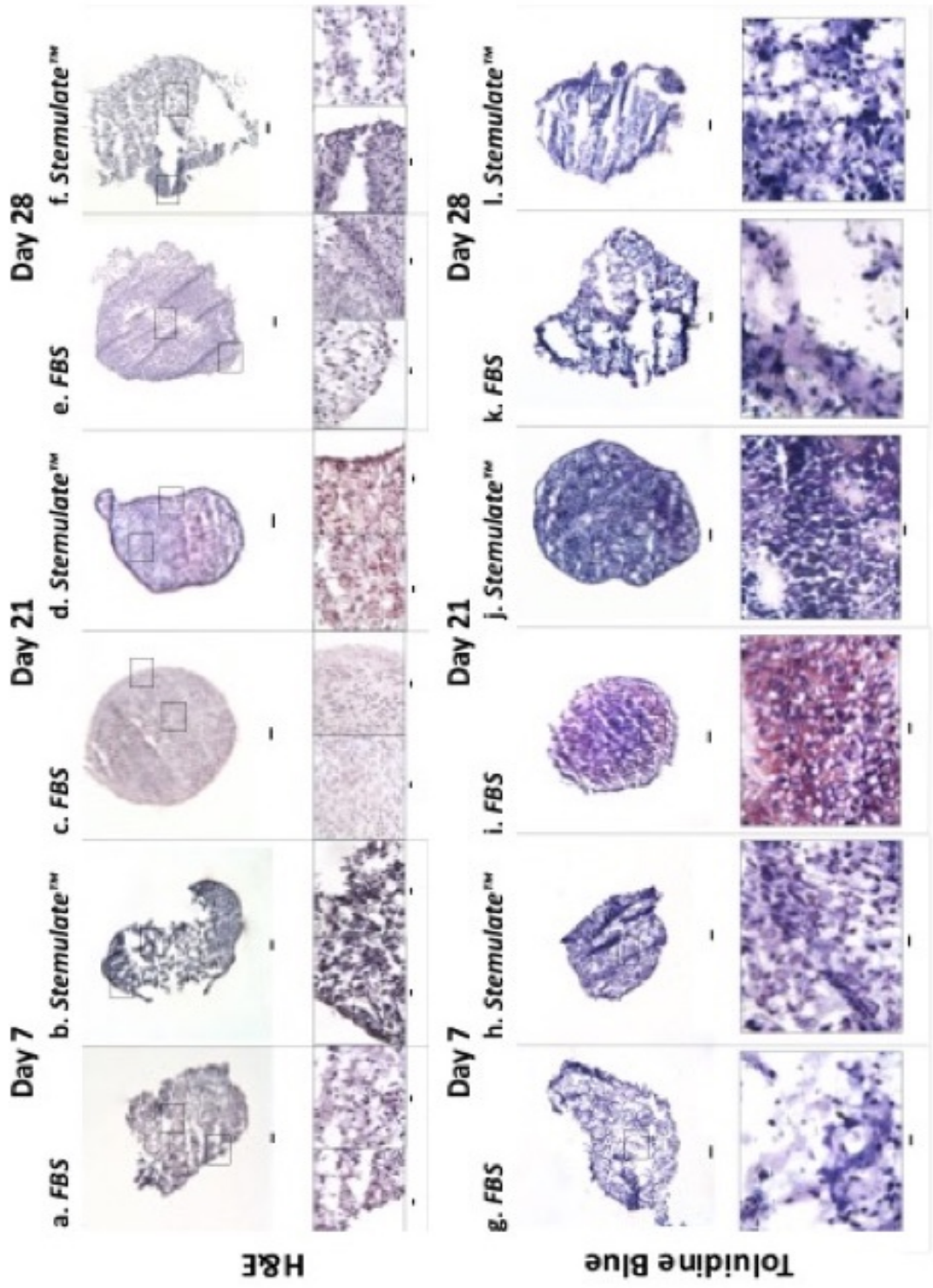


Figure 22: Representative histological images from the centre of FBS-expanded chondrogenic pellets and Stemulate™-expanded chondrogenic pellets across the 28-day time course, stained with H&E to demonstrate morphology and toluidine blue to demonstrate proteoglycan content. A-F: Pellets stained with H&E to assess overall morphology. G-L: Pellets stained with toluidine blue to assess proteoglycan content. Insert images are higher-powered images of the regions marked with a solid line to demonstrate the centre and edges of the chondrogenic pellets. A, B, G, H are pellets at day 7. C, D, I, J are pellets from day 21. E, F, K, L are pellets from day 28. All scale bars represent 100 μm.

The toluidine blue staining intensity was semi-quantified in both sera supplement types using ImageJ analysis as previously described (Figure 23). A similar trend was observed in both sera supplement types, in which the staining intensity of the chondrogenic pellets increased over time. Pellets created from FBS-expanded chondrocytes demonstrated an increase in metachromasia indicated by a decreased staining intensity in Figure 23.

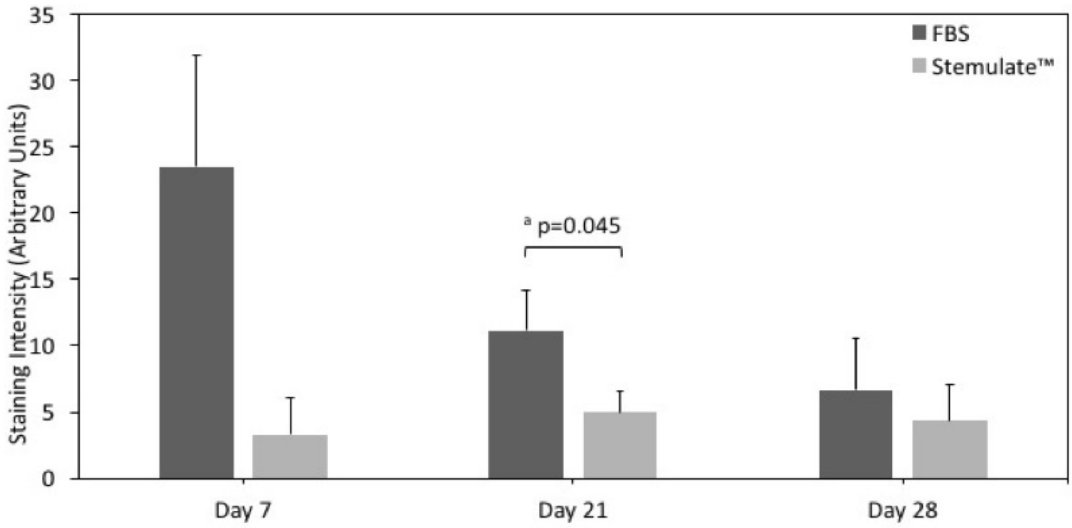


Figure 23: Semi-quantitation of toluidine blue-stained chondrogenic pellets demonstrating an increase in metachromasia, determined by integrated density analysis using ImageJ imaging software (National Institute of Health, Washington, USA; version: 24). [a] denotes significance of staining intensity at day 21. Data is presented as the mean ± SEM and was obtained from five patients for days 0-21 and three patients up to day 28.

This increase was semi-quantitated using ImageJ software and confirmed that FBS-expanded chondrocytes had a significantly more intense

staining at day 21 compared to Stemulate™-expanded chondrocytes at the same time point (p=0.045). The staining intensity was at comparable levels for both sera supplement types at day 28 (p=0.911). Despite there being obviously a higher staining intensity in FBS-expanded chondrocytes compared to Stemulate™-expanded chondrocytes at day 0, this was shown to be non-significant statistically due to high variation in pellet staining (p=0.269), which could be possibly due to an anomalous patient at day 0 and day 21, which was then removed at day 28 resulting in tighter error bars and no statistical significance. As with all the other analysis, there was no evidence of variability between patients for staining intensity (p=0.48).

3.4 Discussion

This study focussed on two sera supplement types: FBS and Stemulate™ and investigated their effects on human chondrocyte proliferation and the subsequent recovery of their chondrogenic capacity using standard chondrogenic pellet differentiation protocols. Our findings provide a detailed analysis of ECM production from five individual patients and to our knowledge there is no other study available providing such insights.

Five key points were demonstrated during this study. First, when in monolayer culture Stemulate™ increased chondrocyte proliferation compared to FBS as highlighted significantly quicker PDT. Stemulate™ had a PDT of 13.10 days ± 2.57 compared to FBS which had a PDT of 25.07 days ± 6.98 (p=0.050). This is not surprising, as it was expected that Stemulate™ would cause similar results to HPL, that has been reported by several studies to increase cell proliferation in

many cell types. However, this is the first study to show Stemulate™ causes an increased cell proliferation in human chondrocytes. The second key finding was that monolayer expansion of human chondrocytes in Stemulate™ negatively influenced the stability of the three-dimensional pellets compared to human chondrocytes expanded in FBS. Chondrogenic pellets formed from Stemulate™-expanded chondrocytes were more fragile and seemed to take longer to become stable. This was particularly evident in the cells occupying the periphery of the pellets in the which appeared to be more fibroblastic in nature within the Stemulate™-pellets compared to the FBS pellets. The third key finding of this study was that total sGAG and total collagen synthesis by Stemulate™-expanded chondrocytes in chondrogenic pellet culture lagged behind FBS-expanded human chondrocytes in chondrogenic pellet culture. Despite both sera supplement types following a similar trend of increasing sGAG synthesis throughout the time course ($p<0.001$), Stemulate™-expanded chondrocytes consistently displayed lower sGAG production compared to FBS-expanded chondrocytes. FBS-expanded chondrocytes had a significantly sharp increase in sGAG production between day 7 and day 14 ($p<0.001$) whereas Stemulate™-expanded chondrocytes only the sharpest increase between day 21 and day 28. This was further supported by total collagen synthesis also increasing over time ($p=0.001$). Total collagen synthesis was comparable levels for both sera supplement types until day 28, where total collagen synthesis displayed an increase in both sera supplement types. Similar to sGAG synthesis, there was a significantly sharper increase in total collagen synthesis at day 28 in the FBS-expanded chondrocytes compared to the Stemulate™-expanded chondrocytes. ($p=0.001$)

The fourth key finding of this study was that expanding chondrocytes in Stemulate™ negatively influenced chondrogenic gene expression in three-dimensional pellets compared to expansion in FBS. Markers of hypertrophy and de-differentiation were not seen in either sera supplement. There were striking difference in gene expression of key chondrogenic markers between the two sera supplement types. Although ACAN expression increased over time in both supplement types ($p<0.001$), it was significantly higher in FBS-expanded pellets compared to Stemulate™-expanded pellets at day 0 ($p>0.001$) and day 21 ($p=0.013$). Whereas, COL2A1 expression remained consistent across time in both sera supplement type but was significantly higher in FBS-expanded pellets compared to Stemulate™-expanded pellets at day 0 ($p=0.033$) and day 21 ($p=0.034$). In contrast, SOX9 expression remained fairly consistent across time in both sera supplement types but was consistently higher in FBS-expanded pellets compared to Stemulate™-expanded pellets at day 21 ($p=0.012$) and day 28 ($p=0.002$).

The final key finding of this study was that FBS-expanded chondrocytes produce higher quality chondrogenic pellets compared to Stemulate™-expanded pellets, when assessed histologically. This supports the findings of higher total sGAG and total collagen synthesis in FBS-expanded pellets and is evident in the BERN scores and the semi-quantitative analysis of the toluidine blue stained pellets.

Studies investigating the effects of HPL have been performed using MSCs. These studies have provided clear evidence to confirm that 5-10% (v/v) HPL is more effective at supporting monolayer expansion of human MSCs than both

FBS and autologous serum at the same concentration range (Bieback et al., 2009; Juhl et al., 2016; Trojahn Kølle et al., 2013). Further, both Bieback et al. (2009) and Juhl et al. (2016) reported that HPL supported long term monolayer expansion whilst still maintaining the MSCs phenotype and differentiation potential. Currently, no published studies have used Stemulate™ for chondrocyte expansion but increased proliferation has been shown using derivatives of HPL (Bieback et al., 2009; Juhl et al., 2016). Given these positive data in MSCs and lack of published data in chondrocytes, we chose to investigate Stemulate™ in human articular chondrocytes. Herein we established that Stemulate™ was better than FBS when it came to increasing chondrocyte proliferation in monolayer culture.

Studies using HPL derivatives have provided a range of contrasting results. Spreafico et al. (2009) compared the effects of platelet rich plasma, platelet poor plasma and FBS on human chondrocytes over a 20-day cell proliferation time course (Spreafico et al., 2009). Platelet rich plasma was found to be the most stimulatory in terms of chondrocyte proliferation. More recently, Hildner et al. (2015) reported that 5-10% HPL significantly increased human chondrocyte proliferation compared to FBS (Hildner et al., 2015). From these studies and our own, it is evident that HPL increases the proliferation of human chondrocytes in monolayer culture.

In our study, we found no morphological differences in human chondrocytes that were monolayer expanded in Stemulate™ or FBS. No other published study has investigated morphological differences between human chondrocytes expanded in HPL or FBS. However, Trojahn-Kolle et al. (2013)

reported morphological differences with monolayer expanded MSCs appearing smaller and less spindle-like when cultured in HPL compared with FBS (Trojahn Kølle et al., 2013). It was noted from our study, that three-dimensional pellets created from human chondrocytes monolayer expanded in Stemulate™ did create more fragile, less compact pellets compared to those expanded in FBS.

Very few studies have used HPL with human chondrocytes and even fewer have investigated the quality of the ECM produced in three-dimensional pellet cultures. Following a published three-dimensional pellet chondrogenic differentiation protocol (Johnstone et al., 1998; Pittenger, 1999) this study demonstrated that FBS-expanded human chondrocytes generated significantly more sGAG than Stemulate™-expanded human chondrocytes across a 28-day time course. These results oppose the findings of Hildner et al. (2015) who reported that HPL-expanded chondrocytes produced more sGAG than FBS-expanded chondrocytes in micromass pellets over a 5-week period, created using the same chondrogenic differentiation protocol that was used in our study (Hildner et al., 2015). These contrasting differences could be accounted for by the difference in supplement types since we used Stemulate™ whereas Hildner et al. used HPL generated in their own laboratory from platelet rich plasma obtained from 36 expired thrombocyte concentrates produced at a transfusion blood centre. Five batches of HPL were characterised by Quantikine ELISA for these experiments but no further details on the characterisation was given (Hildner et al., 2015). Another difference is that in our study, the three-dimensional pellets were cultured in chondrogenic medium without HPL whereas Hildner et al. continued to add HPL into three-dimensional pellet

cultures.

In contrast to sGAG synthesis levels, this study found total collagen production levels to be quite low throughout the three-dimensional pellet culture 28-day time course. Despite this, it was observed that total collagen production by Stemulate™-expanded human chondrocytes appeared to lag behind FBS-expanded human chondrocytes. Gaissmaier et al. (2005) also found HPL-expanded chondrocytes seeded into three-dimensional alginate beads produced low levels of collagen over a shorter time course of 14 days (Gaissmaier et al., 2005).

In the three-dimensional pellet cultures, we also investigated a range of genes which determine chondrocyte differentiation state. We investigated de-differentiation through the expression of COL1A1 (Benya et al., 1978; Brew et al., 2010) and hypertrophy through the expression of COL10A1 (Caron et al., 2012; Dell'Accio et al., 2001) and ALK1 (Blaney Davidson et al., 2009; Dell'Accio et al., 2001). ALK1 is up-regulated during monolayer expansion due to a loss of *in vivo* cartilage formation potential and is associated with irreversible chondrocyte de-differentiation (Blaney Davidson et al., 2007; Blaney Davidson et al., 2009; Dell'Accio et al., 2001). None of these genes were detected in three-dimensional pellets derived from either Stemulate™ or FBS suggesting that our human chondrocytes had not undergone de-differentiation or hypertrophy. In addition, we assessed chondrogenic capacity through the expression of COL2A1, ACAN and SOX9. COL2A1 and ACAN are necessary for ECM formation and the transcription factor SOX9 regulates chondrocyte proliferation, chondrogenesis and transition to a non-hypertrophic state. Expression of COL2A1 and ACAN

genes are known to decline following prolonged monolayer culture, particularly after passage 4 (Schulze-Tanzil et al., 2002). For both these ECM markers, we found an increase across our 4-week time course. However, we expected a much larger increase in gene expression, especially by d 28 as found in similar published studies (Hildner et al., 2015). One reason for this could be that we were approaching nutrient saturation before d 28. Overall, SOX9 gene expression was more striking in FBS three-dimensional pellets compared to those pellets maintained in Stemulate™, indicating that the FBS-expanded human chondrocytes recovered chondrogenic potential more quickly. Taken together, our findings suggest that Stemulate™ causes a delay in the recovery of chondrogenic phenotype following monolayer expansion. It would be interesting for future studies to explore gene expression levels in non-cultured chondrocytes so comparisons could be made between freshly isolated chondrocytes and cultured chondrocytes to determine culture effects on these gene expression levels.

The multilevel modelling analyses performed in our study have allowed us to compare between the sera supplement types over the time course and explore the impact of patient variability (Hox, 2010; Vaughn, 2008). One limitation of this study was that the cartilage obtained was from joints that were potentially osteoarthritic and undergoing joint replacement surgery. Osteoarthritis is classified as a whole joint disease and so although the cartilage was taken from macroscopically healthy areas, chondrocytes could still have a slightly altered phenotype and this should be taken into consideration when analysing the results (Poole, 2012). To explore this possibility further, ALK1 gene

expression was investigated as previous studies have shown that chondrocytes that are osteoarthritic or undergoing hypertrophy express higher levels of ALK1. In this study, ALK1 expression was not detected at day 0, day 21 or day 28 in monolayer-expanded chondrocytes expanded in either sera supplement (Blaney Davidson et al., 2009; Finnson et al., 2008).

Some patient variability was expected within our results as the samples being used were human derived, but in these experiments, we found no statistical evidence for a patient-specific influence on any of the outcomes measured. However, patient variability within this study was assessed across a small sample size of just five patients. The sample size of five patients is one of the limitations of this study. If the sample size was increased to a larger donor pool the patient variability assessed would be more reliable and may alter the significance of patient variability. However, we found no statistical evidence for a patient-specific influence on any of the outcomes measured within this study. An example of further investigation that could elucidate some findings further, would be to utilise patient demographics further, for example BMI, smoking status and activity levels. These were not determined for this study, but could be obtained from patient electronic medical records and used in future investigations.

There were other limitations to this project. One limitation is that we were not able to compare autologous serum for each individual because our existing ethically approved protocol did not allow us to take a sufficient volume of blood to provide sera that would support monolayer *in vitro* expansion to the end of passage 2 to ensure efficient cell numbers for our experiments. Another

limitation is that we have only evaluated chondrocytes obtained from total knee replacement surgery. This means that we should be cautious in how we interpret these data compared with chondrocytes sourced from patients undergoing ACI, as the patient populations for these treatments differ. For example, patients undergoing ACI tend to be younger than those undergoing joint replacement surgery. Despite these limitations, we were able to demonstrate the five key points mentioned above.

The findings of this thesis chapter demonstrate, for the first time, that Stemulate™ does increase the proliferation rates of human articular chondrocytes in monolayer. Stemulate™ has also been proven to negatively impact upon the re-differentiation potential when the expanded chondrocytes were seeded into three-dimensional pellet cultures, evidenced by an altered phenotype mimicking a more fibroblastic nature. The altered phenotype resulting from expansion in Stemulate™ supplement sera was characterised by negatively influenced ECM production compared to FBS-expanded chondrocytes, determined via reduced and delayed synthesis of sGAG, delayed synthesis of total collagen production and reduced proteoglycan content apparent in the reduced toluidine blue staining. The altered phenotype of the Stemulate™-expanded chondrocytes resulted in poorer pellet quality evident in the BERN scores and a more fibroblastic gene expression profile determined by reduced ACAN expression, reduced COL2A1 expression and reduced SOX9 expression. Therefore, these findings provide evidence that Stemulate™ should be used with caution when expanding chondrocytes for clinical use, for example for use in cell therapies including ACI.

There is more work to be done to expand on the findings presented thus far. It would be interesting to perform flow cytometry on the expanded chondrocytes to determine if the cell marker profiles differ and to explore cell cycle changes between the two sera supplement types. Also, expanding qRT-PCR analysis to incorporate the other time points of day 7 and day 14, and show the fold change of expression relative to baseline (day 0) would be interesting, to pinpoint more accurately where the gene is expressed. It is noteworthy that there were apparent vesicles present in the light microscopy images of the monolayer expanded chondrocytes which are of unknown composition and/or origin. Exploring this further could be interesting, especially as the full composition of Stemulate™ is still not disclosed or published. Therefore, one theory could be that these vesicles could be present in the Stemulate™ itself and causing proliferative effects in the chondrocytes, or another theory is that the vesicles are produced by the chondrocytes in response to Stemulate™. Finally, immunohistochemistry for collagen types I and II would provide further information to support the current collagen findings within this thesis chapter, as the collagen findings are for the total collagen production and not the individual collagen types. By characterising between the collagen types, it would provide further information into whether the chondrocytes are undergoing de-differentiation and the quality of the ECM produced, as it is known that collagen type I expression is indicative of de-differentiation and collagen type II is a hallmark of hyaline cartilage.

Future work into the impact of Stemulate™ on the chondrogenic capacity of human articular chondrocytes should cater towards whether other versions of

HPL have a similar or contrasting impact on the chondrogenic phenotype, or whether Stemulate™ does negatively impact the chondrogenic capacity on a short time-scale and needs longer to recover chondrogenic phenotype *in vitro.*

In conclusion, we have demonstrated that although Stemulate™ leads to rapid proliferation of human articular chondrocytes in monolayer, it impacts upon the re-differentiation potential when seeded into three-dimensional pellet cultures (Sykes et al., 2018). These presented findings, the first of their kind, demonstrate that Stemulate™ induces an altered phenotype or enhances monolayer induced chondrocyte de-differentiation and reduces chondrogenic capacity. These findings are clinically important, particularly in cell therapies, as it provides new evidence that Stemulate™ should be used with caution when expanding chondrocytes for clinical use as the altered phenotype results in a lesser quality ECM. This in turn would cause speculation that a lesser quality ECM would result in lesser quality cartilage formation and so would be not be ideal for cartilage repair techniques, including cell therapies such as ACI.

Chapter 4: Characterising the chondrogenic capacity of chondrocytes isolated from human ankle and knee joints.

4.1 Introduction

Over the past 17 years, ACI has been expanded to treat cartilage defects of the foot and ankle joint (Brittberg et al., 2003; Giannini et al., 2001; Thermann et al., 2014; Whittaker et al., 2005). For foot and ankle ACI, chondrocytes are sourced from either knee (McCarthy, Helen and Roberts, 2013; Whittaker et al., 2005) or ankle cartilage (Giannini et al., 2005).

Only a few research groups have compared the clinical outcome of ACI using different cartilage sources. There have been reports that have assessed the joint morbidity of cartilage taken from the knee to treat other joints, including the ankle (Ahmad et al., 2002; Paul et al., 2009; Reddy et al., 2007; Valderrabano et al., 2009; Whittaker et al., 2005), the hip (McCarthy et al., 2015) and the elbow (Iwasaki et al., 2007). No correlation between the number of harvests or the size of harvest on joint morbidity or treatment outcome were reported in the knee (Paul et al., 2009; Reddy et al., 2007). There are indications of joint morbidity, degenerative changes and poor repair histologically at the harvest site in the knee and ankle (Reddy et al., 2007; Valderrabano et al., 2009).

Several groups have demonstrated recent success in ACI being applied to defects of the ankle joint using healthy biopsies sourced from either knee or ankle cartilage (Aurich et al., 2011; Giannini et al., 2014; Giza et al., 2010; Nam et al., 2009; Schneider and Karaikudi, 2009; Thermann et al., 2014; Whittaker et al., 2005).

Despite all this research, there is a paucity of information to determine whether there are any differences in the growth kinetics or the cartilage-forming capacity of chondrocytes isolated from the ankle and the knee. Making ACI truly successful in both knee and ankle joints requires a better understanding of the source of chondrocytes to determine whether joint specificity offers any advantages. Herein, we have made steps to characterise these chondrocytes isolated from ankle and knee cartilage tissue with a long-term goal to improve the efficacy of ACI.

There are differences in the disease susceptibility of the knee and ankle joints (Arthritis Research UK, 2013). In terms of osteoarthritis, knee osteoarthritis tends to be primary in nature and the knee is the most commonly affected joint in the UK, with an estimated 4.71 million individuals over the age of 45 years having osteoarthritis of the knee (Arthritis Research UK, 2013; Jordan et al., 2007; Jordan et al., 2010). By comparison from the same reports, osteoarthritis of the ankle joint is of a lower prevalence with an estimated 1.77 million individuals over the age of 45 years having ankle osteoarthritis. Another UK study coincides with these findings, demonstrating that 41.2% of the 500 patients observed had symptomatic knee osteoarthritis, whereas only 4.4% had symptomatic ankle osteoarthritis (Cushnaghan and Dieppe, 1991). Compared to knee osteoarthritis, ankle osteoarthritis tends to require less surgical intervention as it is usually of a less severe degeneration (as per international cartilage repair society grading) and is usually post-traumatic osteoarthritis in nature (Muehleman et al., 2010; Takakura et al., 998; Thomas and Daniels,

2003). Muehleman et al. (2010) studied 1,060 adult knee and ankle pairs from 545 cadaveric donors and the findings of this study revealed that severe defects, as per the international cartilage repair society grading, were extremely rare in the ankle with only three reported cases in contrast to 50% of knee joints studied being of severe grading (Brittberg et al., 2002; Muehleman et al., 2010). This might suggest that the ankle has a protective mechanism against cartilage damage progression to osteoarthritis that is either not present, or not as well established, in the knee. However, to date, these differences have been attributed to a combination of joint anatomy and biomechanics (Eger et al., 2002; Huch et al., 1997).

Anatomically, the ankle is made up of 26 bones: five metatarsal bones, 14 phalange bones and seven talar bones (Drake et al., 2015), and is covered in a thin layer of articular cartilage less than 1 mm thick. Most cartilage injuries occur within the talar region of the ankle. Whereas, the knee joint comprises four bones: the femur, fibula, tibia and patella (Drake et al., 2015a), and the articular cartilage is thicker than that of the ankle, ranging from 2-6 mm thick (Al-Ali et al., 2002; Ateshian et al., 1991; Shepherd and Seedhom, 1999). Most cartilage injuries occur on the condyle region of the knee, where the cartilage is up to 6 mm thick. So together, the cartilage thickness as well as the bone topography might have some influence on the type of injury and progression to osteoarthritis.

There are some key biomechanical differences between the two joints. For example, the ankle is a highly congruent joint, and thus it can withstand high

levels of loading and stress despite the small contact area (11-13 cm^2) across the joint. This results in the ankle experiencing lower stress than other joints within the leg, for example the knee or hip (Nordin and Frankel, 2001; Sammarco and Hockenbury, 2001). The knee and ankle joints must be able to withstand high levels of load during everyday activities, such as walking. It has been reported that the ankle can withstand 5x body weight during walking, with most of this load transmitted through the tibiotalar joint and applied to the talar dome (Brockett and Chapman, 2016). Whereas, the knee has been shown to regularly withstand lower levels of loading, of 3-4x body weight when walking (Andriacchi et al., 1980; Morrison, 1969; Naaijkens et al., 2012).

Although anatomical and biomechanical differences have been documented, there has been a single comprehensive study comparing the biochemical and cellular differences in the two joints (Quinn et al., 2013) Using cadaveric donor-matched adult human knee and ankle, Quinn et al. (2013) found elevated cell densities within the superficial zone of the knee but not the ankle. Matrix volume per cell in the superficial zone of the knee was significantly less compared to the ankle. Chondrocyte density per unit of articular cartilage were found to be significantly higher in knee cartilage, compared to ankle cartilage. Whilst this study was thorough in nature, its limitation was that it did not explore aspects relevant to ACI; cell growth kinetics and ECM production in three-dimensional culture. The study focused heavily on qualitative histological findings, which have been suggested to not correlate well with clinical outcome

and so may not be the best indicator to characterise differences in ankle and knee cartilage tissue (McCarthy and Roberts, 2013).

Since ACI requires a harvest of healthy cartilage, it is necessary to determine whether there are any differences in the chondrogenic properties of chondrocytes from different joints. If so, it would be necessary to assess whether chondrocytes from a joint alternative to the one being treated could be of more benefit.

Our study is the first of its kind to compare the growth kinetics of chondrocytes isolated from ankle and knee cartilage with ACI protocol in mind. Herein, we aim to characterise and compare chondrocytes isolated from freshly obtained human ankle and knee cartilage tissue donated following total joint replacement surgery, by assessing chondrocyte expansion through growth characteristics and cell morphology. Initial cartilage structure will be assessed using histological analysis of core biopsies. Furthermore, three-dimensional pellet culture will be utilised to assess the ECM through biochemical, histological and gene expression analyses.

4.2 Experimental design

Five ankle samples and five knee samples were received from patients undergoing total joint replacement surgery. Demographics were recorded for each patient and are displayed in Table 11 (page 110). Demographics recorded include: the age and gender of the patient and the source of the cartilage

obtained (ie: ankle or knee). All cartilage was taken from the femoral condyle region of the knee joint or from the talus region of the ankle joint.

Table 11: Demographics of the human ankle and knee tissue sample donors from which chondrocytes were obtained. All tissue was obtained following joint replacement surgery at our centre.

Age of donor:	**Gender of donor:**
Ankles -	
44	Male
55	Male
62	Male
59	Female
47	Female
Age range: 44-62 years; mean age: 53.4 years	3 males; 2 females
Knees -	
72	Male
61	Male
64	Male
55	Female
47	Female
Age range: 47-72 years; mean age: 59.8 years	3 males; 2 females

Figure 24 (page 111) illustrates the experimental design for this project.

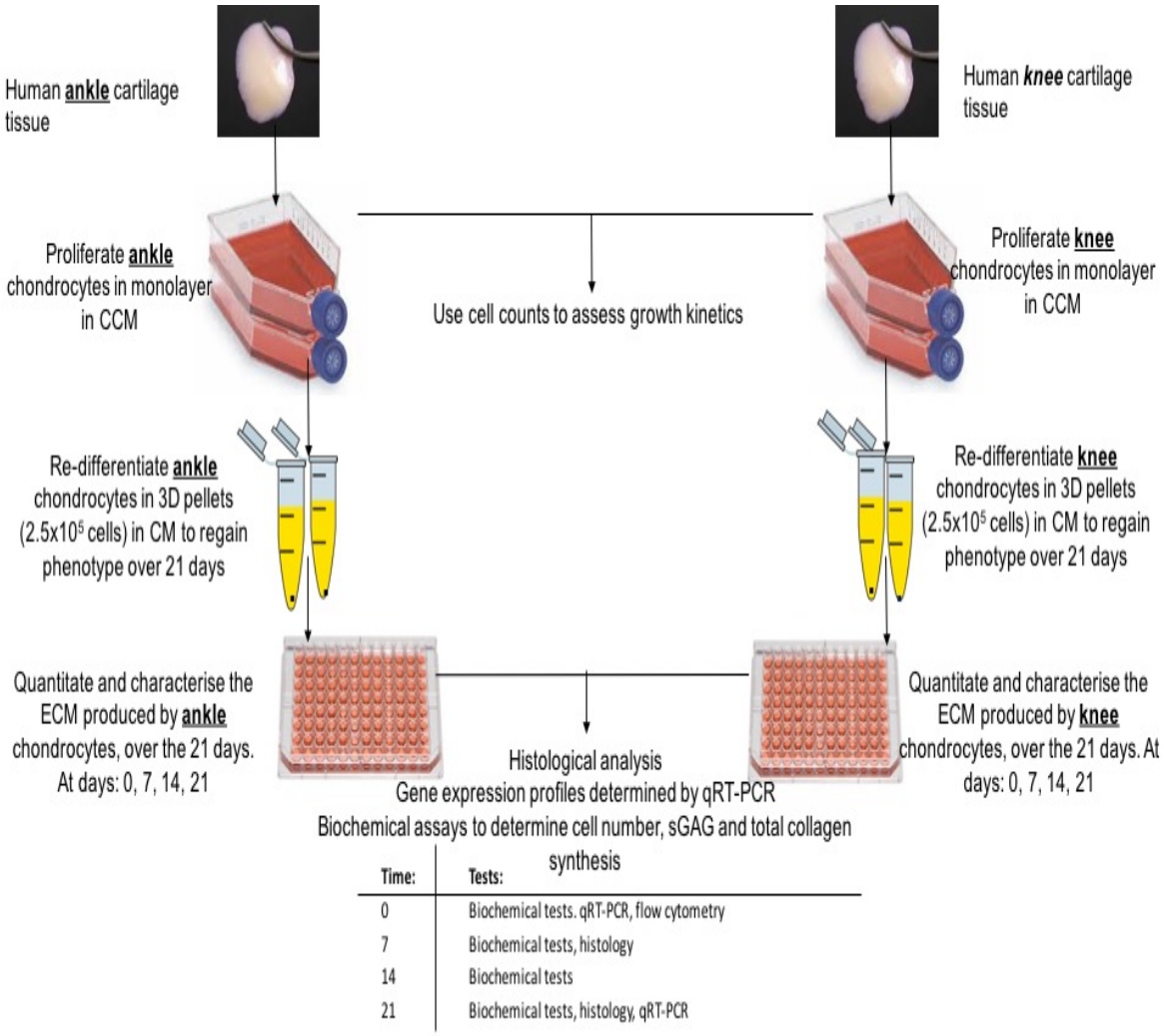


Figure 24: Schematic illustrating the experimental design of the project comparing the chondrogenic capacity of chondrocytes isolated from ankle and knee cartilage.

Chondrocytes were isolated from macroscopically healthy cartilage tissue on the femoral condyle of the knee joint and the talus of the ankle joint, following enzymatic digestion (section 2.1.3, page 53). Representative full depth core biopsies were taken from macroscopically healthy areas, assessed by eye, of the condyle of one knee cartilage tissue sample and the talus of one ankle cartilage tissue sample. Core biopsies were snap-frozen in liquid nitrogen and

stored at -80^{o} until histologically stained with H&E and toluidine blue to illustrate morphological differences and proteoglycan content of ankle and knee full depth cartilage tissues.

Chondrocytes were expanded in monolayer up to passage 2. During monolayer culture, growth kinetics were recorded and phase contrast images were taken to observe morphology using a TS100 Eclipse Nikon microscope and acquired using IP Lab 3.2.6 software. (section 2.1.6, page 54). Once confluent at passage 2, chondrocytes ($4x10^4$) from one ankle and one knee patient, were prepared for flow cytometry analyses (section 2.1.7, page 55).

Remaining cells were passaged and seeded into three-dimensional pellets ($2.5x10^5$) in chondrogenic media, using methods outlined in section 2.1.4 (page 49). Pellets were maintained in culture for up to 21 days for all patients, with assessments being carried out at days 0, 7, 14 and 21 as outlined in Figure 24 (page 111). Chondrogenic pellets (n>4) were digested in proteinase K at days 0, 7, 14 and 21 and processed for biochemical analyses (determining cell number, sGAG synthesis and total collagen synthesis). Chondrogenic pellets (n>3) were snap frozen at days 7 and 21 for histological analysis, and were sectioned and stained with toluidine blue (section 2.1.14.3, page 70) and H&E (section 2.1.14.2, page 69). Chondrogenic pellets (n>3) at days 0 and 21 were homogenised in RLT buffer containing 1% (v/v) β-mercaptoethanol and stored at -80^{o}C until processed for RNA extraction and subsequent qRT-PCR analysis (section 2.1.13.3, page 65), specifically for markers of chondrogenesis, de-differentiation and hypertrophy.

4.3 Results

4.3.1 Chondrocyte yield appears more variable across the age range of ankle cartilage

Across the age-range, the average chondrocyte yield was 1658.39 ± 266.47 cells per mg for knee and 2514.34 ± 487.43 cells per mg for ankle (Table 12).

Table 12: Details of cell yields and wet weight of tissue obtained from the cartilage extraction of joint tissue received.

Age of donor (years):	Cell yield:	Wet weight of tissue (mg):	Cell yield per wet weight of tissue (cells/mg):
Ankles -			
44	820,000	392	2091.84
47	422,222	96	4398.15
55	750,000	456	1644.74
59	640,000	212	3018.87
62	580,000	409	1418.09
Knees -			
47	3,720,000	1950	1907.69
55	1,890,000	1200	1575
61	2,330,000	2728	854.11
64	625,000	472	1323.59
72	1,000,000	380	2631.58

These summary data are graphically represented in Figure 25 (page 114). In the five knee samples, chondrocyte yield per mg of cartilage tissue appeared to be consistent across the age range, whereas in the five ankle samples chondrocyte yield per mg of cartilage tissue appeared variable with

increasing age (Figure 25). The mean cell yield per wet weight of tissue was significantly higher in ankle cartilage compared to knee cartilage (p=0.009).

Flow cytometry analysis was performed to determine the absence or presence of chondrogenic markers (Figure 26; page 115). Immunoprofiling of one representative ankle and one representative knee sample showed varying degrees of expression for CD44, CD49c, CD39 and CD166.

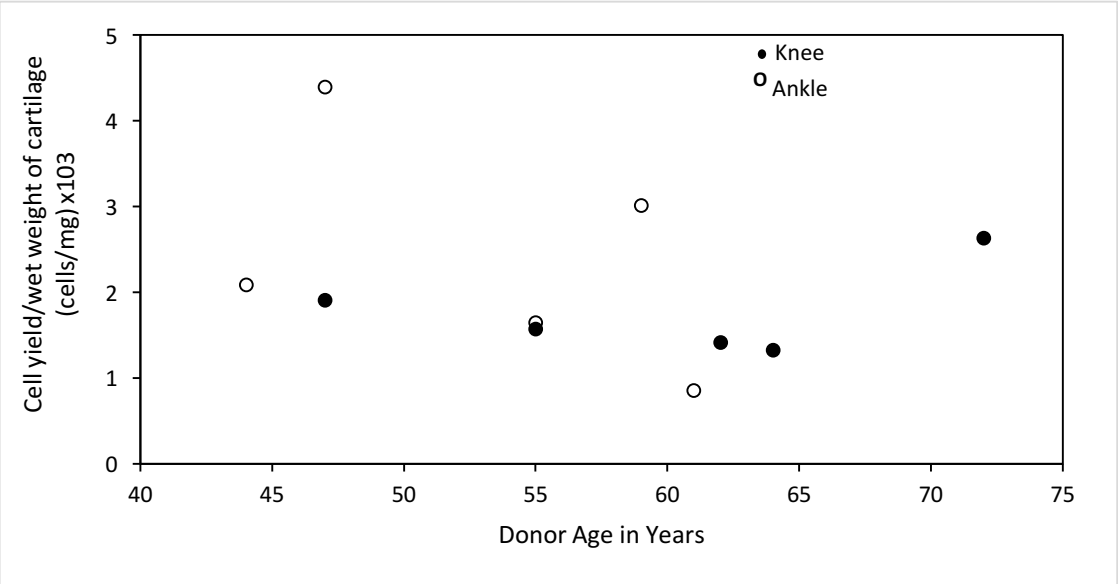


Figure 25: Cell yield per wet weight of cartilage tissue displayed at each age of the donors, for ankle cartilage and knee cartilage.

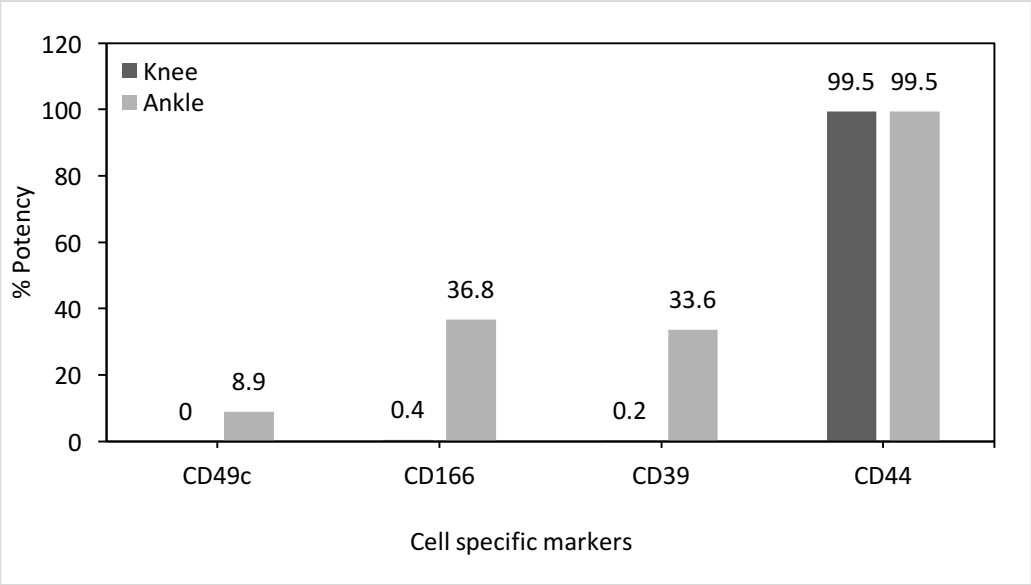


Figure 26: Representative potency profiles of key cell surface markers to determine chondrogenic potency of the chondrocytes extracted from cartilage obtained from one ankle and one knee joint.

Immunoprofiling revealed that both ankle and knee chondrocytes were >98% positive for CD44. Ankle chondrocytes exhibited a more potent expression of CD49c compared to knee chondrocytes which did not demonstrate any detectable expression of CD49c. Ankle chondrocytes had higher cell surface expression of CD39 at 36.8% compared to 0.4% in knee chondrocytes. CD39, also known as ENTPD1, is a cell surface-located enzyme member of the enzyme family expressed on a variety of cell types, which is highly associated with suppression of inflammation and regulation of the immune system (Kaczmarek et al., 1996; Kukulski et al., 2005). CD166 was expressed by ankle chondrocytes (33.6%) but very little expression (0.2%) was observed in knee chondrocytes.

4.3.2 Cartilage source does influence the growth kinetics of chondrocytes during monolayer expansion

Figure 27 illustrated no morphological differences between the chondrocytes isolated from ankle and knee cartilage. There appeared to be no differences in cell shape or size, as both sets of chondrocytes had an elongated flattened shape characteristic of adhered chondrocytes. However, chondrocytes isolated from ankle cartilage appeared to be more clustered, compared to chondrocytes isolated from knee cartilage where the chondrocytes appeared to be more homogeneously dispersed.

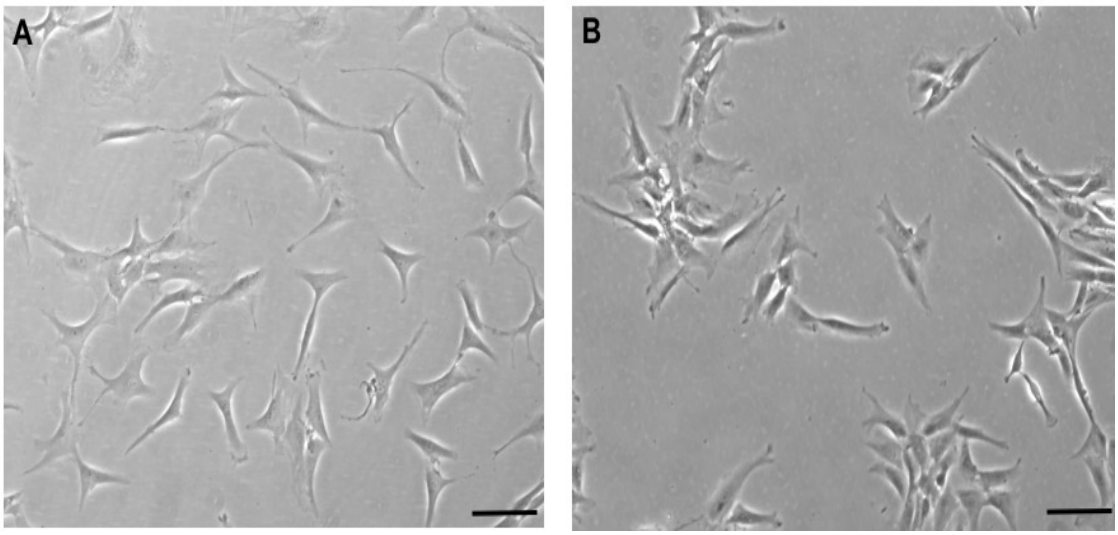


Figure 27: Representative images of chondrocytes at passage 1, 3 days after seeding, isolated from (A) knee cartilage and (B) ankle cartilage tissue. Images were taken using the Nikon TS100 light microscope, Hamamatsu C4742-95 camera and a ×10 magnification lens. Images were acquired using IPLab 3.2.6 software. Scale bars represent 100 µm.

Average absolute PDs demonstrated that ankle chondrocytes grew at a significantly slower rate (p=0.001) compared to knee chondrocytes, across all three passages (passage p=0.132), as shown in Figure 28. Ankle chondrocytes underwent less than 1 PD at all three passages, whereas knee chondrocytes demonstrated more variable growth kinetics, and underwent more PDs than ankle chondrocytes, at less than 1.5 absolute PDs at any of three passages.

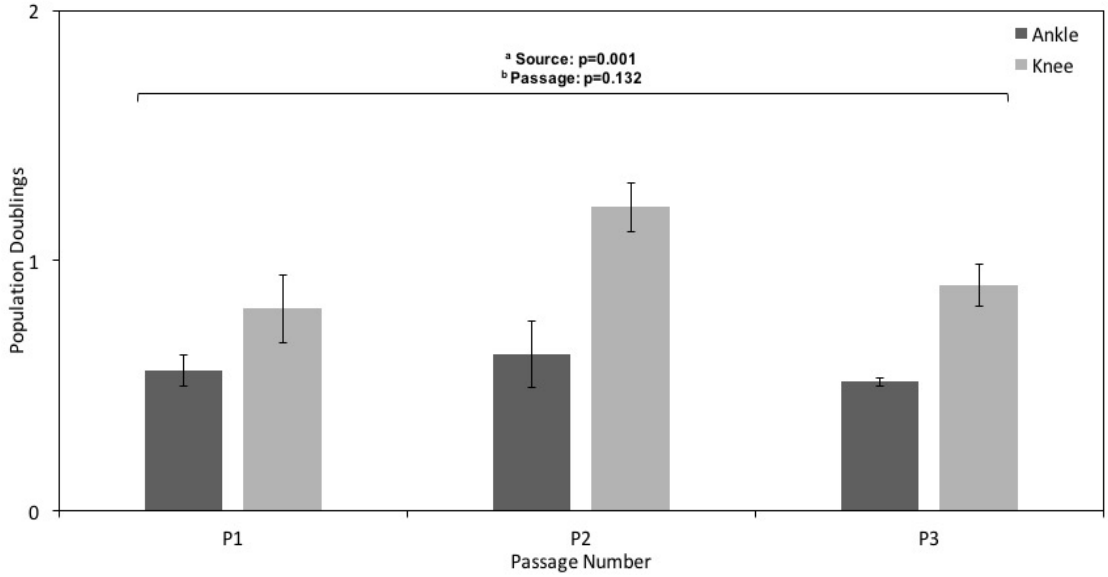


Figure 28: Absolute population doublings (PDs) of chondrocytes expanded from ankle and knee cartilage tissue, at each of the three passages. Data is presented as mean ± SEM and was obtained from five ankle patients and five knee patients in total.

Cell number obtained during monolayer expansion showed that cell number increased over time for both cartilage sources, as expected (p<0.001) but was significantly higher in knee chondrocytes across all passages except

passage 3 (p=0.007), as supported by the increased PDTs. Patient variability was deemed to be not significant (p=0.149) in affecting cell number during monolayer expansion.

4.3.3 Cartilage source does not influence the re-differentiation potential in three-dimensional pellet culture

To determine whether ankle-derived chondrocytes produced higher or lower quantities of ECM than knee-derived chondrocytes, three-dimensional chondrogenic pellets were prepared and maintained over a 21-day time course. Pellets were characterised by assessing total cell number (Figure 29A; page 120), total sGAG production (Figure 29B; page 120), total collagen production (Figure 29C; page 120), histological appearance and gene expression profiles as determined by qRT-PCR.

Pellet cell number (Figure 29A; page 120) remained constant for both cartilage sources (p=0.963) and across all four experimental time points (p=1.0). Patient variability was deemed to be not significant in affecting pellet cell number over time (p=0.728). Total sGAG production (Figure 29B; page 112) was normalised to pellet cell number. Chondrocytes sourced from both the ankle and knee joints demonstrated a steady increase in sGAG production across all time points. sGAG concentration was higher in knee pellets compared to ankle pellets at days 0 and 7, but levelled off at days 14 and 21. There was no significant difference in sGAG production observed between chondrocyte source (p=0834), but both sets of chondrocytes exhibited a significant trend of increasing over

time (p=0.003).

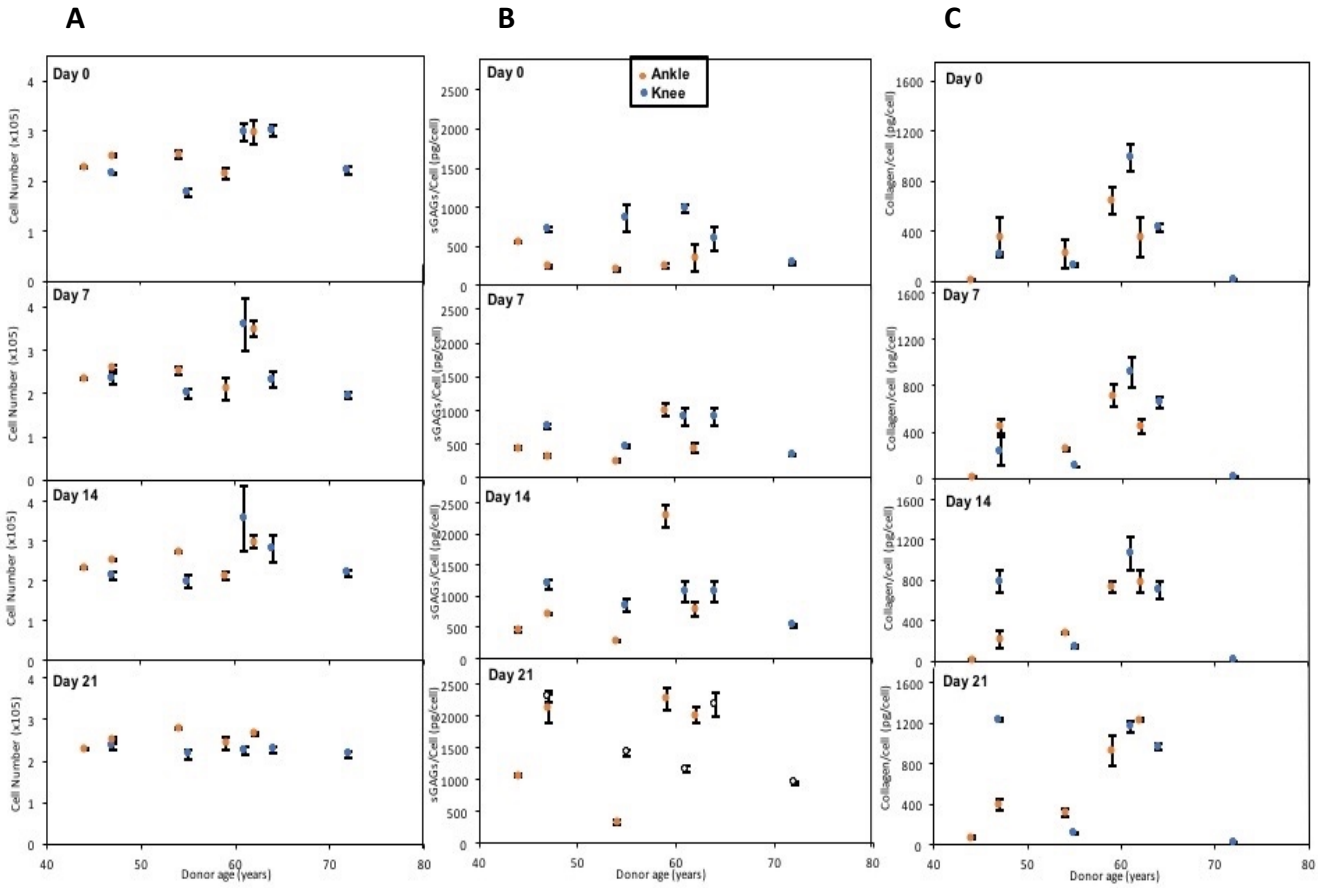


Figure 29: (A) Cell number, (B) sGAG production per cell and (C) collagen production per cell, in pellets created from chondrocytes isolated from ankle and knee cartilage tissue over a 21-day time course. Date is presented as the mean of each patient ± SEM for each time point, plotted against donor age. Data was obtained from five ankles and five knee patients in total.

In this instance, there was some evidence of variability between individual patients (p=0.023). Interestingly, there was one ankle (donor age 55 years) and one knee (donor age 72) that consistently did not produce higher quantities of sGAGs at any of the time points tested.

Total collagen production (Figure 29C; page 120) was normalised to pellet cell number. Total collagen production demonstrated no significance in chondrocyte source (p=0.779), and showed no significance in the trend of increasing over time (p=0.108). However, total collagen production remained higher in ankle chondrogenic pellets compared to knee chondrogenic pellets at day 0 and day 7, before levelling off at day 14 and becoming lower than knee chondrogenic pellets at day 21. Again, there was no evidence of variability between individual patients at any time point (p=0.267). Interestingly, one ankle (donor age 47 years) and one knee (donor age 72 years) demonstrated strikingly low collagen production across the full time course (8.29-69.95 pg/cell for ankle chondrocytes and 3.83-15.63 pg/cell for knee chondrocytes).

4.3.4 Ankle chondrocytes produce a higher quality ECM in three-dimensional pellet culture

Representative core biopsies were taken of both sets of tissue from fresh surgical samples, and were stained with H&E to assess morphology and toluidine blue to assess proteoglycan content (Figure 30; page 122).

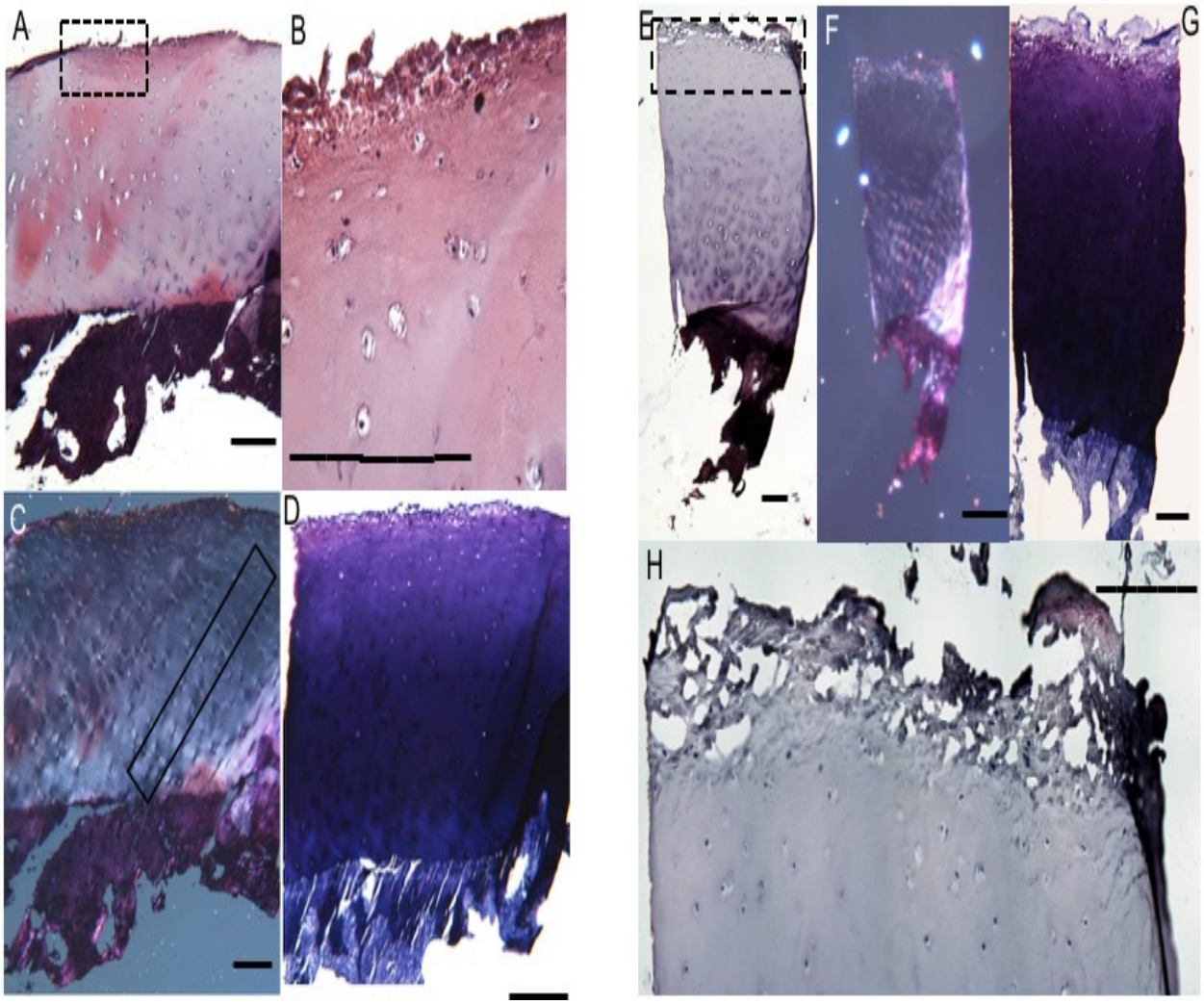


Figure 30: Representative histological images of core biopsies of ankle cartilage taken following fusion (A-D) and knee cartilage taken following total knee replacement (E-H). Both were stained with H&E (A-C,E-F,H) and toluidine blue (D,G). Cartilage and bone were shown to have good integration (A,E). Higher powered images (B,H) of the dashed line region in (A,E) demonstrates cells at the surface. Polarised light (C,F) revealed hyaline cartilage in both tissues. Differences were observed in the arrangement of the collagen fibres, which appeared in a diagonal linear alignment in ankle cartilage and arranged randomly within knee cartilage.

Visualising the core biopsies using polarised light demonstrated that both tissues were hyaline cartilage. Differences were observed in the arrangement of the collagen fibres, which appeared in a diagonal linear alignment in ankle cartilage tissue and arranged randomly within knee cartilage tissue. Both tissues demonstrated good integration between bone and cartilage.

Histological quality of the pellets at day 7 and day 21 were assessed using toluidine blue stained pellets and determined using the BERN score, and demonstrated no significant difference in histological quality between the two cartilage sources (Table 13).

Table 13: Grading of the pellets isolated from ankle and knee cartilage tissue at day 7 and day 21.

Time (days):	BERN score
Ankles -	
7 21	8 9
7 21	/ 6
7 21	9 /
7 21	8 7
7 21	7 8
Knees -	
7 21	8 8
7 21	6 7
7 21	/ 8
7 21	9 7

Histological grading of quality remained consistent at both time points in both sets of pellets (p=0.721 for day 7, p=0.945 for day 21). The average histological grade for the ankle pellets at day 7 was 8 (range: 7-9) and at day 21 was 7.5 (range: 6-9), whereas the average histological grade for knee pellets at day 7 was 7.6 (range: 6-9) and 7.5 at day 21 (range: 7-8).

Figure 31 (page 125) illustrates representative histology data for both knee and ankle chondrogenic pellets at both day 7 and day 21 time points.

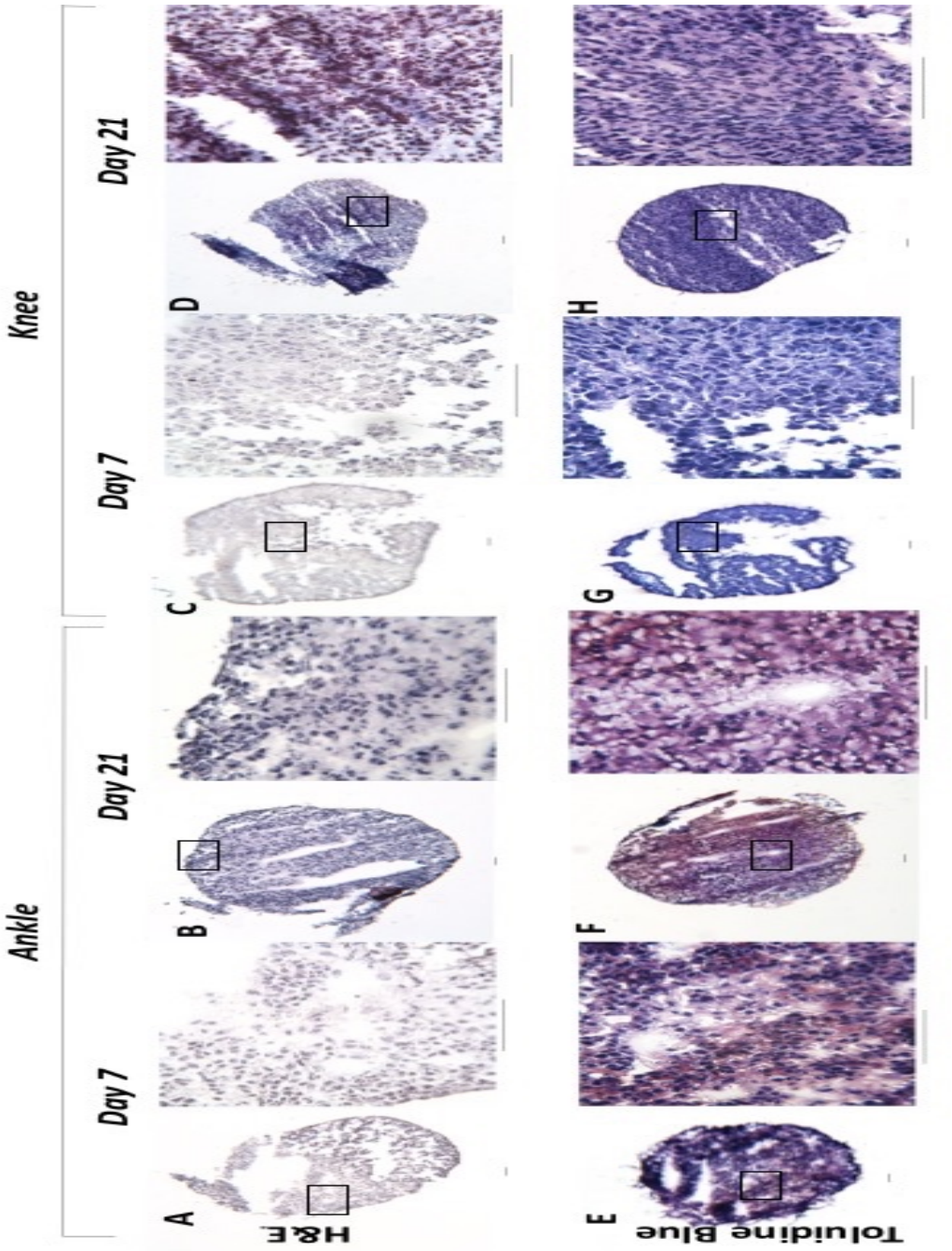


Figure 31: Representative histological images from the centre of (A,B,E,F) ankle pellets and (C,D,G,H) knee pellets at (A,C,E,G) day 7 and (B,D,F,H) day 21. Pellets stained with (A-D) H&E and(E-H) toluidine blue. Insert images are higher-powered images of the regions marked with a solid line to demonstrate the pellet centre and edge. All scale bars represent 100 µm.

Chondrocytes isolated from ankle cartilage appeared to form more robust pellets than those created from knee chondrocytes. In both sets of pellets, it appeared there was no difference in matrix being produced over time. Pellets created from ankle chondrocytes displayed a higher level of metachromasia, indicating that ankle chondrocytes had more ECM produced at both time points assessed.

Staining intensity of the toluidine blue stained chondrogenic pellets (section 2.1.14.4, page 69) demonstrated no significant differences between cartilage sources (Figure 32, page 127; p=0.837) but showed significance of intensity changing over time (p=0.004). A higher staining intensity in the ankle pellets was observed at day 7 (p=0.001). This is a reversely proportional relationship due to metachromasia.

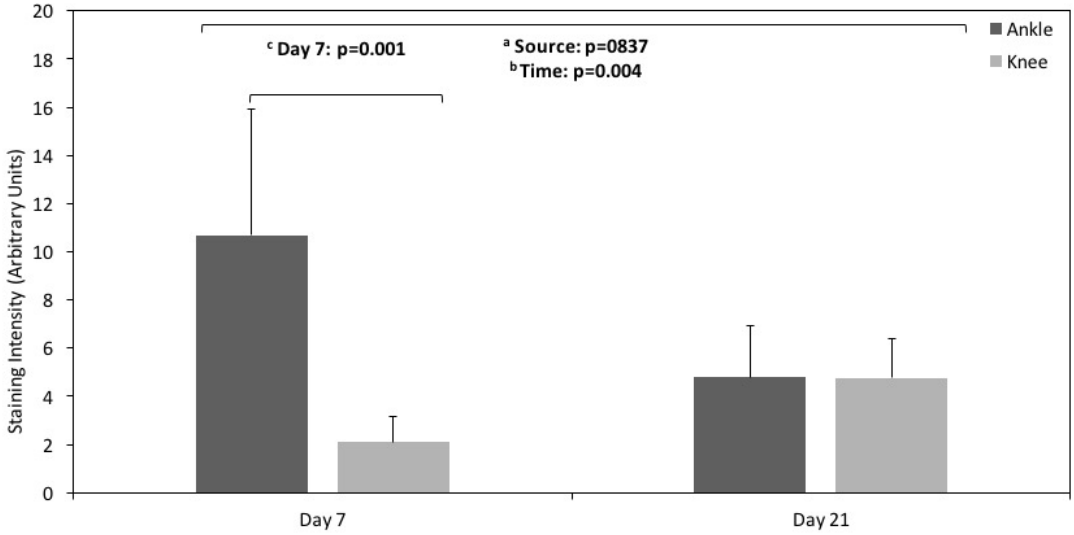


Figure 32: Semi-quantitation of toluidine blue-stained chondrogenic pellets demonstrating an increase in metachromasia, determined by integrated density analysis using ImageJ imaging software (National Institute of Health, Washington, USA; version: 24). [a,b,c] denotes significance of cartilage source on staining intensity, time and specifically at day 7 respectively. Data is presented as the mean ± SEM and was obtained from five ankles and five knee patients.

Time significantly affected the staining intensity of the pellets (p=0.004), with ankle pellet staining intensity decreasing over time, but the reverse occurring in knee pellets where staining intensity increased over time. Again, there was no evidence of variability between individual patients at any time point (p=0.194). This data is reflected in sGAG synthesis (Figure 29B; page 120), toluidine blue stain intensity (Figure 32) and BERN scores (Table 13; page 123).

4.3.5 Chondrocyte source influences gene expression profiles in chondrogenic three-dimensional pellets

qRT-PCR was performed at day 0 and day 21, relative to the two reference genes: hypoxanthine-guanine phosphoribosyltransfrase-1 and TATA box-binding protein, determined by the comparative C_T method (Schmittgen and Livak, 2008) and normalised to pellet cell number. Expression of COL1A1, COL10A1 or ALK1 were not detected in either cartilage source, indicating no evidence of hypertrophy or de-differentiation. For the key chondrogenic markers: ACAN, COL2A1 and SOX9, there were striking differences between gene expression profiles in ankle and knee chondrogenic pellets (Figure 33; page 129).

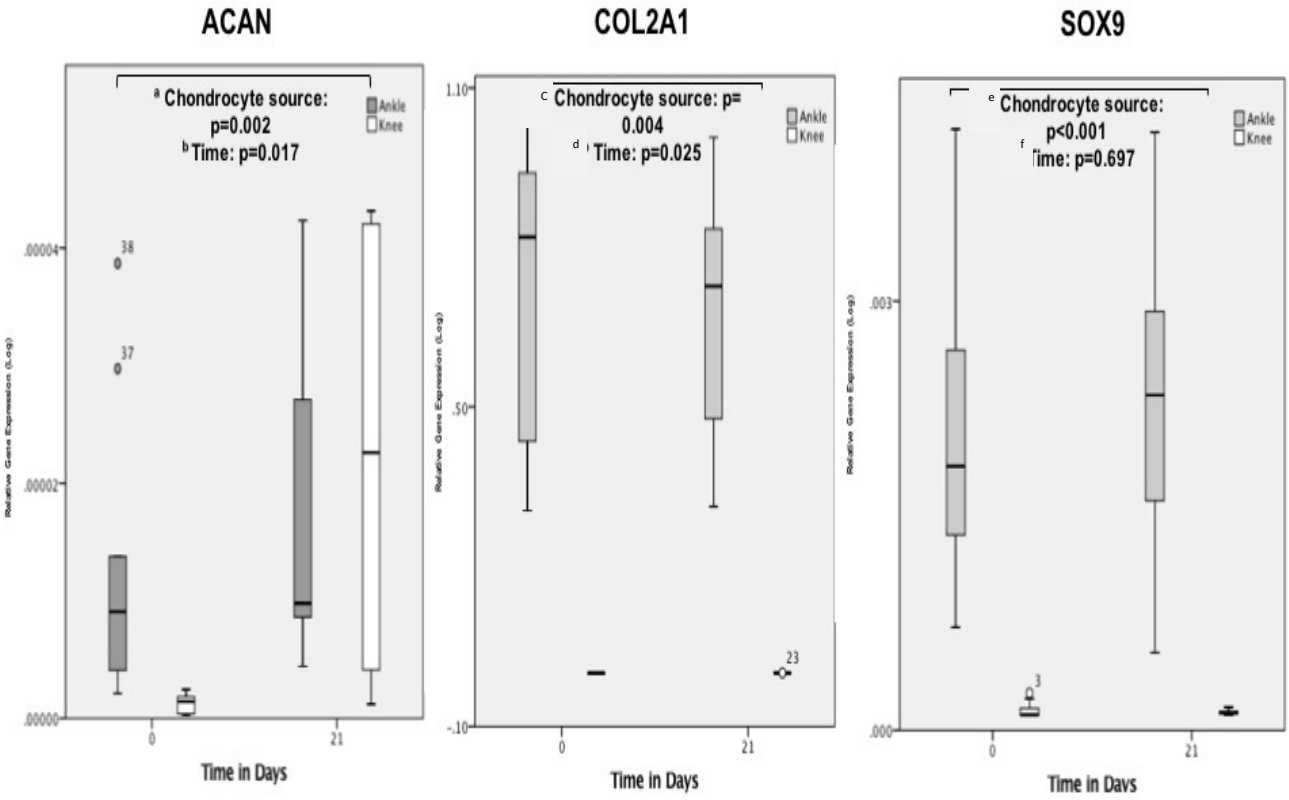


Figure 33: Box and whisker plots of the gene expression profiles of the chondrogenic pellets created from chondrocytes isolated from ankle and knee cartilage. Data is expressed relative to two reference genes, hypoxanthine-guanine phosphoribosyltransfrase-1 and TATA box-binding proteins, obtained from five ankles and five knee patients using three experimental replicated and normalised to cell number. [a, b] denotes statistical significance of ACAN expression between cartilage sources and significance of ACAN expression over time respectively. [c, d] denote statistical significance of COL2A1 expression between cartilage source and significance of COL2A1 expression over time respectively. [e, f] denote significance of SOX9 expression between cartilage source and significance of SOX9 expression over time respectively.

ACAN expression increased over the time course (p=0.017) and showed significant differences between the two chondrocyte sources (p=0.002). There was no evidence of variability between individual patients for ACAN expression (p=0.921). There was a significant change in COL2A1 expression over time (p=0.025), and COL2A1 expression was significantly higher in ankle pellets compared to that seen in knee pellets at both time points (p=0.004 for source), and a trend was seen for change of expression over time between sources of cartilage (p=0.017). There was no evidence of variability between individual patients for COL2A1 expression (p=0.084). In contrast, there was no evidence for a general change over time in SOX9 expression (p=0.695). However, ankle pellets demonstrated significantly higher expression of SOX9 than knee pellets (p<0.001). As with all three chondrogenic markers assessed, there was no evidence of variability between individual patients for SOX9 expression (p=0.079).

4.4 Discussion

This study has characterised primary-isolated articular chondrocytes from the ankle and knee joints of human donors, to determine whether cartilage tissue location has any effect on human chondrocyte growth kinetics and chondrogenic capacity using standard chondrogenic pellet differentiation protocols. Our findings provide a detailed analysis of ECM production from five individual ankle patients and five individual knee patients from a similar mean age (53.4 years for

ankle, 59.8 years for knee).

Several key points were demonstrated during this study. First, cartilage source did influence growth characteristics of the chondrocytes, as ankle-derived chondrocytes grew at a slower rate than knee chondrocytes. Ankle chondrocytes underwent significantly less PDs, despite having a similar PDTs to knee chondrocytes, and knee chondrocytes achieved significantly higher cell numbers during monolayer culture compared to ankle chondrocytes. The second key finding was that no significant differences in sGAG or total collagen production were observed between the ankle and knee chondrocytes, but both exhibited the same trends over time. Histologically, representative images showed more ECM was observed in the chondrogenic pellets created from ankle cartilage tissue as seen by the increased induction of metachromasia between day 7 and day 21, yet there was no significant difference in the histological quality of the pellets or in the overall staining intensity across the full-time course. However, at day 7 knee chondrocytes had a significantly lower staining intensity than ankle chondrocytes, suggesting earlier time points are more indicative of ECM production in ankle cartilage. Finally, the last key finding was that chondrocyte source was deemed to be significant metabolically, as assessed in the gene expression profiles of the three key chondrogenic markers: ACAN, COL2A1 and SOX9. Collectively, these findings were interesting as many studies have reported differences between these two joints, both biomechanically and anatomically, but limited investigations have occurred biochemically, metabolically or at a cellular level. It could be hypothesised that there would be

significant biochemical differences in the production of key ECM components, but this was not found in our studies.

Investigations into ankle and knee chondrocytes have been performed across several studies but none, to our knowledge, have used freshly isolated human cartilage tissue. One study by Candrian evaluated ten donor matched chondrocytes isolated from the talus and femoral head of cadavers, performing a similar range of tests to our study (Candrian et al., 2009). This study reported similar findings to ours in growth kinetics, showing similar proliferation rates exhibited between ankle and knee chondrocytes, up to the second passage. However, this was assessed using doublings per day, as opposed to the method adopted in our study of using PDTs and absolute PDs. One difference was Candrian reported similar cell yields for the two sets of chondrocytes at harvesting which contrasts our findings, as we showed significantly higher cell yields from knee cartilage at harvest, compared to ankle cartilage. We found this unsurprising, as the chondrocytes isolated during our study were from macroscopically healthy cartilage tissue samples following total joint replacement and so less cartilage was available for harvest. Our finding of different growth kinetic profiles between the chondrocytes isolated from these two joints, was further supported by a study investigating chondrocytes from various joints and animal donors (Akens and Hurtig, 2005). Representative potency profiles of the ankle and knee chondrocytes differed greatly, with ankle chondrocytes exhibiting a more potent profile as illustrated by higher percentages of the key chondrogenic cell surface markers.

Our study investigated the synthesis of key cartilage matrix components in primary isolated chondrocytes from freshly harvested human cartilage tissue. Conflicting results have been seen throughout numerous studies, using chondrocytes sourced from cadavers. Some studies demonstrate that the ankle chondrocytes exhibit higher proteoglycan synthesis levels in both cartilage slices and alginate chondrocyte cultures, compared to the donor matched knee samples (Eger et al., 2002; Huch, 2001). Further studies have shown chondrocytes isolated from the ankle joint to have higher synthesis rates of cartilage matrix proteins compared to chondrocytes isolated from the knee joint, producing a denser ECM (Kuettner and Cole, 2005; Treppo et al., 2000). These findings could explain the reason why ankle cartilage is less susceptible to degenerative damage or has a higher capacity for repairing cartilage damage, preventing progression to more permanent degenerative changes, including osteoarthritis. However, other studies have found that ankle and knee cartilage synthesises sGAGs at similar rates (Aurich et al., 2002; Candrian et al., 2009), with one particular study reporting that, in tissue explants ankle cartilage had a higher sGAG content than knee cartilage, but these levels became similar when the cartilage was seeded into alginate cultures (Kuettner and Cole, 2005). These studies support the findings of this study.

Similar results from our study were seen with collagen synthesis, in which both sets of chondrocytes synthesised similar levels of total collagen. This finding was supported by several studies, all of which showed no difference in collagen synthesis between ankle and knee chondrocytes (Candrian et al., 2009; Kuettner

and Cole, 2005). Collectively, these findings suggest that once chondrocytes are isolated and cultured, they lose some differences derived from the microenvironment of the native cartilage source. Therefore, the changes found in freshly isolated cartilage tissue may not be translated to chondrocytes following monolayer expansion, or the chondrocytes possibly need longer than the time frame investigated in this study to fully recover their phenotype and exhibit the differences seen in these native cartilage tissues.

We also reported histological differences between human ankle and human knee cartilage as both native tissue and as chondrogenic three-dimensional pellets. Representative core biopsies of native cartilage tissue demonstrated both sets of cartilage tissue were hyaline cartilage and had good integration between the bone and cartilage tissue. The only evident difference in cartilage structure was the collagen fibre arrangement. When visualised under polarise light, ankle cartilage had an organised arrangement of collagen fibres aligned in a diagonal linear arrangement, whereas knee cartilage had a more random arrangement of collagen fibres. This is unsurprising as the joints have different loads and stress applied to them, which is typically resisted by the collagen fibre arrangement and meshwork. Representative core biopsies also illustrated the difference in 'healthy' tissue. Despite chondrocytes being extracted from macroscopically healthy areas of harvested joint tissue, histologically the cartilage biopsies for the knee tissue did not appear healthy but had a rough fragmented surface edge. This was not surprising, as the tissue obtained was following total joint replacement, so would likely be osteoarthritic

in nature. These findings also supports reports that state histological analysis does not always align with other clinical findings, such as radiological analysis (McCarthy et al., 2015; McCarthy and Roberts, 2013).

Our study showed no expression for de-differentiation or hypertrophy of ankle or knee chondrocytes through assessment of ALK1, COL1A1 and COL10A1. Differing gene expression profiles between the knee and ankle chondrocytes were observed, with ankle chondrocytes expressing significantly higher levels of COL2A1 (p=0.004) and SOX9 (p<0.001) across both time points, compared with knee chondrocytes, and these findings are supported by several studies (Chubinskaya et al., 1999; Kang et al., 1998; Miller et al., 2017). Ankle chondrocytes were also found to have higher levels of ACAN at day 0, indicating early expression of the key component of the ECM, aggrecan. Collectively, these gene expression profiles indicate that ankle chondrocytes express not only higher levels of genes responsible for synthesising aggrecan, collagen type II and SOX9, but also express these genes earlier compared with knee chondrocytes. Our findings coincide with an ongoing study being performed by Miller, in which next generation sequencing has been used to map the transcriptome of knee and ankle samples obtained following amputation. Early findings from that study illustrate expression of 809 genes were found to differ between the two chondrocyte sources, with 781 genes being upregulated and 27 genes being downregulated in ankle chondrocytes when compared to knee chondrocytes (Miller et al., 2017). Another study coincides with these findings and found that mRNA expression of matrix metalloproteinase-8, a key enzyme in degrading ECM

proteins, was not detected in normal ankle cartilage but was present in normal knee cartilage (Chubinskaya et al., 1999). Differing gene expression profiles could be one possible reason for ankle cartilage being more resistant to degenerative damage when compared with knee cartilage.

Representative immunoprofiling of one ankle and one knee sample demonstrated differences in the chondrogenic potency illustrated by the expression of cell surface markers. We are the first study, to our knowledge, that has directly compared ankle and knee chondrocytes in this way. We assessed four key cell surface markers: CD49c, CD39, CD44 and CD166 (Garcia et al., 2016). CD44 is major cell surface receptor for HA and is homologous to cartilage link proteins (Ishida et al., 1997). It is a cell surface receptor that is associated with matrix-cell-cytoskeleton interactions (Knudson and Loeser, 2002). HA adheres to CD44 which regulates proliferation and matrix synthesis (Ishida et al., 1997). HA-CD44 is required for the retention of proteoglycans within the cartilage ECM (Chow et al., 1998; Knudson et al., 2000; Nishida et al., 2004). CD44 is indicative of chondrogenic potency, as high expression levels have been associated with high chondrogenic capacity and increased signalling of CD44 has been reported in high levels of GAG/DNA and type II collagen (Garcia et al., 2016; Mennan et al., 2018). Whereas, CD49c is an α-3 integrin subunit, which is considered an important mediator for mesenchymal condensation, which in turn is necessary for the initiation of chondrogenesis (Delise et al., 2000; Goldring et al., 2006). CD49c is associated with cell-cell and cell-matrix interactions, and functions as a cell surface adhesion molecule. It has also been shown to interact

with ECM proteins, and high signalling of CD49c is associated with high synthesis of GAGs and type II collagen (Grogan et al., 2007). Additionally, CD39 is considered a key MSC marker and is predictive of chondrogenic potency responses. It acts by hydrolysing adenosine triphosphate and adenosine diphosphate. It is more recently being considered an immune system mediator that interferes with anti-tumour and anti-inflammatory immune responses (Tan et al., 2016). Finally, CD166 belongs to the immunoglobulin superfamily, and is an activated leukocyte cell adhesion molecule located on the cell-surface. It is considered a key surface marker of human MSCs which increases in expression during monolayer culture and is therefore indicative of chondrocyte de-differentiation. However, expression has been shown to decrease following culture in three-dimensional culture system, as the re-differentiation process occurs (Lee et al., 2009). It has been reported to mediate cell-cell interactions and expression levels are suggested to reflect onset of cellular programming for homeostatic control of growth, as it is involved with dynamic growth and migration (Lee et al., 2009; Swart, 2002). it binds to cell surfaces and is suggested to be associated with dedifferentiation and chondrogenic potency (Brady et al., 2015; Garcia et al., 2016a).

A previous study conducted at our centre concluded that CD49c and CD39 immunopositivity positively predicts GAG production and histological score in cell pellets (Garcia et al., 2016). Positivity of CD49c and CD39 have also been shown to be associated with increased *in vitro* chondrogenic potential (Grogan et al., 2007; Gullo and De Bari, 2013). High signals of CD44 and CD49c have been

reported as correlating with high levels of GAG/DNA and high levels of type II collagen (Garcia et al., 2016; Mennan et al., 2018). It would be useful to obtain a higher number of cartilage samples for the flow cytometry analyses to allow for a more definitive assessment of the differences in chondrogenic potency between ankle and knee chondrocytes and would allow for statistical calculations to be performed; however, we were limited due to the number of cells required to perform all assessments. As we were only able to obtain representative immunoprofiling for one ankle and one knee sample, it is difficult to draw definitive conclusions from these results, and further samples would be required.

However, as knee chondrocytes expressed little CD39, CD166 and CD49c in this study, it could be speculated that these potency markers may not be the best representative markers of chondrogenic potency in knee chondrocytes and could be altered. Further work would be required to determine more accurate or representative potency markers from a wide panel of available markers. Furthermore, these results could speculate that the source of chondrocytes (ie: from knee or ankle) could lead to altered profiles of potency markers, and this would be an interesting avenue to explore in future research studies by determining the most representative panel of potency markers for each different sources of chondrocytes that would be the most representative for that location.

We expected some patient variability within our results but found no statistical evidence for a patient-specific influence on any of the outcomes measured in these experiments, as assessed by multilevel modelling analysis (Hox, 2010; Vaughn, 2008). More work could be done to expand these findings

further and delve deeper into the differences between ankle and knee cartilage to discover variances in osteoarthritis development with ankle osteoarthritis characteristically being post-traumatic osteoarthritis compared to knee osteoarthritis usually being primary in nature. An example of further investigation that could elucidate some findings further, would be to utilise patient demographics further, for example BMI, smoking status and activity levels. These were not determined for this study, but could be obtained from patient electronic medical records and used in future investigations.

These results should be taken with caution when comparing to cell therapy patients, as the samples were obtained following total joint replacement surgery. Due to the nature of this study, human primary cartilage tissue was obtained from total joint replacement surgeries which poses a limitation to our study, as the cartilage obtained could potentially be osteoarthritic. Osteoarthritis is classified as a whole joint disease and although care was taken to ensure cartilage was taken from macroscopically healthy areas, chondrocytes could still have a sub-chondrogenic phenotype upon differentiation, as indicated with our core biopsy images. However, we did not detect any ALK1 expression in monolayer-expanded chondrocytes harvested from either cartilage source, indicating that the tissue extracted was not of an osteoarthritic nature. Furthermore, as these samples were from patients undergoing total joint replacement, caution should be taken when comparing these findings with those from patients undergoing ACI.

In conclusion, to our knowledge there is no other study available providing such insights into characterising the chondrogenic potential of chondrocytes isolated from the knee and ankle. This study clearly demonstrates that although there were no evident significant differences in proliferation rates or in the synthesis of total sGAGs and total collagen, there were significant differences in the gene expression profiles between ankle and knee chondrocytes. These findings demonstrate that the differences exhibited are elucidated from non-biomechanical factors and are due to the chondrocytes themselves independent of biomechanical stimuli or their microenvironment. However, differences reported between ankle and knee cartilage tissue and in osteoarthritis development of these two joints, are likely due to synergic effects of anatomical, biochemical and biomechanical differences. Further research into ankle and knee cartilage pathophysiology and their regenerative capacity is required to improve our knowledge of these joints and advance cartilage repair techniques.

Chapter 5: Clinical methods

5.1 Clinical tests

The methodology to design the PROM was adopted from the three-step approach recommended for best practice by the Food and Drug Administration (U.S. Department of Health and Human Services Food and Drug Administration, 2009). The three steps are: item generation, scale generation and test properties. Patient selection was also considered in the design of the PROM.

5.1.1 Item generation

Given the range of foot and ankle scoring systems, it seemed unwise to design a completely new PROM. However, none of the existing PROMs seem to address all aspects that patients and clinicians find important (Devlin, 2010; Pinsker et al., 2013). The newly devised PROM, named the 'Oswestry Ankle Score (OsAS)' was designed by selecting items through a process of reviewing the existing foot and ankle PROMs and ensuring that all items considered important for assessing clinic outcome from a patient (Pinsker et al., 2013) or health professional perspective were selected for the newly devised scoring system. Where needed, the phrasing of items was adapted to ensure consistency of style and formatting for the score to be solely patient-reported.

Further feedback was gained from appropriate healthcare professionals, namely foot and ankle consultant surgeons and physiotherapists, to ensure that the questions addressed aspects that they found important in thoroughly assessing the treatment outcome and rehabilitation process. This feedback process also allowed for the PROM to be foot and ankle specific.

5.1.2 Scale generation

The scale system for use in the questionnaire was chosen to allow for a wide application and ease of answering the score. A Likert scale, consisting of five-point Likert-type items was selected to capture and measure patient's perception of their ankle health. (Kroenkea et al., 2015; Likert, 1932). Likert scale was selected, due to the ease of answering by patients.

5.1.3 Patient selection

Based on statistical power analyses described in Table 14 (page 143), it was determined that 219 patients would be required to perform all the reliability, validity and responsiveness assessments. A formal power calculation was not performed, but a sample size was selected based on sample sizes needed for all necessary analysis outlined in Table 14 (page 143). Based on previous questionnaire-based studies performed at our centre, a response rate of approximately 85% would be expected. Therefore, based on this we aimed to consent approximately 260 patients, to receive 219 completed questionnaires.

Table 14: Measurement properties and analyses used in this study, together with the sample sizes, to determine the measurement properties of the newly developed score. The sample sizes were decided in deliberation with a biostatistician and with consideration of published guidelines (Frost, et al., 2007; Rothman, et al., 2009).

Total sample size = 219			
Property:	**Analysis:**	**Sample size:**	**Criterion determined sample size:**
Construct validity	Patient focus groups	20-30	Allows for reliable and valuable feedback
Internal consistency	- Rasch analysis - Multilevel factor analysis	200 300	Sample size determined from Frost, *et al.,* 2007
Reliability	Test-retest	105	Allows for 95% confidence interval (CI) of ±0.1 or smaller, assuming the coefficient is at least 0.7, which is reasonable based on similar PROM instruments.
Sensitivity to change	Changes in treatment scores prior and post-surgery	109	Allowing assessments with a 95% CI of ±0.2, assuming a responsiveness of 0.5 and a correlation of 0.5 between baseline and post-treatment score.
Responsiveness	- Cohen's effect size measure - Focus groups	109 20-30	Allowing assessments with a 95% CI of ±0.2, assuming a responsiveness of 0.5 and a correlation of 0.5 between baseline and post-treatment score.
Correlation with other scores	Pearson moment correlation coefficient	219	Allows for assessments with a correlation coefficient with a 95% CI of ±0.1 or smaller, assuming the coefficient is at least 0.5, or a 95% CI of ±0.15 if the coefficient is smaller.

5.1.3.1 Eligibility criteria

To be eligible, participants had to be an adult, that is over the age of 18, and able and willing to provide written informed consent after reading and understanding the patient information sheet. Participants either had to present at a first ankle clinic with symptoms suggesting cartilage defects or osteoarthritis, be awaiting treatment or have already received treatment for ankle cartilage defects or osteoarthritis using any type of treatment, including cell therapy, microfracture, fusion or total ankle replacement.

Participants would be excluded if they were unable or unwilling to read and understand the patient information sheet, unable or unwilling to provide written informed consent for themselves, be under the age of 18, be unable or unwilling to complete all questionnaires for the duration of the study or had symptoms or require treatment for any condition other than cartilage defects or osteoarthritis of the ankle joint.

There were high levels of patient variability in the sample due to the nature of the study and patient population. Variations occurred through age, gender and activity levels (activity levels were not recorded for this study). Further demographics could be obtained from patient electronic records and utilised further if required.

5.1.4 Test properties

To confirm measurement properties of the OsAS PROM, relevant recommendations of consensus-based standards for the selection of health measurement instruments (COSMIN) were followed to examine each subscale in

the whole sample and by subgroup.

The OsAS PROM was designed to measure the patient-perceived clinical outcome of ankle treatments, including cell therapy and total ankle replacement surgery. It aims to address important aspects of the outcome following ankle treatments, including: symptoms, ankle function, activities, pain and quality of life. Patients completed the score both pre-operatively and post-operatively to allow the PROM to capture and determine the outcome of the treatment by a change in PROM score following intervention (Revicki et al., 2008).

The score was assessed for all recommended aspects to determine its design quality and applicability, including validity, reliability and responsiveness (Cöster et al., 2014b; Frost et al., 2007; Revicki et al., 2008). Minor adaptations of existing validated scores do not need an extensive "revalidation" (Snyder, *et al.,* 2010; FDA, 2009). However, since our new PROM combined items from many existing PROMs a full validation seemed to be warranted. The psychometric validation of the score, in particular the factor analysis and Rasch model, was used to select the most appropriate items, thus reducing the length of the PROM and its administrative burden. Table 14 (page 143) describes the different assessments that were used to validate the OsAS PROM.

Internal consistency was assessed using factor analysis and a Rasch analysis. The Rasch analysis required a minimum sample size of 200 participants (Frost, *et al.,* 2007). The factor analysis required around 300 patients (Frost et al., 2007), but at least 100 of the participants in this study were expected to provide two scores, a score at baseline and a score post-treatment.

Test-retest reliability required a sample size of 105 to estimate the correlation coefficient with a 95% CI of ±0.1 or smaller, assuming the coefficient was at least 0.7, a reasonable figure based on similar PROM instruments. The correlation with other instruments, a general health score and a specific validated foot and ankle measure, namely the Veteran Rand-12 questions (VR-12; Appendix VI, page 234) and the Manchester Oxford Foot Questionnaire (MOxFQ; Appendix V; page 232) respectively, was determined using the Pearson-moment correlation coefficient (Morley et al., 2013; Selim et al., 2009). Assessing this correlation required a sample size of 219 people to estimate the correlation coefficient with a 95% CI of ±0.1 or smaller, assuming the coefficient was at least 0.5, or a 95% CI of ±0.15 if the coefficient was smaller. Correlation with other scores is the chosen method for assessing validation, but there is a plethora of methods that could be used (Litwin, 1995).

A sample size of 109 patients was required to determine the responsiveness to change as defined by Cohen's effect size, which is defined as the change in mean baseline to post-treatment score, divided by the standard deviation of the baseline score (Cohen, 1992a; Cohen, 1992b). This assessment required 109 patients to achieve a 95% CI of ±0.2, assuming a responsiveness of 0.5 and a correlation of 0.5 between baseline and post-treatment score. For all analyses, 95% CI were used to summarise results. The assessments are summarised in Table 9.

5.1.5 Ethical considerations

The study met all regulations and ethical considerations set by the sponsor, Keele University, and the host study centre: The RJAH Orthopaedic hospital in Oswestry. For the OsAS questionnaire to be approved by the relevant ethics committee, an ethics application was submitted through the integrated research application system to the health research authority, and ethical approval was obtained from the Preston Ethical Committee in May 2017 (REC reference: 17/NW/0063; Appendix II, page 226). During the first 12-18 months of my PhD study, all drafting of questionnaires, letters, documentation and correspondence in relation to the OsAS score were carried by myself with guidance from the supervisory team.

5.1.6 Patient focus groups

The initial draft of the questionnaire was presented to a patient focus group of 19 people, for their feedback. Feedback was obtained on the content of the questionnaire, the length and duration of completing the score, ease of understanding and answering the questions and if anything they considered important was not addressed in the questionnaire.

Chapter 6: Design and psychometric testing of a new patient-reported outcome measure for ankle treatment.

6.1 Introduction

Patient-reported outcome measures (PROMs) are defined by the US Food and Drug Administration as 'any report of the status of a patient's health condition that comes directly from the patient, without interpretation of patient response by a clinician or anyone else' (U.S. Department of Health and Human Services Food and Drug Administration, 2009). Health care professionals increasingly use PROMs to assess treatment outcome from the patient perspective (Harris et al., 2013). The patient usually fills these out at set stages of their treatment, typically pre-operatively and post-operatively, which allows surgeons, doctors and administrators to monitor the progress of the treatment on a patient-by-patient basis. PROMs also allow for collective data to be used to assess feelings towards the treatment as a whole, indicating any key areas that may need to be addressed, for example through rehabilitation or other areas of recovery (Harris, *et al.,* 2013; Devlin, 2010).

The main advantages of PROMs in surgery is that they allow capturing aspects of the treatment and recovery that are important to the patient, allowing patients to feel more involved in their care and treatment plan, and allow treatment strategies to be assessed on a patient-by-patient basis (Martin and Irrgang, 2007). The limitations associated with the use of PROMs include the potential for a patient to exaggerate or understate their symptoms, the time needed to collect and manage the data and the difficulty for a PROM to capture what healthcare professionals and patients find important for assessing the

treatment outcome (Pinsker, *et al.*, 2013; Cook & Beckman, 2006). As each PROM has its own strengths and limitations, each healthcare professional tends to use a PROM they find important in capturing outcome, which differs between healthcare professionals.

There are barriers associated with the wide application of PROMs, with one being that the measures can prove difficult to administer to the patient. These difficulties may be related to the length of time to complete the PROM; the administrative burden on medical personnel to collect and analyse the data or difficulties in interpreting the measure by medical personnel or by patients. These administrative difficulties lead to a poor implementation of the PROM and problems in determining a final outcome from the measure. Consequently, a major limitation associated with PROMs is that often too many measures are used, all assessing various aspects of the condition or disease, yet no gold standard measure exists to assess all aspects simultaneously. Therefore, selecting an appropriate outcome measure proves to be difficult and usually more than one score is required to assess every important aspect. In turn, this means that completing the score sheets is time consuming for the patient and the analysis is time consuming for the physician. Furthermore, not all scores are validated for a specific use and this can limit their applicability (Nelson & Berwick, 1989; McHorney & Tarlov, 1995; Martin, *et al.,* 1997; Binkley, *et al.,* 1999).

Score:	Type:	Validation:
American academy of orthopaedic surgeon's lower limb outcome assessment instruments foot and ankle modules	RS	Not enough evidence to support full validation for use in ankle.
Ankle osteoarthritis scale	DisS; RS	Not enough evidence to support full validation for use in ankle.
Bristol foot score	RS	Not enough evidence to support full validation for use in ankle.
Foot and ankle ability measure	DimS; RS	Valid for assessing physical performance of foot and ankle musculoskeletal disorders.
Foot and ankle disease index (FADI)	RS	Not enough evidence to support full validation for use in ankle.
Foot and ankle outcome score (FAOS)	RS	Not enough evidence to support full validation for use in foot and ankle.

Foot function index (FFI)	DimS; RS	Valid for assessing foot related pathological disorders.
Foot health status questionnaire (FHSQ)	RS	Valid for assessing surgical treatment in podiatry for foot problems.
Juvenile arthritis foot disability index (JAFI)	PopS; DisS; RS	Valid for assessing arthritis of the foot in juveniles.
Karlsson ankle function score (KAFS)	RS DimS	Not enough evidence to support full validation for use in foot and ankle.
Lower extremity function scale (LEFS)	RS; DisS	Valid for assessing musculoskeletal disorders of the hip, knee, ankle, foot
Maryland foot score (MFS)	RS	Not enough evidence to support full validation for use in ankle.

Manchester-Oxford foot questionnaire (MOxFQ)	RS	Valid for assessing surgical outcome of a variety of foot disorders.
Olerud scoring scale (OSS)	RS	Not enough evidence to support full validation for use in ankle.
Rowan foot pain assessment questionnaire (ROFPAQ)	DimS RS	Not enough evidence to support full validation for use in ankle.
Sports ankle rating system quality of life (SARQOL)	DimS RS	Valid for assessing quality of life after ankle injury.

6.1.1 Current patient-reported outcome measures for use in the foot and ankle

There are currently 16 scoring systems used for assessing foot and ankle conditions (Table 15).

Table 15: The sixteen foot and ankle scores currently used to assess foot and ankle conditions. Table adapted from (Martin and Irrgang, 2007).

Key: DisS = disease specific; DimS = dimension specific; RS = region specific; PopS = population specific

All assess different populations, conditions and diseases, and therefore no score is considered the 'gold-standard' (Pinsker, *et al.,* 2015; Harris,

et al., 2013). Different surgeons use different outcome measures depending on what they are trying to assess and their own personal preference. Despite 16 scores being in use, only six of these are validated for assessing the foot and ankle. This is related to some scores having been devised through adapting other scores, some scores having been developed to assess areas other than the foot and ankle and some scores not being sensitive to capturing foot and ankle issues. None of the existing PROMs were developed to assess the clinical outcome of ankle cartilage surgery and none of these PROMs address all major areas of concern for ankle patients, namely pain, swelling and recreation and leisure, specifically relating to sports (Pinsker et al., 2013).

6.2 Aims

This study aimed to develop and psychometrically refine a new PROM that can be used to assess the patient-perceived outcome of treatments for ankle cartilage injuries or diseases, such as cell therapy or ankle replacement.

6.3 Methods and materials

6.3.1 Design of the Oswestry Ankle Score (OsAS).

An initial version of the questionnaire (Appendix III, page 227) was developed based on questions taken from nine existing questionnaires, which focused on four specific areas of interest known to be important to ankle patients, namely pain, swelling, activities and daily life (Table 16; page 155). These areas and questions were chosen (section 5.1.1, page 141) in collaboration with four foot and ankle consultant surgeons and a specialist physiotherapist. Next, the draft

questionnaire was presented to a focus group consisting of ankle patients who visited the outpatient clinic before or after treatment in order to gain patient feedback and refine the questionnaire. This method of questionnaire design followed relevant guidelines (Frost et al., 2007; Rothman et al., 2007; Rothman et al., 2009)

Table 16: Nine patient-reported outcome measures assessed and used for questions to draft our initial questionnaire: the Oswestry ankle score (OsAS).

Score:	Score name:	Reference(s):
FHSQ	Foot Health Status Questionnaire	Palomo-López et al., 2017; Bennett et al., 1998.
FFI	Foot Function Index	Madeley et al., 2012; Budiman-Mak et al., 2013; Budiman-Mak et al., 1991.
SARQOL	Sports Ankle Related Quality of Life	Beaudart et al., 2017; Beaudart et al., 2017.
OHS	Oxford Hip Score	Wylde et al., 2005; Murray et al., 2007; Cöster et al., 2014.
LKS	Lysholm Knee Score	Smith et al., 2009; Tegner & Lysholm, 1985.
LEFS	Lower Extremity Function Scale	Binkley et al., 1999; Dingemans et al., 2017.
FAOS	Foot and Ankle Outcome Score	Cöster et al., 2014; Roos et al., 2001; Dawson et al., 2006.
FAAM	Foot and Ankle Ability Measure	Martin et al., 2005; Martin & Irrgang, 2007.
SMFA	Short Musculoskeletal Function Assessment Questionnaire	Williams, 2016; Martin et al., 1997.

6.3.2 Content validity

This questionnaire was trialled in a pre-operative assessment clinic on approximately 20 patients to gain patient feedback following Frost's guidelines (Frost et al., 2007) for assessing content validity, and on a physiotherapist and four foot and ankle consultant surgeons to gain clinician input. This was to ensure that our newly developed outcome measure captured both what patients and clinicians considered important when assessing clinical outcome of surgical intervention. Patients were asked about the length of questionnaire, the time taken to complete the questionnaire, wording of questions, ease of understanding, the relevance of questions and if they found anything important to be missing from the questionnaire. Any valuable feedback was noted and the score was adapted to include any suggestions, if applicable.

6.3.3 Psychometric refinement

Eligible patients (section 5.1.3.1, page 144) were invited into the study by sending them an invitation pack consisting of an invitation letter, a patient information sheet, a consent form and a prepaid envelope. The invitation letter explained the reasons for doing the study and what would be required of the participants. Participants were given the opportunity to ask questions by contacting us either by email, post or telephone. If happy to partake in the study, participants would complete the consent form by initialling each box on the form to confirm agreement to each statement and signing and dating the bottom of the form to confirm their participation. The consent form was then returned

using the stamped addressed envelope provided. If the returned consent forms were completed incorrectly, the forms were sent back to the participants to be amended before enrolment into the study. We sent a reminder letter to the participants after approximately 2-3 weeks if we did not get a response. Once enrolled, the consent form was also signed and dated by me and a copy was sent back to the patient for their records. The original was scanned into the electronic patient records, with a study enrolment log, and then filed into the study notes. All further correspondence for the duration of the study (ie: sending or receiving questionnaires) was documented in the electronic patient records. If patients did not wish to partake, they could send the blank forms back using the prepaid envelope provided.

6.3.4 Inclusion and exclusion criteria

Patients could only be enrolled into the study, if they met the following inclusion criteria:

- Over the age of 18
- Either: (1) Attending a first ankle clinic with symptoms suggesting cartilage defects or osteoarthritis, (2) Scheduled for treatment of ankle cartilage defects or osteoarthritis, (3) Having had treatment for ankle cartilage defects or osteoarthritis
- Prospective or actual treatment must be ankle surgery for cartilage injury or disease, including but not limited to: cell therapy, microfracture, fusion and total ankle replacement.

If a patient met all inclusion criteria, they were sent a patient Invitation letter.

Patients were excluded if they were:

- Unable to read and understand the patient information sheet
- Unable or unwilling to provide written informed consent for themselves
- Unlikely to complete the duration of the study for whatever reasons
- Unable to adequately understand verbal explanations or written information given in English or have special communication needs.

6.3.5 Statistical analysis plan

Content validity and the response process of our questionnaire was investigated using two focus groups, one at the development stage prior to the psychometric study and a second focus group after all data was collected, in line with current guidance (Rothman, *et al.,* 2009). The internal consistency of the instrument/questionnaire was determined using a principal component analysis and a Rasch analysis. The statistical analyses were performed using IBM-SPSS Vs 24 (IBM, New York, USA) for the factor analysis, and jMetrik vs 4.1.1 (jMetrik Item Analysis, Virginia, USA) for the Rasch analysis.

6.3.5.1 Factor analysis

Factor analysis is a statistical reduction technique that analyses correlations among multiple outcomes in this case the answers to the separate questions within the questionnaire. It is used to discover which questions in the questionnaire form coherent subsets that are relatively independent from each

other (Tabachnick and Fidell, 2006). The purpose of this analysis within our study was to determine if all questions addressed the same concept or whether the questions would address distinct aspects, such as pain and activity. In the latter case, we would consider eliminating isolated questions that addressed distinct aspects or questions that addressed aspects deemed less important. In the factor analysis, all factors with an eigenvalue larger than 1 were retained.

6.3.5.2 Rasch analysis

The Rasch analysis was performed to determine the internal consistency of the score and followed published guidelines (Linacre and Wright, 1998; Meyer, 2017; Smith, 1996). WMS (Infit mean square) and UMS (outfit mean square) values were used to determine which questions were considered important and which were deemed unimportant for the OsAS score. WMS is defined as an inlier sensitive weighted fit statistic, whereas UMS is defined as an outlier sensitive non-weighted fit statistic. Both are based on the squared standardised residual between what is observed and what would be expected on the basis of the Rasch model (Linacre, 2002). The range of UMS and WMS values is 0 to infinity, with 1 being the ideal value. Values significantly higher than 1 indicate noise within the dataset, whereas values significantly lower than 1 indicate dependency (Bond and Fox, 2007; Wright and Linacre, 1994). Importance was considered based on guidelines from Smith (Smith, 1996), which state 0.5-1.5 are deemed the most suitable cut off values for determining the fit of the questions within the overall score and are deemed productive for measurement (Smith, 1996; Wright and

Linacre, 1994). Questions whose answers did not fit the Rasch model according to these criteria were either eliminated or their scale was collapsed or reordered.

6.3.6 Content validity of revised questionnaire

Based on the results of the factor analysis and the Rasch analysis, some questions were eliminated or had their scales revised. Scales were collapsed, were necessary, based on theta plots (Figure 30; page 122). The analysis could also identify overlapping questions, which were candidates for elimination. The revised questionnaire was then discussed again with the clinical team (consultant surgeons and physiotherapists) and sent to a patient focus group of 13 patients to determine if the revised questionnaire still addressed all aspects deemed important to patients and clinicians. Both groups were also asked which questions (if any) could be eliminated from the revised questionnaire.

6.3.7 Sample size and power

A sample size of 219 was decided upon for all psychometric and further validation analyses using a combination of formal power analysis and relevant guidelines (Frost, *et al.,* 2007; Rothman, *et al.,* 2009; see also Chapter 5, page 139). These guidelines suggest a sample size of 200 for a Rasch analysis and 300 cases for a factor analysis, but decline to advise on sample sizes for focus groups. A formal power calculation was not performed, but a sample size was selected based on sample sizes needed for all necessary analysis outlined in Table 14 (page 143).

6.4 Results

6.4.1 First development and content validation

The initial OsAS score (Version 2) was designed using existing questions from nine questionnaires: foot and ankle outcome score, sports ankle rating system quality of life, foot health status questionnaire, foot function index, foot and ankle ability measure, lower extremity function scale, short musculoskeletal function assessment questionnaire, the Oxford hip score and the Lysholm knee score (section 5.1.1, page 141). Once designed, the initial version of the OsAS questionnaire was given to a patient focus group of 19 patients attending the outpatient clinic at RJAH. All participating patients agreed that the length of the questionnaire was adequate, the time taken to complete the questionnaire was acceptable and the wording of the questions was appropriate. They agreed that everything they considered to be important in assessing the outcome of their surgery was addressed in the OsAS questionnaire. The feedback received from the healthcare professional focus group mimicked that of the patient focus group. It was suggested, by both patients and healthcare professionals, that a question should be added to address any other conditions which could affect overall outcome, for example foot drop could affect the surgical outcome of ankle surgery or depression could affect the patient perspective of their outcome. Furthermore, two questions about the range of movement of the joint were suggested by the physiotherapist. Following feedback received from patients and healthcare professionals, set time frames were given for the questions (i.e.: “in the last two weeks”). The initial questionnaire was amended

to version 3 (Appendix III, page 227), to include the suggested added questions, and the time frames, and is shown below.

6.4.2 Psychometric validation and refinement

We screened more than 5,400 patients across four surgeon clinic-lists between the months of September 2017-June 2018. This is based on 150 mean patients per clinic list, for each of the four surgeons. A total of 360 patients were eligible for the OsAS study, all of which were sent study information. We had a recruitment rate of 57.2%, having successfully recruited 206 patients over a 9-month period, between September 2017 and June 2018. Recruited patients consisted of a wide age range (18-91 years, mean age: 62 years), relatively equal split of gender (49.51% female: 50.49% male split, consisting of 102 females and 104 males). A relatively equal split of treatment status was observed in the participants, (60.68% post-operative: 39.32% pre-operative, consisting of 125 post-operative patients and 81 pre-operative patients). All demographics for all patients are recorded in Table 17 (page 164). Four patients withdrew from the study after consenting, giving a low withdrawal rate of 1.94%, but data collected during their time in the study remained within the data analysis, as per study protocol.

The factor analysis indicated that the 29 questions loaded onto four factors (Figure 37, page 172, Table 18, page 166), which corresponds to (1) daily life, (2) activities, (3) pain/sleep and (4) movement. Whereas the questions that comprised factors 2 and 3 also loaded onto factor 1, the questions that comprised factor 4 (symptoms questions 5 and 6 and activity question 5) loaded entirely onto their own category.

Table 17: Demographics collected from consented patients in the OsAS study.

Patient demographics:						
Gender:		***Age:***		***Status of treatment:***		
Female	Male	Age range	Mean age	Pre-operative	Post-operative	
102	104	18-91 years	62 years	81	125	
Symptoms:						
Pain	Osteochondral lesions / defects	Arthritis / Degenerative changes	Instability or discomfort	Previous trauma	Other	Unknown
32	24	136	6	4	5	10
Treatments:						
TAR	Fusion/ arthrodesis	CCR	Microfracture	Arthroscopy / debridement	Osteotomy	Unknown
34	64	6	7	12	3	1

TAR = total ankle replacement; CCR = complete cartilage regeneration

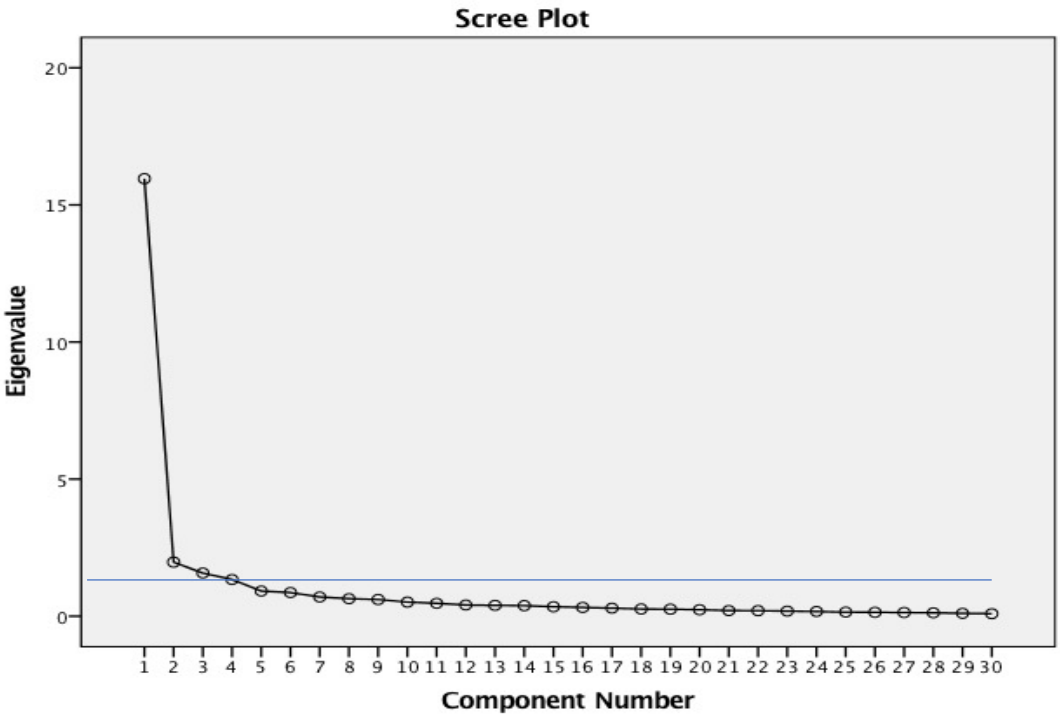


Figure 34: Scree plot of the factor analysis performed on the OsAS questionnaire, demonstrating that all questions fell under one main category, while four questions had an eigenvalue above 1.0 (indicated by the blue horizontal line).

Table 18: Factor analysis performed on the Oswestry Ankle Score questionnaire, demonstrating that all questions fell under four categories.

Rotated Component Matrix[a]				
	Component			
	1	2	3	4
PainQ1	.230	.331	**.767**	.007
PainQ2	.139	.417	**.748**	.118
PainQ3	.322	.337	**.740**	-.026
SymptomsQ1	**.674**	.344	.449	.072
SymptomsQ2	**.661**	.359	.455	.101
SymptomsQ3	**.710**	.308	.325	.023
SymptomsQ4	**.659**	.300	.443	.052
SymptomsQ5	-.088	-.187	-.050	**-.910**
SymptomsQ6	-.099	-.086	-.147	**-.910**
SymptomsQ7	.252	-.015	**.645**	.141
SymptomsQ8	.369	.123	**.584**	.235
ActivitiesQ1	**.538**	**.544**	.263	.200
ActivitiesQ2	.067	**.652**	.435	.118
ActivitiesQ3	.451	**.635**	.364	.097
ActivitiesQ4	**.591**	**.501**	.284	.149
ActivitiesQ5	.350	.343	.290	**.532**
ActivitiesQ6	.446	**.624**	.324	.262
ActivitiesQ7	.282	**.756**	.239	.137
ActivitiesQ8	.338	**.726**	.167	.162
ActivitiesQ9	.422	**.760**	.189	.155
ActivitiesQ10	.468	**.697**	.087	.113
ActivitiesQ11	.203	**.574**	**.548**	.137
ActivitiesQ12	**.594**	.430	.212	.161
ActivitiesQ13	**.518**	**.605**	.206	.102
ActivitiesQ14	**.715**	.306	.366	.108
ActivitiesQ15	**.710**	.372	.247	.031
ActivitiesQ16	**.628**	.330	.292	.154
ActivitiesQ17	**.532**	.282	-.033	.295
ActivitiesQ18	**.542**	.372	-.085	.297
ActivitiesQ19	**.701**	.038	.369	.074

Note: The largest factor loading of each question has been formatted in bold to illustrate which factor it mainly loaded onto.

We felt factor 4 corresponded with movement as these three questions focused on range of motion, for example: 'what difficulty have you experienced from your ankle when raising your heel to stand on tip toes.' When these three

questions were excluded and the factor analysis was run again, all questions loaded one factor (Table 19).

Table 19: Factor analysis performed on the OsAS questionnaire following the elimination of questions highlighted in the initial factor analysis in Table 18 (page 166).

Component Matrix[a]			
	Component		
	1	2	3
PainQ1	**.702**	.495	-.118
PainQ2	**.700**	.454	-.259
PainQ3	**.744**	.447	-.049
SymptomsQ1	**.847**	.075	.219
SymptomsQ2	**.862**	.076	.189
SymptomsQ3	**.784**	-.023	.285
SymptomsQ4	**.809**	.093	.237
SymptomsQ7	**.473**	.466	.113
SymptomsQ8	**.617**	.323	.133
ActivitiesQ1	**.809**	-.144	-.018
ActivitiesQ2	**.654**	.109	-.444
ActivitiesQ3	**.848**	-.053	-.156
ActivitiesQ4	**.821**	-.119	.056
ActivitiesQ6	**.835**	-.102	-.170
ActivitiesQ7	**.757**	-.159	-.347
ActivitiesQ8	**.746**	-.226	-.283
ActivitiesQ9	**.829**	-.242	-.251
ActivitiesQ10	**.764**	-.319	-.156
ActivitiesQ11	**.741**	.201	-.316
ActivitiesQ12	**.750**	-.148	.084
ActivitiesQ13	**.793**	-.186	-.079
ActivitiesQ14	**.822**	-.006	.272
ActivitiesQ15	**.792**	-.120	.231
ActivitiesQ16	**.756**	-.062	.176
ActivitiesQ17	**.535**	-.327	.164
ActivitiesQ18	**.570**	-.405	.090
ActivitiesQ19	**.646**	.101	.448

Note: The largest factor loading of each question has been formatted in bold to illustrate which factor it mainly loaded onto.

This factor analysis demonstrated that all remaining 27 questions fell under one main category, which could be termed “patient-perceived ankle impairment and activity”.

An initial Rasch analysis was performed on all remaining questions following the factor analysis. The results of the Rasch analysis suggested that all questions apart from one symptom question (question 7) and one activity question (question 18) fitted the Rasch model. The two questions that did not fit the model had WMS or UMS values outside the 0.5-1.5 fit guidelines and therefore did not fit well with the total score (Table 20; page 169). These questions were: ‘how often did you experience swelling in your ankle, during the last week?’ and ‘how often did you feel the need to use a walking aid (for example: crutches, walker)?’ and were removed and the Rasch analysis was performed again to ensure that the remaining 25 questions showed a ‘good fit.’

Table 20: Values of fit of the Rasch model with the 27 OsAS PROM questions.

Question	WMS value	UMS value
Pain 1	0.81182852	0.81201876
Pain 2	0.78184903	0.72738411
Pain 3	0.89179109	0.88843466
Symptoms 1	0.60296132	0.60239852
Symptoms 2	0.54902974	0.55101998
Symptoms 3	0.70112567	0.70445605
Symptoms 4	0.6768304	0.68898128
Symptoms 7	**1.55262554**	**1.65873145**
Symptoms 8	1.0880462	1.10843352
Activities 1	0.69064299	0.67700314
Activities 2	0.88987609	0.83323497
Activities 3	0.52647104	0.52333665
Activities 4	0.61764944	0.59363475
Activities 6	0.60779212	0.60413362
Activities 7	0.78222338	0.75304616
Activities 8	0.77736046	0.7640934
Activities 9	0.60129212	0.5876738
Activities 10	0.74453717	0.74254932
Activities 11	0.69061886	0.64266852
Activities 12	0.86571299	0.87680084
Activities 13	0.70046456	0.67095696
Activities 14	0.60794945	0.58626078
Activities 15	0.72988718	0.74599126
Activities 16	0.79257873	0.7657765
Activities 17	1.42503286	1.48904447
Activities 18	1.34985453	**1.68511376**
Activities 19	0.98234759	1.09978342

Note: Bold font indicates values outside the guideline values of 0.5-1.5.

Theta plots of all 30 questions of the initial OsAS PROM (Figure 35; page 170) illustrated which questions did not fit the Rasch model and how the question scales could be collapsed for many questions to provide answer options that were arranged better. Broadly, the theta plots tend to follow the same trend for each of the three subsections: pain, symptoms and activities.

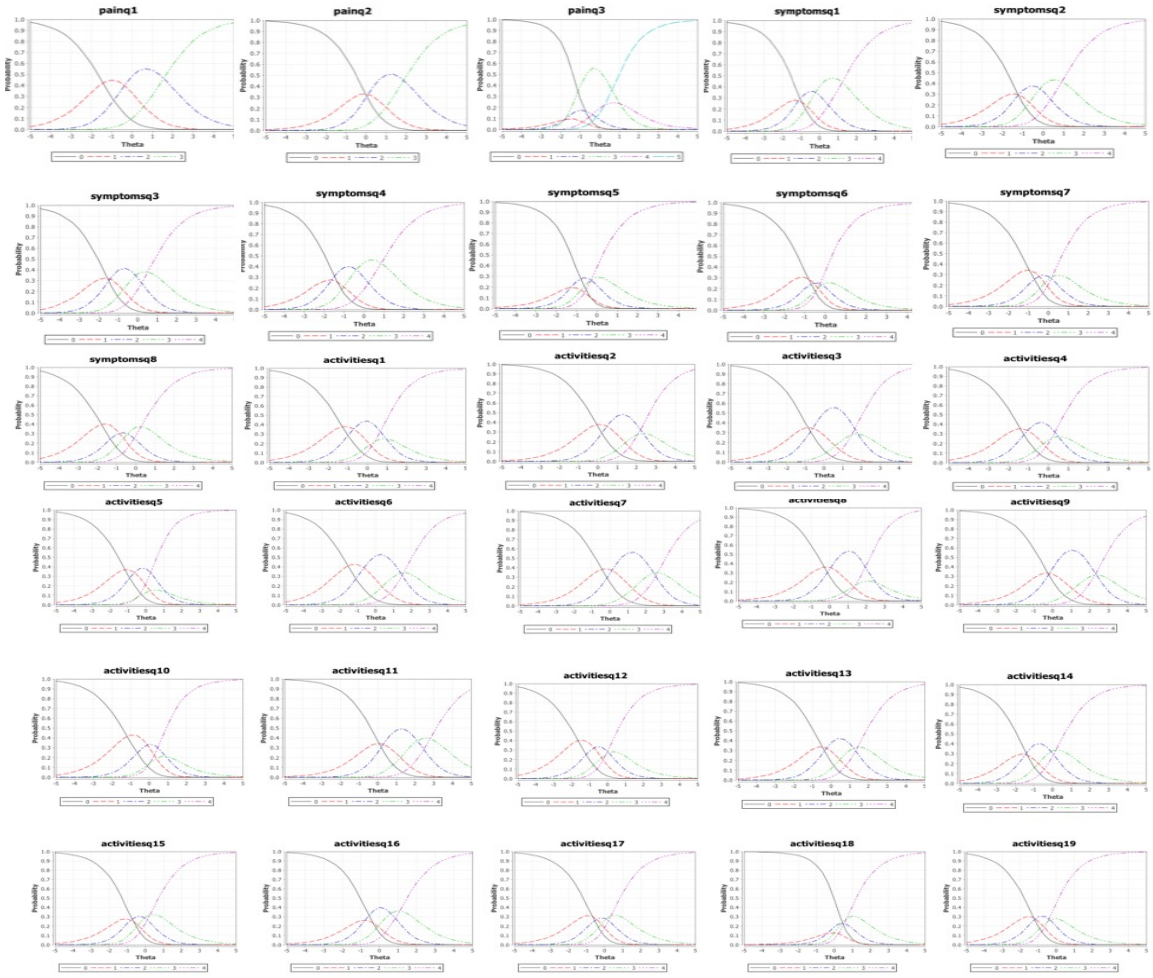


Figure 35: Theta plots for each of the 30 questions of the OsAS questionnaire generated from a Rasch analysis. These plots demonstrate the relevance of each answer option, and the data was used to collapse the scales.

Most questions demonstrated a good fit to the Rasch model, indicated by distinctive peaks for each answer option with minimal overlap and a consistent trend. An example is pain question 1 (Figure 36).

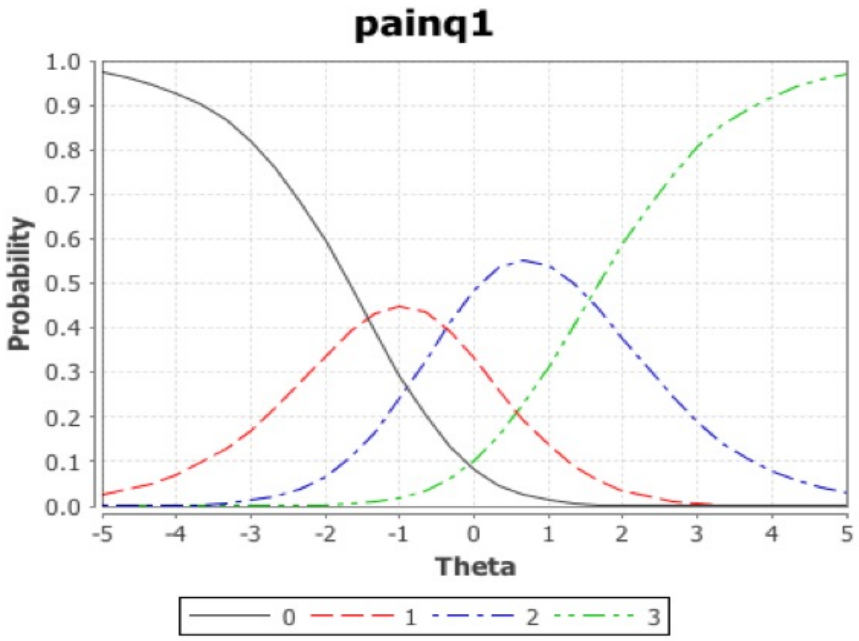


Figure 36: A theta plot demonstrating good fit to the Rasch model, illustrated by distinct peaks with minimal overlap.

These questions required no manipulation with the question or scale. Other questions demonstrated borderline fit to the Rasch model (Figure 37; page 172), indicated by some overlap between the peaks. These questions required

scale collapse to provide more distinct answer options and thus better fit to the Rasch model.

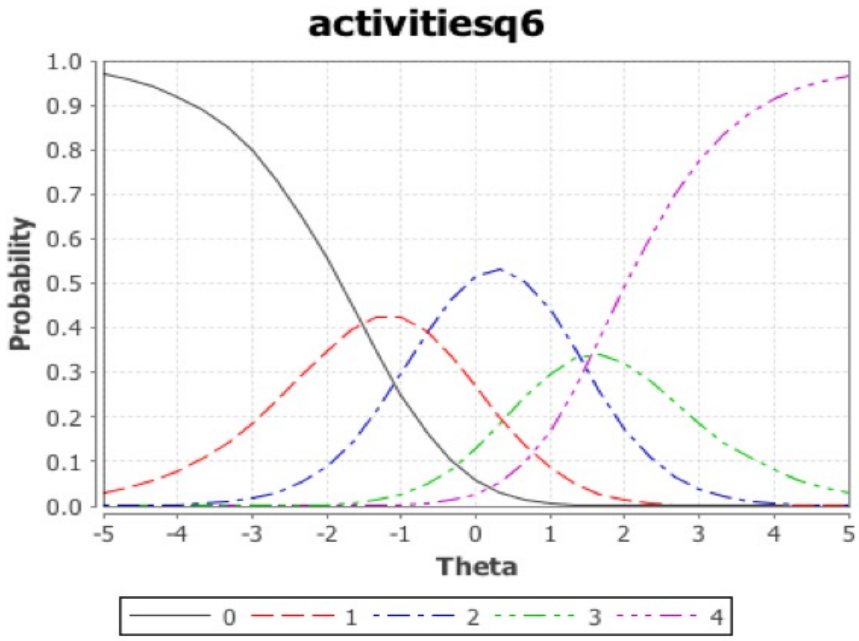


Figure 37: A theta plot demonstrating borderline fit to the Rasch model, illustrated by distinct peaks with some significant overlap, as indicated by the green peak which is completely overlapped by the blue and purple peak.

Five questions demonstrated poor fit to the Rasch model. These questions identified in the factor analysis (Table 18; page 166) and the two in the initial Rasch analysis (Table 20; page 169): symptom questions 5, 6 and 7, and activity questions 5 and 18. These questions were therefore eliminated from the OsAS questionnaire due to the poor fit. An example is shown in Figure 38 (page 173).

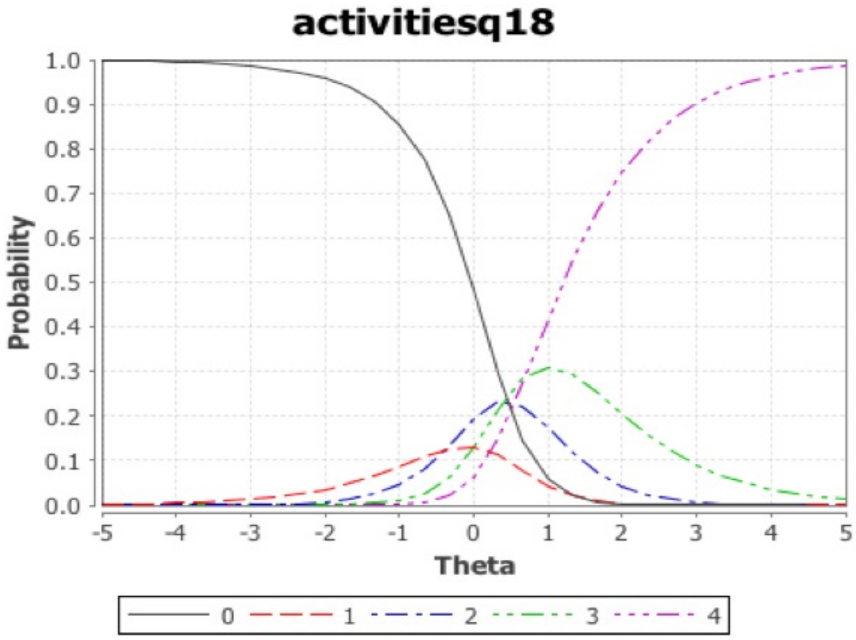


Figure 38: A theta plot demonstrating poor fit to the Rasch model, illustrated by small peaks that completely overlap one another with no consistent pattern, as indicated by the red, blue and green peaks all completely overlapped by the black and purple lines.

6.4.3 Development and content validity of the final version

All 25 remaining questions had similar UMS and WMS values and showed a similar weight of importance. Close examination of the remaining questions suggested that the questionnaire could be condensed further as many questions were similar in nature, such as symptom questions 1 and 2 both ask about the effect on daily life, and symptom questions 3 and 4 both ask about being limited with certain activities. We therefore decided to perform a second focus group round, asking specifically which questions were considered relevant. This group comprised the five clinicians who helped design the original questionnaire, plus

13 patients. The 13 patients completed content validity feedback on the condensed OsAS (Version 3.3; Appendix IV; page 230), which contains 18 questions. All feedback received was in response to importance, which ranged between 76.9-100% (Table 21).

Table 21: Content validity for the condensed OsAS measure (Version 3.3), determined by asking a focus group of 13 patients to rate if a question addressed an important aspect.

Question:	Number of patients considering this question important:	Importance (%):
Pain question 1	13	100
Pain question 2	12	92.3
Symptoms question 1	12	92.3
Symptoms question 2	12	92.3
Symptoms question 3	11	84.6
Symptoms question 4	11	84.6
Symptoms question 5	12	92.3
Activities question 1	11	84.6
Activities question 2	10	76.9
Activities question 3	13	100
Activities question 4	12	92.3
Activities question 5	10	76.9
Activities question 6	11	84.6
Activities question 7	11	84.6
Activities question 8	12	92.3
Activities question 9	12	92.3
Activities question 10	11	84.6
Activities question 11	13	100

The condensed and final version of the OsAS questionnaire, with the collapsed scales, was the version used for all subsequent data analysis and validation purposes (Version 3.3, Appendix IV, page 230).

Based on their suggestions, the OsAS questionnaire was condensed from 25 questions across 3 pages, to 19 questions across 2 pages. This condensed and final version of the OsAS questionnaire, with the collapsed scales, was the version used for all subsequent data analysis and validation purposes (Version 3.3, Appendix V, page 232).

6.5 Discussion

We have successfully developed and psychometrically refined a new PROM entitled the 'Oswestry Ankle Score (OsAS)'. The questionnaire is aimed specifically at patients with ankle problems, before or after treatment. The questionnaire has a good internal consistency, as demonstrated by its fit to the Rasch model, and good content validity, as indicated by patient focus groups and clinicians. The questionnaire was designed with assistance from two patient focus groups, the first consisting of 19 patients, and the second consisting of 13 patients, our foot and ankle clinical care team consisting of four foot and ankle surgical consultants and one specialist physiotherapist, and a biostatistician. The patient focus groups and the foot and ankle clinical care team found that the questionnaire addressed all aspects considered important, from both a clinical and patient perspective. The first patient focus group also stated that they felt the questionnaire was of adequate length, easy to understand, easy to complete and of adequate time for completion.

As with every study design, there were a few limitations. One potential limitation to our study was that the study only occurred at one site (a large tertiary referral centre at a rural location), which could have skewed the patient

population. However, this hospital admits a mixture of patients, consisting of referrals from the local area that are consistent with referrals to other secondary centres, and referrals from a wider area that require specialised care. A second concern associated with this study might be the response rate. As with all paper-based studies, there are some participants who do not return the questionnaires or lose them.

Now developed, we aim to further validate the OsAS measure, using test-retest reliability, responsiveness and construct validity in the form of hypothesis testing regarding correlation with two existing scores: the MOxFQ for assessing foot health (Appendix V, page 232) and the VR-12 for assessing general health (Appendix VI, page 234). These are reported in the next chapter.

Chapter 7: Validation of the Oswestry Ankle Score (OsAS).

7.1 Introduction

Many surgical treatments for cartilage disorders in the ankle are in use, including ankle fusion, debridement, microfracture, autologous chondrocyte implantation (ACI), matrix-assisted chondrocyte implantation (MACI), implantation of a stem-cell concentrate and total ankle replacement. However, assessing and comparing the outcomes of these treatments is problematic because there are very few ankle-focused PROMs. More specifically, there are sixteen PROMs for assessing the foot and ankle (Table 16; page 155), of which only six have been successfully validated. Furthermore, these six validated scores have all been developed for assessing different conditions, age-ranges and populations. This limits the use of the scores and thus has resulted in there being no 'gold-standard' for assessing clinical outcome of the foot and ankle.

With between a quarter and a third of all patients stating they are dissatisfied with their surgery (Dawson et al., 2006), this highlights the demand for a new validated PROM to be developed for assessing clinical outcome of ankle surgery for treating cartilage disorders, which includes cell therapy surgery. The new outcome score would also aim to address areas to improve that could increase patient satisfaction of their surgery. We developed a new PROM, the Oswestry Ankle Score (OsAS), to assess clinical outcome of ankle surgery. In the previous chapter (Chapter 6) we described the development stages and item selection for the score in detail and demonstrated its good internal consistency and content validity.

For the OsAS to be used clinically, it needs to be valid, reliable and responsive (Mokkink et al., 2010). Validity is defined as the extent to which a measure measures the construct it purports to measure. The content must capture what the score aims to capture and must be considered important from the patient perspective. The OsAS must also be responsive, that is sensitive to changes in clinical outcome. Therefore, it must be able to accurately detect when a patient's clinical outcome is improving or declining. Finally, the score must be reliable. More specifically, if no change has occurred in clinical outcome then the OsAS score should remain relatively constant. The quality of the OsAS in these three domains can be assessed using the parameters listed in Chapter 6 (Table 14; page 143).

This study aimed to validate the final 18-item version of the OsAS questionnaire described in the previous chapter. Specifically, it aimed to determine if the OsAS is valid, responsive and reliable using the statistical analyses recommended in the checklist from COSMIN (consensus-based standards for the selection of health status measurement instruments; Mokkink et al., 2010).

7.2 Methods and materials

The 18-question OsAS was sent to patients either pre-operatively or post-operatively, together with two other relevant PROMs, the Manchester Oxford Foot Questionnaire (MOxFQ; Appendix V; Dawson et al. 2006, and the Veterans Rand 12 (VR-12; Appendix VI; Selim et al., 2009). The MOxFQ is a 16-question instrument developed to assess the outcomes of foot surgery, which determines

the outcome in three separate dimensions: walking/standing, pain and social interactions, each scored 0 (best) to 100 (worst) (Dawson et al., 2006). More recently, a method to derive a single summarising MOxFQ score was described, scored from 0 (worst) to 100 (best) (Morley et al., 2013), which we also determined. The VR-12 is a 12-question general health-related quality of life instrument, which summarises the general health along two dimensions: a physical component score (PCS) and a mental component score (MCS), each scored 0 (worst) to 1 (best) (Selim et al., 2009). If sent pre-operatively, where appropriate and relevant we sent a further OsAS score to the patient at least three months after their operation.

7.2.1 Responsiveness to change

We hypothesised that a minimum two-month time frame was sufficient to allow recovery and that the patients' outcome would have changed. Patients who had completed both a pre-operative and post-operative OsAS score were used to analyse the responsiveness of the score (sensitivity to change). It was hypothesised that if the condition of the patient had changed, for example through surgical intervention, the OsAS results would also change. The responsiveness was analysed by performing a paired t-test and determine Cohen's effect size, which is the ratio of the difference in score and the baseline standard deviation (Cohen, 1992). We aimed to obtain a sample size of 109 patients to assess the Cohen's effect size with a 95% CI of ±0.2, assuming a responsiveness of 0.5 and a correlation of 0.5 between baseline and post-treatment score.

7.2.2 Test-retest reliability

Two OsAS scores were sent to patients within a short time-period, less than six weeks apart. We hypothesised that in this small time-frame the patients' condition would not change, provided there had not been an intervention in the meantime. The scores from patients who had completed two scores within an 8-week timeframe and who had no intervention in that time were used to determine the test-retest reliability using the intra-class correlation coefficient (ICC) based on a 1-way mixed ANOVA model with "patient" as the random factor. We aimed to obtain a sample size of 105 patients to estimate the test-retest correlation coefficient with a 95% CI of ±0.1 or smaller, assuming that the coefficient was at least 0.7, a reasonable number based on similar PROM instruments such as the MOXFQ.

7.2.3 Construct and criterion validity

The correlation of the OsAS score with scores on two other relevant PROMs was determined, namely the MoXFQ and the VR-12. Higher scores on both the OsAS and the MOxFQ instrument denote poorer outcomes, whereas they denote better outcomes on the VR-12. We therefore hypothesised that the OsAS would have a positive correlation with the three domains of the MOxFQ but a negative correlation with the two components of the VR-12. We also hypothesised that the correlation with the MOxFQ (a region-specific PROM) would be stronger than that with the VR-12-PCS (a general health-related PROM), and that the correlation with the VR-12 PCS would be stronger than that with the VR-12 MCS.

We aimed to obtain a sample size of 219 people to complete the three questionnaires, based on estimating the Pearson product-moment correlation coefficient with a 95% CI of ±0.1 or smaller, assuming that the coefficient was at least 0.5, or a 95% CI of ±0.15 if the coefficient was smaller. Due to the study design, several patients would submit more than one set of questionnaires. However, to avoid bias we used one set of questionnaires per patients for the correlation analysis. All statistical analyses were carried out using SPSS vs 24 (IBM, New York, USA).

7.3 Results

We used the same cohort of patients from Chapter 6.

7.3.1 Test-retest reliability

We obtained responses from 106 patients for the reliability (test-retest) analysis (section 7.2.2, page 180). The two sets of questionnaires were received within approximately 8 weeks of each other. The mean age of the sample population for the test-retest reliability analysis was 64 years ± SD 1.13; age range: 18-88 years).

The OsAS had a test-retest ICC of 0.93 (95% CI 0.89 to 0.95; Table 22; page 182). In comparison, the test-retest ICC for the MOxFQ sub-scales for pain, symptoms and activity were 0.75 (95% CI 0.65-0.83), 0.80 (0.70-0.86) and 0.78 (95%CI 0.67-0.85) respectively (Table 22; page 182).

Table 22: The test-retest intraclass correlation coefficient (ICC) calculated using a 1-way mixed ANOVA model with "patient" as random factor.

	ICC:	**95% CI:**	
		Lower bound:	**Upper bound:**
OsAS	0.928	0.891	0.952
MOxFQ Pain	0.757	0.652	0.834
MOxFQ Symptoms	0.795	0.704	0.861
MOxFQ Activities	0.782	0.686	0.852

7.3.2 Responsiveness

We obtained 112 sets of questionnaires from 61 patients suitable for the analysis of responsiveness (sensitivity to change). The two sets received for each patient were obtained pre-operatively and a minimum of two-months post-operatively (mean time after surgery 3.36 months ± 0.23 SD, range 2-9 months). The mean age of the sample population for the responsiveness analysis was 60.46 years ± SD 1.68; age range: 19-92 years). The mean outcomes on all three instruments (OsAS, MOxFQ and VR-12) changed following surgery, except the VR-12 MCS (Table 23; page 183). When expressed as Cohen's effect size, the OsAS was found the most responsive (effect size 1.59; 95% CI 1.21 to 1.97), which was 2-4 times higher than the standardised effect size obtained from the three components of the MOxFQ, and more than 10 times higher than that obtained for the VR-12 (Table 23; page 183). The higher effect size of OsAS, compared to the MOxFQ and VR-12, could be due to the OsAS being sensitive than the other two scores or could be because the effect size was inflated.

Table 23: Responsiveness of the OsAS, MOxFQ and VR-12 PROMs, including each sub-scale for the MOxFQ and VR-12.

Score:	Mean score at baseline (±SD)	Mean score at post-operative (±SD)	Mean difference (±SD)	p value	Effect size	95% CI	
						Lower bound	Upper bound
OsAS	56.83 ± 20.30	40.05 ± 20.96	16.78 ± 20.63	< 0.001	1.59	1.21	1.97
MOxFQ Pain	68.56 ± 28.64	55.74 ± 30.61	12.82 ± 3.76	< 0.001	0.62	0.35	0.89
MOxFQ Symptoms	52.62 ± 26.72	45.66 ± 28.53	9.67 ± 3.63	0.001	0.37	0.11	0.63
MOxFQ Activities	45.33 ± 27.51	35.66 ± 29.32	6.97 ± 3.53	0.006	0.45	0.19	0.72
VR-12 PCS	44.60 ± 8.66	43.32 ± 9.37	1.28 ± 1.16	< 0.001	0.148	-0.11	0.40
VR-12 MCS	37.86 ± 5.38	38.02 ± 4.28	-0.16 ± 0.62	0.821	0.030	0.00	0.19

7.3.3 Construct and criterion validity (correlation with other instruments)

In all, 159 patients returned a full set of three instruments (OsAS, MOxFQ and VR-12). Our analyses of the correlation of the OsAS with the other instruments were therefore based on a sample size of 159 patients. The mean age of the sample population for construct and criterion analysis was 62 years ± SD 1.62; age range: 19-88 years).

The correlation of the OsAS, the total MOxFQ score and each domain of the MOxFQ (pain, walking/standing and social interaction) varied from 0.78 (95%

CI 0.72-0.83) for the social interactions domain to 0.85 (95% CI 0.80-0.89) for the pain domain (Table 24). The positive directions of these correlations were in line with our hypothesised direction.

Table 24: Correlation between the OsAS and the MOxFQ scores, based on 159 responses, using one scores per patient.

Correlations:		**MOxFQ total**	**MOxFQ pain**	**MOxFQ walking/ standing**	**MOxFQ social interactions**
OsAS	Pearson correlation	0.810	0.850	0.828	0.780
	95% Lower CI	0.729	0.802	0.770	0.723
	95% Upper CI	0.868	0.889	0.879	0.829

The correlation coefficient of the OsAS with the two domains of the VR-12 were -0.68 (95% CI -0.76 to -0.60) for the PCS and -0.08 (95% CI -0.26-0.09) for the MCS (Table 25). The non-overlapping 95% CIs demonstrated a stronger correlation between the OsAS and the PCS than between the OsAS and the MCS. The negative directions of the correlations and the stronger correlation of the PCS were in line with the hypothesised direction and size.

Table 25: Correlation between the OsAS and the VR-12

Correlations:		**VR-12 MCS**	**VR-12 PCS**
OsAS	Pearson correlation	-0.079	-0.684
	95% Lower CI	-0.257	-0.758
	95% Upper CI	0.092	-0.599

The non-overlapping 95% CIs demonstrated that the correlations between the OsAS and two domain scores of the MOxFQ (pain and walking/standing) were stronger than those between the OsAS and the VR-12 PCS score. This was in line with the hypothesised order.

7.4 Discussion

The Oswestry Ankle Score (OsAS) is a new 18-item PROM to assess outcome of patients following treatment of cartilage disorders in the ankle. The OsAS has good content validity and internal consistency (Chapter 6, page number 146). This study aimed to determine its construct and criterion validity, test-retest reliability and responsiveness to change. The OsAS was found to have a good construct and criterion validity, demonstrated by a very strong correlation with a validated questionnaire for outcomes of foot surgery (MOxFQ), which can be regarded a “gold standard”, and a general health-related quality of life instrument (VR-12). The OsAS also had an excellent test-retest reliability (ICC=0.93) and a high responsiveness. Furthermore, our study assessed the OsAS across both genders, a wide age range (18-91 years) and a variety of presenting symptoms and ankle treatments. Based on our analyses, the 18-item OsAS score is suitable for clinical use to assess the patient-perceived outcome for a wide range of treatments of ankle cartilage disorders.

The OsAS was found to have an excellent test-retest reliability. Its value of 0.93 was above the value of 0.75 commonly used to indicate excellent agreement (Fleiss, 1999). An excellent test-retest reliability above 0.75 was also found for the MOxFQ instrument. The values for the test-retest ICC of the

MOxFQ found in this study (0.76 to 0.78) were slightly smaller than those found by the originators of the MOxFQ, who found values in the range of 0.93 to 0.95 in a group of ankle patients (Dawson et al., 2011). Their sample had a lower mean age than our sample (50 years versus 62 years) and a slightly lower proportion of women (46% vs 50%), which may explain some of the differences. On the other hand, to our knowledge our study is the first external study to confirm the excellent test-retest reliability of the MOxFQ score.

The OsAS instrument was found to have a high sensitivity to change following interventions to help patients with cartilage disorders in the ankle, such as total ankle replacement or an ankle arthrodesis. In our study, the OsAS had a standardised effect size that was two to four times higher than the current PROM used at our centre to assess the outcome of foot and ankle surgery, namely the MOxFQ, and more than ten times higher than a general health-related quality of life instrument, the VR-12. In line with our hypothesised responsiveness, we found that the effect size found using the OsAS, an ankle-specific outcome measure, was larger than that found by the MOxFQ, which is aimed at measuring outcome of foot and ankle surgery and is therefore less specific. Both the OsAS and the MOxFQ showed a larger standardised effect size than the PCS and particularly the MCS of the VR-12. The VR-12 is a general health-related questionnaire and thus less specific to outcomes of ankle or foot and ankle surgery, which would explain its smaller responsiveness. An earlier study also found that the MOxFQ was more responsive to change after ankle surgery than a general health-related quality of life instrument, in that case the SF-36 (Dawson et al., 2012). In that study, a larger standardised effect size was

reported for the MOxFQ, namely 0.86 to 1.10 for the three domains versus 0.26-0.45 in our study. The lower responsiveness of the MOxFQ in our study may be related to the shorter mean time after surgery, which was 3.36 months in our study and 9 months in the study by Dawson et al. (Dawson et al., 2012). One would expect more improvement and thus a larger standardised effect size after a larger period. Nevertheless, our results do suggest that the OsAS is more sensitive to ankle-specific changes than more generic foot-ankle scale.

The OsAS instrument was also found to have a good construct and criterion validity. The OsAS correlated well with the three domains of the MOxFQ and its summary total score, with correlation coefficients ranging from 0.78-0.85. Such a strong positive correlation would be expected for instruments that are specific to similar parts of the body, in this case the ankle and the foot/ankle. The items in the OsAS measure cover the content of the three domains of the MOxFQ, namely pain, walking/standing and social interactions, but on a single uni-dimensional scale, and we therefore had hypothesised this strong correlation. Since the MOxFQ can be regarded a standard instrument, this strong correlation is a good indicator of the criterion validity of the OsAS. The OsAS score also correlated with the VR-12 PCS, which quantifies general physical health-related quality of life, but less well than with the MOxFQ. However, the OsAS correlated poorly with the VR-12 MCS, which quantifies general mental health-related quality of life. These finding supported our prior hypotheses regarding these correlations. Taken together, they provide strong support for the construct validity of the OsAs because they indicate that the OsAS measures a

physical health-related construct that is more region-specific than that obtained by a general health-related instrument.

Our study does have some limitations. Firstly, we did not manage to recruit the number of patients that were needed for each analysis, most notably the correlation with other scores (56% of the aimed recruitment rate) and the correlation with other scores (73% of the aimed recruitment rate). However, as we had CI's narrower than planned, the lower recruitment rate would not impact on the conclusions obtained from our study. Secondly, the time since surgery for the sensitivity to change analysis was relatively short (mean time 3.26 months; range: 2-9 months) and patients could still have been undergoing recovery following their surgery. Thus, the sensitivity to change analysis performed may be higher than calculated. However, the sensitivity to change calculations demonstrated that the changes in score within this short time frame was still large enough to measure an effect. Future studies using the OsAS PROM would be able to calculate the responsiveness after a longer time-period between pre-operative and post-operative intervention. Our score could potentially be used to individualise treatment, by assessing the treatment outcome on a patient-by-patient basis and from the patient perspective. However, a PROM approach is not always the purest analysis methods for predicting patient outcome. The OsAS would not be able to determine the most appropriate treatment for a personalised approach, but assess the outcome of the treatment given.

The OsAS was designed by clinicians and researchers, with patient input from the beginning and throughout. Doing so ensures that the OsAS captures what clinicians and patient find important when assessing their clinical outcome and surgical success (Pinsker et al., 2013). Moreover, this continuous input of patients and clinicians resulted in a unidimensional scale with a high internal consistency between the 18 questions (chapter 6, page number 146). The unidimensional nature means that the OsAS gives a single outcome reflection the patient-perceived “ankle quality”, which will facilitate its use in clinical practice and research. The results from Chapters 5 (page number 140) and this chapter indicate that the OsAS measure can be used as a PROM for assessing the outcome of a wide range of treatments for ankle cartilage disorders, probably with a higher sensitivity to change than the MOxFQ score (Dawson et al., 2011; Morley et al., 2013)

Chapter 8: General Discussion

Damaged articular cartilage has a limited potential for healing and untreated cartilage defects often progress to osteoarthritis. In the UK, osteoarthritis affects 8.75 million people costing the NHS £850 million annually. High hopes have been pinned on regenerative medicine strategies to meet the challenge of preventing progress to osteoarthritis. The ACI procedure has evolved over the last twenty years but it still requires additional refinement to further improve upon the technique. Optimising the expansion and chondrogenic capacity of cultured human articular chondrocytes is one of these refinements.

We investigated an alternative serum supplement to the current gold-standard used in research; FBS. Stemulate™, a commercially available human platelet lysate, has been previously utilised to improve MSC expansion. With freshly isolated human chondrocytes, we reported an increase in proliferation in monolayer culture, however, despite a 28-day three-dimensional culture period the chondrocytes did not recover their chondrogenic capacity to the extent of those cells cultured in the presence of FBS. Although this research, which has been published (Sykes et al 2018; page 238), determined that Stemulate™ was less effective than FBS, it might still have some place in the ACI protocol. We would need to find methods to enhance chondrogenic capacity after monolayer expansion. This could involve loading three-dimensional pellets to enhance ECM production or using the addition of ECM components such as HA to encapsulate the three-dimensional pellet.

The long-term strategy for ACI is to translate the technique to treat other joints, including the ankle and the hip. With this in mind, it is important to explore both cell type and cell source. Herein, we chose to investigate whether

chondrocytes sourced from ankle cartilage could be of 'better quality' than chondrocytes sourced from knee cartilage. Our research showed that ankle and knee chondrocytes differed metabolically and histologically, with ankle chondrocytes demonstrating a slower growth in monolayer culture but higher chondrogenic capacity compared to knee chondrocytes, irrespective of the microenvironment or biomechanical stimuli. This indicates that ankle cartilage could be harvested to treat other injured joints.

As well as refining the current ACI protocol, we also set out to design a novel PROM to assess clinical outcome of ankle treatments, including ACI. This was a challenge since there are very few published validated PROMs and those that do exist are not designed for ankle. Our novel approach consisted of designing a simple, short questionnaire, with both initial patients and clinician input, which could be used in the clinic prior to surgery and then followed up at home after surgery. The success of this approach was confirmed by the rapid recruitment of 206 patients within a short time frame of a few months. With this response rate, we were able to show that our PROM had proven reliability and responsiveness to change, much higher than the currently used PROMs used at our centre: the MOxFQ and the VR-12. The OsAS also demonstrated high internal consistency and good correlation with the MOxFQ and the VR-12. The exciting outcome is that our foot and ankle clinicians are implementing the OsAS to monitor clinical outcomes. With the publication of two back to back papers (currently in preparation) we will be able to engage with other clinicians both nationally and internationally.

In conclusion, the results presented here provides new insights into the biology of chondrocytes including the characterisation of ankle and knee

chondrocytes and provides further insight into the optimisations of the ACI technique. The development of OsAS has provided a new method of assessing clinical outcome of ankle treatments, including ACI, which can be utilised by clinicians and patients alike from herein. Therefore, this work has increased the knowledge in this field and provided information into the potential implications in the development and assessment of cell therapies for cartilage regeneration, and in particularly ACI.

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Appendices

Appendix I

NHS
Health Research Authority

NRES Committee North West - Liverpool East
North West REC Centre
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7832
Facsimile: 0161 625 7299

10 January 2012

Professor Sally Roberts
Director of Spinal Research
ISTM, Keele University
ARC/TORCH Building
RJAH Orthopaedic Hospital
SY10 7AG

Dear Professor Roberts

Study title:	**Investigating the potential for cells and molecules isolated from orthopaedic patients for modelling and understanding pathogenic conditions and developing diagnostic markers and therapies for musculoskeletal disorders and spinal cord injury**
REC reference:	**11/NW/0875**
Protocol number:	**None**

Thank you for your letter of 03 January 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice-Chair and Mr Peter Owen.

Confirmation of ethical opinion

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

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

Appendix II

Health Research Authority

Miss Jessica Sykes
Institute for Science & Technology in Medicine,
University of Keele,
Arthritis Research Centre, Robert Jones & Agnes Hunt
Orthopaedic NHS Hospital Trust
Oswestry, Shropshire
SY10 7AG

Email: hra.approval@nhs.net

10 May 2017

Dear Miss Sykes,

Letter of HRA Approval

Study title:	**A study to develop a new and validated questionnaire to assess the outcomes for foot and ankle surgical treatments at The Robert Jones & Agnes Hunt (RJAH) Orthopaedic NHS Hospital.**
IRAS project ID:	**216460**
Protocol number:	**RG-0048-16-ISTM**
REC reference:	**17/NW/0063**
Sponsor	**Keele University**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Appendix III

Orthopaedic Institute

The Robert Jones and Agnes Hunt Orthopaedic Hospital NHS Foundation Trust

Keele University

Version 3
19th May 2016
Oswestry Ankle Score (OsAS)

The Oswestry Ankle Score (OsAS) has been devised to provide information on the patient's opinion of their ankle condition. The information will help clinicians keep track of how the patient feels about their condition, can be used to evaluate functional impairment, and monitor the effectiveness of interventions. Please answer every question regarding your ankle problem specifically, by selecting the most appropriate answer.

Pain:
The following questions refer to the pain you currently experience from your ankle.

1. During the last week, what level of pain have you experienced from your ankle?
Severe moderate mild none

2. During the last week, what level of pain have you experienced from yourankle at night?
Severe moderate mild none

3. During the last week, how often did you experience pain from your ankle?
Constantly hourly daily weekly monthly never

Symptoms:
The following questions refer to the symptoms you currently experience from your foot/ankle, during the last week.

1. To what extent have you had to alter your daily lifestyle due to the pain and/or difficulty you experience from your ankle?
Always Often Sometimes Rarely Never

2. To what extent did your ankle problem affect your daily life?
Always Often Sometimes Rarely Never

3. To what extent did you avoid performing certain activities that you felt could damage or cause pain in your ankle?
Always Often Sometimes Rarely Never

4. To what extent did you feel limited in certain activities due to the difficulty or pain you experience in your ankle?
Always Often Sometimes Rarely
Never

5. How often were you able to point your foot away from you?
Always Often Sometimes Rarely
Never

6. How often were you able to pull your foot up towards you?
Always Often Sometimes Rarely
Never

7. How often did you experience swelling in your ankle, during the last week?
Always often sometimes rarely
never

8. How often did you experience stiffness in your ankle, during the last week?
Always often sometimes rarely
never

Activities:
The following questions refer to the difficulty you have experienced from your ankle during the listed activities, in the last week.

1. Standing for a long period of time (for example: 1 hour)
Unable extreme moderate mild none

2. Sitting for a long period of time (for example: 1 hour)
Unable extreme moderate mild none

3. Walking for a short period of time (for example: 1-15 minutes)
Unable extreme moderate mild none

4. Walking a long distance (for example: greater than 1 kilometre)
Unable extreme moderate mild none

5. Raising your heel to stand on your tip toes
Unable extreme moderate mild none

6. Climbing up and down stairs
Unable extreme moderate mild none

7. Getting in and out of the car or public transportation
Unable extreme moderate mild none

8. Getting in and out of the shower or bath
Unable extreme moderate mild none

9. Performing light daily household activities (for example: cooking)
Unable extreme moderate mild none

10. Performing heavy daily household activities (for example: scrubbing floors)
Unable extreme moderate mild none

11. Sleeping
Unable extreme moderate mild none

12. How difficult is it for you to continue with your usual physical recreational activities, such as bicycling, jogging, swimming, due to your ankle?
Unable extreme moderate mild none

13. How difficult is it for you to continue with your usual leisure recreational activities, such as hobbies and seeing friends, due to your ankle?
Unable extreme moderate mild none

14. To what extent did your ankle problem affect your daily life?
Never rarely sometimes often always

15. To what extent did your ankle affect your usual work activities?
Never rarely sometimes often always

16. To what extent did your ankle affect your emotional wellbeing or social activities?
Never rarely sometimes often always

17. To what extent did you struggle to find shoes that fit and/or don't hurt your feet?
Never rarely sometimes often always

18. How often did you feel the need to use a walking aid (for example: crutches, walker)?
Never rarely sometimes often always don't have any

19. How often did you walk with a limp due to your ankle problem?
Never rarely sometimes often always

General:
The final question refers to your general health, outside of your ankle problem.

19. Do you have any other issues with your general health that may have an affect your ankle problem, for example: a spinal condition, chronic fatigue etc…?
Yes – please give details below No

Appendix IV

Orthopaedic Institute

The Robert Jones and Agnes Hunt Orthopaedic Hospital NHS Foundation Trust

Keele University

Oswestry Questionnaire
Version 3.3
19th May 2016

Oswestry Ankle Score (OsAS)

The Oswestry Ankle Score (OsAS) has been devised to provide information on the patient's opinion of their ankle condition. The information will help clinicians keep track of how the patient feels about their condition, can be used to evaluate functional impairment, and monitor the effectiveness of interventions. Please answer every question regarding your ankle problem specifically, by selecting the most appropriate answer.

Pain:

The following questions refer to the pain you currently experience from your ankle.

1. During the last week, what level of pain have you experienced from your ankle?

Severe Moderate Mild None

2. During the last week, how often did you experience pain from your ankle?

Constantly Sometimes Rarely Never

Symptoms:

The following questions refer to the symptoms you currently experience from your ankle, during the last week.

3. To what extent have you had to alter your daily lifestyle due to the pain and/or difficulty you experience from your ankle?

Always Often Rarely Never

4. To what extent did your ankle problem affect your daily life?

Always Often Rarely Never

5. To what extent did you avoid performing certain activities that you felt could damage or cause pain in your ankle?

Always Often Rarely Never

6. To what extent did you feel limited in certain activities due to the difficulty or pain you experience in your ankle?

Always Often Rarely Never

7. How often did you experience stiffness in your ankle, during the last week?

Always Often Rarely Never

Activities:

The following questions refer to the difficulty you have experienced from your ankle during the listed activities, in the last week.

8. Standing for a long period of time (for example: 1 hour)

Unable Extreme Moderate Mild None

9. Sitting for a long period of time (for example: 1 hour)

Unable Extreme Moderate Mild None

10. Walking

Unable Extreme Moderate Mild None

11. Climbing up and down stairs

Unable Extreme Moderate Mild None

12. Getting in and out of the car or public transportation

Unable Extreme Moderate Mild None

13. Getting in and out of the shower or bath

Unable Extreme Moderate Mild None

14. Performing daily household activities (for example: cooking, scrubbing floors)

Unable Extreme Moderate Mild None

15. Sleeping

Unable Extreme Moderate Mild None

16. How difficult is it for you to continue with your usual activities, such as cycling, jogging, swimming, seeing friends, doing usual hobbies, due to your ankle?

Unable Extreme Moderate Mild None

17. To what extent did your ankle affect your emotional wellbeing?

Never Rarely Sometimes Often Always

18. How often did you walk with a limp due to your ankle problem?

Never Rarely Sometimes Often Always

General:

The final question refers to your general health, outside of your foot and ankle problem.

19. Do you have any other issues with your general health that may have an affect your ankle problem, for example: a spinal condition, chronic fatigue etc…?

Yes – please give details below No

Appendix V

MANCHESTER-OXFORD FOOT QUESTIONNAIRE (MOXFQ)

Circle as appropriate: **RIGHT / LEFT** ***During the past 4 weeks this has applied to me:***	*Please tick ✓ one box for each statement*				
	None of the time	**Rarely**	**Some of the time**	**Most of the time**	**All of the time**
1. I have pain in my foot/ankle	❑	❑	❑	❑	❑
2. I avoid walking long distances because of pain in my foot/ankle	❑	❑	❑	❑	❑
3. I change the way I walk due to pain in my foot/ankle	❑	❑	❑	❑	❑
4. I walk slowly because of pain in my foot/ankle	❑	❑	❑	❑	❑
5. I have to stop and rest my foot/ankle because of pain	❑	❑	❑	❑	❑
6. I avoid some hard or rough surfaces because of pain in my foot/ankle	❑	❑	❑	❑	❑
7. I avoid standing for a long time because of pain in my foot/ankle	❑	❑	❑	❑	❑
8. I catch the bus or use the car instead of walking, because of pain in my foot/ankle	❑	❑	❑	❑	❑
9. I feel self-conscious about my foot/ankle	❑	❑	❑	❑	❑
10. I feel self-conscious about the shoes I have to wear	❑	❑	❑	❑	❑

Please turn to next page

MOXFQ/ foot continued

***During the past 4 weeks* this has applied to me:**	*Please tick ✓ one box for each statement*				
	None of the time	**Rarely**	**Some of the time**	**Most of the time**	**All of the time**
11. The pain in my foot/ankle is more painful in the evening	❑	❑	❑	❑	❑
12. I get shooting pains in my foot/ankle	❑	❑	❑	❑	❑
13. The pain in my foot/ankle prevents me from carrying out my work/everyday activities	❑	❑	❑	❑	❑
14. I am unable to do all my social or recreational activities because of pain in my foot/ankle	❑	❑	❑	❑	❑

15. During the past 4 weeks how would you describe the pain you usually have in your foot/ankle? *(please tick one box)*

None	Very mild	Mild	Moderate	Severe
❑	❑	❑	❑	❑

16. During the past 4 weeks have you been troubled by pain from your foot/ankle in bed at night? *(please tick one box)*

No nights	Only 1 or 2 nights	Some nights	Most nights	Every night
❑	❑	❑	❑	❑

Finally, please check that you have answered every question

Thank you very much

Appendix VI

Self-Administered

OMB ###-####

Administrative use only:

Local Identifier

Study ID: _____ **Navigator:** _____

Date: _____

THE VETERANS RAND 12-ITEM HEALTH SURVEY (VR-12)

The following questions ask for your views about your health—how you feel and how well you are able to do your usual activities. All kinds of people across the country are being asked these same questions. Their answers and yours will help to improve health care for everyone. There are no right or wrong answers; please choose the answer that best fits your life right now.

Answer each question by marking an 'X' next to the best response. For example:

What is your gender?

- ☐ Male
- ☒ Female

Q1. In general, would you say your health is:

- ❑ Excellent
- ❑ Very good
- ❑ Good
- ❑ Fair
- ❑ Poor

Q2. The following questions are about activities you might do during a typical day. Does **your health now limit you** in these activities? If so, how much?

a. **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling or playing golf?

- ❑ Yes, limited a lot
- ❑ Yes, limited a little
- ❑ No, not limited at all

b. Climbing **several** flights of stairs?

- ❑ Yes, limited a lot
- ❑ Yes, limited a little
- ❑ No, not limited at all

Self-Administered

Q3. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities **as a result of your physical health**?

a. Accomplished **less** than you would like.

- ❑ No, none of the time
- ❑ Yes, a little of the time
- ❑ Yes, some of the time
- ❑ Yes, most of the time
- ❑ Yes, all of the time

b. Were limited in the **kind** of work or other activities.

- ❑ No, none of the time
- ❑ Yes, a little of the time
- ❑ Yes, some of the time
- ❑ Yes, most of the time
- ❑ Yes, all of the time

Q4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities **as a result of any emotional problems** (such as feeling depressed or anxious)?

a. **Accomplished less** than you would like.

- ❑ No, none of the time
- ❑ Yes, a little of the time
- ❑ Yes, some of the time
- ❑ Yes, most of the time
- ❑ Yes, all of the time

b. Didn't do work or other activities as **carefully** as usual.

- ❑ No, none of the time
- ❑ Yes, a little of the time
- ❑ Yes, some of the time
- ❑ Yes, most of the time
- ❑ Yes, all of the time

↳Continue to next page

Self-Administered

Q5. During the past 4 weeks, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

- ❑ Not at all
- ❑ A little bit
- ❑ Moderately
- ❑ Quite a bit
- ❑ Extremely

These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling.

Q6a. How much of the time during the past 4 weeks:

Have you felt calm and peaceful?

- ❑ All of the time
- ❑ Most of the time
- ❑ A good bit of the time
- ❑ Some of the time
- ❑ A little of the time
- ❑ None of the time

Q6b. How much of the time during the past 4 weeks:

Did you have a lot of energy?

- ❑ All of the time
- ❑ Most of the time
- ❑ A good bit of the time
- ❑ Some of the time
- ❑ A little of the time
- ❑ None of the time

Q6c. How much of the time during the past 4 weeks:

Have you felt downhearted and blue?

- ❑ All of the time
- ❑ Most of the time
- ❑ A good bit of the time
- ❑ Some of the time
- ❑ A little of the time
- ❑ None of the time

Continue to next page

Self-Administered

Q7. During the past 4 weeks, how much of the time has your **physical health or emotional problems** interfered with your social activities (like visiting with friends, relatives, etc.)?

- ❑ All of the time
- ❑ Most of the time
- ❑ Some of the time
- ❑ A little of the time
- ❑ None of the time

Now, we'd like to ask you some questions about how your health may have changed.

Q8. Compared to one year ago, how would you rate your **physical health** in general now?

- ❑ Much better
- ❑ Slightly better
- ❑ About the same
- ❑ Slightly worse
- ❑ Much worse

Q9. Compared to one year ago, how would you rate your **emotional problems** (such as feeling anxious, depressed or irritable) **now?**

- ❑ Much better
- ❑ Slightly better
- ❑ About the same
- ❑ Slightly worse
- ❑ Much worse

Your answers are important!

Thank you for completing this questionnaire!

Publications associated with this thesis

European Cells and Materials Vol. 35 2018 (pages 255-267) DOI: 10.22203/eCM.v035a18 ISSN 1473-2262

IMPACT OF HUMAN PLATELET LYSATE ON THE EXPANSION AND CHONDROGENIC CAPACITY OF CULTURED HUMAN CHONDROCYTES FOR CARTILAGE CELL THERAPY

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†Deceased

Abstract

High hopes have been pinned on regenerative medicine strategies in order to prevent the progression of cartilage damage to osteoarthritis, particularly by autologous chondrocyte implantation (ACI). The loss of chondrocyte phenotype during *in vitro* monolayer expansion, a necessary step to obtain sufficient cell numbers, may be a key limitation in ACI. In this study, it was determined whether a shorter monolayer expansion approach could improve chondrogenic differentiation. The effects of two supplement types, foetal bovine serum (FBS) and Stemulate™ (a commercial source of human platelet lysate), on the expansion and re-differentiation potential of human chondrocytes, isolated from five individuals, were compared. Chondrocytes were expanded with 10 % FBS or 10 % Stemulate™. Pellets were cultured for 28 d in chondrogenic differentiation medium and assessed for the presence of cartilage matrix molecules and genes associated with chondrogenicity. Stemulate™ significantly enhanced the proliferation rate [average population doubling times: FBS, 25.07 ± 6.98 d (standard error of the mean, SEM) *vs.* Stemulate™, 13.10 ± 2.57 d (SEM)]. Sulphated glycosaminoglycans (sGAG), total collagen and qRT-PCR analyses of cartilage genes showed that FBS-expanded chondrocytes demonstrated significantly better chondrogenic capacity than Stemulate™-expanded chondrocytes. Histologically, FBS-expanded chondrocyte pellets appeared to be more stable, with a more intense staining for toluidine blue, indicating a greater chondrogenic capacity. Although Stemulate™ positively influenced chondrocyte proliferation, it had a negative effect on chondrogenic differentiation potential. This suggested that, in the treatment of cartilage defects, Stemulate™ might not be the ideal supplement for expanding chondrocytes (which maintained a chondrocyte phenotype) and, hence, for cell therapies (including ACI).

Keywords: Human platelet lysate, Stemulate™, foetal bovine serum, human chondrocytes, cartilage, autologous chondrocyte implantation.

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Introduction

Damaged articular cartilage has a limited potential for healing and untreated cartilage defects often progress to osteoarthritis (OA) (Davies-Tuck *et al.*, 2008). Regenerative medicine strategies have been formulated to meet the challenge of preventing progression to OA; one such strategy is autologous chondrocyte implantation (ACI) (Nawaz *et al.*, 2014). ACI has evolved over 20 years, but chondrocytes are still the preferred cell choice. In brief, autologous chondrocytes are harvested by arthroscopy from a lesser weight-bearing area of the joint, expanded in monolayer culture and re-implanted into the damaged area under a natural or synthetic membrane by an open joint surgical procedure (Brittberg *et al.*, 1994). Robert Jones & Agnes Hunt (RJAH) Hospital, Oswestry and others report a high percentage of good to excellent clinical results (Bhosale *et al.*, 2009; Henderson *et al.*, 2003). Although there is limited long-term follow-up data, especially from the modern methods of ACI, positive clinical results are beginning to emerge. Nawaz *et al.* (2014) report a 78 % at 5 years and 51 % at 10 years ACI graft survival

3D culture, the aim was to assess the recovery of chondrocytes and determine their ability to produce ECM.

Materials and Methods

Chondrocyte isolation and monolayer expansion

Fully informed consent was obtained from five individuals undergoing total knee replacement surgery at RJAH Hospital, Oswestry, with ethical approval (11/NW/0875). Full depth cartilage was removed from macroscopically normal areas of the femoral condyle from three male and two female individuals with a mean age of 67 years (range: 55-75 years). Chondrocytes were isolated following an established protocol (Harrison *et al.*, 2000). Briefly, 100-300 mg of cartilage tissue were placed into 10 mL Dulbecco's modified Eagle's medium (DMEM)/F12 (11330-057; Gibco) containing 0.03 mg/mL deoxyribonuclease I (D4263; Sigma-Aldrich) and 0.83 mg/mL type XI collagenase (C9407; Sigma-Aldrich). Digestion was performed for 16 h in a T25 tissue culture flask (Sarstedt) at 37 °C and 5 % CO_2. The digest was filtered through a 0.2 µm cell strainer and chondrocytes were pelleted at 750 ×*g* for 10 min. After that > 98 % cell viability was verified using trypan blue (T8154; Sigma-Aldrich), the chondrocytes were plated into T175 tissue-culture flasks at a density of 5×10^4 cells/cm^2 in DMEM/F12 supplemented with 0.5 % (w/v) penicillin-streptomycin-amphotericin (17-745E; Lonza), 0.5 % (w/v) ascorbate (A4544; Sigma-Aldrich) and either 10 % (v/v) FBS (10500-064; Gibco) or 10 % (v/v) Stemulate™ (PL-SP-100; Cook Regentec). Media were replenished three times weekly. Once 80 % confluency was reached, the chondrocytes were passaged and re-seeded at a density of 5×10^4 cells/cm^2. Chondrocytes were cultured in monolayer until the end of passage 2.

Growth kinetics during monolayer expansion

To assess growth kinetics during monolayer expansion, population doubling times (PDTs) were recorded at each passage and calculated using the following formula (McAteer and Davis, 2002):

$$PDT = \frac{1}{multiplication\ rate}$$

$$\text{Multiplication rate} = \frac{3.32 \times [\log(final\ cell\ number) - \log(initial\ cell\ number)]}{time\ difference}$$

Re-differentiation in 3D pellet culture in chondrogenic medium

At the end of passage 2, the chondrocytes were detached and re-suspended in chondrogenic re-differentiation medium optimised from the Johnstone method (Johnstone *et al.*, 1998), comprising DMEM/F12 supplemented with 1 % (v/v) FBS, 1 % (v/v) non-essential amino acids (11140-035; Gibco), 1 % (v/v) L-glutamine (G7513; Sigma-Aldrich), 0.5 % (v/v) penicillin-streptomycin-amphotericin (17-745E; Lonza), 0.5 % (w/v) ascorbate (A4544; Sigma-Aldrich), 1 % (v/v) insulin-transferrin-selenium (51500-056; Gibco), 20 ng/mL transforming growth factor-β1 (100-21; Peprotech), 10 nM dexamethasone (D4902; Sigma-Aldrich) and 3.2 µg/mL L-proline (P0380; Sigma-Aldrich). The chondrocytes (2.5×10^5 cells) were transferred to 1.5 mL Eppendorf® tubes and centrifuged at 750 ×*g* for 7 min, to create spherical pellets. Pellets were cultured in 500 µL of chondrogenic medium. Medium was replenished twice weekly.

Pellet processing for analyses

Pellets were assessed at day 0, 7, 14, 21 and 28. For biochemical assays, pellets (n = 5) were digested in 1 mg/mL proteinase K (25530-015; Ambion) in 100 mM ammonium acetate (09691; Sigma-Aldrich) for 2 h at 60 °C. Proteinase K was inactivated for 5 min at 100 °C and samples were stored at − 20 °C. For histology, pellets (n = 4) were placed onto Whatman filter paper (Whatman, Kent, UK), snap frozen in liquid-nitrogen-cooled n-hexane and stored at − 80 °C. For gene expression analysis, pellets (n = 3) were prepared for RNA extraction by homogenisation in 350 µL RLT lysis buffer containing 1 % (v/v) β-mercaptoethanol (M6250; Sigma-Aldrich) using a 21 G needle and syringe (1053393; QIAGEN) and stored at − 80 °C.

Pellet cell number

Cell number was determined using the PicoGreen® fluorescent DNA quantification kit (17916; Invitrogen), with lambda DNA as standard (1 ng/mL-1 µg/mL). Duplicate samples and standards were transferred to a 96-well plate and fluorescence was read at an excitation of 480 nm and emission of 520 nm using a FLUOstar Omega microplate reader (BMG, Ortenberg, Germany). Cell number was calculated using the widely reported value of 7.7 pg of DNA per chondrocyte (Kim *et al.*, 1988).

Total sGAG content in the pellets

The total sGAG was determined using 1,9-dimethylmethylene blue (DMMB) (Farndale *et al.*, 1986). All reagents were procured from Sigma-Aldrich. Chondroitin sulphate A from bovine trachea (C9819) was used as standard (0-200 µg/mL). In brief, 4 mg DMMB (341088) were diluted in 250 mL distilled water containing 0.76 g glycine (G5516) and 0.595 g sodium chloride (S5886) to a final pH of 3.0. Duplicate samples and standards were transferred to a 96-well plate and mixed with 200 µL of DMMB dye. Absorbance was read at 530 nm using a FLUOstar Omega microplate reader (BMG).

Total collagen content in the pellets

The total collagen content was determined by a hydroxyproline assay (Brown *et al.*, 2001). All reagents were procured from Sigma-Aldrich. Hydroxyproline standards (0-0.1 µg/mL; H5,440-9) were prepared in distilled water. 100 µL of each sample were hydrolysed

for 827 patients. A robust economic model, based on a systematic review, indicates that ACI is cost-effective as compared with alternative procedures, providing long-lasting improvements in symptoms and eventually reducing the need for knee joint replacements (Mistry *et al.*, 2017; Web Ref. 1). Overall, the consensus is that ACI is more effective if used early following a cartilage injury and if it is the first attempt at a surgical intervention. Further refinement of ACI will ultimately enhance clinical outcomes and this, in part, is one of the goals of the present study.

To obtain sufficient cell numbers from small cartilage biopsies, the chondrocytes need to undergo *in vitro* expansion. The loss of chondrocyte phenotype, known as de-differentiation (Benya and Shaffer, 1982; Schnabel *et al.*, 2002), during *in vitro* expansion on tissue culture plastic is a key limitation of ACI. During de-differentiation, chondrocytes lose their rounded morphology and decrease gene and protein expression of collagen II and aggrecan, both key components of the articular cartilage extracellular matrix (ECM). Consequently, de-differentiation decreases the capacity of re-implanted chondrocytes to regenerate functional cartilage (Schulze-Tanzil, 2009). At the RJAH Hospital, Oswestry, most of the freshly isolated chondrocytes usually undergo two passages because de-differentiation is known to occur in as few as three passages (Kang *et al.*, 2007). Nevertheless, further minimising the conditions that promote chondrocyte de-differentiation may improve ACI.

Current standard protocols for chondrocyte expansion involve growth medium supplemented with foetal bovine serum (FBS), human autologous serum (AS) or serum-free medium. While FBS is used traditionally for *in vitro* chondrocyte expansion, an increase in demand and limited stocks are constantly increasing its price. FBS is a poorly defined mixture of components that can contain endotoxins, prion and viral proteins (Jochems *et al.*, 2002; Selvaggi *et al.*, 1997; van der Valk *et al.*, 2010). Current good manufacturing practice (GMP) guidelines state that animal products should be replaced by human alternatives, wherever possible, for translating into cell therapies (Giancola *et al.*, 2012; Unger *et al.*, 2008; World Health Organisation, 1992; World Health Organization, 1991). Human AS would seem the ideal solution, but patient-to-patient variability can negatively impact upon key parameters, such as chondrocyte growth and quality (Harrison *et al.*, 2000). Further, a relatively large volume of blood is required to perform chondrocyte expansion for ACI and this can be uncomfortable for the patients. A preferred alternative is defined serum-free growth media, but these media are supplemented with expensive growth factors that need to be replenished regularly during chondrocyte expansion. Given the issues described, other options are needed.

The use of platelet lysate for *in vitro* cell proliferation began in the 1980s (Choi *et al.*, 1980). Doucet *et al.* (2005) describe the preparation and application of human platelet lysate (HPL) for expanding mesenchymal stem cells. Further studies show both autologous and allogeneic HPL to be superior to FBS in enhancing cell proliferation (Burnouf *et al.*, 2016; Hildner *et al.*, 2015; Hofbauer *et al.*, 2014; Kim *et al.*, 2015; Trojahn Kølle *et al.*, 2013). HPL eliminates the risk of cross-species contamination, solves standing ethical considerations and meets the GMP guidelines. In addition, it contains high levels of growth factors and cytokines and a range of vitamins and minerals that are also present in FBS and human AS (Hemeda *et al.*, 2014; Shih and Burnouf, 2015). HPL is created from large donor pools, reducing the variability observed with other serum types. However, there are limitations to HPL use, including the potential risk of contamination with human viruses, some of which are screened for, including HIV. Many studies show that 5-10 % (v/v) HPL is superior to 10 % (v/v) FBS in supporting cell expansion in several different cell types (King and Buchwald, 1984; Mojica-Henshaw *et al.*, 2013; Muraglia *et al.*, 2014; Reinisch *et al.*, 2007; Trojahn Kølle *et al.*, 2013; Witzeneder *et al.*, 2013). A HPL concentration of 10 % (v/v) supports cell proliferation at levels comparable to FBS (Burnouf *et al.*, 2016; Hemeda *et al.*, 2014; Hildner *et al.*, 2015; Hofbauer *et al.*, 2014). Only a handful of studies use HPL as an *in vitro* tool for human chondrocyte expansion (Gaissmaier *et al.*, 2005; Hildner *et al.*, 2015; Moreira Teixeira *et al.*, 2012; Muraglia *et al.*, 2014; Spreafico *et al.*, 2009). Even fewer investigate the recovery of chondrocyte phenotype, known as re-differentiation, in 3-dimensional (3D) culture when the HPL is excluded from the re-differentiation media (Hildner *et al.*, 2015). With ACI therapy in mind, it is important to ensure that the expanded chondrocytes have a chance to recover their chondrogenic phenotype in 3D culture without further exposure to relatively high levels of platelet-derived growth factors, which increase cell proliferation.

Stemulate™ (Cook Regentec, Indianapolis, IN, USA), a commercially available source of HPL, is prepared according to GMP guidelines, using large donor pools from accredited blood centres. Given that Stemulate™ is used to expand a range of cell types, including adipose-derived mesenchymal stem cells (MSCs) and bone-marrow MSCs, it could be an alternative supplement to increase chondrocyte proliferation for cartilage cell therapy (Badowski *et al.*, 2017; Juhl *et al.*, 2016; Mangum *et al.*, 2017; Riis *et al.*, 2016; Søndergaard *et al.*, 2017). Stemulate™ could reduce time in monolayer culture and reduce exposure to tissue-culture plastic, which in turn could reduce the risk of de-differentiation. The aim of the current work was to determine whether Stemulate™ could be a suitable alternative serum supplement to FBS for human chondrocyte expansion by assessing growth characteristics and cell morphology. Further, by using chondrogenic medium for both sets of chondrogenic pellets during re-differentiation in

Table 1. Genes used in qPCR analysis. Genes were selected to determine the chondrogenic phenotype of the cells and if the chondrocytes had de-differentiated to a hypertrophic state.

Gene	Official name and species	Amplicon length	Entrez ID
Reference genes			
HPRT1	Hypoxanthine phosphoribosyltransferase 1 (human)	130 (NM_000194)	3251
TBP	TATA box binding protein (human)	132 (NM_001172085) 132 (NM_003194)	6908
Chondrogenic markers			
ACAN	Aggrecan (human)	62 (NM_001135) 62 (NM_013227) 62 (XM_001131727) 62 (XM_001131734) 62 (XM_00670419)	176
COL2A1	Collagen type 2 alpha 1 (human)	95 (NM_001844) / 95 (XM_006719242)	1280
SOX9	SRY (sex determining region Y)-box 9 (human)	112 (NM_000346)	6662
De-differentiation markers			
COL1A1	Collagen type 1 alpha 1 (human)	118 (NM_000088) 118 (NM_003257058) 118 (XM_005257059) 118 (XM_006721703)	1277
Hypertrophic markers			
ALK1/ACVRL1	Activin A receptor type II-like 1 (human)	74 (NM_000020)	94
COL10A1	Collagen type 10 alpha 1 (human)	91 (NM_000493)	1300

in 100 µL of 10 M hydrochloric acid (19068) for 16 h at 108 °C. On cooling, the samples were neutralised with 800 µL of 1 M sodium hydroxide (S5881). Duplicate samples and standards were transferred to a 96-well plate. 100 µL of oxidation buffer [600 mL isopropyl alcohol, 330 mL distilled water, 390 mL citrate/acetate buffer (1 L consists of 34 g sodium hydroxide (S5881), 34 g citric acid monohydrate (C7129) and 120 g sodium acetate trihydrate (32318-M)), pH 6.0] were used as a negative control. Oxidation solution [100 µL; 300 mg chloramine T (402869) dissolved in 50 mL oxidation buffer] was added to all samples and standards. The plate was incubated at room temperature for 5 min. Ehrlich's reagent [100 µL; 6 g p-dimethylaminobenzaldehyde (156477) dissolved in a mixture of 16 mL of 50 % perchloric acid (244252) and 52 mL of isopropyl alcohol] was added to each well and, then, the plate was sealed and incubated in a 60 °C water bath for 45 min. Absorbance was measured at 570 nm using a FLUOstar Omega microplate reader (BMG) and total collagen was calculated using the widely reported multiplication factor of 7 (Sims *et al.*, 2000).

qRT-PCR to assess gene expression in the pellets

mRNA was extracted using the QIAGEN RNeasy mini kit as per manufacturer's instructions. mRNA was reverse-transcribed in a Progene thermocycler (Techne, Staffordshire, UK) using a high-capacity cDNA reverse transcription kit (4368814; Applied Biosystems) following the manufacturer's instructions. qRT-PCR was performed on three pellet replicates (from each patient) to determine gene expression relative to two reference genes, according to published guidelines (Bustin *et al.*, 2009): hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT1 human; RT00059066) (Foldager *et al.*, 2009; Rushton *et al.*, 2014) and TATA box-binding protein (TBP; QT00000721) (Foldager *et al.*, 2009; Pombo-Suarez *et al.*, 2008) were selected based on low co-efficiencies of variance across both supplement types (data not shown). Several genes (Table 1) were chosen to determine the chondrogenic phenotype after re-differentiation. All genes, including reference genes, were procured from QIAGEN. Gene expression was measured relative to the reference genes and normalised to the cell number. Relative gene expression profiles were determined by the comparative C_T method (Schmittgen and Livak, 2008).

Histology of the frozen pellets

The frozen pellets were mounted onto pre-cooled chucks with optimal cutting temperature (OCT) compound (00.090.112; Sakura Finetek, Zoeterwoude, the Netherlands). All stains were procured from VWR International. 7 µm-thick sections were collected onto poly-L-lysine pre-coated slides (MBC-0102-54A; Cell Path, Newtown, UK) and stained with haematoxylin (350604T) and eosin (34197) or toluidine blue (340774Y), according to Roberts and Menage (2004). Sections were imaged by light microscopy (Leitz, Wetzlar, Leica Microsystems GmbH, Stuttgart, Germany) using ×6.3, ×25 and ×40 objective lenses. Images were captured using

a DS-Fi1 camera and analysed with NIS-Elements BR imaging software (Nikon). Images were white-balanced using Photoshop Elements (Adobe). To semi-quantify the toluidine blue staining intensity, four central images of each pellet were randomly selected and the integrated density was determined using ImageJ Software (NIH) according to the published methodology (Jensen, 2013; Owida *et al.*, 2017; Prasad and Prabhu, 2012).

Statistical analyses

All independent outcomes (PDTs, cell number, sGAG, total collagen, qPCR and staining intensity), patient variability and differences between the two supplement types over the time course were analysed using a multilevel model. Supplement type and time points were considered fixed variables and the patient was considered a random variable (random intercept). All qPCR data were log-transformed before the analysis, to make the data fit a normal distribution.

Cell number, sGAG and total collagen data ($n \geq 4$ per time point per patient, 5 patients in total) were expressed as mean ± standard error of the mean (SEM). Gene expression data ($n = 3$ per time point per patient, 5 patients in total) were shown as a box plot, with the box representing the second and third quartiles, the horizontal line inside the box representing the median and the whiskers either side of the box representing the lower and upper quartiles. All statistical analyses were performed with SPSS statistical software (version 24; IBM) and, for all analyses, $p < 0.05$ was deemed to denote statistical significance.

Results

Stemulate™ positively influenced proliferation of chondrocytes in monolayer culture

For all 5 patients, the chondrocytes expanded in Stemulate™ (mean PDT: 13.10 ± 2.57 d) proliferated quicker than the chondrocytes expanded in FBS (mean PDT: 25.07 ± 6.98 d, $p = 0.050$). Patient variability was considered not statistically significant ($p = 0.48$) in affecting the PDT. Both sets of chondrocytes underwent less than 2 absolute population doublings throughout passages 1, 2 and 3 (Fig. 1). No statistically significant difference was measured in population doublings between the two sera supplements ($p = 0.527$) or at any passage ($p = 0.50$). Patient variability was considered not statistically significant ($p = 0.445$) in affecting population doublings.

Chondrocytes expanded in both FBS and Stemulate™ were seeded at an equal cell density upon chondrocyte isolation, after which both sets of chondrocytes showed a significant increase in cell number over time ($p < 0.001$; Fig. 2). Chondrocytes expanded in Stemulate™ demonstrated a significantly larger increase in cell number as compared to chondrocytes expanded in FBS ($p = 0.014$). Patient variability was considered not statistically significant ($p = 0.296$) in affecting cell number in monolayer.

Fig. 3 shows the morphology of the human chondrocytes (day 4 of each culture) in the presence of either Stemulate™ or FBS during passage 0, 1 and 2. For Stemulate™-expanded chondrocytes, markedly more chondrocytes were present and they appeared to cluster together at all passages. For both supplement types, chondrocytes appeared elongated and flattened, with little difference in their cell shape at all passages.

Stemulate™ negatively influenced the re-differentiation potential of chondrocytes in 3D pellet culture

To investigate whether the removal of Stemulate™ at the end of chondrocyte monolayer expansion resulted in delayed ECM production in 3D pellet culture, a 28 d time course was performed. 3D pellets were assessed for cell number (Fig. 4), total sGAG (Fig. 5), total collagen (Fig. 6) and gene expression by q-PCR (Fig. 7).

For both supplement types, cell number (Fig. 4) remained consistent throughout the time course ($p = 0.84$). This was expected since the 3D pellet conformation should enable the chondrocytes to recover their morphology and prevent further

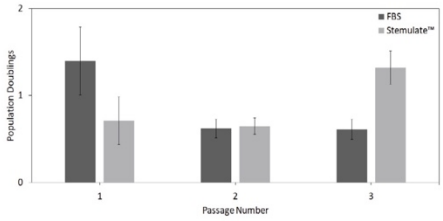

Fig. **1.** Absolute population doublings of chondrocytes expanded in FBS and Stemulate™ sera at each of the three passages. Data are presented as mean ± SEM ($n = 5$).

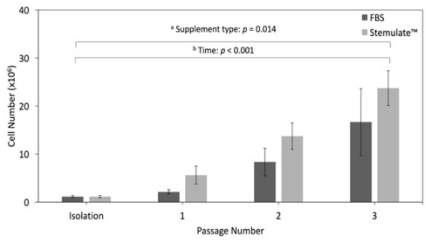

Fig. **2.** Cell number during monolayer expansion of chondrocytes in FBS- and Stemulate™-supplemented sera. Cell number was calculated from cell counts during chondrocyte (seeded at an equal cell density) isolation and across three passages. Data are presented as mean ± SEM ($n = 5$).

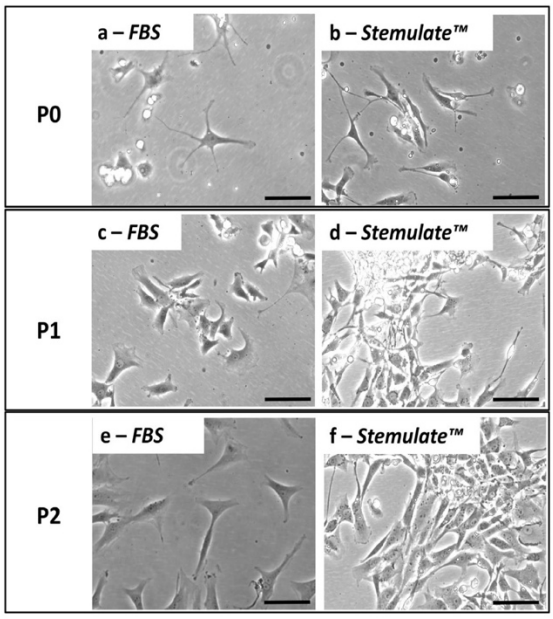

Fig. 3. Representative images of chondrocytes expanded in culture media supplemented with 10 % (**a**,**c**,**e**) FBS and (**b**,**d**,**f**) Stemulate™ at passage 0-2 (P 0-P 2). Images were taken at day 4 of each passage to allow the chondrocytes to adhere. All images were taken using the Nikon TS100 light microscope and ×20 magnification. Images were acquired using the IPLab 3.2.6 software. Scale bars: 10 μm.

proliferation. Total sGAG (Fig. 5) and total collagen (Fig. 6) production were normalised to cell number. Chondrocytes expanded in both supplement types demonstrated a steady and significant increase in sGAG production across all time points ($p < 0.001$) (Fig. 5). sGAG concentration was higher in FBS as compared to Stemulate™ across all time points ($p = 0.009$), as determined by multi-level modelling. A significant sharp increase in sGAG concentration at day 14 was observed for FBS-expanded chondrocytes ($p < 0.001$), with a slight levelling off in sGAG production at day 28 for both supplements. No evidence of variability among individual patients at any time point was detected ($p = 0.60$) (Fig. 5).

Collagen production demonstrated a significant interaction between treatment and time ($p = 0.001$) (Fig. 6). Both supplement types showed a similar pattern of relatively steady levels until a sharp increase between day 21 and 28, with levels being significantly higher in FBS as compared to Stemulate™ ($p = 0.001$). Again, there was no evidence of variability among individual patients at any time point ($p = 0.26$) (Fig. 6).

RT-qPCR was performed at day 0, 21 and 28; relative gene expression profiles were determined by the comparative C_T method (Schmittgen and Livak, 2008) and normalised to the cell number. Expression of *COL1A1*, *COL10A1* or *ALK1* was not detected in either supplement type. For the key chondrogenic markers *ACAN*, *COL2A1* and *SOX9* there were striking differences between gene expression profiles in each supplement. *ACAN* expression increased over the time course in both supplements ($p < 0.001$ for time), but was significantly higher in FBS- than Stemulate™-expanded pellets at day 0 ($p < 0.001$) and day 21 ($p = 0.013$). *COL2A1* expression was significantly higher in FBS- than in Stemulate™-expanded pellets at day 0 ($p = 0.033$) and day 21 ($p = 0.034$). FBS-expanded pellets demonstrated a significantly higher expression of *SOX9* at day 21 ($p = 0.012$) and day 28 ($p = 0.002$) when compared with Stemulate™-expanded pellets. There was no evidence of variability among patients for *ACAN* ($p = 0.48$) and *COL2A1* expression ($p = 0.33$) or for a general change in *COL2A1* ($p = 0.20$) and *SOX9* expression ($p = 0.15$) over time (Fig. 7).

FBS improved the quality of the ECM produced in 3D pellet culture

Frozen chondrogenic pellets were sectioned (7 μm) and stained with haematoxylin and eosin (H&E) or toluidine blue to visualise general morphology and proteoglycan distribution, respectively (Fig. 8). At day 7, chondrocytes expanded in Stemulate™ appeared to form less stable and more fragile pellets when compared with chondrocytes expanded in FBS. Morphological differences seemed apparent in the cells occupying the periphery of the pellets. These cells appeared to be more fibroblastic in pellets created from the Stemulate™-expanded chondrocytes as compared to those created from FBS-expanded chondrocytes (Fig. 8). In both supplement types, there was a trend for the staining intensity of the chondrogenic pellets to increase over time. Pellets from FBS-expanded chondrocytes demonstrated an increase in metachromasia. This increase was quantitated using ImageJ software (Fig. 9), to confirm that, at day 21, FBS-expanded chondrocytes had a significantly more intense staining as compared to Stemulate™-expanded chondrocytes ($p = 0.045$).

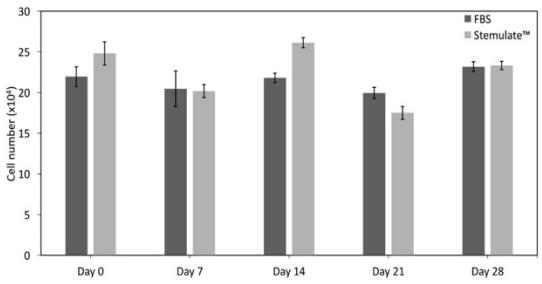

Fig. **4.** Cell number in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 ($n = 5$) and up to day 28 ($n = 3$).

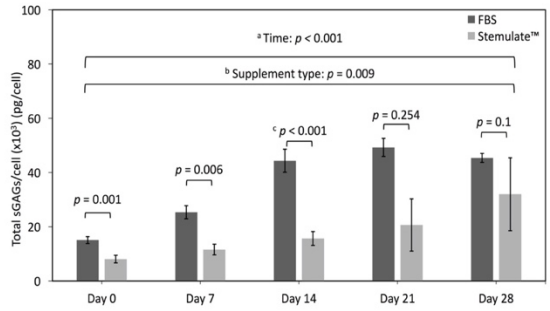

Fig. **5.** sGAG production per cell in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplmented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 ($n = 5$) and up to day 28 ($n = 3$). [a] denotes statistical significance for time. [b] denotes statistical significance for supplement type. [c] denotes statistical significance at day 14.

Again, there was no evidence of variability among patients in staining intensity ($p = 0.48$).

Discussion

This study focused on two sera supplements and their effects on human chondrocyte proliferation and the chondrocytes' subsequent recovery using standard chondrogenic differentiation protocols. To our knowledge, this is the only study providing a detailed analysis of ECM production from 5 individuals. However, it was not possible to compare AS among donors because the existing ethically approved protocol does not allow taking a sufficient volume of blood to provide sera for monolayer *in vitro* expansion to the end of passage 2, representing a limitation of the study. Another limitation was that only chondrocytes obtained from total knee replacement were evaluated. Therefore, caution must be used when interpreting these data as compared with chondrocytes sourced from patients undergoing ACI, as the patient populations for these treatments differ (for example, patients undergoing ACI tend to be younger than those undergoing joint replacement surgery). Despite these limitations, four key points were demonstrated. First, Stemulate™ increased chondrocyte proliferation when compared to FBS. This result was not surprising since several studies report that HPL increases cell proliferation in many cell types (Crespo-Diaz *et al.*, 2011; Hemeda *et al.*, 2014; Hildner *et al.*, 2015; Luttenberger *et al.*, 2000; Mojica-Henshaw *et al.*, 2013). Second, monolayer expansion of human chondrocytes in Stemulate™ negatively influenced the stability of the 3D pellets as compared to expansion in FBS: these chondrogenic pellets disintegrated easily when sectioned, slightly losing the pellet form – especially in the cells occupying the periphery, which appeared to be more fibroblastic. Third, total sGAG synthesis by Stemulate™-expanded chondrocytes lagged behind the FBS-expanded ones. Fourth, expansion in Stemulate™ negatively influenced chondrogenic gene expression in 3D pellets as compared to FBS.

There is clear evidence to confirm that 5-10 % (v/v) HPL is more effective in supporting monolayer expansion of human MSCs than both FBS and AS at the same concentration range (Bieback *et al.*, 2009; Juhl *et al.*, 2016; Trojahn Kølle *et al.*, 2013). Further, both Bieback *et al.* (2009) and Juhl *et al.* (2016) report that HPL supported long term monolayer expansion whilst still maintaining the MSCs phenotype and their differentiation potential. Given these positive data, herein it was established that Stemulate™ was better than FBS at increasing chondrocyte proliferation in monolayer culture. No published studies have used Stemulate™ for chondrocyte expansion, but increased proliferation is shown when using derivatives of HPL. Spreafico *et al.* (2009) compare the effects of platelet rich plasma (PRP), platelet poor plasma (PPP) and FBS on human chondrocytes over a 20 d cell proliferation time course, with PRP resulting the most stimulatory in terms of chondrocyte proliferation. Hildner *et al.* (2015) report that 5 %-10 % HPL significantly increase human chondrocyte proliferation as compared to FBS. From

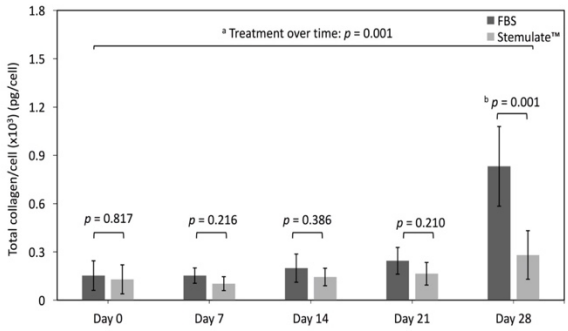

Fig. **6.** Total collagen production per cell in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 (n = 5) and up to day 28 (n = 3). [a] denotes statistical significance for time. [b] denotes statistical significance at day 28.

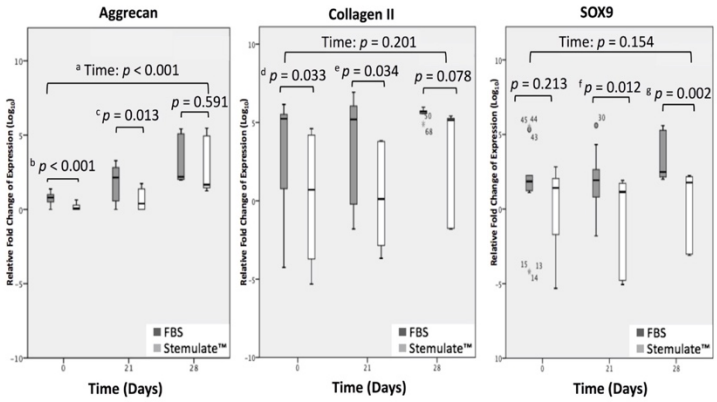

Fig. **7.** Box and whisker plot of gene expression profiles of the chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera. Data are expressed relative to the reference genes, obtained from 5 patients using 3 experimental pellet replicates per patient, and determined by using the comparative C_T method. [a] denotes statistical significance for time. [b] denotes statistical significance for aggrecan expression at day 0. [c] denotes statistical significance for aggrecan expression between supplement types at day 21. [d] denotes statistical significance for collagen II expression between supplement types at day 0. [e] denotes statistical significance for collagen II expression between supplement types at day 21. [f] denotes statistical significance for *SOX9* expression between supplement types at day 21. [g] denotes statistical significance for *SOX9* expression between supplement types at day 28.

these studies and the current, it is evident that HPL increases the proliferation of human chondrocytes in monolayer culture.

In the current study, no morphological differences were observed in human chondrocytes that were monolayer-expanded in Stemulate™ or FBS. No other published study has investigated morphological differences between human chondrocytes expanded in HPL or FBS. However, Trojahn-Kølle *et al.* (2013) report morphological differences in monolayer-expanded MSCs, appearing smaller and less spindle-like when cultured in HPL as compared with FBS. In the current study, 3D pellets from human chondrocytes expanded in monolayer in Stemulate™ created more fragile, less compact pellets as compared to those expanded in FBS, as seen by qualitative histological assessment. These chondrogenic pellets disintegrated easily, losing their pellet form, when prepared for histological analysis, resulting in a more difficult sectioning of the Stemulate™-expanded pellets at day 7. This was not assessed quantitatively but was a consistent observation throughout the study.

Very few studies use HPL with human chondrocytes and even fewer investigate sGAG production in 3D pellets. Following a published 3D pellet chondrogenic differentiation protocol (Johnstone *et al.*, 1998; Pittenger, 1999), it was demonstrated that FBS-expanded human chondrocytes generated significantly more sGAG than Stemulate™-expanded human chondrocytes across a 4-week time course. These results were opposite to the findings of Hildner *et al.* (2015), who report that HPL-expanded chondrocytes produce more sGAG than FBS-expanded chondrocytes in micromass pellets created from the same chondrogenic differentiation

protocol used in this study, over a 5-week period. After a 5-week culture period, a more intense sGAG staining is observed in HPL-expanded human chondrogenic pellets, compared to FBS pellets (Hildner *et al.*, 2015). These differences could be due to the supplement types, since Stemulate™ was used in the current study, whereas Hildner *et al.* (2015) use a HPL generated in their own laboratory from PRP obtained from 36 expired thrombocyte concentrates produced at a transfusion blood centre. 5 batches of HPL are characterised by Quantikine ELISA for these experiments, but no further details on the characterisation are given (Hildner *et al.*, 2015). Furthermore, in the current study, the 3D pellets were cultured in chondrogenic medium without HPL, whereas Hildner *et al.* (2015) add HPL into 3D pellet cultures.

In contrast to sGAG, total collagen production levels were found to be quite low throughout the 3D pellet culture 4-week time course. Despite this, collagen production by Stemulate™-expanded human chondrocytes appeared to lag behind FBS-expanded human chondrocytes. Gaissmaier *et al.* (2005) also observe that HPL-expanded chondrocytes seeded into 3D alginate beads produce low levels of collagen over a shorter time course of 14 d.

In the 3D pellet cultures, genes that determine chondrocyte differentiation state were investigated. De-differentiation was investigated through the relative expression of *COL1A1* (Benya *et al.*, 1978; Brew *et al.*, 2010) and hypertrophy through the relative expression of *COL10A1* (Caron *et al.*, 2012; Dell'Accio *et al.*, 2001) and *ALK1* (Blaney Davidson *et al.*, 2009; Dell'Accio *et al.*, 2001). *ALK1* is up-regulated during monolayer expansion, due to a loss of *in vivo* cartilage formation potential, and is associated with irreversible chondrocyte de-differentiation (Blaney Davidson *et al.*, 2007; Blaney Davidson *et al.*, 2009; Dell'Accio *et al.*, 2001). None of these genes were detected in 3D pellets derived from either Stemulate™- or FBS-expanded human chondrocytes, suggesting that the human chondrocytes had not undergone de-differentiation or hypertrophy. In addition, chondrogenic capacity was assessed through the relative expression of *COL2A1*, *ACAN* and *SOX9*. *COL2A1* and *ACAN* are necessary for ECM formation and the transcription factor *SOX9* regulates chondrocyte proliferation, chondrogenesis and transition to a non-hypertrophic state. Expression of *COL2A1* and *ACAN* declines following prolonged monolayer culture, particularly after passage 4 (Schulze-Tanzil *et al.*, 2002). For both these ECM markers, an increase was found across the 4-week time course. However, a much larger increase in relative gene expression was expected, especially by day 28, as this is found in similar published studies (Hildner *et al.*, 2015). An explanation could be that nutrient saturation was approached

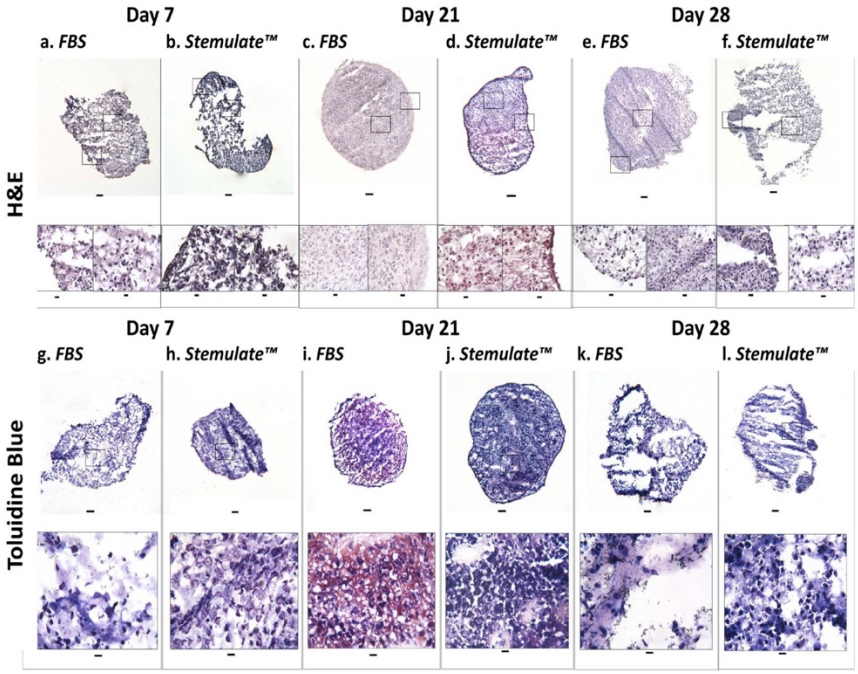

Fig. 8. Representative histological images from the centre of the FBS- and Stemulate™-expanded chondrogenic pellets across the 28 d time course. (**a-f**) Pellets stained with haematoxylin and eosin (H&E) to assess overall morphology. (**g-l**) Pellets stained with toluidine blue to assess proteoglycan content. Insert images are higher-powered images of the regions marked with a solid line. Scale bars: 100 μm.

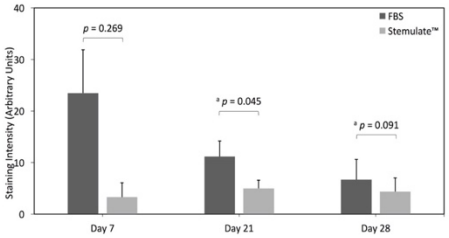

Fig. **9.** Semi-quantification of toluidine-blue-stained chondrogenic pellets, demonstrating an increase in metachromasia, determined by integrated density analysis using ImageJ imaging software (version 24). Data are presented as mean ± SEM for day 0-21 (n = 5) and up to day 28 (n = 3). [a] denotes statistical significance for staining intensity between supplement types at day 21.

before day 28. Overall, *SOX9* gene expression was more striking in FBS 3D pellets when compared to Stemulate™, indicating that the FBS-expanded human chondrocytes recovered chondrogenic potential more quickly. Taken together, these findings suggest that Stemulate™ caused a delay in the recovery of chondrogenic phenotype following monolayer expansion. It would have been interesting to perform q-PCR on the monolayer chondrocytes prior to seeding into pellets and to compare the relative gene expression of the chondrocytes before and during pellet culture. This would have determined how the relative gene expression levels would have changed following chondrogenic differentiation. However, the chondrocytes used were primary isolated chondrocytes and monolayer-expanded up to passage 2, which limited cell number and prohibited the use of q-PCR analysis on the monolayer chondrocytes.

The multilevel modelling analyses performed allowed the comparison of the treatments over the time course and the exploration of the impact of patient variability (Hox, 2010; Vaughn, 2008). One of the limitations of the study was that cartilage was obtained from joints that were potentially osteoarthritic. OA is classified as a whole-joint disease and, although the cartilage was taken from macroscopically normal areas, chondrocytes could still have had a sub-chondrogenic phenotype upon differentiation. A previous study shows that osteoarthritic chondrocytes express higher levels of *ALK1* (Blaney Davidson *et al.*, 2009), but *ALK1* expression was not detected in the monolayer-expanded chondrocytes. Patient variability was expected, but no statistical evidence was found for a patient-specific influence on any of the measured outcomes. Clearly more work needs to be done, but the current work demonstrated that although Stemulate™ led to rapid proliferation, it affected the re-differentiation potential of human chondrocytes. These findings demonstrated that Stemulate™ induced a sub-chondrogenic phenotype upon differentiation or enhanced monolayer-induced chondrocyte de-differentiation and reduced chondrogenic capacity. Therefore, it should be used with caution when expanding chondrocytes for clinical use.

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1. https://www.nice.org.uk/guidance/ta477 [16-04-2018]

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