General introduction, aims and outline of the thesis

The Knee Joint: osteochondral unit and articular cartilage defects

The knee is the largest joint in the body, vital for movement and shock absorption due to its unique properties to allow smooth gliding and even distribution of load transmission across the joint surface. Articular cartilage lines the end of long bones, which in turn are mutually held by four ligaments and dense fibrous connective tissue forming the joint capsule [1, 2].

The term osteochondral unit reflects the constraint among the articular cartilage, calcified cartilage and subchondral bone in term of mechanical and biochemical functional association [3]. Articular cartilage is an exceptional tissue in which the cells -chondrocytes- form 1-2% of the total volume and are dispersed in a dense extracellular matrix (ECM) in absence of blood vessels and innervations. Chondrocyte morphology and orientation is dictated by their localization in the superficial, middle and deep zones. Next to chondrocytes, collagen fibers in the matrix are also highly organized and designed to repeatedly withstand and distribute loads. Type 2 collagen is the predominant collagen fiber (90-95%) in the ECM and contributes to the resistance to tensile loads. In lower quantities, other collagen types such as types I, III, IV, VI, IX and XI, are also part of the fiber network [4]. Proteoglycans are also an essential component of cartilage matrix, aggrecan is the major one composed by a core protein and hydrophilic glycosaminoglycan side chains. Several units of aggrecan attach to a polymer hyaluronic acid chain by link proteins and forms proteoglycan aggregates. This structure due to their high polarity tend to attract, retain and extrude water (65-80% wet weight), conferring to the tissue its resilience in compression forces [5]. A small amount of other non-collagenous proteins, such as lubricin provides frictionless motion to the tissue. Located below articular cartilage and separated by the tidemark lies the calcified cartilage layer, which represents the mineralized transition zone. It acts as stress relief between the much stiffer bone and cartilage, thereby transmitting force and limiting diffusion to the deeper cartilage layer [6]. The subchondral bone, situated under the calcified cartilage, is able to transform shear stresses into compressive and tensile stresses during motion [7]. The subchondral bone distributes 30% of loads through the joints, while only 1-3% is transmitted by the cartilage, and consists of the subchondral bone plate in which bony lamella separate cartilage from the marrow cavity, to converge underneath into subarticular spongiosa region (Figure 1). This area is formed by porous trabecular bone that contains blood vessels and innervation [8].

Injury of articular cartilage due to trauma or degenerative damage is the major cause of disability in the aging population, therefore leading to a concomitant increase in the economic burden worldwide [9]. The lack of self-healing is impacted by the intrinsic avascular nature of the tissue and numerous factors, including the reduced repair ability in elderly patients, failure of the defect to cross the subchondral bone [10] in order to form fibrin clot as scaffold [11, 12], as well as the scarce migration of chondrocytes

immersed in a dense matrix barrier. In addition, increasing wound size was found to be correlated with a decreased spontaneous healing [13]. Recently, arthroscopic procedures revealed the presence of untreated acute injuries associated principally with meniscus tear and cruciate ligament rupture in more than 60% of patients [14], including young and healthy persons, with incidence rates nearly tripled between 1996 and 2011 [15]. These injuries tend to progress towards the degenerative path of osteoarthritis with diminished chances of success of intervention.

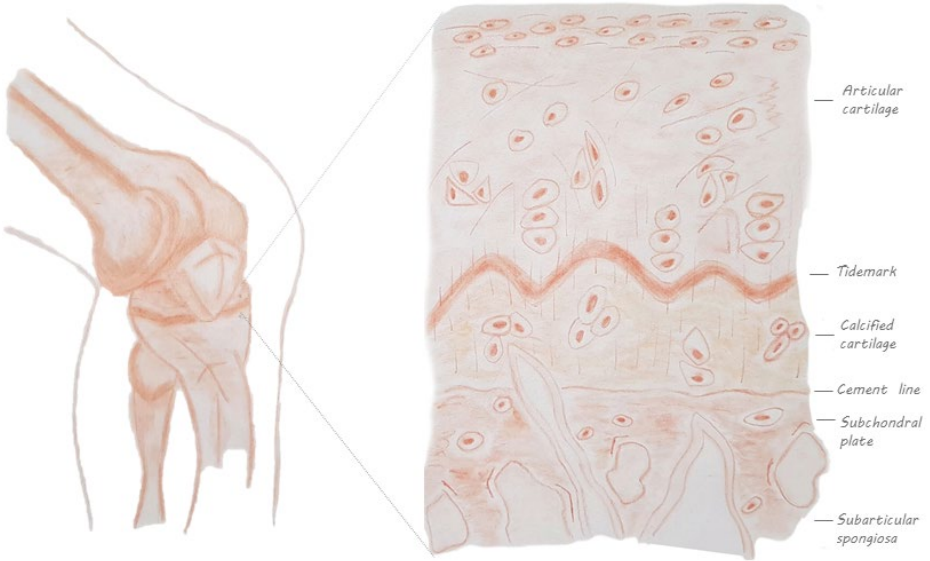

Figure 1 Human knee joint representation. Hierarchical architecture of osteochondral unit. The layers including articular cartilage, calcified cartilage and subchondral bone are shown.

Current and emerging clinical treatments for cartilage injury

When conventional pain management for focal cartilage defects is no longer effective, surgical invasive procedures based on microfracture and transplantation of cartilage/ subchondral graft are considered. The major drawbacks of these methods rely on the poor recreation of long-lasting hyaline cartilage and scarce availability of the autologous graft and its integration with injury site [16-18]. Encouragingly cell-based approaches have been introduced during the years, such as autologous chondrocytes implantation (ACI) [19], matrix assisted ACI (MACI) [20] and mesenchymal stromal cell (MSCs) transplantation [21]. ACI and MACI require a two-step procedure due to isolation, expansion and reimplantation of cells under periosteal flap or scaffold, while autologous and allogenic MSCs transplantation results in a single step joint surgery and has proven its safety and efficacy in terms of chondrogenic potential [22]. Randomized clinical trials comparing these techniques have shown similar results, yet the short follow up periods

do not justify their long-term efficacy [23-25]. Whether these techniques have shown improved outcome over the other methodologies (e.g. microfracture) remains unclear due to conflicting data regarding the durability of different cartilage repair strategies [26-30]. Nonetheless cell therapies face certain limitations in term of cost and regulatory issues [31], they still present failure rates with common comorbidities [9]. The standard surgical treatment after repair failure is total knee arthroplasty (TKA). However, prostheses provide only temporary alleviation of pain (excluding approximately 20% of patients reporting unfavourable chronic pain as adverse outcome at six months after surgery [32]) due to limited lifetime and, particularly in the group of younger patients revision surgeries with higher failure risks are required [33]. This poses pressing need for improvement of cartilage restoration technologies. A holy grail of modern research would be to re-establish native tissue properties by enhancing the body's intrinsic healing ability. Taking advantage from microfracture and autologous matrix-induced chondrogenesis, reconstruction of the cartilage niche may be achieved by persuading cells present in the wound site to regenerate their own damaged tissue structure.

Regenerative Medicine: A time travel

It all started back in the year 600 B.C. with the Greek myth of The Titan Prometheus. Known as benefactor of mankind, he stole fire from Olympus. Zeus, provoked by this theft, punished him to be chained to a rock; where, for 30,000 years an eagle came each night to feed upon his liver. Destined for eternity, everyday his liver grew just to meet the same fate the evening after. Nowadays it is a bona fide fact, the liver is one of the organs in the adult life that possess spontaneous regenerative ability after injury [34].

A thousand years later a lesson from a worm was given by Trembley (1710) and could be found in the early 1900's in the book "Regeneration" published by Thomas Hunt Morgan. They observed a hydra after experimental resection, introducing the fascinating concept of bidirectional regeneration by demonstrating the return of body function from the amputation site. These pioneering works are the basis of tissue engineering and regeneration [35, 36]. Beside the bidirectional regeneration concept, the introduction of unidirectional regeneration in more complex organisms (e.g. Salamander) [37] and the introduction of stem cells during the 1980's [38], have extended possibilities for tissue reconstruction. However, compared to many phyla that permanently renew, regeneration in humans is limited or even absent.

The fast-paced advancement that occurred in the past 70 years has expanded our knowledge of regenerative medicine, highlighting that remodeling mechanisms are not universal or shared among individual body tissues implying inevitable tissue-specific processes. In addition, a diversity of factors constrain regeneration, such as immune responses, extracellular matrix composition, age, injury type, physiological adaptation, angiogenic and neurogenic capacity [39].

Biomaterials in cartilage regeneration

Biomaterials used to restore or re-establish normal body function have changed during the years. From seashells to replace missing teeth during the Mayan civilization, via off-the-shelf materials for joint replacement post World War II such as metals, polymers, and ceramics, to biocompatible and biodegradable engineered materials in the modern era.

Biomaterials shall provide the 3D templates for cartilage regeneration, ideally directing cell migration and differentiation into chondrocyte phenotype [40], mimicking the niche by providing structural and mechanical cues to guide remodeling, reproducing its zonal organization and lastly facilitating integration with the native tissue. Based on these versatile properties a variety of scaffolds have been developed and generally classified in synthetic and natural scaffolds according to their composition. To cite few: degradable synthetic polymers (e.g. polyethylene glycol (PEG), polylactic acid (PLA), polyglycolic acid (PGA) and their co-polymers), natural scaffolds such as protein gels including, collagen, fibrin [41-44] and polysaccharide based materials comprising chitosan, hyaluronic acid, agarose and alginate gels [45, 46] are largely reported in the literature. Synthetic materials have been manufactured to control conditions such as macroporosity to allow cell seeding, instructive topography to promote cell orientation and directions [47], mechanical resistance to withstand compressive forces [48] and attempted three zonal chondrocytes distribution to foster organized matrix deposition [49, 50]. Although there are clear advantages regarding the use of these matrices, bioincompatibility and unfavourable byproducts may impede tissue regeneration [51]. As a result, natural materials, hydrated polymeric networks which recapitulate chemical and biological features of ECM, have been introduced. Their ability of *in situ* gelation render these systems safely injectable and less invasive, which make them ideal candidates to fill every type of focal cartilage defect, enabling higher integration with injured tissue compared to synthetic scaffolds with defined shape [52]. Despite these classes of gels are less reproducible in production and not ideal to resist the complex loading during joint motion as experienced *in vivo*, they rarely induce cellular immune response and toxicity. To overcome the lack of mechanical strength, hybrid natural and synthetic materials have been developed by polymer modifications with functional groups to form hydrophilic structures with increased crosslinking density [53, 54].

This thesis focuses on the use of hybrid modified hydrogels, in particular on the selection of a suitable hyaluronic acid-based hydrogel, which allows us to study early endogenous cell migration processes and to assess its response to complex load when filled into an osteochondral defect model.

Hyaluronan (HA) is a natural cartilage matrix component, possessing both chondro-inductive and chondro-protective properties [55], its unique biochemical composition may favor regenerative processes recalling the embryonic-like

microenvironment [56]. HA-Tyramine (HA-Tyr) conjugate hydrogels were introduced as drug delivery system [57, 58] and for tissue engineering applications [59]. The oxidative reaction catalyzed by hydrogen peroxide and horseradish peroxidase allows crosslinking of tyramine moieties, providing independent tuning of mechanical strength (e.g. crosslinking densities) and gelation rate, thereby adding an increased level of control needed to enhance migration and to create an amenable microenvironment for cartilage (re)generation. The modulation of these properties combined with distinct material stiffness and mesh size can influence cell migration, differentiation and matrix synthesis, making HA-Tyr hydrogels a particularly attractive materials for osteochondral restoration purposes.

Fibrin-HA (FB/HA) hydrogels have extensively been studied for cartilage and intervertebral disc regeneration [41, 60]. Although its network is insufficient to withstand dynamic load at high magnitude, the conjugation of FB/HA (Regenogel™) has shown to remarkably lower the degradation rate of fibrin and enable cell migration by cause of a larger mesh size. The fine tuning between the two components provides sufficient non adhesive and adhesive surfaces (due to HA) to allow cell infiltration and support the ability to move more freely within the fibrin framework forming fibers [60-62]. The achievement of adequate cell density and uniform cell distribution along the hydrogel is a demanding step but crucial to ultimately culminate into neo-tissue formation. Therefore, FB/HA is considered an excellent hydrogel candidate for studying osteochondral repair strategies.

Inductive cues: chemoattractants, growth factors and hydrogel micromechanics

The new generation of biomaterials which are both tolerated by the body and have functional properties, called biofunctional matrices, can be tuned and used to induce a response, e.g. cell migration. This response is influenced by integrating cues, biochemical and biophysical, that foster or improve cell ingress into the wound site and mechanical remodeling of the microenvironment [63]. A requisite stage of migration is cell adhesion enabled by ligands (e.g. integrins) present within the biomaterial; the binding initiates a signalling cascade dependent on the ligand type and concentration. Fibrin and collagen gels are natural materials well-known for their distinguished cell adhesion capability [64]; however, inert materials can be conjugated with small oligopeptide sequences (e.g. RGD) functioning as adhesive ligands to support cell infiltration [65]. To replicate *in vivo* situations more closely, materials are used as suitable carrier to modulate the bioavailability and gradient concentrations of bioactive agents essential for cell homing. Indeed, chemoattractants and growth factors can be encapsulated to provide initial and sustained release, enhance cell sensing and influence matrix deposition. Fibroblasts, endothelial cells, MSCs and cartilage progenitors home towards a wide

variety of biomolecules including stromal cell-derived factor 1 (SDF-1) [66], chemokine ligand 5 (CCL5 or RANTES) [67] and platelet-derived growth factor BB (PDGF-BB) [68]. Additionally, chondrocytes proliferate and increase matrix biosynthesis in presence of fibroblast growth factor 18 (FGF-18) [69].

The design of hydrogels appropriate for osteochondral regeneration is critical since they should also handle the demands of the joint microenvironment to withstand dynamic compressive and shear loads. An attractive option is to use crosslinkable hydrogels, in which for example functional groups of Tyr are grafted to HA backbone to form a hydrophilic network suitable for coping with the physiological environment [70]. Depending on the modifications present along the backbone, the concentration of the polymer and the fine modulation of the crosslinking density, the bulk mechanical properties of a hydrogel (such as compressive and shear moduli) can be tailored. Furthermore, polymers can be drawn into aligned fibre nanostructures to replicate the organization of dense tissues. Although these materials can resist mechanical loading, the stiffness and organized polymer matrix deposition could present an extraordinary physical barrier to cell migration, thereby limiting matrix accumulation [71]. This is due to the inability of the cells to overcome steric hindrance by body deformation or MMP-mediated degradation when matrix stiffness is too high. Despite the extensive body of literature is paving promising approaches, the use of combined bioactive factors in association with the emerging techniques to improve material properties, cartilage regeneration is still limited. Further research is needed to study the efficacy of these delivery systems and the spatiotemporal migration kinetics in the context of a joint-like mechanical setting.

Multiaxial shear and compression load

All connective tissues are exposed to mechanical loading, while their biological and mechanical functions differ due to the heterogeneity and hierarchical nature of protein building blocks. During development mechanical forces contribute to patterning and organogenesis [72], but these physical cues are also pivotal in adult tissues to maintain homeostasis and influence repair.

Because articular cartilage is avascular, aneural, and alymphatic, synovial fluid is the principal means by which the tissue obtains nutrients through diffusion. During mechanical loading, the displacement of water in and out provides an enhancement of flow progression at which chondrocytes receive the nutrients. Indeed, it has been shown that larger solutes (e.g. growth factors, hormones and enzymes) are transported to the cells at different rates, influenced by loading and fluid movement [73]. Loading and movement, also increase synovial fluid production and aid waste products removal via the synovial membrane. Changes in chondrocyte's catabolic and anabolic activities are also considered to occur by transduction of mechanical signal into metabolic events

and structural adjustments [74, 75]. *In vivo* studies have demonstrated that the absence of loading through joint immobilization culminates into degenerative changes defined mainly by loss of sulfated glycosaminoglycan (sGAG) [76]. When autologous grafts are used to resurface synovial joint defects, motility has been shown to be necessary for regulation of chondrogenesis [77]. It is well known that engineered cartilage constructs generally progress towards the endochondral ossification route, in which chondrocytes undergo hypertrophy to then are replaced by bone. However, under certain conditions, especially under the influence of a joint-like mechanical environment, e.g. cyclic hydrostatic pressure, a more stable cartilage adopting an articular phenotype is generated [78]. Numerous studies have sought to demonstrate the effect of mechanical force on cultured chondrocytes and cartilage explants by developing bioreactors that mimic one or more components of the mechanical environment, in order to direct tissue development through mechanical stimulation. As a result, many groups developed dynamic uniaxial or multiaxial compression and shear bioreactors to monitor the metabolic and biochemical responses of chondrocytes and MSCs within scaffolds and cartilage explants using various loading regimes. The major finding was that dynamic compression promoted, whereas static compression inhibited the synthesis of anabolic factors [79]. Due to the complexity of building bioreactors applying combinations of dynamic and shear stimuli only few groups have investigated the supplementary effect of the shear load. *Waldman et al.* [80] investigated the effect of dynamic compression on bovine chondrocytes on ceramic surface, observing only a slight increase in proteoglycan and collagen compared to scaffold free tissues, whereas the addition of shear to the dynamic compressive load significantly enhanced these outcomes. Then, *Grad et al.* [81] demonstrated the application of multiaxial loads on bovine chondrocytes seeded in polyurethane scaffolds comparing dynamic compression alone, with dynamic uniaxial shear (scaffold rotation around its axis) and the combination of compression and uniaxial and multiaxial shear loads (scaffold rotation and ball oscillation over the scaffold surface. They reported that cyclic compression combined with either uniaxial or multiaxial shear stresses could significantly modulate the amount of ECM, promote a functional surface and downregulate matrix degrading enzymes. Other studies using the same system have shown chondrocytes maturation depended on cell passage and onset of loading [82], and by reducing oxygen levels, increased levels of GAG/DNA and reduced collagen 1 gene expression were achieved, further favoring the articular chondrocytes phenotype [83]. Other groups using different materials [84] or introducing a combination of loads and electromagnetic fields showed similar results [85]. When the substrate (cells), as an essential variable, was changed to using human MSCs instead of primary bovine chondrocytes in the scaffolds, the implementation of multiaxial loads acted as a promoter of transforming growth factor beta (TGF-β1) production and activation, thereby inducing MSCs chondrogenic differentiation [86].

These studies suggest that appropriate environmental conditions could maintain or promote the chondrogenic phenotype and could facilitate the generation of tissue engineered cartilage template for the development of the different layer types that make up the osteochondral unit.

Although tremendous progress has been made recently to generate functional tissue and several biomaterials have been suggested [87, 88], there is still much to be learned. To date inadequate biomechanical stability of the graft has been observed [89], and the mismatch between repair and native tissue following surgery still remains one of the major clinical challenges contributing to a continued disruption in joint biomechanics and repair failure. This demonstrates the need for improved treatments. Since translation toward clinics need proof of functionality, the development of *ex vivo* models under mechanical stimuli, closely representing an articulating joint *in vivo,* would allow an accurate screening of materials and factors in order to only select promising conditions for *in vivo* models (figure 2).

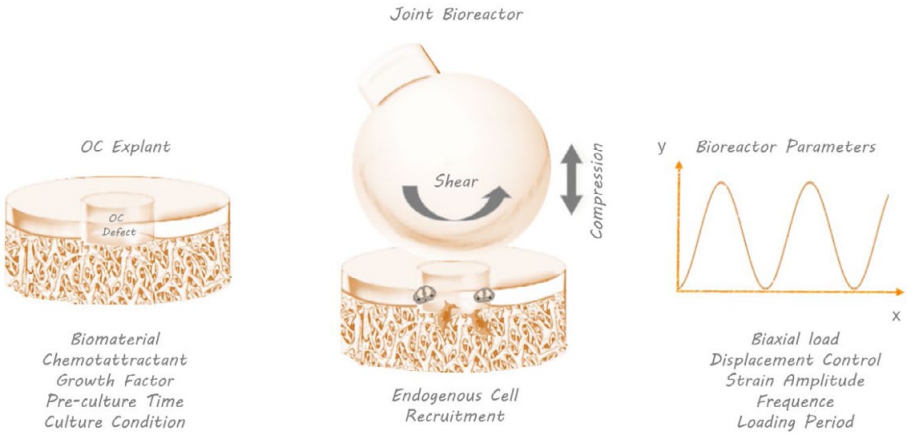

Figure 2 Schematic of bioreactor-based model to investigate cell response to mechanical stimulation. Input variables under consideration in the study design. OC: Osteochondral Explant

AIM AND OUTLINE OF THE THESIS

Articular cartilage injury poses a significant clinical challenge in orthopaedics. Advances in the recent decades are placing cartilage regeneration in the spotlight, paving the potential to overcome limitations of current treatments. The main objective of this thesis is to improve cartilage restoration using a hydrogel-assisted cell-free approach considering the physiological joint-like microenvironment. To achieve this, an ideal hydrogel is first selected for the ability to support cell migration and cartilage formation,

further prompted by diffusible chemotactic agents that create extracellular gradients to eventually accelerate these biological processes. Then dynamic load is applied to the hydrogel to investigate chondrocytes mechano-transduction on the generation of a cartilaginous network. To closely resemble the joint microenvironment, a mechanically stimulated osteochondral defect model is developed. The model is used as pre-clinical testing tool to screen hydrogels and biomolecules for their ability to promote the body's intrinsic healing capacity, under relevant complex mechanical stimuli.

Cell migration towards the cartilage injury site and cell fate can vary under the influence of different soluble factors, mechanical signals, and the composition of an implanted hydrogel. The ideal hydrogel that provides cell support and guides cartilage remodeling has not been identified yet. Hence, we evaluate in **Chapter 2** the *in vitro* and *in vivo* effects of hyaluronan-based hydrogels on cell recruitment, by using a 3D spheroid-based migration assay and a bovine osteochondral defect model. Cell infiltration into the hydrogels was fostered via the creation of chemotactic and growth factor gradients to provide a driving force to ideally complete this biological process. This may contribute to the selection of an optimal microenvironment and soluble signalling factor to support and enhance the delicate step of early endogenous stem and progenitor cell recruitment from cartilage and bone. The tested biomaterials represent a natural derived class of gels frequently used in regenerative medicine and in clinics.

Mechanical loading plays a pivotal role in joint development, pathogenesis, and regeneration. As cartilage is a load-bearing tissue, understanding the influence of load could be an approach to develop new strategies to improve the quality of the regenerated tissue. Therefore, the 3D selected hydrogel should also be capable of transducing mechanical loads and delivering bioactive factors to better facilitate and regulate cell colonization and differentiation in the injured tissue. In **Chapter 3** we aim to test the interplay between mechanical and biochemical signals affecting proliferation and differentiation of primary bovine chondrocytes embedded in a Fibrin-HA hydrogel. This may decode the interdependence of multifactorial determinants elucidating signalling pathways implicated on cartilage homeostasis and repair.

While bioreactor studies, as the one presented in **Chapter 3,** have mostly investigated the effects of mechanical stimuli on isolated scaffolds or hydrogels in unconfined mode, the possibility to consider a confined microenvironment within the tissue as it is experienced *in vivo* is explored in **Chapter 4**. Here, we aim at combining an osteochondral *ex vivo* culture model, in which reproducible osteochondral or chondral defects can be filled with a biomaterial, with mechanical compression and shear load to simulate physiological joint kinematics. Osteochondral *ex vivo* models, in which defects can be generated, are of great value for translational research; the possibility to study integration within the surrounding cartilage and the crosstalk between cartilage and bone are fundamental to the improvement of joint therapies and tissue restoration.

This may enable more predictive pre-clinical screening of new therapies and biomaterial implants likely replacing or reducing pre-clinical *in vivo* studies.

Nowadays it is unclear how mechanical stimuli affect neo tissue formation, as in *in vivo* studies the ability to monitor cell responses to loads that new tissues experience is limited. A sequence of events occurs after injury, including cell recruitment into the site, differentiation into the desired cell type and secretion of factors to promote tissue repair. However, the endogenous cartilage healing is a critical process not very well understood. Thus, it becomes imperative to consider the delicate balance that exists between loading and remodeling to be capable of restoring articular cartilage homeostasis and structural integrity. In this scenario, the possibility to study these phenomena and observe the influence of mechanical stimuli on endogenous cell recruitment over time can be accomplished by using the osteochondral model presented in **Chapter 4**, where an injury site is filled with a biomaterial and chemoattractants. This is explored in **Chapter 5**, with the view to obtain tuned modulation of extracellular signals and their use in cellular decision making. Determining the loading effect over time and exploring whether the implementation of factors could enhance the joint regeneration process, may provide insight on the optimal time to apply dynamic loading after surgical intervention.

Finally, **Chapter 6** provides a general overview of the findings presented in this thesis and addresses future directions on the use of the model as a potential tool to either improve tissue regeneration strategies or inhibit tissue degeneration, in order to promote joint preservation as an attractive alternative to metal/plastic joint replacement.