Towards Cartilage Regeneration:
Biomaterial-Assisted Cell-Free Approach in a Physiological Joint-like Environment

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Towards Cartilage Regeneration:
Biomaterial-Assisted Cell-Free Approach in a Physiological Joint-like Environment

Op weg naar kraakbeen regeneratie: door biomateriaal ondersteunde celvrije aanpak in een gewricht simulerende omgeving

Thesis

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“Mai nulla di splendido è stato realizzato se non da chi ha osato credere che dentro di sé ci fosse qualcosa di più grande delle circostanze»
–Bruce Barton–

A mio padre
Per avermi insegnato a sognare sempre in grande.

A mia madre
Per sostenermi ed incoraggiarmi a dare di più senza mollare.

Ai miei fratelli
Per essere così piacevolmente e meravigliosamente tanto diversi da me.

Con sconfinata gratitudine, questa tesi è dedicata a voi che siete la mia grande ricchezza.

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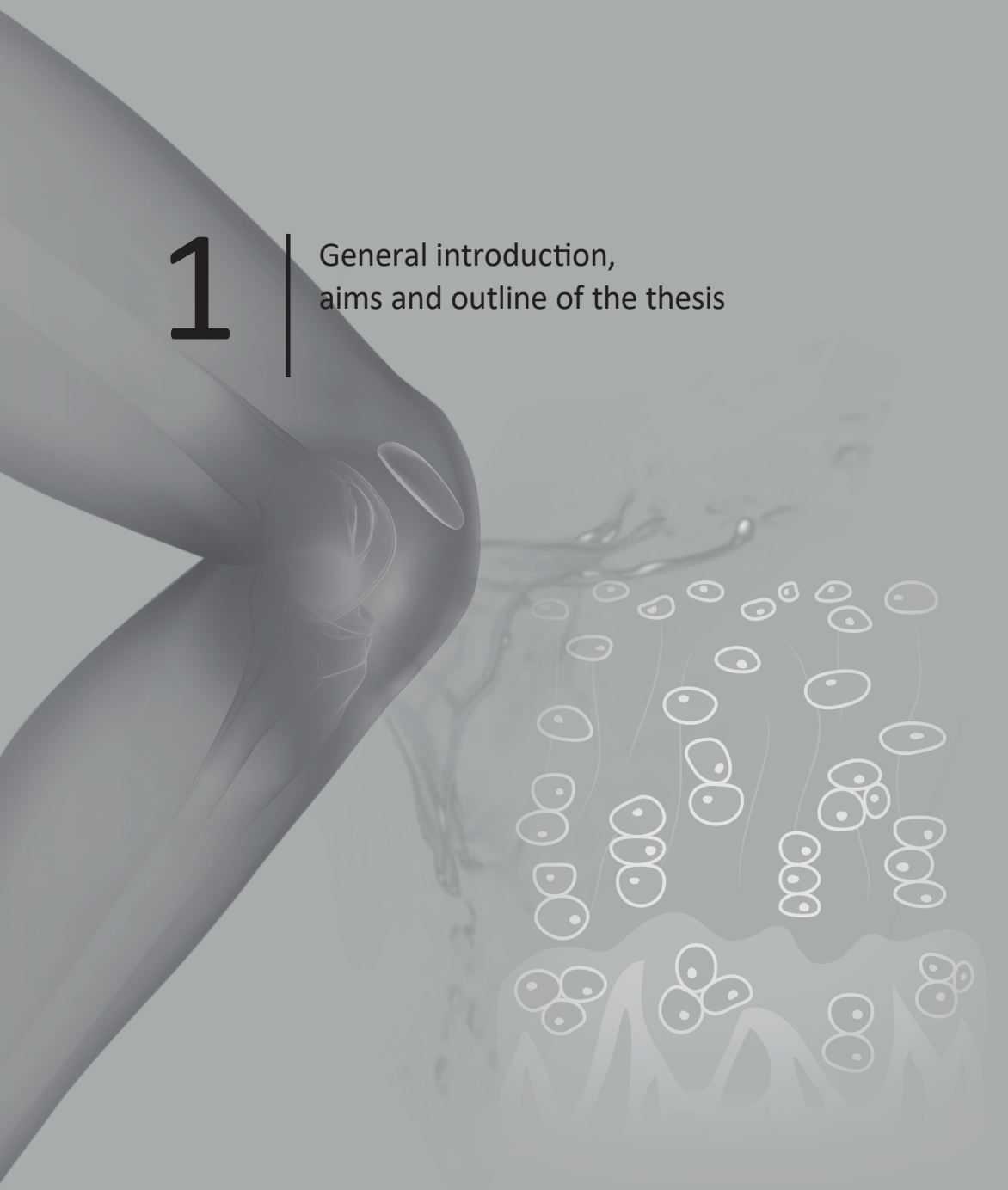
1 General introduction, aims and outline of the thesis



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.

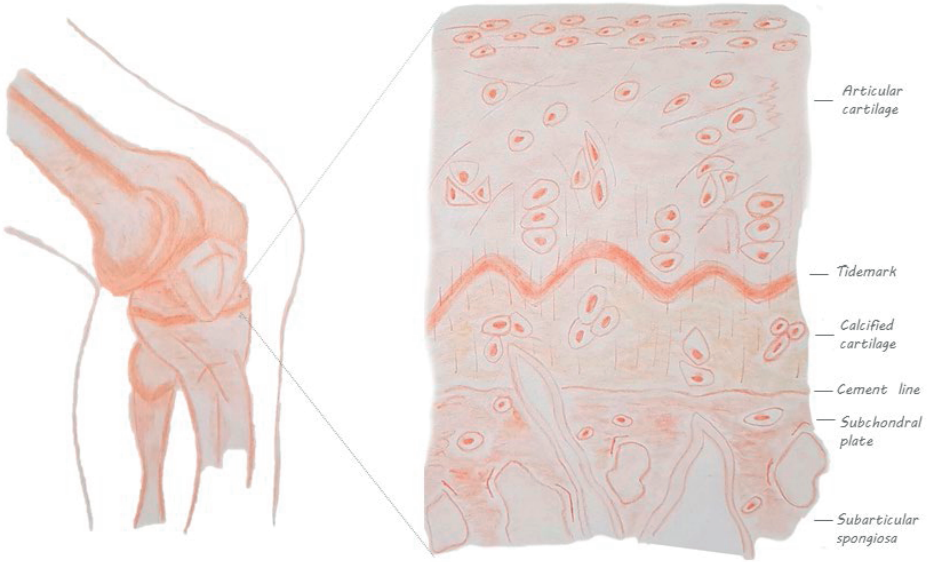


Figure 1 Human knee joint representation. Hierarchical architecture of osteochondral unit. The layers including articular cartilage, calcified cartilage and subchondral bone are shown.

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).

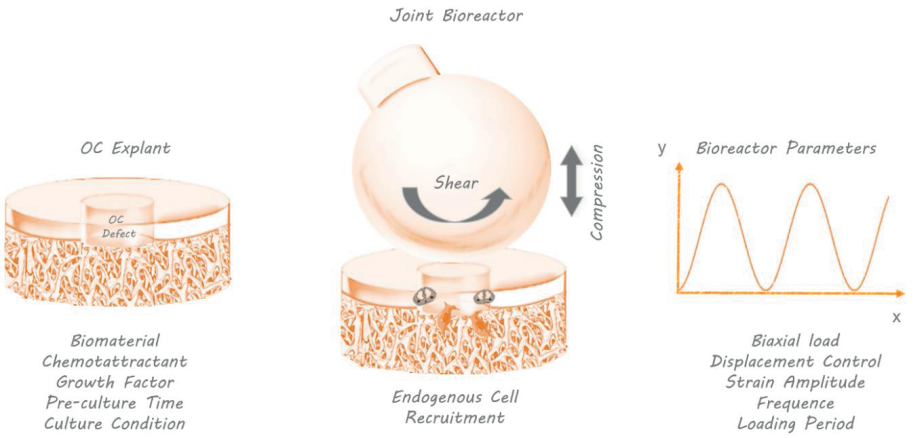


Figure 2 Schematic of bioreactor-based model to investigate cell response to mechanical stimulation. Input variables under consideration in the study design. OC: Osteochondral Explant

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.

2 Evaluation of biomimetic hyaluronic-based hydrogels with enhanced endogenous cell recruitment and cartilage matrix formation

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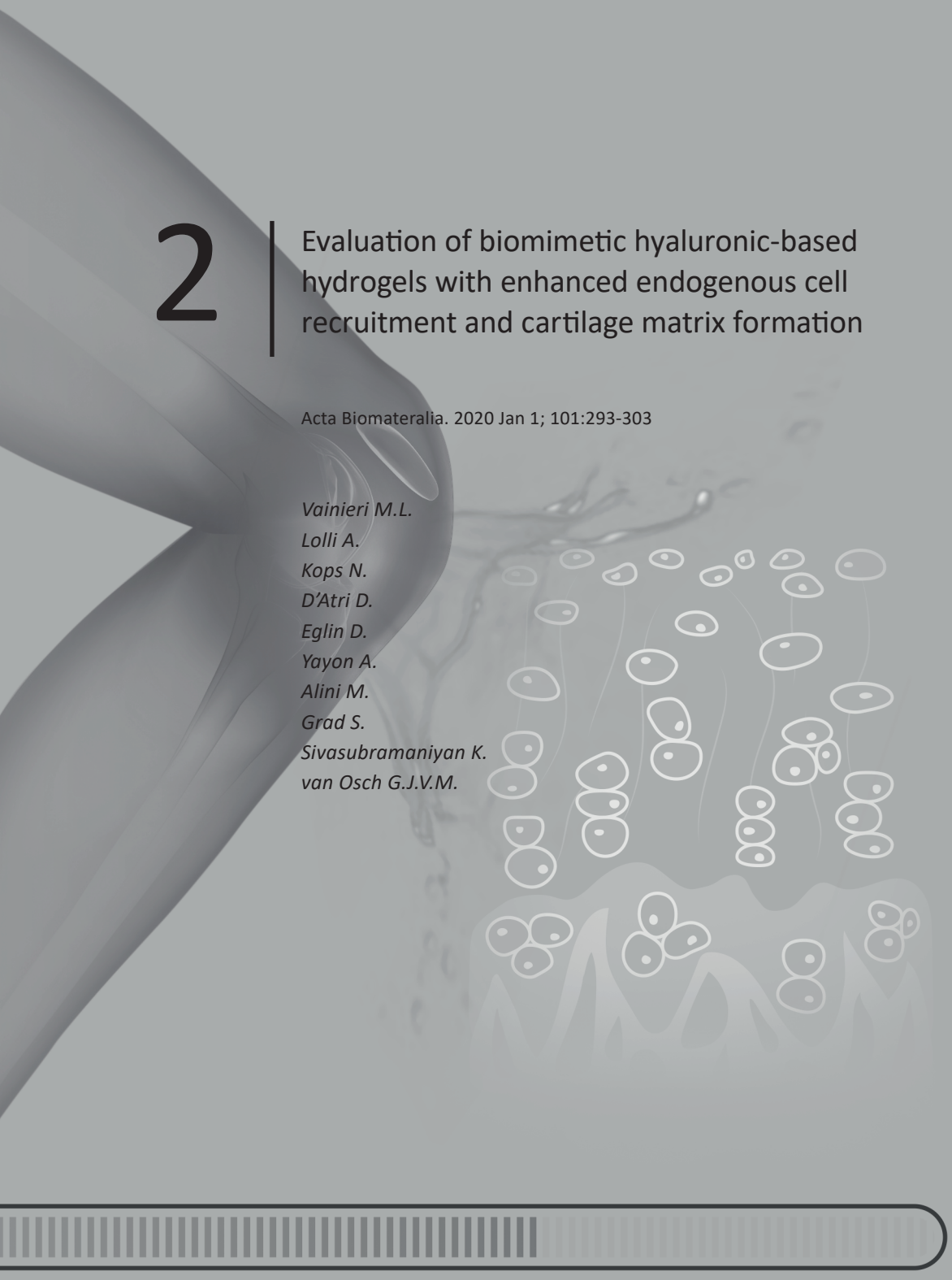


ABSTRACT

Biomaterials play a pivotal role in cell-free cartilage repair approaches, where cells must migrate through the scaffold, fill the defect, and then proliferate and differentiate facilitating tissue remodeling. Here we used multiple assays to test the influence of chemokines and growth factors on cell migration and cartilage repair in two different hyaluronan (HA)-based hydrogels. We first investigated bone marrow Mesenchymal Stromal Cells (BMSC) migration *in vitro*, in response to different concentrations of platelet-derived growth factor-BB (PDGF-BB), chemokine ligand 5 (CCL5/RANTES) and stromal cell-derived factor 1 (SDF-1), using a 3D spheroid-based assay. PDGF-BB was selected as most favourable chemotactic agent, and MSC migration was assessed in the context of physical impediment to cell recruitment by testing Fibrin-HA and HA-Tyramine hydrogels of different cross-linking densities. Supplementation of PDGF-BB stimulated progressive migration of MSC through the gels over time. We then investigated *in situ* cell migration into the hydrogels with and without PDGF-BB, using a cartilage-bone explant model implanted subcutaneously in athymic mice. *In vivo* studies show that when placed into an osteochondral defect, both hydrogels supported endogenous cell infiltration and provided an amenable microenvironment for cartilage production. These processes were best supported in Fibrin-HA hydrogel in the absence of PDGF-BB. This study used an advanced preclinical testing platform to select an appropriate microenvironment provided by implanted hydrogels, demonstrating that HA-based hydrogels can promote the initial and critical step of endogenous cell recruitment and circumvent some of the clinical challenges in cartilage tissue repair.

Keywords: Biomaterials, cell migration, osteochondral defect model, endogenous cell recruitment, cartilage repair

Statement of significance

The challenge of articular cartilage repair arises from its complex structure and architecture, which confers the unique mechanical behavior of the extracellular matrix. The aim of our research is to identify biomaterials for implants that can support migration of endogenous stem and progenitor cell populations from cartilage and bone tissue, in order to permanently replace damaged cartilage with the original hyaline structure.

Here, we present an *in vitro* 3D spheroid-based migration assay and an osteochondral defect model, which provide the opportunity to assess biomaterials and biomolecules**,** and to get stronger experimental evidence of the not well-characterized dynamic process of endogenous cells colonization in an osteochondral defect. Furthermore, the delicate step of early cell migration into biomaterials towards functional tissue engineering is reproduced. These tests can be used for pre-clinical testing of newly developed material designs in the field of scaffold engineering.

INTRODUCTION

Articular cartilage is a load bearing tissue with a unique composition and structure. Once damaged, its poor intrinsic repair ability results in permanent functional impairment, which often leads to osteoarthritis in absence of treatment [90, 91]. A number of studies have shown limited cell infiltration of the cartilage tissue as an impediment to endogenous repair [88]. Effective management of cartilage lesions can be challenging, creating a burden for both patients and clinicians. With conservative treatment being unsuccessful, surgical interventions are proposed for articular cartilage lesions, such as microfracture or osteochondral allograft transplantation. When perforations penetrating the subchondral bone are created in the context of marrow stimulating techniques (microfracture), invading cells often exhibit limit healing potential, producing a fibrocartilaginous tissue with poor mechanical properties, rather than hyaline cartilage [16]. To overcome this issue, cell delivery approaches like autologous chondrocyte implantation and matrix-induced autologous chondrocyte implantation (ACI, MACI), have been established as cartilage repair methods [5-7]. Recently clinical interventions based on autologous mesenchymal stromal cells (MSCs) transplantation have been proposed [92-94]. When evaluating studies comparing patients treated with ACI/MACI and with autologous bone marrow derived MSCs, similar improvements were reported in relation to clinical outcome and pain score [23, 24]. Nonetheless these cell-based therapies face important limitations, due to enormous costs for the patients as well as cell handling, time and regulation related to safety [31]

The extracellular matrix (ECM) of articular cartilage is a highly functional dense connective tissue, but its restrictive barriers impede endogenous cell migration. Partial degradation of the ECM at the wound edge of the cartilage has been proposed to reduce its stiffness [95-97] and enhance endogenous cell migration. Although these findings have shown that cells, that are normally trapped in the dense ECM, are capable of initiating tissue repair once they reach the edge of the lesion, in term of diarthroidal joint studies have suggested that cells might normally come from bone and bone marrow side and even from synovium [98, 99]. The induction of cell mobility to recruit cells into the defect is an attractive option that has already been described for *in situ* regeneration of multiple tissues [66, 98], in order to circumvent issues related to cell-based therapy. There is a pressing need to identify the optimal biomaterials [100] and recruitment factors that can be used as cell free approach for cartilage regeneration strategies. A variety of scaffolds and bioactive compounds were shown to promote stem and progenitor cell recruitment and improve cell differentiation [88, 101]. However, to date, no studies have identified the effective biomaterial that would promote the recruitment and differentiation of endogenous stem/progenitor cells to achieve functional cartilage regeneration *in situ.*

Hyaluronan (HA) is a component of the cartilage matrix that has both chondro-protective and chondro-inductive properties [55]. HA-based biomaterials have been shown to enhance healing processes in osteochondral defects in rabbit and minipig models [102, 103]. They possess a unique biochemical composition that recreate the embryonic-like microenvironment [56], which may be favorable for the regenerative process. HA-based hydrogels can be enzymatically cross-linked *in situ*, rendering the system safely injectable and non-invasive [60, 104]. HA-Tyramine (HA-Tyr) hydrogels have been developed as drug carriers for protein delivery [57, 105] and for tissue engineering applications [58]. It is known that by tuning the hydrogel the microenvironment can be modulated, which in turn can regulate spatial cell organization and matrix biosynthesis. HA-Tyr conjugates have advantageous material chemistry perspectives, the system is enzymatically cross-linked via a reaction catalyzed by horseradish peroxidase (HRP) using hydrogen peroxide (H_2O_2) as substrate. The fine tuning of its mechanical strength can be achieved by the H_2O_2 concentration without affecting the gelation rate [105]. It has been shown that varying the HA-Tyr hydrogel cross-linking density can modulate MSC differentiation and matrix biosynthesis [59]. Fibrin-HA (FB/HA) combination is widely used in tissue engineering and the specific conjugation of FB/HA hydrogel (Regenogel^{TM}), is well known for its applications in the regeneration of various tissues, such as cartilage and intervertebral disc [60, 66, 106]. The particular method employed for conjugation of HA to fibrinogen allows high versatility of the resulting hydrogels by alternating the molecular weight of HA, degree of activation and Fibrinogen/HA ratio. The resulting hydrogels are particularly stable compared to other Fibrin based scaffolds with remarkably lower rate of degradation of fibrin and a lager mesh size, thus allowing better cell migration and ECM deposition [60].

This study uses a sequence of assays to compare different HA-containing hydrogels for cell mobility, differentiation and matrix deposition. We evaluated the ability of human bone marrow stromal cells (hBMSCs) to migrate in a hydrogel under the influence of different chemokines, i.e. PDGF-BB, RANTES and SDF-1 in a 3D spheroid-based assay. After selecting PDGF-BB as strongest stimulator of MSC migration, we tested the injectable FB/HA formulation and HA-Tyr hydrogels with different cross-linking densities for their ability to allow cell migration and support chondrogenic differentiation *in vitro*. Finally, a bovine osteochondral explant model was used as hydrogel testing platform to monitor the recruitment of endogenous cells to the injury site in an *in vivo* mouse model**.**

MATERIAL AND METHODS

Cell isolation and culture

Bone marrow aspirates were collected from 6 patients undergoing total hip replacement (age 50-78 years) after informed consent (approved by the local Medical Ethical Committees of Erasmus MC: protocol MEC-2015-644; and Albert Schweizer Hospital: protocol 2011.07). Mesenchymal stem cells were isolated from leftover iliac crest bone chip material obtained from 1 patient (age 13 years) undergoing alveolar bone graft surgery (as leftover material with approval of local Medical Committee of Erasmus MC: MEC-2014-16). Human bone marrow stromal cells (hBMSCs) were expanded at a seeding density of 2,300 cells/cm^2 in alpha-Minimum Essential Medium (α-MEM; Gibco, Carlsbad, California, United States) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, California, United States), 50 µg/mL gentamycin (Gibco, Carlsbad, California, United States), 1.5 µg/mL fungizone (Gibco, Carlsbad, California, United States), 1 ng/mL fibroblast growth factor 2 (FGF2; AbD Serotec, Puchheim, Germany) and 25 µg/mL ascorbic acid-2-phosphate (AA-2-P, Sigma-Aldrich, Saint Louis, MO). Medium was renewed twice a week. Passage 3 or 4 hBMSCs were used for *in vitro* experiments.

Synthesis of Fibrinogen-HA and HA-Tyramine conjugates and hydrogel formation

FB/HA conjugates were synthesized by the reaction of a buffered fibrinogen solution with a HA-active ester solution using HA molecular weight of 235kDa (LifeCore Biomedical, LLC, Chaska, MN, USA) at FB/HA w/v ratio of 3.2:1 (6.25mg/mL and 1.96mg/mL respectively, ProCore Ltd. Ness Ziona, Israel) [60]. For hydrogel formation, a thrombin solution (50U/mL, Sigma-Aldrich, Missouri, USA) containing calcium chloride (1M $CaCl_2$) was mixed to FB/HA conjugate and vortexed, that was polymerized at 37°C for 30 minutes. A similar formulation of FB/HA hydrogel, containing higher molecular weight HA, is approved for clinical use for the treatment of Osteoarthritis and associated pain (www.RegenoGel.com by ProCore Ltd, Israel).

HA-Tyramine was synthesized as previously described [70]. Briefly, sodium hyaluronate (500mg, 1.25mmol of carboxyl groups, 280/290 kDa, Contipro Biotech S.R.O., Dolni Dobrouc, Czech Republic) was dissolved in deionized H_2O (1% w/v) and were used for amidation reactions of carboxylic acid groups of HA with amine groups of Tyr. HA-Tyramine conjugates were prepared in a one-step reaction by adding 23.4 mmol 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, TCI Europe) as coupling agent and subsequently 25 mmol tyramine hydrochloride (Tyr, Sigma Aldrich, Buchs, Switzerland) dropwise to the solution. After precipitation, lyophilization and reconstitution in PBS, UV–vis analyses were performed to confirm

substitution of tyramine on HA (DS_{mol} 14%). In a typical setup for hydrogel formation, freshly prepared solution of 0.5 U/ml HRP in phosphate buffered saline (PBS) was added to an aqueous solution of 3.5% (w/v) of HA-Tyr conjugates, and rotated overnight at 4 °C. To induce hydrogel formation, different H_2O_2 concentrations (150, 300, 600 µM) were added and immediately vortexed to form HA-Tyr hydrogels with different cross-linking densities (HA-Tyr 150, HA-Tyr 300 and HA-Tyr 600, respectively), in order to provide a homogeneous distribution within the pre-hydrogel solution before gelation occurs. The concentrations of H_2O_2 were determined based on cross-linking density achievement and cell survival [59].

For the screening of factors to stimulate migration of MSCs, rat tail collagen I hydrogel (Life Technologies, Carlsbad, California, United States) was used. Briefly, 0.6% w/v Collagen hydrogels were prepared on ice by mixing 10X PBS, dH_2O and 1N sodium hydroxide (NaOH) and incubating at 37°C, 5% CO_2, for 30–40 minutes until a firm gel was formed, according to manufacturer's instructions.

Rheological measurements

For rheological study, all hydrogels (400µL) were prepared in 12 well plates. Oscillatory tests (amplitude and time sweep) were performed at 37°C using an Anton-Paar MCR-302 rheometer equipped with a Peltier controller and 25mm plate-plate geometry. To monitor shear elastic moduli (G') and loss of moduli (G'') of the hydrogels a humid chamber was created by placing water drops around the rheometer platform containing the gels removed from the plates and a chamber cover on top. The storage modulus (G') was measured at a strain of 1%, which was determined to be within the linear viscoelastic region.

Swelling ratio studies and mesh size calculation

200µL hydrogel disks were swollen in PBS for 72h at 37°C. Then the hydrogel were gently blotted dry with Kimwipe and weighed immediately after. The disks were lyophilized overnight to obtain the dry weight. The swelling ratio was calculated by the following equation:

Swelling ratio (*QM*)= Ws/Wd

where Ws is the swollen weight and Wd is the dry weight

The mesh size of HA-Tyr 150, 300 and 600 was calculated based on equilibrium swelling theory, using Flory-Rehner model, as reported by Leach [107-109]. The average molecular weight between crosslinks, M, was calculated using a simplification of the Flory-Rehner equation:

$$Q_v^{5/3} \cong \frac{\overline{\nu M_c}}{V_1}\left(\frac{1}{2} - \chi\right)$$

where *Qv* is the volumetric swelling ratio, *v* is the specific volume of the dry polymer, Mc is the average molecular weight between crosslinks, *V*1 is the molar volume of the solvent (18 mol/cm^3 for water), and χ is the Flory polymer solvent interaction parameter.

Qv was determined from the degree of mass swelling ratio, *QM*:

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_m - 1)$$

where ρ_p is the density of the dry polymer (1.229 g/cm^3) and ρ_s is the density of the solvent (1 g/cm^3 for water). *QM* values were determined experimentally and used to calculate *Qv*.

The value of χ for HA was estimated to be 0.473, based on several assumptions. First, it was assumed that χ for HA is comparable to that for dextran, a well-studied polysaccharide, because HA and dextran have similar chemical structures. Furthermore, χ estimates for HA that were based on an analysis similar to those published by Gekko [110] gave values within 2% of the value of χ for dextran. Finally, differences between soluble, unmodified polysaccharides and crosslinked polymers were assumed to be negligible.

The swollen hydrogel mesh size, ξ, was determined with the following equation [109, 111]:

$$\xi = 0.1748\sqrt{M_c}Q_v^{1/3}$$

Due to approximations made in the Flory-Rehner calculations, the values calculated (e.g. Mc, ξ) were considered approximations. However, these values were useful for making magnitude order comparisons of the HA-Tyr chemistries in biologically relevant features, such as mesh size.

In vitro 3D spheroid-based migration assay

3D spheroid migration assay was used to evaluate cell migration as a function of different chemoattractants and varying concentrations of hydrogel. Micro-molds (Micro Tissues 3D Petri Dish, Sigma Aldrich, Missouri, USA) were used to cast 3D agarose Petri Dish, in order to form uniform size spheroids (Fig. 1A). Each micro-mold forms 256 circular micro-wells (diam. 400 μm x 800 μm) in a 16 x 16 array. After gelation, the agarose micro molds were placed in a 12 well-plate and equilibrated with α-MEM supplemented with 10%FBS, 25ug/ml AA-2P at 37 °C, 5% CO_2 for 1h. In parallel, hBMSCs (1x10^6 cell/ml) were fluorescently labelled according to the manufacturer's instructions (Vybrant CFDA-SE Cell tracer Kit, Thermo Fisher, Carlsbad, California, United States). Suspensions of CFDA-SE labelled hBMSCs (1.28x10^5 cells) were seeded into each agarose micro-mold and incubated at 37 °C, 5% CO_2 for 24h in culture media, in order to form spheroids

containing 500 cells per micro-well. The next day, spheroids formation was assessed using a standard inverted microscope to exclude the 3D Petri dishes containing uneven size spheroids or the presence of individual cells. To collect spheroids, the micro molds were inverted in a new 12 well-plate containing α-MEM supplemented with 1% insulin, transferrin and selenium (ITS+, Sigma Aldrich, Missouri, United States), 25ug/mL AA-2P (called from now on serum free medium, SF) and centrifuged for 5 minutes at 120g. The SF medium containing the harvested spheroids was transferred in falcon tube and centrifuged for 30sec at 300g to remove the supernatant. Then spheroids seeding was done after partial gelation on collagen gel (as it gels slowly), in order to avoid spheroids settling to the bottom of the gel and consequently fusion. Chambers slides (Nunc cell culture imaging 8 wells; Thermo Fisher, Carlsbad, California, United States) were used to polymerize collagen gel, 125µl were added to each well (9.4mm and 10.7mm in size with a thickness of 1.2mm).

In FB/HA and HA-Tyr hydrogels (HA-Tyr 150, 300 and 600), the spheroids were uniformly resuspended in the hydrogel prior to gelation. As these hydrogels polymerize rapidly, there was no problem of spheroid settling or fusion. 1mL containing 240 spheroids was transferred into each well of 12 well-plate (diameter of 22.8mm and height of 2.4mm) and incubated for gelation at 37^{o}C respectively for 30 min.

Collagen 1 hydrogels containing about 30 spheroids were cultured for 48h in SF medium in the presence or absence of 50 or 100ng/mL of chemokine (C-C motif) ligand 5 (CCL5/RANTES), stromal derived factor 1 (SDF-1) or platelet derived growth factor BB (PDGF-BB, Peprotech, NJ, USA); whereas HA-based hydrogels were cultured with or without addition of 50ng/mL PDGF-BB for 24h, 48h and 72h [112]. For FB/HA hydrogels, Aprotinin (500kIU/ml, Sigma Aldrich, Missouri, USA) was supplemented into the media to prevent early degradation during culture. To monitor cell migration, a confocal microscope was used (Leica SP5, 10X magnification, FITC channel). Through the z-stack option of the confocal microscope all the spheroids were imaged from the top to the bottom in order to consider every single cell path, enabling individual cells tracking (3D reconstruction, Suppl. Fig.1A). Cell migration area from the core was quantified by averaging automated counts from 5 random spheroids running a macro developed in house using Fiji image processing software. The macro developed in house measures the cell migratory area in 2D, by running the Z Project function the software projects at maximum intensity all the stacks acquired covering the whole 3D area (Suppl. Fig 1B). From these pictures the macro excludes the core and sprouting cells (red) and count the area of cell migrating from the core (green), making concentric circles of 10µm radius (yellow). The algorithm generated concentric circles, each of increasing 10µm radius and tracked the cells present in each of these circles. Then the area of migrating cells and distance of cells in each of these circles with respect to the core was calculated and the area of all migratory cells was summed up to get the total migratory area.

HA-based hydrogels invasion assay

To better mimic cell infiltration from the periphery into the gel, all HA-based hydrogels were formed in the presence or absence of 100 ng/mL PDGF-BB, this concentration was chosen to on our initial dose-response experiment and on a previous study [112]. 1mL of hydrogel was polymerized into each well of 12 well-plate (diameter of 22.8mm and height of 2.4mm), then the hydrogel was cut in an equal quarter of a circle. Each quarter of 250µL of HA-based hydrogel with a thickness of 2.4mm was used for the invasion assay. Then the gels (250µL) were maintained in suspension in 50 mL falcon tubes for 3h at 37°C in 500µL of SF medium containing CFDA-SE labelled hBMSCs (5 x 10^5 cells) under gentle shaking to avoid cell settlement and to allow cell adhesion to the gels (n=3/group). Afterwards 1.5mL of fresh SF medium was added in each gel per tube and gels were cultured for 7 days at 37°C (Suppl. Fig. 2A). Medium was changed every second day. For cell ingrowth detection, all gels were imaged at confocal microscope. For HA-Tyr gels multiple z-stacks of 5µm intervals were acquired (10X magnification), whereas for FB/HA hydrogels due to the opacity, tile scans were performed in the center after cutting the hydrogel in the middle; cell infiltration was visualized in the FITC channel.

PDGF-BB release from hydrogels

The release of PDGF-BB from HA-based hydrogels *in vitro* was assessed as previously described [113]. The selected dose of PDGF-BB used in this assay was similar to *in vivo* experiment (see paragraph 2.10). Briefly, 300µL of hydrogels (diameter of 10.7mm and thickness of 3.4mm) loaded with 2ng/µL PDGF-BB, were formed in 48 well-plates at 37°C. Then 600µL of buffer (PBS, 0.5%BSA) was added to the plate and incubated at 37°C for 7 days. At pre-determined time points – 0, 2h, 8h, 24h, 72h, 120h, 168h – half of the medium (300µL) was collected and replaced by the same volume of fresh medium. Cumulative release of PDGF-BB was measured by quantifying the chemokine in the medium using an ELISA kit (human PDGF-BB, DuoSet ELISA, R&D System, Minnesota, USA).

hBMSCs encapsulation and chondrogenic differentiation in HA-based hydrogels

Briefly, hBMSC-FB/HA and hBMSC-HA-Tyr suspensions were mixed with thrombin solution and with 0.5U/mL HRP and varying concentrations of H_2O_2 (150, 300, 600 µM) respectively, to form cell-hydrogels constructs with a cell density of $3x10^6$ cells/mL in 12 well-plates. After gelation for 30 min, the constructs were cultured in complete chondrogenic medium (CCM) for four weeks (day 0 was used as control); medium was changed every second day. The CCM consisted in Dulbecco's modified Eagle's medium with Glutamax (DMEM-HG; Gibco, Carlsbad, California, United States) supplemented

with 1% ITS, 50 µg/mL fungizone, 1.5µg/mL gentamicyn, 1mM sodium pyruvate (Gibco, Carlsbad, California, United States), 40µg/mL proline (Sigma Aldrich, Missouri, USA), 100nM Dexamethasone (Sigma Aldrich, Missouri, USA), 10ng/mL recombinant human transforming growth factor beta 1 (TGF-β1; R&D System, Minnesota, USA). The hBMSC used in this assay were isolated from patients undergoing total hip replacement and from the iliac crest chip of 1 patient. Samples were collected for RNA isolation or histological analysis on day 28.

RNA isolation and qRT-PCR

After 28 days of culture, hBMSC/HA-Fibrin and hBMSC/HA-Tyr constructs were manually homogenized using a pellet pestle, further digested with hyaluronidase, and total RNA was extracted using the miRNeasy micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration and quality were measured using NanoDrop ND100 UV-VIS spectrophotometer (Isogen Life Science B.V, de Meern, the Netherlands). cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, Carlsbad, California, United States) according to the manufacturer's instructions. qRT-PCR was performed in 20µL reactions on ABI Prism 7000 system (Applied Biosystem, Foster City, CA, USA) using either Taqman Universal PCR mastermix (Applied Biosystem, Foster City, CA, USA) or SyberGreen (Eurogenetc, Seraing, Belgium). The expression of collagen type 2 (*COL2*) and aggrecan (*ACAN*) was determined. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as reference gene after comparison with other housekeeping genes [114]. Data were calculated as relative mRNA values.

In-vivo subcutaneous osteochondral defect model

To evaluate the effect of the hydrogels on cartilage repair via ingrowth of endogenous cells, an *in vivo* subcutaneous mouse model was used. All animal experiments were approved by the local animal committee (EMC3284, protocol number 116-14-02). Osteochondral defects were created in osteochondral biopsies (8mm diameter, 5mm height) harvested from metacarpal-phalangeal joints of 3 to 8 months old calves as described previously [115]. Four mm diameter dermal biopsy punches (Stiefel Laboratories, Munich, Germany) and scalpels were used to create osteochondral defects. The defects in the osteochondral plugs were either left unfilled (empty) or filled with 50 µl FB/HA or HA-Tyr 150 hydrogels with or without 1µg/mL of PDGF-BB (n=5/group). In comparison to *in vitro* migration and invasion assay, a higher concentration of PDGF-BB was employed since the growth factor *in vivo* is expected to be released over several days and more easily degraded by proteases [68]. Osteochondral explants were covered with Neuro-Patch membrane (Braun, Melsungen, Germany) to prevent host cell ingrowth and were implanted subcutaneously in female NMRI nu/nu mice (Charles

River, Wilmington, MA, USA) under isoflurane anaesthesia. Four incisions were made on the dorsum of each animal, and subcutaneous' pockets were created using a blunt blade; one construct was placed in every pocket and the incisions were closed with wound clips.

After 4 weeks, mice were euthanized by cervical dislocation, the constructs carefully removed and fixed in 4% formalin for 5 days. Then samples were decalcified in 10% EDTA for two weeks, subsequently embedded in paraffin and subjected to histology (Thionin staining) or immunohistochemistry (Collagen type 2 deposition).

Histology and Immunohistochemistry

Retrieved samples were embedded in paraffin and sectioned (6 μm sections). Slides were deparaffinised and stained with Thionin to visualize glycosaminoglycans in the extracellular matrix. Briefly, slides were first stained with Thionin solution (0.04% Thionin in 0.01M aqueous sodium acetate, Sigma-Aldrich, Missouri, United States) for 8 min and differentiated in 70% ethanol for 8 sec. The cross-sectional area of the osteochondral defect, the number of the infiltrated cells and the area of newly formed Thionin positive tissue were determined using Fiji software (National Institutes of Health, Bethesda, MA, USA). Cell ingrowth into the hydrogels was assessed by counting the number of cell nuclei infiltrating the defect area of the cartilage layer (CL) and the defect area of the subchondral bone (SB) in Thionin stained cross-sections (n=5/group, n=3sections/sample). To convert RGB images in 8 bit we used a trainable weka segmentation plugin, in order to train the software to define the classes of area to exclude (different shadows of background, Thionin staining) and select the area of interest (in this case the cells nuclei). After extracting the results, we select the best threshold to extract the desired objects (cell nuclei). Cartilage formation was quantified as percentage of positive area by dividing the Thionin signal intensity in the defect (glycosaminoglycan deposition) by the defect area of the cartilage layer (CL) and the defect area of the subchondral bone (SB) as measured in full Thionin-stained cross-sections (n=5/group, n=3sections/sample). Fiji image processing software was used to identify areas using a protocol previously described [116].

For immunohistochemical analysis, deparaffinized sections from hydrogel samples were probed with mouse anti-human collagen type 2 antibody (II-II6B3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Antigen retrieval was performed by incubation in 0.1% pronase (Sigma-Aldrich, Missouri, USA) in PBS for 30 min at 37°C. Then slides were incubated with 1% hyaluronidase (Sigma-Aldrich) in PBS for 30 min at 37°C and subsequently with 10% goat serum (Sigma-Aldrich) to block non-specific binding. The primary antibody against collagen type II (1:100 dilution) or mouse IgG1 negative control (Serotech Ltd, Oxford, UK) in PBS containing 0.1% BSA was incubated overnight at 4°C coupled with biotinylated F(ab)2-labeled goat-anti mouse secondary

antibody (#115-066-062; Jackson ImmunoResearch Europe) to prevent cross-reaction with mouse antigens. Excessive primary antibody was captured by addition of 0.1% normal mouse serum prior to the overnight incubation at 4°C with the sections. The reaction was catalyzed by an alkaline-phosphatase-label conjugate (Label, HK-321-UK, Biogenex, CA, USA) diluted 1:100 in PBS/BSA and visualized by subsequent incubation of Neu Fuchsin substrate (Chroma, Kongen, Germany). Slides were counterstained with Haematoxylin.

STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation (SD). For the 3D spheroids-based migration assay in collagen gel, experiments were performed using 4 different hBMSC donors and triplicate per donor, a linear mixed model was used for migratory cell area data. Multiple comparisons were analysed with Sidak post hoc test. Conditions and donors were considered as fixed and random parameters, respectively. Normal distribution of the data or the residuals of the data were confirmed by both Kolmogorov-Smirnov and Shapiro-Wilk tests. In the 3D migration assay in HA-hydrogels, experiments were performed using 3 different donors and triplicate per donor, data were not normally distributed and Kruskal-Wallis test was performed. For the quantification of cartilage repair in the osteochondral samples, experiments were performed using 5 explants per group and 3 sections per sample, statistically significant differences between untreated and hydrogel treated groups were determined by one-way ANOVA, Tukey test for multiple comparison. All tests were performed using SPSS software. Differences were considered statistically significant for $p< 0.05$.

RESULTS

Effect of different chemotactic factors on hBMSC spheroid migration in hydrogel

Although monolayer cell migration has commonly been used for migration studies, recent research shifted toward 3D culture as a more relevant biochemical and biomechanical microenvironment [117]. Here, we used a spheroid-based migration assay to examine the effect of chemotactic factors on the migration of hBMSCs. CDFA-SE fluorescently labelled hBMSC spheroids with an average diameter of 125 µm were generated (Fig. 1 A), placed in a collagen hydrogel and cultured in the absence or in the presence of 50 or 100ng/ml PDGF-BB, RANTES or SDF-1. After 48h, cell migration was imaged (Fig. 1B). Exposure of SDF-1 (50 and 100ng/mL), PDGF-BB (50ng/mL) or RANTES (50ng/mL)

increased hBMSC migration compared to control (***p<0.001, **p<0.01, *p<0.05), except for 100ng/mL RANTES, although variability among donors was observed (Fig. 1C). These results demonstrate that 50ng/mL of PDGF-BB was the most favourable of the tested factors based on higher tendency to increase among all tested donors.

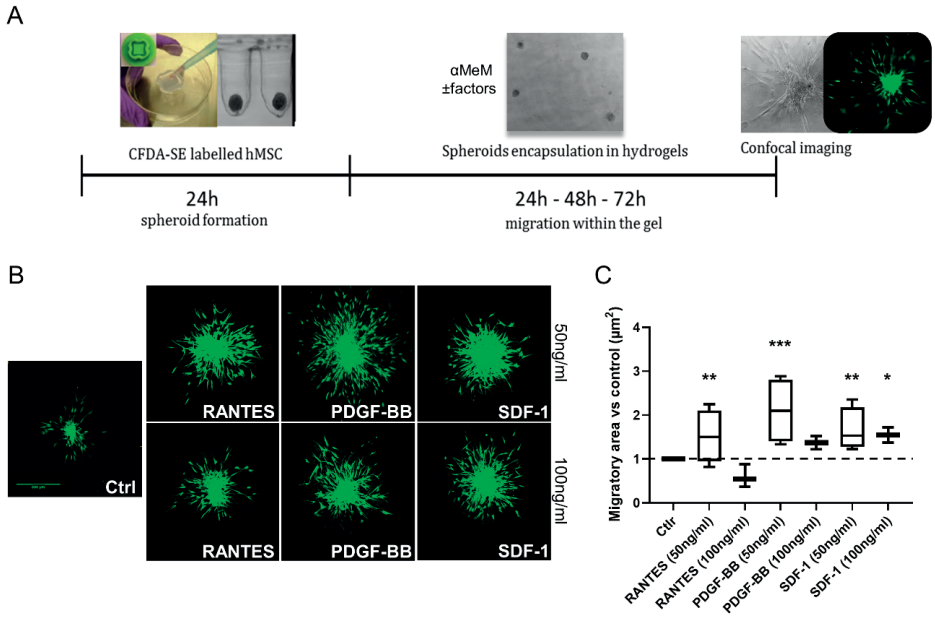


Figure 1. Dose response-study of factors on hBMSCs migration in collagen hydrogel. A) Scheme of 3D spheroids formation and migration assay in hydrogels. **B)** Representative images of BMSCs migrating from the spheroids core at 48h of culture in absence or in presence of 50 or 100ng/mL of RANTES, PDGF-BB or SDF-1; 10X magnification, scale bar indicates 500μm. **C)** Migratory area (mm^2) of hBMSCs encapsulated in collagen hydrogel in absence or in presence of 50 or 100ng/mL of RANTES, PDGF-BB and SDF-1. Results from 4 hBMSC donors assessed in triplicate (donor 2) and quadruplicate (donors 1, 3 and 4) are shown; *p<0.05, **p<0.01, ***p<0.001

PDGF-BB promotes hBMSC migration in FB/HA and HA-Tyr hydrogels with different cross-linking densities *in vitro*

In terms of physical impediment to 3D cell recruitment, the migration of CDFA-SE labelled hBMSCs was assessed in HA-Tyr hydrogels with different crosslinking densities (HA-Tyr 150, 300 and 600 μM H_2O_2) and FB/HA hydrogel, with or without PDGF-BB exposure, using a 3D spheroid assay. Confocal imaging revealed that the area of cell migration from the spheroids in all HA hydrogels progressively increased over three days culture in the presence of 50ng/mL PDGF-BB, except for the stiffer HA-Tyr hydrogels (HA-Tyr 600, Fig. 2A, B) that showed no migration at all. FB/HA hydrogels supported the widest cell migration area in presence of PDGF-BB (4-fold increase compared to FB/HA

only hydrogels after 72h; Fig. 2A, B). In FB/HA gels the cells exhibited spindle-shaped morphology (Fig. 2B), which might have facilitated faster migration, whereas in HA-Tyr hydrogels (HA-Tyr 150, 300) cells showed populations of both spindle and rounded shaped morphology that might have reduced the migration ability.

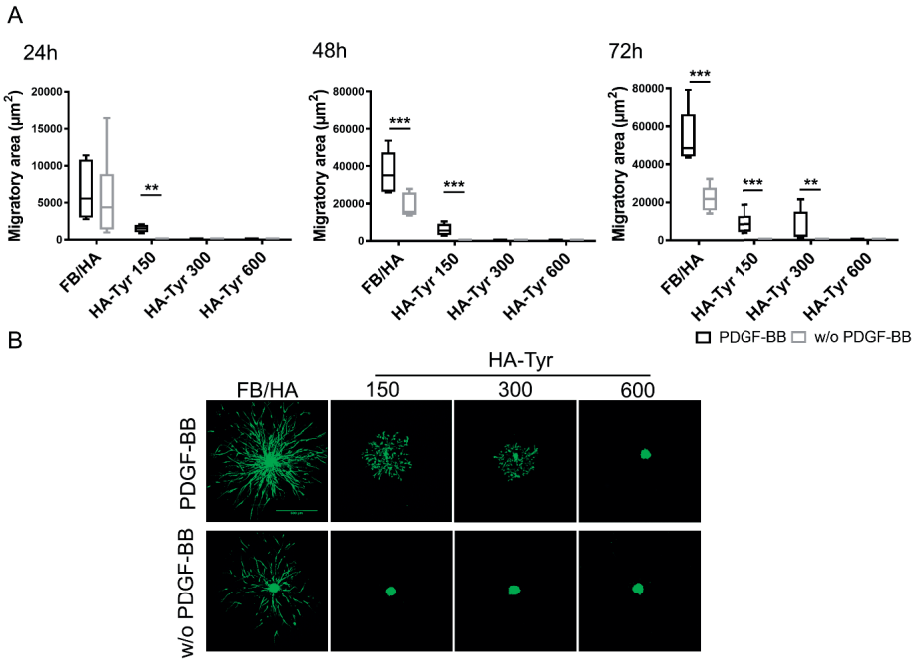


Figure 2. PDGF-BB induces progressive increase of cell migration in FB-HA and HA-Tyr with different crosslinking densities. A) Migratory area (mm^2) of hBMSCs encapsulated in FB/HA and HA-Tyr 150, 300 and 600 at 24h, 48h and 72h of culture in absence or in presence of 50ng/mL of PDGF-BB. Results from 3 hBMSC donors assessed in quintuplicate are shown (**p<0.01 ***p<0.001. **B)** Representative images of BMSCs migrating from the spheroids core at 72h of culture in absence or in presence of 50 and 100ng/mL of PDGF-BB; 10X magnification, scale bar indicates 500µm.

The migration in HA-Tyr hydrogels with different cross-linking density was found to be inversely correlated with the storage modulus of the hydrogel (G'; Suppl. Fig. 2).

Increased migration in the presence of PDGF-BB was observed at 24h and 48h for HA-Tyr hydrogels with lower crosslinking (HA-Tyr 150), which were softer and fostered faster migration than stiffer gels (HA-Tyr 300, 600; **p<0.01 and ***p<0.001 respectively; Fig. 2A). Based on the mesh size calculation described by *Leach et al.* [109], and assuming that HA-Tyr had the same density as HA, the mesh sizes were calculated to be 184.99±8.03, 160.60±5.04 and 130.85±7.04 nm for the HA-Tyr 150, 300 and 600, respectively. At increased cross-linking density, the mesh size decreased according to a decreased swelling ratio (Suppl. fig.3A. B), resulting in a reduced migration kinetic.

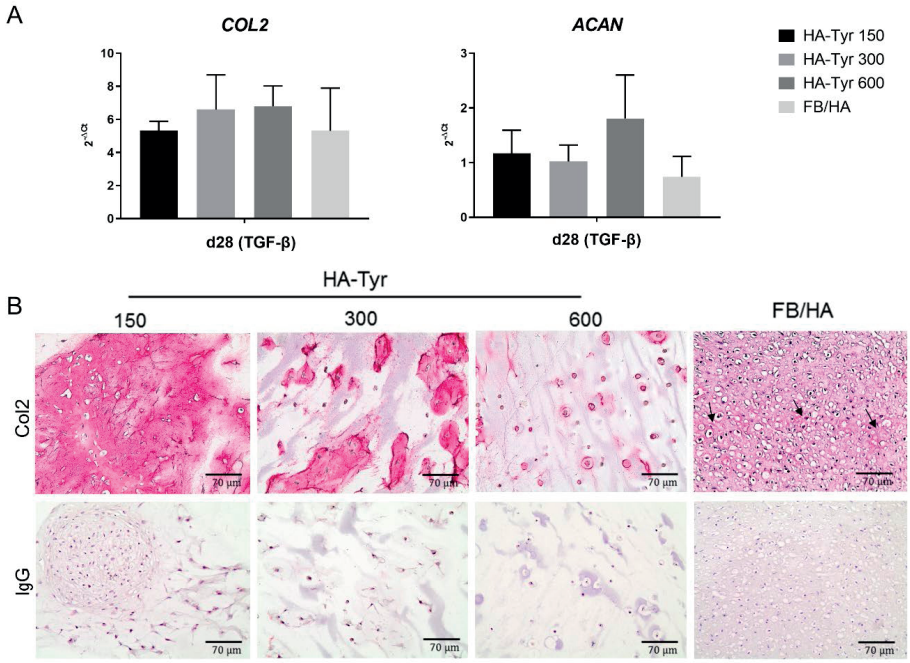


Figure 3. The HA-hydrogels support chondrogenesis *in vitro*. A) hBMSCs encapsulated in HA-Tyr 150, 300, 600 and FB/HA hydrogels were cultured for 28 days in chondrogenic medium in presence of TGF-β1. Relative mRNA levels of *COL2* and *ACAN* were assessed by qRT-PCR and normalized to GAPDH ($2^{-\Delta Ct}$). Data are from 1 hBMSCs donor and presented in biological triplicate as means+/-SD. **B)** Immunohistochemical staining of collagen type II assessed in all hydrogel groups after 28 days of culture in chondrogenic medium in presence of TGF- β1. In FB/HA and HA-Tyr hydrogels the collagen type II deposition is presented by the pink staining (black arrows in FB/HA hydrogel). IgG isotype controls demonstrate collagen type II specificity. 10X magnification, scale bar indicates 70µm.

To better mimic the process of hydrogel invasion by endogenous cells, CDFA-SE labelled hBMSCs in suspension were incubated with free-floating HA-based hydrogels, polymerized with or without 100ng/mL of PDGF-BB, then cultured for 7 days (Suppl. Fig. 4A). Imaging of the hydrogels at day 7 suggested higher cell infiltration in FB/HA hydrogel with PDGF-BB compared to FB/HA only. In HA-Tyr hydrogels, cell migration looks to be higher in presence of the chemokine rather than without and dependent of crosslinking densities (Suppl. Fig. 4B).

HA-based hydrogels support chondrogenesis *in vitro*

With the aim to investigate whether FB/HA and HA-Tyr-hydrogels are suitable for cartilage engineering purposes, their ability to support chondrogenic capacity of hBMSCs was evaluated. hBMSC-loaded hydrogels were cultured for 28 days in CCM and gene expression at day 0 was used as control; hBMSC differentiation was further assessed by IHC. At day

0 gene *COL2A1* expression was undetectable and *ACAN* expression was very low with Ct values between 34 and 37. The results indicate that hBMSC-FB/HA and hBMSC/HA-Tyr constructs similarly allowed chondrogenic differentiation, as demonstrated by clear cartilage marker expression at day 28 (Fig. 3A). Immunohistochemical staining for type II collagen confirmed that FB/HA constructs exhibited areas of newly synthesized matrix and cells with a chondrocyte-like morphology (i.e. rounded and residing within lacunae Fig. 3B). Interestingly, type II collagen deposition in HA-Tyr hydrogels was limited to the pericellular space except for HA-Tyr 150 where the staining was diffuse in the matrix (Fig. 3B), indicating an inverse relationship between the cross-linking density and the extent of neo-cartilage tissue deposition. Since improved cell migration and cartilage matrix formation were observed in HA-Tyr hydrogels with the lowest cross-linking density, HA-Tyr 150 and FB/HA hydrogels were selected for further *in vivo* experiments.

FB/HA hydrogels improve endogenous cartilage tissue repair in an *in vivo* subcutaneous model

To evaluate the ability of the selected hydrogels to support endogenous cartilage repair *in vivo*, osteochondral explants with simulated defects were filled with FB/HA and HA-Tyr, both loaded or not with PDGF-BB, and implanted subcutaneously in nude mice (Fig. 4A). After 4 weeks, osteochondral explants were collected and analyzed by histology. Data revealed differences between the groups in number of cells colonizing the defect through full-depth cartilage and subchondral bone (Fig. 4B, C, Suppl Fig. 5). While cells were evenly distributed throughout the whole area of FB/HA gels (both cartilage and subchondral zone), cell ingrowth was limited to the periphery in HA-Tyr gels in the vast majority of the gels (Fig 4B). Cell infiltration was significantly higher in the cartilage layer and subchondral bone of FB/HA gel group without PDGF-BB compared to HA-Tyr group without PDGF-BB (*$p<0.05$ and ***$p<0.001$: Fig. 4C). Scarce cell infiltration was noticed in only 2 out of 5 samples in the empty defect group (data not shown). When FB/HA and HA/Tyr hydrogels were loaded with PDGF-BB, no significant differences in endogenous cell ingrowth were observed in comparison to untreated hydrogels (Fig. 4C), despite approximately 35% of PDGF-BB was released in FB/HA after one week *in vitro* (Suppl. Fig. 6). This suggest that the factor release did not exert any appreciable effect on migration in the hydrogels (Fig. 4B, C).

In addition to higher cell infiltration in FB/HA gels, cell mediated matrix production was significantly enhanced in the cartilage areas of untreated FB/HA gels in comparison to HA-Tyr gels (*$p<0.05$, Fig 4B, D). The subchondral bone areas showed similar GAG production and no statistical differences were observed. Interestingly, the addition of PDGF-BB to FB/HA gels inhibited cartilage formation (*$p<0.05$, Fig. 4B, D), while in HA-Tyr gels no significant differences were observed when PDGF-BB was added (Fig.

4B, D). Finally, deposition of type II collagen *in vivo* confirmed a similar pattern to GAG deposition (Fig. 4E).

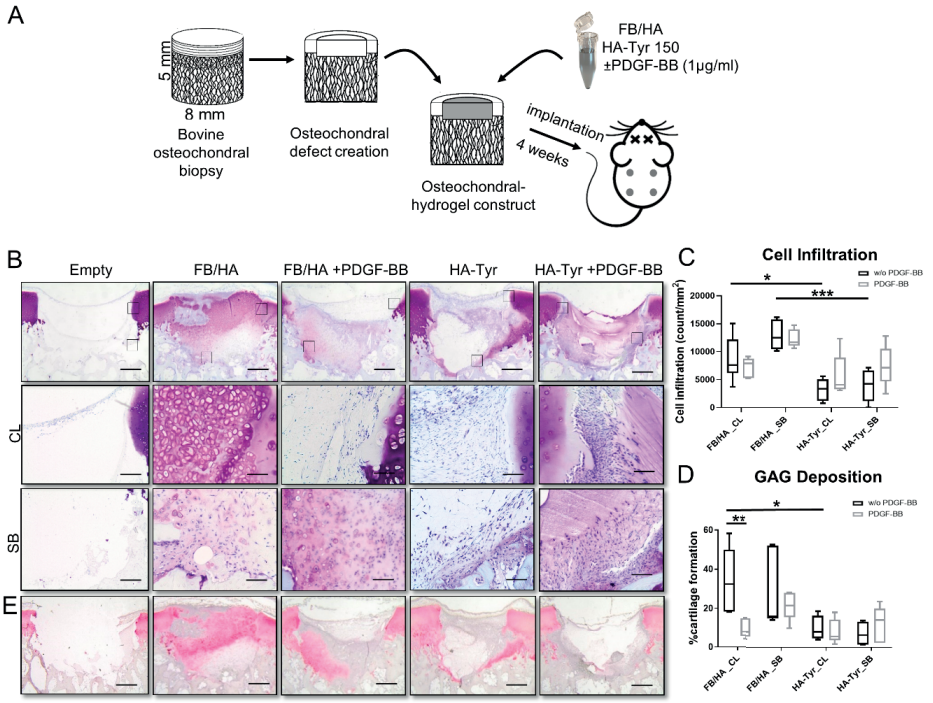


Figure 4. Improved tissue repair of osteochondral defects filled with HA-based hydrogels in an *in vivo* subcutaneous tissue model. A) Scheme of an osteochondral repair explant filled with FB/HA and HA-Tyr 150 hydrogel in presence or absence of 1µg/mL of PDGF-BB, implanted in the subcutaneously in athymic mice. **B)** Representative images of the repair constructs stained with Thionin (pink=GAG) showing cells and matrix deposition within the osteochondral defects after 4 weeks of implantation. 10X magnification scale bars indicate 1mm and 70µm, respectively. **C)** Cell infiltration (count per mm^2) in the CL and SB layers of the osteochondral defects *p<0.05, **p<0.01 and ***p<0.001. **D)** Percentage of cartilage formation in hydrogels indicated by GAG deposition in CL and SB. *p<0.05 and **p<0.01. **E)** Representative images of repair constructs stained with Collagen type II by immunohistochemistry after 4 weeks of implantation. 10X magnification; scale bars indicate 1mm.

DISCUSSION

In this study we compared different types of HA-containing hydrogels for their capacity to support cell migration and chondrogenesis of hBMSC *in vitro,* and to promote recruitment of endogenous cells to the wound site, followed by cartilage repair in an osteochondral defect model *in vivo*. We showed that the FB/HA conjugated formulation

enhanced a spontaneous cellular healing response and was more supportive for cartilage repair compared to the HA-Tyr hydrogel. In addition, the provision of PDGF-BB, chosen as the most favorable chemotactic agent, did not increase cell infiltration into the tested hydrogels but impaired chondrogenesis *in vivo*.

Our approach is based on the use of an advanced platform employed as a pre-clinical tool to screen new biomaterials and biomolecules for their potential to support endogenous cartilage repair. The advantage of this strategy is the application of more relevant experimental models due to the use of an *in vitro* 3D spheroids-based migration assay and an osteochondral defect model, which bring our approach a step closer to physiologically relevant systems. This could be widely applied to achieve stronger experimental evidence of the not well-characterized dynamic process of cell homing and to uncover the delicate step of early cell migration into biomaterials.

The ideal hydrogel should allow cell adhesion, migration and differentiation to favor the synthesis of extracellular matrix components necessary to mimic the native properties of cartilage. However, tuning the gels to match not only cartilage composition and architecture but also mechanical properties to sustain the load, may prevent cell ingrowth that is necessary for the first steps of endogenous tissue repair. Consistent with other *in vitro* studies [59], we found that changes in mechanical properties influenced cell spreading, migration and differentiation. The modulation of HA-Tyr cross-linking degrees (150, 300 and 600 μM H_2O_2), while keeping HA-Tyr and HRP concentrations constant, was the major determinant for both cell migration and matrix synthesis during hBMSC chondrogenesis.

The resulting hydrogels ranged in storage modulus from 80 to 3,000 Pa, which mimics the mechanical properties of certain native cartilaginous tissues like the nucleus pulposus of the intervertebral disc (3-8kPa; [118]), but is lower than the value reported for bovine adult cartilage (range 300-800kPa, [119]). However, cell migration was inhibited in HA-Tyr 600 hydrogels, indicating cell spreading limitations in stiffer highly crosslinked materials [120]. These stiffer gels, however, also have lower mesh size. In general, migration was strongly dependent on crosslinking, indeed gels with low crosslink density have both lower stiffness and higher mesh size (HA-Tyr 150), and foster faster migration; albeit the migration was always less in HA-Tyr than in FB/HA gels. Despite the similar stiffness (G') of FB/HA and HA-Tyr 150, cell migration was profoundly affected by the different HA concentration as well as by the different network and the presence of components that improve cell adherence.

To ameliorate cell migration on HA-Tyr hydrogels further cues could be implemented, such as the Arg-Gly-Asp (RGD) binding sequences to improve the integrin-mediated cell attachment [121].

Chondrogenesis occurred in all tested hydrogels, as indicated by comparable gene expression levels of *COL2* and *ACAN*. While FB/HA, HA-Tyr 150 and 300 hydrogels

showed collagen type II-rich matrix production, HA-Tyr 600 microenvironments showed only pericellular collagen type II deposition. Previous works [59] demonstrated extensive collagen type II deposition in the newly formed HA-Tyr matrix. Although G' values of our gels were much lower (0.08 and 0.45kPa in HA-Tyr 150 and 300, respectively), this suggests that mechanical stiffness is not the only factor that influences stem cell fate [122]. It is possible that the higher amount of HA-Tyr used to form the hydrogel (3.5%w/v vs 2%w/v) increased its density, which has been shown to negatively affect matrix deposition by hBMSCs [123]. Furthermore, the higher concentration may have increased its viscosity, which in turn may have hindered the diffusion of nutrients and growth factors in all the HA-Tyr constructs, which is known to influence the effectiveness of hBMSC chondrogenesis [124], thereby decreasing GAG deposition. The combination of those factors may have decreased the ability of those hydrogels to allow migration and support matrix accumulation. To further support our observations, a recent study has shown that increased HA crosslinking density resulted in an overall more restricted matrix distribution while detecting no statistically significant differences in collagen type II expression among all the groups [125].

Consistently with other reports [68, 126], we found that PDGF-BB was the most effective chemoattractant of hBMSCs in hydrogels, among the factors tested. PDGF-BB is a well-known mitogen and we cannot thus completely rule out a contribution of cell proliferation in the 3D spheroid in vitro assay. Nevertheless, cells that detached from the core were identified as migrating cells and proliferation did not influence this measurement as it would have been reflected as an increased the size of the core. It should also be considered that both processes are desirable and necessary in a context of endogenous tissue repair *in vivo* to guarantee proper cell colonization of the site of injury.

It is worth noting that the PDGF-BB gradient enhanced short-term spheroid migration *in vitro* (3 days) and improved cell infiltration of hydrogels at 7 days of culture in HA-based hydrogels. Our release study of PDGF-BB over 7 days suggested that a chemotaxis gradient might have been less pronounced within HA-Tyr hydrogels than with FB/HA gels. Earlier studies exploring the influence of mechanical strength of HA-Tyr hydrogels on protein release demonstrated that release profile of the molecules depended on mesh size, with the release rate decreasing with decrease in mesh size [105]. The higher concentration of HA within HA-Tyr hydrogels, however, might also have increased the electrostatic interactions among their hydrophilic groups and the charged amino acid residues of the PDGF-BB [127], impeding a sustained delivery.

Although 3D migration studies can provide valuable information, the majority of conventional *in vitro* hydrogel culture systems do not recapitulate the native tissue properties [128]. Furthermore, a recent study has shown that BMMSC gives a distinct response when placed in a different environment, demonstrating that the

microenvironment plays an important role in the induction of cell differentiation highlighting its importance when evaluating the applicability of biomaterials for cartilage repair [129]. Therefore, a fundamental prerequisite is the testing of 3D cell migration and differentiation in a more relevant osteochondral-like system, in order to closely mimic a joint-like microenvironment. To validate our *in vitro* findings, FB/HA and HA-Tyr 150, with or without PDGF-BB, were placed in osteochondral defects in an explant model and implanted subcutaneously *in vivo*. Cellular invasion was evident by 4 weeks in both hydrogels, though infiltration was most advanced in the FB/HA hydrogels, which allowed a uniform distribution of cells. Interestingly, the bridging tissue in the untreated FB/HA constructs, closing over 85-90% of the osteochondral gap compared to HA-Tyr hydrogels, resulted also in increased cartilage matrix formation (*$p<0.05$ and **$p<0.01$), and subsequently more collagen II. A possible explanation for the extensive differences in cell infiltration between the FB/HA and HA-Tyr hydrogels might be that the high fibrin content (FB/HA 3.2:1) favoured binding of cells to its 3D architecture and accelerated cell migration in the porous clots containing hyaluronan [130]. The HA in the FB/HA hydrogels may have influenced the behavior and function of cells involved in the remodeling of the damaged tissue [131]. Whereas the high content of low molecular weight HA (280kDa) in the HA-Tyr gels may have acted as a barrier to cell adhesion and migration [132], therefore slowing this process. We also noticed that 2 out of 5 osteochondral empty defects implanted as controls were partially colonized by cells. Despite the presence of a membrane patch it is possible that liquid or blood after surgery reached the osteochondral defects. Indeed, part of the infiltrated cells were red blood cells. Furthermore, as the bovine explants were harvested from calves aged 6-8 months, the young and healthy material is likely to provide a favorable environment for repair.

This study showed a reduction in the size of cartilage lesion and enhanced regeneration of the cartilage using FB/HA hydrogels without exposure to growth factor before implantation. Interestingly, addition of PDGF-BB worsened the repair of cartilage. A previous study on osteochondral repair in a rat model, demonstrated no significant presence of cartilage matrix deposition when the defect was filled only with PDGF-BB loaded in heparin-conjugated fibrin gels [68]. Although this study used higher concentrations of PDGF-BB (8.5µg/mL and 17µg/mL) compared to the present study (1µg/mL), these findings are in line with our observations, suggesting that the presence of PDGF-BB, although not influencing cell recruitment, diminished chondrogenic differentiation leading to more fibrous tissue formation. Future studies will be performed to evaluate a dose-dependent effect of PDGF-BB on cartilage repair after *in vivo* transplantation and explore the efficacy of other chemotactic agents [66]. To further improve the quantity or quality of the matrix produced by the recruited cells,

our system can be functionalized, for example by adding pro-chondrogenic factors [133, 134], that can stimulate cartilage formation or inhibit hypertrophy.

Our findings with the osteochondral explant model are partially in line with clinical outcomes of the microfracture procedure [16, 135]: cells are recruited without supplementation of exogenous factors and spontaneously generate a cartilaginous tissue, albeit this is a mixed hyaline/fibrous tissue with non-favorable long term outcome. Although the origin of these reparative cells needs further analysis, we suppose that the migrating cells are either endogenous stem/progenitor cells from the subchondral bone region, either perivascular or bone lining cells, that have differentiated towards the chondrocyte lineage, since the newly generated tissue was GAG and collagen type II positive. It is clear that the construct is revascularized upon implantation, meaning a connection is made with the mouse system [136]. Hence, we cannot exclude the presence of cells from murine origin in our plugs. Further investigations of the origin of these cells would be interesting, although this poses significant challenges in discriminating mouse and bovine cells in decalcified sections.

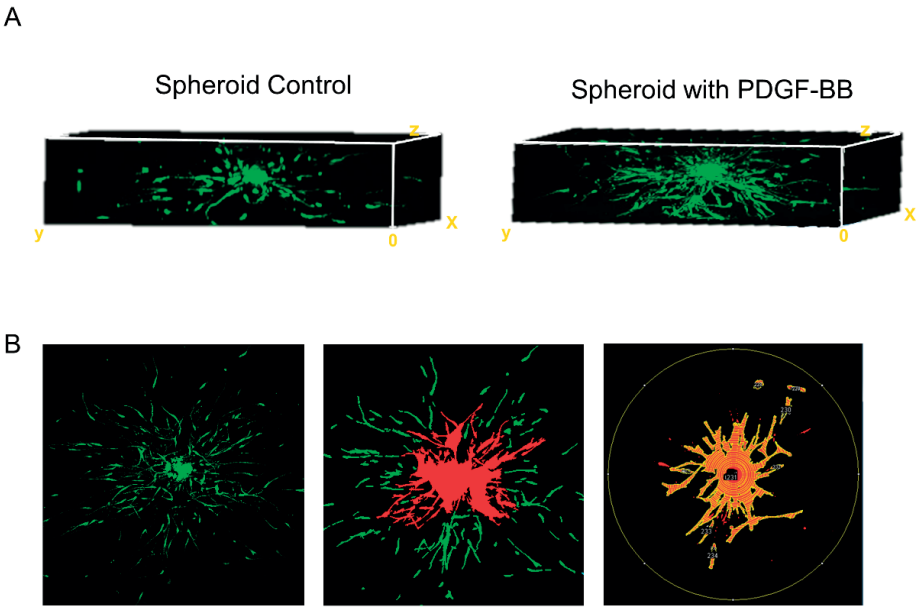
Subcutaneous implantation does not entirely recapitulate the diarthroidal joint in terms of cellular components, immunologic response and mechanical stimuli and allows only short-term evaluation of cell colonization and matrix production *in vivo*. To study the effect of mechanical stimuli to our endogenous cartilage repair system, the use of a bioreactor system to simulate physiological joint kinematics *in vitro* can be useful [137]. Multiaxial loading was shown to induce production and activation of transforming growth factor-beta (TGF-β1), thereby promoting chondrogenesis of BMSCs [138]. Since the process of endogenous cartilage repair and the involved cell populations are still not well characterized, this system can be implemented to carry out additional studies including pre-clinical screening of targeted therapies and biomaterial-based implants. Eventually, the long-term therapeutic effects will need to be validated in large animal models of osteochondral injury.

CONCLUSION

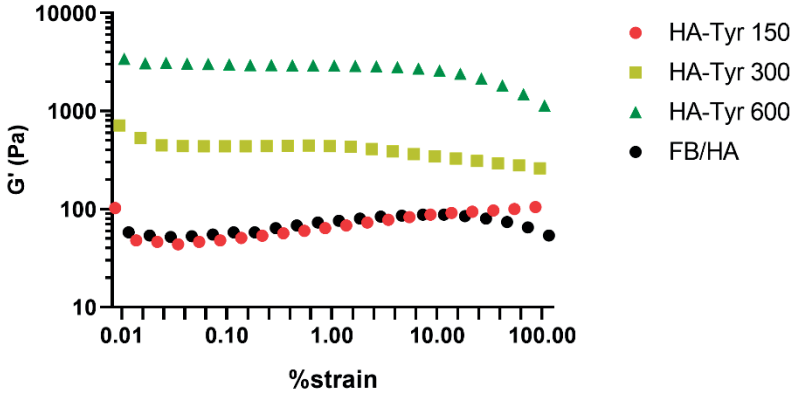
The manuscript emphasizes the application of an advanced preclinical biomaterial testing platform to select the most promising hydrogel to support cell migration and differentiation for cartilage regenerative strategies, posing interesting features in the use of FB/HA conjugated hydrogel, even in the absence of the factor stimulating migration. It is worth noting that both *in vitro* and *vivo* findings indicate that in the FB/HA hydrogel the use of stimulating factors was not necessary to create a local ECM microenvironment amenable for endogenous cell recruitment in both cartilage and bone layers. Particular consideration should be given on creating an environment where

cues may be introduced to stimulate matrix deposition and improve the quality of the newly-formed cartilage, e.g. by silencing anti-chondrogenic factors [139], and promote proper collagen fiber alignment [140]. Combination of these processes will lead to an ideal situation where different but complementary regulators create an optimal microenvironment for cartilage repair.

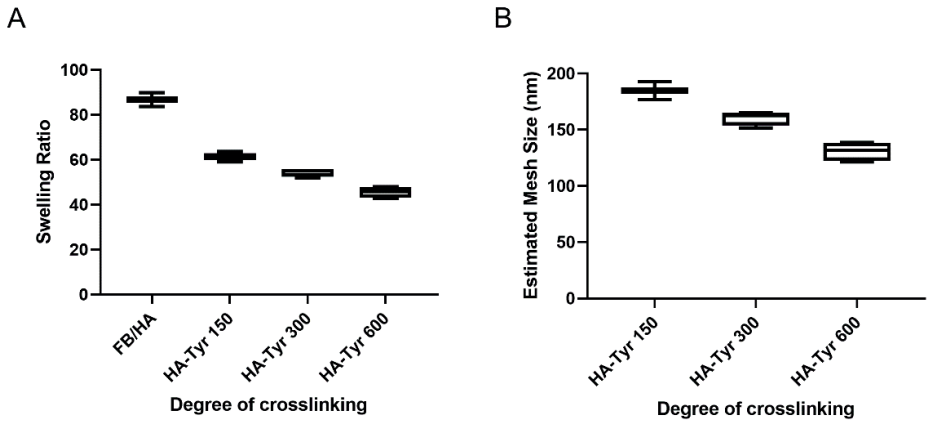
SUPPLEMENTARY DATA



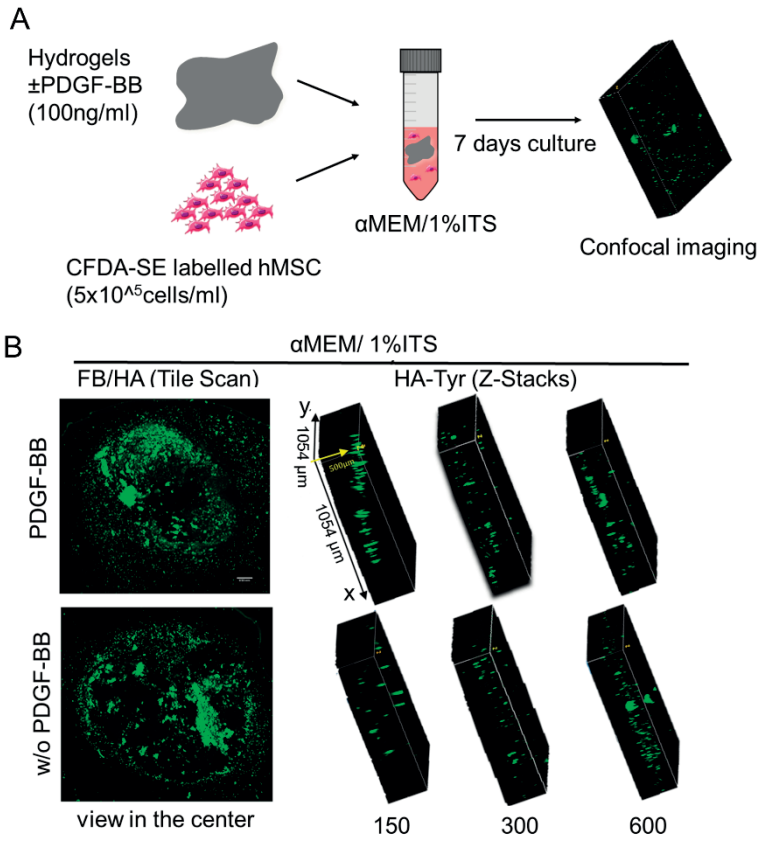
Supplementary Figure 1. Decomposition of a single spheroid after 72h of migration. (A) Representative images of spheroids cultured with and without PDGF-BB, 3D reconstruction using Fiji software. **(B)** Representative image of spheroid to distinguish core and sprouting cell (red) and migrating cells (green). Concentric circles of 10µm radius to calculate the migrating cells and distance of cells with respect to the core to obtain the total migratory area (yellow).



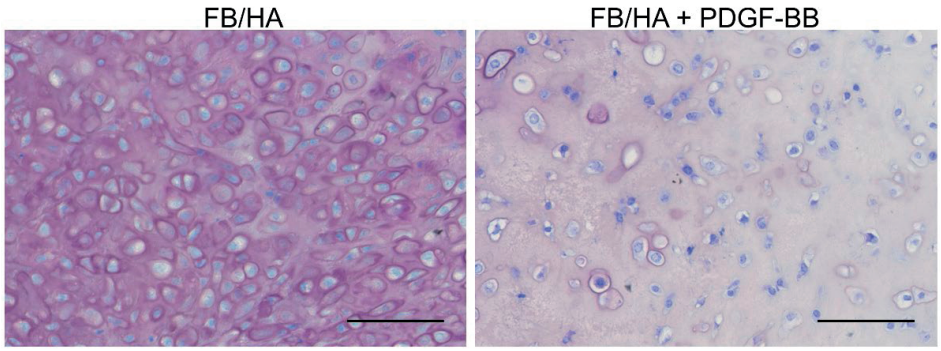
Supplementary Figure 2. Rheological measurement. Typical evolution of the storage modulus G' of HA-Tyr hydrogels formed with 0.5U/ml of HRP and cross-linked at varying concentrations of H_2O_2, and FB/HA hydrogels. The measurement was taken with constant deformation of 1% at 1Hz and 37°C.



Supplementary Figure 3. Effect of crosslinking degree on swelling ratio and mesh size. (A) Crosslinking degree of FB/HA and HA-Tyr hydrogels at varying concentrations of H_20_2 modulate the equilibrium swelling ratio. Hydrogels were swollen for 72h at 37°C. Means+/-SD; n=4/group. **(B)** Estimated mesh size calculation based on equilibrium swelling theory. Means+/-SD; n=4/group.



Supplementary Figure 4. HA-hydrogels allow hBMSCs ingrowth *in vitro.* (A) Scheme of cell invasion assay, hBMSC together with hydrogels (polymerized in presence or absence of PDGF-BB) maintained in culture. **(B)** Representative images of cells invasion into HA-Tyr hydrogels formed with 0.5U/ml of HRP and cross-linked at varying concentrations of H2O2, and FB/HA hydrogels after 7 days of culture. Scale bars indicate x=1054 µm, y=1054 µm, z=500µm



Supplementary Figure 5. FB/HA hydrogels remodeling in osteochondral defect explants after *in vivo* subcutaneous implantation. Representative images of FB/HA in absence or presence of PDGF-BB stained with Thionin (pink=GAG). Cells, nuclei and matrix deposition are distinguishable within the osteochondral defect after 4 weeks implantation. 20X magnification; scale bars indicate 100 μm.

3 Enhanced chondrogenic phenotype of primary bovine articular chondrocytes in Fibrin-Hyaluronan hydrogel by multi-axial mechanical loading and FGF18

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ABSTRACT

Current treatments for cartilage lesions are often associated with fibrocartilage formation and donor site morbidity. Mechanical and biochemical stimuli play an important role in hyaline cartilage formation. Biocompatible scaffolds capable of transducing mechanical loads and delivering bioactive instructive factors may better support cartilage regeneration.

In this study we aimed to test the interplay between mechanical and FGF-18 mediated biochemical signals on the proliferation and differentiation of primary bovine articular chondrocytes embedded in a chondro-conductive Fibrin-Hyaluronan (FB/HA) based hydrogel.

Chondrocytes seeded in a Fibrin-HA hydrogel, with or without a chondro-inductive, FGFR3 selective FGF18 variant (FGF-18v) were loaded into a joint-mimicking bioreactor applying controlled, multi-axial movements, simulating the natural movements of articular joints. Samples were evaluated for DNA content, sulphated glycosaminoglycan (sGAG) accumulation, key chondrogenic gene expression markers and histology.

Under moderate loading samples produced particularly significant amounts of sGAG/DNA compared to unloaded controls. Interestingly there was no significant effect of FGF-18v on cartilage gene expression at rest. Following moderate multi-axial loading, FGF-18v upregulated the expression of Aggrecan (ACAN), Cartilage Oligomeric Matrix Protein (COMP), type II collagen (COL2) and Lubricin (PRG4). Moreover, the combination of load and FGF-18v, significantly downregulated Matrix Metalloproteinase-9 (MMP-9) and Matrix Metaloproteinase-13 (MMP-13), two of the most important factors contributing to joint destruction in OA.

Biomimetic mechanical signals and FGF-18 may work in concert to support hyaline cartilage regeneration and repair.

Keywords: chondrogenic differentiation, fibrin-hyaluronan hydrogel, fibroblast growth factor-18, multi-axial loading.

Statement of significance

Articular cartilage has very limited repair potential and focal cartilage lesions constitute a challenge for current standard clinical procedures. The aim of the present research was to explore novel procedures and constructs, based on biomaterials and biomechanical algorithms that can better mimic joints mechanical and biochemical stimulation to promote regeneration of damaged cartilage.

Using a hydrogel-based platform for chondrocyte 3D culture revealed a synergy between mechanical forces and growth factors. Exploring the mechanisms underlying this mechano-biochemical interplay may enhance our understanding of cartilage remodeling and the development of new strategies for cartilage repair and regeneration.

INTRODUCTION

Articular cartilage is a highly specialized tissue that provides low friction and allows for efficient load bearing and distribution. The major cartilage constituents comprise a highly hydrated and organized extracellular matrix (ECM), consisting mostly of collagen fibres and proteoglycans, and a low density of specialized chondrocytes [141]. Articular cartilage is non-vascularized, non-innervated and lacks a supporting perichondrial layer, therefore, once damaged, it does not elicit effective tissue repair responses. Cartilage damage and associated catabolic processes are usually irreversible and often lead to permanent cartilage loss and osteoarthritis (OA) [142].

Different strategies over the years have attempted to regenerate cartilaginous tissue. Surgical techniques, such as abrasive chondroplasty, microfracture and spongialisation, failed to achieve authentic tissue repair, but, instead, formed fibrocartilaginous tissue, which does not possess the mechanical properties of normal healthy cartilage [143, 144]. Another procedure receiving much attention is autologous chondrocyte implantation (ACI). However, ACI usually requires multiple surgeries, along with long periods of recovery and rehabilitation. On the other hand, matrix-associated ACI (MACI), applies an exogenous matrix that can improve the mechanical stability and durability of the implanted cells as well as provide a proper stimulus for chondrogenic differentiation and cartilage regeneration [145, 146].

Several studies have demonstrated that fibrin-based hydrogels provide a most suitable environment for multiple cell functions, i.e. migration, proliferation and differentiation [60, 147, 148]. Chondrocytes embedded in fibrin hydrogels retain their rounded differentiated morphology and produce cartilaginous ECM [149, 150]. However, fibrin particularly when subjected to the harsh environment of OA, undergoes fibrinolysis and loss of scaffold stability [151]. While rapid degradation can be an advantage in some applications (e.g. wound dressing), it represents a limitation for cartilage repair. Long-term stability of the scaffold is required to provide enough time for cell proliferation, differentiation and matrix production [152].

Efforts were therefore made to add bio-macromolecules to the fibrin hydrogel to improve its stability [153]. Incorporation of hyaluronic acid (HA) into fibrin-based scaffolds decreases the fibrinolysis rate and improves the mechanical and biological properties *in-vitro* and *in-vivo* [154-156]. HA, a major component of articular cartilage and synovial fluid, supports cell proliferation and maintains the chondrogenic phenotype, increasing the production of cartilaginous ECM [157-160].

Fibrin-Hyaluronan hydrogels have been described as adequate platforms for cartilage regeneration, able to crosslink in situ, at body temperature, rendering the system safely injectable and minimally invasive [60, 61, 155, 161, 162]. These have been shown to increase the secretion of extracellular matrix components, such as GAG

and collagen, when compared to chondrocytes embedded in agarose or alginate gels [153]. Cell-hydrogel constructs develop increased mechanical strength following the deposition of extracellular matrix enriched in collagen type II, a hallmark of hyaline cartilage [155]. In gel matrix deposition may facilitate the conductance of intraarticular mechanical stimuli which have been shown to be of critical importance in stimulating the development of normal articular hyaline cartilage [155].

Various anabolic compounds have been evaluated to promote cartilage regeneration [163, 164]. In mature articular chondrocytes, fibroblast growth factor-18 (FGF-18) exhibits mitogenic activities in addition to increased ECM production, thereby promoting cartilage repair, in both in vitro and in vivo models [165-169]. N-terminal truncated FGF-18 variant (FGF-18v) was shown to have improved specificity for FGF receptor-3 (FGFR-3), the major FGFR isotype involved in chondrocytes differentiation and maturation [170, 171]. Correa et al. showed a clearly enhanced anabolic effect of mutated version FGF-18, signalling exclusively through FGFR-3, increasing the production and expression of ECM components (e.g. glycosaminoglycans, aggrecan, type II collagen), in comparison with wild-type FGF-18 and TGF-β [172].

Biomechanical studies have aimed to regenerate neo-tissue to resemble native healthy cartilage [164, 173, 174]. Mechanical stimulation has been shown to transcriptionally activate the expression of genes associated with various cellular processes in chondrocytes, including matrix accumulation and pro-inflammatory gene suppression [175]. Different bioreactors and loading devices have been designed to stimulate neo articular cartilage development, by providing chondrocytes with needed mechanical cues [176, 177]. Multi-axial loading has been shown to effectively stimulate the synthesis of cartilaginous ECM macromolecules in chondrocytes cultured in 3D scaffolds [164]. Specifically, intermittent dynamic compression and sliding surface motion, applied by a ceramic ball, has been shown to improve the gene expression and the synthesis of cartilage specific matrix molecules in chondrocytes-scaffold constructs [83, 137, 178-180]. Both lubricin and cartilage oligomeric matrix protein gene expression are markedly enhanced by applying sliding motion to the surface of a three-dimensional scaffold, whereas the upregulation of collagen Type II and aggrecan was more associated with the application of compression [164].

Previous studies have combined mechanical loading with growth factor supplementation (e.g. fibroblast growth factor-2, transforming growth factor-β, insulin-like growth factor-1, osteogenic protein-1), to modulate chondrocytes phenotype, proliferation and biosynthetic rates [181-184]. However, to the best of our knowledge, the interplay between mechanical stimuli and FGF-18 supplementation is still unknown. We therefore investigated the effect of FGF-18v on primary chondrocytes seeded in a 3D fibrin:hyaluronan (FB/HA)-based hydrogel in free swelling conditions and under mechanical loading. Cell-hydrogel constructs, in the presence or absence

of FGF-18v, were loaded in a custom made joint-mimicking bioreactor. Anabolic and catabolic gene expression and production of ECM were used to evaluate the treatments. This system provides an efficient, pre-clinical testing tool of cartilage therapeutics as a critical step prior clinical translation.

MATERIALS AND METHODS

Fibrin-HA hydrogel production

The FB/HA hydrogel (3.2:1 w/v ratio; 6.25mg/mL and 1.96mg/mL respectively) was manufactured and provided by ProCore Biomed Inc. (Ness Ziona, Israel), at final concentrations of 6.21 mg/mL and 1.94 mg/mL of fibrinogen and HA, respectively. Fibrinogen:HA conjugates were synthesized via a two-step procedure as previously described [185]. Briefly, HA (1.55 MDa; Lifecore Biomedical, Minnesota, USA) was initially reacted with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma, Israel) and *N*-hydroxysuccinimide (NHS; Sigma, Israel) to convert part of its carboxylic groups to NHS-active ester moieties. In a second step, a buffered solution of fibrinogen (Omrix, Israel) was reacted with the HA active ester solution to produce a clear fibrinogen:HA conjugate solution.

Chondrocyte isolation and culture conditions

Chondrocytes were isolated from full thickness fetlock joint cartilage of 4-8 months old calves, using sequential pronase (Roche, Mannheim, Germany) and collagenase (Worthington Biochemical Corporation, NJ, USA) digestion [176]. Isolated chondrocytes ($7.5x10^6$ cells/construct) were suspended in the fibrinogen:HA conjugate (330 µL/construct) and thrombin solution (Omrix, Israel) (22 µL/construct; 50 U/mL) was added at a volume ratio of 1:15 (final concentrations, FB: 5.86 mg/mL; HA: 2.34 mg/mL; thrombin: 3.13 U/mL). Upon, thrombin addition, the suspension was mixed to achieve optimal cell distribution, placed in polyurethane moulds and allowed to crosslink. Cell-hydrogel constructs (8 mm diameter; 4 mm height) were then placed into bioreactor sample holders and incubated for 30 min at 37 °C and 5% CO_2, to allow complete gelation. The constructs were then cultured in growth medium (Dulbecco's Modified Eagle's medium, high glucose (DMEM-HG), 4.5 g/L-glucose; Gibco), supplemented with penicillin/streptomycin (1% P/S, Gibco), 50 µg/mL ascorbic acid-2 phosphate (AA-2P, Sigma), 1% insulin-transferrin-selenium (ITS) and non-essential amino acids. Constructs were exposed to 10 ng/mL or 100 ng/mL of FGF-18v (Procore Bio Med, Ness Ziona, Israel), added to culture media and replenished on every medium exchange. Controls not exposed to FGF-18v were included. FGF18v is a truncated version of FGF-18 lacking the amino-terminal last 50 amino acids of the ligand and has the first methionine replace

glutamine 51 [170]. The medium was changed every second day, and conditioned medium was collected for analysis of sulphated glycosaminoglycans (sGAG) (section 2.4).

Mechanical loading

The hydrogel-chondrocytes constructs were cultured under free swelling conditions for 5 days, to allow cell attachment, colonization and initiation of ECM deposition, similar to previously published and optimised protocol [82, 186, 187]. Subsequently, constructs were exposed to mechanical loading, in the presence or absence of different concentrations of FGF-18v. Mechanical stimuli were applied using a four-station bioreactor system, installed in an incubator at 37°C, 5% CO_2, 85% humidity. At each station, a commercially available ceramic hip ball (32 mm in diameter) was pressed onto a cell-seeded hydrogel to provide a constant displacement of 0.4 mm or 10% of the scaffold height (measured in the construct centre). The ball oscillated vertically in a sinusoidal manner between 0.4 mm and 0.45 mm, i.e., between 10% and 11.25% of the construct height, at a frequency of 0.5 Hz. In addition to the cyclic compressive loading, reciprocate rotation of the ball about an axis perpendicular to the construct axis was promoted, at an amplitude of 25 degrees and a frequency of 0.5 Hz (Fig. 1). This regime of dynamic axial compression with superimposed sliding motion simulates joint articulation more closely compared to axial compression alone [176].

One hour of mechanical loading was performed daily for 14 days. In between loading cycles, the constructs were kept in a free swelling condition (without ball contact). Construct analysis was performed after a total culture time of 19 days. Unloaded scaffolds served as controls.

Biochemical assays

Cell-loaded hydrogels were digested overnight with 0.5 mg/mL of proteinase K, at 56 °C (2.5 U/mg, chromozyme assay; Roche, Mannheim, Germany). The PicoGreen® Assay (Molecular Probes, Life Technologies) was used to assess the DNA content as per manufacturer's guidelines. The sample fluorescence was measured using a microplate reader (VICTOR3 V' Multilabel Counter, PerkinElmer BioSignal Inc, USA) at 480 nm excitation and 520 nm emission. The amount of sulphated glycosaminoglycans (sGAG) was determined by a dimethylmethylene blue dye assay using DMMB solution at pH 1.5 and bovine chondroitin sulfate as a standard. Total sGAG content of the culture media was also measured to assess the release of matrix molecules from the constructs into the media.

Gene expression analysis

Total RNA was extracted from homogenized constructs using TRI Reagent (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed with TaqMan™ reverse transcription reagents (Thermo Fisher Scientific, Reinach, Switzerland), using random hexamer primers and 500 ng of total RNA. PCR was performed using a QuantStudio™ 6 real-time PCR instrument (Applied Biosystems) and TaqMan™ Gene Expression Master Mix. Table 1 shows the sequences of bovine primers and TaqMan™ probes for aggrecan (ACAN), collagen type-I (COL1), type-II (COL2), type-X (COL10), cartilage oligomeric matrix protein (COMP), proteoglycan 4 (PRG4/Lubricin), matrix metalloproteinases -3, -9 and -13 (MMP-3, -9 and -13). Primers and probe for amplification of 60S acidic ribosomal protein lateral stalk P0 (RPLP0, Bt03218086_m1) were acquired from Applied Biosystems (Rotkreutz, Switzerland). Relative quantification of target mRNA was performed according to the comparative CT method, using RPLP0 as an endogenous control [188].

Histology

Histological samples were fixed in 4% buffered formaldehyde (Formafix AG, Hittnau, CH) for 24h, embedded in paraffin and sectioned in 5 µm sections. For staining, slides were deparaffinised using xylene and subsequently hydrated. Safranin-O/Fast green staining was performed to visualize proteoglycan and collagen deposition. Briefly, slides were first stained with Weigert's haematoxylin for 10 min, blued in tap water for 10 min, stained with 0.002% Fast green in deionized water for 5 min and washed in 1% acetic acid. Sections were then stained with 0.1% Safranin-O for 12 min and then imaged (Zeiss Axiovert 200M, Switzerland). ImageJ software (National Institutes of Health, Bethesda, MD) was used for automated quantification of the intensity of red-stained sections, by colour thresholding the regions of interest and calculating the percentage of stained area.

Immunohistochemistry

For immunohistochemical analysis, samples were fixed in 4% buffered formaldehyde (Formafix AG, Hittnau, CH) for 24h, embedded in paraffin and sectioned in 5 µm sections. Before immunolabelling for the aggrecan protein could be conducted, reduction and alkylation steps were necessary to expose a neo-epitope. The endogenous peroxidase activity was blocked with 0.3% peroxidase in 100% methanol and the sections were enzymatically pre-treated (0.25 U/ml of Chondroitinase ABC and 25 mg/mL of hyaluronidase; both Sigma, St.Louis, MO). Next, sections were blocked with horse serum (1:20 in PBS-T) and, subsequently, incubated with the primary antibodies (overnight at 4 °C) against Aggrecan (12/21/1-C-6, 4 µg/ml) and COL2 (CIICI, 2 µg/ml IgG) (both Developmental Studies Hybridoma bank, University of Iowa, Iowa City, IA). The Vectastain elite ABC kit mouse IgG and the ImmPACT DAB peroxidase substrate were used as detection system (both Vector Laboratories, Burlingame, CA). The cell nuclei were stained with Mayer's haematoxylin.

STATISTICAL ANALYSIS

The results are expressed as mean +/- standard deviation (SD) of four independent experiments using four chondrocyte donors (n=12). As sGAG, DNA content and qPCR data did not follow normal distribution when analysed using the Shapiro-Wilk test, statistical analysis using, non-parametric, Kruskal-Wallis analysis was performed, followed by a post-hoc Dunn's comparison test. Differences were considered statistically significant for $p < 0.05$.
The COL2/COL1 ratio was calculated as 2^ (-ΔCt (COL2))/2^ (-ΔCt (COL1)).

RESULTS

Mechanical stimulation promotes sGAG production

To test the biological response of chondrocytes seeded in hydrogels to mechanical loading and FGF-18v stimulation, DNA and sGAG content were quantified after 19 days in culture. All samples, independent of the application of mechanical loading and/or FGF-18v showed similar DNA content when compared to control samples (Day 5 - before loading; Fig 1A). Sample groups exposed to mechanical loading produced significantly more sGAG (normalized to DNA content) when compared to unloaded samples, both in the presence or absence of FGF-18v ($p<0.01$ for no F18 and F18 10ng groups; $p<0.001$ for F18 100ng group) (Fig. 1B). The sole exposure of the constructs to FGF-18v did not significantly affect sGAG production at either concentration in comparison to the samples without F18v.

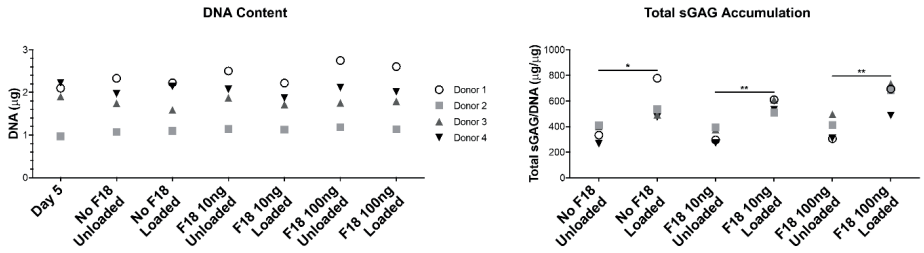


Fig. 1 DNA content (A) and Total sGAG (accumulated in the media and in the construct) per DNA ratio (B) of unloaded and loaded chondrocytes seeded Fibrin/HA hydrogels after 14 days, in presence (F18) or absence (no F18) of FGF-18v supplementation. FGF-18v supplementation featured two concentrations, 10 ng/mL or 100 ng/mL (10ng and 100ng, respectively). Results from 4 chondrocyte donors, assessed in triplicates, are shown; * $p < 0.05$, ** $p < 0.01$.

To further assess the effect of the treatments on sGAG production, Safranin-O/Fast Green staining was performed (Fig. 2) and quantified (Fig. S3). Mechanical stimulation led to increased GAG deposition, when compared to unloaded controls (with and without FGF-18v supplementation). This finding is in line with sGAG/DNA results. In addition, FGF-18v supplementation did not increase proteoglycan deposition, as previously seen in the DMMB assay.

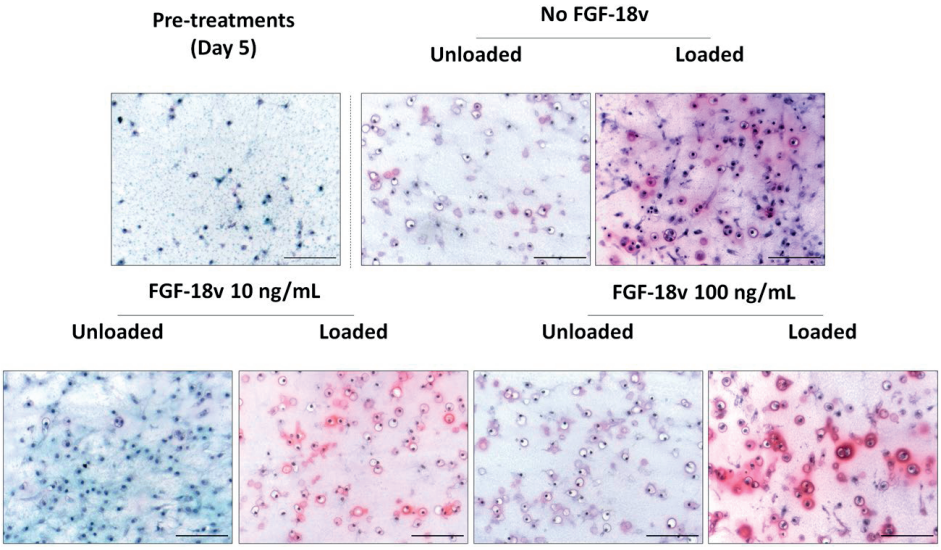


Fig. 2 Representative Safranin-O/Fast Green stained chondrocyte-seeded Fibrin/HA hydrogels, after 5 days (pre-treatments) and 19 days (post-treatments) in culture; 20x magnification, scale bars indicate 100 µm.

Furthermore, ACAN immunohistochemistry staining was assessed (Fig. 3). Results were found in line with those of sGAG/DNA and Safranin-O/Fast Green, displaying increased ACAN production and deposition, when exposed to mechanical loading. The presence of an increased ACAN deposition was more evident when the application of mechanical loading was combined with 100 ng/mL FGF-18v and without FGF-18v supplementation.

Synergistic effect of mechanical loading and FGF-18v supplementation on cartilage gene expression

To assess the effects of FGF-18v and the applied stimuli on the phenotype of primary chondrocytes embedded in FB/HA hydrogels, mRNA expression was evaluated after loading. Gene expression of ACAN, COMP and PRG4 was increased under loading (Fig. 4) in an FGF-18v dependent manner. Thus, the combination of mechanical loading and low FGF-18v concentration (10 ng/mL) significantly upregulated ACAN expression (Fig. 4A), when compared to loaded samples without FGF-18v (no F18 loaded; $p<0.001$). Moreover,

both FGF-18v concentrations, in combination with mechanical loading, significantly upregulated COMP expression (Fig. 4B). FGF18v at 10 ng/mL, in mechanically loaded samples, showed a significant effect when compared to unloaded samples without FGF-18 (no F18 unloaded; $p<0.01$). FGF18v at 100 ng/mL with mechanical loading showed a significant COMP upregulation over unloaded samples without FGF-18v (no F18 unloaded; $p<0.001$), unloaded samples with 10 ng/mL FGF-18v (F18 10ng unloaded; $p<0.05$) and unloaded samples with 100 ng/mL FGF-18v (F18 100ng unloaded; $p<0.0001$). PRG4 upregulation under load was noticeable at high FGF-18v concentration (100 ng/mL, Fig. 4C), in comparison to no F18 unloaded and unloaded samples exposed to 100 ng/mL FGF-18v (F18 100ng unloaded). The comparisons found no statistical significance.

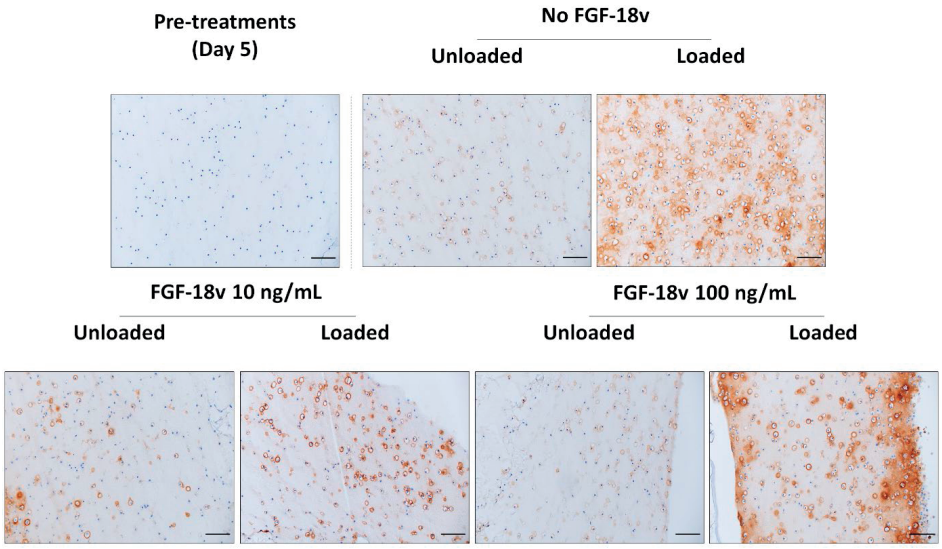


Fig. 3 Representative aggrecan IHC staining of chondrocyte-seeded Fibrin/HA hydrogels, after 5 days (pre-treatments) and 19 days (post-treatments) in culture; 20x magnification, scale bars indicate 100 µm.

Mechanical stimulation and 100 ng/mL FGF-18v supplementation significantly upregulated COL2 expression over no F18 loaded ($p<0.05$; Fig. 5B). On the other hand, treatments did not exert significant effects on COL1 and 10 expression (Fig. 5A and C, respectively).

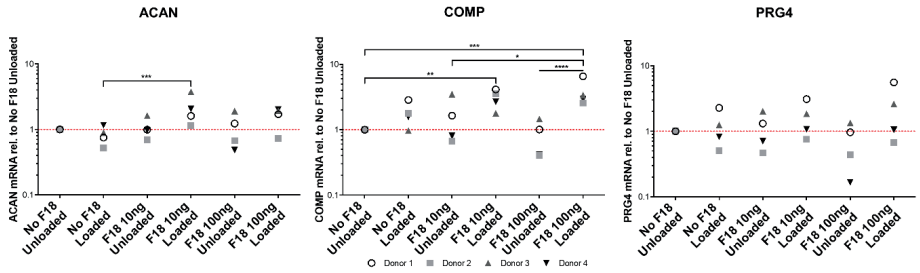


Fig. 4 ACAN (A), COMP (B) and PRG4 (C) mRNA expression of chondrocytes seeded into Fibrin/HA hydrogels, exposed to mechanical stimulation and FGF-18v. Data are expressed relative to mRNA levels of unloaded constructs (No F18 Unloaded). Results from 4 chondrocyte donors assessed in triplicates are shown; *p < 0.05, **p < 0,01, ***p < 0.001, ****p < 0,0001.

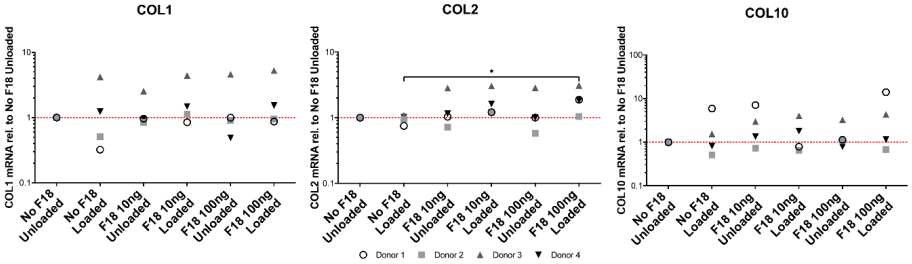


Fig. 5 COL1 (A), COL2 (B) and COL10 (C) mRNA expression of chondrocytes seeded into Fibrin/HA hydrogels, exposed to mechanical stimulation and FGF-18v. Data are expressed relative to mRNA levels of unloaded constructs (No F18 Unloaded). Results from 4 chondrocyte donors assessed in triplicates are shown; *p < 0.05.

When evaluating expression levels normalized to the reference gene (absolute expression; -ΔCt), the expression of COL2 was always higher than that of COL1 (Fig. S1A), for all time points and sample groups, which indicates a COL2/COL1 ratio favourable to COL2. To support this, COL2/COL1 ratio was calculated (Fig. S1B). Moreover, the absolute expression of COL10 was seen to drop after seeding in the hydrogel scaffolds (Day 5 – untreated control), further decreasing through time, at the end of the experiment. To complement COL2 expression, IHC staining was performed (Fig. 6). In line with gene expression findings, IHC staining revealed increased production and deposition of COL2 in loaded samples supplemented with 100 ng/mL FGF-18v.

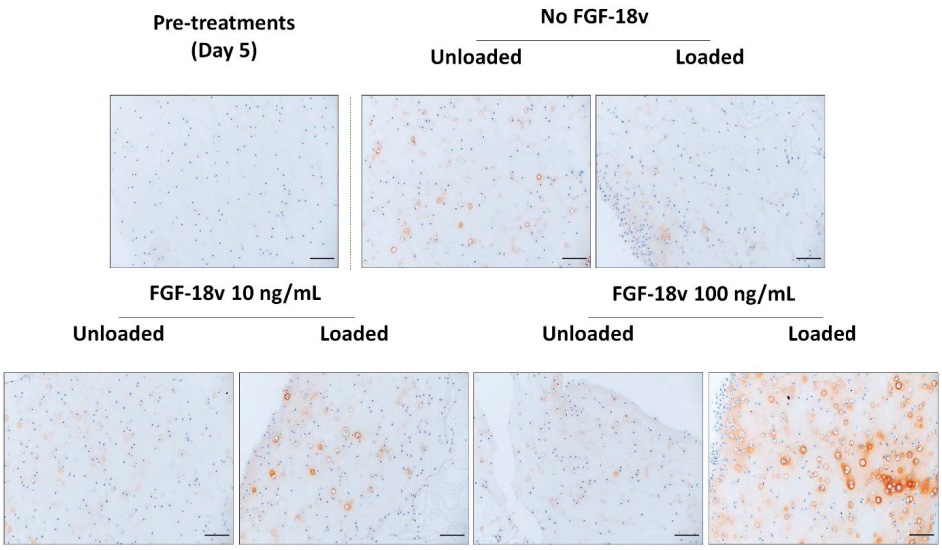


Fig. 6 Representative type II collagen IHC staining of chondrocyte-seeded Fibrin/HA hydrogels, after 5 days (pre-treatments) and 19 days (post-treatments) in culture; 20x magnification, scale bars indicate 100 µm.

When analysing MMP-9 and MMP-13 expression (Fig. 7 B-C), loading significantly decreased the expression of both genes, in the presence and absence of FGF-18v. Mechanical loading, by itself, and in combination with 100 ng/mL FGF-18v was able to significantly downregulate MMP-9, in comparison with no F18 unloaded ($p<0.001$ and $p<0.05$, respectively). In addition, mechanical loading in combination with both FGF-18v concentrations was able to significantly downregulate MMP-9, in comparison with F18 10ng unloaded (F18 10ng loaded, $p<0.05$; F18 100ng loaded, $p<0.01$). MMP-13 expression was significantly downregulated by mechanical loading, by itself, and in combination with 10 ng/mL FGF-18v, in comparison with no F18 unloaded ($p<0.05$ and $p<0.01$, respectively). Moreover, mechanical loading in combination with 10 ng/mL FGF-18v was able to significantly downregulate MMP-9, in comparison with F18 10ng unloaded ($p<0.01$). When analysing MMP-3 expression, despite most of the donors showing the same profile seen in MMP-9 and -13, no significant differences were found as an outcome of the treatments (Fig. 7A).

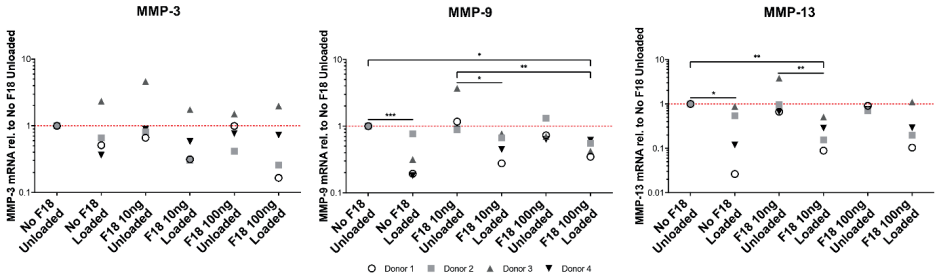


Fig. 7 MMP-3 (A), MMP-9 (B) and MMP-13 (C) mRNA expression of chondrocytes seeded into Fib:HA hydrogels, exposed to mechanical stimulation and FGF-18v. Data are expressed relative to mRNA levels of unloaded constructs (Fib:HA Unloaded). Results from 4 chondrocyte donors assessed in triplicates are shown; *p < 0.05, **p < 0,01, ***p < 0.001.

DISCUSSION

The rationale driving this study was to create a controlled mechanochemical environment *in vitro* and investigate a potential synergistic effect between bi-axial mechanical stimulation and FGF-18v supplementation on primary bovine chondrocytes embedded in a FB/HA hydrogel-based 3D platform. The effect of the combined treatments on the expression of cartilage genes, matrix production and phenotype of primary bovine chondrocytes was explored in comparison to untreated controls. The combination of mechanical stimulation together with FGF-18v supplementation resulted in the upregulation of ACAN, COMP, COL2 and PRG4, while down-regulating MMPs expression. In addition, histological analysis showed increased ACAN, COL2 and GAG deposition. To the best of our knowledge, the interaction of biomimetic mechanical and FGF-18v biochemical stimuli has not been tested before, providing experimental evidence for the benefits of FGF-18v application in cartilage repair, in combination with mechanical loading.

Complex mechanical motion plays a crucial role in the development of cartilage and maintenance of the chondrogenic phenotype [137, 164, 176]. The loading protocol we used originated from protocols previously described by Grad et al. [176]. The original protocol featured the mechanical stimulation of chondrocyte-seeded polyurethane (PU) scaffolds, by applying a cyclical regime consisting of dynamic compression with superimposed sliding motion (shear). Being mechanically stiffer, PU scaffolds were subject to higher intensity set-ups, with a constant compression displacement of 10% of the scaffold's height (and a dynamic oscillation between 10% and 20%), together with ±25° perpendicular shear movement, both at the frequency of 1 Hz. Fibrin-based hydrogels used here feature lower resilience than PU scaffolds. We therefore tuned

down the mechanical loading set-up to fit the mechanical profile of our constructs. Additionally, applying controlled, sub-maximal mechanical stimulation may have revealed a differential response of cell differentiation markers to mechanical stimulation as well as uncovered the mechanical requirements for preconditioning cells to respond to biochemical signals such that of FGF-18. Moderate multi-axial loading may better represent the limited, partial weight bearing loads exerted on articular joints of patients suffering from OA and undergoing various treatment protocols, including intraarticular injection of the presented Fibrin-HA hydrogel (Regenogel) [189-192]. Despite using a lower intensity set-up, it is worth noting that the mechanical loading resulted in a significant increase in total sGAG production, further supported by Safranin-O/Fast Green and ACAN IHC staining, when compared to unloaded controls, as previously seen for the higher intensity set-up [176]. Hence, using FB/HA as a 3D platform, a similar increase in total sGAG production was observed compared to previous studies with PU scaffolds, validating this hydrogel as a proper environment for mechanical stimulation of chondrocytes [137, 176].

The provision of FGF-18v did not result in any significant changes in total sGAG production, with or without mechanical loading. Gigout et al. showed that porcine chondrocytes, in 3D pellet culture, when exposed to recombinant FGF-18 in non-continuous fashions (one-week exposure, once/week exposure) for 5 weeks, resulted in higher matrix deposition compared to the continuously exposed ones [165]. This is called a “hit and run” effect, where short exposure periods tend to initiate a cascade response more effectively, inducing an anabolic effect. Intermittent provision of FGF-18v (once/week) was tested in the present system, for Donor 1, failing to increase sGAG production (in combination with or without mechanical loading), and therefore was dropped for the remaining donors (Fig. S2). The fact that our experiment ran for 3 weeks against the 5-week span featured in Gigout et al., may have played a role in the differences observed. Furthermore, the cell type used, the age and health of the donor should also be considered relevant to the outcome of the study.

Mechanical loading and FGF-18 supplementation have individually shown promise, in several *in vitro* studies, in maintaining chondrogenic phenotype and enhancing matrix production [81, 137, 165, 168, 172, 176]. Previous studies have shown that compression was associated with an upregulation of ACAN, whereas COMP and PRG4 gene expression were markedly enhanced by shear motion at the surface of cell-seeded constructs [164]. Nonetheless, in our study mechanical stimulation by itself was not able to significantly upregulate these genes compared to unloaded samples, most likely due to the combination of lower dynamic compression applied and the low-frequency shear modulus (0.5 Hz instead of 1 Hz). The only addition of FGF-18v in unloaded samples have shown the same trend, however when mechanical stimuli were combined with the factor a marked increase of cartilage matrix genes expression

was observed suggesting a synergistic interaction (i.e. ACAN, COMP, COL2 and PRG4). Huang et al. described the upregulation of ACAN, COMP and PRG4 by FGF-18 on human adipose-derived stem cells, suggesting that a higher concentration of 100 ng/mL was more effective than a lower one (10 ng/mL) [193]. Although working on a different set of cells (i.e. primary bovine chondrocytes), by combining FGF-18v supplementation with mechanical loading we were able to achieve upregulation of ACAN and COMP on lower FGF-18v concentration (10 ng/mL). Conversely, and despite no statistical significance found, PRG4 showed increased upregulation at the highest FGF-18v concentration used. Furthermore, Correa et al. described a study, where human mesenchymal stem cells were supplemented with TGF-β and the same variant FGF-18 herein used [172]. In a time-frame similar to ours (21 days experiment, starting FGF-18v exposure on day 7; continuous supplementation), no ACAN upregulation was achieved, indicating that mechanical loading is important and necessary to induce expression of cartilage matrix genes.

While the expression of COL1 and 10 showed no significant differences between treatment groups (Fig. 5A and C, respectively), the synergistic action of mechanical loading and 100 ng/mL FGF-18v led to significant COL2 upregulation (Fig. 5B), further supported by IHC analysis (Fig. 6). Furthermore, when looking at unnormalized gene expression data (Fig. S1A), COL2 overall expression was higher than COL1 expression for all groups. Thus, we calculated COL2/COL1 absolute expression ratio (Fig. S1B), confirming a ratio favourable to COL2, suggesting that the chondrocytic phenotype was maintained within the 3D environment in both treated and untreated conditions. This should lead to the production of hyaline cartilaginous tissue in favour of fibrocartilage [194]. In addition, in our system COL10 expression decreased after seeding into the hydrogel (Fig S1A), further suggesting the preservation of the differentiated state of chondrocytes, not leading to hypertrophy, characteristic of OA cartilage [195].

Mechanical stimulation is known to be critical for maintaining tissue homeostasis, being a key factor in regulating the balance between chondrocyte anabolic and catabolic processes [137, 164, 176, 179, 196]. Consistent with other reports [81, 197, 198], our findings showed a decrease in the expression of the matrix degrading enzymes, MMP-9 and -13, thereby limiting ECM degradation associated with joint pathologies. This means that our treatment did not foster collagenase-induced ECM degradation, but even down-regulated the expression of matrix degrading agents. Additionally, other studies have shown that FGF-18 supplementation led to a downregulation of MMP expression (e.g. MMP-2, -3, -9 and -13), which corroborates our findings [199-201]. Mori and associates suggested that such inhibitory effects are indirect, via the induction of tissue inhibitor of metalloproteinases (TIMPs), that execute anti-catabolic actions [201]. These endogenous inhibitors are paramount in the regulation of the MMP activity, creating a balance between the production of active enzymes and their inhibition, thus regulating

ECM turnover, tissue remodeling and cellular behaviour [202]. The study performed by Mori et al., showed a decrease in MMP-9 and -13 expression by exposing articular chondrocytes to high concentration FGF-18, for a short period of time, while increasing, significantly, TIMP-1 expression [201]. While the present study ran for a longer span of time, lower dosage FGF-18v combined with mechanical loading, significantly down-regulated MMP-9 and -13 expression. This further confirm the feasibility of the 3D FB/HA platform as responsive system under load.

Biomechanical data demonstrating the superior mechanical properties of these FB/HA hydrogels, in comparison to Fibrin alone, have been previously described, including long term stability of gels containing cells in vitro, frequency-dependent storage moduli (G') and the ratio between storage and loss moduli (G'/G'') overall indicating a solid-elastic character [60, 161]. Moreover, as by definition, hydrogels are characterized by the water-retaining capacity of their polymeric networks [203]. These specific FB/HA hydrogels were found to retain more than 90% of their original water content due to the unique HA conjugation overcoming clot retraction, a physiologically inherent property of all Fibrin networks (unpublished data).

Moreover, degradation of biomaterial-based scaffolds heavily depends on the enzymatic milieu determined by tissue and cell type, and in particular by the action of matrix degrading enzymes, such as MMPs [204]. FB/HA hydrogels containing chondrocytes, or other differentiated cell types, maintain their overall structure for, at least, 4-5 weeks in vitro and more than 3 months in vivo (unpublished data), with no sign of degradation [ref]. Similarly, we did not observe any significant changes in mass of the constructs over time. Moreover, the presented increased production of matrix components and downregulation of matrix degrading enzymes, MMP-9, and MMP-13, under loading, may further stabilise the hydrogels from biological degradation under the employed experimental conditions.

Having found the results of our study encouraging, nonetheless, the system faces certain limitations. The growth factor was not part of the regenerative system but was only added to the culture medium. For *in vivo* application of this formulation in cartilage injuries, FGF-18v would preferably be integrated within the hydrogel system rather than injected freely in the synovial fluid [205]. By integrating the growth factor within the hydrogel, a controlled delivery to the damaged area could be achieved, thus enhancing the regenerative process. It's also important to mention that the system does not fully mimic an in vivo scenario, since the mechanical loading introduced only featured compression and shear, not contemplating rotation force, which is featured in native articular motion [81, 164]. Moreover, the work displayed in this manuscript does not account for the scenario following a trauma, in vivo, specifically the resulting synovial inflammation, and all the agents influencing this process. Additionally, the system herein featured, does not describe a confined system, as the cell-hydrogel construct is not

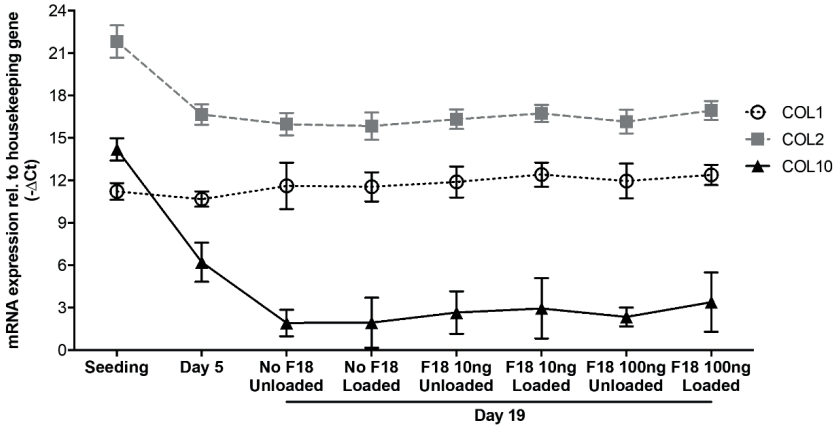
surrounded by tissue (i.e. cartilage and/or bone). Thus, moving forward, progressing from an *in vitro* setting to an *ex vivo*, and ultimately, to an *in vivo* setting, would offer further insight about the potential regenerative effective of this platform of articular cartilage. The osteochondral defect model developed and presented in chapter 4 could be an interesting *ex vivo* platform to continue studying the effects of the FB/HA platform studied, mechanical loading and FGF-18v supplementation [137].

Furthermore, FB/HA hydrogels are highly porous matrixes, with no diffusion limit for molecules until 10 MDa (unpublished data) in size, therefore hydrostatic pressure built-up would be virtually negligible. Nonetheless, since this is not a fully confined system, we cannot completely rule out a contribution of fluid movement around the chondrocytes, which has been shown to promote chondrogenesis, although the effect of pure hydrostatic pressure on the expression of mechano-regulated proteins, such as PRG4 or COMP, has not been shown [206, 207]. It is also noteworthy that, the portrayed model features young chondrocytes from calf and not cells from older, diseased, tissues (e.g. osteoarthritic chondrocytes). While the present study is not a model for osteoarthritis, there is merit in translating the current work to osteoarthritic cells and investigate the re-differentiation potential of the presented platform.

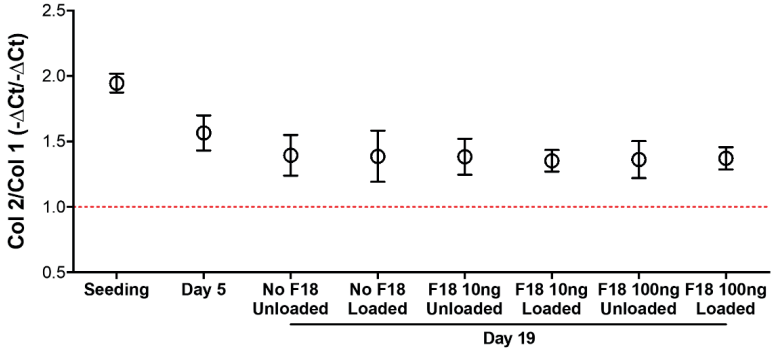
CONCLUSION

In conclusion, our study revealed a synergism between multi-axial mechanical stimulation and biochemical signals delivered by FGF-18v in a fibrin-hyaluronan based hydrogel and their potential to enhance cartilage matrix deposition. This model may be most valuable in decoding the interplay between cells, scaffolds and cartilage guiding factors, elucidating signalling pathways implicated in cartilage homeostasis and repair. There may be merit in the clinical application of a hydrogel-based platform combined with a selective FGF-18 variant, particularly when combined with moderate partial weight bearing rehabilitation protocols. In light to the inherent advantages of each of the different applied stimuli, the fact that this platform can be injected, and crosslinked *in situ*, in an outpatient minimally invasive procedure, makes it an attractive, affordable and easily translatable platform for clinical application.

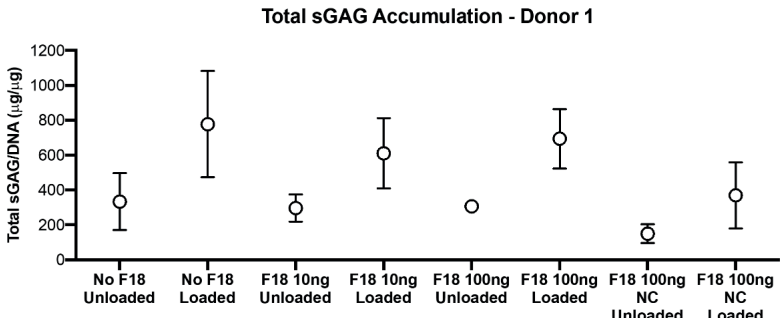
SUPPLEMENTARY DATA



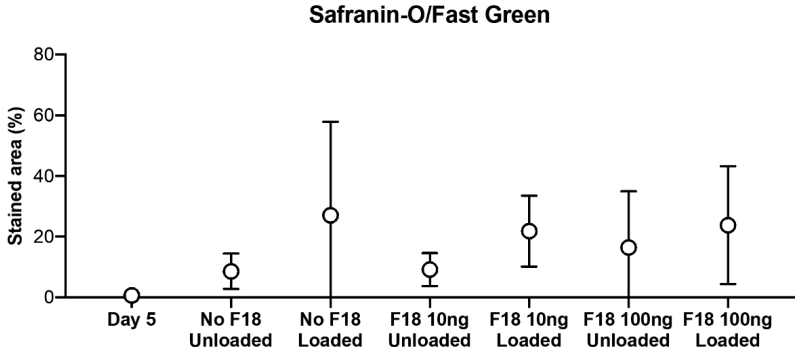
Supplementary Fig. 1 Collagen 1, 2 and 10 mRNA expression of chondrocytes seeded into Fibrin/HA hydrogels, exposed to mechanical stimulation and FGF-18v. Data are expressed as non-normalized mRNA levels. Results from 4 chondrocyte donors assessed in triplicates are shown.



Supplementary Fig. 2 Collagen 2/Collagen 1 expression ratio of chondrocytes seeded into Fibrin/HA hydrogels, exposed to mechanical stimulation and FGF-18v. Data are expressed as the ratio of non-normalized mRNA levels. Results from 4 chondrocyte donors assessed in triplicates are shown.



Supplementary Fig. S3 Total sGAG (accumulated in the media and in the construct) per DNA ratio of unloaded and loaded chondrocytes seeded Fibrin/HA hydrogels after 14 days, in presence (F18) or absence (no F18) of FGF-18v supplementation. FGF-18v supplementation featured two concentrations, 10 ng/mL or 100 ng/mL (10ng and 100ng, respectively) and a non-continuous (NC) fashion.



Supplementary Fig. S4 Safranin-O/Fast Green histological quantification of chondrocyte-seeded Fibrin/HA hydrogels, after 5 days (pre-treatments) and 19 days (post-treatments) in culture; Results from 4 chondrocyte donors assessed in triplicates are shown.

4 Mechanically stimulated osteochondral organ culture for evaluation of biomaterials in cartilage repair studies

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ABSTRACT

Surgical procedures such as microfracture or autologous chondrocyte implantation have been used to treat articular cartilage lesions; however, repair often fails in terms of matrix organization and mechanical behaviour. Advanced biomaterials and tissue engineered constructs have been developed to improve cartilage repair; nevertheless, their clinical translation has been hampered by the lack of reliable *in vitro* models suitable for pre-clinical screening of new implants and compounds.

In this study, an osteochondral defect model in a bioreactor that mimics the multi-axial motion of an articulating joint, was developed. Osteochondral explants were obtained from bovine stifle joints, and cartilage defects of 4 mm diameter were created. The explants were used as an interface against a ceramic ball applying dynamic compressive and shear loading. Osteochondral defects were filled with chondrocytes-seeded fibrin-polyurethane constructs and subjected to mechanical stimulation. Cartilage viability, proteoglycan accumulation and gene expression of seeded chondrocytes were compared to free swelling controls. Cells within both cartilage and bone remained viable throughout the 10-day culture period. Loading did not wear the cartilage, as indicated by histological evaluation and glycosaminoglycan release. The gene expression of seeded chondrocytes indicated a chondrogenic response to the mechanical stimulation. Proteoglycan 4 and cartilage oligomeric matrix protein were markedly increased, while mRNA ratios of collagen type II to type I and aggrecan to versican were also enhanced. This mechanically stimulated osteochondral defect culture model provides a viable microenvironment and will be a useful pre-clinical tool to screen new biomaterials and biological regenerative therapies under relevant complex mechanical stimuli.

Keywords: Articular cartilage; osteochondral defect; bioreactor; ex vivo model; biomaterials

Statement of Significance

Articular cartilage lesions have a poor healing capacity and reflect one of the most challenging problems in orthopedic clinical practice. The aim of current research is to develop a testing system to assess biomaterials for implants, that can permanently replace damaged cartilage with the original hyaline structure and can withstand the mechanical forces long term.

Here, we present an osteochondral *ex vivo* culture model within a cartilage bioreactor, which mimics the complex motion of an articulating joint *in vivo*. The implementation of mechanical forces is essential for pre-clinical testing of novel technologies in the field of cartilage repair, biomaterial engineering and regenerative medicine. Our model provides a unique opportunity to investigate healing of articular cartilage defects in a physiological joint-like environment.

INTRODUCTION

Articular cartilage is a unique tissue, allowing low-friction movement of an articulating joint and withstanding considerable stress and repeat loading, thereby preserving the joint homeostasis. Damage of articular cartilage is prone to progression into early osteoarthritis (OA); due to the limited repair ability, surgical procedures are required to treat cartilage lesions [90, 91]. The two most common approaches to regenerate neocartilage *in situ*, microfracture and autologous chondrocytes implantation (ACI), are well-established procedures for such defects [16, 19, 208-211]. However, cartilage repair outcome after microfracture faces high inter-patient variability [212, 213]. In most cases, little or no hyaline cartilage is regenerated, and the generated hyaline cartilage may turn into a weaker fibrocartilage unable to withstand the compression and shear forces [214-216]. On the other hand, the ACI procedure implies multiple surgeries and requires long recovery time; moreover, the chondrocytes dedifferentiate during *in vitro* expansion and their decreased number and activity with aging may impair the healing or result in failure of repair [217, 218]. Improved understanding about the mechanisms that are involved in the formation of repair tissue is needed to further develop these procedures.

Current research aims to improve the biological and functional outcome of cartilage repair treatments; for example, functional cartilage tissue engineering aims to generate neo-tissue *in situ* with an articular surface similar to that of native cartilage [87]. For *ex vivo* investigations, the use of bioreactors has been introduced to mimic the multi-axial motion of an articulating joint and reproduce the kinematics of mechanical loading experienced by chondrocytes *in vivo* [164]. It has been reported that cyclic compression combined with shear stresses act as modulators of the amount and type of extracellular matrix (ECM) synthesized [83], as promotors of functional articular surfaces [81, 219], and as an inducer of transforming growth factor-beta (TGF-β1) production and activation, thereby promoting chondrogenesis of mesenchymal stem cells [86, 138, 220, 221]. Therefore, implementation of mechanical forces is essential for the development and maintenance of articular cartilage and is required for more predictive pre-clinical *ex vivo* research.

Osteochondral *ex vivo* models [115] in which chondral or osteochondral defects can be generated, are of great value for translational research. In contrast to cell culture models, the osteochondral explant culture model allows investigations of the interplay between cellular and extracellular signals involved in cartilage repair. Moreover, osteochondral defect models are invaluable for assessing integration of a tissue engineered graft with the surrounding cartilage, which is critical for its function and presents a significant challenge in the field. Several materials have been proposed to improve cartilage integration; nevertheless, inadequate biomechanical stability of the

graft has often been observed, which is likely to be the determining factor in the clinical success of the repaired tissue [222], demonstrating the need for improved treatments [138, 223].

Another parameter that needs more rigorous pre-clinical testing is the pre-culture time of an engineered cartilaginous construct, as this was shown to play a pivotal role for both maturation and tissue integration upon implantation [224]. The ideal stage of development is still unknown, and it is most likely scaffold dependent. Different factors, such as perturbations in cell source, migration, differentiation and apoptosis [89], impede cartilage integration by affecting proteoglycan and collagen deposition thus forming a compromised tissue that often lead to mechanical failure. Biomechanical stability is an essential requirement for suitable cartilage integration, which is enhanced by mechanical loading. This highlights the importance of load on maintenance of cartilage health. However, common osteochondral *ex vivo* models have not taken into account the mechanical component and therefore lack an important physiological stimulus; while standard bioreactor studies have applied load to isolated hydrogels or scaffolds mostly in unconfined mode and have not considered the confined environment within the tissue that is experienced *in vivo*. The present study for the first time combines an osteochondral defect model with mechanical compression and shear load that simulates physiological joint kinematics. Addition of multiaxial mechanical load to this model represents an important advancement, enabling more predictive pre-clinical screening of novel therapies and biomaterial-based implants, thereby replacing or reducing pre-clinical *in vivo* animal studies.

Furthermore, using functioning human cells or tissues to screen treatment candidates could accelerate the development process and provide key tools for more clinically relevant research. Organ culture bioreactors therefore hold the potential to provide a testing platform that is more predictable of the whole tissue response, facilitating the therapy screening before starting the clinical trial [225].

The aims of this study were 1) to evaluate the osteochondral explant vitality and cartilage integrity under combined compression and shear load and (2) to assess the early cellular responses to multiaxial load in a confined microenvironment, using an established fibrin-polyurethane scaffold. We demonstrate that cartilage and bone remain viable over the 10 days culture period. Furthermore, we confirm the applicability of the osteochondral defect model with a cell-scaffold construct under mechanical stimuli in a joint bioreactor system.

MATERIAL AND METHODS

Osteochondral tissue harvest, defect creation and culture

Osteochondral explants were harvested from stifle joints of 3 to 5-months-old calves, obtained from a local abattoir (Metzgerei Angst AG, Zurich, CH) within 48 hours of slaughter. Joints were dissected to expose the patellar groove and examined for absence of cartilage bruising and blood tint. Cylindrical osteochondral explants were obtained with an 8 mm diameter diamond coated custom-made trephine drill (Peertools AG, Ftan, CH), using a Bosch compact drill press, saline irrigation and a manual circular saw (Fig 1A). The machine was equipped with an adjustable table for round and angular stifle joint positioning and achieved vertical cutting. Variable speed control and digital drilling depth monitoring facilitated obtainment of a flat articular cartilage surface in a reproducible manner. The subchondral bone part was trimmed to obtain a final osteochondral explant height of 6 mm. From each stifle joint, 5 osteochondral explants were obtained (Fig. 1B).

To generate osteochondral defects, a 4 mm trephine drill was used (Brutsch-Ruegger, Urdorf, CH, Fig. 1C) to centrally remove a full thickness circular cartilage biopsy including part of the subchondral bone (Fig. 1D). To determine the consistency of the harvesting, diameter and length of osteochondral explants were measured using a calliper (Mitutoyo Absolute Digimatic Caliper range 0/200mm). Subsequently, explants were cultured in Dulbecco's modified eagle medium (DMEM-HG, 4.5 g/L-glucose; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (1% P/S, Gibco), at 37°C and 5% CO_2. Intact osteochondral explants and osteochondral defect models were incubated overnight to ensure sterility. Then they were placed in well plates containing 2% low-gelling agarose (SeaPlaque Agarose, Lonza, Rockland, USA), to cover the bone part and prevent cell outgrowth from the subchondral bone, and cultured in DMEM-HG, 1% insulin-transferrin-selenium (ITS), non-essential amino acids and 1% P/S. The medium, referred to as chondro-permissive medium, was changed three times per week.

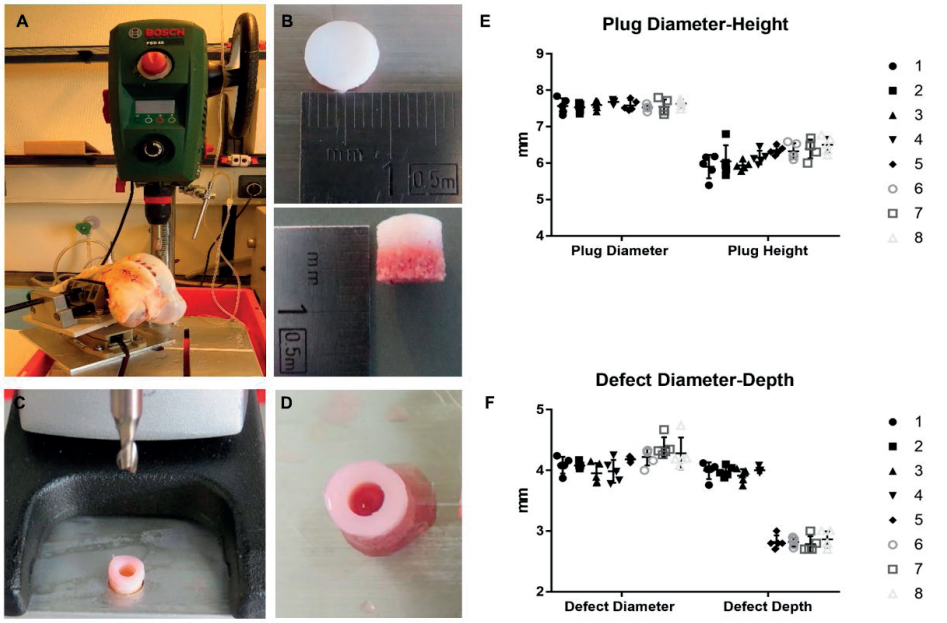

Figure 1. Reproducibility of osteochondral harvesting and defect creation. A) Compact drill press, which achieves vertical cutting, and an angle adjustable table for bovine stifle joint positioning. B) Representative image of an osteochondral explant harvested from the femoral groove; the bone was trimmed to reach the desired height. C) The trephine (4 mm) is adjusted for creating the desired depth of the circular groove, controlled by the digital drilling press. D) Representative image of osteochondral defect model. E) Results of intact osteochondral explant diameter and height achieved after drilling and trimming F) Results of osteochondral defect diameter and depth, showing two different cartilage height groups. Data are presented as mean +/- SD (from 8 stifle joints, n=5 per joint).

Chondrocytes-polyurethane scaffold constructs

Cylindrical (4.15 mm x 2.3 mm and 4.15 mm x 4.3 mm) polyurethane (PU) scaffolds (average pore size 150-300 µm) were prepared as described previously [226-228]. Scaffolds were sterilized by ethylene oxide exposure for 4 h at 37°C and subsequently degassed at 45°C and 150 mbar for 4 days. Before cell seeding, scaffolds were pre-incubated in DMEM HG supplemented with 1% P/S for 1 h to wet the hydrophobic polymer. Chondrocytes were isolated from the left femoral condyles [229], using Pronase and sequential Collagenase digestion as previously described [230]. Upon isolation, primary bovine chondrocytes were suspended in fibrinogen solution and then mixed with thrombin solution (both from Baxter, Vienna) immediately prior to seeding into the PU scaffold at cell density $5x10^7$/mL. The final concentrations of the fibrin gel components were 17 mg/mL fibrinogen and 0.5 U/mL thrombin [231]. Constructs were incubated for 1 h at 37°C, 5% CO_2 to permit fibrin gelation before adding them into the osteochondral defect and were then cultured in chondro-permissive medium, containing

500 kIU/mL aprotinin to prevent fibrin degradation (Fluka, Buchs, Switzerland). The addition of 50 µg/mL ascorbic acid was delayed until 5 days post-seeding, in order to avoid cell damage due to oxidative stress directly after enzymatic digestion during chondrocytes isolation [232].

Mechanical loading

After 5 days of pre-culture, osteochondral defect models filled with cell-scaffold constructs underwent mechanical stimulation using our four-station bioreactor system, installed in a CO_2 incubator at 37°C, 5% CO_2, 85% humidity [180]. A commercially available ceramic hip ball (32 mm in diameter) was pressed onto the osteochondral explants to provide a constant displacement of 0.4 mm or 10% to 14% of the cartilage height (in the centre), to fully maintain the contact of the ball with the cell-scaffold constructs and the surrounding cartilage. Loading groups were exposed to axial compression in a sinusoidal manner between 0.4 mm and 0.8 mm, resulting in an actual strain amplitude of 10-20% or 14-26% of the cartilage explant height (depending on the cartilage height group; see 3.1) at a frequency of 0.5 Hz and simultaneous shear motion by ball oscillation at ±25° and 0.5 Hz. The maximal mechanical loads applied corresponded to 15 N or approximately 0.35 MPa (Fig. 2A, B). This regime of dynamic axial compression with superimposed sliding motion is suggested to more closely simulate joint articulation compared to axial compression [233].

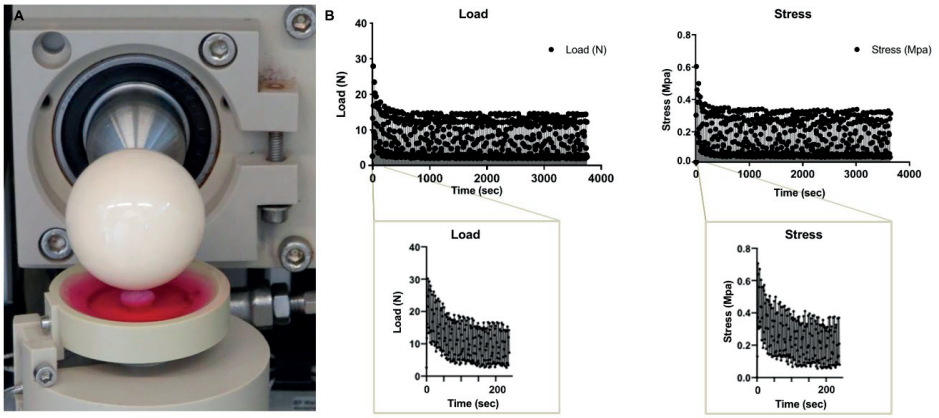

Figure 2. Loading applied to osteochondral defect model. A) Representative image of one station of the joint bioreactor that allows for application of joint specific biomechanical stimuli to osteochondral defect models. Cartilage defect was made and filled with chondrocytes-seeded scaffold. B) Maximal mechanical load applied to the osteochondral explant measured in newton and respective stress in megapascal. Representative graphs of one osteochondral defect model at day 10 of culture (5 days of mechanical loading), after one hour of loading and higher magnification of initial time points.

One hour of mechanical loading was performed twice per day (8 hours free swelling between loading cycles) over 5 consecutive days. In between loading cycles, samples were kept in free-swelling condition (without ball contact) and medium was collected at the end of the experiment to assess the GAG release. Free-swelling osteochondral defect models with cell-scaffold constructs served as controls.

Validation of the model: viability assay

Cell viability of the osteochondral explants was monitored at day 0 (directly after harvesting) and at day 10 by lactate dehydrogenase (LDH) – ethidium homodimer staining. For thin sections, samples were snap-frozen and stored at -80°C. A tungsten carbide D-blade (MICROM, 16 cm, cat. num. 152120) was used to obtain sagittal cryo-sections (20 µm) of undecalcified osteochondral defect models and intact osteochondral explants. Briefly, slides were rinsed in phosphate buffered saline (PBS) and incubated with ethidium-homodimer (46043 SMG-F, Sigma) in PBS for 30 min at 37°C. Subsequently, sections were rinsed in PBS and stained with 40% Polypep-based LDH solution using the salt nitroblue tetrazolium (NBT) as third substrate next to lactate and nicotinamide adenine dinucleotide (NAD) for 3 hours at 37°C. To assess bone viability, thick sections (250 µm) were cut with an annular saw (Leica) and stained with 5% Polypep-based LDH solution using the above-mentioned substrates [234]. Sections were mounted with water based mountant and imaged using a fluorescence microscope to assess the presence of dark stained chondrocytes and osteocytes.

Histology

Histological samples were fixed in 4% buffered formaldehyde (Formafix AG, Hittnau, CH) for 48 hours, decalcified in 10% formic acid (Fluka, cat.num.06460) for 6 days, then embedded in paraffin and sectioned in 5 µm sections. For staining, slides were deparaffinised using xylene and subsequently hydrated. Safranin O/Fast green staining was performed to visualize proteoglycan and collagen content. Briefly, slides were first stained with Weigert's Haematoxylin for 10 min, blued in tap water for 10 min, stained with 0.002% Fast green in deionized water for 5 min and washed in 1% acetic acid. Then, sections were stained with 0.1% Safranin O for 12 min.

RNA extraction and gene expression analysis

At the end of the experiment, cell-scaffold constructs were removed from the osteochondral defect model, homogenized using the Tissue Lyser system (Quiagen, Retsch, Germany), and total RNA was extracted using TRI Reagent® (Molecular Research Center, Cincinnati, OH). Reverse transcription was performed with TaqMan® reverse transcription reagents (Thermo Fisher Scientific, Reinach, Switzerland), using random hexamer primers and 1 µg of total RNA. Table 1 shows the sequences of bovine primers

and TaqMan probes for collagens type-I (*COL1A2*), type-II (*COL2A1*), aggrecan (*ACAN*), cartilage oligomeric matrix protein (*COMP*), proteoglycan 4 (*PRG4*/Lubricin), matrix metalloproteinase 3 (*MMP-3*) and *MMP-13*. Primers and probe for amplification of ribosomal protein lateral stalk subunit P0 (*RPLP0*, Bt03218086_m1) and Versican (*VCAN*, Bt03217632_m1) were from Applied Biosystems (Rotkreuz, Switzerland). Relative quantification of target mRNA was performed according to the comparative C_T method with bovine *RPLP0* as the endogenous control. For a given amount of total RNA, b*RPLP0* values did not vary among the different groups, confirming *RPLP0* was an appropriate endogenous control for chondrocytes-PU constructs subjected to mechanical stimuli. Data were further normalized to the values of the unloaded controls and converted to relative mRNA values using the $2^{-\Delta\Delta CT}$ method [188].

Table 1. Oligonucleotide primers and probes used for qRT-PCR

Gene	Primer forward (5'-3')	Primer reverse (5'- 3')	Probe (5'FAM- 3'TAMRA)
Collagen 1A2	TGC AGT AAC TTC GTG CCT AGC A	CGC GTG GTC CTC TAT CTC CA	CAT GCC AAT CCT TAC AAG AGG CAA CTG C
Collagen 2A1	AAG AAA CAC ATC TGG TTT GGA GAA A	TGG GAG CCA GGT TGT CAT C	CAA CGG TGG CTT CCA CTT CAG CTA TGG
Aggrecan	CCA ACG AAA CCT ATG ACG TGT ACT	GCA CTC GTT GGC TGC CTC	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
MMP-3	GGC TGC AAG GGA CAA GGA A	CAA ACT GTT TCG TAT CCT TTG CAA	CAC CAT GGA GCT TGT TCA GCA ATA TCT AGA AAA C
MMP-13	CCA TCT ACA CCT ACA CTG GCA AAA G	GTC TGG CGT TTT GGG ATG TT	TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
COMP	CCA GAA GAA CGA CGA CCA GAA	TCT GAT CTG AGT TGG GCA CCT T	ACG GCG ACC GGA TCC GCA A
PRG4	GAG CAG ACC TGA ATC CGT GTA TT	GGT GGG TTC CTG TTT GTA AGT GTA	CTG AAC GCT GCC ACC TCT CTT GAA A

Biochemical analysis: s-GAG and DNA content

Cell-scaffold constructs (removed from osteochondral defect models) and media were collected for biochemical analysis. Chondrocytes-scaffold constructs were digested overnight in 0.5 mg/mL proteinase K at 56°C (2.5 U/mg, chromozyme assay; Roche, Mannheim, Germany). DNA content was measured using QUANT-iT® Picogreen, ds assay kit (Molecular Probes, Life Technologies), and values were normalized per scaffold volume. The total amount of sulfated glycosaminoglycan (s-GAG) retained within the scaffold constructs and released from the osteochondral defect models into the media were determined by the dimethylmethylene blue (DMMB) dye-binding assay [235].

STATISTICAL ANALYSIS

The results are expressed as mean +/- standard deviation (SD) of 3 experiments with different chondrocyte donors. s-GAG, DNA content and qPCR data were statistically analysed using non-parametric testing (Mann Whitney U test), since the data were not normally distributed. Differences were considered statistically significant for $p < 0.05$.

RESULTS

Validation of the osteochondral model generation

To validate the generation of the osteochondral defect model, measurements were taken to evaluate the osteochondral explant obtainment and the reproducibility of the defect creation. It is worth noting that having this procedure standardized is of critical importance for the mechanical loading set up. Explants obtained from stifle joints harvested from 8 calves had an average diameter of 7.60 mm and length of 6.10 mm, with standard deviations of 0.14 mm and 0.29 mm, respectively (Fig. 1E). Cartilage height was measured to define the depth of osteochondral defects; the height varied between approximately 3 mm and 4 mm, depending on the bovine donor. Cylindrical holes were created with depths of 2.85 mm +/- 0.11 mm for the 3 mm cartilage height group, or 3.75 mm +/- 0.30 mm for the 4 mm cartilage height group and were 4.30 mm +/- 0.16 mm in diameter (Fig. 1F). Depths were measured from the upper rim of the cartilage by a custom-made crown mill to the level of the circular groove made. In all cases measured, a reproducible procedure was observed. Coefficients of variation of the intact osteochondral explant diameter and height (n=40), and defect diameter and depth are shown in table 2.

Table 2. Coefficients of variation of intact osteochondral explants and osteochondral defect models

Osteochondral Explant	Coefficient of Variation (%)
Height	4.99
Diameter	1.76
Osteochondral Defect Model Diameter	4.78
Osteochondral Defect Model Depth (High)	2.47
Osteochondral Defect Model Depth (Low)	3.76

Evaluation of osteochondral cells morphology and viability after mechanical stimulation

To verify the viability of the cells in the osteochondral defect models, with or without exposure to mechanical stimuli, LDH/ethidium homodimer positive cells were

determined after samples collection at day 0 and after 10 days of culture. Cells within both cartilage and bone regions remained viable throughout the culture period (Fig. 3A, B), except for a small zone of cell death in the outermost cell layer at the cut edges of the cartilage and the edges of the defect for all samples (Fig. 3A).

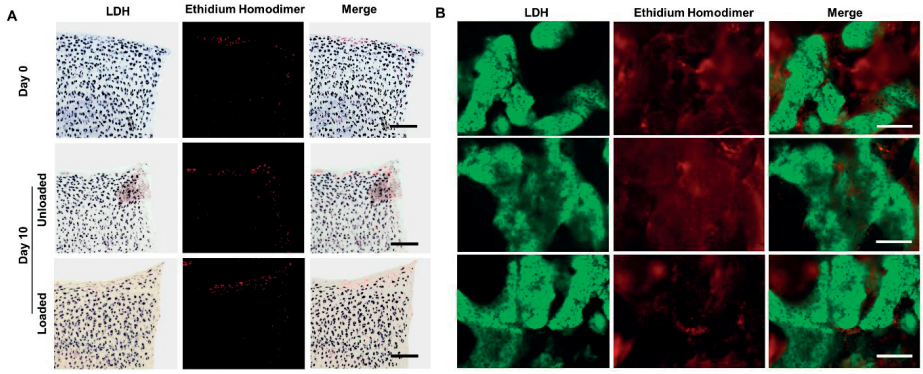

Figure 3. Osteochondral explant viability. Representative images of LDH/Ethidium homodimer stained cells at day 0 and day 10 for unloaded and loaded samples, A) in the cartilage B) in the bone. Scale bars indicate 200 µm (dark-blue cells and double-stained cells = LDH positive cells, representing living cells; red cells = Ethidium Homodimer positive cells, representing dead cells; green is bone autofluorescence, 515-565 nm emission filter [234]).

The loading regime did not affect cell viability in comparison to the free-swelling controls. Safranin O Fast Green staining revealed normal proteoglycan distribution (Fig. 4A), GAG measurement in the medium indicated that the mechanical stimuli did not wear out the cartilage GAG, as no difference in GAG release into the media was detected in comparison to the free swelling controls (Fig. 4B). Chondrocytes maintained their typical morphology, with rounded and polygonal shape in both conditions (Fig. 4A).

Physical stimulation of chondrocytes-seeded scaffold in osteochondral defect models

To test the biological response of chondrocytes-seeded scaffolds implanted into the osteochondral defect model to mechanical loading, DNA, GAG content and mRNA expression levels of the constructs were quantified after 5 days of loading (Figs. 5, 6). Cell-scaffold constructs which underwent loading contained similar amounts of DNA as the unloaded controls (Fig. 5A). GAG content normalised to DNA was stable between the groups, indicating no effect of the loading on GAG production per cell (Fig. 5B). This was confirmed by Safranin O/Fast Green staining of scaffold constructs, where similar amounts of matrix deposition were observed in both groups (Fig. 5C).

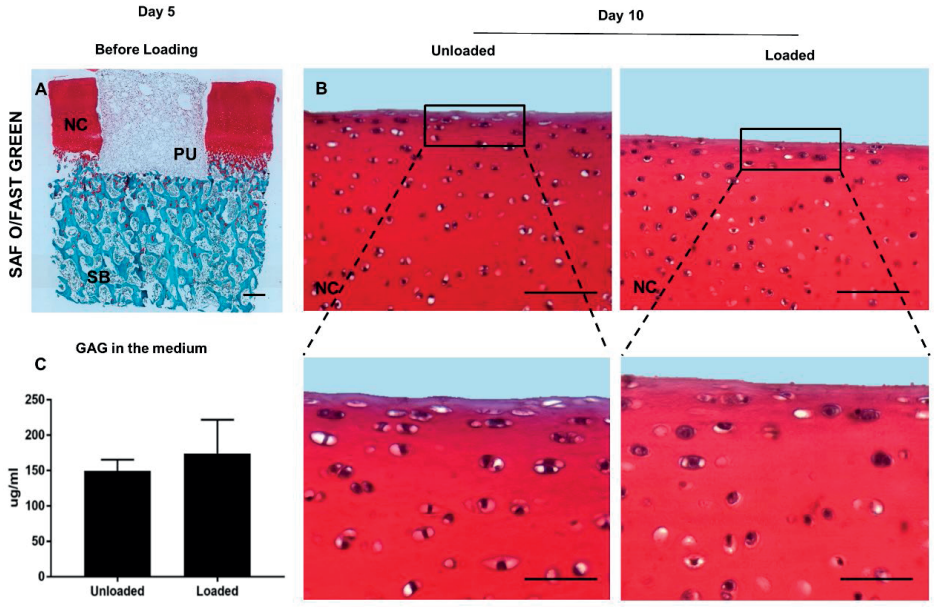

Figure 4. Effect of loading on articular cartilage. A) SAF-O/Fast Green stained osteochondral biopsies. Detail images of articular cartilage after 10 days of culture for unloaded and loaded samples, respectively; 20x magnification, scale bars indicate 100 µm. Dashed lines indicate sections at 40x magnification, scale bars are 50 µm. B) GAG release into the medium at day 10 of culture. Data are presented as mean +/- SD (3 donors, n=12 per group).

The mRNA expression levels of *PRG4/*Lubricin and *COMP* were significantly enhanced in cell-scaffold constructs exposed to complex load as compared to unloaded controls (8.4 and 9-fold increase, respectively; $p<0.001$. Fig. 6A, B). The mRNA ratios of *COL2A1* to *COL1A2* and of *ACAN* to *VCAN*, defined as indices of chondrocytes differentiation, were significantly higher in loaded samples compared to free-swelling controls ($p=0.015$ and $p<0.001$, respectively) (Fig. 6C, D). The gene expression levels of metalloproteinases *MMP3* and *MMP13* remained relatively stable and considering donor variations no significant differences were observed between the groups (Fig. 6E, F).

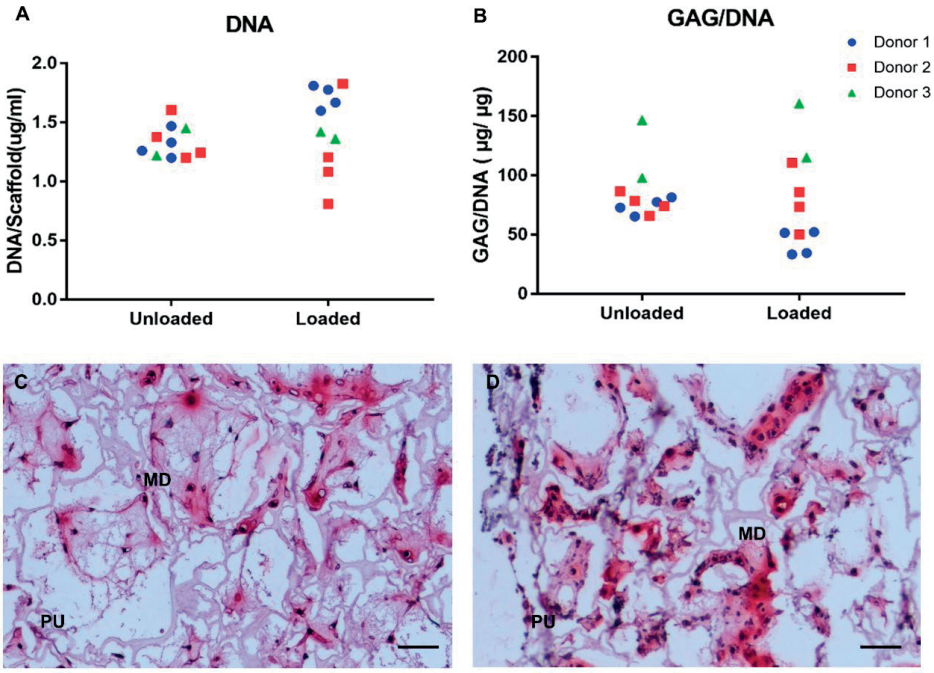

Figure 5. DNA and GAG content in chondrocyte seeded scaffolds in osteochondral defect models cultured in the bioreactor. A) DNA content of unloaded and loaded chondrocytes seeded polyurethane scaffolds cultured for 10 days. B) GAG per DNA ratio of unloaded and loaded chondrocytes seeded polyurethane scaffolds cultured for 10 days. C, D) detail images Safranin O/ Fast Green stained sections of PU scaffolds after 10 days of culture for unloaded (C) and loaded samples (D), respectively; scale bars indicate 50 µm. Results from 3 chondrocyte donors assessed in duplicates (donor 3) or quadruplicates (donors 1 and 2) are shown. SB: Subchondral bone, NC: Native Cartilage, PU: Polyurethane scaffold, MD: Matrix deposition.

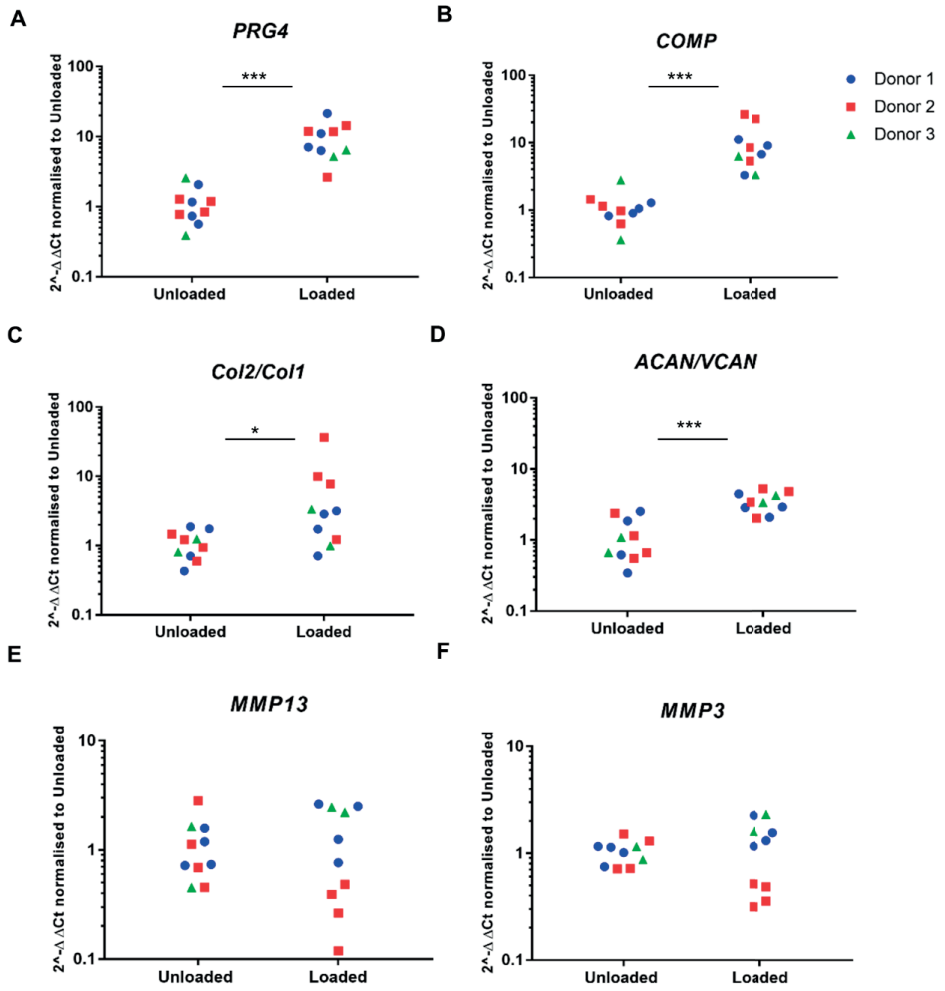

Figure 6. Effect of articular motion on the chondrocytic phenotype. A-F) mRNA expression of chondrocytes seeded into polyurethane scaffolds, implanted in the osteochondral defect, and exposed to dynamic compression and surface motion. Data are expressed relative to mRNA levels of unloaded constructs. Results from 3 chondrocyte donors assessed in duplicates (donor 3) or quadruplicates (donors 1 and 2) are shown; *p<0.05.

DISCUSSION

Considering articular cartilage as load bearing tissue, multi-axial stimuli applied to an *ex vivo* osteochondral defect model are important parameters to investigate their effects on the cartilage repair process. The present study showed that osteochondral defect models, in which mechanical loads were applied: 1) were viable after 5 days of pre-culture

and 5 days within the complex motion bioreactor culture based on LDH staining, 2) did not exhibit articular surface wear as assessed by Safranin O/Fast Green staining and GAG release into the media; and 3) maintained typical gene expression responses (Lubricin and COMP) to load in primary chondrocytes seeded into polyurethane scaffolds filling the osteochondral defects. Further, we showed that reproducible defects could be created at the desired depth. This was reflected by the coefficients of variation that were less than 4% for both depth and diameter. Earlier described osteochondral defect models did not precisely document the control of the defect depth [115, 236, 237]; others directly implanted the osteochondral biopsies *in vivo* [238, 239]; but to our knowledge none of them were intended for *ex vivo* mechanobiology and regenerative studies under complex articulating motion [240-242].

No detrimental effect of mechanical load on the cartilage samples was observed. Both histology and GAG release into the medium of the ceramic ball compression/ articulation group did not show significant differences compared to the free swelling control group. This finding indicates more favorable outcomes in terms of matrix wear and tissue preservation compared to other *ex vivo* studies with complex motion [243] or animal models [244, 245], which used metal articulating indenters or implants to mimic the situation after surgery (e.g. hemiarthroplasty). Surface chemistry and roughness of metal implants can increase friction against articular cartilage and can have significant influence on tissue integrity [246]. Overall, chondrocytes viability was mostly preserved by using saline irrigation for cooling when generating the explants, and no difference in cell death was found in osteochondral explants that underwent compression and shear stress compared to controls; nevertheless, minimal chondrocytes death was observed at the outermost edges of the osteochondral explant and of the defect, as previous works already described [247, 248]. This observation might be representative of a clinical cartilage defect where dead cells have been found along the edges of the injury following joint trauma [249]. Furthermore, a zone of chondrocyte death has been described in and around the periphery of osteochondral grafts that could be reduced by the application of growth factor and collagenase [250]. Therefore, this model could also be very interesting to study such clinically relevant aspects.

Although our model still does not match the native whole joint situation, the application of complex motion patterns using a ceramic ball and the implementation of osteochondral defect models, makes it one step closer to a more physiologically relevant system compared to cartilage explants alone [233]. Dynamic compression and ball oscillation drag flow into the osteochondral defect filled with chondrocytes-PU constructs, resulting in the activation of mechano-transduction pathways which severely depend on the type of load [251]. The loading protocol used in this study was chosen based on a protocol previously described by our group: Grad. et al. investigated the effect of unidirectional and multidirectional motion patterns on gene expression

and molecule release of bovine chondrocytes-seeded polyurethane scaffolds [81]. After 5 days of loading, as in the present work, results showed that multidirectional loading consisting of axial compression and ball oscillation, promoted to the maintenance of the chondrocytic phenotype thorough upregulation of chondrogenic gene expression.

In the present study, the oscillation frequency was set at 0.5 Hz, which is higher than the 0.1 Hz used in the previous study. Our previous evaluation of the effect of sliding velocity on the response of chondrocytes in 3D scaffolds revealed that higher frequency generally triggered a more pronounced response [187]. Accordingly, 1 Hz frequency which approximates the frequency of compressive loading the human articular cartilage experiences during walking and running conditions, induced greatest gene expression upregulation. Interestingly, increasing the frequency from 0.1 to 1 Hz also improved the induction of chondrogenesis in mesenchymal stem cell seeded scaffolds exposed to multiaxial loading [86]. Here, we slightly reduced the loading frequency to 0.5 Hz to minimize the articular cartilage surface injury [252] in the osteochondral model.

The results of this study demonstrated a marked increase in PRG4 gene expression in the loaded group (9-fold higher compared to unloaded), which is in line with previous studies on the effects of complex load on articular chondrocytes [81, 219]. The higher response of PRG4 observed in this study compared to previous results may be related to: a different oscillation frequency of the ceramic ball, different impact of the sliding velocity at the new articular cartilage-PU interface [187] and the more confined system, in which the chondrocytes could sense different stress distribution within the scaffold [253]. In the fibrin–polyurethane composite scaffolds used in the present study, hydrostatic pressure buildup due to the application of external loading would be negligible in an unconfined system because of the high permeability of the scaffolds [253]. In the current confined system, certain hydrostatic pressure is built up, which has previously been shown to promote chondrogenesis, though the effect of pure hydrostatic pressure on PRG4 expression of chondrocytes has not been investigated [254]. Consistent with previous findings [81], the influence of oscillating surface motion also promoted the upregulation of COMP (8.4-fold increase), one of the most abundant non-collagenous proteins of the cartilage ECM. Earlier studies exploring the influence of uni- and multi-axial loading on gene expression in chondrocytes-seeded polyurethane scaffolds demonstrated that the induction of COMP gene expression depended on the loading type and velocity. Axial compression alone did not affect COMP mRNA expression, whereas compression and superimposed sliding motion by ball oscillation significantly increased COMP mRNA levels [81]. Besides, increasing sliding velocity triggered more pronounced up-regulation of COMP gene expression [187]. Furthermore, an increase in the mRNA ratios of Collagen 2 to Collagen 1 and Aggrecan to Versican, defined as markers of chondrocytes differentiation [194], was also associated with the application of compression and shear. These data suggest that physiological stimuli are essential for

stimulation of the chondrogenic phenotype and, more indirectly, for cartilage matrix formation and organization, despite the total GAG per DNA did not show variations between the two groups. Longer term repetitive loading may be necessary to induce significant effects on matrix production [83]. The joint motion simulator did not affect the gene expression of the two matrix degrading enzymes, MMP3 and MMP13, that are involved in joint pathologies. This indicates that mechanical stimuli did not specifically foster collagenase-induced extracellular matrix degradation in chondrocytes implanted into osteochondral biopsies.

A chondrocytes-seeded hybrid fibrin-polyurethane scaffold was used as a model implant in this study. The fibrin component served to improve the cell and matrix retention and to better promote the chondrocytic phenotype compared to the macro-porous PU structure alone [231]; while the elastic PU scaffold has been shown to favorably transmit the applied dynamic mechanical loads [251]. Nevertheless, this material faces some limitations, such as the slow rate of ECM accumulation in the construct center. Other promising materials, for instance injectable thermo-reversible methylcellulose-based hydrogels [255], modified hyaluronic acid hydrogels functionalized with biochemical gradients [256] or biopolymers with improved tissue adhesion properties [257] will be envisaged in future studies.

The model has the advantage of having the cartilage-bone unit intact. Cartilage and bone have been demonstrated to influence each other, and it is known that not only the cartilage but also bone responds to mechanical stimulation to preserve the mechanical strength and impede demineralization [258]; thus, this loaded osteochondral model provides the possibility to recapitulate the healing process in a joint-like microenvironment. There is a body of evidence suggesting that these tissues can communicate. For example, the interface between the subchondral bone and calcified cartilage contains numerous vascular canals suggesting a potential route for molecular diffusion between the two compartments [259-261]. The model can also be used to study the effect of bone changes (that occur in joint diseases such as marrow lesions or subchondral sclerosis) on cartilage repair. Further optimization of the model might include the addition of hyaluronic acid in the bioreactor culture to better mimic the joint space [262]. Another adaptation to the model that can be envisaged is the development of a cartilage-on-cartilage articulating motion system [233], which will better resemble the natural joint niche and will reduce the friction of the testing system. While the present study allowed us to assess the early cellular response to multiaxial load in a confined system, longer studies over at least 3 weeks would be required to achieve neo-cartilage formation by accumulation of significant amounts of extracellular matrix within the cell-seed implants. A further limitation of the short observation time is the inability to draw conclusions about the implant integration into the host tissue. Nevertheless, we showed the medium-term survival and integrity of the cartilage-bone

explant and the reaction of the implanted cells to the applied load, which warrants future long-term studies with advanced cell-material constructs.

Several applications are possible: cartilage repair treatments could be screened *ex vivo*, for example to test the potential of different cell sources and new biomaterials denoting their capabilities to promote chondrogenesis and to integrate into the native tissue. In addition, recruitment of endogenous cells from cartilage or underlying bone into the osteochondral defect and migration of these cells into a biomaterial can be studied with or without the addition of chemokines or growth factors to stimulate tissue repair as cell-free cartilage repair strategy [263]. The system can also perform continuous passive motion with intermittent active motion and hence improve our understanding on the post-operative management of joint injuries and on the time of convalescence, since post-operative loading also affects the quality of the cartilage surgery outcome [264].

We also believe that the *ex vivo* bioreactor-osteochondral culture model may represent an alternative pre-clinical testing system to evaluate the potential and limitations of the different treatment approaches prior moving to *in vivo* testing, in order to minimize the number of animals needed. Where animal testing cannot be replaced, *ex vivo* bioreactor cultures could contribute to identify the proper animal species, the suitable number of animals and to find biologically and statically relevant differences among groups [225]. A multi-center analysis has shown low correlation between *in vitro* cell culture and *in vivo* biomaterial testing for bone regeneration [265]; hence, pilot studies could be performed *ex vivo* with explants from animals of the same species to help bridging this gap. Furthermore, with this model human tissue can be tested, which is an unprecedented opportunity to be human relevant.

Nonetheless, the osteochondral defect model under load faces certain limitations. As it does not resemble the entire diarthrosis, it is not possible to replicate the whole range of events determining the body's healing response in cartilage repair in vivo. The wound healing process is significantly affected by the synovium and synovial fluid, which play a significant role in nutrient supply, metabolic by-product clearance and immune response, thereby influencing matrix production [266]. A critical element in cartilage healing is also the defect size. A rabbit model has shown that different diameters of osteochondral defects heal differently [13]. Our present system only partially reproduces human osteochondral lesions, which can be at least 2 cm^2; therefore, the smaller defect repair may not exactly indicate the cell behaviour adopted in a larger defect. Minor modification of our current model will be required to address also large defect sizes. Last, our bioreactor does not perfectly mimic the complex joint kinematics; rolling or moving contact has not been implemented in our system, which is another important motion component.

In conclusion, we have established a novel *ex vivo* osteochondral defect culture model in a mechanically stimulated microenvironment. Such a model has both experimental and clinical relevance; it can serve to further elucidate the biological and physical crosstalk among the subchondral bone and cartilage in the recovery of osteochondral defects and may help to reveal the molecular signaling involved in the repair in response to a treatment. It will also prove its efficiency regarding controlling cartilage repair under the influence of different loading protocols. Longer-term studies over several weeks will be performed to monitor and evaluate cell and biomaterial-guided neo-cartilage formation and neo-tissue integration using novel cartilage repair methods.

5 Mechanical stress inhibits early stages of endogenous cells migration: a pilot study in *ex vivo* osteochondral model

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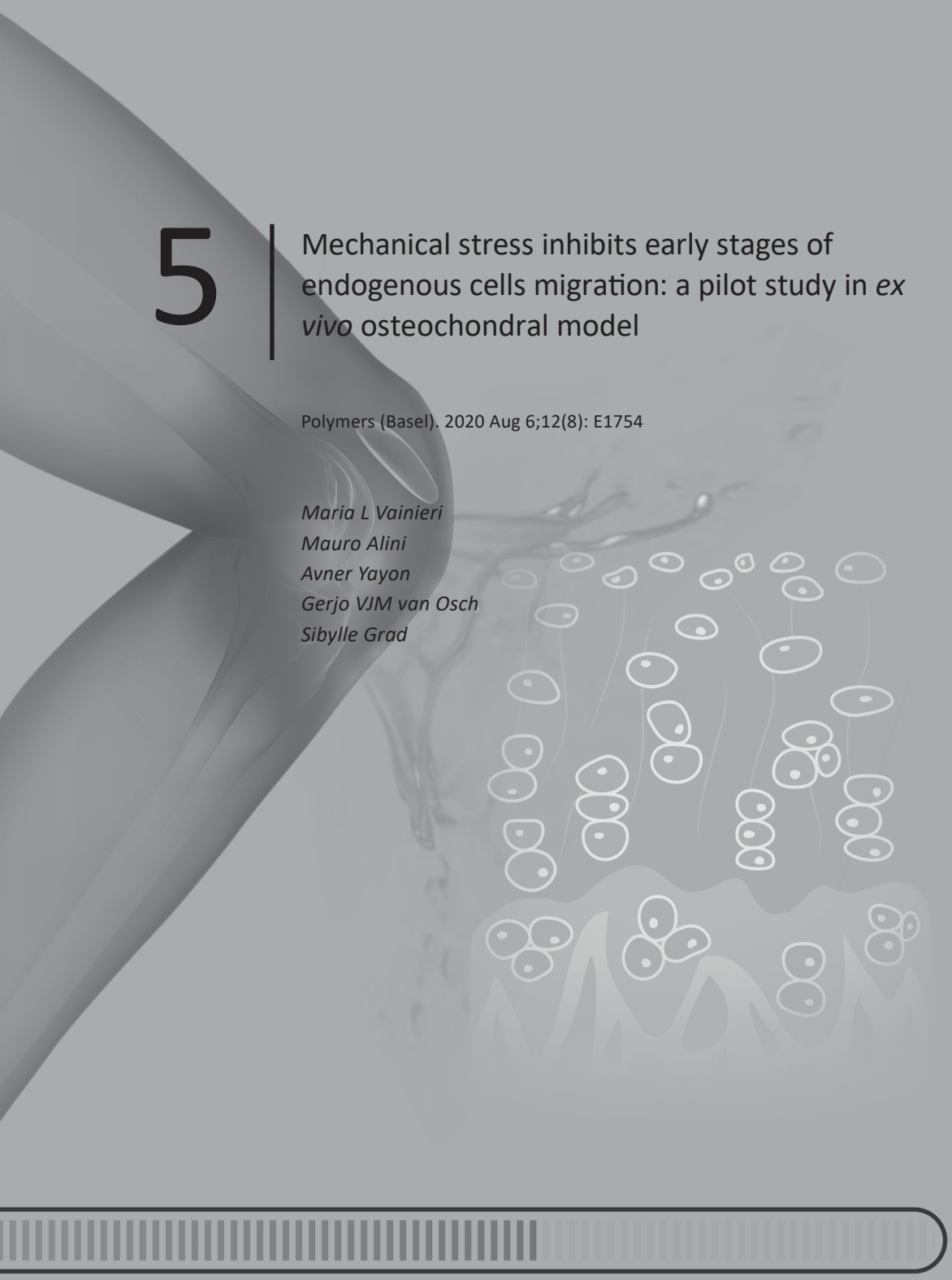


ABSTRACT

Cell migration has a central role in osteochondral defect repair initiation and biomaterial-mediated regeneration. New advancements to reestablish tissue function include biomaterials and factors promoting cell recruitment, differentiation and tissue integration, but little is known about responses to mechanical stimuli. In the present pilot study, we tested the influence of extrinsic forces in combination with biomaterials releasing chemoattractant signals on cell migration. We used an *ex vivo* mechanically stimulated osteochondral defect explant filled with fibrin/hyaluronan hydrogel, in presence or absence of platelet-derived growth factor-BB or stromal cell-derived factor 1, to assess endogenous cell recruitment into the wound site. Periodic mechanical stress at early time point negatively influenced cell infiltration compared to unloaded samples, and the implementation of chemokines to increase cell migration was not efficient to overcome this negative effect. The gene expression at 15-day of culture indicated a marked downregulation of matrix metalloproteinase *(MMP)13* and *MMP3,* a decrease of *β1* integrin and increased mRNA levels of actin in osteochondral samples exposed to complex load. This work using an *ex vivo* osteochondral mechanically stimulated advanced platform demonstrated that recurrent mechanical stress at early time point impeded cell migration into the hydrogel, providing a unique opportunity to improve our understanding on management of joint injury.

Keywords: Biomaterial; hydrogel; cartilage; osteochondral; mechanical loading; endogenous cell recruitment

INTRODUCTION

Articular cartilage plays a key role in the function of joints, and when damaged it becomes inefficient to withstand harsh conditions over time, posing a significant challenge among clinicians. The very poor intrinsic healing capacity of this tissue in combination with the high incidence of trauma place at risk many asymptomatic young and healthy patients toward the evolution of degenerative conditions with reduced possibility of interventions [267]. Surgical procedures including microfracture and osteochondral allografts are being applied in clinical practice. While the former is far from being successful in replacing the damaged cartilage by repair tissue with long-lasting hyaline properties [268], the latter is often a last resource revision surgery after failed attempts of cartilage reconstruction [269], in order to address the subchondral changes seen in the revision setting. The invasiveness of this procedure due to the removal of a healthy cartilage portion together with the potential graft-size mismatch, may hamper the efficacy of this intervention. Emerging opportunities with cell-based repair approaches are considered, such as autologous chondrocytes implantation (ACI) [19], matrix-assisted ACI (MACI) [270] and transplantation of autologous mesenchymal stem cells (MSCs) [93]. Studies comparing patients treated with these strategies have shown similar improvements in term of clinical outcome, although longer periods of randomized trials are required to conclude effective regeneration [23, 24]. Nevertheless, cell therapy faces limitations in clinics in term of costs, safety and quality controls [31]. With the perspective to circumvent these issues and take advantage of bone marrow and bone lining stem/progenitor cells, biomaterial implantation is used to enhance the natural healing process that microfracture affords. An example of this procedure called autologous matrix-induced chondrogenesis (AMIC) is using a collagen membrane to enhance cartilage repair by endogenous progenitor cells [271]. While no difference was found in the outcome between ACI and AMIC for treating cartilage defects in a 2-years follow-up [272], this technique promoted cell-free alternatives via the conception of an instructed microenvironment toward regeneration .

In recent years the modulation of biochemical and biophysical cues, when considering the design of biomaterials used as 3D templates for tissue regeneration, have advanced our understanding of cartilage repair processes [63]. These determinants control both extracellular matrix environment and cell behavior such as cell adhesion, migration and differentiation, which are key processes for successful formation of functional tissues. Indeed, a number of studies have demonstrated that incorporation of small oligopeptides (such as RGD, [273, 274]) conjugated to the backbone of polymers can improve their function as adhesive materials; the presence of chemotactic stimuli in hydrogels, such as platelet-derived growth factor-BB (PDGF-BB) or stromal cell-derived factor 1 (SDF-1α) [62, 66], can enhance cellular migration. Notably, the modulation of

matrix-metalloproteinase (MMP) activity combined with RGD peptides or the addition of micro-RNAs were shown to be able to promote endogenous cell recruited cartilage repair [61, 275], while the introduction of MMPs can also enhance graft integration to the wound site [276].

In agreement with a body of evidence from literature, our previous work on cartilage healing using *in vitro* studies and a model for osteochondral defect repair after subcutaneous implantation in mice suggest that stiffer materials represent a barrier to endogenous healing [62], where matrix limits infiltration and remodeling near injury sites. Cell migration has a critical role in the early process of biomaterial-assisted tissue repair. While several factors are important for cartilage repair success, to render cell-free technologies clinically feasible, mechanical factors should be considered to evaluate their performance in a physiological joint environment. Mechanical loading plays an important role for spontaneous and biomaterial guided chondral and osteochondral defect repair. Several *in vitro* studies have demonstrated that compressive and/or shear load promoted the anabolic phenotype and cartilaginous matrix synthesis of articular chondrocytes and induced chondrogenic differentiation in mesenchymal stem cells [176, 277-280]; these findings led to the definition of regenerative rehabilitation principles in translational orthopedics [281]. Gene expression is affected by mechano-transduction, which results in rapid and long-term cellular changes mediated by integrin-dependent RhoA signaling and downstream actin dynamics. Mechanical compression of glycoprotein-polysaccharide complexes (present on cell surface), which exert electrosteric repulsion to the ECM, around integrin-ligand complexes promote integrin activation and clustering in a kinetic trap manner [282]. This process facilitates focal adhesion activation to the matrix and contraction, which result in different cell responses depending on ECM stiffness, cell distribution and density to control proliferation and differentiation [283, 284]. Matrix is actively organized by cells through their integrins, with the actomyosin machinery allowing them to pull or push on collagen fibers to then establish a new mechanical state [285]. In condition of high tension, tenascin's transcription increase and reduce cellular interaction by decreasing Rho activity and gel contraction by the cell, suggesting a key role of this protein in the negative feedback loop to promote mechanical homeostasis under high stress condition [286, 287]. These results suggest that an appropriate loading regime could facilitate the development of a stable cartilage phenotype. In an *in vivo* rabbit osteochondral defect model treated with cell-free porous poly(lactic-co-glycolic acid) graft implants, daily treadmill exercise resulted in improved outcome in terms of hyaline cartilage tissue formation [288]. However, the effect of early mechanical stimulation on the recruitment of endogenous cells for cartilage and osteochondral defect repair remains largely unknown [263].

The success of material-based systems for osteochondral defect repair depends on the ability of the scaffolds to sustain compressive, shear and tensile forces during joint loading. Although most hydrogels are not ideal materials to resist complex motion, mechanical properties can be enhanced by modifying polymers with functional groups to form hydrophilic structures and increase the crosslinking density in the network [53]. Fibrin/hyaluronan (FB/HA) hydrogel formulation had previously been investigated *in vitro* and *in vivo* as suitable material for cell infiltration for repair of articular cartilage defects and to withstand mechanical loading [61, 62, 289]. With such regenerative tools in our hands, our goal was to test the influence of applied extrinsic forces on the endogenous cell recruitment process by using our custom-made joint bioreactor. To achieve that, we used an *ex vivo* mechanically stimulated osteochondral defect explant model filled with (FB/HA) hydrogel in the presence or absence of PDGF-BB or SDF-1α to further enhance cell infiltration. In the present study, we hypothesize that mechanical compression and shear would modulate early endogenous cell migration.

MATERIALS AND METHODS

Osteochondral tissue harvest and culture

Osteochondral explants were harvested from stifle joints of 5 to 8-months-old calves, obtained from a local abattoir (Metzgerei Angst AG, Zurich, CH) within 48 hours of slaughter. Previous studies using the same timeframe have shown explant viability preservation for up to 28 days [115, 137]. Cylindrical osteochondral plugs were obtained as previously described [137] with an 8 mm diameter custom-made coated trephine drill (Peertools AG, Ftan, CH). The subchondral bone part was trimmed to obtain a final explant height of 6 mm. To generate osteochondral defects of 3 mm depth, a 4 mm diameter trephine drill was used (Brutsch-Ruegger, Urdorf, CH). Subsequently osteochondral explants were placed in bioreactor holders containing 2% low-gelling agarose (SeaPlaque Agarose, Lonza, Rockland, USA), to cover the bone part and prevent cell outgrowth from the subchondral bone. Then, explants were cultured in Dulbecco's modified Eagle medium (DMEM-HG, 4.5 g/L-glucose; Gibco) supplemented with 1% insulin-transferrin-selenium (ITS, Corning), non-essential amino acids, 1% penicillin-streptomycin (Gibco), 25 µg/mL ascorbic acid-2-phosphate (AA-2-P, Sigma-Aldrich, Saint Louis, MO), amino caproic acid (Sigma) and 100 nM dexamethasone (Sigma) at 37°C and 5% CO_2. The medium, referred to as chondro-permissive medium, was changed three times per week.

Fibrin-HA hydrogel preparation and incorporation of PDGF-BB or SDF1α

FB/HA conjugates were synthesized via a two-step reaction as previously described [60]. Final concentrations of 6.25 mg/mL FB and 1.96 mg/mL of HA-active ester solution (FB/HA w/v ratio of 3.2:1) were used with HA molecular weight of 235 kDa (LifeCore Biomedical, LLC, Chaska, MN, USA). Briefly, HA was first reacted with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma, Israel) and *N*-hydroxysuccinimide (NHS; Sigma, Israel) to convert part of its carboxylic groups to NHS-active ester moieties. In a second step, a buffered solution of fibrinogen (Omrix, Israel) was reacted with the HA active ester solution to produce a clear FB/HA conjugate solution. Hydrogels were then prepared by mixing thrombin solution (50 U/mL, Sigma-Aldrich) containing calcium chloride (1M $CaCl_2$) with FB/HA conjugate and polymerizing at 37°C for 30 minutes. The rheological features of the resulting hydrogels were characterized in previous work [60, 62].

PDGF-BB or SDF-1α (both Peprotech, London UK) were added to the FB/HA conjugate solution prior to polymerization to obtain final concentrations of 2 µg/mL of PDGF-BB or 10 µg/mL of SDF-1α, respectively. The selected dose of PDGF-BB used in the following experiments was chosen based on our previous FB/HA hydrogel release study [62], since the factor in the *ex vivo* osteochondral model was expected to be released over several days; while the SDF-1α concentration was chosen based on our previous study demonstrating that the factor could enhance MSCs migration in the intervertebral disc [290].

Ex *vivo* osteochondral defect model for endogenous cell recruitment under mechanical loading

For *ex vivo* explant culture, 50 µL of FB/HA or FB/HA carrying chemotactic factors were cast into the osteochondral explants after defect creation. Then, osteochondral explant constructs were cultured in 3 mL of chondro-permissive medium and loaded in our bioreactor system. Osteochondral plugs underwent mechanical stimulation using a four-station bioreactor system, installed in a CO_2 incubator at 37°C, 5% CO_2, 85% humidity [180]. A ceramic hip ball (32 mm in diameter) was pressed onto the osteochondral plugs to provide a constant displacement of 0.4 mm or 10% to 14% of the cartilage height (~ 3 to 4 mm), to fully maintain the contact of the ball with the hydrogel and the surrounding cartilage. Loading groups were exposed to axial compression in a sinusoidal manner between 0.4 mm and 0.55 mm, resulting in an actual strain amplitude of 10-13.7% or 14-18.3% of the cartilage height at a frequency of 0.5 Hz and contemporary shear motion by ball oscillation at ±25° and 0.5 Hz.

One hour of mechanical loading was performed per day over either 6 days or 15 days from the start of the culture (experimental scheme is represented in Figure 1A). In between loading cycles, samples were kept in free-swelling condition (no contact

with ceramic ball). Unloaded explants with hydrogel served as controls. After loading, osteochondral explants were collected for DNA and RNA isolation or histological analysis.

Histology

Samples for histology were fixed in 4% buffered formaldehyde (Formafix AG, Hittnau, CH) for 24 hours, dehydrated until absolute ethanol, then embedded in methyl methacrylate (MMA) and sectioned in 130 µm sections. For staining, slides were treated with 1% formic acid and subsequently rinsed in tap water and dH_2O. Toluidine blue staining was performed to visualize migrated cells and cartilage matrix. Briefly, slides were stained with 1% Toluidine blue for 1 min while heated at 55-60°C on hot plate, rinsed in deionized water for 1 min and blot dried. Images were acquired using an optical microscope (Olympus).

The number of the infiltrated cells was determined using Fiji software (National Institutes of Health, Bethesda, MA, USA). Cell colonization into the defect was assessed at day 15 by counting cell infiltration number in the defect area following specific criteria. Osteochondral defects of Toluidine blue stained cross-sections (n=3/group) were divided in three subsections of 1 mm height (S1, S2 and S3; Figure 2A). The number of migrated cells per explant was defined as the sum of the numbers of migrated cells in three sagittal sections of the explant. RGB images were converted in 8-bit by using a trainable Weka segmentation plugin, in order to extract results by excluding the background (Toluidine blue staining) and selecting the area of interest (in this case the cells), as previously described [62].

RNA extraction and gene expression analysis

After 15 days of culture, FB/HA hydrogels were removed from the explant, homogenized using the Tissue Lyser system (Qiagen, Retsch, Germany), and total RNA of the migrated cells was extracted using AllPrep DNA/RNA Micro Kit (Qiagen). RNA concentration and quality were measured using NanoDrop 1000 spectrophotometer (Thermo). cDNA was prepared using SuperScript Vilo IV Master Mix (ThermoFisher) according to the manufacturer's instructions and real time PCR was performed on a Quant Studio Flex 6 instrument (ThermoFisher). Table 1 shows the sequences of bovine primers and TaqMan® probes for collagens type-I (*COL1A2*), type-II (*COL2A1*), aggrecan (*ACAN*), matrix metalloproteinase 3 (*MMP-3*), *MMP-13,* and the catalogue numbers of the gene expression assays used for amplification of ribosomal protein lateral stalk subunit P0 (*RPLP0*), versican (*VCAN*), *β*1-integrin (*TFB1M*), and beta-actin (*ACTB*) (Applied Biosystems, Rotkreuz, Switzerland). Data collected at day 15 were expressed as relative values of target mRNA and determined according to the comparative C_T method. First the target gene expression was normalized to the expression of the reference gene *RPLP0*. This reference gene had been shown to remain stable under mechanical loading

conditions, whereas other commonly used reference genes such as glyceraldehyde 3-phosphate dehydrogenase *GAPDH* may be affected by mechanical load [291]c. In a second step the normalized target gene expression levels of samples treated by load and/or chemokine were expressed relative to the corresponding control sample for each donor. The control sample was neither treated by load nor by chemokine delivery. In this way, inter-donor variation was excluded, while only the effect of load and/or chemokine was assessed.

Table 1. Oligonucleotide primers and probes used for qRT-PCR. MMP: Matrix metalloproteinase; VCAN: Versican; TFB1M: Beta-1-integrin; ACTB: Beta-actin. FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.

Gene		Sequence or cat. nr.
Collagen 1A2	*Primer forward (5'-3')*	TGC AGT AAC TTC GTG CCT AGC A
	Primer reverse (5'- 3')	CGC GTG GTC CTC TAT CTC CA
	Probe (5'FAM- 3'TAMRA)	CAT GCC AAT CCT TAC AAG AGG CAA CTG C
Collagen 2A1	*Primer forward (5'-3')*	AAG AAA CAC ATC TGG TTT GGA GAA A
	Primer reverse (5'- 3')	TGG GAG CCA GGT TGT CAT C
	Probe (5'FAM- 3'TAMRA)	CAA CGG TGG CTT CCA CTT CAG CTA TGG
Aggrecan	*Primer forward (5'-3')*	CCA ACG AAA CCT ATG ACG TGT ACT
	Primer reverse (5'- 3')	GCA CTC GTT GGC TGC CTC
	Probe (5'FAM- 3'TAMRA)	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
MMP-3	*Primer forward (5'-3')*	GGC TGC AAG GGA CAA GGA A
	Primer reverse (5'- 3')	CAA ACT GTT TCG TAT CCT TTG CAA
	Probe (5'FAM- 3'TAMRA)	CAC CAT GGA GCT TGT TCA GCA ATA TCT AGA AAA C
MMP-13	*Primer forward (5'-3')*	CCA TCT ACA CCT ACA CTG GCA AAA G
	Primer reverse (5'- 3')	GTC TGG CGT TTT GGG ATG TT
	Probe (5'FAM- 3'TAMRA)	TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
VCAN	Cat. nr.	Bt03217632_m1
TFB1M	Cat. nr.	Bt03269747_m1
ACTB	Cat. nr.	Bt03279174_g1
RPLP0	Cat. nr.	Bt03218086_m1

DNA content measurement

Hydrogels were assessed for DNA content after removing the FB/HA hydrogel from the osteochondral explants followed by homogenization in a Tissue Lyzer for sample disruption (Qiagen, Retsch, Germany). DNA was purified using AllPrep DNA/RNA Micro Kit (Qiagen), and its content measured by Qubit 1X dsDNA HS assay kit following manufacturer's instruction (Qubit 4.0 Fluorometer Invitrogen).

STATISTICAL ANALYSIS

Data were analysed by using SPSS software, and the results are expressed as mean ± standard deviation (SD). Two independent experiments were performed using triplicates per group for early cell migration studies at day 6 and 15. Due to the non-symmetrical data distribution, a non-parametric test was selected to analyze the DNA content and the gene expression data. DNA amounts of samples treated with chemoattractant or mechanical load were expressed relative to the DNA content of untreated control samples from the same bovine donor to normalize for donor variation in basal cell migration. Similarly, gene expression data of samples treated with chemoattractant or mechanical load were expressed relative to the levels of untreated control samples. Independent samples were then statistically assessed by Kruskal-Wallis test and pairwise comparisons. For quantification of cells migrated into the osteochondral samples after 15 days of culture, 3 explants per group and 3 sections per sample were used; statistically significant differences between unloaded and loaded groups were determined by Kruskal-Wallis test and pairwise comparisons. Statistical significance was considered for $p < 0.05$.

RESULTS

Mechanical stimuli affect early cell migration in an ex vivo osteochondral culture model

To determine the effect of loading on defect colonization and evaluate PDGF-BB and SDF-1α as efficient chemotactic factors for cells present in the ex vivo osteochondral explants, migrated cells were assessed as function of mechanical stress and chemoattractant delivery. To achieve that, osteochondral defect plugs filled with FB/HA hydrogel in presence or absence of 2 µg/mL PDGF-BB or 10 µg/mL SDF-1α were cultured for 6 and 15 days with or without exposure to mechanical stimuli. Toluidine blue staining revealed that endogenous cells interacted with FB/HA hydrogel; cells started adhering and infiltrating the defect within 15 days, while no or very few cells were visible at 6 days (Fig. 1B). Mechanical loading seemed to influence the morphology of cells infiltrating the defect (day 15, Fig. 1B).

To quantitatively assess the invasion of endogenous cells into the hydrogel delivered to the osteochondral defect explants, DNA measurement and cell counting were performed. DNA content analysis suggested that the exposure to mechanical stimuli tended to decrease cell recruitment at day 6 and day 15. Although a slight increase in DNA was found in the loaded compared to the unloaded control samples, these differences were not statistically significant (Fig. 1C, D). The addition of chemotactic factors and their combination with mechanical stimuli did not show any effect on cell recruitment.

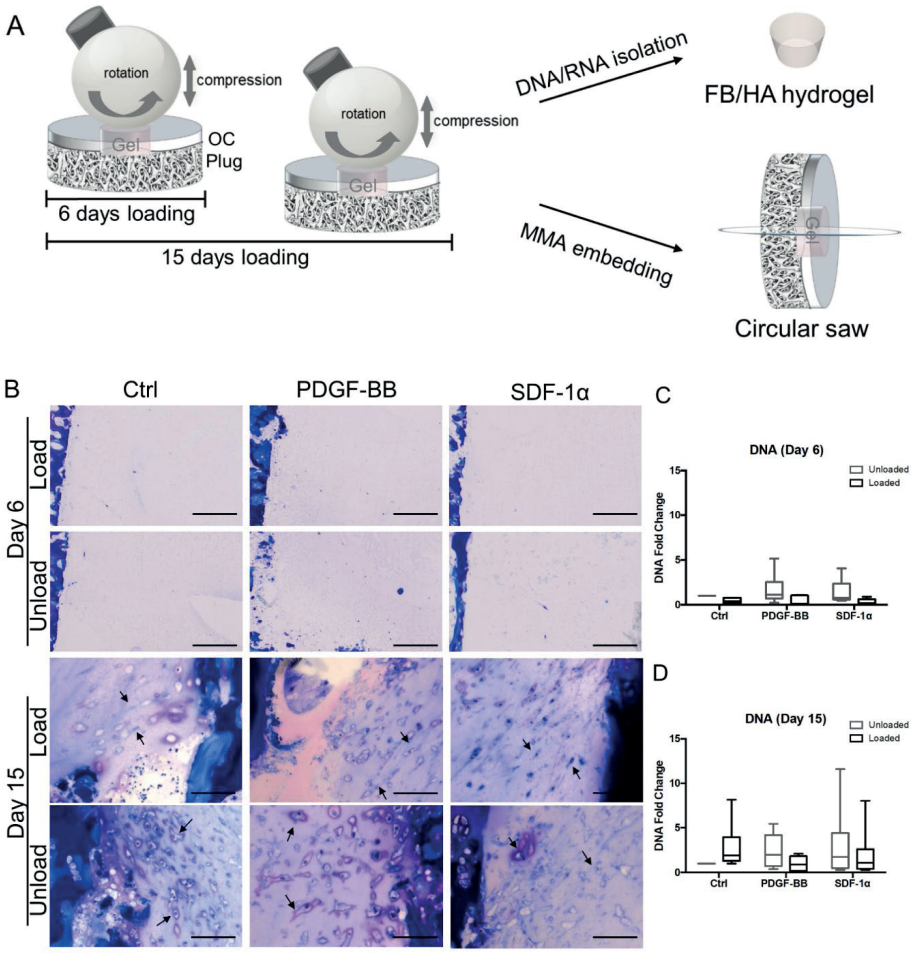


Figure 1. Effect of mechanical stimuli on cells migrating into an ex vivo osteochondral defect filled with FB/HA hydrogels. (A) Schematic representation of the experimental design used for cell migration experiments; OC: Osteochondral explants; Gel: FB/HA hydrogel. **(B)** Representative images of osteochondral constructs stained with Toluidine blue (purple=glycosaminoglycan) showing cells infiltrating the defect after 6 and 15 days in presence or absence of mechanical stimuli. Arrows indicate migrated cells into the defect, a spindle-shape morphology is observed in presence of loading compared to a more rounded shape in unloaded samples; 20X magnification; scale bar indicates 100 µm. **(C, D)** Relative DNA content of unloaded and loaded cell infiltrating FB/HA hydrogels casted in the osteochondral defect models cultured for 6 and 15 days. Data were normalized to the DNA content of unloaded samples without chemokine addition. Results of 6 donors (day 6) and 10 donors (day 15) (one osteochondral explant per donor) are shown.

The cell colonization along the osteochondral explant depth at day 15 was further evaluated by histology. Sagittal sections of explants were cut to permit cell counting in order to explore endogenous cell migration in the entire defect (Fig. 2A) and in three

distinct depths of the defect (bone layer S1, interface layer S2 between calcified cartilage and bone, cartilage layer S3; Fig. 2B). Total cell ingrowth was significantly lower in loaded control explants (without chemokine treatment) compared to unloaded controls ($p<0.05$; Fig. 2A). The numbers of migrating cells were generally more abundant at the interface layer between calcified cartilage and bone; indeed a significantly lower number of cells was observed in loaded control groups (S2) when compared to unloaded controls in the three different depths (S1, S2, S3; $p<0.05$, Fig. 2C). In the adjacent layers (S1 and S3) of control constructs cell ingrowth was limited, and no significant differences were found. The addition of PDGF-BB into FB/HA hydrogel-constructs appeared to slightly increase cell infiltration in unloaded samples compared to the loaded plugs, albeit no significant differences were detected among the conditions tested (Fig. 2A, D). The provision of SDF-1α had no effect on cell recruitment in unloaded samples, nor did it in loaded ones. Overall these results suggested that neither in presence nor in absence of applied stimuli, the chemotactic factors at the concentrations tested exerted any appreciable effects compared to control osteochondral constructs.

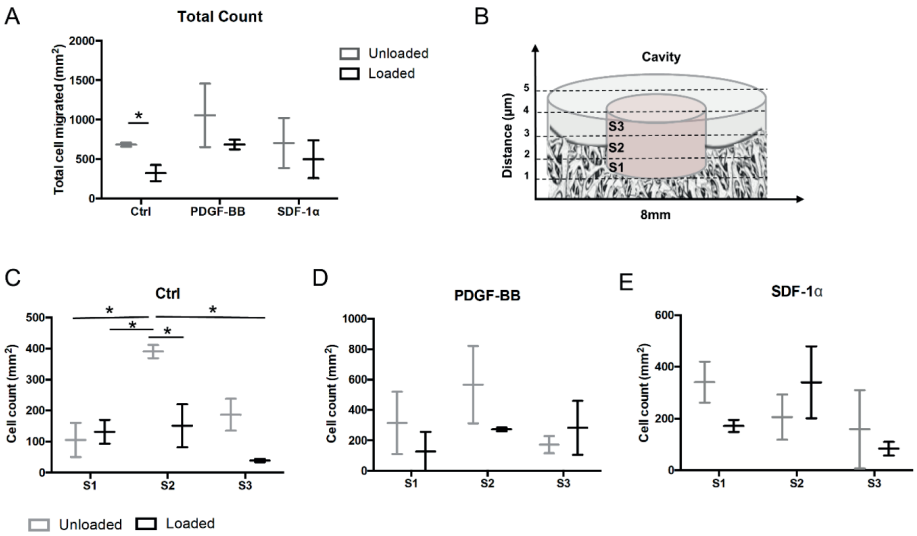


Figure 2. Cell colonization along the osteochondral defect depth. (A) Total count of cells invading FB/HA hydrogels into the osteochondral defect explants at day 15 of culture; *$p<0.05$. **(B)** Schematic representation of the three different depth areas of the defect, S1, S2 and S3. **(C-E)** Cell count on histological sections alongside the bone layer S1, intermediate layer S2 and the cartilage layer S3 in the osteochondral defects at 15 days of culture in presence or absence of PDGF-BB or SDF-1α; *$p<0.05$. Results from 3 donors (one explant per donor) are shown.

Biophysical and biochemical cues influenced gene expression within the osteochondral defect at early time point

To test the phenotypic response of endogenous cells recruited in the FB/HA gel casted into osteochondral explants and uncover more closely the endogenous cartilage repair process, mRNA expression levels of the cells that migrated into the constructs were quantified after 15 days of loading (Fig. 3). Ex vivo exposure of osteochondral explants to complex load led to strong decrease of catabolic markers in migrated cells by day 15. The effect was most evident for gene expression levels of MMP13 in loaded constructs without or with SDF-1α compared to their respective unloaded samples ($p<0.01$, Fig. 3A); for mRNA levels of MMP3, only cells recruited in loaded control group showed significantly reduced expression ($p<0.05$, Fig. 3B). The mRNA ratios of COL2A1 to COL1A2 and ACAN to VCAN remained relatively stable (Fig. 3C, D), whereas a significant reduction in β1 integrin and increase in actin expression were observed in loaded samples in absence or in presence of PDGF-BB compared to the unloaded control ($p<0.05$, Fig. 3E, F).

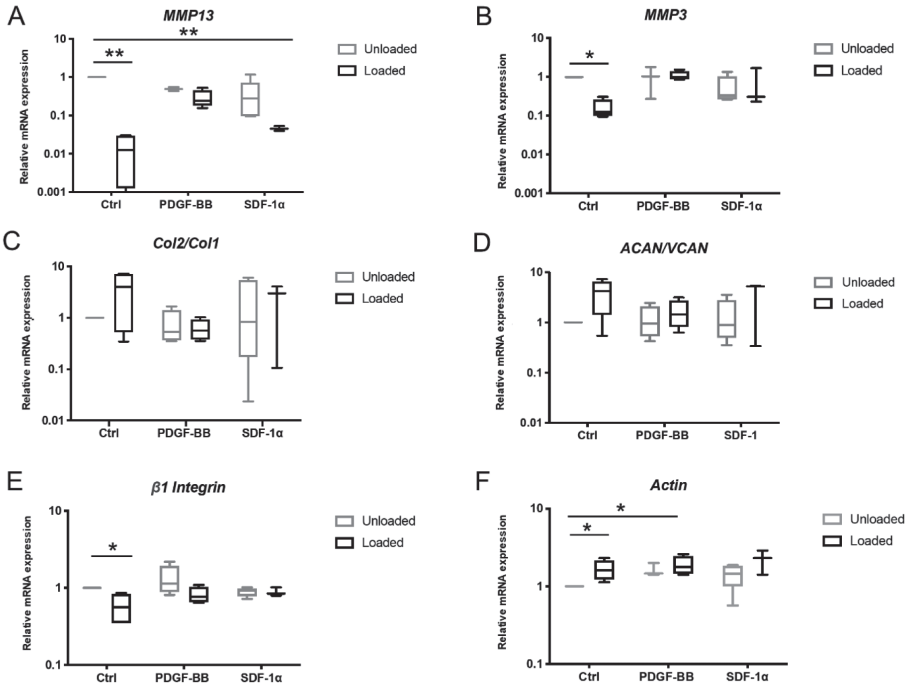


Figure 3. Effect of articular load and motion on phenotype of cells recruited into the wound site. (A-F) mRNA expression of cells infiltrating FB/HA hydrogels implanted into the osteochondral defect explant and exposed to complex load for 15 days. Data are expressed relative to mRNA levels of unloaded samples presented in the graphs by the first line. Results from 4 different donors (one osteochondral explant per donor) are shown; *p<0.05, **p<0.01.

These findings suggest that early applied mechanical stimuli altered the pro-adhesive phenotypic response of cells recruited into the osteochondral defect, thereby disfavoring the migration process.

DISCUSSION

This study showed that complex articulating motion applied to an *ex vivo* osteochondral defect model, filled with hydrogel in presence or absence of chemoattractant, had a negative impact on endogenous cell recruitment into the wound site at early time point. In addition, the provided bioactive agents did not affect this process.

Our approach is based on the use of a previously described advanced platform [137], employed to monitor the spatiotemporal cell infiltration into the injury site under application of multiaxial compression and shear forces, using FB/HA hydrogels as matrix template and delivery carrier. It is widely accepted that mechanical loads are pivotal for cartilage regeneration; earlier described bioreactor studies focused on mechanical loading-based engineered tissue grafts for implantation *in vivo* or on mechanically stimulated osteochondral biopsy-related tissue maturation not combining complex motion patterns [292, 293]. Others focused on a direct implantation of the osteochondral defect models *in vivo* [61, 238, 239]; to our knowledge there is no study intended to document *ex vivo* the influence of multiaxial stimuli on cell defect colonization. The advantage of using this pre-clinical tool is not merely to screen biomaterials and biomolecules, but also to closely study the dynamic process of cell homing, as *in vivo* experiments impede the ability to monitor cell migration and to detect the loads the tissues experience.

Histological analysis revealed that cells started migrating into the defect within two weeks of culture. Mechanical loading seemed to influence the morphology of the cells colonizing the defect; indeed, migrated cells in loaded samples assumed more spindle-shape morphology compared to samples which did not undergo loading that exhibited typical rounded and polygonal shape. Their different morphology suggests that mechanical input is one of the factors governing the mode of migration, in addition to cell type and hydrogel properties. Mesenchymal movement, used by spindle-shaped cells (such as fibroblasts) [294], appears to be dictated by the implementation of mechanical stimuli; whereas ameboid movement, both blebby and pseudopodal, which is used by elliptical like-shape cells [63], may be more predominant in unloaded samples.

Quantitative DNA measurements did not show statistically significative differences at day 6 and day 15, even though fewer cells seemed to populate FB/HA hydrogels exposed to complex load. In support of our observations, total cell count indicated that complex articulating motion significantly decreased cell invasion in loaded control

plugs compared to unloaded controls. These results suggested that mechanical stimuli negatively influenced cell migration by slowing down this process at early time points. We can, however, not exclude that this effect could be due to an inhibition of cell proliferation or an enhanced cell death [295].

It is important to mention that the SDF-1α and PDGF-BB gradients did not significatively enhance migration in our *ex vivo* model. Our previous *in vivo* study on osteochondral repair showed that the exposure of osteochondral defect explants with FB/HA hydrogels to 1 µg/mL PDGF-BB before implantation did not significantly enhance cell recruitment compared to untreated constructs [62]. Although that study used lower concentrations of PDGF-BB compared to the present study (2 µg/mL), the present findings are in line with our earlier observations indicating that cells colonize the defect without factor implementation and the tested factors do not improve cell recruitment. Interestingly, higher variations of numbers of migrating cells were noticed in the chemoattractant groups compared to the control groups without chemoattractant delivery (Fig. 2). This may be attributable to different cellular responses to the chemotactic factors. The chemoattractant effect likely depends on the individual donor explant and on the presence of different proportions of cell types within the explants. In particular, stem and progenitor cells are known to be more responsive to chemotactic factors compared to mature cells [296].

The interface layer (S2) showed the highest cell invasion in unloaded control constructs in comparison to the adjacent layers, suggesting a new potential pattern of migration in the osteochondral unit where either cells present in the subchondral bone or in the calcified cartilage highly participate in defect restoration [98, 297, 298]. Previous models of cell recruitment in osteochondral defects mainly studied the migration of chondrocytes and subchondral bone derived cells, whereby the latter may include osteoblasts, osteoclasts, MSCs or even hematopoietic stem cells [299, 300]. It is generally accepted that stem cells have the highest migration and proliferation rate, osteoblasts are assigned an intermediate rate, while chondrocytes undergo little migration or proliferation [300]. Nevertheless, certain growth factors have been shown to enhance chondrocyte migration [301]. Interestingly, fibrin sealant could promote migration of human chondrocytes in vitro, suggesting that the fibrin-based hydrogel supported the activity of the chondrocytes in our study [302]. Since the layers S1/S2 are mainly exposed to bone derived cells, while S2/S3 are affected by migrating chondrocytes, the interplay between the different cell types that includes autocrine and paracrine signaling may have promoted the cellular activity in the S2 area [298]. Nevertheless, although the migration of chondrocytes from pure cartilage explants is known to be slow, colonization of cells including progenitor-like cells could be demonstrated in a human cartilage explant model using a cell-free implant [303]. Finally, different cell migration rates may further be correlated with different timing of subchondral bone reconstitution and articular cartilage repair, which has been shown

in an *in vivo* rabbit model of spontaneous osteochondral defect healing [304]. Future studies of osteochondral repair should focus on the origin of the reparative cells and mechanisms of cartilage and bone repair interactions over time. It is important to consider that subchondral bone and overlying hyaline cartilage are not two separate structures but a biological unit not only during embryogenesis, but also in adult life in support of the remodeling process.

The loading protocol was chosen based on previous protocols tested by our group. Antunes *et al.* investigated the effect of low intensity motion set-ups and a bioactive agent on gene expression of primary bovine chondrocytes seeded FB/HA hydrogels; due to the low resilience of the hydrogel, samples were subjected to an offset displacement of 10% and low amplitude dynamic axial compression between 10% and 11.5% [289]. Conversely, our previous work on the mechanically stimulated osteochondral explant culture model featured higher mechanical loading set-ups; due to mechanically stiffer polyurethane scaffolds and the use of osteochondral explants as more confined system, dynamic compression was applied at a strain amplitude between 10% to 20% or 14% to 26% [62]. We therefore tuned the mechanical loading protocol with applied complex motion of 10%-14% to fit the mechanical profile of the osteochondral defect constructs containing FB/HA hydrogel. In line with a previous study [81], the influence of external mechanical forces could dictate cell response by dampening matrix degrading collagenases involved in joint pathologies. It is worth noting that the effects of load on *MMP13* and *MMP3* gene expression were no longer present upon the addition of the chemokines. The joint motion simulator did not affect the mRNA ratio of Collagen II to Collagen I and Aggrecan to Versican, indicating minimal influence on the chondrocytic phenotype [305]. Our cell counting data indicated that cell infiltration was still low after 15 days, and due to their uneven distribution cells may not have been accessible to undergo strain-mediated chondrocytic differentiation and matrix remodeling.

Periodic mechanical stress may induce a reduction of endogenous cells adhesion in the defect at early time points by downregulating the expression of β1 integrin and upregulating actin expression. Since integrins provide the main molecular link attaching cells to extracellular matrix, and the bonds that link actin cytoskeleton to integrins dynamically break and reform [306], it is possible that extrinsic mechanical forces decreased integrin function resulting in a reduced adhesion and altered mechano-sensing response as crucial determinants for cell migration.

Although any building blocks need physical forces in order to assemble and hold themselves together [307], we cannot exclude that the application of complex mechanical stimuli at early time point could trigger an altered biological outcome by physically breaking down early matrix organizational network that cells build up in favor of their migration. This is particularly enhanced in a hydrogel system set up, insufficient to counteract the imbalance of cell-generated tissue tension and dynamic load at high

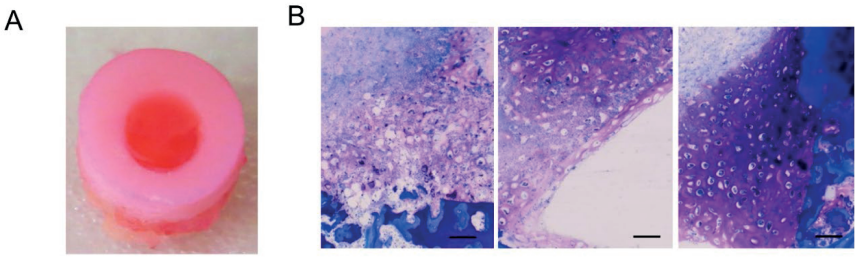
magnitude. Nonetheless, the mechanically stimulated osteochondral defect model mimics the entire joint only approximately, hence we cannot completely replicate the endogenous healing process as it happens *in vivo*. Cell migration is significantly influenced by the synovial microenvironment responsible of inflammatory cytokines and chemokines production, which in turn trigger the cascade of events that could lead to invasion of endogenous reparative cells into the wound site [88].

Taken together these data suggest that the applied mechanical stimuli did not enhance cell recruitment into the osteochondral defect at early time point and the provided chemotactic agents did not influence this process. This might indicate that a well-orchestrated mechanical loading over time is crucial for successful design of endogenous cell recruitment and cartilage healing studies. After an observation period of 15 days, cell infiltration was evident, while the number of migrated cells was still limited. Due to this limitation, no extended evaluation of cell types, matrix synthetic activity and matrix composition could be performed besides the gene expression analysis. A parameter that needs more rigorous attention is the pre-culture time of the osteochondral hydrogel constructs, as it can play a pivotal role for tissue maturation and integration [82]. Indeed, a preliminary experiment showed that longer pre-culture of 5 weeks allowed more cell infiltration and matrix deposition into the injured site (Suppl. Fig. 1). To further observe this phenomenon, future studies will focus on the use of osteochondral explants filled with FB/HA hydrogel pre-cultured for longer time to assess load free effects before being subjected to mechanical stimuli.

CONCLUSIONS

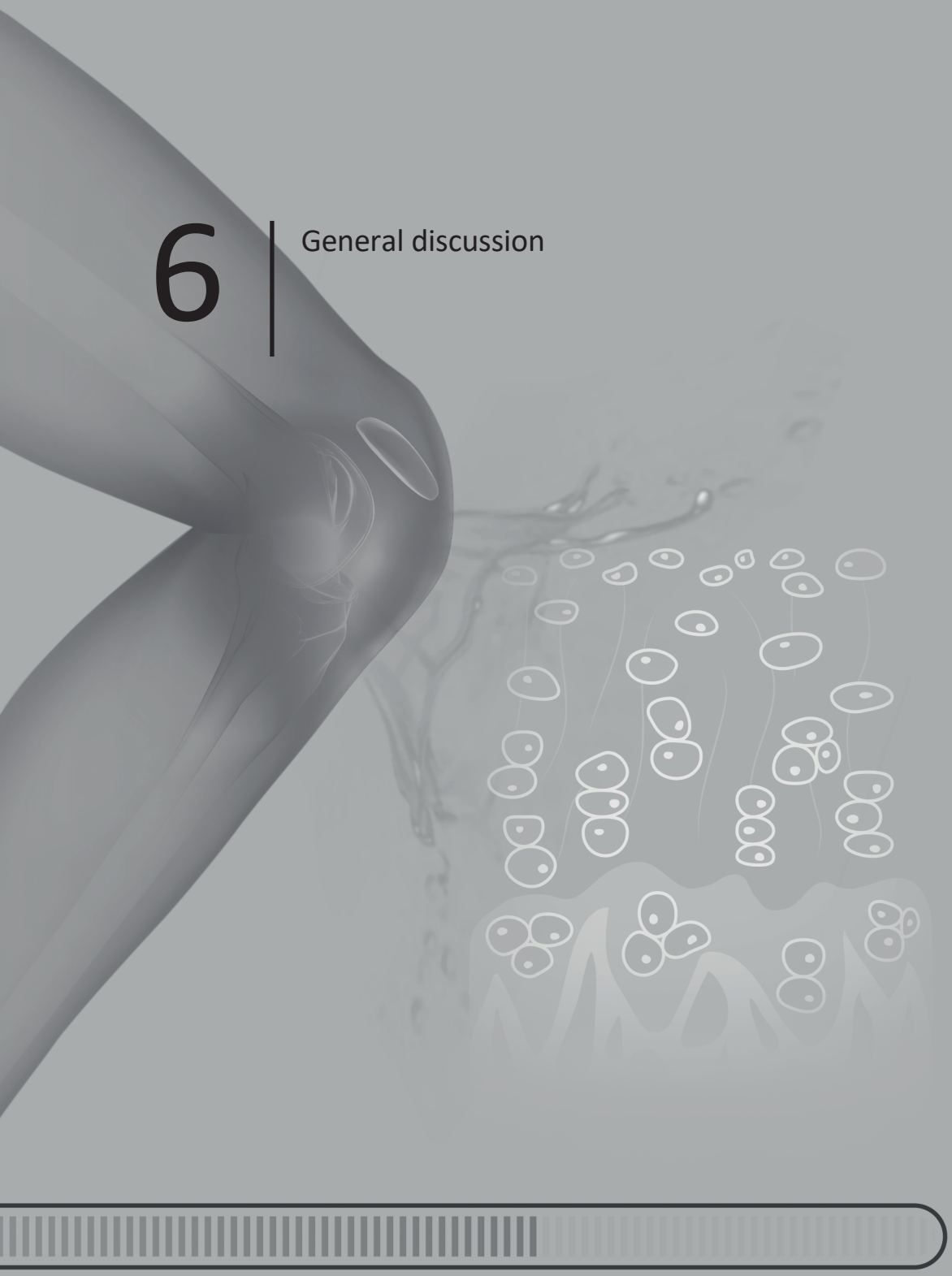
The present short report details the temporal and spatial migration pattern in a mechanically stimulated *ex vivo* osteochondral defect explant filled with FB/HA hydrogel, demonstrating that loading post defect creation might inhibit the endogenous cell migration potential. The implementation of chemokines to increase cell migration was not efficient to overcome this negative effect. This study highlights a significant improvement in the understanding of osteochondral wound healing, suggesting that well-orchestrated mechanical application over time could be the prelude for enhancing cell mobilization and differentiation. The model is useful to decode the interplay between cells, hydrogel, mechanical and biochemical factors; it may unravel the dynamic process of endogenous cell recruitment and signaling pathways implicated in the repair. In light of the inherent advantages that could be utilized based on the modulation of different stimuli, the model represents an attractive system to improve our understanding about the management of joint injury and rehabilitation protocols. Longer-term studies will be required to assess hydrogel-guided neo-cartilage formation and neo tissue integration.

SUPPLEMENTARY DATA



Supplementary Figure S1. Osteochondral explant overview. (A) Macroscopic images of osteochondral explant after 5 weeks of culture. FB/HA hydrogel becomes opaque in presence of cell invasion (left side). **(B)** Toluidine blue staining to assess endogenous cell migration after 5 weeks of culture. Cartilage formation in hydrogel is presented by pink/violet staining. 20X magnification, scale bar indicates 100µm.

6 General discussion



GENERAL DISCUSSION

In diarthrodial joints, articular cartilage, calcified cartilage and subchondral bone form the osteochondral unit, intended as a functional unit that is uniquely integrated to ensure load transfer. During traumatic injuries functional and structural properties of the targeted joint tissue undergo considerable alterations, that will ultimately affect all the components of the osteochondral unit. The complex architecture of the knee joints, beside the very limited self-healing ability of articular cartilage, decreases the chance of complete regeneration. Currently, traumatic defects are repaired with microfracture, ACI and its derivates, autologous or allogeneic MSCs transplantation and osteochondral grafts [308]. Although these procedures have been used successfully and may slow down the onset of OA, it is well-known that pain often returns after an initial relief, due to inferior replication of hyaline cartilaginous tissue and partial or total failure of neo-tissue integration to the injury site [17]. Nevertheless, the use of cell-based therapies faces some disadvantages including the enormous costs for patients and health care systems, cell handling, safety and related regulatory issues [309]. This implicates that cell-free alternative strategies using biomaterials, need to be established to circumvent these impediments. The diarthrodial joints carry and distribute loads; hence, it appears to be inevitable to take also mechanical load into account for successful cartilage regeneration. Therefore, the evaluation of cell-free osteochondral regenerative approaches in combination with application of complex mechanical loading are of vital importance prior translation to clinical application. This thesis focuses on the development of a mechanically stimulated osteochondral defect model, which closely recapitulates an articulating joint microenvironment, to provide great advantages as preclinical screening tool for biomaterials and biomolecules. Furthermore, it evaluates the critical but challenging step of early endogenous cell recruitment into a hydrogel when subjected to joint movement.

Natural hyaluronic acid hydrogel as suitable candidate for cell-free cartilage repair approaches

Replacing damaged cartilage by using a 3D template hydrogel relies on important features that provide either adequate stiffness to resist repetitive loading or biochemical cues such as adhesive and chemotactic stimuli necessary for cell homing and directed cell migration. A key concept in regenerative medicine is the selection of a hydrogel that supports cell infiltration, proliferation, differentiation and guided tissue remodeling.

The hydrogel will form the cells› environment and should mimic the natural micro-architecture supporting cartilage repair. Instinctively, ECM has the potential to be the ideal hydrogel to provide the framework for tissue formation, therefore natural polymers which display inherent bioactivity are often favored as plausible biomaterials.

The properties of a hydrogel are established by the polymer chemistry from which it is synthesized [310]. GAGs are pivotal ECM building blocks used for joint lubrication and protein binding; the uniqueness of HA amongst this family is in the non-sulphated polymer backbone. The carboxylic acid present within the repeating units of glucuronic acid disaccharide confers very high negative charge densities ensuring the formation of HA gels with high swelling ratio and water content. HA can also strongly influence biomolecules, cell diffusion and differentiation by forming non-covalent bonds with cell-surface proteins and complexes (e.g. CD44) [311, 312], having a role in cartilage matrix stabilization. These interactions highly depend on its molecular weight and concentration. Also, an important factor of the HA scaffold bioactivity is its crosslinking density. In tissue engineered applications HA chemical crosslinking is typically required to reinforce its poor mechanical properties (due to high hydration and swelling) and reduce rapid degradation (due to the presence of hyaluronidase enzymes in native tissue) [313, 314]. Tunable hydrogel designs have enabled diverse studies including investigations of screening platforms for probing cell responses by dynamically stiffening or softening hydrogels to modulate cell phenotypic differentiation [315]. Taking advantage of HA hydrogel's tunable mechanics in a user-directed manner, we developed in **chapter 2** an advanced platform to screen biomaterials and biomolecules for their potential to support cell migration by using a 3D spheroid-based migration assay *in vitro* and an osteochondral defect model *in vivo.*

Cell spreading, migration and differentiation were found to be inversely proportional to the increase of HA-Tyr cross-linking degrees (150, 300 and 600 µM H_2O_2; **chapter 2**). Generally, the stiffer gels (highly crosslinked) have lower mesh size and lower cell flux throughout the matrix. When FB/HA hydrogel was used, hMSCs migrated much more in comparison to HA-Tyr hydrogels, in *in vitro* assays. Upon subcutaneous implantation by using an osteochondral model, this effect was even stronger as assessed by toluidine blue and collagen type II staining after 4 weeks.

Despite the similar stiffness (G') of FB/HA and HA-Tyr 150 and similar molecular weight of HA, it is likely that different HA concentrations and polymer backbone modifications drastically affected cell-material interactions, thereby influencing either cell adhesion and migration or differentiation among HA-Tyr and FB/HA hydrogels.

Beyond biophysical properties, hydrogels that more accurately recreate the complex milieus found in tissues include spatial patterning of numerous biochemical signals introduced to deliver factors to control cell response [316]. The encapsulation of PDGF-BB into HA-based hydrogels and its release described in **chapter 2**, chosen as the most potent chemoattractant after screening, differs among different HA-Tyr crosslinking densities and FB/HA gels. This has caused different exposure of hMSCs to the factor and in turn different migration responses *in vitro*. When applied *in vivo*, although the migration profiles of both HA hydrogels reflected the *in vitro* assay, the presence of the

factor did not increase cell infiltration in the osteochondral defect model. Moreover, it impaired chondrogenic differentiation in FB/HA gels. It is possible that the presence of PDGF-BB in those gels at this concentration and at this stage of remodeling stimulated an environment that prevents GAG deposition**.** Although the bioactive factor was not tested during chondrogenesis *in vitro* and a study had shown that the effect of PDGF-BB *in vitro* is to enhance matrix production [317], it is possible that when implanted *in vivo* the biomolecule is exposed to a multitude of different stimuli, may undergo exchange with its surroundings, may be washed out, recruited or may attract different cells from the neighboring tissues. This can explain the diminished chondrogenic differentiation observed in **chapter 2** in FB/HA gels containing PDGF-BB in osteochondral models after *in vivo* transplantation**.** These findings suggest that the factor may be important for endogenous MSC recruitment but may also impair chondrocytes differentiation, necessary for proper cartilage matrix reconstruction. Further studies will be needed to evaluate the dose-dependent effect of PDGF-BB on migration, differentiation and cartilage repair after *in vivo* transplantation.

Despite recent advancement in polymer design there is no single hydrogel that is ideal in every tissue engineering scenario. For instance, a hydrogel used as scaffold replacement for bone growth should be different from cartilage that does not require any blood vessel for growth. Many different biomaterials or bi-tri layer scaffolds are being developed by additive manufacturing to mimic different micro-architecture of cartilage [318], but the tissue complexity sets high standards to hydrogel design. In **chapter 2** the concept to evaluate biomaterials and biomolecules with enhanced endogenous recruitment and cartilage matrix formation was introduced by developing an advanced testing platform *in vitro.* It suggests that stiffer materials represent an obstacle to endogenous healing, thereby limiting matrix accumulation and neo-tissue integration, providing a further gel pre-selection prior to animal transplantation. By adjusting the mechanical properties of the hydrogel and perhaps varying polymer concentrations and crosslinking densities [50] or introducing porogens as sacrificial material (salt particle, fibers) with high degradation rate [319], stiffness can be modulated and porosity can increase to encourage cell infiltration, support differentiation and formation of either cartilage or bone tissue. Clinical translation needs extensive proof of functionality and safety, hence animal studies are a prerogative. To validate our *in vitro* findings, we have used ectopic implantation of osteochondral explants as a model for osteochondral defect repair to study early events of cell infiltration separated from influence of the joint host. A further investigation of the mechanical properties of the newly formed cartilage in an orthotopic defect model, where articular cartilage is subjected to proper mechanical stimuli, is surely of importance to assess long-term construct stability.

Mechanical stimulation of the selected matrix as an inevitable step for successful *in vitro* cartilage regeneration

The role of mechanical forces during development is well known [320] and physical stimuli are now considered as crucial as chemical factors in modulating cell fate and influencing tissue development. Researchers have hypothesized that a structure–function relationship influenced by dynamic loading could be the success for *in vitro* articular cartilage regeneration. To test these speculations the use of bioreactor systems experienced a rapid spread in the field of cartilage tissue engineering, to more closely mimic the mechanical stimuli experienced by chondrocytes within the joint. The responses of chondrocytes and MSCs to mechanical stimuli have been extensively reviewed by Johnstone *et al.* and Panadero *et al.* [321, 322]. They reported how changing input parameters, including hydrogel substrate, cells, growth factors, type of bioreactor and axes in combination with different loading regime, can dramatically influence the biochemical and biomechanical outcomes.

Generally, it has been shown that dynamic loading increases chondrogenic gene expression, proteoglycan content and biomechanical moduli. Mechanical compression applied to primary bovine chondrocytes seeded in agarose was found to enhance matrix production in a loading duration dependent manner [184]. A superior proteoglycan production was also observed when intermittent loading regime was applied to chondrocytes in comparison to unloaded controls [323]. Further contribution to these positive effects makes the pre-culture period of the construct followed by delayed loading; indeed it seems to be a prerogative [324, 325] that allows cells to build pericellular matrix necessary for effective mechano-transduction. However, the effect of the sole dynamic compression was not sufficient to produce tissue specific collagen. Therefore, the need to mimic the complex movement reminiscent more accurately of articular cartilage was considered to be a must. The supplementary application of dynamic uniaxial or multiaxial shear in combination with a compressive loading regime in chondrocytes cultured on ceramic surface or polyurethane scaffolds, pioneered by Waldman *et al.* and Grad *et al.* [80, 81, 219], confirmed the hypothesis that complex motion encourages the chondrocytic phenotype and the expression of genes associated with hyaline cartilage formation (COL2, ACAN, PRG4, COMP).

In analogy with the application of extrinsic mechanical loading, other reports have shown how diverse biomaterials can differently transmit mechanical signals to the embedded cell [138]. Based on biochemical data, results were not promising for cartilage tissue formation when bovine chondrocytes seeded in fibrin gels underwent mechanical stimuli; a reduced ECM deposition in comparison to unloaded controls showed an opposite trend to the agarose gel [326]. Moreover, the properties of both hydrogels are mutually different. **Chapter 3** emphasizes that a low intensity complex motion set-up promotes significant amount of total GAG when chondrocytes are

seeded into previously selected FB/HA hydrogels. The cellular response to load proved to be dependent on hydrogel substrates and highlights the importance of network crosslinking density and cell-matrix interaction, suggesting to some extend a positive effect of loading on matrix production when hyaluronic acid is conjugated with fibrin. This implies superior mechanical properties, longer term *in vitro* stability of gels containing encapsulated cells and an overall solid-elastic character (G'/G'') [60], which indicates higher mechano-resilience than fibrin hydrogels alone. Nevertheless, we could only apply very gentle loads, compared to e.g. fibrin-polyurethane scaffold; integrating cues that permit to recapitulate mechanical properties of the dense irregular cartilage tissue (synthetic polymers, such as aligned nanofibers of poly-(L-lactic acid), PLLA) in combination with e.g. cleavable linkage of natural ECM components to soften the matrix in presence of cells are hypothesized to be advantageous to find a good compromise to maintain a pro-regenerative environment.

Growth factors and chondroblasts, which act as mechanosensors, play a key role during the cartilage formation [327]. Many *in vitro* and *in vivo* studies with soluble factors are being performed without loading in order to enhance cell behavior, differentiation and tissue development [328] as potential way to ameliorate the properties of a hydrogel. However, little is known about the combinatorial mechanisms by which growth factors and loading elicit their effects on chondrocytes metabolism. In the works reported by Gigout and Eckstein, addition of the growth factor FGF-18 in gels, which is associated with mitogenic activity and increased ECM production, resulted in increased cartilage repair in both *in vitro* and *in vivo* models [165, 329]. The development of the N-terminal truncated variant FGF-18v increased the specificity for FGFR-3, the main receptor involved in chondrocyte differentiation and maturation [330]. Moreover, no studies have appreciated or reported the added effects of this factor in a tissue engineered construct within a dynamic loading environment. Therefore, we further complete the former research and conclude that intermitted and delayed biaxial motion in combination of FGF18v supplementation play a crucial role in cartilage development and maintenance of chondrocytic phenotype when seeded in FB/HA hydrogels (**Chapter 3**). The addition of FGF-18v alone was found to be insufficient to promote upregulation of cartilage matrix genes (e.g. ACAN, COMP, COL2, PRG4) in unloaded samples, conversely a marked increase was appreciated when dynamic loading was associated with the soluble factor. At tissue-level this effect was even stronger since safranin O positive repair tissue was formed by bovine chondrocytes in loaded FB/HA gels at FGF-18v concentrations tested, additionally confirmed by aggrecan and collagen type II production. These data suggest that differentiated chondrocytes can synergistically adapt to changing biochemical components and mechanical environment. In agreement with other literature [81, 198], mechanical stimulation in absence or presence of the factor downregulated matrix

metalloproteases involved in joint pathologies, thereby limiting ECM degradation and further proving the responsiveness of our gel platform under load.

The tendency of mature articular chondrocytes to undergo dedifferentiation when isolated and embedded in hydrogels is undesired for cartilage regeneration. Although COL1 and COL10 expression showed no significant differences between groups, it is worth noting that the ratio COL2/COL1 increased and COL10 expression markedly decreased after FB/HA hydrogel seeding. This suggests preservation of the differentiated state of chondrocytes within the 3D environment in loaded and unloaded conditions, likely preventing hypertrophy and in turn providing an environment that favors stable cartilage formation. Better insight into the newly formed cartilage matrix will provide valuable information; especially an accurate assessment of the cartilage tissue phenotype should include collagen protein quantification and differential proteoglycan and collagen type characterization, by quantitative methods such as hydroxyproline based collagen assay, PAGE, LC-MS and ELISA [331-333]. The density and degree of alignment of collagen fibers can be quantified by second harmonic generation images [334].

Longer culture time and total load duration would greatly favor increased collagen production and collagen fiber orientation, which is highly relevant for functional ECM formation important to fulfil the biomechanical role of articular cartilage in diarthrosis.

Taken together these data suggest the additive effect driven by the exogenous FGF-18v in a loaded FB/HA environment, which interdependently influence cellular metabolism by increasing the quality of the tissue engineered cartilaginous construct. Our finding also highlights the importance of the mechanical environment for cartilage tissue remodeling.

The effect of complex articulating motion on cell behaviour in an osteochondral environment

While a large body of literature has established that biomaterials, cells, biomolecules and loading regime are the main elements necessary to define tissue engineering within a dynamic loading environment, significant research is required in a more confined system to understand and predict hydrogel-cell construct behavior when surrounded by tissue during loading. Osteochondral *ex vivo* models [115] in which chondral and osteochondral defects can be generated, have been established with the view to study cartilage-bone interplay and select factors eliciting a healing process towards stable cartilage formation. However, these models have not considered the mechanical component as more predictable platform of the whole tissue response. Therefore, in **chapter 4** a mechanically stimulated *ex vivo* osteochondral defect model is presented and its response to complex motion pattern is validated using bovine chondrocytes embedded in a polyurethane-fibrin scaffold. Addition of compression and shear load provides the necessary improvement to enable more selective screening

of biomolecules and biomaterials for implants and evaluate possible constraints prior to moving to pre-clinical *in vivo* testing, thereby decreasing the number of animals to the essential. In line with previous findings [81, 219, 335], complex articulating motion promoted mechano-responsive articular surface protein and associated chondrogenic gene expression. The platform presented in chapter 4 possesses an advanced capacity of mechano-induced cartilage matrix gene expression due to a certain hydrostatic pressure buildup via external loading within the confined system. The addition of physiological joint kinematics also involves fluid pressurization, which has been shown to increase anabolic effects upon application to articular chondrocytes [336]. In polyurethane-fibrin scaffold these pressures would be imperceptible in an unconfined system because of high permeability of biomaterials.

It has become more and more evident that mechanical motion plays an essential role in the formation of new osteochondral tissues. Albeit the use of cell-laden scaffolds to certain extent might regenerate cartilage in defects after implantation, these approaches are still restricted by high costs, reduced cell sources, risks of disease transmission and complex manufacturing procedure [337]. Recent advancement tries to circumvent these drawbacks by developing acellular biomaterials that rely on endogenous cell recruitment to the wound sites offering great promise for *in situ* osteochondral regeneration. *In vivo* studies have monitored cell recruitment and defined different cell subpopulations involved in the migration process in a joint environment [338-344]. It is thought that providing an appropriate 3D template amenable for migration and differentiation, controlled and prolonged release of chemoattractant to enhance sufficient stem-progenitor cell recruitment to the injury site (**chapter 2**) and appropriate biochemical and biomechanical stimuli (**chapter 3**) are the key requirements for endogenous cartilage healing. However, it is still unknown how cells sense and respond to loads due to *in vivo* study limitations. Therefore, in **chapter 5** we employed the mechanically stimulated osteochondral defect model presented (**chapter 4**), filled with FB/HA hydrogel in presence or absence of PDGF-BB or SDF-1α gradients. This demonstrated that periodic mechanical stress inhibits early stages of endogenous cell infiltration.

Despite physical forces are important factors orchestrating the dynamic process of remodeling [307], it is likely that the early application of complex mechanical stimuli physically breaks down the newly synthesized matrix components, hence triggering an altered biological outcome. This effect is amplified in our hydrogel set up, where the biophysical characteristics are not sufficient to concurrently withstand load and attenuate tissue tension generated by the cell at early time point. However, we cannot exclude that the decreased cell migration number could be the result of a cell proliferation inhibition or an enhanced cell death [295]. . Upon loading cells colonizing the defect exhibit more spindle shape morphology, recalling a mesenchymal movement

[294] compared to the rounded and polygonal shape prevalent in unloaded samples, which could be more associated with ameboid migration phenotype [63]. This implies that mechanical input, besides hydrogel substrate and cell type, is also a determinant dictating the mode of migration.

The influence of external mechanical forces could dictate cell response in our system, however cell infiltration was still low and their uneven distribution may have limited strain-mediated chondrogenic differentiation and matrix remodeling, which might indicate that a well-orchestrated loading over time is crucial for successful endogenous cell remodeling. Obtaining homogeneous cell density and distribution within the hydrogel is a demanding but crucial step towards neo tissue formation [71, 345]. Similarly attracting the correct cell population (e.g. endogenous progenitor) while impeding immune cell infiltration should be a requirement for long-term implant survival. Interestingly, the provision of soluble chemical factors was not enough to augment cell chemotaxis and reverse the negative effect of loading on cell migration. Particular consideration should be given to determining and creating the right gradient concentration that would be effective and safe for enhancing cell mobilization. Another option could be to adapt hydrogel properties by reducing the volume/weight of HA in HA-Tyr, thereby reducing electrostatic interaction among their hydrophilic groups and the charged amino acid residues of the PDGF-BB, or by inserting degrading HA nanofibers to obtain a sustained fashion release and improve cell migration. Therefore, further testing in the model described is required.

The mechanically stimulated ex vivo osteochondral defect model has shown a potential route of cell migration in **chapter 5**, where cells either present in the subchondral bone or in the calcified cartilage cooperate to repair damage. In the context of the osteochondral model, the bone marrow and the contribution of bone lining cells, perivascular cells and cartilage progenitor cells cannot be ruled out, speculating that cross-talks among autocrine and paracrine factors secreted by different cell types may potentiate cellular activity. Nonetheless, the osteochondral defect model under load faces certain limitations. As it does not recapitulate the entire diarthrosis, it is not possible to replicate the whole range of events determining the body's healing response in cartilage repair *in vivo*. The wound healing process is significantly affected by the synovium and synovial fluid, which play a significant role in mesenchymal cell migration to the injury site [346], nutrient supply and metabolic by-product clearance, thereby influencing matrix production.

FUTURE DIRECTIONS

Although the research described in this thesis could not directly be translated into a clinical regenerative approach, important issues have been addressed for future developmental strategies. The selection of FB/HA hydrogel as ideal material to support endogenous cells to migrate into the wound site and trigger chondrogenic differentiation holds great promise for cell-free cartilage repair. A similar formulation of FB/HA hydrogel, containing higher molecular weight HA, is already approved as therapy for the treatment of osteoarthritis and associated pain. Longer *in vivo* studies should assess absence of hypertrophy and bone formation, which are the two most prominent drawbacks that impede stable cartilage formation. This limitation can be further circumvented by using specific pro-chondrogenic molecules such as antimir-221 [61], developing gene-activated matrices to orient progenitor cell fate *in situ* [347] or advances in genome engineering (CRISP-Cas9), which have made it easier to reprogram the intrinsic endogenous cell pathways by knocking down *MMP13* and enhancing collagen type II expression [348]. Despite this hydrogel presents a formidable physical assistance to cell ingress, resistance to high compressive load is critical. Reinforcement of the gel by combination of aligned fibers to promote organized matrix deposition [349], thereby inducing cell polarization and collagen alignment would lead to higher bulk mechanical properties while expediting cell infiltration.

Beside the identification of a multifaceted set of biochemical and biophysical factors with the aim to promote cell infiltration, modulating cell behavior to stimulate this process would be preferable. The entrapment of cargo molecules into the hydrogel is imperative to either attract the appropriate cells to colonize the defect or to enhance collagen deposition and bio-integration. However, no consensus on the ideal factor and its optimal dosage is yet available. A next step could be investigating the functional effects of different factors in a dose response dependent manner to increase cell density by prolonging the effect of the exogenous bioactive without inhibiting cartilage matrix deposition. At the same time, implant integration with the injured tissue could be promoted, for instance, by sequentially releasing collagenase and chemotactic factor in a coordinated and localized manner [350]. Methods to specifically label and track live cells over time are indispensable to define cell populations colonizing the defect, which would be beneficial for elucidating the underlying mechanisms of endogenous recruitment and regeneration. However, the imaging of the different cell types involved in joint defect restoration to distinguish each cell phenotype while differentiating remains a huge challenge.

At present, it is unclear how the mechanical loads affect endogenous reparative cells beneficial for neocartilage tissue formation, as *in vivo* studies are limited by the inability to monitor cell responses and determine the loads that the neo-tissue experience. In

this thesis the implementation of complex loading into osteochondral defect explants (**chapter 5**) shows temporal and spatial migration patterns in an osteochondral defect model, providing a perspective on how infiltrating cells transduce mechanical load into directed migration. This process is slowed down and negatively influenced by dynamic stimulation at early time point, highlighting a significant improvement in the understanding of osteochondral wound healing. Speculation remains on the timing at which the load induces the most beneficial response and whether hypertrophy can be prevented. While many diverse biomaterials are being developed to be tailored in a way to promote migration, differentiation and spatial organization, further testing for their suitability focusing on environmental factors that affect or potentiate cell infiltration under complex load is required. The underlying mechanisms governing the mechanical stress response need to be decoded and may offer new targets for therapies if novel pathways that exert its positive effects are discovered. The model described in this thesis provides an extensive platform that can be used for such screenings of materials in focal cartilage lesions to advance joint regeneration. The delicate balance between osteochondral remodeling and the application of mechanical loading was found to be vital in this highly dynamic process. No information is reported to date on the effectiveness of applying mechanical loading *in vivo* on cell-free implanted hydrogel with respect to cartilage and bone formation in an osteochondral site.

Since the healing of articular cartilage lesions in the knee depends on what happens in the period after surgery is performed, the model holds great promise to mimic surgeons' postoperative restrictions, recovery recommendations and rehabilitation protocols. The immediate post-operative care typically lasts 5-6 weeks (phase I) where the patient is non-weightbearing, while the phase II of rehabilitation (6-12 weeks) is marked by the progressive addition of weight bearing forces [351-353]. Time considered for the osteochondral explant maturation needs more attention, as it has been shown to play a critical role in tissue growth and integration [82]. To approximately resemble the rehabilitation practice, we have performed a preliminary experiment that showed that 5 weeks of explant pre-culture allowed high cell distribution and matrix deposition (**chapter 5**). Future research needs to clarify the effect of longer preculture time in the osteochondral explant before being subjected to mechanical load. Ideally, this model could provide useful insights to either prevent cartilage damage progression or to assess tissue remodeling in a controllable setting, so that mechanical modulation-based regeneration could be applied efficiently.

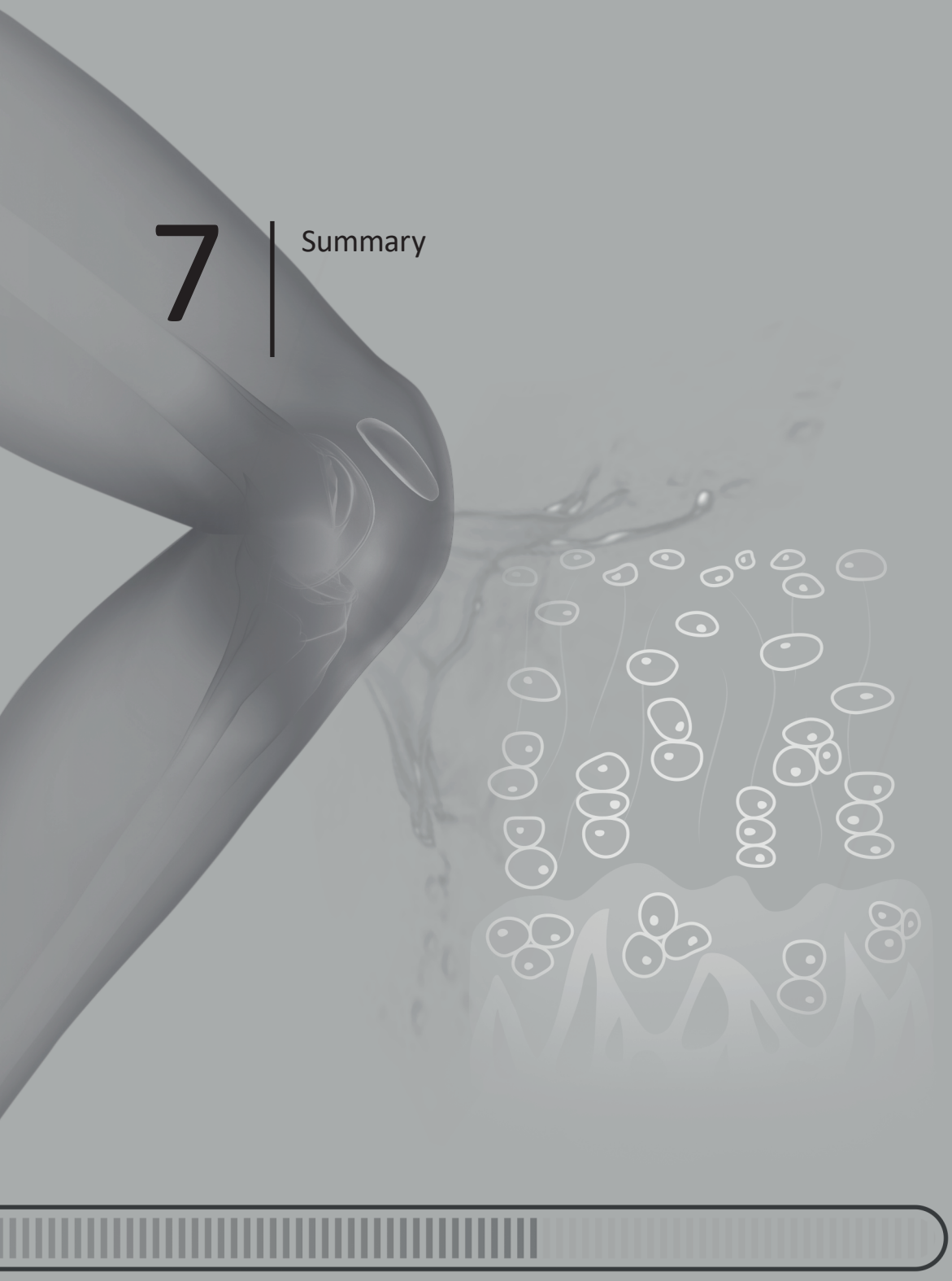
At the time the correct cells have colonized the defect, the success of long-term tissue regeneration is governed by the host immune response to the graft, since inflammatory cells invasion is often the prelude of graft-rejection or fibrotic tissue formation. With the view of translating a cell-free therapeutic approach, a mixed delivery of anabolic and inflammation modulating factors [53] could be more effective by e.g. promoting

spatial cell organization (gene delivery mediated or genome editing), while suppressing immune cell infiltration [354, 355]. The emerging field of immune-engineering seeks to promote tissue regeneration by modulating immune cells through engineering materials designed to release cytokines to drive macrophage polarization towards pro-healing M2 phenotype or present protein antigens conjugation to tune immune cell tolerance, memory and cytotoxicity [356, 357].

It is evident that the model under physiological stimuli has characteristics that only partially recapitulate the entire joint; the synovium and synovial fluid have also a significant influence on cell migration. Hence, mimicking the microenvironment by perhaps pre-exposing the explants to the synovium or synovium conditioned medium would be interesting to closely replicate the cascade of events that could lead to invasion of endogenous reparative cells into the lesion as it happens *in vivo*. An important component of joint kinematics, though less addressed in this thesis, is moving contact or rolling. These features of moving, accompanied by development of a cartilage-on-cartilage articulating motion system, would better resemble the joint niche and reduce friction of the loading system [233]. With the view of resembling traumatic defect as targeted tissue of the population intended for focal defect repair, the use of healthy human osteochondral explants could be an excellent step towards future clinical relevance, although the low donor availability and its high variability would pose a challenge. Nonetheless, the shortage of human explants could impede its clinical therapy, though currently there are no FDA approved engineered constructs grown in dynamic shear and compressive bioreactors. Considerations must be given in term of protocol standardization to entail high reproducibility and scalability

This thesis provided important biochemical and biophysical cues on hydrogel dynamics that resemble the optimal microenvironmental niche for directing osteochondral tissue growth and maintenance over time. The implementation of complex joint motion patterns should not be undervalued; the model described in this thesis can lead to a better understanding of the effects of mechanical stimuli on cell migration and differentiation in an osteochondral environment, and perhaps even boost, the natural biological cues to advance joint regeneration. This model demonstrates that *ex vivo* explants are new and interesting platforms that can help to bridge the still present low correlation between *in vitro* culture and *in vivo* biomaterial testing.

7 Summary



SUMMARY

Articular cartilage injury poses a significant clinical challenge in orthopaedics. Advancements in the recent decades are placing cartilage regeneration in the spotlight, paving the way to overcome limitations of current treatments. In order to improve clinical outcomes and develop new treatment strategies, this thesis aimed to provide models necessary for testing future biomaterial-assisted cell-free repair approaches in the knee joint. The suitability of hydrogels as 3D templates to support infiltration of chondroprogenitor cells, their differentiation, and to deliver biomolecules to enhance these processes were evaluated using spheroids-based migration assay as well as an *ex vivo* mechanically stimulated osteochondral defect explant model.

Cell migration has a critical role in the early process of biomaterial-assisted tissue repair. Multiple biochemical and biophysical factors, reliant on cellular and extracellular matrix (ECM) properties, influence migration efficiency. Cells are dynamically sensitive to biomaterial composition, stiffness and structure as well as to bioactive gradients, which can potentiate cell locomotion and movement. In **Chapter 2** different formulations and cross-linking densities of hyaluronic acid (HA)-based hydrogels were tested *in vitro* and *in vivo*, with the aim to select the most suitable implant template for endogenous regenerative therapies. We found that changes in mechanical properties influenced cell spreading, migration and differentiation. Fibrin conjugation with HA was much more different than the different modulation of the HA-Tyramine cross-linking degree (by using 150, 300 or 600 µM H_2O_2). The H_2O_2 concentration, while keeping HA-Tyramine and horseradish peroxidase concentrations constant, was the major determinant for both cell migration and matrix synthesis during mesenchymal stem cell chondrogenesis. Migration was observed to be inversely correlated with the storage modulus of the gel in the presence of a platelet-derived growth factor BB gradient. This means the softer gel fostered faster migration than the stiffer one. Fibrin-hyaluronic acid (FB/HA) gels, however, always revealed the highest cell migration potential, both in the presence and in the absence of chemoattractant *in vitro*, and also favored chondrogenic differentiation. An osteochondral explant model implanted subcutaneously *in vivo* further confirmed the endogenous cell recruitment, even in the absence of the stimulating factor. This stresses the importance of the microenvironment and the hydrogel substrate in which cells are recruited, as a crucial stepping-stone towards engineering functional musculoskeletal tissues.

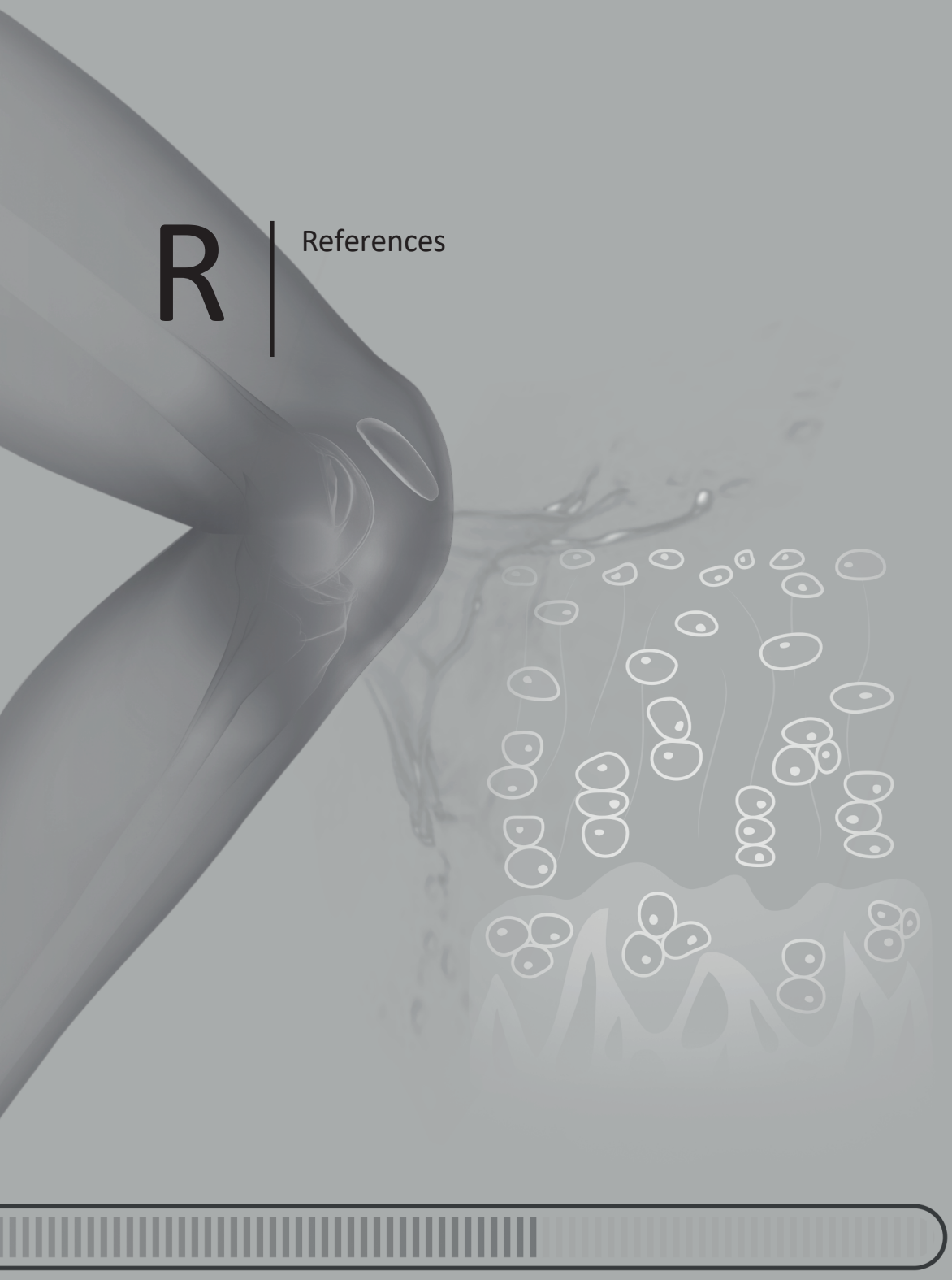
As defects in the osteochondral unit often occur in the weight bearing region, it appears intuitive that repair of focal traumatic lesions should take into account mechanical loading as an essential factor influencing osteochondral tissue regeneration. The success of material-based systems depends on the ability of cells and hydrogels to sustain compressive and shear forces during loading. In **Chapter 3** it was demonstrated that low

intensity complex motion applied to chondrocytes-seeded FB/HA gels had a positive effect on maintenance of chondrogenic phenotype and cartilage matrix production. Cellular mechano-transduction was more effective when exogenous fibroblast growth factor-18 variant (FGF-18v) was added to our *in vitro* culture model when subjected to load, which interdependently influences cellular metabolism by increasing the quality of the tissue engineered cartilaginous construct . We showed that chondrocytes could synergistically adapt to a changing biochemical and mechanical microenvironment by modulating the amount of ECM, downregulating matrix degrading enzymes and promoting a functional surface.

Conventional bioreactor studies for cartilage regeneration often do not take the osteochondral tissue into account. With the aim to render biomaterial-assistant osteochondral defect repair technologies clinically feasible, we applied mechanical stimuli to biomaterials in a more confined environment to evaluate their function within the tissue as experienced *in vivo.* In **Chapter 4** we first described the development and validation of an *ex vivo* osteochondral defect model under mechanical compression and shear that provides a representative physiological joint-like environment to allow reproducible prediction of biomaterials performance and of biomolecule treatment efficacy. This model was used in **Chapter 5** to improve our understanding of the mechanisms governing biomaterial-assisted endogenous cell-mediated repair over time. Mechanical loading was identified as inhibitor of cellular infiltration into the wound site, suggesting that the implementation of mechanical stimuli to this system was not necessary at early time point. Interestingly, the addition of chemotactic factors did not counteract this inhibitory effect. Furthermore, the model provided the opportunity to uncover a potential cell migration route at the interface layer of the osteochondral explant; either cells present in the subchondral bone or in the calcified cartilage highly participate in defect restoration, highlighting the importance of the osteochondral unit when evaluating joint tissue repair strategies. The absence of a strong cell response to external mechanical forces at early time point indicated that tuning the extracellular signals over time would be necessary to optimize their use for cellular decision making. These results underline the essence of the use of representative models to provide insight in the optimal time to apply dynamic loading after surgical intervention.

In this thesis we have provided important clues for future improvement of biomaterial-assisted cell-free cartilage repair approaches. In **Chapter 6** it is postulated that a multifactorial approach is pivotal to ameliorate the current strategies. To achieve successful cartilage regeneration, fine tuning of hydrogel design to allow organized endogenous cell infiltration and differentiation, while recapitulating the bulk mechanical properties of the native tissue, needs particular attention, along with a well-coordinated mechanical loading to provide optimal conditions to enhance cartilage remodeling.

R | References



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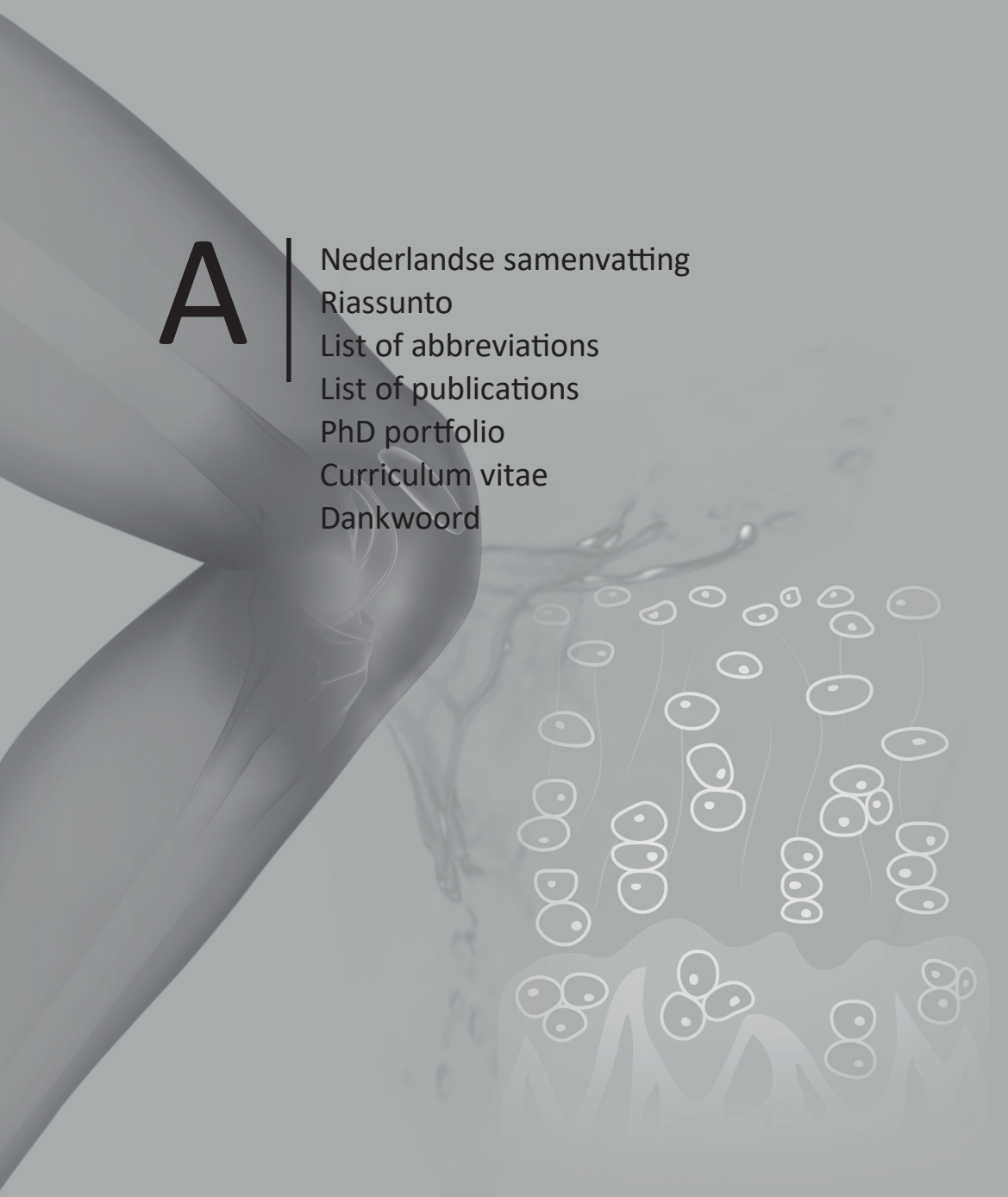

NEDERLANDSE SAMENVATTING

Gewrichtskraakbeenletsel vormt een grote klinische uitdaging in de orthopedie. De vooruitgang in de afgelopen decennia heeft de regeneratie van kraakbeen in de schijnwerpers gezet en de weg vrijgemaakt om de beperkingen van de huidige behandelingen te overwinnen. Om de klinische resultaten te verbeteren en nieuwe behandelstrategieën te ontwikkelen, beoogde dit proefschrift laboratoriummodellen te ontwikkelen die nodig zijn voor het testen van toekomstige behandelingen met biomaterialen zoals hydrogels, om kraakbeenletsel te hertellen. De geschiktheid van hydrogels als 3D-matrix om de ingroei van kraakbeenvoorlopercellen en hun differentiatie te ondersteunen en om biomoleculen te leveren om deze processen te verbeteren, werd geëvalueerd in onze modellen: een celmigratie-test in hydrogelen en een model van een kraakbeen-botexplantaat met een defect dat in kweek mechanisch belast werd in een bioreactor.

Celmigratie speelt een cruciale rol in het vroege proces van weefselherstel. Meerdere biochemische en biofysische factoren, afhankelijk van cel- en weefsel-eigenschappen, beïnvloeden migratie-efficiëntie. Cellen zijn dynamisch gevoelig voor de samenstelling, stijfheid en structuur van het biomateriaal wat gebruikt wordt om het defect te vullen, evenals voor bioactieve gradiënten, die celingroei kunnen versterken. In **Hoofdstuk 2** zijn verschillende formuleringen van hyaluronzuur (HA)-gebaseerde hydrogels in vitro en in vivo getest, met als doel het meest geschikte materiaal te selecteren voor regeneratieve therapieën die herstel door cellen in het lichaam stimuleren. We ontdekten dat veranderingen in mechanische eigenschappen de ingroei en differentiatie van cellen, en dus de productie van kraakbeenmatrix, beïnvloedden. Celingroei was omgekeerd-evenredig gecorreleerd met de stijfheid van de gel. Fibrine-hyaluronzuur (FB/HA)-gels lieten echter altijd de meest celingroei zien in vitro, zowel met als zonder migratiestimulerende factoren, en ook de beste chondrogene differentiatie. Onderhuidse implantatie van een kraakbeen-bot explantaat waarin het defect gevuld was met hydrogel bevestigde dit resultaat en liet endogene celrecrutering zien, zelfs in afwezigheid van migratie-stimulerende factor. Dit benadrukt het belang van de micro-omgeving en de hydrogelmatrix waarin cellen worden gerekruteerd, als een cruciale opstap naar het maken van functionele skeletale weefsels.

Aangezien letsels van kraakbeen en bot in het gewricht vaak optreden in het gewichtdragende gebied, lijkt het intuïtief dat er rekening moet worden gehouden met mechanische belasting als een essentiële factor die het weefselherstel beïnvloedt. Het succes van herstelprocedures die gebruik maken van hydrogels hangt af van het vermogen van cellen en hydrogels om druk- en schuifkrachten tijdens het belasten te weerstaan. In **Hoofdstuk 3** werd aangetoond dat complexe beweging bestaande uit druk-en schuifkrachten, met lage intensiteit uitgevoerd op FB/HA-gels met kraakbeencellen,

een positief effect had op het behoud van het kraakbeencel fenotype en de productie van kraakbeenmatrix. De reactie van cellen op deze krachten was effectiever wanneer de groeifactor FGF-18v werd toegevoegd aan ons in vitro kweekmodel. We toonden aan dat kraakbeencellen zich konden aanpassen aan een veranderende biochemische en mechanische micro-omgeving door de hoeveelheid en samenstelling van de kraakbeenmatrix aan te passen.

Conventionele bioreactorstudies voor kraakbeenregeneratie houden vaak geen rekening met het omliggende kraakbeen- en botweefsel. Met het doel om hydrogels geschikt te maken voor het herstel van kraakbeen- en botletsels in patienten, pasten we mechanische stimuli toe op hydrogels in een defect omgeven door kraakbeen en bot, om hun functioneren te evalueren. In **Hoofdstuk 4** hebben we eerst de ontwikkeling en validatie beschreven van een laboratorium model voor kraakbeen-botexplantaten onder mechanische druk- en schuifkrachten. Dit model biedt een representatieve fysiologische gewrichtachtige omgeving om reproduceerbare voorspellingen te doen van de prestatie van biomaterialen en van de effectiviteit van biomolecuulbehandeling. Dit model werd in **Hoofdstuk 5** gebruikt om de weefselherstelreacties na het inbrengen van een hydrogel in een kraakbeenbotdefect beter te begrijpen. Mechanische belasting werd geïdentificeerd als remmer van celingroei in de wondplaats, wat suggereert dat het aanbrengen van mechanische stimuli op een vroeg tijdstip niet nodig was. Interessant is dat het toevoegen van celmigratie-stimulerende factoren dit remmende effect niet tegenwerkte. Bovendien bood dit model de mogelijkheid om een potentiële celmigratieroute te ontdekken op de grenslaag tussen kraakbeen en bot; cellen die aanwezig zijn in het subchondrale bot of in het verkalkte kraakbeen namen in hoge mate deel aan het herstel van de defecten, wat het belang van de kraakbeen-bot eenheid benadrukt bij het evalueren van strategieën voor het herstel van gewrichtsletsel. De afwezigheid van een sterke celreactie op externe mechanische krachten op het vroege tijdstip gaf aan dat het afstemmen van signalen in de loop van de tijd nodig is om herstelprocessen te optimaliseren. Deze resultaten onderstrepen de essentie van het gebruik van representatieve modellen om inzicht te krijgen in de periode om dynamische belasting toe te passen na chirurgische ingrepen.

In dit proefschrift hebben we belangrijke aanwijzingen gegeven voor toekomstige verbetering van de behandeling van kraakbeenletsels met hydrogels. In **Hoofdstuk 6** wordt gepostuleerd dat een multifactoriële benadering cruciaal is om de huidige strategieën te verbeteren. Voor succesvol herstel van kraakbeenletsel het hydrogelontwerp nodig om georganiseerde endogene celingroei en differentiatie mogelijk te maken nauwkeurig worden afgestemd. Hierbij verdienen het nabootsen van de mechanische eigenschappen van het natuurlijke weefsel en een goed gecoördineerde mechanische belasting, bijzondere aandacht, derhalve de optimale omstandigheden te bieden voor kraakbeenregeneratie.

RIASSUNTO

La lesione della cartilagine articolare rappresenta una sfida significativa in clinica nel l'ambito ortopedico. I progressi degli ultimi decenni puntano i riflettori sulla rigenerazione della cartilagine, aprendo così la strada al superamento degli attuali limiti nei trattamenti odierni. Al fine di migliorare i risultati clinici e sviluppare nuove strategie di trattamento, questa tesi mira a fornire i modelli necessari per testare futuri approcci acellulari di riparazione assistita tramite l'utilizzo di biomateriali nell'articolazione del ginocchio. Gli idrogel, usati come modelli 3D, sono stati testati al fine di valutare la loro capacità di supportare l'infiltrazione di cellule condroprogenitrici e la loro differenziazione, nonché di rilasciare biomolecole per rafforzare questi processi valendosi di un saggio di migrazione basato sugli sferoidi e di un espianto di tessuto osteo-cartilagineo con difetto osteocondrale stimolato meccanicamente *ex vivo*.

La migrazione cellulare ha un ruolo critico nel processo iniziale di riparazione del tessuto a seguito dell'impianto di biomateriali, e la sua efficienza è influenzata dal microambiente attraverso una serie di fattori biochimici e biofisici che dipendono dalle proprietà cellulari e dalla matrice extracellulare utilizzata (ECM). Le cellule sono molto sensibili sia alla composizione del biomateriale che alla sua rigidità e struttura, nonché ai gradienti bioattivi, i quali possono potenziare la locomozione ed il movimento cellulare. Nel **Capitolo 2** sono state testate *in vitro* e *in vivo* diverse formulazioni di idrogel a base di acido ialuronico (HA) e diverse densità di reticolazione, con l'obiettivo di selezionare il gel più adatto a favorire terapie rigenerative che sfruttino le cellule endogene residenti. Abbiamo dimostrato che cambiamenti nelle proprietà meccaniche dell'idrogel influenzano la diffusione, la migrazione e la differenziazione cellulare. La coniugazione della fibrina con l'acido ialuronico era molto differente in termini di formulazione dell'idrogel e di concentrazione percentuale peso/volume rispetto al gel di HA-Tiramina ed alle sue diverse cinetiche di reticolazione (utilizzando 150, 300 o 600 µM di perossido di idrogeno, H_2O_2). Abbiamo osservato che la variazione della concentrazione di uno dei due agenti reticolanti il H_2O_2, mantenendo costante la concentrazione del gel di HA-Tiramina e dell'altro agente reticolante la perossidasi di rafano, è stato il principale fattore che ha influenzato sia la migrazione cellulare che la sintesi della matrice durante la condrogenesi delle cellule staminali mesenchimali. La migrazione era inversamente correlata con il modulo elastico del gel di HA-Tiramina in presenza del fattore di crescita derivato dalle piastrine BB. Ciò significa che il gel più morbido ha favorito una maggiore migrazione rispetto al gel più rigido. Tuttavia, gli idrogel di fibrina ed acido ialuronico (FB/HA) hanno sempre mostrato il più alto potenziale di migrazione cellulare, sia in presenza che in assenza dell'agente chemioattrattante *in vitro*, ed hanno anche favorito la differenziazione condrogenica. Il modello di espianto osteocondrale, impiantato sottocute *in vivo*, ha ulteriormente confermato il reclutamento di cellule endogene

nei gel, anche in assenza del fattore stimolante. Questo sottolinea l'importanza del microambiente e dell'idrogel usato come substrato nel quale vengono reclutate le cellule, rappresentando un cruciale trampolino di lancio verso l'ingegneria tissutale funzionale del tessuto musculare scheletrico.

Poiché i difetti dell'unità osteocondrale spesso si verificano nella regione che sostiente il peso, appare intuitivo che la riparazione delle lesioni focali dovrebbe tenere conto del carico meccanico come fattore essenziale influenzante la rigenerazione dei tessuti osteocondrali. Il successo dei trattamenti basati sull'utilizzo di biomateriali dipende dalla capacità delle cellule e degli idrogel di sostenere le forze di compressione e di taglio durante il carico. Nel **Capitolo 3** è stato dimostrato che l'applicazione meccanica del carico, ad orientamento biassiale ed a bassa intensità, sui gel di FB/HA miscelati con condrociti ha avuto un effetto positivo sul mantenimento del fenotipo condrogenico e sulla produzione della matrice cartilaginea. La meccanotrasduzione si è rivelata più efficace quando una variante esogena del fattore di crescita dei fibroblasti 18 (FGF-18v) è stata aggiunta al nostro modello nel mezzo di coltura *in vitro*, influenzando in modo interdipendente il metabolismo cellulare ed aumentando la qualità del tessuto costruito cartilagineo. Abbiamo dimostrato che i condrociti potrebbero sinergicamente adattarsi ad un microambiente biochimico e meccanico, modulando la quantità di ECM, down regolando enzimi degradanti la matrice e promuovendo una superficie articolare funzionale.

Studi convenzionali sui bioreattori che promuovono la rigenerazione della cartilagine spesso non tengono conto del tessuto osteocondrale. Con l'obiettivo di rendere clinicamente possibili le tecnologie di riparazione del difetto osteocondrale assistito da biomateriali, abbiamo applicato stimoli meccanici sui gel in un ambiente più confinato in modo da valutare la loro funzione all'interno del tessuto, proprio come accade in vivo. Nel **Capitolo 4** abbiamo descritto per la prima volta lo sviluppo e la validazione di un modello di difetto osteocondrale *ex vivo* sotto carico di compressione e taglio, che mima l'ambiente articolare del ginocchio al fine di consentire una riproducibile previsione delle prestazioni dei biomateriali e dell'efficacia del trattamento dei fattori di crescita utilizzati. Questo modello è stato utilizzato nel **Capitolo 5** per migliorare la nostra comprensione dei meccanismi che regolano la riparazione mediata da cellule endogene quando assistita da biomateriali in seguito a trauma. Il carico meccanico è stato identificato come un inibitore dell'infiltrazione cellulare nella zona della ferita, suggerendo che l'aggiunta di stimoli meccanici in questo sistema all'inizio del time point non era necessaria. È interessante notare che l'aggiunta di fattori chemiotattici non ha contrastato questo effetto inibitorio. Inoltre, il modello ha fornito l'opportunità di scoprire una potenziale via di migrazione cellulare nello strato di interfaccia dell'espianto osteocondrale; le cellule presenti nell'osso subcondrale o nella cartilagine calcificata partecipano fortemente al ripristino del difetto, evidenziando l'importanza

dell'unità osteocondrale nella valutazione delle strategie di riparazione dei tessuti articolari. L'assenza di una forte risposta cellulare in seguito all'applicazione di forze meccaniche esterne all'inizio del time point ha indicato che una fine regolazione dei segnali extracellulari nel tempo sarebbe necessaria per ottimizzare il loro utilizzo per modulare il processo decisionale cellulare. Questi risultati sottolineano l'essenza dell'uso di modelli rappresentativi per fornire un'idea sui tempi ottimali in cui applicare il carico dinamico dopo un intervento chirurgico.

In questa tesi abbiamo fornito importanti indizi per il miglioramento futuro di approcci acellulari di riparazione della cartilagine tramite l'utilizzo di biomateriali. Nel **Capitolo 6** si postula che un approccio multifattoriale è fondamentale per migliorare le strategie attuali. Per ottenere con successo la rigenerazione della cartilagine, la messa a punto del design di un idrogel richiede particolare attenzione al fine di consentire l'infiltrazione organizzata delle cellule endogene e la loro differenziazione, mentre riepiloga le proprietà meccaniche del tessuto nativo. In aggiunta il carico meccanico deve essere ben coordinato per fornire le condizioni ottimali per migliorare il rimodellamento della cartilagine.

LIST OF ABBREVIATIONS

AA-2-P	Ascorbic acid-2-phosphate
ACAN	Aggrecan
ACI	Autologous chondrocytes implantation
α-MEM	alpha-Minimum Essential Medium
AMIC	autologous matrix-induced chondrogenesis
BMSC	bone marrow mesenchymal stromal cell
CCM	Complete chondrogenic medium
CDFA-SE	Carboxyfluorescein Diacetate Succinimidyl Ester
CCL5	Chemokine ligand 5 or RANTES
CL	Cartilage layer
COL1A2	Collagen 1
COL2A1	Collagen 2
COL10	Collagen 10
COMP	Cartilage oligomeric matrix protein
DMEM-HG	Dulbecco's modified Eagle's medium
ECM	Extracellular Matrix
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor 2
FGF-18v	Fibroblast growth factor 18 variant
FGFR-3	Fibroblast growth factor receptor-3
FB/HA	Fibrin-Hyaluronan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G'	Storage modulus
G''	Loss modulus
HA	Hyaluronan
HA-Tyr	Hyaluronan-Tyramine
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
ITS+	Insulin, transferrin and selenium
LDH	Lactate dehydrogenase
MACI	Matrix-induced/assisted ACI
MMPs	Matrix Metalloproteinases
MSCs	Mesenchymal stromal cells
OA	Osteoarthritis
PBS	Phosphate buffered saline
PDFG-BB	Platelet-derived growth factor BB
PEG	Polyethylene glycol

PGA	Polyglycolic acid
PLA	Polylactic acid
PRG4	Proteoglycan 4/Lubricin
P/S	Penicillin/Streptomycin
PU	Polyurethane
qRT-PCR	Quantitative real-time polymerase chain reaction
RPLP0	Ribosomal protein lateral stalk sub-unit P0
RGD	Arginin-Glycin-Aspartate
SB	Subchondral bone
SDF-1	Stromal cell-derived factor 1
SF	Serum free medium
sGAG	sulphated-Glycosaminoglycan
TGF-β1	Transforming growth factor beta 1
TKA	Total knee arthroplasty
VCAN	Versican

LIST OF PUBLICATIONS

[1] **M.L. Vainieri**, A.M. Blagborough, A.L. MacLean, M.L. Haltalli, N. Ruivo, H.A. Fletcher, M.P. Stumpf, R.E. Sinden, C.L. Celso, Systematic tracking of altered haematopoiesis during sporozoite-mediated malaria development reveals multiple response points, Open Biol 6(6) (2016).

[2] **M.L. Vainieri**, D. Wahl, M. Alini, G. van Osch, S. Grad, Mechanically stimulated osteochondral organ culture for evaluation of biomaterials in cartilage repair studies, Acta biomaterialia 81 (2018) 256-266.

[3] A. Lolli, K. Sivasubramaniyan, **M.L. Vainieri**, J. Oieni, N. Kops, A. Yayon, G. van Osch, Hydrogel-based delivery of antimiR-221 enhances cartilage regeneration by endogenous cells, Journal of controlled release : official journal of the Controlled Release Society 309 (2019) 220-230.

[4] **M.L. Vainieri**, A. Lolli, N. Kops, D. D'Atri, D. Eglin, A. Yayon, M. Alini, S. Grad, K. Sivasubramaniyan, G. van Osch, Evaluation of biomimetic hyaluronic-based hydrogels with enhanced endogenous cell recruitment and cartilage matrix formation, Acta biomaterialia 101 (2020) 293-303.

[5] B.P. Antunes, **M.L. Vainieri**, M. Alini, E. Monsonego-Ornan, S. Grad, A. Yayon, Enhanced chondrogenic phenotype of primary bovine articular chondrocytes in Fibrin-Hyaluronan hydrogel by multi-axial mechanical loading and FGF18, Acta biomaterialia 105 (2020) 170-179.

[6] F. Colella, J.P. Garcia, M. Sorbona, A. Lolli, B. Antunes, D. D'Atri, F.P.Y. Barré, J. Oieni, **M.L. Vainieri**, L. Zerrillo, S. Capar, S. Häckel, Y. Cai, L.B. Creemers, Drug delivery in intervertebral disc degeneration and osteoarthritis: Selecting the optimal platform for the delivery of disease-modifying agents, Journal of controlled release : official journal of the Controlled Release Society (2020).

[7] S.W. Myriam L.R. Haltalli, Nicola K. Wilson, Kira Eilers, Alexander Lipien, Heather Ang, Flora Birch, Sara Gonzalez Anton, Chiara Pirillo, Nicola Ruivo, **Maria L. Vainieri**, Constandina Pospori, Robert E. Sinden, Tiago C. Luis, Jean Langhorne, Ken R. Duffy, Berthold Göttgens, Andrew M. Blagborough, Cristina Lo Celso Manipulating niche composition limits damage to haematopoietic stem cells during Plasmodium infection, Nature Cell Biology (2020).

[8] **M.L. Vainieri**, M. Alini, A. Yayon, G. van Osch, S. Grad, Mechanical Stress Inhibits Early Stages of Endogenous Cell Migration: A Pilot Study in an Ex Vivo Osteochondral Model, Polymers (Basel) 12(8) (2020).

This thesis is based on the following publications: 2, 4, 5 and 8.

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Maria Letizia Vainieri	**PhD period:** September 2015 – December 2019
Erasmus MC Department: Orthopaedics	**Promotor:** Prof. Gerjo J.V.M. van Osch, PhD
Research School: MolMed	**Co-promotor:** Sibylle Grad, PhD

1. PhD training

		Year	Workload (ECTS)
Courses			
-	ESR Nanomedicine Training Session (Imperial College London, UK)	10-2015	0.1
-	Scientific Presentation (Academia Retica, Davos, CH)	12-2015	0.5
-	ESRs RNA interference Training Session (iNANO, Aarhus University, DK)	05-2016	0.1
-	Statistics for Biology and Medical Research (SIAF Institute, Davos, CH)	06-2016	0.6
-	Personalised Therapy Symposium at TERMIS-EU Conference (Davos, CH)	07-2016	0.1
-	Enabling Technologies (Utrecht University, NL)	10-2016	1.5
-	Image J Analysis Workshop (Utrecht University, NL)	10-2016	0.1
-	ESRs Stem Cells and Organoids Training Session (Utrecht University, NL)	12-2016	0.1
-	German Course (ARI, Davos, CH)	2016	4
-	Proposal Writing & Funding (Graduate School Graubunden, Davos, CH)	12-2017	0.3
-	Summer School: Complex Cell Systems (6th SBMS, Interlaken, CH)	05-2017	0.5
-	ESRs Training on Patents and IP strategies (Technion University, Haifa, IL)	01-2018	0.1
-	Summer School: Complex Cell Systems (6th SBMS, Interlaken, CH)	05-2018	0.5
-	Data Processing Advanced Course with Graph Pad Prism (ARI, Davos, CH)	07-2018	0.6
-	ESRs Preclinical Imaging Training Session (University of Leiden, NL)	07-2018	0.1
-	Research Integrity (Erasmus MC, Rotterdam, NL)	02-2019	0.3
-	Summer School: The Osteochondral Interface (7th SBMS, Interlaken, CH)	05-2019	0.5
Conferences – Podium Presentations			
-	5th Graubunden Forscht Conference, Davos, CH:	09-2016	1
	Investigating the homing behavior of endogenous stem cells in a joint bioreactor to regenerate articular cartilage.		
-	SSB+RM Conference, Fribourg, CH:	05-2018	1
	Novel hyaluronan-based hydrogels to support endogenous cartilage repair.		
-	6th Graubunden Forscht Conference, Davos, CH:	09-2018	1
	HA-based hydrogels for cartilage tissue engineering		
-	TERMIS-EU Conference, Rodhes, GR :	05-2019	1
	Biomimetic hyaluronic acid-hydrogel enhances endogenous cell recruitment and healing process of cartilage lesions		
Conferences – Poster Presentations			
-	Gordon Research Conference Cartilage Biology, Lucca, IT:	04-2017	1
	Novel ex-vivo osteochondral defect model in a joint bioreactor system for articular cartilage repair studies		

-	TERMIS-EU, Davos, CH: *Novel ex-vivo osteochondral defect model in a joint bioreactor system for articular cartilage repair studies*	06/2017	1
-	ICRS 14th World Congress, Macau, China: *Hyaluronic acid-based hydrogels promote mesenchymal stem cell ingrowth and cartilage production in vitroand in vivo.*	04/2018	1
-	eCM XVIII Conference Cartilage and Disc Repair, Davos, CH: *Novel ex-vivo osteochondral model for cartilage repair in mechanical stimulated bioreactor*	06/2018	1
-	ORS Annual Meeting, Austin, TX, USA: *Effect of mechanical stimulation combined with FGF-18 on bovine chondrocytes embedded in a novel Fibrin:Hyaluronan hydrogel*	02/2019	1
Presentations and meetings			
-	Lab Meetings IVD/Cartilage (weekly)	2015-2019	2
-	Muskuloskeletal Regeneration Programme Meetings (monthly)	2015-2019	1
-	Journal club (monthly)	2015-2018	1
-	WP4 TargetCare Meetings (monthly)	2016	0.2
-	Lab Meetings dept. Orthopaedics (weekly)	05-09 '18	0.1
-	Research Meetings dept. Internal Medicine/Skeleton Meetings (weekly)	05-09 '18	0.1
-	ESRs TargetCare Consortium Meetings (biannually)	2015-2019	2
2. Teaching			
		Year	**Workload (ECTS)**
-	Workshop "Synovial Joint and Articular Cartilage" for ETH and Winterthur Master Students, ARI Davos, CH (annually)	2016-2019	2
Supervising and Tutoring			
-	Partial Supervision Master Student, Dal Fabbro Lea Tiziana, Winterthur, CH, (3 months)	2017	1
-	Co-Supervision PhD Student, Bernardo Antunes, Hebrew University, IL (8 months)	2019	3
Miscellaneous			
-	Active Board Member of Young Scientists (YS) of Swiss Society of Biomaterial and Regenerative Medicine (SSB+RM)	2016-2020	
-	Organization YS Lab-Networking event at RMS Foundation, Bettlach, CH	01-2017	0.5
-	Organization Pre-Conference SSB+RM YS Workshop "Training for scientific writing: Tips and Tricks"	05-2017	0.5
-	Co-Chair Session TargetCare Symposium at TERMIS-EU, Davos, CH	06-2017	
-	Organization YS Symposium Universitäts Spital Zürich	11-2017	5
-	Review of scientific papers (2x)	2017	0.3
-	Organization YS Lab-Networking event at AO Foundation, Davos, CH	04-2018	0.5
-	Visiting PhD Student at the Connective Tissue Regeneration Lab, Erasmus MC Rotterdam, NL (5 months)	05-09 '18	5
-	Co-chair Session Delivery and Imaging System at eCM XVIII Conference, Davos Congress, CH	06-2018	

-	Organization Pre-Conference SSB+RM YS Workshop “You and Your career”	06-2018	0.5
-	Visiting PhD Student at Procore Ltd, Weissman Park, Ness Ziona, IL	01-2019	1
-	Organization YS Lab-Networking event at Geistlich Pharma, Wolhusen, CH	01-2019	0.5
-	Organization Pre-Conference Workshop “Patient-Specific Implants produced By Additive Manufacturing”	05-2019	0.5
Other			
-	Die Südostschweiz Newspaper Interview on PhD life and challenges	03-2016	
-	Organization Group Activity Muskuloskeletal Program (5 days)	2016	5
-	Organization Group Activity Muskuloskeletal Program (5 days)	2019	5
Grant and Award			
-	Best Oral Presentation Award, 5th Conference Graubunden Forscht “Young Scientist in Contest”, Davos, CH	2016	
-	Swiss Bone Mineral Society Travel Fellowship, Bern, CH	2019	

CURRICULUM VITAE

Maria Letizia Vainieri was born on the 22nd of December 1983 in Melfi (PZ), Italy. After graduating from Scientific Lyceum "Federico II di Svevia", she started Biotechnology at La Sapienza University of Rome. For her bachelor assignment, she gained her first experience with scientific research during the 8 months internship at Biochemistry Department of La Sapienza University. This resulted in the bachelor thesis "ERp57 and the oxidative stress" in November 2008. In 2009 she started the Master in Molecular, Cellular and Medical Biotechnology at La Sapienza University of Rome. In 2011 she was given the opportunity to conduct a year study on the establishment of bone marrow and hematopoietic niche *in vivo* by reversion of chondrocytes differentiation of human bone marrow stromal cells. This research was carried out under supervision of Prof. Paolo Bianco in the bone tissue regeneration group at University Hospital Policlinico Umberto I in Rome. Her interest in cartilage, bone and bone marrow led to the graduation research entitled "New heterotopic transplantation protocol of human mesenchymal stem cells" obtaining a master's degree in October 2012. In 2013 she won two scholarships and moved to Imperial College London to investigate how Malaria infection affects hematopoietic stem cells and bone marrow. Then in September 2015 her PhD training began as a Marie Curie ITN Fellow (TargetCare Consortium) at Musculoskeletal Regeneration group at AO Foundation in Davos (CH) under supervision of dr. Sybille Grad and at Connective Tissue Repair group at the Erasmus MC, University Medical Center in Rotterdam (NL) under supervision of prof. dr. Gerjo J.V.M. van Osch. As of January 2020, she is working as education project coordinator at AO Foundation in Davos (CH).

DANKWOORD

And here it comes….after 5 intense years this day finally arrived: the last touch of this thesis, the acknowledgements. It has been an epic journey of deep learning both at scientific and personal level, which resulted in a strong impact on my personality. Doing a PhD in a such remote place in the middle of Alpes push your boundaries, an unforgettable experience which I would certainly repeat. This thesis, far from being the final goal of my PhD, is the expression of a fruitful human and scientific experience disseminated from meeting many people of different cultures and special places. While writing, I realized the PhD is about people you had fortune to meet and I should say I had the chance to encounter so many pioneers in the field from cartilage matrix development to stem cells and tissue engineering, each of them inspired me and helped me to find solutions to my questions, belief and ideas.

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but also, despite the distance, the Post-Doc I missed on my side during this journey. I enjoyed the time spent in Rotterdam, thanks also for hosting and "standing" me.

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