

**MICRO-ENVIRONMENTAL AND  
MACRO-ENVIRONMENTAL**

improvement of joint quality in  
knee joint pathologies

UFUK TAN TIMUR



Micro-Environmental and Macro-Environmental  
Improvement of Joint Quality  
in Knee Joint Pathologies

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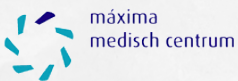
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# **Micro-Environmental and Macro-Environmental Improvement of Joint Quality in Knee Joint Pathologies**

## **PROEFSCHRIFT**

Ter verkrijging van de graad van doctor aan de  
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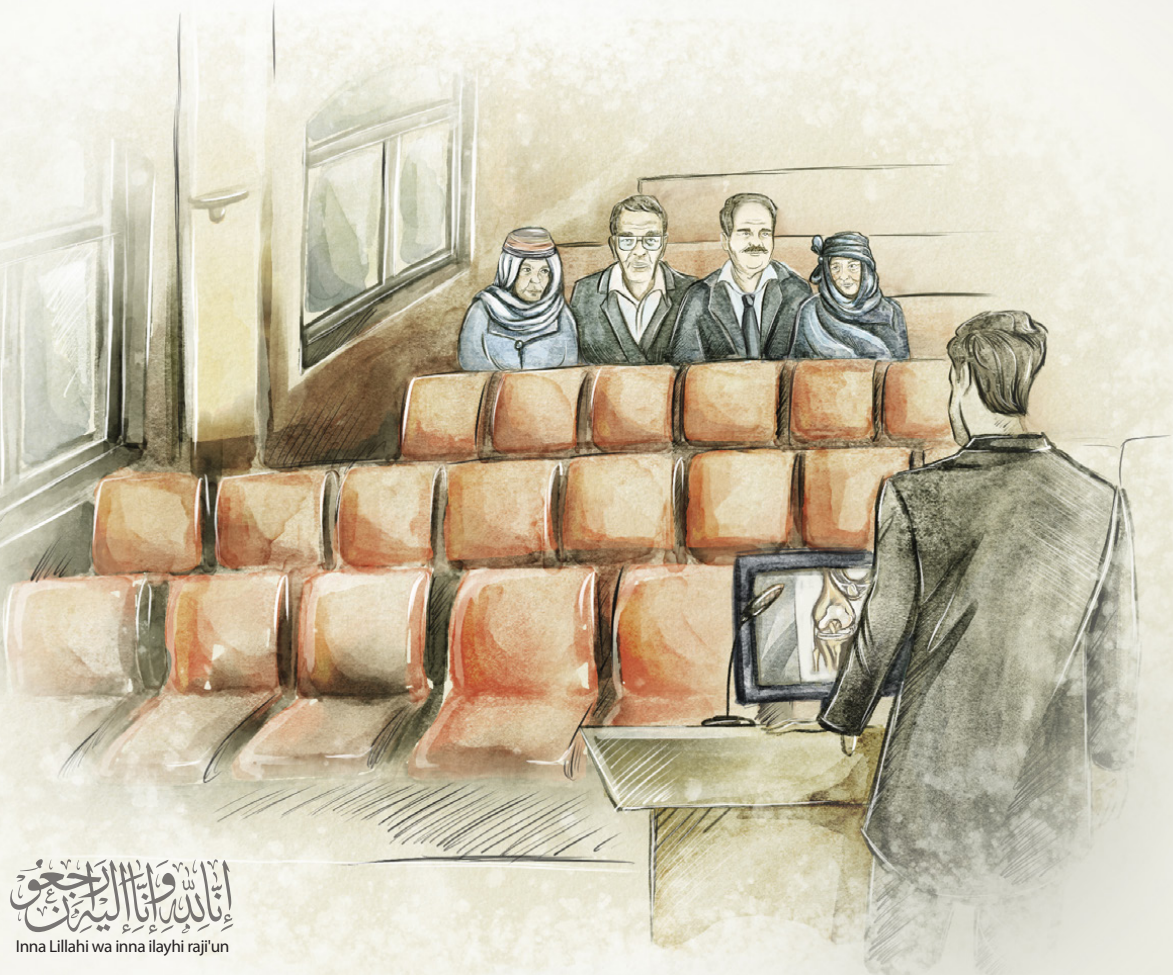
Prof. dr. L.J.C. van Loon

Prof. dr. M. Poeze

For my family, voor mijn familie, ailem için

# بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِیْمِ

———— Bismillahirrahmanirrahim ————



إِنَّا لِلّٰهِ وَإِنَّا إِلَيْهِ رَاجِعُونَ  
Inna Lillahi wa inna ilayhi raji'un



## Voorwoord

---

Het was donderdag 7 november 2002. Ik herinner me het moment dat mijn leraar Latijn onderwijs gaf over de overtreffende trap, ofwel de comparativus in Latijn. Hij legde uit dat de comparativus wordt gevormd door achter de stam van het woord –ior te plaatsen. Een doctorandus was letterlijk vertaald ‘hij die geleerder moet worden’, en doctor was ‘geleerder’. Je kon pas doctor worden als je een boek zou schrijven. Ik dacht bij mezelf, alsof al die proefwerken en verplichtingen al niet genoeg zijn, ook nog een boek schrijven? Ik keek zuchtend uit het raam, dwaalde af met mijn gedachten en ging aan leuke dingen denken, zoals de 6-0 overwinning van mijn jeugdliefde Fenerbahce op Galatasaray van gisterenavond. Wie had ooit gedacht dat ik precies 20 jaar later als zoon van gastarbeiders, opgegroeid in een flat in de Noordstraat, beter bekend als ‘klein Turkije’, daadwerkelijk dat boek zou schrijven? Lieve familie, vrienden en collega’s, voor u ligt het boek wat ik heb geschreven en waar ik veel energie en tijd in heb gestoken. Het was een lange rit, met vallen en opstaan. Het laboratorium is een plek waar veel geduld voor nodig is. De resultaten beschreven in dit boek zijn misschien 10 % van experimenten die succesvol zijn geweest, maar er zijn ook grotendeels experimenten die helaas minder succesvol zijn geweest. Experimenten waarvoor ik soms in de avonduren of in de nacht in het laboratorium heb moeten werken, experimenten waarvoor ik in de sneeuw op een trein met vertraging heb moeten wachten, experimenten waarvoor ik lange ritten heb moeten afleggen van Rotterdam naar Maastricht en Aachen.

*‘Het schrijven van dit boek heeft me geleerd dat wetenschap topsport is, geef nooit op en de overwinning is van degene die erin gelooft!’*

PS: Niet stiekem nu al naar mijn dankwoord bladeren hè!



## Preface

---

It was Thursday, November 7, 2002. I remember the moment my Latin teacher was teaching about the superlative, or comparativus in Latin. He explained that the comparative is formed by placing an -ior after the stem of the word. A doctorandus was literally translated 'he who must become a learned', and doctor was 'more learned'. You couldn't become a doctor until you wrote a book. I thought to myself, as if all those tests and obligations weren't enough, write a book too? I looked out the window with a sigh, lost my mind and started thinking about fun things, such as my childhood sweetheart Fenerbahce's 6-0 victory over Galatasaray of last night. Who would have ever thought that exactly 20 years later, me as the son of guest workers, raised in a flat in the Noordstraat, better known as 'small Turkey', would actually write that book? Dear family, friends and colleagues, this is the book that I have written and in which I have invested a lot of energy and time. It was a long ride, with trial and error. The laboratory is a place that requires a lot of patience. The results described in this book may be 10% of experiments that have been successful, but there have been a lot of experiments that have unfortunately been less successful. Experiments for which I sometimes had to work in the laboratory in the evenings or at night, experiments for which I had to wait in the snow for a train that was delayed, experiments for which I had to make long journeys from Rotterdam to Maastricht and Aachen.

*'Writing this book has taught me that science is professional sport, never give up  
and the victory belongs to the one who believes in it!'*

PS: Don't sneakily scroll to my acknowledgements!

## Önsöz

Bir perşembe günüydü, tarihlerden 7 Kasım 2002. Latince öğretmenimin Latince'de en üstün veya karşılaştırmalı sözcükleri öğrettiği anı hatırlıyorum. Latince'de karşılaştırmaların -ior kelimesinin kökünden sonra yerleştirilerek oluşturulduğunu öğretti. Bir 'doktorandus', kelimenin tam anlamıyla 'bilgili olması gereken kişi' olarak tercüme edilir ve doktor 'daha bilgili' idi. Bir kitap yazana kadar doktor olamassınız. Bunca imtihan ve yükümlülükler yetmezmiş gibi birde kitap mi yazacağım diye düşündüm. Pencereden derin bir iç çekerek dışarı baktım, aklımı kaybettim ve çocukluk aşkımla Fenerbahçe'nin dün gece Galatasaray'ı 6-0 yendiği gibi eğlenceli şeyler düşünmeye başladım. Tam 20 yıl sonra, Noordstraat'ta, daha çok 'küçük Türkiye' olarak bilinen bir apartman dairesinde büyümüş bir misafir işçinin oğlu olarak, o kitabı gerçekten yazacağım kimin aklına gelirdi?

Sevgili ailem, arkadaşlarım ve meslektaşlarım, yazdığım ve çok fazla enerji ve zaman harcadığım kitap önünüzde duruyor. Düşse kalka geçirdiğim uzun bir yolculuktu. Laboratuvar çok sabır gerektiren bir yerdir. Bu kitapta açıklanan sonuçlar, başarılı olan deneylerin %10'u olabilir, ancak ne yazık ki kariyerimde daha az başarılı olan deneyler çoğunluktadır. Bazen akşamları veya geceleri laboratuvarında çalışmak zorunda kaldığım deneyler, gecikmeli bir tren için karda beklemek zorunda kaldığım deneyler, Rotterdam'dan Maastricht ve Aachen'e uzun yolculuklar yapmak zorunda kaldığım deneyler.

*'Bu kitabı yazmak bana bilimin profesyonel bir spor olduğunu öğretti, asla pes etme ve zafer inananlarındır!'*

PS: Lütfen kitabı bastan okuyunuz ve simdiden gizlice teşekkür sözü bölümüne geçmeyiniz!






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An anatomical illustration of a human knee joint, showing the femur (thigh bone) at the top, the tibia (shin bone) at the bottom, and the patella (kneecap) in the center. The bones are rendered in a light, realistic style with some shading to show depth. The background is a soft, light gray.

*Chapter*  
**01**

**General Introduction and  
Aims of This Thesis**

## The Wisdom of the Knee Joint

---

Humans are homeotherms which are capable of withstanding extreme temperatures<sup>1</sup>. Expose a healthy human being to high temperatures, his body temperature will not rise above normal. Expose an arctic mammal to cold as low as 25 degrees Centigrade below freezing, its body temperature will hardly show a noteworthy fall<sup>1</sup>. In 1929, it was Walter Bradford Cannon, coining in the concept of homeostasis<sup>2</sup>:

*'The coordinated physiological processes which maintain most of the steady states in the organism are so complex and so peculiar to living beings - involving, as they may, the brain and nerves, the heart, lungs, kidneys and spleen, all working cooperatively - that I have suggested a special designation for these states, homeostasis. The word does not imply something set and immobile, a stagnation. It means a condition - a condition which may vary, but which is relatively constant'<sup>2</sup>.*

The concept of homeostasis owes much to the endeavors made by ancient physicians such as Claudius Galenus from Bergama, Hippocrates, Paul of Aegina and Avicenna<sup>3</sup>. Claudius Galenus already described that health is a state which helps to maintain the functions of the human body through proper balance of its temperament and composition in a correct manner, while disease is contrary to the aforementioned state<sup>3</sup>. This concept has, amongst others, also been acknowledged by Ibn Sina (Avicenna), which was one of the pioneers of modern medicine<sup>4</sup>. The concept of homeostasis also holds true for the knee joint in the human body. The knee joint consists of two articulations formed by three bones: the tibia, femur and patella<sup>5</sup>. One articulation is present between the femur and patella (i.e. patellofemoral joint) and one between the femur and the tibia (i.e. tibiofemoral joint). The knee joint is a synovial joint containing articular elements, enclosed in an articular capsule. These intra-articular elements are in close contact with each other and maintain 'joint homeostasis'.



## Joint Homeostasis in the Healthy Knee Joint

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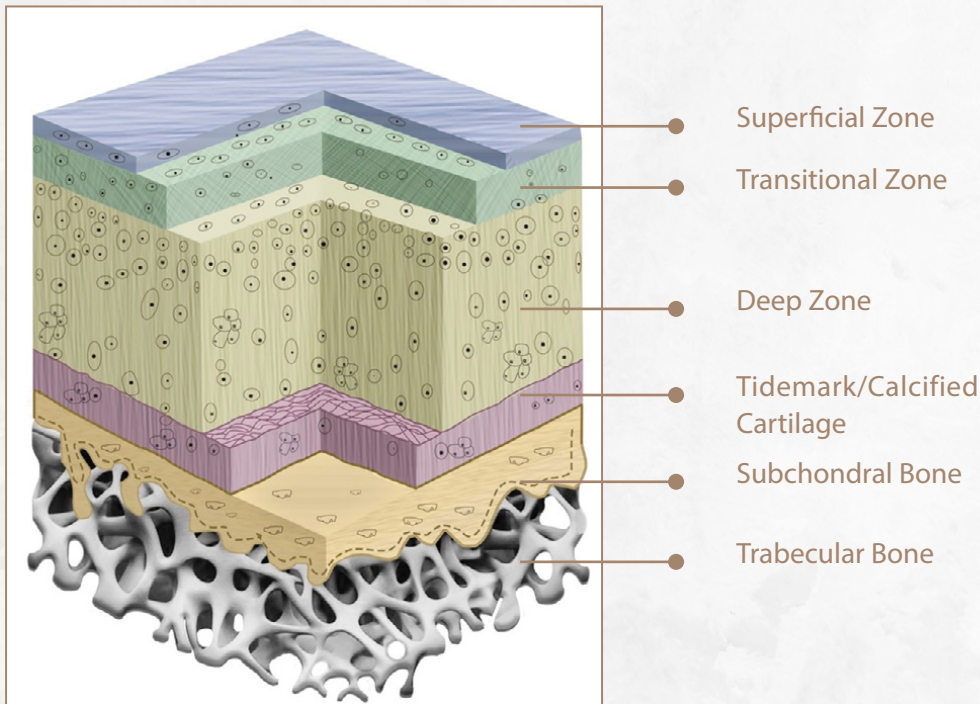
The specific anatomy of a human knee joint makes movement possible even under the most challenging circumstances such as marathon walking or heavy weight lifting. The knee joint comprises a closed cavity and different intra-articular structures can be identified. These different intra-articular tissues critically interact with each other to maintain 'joint homeostasis'. The articulating ends of each bone are covered by a thin layer of connective tissue, which is called articular cartilage. The principal function of articular cartilage is to provide a smooth, lubricated surface for the physical articulation and to facilitate the transmission of loads between bony skeletal elements with a low frictional coefficient. Articular cartilage is a hyaline cartilaginous connective tissue and is composed of water (65-80%), collagens (12-21%), proteoglycans (6-10%) and other glycoproteins (2-3.5%)<sup>6</sup>. The extracellular matrix (ECM) of hyaline articular cartilage is produced by the main cell type found in cartilage: the articular chondrocyte<sup>6</sup>. In healthy cartilage, a balance exists in anabolism and catabolism resulting in articular cartilage homeostasis<sup>7</sup>.

The intriguing characteristics of cartilage have already been recognized by Ogston and colleagues<sup>8</sup> and date back to the 19<sup>th</sup> century, underscoring the fact that it was hard to understand that cartilage is not worn away despite high forces the tissue has to resist, while on the other hand the enamel of teeth shows effects of friction in a short period of time. Reeves analyzed articular hyaline cartilage in 1876 and made horizontal thin sections of it, noting the appearance of straight bands running in various directions and planes<sup>9</sup>. Hence, reports in the 19<sup>th</sup> century already described that based on chondrocyte shape, chondrocyte distribution, and collagen fiber orientation, different zones can be distinguished in articular cartilage.

Articular cartilage can be divided in the superficial zone, the transitional zone, the deep zone and the calcified zone. The calcified zone is separated from the deep zone by the 'tidemark'<sup>10</sup>. While chondrocytes in the superficial layer of articular cartilage have a flattened fibroblast-like morphology, chondrocytes become larger in size with a hypertrophic appearance towards the deeper layers of articular cartilage<sup>10</sup>. The superficial

## Chapter 1

zone comprises 10 to 20 % of articular cartilage thickness and protects deeper layers from shear stresses<sup>10</sup>. Here, chondrocytes are flattened and collagen fibers are oriented parallel to the articular surface contributing to resistance to shear forces and to distribute load towards the subchondral bone<sup>10</sup> (Figure 1). The transitional zone is the next layer, representing 40-60 % of articular cartilage thickness<sup>6</sup>. It contains proteoglycans and randomly organized thick collagen fibrils and has been thought to be the first zone to resist compressive forces imposed by articulation<sup>10</sup>. Closer to the bone than the transition zone is the deep zone, which contains the highest proteoglycan content and the lowest water concentration representing approximately 30 % of the articular cartilage thickness. In this zone, chondrocytes are organized in column-like structures (Figure 1), while the collagen fibers are oriented perpendicular to the joint surface providing the greatest resistance to compressive forces<sup>10</sup>. The deep zone is separated from the next layer, the calcified cartilage zone, by the tidemark. This zone is scarcely populated by chondrocytes and chondrocytes present with a hypertrophic phenotype (Figure 1). The calcified cartilage plays an essential role in securing the layer of articular cartilage to the underlying subchondral bone by anchoring the collagen fibrils to the underlying subchondral bone tissue<sup>6</sup>.



**Figure 1: Layers of Articular Cartilage.**

Articular cartilage can be divided into the superficial zone, transitional zone, the deep zone and the calcified cartilage, which is separated from the deep zone by the tidemark. While chondrocytes are flattened and parallel to the joint surface in the superficial zone, collagen fibers become tangential to the joint surface in deeper layers of cartilage providing resistance to compressive forces.

## Articular Cartilage: A Tissue with Unique Traits

---

Functional adaptation is a concept that can be described as the ability of tissues to adjust structure in accordance with specific environmental requirements<sup>11</sup>. This concept has been established to occur in bone (Wolff's law)<sup>12</sup>. Given the unique architecture of cartilage and its intriguing ability to withstand compressive forces, this concept of 'functional adaptation' also may hold true for cartilage. Environmental factors such as mechanics aid in the specific structural architecture of cartilage. Indeed, several studies show that chondrocytes are responsive to mechanical stimuli<sup>13,14</sup>. Furthermore, the thickness of articular cartilage can change in response to mechanical stimuli, as evidenced by a study which showed reduced cartilage thickness in patients with reduced load bearing due to paraplegia<sup>15</sup> or in a study showing reduced cartilage thickness in patients with reduced load bearing due to an ankle fracture<sup>16</sup>.

Articular chondrocytes are distinctively adapted to respond to biomechanical stress<sup>17</sup>. Compared to other cell types, articular chondrocytes have less negative resting membrane potential, which permits the chondrocyte to efflux positively charged potassium ions rapidly enough to limit cell-volume increase under conditions of reduced osmolarity<sup>17</sup>. This improved control of cell volume is crucial for the maintenance of optimal chondrocyte metabolism and matrix biosynthesis<sup>18</sup>.

Another unique feature for articular cartilage is that loading is needed for articular cartilage homeostasis. During loading, the displacement of water in and out of the articular cartilage increases the rate at which chondrocytes receive nutrients<sup>19</sup>. Moreover movement also assists in cartilage nutrition by increasing production of synovial fluid and aiding in the removal of waste products through the synovial membrane<sup>19</sup>. Furthermore, biomechanical



stresses have been shown to activate latent TGF- $\beta$ , a morphogen residing in the ECM of articular cartilage which plays a crucial role in articular cartilage homeostasis<sup>20</sup>. Importantly, it was shown that biomechanical forces can lead to activation of SMAD2/3 signaling, which is known to be protective for articular cartilage, again demonstrating the importance of movement for articular cartilage homeostasis<sup>21</sup>.

## Biophysiological Environment of Articular Cartilage

---

Articular cartilage remains functional through life if not affected by pathology<sup>10</sup>. It differs from other cartilaginous tissues such as the growth plate or fracture callus, which undergo a process defined as endochondral ossification<sup>22,23</sup>. This process guides longitudinal skeletal growth and fracture repair and is characterized by chondrocytes that proliferate, become hypertrophic and that eventually are being replaced by bony tissue through the actions of invading osteoclasts and osteoblasts<sup>22,24,25</sup>. The differential behavior of chondrocytes in articular cartilage and chondrocytes in a growth plate or fracture callus may be related to their physiological environment<sup>25</sup>. Two biophysiological conditions are characteristic for articular cartilage: its lower oxygen tension levels and higher osmolarity in comparison to many other tissues. These biophysiological conditions are known to be active regulators of the chondrocyte and have been described to prevent the chondrocyte to undergo hypertrophic differentiation<sup>26,27,28</sup>.

Cartilage is not vascularized, and therefore oxygen supply occurs through diffusion from the synovial fluid, but also from the subchondral bone<sup>29,30</sup>. This causes relatively lower oxygen tension in cartilage in comparison to other tissue types<sup>31</sup>. It has been suggested that oxygen tension in cartilage ranges from 1% to 10% and oxygen tension decreases from the surface towards deeper regions of the cartilage<sup>29,31,32,33,34,35</sup>. Chondrocytes are well-adapted to this hypoxic environment and primarily employ anaerobic metabolism to generate ATP<sup>36</sup>.

Next to the hypoxic environment in articular cartilage, another characteristic present in articular cartilage is the higher osmolarity in comparison to other tissues. Osmolarity in the



interstitial fluid of cartilage ranges between 380 and 450 mOsm in healthy cartilage (i.e. physiological osmolarity)<sup>18,37,38,39,40,41,42</sup>. The structure and composition of the ECM causes a high osmotic value in the interstitial fluid, which gives articular cartilage its unique essential biomechanical properties, able to withstand compressive forces which occur in the knee joint. The ECM of articular cartilage is mainly composed of proteoglycans (like aggrecan) entangled in a network of collagen fibers (mainly collagen type II) and large amounts of water<sup>43,44</sup>. Water and inorganic salts represent the bulk mass wet weight portion (65-80%), with collagens representing the majority (i.e., 15-20%) of the remaining fraction<sup>44</sup>. Cartilage requires a high osmotic value of the interstitial fluid to maintain its hydrostatic pressure and viscoelastic properties, for which negatively charged glycosaminoglycan side chains of the proteoglycans are crucial to attract mobile cations and water into the ECM environment resulting in a high osmotic value of the interstitial fluid. The intact collagen network, in contrast, restricts the inherent swelling force of the ECM and determines the relatively high osmolarity of this interstitial fluid<sup>18</sup>. Loading of the knee joint, for example during walking, causes water to squeeze out of the cartilage. In contrast to water, proteoglycans remain in the ECM, which causes water immediately to be osmotically attracted back into the ECM when compressive forces are reduced again. The perpetuation of hydrostatic pressure gives cartilage its unique ability to absorb forces up to 20 MPa<sup>45</sup>.

## The Concept of Micro- and Macroenvironment

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Chondrocytes which reside in articular cartilage resemble a specific phenotype, responsible for the aforementioned unique properties of articular cartilage<sup>8</sup>. Unraveling the chondrocyte phenotype on a micro-environmental level will aid in understanding joint homeostasis. However, the chondrocyte is not the only cell type in the knee joint. The knee joint can be seen as an organ with a complex design, made up of smaller essential components, each exhibiting their own dynamic behavior. These smaller essential components combine into a large functioning unit, the knee joint macroenvironment, leading to the emergence of unique properties of the knee joint such as the ability to withstand biomechanical forces and support motion. The next paragraph will describe the smaller essential components which combine to form the knee joint macroenvironment.

## Intra- and Extra-Articular Tissues in a Healthy Knee Joint

---

The knee joint is surrounded by a joint capsule, which consists of an outer layer of fibrous connective tissue. This outer layer is lined on the inner side by the synovial membrane. The synovial membrane is composed of a thin layer of type A (macrophages) and type B (fibroblasts) synoviocytes<sup>46</sup>. The synoviocytes are important contributors to the biomolecular content found in synovial fluid, which contains compounds such as hyaluronic acid in order to lubricate the knee and to reduce wear and friction<sup>46</sup>. While cartilage is aneural and non-vascularized, the synovium is a highly vascularized, but also highly innervated tissue with sensory neurons<sup>47</sup>. Small molecules are able to diffuse through the synovium into the synovial fluid due to extensive vascularization of the synovium, providing cartilage with necessary nutrients, thereby maintaining joint homeostasis<sup>48</sup>.

Next to hyaline cartilage and synovial tissue, several other intra-articular tissues are present in the knee joint, each with their own function. Two menisci are situated between the femoral condyle and tibial plateau at both the medial and lateral aspects of the knee joint. These menisci withstand forces such as compression, shear and tension and play crucial roles in load transmission, shock absorption and load-bearing<sup>49</sup>. Another intra-articular tissue is Hoffa's fat pad (HFP). While four fat pads are present in the knee, the HFP is the largest in size<sup>50</sup>. Its position is posterior to the patellar tendon and inferior to the patella. The HFP is interposed between the capsular layer and the synovium and is thus located intra-articularly but extra-synovially, being in close proximity with the articular cartilage<sup>51</sup>. It is acknowledged that, besides acting as a reservoir for excess calories, adipose tissue can play a functional role in homeostatic processes. For instance, the omentum is well known as a visceral fat depot, but has also been described as 'the policeman of the abdomen' due to its ability to promote surgical wound healing and to attenuate peritonitis<sup>52</sup>. In fact, omental tissue has been described to possess angiogenic, fibrotic and immunologic activities which can promote vascularization, accelerate wound healing and limit infection<sup>53,54,55</sup>. Similarly, the HFP possesses angiogenic, fibrotic and immunologic activities which may play a role in maintaining knee joint homeostasis<sup>56,57,58</sup>. Besides its involvement in shock absorption in the anterior region of the knee<sup>51</sup>, scientific evidence regarding its potential role in knee

joint homeostasis is now accumulating<sup>51,56,57,58</sup>. Next to the aforementioned intra-articular tissues, also other intra- and extra-articular tissues such as the cruciate ligaments, collateral ligaments, other small fat pads, peri-articular muscles and subchondral bone all act in concert and combine into a large functioning joint unit.

## Articular Cartilage Defects

---

One of the joint pathologies which can compromise joint homeostasis in a healthy knee joint are articular cartilage defects<sup>59</sup>. Articular cartilage can become damaged due to trauma, degeneration, osteonecrosis of subchondral bone, septic arthritis, aging and osteochondritis dissecans<sup>60</sup>. Damage to articular cartilage can be graded from mild to severe according to the ICRS cartilage grading system (**Table 1**)<sup>61</sup>.



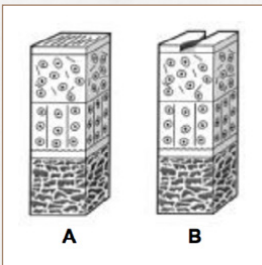
**Table 1: The ICRS cartilage grading system for cartilage defects.**

*The International Cartilage Repair Society (ICRS) Cartilage Lesion Classification System. Published with permission from the ICRS Cartilage Injury Evaluation Package (<http://www.cartilage.org/>).*



**GRADE 0**

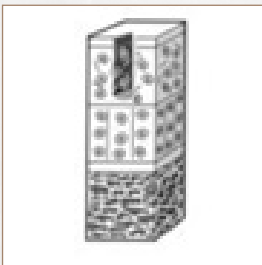
Normal Cartilage



**GRADE 1**

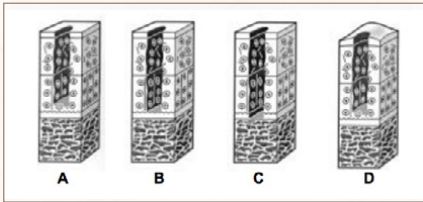
Nearly Normal:

Soft indentation (A) and/or superficial fissures and cracks (B).



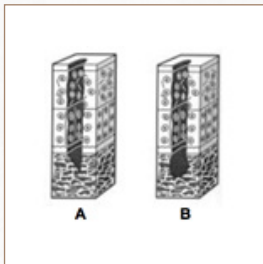
**GRADE 2**

Abnormal (lesions extending down to < 50 % of cartilage depth)



### GRADE 3

Severely abnormal: cartilage defects > 50 % of cartilage depth (A), as well as down to calcified layer (B) and down to but not through the subchondral bone (C). Blisters are included in this grade (D).



### GRADE 4

Severely abnormal: Lesions through the subchondral bone

Cartilage is aneural and therefore superficial defects (ICRS grade 1+2) do not result in pain<sup>62</sup>. However, defects that extend deeper in the cartilage may eventually become symptomatic<sup>59</sup>. Symptoms include pain, joint locking and functional impairment<sup>59</sup>. Cartilage has very limited self-repair capacity<sup>63</sup> which was already well appreciated in 1743 by William Hunter who famously quoted:

*'If we consult the standard chirurgical writers from Hippocrates down to the present age we shall find, that an ulcerated cartilage is universally allowed to be a very troublesome disease and that once destroyed it is never recovered'*<sup>7</sup>.

Cartilage defects have been established to be an independent risk factor for osteoarthritis (OA) development<sup>64</sup> and therefore it is essential to develop effective treatment strategies. Approaches in treatment of cartilage defects can be divided into several operative approaches (**Table 2**). A major disadvantage of microfracturing and subchondral drilling is that in the majority of cases fibrocartilage forms with inferior biomechanical properties

compared to healthy hyaline cartilage<sup>65</sup>. Autologous chondrocyte implantation<sup>66</sup> and Matrix-induced autologous chondrocyte implantation have superior clinical outcomes compared to microfracture<sup>67</sup>, but disadvantages of these techniques include the high economic burden, transplant failure and graft hypertrophy<sup>68</sup>. A limitation of the osteochondral autograft/allografting technique is that the size of the cartilage defect is a limit to use the technique and morbidity is induced to donor site when using autografts<sup>69</sup>. Non-biological resurfacing techniques rely on osseointegration rather than chondrogenesis and may be considered in the middle-aged patient<sup>70</sup>.

**Table 2: Operative approaches in the treatment of cartilage defects.**

OPERATIVE APPROACHES	
Regenerative Techniques	Resurfacing Techniques
<p><b>Microfracture and subchondral drilling:</b> subchondral bone is penetrated with a drill to establish access of blood flow and influx of bone marrow stem cells into the defect which can initiate a repair response in the defect<sup>71</sup>.</p>	<p><b>Osteochondral auto- and allografting<sup>60,72</sup>:</b> harvesting osteochondral plugs from a non-weight bearing surface in the ipsilateral joint (autograft) or harvesting from another donor (allograft) and replacing it into the defect.</p>
<p><b>ACI and MACI:</b> harvesting cartilage from a non-weight bearing surface of the ipsilateral joint. Next, chondrocytes are isolated and expanded in a laboratory after which the expanded chondrocytes are placed either unseeded (ACI) or pre-seeded into a matrix (MACI) into the defect during a second surgical procedure<sup>59</sup>.</p>	<p><b>Non-biological resurfacing techniques:</b> focal knee resurfacing implants (FKRI)<sup>73,74,75,76,77</sup>.</p>

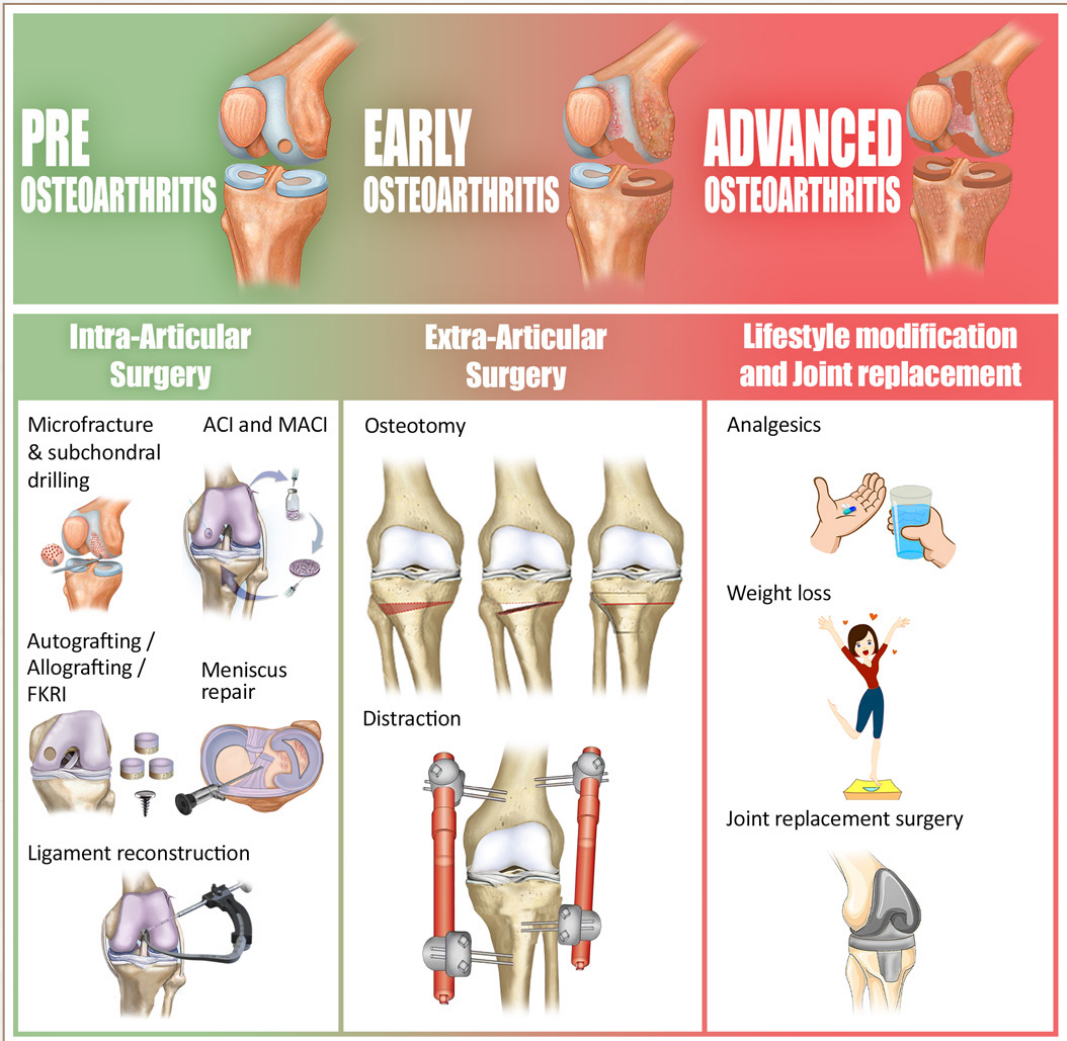
**ACI:** autologous chondrocyte implantation. **MACI:** matrix-induced autologous chondrocyte implantation



## Homeostatic Failure of a Musculoskeletal Organ: Knee Osteoarthritis

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A disturbance in the balance between loading and load capacity may occur due to several reasons such as cartilage defects, an ACL rupture, varus or valgus leg malalignment, meniscus pathology, obesity, or an increasing age<sup>78</sup>. Various intra-articular joint preserving treatments such as cartilage repair surgery (**Table 2**), ACL reconstruction surgery or meniscal repair surgery may be performed to improve knee joint quality (Figure 2). Over time, these surgical strategies may fail or not suffice anymore leading to a disbalance in the knee joint between loading and load capacity. A disbalance between loading and load capacity will drive the knee joint into a catabolic condition leading to a compromised knee joint homeostasis with decreased knee joint quality<sup>79</sup>. When external and internal factors outpace physiological limits and the capacity for adaptive responses, 'joint homeostasis' is disturbed and knee osteoarthritis (OA) is prone to develop<sup>80,81</sup>. This development is a process of many years<sup>82</sup>. Early in the disease process (early-OA), extra-articular joint preserving treatments can be performed to delay progression of the disease (Figure 2). Examples of these treatments are an osteotomy, which is a surgical procedure realigning the knee joint thereby decreasing excessive forces on the medial or lateral side of the knee joint<sup>83</sup>. Another example is knee joint distraction, which is a technique that unloads the tibiofemoral joint using an external fixation frame<sup>84</sup>. Eventually, when joint preserving treatments fail and knee OA reaches an end-stage, total knee joint replacement surgery can be performed to relieve pain (Figure 2).



**Figure 2: Approaches in the treatment of decreased knee joint quality.**

Approaches in joint preserving treatment can be divided into different operative approaches. Joint trauma can lead to damage to intra-articular structures. When osteoarthritis is not present (pre-OA), this damage can be combatted by means of intra-articular surgery such as cartilage repair strategies, meniscus repair or ligament reconstruction surgery. Over time, these strategies may not suffice and osteoarthritis can develop. Early in the disease process (early-OA), extra-articular surgery such as a pressure relieving osteotomy or distraction surgery can be performed. These strategies may fail over time and osteoarthritis can proceed to a more advanced stage (advanced-OA). During this phase, conservative strategies such as weight loss and the use of analgesics can be used to combat pain. These strategies may also be used in earlier phases of the disease (pre-OA and early-OA). Eventually, when osteoarthritis progresses to an advanced stage, joint replacement surgery becomes inevitable.

Knee OA is the most common joint disease of adults worldwide while the average age for developing knee OA is currently decreasing<sup>85,86</sup>. The most common symptoms are joint pain and joint stiffness. While the etiopathogenesis of knee OA remains obscure, it is now well appreciated that OA should not be considered as one disease, but rather as a final common pathway secondary to many predisposing factors. This 'final common pathway' is characterized by one of the most obvious features of OA: cartilage degeneration. Characteristic for the disease is the compromised balance in anabolic and catabolic activities in the cartilage tissue, with a shift towards catabolic activities, resulting in tissue degradation predominating over the capacity of tissue repair<sup>79</sup>. Clinically, joint cartilage degeneration is indirectly evaluated using the Kellgren & Lawrence scoring system, which evaluates the presence of osteophytes and joint space narrowing (due to articular cartilage loss) on knee X-rays<sup>87</sup>. However, more sensitive imaging methods such as MRI are emerging<sup>88</sup>.

In the last decade, it became clear that, next to cartilage, other intra-articular tissues such as the subchondral bone, meniscus, synovium, Hoffa's fat pad and peri-articular muscles participate in the disease process leading to 'joint failure'<sup>89</sup>. Thus, despite being considered as a cartilage problem, observations in these joint tissues are the impetus to consider OA as a total joint disease. Given the anatomical juxtaposition of intra-articular tissues such as articular cartilage, synovium, Hoffa's fat pad and meniscus, studying articular cartilage while taking the entire joint into account, is expected to aid in understanding cartilage pathology. Primarily focusing on the articular cartilage in joint pathologies would fail to notice the well-accepted phenomenon that living cells and tissues act in concert to maintain a constant milieu interne<sup>2</sup>. The following chapters will illustrate the pathological changes that occur in different intra-articular tissues in knee OA.



## Pathological and Structural Changes in Osteoarthritic Cartilage

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While healthy articular cartilage has a smooth surface, in OA the articular cartilage tissue becomes disorganized. Typical features of osteoarthritic cartilage are surface fibrillation, fissures and erosions<sup>90</sup>. In the healthy state, chondrocytes in normal adult cartilage display a quiescent phenotype with little turnover of their matrix<sup>91</sup>. When OA develops, chondrocytes turn from a quiescent state into an activated state<sup>92</sup>. Characteristic of this activation are chondrocyte proliferation, cluster formation and increased synthesis of matrix proteins but also matrix degrading enzymes<sup>90</sup>. It has been suggested that this activation may be regarded as an ectopic recapitulation of endochondral ossification, since the osteoarthritic chondrocyte displays features of hypertrophic chondrocytes also seen in the growth plate, and is typically characterized by collagen type X expression<sup>25,93,94,95,96,97</sup>.

The activation of the chondrocyte and the consequent increase in synthesis of matrix-degrading enzymes results in a misbalance, shifted towards a catabolic phenotype. The osteoarthritic chondrocyte increases the synthesis of proteinases that are able to degrade the main cartilage ECM components, aggrecan and collagen. These proteinases include aggrecanases of the A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) family and metalloproteinases, but also other proteinases such as serine proteinases and cysteine proteinases<sup>98</sup>. Central roles have been attributed to metalloproteinase 13 (MMP-13) in degrading collagen type 2 and the aggrecanases ADAMTS4 and ADAMTS5 in degrading aggrecan<sup>98</sup>.

Next to the expression of matrix degrading proteinases, the osteoarthritic chondrocyte is characterized by activation of several pathways involved in metabolic and inflammatory processes, which can enhance catabolic events<sup>92</sup>. These include the mitogen-induced protein kinase pathways and canonical NF- $\kappa$ B (p65/p50) signaling pathway. Activation of these pathways can subsequently lead to expression of inflammatory molecules such as nitric oxide synthase (NOS) 2, cyclooxygenase (COX) 2 and interleukin-1 beta (IL-1 $\beta$ )<sup>92</sup>. Furthermore, breakdown products of cartilage matrix proteins (damage-associated

molecular patterns (DAMPs)<sup>99</sup> are generated in osteoarthritic cartilage<sup>100,101</sup>, which can subsequently stimulate the production of inflammatory cytokines and MMPs by articular chondrocytes resulting in a vicious circle loop<sup>100,102,103</sup>.

## Pathological and Structural Changes in Osteoarthritic Synovium

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Traditionally being classified as a non-inflammatory arthropathy, research in the last decades led to the currently held doctrine postulating that OA contains an inflammatory component<sup>104</sup>. A mild inflammatory infiltrate has been described in synovial samples of knee OA patients<sup>105</sup>. Typical histological observations related to the synovitis seen in knee OA include infiltration of lymphocytes and macrophages, villous hyperplasia and subintimal thickening<sup>106</sup>. Macrophage infiltration in the synovium is increased in early OA patients compared to more advanced disease<sup>107</sup>. It could be hypothesized that the inflammation in the synovium is a reaction to degraded cartilage debris, which leads to the production of inflammatory cytokines by the irritated synovial tissue, which will lead to more cartilage breakdown. On the other hand, the inflammation seen in the synovium might be the initiating etiological factor leading to cartilage breakdown. Several studies have been published supporting the role of an inflamed synovium in cartilage breakdown. Patients with progressive knee OA display higher CRP serum levels compared to patients without OA<sup>108</sup> and semi-quantitative synovitis scoring on contrast-enhanced MRI was associated with OA disease severity as assessed by radiographs<sup>109</sup>. In addition, in a study with 422 knee OA patients enrolled, inflammatory changes observed by arthroscopy in perimeniscal synovium were associated with more severe worsening of medial chondropathy<sup>110</sup>. Further support comes from a study performed with patients without cartilage damage on MRI or without radiographic OA at baseline, in which a significant association was shown between baseline effusion synovitis and future cartilage damage<sup>111</sup>. Synovitis is not only found in late OA, but also in early OA patients. In 2002, Oehler *et al.* found synovial pathology in early OA patients, suggesting the synovium to be pathogenically involved in the OA disease process<sup>46</sup>. Pessler *et al.* showed increased infiltration of macrophages

and T-cells in OA synovium compared to synovium of healthy subjects<sup>112</sup> and Scanzello *et al.* reported increased levels of the inflammatory cytokine IL-15 in the synovial fluid of early OA patients<sup>113</sup>. Synovitis in OA has therapeutical implications from 2 points of view. As the synovium contains nerve fibers, the inflammation in the synovium itself can generate pain. Indeed, a correlation has been demonstrated between synovial inflammation and pain in knee OA<sup>114</sup>. On the other hand, synovial inflammation can lead to joint stiffness and more severe cartilage degeneration, which will cause denuded bony surfaces abutting each other causing pain. Next to associations found between synovitis and OA disease progression, several studies also reported correlations between synovitis and symptoms in knee OA patients<sup>115</sup>, indicating that treatments targeting the inflamed synovium in OA patients may have pain killing effects<sup>116</sup>.

## Pathological and Structural Changes in Hoffa's Fat Pad

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Adipose tissue has been described to possess more functions than solely acting to store energy from excess calories. Disturbances in the immunological functions of omental tissue may lead to an impairment in the defense against pathogens or mechanical damage<sup>117</sup>. Similarly, one can hypothesize that disturbances in intra-articular adipose tissues may lead to pathological joint diseases such as OA. The HFP is the largest sized adipose tissue in the knee joint and has long been believed to have a structural function without a metabolic role. Nevertheless, this fatty tissue could be an important participant in knee OA<sup>51</sup>. Besides adipocytes, this tissue also contains immune cells such as granulocytes, macrophages and lymphocytes<sup>50</sup>. The HFP contains a higher concentration of immune cells than subcutaneous adipose tissue, pointing to a possible inflammatory role of this tissue<sup>118</sup>.

Similar to synovium, inflammatory changes have been found in osteoarthritic HFP. Evidence is accumulating that inflammatory changes in the HFP itself may be involved in the OA disease process. For instance, superolateral edema in the HFP has been shown to be associated with future patellar cartilage loss<sup>119</sup>. Furthermore, Clockaerts *et al.* already showed that the HFP is capable of secreting large amounts of inflammatory cytokines such as IL-1 $\beta$  and Tumor Necrosis Factor- $\alpha$  (TNF $\alpha$ )<sup>120</sup>. These inflammatory cytokines are known



to play key roles in degradation of the cartilage ECM. They are able to stimulate expression and release of proteinases such as collagenases and aggrecanases, which degrade collagen and aggrecan<sup>121</sup>. It was also shown that, when an inflammatory stimulus (IL-1 $\beta$ ) is applied to the HFP, it responds with an increased cytokine production<sup>120</sup>. It is likely that production and release of these inflammatory mediators by the HFP can lead to inflammatory and destructive responses in OA cartilage.

Signal intensity alterations in HFP have been detected on MRI, which may be related to inflammatory changes in the HFP tissue<sup>122</sup>. Given the presence of an extensive network of nerve fibers in the HFP<sup>123,124</sup>, the association of signal intensity alterations on MRI with clinical symptoms in patients with knee OA<sup>125</sup> is not surprising and points to another potential intra-articular source of pain in patients with knee OA.

In conclusion, next to cartilage and synovium, there is abundant evidence that the HFP is another intra-articular tissue that actively participates in the OA disease process and should be considered when developing treatment strategies to improve knee joint homeostasis and slow down knee OA progression.

## Pathological and Structural Changes in the Meniscus

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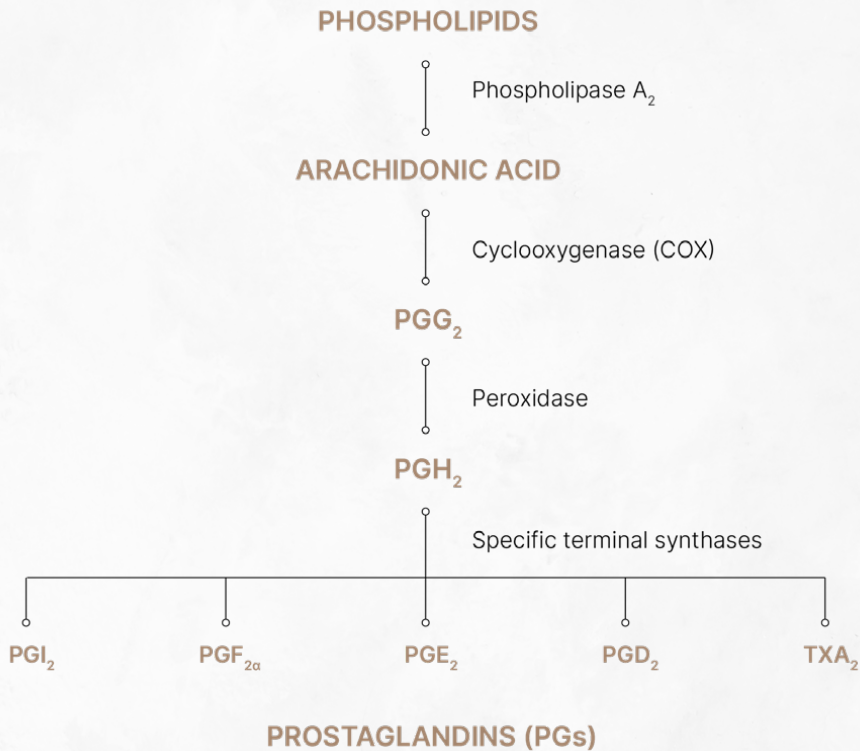
While it has been well established that damage to the meniscus increases the risk of developing knee OA<sup>126</sup>, it is now recognized that the meniscus itself shows pathologic changes which resemble the pathologic changes seen in osteoarthritic cartilage. Like in osteoarthritic cartilage, the meniscus displays changes such as matrix fibrillation, calcification, cell clusters and cell death<sup>127,128</sup>. In view of the essential biomechanical role of menisci in preserving adequate knee function, pathological changes in meniscus structure indeed may contribute to knee OA progression from a biomechanical point of view. In addition, it is also essential to realize that the meniscus has been shown to display molecular and cellular alterations, which could, via secreting catabolic mediators in the synovial fluid compromise knee joint homeostasis<sup>129</sup>.

## Improving Knee Joint Macro-Environment: A Role for Targeting Inflammatory Pathways

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Reduction in load bearing capacity of the knee joint, due to cartilage damage, meniscal damage or any other factor, will lead to joint inflammation<sup>130</sup> and drive the knee joint into a catabolic direction, making it prone for knee OA<sup>131</sup>. Knee OA is not yet a curable disease. With the aging of the world population, preventive measures and treatment strategies that can slow down the progression of knee OA are warranted. Since knee OA has an inflammatory component, targeting inflammatory stimuli that lead to catabolic responses in OA is a potential approach to provide effective treatments to slow down progression of the disease. It is now well established that some signaling pathways are specifically induced in an inflammatory environment. In this context, cyclooxygenase-2 (COX-2) has been demonstrated to be involved in inflammation<sup>132</sup>. Cyclooxygenases (COXs) are enzymes responsible for the oxidation of arachidonic acid (AA), a poly unsaturated omega-6 fatty acid, which results in the conversion to prostaglandin G<sub>2</sub> (PGG<sub>2</sub>)<sup>133</sup>. Next, the peroxidase activity of the COX enzyme drives the reduction of PGG<sub>2</sub> to PGH<sub>2</sub> (Figure 3). Specific synthases then convert PGH<sub>2</sub> to the main prostaglandin subgroups: PGE<sub>2</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, PGF<sub>2α</sub> and Thromboxane A<sub>2</sub> (TXA<sub>2</sub>)<sup>134</sup>. These prostaglandins act as autocrine and paracrine lipid mediators to maintain local homeostasis in the body<sup>135,136</sup>. Three COX isoforms have been described: COX-1, COX-2 and COX-3<sup>137,138</sup>. COX-1 is constitutively expressed by most mammalian cells and regarded as the 'housekeeping' cyclooxygenase, responsible for the production of basal prostaglandin levels which maintain local homeostasis. COX-3 is a splice variant of COX-1 and virtually nothing is known about its role in musculoskeletal tissues<sup>138</sup>. COX-2 is considered a pro-inflammatory enzyme, a main target for the treatment of inflammatory diseases and it is encoded by the gene *PTGS2*<sup>132,139</sup>. While the COX-1 gene contains cis-regulatory elements contributing to the constitutive expression of COX-1<sup>139</sup>, the promotor of the COX-2 gene contains several inducible enhancer elements<sup>132</sup>. COX-2 expression is upregulated in inflamed joint tissues<sup>140,141,142</sup> and its expression is induced in cartilage, synovium and also in the HFP upon stimulation with inflammatory mediators<sup>143,144,145</sup>. The main reason for considering COX-2 as a pathological enzyme

is that it can be induced by inflammation-associated factors pointing to a possible role in knee OA<sup>146</sup>.



**Figure 3: Biosynthesis of Prostaglandins.**

Membrane-bound phospholipids are converted into Arachidonic Acid (AA) by phospholipase A<sub>2</sub> (PLA<sub>2</sub>). COX first converts AA to PGG<sub>2</sub> and then to PGH<sub>2</sub>. Distinct terminal synthases convert PGH<sub>2</sub> to TXA<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGE<sub>2</sub>, or prostacyclin (PGI<sub>2</sub>).



Given the inflammatory component in knee OA and considering the fact that COX-2 has been described as an inflammatory enzyme, it has been suggested that COX-2 inhibitors may be used as a therapeutic agent in OA. Traditionally, COX-inhibitors (belonging to a class of drugs called non-steroidal anti-inflammatory drugs (NSAID)) have been used to provide anti-analgesic effects. Inhibition of COX enzymes results in reduced production of prostaglandins, which besides having physiological functions, are important mediators of inflammation and pain<sup>147</sup>. However, side effects, such as gastrointestinal ulceration and bleeding, were accepted as inevitable consequences of the inhibition of the activity of not only the inflammatory COX-2 but also COX-1. Because COX-2 is clearly associated with inflammation, and COX-1 is considered to fulfill a physiological role, selective COX-2 inhibitors have been designed<sup>148</sup>. Celecoxib is a selective COX-2 inhibitor which is currently used as an analgesic by patients with inflammatory arthritic diseases such as rheumatoid arthritis and OA<sup>149</sup>.

Taken together, there is a growing body of evidence showing that once joint homeostasis is disturbed, joint pathologies such as OA can develop. OA should be considered as a total joint disease in which joint homeostasis is compromised and in which different intra-articular tissues are involved, which will lead to impaired knee joint quality. Since experimental evidence clearly shows the association between inflammation and cartilage degeneration, developing therapies for joint pathologies such as cartilage defects and OA should focus on inflammation in the joint as a whole. Insights into modulating inflammation in different intra-articular tissues will further contribute to developing new treatment strategies that can aid in cartilage repair strategies and slow down progression of knee OA.

## Aims and Outline of This Thesis

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### 1. To Investigate the Molecular Mechanisms Involved in the Micro-Environmental Improvement of the Chondrocyte Phenotype

Cartilage defects are a major risk factor for knee OA and *in vitro* expansion of human articular chondrocytes can be utilized for regenerative medicine purposes to restore cartilage tissue<sup>59</sup>. For tissue engineering purposes, it is of utmost importance that after *in vitro* expansion, the chondrocyte is reimplanted in the knee joint in a cellular phenotype that resembles the native chondrocyte as good as possible. In **Chapter 2** and **Chapter 3** we have evaluated methods to improve knee joint quality on the cellular level. In **Chapter 2** we aimed to elucidate the molecular mechanisms involved in the improvement of the chondrocyte phenotype after *in vitro* expansion using an oxygen tension closely resembling the tension in cartilage *in vivo* environment, i.e. 1 % pO<sub>2</sub>. We have evaluated the effects of oxygen tension on TGF- $\beta$  signaling, which is known to play a key role in regulating the chondrocyte phenotype<sup>150</sup>. Next to oxygen tension, osmolarity is another factor that has been demonstrated to improve the chondrocyte phenotype *in vitro*<sup>151</sup>. In **Chapter 3**, we investigated the molecular mechanisms involved in the improvement of the chondrocyte phenotype after *in vitro* expansion under osmolarity closely resembling values found in the *in vivo* environment, i.e. 380 mOsm.

### 2. To Investigate and to Modulate the Macro-Environment in the Knee Joint

While the chondrocyte phenotype is important when cells are reimplanted into a knee joint, it is also essential to reimplant chondrocytes in a suitable 'macro-environment', because disturbance of this macro-environment has been shown to lead to cartilage degradation<sup>152</sup>. This disturbance of the joint macro-environment may be caused by a disturbed equilibrium in knee joint loading and knee joint load capacity due to for instance cartilage or meniscal pathology. Such a biomechanical mismatch can lead to inflammation in the entire knee joint<sup>130</sup>, eventually leading to a self-perpetuating inflammatory vicious circle resulting in 'total organ failure'<sup>90</sup>. After our research in **Chapter 2** and **Chapter 3**, which focused on

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methods to improve knee joint quality on the cellular level, we continued to investigate methods to improve knee joint quality on the tissue level. One of the intra-articular tissues which has been suggested to be a contributor to a self-perpetuating inflammatory vicious cycle participating in the knee OA process is the HFP<sup>50</sup>. In **Chapter 4**, we hypothesized that the HFP from OA patients releases higher amounts of prostanoids than the HFP from non-OA patients. Prostanoids are important factors involved in inflammation, and we evaluated whether we could modulate the prostanoid release profile by the OA HFP with the anti-inflammatory COX-2 selective drug celecoxib.

We finally continued to investigate methods to improve knee joint quality on the total joint level. In **Chapter 5**, we have conducted a systematic review to investigate the ability of selective COX-2 inhibitors to act chondroprotective *in vivo*, depending on their route of administration. We reviewed whether compared to systemic oral administration, the intra-articular administration of selective COX-2 inhibitors is more potent in modulating knee joint pathological changes. To target the inflamed knee joint, thereby potentially improving knee joint macro-environment, a drug-delivery system (DDS) is an effective means to support an intra-articular therapy. In comparison to bolus drug injections, a DDS offers the advantage of minimizing systemic toxicity, avoiding the need for frequent intra-articular injections and allowing for prolonged drug residence time for long-term treatment in a chronic disease such as knee OA. We therefore investigated release behavior, biocompatibility and degradation of polyester amide (PEA) microspheres loaded with celecoxib in **Chapter 6** in order to evaluate the potential of this DDS to be used for anti-inflammatory treatment in knee OA.

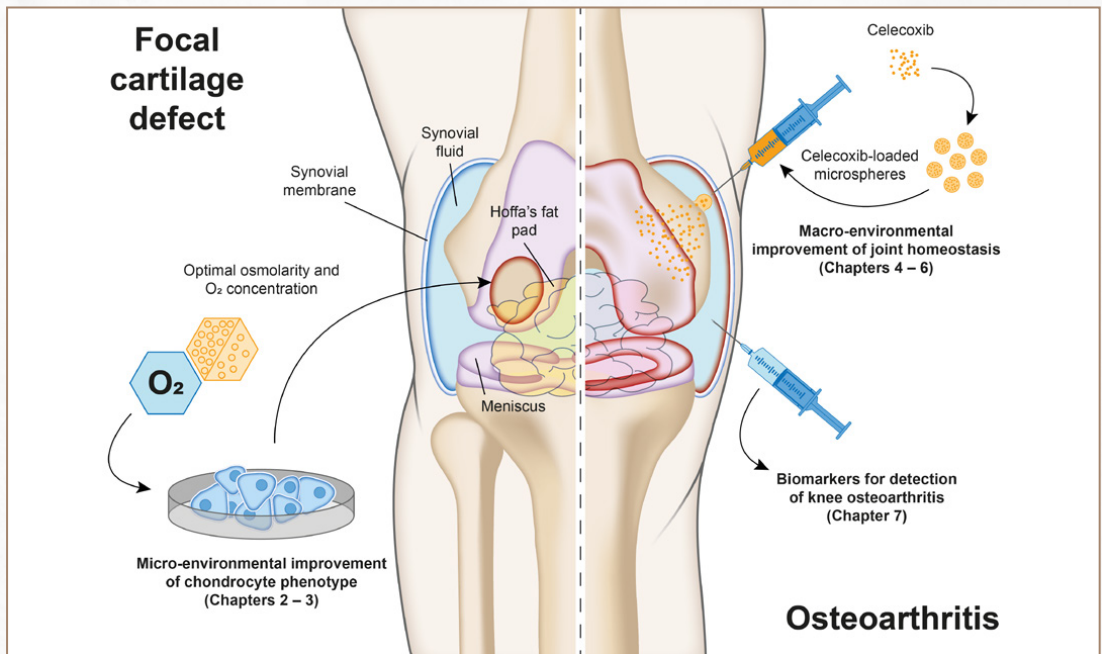
Because the diagnosis of knee OA is made when the disease has reached an end-stage stadium, early intervention in order to slow down knee OA disease progression is currently not possible. In a total joint disease such as knee OA, identification of intra-articular tissue type-dependent and OA-associated protein species in the synovial fluid is expected to fuel the development of synovial fluid-based diagnostic approaches. This would enable the grading and typing of knee OA on a molecular basis, and can potentially improve efficacy of treatment strategies. In **Chapter 7**, we therefore analyzed the protein secretome of different intra-articular tissues by means of mass-spectrometry analysis and subsequently



evaluated whether tissue-dependent secreted proteins were OA-specific by comparing their abundance in OA versus non-OA synovial fluid.

Finally, **Chapter 8** and **Chapter 9** present the general discussion, the conclusion and the impact paragraph in which the results of this thesis are discussed and summarized and in which potential clinical applications are highlighted.

In Figure 4 a schematic overview can be found demonstrating the specific aims of this thesis per chapter.



**Figure 4: Schematic overview of the specific aims per chapter.**

Chapter 2 and 3 focuses on improving knee joint homeostasis on the cellular level. Actions of micro-environmental influences such as hypoxia and hyperosmolarity on the chondrocyte phenotype were investigated. Chapter 4 focuses on improving knee joint homeostasis on the tissue level by treating HFP of OA and cartilage defect patients with celecoxib. Chapters 5 and 6 focused on improving knee joint homeostasis on a total joint level by means of intra-articular treatment with celecoxib. Finally, Chapter 7 investigated possible identification of intra-articular tissue type-dependent and OA-associated protein species in the synovial fluid.

## References

- 1 J.A. A. Homeostatic Processes for Thermoregulation. *Nature Education Knowledge*. 2011;3(10):7.
- 2 Cannon WB. *The wisdom of the body*. New York,: W.W. Norton & Company; 1939. xviii, 19-333 p. p.
- 3 Anderson EM. The Prince of Medicine: Galen in the Roman Empire. *Library Journal*. 2013;138(11):98.
- 4 Sina I. *Al-Qanun fi al-Tibb*. 1025.
- 5 Gray H, Warwick R, Williams PL. *Gray's anatomy*. 35th ed. London: Longman; 1973. xvi, 1471 p. p.
- 6 Hunziker EB, Quinn TM, Hauselmann HJ. Quantitative structural organization of normal adult human articular cartilage. *Osteoarthritis Cartilage*. 2002;10(7):564-72.
- 7 Hunter W. Of the structure and disease of articulating cartilages. 1743. *Clin Orthop Relat Res*. 1995(317):3-6.
- 8 Ogston A. On Articular Cartilage. *J Anat Physiol*. 1875;10(Pt 1):48 1-74.
- 9 Reeves HA. On the Structure of the Matrix of Human Articular Cartilage. *Br Med J*. 1876;2(828):616.
- 10 Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect*. 1998;47:477-86.
- 11 Frost HM. The regional acceleratory phenomenon: a review. *Henry Ford Hosp Med J*. 1983;31(1):3-9.
- 12 Wolff J. Das gesetz der transformation der knochen. *A Hirshwald*. 1892;1:1-152.
- 13 Kim YJ, Bonassar LJ, Grodzinsky AJ. The role of cartilage streaming potential, fluid flow and pressure in the stimulation of chondrocyte biosynthesis during dynamic compression. *J Biomech*. 1995;28(9):1055-66.
- 14 Waldman SD, Spiteri CG, Grynepas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed *in vitro*. *J Orthop Res*. 2003;21(4):590-6.
- 15 Vanwanseele B, Eckstein F, Knecht H, Spaepen A, Stussi E. Longitudinal analysis of cartilage atrophy in the knees of patients with spinal cord injury. *Arthritis Rheum*. 2003;48(12):3377-81.
- 16 Hinterwimmer S, Krammer M, Krotz M, Glaser C, Baumgart R, Reiser M, *et al*. Cartilage atrophy in the knees of patients

- after seven weeks of partial load bearing. *Arthritis Rheum.* 2004;50(8):2516-20.
- 17 Lewis R, Asplin KE, Bruce G, Dart C, Mobasher A, Barrett-Jolley R. The role of the membrane potential in chondrocyte volume regulation. *J Cell Physiol.* 2011;226(11):2979-86.
- 18 Urban JP, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol.* 1993;154(2):262-70.
- 19 O'Hara BP, Urban JP, Maroudas A. Influence of cyclic loading on the nutrition of articular cartilage. *Ann Rheum Dis.* 1990;49(7):536-9.
- 20 Morales TI, Joyce ME, Sobel ME, Danielpour D, Roberts AB. Transforming growth factor-beta in calf articular cartilage organ cultures: synthesis and distribution. *Arch Biochem Biophys.* 1991;288(2):397-405.
- 21 Madej W, van Caam A, Blaney Davidson EN, van der Kraan PM, Buma P. Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling. *Osteoarthritis Cartilage.* 2014;22(7):1018-25.
- 22 White A, Wallis G. Endochondral ossification: a delicate balance between growth and mineralisation. *Curr Biol.* 2001;11(15):R589-91.
- 23 Ford JL, Robinson DE, Scammell BE. Endochondral ossification in fracture callus during long bone repair: the localisation of 'cavity-lining cells' within the cartilage. *J Orthop Res.* 2004;22(2):368-75.
- 24 Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol.* 2008;40(1):46-62.
- 25 Ripmeester EGJ, Timur UT, Caron MMJ, Welting TJM. Recent Insights into the Contribution of the Changing Hypertrophic Chondrocyte Phenotype in the Development and Progression of Osteoarthritis. *Front Bioeng Biotechnol.* 2018;6:18.
- 26 Murphy CL, Sambanis A. Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes. *Tissue Eng.* 2001;7(6):791-803.
- 27 Leijten JC, Moreira Teixeira LS, Landman EB, van Blitterswijk CA, Karperien M. Hypoxia inhibits hypertrophic differentiation and endochondral ossification in explanted tibiae. *PLoS One.* 2012;7(11):e49896.
- 28 van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, *et al.* Physiological



## Chapter 1

- tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 *in vitro*. *Arthritis Res Ther*. 2010;12(3):R100.
- 29 Zhou S, Cui Z, Urban JP. Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study. *Arthritis Rheum*. 2004;50(12):3915-24.
- 30 Hodge JA, McKibbin B. The nutrition of mature and immature cartilage in rabbits. An autoradiographic study. *J Bone Joint Surg Br*. 1969;51(1):140-7.
- 31 Silver IA. Measurement of pH and ionic composition of pericellular sites. *Philos Trans R Soc Lond B Biol Sci*. 1975;271(912):261-72.
- 32 Anderson DE, Markway BD, Weekes KJ, McCarthy HE, Johnstone B. Physioxia Promotes the Articular Chondrocyte-Like Phenotype in Human Chondroprogenitor-Derived Self-Organized Tissue. *Tissue Eng Part A*. 2017.
- 33 Kellner K, Liebsch G, Klimant I, Wolfbeis OS, Blunk T, Schulz MB, *et al*. Determination of oxygen gradients in engineered tissue using a fluorescent sensor. *Biotechnol Bioeng*. 2002;80(1):73-83.
- 34 Lund-Olesen K. Oxygen tension in synovial fluids. *Arthritis Rheum*. 1970;13(6):769-76.
- 35 Brighton CT, Heppenstall RB. Oxygen tension in zones of the epiphyseal plate, the metaphysis and diaphysis. An *in vitro* and *in vivo* study in rats and rabbits. *J Bone Joint Surg Am*. 1971;53(4):719-28.
- 36 Grimshaw MJ, Mason RM. Bovine articular chondrocyte function *in vitro* depends upon oxygen tension. *Osteoarthritis Cartilage*. 2000;8(5):386-92.
- 37 Maroudas A, Venn M. Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. II. Swelling. *Ann Rheum Dis*. 1977;36(5):399-406.
- 38 Buckwalter JA, Mankin HJ, Grodzinsky AJ. Articular cartilage and osteoarthritis. *Instr Course Lect*. 2005;54:465-80.
- 39 Bhalla A, Sankaralingam S, Dundas R, Swaminathan R, Wolfe CD, Rudd AG. Influence of raised plasma osmolality on clinical outcome after acute stroke. *Stroke*. 2000;31(9):2043-8.
- 40 Charkoudian N, Eisenach JH, Joyner MJ, Roberts SK, Wick DE. Interactions of plasma osmolality with arterial and central venous pressures in control of sympathetic activity and heart rate in humans. *Am J Physiol Heart Circ Physiol*. 2005;289(6):H2456-60.
- 41 Urban JP. The chondrocyte: a cell under pressure. *Br J Rheumatol*. 1994;33(10):901-8.

- 42 Urban JP, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol.* 1993;154(2):262-70.
- 43 Lesperance LM, Gray ML, Burstein D. Determination of fixed charge density in cartilage using nuclear magnetic resonance. *J Orthop Res.* 1992;10(1):1-13.
- 44 Venn M, Maroudas A. Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. I. Chemical composition. *Ann Rheum Dis.* 1977;36(2):121-9.
- 45 Adams MA. The mechanical environment of chondrocytes in articular cartilage. *Biorheology.* 2006;43(3-4):537-45.
- 46 Oehler S, Neureiter D, Meyer-Scholten C, Aigner T. Subtyping of osteoarthritic synoviopathy. *Clin Exp Rheumatol.* 2002;20(5):633-40.
- 47 Smith MD. The normal synovium. *Open Rheumatol J.* 2011;5:100-6.
- 48 Smith MD. Immunohistochemistry of normal synovium. *Ann Rheum Dis.* 2004;63(11):1532-3; author reply 3.
- 49 Makris EA, Hadidi P, Athanasiou KA. The knee meniscus: structure-function, pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials.* 2011;32(30):7411-31.
- 50 Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, Van Osch GJ, Van Offel JF, Verhaar JA, *et al.* The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthritis Cartilage.* 2010;18(7):876-82.
- 51 Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, Van Osch GJ, Van Offel JF, Verhaar JA, *et al.* The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthritis Cartilage.* 2010;18(7):876-82.
- 52 Morrison R. On the functional aspects of the greater omentum. *BMJ.* 1906;1:76-8.
- 53 Rangel-Moreno J, Moyron-Quiroz JE, Carragher DM, Kusser K, Hartson L, Moquin A, *et al.* Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity.* 2009;30(5):731-43.
- 54 Garcia-Gomez I, Goldsmith HS, Angulo J, Prados A, Lopez-Hervas P, Cuevas B, *et al.* Angiogenic capacity of human omental stem cells. *Neurol Res.* 2005;27(8):807-11.
- 55 Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, *et al.* Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat

- mass loss. *Diabetes*. 2010;59(11):2817-25.
- 56 do Amaral R, Almeida HV, Kelly DJ, O'Brien FJ, Kearney CJ. Infrapatellar Fat Pad Stem Cells: From Developmental Biology to Cell Therapy. *Stem Cells Int*. 2017;2017:6843727.
- 57 Klein-Wieringa IR, Kloppenburg M, Bastiaansen-Jenniskens YM, Yusuf E, Kwekkeboom JC, El-Bannoudi H, *et al*. The infrapatellar fat pad of patients with osteoarthritis has an inflammatory phenotype. *Ann Rheum Dis*. 2011;70(5):851-7.
- 58 Bastiaansen-Jenniskens YM, Wei W, Feijt C, Waarsing JH, Verhaar JA, Zuurmond AM, *et al*. Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: a possible role for prostaglandin f2alpha. *Arthritis Rheum*. 2013;65(8):2070-80.
- 59 Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med*. 1994;331(14):889-95.
- 60 Falah M, Nierenberg G, Soudry M, Hayden M, Volpin G. Treatment of articular cartilage lesions of the knee. *Int Orthop*. 2010;34(5):621-30.
- 61 van den Borne MP, Raijmakers NJ, Vanlauwe J, Victor J, de Jong SN, Bellemans J, *et al*. International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture. *Osteoarthritis Cartilage*. 2007;15(12):1397-402.
- 62 Buckwalter JA, Mankin HJ. Articular cartilage repair and transplantation. *Arthritis Rheum*. 1998;41(8):1331-42.
- 63 Haldeman KO, Soto-Hall R. The Response of Articular Cartilage to Trauma: With Special Reference to the Knee Joint. *Calif Med*. 1948;69(3):179-82.
- 64 Crema MD, Nevitt MC, Guermazi A, Felson DT, Wang K, Lynch JA, *et al*. Progression of cartilage damage and meniscal pathology over 30 months is associated with an increase in radiographic tibiofemoral joint space narrowing in persons with knee OA—the MOST study. *Osteoarthritis Cartilage*. 2014;22(10):1743-7.
- 65 Steinwachs MR, Guggi T, Kreuz PC. Marrow stimulation techniques. *Injury*. 2008;39 Suppl 1:S26-31.
- 66 Ritter SY, Subbaiah R, Bebek G, Crish J, Scanzello CR, Krastins B, *et al*. Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis Rheum*. 2013;65(4):981-92.
- 67 Brittberg M, Recker D, Ilgenfritz J,



- Saris DBF, Group SES. Matrix-Applied Characterized Autologous Cultured Chondrocytes Versus Microfracture: Five-Year Follow-up of a Prospective Randomized Trial. *Am J Sports Med.* 2018;46(6):1343-51.
- 68 Harris JD, Siston RA, Brophy RH, Lattermann C, Carey JL, Flanigan DC. Failures, re-operations, and complications after autologous chondrocyte implantation--a systematic review. *Osteoarthritis Cartilage.* 2011;19(7):779-91.
- 69 Hangody L, Rathonyi GK, Duska Z, Vasarhelyi G, Fules P, Modis L. Autologous osteochondral mosaicplasty. Surgical technique. *J Bone Joint Surg Am.* 2004;86-A Suppl 1:65-72.
- 70 Jeuken RM, van Hugten PPW, Roth AK, Timur UT *et al.* A Systematic Review of Focal Cartilage Defect Treatments in Middle-Aged Versus Younger Patients. *Orthopaedic Journal of Sports Medicine.* October 2021. doi:10.1177/23259671211031244
- 71 Passler HH. [Microfracture for treatment of cartilage defects]. *Zentralbl Chir.* 2000;125(6):500-4.
- 72 Jeuken RM, Roth AK, Peters R, Van Donkelaar CC, Thies JC, Van Rhijn LW, *et al.* Polymers in Cartilage Defect Repair of the Knee: Current Status and Future Prospects. *Polymers (Basel).* 2016;8(6).
- 73 Becher C, Cantiller EB. Focal articular prosthetic resurfacing for the treatment of full-thickness articular cartilage defects in the knee: 12-year follow-up of two cases and review of the literature. *Arch Orthop Trauma Surg.* 2017;137(9):1307-17.
- 74 Nathwani D, McNicholas M, Hart A, Miles J, Bobic V. Partial Resurfacing of the Knee with the BioPoly Implant: Interim Report at 2 Years. *JB JS Open Access.* 2017;2(2):e0011.
- 75 Martinez-Carranza N, Hultenby K, Lagerstedt AS, Schupbach P, Berg HE. Cartilage Health in Knees Treated with Metal Resurfacing Implants or Untreated Focal Cartilage Lesions: A Preclinical Study in Sheep. *Cartilage.* 2019;10(1):120-8.
- 76 Cole BJ, Pascual-Garrido C, Grumet RC. Surgical management of articular cartilage defects in the knee. *Instr Course Lect.* 2010;59:181-204.
- 77 Pascual-Garrido C, Daley E, Verma NN, Cole BJ. A Comparison of the Outcomes for Cartilage Defects of the Knee Treated With Biologic Resurfacing Versus Focal Metallic Implants. *Arthroscopy.* 2017;33(2):364-73.
- 78 Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum.* 2012;64(6):1697-707.

## Chapter 1

- 79 Hunter DJ, Felson DT. Osteoarthritis. *BMJ*. 2006;332(7542):639-42.
- 80 Saris DB, Dhert WJ, Verboort AJ. Joint homeostasis. The discrepancy between old and fresh defects in cartilage repair. *J Bone Joint Surg Br*. 2003;85(7):1067-76.
- 81 Samuels J, Krasnokutsky S, Abramson SB. Osteoarthritis: a tale of three tissues. *Bull NYU Hosp Jt Dis*. 2008;66(3):244-50.
- 82 Ryd L, Brittberg M, Eriksson K, Jurvelin JS, Lindahl A, Marlovits S, *et al*. Pre-Osteoarthritis: Definition and Diagnosis of an Elusive Clinical Entity. *Cartilage*. 2015;6(3):156-65.
- 83 Insall JN. High tibial osteotomy in the treatment of osteoarthritis of the knee. *Surg Annu*. 1975;7:347-59.
- 84 van der Woude JA, Nair SC, Custers RJ, van Laar JM, Kuchuck NO, Lafeber FP, *et al*. Knee Joint Distraction Compared to Total Knee Arthroplasty for Treatment of End Stage Osteoarthritis: Simulating Long-Term Outcomes and Cost-Effectiveness. *PLoS One*. 2016;11(5):e0155524.
- 85 Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clinics in geriatric medicine*. 2010;26(3):355-69.
- 86 van der Kraan PM, Blaney Davidson EN, Blom A, van den Berg WB. TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. *Osteoarthritis Cartilage*. 2009;17(12):1539-45.
- 87 Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. *Ann Rheum Dis*. 1957;16(4):494-502.
- 88 Oei EHG, Wick MC, Muller-Lutz A, Schleich C, Miese FR. Cartilage Imaging: Techniques and Developments. *Semin Musculoskelet Radiol*. 2018;22(2):245-60.
- 89 Felson DT. Developments in the clinical understanding of osteoarthritis. *Arthritis Res Ther*. 2009;11(1):203.
- 90 Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum*. 2012;64(6):1697-707.
- 91 Repo RU, Mitchell N. Collagen synthesis in mature articular cartilage of the rabbit. *J Bone Joint Surg Br*. 1971;53(3):541-8.
- 92 Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther*. 2009;11(3):224.
- 93 Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, *et al*. Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. *Bone Miner*. 1991;15(2):151-63.
- 94 Aigner T, Frischholz S, Dertinger S, Beier F, Girkontaite I, von der Mark K. Type X

- collagen expression and hypertrophic differentiation in chondrogenic neoplasias. *Histochem Cell Biol.* 1997;107(6):435-40.
- 95 Aigner T, Reichenberger E, Bertling W, Kirsch T, Stoss H, von der Mark K. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. *Virchows Arch B Cell Pathol Incl Mol Pathol.* 1993;63(4):205-11.
- 96 von der Mark K, Frischholz S, Aigner T, Beier F, Belke J, Erdmann S, *et al.* Upregulation of type X collagen expression in osteoarthritic cartilage. *Acta Orthop Scand Suppl.* 1995;266:125-9.
- 97 von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, *et al.* Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. *Arthritis Rheum.* 1992;35(7):806-11.
- 98 Troeberg L, Nagase H. Proteases involved in cartilage matrix degradation in osteoarthritis. *Biochim Biophys Acta.* 2012;1824(1):133-45.
- 99 Rosenberg JH, Rai V, Dilisio MF, Agrawal DK. Damage-associated molecular patterns in the pathogenesis of osteoarthritis: potentially novel therapeutic targets. *Mol Cell Biochem.* 2017;434(1-2):171-9.
- 100 Homandberg GA, Wen C, Hui F. Cartilage damaging activities of fibronectin fragments derived from cartilage and synovial fluid. *Osteoarthr Cartilage.* 1998;6(4):231-44.
- 101 Zack MD, Arner EC, Anglin CP, Alston JT, Malfait AM, Tortorella MD. Identification of fibronectin neoepitopes present in human osteoarthritic cartilage. *Arthritis Rheum.* 2006;54(9):2912-22.
- 102 Pulai JI, Chen H, Im HJ, Kumar S, Hanning C, Hegde PS, *et al.* NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. *J Immunol.* 2005;174(9):5781-8.
- 103 Fichter M, Korner U, Schomburg J, Jennings L, Cole AA, Mollenhauer J. Collagen degradation products modulate matrix metalloproteinase expression in cultured articular chondrocytes. *J Orthop Res.* 2006;24(1):63-70.
- 104 Berenbaum F, van den Berg WB. Inflammation in osteoarthritis: changing views. *Osteoarthritis Cartilage.* 2015;23(11):1823-4.
- 105 Pessler F, Dai L, Diaz-Torne C, Gomez-Vaquero C, Paessler ME, Zheng DH, *et al.* The synovitis of "non-inflammatory" orthopaedic arthropathies: a quantitative histological and immunohistochemical analysis. *Ann Rheum Dis.* 2008;67(8):1184-7.



## Chapter 1

- 106 Pearle AD, Scanzello CR, George S, Mandl LA, DiCarlo EF, Peterson M, *et al.* Elevated high-sensitivity C-reactive protein levels are associated with local inflammatory findings in patients with osteoarthritis. *Osteoarthr Cartilage.* 2007;15(5):516-23.
- 107 Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis.* 2005;64(9):1263-7.
- 108 Spector TD, Hart DJ, Nandra D, Doyle DV, Mackillop N, Gallimore JR, *et al.* Low-level increases in serum C-reactive protein are present in early osteoarthritis of the knee and predict progressive disease. *Arthritis Rheum.* 1997;40(4):723-7.
- 109 Krasnokutsky S, Belitskaya-Levy I, Bencardino J, Samuels J, Attur M, Regatte R, *et al.* Quantitative magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis. *Arthritis Rheum.* 2011;63(10):2983-91.
- 110 Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. *Osteoarthr Cartilage.* 2005;13(5):361-7.
- 111 Roemer FW, Guermazi A, Felson DT, Niu J, Nevitt MC, Crema MD, *et al.* Presence of MRI-detected joint effusion and synovitis increases the risk of cartilage loss in knees without osteoarthritis at 30-month follow-up: the MOST study. *Ann Rheum Dis.* 2011;70(10):1804-9.
- 112 Pessler F, Chen LX, Dai L, Gomez-Vaquero C, Diaz-Torne C, Paessler ME, *et al.* A histomorphometric analysis of synovial biopsies from individuals with Gulf War Veterans' Illness and joint pain compared to normal and osteoarthritis synovium. *Clin Rheumatol.* 2008;27(9):1127-34.
- 113 Scanzello CR, Umoh E, Pessler F, Diaz-Torne C, Miles T, Dicarolo E, *et al.* Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. *Osteoarthritis Cartilage.* 2009;17(8):1040-8.
- 114 Torres L, Dunlop DD, Peterfy C, Guermazi A, Prasad P, Hayes KW, *et al.* The relationship between specific tissue lesions and pain severity in persons with knee osteoarthritis. *Osteoarthritis Cartilage.* 2006;14(10):1033-40.
- 115 Hill CL, Hunter DJ, Niu J, Clancy M, Guermazi A, Genant H, *et al.* Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis. *Ann Rheum Dis.* 2007;66(12):1599-603.

- 116 Torres L, Dunlop DD, Peterfy C, Guermazi A, Prasad P, Hayes KW, *et al.* The relationship between specific tissue lesions and pain severity in persons with knee osteoarthritis. *Osteoarthr Cartilage.* 2006;14(10):1033-40.
- 117 Liu M, Silva-Sanchez A, Randall TD, Meza-Perez S. Specialized immune responses in the peritoneal cavity and omentum. *J Leukoc Biol.* 2020.
- 118 Klein-Wieringa IR, Kloppenburg M, Bastiaansen-Jenniskens YM, Yusuf E, Kwেকেboom JC, El-Bannoudi H, *et al.* The infrapatellar fat pad of patients with osteoarthritis has an inflammatory phenotype. *Ann Rheum Dis.* 2011;70(5):851-7.
- 119 Haj-Mirzaian A, Guermazi A, Hafezi-Nejad N, Sereni C, Hakky M, Hunter DJ, *et al.* Superolateral Hoffa's fat pad (SHFP) oedema and patellar cartilage volume loss: quantitative analysis using longitudinal data from the Foundation for the National Institute of Health (FNIH) Osteoarthritis Biomarkers Consortium. *Eur Radiol.* 2018;28(10):4134-45.
- 120 Clockaerts S, Bastiaansen-Jenniskens YM, Feijt C, De Clerck L, Verhaar JA, Zuurmond AM, *et al.* Cytokine production by infrapatellar fat pad can be stimulated by interleukin 1beta and inhibited by peroxisome proliferator activated receptor alpha agonist. *Ann Rheum Dis.* 2012;71(6):1012-8.
- 121 Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.
- 122 Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.
- 123 Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.
- 124 Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.

## Chapter 1

- 125 Han W, Aitken D, Zhu Z, Halliday A, Wang X, Antony B, *et al.* Signal intensity alteration in the infrapatellar fat pad at baseline for the prediction of knee symptoms and structure in older adults: a cohort study. *Ann Rheum Dis.* 2016;75(10):1783-8.
- 126 Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, *et al.* Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med.* 2000;133(8):635-46.
- 127 Katsuragawa Y, Saitoh K, Tanaka N, Wake M, Ikeda Y, Furukawa H, *et al.* Changes of human menisci in osteoarthritic knee joints. *Osteoarthr Cartilage.* 2010;18(9):1133-43.
- 128 Pauli C, Grogan SP, Patil S, Otsuki S, Hasegawa A, Koziol J, *et al.* Macroscopic and histopathologic analysis of human knee menisci in aging and osteoarthritis. *Osteoarthr Cartilage.* 2011;19(9):1132-41.
- 129 Lopez-Franco M, Gomez-Barrena E. Cellular and molecular meniscal changes in the degenerative knee: a review. *J Exp Orthop.* 2018;5(1):11.
- 130 Cambre I, Gaublonne D, Burssens A, Jacques P, Schryvers N, De Muynck A, *et al.* Mechanical strain determines the site-specific localization of inflammation and tissue damage in arthritis. *Nat Commun.* 2018;9(1):4613.
- 131 Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, *et al.* Osteoarthritis. *Lancet.* 2015;386(9991):376-87.
- 132 Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum.* 2003;33(3):155-67.
- 133 Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowitz JB. Arachidonic acid metabolism. *Annu Rev Biochem.* 1986;55:69-102.
- 134 Samuelsson B, Goldyne M, Granstrom E, Hamberg M, Hammarstrom S, Malmsten C. Prostaglandins and thromboxanes. *Annu Rev Biochem.* 1978;47:997-1029.
- 135 Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol.* 1998;38:97-120.
- 136 Varga J, Diaz-Perez A, Rosenbloom J, Jimenez SA. PGE2 causes a coordinate decrease in the steady state levels of fibronectin and types I and III procollagen mRNAs in normal human dermal fibroblasts. *Biochem Biophys Res Commun.* 1987;147(3):1282-8.
- 137 Toussiroit E, Streit G, Wendling D. The contribution of adipose tissue and adipokines to inflammation in joint diseases. *Curr Med Chem.* 2007;14(10):1095-100.



- 138 Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS, *et al.* COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A.* 2002;99(21):13926-31.
- 139 Xu XM, Tang JL, Chen X, Wang LH, Wu KK. Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity. *J Biol Chem.* 1997;272(11):6943-50.
- 140 Anderson GD, Hauser SD, McGarity KL, Bremer ME, Isakson PC, Gregory SA. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J Clin Invest.* 1996;97(11):2672-9.
- 141 Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K, *et al.* Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production *in vivo*. *J Exp Med.* 1996;184(3):883-91.
- 142 Siegle I, Klein T, Backman JT, Saal JG, Nusing RM, Fritz P. Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: differential elevation of cyclooxygenase 2 in inflammatory joint diseases. *Arthritis Rheum.* 1998;41(1):122-9.
- 143 Clockaerts S, Bastiaansen-Jenniskens YM, Feijt C, De Clerck L, Verhaar JA, Zuurmond AM, *et al.* Cytokine production by infrapatellar fat pad can be stimulated by interleukin 1beta and inhibited by peroxisome proliferator activated receptor alpha agonist. *Ann Rheum Dis.* 2012;71(6):1012-8.
- 144 Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, *et al.* Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *J Clin Invest.* 1997;99(6):1231-7.
- 145 Pelletier JP, Mineau F, Fernandes JC, Duval N, Martel-Pelletier J. Diacerein and rhein reduce the interleukin 1beta stimulated inducible nitric oxide synthesis level and activity while stimulating cyclooxygenase-2 synthesis in human osteoarthritic chondrocytes. *J Rheumatol.* 1998;25(12):2417-24.
- 146 Seibert K, Masferrer JL. Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor.* 1994;4(1):17-23.
- 147 Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol.* 2011;31(5):986-1000.
- 148 Lipsky PE. The clinical potential of cyclooxygenase-2-specific inhibitors. *Am J Med.* 1999;106(5B):51S-7S.
- 149 Clemett D, Goa KL. Celecoxib: a review

## Chapter 1

---

of its use in osteoarthritis, rheumatoid arthritis and acute pain. *Drugs*. 2000;59(4):957-80.

- 150 Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. *J Bone Miner Res*. 2008;23(6):896-906.
- 151 van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, *et al*. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 *in vitro*. *Arthritis Res Ther*. 2010;12(3):R100.
- 152 Saris DB, Dhert WJ, Verbout AJ. Joint homeostasis. The discrepancy between old and fresh defects in cartilage repair. *J Bone Joint Surg Br*. 2003;85(7):1067-76.





Chapter  
02



**TGF- $\beta$ 2 is Involved in the Preservation  
of the Chondrocyte Phenotype Under  
Hypoxic Conditions.**

*Das, R.; **Timur, UT.**; Edip, S.; Haak, E.; Wruck, C.; Weinans, H.; Jahr, H.(2015)  
Annals of Anatomy, volume 198, pp. 1 - 10*

# Abstract

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### Objective:

Culturing chondrocytes under oxygen tension closely resembling their *in vivo* environment has been shown to have positive effects on matrix synthesis. In redifferentiation of expanded chondrocytes, hypoxia increased collagen type II expression. However, the mechanism by which hypoxia enhances redifferentiation is still unknown. We employed novel bioreactor technology to investigate the role of TGF- $\beta$ , a growth factor heavily implicated in matrix production, in chondrocytes under hypoxia.

### Material & Methods:

Dedifferentiated chondrocytes in alginate were cultured for 48 h under hypoxic (1% pO<sub>2</sub>) or normoxic (20%) conditions, using specialized bioreactor technology. Hypoxia induced gene expression (*GDF1-*, *PHD3*, *HAS2*, *VEGF*, *COX2*), chondrocyte markers (*SOX9*, *COL2*, *COL1*, *AGC1* and *MMP13*), as well as components of the TGF- $\beta$  signaling pathway (TGF- $\beta$  isoforms, receptors, and downstream effectors) were analyzed by qPCR after 48 h. In addition, protein expression of COL2 and TGF- $\beta$ 2 were evaluated. To further elucidate the involvement of the TGF- $\beta$ 2, we used siRNA and ALK5 inhibition.

### Results:

Hypoxic culture showed robust upregulation of hypoxic markers as well as upregulation of *SOX9* and *COL2* expression. Of all TGF- $\beta$  isoforms, only TGF- $\beta$ 2 was upregulated under hypoxia on both gene and protein level. In addition, both type I receptors (ALK1 and ALK5) were upregulated under hypoxia, but type II and III receptors were not. TGF- $\beta$ 2 downregulation *via* siRNA abrogated the hypoxia-induced COL2 expression, as did ALK5 inhibition, giving a strong indication that this pathway is involved in chondrocyte redifferentiation under low oxygen tension.



**Conclusion:**

Hypoxic culture is a common approach for cartilage tissue engineering, but its underlying mechanisms are still poorly understood. Here, we show that increased TGF- $\beta$ 2 signaling through ALK5 plays a role in hypoxia-induced redifferentiation of chondrocytes.

## 1. Introduction

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Articular cartilage covers the surface of all diarthrodial joints in the body, where it provides frictionless articulation and resistance to compressive forces. Due to the avascularity of this tissue, chondrocytes rely on diffusion primarily from the surrounding synovial fluid for their supply of oxygen and nutrients. Consequently, articular chondrocytes reside in an environment that is naturally hypoxic compared to other tissues. A  $pO_2$  of 7–10% is estimated for the pericellular regions in the superficial zone of articular cartilage, while the  $pO_2$  might be as low as 1% in the deeper zones near the calcified cartilage<sup>1,2,3,4</sup>. Chondrocytes are well adapted for survival in this hypoxic environment. For example, they mainly use anaerobic metabolism for the generation of ATP, thereby minimizing consumption of oxygen<sup>5</sup>. However, chondrocyte isolation and subsequent expansion exposes these cells to oxygen tension that far exceed physiological values. Culturing chondrocytes under oxygen tensions that more closely resemble their *in vivo* environment (i.e. “hypoxic”) has been shown to have positive effects on matrix synthesis. Increased proteoglycans (PG) and collagen synthesis has been found in several studies, although findings are not always conclusive. In fact, effects appear to depend on cell source, species and exact oxygen tension<sup>6</sup>. In healthy bovine chondrocytes, hypoxia showed increased mRNA expression of aggrecan (AGC1) and TIMP-1, but a slight decrease in collagen type II (COL2) mRNA at near anoxic condition in one study<sup>7</sup>. On the other hand, increased proteoglycan deposition and COL2 synthesis was found in another study<sup>8</sup>. During redifferentiation of expanded chondrocytes, low oxygen tension also appears to increase collagen type II immunostaining<sup>9</sup>.

TGF- $\beta$  appears to play a major role in cartilage repair and multiple micro-environmental factors potentially regulate the chondrocytes phenotype directly or indirectly by modulating TGF- $\beta$  signaling. TGF- $\beta$  is the prototypic member of the TGF- $\beta$  superfamily

of growth factors, which further includes, among others, activins and inhibins, nodal, myostatin and bone morphogenetic proteins (BMPs)<sup>10</sup>. Three mammalian isoforms of TGF- $\beta$ <sup>1,2,3</sup> exist and while their active forms mostly have similar actions on cells *in vitro*, they also have non-redundant functions *in vivo*<sup>11,12</sup>. This highly pleiotropic growth factor plays a key role in various cellular processes, including inhibition of cell growth, induction of apoptosis and production of extracellular matrix proteins.

In endothelial cells, hypoxia (1% pO<sub>2</sub>) was shown to upregulate TGF- $\beta$ 2 at the transcriptional level, peaking after 48 h<sup>13</sup>, while, in dermal fibroblasts, hypoxia upregulates the synthesis of the  $\beta$ 1 isoform<sup>14</sup>. In redifferentiating bovine chondrocytes, complete anoxia appeared to slightly affect TGF- $\beta$  expression<sup>7</sup>, but this study did not distinguish between the different isoforms, nor did it investigate oxygen tensions between 0% and 5% pO<sub>2</sub>. Martin *et al.* showed that, in fresh bovine chondrocytes, short term hypoxia (5% pO<sub>2</sub>) had a modulatory effect on TGF- $\beta$  expression, but no long-term effects were investigated, nor did the authors examine effects in human OA chondrocytes<sup>15</sup>. Other extracellular parameters also affect TGF- $\beta$  signaling; while low pH can facilitate TGF- $\beta$  signaling by activating its latent form<sup>16,17</sup>. Other parameters, such as plasmin-mediated proteolysis<sup>18</sup>, can also influence this signaling pathway<sup>19</sup>. The controversy about ambivalent effects of TGF- $\beta$  on articular chondrocytes has been recently reviewed<sup>20,21</sup> and appears to depend on the context of the culture: the presence of cytokines, the culture method (monolayer versus 3D culture)<sup>22</sup> or even the phase of the cell cycle<sup>23</sup>. In chondrocytes, TGF- $\beta$  has been mainly implicated in the production of COL2<sup>21,24,25</sup>. TGF- $\beta$ 2 has been described as the more potent stimulatory isoform (compared to TGF- $\beta$ 1) for chondrogenesis, proteoglycan synthesis and cell proliferation in rabbit chondrocytes<sup>26</sup>. TGF- $\beta$ 2 is also unique among the three isoforms in that it lacks a RGD integrin-binding sequence in its precursor and is the only isoform with multiple CRE elements immediately upstream of its P1 promoter<sup>11</sup>. Of note, the CREB family of transcription factors has recently been shown to be activated by hypoxia<sup>27</sup>.

Canonically, TGF- $\beta$  ligands bind to the TGF- $\beta$  type II receptor (TGFBRII) which, in most cells, then recruits the type I receptor activin-like kinase 5 (ALK5). The tetrameric receptor complex subsequently phosphorylates the intracellular effector molecules Smad-2 and -3, which, after translocation to the nucleus, regulate transcriptional activity in the target cell<sup>28</sup>.

However, recently it was shown that in some cells (e.g. endothelial cells and chondrocytes) TGF- $\beta$  can also signal through an alternative receptor, ALK1<sup>29</sup>. Signaling via this receptor leads to phosphorylation of Smad1/5/8 rather than Smad2/3. It was postulated that the ratio of ALK5 and ALK1 signaling is responsible for the seemingly contradictory effects of TGF- $\beta$  in chondrocytes<sup>30</sup>. In addition, Smad-independent pathways may fine-tune the response to TGF- $\beta$  in different contexts<sup>21</sup>.

In this study we hypothesize that hypoxia affects TGF- $\beta$  signaling in redifferentiating human chondrocytes. Moreover, we postulate that the positive effects on COL2 expression in hypoxic culture of redifferentiating chondrocytes are caused by upregulation of components of the TGF- $\beta$  signaling pathway. The different TGF- $\beta$  isoforms as well as their receptors are investigated at the gene expression and protein levels, employing pharmacologic receptor inhibition and TGF- $\beta$ 2 RNAi to shed light on the underlying molecular effects.

## 2. Materials & Methods

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### 2.1 Cell Culture

Cartilage biopsies of 8 patients undergoing total knee replacement surgery (after approval by the local ethics committee; MEC2004-322) were used for this study. Full thickness cartilage was harvested, treated with 7 U/mL protease in physiological saline solution (Sigma-Aldrich, Zwijndrecht, Netherlands) for 90 min and subsequently digested overnight in basal medium (DMEM, 4.5 g/L glucose with 10% Fetal Calf Serum (FCS), 50  $\mu$ g/ml gentamicin and 1.5  $\mu$ g/ml fungizone (all Invitrogen, Scotland, UK)) supplemented with 15 U/mL collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a haemocytometer. The primary chondrocytes were then seeded at a density of 7500 cell/cm<sup>2</sup> in T175 culture flasks for expansion culture. The cells plated for expansion were cultured for three passages. Alginate beads were created with second passage chondrocytes in 1.2% low viscosity alginate (Keltone LV, Kelco, Surrey, UK) by dripping a 1.2% alginate suspension with 4 million cells per ml through a 23 gauge needle into a CaCl<sub>2</sub> solution. After 10 min, the beads were washed twice with physiological saline



and once with basal medium and transferred a T175 culture flask with 150 ml basal medium with 25 mM l-ascorbic acid. After five days of pre-culture, beads were transferred to a  $\mu$ 24 bioreactor (Applikon Biotechnology, Schiedam, The Netherlands). Chondrocytes were also stimulated with 10 ng/ml TGF- $\beta$ 2 for 48 h and 7 days under 1% or 20% pO<sub>2</sub> and expression levels of key chondrocyte marker COL2 were compared to unstimulated controls.

### 2.2 Bioreactor Culture

The bioreactor system used for hypoxic culture has been previously described by our group<sup>31</sup>. Briefly, 30 beads were transferred to every well of a specific 24 well cassette, designed for use with the  $\mu$ 24 bioreactor. Four milliliter of redifferentiation medium (DMEM, 4.5 g/L glucose with 1:100 ITS+ premix, 10 ng/ml IGF-1, 25  $\mu$ g/ml l-ascorbic acid and 50  $\mu$ g/ml gentamicin and 1.5  $\mu$ g/ml fungizone) was added to every well. Oxygen tension was lowered by continuous sparging with nitrogen. Oxygen tension was maintained at the desired set-point (either 1%, 5% or 20% pO<sub>2</sub>) by using air as a counter gas. Medium pH was maintained at 7.2 using CO<sub>2</sub>.

### 2.3 RNA Isolation and Quantitative RT-PCR

Alginate beads were produced as described earlier by our group<sup>32,33</sup> and after dissolution, cells were resuspended in 350  $\mu$ L RNABee (TEL-TEST Inc., Friendswood, TX, USA). After addition of 60  $\mu$ L chloroform, samples were spun down for 20 min at 13,000  $\times$  g. Total RNA was isolated from the supernatant using the Qiagen RNA Micro Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop<sup>®</sup> ND1000, Isogen Life Science, IJsselstein, The Netherlands). In compliance with MIQE guidelines<sup>34</sup>, good RNA integrity (RIN  $\geq$  8.8; BioAnalyzr 2100, Agilent Technologies, Amstelveen, Netherlands) and absence of PCR inhibitors (data not shown) was ensured. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Rockford, IL) with mixed priming (oligo-d(T):random hexamers, 3:1, v/v) according to the supplier's instructions. Both cDNA synthesis (0.5–1  $\mu$ g total RNA/reaction) and real-time quantitative PCR (10–15 ng cDNA/reaction) method, with specificity and efficiency controls, as well as

data normalization to a reference gene index were described in detail elsewhere<sup>35</sup>. Briefly, the geometric mean of CT-values of pre-evaluated endogenous calibrators 18S rRNA, ubiquitin (UBC), beta-actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and beta-2 microglobulin (B2M)<sup>36</sup> was used to normalize expression data. To choose the “best housekeeper index”, the BestKeeper macro was used as established tool<sup>37</sup>. GAPDH, UBC and HPRT1 were chosen for the index for their stable expression across experimental conditions. The relative fold-change in gene expression was then calculated according to the  $2^{-\Delta Ct}$  method as reported by Livak and Schmittgen<sup>38</sup>. Individual differences were first averaged and replicates then used to calculate the relative expression, normalized to the internal control, as described previously by us<sup>35</sup>. RT-PCR primers of extracellular matrix (ECM) components (aggrecan, collagen type I and type II (COL1(A1) and COL2(A1) resp.), sex determining region Y-box 9 (SOX9), matrix metalloproteinase-13 (MMP13), markers of hypoxia (prolyl hydroxylase 3 (PHD3), vascular endothelial growth factor (VEGF), growth differentiation factor 10 (GDF10), hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (COX2)), TGF- $\beta$  isoforms (TGF- $\beta$ 1, - $\beta$ 2 and  $\beta$ 3), TGF- $\beta$  receptors (activin-like receptor kinases (ALK1 and ALK5), TGF- $\beta$  receptor 2 (TGFB2), betaglycan (BGCAN) and endoglin (EGN)) and targets of TGF- $\beta$  signaling (plasminogen activator inhibitor-1 (PAI-1) and inhibitor of DNA binding 1 (ID1)) were also previously described<sup>35,39,40,41</sup>. Cycling was performed on an ABI7000 (Applied Biosystems) with 60 °C annealing and a standard two-step protocol.

### 2.4 Immunoblotting

After RNA isolation, polypeptides were purified from the phenol-ethanol supernatant layer left over by dialysis. Briefly, upon 1:4 dilution, pooled phenol layers from three independent bioreactor experiments were dialyzed against 0.2% (w/v) SDS for 16, 4, and 2 h, respectively, using Spectra/Por®3 (Carl Roth GmbH & Co.KG, Schoemperlenstr., Karlsruhe, Germany). Subsequently, dialysates were centrifuged (10,000  $\times$  g, 10 min, 4 °C) and supernatants concentrated using Centriprep YM-3 (Millipore, Billerica, MA) according to the manufacturer's instructions. Polypeptide concentration was determined using routine bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific Inc., Rockford, IL) as described earlier<sup>42</sup> and immunoblotting was performed as described elsewhere by our group<sup>43</sup>. Concisely, equal amounts of polypeptides were separated by routine SDS-PAGE,

subsequently transferred by electro-blotting and detected on an Odyssey infrared imaging system (Li-Cor Biosciences) as described earlier<sup>44</sup>, but using primary polyclonal goat anti-*COL2A1*, polyclonal rabbit anti-Sox9<sup>45</sup> and mouse monoclonal anti- $\beta$ -tubulin (G-8; Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibodies. For dot blot analyses, the pooled polypeptide fractions were spotted onto 0.45  $\mu$ m nitrocellulose membranes using a Bio-Dot<sup>®</sup> Apparatus (both: Bio-Rad, München, Germany) and processed as described above for Western blots, except that an anti-human TGF- $\beta$ 2-specific antibody (Proteintech, Chicago, IL; 1:1000) was used. Densitometric quantification data (ImageJ software, version 1.47) were normalized to  $\beta$ -tubulin and displayed relative to controls, as reported by Caron *et al.*<sup>45</sup>.

### 2.5 ALK5 Inhibition

ALK5 activity was inhibited using a novel, specific ALK5 inhibitor (SB-525334, Sigma–Aldrich; 10  $\mu$ M)<sup>46</sup> added 2 h prior to induction of hypoxia (1% pO<sub>2</sub>). Ten beads were harvested after 48 h of hypoxia for RNA isolation as described above. Specificity of the inhibitor was confirmed using qRT-PCR analysis on ALK5 and ALK1 specific target genes PAI-1<sup>47,48</sup> and ID1<sup>49,50</sup>, respectively.

### 2.6 RNA Interference (RNAi)

We used our established lentiviral vector technology for nontransient shRNA-mediated gene silencing in primary chondrocytes<sup>35</sup>, employing commercially available, pre-validated shRNA clones (Sigma–Aldrich, MISSION shRNA library). Briefly, BamHI/MunI restriction fragments of the parental pLKO.1-puro vector containing one out of five different, sequence-verified anti-human TGF- $\beta$ 2 shDNAs were subcloned into corresponding restriction sites of recipient vector pRRL.PPT.PGK.GFPpre. Lentiviral particles were produced in HEK293T cells by transient transfection using a calcium phosphate protocol. Cells transduced with a lentiviral vector lacking the TGF $\beta$ 2-specific shRNA expression cassette (CTRL) and a non-targeting scrambled shRNA sequence (SCR), which did not significantly differ from CTRL (not shown)<sup>35</sup>, served as controls. Quantitative RT-PCR was subsequently used to determine the knockdown efficacies: TRCN0000033425 (RNAi-1) and TRCN0000033427 (RNAi-2) were selected as best performing shDNA clones, reaching 75–80% of NM\_003238 mean knock-

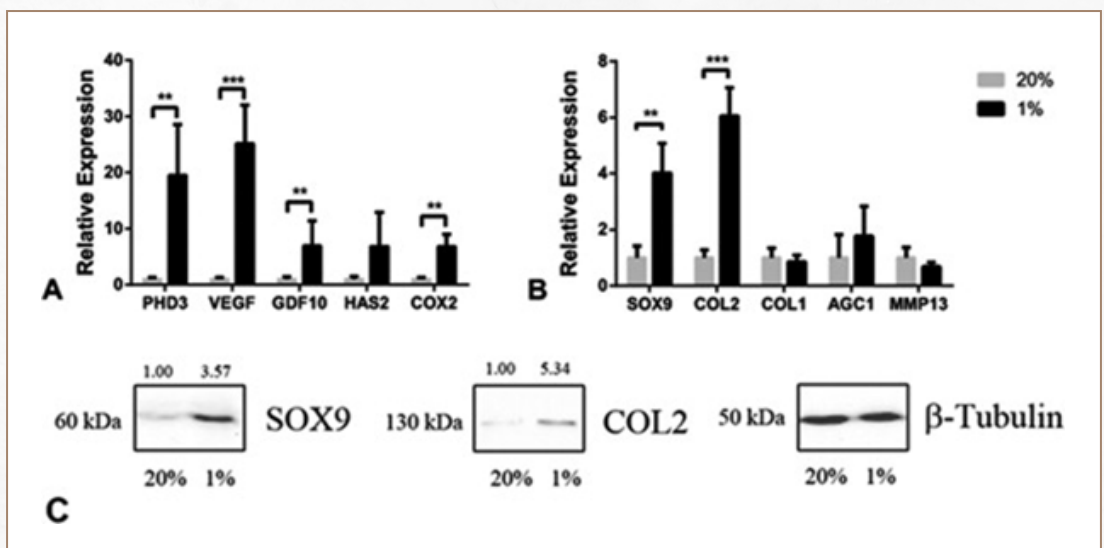


down efficacy at a MOI of 1.5 in HEK29T cells (data not shown). In subsequent experiments, P1 OAHACs from three donors were seeded and transduced at a MOI of 2 with RNAi-1 and RNAi-2 for  $\pm 18$  h as described earlier<sup>35</sup>. Cells were then used as passage 2 cells in monolayer culture (10,000 cells/cm<sup>2</sup>) and cultured under hypoxia or normoxia for 48 h.

### 3. Results

#### 3.1 Hypoxia and Chondrocyte Marker Expression

To validate our culture system, we first confirmed the expression of accepted marker genes that have been shown to be hypoxia-responsive in different species and *in vitro* settings<sup>41,51,52,53</sup>. We, therefore, used these genes as robust indicators of cellular hypoxia on mRNA level: a highly significant upregulation was found for four out of five of these markers under 1% pO<sub>2</sub> (Figure 1A). When compared to the 20% pO<sub>2</sub> “normoxic” controls, *PHD3* and *VEGF* were the most hypoxia responsive, displaying a more than 20-fold upregulation under 1% pO<sub>2</sub>, while *GDF10* and *COX2* were upregulated almost 7-fold. Expression of *HAS2* was not significantly affected by hypoxia, although data suggested its responsiveness to hypoxia in chondrocytes<sup>41,54</sup>.



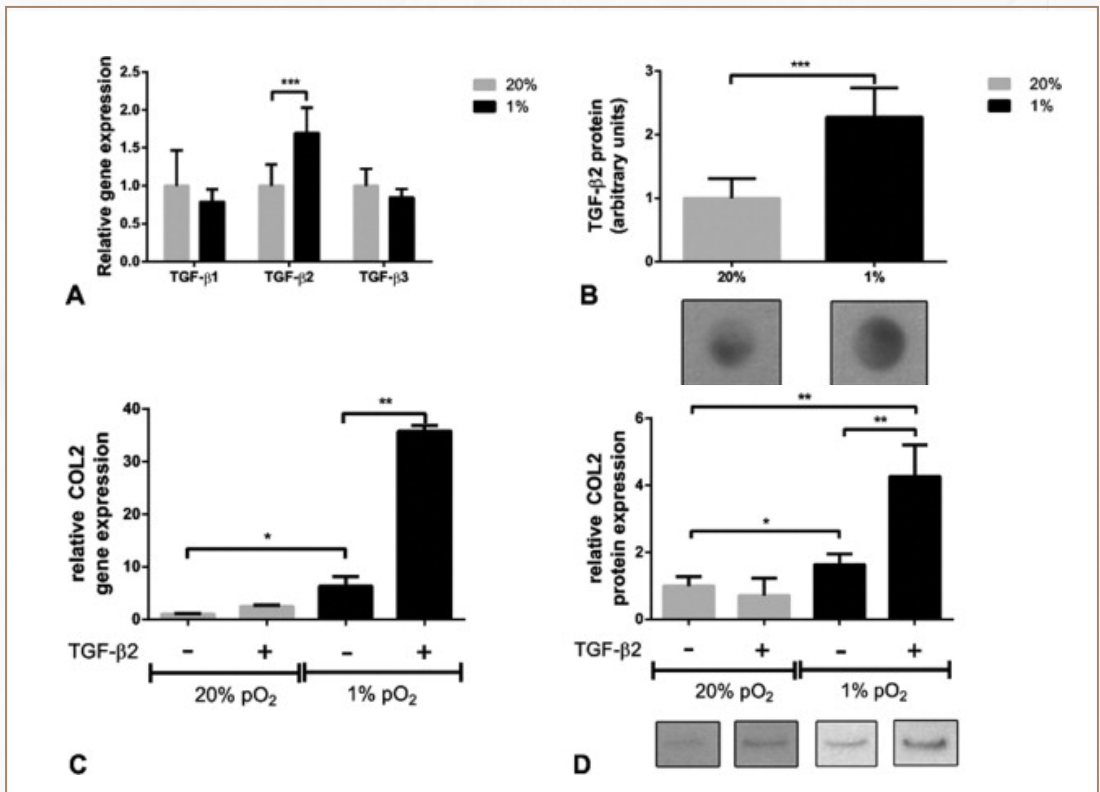
### **Figure 1: Relative gene expression of hypoxia- and chondrocyte-specific markers.**

Expression of accepted indicators of hypoxia in chondrocytes (A) next to that of chondrocyte differentiation-specific markers (B) after 48 h in bioreactor culture. Representative protein expression of SOX9, collagen type II (COL2A1) and  $\beta$ -tubulin (loading control) (C). Hypoxia (1% pO<sub>2</sub>, black bars) and normoxia (20% pO<sub>2</sub>, gray bars). Molecular weight markers (kDa) are indicated on the left and normalized quantitative data above respective bands. \*\*p < 0.01, \*\*\*p < 0.001.

Next, we studied the expression levels of selected chondrocyte differentiation stage specific markers: hypoxia induced COL2-specific transcription factor SOX9<sup>55,56,57</sup> significantly (4-fold), along with that of COL2 (6-fold) itself. In contrast, collagen type I (COL1) expression was downregulated under the same condition (Figure 1B), leading to an improved COL2/COL1 ratio (data not shown). Interestingly, we could only detect a non-significant trend toward upregulation of the core protein of key proteoglycan (PG) aggrecan (AGC1) after 48 h of low oxygen pressure. Of note, the key chondrocyte hypertrophy marker matrix metalloproteinase 13 (MMP13)<sup>58</sup> showed a non-significant trend toward downregulation under these conditions. We further verified the expression of key chondrocyte phenotype markers, SOX9 and COL2, at protein level by Western blot analysis and confirmed a significant, more than 3-fold, upregulation of both (Figure 1C).

### **3.2 Hypoxia Mediated TGF- $\beta$ Isoform Expression**

Since the TGF- $\beta$  system plays a crucial role in cartilage homeostasis and repair, we investigated its oxygen pressure-dependent regulation in articular chondrocytes. Of all three human isoforms, hypoxia specifically induced TGF- $\beta$ 2 expression in human articular chondrocytes: Following 48 h of 1% pO<sub>2</sub> TGF- $\beta$ 2 mRNA was significantly up-regulated by 60% as compared to the normoxic control (20% pO<sub>2</sub>). Expression of the other two isoforms (TGF- $\beta$ 1 and - $\beta$ 3) was not altered under these conditions (Figure 2A). Subjecting human chondrocytes to 5% pO<sub>2</sub> also did not significantly alter the expression of TGF- $\beta$  isoforms (data not shown). Consequently, we further focused on comparing 1% pO<sub>2</sub> to 20% pO<sub>2</sub>. To verify whether the detected changes on mRNA level could be biologically meaningful, we also performed TGF- $\beta$ 2 immunoblots to demonstrate a similar magnitude of regulation at the protein level (Figure 2B).



**Figure 2: Oxygen tension dependent changes in gene expression of TGF- $\beta$  and collagen.**

Relative mRNA abundance of the three mammalian TGF- $\beta$  isoforms after 48 h (A). Expression of TGF- $\beta$ 2 protein under hypoxia and normoxia with representative blots below (B). Effects of hypoxia and stimulation with exogenous TGF- $\beta$ 2 (10 ng/ml) on the induction of COL2A1 mRNA (C) and COL2 protein expression (D). Representative immunoblot signals are shown below their respective quantification chart. Hypoxia (1% pO<sub>2</sub>, black bars) and normoxia (20% pO<sub>2</sub>, gray bars). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

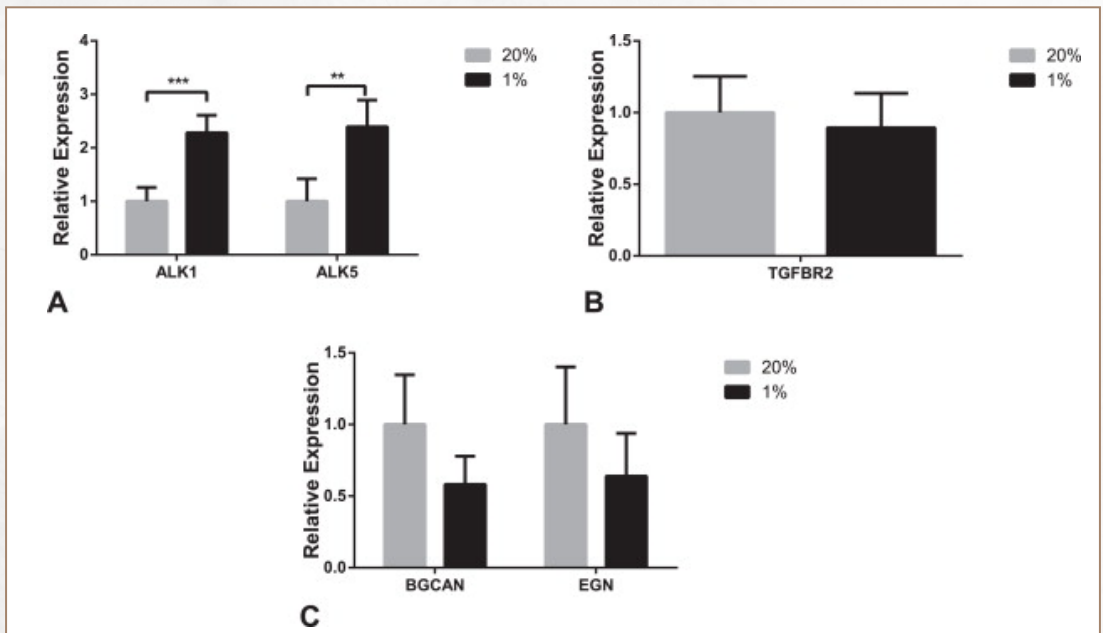
Exogenously applied TGF- $\beta$ 2 is known to induce COL2 expression in rabbit perichondrial cells cultured in alginate beads<sup>59</sup>, but little is known about its effects under hypoxic conditions. Therefore, we tested whether this isoform would also be able to induce COL2 expression in human articular chondrocytes. Under normoxic conditions (20% pO<sub>2</sub>) exogenous TGF- $\beta$ 2 did not significantly upregulate COL2 mRNA expression (Figure 2C). In contrast, as shown previously (Figure 1B), hypoxia itself was able to induce COL2 expression to some extent. However, there was a prominent synergistic induction once exogenous TGF- $\beta$ 2 was applied



under hypoxic conditions: *COL2* mRNA was significantly induced 5.7-fold. At the protein level, a similar trend was confirmed, resulting in a synergistic 4-fold induction of collagen type II under low oxygen pressure in the presence of TGF- $\beta$ 2 (Figure 2D).

### 3.3 Hypoxia and TGF- $\beta$ Receptor Expression

To further shed light on the hypoxia-mediated TGF- $\beta$  signaling, we looked into the expression of relevant TGF- $\beta$  receptors. Hypoxic culture led to a significant upregulation of the expression of both type-1 receptors, ALK1 and ALK5 (Figure 3A), which more than doubled (approximately 120% increase) compared to the normoxic condition. In contrast, expression of the type II (TGFBR2) and type III receptors (BGCAN and EGN) was unaffected by changes in oxygenation levels (Figure 3B and C).



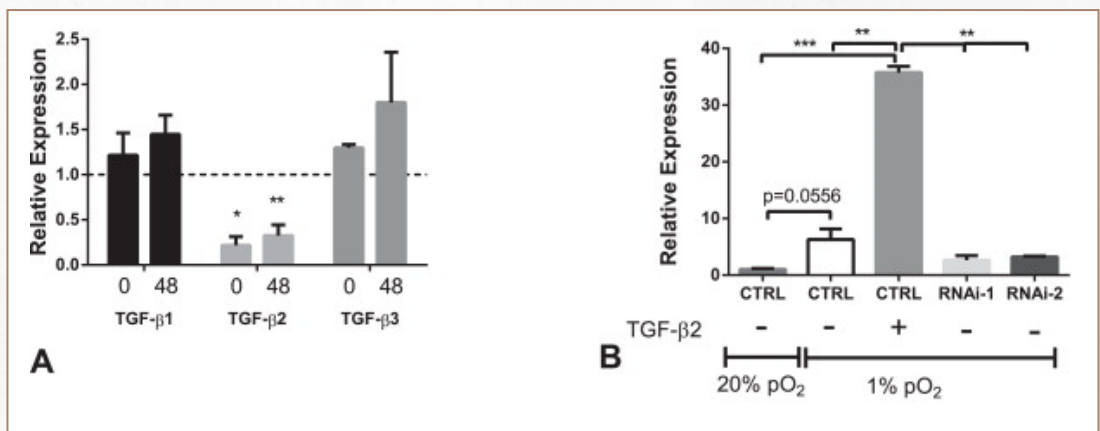
**Figure 3: Oxygenation-dependent expression of TGF- $\beta$  receptors.**

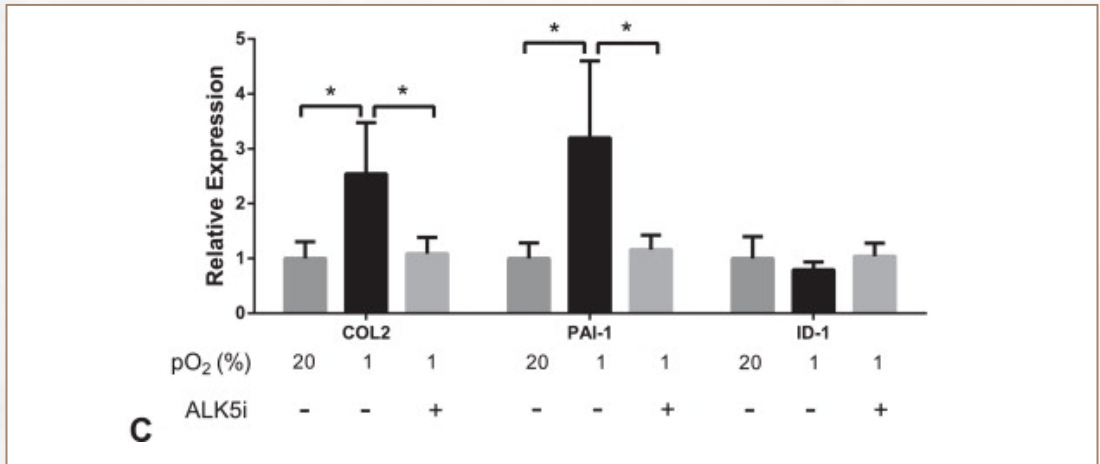
Type I receptors ALK1 and ALK5 (A) next to type II receptor (B) and type III receptors (C). Hypoxia (1% pO<sub>2</sub>, black bars) and normoxia (20% pO<sub>2</sub>, gray bars). \*\*p < 0.01, \*\*\*p < 0.001.

### 3.4 TGF- $\beta$ Signaling Interference

Hypoxia induced COL2 expression in human articular chondrocytes *in vitro* and TGF- $\beta$ 2 was the only isoform of this cytokine that was also induced upon oxygen deprivation in these cells. In addition, applying exogenous TGF- $\beta$ 2 (10 ng/ml) to these cells synergistically induced collagen type II expression at the mRNA and protein levels (Figure 2C and D) exclusively under hypoxic conditions. Therefore, we used isoform-specific shRNAs to investigate whether a specific knockdown of TGF- $\beta$ 2 could selectively suppress COL2 levels under hypoxic conditions.

First, we evaluated the knockdown efficacy of five commercially available shRNA sequences by screening residual TGF- $\beta$ 2 expression levels in HEK293 cells as reported earlier<sup>35</sup>. In our study, only two constructs (RNAi-1 and -2) reached at least 70% knockdown efficacy (data not shown) and were chosen for further experiments using primary human articular chondrocytes. In primary chondrocytes we first verified the TGF- $\beta$  isoform specificity and knockdown efficacy of the two most efficient shDNAs constructs 48 and 96 h, respectively, after lentiviral transduction (Figure 4A, only RNAi-1 is shown). RNAi selectively suppressed only TGF- $\beta$ 2 expression without negatively affecting the other two mammalian TGF- $\beta$  isoforms. The shRNA expression was shown to result in a stable, about 70% (as compared to non-targeting controls, normalized to 1), efficient knockdown in cells 48 h after transduction and 48 h later (96 h) in alginate beads.





**Figure 4: Specificity and efficacy of TGF- $\beta$  RNAi and TGF- $\beta$  receptor inhibition.**

Confirmation of the isoform specificity and knockdown efficacy of the lentiviral shRNA vectors in primary chondrocytes (A). Suppression of hypoxia-induced TGF- $\beta$  mediated COL2 expression in primary chondrocytes by TGF- $\beta$  RNAi (B). A specific small molecular receptor antagonist (SB-525334, ALK5i) was used to confirm ALK5 participation in the TGF- $\beta$  mediated downstream signaling. PAI-1 and ID-1 served as established TGF- $\beta$  inducible, but strictly ALK5- and ALK1 dependent, controls (C). Hypoxia (1% pO<sub>2</sub>, black bars) and normoxia (20% pO<sub>2</sub>, gray bars). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

In primary human chondrocytes in alginate beads in a bioreactor culture system, hypoxic conditions alone non-significantly ( $p = 0.0556$ ) upregulated COL2 mRNA expression in the non-targeting controls (CTRL). Addition of exogenous TGF- $\beta$ 2 (10 ng/ml) to these hypoxic cells prominently and synergistically increased COL2 expression even further (Figure 4B). In contrast, effective TGF- $\beta$ 2 RNAi by means of non-transient lentiviral shRNA expression (RNAi-1 and -2) significantly suppressed this induction in COL2 expression. The TGF- $\beta$ 2 RNAi suppressed the COL2 expression to below that of the non-targeting control (Figure 4B).

Finally, we aimed to identify the receptor that mediates this TGF- $\beta$ 2-dependent upregulation of COL2 expression under hypoxic conditions. To this end, we used a highly ALK5-specific, novel type I receptor inhibitor under the same experimental conditions. While hypoxia, again, reproducibly induced COL2 expression under hypoxic conditions, the presence of the small molecular ALK5 antagonist SB-525334 (ALK5i) fully abrogated this effect (Figure 4C): COL2 induction was suppressed to control levels. Interestingly, in contrast to blocking



TGF- $\beta$ 2 itself, COL2 mRNA levels were indistinguishable between normoxic controls and the hypoxic condition with ALK5i. The established TGF- $\beta$ -inducible and ALK5-dependent expression of *PAI-1*<sup>49,60</sup> revealed a very similar pattern to that of *COL2*. The expression of *ID-1*, which is reported to be TGF- $\beta$ -inducible but in chondrocytes ALK1-dependent<sup>48</sup>, was not induced by hypoxia nor affected by ALK5i (Figure 4C).

## 4. Discussion

In the present study, we used a sophisticated bioreactor system to stringently feedback-control the dissolved oxygen concentration in culture media of 3D-cultured chondrocytes to show that hypoxia (1% pO<sub>2</sub>) induces *COL2* expression. We further hypothesized that hypoxia facilitates TGF- $\beta$  signaling, which *via* ALK5, synergistically contributes to an improved phenotype by inducing the expression of key chondrocyte marker *COL2A1* in redifferentiating human chondrocytes.

We first validated our culture system and confirmed the robust expression of well established hypoxia-responsive marker genes (Figure 1A). Hypoxia inducible factor, HIF, expression and transcriptional activity are regulated by oxygen-sensitive prolyl hydroxylases (PHD1-3), of which PHD3 was shown to be most prominently regulated by sustained hypoxic culture in other cells<sup>61</sup>. We confirmed its strong, 20-fold, induction in hypoxic chondrocytes. Others reported *VEGF*, *GDF10*, *HAS2* and *COX-2* (*PTGS2*) as hypoxia responsive genes<sup>41,51,52,53,62</sup>. Expression of vascular endothelial growth factor, *VEGF*, is hypoxia-inducible in cartilage cells<sup>63</sup>, but the magnitude of its induction (20-fold, Figure 1B) was unexpected. Lafont *et al.*<sup>41</sup> earlier reported a rather moderate, approximately 3-fold induction, of this gene, which may be an underestimation due to their microarray-based analysis or result from the fact that these authors analyzed healthy human chondrocytes from osteosarcomas, since *VEGF* expression is known to be higher in OA<sup>64</sup>. The fold-change of *GDF10* and *COX-2* expression in the present study is in nice agreement with earlier reported QPCR data<sup>41</sup>.

Hyaluronan, a key component of the chondrocytes' ECM, is synthesized by a family of hyaluronic acid synthetases (HAS). *HAS2*, which is a particularly important isoform, was

upregulated by hypoxia in our cultures, but just not significantly due to a large standard deviation. Others<sup>41,54</sup> have also reported its positive hypoxia-dependency, although the fold change of hypoxia-induced *HAS2* regulation was moderate (<3-fold). We further evaluated the hypoxia-dependency of selected other components of the chondrocytes' ECM, like *SOX9*, collagens, key proteoglycan aggrecan and hypertrophy marker *MMP13* (Figure 1B). *COL1* expression was not-significantly regulated in our study, but slightly, about 50%, suppressed in another study using human chondrocytes in hypoxic 3D-culture<sup>54</sup>. The trend toward upregulation of *AGC1* under 1% pO<sub>2</sub> in our study was not significant. This may be a timing issue as *AGC1* expression seems to respond early<sup>41</sup>. Overall, *AGC1* seems to be only moderately induced by hypoxia<sup>41,54</sup>, which is apparently sufficient to double the sGAG content in cultured human chondrocyte pellets<sup>54</sup> and the trend in our data may thus still be biologically meaningful. The magnitude of hypoxia-induced fold changes in *SOX9* and *COL2A1* expression in human chondrocytes seem to be rather robust and there are similarities between our data and earlier studies<sup>41,54</sup>. Therefore, we propose that these genes can be viewed as robust indicators of cellular hypoxia at the mRNA level. A plethora of data suggests that, for tissue engineering, being able to improve *COL2A1* expression may be more relevant than controlling *AGC1*. Since *COL2A1* can be regarded as the prime marker for redifferentiation, with *SOX9* driving its expression, we verified the induction of both key factors at the protein level.

TGF- $\beta$  ligands have already been described as secreted polypeptides capable of inducing collagen production in general<sup>65</sup> and in cartilage in particular<sup>24,25</sup>. We found a significant and selective upregulation of specifically the TGF- $\beta$ 2 isoform, which was verified at protein expression level (Figure 2A and B). This led us to postulate that the endogenously produced TGF- $\beta$ 2 isoform is critically involved in the hypoxia-driven induction of COL2. Exogenous TGF- $\beta$ 2 is known to upregulate COL2 in redifferentiating chondrocytes<sup>24,25</sup> and its expression has also been shown to be hypoxia-driven in other cells<sup>13</sup>. Subjecting human chondrocytes to 5% pO<sub>2</sub>, instead of 1% pO<sub>2</sub>, did not significantly alter the expression of TGF- $\beta$ 2 (data not shown), which suggests a potential contribution of hypoxia inducible factors (HIF) to hypoxic COL2 $\alpha$ 1 production. The promoter regions of the TGF- $\beta$  isoforms are appreciably different from each other and characterization of the 5' UTR of the human TGF- $\beta$  genes

also revealed that TGF- $\beta$ 2 has the longest regulatory region of all isoforms to regulate its transcription and translation<sup>11</sup>. TGF- $\beta$ 2 was shown to auto-induce its own mRNA after only 3 h<sup>66</sup>. Of note, blocking HIF-1 $\alpha$  can also reduce TGF- $\beta$  stimulated collagen production<sup>67</sup>. TGF- $\beta$ 2 is also different from the other two isoforms in that it needs betaglycan for signaling. TGF- $\beta$ /Smad3 signaling is then able to inhibit terminal hypertrophic differentiation of chondrocytes<sup>68</sup>. Even low concentrations of 1 ng/ml of TGF- $\beta$  can quickly upregulate its own receptors. In our study, protein levels of TGF- $\beta$ 2 were also increased under hypoxia over two-fold (Figure 2B). Since low concentrations already have biological relevance, this increase can cause further downstream effects.

To further shed light on the hypoxia-mediated TGF- $\beta$  signaling, we also screened the expression of relevant TGF- $\beta$  receptors. Hypoxic culture significantly increased expression levels of both type I receptors that are involved in TGF- $\beta$  signaling in chondrocytes (*ALK1* and *ALK5*<sup>49,60</sup>), but did not alter the expression of *TGFBR2* or *BGCAN* and *EGN* (Figure 3). We cannot exclude that post-transcriptional regulation affected receptor protein levels differently as we were not able to investigate this aspect; this will be addressed in future studies. A previous study also reported upregulation of one type I receptor under short term hypoxia (5% pO<sub>2</sub><sup>15</sup>), but only looked at *ALK5*. While *BGCAN* favors TGF- $\beta$ 2-mediated signaling, *EGN* regulates TGF- $\beta$  receptor endocytosis and inhibits TGF- $\beta$  signaling<sup>69</sup>. *TGFBR2B* (TbetaRII-B), a splice variant of *TGFBR2*, is also prominently expressed by osteoarthritic human chondrocytes and can form heteromeric complexes with all type I, II and III receptors and can thereby enhance TGF- $\beta$  signaling in chondrocytes when being overexpressed<sup>70</sup>. However, expression of this variant is another unexplored aspect that may have contributed to the upregulation of *COL2* levels under hypoxia. Interestingly, it is the ratio between the two type I receptors that is suggested to dictate the TGF- $\beta$  response in chondrocytes<sup>29,49</sup>. Although hypoxia upregulated both *ALK1* and *ALK5*, post transcriptional effects of hypoxia on either type I receptor might shift that balance at the cell signaling level. Additionally, *EGN*, which binds TGF- $\beta$ 1 and - $\beta$ 3, but not TGF- $\beta$ 2<sup>71,72</sup>, can inhibit the *ALK5*-mediated Smad2 phosphorylation (Smad2-P) in human chondrocytes<sup>73</sup>. Interestingly, hypoxia non-significantly decreased *EGN* expression, which would be in line with an *ALK5*-Smad2-P dominance between these two signaling routes. Future experiments should



look into phosphorylation of Smads in this model to elucidate the active signaling routes involved.

Next, we employed RNAi to confirm a crucial contribution of TGF- $\beta$ 2 to the hypoxia-induced COL2 production. Upon confirmation of the isoform specificity of the anti-TGF- $\beta$ 2 shRNAs (Figure 4A), we showed that, in primary human chondrocytes, severely hypoxic conditions (1% pO<sub>2</sub>) alone only just non-significantly upregulated COL2 mRNA expression in non-targeting controls (CTRL, Figure 4B). The addition of exogenous TGF- $\beta$ 2 to these hypoxic cells, again prominently and synergistically increased COL2 expression. Non-transient lentiviral anti-TGF- $\beta$ 2 shRNA expression significantly suppressed this induction in COL2 expression to below that of the non-targeting control. This is in line with a cell signaling model in which hypoxic conditions induce TGF- $\beta$ 2 synthesis in order to maintain an autoregulatory loop that induces COL2 expression in these cells. However, in cells which actively express anti-TGF- $\beta$ 2 shRNAs, the hypoxia-induced nascent TGF- $\beta$ 2 mRNAs will be readily degraded. In these cells any positive contribution of TGF- $\beta$ 2 signaling to the COL2 expression is inhibited and cannot reach the same level as in the untreated hypoxic cells. On the other hand, as the knockdown is never 100% efficient, the residual TGF- $\beta$ 2 mRNAs in these cells may be sufficient to induce COL2 expression to just above normoxic control levels (Figure 4B).

We further inhibited ALK5 to elucidate its role during the TGF- $\beta$ 2-mediated hypoxic induction of COL2 synthesis in human chondrocytes: while hypoxia induced COL2, ALK5i suppressed it (Figure 4C). In chondrocytes, TGF- $\beta$  signaling promotes HIF1 $\alpha$  stabilization *via* ALK5<sup>74</sup>, which would establish a positive feedback loop between hypoxia and TGF- $\beta$  signaling.

When demonstrating the hypoxia- and TGF- $\beta$ 2-dependency of COL2 expression, we further used PAI-1 as an internal control: its expression was recently shown to depend on both, TGF- $\beta$  and HIF1A, in other cells<sup>75</sup>. Also, induction of PAI-1 crucially depends on SMAD3 and SMAD4 binding<sup>76</sup>. Exposure of HUVEC cells to 1% pO<sub>2</sub> resulted in SMAD binding to the -77 to -40 bp region ("CAGA box") in the TGF- $\beta$ 2 promoter and increased TGF- $\beta$ 2 mRNA levels as compared to normoxic controls (20% pO<sub>2</sub>). These authors used a 3TP-lux reporter,

a Smad2/3-responsive construct<sup>77</sup> similar to the Smad3-responsive CAGA<sub>12</sub>-lux reporter construct<sup>78</sup>, which is basically derived from the minimal SREs of the PAI-1 promoter, to show the SMAD-dependency of TGF- $\beta$ 2 signaling in their system. Also here, TGF- $\beta$ 2 mRNA was the only specifically induced TGF- $\beta$  isoform<sup>13</sup>. Finsson *et al.*<sup>60</sup> showed that COL2 and PAI-1 is stimulated by exogenous TGF- $\beta$ 1 in chondrocytes. It has also been shown in hepatocytes that the HRE site in the PAI-1 promoter could be a target for CREB binding to mediate PAI-1 gene induction<sup>79,80</sup>. Interestingly, TGF- $\beta$ 2 is also the only isoform with multiple, hypoxia-sensitive, CRE elements within 400 nucleotides upstream of its P1 promoter<sup>11</sup>. Although TGF- $\beta$ 2 is structurally similar to TGF- $\beta$ 1, their biological responses may differ dependent upon cell type dependently. Interestingly, initial data suggest that cartilaginous effects of TGF- $\beta$  at low concentration (1 ng/ml) are especially dependent on low oxygenation levels below 5%<sup>56</sup>.

Recently, Finsson *et al.*<sup>60</sup> also showed that endoglin enhanced TGF- $\beta$ 1-induced Smad1/5 phosphorylation, but inhibits TGF- $\beta$ -induced Smad2 phosphorylation, and Smad3-driven transcriptional COL2 and PAI-1 activation in chondrocytes. Our data support the notion that hypoxia induces the ALK5-SMAD2/3 pathway rather than ALK1-Smad1/5 signaling. We further used suppression of inhibitor of differentiation 1 (Id1), a downstream target of the BMPR2 pathway<sup>81</sup> to show that the specificity of ALK5: ID1 expression is not induced by hypoxia and not affected by ALK5i. ID-1 has been shown to be an ALK1 downstream target in endothelial cells<sup>50</sup>. It is interesting that ALK1 also stimulates MMP13 expression and we found a (non-significant) trend toward down regulation at MMP13 mRNA levels. The ID-1/PAI-1 ratio also increases with OA progression (*i.e.* cartilage degradation) and that this is in line with a shift from ALK5 to predominant ALK1 signaling<sup>49</sup>. From Figure 4C it is apparent that hypoxia increases the PAI-1/ID-1 ratio. We propose that this indicates a hypoxia-mediated shift in TGF $\beta$  signaling in favor of anabolic ALK5 signaling and ECM production. In addition, the Sp1/Sp3 ratio is important for COL2 expression by chondrocytes, but was not studied by us. The levels of both transcription factors decrease with dedifferentiation<sup>82</sup> and Sp1 overexpression increases COL2 expression. TGF- $\beta$ /Smad3 signals are essential for repressing articular chondrocyte differentiation. Without these inhibition signals, chondrocytes leave the quiescent state and undergo terminal differentiation<sup>83</sup>. It is interesting that Sp1 has

been reported to be down-regulated by TGF- $\beta$ 1<sup>39</sup>; it would be interesting to see if this also holds for TGF- $\beta$ 2 as our data would suggest the opposite.

In summary, our findings strongly suggest that the hypoxia-induced TGF- $\beta$ 2 expression in human articular chondrocytes *in vitro* is responsible for the subsequent induction of type II collagen by these cells. We further provide data supporting the notion that ALK5 is critically involved in this process. This study suggests that hypoxic culture conditions for cartilage tissue engineering may benefit from an auto-regulatory TGF- $\beta$  signaling loop by the chondrocytes.

## 5. Acknowledgements

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## 6. Supplementary data

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Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aanat.2014.11.003>.



## References

- 1 Falchuk KH, Goetzel EJ, Kulka JP. Respiratory gases of synovial fluids. An approach to synovial tissue circulatory-metabolic imbalance in rheumatoid arthritis. *Am J Med.* 1970;49(2):223-31.
- 2 Lund-Olesen K. Oxygen tension in synovial fluids. *Arthritis Rheum.* 1970;13(6):769-76.
- 3 Silver IA. Measurement of pH and ionic composition of pericellular sites. *Philos Trans R Soc Lond B Biol Sci.* 1975;271(912):261-72.
- 4 Treuhaft PS, DJ MC. Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. *Arthritis Rheum.* 1971;14(4):475-84.
- 5 Grimshaw MJ, Mason RM. Bovine articular chondrocyte function *in vitro* depends upon oxygen tension. *Osteoarthritis Cartilage.* 2000;8(5):386-92.
- 6 Malda J, Martens DE, Tramper J, van Blitterswijk CA, Riesle J. Cartilage tissue engineering: controversy in the effect of oxygen. *Crit Rev Biotechnol.* 2003;23(3):175-94.
- 7 Grimshaw MJ, Mason RM. Modulation of bovine articular chondrocyte gene expression *in vitro* by oxygen tension. *Osteoarthritis Cartilage.* 2001;9(4):357-64.
- 8 Coyle CH, Izzo NJ, Chu CR. Sustained hypoxia enhances chondrocyte matrix synthesis. *J Orthop Res.* 2009;27(6):793-9.
- 9 Domm C, Schunke M, Steinhagen J, Freitag S, Kurz B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine articular chondrocytes in alginate bead culture under high and low oxygen tension. *Tissue Eng.* 2004;10(11-12):1796-805.
- 10 Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells.* 2002;7(12):1191-204.
- 11 Roberts AB, Sporn MB. Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol Reprod Dev.* 1992;32(2):91-8.
- 12 Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, *et al.* TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development.* 1997;124(13):2659-70.
- 13 Akman HO, Zhang H, Siddiqui MA, Solomon W, Smith EL, Batuman

## Chapter 2

- OA. Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. *Blood*. 2001;98(12):3324-31.
- 14 Falanga V, Qian SW, Danielpour D, Katz MH, Roberts AB, Sporn MB. Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. *J Invest Dermatol*. 1991;97(4):634-7.
- 15 Martin G, Andriamanalijaona R, Grassel S, Dreier R, Mathy-Hartert M, Bogdanowicz P, *et al*. Effect of hypoxia and reoxygenation on gene expression and response to interleukin-1 in cultured articular chondrocytes. *Arthritis Rheum*. 2004;50(11):3549-60.
- 16 Jullien P, Berg TM, Lawrence DA. Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. *Int J Cancer*. 1989;43(5):886-91.
- 17 Lyons RM, Keski-Oja J, Moses HL. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J Cell Biol*. 1988;106(5):1659-65.
- 18 Yee JA, Yan L, Dominguez JC, Allan EH, Martin TJ. Plasminogen-dependent activation of latent transforming growth factor beta (TGF beta) by growing cultures of osteoblast-like cells. *J Cell Physiol*. 1993;157(3):528-34.
- 19 Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci*. 2003;116(Pt 2):217-24.
- 20 O'Connor WJ, Botti T, Khan SN, Lane JM. The use of growth factors in cartilage repair. *Orthop Clin North Am*. 2000;31(3):399-410.
- 21 van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. *Osteoarthritis Cartilage*. 2002;10(8):631-7.
- 22 Demoor-Fossard M, Boittin M, Redini F, Pujol JP. Differential effects of interleukin-1 and transforming growth factor beta on the synthesis of small proteoglycans by rabbit articular chondrocytes cultured in alginate beads as compared to monolayers. *Mol Cell Biochem*. 1999;199(1-2):69-80.
- 23 Vivien D, Galera P, Lebrun E, Loyau G, Pujol JP. Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes. *J Cell Physiol*. 1990;143(3):534-45.
- 24 van Osch GJ, Mandl EW, Marijnissen WJ, van der Veen SW, Verwoerd-Verhoef HL, Verhaar JA. Growth factors in cartilage tissue engineering. *Biorheology*. 2002;39(1-2):215-20.

- 25 Yaeger PC, Masi TL, de Ortiz JL, Binette F, Tubo R, McPherson JM. Synergistic action of transforming growth factor-beta and insulin-like growth factor-I induces expression of type II collagen and aggrecan genes in adult human articular chondrocytes. *Exp Cell Res.* 1997;237(2):318-25.
- 26 Okazaki R, Sakai A, Nakamura T, Kunugita N, Norimura T, Suzuki K. Effects of transforming growth factor beta s and basic fibroblast growth factor on articular chondrocytes obtained from immobilised rabbit knees. *Ann Rheum Dis.* 1996;55(3):181-6.
- 27 Leonard MO, Howell K, Madden SF, Costello CM, Higgins DG, Taylor CT, *et al.* Hypoxia selectively activates the CREB family of transcription factors in the in vivo lung. *Am J Respir Crit Care Med.* 2008;178(9):977-83.
- 28 Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature.* 1997;390(6659):465-71.
- 29 Finnsen KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. *J Bone Miner Res.* 2008;23(6):896-906.
- 30 Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, *et al.* Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J Immunol.* 2009;182(12):7937-45.
- 31 Das RH, van Osch GJ, Kreukniet M, Oostra J, Weinans H, Jahr H. Effects of individual control of pH and hypoxia in chondrocyte culture. *J Orthop Res.* 2010;28(4):537-45.
- 32 Uitterlinden EJ, Jahr H, Koevoet JL, Jenniskens YM, Bierma-Zeinstra SM, Degroot J, *et al.* Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants. *Osteoarthritis Cartilage.* 2006;14(3):250-7.
- 33 Uitterlinden EJ, Jahr H, Koevoet JL, Bierma-Zeinstra SM, Verhaar JA, Weinans H, *et al.* Glucosamine reduces anabolic as well as catabolic processes in bovine chondrocytes cultured in alginate. *Osteoarthritis Cartilage.* 2007;15(11):1267-74.
- 34 Bustin SA, Benes V, Garson JA, Helleman J, Huggett J, Kubista M, *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55(4):611-22.
- 35 van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, *et al.* Physiological tonicity improves human chondrogenic



## Chapter 2

- marker expression through nuclear factor of activated T-cells 5 *in vitro*. *Arthritis Res Ther*. 2010;12(3):R100.
- 36 Foldager CB, Munir S, Ulrik-Vinther M, Soballe K, Bunger C, Lind M. Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. *BMC Mol Biol*. 2009;10:94.
- 37 Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol Lett*. 2004;26(6):509-15.
- 38 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>( $\Delta\Delta C_T$ ) Method. *Methods*. 2001;25(4):402-8.
- 39 Bauge C, Cauvard O, Leclercq S, Galera P, Boumediene K. Modulation of transforming growth factor beta signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of Sp1 in both early and late response cells to transforming growth factor beta. *Arthritis Res Ther*. 2011;13(1):R23.
- 40 Das RH, Jahr H, Verhaar JA, van der Linden JC, van Osch GJ, Weinans H. *In vitro* expansion affects the response of chondrocytes to mechanical stimulation. *Osteoarthritis Cartilage*. 2008;16(3):385-91.
- 41 Lafont JE, Talma S, Hopfgarten C, Murphy CL. Hypoxia promotes the differentiated human articular chondrocyte phenotype through SOX9-dependent and -independent pathways. *J Biol Chem*. 2008;283(8):4778-86.
- 42 van der Windt AE, Haak E, Kops N, Verhaar JA, Weinans H, Jahr H. Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. *Arthritis Rheum*. 2012;64(6):1929-39.
- 43 Caron MM, Emans PJ, Coolsen MM, Voss L, Surtel DA, Cremers A, *et al*. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2012;20(10):1170-8.
- 44 Siebelt M, Jahr H, Groen HC, Sandker M, Waarsing JH, Kops N, *et al*. Hsp90 inhibition protects against biomechanically induced osteoarthritis in rats. *Arthritis Rheum*. 2013;65(8):2102-12.
- 45 Caron MM, van der Windt AE, Emans PJ, van Rhijn LW, Jahr H, Welting TJ. Osmolarity determines the *in vitro* chondrogenic differentiation capacity

- of progenitor cells via nuclear factor of activated T-cells 5. *Bone*. 2013;53(1):94-102.
- 46 Grygielko ET, Martin WM, Tweed C, Thornton P, Harling J, Brooks DP, *et al*. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-beta type I receptor kinase in puromycin-induced nephritis. *J Pharmacol Exp Ther*. 2005;313(3):943-51.
- 47 Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J*. 1998;17(11):3091-100.
- 48 Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J*. 2002;21(7):1743-53.
- 49 Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, *et al*. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J Immunol*. 2009;182(12):7937-45.
- 50 Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, *et al*. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *Embo J*. 2004;23(20):4018-28.
- 51 Fujita N, Markova D, Anderson DG, Chiba K, Toyama Y, Shapiro IM, *et al*. Expression of prolyl hydroxylases (PHDs) is selectively controlled by HIF-1 and HIF-2 proteins in nucleus pulposus cells of the intervertebral disc: distinct roles of PHD2 and PHD3 proteins in controlling HIF-1alpha activity in hypoxia. *J Biol Chem*. 2012;287(20):16975-86.
- 52 Hashimoto K, Fukuda K, Yamazaki K, Yamamoto N, Matsushita T, Hayakawa S, *et al*. Hypoxia-induced hyaluronan synthesis by articular chondrocytes: the role of nitric oxide. *Inflamm Res*. 2006;55(2):72-7.
- 53 Lee JJ, Natsuizaka M, Ohashi S, Wong GS, Takaoka M, Michaylira CZ, *et al*. Hypoxia activates the cyclooxygenase-2-prostaglandin E synthase axis. *Carcinogenesis*. 2010;31(3):427-34.
- 54 Markway BD, Cho H, Johnstone B. Hypoxia promotes redifferentiation and suppresses markers of hypertrophy and degeneration in both healthy and osteoarthritic chondrocytes. *Arthritis Res Ther*. 2013;15(4):R92.
- 55 Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugge B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro

- alpha1(II) collagen gene. *Mol Cell Biol.* 1997;17(4):2336-46.
- 56 Murphy CL, Thoms BL, Vaghjiani RJ, Lafont JE. Hypoxia. HIF-mediated articular chondrocyte function: prospects for cartilage repair. *Arthritis Res Ther.* 2009;11(1):213.
- 57 Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA. Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. *Biochem J.* 2001;360(Pt 2):461-70.
- 58 D'Angelo M, Yan Z, Nooreyazdan M, Pacifici M, Sarment DS, Billings PC, *et al.* MMP-13 is induced during chondrocyte hypertrophy. *J Cell Biochem.* 2000;77(4):678-93.
- 59 Van Osch GJ, Van Der Veen SW, Burger EH, Verwoerd-Verhoef HL. Chondrogenic potential of *in vitro* multiplied rabbit perichondrium cells cultured in alginate beads in defined medium. *Tissue Eng.* 2000;6(4):321-30.
- 60 Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. *J Bone Miner Res.* 2008;23(6):896-906.
- 61 Walmsley SR, Chilvers ER, Thompson AA, Vaughan K, Marriott HM, Parker LC, *et al.* Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. *J Clin Invest.* 2011;121(3):1053-63.
- 62 Marxsen JH, Schmitt O, Metzen E, Jelkmann W, Hellwig-Burgel T. Vascular endothelial growth factor gene expression in the human breast cancer cell line MX-1 is controlled by O<sub>2</sub> availability *in vitro* and *in vivo*. *Ann Anat.* 2001;183(3):243-9.
- 63 Zhang C, Li Y, Cornelia R, Swisher S, Kim H. Regulation of VEGF expression by HIF-1alpha in the femoral head cartilage following ischemia osteonecrosis. *Sci Rep.* 2012;2:650.
- 64 Jansen H, Meffert RH, Birkenfeld F, Petersen W, Pufe T. Detection of vascular endothelial growth factor (VEGF) in moderate osteoarthritis in a rabbit model. *Ann Anat.* 2012;194(5):452-6.
- 65 Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, *et al.* Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci U S A.* 1986;83(12):4167-71.
- 66 Miura T, Shiota K. TGFbeta2 acts as an "activator" molecule in reaction-diffusion



- model and is involved in cell sorting phenomenon in mouse limb micromass culture. *Dev Dyn.* 2000;217(3):241-9.
- 67 Basu RK, Hubchak S, Hayashida T, Runyan CE, Schumacker PT, Schnaper HW. Interdependence of HIF-1alpha and TGF-beta/Smad3 signaling in normoxic and hypoxic renal epithelial cell collagen expression. *Am J Physiol Renal Physiol.* 2011;300(4):F898-905.
- 68 Grimaud E, Heymann D, Redini F. Recent advances in TGF-beta effects on chondrocyte metabolism. Potential therapeutic roles of TGF-beta in cartilage disorders. *Cytokine Growth Factor Rev.* 2002;13(3):241-57.
- 69 Bizet AA, Liu K, Tran-Khanh N, Saksena A, Vorstenbosch J, Finnson KW, *et al.* The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. *Biochim Biophys Acta.* 2011;1813(5):742-53.
- 70 Parker WL, Finnson KW, Soe-Lin H, Knaus P, Philip A. Expression and function of TbetaRII-B, a variant of the type II TGF-beta receptor, in human chondrocytes. *Osteoarthritis Cartilage.* 2007;15(4):442-53.
- 71 Brown CB, Boyer AS, Runyan RB, Barnett JV. Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science.* 1999;283(5410):2080-2.
- 72 Sankar S, Mahooti-Brooks N, Centrella M, McCarthy TL, Madri JA. Expression of transforming growth factor type III receptor in vascular endothelial cells increases their responsiveness to transforming growth factor beta 2. *J Biol Chem.* 1995;270(22):13567-72.
- 73 Finnson KW, Parker WL, Chi Y, Hoemann CD, Goldring MB, Antoniou J, *et al.* Endoglin differentially regulates TGF-beta-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes. *Osteoarthritis Cartilage.* 2010;18(11):1518-27.
- 74 Estrada KD, Wang W, Retting KN, Chien CT, Elkhoury FF, Heuchel R, *et al.* Smad7 regulates terminal maturation of chondrocytes in the growth plate. *Dev Biol.* 2013;382(2):375-84.
- 75 Ueno M, Maeno T, Nomura M, Aoyagi-Ikeda K, Matsui H, Hara K, *et al.* Hypoxia-inducible factor-1alpha mediates TGF-beta-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol.* 2011;300(5):L740-52.
- 76 Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-

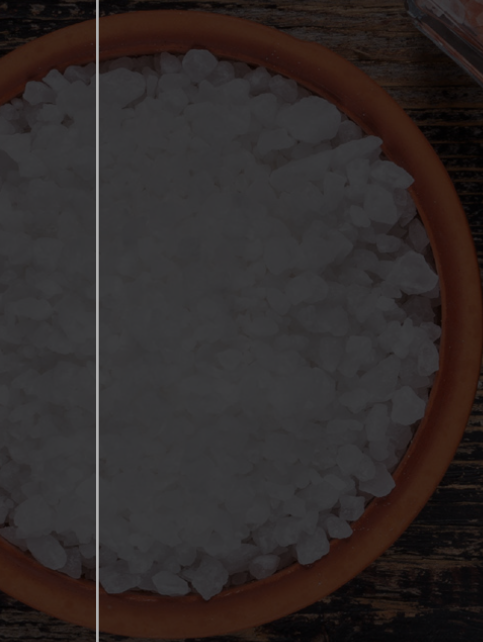
## Chapter 2

- inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J.* 1998;17(11):3091-100.
- 77 Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, *et al.* TGF beta signals through a heteromeric protein kinase receptor complex. *Cell.* 1992;71(6):1003-14.
- 78 Dennler S, Huet S, Gauthier JM. A short amino-acid sequence in MH1 domain is responsible for functional differences between Smad2 and Smad3. *Oncogene.* 1999;18(8):1643-8.
- 79 Dimova EY, Jakubowska MM, Kietzmann T. CREB binding to the hypoxia-inducible factor-1 responsive elements in the plasminogen activator inhibitor-1 promoter mediates the glucagon effect. *Thromb Haemost.* 2007;98(2):296-303.
- 80 Kvietikova I, Wenger RH, Marti HH, Gassmann M. The hypoxia-inducible factor-1 DNA recognition site is cAMP-responsive. *Kidney Int.* 1997;51(2):564-6.
- 81 Wu X, Chang MS, Mitsialis SA, Kourembanas S. Hypoxia regulates bone morphogenetic protein signaling through C-terminal-binding protein 1. *Circ Res.* 2006;99(3):240-7.
- 82 Ghayor C, Chadjichristos C, Herrouin JF, Ala-Kokko L, Suske G, Pujol JP, *et al.* Sp3 represses the Sp1-mediated transactivation of the human COL2A1 gene in primary and de-differentiated chondrocytes. *J Biol Chem.* 2001;276(40):36881-95.
- 83 Yang X, Chen L, Xu X, Li C, Huang C, Deng CX. TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. *J Cell Biol.* 2001;153(1):35-46.





Chapter  
03



**Increased TGF- $\beta$  and BMP Levels and Improved Chondrocyte-Specific Marker Expression *In Vitro* under Cartilage-Specific Physiological Osmolarity.**

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# Abstract

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### Objective:

During standard expansion culture (i.e., plasma osmolarity, 280 mOsm) human articular chondrocytes dedifferentiate, making them inappropriate for autologous chondrocyte implantation to treat cartilage defects. Increasing the osmolarity of culture media to physiological osmolarity levels of cartilage (i.e., 380 mOsm), increases collagen type II (*COL2A1*) expression of human articular chondrocytes *in vitro*, but the underlying molecular mechanism is not fully understood. We hypothesized that TGF- $\beta$  superfamily signaling may drive expression of *COL2A1* under physiological osmolarity culture conditions.

### Material & Methods:

Human articular chondrocytes were cultured in cytokine-free medium of 280 or 380 mOsm with or without siRNA mediated TGF- $\beta$ 2 knockdown (RNAi). Expression of TGF- $\beta$  isoforms, and collagen type II was evaluated by RT-qPCR and immunoblotting. TGF- $\beta$ 2 protein secretion was evaluated using ELISA and TGF- $\beta$  bioactivity was determined using an established reporter assay. Involvement of BMP signaling was investigated by culturing human articular chondrocytes in the presence or absence of BMP inhibitor dorsomorphin and BMP bioactivity was determined using an established reporter assay.

### Results:

Physiological cartilage osmolarity (i.e., physosmolarity) most prominently increased TGF- $\beta$ 2 mRNA expression and protein secretion as well as TGF- $\beta$  bioactivity. Upon TGF- $\beta$ 2 isoform-specific knockdown, gene expression of chondrocyte marker *COL2A1* was induced. TGF- $\beta$ 2 RNAi under physosmolarity enhanced TGF- $\beta$  bioactivity. BMP bioactivity increased upon physosmotic treatment, but was not related to TGF- $\beta$ 2 RNAi. In contrast, dorsomorphin inhibited *COL2A1* mRNA expression in human articular chondrocytes independent of the osmotic condition.



### Conclusion:

Our data suggest a role for TGF- $\beta$  superfamily member signaling in physosmolarity-induced mRNA expression of collagen type II. As physosmotic conditions favor the expression of *COL2A1* independent of our manipulations, contribution of other metabolic, post-transcriptional or epigenetic factors cannot be excluded in the underlying complex and interdependent regulation of marker gene expression. Dissecting these molecular mechanisms holds potential to further improve future cell-based chondral repair strategies.

### Keywords:

Chondrocyte, osmolarity, TGF- $\beta$  superfamily, signalling, collagen type II, bone morphogenetic proteins.

## 1. Introduction

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Articular cartilage defects do not heal spontaneously and recent data suggest that treatment of these cartilage defects by microfracture (MF) procedures are inferior to autologous chondrocyte implantation (ACI)<sup>1,2</sup> due to biomechanically inferior repair tissue<sup>3</sup>. Despite ACI being superior to microfracture, it is not always characterized by hyaline cartilage repair tissue<sup>4</sup>. Further improvements of currently available chondrocyte-based treatment approaches such as the ACI are thus needed.

Chondrocytes are the main cell type found in cartilage (i.e., about 1–5% v/v) and responsible for maintaining the cartilage extracellular matrix (ECM)<sup>5</sup>. ACI involves *in vitro* expansion of human articular chondrocytes (HACs) prior to re-implantation into the cartilage defect. During *in vitro* expansion chondrocytes inevitably lose their specific phenotype and dedifferentiate with increasing passages making them gradually less suitable for autologous cell therapies<sup>6</sup>. Dedifferentiation of HACs is characterized by reduced expression of aggrecan (ACAN) and collagen type II (*COL2A1*) mRNA, probably being the most important cartilage-specific markers, and accompanied by an increased fibroblast-like phenotype<sup>6</sup>.

Cartilage requires a high osmotic value of its interstitial fluid to maintain its hydrostatic pressure and viscoelastic properties, for which negatively charged glycosaminoglycan side chains of the proteoglycans (PGs) are crucial to attract mobile cations and water into the ECM environment. The intact collagen network, in contrast, restricts the inherent swelling force of the ECM and determines the relatively high osmolarity of this extracellular fluid<sup>5</sup>. The latter ranges between 380 and 450 mOsm in healthy cartilage (i.e., physiological osmolarity)<sup>5</sup>, which is markedly higher than that of plasma levels or standard culture medium (around 280 mOsm)<sup>7</sup>. In analogy to using “physoxia” to describe physiological oxygenation levels in cartilage, avoiding the term hypoxia<sup>8</sup>, we would like to introduce physiological osmolarity as “physosmotic”. We and others have already shown that cultured chondrocytes are osmo-responsive and improve ECM synthesis accordingly<sup>5,9,10</sup>. However, the underlying molecular mechanism is poorly understood.

Transforming growth factor (TGF- $\beta$ ) plays an indispensable role in cartilage repair and homeostasis<sup>11,12</sup>. This pleiotropic growth factor is the prototypic member of the TGF- $\beta$  superfamily, which further includes, among others, the large subfamily of bone morphogenetic proteins (BMPs)<sup>13</sup>. Three mammalian isoforms of TGF- $\beta$  have been described, with non-redundant functions *in vivo*<sup>14,15</sup>. In chondrocytes, TGF- $\beta$  has been implicated in the transcription of collagen type II<sup>16</sup>. Canonically, TGF- $\beta$  ligands bind to the TGF- $\beta$  type II receptor (TGFBRII) which, in most cells, then recruits the type I receptor activin-like kinase 5 (ALK5) to phosphorylate intracellular effector molecules SMAD-2 and -3 to regulate transcriptional activity<sup>17</sup>. It was shown that in chondrocytes TGF- $\beta$  can also signal through an alternative receptor, ALK1<sup>18</sup> to phosphorylate SMAD1/5/8, rather than SMAD2/3. The ALK5/ALK1 ratio now appears to be responsible for the seemingly contradictory actions of TGF- $\beta$  in chondrocytes<sup>19</sup>. Context-dependent ambivalent actions of TGF- $\beta$  on articular chondrocytes have been reviewed<sup>12</sup>, underscoring its key role in chondrocyte ECM synthesis and homeostasis.

Since TGF- $\beta$  is known to induce *COL2A1* expression in chondrocytes<sup>18</sup>, it is tempting to speculate that increased *COL2A1* expression in *in vitro* HAC cultures upon physosmotic treatment, may be caused by stimulated TGF- $\beta$  signaling. Currently, however, little is known about how osmolarity may induce endogenous TGF- $\beta$  signalling, which holds especially

for chondrocytes. Dissecting molecular mechanisms underlying physosmotic induction of chondrocyte markers might aid in further improving cell-based chondral repair strategies, as well as improving HAC culture conditions for research purposes<sup>20</sup>.

In the present study, we therefore aim to elucidate whether changes in TGF- $\beta$  signalling underlie the cartilage physosmolar induction of chondrocyte marker gene expression in *in vitro* HAC cultures.

## 2. Materials and Methods

### 2.1 Cartilage and Chondrocyte Isolation

Human articular cartilage was explanted from macroscopically normal areas of the femoral condyles and tibial plateau of nine patients undergoing total knee replacement surgery for osteoarthritis (OA). The Erasmus MC and the Maastricht University Medical Centre institutional policy on the use of residual human surgical material specifically states that no informed consent is needed in the case of residual surgical material. However, an approval from the institutional Medical Ethical Committee (MEC) for the use of this material is required. The MEC approved this study and assigned approval ID: MEC2004-322 and MEC08-4-028 (23 June 2008).

Cartilage was separated from the subchondral bone and cut into small pieces using a sterile surgical blade. Cartilage pieces were digested overnight at 37 °C in collagenase type II solution (300 U/mL in HEPES buffered DMEM/F12 supplemented with antibiotics (Invitrogen, Carlsbad, CA, USA), 280 mOsm) under continuous agitation<sup>10,46,47,48</sup>. Medium osmolarity was experimentally adjusted to 380 mOsm (physosmotic medium i.e., PM) by adding sterile NaCl as reported earlier<sup>10</sup>. The preparation was rinsed with 0.9% NaCl over a 70  $\mu$ m cell strainer and plated in culture flasks.



### 2.2 Chondrocyte Expansion and Culturing

Briefly, primary HACs were cultured (humidified atmosphere at 37 °C, 5% CO<sub>2</sub>) for expansion in monolayers at a seeding density of 7500 cells/cm<sup>2</sup> in culture medium consisting of DMEM/F12 (Invitrogen) with 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic/antimycotic (Invitrogen) corresponding to their isolation osmolarity; 280 mOsm (osmotic control medium, i.e., OCM) or 380 mOsm (physosmotic medium i.e., PM). Once cells reached confluency, they were trypsinized, resuspended, and replated into 175 cm<sup>2</sup> flasks. Within four weeks after harvest early passage HACs (P1, P2) were seeded in high-density monolayers (20,000 cells/cm<sup>2</sup>) and were cultured in OCM or PM, respectively, for an additional 7 days before analysis. Moreover, HACs were cultured for 7 days, including a 72 h serum free period in order to collect conditioned medium for TGF-β protein analyses (TGF-β2 isoform-specific ELISA and TGF-β bioassay). Experiments were performed in technical replicates from six donors.

### 2.3 RNA Expression Analysis

According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines<sup>49</sup>, we subsequently aim at reporting suggested essential information: (i) the experimental design is outlined elsewhere in this section, with the numbers within each group reported as “n” per analyses, (ii) the sample dissection, processing and storage has been reported earlier by us<sup>10</sup>, (iii) total RNA extraction, purification, and quantification thereof are also reported in much detail<sup>10</sup>, as was (iv) the RT reaction, both using commercially available Eurogentec kits. Other essential details on the reaction conditions of the cDNA synthesis has also been previously reported<sup>10</sup>. With respect to (v) qPCR target information, we aimed at using the latest Entrez Gene Symbols, designed our assays using the National Center for Biotechnology Information (NCBI)’s database RefSeq sequences<sup>50</sup>, performed NCBI’s Primer-Blast to verify specificity *in silico* and report NCBI Gene IDs. Real-time quantitative PCR (RT-qPCR) was performed using Mesagreen™ qPCR mastermix plus for SYBR Green (Eurogentec, Maastricht, The Netherlands) and a CFX96 Real-Time PCR Detection system (Biorad, Hercules, CA, USA for amplification with the following profile: initial denaturation 10 min at 95 °C, followed by 50 cycles of amplification (15 s at

95 °C and 1 min at 60 °C), and followed by a dissociation curve. Specificity of the Primer Designer™ (ThermoFisher Scientific, Waltham, MA, USA) designed and mfold-checked amplicons (> 70 < 250 bp; default secondary structure exclusion settings) were checked by agarose gel electrophoresis to exclude primer dimers and post run melting curve evaluation and assays were selected to have PCR efficiencies of >0.95. Data analysis: data were normalized to an index of stably-expressed reference genes that were pre-evaluated to be stably expressed across samples by BestKeeper software (Microsoft, Redmond, WA, USA) as was reported elsewhere by our group<sup>2,10</sup>. All NTCs had  $C_q \leq 39$  and GOIs with  $C_q \geq 35$  were excluded from analyses. Relative expression was calculated according to the delta-delta  $C_T$  method. Not earlier reported oligonucleotide sequences are listed in Table 1 and the statistical methods are reported elsewhere in this section.

**Table 1: oligonucleotide sequences**

GENE AMPLICON	ID T <sub>M</sub>	FORWARD/SENSE (5' -3') NT POSITIONS	REVERSE/ANTISENSE (5' -3') NT POSITIONS
COL2A1 44 bp	1280 62.0/62.4	TGGACGATCAG GCGAAACC 3570–3588	GCTGCGGATGCT CTCAATCT 3813–3794
TGFB1 209 bp	7040 61.4/60.7	CTAATGGTGGGA AACCCACAACG 334–355	TATCGCCAGGAAT TGTTGCTG 542–522
TGFB2 154 bp	7042 2.3/62.9	CCATCCCGCCC ACTTTCTAC 434–453	AGCTCAATCCGTT GTTTCAGGC 587–567
TGFB3 121 bp	7043 0.9/60.3	GGAAAACACCGA GTCGGAATAC 279–300	GCGGAAAACCTT GGAGGTAAT 399–379
ALK1 194 bp	94 2.3/62.3	CATCGCCTCAG ACATGACCTC 777–797	GTTTGCCTGTG TACCGAAGA 970–950
ALK5 167 bp	7046 60.6/61.1	ACGGCGTTACAGT GTTTCTG 94–113	GCACATACAAAC GGCCTATCTC 260–239
GAPDH 101 bp	2597 2.0/62.9	CTGGGCTACTACT GAGCACC 694–712	AAGTGGTCGTTG AGGGCAATG 794–774
TGF- $\beta$ 2 siRNA	-	CTAATGGTGGAAA CCCACAACG	TATCGCCAGGAA TTGTTGCTG

Primer sequences for RT-qPCR and oligonucleotide sequences for RNAi are listed in the 5'–3' direction. Gene, NCBI Gene Symbol; ID, NCBI Gene ID; bp, base pairs; nt positions, location of primers. Note the following newer aliases for above-listed gene symbols: ALK1 (ACVRL1), ALK5 (TGFB1).

## 2.4 Immunoblotting

HACs were washed with 0.9% NaCl and lysed in RIPA buffer. Extracts were sonicated on ice using the Soniprep 150 (MSE, London, UK). Insoluble material was removed by centrifugation (13,000 $\times$  g, 4 °C). The BCA protein assay (Sigma-Aldrich) was used to determine protein concentration. Polypeptides were separated by SDS-PAGE (sodium



dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes by electroblotting. Primary antibodies (all 1:100 dilution) for immunodetection were polyclonal goat anti-*COL2A1* 1320-01, Southern Biotech, Birmingham, AL, USA), and mouse monoclonal anti-GAPDH (10R-1261096, Fitzgerald, MA, USA). Bound primary antibodies were detected with secondary immunoglobulins conjugated with horseradish peroxidase (Dako) and visualized by enhanced chemiluminescence (ECL). ECL signals were quantified using ImageJ 1.46f software (National Institutes of Health, Bethesda, MA, USA), and relative differences, corrected for background and housekeeper, were determined as compared to control conditions.

### 2.5 TGF- $\beta$ 2 ELISA

To determine TGF- $\beta$ 2 in the secretome, medium was collected and centrifuged at 1200 rpm for 8 min to remove cell debris. The cleared supernatant was collected and stored at  $-80^{\circ}\text{C}$  until further use. TGF- $\beta$ 2 concentration was quantified spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA; Demeditec Diagnostics GmbH, Kiel, Germany). TGF- $\beta$ 2 concentrations in the samples were calculated from a standard curve according to manufacturer's instructions.

### 2.6 TGF- $\beta$ Bioassay

To verify that the secreted TGF- $\beta$  as measured by ELISA is biologically active, we next employed an established luciferase assay, using the CAGA-luc Smad2/3 reporter. Human embryonic kidney cells were seeded at  $3 \times 10^4$ /well in 24-well plates and transiently transfected with 150 ng of the reporter construct and 75 ng of pRL-TK vector (Promega, Madison, WI, USA), an internal control for transfection efficiency, using FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Twenty-four hours after transfection, cells were incubated for 2 h in medium containing 0.2% FCS, followed by 16 h incubation with 10 ng/mL TGF- $\beta$ 2 as a positive control, DMEM/F12 as a negative control or 300  $\mu\text{L}$  conditioned medium (see above, ELISA). Twenty-four hours after stimulation, the firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

### 2.7 RNAi Experiments

Primary HACs from three OA patients were cultured at OCM for two weeks and then seeded in high-density monolayers (20,000 cells/cm<sup>2</sup>). A TGF- $\beta$ 2-specific siRNA duplex (Table 1) and a scrambled siRNA control duplex were used (Eurogentec, Seraing, Belgium)<sup>47</sup>. HACs were transfected with siRNAs (100 nM) at OCM using HiPerFect (Qiagen, Hilden, Germany) according to the manufacturer's protocol. One day after transfection, HACs were refreshed with either OCM or PM in the presence or absence of dorsomorphin (10  $\mu$ M, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Forty-eight hours after stimulation cells were harvested for mRNA expression or protein analyses. HACs were also seeded and cultured with either DMSO (1:1000) or dorsomorphin (10  $\mu$ M). One day after seeding, cells were either refreshed with OCM or PM, and 48 h later harvested for gene expression analyses. In another experiment, SW1353 cells were seeded in high-density monolayers (30,000 cells/cm<sup>2</sup>). Cells were transfected with the previously described CAGA-luc Smad2/3 reporter or the BRE-luc reporter using FuGene transfection reagent (Promega, Madison, WI, USA) at OCM<sup>51</sup>. The gaussia luciferase reporter construct was used as an internal control for transfection efficiency. One day after transfection of the reporters, the previously described TGF- $\beta$ 2-specific siRNA duplex and a scrambled siRNA control duplex were transfected (100 nM) at OCM using HiPerFect (Qiagen, Hilder, Germany). Five hours after siRNA transfection medium was refreshed with either OCM or PM. Forty-eight hours later, cells were processed and their luciferase activity was measured by a luciferase reporter assay system (Promega, Madison, WI, USA).

### 2.8 Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were tested for normality using Kolmogorov-Smirnov test and normality plots were visually assessed for skewness. All samples for gene expression analysis and TGF- $\beta$ 2 measurements in medium were processed and analyzed individually with replicate measurements per donor. Gene expression levels, TGF- $\beta$ 2 protein expression and luciferase activity between HACs cultured in PM or OCM were compared by an unpaired *t*-test. Data obtained from RNAi experiments were analyzed by a mixed linear model test. In

the model, conditions with or without TGF- $\beta$ 2 knockdown and with or without physosmotic treatment were considered as fixed factors, while the cartilage donor was considered as a random factor. Adjustment for multiple comparisons was made by a Bonferonni's post hoc comparison test.  $p < 0.05$  was considered to indicate levels of statistically significant difference.

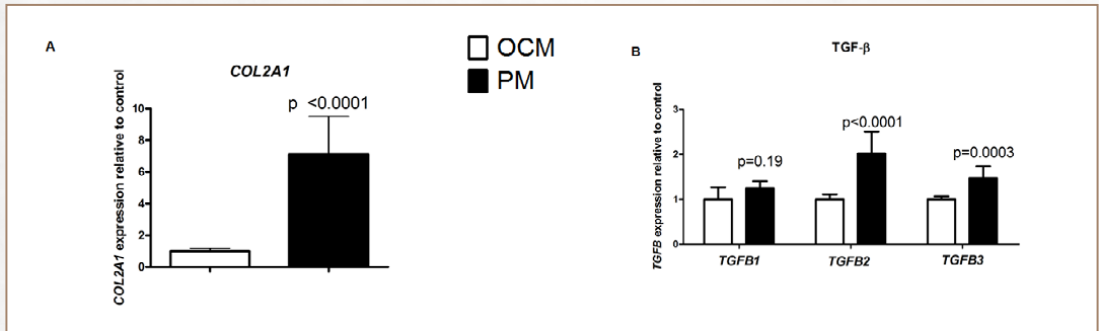
### 3. Results

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#### 3.1 Cartilage Physosmotic Culture Induces Specific TGF- $\beta$ Isoform Expression in HACs

Confirming our earlier results<sup>10</sup>, culturing HACs for seven days in physosmotic conditions (380 mOsm, physosmotic medium, PM) results in significantly ( $p \leq 0.0001$ ) elevated *COL2A1* mRNA expression, as compared to 280 mOsm (i.e., osmotic control medium, OCM) (Figure 1A). To determine whether this increased *COL2A1* expression upon physosmotic treatment may be caused by stimulated specific TGF- $\beta$  isoform induced signalling, mRNA expression of the three human TGF- $\beta$  isoforms (*TGFB1-3*) was measured. PM selectively induced expression of *TGFB2* (two-fold,  $p < 0.0001$ ) and *TGFB3* (1.5-fold,  $p < 0.0001$ ), respectively. In contrast, expression of *TGFB1* was not significantly altered by changes in medium osmolarity (Figure 1B).



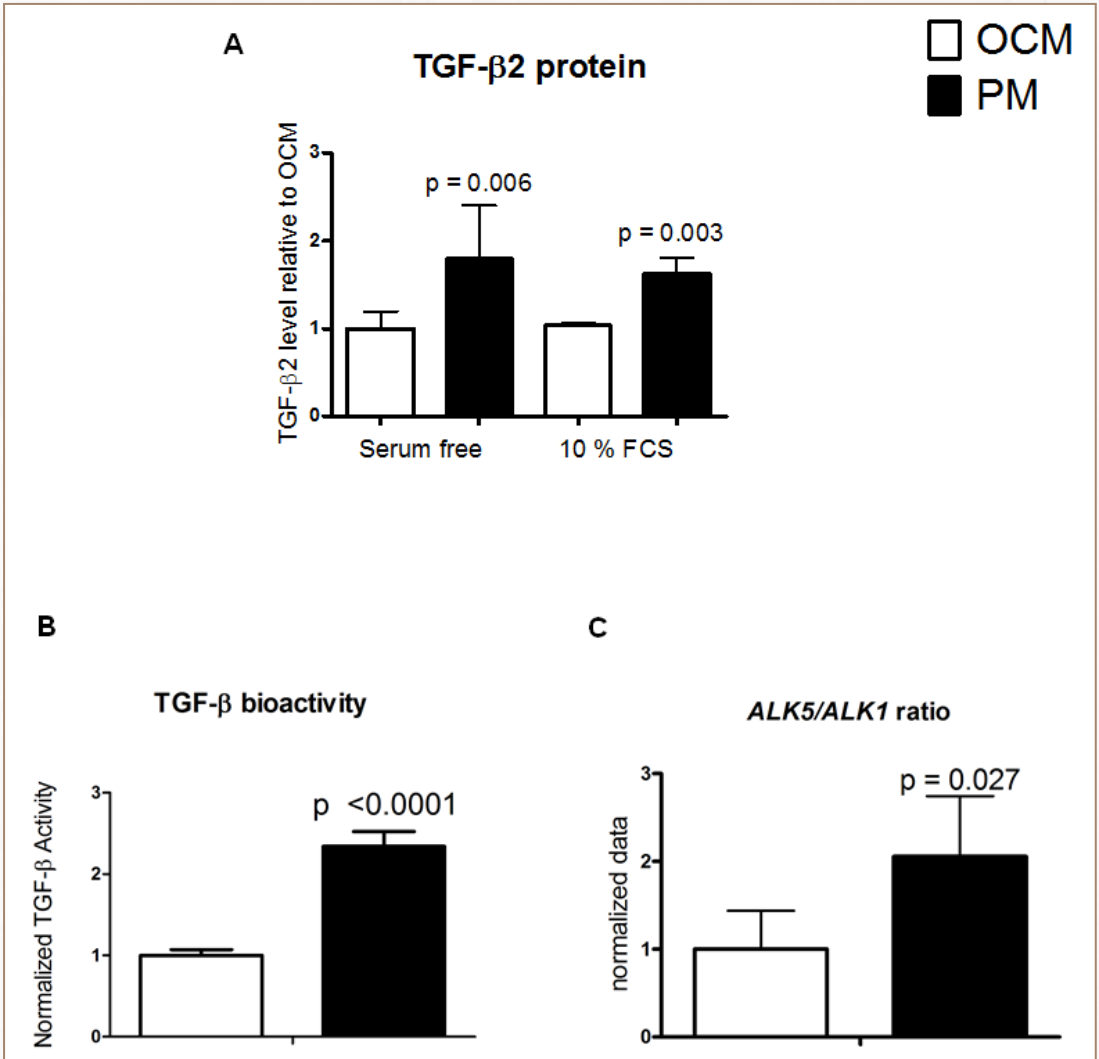


**Figure 1: Physosmolarity-induced changes in gene expression of chondrocytes in vitro.**

Isolating and expanding HACs at PM for 7 days (black bars) significantly increased gene expression of COL2A1 (A), as well as TGFβ2 and TGFβ3 as compared to control (OCM; white bars) (B). Gene expression of TGFβ1 was not significantly affected (B). mRNA levels were determined relative to control OCM conditions by RT-qPCR (normalized for housekeeper expression) in HACs. Data are from six donors measured in duplicate and presented as the mean  $\pm$  standard deviation.

### 3.2 Physosmolarity Increases Secretion of Bioactive TGF-β2

As PM most prominently increased TGFβ2 mRNA abundance, we next aimed at confirming if this resulted in increased TGF-β2 protein level, using a TGF-β2 isoform-specific ELISA assay. HACs were cultured in either serum free (SF) medium or with 10% FCS and TGF-β2 secretion in culture supernatants was measured after seven days. TGF-β2 secretion was largely independent of the presence of serum in the culture medium and showed a 1.6-fold (10% FCS,  $p = 0.003$ ) and two-fold (serum-free,  $p = 0.006$ ) increase in PM as compared to OCM (Figure 2A). The osmolarity-dependent change in TGFβ2 gene expression is thus predictive for TGF-β2 protein secretion in culture supernatant in response to PM. To determine whether the secreted TGF-β2 is also bioactive, we performed an established bioassay that reports a TGF-β specific activation of the TGF-β signaling pathway. Briefly, the TGF-β-responsive SMAD response element-mediated firefly luciferase signal is normalized to a constitutively expressed Renilla luciferase signal, to provide relative activity<sup>21</sup>. In agreement with increased TGF-β2 secretion in PM, this conditioned PM had a significantly ( $p < 0.0001$ ) increased TGF-β bioactivity (2.3-fold; Figure 2B), as compared to conditioned OCM.



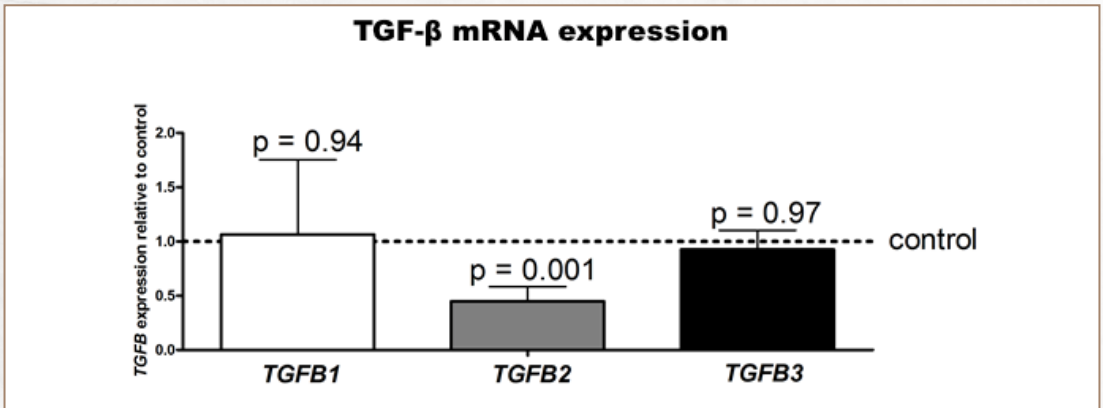
**Figure 2: Physosmotic culture medium (PM) increases TGF- $\beta$ 2 secretion and TGF- $\beta$  bioactivity in HACs.**

In HACs cultured for seven days (serum-free cultures on the left and 10% FCS cultures on the right) TGF- $\beta$ 2 protein levels were determined by specific TGF- $\beta$ 2 ELISA in PM (black bars) or OCM (white bars) (A). TGF- $\beta$  bioactivity was determined by TGF- $\beta$  bioassay in 72 h serum starved conditioned medium from PM and OCM HAC cultures (B). Corresponding ALK5/ALK1 gene expression ratio was determined in samples from Figure 1 (C). Data are from 3 donors measured in duplicate and are presented as mean  $\pm$  standard deviation relative to control OCM conditions.

On the receptor level ALK5, but not ALK1, appears to be essential for TGF- $\beta$  induced phosphorylation of SMAD3<sup>18</sup>, and therefore we further investigated the expression ratio of *ALK5/ALK1* in response to PM to get an indication if the increased TGF- $\beta$  might be able to induce canonical signaling. In accordance with the TGF- $\beta$  bioassay, the *ALK5/ALK1* expression ratio was significantly shifted in favor of increased ALK5 expression in chondrocytes cultured in PM as compared to OCM ( $p = 0.027$ ) (Figure 2C). These data indicate that physosmotic culture conditions improve activation of ALK5 driven TGF- $\beta$  signaling.

### 3.3 TGF- $\beta$ 2 Isoform-Specific Knockdown in HAC Cultures *in vitro*

To investigate whether a causal relation exists between the increased *COL2A1* expression and TGF- $\beta$ 2 secretion after treatment with PM, we performed TGF- $\beta$ 2 knockdown experiments using RNAi (siRNA) in our HAC cultures. First, we evaluated the isoform-specificity of RNAi before treatment with PM at  $t = 0$ : TGF- $\beta$ 2 gene expression was specifically and significantly down-regulated upon RNAi when compared to control and other TGF- $\beta$  isoforms (Figure 3) ( $p = 0.001$ ).



**Figure 3: TGF- $\beta$ 2 RNAi efficacy and specificity in HACs.**

*TGFB1*, *TGFB2*, and *TGFB3* gene expression was determined by RT-qPCR upon TGF- $\beta$ 2-specific knockdown using transfected siRNAs (in OCM conditions). Data are the means  $\pm$  standard deviation of duplicate measurements from three donors relative to control conditions (scrambled siRNA) and were normalized to housekeeper expression. Control condition is indicated by the dotted line (set to 1).



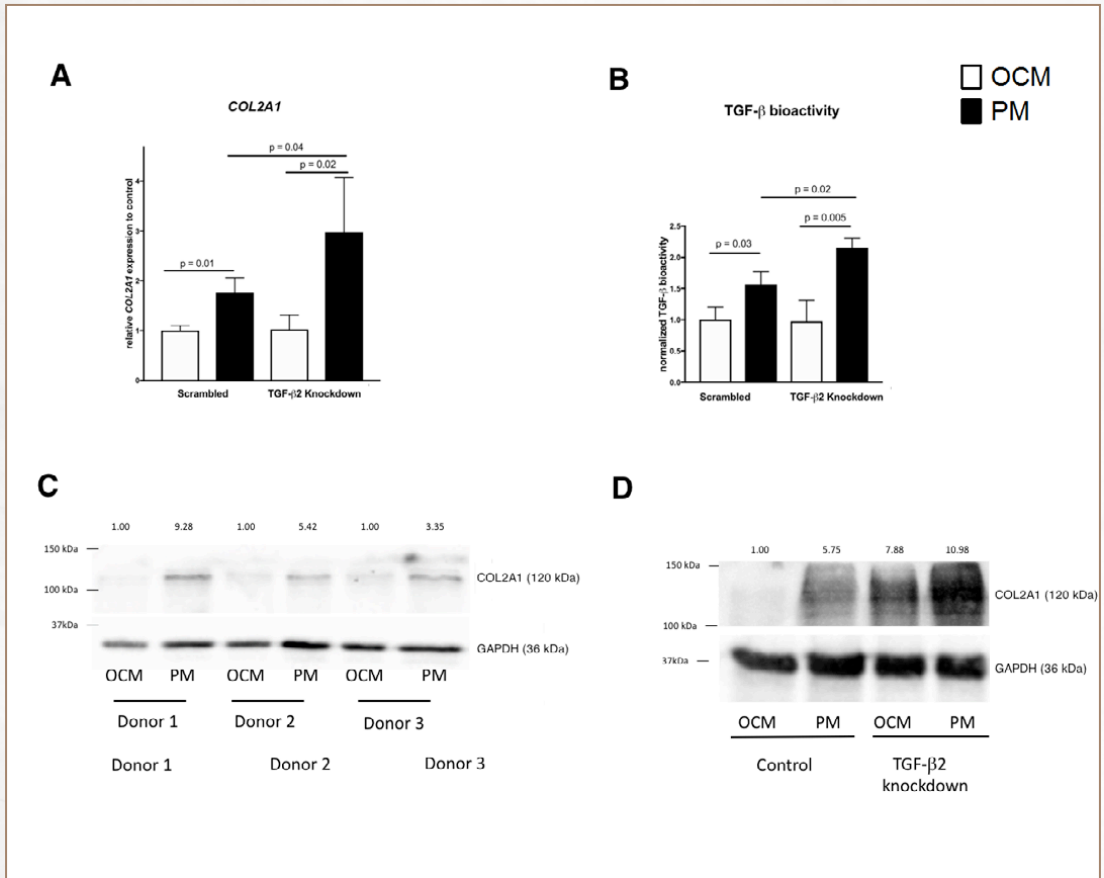
### 3.4 TGF- $\beta$ RNAi Combined with Physosmotic Treatment Increases *COL2A1* Gene Expression in HACs

We subsequently determined the consequence of TGF- $\beta$  RNAi on *COL2A1* gene expression 48 h upon stimulation with PM as compared to OCM. PM significantly increased *COL2A1* after 48 h (i.e., 1.8-fold,  $p = 0.01$ ) (Figure 4A).

TGF- $\beta$  knockdown, did not alter *COL2A1* expression in OCM (Figure 4A). However, TGF- $\beta$  RNAi with PM stimulation after 48 h resulted in higher *COL2A1* expression compared to OA HACs cultured at PM without TGF- $\beta$  RNAi ( $p = 0.04$ ) (Figure 4A).

To determine if the increased *COL2A1* expression upon TGF- $\beta$  knockdown under physosmotic conditions (i.e., in PM) was accompanied by an increased reporter assay activity, we repeated the experiment in the established chondrosarcoma cell line SW1353<sup>22,23</sup>. We measured CAGA-12 luciferase activity 48 h after stimulation with PM with TGF- $\beta$  RNAi. Bioactivity of TGF- $\beta$  displayed a similar pattern as *COL2A1* expression: stimulation with PM increased CAGA-12 luciferase activity ( $p = 0.03$ ) as compared to the control condition (i.e., OCM, Figure 4B). While TGF- $\beta$  knockdown at OCM did not alter CAGA-12 luciferase activity, stimulation with PM in combination with TGF- $\beta$  knockdown significantly increased the activity of this TGF- $\beta$  signaling-specific reporter as compared to the same osmotic stimulation without TGF- $\beta$  knockdown ( $p = 0.02$ ).

*COL2A1* gene expression data were confirmed on protein level. As shown in Figure 4C, PM increased *COL2A1* protein levels. In addition, *COL2A1* protein levels were higher at PM combined with TGF- $\beta$  knockdown than in HACs cultured at PM alone (Figure 4D).



**Figure 4: TGF- $\beta$ 2 RNAi combined with physosmotic treatment increases COL2A1 gene expression and TGF- $\beta$  bioactivity in HACs.**

Expression of COL2A1 was measured by RT-qPCR replicates ( $n = 3$ ) in control conditions (left) and after TGF- $\beta$ 2 RNAi (right) in OCM (white bars) or PM (black bar) conditions 48 h after physosmotic stimulation (A). TGF- $\beta$  bioactivity in media from SW1353 cultures with or without TGF- $\beta$  knockdown from PM and OCM cultures. Data are normalized means  $\pm$  standard deviation relative to control OCM condition (B). Collagen type II (COL2A1) protein expression was confirmed by immunoblotting in HACs from three donors. Molecular weight markers in kDa are shown on the left. The numbers across the top depict the relative quantity (C, D).

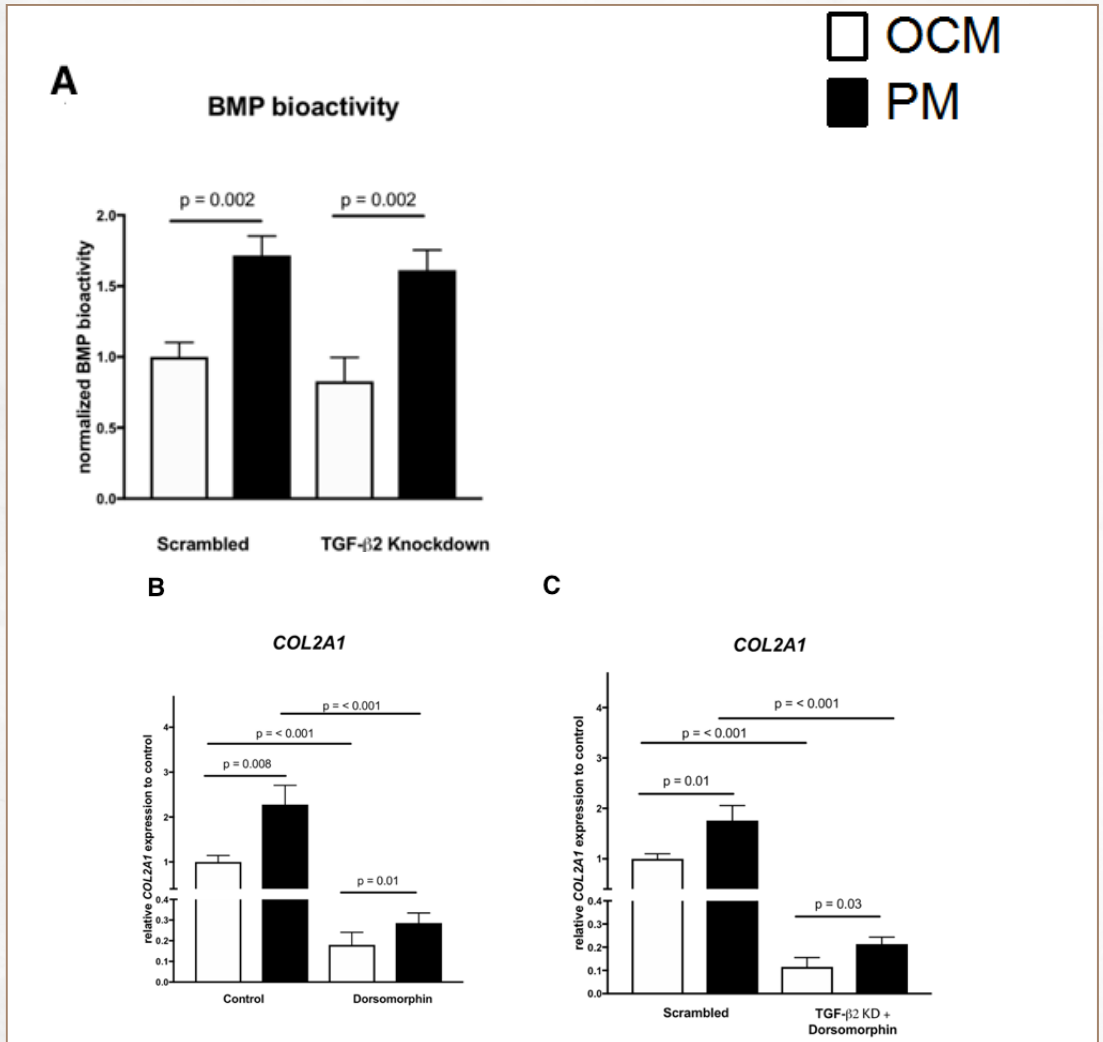
Since BMP signaling has been shown to be also involved in COL2A1 expression, we next evaluated BMP-specific BRE reporter activity under the same *in vitro* conditions. As shown in Figure 5A, PM significantly increased BRE activity compared to the OCM control condition ( $p = 0.002$ ). In contrast, TGF- $\beta$ 2 knockdown did not alter BRE activity at both PM and OCM

compared to conditions without TGF- $\beta$ 2 knockdown. Under TGF- $\beta$ 2 knockdown, PM again increased BMP reporter activity as compared to OCM ( $p = 0.002$ , Figure 5A).

We further evaluated the effect of an established BMP signaling inhibitor, Dorsomorphin, on *COL2A1* expression levels when TGF- $\beta$ 2 knockdown was combined with physosmotic stimulation.

First, we confirmed the attenuating effect of inhibiting BMP signaling under culture conditions without TGF- $\beta$ 2 knockdown. BMP signaling inhibition significantly decreased *COL2A1* expression levels at both PM and OCM compared to vehicle controls, while *COL2A1* expression levels at PM were still significantly elevated compared to OCM (Figure 5B). Under culture conditions with TGF- $\beta$ 2 knockdown, dorsomorphin again decreased *COL2A1* expression, while *COL2A1* expression was again significantly elevated at PM as compared to OCM (Figure 5B). *COL2A1* expression levels in conditions with TGF- $\beta$ 2-specific knockdown in combination with blocked BMP signaling were suppressed in both OCM and PM, while *COL2A1* expression remained relatively always higher in PM as compared to OCM irrespective of the TGF- $\beta$ 2 knockdown and blocked BMP signalling (Figure 5C).





**Figure 5: Blocking of BMP signalling decreases COL2A1 expression of chondrocytes irrespective of osmolarity.**

Bioassays in SW1353 cells show a largely TGF- $\beta$ 2 knockdown-independent, but osmolarity-dependent induction of BMP-responsiveness. Data are the normalized means  $\pm$  standard deviation ( $n = 3$ ) relative to control OCM condition (A). Physosmolar conditions further significantly elevated COL2A1 expression as compared to vehicle controls at OCM (B). Dorsomorphin (10  $\mu$ M)-mediated inhibition of BMP signaling significantly suppressed COL2A1 expression in both osmolarities (B), but COL2A1 expression remained relatively higher in PM as compared to OCM irrespective of BMP signaling. Combining TGF- $\beta$ 2 knockdown and dorsomorphin showed the same pattern as dorsomorphin alone (C). Data from five donors were measured in duplicates and are presented as normalized means  $\pm$  standard deviation relative to control OCM condition. Data is normalized means  $\pm$  standard relative to control OCM condition (replicate measurements, three donors).

## 4. Discussion

In the present study, we investigated whether physiological osmolarity (PM) is able to improve expression of key cartilage ECM marker *COL2A1* in chondrocytes (HACs), through facilitating TGF- $\beta$  superfamily signalling.

We reported earlier that expansion of HACs in PM (i.e., 380 mOsm) improved *COL2A1* marker gene and protein expression<sup>10</sup> and now reveal a correlation between improved expression of key chondrocyte marker *COL2A1* and upregulated TGF- $\beta$  family member ligands (e.g., TGF- $\beta$ 2, - $\beta$ 3) in PM. Interestingly, of all three prototypic isoforms of this family, only TGF- $\beta$ 2 was prominently osmo-dependently regulated in our study. Of all three human isoforms, several reports support a relatively more potent role of TGF- $\beta$ 2 in stimulating ECM synthesis: first, expression of TGF- $\beta$ 2, but not TGF- $\beta$ 1, is reduced in cartilage of old compared to young mice, suggesting a possible chondroprotective role<sup>24</sup>. Secondly, only TGF- $\beta$ 2 is characterized by unique upstream response elements in its P1 promoter<sup>25</sup>, suggesting its isoform specific regulation. TGF- $\beta$ 2 can further suppress collagenase mediated proteolytic degradation of collagen type II in human cartilage<sup>26</sup> and preserved the chondrocyte phenotype in physoxic high density *in vitro* cultures<sup>2</sup>. TGF- $\beta$ 2 also more potently induces SMAD2 phosphorylation at 10 and 250 pM as compared to TGF- $\beta$ 1 and TGF- $\beta$ 3<sup>16</sup>. Furthermore, TGF- $\beta$ 1 and TGF- $\beta$ 3, but not TGF- $\beta$ 2, bind to endoglin<sup>27</sup>, which is known to inhibit SMAD3 driven ECM production in chondrocytes<sup>28</sup>. Collectively, this prompted us to postulate that TGF- $\beta$ 2 signalling might trigger the increased collagen type II expression.

More than twice as much TGF- $\beta$ 2 protein was measured in conditioned PM compared to medium of plasma osmolarity (OCM). As TGF- $\beta$  can be secreted noncovalently attached to its prodomain, requiring additional processing to be activated from latency<sup>16</sup>, we subsequently used a functional bio-assay to investigate TGF- $\beta$  activity. A twofold increase in TGF- $\beta$  activity in conditioned PM was observed, confirming our gene- and protein-expression data. We next used a functional TGF- $\beta$  bioassay with a SMAD3 responsive element as TGF- $\beta$ 2 induced SMAD3-driven transcriptional activity may cause subsequent expression

of *COL2A1* in chondrocytes<sup>18</sup>. The endogenous TGF- $\beta$  activity measured in the conditioned PM reached 50% of the activity of the exogenously added positive TGF- $\beta$  control (10 ng/mL) used in the bioassay. This translates to a concentration of about 5 ng/mL active TGF- $\beta$  in conditioned PM, a concentration which is sufficient for SMAD3 phosphorylation<sup>29</sup>. As the TGF- $\beta$  bioassay is not isoform specific, we are unfortunately unable to exclude a potential contribution of other TGF- $\beta$  isoforms to the observed effects.

Thus, a limitation of our study is that we cannot rule out a contribution of antagonistic TGFBR1-like pseudo-receptors, MAPKs, like p38 MAPK or ERK1/2, in our experiments<sup>30</sup>. These have been shown to contribute to effects of hyperosmotic stimulation in chondrocytes<sup>30</sup>, at least at a high osmotic value (380 vs. 550 mOsm). Tew *et al.* further underscored the importance of post-transcriptional regulation in response to osmotic changes and, interestingly, while downstream transcriptional regulators of TGF- $\beta$  signaling were induced, expression of TGF- $\beta$  receptors (i.e., TGFBR2) and certain SMADs were downregulated<sup>30</sup>.

Of note, our physosmotic treatment also shifted receptor expression from TGF- $\beta$  type I receptor ALK1 towards relatively more canonical TGF- $\beta$  type I receptor ALK5 abundance. This is in agreement with our TGF- $\beta$  bioassay, since it is well established that ALK5 potentiates TGF- $\beta$ -induced SMAD3-driven transcriptional activity of the *COL2A1* promoter<sup>18</sup>. Cartilage-specific deletion of ALK5 induces an osteoarthritis-like phenotype in mice, underscoring its chondroprotective function in cartilage<sup>31</sup>. A limitation of the present study is that we could not study receptor expression on protein level to answer this, due to their low expression levels<sup>16</sup>.

A bioinformatics-driven approach suggested an involvement of TGF- $\beta$ 3 at high end osmotic values<sup>30</sup>, which we cannot confirm nor rule out as we focused on TGF- $\beta$ 2. The latter appeared to be the most prominently regulated isoform under physiological conditions. High osmolarity is able to down-regulate not only TGFRI mRNA expression, but also TAB1, SMAD3 and BMP signaling inhibitor Smad7<sup>30</sup>. Altogether, the response to extracellular osmotic values appears complex and seems to depend not only on the species, but also on cell type and its pathological state. Even the exact zonal location the chondrocytes are derived from may also influence the extent of the response to osmotic stimulation<sup>32</sup>.



We further aimed to elucidate the role of physosmotic-driven TGF- $\beta$ 2 signaling on collagen expression by a ligand-specific knockdown approach. Unexpectedly, anti-TGF- $\beta$ 2 RNAi increased collagen type II expression upon TGF- $\beta$ 2 specific knockdown. We, therefore, next investigated if a counter-regulatory increase in TGF- $\beta$  bioactivity might have caused the increased *COL2A1* expression and evaluated SMAD3 driven transcriptional activity. We did this in SW1353 chondrosarcoma cells as a model for human chondrocytes<sup>23</sup>, which resulted in a similarly increased TGF- $\beta$  bioactivity. Interestingly, however, the bioactivity increased even further when physosmotic treatment was combined with TGF- $\beta$ 2 knockdown as compared to physosmotic treatment alone. Given that SMAD3 can transcriptionally induce *COL2A1* expression<sup>18</sup>, this may indicate a compensatory increase in TGF- $\beta$  bioactivity upon TGF- $\beta$ 2 knockdown under physosmotic conditions. Post-transcriptional processes may also interfere with collagen expression under these conditions and may explain the increase in type II collagen protein. Earlier, SOX9 mRNA decay was shown to be influenced by high osmolarity. This led to a p38 MAPK-dependent increase in SOX9 protein and consequently enhanced *COL2A1* transcription. Of note, high end osmolarity destabilized *COL2A1* mRNA and reduced its overall expression<sup>30,33</sup>. In freshly isolated chondrocytes, osmotic stimulation thus increased SOX9, but decreased *COL2A1*, mRNA levels post-transcriptionally<sup>33</sup>. In our study, for some experiments we used SW1353 and not freshly isolated chondrocytes. Furthermore, we shifted the osmotic environment from plasma level to a (moderate) cartilage-specific physosmotic level and not from 380mOsm to high end osmolarity (>500 mOsm) as in the latter study. Together, differences in the osmotic baseline, the quality of the osmotic shift, and a different timing may at least partially explain contradictions between reported results.

Since BMP-signalling also regulates *COL2A1* expression in chondrocytes, we additionally evaluated BMP bioactivity under physosmotic conditions in combination with TGF- $\beta$ 2 knockdown. Cross-talk between different TGF- $\beta$  superfamily members can have synergistic or antagonistic effects<sup>34</sup>. Physosmotic treatment significantly increased BMP responsive element (BRE) activity under both *in vitro* culture conditions, with or without knockdown. There was no TGF- $\beta$ 2 knockdown specific effect on BRE signaling, while BMP-signaling inhibitor dorsomorphin was able to attenuate *COL2A1* expression in both conditions.

When combining TGF- $\beta$ 2 RNAi and pharmacological inhibition (i.e., dorsomorphin), *COL2A1* expression appeared to be largely BMP-dependent (Figure 5) as BMP signaling inhibition reduced *COL2A1* expression to 20% of the baseline level and 10% of the TGF- $\beta$ 2 knockdown control. Thus, while TGF- $\beta$  superfamily members interdependently influence expression of this chondrocyte marker, physosmotic conditions apparently always favor its expression independent of our means of TGF- $\beta$  superfamily signalling manipulation.

The quantity of the osmotic pressure appears to be as important as its quality. To this end, recent studies confirm that using physiological salt solutions may be more appropriate than using sorbitol. Hyperosmolar potassium treatment attenuate protein production of catabolic and inflammatory OA markers in a 3D *in vitro* model, at values of  $\sim$ 490 mOsm<sup>35</sup>. A study of experimentally injured rat and bovine cartilage reported chondroprotection by raising osmolarity of irrigation solutions<sup>36</sup>. However, also here normal saline (300 mOsm) was compared to sucrose-mediated hyperosmolar saline solution (600 mOsm)<sup>36</sup>. We reported earlier that sodium is specifically enriched in the ECM of cartilage<sup>10,37,38</sup> and using NaCl to alter osmotic values appears to be more physiological than using e.g., sorbitol or sucrose solutions. Although chondrocytes *in vitro* do not tolerate hyperosmolar stresses above about 380 mOsm for too long<sup>10</sup>, *in vivo* the situation may be different. All experiments of the present study were performed in 2D, while results might differ in 3D cultures<sup>32</sup>, which is a limitation.

We showed that expression of several TGF- $\beta$  superfamily member ligands, their receptors and target genes is altered in cultured human chondrocytes in physosmolar *in vitro* culture. However, the cellular responses to extracellular osmotic changes are complex<sup>39</sup> and both, macromolecular crowding and high ionic strength, may trigger a plethora of cell signaling cascades. High intracellular ionic strength enhances NFAT5 activity in chondrocytic cells<sup>10,40</sup> and more universally in mammalian cells<sup>39</sup>. Basically, elevating the extracellular osmolarity causes, at least temporarily, a state of molecular crowding from the presence of a total high weight per volume concentration of functionally unrelated soluble macromolecules<sup>41</sup>. This may indirectly affect interactions of soluble macromolecules with membranes, other structural elements or molecular chaperones.

In summary, physosmotic conditions preserve the phenotype of articular chondrocytes *in vitro*, a process to which TGF- $\beta$  superfamily members seem to contribute. Although we could not proof a link between increased activity of this superfamily and increased *COL2A1* expression in physosmotic cultures, the osmotic component appears prevailing. The TGF- $\beta$  superfamily<sup>42</sup> consists of more than 35 members of which we only analyzed prototypic members (i.e., TGF- $\beta$ s and BMPs) of each major branch<sup>43</sup> in more detail. Within this setting, our rational approach further cannot exclude TGF- $\beta$  mediated responses that are independent of Smad proteins<sup>44</sup>. Molecular crowding might affect protein structure, folding, shape, conformational stability, binding of small molecules, enzymatic activity, protein-protein interactions, protein-nucleic acid interactions, and pathological aggregation<sup>45</sup> and should be addressed in future studies.

### 5. Author Contributions

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U.T.T., M.C., G.v.d.A., T.W., and H.J. designed the study. U.T.T. and M.C. performed the experiments. U.T.T. wrote the manuscript, and analyzed and interpreted the data. M.C., A.v.d.W., T.W., H.J., P.E., L.v.R., H.W., and J.V. analyzed and interpreted the data, and critically revised the manuscript. All authors approved the final version of the manuscript.

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### 7. Conflicts of Interest

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The authors declare that they have no conflicts of interest.



## References

- 1 Brittberg M. Autologous chondrocyte transplantation. *Clin. Orthop. Relat. Res.* 1999;147–155. doi: 10.1097/00003086-199910001-00016.
- 2 Das R., Timur U.T., Edip S., Haak E., Wruck C., Weinans H., Jahr H. Tgf-beta2 is involved in the preservation of the chondrocyte phenotype under hypoxic conditions. *Ann. Anat.* 2015;198:1–10. doi: 10.1016/j.aanat.2014.11.003.
- 3 Chawla A., Twycross-Lewis R., Maffulli N. Microfracture produces inferior outcomes to other cartilage repair techniques in chondral injuries in the paediatric knee. *Br. Med. Bull.* 2015;116:93–103. doi: 10.1093/bmb/ldv040.
- 4 Falah M., Nierenberg G., Soudry M., Hayden M., Volpin G. Treatment of articular cartilage lesions of the knee. *Int. Orthop.* 2010;34:621–630. doi: 10.1007/s00264-010-0959-y.5 Urban J.P., Hall A.C., Gehl K.A. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J. Cell. Physiol.* 1993;154:262–270. doi: 10.1002/jcp.1041540208.
- 6 Benya P.D., Padilla S.R., Nimni M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell.* 1978;15:1313–1321. doi: 10.1016/0092-8674(78)90056-9.
- 7 Bhalla A., Sankaralingam S., Dundas R., Swaminathan R., Wolfe C.D., Rudd A.G. Influence of raised plasma osmolality on clinical outcome after acute stroke. *Stroke.* 2000;31:2043–2048. doi: 10.1161/01.STR.31.9.2043.
- 8 Anderson D.E., Markway B.D., Weekes K.J., McCarthy H.E., Johnstone B. Physioxia promotes the articular chondrocyte-like phenotype in human chondroprogenitor-derived self-organized tissue. *Tissue Eng. Part A.* 2017 doi: 10.1089/ten.tea.2016.0510.
- 9 Johnson Z.I., Shapiro I.M., Risbud M.V. Extracellular osmolarity regulates matrix homeostasis in the intervertebral disc and articular cartilage: Evolving role of tonebp. *Matrix Biol.* 2014;40:10–16. doi: 10.1016/j.matbio.2014.08.014.
- 10 Van der Windt A.E., Haak E., Das R.H., Kops N., Welting T.J., Caron M.M., van Til N.P., Verhaar J.A., Weinans H., Jahr H. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated t-cells 5 *in vitro*. *Arthritis. Res. Ther.* 2010;12:R100. doi:

- 10.1186/ar3031.
- 11 Van der Kraan P.M., Buma P., van Kuppevelt T., van den Berg W.B. Interaction of chondrocytes, extracellular matrix and growth factors: Relevance for articular cartilage tissue engineering. *Osteoarthr. Cartil.* 2002;10:631–637. doi: 10.1053/joca.2002.0806.
  - 12 Van der Kraan P.M., Blaney Davidson E.N., Blom A., van den Berg W.B. Tgf-beta signaling in chondrocyte terminal differentiation and osteoarthritis: Modulation and integration of signaling pathways through receptor-smads. *Osteoarthr. Cartil.* 2009;17:1539–1545. doi: 10.1016/j.joca.2009.06.008.
  - 13 Miyazawa K., Shinozaki M., Hara T., Furuya T., Miyazono K. Two major smad pathways in tgf-beta superfamily signalling. *Genes Cells.* 2002;7:1191–1204. doi: 10.1046/j.1365-2443.2002.00599.x.
  - 14 Roberts A.B., Sporn M.B., Assoian R.K., Smith J.M., Roche N.S., Wakefield L.M., Heine U.I., Liotta L.A., Falanga V., Kehrl J.H., *et al.* Transforming growth factor type beta: Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc. Natl. Acad. Sci. USA.* 1986;83:4167–4171. doi: 10.1073/pnas.83.12.4167.
  - 15 Sanford L.P., Ormsby I., Gittenberger-de Groot A.C., Sariola H., Friedman R., Boivin G.P., Cardell E.L., Doetschman T. Tgfbeta2 knockout mice have multiple developmental defects that are non-overlapping with other tgfbeta knockout phenotypes. *Development.* 1997;124:2659–2670.
  - 16 Parker W.L. *Tgf-β Receptors on Human Chondrocytes: Hetero-Oligomerization and Function.* McGill University; Montreal, QC, Canada: 2003.
  - 17 Heldin C.H., Miyazono K., ten Dijke P. Tgf-beta signalling from cell membrane to nucleus through smad proteins. *Nature.* 1997;390:465–471. doi: 10.1038/37284.
  - 18 Finnson K.W., Parker W.L., ten Dijke P., Thorikay M., Philip A. Alk1 opposes alk5/smad3 signaling and expression of extracellular matrix components in human chondrocytes. *J. Bone Miner. Res.* 2008;23:896–906. doi: 10.1359/jbmr.080209.
  - 19 Blaney Davidson E.N., Remst D.F., Vitters E.L., van Beuningen H.M., Blom A.B., Goumans M.J., van den Berg W.B., van der Kraan P.M. Increase in alk1/alk5 ratio as a cause for elevated mmp-13 expression in osteoarthritis in humans and mice. *J. Immunol.* 2009;182:7937–7945. doi: 10.4049/jimmunol.0803991.
  - 20 Harrison P.E., Ashton I.K., Johnson W.E., Turner S.L., Richardson J.B., Ashton B.A. The *in vitro* growth

## Chapter 3

- of human chondrocytes. *Cell Tissue Bank*. 2000;1:255–260. doi: 10.1023/A:1010131729208.
- 21 Dennler S., Itoh S., Vivien D., ten Dijke P., Huet S., Gauthier J.M. Direct binding of smad3 and smad4 to critical tgf beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J*. 1998;17:3091–3100. doi: 10.1093/emboj/17.11.3091.
- 22 Zheng Z., Le H., Chen W., Shen W., Ouyang H. Progress on treatment of tendinopathy with platelet-enriched plasma. *Zhejiang Da Xue Xue Bao Yi Xue Ban*. 2016;45:179–186.
- 23 Gebauer M., Saas J., Sohler F., Haag J., Söder S., Pieper M., Bartnik E., Beninga J., Zimmer R., Aigner T. Comparison of the chondrosarcoma cell line sw1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to il-1beta. *Osteoarthr. Cartil*. 2005;13:697–708. doi: 10.1016/j.joca.2005.04.004.
- 24 Blaney Davidson E.N., Scharstuhl A., Vitters E.L., van der Kraan P.M., van den Berg W.B. Reduced transforming growth factor-beta signaling in cartilage of old mice: Role in impaired repair capacity. *Arthritis Res. Ther*. 2005;7:R1338–R1347. doi: 10.1186/ar1833.
- 25 Roberts A.B., Sporn M.B. Differential expression of the tgf-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol. Reprod. Dev*. 1992;32:91–98. doi: 10.1002/mrd.1080320203.
- 26 Tchetina E.V., Antoniou J., Tanzer M., Zukor D.J., Poole A.R. Transforming growth factor-beta2 suppresses collagen cleavage in cultured human osteoarthritic cartilage, reduces expression of genes associated with chondrocyte hypertrophy and degradation, and increases prostaglandin e(2) production. *Am. J. Pathol*. 2006;168:131–140. doi: 10.2353/ajpath.2006.050369.
- 27 Cheifetz S., Bellon T., Cales C., Vera S., Bernabeu C., Massague J., Letarte M. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J. Biol. Chem*. 1992;267:19027–19030.
- 28 Finnson K.W., Parker W.L., Chi Y., Hoemann C.D., Goldring M.B., Antoniou J., Philip A. Endoglin differentially regulates tgf-beta-induced smad2/3 and smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes. *Osteoarthr. Cartil*. 2010;18:1518–1527. doi: 10.1016/j.joca.2010.09.002.
- 29 Remst D.F., Blaney Davidson E.N., Vitters E.L., Bank R.A., van den Berg W.B., van der



- Kraan P.M. Tgf-ss induces lysyl hydroxylase 2b in human synovial osteoarthritic fibroblasts through alk5 signaling. *Cell Tissue Res.* 2014;355:163–171. doi: 10.1007/s00441-013-1740-5.
- 30 Tew S.R., Vasieva O., Peffers M.J., Clegg P.D. Post-transcriptional gene regulation following exposure of osteoarthritic human articular chondrocytes to hyperosmotic conditions. *Osteoarthr. Cartil.* 2011;19:1036–1046. doi: 10.1016/j.joca.2011.04.015.
- 31 Wang Q., Tan Q., Xu W., Qi H., Chen D., Zhou S., Ni Z., Kuang L., Guo J., Huang J. Cartilage-specific deletion of alk5 gene results in a progressive osteoarthritis-like phenotype in mice. *Osteoarthr. Cartil.* 2017;25:1868–1879. doi: 10.1016/j.joca.2017.07.010.
- 32 Takada E., Mizuno S. Reproduction of characteristics of extracellular matrices in specific longitudinal depth zone cartilage within spherical organoids in response to changes in osmotic pressure. *Int. J. Mol. Sci.* 2018;19:1507. doi: 10.3390/ijms19051507.
- 33 Tew S.R., Peffers M.J., McKay T.R., Lowe E.T., Khan W.S., Hardingham T.E., Clegg P.D. Hyperosmolarity regulates sox9 mrna posttranscriptionally in human articular chondrocytes. *Am. J. Physiol. Cell Physiol.* 2009;297:C898–C906. doi: 10.1152/ajpcell.00571.2008.
- 34 Guo X., Wang X.F. Signaling cross-talk between tgf-beta/bmp and other pathways. *Cell Res.* 2009;19:71–88. doi: 10.1038/cr.2008.302.
- 35 Erndt-Marino J., Trinkle E., Hahn M.S. Hyperosmolar potassium (K+) Treatment Suppresses Osteoarthritic Chondrocyte Catabolic and Inflammatory Protein Production in a 3-Dimensional *In vitro* Model. *Cartilage.* 2017 doi: 10.1177/1947603517734028.
- 36 Eltawil N.M., Ahmed S., Chan L.H., Simpson A.H.R.W., Hall A.C. Chondroprotection in models of cartilage injury by raising the temperature and osmolarity of irrigation solutions. *Cartilage.* 2018;9:313–320. doi: 10.1177/1947603516688511.
- 37 Van der Windt A.E., Jahr H., Farrell E., Verhaar J.A., Weinans H., van Osch G.J. Calcineurin inhibitors promote chondrogenic marker expression of dedifferentiated human adult chondrocytes via stimulation of endogenous tgfbeta1 production. *Tissue Eng. Part A.* 2010;16:1–10. doi: 10.1089/ten.tea.2009.0082.
- 38 Van der Windt A.E., Haak E., Kops N., Verhaar J.A., Weinans H., Jahr H. Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. *Arthritis Rheum.* 2012;64:1929–1939. doi: 10.1002/art.34369.

- 39 Bounedjah O., Hamon L., Savarin P., Desforges B., Curmi P.A., Pastré D. Macromolecular crowding regulates assembly of mrna stress granules after osmotic stress: New role for compatible osmolytes. *J. Biol. Chem.* 2012;287:2446–2458. doi: 10.1074/jbc.M111.292748.
- 40 Caron M.M., van der Windt A.E., Emans P.J., van Rhijn L.W., Jahr H., Welting T.J. Osmolarity determines the *in vitro* chondrogenic differentiation capacity of progenitor cells via nuclear factor of activated t-cells 5. *Bone.* 2013;53:94–102. doi: 10.1016/j.bone.2012.11.032.
- 41 Rivas G., Minton A.P. Macromolecular crowding *in vitro*, *in vivo*, and in between. *Trends Biochem. Sci.* 2016;41:970–981. doi: 10.1016/j.tibs.2016.08.013.
- 42 Massagué J. How cells read tgf-beta signals. *Nat. Rev. Mol. Cell Biol.* 2000;1:169–178. doi: 10.1038/35043051.
- 43 Van der Kraan P.M. The changing role of tgfb in healthy, ageing and osteoarthritic joints. *Nat. Rev. Rheumatol.* 2017;13:155–163. doi: 10.1038/nrrheum.2016.219.
- 44 Shi Y., Massagué J. Mechanisms of tgfbeta signaling from cell membrane to the nucleus. *Cell.* 2003;113:685–700. doi: 10.1016/S0092-8674(03)00432-X.
- 45 Kuznetsova I.M., Turoverov K.K., Uversky V.N. What macromolecular crowding can do to a protein. *Int. J. Mol. Sci.* 2014;15:23090–23140. doi: 10.3390/ijms151223090.
- 46 Caron M.M., Emans P.J., Coolen M.M., Voss L., Surtel D.A., Cremers A., van Rhijn L.W., Welting T.J. Redifferentiation of dedifferentiated human articular chondrocytes: Comparison of 2d and 3d cultures. *Osteoarthr. Cartil.* 2012;20:1170–1178. doi: 10.1016/j.joca.2012.06.016.
- 47 Caron M.M., Emans P.J., Surtel D.A., van der Kraan P.M., van Rhijn L.W., Welting T.J. Bapx-1/nkx-3.2 acts as a chondrocyte hypertrophy molecular switch in osteoarthritis. *Arthritis Rheumatol.* 2015;67:2944–2956. doi: 10.1002/art.39293.
- 48 Das R.H., van Osch G.J., Kreukniet M., Oostra J., Weinans H., Jahr H. Effects of individual control of ph and hypoxia in chondrocyte culture. *J. Orthop. Res.* 2009. doi: 10.1002/jor.20994.
- 49 Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., *et al.* The miq guidelines: Minimum information for publication of quantitative real-time pcr experiments. *Clin. Chem.* 2009;55:611–622. doi: 10.1373/clinchem.2008.112797.
- 50 Maglott D., Ostell J., Pruitt K.D., Tatusova T. Entrez gene: Gene-centered information at ncbi. *Nucleic Acids Res.* 2011;39:D52–

D57. doi: 10.1093/nar/gkq1237.

- 51 Korchynskyi O., ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the id1 promoter. *J. Biol. Chem.* 2002;277:4883–4891. doi: 10.1074/jbc.M111023200.



Chapter  
04



**Celecoxib-Mediated Reduction of Prostanoid Release in Hoffa's Fat Pad from Donors with Cartilage Pathology Results in an Attenuated Inflammatory Phenotype.**

**Timur UT**, Caron MMJ, Bastiaansen-Jenniskens YM, Welting TJM, van Rhijn LW, van Osch G, Emans PJ *Osteoarthritis Cartilage*. 2018;26(5):697-706.

## Abstract

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### **Objective:**

The Hoffa's fat pad (HFP) is an intra-articular adipose tissue which is situated under and behind the patella. It contains immune cells next to adipocytes and secretes inflammatory factors during osteoarthritis (OA). In this study, we compared the release profile of prostanoids, which are involved in inflammation, of HFP from OA patients vs patients with a focal cartilage defect (CD) without evidence for OA on MRI and investigated the prostanoid modulatory anti-inflammatory action of celecoxib on HFP.

### **Material & Methods:**

Prostanoid release was analyzed in conditioned medium of HFP explant cultures from 17 osteoarthritic patients and 12 CD patients, in the presence or absence of celecoxib. Furthermore, gene expression of COX enzymes and expression of genes indicative of a pro-inflammatory or anti-inflammatory phenotype of HFP was analyzed.

### **Results:**

Prostanoid release by HFP from knee OA patients clustered in two subgroups with high and low prostanoid producers. HFP from high prostanoid producers released higher amounts of PGE<sub>2</sub>, PGF<sub>2α</sub> and PGD<sub>2</sub> compared to HFP from CD patients. PGE<sub>2</sub> release by OA HFP was positively associated with expression of genes known to be expressed by M1 macrophages, indicating a role for macrophages. Celecoxib modulated prostanoid release by HFP, and also modulated the inflammation ratio towards a more favorable anti-inflammatory M2 phenotype, most effectively in patients with higher prostanoid release profiles.

### **Conclusion:**

In knee OA patients with inflamed HFP's, celecoxib may exert positive effects in the knee joint via decreasing the release of prostanoids produced by the HFP and by favorably modulating the anti-inflammatory marker expression in HFP.



## 1. Introduction

Knee osteoarthritis (OA) is a multifactorial disease characterized by cartilage deterioration and subchondral sclerosis<sup>1</sup>. An inflammatory component in this disease is now acknowledged<sup>2</sup>. Next to cartilage and synovium, studies have shown the ability of the Hoffa's fat pad (HFP) to secrete inflammatory mediators able to induce inflammatory and destructive responses in knee OA<sup>3,4</sup>. The HFP is an adipose tissue located intra-articularly behind and under the patella<sup>4</sup>. Important molecules involved in inflammatory processes are prostanoids. While prostanoids have been shown to be secreted by the HFP<sup>5</sup>, modulation of prostanoid secretion by HFP and potential consequences of such modulation on inflammatory processes in HFP has not been investigated.

The major rate-limiting step in the biosynthesis of prostanoids is the cyclooxygenase (COX) dependent production of PGH<sub>2</sub><sup>6</sup>. The prostanoid family consists of five different subtypes: PGE<sub>2</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, PGF<sub>2α</sub> and Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and these subtypes are synthesized from PGH<sub>2</sub> by dedicated enzymes<sup>7</sup>. COX-1 is regarded as the 'housekeeping' COX, responsible for the production of basal prostanoid levels, which are thought to maintain local homeostasis<sup>8</sup>. On the other hand, COX-2 is considered to be inflammatory, since its expression is upregulated in inflamed joint tissues<sup>8</sup>. Indeed, COX-2 expression in the HFP has been shown to be increased upon inflammatory stimulation<sup>3</sup>, and while it is now well established that COX-2 expression is upregulated in inflamed joint tissues<sup>8</sup>, the consequences of altered COX-2 expression on the production of its main downstream prostanoid products are poorly documented in OA. Different prostanoid subtypes such as PGE<sub>2</sub> and PGF<sub>2α</sub> have been shown to be involved in catabolic and pro-fibrotic responses in knee OA<sup>9,10,11</sup>, and thus a broader insight into the involvement of specific prostanoids in OA-related inflammatory processes will be essential to understand their contribution in OA. Since the HFP contains nerve fibers<sup>4</sup> and considering the pronociceptive effects of different prostanoid subtypes<sup>12,13,14</sup>, prostanoids produced by the HFP may provide valuable targets for anti-inflammatory and pain therapy in the OA knee joint. Several risk factors have been described which predispose for knee OA, one of which are cartilage defects (CDs)<sup>15</sup>. Defining the difference between a CD and 'pre-osteoarthritis' still represents a major

challenge. Although Gierman and coworker have shown that there was a slight difference in the inflammatory profile of HFP between OA and non-OA donors<sup>5</sup>, the HFP from patients with a CD vs HFP from OA patients has never been studied to our knowledge and may provide further insight in the inflammatory pathogenesis of both overlapping diseases OA and CDs. Therefore, the main goal of this study was to evaluate the secretion of prostanoid subtypes by the HFP from knee OA patients and compare with prostanoid secretion by the HFP of CD patients. In addition, correlations were investigated between prostanoid secretion from HFP and expression of inflammatory markers in the HFP, to evaluate the general inflammatory status of the tissue. Finally, since COX-2 has been considered as an important inflammatory factor in HFP<sup>3</sup>, we sought to determine the anti-inflammatory actions of celecoxib, a specific COX-2 inhibitor, on HFP.

## 2. Material & Methods

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### 2.1 Preparation of Hoffa's Fat Pad Conditioned Medium

The Medical Ethical Committee (MEC) of Maastricht University approved this study and assigned approval ID: MEC 11-4-040.17. HFPs were obtained as anonymous material from human subjects with endstage OA (Kellgren & Lawrence grade three–four) who underwent total knee arthroplasty (TKA). Since the autologous chondrocyte transplantation (ACT) procedure is performed less frequently than a TKA for endstage OA, 12 HFPs were obtained from human subjects with a single CD who underwent an ACT procedure (ACT; Chondroelect Tigenix, Leuven Belgium). CDs were either due to trauma or osteochondritis dissecans and patients did not show any signs of OA on X-ray and MRI. These HFP's were used to generate HFP conditioned medium (HCM) at a concentration of 100 mg tissue/mL using DMEM/F12 (Life Technologies, Carlsbad US), 1% ITS, 1% antibiotic/antimycotic (Life Technologies) in a humidified atmosphere at 37°C, 5% CO<sub>2</sub> as reported previously<sup>16</sup>. The inner part of HFP was cut into small explants to avoid the presence of synovial tissue. Explants were selected randomly throughout the entire tissue and used at a concentration of 100 mg tissue/mL medium to create OA HCM or CD HCM. A stock solution of 10 mM celecoxib (LC Laboratories, Woburn, MA, US) was made in dimethylsulfoxide (DMSO, Sigma

Aldrich, St. Louis, MO, US) and added 1:1,000 to medium with pieces of HFP to obtain a 10  $\mu\text{M}$  concentration. DMSO was also added 1:1,000 to HFP cultures without celecoxib as a control. In addition, three patients in the OA group were cultured with 2.5, 10 and 100  $\mu\text{M}$  celecoxib to examine a dose response. In accordance with the method described by Clockaerts *et al.*, medium was harvested after 24 h, centrifuged at 1,200 RPM for 8 min and the supernatant was frozen at  $-80^{\circ}\text{C}$ <sup>16</sup>. Media were stored for a maximum of 8 weeks before analysis. HFP explants were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until being processed for RNA isolation.

## 2.2 Prostanoid, Interleukin-6 and Interleukin-1 $\beta$ Measurement in HCM

Concentrations of  $\text{PGE}_{2}$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGD}_{2}$  and  $\text{TXB}_{2}$  (a stable metabolite of  $\text{TXA}_{2}$ ) were determined in OA HCM and CD HCM by a competitive Enzyme-Linked Immunosorbent Assay according to the manufacturer's instructions (ELISA, Cayman Chemicals, Ann Arbor, MI, US) as reported previously<sup>17</sup>. Concentrations of interleukin-6 and interleukin-1 $\beta$  in HCM was determined according to the manufacturer's instructions of the human IL-6 and IL-1 $\beta$  Quantikine ELISA Kits (R&D Systems, MI, US).

## 2.3 RNA Isolation and Quantitative RT-PCR

Frozen HFP was homogenized with a Mikro-Dismembrator and suspended in 1 ml TRIzol/100 mg tissue. RNA was extracted and cDNA was made as reported previously<sup>16</sup>. Real time quantitative PCR (RT-qPCR) was performed using Mesagreen qPCR mastermix plus for SYBR Green (Eurogentec). A CFX96 Real-Time PCR Detection system (Biorad) was used for amplification: initial denaturation  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of amplification (denaturing 15 s at  $95^{\circ}\text{C}$  and annealing 1 min at  $60^{\circ}\text{C}$ ) as reported previously<sup>17</sup>. Validated primer sequences can be found in Supplementary Table 1. Gene expression was normalized to a best housekeeper ratio based on gene expression levels of *GAPDH*, *PPIA* and *28S*<sup>8</sup> and data were analyzed according to the  $2^{-\Delta\Delta\text{CT}}$  method<sup>19</sup>. Housekeeper gene expression was found to be stable (data not shown).



### 2.4 Statistical Analysis

SPSS Inc., Chicago, USA (version 20) was used for statistical analyses. A minimum of five explants was used per experimental condition. All samples for gene expression analysis and cytokine measurements in medium were processed and analyzed individually with a single measurement per donor. Continuous variables were tested for normality using Kolmogorov–Smirnov test.

PGE<sub>2</sub> release data were not normally distributed and a Mann–Whitney *U* test was performed to evaluate differences between PGE<sub>2</sub> release by OA HFP and CD HFP. Based on PGE<sub>2</sub> release data, OA patients were further divided into two subgroups, which was based on an arbitrarily chosen PGE<sub>2</sub> release threshold. An inflammation ratio was calculated, using *IL6*, *IL1B*, *CD86* and *TNFA* as markers associated with pro-inflammatory or M1 macrophages and *CD206*, *IL10*, *CD163* and *CCL18* as markers associated with anti-inflammatory or M2 macrophages<sup>20</sup>. The mean of the relative expression of pro-inflammatory genes per sample, was divided over the mean of the relative expression of anti-inflammatory genes per sample. Gene expression data, the inflammation ratio, prostanoid release and cytokine release data of these two OA subgroups and CD patients were then log transformed and subjected into a general linear model. For celecoxib treatments in the patient groups, the celecoxib treatment itself was defined as a fixed factor in a general linear model. Correlations between prostanoid levels and expression of markers associated with inflammation were calculated using the Spearman's rank correlation test.

Differences in clinical parameters BMI, and age between OA and CD patients were analyzed using an independent Student's *t*-test, differences in duration of symptoms and K&L grade between OA and CD patients were analyzed using a Mann–Whitney *U* test and differences in gender using a Pearson Chi-square test. Bonferroni's *post hoc* comparisons test was used to adjust for multiple comparisons. Mean ± 95% coincidence intervals are included in the figures.

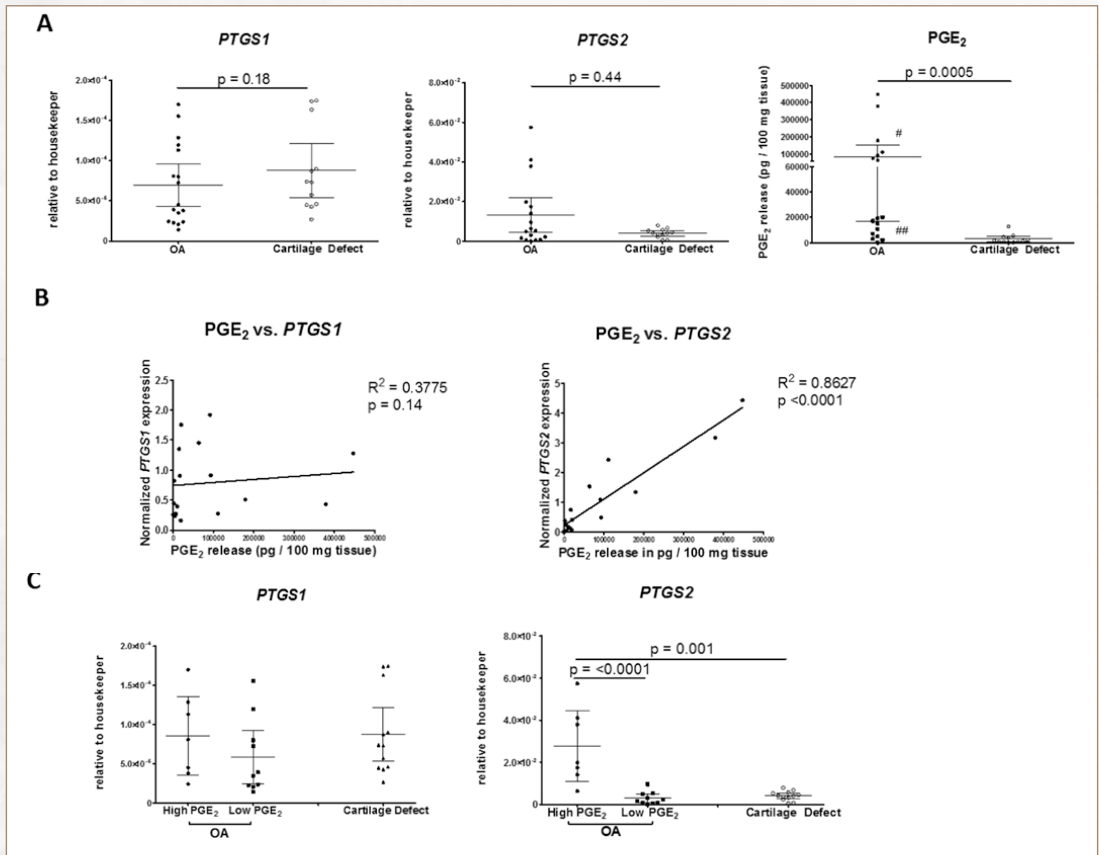
### 3. Results

#### 3.1 Hoffa's Fat Pad from OA Patients Releases Higher Amounts of Prostanoids than Hoffa's Fat Pad from CD Patients, which is correlated to PTGS2 Expression

Demographical data of HFP from OA and CD patients can be found in Table I. Age and K&L grade were the only significantly different factors between the two patient groups ( $p < 0.001$ ) for both parameters. The mean age of OA patients was  $63.8 \pm 1.9$  years and the mean age of CD patients was  $26.1 \pm 7.9$  years, while the mean K&L grade was 3.3 for OA patients and 0 for CD patients (Table I). In the HFP of OA and CD patients we first examined mRNA expression levels of the prostanoid synthesizing COX-1 and COX-2 enzymes (encoded by *PTGS1* and *PTGS2*). No significant differences were observed in *PTGS1* and *PTGS2* mRNA expression between HFP of OA and CD patients (Figure 1A). To investigate COX enzyme activity, we next analyzed PGE<sub>2</sub> release (the major prostanoid subtype synthesized by different intra-articular tissues and generally acknowledged to be the most inflammatory prostanoid subtype<sup>21,22</sup>) in culture supernatants of OA and CD HFP. Release of PGE<sub>2</sub> was 30-fold higher by OA HFP in comparison to CD HFP ( $p = 0.0005$ ) (Figure 1A). However, a wide variation in PGE<sub>2</sub> release by OA donors was observed, and the donors clustered into two groups: a subgroup with higher (higher than 25,000 pg/ml PGE<sub>2</sub>: high PGE<sub>2</sub> OA group, designated with # in Figure 1A) or lower (lower than 25,000 pg/ml: low PGE<sub>2</sub> OA group, designated with ## in Figure 1A) PGE<sub>2</sub> release profiles.

**Table I Clinical Characteristics**

	OA PATIENTS	CD PATIENTS
Number	17	12
Male:Female	8:9	8:4
Age, mean $\pm$ SD (in years)	63.8 $\pm$ 1.9	26.1 $\pm$ 7.9
BMI, mean $\pm$ SD	29 $\pm$ 6.4	24 $\pm$ 3
Kellgren & Lawrence, mean $\pm$ SD	3.3 $\pm$ 0.5	0 $\pm$ 0



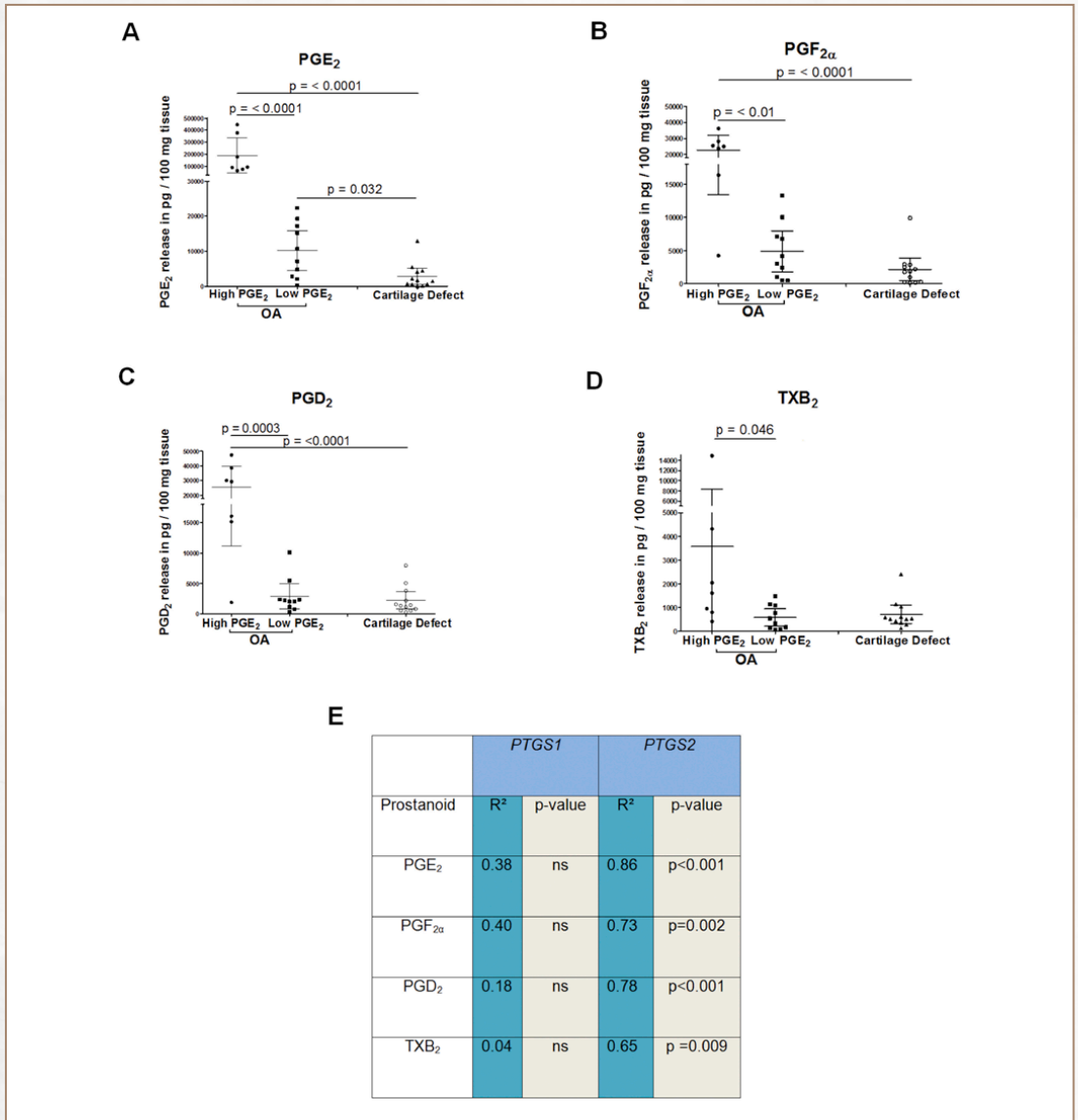


**Figure 1: Higher PGE<sub>2</sub> release from OA Hoffa's fat pad (HFP) than cartilage defect (CD) HFP.**

Relative PTGS1 (left figure), PTGS2 (middle figure) mRNA expression and PGE<sub>2</sub> release (right figure) by osteoarthritis (OA) and CD HFP 24 h after culturing. In the right figure, the high PGE<sub>2</sub> OA group is designated with # and the low PGE<sub>2</sub> OA group is designated with ##. Each dot or square represents normalized gene expression data or absolute PGE<sub>2</sub> levels released in culture medium in pg/100 mg tissue from separate patients. Data is presented in scatter dot plots with mean  $\pm$  95% CI (A). Spearman correlation coefficients between PGE<sub>2</sub> release in culture medium and PTGS1 (left figure) or PTGS2 (right figure) mRNA expression in HFP at 24 h after culturing. R<sup>2</sup> and p values are provided within each graph (B). PTGS1 mRNA (left figure) and PTGS2 mRNA (right figure) expression in HFP from the high PGE<sub>2</sub> OA, low PGE<sub>2</sub> OA and CD patients. Data is presented in scatter dot plots with mean  $\pm$  95% CI (C). N = 17 for OA HFP and N = 12 for CD HFP.

While we did not find a correlation between PGE<sub>2</sub> release by OA HFP and PTGS1 expression ( $R^2 = 0.337$ ,  $p = 0.14$ ), a strong correlation between PGE<sub>2</sub> release and PTGS2 expression in HFP ( $R^2 = 0.86$ ,  $p \leq 0.0001$ ) was observed (Figure 1B). In accordance with these data, patients in the high PGE<sub>2</sub> OA group displayed 9.6-fold higher PTGS2 (but not PTGS1) expression levels in comparison to patients from the low PGE<sub>2</sub> OA subgroup ( $p \leq 0.0001$ ) and 6.8-fold higher in comparison to CD patients ( $p = 0.001$ ) (Figure 1C).

HFP from both the high and the low PGE<sub>2</sub> OA group released more PGE<sub>2</sub> than HFP from CD patients (Figure 2A). Release of prostanoid subtypes PGF<sub>2 $\alpha$</sub>  and PGD<sub>2</sub> followed similar dynamics as PGE<sub>2</sub> and release of these prostanoids was significantly higher in HFP from the high PGE<sub>2</sub> OA group, while release of TXB<sub>2</sub> by HFP was not significantly different between OA and CD patients (Figure 2A-D). Significant correlations were found between PGF<sub>2 $\alpha$</sub> , PGD<sub>2</sub> and TXB<sub>2</sub> release and the inflammation-induced PTGS2 expression in HFP (Figure 2E). No significant correlations were found between PGF<sub>2 $\alpha$</sub> , PGD<sub>2</sub> and TXB<sub>2</sub> release and PTGS1 levels (Figure 2E). The differential prostanoid release between OA patients could not be related to several clinical parameters such as age, duration of symptoms, gender, BMI or K&L grade (Supplementary Figure 1).



**Figure 2: Higher prostanoid release from OA HFP than from CD HFP.**

PGE<sub>2</sub> (A), PGF<sub>2α</sub> (B), PGD<sub>2</sub> (C) and TXB<sub>2</sub> (D) release from HFP in pg/100 mg tissue. Data is presented in scatter dot plots with mean ± 95% CI. Spearman correlation coefficients between PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, TXB<sub>2</sub> release and PTGS mRNA expression in OA HFP (E). N = 17 for OA HFP and N = 12 for CD HFP.

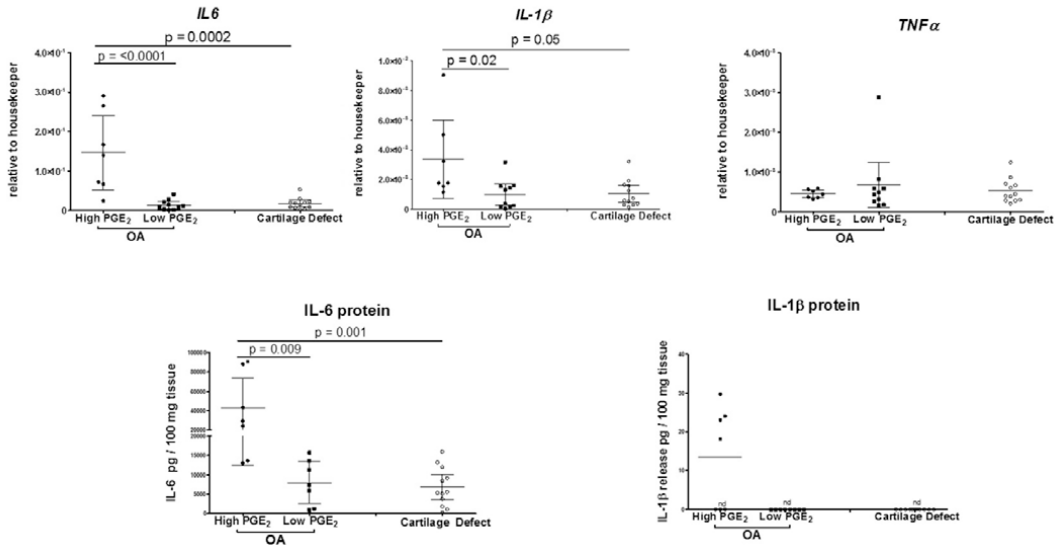
### 3.2 High Prostanoid Release by OA HFP is Accompanied by High Expression of Inflammatory Markers

We next investigated whether the high prostanoid release from OA HFP was accompanied by alterations in other markers involved in inflammation. Since it has been shown that HFP contains macrophages<sup>16</sup>, we specifically included the expression of markers in the HFP associated with a pro-inflammatory macrophage phenotype, and markers associated with an anti-inflammatory macrophage phenotype<sup>20,21,22,23</sup>. HFP from the high PGE<sub>2</sub> OA group displayed 10.5-fold higher gene expression levels of pro-inflammatory marker *IL6* compared to the low PGE<sub>2</sub> OA group ( $p \leq 0.0001$ ) and 8.3-fold higher compared to CD patients ( $p = 0.0002$ ). Another inflammatory marker, *IL1B*, showed similar expression patterns between the three patient groups with 3.4-fold higher expression levels in the high PGE<sub>2</sub> OA group compared to the low PGE<sub>2</sub> OA group ( $p = 0.02$ ) and 3.2-fold higher compared to CD patients ( $p = 0.05$ ) (Figure 3A). IL-6 protein release by HFP showed the same pattern as mRNA data (Figure 3A). IL-1 $\beta$  protein release by HFP could only be detected in four samples, which were all conditioned media collected from patients in the high PGE<sub>2</sub> OA group (Figure 3A). Gene expression levels of the pro-inflammatory marker *TNFA* was not different between the HFPs of the three patient groups (Figure 3A).



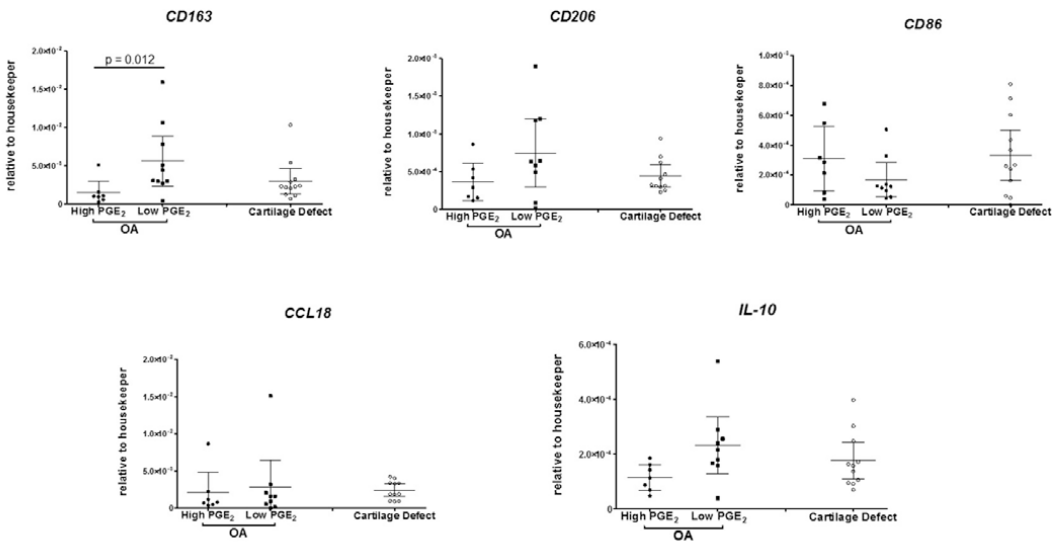
**A**

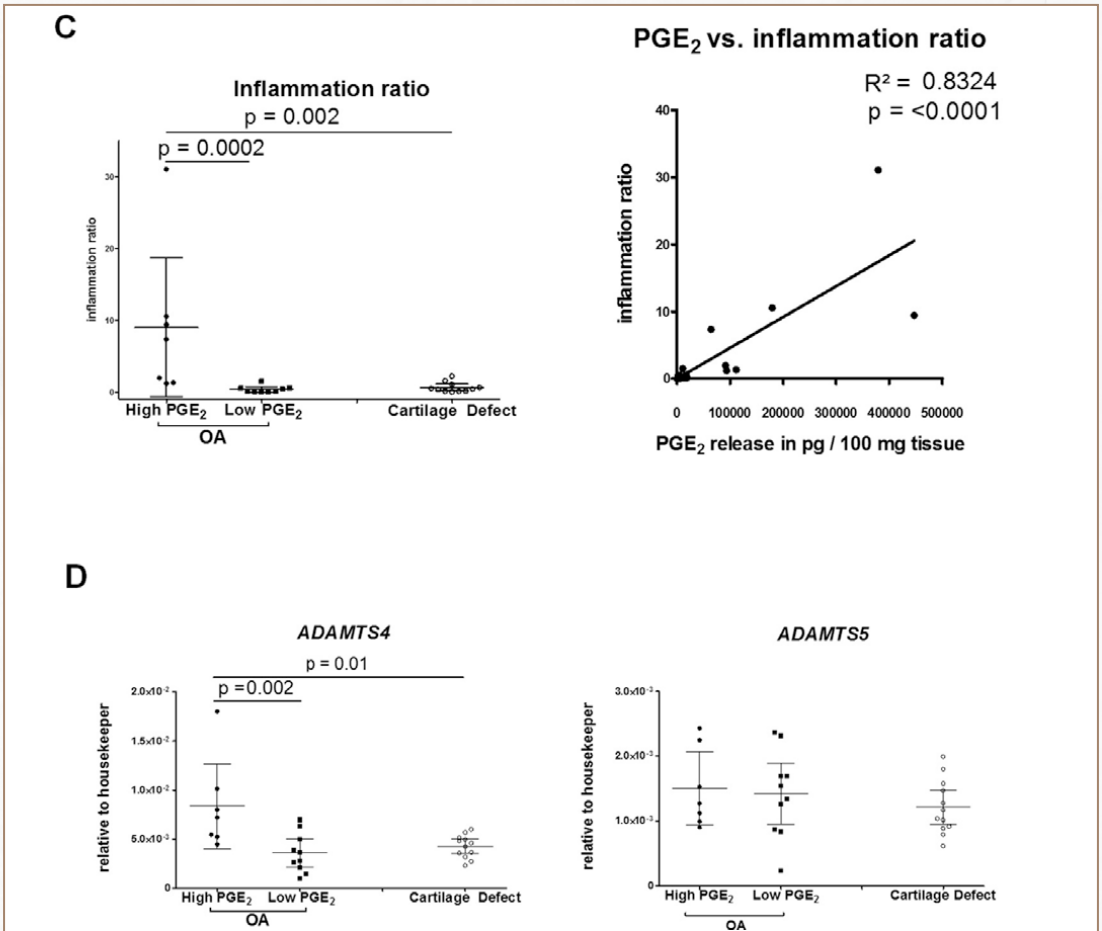
**Pro-inflammatory markers**



**B**

**Markers associated with macrophages**





**Figure 3: More inflammatory phenotype OA HFP than CD HFP.**

Gene expression levels of pro-inflammatory markers (*IL6*, *IL1B*, *TNFA*) and protein release levels of *IL-6* and *IL-1 $\beta$*  (A) and markers associated with macrophages (*CD86*, *CD163*, *IL10*, *CCL18*, *CD206*) (B) in HFP from the high PGE<sub>2</sub> OA group, low PGE<sub>2</sub> OA group and Cartilage Defect group. Data is presented in scatter dot plots with mean  $\pm$  95% CI. Inflammation ratio in HFP from the high PGE<sub>2</sub> OA group, low PGE<sub>2</sub> OA group and Cartilage Defect group (left figure, data represented in scatter dot plots with mean  $\pm$  95% CI) and association PGE<sub>2</sub> release and inflammation ratio in HFP (right figure). Each dot represents data from separate patients (C). Gene expression levels of the inflammatory aggrecanase *ADAMTS4* and non-inflammatory aggrecanase *ADAMTS5* in high PGE<sub>2</sub> OA group, low PGE<sub>2</sub> OA group and Cartilage Defect group. Data is presented in scatter dot plots with mean  $\pm$  95% CI (D). N = 17 for OA HFP and N = 12 for CD HFP.

Gene expression levels of the marker associated with anti-inflammatory macrophages, *CD163* was 3.7-fold lower ( $p = 0.012$ ) in HFP from the high PGE<sub>2</sub> OA group compared to HFP from the low PGE<sub>2</sub> OA group (Figure 3B). Gene expression levels of the pro-inflammatory marker *CD86* and of the anti-inflammatory markers *IL-10*, *CCL18* and *CD206* were not statistically different between HFPs from OA or CD patients (Figure 3B). The higher pro-inflammatory and the lower anti-inflammatory marker gene expression in HFP from the high PGE<sub>2</sub> OA group resulted in a 21.4-fold higher inflammation ratio than the HFP from the low PGE<sub>2</sub> OA group ( $p = 0.0002$ ) and 9.7-fold higher than the HFP from CD patients ( $p = 0.002$ ) (Figure 3C). PGE<sub>2</sub> release was positively associated with expression of pro-inflammatory markers and negatively associated with expression of anti-inflammatory markers, resulting in a strong correlation ( $R^2 = 0.83$ ,  $p \leq 0.0001$ ) of the inflammation ratio with PGE<sub>2</sub> release by HFP (Figure 3C; separate correlation of pro- and anti-inflammatory markers with PGE<sub>2</sub> release can be found in Supplementary Figure 2).

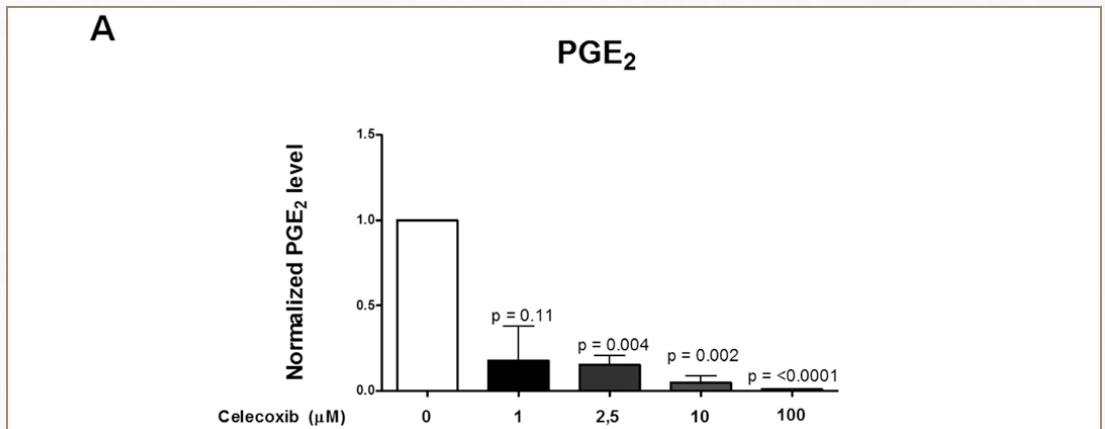
Since inflammation in OA is often accompanied by expression of proteolytic enzymes<sup>24</sup> and transcription of the extracellular proteolytic enzyme *ADAMTS4* is responsive to inflammatory stimuli and *ADAMTS5* is being considered as a non-inflammatory aggrecanase<sup>25,26</sup>, we evaluated the mRNA expression levels of these two aggrecanases in the HFP. The mRNA expression of *ADAMTS4*, but not *ADAMTS5*, was 2.3-fold higher in HFP from the high PGE<sub>2</sub> OA group than the HFP from the low PGE<sub>2</sub> OA group ( $p = 0.002$ ) and 2-fold higher than the HFP from CD patients ( $p = 0.01$ ), which is in concert with an inflammatory and non-inflammatory association of *ADAMTS4* and *ADAMTS5* respectively (Figure 3D).

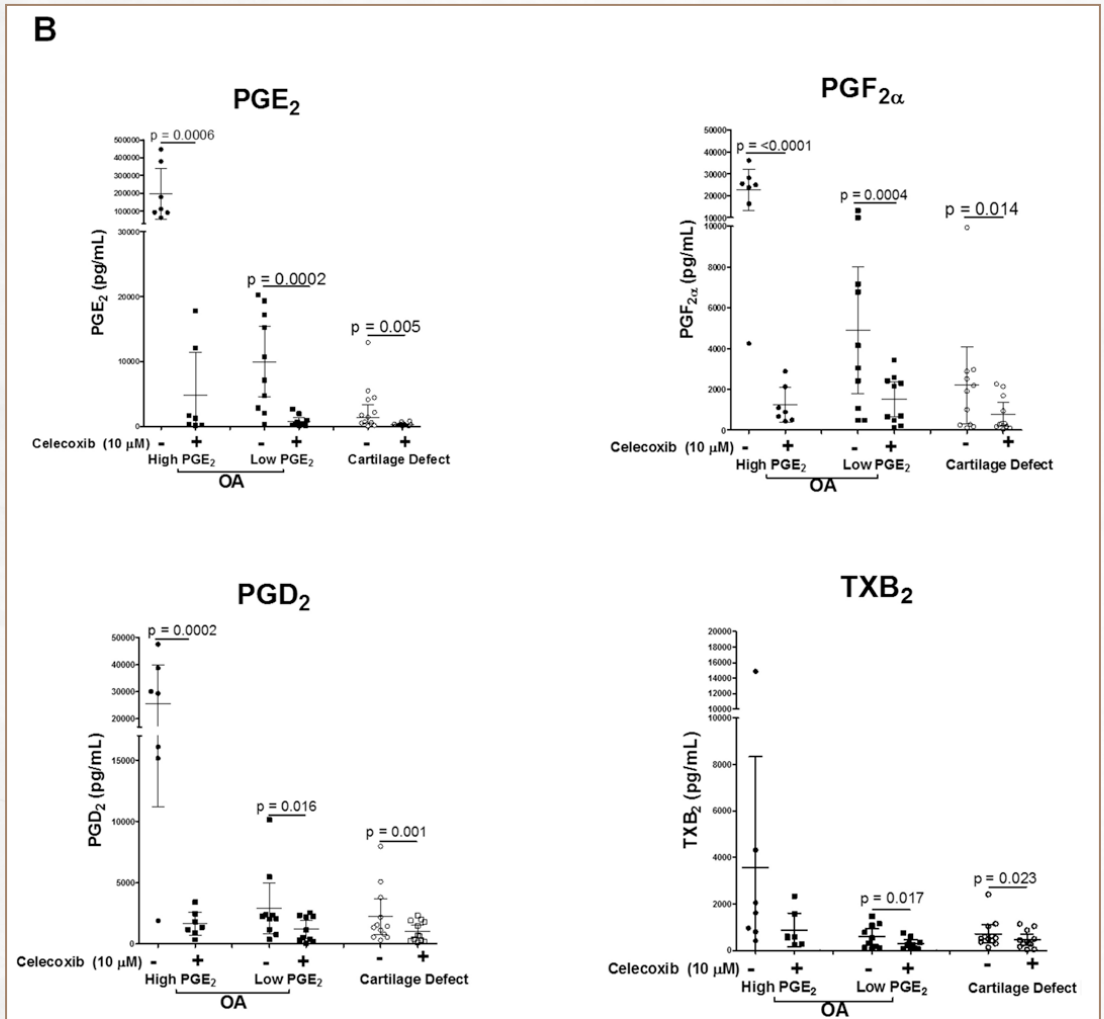
### 3.3 Celecoxib Reduces Prostanoid Release by OA and CD HFP

We next asked whether specific COX-2 inhibition by celecoxib in OA and CD HFPs has inflammation-attenuating effects. First, the concentration of celecoxib that is able to most efficiently reduce prostanoid release was determined by culturing HFP of three OA patients in the presence of 2.5, 10 or 100  $\mu\text{M}$  celecoxib. These three patients were selected randomly, without information about their distribution to either low or high PGE<sub>2</sub> OA group. Celecoxib at a dose of 100  $\mu\text{M}$  was highly effective in reducing PGE<sub>2</sub> release by HFP ( $p \leq 0.0001$ ), but considering the possibility that higher doses of celecoxib may result in reduced COX-2



selectivity<sup>27</sup>, we chose to use 10  $\mu\text{M}$  celecoxib in our experiments as this concentration was able to significantly reduce  $\text{PGE}_2$  release by at least 85% from HFP from all three patients ( $p = 0.002$ ) (Figure 4A).





**Figure 4: Celecoxib reduces prostanoïd release by OA and CD HFP.**

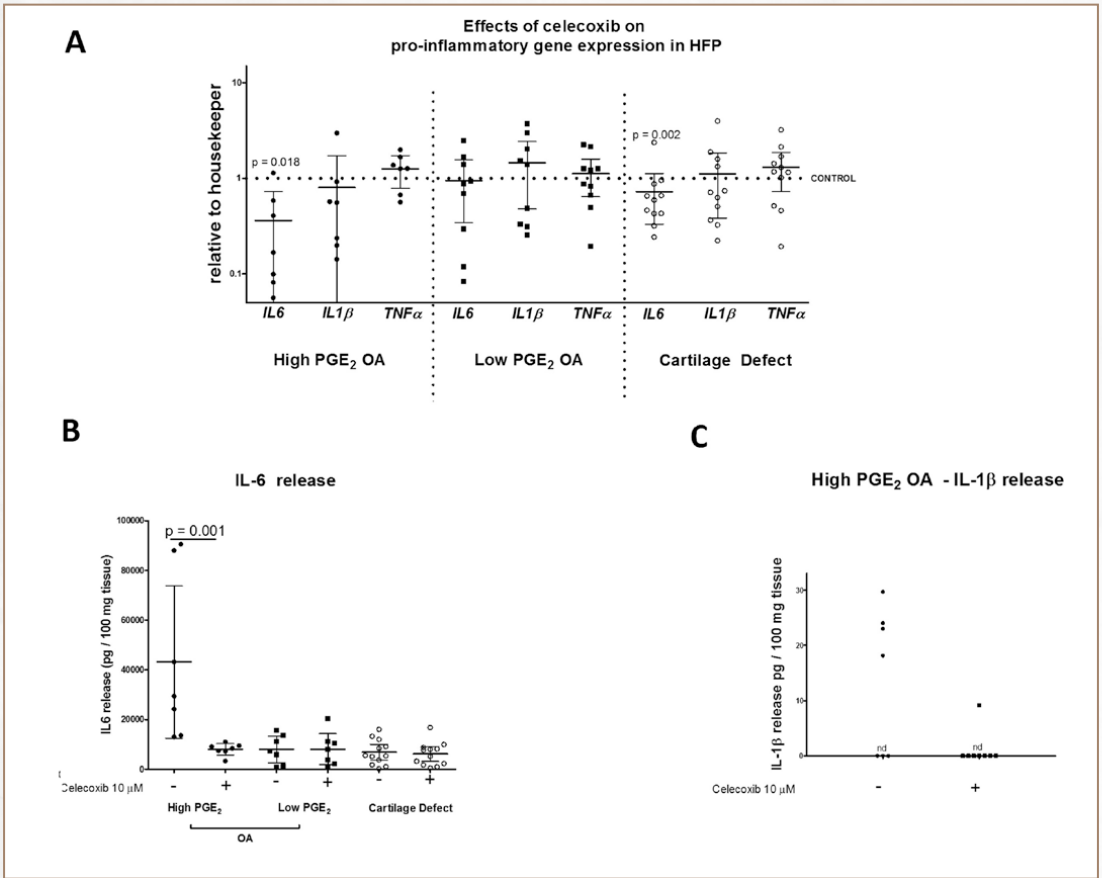
PGE<sub>2</sub> release in culture medium by OA HFP from three patients with increasing concentrations of celecoxib. Data represent normalized PGE<sub>2</sub> levels in three OA patients. The PGE<sub>2</sub> release in culture medium of HFP cultured without celecoxib was set to 1. Bars show the mean ± SD (A). Effects of celecoxib on PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub> and TXB<sub>2</sub> release in Hoffa's fat pad conditioned medium (HCM) from the high and low PGE<sub>2</sub> OA groups and CD group. Data is presented in scatter dot plots with mean ± 95% CI (B). N = 17 for OA HFP and N = 12 for CD HFP.

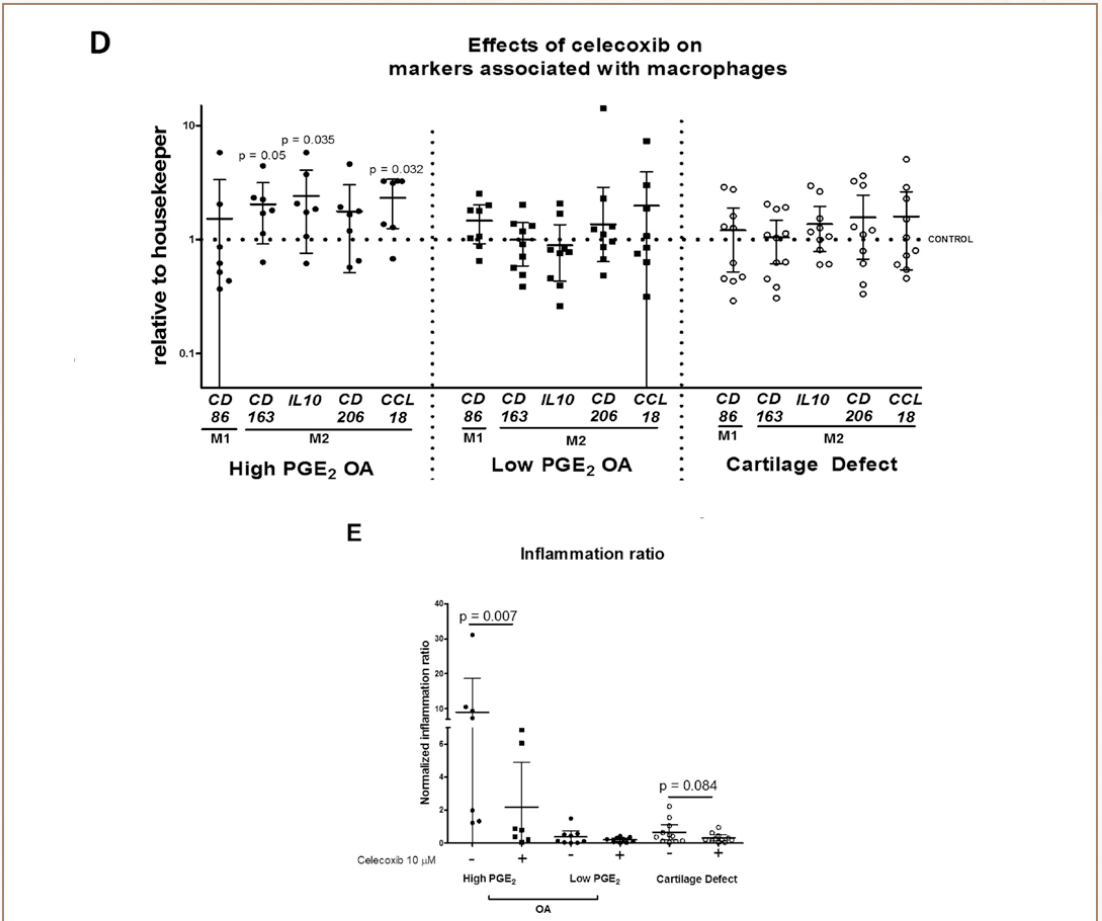
Celecoxib significantly reduced PGE<sub>2</sub>, PGF<sub>2α</sub> and PGD<sub>2</sub> release from HFP from the high PGE<sub>2</sub> (40.6-fold,  $p = 0.0006$ , 18.5-fold,  $p < 0.0001$  and 15.5-fold,  $p = 0.0002$  respectively), low PGE<sub>2</sub> (12.1-fold,  $p = 0.0002$ , 3.2-fold,  $p = 0.0004$  and 2.4-fold,  $p = 0.016$  respectively) and CD group ( $p = 0.005$ ,  $p = 0.014$  and  $p = 0.001$  respectively). Celecoxib also significantly reduced TXB<sub>2</sub> release by HFP from the low PGE<sub>2</sub> group (2.1-fold,  $p = 0.017$ ) and HFP from CD patients (1.6-fold,  $p = 0.023$ ) (Figure 4B).

### 3.4 Celecoxib Acts Anti-Inflammatory on the Inflamed OA HFP

Since PGE<sub>2</sub> release was positively associated with the inflammation ratio, we next investigated the consequences of reducing prostanoid release on the expression of markers associated with inflammation. Celecoxib reduced *IL6* expression by 3.5-fold ( $p = 0.018$ ) (Figure 5A) and IL-6 protein release by 3.9-fold ( $p = 0.001$ ) (Figure 5B) in HFP from the high PGE<sub>2</sub> OA group. No significant alterations in *IL6* expression and IL-6 protein release were observed in HFP from the low PGE<sub>2</sub> OA group (Figure 5A and 5B). In HFP from CD patients, celecoxib decreased *IL6* mRNA expression by 1.3-fold ( $p = 0.002$ ), but no significant effect was observed on IL-6 protein release after celecoxib treatment (Figure 5A and 5B). Celecoxib also reduced IL-1 $\beta$  protein release to non-detectable levels by HFP from the four patients (Figure 3A) in the high PGE<sub>2</sub> OA group, in which IL-1 $\beta$  was detectable (Figure 5C). Furthermore, celecoxib increased gene expression of *CD163* by 2.1-fold ( $p = 0.05$ ), *IL10* by 2-fold ( $p = 0.035$ ) and *CCL18* by 1.5-fold ( $p = 0.032$ ) in HFP from the high PGE<sub>2</sub> OA group (Figure 5D). The specific modulation of gene expression levels of pro- and anti-inflammatory markers in HFP from the high PGE<sub>2</sub> OA group resulted in significant modulation of the inflammation ratio in HFP from the high PGE<sub>2</sub> OA group by 5-fold ( $p = 0.007$ ), while no significant alteration in this ratio in HFP from the low PGE<sub>2</sub> OA group ( $p = 0.259$ ) and CD patients ( $p = 0.084$ ) (Figure 5E) was observed.







**Figure 5: Anti-inflammatory effects of celecoxib in inflamed HFP's.**

Effects of celecoxib on gene expression levels of pro-inflammatory markers (IL6, IL1B and TNFα) Normalized gene expression level of the control condition was set to 1 (horizontal dotted line) (A). Effects of celecoxib on protein levels of pro-inflammatory markers IL-6 (B) and IL-1β (C). Effects of celecoxib on gene expression levels of markers associated with macrophages (CD86, CD163, IL10, CD206 and CCL18) Normalized gene expression level of the control condition was set to 1 (horizontal dotted line) (D). Data is presented in scatter dot plots with mean ± 95% CI. Each dot or square represents separate patients. Effects of celecoxib on the inflammation ratio in HFP from the high and low PGE<sub>2</sub> OA group and in HFP from CD patients. Data is presented in scatter dot plots with mean ± 95% CI (E). N = 16 for OA HFP and N = 10 for CD HFP.

## 4. Discussion

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To get a broader insight into the involvement of specific prostanoids in OA-related inflammatory processes we compared the release profile of prostanoids between HFP from OA patients vs HFP from patients without OA but with a focal CD, and investigated the modulatory effects of celecoxib on prostanoid production by HFP.

We here show that specific prostanoids PGE<sub>2</sub>, PGF<sub>2α</sub> and PGD<sub>2</sub> are released in higher amounts by HFP from OA patients compared to HFP from patients with CDs. Prostanoid release by OA HFP clustered in two subgroups with high and low prostanoid producers and celecoxib was able to attenuate the inflammatory phenotype in OA HFP most effectively in donors with a more inflammatory phenotype.

Our findings are in accordance with previous work from Massicotte *et al.* showing variable secretion of PGE<sub>2</sub> by subchondral osteoblasts from knee OA patients, which distributed to 2 OA subgroups<sup>28</sup>. In our study, in addition to PGE<sub>2</sub>, release of two other prostanoid subtypes PGF<sub>2α</sub> and PGD<sub>2</sub>, but not TXB<sub>2</sub>, were higher in the high PGE<sub>2</sub> OA group than the CD group, and correlated with increased COX-2 expression. Our data show that specific prostanoid subtypes are released from the OA HFP and may participate in the inflammatory response in the HFP. These findings indicate that depending on its inflammatory status, the HFP is an important contributor of inflammatory mediators found in synovial fluid of OA patients<sup>29</sup>.

Different possible explanations for the observed differential inflammatory phenotype between OA patients can be given. First, a different subtype of knee OA patients might exist. We correlated our findings to several parameters such as sex, age, BMI and K&L grade, but did not find any associations (Supplementary Figure 1). Possible associations between these parameters and HFP inflammation will be valuable to investigate in the future with greater experimental groups. Secondly, the duration of OA development or phase in OA disease progression can be different between patients. Unfortunately, information about the duration of symptoms was sometimes missing, and if reported, described concisely. Third, the HFP is reported to contain immune cells, and in particular macrophages<sup>16</sup>.



As the distribution of all the different cells present in HFP might not be homogeneous, different inflammatory release profiles between OA patients might be caused by this inhomogeneity. To circumvent this, the entire obtained HFP was cut into small pieces and explants were randomly divided over the different samples.

One other study compared prostanoid release profiles by HFP between OA and non-OA patients<sup>5</sup>. In that study, the precursor of prostanoid subtypes, arachidonic acid, was shown to be higher secreted by OA vs non-OA HFP. In our study, the difference in downstream prostanoid metabolites of arachidonic acid was more prominent between OA and non-OA donors, which was partly unexpected because CD patients have been demonstrated to display elevated levels of inflammatory cytokines such as IL-6 and IFN- $\gamma$  in synovial fluid compared to a healthy control group<sup>30</sup>. Possible explanations between our results and the results of Gierman *et al.* may be related to the different method of prostanoid analysis, the variation in prostanoid release in OA patients or age.

PGE<sub>2</sub> release by OA HFP was positively associated with a calculated inflammation ratio of the HFP, and celecoxib modulated this inflammation ratio specifically in patients with higher prostanoid release profiles (the high PGE<sub>2</sub> OA group). This indicates that this modulatory capacity is dependent on the level of inflammation. Skewing of the inflammation ratio in HFP by celecoxib might potentially have an effect on pain sensation. Inflammatory factors can contribute to sensitization of the joint<sup>31</sup>, while anti-inflammatory factors can have analgesic effects<sup>32</sup>. Indeed, in addition to the synovium, HFP in knee OA patients is gaining interest as a potential source of pain in knee OA<sup>4</sup>.

Adipocytes in adipose tissue have been shown to produce mediators such as IL-6, IL-1 $\beta$  and IL-10 and therefore adipocytes may be the cell source responsible for the increased prostanoids, IL-1 $\beta$  and IL-6 release we have observed in the high PGE<sub>2</sub> OA group. Future experiments will further elucidate the cell source responsible for this increased release and will also provide further insights into the source of cells mostly affected by the action of celecoxib. Moreover longer incubation periods with celecoxib may result in more pronounced anti-inflammatory effects and influencing the inflammatory status of the

adipocyte in the tissue may also have indirect anti-inflammatory effects on other cell types in the HFP.

Celecoxib also reduced prostanoid release in HFP from CD patients. Since targeting pro-inflammatory cytokines has been shown to prevent cartilage degradation *in vivo* following knee injury<sup>33</sup> and the link between pro-inflammatory factors and cartilage degradation is increasingly being recognized<sup>24,33</sup>, celecoxib may be used to improve the outcome of cartilage regeneration strategies or act preventive on progression into OA in patients with CDs.

Taken together, we here show for the first time that patients with knee OA display a differential inflammatory profile in their HFP compared to patients with CDs, which may be of use when developing future therapies (e.g., pharmaceutical and regenerative). Decreasing prostanoid release by OA HFP with celecoxib can modulate the general inflammatory phenotype in HFP. We expect that in a subset of knee OA patients presenting with inflamed HFP's, celecoxib may have the potency to exert an anti-inflammatory action via decreasing the release of prostanoids produced by the HFP and modulate the inflammatory phenotype of the HFP.

## 5. Author Contributions

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U.T.T., conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript, M.C., Y.BJ., T.W., L.R., G.O., P.E., conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript.

## 6. Competing Interests

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Prof. Dr LW. Van Rhijn, dr. TJM. Welting and dr. PJ. Emans have a patent US 20130123314 A1 issued. The patent describes an improved method for repairing damaged cartilage and/or preventing cartilage degeneration in tissue, in particular in a joint by administering a

pharmaceutically active agent not in the intra-articular cavity but directly into the fat pad of a joint.

## 7. Acknowledgements

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## 8. Supplementary Data

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The supplementary data belonging to this article can be found online ([https://www.oarsijournal.com/article/S1063-4584\(18\)30094-3/fulltext#supplementaryMaterial](https://www.oarsijournal.com/article/S1063-4584(18)30094-3/fulltext#supplementaryMaterial)).



## References

- 1 Hunter D.J., Felson D.T.: Osteoarthritis. *BMJ* 2006; 332: pp. 639-642.
- 2 Berenbaum F.: Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthritis Cartilage* 2013; 21: pp. 16-21.
- 3 Clockaerts S., Bastiaansen-Jenniskens Y.M., Feijt C., de Clerck L., Verhaar J.A., Zuurmond A.M., *et. al.*: Cytokine production by infrapatellar fat pad can be stimulated by interleukin 1beta and inhibited by peroxisome proliferator activated receptor alpha agonist. *Ann Rheum Dis* 2012; 71: pp. 1012-1018.
- 4 Clockaerts S., Bastiaansen-Jenniskens Y.M., Runhaar J., van Osch G.J., van Offel J.F., Verhaar J.A., *et. al.*: The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthr Cartil* 2010; 18: pp. 876-882.
- 5 Gierman L.M., Wopereis S., van El B., Verheij E.R., Werff-van der Vat B.J., Bastiaansen-Jenniskens Y.M., *et. al.*: Metabolic profiling reveals differences in concentrations of oxylipins and fatty acids secreted by the infrapatellar fat pad of donors with end-stage osteoarthritis and normal donors. *Arthritis Rheum* 2013; 65: pp. 2606-2614.
- 6 Pelletier J.P., DiBattista J.A., Roughley P., McCollum R., Martel-Pelletier J.: Cytokines and inflammation in cartilage degradation. *Rheum Dis Clin N Am* 1993; 19: pp. 545-568.
- 7 Ricciotti E., FitzGerald G.A.: Prostaglandins and inflammation. *Arterioscl Thromb Vasc Biol* 2011; 31: pp. 986-1000.
- 8 Martel-Pelletier J., Pelletier J.P., Fahmi H.: Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum* 2003; 33: pp. 155-167.
- 9 Attur M., Al-Mussawir H.E., Patel J., Kitay A., Dave M., Palmer G., *et. al.*: Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor. *J Immunol* 2008; 181: pp. 5082-5088.
- 10 Eymard F., Pigenet A., Citadelle D., Flouzat-Lachaniette C.H., Poignard A., Benelli C., *et. al.*: Induction of an inflammatory and prodegradative phenotype in autologous fibroblast-like synoviocytes by the infrapatellar fat pad from patients with knee osteoarthritis. *Arthritis Rheum* 2014; 66: pp. 2165-2174.
- 11 Bastiaansen-Jenniskens Y.M., Wei W., Feijt


- C., Waarsing J.H., Verhaar J.A., Zuurmond A.M., *et. al.*: Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: a possible role for prostaglandin f2alpha. *Arthritis Rheum* 2013; 65: pp. 2070-2080.
- 12 Uda R., Horiguchi S., Ito S., Hyodo M., Hayaishi O.: Nociceptive effects induced by intrathecal administration of prostaglandin D2, E2, or F2 alpha to conscious mice. *Brain Res* 1990; 510: pp. 26-32.
- 13 Reinold H., Ahmadi S., Depner U.B., Layh B., Heindl C., Hamza M., *et. al.*: Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *J Clin Invest* 2005; 115: pp. 673-679.
- 14 Minami T., Uda R., Horiguchi S., Ito S., Hyodo M., Hayaishi O., *et. al.*: Allodynia evoked by intrathecal administration of prostaglandin F2 alpha to conscious mice. *Pain* 1992; 50: pp. 223-229.
- 15 Ryd L., Brittberg M., Eriksson K., Jurvelin J.S., Lindahl A., Marlovits S., *et. al.*: Pre-osteoarthritis: definition and diagnosis of an elusive clinical entity. *Cartilage* 2015; 6: pp. 156-165.
- 16 Bastiaansen-Jenniskens Y.M., Clockaerts S., Feijt C., Zuurmond A.M., Stojanovic-Susulic V., Bridts C., *et. al.*: Infrapatellar fat pad of patients with end-stage osteoarthritis inhibits catabolic mediators in cartilage. *Ann Rheum Dis* 2012; 71: pp. 288-294.
- 17 Caron M.M., Emans P.J., Cremers A., Surtel D.A.M., van Rhijn L.W., Welting T.J.M., *et. al.*: Indomethacin induces differential effects on *in vitro* endochondral ossification depending on the chondrocyte's differentiation stage. *J Orthop Res Off Publ Orthop Res Soc* 2016;
- 18 Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P.: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol Lett* 2004; 26: pp. 509-515.
- 19 Livak K.J., Schmittgen T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: pp. 402-408. S1046-S2023 (01)91262-9 [pii]
- 20 Utomo L., Bastiaansen-Jenniskens Y.M., Verhaar J.A., van Osch G.J.: Cartilage inflammation and degeneration is enhanced by pro-inflammatory (M1) macrophages *in vitro*, but not inhibited directly by anti-inflammatory (M2) macrophages. *Osteoarthritis Cartilage* 2016; 24: pp. 2162-2170.
- 21 Brock T.G., McNish R.W., Peters-Golden

## Chapter 4

- M.: Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. *J Biol Chem* 1999; 274: pp. 11660-11666.
- 22 Knott I., *et. al.*: Induction of cyclooxygenase by interleukin 1: comparative study between human synovial cells and chondrocytes. *J Rheumatol* 1994; 21: pp. 462-466.
- 23 Mosser D.M., Edwards J.P.: Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; 8: pp. 958-969.
- 24 Goldring M.B., *et. al.*: Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cell Mater* 2011; 21: pp. 202-220.
- 25 Roberts S., Evans H., Wright K., van Niekerk L., Caterson B., Richardson J.B., *et. al.*: ADAMTS-4 activity in synovial fluid as a biomarker of inflammation and effusion. *Osteoarthr Cartil* 2015; 23: pp. 1622-1626.
- 26 Bau B., Gebhard P.M., Haag J., Knorr T., Bartnik E., Aigner T., *et. al.*: Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes *in vivo* and *in vitro*. *Arthritis Rheum* 2002; 46: pp. 2648-2657.
- 27 Welting T.J., Caron M.M., Emans P.J., Janssen M.P., Sanen K., Coolsen M.M., *et. al.*: Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater* 2011; 22: pp. 420-436. discussion 436-427
- 28 Massicotte F., Lajeunesse D., Benderdour M., Pelletier J.P., Hilal G., Duval N., *et. al.*: Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthr Cartil* 2002; 10: pp. 491-500.
- 29 Brenner S.S., Klotz U., Alscher D.M., Mais A., Lauer G., Schweer H., *et. al.*: Osteoarthritis of the knee—clinical assessments and inflammatory markers. *Osteoarthr Cartil* 2004; 12: pp. 469-475.
- 30 Tsuchida A.I., Beekhuizen M., t Hart M.C., Radstake T.R., Dhert W.J., Saris D.B., *et. al.*: Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Arthritis Res Ther* 2014; 16: pp. 441.
- 31 Miller R.E., Miller R.J., Malfait A.M.: Osteoarthritis joint pain: the cytokine connection. *Cytokine* 2014; 70: pp. 185-193.



- 32 Pannell M., *et. al.*: Adoptive transfer of M2 macrophages reduces neuropathic pain via opioid peptides. *J Neuroinflammation* 2016; 13: pp. 262.
- 33 Furman B.D., Mangiapani D.S., Zeitler E., Bailey K.N., Horne P.H., Huebner J.L., *et. al.*: Targeting pro-inflammatory cytokines following joint injury: acute intra-articular inhibition of interleukin-1 following knee injury prevents post-traumatic arthritis. *Arthritis Res Ther* 2014; 16: pp. R134.

An anatomical illustration of a human leg, showing the skeletal structure and a complex network of glowing blue vessels (arteries and veins) that run through the leg. The vessels are highlighted in a bright blue color, contrasting with the darker, semi-transparent skin and muscle tones of the leg. The overall image has a dark, almost black background, which makes the glowing vessels and the white text stand out prominently.

Chapter  
05

# Chondroprotective Actions of Selective COX-2 Inhibitors *In Vivo*: A Systematic Review.

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Welting T.J.M., van Rhijn L.W., van Osch G.J.V.M., Emans P.J. *Int. J Mol  
Sci.* 2020 21(18).



## Abstract

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### **Objective:**

Knee osteoarthritis (OA) is a condition mainly characterized by cartilage degradation. Currently, no effective treatment exists to slow down the progression of OA-related cartilage damage. Selective COX-2 inhibitors may, next to their pain killing properties, act chondroprotective *in vivo*.

### **Material & Methods:**

To determine whether the route of administration is important for the efficacy of the chondroprotective properties of selective COX-2 inhibitors, a systematic review was performed according to the PRISMA guidelines. Studies investigating OA-related cartilage damage of selective COX-2 inhibitors *in vivo* were included.

### **Results:**

Nine of the fourteen preclinical studies demonstrated chondroprotective effects of selective COX-2 inhibitors using systemic administration. Five clinical studies were included and, although in general non-randomized, failed to demonstrate chondroprotective actions of oral selective COX-2 inhibitors. All of the four preclinical studies using bolus intra-articular injections demonstrated chondroprotective actions, while one of the three preclinical studies using a slow release system demonstrated chondroprotective actions.

### **Conclusion:**

Despite the limited evidence in clinical studies that have used the oral administration route, there seems to be a preclinical basis for considering selective COX-2 inhibitors as disease modifying osteoarthritis drugs when used intra-articularly. Intra-articularly injected selective COX-2 inhibitors may hold the potential to provide chondroprotective effects *in vivo* in clinical studies.

### **Keywords:**

Selective COX-2 inhibitors, intra-articular injection, knee osteoarthritis, DMOADs

## 1. Introduction

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Knee osteoarthritis (OA) is a condition that leads to pain and is mainly characterized by cartilage degradation<sup>1</sup>. Currently, drug treatments provide symptomatic pain relief but no effective treatment exists to slow down progression of OA-related cartilage damage<sup>1</sup>. Pain-killing drug therapies include non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs provide pain relief by blocking cyclooxygenase (COX)-dependent prostanoid synthesis. Prostanoids are an important family of signaling molecules present in synovial fluid<sup>2</sup>. At least two COX isoforms have been described, COX-1 and COX-2, the latter being considered as the inflammatory isoform<sup>3</sup>. Selective COX-2 inhibitors have been developed to specifically target the inflammatory COX-2 while circumventing inhibition of the COX-1 isoform. While selective COX-2 inhibitors may provide an effective means for pain relief, targeting the inflammatory COX-2 may be also a promising approach to inhibit cartilage degradation and thereby slow down knee OA progression<sup>3</sup>. This hypothesis is supported by the accumulating evidence showing that inflammation precedes OA disease progression<sup>3,4</sup>.

From the anti-nerve growth factor clinical trials it became clear that next to a substantial improvement in pain, patients also displayed structural OA disease progression<sup>5</sup>. This emphasizes the importance of treatment strategies not only providing pain relief, which will lead to a vicious self-perpetuating cycle of joint overloading and OA progression, but also providing the ability for disease modification.

The actions of non-selective COX inhibitors on cartilage degradation *in vitro* and *in vivo* have been excellently reviewed in the past<sup>6</sup>. Moreover, *ex vivo* and *in vivo* actions of the selective COX-2 inhibitor celecoxib on cartilage degradation, synovial inflammation and osteoclast metabolism have also been reviewed and date back to 2011<sup>7</sup>. Recently, an updated review of studies published until 2016 has been conducted on the OA disease-modifying actions of celecoxib on different OA tissues. This review confirms the contradictive reports regarding the chondroprotective actions of celecoxib both *ex vivo* and *in vivo*. Based on these reviews, it still remains obscure whether selective COX-2 inhibitors can be used *in vivo* to protect cartilage and slow down the progression of knee OA. One of the explanations of

the contradictory reports found in the literature regarding the potential of selective COX-2 inhibitors may be related to the route of administration. Specifically, scarcely vascularized tissues such as cartilage and meniscus, which are important participants in knee OA, may be modified differentially after systemic oral treatment compared to intra-articular treatment<sup>8,9</sup>. To date, the role of the route of administration on the chondroprotective effects of selective COX-2 inhibitors remains unclear. Therefore, the objective of this review was to systematically review available literature on chondroprotective properties of selective COX-2 inhibitors in preclinical models for OA or in clinical OA studies depending on the route of administration.

## 2. Materials and Methods

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### 2.1 Search Strategy and Data Extraction

MEDLINE and EMBASE databases were systematically searched on all studies relating intra-articular or oral treatment of knee OA patients with selective COX-2 inhibitors. The search was conducted in May 2020 according to the search strategy and data collection guidelines of the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement. A manual search of the Cochrane library yielded no relevant articles. Upon reading the full-text key papers snowballing searching manually reference lists of the included articles was allowed. Disagreements between the reviewers were resolved by consensus.

After disregarding duplicates, the title and the abstract of articles were independently screened by two observers according to predefined criteria. The search query was as follows:

```
("Osteoarthritis"[Mesh] OR OA OR osteoarthritis) AND ("Knee"[Mesh] OR Knee [tiab]) AND  
(((COX-2 [tiab] OR COX2 [tiab] OR Cyclooxygenase 2 [tiab] OR "Cyclooxygenase 2"[Mesh])  
AND (inhibitor [tiab] or inhibition [tiab] or limitation [tiab] or limiting [tiab])) OR celecoxib  
[tiab] OR etoricoxib [tiab] OR rofecoxib [tiab] or valdecoxib [tiab] or lumiracoxib [tiab] or
```



mavacoxib [tiab] or meloxicam [tiab] or VA441 [tiab] or MK-0966 [tiab] or gw406381 [tiab] or SC-58635 [tiab] or VA692 [tiab] or VA694 [tiab] or SC-236 [tiab]) AND ((oral [tiab] AND (suppletion [tiab] OR supplements [tiab] OR supplementation [tiab] OR treatment [tiab] OR ingestion [tiab] OR medication [tiab] OR tablets [tiab] OR intake [tiab] OR absorption [tiab])) OR treatment OR ((intraarticular [tiab] OR intra-articular [tiab]) AND (injection [tiab] OR therapy [tiab] OR supplementation [tiab] OR suppletion [tiab])))).

## 2.2 Articles Were Selected Based on Inclusion and Exclusion Criteria

### Inclusion Criteria:

- i. Presenting data about chondroprotective effects of COX-2 inhibitors;  
The chondroprotective effect is defined as any effect that leads to significantly less cartilage degradation evidenced either through imaging, biochemical analysis or on histology.
- ii. Either *in vivo* animal studies or clinical studies involving human knee OA patients;
- iii. Intra-articular therapies with COX-2 inhibitors;
- iv. Systemic therapies with COX-2 inhibitors.

### Exclusion Criteria:

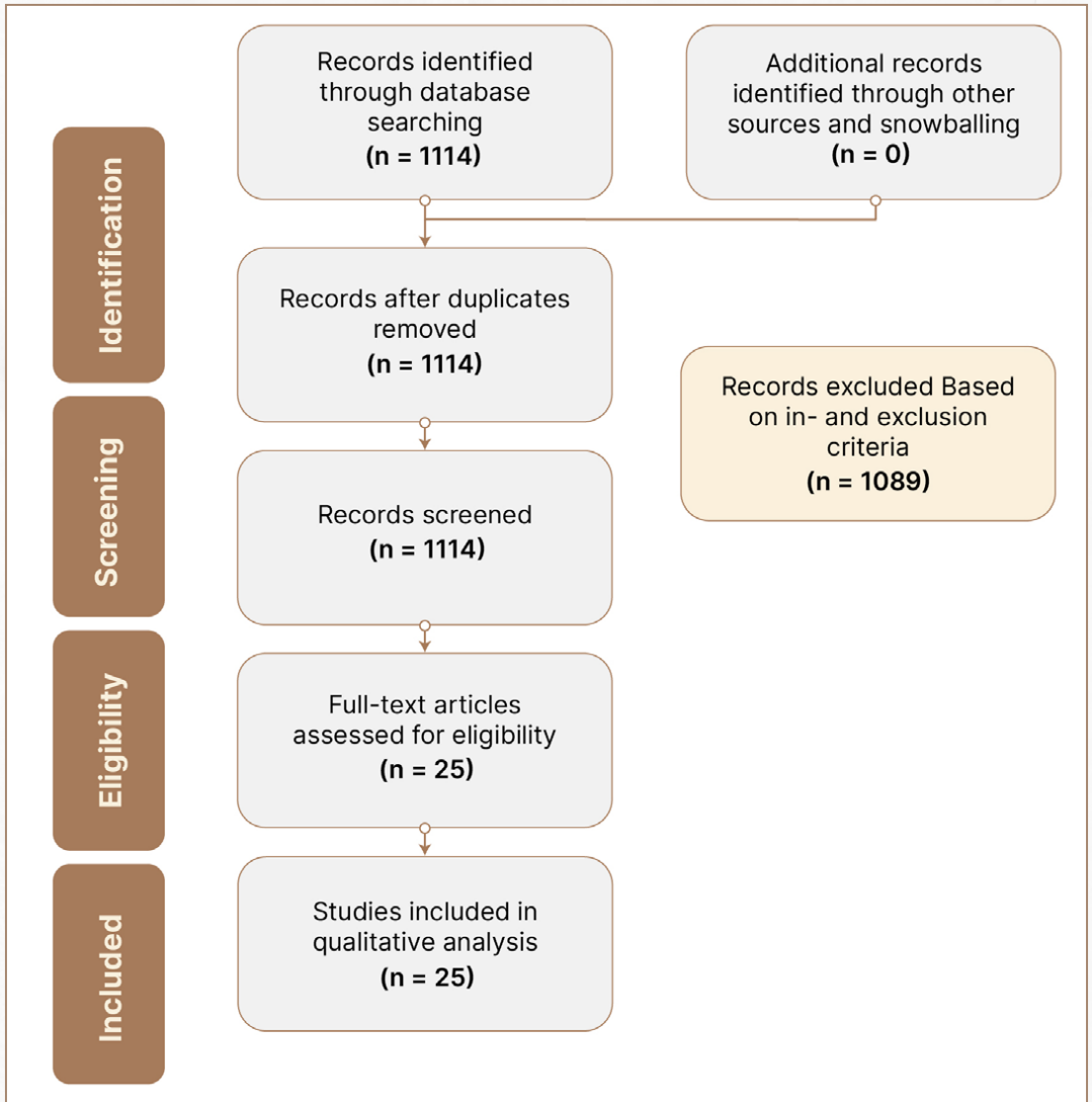
- i. Studies solely investigating non-selective cox-inhibitors;
- ii. Studies investigating actions of celecoxib inhibitors on pain modulation in knee OA;
- iii. Studies investigating other joints than the knee;
- iv. Studies reporting solely *in vitro* data.

Two observers systematically extracted study data based on the inclusion and exclusion criteria. The risk of bias of the included studies was evaluated using the SYRCLE's risk of bias tool for animal studies<sup>10</sup> and the Cochrane risk of bias tool<sup>11</sup> for human studies.

### 3. Results

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The search strategy yielded 25 articles for inclusion (Figure 1). We identified preclinical and clinical studies investigating chondroprotective actions of selective COX-2 inhibitors either via systemic administration or via intra-articular delivery. Using the SYRCLE's risk of bias tool for preclinical studies, we observed that outcome measurements were in general blinded, but randomization sequences were not always reported increasing the risk for selection bias (supplementary data)<sup>10</sup>. For clinical studies, the Cochrane risk bias tool revealed that clinical studies were non-randomized, displaying a high risk of selection and performance bias (supplementary data)<sup>11</sup>.



**Figure 1: PRISMA flowchart.**

PRISMA flowchart showing the yield of the search and inclusion of studies leading to the 25 included studies.



The following sections will discuss the main results of the included 25 studies and compare chondroprotective actions of selective COX-2 inhibitors when used systemically or intra-articularly.

### 3.1 Preclinical Studies: Oral and Intraperitoneal Administration

Fourteen of the included studies have investigated chondroprotective actions of selective COX-2 inhibitors after systemic administration (Table 1). Most studies focused on chondroprotective actions of the selective COX-2 inhibitor celecoxib<sup>12,13,14,15,16,17,18,19,20,21</sup>, while other studies investigated chondroprotective actions of meloxicam<sup>22,23</sup> and etoricoxib<sup>24,25</sup>. Four studies that investigated chondroprotective actions of selective COX-2 inhibitors in surgically induced OA models failed to demonstrate chondroprotective actions when the drug was administered directly after OA induction<sup>12,14,18,22</sup>. On the other hand, Dai *et al.* demonstrated chondroprotective actions of celecoxib, as evidenced by an improved macroscopic and histological OARSI score, in a surgically induced pig OA model, in which treatment was started one week after surgery<sup>20</sup>. Further support of chondroprotective actions of selective COX-2 inhibitors comes from a study in a surgically induced rat OA model, where treatment with oral etoricoxib decreased OA-related cartilage damage as evidenced by an improved Pritzker score compared to controls<sup>24</sup>. Chondroprotective actions of celecoxib were also shown in a study in which the Achilles tendon transection rat knee OA model was used<sup>15</sup>. It was shown that compared to controls, chondrocyte apoptosis was lower after oral administration of celecoxib<sup>15</sup>.

**Table 1: The schematic of studies investigating chondroprotective effects of systemically administered selective COX-2 inhibitors.**

PRECLINICAL STUDIES USING ORAL ADMINISTRATION								
Authors	Species	COX-2 Inhibitor	OA Model	Dosing Regime	Start of Treatment	Timepoint of Evaluation	Evaluation of cartilage Degradation	Main Findings
Mastbergen <i>et al.</i> <sup>12</sup>	Canine	Celecoxib	Groove	Daily 200 mg	Directly after surgery	15 weeks after OA induction	Histology: Modified Mankin Biochemistry: PG content, synthesis and release	No difference in histological scores, no difference in PG content, synthesis or release
Huh <i>et al.</i> <sup>13</sup>	Rabbit	Celecoxib	Collagenase	Daily 100 mg/kg	Directly after OA induction	4 weeks after OA induction	Histology: Colombo score for cartilage and synovitis score for synoviummRNA analysis <i>PTGS1</i> , <i>PTGS2</i> , <i>MMP1</i> and <i>MMP3</i>	Improved histological score in celecoxib group vs. control, decreased MMP-1 mRNA expression in celecoxib group vs. control
Jones <i>et al.</i> <sup>22</sup>	Rat	Meloxicam	MCLT + ACLT + MMx	Daily 3 mg/kg	Directly after OA induction	8 weeks after OA induction	Histology: Modified Mankin	No difference in histological score meloxicam vs. control
Fukai <i>et al.</i> <sup>14</sup>	Mouse	Celecoxib	MCLT + MMx	Daily 10 mg/kg or 30 mg/kg	Directly after surgery	12 weeks after OA induction	Histology: Pritzker score	No difference in Pritzker score celecoxib vs. control.
Ou <i>et al.</i> <sup>15</sup>	Rat	Celecoxib	Achilles tendon transection	Daily 24 mg/kg	Directly after surgery		Histology: Type II collagen Tunel staining	No difference in type II collagen content in celecoxib vs. control, decreased chondrocyte apoptosis in celecoxib group.
Ashkavand <i>et al.</i> <sup>16</sup>	Rat	Celecoxib	MIA	Daily 100 mg/kg	Directly after OA induction	15 days after OA induction	Histology: Own developed score	Improved histological score in the celecoxib group. Superior chondroprotective effects when celecoxib is combined with silymarin

PRECLINICAL STUDIES USING ORAL ADMINISTRATION								
Authors	Species	COX-2 Inhibitor	OA Model	Dosing Regime	Start of Treatment	Timepoint of Evaluation	Evaluation of cartilage Degradation	Main Findings
Moon <i>et al.</i> <sup>17</sup>	Rat	Celecoxib	MIA	Daily 2.5 mg/kg	Directly after OA induction	7 days after OA induction	Histology: Modified Mankin	No difference in Modified Mankin celecoxib vs. control. Synergistic beneficial action when celecoxib is added to rebamipide
Panahifar <i>et al.</i> <sup>18</sup>	Rat	Celecoxib	ACLT + MCLT + MMx	Daily 2.86 mg/kg	Directly after surgery	4,8 and 12 weeks after OA induction	Histology: Modified Mankin	No difference in histological score celecoxib vs. control.
Li <i>et al.</i> <sup>19</sup>	Rat	Celecoxib	Collagenase	Daily 0.25 mg	6 weeks after surgery	10 weeks after OA induction	Histology: Colombo score and Biochemistry: CTX-II content in serum, Caspase 3 activity in tissue homogenate	Improved histological score in celecoxib group vs. control, higher CTX-II content in celecoxib group, lower Caspase 3 activity in celecoxib group. More pronounced effects when celecoxib is combined with diacerein
Dai <i>et al.</i> <sup>20</sup>	Pig	Celecoxib	MMx	20 mg/kg daily	1 week after surgery	12 weeks after surgery	Macroscopic:  OARSI score Histology: OARSI score COL-II and AGC immunohistochemistry	Improved macroscopic and histological score celecoxib group vs. control, no difference in COL-II and AGC expression
Nagy <i>et al.</i> <sup>23</sup>	Rat	Meloxicam	MIA	Daily 0.2 mg/kg or 1 mg/kg	3 weeks after OA induction	11 weeks after OA induction	Histology: OARSI score	Improved histological score at both doses meloxicam compared to control
Tu <i>et al.</i> <sup>21</sup>	Mouse	Celecoxib	Spontaneous OA (STR/Ort mouse)	Daily 8 mg/kg	3 months old mice	4 weeks after start treatment	Histology: OARSI score	Improved OARSI score in celecoxib group vs. controls,



**PRECLINICAL STUDIES USING ORAL ADMINISTRATION**

Authors	Species	COX-2 Inhibitor	OA Model	Dosing Regime	Start of Treatment	Timepoint of Evaluation	Evaluation of cartilage Degradation	Main Findings
Wen <i>et al.</i> <sup>24</sup>	Rat	Etoricoxib	ACL	6.7 mg/kg or 33.3 mg/kg three times per week	8 weeks after surgery	21 weeks after surgery	Histology: Pritzker score for cartilage	Improved histological score in the etoricoxib group versus control

**PRECLINICAL STUDIES USING INTRA-PERITONEAL INJECTIONS**

Liu <i>et al.</i> <sup>25</sup>	Mouse	Etoricoxib	DMM	5 mg/kg, 10 mg/kg, 20 mg/kg three times per week	2 days after OA induction	30 days after surgery	Histology: OARSI score	No difference in histological score etoricoxib versus control.
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**ACL**T: Anterior Cruciate Ligament Transection, **CLX**: celecoxib, **DMM**: destabilization medial meniscus, **ETX**: etoricoxib, **MCLT**: Medial Collateral Ligament Transection, **MIAI**: Monosodium Iodoacetate, **MMx**: medial meniscectomy, **NP**: nanoparticle, **pMMx**: partial medial meniscectomy, **PG**: proteoglycan.

Next to surgically induced OA models, the monosodium iodoacetate (MIA) rat OA model<sup>16,17,23</sup>, the collagenase OA model<sup>13,19</sup> and the spontaneous OA mouse model (STR/Ort)<sup>21</sup> were also used to investigate chondroprotective actions of selective COX-2 inhibitors celecoxib<sup>13,16,17,19,21</sup> and meloxicam<sup>23</sup>. In these studies, chondroprotective actions of selective COX-2 inhibitors were observed after oral administration as evidenced by improved histological scores compared to the controls.

While the aforementioned studies investigated chondroprotective actions of drugs administered orally, one study investigated chondroprotective actions of etoricoxib when administered intraperitoneally<sup>25</sup>. In this study, treatment with intraperitoneal etoricoxib injections was started two days after surgically inducing OA using the destabilization of the medial meniscus (DMM) mouse model<sup>25</sup> and histological analysis did not demonstrate a reduction in OA-related cartilage damage.

In conclusion, there is conflicting evidence regarding the chondroprotective actions of systemically administered selective COX-2 inhibitors since five studies did not show

chondroprotective actions, while nine studies showed chondroprotective actions after systemic administration with selective COX-2 inhibitors.

### 3.2 Preclinical Studies: Intra-Articular Administration

Next to *in vivo* studies investigating chondroprotective actions of selective COX-2 inhibitors administered orally, seven studies focused on their chondroprotective actions when used intra-articularly (Table 2). These studies evaluated several selective COX-2 inhibitors such as celecoxib<sup>26,27,28,29</sup>, parecoxib<sup>30</sup>, meloxicam<sup>31</sup> and etoricoxib<sup>32</sup>. All studies used surgically induced OA models. Chondroprotective actions, as evidenced by improved histological scores, were shown in three studies after intra-articular administration with selective COX-2 inhibitors<sup>26,30,31</sup>. Four studies investigated chondroprotective actions of selective COX-2 inhibitors when incorporated in a drug delivery system (DDS). Dong *et al.* used celecoxib-loaded liposomes or liposomes loaded with a combination of celecoxib and hyaluronic acid to study OA-related cartilage damage in a surgically induced rabbit OA model<sup>27</sup>. No difference in histological scores was reported when rats received celecoxib-loaded liposomes, while liposomes loaded with both celecoxib and hyaluronic acid exerted a chondroprotective effect compared to control injections<sup>27</sup>.

**Table 2: The schematic of studies investigating chondroprotective effects of intra-articularly administered selective COX-2 inhibitors.**

PRECLINICAL STUDIES USING INTRA-ARTICULAR INJECTIONS								
Authors	Species	COX-2 Inhibitor	OA Model	Dosing Regime	Start of Treatment	Timepoint of Evaluation	Evaluation of Cartilage Damage	Main Findings
Jean <i>et al.</i> <sup>30</sup>	Rat	Parecoxib	ACLT	Weekly 100 µg parecoxib for 5 consecutive weeks	Eight weeks after surgery	20 weeks after surgery	Histology: Mankin score	Improved histological scores in the parecoxib group compared to controls
Jiang <i>et al.</i> <sup>26</sup>	Rabbit	Celecoxib	ACLT+ PCLT + MMx	Weekly 1.2 mg celecoxib for 5 consecutive weeks	Directly after surgery	12 weeks after surgery	Histology: Mankin score	Improved histological scores in the Celecoxib group compared to controls

PRECLINICAL STUDIES USING INTRA-ARTICULAR INJECTIONS

Authors	Species	COX-2 Inhibitor	OA Model	Dosing Regime	Start of Treatment	Timepoint of Evaluation	Evaluation of Cartilage Damage	Main Findings
Dong <i>et al.</i> <sup>27</sup>	Rat	Celecoxib	ACL+ PCLT+ MCLT + MMx	Single injection: 0.15 mg celecoxib incorporated in DDS	One week after surgery	2 weeks after surgery	Histology: Colombo score	Improved histological score only when celecoxib is combined with HA in a DDS compared to controls
Wen <i>et al.</i> <sup>31</sup>	Rat	Meloxicam	ACL	Weekly 0.25 or 1 mg meloxicam for 5 consecutive weeks	Five weeks after surgery	20 weeks after surgery	Histology: Pritzker score	Lower Pritzker score in the meloxicam group versus control
Janssen <i>et al.</i> <sup>28</sup>	Rat	Celecoxib	ACL + pMMx	Single injection: 0.015 mg celecoxib incorporated in DDS	Four weeks after surgery	16 weeks after surgery	Histology: OARSI score	No difference in histological score in celecoxib loaded microspheres vs. control
Tellegen <i>et al.</i> <sup>29</sup>	Rat	Celecoxib	ACL + pMMx	Single injection: 0.015, 0.115 or 0.195 mg celecoxib incorporated in DDS	Four weeks after surgery	16 weeks after surgery	Histology: OARSI score	No difference in histological score in celecoxib loaded microspheres vs. control
Liu <i>et al.</i> <sup>32</sup>	Rat	Etoricoxib	ACL	Three injections: - 10 µM etoricoxib in 100 µL NaCl- 6.93 µg ETX-NPs, drug loading unclear	Three, six and nine weeks after surgery	12 weeks after surgery	Histology: OARSI score Immunohistochemistry	Improved histological score in ETX-NP, but not ETX, compared to control.

**ACL**: Anterior Cruciate Ligament Transection, **CLX**: celecoxib, **DDS**: drug delivery system, **ETX**: etoricoxib, **HA**: hyaluronic acid, **MCLT**: Medial Collateral Ligament Transection, **MMS**: modified mankin score, **MMx**: medial meniscectomy, **NP**: nanoparticle, **pMMx**: partial medial meniscectomy.

In an earlier study<sup>28</sup>, our group has investigated chondroprotective effects of celecoxib-loaded polyesteramide (PEA) microspheres in a surgically induced rat OA model. While celecoxib-loaded microspheres reduced PGE<sub>2</sub> as measured in homogenates from knees in this experiment, we could not find chondroprotective effects of celecoxib-loaded



PEA microspheres as measured by the OARSI score<sup>28</sup>. In another study, Tellegen *et al.*<sup>29</sup> evaluated effects of the aforementioned celecoxib-loaded PEA microspheres on cartilage degeneration in a surgically induced rat OA model. Consistent with our study, no chondroprotective action of celecoxib-loaded PEA microspheres was observed. In contrast to the findings of the aforementioned two studies, one study investigating controlled release of etoricoxib demonstrated chondroprotective actions as evidenced by improved histological scores compared to controls<sup>32</sup>.

In conclusion, when used intra-articularly, three studies report chondroprotective actions of selective COX-2 inhibitors. On the other hand, there is conflicting evidence regarding chondroprotective actions of selective COX-2 inhibitors incorporated in intra-articular drug delivery systems: two studies failed to demonstrate chondroprotective actions, one study shows chondroprotective actions only when celecoxib is combined with HA and one study demonstrated chondroprotective activity.

### **3.3 Clinical Studies**

Four clinical studies investigated chondroprotective actions of selective COX-2 inhibitors via oral administration, while there were no studies investigating chondroprotective actions of selective COX-2 inhibitors after intra-articular administration (Table 3).

**Table 3: The schematic overview of clinical studies investigating chondroprotective effects of systemically administered selective COX-2 inhibitors.**

CLINICAL STUDIES USING ORAL ADMINISTRATION							
Authors	Species	COX-2 Inhibitor	OA Grade	Dosing Regime	Treatment Duration	Evaluation of Cartilage Damage	Chondroprotection
Tindall <i>et al.</i> <sup>33</sup>	Human	Celecoxib	K&L 2 and 3	Daily 200 or 400 mg	12 months	Radiographs of the knee	No difference in JSW, subchondral sclerosis, cysts, malalignment or tilting after 12 months of treatment with celecoxib
Sawitzke <i>et al.</i> <sup>34</sup>	Human	Celecoxib	K&L 2 and 3	Daily 200 mg	24 months	Radiographs of the knee	No significant differences in JSW loss in celecoxib vs. controls
De Boer <i>et al.</i> <sup>35</sup>	Human	Celecoxib	Not specified	Daily 200 mg	4 weeks	Biochemical cartilage analysis:PG synthesis rate, PG release and PG content	Increased PG synthesis rate, decreased PG release and increased PG content in the celecoxib group vs. controls. Decreased release of il-1, TNF- $\alpha$ and mmp-activity in celecoxib group vs. control
Raynauld <i>et al.</i> <sup>36</sup>	Human	Celecoxib	K&L 2 and 3	Daily 200 mg	12 months	Quantitative MRI	No difference in cartilage volume loss in celecoxib group vs. a historical cohort control group

**JSW:** Joint space width, **K&L:** Kellgren and Lawrence, **PG:** proteoglycan.

Tindall *et al.*<sup>33</sup> included patients diagnosed with knee OA in a prospective open-label trial. Patients with OA received celecoxib orally at two dosages throughout a 12-month study period. Long-term celecoxib treatment did not result in significant changes of joint space width (JSW) at 1 year after treatment as assessed radiographically. Moreover, when radiographic knee OA progression during the study was compared to radiographic knee OA progression prior to the start of the study, no significant differences were detected<sup>33</sup>.

Sawitzke *et al.*<sup>34</sup> conducted a placebo-controlled study of patients with knee OA in which patients received placebo or celecoxib orally on a daily basis. No significant differences were found in JSW loss compared to placebo at 24 months, while Kellgren and Lawrence grade 2 knees showed a trend towards less JSW loss. In contrast to the findings of the

aforementioned clinical studies, a study performed by de Boer *et al.* suggested that celecoxib might exert chondroprotective actions<sup>35</sup>. In this study, patients with end-stage knee OA were treated with orally supplemented celecoxib 28 days prior to total knee replacement surgery. Compared to patients that did not receive celecoxib, cartilage samples of patients that received celecoxib showed increased proteoglycan (PG) synthesis, decreased PG release and an increased PG content.

Raynould *et al.* conducted a study in which patients with knee OA were enrolled receiving celecoxib orally throughout a 12-month study period<sup>36</sup>. After correcting for potential confounders, celecoxib treatment did not show any protective effect on cartilage volume loss compared to a historical knee OA cohort loss, as assessed by quantitative MRI.

In conclusion, evidence of chondroprotective actions of selective COX-2 inhibitors in human patients is low, since three human *in vivo* studies failed to show chondroprotective effects of orally administered celecoxib, while only one study suggests that celecoxib may act chondroprotective in humans.

The selectivity of the COX-2 inhibitors that were used in the preclinical and clinical studies described in this review has been compared by Riendeau *et al.*<sup>37</sup>. The COX-2 selectivity may play a role in the chondroprotective outcome of a selective COX-2 inhibitor, but the number of studies described in this systematic review was too small to draw any conclusions. An overview of the half maximal inhibitory concentration ( $IC_{50}$ ) values for COX-1 and COX-2 of these selective COX-2 inhibitors together with the COX-1/COX-2  $IC_{50}$  ratio is provided in Table 4.



**Table 4: The schematic overview of the half maximal inhibitory concentration ( $IC_{50}$ ) values and the COX-1/COX-2  $IC_{50}$  ratio of various selective COX-2 inhibitors. Adapted from<sup>37</sup>.**

DRUG	$IC_{50}$ COX-1 ( $\mu$ M)	$IC_{50}$ COX-2 ( $\mu$ M)	COX-1/COX-2 $IC_{50}$ RATIO
Meloxicam	1.4	0.7	2
Celecoxib	6.7	0.87	7.7
Etoricoxib	116	1.1	105
Parecoxib	26.1	0.87	30

## 4. Discussion

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The main finding of this systematic review is that the administration route plays a major role in determining chondroprotective actions of selective COX-2 inhibitors. The intra-articular administration route may be promising, since studies using bolus intra-articular administration of selective COX-2 inhibitors show chondroprotective effects. On the other hand, conflicting evidence exists when selective COX-2 inhibitors are incorporated into a drug delivery system. While preclinical studies point out to a potential chondroprotective role of COX-2 inhibitors, clinical studies did not investigate the intra-articular administration route and failed to confirm chondroprotective actions of systemically administered selective COX-2 inhibitors.

Discrepancies in chondroprotective actions of selective COX-2 inhibitors observed in the preclinical studies may be related to the route of administration. While six of the fourteen studies using systemic administration failed to demonstrate chondroprotective actions of selective COX-2 inhibitors, all studies that applied intra-articular bolus injections demonstrated chondroprotective actions. Interestingly, all clinical studies included in this systematic review evaluated chondroprotective actions using the systemic administration route. Since these studies failed to demonstrate chondroprotective actions, it will be of interest to investigate the chondroprotective actions of selective COX-2 inhibitors using the intra-articular administration route for clinical studies. We did not encounter studies comparing chondroprotective effects of systemic versus intra-articularly administration of selective COX-2 inhibitors, but we believe this will be of interest to investigate in the future.

Improved chondroprotection of intra-articular injections with selective COX-2 inhibitor compared to a saline control condition may be related to increased bioavailability of the drug in the knee joint compared to systemic administrations. In addition, in a total joint disease such as knee OA<sup>38,39,40,41</sup>, intra-articular administration of drugs may be more effective compared to systemic treatment due to the presence of scarcely vascularized tissues such as cartilage and meniscus<sup>8</sup>. Since COX-2 inhibitors can have an effect on all joint tissues, it is unclear whether the chondroprotective effect is a direct result of COX-2

inhibition in chondrocytes or an effect of COX-2 inhibition of other joint tissues.

Others and we failed to show a reduction of OA-related cartilage damage by celecoxib, when incorporated in an intra-articular drug delivery system<sup>28,29</sup>. It is a possibility that prolonged release of celecoxib may counteract potential chondroprotective effects due to increased loading of the affected joint indirectly caused by the analgesic effects of COX-2 inhibitors. These findings corroborate earlier observations in clinical studies, in which patients treated with anti-NGF demonstrated an increase in OA-related cartilage damage possibly due to joint overloading<sup>5</sup>.

COX-2 inhibitors are expected to have anti-inflammatory effects by inhibiting the synthesis of prostanoids. Prostanoid subtypes are considered as inflammatory mediators<sup>2</sup> and are involved in cartilage degradation<sup>3</sup>, but also in other pathophysiologic OA processes in different joint tissues such as synovial fibrosis and chondrocyte hypertrophy<sup>42,43</sup>. However, also anti-inflammatory actions of certain prostanoid subtypes have been shown<sup>2</sup>, and therefore identifying downstream targets of the COX-2 pathway may further aid in anti-inflammatory treatment for knee OA. Inflammatory processes have been suggested to precede knee OA<sup>4,44</sup> and to be involved in structural disease progression<sup>4</sup>. A window of opportunity may exist, in which modulating the inflammatory status of the knee joint via intra-articular treatment with selective COX-2 inhibitors may lead to OA disease modification. The initiation of treatment may thus be important for the treatment outcome. The studies performed by Wen *et al.*<sup>24</sup> and Dai *et al.*<sup>20</sup> did not start treatment shortly after surgery and demonstrated chondroprotective actions of orally administered COX-2 inhibitors. It can be hypothesized that inhibiting inflammation directly after inducing a joint trauma may compromise cartilage regeneration, since inflammation is part of the early phases of natural tissue regeneration after a trauma<sup>45</sup>. This may explain why studies in surgical OA models fail to show chondroprotective effects when starting treatment directly after surgery. Moreover, the lack of a chondroprotective effect in clinical studies may be related to the stage of disease. Patients with knee OA are diagnosed in a stadium when the disease has progressed towards its end-stage<sup>46</sup>, and it can be hypothesized that at this stage the disease is in an irreversible stadium where drug-based disease modification is not effective anymore. In addition, knee OA is a heterogeneous disease showing variability in



the rate of disease progression in human subjects<sup>47</sup>, while also the existence of distinct OA subtypes has been suggested<sup>18,48</sup>, suggesting that patient-tailored drug treatment needs to be developed. Finally, the outcome measurements of clinical studies, such as joint space narrowing on conventional radiography, may not be sensitive enough. With the ongoing advancements in cartilage imaging<sup>49</sup>, advanced techniques such as 7-tesla MRI imaging<sup>49</sup> may provide a more sensitive means to evaluate multiple outcome domains relevant to the clinical and pathophysiological aspects of disease modifying osteoarthritis drug (DMOAD)-mediated disease modification.

## 5. Conclusions

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To date there is conflicting evidence regarding the ability of selective COX-2 inhibitors to be used as DMOADs. Preclinical studies have used different routes of administration, which may alter the chondroprotective outcome of selective COX-2 inhibitors in the knee joint where scarcely vascularized tissues are present. Other factors such as the OA model type, type of selective COX-2 inhibitor and disease stage seem also to be involved in the chondroprotective outcome of selective COX-2 inhibitors. Despite the limited evidence of data in clinical studies, there seems to be a preclinical basis for considering selective COX-2 inhibitors as DMOADs specifically when used intra-articularly.

## 6. Patents

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TJM Welting is listed as inventor on patents: WO2017178251, WO2017178253 and US 20130123314. PJ Emans and LW van Rhijn are listed as inventors on patent US 20130123314. LW van Rhijn, PJ Emans and TJM Welting have shares in Chondropeptix and are CDO, CMO and CSO of Chondropeptix, respectively.

## 7. Supplementary Materials

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Supplementary materials can be found at <https://www.mdpi.com/1422-0067/21/18/6962/s1>.

## References

- 1 Hunter D.J., Felson D.T. Osteoarthritis. *BMJ*. 2006;332:639–642. doi: 10.1136/bmj.332.7542.639.
- 2 Ricciotti E., FitzGerald G.A. Prostaglandins and inflammation. *Arterioscler Thromb Vasc. Biol.* 2011;31:986–1000. doi: 10.1161/ATVBAHA.110.207449.
- 3 Martel-Pelletier J., Pelletier J.P., Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin. Arthritis Rheum.* 2003;33:155–167. doi: 10.1016/S0049-0172(03)00134-3.
- 4 Ayril X., Pickering E.H., Woodworth T.G., Mackillop N., Dougados M. Synovitis: A potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis—Results of a 1 year longitudinal arthroscopic study in 422 patients. *Osteoarthritis Cartilage.* 2005;13:361–367. doi: 10.1016/j.joca.2005.01.005.
- 5 Miller R.E., Block J.A., Malfait A.M. Nerve growth factor blockade for the management of osteoarthritis pain: What can we learn from clinical trials and preclinical models? *Curr. Opin. Rheumatol.* 2017;29:110–118. doi: 10.1097/BOR.0000000000000354.
- 6 Ding C. Do NSAIDs affect the progression of osteoarthritis? *Inflammation.* 2002;26:139–142. doi: 10.1023/A:1015504632021.
- 7 Zweers M.C., De Boer T.N., Van Roon J., Bijlsma J.W., Lafeber F.P., Mastbergen S.C. Celecoxib: Considerations regarding its potential disease-modifying properties in osteoarthritis. *Arthritis Res. Ther.* 2011;13:239. doi: 10.1186/ar3437.
- 8 Buckwalter J.A., Mankin H.J. Articular cartilage: Tissue design and chondrocyte-matrix interactions. *Instr. Course Lect.* 1998;47:477–486.
- 9 Makris E.A., Hadidi P., Athanasiou K.A. The knee meniscus: Structure-function, pathophysiology, current repair techniques, and prospects for regeneration. *Biomaterials.* 2011;32:7411–7431. doi: 10.1016/j.biomaterials.2011.06.037.
- 10 Hooijmans C.R., Rovers M.M., De Vries R.B., Leenaars M., Ritskes-Hoitinga M., Langendam M.W. SYRCLE's risk of bias tool for animal studies. *BMC Med. Res. Methodol.* 2014;14:43. doi: 10.1186/1471-2288-14-43.
- 11 Van der Kraan P.M., Buma P., Van Kuppevelt



- T., Van den Berg W.B. Interaction of chondrocytes, extracellular matrix and growth factors: Relevance for articular cartilage tissue engineering. *Osteoarthritis Cartilage*. 2002;10:631–637. doi: 10.1053/joca.2002.0806.
- 12 Mastbergen S.C., Marijnissen A.C., Vianen M.E., Zoer B., Van Roermund P.M., Bijlsma J.W., Lafeber F.P. Inhibition of COX-2 by celecoxib in the canine groove model of osteoarthritis. *Rheumatology*. 2006;45:405–413. doi: 10.1093/rheumatology/kei187.
- 13 Huh J.E., Baek Y.H., Kim Y.J., Lee J.D., Choi D.Y., Park D.S. Protective effects of butanol fraction from *Betula platyphyla* var. *japonica* on cartilage alterations in a rabbit collagenase-induced osteoarthritis. *J. Ethnopharmacol.* 2009;123:515–521. doi: 10.1016/j.jep.2008.08.028.
- 14 Fukai A., Kamekura S., Chikazu D., Nakagawa T., Hirata M., Saito T., Hosaka Y., Ikeda T., Nakamura K., Chung U., *et al.* Lack of a chondroprotective effect of cyclooxygenase 2 inhibition in a surgically induced model of osteoarthritis in mice. *Arthritis Rheum.* 2012;64:198–203. doi: 10.1002/art.33324.
- 15 Ou Y., Tan C., An H., Jiang D., Quan Z., Tang K., Luo X. Selective COX-2 inhibitor ameliorates osteoarthritis by repressing apoptosis of chondrocyte. *Med. Sci. Monit.* 2012;18:BR247–BR252. doi: 10.12659/MSM.882901.
- 16 Ashkavand Z., Malekinejad H., Amniattalab A., Rezaei-Golmishesh A., Vishwanath B.S. Silymarin potentiates the anti-inflammatory effects of Celecoxib on chemically induced osteoarthritis in rats. *Phytomedicine*. 2012;19:1200–1205. doi: 10.1016/j.phymed.2012.07.008.
- 17 Moon S.J., Park J.S., Jeong J.H., Yang E.J., Park M.K., Kim E.K., Park S.H., Kim H.Y., Cho M.L., Min J.K. Augmented chondroprotective effect of coadministration of celecoxib and rebamipide in the monosodium iodoacetate rat model of osteoarthritis. *Arch. Pharm. Res.* 2013;36:116–124. doi: 10.1007/s12272-013-0010-0.
- 18 Panahifar A., Jaremko J.L., Tessier A.G., Lambert R.G., Maksymowych W.P., Fallone B.G., Doschak M.R. Development and reliability of a multi-modality scoring system for evaluation of disease progression in pre-clinical models of osteoarthritis: Celecoxib may possess disease-modifying properties. *Osteoarthritis Cartilage*. 2014;22:1639–1650. doi: 10.1016/j.joca.2014.06.013.
- 19 Li Z., Meng D., Li G., Xu J., Tian K., Li Y. Celecoxib Combined with Diacerein Effectively Alleviates Osteoarthritis in Rats via Regulating JNK and p38MAPK Signaling Pathways. *Inflammation*. 2015;38:1563–1572. doi: 10.1007/s10753-

- 015-0131-3.
- 20 Dai M.W., Chu J.G., Tian F.M., Song H.P., Wang Y., Zhang Y.Z., Zhang L. Parathyroid hormone(1-34) exhibits more comprehensive effects than celecoxib in cartilage metabolism and maintaining subchondral bone micro-architecture in meniscectomized guinea pigs. *Osteoarthritis Cartilage*. 2016;24:1103–1112. doi: 10.1016/j.joca.2016.01.007.
- 21 Tu M., Yang M., Yu N., Zhen G., Wan M., Liu W., Ji B., Ma H., Guo Q., Tong P., *et al*. Inhibition of cyclooxygenase-2 activity in subchondral bone modifies a subtype of osteoarthritis. *Bone Res*. 2019;7:29. doi: 10.1038/s41413-019-0071-x.
- 22 Jones M.D., Tran C.W., Li G., Maksymowych W.P., Zernicke R.F., Doschak M.R. *In vivo* microfocal computed tomography and micro-magnetic resonance imaging evaluation of antiresorptive and antiinflammatory drugs as preventive treatments of osteoarthritis in the rat. *Arthritis Rheum*. 2010;62:2726–2735. doi: 10.1002/art.27595.
- 23 Nagy E., Vajda E., Vari C., Sipka S., Farr A.M., Horvath E. Meloxicam ameliorates the cartilage and subchondral bone deterioration in monoiodoacetate-induced rat osteoarthritis. *PeerJ*. 2017;5:e3185. doi: 10.7717/peerj.3185.
- 24 Wen Z.H., Lin Y.Y., Chang Y.C., Tang C.C., Hsieh S.P., Lee H.P., Sung C.S., Chen W.F., Lee C.H., Jean J.H. The COX-2 inhibitor etoricoxib reduces experimental osteoarthritis and nociception in rats: The roles of TGF-beta1 and NGF expressions in chondrocytes. *Eur. J. Pain*. 2020;24:209–222. doi: 10.1002/ejp.1478.
- 25 Liu B., Ji C., Shao Y., Liang T., He J., Jiang H., Chen G., Luo Z. Etoricoxib decreases subchondral bone mass and attenuates biomechanical properties at the early stage of osteoarthritis in a mouse model. *Biomed Pharmacother*. 2020;127:110144. doi: 10.1016/j.biopha.2020.110144.
- 26 Jiang D., Zou J., Huang L., Shi Q., Zhu X., Wang G., Yang H. Efficacy of intra-articular injection of celecoxib in a rabbit model of osteoarthritis. *Int. J. Mol. Sci*. 2010;11:4106. doi: 10.3390/ijms11104106.
- 27 Dong J., Jiang D., Wang Z., Wu G., Miao L., Huang L. Intra-articular delivery of liposomal celecoxib-hyaluronate combination for the treatment of osteoarthritis in rabbit model. *Int. J. Pharm*. 2013;441:285–290. doi: 10.1016/j.ijpharm.2012.11.031.
- 28 Janssen M., Timur U.T., Woike N., Welting T.J., Draaisma G., Gijbels M., van Rhijn L.W., Mihov G., Thies J.C., Emans P.J. Celecoxib-loaded PEA microspheres as an auto regulatory drug-delivery system after intra-articular injection. *J. Control Release*. 2016;244:30–40. doi: 10.1016/j.

- jconrel.2016.11.003.
- 29 Tellegen A.R., Rudnik-Jansen I., Pouran B., De Visser H.M., Weinans H.H., Thomas R.E., Kik M.J.L., Grinwis G.C.M., Thies J.C., Woike N., *et al.* Controlled release of celecoxib inhibits inflammation, bone cysts and osteophyte formation in a preclinical model of osteoarthritis. *Drug Deliv.* 2018;25:1438–1447. doi: 10.1080/10717544.2018.1482971.
- 30 Jean Y.H., Wen Z.H., Chang Y.C., Hsieh S.P., Tang C.C., Wang Y.H., Wong C.S. Intra-articular injection of the cyclooxygenase-2 inhibitor parecoxib attenuates osteoarthritis progression in anterior cruciate ligament-transected knee in rats: Role of excitatory amino acids. *Osteoarthritis Cartilage.* 2007;15:638–645. doi: 10.1016/j.joca.2006.11.008.
- 31 Wen Z.H., Tang C.C., Chang Y.C., Huang S.Y., Chen C.H., Wu S.C., Hsieh S.P., Hsieh C.S., Wang K.Y., Lin S.Y., *et al.* Intra-articular injection of the selective cyclooxygenase-2 inhibitor meloxicam (Mobic) reduces experimental osteoarthritis and nociception in rats. *Osteoarthritis Cartilage.* 2013;21:1976–1986. doi: 10.1016/j.joca.2013.09.005.
- 32 Liu P., Gu L., Ren L., Chen J., Li T., Wang X., Yang J., Chen C., Sun L. Intra-articular injection of etoricoxib-loaded PLGA-PEG-PLGA triblock copolymeric nanoparticles attenuates osteoarthritis progression. *Am. J. Transl. Res.* 2019;11:6775–6789.
- 33 Tindall E.A., Sharp J.T., Burr A., Katz T.K., Wallemark C.B., Verburg K., Lefkowitz J. A 12-month, multicenter, prospective, open-label trial of radiographic analysis of disease progression in osteoarthritis of the knee or hip in patients receiving celecoxib. *Clin Ther.* 2002;24:2051–2063. doi: 10.1016/S0149-2918(02)80096-0.
- 34 Sawitzke A.D., Shi H., Finco M.F., Dunlop D.D., 3rd Bingham C.O., Harris C.L., Singer N.G., Bradley J.D., Silver D., Jackson C.G., *et al.* The effect of glucosamine and/or chondroitin sulfate on the progression of knee osteoarthritis: A report from the glucosamine/chondroitin arthritis intervention trial. *Arthritis Rheum.* 2008;58:3183–3191. doi: 10.1002/art.23973.
- 35 De Boer T.N., Huisman A.M., Polak A.A., Niehoff A.G., Van Rinsum A.C., Saris D., Bijlsma J.W.J., Lafeber F.J.P.G., Mastbergen S.C. The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: Ex vivo evaluation of human cartilage tissue after *in vivo* treatment. *Osteoarthritis Cartilage.* 2009;17:482–488. doi: 10.1016/j.joca.2008.09.002
- 36 Raynauld J.P., Martel-Pelletier J., Beaulieu A., Bessette L., Morin F., Choquette D., Haraoui B., Abram F., Pelletier J.P. An open-label pilot study evaluating by magnetic resonance imaging the



- potential for a disease-modifying effect of celecoxib compared to a modeled historical control cohort in the treatment of knee osteoarthritis. *Semin. Arthritis Rheum.* 2010;40:185–192. doi: 10.1016/j.semarthrit.2009.10.003.
- 37 Riendeau D., Percival M.D., Brideau C., Charleson S., Dubé D., Ethier D., Falgoutret J.P., Friesen R.W., Gordon R., Greig G., *et al.* Etoricoxib (MK-0663): Preclinical Profile and Comparison with other agents that selectively inhibit Cyclooxygenase-2. *J. Pharmacol. Exp. Ther.* 2001;296:558–566.
- 38 Loeser R.F., Goldring S.R., Scanzello C.R., Goldring M.B. Osteoarthritis: A disease of the joint as an organ. *Arthritis Rheum.* 2012;64:1697–1707. doi: 10.1002/art.34453.
- 39 Clockaerts S., Bastiaansen-Jenniskens Y.M., Runhaar J., Van Osch G.J., Van Offel J.F., Verhaar J.A., de Clerck L.S., Somville J. The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: A narrative review. *Osteoarthritis Cartilage.* 2010;18:876–882. doi: 10.1016/j.joca.2010.03.014.
- 40 Weinberg J.B., Fermor B., Guilak F. Nitric oxide synthase and cyclooxygenase interactions in cartilage and meniscus: Relationships to joint physiology, arthritis, and tissue repair. *Subcell Biochem.* 2007;42:31–62.
- 41 De Lange-Brokaar B.J., Ioan-Facsinay A., Van Osch G.J., Zuurmond A.M., Schoones J., Toes R.E., Huizinga T.W.J., Kloppenburg M. Synovial inflammation, immune cells and their cytokines in osteoarthritis: A review. *Osteoarthritis Cartilage.* 2012;20:1484–1499. doi: 10.1016/j.joca.2012.08.027.
- 42 Bastiaansen-Jenniskens Y.M., Wei W., Feijt C., Waarsing J.H., Verhaar J.A., Zuurmond A.M., Hanemaaijer R., Stoop R., van Osch G.J.V.M. Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: A possible role for prostaglandin f2alpha. *Arthritis Rheum.* 2013;65:2070–2080. doi: 10.1002/art.37996.
- 43 Welting T.J., Caron M.M., Emans P.J., Janssen M.P., Sanen K., Coolen M.M., Voss L., Surtel D.A., Cremers A., Voncken J., *et al.* Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur. Cell Mater.* 2011;22:420–437. doi: 10.22203/eCM.v022a31.
- 44 Felson D.T., Niu J., Neogi T., Goggins J., Nevitt M.C., Roemer F., Torner J., Lewis C.E., Guermazi A. Synovitis and the risk of knee osteoarthritis: The MOST Study. *Osteoarthritis Cartilage.* 2016;24:458–464. doi: 10.1016/j.joca.2015.09.013.
- 45 Karin M., Clevers H. Reparative

inflammation takes charge of tissue  
regeneration. *Nature*. 2016;529:307–315.  
doi: 10.1038/nature17039.

- 46 Glyn-Jones S., Palmer A.J., Agricola R.,  
Price A.J., Vincent T.L., Weinans H., Carr A.J.  
Osteoarthritis. *Lancet*. 2015;386:376–387.  
doi: 10.1016/S0140-6736(14)60802-3.
- 47 Karsdal M.A., Christiansen C., Ladel  
C., Henriksen K., Kraus V.B., Bay-  
Jensen A.C. Osteoarthritis—a case for  
personalized health care? *Osteoarthritis  
Cartilage*. 2014;22:7–16. doi: 10.1016/j.  
joca.2013.10.018.
- 48 Waarsing J.H., Bierma-Zeinstra S.M.,  
Weinans H. Distinct subtypes of knee  
osteoarthritis: Data from the Osteoarthritis  
Initiative. *Rheumatology*. 2015;54:1650–  
1658. doi: 10.1093/rheumatology/kev100.
- 49 Oei E.H.G., Wick M.C., Muller-Lutz A.,  
Schleich C., Miese F.R. Cartilage Imaging:  
Techniques and Developments. *Semin.  
Musculoskelet. Radiol.* 2018;22:245–260.  
doi: 10.1055/s-0038-1639471.

# Chapter 06





## Celecoxib-Loaded PEA Microspheres as an Auto Regulatory Drug-Delivery System After Intra-Articular Injection

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# Abstract

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### **Objective:**

In this study, we investigated the potential of celecoxib-loaded polyester amide (PEA) microspheres as an auto-regulating drug delivery system for the treatment of pain associated with knee osteoarthritis (OA).

### **Material & Methods:**

Celecoxib release from PEA microspheres and inflammation responsive release of a small molecule from PEA was investigated *in vitro*. To investigate biocompatibility and degradation behavior *in vivo*, celecoxib-loaded PEA microspheres were injected in OA-induced (ACLT+pMMx) or contralateral healthy knee joints of male Lewis rats. Bioactivity of celecoxib from loaded PEA microspheres was confirmed by PGE<sub>2</sub> measurements in total rat knee homogenates.

### **Results:**

Inflammation responsive release of a small molecule from PEA was observed when PEA was exposed to cell lysates obtained from a neutrophil-like HI-60 cell line. Following a short initial burst release of ~15% of the total drug load in the first days, celecoxib was slowly released throughout a period of >80days. Intra-articular biocompatibility was demonstrated histologically, where no cartilage damage or synovial thickening and necrosis were observed after intra-articular injections with PEA microspheres. Degradation of PEA microspheres was significantly higher in OA induced knees compared to contralateral healthy knee joints, while loading the PEA microspheres with celecoxib significantly inhibited degradation, indicating a drug delivery system with auto regulatory behavior.

**Conclusion:**

In conclusion, this study suggests the potential of celecoxib-loaded PEA microspheres to be used as a safe drug delivery system with auto regulatory behavior for treatment of pain associated with OA of the knee.

**Keywords:**

Autoregulation; Drug-delivery; Microspheres; Osteoarthritis; Polyester amide; Slow release.

## 1. Introduction

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Osteoarthritis (OA) is the most common form of arthritis and constitutes a large medical healthcare economic burden worldwide, leading to pain and physical disability<sup>1</sup>. Systemic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) has shown to provide effective pain relief in patients with knee OA, but its systemic use is associated with gastrointestinal and cardiac adverse effects<sup>2,3</sup>. This limits its potential use in a chronic disease such as OA, where long term treatment is required. Therefore, an intra-articular drug delivery system (DDS) is necessary, which circumvents side effects associated with systemic treatment and allows prolonged local drug residence time<sup>4</sup>. Because OA is a chronic disease characterized by a variation in inflammation intensity<sup>4</sup> an attractive therapeutic approach would be intra-articular injection with a DDS which is inflammation-responsive. Loading an inflammation-responsive DDS with an anti-inflammatory drug may result in an auto regulatory DDS: the level of inflammation will impact degradation of the DDS and drug release, quenching inflammation, decreasing degradation and thus dosing the release.

Importantly, a DDS which can be used as an intra-articular treatment for OA should present a set of properties<sup>5</sup>. Firstly, the DDS should be responsive to the osteoarthritic disease process and able to slowly release a drug throughout time. Secondly, the DDS should be biocompatible and able to safely degrade in a knee joint. Finally, drug release from the DDS would be desired to slow the rate of its own release.



A candidate for such a DDS with auto regulatory behavior is a polyester amide (PEA) based injectable microsphere formulation. PEA polymers are based on  $\alpha$ -amino acids, aliphatic dicarboxylic acids, and aliphatic  $\alpha$ - $\omega$  diols<sup>6</sup>. The presence of amino acids in PEA makes it susceptible to enzymatic degradation by proteolytic enzymes. Several studies have reported this mechanism of degradation of PEA by enzymes such as  $\alpha$ -chymotrypsin, elastase, papain and protease K, which are enzymes belonging to the serine protease family<sup>7,8,9,10,11</sup>. Since serine proteases are present in synovial fluid and a key component of the inflammatory response, drug release from a PEA based DDS is potentially reactive to the disease process in inflammation related conditions such as OA<sup>12,13</sup>.

A candidate anti-inflammatory drug to incorporate into PEA microspheres is the COX-2 inhibitor celecoxib, which is an anti-inflammatory drug that has been shown to be an effective analgesic for OA related pain<sup>14</sup>. Celecoxib, when administered systemically, has been reported to raise the risk for cardiovascular events, however incorporating the drug in a PEA based DDS for intra-articular administration can circumvent these side effects<sup>15</sup>. PEA has already been demonstrated to have a good biocompatibility, is applied clinically in drug eluting stents and is being investigated for ophthalmologic indications<sup>16,17</sup>. However its use in the treatment of arthritic diseases has not yet been described.

In this study, we investigated the use of PEA microspheres as an auto-regulatory intra-articular DDS in OA treatment. First, we examined inflammation-responsive release of a small molecule from PEA *in vitro* in the presence or absence of a serine protease inhibitor. Next, biocompatibility, degradation and effects on OA progression of celecoxib-loaded PEA microspheres were investigated in experimental OA *in vivo*.

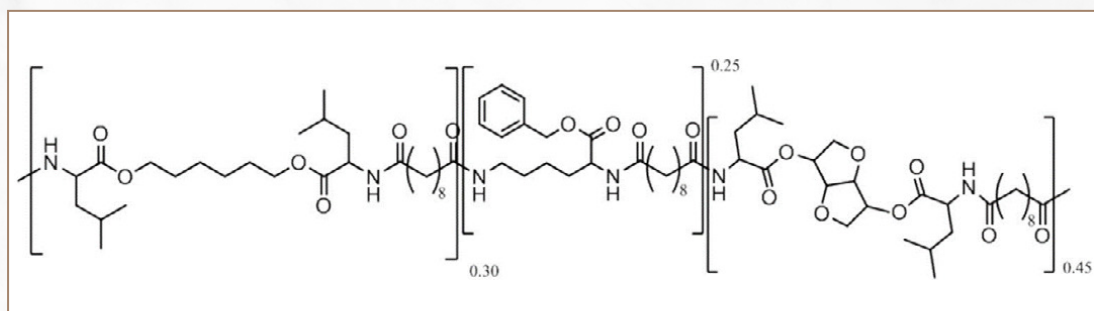
## 2. Material & Methods

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### 2.1 Synthesis of Polymer and Preparation of PEA Microspheres

PEA was synthesized in accordance to procedures reported previously<sup>16,18,19</sup>. The selected PEA is depicted on Figure 1 and it comprises three types of building blocks randomly

distributed along the polymer chain. Polymer characterization can be found in Table 1. For the preparation of microspheres, PEA was dissolved in dichloromethane (Merck Millipore). 5 wt% celecoxib was added to the solution and homogenized by sonication. The suspension was added to 20 mL of cold water containing 1 wt% of poly(vinyl alcohol) (Sigma Aldrich) under high shear, using an ultra-Turrax. After a stable suspension was obtained the particles were let to harden in 100 mL of water containing 1 wt% of poly(vinyl alcohol) for 12 h. Excess of water and surfactant was removed by rinsing and centrifugation. Finally, particles were frozen, dried under vacuum and stored at  $-15\text{ }^{\circ}\text{C}$  until being used.



**Figure 1: Structure of PEA III Ac Bz, random copolymer consisting of building blocks A, B and C.**

**Table 1. Polymer characterization. The relative ratio between the polymer building blocks was determined by  $^1\text{H}$  NMR.  $T_g$  of the polymer was determined under dry conditions.**

	MN (KDA)	POLYDISPERSITY INDEX (PDI)	GLASS TRANSITION TEMPERATURE ( $T_g$ )	RELATIVE RATIO A:B:C
PEA III Ac Bz	55	1.6	$57\text{ }^{\circ}\text{C}$	0.31:0.26:0.43

### 2.2 Determining Particle Size, Particle Morphology and Loading Efficiency of Celecoxib in PEA Microspheres

Size distribution of particles was measured using a Malvern Mastersizer 2000. Morphological examination of PEA microspheres was performed using SEM (Versa 3D FEG-ESEM). For determination of celecoxib loading efficiency, 10 mg of freeze dried microparticles were weighed and dissolved in methanol and shaken until a clear solution was obtained. Next, samples were subjected to analysis with High Performance Liquid Chromatography (HPLC) using a Waters e2695 Alliance HPLC with a UV detector. The method was obtained from the pharmacopeia collection.

### 2.3 Release kinetics of PEA based Celecoxib-Loaded Microspheres *In Vitro*

For the evaluation of the *in vitro* release kinetics of celecoxib from PEA microspheres, at least 10 mg of microspheres were placed in centrifuge tubes and immersed in phosphate buffered saline (PBS) at 37 °C under gentle shaking. After centrifugation, part of the buffer was removed and replaced with fresh buffer at defined time points such as 1 h, 4 h, 1 day, 2 days, 4 days, and 7 days until completion of the release study. The PBS solution with released API was transferred to HPLC vials and stored in the freezer until HPLC analysis.

### 2.4 HI-60 Cell Culture and Cell Lysate

The promyelocytic cell-line HI-60 (98070106) was purchased at the European Collection of Cell Cultures (ECACC). HI-60 cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Sigma Aldrich R8758). The culture medium was supplemented with 10% sterile filtered fetal bovine serum (Sigma Aldrich F2442) and 100 U/mL streptomycin and 100 U/mL penicillin. Cells were cultured at 37 °C and 5% CO<sub>2</sub> without any shaking and were passaged every 2–3 days to keep cell concentration between  $0.1 \times 10^6$  and  $1 \times 10^6$  cells/mL. During passaging, cells were centrifuged a  $300 \times g$  and seeded in fresh full medium at a concentration of  $0.1\text{--}0.2 \times 10^6$  cells/mL.



### 2.4.1 Viability and Cell Counting

Cells were counted using the Guava Viacount reagent (Merck Millipore # 4000-0040) on a Guava EasyCyte plus flow cytometer according to the manufacturer's instructions. Viability was also determined within the same method.

### 2.4.2 Differentiation

HI-60 cells were differentiated toward neutrophil like cells using the DMSO method. For this method cells were incubated in full medium supplemented with 1.25% sterile filtered DMSO for 5 days. Cells were seeded at a concentration of  $0.2 \times 10^6$  cells/mL at the start of the differentiation process and reached  $\pm 1 \times 10^6$  cells/mL after 5 days.

### 2.4.3 Lysates

Cells were lysed by 3–5 freeze-thaw cycles from liquid nitrogen to a 37 °C water bath. Cells were generally kept for 10 min in liquid nitrogen and 10 min in the water bath. Cell lysis was confirmed by microscopic analysis. The process was repeated till  $\pm 100\%$  lysed cells were obtained.

## 2.5 *In Vitro* Fluorescein Release from PEA Films

Degradation driven release of PEA *in vitro* was investigated by loading PEA III Ac Bz films with 9.1 wt% fluorescein. 101.3 mg fluorescein and 999.3 mg PEA III Ac Bz were dissolved in 19 mL ethanol. The solution was left overnight to dissolve under gentle agitation on an orbital shaker. 8 mL of the polymer fluorescein solution was pipetted in a Teflon mold, with a diameter of 5 cm and placed in a desiccator. Under a gentle nitrogen flow the solvent was allowed to evaporate in 18 h. The nitrogen flow dried films were removed and dried further under vacuum at 70 °C for 48 h. A sample of the film was analyzed for residual ethanol by  $^1\text{H}$  NMR analysis in  $\text{CDCl}_3$ , the characteristic methylene quartet at 3.7 ppm and methyl triplet at 1.2 ppm of ethanol were not observed. 6 mm round disks were punched out of the dried film and were used for the release experiment. Two release series were started both in triplicate for 60 days. At each time point the solutions were refreshed. Series

1 released in PBS buffer for the entire period (diffusion driven release). Series 2 started with release in PBS buffer for 26 days. After 26 days, a HI-60 neutrophil like cell lysate was added for a period of 7 days. Next, a serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (0.5 mM) was added to the lysate solution for a duration of 14 days and finally a HI-60 neutrophil like cell lysate without serine protease inhibitor was added during the final stages of the release experiment. Fluorescein release was quantified with a spectrophotometric assay.

### **2.6 Collection of Synovial Fluid and Synovium**

Synovial fluid and synovial tissues were obtained as anonymous left-over material from 5 patients with knee OA undergoing total knee replacement. This study was approved by the local ethical committee (MEC 08-4-028). Synovial fluid was directly centrifuged at 1200 rpm for 8 min and the supernatant was aliquoted and stored at  $-80^{\circ}\text{C}$ . Synovial tissue was washed thoroughly with 0.9% NaCl and cut into pieces of approximately 50 mg and cultured at a concentration of 100 mg/mL in Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 1% antibiotic/antimycotic. After 72 h, synovium conditioned medium was collected, centrifuged at 1200 rpm for 8 min and the supernatant was aliquoted and stored at  $-80^{\circ}\text{C}$ .

### **2.7 Protease Assay in Synovial Fluid and Synovium Conditioned Media**

A protease assay kit (EnzChek Protease Assay Kit for green fluorescence, Thermo Fisher) was used in accordance with the manufacturer's instructions to determine proteolytic activity in synovial fluid and synovium conditioned media.

First, 10  $\mu\text{L}$  of AEBSF (Sigma Aldrich) (10 mM) or 10  $\mu\text{L}$  of a Tris-HCl buffer (10 mM Tris-HCl, pH 7.8, 0.2 mM sodium azide) was added to 100  $\mu\text{L}$  of synovial fluid (ten times diluted in  $1 \times$  digestion buffer), synovium conditioned medium samples, positive control (1  $\mu\text{g}/\text{mL}$  chymotrypsin, dissolved in DMEM medium) or negative control (DMEM medium only) and pre-incubated for 1 h at room temperature.

Then, pre-incubated samples or controls were incubated at 37 °C with a fluorescently labeled casein solution, which was prepared in Tris-HCl buffer, in an end concentration of 5 µg casein/mL. After 24 h, fluorescence was determined in a Spectramax M2 microplate reader (Molecular Devices): excitation = 485 nm, emission = 530 nm.

## 2.8 *In Vivo* Studies

### 2.8.1 Induction of Osteoarthritis *In Vivo*

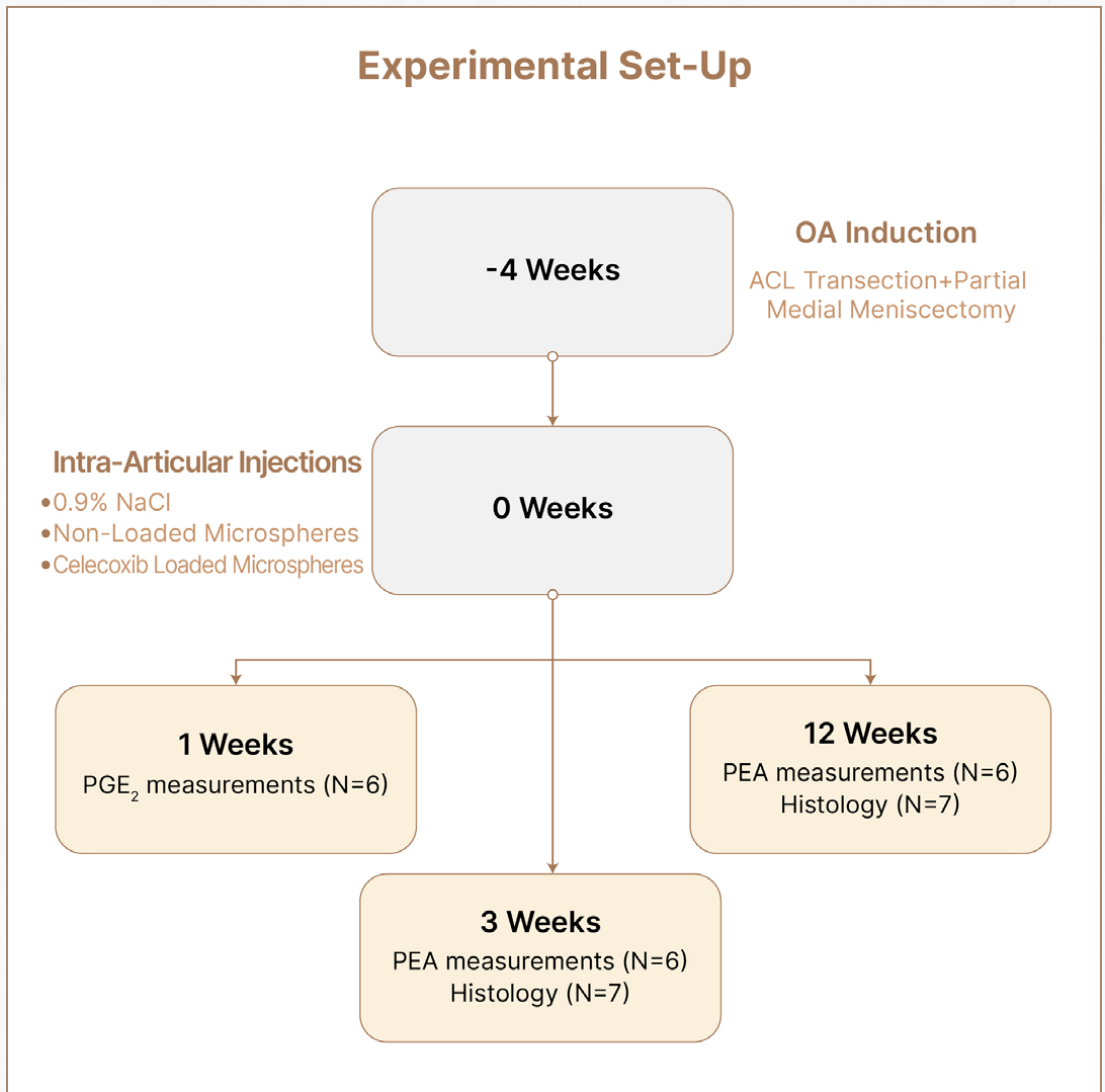
All animal experimental protocols were approved by the Maastricht University Animal Ethics Committee (DEC13-052). Eighty-six skeletally mature, 12 weeks old male Lewis rats (Charles River Laboratories) were allowed to acclimatize for 1 week before the initial start of the experiments. Animals were housed in groups of 2 and fed *ad libitum*. Osteoarthritis was surgically induced in the right knee of eighty-six rats according to a previously described method<sup>20</sup>. Rats were anesthetized in a chamber containing 3% isoflurane (Isoflo, Abbott Laboratories, USA). The knee joint was shaved, cleaned and disinfected with iodine (Eurovet Animal Health, the Netherlands). The skin was incised with a longitudinal incision over the knee joint. A medial parapatellar approach was used. In short, the joint capsule was incised on the medial side of the patella, which provided access to the joint space. The patella was dislocated laterally and the anterior cruciate ligament (ACL) was transected using a surgical blade (size 11). Transection of the ligament was confirmed by a manually performed anterior drawer test. In addition, the anterior part of the medial meniscus was removed using a surgical scissor. The joint capsule and skin were closed with Vycril 4-0 suture. No wound infection was noticed after ACLT and pMMx surgery. The wound healed within 1 week and no difference in use of the operated and non-operated leg was observed. Animals were allowed to move freely in their cage and were checked daily for general health and experiment-related discomfort throughout the experiment.

### 2.8.2 Intra-Articular Injections

Four weeks after surgery, rats were randomly assigned to 3 experimental groups which consisted of intra-articular injections of 25 µL in both the operated and non-operated



leg with 0.9% NaCl, non-loaded microspheres (15 mg particles/mL) or celecoxib-loaded microspheres (15 mg particles/mL, loaded with 3.9 wt.% celecoxib). One, three and twelve weeks after injection, rats were anesthetized with 1% isoflurane and sacrificed by cervical dislocation. Rats were divided in three analysis groups; 1. analysis of *in vivo* celecoxib release by measuring PGE<sub>2</sub> content in total rat knee homogenates (n = 7 per experimental group), 2. analysis of PEA degradation by measuring PEA content in knee joints (n = 6 per experimental group), 3. analysis of biocompatibility and OA severity by scoring histological sections of rat knee joints semi-quantitatively and using the OARSI histopathology initiative for the rat (n = 7 per experimental group) (Figure 2).



**Figure 2: Experimental set-up in vivo study.**

OA was induced in the right knee of 12 weeks old Lewis rats by ACLT + pMMx surgery. After 4 weeks, OA-induced and contralateral healthy knees were injected with 0.9% NaCl (control), non-loaded microspheres and celecoxib-loaded microspheres. One week after intra-articular injections rats were sacrificed for PGE<sub>2</sub> measurements. Directly, three and twelve weeks after intra-articular injections rats were sacrificed for PEA measurements and histology.

### 2.8.3 PGE<sub>2</sub> Measurement in Knee Joints

Rat knee joints were carefully resected and snap frozen in liquid nitrogen. Total rat knee joints were homogenized using a Mikro-Dismembrator, weighed and dissolved in 1 mL of 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 10  $\mu$ M indomethacin per 100 mg of tissue. Tissue homogenates were then spun down at 15,000 rpm for 10 min after which 50  $\mu$ L of the supernatant was used for PGE<sub>2</sub> measurements with an ELISA (Cayman Chemical) according to the manufacturer's instructions. PGE<sub>2</sub> content (in mg) was estimated from a calibration curve PGE<sub>2</sub>.

### 2.8.4 PEA Measurement in Knee Joints

To evaluate *in vivo* degradation of microspheres, rats were sacrificed directly, three or twelve weeks after intra-articular injections. Right and left knee joints were roughly cleared from muscle. Femur and tibia were cut on a 1.5 cm distance from the knee joint, while leaving the joint capsule intact. Knee joints were hydrolyzed by adding 8 mL of 6 N HCl and incubated at 90 °C for  $\pm$  40 h. Next, samples were subjected to analysis by liquid chromatography combined with mass spectrometry. PEA content (in mg) was estimated from a calibration curve with PEA (DSM Biomedicals).

### 2.8.5 Tissue Preparation and Histology

Rat knee joints were carefully resected and fixed with 3.7% paraformaldehyde in 0.1 M phosphate buffered saline at 4 °C for 1 week. Next, tissues were decalcified in 0.5 M EDTA solution (pH 7.8) for 8 weeks. After confirmation of decalcification on X-ray, knee joints were cut in two equal halves along the medial collateral ligament in the frontal plane in order to directly get access to the central weight bearing region of the joint. The posterior halves of the knees were dehydrated by transferring it through solutions of increasing ethanol concentration until 100% ethanol was reached. After a final 24 h dehydration step in cold 100% acetone at 4 °C, specimens were infiltrated with Technovit 8100 (Werheim, Germany) at 4 °C for 4 weeks. After this, specimens were placed into polyethylene-embedding molds. Polymerization solution, prepared according to the protocol of the manufacturer,



was poured into the molds and air-contact was prevented by covering the cavities with plastic films. The embedding form was placed on a thin layer of ice and polymerization was allowed for 24 h at 4 °C. After hardening was complete, specimens were blocked with Histobloc and Technovit 3040 (Werheim, Germany) and removed from the molds. Sections (5 µm) were cut from the blocks using a rotation microtome (Leica), stretched on distilled water and mounted on uncoated glass slides at 80 °C. Slides were subjected to Thionin staining for routine histological examination by light microscopy (Axioscope A1, Axiovision LE release 4.8.2, Carl Zeiss, Germany).

### **2.8.6 Biocompatibility Scoring**

Synovium and tissue around microspheres in histological sections were evaluated semi-quantitatively for the presence of giant cells, macrophages and fibroblast like cells at three and twelve weeks after intra-articular injections by an experienced animal pathologist microscopically (Nikon digital camera DMX1200 and ACT-1 v2.63 software, Nikon Instruments Europe, Amstelveen, The Netherlands).

### **2.8.7 OA Scoring**

Thionin stained sections were scored according to the OARSI histopathology initiative for the rat<sup>20</sup>. The OARSI score evaluates the medial tibial plateau of a knee joint, because this is the region with the most prominent OA features in the ACLT + pMMx model<sup>20</sup>. Measurements of parameters needed for the OARSI score were made using the Axiovision software.

### **2.8.8 Data Analyses and Statistics**

Protease activity in synovial fluid or synovium conditioned medium samples with or without a specific serine protease inhibitor AEBSF was compared using a paired one tailed *t*-test. PGE<sub>2</sub> content in total rat knee homogenates between knees injected with celecoxib-loaded or non-loaded microspheres was compared using an unpaired one tailed *t*-test. Semi-quantitative scores on histological sections intra-articular injected with non-loaded

or celecoxib-loaded microspheres were compared using a chi-squared test. PEA content in intra-articular injected OA and healthy knees at different time points were compared using a Kruskal-Wallis test, followed by a *post hoc* analysis. Differences between healthy and OA knees in knees injected with non-loaded or loaded microspheres were evaluated using a Wilcoxon's matched pairs signed rank test. Statistical differences in histology scores in OA-induced knees at 3 and 12 weeks after intra-articular injections were analyzed using a Kruskal-Wallis test.

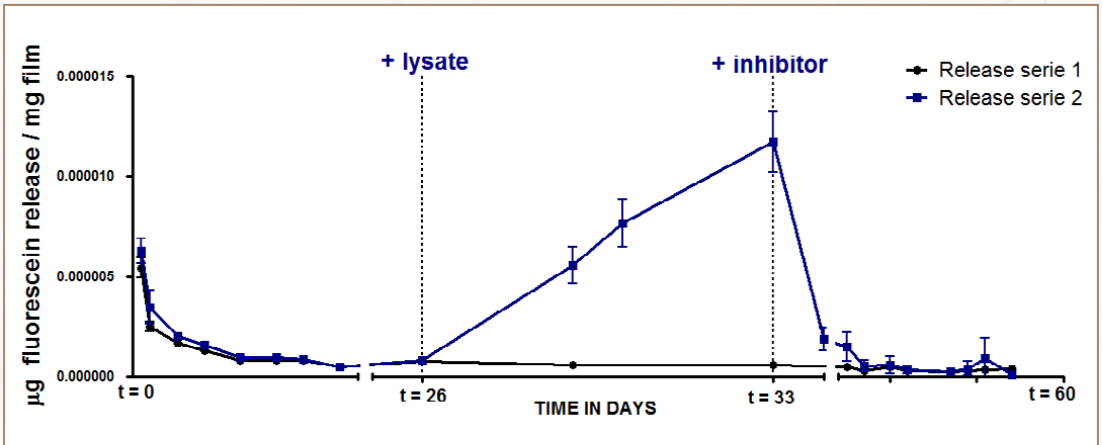
### 3. Results

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#### 3.1 Release of a Small Molecule from PEA is Inflammation Responsive *In Vitro*

An effective tool to study the potential inflammation-responsive drug release from PEA *in vitro*, is the HI-60 neutrophil like cell line, which is a commonly used model to study neutrophil functions and inflammatory cell responses<sup>21</sup>. The hypothesis for an inflammation responsive release rate of a small molecule from PEA was challenged by loading the polymer with fluorescein and evaluating the fluorescein release rate after exposure of the fluorescein-loaded PEA to HI-60 cell lysates *in vitro*.

Following an initial burst release, release of fluorescein in PBS buffer was low and followed zero order release kinetics (Figure 3). After zero order release kinetics was reached, a HI-60 cell lysate was added to the fluorescein loaded polymer. As shown in Figure 3, this resulted in increased release of fluorescein. Subsequent addition of a serine protease inhibitor, 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), to the cell lysate reduced the fluorescein release rate to release rates seen in PBS buffer. In conclusion, release of a small molecule from PEA was responsive to inflammation-related serine protease derived PEA degradation.



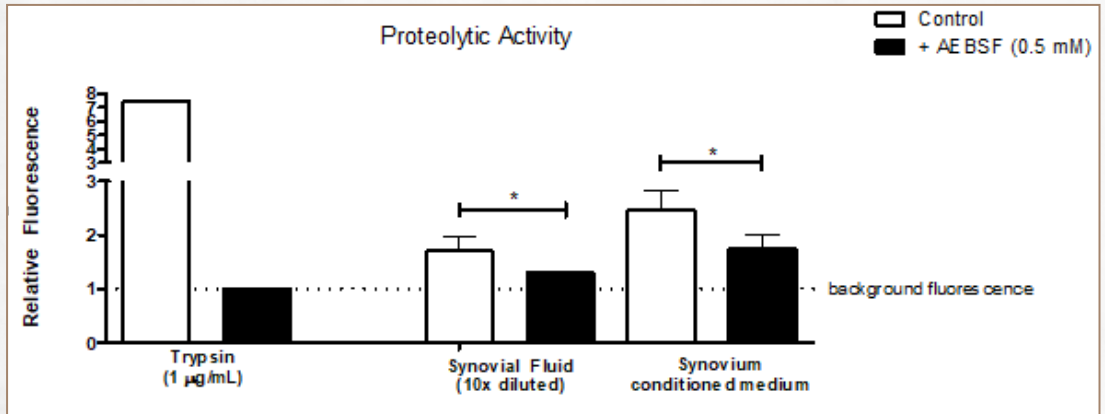
**Figure 3: Inflammation responsive release of a small molecule from PEA.**

PEA was loaded with fluorescein and incubated in PBS buffer for 60 days (release series 1). Alternatively, fluorescein loaded PEA was incubated in PBS buffer, a HI-60 neutrophil like cell lysate was added after 26 days and a HI-60 neutrophil like cell lysate + a serine protease inhibitor AEBSF (0.5 mM) after 33 days. Finally, lysate without inhibitor was added after 47 days (release series 2). Absorbance was measured spectrophotometrically at 490 nm. Data are mean  $\pm$  SD, N = 3 per time point.

### 3.2 Serine Proteases are Present Intra-Articular and Produced by Osteoarthritic Synovial Tissue *In Vitro*

After confirming inflammation-responsive release of a small molecule from PEA by serine proteases, we next evaluated serine protease activity in synovial fluid and synovium conditioned medium of OA patients. Synovial fluid or synovium conditioned media contained proteolytic activity and a specific serine protease inhibitor AEBSF was able to significantly reduce proteolytic activity in synovial fluid and synovium conditioned medium, indicating that at least a part of the proteolytic activity detected is serine protease driven (Figure 4).

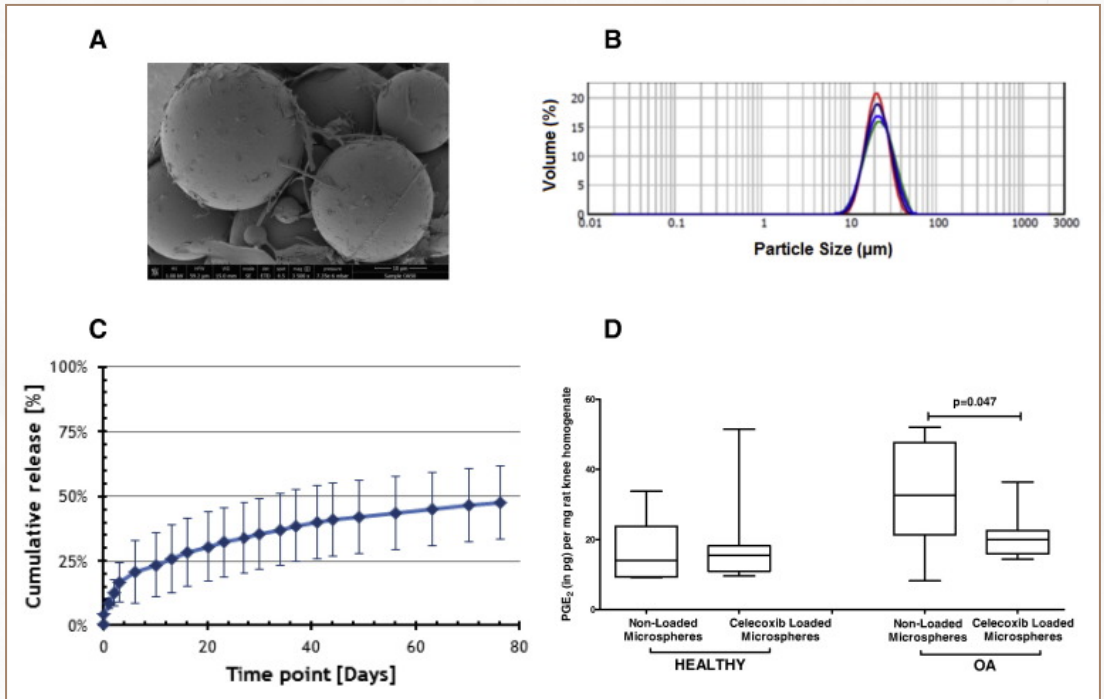




**Figure 4: Serine protease activity in synovial fluid and synovial tissue from OA patients.** Synovial fluid (ten times diluted) or synovium conditioned media were incubated with a fluorescein labeled casein and fluorescence was measured 24 h after incubation. Trypsin (1 µg/mL) was used as a positive control.

### 3.3 Celecoxib is Released from PEA Microspheres *In Vitro* and Bioactive *In Vivo*

Celecoxib-loaded PEA microspheres were prepared and SEM analysis showed spherical structures (Figure 5A). Particle size was found to be 10–100 µm (Figure 5B).



**Figure 5: Celecoxib from celecoxib-loaded PEA microspheres is released *in vitro* and bioactive *in vivo*.**

Scanning electron microscope image of PEA microspheres (A) and size distribution of PEA microspheres (B). The concentration of celecoxib release in PBS medium as determined by HPLC throughout time. The release is expressed in percentage of theoretical celecoxib load (C). PGE<sub>2</sub> content (in pg per mg knee homogenate) in total OA rat knee homogenates 1 week after injections with non-loaded or celecoxib-loaded microspheres ( $n = 7$  for non-loaded microspheres and  $n = 7$  for celecoxib-loaded microspheres) (D).

To investigate whether celecoxib-loaded PEA microspheres are able to slowly release celecoxib, *in vitro* release performance was determined throughout 80 days. Following a short initial burst release of ~ 15% of total drug load in the first days, celecoxib was slowly released throughout time (Figure 5C).

Celecoxib release and bioactivity *in vivo* were evaluated by measuring PGE<sub>2</sub> content in healthy or OA-induced total rat knee homogenates, injected with non-loaded or celecoxib-loaded PEA microspheres. One week after injections with celecoxib-loaded PEA microspheres, PGE<sub>2</sub> content was significantly lower ( $p = 0.047$ ) in OA knees injected

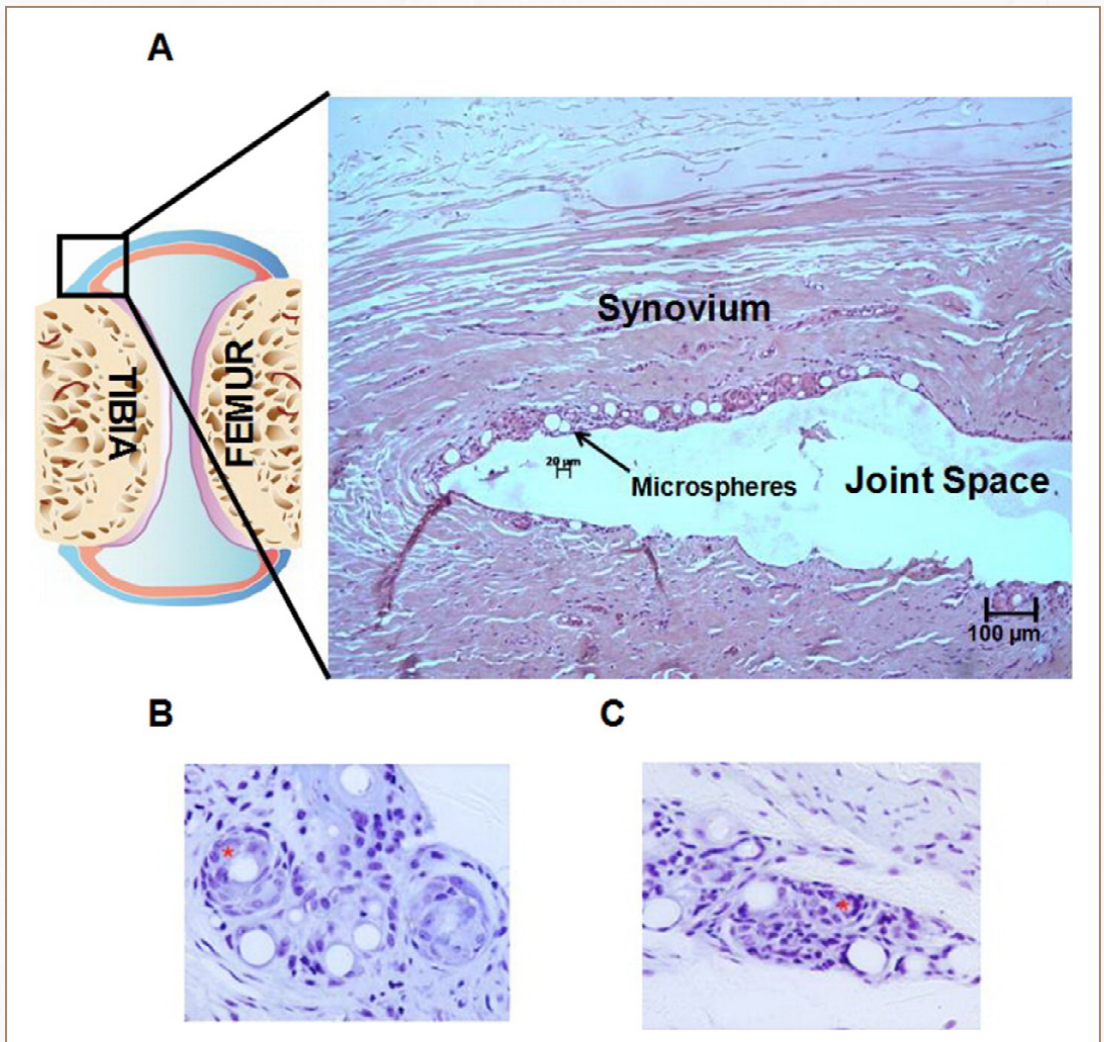
with celecoxib-loaded microspheres compared to OA knees injected with non-loaded microspheres, indicating that celecoxib is released from PEA microspheres and bioactive *in vivo* (Figure 5D).

### **3.4 Entrapment of Microspheres within the Synovial Membrane and Biocompatibility**

Intra-articular biocompatibility of PEA microspheres was investigated by scoring histological sections of rat knees injected with non-loaded or celecoxib-loaded microspheres semi-quantitatively.

Non-loaded and loaded microspheres were found in all OA and healthy knees 3 weeks after injections. The microspheres were found to be entrapped in the synovium and no necrosis or thickening of the synovium as a reaction to the injection of the microspheres was observed (Figure 6A). Microspheres were surrounded by mononuclear inflammatory cells and giant cells (Figure 6B and C). Small microspheres 5  $\mu\text{m}$  in size were seen, but also bigger microspheres 15–20  $\mu\text{m}$  in size. Twelve weeks after injection, microspheres were smaller in size and not found in all knees. Microspheres could be found in 2 OA-induced and 2 healthy knees injected with non-loaded microspheres and 3 OA-induced and 4 healthy knees injected with celecoxib-loaded microspheres. Semi-quantitative scoring of histological sections revealed a slight to moderate presence of giant cells, macrophages and fibroblast-like cells surrounding the microspheres in a greater part of the animals. No significant differences were observed in semi-quantitative scoring of histological sections between rats injected with loaded or non-loaded PEA microspheres (Table 2).





**Figure 6: Entrapment of microspheres in the synovium.**

Haematoxylin & eosin stained section of healthy knee 3 weeks after injection with non-loaded microspheres (A). Thionin stained sections of OA-induced knees 3 weeks after injection non-loaded microspheres (40 × magnification) (B) or celecoxib-loaded microspheres (40 × magnification) (C). Red asterisks in (B) and (C) depict a giant cell.

**Table 2. Semi-quantitative histological assessment of the amount of giant cells, macrophages or fibroblast like cells surrounding the microspheres. Numbers in table indicate amount of animals.**

THREE WEEKS AFTER INTRA- ARTICULAR INJECTIONS	Giant cells (number of animals)		Macrophages (number of animals)		Fibroblast like cells (number of animals)	
	Non-loaded microspheres	Loaded microspheres	Non-loaded microspheres	Loaded microspheres	Non-loaded microspheres	Loaded microspheres
<b>OA KNEES</b>						
Absent	0	0	0	2	1	1
Slightly present	3	3	2	1	4	4
Moderately present	3	2	3	3	1	1
Very abundantly present	0	1	1	0	0	0
<b>HEALTHY KNEES</b>						
Absent	0	0	0	1	4	6
Slightly present	1	2	2	0	1	0
Moderately present	4	3	2	4	0	0
Very abundantly present	0	1	1	1	0	0

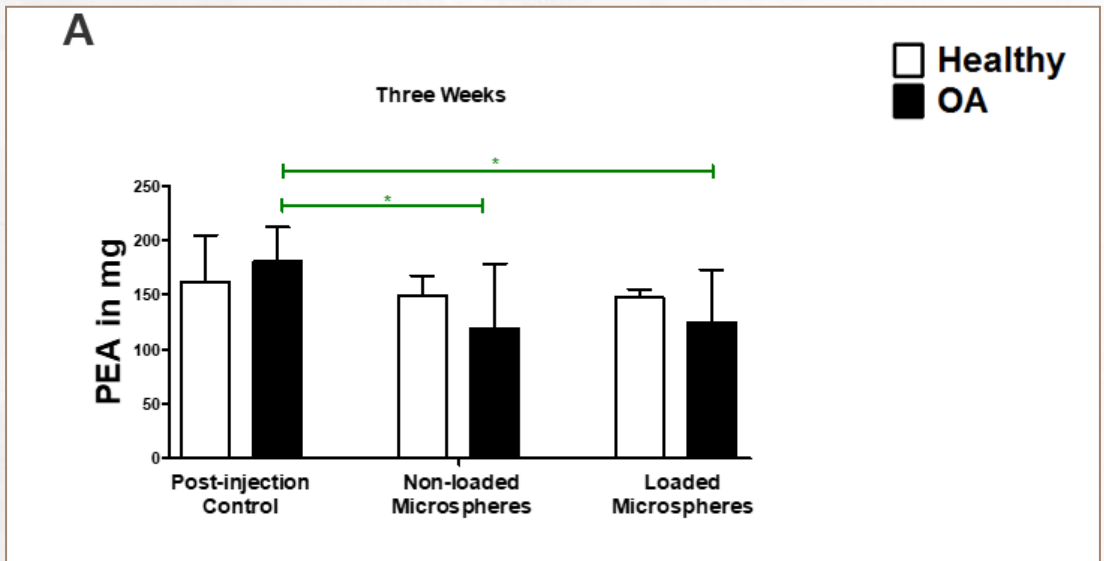
**Celecoxib-Loaded PEA Microspheres as an Auto Regulatory Drug-Delivery System after Intra-Articular Injection**

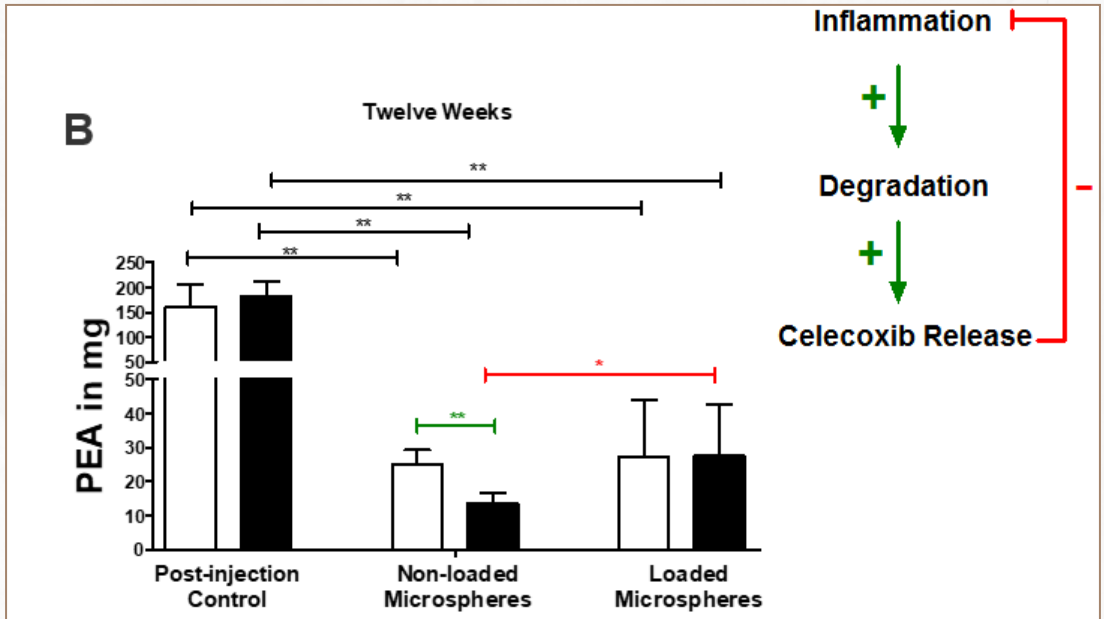
<b>TWELVE WEEKS AFTER INTRA- ARTICULAR INJECTIONS</b>	<b>Giant cells (number of animals)</b>		<b>Macrophages (number of animals)</b>		<b>Fibroblast like cells (number of animals)</b>	
	<b>Non-loaded microspheres</b>	<b>Loaded microspheres</b>	<b>Non-loaded microspheres</b>	<b>Loaded microspheres</b>	<b>Non-loaded microspheres</b>	<b>Loaded microspheres</b>
<b>OA KNEES</b>						
Absent	0	2	0	0	0	1
Slightly present	0	1	1	0	1	1
Moderately present	1	0	1	3	1	1
Very abundantly present	1	0	0	0	0	0
<b>HEALTHY KNEES</b>						
Absent	0	0	0	1	1	2
Slightly present	2	3	2	1	1	2
Moderately present	0	1	0	2	0	0
Very abundantly present	0	0	0	0	0	0



### 3.5 PEA Microspheres Degrade *In Vivo* and Show Auto-Regulatory Degrading Behavior

To evaluate *in vivo* degradation of the microspheres, the amount of PEA was measured directly, three or twelve weeks after intra-articular injections with PEA microspheres. PEA content was not significantly different between healthy or OA-induced knees directly after injections with microspheres. Three weeks after intra-articular injections, PEA content was significantly reduced in OA-induced knees injected with both non-loaded and loaded microspheres by 34% and 31% respectively while PEA content in healthy knees injected with non-loaded and loaded microspheres was reduced by 7% and 9% respectively, not reaching statistical significance (Figure 7A).





**Figure 7: *In vivo* degradation of PEA microspheres and auto regulatory behavior of celecoxib-loaded PEA microspheres.**

PEA levels in knees measured directly after injection (post-injection control), three weeks and twelve weeks after injection. Data are mean  $\pm$  SEM,  $N = 6$ . \*  $P < 0.05$ , \*\*  $P < 0.005$ . Because no degradation is to be expected directly post injection, we injected celecoxib-loaded microspheres as controls at this time point (post-injection control).

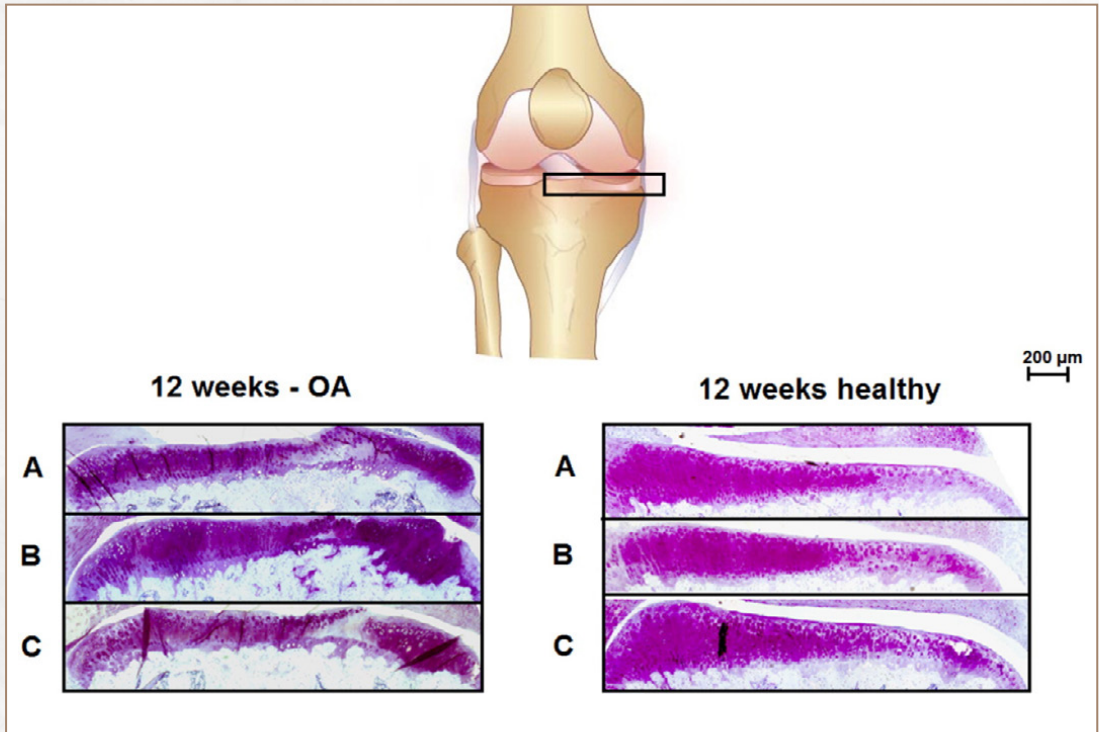
Twelve weeks after intra-articular injections, healthy and OA-induced knees injected with both non-loaded and loaded microspheres showed significantly reduced PEA-levels (> 80%), indicating that *in vivo* degradation of the microspheres occurred (Figure 7B).

Interestingly, PEA degradation was significantly higher in OA-induced knees injected with non-loaded microspheres, compared to healthy knees injected with non-loaded microspheres (Figure 7B). On the other hand, no significant differences were observed in PEA levels in OA-induced and healthy knees injected with celecoxib-loaded microspheres. Moreover, loading PEA microspheres with celecoxib significantly inhibited PEA degradation in OA-induced knees (Figure 7B).

**3.6 In Vivo Effects of Intra-Articular Injections with Celecoxib on Joint Pathology**

After confirming *in vivo* degradation of PEA microspheres, we next evaluated the effects of injections with celecoxib-loaded microspheres on joint pathology in OA-induced rat knees.

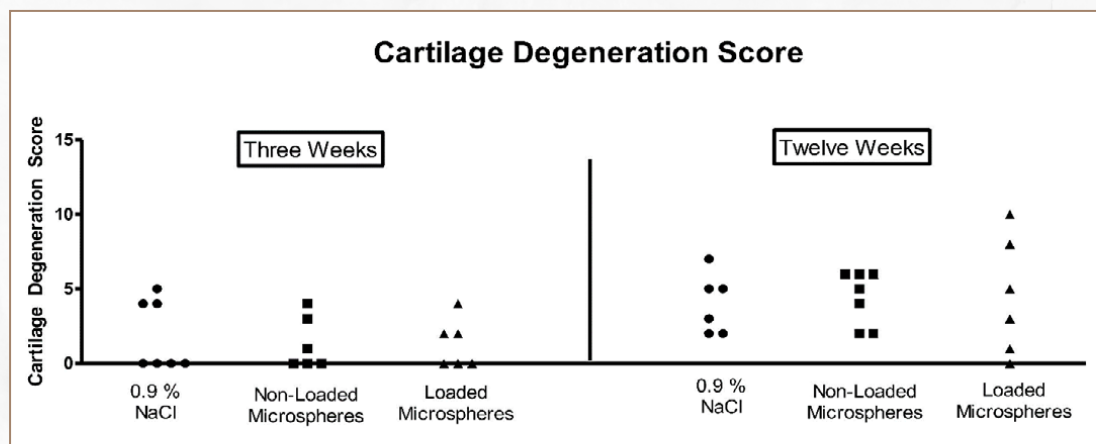
Whole-joint histology revealed healthy cartilage in healthy knees (Figure 8), while OA-induced knees displayed OA-like changes twelve weeks after intra-articular injections (sixteen weeks after OA-induction) (Figure 8). No significant difference was observed in the OARSI cartilage degeneration score (Figure 9) between rats injected with 0.9% NaCl, non-loaded microspheres and celecoxib-loaded microspheres.



**Figure 8: Twelve weeks after intra-articular injections with microspheres in OA-induced or healthy knees.**

*Thionin stained Histological sections of the medial tibial plateau of OA (left panel, 16 weeks after OA induction) or healthy knees (right panel) 12 weeks after injections with 0.9% NaCl (A), non-loaded microspheres (B) and celecoxib-loaded microspheres (C). OA severity was comparable between all groups and injections with microspheres did not result in cartilage damage.*





**Figure 9: Effects of intra-articular injections with celecoxib on cartilage pathology in OA-induced rat knees.**

Thionin stained histological sections of the medial tibial plateau of right rat knees were scored for cartilage degeneration score at 3 and 12 weeks after intra-articular injection with 0.9% NaCl, non-loaded microspheres or celecoxib-loaded microspheres. No significant differences were seen between groups. Each dot, square or triangle represents a separate animal. N = 7 for control, N = 6 for non-loaded microspheres, N = 6 for loaded microspheres at week 3 and N = 6 for control, N = 7 for non-loaded microspheres, N = 6 for loaded microspheres at week 12.

## 4. Discussion

In this study we have investigated the potential to use PEA as a biomaterial for an auto regulatory intra articular drug delivery system. First, inflammation-responsive release of a small molecule was confirmed *in vitro*. Next, PEA microspheres were loaded with an anti-inflammatory drug celecoxib and investigated *in vivo*.

Release of a small molecule fluorescein from PEA was increased after exposure to lysates from the neutrophilic cell line HI-60 and addition of a specific serine protease inhibitor reduced fluorescein release. This result indicates release of fluorescein from PEA after inflammation-related enzymatic degradation. Since serine proteases are key components of the inflammatory response<sup>12</sup>, these findings highlight the potential of PEA drug delivery systems to be used in drug delivery systems for the treatment of diseases with an

inflammatory component such as osteoarthritis, in which drug release will be reactive to the disease process.

After conforming the inflammation responsive properties of PEA, biocompatibility of PEA microspheres was investigated *in vivo*. Intra-articular injection of microspheres was well tolerated: no abnormal behavior was observed in rats and also no infections occurred. All rats gained in weight equally (data not shown) and no death was observed. Three weeks after intra-articular injections, microspheres were found back in the knees of all rats injected with microspheres. The microspheres were entrapped in the synovium surrounded by giant cells, mononuclear inflammatory cells and fibroblast like cells. Entrapment of microspheres in the synovium has been shown earlier in rat and rabbit knees<sup>22,23</sup>. The synovial membrane displays ability to remove foreign particles, which is the most likely explanation for this phenomenon we observed<sup>24</sup>. Macrophages have been shown to take up particles in the micrometer size range<sup>25</sup>: in our study we did not observe endocytosis of our microspheres. We did not see any necrosis or thickening of the synovium as a reaction to the injection of the microspheres, confirming biocompatibility of PEA microspheres. In short, we conclude a normal and mild foreign body response.

The fact that we found fewer microspheres twelve weeks after injections with PEA microspheres compared to three weeks after injections indicated *in vivo* degradation of the polymer, but we also evaluated degradation quantitatively by measuring PEA levels in total rat knee joints. PEA microspheres degraded *in vivo* and PEA levels were reduced to 20% twelve weeks after intra-articular injections. As OA is considered to have an inflammatory component, our hypothesis was that there would be more degradation of microspheres in an OA-induced knee. Consistent with this hypothesis, PEA degradation was significantly increased in an OA-induced knee compared to a healthy knee. Interestingly, loading the microspheres with celecoxib significantly reduced degradation as PEA levels were significantly higher in OA-induced knees injected with celecoxib-loaded microspheres compared to OA-induced knees injected with non-loaded microspheres. These *in vivo* data are in accordance with our *in vitro* data indicating that inflammatory cells are able to enzymatically degrade PEA microspheres, and that celecoxib is able to inhibit this process,

highlighting the potential of celecoxib-loaded microspheres to use as a controlled DDS with an auto regulatory behavior.

Different carriers such as microspheres, liposomes and hydrogels have been investigated in intra-articular treatment with use of synthetic and natural polymers<sup>26,27,28,29,30,31,32,33,34,35,36,37</sup>. While most studies report intra-articular biocompatibility of the DDS investigated, information about degradation behavior of the DDS investigated *in vivo* is lacking. Bedouet *et al.* investigated PEG-hydrogel based microspheres and observed slow degradation over 60 days *in vitro*, but did not investigate degradation behavior *in vivo*<sup>22</sup>. In another study performed by Sandker *et al.*, an acyl-capped PCLA-PEG-PCLA polymer based hydrogel depot was investigated<sup>38</sup>. Fast degradation of the hydrogel after intra-articular injection occurred and the depot could not be detected after 3 weeks. Arankumar *et al.* show intra-articular retention of PCL microparticles for 6 weeks, by using *in vivo* fluorescence imaging<sup>39</sup>. We here show retention of PEA microspheres for at least 12 weeks after intra-articular injection and in addition show an auto regulatory degradation behavior of PEA microspheres: an intra-articular DDS degradation mechanism not earlier described. This auto regulatory degradation behavior is beneficial from 2 points of view. Firstly, degradation and thus drug release will follow disease activity. Secondly, the microspheres potentially reside in the joint for a longer period of time and thus fewer injections will be needed.

Our PEA degradation data *in vivo* indicate slow and bioactive release of celecoxib over a period of 12 weeks. Bioactivity of celecoxib from PEA microspheres *in vivo* was also proven by performing PGE<sub>2</sub> measurements in total rat knee homogenates. Rat knees injected with celecoxib-loaded PEA microspheres contained significantly lower PGE<sub>2</sub> levels compared to rat knees injected with non-loaded PEA microspheres.

Based on *in vitro* release rates and average synovial fluid volume of a rat, *in vivo* celecoxib concentrations in the knee joint were calculated to be 10 μM, the concentration celecoxib in which efficient anti-inflammatory effects are shown in various intra-articular tissues<sup>40</sup>. However, extrapolation of release rates from *in vitro* to *in vivo* is complicated. In addition to diffusion derived release, degradation derived release is another important component influencing drug release *in vivo*.



Moreover drug release *in vivo* is influenced by the highly viscous synovial fluid<sup>41</sup>. Due to the small synovial fluid volumes in a rat knee, we were unable to investigate local celecoxib concentration in the synovial fluid. To determine celecoxib release *in vivo* and evaluate systemic celecoxib concentrations, we performed a celecoxib-specific ELISA on plasma samples of rats which received non-loaded and celecoxib-loaded PEA microspheres three, six, nine and twelve weeks after injections. The detection limit of this ELISA is ~ 0.25 ng/mL and 10 out of 42 tested plasma samples of rats injected with loaded microspheres were just above detection limit *versus* 3 out of 42 tested plasma samples of rats injected with non-loaded microspheres. While we can only speculate about absolute celecoxib concentrations in plasma, these data indicate low systemic concentrations of celecoxib *in vivo* (< 0.5 ng/mL), which will result in reduced risk of systemic side effects. Radioactively labeling drugs such as celecoxib and examining release behavior *in vivo* may provide valuable data to predict release behavior of drugs loaded in a DDS in future experiments.

Because several studies have shown chondroprotective effects of celecoxib *in vivo*, we also evaluated the effects of intra-articular injections with celecoxib-loaded microspheres on OA progression<sup>42,43,44</sup>. Intra-articular injections with celecoxib-loaded microspheres did not result in reduced OA pathology as evaluated by histological analysis in cartilage, consistent with two other studies showing chondroneutral effects of celecoxib *in vivo*<sup>32,45</sup>. Contradictory reports on OA modifying properties of celecoxib may be caused by differences in OA model used. Beneficial effects of celecoxib in OA models based on mechanical instability can be restrained by increased loading of the affected joint due to analgesic effects of celecoxib. Moreover, the amount of synovial inflammation is different in several OA models, and the effectiveness of an anti-inflammatory drug like celecoxib may be proportional to the amount of inflammation present. Although we investigated the amount of synovial inflammation in our OA model by scoring sub synovial proliferation and inflammatory cell infiltration on histological sections, synovitis at the time points we evaluated was too minimal even in untreated OA-induced knees, consistent with earlier reports<sup>46</sup>. Other models which display more inflammation<sup>47,48</sup>, may be useful to investigate the role of celecoxib on (synovial) inflammation.

## 5. Conclusion

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PEA microspheres displayed good intra-articular biocompatibility and degraded in an *in vivo* OA model. Degradation of PEA microspheres was significantly higher in an inflammatory environment, while loading the microspheres with celecoxib decreased PEA degradation suggesting a DDS with auto regulatory behavior. We propose the use of PEA microspheres loaded with an anti-inflammatory drug as an auto regulatory drug delivery system to evaluate analgesic and OA disease modifying effects of a broad range of drugs in knee OA.

## 6. Acknowledgments

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## References

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- 1 Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clin Geriatr Med*. 2010;26(3):355-69.
- 2 De Vecchis R, Baldi C, Di Biase G, Ariano C, Cioppa C, Giasi A, *et al*. Cardiovascular risk associated with celecoxib or etoricoxib: a meta-analysis of randomized controlled trials which adopted comparison with placebo or naproxen. *Minerva Cardioangiol*. 2014;62(6):437-48.
- 3 Mantry P, Shah A, Sundaram U. Celecoxib associated esophagitis: review of gastrointestinal side effects from cox-2 inhibitors. *J Clin Gastroenterol*. 2003;37(1):61-3.
- 4 Martel-Pelletier J. Pathophysiology of osteoarthritis. *Osteoarthritis Cartilage*. 2004;12 Suppl A:S31-3.
- 5 Janssen M, Mihov G, Welting T, Thies J, Emans P. Drugs and polymers for delivery systems in OA joints: clinical needs and opportunities. *Polymers*. 2014;6(3):799-819.
- 6 Castaldo L CP, Maglio G, Palumbo R. Synthesis and preliminary characterization of polyesteramides containing enzymatically degradable amide bonds. *Polym Bull*. 1992;28:301-7.
- 7 Han S-I, Kim B-S, Kang S-W, Shirai H, Im SS. Cellular interactions and degradation of aliphatic poly (ester amide) s derived from glycine and/or 4-amino butyric acid. *Biomaterials*. 2003;24(20):3453-62.
- 8 Murase SK, Lv L-P, Kaltbeitzel A, Landfester K, del Valle LJ, Katsarava R, *et al*. Amino acid-based poly (ester amide) nanofibers for tailored enzymatic degradation prepared by miniemulsion-electrospinning. *RSC Advances*. 2015;5(68):55006-14.
- 9 Botines E, Franco L, Puiggali J. Thermal stability and degradation studies of alternating poly (ester amide) s derived from glycolic acid and  $\omega$ -amino acids. *Journal of applied polymer science*. 2006;102(6):5545-58.
- 10 Fan Y, Kobayashi M, Kise H. Synthesis and biodegradability of new polyesteramides containing peptide linkages. *Polymer Journal*. 2000;32(10):817-22.
- 11 Saotome Y, Miyazawa T, Endo T. Novel enzymatically degradable polymers comprising. ALPHA.-amino acid, 1, 2-ethanediol, and adipic acid. *Chemistry Letters*. 1991(1):21-4.
- 12 Sharony R, Yu P-J, Park J, Galloway AC,



- Mignatti P, Pintucci G. Protein targets of inflammatory serine proteases and cardiovascular disease. *Journal of Inflammation*. 2010;7(1):1.
- 13 Nakano S, Ikata T, Kinoshita I, Kanematsu J, Yasuoka S. Characteristics of the protease activity in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. *Clin Exp Rheumatol*. 1999;17(2):161-70.
- 14 Williams GW, Hubbard RC, Yu SS, Zhao W, Geis GS. Comparison of once-daily and twice-daily administration of celecoxib for the treatment of osteoarthritis of the knee. *Clin Ther*. 2001;23(2):213-27.
- 15 Brophy JM. Celecoxib and cardiovascular risks. *Expert Opin Drug Saf*. 2005;4(6):1005-15.
- 16 Andrés-Guerrero V, Zong M, Ramsay E, Rojas B, Sarkhel S, Gallego B, *et al*. Novel biodegradable polyesteramide microspheres for controlled drug delivery in ophthalmology. *Journal of Controlled Release*. 2015;211:105-17.
- 17 Liu X, Costantini C, Bonnier H, Londero H, De Scheerder I. P535 Nitric oxide through biodegradable layer elective study for safety and efficacy (NOBLESSE)-first in man pilot trial. *European Heart Journal*. 2003;5(24):86.
- 18 Katsarava R, Beridze V, Arabuli N, Kharadze D, Chu C, Won C. Amino acid-based bioanalogous polymers. Synthesis, and study of regular poly (ester amide) s based on bis ( $\alpha$ -amino acid)  $\alpha$ ,  $\omega$ -alkylene diesters, and aliphatic dicarboxylic acids. *Journal of Polymer Science Part A: Polymer Chemistry*. 1999;37(4):391-407.
- 19 Willems N, Mihov G, Grinwis G, van Dijk M, Schumann D, Bos C, *et al*. Safety of intradiscal injection and biocompatibility of polyester amide microspheres in a canine model predisposed to intervertebral disc degeneration. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 2015.
- 20 Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the rat. *Osteoarthritis Cartilage*. 2010;18 Suppl 3:S24-34.
- 21 Carrigan SO, Weppler AL, Issekutz AC, Stadnyk AW. Neutrophil differentiated HL-60 cells model Mac-1 (CD11b/CD18)-independent neutrophil transepithelial migration. *Immunology*. 2005;115(1):108-17.
- 22 Bedouet L, Pascale F, Moine L, Wassef M, Ghegediban SH, Nguyen VN, *et al*. Intra-articular fate of degradable poly(ethyleneglycol)-hydrogel microspheres as carriers for sustained drug delivery. *Int J Pharm*. 2013;456(2):536-44.

## Chapter 6

- 23 Horisawa E, Kubota K, Tuboi I, Sato K, Yamamoto H, Takeuchi H, *et al.* Size-dependency of DL-lactide/glycolide copolymer particulates for intra-articular delivery system on phagocytosis in rat synovium. *Pharm Res.* 2002;19(2):132-9.
- 24 Iwanaga T, Shikichi M, Kitamura H, Yanase H, Nozawa-Inoue K. Morphology and functional roles of synoviocytes in the joint. *Arch Histol Cytol.* 2000;63(1):17-31.
- 25 Tuovinen L, Ruhanen E, Kinnarinen T, Ronkko S, Pelkonen J, Urtti A, *et al.* Starch acetate microparticles for drug delivery into retinal pigment epithelium-*in vitro* study. *J Control Release.* 2004;98(3):407-13.
- 26 Trif M, Guillen C, Vaughan DM, Telfer JM, Brewer JM, Roseanu A, *et al.* Liposomes as possible carriers for lactoferrin in the local treatment of inflammatory diseases. *Exp Biol Med (Maywood).* 2001;226(6):559-64.
- 27 Zhang Z, Huang G. Intra-articular lornoxicam loaded PLGA microspheres: enhanced therapeutic efficiency and decreased systemic toxicity in the treatment of osteoarthritis. *Drug Deliv.* 2012;19(5):255-63.
- 28 Tuncay M, Calis S, Kas HS, Ercan MT, Peksoy I, Hincal AA. *In vitro* and *in vivo* evaluation of diclofenac sodium loaded albumin microspheres. *J Microencapsul.* 2000;17(2):145-55.
- 29 Bozdag S, Calis S, Kas HS, Ercan MT, Peksoy I, Hincal AA. *In vitro* evaluation and intra-articular administration of biodegradable microspheres containing naproxen sodium. *J Microencapsul.* 2001;18(4):443-56.
- 30 Fernandez-Carballido A, Herrero-Vanrell R, Molina-Martinez IT, Pastoriza P. Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration. Effect of Labrafil addition on release *in vitro*. *Int J Pharm.* 2004;279(1-2):33-41.
- 31 Thakkar H, Sharma RK, Mishra AK, Chuttani K, Murthy RS. Celecoxib incorporated chitosan microspheres: *in vitro* and *in vivo* evaluation. *J Drug Target.* 2004;12(9-10):549-57.
- 32 Dong J, Jiang D, Wang Z, Wu G, Miao L, Huang L. Intra-articular delivery of liposomal celecoxib-hyaluronate combination for the treatment of osteoarthritis in rabbit model. *Int J Pharm.* 2013;441(1-2):285-90.
- 33 Butoescu N, Jordan O, Burdet P, Stadelmann P, Petri-Fink A, Hofmann H, *et al.* Dexamethasone-containing biodegradable superparamagnetic microparticles for intra-articular administration: physicochemical and magnetic properties, *in vitro* and *in vivo* drug release. *Eur J Pharm Biopharm.* 2009;72(3):529-38.

- 34 Elron-Gross I, Glucksam Y, Biton IE, Margalit R. A novel Diclofenac-carrier for local treatment of osteoarthritis applying live-animal MRI. *J Control Release*. 2009;135(1):65-70.
- 35 Petit A, Sandker M, Muller B, Meyboom R, van Midwoud P, Bruin P, *et al*. Release behavior and intra-articular biocompatibility of celecoxib-loaded acetyl-capped PCLA-PEG-PCLA thermogels. *Biomaterials*. 2014;35(27):7919-28.
- 36 Petit A, Redout EM, van de Lest CH, de Grauw JC, Muller B, Meyboom R, *et al*. Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. *Biomaterials*. 2015;53:426-36.
- 37 Aydin O, Korkusuz F, Korkusuz P, Tezcaner A, Bilgic E, Yaprakci V, *et al*. *In vitro* and *in vivo* evaluation of doxycycline-chondroitin sulfate/PCLmicrospheres for intraarticular treatment of osteoarthritis. *J Biomed Mater Res B Appl Biomater*. 2015;103(6):1238-48.
- 38 Sandker MJ, Petit A, Redout EM, Siebelt M, Müller B, Bruin P, *et al*. In situ forming acyl-capped PCLA-PEG-PCLA triblock copolymer based hydrogels. *Biomaterials*. 2013;34(32):8002-11.
- 39 Arunkumar P, Indulekha S, Vijayalakshmi S, Srivastava R. Synthesis, characterizations, *in vitro* and *in vivo* evaluation of Etoricoxib-loaded Poly (Caprolactone) microparticles - a potential Intra-articular drug delivery system for the treatment of Osteoarthritis. *J Biomater Sci Polym Ed*. 2016;27(4):303-16.
- 40 Timur U, Caron M, Bastiaansen-Jenniskens Y, Welting T, van Osch G, Emans P. PGE2 and PGF2 $\alpha$  are secreted by the osteoarthritic infrapatellar fat pad and their release can be modulated by celecoxib. *Osteoarthritis and Cartilage*. 2016(24):S339.
- 41 Madsen CG, Skov A, Baldursdottir S, Rades T, Jorgensen L, Medlicott NJ. Simple measurements for prediction of drug release from polymer matrices—Solubility parameters and intrinsic viscosity. *European Journal of Pharmaceutics and Biopharmaceutics*. 2015;92:1-7.
- 42 Jiang D, Zou J, Huang L, Shi Q, Zhu X, Wang G, *et al*. Efficacy of intra-articular injection of celecoxib in a rabbit model of osteoarthritis. *Int J Mol Sci*. 2010;11(10):4106-13.
- 43 Ou Y, Tan C, An H, Jiang D, Quan Z, Tang K, *et al*. Selective COX-2 inhibitor ameliorates osteoarthritis by repressing apoptosis of chondrocyte. *Med Sci Monit*. 2012;18(6):BR247-52.
- 44 Anderson GD, Hauser SD, McGarity KL,



## Chapter 6

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- Bremer ME, Isakson PC, Gregory SA. Selective inhibition of cyclooxygenase (COX)-2 reverses inflammation and expression of COX-2 and interleukin 6 in rat adjuvant arthritis. *J Clin Invest.* 1996;97(11):2672-9.
- 45 Mastbergen SC, Marijnissen AC, Vianen ME, Zoer B, van Roermund PM, Bijlsma JW, *et al.* Inhibition of COX-2 by celecoxib in the canine groove model of osteoarthritis. *Rheumatology (Oxford).* 2006;45(4):405-13.
- 46 Jukka Morko ZP, Jukka Vaaraniemi, Katja M Fagerlund, Jukka P Rissanen, Jenni Bernoulli and Jussi M Halleen Degenerative Changes in Knee Joints and Joint Pain in Surgically and Chemically Induced Rat Models of Osteoarthritis. *ASBMR; Baltimore, Maryland, USA2013.*
- 47 Adaes S, Mendonca M, Santos TN, Castro-Lopes JM, Ferreira-Gomes J, Neto FL. Intra-articular injection of collagenase in the knee of rats as an alternative model to study nociception associated with osteoarthritis. *Arthritis Res Ther.* 2014;16(1):R10.
- 48 Ross TN, Kisiday JD, Hess T, McIlwraith CW. Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide. *Osteoarthritis Cartilage.* 2012;20(12):1583-90.



# Chapter 07





## Identification of Tissue Type-Dependent Proteins in Knee OA Synovial Fluid.

*Timur UT, Jahr H, Anderson J., Green D.C., Emans P.J., Smagul A., van Rhijn L.W., Peffers M.J., Welting T.J.M. Osteoarthritis Cartilage. 2020.*

# Abstract

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### **Objective:**

For many proteins from osteoarthritic synovial fluid, their intra-articular tissue of origin remains unknown. In this study we performed comparative proteomics to identify osteoarthritis-specific and joint tissue-dependent secreted proteins that may serve as candidates for osteoarthritis biomarker development on a tissue-specific basis.

### **Material & Methods:**

Protein secretomes of cartilage, synovium, Hoffa's fat pad and meniscus from knee osteoarthritis patients were determined using liquid chromatography tandem mass spectrometry, followed by label-free quantification. Validation of tissue-dependent protein species was conducted by ELISA on independent samples. Differential proteomes of osteoarthritic and non-osteoarthritic knee synovial fluids were obtained via similar proteomics approach, followed by ELISA validation.

### **Results:**

Proteomics revealed 64 proteins highly secreted from cartilage, 94 from synovium, 37 from Hoffa's fat pad and 21 from meniscus. Proteomic analyses of osteoarthritic vs non-osteoarthritic knee synovial fluid revealed 70 proteins with a relatively higher abundance and 264 proteins with a relatively lower abundance in osteoarthritic synovial fluid. Of the 70 higher abundance proteins, 23 were amongst the most highly expressed in the secretomes of a specific intra-articular tissue measured. Tissue-dependent release was validated for SLPI, C8, CLU, FN1, RARRES2, MATN3, MMP3 and TNC. Abundance in synovial fluid of tissue-dependent proteins was validated for IGF2, AHSG, FN1, CFB, KNG and C8.

### **Conclusion:**

We identified proteins with a tissue-dependent release from intra-articular human knee OA tissues. A number of these proteins also had an osteoarthritis-specific abundance in knee synovial fluid. These proteins may serve as novel candidates for osteoarthritis biomarker development on a tissue-specific basis.

## **1. Introduction**

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Knee osteoarthritis (OA) is an increasingly prevalent disabling condition with serious socioeconomic impact on a global scale<sup>1,2</sup>. Currently, no effective treatment exists to attenuate OA progression, and the current standard of care involves conservative pain-killing drug therapies, physiotherapy and visco-supplementation of the affected joint. These therapies are all aimed at postponing total joint arthroplasty, which is an invasive surgical approach to alleviate the patient's OA-related pain and regain mobility when the condition reaches end-stage. Knee OA is currently diagnosed radiographically by significant joint space narrowing, osteophyte formation and subchondral bone lesioning<sup>3</sup>. However, these macroscopic changes are indicative of the disease progressing towards its end-stage, and often the patient presents with symptoms such as pain, joint stiffness and debilitating mobility limitations prior to diagnosis<sup>4</sup>.

The development of OA is multifactorial, comprising genetic, biomechanical, ageing, environmental, epigenetic, cellular, and metabolic factors contributing to the intra-articular disease process and ultimately leading to a final common pathway of articular cartilage degeneration<sup>4,5,6,7,8</sup>. Despite the fact that OA is recognized as a total-joint disease, intra-articular pathobiological processes occur in a tissue-specific manner<sup>5</sup>. Since the synovial fluid is in contact with many of the important intra-articular tissues, it represents a promising body fluid for developing OA biomolecular diagnostics to access the condition of the joint as whole, as well as with a potential for joint tissue-specific diagnostics<sup>9,10</sup>.



In the search of diagnostic targets that are capable of identifying biomolecular changes in knee joint homeostasis, efforts have been made to understand the biomolecular composition of synovial fluid and to identify OA-related changes in its composition. Research has previously characterised OA-dependent proteomic, transcriptomic, metabolomic and lipidomic changes in the synovial fluid of the human knee joint<sup>10,11,12,13</sup>. From these studies it became evident that the knee joint synovial fluid composition undergoes a great number of detectable biomolecular changes specific for OA, and depending on distinct OA subtypes, but also related to the severity of cartilage damage<sup>14,15,16,17</sup>. However, the origin of OA-driven alterations in the biomolecular composition of synovial fluid is poorly documented.

In this study, we aimed to delineate potential tissue-dependent proteins in the synovial fluid of knee OA patients. To this end, we performed a comparative proteomic analysis of the secretomes of Hoffa's fat pad, synovium, meniscus and articular cartilage of OA knees. Proteins with a tissue-dependent amount present in the secretomes were identified and the OA-specificity of their abundance was determined in knee synovial fluid. We expect that the identification of tissue-dependent and OA-related protein species from specific intra-articular tissues implicated in knee OA pathogenesis will fuel the development of novel synovial fluid-based diagnostic approaches aiming at grading and subtyping knee OA.

## 2. Materials and Methods

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### 2.1 OA Knee Tissue Secretomes

Full thickness cartilage explants from medial and lateral femoral condyles, a synovial explant, an explant from Hoffa's fat pad (obtained from the core of the fat pad and avoiding synovial tissue adjacent to the fat pad) and explants from the medial and lateral menisci (avoiding synovial tissue present at the outer margins of the meniscus) were obtained as waste material following total knee arthroplasty for each of the four donors with end-stage knee OA (MEC approval 2017-0183) (Supplementary Table 1). These samples were designated

as the dependent cohort. The same tissue explants were also collected from an additional 12 knee OA patients who underwent total knee arthroplasty and were designated as an independent cohort ( Supplementary Table 2). Tissue explants were rinsed thoroughly in 0.9% NaCl and then pre-incubated at 37°C, 5% CO<sub>2</sub> for 24 h in a tissue/media ratio of 150 mg tissue/ml medium (Dulbecco's Modified Eagle's Medium-F12 without phenol red (Thermofisher, Massachusetts, USA) supplemented with 1% Glutamax (Thermofisher, Massachusetts, USA) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, US)). After 24 h of pre-incubation, medium was fully refreshed at identical tissue/media ratio. Secretomes were then obtained following 48 h conditioning of these cultures. Secretomes were freshly processed by differential centrifugation (5 min at 1200 RPM, 10 min 15,000 RPM, room temperature). To inhibit proteolytic degradation of supernatants protease inhibitors were added (Complete Mini, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich, Saint Louis, USA). Secretomes samples were aliquoted and stored at -80°C until further analysis.

### 2.2 Synovial Fluid Collection and Processing

Knee OA synovial fluid was used from three out of the four patients from the dependent cohort and 10 out of the 12 patients from the independent cohort (MEC approval 2017-0183) ( Supplementary Table 1 , patient 2, 3 and 4; Supplementary Table 2 , patient 6, 7, 8, 9, 11, 12, 13, 14, 15 and 16. K&L scores are indicated in these tables.). Knee OA synovial fluids were processed within half an hour of collection. Samples were processed by differential centrifugation (5 min at 1200 RPM, 10 min 15,000 RPM, room temperature). Non-OA knee synovial fluid from 10 individuals was purchased from Articular Engineering, Illinois, USA. Synovial fluid was collected directly from post mortem macroscopically normal joints and stored in aliquots at -80°C until shipping. Details including race, age, sex and cause of death are included in Supplementary Table 3. To inhibit proteolytic degradation protease inhibitors were added (Complete Mini, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich, Saint Louis, USA). Synovial fluid samples were aliquoted and stored at -80°C until further analysis.

### 2.3 Total Protein Content Measurements

Total protein content was measured in samples using the Nano Orange Protein Quantification kit according to manufacturer's instructions (Invitrogen, Carlsbad, USA). Fluorescence was measured using a Tristar LB 942 (Berthold, Bald Wildbad, Germany) at 485 nm excitation and 590 nm emission.

### 2.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Silver Staining

Secretome samples (containing 1.25 µg of total protein) of the dependent cohort were loaded on a 13% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Protean II xi cell, Bio-Rad, Hercules, USA) and proteins were resolved by electrophoresis. The gel was silver stained to visualize protein bands (Pierce Silver Stain Kit, Thermofisher, Massachusetts, USA) according to the manufacturer's instructions.

### 2.5 Enzyme-Linked Immune Sorbent Assay (ELISA) Validation

Differential abundance of selected protein species in secretomes or synovial fluids was validated by ELISAs from various manufacturers (Supplementary Table 4). Measurements were performed according to the manufacturer's instructions.

### 2.6 Liquid Chromatography Tandem Mass Spectrometry and Label-Free Quantification of Tissue Secretome Proteomes

Proteolytic digestion was performed on 10 µg protein of each individual sample using 10 µl Strataclean (Agilent, Genomics, UK). In-solution tryptic digestion of protein samples was carried out following sequential reduction and alkylation in 3mM DTT (60 °C for 10 min) and then 9mM iodoacetamide (30 min in the dark at room temperature) with trypsin at a ratio of 1:50 protein: trypsin ratio overnight at 37 °C<sup>18</sup>. Samples were randomized for loading onto the instrument using Excel (Microsoft, USA). All samples were run in a single batch. 500 ng of each tryptic digest was subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS), using a 2 h gradient. Data-dependent analyses were conducted on a QExactive



HF quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 RSLC nano-liquid chromatograph (Hemel Hempstead, UK). Sample digests were loaded onto a trapping column (Acclaim PepMap 100 C18, 75  $\mu\text{m} \times 2 \text{ cm}$ , 3  $\mu\text{m}$  packing material, 100  $\text{\AA}$ ) using a loading buffer of 0.1% (v/v) TFA, 2% (v/v) acetonitrile in water for 7 min at a flow rate of 12  $\mu\text{L min}^{-1}$ . The trapping column was then set in-line with an analytical column (EASY-Spray PepMap RSLC C18, 75  $\mu\text{m} \times 50 \text{ cm}$ , 2  $\mu\text{m}$  packing material, 100  $\text{\AA}$ ) and the peptides eluted using a linear gradient of 96.2% A (0.1% [v/v] formic acid):3.8% B (0.1% [v/v] [formic acid in water/acetonitrile [80/20] [v/v]) to 50% A:50% B over 90 min at a flow rate of 300  $\text{nL min}^{-1}$ , followed by washing at 1% A:99% B for 5 min and re-equilibration of the column to starting conditions. The column was maintained at 40°C, and the effluent introduced directly into the integrated nano-electrospray ionisation source operating in positive ion mode. The mass spectrometer was operated in data dependent acquisition mode with survey scans between  $m/z$  350–2000 acquired at a mass resolution of 60,000 (FWHM) at  $m/z$  200. The maximum injection time was 100 ms, and the automatic gain control was set to  $3e^6$ . The 12 most intense precursor ions with charges states of between 2+ and 5+ were selected for MS/MS with an isolation window of 2  $m/z$  units. The maximum injection time was 100 ms, and the automatic gain control was set to  $1e^5$ . Fragmentation of the peptides was by higher-energy collisional dissociation using normalized collision energy of 30%. Dynamic exclusion of mass/charge values to prevent repeated fragmentation of the same peptide was used with an exclusion time of 20 s. For label-free quantification, the raw files of the acquired spectra were analyzed by the Progenesis Q1 software (Waters, Manchester, UK)<sup>19</sup> which aligns the files and then peak picks for quantification by peptide abundance. Briefly, the top five spectra for each feature were exported from Progenesis Q1 and utilised for peptide identification with our local Mascot server (Version 2.6.2), searching against the Unihuman Reviewed database with carbamidomethyl cysteine as a fixed modification and methionine oxidation as a variable modification, peptide mass tolerance of 10 ppm and fragment tolerance of 0.01 Da.

### **2.7 Liquid Chromatography Tandem Mass Spectrometry and Label-Free Quantification of Synovial Fluid Samples**

We utilized our previously optimised method for synovial fluid proteomics using Proteominer beads<sup>20</sup>. Briefly, individual synovial fluid samples were thawed and treated with 1 µg/mL hyaluronidase as previously described<sup>19</sup> and centrifuged at 10000g for 10 min to remove any particulates. Protein concentrations of synovial fluid were determined by Bradford assay (Thermofisher, Massachusetts, USA). A volume of sample equivalent to 5 mg of protein was added to 10 µL of ProteoMiner beads (BioRad, UK) according to the manufacturer's instructions. Beads were re-suspended in 80 µL of 25mM ammonium bicarbonate and 5 µL of 1% (w/v) Rapigest SF (Waters, UK) added and the sample heated at 80°C for 10 min. On bead trypsin digestion was undertaken<sup>19</sup>. Tryptic digests were subjected to LC-MS/MS, using a 2 h gradient using methods described above. Label-free quantification was undertaken using methods described above.

### **2.8 Mass Spectrometry Proteomics Data**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014321 and 10.6019/PXD014321<sup>21</sup>.

### **2.9 Statistical Analyses**

Statistical analysis was undertaken in Progenesis QI software (Waters, Manchester, UK). Proteins identified following label-free quantification were retained if they had at least two unique peptides. Protein abundances were logarithm (base 10) transformed and normalised relative to a selected reference sample.

To assess differences in normalised abundance in the dependent cohort between cartilage, Hoffa's fat pad, synovium and meniscus secretomes, we implemented repeated measures one-way analysis of variance (RM-ANOVA). For the RM-ANOVA, we specified our factor variable to be tissue and our within-subject dependent variable as the donors. No

shrinkage methods were applied. Differentially expressed proteins between tissue-specific secretomes were defined as those with a false discovery rate (FDR)  $q$  value  $< 0.05$  and fold-change that exceeded 2 for any tissue<sup>22</sup>.

To assess differences in normalised protein abundance between the synovial fluid of OA and non-OA donors, we implemented a one-way analysis of variance (ANOVA). The condition factor was set according to whether the protein originated from OA or non-OA synovial fluid. OA and non-OA synovial fluid donors were independent donors. Differentially expressed proteins between OA and non-OA synovial fluid were defined as those with an FDR  $q$  value  $< 0.05$  and a fold change that exceeded 2 between conditions<sup>22</sup>.

95% confidence intervals for pairwise differences between means were generated for any statistically significant proteins as determined by the above criteria (R, Version 4.0.1). The 95% confidence intervals were adjusted based on parameters of selection to allow for control of false coverage rate (FCR)<sup>23,24</sup>.

Statistical analysis of tissue secretome Enzyme-linked immune sorbent assay (ELISA) data was performed using Graphpad Prism (version 7, La Jolla, California, USA). Data were log-transformed and protein abundances between cartilage, Hoffa's fat pad, synovium and meniscus were compared using a repeated measures ANOVA both for the dependent and the independent cohort. Multiple comparisons between tissues were performed using a Fisher's LSD test. Significance was accepted at a  $P$  value  $< 0.05$ . For the synovial fluid ELISA data, protein abundances in synovial fluid samples of OA and non-OA donors were compared using a Mann–Whitney  $U$  test. Significance was defined as a  $P$ -value of  $< 0.05$ .

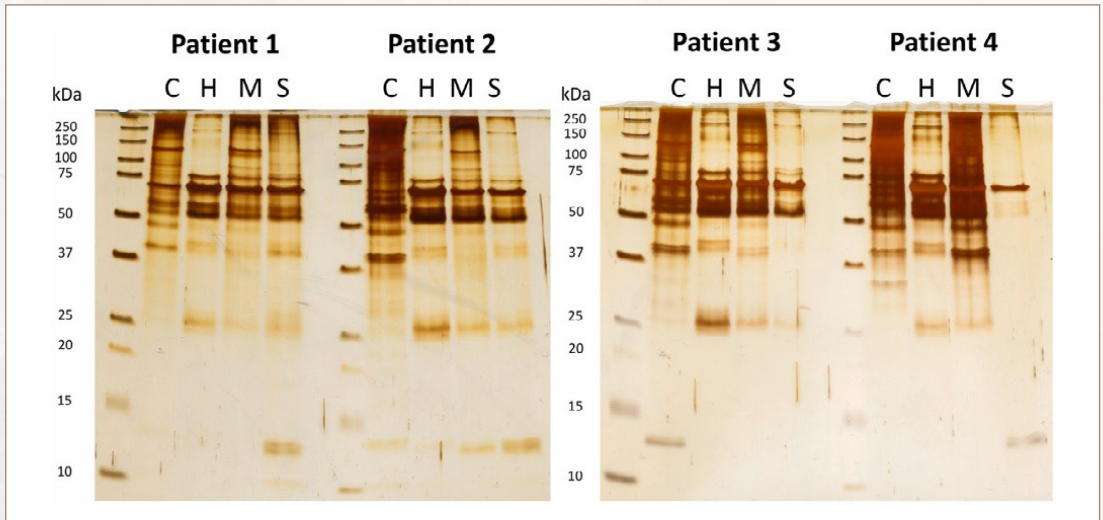
All principal component analysis calculations (PCA) were performed in R (Version 4.0.1). Log transformed normalised protein abundances were centered and scaled. For OA vs non-OA synovial fluid we used the `prcomp` function within the `stats` package to extract principal components<sup>25</sup>. For the multiple tissue secretomes dataset, due to the repeated measures design, we used the PCA function within the `mixOmics` package to generate a multilevel PCA plot that first calculates within subject variance prior to dimensionality reduction<sup>26</sup>.



### 3. Results

#### 3.1 Preparation of OA Knee Tissue-Derived Secretomes

Mean total protein concentration of cartilage secretomes samples was 233.4  $\mu\text{g/mL}$  (range 191–285  $\mu\text{g/mL}$ ), of Hoffa's fat pad secretomes 298.7  $\mu\text{g/mL}$  (range 203–444  $\mu\text{g/mL}$ ), of synovium secretomes 326.5  $\mu\text{g/mL}$  (range 253–404  $\mu\text{g/mL}$ ) and of meniscus secretomes samples 275.0  $\mu\text{g/mL}$  (range 167–362)  $\mu\text{g/mL}$ . To evaluate sample quality and potential gross differences in protein profiles in the different secretomes a silver stained SDS-PAGE demonstrated sharp, distinct bands, confirming sample quality (Figure 1). In addition, tissue-dependent enrichment of specific protein bands was observed. This indicates the presence of tissue-dependent proteins in the OA knee tissue secretomes.

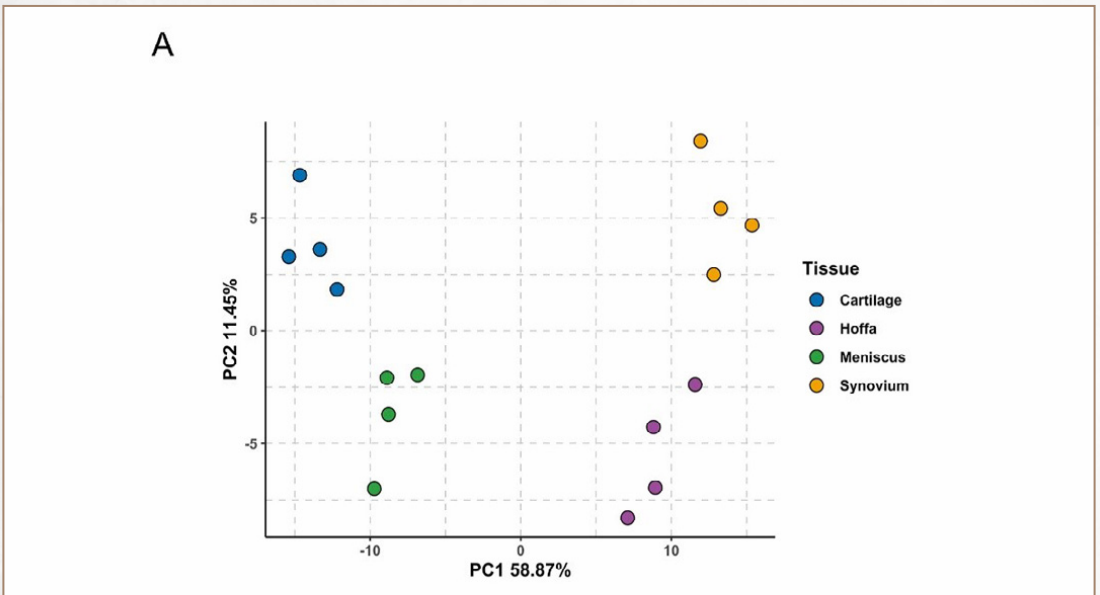


**Figure 1: Confirmation of sample quality and gross differences in protein profiles in secretomes.**

Scans of silver-stained acrylamide gels with patient numbers indicated. C = cartilage, H = Hoffa's fat pad, M = meniscus, S = synovium. Protein molecular weights (kDa) are indicated on the left of both gels.

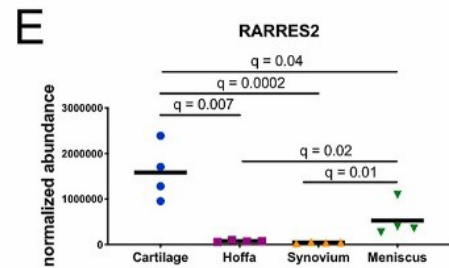
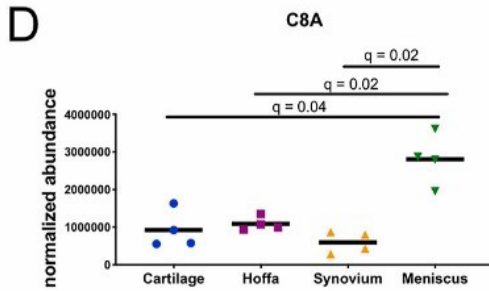
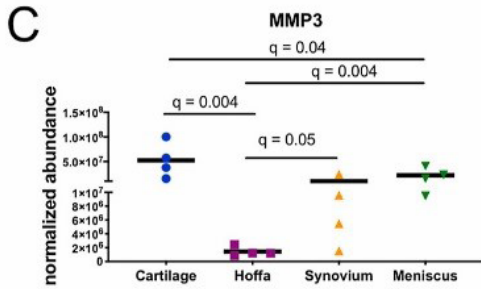
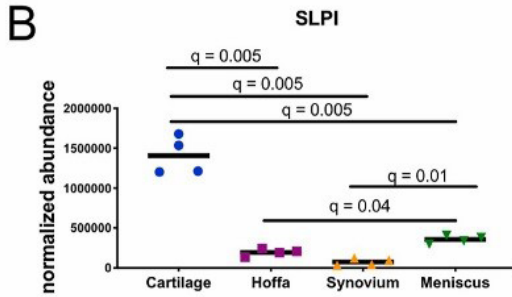
### 3.2 Identification of Protein Species in OA Knee Tissue Secretomes

To identify the protein species in the OA knee tissue secretomes, label-free mass spectrometry proteomic analysis was undertaken. Principal component analysis of the acquired proteomes demonstrated tissue-dependent protein clustering (Figure 2A). A total of 216 proteins were identified with highest mean significant detection in one of the tissue type-dependent secretomes (Supplementary Table 5). From these 216 protein species, 64 were highest secreted from cartilage, 94 from synovium, 37 from Hoffa's fat pad and 21 from meniscus. Four examples of proteins with a tissue-dependent amount detected in the secretomes are shown in Figure 2B–E. The amount of Antileukoproteinase (SLPI) was highest in the secretome of cartilage and lowest in the secretome of synovium (Figure 2B). The lowest amount of Matrix metalloproteinase 3 (MMP3) was found in the secretome of Hoffa's fat pad and highest of cartilage (Figure 2C). The amount of Complement C8 alpha chain (C8A) was highest in the meniscus secretome and lowest in secretome of synovium (Figure 2D). Finally, the amount of Retinoic acid receptor responder protein 2 (RARRES2) was highest in the secretome of cartilage and lowest in synovium secretome (Figure 2E). The OA knee tissue-dependent levels of these four protein species in the acquired secretomes was validated by means of ELISA (Figure 2F–I). A full list of proteins with highest significant amount per tissue-specific secretome is provided in Supplementary Table 5 and a full list of differentially expressed proteins with the corresponding confidence intervals for each tissue is in Supplementary Table 6.

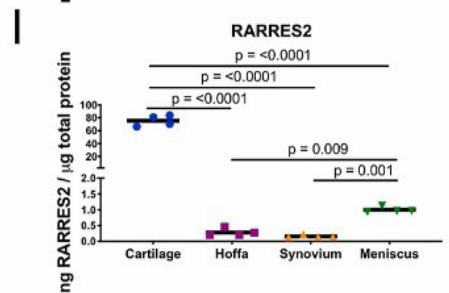
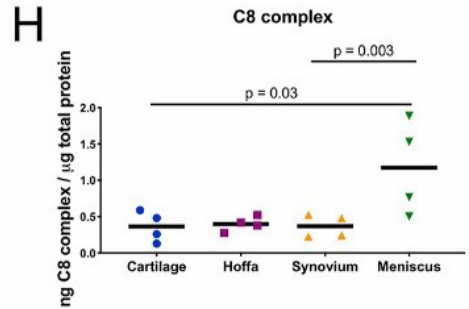
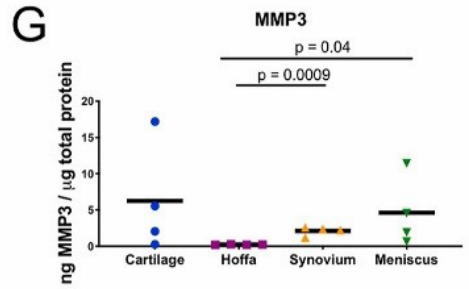
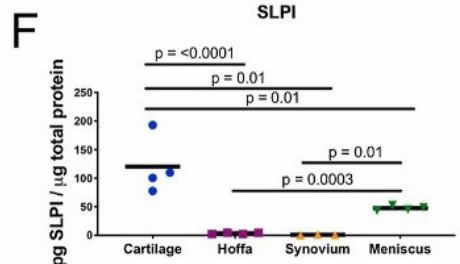




Mass Spectrometry



ELISA



### **Figure 2: PCA analysis and ELISA validation of differentially expressed proteins in tissue-specific secretomes.**

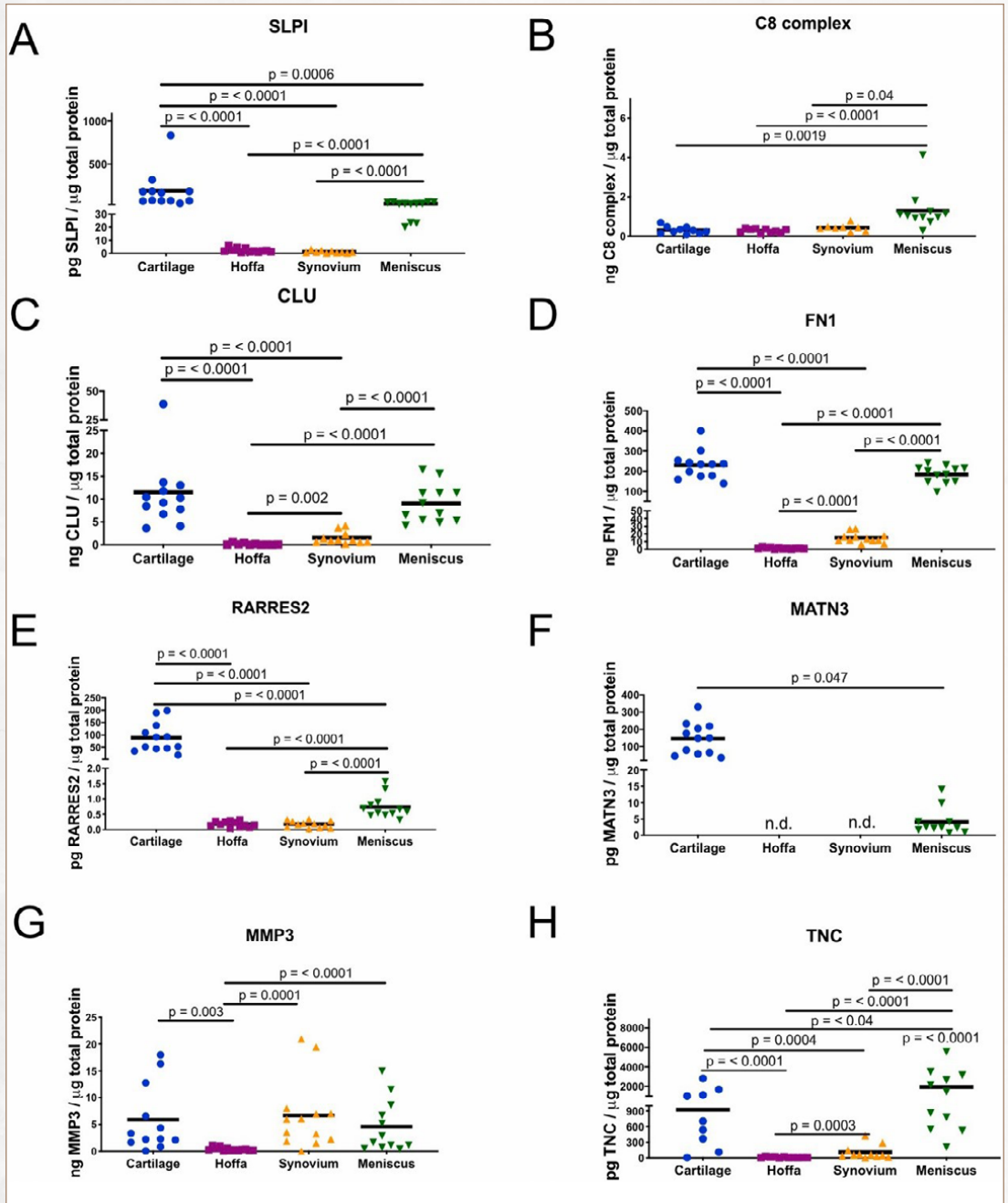
*Principal Component Analyses following label-free quantification of secretome proteomes from the dependent cohort (A). Graphical representation of normalized abundance values of mass spectrometry data of four selected protein species which were found to be differentially expressed in a tissue-dependent fashion: SLPI (B), MMP3 (C), C8A (C), RARRES2 (D). ELISA-based validation of levels of SLPI (F), MMP3 (G), C8A (H), RARRES2 (I) in the tissue-dependent secretomes. Data are presented in scatter plots (individual patients are shown as separate dots) with mean values indicated as a horizontal line. Statistics following label-free quantification were calculated using a repeated measures ANOVA. Q values were generated from resulting P values using FDR correction for multiple testing. Statistical significance following ELISA analysis was calculated using a repeated measures ANOVA after log-transformation, followed by a Fisher's LSD test. Statistical significance is indicated in the figures.*

### **3.3 Validation of Tissue-Dependent Secretome Proteins in an Independent Cohort**

We next investigated whether our mass spectrometry data is applicable within a general knee OA population. To this end, the proteins measured in Figure 2 , and an additional selection of proteins with a highest amount in the secretomes of the tissues from Supplementary Table 5 was measured by ELISA in the tissue secretomes of 12 independent knee OA patients (the independent cohort; Supplementary Table 2). ELISA measurements were performed for SLPI, C8 complex, CLU, FN1, RARRES2, MATN3, MMP3 and Tenascin C (TNC) (Figure 3). The amount of SLPI in the secretome was highest from cartilage and lowest from synovium as revealed by mass spectrometry analysis (Supplementary Table 5) and this was confirmed by ELISA measurements in the independent cohort (Figure 3A). The C8A amount was highest in the secretome of meniscus and lowest in the secretome of synovium in our mass spectrometry data. ELISA analysis of C8 complex in the independent cohort confirmed the highest amount originating from meniscus (Figure 3B). The amount of Clusterin (CLU) was highest in the cartilage secretome and lowest in the Hoffa's fat pad secretome in the mass spectrometry data. ELISA analysis of the independent cohort validated this observation (Figure 3C). Mean Fibronectin 1 (FN1) amount was highest in the cartilage secretome and lowest in the secretomes of Hoffa's fat pad as concluded from mass spectrometry data and ELISA analysis validated this (Figure 3D). ELISA analysis

further confirmed mass spectrometry data for RARRES2 amount, which was highest in the cartilage secretome and lowest in the synovium secretome (Figure 3E). Mass spectrometry data showed that Matrilin 3 (MATN3) amount was highest the secretome of cartilage and lowest in the Hoffa's fat pad secretome. The ELISA analysis of the independent cohort confirmed that MATN3 amount was highest in the cartilage secretome. However, this was not detectable in the secretome of Hoffa's fat pad or synovium (Figure 3F). The amount of MMP3 was lowest in the secretome of Hoffa's fat pad and highest the cartilage secretome in mass spectrometry data. Lowest MMP3 amount in tissue secretomes was also confirmed for Hoffa's fat pad by ELISA analysis of the independent cohort (Figure 3G). Finally, mean TNC amount was highest in the secretome of meniscus and lowest the Hoffa's fat pad secretomes in mass spectrometry analysis and this was confirmed by ELISA (Figure 3H). In conclusion, protein mass spectrometry analysis and ELISA validation of OA knee joint tissue-specific secretomes revealed the presence of many tissue-dependent proteins.





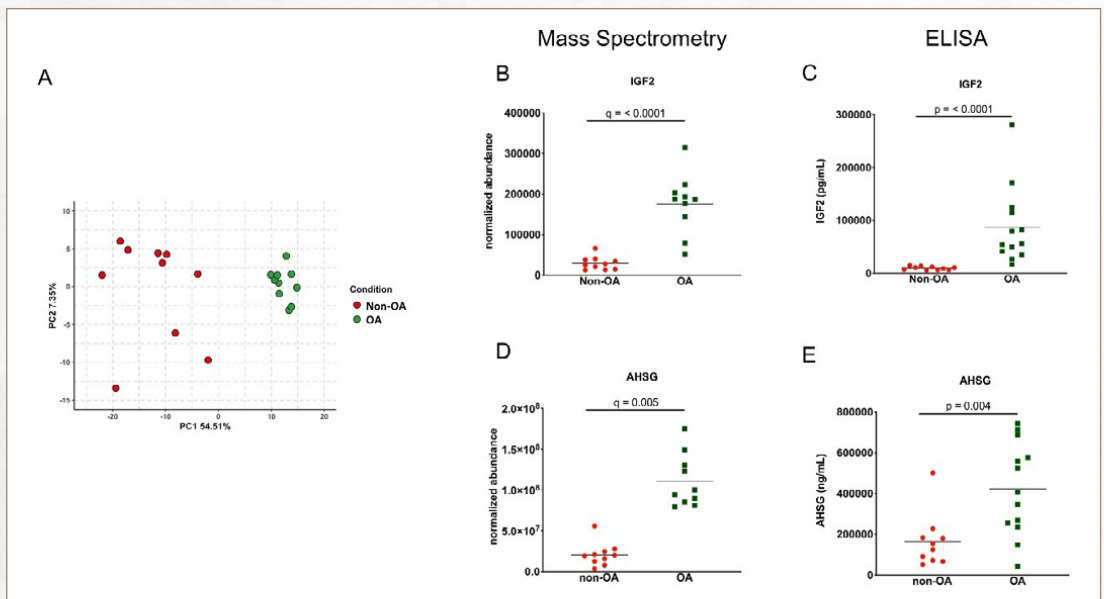
### **Figure 3: Independent validation of secretome tissue specificity.**

ELISA-based validation of eight selected protein species which were found to be differentially expressed in a tissue-dependent fashion in the mass spectrometry data. SLPI (A), C8 complex (B), CLU (C), FN1 (D), RARRES2 (E), MATN3 (F), MMP3 (G), TNC (H). Data are presented in scatter plots (individual donors are shown as separate dots) with mean values indicated as a horizontal line. Statistical significance was calculated using a repeated measures ANOVA after log-transformation, followed by a Fisher's LSD test and is indicated in the figures.

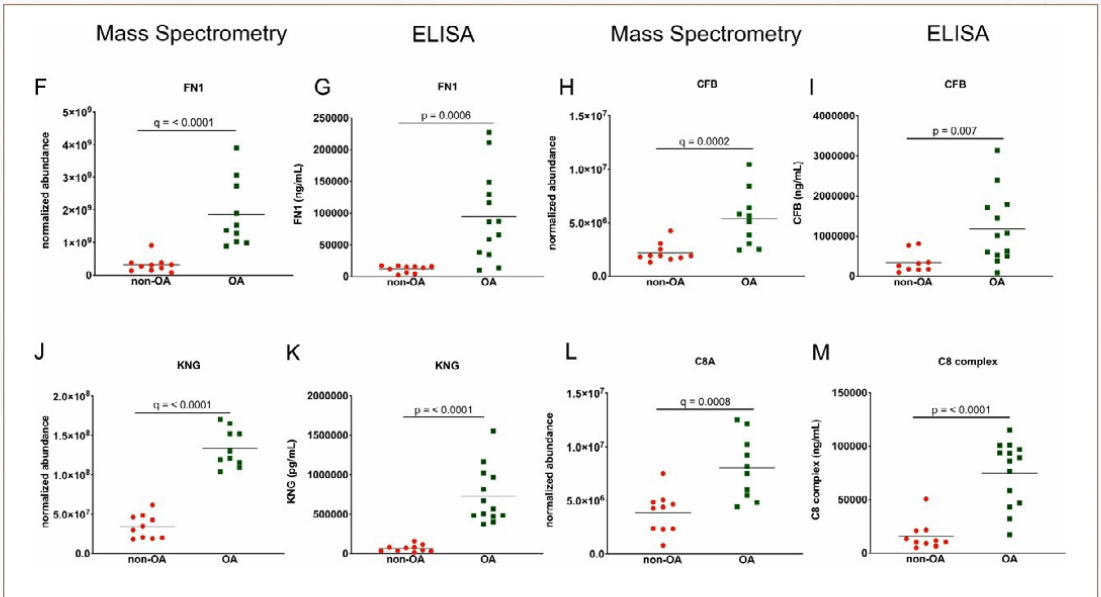
### **3.4 OA-Specific Abundance of Protein Species in Synovial Fluid**

Next, we sought to determine whether the OA knee joint tissue-dependent proteins identified above (Supplementary Table 5) showed an OA-specific abundance in knee joint synovial fluid. To address this question, we performed protein mass spectrometry and label-free quantification on knee joint synovial fluid from 10 patients with knee OA and from 10 individuals without OA and compared their proteomes. Principal component analysis of the acquired proteomes revealed defined group-specific clustering (Figure 4A). The abundance of 62 protein species was found to be significantly increased in OA synovial fluid compared to non-OA synovial fluid. A list of synovial fluid proteins with OA-specific enrichment is presented in Supplementary Table 7. Of these 62 protein species, 39 were also detected in the secretomes above. Of these 39 proteins, 19 were present in the secretomes in an OA knee joint tissue-dependent manner (Supplementary Tables 7 and 8). In addition, 234 protein species were present in significantly lower abundance in OA synovial fluid compared to non-OA synovial fluid. Of these 234 protein species, 56 were also detected in the secretomes. Of these 56 proteins, 54 were found to be present in the secretomes in a tissue-dependent manner (Supplementary Table 9). Next, we validated mass spectrometry results of the synovial fluid proteomes using ELISA assays. Validation was performed using knee OA synovial fluid samples of the knee OA patients from which we also used the tissues to generate tissue-conditioned media ( $n = 13$ ), and using the non-OA knee synovial fluid samples which were also used for the synovial fluid mass spectrometry analysis ( $n = 10$ ). Six proteins that were significantly higher in OA synovial fluid compared to non-OA synovial fluid (and some with an OA knee joint tissue-dependent release; Supplementary Tables 5 and 7) were selected for ELISA validation (IGF2, AHSG, FN1, CFB, KNG, C8 complex). For these proteins data following ELISA validation were highly concordant with our mass

spectrometry data (Figure 4). In addition to proteins with a higher abundance in OA synovial fluid, we also validated mass spectrometry data of two proteins with different expression dynamics. In the mass spectrometry data, the abundance of DCN (Decorin) was found to be significantly lower in OA synovial fluid compared to non-OA synovial fluid. The lower abundance of DCN in OA synovial fluid was validated by ELISA (Figure 5A and 5B). Furthermore, the abundance of ANG (Angiogenin) was not significantly different between OA and non-OA synovial fluid and this was confirmed with ELISA (Figure 5C and 5D).

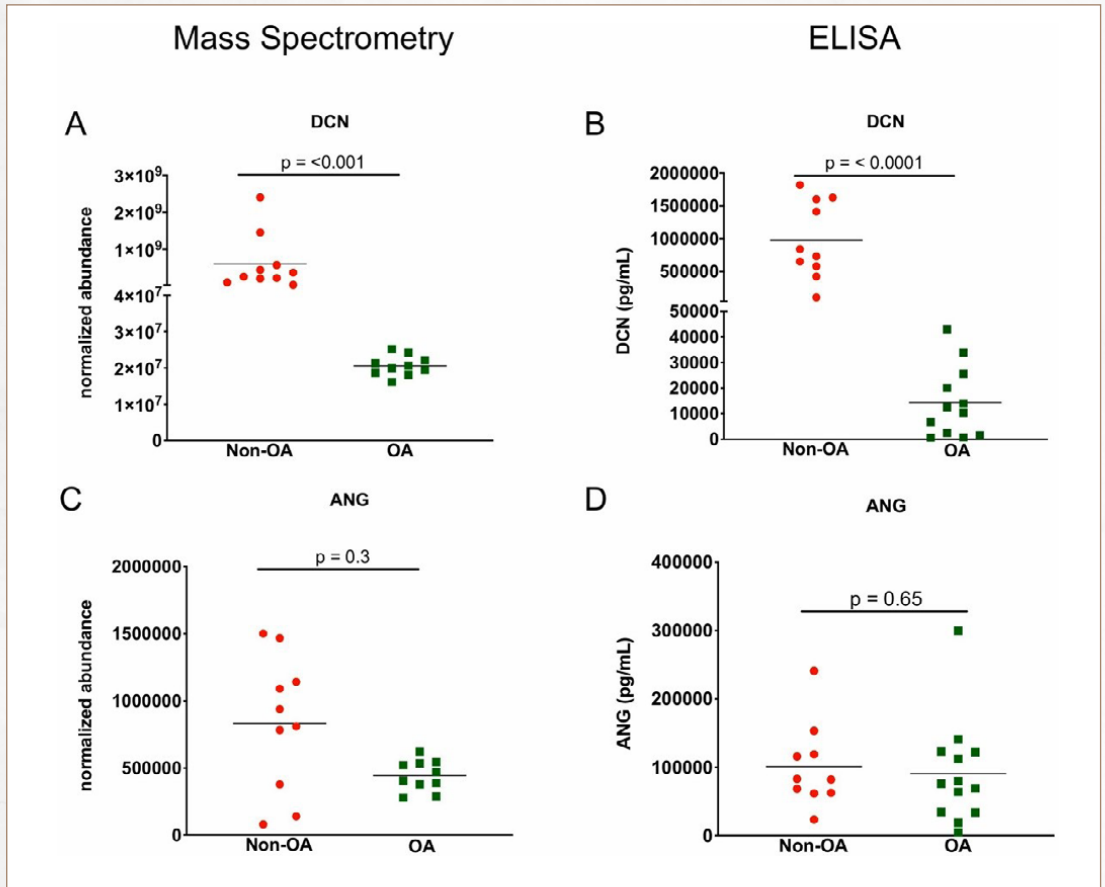






**Figure 4: PCA analysis and ELISA validation of synovial fluid mass spectrometry data.**

Principal Component Analyses following label-free quantification of knee OA and non-OA synovial fluids (A). Graphical representation of normalized abundance values of mass spectrometry data and accompanying ELISA validation of selected protein species that were found to be present in higher abundance in OA vs non-OA synovial fluids (based on mass spectrometry analysis (Supplementary Table 7)). IGF2 (B/C), AHSG (D/E) FN1 (F/G), CFB (H/I), KNG (J/K), C8A and C8 complex (L/M). Data are presented in scatter plots (individual donors are shown as separate dots), with mean values indicated as a horizontal line. Statistics following label-free quantification were calculated using an ANOVA. Q values were generated from resulting P values using FDR correction for multiple testing. Statistical significance following ELISA analysis was calculated using a Mann–Whitney U test. Statistical significance is indicated in the figures.



**Figure 5: ELISA validation of synovial fluid mass spectrometry data.**

Graphical representation of normalized abundance values of mass spectrometry data and accompanying ELISA validation of selected protein species that were found to be present in lower and equal abundance in OA vs non-OA synovial fluids (based on mass spectrometry analysis). DCM (A/B), ANG (C/D). Data are presented in scatter plots (individual donors are shown as separate dots), with mean values indicated as a horizontal line. Statistical significance was calculated using a Mann–Whitney U test and is indicated in the figures.

## 4. Discussion

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In the search of diagnostic targets that are capable of identifying biomolecular changes in knee joint homeostasis, important efforts have been made to understand the proteomic composition of synovial fluid and to identify OA-related changes in its composition<sup>10,19,27,28,29</sup>. However, the origin of OA-related alterations in the proteomic composition of synovial fluid remained poorly documented and biochemical markers generally lacked specificity to certain joint tissues<sup>30</sup>. In our study we identified 62 proteins with a significantly higher abundance in synovial fluids of osteoarthritic knees. A large part of the synovial fluid is created by ultrafiltration of plasma through the synovial membrane. Synovial fluid is in direct contact with different intra-articular tissues such as cartilage, synovium and meniscus as well as at an intimate distance from Hoffa's fat pad<sup>31</sup>. Thus, the source of protein species in the synovial fluid may be from the blood stream, proximity tissues or released by different intra-articular joint tissues into the synovial fluid. From the 62 proteins with an OA-specific higher abundance in knee synovial fluid, 39 were present in the secretomes of the investigated knee joint tissues. This indicates that their abundance in knee synovial fluid may depend on their release from Hoffa's fat pad, synovium, meniscus and/or articular cartilage. From these 39 proteins, 19 are of particular interest, since their amount is specifically highest in the secretome of one of the investigated intra-articular tissues (Supplementary Table 8). Our results strongly suggest that specific OA knee joint tissues differentially contribute to OA-related changes in the synovial fluid proteome of the knee joint. Earlier publications reported clear differences in protein profiles between OA and non-OA synovial fluids<sup>10,27,32</sup>. Specific joint tissues, such as cartilage and synovium, have been individually investigated as contributors to the levels of OA-related proteins (and protein fragments) in synovial fluid<sup>10,27,33,34,35</sup>. We have determined the protein secretomes of four different knee joint tissues and linked this to OA-specific changes in the synovial fluid proteome of the knee joint. Our findings therefore provide a comprehensive insight into the tissue-dependent origin of proteins in knee joint synovial fluid with an OA-specific abundance.



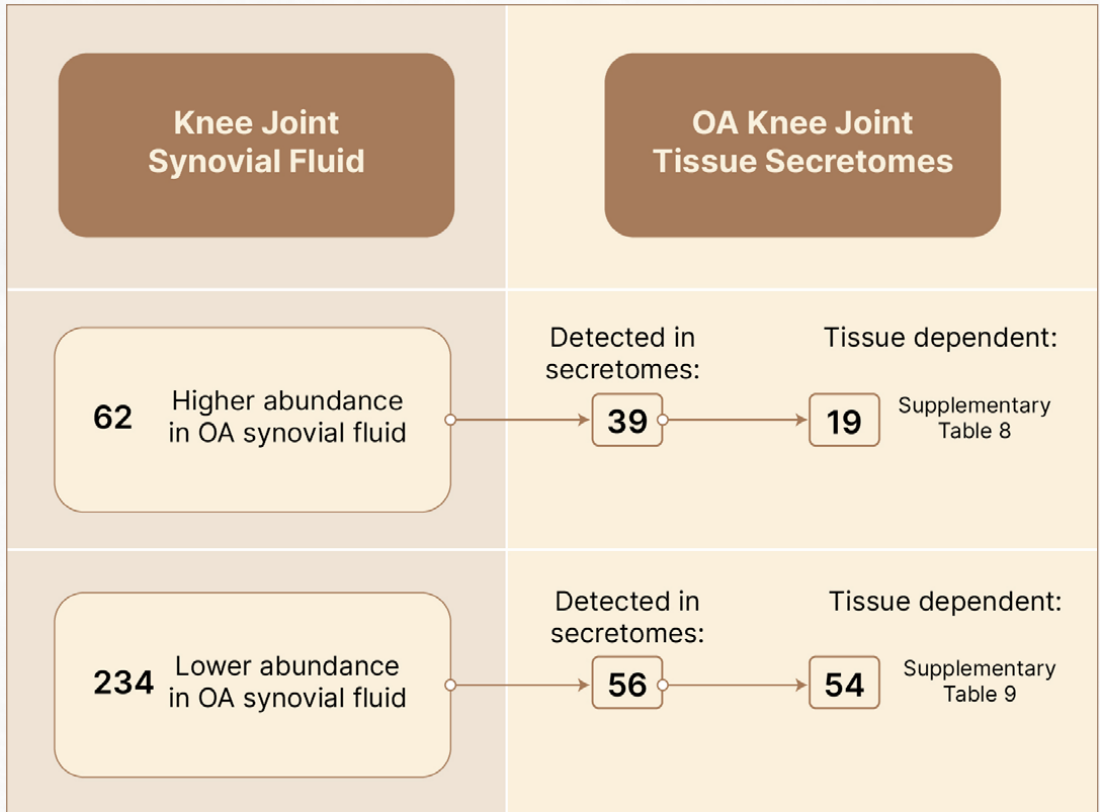
Knowledge of the main tissue source of OA-specific proteins present in knee joint synovial fluid is expected to have important implications for understanding the progression of knee OA on the tissue level, as well as for the development of molecular diagnostics that may aid in the sub-/endotyping of knee OA. Also, efficacy studies of novel (experimental) treatments for knee OA will benefit from biomarkers that are able to report about the joint's health status on the tissue level. For instance, accumulating evidence suggests that complement system components play a key role in the pathogenesis of knee OA<sup>36,37</sup>. Of the four different explanted OA knee tissues the secretome of explanted OA meniscal tissue had the highest amount of Complement component C8. This suggests that C8 might be put forward as a potential synovial fluid biomarker to evaluate the efficacy of therapeutic interventions in knee OA due to meniscal pathology. Another protein whose abundance has been shown previously to be increased in OA synovial fluid is Clusterin<sup>27,36</sup>. While we confirmed this finding in our present study, our data additionally demonstrates the greatest amount of Clusterin is in the explant secretomes of cartilage and meniscal tissues. This points towards cartilage and meniscus as the tissues-of-origin of Clusterin levels in OA synovial fluid. The same holds true for Fibronectin, which has been shown to be more abundant in OA synovial fluid compared to non-OA synovial fluid<sup>27,36</sup>.

As a general study limitation we cannot exclude that some proteins in the tissue-specific secretomes and synovial fluid may originate from degradation products of cell death, extracellular matrix or the blood stream, respectively. However, regarding cell death, this is unlikely to play a major role since clear signs of viability have been reported for similar explant cultures<sup>14,38,39</sup>. Future investigations will further clarify the contribution of the intra-articular tissues to specific proteins in the synovial fluid.

A schematic overview demonstrating the numbers of proteins with a highest secretion from one of the investigated knee joint tissues and their OA-specific abundance in knee synovial fluid is shown in Figure 6. In this respect, it is interesting to note that our study revealed that in addition to cartilage and synovium, the meniscus and Hoffa's fat pad are also likely to be significant contributors to the OA-specific protein composition of the synovial fluid of the knee joint. We expect that improved knowledge of the tissue-specific origin of OA-specific synovial fluid biomarkers will propel the understanding of the role of

## Identification of Tissue Type-Dependent Proteins in Knee OA Synovial Fluid

specific tissues in the progression of OA and fuel the development of improved synovial fluid-based diagnostic approaches aiming at grading and subtyping OA<sup>40,41</sup>.



**Figure 6: Graphical data summary.**

Numbers of protein species with an OA-specific abundance in knee synovial fluid, the number of protein species from these that were detected in the OA knee joint tissue secretomes, and the number of these with a tissue-dependent release into the OA knee joint tissue secretomes (Supplementary Tables 5, 7, 8, 9).

## 5. Supplementary Data

**Supplementary Table 8: Protein species which are present in higher abundance in OA synovial fluid compared to non-OA synovial fluid and present in the secretomes in a tissue-dependent manner**

PROTEIN ACC. NUMBER	PROTEIN NAME
P02751 FINC_HUMAN	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4
P24821 TENA_HUMAN	Tenascin OS=Homo sapiens GN=TNC PE=1 SV=3
Q92743 HTRA1_HUMAN	Serine protease HTRA1 OS=Homo sapiens GN=HTRA1 PE=1 SV=1
P02765 FETUA_HUMAN	Alpha-2-HS-glycoprotein OS=Homo sapiens GN=AHSG PE=1 SV=1
P19827 ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1 OS=Homo sapiens GN=ITIH1 PE=1 SV=3
P01871 IGHM_HUMAN	Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3
P07357 CO8A_HUMAN	Complement component C8 alpha chain OS=Homo sapiens GN=C8A PE=1 SV=2
P02753 RET4_HUMAN	Retinol-binding protein 4 OS=Homo sapiens GN=RBP4 PE=1 SV=3
P01591 IGJ_HUMAN	Immunoglobulin J chain OS=Homo sapiens GN=JCHAIN PE=1 SV=4
P02768 ALBU_HUMAN	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
Q96PD5 PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase OS=Homo sapiens GN=PGLYRP2 PE=1 SV=1
P04196 HRG_HUMAN	Histidine-rich glycoprotein OS=Homo sapiens GN=HRG PE=1 SV=1
P07360 CO8G_HUMAN	Complement component C8 gamma chain OS=Homo sapiens GN=C8G PE=1 SV=3
A1L4H1 SRCRL_HUMAN	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D OS=Homo sapiens GN=SSC5D PE=1 SV=3
Q9NQ79 CRAC1_HUMAN	Cartilage acidic protein 1 OS=Homo sapiens GN=CRTAC1 PE=1 SV=2



PROTEIN ACC. NUMBER	PROTEIN NAME
P0CG05 LAC2_HUMAN	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1
B9A064 IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens GN=IGLL5 PE=2 SV=2
P17936 IBP3_HUMAN	Insulin-like growth factor-binding protein 3 OS=Homo sapiens GN=IGFBP3 PE=1 SV=2
P01714 LV301_HUMAN	Ig lambda chain V-III region SH OS=Homo sapiens PE=1 SV=1

Protein acc. Number = Protein accession Number.

**Supplementary Table 9: Protein species which are present in lower amounts in OA synovial fluid compared to non-OA synovial fluid, as well as present in the secretomes in a tissue-dependent manner.**

PROTEIN ACC. NUMBER	PROTEIN NAME
O75339 CILP1_HUMAN	Cartilage intermediate layer protein 1 OS=Homo sapiens GN=CILP PE=1 SV=4
Q8IUL8 CILP2_HUMAN	Cartilage intermediate layer protein 2 OS=Homo sapiens GN=CILP2 PE=2 SV=2
Q14767 LTBP2_HUMAN	Latent-transforming growth factor beta-binding protein 2 OS=Homo sapiens GN=LTBP2 PE=1 SV=3
P05387 RLA2_HUMAN	60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1
P00325 ADH1B_HUMAN	Alcohol dehydrogenase 1B OS=Homo sapiens GN=ADH1B PE=1 SV=2
P03973 SLPI_HUMAN	Antileukoproteinase OS=Homo sapiens GN=SLPI PE=1 SV=2
P16112 PGCA_HUMAN	Aggrecan core protein OS=Homo sapiens GN=ACAN PE=1 SV=2
Q06828 FMOD_HUMAN	Fibromodulin OS=Homo sapiens GN=FMOD PE=1 SV=2
P21333 FLNA_HUMAN	Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4
Q13642 FHL1_HUMAN	Four and a half LIM domains protein 1 OS=Homo sapiens GN=FHL1 PE=1 SV=4
P40121 CAPG_HUMAN	Macrophage-capping protein OS=Homo sapiens GN=CAPG PE=1 SV=2
P06753 TPM3_HUMAN	Tropomyosin alpha-3 chain OS=Homo sapiens GN=TPM3 PE=1 SV=2
P19971 TYPH_HUMAN	Thymidine phosphorylase OS=Homo sapiens GN=TYMP PE=1 SV=2
P37802 TAGL2_HUMAN	Transgelin-2 OS=Homo sapiens GN=TAGLN2 PE=1 SV=3
P34096 RNASE4_HUMAN	Ribonuclease 4 OS=Homo sapiens GN=RNASE4 PE=1 SV=3
P29401 TKT_HUMAN	Transketolase OS=Homo sapiens GN=TKT PE=1 SV=3

## Identification of Tissue Type-Dependent Proteins in Knee OA Synovial Fluid

PROTEIN ACC. NUMBER	PROTEIN NAME
Q05707 COEA1_HUMAN	Collagen alpha-1(XIV) chain OS=Homo sapiens GN=COL14A1 PE=1 SV=3
Q01518 CAP1_HUMAN	Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=5
P07585 PGS2_HUMAN	Decorin OS=Homo sapiens GN=DCN PE=1 SV=1
P13489 RINI_HUMAN	Ribonuclease inhibitor OS=Homo sapiens GN=RNH1 PE=1 SV=2
P14555 PA2GA_HUMAN	Phospholipase A2, membrane associated OS=Homo sapiens GN=PLA2G2A PE=1 SV=2
Q14195 DPYL3_HUMAN	Dihydropyrimidinase-related protein 3 OS=Homo sapiens GN=DPYSL3 PE=1 SV=1
P62805 H4_HUMAN	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2
P19338 NUCL_HUMAN	Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3
P00488 F13A_HUMAN	Coagulation factor XIII A chain OS=Homo sapiens GN=F13A1 PE=1 SV=4
P98095 FBLN2_HUMAN	Fibulin-2 OS=Homo sapiens GN=FBLN2 PE=1 SV=2
Q96Q06 PLIN4_HUMAN	Perilipin-4 OS=Homo sapiens GN=PLIN4 PE=1 SV=2
P14618 KPYM_HUMAN	Pyruvate kinase PKM OS=Homo sapiens GN=PKM PE=1 SV=4
Q9BXR6 FHR5_HUMAN	Complement factor H-related protein 5 OS=Homo sapiens GN=CFHR5 PE=1 SV=1
P08238 HS90B_HUMAN	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4
Q8IUX7 AEBP1_HUMAN	Adipocyte enhancer-binding protein 1 OS=Homo sapiens GN=AEBP1 PE=1 SV=1
Q9ULV4 COR1C_HUMAN	Coronin-1C OS=Homo sapiens GN=CORO1C PE=1 SV=1
Q06830 PRDX1_HUMAN	Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1
P46940 IQGA1_HUMAN	Ras GTPase-activating-like protein IQGAP1 OS=Homo sapiens GN=IQGAP1 PE=1 SV=1



PROTEIN ACC. NUMBER	PROTEIN NAME
P15090 FABP4_HUMAN	Fatty acid-binding protein, adipocyte OS=Homo sapiens GN=FABP4 PE=1 SV=3
P40925 MDHC_HUMAN	Malate dehydrogenase, cytoplasmic OS=Homo sapiens GN=MDH1 PE=1 SV=4
P08670 VIME_HUMAN	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4
O75369 FLNB_HUMAN	Filamin-B OS=Homo sapiens GN=FLNB PE=1 SV=2
P02743 SAMP_HUMAN	Serum amyloid P-component OS=Homo sapiens GN=APCS PE=1 SV=2
O75874 IDHC_HUMAN	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Homo sapiens GN=IDH1 PE=1 SV=2
Q9UKZ9 PCOC2_HUMAN	Procollagen C-endopeptidase enhancer 2 OS=Homo sapiens GN=PCOLCE2 PE=1 SV=1
P37802 TAGL2_HUMAN	Transgelin-2 OS=Homo sapiens GN=TAGLN2 PE=1 SV=3
Q14195 DPYL3_HUMAN	Dihydropyrimidinase-related protein 3 OS=Homo sapiens GN=DPYSL3 PE=1 SV=1
Q8N335 GPD1L_HUMAN	Glycerol-3-phosphate dehydrogenase 1-like protein OS=Homo sapiens GN=GPD1L PE=1 SV=1
P00338 LDHA_HUMAN	L-lactate dehydrogenase A chain OS=Homo sapiens GN=LDHA PE=1 SV=2
P35443 TSP4_HUMAN	Thrombospondin-4 OS=Homo sapiens GN=THBS4 PE=1 SV=2
P52209 6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE=1 SV=3
Q99497 PARK7_HUMAN	Protein deglycase DJ-1 OS=Homo sapiens GN=PARK7 PE=1 SV=2
P04075 ALDOA_HUMAN	Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2
P30041 PRDX6_HUMAN	Peroxiredoxin-6 OS=Homo sapiens GN=PRDX6 PE=1 SV=3
P08294 SODE_HUMAN	Extracellular superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD3 PE=1 SV=2

PROTEIN ACC. NUMBER	PROTEIN NAME
P07998 RNAS1_HUMAN	Ribonuclease pancreatic OS=Homo sapiens GN=RNASE1 PE=1 SV=4
P21399 ACOC_HUMAN	Cytoplasmic aconitate hydratase OS=Homo sapiens GN=ACO1 PE=1 SV=3
Q9Y279 VSIG4_HUMAN	V-set and immunoglobulin domain-containing protein 4 OS=Homo sapiens GN=VSIG4 PE=1 SV=1

Protein acc. Number = Protein accession Number.

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## References

- 1 Hunter D.J., Felson D.T.: Osteoarthritis. *BMJ* 2006; 332: pp. 639-642.
- 2 van der Kraan P.M., Blaney Davidson E.N., Blom A., van den Berg W.B.: TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. *Osteoarthritis Cartilage* 2009; 17: pp. 1539-1545.
- 3 Petersson I.F., Boegard T., Saxne T., Silman A.J., Svensson B.: Radiographic osteoarthritis of the knee classified by the Ahlback and Kellgren & Lawrence systems for the tibiofemoral joint in people aged 35-54 years with chronic knee pain. *Ann Rheum Dis* 1997; 56: pp. 493-496.
- 4 Glyn-Jones S., Palmer A.J., Agricola R., Price A.J., Vincent T.L., Weinans H., *et. al.*: Osteoarthritis. *Lancet* 2015; 386: pp. 376-387.
- 5 Loeser R.F., Goldring S.R., Scanzello C.R., Goldring M.B.: Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum* 2012; 64: pp. 1697-1707.
- 6 Ripmeester E.G.J., Timur U.T., Caron M.M.J., Welting T.J.M.: Recent insights into the contribution of the changing hypertrophic chondrocyte phenotype in the development and progression of osteoarthritis. *Front Bioeng Biotechnol* 2018; 6: pp. 18.
- 7 Peffers M.J., Balaskas P., Smagul A.: Osteoarthritis year in review 2017: genetics and epigenetics. *Osteoarthritis Cartilage* 2018; 26: pp. 304-311.
- 8 Zhai G.: Alteration of metabolic pathways in osteoarthritis. *Metabolites* 2019; 9:
- 9 Nguyen L.T., Sharma A.R., Chakraborty C., Saibaba B., Ahn M.E., Lee S.S.: Review of prospects of biological fluid biomarkers in osteoarthritis. *Int J Mol Sci* 2017; 18:
- 10 Ritter S.Y., Subbaiah R., Bebek G., Crish J., Scanzello C.R., Krastins B., *et. al.*: Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis Rheum* 2013; 65: pp. 981-992.
- 11 Del Rey M.J., Usategui A., Izquierdo E., Canete J.D., Blanco F.J., Criado G., *et. al.*: Transcriptome analysis reveals specific changes in osteoarthritis synovial fibroblasts. *Ann Rheum Dis* 2012; 71: pp. 275-280.
- 12 Carlson A.K., Rawle R.A., Wallace C.W., Brooks E.G., Adams E., Greenwood M.C.,



- et. al.*: Characterization of synovial fluid metabolomic phenotypes of cartilage morphological changes associated with osteoarthritis. *Osteoarthritis Cartilage* 2019; 27: pp. 1174-1184.
- 13 Kosinska M.K., Mastbergen S.C., Liebisch G., Wilhelm J., Dettmeyer R.B., Ishaque B., *et. al.*: Comparative lipidomic analysis of synovial fluid in human and canine osteoarthritis. *Osteoarthritis Cartilage* 2016; 24: pp. 1470-1478.
- 14 Favero M., Belluzzi E., Frallonardo P., Peruzzo L., Tauro L., Oliviero F., *et. al.*: Synovial fluid fetuin-A levels in patients affected by osteoarthritis with or without evidence of calcium crystals. *Rheumatology* 2019; 58: pp. 729-730.
- 15 Dong N., Gao Y.H., Liu B., Zhao C.W., Yang C., Li S.Q., *et. al.*: Differential expression of adipokines in knee osteoarthritis patients with and without metabolic syndrome. *Int Orthop* 2018; 42: pp. 1283-1289.
- 16 Arellano Perez Vertti R.D., Aguilar Muniz L.S., Moran Martinez J., Gonzalez Galarza F.F., Arguello Astorga R.: Cartilage oligomeric matrix protein levels in type 2 diabetes associated with primary knee osteoarthritis patients. *Genet Test Mol Biomarkers* 2019; 23: pp. 16-22.
- 17 Clair A.J., Kingery M.T., Anil U., Kenny L., Kirsch T., Strauss E.J.: Alterations in synovial fluid biomarker levels in knees with meniscal injury as compared with asymptomatic contralateral knees. *Am J Sports Med* 2019; 47: pp. 847-856.
- 18 Peffers M.J., Beynon R.J., Clegg P.D.: Absolute quantification of selected proteins in the human osteoarthritic secretome. *Int J Mol Sci* 2013; 14: pp. 20658-20681.
- 19 Peffers M.J., McDermott B., Clegg P.D., Riggs C.M.: Comprehensive protein profiling of synovial fluid in osteoarthritis following protein equalization. *Osteoarthritis Cartilage* 2015; 23: pp. 1204-1213.
- 20 Anderson J.R., Phelan M.M., Rubio-Martinez L.M., Fitzgerald M.M., Jones S.W., Clegg P.D., *et. al.*: Optimization of synovial fluid collection and processing for NMR metabolomics and LC-MS/MS proteomics. *J Proteome Res* 2020 Jul 2; 19: pp. 2585-2597..
- 21 Perez-Riverol Y., Csordas A., Bai J., Bernal-Llinares M., Hewapathirana S., Kundu D.J., *et. al.*: The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res* 2019; 47: pp. D442-D450.
- 22 Storey J.D.: A direct approach to false discovery rates. *J Roy Stat Soc B* 2002; 64: pp. 479-498.
- 23 Benjamini Y., Yekutieli D.: Quantitative trait Loci analysis using the false discovery rate.

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- Genetics 2005; 171: pp. 783-790.
- 24 Storey J.D., Tibshirani R.: Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A* 2003; 100: pp. 9440-9445.
- 25 Core Team: R: A Language and Environment for Statistical Computing. 2018. R Foundation for Statistical Computing Vienna, Austria Available online at: <https://www.R-project.org/>
- 26 Rohart F., Gautier B., Singh A., Le Cao K.A.: mixOmics: an R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol* 2017; 13:
- 27 Gobezie R., Kho A., Krastins B., Sarracino D.A., Thornhill T.S., Chase M., *et al.*: High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis Res Ther* 2007; 9: pp. R36
- 28 Mobasheri A., Henrotin Y.: Biomarkers of (osteo)arthritis. *Biomarkers* 2015; 20: pp. 513-518.
- 29 Kraus V., Hsueh M.: Chapter 22 in *Genomic and Precision Medicine*. 3rd edn. 2019. Infectious and Inflammatory Disease pp. 429-444.
- 30 van Spil W.E., Szilagyi I.A.: Osteoarthritis year in review 2019: biomarkers (biochemical markers). *Osteoarthritis Cartilage* 2020; 28: pp. 296-315.
- 31 Davies D.V.: Synovial membrane and synovial fluid of joints. *Lancet* 1946; 2: pp. 815-819.
- 32 Balakrishnan L., Nirujogi R.S., Ahmad S., Bhattacharjee M., Manda S.S., Renuse S., *et al.*: Proteomic analysis of human osteoarthritis synovial fluid. *Clin Proteomics* 2014; 11: pp. 6.
- 33 Scanzello C.R., Umoh E., Pessler F., Diaz-Torne C., Miles T., Dicarolo E., *et al.*: Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. *Osteoarthritis Cartilage* 2009; 17: pp. 1040-1048.
- 34 Kamphorst J.J., van der Heijden R., DeGroot J., Lafeber F.P., Reijmers T.H., van El B., *et al.*: Profiling of endogenous peptides in human synovial fluid by NanoLC-MS: method validation and peptide identification. *J Proteome Res* 2007; 6: pp. 4388-4396.
- 35 Pan X., Huang L., Chen J., Dai Y., Chen X.: Analysis of synovial fluid in knee joint of osteoarthritis: 5 proteome patterns of joint inflammation based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Int Orthop* 2012; 36: pp. 57-64.
- 36 Ritter S.Y., Subbaiah R., Bebek G., Crish J., Scanzello C.R., Krastins B., *et al.*:

- Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis Rheum* 2013; 65: pp. 981-992.
- 37 Wang Q., Rozelle A.L., Lepus C.M., Scanzello C.R., Song J.J., Larsen D.M., *et. al.*: Identification of a central role for complement in osteoarthritis. *Nat Med* 2011; 17: pp. 1674-1679.
- 38 Bastiaansen-Jenniskens Y.M., Wei W., Feijt C., Waarsing J.H., Verhaar J.A., Zuurmond A.M., *et. al.*: Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: a possible role for prostaglandin f2alpha. *Arthritis Rheum* 2013; 65: pp. 2070-2080.
- 39 Chu S.C., Yang S.F., Lue K.H., Hsieh Y.S., Li T.J., Lu K.H.: Naproxen, meloxicam and methylprednisolone inhibit urokinase plasminogen activator and inhibitor and gelatinases expression during the early stage of osteoarthritis. *Clin Chim Acta* 2008; 387: pp. 90-96.
- 40 Waarsing J.H., Bierma-Zeinstra S.M., Weinans H.: Distinct subtypes of knee osteoarthritis: data from the Osteoarthritis Initiative. *Rheumatology* 2015; 54: pp. 1650-1658.
- 41 Nelson A.E., Fang F., Arbeeve L., Cleveland R.J., Schwartz T.A., Callahan L.F., *et. al.*: A machine learning approach to knee osteoarthritis phenotyping: data from the FNIH Biomarkers Consortium. *Osteoarthritis Cartilage* 2019; 27: pp. 994-1001.



Chapter

08



**General Discussion, Conclusion and Clinical Perspectives for the Future**

The knee joint is exposed to biomechanical demands and complex interactions exist between its biological composition<sup>1</sup>. From a micro-environmental point of view, distinct cell types exist within the different intra-articular tissues, which are responsible for a physiological joint functioning. From a macro-environmental point of view, all these distinct cell types and joint tissues form a large functioning unit, the knee joint, which is able to withstand biomechanical forces and support locomotion. External influences on the knee joint can exceed physiological limits, but also internal derangements can lower the capacity for adaptive responses<sup>2</sup>. This will lead to a disruption in knee joint homeostasis leading to an impaired knee joint quality<sup>3</sup>. Knee joint quality depends on the equilibrium between intra-articular tissues such as cartilage, synovium, Hoffa's fat pad (HFP), meniscus, but also extra-articular tissues such as peri-articular muscles<sup>2,4</sup>. Risk factors such as ageing, inflammatory diseases, sex, race, obesity, genetics, joint trauma or loading on a maligned joint are examples of external or internal influences which can induce degenerative changes in joint tissues and cells within the joint<sup>3,5,6,7</sup>. This will consequently lead to a loss of joint tissue quality. If tissue quality is compromised and cells within these tissues cannot compensate by means of tissue remodeling processes, the equilibrium within the knee joint becomes chronically disturbed and even normal daily activity levels may become pathological, leading to inferior 'knee joint quality'. In this thesis, we aimed to gain new insights in novel strategies to improve 'knee joint quality' either on a cellular, tissue or total knee joint level.



# 1. Improving Knee Joint Quality on the Cellular Level

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### 1.1 Improving the Chondrocyte Phenotype through Culturing Chondrocytes under Conditions Resembling their *In Vivo* Physiological Environment

Focal cartilage defects may lead to inferior knee joint quality due to distorted joint biomechanics, an increased inflammatory intra-articular environment, and ultimately lead to osteoarthritis (OA)<sup>8</sup>. Therefore, we have evaluated molecular mechanisms involved in the phenotypic improvement of human articular chondrocytes. This may lead to improvements in cell-based cartilage repair strategies and thereby supporting post-treatment knee joint quality<sup>9</sup>. In **Chapter 2**, we showed that culturing human articular chondrocytes under oxygen tension closely resembling their physiological *in vivo* environment, improves collagen type II expression via TGF- $\beta$ <sup>10</sup>. Given the fact that hypoxia has been shown to upregulate mRNA expression of TGF- $\beta$  superfamily members in different cell types<sup>11,12,13</sup>, we hypothesized that TGF- $\beta$  is involved in improving the chondrocyte phenotype in hypoxic cultures. Using specialized bioreactor technology, human articular chondrocytes were cultured under hypoxic (1% pO<sub>2</sub>) or normoxic (20 % pO<sub>2</sub>) conditions. We first confirmed that culturing chondrocytes under hypoxic conditions improves the chondrocyte phenotype. Interestingly, of all three TGF- $\beta$  isoforms, TGF- $\beta$ 2 expression was specifically upregulated under hypoxia on both gene and protein level, as were TGF- $\beta$  type I receptors ALK 1 and ALK5. Characterization of the human TGF- $\beta$  isoforms revealed that TGF- $\beta$ 2 possesses the longest 5' untranslated region (UTR), which hints towards a unique post-transcriptional regulation of its protein translation<sup>14</sup>. TGF- $\beta$ 2 further differs from the other two isoforms by its binding affinity towards the TGF- $\beta$  type II receptor. In contrast to TGF- $\beta$ 1 and TGF- $\beta$ 3, its affinity for TGF- $\beta$  type II receptors is weaker and requires the type III receptor betaglycan<sup>15</sup>. While in this study we were not able to show regulation of betaglycan under hypoxic conditions at the mRNA level, post-transcriptional regulation may regulate betaglycan protein levels, which may lead to improved TGF- $\beta$ 2 ligand signaling. Indeed, in another study it was found that under hypoxia, endoglin expression was reduced in chondrocytes while faint betaglycan immunoblotting signals appeared increased, hinting towards a role of this assessor receptor in TGF- $\beta$  mediated *COL2A1* expression<sup>16</sup>.

As cartilage is generally considered to be "hypoxic and hyperosmotic"<sup>17,18</sup>, the involvement of the TGF- $\beta$  pathway in the improvement of the chondrocyte phenotype under hypoxic

conditions prompted us to also investigate its role under hyperosmotic conditions in **Chapter 3**. We could confirm an improved chondrocyte phenotype under hyperosmotic conditions. Importantly, we reported earlier that this is not related to accumulation of specific inorganic ions but due to specific osmolarity-mediated effects<sup>19</sup>. Interestingly, hyperosmotic conditions also most prominently increased TGF- $\beta$ 2 mRNA expression, which was in concordance with our experiments under hypoxic conditions. This may indicate that of the three TGF- $\beta$  isoforms, TGF- $\beta$ 2 has a prominent role in regulating the chondrocyte phenotype under hyperosmotic and hypoxic conditions.

Crosstalk between TGF- $\beta$  superfamily members has been shown to exert synergistic or antagonistic effects on a myriad of events, such as cell proliferation, migration and matrix synthesis in different cell types such as epithelial cells<sup>20</sup>, but also in chondrocytes<sup>21</sup>. Bone morphogenetic proteins (BMPs) are members of the TGF- $\beta$  superfamily<sup>22</sup>. By using the BMP-signaling inhibitor dorsomorphin, we further elucidated the influence of TGF- $\beta$  superfamily members on *COL2A1* expression in human articular chondrocytes under TGF- $\beta$ 2 knockdown conditions. While *COL2A1* expression appeared to be largely BMP-signaling dependent in human articular chondrocytes (HACs), we found higher *COL2A1* expression levels under hyperosmotic condition compared to non-hyperosmotic conditions under dorsomorphin treatment. Apparently, hyperosmotic conditions stimulate *COL2A1* expression independently of TGF- $\beta$  superfamily signaling. While many of the signaling responses induced by TGF- $\beta$  are mediated by SMAD proteins, evidence is accumulating that TGF- $\beta$  can signal via SMAD-independent pathways, such as MAP kinases<sup>23</sup>. To understand the mechanisms involved in this specific TGF- $\beta$  signalling, it is crucial to unravel the intracellular events that provide a link between activated TGF- $\beta$  on the cell surface and its downstream effects. The mitogen-activated protein kinase (MAPK) pathway consists of several phosphorylation cascades, each of which modulates different signalling events, either alone or in concert with other pathways<sup>24</sup>. Three major subgroups of the MAPK super family, Extracellular signal-regulated kinases (ERK), p38 MAP kinase and c-Jun N-terminal kinase (JNK), have been found to participate in TGF- $\beta$  signalling<sup>25,26,27</sup>. These kinases can be activated by various extracellular stimuli, including environmental stressors such as hypoxia and hyperosmolarity, and they play an essential role in the signal transduction



cascades that alter cell growth and other key cell functions<sup>28,29,30</sup>. In future experiments, it will be of interest to investigate the influence of hyperosmotic and hypoxic conditions on these alternative TGF- $\beta$  signalling components. Targeting downstream kinase targets such as ERK, p38 and JNK in future experiments may give more insights into hypoxia and osmolarity-mediated effects on the chondrocyte phenotype.

Stresses such as osmolarity have been shown to enhance susceptibility to TGF- $\beta$  by increasing TGF- $\beta$  type I receptor protein levels and downregulating the expression of TGF- $\beta$  signalling inhibiting SMAD proteins (i.e. SMAD7)<sup>31</sup>. This increased susceptibility to TGF- $\beta$  signalling may be a contributing mechanism and potential explanation for the increased TGF- $\beta$  bioactivity we have demonstrated under hyperosmotic conditions. Furthermore, cytoskeletal changes may activate latent TGF- $\beta$ , thereby releasing TGF- $\beta$  from extracellular matrix (ECM) stores<sup>32</sup>. Latent TGF- $\beta$  activation requires a contractile cytoskeleton<sup>33</sup>. In kidney cells, it has been shown that osmotic stress can activate latent TGF- $\beta$ <sup>34</sup>, and this might not be different in other cell types such as the chondrocyte. We speculate that cytoskeletal changes caused by an increase in osmolarity<sup>18</sup> may lead to contractile forces in the cytoskeleton potentially leading to increased TGF $\beta$  activity. Future experiments have to elucidate the exact mechanisms involved in the osmolarity mediated activation of latent TGF- $\beta$ .

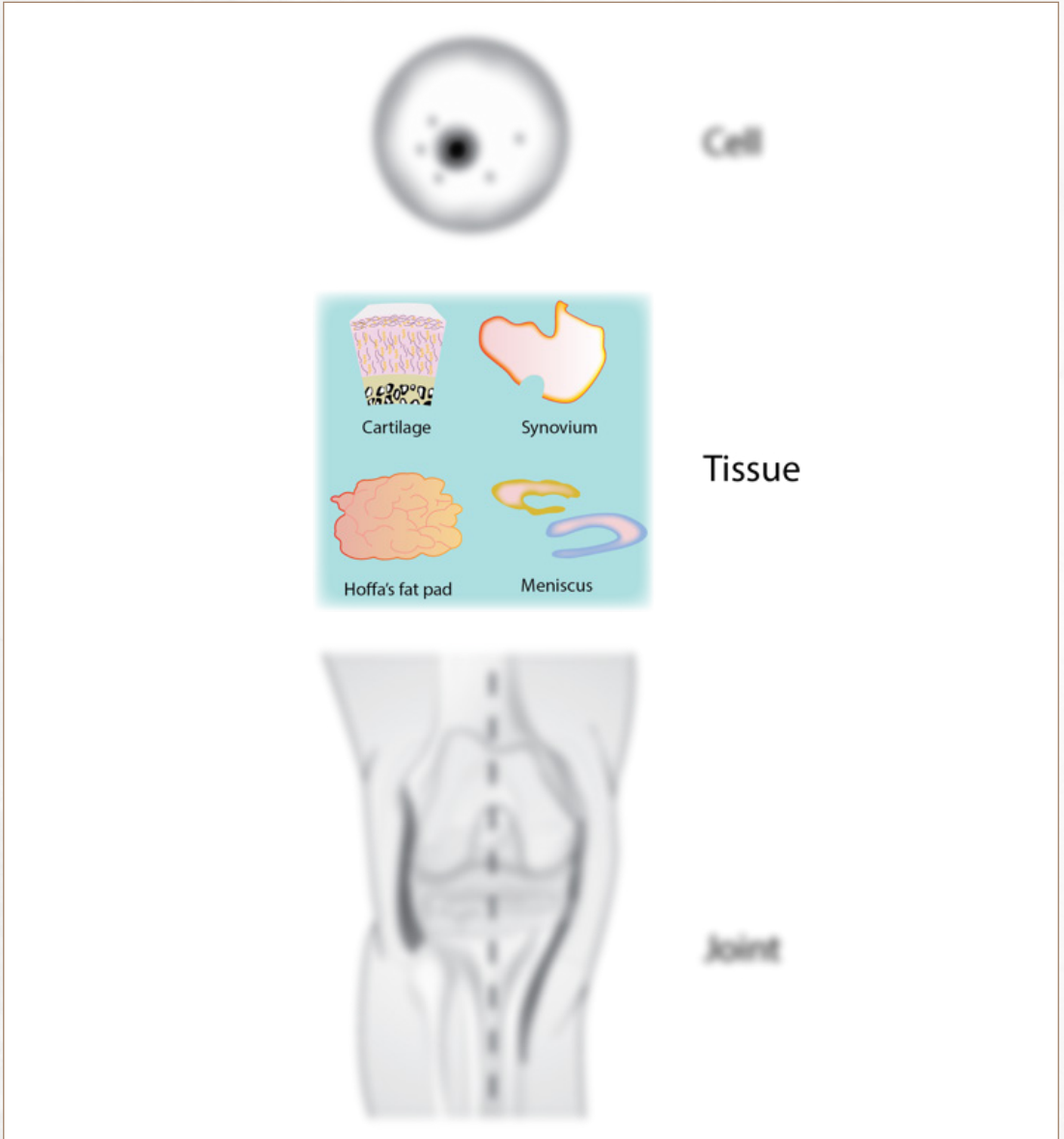
We do acknowledge that the hyperosmotic and hypoxic culture conditions we have described in **Chapter 2** and **Chapter 3** can increase the expression of hypertrophic chondrocyte markers such as collagen type X<sup>36,37</sup>. This notion has important clinical implications, since another concern described for cartilage regenerative approaches is the hypertrophic differentiation of the cartilage graft<sup>38</sup>. Hypertrophic differentiation of chondrocytes has been described for ACI, perichondrium transplantation and microfracture<sup>39,40</sup>. Moreover, we have published a review in which we show that OA chondrocytes display features of hypertrophic differentiation showing changes which resemble the process of endochondral ossification<sup>41</sup>. Importantly, targeting chondrocyte hypertrophic processes may aid in improving cartilage-repair strategies and slow down knee OA progression. Our group previously published findings showed that COX-2 inhibition during chondrogenic differentiation of progenitor cells effectively decreased the level of chondrocyte

hypertrophic differentiation in *in vitro*, *ex vivo* and *in vivo* chondrogenic models<sup>42</sup>. While we showed that TGF- $\beta$  signaling is involved in chondrocyte phenotype improvement, TGF- $\beta$  has also been associated with an induction of COX-2 expression<sup>43,44</sup>. This induction may lead to hypertrophic differentiation of chondrocytes. Therefore, combining hyperosmotic or hypoxic chondrocyte treatment with selective COX-2 inhibitors may be used to prevent hypertrophic differentiation under these environmental conditions.

In conclusion, based on our results and previous literature, our data from **Chapter 2 and 3** further support existing evidence that TGF- $\beta$  superfamily members are in a tight interplay in regulating the chondrocyte phenotype in environmental conditions relevant for the articular chondrocyte. These environmental conditions describing physiological oxygenation and osmolarity levels in cartilage, could in future research be described as 'physoxia' and 'physosmolarity'.

## 2. Improving Joint Quality on the Tissue Level

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## 2.1 Hoffa's Fat Pad: An Important Participant in Determining Knee Joint Quality

One of the knee joint components which is increasingly being recognized as an important actor in joint pathologies such as knee OA<sup>45</sup>, is the HFP. In **Chapter 4** of this thesis, we have focused on this specific knee joint component with a specific focus on inflammatory processes. Inflammation is thought to be a physiological response to a disruption in the homeostatic balance and can be defined as an activation of effector mechanisms in an attempt to restore homeostatic balance and return the system to the status quo<sup>46</sup>. Gaining insights into the role of these inflammatory processes for knee joint quality may yield important clues to develop new treatment strategies to improve knee joint quality.

In **Chapter 4**, we have compared release of prostaglandins, important molecules involved in inflammation<sup>47</sup>, between HFP from OA and cartilage defect patients. Next to comparing the inflammatory profile between OA and cartilage defect patients, we also investigated the ability to modulate inflammation in HFP from these patients by means of inhibiting prostaglandin production by celecoxib treatment. Our data support the notion that the HFP is an important contributor to inflammation in joint pathologies such as knee OA but also cartilage defects and should not solely be considered as a shock absorber in the knee joint<sup>45</sup>. The secretion of prostanoid subtypes from the HFP from knee OA patients distributed into 2 OA subgroups. Subgrouping may be related to a different subtype of knee OA, duration of OA development and/or inhomogeneity of HFP tissue with differentially distributed immune cells. Of note, a recent study demonstrated that knee OA patients with hypercholesterolemia have altered expression levels of the downstream PGE<sub>2</sub> synthase, mPGES, in HFP<sup>48</sup>. Thus, patient specific characteristics, such as hypercholesterolemia, appear to result in differential expression levels of enzymes involved in inflammation. Our findings in **Chapter 4** showing variable secretion of prostanoid subtypes distributing to 2 OA subgroups is further in accordance with previous work from Massicotte *et al.* showing variable secretion of PGE<sub>2</sub> by subchondral osteoblasts from knee OA patients, which distributed to 2 OA subgroups<sup>49</sup>. In our study, in addition to PGE<sub>2</sub>, release of two other prostanoid subtypes PGF<sub>2α</sub> and PGD<sub>2</sub>, but not TXB<sub>2</sub>, was higher in the high-PGE<sub>2</sub> OA group than the cartilage defects group, and correlated with increased COX-2 expression. Hence, specific prostanoid subtypes are released from the OA HFP and may participate in the

inflammatory response in the HFP.

Given the fact that obesity leads to inflammatory changes in adipose tissue<sup>50</sup>, one can hypothesize that similar inflammatory changes may also be present in HFP of obese patients which eventually may lead to articular cartilage pathology. Kisand and colleagues showed prominent associations between OA in the patellofemoral joint in woman, BMI and the adipokine leptin<sup>51</sup>. However, in our study we did not identify a correlation between prostanoid levels released by HFP and BMI in **Chapter 4**. Also other colleagues in the field do not show an association of BMI and inflammatory changes in HFP<sup>52,53</sup>. Moreover, in contrast to subcutaneous adipose tissue, nor HFP size neither size of the adipocytes in the osteoarthritic HFP is correlated to body mass index<sup>52,54,55,56</sup>. These findings emphasize that the HFP should be considered as a unique adipose tissue, which is distinct from subcutaneous adipose tissue, and suggests that HFP may not be involved in obesity-related joint environmental changes in knee OA.

To evaluate the possibility of improving joint homeostasis via influencing the HFP inflammatory status, we have used the specific COX-2 inhibitor celecoxib to target the inflamed HFP in **Chapter 4**. Consistent with our data showing a correlation between *PTGS2* and PGE<sub>2</sub> release in HFP, the COX-2 inhibitor celecoxib was able to effectively reduce PGE<sub>2</sub> release by HFP by at least 80 %. These data highlight the potential of celecoxib to target the inflamed HFP and reduce release of prostanoids known to be involved in inflammation<sup>57</sup>. We did not evaluate the action of celecoxib on specific cell types present within the HFP, but our experiments in **Chapter 4** showed that celecoxib treatment of the inflamed HFP modulated expression of factors associated with M1 and M2 macrophages. Furthermore, celecoxib was able to skew the expression ratio of M1/M2 markers towards a more favorable M2 phenotype most effectively in HFP tissue with higher prostanoid release. This indicates that the capacity of celecoxib to skew towards the expression of markers associated with M2 macrophages in HFP tissue is dependent on the amount of inflammation present. Future research should address celecoxib mediated effects on macrophage phenotype in the HFP using flow-cytometry. Furthermore, it will also be interesting to investigate anti-inflammatory effects of celecoxib on other immune cells such as lymphocytes and mast cells which have been shown to be present in HFP<sup>45</sup>.

From **Chapter 4** it became evident that the HFP of patients with cartilage defects produce prostanoid subtypes which are involved in inflammation. This supports the theory that the HFP of patients with cartilage defects is an important contributor to inflammatory factors found in synovial fluid of this patient group. The positive association between pro-inflammatory factors in synovial fluid and cartilage degradation is increasingly being recognized<sup>46,58,59</sup>. Considering reports showing that targeting pro-inflammatory cytokines in knee joint synovial fluid may attenuate cartilage degradation *in vivo* following knee injury<sup>58</sup>, it is likely that an increased inflammatory profile in knee joint synovial fluid negatively affects the outcome of cartilage repair strategies. Therefore, our findings in **Chapter 4** demonstrating a difference in prostanoid release profile of HFPs from patients suffering from a cartilage defect may have important therapeutical implications. Specifically, it may implicate that certain patients with an increased inflammatory profile in their HFP may not optimally benefit from cartilage repair approaches. Next to aiding in decision-making for cartilage repair surgery, the level of HFP inflammation may also be used to predict the outcome of cartilage repair surgery.

In conclusion, the HFP is an important actor in determining knee joint quality and by secreting prostanoids and other inflammatory molecules participates in the inflammatory process observed in joint pathologies such as knee OA and cartilage defects. Specific prostanoids secreted by the HFP may serve as biomarkers in synovial fluid to assess the health status of the knee joint, and celecoxib may be used to improve knee joint quality via modulating HFP inflammation.

## 2.2 The Influence of other Intra-Articular Tissues on Knee Joint Quality

Knee joint quality is not attributable to a single joint tissue, but is characterized by the interplay of different intra-articular and extra-articular tissues. Although the work in this thesis has focused on anti-inflammatory modulation of the HFP, we do acknowledge the role of other intra-articular tissues in determining knee joint quality. Next to the HFP other fat pads are present in the knee joint: the posterior fat pad, anterior suprapatellar fat pad and the posterior suprapatellar fat pad<sup>45</sup>. Interestingly, the suprapatellar fat pad together with another fat pad located in the hip joint, the acetabular fat pad, has been shown to



release higher amounts of inflammatory mediators such as IL-7, IL-8 and PGE<sub>2</sub> compared to subcutaneous adipose tissue<sup>60</sup>. Moreover, stimulation of fibroblast-like synoviocytes with medium conditioned by the HFP and the suprapatellar fat pad from knee OA patients induces an inflammatory response in synoviocytes<sup>60</sup>. This indicates cross-talk between intra-articular adipose tissue and synovium within the joint, which may be involved in joint inflammation. In addition, it has been shown that ACL rupture in sheep results in inflammatory changes in the HFP<sup>61</sup>, and also inflammatory changes in synovium have been described<sup>62</sup>. Therefore, anti-inflammatory actions of celecoxib on these tissues *in vivo* may lead to improved joint homeostasis.

The subchondral bone is another functional unit in the knee joint, releasing factors in to the synovial fluid via diffusion through cartilage<sup>63</sup>. The subchondral bone displays a broad range of functional disarrangements in knee OA. At the osteochondral junction of osteoarthritic cartilage, a duplication and sometimes multiplication of the tidemark and vascular channels entering the non-calcified cartilage have been observed<sup>64,65</sup>. The vascular invasion results in advancement of the calcified cartilage layer into the deep zones of the articular cartilage, which leads to thinning of the articular cartilage<sup>66,67</sup>. The subchondral bone in OA cartilage has been shown to display altered biomechanical properties due to bone resorption at the osteochondral junction<sup>68,69</sup>. Moreover, pain in knee OA may be related to the increased osteochondral vascularity which is observed in knee OA<sup>64</sup>. In addition, bone marrow lesions in the subchondral bone have also been described which may be a cause of pain in OA patients<sup>70,71</sup>. These lesions are characterized by a focally increased signal on fat-suppressed T2-weighted MRI imaging<sup>70</sup>. Histological evaluation of these lesions demonstrate microfractures of the trabecular bone structure, fibrosis, medullary fat necrosis, hypervascularity and poor mineralization<sup>72</sup>. A novel treatment approach to target bone marrow lesions is subchondroplasty<sup>73</sup>. This technique involves the injection of calcium phosphate into the space between trabeculae of cancellous bone in the subchondral bone where the bone marrow lesion is present. It has been suggested to result in pain reduction in knee OA patients<sup>73,74</sup>.

The anterior cruciate ligament (ACL) is another tissue present in the knee joint which plays an important role in knee joint quality. The ACL is essential for knee joint kinematics and

acts as an anterior/posterior stabilizer but also provides knee joint stability during rotatory movements<sup>75</sup>. An ACL rupture makes the knee joint more susceptible to damage to important joint structures such as articular cartilage and the meniscus<sup>76</sup>. It is frequently associated with bone bruises in the dorsal lateral tibia plateau and the anterior femoral condyle leading to subchondral fractures, which may lead to future cartilage damage<sup>77</sup>. In addition, altered knee joint kinematics leading to a shift in tibiofemoral cartilage loading has been shown in an ACL-deficient knee<sup>78</sup>. This may on the long-term lead to cartilage deterioration and future OA. Next to alterations in knee joint kinematics, studies have also shown that the ACL is altered at the molecular and cellular level. For instance, neovascularization, leucocyte infiltration, a disorganization of collagen fibers and chondroid metaplasia are well-known features of the ACL that occur during joint pathologies such as knee OA<sup>79,80,81</sup>.

The meniscus plays a major role in knee joint homeostatic processes, both from a mechanical and biochemical point of view. Traditionally, it has been thought that the meniscus plays an important role in maintaining joint homeostasis due to its ability to absorb shocks and to increase joint congruency<sup>82</sup> and huge scientific efforts have been made in order to develop treatment strategies for meniscal tears, given its association with future OA. However, less focus has been paid on the meniscus as a contributor to molecular mediators in synovial fluid that can compromise joint homeostasis. The meniscus is not merely a bystander in knee OA: it is an active participant in knee OA by secreting mediators involved in inflammatory processes<sup>83</sup>. This is further supported by reports from the literature. For instance, it has been shown that when exposed to impact injury the meniscus releases different mediators involved in inflammation such as PGE<sub>2</sub> and IL-1 $\beta$ <sup>84</sup>. In addition, stimulating the meniscus with IL-1 $\beta$  leads to the secretion of proinflammatory and degradative mediators such as MMPs<sup>84</sup>. Furthermore, in the light of the paracrine interactions that may occur between intra-articular tissues, Favero *et al.* showed cross-talk between synovial and meniscal explants from knee OA patients and demonstrated induction of different metalloproteases such as MMP3 and MMP-10 involved in OA pathology<sup>85</sup>. Meniscal tissue has been suggested to be more susceptible to adipokine-stimulated catabolism than cartilage<sup>86</sup>. Considering the fact that intra-articular joint tissues such as the HFP release adipokines, this may implicate that joint injury or other factors leading to increased adipokine release in the knee joint may

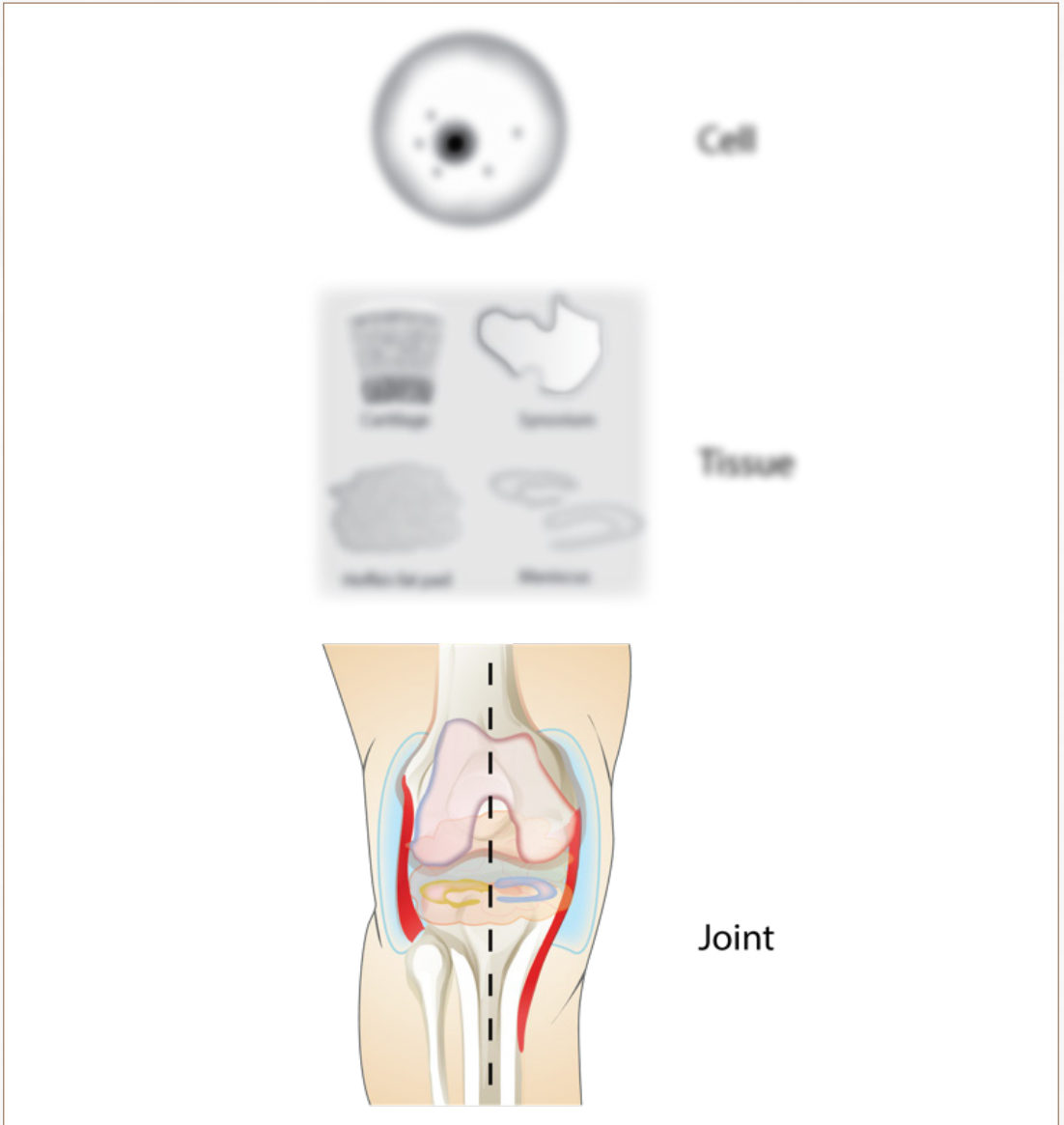
alter the mechanical integrity of the meniscus, thereby increasing susceptibility to knee OA development.

In conclusion, several intra-articular tissues such as cartilage, synovium, HFP, meniscus, smaller fat pads, the subchondral bone and the ACL are present in the knee joint. These joint tissues can compromise knee joint homeostasis via secretion of catabolic molecules into the synovial fluid. Moreover, paracrine functional interactions which occur between the different joint tissues can further compromise knee joint homeostasis leading to a catabolic joint environment. A catabolic environment in the knee joint may lead to inferior quality of intra-articular tissues such as the cartilage and the meniscus, which fulfill an important biomechanical role<sup>82</sup>. This in turn will lead to a self-perpetuating vicious cycle of tissue damage and biomechanical knee joint failure. Therefore, these joint components are important subjects for future research and to determine whether they can be targeted for the treatment of knee joint pathologies.



### 3. Improving Joint Quality on a 'Total Joint' Level

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### 3.1 Knee Joint Inflammation: Physiologic or Pathologic?

Knee joint homeostasis can become disturbed and lead to inflammatory changes in intra-articular tissues due to different joint pathologies<sup>87</sup>. One example of a joint pathology that can lead to knee joint inflammation is a focal cartilage defect, as also demonstrated in **Chapter 4**<sup>88</sup>. These focal cartilage defects are a predisposition for the development of knee OA later in life<sup>8</sup>. The notion that untreated cartilage defects can lead to OA development later in life can be explained from a biomechanical point of view<sup>89</sup>. However, abnormal mechanical loading may, next to purely mechanical damage, also contribute to knee joint inflammation via translation of abnormal mechanical stimuli into inflammatory signaling pathways. This process has been referred to as mechanoinflammation<sup>90</sup>. In concert with this notion, it has been described that subchondral bone changes are associated with early osteoarthritis development<sup>91</sup>. Biomechanical disturbances caused by these changes in subchondral bone architecture can lead to suboptimal biomechanics potentially contributing to a mechanoinflammatory response leading to a self-perpetuating vicious cycle of mechanoinflammation<sup>91</sup>. Given the fact that inflammatory processes may lead to cartilage degradation<sup>1</sup>, it can be hypothesized that a mechanoinflammatory response may potentially cause failure of tissue-engineering strategies to treat cartilage defects. Several reports in literature support the theory that cell-based strategies for cartilage repair are more likely to be successful when implanted into a knee joint with 'normal homeostasis'<sup>4,92,93</sup>. For instance, late treated cartilage defects showed worse outcomes than early treated defects in an *in vivo* model<sup>4</sup>. This suggests that alterations in joint homeostasis (an increased inflammatory joint environment) specific to the late treated group may have hampered cartilage repair<sup>4</sup>. Moreover, synovial fluid from injured knee joints versus non-injured knee joints negatively affected *in vitro* chondrogenesis<sup>92,93</sup>. Rodrigo *et al.* demonstrated that synovial fluid from 'chronically' injured knee joints inhibited the proteoglycan synthesis rate in a chicken limb bud assay<sup>93</sup>. It is tempting to speculate that the synovial fluid of chronically injured knee joints has a specific inflammatory profile inducing catabolic effects on cartilage. However, in the same study it was also shown that the synovial fluid from 'acutely' injured knee joints stimulated proteoglycan synthesis<sup>93</sup>. Thus, it can be concluded that an inflammatory response is not necessarily detrimental for the knee joint

and is not always related to impaired tissue repair. In fact, inflammation is considered as a normal response of the body to tissue alterations<sup>94</sup>. It has been suggested to be the sum of effector mechanisms activated in response to external or internal noxious stimuli in an endeavor to restore homeostatic balance<sup>95</sup>. Inflammation is a central element of healing processes<sup>96</sup>. As a matter of fact, the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*) is, next to its role in catabolic inflammatory processes, involved in bone fracture repair and has an essential role in chondrogenesis<sup>97</sup>. Lessons we have learned from the role of inflammation in bone fracture healing and the endochondral ossification process have provided important insights in the signaling networks driving cartilage repair processes<sup>42</sup>. Nevertheless, important challenging questions remain within the phenomenon of knee joint inflammation and cartilage repair strategies. When can we consider inflammation as a physiological process attempting to restore homeostatic balance in the knee joint? When should we consider knee joint inflammation as chronic and harmful, leading to knee joint degeneration and inferior knee joint quality? Currently, decision making in treatment for cartilage defects is based on the age of a patient<sup>98</sup>, illustrating the shortcomings in current clinical decision making for this patient group. However, an individual's joint regenerative potential is not necessarily related to age. An individual 35 years of age with multiple comorbidities such as high BMI, diabetes and a mechanically instable knee joint can have a more compromised knee joint homeostasis than an individual 50 years of age with a mechanically stable knee, normal BMI and without diabetes<sup>99</sup>. The future challenge will be to develop a biomarker set for patients with cartilage defects that is able to objectively assess the status of the homeostasis of the joint. Identifying the inflammatory status of the knee joint, for instance by using the prostanoids that we have measured in **Chapter 4** or inflammatory factors that we have found in the synovial fluid of knee OA patients in **Chapter 7**, may be part of this set of biomarkers. This identification may lead to better selection of patient groups which will benefit from cartilage repair strategies or alternatively might better be treated using anti-inflammatory strategies to modulate joint homeostasis and to create an environment more permissive for cartilage repair.



In conclusion, determining the level of knee joint inflammation may aid in patient selection for cartilage repair strategies. A level of knee joint inflammation which does not spontaneously resolve may lead to sustained functional joint problems, which may be treated by means of pharmaceutically active agents that target inflammatory processes. Determining when joint inflammation is not beneficial anymore and investigating factors leading to prolonged and pathologic inflammation will further aid in developing anti-inflammatory interventions for joint pathologies.

### 3.2 Gaining Control Over Knee Joint Homeostasis

Joint homeostasis may become disrupted and knee joint quality may become impaired in an inflamed knee joint. To combat this, different methods can be exploited. An intra-articular administration may be an effective means, since this means that the drug will be in direct contact with the different knee joint tissues. In **Chapter 5**, we have systemically reviewed the evidence for potential *in vivo* chondroprotective actions of the selective COX-2 inhibitors. We reviewed the use of selective COX-2 inhibitors either systemically or intra-articularly. From the systematic review (**Chapter 5**) it became clear that direct intra-articular administration may be a more effective means to use selective COX-2 inhibitors such as celecoxib to improve joint quality than systemic administration. Importantly, celecoxib is hydrophobic and we speculate that the drug may become entrapped in the Hoffa's fat pad, or other intra-articular adipose tissues which are also present in synovial joints such as the hip and ankle joints<sup>100</sup>. To investigate this possibility, it will be of interest to examine whether these adipose tissues can act as natural drug releasing depots for pharmaceutically active agents, thereby providing anti-inflammatory and chondroprotective effects. While our findings in Chapter 5 suggested superiority of intra-articular injections with selective COX-2 inhibitors over systemic treatment, a drug delivery system may provide additional benefits compared to bolus injections. A drug delivery system may circumvent side effects associated with systemic treatment, allowing a prolonged local drug residence time, increasing patient compliance and preventing frequent intra-articular injections. In this context, the use of a 'smart' drug delivery system in which drug release will be reactive to the disease process, may be an effective means to improve knee joint quality in joint pathologies such as for instance knee OA. In **Chapter 6**, we have investigated whether celecoxib-loaded

PEA was chondroprotective. While in **Chapter 5** chondroprotective effects of celecoxib were suggested, this was not observed in rat knees injected with celecoxib-loaded PEA microspheres. Since we have used a mechanical instability model to induce experimental OA, animals mobilizing more may have more severe OA induction. It can therefore be hypothesized that animals which received celecoxib-loaded PEA microspheres loaded the affected joint more compared to animals injected with non-loaded microspheres. This could be attributable to analgesic effects of continuously available celecoxib, and thereby counteracting potential chondroprotective effects of celecoxib.

The auto-regulatory behavior of the drug delivery system we describe in **Chapter 6** is of particular interest if we consider our results in **Chapter 4**. In **Chapter 4**, we identified 2 subgroups of knee OA patients having a high or low inflammatory profile in their HFPs. It will be interesting to investigate whether celecoxib release from our auto regulatory drug delivery system will be more efficient in the patient group with higher inflamed HFPs and whether it will exert its desired anti-inflammatory effect on the HFP and other intra-articular tissues at the moment when inflammation in the tissue flares up.

### 3.3 Molecular Diagnostics of Total Knee Joint Homeostasis

Currently, when assessing joint homeostasis, most techniques focus on only one specific part of the joint. Imaging techniques investigate structural tissue integrity, experimental *ex vivo* studies focus on articular cartilage specimens, or *in vitro* studies examine cellular or tissue responses. However, such reductionistic approaches hamper the possibility to fully comprehend the homeostatic nature of a joint and provides a relatively fragmented picture. In **Chapter 7**, we hypothesized that the synovial fluid, which bathes important intra-articular tissues, represents a promising body fluid to access the homeostatic condition of the knee joint as a whole<sup>47,101,102</sup>. We performed comparative full-proteome analysis of the synovial fluid of OA patients versus non-OA individuals, as well as of the secretomes of Hoffa's fat pad, synovium, meniscus and articular cartilage of osteoarthritic knees. We identified several protein species with a tissue type-dependent release and also demonstrated their OA-specificity. Hence different from hypotheses in literature suggesting that biochemical markers might lack specificity to joint tissues<sup>103</sup>, our findings in **Chapter**

**7** highlighted the presence of tissue type-dependent release of OA-specific proteins in OA knee joint synovial fluid.

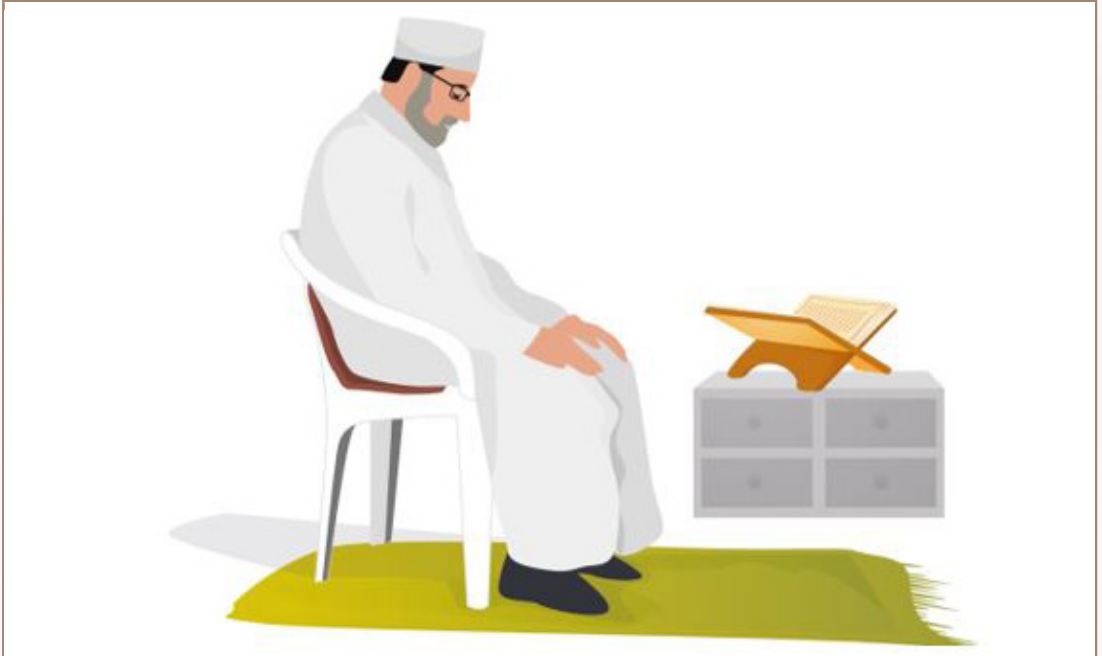
Our findings in **Chapter 7** showing a comprehensive insight into the tissue-dependent origin of OA-specific protein species found in knee joint synovial fluid are of particular interest from several points of view. Firstly, considering the current diagnostic limitations in knee OA and given the increasing recognition that several OA subtypes exist<sup>103,104,105</sup>, our findings may aid in the development of molecular diagnostics in sub-/endotyping knee OA. Associations of biochemical markers with severity of knee OA have been shown to correlate to gender, but also even to OA severity in distinct joint compartments such as the tibiofemoral and the patellofemoral joint<sup>51</sup>. The low success rates in clinical trials with new intra-articular treatments to treat joint pathologies such as knee OA may be related to patient heterogeneity and lack of proper patient selection. Therefore, the sub-/endotyping of knee OA holds potential to improve treatment outcomes in clinical trials<sup>106</sup> (Figure 1). However, it remains to be determined whether biomarkers in synovial fluid can be used as 'stand-alone markers'. Results obtained from measurements of protein biomarkers in the synovial fluid are a snapshot in time, and events such as knee joint trauma might cause important fluctuations in biomarker levels. This will pose specific challenges when using such biomarkers for diagnostics. Nevertheless, these protein biomarkers may aid in clinical decision making in knee OA patients by combining them with other data such as demographical, clinical and imaging data.

Secondly, efficacy studies of novel experimental treatments for knee OA will benefit from biomarkers able to report about the joint's health status on the tissue type level. For instance, cartilage specific biomarkers may aid in evaluating treatments in cartilage repair strategies, while meniscus specific biomarkers may aid in evaluating treatments in meniscus repair strategies. Thirdly, knowledge about the main tissue source of OA-specific proteins present in knee joint synovial fluid is expected to have important implications for understanding knee OA pathophysiology and the tissue type-dependent influence on its progression.

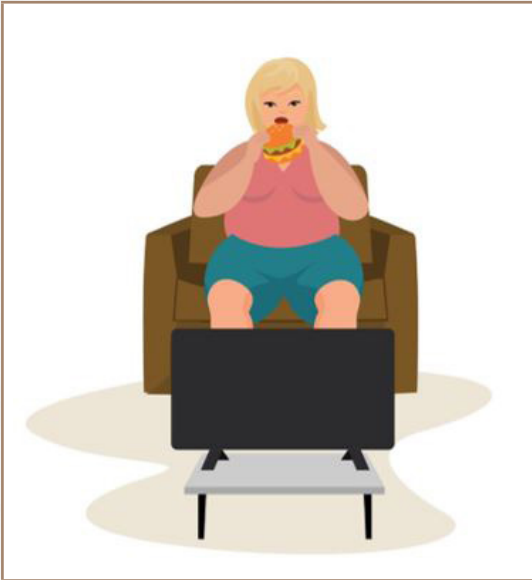


## Knee Osteoarthritis: A Heterogeneous Disease

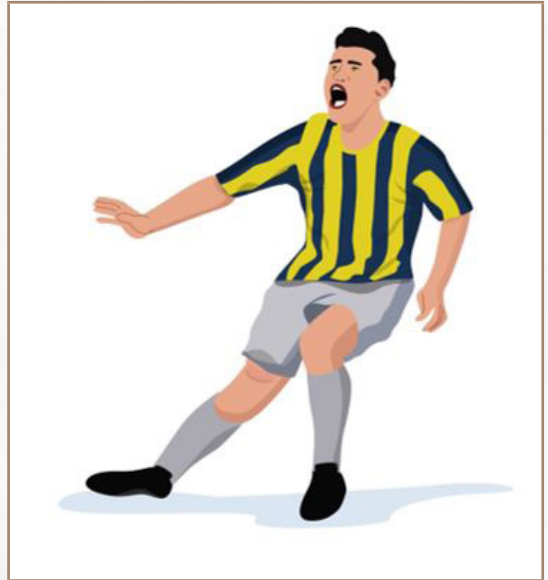
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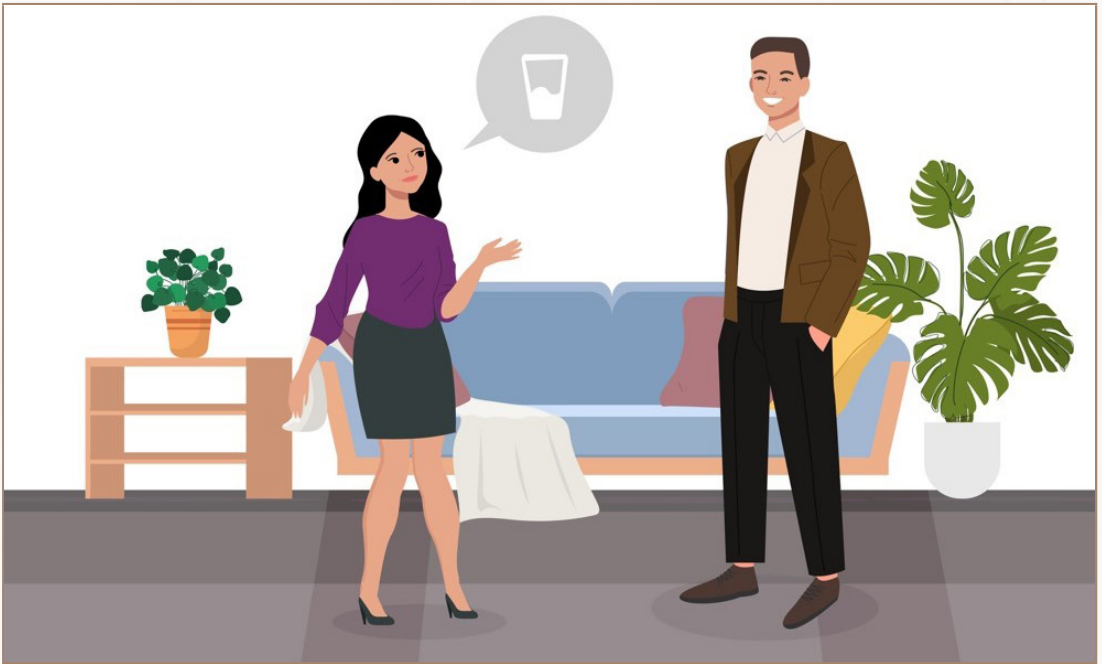
Hamit is a 97-year old man who used to live in Adana. Despite living in poverty, he raised up 12 children and his backbreaking work became a labor of love. Unfortunately, he was not able to do his ritual praying by prostrating on the floor due to his bilateral total knee prostheses.



Jennifer is 45 years old and suffers from metabolic syndrome. Unfortunately, she is not able to live a healthy lifestyle and do exercises for weight loss due to pain in her right knee. She has recently been diagnosed with early knee OA.



Alex is a 27-year old soccer player, who suffered a knee distortion of his right knee joint 2 years ago. He was diagnosed with an unhappy triad and now complains about right knee pain despite successful ligament reconstruction surgery.



Sarah is 39-years old and used to have recurrent patellar luxations. She has a bilateral valgus leg axis and is starting to have anterior knee pain, particularly during stair climbing. She was recently diagnosed with patellofemoral knee OA.





John is a 47-year old construction worker with a bilateral varus leg axis. On the construction site, he tries to listen to the instructions of his boss carefully. Unfortunately, he is not able to concentrate because he can not ignore the medial knee pain he is suffering from the last few months.

**Figure 1: Knee osteoarthritis is a heterogeneous disease.**

*To date, clinical trials have failed in developing a therapeutic tool which is able to postpone total knee arthroplasty. Clinical trials use a heterogeneous clinical population for whom interventions often are ineffective to modulate OA disease or to relieve pain. Knee osteoarthritis is a final common pathway of cartilage degradation, but should be considered as a total joint disease. In future clinical trials the heterogeneity of the disease should be considered to demonstrate therapeutic effects on disease modification or pain relief.*

Interestingly, TGF- $\beta$ 2 was the only cytokine from the TGF- $\beta$  superfamily we detected in our synovial fluid proteomic analysis, as well as in our analyses of the secretomes of cartilage, Hoffa's fat pad, meniscus and synovium. In **Chapter 2** and **Chapter 3** we have already demonstrated that this specific TGF- $\beta$  isoform may be of crucial importance for the chondrocyte phenotype. Our protein mass spectrometry results detecting only this specific isoform may point to additional evidence that this specific isoform is important when investigating pathophysiological mechanisms in knee joint pathologies.

Our proteomic analysis of joint tissues in **Chapter 7** revealed the secretion of several serine proteases such as HTRA, kallikrein and elastase, but also confirmed their presence in synovial fluid. While matrix metalloproteinases are widely believed to be involved in inflammation driven proteolysis in joint pathologies<sup>46</sup>, serine proteases are emerging as important players in degenerative joint pathologies such as osteoarthritis<sup>107</sup>. Therefore, our findings in **Chapter 7** highlight the potential of our serine protease-reactive PEA microspheres described in **Chapter 6** to be used in joint pathologies and may promise an attractive opportunity to be used as an auto-regulatory drug delivery system with a serine protease activity dependent drug release behavior.

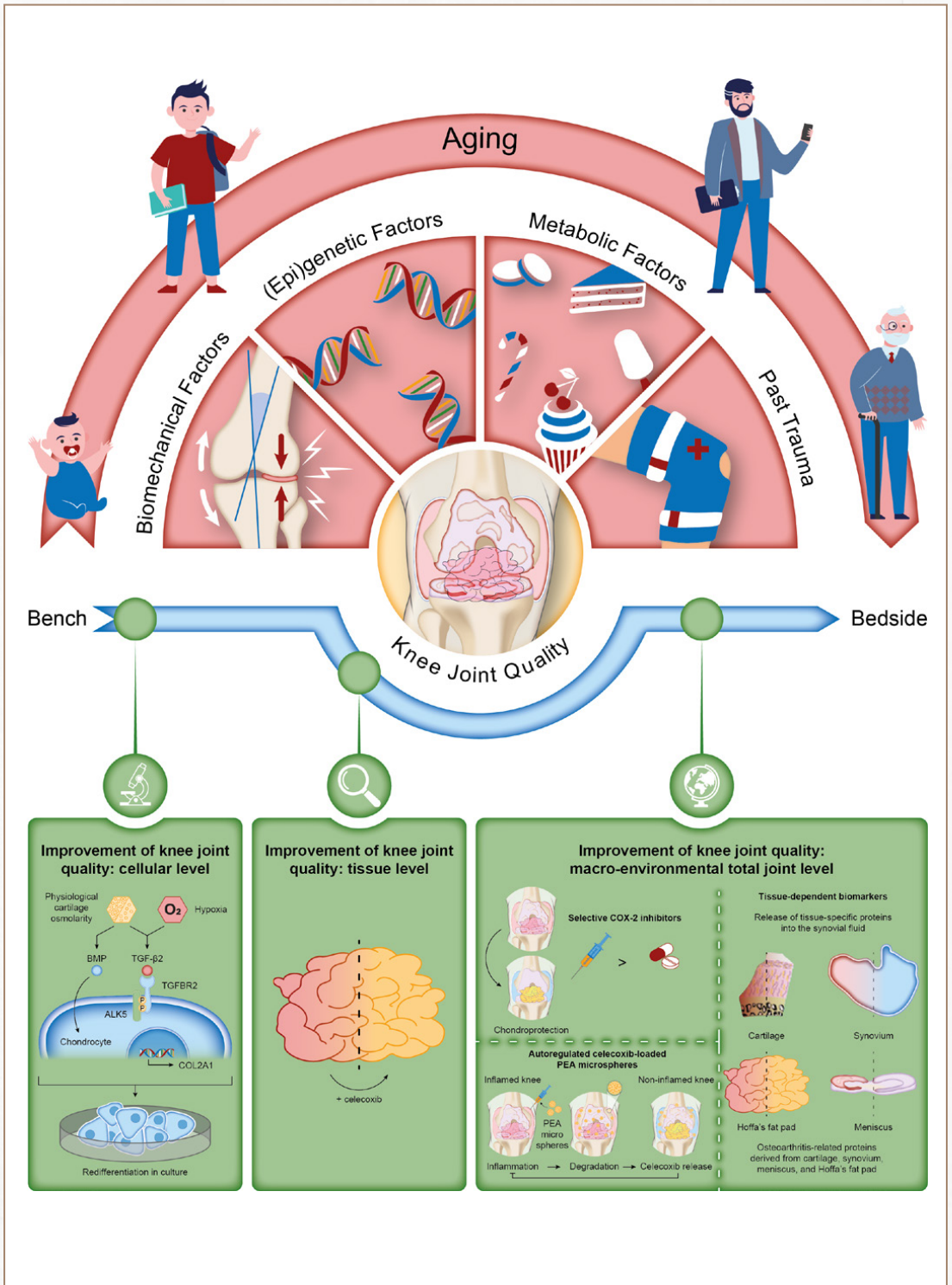
From discovery studies it became clear that the knee joint synovial fluid composition undergoes a great number of detectable biomolecular changes specific for OA and depending on distinct OA subtypes<sup>87,108,109,110,111,112,113,114</sup>. In addition to our proteomic approach, future comparison of release profiles of different joint tissues on the metabolomic, transcriptomic and lipidomic level will provide additional insight in the tissue-type dependent contribution to the development of knee joint pathology.

Taken together, our findings presented in this dissertation demonstrate novel insights in the molecular mechanisms involved in chondrocyte phenotype improvement after hyperosmotic and hypoxic cultures and demonstrate the importance of TGF- $\beta$  signalling in the chondrocyte phenotype. Moreover, the HFP of patients with joint pathologies such as cartilage defects and knee OA has a substantial contribution in the prostanoid content found in synovial fluid. Targeting the knee joint by means of intra-articular injections instead of systemic administration of drugs may be more effective in improving knee joint

homeostasis. Furthermore, celecoxib, either injected as a bolus drug or incorporated in a smart drug release system, such as PEA microspheres, is an attractive novel strategy to improve joint homeostasis. Finally, we uncovered tissue type-dependent release of OA-specific proteins in knee joint synovial fluid. These proteins may be used as biomarkers to aid in developing treatment strategies to improve joint homeostasis for cartilage repair strategies or slowing down knee OA.

In Figure 2, a schematic representation of the main results of this thesis is illustrated.





**Figure 2: Schematic representation of the main results of this thesis.**

Knee joint quality can become compromised due to risk factors such as aging, (epi)genetic factors, metabolic factors, biomechanical factors or past joint trauma. In **Chapter 2 and 3** we have investigated the influence of biophysical environmental factors such as hypoxia and hyperosmolarity on the chondrocyte phenotype. We show that chondrocyte phenotype can be improved when culturing chondrocyte under these conditions and that TGF- $\beta$  superfamily signaling is involved in controlling the chondrocyte phenotype under these conditions. In **Chapter 4**, we show that the HFP can contribute to the inflammatory mediators found in synovial fluid in joint pathologies such as cartilage defects and generalized knee osteoarthritis and that celecoxib is able to modulate HFP inflammation. The **Chapters 5,6 and 7** focuses on improving knee joint quality from a more comprehensive macro-environmental point of view. In **Chapter 5**, our systematic review clarified that direct intra-articular treatment with anti-inflammatory selective COX-2 inhibitor may hold more potential compared to systemic treatment to improve knee joint quality. In **Chapter 6**, we show that celecoxib loaded PEA microspheres hold potential to be used as an auto regulatory drug release system to improve knee joint quality in inflammatory pathologies such as knee OA. Finally, in **Chapter 7** we show tissue type dependent release of OA specific proteins in knee joint synovial fluid which may be used as biomarkers to aid in developing treatment strategies to optimize macro-environmental joint homeostasis.

## References

- 1 Houard X, Goldring MB, Berenbaum F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Curr Rheumatol Rep.* 2013;15(11):375.
- 2 Brandt KD, Radin EL, Dieppe PA, van de Putte L. Yet more evidence that osteoarthritis is not a cartilage disease. *Ann Rheum Dis.* 2006;65(10):1261-4.
- 3 Heinegard D, Saxne T. The role of the cartilage matrix in osteoarthritis. *Nat Rev Rheumatol.* 2011;7(1):50-6.
- 4 Saris DB, Dhert WJ, Verbout AJ. Joint homeostasis. The discrepancy between old and fresh defects in cartilage repair. *J Bone Joint Surg Br.* 2003;85(7):1067-76.
- 5 Sandell LJ. Etiology of osteoarthritis: genetics and synovial joint development. *Nat Rev Rheumatol.* 2012;8(2):77-89.
- 6 Zhuo Q, Yang W, Chen J, Wang Y. Metabolic syndrome meets osteoarthritis. *Nat Rev Rheumatol.* 2012;8(12):729-37.
- 7 Wluka AE, Lombard CB, Cicuttini FM. Tackling obesity in knee osteoarthritis. *Nat Rev Rheumatol.* 2013;9(4):225-35.
- 8 Cicuttini F, Ding C, Wluka A, Davis S, Ebeling PR, Jones G. Association of cartilage defects with loss of knee cartilage in healthy, middle-age adults: a prospective study. *Arthritis Rheum.* 2005;52(7):2033-9.
- 9 Hunziker EB, Lippuner K, Keel MJ, Shintani N. An educational review of cartilage repair: precepts & practice--myths & misconceptions--progress & prospects. *Osteoarthritis Cartilage.* 2015;23(3):334-50.
- 10 Pujol JP, Galera P, Redini F, Mauviel A, Loyau G. Role of cytokines in osteoarthritis: comparative effects of interleukin 1 and transforming growth factor-beta on cultured rabbit articular chondrocytes. *J Rheumatol Suppl.* 1991;27:76-9.
- 11 Akman HO, Zhang H, Siddiqui MA, Solomon W, Smith EL, Batuman OA. Response to hypoxia involves transforming growth factor-beta2 and Smad proteins in human endothelial cells. *Blood.* 2001;98(12):3324-31.
- 12 Falanga V, Qian SW, Danielpour D, Katz MH, Roberts AB, Sporn MB. Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. *J Invest Dermatol.* 1991;97(4):634-7.



## Chapter 8

- 13 Grimshaw MJ, Mason RM. Modulation of bovine articular chondrocyte gene expression *in vitro* by oxygen tension. *Osteoarthritis Cartilage*. 2001;9(4):357-64.
- 14 Roberts AB, Sporn MB. Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol Reprod Dev*. 1992;32(2):91-8.
- 15 Bilandzic M, Stenvers KL. Betaglycan: a multifunctional accessory. *Mol Cell Endocrinol*. 2011;339(1-2):180-9.
- 16 Jahr H, Gunes S, Kuhn AR, Nebelung S, Pufe T. Bioreactor-Controlled Physoxia Regulates TGF-beta Signaling to Alter Extracellular Matrix Synthesis by Human Chondrocytes. *Int J Mol Sci*. 2019;20(7).
- 17 Zhou S, Cui Z, Urban JP. Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study. *Arthritis Rheum*. 2004;50(12):3915-24.
- 18 Urban JP, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol*. 1993;154(2):262-70.
- 19 van der Windt AE, Haak E, Das RH, Kops N, Welting TJ, Caron MM, *et al*. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 *in vitro*. *Arthritis Res Ther*. 2010;12(3):R100.
- 20 Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res*. 2009;19(1):71-88.
- 21 Li TF, O'Keefe RJ, Chen D. TGF-beta signaling in chondrocytes. *Front Biosci*. 2005;10:681-8.
- 22 Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res*. 2009;19(1):71-88.
- 23 Moustakas A, Heldin CH. Non-Smad TGF-beta signals. *J Cell Sci*. 2005;118(Pt 16):3573-84.
- 24 Seger R, Krebs EG. The MAPK signaling cascade. *FASEB J*. 1995;9(9):726-35.
- 25 Lee MC, Goomer RS, Takahashi K, Harwood FL, Amiel M, Amiel D. Transforming growth factor beta one (TGF-beta 1) enhancement of the chondrocytic phenotype in aged perichondrial cells: an *in vitro* study. *Iowa Orthop J*. 2000;20:11-6.
- 26 Wang W, Zhou G, Hu MC, Yao Z, Tan TH. Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. *J Biol Chem*. 1997;272(36):22771-5.

- 27 Simeone DM, Zhang L, Graziano K, Nicke B, Pham T, Schaefer C, *et al.* Smad4 mediates activation of mitogen-activated protein kinases by TGF-beta in pancreatic acinar cells. *Am J Physiol Cell Physiol.* 2001;281(1):C311-9.
- 28 Bokemeyer D, Sorokin A, Dunn MJ. Multiple intracellular MAP kinase signaling cascades. *Kidney Int.* 1996;49(5):1187-98.
- 29 Park EC, Rongo C. The p38 MAP kinase pathway modulates the hypoxia response and glutamate receptor trafficking in aging neurons. *Elife.* 2016;5.
- 30 Racz B, Reglodi D, Fodor B, Gasz B, Lubics A, Gallyas F, Jr., *et al.* Hyperosmotic stress-induced apoptotic signaling pathways in chondrocytes. *Bone.* 2007;40(6):1536-43.
- 31 Chiang TA, Yang YL, Yang YY, Hu MH, Wu PF, Liu SF, *et al.* Hyperosmolarity enhanced susceptibility to renal tubular fibrosis by modulating catabolism of type I transforming growth factor-beta receptors. *J Cell Biochem.* 2010;109(4):663-71.
- 32 Khalil N. TGF-beta: from latent to active. *Microbes Infect.* 1999;1(15):1255-63.
- 33 Wipff PJ, Hinz B. Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. *Eur J Cell Biol.* 2008;87(8-9):601-15.
- 34 Sugiura T, Yamauchi A, Kitamura H, Matusoka Y, Horio M, Imai E, *et al.* Effects of hypertonic stress on transforming growth factor-beta activity in normal rat kidney cells. *Kidney Int.* 1998;53(6):1654-60.
- 35 Redini F, Galera P, Mauviel A, Loyau G, Pujol JP. Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. *FEBS Lett.* 1988;234(1):172-6.
- 36 Hirao M, Tamai N, Tsumaki N, Yoshikawa H, Myoui A. Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. *J Biol Chem.* 2006;281(41):31079-92.
- 37 van der Windt AE, Haak E, Kops N, Verhaar JA, Weinans H, Jahr H. Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. *Arthritis Rheum.* 2012;64(6):1929-39.
- 38 Kreuz PC, Steinwachs M, Erggelet C, Krause SJ, Ossendorf C, Maier D, *et al.* Classification of graft hypertrophy after autologous chondrocyte implantation of full-thickness chondral defects in the knee. *Osteoarthritis Cartilage.* 2007;15(12):1339-47.
- 39 Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with

- autologous chondrocyte transplantation. *N Engl J Med.* 1994;331(14):889-95.
- 40 Hayes DW, Jr., Averett RK. Articular cartilage transplantation. Current and future limitations and solutions. *Clin Podiatr Med Surg.* 2001;18(1):161-76.
- 41 Ripmeester EGJ, Timur UT, Caron MMJ, Welting TJM. Recent Insights into the Contribution of the Changing Hypertrophic Chondrocyte Phenotype in the Development and Progression of Osteoarthritis. *Front Bioeng Biotechnol.* 2018;6:18.
- 42 Welting TJ, Caron MM, Emans PJ, Janssen MP, Sanen K, Coolsen MM, *et al.* Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater.* 2011;22:420-36; discussion 36-7.
- 43 Fang L, Chang HM, Cheng JC, Leung PC, Sun YP. TGF-beta1 induces COX-2 expression and PGE2 production in human granulosa cells through Smad signaling pathways. *J Clin Endocrinol Metab.* 2014;99(7):E1217-26.
- 44 Harding P, Balasubramanian L, Swegan J, Stevens A, Glass WF, 2nd. Transforming growth factor beta regulates cyclooxygenase-2 in glomerular mesangial cells. *Kidney Int.* 2006;69(9):1578-85.
- 45 Clockaerts S, Bastiaansen-Jenniskens YM, Runhaar J, Van Osch GJ, Van Offel JF, Verhaar JA, *et al.* The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review. *Osteoarthritis Cartilage.* 2010;18(7):876-82.
- 46 Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El Hachem K, *et al.* Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cell Mater.* 2011;21:202-20.
- 47 Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum.* 2003;33(3):155-67.
- 48 Mukai M, Uchida K, Takano S, Iwase D, Aikawa J, Inoue G, *et al.* Down-regulation of microsomal prostaglandin E2 synthase-1 in the infrapatellar fat pad of osteoarthritis patients with hypercholesterolemia. *Lipids Health Dis.* 2018;17(1):137.
- 49 Massicotte F, Lajeunesse D, Benderdour M, Pelletier JP, Hilal G, Duval N, *et al.* Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthritis Cartilage.* 2002;10(6):491-500.



- 50 Ni Y, Ni L, Zhuge F, Xu L, Fu Z, Ota T. Adipose Tissue Macrophage Phenotypes and Characteristics: The Key to Insulin Resistance in Obesity and Metabolic Disorders. *Obesity (Silver Spring)*. 2020;28(2):225-34.
- 51 Kisand K, Tamm AE, Lintrop M, Tamm AO. New insights into the natural course of knee osteoarthritis: early regulation of cytokines and growth factors, with emphasis on sex-dependent angiogenesis and tissue remodeling. A pilot study. *Osteoarthritis Cartilage*. 2018;26(8):1045-54.
- 52 Wei W, Rudjito E, Fahy N, Verhaar JA, Clockaerts S, Bastiaansen-Jenniskens YM, *et al*. The infrapatellar fat pad from diseased joints inhibits chondrogenesis of mesenchymal stem cells. *Eur Cell Mater*. 2015;30:303-14.
- 53 Bastiaansen-Jenniskens YM, Clockaerts S, Feijt C, Zuurmond AM, Stojanovic-Susulic V, Bridts C, *et al*. Infrapatellar fat pad of patients with end-stage osteoarthritis inhibits catabolic mediators in cartilage. *Ann Rheum Dis*. 2012;71(2):288-94.
- 54 Cai J, Xu J, Wang K, Zheng S, He F, Huan S, *et al*. Association Between Infrapatellar Fat Pad Volume and Knee Structural Changes in Patients with Knee Osteoarthritis. *J Rheumatol*. 2015;42(10):1878-84.
- 55 Chuckpaiwong B, Charles HC, Kraus VB, Guilak F, Nunley JA. Age-associated increases in the size of the infrapatellar fat pad in knee osteoarthritis as measured by 3T MRI. *J Orthop Res*. 2010;28(9):1149-54.
- 56 Duran S, Aksahin E, Kocadal O, Aktekin CN, Hapa O, Gencturk ZB. Effects of body mass index, infrapatellar fat pad volume and age on patellar cartilage defect. *Acta Orthop Belg*. 2015;81(1):41-6.
- 57 Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31(5):986-1000.
- 58 Furman BD, Mangiapani DS, Zeitler E, Bailey KN, Horne PH, Huebner JL, *et al*. Targeting pro-inflammatory cytokines following joint injury: acute intra-articular inhibition of interleukin-1 following knee injury prevents post-traumatic arthritis. *Arthritis Res Ther*. 2014;16(3):R134.
- 59 Lieberthal J, Sambamurthy N, Scanzello CR. Inflammation in joint injury and post-traumatic osteoarthritis. *Osteoarthritis Cartilage*. 2015;23(11):1825-34.
- 60 Eymard F, Pigenet A, Citadelle D, Tordjman J, Foucher L, Rose C, *et al*. Knee and hip intra-articular adipose tissues (IAATs) compared with autologous subcutaneous adipose tissue: a specific phenotype for a central player in osteoarthritis. *Ann Rheum Dis*. 2017;76(6):1142-8.
- 61 Solbak NM, Heard BJ, Achari Y, Chung M, Shrive NG, Frank CB, *et al*.

## Chapter 8

- Alterations in Hoffa's fat pad induced by an inflammatory response following idealized anterior cruciate ligament surgery. *Inflamm Res.* 2015;64(8):615-26.
- 62 Tellegen AR, Rudnik-Jansen I, Pouran B, de Visser HM, Weinans HH, Thomas RE, *et al.* Controlled release of celecoxib inhibits inflammation, bone cysts and osteophyte formation in a preclinical model of osteoarthritis. *Drug Deliv.* 2018;25(1):1438-47.
- 63 Findlay DM, Kuliwaba JS. Bone-cartilage crosstalk: a conversation for understanding osteoarthritis. *Bone Res.* 2016;4:16028.
- 64 Walsh DA, Bonnet CS, Turner EL, Wilson D, Situ M, McWilliams DF. Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis. *Osteoarthr Cartilage.* 2007;15(7):743-51.
- 65 Pritzker KP, Aigner T. Terminology of osteoarthritis cartilage and bone histopathology - a proposal for a consensus. *Osteoarthritis Cartilage.* 2010;18 Suppl 3:S7-9.
- 66 Lane LB, Villacin A, Bullough PG. The vascularity and remodelling of subchondrial bone and calcified cartilage in adult human femoral and humeral heads. An age- and stress-related phenomenon. *J Bone Joint Surg Br.* 1977;59(3):272-8.
- 67 Burr DB, Schaffler MB. The involvement of subchondral mineralized tissues in osteoarthritis: quantitative microscopic evidence. *Microsc Res Tech.* 1997;37(4):343-57.
- 68 Fazzalari NL, Parkinson IH. Fractal properties of subchondral cancellous bone in severe osteoarthritis of the hip. *J Bone Miner Res.* 1997;12(4):632-40.
- 69 Grynblas MD, Alpert B, Katz I, Lieberman I, Pritzker KP. Subchondral bone in osteoarthritis. *Calcif Tissue Int.* 1991;49(1):20-6.
- 70 Roemer FW, Guermazi A, Javaid MK, Lynch JA, Niu J, Zhang Y, *et al.* Change in MRI-detected subchondral bone marrow lesions is associated with cartilage loss: the MOST Study. A longitudinal multicentre study of knee osteoarthritis. *Ann Rheum Dis.* 2009;68(9):1461-5.
- 71 Hunter DJ, Zhang Y, Niu J, Goggins J, Amin S, LaValley MP, *et al.* Increase in bone marrow lesions associated with cartilage loss: a longitudinal magnetic resonance imaging study of knee osteoarthritis. *Arthritis Rheum.* 2006;54(5):1529-35.
- 72 Kazakia GJ, Kuo D, Schooler J, Siddiqui S, Shanbhag S, Bernstein G, *et al.* Bone and cartilage demonstrate changes localized to bone marrow edema-like lesions within osteoarthritic knees. *Osteoarthritis Cartilage.* 2013;21(1):94-101.

- 73 Chua K, Kang JYB, Ng FDJ, Pang HN, Lie DTT, Silva A, *et al.* Subchondroplasty for Bone Marrow Lesions in the Arthritic Knee Results in Pain Relief and Improvement in Function. *J Knee Surg.* 2019.
- 74 Nairn LN, Subramaniam M, Ekhtiari S, Axelrod DE, Grant JA, Khan M. Safety and early results of Subchondroplasty(R) for the treatment of bone marrow lesions in osteoarthritis: a systematic review. *Knee Surg Sports Traumatol Arthrosc.* 2020.
- 75 Woo SL, Livesay GA, Engle C. Biomechanics of the human anterior cruciate ligament. Muscle stabilization and ACL reconstruction. *Orthop Rev.* 1992;21(8):935-41.
- 76 Louboutin H, Debarge R, Richou J, Selmi TA, Donell ST, Neyret P, *et al.* Osteoarthritis in patients with anterior cruciate ligament rupture: a review of risk factors. *Knee.* 2009;16(4):239-44.
- 77 Patel SA, Hageman J, Quatman CE, Wordeman SC, Hewett TE. Prevalence and location of bone bruises associated with anterior cruciate ligament injury and implications for mechanism of injury: a systematic review. *Sports Med.* 2014;44(2):281-93.
- 78 Andriacchi TP, Mundermann A, Smith RL, Alexander EJ, Dyrby CO, Koo S. A framework for the *in vivo* pathomechanics of osteoarthritis at the knee. *Ann Biomed Eng.* 2004;32(3):447-57.
- 79 Brophy RH, Tycksen ED, Sandell LJ, Rai MF. Changes in Transcriptome-Wide Gene Expression of Anterior Cruciate Ligament Tears Based on Time From Injury. *Am J Sports Med.* 2016;44(8):2064-75.
- 80 McIlwraith CW, Kawcak CE, Frisbie DD, Little CB, Clegg PD, Peffers MJ, *et al.* Biomarkers for equine joint injury and osteoarthritis. *J Orthop Res.* 2018;36(3):823-31.
- 81 Hasegawa A, Otsuki S, Pauli C, Miyaki S, Patil S, Steklov N, *et al.* Anterior cruciate ligament changes in the human knee joint in aging and osteoarthritis. *Arthritis Rheum.* 2012;64(3):696-704.
- 82 Chang PS, Brophy RH. As Goes the Meniscus Goes the Knee: Early, Intermediate, and Late Evidence for the Detrimental Effect of Meniscus Tears. *Clin Sports Med.* 2020;39(1):29-36.
- 83 Hsieh YS, Yang SF, Chu SC, Chen PN, Chou MC, Hsu MC, *et al.* Expression changes of gelatinases in human osteoarthritic knees and arthroscopic debridement. *Arthroscopy.* 2004;20(5):482-8.
- 84 Cook AE, Stoker AM, Leary EV, Pfeiffer FM, Cook JL. Metabolic responses of meniscal explants to injury and inflammation *ex vivo*. *J Orthop Res.* 2018;36(10):2657-63.
- 85 Favero M, Belluzzi E, Trisolino G, Goldring



## Chapter 8

- MB, Goldring SR, Cigolotti A, *et al.* Inflammatory molecules produced by meniscus and synovium in early and end-stage osteoarthritis: a coculture study. *J Cell Physiol.* 2019;234(7):11176-87.
- 86 Nishimuta JF, Levenston ME. Meniscus is more susceptible than cartilage to catabolic and anti-anabolic effects of adipokines. *Osteoarthritis Cartilage.* 2015;23(9):1551-62.
- 87 Ritter SY, Subbaiah R, Bebek G, Crish J, Scanzello CR, Krastins B, *et al.* Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis Rheum.* 2013;65(4):981-92.
- 88 Tsuchida AI, Beekhuizen M, t Hart MC, Radstake TR, Dhert WJ, Saris DB, *et al.* Cytokine profiles in the joint depend on pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Arthritis research & therapy.* 2014;16(5):441.
- 89 Guettler JH, Demetropoulos CK, Yang KH, Jurist KA. Osteochondral defects in the human knee: influence of defect size on cartilage rim stress and load redistribution to surrounding cartilage. *Am J Sports Med.* 2004;32(6):1451-8.
- 90 Hugle T, Geurts J. What drives osteoarthritis?-synovial versus subchondral bone pathology. *Rheumatology (Oxford).* 2017;56(9):1461-71.
- 91 Donell S. Subchondral bone remodelling in osteoarthritis. *EFORT Open Rev.* 2019;4(6):221-9.
- 92 Yang KG, Saris DB, Verbout AJ, Creemers LB, Dhert WJ. The effect of synovial fluid from injured knee joints on *in vitro* chondrogenesis. *Tissue Eng.* 2006;12(10):2957-64.
- 93 Rodrigo JJ, Steadman JR, Syftestad G, Benton H, Silliman J. Effects of human knee synovial fluid on chondrogenesis *in vitro*. *Am J Knee Surg.* 1995;8(4):124-9.
- 94 Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol.* 2007;127(3):514-25.
- 95 Kotas ME, Medzhitov R. Homeostasis, inflammation, and disease susceptibility. *Cell.* 2015;160(5):816-27.
- 96 van der Kraan PM. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng Regen Med.* 2019;16(4):327-34.
- 97 Caron MM, Emans PJ, Surtel DA, Cremers A, Voncken JW, Welting TJ, *et al.* Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. *PLoS One.* 2012;7(3):e33467.

- 98 Martin AR, Patel JM, Zlotnick HM, Carey JL, Mauck RL. Emerging therapies for cartilage regeneration in currently excluded 'red knee' populations. *NPJ Regen Med.* 2019;4:12.
- 99 Taskiran E, Taskiran D, Duran T, Lok V. Articular cartilage homeostasis after anterior cruciate ligament reconstruction. *Knee Surg Sports Traumatol Arthrosc.* 1998;6(2):93-8.
- 100 Davies DV, White JE. The structure and weight of synovial fat pads. *J Anat.* 1961;95:30-7.
- 101 Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. *Biorheology.* 2002;39(1-2):237-46.
- 102 Wu CL, Kimmerling KA, Little D, Guilak F. Serum and synovial fluid lipidomic profiles predict obesity-associated osteoarthritis, synovitis, and wound repair. *Sci Rep.* 2017;7:44315.
- 103 van Spil WE, Szilagyí IA. Osteoarthritis year in review 2019: biomarkers (biochemical markers). *Osteoarthritis Cartilage.* 2020;28(3):296-315.
- 104 Waarsing JH, Bierma-Zeinstra SM, Weinans H. Distinct subtypes of knee osteoarthritis: data from the Osteoarthritis Initiative. *Rheumatology (Oxford).* 2015;54(9):1650-8.
- 105 Nelson AE, Fang F, Arbeevee L, Cleveland RJ, Schwartz TA, Callahan LF, *et al.* A machine learning approach to knee osteoarthritis phenotyping: data from the FNIH Biomarkers Consortium. *Osteoarthritis Cartilage.* 2019;27(7):994-1001.
- 106 Jones IA, Togashi R, Wilson ML, Heckmann N, Vangsness CT, Jr. Intra-articular treatment options for knee osteoarthritis. *Nat Rev Rheumatol.* 2019;15(2):77-90.
- 107 Milner JM, Patel A, Rowan AD. Emerging roles of serine proteinases in tissue turnover in arthritis. *Arthritis Rheum.* 2008;58(12):3644-56.
- 108 Del Rey MJ, Usategui A, Izquierdo E, Canete JD, Blanco FJ, Criado G, *et al.* Transcriptome analysis reveals specific changes in osteoarthritis synovial fibroblasts. *Ann Rheum Dis.* 2012;71(2):275-80.
- 109 Carlson AK, Rawle RA, Wallace CW, Brooks EG, Adams E, Greenwood MC, *et al.* Characterization of synovial fluid metabolomic phenotypes of cartilage morphological changes associated with

- osteoarthritis. *Osteoarthritis Cartilage*. 2019;27(8):1174-84.
- 110 Kosinska MK, Mastbergen SC, Liebisch G, Wilhelm J, Dettmeyer RB, Ishaque B, *et al*. Comparative lipidomic analysis of synovial fluid in human and canine osteoarthritis. *Osteoarthritis Cartilage*. 2016;24(8):1470-8.
- 111 Favero M, Belluzzi E, Frallonardo P, Peruzzo L, Tauro L, Oliviero F, *et al*. Synovial fluid fetuin-A levels in patients affected by osteoarthritis with or without evidence of calcium crystals. *Rheumatology (Oxford)*. 2019;58(4):729-30.
- 112 Dong N, Gao YH, Liu B, Zhao CW, Yang C, Li SQ, *et al*. Differential expression of adipokines in knee osteoarthritis patients with and without metabolic syndrome. *Int Orthop*. 2018;42(6):1283-9.
- 113 Arellano Perez Vertti RD, Aguilar Muniz LS, Moran Martinez J, Gonzalez Galarza FF, Arguello Astorga R. Cartilage Oligomeric Matrix Protein Levels in Type 2 Diabetes Associated with Primary Knee Osteoarthritis Patients. *Genet Test Mol Biomarkers*. 2019;23(1):16-22.
- 114 Clair AJ, Kingery MT, Anil U, Kenny L, Kirsch T, Strauss EJ. Alterations in Synovial Fluid Biomarker Levels in Knees With Meniscal Injury as Compared With Asymptomatic Contralateral Knees. *Am J Sports Med*. 2019;47(4):847-56.





Chapter

09



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## 1. Joint Pathologies: A Major Socioeconomic Burden

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Joint pathologies such as knee osteoarthritis (OA) are a major public healthcare problem and cause chronic pain and disability among elderly worldwide<sup>1</sup>. These pathologies have a huge impact on the health-related quality of life<sup>1</sup>. OA is the single most common cause of disability in older adults<sup>2</sup>. Factors such as population ageing and increasing numbers of obese individuals are examples which explain the increasing prevalence of joint pathologies such as OA worldwide<sup>2</sup>. Current management modalities target symptoms such as pain, and depend on the severity of the symptoms and structural disease, but eventually surgical intervention with joint replacement is inevitable<sup>3</sup>. Pharmaceutical treatments are being investigated to reduce the burden of disease in knee OA<sup>3</sup>. In addition, drugs are evaluated in their ability to modify the natural course of knee OA by slowing down or stopping the biological processes underlying tissue damage<sup>3</sup>. Currently, no disease modifying OA drug (DMOAD) is available to prevent OA progression. This leads to a major unmet medical need in the treatment of knee OA<sup>4</sup>. Global health metric data show that the number of disability adjusted life years (DALYs) has increased by 2-fold due to OA during the past 30 years<sup>5</sup>. Furthermore, OA is one of the top leading causes of disability in patients aged from 50-75 years<sup>5</sup>. Given the fact that a large proportion of patients in this age group represent the working population, disability caused by OA causes a great socioeconomic burden. Therefore, optimizing treatment for joint pathologies such as OA are of utmost importance.

Unfortunately, the development of treatments to improve knee joint quality and to modify disease progress in joint pathologies such as OA has not made comparable progress with that of many other chronic non-communicable diseases<sup>4</sup>. This may be related to several challenges that exists in joint preserving treatment strategies. In this dissertation, these challenges are described and a scientific basis is provided that can help to impact these challenges.

## 2. Improving Cartilage Repair Strategies to Improve Knee Joint Quality

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In the first part of this thesis (**Chapter 2 and 3**) we have investigated the chondrocyte phenotype in order to improve cell-based cartilage repair strategies in patients with cartilage defects. We have used chondrocyte culture conditions closely resembling the *in vivo* biophysical environment of the chondrocyte. Our findings published in **Chapter 2 and 3** may stimulate others in the field to take the biophysical environment of the chondrocyte into account when optimizing cellular therapies. Since focal cartilage defects are well-known risk factors for knee OA<sup>6,7</sup>, combatting cartilage defects with cellular therapies may lead to preventing or at least delaying in knee OA progression.

Next to an improvement of the chondrocyte phenotype *in vitro*, we also expect that our findings will have implications on the intra-articular joint environment *in vivo*. Knee joint osmolarity is decreased in knee OA<sup>8</sup>. Considering studies showing an *in vitro* anti-inflammatory action of biophysical medium osmolarity on human OA chondrocytes<sup>9,10</sup>, we expect that increasing intra-articular joint osmolarity to biophysical levels will act anti-inflammatory on the knee joint *in vivo*. This in turn can lead to improved cellular therapies, but may also delay knee OA progression. Furthermore, a biophysical osmotic environment in the knee joint is expected to protect against iatrogenic injuries to cartilage due to sharp surgical instruments or edges of the arthroscopic sheath during routine orthopaedic arthroscopic surgery<sup>11</sup>. This is supported by an *in vivo* study showing that articular chondrocytes are less prone to iatrogenic injury due to surgical instruments when the osmotic pressure of the irrigation fluid used during routine orthopaedic surgery is increased<sup>11</sup>.

In conclusion, our findings published in **Chapter 2 and 3** provide methods to improve the chondrocyte phenotype and this is expected to facilitate others in optimizing cellular therapies for transition of *in vitro* findings to *in vivo* patient care.

### 3. Anti-Inflammatory Knee Joint Treatment to Improve Knee Joint Quality

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In knee joint pathologies such as knee OA, joint homeostasis becomes disturbed and pathologic changes can be observed in other joint tissues<sup>4</sup>. Our findings in **Chapter 4**, showing secretion of prostanoids by HFP from cartilage defect patients, have important implications. Prostanoids are involved in inflammation and cartilage degradation<sup>12</sup>. The increased presence of prostanoids in the knee joint may be a sign of pre-OA, where regenerative cartilage treatment strategies are less likely to succeed<sup>13</sup>. Therefore, our findings can stimulate the field in future research to correlate prostanoid levels produced by joint tissues to clinical outcomes of cartilage repair surgeries. Moreover, we showed that celecoxib can act anti-inflammatory on the HFP of patients with OA and cartilage defects. Given the fact that an inflamed joint environment is associated with cartilage degradation<sup>13</sup>, anti-inflammatory treatment with celecoxib holds potential to create a joint environment more permissive for cartilage repair. Our findings presented in **Chapter 4**, showing secretion of different prostanoid subtypes, can also have important implications as biomarkers for specific processes such as fibrosis in knee OA<sup>14</sup>. Prostanoid subtypes are known to be involved in specific pathophysiological processes of the knee joint: for instance, the prostanoid subtype  $\text{PGF}_{2\alpha}$  has been shown to be involved in fibrosis in the knee joint<sup>15</sup>. This specific prostanoid subtype thus holds potential as a target to improve knee joint quality in joint pathologies such as knee OA, where fibrosis has been shown to occur<sup>15</sup>. Furthermore, post-operative fibrosis in the knee joint, i.e. arthrofibrosis, has been described to occur after knee surgeries such as anterior cruciate ligament reconstruction surgeries<sup>16</sup>. Therefore, a modulation of  $\text{PGF}_{2\alpha}$  release in the knee joint holds potential to target post-operative arthrofibrosis.

As earlier mentioned, the development of pharmacological treatments to improve knee joint quality and to modify disease progress in joint pathologies such as OA has not made comparable progress with that of many other chronic non-communicable diseases<sup>4</sup>. This is related to several challenges that exist in developing DMOADs which are inherent



to the disease. Firstly, OA has a slow and long-term natural course, which requires long follow-up periods for DMOADs to detect OA disease modifying effects<sup>1</sup>. Secondly, OA is a heterogenous disease characterized by multiple phenotypes, with a substantial variability in its clinical and radiographic presentation<sup>17</sup>. In **Chapter 4**, we have found a differential inflammatory profile in the HFP from OA patients, which distributed to 2 OA subgroups. It will be interesting to determine whether the different HFP inflammatory states correlate to pain sensation. If this will be the case, then this has a potential impact on the patient specific use of anti-inflammatory treatments for pain relief in knee OA. Considering the fact that also other synovial joints such as for instance the hip, ankle and the elbow joint contain an intra-articular fat pad<sup>18</sup>, it is tempting to speculate that these intra-articular fat depots can be a source of pain in patients that have OA in these specific synovial joints. We hypothesize that these intra-articular fat depots may act as internal homeostatic joint regulators and can become a potential source of pain in joint pathologies such as OA.

In **Chapter 4**, we described that celecoxib holds potential as an anti-inflammatory drug that can improve knee joint quality via decreasing secretion of inflammatory mediators by the HFP. In **Chapter 5**, we present a systematic review about the chondroprotective actions of selective COX-2 inhibitors when used systemically or intra-articularly. Our review in **Chapter 5** has provided important insights into the chondroprotective actions of selective COX-2 inhibitors: their chondroprotective action may depend on their administration route. This is a possible explanation why clinical trials, which used the systemic administration route<sup>19,20</sup>, failed to show *in vivo* chondroprotective actions with selective COX-2 inhibitors. The intra-articular administration route may be a more effective means, and this can also hold true for other DMOADs. A disadvantage of the intra-articular administration route is the need of frequent injections, which will increase the risk of bacterial knee joint infection<sup>21</sup>. However, it still remains the question whether frequent injections are needed, or whether drug-delivery systems are paramount to incorporate DMOADs in. Moreover, a DMOAD may only be effective in disease modification for a certain OA subtype. Nevertheless, it is likely that drug delivery systems may become important tools to incorporate DMOADs in, considering the long-term natural course of knee OA<sup>3</sup>. Therefore, intra-articular drug delivery system are being developed and are under investigation for treatment in joint pathologies such as

knee OA<sup>22</sup>. Intra-articular drug delivery has a number of advantages over systemic delivery, including increased local bioavailability, reduced systemic exposure, fewer adverse events and reduced cost<sup>22</sup>.

In **Chapter 6**, we have evaluated an auto-regulatory drug delivery system and demonstrated its biocompatibility and auto-regulatory release behavior. Considering the pain-killing effects of selective COX-2 inhibitors such as celecoxib which we have investigated in **Chapter 4** and **5**, one has to consider the delicate balance between the pain-killing and structural disease-modifying effects. The auto-regulatory drug delivery system that we have described in **Chapter 6** may provide important advantages compared to a drug delivery system without auto-regulation, especially considering the adverse progression of knee OA that occurred in the clinical trials with for instance anti-NGF<sup>23</sup>. This unwanted adverse progression of OA was possibly due to the fact that a continuously analgesic effect of the anti-NGF caused patients to increase their physical activity and thereby overloading their joints. The FDA has rejected the introduction of anti-NGF into the clinical practice due to the associated risk for joint destruction and rapidly progressive OA. We expect that an auto-regulatory drug release approach will prevent a continuous suppression of pain sensation. This in turn will likely prevent patients to continuously overload their joints, and avoid adverse effects of the treatment. Thus, an auto-regulatory drug release approach provides an attractive balance between drug release and local knee joint trauma. Before implementation of our investigated drug delivery system into the clinical practice, important challenges remain. Firstly, a DMOAD has to be developed that can be bio-actively released from the carrier. As an alternative, an analgesic without adverse effects on knee joint quality may be used. Secondly, sterilization of the system may alter its drug release behavior and few studies pay attention to sterilization of the drug delivery system<sup>21</sup>. Furthermore, contamination with microbial components such as lipopolysaccharides has been described in engineered drug delivery systems and can lead to unwanted toxic side effects<sup>24</sup>. Fourthly, manufacturers of drug delivery systems face regulatory challenges. No uniform definition exists which can classify a certain drug delivery system under a specific category from pharmaceutical science<sup>25</sup>. This prevents the application of a uniform regulation, and certain drug delivery systems may be subject to the European

Union Medical Device Regulation, introducing specific requirements for medicines with an integral device<sup>25</sup>. Cell therapies, coatings and injectables with drug release behavior are upcoming technologies for which new regulations as well as collaborations between public and private partners are paramount.

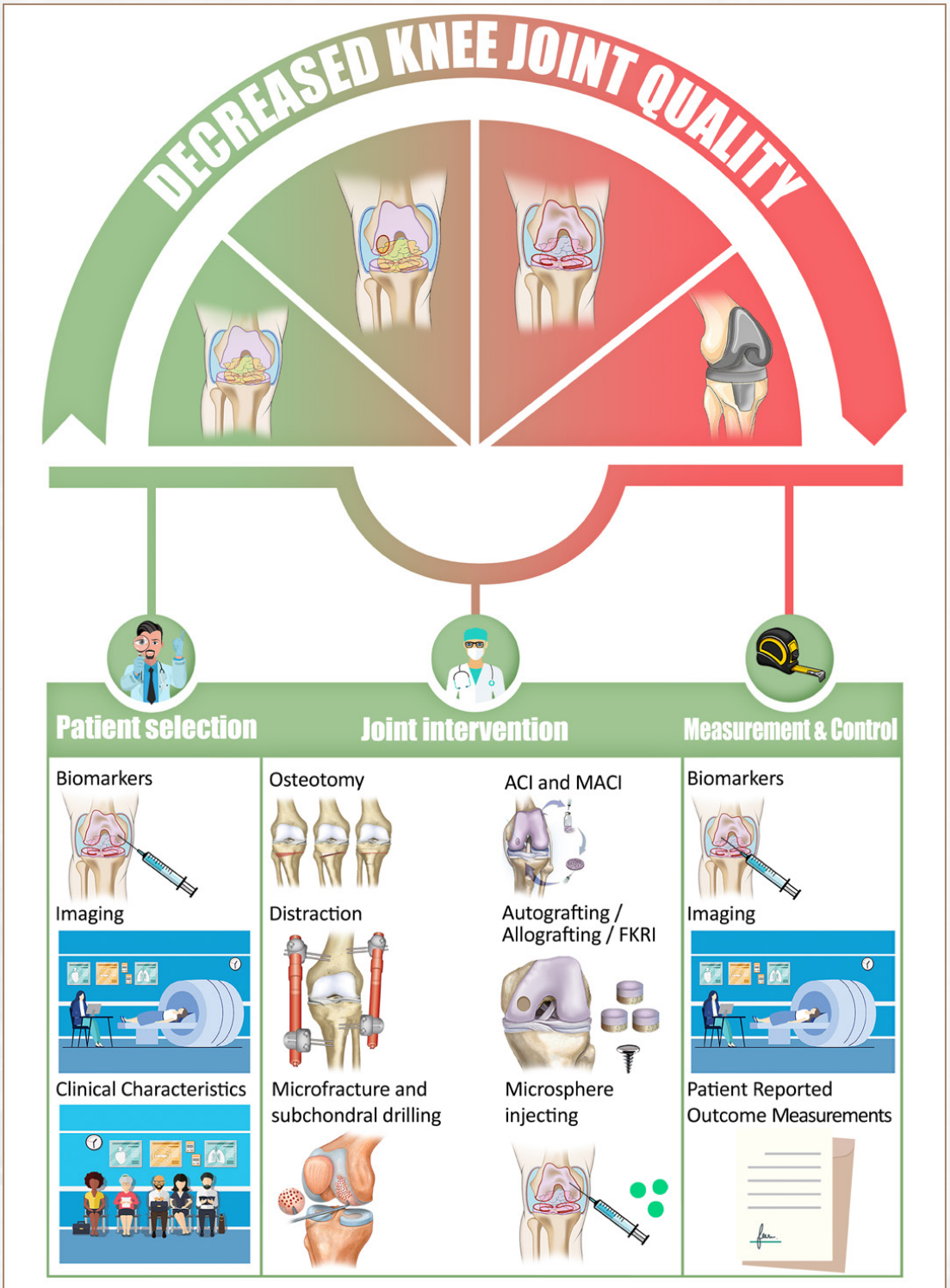
## Patient Selection and Control of Clinical Outcome in Therapies Targeting Knee Joint Quality

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In this thesis we have described important strategies to improve knee joint quality. Two important considerations regarding these joint-preserving strategies have to be addressed (Figure 1):

1. Selecting patients that will benefit from the joint preserving intervention
2. Measurement of and control over the clinical outcomes of the joint preserving intervention





**Figure 1: Patient selection, joint intervention and outcome measurement.**

*Total knee replacement surgery can be performed when knee joint quality is decreased. To postpone a total knee replacement surgery, it is important to select patients that will benefit from a joint preserving intervention. Clinical characteristics, imaging data and synovial fluid biomarkers are attractive candidates to utilize in patient selection. To evaluate the outcomes of joint preserving interventions, synovial fluid biomarkers, imaging data and patient reported outcome measurements may be combined.*

The existence of OA phenotypes has to be considered when selecting patients that can potentially benefit from a specific joint preserving intervention<sup>26</sup>. Currently, it is unclear how the various OA phenotypes can be applied to predict outcome of the disease or specific response to joint preserving treatments. Post-traumatic OA, for instance OA developing in patients with cartilage defects, is one example of an OA subtype<sup>6</sup>. Since patients with cartilage defects can develop OA<sup>6</sup>, patient selection for joint preserving interventions such as cartilage repair surgery is important to delay or even prevent OA progression in this patient group. Several prognostic factors have already been described for these patients undergoing cartilage repair surgery. Earlier it was demonstrated that factors such as defect location, patient age, the number of treatments and defect age can predict clinical outcomes after cartilage repair<sup>27,28</sup>. For defect age, it was shown that patients with older defects have decreased improvements on clinical outcome after cartilage repair surgery<sup>27</sup>. This is likely to be related to a more disturbed knee joint homeostasis in this patient group, which is expected to inhibit the reparative response in the knee joint. This disturbed knee joint homeostasis can be evaluated by means of certain biomolecular biomarkers. Biomolecular markers may aid in selecting a specific patient group that will benefit from a joint preserving treatment. To date, no single biomarker has been developed to be sufficient for diagnosis or prognosis of joint pathologies such as cartilage defects or knee OA<sup>17</sup>. It is unlikely that in joint pathologies such as knee OA, a single biomarker can be used as a stand-alone measurement that will guide therapeutic strategies. However, these biomolecular markers can be used as an additional tool next to clinical, demographical and radiographical data to guide treatment decisions.

Biomolecular markers can be measured in various body fluids such as blood plasma, urine, serum, lacrimal secretion or synovial fluid. Specifically, the synovial fluid represents a promising body fluid for developing OA biomolecular diagnostics to access the condition of the joint as whole. It is in contact with many of the important intra-articular tissues, and directly represents the status of the knee joint, providing a clear evaluation opportunity of knee joint homeostasis. In **Chapter 7** we have identified the intra-articular tissue of origin for many proteins which are known to be increased in synovial fluid from OA patients compared to non-OA patients. Moreover, we found tissue-specific proteins which were increased in OA synovial fluid compared to non-OA synovial fluid. These proteins are potential candidates as biomarkers in efficacy studies of novel experimental treatments for knee OA, and may be able to report about the joint's health status on the tissue level. Cartilage-specific proteins can be used to aid in clinical decision making for patients with a cartilage defect. Furthermore, these tissue specific proteins hold potential to be used as treatment response biomarkers, aiding in the evaluation of outcomes in cartilage and meniscal repair surgery. In addition, our findings published in **Chapter 7** will fuel the development of novel synovial fluid-based approaches aiming at grading and subtyping knee OA. We expect that grading and subtyping joint pathologies such as knee OA will help to select patients which will benefit from specific treatments such as intra-articular injections with pharmaceuticals or regenerative cartilage repair strategies.

In conclusion, findings published in this thesis hold the potential to limit iatrogenic injuries during routine orthopaedic surgery, and may help to improve cell-based cartilage repair strategies, which can potentially delay knee OA progression. Furthermore, we provided evidence that anti-inflammatory treatment of the inflamed HFP can potentially lead to an improved knee joint quality. Moreover, we reported that the chondroprotective effects of selective COX-2 inhibitors may depend on their administration route. The auto-regulatory drug delivery system that we have described, holds the potential to incorporate analgesics, or DMOADs, to treat pain or improve structural changes in knee OA. Finally, we identified novel potential biomolecular markers that are released from different intra-articular tissues and may be used to assess knee joint quality on a tissue-specific basis. These biomarkers can aid in clinical decision making in joint pathologies such as knee OA and cartilage defects,



by subtyping knee OA and selecting patients that may benefit from the anti-inflammatory treatment with a drug delivery system. For example, the one that we have described in this thesis.

## References

- 1 Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, *et al.* Osteoarthritis. *Lancet.* 2015;386(9991):376-87.
- 2 Peat G, Thomas MJ. Osteoarthritis year in review 2020: epidemiology & therapy. *Osteoarthritis Cartilage.* 2021;29(2):180-9.
- 3 Latourte A, Kloppenburg M, Richette P. Emerging pharmaceutical therapies for osteoarthritis. *Nat Rev Rheumatol.* 2020;16(12):673-88.
- 4 Hunter DJ, March L, Chew M. Osteoarthritis in 2020 and beyond: a Lancet Commission. *Lancet.* 2020;396(10264):1711-2.
- 5 Diseases GBD, Injuries C. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet.* 2020;396(10258):1204-22.
- 6 Cicuttini F, Ding C, Wluka A, Davis S, Ebeling PR, Jones G. Association of cartilage defects with loss of knee cartilage in healthy, middle-age adults: a prospective study. *Arthritis Rheum.* 2005;52(7):2033-9.
- 7 Carey JL, Shea KG, Lindahl A, Vasiliadis HS, Lindahl C, Peterson L. Autologous Chondrocyte Implantation as Treatment for Unsalvageable Osteochondritis Dissecans: 10- to 25-Year Follow-up. *Am J Sports Med.* 2020;48(5):1134-40.
- 8 Shanfield S, Campbell P, Baumgarten M, Bloebaum R, Sarmiento A. Synovial fluid osmolality in osteoarthritis and rheumatoid arthritis. *Clin Orthop Relat Res.* 1988(235):289-95.
- 9 Alinezhad-Bermi S, Kabiri M, Rad I, Irani S, Hanaee-Ahvaz H. Hyperosmolarity benefits cartilage regeneration by enhancing expression of chondrogenic markers and reducing inflammatory markers. *In Vitro Cell Dev Biol Anim.* 2021;57(3):290-9.
- 10 Mang T, Lindemann S, Gigout A. Increasing the Medium Osmolarity Reduces the Inflammatory Status of Human OA Chondrocytes and Increases Their Responsiveness to GDF-5. *Int J Mol Sci.* 2020;21(2).
- 11 Eltawil NM, Ahmed S, Chan LH, Simpson A, Hall AC. Chondroprotection in Models of Cartilage Injury by Raising the Temperature and Osmolarity of Irrigation Solutions. *Cartilage.* 2018;9(3):313-20.

- 12 Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum.* 2003;33(3):155-67.
- 13 van der Kraan PM. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng Regen Med.* 2019;16(4):327-34.
- 14 Usher KM, Zhu S, Mavropalias G, Carrino JA, Zhao J, Xu J. Pathological mechanisms and therapeutic outlooks for arthrofibrosis. *Bone Res.* 2019;7:9.
- 15 Bastiaansen-Jenniskens YM, Wei W, Feijt C, Waarsing JH, Verhaar JA, Zuurmond AM, *et al.* Stimulation of fibrotic processes by the infrapatellar fat pad in cultured synoviocytes from patients with osteoarthritis: a possible role for prostaglandin f2alpha. *Arthritis Rheum.* 2013;65(8):2070-80.
- 16 Shelbourne KD, Wilckens JH, Mollabashy A, DeCarlo M. Arthrofibrosis in acute anterior cruciate ligament reconstruction. The effect of timing of reconstruction and rehabilitation. *Am J Sports Med.* 1991;19(4):332-6.
- 17 Deveza LA, Nelson AE, Loeser RF. Phenotypes of osteoarthritis: current state and future implications. *Clin Exp Rheumatol.* 2019;37 Suppl 120(5):64-72.
- 18 Labusca L, Zugun-Eloae F. The Unexplored Role of Intra-articular Adipose Tissue in the Homeostasis and Pathology of Articular Joints. *Front Vet Sci.* 2018;5:35.
- 19 Tindall EA, Sharp JT, Burr A, Katz TK, Wallemark CB, Verburg K, *et al.* A 12-month, multicenter, prospective, open-label trial of radiographic analysis of disease progression in osteoarthritis of the knee or hip in patients receiving celecoxib. *Clin Ther.* 2002;24(12):2051-63.
- 20 Raynauld JP, Martel-Pelletier J, Beaulieu A, Bessette L, Morin F, Choquette D, *et al.* An open-label pilot study evaluating by magnetic resonance imaging the potential for a disease-modifying effect of celecoxib compared to a modeled historical control cohort in the treatment of knee osteoarthritis. *Semin Arthritis Rheum.* 2010;40(3):185-92.
- 21 Kou L, Xiao S, Sun R, Bao S, Yao Q, Chen R. Biomaterial-engineered intra-articular drug delivery systems for osteoarthritis therapy. *Drug Deliv.* 2019;26(1):870-85.
- 22 Jones IA, Togashi R, Wilson ML, Heckmann N, Vangsness CT, Jr. Intra-articular treatment options for knee osteoarthritis. *Nat Rev Rheumatol.* 2019;15(2):77-90.
- 23 Lane NE, Schnitzer TJ, Birbara CA, Mokhtarani M, Shelton DL, Smith MD, *et al.* Tanezumab for the treatment of pain from osteoarthritis of the knee. *N Engl J Med.* 2010;363(16):1521-31.



## Chapter 9

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- 24 Li Y, Boraschi D. Endotoxin contamination: a key element in the interpretation of nanosafety studies. *Nanomedicine (Lond)*. 2016;11(3):269-87.
- 25 Al-Jawadi S, Capasso P, Sharma M. The road to market implantable drug delivery systems: a review on US FDA's regulatory framework and quality control requirements. *Pharm Dev Technol*. 2018;23(10):953-63.
- 26 Yuan C, Pan Z, Zhao K, Li J, Sheng Z, Yao X, *et al*. Classification of four distinct osteoarthritis subtypes with a knee joint tissue transcriptome atlas. *Bone Res*. 2020;8(1):38.
- 27 de Windt TS, Bekkers JE, Creemers LB, Dhert WJ, Saris DB. Patient profiling in cartilage regeneration: prognostic factors determining success of treatment for cartilage defects. *Am J Sports Med*. 2009;37 Suppl 1:58S-62S.
- 28 Janssen MPF, van der Linden EGM, Boymans T, Welting TJM, van Rhijn LW, Bulstra SK, *et al*. Twenty-Two-Year Outcome of Cartilage Repair Surgery by Perichondrium Transplantation. *Cartilage*. 2020:1947603520958146.



# English Summary



The research that is reported in this dissertation aimed at gaining new insights in novel strategies to improve 'knee joint quality' either on a cellular, tissue or articular joint level.

Focal cartilage defects may lead to inferior knee joint quality due to distorted joint biomechanics, leading to an increased inflammatory intra-articular environment. Ultimately this environment will lead to a joint disease known as osteoarthritis (OA). Therefore, we have evaluated molecular mechanisms involved in the phenotypic improvement of human articular chondrocytes. Generated knowledge is expected to aid in improvements in cell-based cartilage repair strategies and supporting post-treatment knee joint quality. In **Chapter 2**, we showed that culturing human articular chondrocytes under oxygen tension closely resembling their physiological *in vivo* environment, improves collagen type II expression via TGF- $\beta$ . Besides being hypoxic, cartilage is also generally considered to be hyperosmotic. The involvement of the TGF- $\beta$  pathway in the improvement of the chondrocyte phenotype under hypoxic conditions prompted us to also investigate its role under hyperosmotic conditions in **Chapter 3**. We could confirm an improved chondrocyte phenotype under hyperosmotic conditions. Interestingly, hyperosmotic conditions also most prominently increased TGF- $\beta$ 2 mRNA expression, which was in concordance with our experiments under hypoxic conditions. This may indicate that of the three TGF- $\beta$  isoforms, TGF- $\beta$ 2 has a prominent role in regulating the chondrocyte phenotype under hyperosmotic and hypoxic conditions.

Inflammatory processes have been linked to increased articular cartilage deterioration. Gaining insights into the role of such inflammatory processes for knee joint quality may yield important clues to develop new treatment strategies to improve cartilage repair strategies. In **Chapter 4**, we showed that prostanoid release by HFP from end-stage OA patients was on average higher than prostanoid release by HFP from patients with a cartilage defect. The secretion of prostanoid subtypes from the HFP from knee OA patients distributed into 2 OA subgroups. Furthermore, the COX-2 inhibitor celecoxib was able to effectively reduce prostanoid release by HFP by at least 80 %. In addition, celecoxib was able to skew the expression ratio of M1/M2 macrophage markers towards a more favorable M2 phenotype most effectively in HFP tissue with higher prostanoid release. This indicates that the capacity of celecoxib to skew towards the expression of markers associated with

M2 macrophages in HFP tissue is dependent on the magnitude of inflammation present.

Joint homeostasis may become disrupted and knee joint quality may become impaired in an inflamed knee joint. To combat this, different methods can be exploited. An intra-articular administration may be an effective means, since this means that the drug will be in direct contact with the different knee joint tissues. In **Chapter 5**, we have systemically reviewed the literature evidence for potential *in vivo* chondroprotective actions of selective COX-2 inhibitors. We reviewed the use of selective COX-2 inhibitors either systemically or intra-articularly. From this review it became clear that intra-articular administration, either as a bolus drug or incorporated in a drug delivery system (DDS) may be a more effective means to use selective COX-2 inhibitors to improve joint quality than systemic administration.

A DDS may circumvent side effects associated with systemic treatment, allowing a prolonged local drug residence time, increasing patient compliance and preventing frequent intra-articular injections. In this context, the use of a 'smart' drug delivery system from which drug release is reactive to the disease process, may be an effective means to treat joint pathologies such as knee OA. In **Chapter 6**, we have investigated the use of celecoxib-loaded poly(esteramide) (PEA) microspheres as a smart intra-articular DDS for OA treatment. PEA microspheres demonstrated good intra-articular biocompatibility and degraded in an *in vivo* OA model. Degradation of PEA microspheres was significantly higher in an inflammatory environment, while loading the microspheres with celecoxib decreased PEA degradation which may indicate a DDS with an auto regulatory behavior.

In **Chapter 7**, we hypothesized that knee joint synovial fluid, which is in contact with important intra-articular tissues, represents a promising body fluid to access the homeostatic condition of the knee joint as a whole. We performed comparative full-proteome analysis of the synovial fluid of OA patients versus non-OA individuals, as well as of the protein secretomes of Hoffa's fat pad, synovium, meniscus and articular cartilage of osteoarthritic knees. We identified several protein species with a tissue type-dependent release and also demonstrated their OA-specificity. Our findings demonstrated the presence of tissue type-dependent release of OA-specific proteins in OA knee joint synovial fluid.

Collectively, our findings presented in this dissertation demonstrate novel insights in the molecular mechanisms involved in chondrocyte phenotype improvement after hyperosmotic and hypoxic cultures and demonstrate the importance of TGF- $\beta$  signaling in the context of the chondrocyte phenotype. The HFP of patients with joint pathologies such as cartilage defects and knee OA has a substantial contribution to the prostanoid content found in synovial fluid. Targeting the knee joint by means of intra-articular injections instead of systemic administration of drugs may be more effective in improving knee joint homeostasis. Furthermore, celecoxib, either injected as a bolus drug or incorporated in a smart drug release system is an attractive novel strategy for treatment of joint diseases such as OA. Finally, we uncovered tissue type-dependent release of OA-specific proteins in knee joint synovial fluid. These proteins may be used as biomarkers to aid in developing treatment strategies to improve joint homeostasis for cartilage repair strategies or slowing down knee OA. Taken together, the findings published in this thesis in describing insights in improving knee joint quality on the cellular, tissue or total joint level will contribute to our understanding of joint pathologies such as knee OA and it is expected to aid in developing strategies to treat these joint pathologies.



## **Nederlandse Samenvatting**

Het onderzoek dat in dit proefschrift wordt beschreven, is gericht op het verkrijgen van nieuwe inzichten in nieuwe strategieën om de 'kniegewrichtskwaliteit' te verbeteren op cellulair, weefsel- of totaal kniegewrichtsniveau.

Focale kraakbeendefecten kunnen leiden tot een inferieure kwaliteit van het kniegewricht als gevolg van verstoorde gewrichtsbiomechanica. Een verstoring in de gewrichtsbiomechanica kan leiden tot een verhoogde inflammatoire intra-articulaire omgeving, wat uiteindelijk kan leiden tot artrose. Daarom hebben we moleculaire mechanismen geëvalueerd die betrokken zijn bij de fenotypische verbetering van menselijke articulaire chondrocyten. De verkregen wetenschappelijke inzichten kunnen leiden tot verbeteringen in celgebaseerde kraakbeenherstelstrategieën en het verbeteren van de kwaliteit van het kniegewricht. In **Hoofdstuk 2** hebben we aangetoond dat het kweken van menselijke articulaire chondrocyten onder zuurstofspanning die sterk lijkt op hun fysiologische *in vivo* omgeving, de expressie van collageen type II via TGF- $\beta$  verhoogt. Behalve dat het hypoxisch is, wordt kraakbeen over het algemeen ook als hyperosmotisch beschouwd. De betrokkenheid van de TGF- $\beta$  pathway bij de verbetering van het chondrocytfenotype onder hypoxische condities heeft ons ertoe aangezet om ook de rol ervan onder hyperosmotische condities te onderzoeken in **Hoofdstuk 3**. We konden een verbeterd chondrocytfenotype onder hyperosmotische condities bevestigen. Interessant is dat hyperosmotische omstandigheden ook het meest de TGF- $\beta$ 2-mRNA-expressie verhoogden, wat in overeenstemming was met onze experimenten onder hypoxische omstandigheden. Dit kan erop wijzen dat van de drie TGF- $\beta$ -isovormen, TGF- $\beta$ 2 een prominente rol speelt bij het reguleren van het chondrocytfenotype onder hyperosmotische en hypoxische omstandigheden.

Inflammatoire processen zijn in verband gebracht met een verhoogde afbraak van kraakbeen. Het verkrijgen van inzicht in de rol van deze inflammatoire processen voor de kwaliteit van het kniegewricht kan belangrijke aanwijzingen opleveren voor het ontwikkelen van nieuwe behandelingsstrategieën gericht op kraakbeenherstel. In **Hoofdstuk 4** hebben we laten zien dat de afgifte van prostanoiden door het Hoffa's vetlichaam (HFP) van artrosepatiënten gemiddeld hoger was dan de afgifte van prostanoiden door HFP van patiënten met een kraakbeendefect. De afscheiding van prostanoidesubtypen uit

de HFP van patiënten met knieartrose toonde een verdeling over 2 artrose-subgroepen. Bovendien was de COX-2-remmer celecoxib in staat om de PGE<sub>2</sub>-afgifte door HFP met ten minste 80% te verminderen. Tevens was celecoxib in staat om de expressieverhouding van M1/M2 macrofagenmarkers te veranderen naar een gunstiger M2 fenotype, het meest effectief in HFP-weefsel met hogere prostanoïde-afgifte. Dit geeft aan dat het vermogen van celecoxib om de expressie van markers die zijn geassocieerd met M2-macrofagen in HFP-weefsel te beïnvloeden afhankelijk is van de hoeveelheid aanwezige inflammatie.

De gewrichtshomeostase kan worden verstoord en de kwaliteit van het kniegewricht kan worden aangetast bij een ontstoken kniegewricht. Om dit tegen te gaan, kunnen verschillende methoden worden gebruikt. Een intra-articulaire toediening kan een effectieve methode zijn, aangezien dit betekent dat het medicijn direct in contact komt met de verschillende kniegewrichtsweefsels. In **Hoofdstuk 5** hebben we het bewijs voor mogelijke *in vivo* chondroprotectieve acties van ontstekingsremmende selectieve COX-2 remmers onderzocht. We hebben het gebruik van selectieve COX-2 remmers systemisch of intra-articulair beoordeeld. Uit deze review werd duidelijk dat directe intra-articulaire toediening een effectievere manier kan zijn om selectieve COX-2 remmers te gebruiken om de gewrichtskwaliteit te verbeteren in vergelijking met systemische toediening.

Een drug delivery systeem (DDS) kan bijwerkingen als gevolg van een systemische behandeling omzeilen, waardoor een langere lokale verblijftijd van het medicijn mogelijk is, de therapietrouw van de patiënt toeneemt en frequente intra-articulaire injecties worden vermeden. In deze context kan het gebruik van een 'slim' DDS waarin de medicijnafgifte reageert op het ziekteproces, een effectief middel zijn om de kwaliteit van het kniegewricht te verbeteren bij gewrichtspathologieën zoals bijvoorbeeld knieartrose. In Hoofdstuk 6 hebben we het gebruik van celecoxib-geladen polyesteramide (PEA) microsferen onderzocht als een 'slim' intra-articulaire DDS bij artrosebehandeling. PEA-microsferen vertoonden een goede intra-articulaire biocompatibiliteit en werden afgebroken in een *in vivo* OA-model. De afbraak van PEA-microsferen was significant hoger in een inflammatoire omgeving, terwijl het laden van de microsferen met celecoxib de PEA-afbraak verminderde, wat zou kunnen wijzen op een DDS met auto-regulerend gedrag.

In **Hoofdstuk 7** stelden we de hypothese op dat de synoviale vloeistof uit het knie gewricht,



die in contact is met belangrijke intra-articulaire weefsels, een veelbelovende lichaamsvloeistof vertegenwoordigt om inzichten te verkrijgen in de homeostatische toestand van het hele kniegewricht. We hebben een vergelijkende volledige proteoomanalyse uitgevoerd van de synoviale vloeistof van artrosepatiënten versus niet-artrosepatiënten, evenals van de eiwitsecretomen van HFP, synovium, meniscus en gewrichtskraakbeen van knieën met OA. We identificeerden verschillende eiwitsoorten met een weefseltype-afhankelijke afgifte en toonden ook hun OA-specificiteit aan. Onze bevindingen in **Hoofdstuk 7** benadrukten de aanwezigheid van weefseltype-afhankelijke afgifte van OA-specifieke eiwitten in synoviaal vocht van het kniegewricht van OA-patiënten.

Samenvattend demonstreren onze bevindingen gepresenteerd in dit proefschrift nieuwe inzichten in de moleculaire mechanismen die betrokken zijn bij de verbetering van het chondrocytfenotype na hyperosmotische en hypoxische culturen en demonstreren ze het belang van de TGF- $\beta$  pathway in het chondrocytfenotype. Bovendien levert de HFP van patiënten met gewrichtspathologieën zoals kraakbeendefecten en knieartrose een substantiële bijdrage aan het gehalte aan prostanoiden in gewrichtsvloeistof. Het behandelen van pathologieën in het kniegewricht door middel van intra-articulaire injecties in plaats van systemische toediening van medicijnen kan effectiever zijn bij het verbeteren van de homeostase van het kniegewricht. Celecoxib is, geïnjecteerd als een bolus medicijn of geïncorporeerd in een slim DDS, zoals PEA-microsferen, een aantrekkelijke nieuwe strategie om de gewrichtshomeostase te verbeteren. Tot slot hebben we weefseltype-afhankelijke afgifte van OA-specifieke eiwitten in het synoviale vocht van het kniegewricht aangetoond. Deze eiwitten kunnen worden gebruikt als biomarkers om te helpen bij het ontwikkelen van behandelingsstrategieën om de gewrichtshomeostase te verbeteren voor kraakbeenherstelstrategieën of om knieartrose te vertragen.

Concluderend zullen de bevindingen die in dit proefschrift zijn gepubliceerd over het verkrijgen van inzichten in het verbeteren van de kwaliteit van het kniegewricht op cellulair-, weefsel- en gewrichtsniveau bijdragen aan ons begrip van gewrichtspathologieën zoals knieartrose en zal naar verwachting helpen bij het ontwikkelen van strategieën om deze te behandelen.

## Türkçe Özet

Bu tezde sunulan araştırmanın amacı; hücresele, doku veya eklem seviyesinde 'diz eklemi kalitesini' geliştirmek için kullanılacak olan yeni stratejiler için yeni öngörüler ve kazanımlar elde etmektir. Fokal kıkırdak defektleri bozulmuş eklem biyomekaniği ve düşük diz eklemi kalitesine neden olabilmekte ve eklem içi artan bir inflamatuvar ortama yol açabilmektedir. Bu artan inflamatuvar ortam, osteoartrit diye tabir ettiğimiz bir eklem hastalığına neden olmaktadır. Dolayısıyla bu çalışma, insanlardaki kıkırdak hücrelerin fenotipik gelişiminde rol oynayan moleküler mekanizmalar üzerine odaklanmaktadır. Bu üretilen bilginin hücre bazlı kıkırdak onarım stratejilerinde ve tedavi sonrası diz eklemi kalitesini iyileştirmede yardımcı olması bekleniyor. Tezin **İkinci Bölümünde**, kıkırdak hücrelerinin, kıkırdağın insan vücudundaki fizyolojik olarak görülen hipoksik oksijen gerilim oranıyla kültüre edilmesiyle ilgili bilgilere yer verilmektedir. Bu araştırmalar neticesinde, kıkırdak hücrelerinde, kıkırdak dokusunda önemli bir protein olan kolajen tip II ekspresyonunun iyileştiği gözlemlenmiş ve bu iyileştirmede TGF- $\beta$  protein'in önemli bir rol üstlendiği tespit edilmiştir.

Kıkırdak hipoksik olmanın yanı sıra genellikle hiperozmotik olarak değerlendirilir. TGF- $\beta$  protein'in hipoksik gerilim ortamında kıkırdak hücrelerindeki fenotopik gelişimindeki rolü, bizleri Tezin **Üçüncü Bölümünde** bahsedilen, bu protein'in hiperosmotik ortamlarında kültüre edilmiş kıkırdak hücrelerin fenotipik gelişimindeki rolünü araştırmaya teşvik etmiştir. Bu amaçla öncelikle, hiperosmotik ortamlarında kültüre edilmiş kıkırdak hücrelerindeki fenotipik gelişimi doğrulanmıştır. İlginç bir şekilde, tıpkı hipoksik koşullarda kültüre edilmiş kıkırdak hücrelerinde gözlemediğimiz gibi, hiperozmotik koşullar da en belirgin şekilde artan TGF- $\beta$ 2 mRNA ekspresyonu olduğu gözlemlenmiştir. Bu bulgu, üç TGF- $\beta$  izoformu arasında, TGF- $\beta$ 2 izoformunun kıkırdak hücrelerindeki fenotipik gelişiminde önemli bir rol aldığını gösterebilir.

Kıkırdak hasarları inflamatuvar bir ortam ile ilişkilendirilmiştir. Bu inflamatuvar ortamın diz eklemi kalitesine etkisini araştırmak, yeni kıkırdak onarım tedavileri geliştirmek için önemli ipuçları verebilir. Tezin **Dördüncü Bölümünde** bahsedildiği üzere, osteoartrit hastaların Hoffa yağ yastığı tarafından salgılanan prostanoid oranının, kıkırdak defekt hastaların Hoffa yağ yastığından salgılanan prostanoid oranından, daha yüksek olduğu görülmüştür. Araştırmamızda, osteoartrit hastaların Hoffa yağ yastığından salgılanan prostanoid oranının 2 alt gruba ayrıldığı da gözlemlenmiştir. Ayrıca, COX-2 inhibitörü



selekoksib, Hoffa yağ yastığı tarafından prostanoid salınımını en az %80 oranında etkili bir şekilde azaltabilmiştir. Ek olarak, selekoksib M1/M2 makrofaj ekspresyon oranını daha uygun olan M2 fenotipine yönlendirmiştir. Bu M2 fenotipine en etkili yönlendirmenin, prostanoid salınımı yüksek olan Hoffa yağ yastıklarında olduğu gözlemlenmiştir. Bu bulgu, selekoksibin, M2 makrofajları ile ilişkili olan gen ekspresyonuna yönlendirme kapasitesi, Hoffa yağ yastığında inflamasyon oranına bağlı olduğunu gösterebilir.

Eklem homeostazı bozulabilir ve inflamasyon oranı yüksek olan bir diz eklemde diz eklem kalitesi düşebilir. Bununla mücadele etmek için farklı yöntemlerden yararlanılabilir. Eklem içi bir enjeksiyon etkili bir araç olabilir, çünkü bu, ilacın farklı diz eklem dokuları ile doğrudan temas halinde olacağı anlamına gelecektir. Tezin **Beşinci Bölümünde**, sistematik bir derleme ile selektif COX-2 inhibitörlerin potansiyel *in vivo* kondroprotektif etkileriyle ilgili araştırma bilgilerine yer verilmektedir. Bu sistematik derlemede selektif COX-2 inhibitörlerin sistemik veya eklem içi kullanımı incelenmiştir. Bu sistematik derlemeden elde edilen bulgular, selektif COX-2 inhibitörlerin sistemik tedaviye nazaran, eklem içi bolus bir ilaç veya bir ilaç dağıtım sistemine dahil edilmiş olarak kullanıldığında, eklem kalitesini geliştirmek için, daha etkili olabileceğini göstermektedir. Eklem hastalıklarında tedavi için, kontrollü bir ilaç salım sistemi daha avantajlı olabilir. Kontrollü ilaç salım sistemi, sistemik tedaviyle ilişkili yan etkilerin üstesinden gelebilir. Ayrıca kontrollü ilaç salım sistemi, ilacın eklem içinde daha uzun kalması, hastanın kullanımından kaynaklı ilaç kaybının engellenmesi ve gerek duyulan eklem içi enjeksiyonları minimum seviyesine indirmesi gibi avantajları vardır. Bu bağlamda, eklem içinde meydana gelen patolojik bulgulara ilaç salımı ile cevap verebilme yeteneği olan 'akıllı' bir kontrollü ilaç salım sistemi, osteoartrit gibi bir diz eklem hastalığında, bir avantaj getirebilir. Tezin **Altıncı Bölümünde**, selekoksib yüklü polyesteramid (PEA) mikrokürelerin osteoartrit tedavisinde eklem içi 'akıllı' bir kontrollü ilaç salım sistemi olarak uygulanabilirliği ile ilgili araştırma bilgilerine yer verilmektedir. Bu PEA mikroküreler, eklem içi biyouyumluluk gösterdi ve *in vivo* bir osteoartrit laboratuvar hayvan modelinde eklem içi degradasyon göstermiştir. PEA mikrokürelerinin degradasyonu, inflamatuvar bir ortamda daha yüksekken, mikroküreleri selekoksib ile yüklemek degradasyonu azaltmıştır. Bu bulgular, PEA mikrokürelerin otheregülasyon davranışı gösterebileceğine dair bir işarettir.

Tezin **Yedinci Bölümünde**, diz eklemi içinde bütün dokular ile temas halinde olan sinovyal sıvısı'nın, diz eklemine homeostatik durumu hakkında bilgi edinmek için umut verici bir sıvı olduğuna dair bir hipotez geliştirilmiştir. Hem osteoartrit hastaların, hem osteoartrit hastalığı olmayan bireylerin sinovyal sıvılarında proteomik analizlerimizin yanı sıra, Hoffa'nın yağ yastığı, sinovyum, menisküs ve eklem kıkırdağının sekretomlarında proteom analizleri gerçekleştirilmiştir. Analizler de doku-spesifik proteinler bulunmuştur ve bu proteinlerin OA-spesifik özellikleri olduğu gösterilmiştir. Bulgularımız, osteoartrit diz eklemi'nin sinovyal sıvısında doku-spesifik ve osteoartrite özgü proteinlerin varlığını göstermiştir.

Toplu olarak, bu tezde sunulan bulgular, hiperozmotik ve hipoksik kültürlerden sonra kıkırdak hücrelerdeki fenotip gelişiminde rol alan moleküler mekanizmalarda yeni anlayışlar göstermekte ve kıkırdak hücrelerin fenotipinde TGF- $\beta$ 'nin önemli rol oynadığını belirtmektedir. Sinovyal sıvıda bulunan prostanoid içeriğine, kıkırdak defektleri ve osteoartrit gibi eklem patolojileri olan hastaların Hoffa yağ yastığının önemli bir katkısı vardır. İlaçların sistemik uygulanması yerine, eklem içi enjeksiyonlar yoluyla diz eklemine hedeflenmesi, diz eklemi homeostazını iyileştirmede daha etkili olabilecektir. Ayrıca, bolus ilaç olarak enjekte edilen veya akıllı bir ilaç salım sistemine dahil edilen selekoksib, osteoartrit gibi eklem hastalıklarının tedavisi için çekici bir yeni stratejidir. Son olarak, diz eklemi sinovyal sıvısında osteoartrite özgü proteinlerin doku tipine bağlı salınımı gösterilmiştir. Bu proteinler, kıkırdak onarım stratejileri için eklem homeostazını iyileştirmek veya osteoartriti yavaşlatmak için tedavi stratejileri geliştirmeye yardımcı olmak için biyobelirteçler olarak kullanılabilir.

Birlikte ele alındığında, bu tezde yayınlanan ve hücresele, doku ve total eklem seviyesinde sunulan ve diz eklemi kalitesinin geliştirebilecek yeni bulgular, osteoartrit gibi eklem patolojilerini anlamamıza katkıda bulunacaktır ve bu eklemleri tedavi etmek için stratejilerin geliştirilmesine yardımcı olacaktır.

## List of Publications and Presentations



## List of Publications

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1. Das R, **Timur UT**, Edip S, Haak E, Wruck C, Weinans H, *et al.* TGF-beta2 is involved in the preservation of the chondrocyte phenotype under hypoxic conditions. *Ann Anat.* 2015;198:1-10.
2. **Timur UT**, van Herwaarden JA, Mihajlovic D, De Jong P, Mali W, Moll FL. (18)F-FDG PET scanning of abdominal aortic aneurysms and correlation with molecular characteristics: a systematic review. *EJNMMI Res.* 2015;5(1):76.
3. **Timur UT**, Janssen MPF, Woike N, Welting TJ, Draaisma G, Gijbels M, *et al.* Celecoxib-loaded PEA microspheres as an auto regulatory drug-delivery system after intra-articular injection. *J Control Release.* 2016;244(Pt A):30-40. **(Shared First Author)**
4. **Timur UT**, Ripmeester EGJ, Caron MMJ, Welting TJM. Recent Insights into the Contribution of the Changing Hypertrophic Chondrocyte Phenotype in the Development and Progression of Osteoarthritis. *Front Bioeng Biotechnol.* 2018;6:18. **(Shared First Author)**
5. **Timur UT**, Caron MMJ, Bastiaansen-Jenniskens YM, Welting TJM, van Rhijn LW, van Osch G, *et al.* Celecoxib-mediated reduction of prostanoid release in Hoffa's fat pad from donors with cartilage pathology results in an attenuated inflammatory phenotype. *Osteoarthritis Cartilage.* 2018;26(5):697-706.
6. **Timur UT**, Caron M, van den Akker G, van der Windt A, Visser J, van Rhijn L, *et al.* Increased TGF-beta and BMP Levels and Improved Chondrocyte-Specific Marker Expression *In Vitro* under Cartilage-Specific Physiological Osmolarity. *Int J Mol Sci.* 2019;20(4).
7. **Timur UT**, Caron MMJ, Jeuken RM, Bastiaansen-Jenniskens YM, Welting TJM, van Rhijn LW, *et al.* Chondroprotective Actions of Selective COX-2 Inhibitors *In Vivo*: A Systematic Review. *Int J Mol Sci.* 2020;21(18).

## List of Publications and Presentations

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8. **Timur UT**, Jahr H, Anderson J, Green DC, Emans PJ, Smagul A, *et al.* Identification of tissue-dependent proteins in knee OA synovial fluid. *Osteoarthritis Cartilage*. 2021;29(1):124-33.
9. **Timur UT**, Jaspars CCMM, van Baardwijk L. Behandeling van recidiverende hemartros na totale knieprothese. *Medisch Journaal MMC, Najaarseditie* 2021.
10. Jeuken R, v Hugten P, Roth AK, **Timur UT**, Boymans TAEJ, van Rhijn LW, Bugbee WD, Emans PJ. A Systematic Review of Focal Cartilage Defect Treatments in Middle-Aged versus Younger Patients. *OJSM* 2021.

## Conferences with Oral Presentations

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1. **Timur UT**, van der Windt AE, Caron MC, Haak E, Visser J, Weinans H, Welting TJM, Emans PJ, Jahr H. TGF $\beta$ 2 knockdown under physiological osmolarity improves COL2 expression in chondrocytes *in vitro*. **(EORS 2014, Nantes)**
2. **Timur UT**, van der Windt AE, Caron MC, Haak E, Visser J, Weinans H, Welting TJM, Emans PJ, Jahr H. TGF $\beta$ 2 knockdown under physiological osmolarity improves COL2 expression in chondrocytes *in vitro*. **(NBTE 2014, Lunteren)**
3. **Timur UT**, van der Windt AE, Haak E, Visser J, Caron MC, Weinans H, Welting TJM, Emans PJ, Jahr H. Physiological osmolarity combined with TGF-  $\beta$ 2 knockdown improves collagen type II expression in chondrocytes *in vitro*. **(EORS 2015, Bristol, best oral presentation award)**
4. **Timur UT**, van der Windt AE, Caron MC, Weinans H, Welting TJM, Emans PJ, Jahr H. TGF- $\beta$ 2 knockdown under physiological osmolarity improves COL2 expression in chondrocytes *in vitro*. **(DKOU 2015, Berlin)**
5. **Timur UT**, Janssen M, Berard J, Welting TJM, van Rhijn L, Mihov G, Thies J, Emans PJ. Biocompatibiliteit en afbraak van celecoxib geladen microspheres na intra-articulaire injectie bij osteoartrose. **(NOV jaarcongres 2016, Den Bosch, nominated for the prof. dr. ir. Rik Huiskeprijs)**

6. **Timur UT**, Janssen M, Berard J, Welting TJM, van Rhijn L, Mihov G, Thies J, Emans PJ. Biocompatibiliteit en afbraak van celecoxib geladen microspheres na intra-articulaire injectie bij osteoartrose. **(NOV jaarcongres 2016, Den Bosch)**
7. **Timur UT**, Caron MC, Bastiaansen-Jenniskens Y, Welting TJM, van Osch GJVM, Emans PJ. PGE<sub>2</sub> and PGF<sub>2a</sub> are secreted by the osteoarthritic infrapatellar fat pad and their release can be modulated by celecoxib. **(ICRS 2016, Sorrento)**
8. **Timur UT**, Janssen MPF, Gijbbels M, Draaisma G, Mihov G, Welting T, van Rhijn L, Thies J, Emans PJ. Celecoxib loaded PEA-based microspheres as an auto regulatory drug-delivery system after intra-articular injection. **(ICRS 2016, Sorrento)**
9. **Timur UT**, Caron M, van der Windt A, Emans P, Welting T, Jahr H. TGFβ superfamily-dependent and osmolarity-mediated type II collagen expression by chondrocytes *in vitro*. *(ICORS 2016, Xian)*
10. **Timur UT**, van der Windt AE, Caron MC, Weinans H, Welting TJM, Emans PJ, Jahr H. Osmolarity mediated and TGF-β2 dependent BMP and COL2 expression in chondrocytes *in vitro*. **(EORS 2016, Bologna)**
11. **Timur UT**, Caron MC, van der Windt AE, Weinans H, Welting TJM, Emans PJ, Jahr H. BMP signalling in human chondrocytes is involved in the expression of COL2 in human chondrocytes. **(EORS 2017, Munich)**
12. **Timur UT**, Emans PJ, van Rhijn L, Welting TJM. CART-1: a novel chondrogenic regulator. **(EORS 2017, Munich)**
13. **Timur UT**, Caron MC, Welting TJM, van Rhijn L., Emans PJ, Jahr H. BMP signaling in human chondrocytes is involved in the expression of COL2 in human chondrocytes. **(EORS 2017, Munich)**
14. **Timur UT**, Caron MC, Bastiaansen-Jenniskens Y, Welting TJM, van Osch GJVM, Emans PJ. Celecoxib vermindert prostanoïde uitscheiding door verschillende intra-articulaire gewrichtsweefsels. **(NOV Najaarsvergadering 2017, Veldhoven)**



## List of Publications and Presentations

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15. **Timur UT**, Jahr H, van Rhijn L, Emans PJ, Peffers MJ, Welting TJM. Identification of tissue-specific biomarkers in knee OA synovial fluid: a proteomic approach. **(OARSI 2018, Liverpool)**

## Conferences with Poster Presentations

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1. **Timur UT**, van der Windt AE, Caron MC, Weinans H, Welting TJM, Emans PJ, Jahr H. Improved collagen type II expression in chondrocytes under physiological osmolarity upon TGF- $\beta$ 2 knockdown. **(ORS 2015, Las Vegas)**
2. **Timur UT**, Caron MC, Bastiaansen-Jenniskens Y, Welting TJM, van Osch GJVM, Emans PJ. PGE<sub>2</sub> and PGF<sub>2a</sub> are secreted by the osteoarthritic infrapatellar fat pad and their release can be stimulated by celecoxib. **(OARSI 2016, Amsterdam)**
3. **Timur UT**, Caron MC, Bastiaansen-Jenniskens Y, Welting TJM, van Osch GJVM, Emans PJ. Synovium, Hoffa's fat pad and meniscus of knee osteoarthritis patients release higher levels of prostaglandin subtypes than cartilage and their release can be modulated by celecoxib. **(ORS 2017, San Diego)**
4. **Timur UT**, Caron MC, Emans PJ, van Rhijn L, Welting TJM, Jahr H. TAK1 inhibition improves chondrocyte marker expression independent of osmolarity. **(EORS 2017, Munich)**
5. **Timur UT**, Caron MC, Bastiaansen-Jenniskens Y, Welting TJM, van Osch GJVM, Emans PJ. Celecoxib modulates release of different prostanoid subtypes by intra-articular tissues: towards celecoxib based intra-articular treatment for OA modulation? **(OARSI 2018, Liverpool)**

## Other Achievements

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Winner of the Mosacell microscopy contest: Mass-spec imaging of a rat knee by Ufuk Tan Timur (Orthopedic Surgery), Nina Ogrinc Potocnik (M4I) and Florian Barré (M4I).



**Dankwoord**



## Anneme ve Babama

Sevgili anne ve baba, bu kitap sizlerin sayesinde mümkün oldu. Benim için ne anlama geldiğini ve ne ifade ettiğini anlatmaya kelimeler yetmez ama yine de deneyeceğim.

Sevgili annem,

Bana anlattığın bir hikaye ile başlamak istiyorum. Hamileyken anneannemin merdivenlerini silerken dua etmissin, inşallah bir oğlum olur, gözleri yeşil. Oğlan olmasa da gözleri yeşil olsun yeter demissin. Allah sana ikisini birden nasip etmiş. Seni hiç bir zaman üzmedim, ama senin gibi bir anne'yi kim üzebilir ki? Allah herkese senin gibi bir super anne nasip etsin.

Sabahın erken saatlerinde, bazen akşam geç saatlere kadar vardiyeli çalıştığın fulltime bir işe ek olarak, fulltime bir anne olarak benimle ilgilendin. İlkokul ve ortaokuldayken kendimi geliştirmem için her zaman en iyi şartları sağladın. Türk annelerin klişe bir lafı vardır, oğlum okuyun da bizim gibi olmayın, eskiden anlamıyorduk belki, ama şimdi çok daha iyi anlıyorum. Senin gibi kötü çalışma koşullarında çalışmak zorunda kalmamamız için bana her zaman destek oldun ve iyi bir eğitimin önemini anlattın. Her zaman okulda elimden gelenin en iyisini yapmamı isterdin. Hiç ödev yapmadığım için sık sık tartışmış olsak da, 'her şey çok güzel olacak medoş' dediğimde gerçekten her şeyin yolunda gittiğini gördün, bu kitabimi sana ve babama armağan ediyorum, bu kitap sizlerin sayesinde yazıldı. Okuldan iyi bir notla geldiğimde gözlerindeki neşe ve coşkuyu asla unutmuyacağım. Kendimi maksimum seviyede geliştirmem gerekme motivasyonun beni olduğum yere getirdi. Çalıştığın fabrikada bir öğrenci olarak bir iş bulduğumda ve geçici olarak meslektaşın olduğumda, nasıl bir süper anne olduğunu gerçekten anladım. Sadece bir süper anne her gün bu işi yapabilir ve aynı zamanda 2 çocuk yetiştirebilir. Rüzgara karşı, yağmur, çamur demeden, beni bisikletle basketbol antrenmanlarıma bıraktın (neyse ki o zamanlar o kadar da kilolu değildim). Benim için en lezzetli yemekleri yaptın. Zor kazanılan paralarla bana en güzel Nike Air Max ayakkabılarını aldın. Beni zorlamadan, inancımız hakkında mümkün olduğunca çok şey öğrenebilmem için beni Camii'ye gönderdin ama aynı zamanda bana her zaman diğer insanlara, dinlere ve kültürlere saygı duymayı da öğrettin.

Sevgili babam,

35 yıl önce Hollanda'ya misafir işçi olarak geldin. İki küçük çocuk, bir fabrikada fulltime bir iş ve aynı zamanda Fontys'de öğretmenlik eğitimi, pek çok insan seni taklit edemez!

Bir Kürt çocuğu olarak Türk bir ailede damat olmanın senin için kolay olmadığını farkındayım. Azmin harika ve genlerimize kazınmış. Bana bunu verdiğin için teşekkür ederim. Herkese yardımsever yaklaşımın benim için bir gurur kaynağı, seninle gurur duyuyorum. Yaptığım her şeyde beni her zaman destekledin ve hayat derslerin beni bugün olduğum yere getirdi. Bu yaşıma kadar ne yaptıysam, hatalarımla birlikte, hep arkamda dağ gibi, bir baba gibi durdun. Beni Fenerbahçe taraftarı yaptığın için sana teşekkür ederim. 6 Kasım 2002'de sadece ilk yarısını izlememe izin verdin, ama sonra uyumam gerektiğini söyledin, çünkü ertesi gün okulum olduğu için uyumam gerekiyordu. Yine de o gece beni uyandırıp 6-0 kazandık oğlum dediğini dün gibi hatırlıyorum.

18 yaşına basıp tıp'ı kazandığımda beni tatile Türkiye'ye gönderdin. Bu arada, annem ve sen, tatilimden hemen sonra taşınabileceğim Rotterdam'daki daireyi döşuyordunuz! Bir baba figürü olarak hayatım boyunca benim için gerçekten her şeyi nasıl ayarladın, kafamdaki kaosumu düzenlemeyi nasıl sağladın, tüm bunlar olmasaydı, bunu asla başaramazdım.

Sevgili annem ve babam,

Hayatımda bir olay var, hiç unutamiyorum, size şimdiye kadar hiç bahsetmedim, ama bugüne kısmetmiş. Bir gün hastaydim ve yüzümü yıkamak için banyoya gittim, benim için endişelendiniz, aynaya baktım, sağ tarafımda babamın sol eli omuzumda, sol tarafımda annemin sağ eli omuzumda, işte bu görüntü sizlerin benim hayatımdaki yeri için bir mecazi.

“

Gurbet Elde Bir Hal Geldi Başıma,

Ağlama Gözlerim Mevlâ Kerimdir.

Derman Arar İken Derde Düş Oldum,

Ağlama Gözlerim Mevlâ Kerimdir ”

Sizleri çok ama çok seviyorum, iyi ki benim annem ve babamsınız!



## Dankwoord

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Beste collega's, vrienden en familie. Jammer dat u meteen weer het dankwoord opent, in mijn voorwoord had ik al gewaarschuwd, maar het is u vergeven. Het was een lange rit, maar mijn boek is eindelijk af! Dit boek heeft bijgedragen aan mijn wetenschappelijke ontwikkeling, maar ook aan mijn persoonlijke ontwikkeling. Ik wil iedereen bedanken voor hun interesse en hulp in het tot stand komen van dit proefschrift, het is en blijft een team effort. Er zijn een aantal mensen die ik in het bijzonder wil bedanken voor hun bijdrage.

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Mijn Co-Promotor **dr. P.J. Emans**, beste **Pieter**, jij was de elektromotor van dit proefschrift, de drijfkracht. Toen ik op 06-03-2014 jouw kamer binnenstapte was ik al gelijk onder de indruk van jouw enthousiasme over wetenschappelijk onderzoek. Je hebt altijd in mij geloofd en hebt me wijze lessen geleerd over onderzoek doen. Je weet altijd overal een praktische oplossing op te bedenken, ook bij tegenslagen:

“ *Promoveren is als trouwen met de universiteit zei je, maar je moet later ook zelf trouwen met een droomvrouw, want achter elk succesvolle man staat een succesvolle vrouw!* ”

Mijn moeder is nog steeds dankbaar en benieuwd naar wie deze fantastische co-promotor van mij is. Jouw quotes en oneliners zijn inspirerend en ik sta nog steeds versteld van jouw kennis, niet alleen op medisch gebied, maar ook op allerlei andere gebieden zoals de politiek, cultuur of zelfs voetbal. Je kan werkelijk waar over alles meepraten en je bent een lopende encyclopedie. Nadat ik bij jou uit de kamer stapte na een gesprek had ik soms wel kortsluiting in mijn brein, omdat ik veel informatie kreeg en het deels begreep, maar niet alles. Hoe bijzonder en fascinerend is het dat ik werkelijk waar na elke meeting altijd de dingen ben tegengekomen waar jij het over had: 'Oh, dit had Pieter al gezegd, dus dit

was het wat hij bedoelde! Woorden schieten te kort om te omschrijven hoeveel je voor mijn carrière hebt betekend en nog steeds betekent. Als ik ergens mee zit bied je altijd een luisterend oor, jouw gesprekken hebben ervoor gezorgd dat ik mezelf ben blijven ontwikkelen en in mezelf ben blijven geloven! Je vervult niet alleen een voorbeeldfunctie voor mij op werk, maar ook een voorbeeldfunctie als mens en vader. Ik zal onze rit in de Tesla over de straten van Bemelen samen met Lotte en Jaap nooit vergeten (Jij sliep al Puk). Ik zou ook **Lonneke** willen bedanken voor de gezellige etentjes en de effectieve meetings bij jullie thuis, dank voor de gastvrijheid!

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## Dankwoord

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Rotterdam en ik kreeg meteen een hele tekening over TGF-beta signalling op de whiteboard. Een tekening met SMAD-eiwitten, TGF-beta receptoren, noggin eiwit etc. Ik snapte er niks van. Gelukkig begrijp ik het nu allemaal een stuk beter. Bedankt voor alle leuke momenten op congressen in Berlijn, Nantes en Bristol. Bedankt dat je me wakker hebt gehouden op onze terugrit vanuit Nantes naar Maastricht, waarin je merkte dat ik het toch af en toe moeilijk had richting het einde van de rit. Bedankt voor alle lekkere etentjes in Aachen, waaronder ook in het Turkse restaurant waar niemand Turks kon praten.

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“*Waar wrijving is ontstaat warmte.*”

## Dankwoord

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Als AIOS leer ik nog steeds heel veel van jou. Ik vind het mooi om te zien dat je de AIOS die door jou zijn opgeleid in een oogopslag herkent.

“ *Leuk is voor thuis, interessant is het op werk.* ”

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## Dankwoord

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Je bent een fantastische persoon, en hebt het hart op de juiste plek.

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## Dankwoord

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onmisbare waarde geweest en de kwaliteit van de histologische plaatjes wordt nog altijd geroemd in wetenschappelijke presentaties! Alle collega's van het **CPV**, bedankt voor jullie hulp bij alle experimenten die ik heb uitgevoerd!

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## Dankwoord

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## About the Author



## CURRICULUM VITAE

Ufuk Tan Timur was born on the 9<sup>th</sup> of May 1989 in Waalwijk, The Netherlands. After graduating *cum laude* from high school (Gymnasium, Willem van Oranje College, Waalwijk) in 2007, he started with his Medical School Training at the Erasmus University Rotterdam in the Netherlands. During his Medical School Training he was selected for the Erasmus MC Molecular Medicine programme, which is a Master of Science programme which focuses on developments in biomedical science.



As part of his Medical School Training, he started with Orthopaedic Research at the Department of Orthopaedic surgery in Rotterdam, under supervision of prof. dr. ir. H.H. Weinans and dr. H. Jahr. After graduating *cum laude* from medical school at the Erasmus University in Rotterdam in 2014, he has continued with a research year at the Department of Orthopaedic Surgery in Maastricht to finish his last year of the Molecular Medicine Master of Science Programme. The research he has conducted in Rotterdam and Maastricht led to the start of his PhD training Programme in 2015 at the Department of Orthopaedic Surgery in Maastricht (under supervision of prof. dr. L.W. Van Rhijn, prof. dr. T.J.M. Welting and dr. P.J. Emans) and Uniklinik RWTH in Aachen (under supervision of dr. H. Jahr). To gain clinical experience, he has interrupted his PhD training in 2016 to work as a resident-not-in-training at the Department of Orthopaedic Surgery, Maxima Medical Center Veldhoven under supervision of dr. R.P.A. Janssen. In 2017, Ufuk Tan Timur was accepted for the Orthopaedic Surgery Residence Programme in ROGO Zuid. In January 2018, he started his general surgical training at the Catharina Hospital in Eindhoven (under supervision of dr. S.W. Nienhuijs).

## About the Author

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He continued his Orthopaedic Surgery Training at the MUMC+ (under supervision of prof dr. L.W. Van Rhijn and dr. H.M. Staal) and currently prolonged his residency at the Maxima Medical Center in Veldhoven (under supervision of dr. R.P.A. Janssen). He is expected to finish his Orthopaedic Training in 2024.

Ufuk Tan Timur has Turkish and Kurdish Roots. He is engaged to Eslem Coban and the wedding is planned in September 2022.





Welcome to the incredible world of Orthopedic Surgery. Joint pathologies such as knee osteoarthritis are a major public healthcare problem and cause chronic pain and disability worldwide. These pathologies have a huge impact on the health-related quality of life.

In this thesis, author Ufuk Tan Timur offers an insightful look into methods to improve knee joint quality on a micro- and macro-environmental level to combat knee joint pathologies such as cartilage defects and knee osteoarthritis. This thesis will learn you about new insights in improving knee joint quality on the cellular, tissue or total joint level. These insights will contribute to our understanding of joint pathologies and it is expected to aid in developing strategies to treat these joint pathologies.

