Boundary conditions for cartilage regeneration

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Orthopaedie

Universitair Medisch Centrum Utrecht

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Boundary conditions for cartilage regeneration

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(met een samenvatting in het Nederlands)

Proefschrift

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door

Kiem Gie Auw Yang
geboren op 2 februari 1976 te Rotterdam

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Publications contributing to this thesis

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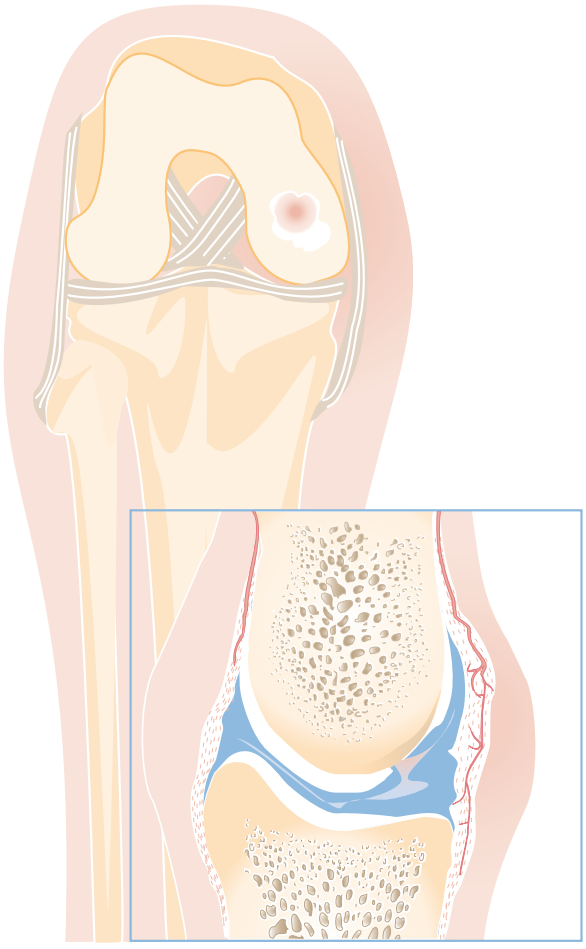
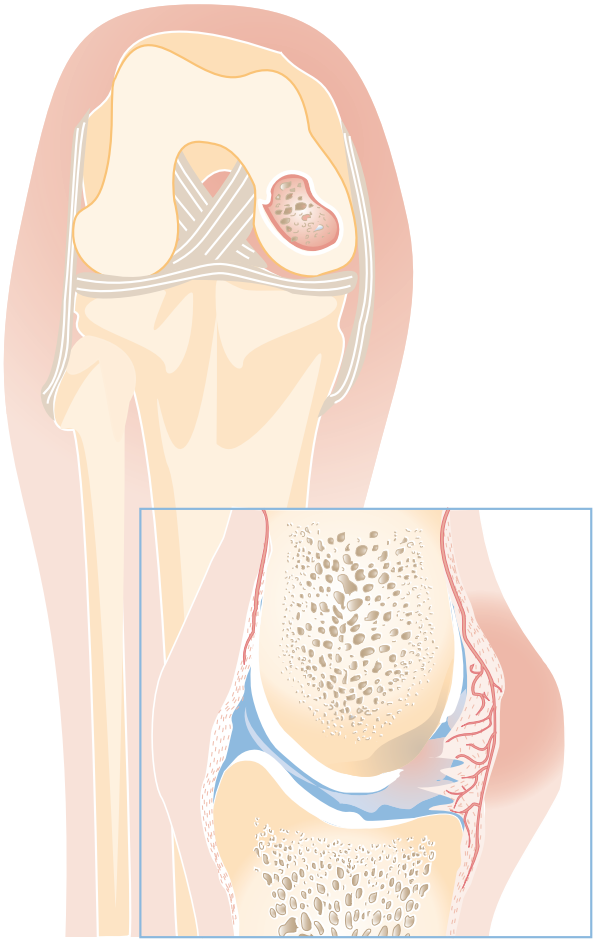
List of abbreviations

ACI	Autologous Chondrocyte Implantation
ACL	Anterior Cruciate Ligament
ADL	Activities in Daily Living
AE	Adverse Event
BEC	Basic Expansion Conditions
bFGF	basic Fibroblastic Growth Factor
BMI	Body Mass Index
BMP-2	Bone Morphogenetic Protein - 2
BMSC	Bone Marrow Stromal Cell
CCI	Characterized Chondrocyte Implantation
COX-2	Cyclo-oxygenase type 2
CM	Culture Model
DMOAD	Disease Modifying Osteoarthritic Drug
ECM	Extracellular matrix
ES	Effect Size
FGFR-3	Fibroblast Growth Factor receptor
GAG	Glycosaminoglycan
GCP	Good Clinical Practice
GFSEC	Growth Factor Supplemented Expansion Conditions
ICC	Intra-Class Correlation coefficient
IGF-1	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL-1	Interleukin-1
IL-1ra	Interleukin-1 receptor antagonist
KNN	K-Nearest neighbour
KOOS	Knee injury and Osteoarthritis Outcome Score
KSCRS	Knee Society Clinical Rating Scale
MACI	Matrix enhanced Autologous Chondrocyte Implantation
MMP	Matrix Metallo-Proteinases
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
n.s.	Non-significant
NSAID	Nonsteroidal Anti-Inflammatory Drug
OA	Osteoarthritis / Osteoarthritic
PAPP-a	Pregnancy Associated Plasma Protein-a
PCR	Polymerase Chain Reaction
PD	Population doublings
PDw MRI	Proton density weighted MRI
PG	Proteoglycan
QOL	Quality of Life
RA	Rheumatoid Arthritis
SD	Standard deviation
SEM	Standard error of mean
SF	Synovial Fluid
siRNA	Small inhibitory RNA
SRM	Standardized Response Mean
THR	Total hip replacement
T1-w MRI	T1-weighted MRI
T2-w MRI	T1-weighted MRI
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
VAS	Visual Analogue Scale
WOMAC	Western Ontario and McMasters Universities Index
WML	White Matter Lesion





Introduction and aims of this thesis

Chapter 01

Introduction

Articular cartilage is a highly specialized tissue covering the articulating ends of bones, which provides a sliding surface for smooth and pain free articulation of joints[1-3]. After joint injury, patients frequently seek medical help because of acute or persistent complaints that can often be attributed to the existence of cartilage defects. The incidence of cartilage defects has been estimated to be 2.6 per 1,000 adults. Even more, upon arthroscopic evaluation of symptomatic knee joints, the incidence of chondral defects has been reported to range from 16% to 63%[4-10]. Unfortunately, cartilage defects larger than 2 millimeter in diameter do not heal and may eventually even result in osteoarthritis (OA) development. Current treatment strategies, such as microfracturing, debridement, subchondral drilling, and osteochondral transplantation result in repair tissue with insufficient structural and mechanical properties as compared to native cartilage[11-19] and, therefore, are thought to provide merely a temporary alleviation of symptoms and insufficient protection against osteoarthritis (OA) development[20]. This composes a strong driving force for the rapid development of the field of regenerative medicine and the application of such strategies to the treatment of cartilage defects.

Tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ"[21].

In the field of cartilage tissue engineering, *in vitro* and *in vivo* studies have provided a thorough insight into the use of different cell sources, the application of scaffold materials, and the mechanism involved in the process of chondrogenesis, such as chondrocyte differentiation, extracellular matrix (ECM) synthesis, and signaling mechanisms. By implementing this knowledge in highly controlled environments, such as culture systems and, to a lesser degree, animal models, it has been proven possible to regenerate hyaline-like cartilage in a reliable and reproducible manner. Along this line, Autologous Chondrocyte Implantation (ACI), which is an example of regenerative medicine in current clinical practice, has been studied as treatment for articular cartilage defects since the early nineties (Figure 1).

The first report, which demonstrated the clinical feasibility of this technique, was published in 1994 by Brittberg *et al*, who reported alleviation of symptoms, such as knee locking, pain and swelling, in all patients. Furthermore, during control-arthroscopy 2 years post-surgery, they observed regeneration tissue with macroscopically good-to-excellent quality in 14 out of 16 patients. Biopsies showed that 11 of the 15 femoral transplants and 1 of the 7 patellar transplants had the appearance of hyaline-like tissue[24].
Since then, ACI is increasingly applied and numerous publications have reported similar promising results[25-33]. In addition, Bentley described that ACI resulted in

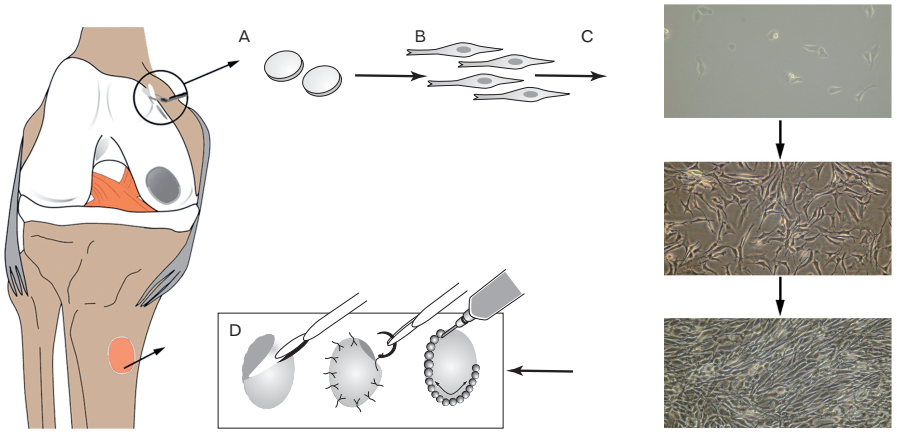


Figure 1
The ACI procedure comprises two-step surgical procedure. (A) First, an arthroscopy is performed during which a cartilage biopsy is taken from a less-weight bearing area of the injured joint. (B) Chondrocytes are isolated from the biopsy through enzymatic digestion of the ECM and, subsequently, (C) cultured in order to obtain sufficient cells for treatment of the defect. During this procedure, chondrocytes dedifferentiate and produce less cartilage-specific extracellular matrix (ECM) proteins[22,23]. (D) Finally, during an open surgical procedure, the cultured chondrocytes are implanted in the cartilage defect, after which the cells need to redifferentiate towards a chondrogenic phenotype in order to synthesize tissue with adequate mechanical properties.

arthroscopically and morphologically superior repair tissue and more improvement in joint function as compared to mosaicplasty[34]. When compared to the current treatment standard, microfracturing, ACI resulted in similar improvement of pain and function as determined by the Lysholm score and the visual analogue scale (VAS) for pain, while microfracturing-treated patients showed significantly more improvement on the short form-36 (SF-36). Furthermore, Knutsen reported that microfracturing and ACI resulted in histologically comparable repair tissue. However, 13 out of 80 patients did not undergo arthroscopy to obtain a biopsy of the repair tissue, which may have skewed the outcome of the data[31]. Performing adequate histological follow-up in cartilage repair studies should be considered essential, as the morphological quality of the regeneration tissue has not only been related to the degree of symptomatic alleviation[33], but in addition, to the longevity of the regeneration tissue and the observed clinical improvement[35]. Altogether, cartilage regeneration techniques such as ACI are increasingly applied, although microfracturing is still considered the treatment of choice for articular cartilage defects by many surgeons. The full scope of information needed for final evaluation of the efficacy of cartilage regeneration techniques remains to be provided in prospective randomized clinical trials.

Exploring the boundaries of successful cartilage regeneration

Despite these promising results, the regeneration tissue resulting from clinically applied ACI is still variable, ranging from hyaline cartilage-like tissue to fibrocartilaginous tissue[27,31,33,36]. Interestingly, a discrepancy in reliability and reproducibility of cartilage regeneration techniques is observed when comparing the outcome of culture techniques and animal models with clinically applied ACI. This suggests that we need to explore the boundary conditions that determine these discrepancies. As shown in figure 1, ACI encompasses a multi-step procedure. Therefore, these boundary conditions are of considerable relevance in a multitude of factors, such as the intra-articular environment, the culture conditions used for chondrocyte expansion and the donor tissue quality.

The discrepancy between clinical and experimental results of cartilage regeneration techniques may, at least in part, be explained by the concept of joint homeostasis[37]. This concept reminds us that treatment of a cartilage defect is not limited to the defect itself, but involves the whole joint as 'an organ system'. The homeostasis of this organ system can be disturbed by the presence of a defect as well as by other intra and extra articular derangement. This theory is supported by Rodrigo and Steadman, who demonstrated in an *in vitro* model that synovial fluid (SF) from newly injured knee joints was stimulatory for proteoglycan (PG) synthesis, while SF samples from knee joints with longer existing injuries were inhibitory[38]. Analogous to these findings, Saris *et al* demonstrated that treating "fresh" cartilage defects in knee joints of Dutch milk goats by periosteal transplantation not only resulted in significantly higher final proteoglycan content, but also improved morphological quality, as compared to "old" defects and had a protective effect on further degeneration of the lateral compartment of the knee[37]. These and the work by Hogervorst *et al* on homeostasis after ACL injury and reconstruction[39], demonstrate that the environment of the regenerating tissue is disturbed by the presence of a cartilage defect, resulting in a condition that is detrimental for the proteoglycan turnover and the morphology of the regenerated tissue in a goat model and *in vitro* chick limb bud assay. It remains to be elucidated if these parameters would be affected similarly in human articular chondrocytes and whether the process of chondrocyte redifferentiation would be altered.

The joint homeostasis, i.e. the intra-articular environment of the regenerating tissue, comprises a variety of tissue types, such as the surrounding cartilage, subchondral bone layer and synovial tissue, which determines largely the composition of synovial fluid. These tissue types may interfere with the process of cartilage regeneration either directly, through the quality of the donor tissue, or indirectly through excretion of mediators, such as growth factors and pro- and anti-inflammatory cytokines. Further research is required to explore the effect of these parameters and to identify possible mediators within these parameters that are responsible for the observed effects. This knowledge would provide new targets for

future improvement of clinically implemented cartilage regeneration techniques or define the boundaries to which cartilage regeneration techniques should be limited.

Relatively little is known about mediators involved in the disturbed homeostasis of joints with cartilage defects, while the process of OA development has been studied more extensively. Although OA and cartilage defects are considered two independent entities with different etiologies and distinct treatment modalities, integration of knowledge gained in both fields may provide feasible solutions for further advancement in the area of cartilage regeneration. Here, one can think of the extensive knowledge on altered growth factor availability, chondrocyte characteristics and mediators involved in disease progression.
One such common parameter could be the role of transforming growth factor-β (TGF-β). Studies by Blom and Blaney Davidson have demonstrated that OA development resulted in an increased TGF-β release from synovial tissue[40,41]. TGF-β is known to be a crucial growth factor during chondrogenesis, not only inducing cell proliferation, but also supporting chondrogenic differentiation[42,43]. Insulin-like growth factor-1 (IGF-1) is known to be another key-growth factor during this process, stimulating chondrocyte proliferation and proteoglycan synthesis[44-47]. When these growth factors are added in combination to culture media of periosteal explant cultures, they induce a synergistic chondrogenic effect[47]. TGF-β has been suggested to regulate the bioavailability of IGF-I through modulation of insulin-like growth factor binding proteins (IGFBPs)[48,49]. However, little is known on how these growth factors and binding proteins affect each other during the process of chondrogenesis.
Another factor that may have a significant effect on the outcome of cartilage regeneration techniques are the characteristics of the chondrocytes in the cartilage biopsy. OA chondrocytes display altered metabolic properties, resulting in increased proteoglycan turnover and expression of proteases that cause matrix degradation. Furthermore, OA chondrocytes have an altered phenotype, expressing increased amounts of collagen type I and X mRNA and decreased amounts of hyaline-cartilage specific collagen type II mRNA, and show an increased responsiveness to chondrogenic growth factors, such as TGF-β[50-56]. Despite these differences, chondrocytes used for *in vitro* research are frequently harvested from OA joints, as this is a readily available source of cells. Furthermore, ACI has been suggested as a possible treatment for early OA[57]. However, from studies comparing chondrocytes with bone marrow derived mesenchymal stem cells (MSCs) and different sources of MSCs, it has been well established that the phenotype and origin of the donor tissue may have a significant effect on outcome of cartilage regeneration techniques[58,59]. To date, no detailed information is available on the differences in chondrogenic potential between healthy and OA chondrocytes, while such studies might provide us with crucial knowledge for correct interpretation of *in vitro* studies that are performed with OA chondrocytes and, in addition, for adequate patient selection, treatment timing, culture adaptation and cell selection.
As discussed above, the intra-articular environment to which the regenerating tis-

sue is exposed, contains various tissue types that may affect the outcome of cartilage regeneration techniques by the excretion of mediators. In the field of OA research these mediators are extensively studied. Various pro- and anti-inflammatory cytokines are thought to mediate the initiation and progression of cartilage degradation[60-62]. Of these cytokines, Interleukin-1β (IL-1β) is proposed to play a key role[60-65]. This pro-inflammatory cytokine is involved in the initial inflammatory response to tissue damage in most tissue types throughout the human body. IL-1β mediates this effect by inducing migration of inflammatory and progenitor cells, wound debridement, ECM synthesis and vasculogenesis, which are all essential steps for adequate wound repair[66,67]. Likewise, cartilage defects will likely induce an intra-articular inflammatory response (Figure 2). However, in the context of articular cartilage, this pro-inflammatory cytokine has a detrimental effect through the induction of matrix metallo-proteinases (MMPs), collagenase and prostaglandin, and by decreasing synthesis of cartilage specific collagens and proteoglycans[61,68-70]. As a result, IL-1β results in degradation of the cartilage ECM.
Therefore, IL-1β likely is a viable target to improve the joint homeostasis and, thereby, the outcome of cartilage regeneration techniques. Interleukin-1 receptor antagonist (IL-1ra) is such a protein that may be implemented to prevent the detrimental effect of IL-1β. It induces its effect by blocking the IL-1 receptor [71]. Recently, a technique was described to induce the excretion of IL-1ra from monocytes in whole human blood (Orthokin)[72]. It has been proposed as a disease-modifying drug that may alter the course of OA (DMOAD)[73].

Outcome tools for cartilage research

Overall, we can conclude that biological joint reconstruction and OA modulation is a viable and realistic goal in regenerative medicine, although further research to improve the outcome of these techniques seems legitimate. In future studies, the cartilage research community needs to strive towards the implementation of uniform and validated research tools. This is essential to ensure comparability between studies and, thereby, rapid progression of our understanding of biological joint reconstruction. In this thesis, we also focused on the validation of clinical, radiological, and histological outcome tools.

To evaluate the clinical outcome of surgical and pharmacological treatment of OA and cartilage regeneration, different knee rating scales have been developed, such as the Knee injury and Osteoarthritis Outcome Score (KOOS) and the Western Ontario and McMaster's Universities Osteoarthritis Index (WOMAC)[74-78]. Both outcome tools are rather extensive, while short questionnaires are known to result in improved patient compliance and survey response rate, and even have been suggested to improve response quality[79-81]. In addition, short questionnaires can be implemented to monitor the outcome of our clinical interventions in daily practice. This does not merely provide us with information concerning our personal competence, but also with large databases regarding the outcome of newly implemented

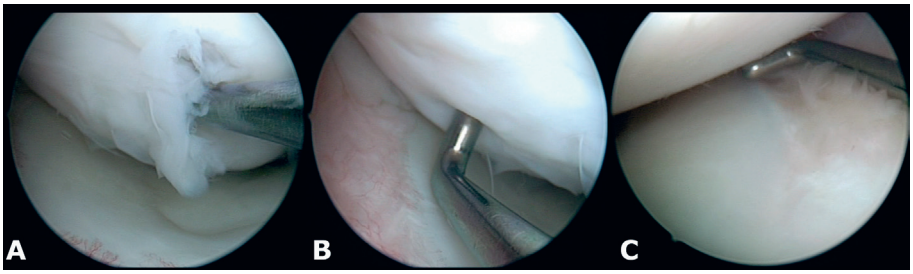


Figure 2
These photographs were taken during an arthroscopical procedure of a knee joint with a chondral defect on the femoral condyle (A). Note the hyper-vascularization of the margin of the meniscus and the joint capsule (B), which suggests that an inflammatory reaction has occurred. (C) At the contra-lateral side of the same knee joint, no signs of inflammation were observed. This example supports the concept of disturbed joint homeostasis induced by cartilage defects.

treatment strategies. Whitehouse developed and validated a reduced WOMAC function scale (short form WOMAC function scale) that consists of 7 function items for the evaluation of total joint replacement[82]. However, in order to extend the applicability of this scoring system, it requires to be validated for a broader range of knee disorders and treatment modalities.

Bone bruises are radiological anomalies, frequently observed on MRIs of patients with (osteo-)chondral defects[83]. Various publications associated this phenomenon with clinical symptoms resulting from a cartilage defect, although this has never convincingly been demonstrated, in part because of the lack of accurate and objective classification systems to determine the severity of such a bone bruise[84-86]. Anbeek recently validated a software application for automated segmentation of MRIs of the brain to determine white matter lesions[87]. A bone bruise specific modification of this application may prove to be a valuable tool for objective and accurate determination of bone bruise volume on MRI.

Henderson demonstrated that the durability of the repair tissue and the symptomatic relief is related to the morphological quality of the regeneration tissue[35]. Therefore, *in vivo* studies investigating cartilage regeneration techniques should strive towards implementing histology as one of their primary outcome parameters. A great variety of histological scoring systems is available of which the systems described by Pineda and by O'Driscoll are widely used[88-94]. However, no validation studies of these outcome scales were previously reported.

Research goals and questions to be addressed in this thesis

The studies presented in this thesis aimed to establish the position of cartilage regeneration techniques for treatment of articular cartilage defects. Subsequently, we aimed to elucidate possible targets for further optimization of cartilage tissue engineering and to determine the *in vivo* efficacy of possible disease modifiers. Finally, this thesis aimed for the development of new tools for future studies in the field of cartilage repair. Therefore, this thesis seeks to answer a number of research questions that can be divided in the following four general aims and subsequent questions:

- **To determine whether ACI provides superior structural regeneration over microfracturing:**
  - Does ACI result in superior tissue regeneration as compared to microfracturing when evaluating histological, immunohistochemical and functional parameters?

- **To elucidate the effect of donor tissue origin, cartilage defect related changes and intra-articular factors on cartilage regeneration:**
  - Does TGF-β modulate the IGF-axis during periosteal chondrogenesis?
  - Do chondrocytes from less affected areas in osteoarthritic knee joints display altered chondrogenic properties as compared to chondrocytes from healthy joints?
  - How does SF from injured knee joints affect *in vitro* chondrogenesis by human articular chondrocytes with respect to tissue morphology, proteoglycan turnover and redifferentiation?

- **To evaluate the clinical efficacy of autologous IL-1ra:**
  - Does autologous IL-1ra effectively reduce symptoms in patients with OA of the knee?

- **To develop new outcome evaluation methods for future OA and cartilage regeneration studies:**
  - Can a reduced WOMAC function scale provide a valid alternative for the traditional WOMAC in evaluating knee OA?
  - Can bone bruise volume be determined by automatic segmentation of MRI?
  - How do two frequently used cartilage repair histological outcome tools compare with respect to their intra- and inter-observer reliability?

Contents of this thesis

In order to properly address above described aims, a set of clinical trials, *in vitro* experiments and a group of validation studies was designed, the outcome of which is represented in the subsequent chapters of this thesis:

Chapter 2 provides a general background with an overview of cartilage biology, etiology of cartilage defects and OA development, and cartilage restoration techniques.

Autologous chondrocyte implantation has been demonstrated to be a promising therapy for treatment of articular cartilage defects, although its superiority as compared to microfracturing, which is currently considered the treatment of choice by many, has never convincingly been demonstrated. Determining the molecular profiles of expanded chondrocytes may give crucial information to predict the outcome of clinically applied ACI and thereby possibly provide a tool for patient selection and future optimization of ACI. **Chapter 3** describes a randomized clinical trial in which microfracturing was compared to this characterized chondrocyte implantation (CCI) treatment to determine whether CCI results in superior tissue regeneration as compared to microfracturing when evaluating histological, immunohistochemical and functional parameters.

In **chapter 4** we provide new insights into regulatory mechanisms involved during chondrogesis with respect to the modulation of the IGF-axis by TGF-β Understanding these mechanisms is essential for future improvement of cartilage regeneration techniques. As IGF is known to be a potent stimulator of periosteal chondrogenesis, this study specifically aimed to elucidate the effect of TGF-β1 on the IGF-axis, i.e. the protein IGF-1 itself, its receptor, inhibitory proteins, and enzymes involved in regulating the bio-availability, and thus the activity, of IGF.

Subsequent *in vitro* investigations are aimed at translation to clinical practice. So human articular chondrocytes are used in order to retain extrapolability to clinically applied ACI as much as possible. However, the most frequently used culture techniques in literature result in tissue with variable histological and biochemical quality when using human chondrocytes. In order to study possible detrimental effects of *in vivo* environmental factors on the outcome of cartilage tissue engineering, a reliable and reproducible *in vitro* chondrogenic model is required. Therefore, in **chapter 5** we compare a frequently applied non-growth-factor-supplemented expansion medium with bFGF-supplemented culture medium, and a pellet culture system with collagen type-II-coated filter redifferentiation system to determine which system provides most optimal *in vitro* chondrogenesis.

The subsequent chapter aims to elucidate the role of donor tissue quality on the outcome of cartilage regeneration techniques, as OA chondrocytes are frequently used for *in vitro* cartilage regeneration studies. In addition, ACI has been suggest-

ed for treatment of early OA, while currently ACI seems merely suitable for treatment of localized articular cartilage defects. Therefore, **chapter 6** compares the chondrogenic capacity of chondrocytes isolated from healthy cartilage with OA cartilage to determine the validity of *in vitro* studies performed with OA chondrocytes.

Usually patients do not seek medical care immediately after cartilage damage has occurred, but continue to use their damaged joints for some time. This will inadvertently lead to a disturbed joint homeostasis and, therefore, may explain the relatively variable outcome of clinically applied ACI when compared to *in vivo* and *in vitro* experimental results. The study described in **chapter 7** aims to clarify the effect of synovial fluid from injured human knee joints on *in vitro* chondrogenesis with respect to chondrocyte redifferentiation, PG turnover, and tissue morphology.

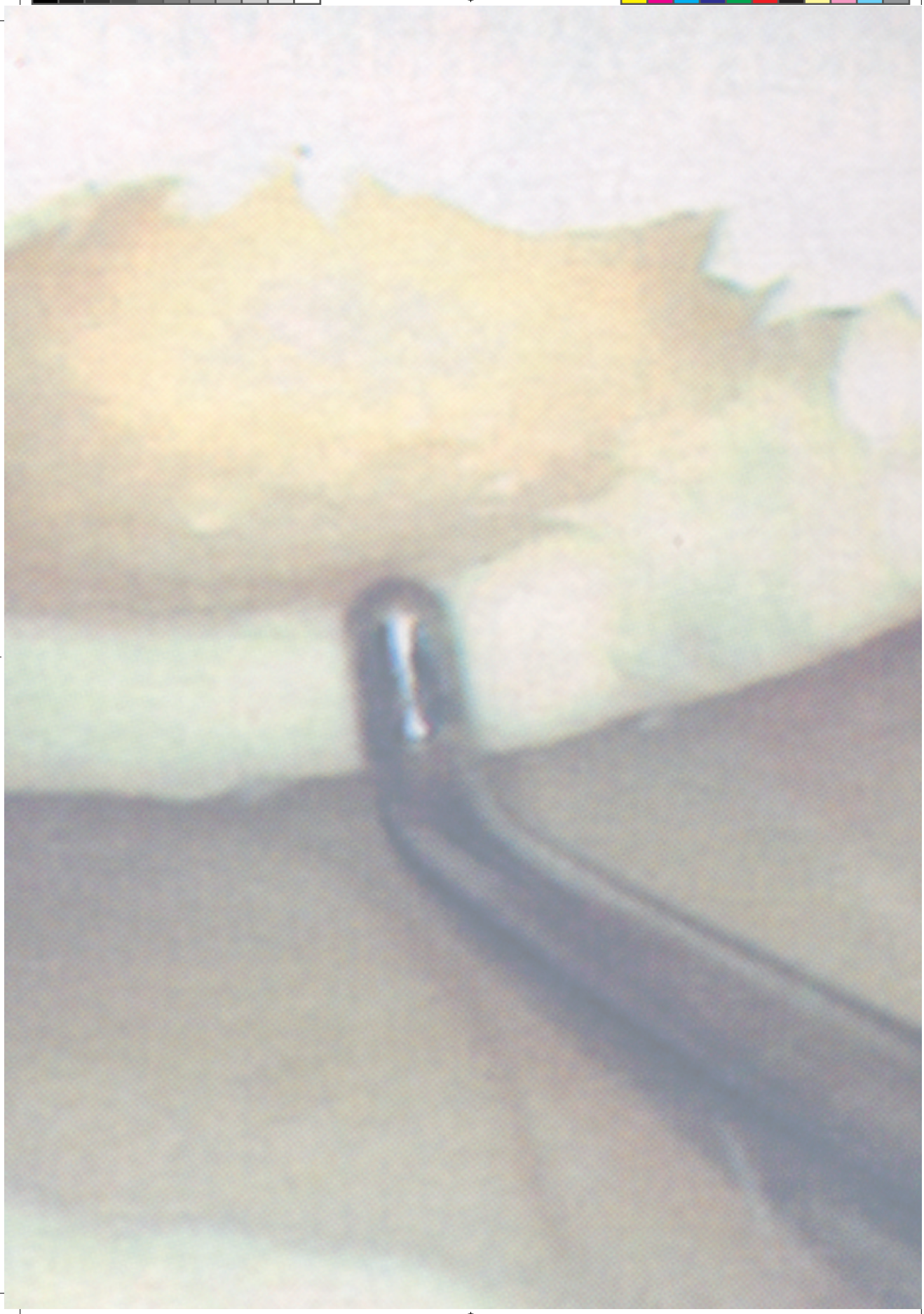
As cartilage defects may lead to the development of OA and IL-1β is known to be an important pro-inflammatory cytokine involved in the process of OA development, Orthokin (a blood product containing high amounts of IL-1ra) treatment may be a viable approach to restore the joint homeostasis, thereby possibly preventing OA development or even optimizing the conditions for cartilage tissue engineering. However, Orthokin was developed for osteoarthritic joint disorders. Before implementing it in the field of cartilage tissue engineering, we performed a multi-center, randomized, double blind, placebo-controlled trial to determine the biological efficacy and safety of Orthokin in symptomatic knee OA as described in **chapter 8**.

The subsequent chapters are devoted to improve fundamental research and clinical evaluation tools and hope to be instrumental in the progress of uniform reporting of cartilage regeneration outcome. **Chapter 9** describes the evaluation of a reduced WOMAC scale for evaluation of patients with non-surgically treated knee OA.

Chapter 10 evaluates a newly developed software application for automated segmentation of bone bruise on MRIs of patients with articular cartilage defects. In addition, by using this application, the relationship between bone bruise and symptoms arising from articular cartilage defects was examined.

In **chapter 11**, we compared two frequently used histological grading systems with respect to their reliability and reproducibility for evaluating cartilage regeneration techniques.

Finally, a summary and discussion of conclusions from the research performed aims to put the clinical implications of these results into perspective, and allows for a proposition of some future directions in research and clinical practice.



General background

Chapter 02

Articular cartilage

Articular cartilage is a highly specialized tissue covering the articulating ends of bones. It has a remarkable stiffness against compression[2,3]. Cartilage provides a sliding surface for smooth and pain free articulation, a property that is even further enhanced by the lubricating properties of synovial fluid[1].
Articular cartilage is a-vascular and a-neural. Therefore, oxygenation and nutrition of the tissue is obtained through diffusion from synovial fluid and the underlying subchondral bone layer. Synovial fluid is continuously renewed by the synovial membrane that covers the inside of the joint. Furthermore, articular cartilage is a highly organized tissue that mainly consists of extracellular matrix (ECM), while the cells contribute for approximately 1% of the total tissue volume. Classically it is thought that cartilage contains merely one cell type, the chondrocyte. However, recent studies have demonstrated that the superficial zone of articular cartilage additionally contains a pluripotent progenitor cell type, which may play an important function in the development and maintenance of the tissue[95].
The ECM consists of two main components, the collagen network (60% of the dry weight) and the glycosaminoglycans (25-35% of the dry weight). Throughout articular cartilage, 4 layers can be recognized, each with their distinct concentration and structural organization of collagens and GAGs, which is essential for the mechanical properties of the tissue (Figure 1). Collagens provide the tensile strength of the tissue. Collagen type II is the main collagen type present in articular cartilage and a stable fibrillar network is created by interlinking these collagen type II molecules by using collagen type IX[96]. The proteoglycans (PGs) consist mainly of highly sulfated aggrecan molecules, which are attached to hyaluronic acid. Due to the negative charge of these PGs, the ECM is hydrophilic and high amounts of water are attracted into the tissue (80% of the wet weight), while the collagen network provides tensile resistance to prevent unlimited expansion[97]. This property provides the tissue with stiffness against compression. Upon high loads, water is forced out of the tissue, which is regained rapidly in unstressed conditions. This sponge-like activity is important for both the nourishment of the tissue and for its load distributing properties to protect the underlying subchondral bone against peak stress.

Cartilage damage

Cartilage damage can induce progressively increasing pain, joint effusion, locking, giving way, crepitations and limited range of motion and, upon prolonged damage, may eventually even result in joint deformation. Pain usually increases after exercise and decreases in rest and is often difficult to localize. Stiffness occurs mainly in the morning after sleep and after prolonged rest and usually disappears within 10 minutes after minimal motion. Joint deformations are caused by benign hypertrophic reactions at the edges of the joints (osteophytes), by effusion or by synovial hypertrophy. A limited range of motion is caused by pain, joint destruction and

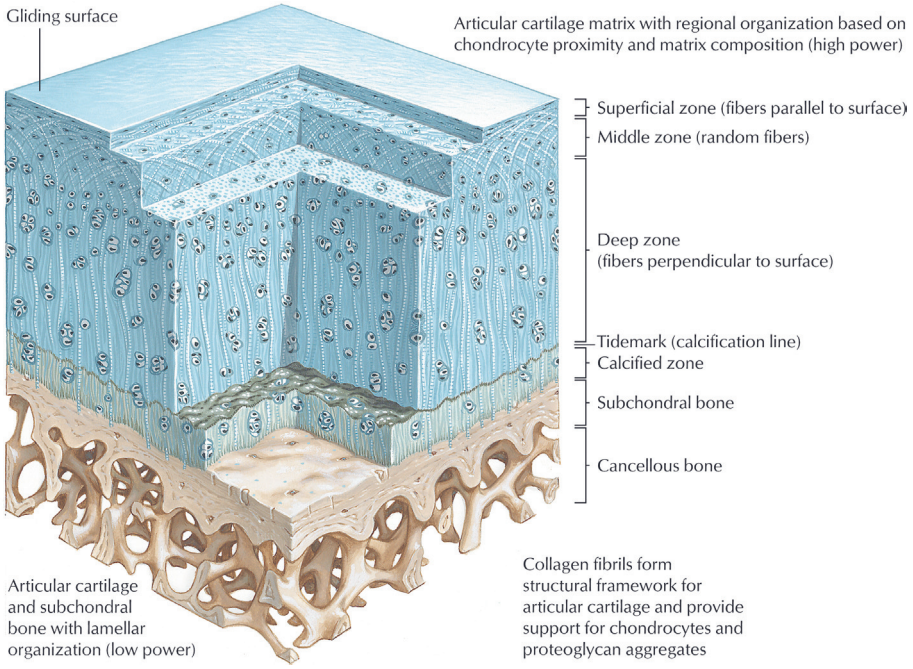


Figure 1
This schematic representation of articular cartilage shows the complex organization of the extracellular matrix and the typical distribution of the chondrocytes within the tissue. The tissue comprises four distinct layers, each with its specific amount and organization of cells and ECM proteins, which is essential for the mechanical properties of the tissue. (Reproduced with permission from netterimages.com, Elsevier).

inflammatory reaction of the soft tissue surrounding the joint.
The incidence rate of cartilage defects has been estimated to be 2.6 patients per 1,000 adults. Even more, upon arthroscopic evaluation of symptomatic knee joints, the incidence of chondral defects has been reported to range from 16% to 63%[4-10]. Chondrocytes are capable of compensating for normal ECM loss during daily use by increasing the synthesis rate of ECM proteins and inducing chondrocyte proliferation. Unfortunately, this compensation mechanism provides insufficient repair activity for defects larger than 2 millimeter in diameter. Furthermore, articular chondrocytes have a limited capacity to migrate within the tissue due to the tight ECM network and, resulting from the avascular nature of the tissue, cartilage lacks a normal inflammatory response to tissue damage, while such a response has been demonstrated to be essential for tissue repair in a variety of tissues throughout the human body, such as skin, cardiac muscle and other musculoskeletal tissue types[66,98-102]. This insufficient healing capacity of articular cartilage was already recognized in the 18th century by Dr. William Hunter, who stated: “Ulcerated car-

tilage is a troublesome thing; once destroyed, it is not repaired"[103]. In addition to the lack of repair activity, a detrimental sequence of tissue reactions following cartilage damage has been described. As a result of cartilage defects, the articulating surface is discontinuous and peak stresses evolve at the wound edges of cartilage defects[104-106]. These peak stresses in turn have been demonstrated to induce both chondrocyte necrosis and apoptosis[107-109]. Furthermore, reactive oxygen species are induced, resulting in increased oxidative stress, which in turn results in accelerated chondrocyte senescence, as reflected by a decrease in proteoglycan synthesis, response to the anabolic cytokine IGF-I, proliferative capacity, and mitochondrial function[110-112]. Altogether, this chondrocyte death and senescence result in an insufficient number of cells and inadequate cell activity to provide tissue repair and to prevent degradation. Therefore, if left untreated, cartilage defects do not heal and even can lead to the development of osteoarthritis (OA)[99,100]. OA chondrocytes are known to display altered characteristics such as an increased proteoglycan turnover rate, resulting from enhanced proteoglycan synthesis and release[50-55], the production of the hypertrophic marker type X collagen, and the expression of matrix degrading proteases such as cathepsin B and MMP-13. Moreover, chondrocytes from OA joints display a shift towards the production of collagen type I at the expense of collagen type II, while the latter collagen type is an essential component of the fibrillar network in hyaline cartilage.

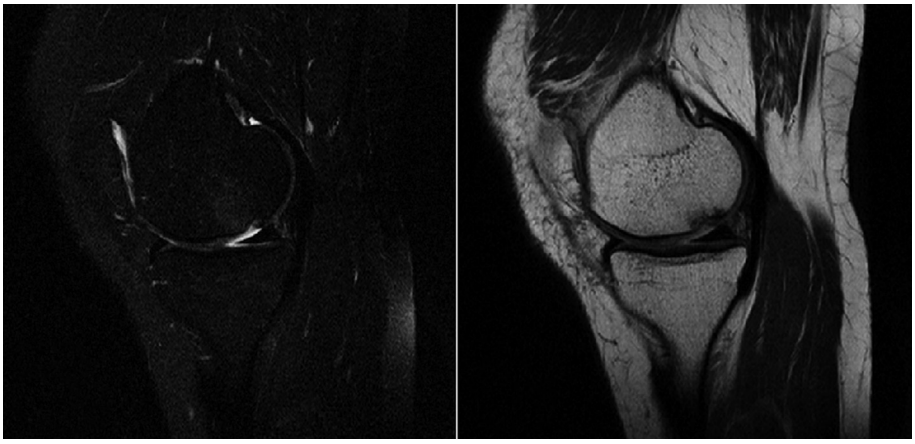
Diagnostics

Plain X-rays have little value for the visualization of chondral defects, especially when the subchondral bone layer is not involved, as the chondral tissue itself cannot be visualized.

In contrast, MRI provides detailed information on the presence and the size of a cartilage defect and possible damage to the underlying bone. In addition, MRI visualizes other intra-articular tissues, such as the menisci and the ligaments. When using proton density weighted (PDw) and T2-weighted (T2-w) Fast Spin Echo (FSE) images, the defect appears brighter than adjacent cartilage, whereas on fat-suppressed T1-weighted (T1-w) three-dimensional spoiled gradient-echo images the fluid-filled defect appears dark[113]. Furthermore, the presence of a focal area of edema-like signal in the subchondral bone marrow beneath a cartilage lesion may be more readily seen than the cartilage lesion itself, as bone bruises are found in 72 – 83% of treatable cartilage lesions[83]. This phenomenon is characterized by diffuse diminished signal intensity on PDw and T1-w MRIs, and, in the acute phase, increased signal intensity on T2-w MRIs (Figure 2).

Recent techniques have opened the field of non-invasive metabolic monitoring since settings have been developed that will allow us to evaluate proteoglycan synthesis and metabolic alterations in relationship with anatomical structure[114-117]. However, these are not standardized techniques and the clinical implications in evaluation of cartilage repair techniques need to be determined.

Despite these benefits of MRIs, it remains difficult to uniformly evaluate aspects



A B

Figure 2
(A) T2-weighted (T2-w) and (B) Proton density-weighted (PDw) MRI scans of the same knee joint with a chondral defect of the femoral condyle. Note the increased signal intensity of the damaged cartilage in both MRI settings. The subchondral bone anomaly, also known as bone bruise or bone marrow edema, is characterized by the increased signal intensity on the T2-w MRI and decreased signal intensity on the PDw MRI.

on MRI such as bone bruise and effusion. Additionally, little is known of how such phenomena relate to the patients' clinical symptoms. As articular cartilage is aneural, other pathways for explanation of clinical symptomatology should be considered. One theory relates these symptoms, at least in part, to bone bruise, although previous studies on this matter were inconclusive[84,118].

Treatment options

Surgical treatment

When medical treatment does not reduce symptoms, arthroscopic debridement is often considered in order to overcome mechanical restrictions resulting from loose and unstable cartilage. This procedure has frequently been reported to reduce pain, however there is no evidence that arthroscopy influences disease progression in OA. In contrast, from a randomized placebo controlled trial involving patients with osteoarthritis of the knee, Moseley suggested that debridement and lavage were no better than placebo treatment (sham surgery) up to 1 year post-treatment[11]. Although this study had several serious limitations concerning patient selection, stratification and insufficient statistical power to demonstrated equality between sham surgery and debridement, it does indicate that mechanical inhibition, and not OA symptoms, should guide our indication for debridement surgery.

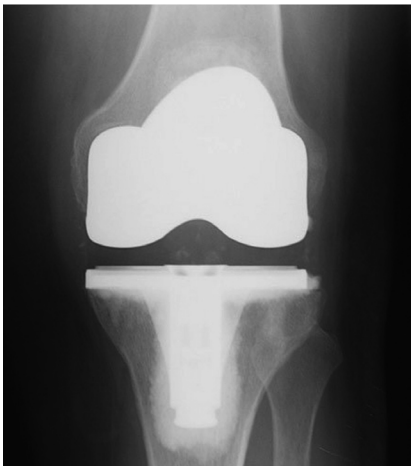


Figure 3
Total knee arthroplasty has proven to be a highly succesful treatment for OA of the knee. However, less suitable for young, more active patients, due to the limited survival of the prothesis.

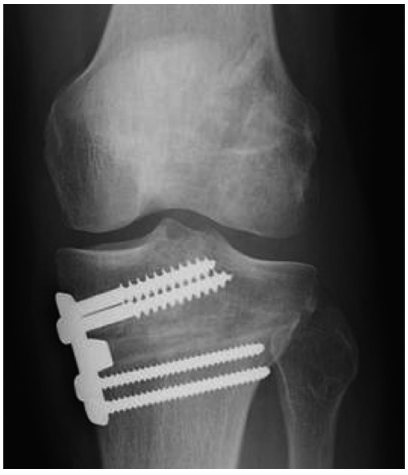


Figure 4
X-ray after an opening wedge valgus producing knee osteotomy. These inventions are an effective (temporary) solution for univompartmental knee OA.

Joint replacement surgery has proven to be a highly successful treatment in elderly patients relieving pain and resulting in restoration of function[119-122] (Figure 3). However, complications, such as (septic) prosthesis loosening, result in high morbidity for the patients, as often multiple surgical interventions are needed to solve such a problem, and revision arthroplasty does not have similar survival rates compared to primary joint replacement interventions. Furthermore, for the younger (less than 60 years of age) active patient, long-term survival of the prosthesis is the major concern and high satisfaction and low revision rates are unattainable, which is caused by higher physical activity of these patients, resulting in wear and loosening of the prosthesis.

Osteotomy is frequently used as a treatment for unicompartmental cartilage damage. The goal of an osteotomy is to alter the biomechanical axis of a joint such that the damaged cartilage is unloaded (Figure 4). Many studies have reported successful results in the application of osteotomy as it can decrease the symptomatology and increase radiological joint space width,[123-126]. Overall valgus producing high tibial osteotomies result in 53-87% of the cases with good or excellent results after 7-10 years follow-up[125]. For varus producing osteotomies, comparable beneficial results are reported[126-128]. Despite these promising results, there is a disagreement on the optimal correction angle for these procedures and these beneficial effects are known to deteriorate over time[129-132]. Furthermore, the mechanism by which the observed improvements occur is not clarified. It might be effectuated simply by the altered mechanical loading of the joint or even by the formation of

newly synthesized fibrocartilaginous tissue.

Arthrodesis, fixation of the affected joint, is highly effective in treatment of pain [133-135]. However, as arthrodesis is very disabling due to immobility of the joint, this treatment is considered undesirable for all joints with the exception of the ankle, wrist, and metatarsal 1. Especially in younger and more active patients, arthrodesis should be reserved for extreme situations in which other treatment options have failed or are not applicable.

None of the above described surgical interventions aims at restoring articular surface. However, it is not unlikely that early treatment of cartilage defects can prevent the development of OA, if the repair tissue can take over the mechanical properties of the original joint surface. During the past decades, the knowledge with respect to biological repair of cartilage defects has increased and various treatment options have become available, such as microfracturing and mosaicplasty, and cartilage regeneration techniques, such as autologous chondrocyte implantation (ACI),

Mosaicplasty is a technique in which osteochondral plugs are harvested from a non- or less-weight bearing site of the joint and implanted in the defect site (Figure 5). Variable results have been published with respect to the repair tissue resulting from mosaicplasty, ranging from fibrocartilaginous tissue to hyaline cartilage[12-15]. In addition, this treatment has been associated with a risk of donor site morbidity, especially in patients with larger defects, and it remains unknown whether the damage to the donor site does not induce the development of osteoarthritis.

Another approach comprises the perforation of the subchondral bone layer in order to provide mesenchymal progenitor cells, which are contained within the bone marrow space, access to the cartilage defect. After a blood cloth has formed in the defect, the mesenchymal progenitor cells contained within the blood cloth need to differentiate in order to synthesize cartilaginous tissue. This technique was first described by Pridie[16] and various variations to this approach have been suggested. Of these microfracturing is, as described by Steadman, currently the most frequently applied technique to perforate the subchondral bone layer (Figure 6)[17]. Therefore, the holes in the subchondral bone layer should be made as close together as possible. Consequently, microfractures occur in the bone bridging the defects. This technique results in increased amounts of repair tissue, which mostly resembles fibrocartilage, compared to untreated cartilage defects and a significant decrease of symptoms has been reported. 80% of the patients treated with microfracturing have significant reduction of symptoms until 5 years after the treatment[136].

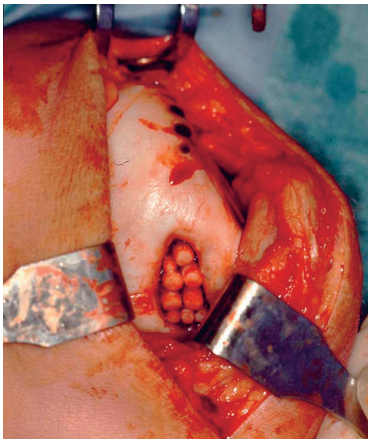


Figure 5
A per-operative example of moisaicplasty. During this procedure osteochondral plugs are harvested from a less weight-bearing area of a knee joints and transplanted into the defect area.

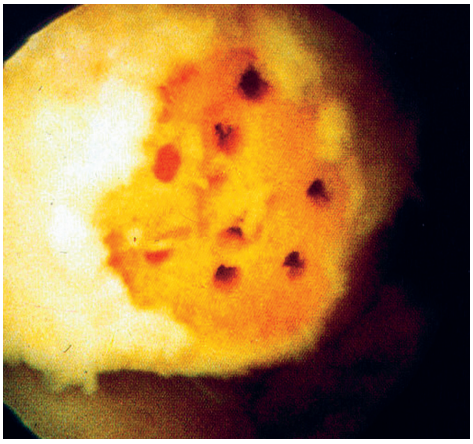


Figure 6
This arthroscopic view during microfracturing treatment demonstrates the perforated subchondral bone layer. This procedure allows bone marrow, which contains mesenchymal progenitor cells, to fill the defect and form a cloth. The repair tissue resulting from microfracturing most frequently resembles fibrocartilaginous tissue.

In conclusion, traditionally successful surgical interventions for treatment of cartilage damage are less applicable for young and active patients. The current biological treatment modalities, such as microfracturing and mosaicplasty, results is significant symptomatic alleviation, although concerns have been raised with respect to possible donor-site morbidity and limited life-expectancy of the repair tissue, which drives current cartilage research towards further exploration of regenerative medicine as treatment for cartilage defects.

Cartilage regeneration

Autologous chondrocyte transplantation (ACI), which is a cartilage regeneration technique, may prove to be a valuable treatment strategy for articular cartilage defects. This therapy has evolved from the numerous *in vivo* and *in vitro* cartilage tissue engineering studies published during the last three decades, which have provided a thorough insight into the process of chondrocyte differentiation.

Embryologically, chondrocytes are derived from mesenchymal progenitor cells. These can differentiate into a variety of cell types which give rise to tissues, such as bone, nerves, muscle, fat, skin and cartilage[137-140]. As a result, these mesenchy-

mal progenitor cells can be found in a variety of tissues, such as bone marrow, the cambium layer of periosteum, perichondrium, and even in more differentiated tissue types such as fat and cartilage[95,141-143]. Despite the chondrogenic capacity of these mesenchymal progenitor cells, ACI is currently the only clinically applied cartilage regeneration technique as these cells provide the most reliable cartilage regeneration. However, the use of autologous chondrocytes has a substantial drawback, which is the limited amount of donor tissue that can be harvested as this procedure introduces a new cartilage defect in the joint from which it is harvested. In addition, due to the low cellularity of the tissue and the merely fractional isolation of chondrocytes by enzymatic digestion[144], insufficient cell numbers are obtained to treat a chondral defect. Therefore, the cells are expanded in monolayer, a process during which chondrocytes dedifferentiate. They lose their rounded shape, produce less cartilage specific matrix proteins, such as collagen type II, and behave more like a fibroblast, producing increased amounts of collagen type I[22]. In order to obtain regenerated tissue with adequate mechanical properties, these cells need to synthesize appropriate amounts of hyaline cartilage-specific proteins. Therefore, it seems desirable either to prevent dedifferentiation or to maintain redifferentiation capacity during chondrocyte expansion. Numerous factors, such as donor age, cell seeding densities, growth factor supplementation, have been identified to affect the proliferative capacity, the degree of dedifferentiation and the chondrogenic capacity of in monolayer expanded cells[145-157]. For example, growth factors, such as Transforming Growth Factor β (TGF-β), Insulin-like Growth Factor (IGF), and basic Fibroblastic Growth Factor (bFGF), have been demonstrated to effectively improve the chondrogenic capacity of in monolayer expanded chondrocytes[146,147,149,158], although contradicting effects of bFGF on the degree of dedifferentiation have been reported[147,150,158]. Besides collagen type II, other chondrogenic markers have been identified to be associated with the cartilage-forming potential and phenotypical stability of expanded chondrocytes. such as high Fibroblastic Growth Factor-3 (FGF-3) and low Activin Receptor-like Kinase 1 (ALK-1) mRNA mRNA levels. These markers may proof to be a valuable tool for future patient selection in ACI treatment[159].

After expansion, the chondrocytes require an environment that provides adequate chondrogenic stimuli in order to induce redifferentiation and synthesis of cartilaginous tissue. Therefore, in *in vitro* models, chondrocytes are brought into high-density cultures, such as pellet cultures or micromass culture systems, which provide the 3-D environment and cell-cell contact that are known to be beneficial for chondrocyte redifferentiation and matrix synthesis[160-162]. In clinically applied ACI, chondrocytes are classically implanted by injecting a cell suspension under a periosteal membrane in order to retain the cells adequately located[24]. In contrast to the highly controlled environment of these culture models, these cells are exposed to the natural mechanical environment and other intra-articular factors, which may have a significant effect on the outcome of cartilage regeneration techniques. A relatively new topic of interest in the era of cartilage tissue engineering is the use of scaffolds as a carrier material for implantation of chondrocytes (Figure 7). Not

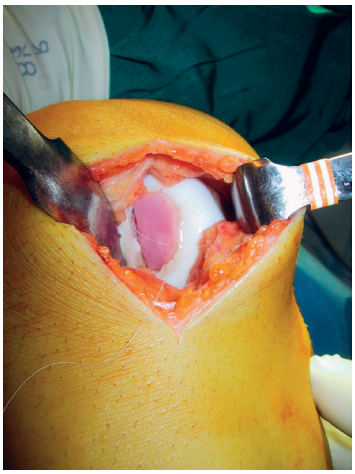


Figure 7
This picture shows a scaffold supported implantation technique. The use of scaffold materials may have a number of benefits for the ACI procedure, as it may increase the ease of the surgical procedure, protect the chondrocytes against the mechanical environment of the joint and support chondrogenic re-differentiation.

only can such scaffolds increase the ease of the surgical procedure of ACI, as it helps to contain the cells in the defect, they can also provide mechanical properties similar to the surrounding cartilage to protect the transplanted chondrocytes against mechanical stress until ECM with adequate mechanical properties has been synthesized. Furthermore, scaffolds may significantly modulate the process of chondrogenesis[163,164], for example through exposure of cell-surface integrins to collagen type II or aggrecans, which is known to result in increased matrix synthesis and an increased response to chondrogenic growth factors such as TGF-β[165,166].
Various growth factors, such as TGF-β and IGF-I, have been demonstrated to play a crucial role in the process of chondrogenesis. These growth factors increase cell proliferation, redifferentiation, as reflected by increased collagen type II expression levels, and proteoglycan synthesis. When combined, these growth factors induce a synergistic chondrogenic effect[42,43,45-47,167,168]. In addition, in cultured human osteoblasts, it has been demonstrated that TGF-β may even further enhance the bioavailability of IGF-1 through dissociation of IGF-I from its inhibitory binding protein, which is likely induced through upregulation of its cleaving enzyme: pregnancy associated plasma protein-A (PAPP-a)[48,169]. However, clinical implementation of these growth factors by direct exogenous supplementation, e.g. through intra-articular injection, may result in significant side-effects such as osteophyte formation[44]. Therefore, new developments in the field of gene-therapy and controlled released from so-called “smart scaffolds” may be a feasible approach to introduce these growth factors in clinical application[170-176].

Altogether, cartilage regeneration seems a viable approach for long-lasting repair of articular cartilage defects. Currently, ACI comprises a multi-step procedure and its outcome can be optimized on various levels, ranging from culture conditions and surgical techniques to patient selection and modulation of the intra-articular environment.

Disease modifying osteoarthritic drugs

Inflammatory cytokine involvement during OA development

As discussed in the introduction of this thesis, pre-treatment of a joint in order to restore the joint homeostasis may be a viable approach to improve cartilage tissue engineering techniques. Given the novelty of this concept, little is known of the factors responsible for the disturbed joint homeostasis. In contrast, the mechanisms involved in OA development have been elucidated in much further detail. While cartilage defects and OA are considered two independent entities with different etiologies and distinct treatment modalities, integration of knowledge gained in the field of OA development may provide feasible targets for restoring joint homeostasis.

Due to its high prevalence (6-12% of the adult population)[177]. OA is associated with high costs for the society, which will increase even more due to the aging of the population. Present day therapy of OA combines non-pharmacologic and pharmacologic treatments aimed at symptom relief. Although there are no medications or surgical interventions yet indisputably proven to alter the course of OA development, there is considerable research currently being directed at disease modification.
Inflammatory cytokines appear to play an important role in this process and IL-1β is frequently proposed to be a key factor[60,63]. In the context of articular tissues, IL-1 was first described by Seckinger who showed that this cytokine induced collagenase and prostaglandin production in synovial fibroblast cultures, which suggests that it stimulates the catabolism of cartilage[70]. Several studies have shown that IL-1 could also contribute to the depletion of the cartilage matrix by decreasing the synthesis of cartilage specific collagens and proteoglycans[61,69]. Matrix metalloproteinase (collagenase, gelatinase and stromelysin) and prostanoid production by IL-1-activated rabbit articular chondrocytes is shown to be suppressed by Interleukin-1-receptor antagonist (IL-1ra), which suggests a beneficial effect of this protein for cartilage metabolism[178]. Analogously, IL-1ra restores dose-dependently the cartilage metabolism in IL-1α and IL-1β stimulated cartilage explants[178]. IL-1ra induces its effect through competitive inhibition of IL-1 by occupying the IL-1 receptor without activating it. Furthermore, in an experimental equine OA model, *in vivo* delivery of the equine IL-1Ra gene results in significant improvement in clinical parameters of pain and disease activity, preservation of articular cartilage, and has beneficial effects on histological parameters of synovial membrane and articular cartilage[71].

Orthokin

Incubation of whole blood with $CrSO_4$-coated glass beads has been demonstrated to stimulate the synthesis of Interleukin-1 receptor antagonist (IL-1ra) and other anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13[72]. This product is known as Orthokin. As IL-1β is suggested to play a key role in OA development, Orthokin might have a beneficial effect on the symptoms and disease progression of OA.

Furthermore, if an intra-articular biological effect can be shown, it might also be implemented to improve the success of cartilage tissue engineering techniques for treatment of cartilage defects by modulating the joint homeostasis into an environment that is beneficial for cartilage regeneration.

Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture

Chapter 03

Abstract

Goal: To determine whether Characterized Chondrocyte Implantation (CCI) provides superior structural repair and non-inferior clinical improvement as compared to microfracturing.

Methods: In a multicenter randomized controlled trial, CCI was compared with microfracture in patients with symptomatic cartilage defects of the femoral condyles. Structural repair was assessed at 1 year and clinical outcome was determined at 12 and 18 months post-surgically.

Results: CCI resulted in better structural repair, as assessed by histomorphometry ($p = 0.003$) and overall histology ($p = 0.012$). A comparable improvement on clinical outcome was found at 12 to 18 months post-surgically. Furthermore, both treatments displayed comparable safety profiles.

Conclusion: CCI was associated with a tissue regenerate that was superior to the repair tissue after microfracture, which may result in an improved long-term clinical benefit with CCI, while the short-term clinical outcome was similar for both treatments. These results warrant further investigations that aim to elucidate and improve the process of ACI.

Introduction

Regenerative medicine holds great promise for the reconstitution of damaged tissues and organs. Articular cartilage injuries are a prime target for regenerative techniques, as spontaneous healing is poor. When untreated, cartilage injuries predispose to osteoarthritis, which is a major cause of disability and represents a huge socioeconomic burden to society[5,9,179,180].

The aim of treatment for cartilage damage is to restore normal, long-term and pain-free motion of the joint, by promoting the formation of repair tissue with the structure and durability of natural hyaline-like articular cartilage[181-183]. Marrow stimulation techniques (such as microfracture), mosaicplasty and autologous chondrocyte implantation (ACI) are currently used joint resurfacing techniques. Although studies have been conducted to evaluate their efficacy, the methodological quality has been generally low[184] and the results conflicting. Consequently, no evidence-based consensus has emerged regarding the best treatment option.

ACI was one of the first techniques to introduce the principle of regenerative medicine. It is a two-step procedure with a cartilage harvest during arthroscopy and implantation of *ex vivo*-expanded chondrocytes during arthrotomy 4-5 weeks later[185]. Since the first publication of this technique 12 years ago[24], more than 12,000 patients have undergone ACI[186]. Convincing clinical evidence regarding its efficacy and safety is still lacking[184], with only one Level 1 study published to date[31]. While hyaline-like repair tissue and good long-term results have been demonstrated following conventional ACI[33], some studies have shown ACI to be associated with the formation of fibrocartilage or mixed hyaline/fibrocartilage[34,187]. Expansion of autologous cells in conventional ACI leads to dedifferentiation and loss of chondrogenic capacity[188-190]. Characterized Chondrocyte Implantation (CCI) has been developed to improve the results of articular regeneration with chondrocyte cell therapy through the use of a cell population capable of making stable hyaline-like cartilage *in vivo*, a feature that is determined by assessing a previously described panel of molecular markers (ChondroCelect®)[159,189,190].

This prospective, randomized, controlled study was designed to compare the efficacy and safety of CCI versus microfracture in the repair of single symptomatic cartilage defects of the femoral condyle. Microfracture was chosen as the comparator as it is the most frequently used first-line treatment for this condition[191]. The dual primary aims of the study were (i) to demonstrate superiority of CCI over microfracture in the overall quality of structural repair 12 months posttreatment (by histomorphometry and an Overall Histology Assessment Score), and (ii) to demonstrate noninferiority of clinical outcome, assessed by the Overall Knee Injury and Osteoarthritis Outcome Score (KOOS) at 12 to 18 months posttreatment. In addition, components of structural repair relating to chondrocyte phenotype and tissue structure were evaluated, as well as short-term clinical outcome according to KOOS subdomains.

Methods

Study Population

The first patient was enrolled in the study on February 21, 2002 and the last patient completed the 18-month assessments on July 25, 2006. Eligible patients were aged between 18 and 50 years, had a symptomatic single cartilage lesion of the femoral condyles (between 1 and 5 cm^2), and agreed to actively participate in a strict rehabilitation and follow-up program.

This was a multicenter study involving 13 orthopedic centers in Belgium, The Netherlands, Germany and Croatia. The study conformed to the Declaration of Helsinki and was conducted according to the International Conference on Harmonization guidelines for Good Clinical Practice (GCP). The protocol and all amendments were approved by the ethics review board at each participating center and all patients gave written informed consent.

Study Design and Treatment Randomization

Following screening, all patients underwent arthroscopy, during which the knee and the suspected cartilage defect were inspected. At this time, patients were randomly allocated to either CCI or microfracture, using a minimization method[192] to achieve treatment balance with respect to operating surgeon, location of lesion and presence or absence of associated lesions.

For both procedures, surgeons and study team personnel were trained extensively in the standardization of surgical and study procedures, including the use of ChondroCelect® (TiGenix NV, Haasrode, Belgium). Patients allocated to the microfracture group were treated according to standard technique[193]. Those allocated to the CCI group were treated as described by Brittberg[24]. Harvested cells were isolated, expanded ex vivo in a Good Manufacturing Practice (GMP)-approved facility, and their phenotype characterized. Each batch of ChondroCelect® was quantitatively graded using a biaxial scoring system based on the quantitative gene expression of a selection of positive and negative markers developed to predict the cells' ability to form stable hyaline cartilage *in vivo*[159,189,190]. CCI was performed 27 days (± 5 days) after chondrocyte harvest who scored positive on the ChondroCelect® biaxial scoring system.

Following surgery, both groups of patients were enrolled in an identical standardized rehabilitation program and attended regular follow-up visits at the clinic. Patients were to refrain from taking pain relief medications for 2 weeks prior to baseline and 2 weeks prior to each subsequent study assessment. Investigators were responsible for the presurgical assessments, surgery, and endpoint biopsy. A coordinating surgeon assured surgical standardization during cartilage harvest, treatment and endpoint biopsy, and an independent evaluator at each study center was responsible for all postsurgical evaluations.

Histological Analysis

Cylindrical full-thickness cartilage biopsies of 2 mm in diameter were obtained from the center of the repair tissue 12 months post treatment. The specimens

were fixed in paraformaldehyde prior to being embedded in paraffin and sectioned at a thickness of 2 to 5 µm. Sections were stained with hematoxylin-eosin (H/E), safranin-O and anti-collagen II antibodies.
Histomorphometry was performed at the Institute for Pathology, University of Bern, Switzerland, by independent personnel blinded to treatment allocation. Stained sections were digitized using a Zeiss digital camera and the stain signal for safranin-O and anti-collagen II antibody determined using Image Pro-plus software (Media Cybernetics, Silver Spring, Maryland, USA). The surface area of the cartilage was estimated using a calibrated macro. The extent of staining with safranin-O and anti-collagen II antibody was expressed as a ratio of the total surface area of the cartilage[194] and the ratios were summed.
The Overall Histology Assessment Scores were determined by two blinded independent histopathologists, specialized in cartilage histology, who assessed the quality of cartilage repair. The histopathologists also evaluated individual components of cartilage repair relating to chondrocyte phenotype (matrix-staining metachromasia, cell morphology), tissue structure (tissue morphology, calcification front/tidemark, basal integration, mid/deep zone assessment, surface architecture, surface/superficial assessment) and negative features of cartilage repair (vascularization, inflammation, subchondral bone abnormalities/cellular infiltration, abnormal calcification/debris). Each item was scored using a 100 mm visual analog scale (VAS).
The histopathologists responsible for the histology scoring were trained to grade sections in a consistent manner before performing the analysis of the sections for this clinical trial.

Assessment of Clinical Outcome

Clinical outcome was assessed using the patient-completed KOOS questionnaire, consisting of five domains: Symptoms/Stiffness, Pain, ADL, Function in Sports and Recreational Activities, and Quality of Life (QoL). Scores for the summed (Overall KOOS) and individual domains were transformed to a 0 - 100 scale, with 0 representing extreme knee problems and 100 representing no knee problems[76]. The sports domain was not included in the Overall KOOS because it could not be assessed in patients who did not participate in sports.

Safety

Safety was assessed by physical examinations, vital signs, electrocardiograms (EKGs), clinical laboratory tests and continuous monitoring of adverse events.

Statistical Analysis

Analyses were performed according to the intention-to-treat principle. Safety analyses included all randomized subjects who received any study treatment (harvest biopsy or microfracture). Efficacy analyses were performed on all randomized subjects who did not withdrew before completion of the surgical treatment.
The sample size for this study was determined using a categorization (success/failure) whereby the presence of hyaline or hyaline-like tissue was recorded as a

success and fibro- or non-cartilage as a failure. It was assumed that 30% of patients allocated to microfracture would be categorized as a success, and that an improvement in this success rate to 60% success with CCI would constitute a clinically significant improvement. On this basis, with 90% power and using a 2.5% one-sided test, it was calculated that a total enrollment of 112 patients (56 per group) would be required.
The sum of histomorphometric scores on safranin-O and collagen II staining and the mean Overall Histology Assessment Score at 12 months were analyzed using fixed effects analysis of covariance (ANCOVA) with treatment, location of lesions and associated lesion as categorical variables and age as a covariate. The differences in adjusted means for the histomorphometric sum of the safranin-O and collagen II endpoint, and for the Overall Histology Assessment Score, were tested independently for equality to zero. Multiple comparison adjustments were controlled at a significance level of 0.05 using the Hochberg procedure[195].
The change from baseline in KOOS score averaged over 12 and 18 months was analyzed using the same method as for structural repair, with baseline KOOS score added to the ANCOVA model as a continuous variable. Noninferiority for the Overall KOOS was defined as having been demonstrated if the lower end of the 95% confidence interval (CI) for the difference between adjusted means in the two treatment groups was above -9 percentage points[31,196,197]. For missing KOOS domain scores due to reintervention of the index lesion (i.e. treatment failure), a last observation carried forward (LOCF) approach was used whereby missing values were replaced with the last previous nonmissing value.
Adverse events that occurred with a frequency of 5% or more in either treatment group were compared using the Fisher's exact test.

Results

Patient Enrollment and Baseline Characteristics

Of the 118 patients enrolled in the study, 57 were randomized to CCI and 61 to microfracture. Patient disposition and the numbers of patients who contributed data to the structural repair and clinical outcome analyses are presented in Figure 1. The study was unblinded 18 months after the last patient had been treated.

Table 1 illustrates that both treatment groups were generally well-matched regarding patient baseline characteristics. Proportionally more patients in the CCI group had undergone previous knee surgery (50/57 [88%] versus 47/61 [77%]), in particular, marrow stimulation, and had undergone two or more previous knee surgeries (21/57 [37%] versus 13/61 [21%]). The median duration of symptoms of patients in the CCI group was slightly longer than that of the microfracture group (1.97 years versus 1.57 years).

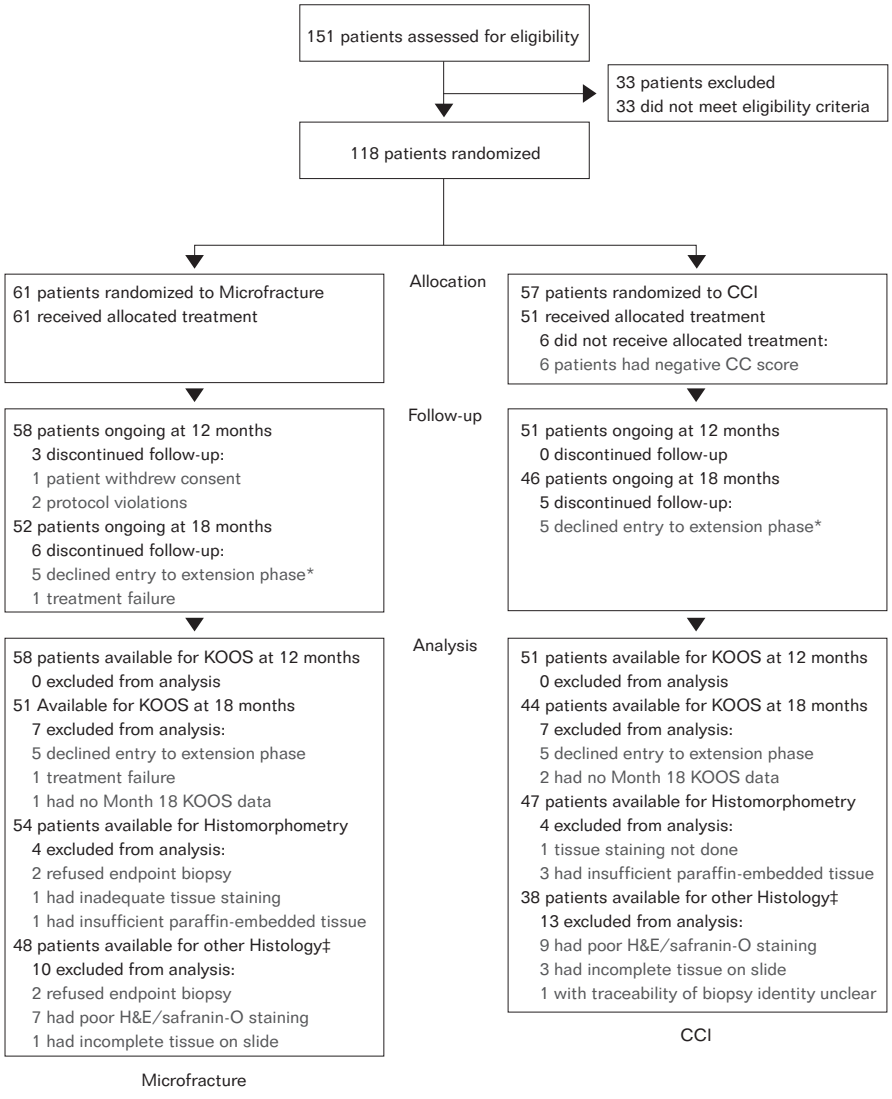


Figure 1
An overview of the enrollment of the patients. * Includes one patient declined entry to extension phase (months 12 to 60) due to re-intervention (i.e. treatment failure). ‡ Note: not all samples were evaluated for all 14 histology scales (e.g. Overall Histology Assessment Score: CCI, n=36, microfracture, n=46; Inflammation scale: CCI, n=28, microfracture, n=36

Structural Repair

The adjusted mean sum of ratios for safranin-O and collagen II staining, determined by histomorphometry, was statistically significantly higher for the CCI group than for the microfracture group (Figure 2A). The mean sum of ratios provides a measure of proteoglycan and collagen II content of the cartilage, which is considered a quantitative and objective measure of good structural cartilage repair[194].
The adjusted mean Overall Histology Assessment Score was statistically significantly higher for the CCI treatment group than for the microfracture group (Figure 2B). Inter-reader agreement for the Overall Histology Assessment Scores was good (Pearson correlation coefficient = 0.78).
Adjusted mean scores for components of structural repair relating to chondrocyte phenotype and some components relating to tissue structure were also significantly higher for the CCI group compared to the microfracture group (Figure 2C). The statistically significant differences between the two treatment groups in the quality of the repair tissue are reflected in the safranin-O- and collagen II-stained biopsy specimens presented in Figure 3.

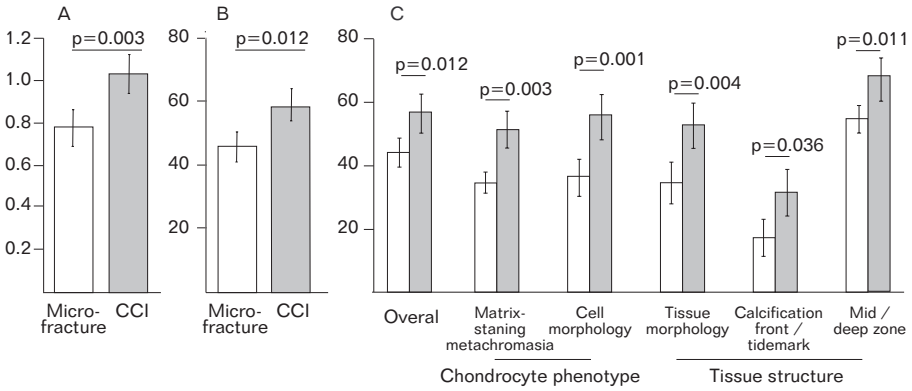


Figure 2

These graphs show the effect of CCI as compared with microfracture on structural repair determined from histological evaluation of biopsy specimens taken 12 months post-surgery. In all panels the clear bars represent microfracture, shaded bars represent CCI. All values presented are adjusted means (± standard error [SE]). P-values are provided for differences in adjusted means between CCI and microfracture. In panel A, columns represent the mean sum of ratios of the computer assisted histomorphometry score. In panel B, columns represent mean scores from two histopathologists, blinded to treatment allocation, for the Overall Histology Assessment Score (scored on 100 mm VAS on which a score of 0 mm was 'bad' and 100 mm was 'good'). In panel C mean scores from two histopathologists, blinded to treatment allocation, for other structural components (scored on 100 mm VAS where a score of 0 indicates a poor repair result and 100 mm is an ideal repair result): Items not presented include: basal integration (CCI 67.7 [SE, 6.7], microfracture 72.2 [SE, 5.7]; p = 0.471); architecture of the surface (CCI 57.7 [SE, 8.4], microfracture 61.9 [SE, 7.0]; p = 0.593); surface/superficial assessment (CCI 37.1 [SE, 5.7], microfracture 33.7 [SE, 4.9]; p = 0.547); subchondral bone abnormalities/cellular infiltration (CCI 81.8 [SE, 4.8], microfracture 74.1 [SE, 4.0]; p = 0.096); inflammation (CCI 99.8 [SE, 1.2], microfracture 98.4 [SE, 1.0]; p = 0.247); vascularization (CCI 97.5 [SE, 4.7], microfracture 91.7 [SE, 4.1]; p = 0.207) and abnormal calcification/debris (CCI 84.7 [SE, 6.2], microfracture 81.9 [SE, 5.2]; p = 0.627).

Table 1
Baseline Characteristics of the Patients

		Microfracture (N=61)	CCI (N=57)
General demographics			
Age category	Age – yr	33.9 ± 8.6	33.9 ± 8.5
	<40 yr – no. (%)	43 (70)	40 (70)
	≥40 yr – no. (%)	18 (30)	17 (30)
Gender category	Height – cm	177.0 ± 8.5	176.5 ± 10.8
	Weight – kg	80.6 ± 13.3	78.3 ± 13.9
	Male – no. (%)	41 (67)	36 (61)
	Female – no. (%)	20 (33)	22 (39)
BMI category	≤25 kg/m^2 – no. (%)	31 (51)	28 (49)
	>25 - ≤30 kg/m^2	24 (39)	26 (46)
	>30 kg/m^2	6 (9.8)	3 (5.3)
Knee condition at screening			
Symptom onset category	Duration since onset – yr (median, range)	1.57 (0-18)	1.97 (0-18)
	Gradual – no. (%)	26 (43)	29 (51)
	Acute – no. (%)	35 (57)	28 (49)
# of previous knee surgeries	Normal opposite knee - no. (%)	50 (82)	38 (67)
	Any previous knee surgery § - no. (%)	47 (77)	50 (88)
	0 – no. (%)	14 (23)	7 (12)
	1 – no. (%)	34 (56)	29 (51)
	≥2 – no. (%)	13 (21)	21 (37)
Femoral condyle at baseline arthroscopy			
	Single cartilage lesion – no. (%)	59 (97)	56 (98)
	Defect size postdebridement – cm^2	2.4 ± 1.2	2.6 ± 1.0
	Cartilage lesion grade IV – no. (%)	45 (74)	47 (82)
	Associated lesions – no. (%)	15 (25)	17 (30)
Concomitant lesions	ACL – no. (%)	4 (6.6)	3 (5.3)
	ML – no. (%)	6 (9.8) †	0 (0)
	ACL + ML – no. (%)	1 (1.6)	1 (1.8)

Values are means ± SD (unless stated otherwise). ACL denotes anterior cruciate ligament, ML meniscal lesion. † In the microfracture group, 5 of the 6 patients categorized as being treated for a meniscal lesion during the study received only shaving or debridement rather than a surgical resection. § In the CCI group, 5 patients had previous microfracture and 3 had previous subchondral drilling. In the microfracture group 1 patient had previous microfracture and 2 had previous subchondral drilling. In addition, 1 patient in each group had previous abrasion arthroplasty.

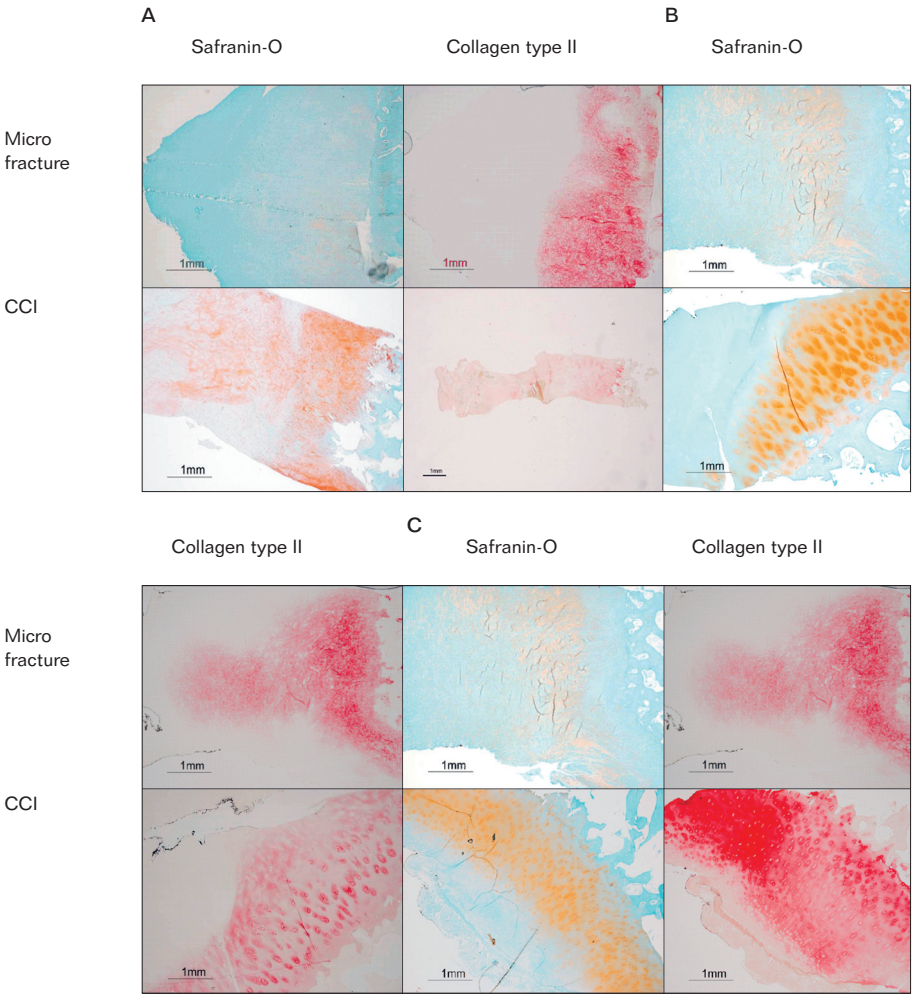


Figure 3
These histological sections demonstrate the effect of CCI as compared with microfracture on Safranin-O and Collagen II staining of 12-month post-surgical biopsies. Sections presented from the CCI and MF treatment groups have been selected based on the Overall Histology Assessment Scores and are representative of the worst (A), median (B), and best (C) images.

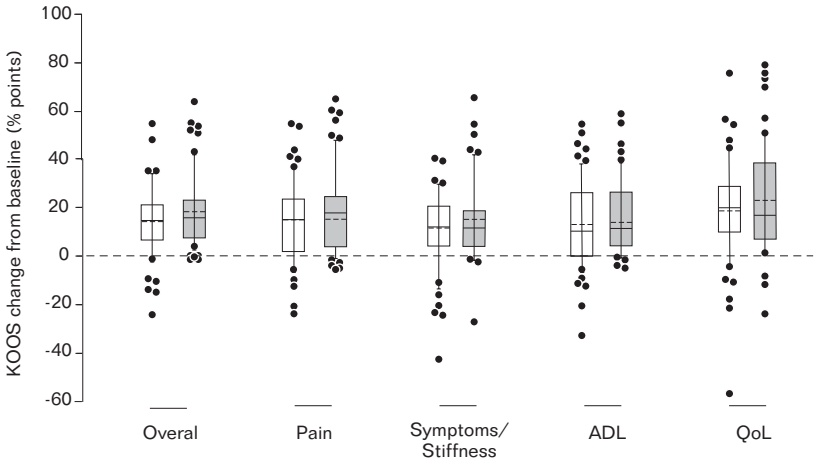


Figure 4
This graph shows the change from baseline in overall KOOS and Individual KOOS domains for the average of 12 and 18 months of post-surgery follow-up. Open bars represent data from the Microfracture group and the shaded bars data from the CCI group. The upper and lower edges of each box plot indicate the 25th and 75th percentiles, the "whiskers" the 10th and 90th percentiles, the solid horizontal line the median, and the dotted line the mean. All outliers are shown as individual data points.

Clinical Outcome

The adjusted means for the change from baseline to the average of 12 and 18 months in Overall KOOS and the subdomains of Pain, Symptoms/Stiffness, ADL and QoL were also similar for the two treatment groups (Figure 4). The lower limit of the 95% CI for the difference between adjusted means (i.e. -3.28 percentage points) in Overall KOOS was above the predefined delta for noninferiority testing, confirming noninferiority of CCI compared to microfracture.

Safety

Similar proportions of patients recorded one or more treatment-emergent (post-randomization) adverse events (AEs) in the CCI group compared to the microfracture group (50/57 [88%] versus 50/61 [82%] patients, respectively).

Overall, significantly more patients in the CCI group experienced joint swelling (11/57 [19%] versus 3/61 [4.9%]; p = 0.022), which occurred between 1 and 14 days after arthrotomy in the CCI group compared to between 6 and 12 weeks after arthroscopy in the microfracture group. Related AEs of joint crepitation were significantly higher in the CCI group compared to the microfracture group (7/57 [12%] versus 1/61 [1.6%]; p = 0.028).

No patients were discontinued from the study due to AEs and there were no clinically important trends on physical examination, vital signs, EKG, hematology or clinical chemistry during the study.

Concerns have been expressed in the literature regarding cartilage hypertrophy following ACI[198]. There was no statistically significant difference in the incidence of hypertrophy between the CCI and microfracture groups (14 patients [25%] versus 8 patients [13%], respectively). Of these, 7/14 patients in the CCI group were asymptomatic, 5/14 were symptomatic and 2/14 had both asymptomatic and symptomatic events. In the microfracture group, 7/8 patients had asymptomatic hypertrophy and one was symptomatic.

Discussion

This is the first prospectively designed and controlled GCP-compliant trial to be conducted to date in the field of cartilage repair. This trial design included various measures to ensure an unbiased comparison including (i) random allocation of patients to treatment group, (ii) implementation of the same rehabilitation program for all patients, (iii) conduct of postoperative assessments by independent evaluators, (iv) assessment of structural repair by independent histopathologists blinded to the patients' treatment allocation, (v) evaluation of clinical outcome according to a patient-reported outcome measure and (vi) the use of a prespecified statistical analysis plan. Although the specialist surgical skills required for ACI make a comparison with standard surgical procedures such as microfracture difficult, the surgeons involved in this trial were fully trained in the CCI technique prior to its commencement. Additionally, a coordinating surgeon was responsible for standardizing surgical procedures. Finally, exploratory analyses revealed no significant effects of surgeon learning on the primary endpoints of this study.

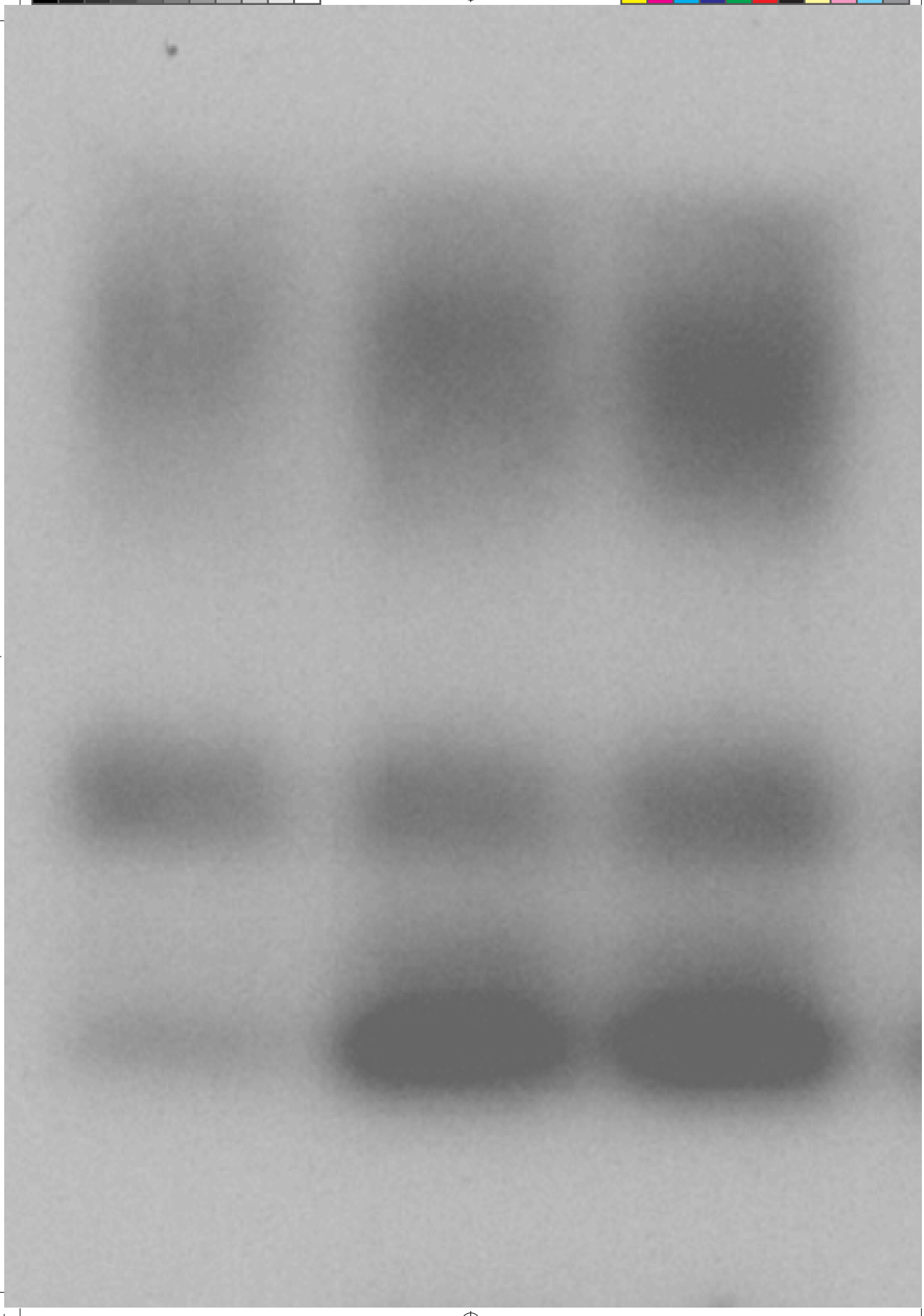
Both treatment groups were generally well balanced for demographic and baseline characteristics. The average defect size treated in this study (approximately 2.5 cm^2) is typically encountered in clinical practice and may be expected to respond well to both microfracture and ACI[199]. In this study, patients in the CCI group had experienced symptoms for longer than patients in the microfracture group. In addition, more patients in the CCI group had undergone knee surgery prior to their participation in this study. It has been shown that it is more difficult to regenerate cartilage of a hyaline-like quality when the patient has more symptoms, has had previous knee surgery or has an older defect[37], and randomization outcome of this study might therefore be interpreted as giving a small bias in favor of the microfracture group.

This study demonstrates that CCI results in superior structural repair compared to microfracture, as determined by analysis of biopsy staining, and this is supported by the significantly higher Overall Histology Assessment Score in the CCI group.

Statistically significant improvements with CCI compared to microfracture were observed for quantitative histomorphometry, overall histology and certain components of structural repair relating to chondrocyte phenotype and tissue structure. Thus, analysis of the tissue regenerate at 12 months post-treatment indicates that CCI is associated with more chondrocyte-like cells and a higher proteoglycan content of the cellular matrix, which confers compressive strength. The repair tissue

of patients treated with CCI was less fibrous and showed elements indicative of higher compressive strength and physiological maturation, in favor of true regeneration and reconstitution of articular cartilage structure.
The Modified O'Driscoll Score (MODS) is a widely used tool for the histological assessment of cartilage repair in animal studies ex vivo[200]. Although it was initially intended to use the MODS in this study, limitations in its use in biopsy specimens of patient cartilage repair studies were reported[36]. The Overall Histology Assessment Score and the individual components of cartilage repair evaluated in this study constitute a novel scoring system derived from the MODS that has been designed to ensure greater applicability to patient studies, soon to be adopted by the International Cartilage Repair Society.
The basic premise of conventional ACI is that implanted chondrocytes are able to form hyaline-like cartilage, which in turn may restore the integrity of the joint surface and promote durable tissue regeneration. The only other randomized controlled trial comparing ACI and microfracture to date failed to show a statistically significant difference between treatments in structural repair at 24 months[31], possibly due to the lower number of patients. The improved structural repair observed with CCI in this study may reflect the use of characterized chondrocytes rather than uncharacterized, dedifferentiated chondrocytes that may have lost their ability to re-express the articular cartilage phenotype *in vivo*. These results suggest that characterization of the cells might be essential, as recently discussed for cardiac cell therapy products[201].
Patients randomized to the CCI group required both an arthroscopy with harvest biopsy and an arthrotomy, whereas those in the microfracture group required arthroscopy only (and no harvest biopsy). It may be expected that open surgery with CCI may be disadvantageous to the pace of physical recovery in the CCI group. However, the results from this study demonstrate that by 6 months following treatment, the patients' clinical status had improved to a similar extent in both groups compared to baseline. In addition, although it may be expected that CCI would be associated with a significantly worse tolerability profile, it was generally well tolerated and did not result in a marked increase in the incidence of AEs when compared to microfracture. The patient retention rate was high for both treatment groups, with 90% and 85% of patients treated with CCI or microfracture, respectively, still in the study at 18 months.
Remodelling and maturation of the cartilage repair tissue following CCI is a process that is thought to occur over time and to continue to improve beyond 18 months following treatment[202,203]. The mechanisms of tissue repair with marrow stimulation techniques, such as microfracture, are quite different from ACI, and the resulting repair tissue is thought to be more 'callus-like' or fibrocartilaginous in nature and likely to be less durable than the original articular cartilage, degenerating over time so that clinical symptoms relapse[182,202,204-207]. In addition, as this method of repair is 'osteochondral', violating the tidemark and subchondral bone plate, it is suggested that the tidemark/bone front may 'move up' and ultimately lead to 'intralesional osteophytes', which potentially contribute to deterioration of symptoms and development of osteoarthritis. In view of this, it may be incorrect to

consider microfracture as a procedure that does not "burn bridges"[208].
The combined structural and clinical outcome measures assessed in this study are, in our view, warranted. Currently, insight into clinically relevant outcomes in these patient populations is limited. Analysis of appropriate reconstitution of tissue structure is expected to be predictive of successful long term outcome, and thus reflects an essential aspect in the assessment of novel regenerative treatments. However, we believe that this is only acceptable if short term clinical outcomes are not inferior to existing treatment methods and are accompanied by significant treatment effects, as observed in this study. The clinical outcome of patients enrolled in this study continues to be evaluated.
In conclusion, the use of characterized chondrocytes in autologous cartilage repair represents a new class of treatment, which is associated with superior structural repair of cartilage tissue, compared to microfracture. In the short-term, the risk-benefit profile for CCI and microfracture appears to be similar, supporting a first-line use. The significantly better structural repair with CCI observed in this trial merits long-term clinical evaluation to determine whether a correlation exists between superior short-term structural repair and long-term clinical outcome.



Regulation of the IGF axis by TGF-β during periosteal chondrogenesis: implications for articular cartilage repair

Chapter 04

Abstract

Goal: TGF-β and IGF-I are key growth factors during chondrogenesis. The bioavailability and activity of IGF-I can be regulated on multiple levels: IGF-I, IGF-I-receptor, inhibitory IGF binding proteins and cleaving enzymes that cleave IGF-I from its binding enzymes. The objective of this study was to determine if TGF-β regulates the IGF-I axis during *in vitro* periosteal chondrogenesis.

Methods: Periosteal explants from 2-month-old rabbits were cultured with or without TGF-β1. Gene expression was determined by real-time quantitative PCR. Conditioned medium was analyzed for IGF binding protein (IGFBP) levels, IGFBP-4 protease activity, and the IGFBP-4 protease pregnancy-associated plasma protein-A (PAPP-A) secretion.

Results: TGF-β1-treated explants contained lower IGFBP-4 mRNA levels and secreted less IGFBP-3 and IGFBP-4. PAPP-A mRNA and protein levels, as well as IGFBP-4 protease activity were increased between 7 and 10 days of culture (the onset of cartilage formation in this model) in TGF-β1-treated explants. IGF-I and IGF-I receptor mRNA levels were also decreased in TGF-β1-treated explants.

Conclusion: Although additional studies are required to determine the net effect of TGF-β1 on IGF bioavailability, this study demonstrates that the expression of multiple members of the IGF-I axis is modulated during *in vitro* TGF-β1-induced periosteal chondrogenesis.

Introduction

Previous studies have suggested that TGF-β can regulate the bioavailability of IGF-I in chondrocytes and osteoblasts through modulation of IGFBP levels[48,49]. Six high affinity IGFBPs have been characterized and designated IGFBP-1 through IGFBP-6 [209]. Using chondrocytes in monolayer culture, Tsukazaki, *et al*. reported that TGF-β treatment decreased production of a 41-kDa IGFBP (presumably IGFBP-3)[49]. Ortiz *et al*. demonstrated that TGF-β regulates IGFBP-4 and increases the expression of the IGFBP-4 protease PAPP-A[48]. PAPP-A is an IGF-dependent-IGFBP-4 protease expressed by human fibroblasts and osteoblasts in culture[210]. IGFBP-4, which is expressed in cartilage and bone, is a negative regulator of local IGF action[211-213]. In bone, IGFBP-4 binds and sequesters IGF-I from its receptors, thereby inhibiting osteoblast proliferation[214,215]. IGFBP-4 bioavailability is determined by gene expression and proteolysis[216-218]. Cleavage of IGFBP-4 at Met[135]-Lys[136] reduces its binding affinity for IGF allowing for greater receptor stimulation and subsequent growth response in cultured cells[217-220].

Periosteum, the connective tissue that surrounds bones, has the potential to regenerate both cartilage and bone[221]. This unique tissue contains two discrete layers: the inner cambium, which contains undifferentiated mesenchymal cells, and an outer fibrous layer[221,222]. During normal bone growth involving intramembranous ossification, mesenchymal stem cells differentiate directly into osteoblasts[223,224]. However, during endochondral ossification or fracture healing, these cells differentiate into chondrocytes[225,226]. The natural osseous and chondro-regenerative capacities of periosteum make it an attractive tissue and cell source for transplantation and tissue engineering approaches in orthopedics[227-229]. One obstacle regarding the use of periosteum, however, is the decrease in the number of mesenchymal stem cells in the cambium that occurs with age[230]. In order to overcome this problem and fully realize the clinical potential of periosteum, it will be important to understand more about the cellular processes involved in the proliferation, differentiation and maturation of this tissue. Using an *in vitro* periosteal organ culture model, we have demonstrated that periosteal proliferation and chondrogenesis can be induced by exogenous treatment with TGF-β1 or IGF-I alone or in combination[42,43,46,47]. Therefore, it may be possible to use TGF-β1 and/or IGF-I to enhance the efficacy of periosteum for cartilage repair.

We hypothesized that TGF-β regulates the IGF-I axis during periosteal chondrogenesis. Such cross talk between TGF-β and IGF signaling may be important in fracture healing and the use of TGF-β and IGF in the application of periosteum for cartilage repair. However, no information regarding the interaction between IGF and TGF-β signaling during periosteal chondrogenesis has been available. Thus, the objective of this study was to determine if TGF-β regulates the IGF-I axis during *in vitro* periosteal chondrogenesis.

Methods

Periosteal Explant harvest and Culture

For the gene expression experiments, 352 explants were harvested from 44 two-month old New Zealand white rabbits. The periosteal explants were cultured in two groups: culture media supplemented with or without 10 ng/mL TGF-β1 during the first two days of culture. Culture periods varied from 3 to 42 days.

For conditioned media (CM) experiments, 184 explants were cultured as described above for 7 days. After 7 days, the serum containing medium was removed and replaced with serum-free medium and cultured for 48, 72, and 96 h. Afterwards, the conditioned medium (CM) was collected for analysis.

Analytical methods

After 3 to 42 days of culture, the IGFBP-4, PAPP-a, IGF-1, IGF-1-receptor and Aggrecan mRNA levels were determined by real time PCR. mRNA levels were expressed relatively to endogenous GAPDH mRNA levels. All PCRs were performed in duplicate.

After 2, 3, or 4 days of culture, the PAPP-A protein levels, IGFBP-4 protease activity levels, and the levels of various IGFBPs were determined in the conditioned media (CM) samples.

Statistical analysis

Quantitative real-time PCR data were analyzed by 1 or 2-factor ANOVA to determine the effect of time and/or TGF-β dosage on the measured RNA levels. Where appropriate, post-hoc testing using Duncan's Multiple Range test was performed to determine significance ($p < 0.05$) between specific time points for a given TGF-β dosage (i.e. 0 or 10 ng/mL). Significance between TGF-β dosage groups for specific time points were determined using means contrast comparisons. PAPP-A protein level secretion (ELISA) and activity (protease assay) results were analyzed using student t-tests. Western ligand blot results for the IGF binding proteins analyzed using a 2-factor ANOVA to determine the effects of TGF-β dosage and/or time. Means contrast comparisons were used to determine significance between groups with and without TGF-β for each specific binding protein at each time point (i.e. 48h and 72h CM culture).

For further details of the materials and methods used, please refer to appendix A.

Results

TGF-β1 Regulation of IGF binding proteins

Periosteal explants were cultured for up to 42 days with or without 10 ng/mL TGF-β1 (for the first two days) as described in Materials and Methods. RNA was ex-

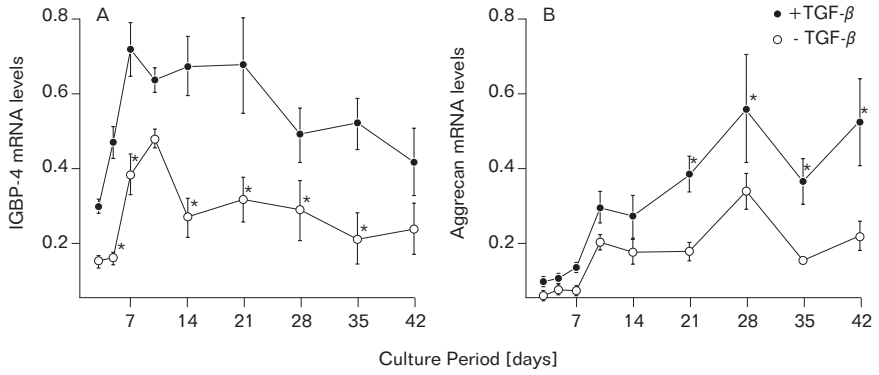


Figure 1
TGF-β1 regulation of IGFBP-4 (A) and aggrecan (B) mRNA levels in periosteal explants cultured with or without 10 ng/mL TGF-β1 (for the first two days). mRNA levels were quantitated by normalizing the target signal with the GAPDH signal. *: $p < 0.05$.

tracted from the explants after 3, 5, 7, 10, 14, 21, 28, 35 and 42 days of culture, reverse transcribed and analyzed using Real Time PCR with rabbit-specific primers and probes for IGFBP-4, aggrecan and GAPDH. As shown in Figure 1A, IGFBP-4 mRNA was detected in all explants. The relative levels of IGFBP-4 mRNA were significantly reduced ($p < 0.05$) in the TGF-β1 treated explants at all time points except day 42. In order to verify that the explants used in this experiment responded in the expected chondrogenic manner to TGF-β1 treatment, aggrecan mRNA levels were analyzed in the same pool of periosteal explants. As seen in Figure 1B, relative aggrecan mRNA levels were significantly increased in the TGF-β1 treated explants compared to controls on days 21-42 of culture ($p < 0.008$).

To determine the effect of TGF-β1 on IGFBP secretion, periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF-β1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 48h later. The conditioned medium (CM) was then analyzed by Western ligand blot. As shown in Figure 2, control explants secreted detectable amounts of intact IGFBP-4 (24 kDa) whereas no secreted IGFBP-4 was detectable in the CM from TGF-β-treated explants. Interestingly, a significant decrease in secreted IGFBP-3 (38/42 kDa) was detected in CM from the TGF-β-treated explants compared to controls ($p < 0.0001$). Bands that may represent the IGFBP-2 (34 kDa) and IGFBP-5 (30 kDa) were also detected in the CM.

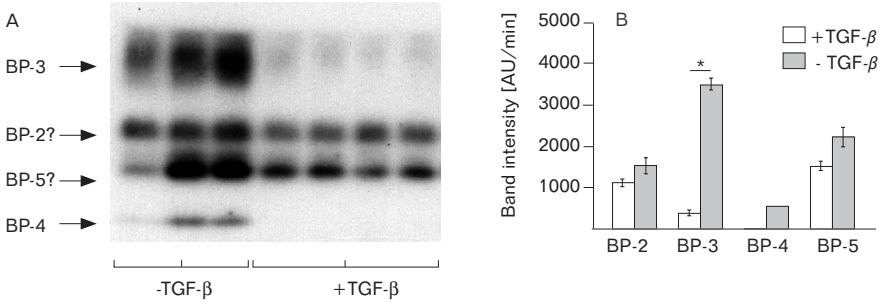


Figure 2
Western ligand blot analysis of IGFBPs in conditioned medium from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF-β1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 48h later. The conditioned medium (CM) was then analyzed by Western ligand blot (A) and densitometry was performed on the bands using NIH Image software (B). *: $p < 0.05$

TGF-β Regulation of PAPP-A and IGBP-4 Proteolysis

Periosteal explants were cultured for up to 42 days with or without 10 ng/mL TGF-β1 (for the first two days) as described in Materials and Methods. RNA was extracted from the explants after 3, 5, 7, 10, 14, 21, 28, 35 and 42 days of culture, reverse transcribed and analyzed using Real Time PCR with rabbit-specific primers and probes for PAPP-A. As shown in Figure 3, PAPP-A mRNA was detected in all explants, and TGF-β1 treated explants contained significantly higher levels of PAPP-A mRNA on days 7 and 10 and lower levels on day 14 ($p < 0.001$).

In order to determine the levels of secreted PAPP-A between days 7 and 10, periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF-β1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. The conditioned medium (CM) was then analyzed by ELISA. As shown in Figure 4, CM from TGF-β1 treated explants contained significantly more PAPP-A protein ($p < 0.0003$).

The effect of TGF-β1 on IGFBP-4 proteolysis in CM from cultured periosteal explants was also determined. In this experiment, aliquots of the same CM described above were analyzed using the IGFBP-4 protease assay as described in the Methods. As shown in Figure 5, addition of 5 nM IGF-II significantly increased IGFBP-4 protease activity in both the control ($p < 0.0001$) and TGF-β1 treated ($p < 0.024$) samples. In addition, TGF-β1 treatment significantly enhanced IGFBP-4 protease activity in both the control ($p < 0.0001$) and IGF-II treated ($p < 0.0005$) samples.

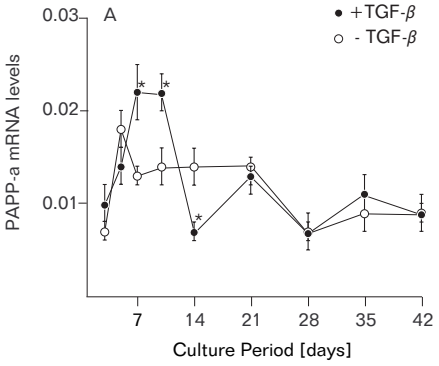


Figure 3
TGF-β1 regulation of PAPP-A mRNA levels in cultured periosteal explants cultured with or without 10 ng/mL TGF-β1 (for the first two days). mRNA levels were quantitated by normalizing the target signal with the GAPDH signal. *: $p < 0.05$.

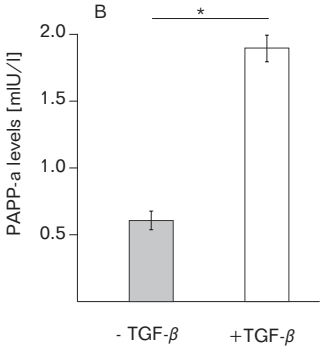


Figure 4
TGF-β1 regulation of PAPP-A secretion from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF-β1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. The conditioned medium (CM) was then analyzed by ELISA. *: $p < 0.05$.

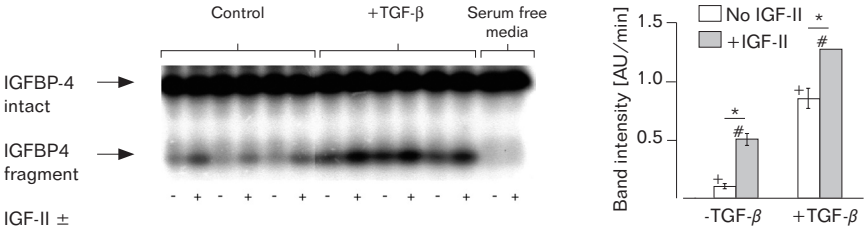


Figure 5
Effect of TGF-β1 on IGFBP-4 proteolysis in conditioned medium from cultured periosteal explants. Periosteal explants were cultured in serum containing medium until day 7 (with or without 10 ng/mL TGF-β1 for the first 48h). The medium was then changed to serum free medium and conditioned medium was collected 96h later. The conditioned medium (CM) was incubated in a microcentrifuge tube containing ^{125}I-IGFBP-4 with or without 5 nM IGF-II at 37 °C for 24 hr, as previously described[210,220]. Reaction products from three different samples for control and TGF-β groups were separated by SDS-PAGE, 7.5-15% gradient and visualized by autoradiography (A). Band intensity was quantitated using an LKB Ultrascan XL laser densitometer (B). *: $p < 0.05$; +: $p < 0.0001$; #: $p < 0.0005$.

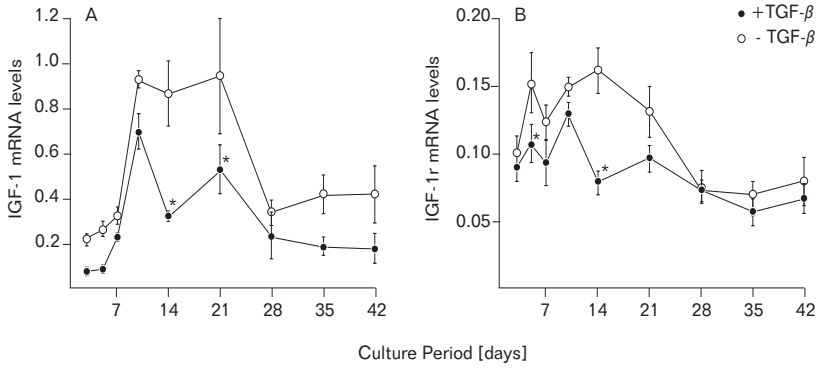


Figure 6
TGF-β1 regulation of IGF-I (A) and IGF-I receptor (B) mRNA levels in cultured periosteal explants. Periosteal explants were cultured for up to 42 days with or without 10 ng/mL TGF-β1 (for the first two days). mRNA levels were quantitated by normalizing the target signal with the GAPDH signal. *: $p < 0.05$.

TGF-β Regulation of IGF-I and IGF-I receptor

Periosteal explants were cultured for up to 42 days with or without 10 ng/mL TGF-β1 (for the first two days) as described in Materials and Methods. RNA was extracted from the explants after 3, 5, 7, 10, 14, 21, 28, 35 and 42 days of culture, reverse transcribed and analyzed using Real Time PCR with rabbit-specific primers and probes for IGF-I, IGF-I receptor and GAPDH. As illustrated in Figure 6, IGF-I and IGF-I receptor mRNA was detected in all periosteal explants. The relative IGF-I mRNA levels in the explants were significantly lower in the TGF-β1-treated explants on days 14 and 21 of culture ($p < 0.002$). IGF-I receptor mRNA levels were also significantly reduced by TGF-β1-treatment at days 5 and 14.

Discussion

In this study we illustrate that TGF-β1 regulates multiple members of the IGF-I axis during *in vitro* periosteal chondrogenesis. Because IGF-I is an important chondrogenic growth factor, regulation of the IGF-I axis may be an essential aspect of TGF-β1 action on periosteum. In these experiments a dramatic decrease in IGFBP-4 mRNA levels was observed throughout the six week culture after treatment with 10 ng/mL TGF-β1 for only the first 48h of culture. In the same set of periosteal explants, aggrecan mRNA levels were increased by TGF-β1 confirming that chondrogenesis was induced by TGF-β1 in these explants. These results are consistent with our previous findings that 48h of 10 ng/mL TGF-β1 treatment is sufficient to stimulate periosteal chondrogenesis and produce sustained effects on gene expression in cultured explants[231,232]. It is not yet known, however, whether the observed decrease in IGFBP-4 mRNA levels is due to decreased gene transcription or increased mRNA turnover.

TGF-β1 treatment also increased the IGBP-4 protease, PAPP-A mRNA levels on days 7 and 10 of culture. These results were confirmed by protein analysis, as a 3-fold increase in secreted PAPP-A protein was detected in CM from TGF-β1 treated explants compared to controls. Furthermore, we demonstrate that the secreted PAPP-A is functional as evident by a significant increase in IGFBP-4 proteolysis in CM from TGF-β1 treated explants. Together these data demonstrate that TGF-β1 stimulates the synthesis and secretion of a functional protein, PAPP-A between 7 and 11 days of culture. This observation is of particular interest because it occurs during the transition from the proliferation to differentiation stage in this *in vitro* periosteal chondrogenesis model[231]. At this time cartilage formation is initiated as chondrocyte precursors begin to differentiate into mature chondrocytes that are capable of matrix synthesis[233]. Therefore, because IGFBP-4 plays a fundamental role in regulating IGF-I bioavailability, alterations in PAPP-A activity during this period may have a functional role in TGF-β1 induced periosteal chondrogenesis. These results are also similar to previously reported findings in cultured human osteoblasts (hOB)[48,217]. Interestingly, in the present study a significant decrease in PAPP-A mRNA levels was observed on day 14 which may be the result of a feedback loop mechanism.

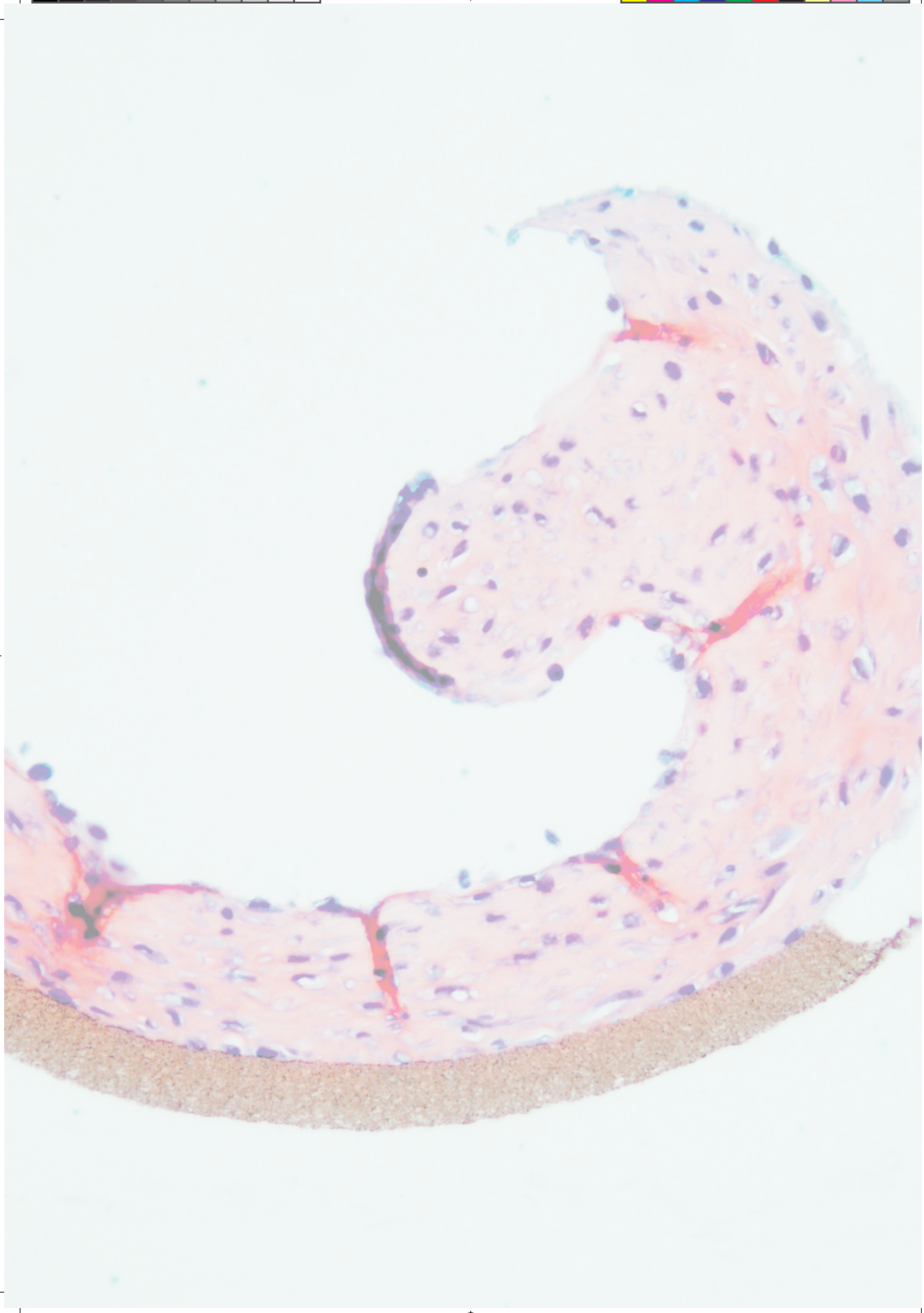
A dramatic decrease in intact IGFBP-3 levels was also observed in CM from TGF-β1 treated periosteal explants between days 7 and 10 of culture. This decrease in IGFBP-3 may lead directly to increased IGF-I availability and also indirectly because IGFBP-3 has been shown to inhibit IGFBP-4 proteolysis[234]. It is important to note, however, that the overall effect of TGF-β treatment on IGF-I bioavailability in cultured periosteum is not yet known. In fact, TGF-β-treatment resulted in decreased levels of IGF-I and IGF-I receptor mRNA levels in cultured periosteal explants. These findings are consistent with previously reported results in cultured rat articular chondrocytes[49]. In this study, TGF-β treatment resulted in decreased IGF-I secretion and mRNA levels, decreased 41-kDa IGFBP levels and decreased IGF-I receptor dephosphorylation, while increasing [^{125}I]IGF-I binding and aggrecan mRNA levels.

Regardless of the overall effect of TGF-β1 on IGF-I bioavailability and IGF-I signaling, the regulation of the IGFBPs by exogenous TGF-β1 is likely to have functional significance in periosteal chondrogenesis. In fact, evidence suggests that the IGFBPs can have IGF-independent effects[235]. For example, IGFBP-3 has IGF-independent effects on proliferation and differentiation of mesenchymal chondroprogenitor cells[236-238]. Specifically, IGFBP-3 inhibits growth, increases apoptosis, and antagonizes TGF-β chondroinductive effects in cultured RCJ3.1C5.18 mesenchymal chondroprogenitors[236-238]. Additional studies demonstrated that TGF-β antagonizes the IGFBP-3 antiproliferative effect on RCJ3.1C5.18 through cross-talk between the IGFBP-3-dependent STAT-1 and TGF-β-dependent ERK pathways[239]. Based on these findings, we hypothesize that the observed decreased levels of IGFBP-3 may result in enhanced periosteal proliferation and chondrogenesis via an IGF-independent mechanism, in addition to modulating IGF-I bioavailability.

It is also important to note that alterations in the IGF-I axis have been reported in synovial fluid from arthritic joints and in osteoarthritic cartilage[240-242]. Osteoarthritc

chondrocytes have been shown to be hyporesponsive to IGF-I despite having increased binding sites[243]. Interestingly, the response of chondrocytes to IGF-I also decreases with age[152]. The decreased response of chondrocytes to IGF may be due to increased levels of IGFBPs[152,242]. Therefore, IGFBP degradation pathways may be suitable targets for articular cartilage repair especially in patients with elevated synovial IGFBP levels as in older patients or those with RA or OA. Therefore, if even short-term TGF-β1 treatment can produce a sustained inhibition of IGFBP production during cartilage repair, this may provide additional benefit to the initial quality and homeostasis of the repair tissue. Altogether, additional studies need to be conducted to determine the net effect of TGF-β1 on the IGF bioavailability and function, the IGF-independent effects of IGFBPs on periosteal chondrogenesis, and the effects of IGFBPs on cartilage repair tissue.

In conclusion, these studies demonstrate that TGF-β1 treatment of cultured periosteal explants results in the regulation of the IGF-I axis. These findings are likely to be relevant to the use of periosteal grafts or periosteal cells for the regeneration of musculoskeletal tissues such as articular cartilage especially if TGF-β1 and/or IGF treatment is used to augment growth and differentiation.



Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes

Chapter 05

Abstract

Goal: To obtain a reliable and reproducible *in vitro* chondrogenic model we investigated the influence of expansion and redifferentiation conditions on final tissue morphology by comparing two expansion and two redifferentiation methods.

Methods: Chondrocytes from human donors were expanded in medium without growth factor supplementation (basic expansion condition [BEC]) or in medium with basic fibroblast growth factor (bFGF) supplementation (growth factor supplemented expansion condition [GFSEC]). After expansion, cells were redifferentiated either in pellet culture or seeded on collagen type II-coated filters.

Results: Expansion in GFSEC resulted in decreased collagen type II, and Sox-5 and -6 mRNA levels compared to BEC, which suggested that GFSEC results in increased chondrocyte dedifferentiation. However, upon redifferentiation GFSEC-expanded chondrocytes resulted in significantly better tissue morphology ($p < 0.05$), and increased proteoglycan (PG) synthesis rate at day 9 ($p < 0.01$), overall PG release ($p < 0.001$), and final PG content at day 28 ($p < 0.05$) compared to BEC. Redifferentiation on collagen-coated filters compared to pellet culture increased PG synthesis rate at day 9 ($p < 0.01$), PG release ($p < 0.01$), and final PG content ($p < 0.01$). Moreover, as visualized via electron microscopy, chondrocytes and organization of extracellular matrix cultured on filters was more similar to those found for hyaline cartilage.

Conclusion: Chondrocyte expansion in GFSEC and redifferentiation on collagen-coated filters resulted in most optimal chondrogenesis. Therefore, this culture model was implemented to investigate the effect of donor tissue quality and joint homeostasis on cartilage regeneration.

Introduction

In this thesis we are examining the boundary conditions for cartilage regeneration. Therefore, we required a reliable and reproducible *in vitro* model. Numerous culture systems have been described. However, similar to regeneration tissue from clinically applied ACI, the morphology of tissue synthesized in culture models varies greatly, frequently displaying fibrocartilaginous characteristics[31,33,91,145,244]. Since current ACI and the *in vitro* models studying its process consist of 2-step procedures during which chondrocytes undergo a process of de- and redifferentiation, both steps provide targets for improving the outcome of cartilage regeneration.
Over the last decades, a variety of chondrocyte expansion conditions have been described, of which recently, the use of basic fibroblast growth factor (bFGF)-supplemented expansion medium has been shown to be beneficial for the chondrogenic capacity of expanded chondrocytes in terms of extracellular matrix (ECM) synthesis and expression of chondrocyte specific phenotype[146,147,149,158].
After expansion, adequate chondrogenic stimuli, such as culturing chondrocytes in a 3-D environment, growth factors, like TGF-β and IGF-1, and carriers consisting of ECM proteins, are applied to achieve optimal redifferentiation. Various redifferentiation methods have been tested until now, of which the most frequently applied is based on pellet culture of expanded chondrocytes. This 3-D environment provides cell-cell contact that is thought to induce redifferentiation and matrix synthesis[160]. However, pellet cultures frequently result in the synthesis of tissue with a fibrocartilaginous morphology[91,146,244]. A previously published culture system in which expanded chondrocytes are redifferentiated on collagen type II coated filters may affect chondrogenesis beneficially, e.g. by improved redifferentiation and increased ECM synthesis, as compared to pellet culture[245], as exposure of integrins to collagen type II induces increased matrix synthesis and an increased response to chondrogenic growth factors such as TGF-β by chondrocytes[165,166].

The goal of this study was to evaluate *in vitro* chondrogenesis departing from two frequently used expansion conditions, one of which is supplemented with bFGF, and two redifferentiation models, the frequently applied pellet culture model and redifferentiation culture on collagen type II-coated filters.

Materials and methods

Culture methods

In vitro chondrogenesis departing from two frequently used expansion conditions, and two redifferentiation models were compared using articular chondrocytes from 9 human donors (average age was 50.3 years; 38 – 68 years old; Figure 1). The chondrocytes were either expanded under classical (non growth factor supplemented) expansion conditions (Basic Expansion Condition or BEC), or
under growth factor supplemented expansion conditions (GFSEC). After the chondrocytes were expanded for 2 passages, the chondrocytes were redifferentiated for 28 days either in pellet culture or on collagen type II-coated filters.

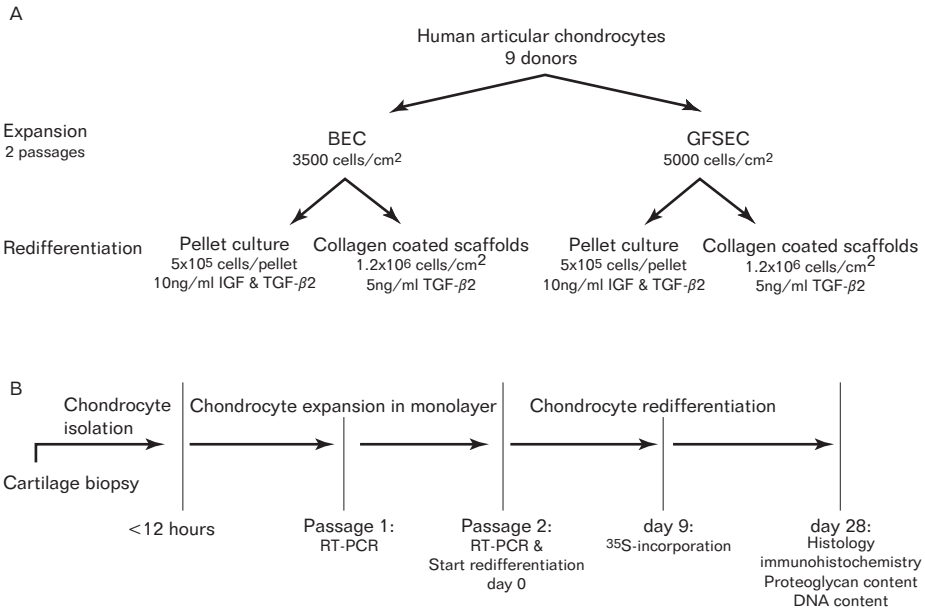


Figure 1
A schematic overview of the culture conditions (A) and analysis techniques (B). For GAG release analysis, culture medium was collected every three days.

Analytical methods

Post-expansion collagen types I and II and aggrecan mRNA levels were determined by real time PCR to evaluate the degree of dedifferentiation. SOX-5, 6 and 9 mRNA levels were assessed to determine whether differences in re-differentiation capacity between the expansion methods could be explained by expression profiles of these transcription factors, which are required for collagen type II expression and thus for the redifferentiation of expanded chondrocytes[246,247]. Since collagen type II is one of the typical structural proteins synthesized by differentiated chondrocytes in hyaline cartilage, as compared to collagen type I that is normally synthesized by dedifferentiated chondrocytes, we defined the ratio of collagen type II and collagen type I mRNA levels as 'differentiation index'.

Collagen type X mRNA levels were measured to determine whether either chondrocyte expansion method directs chondrocytes towards a hypertrophic pathway[248-251]. After 28 days of redifferentiation culture, proteoglycan synthesis and release-rate, cell content, tissue morphology (Safranin O / fast green), immunohistochemistry for collagen types I and II, and electron microscopy were determined to assess cartilage metabolism and final outcome.

Statistical analysis

The data were analyzed by multiple factor analysis of variance (factorial ANOVA) correcting for tissue donor using the general linear model. For post-expansion mRNA levels, the effect of expansion condition and passage number was analyzed. For post-redifferentiation analysis methods, such as the Bern histological score, ^{35}S-sulphate incorporation, GAG release, the effect of expansion conditions and redifferentiation methods were studied. If interaction between variables was observed, i.e. for post-expansion data interaction between passage number and expansion conditions and for post-redifferentiation data interaction between expansion conditions and redifferentiation models, the mean values of the treatment groups were compared using Tukey's post-hoc test. If the data were not normally distributed, a log-transformation was applied. P-values lower than 0.05 were considered statistically significant. Statistical calculations were done with the SPSS (Chicago, IL, USA) version 11.0 software package.

For further details of the materials and methods used, please refer to appendix B and C.

Results

Histology

During cell expansion under either condition, chondrocytes acquired a fibroblastic cell shape without obvious differences in the morphology observed. Despite the higher initial seeding density in GFSEC cultures, chondrocyte expansion in GFSEC and BEC resulted in similar numbers of population doublings (8.9 ± 0.3 versus 8.4 ± 0.4). Both pellet culture and culture on collagen-coated filters resulted, for all donors, in the synthesis of cartilaginous tissue by chondrocytes, as reflected by the rounded appearance of the cells and positive Safranin O-staining of the extracellular matrix of most samples. However, independent of the redifferentiation model used, chondrocyte expansion in GFSEC appeared to result in larger tissue volume, increased Safranin O-staining and more rounded appearance of chondrocytes, which suggests that expansion of chondrocytes in GFSEC improves tissue morphology compared to expansion in BEC (Figure 2A-D).

These histological observations were confirmed by the significantly higher "Bern score" of tissue synthesized by chondrocytes expanded in GFSEC, compared to expansion in BEC; 6.4 ± 0.3 points versus 4.5 ± 0.3 points, respectively, in pellet culture and 6.0 ± 0.4 points versus 4.5 ± 0.3 points, respectively, on collagen-coated filters ($p = 0.003$) (Figure 2E). The morphology of the tissue synthesized was not significantly affected by the redifferentiation technique.

Immunohistochemically all sections stained diffusely positive for collagen type I. Collagen type II appeared mainly synthesized by chondrocytes that were expanded in GFSEC. In addition, tissue synthesized on collagen-coated filters appeared to display an increase in collagen type II immunohistochemical staining (Figure 3).

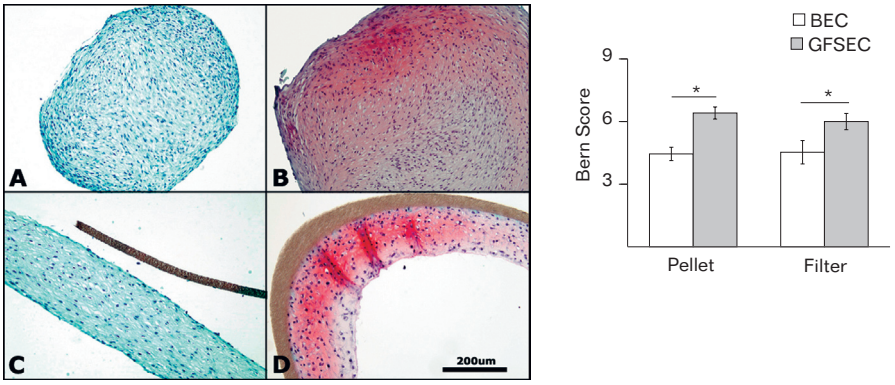


Figure 2
5 µm histological sections with safranin O / fast green staining after 28 days of culture. A/C: Chondrocytes expanded in BEC B/D: Chondrocytes expanded in GFSEC A/B: Expanded chondrocytes redifferentiated in pellet culture C/D: Expanded chondrocytes redifferentiated on collagen-coated filters. E: "Bern" histological score after 28 days of culture. Bars represent average±SEM. Statistically significant differences are indicated by * ($p < 0.05$).

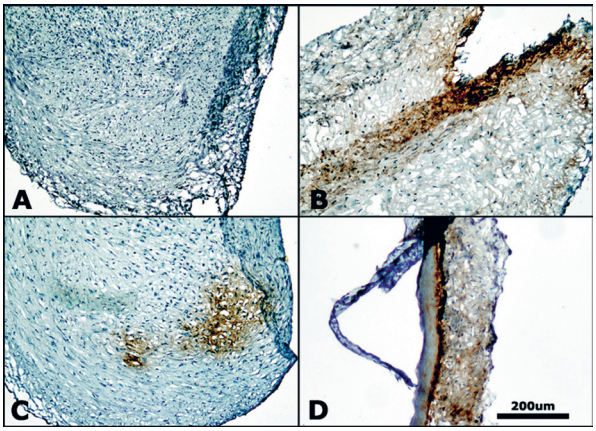


Figure 3
5 µm sections stained immunohistochemically for collagen type II after 28 days of culture. A/C: Chondrocytes expanded in BEC B/D: Chondrocytes expanded in GFSEC A/B: Expanded chondrocytes redifferentiated in pellet culture C/D: Expanded chondrocytes redifferentiated on collagen-coated filters.

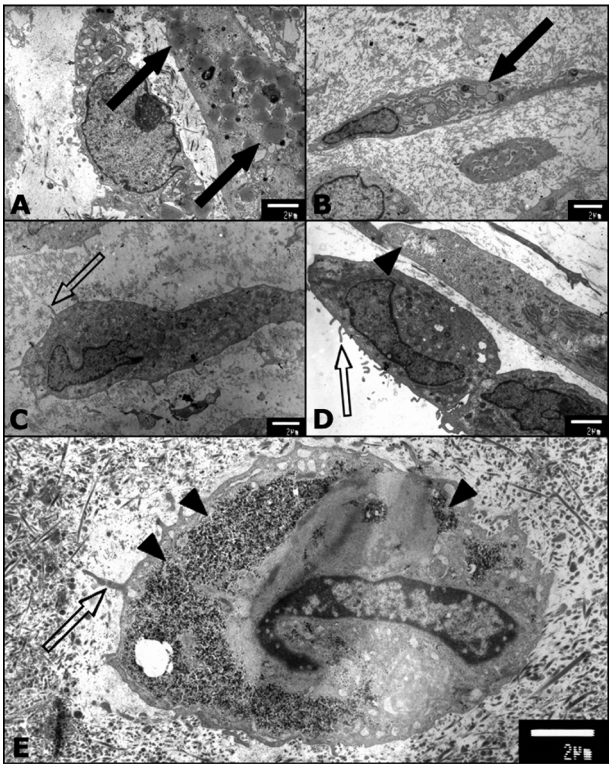


Figure 4
Transmission electron microscopical images of tissue formed after 28 days of culture. A/C: Chondrocytes expanded in BEC B/D: Chondrocytes expanded in GFSEC A/B: Expanded chondrocytes redifferentiated in pellet culture C/D: Expanded chondrocytes redifferentiated on collagen-coated filters. E: Control cartilage harvested from a healthy human femoral condyle. Note the high amount of lipid vacuoles (indicated by black arrows) in the cytoplasm of cells redifferentiated in pellet culture, and the relatively prominent presence of cytoplasmic extensions in the cell membrane (indicated by transparent arrows) and glycogen (black triangles) in control cartilage and tissue synthesized on collagen coated scaffolds.

Electron microscopic evaluation of regenerated cartilage revealed that tissue generated by chondrocytes cultured in pellets showed striking deviations from the morphology of chondrocytes in native articular cartilage. Abundant lipid-containing vacuoles were observed in the cytoplasm, which are seldomly present in chondrocytes of normal hyaline cartilage (Figure 4A), whereas the usual glycogen storage and cytoplasmic extensions were lacking in the pellet chondrocytes.

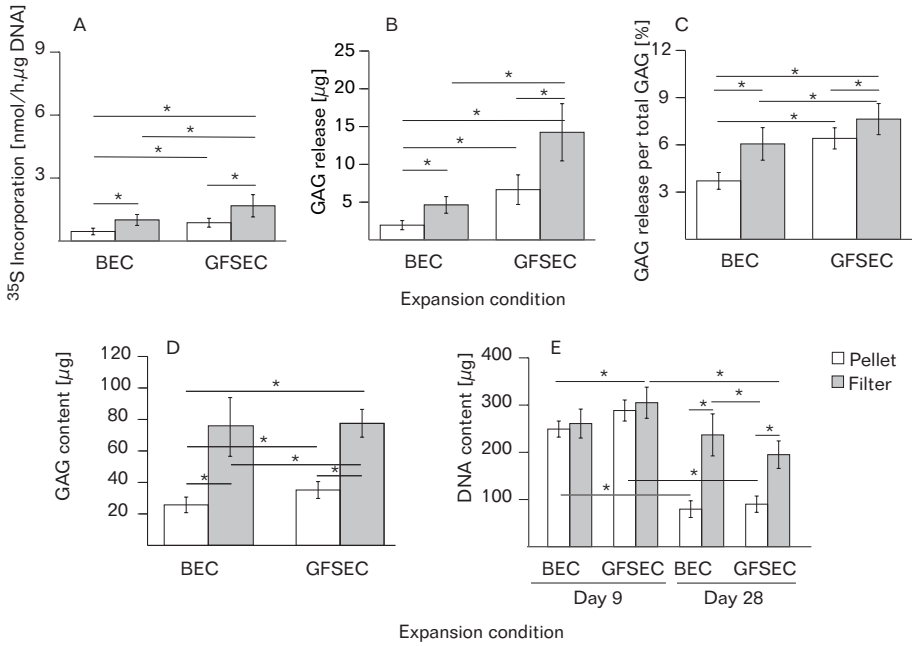


Figure 5
(A) Day-9 ^{35}S-incorporation normalized to DNA. (B) Average GAG release during the whole culture period. (C) Average GAG turnover as measured by GAG release normalized to the total amount of PG synthesized during the culture period (D) Final GAG content after 28 days of culture. (E) Day-9 and day-28 DNA content. Bars represent average±SEM. Statistically significant differences are indicated by * ($p < 0.05$).

In contrast to pellet cultures and similar to chondrocytes found in control tissue (native articular cartilage), fat vacuoles were not observed in chondrocytes cultured on collagen-coated filters, while glycogen and cytoplasmic extensions were more evidently present. No effects of the expansion conditions on cellular morphology were observed (Figure 4).

Matrix turnover

Expansion in GFSEC compared to expansion in BEC resulted in increased proteoglycan synthesis rate at day 9 (4.0-fold in pellet culture and 1.9-fold on collagen-coated filters; $p = 0.003$; Figure 5A); increased proteoglycan release (6.7-fold in pellet culture and 3.2-fold on collagen-coated filters; $p < 0.001$; Figure 5B); increased proteoglycan turnover rate as measured by GAG release normalized to the total amount of PG synthesized during the culture period (2.0-fold in pellet culture and 1.2-fold on collagen-coated filters; $p = 0.008$; Figure 5C); increased final GAG content after 28 days of culture (1.6-fold in pellet culture and 1.5-fold on collagen-coated filters; $p = 0.026$; Figure 5D); and increased final GAG content

normalized to DNA content (1.4-fold in pellet culture and 1.9-fold on collagen-coated filters; $p = 0.026$)
Compared to pellet culture, redifferentiation on collagen-coated filters resulted in increased proteoglycan synthesis rate at day 9 (5.2-fold in BEC-expanded chondrocytes and a 2.6-fold in GFSEC-expanded chondrocytes; $p = 0.005$; Figure 5A); increased proteoglycan release rate (4.2-fold in BEC-expanded chondrocytes and 3.1-fold in GFSEC-expanded chondrocytes; $p = 0{,}002$; Figure 5B); increased proteoglycan turnover rate (1.4-fold in BEC-expanded chondrocytes and 1.2-fold GFSEC-expanded chondrocytes; $p = 0.02$; Figure 5C); and increased final GAG content (1.3-fold in BEC-expanded chondrocytes and 1.9-fold in GFSEC-expanded chondrocytes; $p = 0.001$; Figure 5D). Final GAG content normalized to DNA was not significantly affected by the redifferentiation method used.

DNA content

Between 9 and 28 days of culture, DNA content decreased in both redifferentiation methods. This decrease was significantly higher in pellet culture compared to redifferentiation on collagen-coated filters ($p = 0.025$); 4.7-fold versus 1.0-fold in BEC-expanded chondrocytes and 4.1-fold versus 1.2-fold in GFSEC-expanded chondrocytes (Figure 5E). After 9 days of culture, DNA content was significantly increased for chondrocytes that were expanded in GFSEC and redifferentiated on collagen-coated filters compared to chondrocytes that were expanded in BEC and redifferentiated in pellets ($p = 0.047$). Evaluation of expansion and redifferentiation methods separately did not show statistically significant effects. After 28 days of culture, DNA content was significantly higher after redifferentiation on collagen-coated filters compared to pellet culture ($p = 0.007$), 1.9-fold in BEC-expanded chondrocytes and 2.1-fold in GFSEC-expanded chondrocytes.

mRNA expression levels

Collagen and aggrecan mRNA levels

The efficacy of the PCRs for all molecular markers was established using mRNA isolated from osteoarthritic cartilage. Collagen type I mRNA levels were 54-fold higher in first passage chondrocytes that were expanded in BEC compared to chondrocytes that were expanded in GFSEC ($p < 0.001$). In second passage chondrocytes, we did not observe a statistically significant effect of the expansion conditions on collagen type I mRNA levels (Figure 6A). Collagen type II mRNA levels were significantly more down-regulated in chondrocytes expanded in GFSEC after both the first (5.6-fold) and the second (24-fold) passage ($p = 0.001$), compared to chondrocytes expanded in BEC. Furthermore, collagen type II mRNA levels were significantly lower in second passage chondrocytes compared to first passage chondrocytes ($p < 0.001$); 8.7-fold in chondrocytes expanded in GFSEC and 2.0-fold in chondrocytes expanded in BEC (Figure 6B). The differentiation index was significantly lower in second passage chondrocytes compared to first passage chondrocytes ($p < 0.001$); 69-fold in chondrocytes expanded in GFSEC and 2.3-

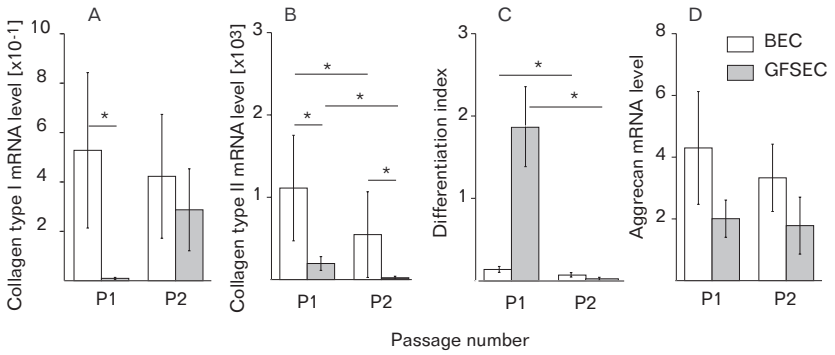


Figure 6
Relative mRNA levels (A) collagen type I, (B) collagen type II, (C) the collagen type II/I ratio (differentiation index), (D) aggrecan in chondrocytes expanded in BEC (-bFGF) or in GFSEC (+bFGF) after the first (P1) and second (P2) passage. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control and normalization to the relative expression of the respective gene of interest in a control sample (mRNA extracted from OA cartilage). Bars represent average±SEM. Statistically significant differences are indicated by * ($p < 0.05$).

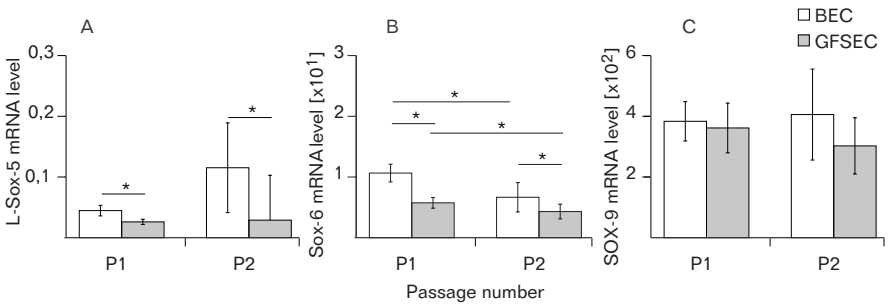


Figure 7
Relative mRNA levels of (A) L-Sox-5, (B) Sox-6, (C) Sox-9 in chondrocytes expanded in BEC (-bFGF) or in GFSEC (+bFGF) after the first (P1) and second (P2) passage. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control and normalization to the relative expression of the respective gene of interest in a control sample (mRNA extracted from OA cartilage). Bars represent average±SEM. Statistically significant differences are indicated by * ($p < 0.05$).

fold in chondrocytes expanded in BEC (Figure 6C). Collagen type X was below detection levels in all chondrocyte expansion cultures (not shown). Aggrecan mRNA levels were not significantly affected either by expansion method or number of passages (Figure 6D).

Sox mRNA levels

L-Sox-5 mRNA levels were significantly lower in chondrocytes expanded in GFSEC compared BEC ($p < 0.05$); 1.7-fold in first passage chondrocytes and 4.0-fold in second passage chondrocytes (Figure 7A). Expansion in GFSEC resulted in 1.9-fold lower Sox-6 mRNA levels in first passage chondrocytes and 1.5-fold lower SOX-6 mRNA in second passage chondrocytes compared to expansion in BEC ($p < 0.05$). Sox 6 mRNA levels decreased significantly with increasing number of passages ($p < 0.05$); 1.3-fold in GFSEC and 1.6-fold in BEC (Figure 7B). Sox 9 mRNA levels were not dependent on either expansion conditions or number of passages (Figure 7C).

Discussion

The current study evaluated *in vitro* chondrogenesis starting from two frequently used expansion conditions and two redifferentiation models, and clearly illustrates the importance of both expansion and redifferentiation conditions on final outcome of *in vitro* cartilage regeneration.

Histologically, cartilaginous tissue was synthesized in all culture models. However, both the expansion conditions and redifferentiation methods studied had significant effects on the quality of the tissue synthesized. As compared to BEC (basic expansion condition), GFSEC (growth factor supplemented expansion condition) resulted in enhanced tissue morphology, as reflected by larger tissue volume, increased Safranin O-staining and more rounded appearance of chondrocytes, and increased collagen type II synthesis. Furthermore GFSEC resulted in increased proteoglycan turnover, final proteoglycan content and final proteoglycan content normalized to cell content. In contrast, GFSEC resulted in decreased collagen type II, L-Sox-5 and Sox-6 mRNA levels in expanded chondrocytes. Redifferentiation on collagen-coated filters appeared to improve collagen type II synthesis and resulted in increased proteoglycan turnover and final proteoglycan content. At the ultrastructural level, chondrocytes cultured on collagen-coated filters demonstrated a stronger resemblance to native articular chondrocytes compared to pellet cultures, as reflected by the absence of lipid-containing vacuoles in the cytoplasm and the presence of glycogen storage and cytoplasmic extensions. At the ultrastructural level, no differences were noted between expansion conditions. Between 9 and 28 days of culture, cell number decreased in both redifferentiation models, but this decrease was stronger in pellet culture. DNA content at day 28 was higher on collagen-coated filters compared to pellet cultures.

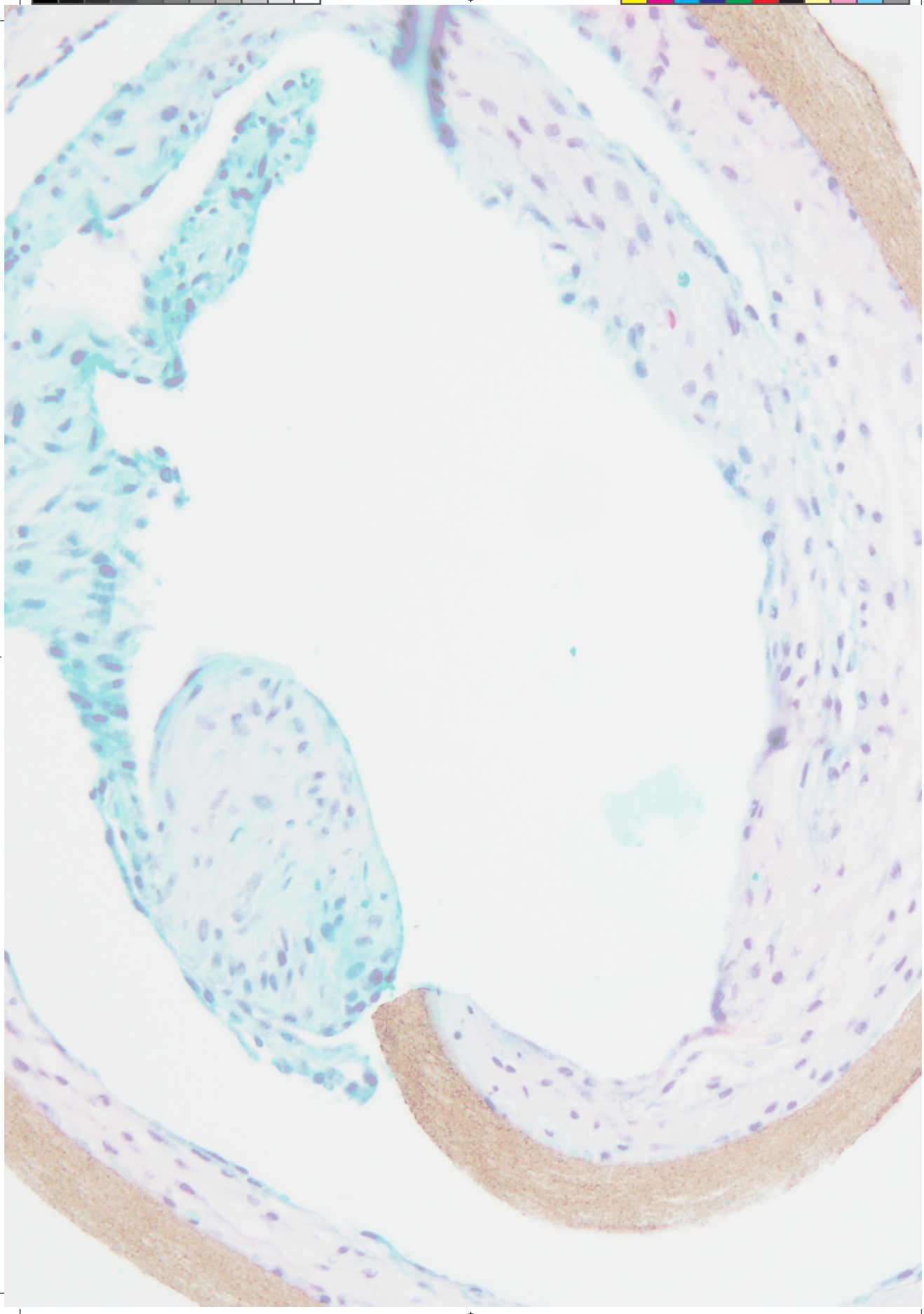
The effect on proteoglycan production of redifferentiation on collagen-coated filters seemed to result from the higher cell number found, rather than from the

higher synthetic activity per cell, as the final proteoglycan content per cell appeared not affected by the redifferentiation methods. It is not clear whether the decline in cell number in pellet cultures is dependent on higher apoptotic activity or a decreased proliferation as compared to filter culture. The absence of detectable collagen type X mRNA levels suggests that neither expansion method directs expanding chondrocytes towards a hypertrophic pathway during chondrocyte expansion. Altogether, various parameters of cartilage metabolism and regeneration appear to be strongly affected by both expansion and redifferentiation. However, pinpointing the exact factors involved in the effects found is difficult, as we have compared previously published expansion and redifferentiation conditions in this study. Considering the differences in expansion conditions, in BEC, which yielded less optimal results in quantitative and qualitative aspects of matrix production, initial seeding density was lower, ascorbic acid and proline were added, whereas no bFGF was added, compared to GFSEC. Lower initial seeding densities have been suggested to increase the degree of dedifferentiation during chondrocyte expansion in monolayer[155]. However, increased dedifferentiation appears to be a result of increased number of population doublings rather than initial seeding density[148]. As the expansion conditions in this study resulted in comparable population doublings, the effect of GFSEC on chondrogenic capacity is not likely due to the slightly higher initial seeding density. This is supported by similar differentiation indices after two passages in both expansion conditions and even lower collagen type II mRNA levels in GFSEC expanded chondrocytes, suggesting that expansion in GFSEC induces at least a similar degree of dedifferentiation as expansion in BEC. Also the effects of proline and ascorbic acid are not likely to have been deleterious to cartilage regeneration. Proline is an essential amino acid constituting the collagen triple helix molecules and is thus essential for chondrogenesis. And although ascorbic acid has been reported to have varying effects, in high density cultures ascorbic acid has been shown to inhibit a decline in collagen type II mRNA levels and promote expression of collagen type II and aggrecan upon resuspension of cultured chondrocytes[252]. These observations suggest that ascorbic acid supplementation to culture media also enhances rather than inhibits preservation of the chondrocyte phenotype and thus is unlikely to be responsible for the lower chondrogenic capacity of chondrocytes expanded in BEC compared to GFSEC.
Taken these considerations together, bFGF supplementation to the culture medium may be responsible for the beneficial effect of expansion in GFSEC on the chondrogenic capacity of monolayer-expanded chondrocytes. Recent publications showed enhancement of the redifferentiation capacity of chondrocytes that were expanded in the presence of bFGF, although contradicting effects were reported with respect to its effect on dedifferentiation during expansion in monolayer; increasing dedifferentiation in serum-containing medium and decreasing dedifferentiation in serum free medium1[47,150,158]. In the current study, the downregulated collagen type II, L-SOX-5 and SOX-6 mRNA levels in GFSEC-expanded chondrocytes, suggest that chondrocyte expansion in GFSEC actually increases the degree of dedifferentiation compared to BEC, while it enhances cartilage regeneration upon redifferentiation.

Also the effects of the redifferentiation models could be attributed to various parameters. Chondrocytes in pellet culture were exposed to a higher concentration of TGF-β2 and in addition to IGF-I. IGF-I is known to enhance proteoglycan synthesis and mitotic activity in chondrocytes and to decrease matrix catabolism[253,254]. TGF-β induces similar effects on proteoglycan synthesis[255,256]. TGF-β and IGF-I synergistically induce reexpression of the cartilage phenotype in dedifferentiated chondrocytes in alginate culture systems[257]. Nevertheless, redifferentiation of expanded chondrocytes on collagen-coated filters resulted in improved cartilage regeneration, compared to redifferentiation in pellet culture. This was reflected by the higher proteoglycan content of the tissue synthesized, mainly caused by the higher cell numbers. Moreover, at the ultrastructural level, cells on filters more closely resembled articular chondrocytes. Strikingly, the abundance of lipid-containing vacuoles and scarcity of glycogen storage in the pellet chondrocytes suggested altered energy expenditure profiles. It will be interesting to quantify and expand this feature of chondrocyte behavior. Altogether chondrocyte redifferentiation on collagen type II-coated scaffolds appears to be more optimal than in pellet culture, which is an important observation as pellet culture is one of the most widely used culture models to study *in vitro* chondrogenesis. An explanation for the effect found could lie in the fact that the layer of cells on the filters is relatively thin compared to cells in pellet culture, which enables optimal diffusion of nutrients in the tissue. On the other hand, low oxygen tension, as found in pellets, stimulates chondrocyte redifferentiation and matrix synthesis, which suggests that pellet cultures should result in better tissue morphology, at least in the center[258-260].

Another explanation for the effects of regeneration on filters might lie in the presence of a collagen type II coating on the filters, providing the cells with a structural protein normally present in healthy hyaline cartilage. Chondrocyte binding of integrins to collagen type II induces increased matrix synthesis and provides stimuli for maintenance of the chondrocyte phenotype[261]. Moreover, it has been demonstrated that chondrocytes show an increased responsiveness to growth factors, such as TGF-β, when bound to collagen type II through integrins[165,166,262,263]. Finally, the collagen type II coating might also have resulted in a cell selection for cells with a higher collagen type II binding integrin expression, e.g. integrin β1 and annexin V, as these cells theoretically have a higher adherence potential. Beta-1 integrins have been shown to be crucial in induction of chondrogenesis during early cartilage differentiation[261,264]. However, to our knowledge it is unknown whether higher expression levels of these collagen binding integrins are also related to a higher chondrogenic capacity of chondrocytes in monolayer-expanded chondrocytes. Whatever the final mechanism, the results in this study suggest that the application of collagen-based scaffolds for implantation of *in vitro* expanded chondrocytes may improve the quality of the tissue synthesized by these cells. Other scaffold materials, like PLA and PGA, should also be considered for clinical application, as these have been demonstrated to support *in vitro* chondrocyte proliferation and chondrogenic differentiation[265]. Future studies should elucidate which most potently stimulates chondrogenesis.

Future studies are required to investigate whether the improved chondrogenic properties of this redifferentiation models is induced through a collagen II-dependent stimulation. This might have important implications for the cells that are used with the clinical application of ACI. Traditionally, expanded chondrocytes are implanted in suspension under a periosteal flap or artificial membrane to prevent cells from migrating out of the cartilage defect site. As a result not all chondrocytes are exposed to cartilage matrix proteins like collagen type II and therefore initially do not have the beneficial effect of collagen-integrin interaction.
In conclusion, of the culture methods studied in this paper, a combination of chondrocyte expansion in GFSEC containing bFGF and redifferentiation on collagen-coated filters is most optimal for the quality of the tissue synthesized.



Altered *in vitro* chondrogenic properties of chondrocytes harvested from unaffected cartilage in osteoarthritic joints

Chapter 06

Abstract

Goal: As chondrocytes isolated from macroscopically normal cartilage biopsied from OA joints cartilage are frequently used for *in vitro* cartilage regeneration studies, the goal of the current study was to investigate whether these chondrocytes display altered chondrogenic properties compared to chondrocytes isolated from healthy cartilage biopsies.

Methods: Chondrocytes isolated from both healthy and OA cartilage biopsies were expanded in monolayer under GFSEC for 2 passages and were subsequently redifferentiated on collagen-coated filters for 28 days.

Results: After chondrocyte expansion, collagen type X mRNA was only detectable in chondrocytes from OA joints at both passages ($P < 0.01$). Upon redifferentiation, tissue regenerated by chondrocytes from healthy joints showed a significantly better morphology ($P < 0.01$) and higher proteoglycan content ($P < 0.05$), compared to chondrocytes from OA joints. Matrix turnover parameters, i.e., proteoglycan synthesis and degradation rate, were not significantly affected by donor tissue origin.

Conclusion: These results demonstrate that clear differences between chondrocytes from healthy and OA joints exist and that these are not completely abolished during the process of de- and redifferentiation. Therefore, *in vitro* cartilage regeneration models, which use chondrocytes from OA joints, should be interpreted with care.

Introduction

Given the fact that regenerative medicine provides superior structural repair as compared to microfracturing and the knowledge that the selected culture condition is of essential influence on the outcome of cartilage regeneration, we will now focus on the role of cartilage biopsy quality.
The starting point of cultures may be of considerable influence on the outcome of both *in vitro* and *in vivo* cartilage regeneration. However, chondrocytes used for *in vitro* research are frequently harvested from less affected areas of osteoarthritic joints, as this is a readily available source of cells. However, it is not clear whether these chondrocytes are actually phenotypically identical to chondrocytes from healthy joints. Transformation to osteoarthritic tissue is not very likely to involve sudden changes, but is more likely to be a gradual and initially microscopically invisible process. Although it may be argued that dedifferentiation, occurring because of expansion of harvested chondrocytes, effaces existing differences, leading to identical tissue compositions upon regeneration, it has never been investigated whether this is actually the case. Chondrocytes harvested from "healthy" areas of osteoarthritic joints may already display characteristics of chondrocytes in osteoarthritic lesions. These characteristics are an increased proteoglycan turnover rate, resulting from enhanced proteoglycan synthesis and release[50-55], the production of the hypertrophic marker type X collagen, and the expression of matrix degrading proteases such as cathepsin B and MMP-13. Moreover, chondrocytes from OA joints display a shift towards the production of collagen type I at the expense of collagen type II. As the chondrocyte phenotype, in particular the expression of collagen II and aggrecan, is dependent on the expression of the transcription factors L-Sox-5, Sox-6 and Sox-9, the diminished production of cartilaginous matrix proteins by the OA chondrocyte might be associated with decreased levels of these transcription factors.

The aim of the current study was to investigate to what extent chondrocytes harvested from OA joints display an altered, more dedifferentiated or hypertrophic, phenotype after expansion, compared to chondrocytes from healthy joints and to what extent this is associated with differences in cartilage regeneration upon dedifferentiation in monolayer and redifferentiation in a previously described *in vitro* model[245].

Materials and methods

Culture methods

Articular cartilage was harvested from 9 healthy (average age 50.3 years; 38-68 years old) and 9 macroscopically less affected areas of OA joints (average age 65 years; 57-73 years old). The chondrocytes were expanded for 2 passages under growth factor supplemented expansion conditions (GFSEC). After the chondrocytes were expanded for 2 passages, the chondrocytes were redifferentiated on collagen type II-coated filters.

Analytical methods

Post-expansion collagen types I and II and aggrecan mRNA levels were determined by real time PCR to evaluate the degree of dedifferentiation. SOX-5, 6 and 9 mRNA levels were assessed to determine whether differences in re-differentiation capacity between the expansion methods could be explained by expression profiles of these transcription factors, which are required for collagen type II expression and thus for the redifferentiation of expanded chondrocytes[246,247]. Collagen type X, MMP-13 and Cathepsin B mRNA levels were measured to determine whether chondrocyte donor type predisposes towards a hypertrophic phenotype and increased proteoglycan turnover rate upon redifferentiation[248-251].

After 28 days of redifferentiation culture, proteoglycan synthesis and release-rate, cell content, tissue morphology (Safranin O / fast green), and immunohistochemistry for collagen types I and II were determined to assess cartilage metabolism and final outcome.

Statistical analysis

For post-expansion mRNA levels and post-redifferentiation cellularity, the effect of donor type and passage number was analyzed by multiple factor analysis of variance (factorial ANOVA) using the general linear model. If interaction between variables was observed, the mean values of the treatment groups were compared using Tukey's post-hoc test. For post-redifferentiation analysis methods, i.e. Bern score, ^{35}S-sulphate incorporation, GAG release and final GAG content, analysis of the data of the various assays was done using Student's t-tests after confirming equal variances of the data. If the data were right skewed instead of normally distributed, a log-transformation was applied before analysis. In cases of unequal variances, the data were analyzed using Mann-Whitney-Wilcoxon rank sum tests (significance level, $p \leq 0.05$). Statistical calculations were done with JMP (Cary, NC, USA), version 5.1, statistical analysis software.

For further details of the materials and methods used, please refer to appendix B and C.

Results

Population doubling

During monolayer culture, no obvious differences in gross morphology between cell types were observed. After two passages 8.7 ± 1.0 and 7.1 ± 2.9 population doublings (PDs) were observed for chondrocytes from healthy and OA joints respectively, which was not significantly different. The average population doubling time was comparable for chondrocytes from healthy (3.0 ± 0.8 days) and OA joints (2.9 ± 1.0 days) (n.s.).

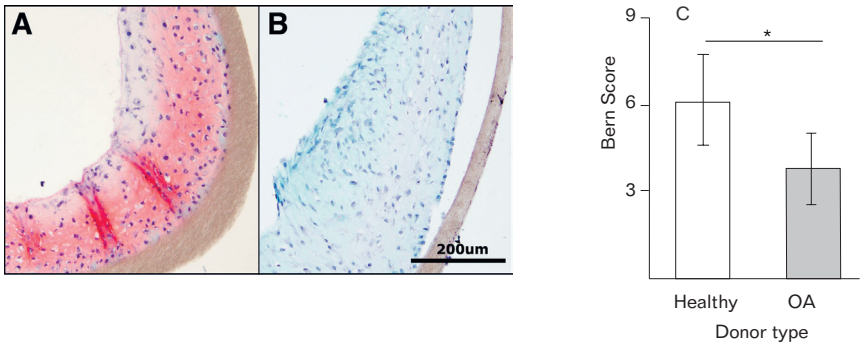


Figure 1
Representative safranin O stained slides of tissue synthesized respectively by (A) healthy chondrocytes and (B) chondrocytes from OA joints after 28 days of culture (C) Bern score of the tissue synthesized 28 days culture support these findings. Bars are means + SEM. * indicates a statistically significant difference ($p < 0.05$).

Histology and Immunohistochemistry

After 28 days of culture, cartilaginous tissue was synthesized by chondrocytes from both healthy and OA joints as determined by the safranin O staining and the rounded morphology of the cells in most samples (Figure 1A and B). However, tissue synthesized by chondrocytes from healthy joints showed a more hyaline cartilage-like morphological aspect, as reflected by the more rounded appearance of the chondrocytes, the lower cellularity, and the more intense safranin O staining of the matrix. These findings were confirmed by blinded histological scoring according to the Bern grading system, as tissue synthesized by chondrocytes from healthy joints scored two-fold higher compared to chondrocytes from OA joints (6.2 ± 1.8 points versus 3.8 ± 1.6, $p = 0.003$; Figure 1C).
All tissues stained diffusely positive for collagen type I. Collagen type II staining was more variable between donors, ranging from intense diffuse staining throughout the whole sample to no staining, though in most samples a diffuse positive staining was found. No visual differences in immunohistochemical staining for collagen type I and type II between chondrocytes from healthy or OA joints was found (Figure 2).

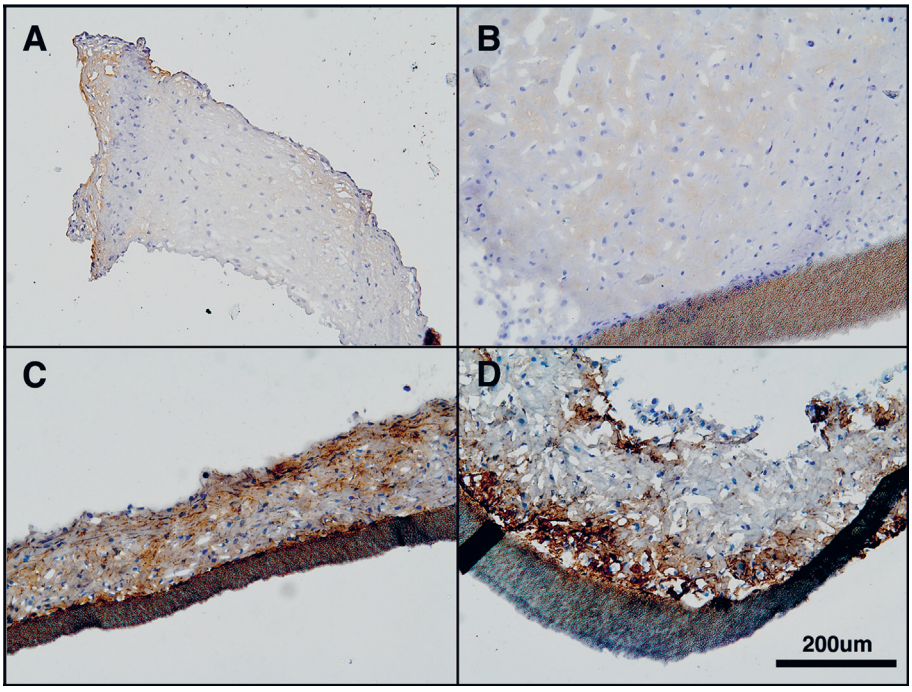


Figure 2
Collagen type I and type II immunohistochemistry of tissue synthesized by healthy and OA expanded chondrocytes after two passages expansion and 28 days redifferentiation culture. (A and B) collagen type I, (C and D) collagen type II, (A and C) tissue synthesized by healthy chondrocytes, and (B and D) tissue synthesized by OA chondrocytes. Brown color represents positive staining.

Matrix turnover

Final proteoglycan content after 28 days of culture was 1.4-fold higher in tissue synthesized by chondrocytes from healthy joints compared to chondrocytes from OA joints ($p < 0.05$; Figure 3A). Upon normalization to DNA content, no statistically significant difference was found between donor origin. Day-9 35S-incorporation normalized to DNA content (Figure 3B), average GAG release expressed as to the total amount of GAG synthesized (sum of total GAG released and final GAG content) (Figure 3C), and total cell numbers, as determined by DNA content (Figure 3D) were not significantly affected by the donor tissue origin.

mRNA expression levels

Collagen type I mRNA levels were not significantly affected by donor type or passage number (Figure 4A). Collagen type II mRNA levels decreased 850-fold in chondrocytes from OA joints between the first and second passage ($p < 0.001$).

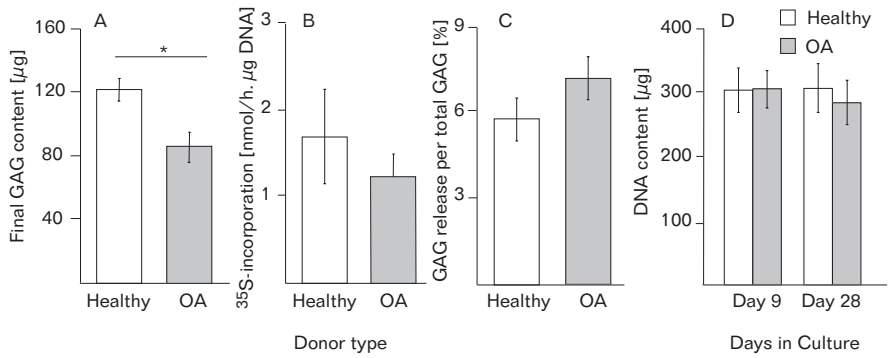


Figure 3
(A) Tissue GAG content after 28 days of culture. (B) Day-9 ^{35}S-incorporation normalized to DNA content (C) Average GAG release per total GAG synthesized during the whole culture period. (D) The DNA content after 9 and 28 days of culture. Bars are means + SEM. Statistically significant differences are indicated by * ($p < 0.05$).

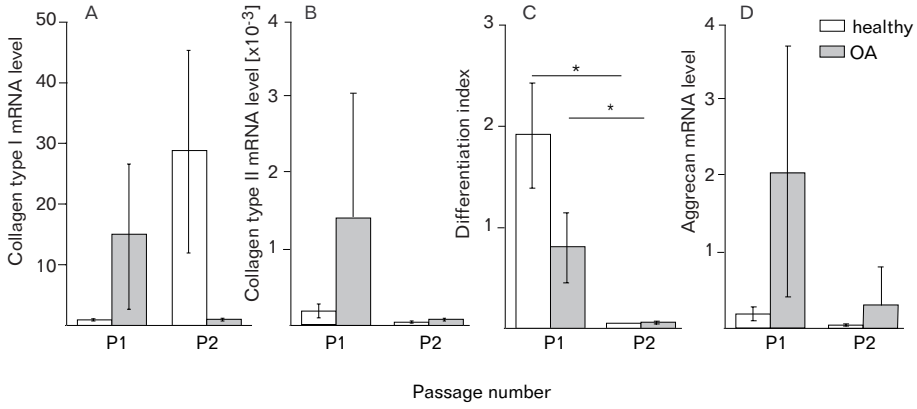


Figure 4
Relative mRNA levels of (A) collagen type I, (B) collagen type II, (C) the collagen type II/I ratio (differentiation index) and (D) aggrecan. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control and normalization to the relative expression of the respective gene of interest in a control sample (mRNA extracted from OA cartilage). Bars are means + SEM. Statistically significant differences are indicated by * ($P < 0.05$).

No significant effect of donor type on collagen type II mRNA levels was observed (Figure 4B). Independent of the passage number, the differentiation index, i.e. the collagen type II/I ratio, appeared lower in chondrocytes from OA joints compared to chondrocytes from healthy joints ($p = 0.067$; Figure 4D).

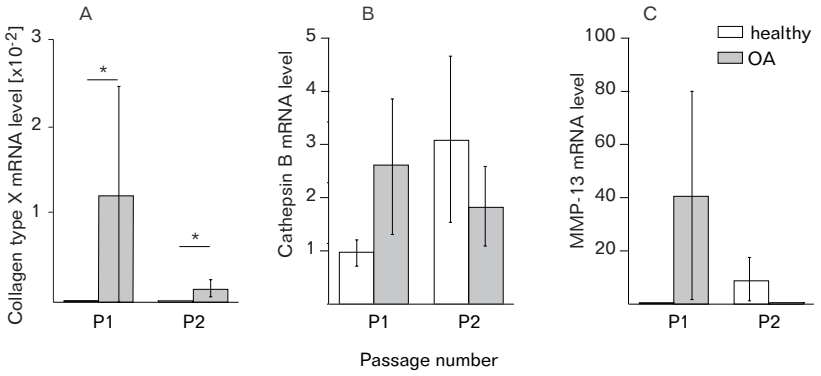


Figure 5
Relative mRNA levels of (A) collagen type X, (B) cathepsin B and (C) MMP-13 in monolayer expanded healthy and OA chondrocytes after the first and second passage. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control and normalization to the relative expression of the respective gene of interest in a control sample (mRNA extracted from OA cartilage). Bars are means ± SEM. Statistically significant differences are indicated by * ($P < 0.05$).

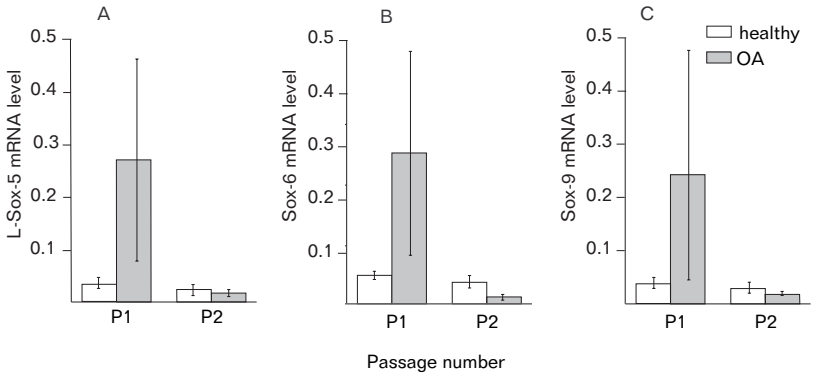


Figure 6
Relative mRNA levels of (A) L-Sox-5, (B) Sox-6 and (C) Sox-9 in monolayer expanded healthy and OA chondrocytes after the first and second passage. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control and normalization to the relative expression of the respective gene of interest in a control sample (mRNA extracted from OA cartilage). Bars are means ± SEM.

Furthermore, the differentiation index decreased significantly between first and second passage for chondrocytes from both healthy (69-fold, $p < 0.05$) and OA joints (52-fold, $p < 0.001$). Collagen type X mRNA levels were below detection limits in chondrocytes from healthy joints, while clearly present in both first and second passage chondrocytes from OA joints of all donors ($p = 0.008$) and appeared to decrease between passage 1 and 2 ($p = 0.073$; Figure 5A). Aggrecan (Figure 4D), Cathepsin B and MMP-13 (Figure 5B-C) mRNA levels were not significantly affected by donor tissue quality or passage number.

L-Sox-5, Sox-6 and Sox-9 mRNA levels were not significantly affected by donor tissue origin or passage number, although the Sox mRNA levels showed strikingly similar trends compared to collagen type II mRNA levels (Figure 6A-C).

Discussion

The current study demonstrates a clear difference in cartilage expression patterns and chondrogenic capacity between chondrocytes from healthy and osteoarthritic joints. Moreover, the process of de- and redifferentiation that occurs during *in vitro* chondrogenesis appears insufficient to completely abolish pre-existing differences between donor types.

Both chondrocytes from normal and OA joints synthesized cartilaginous tissue after expansion in monolayer, although chondrocytes from healthy joints synthesized tissue with significantly better morphology, although no obvious differences in collagen type II synthesis were observed between donor types. The final content of proteoglycans in tissue synthesized by chondrocytes from normal joints was significantly higher compared to chondrocytes from OA joints. A combined effect of the apparently higher proteoglycan synthesis rate, lower proteoglycan release rate and higher cellularity in healthy chondrocytes may be responsible for this observation. The role of cellularity seems supported by the fact that no significant differences in proteoglycan content per cell could be demonstrated.

No significant effect of chondrocyte donor origin on collagen type II mRNA levels was observed. Collagen type II mRNA levels decreased significantly between the first and second passage merely in chondrocytes from OA joints. Independent of chondrocyte origin, the differentiation index decreased significantly between first and second passage. Collagen type X was only detected in chondrocytes from OA joints and appeared to decrease between passage 1 and 2. The absence of expected statistically significant differences of various parameters, like the absence of increasing collagen type I mRNA levels with increasing number of passages, may be due to the limited number of chondrocyte donors used. All PCRs were performed in triplicate. Triplicates were found to be very accurate. Differences in Ct-values (=number of cycles for PCR needed to reach a specified threshold) within

triplicates were generally less than 1 cycle. Obvious outliers were excluded from further analysis and therefore are not responsible for the variation in the data. The spread in the RT-PCR-data underlines that large differences exist among different chondrocyte donors and underlines that (especially non-significant) PCR data should be interpreted with care.

However, the PCR data do demonstrate that chondrocytes from both healthy and OA joints increasingly dedifferentiate between first and second passage, which is in accordance with previous data showing that chondrocytes cultured in monolayer express and synthesize decreased amounts of collagen type II and increased amounts of collagen type I with increasing passage number[22,266]. Despite this trend, the differentiation index decreased to a higher extent and appeared to remain lower in chondrocytes from OA joints compared to chondrocytes from healthy joints, which is induced by the relatively large decrease in collagen type II expression in chondrocytes from OA joints.
Most striking were the clear differences in collagen type X expression. This hypertrophic marker was solely expressed by chondrocytes from OA joints and did not decrease significantly between passage 1 and 2. Surprisingly, this tendency towards hypertrophy could not be explained by decreased levels of the Sox-5, Sox-6 and Sox-9 mRNA levels. This triad of transcription factors is known to be sufficient for cartilage differentiation, amongst others collagen type II expression, and inhibits hypertrophic differentiation. Furthermore, Sox-9 is expressed in all chondrocytes and chondroprogenitor cells, except for hypertrophic chondrocytes[246,247,267]. In contrast, in this study their levels even seem to be increased in chondrocytes from OA joints. Collagen type I or II, or SOX mRNA levels were studied to determine whether differences in tissue quality could be explained by *a priori* differences between chondrocyte donor types, as these genes are required for adult chondrocyte biology. However, no significant differences were observed that might explain the differences in chondrogenic capacity between healthy and OA chondrocytes. Recently, various markers, like BMP2 and FGFR3, have been suggested to be predictive of the chondrogenic potential of expanded chondrocytes, while loss of chondrogenic potential of dedifferentiated chondrocytes has been associated with deficient Shc-Erk interaction[159,268]. Future studies should be performed to elucidate the role of these markers in the observed differences in chondrogenic capacity.

The apparently diminishing differences in mRNA levels from passage 1 to 2 and the limited differences of immunohistochemical and matrix turnover parameters during redifferentiation culture as found in this study suggests that differences between primary healthy and OA chondrocytes might, at least partly, be lost during expansion in monolayer. However, in chondrocytes from OA joints, the presence of collagen type X mRNA after expansion, as well as the poor morphology and lower final proteoglycan content, suggest that the degree of dedifferentiation induced by monolayer expansion and the redifferentiation conditions were insufficient to abolish differences between chondrocytes from healthy and OA joints

completely. It should be noted that the differences in phenotype and chondrogenic capacity between healthy and OA chondrocytes is an overall property observed for a "pool" of cells. None of our analysis-techniques were aimed at studying differences at single cell level, while the chondrocytes studied might actually consist of a heterogenic population. This hypothesis is supported by various studies, which have demonstrated that articular cartilage does not consist of one specific cell type, but rather contains sub-populations of cells, which, for example, can function as progenitor cells[95,142,146,189,269]. These results suggest that the observed differences in the current study might be due to alterations of small cell subsets. Furthermore, this phenomenon might explain the discrepancies between Sox and collagen type X mRNA levels as discussed above.

The observed differences in chondrogenic capacity could be suggested to be attributable to the difference in average donor age between the healthy and OA donors, as reflected by differences in cell viability, cell proliferation rate and chondrogenic capacity. However, the proliferation rate of chondrocytes did not differ between donor types, which is in accordance with previous data failing to show a significant effect of donor age on proliferation rate for the donor ages studied in this paper[145]. In addition, no differences were found previously with respect to the chondrogenic capacity of expanded chondrocytes for these age groups[145]. Therefore, the observed differences are unlikely to be age-related. Finally, as both the OA and healthy chondrocytes underwent similar number of population doublings, differences with respect to the degree of dedifferentiation resulting from expansion are also unlikely.

Altogether, the difference in chondrogenic capacity between chondrocytes from healthy and OA joints is more likely due to an *a priori* altered phenotype of chondrocytes from OA joints, leading to differences in cartilage metabolism, either intrinsic or due to a changed sensitivity for mediators such as growth factors. Importantly, the differences in phenotype and chondrogenic capacity found here have several consequences. Caution is needed when using chondrocytes from OA joints as a model for *in vivo* chondrogenesis, as is done frequently both for research into basic chondrogenic mechanisms and as a model for autologous chondrocyte implantation (ACI) because of the mere availability of cartilage from these joints. This study opposes the assumption that macroscopically less affected cartilage from OA joints contains chondrocytes with identical chondrogenic properties as chondrocytes isolated from healthy joints. In fact, these chondrocytes are likely to display an intrinsically different cartilage metabolism and/or respond differently to applied stimuli than healthy chondrocytes.
Furthermore, a gradual change of a healthy chondrocyte towards an OA chondrocyte may be actual in joints with traumatic cartilage damage, which are often seen to develop towards OA. If this holds true, we hypothesize that cartilage biopsied from the less weightbearing area of a joint in which a defect has been present for a prolonged period of time may have undergone phenotypical changes that may have a significant influence on *in vitro* expansion and the outcome of ACI as previ-

ously discussed in the concept of joint homeostasis[37]. Further research into the properties of cells harvested from joints with articular trauma is required to shed light on this process, and might provide valuable insight in the importance of the timing of this therapy. However, this statement should be put into perspective, as, currently, it is unknown whether changes in phenotype of OA chondrocytes develop as a gradual process or rather occur as a "sudden" alteration. Therefore, it is unclear whether chondrocytes harvested for ACI, which are isolated from biopsies from joints with cartilage defects, should be considered "healthy".

In conclusion, in this study we demonstrated that the use of chondrocytes harvested from joints with OA lesions leads to altered *in vitro* chondrogenesis, despite the procedure *in vitro* expansion and dedifferentiation postulated to level off existing differences.

The effect of synovial fluid from injured knee joints on *in vitro* chondrogenesis

Chapter 07

Abstract

Goal: Synovial fluid (SF) plays a major role in the nutrition and metabolical status of articular cartilage, and, therefore, may play a significant role in the detrimental effect of a disturbed joint homeostasis on cartilage regeneration. The goal of the current study was to investigate the effect of human SF from injured knee joints on *in vitro* chondrogenesis by expanded human chondrocytes.

Methods: Chondrocytes isolated from healthy cartilage biopsies were expanded in monolayer under GFSEC for 2 passages and were subsequently redifferentiated on collagen-coated filters for 14 days either in the presence or absence of 10% SF from injured knee joints.

Results: SF supplementation resulted in a significant downregulation of final proteoglycan (PG) content ($p = 0.0001$), PG content normalized to DNA ($p < 0.05$), collagen type II mRNA levels ($p < 0.001$), and differentiation index ($p < 0.001$) as compared to control culture conditions. Additionally, SF-supplemented media resulted in significantly increased cellularity compared with control media ($p < 0.0001$). Morphology, and collagen type I, X, and aggrecan mRNA levels were not significantly affected.

Conclusion: This study demonstrates that SF from injured human knee joints significantly affects *in vitro* chondrogenesis and therefore may provide a viable target for future improvement of ACI by refinement of culture techniques, patient selection, or pretreatment of affected joints to restore joint homeostasis.

Introduction

Synovial fluid (SF) contains factors that are essential for nutrition and metabolic chondrocyte activity. SF is a dialysate of blood plasma with the addition of components synthesized by synovial tissue and catabolic products from the surrounding tissues, e.g. PG and collagen breakdown products[270]. Furthermore, SF contains growth factors and, dependent on the status of the joint, pro- and anti-inflammatory cytokines[60,271,272]. Therefore, synovial fluid may also affect chondrogenesis in joints with cartilage defects. This has consequences for regenerative medicine since the SF may affect the quality of the biopsy region prior to *in vitro* expansion, but may also play an important role in the detrimental environment the cells encounter on reimplantation in joints with prolonged cartilage defects[37,38]. However, relatively little is known about the role of SF in cartilage turnover and differentiation, in particular during cartilage regeneration after trauma.

The goal of this study was to investigate the effect of human SF from injured knee joints on *in vitro* chondrogenesis by expanded human chondrocytes in a previously validated culture model[273]. Collagen types I and II and aggrecan mRNA levels were determined to evaluate the effect of SF on the degree of re-differentiation. Collagen type X mRNA levels were measured to detect hypertrophic changes[248-251]. Tissue morphology, PG content, and cell content of the extracellular matrix (ECM) were determined to assess the effect of SF on the final tissue quality.

Materials and methods

Synovial fluid collection

Synovial fluid (SF) samples were collected from 12 patients, either during arthroscopy or in the outpatient clinic. Demographic and clinical data of these samples are summarized in table 1. Defect ages were determined from patient history. Diagnoses were determined either by MRI or arthroscopically. The donors had no clinical history of inflammatory or degenerative joint disorders and did not use corticosteroids (systemically or intra-articularly). After aspiration, the samples were spun at 300 x g to remove debris and stored in small aliquots at -80°C until further use.

Culture methods

Articular cartilage was harvested from a femoral condyle of a 48 year-old human female donor. The chondrocytes were expanded for 2 passages under growth factor supplemented expansion conditions (GFSEC). After the chondrocytes were expanded for 2 passages, the chondrocytes were redifferentiated on collagen type II-coated filters in two groups: (A) Control group: “standard” redifferentiation culture as described in chapter 4, or (B) Identical to control condition, but with 10% synovial fluid supplemented to the culture media.
The culture media were renewed at 7 and 10 days of culture. The samples were

harvested after 14 days of culture. For each analysis method, 3 samples per SF donor were cultured. The results of the present study were confirmed by repeating the experiment with chondrocytes isolated from a 51 year-old human male donor. Results that were not consistent with results observed in the first experiment are mentioned explicitly in the results section.

Analytical methods

After 14 days of redifferentiation culture, proteoglycan content, cell content and tissue morphology (Safranin O / fast green) were determined in order to assess the outcome of the two culture conditions. The collagen type I, II, X and aggrecan mRNA levels were determined by real-time PCR. Since collagen type II is one of the typical structural proteins synthesized by differentiated chondrocytes in hyaline cartilage, as compared to collagen type I that is normally synthesized by dedifferentiated chondrocytes, we defined the ratio of collagen type II and collagen type I mRNA levels as “differentiation index”.

Statistical analysis

The effect of synovial fluid supplementation compared to no supplementation was analyzed using a separate-variance T-test. If non-normal distribution or unequal variance was observed among the treatment groups, that specific variable was analyzed by a Wilcoxon Rank Sum test. The graphs show average values in either treatment group; p-values lower than 0.05 were considered statistically significant. Statistical calculations were done with the JMP (Cary, NC, USA) version 5.0 software package.

For further details of the materials and methods used, please refer to appendix B and C.

Table 1
Demographic data of synovial fluid samples

Patient No.	Patient age [Years]	Defect age [Days]	Diagnosis
1	27	5	Knee distortion with pain and swelling: no further tissue damage demonstrated
2	43	7	Knee distortion with pain and swelling: no further tissue damage demonstrated
3	23	8	Cartilage defect: Medial Femoral condyle
4	47	9	Knee distortion with pain and swelling: no further tissue damage demonstrated
5	28	14	Medial meniscus rupture
6	36	14	Cartilage defect: Medial Femoral condyle
7	20	31	Cartilage defect: Medial Femoral condyle and anterior cruciate ligament rupture
8	33	39	Cartilage defect: Medial Femoral condyle
9	46	254	Cartilage defect: Medial Femoral condyle
10	46	341	Cartilage defect: Medial Femoral condyle and medial meniscus rupture
11	44	885	Cartilage defect: Medial Femoral condyle
12	46	>1000	Cartilage defect: Medial Femoral condyle

Results

Tissue morphology

Both culture with and without SF supplementation resulted in the synthesis of fibrocartilaginous tissue by chondrocytes. The extracellular matrix stained positive with Safranin O in all samples and most chondrocytes had a rounded appearance, although a substantial part of the cells had a fibroblastic appearance and lacked lacunae, typical for native chondrocytes in articular cartilage (Figure 1A and B). No differences in tissue morphology between both treatment groups were observed. In order to objectify this finding, three blinded observers scored the morphological quality of the tissue synthesized according to the "Bern score". No statistically significant differences were found between both treatment groups, tissue synthesized under control conditions scoring 4.9 ± 0.7 points versus tissue synthesized in SF supplement culture media, which scored 4.6 ± 0.8 points (Figure 1C).

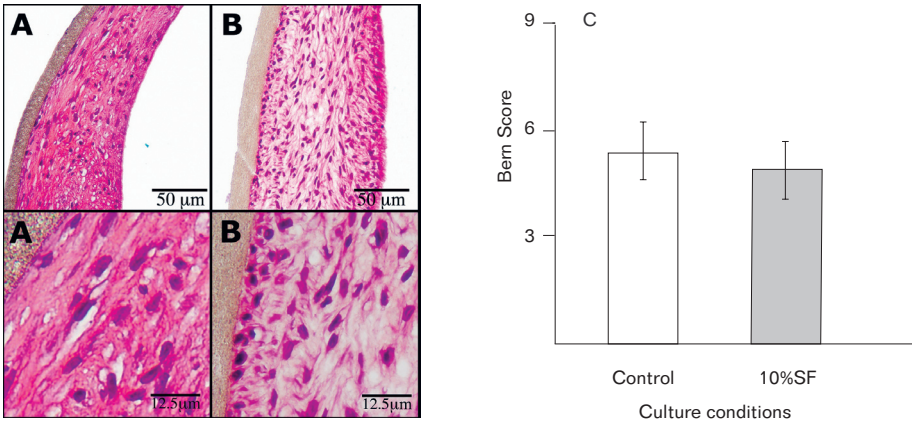


Figure 1
5 µm histological sections with safranin O / fast green staining after 14 days of culture of tissue synthesized by chondrocytes redifferentiated in (A) control media and (B) culture media supplemented with 10% synovial fluid. Original magnification: 40X. (C) "Bern" histological score after 14 days of culture. Bars represent average±SD.

Proteoglycan synthesis and DNA content

Extra-cellular matrix (ECM) synthesis as measured by the final PG content of the tissue was significantly affected by the supplementation of SF to the culture media. Chondrocyte redifferentiation culture under control conditions resulted in a significantly higher amount of PGs synthesized after 14 days of culture compared to SF-supplemented culture conditions (15.6±1.3 mg versus 7.3±1.8 mg respectively: $p = 0.0001$; Figure 2A). PG content normalized to DNA content showed a similar significant downregulation by SF-supplemented culture conditions compared to control culture conditions (0.7±0.5mg/µg versus 3.0±0.6 mg/µg respectively: $p < 0.05$; Figure 2B).
Chondrocyte redifferentiation under SF-supplemented culture conditions resulted in a significantly higher DNA content after 14 days of culture compared to control media (1369±683 µg versus 514±72 µg respectively: $p < 0.0001$; Figure 2C).

Post-redifferentiation mRNA levels

SF supplementation resulted in significant downregulation of relative collagen type II mRNA levels (0.2±0.3 versus 7.0±5.6 respectively) and the differentiation index (the collagen type II/I mRNA ratio; 0.1±0.2 versus 6.0±2.9 respectively) as compared to control media (Figure 3B-C).
SF-supplementation appeared to result in upregulation of collagen type I mRNA levels in both donors, although this effect was not consistently significant (Donor 1: 28.3±61.7 versus 10.6±3.9 respectively: $p = 0.31$ and Donor 2: 10.2±9.1 versus 4.5±1.7 respectively: $p = 0.03$; Figure 3A). Aggrecan mRNA levels were not significantly different between culture conditions (Control 5.1±1.7 versus 10% SF 8.6±13.8; Figure 3D). Collagen type X was below detection level for all samples.

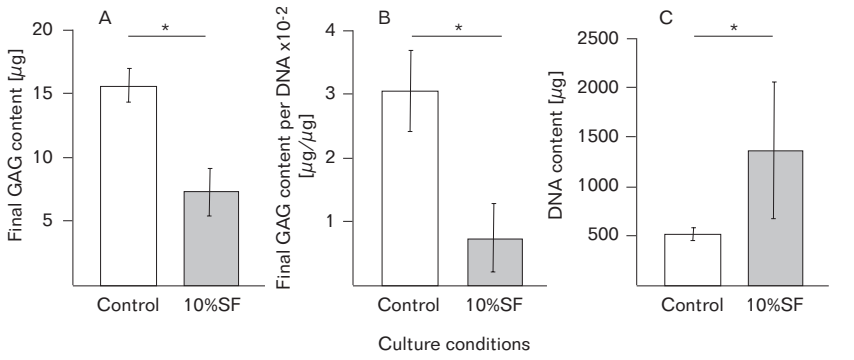


Figure 2
(A) Final GAG content. (B) Final GAG content normalized to DNA content. (C) Final DNA content. Bars represent average±SD. Statistically significant differences are indicated by * ($p < 0.05$). Note that SF supplementation to redifferentiation media significantly inhibits the final proteoglycan content of the tissue synthesized and stimulates the cellularity compared to control redifferentiation conditions.

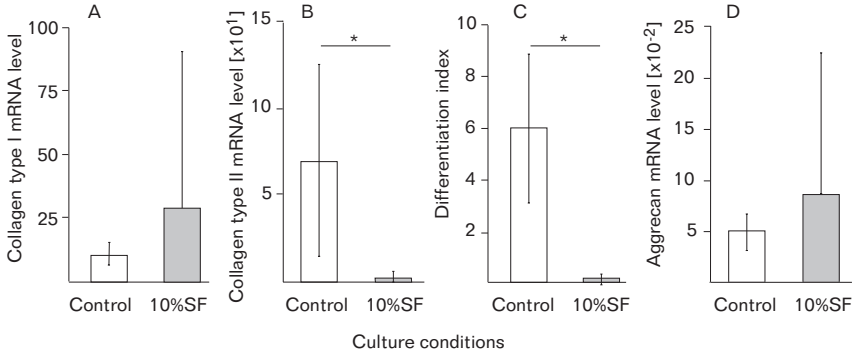


Figure 3
Relative mRNA levels of (A) collagen type I, (B) collagen type II, (C) the collagen type II/I ratio (differentiation index), and (D) aggrecan in tissue synthesized by expanded chondrocytes that were redifferentiated on collagen coated filters for 14 days. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control. Statistically significant differences are indicated by * ($p < 0.05$). Note that SF supplementation results in significantly decreased collagen type II mRNA levels and differentiation index compared to control redifferentiation conditions.

Discussion

In order to optimize the quality of tissue formed after clinically applied ACI, cartilage regeneration has been studied extensively, both *in vitro* and *in vivo*. However, few studies have aimed at elucidating the effect of the *in vivo* environment on chondrogenesis. The current study clearly demonstrates that *in vitro* chondrogenesis is significantly affected by supplementation of culture media with SF obtained from injured knee joints. After 14 days of culture, SF supplementation resulted in a significant decrease of final PG content, significant increase of final cellularity, and a significant inhibition of chondrocyte redifferentiation at the mRNA level. These results suggest that SF will influence the intra-articular environment of chondrocytes at reimplantation and, in addition, may affect the "normal" cartilage taken for culture expansion at the first step in current ACI.
The observed decrease in final PG content by chondrocyte redifferentiation in SF supplemented culture media may be caused either by a higher anabolic activity of chondrocytes cultured in control media, a higher catabolic activity of chondrocytes cultured in SF supplemented culture media, or a combination of these two factors. Either way, these results in combination with decreased redifferentiation suggest that SF from injured knee joints is detrimental to *in vitro* chondrogenesis. Possible factors responsible are chemokines, inflammatory cytokines and matrix metalloproteinases (MMPs). These mediators have been described to be induced as a physiological reaction to damage in a variety of tissues and are known to be crucial for e.g. cutaneous wound healing[274-277]. Likewise, joint injuries might result in the induction of such mediators[278,279]. However, in the context of cartilage biology, these mediators are suggested to be involved in osteoarthritis development and to be detrimental to the ECM of articular cartilage[60,62]. Therefore, they might be responsible for the decreased final PG content in SF-supplemented cultures by inducing increased PG degeneration rate. Future studies should aim at elucidating the factors responsible for the observed effects, as these might provide a possible target for improvement of clinically applied ACI, e.g. by desensitizing chondrocytes for specific cytokines using siRNA, or restoring a beneficial joint homeostasis for chondrogenesis by pre-treatment of joints. Similar treatments have been studied in patients with rheumatoid arthritis, for example tumor necrosis factor-alpha (TNF-α) inhibitors[280,281] and are now a successful example of modern early intervention that has changed the course of the disease[282]

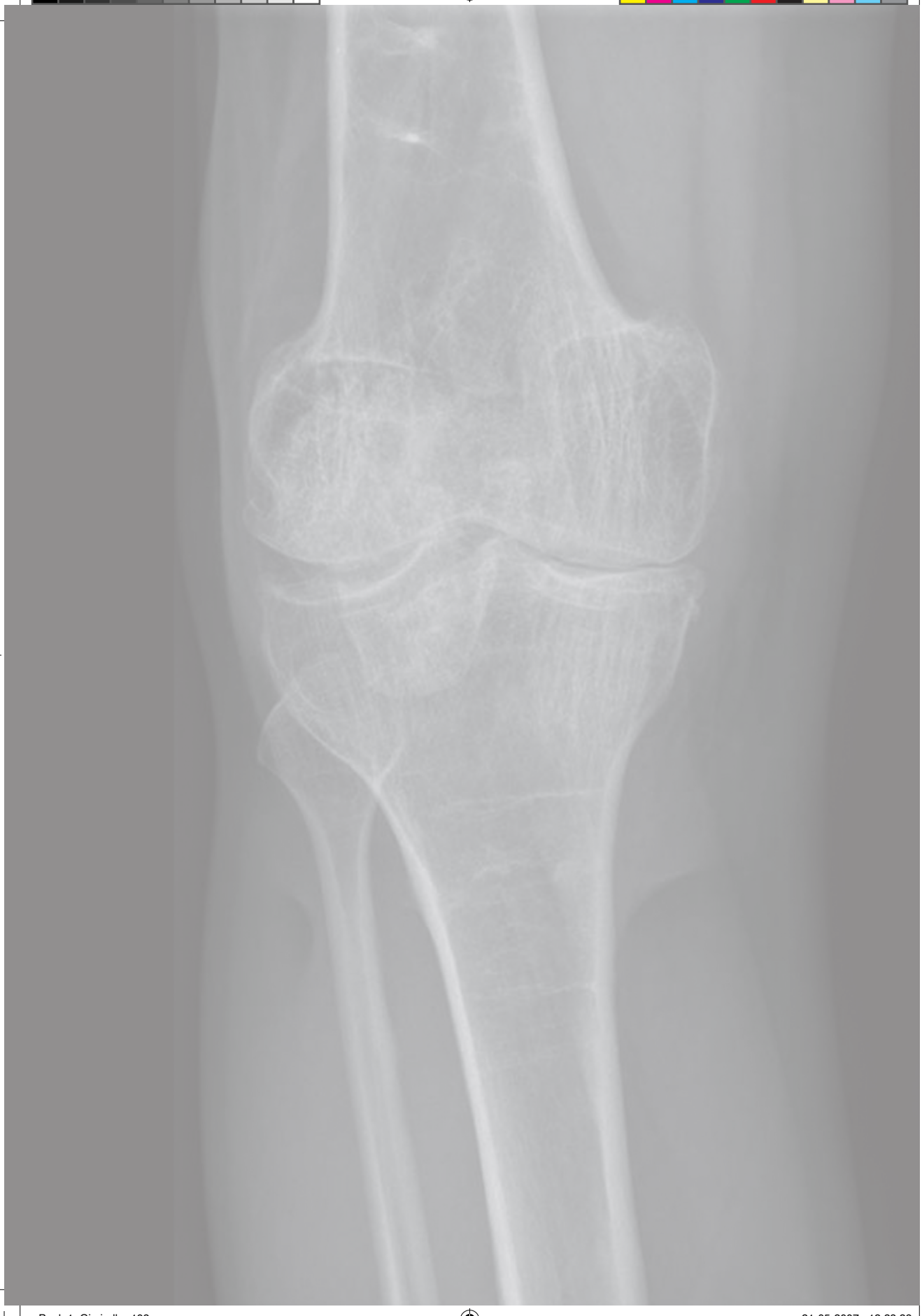
In contrast with our results, Hegewald *et al* demonstrated that autologous SF obtained from healthy equine joints is supportive for *in vitro* chondrogenic differentiation and PG synthesis by equine mesenchymal stem cells[283]. Furthermore, Skoog *et al* presented similar supportive effects for *in vitro* chondrogenesis from perichondral tissue harvested from rabbit rib cartilage[284]. These discrepancies may be explained by the fact that, in the current study, SF samples were harvested from human injured knee joints in contrast to above described studies, which used SF obtained from healthy joints. This hypothesis appears supported by the fact that SF from "chronically" injured knee joints has indeed been demonstrated to inhibit

the PG synthesis rate in a chick limb bud assay[38]. However, in the same study, PG synthesis rate was stimulated by synovial fluid from "acutely" injured knee joints. Likewise, periosteal transplantation in joints with longer existing cartilage defects has been demonstrated to result in regeneration tissue with significantly worse morphology and to affect PG turnover of the treated joint negatively compared to periosteal transplantation in joints with "fresh" cartilage defects[37]. These studies suggest that the effect of SF on chondrogenesis is related to the age-of-injury of the joint from which the SF samples are harvested. Such trauma-age related effects were not observed in the current study. This may be due to various factors, such as the culture model used, differences in chondrocyte species, the limited number of SF samples, or the outcome parameter used, e.g. the above-described studies determined proteoglycan turnover parameters rather than final proteoglycan content, while the latter appears more relevant as this has a direct relation to mechanical properties of the tissue. These studies do confirm that joint injury may result in disturbed joint homeostasis, which may be detrimental for the quality of regenerating tissue after ACI.

Interestingly, SF supplementation resulted in significantly higher cellularity of tissue formed after 14 days of culture. These results are in accordance with a recently published study in which cartilage injuries were demonstrated to result in basic Fibroblastic Growth Factor (bFGF) release[285]. Besides being a potent mitogen, bFGF is known to be a potent inhibitor of the anabolic effects of both IGF-1 and OP-1, i.e. bFGF significantly down-regulated the PG synthesis rate of culture chondrocytes induced by these growth factors[286]. A similar mechanism might be responsible for the observed effects of SF supplementation in the current study. However, in an animal study Fukuda *et al* demonstrated that treatment of osteochondral defects by matrix induced autologous chondrocyte implantation (MACI) resulted in macroscopically, morphologically and mechanically improved tissue regeneration when scaffolds were impregnated with bFGF[287]. These apparently contrasting results may be explained if bFGF-induced proliferation precedes redifferentiation rather than occurring simultaneously. A similar process was previously suggested to occur during *in vitro* periosteal chondrogenesis[231]. Although this hypothesis seems to be supported by the downregulation of redifferentiation parameters in our study, future experiments with extended culture periods should investigate whether this hypothesis holds true. Joint-injury-induced upregulation of intra-articular bFGF levels might actually result in increased amounts of regeneration tissue, rather than to be detrimental as the results of the current study suggest.

Altogether, various parameters of ECM quality and chondrocyte redifferentiation appear to be strongly affected by SF supplementation to redifferentiation media. However, pinpointing the exact factors involved in the effects found is difficult, as various limitations should be considered in this study. All SF tested were obtained from injured knee joints. Therefore, the observed effects cannot be assigned with certainty to the fact that the joints, from which the SF samples were harvested,

were injured. Unfortunately, human SF samples from "healthy " joints are sparsely available. Furthermore, tissue quality was determined at only one time point, limiting the possibility of temporal interpretations of the current data. Whether decreased PG content combined with increased cellularity actually represent detrimental effects induced by SF supplementation, or rather reflects increased amounts of tissue that may be synthesized, remains to be elucidated in future studies with extended culture periods. Finally, SF samples that were supplemented to the redifferentiation media were obtained from other donors than the chondrocytes, which suggests that the adverse effects induced by SF supplementation may be due to an immunological rejection. However, the significantly increased final cellularity induced by SF supplementation contradicts this hypothesis.
In conclusion, synovial fluid supplementation to culture media significantly affects *in vitro* chondrogenesis, suggesting that caution is warranted when investigating *in vitro* chondrocyte redifferentiation without SF supplementation to culture media. Moreover, this study suggests that SF from injured knee joints has a detrimental effect on redifferentiation and extracellular matrix synthesis. Therefore, factors present in the synovial fluid of injured joints may, once identified, provide a viable target for future improvement of ACT by refinement of culture techniques, patient selection or pre-treatment of affected joints in order to restore joint homeostasis.



Autologous Interleukin-1 receptor antagonist for treatment of knee osteoarthritis

Chapter 08

Abstract

Goal: Altering the joint homeostasis can be an important pathway by which we may improve the course of OA and the treatment of cartilage defects. As Interleukin-1β is thought to play a key-role in cartilage degeneration and the development of osteoarthritis (OA), this might be a viable target to improve the joint homeostasis. Therefore, the current study aimed to determine the biological efficacy of a newly developed autologous form of Interleukin-1 receptor antagonist (Orthokin).

Methods: In a multicenter, randomized, double blind, placebo controlled trial, we compared the clinical efficacy of Orthokin and physiological saline. The primary efficacy objective consisted of 30% superiority on the WOMAC at 3, 6, 9, and 12 months post-treatment. Additionally, the patients completed the visual analogue scale for pain (VAS), the Knee injury and Osteoarthritis Outcome Score (KOOS) and Knee Society Clinical Rating System (KSCRS).

Results: Orthokin and placebo treatment resulted in similar improvements on the WOMAC. Orthokin resulted in significantly more improvement for KOOS symptom ($P = 0.002$) and KOOS sport ($P = 0.042$) parameters as compared to placebo treatment. For most other outcome parameters, Orthokin-treated patients consistently showed higher improvement compared to placebo-treated patients (n.s.).

Conclusion: The statistically significant improvement of KOOS symptom and KOOS sport parameters together with the consistently higher, though non-statistically significant, improvement of most other parameters demonstrates that Orthokin clearly induces a beneficial biological response, although the clinical effect when administered under trial conditions is limited.

Introduction

Biological joint reconstruction is a viable and realistic goal in regenerative medicine. In the previous chapter, we have shown the role of SF mediated factors on the outcome of cartilage regeneration. Modulating these factors may be a viable approach to improve cartilage regeneration, although little is known of the factors responsible for the observed effect. Much more is known of the factors involved in OA development.

Osteoarthritis (OA) is a slowly progressive, degenerative, and disabling disease of articulating joints that not only affects the elderly, but also involves younger, more active patients, e.g. post-traumatic or due to prolonged participation in high demanding sports. Treatment of this young population is especially troublesome as current treatment options, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors, do not prevent the progression of OA, although they have been proven to effectively reduce symptoms of OA[288,289]. In addition, prolonged use of these drugs is related to important drawbacks, such as increased risk of upper gastrointestinal bleeding and cardiovascular ischemia through platelet activation[290,291].

Over the last two decades, OA research has increasingly focussed on drugs that not only improve the patients' symptoms, but additionally are capable of altering the course of OA development and consequently postpone or even prevent the need for total joint replacement, the so-called disease modifying osteoarthritis drugs (DMOADs). Best known are glucosamine and chondroitin sulfate. When used in combination, these have recently demonstrated to be effective in reducing clinical symptoms[292]. However, glucosamine and chondroitine sulfate have never been shown to be effective modifiers of OA progression and, in fact, the mechanism through which these should alter the course of OA development remains unclear.

A viable target for DMOADs are pro- and anti-inflammatory cytokines, as these are known to be involved in OA development[60-65]. Of these, Interleukin 1β (IL-1β), a pro-inflammatory cytokine, has been proposed to play a key role[293-295]. It induces the production of collagenase and prostaglandin and results in decreased synthesis of cartilage specific collagens and proteoglycans[61,68-70]. In an experimental equine OA model, *in vivo* delivery of the IL-1Ra gene results in significant improvement in clinical parameters of pain and disease activity, preservation of articular cartilage, and beneficial effects on histological parameters of the synovial membrane and adjacent articular cartilage[71].

Orthokin (Orthogen, Düsseldorf, Germany) is a product in which whole blood is incubated with CrSO4-coated glass beads. This process has been demonstrated to stimulate the synthesis of Interleukin-1 receptor antagonist (IL-1ra) and other anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13[72]. Therefore, Orthokin might have a beneficial effect on the symptoms and disease progression of OA. The aim of this study is to investigate the efficacy of this from of autologous IL-1ra for the relief of symptoms in patients with OA of the knee in a prospective, randomized, double blind, placebo controlled study of intra-articular injection therapy.

Materials & Methods

A prospective double blind placebo controlled randomized multi-centre trial was conducted to evaluate the efficacy of intra-articularly injected Orthokin versus placebo (physiological saline) in reducing symptoms of OA in the knee. This trial was conducted over a period of 30 months; the first patient was included in February 2004 and the last patient completed follow-up in August 2006. The trial was performed at 7 centres in the Netherlands and was approved by the medical ethics committee of the University Medical Center in Utrecht and the local ethics committee at each participating study site.

Trial overview

After the participants were informed both verbally and by a written information brochure, they signed informed consent, and, in order to assess whether the participants met all inclusion criteria, the patients completed all questionnaires and x-rays (weight-bearing antero-posterior, so called notch view, weight-bearing lateral, and patellar skyline). The questionnaires comprised the visual analogues scale (VAS) for pain, the Knee injury and Osteoarthritis Outcome Score (KOOS) and the Knee Society clinical rating scale (KSCRS). The Western Ontario and McMaster Universities Index (WOMAC) scores were deducted from the separate KOOS items. If the participants still met all in- and exclusion criteria, they were randomized to either the Orthokin or the placebo treatment.

After randomization, all participants returned to the local study physician, who performed the treatment. The treatment consisted of an initial peripheral vena punction for production of the Orthokin product, followed by six intra-articular injections in the affected knee joint, which were given in a rigid scheme comprising three weeks: day 0, 3, 7, 10, 14 and 21. In order to ensure blindness to the treatment given, all procedures were identical for both the Orthokin and the placebo (physiological saline) injections.

At 3, 6, 9 and 12 months after the first injection, the patients completed the same questionnaires as at baseline. Subsequently, the participants visited their treating orthopaedic surgeon, who performed a physical examination of the knee, completed the surgeon-part of the KSCRS and checked for adverse events, changes in NSAID and other analgesic use.

Study outcomes

For the WOMAC, the KOOS sub-domains (pain, stiffness, function, sport and Quality of Life (QoL)) and the KSCRS, a higher score represented a better outcome; the possible values ranged from 0 (worst score) to 100 (best score). In contrast, for the VAS for pain, 0 indicated no pain and 100 most severe pain. An absolute increase in the response rate of 30% on the WOMAC scale, as compared to the rate in the placebo treatment was considered a clinically relevant effect. With an expected standard deviation of 40% of the mean, as frequently found in other

OA treatment studies, 100 patients were required to obtain a power of 90%.
Treatment failures were defined as patients who underwent a different treatment of the afflicted knee during the 12 months follow up period; patients randomized in the non-NSAID group who started NSAIDs during the follow-up period; patients randomized in the NSAID group who increased the use of NSAIDs.

Statistical analysis

FileMaker Pro 6.0 for Windows (Filemaker Inc, Santa Clara, CA, USA) and SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data management and statistical analysis.
For comparison of the efficacy of Orthokin versus placebo treatment, a repeated measure analysis was performed. When multivariate analysis showed an interaction between the treatment effect and time, an additional repeated measure analysis was performed in order to study the early (0-3 months) and late (6, 9, and 12 months) treatment effects. Although randomization was stratified for NSAID usage, gender and age (<45 and >45 years old), sub-analysis was performed for these groups. Finally, correlation analysis has been performed to study whether baseline characteristics, such as age, gender, and degree of symptoms, could predict the outcome of the treatment. Patients who were considered treatment failures, were excluded for further follow-up. The datasets of these participants were completed using the last-observation-carried-forward method. Statistical analysis of treatment failure frequency between groups was separately done using the Chi-square test. P-values less than 0.05 were considered statistically significant. All graphs show mean values with Standard error of Mean (SEM).

For further details of the methods used, please refer to appendix D.

Results

Demographics and baseline characteristics

A total of 250 patients were screened for possible inclusion into the study. Of these, 182 met the inclusion and exclusion criteria and were randomized to receive Orthokin (n= 94) or placebo (n= 88). Before injection, 6 patients were excluded and 9 patients withdrew informed consent (Figure 1). Of the remaining 167 patients, 89 patients were randomized to the Orthokin group and 78 were randomized to the placebo group. Subsequently 14 patients were excluded from further analysis, because of serious adverse events and major protocol violations (Figure 1), i.e. 153 patients were analyzed: 80 patients received Orthokin treatment and 73 patients received placebo treatment
The baseline characteristics of the Orthokin and placebo group were comparable (Table 1), no significant differences between the treatment groups were found with respect to age, gender, weight, body-mass index (BMI) or use of NSAIDs before and during the study period.

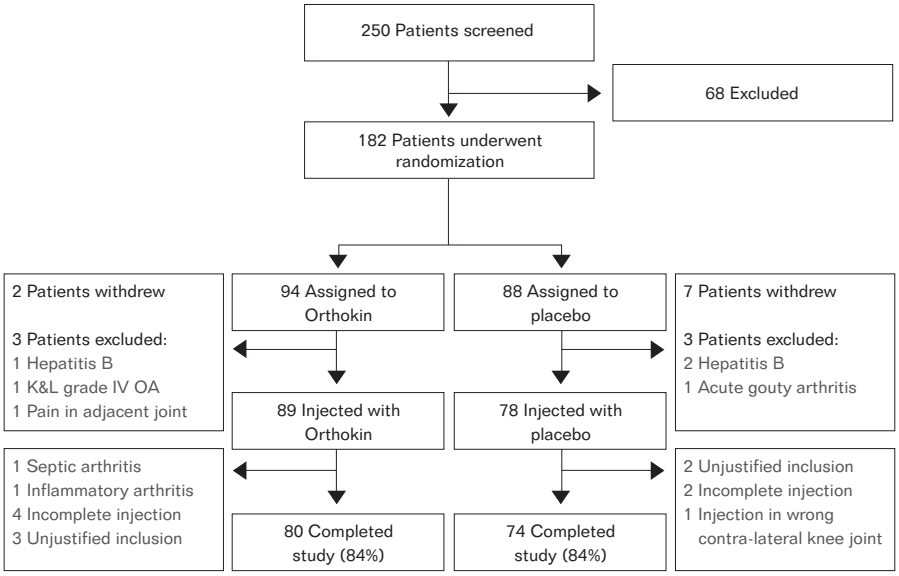


Figure 1
An overview of enrollment of the patients.

Table 1
Baseline demographic and clinical parameters.

	Orthokin n=(80)		Placebo n=(73)	
	n	%	n	%
Male	49	61.25	43	58.90
Analgesic medication use	22	27.50	24	32.88
PCM	12	15.00	17	23.29
NSAID	7	8.75	6	8.22
Other analgesics	3	3.75	1	1.37
	Mean	SD	Mean	SD
Age (years)	54.14	10,75	53.09	10.77
Weight (kg)	83.12	16.01	86.52	14.45
Body mass index (kg/m2)	27.20	4.95	28	14.45
WOMAC	54.49	17.60	50.47	15.61
KOOS pain	47.19	15.93	45.17	14.88
KOOS activity daily life	55.00	18.16	50.87	16.74
KOOS symptoms	54.60	17.63	48.43	16.95
KOOS sport	24.94	19.18	20.82	15.14
KOOS quality of life	28.59	13.63	26.2	13.61
VAS for pain	59.86	20.25	63.44	18.22
KSCRS, patient part	68.97	22.79	67.66	20.38
KSCRS, physician part	47.43	16.38	47.02	12.71

OA=Osteoarthritis, K-L = Kellgren-Lawrence; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; KOOS = Knee injury and Osteoarthritis Outcome Score, VAS = Visual Analogue Scale, KSCRS = Knee Society Clinical Rating Score, NSAID = Non-steroidal anti-inflammatory drugs.

Treatment effect

Both Orthokin and placebo-treated patients showed a significant improvement on all outcome measures ($P < 0.001$), as compared to baseline values. Comparable improvements were found for the Orthokin and placebo treatment on the WOMAC (28% versus 23% at 3 months, 15% versus 18% at 6 months, 14% versus 17% at 9 months, and 19% versus 13% after 12 months; n.s.; Table 2). On all outcome parameters, Orthokin treated patients scored consistently better as compared to placebo treated patients. However, the differences between the two treatment groups were small. With respect to improvement over time, Orthokin resulted in significantly more improvement for KOOS symptomatology ($P = 0.002$) and KOOS sport ($P = 0.042$), as compared to placebo treatment (Figure 2). This coincides with the clinical observation that patients describe an initial effect on pain and subsequently choose for an increase in their activities in daily life (ADL), in sports and in hobby. Furthermore, Orthokin-treated patients consistently showed higher relative improvements compared to placebo-treated patients for all other outcome parameters, except for the VAS at 3 months (21% versus 25%), the KOOS sport at 3 and 9 months (41% versus 43%, and 42% versus 43% respectively), and the KOOS ADL at 6 and 9 months (15% versus 17%, and 13% versus 16% respectively), although none of these differences were statistically significant. Treatment failures were equally distributed over both treatment groups (Orthokin 8, placebo 7; $p = 0.954$), and were mainly due to worsening of symptoms for which these patients were treated either with NSAIDs or by surgical intervention.

Sub-analysis for age (<45 and >45 years old) and gender did not show statistically significant differences in responsiveness between these subgroups. In addition, correlation analysis did not show significant relations between baseline patient characteristics (age, gender, symptom severity) and treatment outcome. Interestingly, the superior improvement resulting from Orthokin treatment, as compared to placebo treatment, appeared even more pronounced upon sub-analysis for the patients who continued using NSAIDs during the trial (Figure 3 and Table 2). For these patients ($n = 15$), repeated measure analysis showed that Orthokin treatment resulted in statistically significant more improvement of the KOOS sport parameters as compared to placebo treatment ($p = 0.011$; Figure 3D). Furthermore, Orthokin resulted in significantly more improvement of the KSCRS, surgeons part: as compared to placebo treatment ($p = 0.005$; Table 2).

Adverse events

During the trial, 219 adverse events were reported (Table 3). 159 AE's were knee related, of which the majority was attributable to (subjective) increase of knee pain (Orthokin: 44 versus Placebo: 50; n.s.). The involved surgeons graded 2 adverse events as serious; both cases were Orthokin-treated patients. The first serious adverse event was due to a patient with a septic arthritis of the knee joint. Because the product was injected through a 0.22 µm sterile filter and because no bacterial contamination was demonstrated by microbiological testing of the sample, it was concluded that this event was caused by the injection procedure and

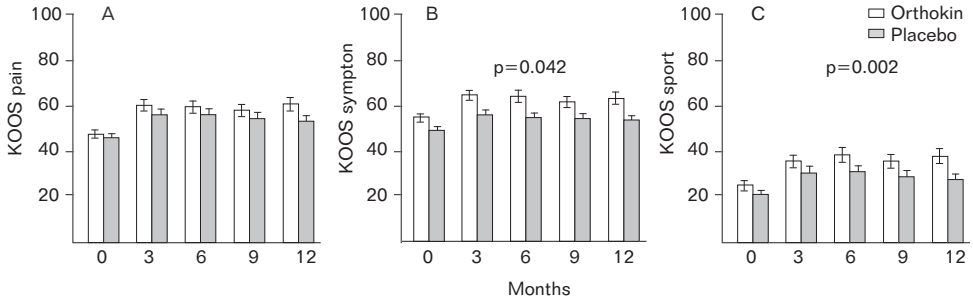


Figure 2
This graph shows the effect of Orthokin and placebo treatment over time for the 5 separate KOOS items. (A) KOOS pain, (B) KOOS symptomatology, and (C) KOOS sport. Note that Orthokin resulted in statistically significant more improvement as compared to placebo treatment for the KOOS symptomatology (C) and KOOS sport items (D). Bars represent mean ± SEM.

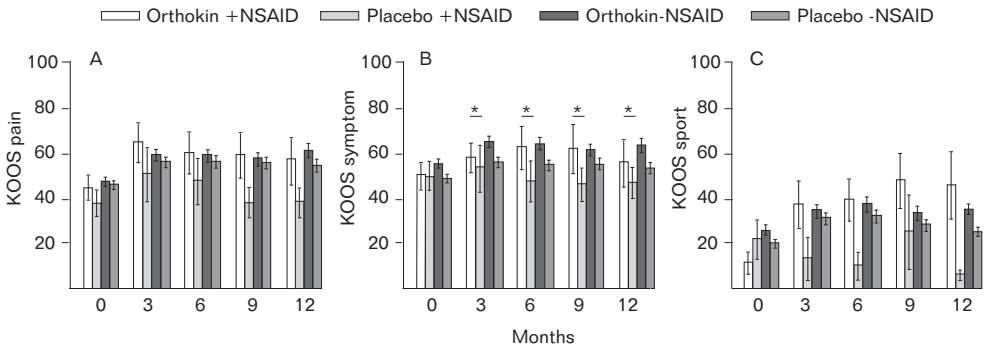


Figure 3
This graph shows the effect of Orthokin and placebo treatment over time for the 5 separate KOOS items subdivided for NSAIDs (+NSAID) and the non-NSAID (-NSAID) strata. (A) KOOS pain, (B) KOOS symptomatology, and (C) KOOS sport. Note that Orthokin resulted in statistically significant more improvement as compared to placebo treatment for the KOOS symptomatology and KOOS sport items. Bars represent mean ± SEM.

not by contamination of the product. The second adverse event was related to a patient with repeated severe inflammatory reactions of the knee joint within hours after the injection, as reflected by severe pain, swelling, and warmth of his knee joint. As a result, this patient discontinued the treatment after 3 injections.

Table 2

Outcome scores per treatment over time. Values are given for the complete dataset (All patients) and for the dataset after stratification to NSAID use during the trial. Note the fact that Orthokin-treated patients score consistently better at most datapoints, suggesting a beneficial biological effect of Orthokin.

	WOMAC		KSCRS patient part		KSCRS physician part		VAS	
All patients (n=153)								
Orthokin	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	54.49	17.6	47.43	16.4	68.98	22.8	59.68	20.2
3mo	63.37	20.6	58.87	19.8	76.53	22.9	43.63	26.5
6mo	62.90	23.7	58.29	21.2	74.81	21.2	48.59	28.5
9mo	61.78	23.4	58.46	21.1	75.52	21.9	48.91	27.7
12mo	65.02	24.1	58.96	20.2	77.17	21.6	47.32	28.0
Placebo	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	50.47	15.6	47.20	12.7	67.66	20.4	63.44	18.2
3mo	59.51	19.6	54.84	19.3	74.13	19.2	47.51	26.5
6mo	60.00	21.6	55.03	19.3	72.86	20.4	49.06	27.4
9mo	59.08	22.2	53.04	20.0	72.27	21.4	50.79	25.4
12mo	57.26	22.3	55.20	18.8	70.76	23.5	49.76	26.7
Non-NSAID- using patients (n=140)								
Orthokin	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	55.14	17.8	69.03	23.2	47.84	16.4	59.88	20.7
3mo	65.32	20.5	76.86	23.0	58.65	19.6	47.23	26.7
6mo	62.77	23.6	75.05	21.4	58.05	21.4	49.21	28.5
9mo	61.53	23.3	75.99	21.7	58.28	20.9	50.03	27.2
12mo	65.57	23.7	77.85	21.1	59.12	20.1	47.79	27.8
Placebo	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	50.75	15.9	67.66	20.9	47.93	12.6	62.61	18.7
3mo	59.90	19.2	75.03	19.1	55.57	19.1	46.14	26.5
6mo	61.05	21.4	74.44	20.3	56.12	19.3	47.66	27.7
9mo	60.31	22.5	73.91	21.4	54.19	20.2	49.38	25.9
12mo	58.49	22.6	72.47	23.4	56.79	18.3	48.45	27.0
NSAID using patients (n=13)								
Orthokin	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	47.77	14.6	68.43	19.6	43.21	16.6	59.71	15.3
3mo	65.92	22.7	73.14	23.0	61.16	23.0	40.57	25.6
6mo	64.29	26.3	72.29	20.8	60.90	21.5	42.29	29.8
9mo	64.29	26.2	70.71	24.4	60.09	24.8	37.43	32.4
12mo	59.52	29.1	70.29	27.3	57.10	24.0	42.57	31.0
Placebo	mean	SD	mean	SD	mean	SD	mean	SD
Before treatment	47.40	12.4	67.67	13.7	36.92	9.5	72.67	7.3
3mo	55.21	24.9	64.33	18.1	47.38	21.3	62.33	24.1
6mo	48.78	21.8	56.17	12.5	44.48	16.2	64.00	20.9
9mo	45.49	13.7	54.50	12.7	41.38	13.3	66.33	12.5
12mo	43.92	14.5	52.50	15.8	36.15	15.4	64.00	18.7

WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; VAS = Visual Analogue Scale; KSCRS = Knee Society Clinical Rating Score.

Table 3

An overview of all adverse events registered during the trial. Prevalences of adverse events were compared using the Chi-square test. No statistically significant differences were found, except for backpain.

	Orthokin n = 80	Placebo n = 73
Knee related adverse events		
Pain during injection	10	8
Irritation after injection	70	67
Increase knee pain	44	50
Septic arthritis	1	0
Severe inflammatory arthritis (non-septic)	1	0
Foreign body sensation	0	2
Crepitations	1	1
Locking	1	3
Irritation / pain	3	3
Swelling / synovitis	10	3
Redness / Warmth	3	1
Stiffness	2	1
Muscle cramps	2	3
Heavy feeling	1	0
Injection in wrong (contra-lateral) knee joint	1	0
Baker's cyst	1	0
Meniscus degeneration	1	0
Ligament ruptures (ACL / MCL)	1	1
Other Musculoskeletal adverse events		
Feet pain	1	1
Back pain (P = 0.009)	0	6
Hip pain	2	1
Shoulderpain / cuff tendinits	2	1
Achilles tendon swelling	0	1
Stiffness of other joints	0	1
Hotspot at cuboid - Metatarsal 5 joint	0	1
Heel pain	0	1
Fall on knee / knee distorsion	9	5
Contra-lateral knee injury	4	1
General adverse events		
Flu	4	1
Pneumonia	1	0
Jaw / Molar inflammation	1	1
Infection	1	1
Headache / migraine	2	1
Nephrolithiasis	2	0
Groin pain	1	0
Hysteroscopy	1	0
Endometrium polyp	1	0
Hypertension	0	1
Admission to rehabilitation clinic	1	0
Hospital admission due	1	0
Weight reduction	0	1
Weight increase	0	1

Discussion

Due to its high prevalence (6-12% of the adult population), OA is associated with high cost for society induced by healthcare consumption and loss of productivity at work[177,296]. These costs are expected to increase even more due to the ageing of the population. Therefore, over the last decades, an increasing interest for drugs that may alter the course of OA development (DMOADs) and thereby possibly delaying or even prevent the need for surgical interventions, such as total joint replacement has developed. Orthokin is an autologous blood product in which the production of various anti-inflammatory cytokines, such as Interleukin-1 receptor antagonist (IL-1ra), have been demonstrated to be upregulated[72]. By competitive inhibition of IL-1β in OA knee joints, this product may have a beneficial effect on the development of degenerative articular changes.

The aim of the current study was to evaluate the efficacy of Orthokin for treatment of knee osteoarthritis in a randomized, multicenter, double-blind, placebo-controlled trial with a primary efficacy objective to demonstrate 30% superiority of the Orthokin treatment on the WOMAC osteoarthritis index at 3, 6, 9, and 12 months post-treatment, as compared to placebo treatment. In the current trial this objective was not met, as the WOMAC showed comparable improvement ratios for Orthokin and placebo treatment. However, absolute values of the WOMAC and most other outcome measures showed that Orthokin treated patients scored consistently better, compared to placebo treated patients. In addition, Orthokin treatment was found to result in statistically significant more improvement on the KOOS symptomatology scores and the KOOS sport parameters. Altogether, these findings suggest a beneficial biological effect of Orthokin on clinical symptoms arising from knee OA.

This hypothesis is further supported by the dataset of patients who continued NSAID use during the trial (NSAID group), in which Orthokin treatment resulted in even more apparent improvement of the KOOS sport parameter and, in addition, induced an improved knee function as measured by the surgeon on the Knee Society Clinical Rating scale. Furthermore, when comparing the absolute values of the NSAID and the non-NSAID group, a striking trend was observed, namely that patients who were treated with Orthokin in combination with NSAIDs showed improvement similar to or more than both Orthokin and placebo treated patients of the non-NSAID group. In contrast, for the placebo treated patients of the NSAID group, improvements were much smaller, or not observed at all. This may be explained by the fact that all patients were required to stop all analgesics, including NSAIDs, one week before completing the questionnaires and visiting their treating surgeon. Patients using NSAIDs experienced more pain during this week than those not requiring NSAIDs, an effect that was not seen in the NSAID group treated with Orthokin. The absence of this trend for Orthokin treated patients is at least interesting, as this finding supports the hypothesis that Orthokin induces a beneficial biological effect.

Despite these findings, incorporation of Orthokin to the standard spectrum of treatment modalities for symptomatic knee OA needs careful consideration.

The clinical benefit found in the current study was small and did not meet the initial trial objective of 30% superiority on the WOMAC. This may be due to several causes.

Based on the WOMAC outcome scores, the placebo effect found in the current study was high, but comparable to other studies studying the efficacy of various oral and intra-articular treatment modalities. The latter observation supports the validity of the current dataset[292,297-299]. However, demonstrating a beneficial effect of new treatment modalities is hampered by such large placebo effects. Furthermore, it was recently demonstrated that the patients' perception of his/her health status and their symptoms of OA changes over time[300].

This phenomenon can have a significant impact on evaluating the effectiveness of interventions, which is known as response shift. This aspect is not taken into account in the current study, which has been demonstrated to increase the risk of a type-2 error[300,301]. On the other hand, it may be argued that the treatment dose was to low, i.e. the small Orthokin volume. However, the treatment was performed strictly according to the manufacturer's instructions. Furthermore, assuming that IL-1ra is the main effective mediator of the Orthokin treatment, 2 ml Orthokin provides sufficient IL-1ra intra-articularly in order to provide complete inhibition of IL-1β[72,271,302,303]. Finally, as OA is a slowly progressive degenerative disease with gradually evolving symtomatology, most OA patients are used to a certain degree of pain. As a result, these patients may increase their activity rate until an acceptable degree of complaints is reached, thereby demonstrating improvement on activity scales (KOOS sport), but suppressing improvements on all other scales.

Altogether, autologous induced synthesis of anti-inflammatory cytokines seems an interesting and possibly effective approach in the treatment of symptomatic knee OA. However, Orthokin is based on human serum and therefore, the quality of the product may be variable among different patients. Also, apart from the upregulation of IL-1ra, IL-4, IL-10, and IL-13, the modulation of numerous other proteins by the incubation of blood with $CrSO_4$ coated glass beads has not been clarified. Furthermore, the clinical relevance of the demonstrated differences is disputable. Other, cheaper, less invasive, and effective treatments, such as NSAIDs and COX-2 inhibitors, are available for reducing symptoms from knee OA[288,289,304]. In addition, results from recent *in vitro* studies suggest that selective COX-2 inhibitors, such as Celecoxib, may be an effective DMOAD[305,306]. Nevertheless, specifically the chondroprotective effect of Orthokin on articular cartilage was not studied in the current trial, while potentially being the most important aspect of this treatment. However, the follow-up period of the current trial was to short to reasonably expect a detectable protective effect on knee radiographs. Therefore, we are in the process of to determining the chondroprotective effect of Orthokin by analysis of knee radiographs after an extended follow-up period, and, in addition, by determining cartilage breakdown products, and pro- and anti-inflammatory cytokines in synovial fluid samples from this patient cohort.

Furthermore, cartilage defects have been demonstrated to result in a disturbed joint homeostasis, which negatively affects the outcome of cartilage tissue engineering techniques[37,38]. As cartilage defects can lead to the development of osteo-

arthritis[99,100], the disturbed joint homeostasis induced by cartilage defects may very well occur through similar pathways as those responsible for OA development. Therefore, Orthokin may provide a feasible approach for further optimization of cartilage tissue engineering techniques, such as autologous chondrocyte implantation (ACI), although this hypothesis remains to be studied in future clinical trials.

In conclusion, Orthokin appears to have a clear beneficial biological effect on symptoms arising from knee OA, although the clinical effect when administered under trial conditions is limited.

KOOS Knie enquete

Datum: / /20 (Dag/Maand/Jaar)

Naam:

Geboortedatum: / /19 (Dag/Maand/Jaar)

Instructies: Deze enquête stelt vragen in verband met uw visie betreffende uw knie. Deze informatie helpt ons te achterhalen hoe u zich voelt en in hoeverre het mogelijk is voor u om uw dagelijkse activiteiten uit te voeren. Beantwoord de onderstaande vragen door **één** antwoord aan te vinken dat voor u van toepassing is. Als u niet geheel zeker bent van uw antwoord, graag toch het best mogelijke antwoord geven.

Belangrijk: Probeer uitsluitend antwoord met betrekking tot het kniegewricht waarvoor u in deze studie behandeld wordt.

Symptomen

Deze vragen dienen te worden beantwoord met betrekking tot de knie symptomen gedurende de afgelopen week.

S1. Is uw knie gezwollen?

O Nooit O Bijna nooit O Soms O Vaak O Altijd

S2. Voelt u knarsen in uw knie of hoort u uw knie klikken of een ander geluid al u uw knie beweegt?

O Nooit O Bijna nooit O Soms O Vaak O Altijd

S3. Blijft uw knie steken of schiet uw knie op slot bij bewegen?

O Nooit O Bijna nooit O Soms O Vaak O Alt

S4. Kan u uw knie volledig strekken?

O Altijd O Vaak O Soms O Bijna nooit O Noo

S5. Kan u uw knie volledig buigen?

O Vaak O Soms O Bijna nooit O N

O Altijd

Validation of the short form WOMAC function scale for evaluating osteoarthritic knee complaints

Chapter 09

Abstract

Goal: As short questionnaires are known to result in improved patient compliance, survey response rate, and even improved response quality, Whitehouse developed and validated a short form WOMAC function scale for evaluation of total joint replacement. The goal of this study was to validate the short form WOMAC function scale for non-operatively treated osteoarthritic knee complaints.

Methods: Prospective data (pre-treatment and 3 and 6 months post-treatment) from non-operatively treated OA patients were used to determine the validity, internal consistency, test-retest reliability, floor- and ceiling-effects, and responsiveness of the Whitehouse short form WOMAC function scale.

Results: The short form WOMAC function scale showed high correlation with the traditional WOMAC and other measures. The internal consistency was good and an excellent test-retest reliability was found. The responsiveness was adequate and comparable with the traditional WOMAC and appeared not significantly affected by floor- or ceiling effects.

Conclusion: This study demonstrates the short form WOMAC function scale to be a valid, reliable and responsive alternative for the traditional WOMAC in the evaluation of non-operatively treated OA patients. The short form WOMAC is simple to use in daily practice and therefore less a burden for patients in clinical trials.

Introduction

To be able to compare results between studies, centers, techniques and populations it is of essential importance that validated uniform outcome tools are used. Therefore, the following three chapters are devoted to our effort to the uniformity of research and clinical evaluation tools in cartilage regeneration and treatment of OA. We will discuss the validation of:

- Patient administered outcome tools for OA and biological joint reconstruction.
- Automated measures of MRI scoring of bone bruise.
- Histological scores for cartilage regeneration.

To evaluate the surgical and pharmacological treatment of OA and cartilage regeneration, different knee rating scales have been developed. Traditionally these scales are based on physical findings, such as range of motion (ROM), knee laxity, joint axis deformation, and radiological variables, which are usually evaluated according to the Kellgren and Lawrence radiological grading system[307,308]. During the last decade, outcome assessment has focused increasingly on analysis from a patients' perspective. This has been an important change and considerable improvement, as treatment of OA is primarily focused at symptomatic relief [309]. The critical evaluation of OA treatment is an increasingly important topic in orthopaedic research due to the increasing prevalence of OA, resulting from the ageing of the population, and the socio-economic consequences[310].
For the evaluation of OA treatment, a considerable number of questionnaires and rating scales are available, among which the Knee Society Clinical Rating Scale, the Knee injury and Osteoarthritis Outcome Score (KOOS) and the Western Ontario and McMaster's Universities Osteoarthritis Index (WOMAC) are most frequently used. The Knee Society Clinical Rating Scale is widely accepted as a reliable and valid measure of knee status in patients undergoing total knee arthroplasty[74]. The Knee Society questionnaire consists of items on functional ability and physical examination. The dual rating system appears to be an advantage as it reflects both the physicians' and patients' perspective. However, this system is more labor intensive, more sensitive to bias by the researchers than one that can be self-assessed by the patient, and it has been shown to be less responsive to changes than the WOMAC[75].
The KOOS is a 42-item self-administered self-explanatory questionnaire that is an extension of the WOMAC and covers 5 dimensions; pain, symptoms, activity in daily living (ADL), sport/recreation and knee-related quality of life (QOL). The KOOS was shown to be a reliable and valid instrument[76]. In younger patients it was found to be even more responsive than the WOMAC[77]. Because of its extensiveness, the KOOS provides detailed insight in different dimensions. However its length (42 items) might be a disadvantage, because it possibly results in reduced patient compliance.
The WOMAC is a self-assessed disease specific measure for patients with osteoarthritis of the knee and hip, comprising 24 items in three dimensions; pain,

function and stiffness[78]. The WOMAC underwent vigorous psychometric validation before the orthopaedic community accepted it as an appropriate measurement tool for determining orthopaedic disability and is currently considered a golden standard. Short questionnaires are known to result in improved patient compliance and survey response rate, and even have been suggested to improve response quality[79-81]. Therefore, Whitehouse developed a reduced WOMAC function scale (short form WOMAC function scale) that consists of 7 function items, which were selected by a clinically driven process conducting a poll among orthopaedic and rheumatology personnel. Furthermore, the limited number of items increases the ease of analysis and the applicability for routine clinical practice. They have shown it to be a practical, valid, reliable and responsive alternative for the full WOMAC function scale for the evaluation of total joint replacement[82]. However, the authors concluded that in order to extend the applicability of this scoring system, it is required to validate this short form WOMAC function scale for a broader range of OA patients, e.g. patients who are treated non-operatively as well as those undergoing THR and revision total joint arthroplasty.

The goal of the current study was to evaluate the short form WOMAC function scale for patients with non-operatively treated osteoarthritic knee complaints. In addition, as the short form WOMAC function scale lacks pain and stiffness items, which were originally incorporated in the traditional WOMAC, we tested whether incorporation of these items with the short form WOMAC function scale (modified short form WOMAC) would result in improved validity, reliability and responsiveness (Table 1).

Patients & Methods

The validity of the short form WOMAC function scale was studied among a controlled outpatient population described in chapter 8, consisting of one hundred OA patients with a varying degree of complaints from knee OA.
All patients completed the traditional WOMAC once and the short form WOMAC function scale twice. The scores of the modified short form WOMAC were deduced from the traditional WOMAC. This dataset was used to calculate the internal consistency, the test-retest reliability of these scoring systems, and the agreement of the short form WOMAC function scale with the traditional WOMAC and the modified short form WOMAC. The first of the duplicate scores of the short form WOMAC function scale was used for the latter assessment. In addition, the data were used to study the responsiveness and the floor- and ceiling effects of these scoring systems. In order to determine the validity of the short form WOMAC function scale and the modified short form WOMAC, we determined the reliability (internal consistency and test-retest reliability), the validity (convergent construct validity and floor/ceiling effect), and responsiveness (its sensitivity to change over time). Statistical calculations were done with the JMP (Cary, NC, USA) version 5.0 software package.

For further details of the methods used, please refer to appendix D and E.

Table 1
A summary of the items that are scored in the three different forms of the WOMAC scale tested in this paper.

	Knee Injury and Osteoarthritis Outcome Score (KOOS)	Traditional WOMAC	Short form WOMAC function scale (Whitehouse)	Modified short form WOMAC
Stiffness	1. Stiffness in the morning	•		•
	2. Stiffness later in the day	•		•
Pain	1. How often pain			
	2. Twisting/pivoting			
	3. Straightening fully			
	4. Bending fully			
	5. Walking on flat surface	•		•
	6. Stair climbing	•		•
	7. At night while in bed	•		•
	8. Sitting or lying	•		•
	9. Standing upright	•		•
Daily activity	1. Descending stairs	•		
	2. Ascending stairs	•	•	•
	3. Rising from sitting	•	•	•
	4. Standing	•		
	5. Bending on floor	•		
	6. Walking on flat	•	•	•
	7. Getting in/out car	•	•	•
	8. Going shopping	•		
	9. Putting on socks	•	•	•
	10. Rising from bed	•	•	•
	11. Taking off socks	•		
	12. Lying in bed	•		
	13. Getting in/out bath	•		
	14. Sitting	•	•	•
	15. Getting on/off toilet	•		
	16. Heavy domestic duties	•		
	17. Light domestic duties	•		

Results

The OA patients consisted of a population with a mean age of 54.9 years (range 34-85 years), 37% was female and 63% was male. Their Body Mass Index (BMI) varied from 21.7 to 40, with a mean of 27.5 (SD 3.9). The 100 patients were included between February 2004 and November 2004. All patients completed the study and no patients were lost to follow up.

Reliability

The Cronbach's alpha, representing the internal consistency of the short form WOMAC function scale was 0.95. Similarly, the Cronbach's alpha values found for the traditional WOMAC and the modified short form WOMAC were 0.98 and 0.97 respectively. Sub-analysis for the baseline, the 3-month and 6-month post-treatment dataset showed similar values (table 2)

Test-retest reliability questionnaires were documented with a mean time interval of 9.1h, (min 4h, max 48h). Lin's concordance coefficient (ρ_c) of each question are shown in table 3 and varied from 0.85 to 0.91. Lin's concordance coefficient (ρ_c) of the total score of the short form WOMAC was even higher, 0.94 (Figure 1A).
The Bland-Altman plot demonstrates that the mean differences between the value of the short form WOMAC function scale and the traditional WOMAC was 0.64 (SD 7.9) (Figure 1B). No funnel effect or skewing of the data was observed, and therefore the accuracy appeared not affected by repeated completion of the questionnaire. In addition, this plot demonstrates that the upper and lower limits of agreement were 16.4 and -15.1. The plot does show striking test-retest differences up to 35 points. Comparing the outcome of both measurements by performing a paired *t*-test did not show statistical significance ($t=0.817$, $p = 0.416$), i.e. no systematic effect of repeated completion of the questionnaire was observed.

Validity

Lin's concordance correlation coefficient (ρ_c) of the short form WOMAC function scale with the traditional WOMAC was 0.96 (Figure 2A). Lin's concordance correlation coefficient (ρ_c) of the modified short form WOMAC with the traditional WOMAC was 0.98 (Figure 2B).
The Bland-Altman plot demonstrates that the mean differences between the value of the short form WOMAC function scale and the traditional WOMAC was 0.23 (SD 5.8) (Figure 3A). No funnel effect or skewing of the data was observed, and therefore the accuracy appeared not dependant on the degree of patients' symptoms. In addition, this plot demonstrates that the upper and lower limits of agreement were 11.8 and -11.4, i.e. the maximum likely difference between a pair of observations in the two scales is 11.8 on a scale that ranges from 0 - 100. The addition of pain and stiffness items to the short form WOMAC function scale (modified short form WOMAC) resulted in similar values with a mean difference of 0.43 (SD 2.4). The lower and upper limits of agreement are -4.37 and 5.23 (Figure

Table 2
Traditional WOMAC, short form WOMAC function scale and modified short form WOMAC means (0 worst, 100 best), SD and Cronbach's alpha values for non-operatively treated OA patients.

	OA patients		
Mean (SD)	Pre-intervention	At 3 months	At 6 months
Traditional WOMAC	53.6 (15.2)	63.9 (20.4)	63.8 (22.0)
Short form WOMAC function scale	55.3 (16.5)	65.9 (21.1)	64.7 (22.6)
Modified short form WOMAC	53.7 (15.0)	64.7 (20.2)	64.1 (21.8)
Cronbach's alpha			
Traditional WOMAC	.95	.98	.98
Short form WOMAC function scale	.88	.93	.95
Modified short form WOMAC	.92	.96	.97

Table 3
Test-retest reliability: Lin's concordance correlation coefficients (ρ_c), means and mean of the difference with 95% confidence interval (CI) of the short form WOMAC function scale.

Items	Test Mean (SD)	Re-test Mean (SD)	Differences Mean (95% CI)	Concordance correlation (ρ_c)
Ascending stairs (2)	1.7 (1.1)	1.7 (1.1)	-0.04 (-0.13-0.05)	0.91
Rising from sitting (3)	1.6 (1.1)	1.6 (1.1)	-0.02 (-0.14-0.10)	0.85
Walking on flat (6)	1.2 (1.0)	1.3 (1.0)	-0.09 (-0.18-0.00)	0.90
Getting in/out car (7)	1.5 (1.0)	1.5 (1.1)	-0.02 (-0.11-0.07)	0.90
Putting on socks (9)	1.2 (0.9)	1.1 (1.0)	0.01 (-0.08-0.10)	0.90
Rising from bed (10)	1.4 (1.1)	1.3 (1.1)	0.08 (-0.04-0.20)	0.85
Sitting (14)	1.1 (1.0)	1.2 (1.0)	-0.10 (-0.02-0.00)	0.86
Total score SF WOMAC FS	65.5 (23.0)	65.0 (23.7)	0.64 (-0.93-2.22)	0.94

3B). Comparing the outcome of both scoring systems with the traditional WOMAC by performing a paired t-test did not show statistical significance (t=0.405, p 0.686 and t= -1.771, p 0.080), for the short form WOMAC function scale and the modified short form WOMAC respectively), i.e. no systematic effect was observed. At baseline, and 3 and 6 month post-treatment, comparisons of the traditional WOMAC and the short form WOMAC function scales gave similar mean values and standard deviations (Table 2).
Furthermore, highly variable concordance correlation coefficients were found when

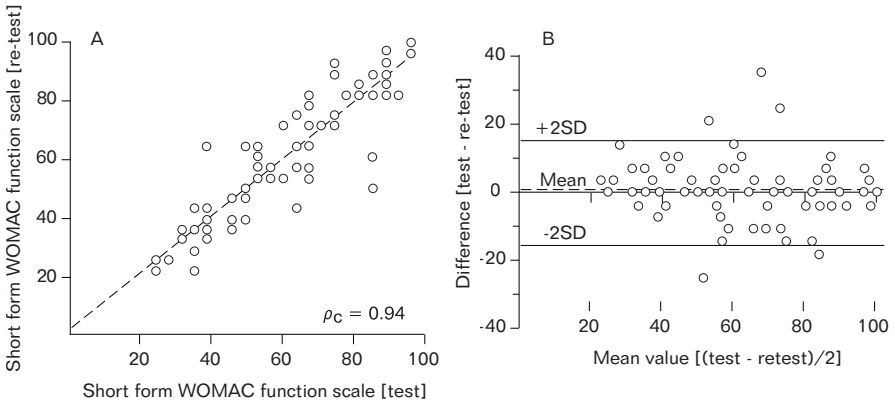


Figure 1
(A) Lin's concordance correlation graph of the test-retest data for the short form WOMAC Function scale. (B) Bland Altman plot showing the difference of the test-retest data of the short form WOMAC function scale plotted against the mean value of these two measurements.

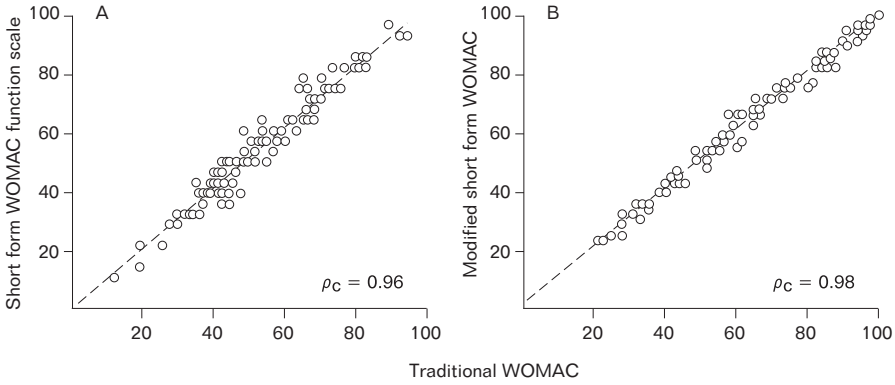


Figure 2
Lin's concordance correlation between (A) the short form WOMAC function scale and the traditional WOMAC and (B) the modified short form WOMAC and the traditional WOMAC.

the traditional WOMAC, the short form WOMAC function scale, and the modified short form WOMAC were related to the separate KOOS items, the VAS for pain and the Knee Society Score (Table 4).
The floor and ceiling effect for all tools ranged from 0% to 7% at baseline as well as at 3 and 6 months post-intervention.

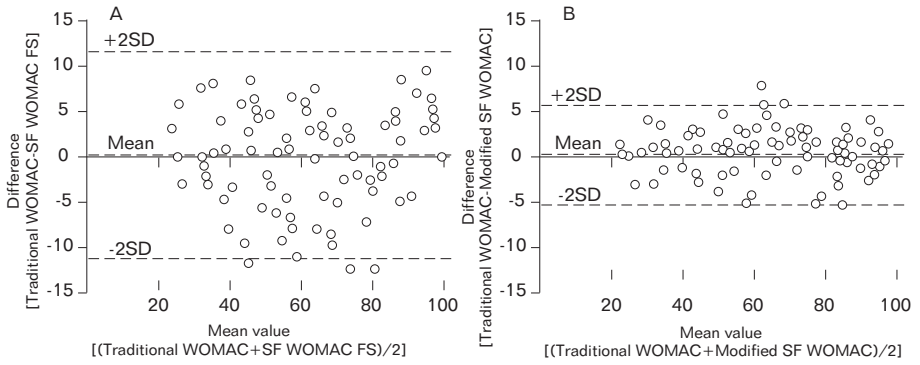


Figure 3
Bland Altman plot: (A) The difference of the traditional WOMAC and the short form WOMAC function scale (SF WOMAC FS) plotted against the mean value of these two scales. (B) The difference of the traditional WOMAC and the modified short form WOMAC (modified SF WOMAC) plotted against the mean value of these two scales. The dotted line marked by mean difference represents the mean difference between both scoring systems.

Responsiveness

Responsiveness is a measure of sensitivity for changes over time. When comparing the baseline, three month post-intervention data, the standardized response means assessed by the short form WOMAC function scale and the traditional WOMAC are 0.56 and 0.58 respectively. The effect sizes were 0.64 and 0.67. The modified short form WOMAC resulted in a standardized response mean (SRM) of 0.62, and effect size (ES) of 0.74 (Table 5). All indices in Table 5 are 'medium' apart from the SRM for the short form WOMAC at six months, which has a value of 0.44, just below 0.5.

Discussion

The current study clearly demonstrates the validity of the short form WOMAC function scale for evaluation of non-operatively treated OA, as shown by a good internal consistency, the high concordance correlation coefficients with the traditional WOMAC, excellent test-retest reliability, and adequate responsiveness. This finding supports our hypothesis that the short form WOMAC function scale captures the functional status as well as the traditional WOMAC for evaluation of non-operatively treated OA. Our data are in line with results published by White-house for total hip and total knee arthroplasty, although his population displayed more severe symptomatology[82]. The mean difference in scores measured by the traditional and the short form WOMAC function scale is small (0.23 on a scale that ranges from 0 -100), appears independent on the degree of symptoms of the patient, and is probably not relevant in larger populations, as in clinical trials.

Table 4
Lin's concordance correlation coefficients (ρ_c), between traditional and short form WOMAC scales and various measures of symptomatology of OA patients, pre-treatment.

	Short form WOMAC function scale	Traditional WOMAC	Modified short form WOMAC
KOOS pain	0.66	0.75	0.79
KOOS stiffness	0.58	0.60	0.61
KOOS ADL	0.97	0.98	0.94
KOOS sport	0.17	0.18	0.15
KOOS quality of life	0.18	0.20	0.20
VAS for pain	0.33	0.37	0.39
Knee Society Score, function score	0.41	0.40	0.37

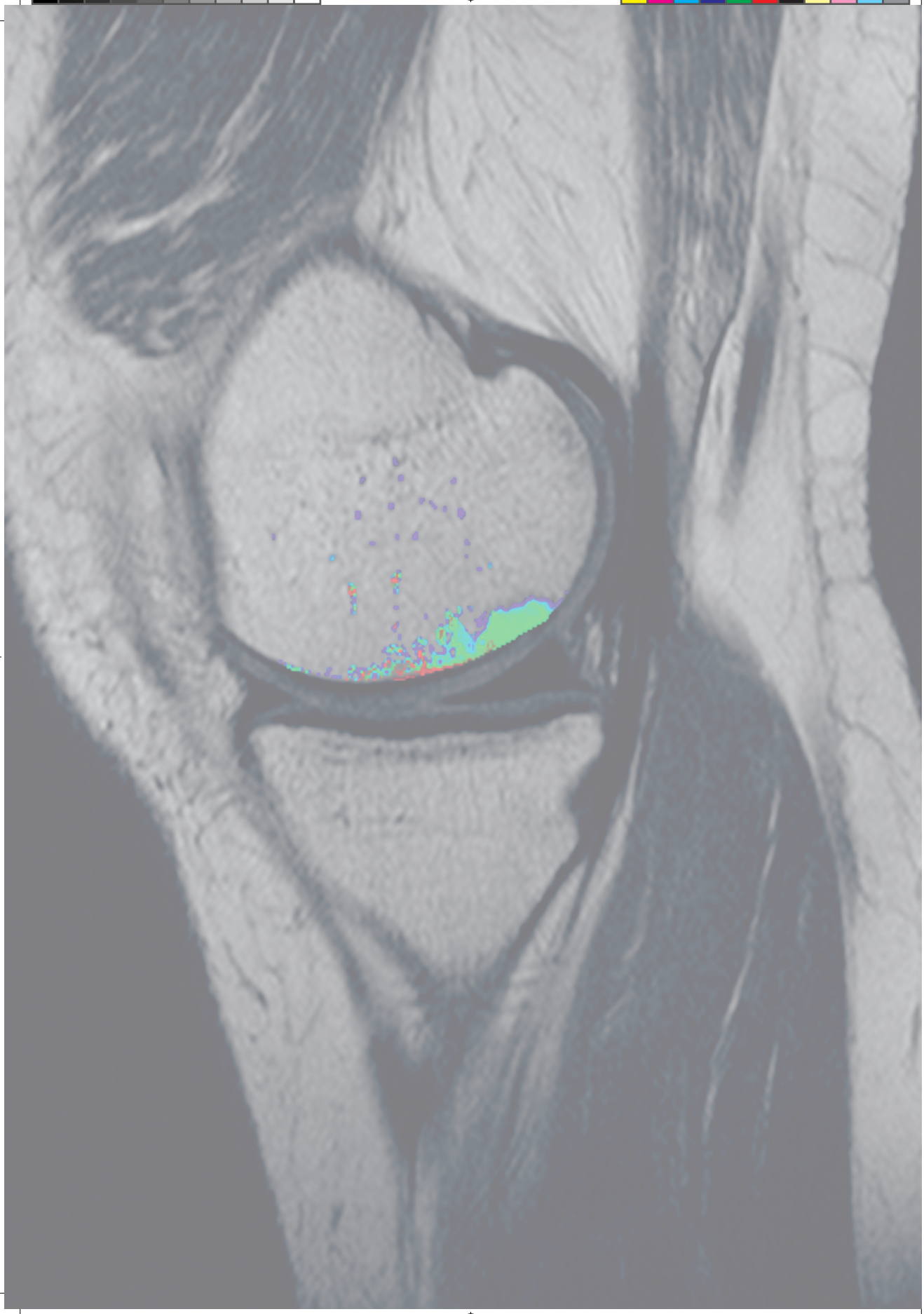
Table 5
Standard response means (SRM) and effect sizes (ES) of the traditional WOMAC, SF WOMAC function scale, modified short form WOMAC at different time points compared to baseline (n=100).

	Traditional WOMAC		Short form WOMAC FS		Modified short form WOMAC	
	SRM	ES	SRM	ES	SRM	ES
OA patients						
3 months	0.58	0.67	0.56	0.64	0.62	0.74
6 months	0.51	0.66	0.44	0.57	0.52	0.69

Furthermore, excellent test-retest reliability was found for the short form WOMAC function scale, for both the complete questionnaire and its individual items. However, caution is needed when evaluating individual patients and smaller groups, as inaccuracies up to 11.8 (mean difference ± [2 x SD of the difference]) compared to the traditional WOMAC, and 16.4 up on repeated completion of the questionnaire can occur, which likely is clinically relevant. These findings are supported by previously published data and seem not to be affected by the fact that the current scale was reduced[311-318]. Although the responsiveness 3 months post-treatment was adequate, 6 months post-treatment it appears to decrease considerably. However, the traditional WOMAC and the modified short form WOMAC showed similar trends and values and therefore can likely be attributed to the efficacy of the treatment rather than shortcomings of the questionnaire. As the responsiveness of the traditional WOMAC has previously been confirmed for a similar population, the observed responsiveness in the current study was not considered a limitation for the short form WOMAC function scale[78,319].

Despite the proven reliability, validity, and apparently adequate responsiveness, investigators should consider the limited extrapolability of both the traditional and the short form WOMAC function scale as demonstrated by the low concordance correlation coefficients of these questionnaires with the separate KOOS items, the VAS for pain and the Knee Society Score, except for the KOOS ADL items.
The addition of pain and stiffness parameters from the traditional WOMAC to the short form WOMAC function scale (modified short form WOMAC) did not result in improved reliability and validity. The addition of pain and stiffness parameters to the short form WOMAC function scale did result in a slight improvement of the concordance correlation coefficient with the KOOS pain and, at 6 months post-treatment, of the responsiveness. However, the modified short form WOMAC has a diminished benefit of a reduced scale compared to the short form WOMAC function scale (14 versus 7 items). In addition, the Cronbach's alpha values suggest that incorporation of these items may actually be redundant. This was previously demonstrated by Ryser, who showed redundancy of these parameters in the traditional WOMAC[320]. Therefore, despite the slightly improved responsiveness and extrapolability, we advised against the use of the modified short form WOMAC and recommend the use of the short form WOMAC function scale, as developed by Whitehouse.
Recently, Tubach *et al* published a different reduced WOMAC scale. Compared to the short form WOMAC function scale, they suggested that their scale would be more applicable for the evaluation of OA patients with less severe symptomatology than the population described by Whitehouse[321]. However, in the current study the short form WOMAC function scale by Whitehouse demonstrates similar validity and reliability as the reduced WOMAC by Tubach, while the baseline symptomatology of the OA population studied in this paper was even less severe than the population studied by Tubach. Uniformity of scoring systems used in orthopaedic research should be pursued. As the short form WOMAC function scale is validated for a greater variety of joint disorders and treatment modalities than the reduced scale described by Tubach, we propose that the use of the short form WOMAC function scale is preferable.

In conclusion, this study shows that the short from WOMAC function scale as developed by Whitehouse is not only a reliable and valid questionnaire for evaluation of total knee and total hip replacement patients, but can also be used in evaluation of non-operatively treated OA patients.



Automated measurement of bone bruise volumes and how this relates to cartilage defect symptoms

Chapter 10

Abstract

Goal: To evaluate automated segmentation software for determining bone bruise on MRI and to improve our understanding of its relationship to clinical symptomatology.

Methods: We obtained pre- and (6 and 12 months) post-operative MRIs of knee joints with solitary cartilage defects. These were graded by 3 observers to evaluate the inter-observer reliability of a subjective MRI assessment scale. The validity of the automated software system was studied by determining its correlation with the subjective MRI assessment scale. We studied how clinical symptoms (Visual Analogue Scale for pain: VAS) related to bone bruise volume.

Results: The subjective MRI assessment scale showed excellent inter-observer reliability (ICC = 0.84). Automated segmentation accurately demarcates bone bruises on PDw scans and has a high correlation with the subjective MRI assessment scale ($r = 0.75$). Bone bruise volume was weakly correlated with the VAS score for the complete ($r = 0.28$) and pre-operative dataset ($r = 0.43$). When post-operative changes in bone bruise volume were related to changes in symptomatology, we observed discrepancies ranging from 0-44% of the cases. Twelve months post-surgically, these discrepancies were only observed in microfracturing-treated patients and were hypothesized to be induced by the subchondral bone damage induced by the treatment.

Conclusion: Bone bruise might be related to clinical symptoms in patients with cartilage defects, although additional longitudinal studies are required to provide definite conclusions. Subjective MRI assessment seems reliable and reproducible, but is limited in the amount of information gained. The automatic segmentation technique is an easy, objective, accurate and useful tool for future investigations that aim to study bone bruise on PDw MRIs.

Introduction

Magnetic Resonance Imaging (MRI) is a valuable diagnostic tool for the detection and evaluation of intra-articular pathology, such as articular cartilage defects[113,322-324]. However, uniform evaluation of aspects such as bone bruise and effusion remains difficult. Additionally, little is known of how such phenomena relate to the patients' clinical symptoms. Patients with chondral defects usually present with varying symptoms, such as pain, catching, giving way and swelling. Pain however, is not only reported during physical activity, but frequently also during rest and at night. As articular cartilage is aneural, other pathways for the explanation of this physical discomfort should also be considered. One theory relates such symptoms to a process in the subchondral bone frequently seen on MRIs, named bone bruise or bone marrow edema. Bone bruises are found in 72 – 83% of treatable cartilage lesions[83] and are characterized by diffuse diminished signal intensity on proton density weighted (PDw) and T1-weighted (T1-w) MRIs, and, in the acute phase, increased signal intensity on T2-weighted (T2-w) MRIs. In tissue with bone bruise on MRI, a variety of histological and biochemical abnormalities have been described ranging from hemorrhages and edema, degeneration of chondrocytes, necrosis of chondrocytes in the subchondral bone, microfractures and repair processes of the cancellous bone, and loss of proteoglycans[325-328].

To the best of our knowledge, no studies are available that investigated the relationship between bone bruise and clinical symptomatology in cartilage defect patients. For patients with other intra-articular pathologies, like OA patients, previous studies have reported contradicting results[84-86]. One problem in such evaluation is the lack of a reliable validated quantification of bone bruise volume. The subjective assessment tool previously described by Felson *et al* is rather basic, grading bone bruise severity based on its volume by counting the numbers of slices on which bone bruise is found, and ranging from grade 0 (no bone bruise) to grade 3 (severe bone bruise)[84]. Other groups have proposed grading scales that were designed to evaluate bone bruise due to other pathologies than articular cartilage defects, like acute ligamentous injuries and occult posttraumatic osteochondral lesions of the knee[329,330]. However, these systems not only grade the severity of the bone bruise, but additionally take other abnormalities, such as cortical line interruption and subchondral bone impaction, into account. The crudeness of these classification systems results in a rather gross estimation of bone bruise severity and limits the sensitivity to change over time. Furthermore, as Felson stated previously, longitudinal studies are required to study the relationship between bone bruise and symptomatology, as such study designs not only allow for evaluation of the direct correlation among these two parameters, but also enable us to determine how changes over time in both parameters relate to each other[84].

Recently, a new method, based on the K-Nearest Neighbor (KNN) classification technique, was developed for fully automated segmentation of white matter lesions (WML) on cranial MRIs[87]. This method could provide a useful tool for accurate determination of bone bruise volumes.

The goals of the current investigation were:

- To develop an automated system for determination of subchondral bone bruise and bone bruise volume calculation.
- To evaluate the reliability of the subjective MRI assessment system described by Felson[84]. Subsequently, the automated system for bone bruise segmentation was validated by determining how it relates to subjective bone bruise assessment.
- To study whether clinical symptoms arising from articular cartilage defects are, at least partly, due to bone bruise.

Materials and methods

Data acquisition

77 MRIs from patients with solitary chondral defects of the femoral condyles were made upon presentation (26), and six (32) and twelve months post-operatively (19). 26 MRIs were obtained in a longitudinal fashion, i.e. baseline and 6 months post-operatively, or baseline and 6 and 12 months post-operatively. After the pre-operative MRI was acquired, the cartilage defects were treated either by autologous chondrocyte implantation (ACI) or by microfracturing. The population consisted of 37 patients with an average age of 31 (19-48), 8 patients were female and 29 were male, 19 patients were treated with ACI and 18 with microfracturing. At the time-points of MRI acquisition, the patients completed the Visual Analogue Scale for pain (VAS) and the Knee Osteoarthritis Outcome Scale (KOOS) from which the traditional WOMAC pain parameters were deduced. These questionnaires were used to study how bone bruise volume relates to clinical symptomatology. In addition, the longitudinal dataset was used to study how post-operative increase or decrease of bone bruise volume relate to post-operative changes in clinical symptoms.

Automatic MRI segmentation

The proposed method for bone bruise segmentation determines the probability of each voxel being bone bruise. The method requires a learning set of MR images for training purpose. In this study, both the PDw and T2-w MRI scans of 8 patients were manually segmented as learning set for the automated segmentation software.

For the final evaluation of the complete dataset, only the PDw MRI scans were invoked for both the manual (learning) and automatic segmentation, as bone bruises appeared most evidently present and most sharply demarcated on this scan type, while including T2-w MRI scans in the learning set decreased the accuracy of the automatic segmentation (Figure 1).

The method used for automatic segmentation of the MRIs takes a probabilistic approach and calculates the likelihood of a voxel being part of a particular tissue type, which in the current study consists of bone bruise[87]. From this segmentation, a probabilistic bone bruise volume and an absolute volume can be calculated. Volumes are given in cm^3.

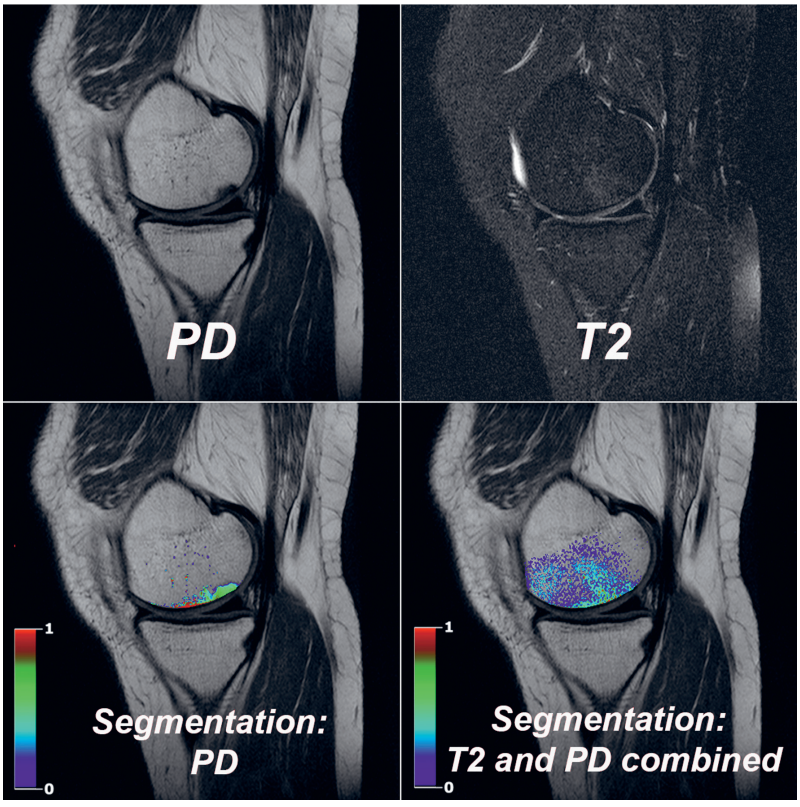


Figure 1
Example of an automatically segmented bone bruise using either the PD-weighted MRI scan alone (Segmentation: PD) or the PD-weighted and T2-weighted MRI scans combined (Segmentation: T2 and PD combined). These results show that when the PD-weighted and T2-weighted MRI scans are combined a more a-specific automatic segmentation of the bone bruise area is acquired. Therefore, T2-weighted MRI scans were excluded from the remaining study.

Clinical rating of MRIs

All MRIs were graded individually by 3 observers with different levels of experience in reading skeletal MRIs, 1 orthopaedic surgeon, 1 orthopaedic resident, and 1 orthopaedic PhD student in his pre-resident phase. The observers were asked to examine and grade the MRIs according to the subjective assessment scale previously published by Felson *et al*[84]. The grading scale ranged from grade 0 (no bone bruise) to grade 3 (severe bone bruise). Grade 1 showed bone bruise on 1 or 2 MRI slices; grade 2 showed bone bruise on 3 slices; and grade 3 showed bone bruise on 4 slices or more. Before examining the experimental cartilage MRIs, the observers were allowed to become familiar with the grading system on sample MRIs that

were not included in the study. The observers were blinded to the patient background and clinical data of the MRIs. All scans were presented randomly. The average score of the three observers was used to determine the construct validity of the automated bone bruise segmentation macro, be calculating Spearman's correlation coefficient with the automatically calculated bone bruise volumes.

Statistical analysis

In order to determine the validity of the method for automated segmentation of bone bruise volumes on MRI, the Spearman's correlation coefficients between the probabilistic bone bruise volume, the absolute (binary) bone bruise volumes, and the clinical rating of the bone bruise was determined. The inter-observer reliability of the subjective MRI assessment scale was assessed by calculating intra-class correlation coefficient (ICC). To visualize the inter-observer reliability, a Bland-Altman plot was used, which plots the difference between observer scores for a given MRI against the average for that specific MRI. Therefore, the Bland-Altman plot gives insight as to whether the accuracy of the subjective MRI assessment scale score is dependent up on the degree of symptomatology[331,332]. Spearman's correlation coefficients between the VAS, the WOMAC pain scale, and the absolute bone bruise volume were calculated in order to determine whether bone bruise might have a causal relationship with the patients' clinical symptoms. Correlations are interpreted as follows: 0.00–0.25 as little to none, 0.25–0.50 as weak, 0.50–0.75 as moderate to good, and 0.75–1.00 good to excellent[333]. Average values were compared by oneway ANOVA and Least Significant Difference (LSD) post-hoc tests as the dataset consisted of three levels (time points)[334]. P-values less than 0.05 were considered statistically significant. Statistical calculations were done with the JMP (Cary, NC, USA) version 5.0 software package.

For further details of the methods used, please refer to appendix F.

Results

Clinical rating of MRIs

In order to study if there was agreement among observers using the subjective MRI assessment system, the intra-class correlation coefficient was determined and was found to be excellent (ICC: 0.84; $p < 0.001$). The Bland-Altman plot demonstrates that the average differences between the observers was 0 points (SD 0.48) (Figure 2). The graph demonstrates excellent agreement at the top and bottom of the scale, i.e. no bone bruise and most severe bone bruise. Between these extremes, the grading system appeared more sensitive for disagreement among observers, with upper- and lower limits of agreement of 0.96 and -0.96 respectively.

Automated MRI segmentation

Automated segmentation of bone bruise volume was possible using the software

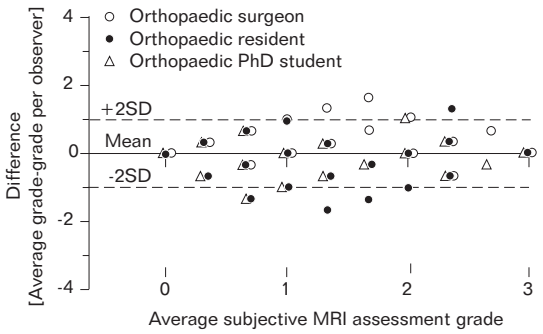


Figure 2
This graph visualizes the inter-observer reliability for the clinical grading system between three observers, which was found to be excellent (ICC = 0.84). The difference between observer scores for a given MRI is plotted against the average for that specific MRI. Each observer is represented by a different symbol. The average difference was 0 points within a 3 point grading system, with a standard deviation of 0.48. Note the excellent agreement at the top and bottom of the scale, i.e. no bone bruise and severe bone bruise. In between, the grading system appeared more sensitive for disagreement, although disagreement among observers, with upper- and lower limits of agreement of 0.96 and -0.96 respectively, stayed comfortably within the required confidence zone.

system developed for this purpose. The automatic segmentation appeared to result in a visual overestimation of the bone bruise volume (Figure 3A and B). However, as shown in figure 1, the color gradient demonstrates that the system considered voxels at the bone bruise borders, which were clinically interpreted as overestimated, as unlikely of being part of the bone bruise. Most a-specific voxels were excluded after setting the threshold at 50%. The area demarcated as being part of the bone bruise resulted in an accurate representation of our clinical interpretation of bone bruise volumes (Figure 3C). Furthermore, the volumes calculated from the probabilistic and the absolute segmentation showed excellent agreement, with no obvious under- or overestimation of the probabilistic segmentation ($r = 0.99$; $p < 0.0001$; $y = 1.004X - 0.04$; Figure 4A). Therefore, in the remaining results section, only the absolute bone bruise volumes are described.

The validity of the automatically calculated bone bruise volume was confirmed by a strong positive correlation between the absolute bone bruise volume and the average subjective MRI assessment grade ($r = 0.75$; $p < 0.0001$) (Figure 4B).Bone bruise volume related to clinical symptomatology

Based on automatically segmented MRIs, a great variety in bone bruise volume changes were observed, with a maximum increase of 99% and maximum decrease of 78% between 0 and 6 months, a maximum increase of 138% and maximum decrease of 45% between 0 and 12 months, and, between 6 and 12 months, a maximum increase of 255% and maximum decrease of 50%. However, no significant differences in average subjective bone bruise grading nor average absolute bone

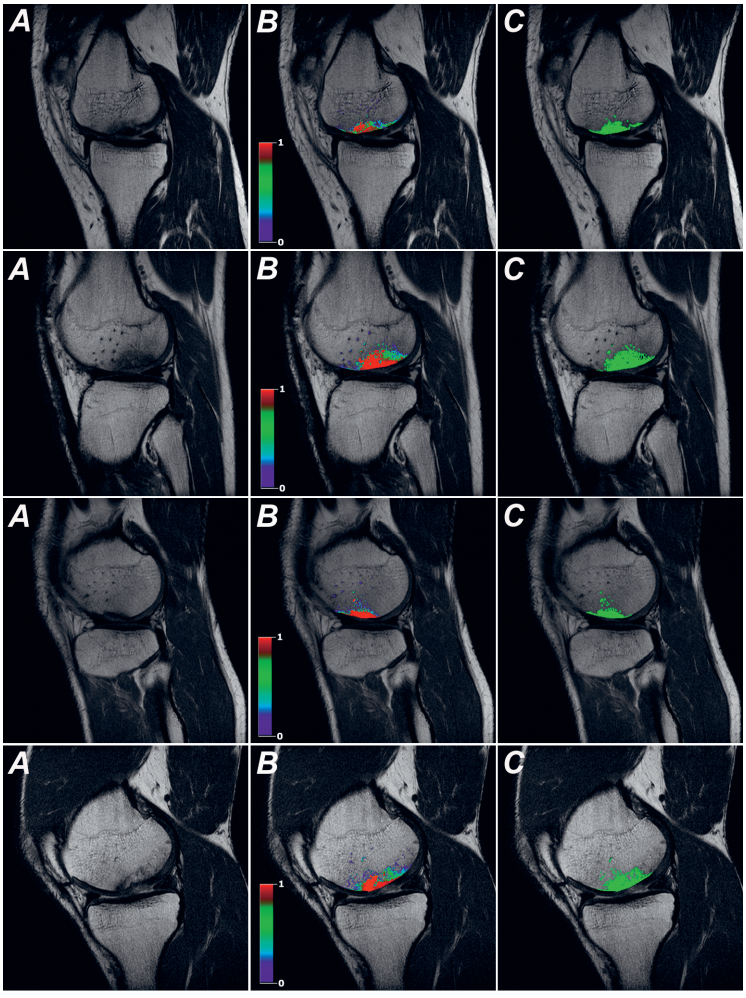


Figure 3
Four examples of automatically segmented bone bruises on PD MRI scans from patients with solitary articular cartilage defects. (Column A) Original PD MRI scan. (Column B) Example of full overlays of the automatic segmentation from which probabilistic volumes were calculated. The automatic segmentation results in a slight overestimation of bone bruise volume. However, note the color gradient indicating the likelihood of a positive voxel being part of the bone bruise as computed by the technique, indicating that the segmentation software considers the a-specific voxels to be unlikely part of the bone bruise. (Column C) Example of overlays after setting a 50% threshold. Note how a-specific voxels are excluded from the overlay.

Table 1
Visual Analogue Scale for pain scores, bone bruise volumes, and bone bruise clinical grades. Values are averages ± standard deviation. P-values are differences compared to pre-operative data (LSD post-hoc). VAS for pain and WOMAC pain scale scores decreased significantly in the microfracturing treated population 6 and 12 months post-surgically while no significant changes were observed for the other parameters. Pre-op = Pre-operative.

		VAS for Pain		WOMAC pain scale		Bone bruise clinical grade		Probabilistic bone bruise volume [cm^3]		Absolute bone bruise volume [cm^3]	
		Mean ±SD	*P-value*	Mean ±SD	*P-value*	Mean ±SD	*P-value*	Mean ±SD	*P-value*	Mean ±SD	*P-value*
All	Pre-op	42.4±31.7		76.3±20.4		1.24±0.95		1.04±0.67		1.01±0.67	
	6 months	25.4±25.3	*0.025*	86.3±12.1	*0.026*	1.29±0.90	*0.847*	1.17±0.78	*0.516*	1.14±0.79	*0.522*
	12 months	25.1±24.0	*0.053*	84.4±16.4	*0.119*	1.11±0.99	*0.627*	1.10±0.78	*0.813*	1.04±0.79	*0.890*
ACI	6 months	28.3±26.0	*0.124*	84.3±9.8	*0.140*	1.02±0.90	*0.458*	0.97±0.68	*0.758*	0.95±0.73	*0.801*
	12 months	38.4±25.6	*0.731*	75.6±19.5	*0.912*	0.70±0.56	*0.132*	0.97±0.55	*0.809*	0.92±0.57	*0.761*
Micro #	6 months	22.8±25.1	*0.028*	87.9±13.8	*0.024*	1.53±0.84	*0.320*	1.35±0.84	*0.189*	1.31±0.82	*0.212*
	12 months	14.8±17.6	*0.011*	92.2±7.5	*0.013*	1.47±1.18	*0.515*	1.21±0.96	*0.554*	1.15±0.96	*0.618*

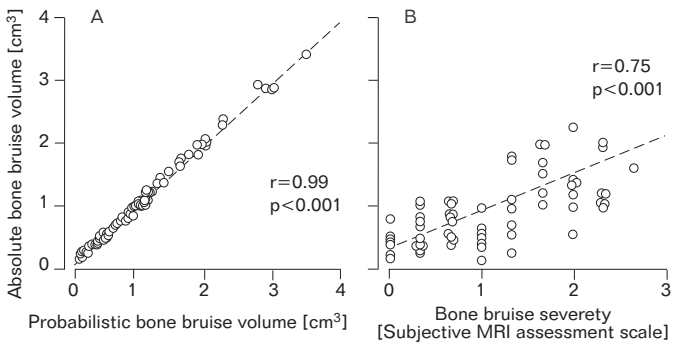


Figure 4
(A) The correlation graph between the probabilistic and absolute (threshold 50%) bone bruise volumes (complete dataset) demonstrates excellent agreement between these two approaches and confirms the accuracy of the probabilistic approach. (B) The correlation graph between absolute bone bruise volume and average clinical grade (complete dataset) shows high agreement between automatically segmented bone bruise volumes and clinical interpretations of the respective MRIs, confirming the accuracy of the automatic segmentation.

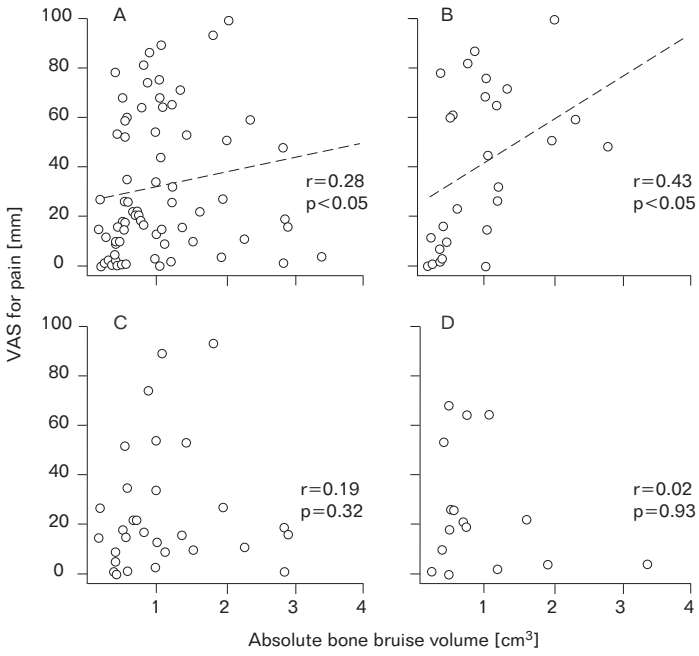


Figure 5
Correlation graphs between the VAS score of the patients and the absolute bone bruise volumes (threshold 50%). Weak, but significant correlations were found for (A) the complete dataset and (B) the pre-operative dataset. In contrast, no significant correlations were found for (C) the six-month follow-up dataset and (D) the twelve-month follow-up dataset, suggesting that bone bruise volume is, at least, a moderate indicator for clinical symptoms arising from articular cartilage defects.

bruise volumes were observed between time points (Table 1).Bone bruise volume appeared not related to cartilage defect surface area as no significant correlation was found between these parameters ($r = 0.23$; $p = 0.27$). Pre-operative, the patients scored 42.4±31.7 mm on de VAS for pain, which improved by 6 months (25.4±25.3; $p < 0.05$) and 12 months (25.1±24.0; $p = 0.053$). A similar improvement was seen for the WOMAC pain scale (Table 1).

Sub-analysis for treatment showed only significant improvement of VAS and WOMAC scores in microfracturing-treated patients, while no significant differences in clinical symptoms were observed for ACI-treated patients. In contrast, post-operatively no significant alterations in bone bruise subjective MRI assessment grade and bone bruise volumes were found compared to pre-operative values (Table 1).

Table 2
Spearman's' correlation coefficients between absolute bone bruise volumes and VAS scores or WOMAC pain scores. Post-operative correlations are also shown for the two surgical techniques separately. A weak, but significant positive correlation was found between bone bruise volumes and the complete and pre-operative dataset. Post-operatively, these parameters showed no significant correlations.

		Complete data set		Pre-operative data		6-month data-set		12-month data-set		6- and 12-month data	
		r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*	r	*p-value*
All	VAS	0.28	*0.0176*	0.43	*0.03*	0.19	*0.33*	0.02	*0.93*	0.11	*0.46*
	WOMAC	0.0594	*0.62*	0.13	*0.52*	0.14	*0.46*	0.15	*0.57*	0.03	*0.86*
ACI	VAS					0.36	*0.21*	0.41	*0.36*	0.37	*0.10*
	WOMAC					0.04	*0.89*	0.49	*0.21*	0.23	*0.30*
Micro #	VAS					0.13	*0.63*	0.06	*0.88*	0.09	*0.66*
	WOMAC					0.07	*0.78*	0.03	*0.93*	0.03	*0.88*

To investigate whether MRI determined bone bruise signal is related to pain experienced by patients with articular cartilage defects, the correlation between bone bruise volumes and VAS for pain was studied. For the complete dataset, only a weak positive correlation was found between the absolute bone bruise volume and the VAS score ($r = 0.28$; $p < 0.05$; Figure 5A). For the pre-operative data-set, the correlation between absolute bone bruise volume and VAS scores was slightly higher ($r = 0.43$; $p < 0.05$; Figure 5B), while 6- and 12-month post-operative VAS scores appeared unrelated to bone bruise volumes ($r = 0.19$; $p = 0.32$ and $r = 0.02$; $p = 0.93$ respectively; Figure 5 C-D). To investigate whether these poor correlation coefficients for the follow-up data were due to subchondral bone damage induced by microfracturing, the 6- and 12-month follow-up data were analyzed separately for ACI and microfracturing (table 2). However, this sub-group analysis did not change the statistical findings. No significant correlations were found between bone bruise volumes and WOMAC pain scores (table 2).

Besides studying the correlation between bone bruise volume and the degree of clinical symptoms, the correspondence in change over time between these two parameters was studied to elucidate whether a possible causal relationship exists. Discrepancies between changes in symptomatology and changes in bone bruise volumes, like increasing bone bruise volumes between successive time points in combination with decreasing VAS scores, and decreasing bone bruise volumes in combination with increasing VAS scores, were found for both the complete dataset as well as for the surgical treatments separately (table 3). For the ACI dataset, the prevalence of these discrepancies was respectively 22% and 11% at 6 months

Table 3
Discrepancies between bone bruise volume changes and VAS score changes over time. Note the fact that the percentage of discrepancies were higher for patients, whose bone bruise volume increased over time. In addition, when studying 12-month changes, these discrepancies were only observed for patients who were treated with microfracturing, which might be due to subchondral bone damage induced by this surgical technique.

		↑ Bone bruise volume combined with ↓ VAS scores		↓ Bone bruise volume combined with ↑ VAS scores	
		#	%	#	%
All	0 – 6 months (n = 22)	5	23%	2	9%
	0 – 12 months (n = 14)	4	29%	0	0%
ACI	0 – 6 months (n = 9)	2	22%	1	11%
	0 – 12 months (n = 5)	0	0%	0	0%
Micro #	0 – 6 months (n = 13)	3	23%	2	15%
	0 – 12 months (n = 9)	4	44%	0	0%

post-operatively. Twelve months post-operatively, no discrepancies were observed for the ACI dataset. For the microfracturing dataset, the prevalence was 23% and 15% respectively at 6 months post-operatively. Twelve months post-operatively the prevalence was 44% and 0% respectively. Strikingly, the percentage of discrepancies was consistently higher for patients with increasing bone bruise volumes combined with decreasing VAS scores compared to patients with decreasing bone bruise volumes combined with increasing VAS scores. Furthermore, discrepancies in changes of bone bruise volume and VAS scores 12 months post-surgically were only observed for patients who were treated with microfracturing.

Discussion

Using this novel automated bone bruise segmentation technique for MRI, we demonstrated that bone bruise on proton density weighted (PDw) MRI scans can be accurately defined in human femoral condyles with solitary cartilage defects.

The clinical rating scale used to determine the severity of bone bruise was found to be reliable among different observers with varying clinical expertise, as shown by the excellent ICC value[84]. The Bland-Altman plot shows that, among observers, disagreement in subjective bone bruise assessment was less than 1 point. Although, this difference appears to be relatively large on a 4 point grading scale, such differences are expected when using such crude grading systems. Until now, various studies have investigated bone bruise and the clinical relevance using a

variety of subjective grading systems[118,329,330,335,336]. Besides being labor intensive, to our knowledge, none of these grading systems have been validated for evaluation of bone bruise. The excellent inter-observer reliability for the subjective MRI assessment scale, which was previously described by Felson, suggests this scale can be implemented more widely and will provide reproducible results among different observers[84]. However, this grading scale is, like other previously described subjective grading systems, rather crude consisting of 4 grades, which has a negative effect on the responsiveness (sensitivity to change over time) and the amount of information that can be gained using these grading scales.

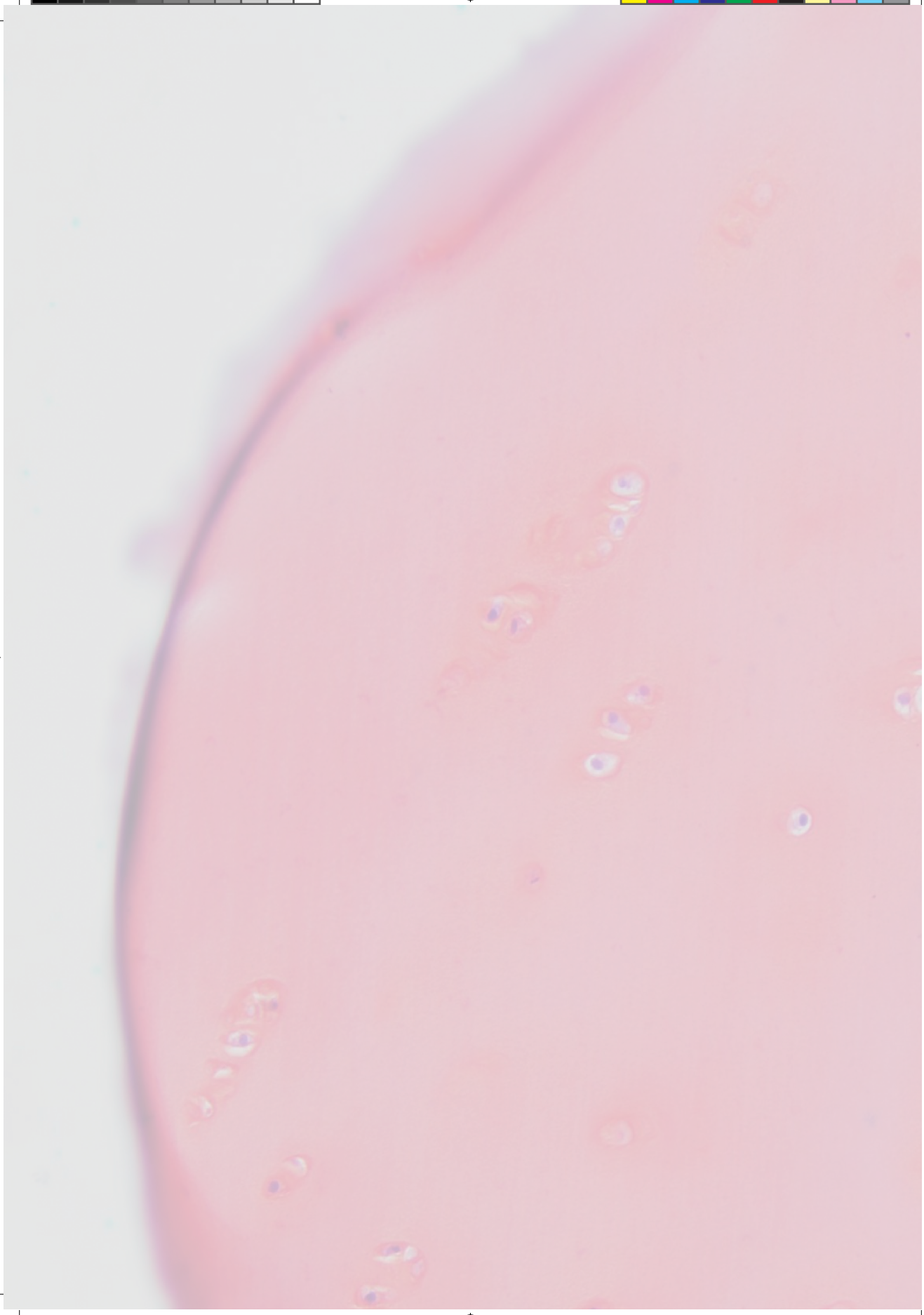
The automatic segmentation software introduced in this paper provides an easy, objective and accurate alternative to determine bone bruise volumes. The excellent correlation of probabilistic bone bruise volumes with absolute bone bruise volumes, which were visually representative for bone bruise, and the high correlation with subjective MRI assessment scale described by Felson confirms the construct validity of the system.
Nevertheless, several limitations should be discussed. The accuracy of the calculated bone bruise volume is dependant on voxel size and MRI slice thickness, i.e. low resolution MRIs and high slice thickness results in low accuracy. Furthermore, the technique requires a learning set of MRIs in which bone bruises are manually segmented before it can be implemented for analysis of the experimental MRIs. Therefore, the system will be biased towards the clinical interpretation of persons who perform the manual segmentations. In this study, the learning-set was fine-tuned until consensus was attained among all authors. The software is not fully automatic, as it requires masks, which are made manually, in order to locate femoral condyles. This technique provides merely information with respect to the bone bruise volume in contrast to various subjective assessment scales that integrate information, such as location, cortical line interruption and, if applicable, fracture types[329,330]. In our study, this was not considered a limitation because our specific aim was to investigate how clinical symptoms relate to bone bruise volumes. Finally, we merely used proton density weighted (PDw) MRI scans, while other studies have suggested that bone bruise can also be visualized on T1- and T2-weighted (T2-w) MRI scans. Using T2-w MRI scans would give additional information with respect to the acuteness of the subchondral bone damage as this sequence is specifically sensitive for the presence of water and thus edema. In the current study, automatic segmentation of PDw MRI scans alone compared to PDw and T2-w MRI scans combined was found to give a more accurate representation of the clinical interpretation of bone bruise. Therefore, T2-w MRI scan were excluded from further evaluation of the relationship between bone bruise volume and clinical symptoms. However, as PDw fat saturation MRI sequences have been demonstrated to be more sensitive in the detection of bone bruise than fast spin-echo T2-w MRI sequences, and PDw MRI provides good spatial resolution for assessment of other structures in the knee[337], the lack of T2-w MRI scans in the evaluation of bone bruise is unlikely to be a relevant limitation of the automatic segmentation software.

Despite these limitations, the automatic segmentation software tested in this paper provides an accurate and elegant tool for studying the role of bone bruise in a broader range of joint disorders, like cruciate ligament rupture, osteoarthritis and transient migrating bone marrow edema on PDw MRI scans. This technique should be further validated in other anatomic locations and should continuously be adapted to developing MRI sequences.

Although numerous studies have described bone bruises on MRIs of patients with articular cartilage defects, no relation has been established, between bone bruise and clinical symptoms in this specific population. Previous studies investigating osteoarthritis (OA) patients have reported contradictory results. In a cross-sectional study, Felson found significantly higher prevalence of bone bruises in patients with knee pain compared to patients without knee pain, although no correlation was found between the size of the lesion and the severity of the symptoms[84]. In contrast to the latter observation, Sowers reported a higher prevalence of painful knee OA in joints with "larger" bone bruise compared to smaller or no bone bruise [118], which suggests that bone bruise severity is, at least in part, related to clinical symptoms arising from OA. These results seem supported by our pre-operative data in which a weak positive correlation between bone bruise volume and clinical symptoms was found.
The presence and alleviation of hip pain due to osteonecrosis of the femoral head, was shown to be strongly associated with respectively the presence and resolution of bone bruises and was even found to be independent of radiological collapse of the hip joint[335]. In contrast, our follow-up data show discrepancies between post-operative bone bruise volume changes and clinical symptom changes. The difference between these two studies may be due to the fact that osteonecrosis of the femoral head is a different entity than articular cartilage defects. On the other hand, it can be hypothesized that, in patients with articular cartilage defects, bone bruise on MRI changes more slowly than clinical symptoms, which seems supported by the higher percentage of patients with increasing bone bruise volume combined with decreasing VAS score compared to patients with decreasing bone bruise volume combined with increasing VAS score. Furthermore, the fact that at 12-months these discrepancies were only found in microfracturing-treated patients suggests that these discrepancies are, at least partly, due to subchondral bone damage, which is induced during surgery in microfracturing-treated knee joints and not in ACI-treated knee joints. In order to study this hypothesis, an additional longitudinal dataset of untreated articular cartilage defects would be required. However, with the current knowledge of the natural (detrimental) course of untreated cartilage defects, its symptomatology, and the success of current treatment options, leaving a known clinically relevant articular cartilage defect untreated should be considered undesirable. Altogether, from a clinical point of view, these results suggest that determination of absolute bone bruise volumes is of little value in determining the pre-operative symptomatology in individual cartilage defect patients. Furthermore, the value of post-operative changes in bone bruise volumes on MRI to determine the success of microfracturing is questionable, at

least up to one year post-surgery. In contrast, our results suggest that determining changes in bone bruise volume are predictive for the success of ACI in the treatment of articular cartilage defects, although this was only observed after one-year follow-up.
Differences in correlation of bone bruise volumes with the VAS for pain and the WOMAC pain scale may be due to differences in types of questions asked in either scoring system, the WOMAC pain scale investigating pain during specific actions, while the VAS for pain asking a more open question, which likely results in increased sensitivity for psychological effects of clinical symptoms on the patients' wellbeing.

In summary, our results suggest that bone bruise might indeed be causally related to clinical symptoms resulting from articular cartilage defects in knee joints. However, larger longitudinal studies, for which microfracturing-treated patients seem not to be an adequate population, are required to provide a definite conclusion. The subjective bone bruise assessment scale seems reliable and reproducible, but gives limited information. Therefore, the automatic segmentation method developed for this study provides an easy, objective, accurate and useful alternative for future investigations that aim to study bone bruise on proton density weighted MRIs.



The correlation and reproducibility of histological scoring systems in cartilage repair

Chapter 11

Abstract

Goal: The objective of this study was to compare two frequently used cartilage repair grading scales, namely, the comprehensive O'Driscoll and the simple Pineda scale.

Methods: We determined the intra- and inter-observer variability of each score as well as the correlation between them. Thirty-eight joint section samples with variable cartilage quality were examined by three observers two time-points.

Results: Statistical analysis showed very good intra- and inter-observer reliability as well as a good correlation between the two scores. For the intra-observer variability of the O'Driscoll scale, we found an average difference of 0.05±0.93 in a 24-point score and a kappavalue of 0.87. For the inter-observer reliability, we observed an average difference was 0.001±2.25, and a kappavalue of 0.92. The Pineda scale showed an average difference of 0.86±1.38 in a 14-point score and a kappavalue of 0.86 for the intra-observer reliability. For the inter-observer reliability, we observed an average difference of 0.82±0.96 and a kappavalue of 0.89. The comparison between the two scales showed a high, inversely related correlation with a correlation coefficient of 0.71.

Conclusion: Both the O'Driscoll and the Pineda scales are reliable semi-quantitative cartilage scoring systems and that acceptance for general use of these two scores will benefit the reliability of literature on tissue engineering for cartilage repair. The added strength of comparison between published study results allows better understanding of cartilage repair publications and increases the impact of their results.

Introduction

As the research activities in the field of cartilage regeneration increased over the last decades, multiple histological scoring systems in cartilage repair research were introduced, modified, and used to evaluate results; two of these scoring systems have, since their introduction, been more frequently reported — the system of Pineda[90] and the score by O'Driscoll[88,89]. Although these scores have been widely used during the past 10 years, there is no publication of their reliability and reproducibility to be found in literature so far. Such an evaluation is, however, essential for the progress and quality of research in this field. The relevance and impact of uniformity in outcome measurement is also discussed and demonstrated in a recent publication by O'Driscoll on the validity of a simple subjective cartilage scoring system as compared to automated histomorphometric analysis. The authors conclude that, although an objective test is always preferable over a subjective one, a subjective histological/histochemical scoring system may be an appropriate tool to use[200].

The purpose of the investigation presented in this paper was to compare two frequently used histological cartilage repair scoring systems for their inter- and intraobserver variability as well as to investigate the correlation between the two systems. This would comprise important knowledge relevant for cartilage repair investigations, since a good qualification of the scoring systems enables researchers to make more reliable comparisons between different publications and different methods in cartilage regeneration. Neither O'Driscoll, nor Pineda included such validation in their original description of the cartilage score, nor were they subsequently compared in literature.

The grading system introduced by Pineda is a simple system that addresses four parameters for 0–14 points[90], whereas the grading system introduced by O'Driscoll is a much more comprehensive system that includes nine different parameters for 0–24 points[88,89] (Tables 1 and 2). Our hypothesis was that the Pineda system would have a better reproducibility, both within and between observers, because it has a smaller number of less complex and possibly more objective parameters. Because of its simplicity, the Pineda system was also estimated to be more user friendly. For the score by O'Driscoll, the larger number of parameters was hypothesized to provide a higher power of discrimination between findings and thereby allow more detailed information about the exact quality of the cartilage.

Table 1
Cartilage repair score (Pineda)

Characteristics	Score
Filling of defect	
125%	1
100%	0
75%	1
50%	2
25%	3
0%	4
Reconstruction of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced staining	1
Significantly reduced staining	2
Faint staining	3
No stain	4
Cell morphology	
Normal	0
Most hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage, but mostly nonchondrocytic cells	3

Methods

Four hundred and fifty six observations of Hematoxilin-Eosin, Alcian Blue-Fast Green, and Safranin O-Fast Green stained slides of articular cartilage were performed by three observers. All samples were examined twice. Between observations, there was an interval of at least 1 week. The group of observers consisted of three orthopedic researchers with different levels of experience in cartilage research, ranging from inexperienced to experienced. The observers were asked to examine and grade the samples according to the two different grading systems. Before examining the experimental cartilage samples, the observers were allowed to become familiar, by self-education, with each of the grading systems using sections of relevant tissue not included in the current investigation. All samples were presented to the observers in a blinded and random order. For further details of the techniques used, please refer to appendix G.

Table 2
Cartilage repair score (O'Driscoll)

Characteristics	Score
Nature of predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures 25–100% of the thickness	1
Severe disruption, including fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	0
Thickness	
100% or normal adjacent cartilage	2
50–100% of normal cartilage	1
0–50% of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Not bonded	0
Freedom from cellular changes of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
<25% of the cells	1
25–100% of the cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0

Statistical analysis

The statistical methods that were used to calculate the intra- and interobserver reproducibility were based on the graphic techniques and calculations as described by Bland and Altman[331]. These methods were found to be reliable for the hypothesis tested in the current investigation. The intraobserver reliability was calculated from the difference between the two scores from that observer as compared to the mean of that section. For the determination of interobserver reliability, we determined the difference between observer scores for a given section versus the mean for that tissue sample. Correlation between the scoring systems suggested by O'Driscoll and the score of Pineda was determined from linear regression analysis of the average scores for each cartilage sample. Correlation and kappavalues are interpreted according to the guidelines described by Landis and Koch[338]: 0.00 as poor, 0.00–0.20 as slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect. For further details of the materials and methods used, please refer to appendix G.

Results

O'Driscoll cartilage repair score

The intraobserver variability was very low (Figure 1). The average difference between the first and the second observation was very small, 0.05 (range 20.08 to 0.18). Standard deviation of the observations was 0.93 (range 0.85 to 1.06) within the 24 points in this scoring system. Kappavalues were high for all three observers; 0.92 for observer A, 0.78 for observer B, and 0.87 for observer C. This results in an overall kappavalue for the O'Driscoll score of 0.87, which is qualified as almost perfect. The overall kappavalue for the interobserver reliability scored even higher, at 0.92, with an average difference of 0.001 and a standard deviation of 2.25. All results are shown in Table 3.

Pineda cartilage repair score

Scoring 0.86, this system had a small overall average difference for the intraobserver reliability (range 20.38 to 0.75). Standard deviation within the 14 point score was somewhat higher at 1.38 (range 0.53–3.17) a compared to that in the O'Driscoll score. This SD is somewhat confounded by the fact that one observer had a SD of 3.17, whereas the other two observers revealed a SD of 0.53 and 0.55, respectively. Again, result show high kappavalues: 0.91 for observer A, 0.86 for observer B, and 0.80 for observer C, leading to an overall kappavalue of 0.86 ($y = 0.92x + 1.28$). For the overall interobserver reliability, the kappavalue was high, at 0.89, with an average difference 0.82 and a standard deviation of 0.96 (Figure 2).

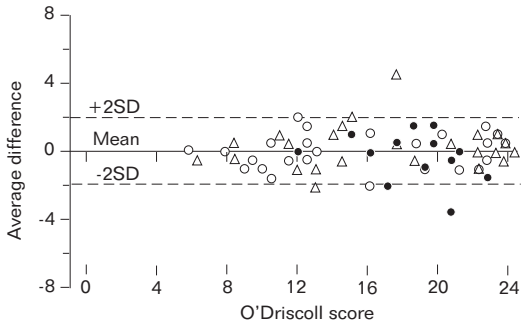


Figure 1
Intraobserver variability of the O'Driscoll system for all three observers. The difference between the first and second observation for each observer is plotted against the average score for that sample. Each observer is represented by a different symbol. The average difference was 0.05 within a 24-point score, with a standard deviation of 0.93, giving a kappavalue of 0.92.

Table 3
Results of the intra-and interobserver reproducibility of the O'Driscoll (0–24 points) and the Pineda (0–14 points) scoring system

	O'Driscoll						Pineda					
	Intraobserver			*Interobserver*			*Intraobserver*			*Interobserver*		
Observer	r^2	Average diff	SD	r^2	Average diff	SD	r^2	Average diff	SD	r^2	Average diff	SD
A	0.92	0.04	0.86	0.98	1.39	1.54	0.91	20.38	0.55	0.88	0.91	0.73
B	0.78	20.08	0.85	0.89	21.89	2.36	0.86	0.75	3.17	0.82	1.21	1.36
C	0.87	0.18	1.06	0.96	0.50	1.22	0.80	0.37	0.53	0.94	0.76	0.98
Overall	0.87	0.05	0.93	0.92	0.00	2.25	0.86	0.86	1.38	0.89	0.82	0.96

Our third goal was the comparison of the results of the O'Driscoll system with the results using the Pineda system and the possible correlation between them. Regression analysis determining correlation between the observation using the mean of observations with each of two scoring systems showed a favorable correlation. The correlation coefficient was 0.71 ($y = 0.49x + 11.64$; Figure 3). According to Landis and Koch, such values are to be considered as representing a substantial correlation.

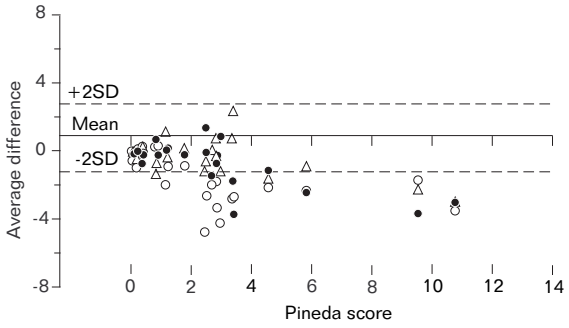


Figure 2
Interobserver variability of the Pineda system for all three observers. In this figure, the difference between observations and the average for all observers is plotted against the mean score for a given sample. Each observer is represented by a different symbol. The average difference was 0.82 within a 14-point score, with a standard deviation of 0.96, giving a kappavalue of 0.89.

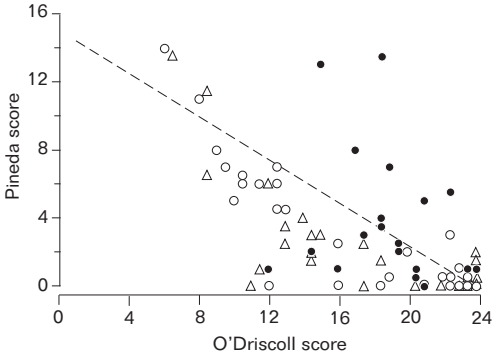


Figure 3
Correlation of the O'Driscoll and Pineda system. O'Driscoll *et al*. score from 0 for poor to 24 for good cartilage quality, whereas Pineda *et al*. score from 14 for poor to 0 for good. All three observers are represented by different symbols. An inversely proportional correlation was found, $r^2 = 0.71$ ($y = -0.49x + 11.64$).

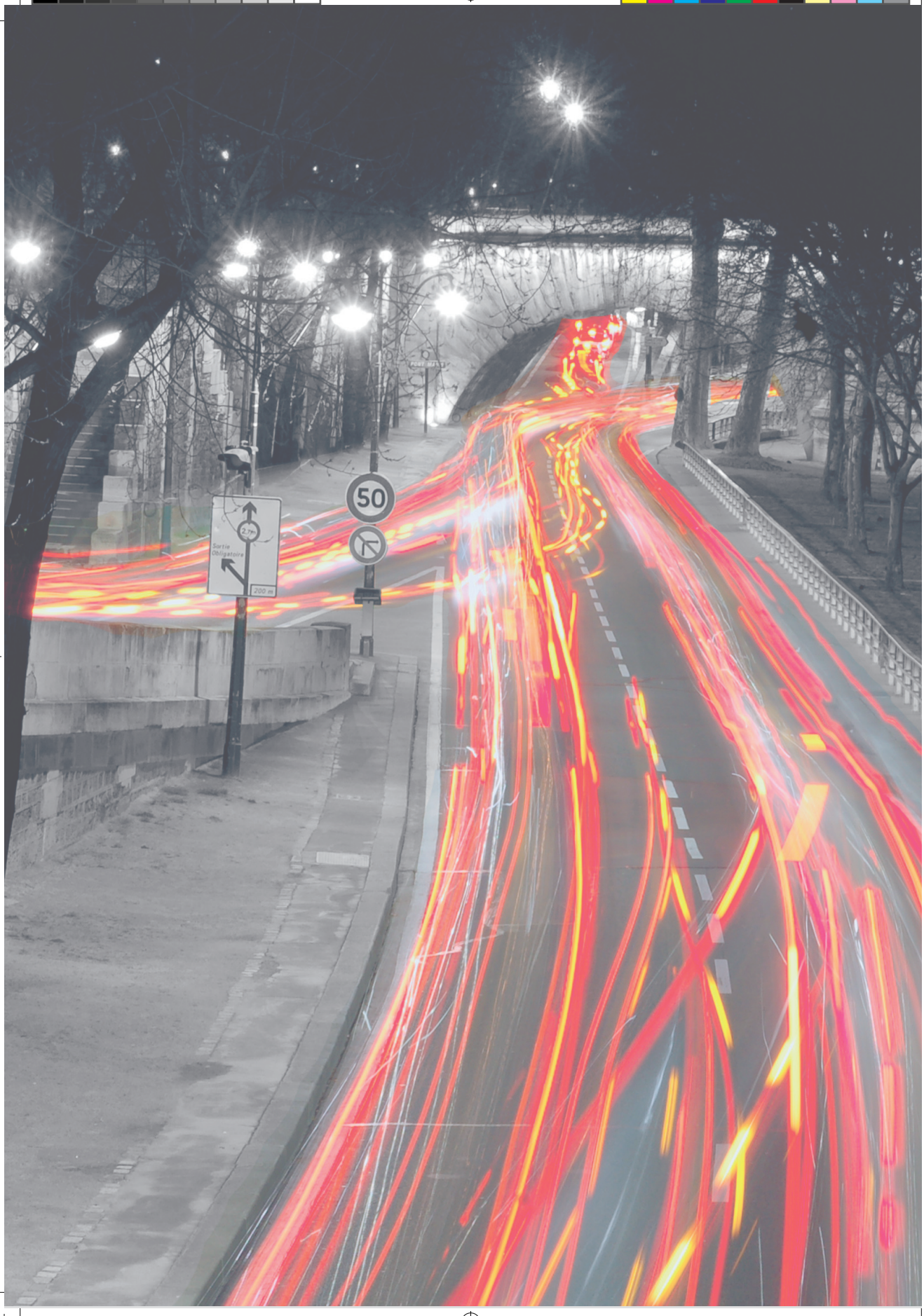
Discussion

Based upon our current observations, the system by O'Driscoll. was determined to be a reliable scoring system for grading cartilage as it was shown to have both very high intra- and interobserver reproducibility. For the system by Pineda, we found comparable values for both items. According to the guidelines described by Landis and Koch[338], all these results can be classified as almost perfect. For both systems, the average difference between observations as well as the standard deviation within the score range per sample were low. The high standard deviation of observer B on the Pineda system may be attributed to the relative lack of experience of this observer with this system. Those results did not lead to significant loss of discerning capability. In conclusion, we may say that both these cartilage repair scoring systems are reliable semi-quantitative systems for grading the healing process of cartilage defects, in attempts at repair by tissue engineering.
We hypothesized that the system by Pineda would show better reproducibility because of simplicity. This hypothesis was shown to be incorrect. The system by

O'Driscoll proved as easy to use with results that were as reliable. We feel that this can in part be explained by the number of parameters included in the overall score. When the observers assess one or two parameters differently, using the O'Driscoll system, this could be compensated by a multitude of other parameters. The Pineda system, with only four parameters, does not allow for such possibilities. To assess the possibility of comparison between the two systems, we looked at the correlation between them; we found them to be inversely proportionally "substantially" correlated.

All observers subsequently judged the more extensive O'Driscoll system to be slightly more complex, because of the larger number of parameters to assess. We feel that investigators should perhaps gain more experience to use it efficiently as compared to the simpler Pineda system. However, the statistical analysis showed adequate reliability, even with an inexperienced observer, and there was no difference when compared with the Pineda system. Finally the O'Driscoll system does provide considerably more information about the exact quality of the cartilage repair due to the larger number of included parameters and allows comparisons on subitems, which may be of interest when comparing the results of different tissue regeneration strategies.

From the results presented in this study, we conclude that both the cartilage repair scoring systems as suggested by O'Driscoll and by Pineda are reliable semi-quantitative cartilage scoring systems with good correlation. There is no apparent need to apply modifications or to develop new systems given their successful application during many years. Using these systems will increase reliability and, to some extent, give us the opportunity of comparisons between studies, which is of benefit for better understanding of cartilage repair publications and the impact of their results.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Summary and conclusions

Chapter 12

Summary and Conclusions

The goals of this thesis were to define boundary conditions for successful cartilage regeneration, to demonstrate methods that improve biological joint reconstruction and may optimize joint homeostasis, and to develop and validate new tools for future research in the field of cartilage repair. In this chapter, the results of the studies that were performed for this thesis are summarized by addressing the aims that were formulated in the introduction of this thesis and discussing the implementation of the knowledge gained.

Aim 1: To determine whether autologous chondrocyte implantation provides superior structural repair over microfracturing

In 1994 Brittberg was the first to report the clinical results of autologous chondrocyte implantation (ACI) in the treatment of articular cartilage defects[24]. Since then, ACI is increasingly applied and numerous publications have described promising results. The randomized clinical trial described in **chapter 3** demonstrates that one year post-surgically, CCI resulted in morphologically significantly better regeneration tissue as compared to traditionally applied microfracturing. This observation was not only true for the overall histological score, but for all individual parameters tested in this study, i.e. PG staining, cell and tissue morphology, tide mark, and the structure of middle/deep zone. The obvious difference between scar formation in microfracturing and tissue reconstruction in CCI was the most striking finding of this investigation. However, it must be noted that the quality of the repair tissue resulting from ACI was still variable, ranging from fibrocartilaginous to more hyaline-like tissue. The clinical outcome at 12 to 18 months with CCI was comparable to microfracturing. Interestingly, this was also found at the earlier follow-up time-points, while ACI is a two-stage surgical procedure, of which the second surgical intervention is an arthrotomy, in contrast to microfracturing, which merely comprises one arthroscopic intervention. In addition, the safety profile was similar for both treatments. From these results, we concluded that CCI represents a new class of treatment, which is associated with superior structural repair of cartilage tissue, compared to microfracturing.

Implications of the data

To our knowledge, this is the first GCP conducted clinical trial with full safety monitoring that actually demonstrates the superiority of ACI for treatment of articular cartilage defects over microfracturing, while the latter is currently considered the treatment of choice. Recently, it has been demonstrated that high morphological quality results in increased longevity of the regenerated tissue and of the observed clinical improvement resulting from ACI, thus suggesting on the long run that the clinical outcome in patients treated with ACI will be better[35]. Taken together, these results support the increasing use of ACI and justify further *in vitro* and *in vivo* studies for ACI optimization.

Several aspects should be addressed in an attempt to improve the outcome of

ACI. Concerns have been expressed with respect to possible occurrence of donor site morbidity or even OA development as a result of the cartilage biopsy required for donor cells. However, numerous clinical ACI trials have been described, some with follow-up periods up to 10 years[33,339], and, to date, none have reported such donor site morbidity. Furthermore, the CCI technique used in the current trial requires two surgical interventions, of which the second intervention is an arthrotomy. Besides being a great burden for the patient, this is likely to induce more disturbance of the joint homeostasis than if ACI would comprise a one step arthroscopical procedure. These reasons justify the ongoing research on the use of alternative cell sources, such as ear and nose chondrocytes, BMSCs and other mesenchymal progenitor cells. Also the development of scaffold materials, which can significantly improve the ease and reproducibility of the surgical procedure, provides a viable approach for improving the outcome of ACI. Furthermore, our results demonstrate that, although significantly superior to the repair tissue resulting from microfracturing, the regeneration tissue ensuing from CCI is still morphologically variable[31,33]. This finding is supported by other previously conducted trials and may be attributable to a variety of factors. The studies presented in **chapter 4, 5, 6 and 7** aim to provide more insight into *in vitro* models used for cartilage tissue engineering studies and the involvement of intra-articular factors, in particular donor tissue quality and synovial fluid (SF) on the process of chondrogenesis and cartilage regeneration techniques.
Altogether, we can conclude that CCI provides a superior treatment modality for articular cartilage defects as compared to microfracturing, since it results in cartilage regeneration rather than repair. Therefore, CCI should be considered a first line of defense and not a last resort.

Aim 2: To elucidate the effect of donor tissue quality, cartilage defect related changes and intra-articular factors on cartilage regeneration

We need a thorough understanding of the mechanisms involved to improve the outcome of cartilage regeneration techniques. Both TGF-β and IGF-I have extensively been demonstrated to be key growth factors during the process of chondrogenesis. They stimulate both cell proliferation and chondrocyte differentiation, which are essential steps in acquiring regeneration tissue with adequate mechanical properties[42,43,46,47]. TGF-β has been suggested to regulate the bioavailability of IGF-I through modulation of IGFBP levels[48,49]. However, little is known on how these growth factors interact during the process of chondrogenesis. Using an *in vitro* periosteal chondrogenesis model, we demonstrated (**chapter 4**) that TGF-β-supplemented culture media affected the IGF-axis by decreasing IGFBP-4 mRNA levels and IGFBP-3 and IGFBP-4 secretion in cultured periosteal explants. Furthermore, PAPP-A mRNA and protein levels, as well as IGFBP-4 protease activity were increased between 7 and 10 days of culture (the onset of cartilage formation in this model) in TGF-β-treated explants. IGF-I and IGF-I receptor mRNA levels were decreased by TGF-β treatment. From these results, we concluded that TGF-β regulates multiple members of the IGF-I axis during *in vitro* periosteal chondrogenesis.

A major factor affecting cartilage regeneration was identified previously to be the synovial fluid (SF), a biological fluid that typically contains a host of cytokines, growth factors and proteolytic enzymes. In order to study the possible detrimental effects of these micro-environmental factors, we needed a reliable and reproducible chondrogenic model. In **chapter 5**, we compared previously published expansion and redifferentiation conditions. When compared to basic expansion conditions (BEC), growth factor supplemented expansion conditions (GFSEC) were found to result in significantly improved *in vitro* chondrogenesis upon redifferentiation (independent of the redifferentiation model used), as demonstrated by the significantly tissue morphology and increased final proteoglycan content. Interestingly, chondrocyte expansion in GFSEC resulted in significantly decreased collagen type II, SOX-5 and SOX-6 mRNA levels as compared to expansion in BEC, which appears to be in contrast with the improved post-redifferentiation outcome. Redifferentiation on collagen-coated filters compared to pellet culture resulted in an increased final PG content and, as observed by electron microscopy, a chondrocyte morphology and extracellular matrix (ECM) organization that were more similar to those found for hyaline cartilage. Even more, the combination of chondrocyte expansion in GFSEC and redifferentiation on collagen-coated scaffolds resulted in a significantly higher cellularity upon redifferentiation as compared to chondrocytes expanded in BEC and redifferentiated in pellet culture.
From this study, we concluded that, of the culture models tested, chondrocyte expansion in GFSEC and redifferentiation on collagen-coated filters resulted in the most optimal chondrogenesis. Therefore, we implemented this model for the studies described in chapter 6 and 7.

Chondrocytes used for *in vitro* research are frequently harvested from less affected areas of OA joints, as this is a readily available source of cells. Furthermore, ACI has been suggested as a possible treatment for early OA[57]. However, it is unclear how phenotypical changes associated with OA development affect the process of cartilage regeneration.
The goal of the study discussed in **Chapter 6** was to investigate to what extent the chondrocytes harvested from OA joints display an altered, more dedifferentiated or hypertrophic phenotype after expansion. Subsequently, we studied the differences between both donor types in biochemical and (immuno-) histological properties of the tissue upon redifferentiation culture.
The differentiation index (Collagen type II/I mRNA ratio) decreased significantly between passage 1 and 2 for both healthy and OA chondrocytes, which is analogous to previously published data by other groups. No statistically significant differences in differentiation index could be demonstrated between donor types (OA vs healthy). In contrast, collagen type X mRNA was evidently expressed in chondrocytes from OA joints, while being undetectable in chondrocytes from healthy joints after both one and two expansion passages. Furthermore, tissue generated by chondrocytes from healthy joints had a significant better morphology and higher proteoglycan content as compared to chondrocytes from OA joints.
From these results, we concluded that chondrocytes from OA joints have altered

in vitro chondrogenic properties as compared to chondrocytes from healthy joints, which is detrimental for the final tissue quality. Therefore, *in vitro* studies that use OA chondrocytes appear not to be a representative model for clinically applied ACI and, therefore, should be interpreted with care. Furthermore, currently ACI does not seem to be an appropriate treatment modality for early OA.

Previous cartilage regeneration studies have demonstrated a detrimental effect of disturbed joint homeostasis on proteoglycan turnover and tissue morphology of the tissue synthesized. In **Chapter 7**, we studied the effect of human synovial fluid (SF) from injured knee joints on cartilage regeneration by expanded human articular chondrocytes with respect to tissue morphology, biochemistry and chondrocyte redifferentiation. After 14 days of culture, SF supplementation to the culture media resulted in a significant downregulation of final proteoglycan (PG) content, PG content normalized to DNA, collagen type II mRNA levels and differentiation index as compared to control culture conditions. Interestingly, SF-supplemented media resulted in significantly increased cellularity, as reflected by DNA content, compared to control conditions. The morphology, and the collagen type I, type X, and aggrecan mRNA levels were not significantly affected by SF supplementation to the culture media.
From this study, we concluded that SF from injured human knee joints has a negative effect on *in vitro* chondrogenesis with respect to the extra-cellular matrix turnover and the level of chondrocyte redifferentiation.

Implications of the data
These studies give increased insight by which cartilage defect-related changes may affect the process of cartilage tissue engineering and to what extent the outcome of cartilage regeneration techniques may be improved by altered culture conditions and possibly by peri-operative modulation of the joint homeostasis.

Our results demonstrate that TGF-β induced chondrogenesis is not only influenced by the direct chondrogenic properties of this growth factor, but also through increased bio-availability of IGF-I by reducing its binding proteins and increasing IGFBP-4 protease (PAPP-a) and the IGFBP-4 protease activity. Whether this phenomenon actually results in increased IGF activity remains to be proven, as we found an additional down-regulation of IGF-I and IGF-I-receptor mRNA levels. Besides further increasing our insight into the mechanism of chondrogenesis in general, these results may have significant implications for clinically applied ACI. Cartilage damage and OA development have been demonstrated to result in an increased TGF-β availability[40,41]. As the main mode of action of IGF-I is mitogenic, this mechanism may explain the increased cellularity induced by supplementation of SF from injured knee joints. Furthermore, for studies that aim to improve cartilage regeneration by increasing the peri-cellular growth factor concentrations, e.g. by gene transfection and controlled release techniques from scaffold coatings, it may be sufficient to limit their focus to TGF-β. This approach would provide a substantial benefit, as exogenous (intra-articular) growth factor supplementation has been associated with serious side effects.

Interestingly, we found a discrepancy between post-expansion collagen type II mRNA levels and chondrogenic potential upon redifferentiation when expanding cells in growth factor-supplemented media, showing that the role of growth factors can be ambiguous. In addition, these results demonstrate that for optimalization of chondrocyte expansion conditions, merely screening collagen type I and type II mRNA levels is not sufficient, as chondrogenic capacity of these chondrocytes appeared unrelated to decreased collagen type II mRNA levels. The observation that *in vitro* cartilage regeneration was further enhanced by culturing on collagen type II-coated filters not only provided us with a reliable and reproducible chondrogenic model, but might implicate that collagen II is a useful matrix for inclusion in the so-called matrix-induced autologous chondrocyte implantation (MACI). This technique not only can support cartilage regeneration and the chondrogenic phenotype, but can also significantly improve the ease of the surgical procedure and even possibly facilitate an arthroscopically assisted cellular cartilage regeneration procedure, e.g. in combination with a self setting collagen type II-based polymer. Furthermore, scaffold materials may provide mechanical support to the implanted cells, which could have significant beneficial consequences for the post-surgical rehabilitation period.

Surgical procedures based on ACI do not yet seem feasible for patients with OA, as we found that chondrocytes from OA knee joints have an altered collagen expression profile upon chondrocyte expansion and a reduced chondrogenic potential upon redifferentiation. On top, this observation has a deep impact on the interpretation of *in vitro* chondrogenesis research, where most investigators use chondrocytes harvested from putative non-affected areas in OA joints. Thus, such studies should be interpreted with great care.
In addition, combined with the fact that the presence of a cartilage defect increases the chance of early onset OA, the difference in chondrogenic potential between chondrocytes from healthy and OA joints suggests that cartilage defects should be treated as soon as possible after a defect has arisen or, at least, before phenotypical changes of the chondrocytes in the surrounding cartilage have occurred. Research on possible phenotypical changes of chondrocytes from joints with long-standing defects and the dynamics of the transition from healthy to OA chondrocytes in damaged knee joints is required to reveal whether this is a sudden or rather a slow, at first macroscopically invisible, process.
Besides the aspect of donor tissue quality, one needs to realize that cartilage damage represents a general joint disorder rather than an isolated cartilage problem, involving a disturbed joint homeostasis. In particular the synovial fluid (SF) harbours a complex mixture of mediators of various anabolic and catabolic pathways.
Indeed SF from injured knee joints, previously shown to negatively affect morphology and the ECM turnover, also significantly inhibited chondrocyte redifferentiation in our study. These results support the hypothesis that the local intra-articular environment may provide a viable target for future improvement of ACI by patient selection, or pretreatment of affected joints to restore joint homeostasis. How-

ever, as SF contains a large variety of proteins, new analysis techniques, such as protein arrays and multiplex ELISA, may prove indispensable to elucidate which factors should be targeted to improve to the intra-articular environment and, thereby, the outcome of cartilage regeneration techniques.

One such target might be IL-1β, as this cytokine is known to play a key role in the inflammatory response to damage in most human tissue types, which, in turn, is essential for adequate wound repair. However, in the context of articular cartilage, IL-1β is associated with degeneration and development of osteoarthritis and rheumatoid arthritis. Therefore, targeting IL-1 in the SF may be a feasible treatment option to treat or maybe even prevent these disorders and, in addition, a viable method to restore a beneficial joint homeostasis for improvement of cartilage regeneration techniques. This approach was tested for the first time in a clinical trial comparing an autologous form of IL-1 receptor antagonist with corticosteroids and placebo treatment[73]. Despite various methodological limitations of this trial, their results seemed to support the hypothesis that targeting IL-1 may have a beneficial effect on the clinical symptoms arising from OA.

Aim 3: To evaluate the clinical efficacy of autologous IL-1ra

Orthokin is an autologous product containing high concentrations of IL-1ra and was originally developed for the treatment of symptomatic knee OA. Therefore, before implementing it to improve the outcome of cartilage regeneration techniques, we conducted a multicenter, prospective, double blind, placebo controlled trial to investigate the clinical efficacy of Orthokin in patients with symptomatic knee OA. Although Orthokin did not meet the primary efficacy objective, which was 30% more improvement on the WOMAC score as compared to placebo treatment, Orthokin induced statistically significant more improvement on the KOOS symptom and KOOS sport parameters. Even more, Orthokin-treated patients consistently scored better on all outcome parameters tested, a trend that was even more pronounced in patients who continued using NSAIDs during the trial. From these results, we concluded that Orthokin has a clear beneficial *in vivo* biological effect, although the clinical effect when administered under trial conditions is limited.

Implications of the data

Despite the limited symptom alleviation, this study clearly demonstrates a biological effect of Orthokin. This finding may prove an important advancement in the field of OA treatment, as to our knowledge, Orthokin is the only available disease modifying osteoarthritic drug (DMOAD) that not only has a beneficial effect on clinical symptoms, but, in addition, has a clear mechanism of action through which it might induce a chondroprotective effect. However, specifically this feature could not be demonstrated in the current study, as no reliable tools are available to monitor *in vivo* disease progression in such, relatively short-term, follow-up studies. Therefore, future *in vitro* and *in vivo* studies and controlled trials with long-term

radiological follow-up are required to determine the disease modifying properties of this therapy. Although various *in vitro* and animal studies have suggested the importance of IL-1β in OA development, to date, no studies have convincingly demonstrated its presence and role in human OA development. Orthokin may show to support this hypothesis. However, it should be kept in mind that Orthokin is developed by exposing whole blood to $CrSO_4$-coated glass beads and that it remains unclear how the composition, besides the upregulation of IL-1ra and other anti-inflammatory cytokines, is affected by this procedure. Further studies should aim at assessment of the factors of interest, in particular IL-1 and IL-1ra, to see what is the actual contribution of IL-1 inhibition to the clinical improvement found. Similar therapies and interventions aiming at interference with the local soluble factors may therefore not only be implemented in the treatment of OA, but in addition provide a feasible tool to improve the outcome of cartilage tissue engineering techniques, such as ACI, by pre-treating the joint and thereby restoring the disturbed joint homeostasis.

Aim 4: To develop new outcome evaluation methods for future OA and cartilage regeneration studies

Regenerative medicine is a rapidly developing field that should now be considered part of our standard orthopaedic armatorium in both cartilage regeneration and OA modulation. However, as became clear in the previous chapters, there is still room for improvement of its outcome. Therefore, the ongoing efforts in this field of research must be stimulated. To be able to compare results between studies, centers, techniques and populations it is of essential importance that validated uniform outcome tools are used. Therefore, in chapters 9, 10 and 11, we developed and validated new research and clinical outcome tools for evaluation of cartilage regeneration and treatment of OA.

Frequently, extensive questionnaires are used to monitor the status of the patient and the efficacy of new treatments in clinical trials. Reduced questionnaires have the benefit that they are easy to use, less time consuming and less labor intensive upon analysis, and, in addition, have been suggested to improve patient compliance. In **chapter 9** we validated the use of the short form WOMAC function scale for evaluation of non-surgically treated knee OA. The short form WOMAC function scale had an excellent internal consistency and test-retest reliability. In addition, a high correlation with the traditional WOMAC was found and the average disagreement between the two measures was minimal (0.23 points on a 100-point scoring scale). It must be noted however, that when evaluating individual patients, inaccuracies of approximately 12 points can be expected. Furthermore, the responsiveness was found to be adequate and comparable with that of the traditional WOMAC. The short form WOMAC function scale lacks two complete categories from the traditional WOMAC (stiffness and pain). However, we observed that incorporating these items did not further improve the validity of the reduced questionnaire.

From this study, we concluded the short form WOMAC function scale to be a valid, reliable and responsive alternative for the traditional WOMAC in the evaluation of non-operatively treated OA patients.

Bone bruise is a radiological phenomenon that is frequently observed on MRIs of knee joints with articular cartilage defects and, although not indisputably proven, is associated with clinical symptoms arising from cartilage defects. We performed a study to validate automated MRI segmentation software to determine bone bruise volume on MRI as described in **chapter 10**. We determined the inter-observer reliability of a previously published subjective grading scale, which was found to be excellent. The automatic segmentation software provided accurate demarcation of bone bruise on proton density weighted (PDw) MRI scans. The incorporation of T2-weighted (T2-w) MRI scans resulted visually in a decreased accuracy of the segmentation and, therefore, were excluded for the automated segmentation procedure. In addition, the automatically determined bone bruise volume had a high correlation with the subjective MRI grading scale, which confirms that the software system demarcates the area that clinically is considered bone bruise area. Furthermore, a preliminary study on the relationship between bone bruise and clinical symptoms showed a weak positive correlation between these parameters. Although not statistically significant, the bone bruise volume appeared to decrease after ACI treatment, while it appeared to increase after microfracturing treatment. Analogous to this observation, we observed that discrepancies in post-operative changes in bone bruise volume and clinical symptoms were mainly found after microfracturing treatment. The increased bone bruise volume was hypothesized to be due to the subchondral bone damage induced during the surgical procedure, which suggests that the automated segmentation software is unable to differentiate between subchondral bone damage and the presence of actual bone bruise.

Three aspects of bone bruise analysis on MRI were studied, from which we concluded:

- The subjective MRI grading scale provides reliable and reproducible information on bone bruise severity, but is limited responsive to changes due to its crudeness;
- The automatic segmentation technique is an easy, objective, accurate and useful tool for future investigations that aim to study bone bruise on PDw MRIs;
- Bone bruise might indeed be related to clinical symptoms resulting from articular cartilage defects in knee joints. However, larger longitudinal studies are required to provide a definite conclusion and reveal whether this hypothesis is true;
- Microfracturing-treated patients seem not to be an adequate population to study the relation between bone bruise volume and clinical symptoms.

Multiple histological scoring systems in cartilage repair research have been introduced and used to evaluate results, although none were validated. In **chapter 11** we compared two histological scoring systems previously published by Pineda and

O'Driscoll[88-90]. We found very good intra- and inter-observer reliabilities as well as a good correlation between the two scores.
For both the intra-observer and inter-observer reliability, we found that the difference among observations and observers was low in both scoring systems. However, the relative difference was slightly smaller for the O'Driscoll scale. This is likely due to the fact that this system was the most extensive of the two scales. Therefore, parameters that were assessed differently were likely compensated for by the multitude of other parameters.
We concluded that both the O'Driscoll and the Pineda scales are reliable semi-quantitative cartilage scoring systems and that acceptance for general use of these two scores will benefit the comparability of literature on tissue engineering for cartilage repair.

Implications of the data

Validation of the short form WOMAC function scale

In our ongoing efforts to establish cartilage regeneration techniques and DMOADs as treatment standards for cartilage repair and symptom alleviation, outcome measures are required that can be implemented both under trial conditions, as well as in daily clinical practice to evaluate the effectiveness of our interventions. The short form WOMAC function scale can be implemented for evaluation of non-surgically treated knee OA and joint replacement surgery of both the hip and the knee. Before implementing this outcome tool for evaluation of other treatment modalities, such as ACI, additional validation studies are required. The short form WOMAC function scale should not be used for evaluation of individual patients. However, when evaluating groups of patients, the data obtained can be compared to studies that have used the traditional WOMAC (currently the golden standard in OA research). This feature should be considered essential when developing new outcome tools, as comparability with other (previously performed) studies in the field increases the value of future studies.

Automated segmentation of bone bruise on MRI

Although bone bruise has been described frequently in the context of articular cartilage defects, to date, the role and consequences of this phenomenon remain unclear. None of the previously used measures to determine bone bruise severity has been validated and their crudeness suggests that these measures are unable to determine subtle changes as observed in the current study up to 12 months post-surgically. The automated segmentation software provides an easy, objective, accurate and useful tool for future investigations that aim to study bone bruise on PDw MRIs. Our results in ACI-treated patients support the hypothesis that bone bruise is related to clinical symptoms in patients with cartilage defects, although additional longitudinal studies are required to draw definite conclusions. However, it should be noted that microfracturing-treated patients appear to provide an inadequate population to study the process and role of bone bruise on PD-w MRI

Histological grading

We compared two frequently used histological scoring systems, the first being a relatively simple system that addresses four parameters[90], whereas the grading system introduced by O'Driscoll is a much more comprehensive system that includes nine different parameters. As both systems showed excellent inter- and intra-observer reliabilities, both systems can be considered reliable and can be implemented for general use. However, several aspects should be considered before using these systems. The system described by Pineda is shorter and, therefore easier to use and less time-consuming. When using the scale described by O'Driscoll, a broader range of information is gained, which might be used when reviewing specific sub-items. Furthermore, both systems require a "regenerate tissue – native cartilage" interface to score sub-items such as "Filling of the defect" and "Bonding to the adjacent cartilage". These limitations make these scales less suitable for clinical trials in which small biopsies are taken from regeneration tissue that is often difficult to distinguish from native cartilage. For this purpose the ICRS II scale as applied in chapter 2 was developed by the ICRS.

General conclusions and future directions

In this thesis, we have clearly demonstrated the superior structural repair resulting from ACI as compared to the traditionally used microfracturing technique. However, one should realize that these beneficial results were found under strict trial conditions with accurately defined in- and exclusion criteria based on previously reported trials. Still, we found that ACI resulted in morphologically variable regeneration tissue-quality, which led us to the subject of this thesis: "exploring the boundaries of successful cartilage regeneration".

In our effort to acquire adequate regenerative conditions, we established that in future clinically applied ACI, chondrocytes should be expanded under growth factor supplemented conditions. Furthermore, upon refining the surgical techniques, scaffold materials should not only be considered for practical reasons, but also because they may significantly enhance the process of chondrogenesis. Further optimization may be attained by *in vitro* and *in vivo* experiments comparing the influence of different scaffold materials on cartilage regeneration by expanded and implanted chondrocytes.

Besides the culture conditions, we have demonstrated the importance of selecting patients with sufficient donor tissue quality. Until the substantial differences in chondrogenic potency between healthy and OA chondrocytes can be overcome, ACI should not be implemented for the treatment of early OA. In addition, effort should be put into elucidating the transition from cartilage defects towards the development of OA and how this affects donor tissue characteristics, as this increases insight into adequate treatment timing and patient selection.

Lastly, we have demonstrated that transferring clinically relevant variables, such as SF from injured knee joints, to the "controlled" environment of *in vitro* experiments significantly alters the process of cartilage regeneration. Therefore, exploring the variables of this environment will likely elucidate mediators unfavorable to *in vivo* cartilage regeneration. These mediators may provide feasible targets for

optimization of joint homeostasis by pre-treating a damaged joint before implementing ACI. Among others, IL-1β might be such a target, as this pro-inflammatory cytokine has proven to be of key importance during wound repair throughout different tissue types in the human body. The biological efficacy of a newly developed autologous form of IL-1ra was demonstrated by clinical improvement found in patients with symptomatic knee OA. Whether this treatment modality actually results in improved cartilage regeneration remains to be proven.

In conclusion, the aim of this thesis was to establish the position and to determine the boundary conditions of cartilage regeneration techniques for the treatment of articular cartilage defects. Based on our results, ACI should now be considered a first approach rather than a last resort for the treatment of articular cartilage defects. Still, we have demonstrated the significant impact of clinically relevant parameters on the outcome of these treatment strategies. Clarifying and targeting the mediators that account for the observed detrimental effects, have the potential to provide the key in our ongoing effort to enhance the clinical outcome of cartilage regeneration techniques.

Appendix Materials and Methods

Appendix A (Chapter 4)

Culture methods for periosteal explants

Periosteal Explant harvest and Culture

For the gene expression experiments, explants (2 x 3 mm^2) were harvested from the medial proximal tibiae of two-month old New Zealand white rabbits using sharp elevation. The explants were cultured in a 0.5 % agarose suspension in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, N.Y.) with 1 mM L-proline (Sigma, St. Louis, MO), 50 µg/mL L-ascorbic acid (BDH Chemicals, Toronto, Canada), penicillin-streptomycin (Sigma, St. Louis, MO) (10% fetal bovine serum (FBS) with or without 10 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN) during the first two days of culture as previously described[43]. The periosteal explants were cultured for various lengths of time that ranged from 3 to 42 days. At harvest, explants were washed in phosphate buffered saline (PBS) and pooled (2 explants/group) flash frozen in liquid nitrogen and stored at -70°C until RNA isolation.

For conditioned media (CM) experiments, explants were cultured as described above for 7 days. After 7 days, the serum containing medium was removed and replaced with serum-free DMEM containing 0.1% BSA for 48, 72, and 96 h. The conditioned medium (CM) was collected and centrifuged at 2000 g at 4°C for 15 minutes and stored at -70°C.

RNA Isolation and cDNA Synthesis

Cultured periosteal explants were pulverized in liquid nitrogen, homogenized using a QIA shredder (Qiagen, Inc., Valencia, CA, USA), incubated for 20 min. at 55°C in 20 mg/mL with proteinase K (Qiagen, Inc., Valencia, CA, USA) and extracted using the Rneasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) including the "on-column" DNase digestion protocol. A second "off-column" Dnase treatment using RQ1 Rnase-Free Dnase (Promega, Madison, WI, USA) followed by re-purification with the Rneasy Mini Kit was used to eliminate residual genomic DNA contamination. The total RNA yield from two cultured periosteal explants ranged from 1 to 3 µg. Approximately 500 ng of total RNA was reverse transcribed with random hexamer primers at 37°C for 60 min.

Quantitative Real-time PCR

Quantitative real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System and software (PE Applied Biosystems, Foster City, CA). Rabbit specific cDNA sequences were obtained using a gene digging technique previously described[340]. These rabbit sequences were used to generate primer and probe sequences using the Primer Express™ (PE Applied Biosystems, Foster City, CA) software. Standard curves were generated from synthetic oligonucleotides of the experimental amplicons to obtain copy number data. All samples (n=6) were run in duplicate and quantitated by normalizing the target signal with the GAPDH signal. The detection level of all real time PCR experiments described in this thesis were set at 40 PCR cycles.

Table 1
Primers sequences used for semi-quantitative real time PCR using the Taqman real time PCR assay. mRNA levels were normalized to GAPDH mRNA levels.

	Primer sequences	Amplicon length
IGFBP-4		
FWD	5' GCA CCC ACG AGG ACC TCT T 3'	86 bp
RVS	5' GGG CTG GGT GAC ACT GCT T 3'	
Probe	6FAM-CGA CCG CAA CGG CAACTT CCA C-MGBNFQ	
Aggrecan		
FWD	5' CTG CTA CGG AGA CAA GGA TGA GT 3'	89 bp
RVS	5' CTG CGA AGC AGT ACA CGT CAT AG 3'	
Probe	6FAM-CCC TGG CGT GAG AAC CTA CGG CA-MGBNFQ	
PAPP-A		
FWD	5' GAC TTG CTT TGA TCC CGA CTC T 3'	88 bp
RVS	5' ATG TGT TGA TCC ATC CAA TTT CAG 3'	
Probe	6FAM-CTC ACA GAG CTT ATC TG-MGBNFQ	
IGF-I		
FWD	5' CGG AGG GCA CCT CAG ACA 3'	73 bp
RSV	5' ACA TCT CCA GCC TCC TCA GAT C 3'	
Probe	6FAM-CAT CGT GGA TGA GTG CTG CTT CCG-MGBNFQ 5'	
IGF-I receptor		
FWD	5' ACG CGC CTG ACG ACT ACA C 3'	63 bp
RSV	5' CGC AGA CGC GGA GAA 3'	
Probe	6FAM-CCT GCG TCG CCT GCC GCT AC-MGBNFQ	
GAPDH		
FWD	5' GAG ACA CGA TGG TGA AGG TCG 3'	61 bp
RVS	5' CTG GTG ACC AGG CGC C 3'	
Probe	6FAM-CCA ATG CGG CCA AAT CCG TTC A-MGBNFQ	

Appendix B (Chapter 5, 6, & 7)

Culture conditions for human articular chondrocytes

Cartilage sample collection

Articular cartilage was harvested post mortem (within 24 h after death) from femoral condyles of human donors. In chapter 4 and 6, the donors had no clinical history of degenerative, inflammatory or crystalline joint disorders and did not use corticosteroids (systemically or intra-articularly). In chapter 5, the cartilage of the experimental group (OA group) was harvested from OA knee joints during joint replacement surgery. For this experiment cartilage was harvested from macroscopically relatively normal appearing areas of the femoral condyles. However, cartilage biopsies from OA donors were more yellowish and had a softer texture compare to cartilage from healthy knee joints. No fibrous scar-like tissue was used.

Chondrocyte isolation and expansion

Within 12 hours after biopsy, chondrocytes were isolated by a 3-hour 0.1% pronase (Roche, Mannheim, Germany) digestion at 37°C followed by an O/N 0.04% colla-

genase (Sigma, St Louis, USA) digestion at 37°C. The cells were expanded in monolayer at 37°C and 5% CO_2 for 2 passages using two different expansion conditions: (A) The chondrocytes were plated at a cell density of 3500 cells/cm^2 and were cultured in DMEM (Gibco) containing L-Glutamin, 10% FBS, 1x non essential amino acids, 10 mM HEPES buffer, 100 µ/ml penicillin, 100 mg/ml streptomycin, 0.2 mM ascorbic acid and 0.4 mM proline (Basic Expansion Condition or BEC). (B) The chondrocytes were plated at a cell density of 5000 cells/cm^2 and were cultured in DMEM (Gibco) containing L-glutamine, 4.5 mg/ml glucose, 25 mM HEPES buffer, 10% FBS, 100 u/ml and penicillin, 100 mg/ml streptomycin. During the first three days of culture, no growth factors were added to the culture media. For the remaining culture period, the media were supplemented with 10 ng/ml bFGF (R&D, Minneapolis, MN, USA) (Growth Factor Supplemented Expansion Condition or GFSEC). In both methods, the culture medium was renewed every 3 days. At confluency, the cells were trypsinized using 0.25% trypsin/EDTA and replated.

Redifferentiation culture methods

After 2 passages, cells from generated through both expansion methods were redifferentiated using two techniques: Pellet culture. Pellets were formed by centrifuging aliquots of 5 x 10^5 cells at 500 x g for two minutes at room temperature. The pellets were cultured in DMEM (Gibco) containing Glutamax, 0.1% human serum albumin, 0.2 mM ascorbic acid, 1x ITS+1 (Sigma, St Louis, USA), 10 ng/ml IGF-I (R&D) and 10 ng/ml TGF-β2 (R&D) (10 ng/ml). Culture on collagen-coated filters. Cells were seeded at a cell density of 1.6 x 10^6 cells/cm^2 on Millicell filters (Millipore Co, Bedford, MA, USA) that were precoated with collagen type II (Sigma). The filters were cultured in DMEM (Gibco) containing, L-glutamine, 2% human serum albumin (Equitech-Bio, Kerrville, TX, USA), 1 x ITSx (Gibco), 5 ng/ml TGF-β2 (R&D) and 0.4 mM ascorbic acid. Both culture systems were incubated at 37°C in 5% CO_2 and culture media were renewed every 3 days.

For the experiments described in chapter 5 and 6, the GFSEC expansion conditions and collagen type-II coated filter technique were used. For the experimental group in chapter 6, 10% of the culture media were replaced with 10% synovial fluid.

Appendix C (Chapter 5, 6, & 7)

Analytical methods for cultures using human chondrocytes

Histology

Samples were fixed in 10% buffered formalin and cut at 5 µm for histological evaluation (Safranin O/Fast green). Safranin O staining was performed as described previously by Rosenberg[341]. The histological quality of the tissue synthesized was evaluated by four blinded observers and scored using a scoring system specifically designed to evaluate *in vitro* synthesized cartilage (Bern Score)[91]. This scoring system evaluates three parameters: (1) the darkness and uniformity of the safra-

nin O staining, (2) the amount and organization of the extracellular matrix, (3) the morphology of the cell. Each item scored minimum 0 points (worse) and maximum 3 points (best), thus the maximum score is 9 points (histologically resembling hyaline cartilage).

Immunohistochemistry for collagen I and II

After 28 days of culture, samples were embedded in Tissuetek O.C.T. compound (Sakura, Torrance, CA, USA), cut at 5 µm using a cryotome, and stained immunohistochemically for collagen type I and type II to assess the degree of redifferentiation. To this end, sections were incubated with monoclonal antibodies against collagen type I and II (1:1500, Omnilabo and 1:100, Developmental Studies Hybridoma Bank respectively) for 1 hour at room temperature (RT). Subsequently the samples were incubated with a biotinylated goat anti-mouse secondary antibody (1:100, DAKO) for 1 hour at RT. Diaminobenzidine (Liquid DAB+ Substrate, DAKO, Glostrup Denmark) was used to obtain a signal in positive cells or matrix. Healthy human skin was used as negative control for collagen type II and the middle and transitional zone of healthy human cartilage as a negative control for collagen type I. Additionally, all sections were stained leaving out the primary antibody to control for aspecific binding of the secondary antibody. Finally the sections were counterstained with haematoxylin.

Electron Microscopy

After 28 days of culture, samples were fixed in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4), post-fixed with 2% osmium tetroxide in cacodylic buffer, dehydrated through a graded alcohol series and embedded in Epon 812. Ultra-thin sections (60 nm) were cut using a Reichert Jung Ultracut E ultramicrotome (Leica, Rueil-Malmaison, France). Sections were stained using uranyl-acetate (7% in 70% methanol) and lead citrate (2%). Ultrathin sections were examined using an electron microscope (Jeol JEM1200 EX, Japan). Hyaline cartilage, obtained post-mortem from a healthy human femoral condyle was cut to provide reference and will be further referred to as native articular cartilage.

Proteoglycan synthesis

Proteoglycan synthesis rate was analyzed by a ^{35}S-incorporation assay after 9 days of culture in three samples per donor by a 4-hour incubation in the presence of 4 µCi $^{35}SO_4^{2-}$ (Na_2 $^{35}SO_4$, carrier-free; NEX-041-H; Dupont). Subsequently, the samples were rinsed three times in 4°C PBS to stop ^{35}S-incorporation and stored at -20°C until further analysis. At analysis, the samples were digested in 12% papain dissolved in 50 mM phosphate buffer, 2 mM N-acetylcysteine and 2 mM Na_2-EDTA, pH 6.5, at 65°C for 2 hours. Part of the digest was used to measure the DNA content and after precipitation of proteoglycans using 290 mM hexadecylpyridinium-chloride (Sigma) the precipitate was dissolved in 3 M NaCl and used to measure ^{35}S-incorporation (Tri-carb 1900CA, Liquid scintillation counter, Packard, USA). ^{35}S-incorporation was normalized to the DNA content in the sample.

Proteoglycan content of conditioned media and tissue samples

The proteoglycan turnover rate was analyzed using a previously described alcian blue assay, which measures the GAGs released in the culture medium[342]. To this end, culture medium was collected every 3 days and stored at –20°C until further analysis. GAGs were precipitated from the medium and stained with an Alcian blue dye solution (Alcian blue 8GX, Sigma, saturated in 0.1 M sodium acetate buffer, containing 0.3 M $MgCl_2$, pH 6.2) for 30 minutes at 37°C. The blue staining of the medium was quantified spectrophotometrically from the change in absorbance at 620 nm. Chondroitin sulphate (Sigma) was used as a reference. As indication of the proteoglycan turnover rate, values were normalized to the total amount of GAGs synthesized during the whole culture period (sum of total GAG content at day 28 and the total GAG release during the whole culture period).

Tissue proteoglycan content

As a parameter of matrix synthesized and contained within the tissue, the proteoglycan content after 28 days of culture was determined in three samples per donor using an Alcian Blue precipitation assay on tissue digests as described previously[55]. To this end, the samples were digested in 12% papain buffer as described above. Separate aliquots of the digest was used to determine DNA content and GAG concentration present in the samples was measured using the above described Alcian Blue assay.

DNA content

DNA content of the samples was determined as a measure of cellularity. In the papain digest samples, DNA was stained with the fluorescent dye HOECHST 33258 as described previously and fluorescence was measured on the Cytofluor[343]. Calf thymus DNA (Sigma) was used as a reference.

Total RNA-isolation

mRNA isolated from cartilage explants from OA human knee joints was used for normalization of gene expression levels in experimental samples (expanded chondrocytes). The cartilage biopsies were crushed using a pestle and mortar filled with liquid nitrogen. The ground cartilage was suspended in RNeasy lysis buffer (RLT buffer, RNeasy Mini Kit, Qiagen, Hilden, Germany). The suspension was homogenized using a syringe and a needle. Subsequently the samples were centrifuged for 10 min at 300 x g to remove debris and the supernatant was used for total RNA extraction as described below. Total RNA was extracted from cells cultured in monolayer after the first and second passage. At confluency, cells were trypsinized in 0.25% trypsin/EDTA. Subsequently the cells were washed in PBS. $5x10^5$ cells were spun at 300 x g at RT to form cell pellets that were stored at -80 °C until further total RNA extraction as described below. In order to determine post-redifferentiation mRNA levels as described in chapter 6, the cells were redifferentiated for 14 days on collagen type-II coated filters. When the culture was stopped, the filters were rinsed using PBS and the filters were cut out of their “cup-holder” using a scalpel. Subsequently, the filters were stored at -80 °C until further total RNA extraction as described below.

Table 2

Primers sequences used for semi-quantitative real time PCR using the Sybr Green assay. mRNA levels were normalized to GAPDH mRNA levels. Collagen type I, II, X and Aggrecan mRNA levels were semi-quantified using a Taqman real time PCR assay and normalized to GAPDH.

	Primer sequences	Amplicon length
Sox 9		
FWD	5' GAC TTC CGC GAC GTG GAC 3'	98 bp
RVS	5' GTT GGG CGG CAG GTA CTG 3'	
L-Sox 5		
FWD	5' ATC CCA ACT ACC ATG GCA GCT 3'	61 bp
RVS	5' TGC AGT TGG AGT GGG CCT A 3'	
Sox 6		
FWD	5' GCA GTG ATC AAC ATG TGG CCT 3'	119 bp
RVS	5' CGC TGT CCC AGT CAG CAT CT 3'	
MMP-13		
FWD	5' AAA TTA TGG AGG AGA TGC CCA TT 3'	125 bp
RSV	5' TCC TTG GAG TGG TCA AGA CCT AA 3'	
Cath B		
FWD	5' TGT GTA TTC GAA CTT CCT GCT 3'	112 bp
RSV	5' GTG TGC CAT TCT CCA CTC C 3'	
Collagen type I		
FWD	5' CAG CCG CTT CAC CTA CAG C 3'	83 bp
RSV	5' TTT TGT ATT CAA TCA CTG TCT TGC C 3'	
Probe	6FAM-CCG GTG TGA CTC GTG CAG CCA TC-MGBNFQ	
Collagen type II		
FWD	5' GGC AAT AGC AGG TTC ACG TAC A 3'	79 bp
RSV	5' CGA TAA CAG TCT TGC CCC ACT T 3'	
Probe	6FAM-CCG GTA TGT TTC GTG CAG CCA TCC T-MGBNFQ	
Collagen type X		
FWD	5' CAA GGC ACC ATC TCC AGG AA 3'	70 bp
RSV	5' AAA GGG TAT TTG TGG CAG CAT ATT 3'	
Probe	6FAM-TCC AGC ACG CAG AAT CCA TCT GA-MGBNFQ	
Aggrecan		
FWD	5' TCG AGG ACA GCG AGG CC 3'	85 bp
RSV	5' TCG AGG GTG TAG CGT GTA GAG A 3'	
Probe	6FAM-ATG GAA CAC GAT GCC TTT CAC CAC GA-MGBNFQ	
GAPDH		
FWD	5' AGA AGG CTG GGG CTC ATT T 3'	135 bp
RVS	5' GAG GCA TTG CTG ATG ATC TTG 3'	

At analysis, the pellets or the cells on the collagen-coated filters were lysed in RNeasy lysis buffer (RLT buffer, RNeasy Mini Kit) and total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol[344]. RNA concentration was measured spectrophotometrically at 260 nm and sufficient RNA purity was assumed if the 260/280 nm ratio was between 1.6 and 2.0. Subsequently, RNA samples were DNase treated and 750 ng of RNA was reverse transcribed using the iScript cDNA synthesis Kit (Bio-Rad, USA).

Semi-quantitative real time RT-PCR analysis

L-SOX-5, SOX-6 and SOX-9, MMP-13 and cathepsin B mRNA levels were determined using a SYBR green assay on the MyiQ thermal cycler (BioRad, USA). Human L-Sox-5, Sox-6 and Sox-9 primer sequences were kindly provided by Dr. A.D. Murdoch (Wellcome Trust Centre for Cell Matrix Research, University of Manchester, UK). The specificity of all primer sets was checked by BLASTN searches. Expression levels were normalized to endogenous GAPDH expression levels and were calculated using the method according to Pfaffl to correct for the efficiency of the reaction[345]. Primer sequences and concentrations are provided in table 1.
Collagen type I, II X and aggrecan mRNA levels were determined using a Taqman assay on the ABI prism 7900HT sequence detection system. Primers and probes were generously provided by Dr. H. Jahr and Dr. G.J.V.M. van Osch (Dept of Orthopaedics, Rotterdam, The Netherlands). Collagen and aggrecan primer and probe sequences are previously published by Martin and Mandl.[147,346]. GAPDH primer and probe endogenous control mix for the Taqman assay is commercially available (Applied Biosystems). The expression levels were normalized to endogenous GAPDH expression levels and were calculated using the 2^{-DDCT} formula (ABI Prism 7700 sequence Detection System User bulletin #2). Since collagen type II is one of the typical structural proteins synthesized by differentiated chondrocytes in hyaline cartilage, as compared to collagen type I that is normally synthesized by dedifferentiated chondrocytes, we defined the ratio of collagen type II and collagen type I mRNA levels as 'differentiation index'.

Appendix D (Chapter 8 and 9)

Detailed trial conditions

Patients

Eligible patients were aged > 18 years and had clinical evidence of OA as judged by the orthopaedic surgeon, defined by the presence of typical knee symptoms (pain, stiffness, disability) and radiographic evidence of OA (grade I-III on the Kellgren – Lawrence index). Other inclusion criteria were knee complaints surpassing the threshold indicated by at least two of the following questionnaires: maximal 60 points out of 100 points on the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), the pain sub-item of the Knee Injury and Osteoarthritis Outcome Score (KOOS), or the Knee Society clinical rating scale (KSCRS), and minimal 40 mm for the 100-mm Visual Analogue Scale(VAS) for pain.
Exclusion criteria were poor general health as judged by the orthopaedic surgeon; concomitant painful or disabling disease of the spine, hips, or lower limbs that would interfere with evaluation of the afflicted knee; suspicion of ipsilateral coxarthrosis and hip prosthesis loosening; any clinically significant or symptomatic vascular or neurological disorder of the lower extremities; crystalline, inflammatory and infectious arthropathies, known human immunodeficiency virus (HIV), hepatitis, cytomegalovirus (CMV) or syphilis infections, current diagnosis of osteomyelitis, alcohol/drug abuse, OA grade IV; known immunodeficiency; participation in

other trials within 3 months of inclusion, surgical and intra-articular pharmacological treatment within six months of inclusion, known coagulopathy; corticosteroid and anti-coagulant usage or morbid obesity.

Randomization

All participants were enrolled between February 2004 and August 2005. They were first seen in the practice of their orthopaedic surgeon, who evaluated eligibility for enrolment based on in- and exclusion criteria. Furthermore, the patients received a written information brochure and were informed verbally after which the patients were given at least 24 hours to consider participation in the trial. Subsequently, all patients were seen by a specially trained local study physician, who provided information concerning the trial, the products, and the alternatives, and obtained written informed consent. Next, in order to assess whether the participants met all inclusion criteria, the patients completed all questionnaires and x-rays (weight-bearing antero-posterior, so called notch view, weight-bearing lateral, and patellar skyline) were obtained. If the participants still met all in- and exclusion criteria, they were randomized. Therefore, a computer-generated randomization code was produced according to the "random-permuted-block within strata principle" by a researcher not affiliated with the trial using SYSTAT for windows (SYSTAT Inc., Evanston, Illinois, USA). The randomization was stratified for gender, age (<45 and >45 years of age) and NSAID-usage. In cases of severe subjective symptoms, patients were able to indicate whether NSAID usage would be continued during the trial. The randomization code was managed by a trial coordinator, who allotted the participants either to the Orthokin of the placebo group in a sequential manner according to the randomization code. The participants and their orthopaedic surgeon were blinded for the treatment to which the patients were assigned throughout the study.

One knee per patient was analyzed. Patients who needed bilateral treatment were randomized as described above and were treated with the same product in both knees. However, since two knees in the same patient cannot be analyzed statistically as independent specimens, an additional randomization step was performed to determine which knee would be analyzed.

Patients were informed that all placebo group patients would be given the opportunity to have the Orthokin treatment if effectiveness was determined at completion of the trial.

Intervention

After randomization, all participants returned to the local study physician, where 50 millilitres of venous blood was obtained using the Orthokin syringe, which contains the $CrSO_4$ coated glass beads. The syringe was gently rotated to ensure complete mixing and maximal contact of beads and blood, immediately stored at 37°C and shipped to the Orthogen laboratory within 24 hours in a designated transport incubator. At the laboratory, the blood samples were tested for Hepatitis A and B, and HIV because of uncertainty of virus titre response to such incubation. If blood samples were found positive for one of these diseases, the patients were

retested using new blood samples. In case of repeated positive test-outcome, the patients were excluded from the study. When the patient were tested negative, the Orthokin product was prepared by the Orthogen laboratory and was returned to the hospital after 14-21 days in 2 ml vials at -20°C. Subsequently, an injection regime of six injections was started in a rigid scheme comprising three weeks: injections were given on day 0, 3, 7, 10, 14 and 21. The participant was placed in a supine position, the knee was disinfected with alcohol draped in a sterile fashion. A sterile 21 gauge needle was placed supero-laterally into the supra-patellar pouch. The synovial fluid present was aspirated to minimize drug dilution. The needle was left in place and 2 ml Orthokin or 2 ml placebo (physiological saline) was injected through a 0.22 µm pore size anti-bacterial sterile filter. All procedures were identical for both the Orthokin and the placebo injections.

Follow-up

At 3, 6, 9 and 12 months after the first injection, the patients completed the same questionnaires as at baseline, namely the VAS for pain, the KOOS and the KSCRS. The WOMAC scores were deducted from the separate KOOS items. All questionnaires were sent by mail and were completed prior to the follow-up visits with the treating orthopaedic surgeon. At these follow-up visits, the treating orthopaedic surgeon performed a physical examination of the knee, completed the surgeon-part of the KSCRS, checked for adverse events and changes in NSAID and other analgesic use.
During the treatment and follow-up period, the patients were allowed to use only Acetaminophen (Paracetamol; maximum of 4 grams / day). For patients stratified into the NSAID-group, additional NSAID use was permitted. However, all patients were asked to stop all analgesics at least 1 week before completing the questionnaires and visiting their treating orthopaedic surgeon.

Appendix E (Chapter 9):

Methods used for validation of the reduced WOMAC scales

Reliability

This term encompasses the internal consistency of a scale, usually measured as Cronbach's alpha, which measures the degree of correlation among items[347]. A Cronbach's alpha of 0.7 is widely accepted to be the cut off point to be acceptable, values between 0.7 and 0.9 are good and above 0.9 are excellent, but also may indicate redundancies in the scale[348,349].
Furthermore, the test-retest reliability of the short form WOMAC function scale was assessed by calculation of the Lin's concordance correlation coefficient (ρ_c) for both the complete questionnaire as well as its items separately.

Validity

To examine the validity of a score or measurement tool, the results from this measurement on an appropriate patient cohort should be tested by calculating the cor-

relation with a measurement, which has been used and accepted in the field as a golden standard. To determine the convergent construct validity of the short form WOMAC function scale and the modified short form WOMAC, Lin's concordance correlation coefficients (ρ_c) with the traditional WOMAC scale was determined. The validity of both short form scoring systems was assessed by comparing the results for the traditional WOMAC (i.e. the gold standard) with the results of the short form WOMAC function scale and the modified short form WOMAC scoring systems by determining Lin's concordance correlation coefficient. This coefficient not only indicates how well pair of measurements conform to, or deviate from, a straight line, but also determines the degree of agreement, i.e. the extend to which the observations conform to a 45° line in a scatter plot when the traditional WOMAC is plotted against either the short form WOMAC function scale or the modified short form WOMAC[350]. Additionally, the Lin's concordance correlation coefficients (rc) of these scales with the separate KOOS items, the VAS for pain and the Knee Society Score were calculated. To visualize whether the accuracy of the short form WOMAC function scale score would be dependent up on the degree of symptomatology, a Bland-Altman plot is used, which plots the difference between the two scores (the traditional WOMAC and the short form WOMAC function scale) against the mean of the two scores[331,332]. Furthermore the Bland Altman plot was used to determine whether the accuracy of the scale would be dependent on the degree of symptomatology by assessing whether a funnel or cone effect is observed when looking at the scatter of the points in the plot, as such an effect would imply a dependency. In addition, in the Blant Altman plot the upper- and lower limits of agreement were indicated. These were calculated by adding and subtracting 2 times the standard deviation of the differences to and from the mean of the difference. A paired- t-test was used to assess whether there is evidence of bias, in which case there would be a systematic difference between the two sets of measurements, with one of the scoring systems tending to give larger values than the other.

The floor and ceiling range of the short form WOMAC function scale, the modified WOMAC and the traditional WOMAC were determined. The floor and ceiling effects were defined as the percentage of respondents that score, respectively, the minimum and maximum levels on that scale. Ideally, no more than 10 % should fall at the bottom or top of the scale[351]. Higher floor/ceiling effects indicates insensitivity of the tool for respectively worsening or improvement of symptoms.

Responsiveness

This term is defined as the sensitivity of an assessment technique to change over time. Responsiveness was determined by examining the effect size (ES) and the standardized response means (SRM). The ES is equal to the mean change in score from baseline to 3 (or 6) months divided by the standard deviation of the baseline score[352]. The SRM is calculated as the mean change in score from baseline to 3 or 6 months follow-up divided by the standard deviation of the change in score[353]. A large value of the ES or SRM indicates that the technique has high sensitivity to

change, and small values indicate either a lack of responsiveness in the technique or no change over time in the population studied. For ES and SRM, a value of 0.2 to 0.5 was regarded as "small", 0.5 to 0.8 as "medium" and those above 0.8 as "large"[352].

Appendix F (Chapter 10)

Methods used for validation of automated bone bruise segmentation

In- and exclusion criteria

The main inclusion criteria were single symptomatic cartilage lesions on the femoral condyles and lesion size between 1 and 5 cm^2 (defect surface area was determined during arthroscopy). These cartilage defects were diagnosed by both MRI and arthroscopy, all lesions were grade IV chondral defects. The main exclusion criteria were surgical and intra-articular pharmacological treatment within six months before surgery, additional chondral defects at other locations in the joint, advanced osteoarthritis (radiological grade 2-3), complex ligamentous instability, lateral meniscal damage or past resection, and current medial meniscal damage or past resection of more than 50%.

MRI Settings

MR images were acquired on a Philips Gyroscan ACS-NT at 1.5T (8-channel, knee coil; Philips Medical Systems, Best, The Netherlands). All MRIs were acquired using the same protocol, consisting of non-dual PDw and T2-w MRI scans. All scans were performed with a 3.3 mm slice thickness, 24 contiguous slices, 150x150 mm field of view and a 512x512 scan matrix. The individual scan parameters were: PDw – repetition time (TR)/echo time (TE) 2244/35 ms; T2-w – TR/TE 4027/70 ms. Intra-patient rigid registration by mutual information was performed on the images in order to achieve spatial correlation between the PDw and T2-w scans[354].

Automatic MRI segmentation

The proposed method for bone-bruise segmentation determines the probability of each voxel being bone bruise, based on the signal intensities and spatial coordinates in one or more MR images. The method used is based on the KNN-classification, which invokes a learning set of MR images for training purpose. In theses images all voxels are manually pre-classified as bone-bruise or normal tissue. In this study, both the PDw and T2-w MRI scans of 8 patients were manually segmented as learning set for the automated segmentation software. These 8 MRIs were also included in the final analysis of bone bruise volumes, but the learning set for segmentation of one patient was built from the voxels of the other 7 patients (the so-called ''leave-one-out'' method).

Before automatic segmentation of the bone bruise, masks, denoting the femoral condyles, were provided to the system by manual input from the programmers, in order to indicate the region of interest for the segmentation.

The method used for automatic segmentation of the MRIs takes a probabilistic approach and calculates the likelihood of a voxel being part of a particular tissue type, which in the current study consists of bone bruise[87]. The probability calculation is based on the grayscale of the voxels compared to the neighboring voxels and spatial information, i.e. the location within the femoral condyle. The volume of the bone bruise was calculated by two different approaches, using either a probabilistic or binary voxel values. For calculation of the probabilistic volume, the calculated likelihood of a voxel being part of the bone bruise was taken into account, e.g. a likelihood of 20% results in 0.2 voxel volume. For calculation of the absolute volume, a binary approach was applied after setting a threshold of >50% likelihood, i.e. each voxel with 50% likelihood or more of being part of the bone bruise counts for 1 voxel volume, each voxel with 49% likelihood or less counts for 0 voxel volume. Voxel volume was calculated by multiplication of voxel surface area with slice thickness. Volumes are given in cm^3.

Appendix G (Chapter 11)

Cartilage samples

For this study, 38 samples were obtained during a large investigation into the effect of early osteoarthritis on cartilage regeneration, using an *in vivo* goat model. The samples represented the full range from normal to severely disrupted articular cartilage[37]. All samples were of rectangular (5 by 10 mm) full-thickness defects from the medial femoral condyl. Goats were divided in three experimental groups: (1) a defect without treatment, (2) a defect with immediate periosteal transplantation, and (3) a defect with a periosteal transplantation after 10 weeks of free loading of the defect. Postoperatively, all knees were loaded without restriction for 10 weeks, after which the cartilage defect tissue and adjacent subchondral bone were harvested[37]. For this study, approval was given by the Animal Experimental Ethics Committee of the University of Utrecht.

Histological processing

Immediately after harvesting, samples were fixed in 4% buffered formalin and decalcified using the method of Christensen followed by processing with a Vacuum Infiltration Processor (VIP) and cedar oil (at 37°C). Following the decalcification process, the cartilage samples were embedded in paraffin and from the center of each sample 3 mm thick sections were cut. All samples were deparaffinized using xylene and stepwise incubation in alcohol (100–96–70%). Hereafter, three slides from each cartilage sample were colored with either Hematoxilin-Eosin, Alcian Blue-Fast Green, or Safranin O-Fast Green.

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Nederlandse samenvatting

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Introductie

Gewrichtskraakbeen is een gespecialiseerd weefsel dat gewrichten voorziet van een glad glijdend oppervlak. Na een gewrichtstrauma zoeken patiënten vaak medische hulp vanwege acute of aanhoudende klachten welke toegeschreven kunnen worden aan kraakbeendefecten. De incidentie van kraakbeendefecten wordt geschat op 2,6 patiënten per 1000 volwassenen. Helaas heeft kraakbeen een beperkte genezingscapaciteit en kunnen defecten zelfs leiden tot het ontwikkelen van gewrichtsslijtage (artrose). Huidige behandelingstechnieken, zoals het schoonmaken van gewrichten, het transplanteren van kraakbeenplugs uit andere gedeelten van het gewricht, of het doorboren van de onderliggende botlaag (microfracturing), resulteren in weefsel met inferieure structurele en mechanische eigenschappen in vergelijking met het oorspronkelijke gewrichtskraakbeen. Dit heeft tot gevolg dat deze technieken resulteren in slechts een tijdelijke verbetering van symptomen en onvoldoende beschermen tegen de ontwikkeling van artrose. Dit heeft een aanzienlijke drijfveer gevormd voor de snelle ontwikkelingen op het gebied van regeneratieve geneeskunde en de implementatie van dergelijke technieken voor de behandeling van kraakbeendefecten.

In vitro en *in vivo* studies hebben aangetoond dat het mogelijk is om betrouwbaar en reproduceerbaar hyaline kraakbeen (gewrichtskraakbeen) te regenereren. Derhalve is begin jaren negentig Autologe Chondrocyten Implantatie (ACI), een voorbeeld van een kraakbeen regeneratie techniek, bestudeerd als behandeling voor kraakbeendefecten. De initiële resultaten waren veelbelovend. Een recente studie heeft aangetoond dat ACI resulteert in een vergelijkbare verbetering van symptomen als microfracturing, wat momenteel als de behandelingsstandaard wordt beschouwd. Echter, deze studie demonstreerde dat beide behandelingen resulteerden in regeneratieweefsel dat varieerde van fibreus (litteken = slecht) weefsel tot hyaline-achtig kraakbeen (goed), alhoewel er ten gevolge van een aanzienlijke loss-to-follow-up geen sluitende conclusies getrokken konden worden met betrekking tot het verschil in histologische kwaliteit tussen ACI en microfracturing. Opvallend is dat de kwaliteit van het regeneratie weefsel meer variabel is dan eerdere *in vitro* en *in vivo* dierexperimentele onderzoeken zouden doen verwachten. Deze discrepanties kunnen mogelijk verklaard worden door het concept van gewrichtshomeostase. Dit concept onderstreept het feit dat de behandeling van kraakbeendefecten niet beperkt is tot het defect zelf, maar dat het gehele gewricht als orgaansysteem beschouwd dient te worden, waarbij een verscheidenheid aan intra-articulaire factoren het proces van kraakbeenregeneratie kunnen beïnvloeden. Dit stimuleert ons aanvullend onderzoek te verrichten naar de oorzaak van de discrepanties tussen klinische en experimentele resultaten, waarbij mogelijk grensvoorwaarden gedefinieerd kunnen worden voor succesvolle kraakbeenregeneratie en mogelijke “targets” geïdentificeerd kunnen worden voor verbetering van deze technieken. Bij dit laatste kan men denken aan zogenaamde “disease modifying drugs” welke als doel hebben het natuurlijk beloop van kraakbeendefecten (zoals de ontwikkeling van artrose) te vertragen/voorkomen en mogelijk zelfs het proces

van kraakbeenregeneratie te verbeteren door het optimaliseren van de gewrichtshomeostase.
Concluderend kan gesteld worden dat, op het gebied van regeneratieve geneeskunde, biologische gewrichtsreconstructie een realistisch doel is, alhoewel toekomstig onderzoek voor optimalisatie van deze technieken legitiem lijkt. Bij toekomstige studies dienen onderzoekers te streven naar het gebruik van uniforme uitkomst parameters, aangezien dit de vergelijkbaarheid tussen studies, en daarmee de voortgang van ons begrip omtrent het proces van biologische gewrichtsreconstructie, begunstigt.

Doelstelling van dit proefschrift
In dit proefschrift worden een aantal studies beschreven met een viertal centrale doelen:
- Om te onderzoeken of ACI resulteert in structureel beter regeneratieweefsel dan microfracturing.
- Om het effect van donorweefsel kwaliteit en kraakbeendefect gerelateerde (intra-articulaire) veranderingen op kraakbeenregeneratie te verduidelijken.
- Om de klinische effectiviteit van een autologe vorm van interleukine-1 antagonist als “disease modifying drug” te evalueren.
- Om nieuwe evaluatie methodes voor toekomstige artrose en kraakbeenregeneratie studies te ontwikkelen en te valideren.

Resultaten en conclusies

Doel 1: Om te onderzoeken of ACI resulteert in structureel beter regeneratieweefsel dan microfracturing.

Brittberg heeft in 1994 de eerste klinische resultaten van autologe chondrocyten implantatie (ACI) voor de behandeling van kraakbeendefecten gepubliceerd. Sindsdien wordt deze techniek in toenemende mate gebruikt en vele publicaties hebben veelbelovende resultaten gerapporteerd. In **hoofdstuk 3** wordt in een prospectief gerandomiseerde klinische trial aangetoond dat ACI significant beter regeneratieweefsel oplevert dan microfracturing, dat momenteel als behandelingsstandaard beschouwd wordt. Desondanks hebben wij waargenomen dat ACI nog altijd resulteert in kraakbeenregeneratie met een variërende morfologische kwaliteit. Klinisch resulteerde ACI 12-18 maanden postoperatief in een vergelijkbare verbetering als microfracturing, terwijl ACI een twee-staps procedure is met als laatste een “open” procedure (arthrotomie) versus de een-staps “kijkoperatie” (arthroscopie) bij microfracturing. Tenslotte werd er gevonden dat de veiligheidsprofielen van beide behandelingen vergelijkbaar waren. Uit deze resultaten hebben wij geconcludeerd dat ACI beschouwd zou moeten worden als een nieuwe behandelingsklasse die resulteert in superieur structureel herstel vergeleken met microfracturing.

Implicaties van de resultaten

Voor zover ons bekend is dit de eerste klinische trial die volledig verricht is volgens "Good Clinical Practice" (GCP) richtlijnen met veiligheidsmonitoring en die het superieure structurele herstel van ACI ten opzichte van microfracturing demonstreert. Echter, microfracturing wordt nog altijd als de behandeling van keuze beschouwd. Dit lijkt incorrect aangezien recentelijk is aangetoond dat de kwaliteit van het regeneratieweefsel gerelateerd is aan de "overleving" van het regeneratieweefsel en aan de geobserveerde klinische symptoomverlichting. Dit suggereert dat de lange-termijnsuitkomst van ACI beter zal zijn dan dat van microfracturing. Al met al ondersteunen deze resultaten het toenemende gebruik van ACI en toekomstige *in vitro* en *in vivo* studies op het gebied van ACI.

Er zijn een aantal aspecten die verduidelijkt dienen te worden en/of mogelijke aanknopingspunten vormen voor de optimalisatie van ACI. Zo worden er frequent zorgen geuit over het mogelijk optreden van "donor site morbidity", of te wel nadelige effecten ten gevolge van het nemen van een kraakbeenbiopt. Er zijn echter al vele klinische trials beschreven en geen van deze rapporteren dergelijke nadelige gevolgen. Verder bestaat de huidige ACI techniek uit een twee-staps procedure waarbij de tweede ingreep een arthrotomie betreft. Naast het feit dat dit een aanzienlijke belasting betekent voor de patiënt, leidt dit waarschijnlijk tot meer verstoring van de gewrichtshomeostase dan wanneer ACI een één-stap arthroscopische procedure zou omvatten. Deze overwegingen ondersteunen het voortgaande onderzoek naar andere bronnen van cellen, bijvoorbeeld oor- of neuskraakbeen, voorlopercellen uit beenmerg (bone marrow stromal cells) en andere kraakbeen voorlopercellen.

Ten slotte suggereert de variatie in morfologische uitkomst van ACI dat er nog ruimte is voor aanzienlijke optimalisatie van deze techniek. De studies beschreven in **hoofdstuk 4, 5, en 6** hebben als doel meer inzicht te bieden in de *in vitro* modellen die gebruikt worden voor kraakbeen tissue engineering studies en de invloed van intra-articulaire factoren, zoals de kwaliteit van het donor weefsel en het synoviaalvocht (SF), op het proces van chondrogenese en kraakbeenregeneratie technieken.

Doel 2: Om het effect van donorweefsel kwaliteit en kraakbeendefect gerelateerde (intra-articulaire) veranderingen op kraakbeenregeneratie te verduidelijken.

Om de uitkomst van kraakbeenregeneratie technieken te kunnen verbeteren is een grondig begrip vereist van de mechanismen die betrokken zijn gedurende dit proces. Transforming Growth Factor-β (TGF-β) en Insulin-like Growth Factor-I (IGF-I) zijn belangrijke celdeling en celdifferentiatie stimulerende groeifactoren bij chondrogenese. Er is gesuggereerd dat TGF-β de *in vivo* beschikbaarheid van IGF-I beïnvloed door het reguleren van IGF-bindende eiwitten (IGF-binding proteins of IGFBPs). Er is echter weinig bekend over hoe deze groeifactoren elkaar beïnvloeden gedurende chondrogenese. In **hoofdstuk 4** wordt aangetoond dat TGF-β gesupplementeerde kweek media de IGF-as reguleren door een afname van IGFBP-4

mRNA levels en afname van de excretie van IGFBP-3 en IGFBP-4 door gecultiveerde periostale explantaten. Verder resulteerde TGF-β in toegenomen Pregnancy Associated Plasma Protein-A (PAPP-A of IBFBP-4 protease) mRNA levels en eiwit niveaus na 7 tot 10 dagen kweken (het begin van kraakbeen formatie in dit model). Tenslotte werden IGF-I en IGF-I receptor mRNA-levels verlaagd door TGF-β. Hieruit is de conclusie getrokken dat TGF-β verschillende delen van de IGF-as reguleert gedurende *in vitro* periostale chondrogenese.

In eerdere studies is vastgesteld dat synoviaal vocht (SF) een belangrijke invloed heeft op kraakbeenregeneratie. SF is een biologische (gewrichts-)vloeistof, die cytokines, groeifactoren en proteolytische enzymen bevat. Om de mogelijke negatieve effecten van deze (micro-)omgevingsfactoren te bestuderen is een betrouwbaar en reproduceerbaar chondrogenetisch model noodzakelijk. In **hoofdstuk 5** zijn eerder gepubliceerde expansie en redifferentiatie condities vergeleken. In vergelijking met de basale expansie condities (BEC) resulteerden groeifactor-gesupplementeerde expansie condities (GFSEC) in een duidelijke verbetering van de *in vitro* kraakbeenvorming (onafhankelijk van het model dat voor de redifferentatie is gebruikt), zoals aangetoond door de significant verbeterde morfologie en hogere proteoglycaan-content van het regeneratieweefsel. Interessant was de observatie dat chondrocyten expansie in GFSEC resulteerde in een significante afname van collageen type II, SOX-5 en SOX-6 mRNA hoeveelheden in vergelijking met expansie in BEC. Dit lijkt in tegenstelling te zijn met het verbeterde post-redifferentiatie resultaat. Verder resulteerde redifferentiatie van chondrocyten op collageen gecoate filters in betere chondrogenese (kraakbeennieuwvorming) in vergelijking met pelletkweken, zoals een vermeerdering van de uiteindelijke PG content, en verbeterde chondrocyt morfologie en extracellulaire matrix (ECM) organisatie.
Uit deze studie hebben we geconcludeerd dat, van de geteste kweekmodellen, chondrocyten expansie in GFSEC en redifferentiatie op collageen gecoate filters resulteerde in de meest optimale chondrogenese. Om die reden is dit model gebruikt in de studies welke zijn beschreven in hoofdstuk 6 en 7.

In vele *in vitro* studies wordt gebruik gemaakt van chondrocyten welke geoogst worden van zogenaamde minder-aangedane gebieden van artrotische gewrichten, aangezien dit makkelijk te verkrijgen is gedurende gewrichtsvervangende chirurgie. Verder is er recentelijk gesuggereerd dat ACI mogelijk een geschikte therapie zou zijn voor vroege stadia van artrose. Echter, het is onduidelijk hoe de aan artrose gerelateerde veranderingen in chondrocyten het proces van chondrogenese beïnvloeden. Het doel van de studie in **hoofdstuk 6** was om te onderzoeken in welke mate OA chondrocyten een veranderd fenotype vertonen na expansie (*in vitro* celdeling) in vergelijking met chondrocyten van gezonde gewrichten. Vervolgens zijn de verschillen in chondrogenetische capaciteit tussen beide donortypen (gezond versus OA) vergeleken. Na celexpansie kwam, het aan kraakbeenhypertrofie gerelateerde, collageen type X duidelijk tot expressie in geëxpandeerde chondrocyten van OA gewrichten, terwijl dit niet detecteerbaar was in chondrocyten uit gezonde gewrichten. Daarbij had het weefsel geregenereerd door chondro-

cyten van gezonde gewrichten een significant betere morfologie en hogere proteoglycaan-content in vergelijking met weefsel geregenereerd door OA chondrocyten. Uit deze resultaten is geconcludeerd dat chondrocyten uit artrotische gewrichten veranderde (verslechterde) chondrogenetische eigenschappen hebben in vergelijking met chondrocyten uit gezonde gewrichten. Om deze reden lijken *in vitro* studies die gebruik maken van OA chondrocyten niet representatief voor klinisch toegepaste ACI en dienen zij met voorzichtigheid geïnterpreteerd en geëxtrapoleerd te worden. Verder lijkt ACI momenteel nog geen geschikte therapie te zijn voor vroege stadia van artrose.

Eerdere studies hebben een nadelig effect aangetoond van verstoorde gewrichtshomeostase op de proteoglycaan turnover en weefsel morfologie van het regeneratieweefsel. In **hoofdstuk 7** hebben we het effect van menselijk synoviaal vocht (SF) van getraumatiseerde kniegewrichten op kraakbeenregeneratie bestudeerd. SF resulteerde in een **significante** vermindering van het definitieve proteoglycaan gehalte, collageen type II mRNA hoeveelheden en differentiatie index in vergelijking met gecontroleerde kweek condities. Interessant hierbij is dat SF resulteerde in een significant hogere cellulariteit van het regeneratieweefsel. De morfologie en de collageen type I, type X en aggrecan mRNA hoeveelheden werden niet beïnvloed door SF toevoeging aan kweekmedia. Uit deze studie is geconcludeerd dat SF van aangedane knie gewrichten een negatief effect heeft op *in vitro* chondrogenese met betrekking tot de ECM-turnover en de mate van chondrocyt redifferentiatie.

Implicaties van de resultaten

Deze studies geven meer inzicht in de wijze waarop kraakbeendefect gerelateerde veranderingen het proces van kraakbeen tissue engineering zouden kunnen beïnvloeden en in welke mate het resultaat van kraakbeenregeneratie technieken verbeterd zou kunnen worden door gewijzigde kweekcondities en mogelijk door peri-operatieve beïnvloeding van de gewrichtshomeostase.

Onze resultaten tonen aan dat TGF-β geïnduceerde chondrogenese niet alleen wordt beïnvloed door de directe chondrogenetische eigenschappen van deze groeifactor, maar ook door de toegenomen hoeveelheid "vrij" beschikbare (bio-available) IGF-1 door verminderde IGFBP-4 concentraties en toegenomen IGFBP-4 protease (PAPP-a) activiteit. Of dit fenomeen daadwerkelijk resulteert in een toegenomen IGF activiteit moet nog worden aangetoond, aangezien we tevens een achteruitgang vonden van IGF-1 en IGF-1-receptor mRNA hoeveelheden. Afgezien van een toegenomen inzicht in het mechanisme van kraakbeenvorming in het algemeen, kunnen deze resultaten van betekenis zijn voor klinisch toegepaste ACI, aangezien van kraakbeenschade en artroseontwikkeling is aangetoond dat zij resulteren in een toegenomen TGF-β beschikbaarheid. Aangezien IGF-1 een mitogeen effect heeft, zou het bovenbeschreven mechanisme de toegenomen cellulariteit (celhoudendheid), geïnduceerd door SF uit getraumatiseerde gewrichten, kunnen verklaren. Verder kunnen technieken, die tot doel hebben om kraakbeenregeneratie te

optimaliseren door een verhoging van de peri-celullaire groeifactor concentraties, bijvoorbeeld door gentherapie en groeifactor release uit scaffoldcoatings, zich mogelijk beperken tot TGF-β. Deze benadering heeft mogelijk een substantieel voordeel, aangezien de toevoeging van exogene (intra-articulaire) groeifactoren in verband is gebracht met aanzienlijke bijwerkingen.
Interessant was de observatie dat er een discrepantie bestaat tussen post-expansie collegeen type II mRNA levels en het chondrogenetisch potentieel van deze geëxpandeerde chondrocyten. Deze resultaten tonen aan dat voor optimalisatie van expansie condities, screening van enkel post-expansie collageen type I en II mRNA levels alleen niet voldoende is.
De observatie dat *in vitro* kraakbeenregeneratie gestimuleerd wordt door redifferentiatie op collageen type II-coated filters verschafte ons niet alleen een betrouwbaar en reproduceerbaar chondrogenetisch model, maar impliceert tevens dat collageen type II een bruikbare matrix is voor zogenaamde "Matrix-induced Autologous Chondrocyte Implantation" (MACI) toepassing.
Deze techniek ondersteunt mogelijk niet alleen kraakbeenregeneratie en het chondrogenetische phenotype, maar kan ook de chirurgische procedure aanzienlijk vereenvoudigen en zou zelfs een artroscopische implantatie procedure kunnen faciliteren, bijvoorbeeld met behulp van een self-setting collageen type II-gebaseerde polymeer. Bovendien kunnen scaffold (cel-drager) materialen mechanische ondersteuning bieden aan de geïmplanteerde cellen, wat significant gunstige consequenties kan hebben voor de post-chirurgische revalidatie periode.
Chirurgische procedures die zijn gebaseerd op ACI lijken niet geschikt te zijn voor patiënten met artrose, aangezien chondrocyten van OA kniegewrichten een veranderd collageen expressie profiel hebben na expansie en een verminderde chondrogenetische potentie na redifferentiatie. Deze observatie heeft een belangrijke impact op de interpretatie van *in vitro* kraakbeenregeneratie studies, welke frequent gebruik maken van chondrocyten geoogst vanuit niet/minder aangedane gebieden van artrotische gewrichten. Aldus zouden dergelijke studies met grote zorgvuldigheid geïnterpreteerd moeten worden. Aangezien kraakbeendefecten kunnen resulteren in vroegtijdige ontwikkeling van OA, suggereren deze resultaten dat kraakbeendefecten zo vroeg mogelijk, nadat het defect is ontstaan, behandeld zouden moeten worden, of, in ieder geval, voordat phenotypische veranderingen van de chondrocyten in het omgevende kraakbeen zijn ontstaan. Nader onderzoek naar mogelijke phenotypische veranderingen van chondrocyten uit gewrichten met al langer bestaande defecten en de dynamica van de overgang van gezonde naar OA chondrocyten in aangedane kniegewrichten is noodzakelijk om vast te stellen of dit een plotseling optredend of een langzaam, in eerste instantie macroscopisch onzichtbaar, proces is.

Naast het aspect van donor weefsel kwaliteit moet men zich realiseren dat kraakbeenschade een aandoening is die het gehele gewricht als orgaansysteem beïnvloedt en niet slechts een geïsoleerde kraakbeenafwijking betreft. Deze aandoening resulteert in een verstoorde gewrichtshomeostase. In het bijzonder bevat het SF een mix van anabole en catabole mediatoren. In het verlengde hiervan is

van SF van beschadigde kniegewrichten eerder aangetoond dat dit een negatief effect heeft op de morfologie en de ECM-turnover. Daarbij werd de chondrocyt redifferentiatie geremd door SF van getraumatiseerde kniegewrichten. Deze resultaten ondersteunen de hypothese dat het lokale intra-articulaire milieu mogelijke "targets" bevat die, in het kader van toekomstige verbetering van ACI, middels patiëntenselectie, of door voorbehandeling van aangedane gewrichten om gewrichtshomeostase te herstellen, beïnvloed kunnen worden. Zo'n "target" zou IL-1β kunnen zijn, aangezien deze cytokine een rol speelt bij de ontstekingsreactie na beschadiging van diverse humane weefseltypes, wat essentieel is voor adequate wondheling. Echter, in de context van gewrichtskraakbeen is IL-1β gerelateerd aan kraakbeendegeneratie en de ontwikkeling van artrose en reumatoïde arthritis. Daarom is interfereren met IL-1β in SF mogelijk een geschikte therapie voor de behandeling of misschien zelfs preventie van artrose. Daarnaast zou dit een geschikte methode kunnen zijn om de gewrichtshomeostase te herstellen met als doel kraakbeenregeneratie technieken te verbeteren. Deze benadering is in een eerdere klinische trial bestudeerd, waarbij een autologe vorm van IL-1 receptor antagonist is vergeleken met corticosteroïden en een placebo behandeling. Ondanks diverse methodologische beperkingen van deze trial, lijken de resultaten de hypothese dat het remmen van IL-1β een gunstig effect heeft op de klinische symptomen bij artrose, te ondersteunen.

Doel 3: Om de klinische effectiviteit van een autologe vorm van interleukine-1 antagonist als "disease modifying drug" te evalueren.

Interleukine-1β (IL-1β) is een pro-inflammatoire cytokine (een mediator welke ontstekingsreacties in het lichaam stimuleert) en wordt in vele verschillende weefsels geïnduceerd als gevolg van weefselschade. Dit proces is essentieel voor herstel van weefselschade, echter in de context van gewrichtskraakbeen heeft dit eiwit een ongunstig effect en is het betrokken bij de initiatie en voortgang van artrose ontwikkeling. Orthokin is een autoloog bloedproduct dat hoge concentraties Interleukine-1 receptor antagonist (IL-1ra) bevat, een eiwit dat de activiteit van IL-1 kan tegengaan door de receptor van IL-1 te blokkeren. Aangezien Orthokin oorspronkelijk ontwikkeld is voor de behandeling van artrose, is een multicenter, prospectieve, dubbelblinde, placebo gecontroleerde trial verricht om de klinische effectiviteit van Orthokin bij patiënten met artrose van de knie te onderzoeken (Hoofdstuk 8). Alhoewel Orthokin niet de beoogde mate van effectiviteit induceerde (30% meer verbetering dan in de controle groep), resulteerde de behandeling wel in significant meer verbetering op het gebied van algemene symptomen (KOOS sport schaal) en de activiteit van de patiënt (KOOS sport schaal) in vergelijking met placebo behandeling. Verder scoorden Orthokin behandelde patiënten consistent beter op alle uitkomst parameters dan placebo behandelde patiënten. Uit deze resultaten hebben wij geconcludeerd dat Orthokin een duidelijk gunstig *in vivo* biologisch effect heeft, alhoewel de klinische effectiviteit onder trial condities beperkt is.

Implicaties van de resultaten

Ondanks de beperkte symptoomverlichting heeft deze studie een duidelijk biologisch effect van Orthokin aangetoond. Deze bevinding is mogelijk een belangrijke stap op het gebied van artrose behandeling, aangezien Orthokin, naar ons beste weten, de enige beschikbare disease modifying osteoarthritic drug (DMOAD) is welke niet alleen een duidelijk gunstig klinisch effect, maar tevens een duidelijk werkingsmechanisme heeft waarmee het mogelijk een kraakbeenbeschermend effect heeft. Men dient zich er echter bewust van te zijn dat juist deze eigenschap niet aangetoond is in de huidige studie door gebrek aan betrouwbare instrumenten om dit te meten en gezien de relatieve korte follow-up termijn van deze studie. Derhalve dienen toekomstige *in vivo* en *in vitro* experimenten en gecontroleerde trials met langetermijn radiologische follow-up dit aspect te verduidelijken.
Alhoewel diverse *in vitro* studies en dierexperimenten de rol van IL-1β bij artrose ontwikkeling lijken te ondersteunen, is de rol van deze cytokine bij humane artrose ontwikkeling nog nooit onomstotelijk vastgesteld. Onze trial lijkt deze hypothese te ondersteunen. Hierbij dient men zich te realiseren dat Orthokin een bewerkt autoloog bloedproduct betreft waarbij naast de stimulatie van IL-1ra mogelijk andere anti-inflammatoire (ontstekingsremmende) cytokines worden gestimuleerd en mogelijk het gunstige effect van Orthokin niet geïnduceerd wordt door remming van IL-1β. Gezien de biologische effectiviteit zou Orthokin, en mogelijk andere behandelingen die zich richten op interferentie met lokale mediatoren, niet alleen geschikt zijn voor behandeling van artrose, maar tevens voor het herstel van de gewrichtshomeostase en optimalisatie van kraakbeenregeneratie technieken zoals ACI.

Doel 4: Om nieuwe evaluatie methodes voor toekomstige artrose en kraakbeenregeneratie studies te ontwikkelen en valideren.

Regeneratieve geneeskunde ontwikkelt zich snel en dient beschouwd te worden als onderdeel van de standaard orthopaedische interventie mogelijkheden voor de behandeling van kraakbeendefecten. Echter, uit voorgaande hoofdstukken is duidelijk geworden dat er nog aanzienlijke ruimte is voor verbetering van deze technieken en dienen toekomstige studies gestimuleerd te worden. Om studies, onderzoekscentra, technieken en patiëntenpopulaties te kunnen vergelijken, is het van essentieel belang om gebruik te maken van gevalideerde uniforme uitkomst parameters/tools. Derhalve hebben we nieuwe onderzoeks- en klinische tools ontwikkeld en gevalideerd voor de evaluatie van kraakbeen regeneratietechnieken en de behandeling van artrose.
Frequent wordt er gebruik gemaakt van uitgebreide vragenlijsten om de status van een patiënt en de effectiviteit van een behandeling in beeld te brengen. Echter, korte vragenlijsten hebben een aantal voordelen: gebruiksgemak, minder tijdrovend voor de patiënt en bij analyse, en verbeterde “compliance” van de patiënt. De Western Ontario McMasters Universities Outcome Scale (WOMAC) wordt momenteel beschouwd als de gouden standaard voor evaluatie van artrose behandeling. In **hoofdstuk 9** hebben we het gebruik van een verkorte vorm van de

WOMAC (short form WOMAC function scale) gevalideerd voor evaluatie van niet-chirurgisch behandelde artrose. Deze vragenlijst toonde een excellente interne consistente en test-hertest betrouwbaarheid, een hoge correlatie met de traditionele WOMAC en adequate "responsiveness" (gevoeligheid om veranderingen over tijd te detecteren). Concluderend biedt de short form WOMAC function scale een valide, betrouwbaar en responsief alternatief voor de traditionele WOMAC voor de evaluatie van niet-chirurgisch behandelde artrose patiënten.

Bone bruise is een radiologisch fenomeen dat frequent gezien wordt op MRI scans van knieën met kraakbeendefecten. Dit fenomeen is geassocieerd met de klinische symptomen die optreden ten gevolge van kraakbeendefecten. In **hoofdstuk 10** hebben we een validatie-studie verricht voor het softwarematig detecteren van bone bruise op MRI scans. Allereerst is een hoge inter-observer betrouwbaarheid van een eerdere gepubliceerde subjectieve bone bruise gradering schaal gedemonstreerd. Vervolgens zijn er een tweetal MRI settings getest (proton densitiy-gewogen en T2-gewogen MRI scans). De incorporatie van T2-gewogen MRI scans resulteerde in een meer a-specifieke afgrenzing van het bone bruise gebied en werd om die reden niet gebruikt bij verdere segmentaties van MRI scans. Automatisch gesegmenteerde bone bruise volumes toonden een hoge positieve correlatie met de subjectieve bone bruise gradering schaal, wat de eerder genoemde subjectieve nauwkeurigheid van de segmentatiesoftware ondersteunt. Tenslotte hebben wij een zwakke positieve correlatie gevonden tussen bone bruise volume en klinische symptomen. Alhoewel niet statistisch significant, leken de bone bruise volumes af te nemen na ACI, terwijl bone bruise volumes leken toe te nemen na microfracturing behandeling. In het verlengde van deze observatie hebben wij vastgesteld, dat de discrepanties in de post-operatieve veranderingen in bone bruise volumes en in klinische symptomen voornamelijk werden gevonden na microfracturing behandeling. Deze observatie kan mogelijk toegeschreven worden aan subchondrale schade, welke optreedt ten gevolge van de chirurgische ingreep, wat suggereert, dat de geautomatiseerde segmentatie software niet in staat is om onderscheid te maken tussen subchondrale schade en werkelijke bone bruise.
Drie aspecten van de bone bruise analyse werden met behulp van MRI bestudeerd, waaruit wij het volgende hebben geconcludeerd:

- De subjectieve bone bruise gradering schaal levert betrouwbare en reproduceerbare informatie over de ernst van de bone bruise, waarbij de hoeveelheid informatie echter beperkt is door de grofheid van de methode;
- De geautomatiseerde segmentatie techniek is een gemakkelijke, objectieve, nauwkeurige en bruikbare methode voor toekomstig onderzoek dat gericht is op bone bruise studies met PD-w MRI scans;
- Bone bruise zou inderdaad gerelateerd kunnen zijn aan klinische symptomen, welke het gevolg zijn van gewrichtskraakbeen defecten in de knie. Meer longitudinale studies zijn vereist om definitieve conclusies te kunnen trekken en om aan het licht te brengen of deze relatie causaal is dan wel louter toevallig;
- Patiënten die met microfracturing zijn behandeld, lijken een niet-bruikbare groep te zijn om het verband tussen bone bruise volumes en klinische symptomen te bestuderen.

In het verleden zijn er meerdere histologische scoringssystemen gebruikt om de resultaten van kraakbeenherstel technieken te evalueren, alhoewel geen enkele gevalideerd is.
In **hoofdstuk 11** hebben we 2 histologische scoringssystemen vergeleken, welke voorheen gepubliceerd zijn door Pineda en O'Driscoll. We vonden een zeer goede intra- en interwaarnemer betrouwbaarheid, als ook een goede correlatie tussen de twee scoringssystemen. Met betrekking tot de intra- en inter-waarnemer betrouwbaarheid, hebben we voor beide scoringssystemen vastgesteld dat het verschil tussen de waarnemingen en de verschillende waarnemers klein was. Alhoewel het relatieve verschil kleiner was voor de O'Driscoll schaal. Dit komt waarschijnlijk doordat dit systeem de meest uitgebreide is van de twee scoringssystemen. Door de veelheid aan andere parameters worden parameters, die afwijkend zijn beoordeeld, waarschijnlijk gecompenseerd.
We concludeerden dat zowel de O'Driscoll schaal als de Pineda schaal betrouwbare semi-kwantitatieve kraakbeen scoringssystemen zijn en dat de acceptatie van deze twee schalen voor algemeen gebruik ten goede zal komen aan de betrouwbaarheid van de literatuur op het gebied van tissue engineering voor kraakbeen herstel.

Implicaties van de resultaten

Validatie van short form WOMAC function scale

In ons streven om de kraakbeen regeneratietechnieken en zogenaamde "Disease Modifying Osteoarthritic Drugs" (DMOADs) als de behandelingsstandaard in te voeren voor het kraakbeen herstel en de verlichting van symptomen bij OA, zijn methoden vereist die zowel in trial omstandigheden als ook in de dagelijkse klinische praktijk geïmplementeerd kunnen worden om de effectiviteit van onze interventies te kunnen evalueren. De short form WOMAC function scale kan geïmplementeerd worden voor zowel de evaluatie van niet chirurgisch behandelde artrose van de knie, als ook voor evaluatie van gewrichtsvervangende ingrepen. Toekomstig aanvullende validatiestudies zijn vereist voordat dit scoringssysteem ook geïmplementeerd kan worden voor de evaluatie van andere behandelingstechnieken, zoals ACI.
De short form WOMAC function scale kan niet gebruikt worden voor de evaluatie van individuele patiënten. Echter, wanneer men groepen van patiënten wil evalueren, kunnen de verkregen data vergeleken worden met de studies waarbij de traditionele WOMAC (momenteel de gouden standaard in OA research) gehanteerd is. Deze essentiële eigenschap zou men in aanmerking moeten nemen bij het ontwikkelen van nieuwe "outcome tools", aangezien dit de vergelijkbaarheid met voorgaande studies op dit gebied verbetert en de waarde van toekomstige studies verhoogt.

Geautomatiseerde segmentatie van bone bruise met behulp van MRI

Alhoewel het fenomeen bone bruise veelvuldig beschreven is in de context van kraakbeendefecten, blijft de rol en de gevolgen van dit fenomeen tot op heden onduidelijk. Geen van de voorheen gebruikte methoden om de ernst van bone

bruise vast te stellen is gevalideerd en, zoals is vastgesteld in de huidige studie, suggereert de grofheid van deze methoden dat het onmogelijk is om subtiele veranderingen waar te nemen.
De geautomatiseerde segmentatie software is een gemakkelijk, objectief, accuraat en nuttig hulpmiddel voor toekomstig onderzoek met het doel om bone bruise te bestuderen op PD-w MRI scans. Onze resultaten bij ACI-behandelde patiënten ondersteunen de hypothese dat bone bruise gerelateerd is aan klinische symptomen bij patiënten met kraakbeendefecten, alhoewel aanvullende longitudinale studies nodig zijn om definitieve conclusies te kunnen trekken. Het dient echter opgemerkt te worden dat patiënten die behandeld zijn met microfracturing een niet geschikte populatie lijken te zijn om het proces en de rol van bone bruise op PD-w MRI scans te bestuderen.

Histologische scoringssystemen
We vergeleken twee frequent gebruikte histologische scoringssystemen. Aangezien beide systemen een uitstekende inter- en intra-waarnemers betrouwbaarheid vertonen, kunnen beide systemen als betrouwbaar beschouwd worden en zouden zij geïmplementeerd kunnen worden voor algemeen gebruik. Enkele aspecten zouden echter nader beschouwd moeten worden voordat men deze systemen gaat gebruiken. Het systeem dat door Pineda is beschreven is korter en om die reden makkelijker te gebruiken en neemt minder tijd in beslag. Het systeem van O'Driscoll levert een bredere range aan informatie, die gebruikt zou kunnen worden wanneer specifieke sub-items beoordeeld worden.
Verder vereisen beide systemen dat het histologische preparaat een "regeneratie weefsel – oorspronkelijk kraakbeen interface" heeft om sub-items zoals "Filling of the defect" en "Bonding to the adjacent cartilage" te kunnen graderen. Deze beperking maakt deze scoringssystemen minder bruikbaar voor klinische trials waarbij kleine biopten genomen worden van regeneratieweefsel, dat vaak moeilijk te onderscheiden is van oorspronkelijk kraakbeen. Voor dit doel is de ICRS II schaal ontwikkeld door ICRS, zoals toegepast in hoofdstuk 2.

Algemene conclusies en toekomstige richtingen

In dit proefschrift hebben we aangetoond dat ACI resulteert in structureel superieur regeneratieweefsel in vergelijking met de traditioneel gebruikte microfracturing techniek. Echter, men moet zich realiseren dat deze gunstige resultaten werden gevonden onder strikte onderzoekscondities met accuraat gedefinieerde in- en exclusie criteria die gebaseerd zijn op eerdere gerapporteerde onderzoeken. Desondanks resulteerde ACI in een morfologisch variabele kwaliteit van het regeneratieweefsel, wat ons tot het onderwerp van dit proefschrift heeft gebracht, namelijk: "het exploreren van de randvoorwaarden voor succesvolle kraakbeen regeneratie".
Onze studies naar adequate *in vitro* regeneratie condities wijzen erop dat in klinisch toegepaste ACI, chondrocyten zouden moeten worden geëxpandeerd onder groeifactor gesupplementeerde condities. Bovendien zouden, om de chirurgische technieken te verfijnen, niet alleen scaffold materialen moeten worden overwogen

vanwege praktische redenen, maar ook omdat zij het proces van chondrogenese significant zouden kunnen verbeteren. Verdere optimalisatie zou bereikt kunnen worden door *in vitro* en *in vivo* experimenten waarbij de invloed van verschillende scaffold materialen op kraakbeenregeneratie door geëxpandeerde en geïmplanteerde chondrocyten wordt vergeleken. Naast de kweekcondities hebben we het belang van het selecteren van patiënten met donorweefsel van voldoende kwaliteit, aangetoond. Totdat de substantiële verschillen in chondrogenetische potentie tussen gezonde en OA chondrocyten verminderd of zelfs volledig opgelost kunnen worden, zou ACI niet moeten worden geïmplementeerd voor de behandeling van (vroege stadia van) OA. Daarnaast zouden toekomstige studies zich dienen te verdiepen in de overgang van kraakbeendefecten naar de ontwikkeling van OA en hoe dit donorweefsel karakteristieken beïnvloedt, aangezien dit ons inzicht in de timing van de behandeling en de patiëntenselectie verhoogd.

Tenslotte hebben we aangetoond dat het overbrengen van klinisch relevante variabelen, zoals synoviaal vocht uit getraumatiseerde kniegewrichten, naar de "gecontroleerde' omgeving van *in vitro* experimenten het proces van kraakbeenregeneratie significant verandert. Om deze reden zouden in toekomstige studies de variabelen, welke verantwoordelijk zijn voor de nadelige effecten op kraakbeenregeneratie, geïdentificeerd dienen te worden. Deze mediatoren bieden mogelijk geschikte "targets" voor de optimalisatie van gewrichtshomeostase door het voorbehandelen van een getraumatiseerd gewricht alvorens tot ACI over te gaan. Onder andere IL-1β zou zo'n doel kunnen zijn, aangezien bekend is dat dit cytokine van doorslaggevende betekenis is gedurende wondheling van verschillende weefseltypes in het menselijk lichaam.
De biologische effectiviteit van een recent ontwikkelde autologe vorm van IL-1ra is aangetoond door de klinische verbetering van patiënten met symptomatische knie OA. Of deze therapie daadwerkelijk resulteert in verbeterde kraakbeenregeneratie door voorbehandeling van getraumatiseerde gewrichten moet nog aangetoond worden.

Dit proefschrift heeft als doelstelling om de positie en de randvoorwaarden van kraakbeenregeneratie technieken voor de behandeling van articulaire kraakbeendefecten te exploreren. Gebaseerd op onze resultaten zou ACI momenteel als een eerste aanpak in plaats van een laatste redmiddel voor de behandeling van kraakbeendefecten overwogen moeten worden. Desalniettemin bestaat er een significante impact van diverse *in vivo* parameters op de uitkomst van dergelijke behandelingsstrategieën. Derhalve is het verduidelijken en vervolgens interfereren met de mediatoren die verantwoordelijk zijn voor de negatieve effecten op kraakbeenregeneratie potentieel dé benadering voor het optimaliseren van klinisch toegepaste kraakbeen regeneratietechnieken.

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