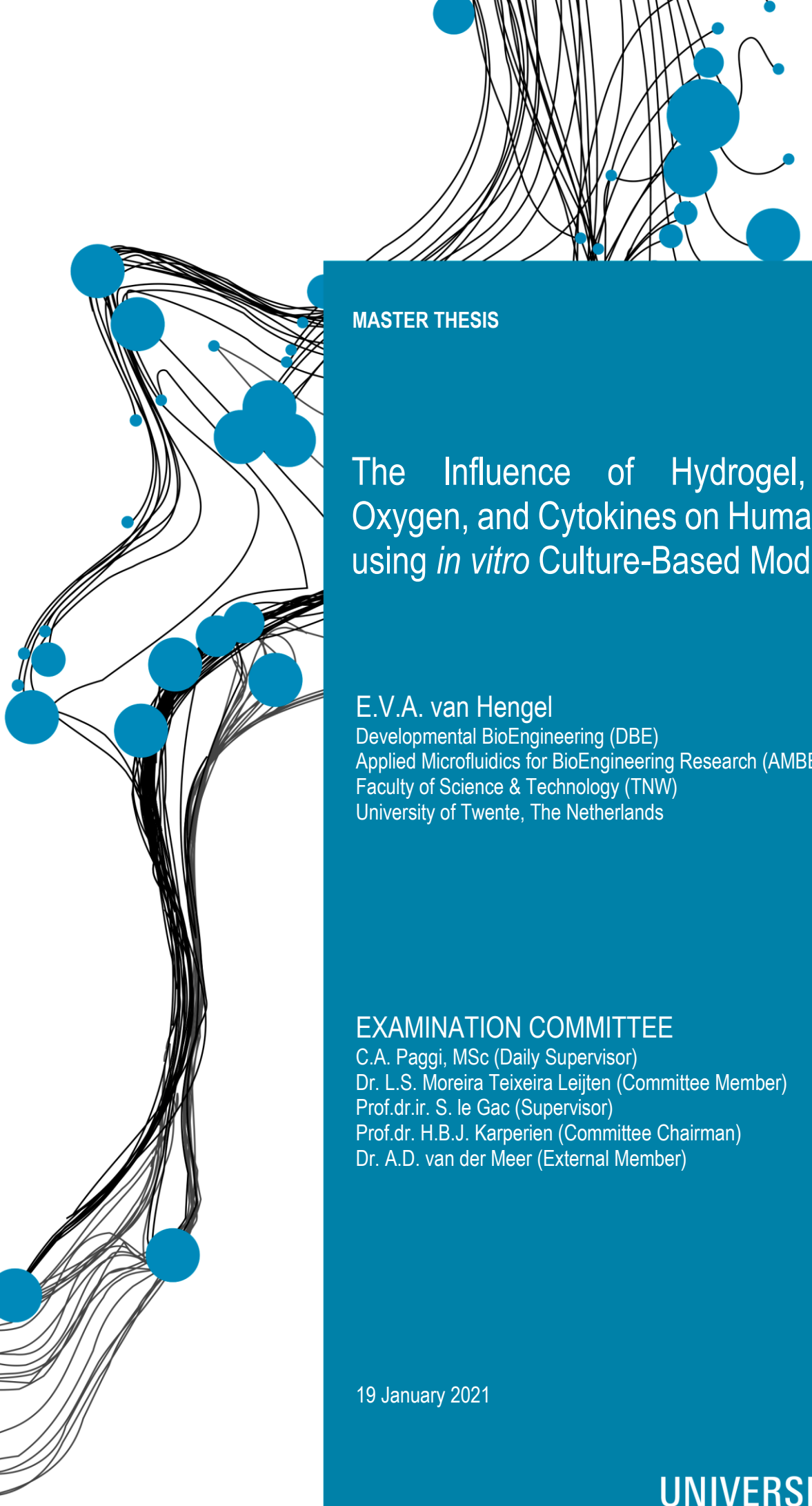

MASTER THESIS

The Influence of Hydrogel, Temperature, Oxygen, and Cytokines on Human Chondrocytes using *in vitro* Culture-Based Models

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Abstract

Osteoarthritis is one of the most common knee cartilage diseases affecting over 1,4 million people in the Netherlands in 2018 and results in cartilage destruction and impaired mobility. Due to the unique structure and characteristics of articular cartilage, there is currently no appropriate *in vitro* model available that fully mimics the *in vivo* situation, limiting the research on disease progress and drug development. Important requirements for a culture model are the low physiological temperature (32,6 °C) and oxygen levels (2,5% O_2) of cartilage, and the ability to culture in a dynamic three-dimensional environment, enabling both mechanical and biochemical stimuli. In this study, the aim was to establish a chondrocyte *in vitro* culture system which comprises all required factors. To this end, the project was split into two sections: the first part to study hydrogels for a dynamic culture, the second part to study a physiological culture environment.

Current protocols rely on agarose as a matrix for chondrocyte culture, but this gel lacks various features, such as cell attachment sites and the ability for cells to proliferate. Therefore, the use of Dex-TA/HA-TA hydrogel was evaluated for a three-dimensional dynamic culture system. Dex/HA chondrocytes showed viability, maintenance of the chondrogenic phenotype, motility, and enhanced production of degradative factors, suggesting the potential as culture matrix for a better representation of *in vivo* cartilage, as an alternative to agarose.

Secondly, we determined the effect of temperature 33 °C (intra-articular), 37 °C (body core), and 39 °C (hyperthermic cartilage) under hypoxic circumstances for monolayer and pellet cultures. A culture temperature between 33 °C and 37 °C under hypoxic circumstances was optimal for chondrocyte culture; positively influencing the chondrocyte-specific genes; cartilage development; and the maintenance of the differentiated phenotype. Lastly, under the same temperature and oxygen conditions, an inflammatory response was triggered by pro-inflammatory cytokine stimulation. The effects of cytokine stimulation were enhanced under hypoxic conditions, resulting in a stress response.

In the future, a reliable platform for chondrocyte culture could be established by combining the Dex/HA hydrogel culture and physiological culture circumstances, this could be applied as a disease model for Osteoarthritis by hyper physiological mechanical and cytokine stimulation.

Keywords: chondrocytes; cartilage-on-chip; hydrogel; mechanical stimulation; temperature and oxygen variations; 2D and 3D models

Samenvatting

Osteoartritis is een van de meest voorkomende ziektes van het knie kraakbeen, met in 2018 ruim 1,4 miljoen gevallen in Nederland, en leidt tot schade aan het gewichtskraakbeen en verslechterde mobiliteit. Door de unieke structuur en kenmerkende eigenschappen van kraakbeenweefsel is er op dit moment geen geschikt *in vitro* model, wat het onderzoek naar het ziekteverloop en behandelingen belemmert. Belangrijke vereisten voor een dergelijk kraakbeenmodel zijn de lage fysiologische omgevingstemperatuur en zuurstofgehalte en de kweek in een dynamische driedimensionale omgeving met de mogelijkheid voor zowel mechanische en biochemische stimulatie. Het doel van dit project was om een *in vitro* model te realiseren dat al deze beschreven factoren in acht neemt. Daarvoor is dit project onderverdeeld in twee secties: eerst een hydrogel studie voor een dynamisch kraakbeen-op-chip platform, en vervolgens een studie betreffende de fysiologische kweekomstandigheden.

Huidige protocollen gebruiken agarose als kweekmatrix voor kraakbeen, maar deze gel kent een aantal tekortkomingen, zoals het gebrek aan cel aanhechtingen en mogelijkheid tot proliferatie. Daarom is het gebruik van een Dex-TA/HA-TA hydrogel getest onder dynamische kweekomstandigheden, wat resulteerde in behoud van het chondrogene fenotype, cel mobiliteit en verhoogde productie van degradatie factoren, waardoor het gebruik van deze hydrogel als kweekmatrix een betere representatie van *in vivo* kraakbeen kan bieden.

In het tweede deel van dit project is het effect van kweektemperatuur, 33 °C (intra-articulair), 37 °C (lichaamskern), en 39 °C (hyper thermisch), onder lage zuurstof omstandigheden (2,5 % O_2) bepaald voor zowel twee als driedimensionale chondrocyt kweek. Een temperatuur tussen 33 °C en 37 °C onder lage zuurstof omstandigheden was het meest optimaal, met een positief effect op kraakbeen-specifieke genen, kraakbeen ontwikkeling en het behoud van het gedifferentieerde fenotype. Tenslotte is onder dezelfde fysiologische kweekomstandigheden een ontstekingsrespons getriggerd door stimulatie met pro-inflammatoire cytokines, welke werd versterkt onder hypoxie kweek, resulterende in een stress reactie.

Een verbeterd kweekplatform voor chondrocyten kan in de toekomst bereikt worden door de hydrogel kweek te combineren met fysiologische kweekomstandigheden en deze te gebruiken als ziektemodel voor osteoartritis door stimulatie met hyper fysiologische mechanische stimulatie en cytokines.

Kernwoorden: chondrocyten; kraakbeen-op-chip; hydrogel; mechanische stimulatie; temperatuur en zuurstof variaties; 2D en 3D modellen

Contents

List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACAN	Aggecan
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AsAP	Ascorbic acid 2-phosphate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDM	Chondrogenic Differentiation Medium
Col	Collagen
Comp.	Compression stimulated
COMP	Cartilage Oligomeric Matrix Protein
CPM	Chondrogenic Proliferation Medium
CT	Cycle threshold
Dex-TA	Dextran-Tyramine
Dex/HA	Dextran-Tyramine/Hyaluronic acid-Tyramine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HA-TA	Hyaluronic acid-Tyramine
HIF-1α	Hypoxia-inducible factor 1-alpha
HK2	Hexokinase 2
HRP	Horse radish peroxidase
HSP	Heat shock protein
IGF	Insulin-like growth factor
IL	Interleukin
MMP	Metalloproteinases
mRNA	Messenger ribonucleic acid
OA	Osteoarthritis
PBS	Phosphate Buffered Saline
PKM2	Pyruvate kinase isozymes
PD	Polydopamine
PDMS	Polydimethylsiloxane
RNA	Ribonucleic acid
RT	Room temperature
SOX9	SRY-Box transcription factor 9
TA	Tyramine
TGF-β	Transforming growth factor-beta
TNF−α	Tumor necrosis factor-alpha
qPCR	Quantitative real-time polymerase chain reaction

1. Introduction

1.1. Articular Cartilage

1.1.1. Knee joint

The joints of the human body connect two or more bones and allow stability and movement of the skeleton. Among the articular joints, the knee is the largest and the most studied due to its importance in the leg movement. [1] The knee is a primary weight- and load-bearing mobile joint, since it carries the mass of our body, and handles load during movement. [2] During movement, the load generated is transferred from the bone to the articular cartilage. [3] Articular cartilage on the surface of the bones prevents grinding and rupture of the bones, to enable withstanding of loading cycles without tissue damage. [2] Moreover, ligaments and the joint capsule provide stability during motion, giving strength and preventing excess or abnormal movements (**Figure 1**). [1][4] Within the joint capsule, the synovial membrane, also called synovium, is located. This connective tissue secretes synovial fluid, containing nutrients for the articular cartilage, and further reduces the friction generated during movement. [2][5]

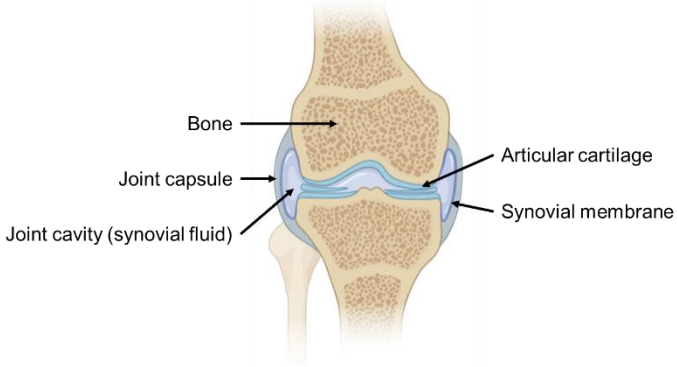


Figure 1. *Schematic representation of the knee joint. The bones are covered with articular cartilage, which are in contact within the joint cavity filled with synovial fluid. Created with BioRender.com.*

1.1.2. Cartilage tissue

Within the articulating knee joint, the friction between bones is prevented by a layer of 2 to 4 mm thickness hyaline cartilage. [5] Healthy cartilage can withstand many loads without degradation or failure and is able to support and transfer load, allowing smooth movement of the bones against each other. [3][5] Articular cartilage is mainly composed of extracellular matrix (ECM) filled with chondrocytes. In healthy cartilage, 65 – 80% of the total ECM weight consists of tissue fluid, which is composed of water with inorganic ions. The solid ECM is responsible for support of single and rapid movements. Its unique properties are due to the characterized structure and subdivision in zones (**Figure 2**). [3][5] The division into superficial, middle, deep, and calcified zones vary in biochemical composition, function, and biomechanical properties.

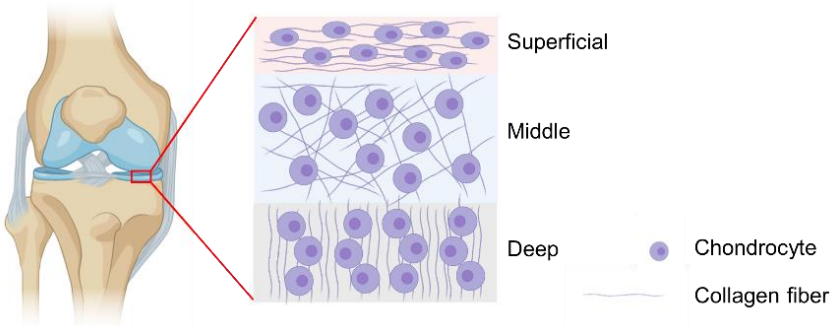


Figure 2. *Schematic representation of human articular cartilage. Orientation of chondrocytes and Collagen fibers are shown for the superficial, transitional and deep zone. Created with BioRender.com.*

Cartilage tissue does not present any nerve and blood vessels. Therefore nutrients are provided by two pathways; diffusion from the bone vessels and diffusion from the synovial fluid. [6] The synovial fluid, containing multiple proteins, provide nutrients through the articular surface to the chondrocytes within the superficial and middle zone. However, only a part of the chondrocytes are provided with nutrients, resulting in a nutrient gradient within the cartilage tissue. During movement, the synovial fluid can reach the bone zone and thereby increase the diffusion of nutrients. [3] Beside the nutrients and waste transportation and the reduction of friction between bones, the synovial fluid is also an important contributor to joint homeostasis. [2][5] The cells located inside the synovium, the synoviocytes, produce albumin and hyaluronic acid (HA), which give the fluid its viscosity and slickness. [7] The viscosity plays an important role in shock absorption and increases due to applied pressure. The reduction of frictional forces is due to the presence of lubricin on the articular surface, which is an important protein for joint lubrication. [2]

Collagen represents 60% of the dry weight of cartilage and forms a fibrous framework that enables shear stress resistance and ECM protection. [5] Collagen type II accounts for over 90% of the collagens within adult articular cartilage. [8] Its synthesis is highest during skeletal growth, and drops thereafter. This major Collagen type crosslinks with type IX and XI to form the fundamental framework of cartilage ECM. The crosslinking and orientation determines the tissue strength.

Proteoglycans, including Aggrecan and fibromodulin, are proteins that are intertwined within the Collagen network. [3] They are synthesized, maintained and secreted into the ECM by chondrocytes and are important for cartilage homeostasis. The proteoglycan metabolism is regulated by a number of growth factors, such as transforming growth factor-β (TGF-β). [3] Furthermore, proteoglycans can form highly hydrated aggregates that provide resistance to compressive loads, due to their negative charge, a swelling pressure occurs. When no external loads are applied on the tissue, the swelling pressure is kept in balance by stresses generated in the cartilage matrix, which are called pre-stresses. The pre-stress and swelling pressure contribute to the mechanical properties of cartilage tissue. [9][10] It is found that over years, the proteoglycan aggregates get smaller due to a decrease in available binding sites. [3][11] This results in a lower hydration of the cartilage ECM, resulting in an increase of compressive stiffness and less force withstanding. [12]

1.1.3. Chondrocytes

Chondrocytes are the cellular constituent of cartilage, they include only 1 – 2% of the total tissue volume. Chondrocytes play a key role in the regulation of ECM synthesis and homeostasis in order to maintain cartilage architecture, composition, and functionality. [5] They monitor the synthesis of components, such as Collagen, glycoproteins, hyaluronic acid, and proteoglycans to keep a physiological balance of the ECM. Chondrocytes maintain a spheroid shape *in vivo*, while being surrounded by a proteoglycan rich precellular matrix. [13] Further, chondrocytes are mainly depended on the anerobic metabolism and they obtain their nutrition via diffusion from the synovial fluid. [3] After cartilage damage, an imbalance in their homeostasis can occur, and the chondrocytes retain only a limited capacity for self-repair and recapitulation of Collagen synthesis. [9][14] Beside this, the chondrocyte organization and functionality is determined by age. Over time, the number of chondrocytes stay the same, but their organization and functionality changes. A decrease in ability to maintain and repair the tissue and a decreased responsiveness to growth factors, results in softening of the tissue and loss of matrix strength and stiffness. [15]

In the human body, chondrocytes are able to move through the cartilage tissue. They show controlled and regulated movements during growth and tissue remodeling, despite the high density and pressure of cartilage matrix. [13] The exact underlying mechanisms of cell motility are unknown, but may be initiated by the presence of chondroprogenitor cells. [16] Movement may also be due to active migration as response to motility cues, weakening of the attachment to the cartilage matrix, or grip-and-pull operation on Collagen fibers by the use of their contractile cell skeleton. [13] Migrating chondrocytes do express chondrogenic-specific genes (e.g. Collagen II), indicating that they maintain their differentiated status. [17]

1.1.4. Joint movement and loading

Motion of the knee results mainly in compression and shear forces on the cartilage tissue, which is withstand by the unique viscoelastic properties of cartilage. The modulus of cartilage depends on the tissue depth and orientation of the tissue. [5] The superficial zone of cartilage contains mainly Collagen fibers that are aligned parallel to the surface, making them functional for sustaining induced shear stresses. Within the middle zone and deep zone, the Collagen fibers are perpendicular organized and therefore offer protection to compression forces. [3][18]

The interaction between the fluid and solid phases of the ECM is important for the mechanical functionality and thereby its deformation characteristics. [5] Within the superficial layers, the fluid content is high, which mainly consists of water and inorganic ions. The porous and permeable ECM is considered as solid phase. Single and fast loading causes an increase in fluid pressure, whereby the fluid is squeezed out of the tissue. During repetitive loading cycles, almost all fluid flows out of the tissue, and the ECM provides resistance to the load. Thereafter, during rest, the fluid is reabsorbed by the ECM. [3][12]

Healthy articular cartilage is maintained by joint motion and load, and research has shown that during low intensity movement the production of ECM is stimulated. In contrast, high loading movements can inhibit the mechanism of ECM synthesis and can even result in tissue degradation. But on the other hand, due to long lasting periods of inactivity, the chondrocytes can become senescence and their responsiveness to growth factor decreases, resulting in altered proteoglycan synthesis and cartilage degradation as well. [3][19][20]

1.1.5. Cartilage disorders

Cartilage can be affected by several injuries and diseases, resulting in degradation and less mobility. Arthritis is the collective name for disorders that affect the joints. Common symptoms are joint, inflammation, stiffness, swelling, and a decreased range of motion of the affected joints. There are over 100 types of arthritis, with Osteoarthritis (OA) as most common type. [21]

Osteoarthritis

In 2018, more than 1,4 million patients in the Netherlands were diagnosed with the degenerative disease Osteoarthritis (OA), of which almost 700.000 affects the knee joint. Since OA mostly affects the elderly, the annual prevalence is expected to increase with 41% in 2040 due to the aging population. [22] The exact mechanism of OA development remains unclear, but biomechanical, anatomic, and functional factors play an important role and risk factors include age, gender, former injuries, trauma and obesity. [23] Due to the slow, but progressive degeneration of the articular tissue, it highly influences the quality of life. [7][23] Cartilage tissue in OA follows a myriad of cascades, only the ones that are most relevant for this project will be appointed in this section.

The cartilage homeostasis, which is regulated by the interplay between mechanical, inflammatory, and biochemical factors, gets imbalanced during OA. [23] The metabolic imbalance results in the breakdown of the fibrillar network, which highly affects the superficial zone of the tissue (**Figure 3**). [24] When cartilage is damaged in healthy tissue, chondrocytes secrete new ECM components, such as Collagen type II and proteoglycans, to repair the damage. However, during OA, chondrocytes undergo phenotypic alterations into a hypertrophic phenotype and thereby produce higher levels of proteolytic enzymes, Collagen type III and X. [25] Hypertrophic cells are considered the end state of the chondrocyte differentiation pathway and stimulate cartilage degradation and the expression of proteolytic enzymes. Furthermore, inflammatory cytokines, chemokines, and other inflammatory mediators are produced, resulting in disruption of the cartilage homeostasis. Because the turnover rates of proteoglycans and collagens are not in balance and tissue degeneration is faster than the regeneration process, this results in loss of cartilaginous tissue over time. [11][19][23]

OA is involved in the entire joint, and also alters the calcified cartilage which is present below the articular hyaline cartilage. In a healthy joint, cartilage is separated from the calcified layer and the underlying subchondral bone by the tidemark. During OA, loss of the network results in the development of fissures in the cartilage tissue, leading to exposure to the calcified cartilage and subchondral bone. [26] Hereby, calcification of the deepest zone occurs by the expansion of calcified cartilage into the articular cartilage and remodeling of the subchondral bone. The subchondral bone increases in volume, but becomes weaker than healthy bone. In addition, osteophytes and bone cysts are developed at the joint margin, contributing to the generation of pain in disease. [27]

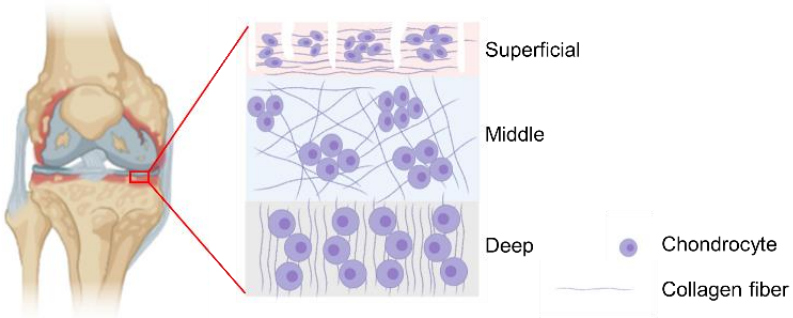


Figure 3. *Schematic representation of Osteoarthritis diseased tissue. Orientation of chondrocytes and Collagen fibers are shown for the superficial, transitional and deep zone. Created with BioRender.com.*

The metabolic activity of chondrocytes is altered by pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor−α (TNF−α), resulting in a metabolic imbalance. The cytokines have catabolic effects, resulting in cartilage degradation and chondrocyte dedifferentiation. [3][11][28] Moreover, the synthesis of matrix degrading enzymes is stimulated, e.g. the Collagenases metalloproteinases (MMPs) and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). [23] ADAMTSs are responsible for Aggrecan and proteoglycan breakage, resulting in a decrease in matrix hydration and thereby an increase in cartilage compressive stiffness, which accelerates the rate of Collagen loss. [29][30] MMP-1, MMP-3, MMP-9 are mainly involved in the breakdown of collagens and other extracellular molecules. MMP-13 is the major catabolic effector of cartilage degradation in OA, and mainly responsible for Collagen II degradation. [31][32] Furthermore, by elevated MMP expression, the synthesis of new ECM components, and thereby the restorage of degraded matrix, is blocked. Progressive loss of collagens and proteoglycans in combination with the disturbance of their normal production results in cartilage degradation and chondrocyte death. [53]

Treatment of Osteoarthritis

Due to the lack of vascularization of the tissue, cartilage has only a limited ability to self-repair and treatment options are important. In early-stages, cure options are mainly focused on pain reducing and reducing the degradation speed. Other options include analgesic and anti-inflammatory medicines, injection of steroid hormones, physiotherapy, or viscosupplementation. [7] Joint functionality may be applied in advanced stages, whereby the joint functionality is restored by a surgical procedure. Arthroplasty includes the resurfacing of bones or the use of an artificial joint. Currently, no options are available to stop or reverse the process of OA, mainly due to the absence of a proper platform for drug studies. [11]

1.2. Models for cartilage culture

A model for disease and drug studies requires to mimic the native cartilage with its specific structure, physiological conditions, and dynamic environment. [33] Hence, different parameters, including the 3D architecture, temperature levels, oxygen tension, and the ability to apply mechanical and biochemical stimulation, were considered in this project.

1.2.1. Three-dimensional culture

Simplified models, consisting of a monolayer of cells, have the disadvantages of alteration in genetic profile and phenotype, due to cell adhesion and dedifferentiation, and are therefore an unsuitable representation of the native environment. During passing, the chondrogenic phenotype is not supported and monolayer cultured chondrocytes express less Collagen type II and less large proteoglycans with respect to 3D culture.

When cultured in 3D, for example using a hydrogel, aggregate or pellet culture, the cell-cell interactions are increased, resulting in a higher chondrogenic potential. [34][35] However, chondrocyte aggregates are not complex enough and are not suitable for applying mechanical stimuli. Further, 3D models containing a controllable and mechanical stable synthetic scaffold do not allow appropriate cell adhesion. Natural scaffolds are highly of interest due to their biocompatibility, but are limited by fabrication and mechanical properties. [36] The choice of hydrogel is important since it highly influences cellular behavior and differentiation capacities. [33] Current 3D protocols rely on the use of agarose 2% hydrogel in combination with human chondrocytes. Agarose, although widely used, presents some drawbacks. Hence, a new hydrogel composed of hyaluronic acid-tyramine (HA-TA) and dextran-tyramine conjugates (Dex-TA), Dex/HA in short, was investigated and compared to the current gold standard.

Agarose

The natural biomaterial agarose (**Figure 4**) is a biocompatible hydrogel, that facilitates cell survival and promotes the maintenance of cell morphology and phenotype *in vitro*. [37][38][39][40] Agarose allows regeneration of cartilage tissue and promotes the synthesis of the extracellular matrix, including the deposition of Collagen type VI and the synthesis of proteoglycan-Collagen constructs. [41][42]

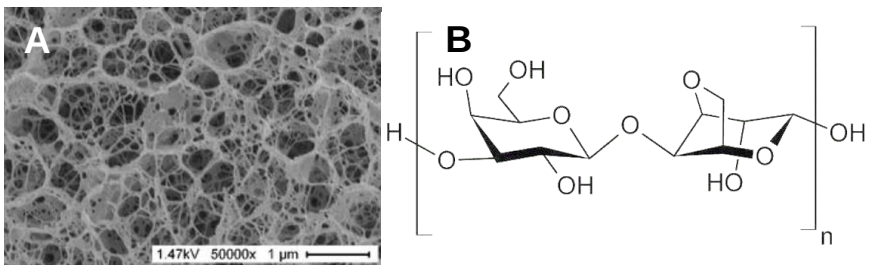


Figure 4. *A) SEM image of Agarose 2% hydrogel at 20 °C performed by Tuvikene et al.* [44], *scalebar: 1 µm. B) Structure of UltraPure Agarose (ThermoFisher Scientific).*

Agarose is suitable for dynamic culture, resulting in high viability levels for cells cultured within static and mechanical stimulated conditions. [33] However, chondrocytes do not proliferate when cultured in agarose and they cannot attach to the hydrogel. Further, the structure agarose is highly different when compared to native cartilage ECM. [37][43]

Dex-HA/HA-TA hydrogel

The Dex-HA/HA-TA hydrogel (Dex/HA) was developed by the Developmental BioEngineering (DBE) group at the University of Twente. The hybrid hydrogel consists of tyramine-substituted dextran (Dex-TA) and hyaluronic acid (HA-TA), in 50/50 ratio (**Figure 5**). The combination of both polysaccharides compose a hydrogel which is based on the molecular structure of proteoglycans found in the native cartilage ECM, and is therefore of interest for cartilage research. [45][46][47] The hydrogel was investigated as injectable and tunable hydrogel for cartilage repair, and was already widely investigated for multiple cartilage tissue engineering purposes. [47][48] Chondrocytes cultured within the gel show high viability, proliferation, metabolic activity, and production of matrix proteins. Further, Dex/HA with chondrocytes incorporated stimulated the regeneration of cartilage. [47][49] Hydrogels containing naturally occurring HA do have viscoelastic properties, allow good water retention, are highly bioactive by providing cell attachment sites, and are biodegradable in the presence of hyaluronidases. [50][52] Contrarily, Dex-TA is non-degradable, biocompatible and provides long term stability. [51]

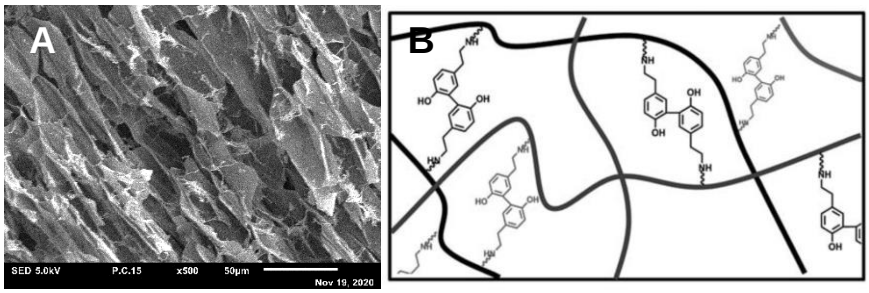


Figure 5. *A) SEM image of Dex/HA 10wt% hydrogel, performed by Malin Becker, scalebar: 50 µm. B) Structure of Dex/HA hydrogel network, obtained from* [49].

Dex/HA hydrogel is prepared by an enzymatic crosslinking reaction of the polymer-tyramine conjugates, via the oxidative coupling of the phenol moieties. The enzyme-catalyzed crosslinking is cell-friendly, easy, and efficient. [46][52] The advantages of using enzymatic crosslinking over other techniques, such as Michael-type addition reactions and Ultraviolet crosslinking, are its relatively fast gelation time and the non-cytotoxicity. The reaction is initiated by horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) as oxidant. [47][49] When the enzyme HRP reacts with H_2O_2, a tyramine moiety (TA) is oxidized, creating a radical. By reaction between two TA radicals, a network between both polysaccharides is formed. The hydrogels mechanical strength, and thereby the degree of crosslinking, depends on the ratio between H_2O_2 and TA moieties. The gelation time of Dex/HA 10wt% is below 20 seconds, but can be altered by varying both H_2O_2 and HRP concentrations. [49]

1.2.2. Temperature levels

Cells are often cultured at 37 ˚C *in vitro*, since this is assumed to mimic the inner body temperature. However, the temperature of a healthy knee joint is 32,6 ˚C at rest, and during high intensity movement, the inter-articular temperature can rise up to 38,8 ˚C. [53] During OA, the articular cartilage loses its resistance to vascularization and blood vessel growth increases. The inflammation drives angiogenesis by macrophage activation, being a critical mechanism in the persistence of OA. [54][55] Inflamed tissue produces warmth by rapid

circulation of the blood in the inflamed part, resulting in an intra-articular temperature of 33,5 ˚C in OA patients and up to 36,0 ˚C for rheumatoid arthritis. [56][57][58]
The effects of a hypothermic or hyperthermic culture environment on chondrocytes was already investigated in earlier research. Kaplan et al. showed a decrease in metabolic activity of chondrocytes cultured at high temperature levels, and established an optimal temperature for cartilage ECM production between 32 ˚C and 37 ˚C. [27] Flour et al. found comparable chondrocyte proliferation rates for 37 ˚C and 40 ˚C culture, but noticed an increase in protein content and cell volume at these temperatures. [59] At high and lowered temperatures, heat shock proteins (HSPs) are produced by the chondrocytes. HSPs are chondroprotective to human chondrocytes, by protecting and aiding the protein folding and enhance the metabolic activity. [60][61] The effects of a thermal environment on chondrocyte culture *in vitro* was investigated by Ito et al., showing an increase in cell proliferation at 37 ˚C, while culturing at 31 ˚C resulted in a reduced cell growth and metabolism. [30] A hyperthermic condition of 42 ˚C resulted in upregulation of Collagen I, Collagen II, Sox9 and destructive enzymes. But, culturing at temperatures above 40 ˚C can result in appropriate protein folding, and when cultured at 43 ˚C, the cell cycle was arrested, resulting in cell death. [62]

1.2.3. Oxygen tension levels

Human chondrocytes are able to survive in a physiologically hypoxic environment, due to the avascularity of cartilage. The concentration of oxygen (O_2) is approximately 6% in the superficial layer and only 1% in the calcified layer (**Figure 6**). The oxygen gradient declines even more in OA tissue, and since oxygen tension modulates cell behavior and phenotype, there is a need to understand the exact response under different oxygen tensions. [63] Hypoxia (2,5% O_2) positively influences chondrocyte phenotype and ECM synthesis *in vitro*. [64][63] Studies have shown that hypoxic conditions results in enhanced cartilage formation during differentiation and stimulates biosynthetic activity, with an increase in Collagen type II, SOX9, and Aggrecan. [65][66][67][68] Hypoxia inducible factor-1alpha (HIF-1α) regulates the anabolic behavior of chondrocytes in a hypoxic environment, for example by the production of chondroprotective HSPs, which further promote the metabolic activity of chondrocytes. [60][69]

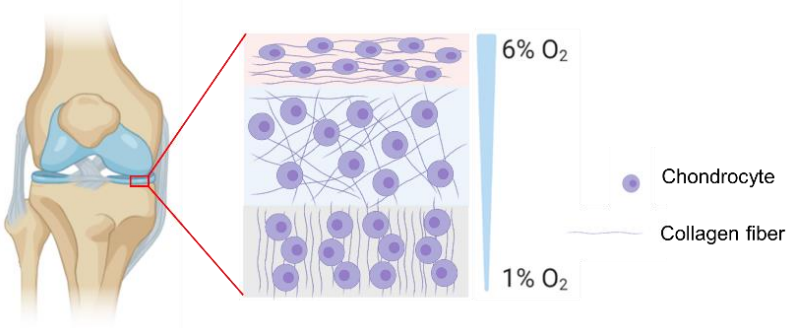


Figure 6. *Schematic representation of human articular cartilage with corresponding oxygen levels. Created with Biorender.com.*

1.2.4. Dynamic culture environment

Since chondrocytes are used to grow in a dynamic environment, a culture system that enables to mimic comparable dynamics is required. The micromechanical environment has a positive effect on chondrocyte growth, differentiation, and the metabolism of the ECM. Physiologic levels of loading have anabolic and anti-inflammatory effects, stimulating the synthesis of ECM components. [70] Hyper-physiological conditions can result in catabolic and pro-inflammatory effects, and in contrast, long lasting periods of inactivity negatively affects the metabolic activity and result in a decreased ECM production. [20][71]

Further, to create a proper cartilage model to study the movement kinetics, an environment enabling cell movement in 3-dimensions is required. Various *in vitro* models have shown that chondrocytes are able to migrate under different stimuli, including bone morphogenetic factors and multiple growth factors. [17][72][73] Furthermore, research has shown that chondrocytes are able to move *in vitro* towards stimulation of chemoattractants, e.g. hyaluronic acid, fibronectin and Collagen l. [72][74][75]

1.3. Cartilage-on-chip

Current *in vitro* models do not meet all requirements necessary to mimic the native cartilage, including the tissue structure, the physiological conditions, and a dynamic culture environment. A better representation of cartilage tissue was aimed by the development of an organ-on-chip platform. Organ-on-chip devices allow miniaturization of an organ by mimicking the microenvironment, mechanics, and physiological conditions of a tissue. Multiple parameters can be easily controlled, such as supply of fluid containing chemical or nutrients. [33]
A cartilage-on-chip was developed by the Applied Microfluidics for BioEngineering Research (AMBER) and Developmental BioEngineering (DBE) departments at the University of Twente, to study both healthy and diseased cartilage conditions. The inclusion of the cartilage structure, nutrients transport, and mechanical stimuli were aimed as important factors to mimic native cartilage tissue. The system enables mimicking of mechanical stresses similar to articular movement, which allow both compression and shear stress simultaneously. Stimuli corresponding to healthy physiological and diseased hyper-physiological conditions can be reproduced. [33]

1.3.1. Cartilage-on-chip design

The microfluidic platform is made of non-toxic silicone-based polymer Polydimethylsiloxane (PDMS). The 254 μm thick chip consists of a mechanical stimulation section with 3 actuation chambers, a cell-hydrogel chamber, and a perfusion channel (**Figure 7**). The mechanical stimulation chamber is separated from the hydrogel and cells by a thin elastic membrane (50 μm thickness).

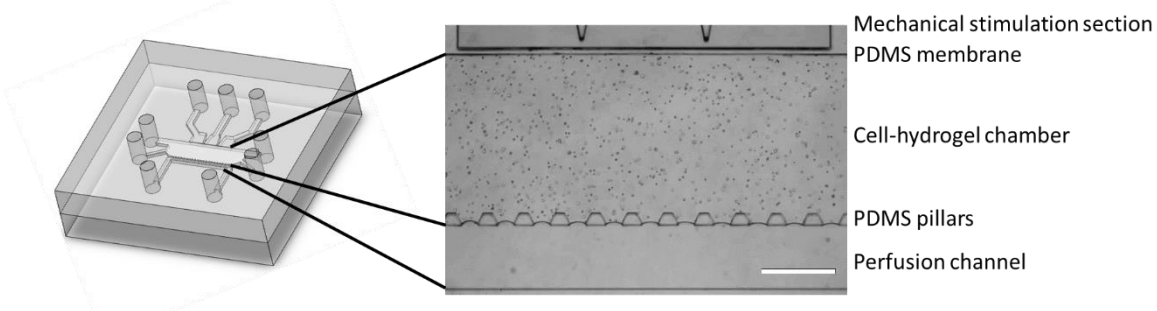


Figure 7. *Cartilage-on-chip design, containing a mechanical stimulation section with 3 actuation chambers, PDMS membrane, cell-hydrogel chamber, pillars, and perfusion channel. Device design provided by C.A. Paggi. Scalebar: 500 μm.*

The mechanical stimulation section is connected to a pressure and vacuum pump. By applying a pressure, the PDMS membrane deforms towards the cell-hydrogel section. Applying a vacuum causes negative deformation, resulting in membrane deformation towards the mechanical stimulation chamber. The microfluidic device can exert compression only or shear forces, by altering the applied pressure or vacuum subsequently to the single chambers (**Figure 8**). The mechanical stimulation generates gradients of gel deformation and cell deformation, and thereby mimic the healthy cartilage during knee articulation. Diseased knee cartilage can be mimicked by applying hyper-physiological compression, resulting in rupture of the cell

membrane and suggested cell death. [33] This project focuses on application of healthy mechanical compression levels.

Hydrogel containing chondrocytes can be injected into the cell-hydrogel section. This section is separated from the perfusion channel by a line of PDMS pillars (100 x 100 or 150 x 150 µm), to prevent diffusion of the injected hydrogel into the perfusion channel.

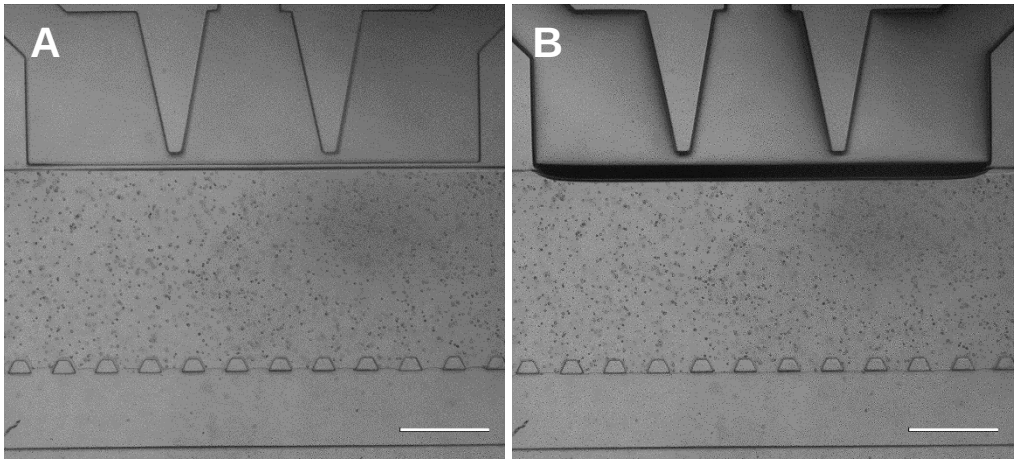


Figure 8. *Cartilage-on-chip device with 3 actuation chambers in static condition (A) and in compressed condition (B). Images provided by C.A. Paggi. Scalebars: 500 µm.*

2. Aim and objectives

2.1. Aim

The aim of this project is to improve chondrocyte culture *in vitro*. The project proceeds in two independent steps, which were handled separately in the results and discussion section. For the first part of the project, a cartilage-on-chip platform is used to mimic the dynamic joint culture. The use of Dextran-Tyramine Hyaluronic Acid-Tyramine hydrogel (Dex/HA) for this cartilage platform will be evaluated. This hydrogel was aimed to closely mimic the composition and structure of articular cartilage ECM. The chondrocyte viability, morphology, behavior, and functionality will be assessed and compared to the currently used agarose hydrogel (**Figure 9**).

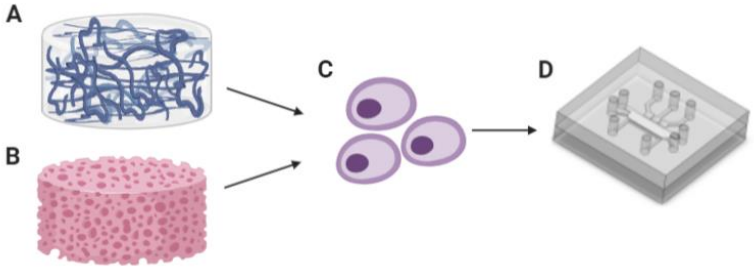


Figure 9. *The Dex/HA hydrogel (A) was proposed as alternative for the currently used agarose hydrogel (B) as matrix for chondrocyte culture (C) within the cartilage-on-chip device (D). Created with BioRender.com.*

Secondly, the effects of temperature and oxygen on monolayer culture were determined to improve the general conditions for healthy chondrocyte culture. A 3D pellet culture was proposed to further improve the chondrocyte culture and the effects of different temperatures in a hypoxic environment. A diseased condition was induced by application of pro-inflammatory cytokines IL-1β and TNF−α. The differences in impact on 2D and 3D cultured cells, and the effects of temperature and oxygen on the diseased cells, was determined. The changes in cellular behavior for all conditions was monitored, including their viability, metabolic activity, gene and protein expression (**Figure 10**).

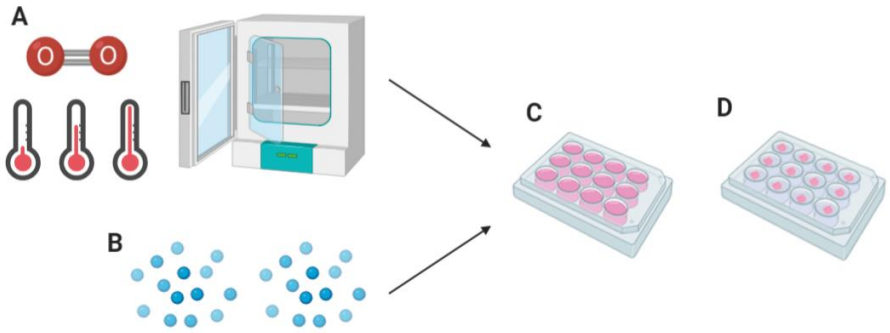


Figure 10. *The effects of oxygen and temperature culture conditions (A) and cytokine stimulation (B) on both chondrocyte monolayer (C) and pellet (D) culture was examined. Created with BioRender.com.*

2.2. Objectives

To achieve the above described aims, the following objectives were formulated:

- Improve cartilage-on-chip by chondrocyte culture in Dex/HA hydrogel:
    - Characterize hydrogel and evaluate behavior under mechanical compression
    - Determine chondrocyte viability, morphology, activity, and behavior at microscopical level
    - Determine chondrocyte behavior, protein synthesis, and gene expression at molecular level
- Improve general chondrocyte culture *in vitro*:
    - Determine the effects of temperature and hypoxia on chondrocyte viability, morphology, activity, protein synthesis, and gene expression in a monolayer culture
        - Intra-articular temperature (33 °C) versus body core temperature (37 °C) versus hyperthermic condition (39 °C)
    - Trigger inflammation response in chondrocytes by application of pro-inflammatory cytokines for the promotion of a diseased condition in monolayer culture
    - Evaluate the effect of pro-inflammatory cytokines under different temperature and hypoxia culture conditions
    - Determine the effects of temperature, oxygen, and cytokine on both monolayer (2D) and pellet (3D) culture systems.

2.3. Research design

The project proceeds in two independent steps, hydrogel testing on-chip and temperature/oxygen culture conditions:

Cartilage-on-chip

First experiments were performed to determine if and whether the use of Dex/HA hydrogel for the dynamic cartilage-on-chip platform had advantages with respect to agarose. Thereafter, the chondrocyte morphology, behavior and activity within the gel was evaluated. Important analysis methods included a viability assay, metabolic activity assay, and qPCR for gene expression analysis.

Temperature/oxygen conditions

The effects of temperature and oxygen on chondrocyte behavior were determined from a viewpoint of cell viability, metabolic activity, protein production, and gene expression. 33 ˚C was considered as normal intra-articular temperature, 37 ˚C as inter-core temperature and 39

˚C as extreme hyperthermic condition, 2,5% O_2 was approached as physiological oxygen tension.

The pro-inflammatory cytokines TNF−α and IL-1β were investigated as inflammatory stimulation for chondrocytes. The interplay between both IL-1β and TNF−α was most effective to induce cartilage degradation in vitro, as described by Zhong et al. [76] Applying both cytokines to human chondrocytes *in vitro* induced cartilage matrix degradation and resorption. [28][77][78] To evaluate the stress induced by the thermal and hypoxia environment, the expression of heat shock proteins (HSPs) was measured. HSPs are responsible for cell protection and recovery and are therefore rapidly produced in a stressful environment, e.g. cytotoxic or pathological conditions, hydrostatic pressure or heat shock. [79] HSPs protect against stresses by increasing the cartilage matrix metabolism and induce pro-inflammatory cytokine production to activate the innate immune system. [80] All experiments were performed in both monolayer and pellet chondrocyte culture systems, to determine the influence of temperature, oxygen, and cytokine.

3. Materials & Methods: Hydrogel testing

This section includes the materials and methods followed for hydrogel testing in the cartilage-on-chip device, including microfluidic device fabrication, the mechanical stimulation set-up, cell-hydrogel culture, and experimental section.

3.1. Microfluidic device fabrication

3.1.1. Mold fabrication

Devices were designed and developed by the Developmental BioEngineering (DBE) and Applied Microfluidics for BioEngineering Research (AMBER) departments at the University of Twente, the Netherlands. Designs were made using Clewin5 software (WieWeb software, The Netherlands) and converted to a quartz photomask using direct-writing lithography. Mask fabrication was described by Paggi et al., [33] according to the following protocol: The mask was used to produce a mold by photolithography on a SU-8 silicon wafer. A 254 um layer of SU-8 100 photoresist (MicroChem, Westborough, USA) was made on the surface of a silicon wafer (Okmetic, Finland) using spin-coating, and baked for 30 minutes. The photomask was used to create a negative mold, by exposing the SU-8 layer to a 365 nm UV light through a photomask. The SU-8 exposed to the UV was photo-polymerized, while the photoresist that was not covered by the photomask did not crosslink. The wafer was post-baked afterwards, and the remaining photoresist was washed away with RER 600 in a sonication system. IPA and isopropanol wash was used for further cleaning.

3.1.2. Cartilage-on-chip fabrication

The organ-on-chip devices were produced using Polydimethylsiloxane (PDMS) soft-lithography. PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, USA) were prepared in weight concentration 20:1 by mixing intensively and degassing for 30 minutes. Prepared PDMS was poured on top of the mold, degassed for 30 minutes, and baked at 60 °C for 24 hours. After baking, the chips were cut out and the feature surface was protected by a layer of tape. 2 reservoirs with a diameter of 2 mm were punched for the cell-hydrogel channel and 1 mm reservoirs were punched for the perfusion channel and mechanical stimuli chamber. PDMS 20:1 empty base chips (4 mm in height) were prepared according to the same protocol. An empty base chip of PDMS and a glass slide were oxidized using plasma treatment (Recipe 2, Cute, Femto Science, South Korea) and bound together. The glass slide with PDMS base and a chip were activated using plasma treatment. Afterwards the feature surface of the device was plasma-bonded on the PDMS base to form the platform (**Figure 11**). The microfluidic device was checked with bright field microscopy to determine the intactness of the membrane and pillars, further baked at 60 °C for 24 hours and stored at room temperature until further use.

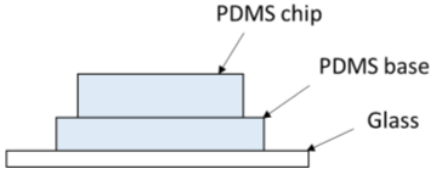


Figure 11. *Cartilage-on-chip platform, consisting of a glass slide, PDMS base, and PDMS chip with feature surface faced down, obtained from* [81].

Polydopamine coating

A 2 mg/mL solution of Dopamine Hydrochloride (Sigma Life Science) in Tris HCl buffer (10 mM, pH 8,5) was prepared (**Figure 12**). 30 µL of PD solution was loaded in the cell-hydrogel section, and incubated at RT for 45 minutes. Afterwards, the channel was washed 3 times with

miliQ water to remove the unbound dopamine molecules and dried overnight at RT. PD treatment was only applied for Dex/HA conditions.

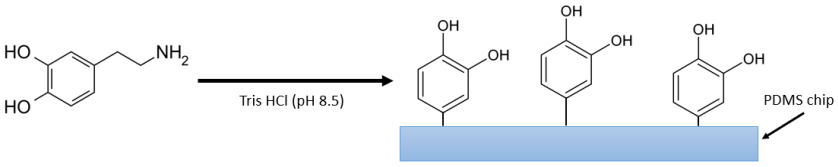


Figure 12. *Schematic overview of polydopamine treatment of PDMS chip. Dopamine structure obtained from* [82]*, image created with BioRender.com.*

3.1.3. Mechanical stimulation set-up

Mechanical stimulation was applied to the microfluidic devices by connection to the mechanical stimulation set-up (**Figure 13**). Fluigent software enabled control of mechanical compression generation and the switching system. A script to control the switching between pressures was written in Fluigent software and the applied pressure was controlled by MAESFLO (Fluigent Software, France). The mechanical compression was established by a nitrogen pump and controlled by the MFCS-EZ pressure controller (Fluigent, France). The Switchboard (Fluigent, France) controlled the switching for the applied pressure. In the 2-Switch system (Fluigent, France), the switching and applied pressure were combined and passed to the mechanical stimulation chamber of the microfluidic device.

An inverted microscope (IX51, Olympus, Japan) with camera (ORCA-flash 4.0 LT, Hamamatsu Photonics, Germany) was used to monitor and image the cartilage-on-chip using HOKAWO imaging software (Hamamatsu Photonics, Germany).

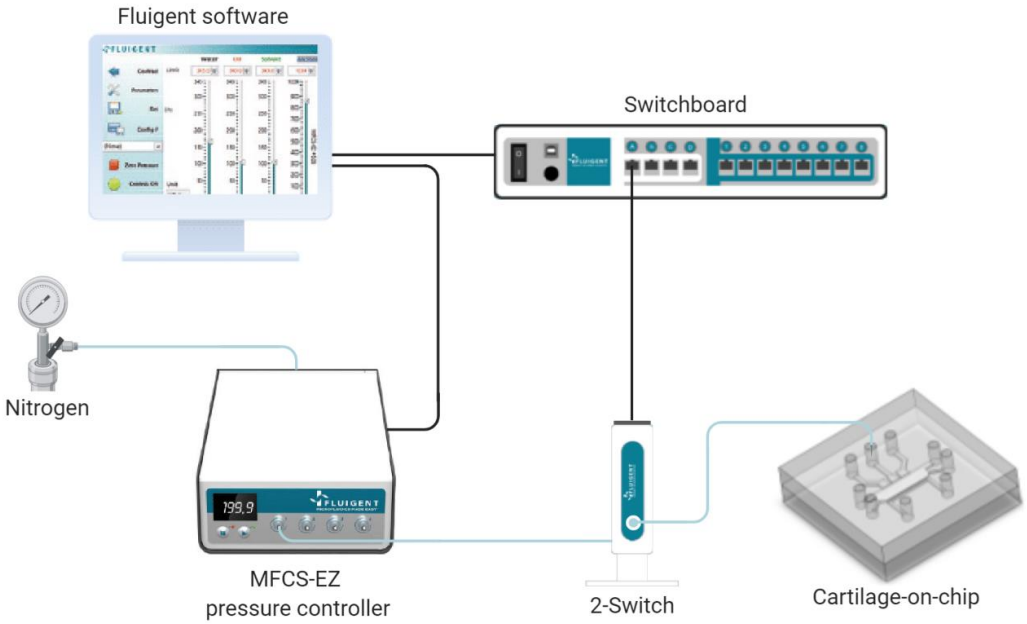


Figure 13. *Schematic representation of the set-up used for generation of mechanical stimulus for one actuation chamber. Nitrogen course is shown with blue connection, software control with black connections. Created with BioRender.com, images obtained from* [83].

3.2. Human chondrocyte culture

Human chondrocytes were obtained from a patient undergoing total knee replacement. Cells were stored in liquid nitrogen and before use defrosted at 37 °C. After defrosting, the cells were dropwise added to 9,5 mL chondrogenic proliferation medium (CPM: DMEM supplemented with FBS, Asorbic acid 2-phosphate, non-essential amino acid penicillin, and streptomycin, as

listed in **Appendix 12.1**). The medium containing cells was centrifuged at 300 rpm for 5 minutes to get rid of the solvent dimethyl sulfoxide (DMSO). After pelleting, the cells were resuspend in 10 mL CPM, transferred to a T175 Cell Culture Flask, and supplemented with 10 mL CPM. After complete cell attachment, the medium was refreshed every 3 days to stimulate chondrocyte proliferation. Cells were passaged when 80% confluency was reached.

Briefly, medium was removed and the cells were washed with 5 mL PBS. Cells were incubated in 5 mL Trypsin-EDTA (1x, Invitrogen, USA) for 5 minutes at 37 °C. When all cells were de-touched from the surface, 5 mL of CPM was added to the flask to stop the trypsinization. The mixture with cells was displaced to a 15 mL tube and the flask was washed one more time with 5 mL CPM. 10 µl was added to a counting chamber, and 4 chambers were counted in duplicate. The total amount of cells was calculated as follows: $Cell\ concentration = \left(\frac{Cell\ count}{Number\ of\ counted\ quadrants}\right) * 10.000 * DF$, with DF the dilution factor (15 mL). The dilution ratio was calculated to obtain $5x10^5$ cells. After centrifuging at 500 rpm for 3 minutes, the cells were resuspended in 10 mL CPM, and transferred to a new T175 Cell Culture Flask. When all cells were attached, the medium was refreshed every 3 days and cells were passaged again when confluent5. All experiments were performed with the same human chondrocytes donor line (D63 Healthy looking), passage 5.

3.3. Cartilage-on-chip culture

3.3.1. Cell-hydrogel culture

Agarose hydrogel

Agarose solution 4wv% was obtained by dissolving 2 gram of low melting point agarose powder (UltraPure Agarose, Invitrogen, USA) in 50 mL of PBS 1x. Dissolving procedure was stimulated by heating in the microwave for 8 seconds time intervals, for approximately 1 minute in total. For further processing, 4wv% agarose solution was diluted in 1:1 ratio with PBS or medium containing cells to obtain a final concentration of 2wv%: Human chondrocytes passage 5 were processed using trypsinization, as described before. The dilution ratio was calculated to obtain $3x10^6$ cells/mL. After pelleting, the cells were suspended within the calculated amount of CPM and mixed 1:1 with 4wv% agarose hydrogel to obtain a cell concentration of 1.500.000 cells/mL and hydrogel concentration of 2wv%.

Dex/HA hydrogel

Dex-TA and HA-TA were synthesized by the DBE department, with degrees of substitution 13 and 10, respectively. Both Dex-HA and HA-TA polymers were dissolved in PBS 1x to obtain 15wt% solutions and stored at -20 °C until further use. 0,15% and 0,3% H_2O_2 solutions were prepared from 30wt% stock solution (Sigma-Aldrich) in PBS 1x. HRP 10 units/mL was prepared by dissolving in PBS 1x. 5wt% and 10wt% (50/50) hydrogels were prepared under sterile conditions by dilution with medium containing cells. For the hydrogel characterization experiments, the hydrogels were prepared with PBS containing microbeads, instead of CPM containing cells.

Human chondrocytes passage 5 were processed using trypsinization, as described before. The dilution ratio was calculated to obtain $2,5x10^6$ cells/mL. After pelleting, the cells were suspended within the calculated amount of CPM. Both polymer components were first mixed together and vortexed to obtain a homogeneous mixture. The polymer mixture was combined with medium containing cells. Lastly, HRP and H_2O_2 were added to the mixture, in 1:10 ratio. Final composition for both hydrogel concentrations can be found in **Table 1**. The mixture was mixed well by pipetting up and down before injection in the microfluidic device.

Table 1. *Composition of 5wt% and 10wt% Dex/HA hydrogels for a total volume of 300 µl. Polymer mixture was prepared first, afterwards medium containing cells was added, and lastly, HRP and H_2O_2 were added.*

	15wt% Dex-TA	**15wt% HA-TA**	**Cells in CPM**	**CPM**	**HRP**	**H_2O_2**
5wt%	30 µL	30 µL	128 µL	52 µL	30 µL	30 µL (0,15%)
10wt%	43 µL	43 µL	128 µL	0 µL	43 µL	43 µL (0,30%)

Cell-hydrogel injection

50 µL of the cell-hydrogel mixture was gently injected within the cell-hydrogel reservoir of the organ-on-chip device. 100 µL of CPM was added to the perfusion channel and additional on the surface of the microfluidic system to prevent dehydration. The devices were positioned in a petri dish and closed with Parafilm to prevent evaporation and placed in a humified incubator containing 20% O_2 and 5% CO_2 at 37 °C .

Culture medium

At day 1, the medium of the devices was replaced to chondrogenic differentiation medium (CDM: DMEM supplemented with pen/strep, ITS, L-Proline, ASAP, Sodium Pyruvate, Dexamethasone, and transforming growth factor β, listed in **Appendix 12.1**) and refreshed daily. Since chondrocytes cultured in 2D often dedifferentiate, their redifferentiation is initiated by using medium containing Dexamethasone, and transforming growth factor β.

3.3.2. Mechanical stimulation

Compressive stimulation was applied to the organ-on-chip, using the set-up described in section 3.1.3. The physiological strain of healthy dynamic knee cartilage was mimicked by applying 350 mbar with a frequency of 1 Hz, for 60 minutes. [33] Devices with no pressure applied were considered as static culture and kept as negative control.

3.4. Experimental set-up cartilage-on-chip experiments

Dex/HA hydrogel as alternative for agarose was evaluated for the cartilage-on-chip platform. Firstly, the characterization of the hydrogels is described. Hydrogel structures were studied using scanning electron microscopy and the crosslinking and degradation of Dex/HA hydrogel was determined. Lastly, the deformation capacities of the hydrogel and the movement of microbeads under mechanical stimulation was investigated.

Next, multiple experiments were performed to evaluate the short- and long term behavior of human chondrocytes within the multiple hydrogels. First experiments were performed for a period of 11 days (**Figure 14**). Hydrogel containing human chondrocytes were seeded in the devices at day 0 and mechanical stimulation was applied on day 1 – 4 and day 7 – 11, for 60 minutes per day. Medium (CDM) was refreshed daily and stored on day 5 and day 11 for MMP activity and SPR analysis. Samples were analyzed using bright-field imaging, viability staining and qPCR. Static conditions were handled following the same protocol.

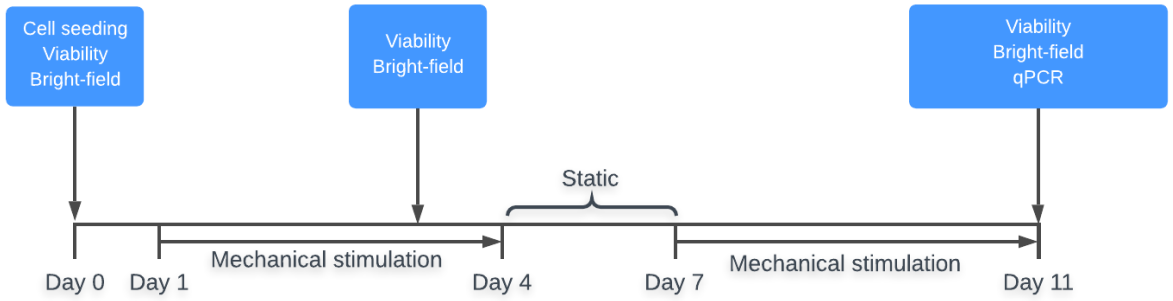


Figure 14. *Long term cartilage-on-chip experiments. Cells were cultured for 11 days, and stimulated with compression load at day 1–4 and day 7–11.*

Short term experiments were performed for a period of three days (**Figure 15**). At day 0, the hydrogel containing human chondrocytes were seeded in the cartilage-on-chip. The compression experiments were performed for 1, 2, and 3 days, respectively. Static conditions were kept 3 days in culture. Medium (CDM) was refreshed daily and stored for MMP activity and SPR analysis. Bright-field images and viability images were made daily. Samples were sacrificed for qPCR and IF on day 1, day 2, and day 3 for the compression conditions and on day 3 for the static conditions.

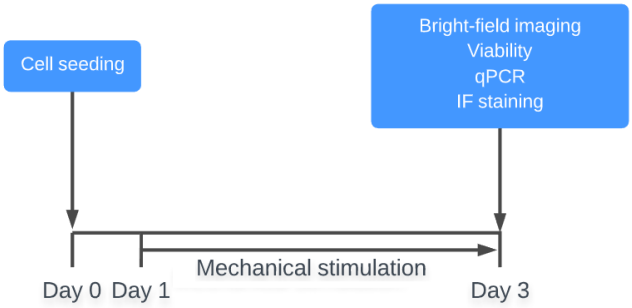


Figure 15. *Short term cartilage-on-chip experiments. Cells were cultured for 3 days, and stimulated with compression load at day 1–3.*

4. Materials & Methods: Culture conditions

This section includes the materials and methods followed for the experiments performed to determine the effects of temperature, oxygen, and cytokine stimulation on human chondrocyte culture, including the chondrocyte culture in both pellet and monolayer systems, and the experimental section.

4.1. Mold fabrication and pellet formation

A PDMS mold (22 mm in diameter) containing 3000 mini wells (200 µm in diameter) was used to create an agarose mold using replica molding (**Figure 16**). Agarose 3wv% solution (Ultrapure Agarose, Invitrogen, USA) was added on top of the PDMS mold until covered. After gelation, the PDMS mold was removed and the agarose chips were cut and placed in the wells of a 12 wells plate (Suspension culture, Greiner Bio One, Germany).

Figure 16. *Preparation of pellets using replica molding technique. Agarose 3% mold is obtained using a PDMS mold. Chondrocytes are added on top of the agarose mold and centrifuged to form pellets in the mini-wells. Created with BioRender.com.*

Human chondrocytes passage 5 were processed using trypsinization, as previously described. 150.000 cells/well in CPM were added to the 12 wells plate and centrifuged (300 rpm, 3 minutes, brake 2) to distribute cells over the mini-wells. Medium was replaced with 1 mL fresh CPM and refreshed daily.

4.2. Monolayer experiments

Human chondrocytes passage 5 were processed using trypsinization, as previously described. 25.000 cells per well were seeded in a cell culture 12-wells plate (Greiner Bio One, Germany) and 1 mL CPM was added.

4.3. Experimental set-up

Cells in both pellet and monolayer systems were cultured at 2.5% O_2, 5% CO_2 and various temperatures (33 °C, 37 °C, or 39 °C). Cells were cultured in a conventional incubator (20% O_2, 5% CO_2 and 37 °C) as control. Unhealthy conditions were proposed by culture in CPM, supplemented with IL-1β (10 ng/mL), TNF−α (10 ng/mL), or IL-1β + TNF−α (10 ng/mL), respectively, starting from day 1 (**Figure 17**). Cytokine solutions were prepared in CPM and refreshed daily for 3 days in total. Bright-field images were made daily. Viability staining was performed at day 0 and day 3. At day 3 the samples were stored for qPCR and IF analysis.

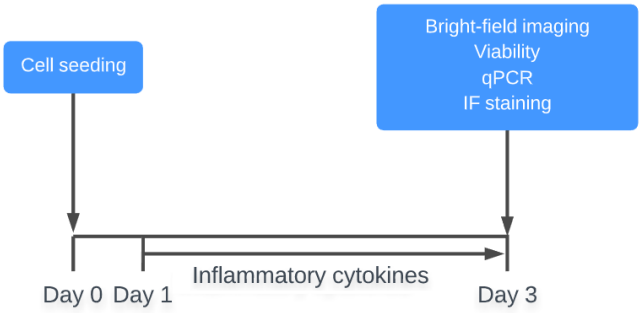


Figure 17. *Experimental scheme for temperature and oxygen experiments under cytokine stimulation.*

5. Analysis methods

This section includes all analysis methods performed for both hydrogel testing and culture condition studies.

5.1.1. Hydrogel characterization

Deformation capacity

15 µm in diameter polystyrene microparticles (Kisker Biotech, Germany) were used to analyze the deformation of the system. The hydrogels were supplemented with microbeads at a concentration of 61 µg/mL in PBS. Displacement of the beads positioned at different positions in the hydrogel were analyzed by taking images at different applied pressures (0, 100, 300, 500, 700, and 800 mbar). The displacement per bead under pressure was determined by measuring the distance between the bead and the membrane. The distance between bead and membrane at rest (0 mbar) was subtracted to the distance under pressure:

$$\text{Bead displacement: } \Delta_X = X_{pressure} - X_{rest}$$

Scanning electron microscopy

Dex/HA hydrogels, with and without addition of microbeads, were stored in MiliQ. and freeze dried in the lyophilizer (IlShin, the Netherlands). Scanning electron microscopy (SEM) images were taken using the Zeiss Merlin HR-SEM (Zeiss, Germany).

5.1.2. Viability assay

Hydrogel and monolayer experiments

Viability assay was performed using Calcein AM (Thermo Fisher, USA) and Ethidium Homodimer-1 (Thermo Fisher, USA) staining. The staining mixture was prepared by mixing 2 µL Calcein AM, 4 µL Ethidium Homodimer-1, and 1 mL of CPM. The medium was removed from the perfusion channel of the cartilage-on-chip systems. 50 µL of the staining solution was added in the perfusion chamber and incubated for 15 minutes at 37 °C.

For the monolayer culture, 2 µL Calcein AM and 4 µL Ethidium Homodimer-1 was directly added per well and incubated for 15 minutes at the experimental condition.

Pellet experiments

The ReadyProbes™ Cell Viability Imaging Kit (ThermoFisher Scientific, USA) was used to determine the cell viability of pellet chondrocytes. 2 Droplets of NucBlue® Live reagent (Hoechst 33342) and 2 droplets of NucGreen® Dead reagent were directly added to the cell culture medium and incubated for 15 minutes at the experimental condition.

The viability of hydrogel, monolayer, and pellet cultures was imaged using the EVOS fluorescence microscope (Thermo Fisher, USA). Cell viability was determined using Fiji ImageJ software by analyzing the fluorescence images. Viability was calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{Amount\ of\ living\ cells - amount\ of\ dead\ cells}{Amount\ of\ living\ cells} * 100\%.$$

5.1.3. Metabolic activity assay

Metabolic activity of the cells cultured in monolayer and pellets was measured using the non-cytotoxic PrestoBlue HS Cell Viability Reagent (Thermo Fisher, USA). 10% solution of PrestoBlue in CPM was prepared. Media was replaced with 1 mL of 10% PrestoBlue solution and incubated for 30 minutes at the experimental conditions. The plate was read on a plate reader (Victor3, PerkinElmer, USA) with an excitation frequency of 535 nm and emission frequency of 595 nm. 3 wells containing 10% PrestoBlue solution were considered as background measurements. The experimental wells were normalized to the average readout of the blank.

5.1.4. Surface Plasmon Resonance

During cartilage-on-chip experiments, replaced medium was stored and kept in freezer (-20 °C). Medium from the device surface and medium from the perfusion channels were stored separately, surface medium was used as control. The presence of IL-6 in the culture supernatant was determined using surface plasmon resonance imaging (SPRi). During SPRi, the proteins in the supernatant are tagged to an antibody, whereby the mutant protein is purified on the sensor surface.

The sensor was prepared according to protocol as described by Jan Hendriks [84], in short: The G-type easy2spot sensor (Ssenv bv, the Netherlands) was washed with MiliQ and incubated with RBD in coupling buffer. After washing with 1% BSA and coupling buffer, the sensor was quenched with ethanol amine. The biotinylated detection antibodies for IL-6 (Biolegend, USA) were spotted on the sensor, followed with neutravidin (Thermo Fisher, USA) and 40 nm biotinylated gold nanoparticles (Cytodiagnostics, Canada), using the Wasatch microfluidics continuous flow spotter (Wasatch Microfluidics, US). Measurements were performed with the

IBIS MX96 (IBIS Technologies, the Netherlands) and evaluated using SUIT software (IBIS Technologies, the Netherlands).

5.1.5. MMP activity assay

MMP activity was measured from culture supernatant for all hydrogel static and compression conditions, after 3 days of culture. A 40 µM solution of Mca-PLGL-Dpa-AR-NH2 Fluorogenic MMP Substrate (ES001, R&D systems, USA) in assay buffer was prepared. Assay buffer was prepared using 50 mM Tris, 10 mM CaCl2, 150 mM NaCl, 0,05wt% Brij35 at pH 7,5. 50 µL of sample was mixed with 25 µL assay buffer and the reaction was started by adding 25 µL substrate solution. Included controls were 100 µL CDM, 100 µL assay buffer, and 75 µL assay buffer + 25 µL substrate solution. Fluorescence was read in kinetic mode every 30 seconds, for 30 minutes, at excitation and emission wavelengths of 320 nm and 405 nm, respectively, using the Tecan infinite M200 pro plate reader (Tecan, Switzerland).

5.1.6. Gene expression

RNA isolation and cDNA synthesis

The cartilage-on-chip were separated from the PDMS base by using a cutting tool, the hydrogel with cells were removed from the system and moved to an Eppendorf tube. Per tube the hydrogel sections of three systems of the same conditions were placed together, washed with PBS 1x, lysed in 350 µL Lysis buffer (Qiagen, Germany), and stored at -80 °C. Ribonucleic acid (RNA) was isolated from the hydrogel samples using the RNeasy Micro Kit (Qiagen 74004, Germany), according to the manufacturer's instructions.

The cells cultured in a wells plate, 350 µL Lysis buffer was added and cells were detached using a cell scraper and moved to an Eppendorf tube. The cultured pellets were removed from the agarose molds by inverting the mold and centrifuging for 3 minutes (300 rpm). 350 µL Lysis buffer was added and transferred to an Eppendorf tube. RNA was isolated from monolayer and pellet samples using the RNeasy Plus Mini Kit (Qiagen 74134, Germany), according to the manufacturer's instructions.

RNA concentration and purity were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, the Netherlands). Complementary DNA (cDNA) was obtained by reverse transcriptase of RNA, using the iScript cDNA synthesis kit from Bio-rad Laboratories (Bio-Rad, USA) and the Bio Rad CFX Connect Real-time system (Bio-Rad, USA).

RT-qPCR

cDNA samples were diluted in RNAse-free water (Lonza, Switzerland) to obtain a final concentration of 5 ng/8 µL. Primers were prepared by diluting the forward and reverse primers in RNAse-free water (Lonza, Switzerland). Primers are listed in **Appendix 12.2**.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta Actin (ACTB) were selected as housekeeping genes. Collagen type II (ColII), SOX9, Aggrecan (ACAN), and Lubricin (PRG4) were selected for chondrocyte activity and functionality. ColI and ColX were selected to investigate chondrocyte dedifferentiation and hypertrophy. Further, fibronectin-1 and hyaluronan (HAS2) were selected as anabolic genes and matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-9, MMP-13, hyaluronidase-1 (HYAL-1), HYAL-2, HYAL-3, a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4), and ADAMTS-5 to determine the catabolic activity.

The diluted cDNA samples, SYBR reagent (SensiMix Bioline Reagents, UK), and primers were loaded in a wells plate (**Table 2**). Analysis was performed using the Bio-Rad CFX Connect Real-time system (Bio-Rad, USA). Obtained Ct values were normalized to the housekeeping genes to obtain ΔCt values. Ct values higher than 38 were considered as undetermined and were left out. $2^{-\Delta Ct}$ values were determined and plotted to show the relative fold gene expression level.

Table 2. *Volumes for qPCR.*

SYBR reagent	10 µL
Primers (Forward + Reverse in water)	2 µL
cDNA (in water)	8 µL
Total	**20 µL**

5.1.7. Immunofluorescence staining

Cells were washed twice with 1x PBS and fixated using 4% fixation buffer (4% paraformaldehyde 0.1M in 1x PBS, pH 7,2 — 7,4) for 15 minutes. After fixation, samples were washed 3 times with PBS 1x and incubated with permeabilization buffer (0,25% Triton X-100 in PBS 1x) for 30 minutes. After washing 3 times with 1x PBS, cells were blocked with blocking buffer (1% BSA in PBS 1x) for 60 minutes at RT. Primary antibodies were diluted in blocking buffer (**Table 3**), and samples were incubated with the primary antibodies overnight at 4 °C. After washing with PBS 1x, the samples were incubated with the secondary antibodies, diluted in blocking buffer**,** for 60 minutes at RT (**Table 4**). The samples were washed 3 times with PBS 1x before incubating with 0,1 ug/mL DAPI for 5 minutes at RT. Samples were stained with Phalloidin for 25 minutes at RT. Samples were stored in PBS 1x protected from light at 4 °C and imaged using the EVOS fluorescence microscope (Thermo Fisher, USA) or NIKON Confocal Laser Microscope A1 (Nikon Instruments, the Netherlands). Images were analyzed using ImageJ software (Fiji, USA).

Table 3. *Primary antibodies.*

Antibody	**Type**	**Dilution**	**Source**
Collagen ll	Rabbit anti-Collagen II	1:100	Abcam, ab34712
Collagen VI	Rabbit anti-Collagen VI	1:100	Abcam, ab182744
Aggrecan	Mouse anti-Aggrecan	1:100	Abcam, ab3778
MMP-13	Rabbit anti-MMP-13	1:100	Abcam, ab219620

Table 4. *Secondary antibodies.*

Product	**Dilution**	**Source**
Alexa Fluor 488 donkey anti-rabbit	1:200	Abcam
Alexa Fluor 647 donkey anti-mouse	1:200	Abcam

5.1.8. Figures and statistical analysis

Figures were created using Microsoft Origin (USA), BioRender (Canada) and Microsoft Powerpoint (USA). Fiji ImageJ software (Java, USA) was used for image progressing, distance, circularity, perimeter and area measurements and determining cell and bead displacement.
Data is shown for means ± SD and for experiments with a minimum of N = 3, statistical analysis was performed using Microsoft Origin (USA) for one-way ANOVA and post hoc Tukey test, with significances $p < 0,1$ (*), $p < 0,05$ (**), $p < 0,01$ (***), and $p < 0,001$ (****).

6. Results: Hydrogel testing

6.1. Hydrogel characterization

6.1.1. Scanning electron microscopy

Hydrogel structures were imaged using scanning electron microscopy (SEM). Pore sizes were larger for Dex/HA 5wt% hydrogel in comparison to 10wt% (**Figure 18**). **Figure 18-C** shows the integration of microbeads within the structure of Dex/HA 5wt% hydrogel.

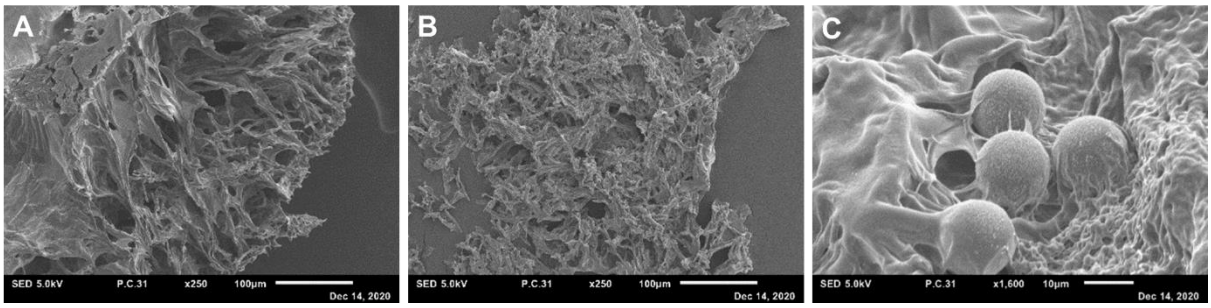


Figure 18. *Scanning electron microscopy images showing the structures of Dex/HA 5wt% (A) and Dex/HA 10wt% (B), scalebars: 100 µm. C) integration of microbeads in Dex/HA 5wt% hydrogel, scalebar: 10 µm.*

6.1.2. Degradation

Before treatment with polydopamine, it was seen that Dex/HA hydrogel degraded over time (**Figure 19**). After 10 days of culture in Dex/HA 10wt% under compression stimulation, only 42,5% of the hydrogels did remain intact (**Figure 19-C**). Applying a polydopamine coating before hydrogel injection was proposed to overcome this problem.

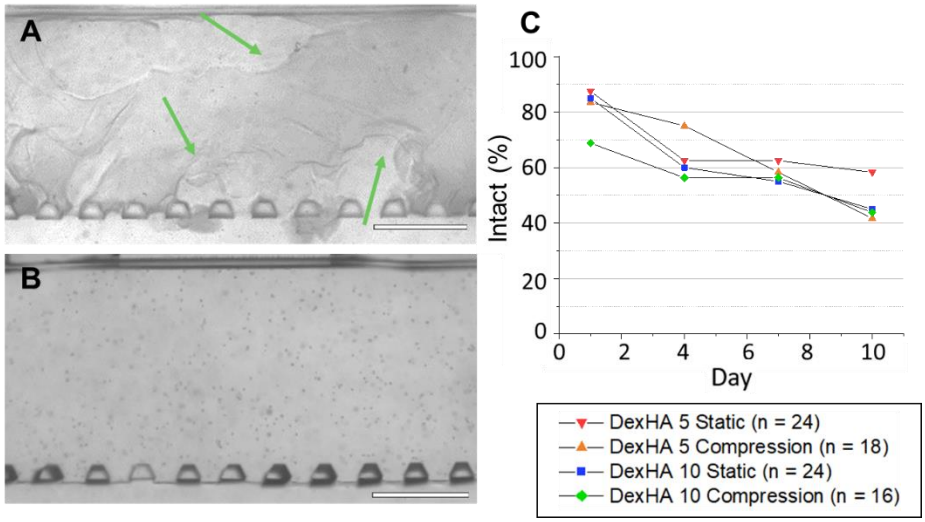


Figure 19. *A) Degradation of Dex/HA 5wt% hydrogel static condition, indicated by green arrows. B) Hydrogel intactsness was proposed by PD treatment before hydrogel injection, scalebars: 500 µm. C) Progress of Dex/HA degradation over time.*

6.1.3. Deformation

Deformation of the hydrogel was determined by applying stimulation of 0, 100, 300, 500, 700, and 800 mbar to the mechanical actuation section (**Figure 20**). The displacement per bead under the various loading was determined.

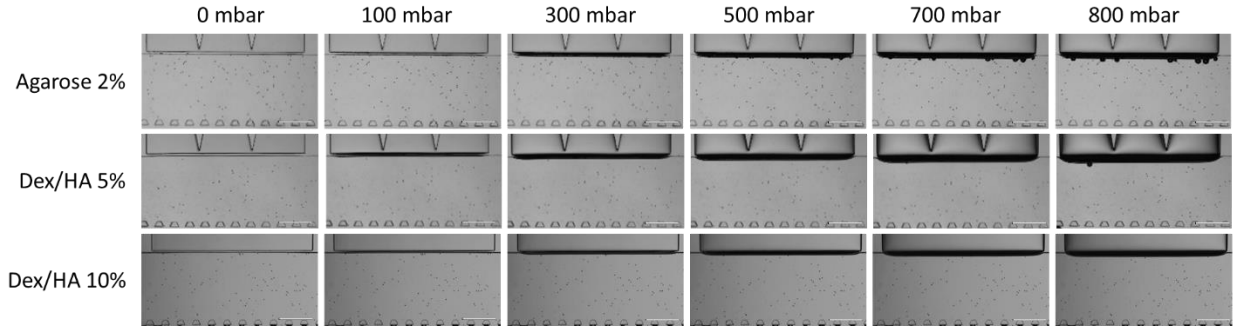


Figure 20. *Deformation of Agarose, Dex/HA 5wt% and Dex/HA 10wt% hydrogels containing microbeads, at 0, 100, 300, 500, 700 and 800 mbar. Scalebars: 500 µm.*

The distance to the membrane of beads positioned at different positions in the hydrogel was plotted against their displacement under applied pressure (**Figure 21**). Bead displacement in the upper region of the hydrogel section, at 0 – 200 µm distance from the PDMS membrane was equivalent for agarose and Dex/HA 10wt%. Dex/HA 5wt% showed more bead displacement in this zone. In the middle section, between 400 and 1000 µm distance from the PDMS membrane, agarose and Dex/HA 5wt% showed comparable deformation capacities with bead displacements between 5 and 25 µm, while Dex/HA 10wt% showed less displacement (below 12,5 µm). In the section most close to the PDMS pillars, at 1000 – 1200 µm distance from the membrane, the beads in agarose hydrogel were still affected by the applied pressure, whereas beads in both Dex/HA hydrogels present nearly no displacement. Data regarding the deformation of Dex/HA without PD treatment is shown in **Appendix 12.3.4.**

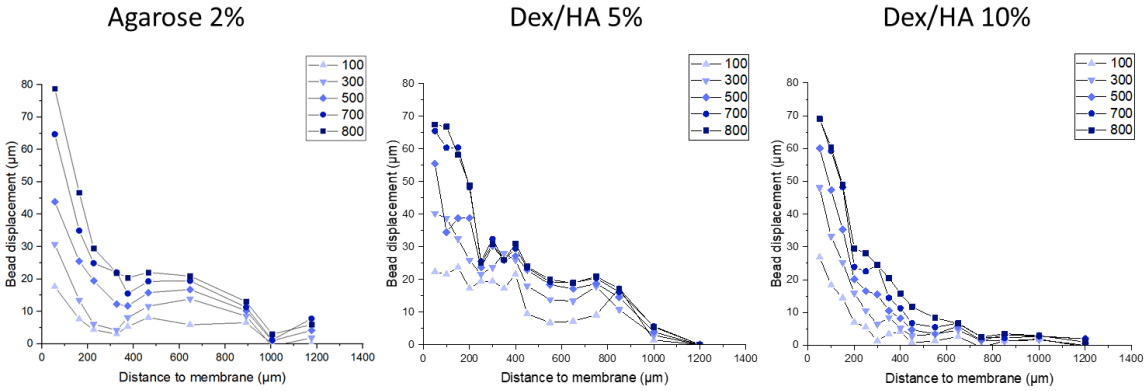


Figure 21. *Displacement of microbeads in agarose, Dex/HA 5wt% and Dex/HA 10wt%. Microbead displacement was measured at 0, 100, 300, 500, 700 and 800 mbar.*

6.1.4. Beads migration

To determine whether the Dex/HA hydrogels containing µ-beads were degrading over time, mechanical stimulation was applied for 3 days, by 60 minutes stimulation (350 mbar) per day. The bright-field images made on day 0, before mechanical stimulation, were compared with the images after 3 days of stimulation, and compared in **Figure 22** for Dex/HA 10wt%. A small bead displacement was detected in the upper zone of the hydrogel, as can be seen in the red section (**Figure 22-D**), but in the other sections the beads did remain on their place.

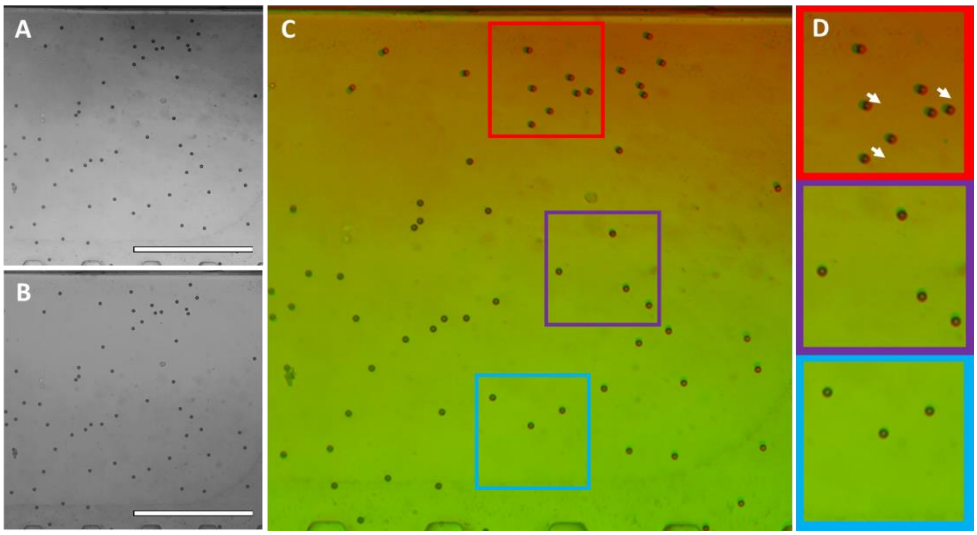


Figure 22. *Bright-field images of Dex/HA 10wt% at day 0 (A) and day 3 (B) for the compression condition. C) Overlay with day 0 (in green) and day 3 (in red). D) Higher magnification for three zones, with bead displacement indicated using white arrows. Scalebars: 250 µm.*

6.2. Cell-hydrogel culture

6.2.1. Bright-field imaging

Bright-field images were used to determine cell distribution and morphology. Chondrocytes did not change cellular morphology over time in both static and compression conditions. Moreover, comparable size and morphology was seen for the 3 hydrogels (**Figure 23**).

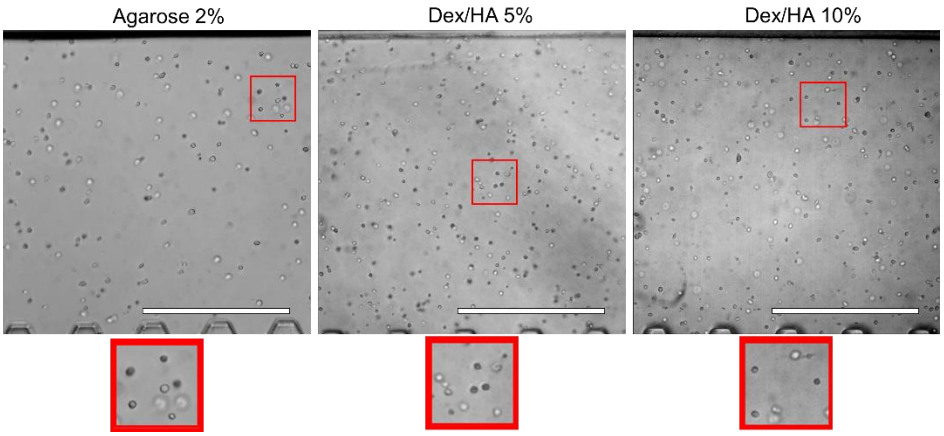


Figure 23. *Bright-field images for agarose and Dex/HA hydrogel compression conditions at day 3. Scalebars: 250 µm.*

6.2.2. Viability

Viability was evaluated using a LIVE/DEAD assay at day 0, day 7 and day 11 for static and compression conditions (**Figure 24 and 25).** For both static and compression conditions, no significant differences were detected between the hydrogels at the time points. A statistically significant reduction in viability from day 0 to day 11 was shown for all conditions. For all hydrogels, the viability was not affected by the mechanical stimuli, since no significant difference was seen between their static and compression conditions.

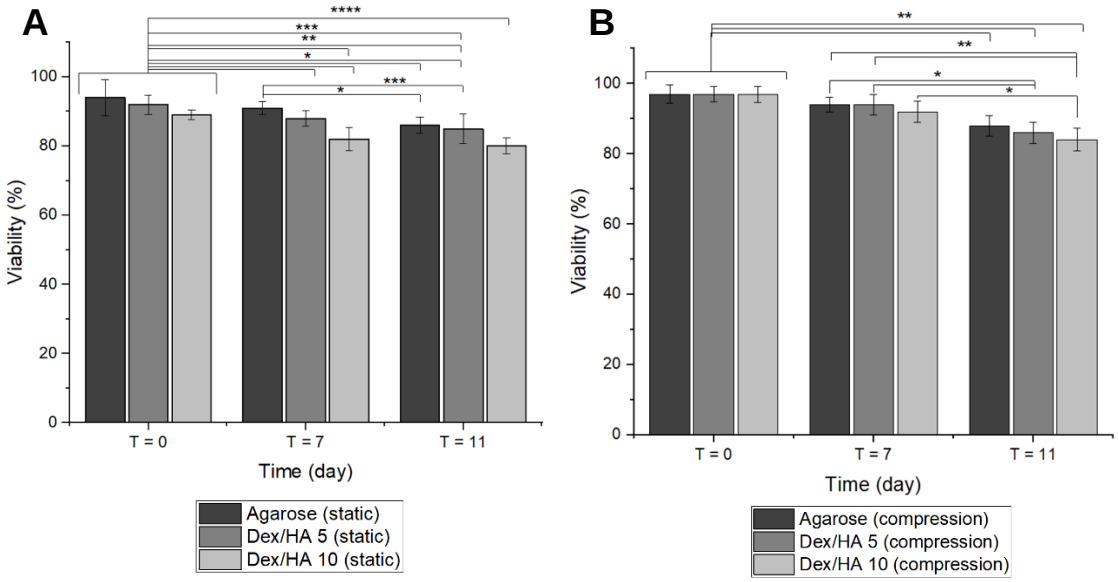


Figure 24*. A) Viability static conditions (N = 12) and B) viability compression conditions (N = 9). Values are expressed with ± SD and p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), p < 0,001 (****).*

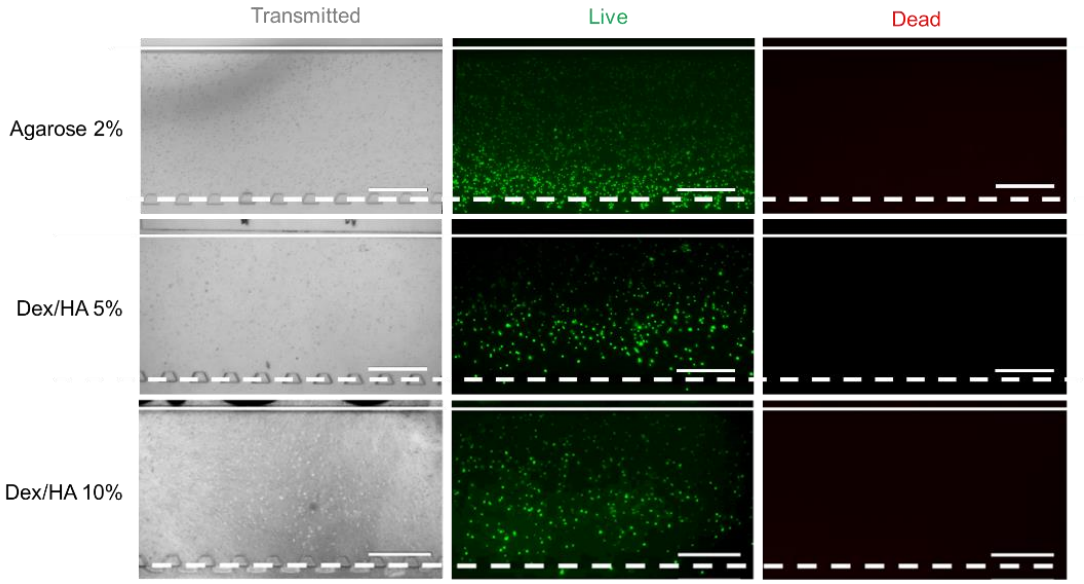


Figure 25. *Viability staining for agarose 2%, Dex/HA 5wt% and Dex/HA 10wt% at day 3. Living cells stained in green and dead cells stained red. Scalebars: 500 µm.*

6.2.3. Cell displacement

Cell displacement during compression loading was evaluated by comparing the bright-field images made in static (0 mbar) and under mechanical stimulation (350 mbar). **Figure 26** shows the overlay of both conditions for Dex/HA 10wt%**.** Close to the membrane, the cell displacement was higher (**Figure 26-D**, red section) with respect to the section close to the pillars (**Figure 26-D**, blue section).

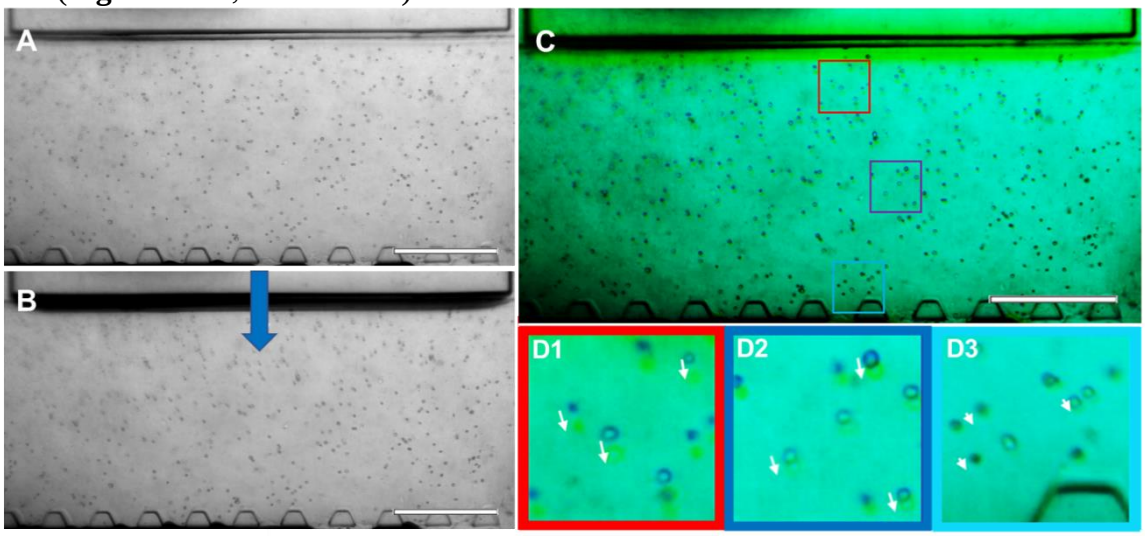


Figure 26. *Bright-field images of Dex/HA 10wt% at 0 mbar (A) and 350 mbar (compression direction indicated with blue arrow) (B). C) Overlay of 0 mbar (in blue) and 350 mbar (in green). D) Magnifications for three selected zones, cell displacement is indicated with white arrows. Scalebars: 500 µm.*

6.2.4. Cell migration

To determine chondrocyte movement, images at day 0, made before exposing to mechanical loading, were compared to images taken after 3 days of mechanical compression. The overlay of both images for Dex/HA 10/wt% hydrogel presented movement of the cells towards the point of mechanical stimulation, centered in the middle of the PDMS membrane (**Figure 27**). Cells in the upper section of the hydrogel, close to the membrane, did move in a lesser extent when compared to the cells in the deeper hydrogel region. Chondrocytes cultured in agarose under compression conditions, showed no cell migration (**Figure 28**). In both Dex/HA 5wt% and 10wt% static cultures, chondrocytes were migrating between different layers of the hydrogel and undirectional (**Figure 29**).

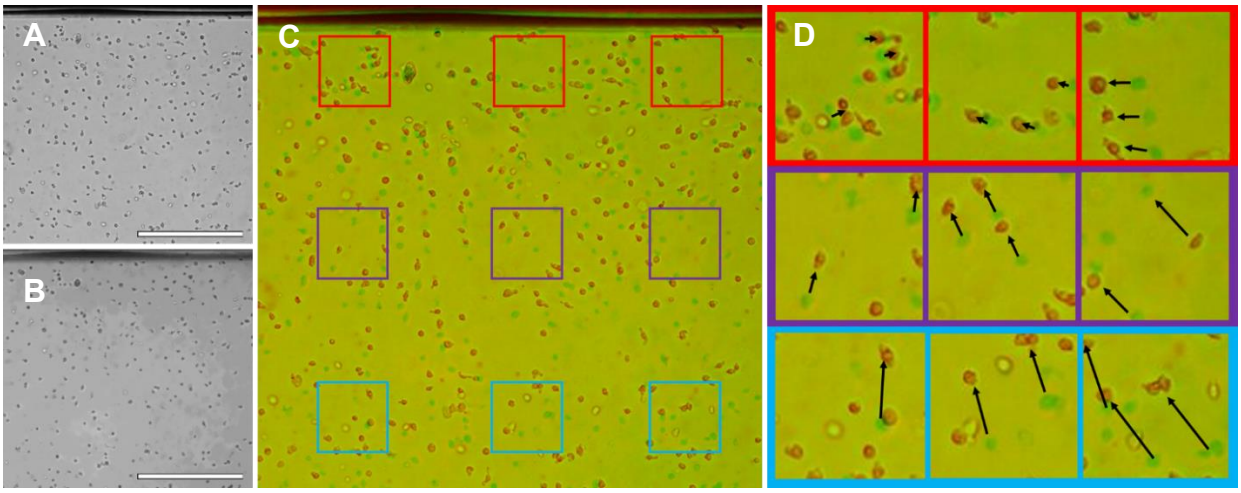


Figure 27. *Bright-field images of Dex/HA 10wt% at day 0 (A) and at day 3 (B) for the compression condition. C) Overlay of day 0 (in green) and day 3 (in red). D) Magnification of 9 selected zones, cell displacement is indicated with black arrows. Scalebars: 250 µm.*

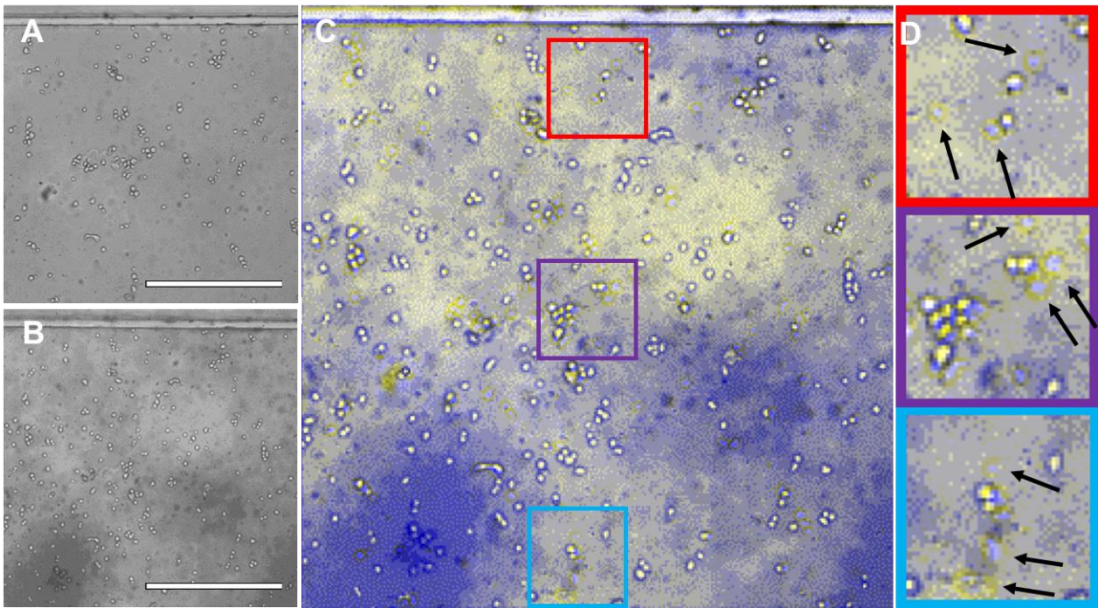


Figure 28. *Bright-field images of Dex/HA 10wt% at day 0 (A) and at day 3 (B) for the static condition. C) Overlay of day 0 (in yellow) and day 3 (in blue). D) Magnification of 3 selected zones, cell displacement is indicated with black arrows. Scalebars: 250 µm.*

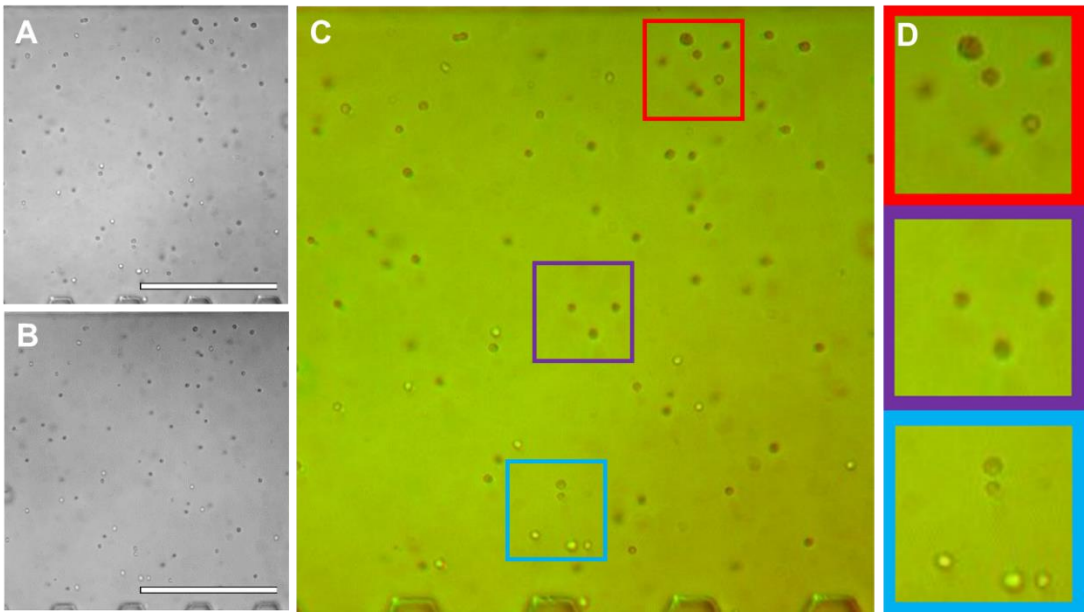


Figure 29. *Bright-field images of agarose 2% at day 0 (A) and at day 3 (B) for the compression condition. C) Overlay of day 0 (in green) and day 3 (in red). D) Magnification of 3 selected zones. Scalebars: 250 µm.*

6.2.5. Surface plasmon resonance

Using SPR, the presence of IL-6 in the culture supernatant after 3 and 7 days of culture in static and compression conditions was determined. For both Dex/HA hydrogels, a reduction in the cytokine presence was seen between day 3 and day 7 after mechanical stimulation (**Figure 30**). Both static conditions did not change over time. Overall, chondrocytes cultured in Dex/HA 5wt% hydrogel did release IL-6 in higher extent, in comparison to Dex/HA 10wt%.

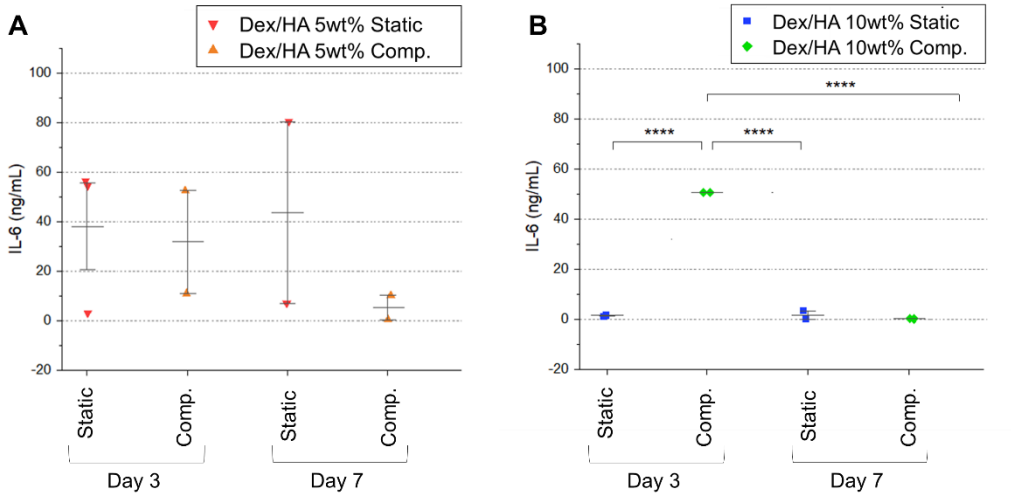


Figure 30. *IL-6 concentration (ng/mL) after 3 and 7 days of culture in both static and compression for Dex/HA 5wt% (A) and Dex/HA 10wt% (B). Comp. = compression, with p < 0,001 (****).*

For agarose hydrogel, only culture supernatant after 3 days in static conditions was analyzed and compared to Dex/HA 5wt% and Dex/HA 10wt% conditions (**Figure 31**)**.** Dex/HA 5wt% showed the highest average IL-6 production, but was not statistically different from agarose.

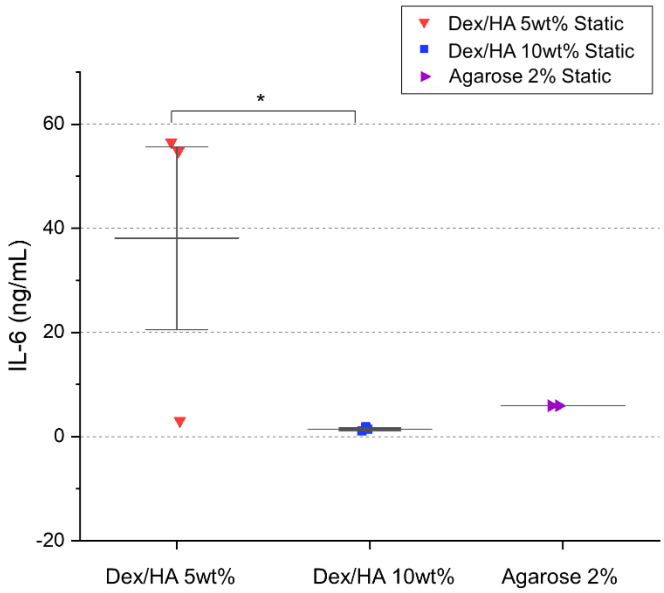


Figure 31. *SPR data for Dex/HA 5%, Dex/HA 10% and Agarose 2%, 3 days of culture in static condition. P < 0,1 (*).*

6.2.6. MMP activity

The presence of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14, MMP-15, and MMP-16 after 3 days of culture was determined using a kinetic MMP activity assay. The MMPs initiate a fluorescence signal, which intensity is plotted over time for all conditions (**Figure 32-A).** All samples reached almost directly their maximum value, indicating a high reaction speed. MMP expression was influenced by both mechanical stimulation and hydrogel choice. The average intensity of the 4 last measured cycles was plotted, showing the highest activity for Dex/HA 10wt% static and compression conditions. Agarose static culture resulted in lowest activity (**Figure 32-B**).

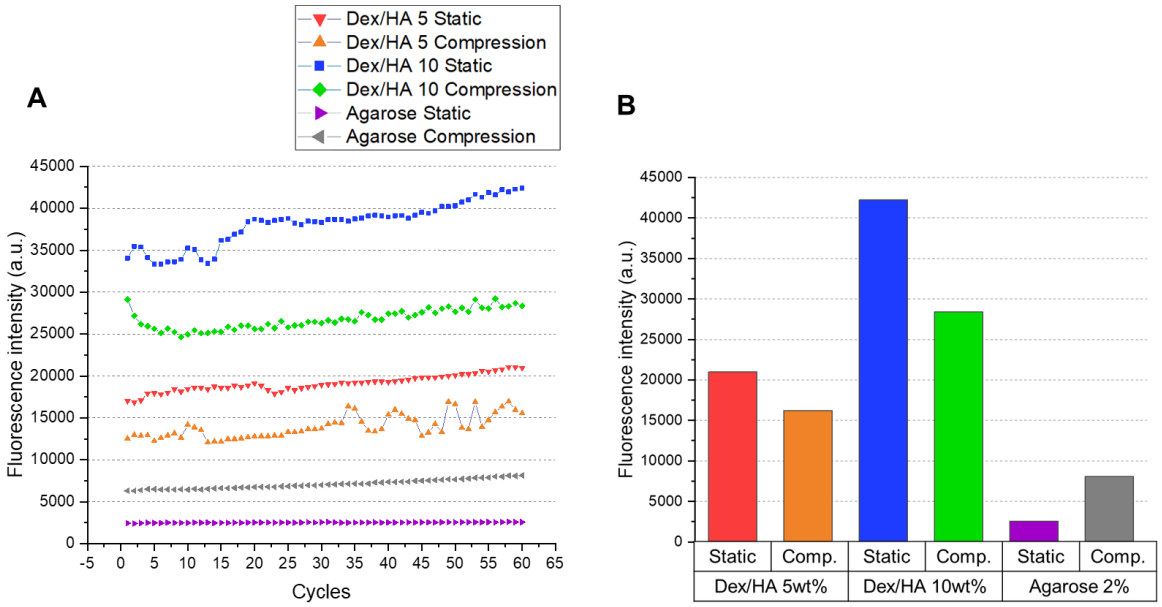


Figure 32. *A) MMP activity for all hydrogel conditions, analyzed from supernatant after 3 days of culture. Measurement was carried out every 30 seconds, for 30 minutes. B) Average intensity of the last 4 measured cycles.*

6.2.7. Gene expression

Relative gene expression was obtained by normalizing the CT values to the values of the housekeeping gene GAPDH, with the statistical analysis for the qPCR data in **Appendix 12.4.7**.

The cartilage-specific gene SOX9, which plays a role in chondrogenesis, showed high expression in Dex/HA 5wt% chondrocytes. For both Dex/HA cultures, there were no significant differences between the static and compression culture. In agarose compression condition the expression of SOX9 was three-fold higher with respect to its static culture. Further, there were no significant differences between the Dex/HA 5wt% compression and agarose 2% compression culture detected.
The cartilage-specific gene Aggrecan (ACAN) was upregulated in Dex/HA 5wt% and agarose compression conditions, with respect to their static cultures. In Dex/HA 10wt% chondrocytes, ACAN was found significantly higher ($P < 0,01$) for the static condition. Lubricin (PRG4), which enables low-friction articulation in a healthy joint, was highly upregulated in agarose compression culture ($P < 0,001$). In contrast to Dex/HA 5wt%, showing an expression twice as high in its static condition with respect to its compression culture ($P < 0,001$).

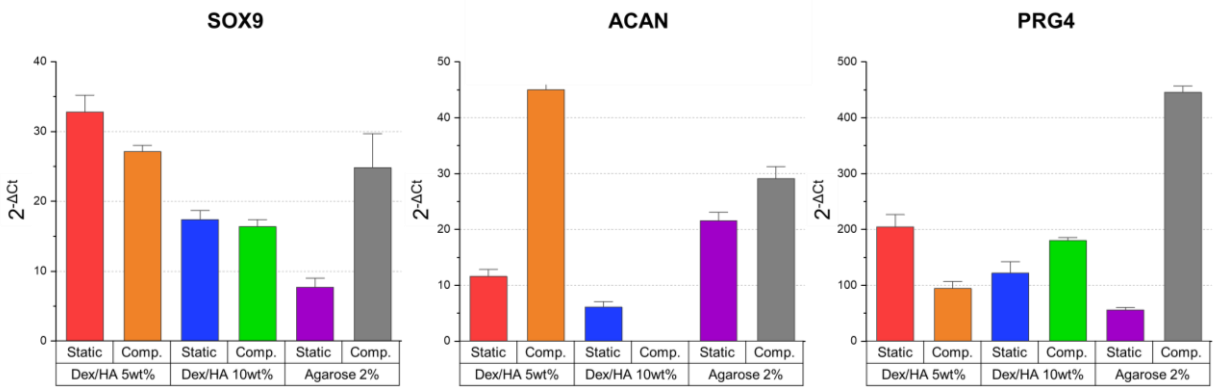


Figure 33. *Relative gene expression of SOX9, ACAN, and PRG4 for all hydrogel conditions. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Mean values ± SD, N = 3.*

The degradation of cartilage proteoglycans, especially Aggrecan, was determined by ADAMTS-4 and ADAMTS-5 expression (**Figure 34**). ADAMTS-4 was upregulated in both Dex/HA compression conditions, with respect to their static cultures ($P < 0,05$ for Dex/HA 5wt% and $P < 0,001$ for Dex/HA 10wt%). Agarose showed high levels of ADAMTS-4 positive cells in static, significant equal to Dex/HA 5wt% static and compression expression. On the other hand, ADAMTS-5 was highly upregulated in Dex/HA 5wt% static condition ($P < 0,001$), while Dex/HA 10wt% and agarose showed an increase for the compression cultures with a low expression in their static cultures (both $P < 0,01$). HA-degrading enzyme HYAL-1 was highly upregulated in Dex/HA 5wt% static and compression cultures and in Dex/HA 10wt% compression cultures. Further, HYAL-3 was highly expressed in Dex/HA 5wt% static and Dex/HA 10wt% compression culture. In agarose static culture, the cells did express both hyaluronidases in a low extent.

Furthermore, the expression of degradative enzymes MMP-1 and MMP-13 were upregulated for both Dex/HA 5wt% and Dex/HA 10wt% compression cultures, and did differ significantly from their static cultures ($P < 0,001$). MMP-13 was not expressed in agarose chondrocytes, which was further confirmed by immunofluorescence staining for hydrogel droplet cultures, showing no presence of MMP-13 in agarose, but a high amount of MMP-13 positive cells in Dex/HA 10wt% (**Appendix 12.4.8, Figure S.9**).

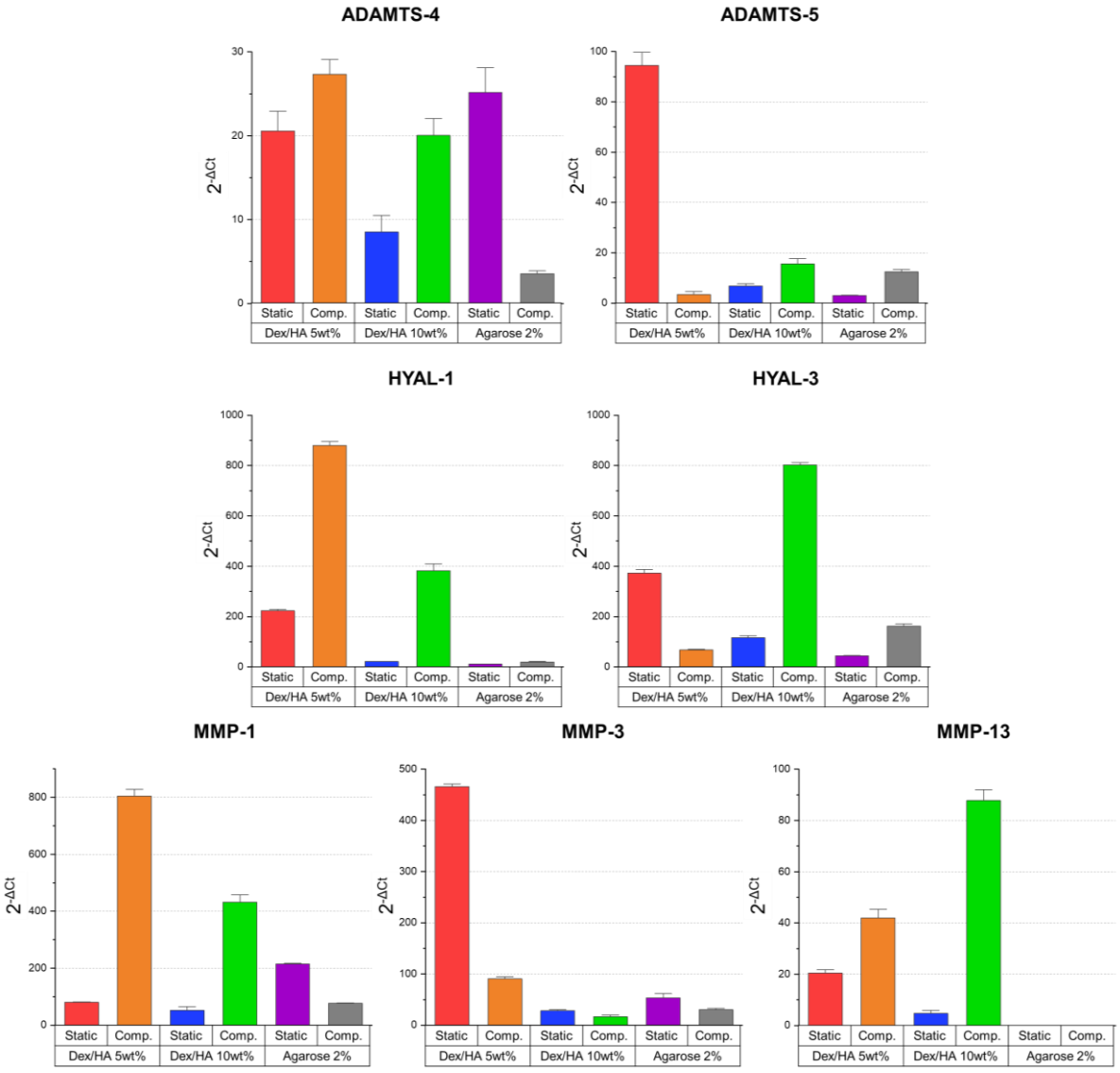


Figure 34. *Relative gene expression of ADAMTSs, HYALs, and MMPs for all hydrogel conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Mean values ± SD, N = 3.*

The cytokine IL-6 was highly expressed in agarose compression conditions (**Figure 35**). Dex/HA 10wt% chondrocytes did not show statistical significance between the static and compression condition, and for Dex/HA 5wt% the expression was almost twice as high in the static with respect to the compression culture ($P < 0{,}001$), suggesting that IL-6 production is not promoted under mechanical stimulation in Dex/HA.

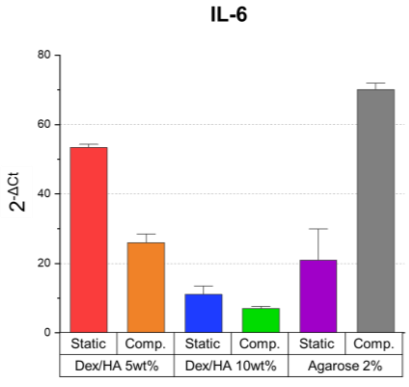


Figure 35. *Relative gene expression of IL-6, for all hydrogel conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Mean values ± SD, N = 3.*

7.1. Results: Culture conditions

7.1.1. Monolayer experiments

The influence of temperature, oxygen, and cytokine stimulation on monolayer chondrocyte culture was evaluated using bright-field imaging, viability assay, immunofluorescence staining, metabolic activity and gene expression. In some sections, only the healthy and IL-1β + TNF−α conditions were considered.

Bright-field imaging

Cell morphology was evaluated using bright-field imaging. The effect of low oxygen tension was determined by comparing the 37 °C hypoxia culture with the 37 °C conventional cultured cells (**Figure 36**). Under hypoxia, the morphology of the cells showed a completely different appearance with respect to the normoxia culture, which was also seen for the 33 °C and 39 °C hypoxic conditions (**Appendix 12.5.1**). The cells in hypoxia culture enlarged their cell area by stretching onto the surface. The contact surface area was further enhanced for the cells after administration of IL-1β and TNF−α for 3 days.

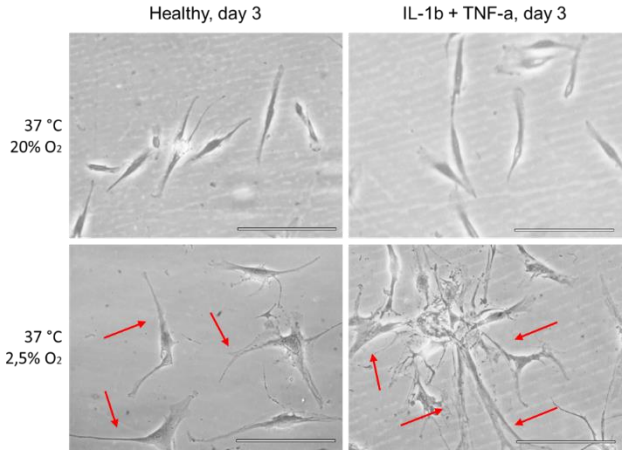


Figure 36. *Bright-field imaging of 37 °C 20% O_2 and 2,5% O_2 culture, for healthy and cytokine stimulated conditions at day 3. Stretching on surface is indicated by red arrows. Scalebars: 200 µm.*

Monolayer viability

Under healthy conditions, cell death was not promoted in 33 °C and 37 °C hypoxic culture (**Figure 37 & 38**). 39 °C did show a significant increase in cell death for healthy culture ($P < 0{,}001$), which was further enhanced by cytokine stimulation. Cytokine stimulation did influence the viability for all hypoxic culture conditions, but did not result in cell death for the conventional culture. No significant difference was detected between 33 °C and conventional healthy and diseased conditions.

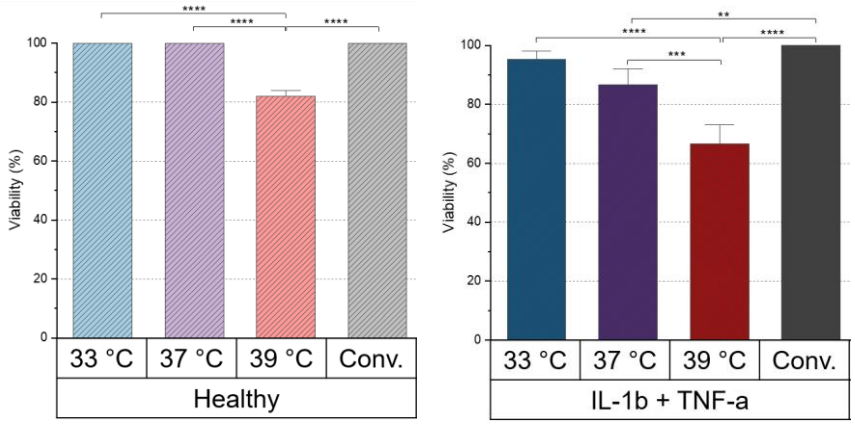


Figure 37. *Chondrocyte viability at day 3 healthy (left) and cytokine stimulated (right) conditions for different temperature and oxygen levels. Values are expressed with ± SD with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), p < 0,001 (****).*

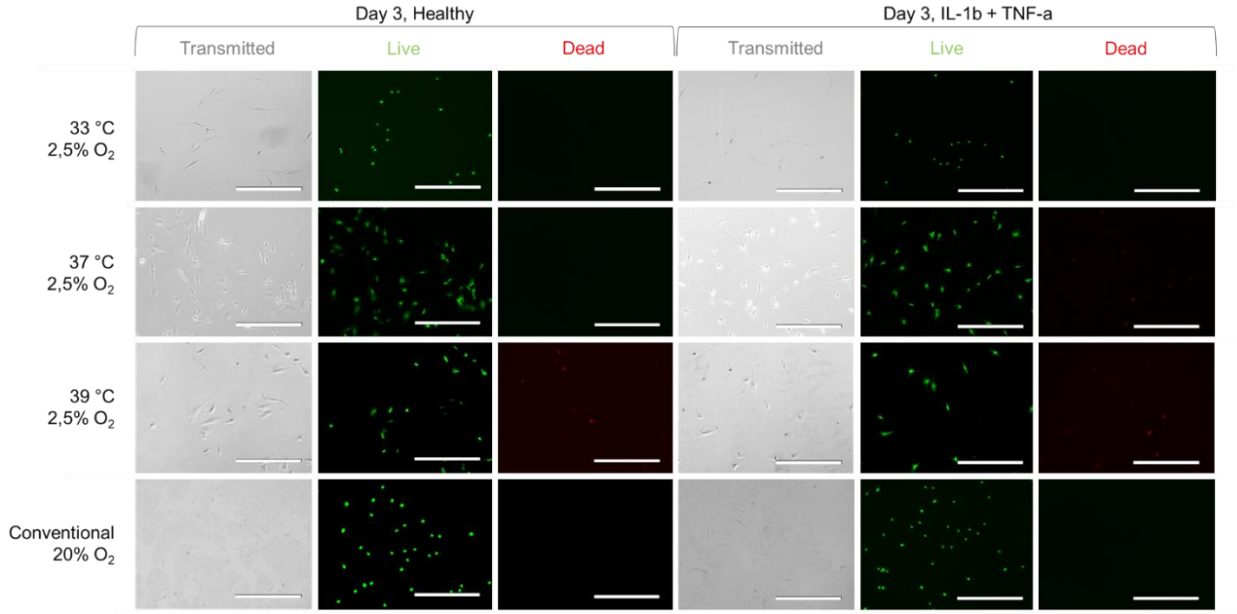


Figure 38. *Chondrocyte viability at day 3 healthy and cytokine stimulated conditions for different temperature and oxygen levels. Scalebars: 400 µm.*

Immunofluorescence staining

Protein expression was detected by immunofluorescence staining, for phalloidin and Aggrecan in 33 °C hypoxic culture (**Figure 39**). Phalloidin staining visualizes the cell contact area under different cytokine stimulations. Aggrecan was detected in a small extent for the cytokine stimulated condition, indicated with white arrows (**Figure 39-D**). Additional images for Collagen VI and Aggrecan staining are shown in **Appendix 12.5.2.**

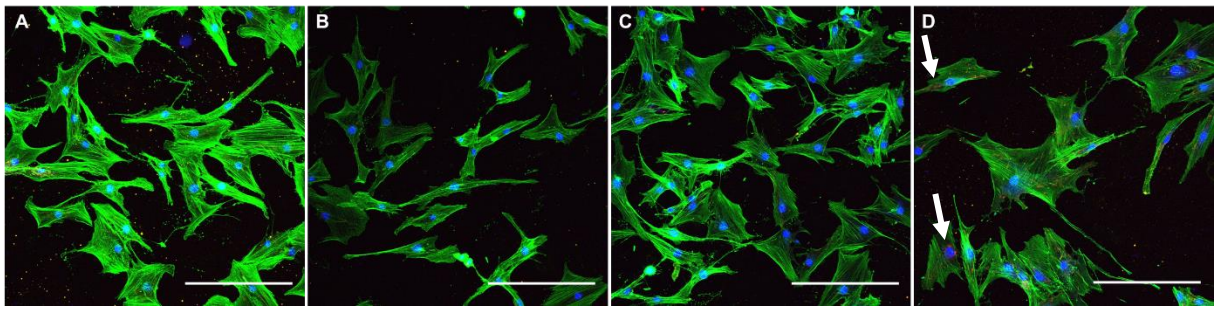


Figure 39. *Phalloidin and Aggrecan immunofluorescence staining of 33 °C hypoxic healthy (A), IL-1 β (B), TNF-α (C), and IL-1 β + TNF- α (D) stimulated conditions at day 3. White arrows indicate presence of Aggrecan. Images taken with Nikon Confocal; green (phalloidin), red (Aggrecan), and blue (nuclear staining, DAPI). Scalebars: 200 µm.*

Cell stretching

Since aberrant cell stretching was seen, the area per cell was determined using the immunofluorescence stained cytoskeleton. A square, including all outermost points per cell, was measured (**Figure 40-B)**. Only single cells were considered, when attached to another cell, it was difficult to determine the separate cytoskeletons. The cell areas were plotted for all conditions to estimate the differences in cell stretching (**Figure 40-A**). The healthy hypoxic cultures resulted in an increase in cell stretching, compared with the healthy conventional culture.

In 33 °C and 37 °C hypoxic cultures, TNF−α and the cytokine cocktail affected the cell areas in greatest extent. 39 °C hypoxia condition affected cell stretching, but the separate cytokines did not highly influence the stretching, and the cytokine cocktail resulted in a slight decrease in surface area.

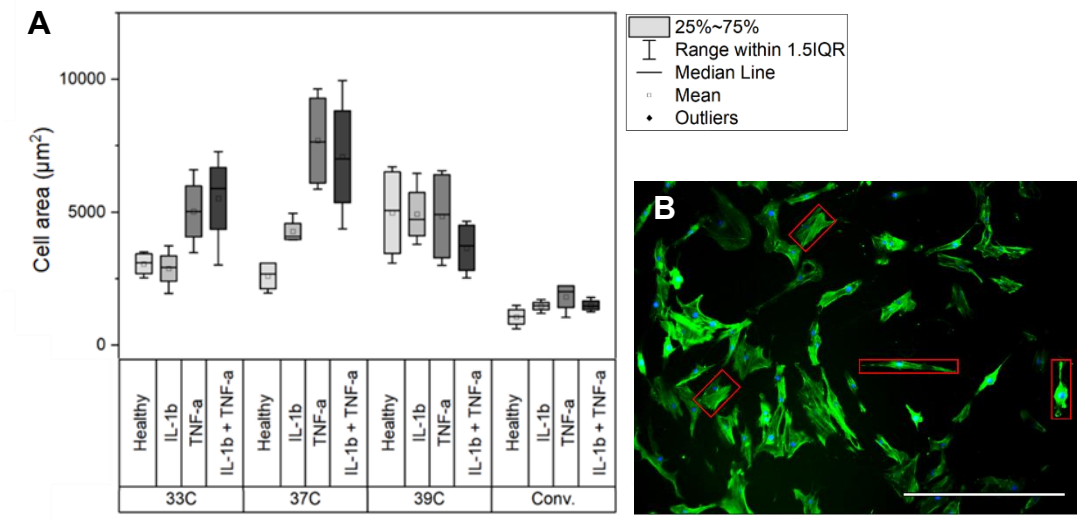


Figure 40. *A) Cell area per condition, N = 8. B) Immunofluorescence staining of phalloidin (green) and DAPI (blue) for quantification of cell area using ImageJ; measured area squares are indicated in red. Scalebar: 400 µm.*

7.1.2. Pellet culture

The influence of temperature, oxygen, and cytokine stimulation on chondrocyte behavior was determined for pellet culture system.

Pellet formation

Chondrocytes were distributed over the mini-wells with an average of 29,4 ± 11,5 cells per pellet (**Figure 41**). At 33 °C hypoxia culture, the pellets formed under healthy circumstances were almost circular, whereas the cytokine stimulation resulted in irregular circumferences (**Figure 42**). Moreover, while in the healthy conditions a single pellet was formed, in the samples treated with cytokines, the single pellets did de-cluster, resulting in an increase in pellet number per well. Comparable behavior was seen for the other conditions after 3 days of culture (**Appendix 12.5.4**).

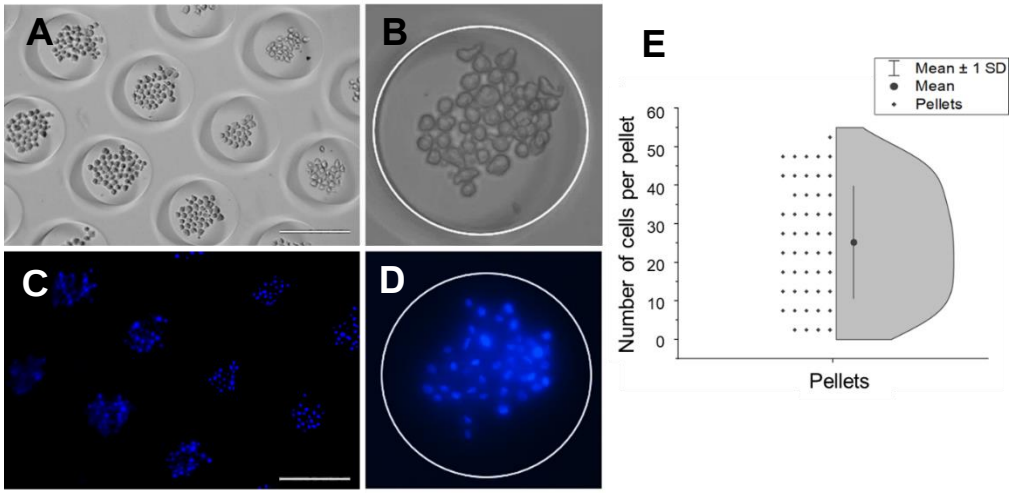


***Figure 41**. Bright-field image of pellet overview (A) and one mini-well containing 1 pellet (B) for 33 °C hypoxic culture. Viability assay with nuclei in blue (DAPI), and dead cells in green (C) and one mini-well containing 1 pellet (D). Average number of cells per pellet was 29 (E). Scalebars of A and C: 200 µm and diameter of mini-wells: 200 µm.*

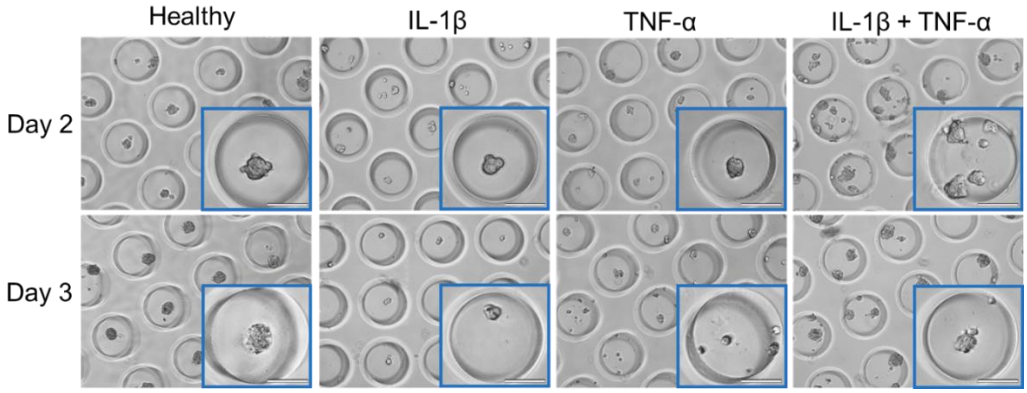


***Figure 42.** Pellets cultured at 33 °C hypoxia culture at day 2 and day 3. Scalebars: 100 µm.*

The differences between the conditions were determined by measuring the circularity, the perimeter, and the number of pellets per mini-well (**Figure 43**). Since the number of cells differ per pellet, we considered per conditions 2 spots, and analyzed per condition 8 pellets in total. Under healthy circumstances, the biggest pellets were formed for the 33 °C hypoxic culture, while the perimeters of 37 °C hypoxic pellets were half of 33 °C pellets perimeter (260 versus 120 µm). Cytokines did not statistically affect the circularity of the formed pellets at 37 °C and 39 °C hypoxic cultures. Pellets fragmented under stimulation of IL-1β and TNF−α, resulting in

a maximum of 6 separate cell aggregates per well for 33 °C, while under healthy conditions all cells did remain together.

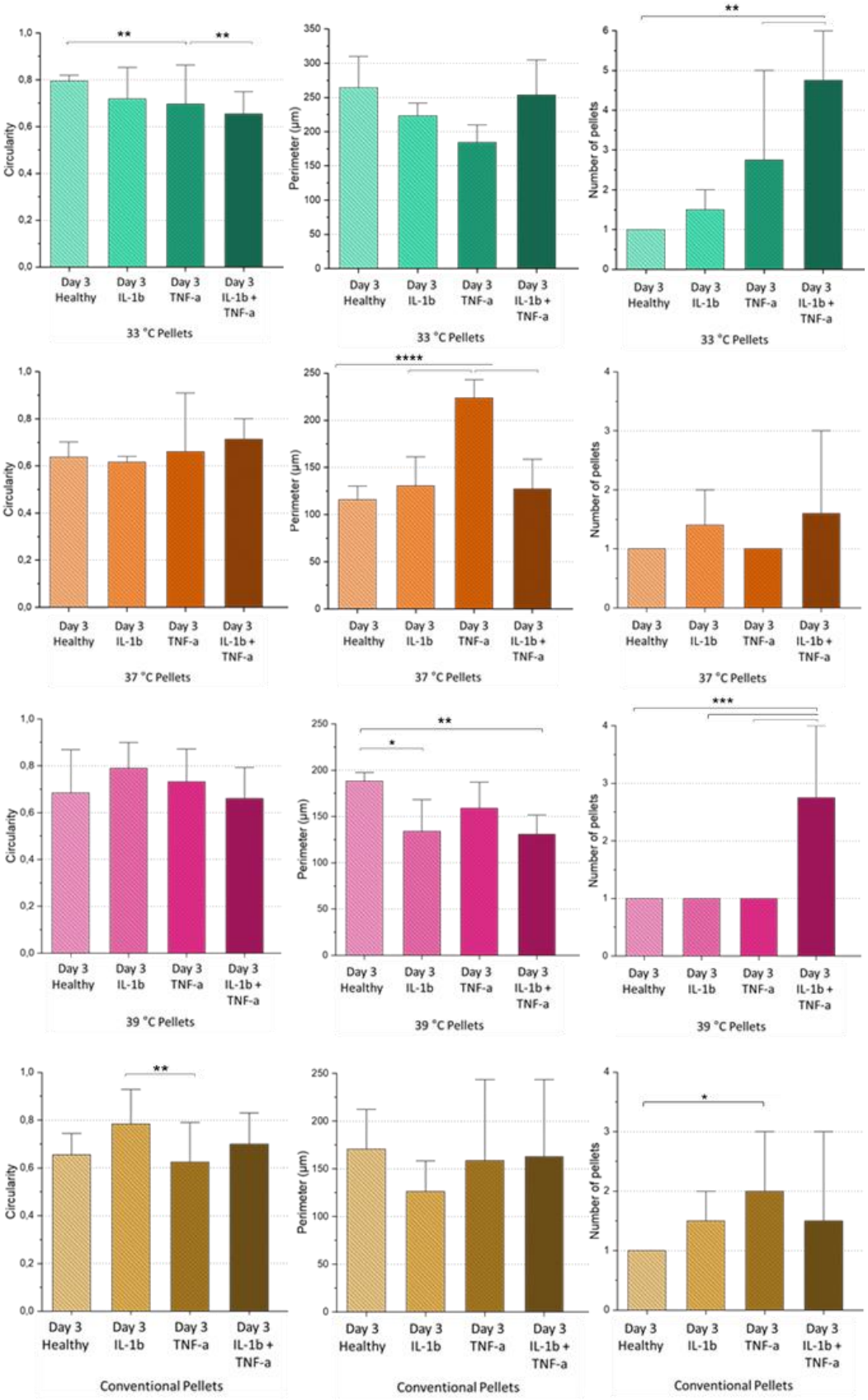


Figure 43. *Circulatory (left), perimeter in µm (middle), and amount of pellets (left) at day 3, for all culture conditions. Values are shown with ± SD. N = 8, p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****).*

Pellet viability

The viability of pellet chondrocytes was determined for all healthy conditions after 3 days of culture (**Figure 44**). Viability at 39 °C and conventional conditions were significant lower with respect to the 33 °C hypoxic culture. Viability of cells cultured in a healthy pellet was not statistically different to cytokine stimulated conditions (**Appendix 12.5.3**).

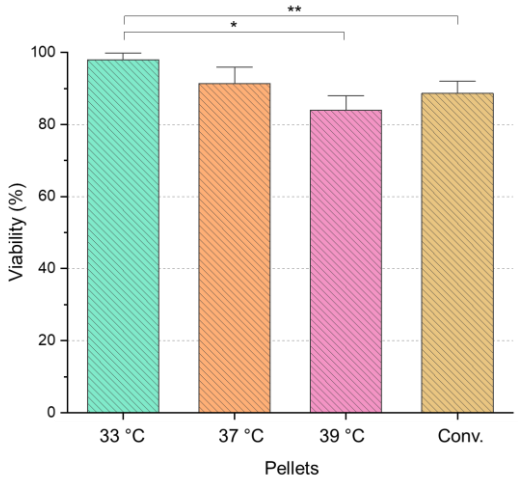


Figure 44. *Pellet viability for healthy conditions at day 3. Values are shown with ± SD and p < 0,05 (**) and p < 0,1 (*).*

7.1.3. Metabolic activity: Monolayer versus pellets

The chondrocyte monolayer and pellet culture were compared for their metabolic activity and the temperature, oxygen, and cytokine influence on both culture systems was approached.

A PrestoBlue assay was performed to determine the metabolic activity of the monolayer and pellet cultured chondrocytes. Healthy conditions for monolayer culture (**Figure 45**) was compared with the pellet culture (**Figure 46**). Due to difference in cell densities, the 2D and pellet cultures cannot be compared 1:1, but only trend wise.

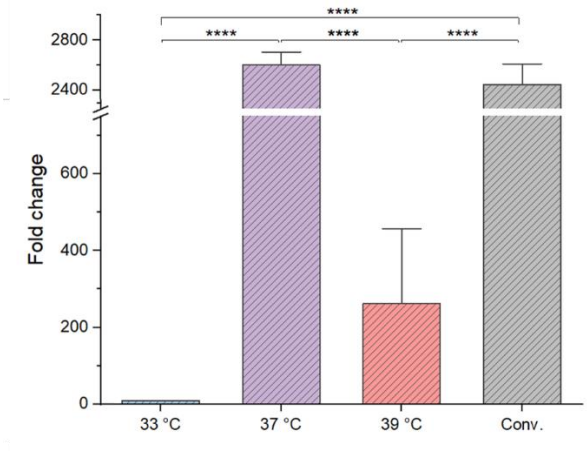


Figure 45. *Fold change of metabolic activity for healthy monolayer cultures, at 33 °C, 37 °C, and 39 °C hypoxic conditions and conventional condition. Values are expressed with ± SD with p < 0,001 (****). N = 3.*

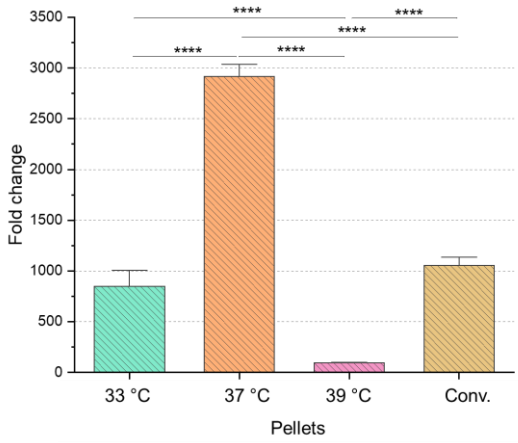


Figure 46. *Fold change of metabolic activity for healthy pellet cultures, at 33 °C, 37 °C, and 39 °C hypoxic conditions and conventional condition. Values are expressed with ± SD with p < 0,001 (****). N = 3.*

Results indicated that the metabolic activity was altered by culture temperature and oxygen tension. In monolayer culture, the metabolic activity was inhibited at 33 °C and 39 °C hypoxic culture. In both monolayer and pellet cultures, highest activity was detected at 37 °C hypoxic culture. Further, the 37 °C hypoxic and conventional culture did not show a significant difference in activity.

Furthermore, the metabolic activity was evaluated for the cytokine stimulated conditions for monolayer cultures (**Figure 47**) and pellet cultures (**Appendix 12.5.5**). Cytokine stimulation did not significantly influence the activity at conventional culture. At 39 °C, the individual cytokines did negatively alter the metabolic activity, while the cytokine cocktail led to an increase in activity. Culture in a pellet at 33 °C and 37 °C hypoxia resulted in a decrease in activity for pellets, with respect to the monolayer culture.

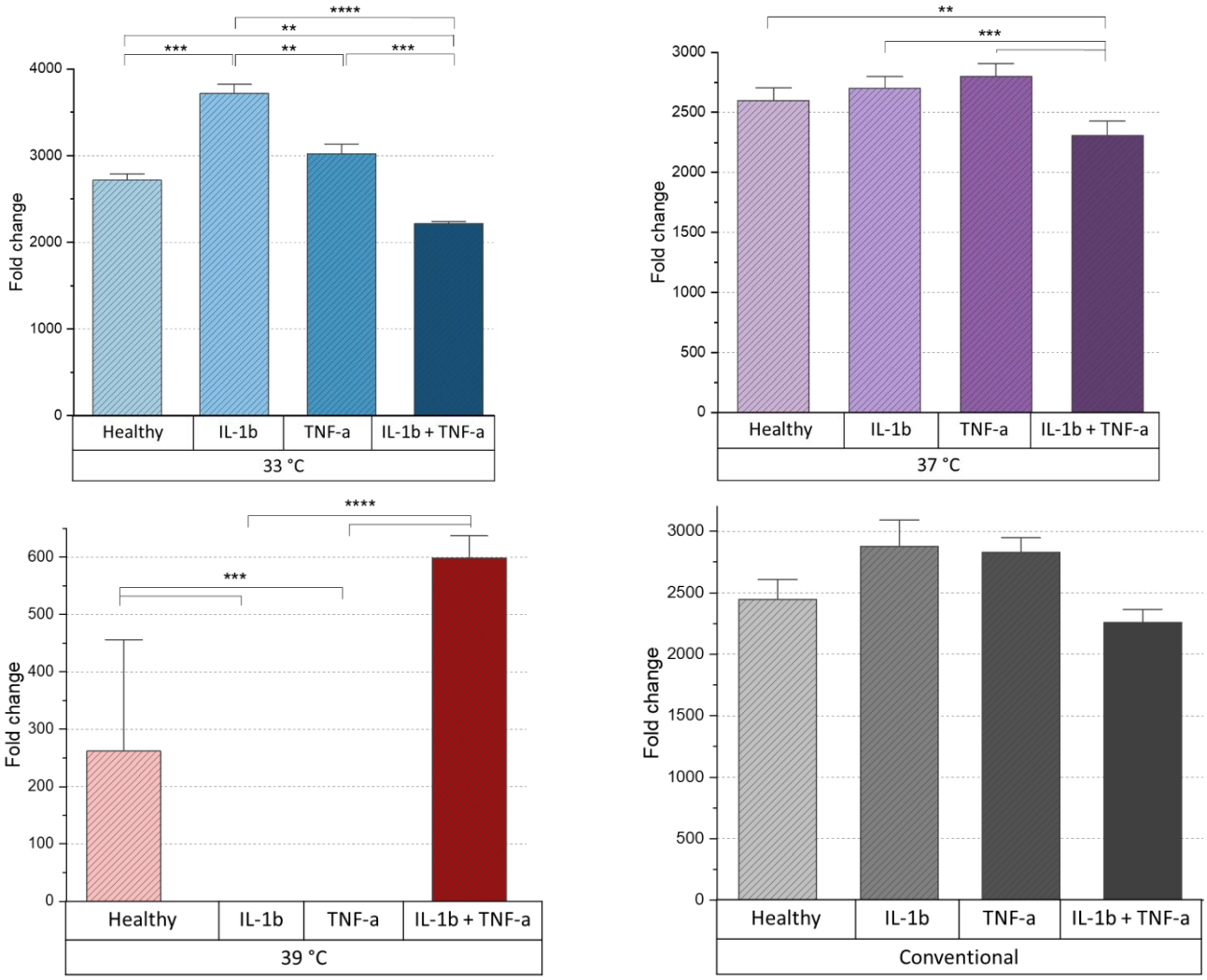


Figure 47. *Fold change of metabolic activity assay for all monolayer cultures, at 33 °C, 37 °C, and 39 °C hypoxic and conventional conditions. Values are expressed with ± SD, N = 3, with significances p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****).*

7.1.4. Gene expression: Monolayer versus pellets

The chondrocyte monolayer and pellet culture were compared for their gene expression and the temperature, oxygen, and cytokine influence on both culture systems was approached.

Glycolysis-related

GAPDH is a metabolic expression-related gene and involved in the catalyzation of the glycolysis, therefore its expression is influenced by culture temperature and oxygen levels (**Figure 48**). Lowest CT values, corresponding to a high GAPDH expression, were determined for the conventional monolayer culture. In all hypoxic conditions, the GAPDH activity was lower, with respect to the conventional control. Highest expression was seen for the 33 °C monolayer culture, corresponding to low GAPDH activity. Significant differences were detected between monolayer and pellet culture for the 33 °C and conventional conditions ($p < 0,05$). No significant differences were detected among all the pellet culture conditions (33 °C, 37 °C, 39 °C, and conventional).

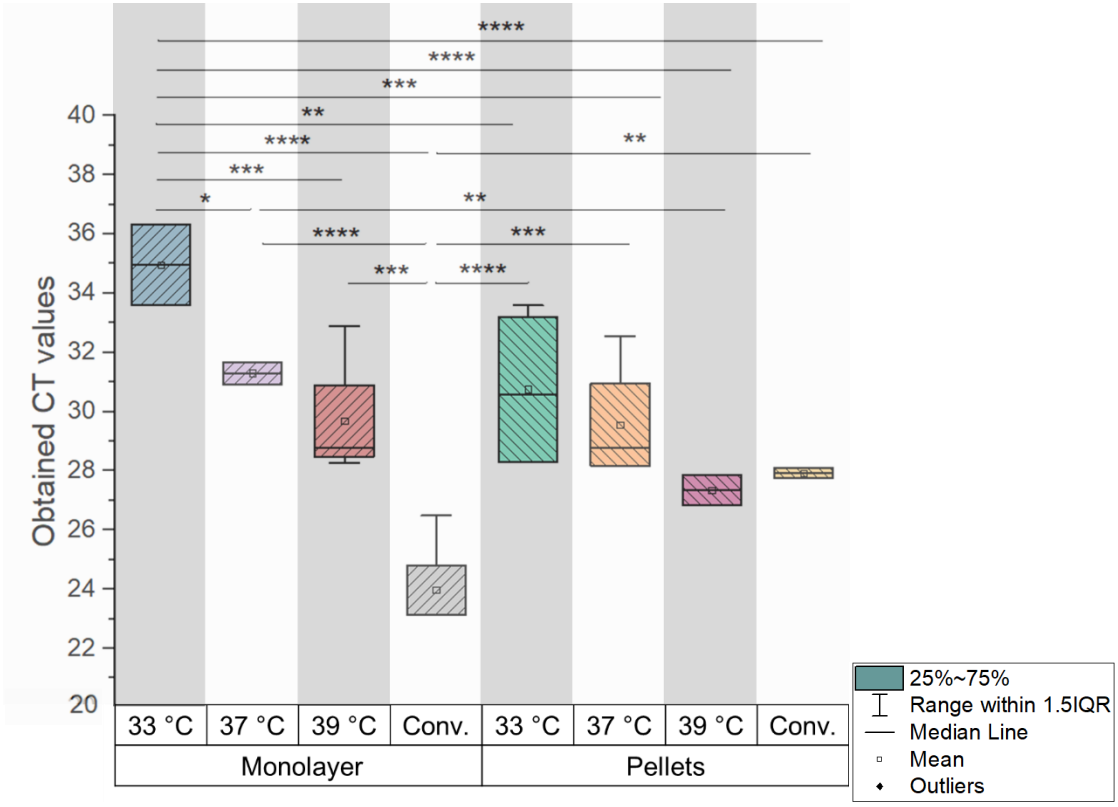


Figure 48. *GAPDH gene expression for healthy monolayer and pellet cultures, at 33 °C, 37 °C, and 39 °C hypoxic and conventional conditions. Values are expressed as median and interquartile range, N = 4, and p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****).*

Despite the variations in GAPDH per condition, it was considered as housekeeping gene by normalizing each individual condition to the obtained GAPDH values for that specific condition. A selection of plots with raw CT values are shown in **Appendix 12.5.6**.

Monolayer qPCR

Transcription factor SOX9 plays a key role in cartilage development, and expressed in a low extent for the hypoxic cultures, compared to the conventional culture (**Figure 49**). The Cartilage Oligomeric Matrix Protein (COMP) was evaluated as marker for cartilage turnover and cartilage metabolism, and was highly expressed at 33 °C.

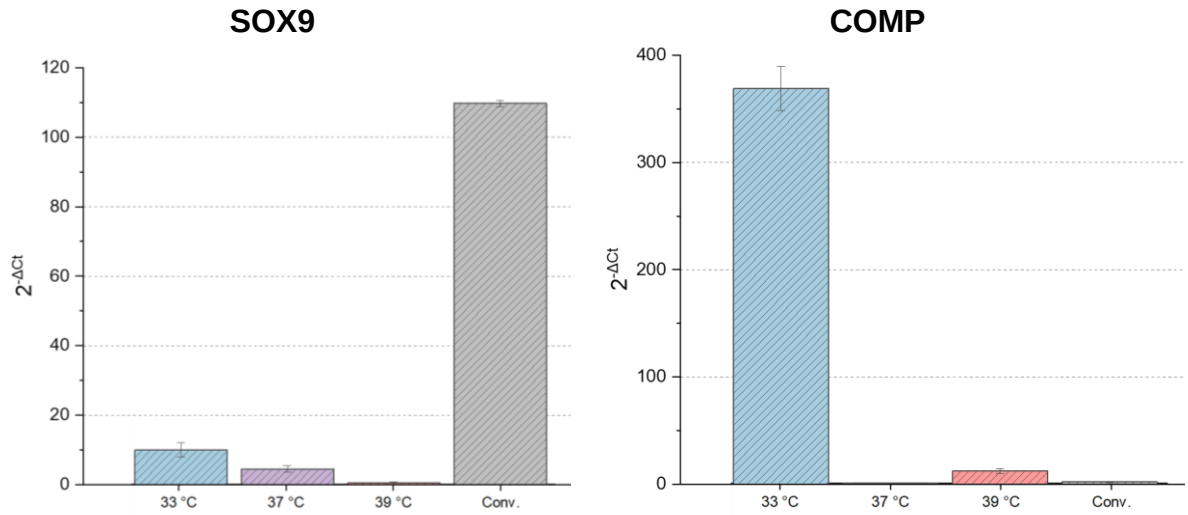


Figure 49. *Relative gene expression of SOX9 and COMP for all healthy monolayer culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

Collagen I expression was upregulated at 39 °C. HIF-1α, an essential transcription factor for the development of cartilage under hypoxic condition, was upregulated in both 37 °C hypoxic and normoxic cultures, but not synthesized under hypothermic (33 °C) and hyperthermic circumstances (39 °C).

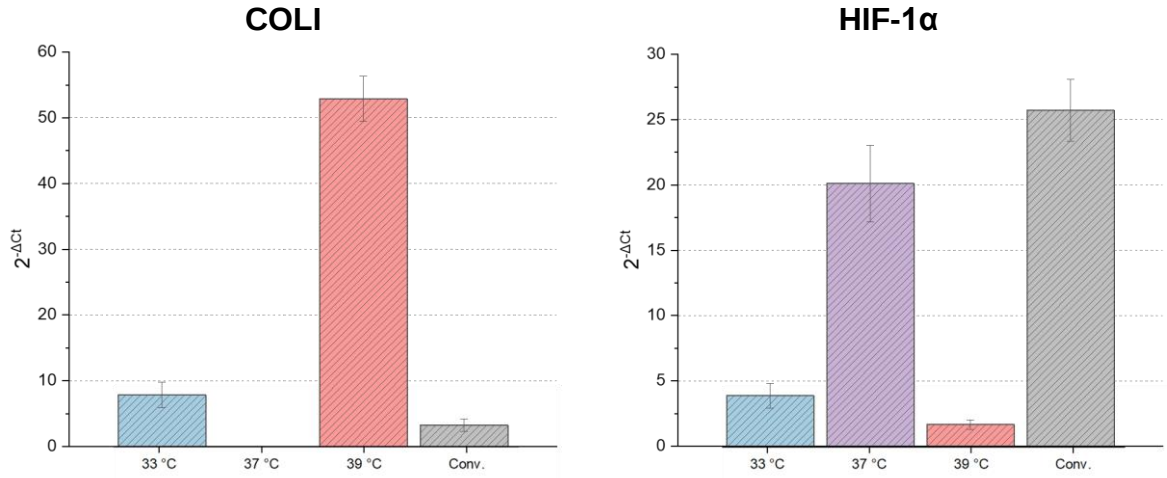


Figure 50. *Relative gene expression of ColI and HIF-1α for all healthy monolayer culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

Next, stress response of the chondrocytes was determined by evaluating the expression of heat shock proteins (HSP). HSP70 was highly expressed at 33 °C (**Figure 51**). HSP90 showed no significant differences for 33 °C, 39 °C and conventional cultures, 37 °C hypoxic culture did not present HSP positive cells.

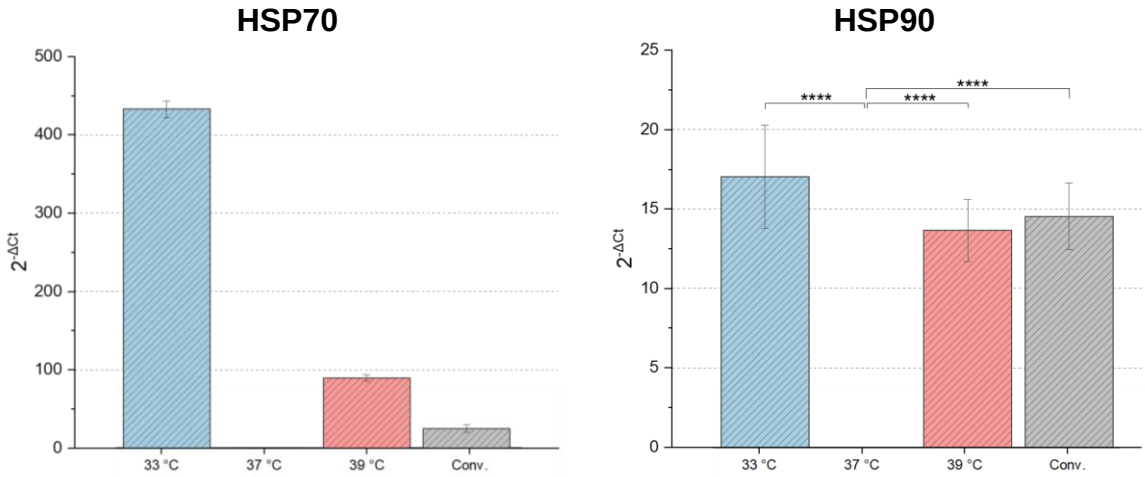


Figure 51. *Relative gene expression of HSP90 and HSP70 for all healthy monolayer culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 3.*

The production of HSP70 was further elaborated by comparing the healthy and diseased conditions (**Figure 52**). The influence of cytokines on the HSP70 expression was temperature related, with the greatest influence of TNF−α at 37 °C, and for the cytokine cocktail at 39 °C. In 33 °C and 37 °C hypoxic conditions, IL-1β application did not affect the HSP70 production and when stimulated with the cytokine cocktail, the expression of HSP70 was lowered for all conditions.

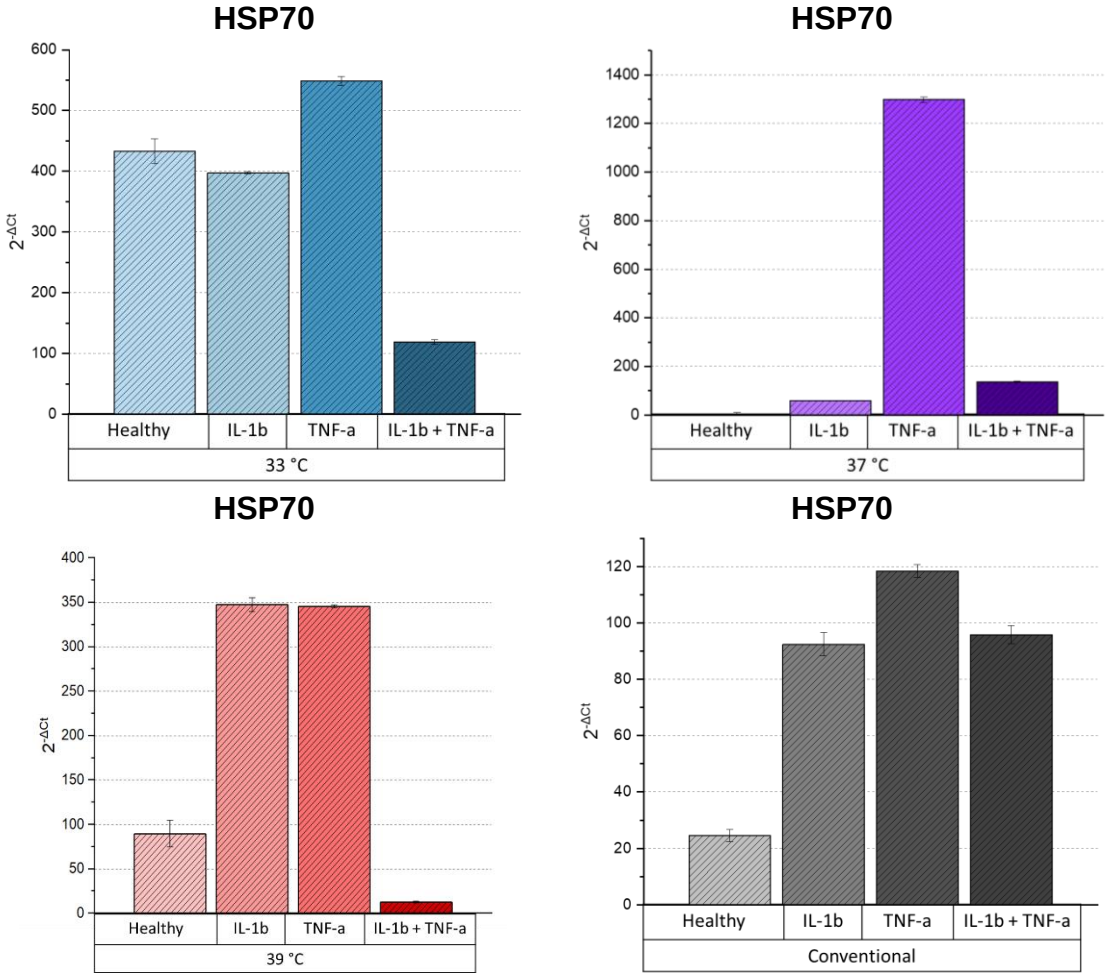


Figure 52. *Relative gene expression of HSP70 for all monolayer culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

The glycolysis-related pyruvate kinase muscle isozyme M2 (PKM2) was investigated as measure for the glycolytic metabolism, and highly expressed in both 33 °C and 37 °C hypoxic cultured chondrocytes, with respect to 39 °C and conventional cultures (**Figure 53**).

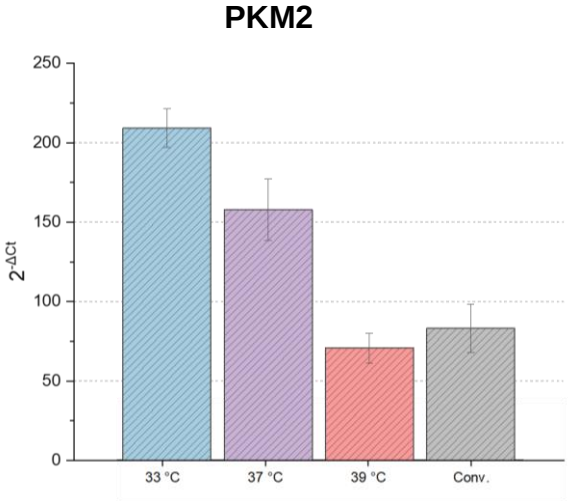
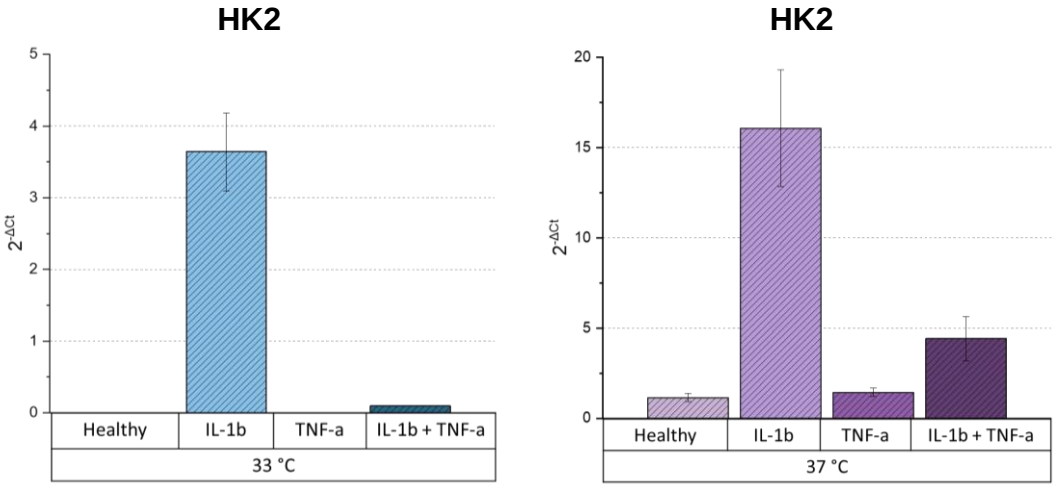


Figure 53. *Relative gene expression of PKM2 for all healthy monolayer culture conditions. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

The glycolytic metabolism was further analyzed using hexokinases (HKs), which are catalyzers of the glucose metabolism. Cytokine stimulation resulted in an increase of HK2 in all hypoxic cultures (**Figure 54**). The effects of IL-1β were highest at 33 °C and 37 °C hypoxic conditions. TNF−α did not promote the HK2 production under hypoxic circumstances.



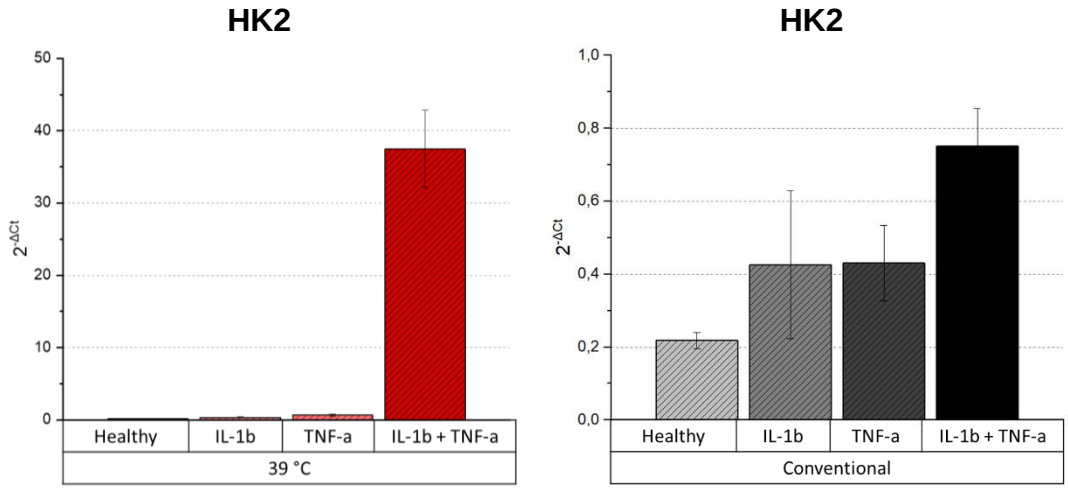


Figure 54. *Relative gene expression of HK2 for all monolayer culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

Pellet qPCR

COMP was highly expressed in 33 °C monolayer culture, but the expression was lower in 33 °C pellet cultures (**Figure 55**). When compared to the monolayer cultures, COMP was upregulated in 37 °C hypoxia and normoxia pellet systems.

At 37 °C hypoxic and conventional culture conditions, higher levels of HSP90 positive cells were found for the pellet system with respect to the monolayer (**Figure 55**). Conventional pellet chondrocytes expressed HSP90 in a higher extent compared to its monolayer. The 33 °C and 39 °C conditions showed a comparable HSP90 expression for both pellet and monolayer cultures.

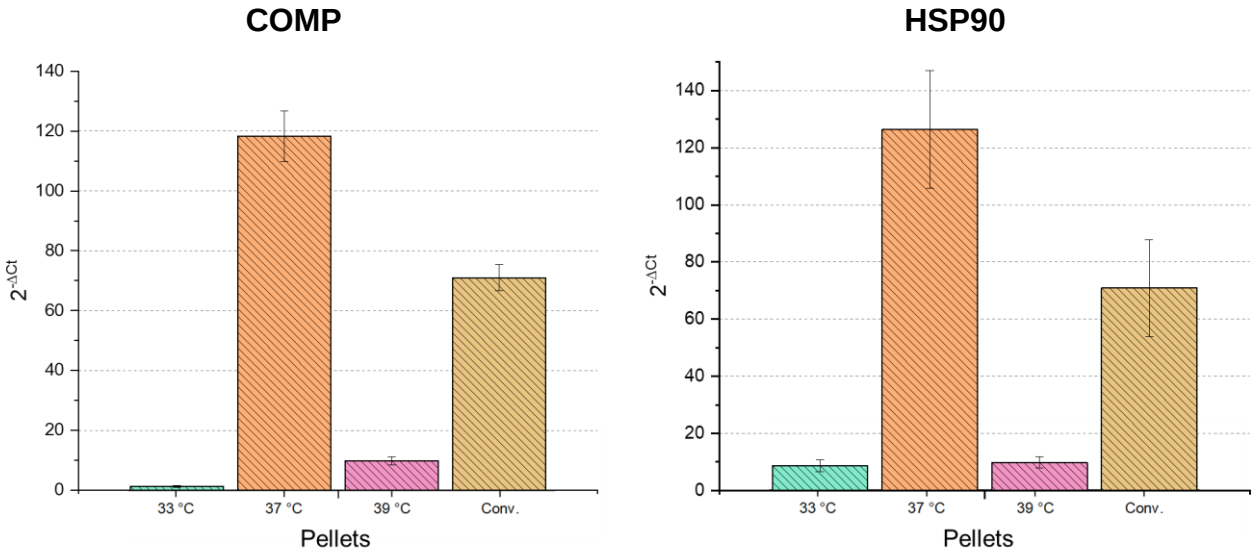


Figure 55. *Relative gene expression of COMP and HSP90 for all healthy pellet cultures. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

Collagen I was detected in all pellet culture systems (**Figure 56**). Collagen I was not expressed in 39C monolayer culture, but was elevated in the pellet chondrocytes. Transcription factor HIF-1α was highly expressed in the conventional pellet culture, suggesting that the chondrocytes experience a hypoxic environment when cultured in a pellet. However, the pellets

that were cultured in the low oxygen levels did not show high expression of HIF-1α, which was equivalent to the expression observed for the monolayer culture.

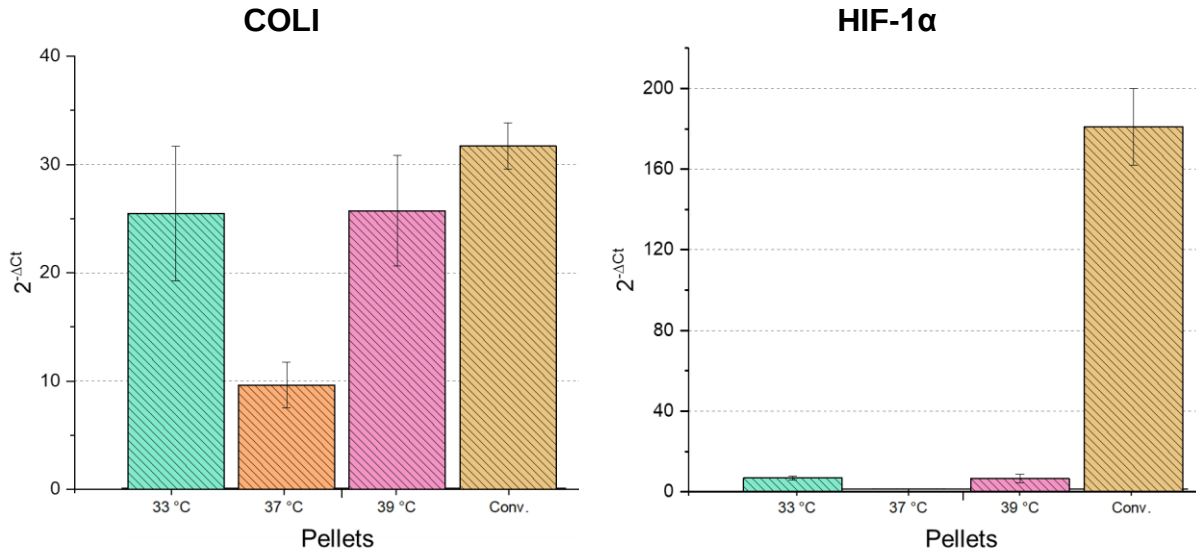


Figure 56. *Relative gene expression of Collagen I and HIF-1α for all healthy pellet cultures. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

The 37 °C hypoxic pellet culture showed upregulation of glycolysis-related genes HK2 and PKM2, which is in contrast to the monolayer culture (**Figure 57**). The other culture conditions presented a comparable expression for both genes.

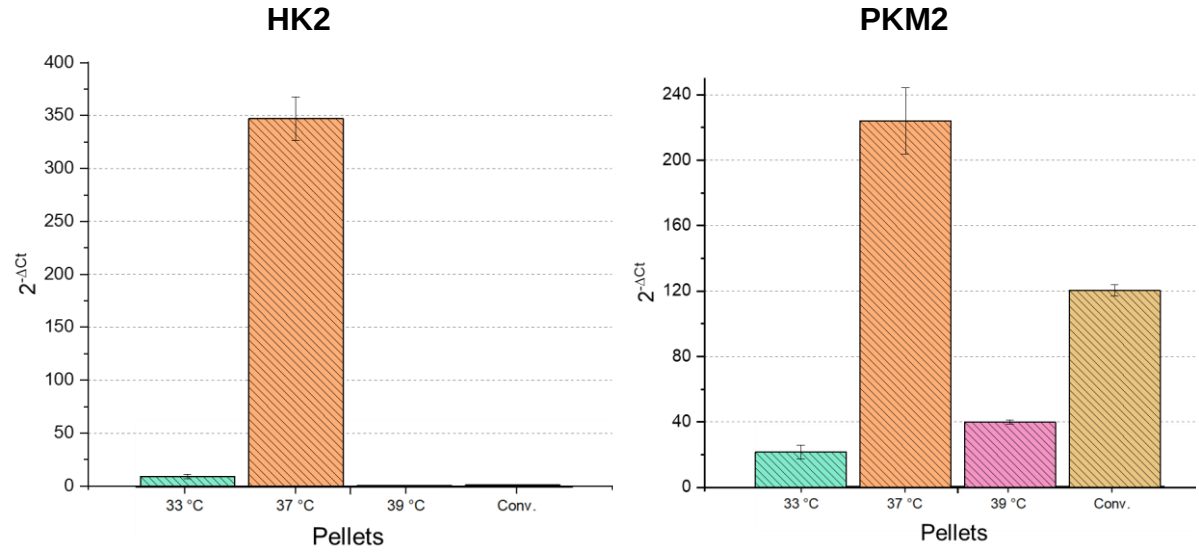


Figure 57. *Relative gene expression of HK2 and PKM2 for all healthy pellet cultures. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

The effects of cytokine stimulation on HK2 production was comparable for pellet and monolayer cultures (**Figure 58**). With respect to the cytokine conditions, the chondrocytes cultured in a healthy hypoxic environment showed low HK2 expression, which was comparable with the obtained monolayer data.

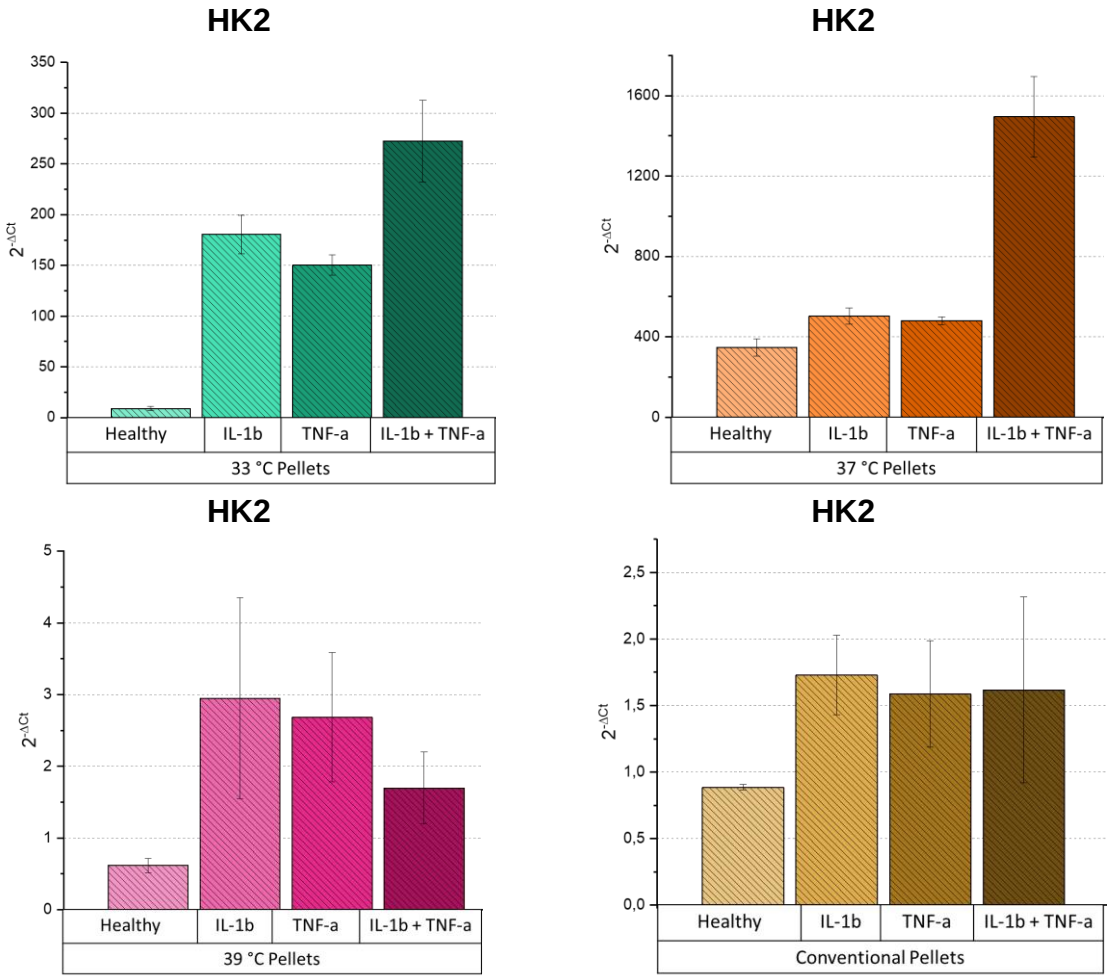


Figure 58. *Relative gene expression of HK2 for all pellet culture conditions. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

Expression of HSP70 was promoted by the separate cytokines, but in a lesser extent by the cytokine cocktail in 37 °C and 39 °C hypoxic cultured pellets (**Figure 59**). Cytokine stimulation did promote HSP production in a lesser extent for 37C hypoxic and conventional conditions, showing higher expression In the healthy conditions. In contrast to the healthy monolayer 37 °C hypoxic and conventional cultures, showing no HSP70 expression.

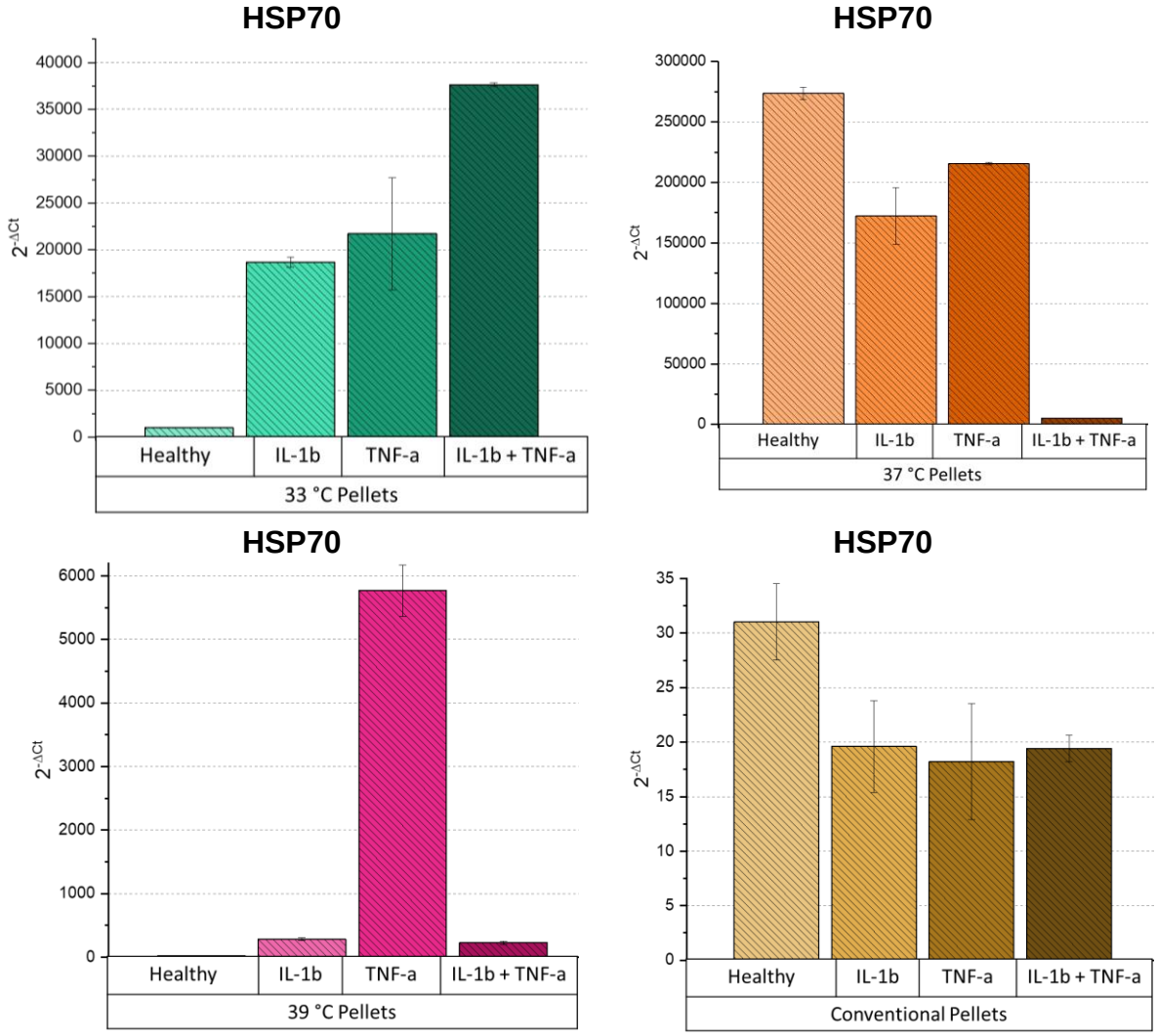


Figure 59. *Relative gene expression of HSP70 for all pellet culture conditions. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 2.*

8. Discussion

Due to the complex structure and unique characteristics of articular cartilage, research on cartilage is hindered by the lack of current *in vitro* models that fully represent the *in vivo* situation. This project evaluated the effects of hydrogel, temperature, oxygen, and cytokines on human chondrocyte *in vitro* culture. By addressing these separate factors, a representative model for healthy and diseased cartilage studies was aimed. First part of the project included testing of the Dex-TA/HA-TA (Dex/HA) hydrogel for dynamic cartilage-on-chip culture. Second part of the project included the evaluation of 33 °C (intra-articular), 37 °C (body core), and 39 °C (hyperthermic) culture under hypoxic circumstances, in both 2D and 3D human chondrocyte culture systems. Lastly, an inflammatory response was triggered by cytokine stimulation under the same temperature and oxygen conditions. In this section, all findings will be discussed and compared with other studies.

Hydrogel

Human chondrocytes behavior when cultured in 3 types of hydrogels (agarose 2%, Dex/HA 5wt%, and Dex/HA 10wt%) was evaluated for cartilage markers and degradative factors using a cartilage-on-chip platform.

Hydrogel degradation

Although biocompatible and easy to use, Dex/HA hydrogel was progressively de-touching and degrading in the system respect to agarose (**Figure 19**). To avoid this problem, surface functionalization of the PDMS was performed using a PD layer to support Dex/HA attachment. PD is a biocompatible coating that can strongly adsorb onto surfaces by covalent conjugation. The electrostatic interaction between PD and HA molecules was described by Xue et al., composing an anti-inflammatory coating for implantable devices. [85] The coating improved the hydrogel binding, resulting in nearly no hydrogel detachment from the microfluidic device. Moreover, the connection between the Dex/HA hydrogel and PDMS did not significantly alter the medium diffusion through the hydrogel as high viability was detected for chondrocytes cultured up to 11 days.

Mechanical stimulation capacities

Next, 15 μm polystyrene beads were used to determine the deformation capacities of the 3 hydrogels in the cartilage-on-chip. First, all hydrogels presented a progressive decrease in hydrogel deformation from the mechanical actuation system to the pillars, suggesting gradient-generation. For both Dex/HA hydrogels, the deformation was lower with respect to agarose, which could be related to the differences in the hydrogel architectures; agarose present a sponge-like structure (**Figure 4**); while Dex/HA gels are composed of a fiber-like network (**Figure 5**), which may affect the way of loading distribution. Further, the higher polysaccharide density and stiffness of Dex/HA 10wt% declares its different behavior with respect to Dex/HA 5wt%. The deformation experiments were not performed using human chondrocytes, but the data for the microbeads suggests that cells in agarose would be more affected by the mechanical loading, resulting in a better sense of compressive stimuli with respect to the Dex/HA hydrogels.

The gradient-generation found during the deformation experiments using microbeads could have altered the sense of loading by the cells. During this project, the cells that were exposed to mechanical loading were not distinguished for their position in the hydrogel. It would be an interesting extension to determine the differences in sensing and behavior between the cells cultured close to the membrane and the cells cultured in the deeper hydrogel section. This could

be achieved by using immunofluorescence staining for chondrocyte specific markers (ColII, ACAN) and anabolic markers (MMPs), or by careful cutting the separate hydrogel sections for gene expression analysis.

Cell movement

Within native cartilage tissue, chondrocytes show mobility under various stimuli, e.g. growth factors or due to a tissue injury. Various research have aimed to mimic the chondrocyte motility *in vitro,* aiming to mimic the highly controlled and specialized movements. [13] Mobility of our Dex/HA chondrocytes was noticed in both static and compressive conditions, in both 5wt% and 10wt% hydrogels (**Figure 27 and 28**). Research has shown that chemoattractants, including hyaluronic acid, can stimulate the migration of chondrocytes. [72] Dex/HA hydrogel provides cell attachment sites due to the presence of biodegradable HA. [50] One plausible reason for cell motility is the activation of the catabolic hyaluronidases from the chondrocytes which could progressively degrade the HA. Besides, Dex/HA chondrocytes produced high levels of MMPs (mRNA and protein level) and ADAMTSs (mRNA level), again demonstrating that the cells were catabolic active. Since MMPs are expressed and secreted in inactive form, it needs to be further elaborated whether the degradative factors were active and degrading the hydrogel. An interesting investigation would be to determine the amount of released HA over time in the cell culture supernatant, by using for example Westernblot, a turbidity assay or Tengblad assay. [86][87][88]

The noticed cell motility in the Dex/HA static cultures could be due to the properties and architecture of the hydrogel, enabling the cells to move through the different planes of the hydrogel. The higher density of Dex/HA 10wt% hydrogel results in a smaller mesh size with smaller pores with respect to Dex/HA 5wt%, according to the images obtained with SEM. A lower hydrogel density, resulting in a greater mesh size, makes it easier for chondrocytes to migrate and proper remodel the ECM. [89] Furthermore, SEM data has shown integration of microbeads in both Dex/HA hydrogels (**Figure 18-C**), but it was not performed for chondrocytes and agarose hydrogel. In addition, the droplet culture suggested that cells were starting to attach to Dex/HA hydrogel, differently from the appearance shown in agarose (**Appendix 12.4.8, Figure S.8**). Further elaboration on the integration of chondrocytes in the hydrogel network will be interesting and changes in membrane structure could give information about movement directionality, and could be investigated by tracking the formation of lamellipodia and filopodia, which are cytoskeletal proteins involved in cell migration. [17]

Our data shows migration of chondrocytes in static and dynamic conditions for both Dex/HA hydrogels with a directionality prevalence for the systems mechanically addressed. In the deep zones of the hydrogel section, close to the pillars, higher cell displacement was seen, with respect to the upper zone, close to the membrane (**Figure 27**). This could be due to the applied pressure, creating an attraction to the point of mechanical stimulation. Determining how the pressure affects the cell motility could be achieved by using a cartilage-on-chip device containing 3 mechanical stimulation chambers, which can be stimulated separately. By only applying pressure on the most right chamber, it should be hypothesized that the cells will move to the upper right corner. Further, the differences in displacement could be due to the removal of non-crosslinked polymers by medium injection, resulting in the hydrogel to become more soft, making it easier for cells to move in a greater extent. However, experiments using microbeads, following the same protocol, did not show movement under compression loading and daily medium application. Since the qPCR data suggests that the movement is related to the production of degradative enzymes, the bonds of the hydrogel network may be cut. The

degraded polymer parts in the deep zone of the hydrogel may thereafter be flushed away by medium change.

Multiple *in vivo* models showed the migration of cells to sites of extensive chondrocyte death or injury sites for tissue regeneration and repair. [90][91][92] *In vitro* cultured OA cartilage slices synthesized high levels of insulin growth factor-1 (IGF-1). IGF-1 is a chemoattractive factor released in traumatized cartilage, and induces a migratory response. In contrast, when OA tissue stimulation using IL-1β and TNF−α did negatively affect the cell migration, declaring the low regenerative potential of OA cartilage tissue. Measuring the gene expression of IGF-1 would provide information about the cause of cell motility; high levels of IGF-1 would suggest that the chondrocytes are sensing an injury-like stress. If the mobility is not, or in a lesser extent, seen after cytokine stimulation, this hypothesis would be confirmed. [92][93][94] However, according to gene expression of chondrogenic specific genes, it is not suspected that diseased chondrocytes were cultured in our Dex/HA hydrogel. More likely, the cell motility is related to the catabolic activity of the chondrocytes, but further research is required.

Cell behavior

Agarose 2%, Dex/HA 5wt% and Dex/HA 10wt% hydrogels were examined for human chondrocyte culture in both static and compressed conditions. The cell behavior was evaluated on viability, gene expression, and synthesis of cytokines and enzymes. The findings of chondrogenic-specific genes SOX9, ACAN, and PRG4 for both agarose cultures suggested the maintenance of the chondrogenic phenotype under mechanical loading (**Figure 33**), suggesting the avoiding of dedifferentiation. [95][96] Further, the 3 genes were expressed in all Dex/HA 5wt% conditions. For Dex/HA 5wt%, the expression of PRG4 was higher in static culture in comparison with its compression, questioning the distribution of compression load to the chondrocytes in Dex/HA 5wt%. The expression of SOX9 was significant comparable for both Dex/HA 5wt% and 10wt% static and compression conditions, suggesting a low influence of mechanical stimulation on SOX9 expression for Dex/HA chondrocytes. Further, research has shown that mechanical stimulation can improve the proliferation of chondrocytes. [95] It is known that cells do not proliferate when cultured in agarose. [37] Evaluating the proliferation capacities of Dex/HA chondrocytes should be considered, for example using an activity assay or by evaluating glycolysis-related factors at mRNA or protein level.

We noticed elevated levels of IL-6 in Dex/HA 5wt% static cultured chondrocytes, with respect to Dex/HA 5wt% compression condition and Dex/HA 10wt% and agarose cultures. Therefore, it was considered that the material choice can trigger the IL-6 synthesis. IL-6 measurements were performed using both SPR (protein level) and qPCR (mRNA level), showing both comparable trends between the conditions, suggesting that the expression at gene level is translated to the actual protein synthesis. Furthermore, highest overall MMP activity was measured for Dex/HA static cultured chondrocytes. The positive correlation between IL-6 and MMP synthesis in OA tissue was widely investigated, but was not seen in our data, suggesting that the synthesis of both was promoted by an external effect; e.g. the fibrous network of Dex/HA hydrogel. [97][98][99]

In addition, elevated levels of catabolic factors ADAMTSs and HYALs were determined in Dex/HA 5wt% static and compression cultures and Dex/HA 10wt% compression culture. The influence of hydrogel structure on chondrocytes culture was described in earlier research using PEG hydrogels. They proved that when cultured in a high density PEG hydrogel, catabolic factors, including MMP-1 and MMP-13, were highly elevated, with respect to lower hydrogel densities. It was suggested that the cells needed to break down the surrounding matrix to create

space for the formation of new matrix. [100][101] Relating this to the expression of both anabolic and catabolic factors by our Dex/HA chondrocytes, suggests that the chondrocytes are trying to remodel the scaffold. Higher catabolic activity in combination with a low degree of ECM deposition will result in softening of the matrix, altering the deformation capacities, influencing the cellular behavior, and the capacity for cell migration. [102] Hence, it would be of interest to determine the matrix production by staining the collagens, or to determine the GAG production over time using a GAG assay, a Safranin-O or DMMB dye, and determine the Collagen deposition using immunohistochemistry. [103][104] Furthermore, long-term experiments are required to investigate the long-term progress of cytokine and MMP synthesis and will provide a better understanding on the cell behavior and matrix production and degradation.

When comparing Dex/HA 5wt% and 10wt% hydrogels, Dex/HA 5wt% showed better deformation capacities and higher expression of chondrogenic-specific genes. Further differences rely on the expression of degradative enzymes, whereas ADAMTSs were higher expressed in Dex/HA 5wt%, presence of hyaluronidases was comparable, and MMP activity was elevated in Dex/HA 10wt%. It is important to determine whether the anabolic and catabolic factors are in balance before validating the hydrogel choice.

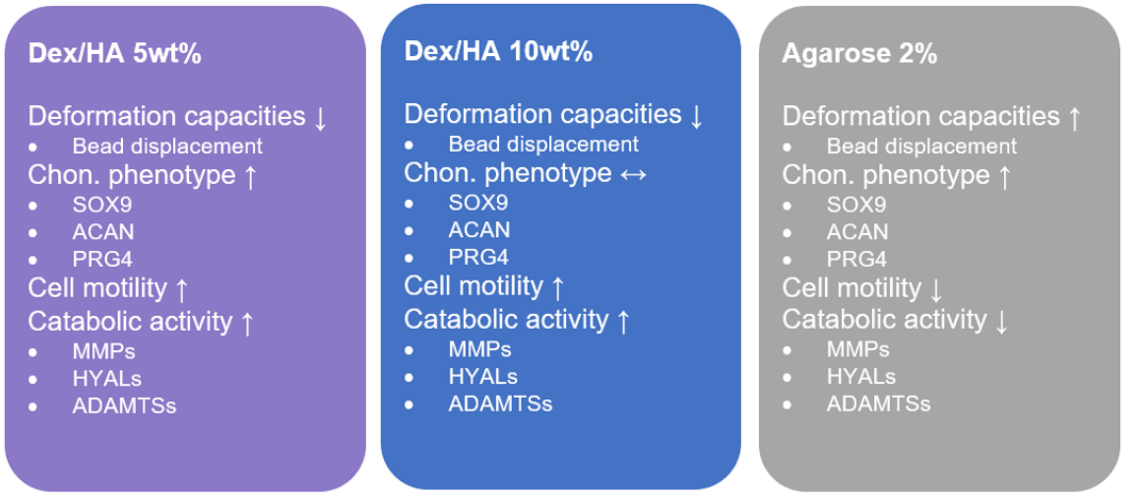


Figure 60. *Overview of Dex/HA 5wt%, Dex/HA 10wt%, and agarose hydrogel behavior. ↑: upregulation, ↔: comparable response, ↓: downregulation/no expression with respect to the other conditions.*

Influence of oxygen and temperature on chondrocyte culture

Hypoxia effects

Human chondrocytes are able to live in a hypoxic environment, since the actual oxygen levels in cartilage tissue are low; from 6% in the superficial zone; to 1% in the deep zone. [63] The behavior of chondrocytes in a hypoxic culture (2,5% O_2) was determined and compared to the conventional culture (20% O_2).

Earlier research described the production of HIF-1α in a hypoxic environment, suggesting to enhance HSP70 and HSP90 synthesis, stimulate anabolic pathways, and reduce differentiation towards a hypertrophic phenotype. [69][105] When comparing the expression of chondrocytes cultured in hypoxia and normoxia conditions, we found upregulation of HIF-1α in hypoxic cultures, but a low expression of HSP70. The exact mechanism of HSP70 regulation by HIF-1α under hypoxic circumstances is unclear, their correlation and relation to the cartilage-specific genes should be further investigated. [60][63][68][69] Next, the promotion of the differentiated phenotype in hypoxia does not fully correlate to the SOX9 expression of our

chondrocytes. Further investigations on chondrocyte-specific genes (ColII, ACAN, PRG4, ACAN) and hypertrophic-related genes (ColX) are necessary to determine the state of differentiation of the chondrocytes in a hypoxic environment. [105]
In addition, according to the PrestoBlue data and PKM2 gene expression, the cells were fully active and remained their energy homeostasis at low oxygen tensions. Research proved that the metabolic activity of chondrocytes in hypoxia is promoted by HSP70 synthesis. However, HSP70 was not expressed in our hypoxic chondrocytes, suggesting that the cell activity was initiated via another pathway. [63] The metabolic activity of chondrocytes could be further determined by measuring the glucose concentrations in culture medium, the ATP production, or by quantifying adenine nucleotides. [64][106][107]

Temperature effects

Another important characteristic of healthy knee cartilage tissue is its low temperature (32,6 ˚C), which can increase due to high intensity movements and disease. [53] We investigated the effects of a hypothermic (33 °C) and hyperthermic (39 °C) environment on human chondrocyte culture, in comparison to the conventional culture temperature (37 °C). It should be mentioned that the temperature experiments were performed at low oxygen tension (2,5% O_2). Therefore, the obtained data might be affected by both temperature and oxygen. To determine which cellular behavior is due to temperature, due to oxygen tension, or due to amplification of both, additional experiments are required. We noticed that HIF-1α, the transcription factor essential for chondrocyte survival in hypoxic circumstances, was temperature dependent and only synthesized in a small extent at 33 °C and 39 °C, but highly expressed in 37 °C hypoxic culture.

According to our data, the temperature influenced the cell viability and metabolic activity, by promoting cell death at 39 °C monolayer culture, which confirms that a high temperature environment promotes cartilage destruction. [62] It should be considered that the difference in cell viability at 39 °C affects the metabolic activity measurements. Further, the metabolic activity was altered at 33 °C, and 37 °C culture was most suitable for the cellular activity according PrestoBlue data. An impaired glucose metabolism can result in chondrocyte dedifferentiation, which is in line with our obtained gene expression for 39 °C culture; no expression of both SOX9 and COMP genes, but high expression of Collagen I. [108][109] This suggests that the chondroprotective origin of HSPs was not fully operating at 39 °C, but only at lowered temperature (33 °C). [60][61] The expression of chondrocyte-related genes (SOX9 and COMP) were upregulated at low temperature, suggesting that the activity of HSPs help restoring the cartilage homeostasis.

It should be noted that GAPDH was considered as housekeeping gene for all temperature and oxygen related experiments. Since GAPDH is a metabolic expression-related gene, which is temperature and oxygen dependent, it was not reliable as stable reference gene. High GAPDH expression for 33 °C chondrocytes did influence the ΔCT values, indicating overall a lower expression for the measured genes (**Figure S.16**). However, the overall progress between the multiple conditions was considered sufficient. Beta2-microglobulin (B2M), human RNA polymerase II (RPII) Ribosomal protein L13a (RPL13A) were described as reliable and stable housekeeping genes under hypoxic circumstances for chondrocyte culture, and should be considered for future experiments. [35][110]

All chondrocytes were initially cultured in a conventional incubator, exposed to 20% O_2 tension at 37 °C degrees, and shifted to the temperature of interest at the start of the experiment. The change in culture environment could trigger a stress response, resulting in synthesis of HSPs at 33 °C and 39 °C. It should be considered that an experiment with a duration of 3 days is not

adequate for chondrocytes to completely adapt to the culture environment and it is recommended to repeat the experiments by directly culturing them at the condition of interest. Besides, having the cells in culture for a longer period of time will give better insights about actual protein production, by detecting their presence using, for example, (immuno)histochemistry staining. [62][111][112]

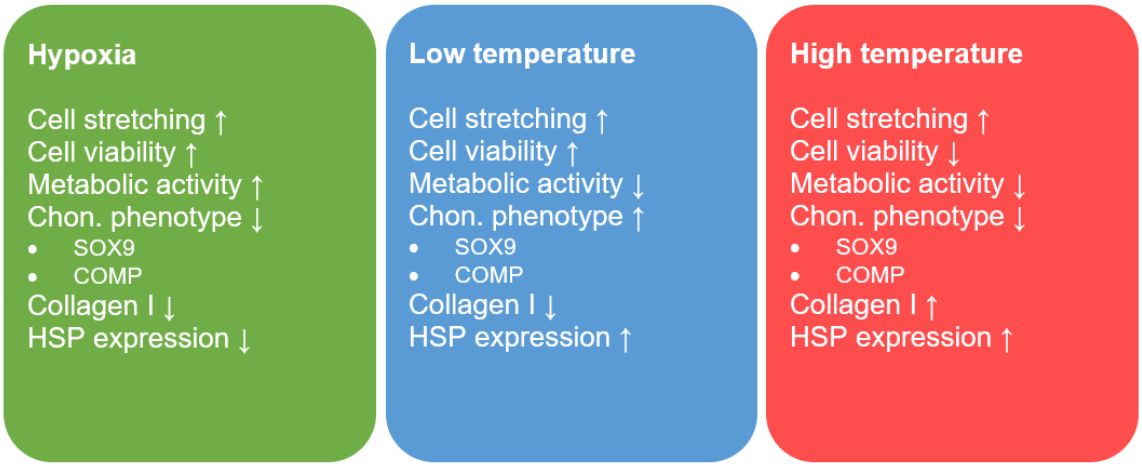


Figure 61. *Overview of hypoxia, and low and high temperature effects on chondrocyte monolayer culture. ↑: upregulation, ↓: downregulation/no expression with respect to conventional culture.*

Cytokine effects

IL-1β and TNF−α are the major pro-inflammatory cytokines present in OA tissue. Both cytokines have a catabolic effect, inducing the synthesis of cartilage degrading enzymes and other pro-inflammatory mediators, resulting in cartilage degeneration. [28][113][114] Stimulation with pro-inflammatory cytokines IL-1β and TNF−α was proposed to trigger an inflammatory response in human chondrocytes, and their effects were evaluated for conventional cultures, and low and high temperatures under hypoxic circumstances. First, the influence of the cytokines will be discussed for the chondrocytes cultured in conventional culture (37 °C, 20% O_2).

The cytokines did not influence the viability, but an increase in metabolic activity was found by both PrestoBlue and HK2 expression. HK2 was expressed in arthritis, and suggested to play a role in the regulation of pro-inflammatory mechanisms, disruption of cell proliferation and provoking cartilage degradation. [115][116] Since the pro-inflammatory cytokines IL-1β and TNF−α are associated with cartilage degradation by stimulation of the synthesis and activity of degradative enzymes, future research should include the determination of catabolic factors, e.g. ADAMTSs and MMPs. The degradative properties of the cytokines initiate a reduce of the proteoglycan content in cartilage tissue. [117] Again, a Safranin-O staining or GAG assay would provide information about the effectivity of cartilage degeneration by IL-1β and TNF−α stimulation. To determine the development and degeneration of the cartilage under stimulation of the cytokines, determining the presence of components of the WNT signaling pathway is an interesting addition. [113]

IL-1β and TNF−α are known to induce the loss of the chondrogenic phenotype, resulting in stimulation of hypertrophy and the initiation of a fibroblast-like dedifferentiation. On mRNA level, we determined upregulation of Collagen I under cytokine stimulation (**Appendix 12.5.7**). [118] However, more data regarding other markers for chondrogenic phenotype (ColII, ACAN, SOX9) and hypertrophy (ColX) are necessary to determine the state of differentiation. Earlier research already described that cytokines IL-1β and TNF−α are associated with the synthesis

of HSP70, e.g. in OA patients and in *in vitro* cultures. [79][119][120][121][122] In our conventional monolayer culture, cytokine stimulation resulted in an increased production of HSPs, confirming that the chondrocytes were conditioned under non-physiological stress.

Our data suggested that the influence of the cytokines is oxygen and temperature related. Cell viability was altered under stimulation in the hypoxic cultures. Further, the synthesis of HSP under cytokine stimulation was elevated at 33 °C. For 37 °C and 39 °C hypoxic cultures, Collagen I was highly upregulated under cytokine stimulation, with respect to the conventional cultures.

Monolayer culture

To investigate the effects of temperature, oxygen tension, and cytokine stimulation on human chondrocyte culture, the cells were cultured in a 2D system. Cell stretching was highly affected under low oxygen tension, resulting in an increase of contact surface area (**Figure 40**). The cell area was further increased at high temperature (39 °C) and was necessary for the maintenance of chondrocyte functionality. [123] Under stimulation of TNF−α and cytokine cocktail, the cell stretching was further enhanced, suggesting that the morphology of the cells is affected by TNF−α, temperature, and oxygen. The influence of inflammatory cytokines on cell morphology was described by Joos et al., showing a comparable change in cell morphology and cytoskeleton organization. [124] During this project, the seeding density for monolayer experiments was low, which could have influenced the behavior and adhesion of the cells due to low cell-cell contact. [125][126] However, we used the same seeding density for the conventional control culture, demonstrating a clear difference with respect to hypoxic cultured chondrocytes.

Pellet culture

Cell-cell interactions are important to create a culture model that is close to the *in vivo* situation. Therefore the human chondrocytes were cultured in a pellet culture, and examined for temperature, oxygen, and cytokine influence.

The formation of pellets was initiated by cell centrifuging at day 0, this new arrangement could have led to expression of HSP70 in the healthy conditions. HSP synthesis was stimulated under influence of inflammatory cytokines, which may have influenced the dis-aggregation of cytokine stimulated pellets over time. The de-clustering of the pellets was further enhanced under influence of both hypoxia and temperature (**Figure 42**). To explore what specific did affect the de-clustering, experiments only considering temperature, oxygen, or cytokines are required.

In chondrocyte 3D cultures the chondrogenic differentiated phenotype is stabilized by i.a. Cartilage Oligomeric Matrix Protein (COMP) expression, as shown in our 37 °C hypoxia and normoxia pellet cultures, with upregulation of COMP in comparison to the monolayer conditions. [127][128] Healthy pellets cultured at 33 °C presented the biggest perimeter after 3 days of culture. However, the metabolic activity of these chondrocytes was lowest, with respect to the other culture environments. Since the enhanced pellet size was presumably not due to cell proliferation, determining the synthesis of matrix components will give important insights on the pellet formation. According to earlier research, a culture temperature between 32 °C and 37 °C is optimal for extracellular matrix production by chondrocytes. [129] Our data indicated an increase in pellet size for 33 °C and 37 °C hypoxic cultures, suggesting the production of ECM components causing an increase in pellet size. To assess the matrix production, insights on pellet composition could be given using qPCR (for Collagen II and Aggrecan); GAG assay; (immuno)histochemistry staining for Collagen II and GAGs.

9. Conclusion

In this project, the potential of a hydrogel composed of dextran-tyramine and hyaluronic acid-tyramine (Dex/HA) was tested for a dynamic cartilage-on-a-chip platform and compared to the currently used agarose hydrogel. Dex/HA chondrocytes resulted in great survival rates, cell morphology, and the expression of chondrocyte-specific markers. In Dex/HA hydrogels the mechanical loading was distributed in a lower extent with respect to agarose. In addition, Dex/HA culture was associated with increased levels of matrix-degrading enzymes. Present findings indicated cell motility in both static and dynamic cultures, but the movement has to be further investigated, to determine what environmental factors are causing this motility. Altogether, the use of Dex/HA 5wt% hydrogel offer a better representation of native cartilage, to study cell motility and both anabolic and catabolic processes, but for now, agarose offers a more reliable culture environment.

Secondly, the effects of temperature and oxygen level on *in vitro* chondrocyte culture were determined. Our results suggested that a culture temperature of 39 °C at 2,5% O_2 resulted in impaired metabolic activity, dedifferentiation, and high levels of cell death. Low temperature (33 °C), which is considered as the normal intra-articular temperature, resulted in maintenance of the chondrogenic phenotype, high metabolic activity and high viability. In addition, our data indicated that when cultured in 37 °C hypoxic environment, the cells retained their phenotype, induce cartilage development, and did not express a stress response. Taken together, the optimal culture condition for chondrocytes may be at low oxygen tension and a culture temperature between 33 °C and 37 °C.

The effects of cytokine administration on chondrocytes culture suggested that their influence is temperature and oxygen related and the highest effect was obtained in 33 °C and 37 °C hypoxic cultures, which offer insights for disease modelling. Cytokine administration resulted in enhanced cell contact area, metabolic activity and HSP and hexokinase production. Additional studies are required to better define the activation of matrix destruction and evaluate the complete inflammatory response.

10. Future perspectives

In this study, several steps were taken to investigate the effects of the culture environment on chondrocytes, including hydrogel, temperature, oxygen, and pro-inflammatory cytokine administration. The use of a hydrogel that more closely mimics the native cartilage environment offers new possibilities to study the behavior of chondrocytes under different circumstances. Examining the matrix metabolism in both Dex/HA 5wt% and Dex/HA 10wt% is important to completely understand the cell motility and expression of degradative factors to determine if there is a balance between catabolic and anabolic factors. To obtain a hydrogel matrix that closely mimics the native cartilage structure, further improvements include the development of a hybrid hydrogel of Dex/HA with decellularized cartilage ECM, containing TA moieties.

By adjustment of temperature and oxygen conditions, a more realistic cartilage model can be created, making the system attractive for different cartilage research purposes. However, to determine the influence of a hyperthermic and hypothermic culture environment, it is required to repeat all experiments under normoxia circumstances. To further improve the temperature experiments, it is important that the cells are initially directly cultured at the temperature of interest, to avoid cell adaptation to conventional circumstances. Lastly, it should be considered that in this study only cells obtained from one individual were used. To generalize the findings, enlarged studies are required.

The use of a hydrogel that more closely mimics the native cartilage environment offers new possibilities to study the behavior of chondrocytes under different circumstances. By application of pro-inflammatory cytokines to the cartilage-on-chip culture, a combination of biochemical and mechanical stimuli can be proposed to offer a convenient platform for the study of cartilage degradation, diseases, drug development, and high-throughput drug screening.

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12. Appendix

12.1. Medium composition

Table S.1. *Medium composition for chondrocyte proliferation medium (CPM).*

DMEM	500 mL
FBS 10%	60 mL
Ascorbic acid 2-phosphate	5,8 mL
Penicillin-Streptomycin	5,8 mL
Non-essential amino acid	5,8 mL
Proline	5,8 mL

Table S.2. *Medium composition for chondrocyte differentiation medium (CDM).*

DMEM	500 mL
Penicillin-Streptomycin	1 mL
Insulin-Transferrin-Selenium mix	1 mL
Proline	1 mL
Ascorbic acid 2-phosphate	1 mL
Sodium Pyruvate	1 mL
TGF-**β**	250 uL
Dexamethasone	500 uL

12.2. Primer sequences for qPCR

Table S.3. *Primer sequences used for qPCR.*

	Forward	**Reverse**
GAPDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT
ACTB	ACTCTTCCAGCCTTCCTTC	GATGTCCACGTCACACTTC
ColIaI	GTCACCCACCGACCAAGAAACC	AAGTCCAGGCTGTCCAGGGATG
ColIIaI	CGTCCAGATGACCTTCCTACG	TGAGCAGGGCCTTCTTGAG
ColXaI	GCAACTAAGGGCCTCAATGG	CTCAGGCATGACTGCTTGAC
SOX9	TGGGCAAGCTCTGGAGACTTC	ATCCGGGTGGTCCTTCTTGTG
COMP	CAACTGTCCCCAGAAGAGCAA	TGGTAGCCAAAGATGATGAAGCCC
PRG4	GATGCAGGGTACCCCAAA	CAGACTTTGGATAAGGTCTGCC
ACAN	AGGCAGCGTGATCCTTACC	GGCCTCTCCAGTCTCATTCTC
FHL2	GGTACCCGCAAGATGGAGTA	TTTGGGGATGAAACTCTTGG
IL-6	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTCATTGAATCC
IL-1β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
Ki-67	CTACGGATTATACCTGGCCTTCC	AGGAAGCTGGATACGGATGTCA
MMP-1	CCAGCAGGAGCAGTTGGTCTT	GGGCCTGGTTGAAAAGCAT
MMP-3	TGGCATTCAGTCCCTCTATGG	AGGACAAAGCAGGATCACAGTT
MMP-9	GGTGATTGACGACGCCTTTGC	CCAGCAGGAGCAGTTGGTCTTC
MMP-13	AAGGAGCATGGCGACTTCT	TGGCCCAGGAGGAAAAGC
ADAMTS-4	CAAGGTCCCATGTGCAACGT	CATCTGCCACCACCAGTGTCT
ADAMTS-5	TGGCTCACGAAATCGGACA	GGAACCAAAGGTCTCTTCACAGA
HYAL-1	CCAGAATGCCAGCCTGATGCC	GTCATTTTGGGCACGGATGCC
HYAL-2	GGCACAATATGAGTTTGAGTTCGC	TTGAGGTACTGGCAGGTCTCCG
HYAL-3	CATTTTCTACAAGAACCAACTCGGCC	CCAATGCAGTTGAGTGTTGCGG
HIF1a	TGCTCATCAGTTGCCACTTC	TGGGCCATTTCTGTGTGTAA
GDF10	AAGACCGCAAGAAGAAAGACCA	TCCAGCACAGTAGTAGGCATCAA
GLUT1	GGTTGTGCCATACTCATGACC	CAGATAGGACATCCAGGGTAGC
HK2	TCCCCTGCCACCAGACTA	TGGACTTGAATCCCTTGGTC
PKM2	CGTCTGAACTTCTCTCATGGAA	ATGGGGTCAGAAGCAAAGC
FN1	AAACTTGCATCTGGAGGCAAACCC	AGCTCTGATCAGCATGGACCACTT

12.3. Requirements chondrocyte *in vitro* model

A model for chondrocyte *in vitro* culture requires to mimic the unique characteristics of articular cartilage tissue, including its structure, physiological conditions, and dynamic environment (**Table S.4**). [33]

Table S.4. *Requirements cartilage in vitro model, healthy and diseased.*

Functionalities	Specification
Healthy model	
3D chondrocyte culture	Hydrogel, pellet or organoid, with ability to migrate
Native-like environment	ECM components; natural or synthetic
Nutrient supply	Through diffusion
Mechanical stimulation	Physiological loading
Physiological culture conditions	Temperature and oxygen
Disease model	
Mechanical stimulation	Hyper physiological loading
Inflammation	Application of inflammatory cytokines
Drug testing	Application of drugs

12.4. Additional data: Hydrogel testing

12.4.1. SEM images

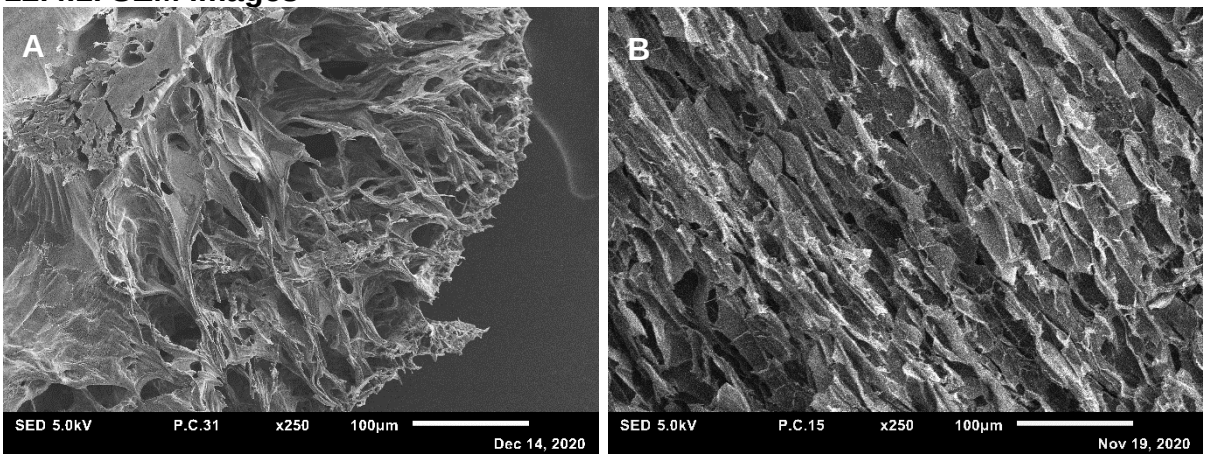

Figure S.1. *Addition SEM images of Dex/HA 5wt% (A) and Dex/HA 10wt% (B), performed by Malin Becker.*

12.4.2. Crosslinking

Hydrophobic crosslinked tyramine moieties were stained red using ethidium homodimer (**Figure S.2**). Hydrogel showed no dark spots, indicating evenly distributed crosslinking of the gel. The staining was not performed for agarose and non-crosslinked gel, therefore the staining cannot be compared with a negative control.

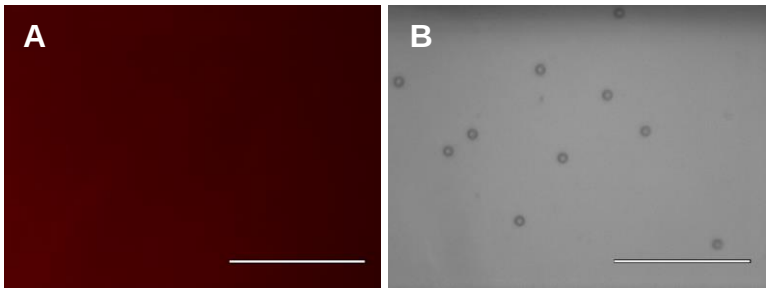

Figure S.2. *Crosslinking of Dex/HA 10wt% hydrogel with 15 µm beads embedded, using ethidium homodimer for staining of crosslinked tyramines (A) and the transmitted image of the same section (B). Scalebars: 200 µm.*

12.4.3. Hydrogel characterization

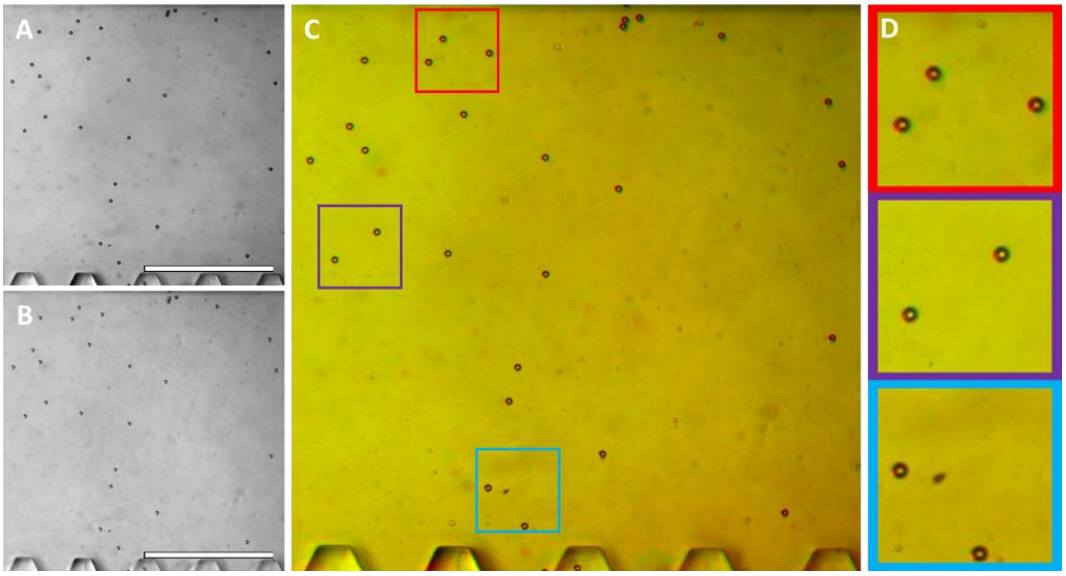

Figure S.3. *Bright-field images of Dex/HA 5wt% at day 0 (A) and day 3 (B) for the compression condition. C) Overlay for day 0 (in green) and day 3 (in red). D) Higher magnification for three zones. Scalebars: 250 µm.*

12.4.4. Hydrogel deformation without PD

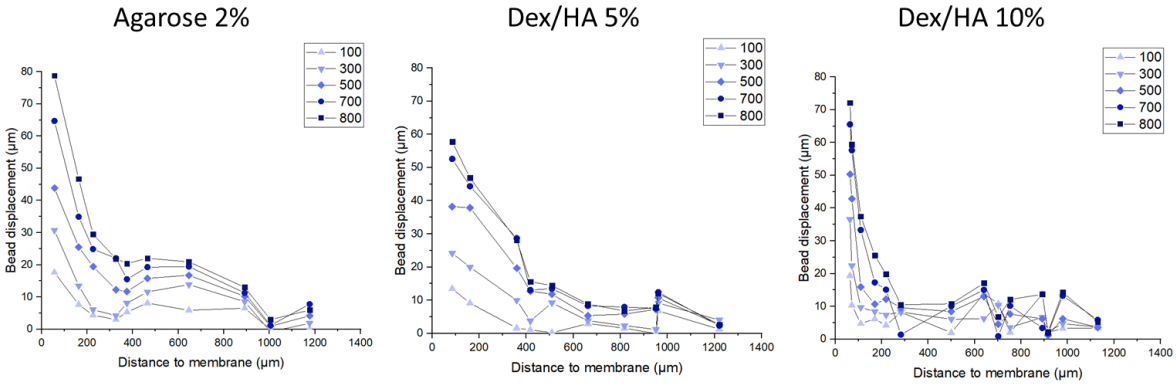

Figure S.4*. Deformation of agarose, Dex/HA 5wt% and Dex/HA 10wt%. Microbead displacement was measured at 0, 100, 300, 500, 700 and 800 mbar. The displacement of multiple microbeads was measured with respect to the membrane.*

12.4.5. Hydrogel viability without PD

Viability for chondrocytes cultured in Dex/HA hydrogel in devices without PD treatment was evaluated. LIVE/DEAD staining can be seen in **Figure S.5**, for both hydrogels in static condition at day 11. Dex/HA 10w/t% conditions shows degradation of the hydrogel.

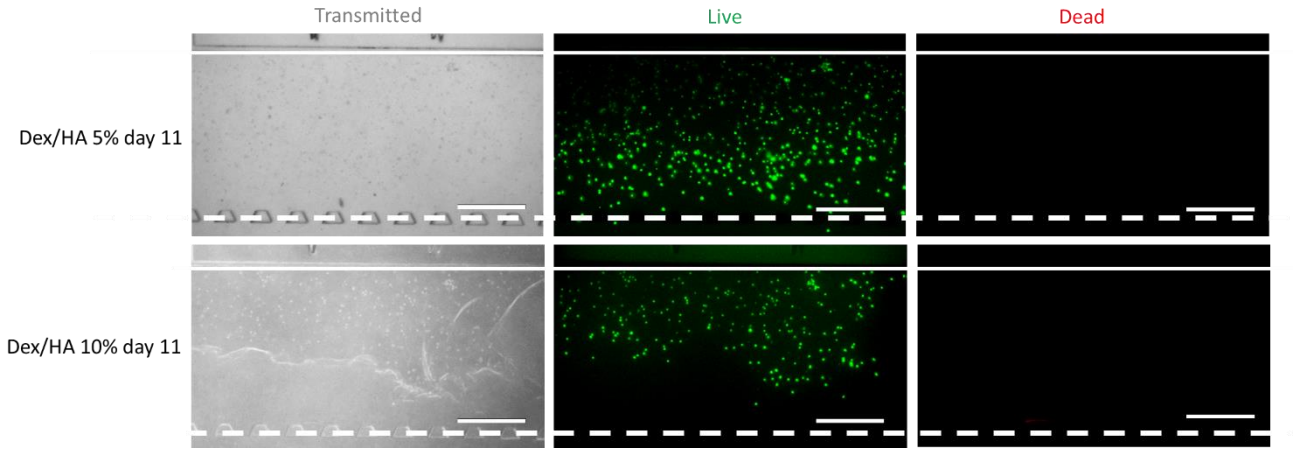

***Figure S.5**. LIVE/DEAD staining for Dex/HA 5wt% and Dex/HA 10wt% at day 11 static conditions, with living cells stained in green and dead cells stained red. Scalebars: 500 µm.*

12.4.6. Cell deformation

Deformation of chondrocytes under stimulation of 350 mbar was determined by comparing the bright-field image to its static condition (0 mbar), as shown in **Figure S.6** for Dex/HA 5wt%. The circular shaped cells close to the membrane were deformed to an oval-shape under mechanical compression (yellow). This effect was not seen in the hydrogel section more close to the pillars, where the cells remained their circular shape (red).

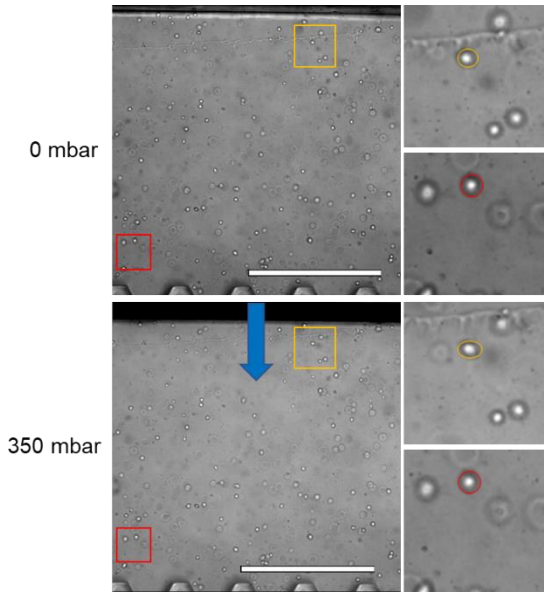

***Figure S.6.** Shape of chondrocytes in Dex/HA 5wt% hydrogel in static condition (0 mbar) versus compression (350 mbar). The direction of the stimulation is indicated with the blue arrow. Scalebars: 250 µm.*

12.4.7. Statistical analysis qPCR

The statistical significance for all gene expression data was determined using one-way ANOVA and post hoc Tukey test, with significances $p < 0,1$ (*), $p < 0,05$ (**), $p < 0,01$ (***), and $p < 0,001$ (****), and listed in **Table S.5**.

Table S.5. *Statistical analysis for all hydrogel conditions and measured gene expression, for α = 0,001, 0,01, 0,05, and 0,1, ns: no significance.*

		SOX9	ACAN	PRG4	IL-6	MMP-1	MMP-3	MMP-13	HYAL-1	HYAL-3	ADAMTS4	ADAMTS5
		α	α	α	α	α	α	α	α	α	α	α
Dex/HA 5 compression	Dex/HA 5 static	ns	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,05	0,001
Dex/HA 10 static	Dex/HA 5 static	0,001	0,05	0,001	0,001	ns	0,001	0,001	0,001	0,001	0,001	0,001
Dex/HA 10 static	Dex/HA 5 compression	0,01	0,001	ns	0,01	0,001	0,001	0,001	0,001	0,001	0,001	0,1
Dex/HA 10 compression	Dex/HA 5 static	0,001	0,001	ns	0,001	0,001	0,001	0,001	0,001	0,001	ns	0,001
Dex/HA 10 compression	Dex/HA 5 compression	0,01	0,001	0,001	0,01	0,001	0,001	0,001	0,001	0,001	0,01	0,001
Dex/HA 10 compression	Dex/HA 10 static	ns	0,01	0,01	ns	0,001	0,1	0,001	0,001	0,001	0,001	0,01
Agarose static	Dex/HA 5 static	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	ns	0,001
Agarose static	Dex/HA 5 compression	0,001	0,001	0,1	ns	0,001	0,001	0,001	0,001	0,1	ns	ns
Agarose static	Dex/HA 10 static	0,01	0,001	0,01	0,1	0,001	0,001	ns	ns	0,001	0,001	ns
Agarose static	Dex/HA 10 compression	0,01	0,001	0,001	0,05	0,001	0,001	0,001	0,001	0,001	0,1	0,001
Agarose compression	Dex/HA 5 static	0,05	0,001	0,001	0,01	ns	0,001	0,001	0,001	0,001	0,001	0,001
Agarose compression	Dex/HA 5 compression	ns	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,01
Agarose compression	Dex/HA 10 static	0,05	0,001	0,001	0,001	ns	ns	ns	ns	0,001	0,1	ns
Agarose compression	Dex/HA 10 compression	0,05	0,001	0,001	0,001	0,001	0,05	0,001	0,001	0,001	0,001	ns
Agarose compression	Agarose static	0,001	0,001	0,001	0,001	0,001	0,001	ns	ns	0,001	0,001	0,01

12.4.8. Droplet culture

A hydrogel droplet culture was performed in addition to the cartilage-on-chip experiments. These experiments were performed to evaluate the behavior of human chondrocytes in a 3D hydrogel, for Dex/HA 10wt% and agarose 2%. A 25 µL hydrogel droplet, containing 150.000 cells, was made in a suspension 12 wells plate (**Figure S.7**). After gelation, CPM was added to the wells and refreshed daily. The hydrogel droplets containing human chondrocytes were cultured for 11 days and analyzed using bright-field imaging and immunofluorescence staining.

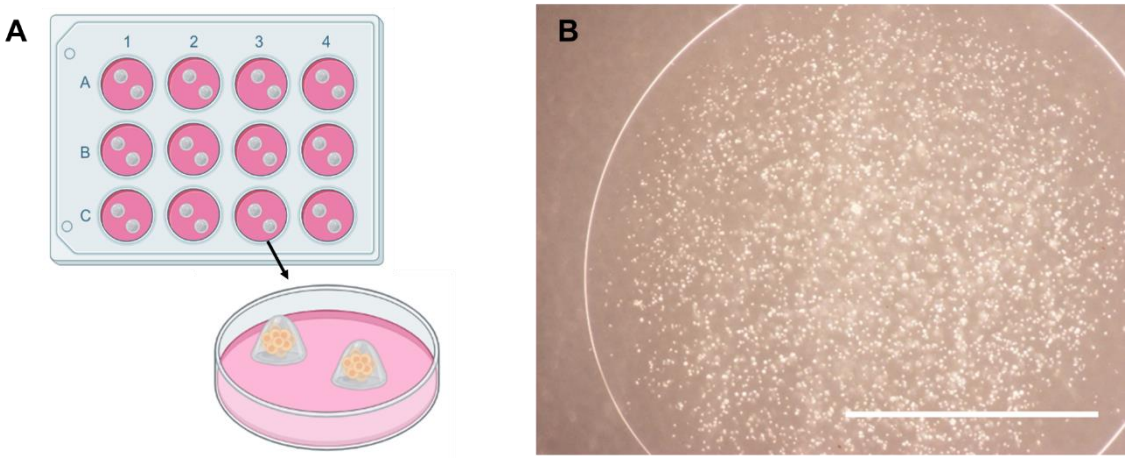

Figure S.7*. A) Schematic overview of hydrogel droplets containing human chondrocytes in a 12 wells plate. B) Overview of Dex/HA 10wt% hydrogel droplet containing human chondrocytes at day 0. Scalebar: 2000 µm.*

Chondrocytes cultured in Dex/HA 10wt% hydrogel did behave differently from the ones cultured in agarose (**Figure S.8**). The changes in cellular surface suggested that Dex/HA chondrocytes did start to attach to the hydrogel matrix.

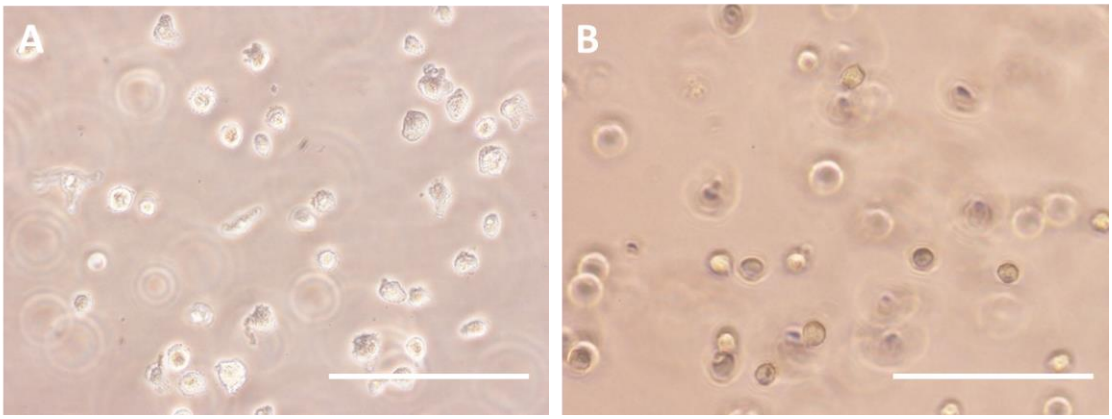

Figure S.8*. Human chondrocyte culture in Dex/HA 10wt% droplet (A) and agarose 2% droplet (B). Scalebars: 200 µm.*

The hydrogel droplets were analyzed using immunofluorescence staining. Staining for Aggrecan, MMP-13 and DAPI indicated elevated levels of MMP-13 for the Dex/HA 10wt% chondrocytes (**Figure S.9**).

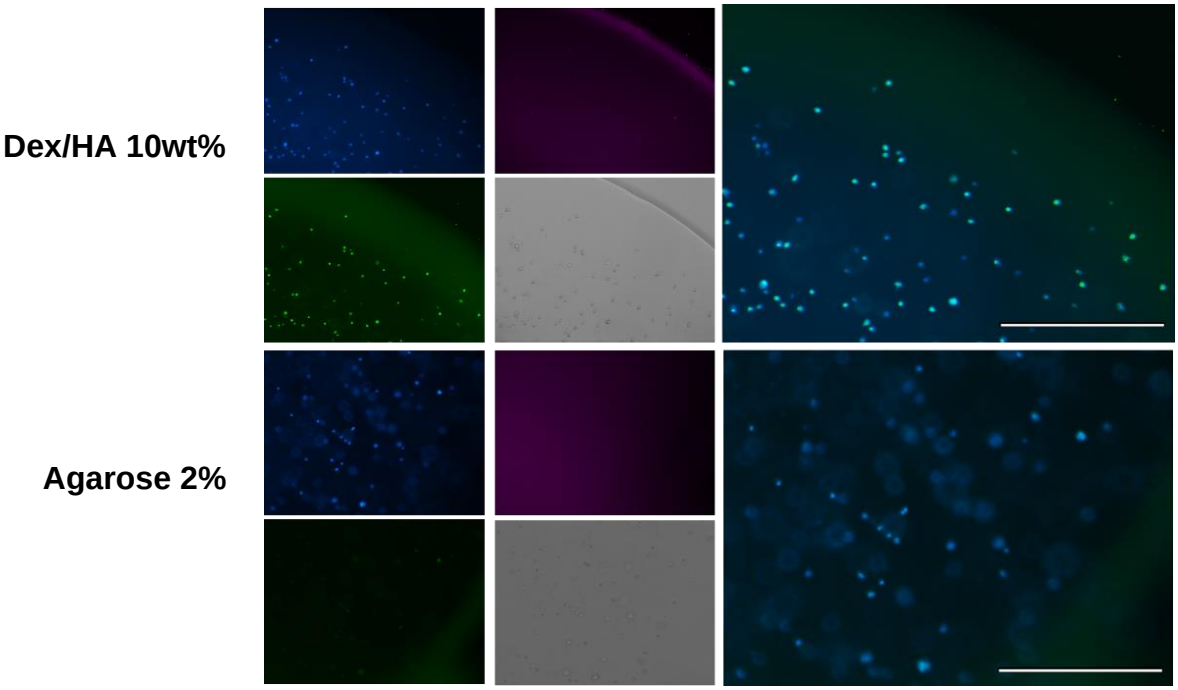

Figure S.9. *Immunofluorescence staining of hydrogel droplets Dex/HA 10wt% and agarose 2% at day 11. Images taken with EVOS fluorescence microscope; green (MMP-13), magenta (Aggrecan), and blue (nuclear staining, DAPI). Scalebars: 400 µm.*

12.5. Additional data: Culture conditions

12.5.1. Bright-field imaging monolayer culture

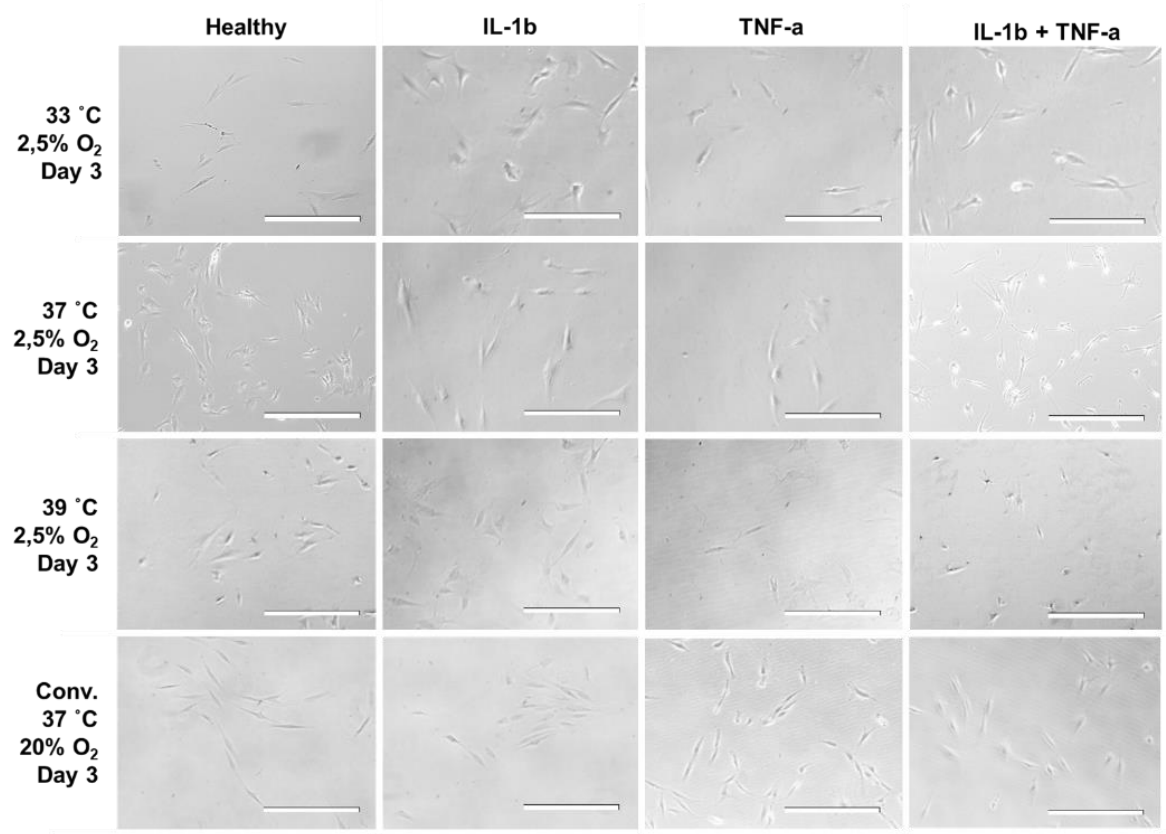

Figure S.10. *Bright-field images of all conditions after 3 days of healthy or cytokine stimulated culture. Scalebars: 400 µm.*

12.5.2. Immunofluorescence staining

Phalloidin and Collagen VI staining was performed for all culture conditions (**Figure S.11**). Presence of Collagen VI, a major component of the chondrocyte peri-cellular matrix, was detected in 37 °C hypoxic cytokine stimulated condition, as indicated with a white arrow. Aggrecan deposition in the cytoskeleton was seen in the 33 °C healthy culture condition (**Figure S.12**).

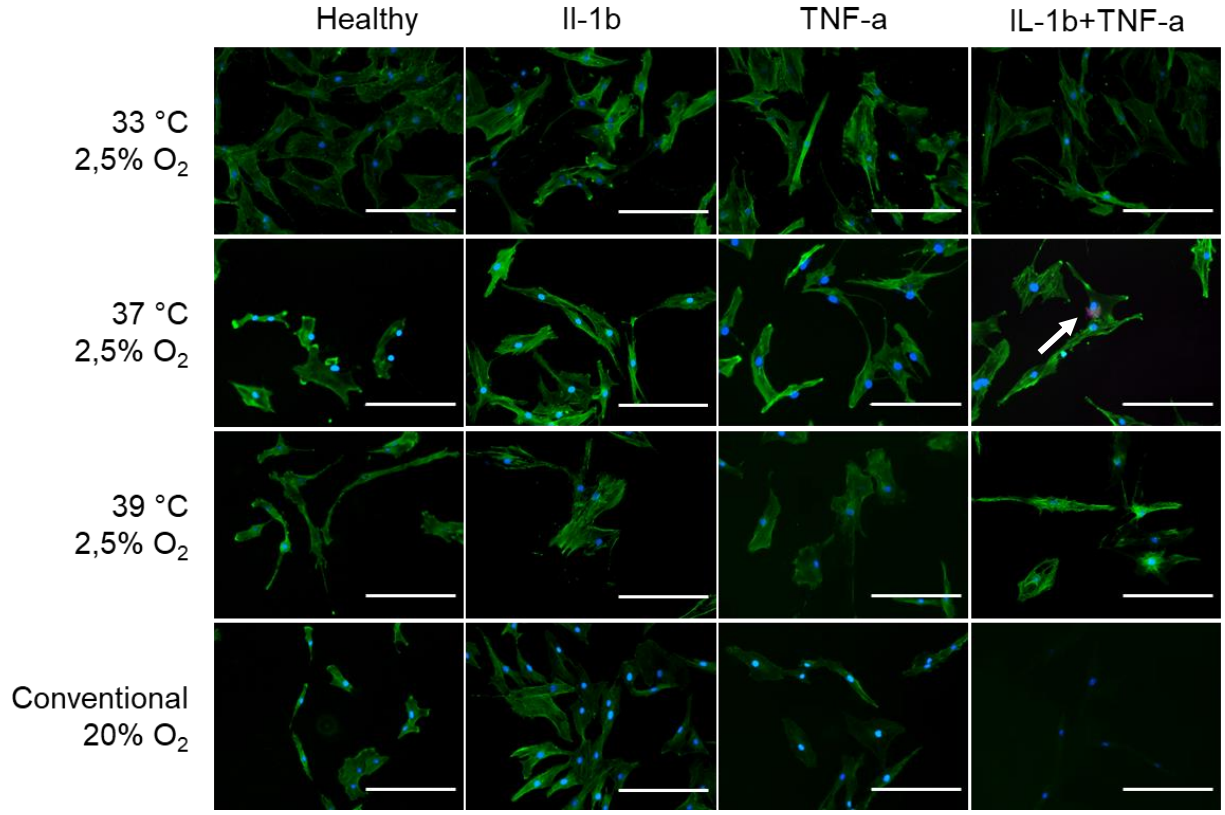

Figure S.11. *Phalloidin and Collagen VI immunofluorescence staining of all culture conditions, healthy and with cytokine stimulation, at day 3. White arrow indicates presence of Collagen VI. Images taken with EVOS fluorescence microscope; green (phalloidin), red (Collagen VI), and blue (nuclear staining, DAPI). Scalebars: 200 µm.*

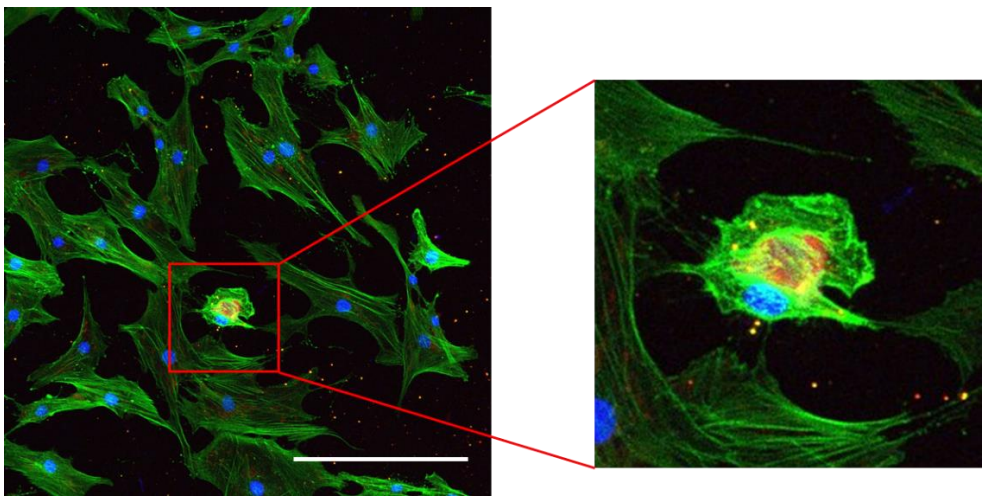

Figure S.12. *Phalloidin and Aggrecan immunofluorescence staining of all 33 °C healthy condition day 3, with focus on Aggrecan accumulation in cytoskeleton. Images taken with Nikon confocal; green (phalloidin), red (Aggrecan), and blue (nuclear staining, DAPI). Scalebars: 200 µm.*

12.5.3. Viability

Viability of human chondrocytes cultured in a pellet system was not highly affected by cytokine stimulation for 33 °C and 37 °C hypoxic cultures (**Figure S.13**). Cytokine stimulation at 39 °C and conventional cultures did result in a significant difference of cell viability over time.

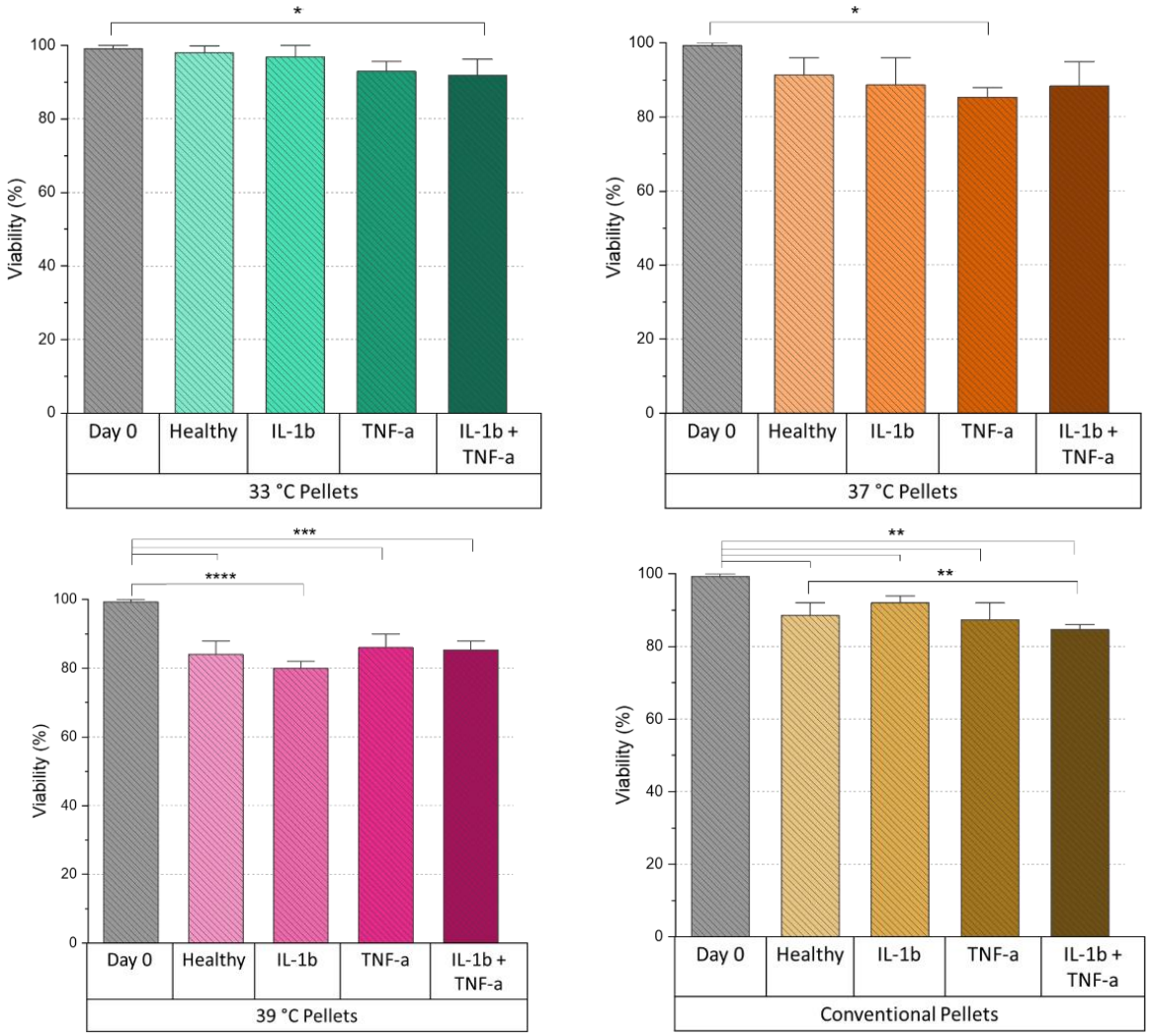

Figure S.13. *Pellet viability at day 0 and all day 3 conditions. Values are shown with ± SD, with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****). N = 4.*

12.5.4. Bright-field imaging pellets

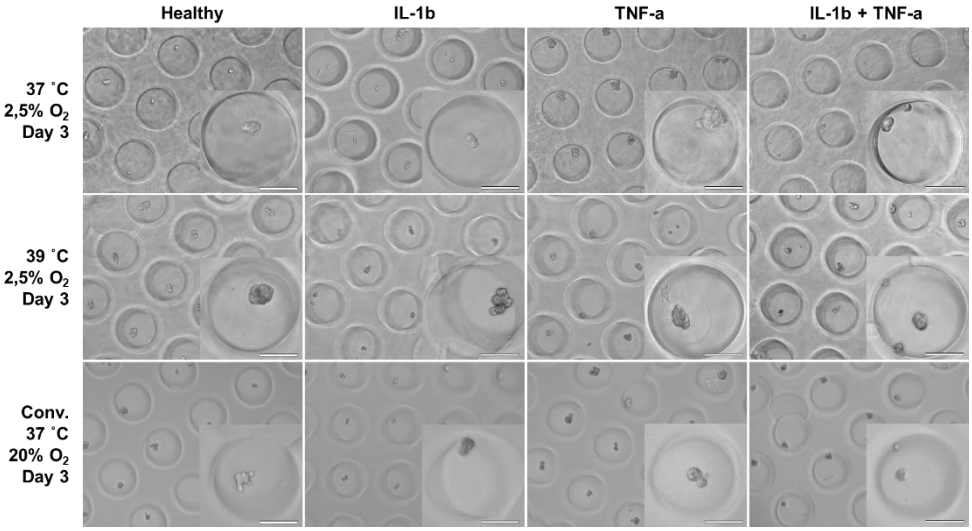

Figure S.14. *Day 3 images of 37 ˚C and 39 ˚C hypoxic cultures and conventional culture in healthy and cytokine stimulated conditions. Scalebars: 100 µm.*

12.5.5. Metabolic activity

Metabolic activity of human chondrocytes cultured in a pellet system was determined using a PrestoBlue assay. Cytokine cocktail promoted the metabolic activity for 39 °C and conventional cultures (**Figure S.15**).

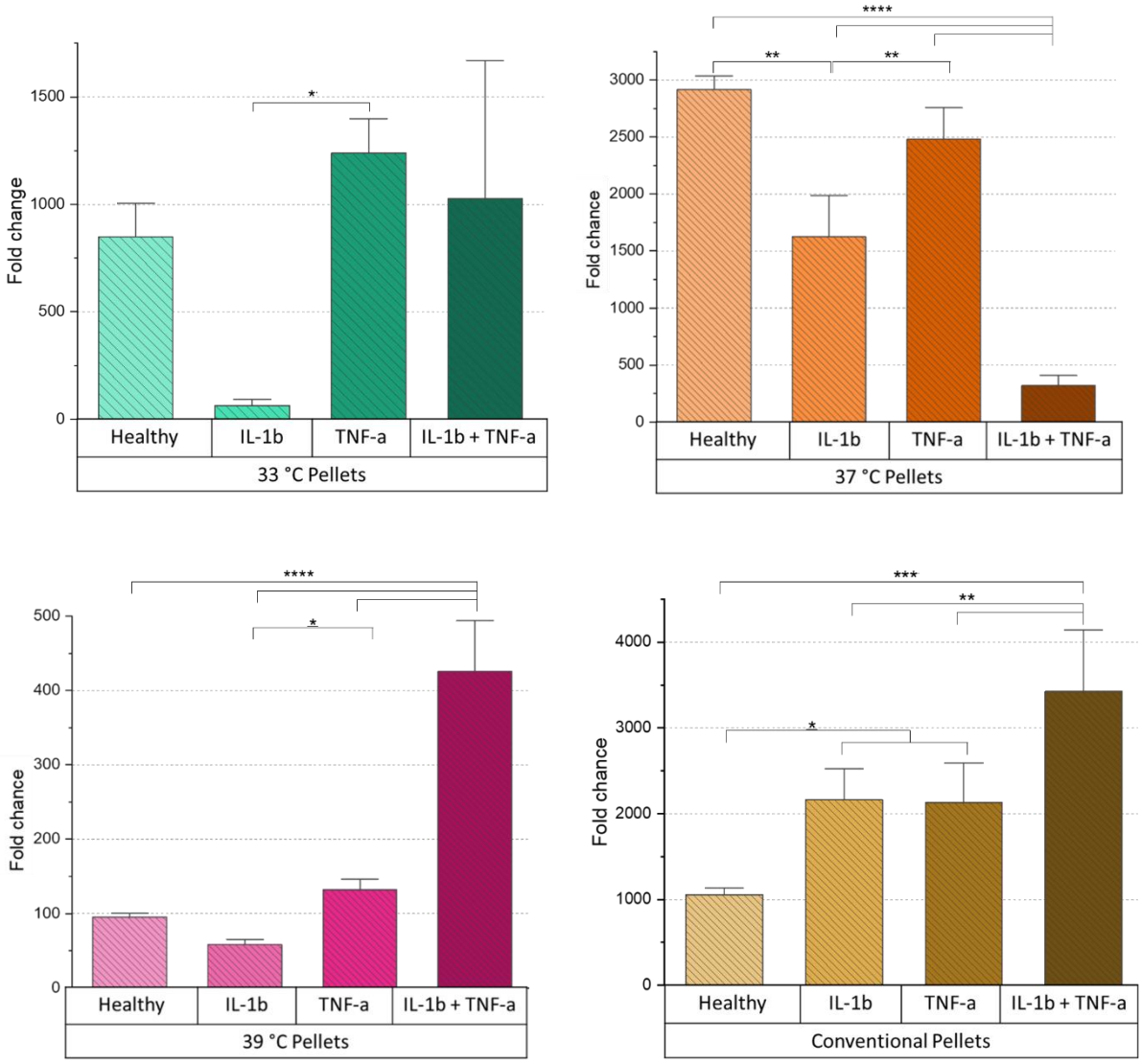

Figure S.15. *Fold change of metabolic activity assay for all pellet cultures, at 33 °C, 37 °C, and 39 °C hypoxic conditions and conventional condition. Values are expressed with ± SD, with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****). N = 3.*

12.5.6. Gene expression – Raw CT values

Due to the potential unreliable origin of GAPDH expression, because of its dependence on the glucose metabolism, raw CT values were considered in this section. High Ct value, meaning a high threshold cycle, indicates a low expression of the gene. Values equal to zero were undetermined, indicating no expression. Overall progress was comparable, but 33 °C data is highly influenced by its high GAPDH expression (**Figure 48**) and may therefore not be completely reliable.

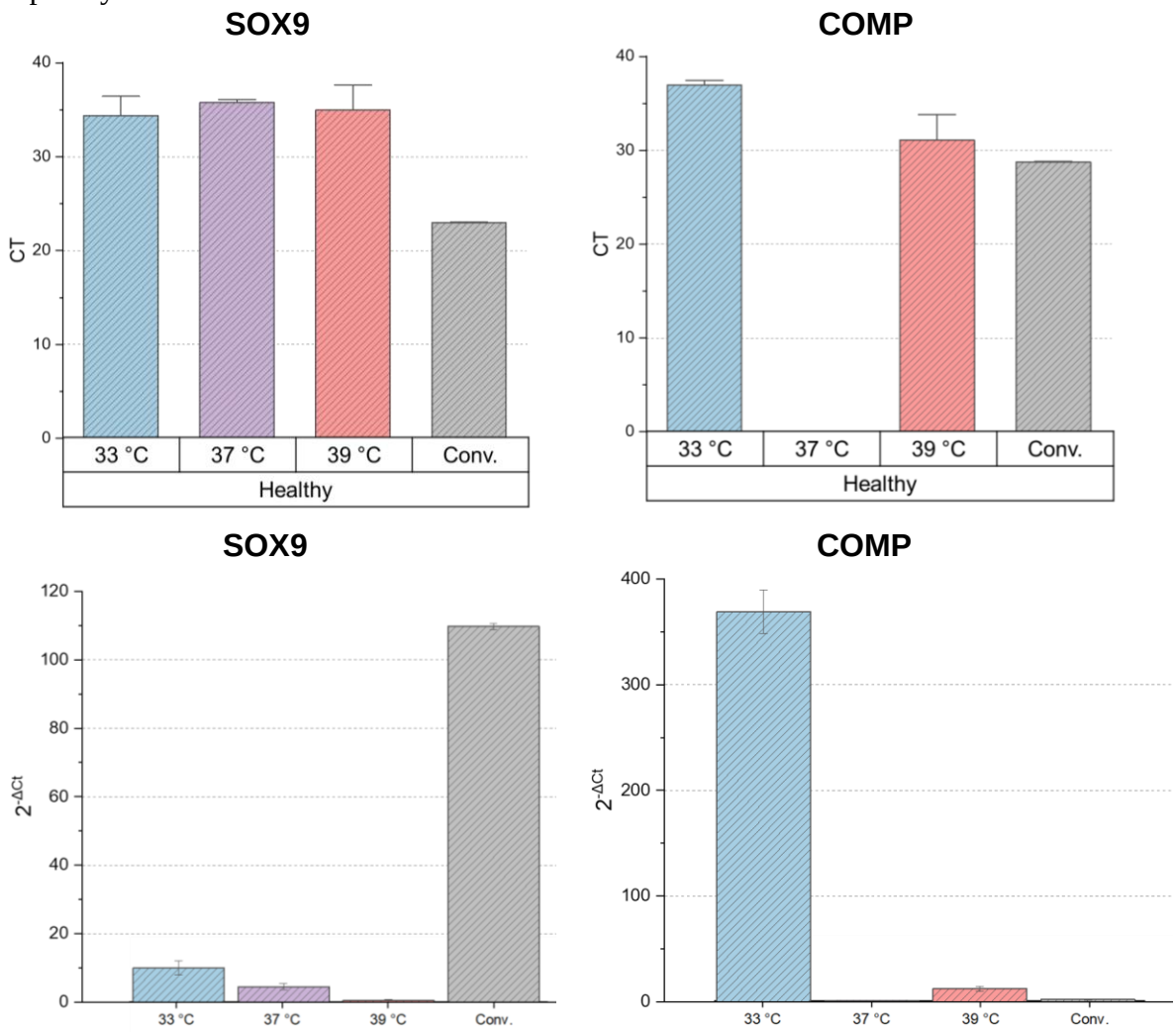

Figure S.16. *Raw CT values (upper row) and relative gene expression ($2^{-\Delta Ct}$, bottom row) for SOX9 and COMP. Values are expressed with ± SD, N = 2.*

12.5.7. Gene expression

SOX9 expression was downregulated under stimulation of IL-1β and TNF−α, whether no significant difference was determined between healthy and cytokine stimulated cultures (**Figure S.17**).

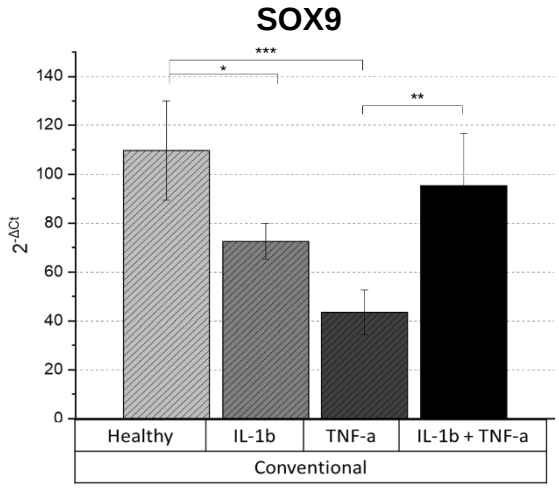

***Figure S.17**. Relative gene expression of SOX9 for the conventional monolayer culture. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****). N = 3.*

Collagen I expression for the monolayer chondrocytes was highly upregulated for healthy 39 °C and IL-1β 37 °C and 39 °C hypoxic conditions (**Figure S.18**). IL-1β did highly influence Collagen I production in hypoxic culture. The data suggests that cells cultured at 37 °C and 39 °C hypoxic conditions are more prone to create hypertrophic tissue, while for 33 °C hypoxic culture and the conventional culture Collagen I was produced in only a small extent. When comparing the Collagen I monolayer expression to the pellet culture (**Figure S.19)**, a remarkable increase in Collagen I positive cells for the 33 °C and conventional conditions were found, suggesting that the cells did become more hypertrophic. Conversely, for the 39 °C hypoxic condition, the Collagen I expression was lower with respect to its monolayer culture, suggesting that the chondrocytes did remain their phenotype. However, more markers should be considered to determine the state of differentiation.

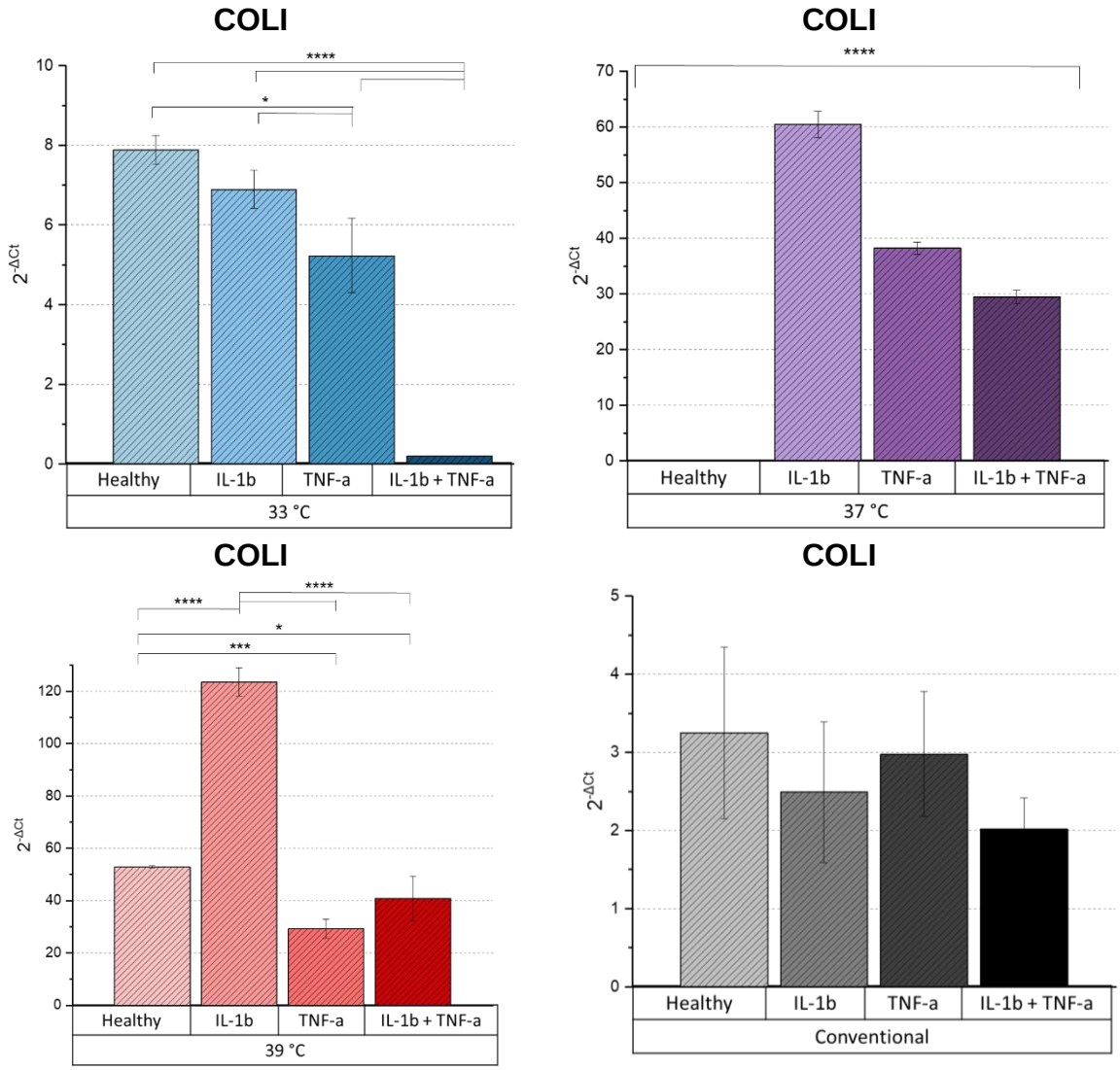

***Figure S.18**. Relative gene expression of Collagen I for all 2D culture conditions. Graphs express the $2^{-\Delta Ct}$ values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 3, with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****).*

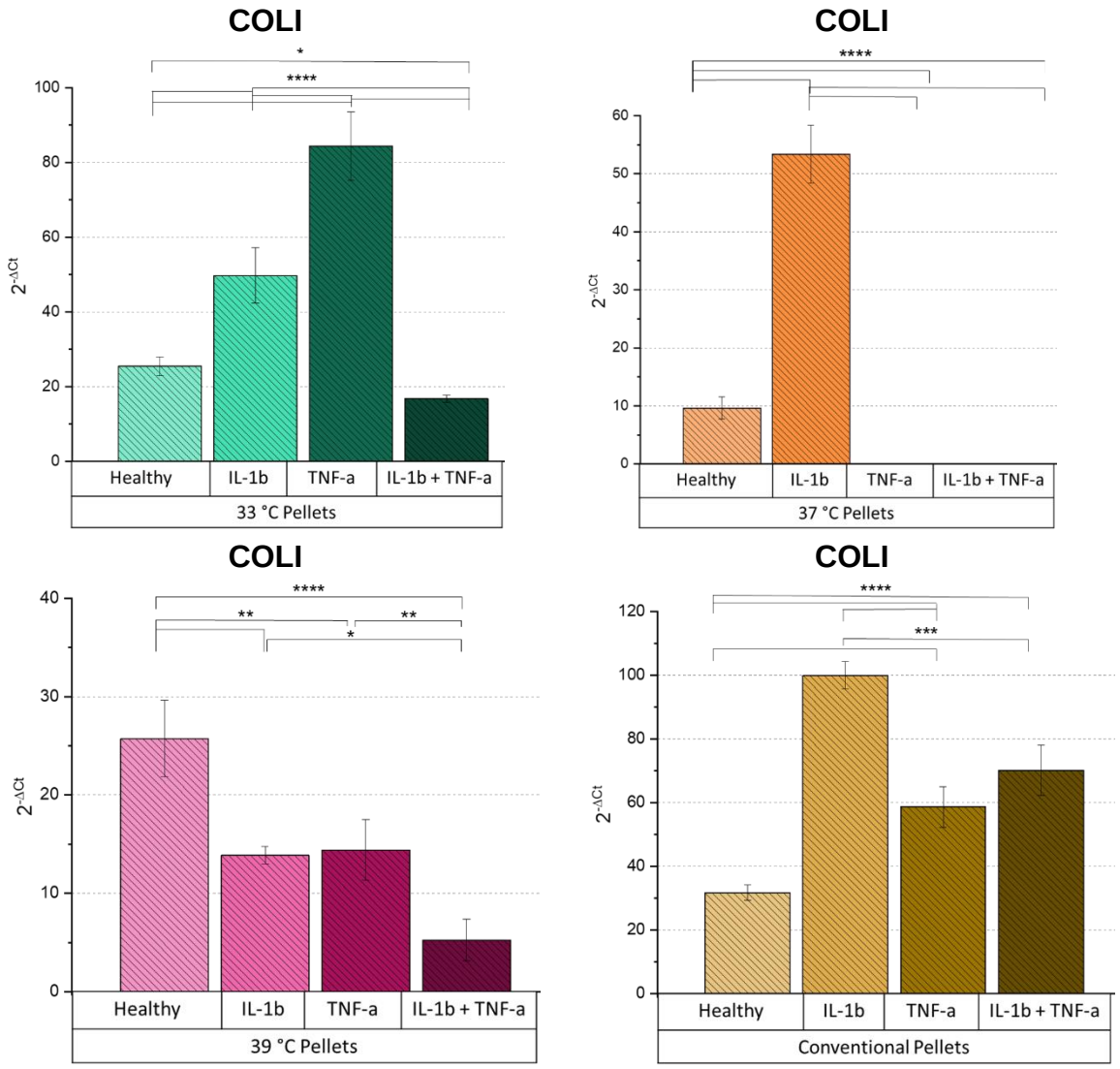

Figure S.19. *Relative gene expression of Collagen I for all Pellet culture conditions. Graphs express the* $2^{-\Delta Ct}$ *values, normalized for housekeeping gene GAPDH. Values are expressed with ± SD, N = 3, with p < 0,1 (*), p < 0,05 (**), p < 0,01 (***), and p < 0,001 (****).*