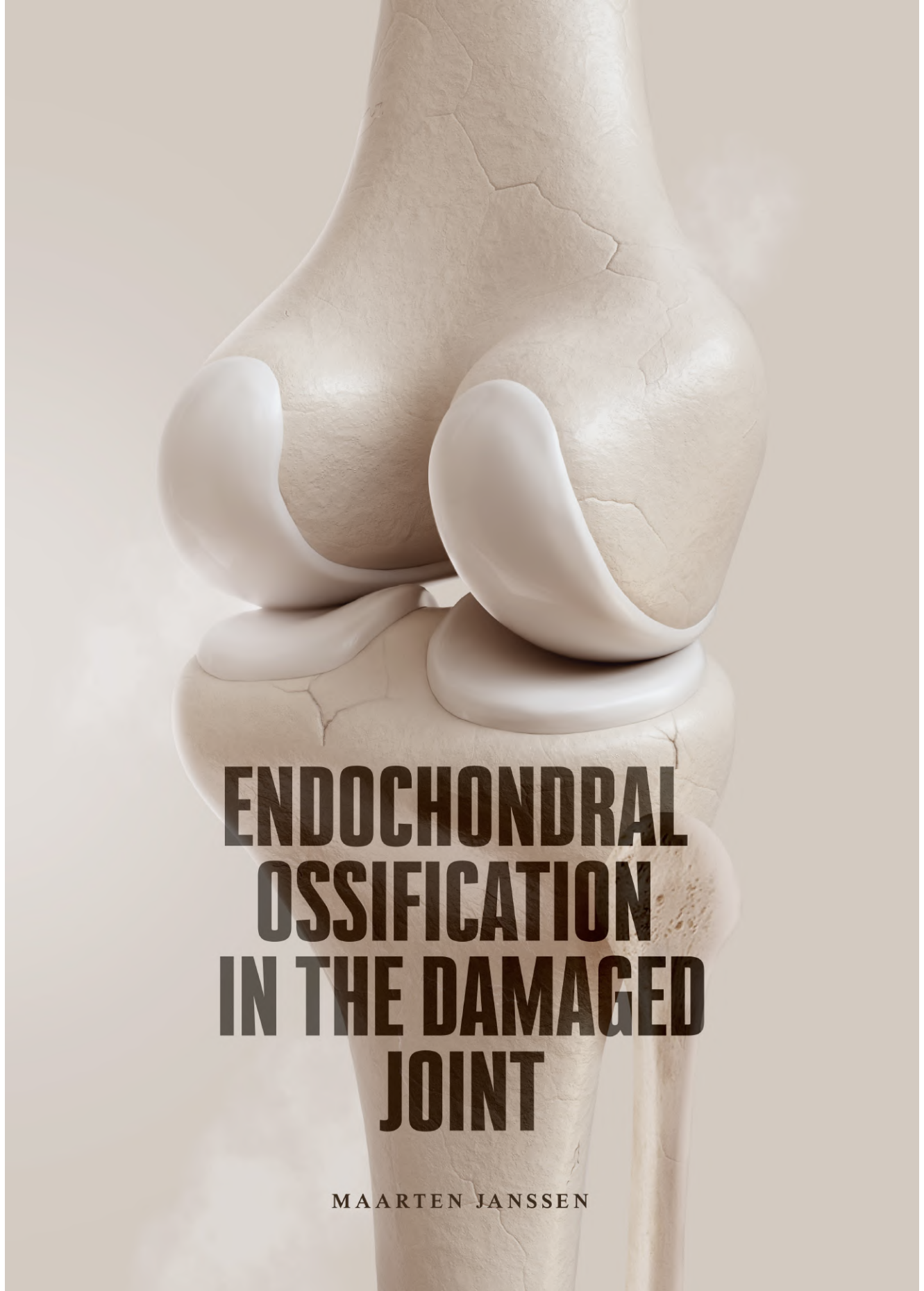

ENDOCHONDRAL OSSIFICATION IN THE DAMAGED JOINT

MAARTEN JANSSEN

Endochondral ossification in the damaged joint

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Contents

General introduction

General introduction

Human life is changing. Life expectancy has increased worldwide and as a way to improve quality of life, physical exercise is recommended, even at an older age.[1-3] The positive effects of exercise on reducing cancer, cardiovascular disease, dementia and also osteoarthritis (OA) have been extensively described in the literature.[4] Ironically, mechanical trauma due to exercise is described as the biggest cause of focal articular cartilage defects.[5] Therefore, increased exercise can also be detrimental due to the extra stress we exert on our musculoskeletal system, which may, especially in combination with increased obesity, result in mechanical and biological joint damage.[6,7]
A great amount of the strength and resilience of the musculoskeletal system is derived from an interplay of tension and compression in the bones, muscles, ligaments and cartilage, a phenomenon called tensegrity (Figure 1.1).[8] Tensegrity seems nature's aim to find the balance between loading and loadbearing capacity and is also found as hydrostatic pressure in the arcade like macromolecular structure of articular cartilage. As a result, these different parts of the human musculoskeletal system have their own characteristics. Articular cartilage in particular, has little regenerative capacity and is prone to continued degeneration.[9] In the process of articular cartilage damage and joint degeneration, an important component is the altered expression of biomolecular factors, amongst others, growth factors and cytokines affecting the homeostasis of articular cartilage and increasing unfavourable endochondral ossification (EO) of the cartilage tissue.[10-12]
The work presented in this thesis focusses on understanding alterations in the role of EO in articular cartilage damage and possibilities to use the active biomolecular mechanisms in EO for the treatment of articular cartilage damage.

Skeletal development

Bones are developed via two different mechanisms: intramembranous and endochondral bone formation.[13] The flat bones of the skull, most of the cranial bones and clavicles are the result of intramembranous bone formation. In this cellular process, immature mesenchymal progenitor cells differentiate directly into bone forming osteoblasts.[14] In contrast, endochondral bone formation occurs by the development of a cartilaginous template, which is later replaced by bone through EO.[15]
The postnatal process of EO is heavily influenced by at least three different levels of biomechanical stimuli that reflect the principles of tensegrity (i.e., cell, muscle, and environmental level).[16-18] In embryonic development, aggregation of mesenchymal stem cells forms the cartilage anlagen. Mesenchymal cells in the non-vascularized centre of the condensation differentiate under the influence of the essential Sex Determining Region Y-Box 9 (SOX9) into chondrocytes and increase the synthesis of, amongst others, aggrecan and collagens type II, IX, and XI.[19] Simultaneously, cells at the periphery of the condensations differentiate into perichondrial cells that express collagen type I and form the bone collar.[19]

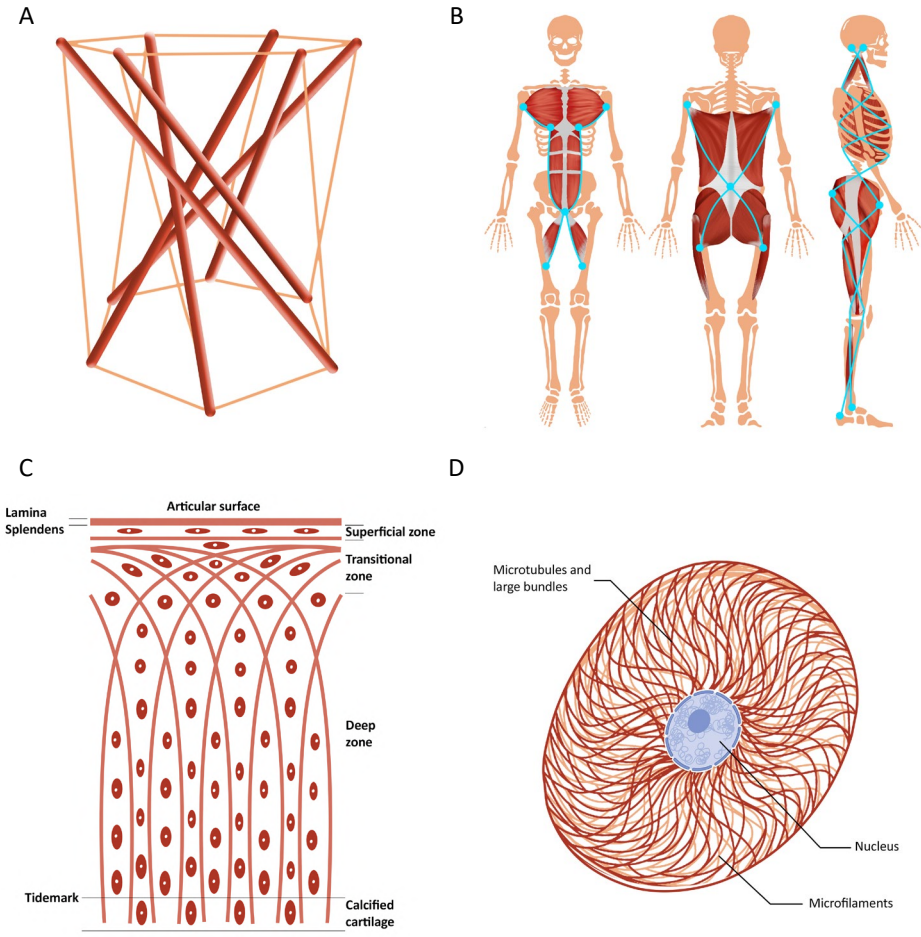

Figure 1.1 The mechanism of tensegrity depicted in several levels.

A. Tensegrity depicted in a 3D-model. The struts counteract the compressive force applied by the strings. Vice versa, the tensile force from the struts is counteracted by the strings resulting in an equilibrium.
B. Tensegrity in the musculoskeletal system of a human. The bones act as struts and counteract the compressive forces created by tensioning of the muscles, tendons and ligaments (the cables).
C. Tensegrity in articular cartilage. Osmotic pressure caused by hydration of proteoglycans pre-tensions the cartilage and counteracts the tensile forces created by a network of collagen fibres.
D. Tensegrity in the cell. Microfilaments from the cytoskeleton cause tensile forces and pull the membrane inwards. Microtubules and large bundles of microfilaments act as struts and counteract the inward forces.

In the resulting cartilage primordium the distinction in growth plate and articular cartilage formation begins with the emergence of the interzone, a tripartite structure consisting of a mid-density inner layer (the central intermediate lamina) and two high-density outer layers (Figure 1.2).[20] Hereafter, two distinct processes continue (i.e., endochondral bone formation and articular cartilage formation), which will be explained below.

Growth plate & endochondral bone formation

Directed by bone morphogenetic protein (BMP) signalling, the chondrocytes at the core of the cartilage primordium proliferate at a high rate.[21] These chondrocytes subsequently become hypertrophic. This is regulated mainly by runt-related transcription factor 2 (Runx2), which drives, amongst others, collagen type X alpha 1 chain (COL10a1) expression.[22] Finally, hypertrophic chondrocytes transdifferentiate into osteoblasts or undergo apoptosis and the surrounding extracellular matrix (ECM) calcifies, setting the stage for endochondral bone formation by vascular invasion and osteoblast activity (i.e., the growth plate) (Figure 1.2).[23,24]
An important factor in regulating the pace of the growth plate development and thereby the speed of EO bone formation is the Parathyroid hormone-related protein (PTHrP) - Indian Hedgehog (Ihh) negative feedback loop.[25]

Ihh is produced by early hypertrophic chondrocytes in the growth plate and stimulates the production of PTHrP by perichondrial cells and resting zone chondrocytes.[26,27] In a concentration-dependent manner, PTHrP inhibits chondrocyte hypertrophy and keeps chondrocytes in a proliferative state.[28] The proliferation of chondrocytes increases the distance between early hypertrophic chondrocytes and the PTHrP-secreting cells in the resting zone. By this increased distance, the local PTHrP concentration decreases, chondrocytes become hypertrophic and again produce Ihh.[29] This resulting PTHrP-Ihh negative feedback loop is crucial in balancing development of the growth plate, but there are multiple other pathways that influence the growth plate (e.g., BMPs, fibroblast growth factor, insulin-like growth factor, Wnt signalling and Runx2) and potentially interact with the PTHrP-Hedgehog negative feedback mechanism.[28,30]

Articular cartilage formation

Occurring by a process similar to the endochondral ossification of the 'true' growth plate, articular cartilage temporarily acts as a surface growth plate and is to some extent responsible for epiphyseal bone growth.[31] However, the process of articular cartilage formation is distinct from growth plate cartilage and subsequent bone formation.[23] As articular cartilage develops within the cartilage anlagen, the anlagen elongates and approaches the adjacent anlagen. However, the anlagen remain separated by the interzone, a distinct, non-cartilaginous region.[32] Chondrocytes at the ends of the anlagen differentiate into the articular cartilage under the influence of Wingless and Int-1 signaling.[23] The chondrocytes in developing articular cartilage are

marked by the expression of Sox5, 6, and 9, which regulate the expression of genes encoding ECM molecules like collagen type II and the proteoglycan aggrecan. This supports the development of hyaluronic acid-rich articular cartilage.[33] The resulting hyaline articular cartilage is an avascular and aneural tissue with an arcade-like macromolecular structure that consists of different zones each with their unique biomechanical and cellular properties. These unique properties provide an environment in which the joint can articulate with very little resistance and at the same time is able to withstand substantial compressive and shear forces.[34] In the superficial articular cartilage zone, collagen fibres are mainly orientated parallel to the joint surface to withstand shear stress.[34] Collagen fibres in the intermediate zone have a more random orientation.[34] In the deep zone the collagen fibres are orientated parallel to the long axis of the bone to withstand compressional forces and to provide a firm anchoring of the articular cartilage to the subchondral bone.[35,36] In close contact to the subchondral bone is the deepest layer, a zone of calcified cartilage, rich in collagen type X and alkaline phosphatase (ALP) which is separated from the non-calcified cartilage by the tidemark (Figure 1.2).[13,37-40]

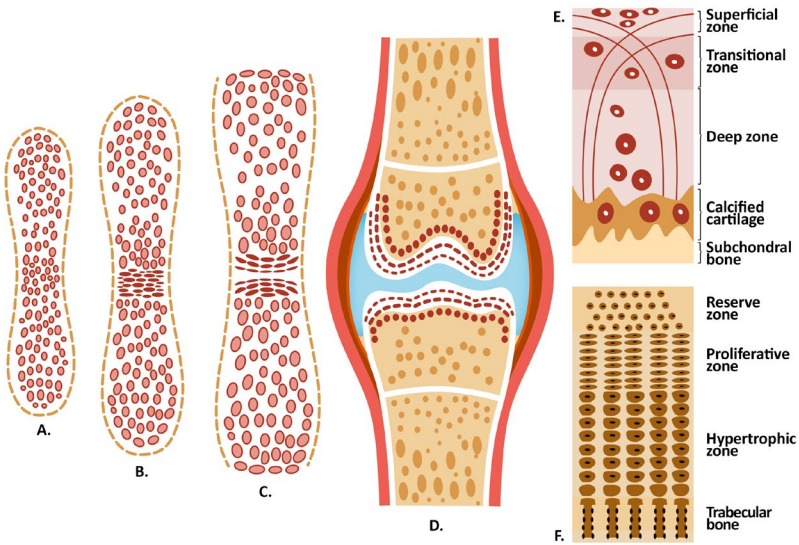

Figure 1.2 Schematic representation of the formation of articular and growth plate cartilage.
(A). Aggregation of mesenchymal cells forms the cartilage anlagen
(B). As the cartilage anlagen elongates, the interzone emerges
(C). The interzone contributes to the formation of the joint cavity and synovial tissue
(D). Bone and joint development continue and the growth plate and articular cartilage arise
(E). The resulting articular cartilage is a highly organized structure of, amongst others, chondrocytes, proteoglycans and collagen fibres which are oriented differently in the superficial, transitional and deep zone. Separated from the non-calcified cartilage by the tidemark is a layer of calcified cartilage which overlies the subchondral bone
(F). A more detailed representation of the growth plate displaying the resting zone, proliferative zone, hypertrophic zone and below the zone of chondrocyte transdifferentiation or apoptosis, vascular invasion and osteoblast activity.

Articular cartilage damage

Articular cartilage has a low cellularity and in addition, a very low number of progenitor cells, as compared to other tissues.[41,42] Also, the highly organized collagen and proteoglycan network debilitates chondrocyte motility.[43] These factors are hypothesized to cause the limited regenerative capacity of articular cartilage.[43-45] Physiological stress on the joint can trigger a beneficial anabolic response for the articular cartilage composition (i.e., increased glycosaminoglycan (GAG) content).[46] However, due to the aforementioned limited regenerative capacity, pathologic (i.e., larger and/or iterative) stresses can either cause a focal cartilage defect, or trigger a catabolic process that disturbs the joint homeostasis and initiate pre-osteoarthritis (pre-OA).[45,46] When not adequately treated, focal cartilage defects, as well as pre-OA, will inevitably lead to OA.[9]

Pre-osteoarthritis

The onset of articular cartilage damage can be defined as pre-OA.[45] At this stage, often before the stage of early OA,[47] a patient has one or more risk factors, but can still be asymptomatic, and damage is microscopic rather than macroscopic. Because joint damage is predominantly at the cellular level.[45] the applied diagnostic modalities have to be able to detect changes at this level. Therefore, biomarkers focused on changes at the tissue level (e.g.: serum cartilage oligomeric matrix protein (s-COMP), adiponectin, matrix metalloproteinases (MMPs), type II collagen C-telopeptide (UCTX-II), and interleukin 6 (IL-6)), and enhanced MRI protocols that can visualize a diminished GAG content in articular cartilage, are extensively studied topics.[45,46] Unfortunately, these techniques have not yet been implemented clinically.[45,46] In addition, when articular cartilage lesions are still confined to the cellular level and no macroscopic damage can be observed (International Cartilage Repair Society (ICRS) score grade 0), arthroscopy has to be enhanced by complementary techniques such as near infrared, ultrasound, or mechanical probing to be able to detect pre-OA.[45]
Although diagnosing pre-OA is difficult, in this stage of OA, only minor changes at a cellular level are present, which might be still reversible (healing without scar formation),[48] or repairable (healing with scar formation but still a functional organ).[47] Therefore, this stage has been proposed as a preferable moment to intervene, rather than when structural damage is already present.[45]

Articular cartilage lesions

Articular cartilage lesions are very common in humans. A review of 42 studies (4322 knees in 3446 asymptomatic adults) reported an overall pooled prevalence of 24%.[49] A more recent prospective cohort study of 230 knees in asymptomatic adults using 3.0T MRI showed articular cartilage lesions in even 62% of the knees.[50] Causes for localized articular cartilage lesions are mostly non-contact trauma in either sports or daily life, but many patients report no history of trauma at all.[5] It is likely that the limit

of physiological stress is exceeded in these patients at some point in time. Patients with articular cartilage lesions are often asymptomatic, but otherwise impaired function, pain, joint effusion, and clicking or locking are most frequently reported.[51] Articular cartilage defects are graded based on the ICRS-score:[52] (Figure 1.3).

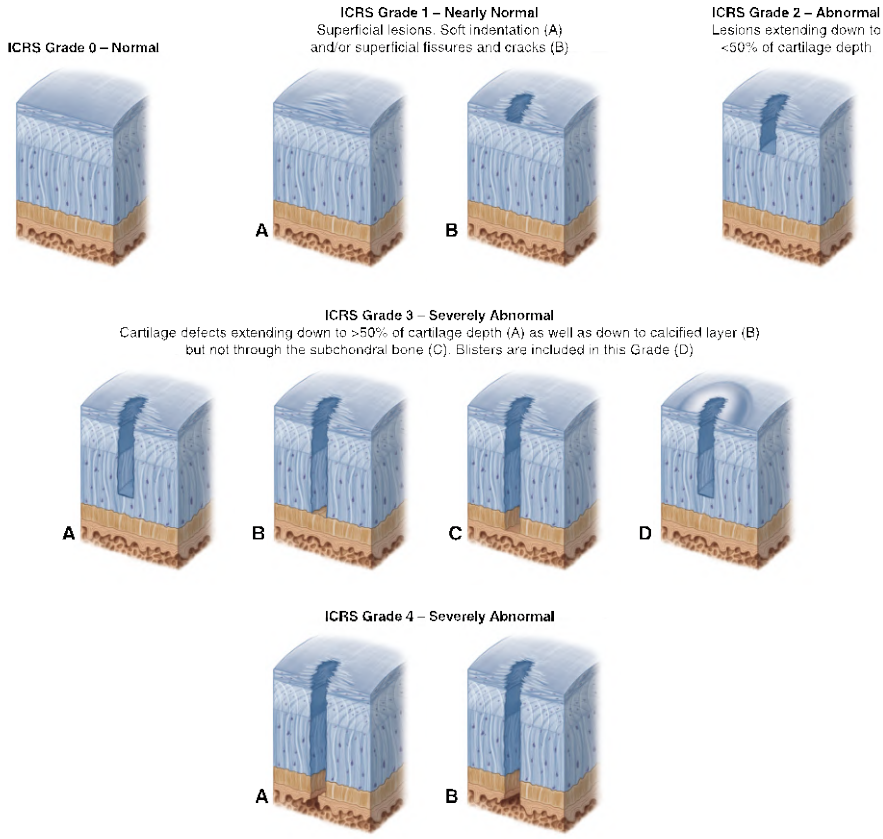

Figure 1.3 ICRS-score (reprint with permission of the International Cartilage Regeneration & Joint Preservation Society)
Grade 0 – Normal
Grade 1 – Softening or superficial cracks.
Grade 2 – Lesions extending <50% of cartilage depth.
Grade 3 – Lesions extending >50% or into the calcified layer, but not in subchondral bone.
Grade 4 – Lesions extending in the subchondral bone.

Articular cartilage defects not only cause visible changes on MRI or arthroscopy, but cellular changes are also present.[53] In a study of Saris et al., within a few weeks after the creation of an articular cartilage defect in the knees of 21 adolescent goats, prostaglandin synthesis increased and GAG content in articular cartilage diminished when the defects were treated 'late' or not at all.[53] An association between articular cartilage defects on MRI and UCTX-II (a marker for collagen type II breakdown) with

only minimal signs of OA was described by Ding et al.[54] The amount of inflammation can differ, but at least some inflammation will be present in the joints of all patients with articular cartilage defects.[55] Proinflammatory cytokine levels (e.g., IL-1-beta, IL-6 and tumor necrosis factor alpha (TNF-alpha)) have shown to inhibit all varieties of tissue repair (including bone and cartilage).[55,56] This notion is supported by the study of Davies-Tuck et al. who used MRI at baseline and 2-year follow-up to show that most articular cartilage defects tend to increase in severity and only a small percentage of defects decrease in severity when left untreated.[9] In the same study, increasing age and a high volume of involved subchondral bone area were found to be risk factors for progression of the defects into OA.[9]

Osteoarthritis

Osteoarthritis (OA) is the most common joint disorder leading to pain and physical disability and constitutes a large medical healthcare economic burden worldwide.[57] Due to the ageing population and increasing prevalence of obesity, this burden will increase even more in the near future.[7,58,59]
Osteoarthritis is a complex and chronic whole joint disease with structural changes in the articular cartilage, subchondral bone, synovium, ligaments and periarticular muscle. This is further characterized by amongst others a pathologic micro-environment that causes cartilage degradation and mineralization,[60] osteophyte formation,[61] subchondral sclerosis,[62] an increased number of tidemarks,[63] and low-grade synovial inflammation with subsequent fibrosis.[64] The cause of osteoarthritis is multifactorial, but the underlying pathogenesis is considered to be an imbalance between degenerative stimuli and repair capacity of the joint tissues.[65] In an OA joint, when the joint homeostasis is disturbed, chondrocytes show an aberrant phenotype and actively produce cartilage-degrading enzymes, such as MMP-13 and aggrecanases.[53,60] Many of the frequently used markers for OA cartilage (type X collagen, MMP-13, Runx-2, ALP, and IHH) indicate a substantial role of endochondral ossification of articular cartilage in OA.[60] Terminal differentiation of chondrocytes, endochondral ossification, and often inflammation, cause alterations that can contribute to the degeneration of cartilage. In the healthy joint, these alterations do not occur in articular cartilage (except in the calcified layer and tidemark),[66] but are found in the osteoarthritic joint.[67] This indicates that EO contributes to the degenerative process in OA cartilage.[67] When left untreated, pre-OA as well as focal articular cartilage defects disturb the joint homeostasis, almost certainly induce osteoarthritis and eventually may even lead to joint replacement surgery.[60,68]

Treatment of articular cartilage damage

Articular cartilage damage can present as focal articular cartilage defects and diffuse cartilage damage (OA). However, an important difference between the two is that OA is a result of long existing and increasing damage and usually encompasses a degenerated joint as a whole. At present there is no treatment known that is able to restore the joint

and its anabolic state.[59] In contrast, a timely and adequate treatment of focal articular cartilage defects is thought to be able to slow down or even reverse a catabolic state of the joint, which prevents deterioration of the joint and subsequent OA.[45,53]
As stated earlier, when left untreated, a focal articular cartilage defect is almost certain to progress into osteoarthritis.[60] Therefore, a variety of interventions to treat focal articular cartilage defects has been developed over the last decades.[69] But, although treatment algorithms have been improved, compared to other areas such as arthroplasty, articular cartilage repair surgery is a relatively emerging field in orthopaedic surgery. Long-term clinical follow-up data is often lacking, hampering the interpretation of clinical efficacy of cartilage repair surgery.[69] An overview of currently available treatments of articular cartilage damage is provided below.

Treatment of focal articular cartilage defects

Tissue Regeneration approaches

Marrow stimulation techniques

Surgery of focal articular cartilage defects in the knee started with reparative procedures such as abrasion chondroplasty, Pridie drilling, microfracture and other marrow stimulation techniques.[70] A more recent advancement is microdrilling, which is a combination of Pridie drilling and microfracture. This is a more controllable technique in which small diameter holes are drilled and no thermal necrosis is found.[69,71] The rationale behind marrow stimulation techniques (MSTs) is to trigger a repair mechanism which results in a defect filled with fibrocartilaginous tissue. The success rate of MSTs is higher in younger patients. This is attributed to a decreasing availability of bone-marrow-derived stem cells in the ageing patient.[69] Although short-term results of MSTs were promising, the resulting tissue lacks durability and the risk of failure on a longer-term follow-up is high.[72] The consensus is that MSTs should be reserved for well-contained chondral lesions smaller than 2-3 cm^2.[73] Moreover, the failure rate of revision surgery by autologous chondrocyte implantation (ACI) is increased after failed previous marrow stimulating techniques and therefore caution is advised for its use in larger lesions.[74]

Tissue engineering and regenerative medicine

The fields of tissue engineering (TE) and regenerative medicine are considered most promising for the treatment of articular cartilage defects.[69] They both aim to create functional (cartilage) tissue by combining cells, scaffolds, and biologically active molecules.[69] Periosteum- and perichondrium transplantation were early forms of TE.[75-78] In these techniques, the harvested tissues included both chondroprogenitor cells and the scaffold.[78,79] While this combination was hypothesized to be ideal for cartilage formation and early results were promising, it was prone to chondrocyte hypertrophy and is no longer used due to unsatisfactory results.[80] The most well-known cell-based

example of TE in articular cartilage surgery is ACI which expands chondrocytes for re-implantation.[81] The cell (chondrocyte) based technique ACI was introduced by Brittberg, Peterson and co-workers in 1994.[82] The technique includes two surgical procedures. During a first surgical procedure, cartilage tissue is harvested from a healthy, non-weight bearing part of the articular cartilage. From this tissue, chondrocytes are retrieved and expanded in a laboratory. During a second procedure, the chondrocytes from the cell culture were injected into the defect beneath a periosteal flap in the first generation of ACI.[83] While ACI has led to good clinical results since its introduction.[83,84] the technique has been refined several times over the years.[69] These refinements include substitution of the periosteal flap by a collagen or hyaluronan matrix (MACI), a selection of chondrocytes with high chondrogenic potential (CCI) and the currently used fourth generation ACI (Chondrosphere/Spherox).[69] Spherox ACI has been found cost-effective based on studies comparing ACI to microfracture and is currently recommended by the National Institute of Health and Care Excellence (NICE) for the treatment of articular cartilage defects (ICRS-score grade III and IV) over 2 cm^2 in patients with minimal OA (i.e., Kellgren & Lawrence grade less than 2) and without previous surgery of the defect.[85,86] Although age is not mentioned as a limiting factor in the NICE guideline, improved outcomes are often demonstrated in younger patients (age <30) for cell-based treatment strategies.[87]

Other cell types for use in TE are mesenchymal stem cells that can be derived from, amongst others, adipose tissue, perichondrium, periosteum and bone marrow and subsequently treated with e.g., growth factors to induce chondrogenic differentiation.[81] Scaffolds can be created from several materials (e.g., collagen, hyaluronan, synthetic) and are also used to contain chondrocytes or other cell sources such as bone marrow stem cells derived from microfracture, drilling or other donor sites.[88] They are designed to resemble the structure and function of articular cartilage and/or subchondral bone.[88] The enormous diversity of tissue properties of articular cartilage and subchondral bone complicates the development of a suitable scaffold for cartilage tissue engineering and to date no engineered construct with acceptable cartilage repair capacity has been produced.[88]

Cartilage tissue resurfacing

To avoid the formation of biomechanically less sustainable fibrocartilage, osteochondral autografting (OATS) and allografting (OCA) were introduced to resurface a defect with articular cartilage.[89] An osteochondral plug is taken from a donor site (autograft in a low- or non-load-bearing location of the knee or allograft) and driven press-fit into the prepared receptor site. Another advantage is its concomitant treatment of the subchondral bone. Both OATS and OCA lead to good results, but a major drawback of autografting is donor site morbidity making osteochondral autografting less suitable for larger lesions.[90,91] In both autograft and allograft, the technique causes tissue damage when hammering the plug into place.[69] The main

drawback of allografting is the ever-present risk for disease transmission, and in Europe its limited availability.[69,92] Substitutes of biological tissue resurfacing are biomaterial-based resurfacing approaches such as focal synthetic implants (e.g., Hemicap and Episurf).[93,94] The goal of these synthetic implants is to slow down or stop degeneration of adjacent cartilage tissue and improve the joint homeostasis. These solutions are mostly used in patients aged 40-60 years, who have less regenerative capacity but are deemed too young for joint replacement.[94] Short-term follow-up (2-7 years) has provided significant improvements in knee function and pain after surgery, but the risk of OA progression remains.[93]

Treatment of osteoarthritis

When a joint has progressed into OA, there is currently no treatment that has demonstrated to be able to biologically restore the joint and its homeostasis.[45,59] When OA progression is severe, but patients are still considered too young for joint arthroplasty, delaying arthroplasty is paramount. In this case, structural changes (e.g., joint preserving surgery, life-style advice and weight-loss) should be considered, supplemented by reducing symptoms (e.g., physical therapy, oral and/or intra-articular analgesic therapy).[59] In order to delay or prevent total joint arthroplasty (which is considered a 'last resort' treatment), alternative treatments can be attempted.[94] Examples of alternative treatments in an OA joint used in current practice or clinical trials are injections (e.g., sprifermin, LNA043),[95,96] joint re-alignment surgery,[97] joint distraction,[98] or focal implants,[99] These treatments, which aim to improve the joint homeostasis by repairing, replacing, or relieving the damaged (part of the) joint, show good results in clinical trials.[100] Creating awareness for the need and further improvement of these joint-preserving treatments is subject of the goal of the International Cartilage Regeneration and Joint Preservation Society (ICRS).[98]

Finally, to adequately treat focal articular cartilage defects as well as osteoarthritis, both conditions should be considered as a disease of the whole joint, instead of the cartilage alone.[101] In addition, patient characteristics are expected to be important factors for the successful outcome of a surgical/biotechnological restoration technique.[102]

Aims and outline of this thesis

Articular cartilage repair surgery is still a relatively emerging field in orthopaedic surgery compared to arthroplasty. To catch-up with knowledge gained in the field of arthroplasty, clinicians will have to collaborate intensively with scientists in the field of cartilage repair. This thesis aims to combine the strengths of a basic scientific approach with the scope of a clinician to contribute to the improvement of articular cartilage repair treatment.

The first part of this thesis focusses on the analysis of articular cartilage defect treatments. **Chapter 2** describes the influence of patient characteristics in articular cartilage repair surgery. The outcome of perichondrium transplantation (PT) was assessed after an average follow-up of 25 years. This long-term follow-up provided the opportunity to analyse failure of PT (defined as revision surgery in which the transplant was removed). The functioning of non-failed patients was evaluated using the International Knee Documentation Committee (IKDC) score. In addition, the influence of patient characteristics was evaluated.
A comparison between PT and the nowadays widely used autologous chondrocyte implantation (ACI) technique is made in **Chapter 3**. Clinical functioning of PT and ACI patients was assessed using the Knee injury and Osteoarthritis Outcome Score (KOOS) and IKDC-score and the current state of the knee was analysed by high-field 7T MRI. The Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score was used to evaluate the MRI-scans and subsequently we assessed the calcifications of the grafts and the influence of these calcifications on the opposing articular cartilage and clinical outcome parameters in more detail.

In **Chapters 4 and 5**, the focus lies on what can be done to improve articular cartilage repair. A better understanding of possible mechanisms that may cause native and repaired articular cartilage to undergo undesired endochondral ossification was sought in **Chapter 4**. The influence of cyclooxygenase-2 (COX-2) inhibition on the cartilaginous phase of three different endochondral ossification scenarios was analysed in an animal model. Fracture healing, growth plate development and the formation of ectopically-induced cartilaginous tissue were analysed by radiography, micro-CT, histology and gene expression analysis.
In **Chapter 5**, we analysed the effect of supplementation of aggrecan and COMP to optimize *in vivo* cartilage formation, a technique previously described to create autologous, cartilaginous tissue for the repair of articular cartilage defects.[103] Supplementation of aggrecan and COMP was studied *in vitro* during chondrogenic differentiation of rabbit periosteum cells and periosteum-derived chondrocytes. In addition, low-melting agarose was supplemented with bovine aggrecan, COMP or vehicle, and was injected *in vivo* between the bone and periosteum at the upper medial side of the tibia of New Zealand white rabbits. In vivo generated subperiosteal cartilage tissue was analysed for weight, GAG and DNA content.

In the next part of this thesis, possible treatment options were explored to optimize the intra-articular environment in order to reduce pain and delay or prevent progression into OA.
In **Chapter 6**, a review of the available literature was performed to assess the currently available 'non-surgical', intra-articular drug therapies. In this review we summarized an overview of candidate drugs for OA treatment and drug delivery systems that are able to carry and release drugs that are potentially beneficial for the treatment of OA.
Because the ideal combination of a drug and a drug delivery system for the intra-articular treatment of OA has not yet been described, we explored a potential novel approach in **Chapter 7**. We describe the development, and *in vitro* and *in vivo* performance of celecoxib-loaded polyester amide-based microspheres as an auto regulatory drug-delivery system for intra-articular injection.

In the general discussion **(Chapter 8)**, findings of the previous chapters are placed into perspective. This provides an overview for implications in future studies on EO in articular cartilage damage and possibilities to use the biomolecular mechanisms in EO in the treatment of articular cartilage damage.

References

1. Max Roser EO-OaHR. Life Expectancy. https://ourworldindata.org/life-expectancy. Published 2013. Accessed.
2. McPhee JS, French DP, Jackson D, Nazroo J, Pendleton N, Degens H. Physical activity in older age: perspectives for healthy ageing and frailty. *Biogerontology.* 2016;17(3):567-580.
3. Garatachea N, Pareja-Galeano H, Sanchis-Gomar F, Santos-Lozano A, Fiuza-Luces C, Moran M, Emanuele E, Joyner MJ, Lucia A. Exercise attenuates the major hallmarks of aging. *Rejuvenation Res.* 2015;18(1):57-89.
4. Hirsch CH, Diehr P, Newman AB, Gerrior SA, Pratt C, Lebowitz MD, Jackson SA. Physical activity and years of healthy life in older adults: results from the cardiovascular health study. *J Aging Phys Act.* 2010;18(3):313-334.
5. Widuchowski W, Widuchowski J, Trzaska T. Articular cartilage defects: study of 25,124 knee arthroscopies. *Knee.* 2007;14(3):177-182.
6. Urquhart DM, Tobing JF, Hanna FS, Berry P, Wluka AE, Ding C, Cicuttini FM. What is the effect of physical activity on the knee joint? A systematic review. *Med Sci Sports Exerc.* 2011;43(3):432-442.
7. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL, Laslett LL, Jones G, Cicuttini F, Osborne R, Vos T, Buchbinder R, Woolf A, March L. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis.* 2014;73(7):1323-1330.
8. Ingber DE. The architecture of life. *Scientific American.* 1998;278(1):48-57.
9. Davies-Tuck ML, Wluka AE, Wang Y, Teichtahl AJ, Jones G, Ding C, Cicuttini FM. The natural history of cartilage defects in people with knee osteoarthritis. *Osteoarthritis Cartilage.* 2008;16(3):337-342.
10. Huang J, Zhao L, Chen D. Growth factor signalling in osteoarthritis. *Growth Factors.* 2018;36(5-6):187-195.
11. Nishimura R, Hata K, Takahata Y, Murakami T, Nakamura E, Ohkawa M, Ruengsinpinya L. Role of Signal Transduction Pathways and Transcription Factors in Cartilage and Joint Diseases. *Int J Mol Sci.* 2020;21(4).
12. Charlier E, Deroyer C, Ciregia F, Malaise O, Neuville S, Plener Z, Malaise M, de Seny D. Chondrocyte dedifferentiation and osteoarthritis (OA). *Biochem Pharmacol.* 2019;165:49-65.
13. Hall BK. *Bones and cartilage: developmental and evolutionary skeletal biology.* Elsevier; 2005.
14. Breeland G, Sinkler MA, Menezes RG. Embryology, Bone Ossification. In: *StatPearls.* Treasure Island (FL)2020.
15. Ono N, Balani DH, Kronenberg HM. Stem and progenitor cells in skeletal development. *Curr Top Dev Biol.* 2019;133:1-24.
16. Carter DR, Wong M, Orr TE. Musculoskeletal ontogeny, phylogeny, and functional adaptation. *Journal of biomechanics.* 1991;24 Suppl 1:3-16.
17. Ingber DE, Folkman J. Tension and Compression as Basic Determinants of Cell Form and Function: Utilization of a Cellular Tensegrity Mechanism. In: Stein WD, Bronner F, eds. *Cell Shape.*1989:3-31.
18. Ingber DE. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci.* 1993;104 (Pt 3):613-627.
19. Allas L, Boumediene K, Bauge C. Epigenetic dynamic during endochondral ossification and articular cartilage development. *Bone.* 2019;120:523-532.
20. Lyons KM, Rosen V. BMPs, TGFbeta, and border security at the interzone. *Curr Top Dev Biol.* 2019;133:153-170.
21. Zou H, Wieser R, Massague J, Niswander L. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* 1997;11(17):2191-2203.
22. Zheng Q, Zhou G, Morello R, Chen Y, Garcia-Rojas X, Lee B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. *J Cell Biol.* 2003;162(5):833-842.
23. Ray A, Singh PN, Sohaskey ML, Harland RM, Bandyopadhyay A. Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation. *Development.* 2015;142(6):1169-1179.
24. Aghajanian P, Mohan S. The art of building bone: emerging role of chondrocyte-to-osteoblast transdifferentiation in endochondral ossification. *Bone Res.* 2018;6:19.

25. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science.* 1996;273(5275):613-622.
26. van Donkelaar CC, Huiskes R. The PTHrP-Ihh feedback loop in the embryonic growth plate allows PTHrP to control hypertrophy and Ihh to regulate proliferation. *Biomech Model Mechanobiol.* 2007;6(1-2): 55-62.
27. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, Bacher JD, Baron J. The role of the resting zone in growth plate chondrogenesis. *Endocrinology.* 2002;143(5):1851-1857.
28. Kronenberg HM. Developmental regulation of the growth plate. *Nature.* 2003;423(6937):332-336.
29. Ohba S. Hedgehog Signaling in Skeletal Development: Roles of Indian Hedgehog and the Mode of Its Action. *Int J Mol Sci.* 2020;21(18).
30. Hallett SA, Ono W, Ono N. Growth Plate Chondrocytes: Skeletal Development, Growth and Beyond. *Int J Mol Sci.* 2019;20(23).
31. Hunziker EB, Kapfinger E, Geiss J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2007;15(4):403-413.
32. Pitsillides AA, Ashhurst DE. A critical evaluation of specific aspects of joint development. *Dev Dyn.* 2008;237(9):2284-2294.
33. Prein C, Beier F. ECM signaling in cartilage development and endochondral ossification. *Curr Top Dev Biol.* 2019;133:25-47.
34. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect.* 1998;47:477-486.
35. Gottardi R, Hansen U, Raiteri R, Loparic M, Duggelin M, Mathys D, Friederich NF, Bruckner P, Stolz M. Supramolecular Organization of Collagen Fibrils in Healthy and Osteoarthritic Human Knee and Hip Joint Cartilage. *PLoS One.* 2016;11(10):e0163552.
36. Hughes LC, Archer CW, ap Gwynn I. The ultrastructure of mouse articular cartilage: collagen orientation and implications for tissue functionality. A polarised light and scanning electron microscope study and review. *Eur Cell Mater.* 2005;9:68-84.
37. Staines KA, Pollard AS, McGonnell IM, Farquharson C, Pitsillides AA. Cartilage to bone transitions in health and disease. *J Endocrinol.* 2013;219(1):R1-R12.
38. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. *Clin Orthop Relat Res.* 2001(391 Suppl):S26-33.
39. Emans PJ, Peterson L. *Developing insights in cartilage repair.* Springer; 2014.
40. Gray H. Antomy of the human body. *Ann Surg.* 1918;68(5):564-566.
41. Kamisan N, Naveen SV, Ahmad RE, Kamarul T. Chondrocyte density, proteoglycan content and gene expressions from native cartilage are species specific and not dependent on cartilage thickness: a comparative analysis between rat, rabbit and goat. *BMC Vet Res.* 2013;9:62.
42. Stockwell RA. The cell density of human articular and costal cartilage. *J Anat.* 1967;101(Pt 4):753-763.
43. Morales TI. Chondrocyte moves: clever strategies? *Osteoarthritis Cartilage.* 2007;15(8):861-871.
44. Hunziker EB, Michel M, Studer D. Ultrastructure of adult human articular cartilage matrix after cryotechnical processing. *Microsc Res Tech.* 1997;37(4):271-284.
45. Ryd L, Brittberg M, Eriksson K, Jurvelin JS, Lindahl A, Marlovits S, Moller P, Richardson JB, Steinwachs M, Zenobi-Wong M. Pre-Osteoarthritis: Definition and Diagnosis of an Elusive Clinical Entity. *Cartilage.* 2015;6(3):156-165.
46. Eckstein F, Hudelmaier M, Putz R. The effects of exercise on human articular cartilage. *J Anat.* 2006;208(4):491-512.
47. Bay-Jensen AC, Hoegh-Madsen S, Dam E, Henriksen K, Sondergaard BC, Pastoureau P, Qvist P, Karsdal MA. Which elements are involved in reversible and irreversible cartilage degradation in osteoarthritis? *Rheumatol Int.* 2010;30(4):435-442.
48. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature.* 2008;453(7193):314-321.
49. Culvenor AG, Oiestad BE, Hart HF, Stefanik JJ, Guermazi A, Crossley KM. Prevalence of knee osteoarthritis features on magnetic resonance imaging in asymptomatic uninjured adults: a systematic review and meta-analysis. *Br J Sports Med.* 2019;53(20):1268-1278.
50. Horga LM, Hirschmann AC, Henckel J, Fotiadou A, Di Laura A, Torlasco C, D'Silva A, Sharma S, Moon JC, Hart AJ. Prevalence of abnormal findings in 230 knees of asymptomatic adults using 3.0 T MRI. *Skeletal Radiol.* 2020;49(7):1099-1107.

51. Messner K, Gillquist J. Cartilage repair. A critical review. *Acta orthopaedica Scandinavica.* 1996;67(5):523-529.
52. Mats Brittberg PA, Ralph Gambardella, Laszlo Hangody, Hans Jörg Hauselmann, Roland P Jakob, David Levine, Stefan Lohmander, Bert R Mandelbaum, Lars Peterson, Hans-Ulrich Staubli. ICRS Cartilage Injury Evaluation Package. https://cartilage.org/society/publications/icrs-score/. Published 2000. Accessed.
53. 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):1067-1076.
54. Ding C, Garnero P, Cicuttini F, Scott F, Cooley H, Jones G. Knee cartilage defects: association with early radiographic osteoarthritis, decreased cartilage volume, increased joint surface area and type II collagen breakdown. *Osteoarthritis Cartilage.* 2005;13(3):198-205.
55. van der Kraan PM. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng Regen Med.* 2019;16(4):327-334.
56. Nakajima S, Naruto T, Miyamae T, Imagawa T, Mori M, Nishimaki S, Yokota S. Interleukin-6 inhibits early differentiation of ATDC5 chondrogenic progenitor cells. *Cytokine.* 2009;47(2):91-97.
57. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clin Geriatr Med.* 2010;26(3):355-369.
58. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet.* 2016;388(10053):1545-1602.
59. Various. OARSI White paper - OA as a Serious Disease. https://www.oarsi.org/education/oarsi-resources/oarsi-white-paper-oa-serious-disease. Published 2016. Accessed.
60. van der Kraan PM, van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartilage.* 2012;20(3):223-232.
61. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nat Rev Rheumatol.* 2016;12(11):632-644.
62. Miller LM, Novatt JT, Hamerman D, Carlson CS. Alterations in mineral composition observed in osteoarthritic joints of cynomolgus monkeys. *Bone.* 2004;35(2):498-506.
63. Mahjoub M, Berenbaum F, Houard X. Why subchondral bone in osteoarthritis? The importance of the cartilage bone interface in osteoarthritis. *Osteoporos Int.* 2012;23 Suppl 8:S841-846.
64. Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. *Arthritis Res Ther.* 2017;19(1):18.
65. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet.* 2019;393(10182):1745-1759.
66. Hoemann CD, Lafantaisie-Favreau CH, Lascau-Coman V, Chen G, Guzman-Morales J. The cartilage-bone interface. *J Knee Surg.* 2012;25(2):85-97.
67. 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.
68. Everhart JS, Abouljoud MM, Kirven JC, Flanigan DC. Full-Thickness Cartilage Defects Are Important Independent Predictive Factors for Progression to Total Knee Arthroplasty in Older Adults with Minimal to Moderate Osteoarthritis: Data from the Osteoarthritis Initiative. *J Bone Joint Surg Am.* 2019;101(1):56-63.
69. 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):334-350.
70. Steadman JR, Rodkey WG, Singleton SB, Briggs KK. Microfracture technique forfull-thickness chondral defects: Technique and clinical results. *Operative techniques in orthopaedics.* 1997;7(4):300-304.
71. Kraeutler MJ, Aliberti GM, Scillia AJ, McCarty EC, Mulcahey MK. Microfracture Versus Drilling of Articular Cartilage Defects: A Systematic Review of the Basic Science Evidence. *Orthop J Sports Med.* 2020;8(8):2325967120945313.
72. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis Cartilage.* 2002;10(6):432-463.
73. Behery O, Siston RA, Harris JD, Flanigan DC. Treatment of cartilage defects of the knee: expanding on the existing algorithm. *Clinical journal of sport medicine : official journal of the Canadian Academy of Sport Medicine.* 2014;24(1):21-30.
74. Minas T, Gomoll AH, Rosenberger R, Royce RO, Bryant T. Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques. *Am J Sports Med.* 2009;37(5):902-908.

75. O'Driscoll SW, Keeley FW, Salter RB. The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. *J Bone Joint Surg Am.* 1986;68(7):1017-1035.
76. Amiel D, Coutts RD, Abel M, Stewart W, Harwood F, Akeson WH. Rib perichondrial grafts for the repair of full-thickness articular-cartilage defects. A morphological and biochemical study in rabbits. *J Bone Joint Surg Am.* 1985;67(6):911-920.
77. Bouwmeester P, Kuijer R, Terwindt-Rouwenhorst E, van der Linden T, Bulstra S. Histological and biochemical evaluation of perichondrial transplants in human articular cartilage defects. *J Orthop Res.* 1999;17(6):843-849.
78. Bulstra SK, Homminga GN, Buurman WA, Terwindt-Rouwenhorst E, van der Linden AJ. The potential of adult human perichondrium to form hyalin cartilage in vitro. *J Orthop Res.* 1990;8(3):328-335.
79. Jansen EJ, Emans PJ, Guldemond NA, van Rhijn LW, Welting TJ, Bulstra SK, Kuijer R. Human periosteum-derived cells from elderly patients as a source for cartilage tissue engineering? *J Tissue Eng Regen Med.* 2008;2(6):331-339.
80. Hunziker EB. Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable? *Osteoarthritis Cartilage.* 1999;7(1):15-28.
81. Makris EA, Gomoll AH, Malizos KN, Hu JC, Athanasiou KA. Repair and tissue engineering techniques for articular cartilage. *Nat Rev Rheumatol.* 2015;11(1):21-34.
82. 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):889-895.
83. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med.* 2010;38(6):1117-1124.
84. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin Orthop Relat Res.* 2000(374):212-234.
85. Health NIf, Excellence C. Autologous chondrocyte implantation using chondrosphere for treating symptomatic articular cartilage defects of the knee. 2018.
86. Armoiry X, Cummins E, Connock M, Metcalfe A, Royle P, Johnston R, Rodrigues J, Waugh N, Mistry H. Autologous Chondrocyte Implantation with Chondrosphere for Treating Articular Cartilage Defects in the Knee: An Evidence Review Group Perspective of a NICE Single Technology Appraisal. *Pharmacoeconomics.* 2019;37(7):879-886.
87. Saris DB, Vanlauwe J, Victor J, Haspl M, Bohnsack M, Fortems Y, Vandekerckhove B, Almqvist KF, Claes T, Handelberg F, Lagae K, van der Bauwhede J, Vandenneucker H, Yang KG, Jelic M, Verdonk R, Veulemans N, Bellemans J, Luyten FP. Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. *Am J Sports Med.* 2008;36(2):235-246.
88. Campos Y, Almirall A, Fuentes G, Bloem HL, Kaijzel EL, Cruz LJ. Tissue Engineering: An Alternative to Repair Cartilage. *Tissue Eng Part B Rev.* 2019;25(4):357-373.
89. Redondo ML, Beer AJ, Yanke AB. Cartilage Restoration: Microfracture and Osteochondral Autograft Transplantation. *J Knee Surg.* 2018;31(3):231-238.
90. Pareek A, Reardon PJ, Macalena JA, Levy BA, Stuart MJ, Williams RJ, 3rd, Krych AJ. Osteochondral Autograft Transfer Versus Microfracture in the Knee: A Meta-analysis of Prospective Comparative Studies at Midterm. *Arthroscopy.* 2016;32(10):2118-2130.
91. Chahal J, Gross AE, Gross C, Mall N, Dwyer T, Chahal A, Whelan DB, Cole BJ. Outcomes of osteochondral allograft transplantation in the knee. *Arthroscopy.* 2013;29(3):575-588.
92. Pisanu G, Cottino U, Rosso F, Blonna D, Marmotti AG, Bertolo C, Rossi R, Bonasia DE. Large Osteochondral Allografts of the Knee: Surgical Technique and Indications. *Joints.* 2018;6(1):42-53.
93. Fuchs A, Eberbach H, Izadpanah K, Bode G, Sudkamp NP, Feucht MJ. Focal metallic inlay resurfacing prosthesis for the treatment of localized cartilage defects of the femoral condyles: a systematic review of clinical studies. *Knee Surg Sports Traumatol Arthrosc.* 2018;26(9):2722-2732.
94. Jeuken RM, Roth AK, Peters R, Van Donkelaar CC, Thies JC, Van Rhijn LW, Emans PJ. Polymers in Cartilage Defect Repair of the Knee: Current Status and Future Prospects. *Polymers (Basel).* 2016;8(6).
95. Eckstein F, Kraines JL, Aydemir A, Wirth W, Maschek S, Hochberg MC. Intra-articular sprifermin reduces cartilage loss in addition to increasing cartilage gain independent of location in the femorotibial joint: post-hoc analysis of a randomised, placebo-controlled phase II clinical trial. *Ann Rheum Dis.* 2020;79(4):525-528.

96. Scotti C, Gimbel J, Laurent D, Madar A, Peters T, Zhang Y, Polus F, Beste M, Vostiar I, Choudhury S, Gerwin N, Goldhahn J, Schieker M, Roubenoff R. LNA043, a Novel Cartilage Regenerative Treatment for Osteoarthritis: Results from a First-In-Human Trial in Patients with Knee Osteoarthritis [abstract]. *Arthritis Rheumatol.* 2020;72 (suppl 10).
97. Brinkman JM, Lobenhoffer P, Agneskirchner JD, Staubli AE, Wymenga AB, van Heerwaarden RJ. Osteotomies around the knee: patient selection, stability of fixation and bone healing in high tibial osteotomies. *J Bone Joint Surg Br.* 2008;90(12):1548-1557.
98. Jansen MP, Boymans T, Custers RJH, Van Geenen RCI, Van Heerwaarden RJ, Huizinga MR, Nellensteijn JM, Sollie R, Spruijt S, Mastbergen SC. Knee Joint Distraction as Treatment for Osteoarthritis Results in Clinical and Structural Benefit: A Systematic Review and Meta-Analysis of the Limited Number of Studies and Patients Available. *Cartilage.* 2020:1947603520942945.
99. Stalman A, Skoldenberg O, Martinez-Carranza N, Roberts D, Hogstrom M, Ryd L. No implant migration and good subjective outcome of a novel customized femoral resurfacing metal implant for focal chondral lesions. *Knee Surg Sports Traumatol Arthrosc.* 2018;26(7):2196-2204.
100. Jansen MP, Mastbergen SC, van Heerwaarden RJ, Spruijt S, van Empelen MD, Kester EC, Lafeber F, Custers RJH. Knee joint distraction in regular care for treatment of knee osteoarthritis: A comparison with clinical trial data. *PLoS One.* 2020;15(1):e0227975.
101. Demange MK, Minas T, von Keudell A, Sodha S, Bryant T, Gomoll AH. Intralesional Osteophyte Regrowth Following Autologous Chondrocyte Implantation after Previous Treatment with Marrow Stimulation Technique. *Cartilage.* 2017;8(2):131-138.
102. de Windt TS, Concaro S, Lindahl A, Saris DB, Brittberg M. Strategies for patient profiling in articular cartilage repair of the knee: a prospective cohort of patients treated by one experienced cartilage surgeon. *Knee Surg Sports Traumatol Arthrosc*. 2012;20(11):2225-2232.
103. Emans PJ, van Rhijn LW, Welting TJ, Cremers A, Wijnands N, Spaapen F, Voncken JW, Shastri VP. Autologous engineering of cartilage. *P Natl Acad Sci USA.* 2010;107(8):3418-3423.

Twenty-two-year outcome of cartilage repair surgery by perichondrium transplantation

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Abstract

Objective
The main purpose of the present study was to assess the risk for major revision surgery after perichondrium transplantation (PT) at a minimum of 22 years postoperatively and to evaluate the influence of patient characteristics.

Design
Primary outcome was treatment success or failure. Failure of PT was defined as revision surgery in which the transplant was removed, such as (unicondylar) knee arthroplasty or patellectomy. The functioning of non-failed patients was evaluated using the International Knee Documentation Committee (IKDC) score. In addition, the influence of patient characteristics was evaluated.

Results
Ninety knees in 88 patients, aged 16 to 55 years with symptomatic cartilage defects were treated by PT. Eighty knees in 78 patients were eligible for analysis and 10 patients were lost to follow-up. Twenty-eight knees in 26 patients had undergone major revision surgery. Previous surgery and a longer time of symptoms prior to perichondrium transplantation were significantly associated with an increased risk for failure of cartilage repair. Functioning of the remaining 52 patients and influence of patient characteristics was analysed using their IKDC score. Their median IKDC score was 39.08, but a relatively young age at transplantation was associated with a higher IKDC score.

Conclusions
This 22-year follow-up study of PT, with objective outcome parameters next to patient reported outcome measurements in a unique group of patients, shows that overall, 66% was without major revision surgery and patient characteristics also influence long-term outcome of cartilage repair surgery.

Introduction

Articular cartilage is a specialised connective tissue that provides a low-friction surface in joints, enabling human movement.[1] However, when damaged, articular cartilage has poor regenerative capacity. When left untreated, cartilage defects eventually catabolically predispose the affected joint for the development of osteoarthritis (OA).[2,3] To be able to treat such cartilage defects, several different articular cartilage repair strategies like microfracture (MF), osteochondral allograft transplantation (OCA), mosaicplasty (MP), perichondrium transplantation (PT), autologous chondrocyte implantation (ACI) and scaffolds, have been developed over the past decades.[4,5] The aim of these techniques is to form hyaline-like cartilage to create a pain-free functioning of the joint and prevent or postpone the development of OA and subsequent joint replacement.[6-9]

Various factors are correlated with a positive outcome of cartilage repair surgery. Examples are younger age, short duration of symptoms,[10,11] and no history of previous surgery on the knee.[12,13] No consensus can be found in the literature on whether the defect location influences outcome, but the occurrence of multiple lesions in one joint is described to impair outcome.[10,14] There has been a gain of knowledge over the years on articular cartilage repair strategies and the importance of adequate patient selection to improve surgical outcome.[14] Therefore, several treatment algorithms were developed to aid in patient selection for cartilage repair surgery.[15-19] However, these algorithms are mostly based on short- and medium-term clinical outcome of cartilage repair surgeries. To our knowledge there are no algorithms based on objective outcome parameters such as major revision surgery on the long term.

From 1986 till 1992, 88 patients with symptomatic cartilage defects in 90 knees were treated by PT.[20] After one-year follow-up Homminga et al. showed that 18 out of 25 patients treated with PT were symptom-free and had resumed their previous work and activities.[21] In 1997, Bouwmeester et al. published the 5-year follow-up results of this study. They described 48 treatment failures, although it should be noted that they applied strict criteria to define a failure: being a re-operation, any change in arthroscopic graft appearance or an HSSS score of <75.[20] In 40 out of 88 patients, there was a fair to good outcome of the procedure (HSSS above 75 and 85 respectively combined with a good graft appearance on arthroscopy).[20] Improved short-term results were described in patients with a single defect, without previous debridement operations, a long history of symptoms, age over 40 years and a grade 2 or worse osteoarthritis.[20] A follow-up study was published in 1999, which presented the histological and biochemical results of these transplants.[22] Because the overall results were found unsatisfactory, PT was only sporadically performed after its introduction. However, the PT-treated patient group is unique because of the 22-year follow-up period, enabling us to analyse the outcome based not only on patient reported outcome measurements but also on objective parameters, such as revision surgery, over-time. The aim of this study was to chart the long-term clinical outcome after 22 years of follow-up after PT and to examine whether patient selection also influences

objective outcome parameters such as major revision surgery next to patient reported outcome measurements (PROMS) in this type of cartilage repair surgery.

Methods

Perichondrium transplantation operative technique

Perichondrium transplantation is a single stage open procedure with two operation sites. Complete study details and early findings were described by Homminga et al. and Bouwmeester et al. in 1990, 1997, 1999 and 2001.[20-23] In short, as described by Bouwmeester et al. in 1997,[20] the procedure starts with debridement of the articular cartilage lesion up to the subchondral bone and a sharp vertical edge will be created on the surrounding cartilage. An oblique incision will be made over the lower part of the left side of the chest. The fascia of the rectus muscle is split transversely and the muscle is split in the line of its fibers. A piece of perichondrium will be dissected from the cartilaginous part of one of the lower ribs and removed together with its chondrogenic layer. The graft will be cut to match the size of the lesion. The perichondrial graft is then placed into the lesion with the chondral side facing up and will be attached with human fibrin glue.[21]

Patients

From September 1986 until December 1992, 90 knees with articular cartilage defects in 88 patients were enrolled in the study. Eligible patients included men and women aged 16 to 55 years with symptomatic cartilage defects of the femoral condyles, patella or trochlea, who were treated by perichondrium transplantation. No exclusion criteria other than age >55 years were used for surgery.
Patient information on pre-operative and short-term postoperative pain and function was retrieved from previous studies for 88 patients (90 knees). Based on these data, we were able to contact 78 patients (80 knees). Five patients were deceased and five patients were unable or unwilling to cooperate. Other than those lost-to-follow-up (n=10), no patients were excluded in this long-term study.

Outcome assessment

Adequately defining the outcome of cartilage repair surgery is hard because no consensus exists on what is successful or non-successful. In previous literature, failure of cartilage repair surgery has been described ranging from no improvement on functional outcome scores to re-intervention in which the graft is removed.[24-27]
For the present study, two different groups were specified. The first group contained the patients who underwent major revision surgery in which the graft was removed and/or arthroplasty was performed. This group that underwent major revision surgery

was defined as treatment failure. Shaving of the transplant was not classified as major revision surgery. Patients who underwent major revision surgery were not asked to complete any questionnaires because their results would reflect the effect of the major revision surgery rather than the effect of the perichondrium transplantation. The time of the perichondrium transplantation and the time of major revision surgery was known and thus the time to failure of the treatment could be calculated. A survival analysis was performed on this data and the influence of patient characteristics on the time to failure was assessed.

Unfortunately, data on pre-operative pain and function was incomplete and could not be used reliably for comparison with our long-term follow-up IKDC-score. Patient characteristics at time of surgery we assessed that might be of influence were based on available literature and those found by Bouwmeester et al. at 52 months follow-up.[10-14,20,25,26] Patient age, sex, number of lesions, lesion size, previous surgery, duration of symptoms, location in the knee and grade of osteoarthritis were described. Preoperative degree of osteoarthritis, location in the knee and type of previous surgery were not included in the cox and linear regression analyses. Only six people had an arthroscopically graded Outerbridge OA score higher than grade 2 in other parts of the knee in this cohort at the time of surgery. Also, group sizes of location in the knee and type of previous surgery were too small for statistical analysis. To identify predictors of outcome, univariate cox regression was performed on possibly important pre-operative factors with the outcome being treatment failure. Parameters with a p-value <0.100 were subsequently analysed in a multivariate cox regression analysis. Because a maximum of 2.8 (n=28/10) characteristics may simultaneously be analysed, an explorative analysis was performed and by stepwise regression excluding the factor with the highest p-value until only characteristics with p-values <0.05 were present.

The second group contained the patients without revision surgery. They were asked to complete the International Knee Documentation Committee (IKDC) questionnaire. The IKDC questionnaire is best suitable to depict overall functioning for this ageing patient population with a long term follow up.[28] This data was analysed by linear regression in a similar way. Missing data, caused by patients that failed to complete the questionnaire was calculated and completed by stochastic regression imputation.

Statistical analysis

Patient characteristics are presented as medians with corresponding interquartile range (IQR) for numerical variables and as number of patients (n and %) for categorical ones. A Kaplan Meier survival analysis was performed to provide insight in the time to failure for these patients. Hazard ratios (HR) were subsequently calculated using univariate and multivariate cox regression analysis. Patients who did not undergo major revision surgery were defined as non-failures and their clinical functioning was evaluated using the IKDC questionnaire. A simple linear regression was calculated to investigate the association between IKDC score based and different patient characteristics. All analyses

were conducted using a significance level of 0.05. Statistical analysis was performed using IBM SPSS statistics for Mac, version 25 (IBM, Armonk, New York).

Ethical approval

This study was approved by the Medical Research Ethics Committee of the Maastricht University Medical Centre+ (METC 13-4-038). Renewed informed consent was obtained prior to participation from all patients for this follow-up.

Results

Patient characteristics

Eighty knees in 78 patients were eligible for analysis. The median follow-up time of this included cohort was 25 years (IQR 25-26 years) with a minimum of 22 years of follow-up. The median age at time of surgery was 31.5 years (IQR 23-39 years). The median age at follow-up was 56.5 years (IQR 48-64 years). Knee cartilage lesions were located on the medial femoral condyle, lateral femoral condyle, patella and trochlea. The median lesion size was 3.0 cm^2 (IQR 2.0-4.0 cm^2). The median time of symptoms before index surgery was 36 months (IQR 24-60 months). Forty-four right and 36 left knees were treated in 47 men and 33 women (Table 2.1).

Table 2.1 Patient characteristics of the 80 knees in 78 patients included in the follow-up cohort. Values are described as a count and percentage of the total 80 knees or as a median with subsequent interquartile range.

Patient characteristics	***n* (%)**	**Median (IQR)**
Age at surgery [years]		31.5 (23-39)
Age at follow-up [years]		56.5 (48-64)
Follow-up time [years]		25 (25-26)
Age <40 years	61 (76%)	
Age ≥40 years	19 (24%)	
Male knees	47 (59%)	
Female knees	33 (41%)	
Defect size (cm^2)		3.0 (2.0-4.0)
Defect location		
Medial femoral condyle	26 (32.5%)	
Lateral femoral condyle	2 (2.5%)	
Patella / trochlea	36 (45%)	
Multiple	16 (20%)	
Time since onset symptoms [months]		36 (24-60)
Knee with previous surgery	61 (76%)	
Knee without previous surgery	19 (24%)	
Arthroscopic degree of osteoarthritis at surgery (Outerbridge classification)		
None (grade 0)	58 (72.5%)	
Little (grade 1-2)	16 (20%)	
Definite (grade 3-4)	6 (7.5%	

n = number of knees, IQR = interquartile range.

Outcome at 22-year follow-up

Twenty-six patients with 28 operated knees (35%) underwent surgery in which the transplant was removed. In 17 patients a total knee arthroplasty was performed, 2 patients underwent a patellofemoral arthroplasty and 1 patient received a unicondylar arthroplasty. Also 6 patients underwent a patellectomy, which was used more frequently at that time as a salvage procedure. Finally, in 1 patient the transplant was removed. These surgeries were defined as major revision surgery and the treatment was classified as a failure. These failures occurred throughout the follow-up period of the study. A Kaplan-Meier survival analysis revealed that 95.0% was still without major revision surgery at one year (SE 2.4%), 83.8% at ten years (SE 4.1%), and 66.3% at twenty years (SE 5.3%) (Figure 2.1).

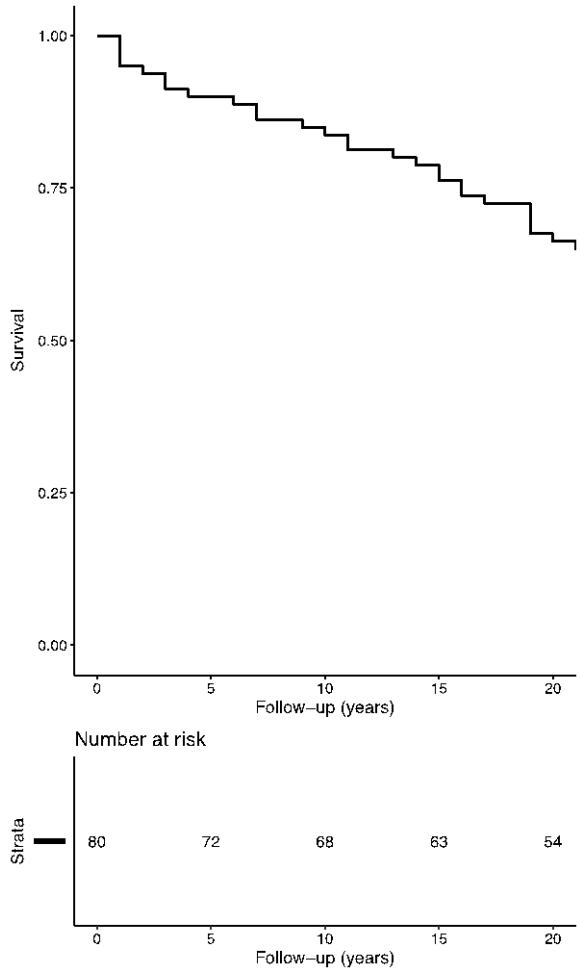

Figure 2.1 A Kaplan-Meier curve depicting graft survival (i.e., patients with no major revision surgery performed) up until the end of our current follow-up time of at least 22 years.

Influence of patient factors on time-to-failure of treatment

In a higher percentage (56%) of patients with multiple lesions treatment failed compared to patients with a single lesion (42%), HR 0.471 (0.213-1.043) p=0.064. Treatment failed in 42% of the female patients and in 30% of the male patients, HR 1.602 (0.763-3.363) p=0.213. In only 30% of patients younger than 40 years at the moment of primary surgery treatment failed *versus* 53% of patients older than 40 years at the moment of primary surgery, HR 0.487 (0.225-1.058) p=0.069. In patients with a lesion size smaller than 3 cm^2 33% failed *versus* 38% in patients with a lesion size greater than 3 cm^2, HR 1.166 (0.999-1.361) p=0.051. In patients without previous surgery only 11% of treatments failed *versus* 43% in patients with previous surgery, HR 4.894 (1.161-20.642) p=0.031 and in patients with symptoms shorter than 24 months there were less treatment failures compared to patients with symptoms longer than 24 months (15% *versus* 45% respectively), HR 1.011 (1.004-1.018) p=0.001 (Table 2.2). This data was analysed by univariate cox regression analysis and subsequently explorative in a multivariate cox regression analysis for characteristics with a p-value <0.100 (duration of symptoms, previous surgery, size of the lesion, age at surgery and surgery on multiple lesions) with the outcome being treatment failure and subsequent major revision surgery. Definite multivariate cox regression was carried out with the characteristics 'previous surgery' and 'time of symptoms'. This definite multivariate cox regression analysis showed that patients who were without previous knee surgery were significantly less at risk for treatment failure, HR 4.390 (95%CI 1.036-18.598; p=0.045). Subsequently, people with a shorter time from onset of symptoms until PT were significantly less at risk for major revision surgery, HR 1.010 (95%CI 1.003-1.017; p=0.003). No significant differences were found for; size of the lesion, age at surgery and number of lesions (Table 2.2).

Influence of patient factors on performance of non-failed grafts at 22 years follow up

Fifty-two PT patients (52 knees) were still without major revision surgery after a minimum follow up of 22 years. To determine their functioning, these remaining patients were analysed using the IKDC score. Their median IKDC score was 39.08 (IQR 25.57 – 53.74). Simple linear regression showed a significant relationship between IKDC and age at surgery (p=0.012). No p-values of <0.100 were found for other factors; number of lesions, previous surgery, time of symptoms and size of the lesion. Therefore, no multivariate testing was performed on these data. (Table 2.3).

Table 2.2 Overview of the percentage of failure of perichondrium transplantation in different patient groups. Parameters with a p-value <0.100 in univariate cox regression analysis were subsequently analysed in an explorative multivariate cox regression analysis (italic text), stepwise excluding characteristics with the highest p-value and definite multivariate cox regression analysis was performed on the characteristics, 'previous surgery' and 'time of symptoms' (plain text).

	Number of knees	Fail	Univariate		Multivariate	
		n (%)	HR (95% CI)	p-value	HR (95% CI)	p-value
Total	80	28 (35%)				
Number of lesions						
Single lesion	*64*	*19 (42%)*	0.471 (0.213-1.043)	0.064	*N.A.*	*N.A.*
Multiple lesions#	*16*	*9 (56%)*				
Patient age at time of surgery						
Age <40	*61*	*18 (30%)*	0.487 (0.225-1.058)	*0.069*	*N.A.*	*N.A.*
Age ≥40#	*19*	*10 (53%)*				
Lesion size						
Size of the lesion <3 cm^{2}#	51	17 (33%)				
Size of the lesion ≥3 cm^{2}	29	11 (38%)	1.166 (0.999-1.361)	*0.051*	*N.A.*	*N.A.*
Previous surgery						
Without previous surgery#	19	2 (11%)				
With previous surgery	61	26 (43%)	4.894 (1.161-20.642)	0.031*	4.390 (1.036-18.598)	0.045*
Duration of symptoms						
Duration of symptoms <24 months#	27	4 (15%)				
Duration of symptoms ≥24 months	53	24 (45%)	1.011 (1.004-1.018)	0.001*	1.010 (1.003-1.017)	0.003*

n: total number and %: percentage of the subgroup that failed, CI: Confidence interval, HR: Hazard ratio *: significant influence, N.A.: not applicable, #: reference group.

Table 2.3 Univariate linear regression of pre-operative factors that possibly correlate with the IKDC score at 22 years of follow up.

	B	95% CI	p-value
Number of lesions	8.163	(-8.887 – 25.213)	0.341
Age at surgery	-0.808	(-1.428 – -0.187)	0.012*
Size of the lesion	-2.496	(-5.674 – 0.682)	0.121
Previous surgery	-6.238	(-18.632 – 6.156)	0.317
Time of symptoms	-0.066	(-0.257 – 0.126)	0.494

CI: Confidence interval, *: significant correlation.

Discussion

The most important finding of this study was that after 22-years of follow-up of cartilage repair surgery in the knee by PT, 66% was still without major revision surgery. Duration of symptoms prior to surgery and previous surgery of the knee are predictors

for undergoing major revision surgery and a younger age at primary cartilage repair surgery is associated with a better functioning as measured by IKDC. In current literature, only limited studies are available with a long-term follow-up of cartilage repair surgery of the knee. Consequently, the outcome on the long-term is mainly available by extrapolating short-term results,[24,29] or in studies with relatively small group sizes.[30,31]

On a shorter follow-up term, Moradi et al., Krishnan et al. and de Windt et al. reported a higher patient age and a longer time of symptoms prior to cartilage repair surgery as a negative factor for successful outcome.[10,11,32] Furthermore, Krishnan et al., Minas et al. and Pestka et al. found previous surgery of the knee as a negative factor for successful outcome.[10,12,13] The follow-up time of many studies is too short for patients to reach an objective endpoint that defines treatment failure (i.e., OA, knee arthroplasty), therefore published results are often based on patient reported outcome measurements, this can however lead to different forms of bias. Knee function deteriorates with increasing age and PROMS, when not corrected for age, can underestimate the outcome.[33,34] Exceptions are the studies of Gobbi et al. who report increased osteoarthritic changes in older patients at 15 years of follow-up and the study of Minas et al. which did include knee arthroplasty, but with a 10-year follow-up period n=210, and 20-year, but with little patients left n=23.[13,30,35] Our survival rate of 84% at 10-year follow-up is lower than the survival rate of 89% found by Gobbi et al. after MF.[35] In contrast to this study, we did not apply exclusion criteria (e.g., lesion size) other than age >55. The 79% survival rate reported of ACI by Minas et al. is even lower, but this study treated patients with a larger average lesion size.[13] The only comparison at 20-year follow-up can be made with the study of Ogura et al. who reported a survival rate of 63% which is similar to our survival rate of 66%.[30] Interestingly, this survival is already reported at their 10-year follow-up, but maintained in their 20-year follow-up. In general, our study has a comparable survival rate and confirms important patient characteristics, but after a longer-term follow-up, in a large patient group and with objective outcome measurements next to patient reported outcome measurements.

A challenging aspect in cartilage surgery remains to define what treatment failure is. Definitions of failure in current literature range from total knee arthroplasty or removal of the implant to a lack of improvement on questionnaires or Visual Analog Scales (VAS) for pain.[29] This wide variety of definitions complicates an adequate comparison of different studies and can be a cause of the great differences in described predictors for success.[10-14,25-27,36,37] Clinical functioning and quality of life is an important outcome factor, and therefore clinical questionnaires were included. However, with increasing age, knee function decreases. A deterioration of the IKDC score as described by Anderson et al.[38] should therefore not be ignored. This is especially important in studies like this with a very long-term follow-up with an ageing population.[34] Ideally a correction for age like the z-score would be calculated and used for a more valid comparison amongst individuals, but unfortunately the z-score can only be calculated

up to the age of 65.[38] When comparing the individuals younger than 65 in this study, the Z-score did not differ between the different age groups 35-50 and 51-65 (z=-1.5 and -1.3 respectively, p-value 0.27). Thus, in this study, when corrected for age, the IKDC score is not worsened in the older patient age group (51-65) compared to the age group 35-50. Furthermore, age was also not found as a confounding factor in the multivariate regression analyses. Still, caution is advised in its interpretation.[34]

We conducted a longitudinal cohort study with 22 years of follow-up. However, a limitation of the present study was that some clinical data has been retrieved retrospectively, especially pre-operative data and questionnaires were incomplete. Without complete pre-operative scores, we considered a comparison with the VAS and HSS Knee Scores at a follow-up of 24 months not reliable and it was not the aim of this article.

Conclusion

We present the long-term survival results of PT. In line with literature presenting mid-term follow-up, a smaller risk of total knee arthroplasty or other major revision surgeries was found in patients with a shorter time of symptoms prior to PT and without previous surgery of the knee. Subsequently a better functional outcome of the knee was found in patients operated at a relatively young age.

References

1. Matsiko A, Levingstone TJ, O'Brien FJ. Advanced Strategies for Articular Cartilage Defect Repair. *Materials (Basel).* 2013;6(2):637-668.
2. Gelber AC, Hochberg MC, Mead LA, Wang NY, Wigley FM, Klag MJ. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. *Ann Intern Med.* 2000;133(5):321-328.
3. 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): 2033-2039.
4. Alford JW, Cole BJ. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am J Sports Med.* 2005;33(2):295-306.
5. Redondo ML, Naveen NB, Liu JN, Tauro TM, Southworth TM, Cole BJ. Preservation of Knee Articular Cartilage. *Sports Med Arthrosc Rev.* 2018;26(4):e23-e30.
6. Billings E, Jr., von Schroeder HP, Mai MT, Aratow M, Amiel D, Woo SL, Coutts RD. Cartilage resurfacing of the rabbit knee. The use of an allogeneic demineralized bone matrix-autogeneic perichondrium composite implant. *Acta orthopaedica Scandinavica.* 1990;61(3):201-206.
7. Coutts RD, Woo SL, Amiel D, von Schroeder HP, Kwan MK. Rib perichondrial autografts in full-thickness articular cartilage defects in rabbits. *Clin Orthop Relat Res.* 1992(275):263-273.
8. Engkvist O, Wilander E. Formation of cartilage from rib perichondrium grafted to an articular defect in the femur condyle of the rabbit. *Scand J Plast Reconstr Surg.* 1979;13(3):371-376.
9. Maruyama Y. An experimental study on cartilage foramation in autogenous perichondrial transplantation in rabbits. *The Keio journal of medicine.* 1979;28(2):63-72.
10. Krishnan SP, Skinner JA, Bartlett W, Carrington RW, Flanagan AM, Briggs TW, Bentley G. Who is the ideal candidate for autologous chondrocyte implantation? *J Bone Joint Surg Br.* 2006;88(1):61-64.
11. 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.
12. Pestka JM, Bode G, Salzmann G, Sudkamp NP, Niemeyer P. Clinical outcome of autologous chondrocyte implantation for failed microfracture treatment of full-thickness cartilage defects of the knee joint. *Am J Sports Med.* 2012;40(2):325-331.
13. Minas T, Von Keudell A, Bryant T, Gomoll AH. The John Insall Award: A minimum 10-year outcome study of autologous chondrocyte implantation. *Clin Orthop Relat Res.* 2014;472(1):41-51.
14. de Windt TS, Concaro S, Lindahl A, Saris DB, Brittberg M. Strategies for patient profiling in articular cartilage repair of the knee: a prospective cohort of patients treated by one experienced cartilage surgeon. *Knee Surg Sports Traumatol Arthrosc.* 2012;20(11):2225-2232.
15. Biant LC, McNicholas MJ, Sprowson AP, Spalding T. The surgical management of symptomatic articular cartilage defects of the knee: Consensus statements from United Kingdom knee surgeons. *Knee.* 2015;22(5):446-449.
16. Alford JW, Cole BJ. Cartilage restoration, part 2: techniques, outcomes, and future directions. *Am J Sports Med.* 2005;33(3):443-460.
17. Cole BJ, Pascual-Garrido C, Grumet RC. Surgical management of articular cartilage defects in the knee. *J Bone Joint Surg Am.* 2009;91(7):1778-1790.
18. Gomoll AH, Farr J, Gillogly SD, Kercher J, Minas T. Surgical management of articular cartilage defects of the knee. *J Bone Joint Surg Am.* 2010;92(14):2470-2490.
19. Makris EA, Gomoll AH, Malizos KN, Hu JC, Athanasiou KA. Repair and tissue engineering techniques for articular cartilage. *Nat Rev Rheumatol.* 2015;11(1):21-34.
20. Bouwmeester SJ, Beckers JM, Kuijer R, van der Linden AJ, Bulstra SK. Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. *Int Orthop.* 1997;21(5):313-317.
21. Homminga GN, Bulstra SK, Bouwmeester PS, van der Linden AJ. Perichondral grafting for cartilage lesions of the knee. *J Bone Joint Surg Br.* 1990;72(6):1003-1007.
22. Bouwmeester P, Kuijer R, Terwindt-Rouwenhorst E, van der Linden T, Bulstra S. Histological and biochemical evaluation of perichondrial transplants in human articular cartilage defects. *J Orthop Res.* 1999;17(6):843-849.

23. Bouwmeester PS, Kuijer R, Homminga GN, Bulstra SK, Geesink RG. A retrospective analysis of two independent prospective cartilage repair studies: autogenous perichondrial grafting versus subchondral drilling 10 years post-surgery. *J Orthop Res.* 2002;20(2):267-273.
24. Nawaz SZ, Bentley G, Briggs TW, Carrington RW, Skinner JA, Gallagher KR, Dhinsa BS. Autologous chondrocyte implantation in the knee: mid-term to long-term results. *J Bone Joint Surg Am.* 2014;96(10):824-830.
25. Vanlauwe J, Saris DB, Victor J, Almqvist KF, Bellemans J, Luyten FP, Tig/Act, Group EXTS. Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. *Am J Sports Med.* 2011;39(12):2566-2574.
26. Knutsen G, Drogset JO, Engebretsen L, Grontvedt T, Isaksen V, Ludvigsen TC, Roberts S, Solheim E, Strand T, Johansen O. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *J Bone Joint Surg Am.* 2007;89(10):2105-2112.
27. Niemeyer P, Porichis S, Steinwachs M, Erggelet C, Kreuz PC, Schmal H, Uhl M, Ghanem N, Sudkamp NP, Salzmann G. Long-term outcomes after first-generation autologous chondrocyte implantation for cartilage defects of the knee. *Am J Sports Med.* 2014;42(1):150-157.
28. Hambly K, Griva K. IKDC or KOOS? Which measures symptoms and disabilities most important to postoperative articular cartilage repair patients? *Am J Sports Med.* 2008;36(9):1695-1704.
29. Mistry H, Connock M, Pink J, Shyangdan D, Clar C, Royle P, Court R, Biant LC, Metcalfe A, Waugh N. Autologous chondrocyte implantation in the knee: systematic review and economic evaluation. *Health Technol Assess.* 2017;21(6):1-294.
30. Ogura T, Mosier BA, Bryant T, Minas T. A 20-Year Follow-up After First-Generation Autologous Chondrocyte Implantation. *Am J Sports Med.* 2017;45(12):2751-2761.
31. Solheim E, Hegna J, Strand T, Harlem T, Inderhaug E. Randomized Study of Long-term (15-17 Years) Outcome After Microfracture Versus Mosaicplasty in Knee Articular Cartilage Defects. *Am J Sports Med.* 2018;46(4):826-831.
32. Moradi B, Schonit E, Nierhoff C, Hagmann S, Oberle D, Gotterbarm T, Schmitt H, Zeifang F. First-generation autologous chondrocyte implantation in patients with cartilage defects of the knee: 7 to 14 years' clinical and magnetic resonance imaging follow-up evaluation. *Arthroscopy.* 2012;28(12): 1851-1861.
33. Rolfson O, Bohm E, Franklin P, Lyman S, Denissen G, Dawson J, Dunn J, Eresian Chenok K, Dunbar M, Overgaard S, Garellick G, Lubbeke A, Patient-Reported Outcome Measures Working Group of the International Society of Arthroplasty R. Patient-reported outcome measures in arthroplasty registries Report of the Patient-Reported Outcome Measures Working Group of the International Society of Arthroplasty Registries Part II. Recommendations for selection, administration, and analysis. *Acta Orthop.* 2016;87 Suppl 1:9-23.
34. Filardo G, Andriolo L, Sessa A, Vannini F, Ferruzzi A, Marcacci M, Kon E. Age Is Not a Contraindication for Cartilage Surgery: A Critical Analysis of Standardized Outcomes at Long-term Follow-up. *Am J Sports Med.* 2017;45(8):1822-1828.
35. Gobbi A, Karnatzikos G, Kumar A. Long-term results after microfracture treatment for full-thickness knee chondral lesions in athletes. *Knee Surg Sports Traumatol Arthrosc.* 2014;22(9):1986-1996.
36. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med.* 2010;38(6):1117-1124.
37. 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):1343-1351.
38. Anderson AF, Irrgang JJ, Kocher MS, Mann BJ, Harrast JJ, International Knee Documentation C. The International Knee Documentation Committee Subjective Knee Evaluation Form: normative data. *Am J Sports Med.* 2006;34(1):128-135.

7 Tesla MRI evaluation of the knee, 25 years after cartilage repair surgery: The influence of intralesional osteophytes on biochemical quality of cartilage

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Abstract

Objective

To evaluate the morphological and biochemical quality of cartilage transplants and surrounding articular cartilage of patients 25 years after perichondrium transplantation (PT) and autologous chondrocyte transplantation (ACT) as measured by ultra-high field 7 Tesla (7T) magnetic resonance imaging (MRI) and to present these findings next to clinical outcome.

Design

Seven PT patients and five ACT patients who underwent surgery on the femoral condyle between 1986 and 1996 were included. Patient reported outcome measures (PROMs) were assessed by the clinical questionnaires: Knee injury and Osteoarthritis Outcome Score (KOOS), International Knee Documentation Committee (IKDC), and Visual Analogue Scale (VAS) for knee pain). The morphological (MOCART score) and biochemical quality (glycosaminoglycans [GAGs] content and collagen integrity) of cartilage transplants and surrounding articular cartilage were analyzed by 7T MRI. The results of the PT and ACT patients were compared. Finally, a detailed morphological analysis of the grafts alone was performed.

Results

No statistically significant difference was found for the PROMs and MOCART scores of PT and ACT patients. Evaluation of the graft alone showed poor repair tissue quality and high prevalence of intralesional osteophyte formation in both the PT and ACT patients. Penetration of the graft surface by the intralesional osteophyte was related to biochemically damaged opposing tibial cartilage; GAG content was significantly lower in patients with an osteophyte penetrating the graft surface.

Conclusions

Both PT and ACT patients have a high incidence of intralesional osteophyte formation 25 years after surgery. The resulting biochemical damage to the opposing tibial cartilage might be dependent on osteophyte morphology.

Introduction

Knee injuries are very common and often seen in otherwise healthy, active patients.[1] Several surgical treatments for focal cartilage defects have been developed aiming to prevent further deterioration of the knee joint, provide pain relief, and increase functional outcomes.[2] Two of these techniques are perichondrium transplantation (PT) and autologous chondrocyte transplantation (ACT), which aimed at restoring the hyaline cartilage tissue using a perichondrium flap or cultured chondrocytes combined with a periosteum flap respectively.[3-7]

Short-term follow-up results of PT were reported by Homminga et al. and Bouwmeester et al. who concluded that the outcome of the surgery was poor.[5,7] Long-term results of PT were described by Janssen et al., who found that patient characteristics (i.e., time of symptoms prior to surgery, previous surgery in the index knee, and patient age) influence the outcome of PT at a follow-up of 22 years.[8] The previous results of ACT were described by Peterson et al., who found that after 10-20 years of follow-up, 92% of the patients were satisfied and would have the surgery again.[9] Intralesional osteophytes occurred frequently after both PT and ACT.[10,11] The cause of the frequent occurrence of intralesional osteophytes was not specifically investigated, but previous marrow stimulation techniques and the osteogenic potential of perichondrial and periosteal tissue are described to increase their occurrence.[12,13] Increased calcification of cartilage repair tissue is known to impair the outcome of the surgery on a short-term follow-up.[14] This impaired outcome is expected to persist at the long-term follow-up, but long-term results of cartilage repair surgery are scarce in literature. However, they are of great value to assess whether the initial goals of surgery were achieved.

Postoperative evaluation of cartilage repair tissue is important to assess the performance of cartilage repair procedures and to evaluate the different phases of repair, function and degradation over time. Close insights in these phases will lead to better understanding of the process and improve cartilage repair strategies. Conventional modalities to follow patients after cartilage repair surgery include plain radiography and magnetic resonance imaging (MRI). Conventional radiography can sometimes visualize intralesional osteophytes and is helpful in grading the degree of late osteoarthritis (OA) as it visualizes joint space narrowing, osteophytes, sclerosis and bony remodeling as a result of cartilage loss. MRI provides direct visualization of articular cartilage and surrounding soft-tissue structures as well as bone marrow edema that can be involved in the OA disease process,[15] and allows for a comprehensive evaluation of repair tissue from the articular joint surface to the bone-cartilage interface and the subchondral bone.

In 2017 the first 7 Tesla (7T) MR scanner (TERRA, Siemens Healthineers, Erlangen, Germany) was approved by the U.S. Food and Drug administration (FDA) and

Conformité Eurpoéenne (CE) certified in Europe, thus translating the so far experimental ultra-high field MR (7 Tesla) into clinical routine examinations of the knee joint. With 7T MR significantly higher signal-to-noise ratios can be achieved compared to 3 Tesla which provides higher spatial resolution in morphological imaging by a mean factor of 2.[16] The higher signal-to-noise ratio allows depiction of small fissures and incomplete cartilage repair tissue integration[17] and the detection of smaller physiological effects. On the downside, challenges of scanning at higher field strength include faster heating of tissue (specific absorption rate [SAR] limits), more intense metallic artifacts and more susceptibility artifacts at the transition between tissues with different densities caused by more field heterogeneities.[18] The added value of 7T MRI lies within dedicated quantitative MR techniques that allow measurement of the biochemical properties of cartilage. Healthy cartilage is characterized by a high concentration of glycosaminoglycans (GAGs) and a well-organized collagen network. Both the GAG content and the organization of the collagen network are important indicators for repair tissue quality after treatment.[15] GAGs carry protons that are in constant chemical exchange with surrounding bulk water protons. Using high-field MRI and a dedicated GAG Chemical Exchange Saturation Transfer (gagCEST) imaging sequence, these protons bound to GAG can be selectively labeled by saturation with a radiofrequency pulse. The label will then be transferred to the bulk water by chemical exchange which results in a reduction of the bulk water signal. This reduction in signal is a measure for the ratio of protons bound to GAG and the bulk water protons and is thereby an indirect measure for the GAG content.[19] An advantage of gagCEST is that it can be performed without a contrast agent and using a regular proton coil, as opposed to dGEMRIC which requires a contrast agent and sodium imaging which requires a sodium coil to assess the GAG content. On the downside, the acquisition and post-processing steps of gagCEST are complex and scanning on high-field MRI is required to be able to detect the small difference between the signal of bulk water protons and GAG bound protons.[20]

Collagen network integrity is measured by T2 mapping. Disruption of the collagen structure increases the mobility of protons and therefore produces higher T2 relaxation times. Furthermore, the well-organized structure of collagen matrix in healthy cartilage gives rise to a zonal difference in T2 relaxation times between the deep layer and the superficial layer which is absent in degenerated cartilage.[21]

The first aim of this study was to evaluate the morphological and biochemical quality of cartilage transplants and the status of the articular cartilage of patients 25 years after PT and ACT as measured by ultra-high field 7T MRI and to present these findings next to clinical outcome. The second aim was to assess intralesional osteophyte formation of the transplants and evaluate its effect on the quality of opposing tibial cartilage, as measured by 7T MRI.

Materials and methods

Patient population

Perichondrium transplantation patients and ACT patients who underwent surgery between 1986 and 1996 were included from two different databases. The PT database consisted of 88 Dutch patients and the ACT database consisted of 400 Swedish patients. To optimize the comparison of the cartilage tissue, only patients with a repaired cartilage defect on the femoral condyle were included. Furthermore, for Dutch PT patients specifically: they needed to be willing to visit the outpatient clinic and undergo a 7T MRI scan in Maastricht; for Swedish ACT patients specifically: they needed to be willing to travel to the Netherlands and undergo a 7T MRI scan in Maastricht. All participants had to approve that coincidental findings would be reported to their general practitioner and approve storing and use of their data for research purposes. Exclusion criteria were knee arthroplasty in the area of the transplant (i.e., total-, hemi-knee arthroplasty); major surgery of transplant in the knee (e.g., patellectomy and microfracture); severe OA (e.g., grade 4 Kellgren and Lawrence classification); contra-indications for 7T MRI scanning. The in- and exclusion criteria, combined with our very long-term follow-up in which a considerable number of patients developed severe OA caused eligibility for only 12 patients to be enrolled in our study.

Perichondrium transplantation patients were notified of plans to perform 7T MRI scanning of the transplants for research purposes at the time of participation in the long-term follow-up study of PT.[8] An information letter to explain the study was sent to eligible patients. A week thereafter the patients were contacted by phone by the research physician (M.J.) to answer questions if any and to ask whether they were willing to participate in the study. Eligible ACT patients were contacted by phone by their surgeon (L.P.) to explain the study and to ask whether they were willing to participate.

This study was performed in accordance with the Helsinki Declaration of 1975, as revised in 2013, and the protocol was accepted by the medical ethical committee of the Maastricht University Medical Center (NL48277.068.14/METC 142039) in which patients gave their written informed consent. Participants from Sweden signed a certified, translated version of the written informed consent, translated by Metamorfose Vertalingen, Utrecht, the Netherlands.

Surgical procedures

A comprehensive description of the surgical procedures has been reported before by Homminga et al., Bouwmeester et al. for PT, and by Peterson et al. for ACT.[5,7,9,10,22] In short, PT is a one-stage procedure. A piece of perichondrium was dissected from the cartilaginous part of one of the lower ribs and removed together with its cambium

layer. The graft was cut to match the size of the defect. Subsequently the perichondral graft was placed in the defect with the chondral side facing up and attached with fibrin glue.[5,10]

Autologous chondrocyte transplantation includes two surgical procedures. During the first surgical procedure, cartilage tissue was harvested from a healthy, non-weight bearing part of the cartilage. From this tissue, chondrocytes were retrieved and cultured in a laboratory for several weeks. During the second surgical procedure, the chondrocytes from the cell culture were injected into the defect under a periosteal flap.[9]

Clinical questionnaires / Patient reported outcome measures

Patients were asked to complete three clinical questionnaires at the time of MRI acquisition: the International Knee Documentation Committee (IKDC),[23] the Knee injury and Osteoarthritis Outcome Score (KOOS)[24] (Validated Swedish version),[25] and the Visual Analogue Scale (VAS) for knee pain.

MRI acquisition

Morphological and biochemical MRI measurements were performed on a 7T MR whole body system (Magnetom, Siemens Healthcare, Erlangen, Germany) using a 28-channel proton knee coil (QED, Electrodynamics LLC, Cleveland, OH). Before acquiring MRI data, the homogeneity of the main magnetic field (B_0) was optimized by a B_0 shim. The radiofrequency pulse (B_1) was optimized by acquiring a B_1 map. To avoid motion artifacts, the leg was stabilized using a vacuum cushion underneath the lower leg.

The morphological protocol included a three-dimensional (3D) T2 dual echo steady state (DESS) sequence. The T2 DESS sequence was obtained for the complete knee in sagittal plane. Furthermore, a two-dimensional (2D) sagittal proton-density (PD) weighted fast spin-echo (FSE) sequence with fat suppression (fatsat) was obtained. The biochemical protocol included T2 mapping and gagCEST sequences.
The T2 relaxation times were obtained from T2 maps that were reconstructed using a multi-echo, spin-echo technique, using a custom written Matlab script.[26] The T2 mapping protocol was obtained in sagittal direction. Due to SAR restrictions, only the femoral condyle containing the cartilage repair tissue region was acquired. The obtained T2 relaxation times are a measure for collagen integrity: the higher the T2 relaxation time, the lower the integrity of the collagen network.[21]

For gagCEST imaging, a 3D radiofrequency (RF) spoiled gradient echo (GRE) sequence including 19 saturation RF pulses was acquired. One additional measurement without the presaturation pulses was acquired. Residual transversal magnetization signal was spoiled by gradient spoiling. The applied B_1 amplitude of the saturation pulses was set to a minimum of 0.8 µT and adapted for each individual to the maximum value possible

in relation to SAR, to achieve optimal saturation. The separate saturation measurements were post-processed into colored GAG maps using a custom made Matlab script which determined the magnetization transfer ratio asymmetry (MTRasym) in the calculated z-spectra.[20] The MTRasym value is a measure for GAG content: the higher the MTRasym value, the higher the GAG content.[19] Imaging parameters for the morphological and biochemical sequences are presented in Table 3.1.

Table 3.1 Imaging parameters for morphological sequences T2 DESS and PD fatsat FSE and for biochemical sequences T2mapping and gagCEST.

	T2 DESS	PD fatsat FSE	T2mapping	gagCEST
Repetition time (ms)	8.90	7440	2200	6.90
Echo time (ms)	2.63	36	13.8, 27.6, 41.4, 55.2, 69.0, 82.8	2.84
Flip angle (°)	18	180	180	9
Field of view ($mm^{2)}$	160 x 160	160 x 160	136 x 160	157 x 180
Matrix size	320 x 320	864 x 864	320 x 272	192 x 168
Voxel size ($mm^{3)}$	0.5 x 0.5 x 0.5	0.4 x 0.4 x 2.5	0.5 x 0.5 x 3.0	0.9 x 0.9 x 2.2
Acceleration factor (GRAPPA)	3	3	2	2
Acquisition time (min)	05:00	08:42	10:57	20:04

DESS = dual echo steady state; PD = proton-density; FSE = fast spin-echo; gagCEST = glycosaminoglycan Chemical Exchange Saturation Transfer.

MRI analysis

The morphological MR data sets were transferred to a freeware JiveX imaging viewer (VISUS Technology Transfer GmbH, Bochum, Germany). The Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART)[27] was used to assess the cartilage transplant tissue and was scored together with the cartilage quality in the rest of the joint by the senior author (S.T.; radiologist with over 25 years of experience in musculoskeletal imaging), in consensus with a resident orthopedic surgeon (M.J.). In case of any uncertainties, an experienced orthopedic surgeon with over 10 years of experience in cartilage repair of the knee (P.E.) was consulted.

The morphological MR data sets as well as the post-processed biochemical T2 maps and GAG maps were transferred to OsiriX imaging software (v.9.0.2, Pixmeo, Switzerland) and analyzed based on a region of interest (ROI) approach. The resolution of the biochemical T2 maps and GAG maps were adapted to the resolution of the morphological DESS images by linear interpolation. As the images were acquired in the same plane, only translation according to their DICOM tags was necessary to register the images. No motion correction was applied, however, the overlay was manually checked by comparing anatomical landmarks in both sequences and adjusted when deemed necessary. Regions of interest were manually drawn in the DESS morphological image of each patient by two independent readers (M.J. and M.P.), the ROIs were finalized after consensus. The inclusion of cartilage pixels only was ensured; no bone

pixels or joint fluid pixels were included in the ROIs. Per patient, regions were selected in a slice showing the defect clearly (Figure 3.1) and regions were selected in a control slice (Figure 3.2). Six ROIs were drawn per patient; a defect ROI (referred to as *defect*) with anterior and posterior adjacent ROIs in the femur (referred to as *adj_A* and *adj_P* respectively); an ROI in the tibia cartilage opposite to the defect between the area covered by the meniscus (referred to as *tibia*); a control ROI in the posterior part of the femur (referred to as *c_femur*); and a control tibia ROI (referred to as *c_tibia*). The ROIs were subsequently transferred to the coregistered GAG maps and T2 maps. In case of the GAG maps, the mean MTRasym value within each ROI was extracted (see Figure 3.1B and Figure 3.2B) as a measure of GAG content. In case of the T2 maps, the mean T2 relaxation time within each ROI as a whole (global T2 relaxation time) as well as within the deep zone and the superficial zone specifically (deep zone T2 relaxation time and superficial zone T2 relaxation time respectively) were extracted (see Figure 3.1C and Figure 3.2C), as a measure for the collagen integrity.

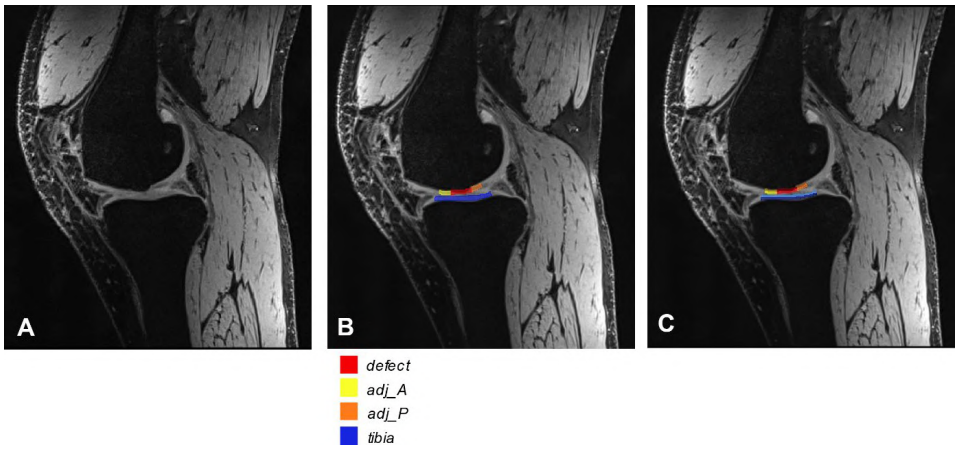

Figure 3.1 Example of ROIs in a slice with defect. **A:** Original image, T2 DESS. **B:** Image with ROIs to obtain MTRasym and global T2 relaxation times: defect ROI in red (*defect*); anterior adjacent ROI in yellow (*adj_A*); posterior adjacent ROI in orange (*adj_P*); tibia ROI in blue (*tibia*). **C:** Image with ROIs divided in a deep zone (dark colors) and a superficial zone (light colors) to obtain zonal T2 relaxation times.
ROI = region of interest; DESS = dual echo steady state; MTRasym = magnetization transfer ratio asymmetry.

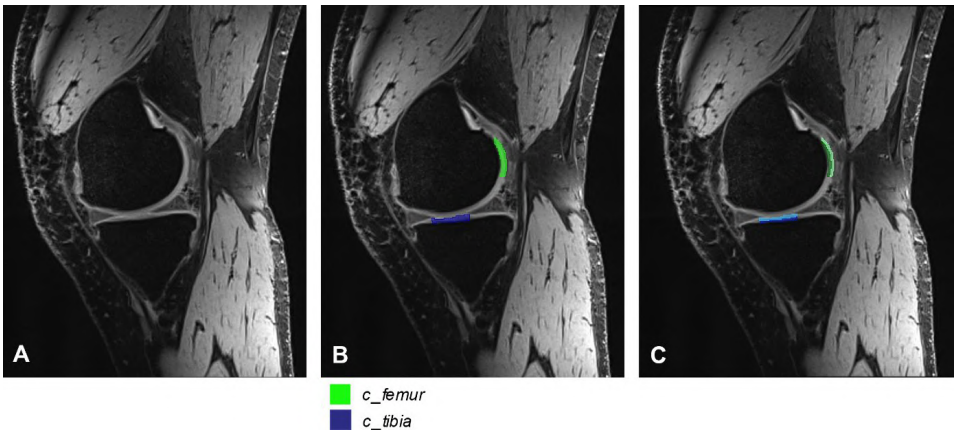

Figure 3.2 Example of ROIs in a control slice. **A:** Original T2 DESS image. **B:** Image with control ROIs to obtain MTRasym and global T2 relaxation times. Control region in the femur is presented in green (*c_femur*), control region for the tibia from meniscus to meniscus is presented in blue (*c_tibia*). **C:** Image with control ROIs divided in a deep zone (dark colors) and a superficial zone (light colors) to obtain zonal T2 relaxation times.
ROI = region of interest; DESS = dual echo steady state; MTRasym = magnetization transfer ratio asymmetry.

Calcification thickness

Calcification was scored in the T2 DESS morphological image of each patient by two independent readers (M.J. and M.P.), the ROIs were finalized after consensus. The used technique is an adaptation from the technique used by Demange and colleagues.[13] An ROI was drawn that included the calcified area of the graft. The *percentage of calcification* was calculated by dividing the number of calcified pixels within the graft by the number of pixels in the total graft. Subsequently, patients with a calcification percentage of less than 50% were given calcification percentage score 0, patients with a calcification percentage of more than 50% were given calcification percentage score 1. Furthermore, the *thickness of the calcification* was scored. Patients with a calcification penetrating the surface of the cartilage layer, thus with the calcification being in direct contact with the opposing tibial cartilage, were given a calcification thickness score of 1. Patients with a calcification that was still covered by a layer of cartilage (regardless of the thickness of that layer of cartilage) preventing direct contact between the calcification and the opposing tibial cartilage were given a calcification thickness score of 0. Examples of the calcification scores are provided in Figure 3.3. Subsequently, the influence of the calcification of the cartilage grafts was compared to the quality of the opposing cartilage tissue.

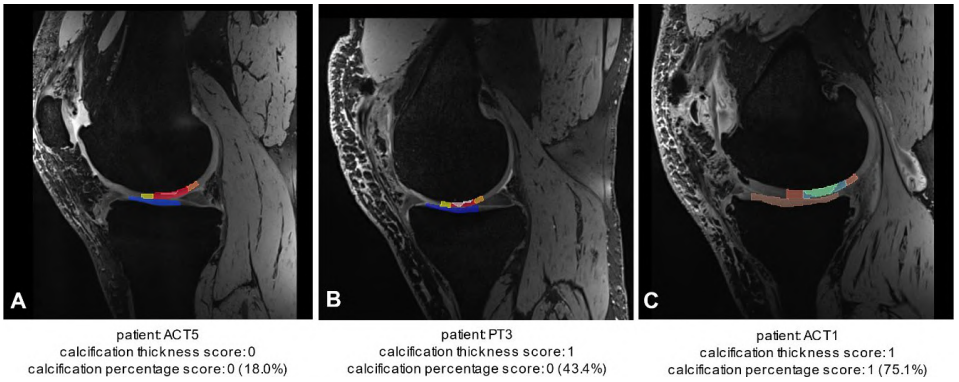

Figure 3.3 Examples of the calcification scoring with the calcification presented in white. **A:** a calcification covering 18% of the defect with a substantial layer of cartilage between the calcification and the opposing tibial cartilage; **B:** a calcification covering less than half of the defect with contact of the calcification with the opposing tibial cartilage; **C:** a calcification covering more than half of the defect with no layer of cartilage between the calcification and the opposing tibial cartilage.
ACT = autologous chondrocyte transplantation; PT = perichondrium transplantation.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics, version 25 (IBM, Armonk, New York). Normality was tested by a Shapiro-Wilk test. Differences between PT and ACT patients were assessed by an independent t-test in case of normality and a Mann-Whitney U-test otherwise. Differences between regions were evaluated by a paired samples t-test in case of normality and a Wilcoxon Signed-Rank test otherwise. Differences were considered statistically significant when the p-value was below 0.05.

Results

Description of patient population

Seven PT patients and five ACT patients were willing to be included in the study. Baseline demographics are provided in Table 3.2. Time between surgery and MRI follow-up was similar for the PT patients and the ACT patients, on average 28.1 years for PT and 24.0 years for ACT (p-value 0.213). Defect size was larger in the ACT patients compared to the PT patients (3.9 cm^2 and 2.1 cm^2, respectively, p-value 0.048). No adverse events or serious adverse events occurred during this study.

Table 3.2 Patient characteristics for the Dutch PT patients (PT1 – PT7) and the five Swedish ACT patients (ACT1 – ACT5) including mean values, standard deviation (SD) and p-values for the numeric characteristics.

	Sex	Age at surgery (years)	BMI (kg/m^2)	Knee	Location defect	Defect size (cm^2)	Follow-up duration (years)
PT1	Male	36	27.5	Right	MFC	2.3	24
PT2	Female	22	23.8	Right	MFC	0.5	25
PT3	Male	45	29.4	Right	MFC	0.8	30
PT4	Male	35	26.3	Left	MFC	2.3	31
PT5	Female	17	23.0	Left	MFC	3.0	29
PT6	Male	23	22.8	Right	MFC	3.0	29
PT7	Male	27	29.1	Left	MFC	3.1	29
mean	-	29.3	26.0		-	2.1	28.1
SD	-	9.8	2.8		-	1.1	2.6
ACT1	Male	24	32.1	Left	MFC	2.0	30
ACT2	Male	27	24.3	Right	LFC	3.0	30
ACT3	Male	32	29.0	Right	MFC	5.2	24
ACT4	Male	28	27.5	Right	MFC	3.3	11
ACT5	Male	27	27.5	Right	MFC	6.0	25
mean	-	27.6	28.0	-	-	3.9	24.0
SD	-	2.9	2.8	-	-	1.6	7.8
p-value		0.719	0.235			0.048	0.213

PT = perichondrium transplantation; ACT = autologous chondrocyte transplantation; kg/m^2 = kilograms per square meter; cm^2 = square centimeter; SD = standard deviation; MFC = medial femoral condyle; LFC = lateral femoral condyle.

Clinical outcome at time of MRI

The IKDC, KOOS and VAS questionnaire scores of each individual patient at the time of MRI acquisition are presented in Table 3.3. No statistically significant difference was found between the questionnaire scores of the PT patients and the ACT patients.

Table 3.3 Individual scoring parameters including the IKDC, KOOS and VAS questionnaire scores were used to assess clinical outcome.The MRI based MOCART score was used to assess the cartilage transplant quality and was scored together with the cartilage quality in the rest of the joint by the senior author (ST). Overall cartilage quality was divided in the categories: no degeneration, early degeneration, moderate degeneration and severe degeneration. Mean values with standard deviation (SD) for the PT patients and the ACT patients were included.

Patient number	IKDC	KOOS					VAS	MOCART score	Cartilage quality in the rest of the joint
		Pain	Other symptoms	Function in daily living	Function in sport and recreation	Knee-related Quality of life			
PT1	86.2	94.4	75.0	100.0	100.0	100.0	5	65	Moderate degeneration
PT2	60.9	84.4	92.9	95.6	75.0	68.8	20	85	Early degeneration
PT3	26.4	16.7	50.0	16.2	0.0	0.0	85	75	Early – moderate degeneration
PT4	88.5	100.0	100.0	100.0	100.0	100.0	5	80	Early degeneration
PT5	73.6	100.0	96.4	100.0	90.0	83.3	0	65	Early degeneration
PT6	75.9	100.0	82.1	100.0	80.0	75.0	0	85	Early – moderate degeneration
PT7	34.5	25.0	28.6	22.1	0.0	12.5	80	60	Early – severe degeneration
mean	**63.7**	**74.4**	**75.0**	**76.3**	**63.6**	**63.8**	**27.9**	**73.6**	**N.A.**
SD	**24.6**	**37.1**	**26.6**	**39.1**	**44.4**	**40.5**	**38.0**	**10.3**	**N.A.**
ACT1	79.3	88.9	53.6	97.1	80.0	56.3	10	80	Early – severe degeneration
ACT2	43.7	77.8	39.3	70.6	35.0	50.0	30	55	Early – severe degeneration
ACT3	74.7	75.0	60.7	92.6	35.0	87.5	0	80	Early – moderate degeneration
ACT4	72.4	91.7	78.6	83.8	45.0	62.5	20	65	Early degeneration
ACT5	80.5	97.2	89.3	100.0	90.0	93.8	30	75	Early – severe degeneration
mean	**70.1**	**86.1**	**64.1**	**88.8**	**57.0**	**70.0**	**18.0**	**71.0**	**N.A.**
SD	**15.1**	**9.4**	**19.9**	**11.9**	**26.1**	**19.5**	**13.8**	**10.8**	**N.A.**
p-value	**0.867**	**0.639**	**0.432**	**0.639**	**0.639**	**0.876**	**0.755**	**0.639**	**N.A.**

IKDC = International Knee Documentation Committee; KOOS = Knee injury and Osteoarthritis Outcome Score; VAS = Visual Analogue Scale; MOCART = Magnetic Resonance Observation of Cartilage Repair Tissue; PT = perichondrium transplantation; N.A. = Not applicable; SD = standard deviation; ACT = autologous chondrocyte transplantation.

Morphological assessment and MOCART score

The morphological MR images of the seven PT patients and the five ACT patients were available for assessment of the transplant by means of the MOCART score. The outcome of the ten MOCART criteria per patient are presented in the Supplemental Table S3.1. The overall MOCART score and the cartilage quality in the rest of the joint are presented in Table 3.3 for each individual patient. The cartilage quality in the rest of the joint was varying from no degeneration to severe degeneration. The overall MOCART score was similar for the PT patients and the ACT patients (mean score 73.6 and 71.0 respectively, p-value = 0.639).
Evaluation of the graft alone showed similar intralesional osteophyte formation in the perichondrium transplants compared to the autologous chondrocyte transplants (Table 3.4). In five of the twelve patients, the grafts were calcified more than 50% and also in five of the twelve patients the calcification penetrated the surface of the graft.

Table 3.4 Calcification scores.

Patient	Calcification percentage		Calcification thickness score
		Score	
PT1	25.9%	0	0
PT2	47.9%	0	0
PT3	43.4%	0	1
PT4	47.0%	0	0
PT5	57.4%	1	1
PT6	73.6%	1	1
PT7	57.4%	1	0
ACT1	75.1%	1	1
ACT2	31.1%	0	0
ACT3	92.4%	1	1
ACT4	22.0%	0	0
ACT5	18.0%	0	0

PT = perichondrium transplantation; ACT = autologous chondrocyte transplantation.

Biochemical assessment

Figure 3.4 shows an overview of the biochemical values of cartilage in the specified ROIs. The biochemical values for each of the six regions are presented next to the overall MOCART score per patient in Supplemental Table S3.2. Paired samples t-test showed that GAG content in the defect region as well as in the adjacent regions was significantly lower than the GAG content in the control ROI. The MTRasym value in the tibia cartilage opposing the defect was similar to the MTRasym value of control tibia cartilage, suggesting similar GAG content in both regions. Paired samples t-test showed significantly higher global T2 relaxation times for the defect region and for anterior adjacent region, compared to the femur control region. The global T2 relaxation times in the tibia region opposing the defect were similar to global T2 relaxation times in the control tibia cartilage, suggesting similar collagen integrity for both regions.

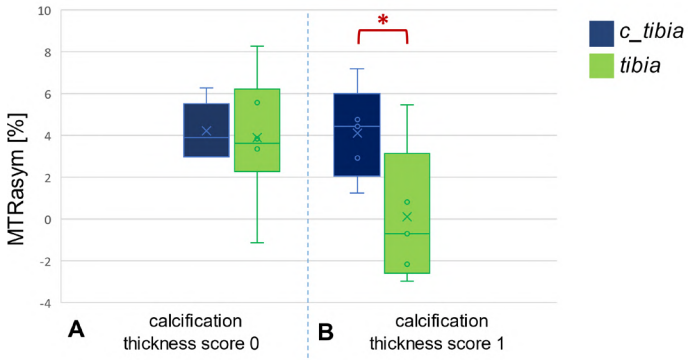

Figure 3.4 MTRasym values (**A**) and global T2 relaxation times (**B**) for the 6 different ROIs. The regions in the femur are displayed on the left side of the dotted line, and regions in the tibia are presented on the right side of the dotted line. Red asterisks represent statistically significant differences between regions. MTRasym = magnetization transfer ratio asymmetry.

Table 3.4 presents the calcification scores for the included patients. The influence of calcification thickness of the transplant on the opposing cartilage is presented in Figure 3.5 for the gagCEST sequence and in Figure 3.6 for T2 mapping. Statistical analysis showed that the tibial cartilage opposing the defect in patients with a calcification that is in contact with the opposing cartilage (calcification thickness score of 1) has significantly lower GAG content (MTRasym value) compared to control tibial cartilage, while the collagen integrity (global T2 relaxation times) was similar for tibial cartilage opposing the defect and control tibial cartilage. Figure 3.6 shows that the zonal variation between the deep layer and the superficial layer of the tibial cartilage opposing the defect is similar to that of the control tibia cartilage. In other words, the collagen integrity of the opposing cartilage was not affected by the calcification thickness of the transplant.

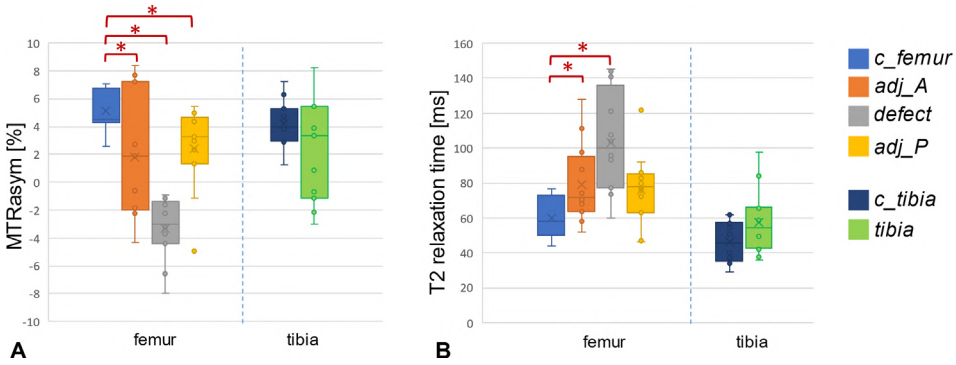

Figure 3.5 MTRasym values for the tibial cartilage opposing the defect (tibia) compared to control tibial cartilage (c_tibia) for patients with calcification thickness score 0 (**A**) and for calcification thickness score 1 (**B**). The red asterisk represents a statistically significant difference between regions. MTRasym = magnetization transfer ratio asymmetry.

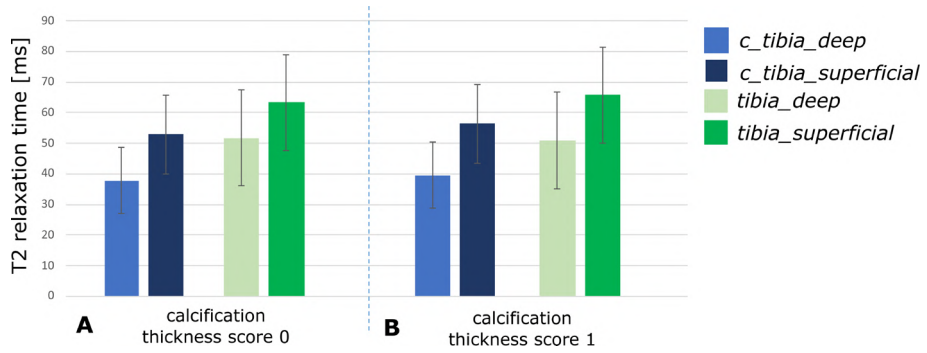

Figure 3.6 T2 relaxation times for the tibial cartilage opposing the defect (tibia) compared to control tibial cartilage (c_tibia) for the calcification thickness score 0 (**A**) and for the calcification thickness score 1 (**B**) in the deep zone and in the superficial zone of the cartilage.

Figure 3.7 illustrates the findings of Figures 3.4, 3.5 and 3.6 in the form of MTR asymmetry and T2 relaxation time overlays for a patient with calcification thickness score of 0 and for a patient with calcification thickness score 1. For both patients, the transplant area shows low MTRasym values and high T2 relaxation times compared to control regions, indicating a lower GAG content and a disturbed collagen network. For the patient with cartilage thickness score 0 (Figure 3.7A and 3.7B) the opposing cartilage is of relatively good quality. The patient with a calcification thickness score of 1 (Figure 3.7C and 3.7D) showed lower GAG content in the opposing cartilage while the collagen integrity does not seem to be influenced.

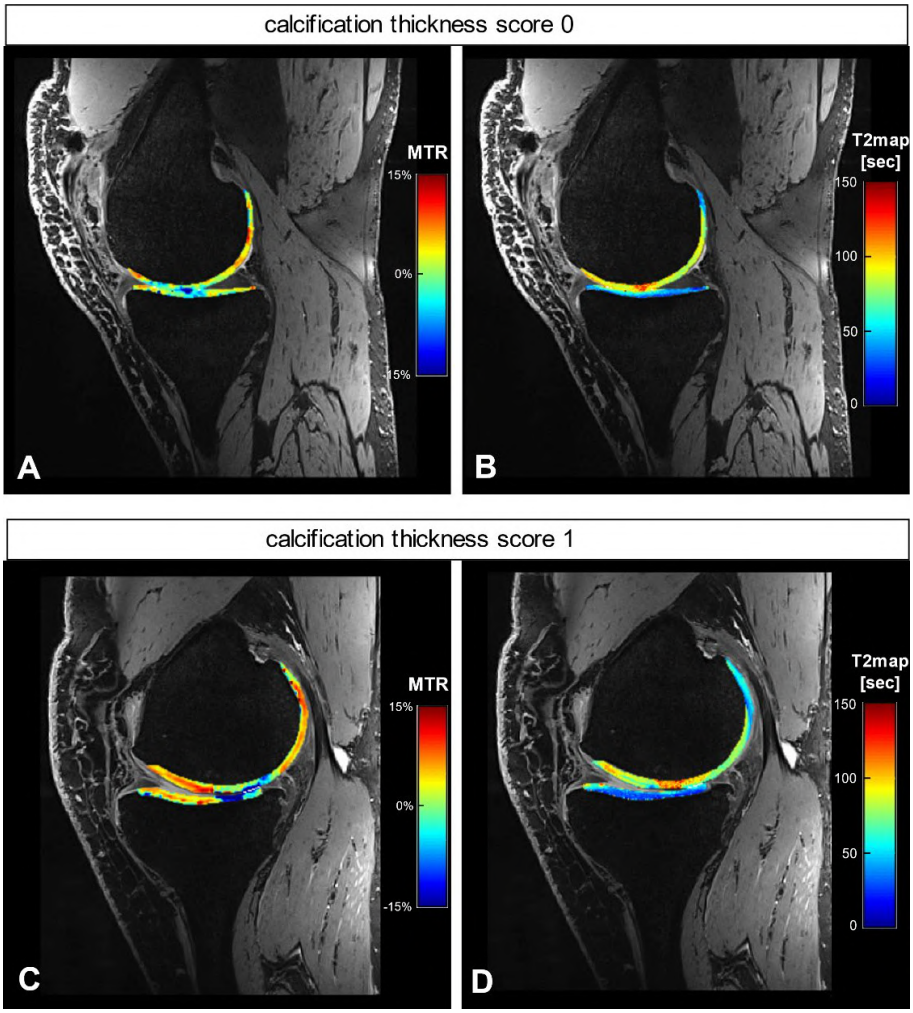

Figure 3.7 Example of a patient with calcification thickness score 0 (**A** and **B**) and a patient with calcification thickness score 1 (**C** and **D**). DESS images are presented with an overlay of MTRasym values and T2 relaxation times. DESS = dual-echo steady state; MTRasym = magnetization transfer ratio asymmetry.

Discussion

In this study, 12 patients were evaluated about 25 years after cartilage repair surgery of the knee by means of clinical questionnaires and 7T MRI. The cartilage tissue in general, the cartilage repair tissue and the opposing tibial cartilage were assessed both morphologically and biochemically by 7T MRI. The quality of the cartilage tissue

throughout the joint was variable. For each of the included cartilage repair patients, the cartilage repair tissue was of poor quality (low GAG content [low MTRasym values] and low collagen integrity (high T2 relaxation times)), regardless of the performed procedure. In line with previous research, we found a high incidence of intralesional osteophytes. The thickness of the calcification in these intralesional osteophytes can influence the opposing tibial cartilage. It was shown that when the intralesional osteophyte penetrates the surface of the graft, the opposing tibial cartilage was biochemically damaged. The damage was more pronounced in the GAG content reflected by the MTRasym values and less in the collagen integrity represented by the intact zonal variation in T2 relaxation times, suggesting that tibial cartilage opposing osteophytes that penetrate the surface showed signs of early OA. A difference in percentage of calcification of the grafts caused no statistically significant difference of opposing cartilage tissue quality. Calcified tissue has an increased stiffness compared to cartilage, which causes higher contact stresses and increased friction.[28] This increased stiffness and friction of a calcification that penetrates the surface of a graft is expected to exert a larger mechanical strain on the opposing tibial cartilage compared to an intact surface and thereby causing its deterioration over time. The 10 to 20-year clinical outcome of cartilage repair surgery has been documented previously by multiple authors, for example by Minas and co-workers with satisfactory results.[29-31] However, to our knowledge there are no studies that describe the biochemical status of the articular cartilage of patients after a follow-up of a mean of 25 years as described in this current paper. Evaluation of articular cartilage by 7T MRI provides the opportunity for its biochemical assessment and a high spatial resolution for detailed morphological assessment. So far, the evaluation of the GAG content in repair tissue, an important marker for the biomechanical properties was restricted to dGEMRIC at lower field MR,[32] which however requires a double dose of intravenous administration of Gadolinium based contrast agents which considering the ongoing discussions of Gadolinium depositions in the brain are problematic. In addition, the standard ionic contrast agent so far used for dGEMRIC, Magnevist, was removed from the European market by the European Medicine Ageny due to the Gadolinium depositions in the human body seen with linear Gadolinium based contrast agents.[33] High spatial resolution can be achieved using new 3T MRI techniques and T2 mapping is available at 3T MRI as well as on 7T MRI, but gagCEST is limited to use at ultra-high field such as 7T MRI.[20] Using gagCEST, the GAG content can be quantified using a regular proton coil (no sodium coil needed) and without the use of a contrast agent (as is the case for dGEMRIC). On the downside, gagCEST is limited to high-field MRI such as 7T MRI, because the spectral resolution on 7T is by a factor of 2 higher compared to 3T, which is needed to separate the small frequency shift between protons bound to GAG and protons in the water pool.[20] Therefore, gagCEST scanning is only feasible at 7T MRI and provides essential biochemical information not available in studies performed with lower field MRI (1.5T or 3.0T).

The occurrence of intralesional osteophytes after cartilage repair surgery has been described before, the incidence of osteophytes rises when the subchondral bone is involved in either the defect or the surgery.[13,14,34] Intralesional osteophytes occur more often after ACT procedures with previous marrow stimulation and in periosteal-covered defects compared to collagen membrane-covered defects,[13] but it is still unclear whether the osteophytes result from a thickening of the subchondral bone or from the progenitor cells in the cambium layer of the periosteal tissue.[35] Kreuz et al. propose an impaired clinical outcome after microfracture caused by a thinner layer of cartilage overlying damaged subchondral bone and subsequent increased shear stresses.[34] However, calcification of the repair tissue was not a part of the MRI scoring systems at the time of publication, nor was calcification described separately in their paper.[34] In addition, Pestka et al. describe an increased failure rate after previous marrow stimulation, but did not directly correlate this to increased intralesional osteophytes.[36]

In a review focusing on the subchondral bone in osteochondral repair, Orth et al. elucidate on the lack of detailed visualization of subchondral bone architecture of repaired cartilage due to technical and ethical limitations. Although there is no absolute lack of studies which assess the repaired cartilage morphologically (often by MRI), a detailed biochemical assessment of repair tissue and evaluation of intralesional osteophytes is less common.[37] Recently the MOCART score has been updated to provide a more detailed assessment of morphologic characteristics of the repaired cartilage resulting in the MOCART 2.0 score,[38] however, at this moment it has only been applied in three clinical cartilage repair studies.[39-41] Only Sessa et al. found a correlation of the MOCART 2.0 score with clinical outcome parameters. However, group sizes in these studies were relatively small and therefore might lack the statistical power to detect correlations.[39-41] Even though our study also assessed a relatively small group of patients, we did find a correlation of surface penetrating intralesional osteophytes which led to opposing cartilage damage. Based on our current data, we are not able to demonstrate an impaired subjective or clinical outcome caused by intralesional osteophyte formation after cartilage repair surgery.

An important limitation of this study was the small sample size. The small numbers of patients for the two surgical procedures did not allow for a long-term comparison between the procedures. The heterogeneity among the included patients was another limitation. Some patients underwent reconstruction of their anterior cruciate ligament in combination with cartilage repair of their defect. Furthermore, it was difficult to select control ROIs in the knees of the patients given that 25 years after surgery the quality of the knee cartilage was in general relatively low. In addition, 7T MRI has only been obtained at a long-term follow-up. To demonstrate the value of clinical evaluation of articular cartilage repair surgery by 7T MRI, larger group sizes and monitoring over several timepoints should be included in future work. It is important to note that ACT has been modified since 1996 to stop the intralesional osteophyte formation by careful removal of the calcified layer down to the subchondral bone plate, release the

tourniquet to detect and stop any bleeding by fibrin glue. Furthermore, the periosteal flap has been replaced by synthetical resorbable membranes.

To conclude, PT and ACT patients have a high incidence of intralesional osteophyte formation 25 years after surgery. The resulting biochemical damage to the opposing tibial cartilage might be dependent on osteophyte morphology.

References

1. Theologis AA, Schairer WW, Carballido-Gamio J, Majumdar S, Li X, Ma CB. Longitudinal analysis of T1rho and T2 quantitative MRI of knee cartilage laminar organization following microfracture surgery. *Knee.* 2012;19(5):652-657.
2. Steadman JR, Rodkey WG, Singleton SB, Briggs KK. Microfracture technique forfull-thickness chondral defects: Technique and clinical results. *Operative techniques in orthopaedics.* 1997;7(4):300-304.
3. Alford JW, Cole BJ. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *Am J Sports Med.* 2005;33(2):295-306.
4. Redondo ML, Naveen NB, Liu JN, Tauro TM, Southworth TM, Cole BJ. Preservation of Knee Articular Cartilage. *Sports Med Arthrosc Rev.* 2018;26(4):e23-e30.
5. Homminga GN, Bulstra SK, Bouwmeester PS, van der Linden AJ. Perichondral grafting for cartilage lesions of the knee. *J Bone Joint Surg Br.* 1990;72(6):1003-1007.
6. 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):889-895.
7. Bouwmeester SJ, Beckers JM, Kuijer R, van der Linden AJ, Bulstra SK. Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. *Int Orthop.* 1997;21(5):313-317.
8. Janssen MPF, van der Linden EGM, Boymans T, Welting TJM, van Rhijn LW, Bulstra SK, Emans PJ. Twenty-Two-Year Outcome of Cartilage Repair Surgery by Perichondrium Transplantation. *Cartilage.* 2020:1947603520958146.
9. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med.* 2010;38(6):1117-1124.
10. Bouwmeester PS, Kuijer R, Homminga GN, Bulstra SK, Geesink RG. A retrospective analysis of two independent prospective cartilage repair studies: autogenous perichondrial grafting versus subchondral drilling 10 years post-surgery. *J Orthop Res.* 2002;20(2):267-273.
11. Vasiliadis HS, Danielson B, Ljungberg M, McKeon B, Lindahl A, Peterson L. Autologous chondrocyte implantation in cartilage lesions of the knee: long-term evaluation with magnetic resonance imaging and delayed gadolinium-enhanced magnetic resonance imaging technique. *Am J Sports Med.* 2010;38(5):943-949.
12. Russlies M, Behrens P, Ehlers EM, Brohl C, Vindigni C, Spector M, Kurz B. Periosteum stimulates subchondral bone densification in autologous chondrocyte transplantation in a sheep model. *Cell Tissue Res.* 2005;319(1):133-142.
13. Demange MK, Minas T, von Keudell A, Sodha S, Bryant T, Gomoll AH. Intralesional Osteophyte Regrowth Following Autologous Chondrocyte Implantation after Previous Treatment with Marrow Stimulation Technique. *Cartilage.* 2017;8(2):131-138.
14. Minas T, Gomoll AH, Rosenberger R, Royce RO, Bryant T. Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques. *Am J Sports Med.* 2009;37(5):902-908.
15. Guermazi A, Roemer FW, Alizai H, Winalski CS, Welsch G, Brittberg M, Trattnig S. State of the Art: MR Imaging after Knee Cartilage Repair Surgery. *Radiology.* 2015;277(1):23-43.
16. Welsch GH, Juras V, Szomolanyi P, Mamisch TC, Baer P, Kronnerwetter C, Blanke M, Fujita H, Trattnig S. Magnetic resonance imaging of the knee at 3 and 7 tesla: a comparison using dedicated multi-channel coils and optimised 2D and 3D protocols. *Eur Radiol.* 2012;22(9):1852-1859.
17. Guermazi A, Alizai H, Crema MD, Trattnig S, Regatte RR, Roemer FW. Compositional MRI techniques for evaluation of cartilage degeneration in osteoarthritis. *Osteoarthritis and cartilage.* 2015;23(10): 1639-1653.
18. Ladd ME, Bachert P, Meyerspeer M, Moser E, Nagel AM, Norris DG, Schmitter S, Speck O, Straub S, Zaiss M. Pros and cons of ultra-high-field MRI/MRS for human application. *Prog Nucl Magn Reson Spectrosc.* 2018;109:1-50.
19. Schmitt B, Brix M, Domayer S. CEST Imaging. *Current Radiology Reports.* 2014;2(3):38.
20. Schreiner MM, Zbýň Š, Schmitt B, Weber M, Domayer S, Windhager R, Trattnig S, Mlynárik V. Reproducibility and regional variations of an improved gagCEST protocol for the in vivo evaluation of knee cartilage at 7 T. *MAGMA.* 2016;29(3):513-521.

21. Apprich S, Mamisch TC, Welsch GH, Stelzeneder D, Albers C, Totzke U, Trattnig S. Quantitative T2 mapping of the patella at 3.0T is sensitive to early cartilage degeneration, but also to loading of the knee. *Eur J Radiol.* 2012;81(4):e438-443.
22. Bouwmeester P, Kuijer R, Terwindt-Rouwenhorst E, van der Linden T, Bulstra S. Histological and biochemical evaluation of perichondrial transplants in human articular cartilage defects. *J Orthop Res.* 1999;17(6):843-849.
23. Irrgang JJ, Anderson AF, Boland AL, Harner CD, Kurosaka M, Neyret P, Richmond JC, Shelborne KD. Development and validation of the international knee documentation committee subjective knee form. *The American journal of sports medicine.* 2001;29(5):600-613.
24. Roos EM, Lohmander LS. The Knee injury and Osteoarthritis Outcome Score (KOOS): from joint injury to osteoarthritis. *Health and quality of life outcomes*. 2003;1:64.
25. Roos EM, Roos HP, Ekdahl C, Lohmander LS. Knee injury and Osteoarthritis Outcome Score (KOOS)--validation of a Swedish version. *Scandinavian journal of medicine & science in sports.* 1998;8(6): 439-448.
26. Hurk Jvd. T1T2T2starmappingtool. https://github.com/jobvandenhurk/T1T2T2starmappingtool. Updated 26-05-2021. Accessed 02-08-2021.
27. Marlovits S, Singer P, Zeller P, Mandl I, Haller J, Trattnig S. Magnetic resonance observation of cartilage repair tissue (MOCART) for the evaluation of autologous chondrocyte transplantation: determination of interobserver variability and correlation to clinical outcome after 2 years. *Eur J Radiol.* 2006;57(1): 16-23.
28. Peters AE, Akhtar R, Comerford EJ, Bates KT. The effect of ageing and osteoarthritis on the mechanical properties of cartilage and bone in the human knee joint. *Sci Rep.* 2018;8(1):5931.
29. Minas T, Von Keudell A, Bryant T, Gomoll AH. The John Insall Award: A minimum 10-year outcome study of autologous chondrocyte implantation. *Clin Orthop Relat Res.* 2014;472(1):41-51.
30. Ogura T, Mosier BA, Bryant T, Minas T. A 20-Year Follow-up After First-Generation Autologous Chondrocyte Implantation. *Am J Sports Med.* 2017;45(12):2751-2761.
31. Gobbi A, Karnatzikos G, Kumar A. Long-term results after microfracture treatment for full-thickness knee chondral lesions in athletes. *Knee Surg Sports Traumatol Arthrosc.* 2014;22(9):1986-1996.
32. Trattnig S, Mamisch TC, Pinker K, Domayer S, Szomolanyi P, Marlovits S, Kutscha-Lissberg F, Welsch GH. Differentiating normal hyaline cartilage from post-surgical repair tissue using fast gradient echo imaging in delayed gadolinium-enhanced MRI (dGEMRIC) at 3 Tesla. *Eur Radiol.* 2008;18(6):1251-1259.
33. Dekkers IA, Roos R, van der Molen AJ. Gadolinium retention after administration of contrast agents based on linear chelators and the recommendations of the European Medicines Agency. *Eur Radiol.* 2018;28(4):1579-1584.
34. Kreuz PC, Steinwachs MR, Erggelet C, Krause SJ, Konrad G, Uhl M, Sudkamp N. Results after microfracture of full-thickness chondral defects in different compartments in the knee. *Osteoarthritis Cartilage.* 2006;14(11):1119-1125.
35. Bruns J, Meyer-Pannwitt U, Silbermann M. The rib perichondrium. An anatomical study in sheep of a tissue used as transplant in the treatment of hyaline-cartilage defects. *Acta Anat (Basel).* 1992;144(3):258-266.
36. Pestka JM, Bode G, Salzmann G, Sudkamp NP, Niemeyer P. Clinical outcome of autologous chondrocyte implantation for failed microfracture treatment of full-thickness cartilage defects of the knee joint. *Am J Sports Med.* 2012;40(2):325-331.
37. Hayashi D, Li X, Murakami AM, Roemer FW, Trattnig S, Guermazi A. Understanding Magnetic Resonance Imaging of Knee Cartilage Repair: A Focus on Clinical Relevance. *Cartilage.* 2018;9(3): 223-236.
38. Schreiner MM, Raudner M, Marlovits S, Bohndorf K, Weber M, Zalaudek M, Rohrich S, Szomolanyi P, Filardo G, Windhager R, Trattnig S. The MOCART (Magnetic Resonance Observation of Cartilage Repair Tissue) 2.0 Knee Score and Atlas. *Cartilage.* 2019:1947603519865308.
39. Casari FA, Germann C, Weigelt L, Wirth S, Viehofer A, Ackermann J. The Role of Magnetic Resonance Imaging in Autologous Matrix-Induced Chondrogenesis for Osteochondral Lesions of the Talus: Analyzing MOCART 1 and 2.0. *Cartilage.* 2020:1947603520946382.
40. Andriolo L, Di Martino A, Altamura SA, Boffa A, Poggi A, Busacca M, Zaffagnini S, Filardo G. Matrix-assisted chondrocyte transplantation with bone grafting for knee osteochondritis dissecans: stable results at 12 years. *Knee Surg Sports Traumatol Arthrosc.* 2020.

41. Sessa A, Romandini I, Andriolo L, Di Martino A, Busacca M, Zaffagnini S, Filardo G. Treatment of Juvenile Knee Osteochondritis Dissecans with a Cell-Free Biomimetic Osteochondral Scaffold: Clinical and MRI Results at Mid-Term Follow-up. *Cartilage.* 2020:1947603520954500.

Table S3.1 Patient individual scores of MOCART variable.

Patient number	Degree of repair and filling of the defect	Integration to border zone	Surface of the repair tissue	Structure of the repair tissue	Signal intensity of the repair tissue 2D PD fatsat FSE	3D T2 DESS	Subchondral lamina	Subchondral bone	Adhesions	Effusion	MOCART score
PT1	15	15	10	0	5	5	5	0	5	5	65
PT2	15	15	10	0	15	15	5	0	5	5	85
PT3	20	15	10	0	15	5	5	0	5	0	75
PT4	20	10	5	0	15	15	5	0	5	5	80
PT5	20	10	5	0	5	15	5	0	5	0	65
PT6	15	15	10	0	15	15	5	0	5	5	85
PT7	20	15	5	0	5	5	5	0	5	0	60
mean	**17.9**	**13.6**	**7.9**	**0**	**10.7**	**10.7**	**5.0**	**0.0**	**2.0**	**2.9**	**73.6**
SD	**2.7**	**2.4**	**2.7**	**0**	**5.3**	**5.3**	**0.0**	**0.0**	**0.0**	**2.7**	**10.3**
ACT1	20	10	10	0	15	15	5	0	5	0	80
ACT2	10	10	10	0	15	N.A.	5	0	5	0	55
ACT3	10	15	10	5	15	15	5	0	5	0	80
ACT4	10	15	5	0	15	5	5	0	5	5	65
ACT5	10	15	10	0	15	15	5	0	5	0	75
mean	**12.0**	**13.0**	**9.0**	**1.0**	**15.0**	**12.5**	**5.0**	**0.0**	**5.0**	**1.0**	**71.0**
SD	**4.5**	**2.7**	**2.2**	**2.2**	**0**	**5.0**	**0.0**	**0.0**	**0.0**	**2.2**	**10.8**
p-value	**0.048**	**0.755**	**0.530**	**0.639**	**0.268**	**0.648**	**1.000**	**1.000**	**1.000**	**0.343**	**0.639**

Table S3.2 For each patient the overall MOCART score is presented next to the biochemical MRI measures for collagen integrity (T2 relaxation time) and GAG content (MTR asymmetry) for the four regions of interest in the defect slice.

		MOCART	T2 relaxation time [ms]						MTR asymmetry [%]					
			defect slice				control slice		defect slice				control slice	
			adjA	defect	adjP	tibiamen	femur	tibiamen	adjA	defect	adjP	tibiamen	femur	tibiamen
NL1	PT1	65	57,78	59,67	72,31	37,83	49,76	57,66	6,31	-2,54	4,36	1,83	4,71	5,29
NL2	PT2	85	111,46	144,19	121,77	84,19	43,89	40,51	-2,25	-0,95	5,40	3,86	4,26	3,80
NL3	PT3	75	87,49	103,81	85,88	49,88	50,87	61,76	-0,64	-3,27	1,35	-2,16	2,60	1,22
NL4	PT4	80	74,38	107,30	63,84	49,31	74,35	56,76	-4,29	-3,71	3,56	8,26	5,10	6,27
NL5	PT5	65	65,62	73,30	46,79	42,16	54,40	33,93	8,36	-6,58	2,35	0,83	6,75	7,21
NL6	PT6	85	70,54	93,51	75,73	35,84	44,25	29,24	7,67	-2,23	4,67	-2,98	7,04	4,78
NL7	PT7	60	67,86	141,15	79,71	60,27	65,75	34,53	7,25	-1,12	2,95	3,37	4,49	3,00
ZW1	ACT1	80	51,83	120,97	82,66	65,59	69,21	61,67	2,73	-4,40	4,98	5,44	4,36	2,91
ZW2	ACT2	55	97,41	96,01	84,18	58,78	52,83	37,08	-1,86	-1,59	-4,98	-1,13	6,63	5,28
ZW4	ACT3	80	128,05	145,03	91,75	97,88	61,74	54,08	2,71	-8,00	4,39	-0,69	6,98	4,41
ZW5	ACT4	65	72,99	77,85	46,30	44,04	76,19	40,42	-2,03	-1,42	-1,13	5,56	4,17	3,99
ZW6	ACT5	75	63,33	77,41	62,84	66,07	76,32	50,40	1,84	-2,99	3,30	3,38	4,27	2,96

adjA= adjacent region anterior to the defect; defect = defect region; adjP = adjacent region posterior to the defect; tibiamen = region in the tibia cartilage opposing the defect; and for the two regions of interest in the control slice ;femur = control region in the femur cartilage; tibiamen = control region in the tibia cartilage opposing the defect.

Impairment of the chondrogenic phase of endochondral ossification in vivo by inhibition of cyclooxygenase-2

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Abstract

Many studies have reported on the effects of cyclooxygenase-2 (COX-2) inhibition on osteogenesis. However, far less is known about the effects of COX-2 inhibition on chondrogenic differentiation. Previous studies conducted by our group show that COX-2 inhibition influences in vitro chondrogenic differentiation. Importantly, this might have consequences on endochondral ossification processes occurring in vivo, such as bone fracture healing, growth plate development and ectopic generation of cartilage. The goal of our study was to investigate, in vivo, the effect of COX-2 inhibition by celecoxib on the cartilaginous phase of three different endochondral ossification scenarios. 10 mg/kg/d celecoxib or placebo were orally administered for 25 d to skeletally-immature New Zealand White rabbits (n=6 per group). Endochondral ossification during fracture healing of a non-critical size defect in the ulna, femoral growth plate and ectopically-induced cartilaginous tissue were examined by radiography, micro-computed tomography (μ-CT), histology and gene expression analysis. Celecoxib treatment resulted in delayed bone fracture healing, alterations in growth plate development and progression of mineralisation. In addition, chondrogenic differentiation of ectopically-induced cartilaginous tissue was severely impaired by celecoxib. In conclusion, we found that celecoxib impaired the chondrogenic phase of endochondral ossification.

Introduction

Bone formation occurs following two mechanisms: intramembranous ossification and endochondral ossification (EO).[1] During intramembranous ossification, mesenchymal stem cells directly differentiate into osteocytes to form new bone without formation of cartilaginous tissue.[2,3] This process mainly takes place during cranial bone formation and healing of highly-stabilised fractures.[2] The other important mechanism is EO, which is responsible for normal long bone formation in the growth plates.[3] Growth plates are populated by highly proliferative chondrocytes, which differentiate into mineralising hypertrophic chondrocytes that either die from apoptosis or transdifferentiate into osteoblasts.[4-6] The remaining mineralised extracellular matrix (ECM) provides a scaffold for osteoblasts and osteoclasts to adhere and remodel, setting the stage for bone apposition and, thus, longitudinal bone growth and limb development.[3,7] Fracture healing through EO starts with the formation of a haematoma in which mesenchymal stem cells condense and subsequently differentiate following the chondrogenic lineage towards chondrocytes. Then, the chondrocytes become hypertrophic and direct the formation of mineralised matrix, promote angiogenesis and finally undergo apoptosis.[1] Then, in a similar way to the growth plate, the leftover mineralised matrix is invaded by osteoblasts and osteoclasts, which will remodel the matrix into woven bone. The woven bone is gradually replaced by lamellar bone and the fracture is united. During the final phase, the newly-formed bone is remodelled to its original shape.[3,8] In addition, cartilaginous tissue, ectopically generated for the purpose of cartilage repair, forms from progenitor cells by an EO process.[9,10] One of the main problems in many cartilage repair strategies is the premature hypertrophic differentiation of the generated cartilage.[11,12] Impaired endochondral ossification can lead to various problems. During EO, disturbances of the growth plate might lead to abnormal skeletal development. Most intensively investigated problems of EO during fracture healing are delayed union and non-union of fractures.[13,14] In contrast, too active EO can cause undesirable effects i.e. heterotopic ossification after joint arthroplasty or trauma[15] and intralesional ossification in cartilage repair surgery.[16,17] To date, the exact biomolecular mechanisms involved in EO are still not fully understood. We only begin to understand the spatiotemporal roles and effects of morphogens or medications on chondrogenic differentiation during EO. However, detailed knowledge on timing and (spatiotemporal) concentration of morphogens and/or medications during chondrogenic differentiation of progenitor cells is important for correct formation of cartilage and bone tissues.[18] Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used systemic inhibitors of inflammatory prostaglandin production induced by cyclooxygenases (COX-1 and COX-2).[19] NSAIDs have inhibitory effects on fracture healing, which mainly depends on the dose and duration of the treatment.[20,21] In addition, most studies that ascribed a role for COX-2 in bone fracture healing, focused on osteogenesis and left chondrogenesis unstudied.[22-26] Therefore, the potential effect of COX-2 inhibitors on the chondrogenic part of EO is incompletely understood. Chondrogenic differentiation precedes the ossification phase during EO and the limiting effect of COX-2 inhibitors on

EO may also be ascribed to impaired chondrogenic differentiation of the mesenchymal progenitor cells. Our previous study shows that specific COX-2 inhibitors decrease chondrocyte hypertrophic differentiation in the growth plate during the chondrogenic phase of EO.[27] However, it is still unknown whether this action is specific for the growth plate or whether it affects the cartilaginous phase of endochondral ossification in a broader way. To further extend our knowledge on the influence of the COX-2 inhibitor celecoxib on the cartilaginous phase of EO, we tested it in three different in vivo compartments of the same rabbit. We studied EO during fracture healing of a non-critical size defect in the ulnae, in the growth plate and in ectopically-induced cartilaginous tissue.[28,29]

Materials and methods

Experimental design and animal model

12 female, skeletally-immature (107 days old, ~1.8 kg), specific-pathogen-free (SPF) New Zealand White (NZW) rabbits were used. The experiment was approved by the Maastricht University animal ethical committee (DEC 2010-027). Sample size was calculated according to the formula of L. Sachs;[30]

n = (expected standard deviation/expected effect size)2 × 15.7

and 6 animals per group were needed. Animals were randomly assigned to the treatment or control group. Throughout the experiment, animals were housed in groups under standard conditions with ad libitum access to water and food and 12 h of light each day. Animal well-being and behaviour (score in response to stimuli, back arch, twitch, wincing, posture, self-care, condition of skin, mobility, limb loading, difficulties in respiration/breathing, dehydration or undernourishment symptoms, colour of the mucous membranes and extremities, oedema/swelling/cold feeling and other notable abnormalities) were checked daily. 10 mg/kg celecoxib (Pfizer, New York, NY, USA) in 1 mL of Critical Care® paste (Oxbow Animal Health, Murdock, NE, USA) were administered orally to the treatment group (n=6), on a daily basis from day 0. Control animals (n=6) received exclusively 1 mL of Critical Care® paste. To label tissue mineralisation, 25 mg/kg calcein green fluorochrome (Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously. Calcein green is a marker of mineralisation that, when injected, is incorporated in newly-formed bone for 24-36 h, while the unincorporated label is excreted by the kidneys within several hours.[31] In previous studies, we show that mineralisation during EO occurs about two weeks after initialisation of EO;[27,29,32] therefore, the injection was performed at day 14 of the experiment (11 days before sacrifice). After 25 d, rabbits were euthanised by an overdose of intravenous pentobarbital. During further processing, the specimens were coded and, thus, the researchers were blinded to the treatment received.

Surgery

Non-critical sized defect

At day 0, a 5 mm non-critical size defect was created in the left ulna of all rabbits.[33] Animals were anaesthetised by isoflurane inhalation. The skin was opened over the ulnae and the diaphysis approached. A 5 mm defect was created 25 mm above the carpal joint in the exposed ulnae using an oscillating saw, which was cooled by irrigation fluid. Due to the fibro-osseous union of ulna and radius proximal and distal to the surgical site, no fixation of the bone fragments was required.[34] Muscles were replaced over the defect and the incision was closed layer by layer with Vicryl Rapide™ 4-0 absorbable sutures (Ethicon, Kirkton, Scotland). The animals were allowed full weight bearing directly after surgery.

Growth plates

The growth plate of the distal femur was examined. No surgery involving the growth plates was performed, no surgery was performed on the femur and the surgery of the tibia was equal in both groups and distal to the tibial growth plate, therefore, it was expected that surgery did not influence the growth plate of the distal femur.

Periosteal endochondral ossification

During the same surgical procedure when the non-critical size defect was created, we used the method described by Emans and colleagues, for ectopically-inducing cartilage formation, in which a subperiosteal space is created to induce periosteal endochondral ossification (PEO).[28,29] PEO was induced on both tibias, as described in literature, with minor modifications.[28,29] The skin was opened over the upper medial side of the tibia, the pes anserinus was identified and the periosteum was incised just medially of the pes anserinus, leaving the semitendinosus tendon untouched. The periosteum was elevated proximally with a probe and 0.2 mL of a 2% (w/v) agarose-based gel were injected between the bone and periosteum. The 2% (w/v) agarose solution was prepared by dissolving 2 g of ultra-pure low-melting agarose granules (Cat no: 10975035, Lot No: MO91807; Invitrogen, Carlsbad, CA, USA) in 100 mL of 0.9% NaCl, followed by steam-sterilization. The preparation was warmed to 40 °C in a water bath to liquefy it prior to use. Next, gelation was accelerated by cooling the PEO location with 5°C sterile 0.9% NaCl. Finally, the wound was closed in separate layers with Vicryl Rapide™ 4-0 absorbable sutures (Ethicon, Kirkton, Scotland). This procedure was repeated on the contra-lateral tibia.[28,29]

Prostaglandin E2 levels

To confirm COX-2 inhibition by celecoxib, we determined prostaglandin E2 (PGE2) levels in blood plasma at day 0 and at sacrifice by standardised enzyme immunoassay (EIA) analysis (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, blood was drawn at day 0

and just before sacrifice and centrifuged at 370 ×g for 5 min. The blood plasma was used to perform an EIA for detection of PGE2 levels.

Radiography

Directly after sacrifice, plain radiographs of the ulnae were obtained with a mammography unit (Philips BV25; Philips, Eindhoven, the Netherlands) to determine the radiologic stage of fracture healing. Bone healing was scored according to the Lane and Sandhu radiological scoring system.[35] To determine formation and mineralization of PEO tissue, also plain radiographs of the tibia were obtained, taking care of positioning the tibia in such way that the site of PEO was visible on the radiograph.

µ-CT

After dissection of the leg, high-resolution images of the fracture region of all 12 affected ulnas (6 per group) were taken using a micro-computed tomography (µCT) scanner (µ-CT80; Scanco Medical, Bruettisellen, Switzerland) at 55 kV. In the fracture region, the scan length was approximately 21 mm and the resolution was set to 36 µm. Based on these images, micro-finite element analyses (FEA) were performed to quantify fracture consolidation, by using an approach similar to that described by Shefelbine et al.[36] First, the resolution was reduced to 108 µm and a section of 14 mm in length, centred on the defect, was selected. Second, a two-level thresholding approach was used to identify three different tissue types based on the Hounsfield unit (HU): cartilaginous tissue (HU: 1000-1999), low-mineralised bone (HU: 2000-2999) and high-mineralised bone (HU ≥3000). Material properties were assigned depending on tissue type, with a Young's modulus of 1 MPa for cartilaginous tissue, 5 GPa for low-mineralised bone and 20 GPa for high-mineralised bone. The Poisson's ratio was set to 0.3 for all tissues. Boundary conditions were applied to represent an axial compression test, an axial torsion test and bending tests in two orthogonal directions. Then, for each test the stiffness of the scanned region was determined (units: N/mm for the compression tests and Nmm/rad for the torsion/bending tests).[37,38]

Histology and image analysis

Non-critical size defect

The left ulnae were isolated and fixed in 4% formalin. After the tissue was fixed, the ulnae were gradually embedded in poly(methyl methacrylate) (PMMA) (Technovit 9100; Heraeus Kulzer, Hanau, Germany). After complete polymerization, 50 µm-sagittal sections were cut using a saw microtome (SP1600; Leica, Wetzlar, Germany). Prior to the cutting of each section, a vonKossa/thionine or Masson-Goldner trichrome (Carl Roth GmbH, Karlsruhe, Germany) staining was performed to visualize different tissue types.[39] Sections were stained for 10 min with 1% silver nitrate (AgNO3 ;VWR Prolabo, Amsterdam, the Netherlands) and for 30 s with 5% sodium thiosulphate (Na_2 S_2 O_3;

VWR Prolabo), rinsed with tap water for 5 min and finally stained for 10 min with a 0.25% thionine solution. The Masson-Goldner trichrome staining was performed according to the manufacturers' protocol. Sections were scored using a modified version of Heiple histologic fracture scoring system.[40]

Femoral growth plates

The left distal femora were isolated and fixed in 4% formalin (VWR Prolabo). Next, the femora were gradually embedded in PMMA (Technovit 9100; Heraeus Kulzer). After complete polymerization, 50 µm-thick sections were cut in the sagittal plane between the condyles, in the anatomical middle of the femur, using a saw microtome (SP1600; Leica). Femora were positioned perpendicular to the microtome saw to obtain reproducible sections and to prevent false measurements due to skewness. Prior to sectioning, haematoxylin/eosin (H&E; Dako, Troy, MI, USA) or no staining was applied by adding acid alcohol for 10 min, 0.6% haematoxylin for 10 min, rinsing for 10 min with tap water and adding 0.2% eosin for 5 min. Then, the H&E-stained sections were further processed for microscopical analysis, using a Zeiss Axioscope A.1 microscope (Zeiss, Oberkochen, Germany), with AxioVision 4.8 software. To prevent skew measurements and improve reproducibility, growth plate sections were placed in a way that the growth plate was aligned horizontally. Then, image frames were standardised to a width of 5.0 mm (2.5 mm left and 2.5 mm right of the anatomical middle of the section). With a custom-written script in MatLab software (MathWorks, Natick, MA, USA), the average height of the proliferative and hypertrophic zone of each growth plate was determined. The unstained sections were processed using a Leica microscope (DM RD; Leica), taking three images for each section with normal light and with filtered light, to assess the calcein green injected. Images were obtained and processed with Leica IM50 software. The three separate colour channels of the RGB images were combined with Adobe Photoshop CS3 software to create one image. Measurements on all sections were taken with AxioVision 4.8 software and a custom-written script in MatLab (the software was calibrated before measurements). The surface area between the mineralization front at day 14 (calcein green front) and at day 25 (status at sacrifice) was measured in a box with a standardised width of 8.0 mm. By dividing the surface area (in μm^2) by the width of 8.0 mm, the total growth after incorporation was calculated for both groups.

Periosteal endochondral ossification

Tibiae were isolated, fixed in 4 % formalin and decalcified for 3 weeks in 0.5 M ethylene diaminetetraacetic acid (EDTA, VWR Prolabo) pH 7.8. An additional brief (20 h) decalcification step was performed using 1:5 diluted Shandon™ TBD-1™ Decalcifier (Thermo Fisher Scientific, Waltham, MA, USA). Next, the tibiae showing PEO were dehydrated with increasing concentration of ethanol and embedded in paraffin wax. Starting from the centre, where PEO occurred, 5 µm-thick sections were cut. Tissue sections were stained with safranin O/fast green (both from Sigma-Aldrich). Slides were

deparaffinised and rehydrated using standard protocols. Proteoglycans were stained with 0.1% safranin O and counterstained with 0.1% fast green. Stained sections were dehydrated and mounted with Histomount (Thermo Fisher Scientific) for subsequent microscopic analysis using a Zeiss Axioscope A.1 (with AxioVision 4.8 software).

Gene expression analysis

Cartilage tissue, ectopically-formed on the tibia or fibrous periosteal tissue (on the tibia where none or little ectopic cartilage was formed), was harvested. Tissue samples where lysed in TRIzol (Life Technologies|Thermo Fisher Scientific, Carlsbad, CA, USA). RNA isolation, RNA quantification by ultraviolet (UV) spectrometry (Biodrop; Isogen Life Sciences, Utrecht, the Netherlands) and cDNA synthesis were performed as described before.[27,41] Real time quantitative PCR (RT-qPCR) was performed using MESAGREEN qPCR MasterMix Plus for SYBR® Assay (Eurogentec, Seraing, Belgium). A CFX96 RealTime PCR Detection system (Biorad, Hercules, CA, USA) was used for amplification with the following protocol: initial denaturation 95°C for 10 min, followed by 40 cycles of amplification (denaturation 15 s at 95°C and annealing 1 min at 60°C). Validated primer sequences used are listed in Table 4.1. Data were analysed using the 2-ΔΔCt method, mRNA expression was normalised to reference genes (28S rRNA, β-actin and GAPDH) and gene expression was calculated as fold change compared to control.

Table 4.1 Primer sequences for RT-qPCR.

Oligo sets	Forward	Reverse
Acan	CGGGACACCAACGAGACCTAT	CTGGCGACGTTGCGTAAAA
Alpl	GGAGGATGTGGCCGTCTTC	CTGCGTAAGCCATCACATGAG
Col1a1	CTGACTGGAAGAGCGGAGAGTAC	CCATGTCGCAGAAGACCTTGA
Col2a1	TGGGTGTTCTATTTATTTATTGTCTTCCT	GCGTTGGACTCACACCAGTTAGT
Col10a1	AACCTGGACAACAGGGACTTACA	CCATATCCTGTTTCCCCTTTCTG
Mmp13	CGATGAAGACCCCAACCCTAA	ACTGGTAATGGCATCAAGGGATA
PTHrP	AAGGGCAAGTCCATCCAAGA	CTCGGCGGTGTGTGGATTTC
Sox9	AGTACCCGCACCTGCACAAC	CGCTTCTCGCTCTCGTTCAG
Runx2	TGATGACACTGCCACCTCTGA	GCACCTGCCTGGCTCTTCT
Vegfa	GTCAGAGAGCAACATCACCA	CATCTGCTGTGCTGTAGGAA
28S rRNA	GCCATGGTAATCCTGCTCAGTAC	GCTCCTCAGCCAAGCACATAC
β-Actin	GACAGGATGCAGAAGGAGATTACTG	CCACCGATCCACACAGAGTACTT
GAPDH	ACTTTGTGAAGCTCATTTCCTGGTA	GTGGTTTGAGGGCTCTTACTCCTT

The 5' to 3' forward and reverse oligonucleotide sequences used for RT-qPCR are listed in the table.

Statistics

Statistical analysis was performed using IBM SPSS 20.0 software (Chicago, IL, USA). Because of our group size, Mann Whitney U test was applied for all measurements. Results with $p<0.05$ were regarded as significant. Data were presented as mean with standard error of the mean (SEM).

Results

Animal well-being and confirmation of COX-2 inhibition by reduced PGE2 levels

To determine the consequences of COX-2 inhibition on endochondral ossification, the skeletally-immature New Zealand white rabbits were systemically treated with celecoxib for 25 d (6 animals in control group and 6 animals in celecoxib group). Analysing blood plasma samples, we confirmed that celecoxib treatment efficiently inhibited in vivo PGE_2 synthesis by 80% after 25 d and, thus, systemically inhibited COX-2 (Figure 4.1A). Throughout the experiment, animal well-being and behaviour were observed daily. We did not observe any difference between the control and celecoxib-treated group. In addition, no significant differences were observed in the body weights during the entire experiment (Figure 4.1B).

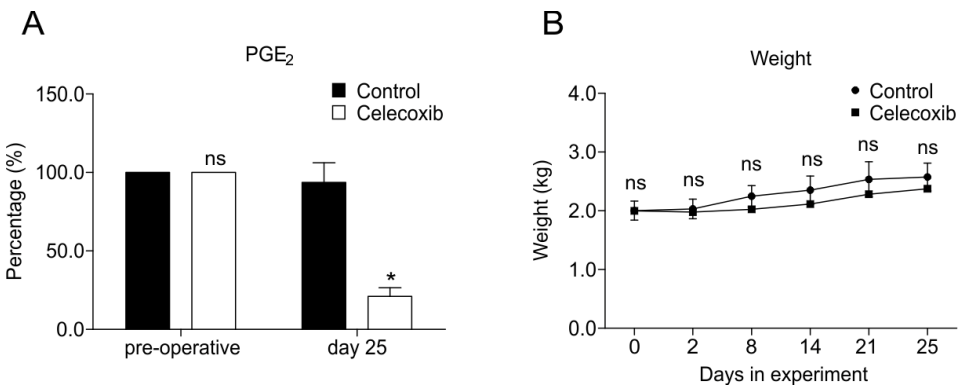


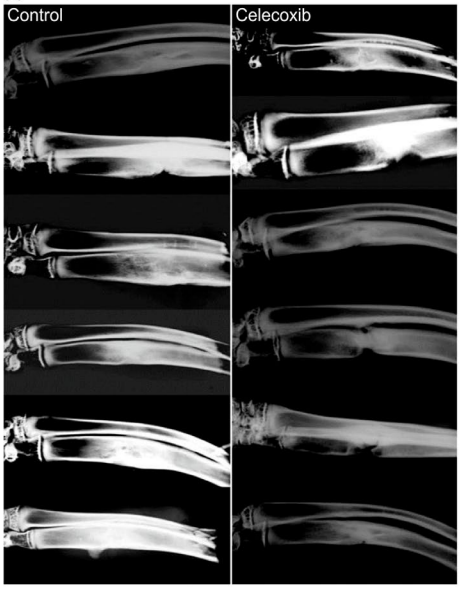
Figure 4.1 Weight and Systemic COX-2 inhibition during the experiment. **(A)** Systemic COX-2 inhibition was confirmed by measuring PGE_2 levels in serum from pre-operative samples and in samples at day 25, just before sacrifice. PGE_2 levels were measured using a PGE_2-specific EIA. Inhibition of PGE_2 synthesis was determined as % decrease as compared to pre-treatment serum samples. **(B)** The weight of the rabbits during the experiment in the control (n=6) and celecoxib group (n=6). Error bars indicate SEM, asterisk (*) p<0.05, ns = not significant.

Ulnar fracture healing and COX-2 inhibition

To determine whether this systemic COX-2 inhibition influenced bone fracture healing, we employed a noncritical size defect model, which has been described before,[42] to study fracture healing capacity. To evaluate radiographical bone fracture healing in control and celecoxib-treated rabbits, plain radiographs were taken after 25 d of follow-up. Overall, these radiographs showed full bony bridging in all but one ulna in the control group. In the celecoxib-treated group only half of the ulnae showed full bony bridging, whereas the other half of the ulnae showed only partial bony bridging (Figure 4.2A). Analysis by the Lane-Sandhu radiologic fracture scoring system, which

measures bone formation, remodelling and union,[43] revealed a significant difference in the average scores: 8.8 (SEM ± 0.8) in the control group vs. 5.7 (SEM ± 0.9) in the celecoxib-treated group (Figure 4.2B). This indicated that fracture healing was indeed impaired in the celecoxib group. To gain more insight in the newly-formed bone structure, bone fracture healing in the ulnae was analysed and evaluated by µ-CT. Reconstructed 3D images confirmed the progression in fracture healing for both groups, as observed in the radiographs (Figure 4.2C). µ-CT data were analysed in greater detail by using a finite element model to predict stiffness of the newly-formed bone (Figure 4.2D). In both groups, one outlier was excluded from analysis due to a refracture of the sample, making the FEA unreliable. A significantly higher average compression stiffness was calculated for the control group as compared to the celecoxib-treated group [21082 N/mm (SEM ± 1172 N/mm) vs. 14707 N/ mm (SEM ± 3101 N/mm), respectively; (p = 0.048)]. However, no significant differences were found in torsional stiffness [4416 Nmm/rad vs. 3477 Nmm/rad (p=0.12)] and bending stiffness around the X-axis [3839 N/mm vs. 2564 N/mm (p=0.09)] or bending stiffness around the Y-axis [2288 N/mm vs. 1949 N/ mm (p=0.13)] (Figure 4.2D). These data supported the observation of impaired fracture healing in the celecoxib-treated rabbits. After 25 d of follow-up, von Kossa/thionine and Masson-Goldner trichrome stained histological samples showed, similar to radiographs and µ-CT analyses, a delayed fracture healing in the celecoxibtreated rabbits (Figure 4.3A-C). The control group showed bony bridging in all sections and reorganisation in most of the sections, only little callus tissue was still present and the cortices reorganised (Figure 4.3A). In the celecoxib-treated group, full bony bridging was only observed in half of the sections. In the other half, more callus tissue was still retained in the fracture region and less remodelling towards woven bone occurred (Figure 4.3A). Furthermore, new bone formation and areas with retained cartilaginous tissue were still present in the fracture area of the celecoxib group whereas advanced reorganization towards woven bone was visible in the control group (Figure 4.3B). In MasonGoldner trichrome stained sections, the celecoxib group showed more connective tissue in the fracture callus, whereas this was largely absent in the control group (Figure 4.3C). When fracture healing was quantified using an existing histological scoring system,[40,44] a significantly decreased score in the celecoxib-treated group was observed. The scoring system takes in account union, formation and remodelling of cartilaginous spongiosa, formation and remodelling of spongious bone, formation, remodelling and continuity of cortical bone (each side scored separately) and bone marrow formation. Each item can be scored from 0 to 4, a total score of 24 means a fully-healed fracture and 0 means no healing occurred at all. A mean score of only 12.00 (SEM ± 1.9) out of 24 was achieved in the celecoxib-treated group, whereas the control group achieved a mean score of 17.67 (SEM ± 1.3) out of 24. An overview of these results is shown in Figure 4.3D. Overall, these data showed that systemic celecoxib treatment for 25 d in a non-critical size bone defect in skeletally-immature rabbits resulted in delayed endochondral bone fracture healing, as determined on plain radiographs, µ-CT images and histology.

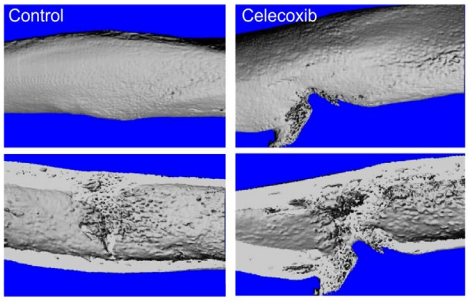
A



B

Lane-Sandhu	Control (*n*=6)	Celecoxib (*n*=6)	*p*-value
Radiographic score	8.8 (± 0.8)	5.7 (± 0.9)	0.03*

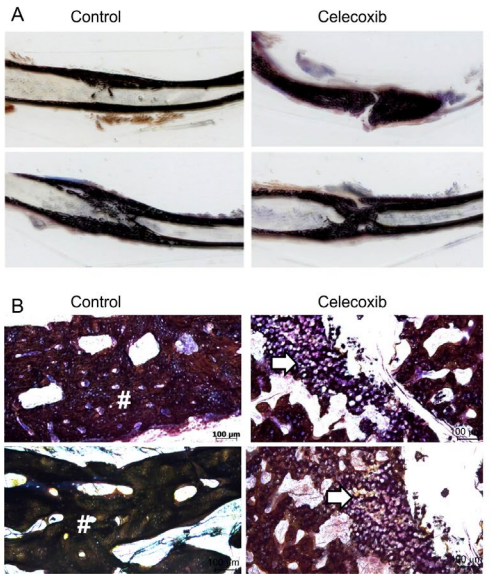
C



D

FEA analysis	Control (*n*=5)	Celecoxib (*n*=5)	*p*-value
Compression stiffness (N/mm)	21082 (±1172)	14707 (±3101)	0.048*
Torsional stiffness (Nmm/rad)	4416 (±937)	3477 (±577)	0.12
Bending stiffness X-axis (N/mm)	3839 (±580)	2564 (±479)	0.09
Bending stiffness Y-axis (N/mm)	2288 (±396)	1949 (±364)	0.13

Figure 4.2 Impaired fracture healing in celecoxib treated rabbits.Impaired fracture healing after celecoxib treatment in an experimental non-critical size defect model. A 5 mm defect was created in the distal ulna, after a follow-up of 25 days fracture healing was examined radiologically. (**A**) Full union in radiography of the ulna of the control group and only partial/delayed union in radiography of the ulna of the celecoxib treated group. (**B**) In radiographic images bone union was assessed using the Lane-Sandhu scoring system. Significantly more union was seen in the control group as compared to the celecoxib treated group. (**C**) 3D reconstruction of micro-CT image of the control group ulna and the celecoxib treated ulna. Note the cortical bridging in the control group and a gap still present in the celecoxib treated group. (**D**) Bone stiffness was assessed by finite element analysis. Either a significant difference (or trend towards significance) was seen between various parameters in control versus celecoxib treated group. Standard error of the mean is depicted between brackets. The * indicate significant p-values.



D

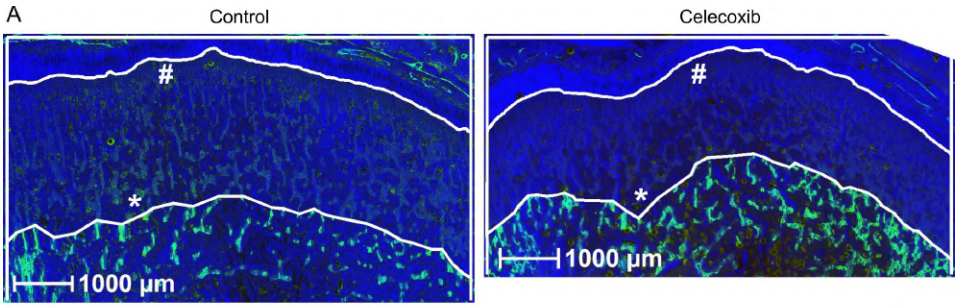
Heiple score	Control (*n*=6)	Celecoxib (*n*=6)	*p*-value
Union	3.33 (±0.21)	2.33 (±0.42)	0.132
Cartilaginous spongiosa	3.00 (±0.26)	2.17 (±0.40)	0.180
Spongious bone	2.33 (±0.21)	1.50 (±0.22)	0.065
Cortex bridging_1	3.00 (±0.00)	2.33 (±0.33)	0.180
Cortex bridging_2	3.00 (±0.45)	2.00 (±0.45)	0.180
Bone marrow	3.00 (±0.37)	1.67 (±0.33)	0.041*
Total	**17.67 (±1.28)**	**12.00 (±1.53)**	**0.041***

Figure 4.3 Impaired histological fracture healing in celecoxib treated rabbits. Delayed fracture healing as seen in histologic sections of the same specimens. **(A)** An overview of VonKossa/Thionine and Masson Goldner trichrome stained sections show bony bridging of the cortex in the control group but in the celecoxib treated group a clear gap is still visible in the region where the defect was created. **(B)** A magnification of the overview pictures (scale bars represent 100 µm) shows advanced reorganization towards woven bone (#) in the control group whereas more cartilaginous tissue (white arrow) is still present and only little reorganization has occurred in the celecoxib treated rabbits. **(C)** Masson Goldner trichrome staining. **(D)** Fracture healing was significantly impaired in the celecoxib treated rabbits when histologically analysed by a modified version of Heiple's histologic scoring system. Standard error of the mean is depicted between brackets. The * indicate significant *p*-values.

Growth plate development and COX-2 inhibition

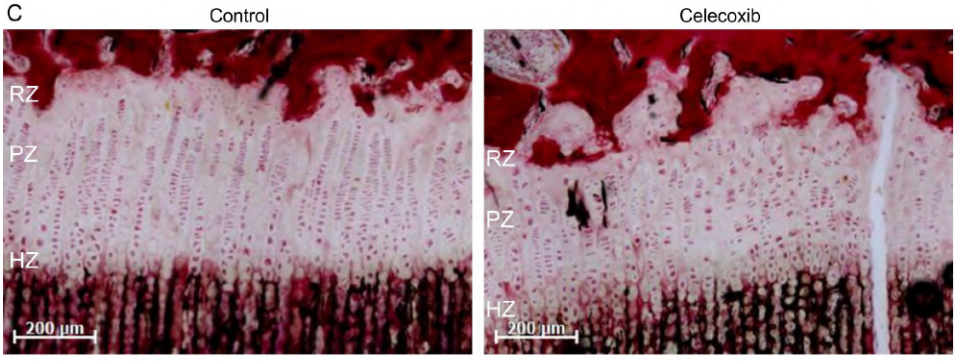
Systemic COX-2 inhibition delayed the bone fracture healing process in our non-critical size defect model, possibly by interfering with the endochondral ossification process. To determine whether COX-2 inhibition caused similar consequences in another scenario of endochondral ossification, we analysed growth plate development in the same rabbits. This had the additional benefit of analysing the effect of COX-2 inhibition on the EO process alone, without clouding of simultaneously occurring intramembranous ossification at the same site, as can be the case during fracture healing. In addition, the progenitor cell source for EO in the growth plate is different from in fracture healing: resting zone chondrocytes versus periosteal- and bone marrow derived mesenchymal cells, respectively. To follow new bone formation originating from endochondral ossification in the growth plate, fluorescent calcium labelling was applied by calcein green injection at day 14 (11 d prior to sacrifice) and analysed in histological sections of the growth plate. Calcium labelling by

fluorochromes, as calcein green, allows to determine the location of active mineralisation at a given time point (time of injection).[31] The injection of calcein green at day 14 caused a clearly visible green fluorescent front at the diaphyseal side of the growth plate of the rabbits' distal femora (mineralisation front) (Figure 4.4A). In the same sections, the transition between proliferative zone and hypertrophic zone of the growth plate could also be observed. The distance between the fluorescent mineralisation front and the proliferative/hypertrophic transition zone was indicative of the growth that occurred between incorporation of calcein green and sacrifice. The growth for the control group was 2436 µm (± 142 µm) and for the celecoxib-treated group 1830 µm (± 54 µm), which was a significantly shorter distance (Figure 4.4B). This showed that celecoxib treatment also inhibited endochondral ossification during the growth plate development. In sequential sections stained with H&E (Figure 4.4C), we further focused on the growth plates of these long bones, to determine whether the inhibition of advancement of the epiphyseal mineralisation front originated from the growth plate (and, thus, the chondrogenic phase of endochondral ossification). The total thickness of the growth plates in the control rabbits was 375.1 µm (± 14.2 µm), whereas the total thickness of the growth plates in the celecoxib-treated rabbits was only 307.9 µm (± 10.34 µm) ($p<0.05$). Also, and in concert with our previous work,[27] the thickness of the hypertrophic zone was significantly decreased in the celecoxib-treated group (Figure 4.4D). Collectively, these results showed that growth plate development was inhibited and, since growth plate development was driven by chondrogenic differentiation, it was likely that at least a part of the reduced growth plate development caused by celecoxib, could be explained by an impaired chondrogenic differentiation.



B

	Control (*n*=6)	Celecoxib (*n*=6)	*p*-value
Advancement of mineralization front	2436 (±142) µm	1830 (±54) µm	0.0043*



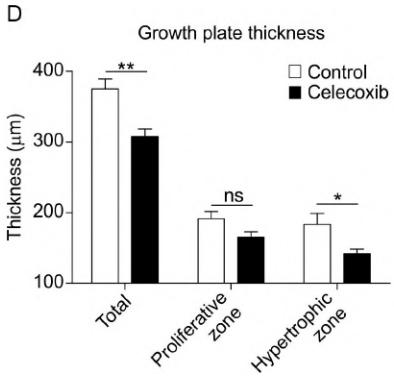
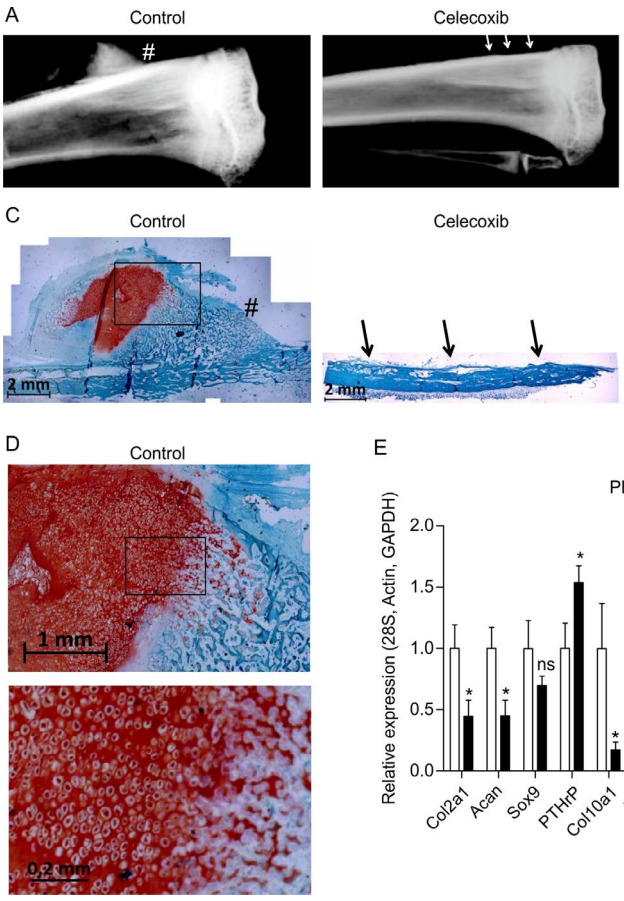


Figure 4.4 Celecoxib inhibits advancement of the mineralization front. The distal femoral growth plate was examined histologically. (**A**) The calcein green mineralization front (*) at day 14 was visualised and compared to the transition between proliferative zone and hypertrophic zone of the growth plate (#) at day 25. The surface of the growth plates was measured over a standard width of 8000µm and the average growth was calculated. The scale bars represent 1000µm. (**B**) Significantly more advancement of the mineralization front was seen in the control group compared to the advancement in the celecoxib treated group. (**C**) In adjacent sections stained with haematoxylin/eosin average thickness was calculated for the total

growth plate, as well as the proliferative zone (PZ) and the hypertrophic zone (HZ). In addition, normal columnar chondrocytes can be seen in the control group growth plate and a less organised structure of the chondrocytes and a less marked transition between resting zone area (RZ) and proliferative zone is seen in the celecoxib treated rabbits. (**D**) A significant decrease in thickness of the total growth plate and in the hypertrophic zone was observed, but no significant difference was present in the proliferative zone. Standard error of the mean is depicted between brackets. The * indicate significant *p*-values ($p<0.05$), ** $p<0.01$, ns = not significant.

PEO and COX-2 inhibition

PEO can be used as a model for studying in vivo endochondral bone formation because, similarly to the growth plate, it sequentially completes the stages of chondrogenic differentiation during endochondral ossification.[29] Different from the growth plate, PEO formation allowed for the specific analysis of the effect of COX-2 inhibition on the newly-formed endochondral ossification processes (as celecoxib treatment started almost simultaneously with induction of PEO) and, thus, study early phases of chondrogenic differentiation during EO. To study whether celecoxib not only had an influence on osteogenic differentiation, but also on chondrogenic differentiation, we implemented this method for ectopically inducing endochondral ossification in the same rabbits used for the noncritical size defect procedure. We analysed whether celecoxib treatment was able to alter PEO formation, as a model for chondrogenic differentiation, through radiography, histology and gene expression analysis. Radiography and histology showed a distinctive 67% PEO formation (4 out of 6 injection sites) in the control group and a 0% distinctive PEO formation (0 of 6 injections sites) in the celecoxib-treated group (Figure 4.5A,B). Safranin O staining, to detect proteoglycans, indicative of cartilage formation (Figure 4.5C,D), showed that rabbits in the celecoxib-treated group did not detectably develop cartilage tissue in the PEO tissue. In Figure 4.5C the tibial cortex and fibrous periosteal tissue could be seen in the celecoxib group, but no PEO tissue was developed. In the control group, cartilaginous tissue was formed, which, at the time of harvest, was gradually ossifying. This could be seen in more detail in Figure 4.5D. Gene expression analysis of the PEO tissue or fibrous periosteal tissue at the site of agarose injection showed that celecoxib significantly impaired the expression of collagen type II (Col2a1), aggrecan (Acan), collagen type X (Col10a1), alkaline phosphatase (Alpl), Runt-related transcription factor 2 (Runx2) and matrix metalloproteinase 13 (Mmp13). Expression of parathyroid hormone-related peptide (PTHrP) was upregulated. No significant differences were found in the expression of SRY box 9 (Sox9), vascular endothelial growth factor alpha (Vegfa) or collagen type I (Col1a1) (Figure 4.5E). Overall, these data indicated that systemic inhibition of COX-2 by celecoxib impaired the formation of PEO tissues, suggesting that, in this model, systemic COX-2 inhibition had a negative effect on the initiation of the chondrogenic phase of endochondral ossification.



Radiography	Control (*n*=6)	Celecoxib (*n*=6)
Percentage PEO formation	67%	0%

Figure 4.5 Impaired PEO formation in celecoxib treated rabbits. A periosteal osteochondral model was used to examine the influence of celecoxib on chondrogenesis. An agarose gel was injected between periosteum and bone of the upper tibia to induce PEO. (**A**) Plain radiographs show (the mineralised part of) PEO (#) in control and celecoxib treated rabbits (white arrows point to the location of injection). (**B**) In 67% of the injected control rabbits and in none of the celecoxib treated rabbits PEO formation was seen. (**C**) Safranin O/Fast green stained histologic section of the same specimen illustrating cartilage formation and mineralization (#) in control group and in the celecoxib treated rabbits again no clear PEO is formed (black arrows point to the location of injection). This shows that not only mineralization is inhibited but the lack of cartilaginous PEO formation indicates an inhibited chondrogenic differentiation. (**D**) A higher magnification of panel C can be seen here where the transition of cartilage into the ossified part is visible in more detail. (**E**) Gene expression analysis of indicated genes was performed by RT-qPCR on PEO tissue in control and celecoxib treated rabbits. Data are presented as relative expression compared to control condition and normalised to a housekeeping index consisting of 28S rRNA, Actin and GAPDH Bars represent mean ± SEM. asterisk (*) $p<0.05$, ns = not significant.

Discussion

We showed that the inhibition of COX-2 by celecoxib influenced the chondrogenic phase of endochondral ossification in vivo, affecting not only fracture healing, but also growth plate development and ectopic periosteal cartilage formation. Fracture healing by EO in a non-critical size defect in the ulna was found to be delayed on radiographs, μ-CT images and histology in the celecoxib-treated animals, which is consistent with previous reports, where COX-2 is selectively inhibited.[24,25,45-47] Remarkably, the non-fused fractures in the celecoxib treated animals were characterised by the presence of retained cartilaginous tissue, which was likely indicative of delayed EO. Impaired bone healing was confirmed by FEA, in which a significant difference in compression strength was observed. Compression strength was the only parameter that differed significantly between control and celecoxib-treated groups. Compression strength largely depends on cortical integrity,[48] and since the celecoxib-treated animals specifically presented lower FEA compression strength, it was likely that the mode of impaired fracture healing due to celecoxib treatment involved impaired cortical healing as a result of delayed endochondral ossification. Indeed, this was confirmed by our histological examinations. Using a calcium-binding fluorochrome, we were able to demonstrate that the mineralisation front in the growth plate advanced less over time in the celecoxib treated animals compared to control. This implicated an impaired advancement of endochondral-driven bone growth and confirmed our previous findings.[27] In the celecoxib-treated rabbits, the growth plates were significantly shorter, which can be mainly attributed to a shorter hypertrophic zone. This shorter hypertrophic zone can be caused by an impaired progression into hypertrophy.[27] Our data strongly indicated that – apart from the possibility that osteogenic remodelling in already developed bones might be affected by celecoxib treatment – the chondrogenic phase of endochondral ossification was sensitive to the celecoxib treatment. In addition to EO in fracture healing and growth plate development, the same animals were used to examine the chondrogenic phase of endochondral ossification, according to a previously described model for in vivo ectopic cartilage formation.[29] We found that no distinctive ectopic cartilage tissue was formed in the celecoxib-treated group. Ectopic periosteal cartilage formation and the formation of cartilaginous fracture callus are de novo initiated cellular processes, requiring an initial inflammatory environment.[49-51] With the systemic celecoxib-dependent reduction of COX-2 activity, an essential inflammatory reaction may be dampened, resulting in an impaired initiation of chondrogenic differentiation or inflammation driven osteogenic remodelling of fracture callus. A challenge is to define at which stage of EO COX-2 is involved and to clarify why EO seemed to be delayed in fracture healing and growth plate development, while being inhibited in the PEO model. EO can be divided into 4 stages: 1) initiation of chondrogenic differentiation of chondroprogenitor cells, 2) chondrocyte proliferation, 3) hypertrophic differentiation, 4) vascularisation and apoptosis.[10,49] Previous work from our group shows a bi-phasic COX-2 expression pattern during chondrogenic differentiation in vitro.[27,52] The two stages, corresponding to COX-2 expression peaks,

resemble early differentiation and chondrocyte hypertrophy. This suggests that inhibition of COX-2 could have an influence on both early chondrogenic differentiation, as well as, late chondrogenic differentiation. Therefore, inhibition of COX-2 could influence both the initiation of chondrogenic differentiation and chondrocyte hypertrophy. According to the present study, growth plate development was an already ongoing process and fracture healing and ectopic periosteal chondrogenic differentiation were initiated simultaneously to COX-2 inhibition. During fracture healing and ectopic periosteal chondrogenic differentiation, the first peak of the bi-phasic COX2 expression was likely inhibited, whereas, in growth plate development, COX-2 inhibition might interfere with the already ongoing chondrogenic differentiation. Therefore, we speculated that it was the inhibition of this biphasic COX-2 expression that was likely to cause differential effects on fracture healing, growth plate development and ectopic periosteal chondrogenic differentiation. The skeletal mechanism of action of arachidonic acid-derived eicosanoids remains a topic of investigation.[23] For instance, the prostanoid receptors of PGE2 (EP-1, EP4) have different roles in chondrocyte and osteocyte differentiation and, therefore, in fracture healing. EP-1-/- mice have enhanced osteoblast differentiation and accelerated fracture repair,[26] whereas EP-2 and EP-4 agonists improve fracture healing.[53] However, in COX-2 -/- mice, fracture healing is severely impaired, but these mice have no reported skeletal abnormalities.[24] The latter is inconsistent with our findings, as we observed an effect of celecoxib on the maturation of the growth plate, implicating that COX2 played a role during endochondral ossification. Finally, in a rat model, reduction of leukotriene synthesis by inhibition of 5-lipoxygenase accelerates fracture healing by increasing COX-2 expression in fracture callus and progression into hypertrophy to complete endochondral ossification.[54,55] COX-2 is a rate-limiting enzyme in the turnover of arachidonic acid to prostaglandins.[56] The decreased levels of PGE2 in the rabbit blood plasma at day 25 confirmed that we were able to inhibit COX-2 activity systemically in vivo. In our experiment, we have chosen to use a celecoxib dose equivalent to 800 mg/d in humans. This is a high dose to be used in orthopaedic conditions and rheumatic diseases, but non-toxic and described as safe for use in a gastrointestinal toxicity study.[57] It is unknown whether the effects of celecoxib, which we reported, would have also been observed if lower concentrations would have been used or with other NSAIDs. Furthermore, local instead of systemic administration of celecoxib might differently influence the course of chondrogenic differentiation, but this will be topic of further studies. Our study design had a limitation, but at the same time an opportunity. Using a single animal for multiple experimental models could be a confounding factor, as we had no control on factors that might influence each other. This could be addressed by using separate animals for each study design. However, considering inter-animal variation when using different animals for different models, we would never have been able to make the comparison as accurate as we did, using this combined model. Moreover, the potential influence of one factor on the other would be expected to be similar in each rabbit. The only difference between the two groups was the administration of celecoxib, which was the variable we aimed to test.

Conclusions

We confirmed previous findings according to which selective COX-2 inhibition (by celecoxib) causes impaired fracture healing,[24] and more importantly, we showed that chondrogenic differentiation during EO was impaired by COX-2 inhibition. This impaired chondrogenic differentiation had an impact on the development of the fracture callus, growth plate development and ectopic cartilage formation. The impaired fracture healing was probably at least partially a result of the effects of celecoxib on the chondrogenic phase of EO, similarly to results found for growth plate development and, therefore, not solely a consequence of impaired osteogenesis. This chondrogenic involvement may have other, yet unknown, implications for the use of COX-2 inhibitors in pregnant women, children and patients suffering from a fracture and necessitates further clinical investigation.

References

1. Marsell R, Einhorn TA. The biology of fracture healing. *Injury*. 2011;42(6):551-555.
2. Thompson Z, Miclau T, Hu D, Helms JA. A model for intramembranous ossification during fracture healing. *J Orthop Res.* 2002;20(5):1091-1098.
3. Kronenberg HM. Developmental regulation of the growth plate. *Nature*. 2003;423(6937):332-336.
4. Park J, Gebhardt M, Golovchenko S, Perez-Branguli F, Hattori T, Hartmann C, Zhou X, deCrombrugghe B, Stock M, Schneider H, von der Mark K. Dual pathways to endochondral osteoblasts: a novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage. *Biol Open.* 2015;4(5): 608-621.
5. Yang G, Zhu L, Hou N, Lan Y, Wu XM, Zhou B, Teng Y, Yang X. Osteogenic fate of hypertrophic chondrocytes. *Cell Res.* 2014;24(10):1266-1269.
6. Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. *PLoS Genet.* 2014;10(12):e1004820.
7. 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.
8. Hadjiargyrou M, O'Keefe RJ. The convergence of fracture repair and stem cells: interplay of genes, aging, environmental factors and disease. *J Bone Miner Res.* 2014;29(11):2307-2322.
9. Caldwell KL, Wang J. Cell-based articular cartilage repair: the link between development and regeneration. *Osteoarthritis Cartilage.* 2014.
10. Caron MM, Welting TJ, van Rhijn LW, Emans PJ. Targetting Inflammatory Processes for Optimization of Cartilage Homeostasis and Tissue Repair Techniques. In: Peterson L, Emans PJ, eds. *ICRS: Developing Insights in Cartilage Repair.* London: Springer-Verlag 2014:43-63.
11. Dickhut A, Pelttari K, Janicki P, Wagner W, Eckstein V, Egermann M, Richter W. Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. *J Cell Physiol.* 2009;219(1):219-226.
12. van Osch GJ, Brittberg M, Dennis JE, Bastiaansen-Jenniskens YM, Erben RG, Konttinen YT, Luyten FP. Cartilage repair: past and future--lessons for regenerative medicine. *Journal of cellular and molecular medicine.* 2009;13(5):792-810.
13. Calori GM, Albisetti W, Agus A, Iori S, Tagliabue L. Risk factors contributing to fracture non-unions. *Injury.* 2007;38 Suppl 2:S11-18.
14. Santolini E, West R, Giannoudis PV. Risk factors for long bone fracture non-union: a stratification approach based on the level of the existing scientific evidence. *Injury.* 2015;46 Suppl 8:S8-S19.
15. Spinarelli A, Patella V, Petrera M, Abate A, Pesce V, Patella S. Heterotopic ossification after total hip arthroplasty: our experience. *Musculoskelet Surg.* 2011;95(1):1-5.
16. Brown WE, Potter HG, Marx RG, Wickiewicz TL, Warren RF. Magnetic resonance imaging appearance of cartilage repair in the knee. *Clinical orthopaedics and related research.* 2004(422):214-223.
17. Bouwmeester SJ, Beckers JM, Kuijer R, van der Linden AJ, Bulstra SK. Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. *Int Orthop.* 1997;21(5):313-317.
18. Emans PJ, Caron MM, van Rhijn LW, Welting TJ. Endochondral Bone Formation as Blueprint for Regenerative Medicine. In: Davies PJ, ed. *Tissue Regeneration - From Basic Biology to Clinical Application.* InTech; 2012:399-424.
19. Brooks P, Emery P, Evans JF, Fenner H, Hawkey CJ, Patrono C, Smolen J, Breedveld F, Day R, Dougados M, Ehrich EW, Gijon-Banos J, Kvien TK, Van Rijswijk MH, Warner T, Zeidler H. Interpreting the clinical significance of the differential inhibition of cyclooxygenase-1 and cyclooxygenase-2. *Rheumatology (Oxford).* 1999;38(8):779-788.
20. Gerner P, O'Connor JP. Impact of analgesia on bone fracture healing. *Anesthesiology.* 2008;108(3): 349-350.
21. Geusens P, Emans PJ, de Jong JJ, van den Bergh J. NSAIDs and fracture healing. *Current opinion in rheumatology*. 2013;25(4):524-531.
22. Lau KH, Kothari V, Das A, Zhang XB, Baylink DJ. Cellular and molecular mechanisms of accelerated fracture healing by COX2 gene therapy: studies in a mouse model of multiple fractures. *Bone.* 2013;53(2):369-381.
23. O'Connor JP, Lysz T. Celecoxib, NSAIDs and the skeleton. *Drugs Today (Barc).* 2008;44(9):693-709.

24. Simon AM, Manigrasso MB, O'Connor JP. Cyclo-oxygenase 2 function is essential for bone fracture healing. *J Bone Miner Res.* 2002;17(6):963-976.
25. Simon AM, O'Connor JP. Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. *J Bone Joint Surg Am.* 2007;89(3):500-511.
26. Zhang M, Ho HC, Sheu TJ, Breyer MD, Flick LM, Jonason JH, Awad HA, Schwarz EM, O'Keefe RJ. EP1(-/-) mice have enhanced osteoblast differentiation and accelerated fracture repair. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2011;26(4):792-802.
27. Welting TJ, Caron MM, Emans PJ, Janssen MP, Sanen K, Coolsen MM, Voss L, Surtel DA, Cremers A, Voncken JW, van Rhijn LW. Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *European cells & materials.* 2011;22:420-436; discussion 436-427.
28. Emans PJ, van Rhijn LW, Welting TJ, Cremers A, Wijnands N, Spaapen F, Voncken JW, Shastri VP. Autologous engineering of cartilage. *Proc Natl Acad Sci U S A.* 2010;107(8):3418-3423.
29. Emans PJ, Spaapen F, Surtel DA, Reilly KM, Cremers A, van Rhijn LW, Bulstra SK, Voncken JW, Kuijer R. A novel in vivo model to study endochondral bone formation; HIF-1alpha activation and BMP expression. *Bone.* 2007;40(2):409-418.
30. Sachs L. *Angewandte statistik: anwendung statistischer methoden.* Springer-Verlag; 2013.
31. van Gaalen SM, Kruyt MC, Geuze RE, de Bruijn JD, Alblas J, Dhert WJ. Use of fluorochrome labels in in vivo bone tissue engineering research. *Tissue engineering Part B, Reviews.* 2010;16(2):209-217.
32. Emans PJ, Hulsbosch M, Wetzels GM, Bulstra SK, Kuijer R. Repair of osteochondral defects in rabbits with ectopically produced cartilage. *Tissue engineering.* 2005;11(11-12):1789-1796.
33. Frame JW. A convenient animal model for testing bone substitute materials. *J Oral Surg.* 1980;38(3):176-180.
34. Matos MA, Araujo FP, Paixao FB. Histomorphometric evaluation of bone healing in rabbit fibular osteotomy model without fixation. *J Orthop Surg Res.* 2008;3:4.
35. Lane JM, Sandhu HS. Current approaches to experimental bone grafting. *Orthop Clin North Am.* 1987;18(2):213-225.
36. Shefelbine SJ, Simon U, Claes L, Gold A, Gabet Y, Bab I, Muller R, Augat P. Prediction of fracture callus mechanical properties using micro-CT images and voxel-based finite element analysis. *Bone.* 2005;36(3):480-488.
37. Isaksson H, Grongroft I, Wilson W, van Donkelaar CC, van Rietbergen B, Tami A, Huiskes R, Ito K. Remodeling of fracture callus in mice is consistent with mechanical loading and bone remodeling theory. *J Orthop Res.* 2009;27(5):664-672.
38. van Rietbergen B, Majumdar S, Pistoia W, Newitt DC, Kothari M, Laib A, Ruegsegger P. Assessment of cancellous bone mechanical properties from micro-FE models based on micro-CT, pQCT and MR images. *Technol Health Care.* 1998;6(5-6):413-420.
39. Bulstra SK, Drukker J, Kuijer R, Buurman WA, van der Linden AJ. Thionin staining of paraffin and plastic embedded sections of cartilage. *Biotech Histochem.* 1993;68(1):20-28.
40. Heiple KG, Goldberg VM, Powell AE, Bos GD, Zika JM. Biology of cancellous bone grafts. *Orthop Clin North Am.* 1987;18(2):179-185.
41. Caron MM, Emans PJ, Coolsen MM, Voss L, Surtel DA, Cremers A, van Rhijn LW, Welting TJ. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2012;20(10):1170-1178.
42. Chai BF, Tang XM. Ultrastructural investigation of experimental fracture healing. Role of osteogenesis played by fibroblasts. *Chin Med J (Engl).* 1986;99(2):126-132.
43. Lane JM, Sandhu HS. Current approaches to experimental bone grafting. *Orthop Clin North Am.* 1987;18(2):213-225.
44. Bos GD, Goldberg VM, Powell AE, Heiple KG, Zika JM. The effect of histocompatibility matching on canine frozen bone allografts. *J Bone Joint Surg Am.* 1983;65(1):89-96.
45. Gerstenfeld LC, Thiede M, Seibert K, Mielke C, Phippard D, Svagr B, Cullinane D, Einhorn TA. Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. *J Orthop Res.* 2003;21(4):670-675.
46. Goodman S, Ma T, Trindade M, Ikenoue T, Matsuura I, Wong N, Fox N, Genovese M, Regula D, Smith RL. COX-2 selective NSAID decreases bone ingrowth in vivo. *J Orthop Res.* 2002;20(6):1164-1169.

47. Herbenick MA, Sprott D, Stills H, Lawless M. Effects of a cyclooxygenase 2 inhibitor on fracture healing in a rat model. *Am J Orthop.* 2008;37(7):E133-137.
48. Fyhrie DP, Vashishth D. Bone stiffness predicts strength similarly for human vertebral cancellous bone in compression and for cortical bone in tension. *Bone.* 2000;26(2):169-173.
49. Caron MM, Emans PJ, Surtel DA, Cremers A, Voncken JW, Welting TJ, van Rhijn LW. Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. *PLoS One.* 2012;7(3):e33467.
50. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, Barnes GL, Graves DT, Einhorn TA. Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res.* 2003;18(9):1584-1592.
51. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *Journal of cellular biochemistry.* 2003;88(5):873-884.
52. Caron MM, Emans PJ, Sanen K, Surtel DA, Cremers A, Ophelders D, van Rhijn LW, Welting TJ. The Role of Prostaglandins and COX-Enzymes in Chondrogenic Differentiation of ATDC5 Progenitor Cells. *PLoS One.* 2016;11(4):e0153162.
53. Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, Grasser WA, Owen TA, Li M, DaSilva-Jardine P, Zhou M, Dunn RL, Dumont F, Korsmeyer R, Krasney P, Brown TA, Plowchalk D, Vukicevic S, Thompson DD. An EP2 receptor-selective prostaglandin E2 agonist induces bone healing. *Proc Natl Acad Sci U S A.* 2003;100(11):6736-6740.
54. Cottrell JA, O'Connor JP. Pharmacological inhibition of 5-lipoxygenase accelerates and enhances fracture-healing. *J Bone Joint Surg Am.* 2009;91(11):2653-2665.
55. Manigrasso MB, O'Connor JP. Accelerated fracture healing in mice lacking the 5-lipoxygenase gene. *Acta Orthop.* 2010;81(6):748-755.
56. Ledwith BJ, Pauley CJ, Wagner LK, Rokos CL, Alberts DW, Manam S. Induction of cyclooxygenase-2 expression by peroxisome proliferators and non-tetradecanoylphorbol 12,13-myristate-type tumor promoters in immortalized mouse liver cells. *J Biol Chem.* 1997;272(6):3707-3714.
57. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA.* 2000;284(10):1247-1255.

Aggrecan and COMP improve periosteal chondrogenesis by delaying chondrocyte hypertrophic maturation

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Abstract

The generation of cartilage from progenitor cells for the purpose of cartilage repair is often hampered by hypertrophic differentiation of the engineered cartilaginous tissue caused by endochondral ossification. Since a healthy cartilage matrix contains high amounts of Aggrecan and COMP, we hypothesized that their supplementation in the biogel used in the generation of subperiosteal cartilage mimics the composition of the cartilage extracellular matrix environment, with beneficial properties for the engineered cartilage. Supplementation of COMP or Aggrecan was studied *in vitro* during chondrogenic differentiation of rabbit periosteum cells and periosteum-derived chondrocytes. Low melting agarose was supplemented with bovine Aggrecan, human recombinant COMP or vehicle and was injected between the bone and periosteum at the upper medial side of the tibia of New Zealand white rabbits. Generated subperiosteal cartilage tissue was analysed for weight, GAG and DNA content and ALP activity. Key markers of different phases of endochondral ossification were measured by RT-qPCR. For the *in vitro* experiments, no significant differences in chondrogenic marker expression were detected following COMP or Aggrecan supplementation, while *in vivo* favourable chondrogenic marker expression was detected. Gene expression levels of hypertrophic markers as well as ALP activity were significantly decreased in the Aggrecan and COMP supplemented conditions compared to controls. The wet weight and GAG content of the *in vivo* generated subperiosteal cartilage tissue was not significantly different between groups. Data demonstrate the potential of Aggrecan and COMP to favourably influence the subperiosteal microenvironment for the *in vivo* generation of cartilage for the optimization of cartilage regenerative approaches.

Introduction

Cartilage lesions can be debilitating, and are a high-risk factor for the development of osteoarthritis (OA) over time.[1] Cartilage lesions can be treated with surgical techniques such as microfracture (MF), mosaicplasty (MP), autologous chondrocyte implantation (ACI) or implantation of a small focal prosthesis.[2] Donor site morbidity, limited donor cartilage availability, high costs, and inferior repair tissue quality (respectively) are just some of the disadvantages of these approaches.[3,4] We proposed a novel paradigm for *de novo* engineering of cartilaginous tissues, the *in vivo* bioreactor (IVB). This is an alternative cartilage repair concept that we aim to further develop.[5] The IVB employs the fracture healing response as a way to generate autologous donor cartilage, suitable for implantation to repair (osteo)chondral defects.[5,6] During bone fracture healing the local periosteum plays an important role in the healing process by providing periosteal mesenchymal progenitor cells, which differentiate into chondrocytes and form the cartilaginous callus tissue that remodels via endochondral ossification to ultimately heal the bone fracture.[7-9] We discovered that local subperiosteal application of an agarose biogel provokes a similar cartilage callus-forming process within the created subperiosteal space, without the need of a fracture.[5] This cartilaginous tissue presents all the hallmarks of hyaline cartilage, and upon transplantation, can heal an osteochondral defect out to 9 months in a rabbit model.[5] However, without further optimization IVB-generated cartilage tissue is prone to further differentiate into hypertrophic cartilage, leading to unwanted ossification.

An important part of the dry-weight of articular cartilage consists of extracellular matrix (ECM) proteins (type II collagen (Col2a1), aggrecan (Acan), cartilage oligomeric matrix protein (COMP), etc.).[10-12] ECM proteins are thus important determinants in cartilage tissue homeostasis and their efficient synthesis is a prerequisite to creating cartilage volume. In addition, these major ECM protein species also condition the cartilage microenvironment in a unique way. Aggrecan plays a key role in generating the cartilage's fixed negative charge due to its glycosaminoglycan content, leading to its water-attracting properties,[13] while COMP provides the cartilage with retention capacity for TGF-β superfamily member growth factors.[14] Therefore, we hypothesized that the supplementation of the IVB biogel with Aggrecan or COMP mimics the composition of the native cartilage extracellular matrix microenvironment, with the potential to gain control over the chondrogenic potential of the IVB.

Materials and methods

Recombinant expression and purification of COMP

Full-length recombinant human (rh)COMP was prepared as previously described.[14] Briefly, human COMP cDNA was cloned into a pQE mammalian expression vector (Qiagen), which was then stably transduced into human HEK293T cells. Cells were

expanded in in DMEM with 10% FBS until 15 cm tissue-culture dishes were 80% confluent, then the FBS was reduced to 0.1% FBS and conditioned media collected and replenished daily for up to 1 week. COMP was purified to near homogeneity from the conditioned culture media using nickel-nitrilotriacetic acid column affinity chromatography (Ni-NTA Agarose, Qiagen). The eluted protein was buffer-exchanged into 20 mM HEPES (pH 7.0), 2 mM $CaCl_2$, and 500 mM NaCl, at approximately 500 ug/ml, with 30% glycerol added prior to storage at -80°C.

Periosteum cell culture

As previously described,[5,15] the periosteum was harvested from the proximal tibia of New Zealand White Rabbits and cut into small pieces using a sterile surgical blade. Post-mortem animals were obtained from an unrelated study; no ethical approval was necessary. Periosteal pieces were digested for three hours at 37°C in collagenase II solution (300 U/ml in HEPES buffered Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, United States) supplemented with 1% antibiotic/antimycotic (Invitrogen)) under continuous agitation. The preparation was rinsed with 0.9% NaCl over a 70 µm cell strainer and plated in culture flasks. Cells were cultured in a humidified atmosphere at 37°C, 5% CO_2 in culture medium consisting of: Minimal Essential Medium (MEM)/D-valine (Invitrogen), 10% fetal calf serum (FCS) (Sigma-Aldrich, St Louis, MO, United States), 1% antibiotic/antimycotic (Invitrogen), 1% non-essential amino acids (NEAA, Invitrogen) and 2mM l-glutamine (Sigma-Aldrich).[8,16] After reaching confluence, cells were passaged 1:2 until passage 2. Passage 2 rabbit periosteal cells from 1 donor were plated at 30.000 cells/cm^2 in triplicates per condition and the next day chondrogenic differentiation was initiated by changing the culture medium to differentiation medium consisting of: Dulbecco's Modified Eagle Medium (DMEM) high glucose (Invitrogen), 10% FCS (Sigma-Aldrich), 1% antibiotic/antimycotic (Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 1% insulin-transferrin-selenite solution (ITS; Sigma-Aldrich), 40 µg/ml L-proline (Sigma-Aldrich), 10 ng/ml TGF-β (Invitrogen), 25 µg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich), and 100 nM dexamethasone. Glycosaminoglycan containing bovine Aggrecan from articular cartilage (Sigma-Aldrich A1960) (Supplementary Figure S5.1) was added at 2 µg/ml concentration and rhCOMP was added at 200 µg/ml. The same volume of 0.9% sodium chloride was added as a control. Differentiation medium was changed every other day and after 0 (baseline measurement) and 21 days cells were harvested for RNA isolation and ALP activity.

Chondrocytes derived from IVB cartilage

Cells were obtained from cartilage out of periosteum tissue generated *in vivo* in New Zealand White Rabbits (DEC2005-159).[6] The IVB cartilage tissue was harvested directly after euthanasia. The autologous IVB cartilage was separated from the periosteum by dissecting with a scalpel and the overlying fibrous tissue was carefully removed. This

cartilage tissue is distinct in phenotype and consistency so risk of contamination with other tissues in the sample is negligible. Tissue was digested for three hours at 37°C in collagenase II solution (300U/ml in HEPES buffered DMEM/F12 (Invitrogen) supplemented with 1% antibiotic/antimycotic (Invitrogen)) under continuous agitation. The preparation was rinsed with 0.9% NaCl over a 70 µm cell strainer and plated in culture flasks. Cells were cultured in a humidified atmosphere at 37°C, 5% CO_2 in culture medium consisting of: DMEM/F12, 10% FCS, 1% antibiotic/antimycotic and 1% NEAA. After reaching confluence, cells were passaged 1:2 until passage 6. Passage 6 cells from 1 donor were plated at 30.000 cells/cm^2 in triplicates per condition and the next day chondrogenic redifferentiation was initiated by changing the culture medium to redifferentiation medium consisting of: DMEM/F12, 1% antibiotic/antimycotic, 1% NEAA, 1% ITS, 10 ng/ml TGF-β and 25 µg/ml L-ascorbic acid-2-phosphate. Bovine Aggrecan was added at a 2 µg/ml concentration and rhCOMP was added at 200 µg/ml. The same volume of 0.9% sodium chloride was added as a control. Differentiation medium was changed every other day and after 0 (baseline measurement) and 7 days cells were harvested for RNA isolation.

Animal study

Twenty-four knees in 12 female, specific-pathogen-free (SPF) New Zealand White Rabbits were used for this experiment (Charles River Laboratories, Wilmington, MA, United States; 107 days old, ~1.8 kg). The experiment was approved by the Maastricht University animal ethical committee (DEC 2012-151) and we confirm that all experiments were performed in accordance with relevant guidelines and regulations (ARRIVE). Throughout the experiment, animals were housed in groups under standard conditions with ad libitum access to water and food and 12 hours of light each day. Animal well-being and behaviour (score in response to stimuli, back arch, twitch, wincing, posture, self-care, condition of skin, mobility, limb loading, difficulties in respiration/breathing, dehydration or undernourishment symptoms, colour of the mucous membranes and extremities, oedema/swelling/cold feeling and other notable abnormalities) were checked daily. The sample size was calculated and corrected for potential dropout, and eight animals per group were included. The IVB method described by Emans and colleagues was used for ectopically-inducing cartilage formation, in which a subperiosteal space is created to induce periosteal endochondral ossification.[5,6,17] In short, the skin was opened over the upper medial side of the tibia, the periosteum was incised just medially of the pes anserinus, leaving the semitendinosus tendon untouched. The periosteum was elevated proximally with a probe and 0.2 ml of a 2% (w/v) agarose-based gel (2 g of ultra-pure low-melting agarose granules (Cat no: 10975035, Lot No: MO91807; Invitrogen) in 100 ml of 0.9% NaCl, followed by steam-sterilization) was injected between the bone and periosteum. Bovine Aggrecan was added at a 2% w/v or rhCOMP was added at 0.5 mg/ml to the agarose-gel. The wound was closed in separate layers with Vicryl Rapide™ 4-0 absorbable sutures (Ethicon, Kirkton, United Kingdom). This procedure was repeated on

the contralateral tibia. After 14 days, rabbits were euthanized by an overdose of intravenous pentobarbital. The IVB cartilage tissue was harvested directly after euthanasia. The autologous IVB cartilage was separated from the periosteum by dissecting with a scalpel and the overlying fibrous tissue was carefully removed. This cartilage tissue is distinct in phenotype and consistency so risk of contamination with other tissues in the sample is negligible. Generated subperiosteal cartilage tissue was analysed for weight, glycosaminoglycan (GAG)- and DNA content. In addition, samples were taken for gene expression analysis and ALP activity assay.

Gene expression analysis

Cells and ectopically-formed cartilage tissue on the tibia were harvested and lysed in TRIzol (Life Technologies|Thermo Fisher Scientific, Carlsbad, CA, United States). RNA isolation, RNA quantification by ultraviolet (UV) spectrometry (Biodrop; Isogen Life Sciences, Utrecht, the Netherlands) and cDNA synthesis were performed as described before.[18,19] Real-time quantitative PCR (RT-qPCR) was performed using Takyon No ROX Sybr® Green MasterMix blue dTTP (Eurogentec, Seraing, Belgium). A CFX96 RealTime PCR Detection system (Biorad, Hercules, CA, United States) was used for amplification with the following protocol: initial denaturation 95°C for 10 minutes, followed by 40 cycles of amplification (denaturation 15 seconds at 95°C and annealing 1 minute at 60°C). Validated primer sequences used are listed in Table 5.1. Data were analysed using the standard curve method, mRNA expression was normalized to the reference gene (28S rRNA) and gene expression was calculated as fold change as compared to baseline conditions (in vitro studies) or control conditions (in vivo study).

Table 5.1 Primer sequences for RT-qPCR.

Oligo sets	Forward	Reverse	-ΔCt in differentiated periosteal cells	-ΔCt in genetated chondro-cytes	-ΔCt in IVB generated cartilage
Acan	CGGGACACCAACGAGACCTAT	CTGGCGACGTTGCGTAAAA	-11,48	-14,88	-16,76
Alpl	GGAGGATGTGGCCGTCTTC	CTGCGTAAGCCATCACATGAG	-14,55	-12,82	-14,09
Nkx3-2	ACCTGGCAGCTTCGCTGAA	AGGTCGGCGGCCATCT	-21,15	-19,85	-25,96
BMP2	AGAAAAGCGTCAAGCGAAACA	GTCCACGTACAAAGGGTGTCTCT	-13,04	-12,67	-19,46
Col1a1	CTGACTGGAAGAGCGGAGAGTAC	CCATGTCGCAGAAGACCTTGA	-14,19	-14,39	-13,85
Col2a1	TGGGTGTTCTATTTATTTATTGTCTTCCT	GCGTTGGACTCACACCAGTTAGT	-11,28	-10,77	-16,57
Col10a1	AACCTGGACAACAGGGACTTACA	CCATATCCTGTTTCCCCTTTCTG	-13,50	-10,59	-18,82
Cox-2	ACCAACATGATGTTTGCATTCTTT	GGTCCCCGCTTAAGATCTGTCT	-17,27	-13,32	-21,20
ID2	CCCGATGAGCCTGCTATACAA	TGGGCACCAGCTCCTTGA	-14,85	-16,21	-17,42
Mmp13	CGATGAAGACCCCAACCCTAA	ACTGGTAATGGCATCAAGGGATA	-13,65	-17,13	-18,45
PTHrP	AAGGGCAAGTCCATCCAAGA	CTCGGCGGTGTGTGGATTTC	-12,53	-14,11	-22,40
Runx2	TGATGACACTGCCACCTCTGA	GCACCTGCCTGGCTCTTCT	-18,25	-13,94	-18,23
Smad7	GCAACCCCCATCACCTTAGTC	GTTTGAGAAAATCCATTGGGTATCTG	-15,33	-13,66	-15,38
Sox9	AGTACCCGCACCTGCACAAC	CGCTTCTCGCTCTCGTTCAG	-15,03	-12,24	-17,86
TGFb3	ACTTGCACCACCTTGGACTTC	GGTCATCACCGTTGGCTCA	-15,85	-11,76	-16,00
28S rRNA	GCCATGGTAATCCTGCTCAGTAC	GCTCCTCAGCCAAGCACATAC	Reference	Reference	Reference

The 5' to 3' forward and reverse oligonucleotide sequences used for RT-qPCR are listed in the table. The -ΔCt values for the control condition in the in vivo IVB generated cartilage tissue, periosteal cells and IVB-derived chondrocytes are shown.

sGAG assay

The total sulphated glycosaminoglycan (GAG) content of the ectopically-formed cartilage tissue was measured using a standardized modified 1,9-dimethyl methylene blue (DMMB) assay (Polysciences).[20,21] The absorbance of samples was read at 540 and 595 nm using a spectrophotometer (Multiskan FC, ThermoFisher Scientific). GAG concentrations were calculated using a standard curve of chondroitin sulphate (Sigma-Aldrich). GAG content was normalized for total DNA content or wet weight of the ectopically-formed cartilage tissue.

DNA quantification

The DNA concentration was determined using SYBR® Green I Nucleic Acid stain (Invitrogen). A serially diluted standard curve of genomic control DNA (calf thymus, Invitrogen) in TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) was included to quantify the DNA concentration in the samples. Before measurement, samples were diluted in TE buffer (1 µl sample and 99 µl TE buffer) and standards were prepared. SYBR® Green was diluted 10,000 times in TE buffer and 100 µl of this solution was added to 100 µl of the above-prepared samples or standards. Fluorescence was determined in standard 96-well ELISA plates in a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, United States): excitation 488 nm and emission 522 nm.

ALP activity assay

Cells or cartilage tissues were lysed in 1.5 M Tris-HCl pH 9.0; 2% (v/v) Triton X-100 and homogenized by sonication (Soniprep 150 MSE). Insoluble material was removed by centrifugation (5 minutes; 13,000 x g; 4°C). Total protein concentration was determined BCA assay (Sigma-Aldrich). ALP enzyme activity in-time was measured by ALP-depend enzymatic conversion of p-nitrophenyl phosphate to p-nitrophenol in buffer containing 1.5 M Tris-HCl; pH 9.0, 1 mM $ZnCl_2$, 1 mM $MgCl_2$ and 7.5 mM p-nitrophenyl phosphate. Substrate conversion was spectrophotometrically quantified at 405 nm and p-nitrophenol concentrations were determined via a p-nitrophenol calibration series. Values were normalized to total protein concentration and ALP enzyme activity was calculated as mmol/min/µg.

Statistics

Statistical significance ($p<0.05$) was determined by student's two-tailed t-test for *in vitro* experiments shown in Figure 5.1, 5.2 and 5.5 using Graphpad PRISM 5.0 (La Jolla, CA, United States). Due to limited sample size (triplicates), normal distribution of input data was assumed as normality could not be reliably tested. For the *in vivo* experiment, normal distribution of input data was tested by D'Agostino-Pearson omnibus normality tests and all data from the in vivo study (Figure 5.3, 5.4 and 5.5) passed the normality

tests. Statistical significance ($p<0.05$) was determined by student's two-tailed t-test. Lines in graphs represent mean ± standard error of the mean (s.e.m.).

Results

Addition of Aggrecan or COMP during chondrogenic differentiation of periosteal progenitor cells inhibits chondrocyte hypertrophy

As the IVB relies on chondrogenic differentiation of the local periosteum, we determined if COMP or Aggrecan could improve the chondrogenic differentiation of periosteum-derived progenitor cells *in vitro*. These two cartilage ECM components were added to the chondrogenic differentiation media of rabbit periosteal derived cells. After 21 days, differences in chondrogenic and hypertrophic gene expression were analysed between groups. Expression of SRY (sex-determining region Y) box9 (Sox9) was significantly increased by Aggrecan (Figure 5.1A), but not significantly by COMP. No significant differences were found in the expression of Col2a1 and Aggrecan by supplementation of either Aggrecan or COMP (Figure 5.1A). In contrast, gene expression of hypertrophic markers was all significantly repressed by Aggrecan or COMP exposure during chondrogenic differentiation of periosteum cells (Figure 5.1B). Runt-related transcription factor 2 (Runx2) expression was reduced in Aggrecan and COMP conditions. Collagen type X (Col10a1) and alkaline phosphatase (Alpl) expression responded similar as Runx2, with decreased gene expression after 21 days of exposure to Aggrecan or COMP (Figure 5.1B). This inhibition of hypertrophic maturation in chondrogenic differentiation of periosteal cells by Aggrecan or COMP was further confirmed by a significant decrease in ALP enzyme activity (Figure 5.1C). No significant differences were detected for fibrotic marker collagen type I (Col1a1) expression between groups (Figure 5.1D). Collectively, these data indicate that exposure of chondrogenically differentiating periosteum cells to supplemented Aggrecan or COMP does not influence the expression of key chondrogenic markers, but specifically suppresses chondrocyte hypertrophic differentiation in these *in vitro* cell cultures.

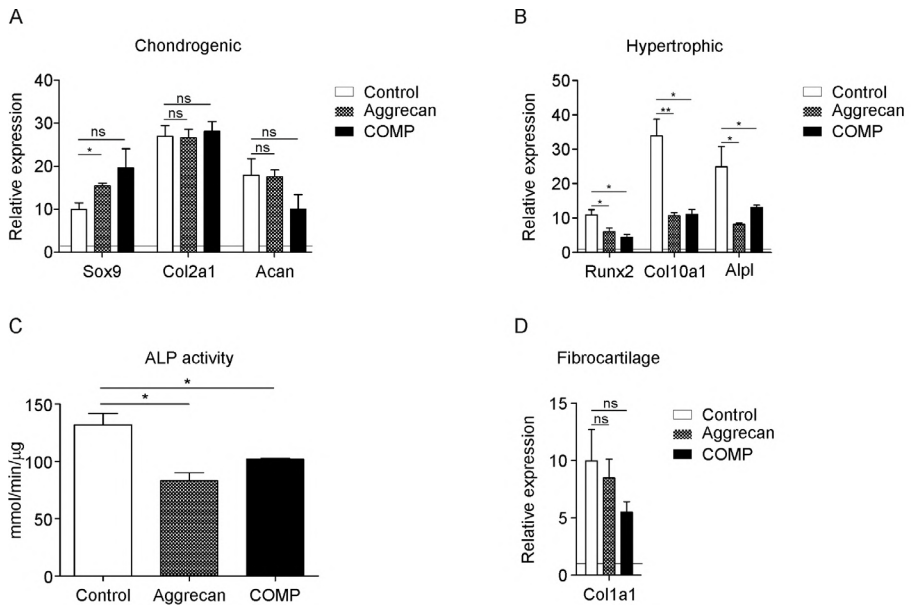

Figure 5.1 Addition of Aggrecan or COMP during chondrogenic differentiation of rabbit periosteal cells results in decreased hypertrophic differentiation. Periosteal derived cells differentiated in chondrogenic lineage under control conditions (white bars) and with Aggrecan (2 µg/ml; dotted bars) or COMP (200 µg/ml; black bars) for 21 days. **A**) Induction of chondrogenic markers Sox9, Col2a1, and Acan mRNA expression was determined by RT-qPCR, normalized for 28S rRNA expression and set relative to baseline (t=0) values (indicated by horizontal line). **B**) Induction of hypertrophic markers Runx2, Col10a1 and Alpl mRNA expression was determined similarly to samples from (A). **C**) ALP enzyme activity in cell lysates of same conditions was determined and normalized to total protein content. **D**) Fibrocartilage marker Col1a1 mRNA expression as determined in similarly to from (A). In graphs, error bars represent mean ± s.e.m. Statistically significant differences ($p<0.05$) are shown by an *, ** $p<0.01$, ns = not significant.

Improved chondrocyte phenotype of chondrocytes derived from IVB cartilage when exposed to Aggrecan or COMP

The biogel (and additives in it) used for the IVB technique is expected to not only influence the initiation of chondrogenic differentiation but also aiding in maintaining or supporting the chondrogenic differentiation status of mature chondrocytes. Therefore, we likewise determined the effect of Aggrecan or COMP on chondrocytes that were isolated from IVB-generated cartilage from a previous *in vivo* experiment.[6] After 7 days of culture with either Aggrecan or COMP, the chondrocyte phenotype was assessed by gene expression analysis. No major differences were observed in mRNA expression of chondrogenic markers Sox9, Col2a1 and Acan following the addition of Aggrecan or COMP to these cultures (Figure 5.2A). However, and in concert with results found above (Figure 5.1), the addition of Aggrecan or COMP to these cultures had a profound

consequence for chondrocyte hypertrophy (Figure 5.2B). Expression of Runx2 was significantly decreased by Aggrecan or by COMP at day 7 in culture (Figure 5.2B). Significant repression of Col10A1 and Alpl was also observed following Aggrecan or COMP supplementation (Figure 5.2B). Col1a1 expression was significantly inhibited by Aggrecan in these cultures, however not by COMP (Figure 5.2C). Together, these data indicate that Aggrecan and COMP improve the chondrocyte phenotype *in vitro* of mature chondrocytes isolated from IVB-generated cartilage by selectively decreasing chondrocyte hypertrophy.

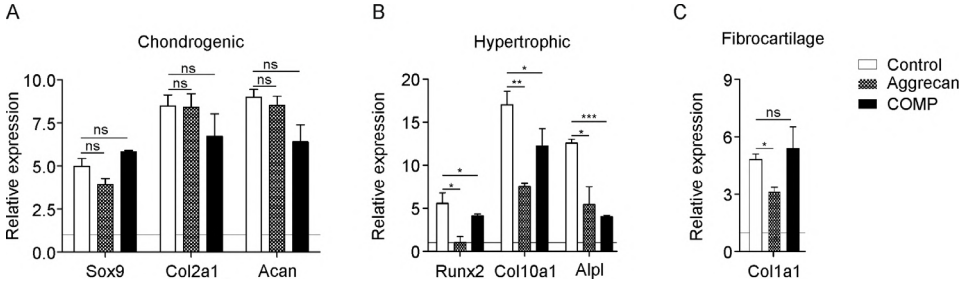

Figure 5.2 Better cartilage quality of chondrocytes generated from periosteal tissue when exposed to Aggrecan or COMP. Chondrocytic cells derived from ectopic generated cartilage out of periosteum tissue were redifferentiated under control conditions (white bars) and with Aggrecan (2 µg/ml; dotted bars) or COMP (200 µg/ml; black bars) for 7 days. **A**) Induction of chondrogenic markers Sox9, Col2a1 and Acan mRNA expression was determined by RT-qPCR, normalized for 28S rRNA expression and set relative to baseline (t=0) values (indicated by horizontal line). **B**) Induction of hypertrophic markers Runx2, Col10a1 and Alpl mRNA expression was determined similarly to samples from (A). **C**) Fibrocartilage marker Col1a1 mRNA expression was determined similarly to samples from (A). In graphs, error bars represent mean ± s.e.m. Statistically significant differences ($p<0.05$) are shown by an *, ** $p<0.01$, *** $p<0.0001$, ns = not significant.

Quality of IVB cartilage generated with Aggrecan or COMP supplementation of the biogel

We next determined if Aggrecan or COMP supplementation to the IVB biogel leads is beneficial for the quality of ectopically generated cartilage in the IVB. We used the IVB technique as described earlier[5,17] and added either Aggrecan (2% w/v; n=8 IVBs) or COMP (0.5 mg/ml; n=8 IVBs) to the agarose biogel, and compared the quality of the cartilage that was generated 14 days after creation of the IVBs with a control group in which only the empty agarose biogel condition was tested (n=8 IVBs). The wet weight of the formed IVB tissues was not significantly different between the empty agarose group versus the IVBs in which the biogel was supplemented with Aggrecan or COMP (Figure 5.3A). Also, no significant differences between the control group and Aggrecan or COMP groups were found in the DNA content of the IVB generated tissues (data not shown). When GAG content in the IVB generated tissues was determined and

normalized for either DNA content or tissue wet weight, again no significant differences were found between the groups (Figure 5.3B).

To analyse the IVB-generated ectopic cartilage tissues in more bio-molecular detail we determined the expression of chondrogenic and chondrocyte hypertrophy genes. Sox9 expression in the generated cartilage tissues was not significantly different between the control and Aggrecan-supplemented or between control and COMP-supplemented groups (Figure 5.4A). Expression of Col2a1 and Acan was significantly increased in the IVBs supplemented with COMP. The IVBs supplemented with Aggrecan showed a significantly increased Col2a1 expression. However, the increase in Acan expression was not significant (Figure 5.4A). In full agreement with data obtained from above *in vitro* cultures of periosteal chondrogenesis (Figure 5.1) and the IVB-derived chondrocytes (Figure 5.2), the most profound differences in gene expression were found for chondrocyte hypertrophy genes (Figure 5.4B). Runx2, Col10a1 and Alpl expression were significantly suppressed in the IVBs supplemented with Aggrecan or COMP (Figure 5.4B). When analysing other chondrocyte hypertrophy-associated genes such as matrix metalloproteinase 13 (MMP13) or cyclooxygenase 2 (COX-2),[19] we observed that MMP13 expression was inhibited in both the Aggrecan and COMP groups, while COX-2 expression was reduced, but not significantly (Figure 5.4B). This inhibition of hypertrophic maturation of the IVB-generated ectopic cartilage by Aggrecan or COMP was further confirmed by a significant decrease in ALP enzyme activity (Figure 5.4C). No significant differences were found for Col1a1 expression between groups (Figure 5.4D). Overall, these results demonstrate that the supplementation of Aggrecan or COMP to the IVB agarose biogel does not change the quantity or GAG content of the generated cartilaginous tissues. However, gene expression analysis shows the development of a favourable cartilage phenotype, with a specific reduction of the magnitude of chondrocyte hypertrophy in the Aggrecan and COMP groups.

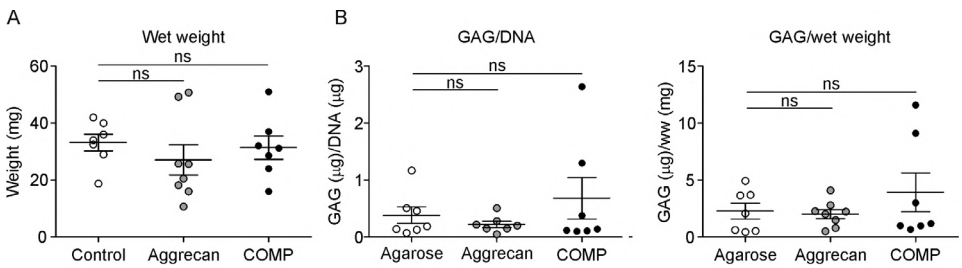

Figure 5.3 Similar GAG content in cartilage generated out of periosteum in vivo. Cartilage formation was ectopically induced by injecting an agarose biogel (n=7) with or without the addition of Aggrecan (2% w/v; n=8) or COMP (0.5mg/ml; n=7) under the tibial periosteum of rabbits and after 14 days generated tissue was harvested for analysis. **A**) Wet weight was determined for each ectopically generated cartilaginous tissue. **B**) GAG content corrected for DNA content (left panel) or for wet weight (right panel) was determined in samples from (A). Each dot represents the determined value for each of these individual generated tissues per group and lines in graphs indicate mean ± s.e.m. Statistically significant differences ($p<0.05$) are shown by an *, ns = not significant.

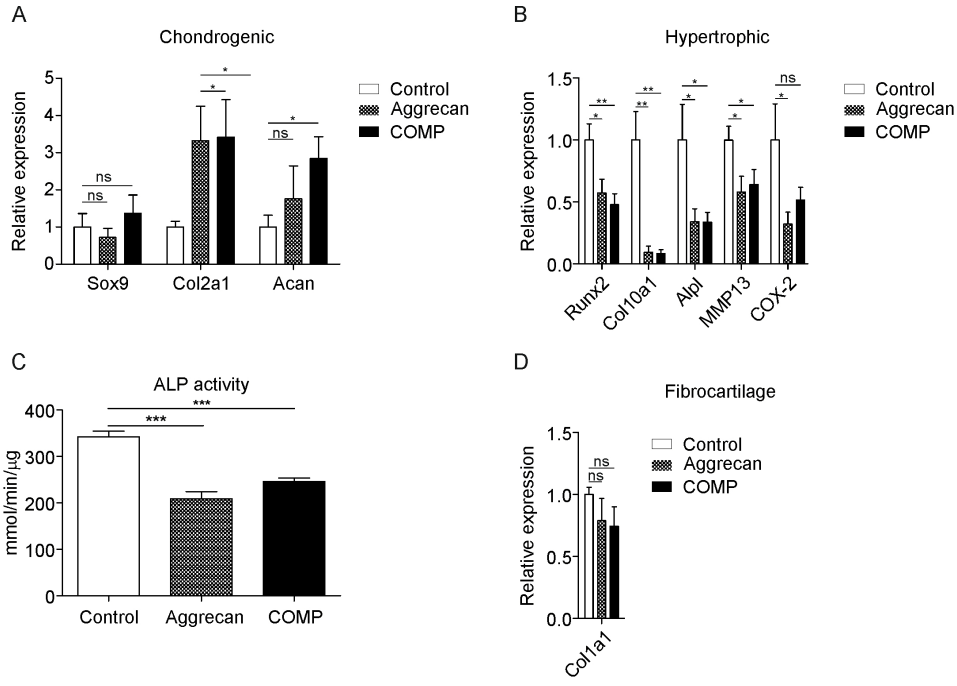

Figure 5.4 Decreased hypertrophic marker expression in in vivo generated cartilage stimulated with Aggrecan or COMP. Cartilage formation was ectopically induced by injecting an agarose biogel (n=8) with or without the addition Aggrecan (2% w/v; n=8) or COMP (0.5 mg/ml; n=8) under the tibial periosteum of rabbits and after 14 days generated tissues were harvested for gene expression analysis. **A**) Induction of chondrogenic markers Sox9, Col2a1, and Acan mRNA expression was determined by RT-qPCR and normalized for 28S rRNA expression. **B**) Induction of hypertrophic markers Runx2, Col10a1 and Alpl mRNA expression was determined by RT-qPCR at day 14 and normalized for 28S rRNA expression. **C**) ALP enzyme activity in tissue lysates of same conditions was determined and normalized to total protein content. **D**) Fibrocartilage marker Col1a1 mRNA expression as determined by RT-qPCR and normalized to 28S rRNA expression. In graphs, error bars represent mean ± s.e.m. Statistically significant differences ($p<0.05$) are shown by an *, ** $p<0.01$, ns = not significant.

Increased NKX3-2 mRNA expression following Aggrecan or COMP supplementation

We next elucidated a potential biomolecular mechanism explaining the observed change in chondrogenic outcome in the chondrogenically differentiating periosteal cells, IVB-derived chondrocytes, and in the newly generated IVB tissues, as a result of exposure to Aggrecan or COMP. To this end, gene expression of important paracrine regulators (PTHrP, TGF-β3, and BMP2) of chondrogenic differentiation was determined.[22,23] In addition, mRNA expression levels of Bagpipe Homeobox Protein Homolog 1 (Bapx1)/ Homeobox Protein NK-3 Homolog B (NKX3-2), a transcriptional

repressor of chondrocyte hypertrophic differentiation,[24,25] was determined in these samples.

At day 21 in *in vitro* chondrogenic differentiation of periosteal cells, expression of parathyroid hormone-related peptide (PTHrP), TGF-β3 and bone morphogenetic protein 2 (BMP2) was not significantly different between groups (Figure 5.5A). However, expression of NKX3-2 mRNA was significantly increased in the chondrogenic cultures supplemented with Aggrecan or COMP ($p=0.0300$) (Figure 5.5A). Mature chondrocytes that were isolated from IVB cartilage and cultured *in vitro* for 7 days in the presence of Aggrecan or COMP did not show any significant responses of PTHrP or TGF-β3 (Figure 5.5B). Exposure of these cultures to COMP resulted in significant inhibition of BMP2 expression, while supplementation of Aggrecan to these cultures did not significantly alter BMP2 expression (Figure 5.5B). However, and similar to chondrogenesis of periosteal cells above, the gene expression of NKX3-2 was significantly increased in cultures supplemented with Aggrecan or COMP (Figure 5.5B).

In the *in vivo* ectopically generated IVB cartilage tissues in which the biogel was supplemented with Aggrecan or COMP, expression of PTHrP was not significantly different when compared to the control empty agarose biogel group (Figure 5.5C). Expression of TGFβ3 and TGFβ target gene Smad7 was not significantly altered in IVBs supplemented with Aggrecan or COMP (Figure 5.5C). BMP signalling, measured by BMP2 and DNA-binding protein inhibitor 2 (Id2) gene expression was not significantly different between groups (Figure 5.5C). Similar to above NKX3-2 expression data and its chondrocyte hypertrophy-suppressive action, NKX3-2 mRNA expression was significantly increased in the IVB cartilage tissues generated from biogel supplemented with Aggrecan or COMP. In conclusion, the gene expression of the chondrocyte hypertrophy transcriptional repressor NKX3-2 was significantly increased in all conditions supplemented with Aggrecan or COMP.

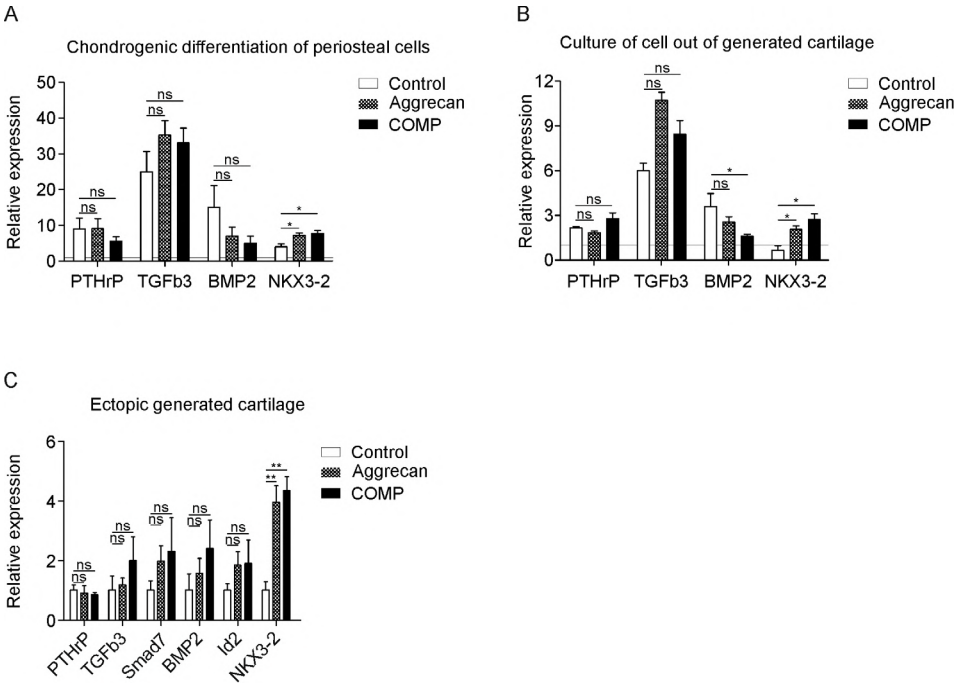

Figure 5.5 Increased NKX3-2 mRNA expression in COMP and Aggrecan generated cartilage in vitro and in vivo. **A**) Expression of PTHrP, TGFb3, BMP2, and NKX3-2 was determined by RT-qPCR, normalized for 28S rRNA expression and set relative to baseline (t=0) values (indicated by horizontal line) in samples from Figure 5.1 (chondrogenic differentiation of periosteal cells). **B**) Expression of PTHrP, TGFb3, BMP2, and NKX3-2 was determined by RT-qPCR, normalized for 28S rRNA expression and set relative to baseline (t=0) values (indicated by horizontal line) in samples from Figure 5.2 (redifferentiation of cells isolated from ectopically generated cartilage). **C**) Expression of PTHrP, TGFb3, Smad7, BMP2, Id2, NKX3-2 was determined by RT-qPCR and normalized for 28S rRNA expression in samples from Figure 5.4 (ectopically generated cartilage *in vivo*). White bars represent the control condition, dotted bars the condition supplemented with Aggrecan and the black bars the condition supplemented with COMP. In graphs, error bars represent mean ± s.e.m. Statistically significant differences ($p<0.05$) are shown by an *, ** $p<0.01$, ns = not significant.

Discussion

The goal of ectopic cartilage regeneration is to create sufficient quantity of hyaline cartilage of good quality to be used for transplantation. Limitations in quantity and progression into hypertrophy remain important drawbacks that need to be addressed in the field. In this study, we showed in three independent models that chondrogenic differentiation and cartilage homeostasis of periosteal cells *in vitro* and *in vivo* can be sustained by the supplementation of Aggrecan or COMP. It specifically leads to suppression of hypertrophic differentiation of the cartilaginous tissue, with possible involvement of NKX3-2.[24,26]

Aggrecan is a key GAG-containing proteoglycan in cartilage and plays an important role in stabilizing the ECM in articular cartilage. Furthermore, due to negatively charged anionic groups of its GAG sidechains, aggrecan creates a large osmotic gradient which draws water into the tissue. This gives cartilage its unique properties.[27] Several studies have shown that articular chondrocytes and chondrogenically differentiating progenitor cells are osmolarity-responsive and increase their ECM synthesis under chondrocyte-physiological osmolarity,[28-30] or after addition of oversulphated polysaccharides.[31] Likewise, plating of fibroblasts on an Aggrecan-coated surface (in the presence of TGF-β) was able to induce chondrogenic differentiation[32] of these cells. We hypothesized that the addition of Aggrecan to the *in vitro* cultures of differentiating periosteal cells, IVB-derived chondrocytes and eventually also the IVB-generated cartilage tissue, would increase the chondrogenic differentiation capacity of these cells. In the *in vitro* cultures we did not observe significantly increased chondrogenic marker expression as measured by Sox9, Col2a1 and Acan, However, in the IVB-generated cartilaginous tissue, the gene expression of Col2a1 was significantly increased by Aggrecan supplementation. GAG-bound TGF-β is able to stimulate neocartilage formation[33] and it was recently shown that under cartilage physiological osmolarity TGF-β signalling was increased. This was associated with an improved chondrocyte phenotype.[34] Indeed, also in our studies we observed increased (but not significant) TGF-β3 expression in the Aggrecan-supplemented conditions. However, we were not able to determine if the actual osmolarity of the culture conditions was significantly increased due to Aggrecan supplementation. Interestingly, in the Aggrecan-supplemented conditions we observed a significant repression of chondrocyte hypertrophy (Runx2, Col10a1, Alpl expression and ALP enzyme activity) in all three models. These data demonstrate that periosteal chondrogenic differentiation *in vitro* and *in vivo* and homeostasis of IVB-derived chondrocytes can be influenced in a hypertrophy-suppressive manner by supplementation with Aggrecan. NKX3-2 is known as a key transcriptional repressor of Runx2 during both early and late chondrogenic differentiation,[25,35] providing control over hypertrophic differentiation. NKX3-2 mRNA expression was significantly upregulated in the aggrecan-supplemented cultures. To the best of our knowledge, it is unknown how Aggrecan would be able to induce the expression of NKX3-2 mRNA in these cells. GAGs are described to be able to bind and regulate activity of growth factors, chemokines, cytokines and adhesion molecules.[36] For instance, FGF and VEGF are stored, stabilized and protected from degradation in the ECM trough interactions with GAGs, and upon stimulation can be released to exert their function.[37,38] We speculate that certain NKX3-2-inducing morphogens, such as Shh, PTHrP or BMPs[25,26,39] are being retained by the GAG-containing supplemented Aggrecan,[40] potentially potentiating their activity and leading to a hypertrophy-suppressing action via NKX3-2. Indeed, our supporting data from ATDC5 chondrogenic differentiation suggest a role for NKX3-2 in hypertrophic differentiation via specific morphogens and increased osmolarity (Supplementary Figure S5.2). However, NKX3-2 data in our present study are limited by a current lack of evidence on the protein level in periosteal cells and needs further investigation to corroborate this hypothesis.

COMP is one of the thrombospondin proteins (TSP-5) that acts as a key component in the synthesis and homeostasis of the cartilage ECM.[41] COMP is essential in chondrogenic growth plate development[42,43] and mutations in COMP are linked to the human skeletal disorders pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED).[44,45] In addition, elevation of COMP levels increased chondrogenic differentiation of human bone marrow stem cells.[41] In this study however, supplementation with COMP did not lead to significant differences in Col2a1 and Acan expression in chondrogenically differentiating periosteal cells or cultures of chondrocytes derived from IVB cartilage tissue *in vitro*. In our *in vivo* study however, IVB biogel supplementation with COMP did significantly increase the expression of chondrogenic markers Col2a1 and Acan. In analogy with the Aggrecan supplemented condition above, supplementation with COMP significant suppressed chondrocyte hypertrophy in all three tested chondrocyte models. COMP is a homopentamer acting as a key intermolecular bridge in cartilaginous tissues.[41] COMP is described to interact with cartilage ECM proteins, including collagen type 2 and Aggrecan, and as such plays a role in matrix assembly and tissue homeostasis. COMP also interacts with endogenous growth factors, such as TGFβs and BMPs, and acts as a lattice for their presentation to cells.[14,41,46] This influences, for instance, growth factor signalling and cell differentiation processes. The activity of TGFβ1 is potentiated when bound to COMP,[14] potentially explaining its prochondrogenic and hypertrophy-suppressing properties in our IVB experiments. It can also be noted that COMP binds BMP7.[14] Previously we reported that BMP7 suppresses chondrocyte hypertrophy in an NKX3.2 dependent fashion,[24,26] and we consider a BMP7 activity-potentiating role for COMP as a possible explanation for our observations. Data supporting a role for TGFβ and BMP7 in the induction of NKX3-2 levels during ATDC5 chondrogenic differentiation are presented in Supplementary Figure S5.2.

Due to a limited quantity of IVB-generated cartilage tissue in this study, we needed to select the most insightful manner of analysis. Although posing a study limitation from a histological perspective, we preferred a quantitative analysis over histology and used gene expression, GAG content, DNA content, ALP activity, and wet weight as primary read-out parameters. Also, we could only test periosteal progenitor cells and chondrocytes from periosteal cartilage from one donor each. Despite these limitations, this study demonstrates in different models that conditioning of the micro-environment with cartilage ECM components Aggrecan or COMP creates a hypertrophy-suppressive niche with prochondrogenic properties for development of cartilaginous tissue in the IVB. A more prolonged analysis of the stability of the IVB neocartilage and investigating potential synergistic consequences of COMP and Aggrecan supplementation will potentially add to the translational value of our observations. This provides novel molecular clues for the optimization of IVB cartilage graft quality for cartilage repair in particular and for endochondral ossification-based cartilage regeneration techniques in general.[47,48] Future work should be able to address the influence of IVB cartilage graft maturation on the pre-clinical outcome of cartilage repair.

References

1. Mollenhauer JA, Erdmann S. Introduction: molecular and biomechanical basis of osteoarthritis. *Cell Mol Life Sci.* 2002;59(1):3-4.
2. Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis Cartilage.* 2002;10(6):432-463.
3. Caldwell KL, Wang J. Cell-based articular cartilage repair: the link between development and regeneration. *Osteoarthritis Cartilage.* 2014.
4. Caron MM, Welting TJ, van Rhijn LW, Emans PJ. Targetting Inflammatory Processes for Optimization of Cartilage Homeostasis and Tissue Repair Techniques. In: Peterson L, Emans PJ, eds. *ICRS: Developing Insights in Cartilage Repair.* London: Springer-Verlag 2014:43-63.
5. Emans PJ, van Rhijn LW, Welting TJ, Cremers A, Wijnands N, Spaapen F, Voncken JW, Shastri VP. Autologous engineering of cartilage. *Proc Natl Acad Sci U S A.* 2010;107(8):3418-3423.
6. Emans PJ, Spaapen F, Surtel DA, Reilly KM, Cremers A, van Rhijn LW, Bulstra SK, Voncken JW, Kuijer R. A novel in vivo model to study endochondral bone formation; HIF-1alpha activation and BMP expression. *Bone.* 2007;40(2):409-418.
7. Bahney CS, Zondervan RL, Allison P, Theologis A, Ashley JW, Ahn J, Miclau T, Marcucio RS, Hankenson KD. Cellular biology of fracture healing. *J Orthop Res.* 2019;37(1):35-50.
8. Jansen EJ, Emans PJ, Guldemond NA, van Rhijn LW, Welting TJ, Bulstra SK, Kuijer R. Human periosteum-derived cells from elderly patients as a source for cartilage tissue engineering? *J Tissue Eng Regen Med.* 2008;2(6):331-339.
9. Nakahara H, Bruder SP, Goldberg VM, Caplan AI. In vivo osteochondrogenic potential of cultured cells derived from the periosteum. *Clin Orthop Relat Res.* 1990(259):223-232.
10. Mankin H, Mow V, Buckwalter J. *Articular cartilage structure, composition, and function.* Rosemont: AAOS; 2000.
11. Moreira-Teixeira LS, Georgi N, Leijten J, Wu L, Karperien M. Cartilage tissue engineering. *Endocr Dev.* 2011;21:102-115.
12. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. *Clin Orthop Relat Res.* 2001(391 Suppl):S26-33.
13. Roughley PJ, Mort JS. The role of aggrecan in normal and osteoarthritic cartilage. *J Exp Orthop.* 2014;1(1):8.
14. Haudenschild DR, Hong E, Yik JH, Chromy B, Morgelin M, Snow KD, Acharya C, Takada Y, Di Cesare PE. Enhanced activity of transforming growth factor beta1 (TGF-beta1) bound to cartilage oligomeric matrix protein. *J Biol Chem.* 2011;286(50):43250-43258.
15. Emans PJ, Pieper J, Hulsbosch MM, Koenders M, Kreijveld E, Surtel DA, van Blitterswijk CA, Bulstra SK, Kuijer R, Riesle J. Differential cell viability of chondrocytes and progenitor cells in tissue-engineered constructs following implantation into osteochondral defects. *Tissue engineering.* 2006;12(6): 1699-1709.
16. Gilbert SF, Migeon BR. D-valine as a selective agent for normal human and rodent epithelial cells in culture. *Cell.* 1975;5(1):11-17.
17. Janssen MP, Caron MM, van Rietbergen B, Surtel DA, van Rhijn LW, Welting TJ, Emans PJ. Impairment of the chondrogenic phase of endochondral ossification in vivo by inhibition of cyclooxygenase-2. *Eur Cell Mater.* 2017;34:202-216.
18. Caron MM, Emans PJ, Coolsen MM, Voss L, Surtel DA, Cremers A, van Rhijn LW, Welting TJ. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2012;20(10):1170-1178.
19. Welting TJ, Caron MM, Emans PJ, Janssen MP, Sanen K, Coolsen MM, Voss L, Surtel DA, Cremers A, Voncken JW, van Rhijn LW. Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater.* 2011;22:420-436; discussion 436-427.
20. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986;883(2):173-177.
21. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res.* 1982;9(4):247-248.
22. Kronenberg HM. Developmental regulation of the growth plate. *Nature.* 2003;423(6937):332-336.

23. 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.
24. Caron MM, Emans PJ, Cremers A, Surtel DA, Coolsen MM, van Rhijn LW, Welting TJ. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis Cartilage.* 2013;21(4):604-613.
25. Provot S, Kempf H, Murtaugh LC, Chung UI, Kim DW, Chyung J, Kronenberg HM, Lassar AB. Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development.* 2006;133(4): 651-662.
26. Caron MM, Emans PJ, Surtel DA, van der Kraan PM, van Rhijn LW, Welting TJ. BAPX-1/NKX-3.2 acts as a chondrocyte hypertrophy molecular switch in osteoarthritis. *Arthritis Rheumatol.* 2015;67(11): 2944-2956.
27. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB. Structure and function of aggrecan. *Cell Res.* 2002;12(1):19-32.
28. 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.
29. Palmer GD, Chao Ph PH, Raia F, Mauck RL, Valhmu WB, Hung CT. Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading. *Osteoarthritis Cartilage.* 2001;9(8):761-770.
30. 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):262-270.
31. Merceron C, Portron S, Vignes-Colombeix C, Rederstorff E, Masson M, Lesoeur J, Sourice S, Sinquin C, Colliec-Jouault S, Weiss P, Vinatier C, Guicheux J. Pharmacological modulation of human mesenchymal stem cell chondrogenesis by a chemically oversulfated polysaccharide of marine origin: potential application to cartilage regenerative medicine. *Stem Cells.* 2012;30(3):471-480.
32. French MM, Rose S, Canseco J, Athanasiou KA. Chondrogenic differentiation of adult dermal fibroblasts. *Ann Biomed Eng.* 2004;32(1):50-56.
33. Park JS, Woo DG, Yang HN, Lim HJ, Chung HM, Park KH. Heparin-bound transforming growth factor-beta3 enhances neocartilage formation by rabbit mesenchymal stem cells. *Transplantation.* 2008;85(4):589-596.
34. Tan Timur U, Caron M, van den Akker G, van der Windt A, Visser J, van Rhijn L, Weinans H, Welting T, Emans P, Jahr H. 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).
35. Rainbow RS, Won HK, Zeng L. The role of Nkx3.2 in chondrogenesis. *Front Biol (Beijing).* 2014;9(5): 376-381.
36. Hadler-Olsen E, Fadnes B, Sylte I, Uhlin-Hansen L, Winberg JO. Regulation of matrix metalloproteinase activity in health and disease. *FEBS J.* 2011;278(1):28-45.
37. Jackson RL, Busch SJ, Cardin AD. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev.* 1991;71(2):481-539.
38. Vlodavsky I, Abboud-Jarrous G, Elkin M, Naggi A, Casu B, Sasisekharan R, Ilan N. The impact of heparanese and heparin on cancer metastasis and angiogenesis. *Pathophysiol Haemost Thromb.* 2006;35(1-2):116-127.
39. Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. *Genes & development.* 2002;16(15):1990-2005.
40. Forsten-Williams K, Chu CL, Fannon M, Buczek-Thomas JA, Nugent MA. Control of growth factor networks by heparan sulfate proteoglycans. *Ann Biomed Eng.* 2008;36(12):2134-2148.
41. Acharya C, Yik JH, Kishore A, Van Dinh V, Di Cesare PE, Haudenschild DR. Cartilage oligomeric matrix protein and its binding partners in the cartilage extracellular matrix: interaction, regulation and role in chondrogenesis. *Matrix Biol.* 2014;37:102-111.
42. DiCesare PE, Morgelin M, Carlson CS, Pasumarti S, Paulsson M. Cartilage oligomeric matrix protein: isolation and characterization from human articular cartilage. *J Orthop Res.* 1995;13(3):422-428.
43. Rock MJ, Holden P, Horton WA, Cohn DH. Cartilage oligomeric matrix protein promotes cell attachment via two independent mechanisms involving CD47 and alphaVbeta3 integrin. *Mol Cell Biochem.* 2010;338(1-2):215-224.

44. Briggs MD, Hoffman SM, King LM, Olsen AS, Mohrenweiser H, Leroy JG, Mortier GR, Rimoin DL, Lachman RS, Gaines ES, et al. Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. *Nat Genet.* 1995;10(3):330-336.
45. Hecht JT, Nelson LD, Crowder E, Wang Y, Elder FF, Harrison WR, Francomano CA, Prange CK, Lennon GG, Deere M, et al. Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. *Nat Genet.* 1995;10(3):325-329.
46. Ishida K, Acharya C, Christiansen BA, Yik JH, DiCesare PE, Haudenschild DR. Cartilage oligomeric matrix protein enhances osteogenesis by directly binding and activating bone morphogenetic protein-2. *Bone.* 2013;55(1):23-35.
47. Emans PJ, Caron MM, van Rhijn LW, Welting TJ. Endochondral Bone Formation as Blueprint for Regenerative Medicine. In: Davies PJ, ed. *Tissue Regeneration - From Basic Biology to Clinical Application.* InTech; 2012:399-424.
48. Emans PJ, Caron MMJ, van Rhijn LW, Shastri VP, Welting TJM. Cartilage Tissue Engineering; Lessons Learned From Periosteum. *Tissue Science & Engineering.* 2011;S2:002.

Supplementary files

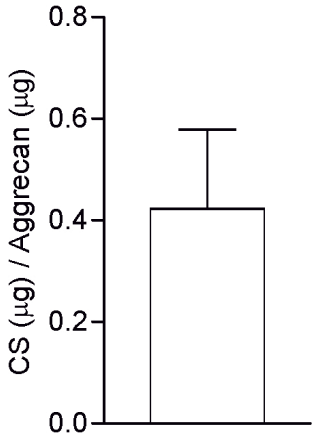

Figure S5.1 Glycosaminoglycan (GAG) content of bovine Aggrecan from articular cartilage. GAG content of bovine Aggrecan from articular cartilage used as supplement in this study was established by Dimethylmethylene Blue Assay (DMMB). The graph shows the chondroitin (CS) equivalent as a measure for GAG content per mass of Aggrecan.

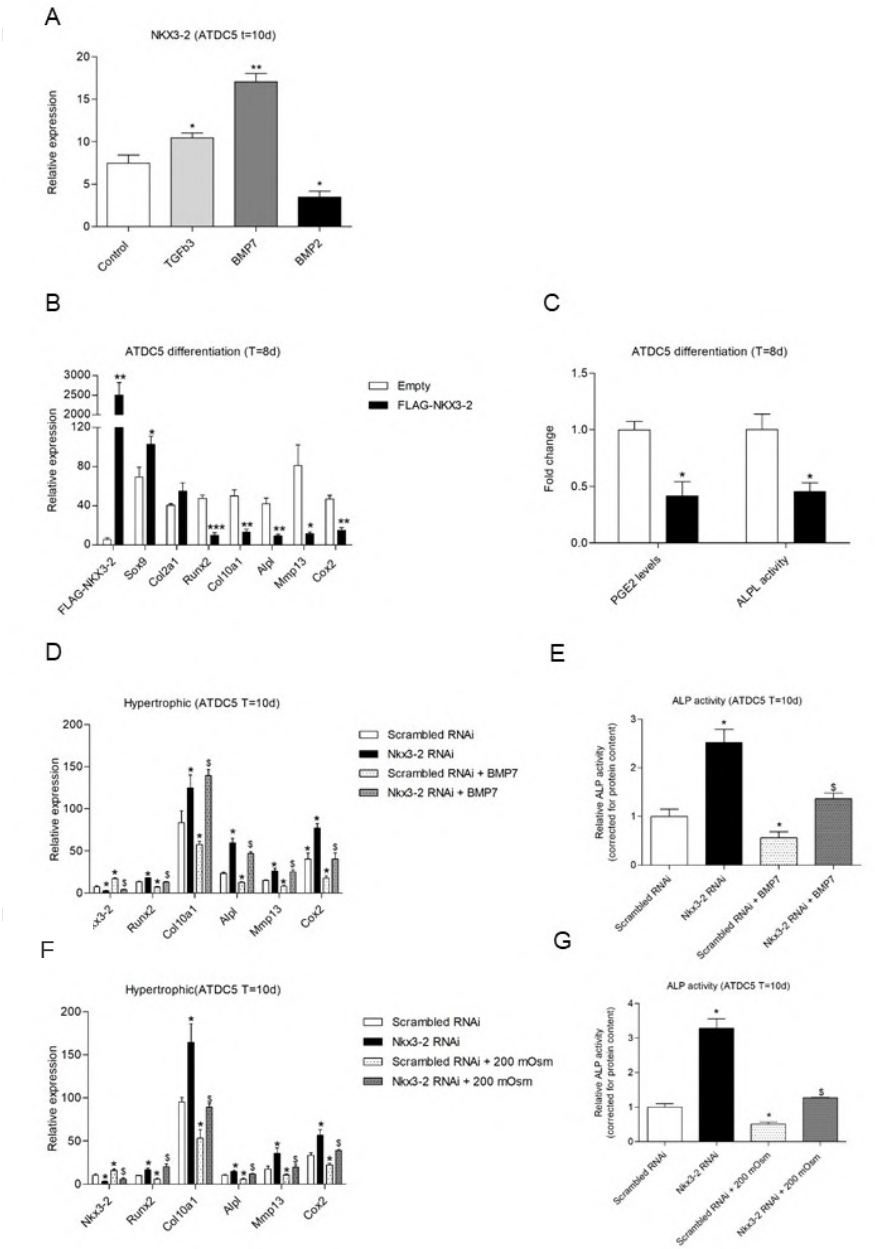

Figure S5.2 Data supporting a role for NKX3-2 in hypertrophic differentiation during chondrogenic differentiation of ATDC5 cells. These are data from experiments using the chondroprogenitor cell line ATDC5, which was differentiated in the chondrogenic lineage showing functional involvement of Nkx3-2 in hypertrophic differentiation in this model. The data show that expression of Nkx3-2 is increased at day 10 in ATDC5 differentiation by the growth factors TGFβ3 and BMP7, but decreased by BMP2 (Figure S5.2A). Overexpression of FLAG-Nkx3-2 by polyethyleneimine-mediated transfection of an Nkx3-2 p3XFLAG-CMV-7.1 expression vector

(1000 ng of plasmid/well) resulted in decreased expression of chondrocyte hypertrophic markers and decreased PGE2 levels in the culture supernatant, as well as reduced ALP enzyme activity (Figure S5.2B and S5.2C). Reducing Nkx3-2 levels by RNAi (100 nM siRNA; transient transfection on day 0 and 4 in ATDC5 differentiation) resulted in increased expression of hypertrophic markers Runx2, Col10a1, Alpl, Mmp13 and Cox-2, as well as functional ALP enzyme activity (Figures 2D-G; white vs. black bars). Addition of BMP7 (Figures S5.2 D/E; white dotted vs. grey dotted) or increasing the osmolarity of the culture medium (Figure S5.2 F/G; white dotted vs. grey dotted) with 200 mOsm (using NaCl) during chondrogenic differentiation reduced hypertrophic differentiation of ATDC5 cells in an Nkx3-2 dependent manner. Considering the conserved and central "switch" function of Nkx3-2 during the hypertrophic phase of chondrogenic differentiation (1-4), and combined with the above ATDC5 data, we speculate that a similar Nkx3-2-dependent mechanism might be active in rabbit periosteal chondrogenesis and potentially providing an underlying mechanism behind our observed COMP and Aggrecan-dependent modulation of chondrocyte hypertrophic differentiation.

References

1. Caron MM, Emans PJ, Cremers A, Surtel DA, Coolsen MM, van Rhijn LW, Welting TJ. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis Cartilage* 2013;21(4): 604-13.
2. Caron MM, Emans PJ, Surtel DA, van der Kraan PM, van Rhijn LW, Welting TJ. BAPX-1/NKX-3.2 acts as a chondrocyte hypertrophy molecular switch in osteoarthritis. *Arthritis Rheumatol* 2015;67(11): 2944-2956.
3. Provot S, Kempf H, Murtaugh LC, Chung UI, Kim DW, J. Chyung J, Kronenberg HM, Lassar AB. Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development* 2006;133(4): 651-662.
4. Yamashita Y, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Exp Cell Res* 2009;315(13):2231-2240.

Drugs and polymers for delivery systems in OA joints: Clinical needs and opportunities

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Abstract

Osteoarthritis (OA) is a big burden of disease worldwide and one of the most common causes of disability in the adult population. Currently applied therapies consist of physical therapy, oral medication, intra-articular injections, and surgical interventions with the main goal to reduce pain and improve function and quality of life. Intra-articular (IA) administration of drugs has potential benefits in OA treatment because it minimizes systemic bioavailability and side effects associated with oral administration of drugs without compromising the therapeutic effect in the joint. However, IA drug residence time is short and there is a clinical need for a vehicle that is able to provide a sustained release long enough for IA therapy to fulfil its promise. This review summarizes the use of different polymeric systems and the incorporated drugs for IA drug delivery in the osteoarthritic joint with a primary focus on clinical needs and opportunities.

Introduction

The osteoarthritic joint

Osteoarthritis (OA) is a progressive disease in which degeneration of joint cartilage and the underlying subchondral bone eventually may cause pain, stiffness, and some inflammation.
The precise cause of OA is unknown, but it is believed to be a combination of both mechanic and biological events affecting the joint.[1] OA mostly affects the knees, hips, hands, feet, and spine, but other joints can also be affected.[2,3] OA is the most common form of arthritis and the leading cause of chronic disability in the United States.[4] It ranks fourth in health impact in women and eighth in men in the western world (US and Europe).[5] Due to aging and increasing life expectancy, OA is expected to become the world's fourth-leading cause of disability in 2020.[6] Because effective treatments are lacking, it is a growing socio-economic problem. The costs (medical and productivity costs) are 871 euro per patient per month in the Netherlands.[6]

Current treatment

Currently available treatment options for OA primarily focus on pain relief and improving function. Non-pharmacologic therapy is widespread but differs per joint and the American College of Rheumatology (ACR) only strongly recommends weight loss if overweight, and participation in either cardiovascular or resistance exercise.[7] Pharmacologic therapy begins with oral administration of paracetamol either combined or substituted with NSAIDs or COX-2 inhibitors and a weak opioid (e.g., tramadol) depending on patient characteristics.[8] Major disadvantages of oral administration of these drugs are the limited bio-availability and the risk of side effects (e.g., liver damage, GI-ulcer/bleeding, and constipation). As OA has a localized nature, intra-articular administration of drugs provides an excellent opportunity to improve treatment. Glucocorticoid and hyaluronic acid (HA) injections are not impeded by the disadvantages of the oral route and are already common practice. However, although these injections provide a fairly good relief of symptoms and improve function over the short- and medium-term, there is little to no disease modification and the beneficial results are often not long-lived.
Therefore, to date OA continues to progress for almost all patients. At end-stage disease, surgical interventions, and finally joint replacement (e.g., total knee arthroplasty [TKA]) is indicated in many patients. However, the exponential increase in knee joint replacements is becoming an inevitable medical and economic problem.[9] The number of TKAs continues to grow each year and as these increase in number, the amount of revision TKAs continues to increase substantially as well.[10] While a primary TKA is cost-effective, revision surgery of TKA has a less favourable outcome for both the healthcare status of the patient and the economic benefit.[11] To prevent this situation a therapy that postpones primary joint arthroplasty is needed.

Clinical needs

To improve treatment of OA there is a need for new strategies. Development of disease modifying osteoarthritis drugs (DMOADs) is one of those strategies. The mechanism of action of DMOADs is directed at reducing, halting or reversing progression of OA or even preventing OA by either inhibiting different causative pathways (catabolic activity) or stimulating repair mechanisms (anabolic activity).[12] To date the pharmaceutical industry has failed to provide effective and safe DMOADs for clinical use.[13] The main reasons are that despite their specific targeted action DMOADs still can cause side effects when administered systemically,[14-16] or when injected intra-articular have a short residence time within the joint.[17,18] It remains unclear how long particular drugs have to remain in the joint for an effective pain relief and/or disease modification after an intra-articular injection. Without a drug delivery system (DDS) synovial disappearance time of a drug in the joint is often short and except for cross-linked HA usually drugs do not reside much longer than 24 hours.[18] Direct intra-articular drug delivery allows for an effective concentration where it is needed with a minimum of drugs. Moreover, it negates the main disadvantages of systemic administration; a low (oral) bioavailability or systemic side effects. However due to the rapid clearance of most intra-articular drugs injected, frequent injections would be needed to maintain an effective concentration.[19] Frequent intra-articular injections are undesired due to the pain and discomfort they may cause and the risk of introducing an infection to the joint. Therefore, a DDS for DMOADs combined with an intra-articular injection seems to be needed to cause prolonged drug residence time and a stable concentration within the therapeutic window with a single injection as compared to repeated injections in which the concentration may vary between a toxic and a subtherapeutic level (Figure 6.1). As a result, this leads to a reduction of side effects and may lead to an improved patient compliance.[20]

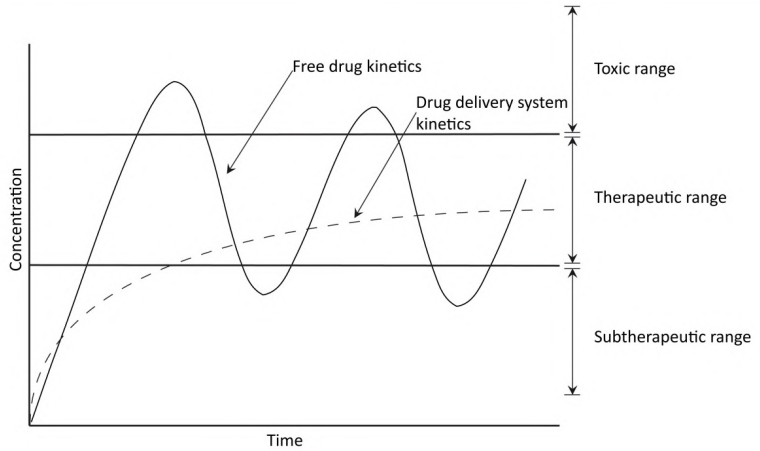

Figure 6.1 **Therapeutic window of administered drugs.** The solid line shows the release profile of a repeatedly dosed free drug with a high variation in available drug concentrations ranging from subtherapeutic to toxic levels. The dashed line shows a possible release profile of a drug delivery system which lies within the therapeutic range.

Furthermore, there is a need for diagnostic improvement, currently the role of biomarkers for diagnosis of OA is still under debate[21]. Regulatory approval in clinical trials still requires changes in radiographic joint space width and an impact on symptoms.[22,23] However, as MRI allows for direct visualization and measurements of cartilage[23,24] the FDA recently recognized the improvement of MRI as an OA imaging biomarker. Other OA associated processes (e.g., osteophytes, subchondral bone changes, and trabecular structure) can likewise be assessed by MRI.[13] With MRI different phenotypes of OA can be identified and the success of treatment may be tailored depending on the phenotype and its effect can be monitored in more detail.[25]
In this review we provide an overview of (candidate) drugs that are needed for an effective OA treatment and can be incorporated in a DDS and which polymers are required to provide for such system.

Candidate drugs for OA treatment

Many different drugs have been investigated for OA treatment. However, there are limitations to which drugs can be incorporated in a DDS. The incorporated drug has to be able to withstand the manufacturing process of the carrier vehicle (i.e., compression, heat, stirring, etc.). As the final goal of manufacturing these vehicles (particles) is injecting them intra-articularly, the DDSs have to be sterilized. Not only should the DDSs be able to withstand this process but so should the incorporated drugs.

NSAIDs, Coxibs, Glucocorticoids and Hyaluronan

Drugs currently used in DDSs in the OA joint are mostly derived from the drugs normally used in OA treatment (NSAIDs, Coxibs, Glucocorticoids and HA). Fourteen studies show incorporation of an NSAID[26-39] and two studies incorporated Celecoxib (Cxb)[40,41] in their carrier. Glucocorticoids were incorporated in six different studies[42-47] and HA in three.[48-50] An overview of these, and other studies is presented in Table 6.1.
The rationale for the use of these drugs is that their mechanism of action has been abundantly investigated in the perspective of OA treatment, their ability to give symptomatic relief and their potential to slow down disease progression. Moreover, these drugs have often already been approved by the FDA for parenteral administration which eases the regulatory process.
An important note however is that these drugs were developed and studied for use in oral OA treatment or an intra-articular injection without a DDS. Since then, great progress has been made in DDSs, and as such more other potential drugs may be used for treatment of OA. Due to systemic side effects, short half time, etc. many of these candidates have been thought not suitable for OA treatment in the past. With the introduction of different drug delivery systems DMOADs and other new candidate drugs may ultimately provide a more effective treatment.

Table 6.1

Author	Year	Type DDS	Composition	Drug	Particle diameter	Model	OA induction	Outcome
Ibim	1998	Microsphere	PolyPhosphazene	Colchicine	Not stated	*In vitro*	N.A.	Prolonged release, possible toxicity
Brown	1998	Microsphere	Gelatin/chondroitin 6-sulfate	14C-catalase, 14C-albumin, 14C-inulin, 14C-diazepam	1-60 µm	*In vitro*/ mice	None	Partially biocompatible
Tuncay	2000	Microsphere	PLGA	Diclofenac	5-10 µm	*In vitro*/ rabbit	Ovalbumin/ FCA	No significant difference in inflammation
Tuncay	2000	Microsphere	Albumin	Diclofenac	± 15 µm	*In vitro*/ rabbit	Ovalbumin/ FCA	Promising at day 30
Bozdag	2001	Microsphere	PLGA, albumin	Naproxen	10 µm	*In vitro*/ rabbit	Ovalbumin/ FCA	PLGA better than albumin
Bragdon	2001	Microsphere	PLGA	Paclitaxel	50 µm	*Ex vivo*/ horse MCP	None	Biocompatible
Horisawa	2002	Nano/ microsphere	PLGA	Fluoresceinamine	265 nm/ 26.5 µm	Rat	None	Phagocytosis is size dependent
Horisawa	2002	Nanosphere	PLGA	Betamethasone	300-490 nm	*In vitro*/ rabbit	Ovalbumin/ FCA	Prolonged efficacy
Liang	2003	Microsphere	PLLA	Methotrexate	83.7-187.6 µm	In vitro/ rabbit	None	Mild inflammation, prolonged release
Fernández-Carballido	2004	Microsphere	PLGA	Ibuprofen, PEG oil (Labrafil)	39.69 µm	*In vitro*	N.A.	Labrafil reduces burst release, prolonged release
Liggins	2004	Microsphere	PLGA, PLA, PCL, Chitosan	Paclitaxel	1-20 µm, 10-35 µm, 35-10 5µm	Rabbit	BSA/FCA, Carrageenan	Chitosan not biocompatible, small PLGA particles give greater inflammation.
Thakkar	2004	Microsphere	Chitosan	Celecoxib	8 µm	Rat	FCA	Chitosan is biocompatible, improved retention
Fernández-Carballido	2004	Microsphere	PLGA	Ibuprofen, PEG oil (Labrafil)	39.31 µm	*In vitro*	N.A.	Storage of PLGA/Ibuprofen particles does not change characteristics
Park	2005	Hydrogel	Hyaluronic acid	Hyaluronic acid Ultrasound	3000 kDA	Rabbit	ACLT/MT	Combination of HYA and US is more effective than monotherapy
Betre	2006	Aggregate	Elastin-like polypeptides	None	N.A.	Rat	None	Biocompatible, prolonged residence time

Table 6.1 (continued)

Author	Year	Type DDS	Composition	Drug	Particle diameter	Model	OA induction	Outcome
Tsai	2007	Nanosphere	Nanogold	None	5, 13 nm	Rat	Collagen	RA reduction
Zhang	2007	Micelle	PNIPAAm/EAB-PPP	Indomethacin	Not stated	*In vitro*/ rat	FCA, Carrageenan	Prolonged release/effect
Hui	2007	Hydrogel	α-CD-EG 4400	Chondroitin sulphate	N.A.	Rabbit	Chondral defect	Biocompatible, improved biomechanical and histologic properties
Lu	2007	Microsphere	Gelatin	Flurbiprofen	2.5-12.3 µm	Rabbit	None	Prolonged residence IA, biocompatibility unclear
Thakkar	2007	Nanoparticles	Glycerol behenate	Celecoxib	257 nm	Rat	FCA	Prolonged residence, biocompatible
Rothenfluh	2008	Nanoparticles	Poly(propylene sulphide)	WYRGRL (Col II-binding peptide)	38, 96 nm	Mice	None	Retention of the small particles in cartilage matrix
Butoescu	2008	Microparticles	PLGA	Dexamethasone / SPIONs	~ 10 µm	*In vitro*	N.A.	Possible to incorporate 2 active substances
Butoescu	2009	Microparticles	PLGA	Dexamethasone / SPIONs	1, 10 µm	Mice	None	Biocompatible, uptake of 1 and 10 µm particles, prolonged action of magnetic particles
Elron-Gross	2009	Collagomers	Collagen:DPPE	Diclofenac	Not stated	Rat	MIA	Better and sustained reduction of inflammation
Butoescu	2009	Microparticles	PLGA	Dexamethasone / SPIONs	~ 10 µm	Mice	N.A. (dorsal air pouch)	Sustained release, first order kinetics
Saravanan	2011	Microsphere	Gelatin	Diclofenac sodium	1-60 µm	Rabbit	None	Prolonged release
Zille	2010	Nanoparticles	PLGA, PLA, HA	FITC–dextran	Not stated	Rat	None	Weak hyperplasia, no inflammation
Zhang	2011	Microspheres	PLGA	Lornoxicam	7.47 µm	Rabbit/ rat	None	Prolonged retention
Panusa	2011	Microspheres	PLGA	Methylprednisolone	3-60 µm	Rat	Carrageenan	Prolonged retention, less inflammation
Zarnescu	2011	Liposomes	PC:DOPE:cholesterol:stearylamine	Chondroitin sulphate	Not stated	*In vitro*	N.A.	Interacts with collagen
Eswaramoorthy	2012	Microspheres	PLGA	Parathyroid hormone	51-85 µm	Rat	Papain/Cystein	Biocompatible, improved GAG and Col II levels
te Boekhorst	2012	Nanoparticles	PLGA	siRNA (against RA)	235-285 nm	Mice	Collagen antibody	Positive effect on RA depending on dose

Table 6.1 (continued)

Author	Year	Type DDS	Composition	Drug	Particle diameter	Model	OA induction	Outcome
Kawadkar	2012	Microspheres	Genipin cross-linked chitosan	Flurbiprofen	5.18-9.74 µm	Rat	Carrageenan	Biocompatible, prolonged retention
Zhang	2012	Microspheres	PLGA	Lornoxicam	Not stated	Rat	Papain	Biocompatible, effect comparable with weekly injections of Lornoxicam
Whitmire	2012	Nanoparticles	TEGM-CHM	Interleukin-1 Ra	300 nm	Rat	MIA	Prolonged retention, no negative effects on cartilage
Gaignaux	2012	Microparticles	PLGA	Clonidine	10-30 µm	*In vitro*	N.A.	Possible to incorporate small hydrophilic drug in PLGA
Présumey	2012	Microspheres	PLGA	Anti-TNF siRNA	23.5 µm	Mice	Collagen	Biocompatible, prolonged inhibition of TNA-α
Chen	2012	Microspheres/ hydrogel	Chitosan	Brucine	0.5-4.5 µm	Rat/rabbit	Collagenase	Prolonged retention of microsphere/hydrogel composite, inhibiting inflammation
Morgen	2012	Nanoparticles	Dextran propionate / MEH-PPV	Fluorescent labelled peptide	100-150 nm	Rat	None	Prolonged retention of peptide, biocompatible
Kawadkar	2013	Microspheres	Genipin cross-linked gelatin	Flurbiprofen	6.39 µm	Rat	Carrageenan	Biocompatible, prolonged release
Ryan	2013	Nanocomplex	HA-chitosan	Salmon calcitonin (sCT)	100-200 nm	Mice	K/BxN serum	sCT-HA-chitosan nanoparticles reduces inflammation and preserves bone and cartilage
Ko	2013	Microspheres	PLGA	Sulforaphane	14.5 µm	Rat	ACLT	Prolonged retention, inhibition of inflammation
Sandker	2013	Hydrogel	PCLA-PEG-PCLA	None	N.A.	Rat	None	Hydrogel degrades after 3+ weeks
Bédouet	2013	Microsphere	PLGA cross-linked PEG	None	40-100 µm	Sheep	None	Slow degradation, little inflammation from MS
Chen	2013	Nanoparticles in microspheres	PLGA-PVA	Brucine	12.38 µm	Rat	None	Prolonged retention, less burst release
Bédouet	2014	Microspheres	PEG-hydrogel	Ibuprofen	40-100 µm	*Ex vivo*/ sheep	LPS	Prolonged retention, less burst release, inhibition of inflammation

DMOADs

Pathological processes in OA consist of inflammation, cartilage degradation and subchondral bone changes[13]. Inflammation can be caused by a variety of cytokines such as Interleukins (ILs)[51], Tumour Necrosis Factors (TNFs), and Nitric Oxide (NO)[52] whereas cartilage degradation is mainly caused by enzymes such as Matrix Metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).[52] Furthermore a strong correlation between subchondral bone changes and OA development has been described.[53,54]

Based on their method of action roughly three groups of DMOADs can be identified: (i) inhibitors of degrading enzymes and inflammation, (ii) growth factors, and (iii) drugs which target subchondral bone changes. Most DMOADs are proteins or protein derived peptides with different properties when applied in therapeutic use (Table 6.2). Diffusion transport of proteins and large peptides is generally slow and due to their weak non-covalent interaction and fragile tertiary structure proteins usually have a low in vivo stability. Enzymatic or proteolytic degradation causes short half-lives when administered without a DDS. In addition, a DDS can protect the protein or peptide against degrading environmental factors when prepared or stored.[55] However, maintaining the structure and function of often fragile protein-based drugs during DDS processing, formulation, sterilization and subsequent degradation and release is far from trivial and as a result very few protein-based DDS products are on the market today. Peptides are already successfully incorporated in DDSs in other fields of research (e.g., Airway and Gastro-intestinal drug delivery).[56,57] These positive results are promising for the application of peptidal DMOADs in a DDS. Even DMOADs and drugs that can be administered systemically or by injection (bisphosphonates and Platelet-rich plasma (PRP) respectively) seem to benefit from a DDS.[58,59] These results also suggest that there might be a beneficial effect of targeting subchondral bone in OA treatment, but more evidence is needed, especially in drug delivery systems.

Cytostatic drugs

Cytostatic drugs are able to inhibit inflammation and can even be chondroprotective,[60] though they are not used in OA treatment because of their high toxicity and often severe side effects when administered systemically. Some studies however showed beneficial effects of IA administration of paclitaxel and methotrexate without apparent toxicity and side effects in an animal model.[61,62] In line with other classes of drugs there is potential for cytostatic drugs when administered via an intra-articular drug delivery system.[61]

When categorizing candidate drugs/DMOADs for use in a DDS, attention should be paid to their chemical nature and the possibilities to incorporate them in a drug delivery system. The complexity in designing effective DDSs for a certain drug increases with the size and complexity of that drug.

Table 6.2 Most investigated disease modifying osteoarthritis drugs (DMOADs) based on their target of action. The chemical nature of a DMOAD is important for incorporation in a DDS.

DMOADs	Chemical nature
Enzyme inhibitors	
MMP inhibitors (TIMP 1-4)	Protein/Peptide
Aggrecanase inhibitors (ADAMTS)	Small molecule
Cytokine inhibitors	
IL-1 inhibitors (IL-1 Ra)	Protein
TNF-α antagonists	Antibody
iNOS inhibitors	Various
Growth factors	
Fibroblast Growth Factor (FGF)-18	Protein/Peptide
Bone morphogenetic protein (BMP)-7	Protein/Peptide
Platelet-rich plasma (PRP)	Plasma
Drugs targeting subchondral bone	
Calcitonin	Peptide
Bisphosphonates	Bisphosphonate

Drug delivery systems

History

The importance of a drug delivery system has long been recognized. In the mid-1960s Folkman discovered that a silicone rubber tube acted as a constant rate drug delivery device in rabbit anaesthesia.[63] In 1987 Ratcliffe et al. provided the first evidence that (albumin) microspheres can delay clearance of a drug from the joint.[64] In the search for a method to provide an ideal (intra-articular) drug delivery system, many different carriers have been investigated. At first focus was on achieving a 'zero order release' usually in macroparticulate systems (e.g., ocular, vaginal, or trans- and, sub-dermal particles). In the 1980s and 1990s a gradual shift towards microparticles and a sustained or long-term drug release occurred[63]. From the 1990s and onwards the development of DDSs went a step further with the introduction of nanoparticles. Conventional techniques such as compression, spray and dip coating, and encapsulation can be used to incorporate drugs in a drug delivery system.[65]

DDSs can have a different structure and morphology, all with different characteristics in drug loading, release and response to the physiological environment (Figure 6.2). In addition, in the case of micro-particulate systems the size of the particles is also important as particles of 1-10μm could be taken up by synoviocytes probably through phagocytosis.[45] Depending on the goal of treatment this can be unwanted. Therefore, when designing a DDS, close attention should be paid to the drug that will be incorporated, physiological environment of the target location, biocompatibility and desired duration of drug release.

An ideal drug delivery system complies with adequate disease modification, biodegradability, and biocompatibility, while responding to feedback and its physiological environment.[65]

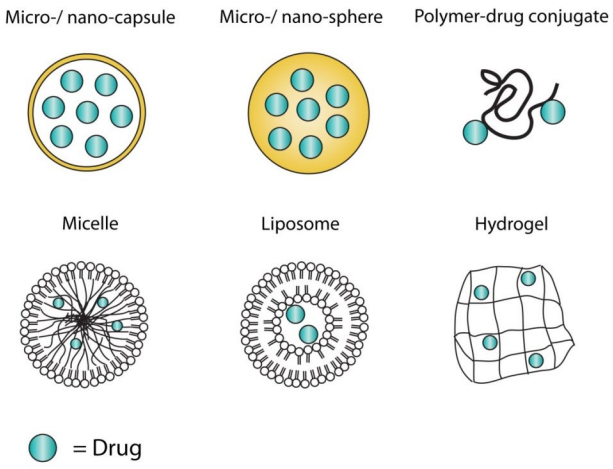

Figure 6.2 Different structures and morphology of DDSs (not-exhaustive). Each structure has its advantages and disadvantages to incorporate and release different types of drugs for intra-articular treatment of OA.

Hurdles in drug delivery system design

Using polymers for intra-articular drug delivery offers a great variety of opportunities to address OA-progression. However, poly(lactic-co-glycolic acid) (PLGA) and NSAIDs emerge more often in different studies, the field of polymers for intra-articular drug delivery is very fragmented. Particle size varies tremendously between particles of only a few nanometres and particles of more than 100µm. Different particle size results in different DDS kinetics and drug release statistics, particles smaller than 10 µm can readily be phagocytized by synoviocytes whereas particles larger than 20 µm can trigger a giant cell response, but not necessarily an inflammatory response. According to Butoescu et al. an optimal particle size for IA drug delivery would be between 5 and 10 µm.[66] Together with size, method of production of a DDS can influence drug characteristics where especially the large proteins are vulnerable to environmental challenges.[67] For clinical application biocompatibility of a drug and DDS in the joint is of great importance. Polyesters like Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and PLGA are already widely used and are deemed biocompatible in drug delivery, but their breakdown products are acidic and can lower the pH in the environment which subsequently can cause drug stability problems and inflammation of the surrounding tissue.[68] Ideally, a drug delivery system has to be fully degradable whereas residue from particles can also cause inflammation of the joint.

Polymers

To avoid inflammation of the injected joint, a polymer carrier has to be biocompatible. The largest group of carriers consists of biodegradable polymeric particles, as well from

natural, synthetic or combined origin. Polymeric particles have the big advantage that they can be altered to fit their purpose. Depending on manufacturing technique particles can either be microcapsules (a reservoir with a separate polymeric shell) or microspheres (matrix type with a homogenous mixture of a polymer and the encapsulated drug). The latter one having excellent sustained release characteristics.[69]
There is a great diversity in both DDSs and in the drugs encapsulated. Natural polymers are widely available and often biodegradable. However, reproducibility is low and they often have a high immunogenicity.[68] Natural polymers investigated for IA drug delivery include Chitosan which was shown to be able to incorporate Cxb or Flurbiprofen and extend their residence time in the joint,[28,41,70] Diclofenac Sodium loaded albumin microspheres provided a significant reduction of arthritis after 30 days of incubation in a rabbit knee,[38] gelatin microspheres are able to incorporate different NSAIDs or proteins and, Saravanan et al. found gelatin microspheres to be more stable than albumin, but residence times are still relatively short.[30,33,71]
Synthetic polymers in general are less biocompatible but their characteristics can easily be altered.[68] For IA drug delivery mostly the polymers that have proven to be biocompatible were investigated. PLA has been shown to be biocompatible in rabbit knees,[61,62] polyethylene glycol (PEG), often combined with other polymers (e.g., polycaprolactone (PCL)) is biocompatible and able to control release characteristics of the incorporated drug[72-75] however by far the most used synthetic polymer is PLGA. This synthetic polymer has a good biocompatibility and is able to incorporate many different types of drugs.[29,31,35-37,39,42-46,50,60,61,72-74,76-84] Several studies have been published on the incorporation of proteins in different DDSs, a common problem in the classical models (e.g., PLGA) however is the initial burst release which can cause local toxic drug concentrations and the acidic breakdown products can influence protein stability followed by a very slow or no release at all.[68,85,86]
The evolution of bio- degradable materials from aliphatic polyesters to nitrogen bearing polymers such as polyurethanes and polyester amides (PEAs) has been accompanied with better control over degradation and release properties. PEAs are based on α-amino acids, aliphatic dicarboxylic acids and aliphatic α-ω diols.[87] Among this class of polymers, it is the AA-BB hetero-chain polymers that offer the greatest versatility in terms of molecular level design to tailor drug release properties. Furthermore, the incorporation of amino acid-based building blocks offers more than providing metabolizable building blocks,[88,89] they provide one or more functional groups along the polymer chain. This allows further modification of the polymer to tailor its physicochemical properties and performance as drug eluting matrices. An important advantage of these polymers is related to the fact that by design they predominantly degrade via an enzymatic mechanism and due to consequential surface erosion, drug release follows nearly zero-order kinetics. PEAs are currently being applied in several developmental DDSs and are in clinical trials for a cardiovascular drug eluting stent.[90]

Liposomes

Liposomes are artificial vesicles composed of one or more concentric phospholipid bilayers and used especially to deliver microscopic drugs to body cells. Liposomes can be used as a carrier for intra-articular drug delivery, but far less research has been done on this carrier as compared to polymer-based microspheres. However, the first reports of liposomes as drug carriers appeared in the 1970s and there are still few results reported on liposomes for intra-articular application. In 2001 Trif et al. reported a positive effect of human Lactoferrin encapsulated in liposomes in collagen-induced arthritis in mice.[91] Elron-Gross et al. reported a reduction of inflammation in a monosodium iodoacetate (MIA) induced OA rat knee after a liposomal dexamethasone and diclofenac combination injection as compared to control assessed by MRI in 2009,[32,92] and Dong *et al.* found a combination of Cxb incorporated liposomes and HA to be more effective in pain control and cartilage protection than a single Cxb injection, Cxb liposome, and HA treatment alone.[93] Although liposomes are well established and are effective and biocompatible, IA residence time is relatively short compared to other DDSs.[18]

Hydrogels

Hydrogels are insoluble, water swollen, cross-linked, three-dimensional structures of polymer chains.[94] HA which is already common practice in many clinics can be seen as a hydrogel. Depending on its molecular weight and whether it is cross-linked or not HA has different characteristics. The working mechanism of HA is believed to depend on its viscosity, lubricity and restoring some of the normal joint physiology. Other than HA, only a few hydrogels are used for IA drug delivery. Bedouet et al. developed a PEG-hydrogel-Microsphere in order to minimize the amount of foreign material injected[73] and in another study by Bedouet et al. they sought to deal with the burst release of intra-articular DDSs by developing a methacrylate derivative of ibuprofen with a hydrophilic PEG-hydrogel which slowly released the ibuprofen.[72] Another method to deal with burst release was provided by Chen et al. by loading brucine in a chitosan microsphere and dispersed that microsphere in a chitosan hydrogel.[95] A more investigative approach was used by Sandker *et al.,* who incorporated 2-(2',3',5',-triiodobenzoyl) moieties (TIB) to make their poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-bpoly(ε-caprolactone-co-lactide) (PCLA-PEG-PCLA) hydrogel radiopaque for long term in vivo visualization.[75]

Discussion

Drug delivery systems have been around for about half a century. Since then, a number of new developments have been made, starting from macroscopic particulates to advanced nanometre sized DDSs that adapt to changes in their physiological environment. Since the discovery of polymeric DDSs as a therapeutic application a

massive increase in citations can be seen on PubMed[68] and an incredible amount of progress has been made in their development. However, it was not until 1987 that the pioneering work of Ratcliffe et al.[64] proposed a DDS for IA treatment of OA and this became an increasing field of interest in the late 1990s. As can be seen in Table 6.1 the most used polymer for DDSs is PLGA, Although PLGA is biocompatible and biodegradable and has been approved by the FDA many years ago, disadvantages are the initial burst release and the acidic microenvironment it creates on its breakdown which could cause inflammation and can lead to stability problems of the incorporated drugs (e.g., proteins).[68,96] The search for improvement of biocompatibility, release characteristics and drug incorporation led to an improved PLGA manufacturing process but also to the discovery of new polymers for intra-articular treatment of OA.[90,96]

The initial treatment was mainly focused on relieving OA symptoms. Most of the incorporated drugs were NSAIDs or glucocorticoids. Drugs which not only target symptoms but also the disease process of OA have been incorporated in DDS more recently. Incorporation of DMOADs is even harder as these drugs are still in a developmental stage and most DMOADs are proteins or peptides (Table 6.2) which makes them vulnerable to environmental challenges in the manufacturing process of DDSs.[13] As such a drug which targets pain such as NSAIDs or glucocorticosteroids released from a DDS are more likely to find their (clinical) application in the near future compared to DMOADs.
The search for the ideal osteoarthritic drug and a biocompatible and biodegradable DDS has been subject of many studies. The focus of most studies was mainly on optimization of DDSs and the ongoing development of the ideal drugs to target OA. To date, this has led to a few ongoing or completed clinical trials on the implementation of polymers for a DDS in OA treatment.[97]

Conclusions

The optimization of existing DDSs is ongoing and new DDSs are still being developed. It seems to be that the ideal DDS for intra-articular OA treatment has not yet been found. However, many hurdles in the developmental process have been taken care of and implementation of DDSs for clinical applications, such as ophthalmology, cardiology, oncology, etc., give us examples of the possibilities. Given the developments in the field of DDS and the increasing number of drugs that may be released from a DDS, it is expected that more clinical trials will start to fulfil the need for OA treatment with a DDS.

References

1. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clin Geriatr Med.* 2010;26(3):355-369.
2. van Saase JL, van Romunde LK, Cats A, Vandenbroucke JP, Valkenburg HA. Epidemiology of osteoarthritis: Zoetermeer survey. Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations. *Ann Rheum Dis.* 1989;48(4):271-280.
3. Buckwalter JA, Saltzman C, Brown T. The impact of osteoarthritis: implications for research. *Clin Orthop Relat Res.* 2004(427 Suppl):S6-15.
4. CDC. Prevalence of doctor-diagnosed arthritis and arthritis-attributable activity limitation --- United States, 2007-2009. *MMWR Morb Mortal Wkly Rep.* 2010;59(39):1261-1265.
5. Haq I, Murphy E, Dacre J. Osteoarthritis. *Postgrad Med J.* 2003;79(933):377-383.
6. Hermans J, Koopmanschap MA, Bierma-Zeinstra SM, van Linge JH, Verhaar JA, Reijman M, Burdorf A. Productivity costs and medical costs among working patients with knee osteoarthritis. *Arthritis Care Res (Hoboken).* 2012;64(6):853-861.
7. Hochberg MC, Altman RD, April KT, Benkhalti M, Guyatt G, McGowan J, Towheed T, Welch V, Wells G, Tugwell P. American College of Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. *Arthritis Care Res (Hoboken).* 2012;64(4):465-474.
8. Zhang W, Doherty M, Arden N, Bannwarth B, Bijlsma J, Gunther KP, Hauselmann HJ, Herrero-Beaumont G, Jordan K, Kaklamanis P, Leeb B, Lequesne M, Lohmander S, Mazieres B, Martin-Mola E, Pavelka K, Pendleton A, Punzi L, Swoboda B, Varatojo R, Verbruggen G, Zimmermann-Gorska I, Dougados M. EULAR evidence based recommendations for the management of hip osteoarthritis: report of a task force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). *Ann Rheum Dis.* 2005;64(5):669-681.
9. Bitton R. The economic burden of osteoarthritis. *The American journal of managed care.* 2009;15(8 Suppl):S230-235.
10. Dixon T, Shaw M, Ebrahim S, Dieppe P. Trends in hip and knee joint replacement: socioeconomic inequalities and projections of need. *Ann Rheum Dis.* 2004;63(7):825-830.
11. Lavernia C, Lee DJ, Hernandez VH. The increasing financial burden of knee revision surgery in the United States. *Clinical orthopaedics and related research.* 2006;446:221-226.
12. Pelletier JP, Martel-Pelletier J, Raynauld JP. Most recent developments in strategies to reduce the progression of structural changes in osteoarthritis: today and tomorrow. *Arthritis research & therapy.* 2006;8(2):206.
13. Qvist P, Bay-Jensen AC, Christiansen C, Dam EB, Pastoureau P, Karsdal MA. The disease modifying osteoarthritis drug (DMOAD): Is it in the horizon? *Pharmacol Res.* 2008;58(1):1-7.
14. King J, Zhao J, Clingan P, Morris D. Randomised double blind placebo control study of adjuvant treatment with the metalloproteinase inhibitor, Marimastat in patients with inoperable colorectal hepatic metastases: significant survival advantage in patients with musculoskeletal side-effects. *Anticancer Res.* 2003;23(1B):639-645.
15. Hudson MP, Armstrong PW, Ruzyllo W, Brum J, Cusmano L, Krzeski P, Lyon R, Quinones M, Theroux P, Sydlowski D, Kim HE, Garcia MJ, Jaber WA, Weaver WD. Effects of selective matrix metalloproteinase inhibitor (PG-116800) to prevent ventricular remodeling after myocardial infarction: results of the PREMIER (Prevention of Myocardial Infarction Early Remodeling) trial. *J Am Coll Cardiol.* 2006;48(1): 15-20.
16. Rudolphi K, Gerwin N, Verzijl N, van der Kraan P, van den Berg W. Pralnacasan, an inhibitor of interleukin-1beta converting enzyme, reduces joint damage in two murine models of osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2003;11(10):738-746.
17. Edwards SH. Intra-articular drug delivery: the challenge to extend drug residence time within the joint. *Vet J.* 2011;190(1):15-21.
18. Larsen C, Ostergaard J, Larsen SW, Jensen H, Jacobsen S, Lindegaard C, Andersen PH. Intra-articular depot formulation principles: role in the management of postoperative pain and arthritic disorders. *J Pharm Sci.* 2008;97(11):4622-4654.
19. Owen SG, Francis HW, Roberts MS. Disappearance kinetics of solutes from synovial fluid after intra-articular injection. *Br J Clin Pharmacol.* 1994;38(4):349-355.

20. Shuid AN, Ibrahim N, Mohd Amin MC, Mohamed IN. Drug delivery systems for prevention and treatment of osteoporotic fracture. *Curr Drug Targets.* 2013;14(13):1558-1564.
21. Lotz M, Martel-Pelletier J, Christiansen C, Brandi ML, Bruyere O, Chapurlat R, Collette J, Cooper C, Giacovelli G, Kanis JA, Karsdal MA, Kraus V, Lems WF, Meulenbelt I, Pelletier JP, Raynauld JP, Reiter-Niesert S, Rizzoli R, Sandell LJ, Van Spil WE, Reginster JY. Value of biomarkers in osteoarthritis: current status and perspectives. *Ann Rheum Dis.* 2013;72(11):1756-1763.
22. Committee for Medicinal Products for Human Use. Guideline on clinical investigation of medicinal products used in the treatment of osteoarthritis. 2010. http://www.ema.europa.eu (accessed 28 January 2014). Accessed.
23. Conaghan PG, Hunter DJ, Maillefert JF, Reichmann WM, Losina E. Summary and recommendations of the OARSI FDA osteoarthritis Assessment of Structural Change Working Group. *Osteoarthritis Cartilage.* 2011;19(5):606-610.
24. Pelletier JP, Cooper C, Peterfy C, Reginster JY, Brandi ML, Bruyere O, Chapurlat R, Cicuttini F, Conaghan PG, Doherty M, Genant H, Giacovelli G, Hochberg MC, Hunter DJ, Kanis JA, Kloppenburg M, Laredo JD, McAlindon T, Nevitt M, Raynauld JP, Rizzoli R, Zilkens C, Roemer FW, Martel-Pelletier J, Guermazi A. What is the predictive value of MRI for the occurrence of knee replacement surgery in knee osteoarthritis? *Ann Rheum Dis.* 2013;72(10):1594-1604.
25. Kinds MB, Marijnissen AC, Viergever MA, Emans PJ, Lafeber FP, Welsing PM. Identifying phenotypes of knee osteoarthritis by separate quantitative radiographic features may improve patient selection for more targeted treatment. *J Rheumatol.* 2013;40(6):891-902.
26. Serra Moreno J, Agas D, Sabbieti MG, Di Magno M, Migliorini A, Loreto MA. Synthesis of novel pyrrolyl-indomethacin derivatives. *Eur J Med Chem.* 2012;57:391-397.
27. Kawadkar J, Jain R, Kishore R, Pathak A, Chauhan MK. Formulation and evaluation of flurbiprofen-loaded genipin cross-linked gelatin microspheres for intra-articular delivery. *J Drug Target.* 2013;21(2):200-210.
28. Kawadkar J, Chauhan MK. Intra-articular delivery of genipin cross-linked chitosan microspheres of flurbiprofen: preparation, characterization, in vitro and in vivo studies. *Eur J Pharm Biopharm.* 2012;81(3):563-572.
29. 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):255-263.
30. Saravanan M, Bhaskar K, Maharajan G, Pillai KS. Development of gelatin microspheres loaded with diclofenac sodium for intra-articular administration. *J Drug Target.* 2011;19(2):96-103.
31. Zhang Z, Bi X, Li H, Huang G. Enhanced targeting efficiency of PLGA microspheres loaded with Lornoxicam for intra-articular administration. *Drug Deliv.* 2011;18(7):536-544.
32. 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.
33. Lu Y, Zhang G, Sun D, Zhong Y. Preparation and evaluation of biodegradable flubiprofen gelatin microspheres for intra-articular administration. *J Microencapsul.* 2007;24(6):515-524.
34. Zhang JX, Yan MQ, Li XH, Qiu LY, Li XD, Li XJ, Jin Y, Zhu KJ. Local delivery of indomethacin to arthritis-bearing rats through polymeric micelles based on amphiphilic polyphosphazenes. *Pharm Res.* 2007;24(10):1944-1953.
35. 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):443-456.
36. Fernandez-Carballido A, Herrero-Vanrell R, Molina-Martinez IT, Pastoriza P. Sterilized ibuprofen-loaded poly(D,L-lactide-co-glycolide) microspheres for intra-articular administration: effect of gamma-irradiation and storage. *J Microencapsul.* 2004;21(6):653-665.
37. 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.
38. 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):145-155.
39. Tuncay M, Calis S, Kas HS, Ercan MT, Peksoy I, Hincal AA. Diclofenac sodium incorporated PLGA (50:50) microspheres: formulation considerations and in vitro/in vivo evaluation. *Int J Pharm.* 2000;195(1-2):179-188.

40. Thakkar H, Kumar Sharma R, Murthy RS. Enhanced retention of celecoxib-loaded solid lipid nanoparticles after intra-articular administration. *Drugs R D.* 2007;8(5):275-285.
41. 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):549-557.
42. Panusa A, Selmin F, Rossoni G, Carini M, Cilurzo F, Aldini G. Methylprednisolone-loaded PLGA microspheres: a new formulation for sustained release via intra-articular administration. A comparison study with methylprednisolone acetate in rats. *J Pharm Sci.* 2011;100(11):4580-4586.
43. Butoescu N, Jordan O, Burdet P, Stadelmann P, Petri-Fink A, Hofmann H, Doelker E. 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):529-538.
44. Butoescu N, Jordan O, Petri-Fink A, Hofmann H, Doelker E. Co-encapsulation of dexamethasone 21-acetate and SPIONs into biodegradable polymeric microparticles designed for intra-articular delivery. *J Microencapsul.* 2008;25(5):339-350.
45. Butoescu N, Seemayer CA, Foti M, Jordan O, Doelker E. Dexamethasone-containing PLGA superparamagnetic microparticles as carriers for the local treatment of arthritis. *Biomaterials.* 2009;30(9):1772-1780.
46. Horisawa E, Hirota T, Kawazoe S, Yamada J, Yamamoto H, Takeuchi H, Kawashima Y. Prolonged anti-inflammatory action of DL-lactide/glycolide copolymer nanospheres containing betamethasone sodium phosphate for an intra-articular delivery system in antigen-induced arthritic rabbit. *Pharm Res.* 2002;19(4):403-410.
47. Pavanetto F, Genta I, Giunchedi P, Conti B, Conte U. Spray-dried albumin microspheres for the intra-articular delivery of dexamethasone. *J Microencapsul.* 1994;11(4):445-454.
48. Morgen M, Tung D, Boras B, Miller W, Malfait AM, Tortorella M. Nanoparticles for improved local retention after intra-articular injection into the knee joint. *Pharm Res.* 2013;30(1):257-268.
49. Park SR, Park SH, Jang KW, Cho HS, Cui JH, An HJ, Choi MJ, Chung SI, Min BH. The effect of sonication on simulated osteoarthritis. Part II: alleviation of osteoarthritis pathogenesis by 1 MHz ultrasound with simultaneous hyaluronate injection. *Ultrasound Med Biol.* 2005;31(11):1559-1566.
50. Zille H, Paquet J, Henrionnet C, Scala-Bertola J, Leonard M, Six JL, Deschamp F, Netter P, Verges J, Gillet P, Grossin L. Evaluation of intra-articular delivery of hyaluronic acid functionalized biopolymeric nanoparticles in healthy rat knees. *Biomed Mater Eng.* 2010;20(3):235-242.
51. Jotanovic Z, Mihelic R, Sestan B, Dembic Z. Role of interleukin-1 inhibitors in osteoarthritis: an evidence-based review. *Drugs Aging.* 2012;29(5):343-358.
52. Lee AS, Ellman MB, Yan D, Kroin JS, Cole BJ, van Wijnen AJ, Im HJ. A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene.* 2013;527(2):440-447.
53. Weinans H, Siebelt M, Agricola R, Botter SM, Piscaer TM, Waarsing JH. Pathophysiology of peri-articular bone changes in osteoarthritis. *Bone.* 2012;51(2):190-196.
54. Intema F, Hazewinkel HA, Gouwens D, Bijlsma JW, Weinans H, Lafeber FP, Mastbergen SC. In early OA, thinning of the subchondral plate is directly related to cartilage damage: results from a canine ACLT-meniscectomy model. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2010;18(5):691-698.
55. Tan ML, Choong PF, Dass CR. Recent developments in liposomes, microparticles and nanoparticles for protein and peptide drug delivery. *Peptides.* 2010;31(1):184-193.
56. Gupta S, Jain A, Chakraborty M, Sahni JK, Ali J, Dang S. Oral delivery of therapeutic proteins and peptides: a review on recent developments. *Drug Deliv.* 2013;20(6):237-246.
57. Jain A, Gulbake A, Shilpi S, Hurkat P, Jain SK. Peptide and protein delivery using new drug delivery systems. *Crit Rev Ther Drug Carrier Syst.* 2013;30(4):293-329.
58. Laslett LL, Kingsbury SR, Hensor EM, Bowes MA, Conaghan PG. Effect of bisphosphonate use in patients with symptomatic and radiographic knee osteoarthritis: data from the Osteoarthritis Initiative. *Ann Rheum Dis.* 2014;73(5):824-830.
59. Saito M, Takahashi KA, Arai Y, Inoue A, Sakao K, Tonomura H, Honjo K, Nakagawa S, Inoue H, Tabata Y, Kubo T. Intraarticular administration of platelet-rich plasma with biodegradable gelatin hydrogel microspheres prevents osteoarthritis progression in the rabbit knee. *Clinical and experimental rheumatology.* 2009;27(2):201-207.

60. Bragdon B, Bertone AL, Hardy J, Simmons EJ, Weisbrode SE. Use of an isolated joint model to detect early changes induced by intra-articular injection of paclitaxel-impregnated polymeric microspheres. *J Invest Surg.* 2001;14(3):169-182.
61. Liggins RT, Cruz T, Min W, Liang L, Hunter WL, Burt HM. Intra-articular treatment of arthritis with microsphere formulations of paclitaxel: biocompatibility and efficacy determinations in rabbits. *Inflamm Res.* 2004;53(8):363-372.
62. Liang LS, Jackson J, Min W, Risovic V, Wasan KM, Burt HM. Methotrexate loaded poly(L-lactic acid) microspheres for intra-articular delivery of methotrexate to the joint. *J Pharm Sci.* 2004;93(4):943-956.
63. Hoffman AS. The origins and evolution of "controlled" drug delivery systems. *J Control Release.* 2008;132(3):153-163.
64. Ratcliffe JH, Hunneyball IM, Wilson CG, Smith A, Davis SS. Albumin microspheres for intra-articular drug delivery: investigation of their retention in normal and arthritic knee joints of rabbits. *J Pharm Pharmacol.* 1987;39(4):290-295.
65. Liechty WB, Kryscio DR, Slaughter BV, Peppas NA. Polymers for drug delivery systems. *Annual review of chemical and biomolecular engineering.* 2010;1:149-173.
66. Butoescu N, Jordan O, Doelker E. Intra-articular drug delivery systems for the treatment of rheumatic diseases: a review of the factors influencing their performance. *Eur J Pharm Biopharm.* 2009;73(2): 205-218.
67. Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. *J Control Release.* 2003;90(3):261-280.
68. Grund S, Bauer M, Fischer D. Polymers in Drug Delivery-State of the Art and Future Trends. *Adv Eng Mater.* 2011;13(3):B61-B87.
69. Wang L, Liu Y, Zhang W, Chen X, Yang T, Ma G. Microspheres and microcapsules for protein delivery: strategies of drug activity retention. *Curr Pharm Des.* 2013;19(35):6340-6352.
70. Ryan SM, McMorrow J, Umerska A, Patel HB, Kornerup KN, Tajber L, Murphy EP, Perretti M, Corrigan OI, Brayden DJ. An intra-articular salmon calcitonin-based nanocomplex reduces experimental inflammatory arthritis. *J Control Release.* 2013;167(2):120-129.
71. Brown KE, Leong K, Huang CH, Dalal R, Green GD, Haimes HB, Jimenez PA, Bathon J. Gelatin/chondroitin 6-sulfate microspheres for the delivery of therapeutic proteins to the joint. *Arthritis and rheumatism.* 1998;41(12):2185-2195.
72. Bedouet L, Moine L, Pascale F, Nguyen VN, Labarre D, Laurent A. Synthesis of hydrophilic intra-articular microspheres conjugated to ibuprofen and evaluation of anti-inflammatory activity on articular explants. *Int J Pharm.* 2014;459(1-2):51-61.
73. Bedouet L, Pascale F, Moine L, Wassef M, Ghegediban SH, Nguyen VN, Bonneau M, Labarre D, Laurent A. Intra-articular fate of degradable poly(ethyleneglycol)-hydrogel microspheres as carriers for sustained drug delivery. *Int J Pharm.* 2013;456(2):536-544.
74. Gaignaux A, Reeff J, Siepmann F, Siepmann J, De Vriese C, Goole J, Amighi K. Development and evaluation of sustained-release clonidine-loaded PLGA microparticles. *Int J Pharm.* 2012;437(1-2): 20-28.
75. Sandker MJ, Petit A, Redout EM, Siebelt M, Muller B, Bruin P, Meyboom R, Vermonden T, Hennink WE, Weinans H. In situ forming acyl-capped PCLA-PEG-PCLA triblock copolymer based hydrogels. *Biomaterials.* 2013;34(32):8002-8011.
76. Chen Z, Liu D, Wang J, Wu L, Li W, Chen J, Cai BC, Cheng H. Development of nanoparticles-in-microparticles system for improved local retention after intra-articular injection. *Drug Deliv.* 2013.
77. Eswaramoorthy R, Chang CC, Wu SC, Wang GJ, Chang JK, Ho ML. Sustained release of PTH(1-34) from PLGA microspheres suppresses osteoarthritis progression in rats. *Acta Biomater.* 2012;8(6):2254-2262.
78. Horisawa E, Kubota K, Tuboi I, Sato K, Yamamoto H, Takeuchi H, Kawashima Y. Size-dependency of DL-lactide/glycolide copolymer particulates for intra-articular delivery system on phagocytosis in rat synovium. *Pharm Res.* 2002;19(2):132-139.
79. Ko JY, Choi YJ, Jeong GJ, Im GI. Sulforaphane-PLGA microspheres for the intra-articular treatment of osteoarthritis. *Biomaterials.* 2013;34(21):5359-5368.
80. Mountziaris PM, Sing DC, Chew SA, Tzouanas SN, Lehman ED, Kasper FK, Mikos AG. Controlled release of anti-inflammatory siRNA from biodegradable polymeric microparticles intended for intra-articular delivery to the temporomandibular joint. *Pharm Res.* 2011;28(6):1370-1384.
81. Mountziaris PM, Sing DC, Mikos AG, Kramer PR. Intra-articular microparticles for drug delivery to the TMJ. *J Dent Res.* 2010;89(10):1039-1044.

82. Mountziaris PM, Tzouanas SN, Sing DC, Kramer PR, Kasper FK, Mikos AG. Intra-articular controlled release of anti-inflammatory siRNA with biodegradable polymer microparticles ameliorates temporomandibular joint inflammation. *Acta Biomater.* 2012;8(10):3552-3560.
83. Presumey J, Salzano G, Courties G, Shires M, Ponchel F, Jorgensen C, Apparailly F, De Rosa G. PLGA microspheres encapsulating siRNA anti-TNFalpha: efficient RNAi-mediated treatment of arthritic joints. *Eur J Pharm Biopharm.* 2012;82(3):457-464.
84. te Boekhorst BC, Jensen LB, Colombo S, Varkouhi AK, Schiffelers RM, Lammers T, Storm G, Nielsen HM, Strijkers GJ, Foged C, Nicolay K. MRI-assessed therapeutic effects of locally administered PLGA nanoparticles loaded with anti-inflammatory siRNA in a murine arthritis model. *J Control Release.* 2012;161(3):772-780.
85. Giteau A, Venier-Julienne MC, Aubert-Pouessel A, Benoit JP. How to achieve sustained and complete protein release from PLGA-based microparticles? *Int J Pharm.* 2008;350(1-2):14-26.
86. Allison SD. Analysis of initial burst in PLGA microparticles. *Expert Opin Drug Deliv.* 2008;5(6):615-628.
87. L. Castaldo PC, G. Maglio, R. Palumbo. *Polymer Bulletin.* 1992;28:301.
88. Dias AJAA, Petit A, Inventors. Microparticles comprising a crosslinked polymer. 27 September 2007.
89. Dias AJAA, Plum BJM, Quaedvlieg PJLM, Wiertz RW, Inventors. Carbamate, thiocarbamate or carbamide comprising a biomolecular moiety. 15 May 2008.
90. Svelte Medical Systems. www.sveltemedical.com/news.php?pid=39&article=113. Accessed.
91. Trif M, Guillen C, Vaughan DM, Telfer JM, Brewer JM, Roseanu A, Brock JH. Liposomes as possible carriers for lactoferrin in the local treatment of inflammatory diseases. *Exp Biol Med (Maywood).* 2001;226(6):559-564.
92. Elron-Gross I, Glucksam Y, Margalit R. Liposomal dexamethasone-diclofenac combinations for local osteoarthritis treatment. *Int J Pharm.* 2009;376(1-2):84-91.
93. 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):285-290.
94. Kopecek J. Hydrogel biomaterials: a smart future? *Biomaterials.* 2007;28(34):5185-5192.
95. Chen ZP, Liu W, Liu D, Xiao YY, Chen HX, Chen J, Li W, Cai H, Cai BC, Pan J. Development of brucine-loaded microsphere/thermally responsive hydrogel combination system for intra-articular administration. *J Control Release.* 2012;162(3):628-635.
96. Athanasiou KA, Niederauer GG, Agrawal CM. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials.* 1996;17(2):93-102.
97. http://clinicaltrials.gov/. Accessed.

Celecoxib-loaded PEA microspheres as an auto regulatory drug-delivery system after intra-articular injection

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Abstract

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). Celecoxib release from PEA microspheres and inflammation responsive release of a small molecule from PEA was investigated in vitro. Inflammation responsive release of a small molecule from PEA was observed when PEA was exposed to cell lysates obtained from a neutrophil-like Hl-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 >80 days. To investigate biocompatibility and degradation behaviour 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_2 measurements in total rat knee homogenates. 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 behaviour. In conclusion, this study suggests the potential of celecoxib-loaded PEA microspheres to be used as a safe drug delivery system with auto regulatory behaviour for treatment of pain associated with OA of the knee.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and constitutes a large medical healthcare economic burden worldwide, leading to pain and physical disability.[1] 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 gastro-intestinal and cardiac adverse effects.[2,3] 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.[4] Because OA is a chronic disease characterized by a variation in inflammation intensity[4] 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.[5] 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 behaviour is a polyester amide (PEA) based injectable microsphere formulation. PEA polymers are based on α-amino acids, aliphatic dicarboxylic acids, and aliphatic α-ω diols.[6] 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 α-chymotrypsin, elastase, papain and protease K, which are enzymes belonging to the serine protease family.[7-11] 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.[12,13]

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.[14] 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.[15] PEA has already been demonstrated to have a good biocompatibility, is applied clinically in drug eluting stents and is being investigated for ophthalmologic indications.[16,17] 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*.

Materials & methods

Synthesis of polymer and preparation of PEA microspheres

PEA was synthesized in accordance to procedures reported previously.[16,18,19] The selected PEA is depicted on Figure 7.1 and it comprises three types of building blocks randomly distributed along the polymer chain. Polymer characterization can be found in Table 7.1. 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°C until being used.

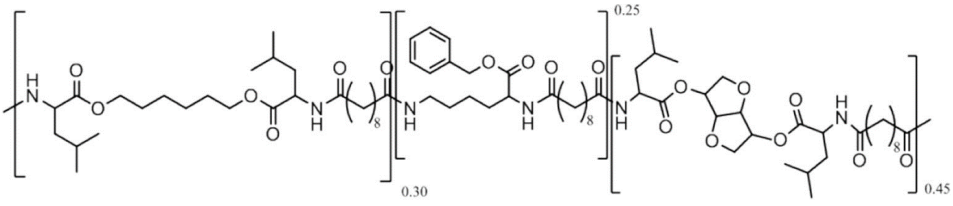

Figure 7.1 Structure of PEA III Ac Bz, random copolymer consisting of building blocks A, B and C.

Table 7.1 Polymer characterization. The relative ratio between the polymer building blocks was determined by ^{1}H NMR. Tg of the polymer was determined under dry conditions.

	Mn (kDa)	Polydispersity index (PDI)	Glass transition temperature (Tg)	Relative ratio A:B:C
PEA III Ac Bz	55	1.6	57 °C	0.31:0.26:0.43

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.

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.

Hl-60 cell culture and cell lysate

The promyelocytic cell-line Hl-60 (98070106) was purchased at the European Collection of Cell Cultures (ECACC). Hl-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_2 without any shaking and were passaged every 2–3 days to keep cell concentration between $0.1 * 10^6$ and $1 * 10^6$ cells/mL. During passaging, cells were centrifuged a $300 \times g$ and seeded in fresh full medium at a concentration of $0.1–0.2 * 10^6$ cells/mL.

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.

Differentiation

Hl-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 $0.2 * 10^6$ cells/mL at the start of the differentiation process and reached $\pm 1 * 10^6$ cells/mL after 5 days.

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 ± 100% lysed cells were obtained.

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 mould, 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 analysed for residual ethanol by ^{1}H NMR analysis in $CDCl_3$, 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 Hl-60 neutrophil like cell lysate was added for a period of 7 days. Next, a serine protease inhibitor 4-(2-aminoethyl)benzene sulfonyl fluoride hydrochloride (AEBSF) (0.5 mM) was added to the lysate solution for a duration of 14 days and finally a Hl-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.

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°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°C.

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 µL of AEBSF (Sigma Aldrich) (10 mM) or 10 µL of a Tris-HCl buffer (10 mM Tris-HCl, pH 7.8, 0.2 mM sodium azide) was added to 100 µL of synovial fluid (ten times diluted in 1 × digestion buffer), synovium conditioned medium samples, positive control (1 µg/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 labelled 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.

In vivo studies

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.[20] 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 Vicryl 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.

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_2 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 7.2)

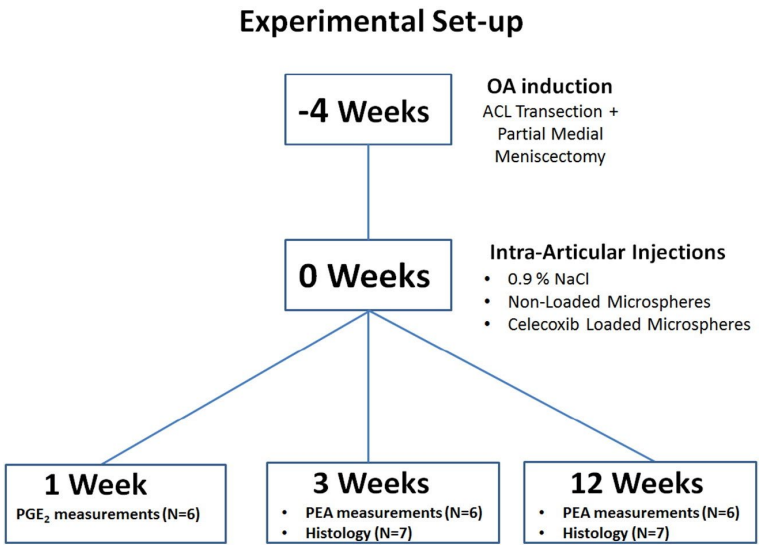

Figure 7.2 Experimental set-up *in vivo* study. OA was induced in the right knee of 12 weeks old Lewis rats by ACLT + pMMx surgery. After 4 weeks, OA-induced and contralateral healthy knees were injected with 0.9% NaCl (control), non-loaded microspheres and celecoxib-loaded microspheres. One week after intra-articular injections rats were sacrificed for PGE_2 measurements. Directly, three and twelve weeks after intra-articular injections rats were sacrificed for PEA measurements and histology.

PGE_2 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 µM indomethacin per 100 mg of tissue. Tissue homogenates were then spun down at 15,000 rpm for 10 min after which 50 µL of the supernatant was used for PGE_2 measurements with an ELISA (Cayman Chemical) according to the manufacturer's instructions. PGE_2 content (in mg) was estimated from a calibration curve PGE_2.

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 hydrolysed by adding 8 mL of 6 N HCl and incubated at 90°C for ± 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).

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 moulds. Polymerization solution, prepared according to the protocol of the manufacturer, was poured into the moulds 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 moulds. 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 Thionine staining for routine histological examination by light microscopy (Axioscope A1, Axiovision LE release 4.8.2, Carl Zeiss, Germany).

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).

OA scoring

Thionine stained sections were scored according to the OARSI histopathology initiative for the rat.[20] 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.[20] Measurements of parameters needed for the OARSI score were made using the Axiovision software.

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_2 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 analysed using a Kruskal-Wallis test.

Results

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 Hl-60 neutrophil like cell line, which is a commonly used model to study neutrophil functions and inflammatory cell responses.[21] 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 Hl-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 7.3). After zero order release kinetics was reached, a Hl-60 cell lysate was added to the fluorescein loaded polymer. As shown in Figure 7.2, 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.

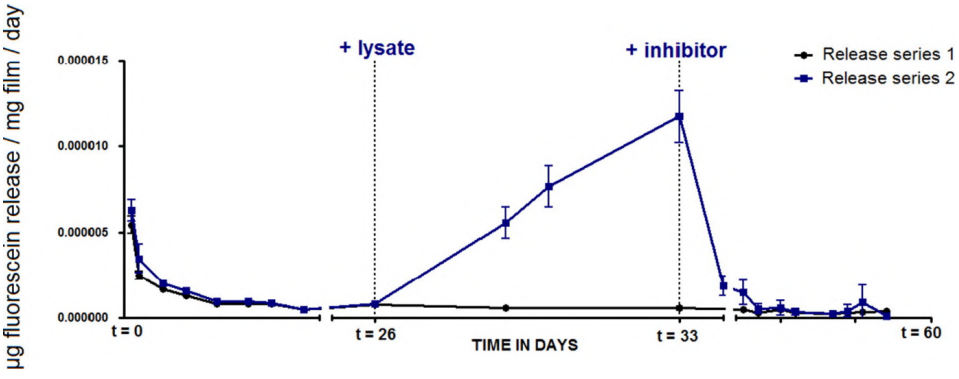

Figure 7.3 Inflammation responsive release of a small molecule from PEA. PEA was loaded with fluorescein and incubated in PBS buffer for 60 days (release series 1). Alternatively, fluorescein loaded PEA was incubated in PBS buffer, a Hl-60 neutrophil like cell lysate was added after 26 days and a Hl-60 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 ± SD, N = 3 per time point.

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 7.4).

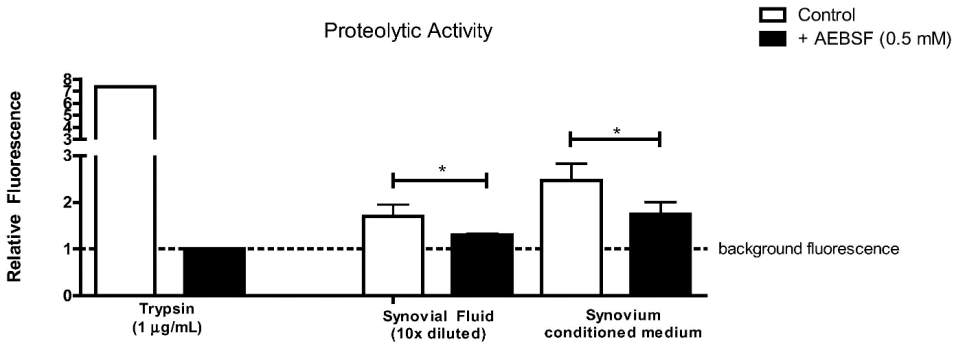

Figure 7.4 Serine protease activity in synovial fluid and synovial tissue from OA patients. Synovial fluid (ten times diluted) or synovium conditioned media were incubated with a fluorescein labelled casein and fluorescence was measured 24 h after incubation. Trypsin (1 µg/mL) was used as a positive control.

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 7.5A). Particle size was found to be 10–100 µm (Figure 7.5B).
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 7.5C).
Celecoxib release and bioactivity *in vivo* were evaluated by measuring PGE_2 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_2 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 7.5D).

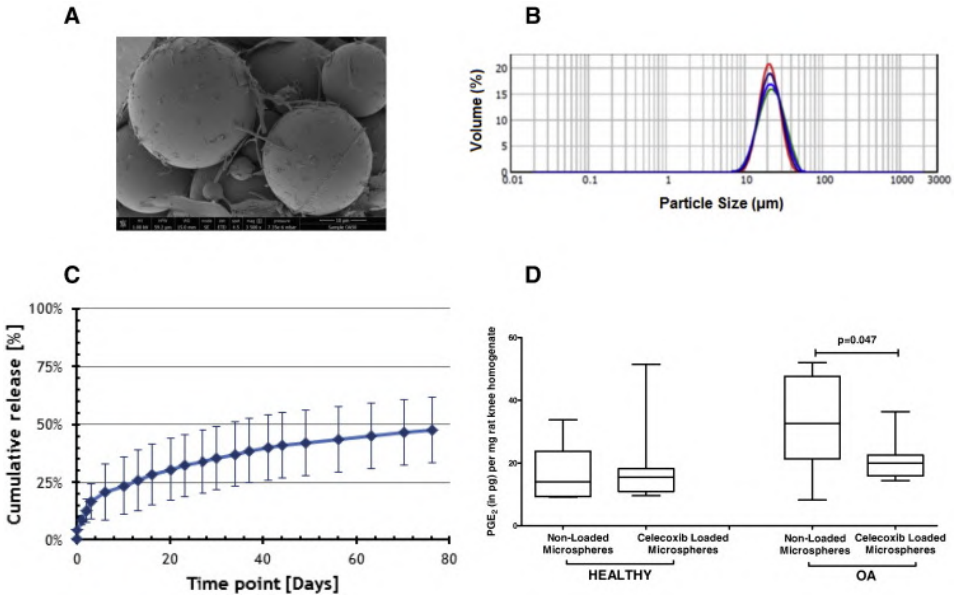

Figure 7.5 Celecoxib from celecoxib-loaded PEA microspheres is released *in vitro* and bioactive *in vivo*. Scanning electron microscope image of PEA microspheres (**A**) and size distribution of PEA microspheres (**B**). The concentration of celecoxib release in PBS medium as determined by HPLC throughout time. The release is expressed in percentage of theoretical celecoxib load (**C**). PGE_2 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**).

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 7.6A). Microspheres were surrounded by mononuclear inflammatory cells and giant cells (Figure 7.6B and 7.6C). Small microspheres 5 µm in size where seen, but also bigger microspheres 15–20 µm 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 7.2).

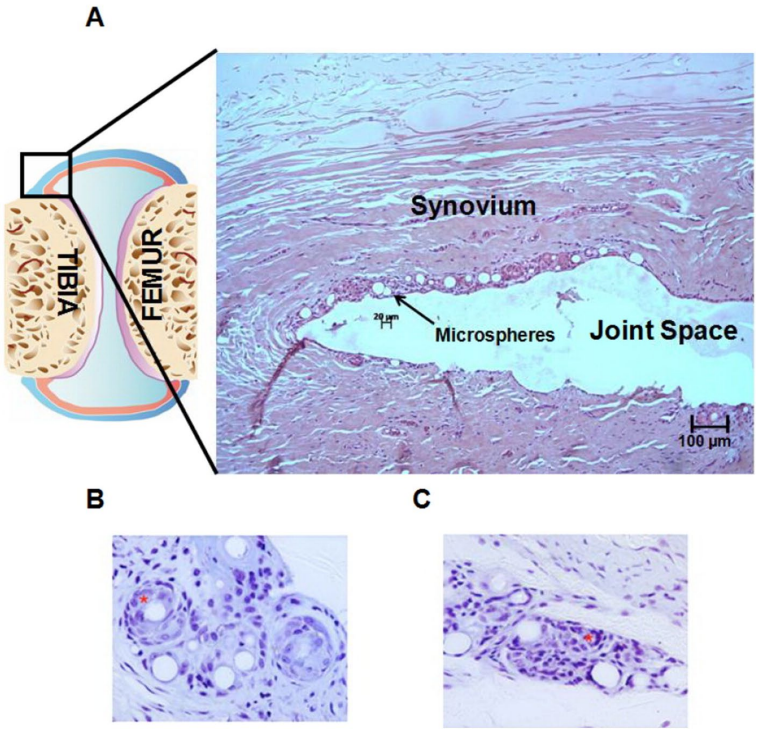

Figure 7.6 Entrapment of microspheres in the synovium. Haematoxylin & eosin-stained section of healthy knee 3 weeks after injection with non-loaded microspheres (**A**). Thionine-stained sections of OA-induced knees 3 weeks after injection non-loaded microspheres (40 × magnification) (**B**) or celecoxib-loaded microspheres (40 × magnification) (**C**). Red asterisks in (B) and (C) depict a giant cell.

Table 7.2 Semi-quantitative histological assessment of the number of giant cells, macrophages or fibroblast like cells surrounding the microspheres. Numbers in table indicate number 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**
OA knees						
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
Healthy knees						
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

Twelve 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**
OA knees						
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
Healthy knees						
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

PEA microspheres degrade *in vivo* and show auto-regulatory degrading behaviour

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 7.7A).

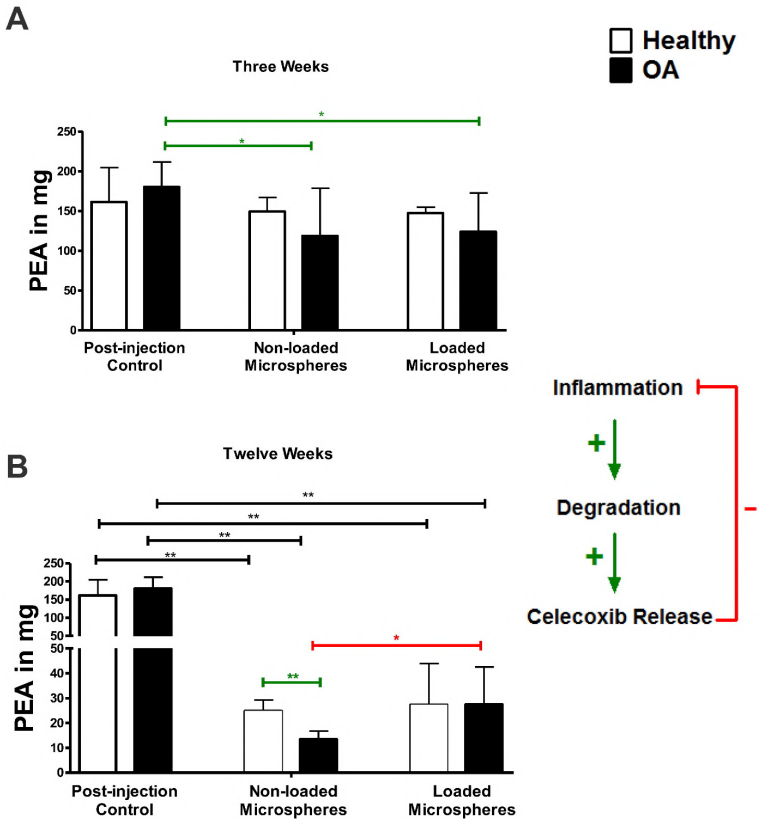

Figure 7.7 *In vivo* degradation of PEA microspheres and auto regulatory behaviour of celecoxib-loaded PEA microspheres. PEA levels in knees measured directly after injection (post-injection control), three weeks and twelve weeks after injection. Data are mean ± SEM, n=6. * $p<0.05$, ** $p<0.005$. Because 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 7.7B).
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 7.7B). 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 7.7B).

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 7.8), while OA-induced knees displayed OA-like changes twelve weeks after intra-articular injections (sixteen weeks after OA-induction) (Figure 7.8). No significant difference was observed in the OARSI cartilage degeneration score (Figure 7.9) between rats injected with 0.9% NaCl, non-loaded microspheres and celecoxib-loaded microspheres.

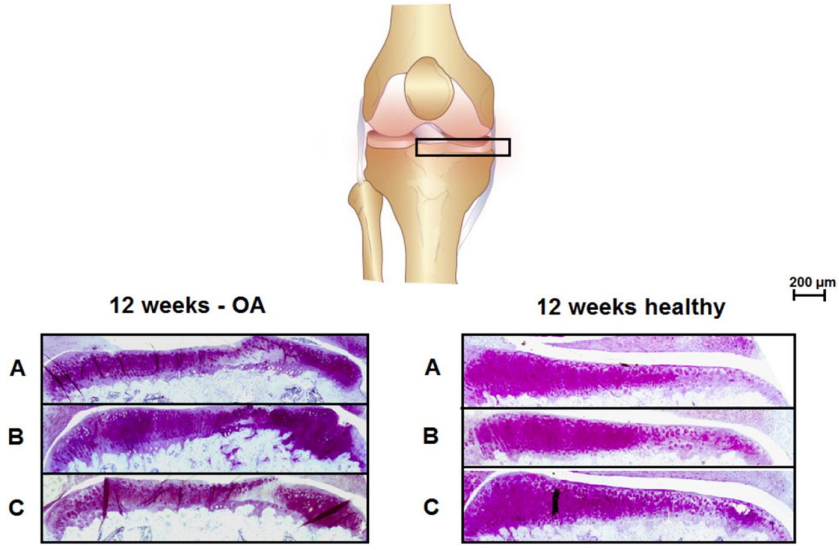

Figure 7.8 Twelve weeks after Intra-articular injections with microspheres in OA-induced or healthy knees. Thionine-stained Histological sections of the medial tibial plateau of OA (left panel, 16 weeks after OA induction) or healthy knees (right panel) 12 weeks after injections with 0.9% NaCl (A), non-loaded microspheres (B) and celecoxib-loaded microspheres (C). OA severity was comparable between all groups and injections with microspheres did not result in cartilage damage.

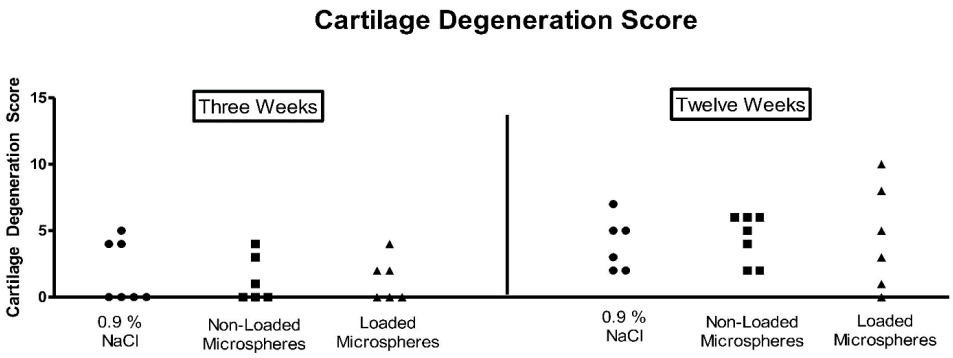

Figure 7.9 Effects of intra-articular injections with celecoxib on cartilage pathology in OA-induced rat knees. Thionine-stained histological sections of the medial tibial plateau of right rat knees were scored for cartilage degeneration score at 3 and 12 weeks after intra-articular injection with 0.9% 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.

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 Hl-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,[12] 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 behaviour 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.[22,23] The synovial membrane displays ability to remove foreign particles, which is the most likely explanation for this phenomenon we observed.[24] Macrophages have been shown to take up particles in the micrometre size range [25]: 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 behaviour.

Different carriers such as microspheres, liposomes and hydrogels have been investigated in intra-articular treatment with use of synthetic and natural polymers.[26-37] While most studies report intra-articular biocompatibility of the DDS investigated, information about degradation behaviour 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 behaviour *in vivo*.[22] In another study performed by Sandker et al., an acyl-capped PCLA-PEG-PCLA polymer based hydrogel depot was investigated.[38] 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.[39] We here show retention of PEA microspheres for at least 12 weeks after intra-articular injection and in addition show an auto regulatory degradation behaviour of PEA microspheres: an intra-articular DDS degradation mechanism not earlier described. This auto regulatory degradation behaviour 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_2 measurements in total rat knee homogenates. Rat knees

injected with celecoxib-loaded PEA microspheres contained significantly lower PGE_2 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.[40] 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.[41] 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 labelling drugs such as celecoxib and examining release behaviour *in vivo* may provide valuable data to predict release behaviour 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.[42-44] 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*.[32,45] 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.[46] Other models which display more inflammation,[47,48] may be useful to investigate the role of celecoxib on (synovial) inflammation.

Conclusion

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 behaviour. 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.

References

1. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clin Geriatr Med.* 2010;26(3):355-369.
2. De Vecchis R, Baldi C, Di Biase G, Ariano C, Cioppa C, Giasi A, Valente L, Cantatrione S. Cardiovascular risk associated with celecoxib or etoricoxib: A meta-analysis of randomized controlled trials which adopted comparison with placebo or naproxen. *Minerva Cardioangiologica.* 2014;62(6):437-448.
3. Mantry PS, Shah A, Sundaram U. Celecoxib associated esophagitis: Review of gastrointestinal side effects from COX-2 inhibitors. *Journal of Clinical Gastroenterology.* 2003;37(1):61-63.
4. Martel-Pelletier J. Pathophysiology of osteoarthritis. *Osteoarthritis and Cartilage.* 2004;12(SUPLL.): S31-S33.
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, Corbo P, Maglio G, Palumbo R. Synthesis and preliminary characterization of polyesteramides containing enzymatically degradable amide bonds. *Polymer Bulletin.* 1992;28(3): 301-307.
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):3453-3462.
8. Murase SK, Lv LP, Kaltbeitzel A, Landfester K, del Valle LJ, Katsarava R, Puiggali J, Crespy D. Amino acid-based poly(ester amide) nanofibers for tailored enzymatic degradation prepared by miniemulsion-electrospinning. *RSC Advances.* 2015;5(68):55006-55014.
9. Botines E, Franco L, Puiggalí J. Thermal stability and degradation studies of alternating poly(ester amide)s derived from glycolic acid and ω-amino acids. *Journal of Applied Polymer Science.* 2006;102(6):5545-5558.
10. Fan Y, Kobayashi M, Kise H. Synthesis and biodegradability of new polyesteramides containing peptide linkages. *Polymer Journal.* 2000;32(10):817-822.
11. Saotome Y, Miyazawa T, Endo T. Novel Enzymatically Degradable Polymers Comprising Alpha-Amino-Acid, 1,2-Ethanediol, and Adipic Acid. *Chem Lett.* 1991(1):21-24.
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):45.
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. *Clinical and Experimental Rheumatology.* 1999;17(2):161-170.
14. Williams GW, Hubbard RC, Yu SS, Zhao W, Steven Geis G. Comparison of once-daily and twice-daily administration of celecoxib for the treatment of osteoarthritis of the knee. *Clinical Therapeutics.* 2001;23(2):213-227.
15. Brophy JM. Celecoxib and cardiovascular risks. *Expert Opinion on Drug Safety.* 2005;4(6):1005-1015.
16. Andrés-Guerrero V, Zong M, Ramsay E, Rojas B, Sarkhel S, Gallego B, de Hoz R, Ramírez AI, Salazar JJ, Triviño A, Ramírez JM, del Amo EM, Cameron N, de-las-Heras B, Urtti A, Mihov G, Dias A, Herrero-Vanrell R. Novel biodegradable polyesteramide microspheres for controlled drug delivery in Ophthalmology. *Journal of Controlled Release.* 2015;211:105-117.
17. Liu X. 'Convention and exhibition economic development positioning and strategy selection of Nanjing'. *Social Science in Nanjing.* 2003;3:86-90.
18. Katsarava R, Beridze V, Arabuli N, Kharadze D, Chu CC, Won CY. Amino Acid-Based Bioanalogous Polymers. Synthesis, and Study of Regular Poly(ester amide)s Based on Bis(a-amino acid) α,ω-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 GCM, van Dijk M, Schumann D, Bos C, Strijkers GJ, Dhert WJA, Meij BP, Creemers LB, Tryfonidou MA. 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.* 2017;105(4):707-714.
20. Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the rat. *Osteoarthritis and Cartilage.* 2010;18:S24-S34.
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):108-117.

22. Bédouet L, Pascale F, Moine L, Wassef M, Ghegediban SH, Nguyen V-N, Bonneau M, Labarre D, Laurent A. Intra-articular fate of degradable poly(ethyleneglycol)-hydrogel microspheres as carriers for sustained drug delivery. *Int J Pharm.* 2013;456(2):536-544.
23. Horisawa E, Kubota K, Tuboi I, Sato K, Yamamoto H, Takeuchi H, Kawashima Y. Size-dependency of DL-lactide/glycolide copolymer particulates for intra-articular delivery system on phagocytosis in rat synovium. *Pharm Res.* 2002;19(2):132-139.
24. Iwanaga T, Shikichi M, Kitamura H, Yanase H, Nozawa-Inoue K. Morphology and Functional Roles of Synoviocytes in the Joint. *Archives of Histology and Cytology.* 2000;63(1):17-31.
25. Tuovinen L, Ruhanen E, Kinnarinen T, Rönkkö S, Pelkonen J, Urtti A, Peltonen S, Järvinen K. Starch acetate microparticles for drug delivery into retinal pigment epithelium—in vitro study. *Journal of Controlled Release.* 2004;98(3):407-413.
26. Trif M, Guillen C, Vaughan DM, Telfer JM, Brewer JM, Roseanu A, Brock JH. Liposomes as Possible Carriers for Lactoferrin in the Local Treatment of Inflammatory Diseases. *Exp Biol Med (Maywood).* 2001;226(6):559-564.
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):255-263.
28. Tunçay M, Çaliş S, Kaş HS, Ercan MT, Peksoy I, Hincal AA. In vitro and in vivo evaluation of diclofenac sodium loaded albumin microspheres. *J Microencapsul.* 2000;17(2):145-155.
29. Bozdağ S, Çaliş S, Kaş 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):443-456.
30. Fernández-Carballido A, Herrero-Vanrell R, Molina-Martínez 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):33-41.
31. Thakkar H, Sharma RK, Mishra AK, Chuttani K, Murthy RSR. Celecoxib Incorporated Chitosan Microspheres: In Vitro and In Vivo Evaluation. *J Drug Target.* 2004;12(9-10):549-557.
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):285-290.
33. Butoescu N, Jordan O, Burdet P, Stadelmann P, Petri-Fink A, Hofmann H, Doelker E. Dexamethasone-containing biodegradable superparamagnetic microparticles for intra-articular administration: Physicochemical and magnetic properties, in vitro and in vivo drug release. *European Journal of Pharmaceutics and Biopharmaceutics.* 2009;72(3):529-538.
34. Elron-Gross I, Glucksam Y, Biton IE, Margalit R. A novel Diclofenac-carrier for local treatment of osteoarthritis applying live-animal MRI. *Journal of Controlled Release.* 2009;135(1):65-70.
35. Petit A, Sandker M, Müller B, Meyboom R, van Midwoud P, Bruin P, Redout EM, Versluijs-Helder M, van der Lest CHA, Buwalda SJ, de Leede LGJ, Vermonden T, Kok RJ, Weinans H, Hennink WE. Release behavior and intra-articular biocompatibility of celecoxib-loaded acetyl-capped PCLA-PEG-PCLA thermogels. *Biomaterials.* 2014;35(27):7919-7928.
36. Petit A, Redout EM, van de Lest CH, de Grauw JC, Müller B, Meyboom R, van Midwoud P, Vermonden T, Hennink WE, René van Weeren P. Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. *Biomaterials.* 2015;53:426-436.
37. Aydin O, Korkusuz F, Korkusuz P, Tezcaner A, Bilgic E, Yaprakci V, Keskin D. In vitro and in vivo evaluation of doxycycline-chondroitin sulfate/PCLmicrospheres for intraarticular treatment of osteoarthritis. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 2015;103(6):1238-1248.
38. Sandker MJ, Petit A, Redout EM, Siebelt M, Müller B, Bruin P, Meyboom R, Vermonden T, Hennink WE, Weinans H. In situ forming acyl-capped PCLA–PEG–PCLA triblock copolymer based hydrogels. *Biomaterials.* 2013;34(32):8002-8011.
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. *Journal of Biomaterials Science, Polymer Edition.* 2016;27(4):303-316.

40. Timur U, Caron M, Bastiaansen-Jenniskens Y, Welting T, van Osch G, Emans P. PGE2 and PGF2α 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, Yang H. Efficacy of Intra-Articular Injection of Celecoxib in a Rabbit Model of Osteoarthritis. *International Journal of Molecular Sciences.* 2010;11(10):4106-4113.
43. 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(6):Br247-252.
44. 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. *The Journal of Clinical Investigation.* 1996;97(11):2672-2679.
45. Mastbergen SC, Marijnissen AC, Vianen ME, Zoer B, van Roermund PM, Bijlsma JW, Lafeber FP. Inhibition of COX-2 by celecoxib in the canine groove model of osteoarthritis. *Rheumatology.* 2006;45(4):405-413.
46. Morko J, Peng Z, Vääräniemi J, Fagerlund KM, Rissanen JP, Bernoulli J, Halleen JM. Degenerative changes in knee joints as determined by OARSI rat scoring system and osteoarthritis-related joint pain in surgically and chemically induced rat models of osteoarthritis. *Osteoarthritis and Cartilage.* 2013;21:S69-S70.
47. Adães S, Mendonça 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 Research & Therapy.* 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 and Cartilage.* 2012;20(12):1583-1590.

8

General discussion

General discussion

As described in the general introduction of this thesis, cartilage damage can occur in different ways. Pathologic stresses can either cause a focal cartilage defect, or trigger a catabolic process that disturbs the joint homeostasis and initiate pre-osteoarthritis (pre-OA).[1,2] When not adequately treated, focal cartilage defects, as well as pre-OA, will inevitably lead to diffuse cartilage damage (i.e., OA).[3]

In this thesis we combined basic science with the scope of a clinician towards a clinical translation of cartilage damage treatment. Endochondral ossification is an indispensable process in the human body. In early life most of our bones form through EO, and in case of major trauma, EO plays a key role in bone fracture healing.[4] Unfortunately, it also often occurs in articular cartilage repair interventions and fuels the development of osteoarthritis.[5,6]

The process of EO in a damaged joint can be influenced on many levels (Figure 8.1) (e.g., patient risk factors, joint and tissue alterations, and growth factors/molecular signalling pathways). Growth factors have multiple, sometimes opposing functions and despite extensive research there is no unambiguous solution on how to use them to direct a signalling pathway to prevent unwanted EO of (repaired) articular cartilage.[7,8]

The focus of the research described in this thesis was to investigate the influence of patient characteristics in focal cartilage damage and repair with a focus on the process of EO. Specifically, to understand the role of EO, and to elucidate at which level interventions have the capacity to prevent undesirable EO in the treatment of focal cartilage damage and diffuse cartilage damage (i.e., osteoarthritis).

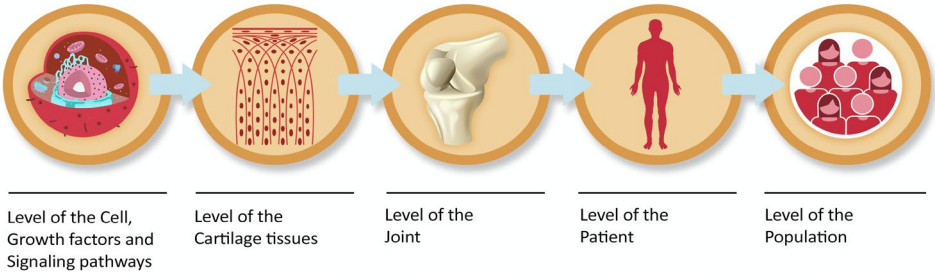

Figure 8.1 The process of endochondral ossification can be influenced on many different levels. From left to right are depicted the levels described in this thesis. (A) the level of the cell, and growth factors and signalling pathways, (B) the cartilage tissue level, (C) the level of the joint, (D) the patient level, and (E) the population level, which is discussed in future perspectives.

The influence of patient characteristics on cartilage repair

Previous research has addressed that patient selection is important and supports in improving the outcome of cartilage repair surgery.[9,10] The work in this thesis confirmed

findings described in literature, but in addition demonstrated in **Chapter 2** that patient characteristics remain of influence on the outcome of cartilage repair surgery by perichondrium transplantation (PT) even after a long-term follow-up. The influence of tissue and patient characteristics as described in **Chapter 2** (i.e., increased age, previous surgery and increased time of symptoms) on the outcome of cartilage repair surgery is linked to an impaired joint homeostasis.[1,9,11] A healthy joint homeostasis is described as the stable equilibrium of all the joint tissues, without inflammation in a well-functioning articulation.[11] However, when the joint homeostasis is impaired, at least the local environment will be in an inflammatory state, which will inherently influence the process of EO.[11,12] Age, previous surgery and increased time of symptoms are non-reversible patient characteristics that are risk factors for a lasting, chronic inflammatory state of the joint. Chronic inflammation is detrimental for the healing process of all tissues and should be avoided.[13] In contrast, an acute inflammatory reaction is found to be essential for the healing of, amongst others, damaged bone, and blocking it will hamper bone healing.[14] Although many different cytokines are linked to either acute inflammation, chronic inflammation, or both,[15] there is no strict point in time that differentiates between acute and chronic inflammation in tissue healing or repair. An interesting topic to analyse in more detail is the different effects of inflammation on EO in fracture healing, cartilage repair surgery, and osteoarthritis (OA). Chronic inflammation in bone fracture healing negatively influences the balance between bone formation and resorption, impairing EO.[16] In cartilage repair surgery the presence of chronic inflammation is linked to an impaired outcome as a result of fibrosis and early OA changes.[11] Interestingly, the formation of osteophytes and subchondral bone sclerosis (as a result of EO) are elements of the structural changes in an OA joint, and the presence of chronic inflammation does not prevent this EO in an OA joint.[17,18] The role of inflammation on chondrogenesis and osteogenesis during EO in fracture healing, focal cartilage damage, and OA is thus complex and can be influenced on many different levels.

In **Chapter 3** we analysed PT-patients from the cohort in **Chapter 2** and compared them to autologous chondrocyte implantation (ACI) patients in more detail using a high-field 7T MRI. Assessment at this level (i.e., obtaining the compositional information of (repaired) cartilage tissue by 7T MRI) enables to explore research topics that would otherwise only be able by the use of (non-ethical) invasive procedures like arthroscopy and biopsies. The 7T MRI facilitates the assessment of the joint and cartilage over several timepoints and is able to detect (biochemical) changes such as decreased GAG content, which would not be detectable in a reliable fashion at lower-field MRI.[19] In our 7T-MRI analysis of this small group of patients, who were still without major revision surgery of the knee, we found a high prevalence of intralesional osteophytes (IO) in both PT and ACI patients. Intralesional osteophytes are the result of (unwanted) EO.[20] We discussed that the most likely cause of IO formation in our population was the use of marrow stimulating techniques (MST) in prior surgery and the use of periosteum and perichondrium as defect cover.

The release of MSCs from the bone marrow cavity after MST, or precursor cells from the periosteum or perichondrium can be a contributing factor to this EO process. The cells from these sources probably still have a high susceptibility to environmental influences (e.g., growth factors) that can induce EO of these cells.[4] The differentiation process of MSCs starts early after surgery but the repair tissue is described to remodel up to 1 year after initiation of the differentiation processs.[21] Possibly this occurs via a similar mechanism of EO in which osteophytes at the peripheral junction of articular cartilage and bone are being formed in the process of OA.[20] The prevalence of intralesional osteophytes in repaired cartilage is increased after MST.[22] Intralesional osteophytes are described to impair the outcome of cartilage repair surgery by increasing the mechanical stiffness of the subchondral bone plate and thereby elevating shear stresses in the cartilage.[23] In addition, intralesional osteophytes are also described to compromise the opposing cartilage tissue.[23] To our knowledge, a detailed evaluation of these morphological and biochemical characteristics of intralesional osteophytes and its effect on the outcome of cartilage surgery has not been described before. A detailed mapping of these detrimental morphological characteristics of the intralesional osteophytes and biochemical assessment of opposing cartilage tissue is expected to aid in the prediction of cartilage repair surgery failure. Subsequently, increased accuracy in failure prediction could facilitate an early re-intervention and possibly prevents further deterioration of the joint. However, IO formation has not yet been assessed comprehensively over multiple timepoints to assess the course of its growth. At this time, it is not yet possible to predict IO formation on an individual level and consensus is that intralesional osteophytes have to be prevented in general.[23]

To prevent intralesional osteophytes as a result of unwanted EO, multiple alterations in cartilage repair procedures have been implemented.[24,25] A small subgroup of PT patients (n=14) was given non-steroidal anti-inflammatory drugs (NSAIDs) (that inhibit COX-1 and COX-2) postoperatively. This decreased the incidence of intralesional ossification.[26] In later generations of ACI, decreased IO was achieved by replacing periosteum tissue for a collagen membrane as a defect cover, as well as the use of selected chondrocytes (2nd generation ACI). The use of scaffolds (3rd generation ACI) and Spherox®, spheroids that contain chondrocytes with a self-synthesized extracellular matrix (4th generation ACI) further decreased IO formation.[25] Furthermore, treatment of the subchondral bone has been critically evaluated by Gomoll and colleagues.[27] As a result, current guidelines advise the use of MST (which are based on the release of MSCs) only in small defects <2-4cm^2 when the risk for revision surgery is low.[25,28] Interestingly, IO formation is present in 25% of these small defects after microfracture.[29] However, two thirds of these defects with IO formation still had a good fill and clinical outcome.[29] Morphology of the IO has not been described systematically in these studies except for their size (small vs. large) in the studies of Demange et al. and Gomoll et al.[23,27,29,30] As we hypothesized that IO morphology (e.g., penetration of the surface by an IO) influences mechanical stress and subsequent joint inflammation,

it would be interesting to investigate the correlation between IO morphology, composition, defect size and (clinical) outcome.
Defect and joint characteristics such as defect size, location and morphology as well as patient characteristics such as increased age, time of symptoms and previous surgery potentially lead to differences in inflammation and joint homeostasis.[1,9,11] The outcome of cartilage repair surgery is inevitably influenced by these factors. In addition, cartilage repair surgery likely induces a 'second inflammatory hit' with a chondrodegenerative process as described in anterior cruciate ligament reconstruction.[31] Increased expression of inflammatory cytokines in synovial fluid can be found up to 5 years after anterior cruciate ligament reconstruction.[32] A chronic inflammatory state of the joint after cartilage repair surgery will impair tissue regeneration. It is therefore important to recognize patient characteristics that increase the risk on a chronic inflammatory state of the joint, and to apply proper patient selection. However, since surgery itself induces chronic inflammation,[31] patient selection alone will not be able to prevent chronic inflammation completely. Therefore, in the next part of this thesis we analysed possible strategies to improve the outcome of cartilage repair surgery on the tissue and cellular level.

Controlling endochondral ossification in cartilage formation

The influence of COX-2 inhibition on the chondrogenic phase of endochondral ossification was demonstrated in previous work from our group.[33,34] In addition, our group showed that COX-2 expression follows a biphasic pattern in EO. The first expression peak is during early chondrocyte differentiation and the second expression peak during chondrocyte hypertrophy.[35] We investigated this mechanism further in **Chapter 4** of this thesis. In a model consisting of bone fracture healing, growth plate development and ectopic cartilage formation, we demonstrated that COX-2 inhibition impaired bone fracture healing, and that COX-2 inhibition impaired chondrogenic differentiation in both ectopic cartilage formation and the growth plate.

As stated in the first part of this discussion, an acute inflammatory response is essential for fracture healing.[12,13] Several studies investigating the influence of COX-2 inhibition on fracture healing describe an effect in the early (inflammatory) phase of the healing process.[36,37] In addition, we proposed an effect on early chondrogenic differentiation to be at least partially responsible for impaired fracture healing. In **Chapter 4**, we found that COX-2 inhibition led to impaired chondrogenesis. This suggests an essential role for inflammation in early chondrogenic differentiation in *in* vivo ectopic cartilage formation, similar to its essential role in fracture healing.[35] Contrasting findings were previously described by Heldens et al., who found that *in vitro* inhibition of inflammation can partially rescue chondrogenesis.[38] It is important to note that still no *in vitro* model exists that resembles the complexity of the *in vivo* EO process.[12] Often only single cell-sources are used for *in vitro* experiments. Furthermore, to induce *in vitro* chondrogenic differentiation, several factors like transforming growth factor beta

(TGF-β) and dexamethasone have to be added.[39] These molecules have a potent effect on inflammation and EO and will inherently influence the model and possibly alter the measured outcome.[12,40-42] In an *in vivo* model, the induction of chondrogenic differentiation by these anti-inflammatory molecules is not needed. Our use of an *in vivo* model could therefore explain the contrasting finding on the inhibition of inflammation on chondrogenesis. The use of different inflammatory molecules (COX-2 versus (interleukin (IL)-1 alpha and tumour necrosis factor (TNF)-alpha) could be another explanation.[38]

Based on the presence of EO in the growth plate, fracture healing, and in our model of newly formed cartilage, we speculate that a similar EO process causes IO formation in MSC-based cartilage repair techniques (e.g., microfracture, PT, and bone marrow aspirate concentrate (BMAC)). The knowledge on EO, gained by *in vitro* as well as *in vivo* models of COX-2 inhibition, can be used to our advantage in order to use celecoxib (CXB) or other selective COX-2 inhibitors to optimize (ectopic) cartilage formation in cartilage repair strategies. At the time of articular (cartilage repair) surgery, inflammation is inherently present in the joint, which may lead to unwanted cartilage degradation and OA changes.[31,43] Next to the inhibition of EO described above, another effect of COX-2 inhibition by celecoxib at the time of, or shortly after cartilage repair surgery is its anti-inflammatory effect on the whole joint.[44] The inhibition of inflammation and conditioning of the joint homeostasis at this timepoint is expected to prevent the joint from reaching a catabolic threshold and thereby improve the outcome of cartilage repair surgery based on the preservation of homeostasis.[45] Ideally, the essential acute inflammation and early chondrogenesis processes should remain unaffected, but the unwanted chondrocyte hypertrophy and possibly chronic inflammation should be inhibited. Depending on the used clinical cartilage repair technique, the cell source can be different. When using MSCs as a cell source compared to (selected) chondrocytes, EO and subsequent (unwanted) chondrocyte hypertrophy are expected to occur at a different point in time after surgery. A possible strategy to preserve the acute inflammation essential for the healing process would be to adjust the timing and extent of COX-2 inhibition. Based on our observations that CXB prevented ISI formation **(Chapter 4)**, immediate post-operative COX-2 inhibition may inhibit the initial chondrogenic differentiation and undesirably prevent cartilage formation in a bone marrow stimulation technique. However, COX-2 inhibition in already differentiated chondrocytes (e.g., ACI, Minced cartilage, and Hedgehog) specifically inhibits hypertrophic differentiation and could be used directly after surgery to prevent chondrocyte hypertrophy and avoid EO and potentially improve the clinical outcome. Therefore, COX-2 inhibition should be properly timed depending on the used cartilage repair technique.

Another strategy to improve cartilage formation is described in **Chapter 5**. We used our model of ectopic periosteal chondrogenic differentiation to investigate whether the addition of the chondrogenic proteins COMP and aggrecan (ACAN) to surgically introduced subperiosteal agarose gel is able to improve cartilage formation. The addition of COMP or aggrecan improved the quality of ectopically generated cartilage

by significantly inhibiting unwanted chondrocyte hypertrophy. Controlling chondrocyte hypertrophy in EO and preventing a chronic inflammatory state of the joint, without inhibiting the 'essential' early phase of inflammation and chondrogenic differentiation provides an interesting starting point to design new strategies to improve cartilage repair. In addition, targeting chondrocyte hypertrophy and EO is increasingly recognized as a possible strategy in the treatment of OA.[46] Therefore, gained knowledge on processes and signalling pathways of EO in cartilage repair also contributes to the improvement of OA treatment.

Drugs and delivery systems targeting EO in cartilage damage

Improving treatment strategies for focal cartilage repair is important to prevent deterioration of the joint into OA, but as discussed earlier in this thesis, after exclusion of non-suitable patients, focal cartilage repair surgery is only applicable in a relatively small group of patients. However, the inhibition of chondrocyte hypertrophy and unwanted EO are potentially applicable on a larger scale in the treatment of OA as well.[46] Prevention is the best treatment of all diseases, including OA. However, prevention will not always be possible. Early diagnosis and treatment are crucial to prevent further irreversible damage to the joint when OA has occurred (secondary prevention), and if possible, treat the reversible damage.[47]

Despite extensive and promising research, to date, no clinically implemented non-surgical OA disease-modifying therapy exists.[48] Currently, non-OA disease-modifying therapies focus on patient education, physical therapy, and pain relief (e.g., oral analgesics, topical analgesics, and intra-articular cortisone or hyaluronic acid) until finally joint arthroplasty is required.[49] In selected cases, surgical options to postpone joint arthroplasty are cartilage or meniscal repair or replacement, re-alignment osteotomies or knee joint distraction.[50] An improved joint homeostasis after knee joint distraction was proposed by Wiegant et al.,[51] and more recently, gene transcriptional changes in synovial fluid that are potentially beneficial for the joint homeostasis were described in more detail by Sanjurjo-Rodriguez et al.[52] A more recent paper confirms that re-alignment osteotomies and knee joint distraction provide clinical benefit to patients and increased radiographic joint space width, but there was no significant improvement of cartilage quality, as determined by delayed gadolinium-enhanced MRI of cartilage (dGEMRIC).[53] An interesting topic of research is therefore the improvement of the joint homeostasis by combining future disease modifying OA treatment that act on the biological level with the optimization of biomechanical properties. The balance between treatment of structural changes (load bearing capacity) versus joint inflammation and pain is also influenced by personalized joint preserving treatments in which lifestyle, activity and coping play an important role in further reducing inflammation.[54] The disturbed joint homeostasis in OA is, amongst others, represented by inflammation and increased chondrocyte hypertrophy.[5,43,55] Therefore, strategies aiming at the inhibition of (unwanted) inflammation and chondrocyte hypertrophy are of interest.

Local pharmacological treatment of joint disease has many advantages over systemic treatment (e.g., better bio-availability and less systemic side-effects). In addition, an intra-articular drug delivery system (DDS) can improve pharmacokinetics of drugs used in OA treatment. Therefore, we provided an overview of (candidate) drugs for an effective OA treatment, what kind of drugs can be incorporated in a DDS, and which polymers are considered suitable to provide for such delivery systems in **Chapter 6**. The ideal drugs for treatment of OA are believed to be disease-modifying osteoarthritic drugs (DMOADs).[48] However, OA is a disease which often progresses slowly over time and may remain asymptomatic until structural joint damage is present.[56] Therefore, it is difficult to provide evidence for the joint preserving properties of DMOADs in clinical trials and getting DMOADs approved for clinical application is challenging.[57] The already clinically applied Glucocorticoid (GC) and hyaluronic acid (HA) injections are believed to provide a fairly good relief of symptoms and improve function over the short- and medium-term.[58,59] However, there is little to no evidence of disease modification by GC and HA injections, and their use is debated in literature.[49] Despite the current decrease in popularity of GC and HA injections, there are some very interesting recent studies that show the potential of intra-articular injections of GC, HA and other drugs. Pre-clinical studies showed that a single, posttraumatic IA injection of dexamethasone has the potency to inhibit joint inflammation and potentially restore joint homeostasis without being chondrotoxic, thereby preventing post-traumatic OA.[60] Intra-articular injections of hyaluronic acid are described to act chondroprotective, possibly by inhibition of TNF-α and thereby suppressing inflammation in the joint.[61] Inflammation is an essential step in EO, and together, the role of inflammation and EO in joint degradation is substantial.[62] Therefore, we investigated the effect of CXB, released from a polyester amide (PEA) drug delivery system in an OA model in **Chapter 7**. The PEA-CXB microsphere caused no adverse reaction. The microsphere degradation and drug release rate of the CXB-PEA microsphere (which is degraded by proteolytic enzymes) suggests an anti-inflammatory and auto-regulatory effect of CXB released from the microsphere. This auto-regulatory effect makes the PEA-CXB microsphere an interesting DDS-drug combination in OA treatment. Data from our animal model did not show histological OA disease modification in a 12-week follow-up after injection of a PEA-CXB microsphere. However, the diminished *in vivo* ectopic cartilage formation in **Chapter 4** indicating an anti-hypertrophic effect of celecoxib on chondrocytes, and the PEA-CXB microsphere degradation and drug release rate described in **Chapter 7** suggested the capacity of the PEA-CXB microsphere to reduce joint inflammation. Increased inflammatory and degeneration biomarkers (e.g., IL-6, MMP13, and COMP), and concurrently hypertrophic biomarkers (e.g., COL10A1 and Runx2) are already present in the stage of early OA.[63] Therefore, the inhibition of chondrocyte hypertrophy, inflammation and possibly subsequent (chronic) pain by the PEA-CXB microsphere can be valuable in the treatment of OA. This might be especially the case when functional and structural alterations leading to persistent inflammation in the joints of OA patients have been addressed.[64-66] In OA, where inflammation is critical, a

treatment with an auto-regulatory behaviour, in which increased inflammation causes increased release of the drug, is very encouraging. In future experiments, combinations of PEA microspheres with other (OA) drugs would be worthwhile to consider. Whether the auto-regulatory effect will remain is likely dependent on the anti-inflammatory action of the incorporated drug. This anti-inflammatory effect can either be a characteristic of the used drug, or an indirect result of an OA disease modifying effect and subsequent reduced inflammation of the joint. The degree of inflammation in the joint, the release of the drug from the PEA-microsphere, and its associated anti-inflammatory effect have to be well balanced in order to create an adequate auto-regulatory effect. The PEA-CXB microsphere could also be valuable in the prevention of post-surgical (chronic) inflammation in patients who underwent cartilage repair surgery. Thereby potentially reducing IO formation or cartilage degradation that results from chronic inflammation.[67] In a setting where patient selection is applied and there are no functional and structural alterations leading to persistent joint inflammation, the inhibition of inflammation and EO by the PEA-CXB microspheres might be more capable of modifying OA disease progression. However, this hypothesis should be tested in future experiments.

Concluding remarks and future perspectives

The worldwide burden of OA is rising rapidly and OA is becoming more prevalent in relatively young patients.[3,68-72] As a result, TKA is performed more and more often in patients younger than 65 years of age, with a subsequent risk for revision surgery later in life.[73,74] Therefore, more attention is needed for joint preserving strategies before proceeding to joint replacement. However, for successful joint preservation, a timely recognition and adequate treatment of cartilage damage is needed. The work presented in this thesis demonstrates, at the 'patient level', that adequate patient selection can improve the long-term outcome of cartilage repair surgery. In the underlying 'cell, tissue and joint level', inflammation and EO are critical processes, not only in the growth plate and fracture healing, but also in 'new' cartilage formation. The right amount of inhibition of inflammation by NSAIDs in a correct temporospatial manner can subsequently inhibit the (often undesired) EO process and improve the treatment of articular cartilage defects and OA. Sustained-release treatment strategies, such as the auto-regulatory PEA-CXB microsphere, that aim at the inhibition of inflammation and EO are potential options to improve the joint homeostasis and intervene at a promising level for the optimization of cartilage repair strategies and early OA treatment. However, because of the complexity of treating the damaged joint it is essential that all preconditions are optimized in order to improve the chance of a successful outcome.

In addition to the suggestions for future research described above (i.e., exploring individualized treatment, inhibition of inflammation and EO, and sustained release of drugs in cartilage repair surgery and OA), orthopaedic surgeons will have to change

their approach to the patient with joint pain. The underlying causes for joint pain (e.g., patient characteristics, psychosocial and biomechanical factors, and cartilage damage (either focal defects or (early) OA)) should be carefully assessed before starting symptomatic treatment. When underlying causes are not addressed, they will lead to persisting inflammation, an impaired joint homeostasis, and continued degradation of the joint. Better use of (high-resolution) MRI, potentially combined with the use of a biomarker, could act as a useful tool for the diagnosis and follow-up of cartilage damage and its treatment. The insight in joint inflammation and homeostasis, which can potentially be improved by intra-articular anti-inflammatory injections may offer novel conservative and operative treatment strategies. As such, immobility is a chronic disease potentially leading to other chronic diseases needing more attention and control in a more personalized manner.[75]

References

1. Ryd L, Brittberg M, Eriksson K, Jurvelin JS, Lindahl A, Marlovits S, Moller P, Richardson JB, Steinwachs M, Zenobi-Wong M. Pre-Osteoarthritis: Definition and Diagnosis of an Elusive Clinical Entity. *Cartilage.* 2015;6(3):156-165.
2. Eckstein F, Hudelmaier M, Putz R. The effects of exercise on human articular cartilage. *J Anat.* 2006;208(4):491-512.
3. Davies-Tuck ML, Wluka AE, Wang Y, Teichtahl AJ, Jones G, Ding C, Cicuttini FM. The natural history of cartilage defects in people with knee osteoarthritis. *Osteoarthritis Cartilage.* 2008;16(3):337-342.
4. Aghajanian P, Mohan S. The art of building bone: emerging role of chondrocyte-to-osteoblast transdifferentiation in endochondral ossification. *Bone Res.* 2018;6:19.
5. Dreier R. Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders. *Arthritis Res Ther.* 2010;12(5):216.
6. Blanke M, Carl HD, Klinger P, Swoboda B, Hennig F, Gelse K. Transplanted chondrocytes inhibit endochondral ossification within cartilage repair tissue. *Calcif Tissue Int.* 2009;85(5):421-433.
7. Shintani N, Siebenrock KA, Hunziker EB. TGF-ss1 enhances the BMP-2-induced chondrogenesis of bovine synovial explants and arrests downstream differentiation at an early stage of hypertrophy. *PLoS One.* 2013;8(1):e53086.
8. Kerkhofs J, Leijten J, Bolander J, Luyten FP, Post JN, Geris L. A Qualitative Model of the Differentiation Network in Chondrocyte Maturation: A Holistic View of Chondrocyte Hypertrophy. *PLoS One.* 2016;11(8):e0162052.
9. de Windt TS, Concaro S, Lindahl A, Saris DB, Brittberg M. Strategies for patient profiling in articular cartilage repair of the knee: a prospective cohort of patients treated by one experienced cartilage surgeon. *Knee Surg Sports Traumatol Arthrosc.* 2012;20(11):2225-2232.
10. 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.
11. 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):1067-1076.
12. van der Kraan PM. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng Regen Med.* 2019;16(4):327-334.
13. Geusens P, Emans PJ, de Jong JJ, van den Bergh J. NSAIDs and fracture healing. *Curr Opin Rheumatol.* 2013;25(4):524-531.
14. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. *Nat Rev Rheumatol.* 2015;11(1):45-54.
15. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. *Frontiers in bioscience : a journal and virtual library.* 1997;2:d12-26.
16. Longoni A, Knezevic L, Schepers K, Weinans H, Rosenberg A, Gawlitta D. The impact of immune response on endochondral bone regeneration. *NPJ Regen Med.* 2018;3:22.
17. Pulsatelli L, Addimanda O, Brusi V, Pavloska B, Meliconi R. New findings in osteoarthritis pathogenesis: therapeutic implications. *Ther Adv Chronic Dis.* 2013;4(1):23-43.
18. Sokolove J, Lepus CM. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis.* 2013;5(2):77-94.
19. Hayashi D, Li X, Murakami AM, Roemer FW, Trattnig S, Guermazi A. Understanding Magnetic Resonance Imaging of Knee Cartilage Repair: A Focus on Clinical Relevance. *Cartilage.* 2018;9(3): 223-236.
20. van der Kraan PM, van den Berg WB. Osteophytes: relevance and biology. *Osteoarthritis Cartilage.* 2007;15(3):237-244.
21. Fortier LA, Cole BJ, McIlwraith CW. Science and animal models of marrow stimulation for cartilage repair. *J Knee Surg.* 2012;25(1):3-8.
22. Minas T, Gomoll AH, Rosenberger R, Royce RO, Bryant T. Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques. *Am J Sports Med.* 2009;37(5):902-908.

23. Demange MK, Minas T, von Keudell A, Sodha S, Bryant T, Gomoll AH. Intralesional Osteophyte Regrowth Following Autologous Chondrocyte Implantation after Previous Treatment with Marrow Stimulation Technique. *Cartilage.* 2017;8(2):131-138.
24. 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):334-350.
25. Caron J, Custers RJH, Emans PJ. Standpunt chirurgische behandeling osteochondrale defecten knie. 2019.
26. Bouwmeester SJ, Beckers JM, Kuijer R, van der Linden AJ, Bulstra SK. Long-term results of rib perichondrial grafts for repair of cartilage defects in the human knee. *Int Orthop.* 1997;21(5):313-317.
27. Gomoll AH, Madry H, Knutsen G, van Dijk N, Seil R, Brittberg M, Kon E. The subchondral bone in articular cartilage repair: current problems in the surgical management. *Knee Surg Sports Traumatol Arthrosc.* 2010;18(4):434-447.
28. Redondo ML, Naveen NB, Liu JN, Tauro TM, Southworth TM, Cole BJ. Preservation of Knee Articular Cartilage. *Sports Med Arthrosc Rev.* 2018;26(4):e23-e30.
29. Mithoefer K, Williams RJ, 3rd, Warren RF, Potter HG, Spock CR, Jones EC, Wickiewicz TL, Marx RG. The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. *J Bone Joint Surg Am.* 2005;87(9):1911-1920.
30. Kreuz PC, Steinwachs MR, Erggelet C, Krause SJ, Konrad G, Uhl M, Sudkamp N. Results after microfracture of full-thickness chondral defects in different compartments in the knee. *Osteoarthritis Cartilage.* 2006;14(11):1119-1125.
31. Hunt ER, Jacobs CA, Conley CE, Ireland ML, Johnson DL, Lattermann C. Anterior cruciate ligament reconstruction reinitiates an inflammatory and chondrodegenerative process in the knee joint. *J Orthop Res.* 2020.
32. Larsson S, Struglics A, Lohmander LS, Frobell R. Surgical reconstruction of ruptured anterior cruciate ligament prolongs trauma-induced increase of inflammatory cytokines in synovial fluid: an exploratory analysis in the KANON trial. *Osteoarthritis Cartilage.* 2017;25(9):1443-1451.
33. Welting TJ, Caron MM, Emans PJ, Janssen MP, Sanen K, Coolsen MM, Voss L, Surtel DA, Cremers A, Voncken JW, van Rhijn LW. Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater.* 2011;22:420-436; discussion 436-427.
34. Caron MM, Emans PJ, Cremers A, Surtel DA, Coolsen MM, van Rhijn LW, Welting TJ. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis Cartilage.* 2013;21(4):604-613.
35. Caron MM, Emans PJ, Surtel DA, Cremers A, Voncken JW, Welting TJ, van Rhijn LW. Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. *PLoS One.* 2012;7(3):e33467.
36. Vuolteenaho K, Moilanen T, Moilanen E. Non-steroidal anti-inflammatory drugs, cyclooxygenase-2 and the bone healing process. *Basic Clin Pharmacol Toxicol.* 2008;102(1):10-14.
37. Simon AM, O'Connor JP. Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. *J Bone Joint Surg Am.* 2007;89(3):500-511.
38. Heldens GT, Blaney Davidson EN, Vitters EL, Schreurs BW, Piek E, van den Berg WB, van der Kraan PM. Catabolic factors and osteoarthritis-conditioned medium inhibit chondrogenesis of human mesenchymal stem cells. *Tissue Eng Part A.* 2012;18(1-2):45-54.
39. Jakobsen RB, Ostrup E, Zhang X, Mikkelsen TS, Brinchmann JE. Analysis of the effects of five factors relevant to in vitro chondrogenesis of human mesenchymal stem cells using factorial design and high throughput mRNA-profiling. *PLoS One.* 2014;9(5):e96615.
40. Cheng X, Yan Y, Chen JL, Ma ZL, Yang RH, Wang G, Chuai M, Ka Ho Lee K, Yang X. Dexamethasone Exposure Accelerates Endochondral Ossification of Chick Embryos Via Angiogenesis. *Toxicol Sci.* 2016;149(1):167-177.
41. Ahmad M, Hachemi Y, Paxian K, Mengele F, Koenen M, Tuckermann J. A Jack of All Trades: Impact of Glucocorticoids on Cellular Cross-Talk in Osteoimmunology. *Front Immunol.* 2019;10:2460.
42. Dangelo M, Sarment DP, Billings PC, Pacifici M. Activation of transforming growth factor beta in chondrocytes undergoing endochondral ossification. *J Bone Miner Res.* 2001;16(12):2339-2347.
43. van der Kraan PM, van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartilage.* 2012;20(3):223-232.
44. Zweers MC, de Boer TN, van Roon J, Bijlsma JW, Lafeber FP, Mastbergen SC. Celecoxib: considerations regarding its potential disease-modifying properties in osteoarthritis. *Arthritis Res Ther.* 2011;13(5):239.

45. Corciulo C, Lendhey M, Wilder T, Schoen H, Cornelissen AS, Chang G, Kennedy OD, Cronstein BN. Endogenous adenosine maintains cartilage homeostasis and exogenous adenosine inhibits osteoarthritis progression. *Nat Commun.* 2017;8:15019.
46. 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.
47. Luyten FP, Bierma-Zeinstra S, Dell'Accio F, Kraus VB, Nakata K, Sekiya I, Arden NK, Lohmander LS. Toward classification criteria for early osteoarthritis of the knee. *Semin Arthritis Rheum.* 2018;47(4):457-463.
48. Oo WM, Hunter DJ. Disease modification in osteoarthritis: are we there yet? *Clinical and experimental rheumatology.* 2019;37 Suppl 120(5):135-140.
49. Bannuru RR, Osani MC, Vaysbrot EE, Arden NK, Bennell K, Bierma-Zeinstra SMA, Kraus VB, Lohmander LS, Abbott JH, Bhandari M, Blanco FJ, Espinosa R, Haugen IK, Lin J, Mandl LA, Moilanen E, Nakamura N, Snyder-Mackler L, Trojian T, Underwood M, McAlindon TE. OARSI guidelines for the non-surgical management of knee, hip, and polyarticular osteoarthritis. *Osteoarthritis Cartilage.* 2019;27(11): 1578-1589.
50. van der Woude JAD, Wiegant K, van Heerwaarden RJ, Spruijt S, van Roermund PM, Custers RJH, Mastbergen SC, Lafeber F. Knee joint distraction compared with high tibial osteotomy: a randomized controlled trial. *Knee Surg Sports Traumatol Arthrosc.* 2017;25(3):876-886.
51. Wiegant K, Heerwaarden R, Van Roermund P, Mastbergen S. Intrinsic joint tissue repair by joint distraction. *OA Arthritis.* 2013;1(1):4.
52. Sanjurjo-Rodriguez C, Altaie A, Mastbergen S, Baboolal T, Welting T, Lafeber F, Pandit H, McGonagle D, Jones E. Gene Expression Signatures of Synovial Fluid Multipotent Stromal Cells in Advanced Knee Osteoarthritis and Following Knee Joint Distraction. *Front Bioeng Biotechnol.* 2020;8:579751.
53. Besselink NJ, Vincken KL, Bartels LW, van Heerwaarden RJ, Concepcion AN, Marijnissen ACA, Spruijt S, Custers RJH, van der Woude JAD, Wiegant K, Welsing PMJ, Mastbergen SC, Lafeber F. Cartilage Quality (dGEMRIC Index) Following Knee Joint Distraction or High Tibial Osteotomy. *Cartilage.* 2020;11(1): 19-31.
54. Dean E, Gormsen Hansen R. Prescribing optimal nutrition and physical activity as "first-line" interventions for best practice management of chronic low-grade inflammation associated with osteoarthritis: evidence synthesis. *Arthritis.* 2012;2012:560634.
55. Caron MM, Emans PJ, Surtel DA, van der Kraan PM, van Rhijn LW, Welting TJ. BAPX-1/NKX-3.2 acts as a chondrocyte hypertrophy molecular switch in osteoarthritis. *Arthritis Rheumatol.* 2015;67(11): 2944-2956.
56. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *The Lancet.* 2019;393(10182):1745-1759.
57. Goldring MB, Berenbaum F. Emerging targets in osteoarthritis therapy. *Curr Opin Pharmacol.* 2015;22:51-63.
58. Hermans J, Reijman M, Goossens LMA, Verburg H, Bierma-Zeinstra SMA, Koopmanschap MA. Cost-Utility Analysis of High Molecular Weight Hyaluronic Acid for Knee Osteoarthritis in Everyday Clinical Care in Patients at a Working Age: An Economic Evaluation of a Randomized Clinical Trial. *Arthritis Care Res (Hoboken).* 2018;70(1):89-97.
59. Kolasinski SL, Neogi T, Hochberg MC, Oatis C, Guyatt G, Block J, Callahan L, Copenhaver C, Dodge C, Felson D, Gellar K, Harvey WF, Hawker G, Herzig E, Kwoh CK, Nelson AE, Samuels J, Scanzello C, White D, Wise B, Altman RD, DiRenzo D, Fontanarosa J, Giradi G, Ishimori M, Misra D, Shah AA, Shmagel AK, Thoma LM, Turgunbaev M, Turner AS, Reston J. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Care Res (Hoboken).* 2020;72(2):149-162.
60. Grodzinsky AJ, Wang Y, Kakar S, Vrahas MS, Evans CH. Intra-articular dexamethasone to inhibit the development of post-traumatic osteoarthritis. *J Orthop Res.* 2017;35(3):406-411.
61. Strauss E, Schachter A, Frenkel S, Rosen J. The efficacy of intra-articular hyaluronan injection after the microfracture technique for the treatment of articular cartilage lesions. *Am J Sports Med.* 2009;37(4):720-726.
62. Zhang Y, Pizzute T, Pei M. Anti-inflammatory strategies in cartilage repair. *Tissue Eng Part B Rev.* 2014;20(6):655-668.

63. Bay-Jensen AC, Hoegh-Madsen S, Dam E, Henriksen K, Sondergaard BC, Pastoureau P, Qvist P, Karsdal MA. Which elements are involved in reversible and irreversible cartilage degradation in osteoarthritis? *Rheumatol Int.* 2010;30(4):435-442.
64. Heard BJ, Achari Y, Chung M, Shrive NG, Frank CB. Early joint tissue changes are highly correlated with a set of inflammatory and degradative synovial biomarkers after ACL autograft and its sham surgery in an ovine model. *J Orthop Res.* 2011;29(8):1185-1192.
65. Timur UT, Caron MMJ, Bastiaansen-Jenniskens YM, Welting TJM, van Rhijn LW, van Osch G, Emans PJ. 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.
66. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis.* 2012;4(4):269-285.
67. Alblowi J, Kayal RA, Siqueira M, McKenzie E, Krothapalli N, McLean J, Conn J, Nikolajczyk B, Einhorn TA, Gerstenfeld L, Graves DT. High levels of tumor necrosis factor-alpha contribute to accelerated loss of cartilage in diabetic fracture healing. *Am J Pathol.* 2009;175(4):1574-1585.
68. Stiebel M, Miller LE, Block JE. Post-traumatic knee osteoarthritis in the young patient: therapeutic dilemmas and emerging technologies. *Open Access J Sports Med.* 2014;5:73-79.
69. Roemer FW, Jarraya M, Niu J, Silva JR, Frobell R, Guermazi A. Increased risk for radiographic osteoarthritis features in young active athletes: a cross-sectional matched case-control study. *Osteoarthritis Cartilage.* 2015;23(2):239-243.
70. 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): 2033-2039.
71. Ding C, Cicuttini F, Scott F, Cooley H, Jones G. Association between age and knee structural change: a cross sectional MRI based study. *Ann Rheum Dis.* 2005;64(4):549-555.
72. Widhalm HK, Seemann R, Hamboeck M, Mittlboeck M, Neuhold A, Friedrich K, Hajdu S, Widhalm K. Osteoarthritis in morbidly obese children and adolescents, an age-matched controlled study. *Knee Surg Sports Traumatol Arthrosc.* 2016;24(3):644-652.
73. Wainwright C, Theis JC, Garneti N, Melloh M. Age at hip or knee joint replacement surgery predicts likelihood of revision surgery. *J Bone Joint Surg Br.* 2011;93(10):1411-1415.
74. Kurtz SM, Lau E, Ong K, Zhao K, Kelly M, Bozic KJ. Future young patient demand for primary and revision joint replacement: national projections from 2010 to 2030. *Clin Orthop Relat Res.* 2009;467(10): 2606-2612.
75. Musich S, Wang SS, Ruiz J, Hawkins K, Wicker E. The impact of mobility limitations on health outcomes among older adults. *Geriatr Nurs.* 2018;39(2):162-169.

Addendum

Summary

Life expectancy has increased worldwide and as a way to improve quality of life, physical exercise is recommended, even at an older age. The positive effects of exercise have been extensively described in the literature. Ironically, mechanical trauma due to exercise is described as the biggest cause of focal articular cartilage defects. When damaged, articular cartilage possesses little regenerative capacity and is prone to continued degeneration. In the process of articular cartilage damage and joint degeneration, an important component is the altered expression of biomolecular factors affecting the homeostasis of articular cartilage and increasing unfavourable endochondral ossification (EO) of the cartilage tissue. The work presented in this thesis focusses on understanding alterations in the role of EO in focal and diffuse articular cartilage damage and possibilities to use the biomolecular mechanisms that are active in EO for the treatment of articular cartilage damage.

In the first part of this thesis, the influence of patient characteristics on the outcome of cartilage repair surgery and the role of EO in this process was investigated. In **Chapter 2**, we analysed patients 25 years after their perichondrium transplantation (PT) and found that the risk of failure (receiving major revision surgery) was lower and knee function (measured by International Knee Documentation Committee (IKDC)-score) was better when adequate patient selection was applied. Factors that significantly changed the risk of failure in our patient study population were: prior surgery to the index knee and a longer time of symptoms prior to cartilage repair surgery. A younger age at the time of surgery was associated with a better IKDC-score at 25 years follow-up. A morphologic and biochemical evaluation of the cartilage repair by 7T MRI was performed in patients from the same PT-cohort and compared to a cohort of autologous chondrocyte transplantation (ACT) patients with a similar follow-up time in **Chapter 3**. We found no correlation between clinical questionnaires and the MOCART (Magnetic Resonance Observation of Cartilage Repair Tissue) score or biochemical impairment of the grafts. Intralesional osteophytes were common in both the PT and ACT patients. These osteophytes can result in biochemical damage to the opposing tibial cartilage. This was more dependent on osteophyte morphology (i.e., an osteophyte extending into the surface of the graft) than the amount of calcification of the graft.

In the second part of this thesis, it was investigated how the process of EO can be influenced. In **Chapter 4**, we showed that Cyclo-oxygenase-2 (COX-2) inhibition not only impaired endochondral ossification in the growth plate and fracture callus, but also in periosteal cartilage formation in an *in vivo* rabbit model. The inhibition of COX-2 was achieved by the use of celecoxib, a selective COX-2 inhibiting drug that belongs to the class of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Supported by previous work from our group that showed a bi-phasic pattern of COX-2 expression in EO, these results indicate that celecoxib impairs not only hypertrophic differentiation, but also

the chondrogenic phase of EO in specific circumstances. Another way to influence the process of EO was investigated in **Chapter 5**. The engineering of cartilage tissue from progenitor cells is often hampered by unwanted EO. To overcome the problem of adverse chondrocyte hypertrophic differentiation, a previously used biogel to induce ectopic cartilage formation was supplemented with the major cartilage proteins aggrecan and cartilage oligomeric matrix protein (COMP). We found that these proteins were able to improve the process of ectopic cartilage formation in an *in* vivo rabbit model by suppressing hypertrophic differentiation of the engineered cartilaginous tissue.

In the final part of this thesis, possible treatment options were explored to improve the intra-articular environment in order to reduce joint pain and to postpone or prevent progression of cartilage damage into osteoarthritis (OA). Currently available oral drug therapy has many disadvantages that can be overcome by intra-articular administration of appropriate drugs. However, drug residence time in the joint is generally short and a drug delivery system (DDS) is needed to improve the efficacy of intra-articular therapy for cartilage damage. Different types of DDSs are: prolonged release systems, controlled release systems, and autoregulatory systems. The literature review performed in **Chapter 6** showed that DDSs can be made from different materials. Since the use of polymers for the development of DDSs, much progress has been made. The use of DDSs for the treatment of OA is promising, but a disease-modifying combination of a DDS and drug has not yet been demonstrated in clinical trials. **Chapter 7** of this thesis describes the development and testing of the polyester amide-celecoxib PEA-CXB-microsphere as a DDS. Pharmacokinetic properties and response to an inflammatory (OA) environment was tested *in vitro* in cell lysates obtained from a neutrophil-like Hl-60 cell line. Subsequently, biocompatibility and degradation of the PEA-CXB microspheres were tested in an *in vivo* rat model where the anterior cruciate ligament was transected and a partial medial meniscectomy was performed to induce post-traumatic OA. The PEA-microsphere caused no adverse reactions and was found suitable as a DDS. The PEA-CXB-microsphere did not show an OA disease-modifying effect. However, increased degradation of the microspheres was present in OA knees, whereas celecoxib loading of the microspheres reduced microsphere degradation, suggesting a DDS with an auto-regulatory behaviour. In **Chapter 8**, key findings of this thesis were put in perspective.

The worldwide burden of OA is rising rapidly, especially in relatively young patients. When total knee arthroplasty is performed in young patients, they are at increased risk for revision arthroplasty later in life. Therefore, more attention for joint preserving strategies is needed. For successful joint preservation, a timely recognition, patient selection, and adequate treatment of cartilage damage and OA are needed. Adequate treatment can be achieved by lifestyle, pharmacological, and surgical interventions and combinations thereof. The work presented in this thesis demonstrates that the improvement of joint preservation can be addressed at these different levels and from

different viewpoints/insights (e.g., cell and patient). However, most important for successful joint-preserving treatment of patients with joint damage is a change in the approach to the clinical problem by orthopaedic surgeons, other healthcare providers and scientists. An approach in which the 'patient journey' of a person with a healthy joint can be followed and guided by the right interdisciplinary collaboration up to joint replacement surgery. This provides the right care at the right time and place to keep people and patients moving and thus prevent other chronic diseases.

Nederlandse samenvatting

De wereldwijde levensverwachting neemt toe en ter verbetering van de kwaliteit van leven wordt lichaamsbeweging geadviseerd, zelfs op gevorderde leeftijd. De positieve effecten van lichaamsbeweging zijn uitgebreid beschreven in wetenschappelijke literatuur. Ironisch genoeg is een mechanisch trauma bij sporten beschreven als de grootste oorzaak van lokale kraakbeendefecten. Kraakbeen heeft weinig regeneratieve capaciteit en een beschadiging van kraakbeen leidt daardoor vaak tot verdere degeneratie. Een belangrijke component in het proces van articulaire kraakbeenschade en degeneratie van het gewricht is de veranderde expressie van biomoleculaire factoren. Deze biomoleculaire factoren beïnvloeden de homeostase van het gewricht en zorgen voor een toename van ongewenste endochondrale ossificatie (EO) van het articulaire kraakbeen en osteofyt vorming.
Het werk in dit proefschrift focust op het begrijpen van veranderingen in de rol van EO in focale en diffuse schade aan articulair kraakbeen en op de mogelijkheden om gebruik te maken van de biomoleculaire mechanismen die actief zijn in EO voor de behandeling van beschadigd articulair kraakbeen.

In het eerste deel van dit proefschrift werd de invloed van patiëntkarakteristieken op het resultaat van kraakbeenchirurgie onderzocht, met ook de rol van EO in dit proces. In **Hoofdstuk 2** werd een groep patiënten 25 jaar na perichondrium transplantatie (PT) geanalyseerd, waarbij perichondrium van de rib getransplanteerd werd naar de knie. We ontdekten dat het risico op falen van deze ingreep (gedefinieerd als het ondergaan van een grote hersteloperatie) lager was en dat de functie van de knie (gemeten door middel van de International Knee Documentation Committee (IKDC) score) beter was wanneer adequate patiënt selectie werd toegepast. Factoren die het risico op falen significant verhoogden, waren een eerdere operatie aan dezelfde knie en een langere duur van de symptomen voorafgaand aan de kraakbeenoperatie. Een jongere leeftijd ten tijde van de operatie werd geassocieerd met een betere IKDC-score na 25 jaar follow-up. Een morfologische en biochemische evaluatie van het herstelde kraakbeen werd door middel van een 7 tesla (7T) MRI verricht in patiënten van hetzelfde PT cohort en de resultaten werden vergeleken met een cohort patiënten die in dezelfde periode een autologe chondrocyten transplantatie (ACT) hadden ondergaan **(Hoofdstuk 3)**. Er werd geen correlatie gevonden tussen klinische vragenlijsten en de MOCART (Magnetic Resonance Observation of Cartilage Repair Tissue) score of biochemische verslechtering van de kraakbeentransplantaten. In zowel PT als ACT patiënten kwamen intralesionale osteofyten frequent voor. Deze osteofyten kunnen leiden tot biochemisch aantoonbare schade aan het tegenoverliggende tibiale kraakbeen. Deze schade was meer afhankelijk van de morfologie van deze osteofyten (i.e., een osteofyt die tot in het oppervlak van een transplantaat groeit) dan van het percentage verkalking van het transplantaat.

In het tweede deel van dit proefschrift onderzochten we hoe het EO-proces beïnvloed kan worden. In **Hoofdstuk 4** toonden we aan dat cyclo-oxygenase-2 (COX-2) inhibitie

niet alleen EO in de groeiplaat en in de fractuurcallus verminderde, maar ook de periostale kraakbeenvorming in een *in vivo* konijnenmodel. De inhibitie van COX-2 werd bereikt door het gebruik van celecoxib, een selectief COX-2 remmend medicijn dat valt in de groep Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Ondersteund door voorgaande studies van onze onderzoeksgroep die een bifasisch patroon van COX-2 expressie in EO aantoonden, laten deze resultaten zien dat celecoxib niet alleen hypertrofe differentiatie van kraakbeencellen remt, maar ook de chondrogene fase van EO in bepaalde omstandigheden kan remmen.
Een andere manier om het EO-proces te beïnvloeden werd onderzocht in **Hoofdstuk 5**. Het vervaardigen van kraakbeen uit progenitorcellen wordt vaak belemmerd door ongewenste EO. Om het probleem van nadelige hypertrofe differentiatie van chondrocyten tijdens ectopische kraakbeenvorming te voorkomen werd een eerder gebruikte biogel aangevuld met de belangrijke kraakbeeneiwitten aggrecan of cartilage oligomeric matrix protein (COMP). Deze eiwitten bleken in staat te zijn om het proces van ectopische kraakbeenvorming te verbeteren in een *in vivo* konijnenmodel, door het onderdrukken van hypertrofe differentiatie van het gevormde kraakbeenweefsel.

In het laatste deel van dit proefschrift werden potentiele behandelopties onderzocht die het intra-articulaire milieu kunnen verbeteren om gewrichtspijn te verminderen en progressie van kraakbeenschade naar artrose uit te stellen of te voorkomen. De huidige beschikbare orale medicamenteuze behandelingen hebben vele nadelen die kunnen worden overwonnen door intra-articulaire toediening van geschikte medicatie. Het medicijn blijft echter vaak maar een korte tijd in het gewricht aanwezig en er is een medicijnafgiftesysteem nodig om de effectiviteit van intra-articulaire therapie voor kraakbeenschade te verbeteren. Verschillende soorten medicijnafgiftesystemen zijn: Verlengde afgifte systemen, gecontroleerde afgifte systemen of autoregulatoire systemen. De literatuurstudie verricht in **Hoofdstuk 6** liet zien dat medicijn-afgiftesystemen gemaakt kunnen worden van verschillende materialen. Sinds het gebruik van polymeren voor de ontwikkeling van medicijnafgiftesystemen is er veel progressie geboekt. Het gebruik van medicijnafgiftesystemen voor de behandeling van artrose is veelbelovend, maar er is nog geen klinische trial die aangetoond heeft dat deze medicijnafgiftesystemen in combinatie met medicijnen in staat zijn het ziekteproces van artrose te veranderen. **Hoofdstuk 7** van dit proefschrift beschrijft de ontwikkeling en het testen van de polyester amide-celecoxib (PEA-CXB) micropartikel als medicijnafgiftesysteem. De farmacokinetische eigenschappen en reactie op een inflammatoire (artrotische) omgeving werden *in vitro* onderzocht in cellysaten verkregen van een neutrofiele Hl-60 cellijn. Vervolgens werden de bio-compatibiliteit en degradatie van de PEA-CXB micropartikel getest in een *in vivo* rat model waarbij post-traumatische artrose was geïnduceerd door middel van het doornemen van de voorste kruisband en gedeeltelijke verwijdering van de mediale meniscus. De PEA-CXB micropartikels veroorzaakten geen detecteerbare bijwerkingen en waren geschikt als medicijnafgiftesysteem. Een verandering van het artroseproces door de PEA-CXB micropartikels werd niet aangetoond. In rattenknieën met artrose was er een

toegenomen degradatie van de micropartikels en de toevoeging van celecoxib aan de partikels zorgde voor een afname van dezelfde degradatie, wat toebedeeld werd aan auto-regulatoire eigenschappen van het medicijnafgiftesysteem. In **Hoofdstuk 8** werden de belangrijkste bevindingen van dit proefschrift in breder perspectief geplaatst.

De wereldwijde ziektelast van artrose stijgt snel, met name in relatief jonge patiënten. Wanneer een totale knieprothese wordt geplaatst bij jonge patiënten is het risico op een revisie operatie groot. Daarom is er meer aandacht nodig voor gewrichtssparende behandelingen. Voor een succesvolle gewrichtssparende behandeling zijn een tijdige herkenning, patiëntselectie en behandeling van kraakbeenschade en artrose nodig. Een adequate behandeling kan bestaan uit leefstijl, farmacologische en/of chirurgische interventies. De bevindingen in dit proefschrift tonen dat de verbetering van gewrichtssparende behandelingen bereikt kunnen worden op verschillende niveaus en vanuit verschillende perspectieven (bijvoorbeeld vanuit de cel en vanuit de patiënt). Echter, het belangrijkste voor een succesvolle gewrichtssparende behandeling van patiënten met gewrichtsschade is een verandering in de benadering van het klinische probleem door orthopedisch chirurgen, andere zorgverleners en wetenschappers. Een benadering waarbij de “patient journey” van een persoon met een gezond gewricht tot aan een gewrichtsvervanging gevolgd en begeleid kan worden door middel van de juiste interdisciplinaire samenwerking. Hierdoor wordt de juiste zorg op de juiste tijd en plaats geboden om mensen en patiënten in beweging te houden en zo andere chronische ziekten te voorkomen.

Impact paragraph

Cartilage is a durable, but flexible tissue that occurs throughout the body. In articular joints, hyaline cartilage comprises a layer that covers the ends of the bones and provides a surface with very low friction that makes movement possible and at the same time functions as a shock absorber. Unfortunately, cartilage has a very low healing capacity. Therefore, damage to articular cartilage is not resolved and often leads to a pathway of joint deterioration and finally osteoarthritis (OA). Articular cartilage lesions are found in up to 62% of the knees of adults without any symptoms of joint pathology.[1] When cartilage degradation becomes symptomatic, or even progresses into OA, this can have an enormous impact on a person's life. Osteoarthritis is a leading cause of disability worldwide and its burden is only expected to increase due to the ageing population and increasing incidence of obesity.[2,3] Furthermore, disabling OA leads to a substantially reduced long-term work participation and is therefore a major economic concern as well.[4] The most frequently applied therapy for end-stage OA is arthroplasty, but the results of total knee arthroplasty (TKA) in working patients are dissatisfying and one third of patients does not return to work after TKA.[5] The lifetime risk of implant revision is increased in younger patients (up to 35% for men in their early 50s).[6] In addition, the median time to revision is significantly shorter in patients who were younger than 60 at the time of TKA.[6] It is thus of great social and economic value to prevent, or at least postpone progression towards end-stage OA and subsequent (early) TKA. Therefore, the main goal of this thesis was to elucidate how the process of endochondral ossification (EO) can be influenced to improve the treatment of damaged cartilage (i.e., focal cartilage defects and OA).

Conclusion of main findings

The process of EO is an essential factor in cartilage damage and repair. The findings in this thesis confirm that patient characteristics can negatively influence the outcome of cartilage repair surgery. Potentially by impairing the joint homeostasis and increasing joint inflammation and subsequent EO of the repaired cartilage tissue. The work in this thesis underlines the potential of inhibiting inflammation and influencing the EO pathway with the aim to improve cartilage repair and OA treatment by reducing undesired chondrocyte hypertrophy.

Implications for research

The influence of inflammation and patient characteristics on the outcome of cartilage repair surgery and OA treatment is widely recognized, but still not fully understood.[7,8] The data in this thesis demonstrate that adequate patient selection can improve the outcome of cartilage repair surgery. In addition, the added value of 7T MRI is underlined. The detailed visualization of morphological and biochemical differences (such as increased calcification of repaired cartilage) suggests that inflammation and EO

can influence the results of articular cartilage repair. This shows that innovations in imaging, such as (high-field) MRI, can aid in an increased understanding of the mechanisms of treatment failure and subsequently provide directions to improve treatment strategies. However, the influence of EO on the quality of cartilage repair tissue and subsequent clinical outcome should be further elucidated in prospectively designed studies. Increased knowledge on the use of 7T MRI also provides a way to evaluate articular cartilage non-invasively and at multiple timepoints, facilitating future clinical research on the influence of EO on articular cartilage damage and repair. This future research could elaborate on the work presented in this thesis that describes the potential improvement of ectopic cartilage tissue formation by influencing inflammation and EO via the inhibition of cyclooxygenase (COX)-2. In addition, the chondrocyte hypertrophy-suppressive effect of aggrecan and cartilage oligomeric protein (COMP) without impairing cartilage formation provides an interesting starting point for future studies.

Implications for individual patients and society

Good surgeons know how to operate, better ones when to operate, and the best when not to operate.[9] This was stated in a BMJ editorial dating back to 1999, but is still applicable. Not performing unnecessary surgical procedures protects patients from avoidable strain. In addition, it decreases hospital costs and all other socioeconomic costs involved with the surgery. Key findings in this thesis increased the knowledge on risk factors and adequate diagnostic tools to detect cartilage defects and (early) OA. Early detection of cartilage damage provides the opportunity to improve the 'patient journey' by starting early with a suitable treatment, preserve a functional joint and prevent loss of mobility in patients. This can subsequently avoid costly procedures in progressed OA such as revision of total knee arthroplasty or socioeconomic costs caused by disability in patients of working age. Furthermore, an increased understanding was obtained on the role of inflammation and EO on the treatment of cartilage damage and OA by the development of the PEA-CXB microsphere. Derived strategies could further elaborate on the inhibition of chondrocyte hypertrophy and inflammation to treat cartilage damage and potentially lead to a reduction of the amount of (early) TKA and subsequent revision TKA. Next to reduced socioeconomic costs, a reduction in the amount of (early) TKA can also decrease secondary (psychological) complaints and improve the quality of life of OA patients.

Implications for health care professionals

Next to the implications for the individual patient and society described above, the research results presented in this thesis are valuable for health care professionals as well. We found that late and multiple surgeries in older patients decrease the chance of success in focal cartilage repair surgery. This underlines the importance of adequate early treatment of articular cartilage damage to prevent further deterioration of the

joint. This can be achieved by educating primary physicians to recognize patients with possible articular cartilage damage that are suitable for early referral to an orthopaedic surgeon. This might be even more applicable for physical therapists, as in the Dutch health care system, physical therapy is often the first line of treatment for patients with (minor) musculoskeletal complaints. Orthopaedic surgeons can benefit from a timely referral and potentially provide less invasive, joint preserving treatments.

Communication towards health care professionals

A timely treatment of articular cartilage damage can prevent further deterioration of the joint.[10,11] In addition, the findings in this thesis showed that a timely treatment of cartilage damage decreases the risk of treatment failure and subsequent TKA. To facilitate this timely treatment of articular cartilage damage, primary healthcare providers involved in the treatment of patients with focal cartilage defects or (early) OA have to be taught that early referral can be joint-preserving. To educate health care providers, publishing research results in peer reviewed journals is essential, but is not enough. Medical information is abundantly available on the internet. However, keeping an overview is complex and the abundant information is impossible to interpret and apply for all different health care providers.[12] For the results of this thesis (and other research) to consistently reach all relevant health care providers, communication will have to be improved. In the following years there will have to be significant advancements in the infrastructure of electronic health records. The currently, not directly linked electronic health records of (amongst others) primary physicians, physical therapists and orthopaedic surgeons will have to be linked or integrated so that all health care providers can have the access to relevant information and are provided with adequate feedback on their treatment actions, ideally supported by scientific research. This can be facilitated by the use of a personal health environment in which personal medical data is owned by the patient and can be shared with different institutions.[13]

References

1. Culvenor AG, Oiestad BE, Hart HF, Stefanik JJ, Guermazi A, Crossley KM. Prevalence of knee osteoarthritis features on magnetic resonance imaging in asymptomatic uninjured adults: a systematic review and meta-analysis. *Br J Sports Med.* 2019;53(20):1268-1278.
2. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet.* 2019;393(10182):1745-1759.
3. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL, Laslett LL, Jones G, Cicuttini F, Osborne R, Vos T, Buchbinder R, Woolf A, March L. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis.* 2014;73(7):1323-1330.
4. Kontio T, Viikari-Juntura E, Solovieva S. Effect of Osteoarthritis on Work Participation and Loss of Working Life-years. *J Rheumatol*. 2020;47(4):597-604.
5. Kievit AJ, van Geenen RC, Kuijer PP, Pahlplatz TM, Blankevoort L, Schafroth MU. Total knee arthroplasty and the unforeseen impact on return to work: a cross-sectional multicenter survey. *J Arthroplasty.* 2014;29(6):1163-1168.
6. Bayliss LE, Culliford D, Monk AP, Glyn-Jones S, Prieto-Alhambra D, Judge A, Cooper C, Carr AJ, Arden NK, Beard DJ, Price AJ. The effect of patient age at intervention on risk of implant revision after total replacement of the hip or knee: a population-based cohort study. *Lancet.* 2017;389(10077):1424-1430.
7. van der Kraan PM. The Interaction between Joint Inflammation and Cartilage Repair. *Tissue Eng Regen Med.* 2019;16(4):327-334.
8. de Windt TS, Concaro S, Lindahl A, Saris DB, Brittberg M. Strategies for patient profiling in articular cartilage repair of the knee: a prospective cohort of patients treated by one experienced cartilage surgeon. *Knee Surg Sports Traumatol Arthrosc.* 2012;20(11):2225-2232.
9. Knowing when not to operate. *Bmj.* 1999;318(7180).
10. Ding C, Cicuttini F, Scott F, Cooley H, Boon C, Jones G. Natural history of knee cartilage defects and factors affecting change. *Arch Intern Med.* 2006;166(6):651-658.
11. Ryd L, Brittberg M, Eriksson K, Jurvelin JS, Lindahl A, Marlovits S, Moller P, Richardson JB, Steinwachs M, Zenobi-Wong M. Pre-Osteoarthritis: Definition and Diagnosis of an Elusive Clinical Entity. *Cartilage.* 2015;6(3):156-165.
12. van der Keylen P, Tomandl J, Wollmann K, Mohler R, Sofroniou M, Maun A, Voigt-Radloff S, Frank L. The Online Health Information Needs of Family Physicians: Systematic Review of Qualitative and Quantitative Studies. *J Med Internet Res.* 2020;22(12):e18816.
13. Kaelber DC, Jha AK, Johnston D, Middleton B, Bates DW. A research agenda for personal health records (PHRs). *J Am Med Inform Assoc.* 2008;15(6):729-736.

List of publications

Publications

Welting, T. J., Caron, M.M., Emans, P.J., **Janssen, M.P.F.**, Sanen, K., Coolsen, M.M., Voss, L., Surtel, D.A., Cremers, A., Voncken J.W. and van Rhijn L.W. (2011). "Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification." Eur Cell Mater 22: 420-436; https://doi.org/10.22203/ecm.v022a31

Janssen M.P.F., 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. https://doi.org/10.3390/polym6030799

Janssen M.P.F., Timur U.T., Woike N., Welting T.J., Draaisma G., Gijbels M., van Rhijn L.W., Mihov G., Thies J., Emans P.J. Celecoxib-loaded PEA microspheres as an auto regulatory drug-delivery system after intra-articular injection. J Control Release. 2016;244(Pt A):30-40. https://doi.org/10.1016/j.jconrel.2016.11.003

Janssen M.P.F., Caron M.M., van Rietbergen B., Surtel D.A., van Rhijn L.W., Welting T.J., Emans P.J. Impairment of the chondrogenic phase of endochondral ossification in vivo by inhibition of cyclooxygenase-2. Eur Cell Mater. 2017;34:202-216. https://doi.org/10.22203/ecm.v034a13

Janssen M.P.F., van der Linden E.G.M., Boymans T.A.E.J., Welting T.J.M., van Rhijn L.W., Bulstra S.K., Emans P.J. Twenty-Two-Year Outcome of Cartilage Repair Surgery by Perichondrium Transplantation. Cartilage. 2021 Dec;13(1_suppl):860S-867S. https://doi.org/10.1177/1947603520958146

Janssen M.P.F., Caron M.M.J., Peeters L., Haudenschild D.R., Cremers A., Surtel D.A.M., van Rhijn L.W., Emans P.J., Welting T.J.M. Aggrecan and COMP Improve Periosteal Chondrogenesis by Delaying Chondrocyte Hypertrophic Maturation. Front Bioeng Biotechnol. 2020;8:1036. https://doi.org/10.3389/fbioe.2020.01036

Janssen M.P.F., Peters M.J.M., Steijvers-Peeters E.G.M., Szomolanyi P., Jutten E.M.C., van Rhijn L.W., Lindahl A., Peterson L., Trattnig S., Emans P.J. 7 Tesla MRI Evaluation of the Knee, 25 Years After Cartilage Repair Surgery: The Influence of Intralesional Osteophytes on Biochemical Quality of Cartilage. Cartilage. 2021 Nov 26:19476035211060506. https://doi.org/10.1177/19476035211060506

Scientific presentations

Janssen M.P.F., Caron M.M., van Rietbergen B., Surtel D.A., van Rhijn L.W., Welting T.J., Emans P.J. Impaired fracture healing by highly selective COX-2 inhibition. *Osteoarthritis Research Society International, Philadelphia, USA (2013)*

Janssen M.P.F., van der Linden E.G.M., Welting T.J.M., van Rhijn L.W., Bulstra S.K., Emans P.J. Human rib perichondrial transplantation: a 20-year follow-up. *International Cartilage Research Society, Izmir, Turkey (2013)*

Janssen M.P.F., Caron M.M., van Rietbergen B., Surtel D.A., van Rhijn L.W., Welting T.J., Emans P.J. Impaired fracture healing by highly selective COX-2 inhibition. *NOV congres, Rotterdam, Netherlands (2014)*
Awarded: Prof. dr. ir. Rik Huiskesprize, best scientific presentation

Janssen M.P.F., Caron M.M., Surtel D.A., van Rhijn L.W., Welting T.J., Emans P.J. Celecoxib released from PLGA microspheres specifically decreases chondrocyte hypertrophy in an in vivo model for generation of cartilage out of periosteum. *European Orthopaedic Research Society, München, Germany (2017)*

Janssen M.P.F., Timur U.T., Woike N., Welting T.J., Draaisma G., Gijbels M., van Rhijn L.W., Mihov G., Thies J., Emans P.J. An auto-regulatory drug delivery system as a long-term, local therapy for osteoarthritis. *Pélerìn symposium, Maastricht, Netherlands (2018)*
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Curriculum vitae

Maarten Janssen was born on 19 July 1987 in Mierlo, the Netherlands. After completing his pre-university education (Were-Di, Valkenswaard) in 2005, he studied medicine at Maastricht University. During his master, he began the work presented in this thesis in his scientific traineeship at the Laboratory for Experimental Orthopedics at the Maastricht University.
After obtaining his medical degree in 2011, he started working as a resident at the department of orthopedic surgery at Maxima Medical Center, Veldhoven. In 2012 Maarten returned as a PhD candidate at the Laboratory for Experimental Orthopedics, within CAPHRI, school of Care and Public Health Research Institute at Maastricht University where the fundaments of this thesis were laid, supervised by dr. P. Emans and prof. dr. T. Welting, and prof. dr. L. van Rhijn.

Maarten combined his further PhD-trajectory with a residency in orthopedic surgery under the supervision of dr. H. Staal. He started his residency in 2014 with his general surgical training at VieCuri Medical Center in Venlo under the supervision of dr. H. Janzing. He continued with his orthopedic surgery training in 2016 at MUMC+ in Maastricht, supervised by dr. H. Staal. In 2017 he continued his training at Maxima Medical Center, Veldhoven supervised by dr. R. Janssen and completed his residency at MUMC+ in Maastricht in March 2021.

Currently, Maarten combines his fellowship ‘hip & knee revision arthroplasty’ at MUMC+ with his work for the medical IT department of MUMC+. In which he focusses on healthcare pathway implementation and the optimization of healthcare data registration and processing.