Towards one-stage cell-based treatment and non-invasive evaluation of cartilage defects

Joris EJ Bekkers

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JEJ Bekkers, M Inklaar, DBF Saris
Treatment selection in articular cartilage lesions of the knee: a systematic review. *Am J Sports Med 2009 Nov; 37 suppl 1:148S-55S*

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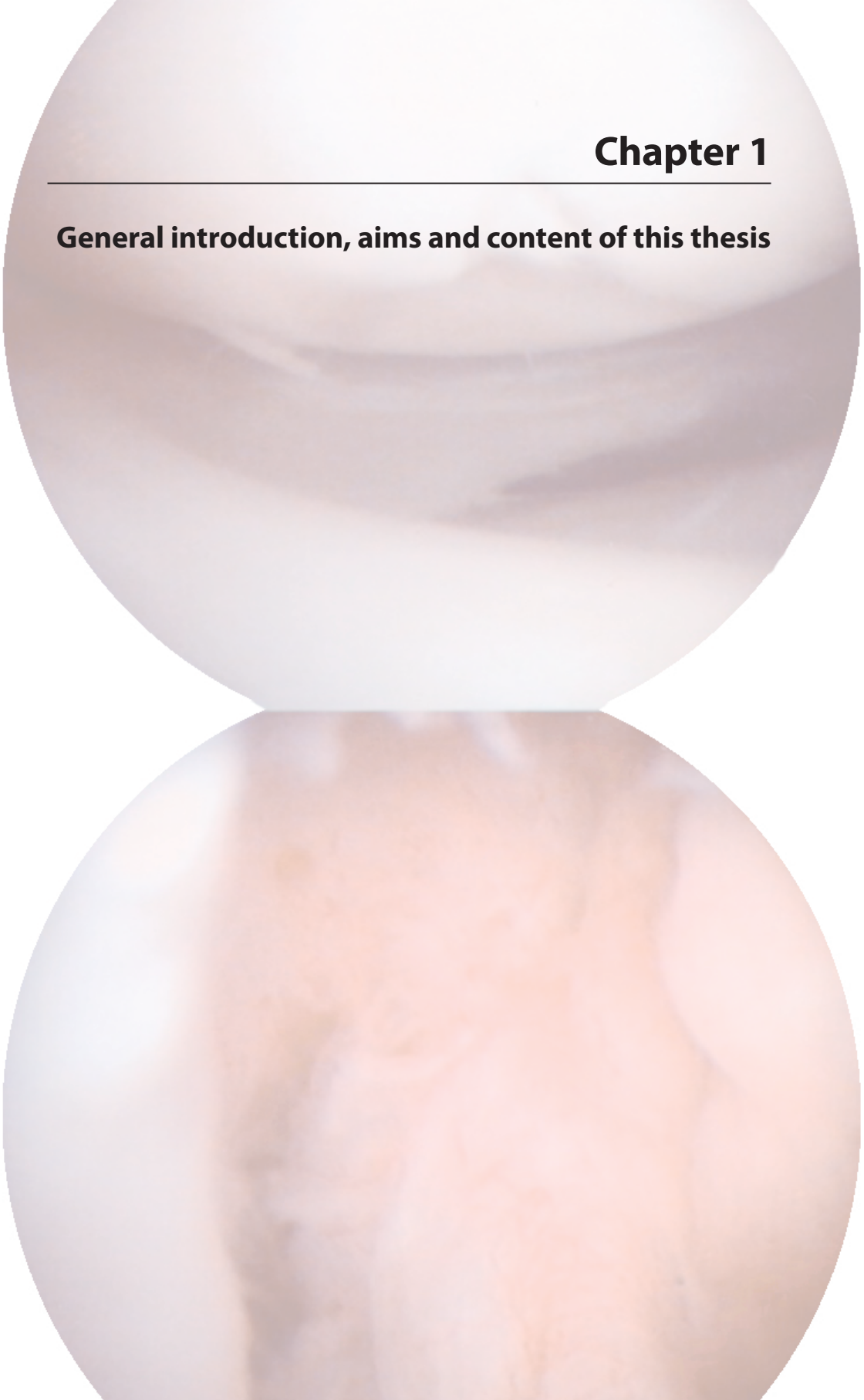
Chapter 1

General introduction, aims and content of this thesis



CARTILAGE TRAUMA; THE CLINICAL PROBLEM

Mobility and sports participation are highly dependent on a normal function of intra-articular structures and are essential determinants of the quality of life. However, on a regular basis, young and middle-aged adults present clinically with knee problems related to damage of the articular surface which cause a serious reduction in their quality of life.
A focal articular cartilage lesion is characterized by an isolated loss of cartilage tissue and function in an otherwise healthy articular joint (Buckwalter and Mankin 1998). Clinically, such lesions are subdivided into several grades, based on their morphological and macroscopic appearance. These grades are defined by normal tissue (grade 0), tissue softening and superficial fissures (grade 1), tissue loss over less than 50% of the tissue depth (grade 2), tissue loss extending towards, but not including, the subchondral bone (grade III) and finally with involvement of the subchondral bone (grade IV) (Outerbridge 2001). Patients younger than 45 years show the highest prevalence (20-40%) of focal cartilage lesions and knee trauma is believed to be an important aetiological factor in the development of such lesions (Aroen et al 2004; Hjelle et al 2002; Widuchowski et al 2007), as judged by the high percentage (45-61%) of patients who are able to relate their knee symptoms to a previously sustained knee trauma (Aroen et al 2004; Hjelle et al 2002; Widuchowski et al 2007). The presence of a focal articular cartilage lesion is mainly a problem for active, young- and middle-aged adults. Symptoms like knee pain, swelling, catching and locking cause a decrease in activities of daily living, sports participation and sports performance (Flanigan et al 2010; Messner and Maletius 1996). In fact, the quality of life of patients with a diagnosed focal cartilage lesion is impaired to the same extent as that of patients with osteoarthritis eligible for arthroplasty (Heir et al 2010). Also the pain, symptoms, activities of daily living and sports subscales of the Knee injury and Osteoarthritis Outcome Score (KOOS) in patients with a focal articular cartilage lesion were shown to be similar to patients with unicompartimental osteoarthritis planned for an osteotomy (Heir et al 2010). These similarities are also reflected by the annual direct (medical) and indirect (absenteeism) costs for society of patients with a focal cartilage lesion ($12,868) and osteoarthritis ($12,200) (Gupta et al 2005; Lindahl et al 2001). This stresses the clinical relevance and socioeconomic consequences of a focal articular cartilage lesion, which should be diagnosed early and treated accurately in order to repair the defect, restore the joint surface and regain an acceptable quality of life.

CURRENT TREATMENT MODALITIES AND THEIR WINDOW FOR IMPROVEMENT

The most frequently applied surgical treatment options for focal articular cartilage lesions are microfracture, osteochondral autologous transplantation (OAT) and autologous chondrocyte implantation (ACI).

The principle of the microfracture technique is to create multiple small holes into the subchondral bone beneath the debrided focal cartilage defect in order to allow bone marrow, containing multipotent stromal cells (MSCs), to enter the intra-articular space. The bone marrow is believed to create a clot in the cartilage defect which is filled with cells and growth factors, that eventually is remodelled to a functional tissue (Steadman et al 2001; Williams III and Harnly 2007). Although microfracture is a relatively easy surgical technique, a follow-up over 2 years and a lesion size over 2 cm^2 are related to a deterioration of clinical success (Bekkers et al 2009; Gobbi et al 2005; Gudas et al 2005; Kon et al 2009). This could possibly be due to the fibrocartilaginous character of the repair tissue (Williams III and Harnly 2007). Fibrocartilaginous repair is characterized by the presence of collagen type I and varying amounts of collagen type II and proteoglycans (Mithoefer et al 2009). This could lead to a matrix which is less stable and, therefore, more vulnerable to damage when exposed to the high intra-articular shear stresses created during physiological joint use. Previous studies (Knutsen et al 2007; Saris et al 2009) noted that a suboptimal histological quality of regenerated tissue does not influence short-term outcome, however, leads to a decrease in clinical outcome at longer follow-up. Also, incomplete defect fill, due to displacement of the initially fragile microfracture clot, and elevation of the subchondral bone, which are both observed after microfracturing (Williams III and Harnly 2007), may influence the biomechanical resistance of the tissue to shear stresses and could, therefore, also contribute to a decline in long-term clinical outcome.

During the OAT procedure, osteochondral plugs are harvested from non-weight-bearing locations in the knee and transplanted to the osteochondral defect (Hangody et al 2004). Despite proven clinical efficacy, the drawbacks of this technique are the limited availability of donor tissue and the donor site morbidity which occurs in at least 5% of patients (Hangody et al 2010). Also the transplanted plugs often fail to integrate into the surrounding cartilage and bone, which may lead to subchondral cysts and decreased graft stability. Attempts to fill the gap between the transplanted plug and the surrounding recipient osteochondral tissue with alginate, containing transforming growth factor-1 (TGF-1) transduced mesenchymal stromal cells, showed some improvement in lateral integration of the transplanted plugs in goats (Sun et al 2009).

Another alternative for the surgical treatment of focal cartilage lesions is autologous chondrocyte implantation (ACI). This treatment was first described by Brittberg et al, and consists of harvest of chondrocytes from non-weight-bearing cartilage in the knee, expansion *in vitro* and reimplantation in the defect during a second surgical procedure

(Brittberg et al 1994). Over the years, this technique has evolved into several generations, all aiming at a more reliable biological response, improved ease of surgical use and a better clinical outcome (Marlovits et al 2006). This evolution from the first to the third generation of ACI could, for a large proportion, be ascribed to the increased knowledge in the biomaterials field. The first generation of ACI used a periosteal flap to cover the defect and keep the re-implanted cells in place. The biological response of the periosteum (periosteal hypertrophy) negatively influenced treatment outcome and urged further development of this technique. Consequently, the second generation of ACI replaced the periosteal flap with a synthetic bioresorbable fleece to cover the cartilage defect. The third generation of ACI is based on the use of 3D biodegradable biomaterials that allow redifferentiation of the cells in a 3D environment.

Innovations of arrived clinical treatments aim at the improvement of therapy quality and reproducibility of the applied treatment and its outcome. One of the more recent approaches to improve the quality of tissue formation after microfracturing, involves the development of several biomaterials that can act as a scaffold for cellular migration and differentiation (introduced as augmented microfracturing) (Strauss et al 2010). Results from this approach are as yet unclear. For OAT alternatives for autologous plugs are sought. Currently, techniques solely based on the application of synthetic biomaterials, are introduced for the treatment of osteochondral lesions of the articular cartilage (Saithna et al 2008; Williams and Gamradt 2008). However, clinical trials on such treatments are lacking. Innovations in ACI are also expected from application of smart biomaterials. Ongoing research focuses on the development of new chondro-conductive scaffolds combined with expanded chondrocytes for cartilage repair. However, the translation of such novel materials to a clinical setting has not yet been achieved (Vinatier et al 2009) and specific attention to the clinical handling and firm fixation of the biomaterial into the defect is lacking. In addition to this, reproducibility of cartilage cell therapy can also be expected from currently defined ATMP and EMA regulations and quality control of reimplanted cells. Although in general the clinical improvement after ACI is at least as good as after microfracture or OAT, the ACI procedure still has some generally acknowledged shortcomings. As the number of chondrocytes obtained from the cartilage biopsy taken for chondrocyte isolation (approximately $0.18\text{-}0.46*10^6$) is limited (Brittberg et al 1994), *in vitro* cell expansion is necessary, which in turn makes two surgeries inevitable. More importantly, expansion causes dedifferentiation of re-implanted chondrocytes, which is characterized by a decreased capacity to produce hyaline cartilage-specific matrix proteins compared to non-expanded differentiated cells (Marlovits et al 2004; Schnabel et al 2002). In conclusion, ACI has several drawbacks that can be addressed in order to further develop this technique. Therefore, the opportunity and impact of further clinical improvement of ACI is expected to be highly relevant and scientifically intriguing.

IMPROVEMENT OF AUTOLOGOUS CHONDROCYTE IMPLANTATION

Addressing the abovementioned challenges of ACI is a good scientific starting point for further development and improvement of this clinical treatment.

Cellular behaviour is controlled by environmental cues and cell-cell and cell-matrix interactions. The communication, via excreted trophic factors, between chondrocytes and adipose, mesenchymal or embryonic stem cells has been suggested to play an important role in cartilage matrix formation (Bigdeli et al 2009; Lee and Im 2010; Wu et al 2011). This notion has led to the suggestion that combinations of chondrocytes with other cell types may be a promising alternative to using expanded and dedifferentiated chondrocytes. Indeed, several 2D and 3D *in vitro* models showed an increase in either articular cartilage-specific matrix production or gene expression when expanded articular chondrocytes were combined with MSCs (Bian et al 2011; Chen et al 2009; Giovannini et al 2010; Tsuchiya et al 2004; Mo et al 2009). This effect was even noted using low numbers of chondrocytes in combination with MSCs and changed depending on the type of biomaterial used as a scaffold for the combined cells (Bian et al 2011; Mo et al 2009). Whether MSCs or other cell types actually differentiate towards the chondrogenic lineage when combined with chondrocytes or whether factors excreted by these cells positively influence the neighbouring chondrocytes remains unclear from these studies. As the presence of MSCs during monolayer expansion of chondrocytes was recently shown to prevent dedifferentiation of chondrocytes during expansion (Chen et al 2009; Tsuchiya et al 2004), at least part of the effects may be mediated by the secretion of soluble factors.

Altogether, these studies clearly show the potential of combining cell populations to improve chondrogenesis from which the current ACI technique could benefit. Cell combinations may allow the application of differentiated chondrocytes which are hypothesized to produce a higher quality cartilage matrix compared to the currently used culture-expanded chondrocytes. This could have a positive influence on the quality of the regenerated tissue and on clinical outcome. Moreover, the direct combination of chondrocytes with other cell types can be performed within one surgical procedure, thereby reducing the number of required surgical interventions, thus reducing surgery-related morbidity, patient and provider acceptation, efficiency and costs.

As these novel therapies will mostly be based on the use of non-expanded chondrocytes, their regenerative capacities, which are likely to be different from expanded cells, should be studied. In addition, the characteristics of non-expanded chondrocytes may depend on factors such as harvest location, but also the isolation procedure. With respect to the harvest location, cartilage characteristics seemed to be dependent on its intra-articular location and thus likely affect the phenotype of freshly isolated chondrocytes (Brommer et al 2005; Bullough et al 1973; Bullough and Walker 1976; Carter et al 2004). Also, different isolation procedures will influence the presence of the pericellular matrix surrounding

chondrocytes (together called the chondron) after digestion. The presence of such a pericellular matrix has been shown to enhance cartilage-specific matrix production.

EVALUATION AND VALIDATION OF OUTCOME TOOLS FOR CARTILAGE REGENERATION

In clinical practice, accurate evaluation of treatment outcome serves several purposes. In addition to clinical success measured as a recovery of functionality and a reduction of symptoms for the patient, measuring biological parameters can assist in understanding the processes occurring inside the body, joint or tissue. This not only allows insight into both mechanisms of disease and recovery during treatment, but also can be used to correlate these processes to the final clinical and biological outcome to determine factors related to a successful outcome. These factors can then be used as new prognostic parameters for individual treatment success. This will help us to improve therapy and build ideal treatment suited patient profiles.

For the evaluation of clinical success after cartilage surgery the Knee injury and Osteoarthritis Outcome Score (KOOS) is often used. However the clinimetric properties of this patient-oriented score have never been tested for validation to measure outcome after treatment of focal cartilage lesions.

In addition to quantification of knee-related symptoms and disabilities, using for example the KOOS score, assessment of joint pathology is also critically important. For this Magnetic Resonance Imaging (MRI) is often used. MRI is applied to visualize several intra-articular pathologies, like a ruptured anterior cruciate ligament (ACL) or torn menisci. Application of MRI for the morphological assessment of the articular cartilage surfaces to diagnose clinically relevant grade III and IV articular cartilage lesions has shown a specificity of 95-97% and a sensitivity of 64-70% (Figueroa et al 2007; Friemert et al 2004; Potter et al 1998). MRI scoring systems, like the WORMS and the KOSS, allow for a longitudinal, semiquantitative, assessment of articular cartilage integrity in a trial setting (Kornaat et al 2005; Peterfy et al 2004). Currently, when used for the evaluation of the structural results after cartilage regeneration therapy, MRI will inform on defect filling. However, this will not differentiate poor from good quality neocartilage, which is critically important for the expected long-term clinical success. Newly developed, quantitative, MRI techniques, such as the delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC), aim at an evaluation of cartilage constituents instead of general cartilage morphology (Eckstein et al 2006). Several MRI sequences, with or without the use of contrast agents, showed to be related to the glycosaminoglycan and collagen content and orientation in the extracellular cartilage matrix and thus its quality (Eckstein et al 2006). Such techniques are promising and could assist in the evaluation of the content and

organization of cartilage constituents after regenerative cartilage surgery and thereby provide a quantitative tool to assess the quality of the regenerated tissue.

PATIENT-ORIENTED TREATMENT SELECTION

The decision for treatment needs to be guided by diagnostic skills, tools, treatment opportunity and expected, desired outcome. Based on the communication between patient and provider an optimal choice needs to be defined and alternatives discussed. The surgical treatment of a focal articular cartilage lesion directly introduces another obstacle to overcome. With several treatments available for the surgical restoration of a focal articular cartilage lesion, the question arises whether parameters could be identified that favour one treatment over the other. The clinical success of microfracture, ACI and OAT differs based on the characteristic of the patient treated and the features of the focal lesion (Bentley et al 2003; Gudas et al 2005; Horas et al 2003; Knutsen et al 2007; Kon et al 2009; Saris et al 2009). For example, age and activity level of the patient were already shown to influence treatment outcome (Knutsen et al 2007). In OAT, transplantated osteochondral tissue is used to heal the defect, whereas in microfracture and ACI the body is stimulated to regenerate new tissue (Brittberg et al 1994; Hangody et al 2004; Steadman et al 2001). The biological response of the body and its ability to regenerate new tissue decreases with age. This could suggest that the latter two treatments would be more suitable for younger patients. Next to this, the delay between the development of an articular cartilage lesion and its treatment also showed to influence the quality of tissue regeneration (Saris et al 2003). Increasing knowledge on such and other, characteristics allow optimal advantage to be taken from the potency of a specific surgical treatment and hence improvement of clinical outcome.

AIMS OF THIS THESIS

The central aim of this thesis is to improve the clinical outcome of patients with a focal articular cartilage lesion treated with autologous chondrocyte implantation, by improvement of the surgical technique, the development of specific treatment algorithms and the evaluation and validation of suited outcome tools. To address this central aim, several specific aims were defined:

Improvement of autologous chondrocyte implantation

Specific aim 1: To investigate the improvement of cartilage-specific matrix production by the interaction of chondrocytes and bone-marrow derived cells.

Specific aim 2: To investigate what biomaterial best supports cartilage formation when a combination of cells is used and how this material should be fixed in a focal cartilage defect.

Specific aim 3: To investigate the influence of biopsy location on chondrogenic potential of isolated cells.

Evaluation and validation of outcome tools for cartilage regeneration

Specific aim 4: To validate the Knee injury and Osteoarthritis Outcome Score (KOOS) to measure the clinical outcome after the treatment of focal articular cartilage lesions.

Specific aim 5: To investigate whether the delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) technique is able to measure -changes in- cartilage matrix constituents after cartilage regeneration and if these changes are predictive for the biological success of regeneration.

Patient-oriented treatment selection

Specific aim 6: To investigate the prognostic value of specific patient- and disease-related characteristics on the clinical outcome after cartilage surgery.

Specific aim 7: To create an evidence-based treatment algorithm to optimize treatment selection for the restoration of articular cartilage lesions in the knee.

CONTENT OF THIS THESIS

The added value of combining chondrocytes and other cell types in cartilage-specific matrix production as the basic principle for the development of a one-stage ACI procedure is first evaluated for different cell combinations *in vitro*. *Chapter 2* describes the effect on cartilage matrix production when non-expanded chondrocytes are combined with mononuclear fraction (MNF) cells. Related to this, *Chapter 3* will evaluate, both *in vitro* and *in vivo*, what type of biomaterial is best suited to act as a scaffold, and promoter of chondrogenesis, for a combination of cells. *Chapter 4* deals with the potential use of chondrocytes derived from debrided defect cartilage to serve as a cell source for ACI. To follow-up on this, *Chapter 5* describes different options for the fixation of a scaffold within an articular cartilage lesion with special focus on fixation technique-related scaffold damage. In *Chapters 6* and *7*, the newly developed one-stage ACI technique is tested in a large animal model compared to microfracturing, for both the combination of non-expanded chondrocytes and MNF cells as well as the non-expanded chondron MSC combination. *Chapter 7* also describes the added value, in terms of cartilage-specific matrix production, of using chondrons over chondrocytes when combined with MSCs.
The evaluation of outcome tools for cartilage regeneration is first introduced in *Chapter 8*. This chapter provides an overview of currently applied and promising new techniques for the evaluation of articular cartilage pathology with a special focus on reliability, clinical value and possible future applications. *Chapter 9* validates the Knee injury and Osteoarthritis Outcome Score for a cohort of patients with a focal cartilage lesion treated by regenerative cartilage surgery. Also newly developed functional MRI techniques could have an additional value to determine the success of an articular cartilage treatment. For this reason *Chapters 10* and *11* describe the added value of dGEMRIC in evaluating articular cartilage regeneration after cartilage surgery.
Finally, identification of patient- and disease-related characteristics, such as defect size and age, patient age and defect location, with a prognostic value for the clinical outcome after cartilage surgery could be the basis for patient-specific treatment selection that provides the best treatment for the individual patient. *Chapter 12* describes several characteristics that influence clinical outcome after cartilage surgery. Following this, such characteristics are used in *Chapter 13* to design a patient-oriented treatment algorithm suited to optimize treatment selection for patients with a focal articular cartilage lesion.

Chapter 2

Coculturing human bone marrow mononuclear cells with primary human articular chondrocytes; differential effects on chondrogenesis

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Submitted for journal publication

ABSTRACT

This study evaluated the effect on chondrogenesis of coculturing unsorted human bone marrow mononuclear fraction cells (MNF) with non-expanded human articular chondrocytes.

Articular chondrocytes were either combined with heterologous or autologous MNF in a pellet coculture at ratios containing 0, 2, 5, 10, 15, 20, 50 or 100% chondrocytes. To verify whether the effects found were cell type-specific and dependent on the presence of the added cells, chondrocytes were also cocultured with fibroblasts or in identical quantities without addition of any other cell type. All cocultures were cultured for 4 weeks and analyzed for GAG and DNA content and (immuno)stained for glycosaminoglycans and collagen type I and II.

The GAG content was lower ($p<0.001$) for all chondrocyte/MNF ratios compared to 100% chondrocyte cultures. However, GAG content normalised to DNA content was higher ($p<0.05$) in the 5-50% coculture pellets compared to cultures with 100% chondrocytes. These effects were not noted when fibroblasts were used. Strikingly, at 2-5% chondrocytes, GAG production was higher in chondrocyte monocultures ($p<0.013$), compared to 2-5% chondrocyte/MNFs cultures, indicating an inhibitory effect of the presence of MNFs. At these low cell numbers, GAG/DNA content was even higher compared to monocultures containing the maximal number of chondrocytes (100%), suggesting an inhibitory effect of cell number on matrix formation. At 15-20% chondrocytes the addition of MNFs did enhance ($p<0.02$) GAG and GAG/DNA content compared to the corresponding number of chondrocytes in monoculture. The high chondrocyte/MNF ratios and chondrocyte monocultures stained positive for glycosaminoglycans and collagen type II while chondrocyte/fibroblast and low ratio chondrocyte/MNF cultures mainly stained positive for collagen type I.

In conclusions, the effect of coculture of MNF cells with chondrocytes is dependent on cell ratios, at low chondrocyte percentages actually inhibiting cartilage formation and leading to fibrocartilage formation, with a stimulatory effect at intermediate chondrocyte content.

INTRODUCTION

Osteoarthritis is generally acknowledged as the end stage of a process of generalized degradation of articular cartilage (Buckwalter and Mankin 1998). Within this degenerative cascade several phases can be identified all having their own disease characteristics and reversibility of inflicted damage (Buckwalter and Mankin 1998). If left untreated, isolated articular cartilage lesions are believed to predispose to generalized osteoarthritis. Such lesions are more and more frequently treated by autologous chondrocyte implantation (ACI). Although ACI, and the newer matrix-associated ACI (mACI) procedures, show good mid-term results (Knutsen et al 2007; Saris et al 2009), the procedure is a burden for the patient, as it requires two surgeries due to the need for expansion of the chondrocytes obtained from the biopsy. Furthermore, this expansion leads to dedifferentiation and loss of function of the reimplanted chondrocytes (Marlovits et al 2004; Schnabel et al 2002).

The combination of chondrocytes with other cell types has previously been proposed as a possible alternative to the use of expanded cells in ACI (Hildner et al 2009; Tsuchiya et al 2004). Primary articular chondrocytes, human embryonic and adipose-derived stem cells have been able to increase cartilage-specific matrix production of expanded articular chondrocytes *in vitro* (Bigdeli et al 2009; Gan and Kandel 2007; Hildner et al 2009). In particular, mesenchymal stem cells (MSCs) have been shown to positively affect chondrogenic differentiation. In several *in vitro* models, direct contact between MSCs and dedifferentiated articular chondrocytes improved the chondrogenic phenotype of dedifferentiated chondrocytes (Chen et al 2009; Tsuchiya et al 2004; Mo et al 2009). Whether MSCs actually take part in chondrogenesis is unclear. Many studies rather suggest morphogenetic signals from the cells in coculture to play a crucial role in directing chondrogenesis (Fischer et al 2010; Hwang et al 2007; Lee and Im 2010; Solursh and Meier 1973).

Despite the clear effects on stimulation of chondrogenesis found, none of these studies have been able to unambiguously prove that the effects were due to the addition of the supporting cell type, rather than reducing the total number of chondrocytes in their system (Gagne et al 2000). Moreover, it is also not clear whether these effects are specific for stem cells, or whether other or more differentiated cell types can also enhance differentiation.

Although the combination of chondrocytes with MSCs holds promise for improving cartilage tissue regeneration, this approach will still require two surgical procedures, as MSCs cannot be obtained within the timeframe of a single surgery. A possible alternative would be the application of the mononuclear fraction (MNF) of bone marrow. Although this cell fraction only harbours a very low number of MSCs (Castro-Malaspina et al 1980), it is likely to be a good source of morphogenic factors that stimulate other

cells to produce their target tissue and can be obtained and applied in the course of one surgical procedure.
This study evaluated the effect on chondrogenesis of a coculture of freshly isolated human bone marrow MNF and non-expanded, primary isolated human articular chondrocytes (PC). Moreover, a comparison was made between MNFs and fibroblasts and primary chondrocytes monoculture to verify whether this effect was specific for MNFs and to what extent the effects were rather related to the final number of primary chondrocytes in culture.

MATERIALS AND METHODS

Patients

All collection of patient material used in this study was approved by the institutional ethical committee. Macroscopically healthy articular cartilage was, under sterile conditions, harvested from the macroscopically healthy knees of 5 different donors during autopsy at the department of Pathology. Whole bone marrow was obtained from 4 patients (age 58-67 years old) undergoing a total hip arthroplasty. Autologous cartilage and bone marrow were also obtained from 10 patients undergoing an anterior cruciate ligament (ACL) reconstruction with notchplasty. During this procedure articular cartilage was taken from waste material collected from notchplasty and bone marrow was harvested by needle aspiration from the intercondylar notch right before drilling of the femoral tunnel for the new ACL graft. Human fibroblasts (FB) were obtained from the foreskin of an 8 year old donor who underwent a routine circumcision.

Cell isolation

Chondrocytes were isolated overnight at 37 °C in a 0.15 % collagenase type II solution (Worthington, USA) in Dulbecco's modified Eagle's medium (DMEM van Gibco, USA) with 1 % penicillin/streptomycin (100 U/ml / 100 µg/ml, Invitrogen, the Netherlands). After enzymatic digestion, the cell suspension was filtered through a 70 µm cell strainer and washed in phosphate-buffered saline (PBS).
The bone marrow cell pellet, obtained from 20 minutes centrifugation at 300g of the whole bone marrow, was diluted 50x in a red blood cell lysing buffer (Sigma-Aldrich, the Netherlands), and incubated at room temperature for 40 minutes. Following this, the remaining cells were spun down by centrifugation and washed twice in PBS.
For fibroblast isolation, the foreskin sample was minced and treated with collagenase IA (Sigma-Aldrich) and hyaluronidase (Sigma-Aldrich) at 37 °C for 45 minutes, then washed with PBS and transferred to a T25 culture flask. The sample was incubated overnight in DMEM with 20 % FBS and 1 % penicillin/streptomycin (100 U/ml / 100 µg/ml, Invit-

rogen) at 37 °C and 5 % CO_2. Adherent skin samples were covered with fresh medium, which was changed every 3 days. When fibroblasts had migrated from the explants, the skin samples were removed and adherent fibroblasts were cultured until they reached subconfluence. Following this, fibroblasts were released by trypsin-EDTA and further expanded in T75 flasks until subconfluence was reached.

Pellet formation

Isolated primary chondrocytes from articular cartilage donors, were either or not combined with the isolated mononuclear fraction of bone marrow or with skin fibroblasts at various ratios and pelleted by centrifugation (n=3 for the heterologous combination and n=2 for the comparison to PC monocultures). For the coculture pellets, a total of 250000 cells per pellet were used containing 0 %, 2 %, 5 %, 10 %, 15 %, 50 % or 100 % hPCs. The pellets containing only PCs (PC monocultures) consisted of pelleted chondrocytes at the same numbers as present in the cocultures, however without addition of any other cell type (n=2).

Per PC/MNF and PC/FB (n=1) ratio, a total of 5 pellets were created and cultured for 4 weeks in culture medium containing DMEM (Gibco), 10 mM HEPES (Invitrogen), 1 % non-essential amino acid solution (NEAA, Sigma-Aldrich), 0.2 mM l-ascorbic acid-2-phosphate (AsAp, Sigma-Aldrich), 0.004 mM proline (Sigma-Aldrich), 10 % fetal bovine serum (FBS) and 1 % pen/strep (100U/100 µg/ml). Medium was renewed twice a week. For the autologous PC/MNF cocultures (n=10) with cells obtained during ACL surgery, cell types were combined at the ratios 10 %, 15 %, 20 %, 35 % and 50 % and only 1 pellet per ratio was formed due to the limited number of chondrocytes available from the waste material after notchplasty.

After 4 weeks of culture, the pellets were digested for analysis of GAG and DNA content or processed for histology and immunohistochemistry.

GAG and DNA content

After overnight digestion in papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 °C, glycosaminoglycan (GAG) content was determined using the dimethylmethylene blue (DMMB) assay (Farndale et al 1986). The ratio of absorption at 540 nm to 595 nm was used to calculate the GAG content, using chondroitin sulphate (shark; Sigma-Aldrich) as a standard.

DNA content of the papain digest was determined using a Picogreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Histological and immunohistochemical evaluation

Pellets were fixed in 4 % buffered formaldehyde, dehydrated in a graded alcohol series, immersed in xylene and embedded in paraffin. For histology, 5 µm sections were depa-

raffinized and rehydrated in xylene and alcohol. Sections were stained with safranin-O (Merck, Germany) for GAG and Weigert's haematoxylin (Klinipath, the Netherlands) and 0.4 % fast green (Merck) to stain nuclei and cytoplasm, respectively.
For collagen I and II immunohistochemistry, 5 µm deparaffinized and rehydrated sections were blocked with 0.3 % H_2O_2 in PBS for 10 minutes. Antigen retrieval was performed by treating sections consecutively with 1 mg/ml pronase in PBS (Roche, the Netherlands) and 10 mg/ml hyaluronidase (Sigma-Aldrich) in PBS for 30 min at 37 ºC. Sections were blocked with 5 % BSA in PBS for 30 min followed by o/n incubation with the primary antibodies for either collagen type I (mouse anti-human IgG 1:100 in 5 % PBS/BSA, CP17L, Merck) or collagen type II. (mouse anti-human IgG, 1:100 in 5 %PBS/BSA, II-II 6B3, DSHB, USA) After overnight incubation at 4 ºC, the slides were washed with PBS-Tween 0.1 % and incubated with the secondary antibody for collagen type I (biotinylated sheep anti-mouse IgG, 1:200, RPN1001V, GE healthcare, the Netherlands) and collagen type II (goat anti-mouse-horse radish peroxidase, 1:100, [HRP], P0447, Dako, the Netherlands) respectively. For collagen type I an enhancement step with 1:500 streptavidin-HRP (PN1M0309, Beckman Coulter, the Netherlands) was included. Peroxidase activity was visualized with 3-diaminobenzidine-solution (DAB, Sigma-Aldrich). The sections were counterstained with Mayer's haematoxylin (Merck), dehydrated and mounted.

Statistical analysis

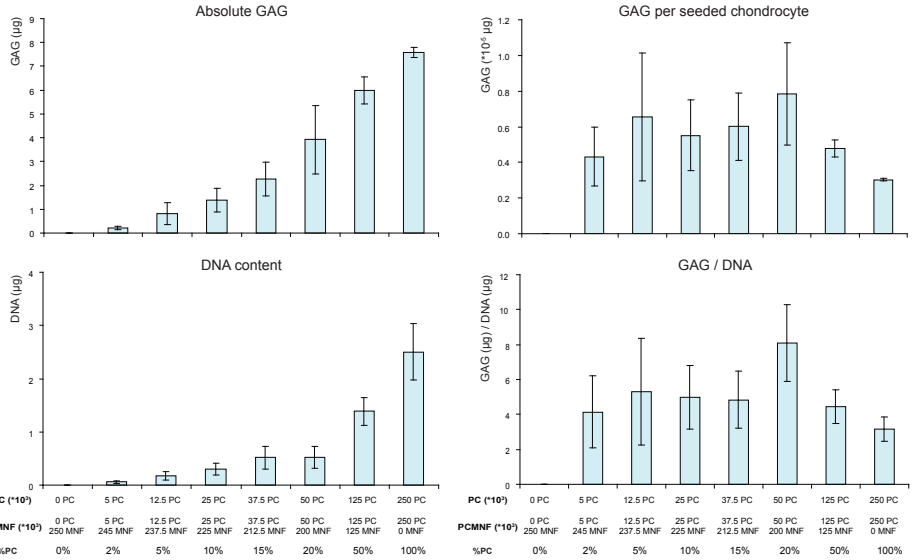
All statistical analyses were performed using SPSS 15.0 software. Differences in GAG/DNA content between the ratios were calculated by a one-way ANOVA with post-hoc LSD test. Differences between the ratios of the PC monocultures and PC/MNF or PC/FB were calculated by a one-way ANOVA with a post-hoc paired samples t-test. A $p<0.05$ was considered statistically significant. Experiments were repeated at least twice.

RESULTS

Coculture heterologous MNF cells and primary chondrocytes

After 4 weeks of culture, absolute GAG content of the pellets was highest ($p<0.001$) in 100 % PC pellets compared to pellets containing any combination of PC and MNF cells (Figure 1A). Pellets consisting of only MNFs hardly formed and immediate cell loss was observed after pellet formation in most PC/MNF coculture conditions. Per number of initially seeded chondrocytes, GAG production was higher ($p<0.05$) in pellets containing 5-50 % chondrocytes compared to those with 100% chondrocytes (Figure 1B). Also GAG production normalized to cell content, after 4 weeks of culture, (Figure 1D) was higher ($p<0.05$) in the 5-50 % coculture range compared to 100% PC pellets. The DNA content (Figure 1C) in the pellets after 4 weeks culture seemed to increase with the number of initially seeded chondrocytes.

Figure 1 Coculture of heterologous human primary chondrocytes and mononuclear fraction cells (PCMNF, n=3 donors).



Absolute GAG (Figure 1A), GAG per initially seeded chondrocytes (Figure 1B), DNA content (Figure 1C), GAG/DNA (Figure 1D) for heterologous PC/MNF coculture.

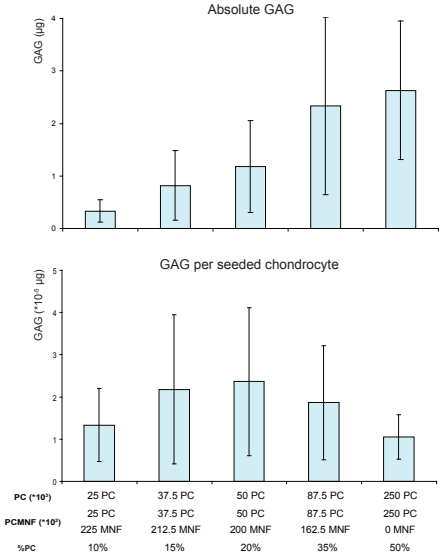
Coculture autologous MNF cells and primary chondrocytes

To verify whether the heterologous PC/MNF results could be reproduced for relevant cell combinations, for 10 donors, autologous PCs and MNF cells isolated from femoral bone marrow were cocultured at the same ratios as in the heterologous cocultures, thereby excluding 0-5% and 100% primary chondrocytes and including pellets containing 35% primary chondrocytes. The effects on absolute GAG content and GAG per seeded chondrocyte were similar as seen for the heterologous cell combinations (Figure 2). Absolute GAG content was higher ($p<0.035$) in pellets containing either 35 or 50% chondrocytes compared to those having 10-15% chondrocytes. Also the pellets containing 15-35% chondrocytes showed a higher, but statistically nonsignificant ($p=0.244$), GAG production per seeded chondrocyte. GAG production per DNA showed a similar trend compared to the heterologous cocultures (data not shown)

Coculture of bone marrow mononuclear cells compared to chondrocyte culture

To determine whether the stimulatory effect of coculture was due to the presence of MNFs, monocultures of primary chondrocytes at the same absolute numbers as present in the coculture with MNFs were compared to cocultures with MNFs. In PC monocultures, at low numbers of PCs (5-12.5*10^3 hPCs) corrresponding to 2-5 % in the cocultures, absolute GAG production was higher than when the same number of PCs was

Figure 2 Coculture of autologous primary chondrocytes and mononuclear fraction cells (PC/MNF) from femoral bone marrow (n=10 donors).



Absolute GAG (Figure 2A), and GAG per initially seeded chondrocytes (Figure 2B) for autologous PC/MNF coculture.

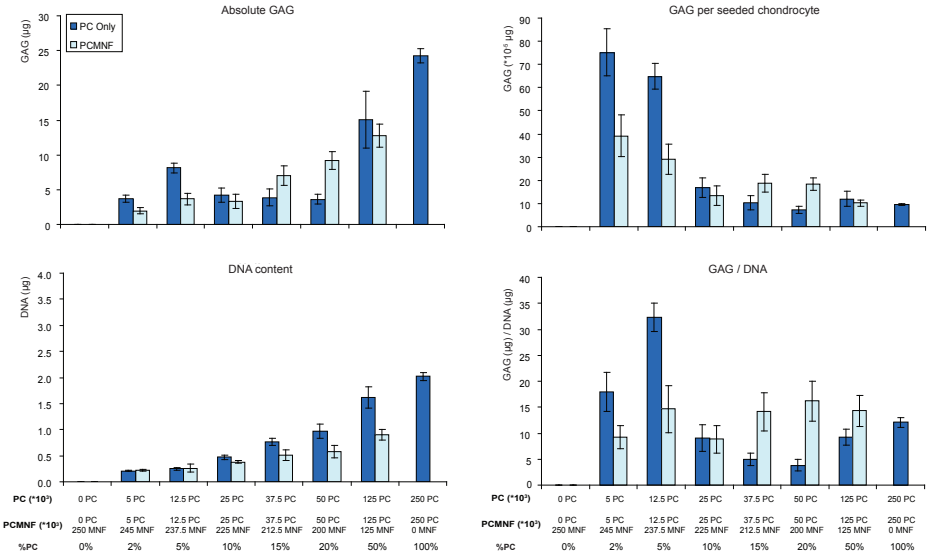
combined with MNFs ($p<0.013$) which indicates an inhibitory effect of the MNFs on GAG production at these ratios (Figure 3A). Moreover, compared to the monoculture of 5 % primary chondrocytes, GAG content was only higher in the 100 % chondrocyte pellets and in the 50 % coculture or 50% monocultures. However, at 15-20 % chondrocytes GAG content was higher ($p<0.02$) compared to chondrocyte monocultures (Figure 1A), indicating a stimulatory effect of MNFs at these percentages. A similar pattern of effects was observed for GAG content per initially seeded chondrocyte. As observed for total GAG content, the amount of GAG produced per initially seeded chondrocyte was higher ($p<0.001$) for the chondrocyte monocultures at ratios 2-5 % when compared to the 2-5 % cocultures, whereas the opposite was true ($p<0.050$) for the 15-20 % ratios (Figure 3B). Also GAG production normalized to DNA content was highest in the PC monocultures at amounts corresponding to 2 and 5 % in coculture, however in the 15-20 % range the PC/MNF coculture pellets showed a higher ($p<0.02$) GAG/DNA when compared to the corresponding PC monocultures.

DNA content was lower at 15-50 % PC/MNF cultures ($p<0.022$) compared to the PC monocultures (Figure 3C).

Coculture with fibroblasts compared to chondrocyte culture

Absolute GAG production in the 5-50 % range was higher ($p<0.05$) in pellets containing only chondrocytes compared to the PC/FB cocultures (Figure 4A). Also, in contrast to the

Figure 3 Coculture of human primary chondrocytes with mononuclear fraction cells (PC/MNF) vs. monocultures of human primary chondrocytes (PC Only), n=2.



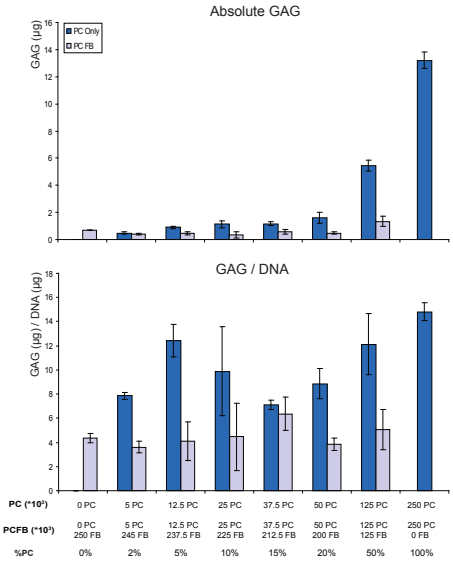
Absolute GAG (Figure 3A), GAG per initially seeded chondrocytes (Figure 3B), DNA content (Figure 3C), GAG/DNA (Figure 3D) for heterologous PC/MNF coculture and PC Only culture.

PC/MNF culture, the PC/FB cultures contained, at all times, less GAG per cell compared to PC monocultures, which was significant for the pellets containing 2 %, 5 %, 20 % and 50 % PCs ($p<0.05$) (Figure 4B). In addition, the low numbers of chondrocytes in the 2-5 % PC monoculture range showed, again, an efficient GAG per cell production. The DNA content followed a similar pattern as previous PC/MNF and PC monocultures, with increasing DNA content with an increasing number of PCs and a higher DNA content for PC monocultures compared to PC/FB cultures (data not shown).

Histological and immunohistochemical evaluation

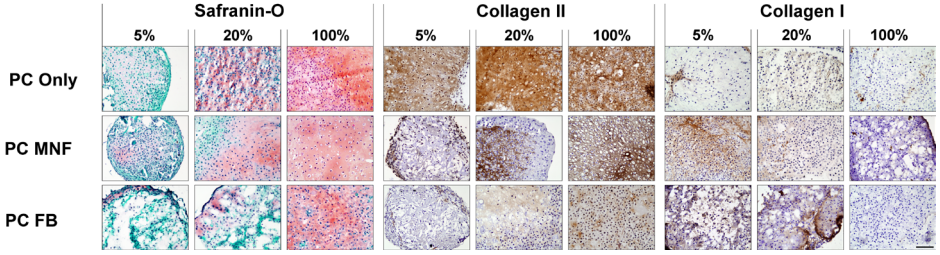
Histology and immunohistochemistry confirmed chondrogenic differentiation in the 20-100 % PC/MNF (co)cultures and PC monocultures, as they both stained positive for safranin-O and collagen type II and negative for collagen type I (Figure 5). However, the 2-15 % PC coculture pellets also were positive for collagen type I, indicative for fibro-cartilaginous differentiation. The 5 % PC monoculture pellets did not show safranin-O staining, but the tissue was positive for collagen type II and negative for collagen type I. Cartilage matrix formation in the PC/FB cocultures was low, as indicated by the negative safranin-O and collagen type II and positive collagen type I staining compared to pellets containing 100 % chondrocytes (Figure 5).

Figure 4 Coculture of human primary chondrocytes with fibroblasts (PC/FB) vs. monocultures of primary chondrocytes (PC Only), (n=1 donor).



Absolute GAG (Figure 4A), and GAG/DNA (Figure 4B) for heterologous PC/FB coculture and PC Only culture.

Figure 5 Staining for cartilage specific matrix production.



Safranin-O and collagen type II stainings are positive for the 20-100% PC Only and PC/MNF cultures whereas the PC/FB cocultures hardly show any safranin-O or collagen type II staining. The collagen type I staining is mainly positive in the PC/FB cultures while also at low percentages of chondrocytes the PC/MNF cocultures show positive collagen type I staining. Scalebar represents 50 µm.

DISCUSSION

This study evaluated the effect on chondrogenesis of coculturing primary human articular chondrocytes with freshly isolated human bone marrow mononuclear cells and showed that the effect of coculture depends on both the absolute number of primary chondrocytes and the ratio of these cells. Low number of cells in chondrocyte monoculture resulted in more cartilage matrix formation, both absolute and normalized to the number of cells when compared to pellets with higher number of chondrocytes. These

results indicate that at these low cell numbers, chondrocyte number per se inhibits chondrocyte proliferation and/or differentiation. Moreover, both the total amount of matrix produced and the amount produced per cell were actually inhibited upon addition of MNFs when cultured at a low chondrocyte to MNF ratio. In contrast, when higher percentages of primary chondrocytes were present in coculture, a stimulatory effect on GAG and GAG/DNA production was noted of addition of MNFs compared to the same numbers of chondrocytes in monoculture. This stimulation seemed to be specific for the MNFs, as at the same combinations, fibroblasts were not able to bring about this stimulation and instead showed a strong inhibition of the total amount of matrix produced as well as on the amount produced on a per cell basis. The presence of fibroblasts in coculture could negatively interfere with the nutrient and waste exchange from and to the chondrocytes thereby inhibiting cartilage matrix formation. Also in contrast to MNFs, fibroblasts are fully differentiated cells with a specific (non-chondrogenic) function and therefore less likely to be able to excrete chondrogenic factors that positively influence cartilage matrix production. Interestingly, at the higher chondrocyte/MNF ratios, the lower cell number at the end of the culture period compared to the chondrocyte monocultures may suggest that the MNFs had inhibited proliferation of the chondrocytes present, in addition to enhancing total matrix synthesis.

Why cartilage formation is enhanced in cultures containing a low number of chondrocytes is unclear. This effect may be ascribed to a lower volume/surface ratio of the tissue in pellet culture, facilitating proper oxygen, nutrient and waste diffusion (Zhou et al 2008), which may also explain the inhibitory effect of adding MNFs to these small quantities of chondrocytes, in spite of any stimulatory effect of, for example, secreted growth factors (Castro-Malaspina et al 1980; Fischer et al 2010; Hwang et al 2007; Lee and Im 2010; Solursh and Meier 1973). How many MNFs were actually incorporated in the pellets remains unclear, as extensive cell loss was observed in the cocultures, in particular at combinations with low number of chondrocytes and high number of MNFs. The stimulation of matrix production by the presence of MNFs combined with a higher number of chondrocytes may indicate that more MSCs were capable of being retained in the pellets, thus tipping the balance from cell number-mediated inhibition towards a relevant growth factor production, leading to a net stimulation of chondrogenesis. However, the presence of MNF at these ratios also appeared to have reduced cell proliferation.

The mechanism through which the combination of MNF cells and chondrocytes influences cartilage formation is still unknown. Excretion of as yet unknown factors may play a role. Within the current experimental set-up, it cannot be inferred whether MNFs were still present at the end of the culture period, nor what their phenotype was. Considering the fact that crude bone marrow in general contains less than 0.01 % of MSCs (Castro-Malaspina et al 1980), their prolonged presence or substantial contribution to chondro-

genesis is unlikely. Although less relevant from the point of view of clinical application, tracking of the MNF cells by e.g. the use of membrane markers (Kastrinaki et al 2008) may provide further insight in their mechanisms of action. In other types of coculture models, the role of added cells to chondrocyte culture has been shown to differ greatly. Enhancement by other cell types of cartilage formation by chondrocytes has mainly been tested for expanded articular chondrocytes. Both MSCs and P0 chondrocytes were able to increase the differentiation potential of passaged articular chondrocytes in vitro (Gan and Kandel 2007; Tsuchiya et al 2004). In the presence of expanded chondrocytes, human embryonic stem cells were able to differentiate towards the mesenchymal lineage and fat-derived stem cells showed a higher relative GAG production when co-cultured with P2 chondrocytes compared to a culture of P2 chondrocytes alone (Bigdeli et al 2009; Hildner et al 2009).

The population of myeloid and lymphoid cells, MSCs, haematopoietic stem cells and endothelial progenitor cells in the MNF (Yeo et al 2009) could be responsible for the production and excretion of stimulatory soluble mediators affecting chondrogenesis. However, the identity of such mediators, their levels and by what stimulus they are produced is unknown and most likely difficult to control in an *in vitro* setting. In general, teasing out factors responsible for any effect on chondrogenesis coculture setups is difficult, as a coculture approach never allows a standardized change of one specific parameter only. The stimulatory effect of culturing very low quantities of chondrocytes without added MNF cells found in the current study demonstrates that caution is required when drawing conclusions from studies in which such-like control conditions are not included.

Although some stimulatory effect of adding MNF cells in the current coculture was noted, altogether the combination of collagen type I staining in most of the cocultures and the relatively limited matrix production do not seem to support the use of MNFs in the current culture setup. As in clinical application of comparable cell combinations only low amounts of chondrocytes will be available, the current combination may not be applicable for a one-stage cell transplantation procedure. However, as a large proportion of the MNF cells did not integrate into the pellet and were lost during culture, a true estimate of the effect of adding these cells cannot be made. A culture model based on cells incorporated in a 3D matrix could determine the true extent and direction of interaction between the cells.

Moreover, it is not clear what parameter best describes tissue quality. Proteoglycan content may be indicative, or the amount of proteoglycan per cell. On the other hand, a high proteoglycan production per cell may also indicate final differentiation, precluding further tissue growth, whereas low proteoglycan production per cell may indicate that growth is still ongoing. As only endpoint measurements were performed in the current study, it cannot be excluded that longer or shorter time points may have revealed different effects of coculture. In addition collagen II production is thought to be important in

the regeneration of true hyaline cartilage, providing a network that entraps the proteoglycans produced (Buckwalter and Mankin 1998). Quantitative analysis of its production may provide further insight in the quality of the tissue formed.

In conclusion, this study showed that the influence of MNF cells on cartilage formation in a coculture model with non-expanded articular chondrocytes depends on the proportion of MNF cells being present in culture. A low chondrocyte to MNF ratio yields fibrocartilaginous tissue while at higher percentages of chondrocytes the MNFs actually seem to stimulate collagen type II and GAG production. In spite of the advantage of using cells that can be applied in the course of a one-step surgical procedure, more research should be done to prove the merits of this approach.

Chapter 3

Scaffold and cell composition influence chondrogenesis in 3D coculture of chondrocytes with mononuclear fraction cells

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Submitted for journal publication

ABSTRACT

The combination of mononuclear fraction cells (MNF) with primary chondrocytes (PC) makes cell expansion in autologous chondrocyte implantation (ACI) redundant and enables the development of a one-stage ACI procedure. This study evaluated the effectiveness of combining MNFs with PCs in scaffolds, compared to chondrocyte monocultures. Different scaffolds (Polyactive, Beriplast®, type II collagen or Hystem®) were seeded with MNF and PCs (PCMNF) or PCs at similar absolute amounts as in the PCMNF cultures without adding MNF (chondrocyte monocultures or PC Only). Scaffolds were cultured *in vitro* or implanted subcutaneously in nude mice and analysed on glycosaminoglycan (GAG) and DNA content and immunostained for collagen types I and II. *In vitro* cytotoxicity was assessed using lactate dehydrogenase (LDH) analysis.

PCMNF cultures in Beriplast®, Polyactive and Hystem® scaffolds resulted in a higher GAG content compared to PC Only cultures, with PCMNF containing 10-20% PCs showing the highest GAG production. Collagen type II staining for PCMNF cultures in Beriplast® and Polyactive scaffolds appeared more intense compared to PC Only cultures. Cytotoxicity was lowest in Beriplast® scaffolds.

The addition of MNFs to PCs increases cartilage-specific matrix production. Beriplast® scaffold seems to enhance this effect and may be the most viable option for a one-stage ACI procedure.

INTRODUCTION

Isolated lesions of articular cartilage are successfully treated by autologous chondrocyte implantation (ACI). This technique comprises two surgical interventions. First, articular cartilage is biopsied from a non-weight-bearing location in the knee. The tissue is digested and isolated chondrocytes are expanded and reimplanted in a second surgical procedure. Primarily to retain the cells in the defect cartilage after implantation, a periosteal flap was used (Brittberg et al 1994), but due to difficulties in surgical handling and the concomitant periosteal hypertrophy, alternatives were sought. Since then, several generations of ACI have been developed using collagen-, hyaluronan-, fibrin- and polymer-based scaffolds (Gobbi et al 2009; Kim et al 2010; Kreuz et al 2009; Nehrer et al 2009; Tohyama et al 2009).

In addition to the fact that two surgical procedures are required for ACI, another disadvantage of this treatment is the need for chondrocyte expansion to obtain sufficient cell numbers, which leads to a loss of chondrogenic phenotype and potential (Barlic et al 2008; Marlovits et al 2004; Schnabel et al 2002). One solution that has raised scientific interest is the addition of other cell types such as mesenchymal, adipose or embryonic stem cells, to non-expanded primary chondrocytes. The role of these added cells may involve differentiation into chondrogenic cells or provision of trophic factors that promote chondrogenesis of the chondrogenic cells present (Wu et al 2011). With other cells available, the number of chondrocytes necessary would be in principle much lower. As a result, the need for expansion is eliminated which allows the implantation of more differentiated cells (Bigdeli et al 2009; Chen et al 2009; Gan and Kandel 2007; Hildner et al 2009; Tsuchiya et al 2004; Mo et al 2009) permitting one surgical intervention and therefore enables the development of a one-stage ACI procedure. In particular, the mononuclear fraction (MNF) from bone marrow has been shown to stimulate chondrogenesis, most likely by the production of morphogenic factors (Chapter 2) and can be obtained and applied within the time frame of one surgical procedure. The MNF could therefore be a promising cell type to be combined with primary isolated chondrocytes in a one-stage ACI procedure.

Retention of cells within the cartilage defect is achieved by a scaffold, as used in the current generation of matrix-assisted ACI (Marlovits et al 2006). Several scaffold characteristics can affect cartilage matrix formation, in particular scaffold architecture and biochemical and biological properties of the material used (Gavenis et al 2006; Jeong and Hollister 2010; Nuernberger et al 2011). Regarding the latter, components of the hyaline cartilage matrix, such as collagen type II or hyaluronic acid, or factors that have a role in wound healing and repair, such as fibrin, might be good candidates. In this respect, Beriplast® (Nycomed, the Netherlands) is a type of fibrin glue which already showed to stimulate redifferentiation of chondrocytes in vitro (Gille et al 2005). In ad-

dition, biphasic scaffolds composed of two materials, where one provides mechanical strength and the other promotes chondrogenesis may hold even more promise.
The aim of this study was to evaluate the effect on cartilage matrix production when mononuclear fraction cells are added to primary chondrocytes and what scaffold material facilitates this interaction best.

MATERIAL AND METHODS

Donor material and cell isolation

All use of patient material in this study was approved by the institutional ethical committee (number: 2008.III.08.082). Macroscopically healthy articular cartilage was harvested from the femoral condyles of 3 donors during autopsy and human bone marrow was obtained from the iliac crest during routine hip arthroplasty.
Primary chondrocytes (PC) were isolated overnight at 37 °C by controlled matrix degradation in a 0.15 % collagenase type II solution (Worthington, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 1 % penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen, The Netherlands). After enzymatic digestion, the cell suspension was filtered using a 70 µm cell strainer, washed in phosphate-buffered saline (PBS) and counted.
The mononuclear fraction (MNF) was isolated from the aspirated bone marrow by 50x dilution of the cell pellet, obtained after 20 min centrifugation (at 300g) of the whole bone marrow, in red blood cell lysing buffer (Sigma-Aldrich, The Netherlands). After 45 minutes incubation at room temperature the remaining cells were spun down by centrifugation (300g), washed twice in PBS and counted.

Preparation of cell-seeded scaffolds

Four different biomaterials, Polyactive, Beriplast®, a type II collagen hydrogel and Hystem®, were seeded with a combination of PCs and MNF cells at various ratios, referred to as PCMNF. Final volume of the scaffolds after seeding was 100 µl for all scaffold types. A total of 250000 cells were seeded per biomaterial containing either 0 (0 %), 12500 (5 %), 25000 (10 %), 37500 (15 %), 50000 (20 %) or 87500 (35 %) chondrocytes. To evaluate the effect of adding MNF cells to articular chondrocytes in scaffolds, the biomaterials were also seeded with chondrocytes at the same absolute amounts as in the PCMNF containing scaffolds, however, without the addition of MNF cells, referred to as PC Only. For the Beriplast® scaffold, the cells were resuspended in 70 µl of the fibrinogen component of the gel, and in a 16 wells chamber slide (Nunc, Germany), combined with 70 µl of the trombin component and allowed to set for 10 minutes at room temperature.

The Polyactive scaffold was prepared as previously described (Bekkers et al 2010). In brief, PEOT/PBT co-polymer granules (PoroGen B.V., The Netherlands) with a 1000/70/30 composition were printed using a 3D fiber deposition technique(Woodfield et al., 2004) with the BioScaffolder system (SysEng, Germany). Fibers were printed in a 0º - 90º orientation with a fiber spacing of 600 µm consisting of 14 layers (2 mm thickness after overnight swelling in PBS). Cylindrical scaffolds with a diameter of 5 mm were punched out and sterilized by 2 h immersion in 70% alcohol followed by 1 hour drying at UV-light exposure. Cells were seeded by incorporation in 45 µl of the fibrinogen component of Beriplast®, which was subsequently injected into the pores of the Polyactive scaffold, followed directly by intraporous injection of 45 µl of the trombin component and allowed to set for 30 minutes.

Hystem® hydrogels (Glycosan, USA) were prepared following the manufacturer's instructions. The thiol-modified hyaluronic acid powder (Hystem® component) was dissolved in 1 ml of degassed, de-ionized water (DG water, Glycosan). Per scaffold the cells were resuspended in 100 µl Hystem® component and combined with 25 µl of the extralink solution (polyethylene-glycol-diacrylate powder dissolved in DG water, Glycosan). The gelation process took place for 3 h at room temperature.

A type II collagen hydrogel was prepared by combining 800 µl of collagen type II solution (AteloCell, Koken, JAP) with 100 µl of 10x concentrated DMEM (Sigma-Aldrich, the Netherlands), 10 µl PBS, 40 µl demineralised water and 50 µl 1 M $NaHCO_3$, on ice. Cells were resuspended in 100 µl of the combined solution and incubated at 37 °C and 5% CO_2 for three hours.

Culture of cell-seeded scaffolds

The scaffolds seeded with PC only or PCMNF ratios (n=5 per ratio) were cultured in DMEM (Invitrogen, the Netherlands) with 0.2 mM l-ascorbic acid-2-phosphate (AsAp, Sigma-Aldrich), 2% Human Serum Albumin (Sanquin, the Netherlands), penicillin/streptomycin (100 U/ml / 100 µg/ml, Invitrogen), 2% ITS-X (Invitrogen). TGF-β_2 (R&D systems, USA) was also added to the medium at a concentration of 10 ng/ml because it is part of our standard culture medium and to be able to compare present cultures with earlier work. This medium was changed twice a week. At days 2 and 21, a medium sample (50 µl) was taken to check for cytotoxicity using a lactate dehydrogenase (LDH) assay. After three weeks of culture, the scaffolds were analyzed for cartilage matrix formation by GAG and DNA quantification, histology and immunohistochemistry. The experiment was repeated to confirm reproducibility.

Histological and immunohistochemical evaluation

After 3 weeks of *in vitro* culturing, one scaffold per combination of cell concentration and biomaterial scaffold was harvested for histology. Samples were embedded in Tissue-Tek

(Qiagen, The Netherlands) and stored at -80 ºC. Cryosections of 5 µm were cut, air-dried o/n and fixed with acetone for 10 minutes and immunostained for collagen types I and II. Endogenous peroxidase was blocked with 0.3 % H_2O_2 in PBS for 10 minutes. Subsequently sections were incubated with 5 % bovine serum albumin (BSA) in PBS for 30 min followed by o/n incubation at 4 ºC with the primary antibodies for either collagen type I (mouse anti-human IgG 1:100 in 5 % PBS/BSA, CP17L, Merck) or collagen type II (mouse anti-human IgG, 1:100 in 5 % PBS/BSA, II-II 6B3, DSHB, USA). Subsequently, the slides were washed with PBS-Tween 0.1 % and incubated with the secondary antibody for collagen type I (biotinylated sheep anti-mouse IgG, 1:200, RPN1001V, GE healthcare, The Netherlands) and collagen type II (goat anti-mouse horse radish peroxidase [HRP], 1:100, P0447, Dako, The Netherlands) respectively. Sections were then washed 3 times for 5 minutes in PBS-Tween and PBS. Peroxidase activity was visualized with 3-diaminobenzidine solution (DAB, Sigma-Aldrich). The sections were counterstained with Mayer's haematoxylin (Merck), dehydrated and mounted.

GAG and DNA analysis

A total of 4 scaffolds per cell concentration per biomaterial were analyzed for GAG and DNA content. The scaffolds were digested o/n in 200 µl papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 ºC. After complexation of the scaffold digest with 1,9-dimethylmethylene blue chloride (DMMB), the GAG content was determined by spectophotometric analysis at 540 nm to 595 nm as described before (Farndale et al 1986) using chondroitin sulphate (shark; Sigma-Aldrich) as a standard. The spectophotometrically measured values were, per scaffold type, corrected by the values of the unseeded 0 % PC Only scaffolds.

The DNA content per scaffold digest was determined using a Picogreen DNA assay (Invitrogen) following the manufacturer's instructions.

LDH analysis

LDH activity, used as a measure of cytotoxicity, was analyzed in conditioned culture medium at days 2 and 21 using a commercially available LDH kit (Roche, USA), following the manufacturers' instructions. The conditioned medium samples of the cell-free scaffolds (0% PC Only) were used as control samples to correct for background LDH signal. The LDH signal at day 21 was corrected for the measured DNA content at day 21.

Subcutaneous implantation in nude mice

To confirm *in vivo* reproducibility of the *in vitro* data, the PCMNF concentrations were also seeded in the four different biomaterials and, for one PC and one MNF donor, subcutaneously implanted in nude mice (NMRI nude mice, FOXn1, Charles River, USA). Animal experiments were conducted in agreements with the committee for animal ex-

periments and following institutional guidelines on the use of laboratory animals (DEC no. 2009.III.05.038).

One day before subcutaneous implantation, the scaffolds (n=5 per biomaterial and PCMNF ratio) were prepared and seeded with cells as described above, with omission of the 15 % PCMNF combination. Before implantation, the mice were anaesthetised with 1.5 % isoflurane. The different cell concentrations and biomaterials were randomized to 5 separate subcutaneous dorsal implantation pockets in 20 female nude mice. During surgery, 8h and 1 day after surgery, the mice were treated with subcutaneous injection of the analgesic buprenorphine (0.05 mg/kg, Merck, Germany). After 1 day of solitary housing, the mice were housed together at the Central Laboratory Animal Institute of the Utrecht University. Three weeks after implantation, the mice were sacrificed by cervical dislocation and the scaffolds were harvested for analysis of GAG and DNA content using the abovementioned protocol. We did not include immunohistochemical stainings of the *in vivo* constructs because our power analysis urged an n=5 per biomaterial per cell combination which did not leave enough cells for additional samples / constructs for immunohistochemical analysis.

Statistical analysis

All statistical analysis was performed using SPSS version 15.0. For both the *in vitro* and *in vivo* experiments, differences in GAG and GAG/DNA between the different scaffold types and between the different chondrocyte concentrations in PCMNF and PC Only cultures were tested using a one-way ANOVA with posthoc Bonferroni test. Differences in GAG and GAG/DNA between the PCMNF and PC Only cultures were tested using a paired samples t-test. A p-value of $p<0.05$ was considered statistically significant.

RESULTS

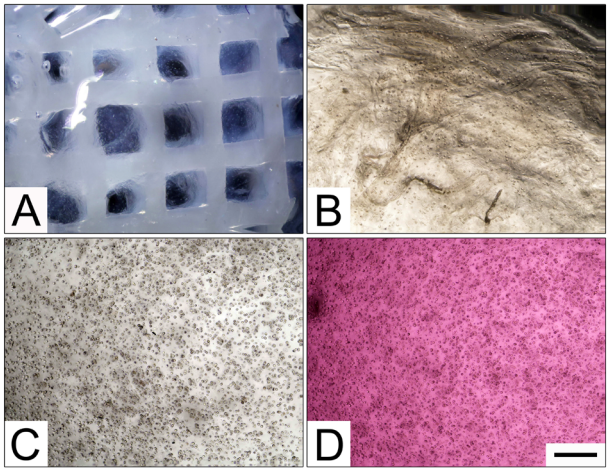
Scaffold production and handling

The scaffolds directly after preparation and cell seeding are shown in Figure 1. In all scaffold materials, the cells could easily be seeded which resulted in a homogenous cell distribution throughout the scaffold. Both the Beriplast® and Polyactive (combined with Beriplast®) scaffolds resulted in firm constructs that could potentially be used directly in a clinical setting. The collagen and, to a lesser extent, Hystem® scaffold remained viscous gels throughout the experiment, which as such seemed unpractical in a clinical setting.

DNA analysis

After 3 weeks of culture, there was no statistical significant difference in DNA content between any of the scaffold types or cell combinations tested (data not shown).

Figure 1



Microscopic pictures of the Polyactive (A) scaffold (loaded with Beriplast®), the Beriplast® (B), Hystem® (C) and Collagen type II (D) hydrogels directly after preparation, showing homogenous cell distribution. Scaling bar represents 500 µm.

In vitro GAG production

The addition of the bone marrow mononuclear fraction to primary chondrocytes increased cartilage matrix production in all scaffold types compared to chondrocyte monocultures (PC Only), except for the collagen scaffold. In terms of absolute GAG the effect was most dominant in Beriplast® scaffolds (5-20 % PCMNF ($p<0.001$)) and in Hystem® scaffolds (10-20 % PCMNF, $p<0.04$). Normalised to DNA content the enhancing effect of cocultures was only significant for Beriplast (5-20% PCMNF ($p<0.001$)) and Polyactive (10-20 %, $p<0.01$) (Figure 2).

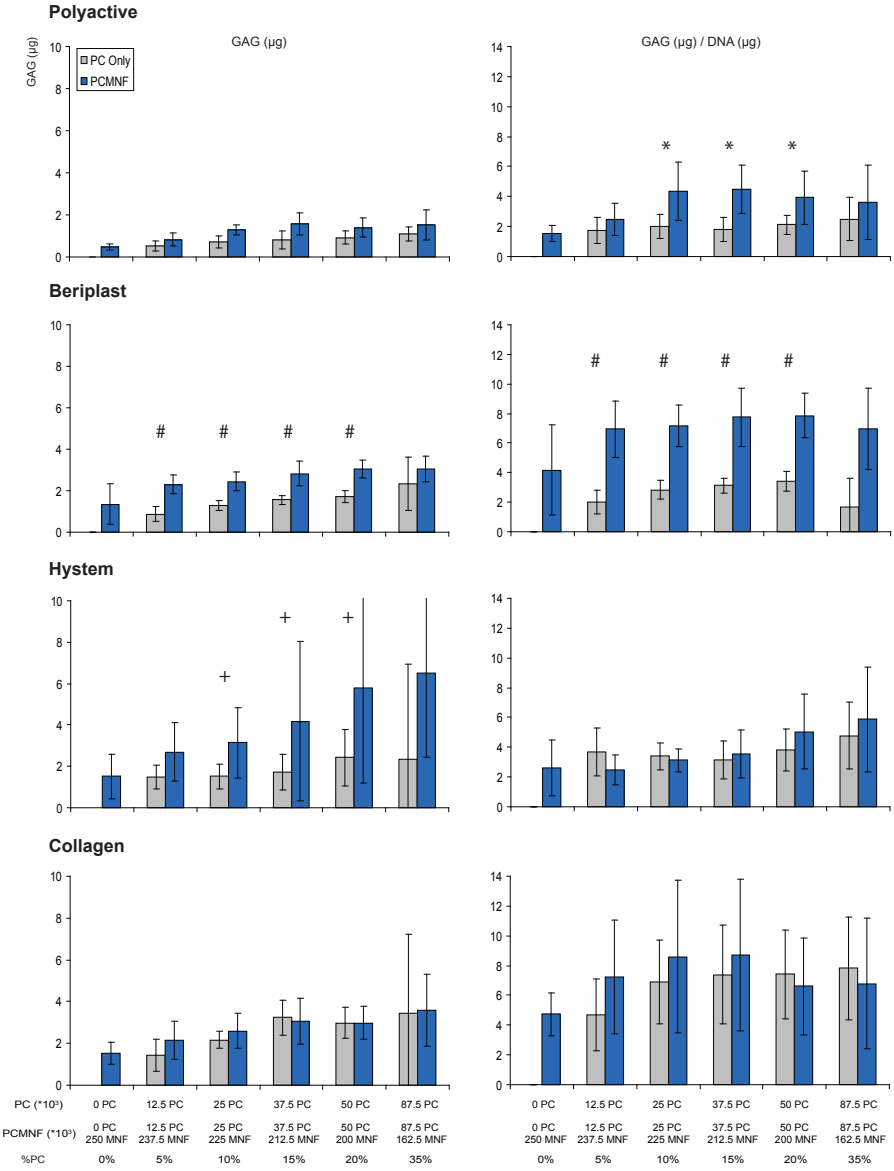
Upon comparison of scaffold-specific effects, absolute GAG production was lowest in Polyactive scaffolds when compared to the Hystem®, Beriplast® and collagen scaffolds for most coculture combinations (5-35 %, $p<0.05$) and also for chondrocyte monocultures (25.000-50.000/10-15 % PCs, $p<0.05$).

Normalised to DNA content, the Beriplast® and collagen scaffolds seeded with cocultures (5-20 % PCMNF, $p<0.04$ and 5-10% PCMNF, $p<0.04$, respectively) and the collagen scaffold with chondrocytes yielded more GAGs per cell compared to the Hystem® and Polyactive scaffolds (Figure 2).

LDH release

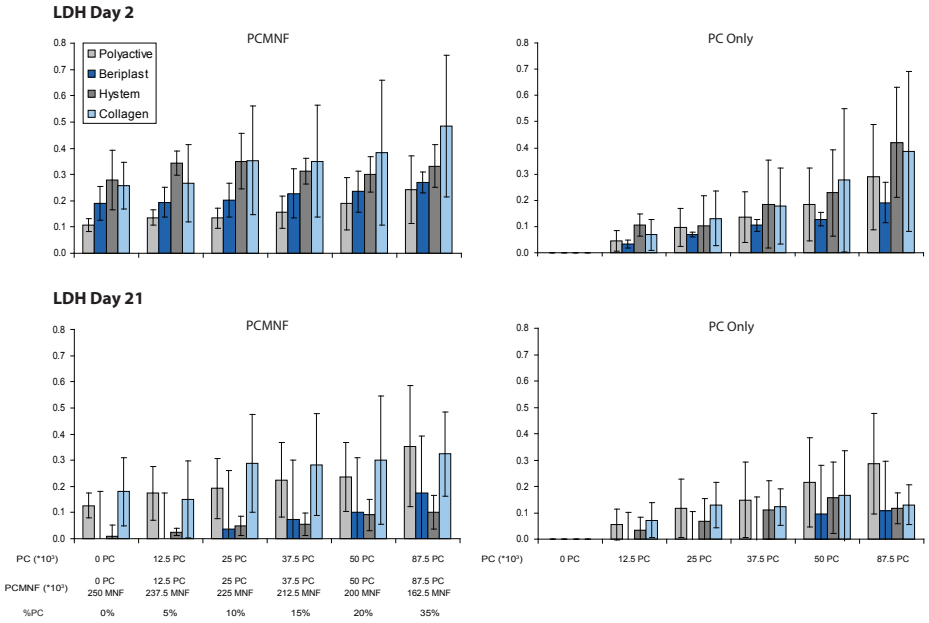
Overall, the immediate cytotoxicity, expressed by the LDH release at day 2, was lowest in Beriplast® and Polyactive (loaded with Beriplast®) scaffolds. In PCMNF cultures, containing low amounts of chondrocytes (0-15 % PCMNF), the Hystem® scaffold released statistical significantly more ($p<0.05$) LDH in the medium compared to the same condi-

Figure 2 *In vitro* GAG and GAG/DNA production for different scaffold types and chondrocyte seeding densities.



A possible effect of combining cells is dependent on the scaffold type used. For Polyactive (loaded with Beriplast®), Beriplast® and to a lesser extend Hystem®, GAG and GAG/DNA production is higher in combined PCMNF cultures compared to related PC Only cultures. The collagen type II scaffold seems to positively influence cartilage formation when only chondrocytes are seeded as those cultures show higher GAG and GAG/DNA compared to the other scaffold types ($^{\#}p<0.001$, $^{+}p<0.04$, $^{*}p<0.01$), (n=3 donors).

Figure 3 LDH measurement (corrected for DNA) in supernatant after 2 and 21 days of culturing.



The Beriplast® scaffold showed a low cytotoxicity at the beginning and end of the *in vitro* culture for all culture conditions and chondrocyte seeding densities, (n=3 donors).

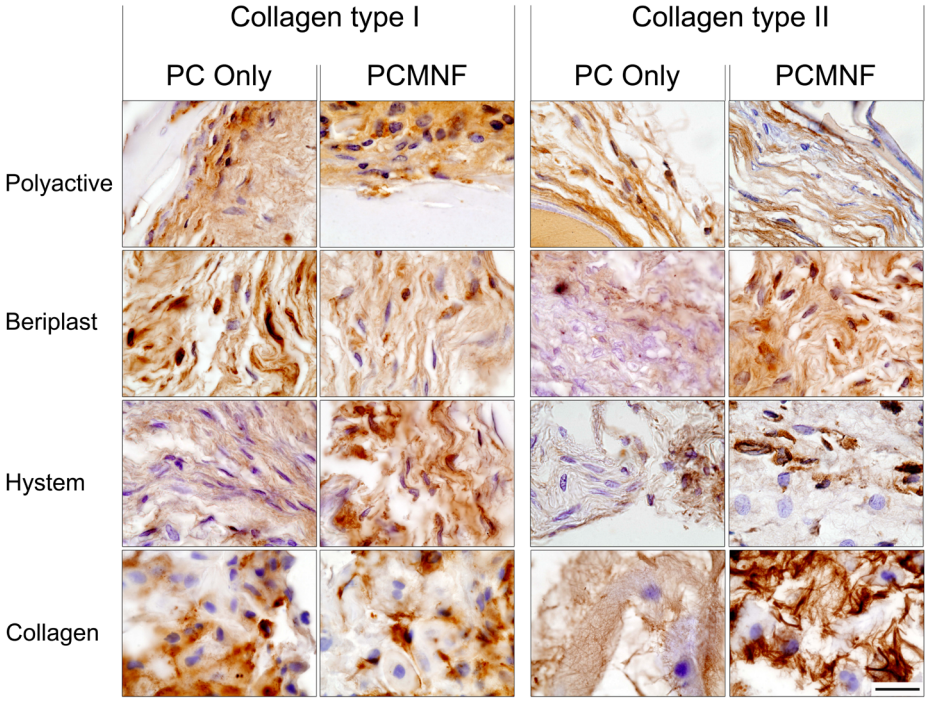
tions in Beriplast® and Polyactive scaffolds. The release of LDH in PC Only cultures, at day 2, was not affected by scaffold type (Figure 3).

After 21 days of culture, the LDH release corrected for DNA, was lowest in the Beriplast® scaffolds and significantly different compared to the collagen scaffold, only in cultures with low amounts of seeded chondrocytes (0-15 % in PCMNF, $p<0.05$, and 12500-25000 PCs for PC Only cultures, $p<0.03$) (Figure 3).

Histological and immunohistochemical evaluation

Staining for collagen type I was negative for all empty scaffolds. Collagen type II staining was, as expected, positive for the empty collagen scaffolds. The immunostainings in PCMNF and PC Only cultures containing 25000 chondrocytes appeared most intense (Figure 4) independent of the scaffold type used. Polyactive (loaded with Beriplast®), Beriplast® and collagen scaffolds showed positive collagen type I staining seeded with both the PC Only and PCMNF. Except for the Polyactive scaffold, all scaffold types appeared to show a more intense collagen type II staining for the PCMNF culture condition compared to PC Only culture conditions.

Figure 4 Immunohistochemical staining for collagen type I and II.



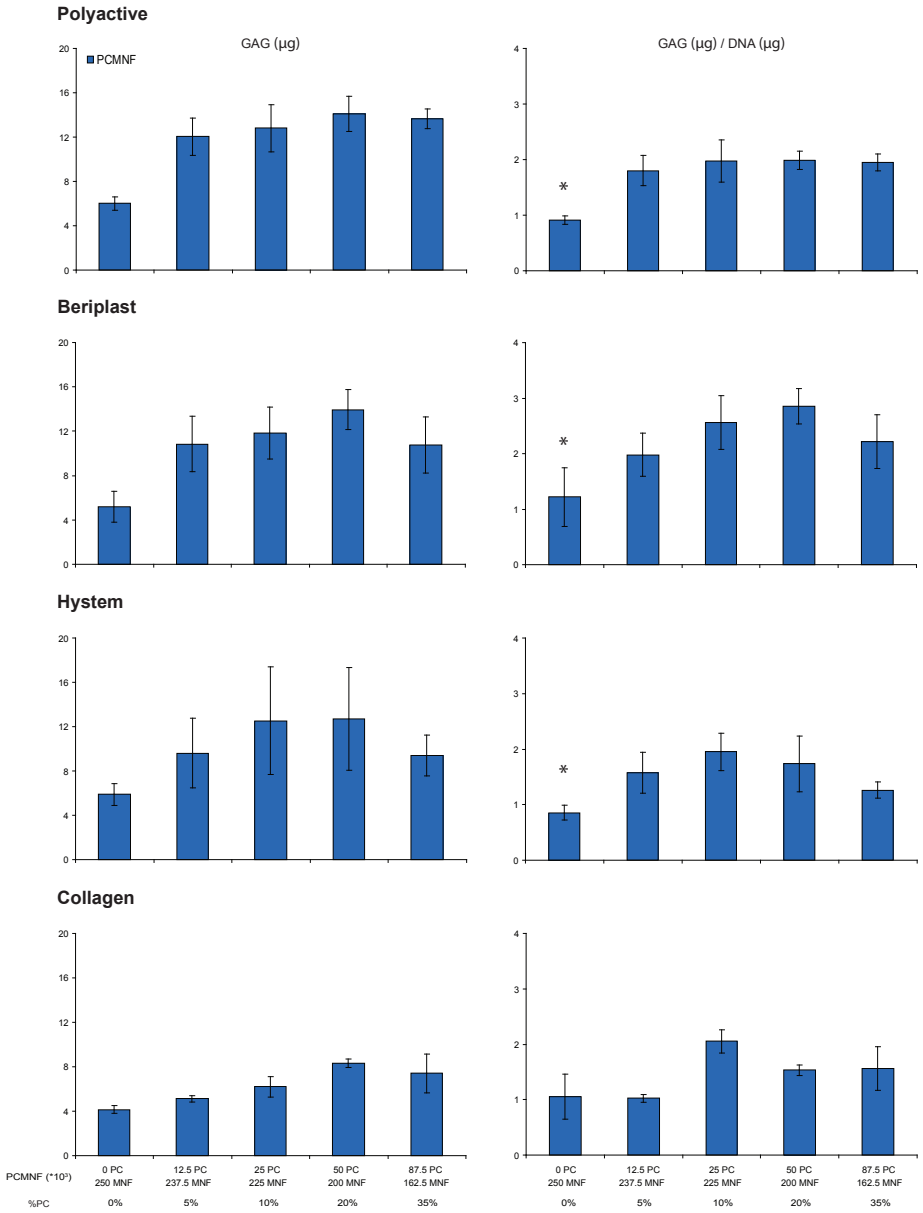
For both the PC Only and PCMNF culture conditions all scaffolds containing 25.000 chondrocytes (10 % PCMNF) showed, except for the Hystem® material, positive staining for collagen type I. All but the Polyactive scaffolds, showed more intense staining for collagen type II in the PCMNF compared to the PC Only culture conditions. The bar at the right bottom represents 20 µm.

In vivo GAG production

Three weeks after subcutaneous implantation in nude mice, no statistically significant differences in DNA content between different scaffolds types and chondrocyte seeding densities were observed (data not shown).

In vivo cartilage matrix production was highest when mononuclear fraction and primary chondrocytes were seeded in Beriplast®, with GAG production in the Beriplast® and Polyactive scaffolds higher (10-35 % PCMNF, $p<0.05$) compared to collagen scaffolds. Normalized to DNA content, at 10-20 % PCMNF, the Beriplast® scaffold outperformed ($p<0.044$) the other scaffold types tested. In the Hystem®, Beriplast® and Polyactive scaffolds, "cocultures" without the addition of chondrocytes (0 % PCMNFs) contained less ($p<0.01$) GAGs and GAGs per DNA compared to the cocultures containing PCs, irrespective of the percentage, For the collagen scaffolds no effect was noted of adding MNFs (Figure 5).

Figure 5 GAG and GAG per DNA production after subcutaneous implantation in nude mice.



On average, the coculture conditions containing 10-20 % primary chondrocytes are most effective in terms of GAG production, also when normalized to cell content (*p<0.01), (n=1 donor).

DISCUSSION

This study examined the influence on chondrogenesis of a coculture approach of bone marrow mononuclear fraction cells and primary chondrocytes compared to using chondrocytes alone in a 3D environment and demonstrated that the added value of coculture depends on the concentration of chondrocytes and the type of scaffold material used. The addition of mononuclear fraction cells to chondrocytes in a 3D environment increased cartilage matrix formation in Beriplast®, Polyactive and Hystem® scaffolds compared to chondrocyte monocultures containing the same amounts of chondrocytes. When corrected for cell number, these differences were maintained in Beriplast® and Polyactive scaffolds. However, when cells were seeded in a collagen type II environment, no effect of adding mononuclear fraction cells was found, although a clear stimulatory effect was found of culturing chondrocytes on this scaffold compared to the other scaffolds. Total GAG production seemed highest in Hystem scaffolds seeded with cocultures at particular ratios, but normalised to cell content, this was highest for Beriplast and collagen scaffolds, in the latter scaffold also for constructs seeded with only chondrocytes. Polyactive scaffolds on the whole seemed least supportive of chondrogenesis. Upon *in vivo* implantation of scaffolds with both cell types, however, this inhibiting effect of Polyactive seemed to be lost, with similar total matrix production in Polyactive, Beriplast® and Hystem®. Also in contrast to the results obtained in vitro, the collagen scaffolds here seemed to be least effective in supporting chondrogenesis, both in absolute terms and on a per cell basis. Moreover, those constructs *in vitro* also showed positive staining for collagen type I. Cytotoxicity was lowest in Beriplast® scaffolds.

In the current study chondrocytes were obtained following overnight isolation in 0.15% collagenase solution. A one-stage ACI approach based on the presented combination of cells is clinically not applicable when the overnight isolation method for chondrocytes is used. However, the primary aim for this study was to show that the combination of P0 chondrocyte and MNF cells positively influences chondrogenesis when compared to chondrocyte monocultures. The promising data of the current study have meanwhile incited research on fast digestion protocols for chondrocytes and we have succeeded in isolating chondrocytes from debrided articular cartilage over a time span of 45 minutes in such amounts and efficacy ($1.37\pm0.50*10^6$ chondrocytes per gram tissue) that would be sufficient for a one-stage ACI procedure (Chapter 7).

Other coculture studies already speculated on the mechanisms of action when chondrocytes were combined with different cell types. Primary articular chondrocytes, human embryonic, mesenchymal and adipose-derived stem cells stimulated cartilage-specific matrix and gene production in coculture with expanded articular chondrocytes *in vitro* (Bigdeli et al 2009; Chen et al 2009; Gan and Kandel 2007; Hildner et al 2009; Tsuchiya et al 2004; Mo et al., 2009). Also mononuclear fraction cells positively influenced cartilage

specific matrix production when combined with primary articular chondrocytes in a pellet study (Chapter 2). However, at low chondrocyte to MNF ratios, the MNF cells seemed to actually inhibit cartilage matrix production (Chapter 2). Cultures consisting of only MNF, embryonic, mesenchymal or adipose-derived cells showed, in most of the abovementioned coculture work, a lower chondrogenic gene expression or cartilage matrix production, compared to the cocultures. Also, MSCs only cultures had a lower collagen II / collagen I ratio and aggrecan expression and higher VEGF expression, suggestive for hyperthrophic differentiation, compared to cocultures with chondrocytes (Chen et al 2009; Tsuchiya et al 2004). This could suggest that MSC or MNF only procedures would not be in favour over combined cultures for future clinical application. It has been reported that members of the FGF, TGF, IGF and BMP family have the ability to stimulate chondrogenesis in articular chondrocytes and are upregulated in bone marrow stroma during the events of chondrogenesis (Rosier et al 1989; Sailor et al 1996; Sekiya et al 2002; Smith et al 1989). Whether the combination of bone marrow derived cells and chondrocytes stimulate chondrogenesis by cell-cell mediated contact or by paracrine effect using trophic factors cannot be elucidated from abovementioned research. However, the ratio at which both cells are present in the construct seems crucial for the effect on cartilage matrix production.

Lysed bone marrow contains approximately 0.01% of MSCs (Castro-Malaspina et al 1980). Recent *in vitro* pellet coculture work shows that MSCs are not present at the end of coculture with chondrocytes (Wu et al 2011). The chance that a population of mainly mature myeloid and lymphoid cells, as used in the current study, persists throughout culture is, therefore, negligible and additional labelling of cells at the start of the culture will, possibly, not provide new insight on that. Consequently, the stimulation of cartilage-specific matrix production upon addition of mononuclear fraction cells to primary chondrocytes is not likely to be instigated directly by MSCs but can most likely be explained by the presence of trophic factors in the mononuclear fraction (Wu et al 2011). In the chondrocyte monocultures the cells were seeded at different densities, whereas for the PCMNF cultures these numbers were complemented with MNFs to a final amount of cells that remained constant. The fact that the absolute amount of DNA at the end of culture did not differ between the PCMNF and equivalent PC Only cultures, could implicate differences in proliferative activity of the cells, but also that chondrocytes were the only cells to survive and/or proliferate. Cells seeded at low density (such as the chondrocyte monoculture) may first have proliferated, where the presence of the mononuclear fraction, may have induced the primary chondrocytes to start producing cartilage matrix earlier. The latter notion is supported by the finding that collagen II staining seemed more intense in combined MNF and chondrocyte cultures compared to chondrocyte monocultures.

Surprisingly, the current study showed that the added value of MNFs appeared dependent on the scaffold material used. In contrast to the other scaffolds, no improvement of matrix production was found in collagen type II gels by the addition of MNFs. Matrix production in the Hystem® scaffold showed intermediate results, where the addition of mononuclear fraction cells did not lead to an extensive increase in matrix production on a per cell basis. Why addition of MNFs to chondrocytes in collagen scaffolds does not increase cartilage matrix production, is not clear. Possibly it is attributable to crosslink density and viscosity of the gels, influencing the exchange and diffusion of molecules (Cheng et al 2011; Lewus and Carta 1999). The Beriplast® gels are more likely to retain the trophic factors excreted by the mononuclear fraction cells compared to the low viscous Hystem® and collagen gel, leading to a prolonged effect on cartilage matrix-formation. Beriplast® scaffolds seeded with PCMNF cells also appeared to stain more intensely for collagen II, compared to those seeded with PC Only. However, collagen type I was also present in these constructs, indicating neocartilage of fibrocartilaginous morphology. Alternatively, the stimulation of chondrocyte matrix production by the collagen scaffolds compared to the other scaffolds may have been at the maximum capacity of the incorporated cells, thus precluding any additional stimulatory effect of the MNFs.

Many biomaterials are already being utilized in a clinical trial setting as carriers for chondrocytes in ACI procedures. Hydrogels and other 3D environments are favourable as they are able to keep the spherical morphology of differentiated chondrocytes and initiate chondrocyte redifferentiation after, expansion-induced, dedifferentiation (Benya and Shaffer 1982; Gille et al 2005; Grigolo et al 2002; Park et al 2009). The use of natural components previously showed to improve cell adhesion and distribution into the scaffold (Nuernberger et al 2011; Schagemann et al 2010). Also materials related to the hyaline cartilage matrix or those involved in wound healing could have a possible stimulatory influence on chondrogenesis as well as being carriers of cells. In this study, the collagen scaffold showed to positively influence GAG production when seeded with low amounts of chondrocytes (PC only cultures) compared to the Hystem® and both Beriplast® containing scaffolds. The presence of a collagen type II matrix might stimulate the production and deposition of GAGs by chondrocytes (Nehrer et al 1997) where this influence could be less outspoken in scaffolds that already contain GAGs (Hystem®) or have no hyaline matrix components (Beriplast®) at all.

Subsequent to the guidance of tissue regeneration, hydrogel cytotoxicity, gelling time and easy (surgical) handling are critical aspects for its clinical application. When primary chondrocytes are being combined with mononuclear fraction cells in a one-stage ACI procedure, Beriplast® seems the most optimal choice as scaffold material because of its low cytotoxicity, easy handling, short gelling time and good cartilage formation. Combining Beriplast® with a solid framework, such as the Polyactive scaffold, did not improve cartilage formation. Moreover, collagen type II staining was less intense and

matrix production was decreased compared to the Beriplast® scaffolds. If, in a one-stage procedure, only chondrocytes were used to regenerate the tissue, the collagen type II scaffold might be preferable as the chondrocyte monocultures showed high cartilage specific matrix production. However, the handling, gelling time and viscosity of the gel need improvement before this technique can be applied in the clinic. For the other scaffolds tested, using chondrocytes only in a one-stage procedure was suboptimal in terms of cartilage matrix production, and clearly required the addition of bone marrow cells.
In conclusion, the results from the current investigation show that in the process of cartilage regeneration the type of scaffold material used determines the regenerative effect of primary chondrocytes and mononuclear fraction cells. Beriplast® seems an excellent option seeded with 10-20% primary chondrocytes and 90-80% mononuclear fraction cells for a one-stage ACI procedure.

Chapter 4

Chondrogenic potential of cells for articular chondrocyte transplantation depends on their original location

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Submitted for journal publication

ABSTRACT

Autologous chondrocyte implantantation (ACI) is a widely used technique for the treatment of focal articular cartilage lesions in de knee. Here, chondrocytes obtained from non-weight-bearing cartilage are, after expansion, re-implanted into the cartilage defect. Already, the cartilage matrix and chondrocyte metabolism showed to differ between anatomical locations in the knee which possibly makes specific locations more suited as a donor site in ACI. This study evaluated the regenerative capacity of chondrocytes derived from debrided defect cartilage compared to healthy cartilage from different weight-bearing regions in the joint in order to determine the best cell source for ACI.

Here we report, for the first time, that non-weight-bearing articular cartilage in the knee is a suboptimal donor site for chondrocytes for ACI, as shown by hypertrophic differentiation and a decreased matrix production. Moreover, chondrocytes from debrided defect cartilage were, in culture, found to outperform cells from non-weight-bearing locations in terms of cartilage-specific matrix production and immunohistochemical staining.

Thus, chondrocytes derived and expanded from debrided defect cartilage can, safely replace cells from the non-weight-bearing region commonly used in ACI without loss of quality of the cartilage formed. Moreover, freshly isolated cells from the latter site of location were clearly shown to have degenerative characteristics, precluding their use in any future treatment based on non-expanded cells.

INTRODUCTION

Although osteoarthritis is a debilitating disease affecting millions of people worldwide, no definite answers have been provided as to its etiopathology. One clear trigger, however, is the presence of focal articular cartilage lesions, which necessitates timely treatment of these defects. Several treatments for focal cartilage defects are based on the transplantation of autologous tissue or cells from areas in the affected joint that are not exposed to mechanical loading (Brittberg et al 1994; Hangody et al 1998). In addition to mosaicplasty, which is based on osteochondral plugs from non-weight-bearing areas transplanted into the defect, a progressively more frequently applied procedure is autologous chondrocyte implantation (ACI) (Brittberg et al 1994). This procedure departs from the harvest of, cartilage biopsies from the non-weight-bearing lateral margin of the trochlea, from which chondrocytes are isolated, expanded and re-implanted into the defect.

However, it is not clear whether the non-weight-bearing region is the appropriate location for harvest of cells or tissues. Adult articular cartilage has distinct biochemical and biomechanical characteristics directed by the influence of mechanical loading (Kurkijarvi et al 2004; Lane et al 2000; Quinn et al 2005; Raimondi et al 2008). By mathematical modelling, already in the late nineties, degenerative changes were predicted in non-weight-bearing areas in the joint (Carter et al 2004). This seemed to be supported by the observation that, in particular, the non-weight-bearing cartilage in macroscopically healthy human joints displayed signs of degeneration as reflected by a reduction in staining for proteoglycan, a major extracellular matrix component of cartilage (Bullough et al 1973; Bullough and Walker 1976). However, it cannot be excluded that this diminished proteoglycan content rather reflected an adapatation to the limited loading. Assessment of the phenotype of the resident cells would provide more insight into a possible irreversible nature of the microscopic changes observed and to what extent this tissue or its resident cells are optimal for transplantation purposes. As cartilage debrided from defects is derived from weight-bearing areas, it can be postulated that this tissue may still be relatively healthy. Chondrocytes from debrided cartilage have been reported to be capable of redifferentiation, but no comparison with healthy cartilage was made, nor were separate defect grades characterised (Biant and Bentley 2007; Chaipinyo et al 2004). The cartilage in Outerbridge grade IV is likely to harbour chondrocytes that are phenotypically different from the cells isolated from the macroscopically intact remnant cartilage matrix in Outerbridge grade III lesions (Outerbridge 2001), but also cartilage cells in grade III may already be predegenerative, compared to cartilage from unaffected locations.

Therefore, the aim of the current study was to determine the regenerative capacity of chondrocytes from non-weight-bearing locations in the knee in comparison to

healthy weight-bearing cartilage and to study the chondrocyte phenotype in cartilage surrounding the two major types of focal cartilage lesions. The results will provide valuable information on the most appropriate cell and tissue type for ACI and other transplantation-based treatments for focal cartilage defects.

MATERIALS AND METHODS

Patient characteristics

All use of patient material in this study was approved by the institutional ethical committee. Macroscopically healthy articular cartilage was, under sterile conditions and within 24h post-mortem, harvested from full-weight and non-weight-bearing locations of the knees of 5 different donors (age 39±9, 3 male, 2 female) during autopsy at the department of Pathology. Non-weight-bearing (NWB) cartilage was obtained from the lateral margin of the trochlea and full-weight-bearing (FWB) cartilage from the weight-bearing femoral condyles.

A total of 20 samples of debrided tissue from focal lesions in 20 patients (age 29±8 years old, 8 male, 12 female) during microfracture or ACI surgery were obtained from March 2009 – April 2010 at the department of Orthopaedics. Among the 20 samples of debrided cartilage, 12 were classified as Outerbridge grade III and 8 grade IV (Outerbridge, 2001).

Isolation and culture of chondrocytes

Cartilage samples were rinsed in PBS, diced into small pieces and digested overnight at 37 °C in a 0.15 % collagenase type II solution (Worthington, Lakewood, USA) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) with penicillin/streptomycin (100 U/ml / 100 µg/ml, Invitrogen, the Netherlands). After enzymatic digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, USA) and washed in phosphate-buffered saline. Isolated chondrocytes were counted and viability was assessed in Trypan Blue (Sigma-Aldrich, the Netherlands) using a Burker-Turk haemocytometer. A total of $1.5*10^6$ isolated chondrocytes were pelleted, by 10 minutes centrifugation (P0 pellets) at 300g, at 250000 cells per pellet. The remaining chondrocytes were expanded in culture flasks at a seeding density of 5000 chondrocytes per cm^2. After expansion, the population doublings per day were calculated based on the initially seeded P0 cells and their final number at passage 1. At passage 2 the expanded chondrocytes were pelleted (P2 pellets) by centrifugation, also at 250000 cells per pellet. All pellets were cultured for 4 weeks in DMEM (Invitrogen), 0.2 mM l-ascorbic acid-2-phosphate (AsAp, Sigma-Aldrich), 2 % Human Serum Albumin (Sanquin, the Netherlands), penicillin/streptomycin (100 U/ml / 100µg/ml, Invitrogen), 2 % ITS-X (Invitrogen) and 10 ng/ml TGF-β_2 (R&D systems, USA). Medium was changed twice a week.

Biochemical analysis

After 4 weeks of culture the pellets were digested overnight in papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 °C followed by determination of the glycosaminoglycan (GAG) content using the dimethylmethylene blue (DMMB, Sigma) assay (Farndale et al 1986). Complexation of GAGs with DMMB was monitored with a spectrophotometer. The ratio of absorption at 540 nm to 595 nm was used to calculate the GAG content, using choindroitin sulphate (shark; Sigma-Aldrich) as a standard.

The DNA content per pellet was determined from the papain digest using a picogreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Histological and immunohistochemical analysis

Histological and immunohistochemical staining was performed on non-weight-bearing, full-weight bearing , debrided grade III and grade IV cartilage tissue and on P0 en P2 pellets cultured from the cells from abovementioned locations.

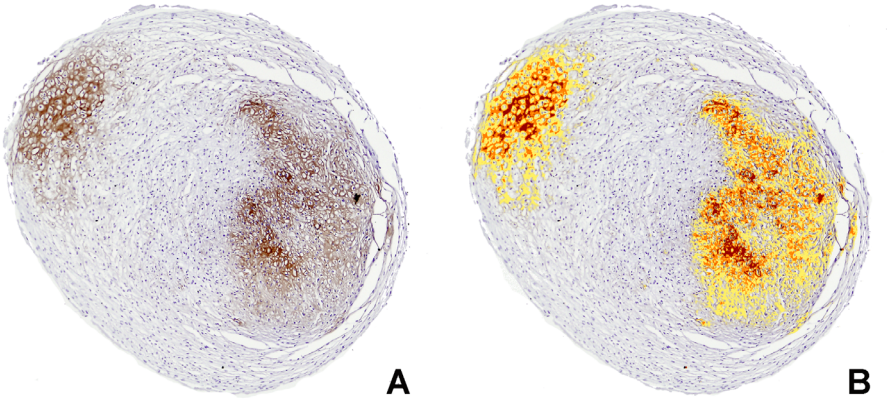
Explants and pellets were fixed in 10 % buffered formalin, dehydrated in alcohol, rinsed in xylene and infiltrated and embedded with paraffin. For histology, 5 µm sections were stained with safranin-O (Merck, Germany) for GAG and counterstained with Weigert's haematoxylin (Klinipath, the Netherlands) and 0.4 % fast green (Merck, Germany) for nuclei and cytoplasm, respectively.

Immunohistochemistry for collagen I, II and X was also performed on 5 µm sections. Antigen retrieval steps, 30 min pronase (Roche, the Netherlands, 1 mg/mL in PBS) followed by 30 min hyaluronidase (Sigma-Aldrich, 10 mg/mL in PBS at 37°C), were performed for both the collagen type I and II staining. For collagen type X staining antigen retrieval was performed by a 2 h pepsin (Sigma-Aldrich, 1 mg/mL in 0.1 N HCl) step at 37 °C followed by hyaluronidase incubation (Sigma-Aldrich, 10 mg/mL in PBS) for 30 min at 37 °C. After antigen retrieval, the sections were blocked using a PBS-BSA 5 % solution for 30 min followed by overnight incubation at 4 °C with the primary antibodies against human collagen type I (Merck, 20 µg/mL in 5% PBS/BSA), collagen type II (DSHB, USA, 1/100 in 5 % PBS/BSA) and collagen type X, clone 53 (Quartett, Germany, 1/20 in 5 % PBS/BSA). For collagen type I and collagen type X, a biotinylated secondary anti-mouse antibody was used (GE Healthcare, UK; 1/200 in 5% PBS/BSA) for 1 h at RT, followed by incubation with streptavidin/peroxidase (Beckman Coulter, USA 1/500 in 5 % PBS/BSA) 1 h at RT. The collagen type II primary antibody was followed by a secondary anti-mouse antibody conjugated with peroxidase (DAKO, the Netherlands, 10 µg/mL, 1 h at RT). Antibody binding was visualized using 3-diaminobenzidine (DAB, Sigma-Aldrich). All immunohistochemical sections were counterstained using Mayer's haematoxylin.

Immunohistochemical staining segmentation

Immunohistochemical staining for collagen type I, II and X was semiquantitatively analyzed using a pixel-intensity staining segmentation (Positive Pixel Count algorithm in ImageScope v8.0; Aperio Technologies, USA). Pixels were identified as positive or negative based on user-defined settings using reference sections. The RGB-code for positive staining was extracted from the average RGB settings (R: 0.15, G: 0.58, B: 0.78) in 15 different randomly selected positively stained region-of-interests (ROIs) from 10 different samples. The intensity of positive staining was subcategorized into weak, moderate or strong based on the positive-pixel-count algorithm settings. These settings were further optimized by testing for false-positive or false-negative pixels in a set of 30 additional ROIs at 20 sections which were stained during the same run. Following this, the optimized algorithm was applied to all sections, which resulted in a color-based staining-intensity picture (Figure 1) and a semiquantitative determination of the percentage of weak, moderate and strongly stained pixels for the different sections. Percentage of positive staining was defined as the sum of weak, moderate and strong positive stain.

Figure 1 Immunohistochemical staining segmentation.



A color-based staining intensity output with the staining intensity on the original picture (A) being categorized to weak (yellow), moderate (orange) and strong (red) in the output picture (B).

Gene expression analysis

To further characterize the isolated chondrocytes from grade III and IV debrided defect cartilage PCR was performed directly after isolation and after 4 weeks pellet culture at P0. Gene expression analysis was performed as previously described (Rapko et al., 2007). All expression levels were normalized to GAPDH expression. A set of 5 genes was selected based on their functional role in cartilage biology (Rapko et al 2007; Rapko et al 2010). Aggrecan (ACAN), cartilage link (HAPLN1) and type II collagen (COL2A1) gene expression were defined as positive markers of chondrocyte biology, whereas microfibrillar associ-

ated protein 5 (MFAP5) and collagen type I (COL1A1) were related to a more fibrocartilaginous phenotype (Rapko et al., 2010). The primer sequences used for the gene expression analysis in 5' to 3' direction were for the GAPDH (forward ATGGGGAAGGTGAAGGTCG, reverse TAAAAGCAGCCCTGGTGACC, probe 6FAM-GCCCAATACGACCAAATCCGTTGAC-MGBNFQ), the ACAN (forward TCGAGGACAGCGAGGCC, reverse TCGAGGGTGTAGCGTGTAGAGA, probe 6FAM-ATGGAACACGATGCCTTTCACCACGA-MGBNFQ), the HAPLN1 (forward TGAAGGATTAGAAGATGATACTGTTGTG, reverse GCCCCAGTCGTGGAAAGTAA, probe VIC-TACAAGGTGTGGTATTCC-MGBNFQ), the COL2A1 (forward GGCAATAGCAGGTTCACGTACA, reverse CGATAACAGTCTTGCCCCACTT, probe 6FAM-CCGGTATGTTTCGTGCAGCCATCCT-MGBNFQ) and the MFAP5 (forward CGAGGAGACGATGTGACTCAAG, reverse AGCGGGATCATTCACCAGAT, probe 6FAM-ACATTCACAGAAGATCC-MGBNFQ). For the COL1A1 a TaqMan gene expression assay (Hs00164004_m1) was used. In addition, the identity index, postulated previously to be positively correlated to the chondrogenic phenotype (Rapko et al 2010), was calculated by extracting MFAP5 expression from HAPLN1 expression.

Statistical analysis

All statistical analysis was performed using SPSS version 15.0. The statistical analysis of the GAG and GAG per DNA and the immunohistochemical staining segmentation analysis was performed using a one-way ANOVA with post-hoc Bonferroni test to test for differences in the performance of cells from different regions. The difference in gene expression between grade III and grade IV cells and pellets was analyzed by a one-way ANOVA also followed by Bonferroni test. A p-value of $p<0.05$ was considered statistically significant.

RESULTS

Cell viability and proliferation

The weight of the debrided cartilage ranged from 0.447-1.551 g and yielded 0.96-3.1*10^6 chondrocytes after overnight isolation (Table 1). The percentage of dead cells after overnight isolation was higher ($p\leq0.001$) in grade IV tissue compared to grade III, NWB and FWB locations (Table 1). Also the total number of cells per gram cartilage was statistically significant lower ($p<0.05$) for the grade IV debrided cartilage when compared to grade III, NWB and FWB cartilage samples (Table 1). The capacity of the freshly isolated P0 cells to expand in culture flasks, as expressed by the population doublings per day, did not show a statistically significant difference ($p\geq0.107$) between the grade IV, grade III, NWB and FWB cells (Table 1).

Table 1 Cell viability and proliferation characteristics.

	Grade III	Grade IV	FWB	NWB
Weight debrided cartilage (gr)	0.95±0.47	1.28±0.50	1.52±0.51	0.97±0.18
No. chondrocytes alive (*10^6)	2.10±1.33	1.79±1.20	3.77±1.40	2.88±0.94
Chondrocytes dead (%)	6.36±1.97	14.30±2.87	9.03±2.29	9.50±1.81
Chondrocytes per gram (*10^6)	2.41±1.21	1.54±0.98	2.68±0.48	3.24±0.89
Population doublings per day	0.36±0.03	0.35±0.13	0.23±0.07	0.27±0.02

DNA content

After 4 weeks of culture, the total amount of DNA did not differ for the P0 ($p \geq 0.076$) nor the P2 ($p \geq 0.217$) pellets between the grade III (P0: 1.06±0.49 µg, P2: 0.67±0.0.32 µg), grade IV (P0: 1.00±0.68 µg, P2: 0.41±0.21 µg), NWB (P0: 0.79±0.02 µg, P2: 0.84±0.36 µg) and FWB (P0: 1.12±0.64 µg, P2: 0.80±0.12 µg) samples.

GAG content

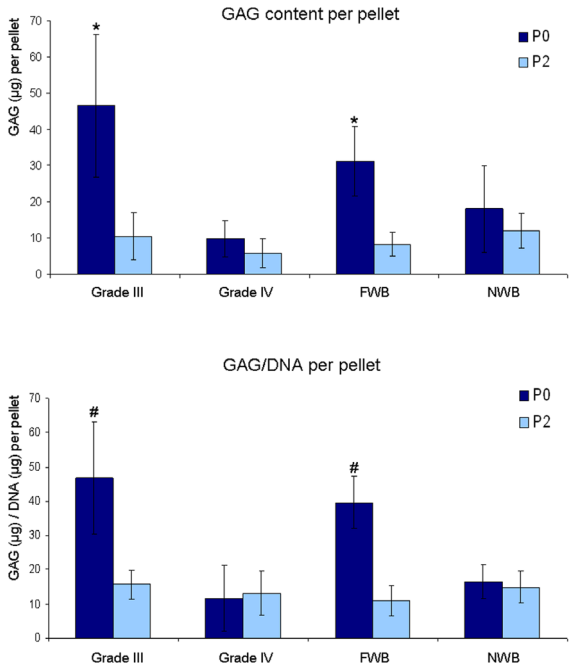
For pellets prepared from P0 chondrocytes, GAG content was statistically significant higher ($p<0.015$) in pellets from grade III (46.45±19.73 µg per pellet) and FWB (31.19±9.57 µg per pellet) chondrocytes compared to pellets from grade IV (9.89±5.04 µg per pellet) and NWB (17.99±11.90 µg per pellet) chondrocytes (Figure 2). Normalized for DNA, similar differences were found for P0 pellets from grade III (46.76±16.41 µg GAG/ µg DNA) and FWB chondrocytes (39.55±7.64 µgGAG/ µg DNA) with more efficient GAG production per cell ($p<0.001$) compared to grade IV (11.54±9.71 µg GAG/ µg DNA) and NWB pellets (16.54±4.95 µg GAG/ µg DNA) (Figure 2). After expansion to P2, GAG content decreased for pellets from all locations, and statistically significant differences between pellets from the different biopsy sites were no longer evident (10.48±6.56 µg for grade III pellets, 5.48±4.06 µg for grade IV pellets, 12.01±4.79 µg for NWB pellets and 8.30±3.34 µg for FWB pellets) ($p \geq 0.105$) (Figure 2). Also normalised to DNA content, no statistically significant differences were observed ($p \geq 0.277$) between the different biopsy sites (Figure 2).

Histological and immunohistochemical staining analysis

Light microscopy of safranin-O-stained tissue sections showed a less intense staining for GAGs for grade IV tissue sections compared to grade III, NWB and FWB tissue. In accordance with the biochemical data, the staining for GAGs in P0 pellets from grade III and FWB chondrocytes appeared more intense compared to the grade IV and NWB P0 chondrocyte pellets. No or only slightly positive safranin-O staining was observed for the regenerated cartilage of the P2 pellets.

Collagen type II staining was strongly positive in grade III, FWB and NWB tissue sections. From light microscopy and semiquantitative analysis, staining of grade IV tissue sec-

Figure 2 GAG content of P0 and P2 pellets at different biopsy sites.



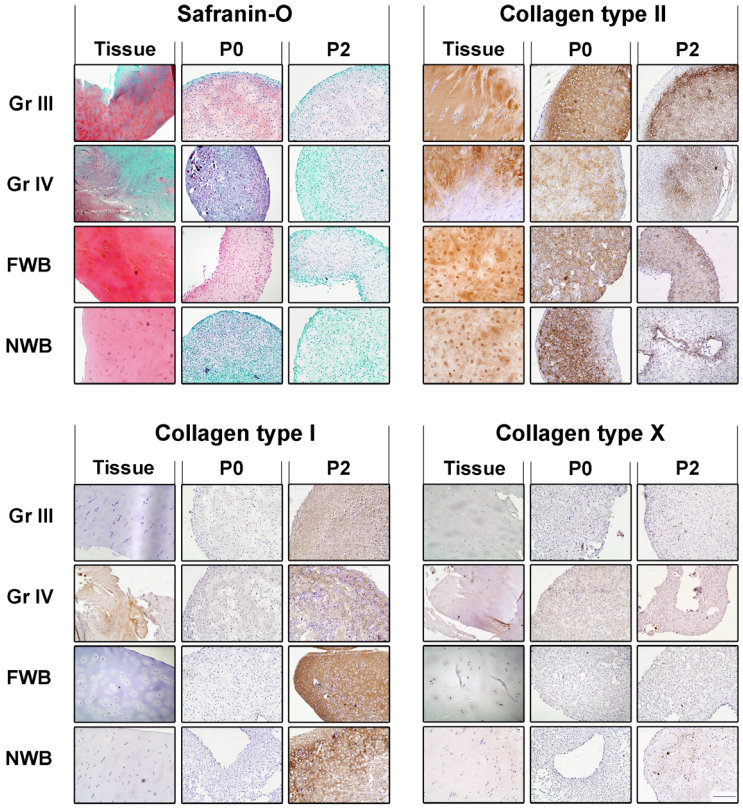
P0 pellets derived from Grade III (n=12 donors) or full-weight bearing (FWB, n=5 donors) chondrocytes produce more GAG (*p<0.001) and have a higher GAG/DNA (#p<0.002) compared to P0 pellets from Grade IV (n=8 donors) and non-weight-bearing (NWB, n=5 donors) chondrocytes.

tions for collagen type II was less positive ($p<0.05$) compared to the other tissue sections (Figure 3, 4). P0 pellets for all the cartilage samples showed a strongly positive collagen type II staining (Figure 3). Staining of the P2 grade IV and NWB pellets appeared less positive (Figure 3) compared to the grade III and FWB P2 pellets, however did not reach statistically significant differences at staining analysis (Figure 3, 4).

Collagen type I staining was strongly positive for grade IV tissue and slightly positive for the P0 pellets, whereas for all the other biopsy sites collagen type I staining was negative for tissue and P0 pellets (Figure 3). Semiquantitative analysis confirmed that the percentage of positive collagen type I staining was significantly higher for grade IV tissue compared to the other tissue samples ($p\leq0.001$) and grade IV P0 pellets versus the pellets derived from the other locations ($p\leq0.015$) respectively (Figure 4). Collagen type I staining for the P2 pellets was positive in all samples, regardless of tissue origin.

Collagen type X staining was negative for tissue and P0 and P2 pellets from grade III and FWB cartilage. Overall, tissue and P0 pellets of grade IV tissue were positive for collagen type X and the staining intensity differed from grade III and FWB tissue sections ($p\leq0.033$) and P0 pellets derived from these locations ($p\leq0.025$) (Figure 3, 4). Interestingly, also the NWB cartilage was positive, which was significantly different ($p\leq0.033$) compared to

Figure 3 Histological and immunohistochemical staining.



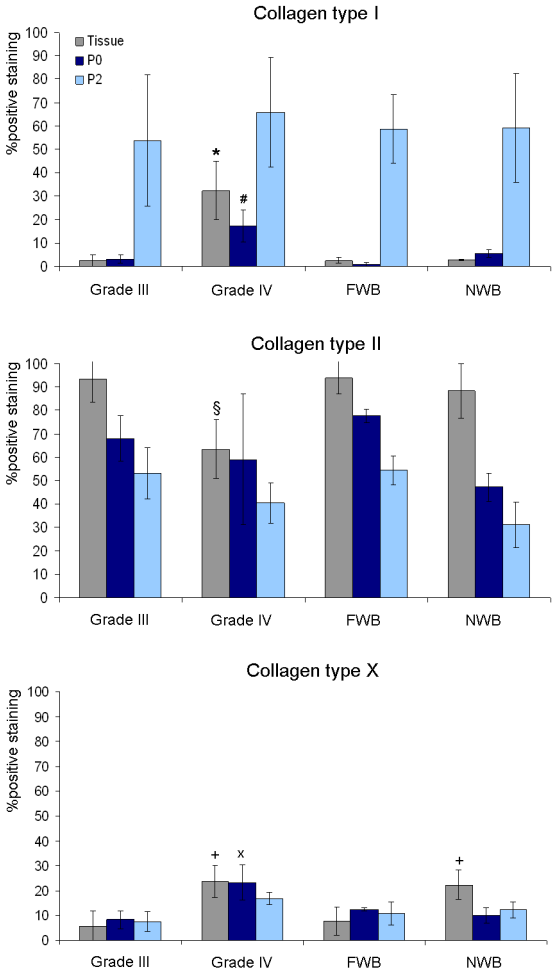
A characteristic decrease in safranin-O and increase in collagen I staining in P2 pellets compared to tissue sections is noted for all the samples from different locations. Tissue sections from Grade IV (Gr IV) defect cartilage show a less intense safranin-O and collagen type II staining compared to Grade III (Gr III) and full-weight-bearing (FWB) tissue sections. Also collagen type X staining is more pronounced in Grade IV and non-weight-bearing (NWB) cartilage when compared to Grade III and full-weight-bearing tissue sections. The scale bar represents 100 µm.

grade III and FWB tissue sections, for collagen type X staining. After culturing P0 or P2 cells in pellets, these differences were less clear (Figure 3).

Gene expression in defect cartilage chondrocytes

To further analyse the nature of the differences in matrix production between the P0 chondrocytes from the defect locations, gene expression analysis for various differentiation and dedifferentiation markers was performed. Gene expression levels of MFAB-5 and collagen type I and collagen type II were higher ($p<0.04$) for freshly isolated P0 grade IV chondrocytes compared to grade III chondrocytes, whereas aggrecan expression and the identity index were lower ($p<0.04$) in the freshly isolated cells from grade

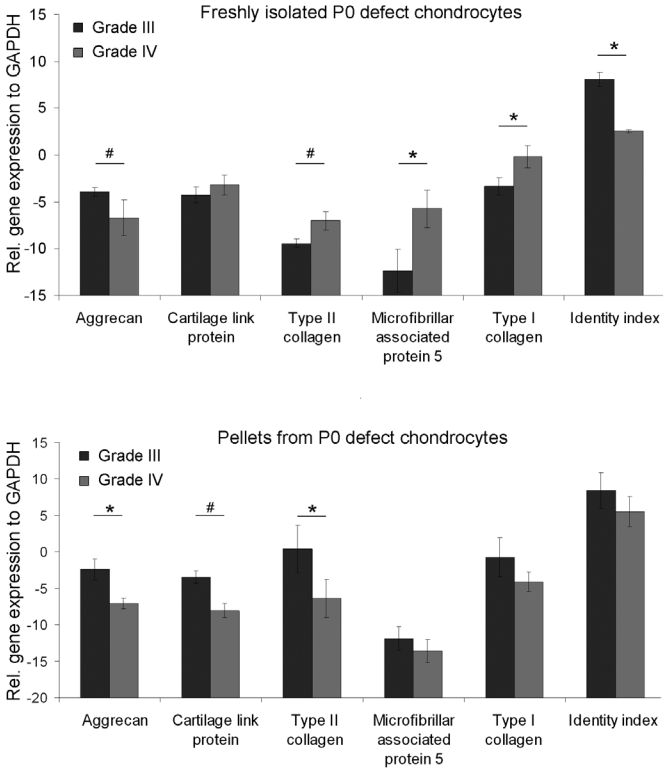
Figure 4 Immunohistochemical staining quantification.



Quantification of immunohistochemical staining (collagen I, II and X) for Grade III (n=12), Grade IV (n=8), non-weight-bearing (NWB, n=5) and full-weight-bearing (FWB, n=5) tissue sections and pellet cultures. Tissue sections (*p≤0.001) and P0 pellet cultures (# p≤0.015) from Grade IV chondrocytes show more collagen type I staining compared to tissue sections and P0 pellets from Grade III, FWB and NWB samples. Also collagen type II staining intensity is lower (§ p<0.05) in Grade IV tissue section compared to the other locations. Collagen type X staining in NWB and Grade IV tissue sections is higher (+ p≤0.033) compared to FWB and Grade III tissue sections. For Grade IV P0 pellets the collagen type X expression is also higher (x p≤0.025) in P0 pellets compared to the other locations.

IV compared to the freshly isolated cells from grade III (Figure 5). The expression pattern of the P0 chondrocytes changed after 4 weeks of pellet culture. In contrast to the freshly isolated cells, the expression of the positive markers for chondrocyte biology (aggrecan, cartilage link protein and collagen type II) were all higher (p<0.04) for the cells in the grade III P0 pellets compared to those in the grade IV P0 pellets (Figure 5).

Figure 5 Gene expression in debrided defect chondrocytes normalized to GAPDH expression.



In freshly isolated Grade IV chondrocytes (n=7) the expression of Microfibrillar associated protein 5 (MFAP5), Collagen type I (Col1A1) and Collagen type II (Col2A1) is higher whereas the Identity index (HAPL1 / MFAP5) and Aggrecan (ACAN) expression is lower compared to Grade III chondrocytes (n=10). The expression of positive markers of chondrocyte biology (ACAN, Cartilage link protein (HAPLN1), Col2A1) is higher in P0 pellets from Grade III compared to Grade IV chondrocytes. #p<0.04, *p<0.001.

DISCUSSION

This study for the first time shows that non-weight-bearing articular cartilage in the knee is a suboptimal source of chondrocytes for autologous chondrocyte transplantation, as shown by hypertrophic differentiation and decreased matrix production of unpassaged cells of this region. Moreover, unpassaged chondrocytes from debrided articular cartilage from Outerbridge grade III focal lesion were found to outperform cells from non-weight-bearing locations in terms of cartilage-specific matrix production. Articular cartilage debrided from grade IV lesions showed, both in native tissue and after pellet culture, more deviations from a hyaline phenotype as judged by higher collagen type I and X and lower GAG content at the biochemical and histological level, compared to grade III and FWB tissue and pellets cultured from cells derived from the latter two locations. Also cartilage-specific gene expression was lower in P0 pellets from grade IV

chondrocytes compared to grade III chondrocytes. Compared to non-weight-bearing cartilage, which is the currently preferred biopsy site for ACI, chondrocytes from grade III lesions produced more GAGs when cultured at P0.

Although not described before, the degenerative aspect of NWB cartilage does not come as a surprise. Articular cartilage adapts to the loading patterns exposed to in life (Lane et al 2000). Habitual disuse of cartilage will lead to hypertrophic and degenerative changes in an overall macroscopically healthy joint, as previously shown for the articular cartilage of the hip and the lateral facet of the tibia (Bullough et al 1973; Bullough and Walker 1976). In this study we showed similar findings for the NWB cartilage of the trochlea, which is the current biopsy site for regenerative cell-based cartilage surgery. Both terminal differentiation, as reflected by collagen type X production, and degeneration, shown by a decreased safranin-O staining, was observed in the NWB trochlear tissue. Moreover, this was accompanied by an actual change in cell phenotype as judged by the decreased capacity of the resident cells to generate hyaline cartilage tissue.

The use of debrided defect cartilage as a cell source for ACI has several advantages over chondrocytes derived from non-weight-bearing cartilage. Firstly, as with passaged cells no difference in redifferentiation capacity was observed between cells from debrided cartilage and non-weight-bearing cartilage, the use of chondrocytes from the debrided tissue from grade III or grade IV defects would be a logical adjustment to the ACI procedure, as no additional damage to the joint cartilage would need to be inflicted. The observed differences in chondrogenic potential between grade III and grade IV derived chondrocytes were, as expected, in line with the macroscopic appearance of the lesions *in vivo*. The fibrous-like morphology of the cartilage remnants in grade IV lesions fitted the observed fibrocartilaginous regeneration in pellets derived from these chondrocytes. Also the high chondrogenic capacity of grade III derived chondrocytes was in line with the healthy appearing remnants of cartilage in these lesions.

A second advantage of using defect chondrocytes is the relatively large number that can be isolated from the debrided material. The standard trochlear cartilage biopsy for ACI yields around $0.18\text{-}0.46*10^6$ chondrocytes (Brittberg et al 1994), requiring at least two passages, whereas the range of $0.96\text{-}3.1*10^6$ cells isolated from the debrided focal defect cartilage in the current study would only need one passage to obtain enough cells for reimplantation. This is likely to enhance tissue quality, as dedifferentiation has been shown to increase and the redifferentiation capacity to decrease with each subsequent passage (Barlic et al 2008; Marlovits et al 2004; Schnabel et al 2002).

For future applications where isolated cells are used without prior expansion, debrided defect cells would be suitable as well. The combination of freshly isolated chondrocytes with various other cell types as part of new one-stage surgical-based approaches towards ACI has recently gained attention and has been shown to improve cartilage matrix production (Biant and Bentley 2007; Bigdeli et al 2009; Hildner et al 2009;

Tsuchiya et al 2004). Partial replacement of articular chondrocytes by, for example, bone marrow or adipose-derived stem cells (Hildner et al 2009; Tsuchiya et al 2004), would directly provide sufficient cells for reimplantation, thereby enabling a one-stage cell-based cartilage therapy. In this case, P0 chondrocytes of debrided grade III cartilage will have a clear advantage over the use of NWB chondrocytes, in addition to circumventing surgery-induced damage to the non-weight-bearing areas. Also grade IV chondrocytes are suitable for such a one-stage procedure as their chondrogenic potential does not seem to be different from NWB chondrocytes. Further evaluation of the behaviour of defect chondrocytes combined with for example bone-marrow-derived stem cells may represent a logical next step towards a one-step cell-based cartilage therapy.

Finally, although this study was aimed at defining the best cell source for ACI, concerns may also be raised about other treatments for cartilage defects, such as mosaicplasty procedures. During this procedure, osteochondral plugs from the NWB region are transplanted to the focal cartilage lesion (Hangody et al 1998). Next to the frequently occurring donor site morbidity, our results suggest that actually cartilage with a hypertrophic degenerative aspect is being used to fill the defect in this procedure. However, alternative harvesting sites are not available in this particular type of treatment, unless of heterologous origin, which would entail other problems, such as risk of disease transmission and reduced functionality due to the required devitalisation procedures. Further research may be needed to indicate the necessity for such alternatives or the use of synthetic plugs (Williams and Gamradt 2008).

In conclusion, this study shows that chondrocytes derived from the common non-weight-bearing location are predegenerative and as such may not be the optimal cell source for ACI. In contrast, cells isolated from debrided defect cartilage represent a good or even better alternative, without loss of quality and quantity of cartilage-specific matrix production. In addition, chondrocytes derived from different lesion grades were showed to harbour different chondrogenic potentials. These two observations will have major implications for future cartilage transplantation procedures, in particular those based on one-stage procedures.

Chapter 5

Quality of scaffold fixation in a human cadaver knee model

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ABSTRACT

Newly developed regenerative cartilage interventions based on the application of 3D-scaffolds require a further evaluation of the surgical techniques involved. The present study compared four different scaffold fixation techniques (fibrin glue, transosseous fixation, biodegradable pin fixation and continuous cartilage sutures) to implant a custom-printed porous PEGT/PBT1000/70/30 scaffold in a human cadaver knee model.
After implantation, the knees were subjected to a vertically-oriented loaded continuous-passive-motion protocol. The fixation techniques were evaluated after 60 and a subsequent 150 motion cycles, focusing on area coverage, outline attachment and scaffold integrity. After the total of 210 cycles, also an endpoint fixation test was performed.
The fixation techniques revealed marginal differences for area coverage and outline attachment after 60 and 150 cycles. The fibrin glue scored higher on scaffold integrity compared to transosseous ($p<0.05$) and cartilage sutures ($p=0.01$). Endpoint fixation was highest for the cartilage sutures, whereas fibrin glue showed a weak final fixation strength ($p=0.01$).
This study showed that optimal fixation cannot be combined always with high scaffold integrity. Special attention devoted to scaffold properties in relation to the fixation technique may result in an improvement of scaffold fixation, and thus clinical cartilage regenerative approaches involving these scaffolds.

INTRODUCTION

Autologous Chondrocyte Implantation (ACI) has proved to be a successful treatment for focal cartilage lesions. Ongoing improvements of this regenerative cartilage therapy have led to the implementation of 3D resorbable scaffolds (Marlovits et al 2006). Several different biomaterials, including collagen type I/III bilayer matrices, Hyalograft® C and BioCartTMII (Bartlett et al 2005; Marcacci et al 2005; Nehrer et al 2008) have already been applied as scaffolds in humans with promising results.

Although good results have been published on the use of natural materials to enhance cartilage formation both *in vitro* (van Susante et al 1995) and *in vivo* (Grigolo et al 2001; Grigolo et al 2002; Park et al 2005), synthetic materials are in favor because of their limited risk on pathogen transfer, lower batch-to-batch variation and their ability to be mass produced (Temenoff and Mikos 2000; Woodfield et al 2002). The most frequently investigated synthetic biomaterials used for cartilage regenerative purposes includes polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-co-glycolic acid (PLGA) and poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEOT/PBT) (Lu et al 2001; Woodfield et al 2002). The PEOT/PBT copolymer showed and enhanced chondrocyte redifferentiation and cartilage matrix formation (Mahmood et al 2006; Miot et al 2005). Varying the amount and the length of the hydrophilic PEOT and hydrophobic PBT block, offers extensive possibilities in the design of polymer systems with tailor-made properties, such as swelling, degradability and mechanical strength (Woodfield et al 2002).

In the development of scaffold-based approaches in cartilage regeneration therapy, the clinical handling and application has been largely overlooked, despite the fact that these are also likely to influence treatment outcome. Therefore, the choice of techniques used for in situ fixation of the scaffolds merits attention. Currently, continuous cartilage sutures are being used for the fixation of collagen membranes in ACI and fibrin glue is applied as an additional sealant. In addition, some new techniques, such as biodegradable pin fixation and transosseous fixation, have recently become available for the fixation of scaffolds (Erggelet et al 2003; Petersen et al., 2008). To the best of our knowledge, there are only two reports that compare several fixation techniques for scaffold implantation in a knee in a human cadaver *ex vivo* model (Drobnic et al 2006; Knecht et al 2007).

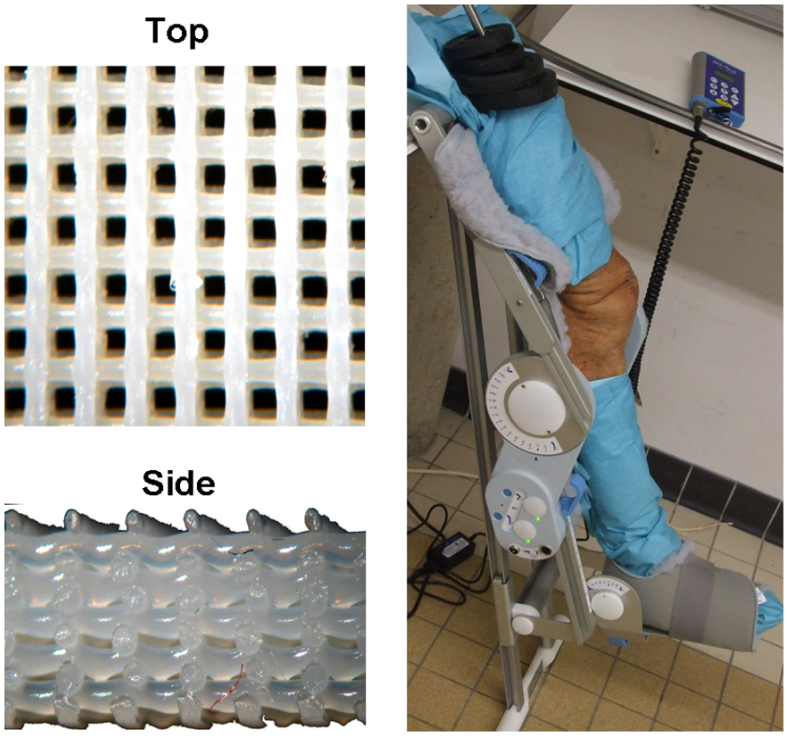
Therefore, we tested a PEOT/PBT copolymer scaffold, combined with four different scaffold fixation techniques, namely cartilage sutures, biodegradable pin fixation, transosseous sutures and fibrin glue, to implant a printed PEOT/PBT 1000/70/30 scaffold in a human cadaver knee model, exposed to a loaded vertically oriented continuous passive motion protocol and focused on scaffold attachment and fixation technique-related scaffold damage.

MATERIAL AND METHODS

Porous scaffold fabrication

PEOT/PBT copolymer granules were obtained from PoroGen B.V. (Bilthoven, The Netherlands) with a composition denoted as 1000/70/30, where 1000 represents the PEG MW (g/mol), 70 and 30 represent the wt% of the PEOT and PBT blocks, respectively. Porous scaffolds were produced using a 3D fiber deposition technique (Woodfield et al 2004) using the BioScaffolder system (SysEng, Hünxe, Germany). Copolymer granules were placed in the dispense head and allowed to melt at a temperature of 200 °C. Molten PEOT/PBT copolymer was forced through a 27 gauge needle (DL Technology, Haverhill, MA) by pressure. The printing of pre-designed scaffolds was controlled by a deposition program (PrimCam version 3.0) via the printer port. Using a printing speed of 262.5 mm/s, a spindle speed of 200 rpm and a fiber spacing of 600 µm, fibers were successively laid down on a heated deposition platform (40 °C) in a 0°–90° orientation, creating a consistent pore size and 100 % interconnecting pore volume (Figure 1). Scaffolds printed according to these settings were previously shown to typically have a porosity of approximately 60 % and a dynamic stiffness of 1 MPa (Woodfield et al 2004). Scaffolds

Figure 1 The PEOT/PBT polymer scaffold and the applied test setup.



were printed either 7 or 14 layers thick, in order to be flush with the created defect. To allow for swelling of the scaffolds, they were immersed in phosphate-buffered saline overnight. After overnight immersion the 7 and 14 layered scaffolds reached a thickness of 1 and 2 mm respectively. The prepared scaffolds were cut out to match exactly the debrided lesions' geometry, using the same template used to create the cartilage defect.

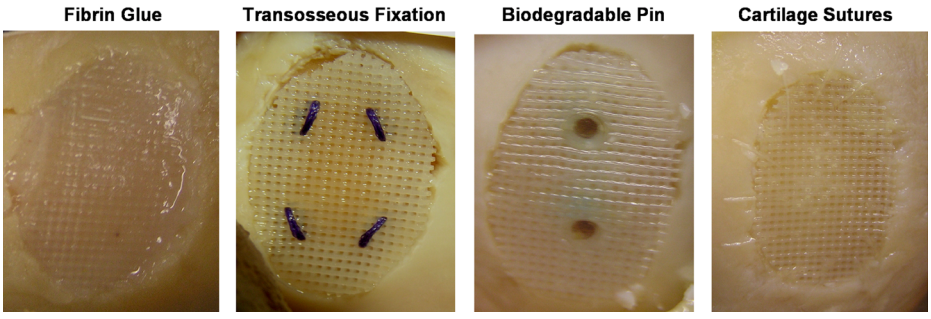
Creation of the full-thickness cartilage defect

Twenty human cadaver legs were obtained from 6 male and 6 female donors (age range: 53-91 years) in accordance with the guidelines of the local ethical committee. Prior to inclusion in the study, the extremities were tested for malalignment, knee instability and full extension and 80º flexion of the knee. The knees were opened using a medial parapatellar approach with lateralization of the patella to obtain a full view of the articular cartilage of the central regions of the femur. Both medial and lateral femur condyles were used for scaffold implantation. A custom-made template with a surface area of 2 cm^2 was used to demarcate the outline of the cartilage defect at the load-bearing portion of each medial and lateral femur condyle. Following this a sharp surgical spoon was used to debride the full-thickness articular cartilage defects. After implantation of the PEOT/PBT scaffolds, the intra-articular environment was filled with phosphate buffered saline, to allow for lubrication during the continuous passive motion (CPM) protocol, and the knees were closed in layers.

Fixation techniques

The four fixation techniques applied in this study, fibrin glue (Tissuecol®, Baxter), modified transosseous fixation (Erggelet et al 2003), biodegradable pin fixation(Petersen et al., 2008) (SmartNail®, ConMed Linvatec) and continuous cartilage sutures (Vicryl®6.0, Ethicon), were randomly assigned to either the medial or lateral femoral condyle of the 20 cadaver knees, thus 10 implantations per fixation technique (Figure 2).

Figure 2 Scaffold fixation techniques.



Fibrin glue: The fibrin glue was kept in hot water (approximately 37 °C) prior to application, following the manufacturer's instructions. The created cartilage defect was dried and the warmed fibrin glue was applied at the bottom of the defect and towards the edges of the cartilage rim. Following this, the scaffold was placed into the defect and additional fibrin glue was added at the cartilage-scaffold interface. Before the knee was closed the fibrin glue fixation was allowed to dry under a continuous hot air flow for 10 minutes with continuous rinsing of the surrounding articular cartilage surface to keep it sufficiently moist.
Cartilage sutures: The scaffold was placed into the defect and sutured to the adjacent cartilage rim by continuous Vicryl® 6.0 bioresorbable sutures comparable to the, previously described, fixation procedure of the ACI technique (Brittberg et al 1994).
Biodegradable pin (SmartNail ®): For this technique a 1.5 mm K-wire was used to drill two 14 mm deep holes into the subchondral bone at 1/3rd and 2/3rd height of the defect. Following this the scaffold was placed into the defect and the SmartNails® were tapped, through the scaffold, into the drilled holes to provide a firm fixation of the scaffold into the created cartilage defect.
Transosseous sutures: A guide wire (1 mm diameter) was used to drill four holes at the 2, 4, 8 and 10 o'clock locations of our defect. At the same locations the scaffold was armed with a resorbable 2.0 thread. At respectively 1 cm and 2 cm from the scaffold, 3 and 2-fold knots were created. The threads were pulled into the defect and through the femoral bone by the guide wires. At the end a short, firm action was necessary to pull the knots into the drilled holes to anchor the scaffold into the cartilage defect.

Continuous passive motion protocol

After implantation of the scaffolds, the legs were strapped onto the CPM devices (Össur, Son en Breugel, the Netherlands and Firma Medical SOT B.V., Losser, the Netherlands) oriented in a vertical position (Figure 1). A total load of 35N was attached on top of each leg to allow for a continuous axial force during motion. One motion cycle was defined as full extension (0°) to 80° flexion to again full extension (0°) and lasted for approximately 60 seconds. After 60 loaded continuous cycles, the knees were reopened and the implantation sites photographed for later macroscopic analysis. Knees were then closed again and exposed to an additional consecutive 150 loaded continuous motion cycles. Subsequently, the knees were opened again and photographs were taken.

Analysis of fixation

Scoring system: After 60 and after a consecutive 150 cycles, the obtained pictures from the implantation sites were evaluated by two independent observers using a modified scoring system (Drobnic et al 2006) focusing on area coverage, outline attachment and scaffold integrity (Table 1). Area coverage and outline attachment were calculated using

Table 1 Modified scoring system.

Outline attachment[1]	Area coverage[2]	Scaffold integrity	Endpoint fixation
Unchanged (5)	Unchanged (100%) (5)	Unchanged (5)	Cannot be detached/suture is pulled through the scaffold. (5)
<25% (4)	75- <100% (4)	Shape deformities or minor fissures that are unrelated to fixation (4)	Detached with 2.5-3 N (4)
25-50% (3)	50-75% (3)	Minor fissures or cracks close to the fixationsite (3)	Detached with 1.5-2N (3)
50-75% (2)	25-50% (2)	Fissures or cracks that jeopardize the fixation of the scaffold (2)	Detached with 0.5 -1 N (2)
75- <100% (1)	<25% (1)	Fissures or cracks jeopardizing the fixation with surrounding scaffold disorganization (1)	Detached with ≤ 0.5 N (1)
100% (0)	0% (0)	Fissures or cracks jeopardizing the fixation with generalized scaffold disorganization (0)	Total self detachment (0)

[1]: % of full circumference that has lost contact with the surrounding cartilage rim.
[2]: % of total cartilage defect that is covered by scaffold.

the AnalySIS 3.0 software. The length of the outer cartilage defect rim was marked to calculate the defect surface area and outline, which were correlated to the scaffold surface area and outline, presented as percentages. Scaffold integrity was determined, on a 5-point scale, by evaluating the severity and location of scaffold fissures and cracks with concomitant focal or generalized scaffold disorganization (Table 1). After 150, cycles, an additional end-point fixation test was performed. A suture was pulled through the center of the scaffold and connected to a pulley-block system to quantitatively measure the pull-off force necessary to dislocate the scaffold.

Scaffold damage

After the loaded CPM test, scaffolds were evaluated macroscopically using a stereomicroscope (Zeiss, Stemi-2000C, Germany) with a 10x magnification. Detailed longitudinal and transverse images of the scaffolds were obtained to show the fixation technique-related scaffold damage.

Statistical analysis

For each individual fixation technique, the average scores and standard deviations per scoring item were calculated after both 60 and 150 motion cycles. Differences in quality of the scaffold fixation techniques, per scoring item, were analyzed for statistical significance by a non-parametric Kruskal-Wallis test followed by a multiple comparison test and Bonferroni correction.

RESULTS

Procedures

Chondral defects were only present in four legs and these defects could easily be debrided during the creation of the full-thickness cartilage defect. All fixation techniques were straightforward, although fixing the scaffolds using transosseous or continuous cartilage sutures was more time consuming compared to the fixation using nails or fibrin glue. Evaluation after 60 and the additional 150 cycles of loaded continuous passive motion (CPM) revealed no significant difference between medially and laterally implanted scaffolds and between 7 and 14 layered scaffolds for any of the scoring items (data not shown), therefore subsequent analyses of the scaffold fixation techniques was done regardless of scaffold thickness or implantation site. None of the specimens showed macroscopic damage at the opposing articular cartilage surface (data not shown).

Area coverage

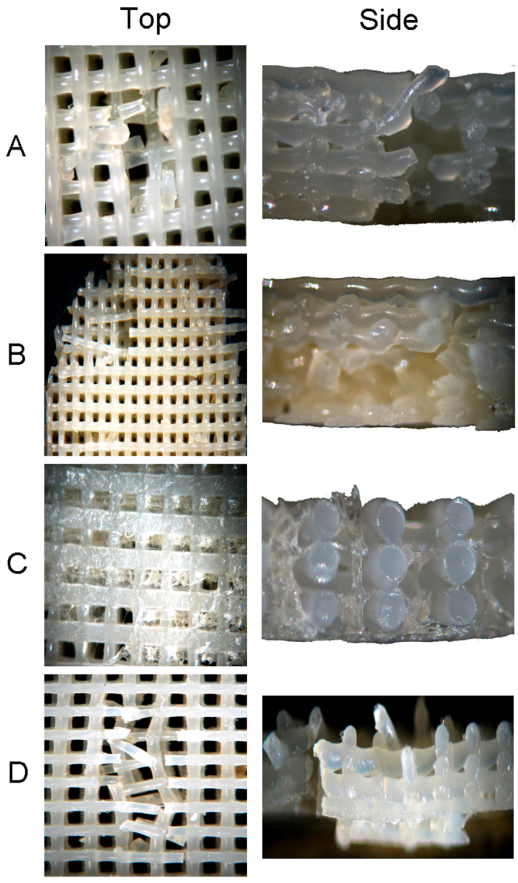
With regard to area coverage, marginal differences were observed between the four different fixation techniques after both 60 and subsequent 150 cycles of loaded CPM (Table 2). After 60 cycles, 3 out of 10 scaffolds fixed with fibrin glue, 2 out of 10 scaffolds fixed with biodegradable pins and 1 out of 10 scaffolds fixed with transosseous sutures, were completely detached. The remaining scaffolds did not detach after the additional 150 cycles. Closer examination of the implantation sites revealed either nearly complete attachment or total detachment of the scaffold, accounting for a large variation in obtained area coverage score for these three techniques (Table 2). If total detachment occurred during the motion cycles, full thickness scaffold fissures were observed at the fixation sites for the biodegradable pin and transosseous fixation techniques (Figure 3),

Table 2 Results after 60 and 150 cycles.

60 cycles average score (± standard deviation)				
	FG	TS	BP	CS
Outline attachment	2.7 (± 1.89)	1.9 (± 1.10)	1.9 (± 1.10)	3.2 (± 0.92)
Area coverage	2.8 (± 1.93)	3.9 (± 1.26)	3.2 (± 1.69)	4.1 (± 0.32)
Integrity	4.3 (± 0.48)*	1.9 (± 1.04)*	2.3 (± 1.34)	2.3 (± 1.51)
150 cycles average score (± standard deviation)				
	FG	TS	BP	CS
Outline attachment	2.4 (± 1.78)	1.8 (± 1.03)	1.6 (± 0.97)	3.0 (± 0.82)
Area coverage	2.8 (± 1.93)#	3.6 (± 1.26)	3.2 (± 1.69)	4.0 (± 0.00)#
Integrity	4.1 (± 0.63)+	1.7 (± 0.95)	2.3 (± 1.34)	1.2 (± 1.13)+

Average per scoring item for the scaffold fixation techniques after 60 and 150 cycles. (*p<0.05, #p=0.01, +p=0.01, FG: Fibrin Glue, TS: Transosseous Fixation, BP: Biodegradable Pin, CS: Cartilage Sutures), (n=10).

Figure 3 Confocal stereomicroscopic pictures of the scaffolds after fixation.



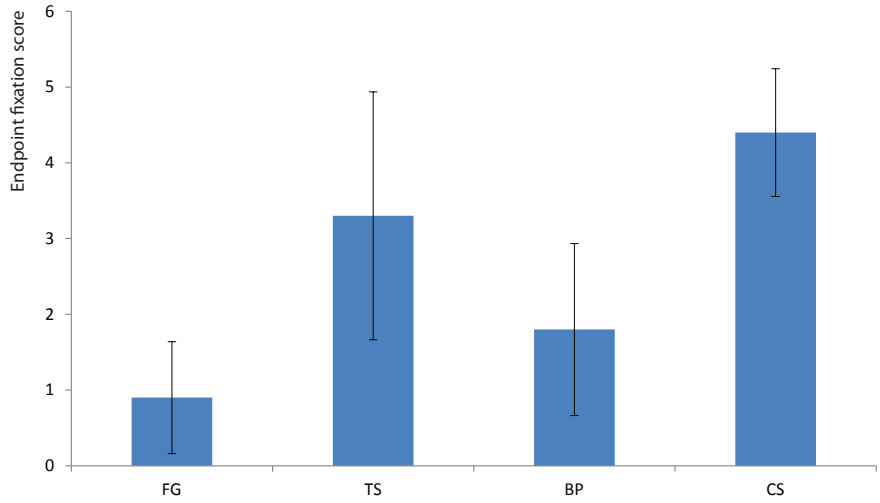
Representative stereomicroscopic pictures of scaffolds after 150 CPM cycles for the applied fixation techniques (biodegradable pin (A), cartilage sutures (B), fibrin glue (C) and transosseous sutures (D)) showing a disorganized collapsed scaffold composition with full thickness fissures or partial scaffold loss for techniques A, B and D.

whereas scaffolds fixed by continuous cartilage sutures remained stable after 60 and subsequent 150 cycles. However, an occasional rupture of a scaffold fibre, caused by the articular cartilage sutures, was observed resulting in loss of scaffold material (Figure 3b) and thus lower area coverage.

Outline attachment

For the biodegradable pin, and to a lesser extent the transosseous suture fixation, folding at the edges was regularly noticed. Small differences were present between the fixation techniques in outline attachment (Table 2). Lower scores, however not statistically significant, were observed for the biodegradable pin and transosseous sutures due to folding of the scaffold and suture rupture respectively. In addition, for the transosseous

Figure 4 Endpoint fixation



Endpoint fixation score per fixation technique (n=10) showing best endpoint fixation when cartilage sutures were used (+p=0.01, FG: Fibrin Glue, TS: Transosseous Fixation, BP: Biodegradable Pin, CS: Cartilage Sutures).

suture technique, a minor discrepancy was noticed between the drilled holes and the site of suture fixation in the scaffold, allowing for dislocation of the scaffold and thus reducing outline attachment. The outline attachment score for articular cartilage sutures was higher compared to the other three techniques, however not significant (Table 2).

Scaffold integrity

The biodegradable pin, transosseous suture and continuous cartilage suture fixation had slightly damaged the scaffold during the fixation procedure. This technique-associated damage increased during loaded CPM and led to subsequent lower scaffold integrity scores (Figure 3). For cartilage sutures, as well as for the biodegradable pin and transosseous sutures, full-thickness scaffold fissures and cracks were noticed after 60 cycles. Moreover, after 150 cycles, generalized scaffold disorganization was regularly noticed when articular cartilage sutures were applied (Figure 3). The fibrin glue fixation technique provided the best scaffold integrity as compared to transosseous sutures after 60 (p=0.04) and 150 (p=0.07) cycles and cartilage sutures (p=0.01) after 150 cycles.

Endpoint fixation

The endpoint fixation test showed a statistically significant better final scaffold attachment (p=0.01) for the continuous cartilage suture compared to the fibrin glue fixation techniques (Figure 4).

DISCUSSION

Secure delivery and retention of a cartilage tissue engineered construct is important for a successful outcome after regenerative cartilage therapy. However, the clinical handling and application of these constructs has been largely overlooked. In the present study, four different scaffold fixation techniques for the implantation of a printed PEOT/PBT scaffold in human cadaver knee joints were compared. We focused on the effect of loaded motion on attachment of the scaffold into the debrided defect and on scaffold damage.

Fibrin glue provided an excellent protection of the scaffold integrity during loaded motion and, if not detached, a good area coverage. The biodegradable pin, transosseous and cartilage suture fixation techniques caused minor damage to the scaffolds during application, leading to further deterioration of the scaffold composition during loaded motion. Elastic properties of the implanted scaffold will determine folding and or dislocation at the outer margins of the defect when a two or four-point fixation technique is applied, since these techniques do not provide any fixation at the outer margins (Drobnic et al 2006). Techniques where fixation occurs at the outer margins of the defect, like cartilage sutures and fibrin glue, do provide good outline attachment. In our study, however, fibrin glue provided a weak attachment when compared to the suturing techniques. This is both remarkable and worrisome, especially when considering its broad clinical application. One explanation for this finding might be that fibrin glue is a biological sealant and therefore the ex vivo performance could be inferior, e.g. lacking the additional fixation by the blood normally present during knee surgery or the lubricating properties of the synovial fluid. In addition, the scaffold used in the current study, which is printed and rather stiff, could be less suitable to be combined with fibrin glue compared to the ones currently being used in clinical practice.

During the endpoint fixation test, the pulley block system applies a force perpendicular to the scaffold surface, which is different from the intra-articular shear stresses created during walking. Although the effect of the cartilage defect rim on scaffold stability is unclear we believe that the pulley block system is a good measure of the actual attachment of the scaffold into the created cartilage defect.

Our findings are in line with previously reported work on scaffold fixation techniques by Knecht et al. Different fixation techniques and scaffold types were compared by uniaxial tensile tests showing that, regardless of the type of scaffold material used, suturing techniques provide better attachment of the scaffold to the cartilage defect when compared to fibrin glue (Knecht et al 2007). This actually would suggest that the scaffold material does not influence fixation quality. However, their study-design, applying non-weight-bearing, static uniaxial tensile tests on in situ cadaver legs, makes the translation of the results to a clinical setting difficult. Therefore, the real behavior of a scaffold fixed by

a certain technique can better be analyzed in an intra-articular environment. Drobnič and colleagues tested the fixation of a collagen fleece with four different techniques in an intra-articular environment by the use of a CPM protocol in a horizontal orientation (Drobnic et al 2006). Similar to our observations on the biodegradable pin and transosseous fixation, they also noticed deformation of the scaffold when applying their 2-point fixation technique. In addition, fibrin glue provided high integrity scores compared to the suturing techniques while the opposite held true for the endpoint fixation. However, the loaded vertically oriented CPM test model of the current study is likely to be a better *ex vivo* approximation of the intra-articular influences on the implanted construct during the postoperative rehabilitation program after cell-based cartilage therapy. Although we did not perform intra-articular pressure measurements, it is likely that the vertical orientation of the cadaver legs with additional loading during motion generated a shear force on the implanted construct, analogous to the *in vivo* situation. This shear force will partially be mediated directly by friction with the opposing cartilage and partially by intra-articular fluid flow. Further research is needed to demonstrate whether perhaps a slightly countersunk scaffold could protect it from the shear forces in vivo, although in one rabbit study it was shown that countersinking metal implants in cartilage defects negatively affected the integrity of the articulating cartilage surfaces (Custers et al 2007). Although the in vitro optimization of conditions for cartilage regeneration is important, the eventual in vivo circumstances might even be more important. For example, several components of the synovial fluid are believed to inhibit the integration of a cartilage construct into the adjacent tissue (Englert et al 2005; Janssen et al 2006; Schaefer et al 2004). In addition, a suboptimal contact between the engineered constructs and the native tissue, e.g. due to folding of the construct at the outline, will negatively influence the integration of the construct with the surrounding tissue. Moreover, failing scaffold fixation can result in a loose intra-articular body, damage to the articulating cartilage surface and loss of reparative cells at the site of the defect. Therefore, stable and lasting in vivo fixation with preservation of the 3D-construct geometry is mandatory to profit from the added value of this scaffold based tissue engineered approach in regenerative cartilage therapy.

The discrepancy between scaffold integrity and endpoint fixation tests in the current study, suggests that good fixation of a scaffold can only be achieved by using techniques that will compromise the scaffold integrity. This raises the question whether the focus for in vivo application of 3D-scaffolds should be optimal fixation or scaffold preservation. Adaptation of scaffold architecture, e.g. preprinted holes when applying the biodegradable pin fixation technique, will most likely limit the scaffold damage as initiated during fixation technique application. This opens the possibility to apply fixation techniques providing a secure fixation while maintaining integrity. Another consideration in the choice of a certain fixation technique may be a reduction of patient morbidity by using

a mini-open arthrotomy or an arthroscopic approach. In contrast to the transosseous sutures, fibrin glue and biodegradable pin fixation, the cartilage suturing technique cannot be applied by either of these approaches and requires an arthrotomy.

The limited number of publications on clinical handling of 3D-scaffolds for *in vivo* application in knee surgery is an indication of the lack of attention in this area. Improvement of the interdisciplinary communication between materials research and surgical technique development has previously been described as a challenge (Malda et al 2008). and is mandatory to improve the translation of basic material science towards clinical concepts. The combination of knowledge on scaffolds for cartilage repair and surgical skills and techniques will lead to optimal scaffold fixation with limited damage to the scaffolds.

In conclusion, this chapter showed that optimal fixation cannot always be combined with high scaffold integrity. Special attention devoted to the effect of the applied fixation technique on the scaffold integrity will result in an excellent scaffold fixation and integrity preservation for future clinical application.

Chapter 6

One-stage focal cartilage defect treatment with mononuclear fraction cells and chondrocytes leads to better cartilage regeneration compared to microfracture in goats

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ABSTRACT

The combination of chondrocytes and mononuclear fraction cells (MNF) might solve the expansion induced dedifferentiation problem of reimplanted cells in autologous chondrocytes implantation as sufficient cells would be available for direct, one-stage, implantation. Earlier *in vitro* work already showed a positive stimulation of cartilage-specific matrix production when chondrocytes and MNF cells were combined (Chapter 2 and 3). Therefore, this chapter aimed to evaluate cartilage regeneration using a one-stage procedure of combining MNF cells and primary chondrocytes for the treatment of focal cartilage lesions in goats compared to microfracture treatment.

Freshly created focal cartilage defect were treated with either a combination of chondrocytes and MNF cells embedded in Beriplast® or microfracture treatment. After 6 months follow-up local regeneration as well as distant joint degeneration were evaluated using validated scores and biochemical assays.

Macroscopic and microscopic scores for the cartilage surface at the treated defect were, after 6 months, higher for the chondrocyteMNF treatment compared to microfracture treated defects. Biochemical GAG evaluation did not reveal differences between the both treatments. After 6 months follow-up both treatments introduced slight to moderate cartilage degeneration at distant locations in the joint.

In conclusion, the treatment of focal articular cartilage lesions using a combination of MNF cells from bone marrow and unexpanded chondrocytes is better compared to microfracture, however needs further fine-tuning to improve defect fill and decrease the negative influence on other joint compartments.

INTRODUCTION

The combination of stem cells and differentiated target cells has recently gained attention to address currently defined challenges in regenerative medicine. For example, vascular endothelial cells and urothelial cells were both able to differentiate adipose derived stem cells into respectively the osteogenic and urothelial lineage in order to solve problems in bone and urothelial tissue engineering (Shi et al 2012; Zhao et al 2012). Also ADAM-9 negative fibroblasts in coculture with melanoma cells revealed important pathofysiological proliferation and apoptosis mechanisms (Abety et al 2012). In cartilage repair the combination of cells has also been introduced as a potential method for improving the quality of tissue regeneration. Articular cartilage matrix production of expanded articular chondrocytes has been shown to be positively influenced by primary chondrocytes and human adipose derived and embryonic stem cells *in vitro* (Bigdeli et al 2009; Gan and Kandel 2007; Hildner et al 2009). In addition, the chondrogenic phenotype of dedifferentiated chondrocytes was improved when cocultured with MSCs (Chen et al 2009; Mo et al 2009; Tsuchiya et al 2004). As these effects were also achieved when conditioned medium and non-contact culture systems, such as transwell culture were used, the underlying mechanism could, in part, be explained by the communication between the cells through trophic factors (Chen et al 2009; Gan and Kandel 2007; Mo et al 2009; Wu et al 2011).

Autologous chondrocyte implantation (ACI) is frequently used to treat focal cartilage lesions in the knee. Due to the limited number of cells that can be harvested from a biopsy, in vitro cell expansion followed by reimplantation of this precultured population is inevitable and considered as the main drawback of this relatively successful procedure. A combination of cells could be an alternative for the expansion of harvested chondrocytes in ACI. When isolated chondrocytes are directly combined with another cell type, that also improves the chondrogenic potential of reimplanted cells, the whole procedure could be performed within one surgery and the quality of regenerated cartilage improved.

The mononuclear fraction (MNF) in bone marrow is a major source of trophic factors and can easily be obtained in the timeframe of one surgery (Balakumaran et al 2010). For this reason, combination of primary isolated chondrocytes and MNF cells could be the basis for a one-stage cell-based regenerative treatment for focal articular cartilage defects. Recent results (Chapter 2) showed that a combination of MNF cells and primary unexpanded chondrocytes improves cartilage-specific matrix production in a pellet culture. These results were also reproducible *in vivo* after subcutaneous implantation of a combination of MNF cells and primary chondrocytes in a Beriplast® carrier in mice (Chapter 3). The next step would be to test whether this cell combination also leads to stable and good cartilage regeneration when applied in a relevant pre-clinical model for cartilage

tissue engineering. Therefore, this chapter aimed to evaluate cartilage regeneration using a one-stage procedure of combining MNF cells and primary chondrocytes for the treatment of focal cartilage lesions in goats compared to microfracture treatment.

MATERIALS AND METHODS

Experimental design

This experiment was approved by and conducted following the guidelines of the animal care committee of the University of Utrecht under number DEC 2011.III.03.026. A fresh standardized 5mm diameter chondral lesion was created in the medial condyle of both knees in female Dutch milk goats. Per goat one defect was treated with a combination of chondrocytes and mononuclear fraction cells in Beriplast® (Nycomed, CSL Behring, the Netherlands) and the other by microfracture. The animals were euthanized at 6 months after surgery. Defect healing and the quality of articular cartilage in all other compartments of the joint were determined using macroscopic, microscopic and biochemical analysis.

Animals

A total of 8 Dutch milk goats (age 3-5 years old, weight 75 ± 10 kg) were used in this study. The necessary number of animal was determined by a power analysis based on the estimated amount of GAG (32.2 ± 2.3 mg / mg tissue) at 6 months after cartilage surgery which was extracted from a previous publication (Saris et al 2003). The relevant mean difference between the two treatments was set at 10%. With a power of 0.90 and a p=0.05 a total of 8 goats was calculated. Once arrived at the animal facility the goats were allowed to acclimatize for at least one week before surgery. During follow-up food was provided ad libitum and general health was assessed by the veterinarian of the animal care facility of the University of Utrecht. Up to two weeks after surgery an additional health scoring system, focusing on wound infection, limping and general activity, was scored by JEJB.

Surgery

One day prior to surgery the goats were weighed and prophylactic pain medication was provided by a fentanyl skin patch. Intravenous premedication (0.4 mg.kg detomidine hydrochloride (Pfizer, the Netherlands) and a single dose of Augmentin was followed by induction anaesthesia using thiopental (6 mg/kg, Rhône Mérieux, France). During surgery, anaesthesia was maintained by a combination of midazolam 0.8 mg/kg (Abbott Laboratories, the Netherlands) and sufenta forte 0.007 mg/kg (ASTPharma, the Netherlands) and, if necessary, isoflurane or propofol (Abbott Laboratories).

Bone marrow was obtained by needle aspiration from the iliac crest. For this a Jamshidi® neelde was tapped into the iliac crest. Bone marrow was aspirated using 20 ml sterile syringes and stored in heparin coated tubes. After this the whole bone marrow was spun down (300g, 10 min) and the cell pellet diluted 50x in red blood cell lysis buffer (Sigma, the Netherlands) during 45 minutes. Following this the remaining cells were spun down by centrifugation and washed twice in PBS, thus producing the MNF fraction.

Surgery was performed under aseptic conditions. A medial parapatellar approach was performed to expose the medial femur condyle. After macroscopic scoring of the medial cartilage surface, using the Mastbergen score (Mastbergen et al 2006), a 5 mm cylindrical chondral lesion was created in the central weight bearing region using a hand-operated drill. A surgical spoon was used to debride the remnants of articular cartilage and to create a stable defect rim. The debrided defect cartilage was digested using a previously validated protocol (Chapter 7). For this, cartilage was cut into small pieces and digested during 45 minutes in 2 % collagenase type II solution (Worthington, Lakewood, USA) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) with penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen, the Netherlands) under continuous shaking at 37 ^{o}C. This digestion protocol resulted in a digestion efficiency of 1.37 ± 0.50*10^{6} viable chondrocytes per gram debrided tissue. Meanwhile, the debrided defect was treated using the microfracture technique by creating 4 holes through the subchondral bone using a 1.5 mm k-wire. Microfracture treatment was confirmed by the presence of blood entering the defect from the drilled holes. Following this the knee was closed in layers.

The other knee was opened, the medial cartilage surface scored and chondral defect created using similar procedures as described above. A mixture of 10% rapidly isolated chondrocytes and 90 % MNF cells were, suspended in the fibrinogen component of Beriplast® (Nycomed) at a final concentration of 1*10^{6} cells per milliliter. The fibrinogen component and thrombin component of the Beriplast® were prepared for application following the manufacturers' protocol and injected into the cartilage defect. After five minutes of gelation time the knee was flexed 10 times to check the stability of the graft into the defect and the knee was closed in layers.

Direct full-weight bearing was allowed following surgery. The animals were housed individually for a period of 24 hours. Additional pain medication and antibiotics were provided, based on the judgment of the veterinarian, with approval of JEJB. The animals were euthanized after 6 months using an overdose of pentobarbital (Euthesate®) and both hind legs explanted for further analysis.

Macroscopic cartilage evaluation

Soft tissues were removed from the explanted hind legs and high resolution pictures obtained from the medial (MFC) and lateral femur (LFC) and tibia cartilage surfaces (medial MTP, lateral LTP) and from the cartilage defect. Pictures were coded for blinded scoring

by two observers. The articular cartilage of the medial and lateral tibia and femur as well as the medial tibia cartilage that directly articulated with the treated defect were scored using the macroscopic Mastbergen score (Mastbergen et al 2006). The Mastbergen score is a 4-point scale ranging from a macroscopically healthy and smooth cartilage surface (0 points) to cartilage degeneration characterized by deep grooves and surrounding cartilage damage (4 points). The close-up pictures from the cartilage defect were scored using the ICRS macroscopic evaluation of cartilage repair score (van den Borne et al 2007). This score (0-12 points scale) evaluates the macroscopic cartilage repair on degree of defect repair and fill, integration into border zone and macroscopic appearance. The higher the score the better the macroscopic cartilage repair. For both macroscopic scoring systems the scores of the two observers were averaged. Where individual items differed more than 2 points between observers consensus was reached.

Microscopic cartilage evaluation

Microscopic evaluation was performed on osteochondral samples derived from the central weight-bearing lateral femur and tibia, the medial tibia cartilage that articulated with the defect and from the posterior half of the defect. Samples were fixed in 10% buffered formalin for 48 h followed by a decalcification process using Luthra's solution (3.2% 11 M HCl, 10.0% formic acid in distilled water). Following this samples were dehydrated by graded alcohol series, immersed in xylene and embedded in paraffin. Sections (5 µm) were stained with safranin-O (Merck, Germany) for GAG. Counterstaining for nuclei and cytoplasm were performed with respectively Weigert's haematoxylin (Klinipath, the Netherlands) and 0.4% fast green (Merck, Germany). The microscopic quality of cartilage regeneration was assessed using the O'Driscoll score (O'Driscoll et al 1986; O'Driscoll et al 1988). This score (range 0-24 points) evaluates the regenerated cartilage on the amount of safranin-O staining in the matrix, cellular morphology and clustering, the structural characteristics of the tissue and degenerative changes in adjacent tissue. The higher the score the better the microscopic cartilage regeneration. All other osteochondral cartilage samples were evaluated using the Mankin score which ranges from normal appearing articular cartilage (0 points) to tissue with complete disorganization, no matrix staining and hypocellularity or cloning (14 points). Similar score processing from the two observers was performed as with the macroscopic scoring.

Biochemical cartilage evaluation

Full-thickness articular cartilage was, if present, obtained from the anterior part of the treated defect, the cartilage that articulated with the defect and from the lateral weight-bearing femur and tibia cartilage. Samples were weighed and digested overnight in papain (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 ºC.GAG content was determined using a dimethylmethylene blue (DMMB) assay

(Farndale et al 1986) where the complexation of GAGs with DMMB was measured spectophotometrically at 540 nm, using 595 nm as a reference. Chondroitin sulphate (shark; Sigma-Aldrich) was used as a standard. Per sample, the DNA content was also determined from the papain digest using a picogreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Statistical analysis

All analysis were performed using SPSS version 15.0 (Chicago, USA). Differences between the two treatments in macroscopic score, microscopic score, GAG / gram tissue and GAG / DNA were tested using a paired samples t-test. A $p<0.05$ was considered statistically significant.

RESULTS

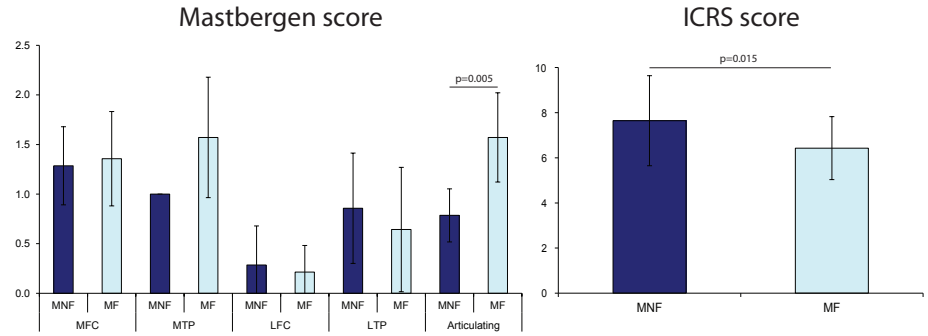
Animal health and follow-up

No signs of surgery related complications, like wound or joint infection, were observed within 2 weeks after surgery and all animals were able to move their joints and load their limbs without limitations. After two weeks no additional pain medication was provided. In total 2 animals died. One animal died directly after surgery due to an already existing, unrecognized, pulmonary infection. This animal was replaced. The other goat was euthanized at 2 months follow-up due to progressive weight loss and increased infection parameters which were, after obduction, related to a large intra-abdominal cyst. After consultation of our statistician we concluded that replacement of this goat was not necessary and would not put the power of the study at risk. The maximal weight loss of the goats that reached 6 months follow-up was 4.5 ± 3.0% compared to their preoperative weight.

Macroscopic evaluation

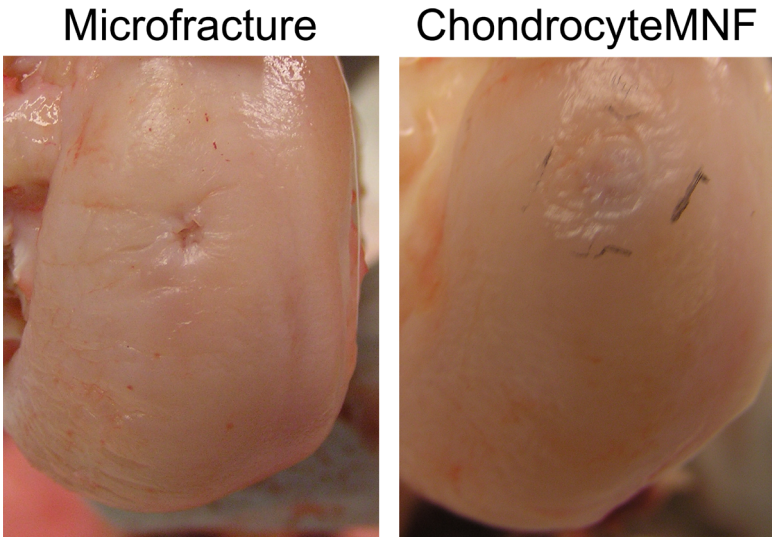
Peroperative macroscopic evaluation of the articular surfaces showed superficial fibrillations that were related to the central weight-bearing cartilage of the medial femoral condyle in 3 cases. Peroperative macroscopic scores of cartilage surfaces did not differ between the two applied treatments ($p=0.62$). Post-treatment macroscopic scores of the articulating tibia cartilage were statistically significant ($p=0.005$) higher in the microfracture treated defects compared to the chondrocyteMNF treated defects. This indicates increased cartilage degeneration of the articulating tibia cartilage following microfracture treatment of a defect in the femur compared to chondrocyteMNF treatment. Other joint locations (lateral tibia and femur cartilage and medial femoral condyle) did not show statistically significant differences, however, the medial compartment showed relatively

Figure 1 Macroscopic scores.



Macroscopic scores show no differences between the treatments at 6 months follow-up for the medial femur (MFC) and tibia (MTP) surfaces, lateral femur (LFC) and tibia (LTP) surfaces. However, the articulating surface showed less degeneration (P=0.005) when in the chondrocyteMNF (MNF) compared to the microfracture treated knees. Also at the treated defect site the chondrocyteMNF treatment (MNF) showed a statistically significant (p=0.015) higher macroscopic ICRS score compared to the microfracture (MF) treated defects (n=7).

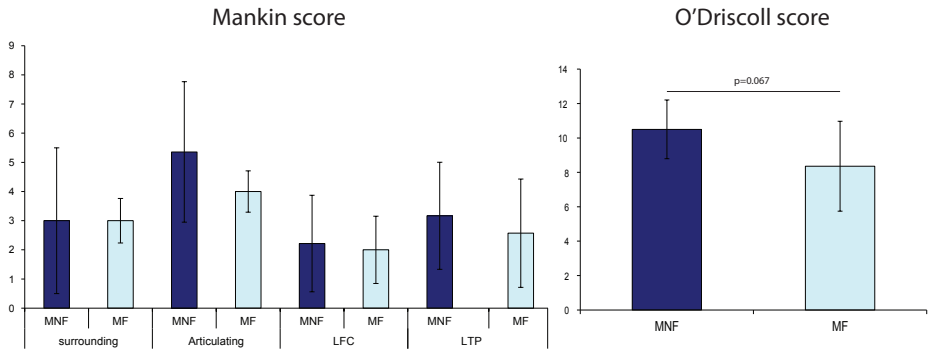
Figure 2 Representative examples of macroscopic repair after 6 months follow-up.



Macroscopic examples of defect fill at 6 months follow-up for both treatments. Microfracture treatment showed incomplete macroscopic defect fill while the chondrocyteMNF treatment showed complete macroscopic fill.

high Mastbergen scores indicative for moderate degeneration (Figure 1). Overall, microfracture treatment showed less defect fill at 6 months compared to chondrocyteMNF treatment.(Figure 2) Also, when the quality of macroscopic cartilage regeneration was

Figure 3 Microscopic scores 6 months after treatment.



Microscopic scores for the articular cartilage surrounding and directly articulating with the treated defect and the lateral femur (LFC) and tibia (LTP) surfaces for both the chondrocyteMNF (MNF) and microfracture (MF) treatment. No statistically significant differences were observed in microscopic score between the two treatment for the mentioned scores. However, a trend towards statistical significance (p=0.067) was observed in microscopic score between chondronMNF and microfracture treatment at the treated defect (n=7).

scored the chondrocyteMNF treatment scored statistically significant (p=0.015) higher compared to the microfracture treated defects.

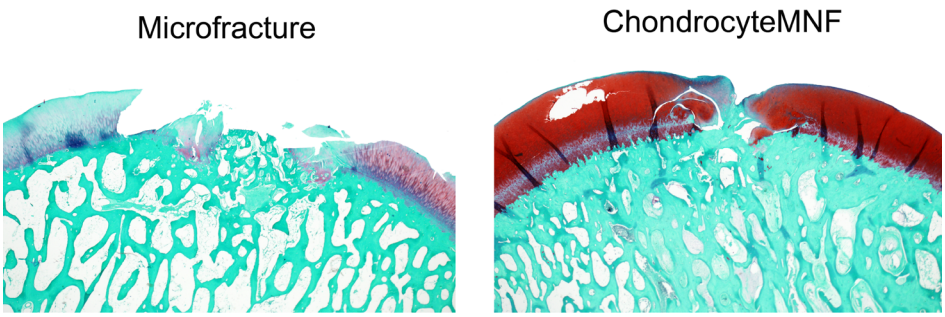
Macroscopic scores

Legend: Macroscopic scores show no differences between the treatments at 6 months follow-up for the medial femur (MFC) and tibia (MTP) surfaces, lateral femur (LFC) and tibia (LTP) surfaces and the articulating defect surface. At the treated defect site the chondrocyteMNF treatment showed a statistically significant (p=0.015) higher macroscopic ICRS score compared to the microfracture treated defects.

Microscopic evaluation

According to the Mankin microscopic score for cartilage degeneration the samples derived from the lateral compartment and cartilage surrounding the defect showed slight cartilage degeneration while those from the articulating medial tibia cartilage showed moderate cartilage degeneration (Figure 3). No statistically significant differences between the cartilage surfaces in the microfracture and chondrocyteMNF treated knees were observed (Figure 3). The microscopic appearance after chondrocyteMNF treatment was better compared to the microfracture treated defects although still incomplete defect fill was present after chondrocyteMNF treatment (Figure 4). The O'Driscoll score for the chondrocyteMNF treated defects was higher (p=0.067) compared to the microfracture treated defects.

Figure 4 Representative examples of microscopic repair after 6 months follow-up.



Representative cross-sectional slices perpendicular to the treated defect at 6 months follow-up showing incomplete / no defect fill for the microfracture treatment while nearly complete defect fill following chondrocyteMNF is observed.

Biochemical evaluation

The produced GAGs per gram regenerated tissue did not show statistically significant differences between the two treatments (25.61 ± 14.95 mg GAG per gram tissue versus 23.51 ± 6.82 mg GAG per gram tissue for the microfracture and chondrocyteMNF respectively). Also for the other joint locations the microfracture treatment (lateral femur 27.26 ± 7.09 mg GAG per gram tissue, lateral tibia 30.90 ± 9.89 mg GAG per gram tissue, articulating cartilage 28.34 ± 10.11 mg GAG per gram tissue) did not show statistically significant differences in produced GAGs per gram tissue compared to chondrocyteMNF treatment (lateral femur 33.87 ± 7.04 mg GAG per gram tissue, lateral tibia 25.04 ± 11.76 mg GAG per gram tissue, articulating cartilage 25.73 ± 10.51 mg GAG per gram tissue). Also for the produced GAG per DNA no difference between the treatments were observed (microfracture: lateral femur 284 ± 113 mg GAG / mg DNA, lateral tibia 390 ± 332 mg GAG / mg DNA, articulating cartilage 462 ± 278 mg GAG / mg DNA vs. chondrocyteMNF: lateral femur 313 ± 144 mg GAG / mg DNA, lateral tibia 215 ± 116 mg GAG / mg DNA, articulating cartilage 416 ± 208 mg GAG / mg DNA).

DISCUSSION

This study shows that treatment of a freshly created cartilage defect using a combination of mononuclear fraction cells and primary chondrocytes, mixed in Beriplast®, leads to better macroscopic, and better microscopic, cartilage regeneration compared to microfracture after 6 months follow-up in a goat model. Also, microfracture treatment showed more macroscopic cartilage degeneration in the directly opposing, articulating, tibia cartilage compared to chondrocyteMNF treatment. After 6 months follow-up both treatments introduced slight to moderate cartilage degeneration at distant locations in the joint. Produced GAG per mg tissue and per DNA did not differ between the two

treatments for the regenerated cartilage nor for cartilage samples from other locations in the joint.

The mononuclear fraction from bone marrow only contains a low percentage of MSCs (Castro-Malaspina et al 1980). Therefore a possible effect on cartilage formation between chondrocytes and the MNF may also be attributed to the presence of stimulatory factors. Recently, Wu et al showed that increased cartilage matrix production in a coculture system between chondrocytes and bone marrow derived MSCs was due to the trophic role of MSCs rather than MSCs undergoing terminal differentiation themselves (Wu et al 2011). Moreover, MSCs actually disappeared during culture. The effect on cartilage matrix production when chondrocytes were cocultured with MSCs was also present in MSC chondrogenic differentiation medium however less obvious when using MSC conditioned medium (Wu et al 2011; Wu et al 2012). Others also showed the positive influence on chondrogenesis when MSCs were combined with chondrocytes (Chen et al 2009; Mo et al 2009; Tsuchiya et al 2004). These studies show that trophic factors play an important role, however, direct cell-cell contact cannot be excluded as a co-factor in the stimulation of cartilage specific matrix production. In this study the combination of MNF and chondrocytes showed a better microscopic and statistically significant macroscopic cartilage regeneration compared to microfracture. The stimulatory effect on cartilage matrix production of chondrocytes instigated by cells from mesenchymal origin could possibly be further enhanced when direct cell-cell contact is also introduced as a factor next to a just pool of trophic factors such as the MNF. For this the MSCs could be an interesting alternative.

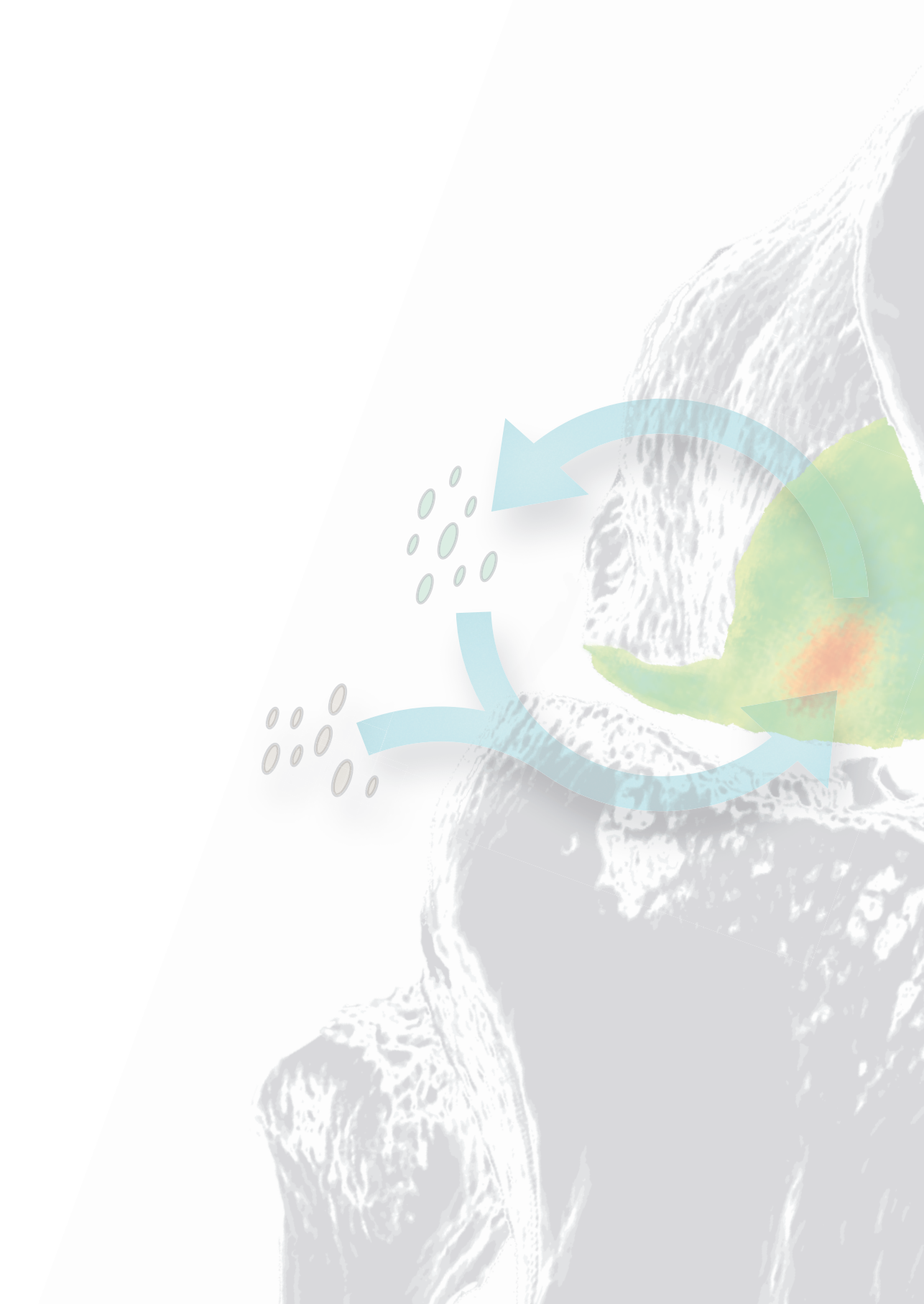
Recent studies have shown that an incongruent cartilage surface introduces high peak forces to the directly articulating cartilage resulting in cartilage degeneration (Becher et al 2008). These results were not only observed after elevated implantation of osteochondral implants but also after microfracture or due to untreated defects (Becher et al 2008; Custers et al 2007; Custers et al 2009; Custers et al 2010). Our results also demonstrate more macroscopic cartilage degeneration following microfracture treatment compared to the chondrocyteMNF treatment. This could have been attributed to an incongruent cartilage surface and incomplete defect fill following microfracture with increased shear forces at the articulating surface thereby introducing cartilage degeneration. For this reason complete defect fill seems imperative not only for local success of treatment but also to preserve the quality at other locations in the knee.

The presence of a focal cartilage lesion negatively influences joint homeostasis (Saris et al 2003). Both in goats and humans treatment of a chronic cartilage lesion was related to a decrease in the treatment success compared to treatment of fresh lesions (de Windt et al 2009; Saris et al 2003). Also, late or no treatment of cartilage defects in goats leads to early signs of osteoarthritis (Saris et al 2003). Therefore early treatment of cartilage defects would, at least in goats, be the ideal situation for two reasons; it improves the

success of cartilage regeneration while it reduces the risk on early osteoarthritis. In this study both treatments were applied in freshly created cartilage defects. Despite this, moderate macroscopic and microscopic cartilage degeneration was observed in the treated, medial, compartment while slight degeneration was present in the lateral compartment. Inability to restore the articular cartilage surface will make the partly regenerated cartilage prone to damage as a non-continuous cartilage surface has a higher tendency to deform which initiates accelerated matrix damage and tissue loss (Braman et al 2005; Gratz et al 2008; Gratz et al 2009). This will initiate the cascade of matrix damage at the articulating surface, the whole treated joint compartment and eventually the contralateral compartment. For this reason the incomplete defect fill in this study could have contributed to the slight to moderate degeneration seen after 6 months follow-up. This is critically important as most treatment failures or disappointing clinical improvement after cartilage therapy can eventually be related to inadequate defect fill and tissue regeneration (Nehrer et al 1999).

Concentrated bone marrow, derived from spinning down whole bone marrow aspirates, showed better macroscopic and microscopic cartilage formation compared to microfracture in an equine model (Fortier et al 2010). Also, bone marrow nucleated cells combined with a collagen hydrogel contributed to the repair of full-thickness focal lesions in minipigs (Zhang et al 2012). In addition to our data, abovementioned studies show that the use of a bone marrow fraction in cartilage repair has potential. However, results so far did not lead to a clinical translation. Further evaluation and modification of the cellular, extracellular and soluble components of bone marrow (Balakumaran et al 2010) before intra-articular application in the treatment of a focal cartilage lesion would be necessary to understand the added value and improve the results of regeneration.

In conclusion, the treatment of focal articular cartilage lesions using a combination of mononuclear fraction cells from bone marrow and unexpanded chondrocytes is better compared to microfracture, however needs further fine-tuning to improve defect fill and decrease the negative influence on other joint compartments.



Chapter 7

One-stage cell-based cartilage regeneration using a combination of chondrons and mesenchymal stromal cells; *in vitro* and *in vivo* results

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ABSTRACT

Autologous chondrocyte implantation (ACI) is a two-step procedure used to repair focal articular cartilage lesions. Using a combination of chondrons and mesenchymal stem cells (MSCs) ACI could be improved towards a one-stage procedure as sufficient cells would be available to fill the defect within one surgery. This chapter evaluates the cartilage formation of a combination of chondrons and MSCs from *in vitro* evaluation to a large animal validation all aiming at the safe and scientific preclinical development of a one-stage cell-based regenerative treatment for focal articular cartilage lesions.

Chondron and MSC combinations were cultured *in vitro* (n=3), implanted subcutaneously in nude mice (n=10) while embedded in Beriplast® and used to fill a freshly created focal cartilage lesion in goats (n=8).

Replacement of MSCs in chondron cultures enhanced cartilage-specific matrix production as combination cultures had higher absolute GAG and GAG corrected for DNA content compared to chondron cultures. Similar results were observed after 4 weeks subcutaneous implantation in nude mice. Also, treatment of freshly created cartilage defects in goats using a combination of chondrons and MSCs in Beriplast® resulted in better microscopic, macroscopic and biochemical cartilage regeneration compared to microfracture treatment.

In conclusion this chapter showed that the combination of chondrons and MSCs positively influences cartilage matrix formation and that this combination of cells can successfully be applied in a one-stage cell-based treatment of focal cartilage lesions which outperforms microfracture in a goat model.

INTRODUCTION

Cartilage cell therapy could be improved by establishing a one-stage technique using cell combinations to circumvent the need for culture expansion. Autologous chondrocyte implantation (ACI) is indicated for isolated lesions of the articular cartilage >2 cm^2 in patients that wish to regain a high level of activity (Bekkers et al 2009). During a first arthroscopic procedure a small biopsy is taken from the non-weight-bearing trochlear margin in the knee. Articular chondrocytes are isolated from this biopsy, expanded *in vitro* and reimplanted in a second mini-open surgical procedure (Brittberg et al 1994). Overall, the clinical improvement is good and patients can reach a satisfying activity level. However, low cellularity of the biopsy necessitates expansion leading to a procedure that requires two surgical interventions and risks cellular dedifferentiation (Marlovits et al 2004; Schnabel et al 2002).

The number of chondrocytes necessary for reimplantation could be reduced by adding another type of cells to the isolated chondrocytes. This will make enough cells available for direct reimplantation in a one-step cell-based treatment. Recently, Wu et al showed that chondrocyte proliferation and cartilage-specific matrix formation are both stimulated by trophic factors derived from MSCs (Wu et al 2011). A stimulation of cartilage matrix production and an upregulation of cartilage-specific matrix genes was observed when chondrocytes were combined with MSCs (Chen et al 2009; Tsuchiya et al 2004). This indicates that MSCs could be an interesting cell population to combine with freshly isolated articular chondrocytes in a one-step procedure. Isolation of autologous MSCs will not be achieved within a single surgical procedure as it takes several days for MSCs to adhere to and expand in culture plastic. Therefore, either autologous MSCs, cultured from an earlier, planned, bone marrow aspiration, or allogeneic MSCs might be used for a one-step procedure. Recent results show that allogeneic MSCs have safely been applied in a number of clinical trials (Griffin et al 2010). Also, when MSCs were encapsulated in hydrogels only a gradual increase in the expression of major histocompatibility complex (MHC) class I and II molecules was observed (Yuan et al 2011).

Within healthy articular cartilage, chondrocytes are surrounded by a pericellular matrix, together called the chondron. This pericellular matrix mainly consists of collagen type VI and has an important role in the metabolic activity of the chondrocyte and the mechanical signalling from and to the extracellular matrix (Graff et al 2003; Larson et al 2002; Wang et al 2010). The presence of a pericellular matrix has been shown to enhance matrix production by chondrocytes, suggesting that an intact pericellular matrix improves cellular cartilage regeneration (Larson et al 2002; Vonk et al 2010). The combination of a pericellular matrix surrounding chondrocytes with MSCs could even further enhance chondrogenesis.

This chapter evaluates the cartilage formation of a combination of chondrons and mesenchymal stromal cells from *in vitro* evaluation to a large animal validation all aiming at the safe and scientific preclinical development of a one-stage cell-based regenerative treatment for focal articular cartilage lesions.

MATERIAL AND METHODS

Donor material and cell isolation

All patient material used in this study was approved by the local ethical committee. The anonymous use of redundant material from patients with focal cartilage lesions undergoing surgery is part of the standard treatment agreement with patients in the University Medical Center Utrecht. For the *in vitro* and the *in vivo* subcutaneous nude mice studies the debrided waste from focal articular cartilage lesions of 6 patients was obtained during ACI surgery. Chondrons from this cartilage tissue were isolated using a rapid digestion protocol. For this, debrided cartilage was cut into small pieces, immersed in PBS with 1% penicillin/streptomycin (100 U/ml / 100 µg/ml, Invitrogen, the Netherlands), followed by 45 minutes digestion at 37 ºC in 2 % collagenase type II solution (Worthington, Lakewood, USA) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) with penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen). MSCs were obtained from and manufactured in our institutional GMP-approved Cell Therapy Facility for a clinical trial in steroid-resistent graft versus host-disease.

To show the presence of a pericellular matrix surrounding the chondrocytes after the rapid digestion protocol, it was compared to the standard procedures for isolation of chondrocytes and chondrons. For this the debrided cartilage from 3 patients was obtained during ACI, minced in small pieces and divided in three for either standard chondrocyte or chondron isolation or the rapid digestion protocol. Chondrocytes were isolated overnight at 37 ºC in 0.15 % collagenase type II solution (Worthington) in DMEM (GIBCO) with penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen). Chondrons were either obtained using the rapid digestion protocol or by an overnight 0.15 % dispase (Gibco) in 0.1 % collagenase (Worthington) and 0.01 % BSA (Roche, Germany) in phosphate buffered saline (PBS) protocol. Isolated cells were cytospinned and stained for safranin-O and collagen type VI (see protocol below).

In vitro culturing

The debrided cartilage from a total of 5 patients was used for the *in vitro* experiments. Chondrons were isolated, using the rapid digestion protocol, and combined with MSCs at different ratios: 0, 5, 10, 20, 50 or 100 % chondrons at a total cell number of 250000 cells. After this, cells were pelleted by centrifugation and cultured for a period of 4 weeks

in DMEM (Invitrogen, the Netherlands) with 0.2 mM l-ascorbic acid-2-phosphate (AsAp, Sigma-Aldrich), 2 % Human Serum Albumin (Sanquin, the Netherlands), penicillin/streptomycin (100U/100µg, Invitrogen), 2 % ITS-X (Invitrogen) and 10 ng/ml TGF-β2 (R&D systems, USA). Medium was changed twice a week.

To directly compare the performance of chondrons in coculture to chondrocytes in coculture the donor material from 3 of the abovementioned 5 patients was divided for half chondron and half chondrocyte isolation. Both cell conditions were cocultured with MSCs at identical ratios and cultured under similar described conditions.

For all *in vitro* experiments a total of 3 pellets were created per coculture ratio. Pellets were analyzed for cartilage matrix production by glycosaminoglycans (GAG) and DNA quantification.

Subcutaneous implantation in nude mice

Animal experiments were conducted in agreements with the committee for animal experiments and following institutional guidelines on the use of laboratory animals (DEC no. 2011.III.03.026). To evaluate the additional effect of MSCs in coculture, chondrons were isolated from the debrided cartilage of one donor, embedded in 100 µl Beriplast® (Nycomed, the Netherlands) and combined with or without MSCs. The coculture Beriplast® scaffolds contained 250000 cells per scaffold with either 0, 5, 10 or 20 % chondrons. The Beriplast® scaffolds containing only chondrons consisted of chondrons at the same absolute number as present in the coculture Beriplast® scaffolds, however without the addition of MSCs. Per ratio, for both the coculture and chondron only group a total of 5 scaffold were created. After preparation, the scaffolds were kept overnight at a 37 ^{o}C, 5 % CO_2 and implanted subcutaneously the next morning.

For implantation the coculture and chondron only Beriplast® scaffolds were randomly assigned to 4 separate subcutaneous dorsal implantation pockets in 10 female nude mice (Hsd-cpb:NMRI-nu, Harlan, the Netherlands). Before surgery, 8 h and 1 day after surgery the mice received pain medication by subcutaneous injection of buprenorphine (0.05 mg/kg, Merck, Germany). After 1 day of solitary housing the mice were housed together (5 mice per cage) during 4 weeks at the Central Laboratory Animal Institute of the Utrecht University. The mice were sacrificed by cervical dislocation, all scaffolds were harvested and analyzed for GAG and DNA content (see protocol below) while one scaffold per ratio was fixed in 10 % buffered formalin overnight and further processed for safranin-O histology and collagen type I and II immunohistochemistry (see protocol below).

One-stage chondronMSC therapy vs. microfracturing in goats

The goat experiments were performed following the guidelines of the local committee for animal experiments and by the institutional guidelines on the use of laboratory

animals (DEC no. 2010.III.09.113). A total of 8 female Dutch milk goats (age 2-4 years old, weight 69 ± 6 kg) were treated with either the ChondronMSC or microfracture treatment. A standardized full thickness chondral defect was created in the medial femoral condyles of the left and right knee. Per goat one defect was treated with the chondronMSC therapy while the other was treated with microfracture. The total study follow-up was 6 months at which food and water was given ad libitum and general health and care conditions were monitored by the laboratory animal welfare officer.

After acclimatization for at least 1 week, and 1 day prior to surgery, a fentanyl patch was placed on the skin as pain medication and the goats were weighed. All animals received intravenous premedication consisting of detomidine hydrochloride (0.04 mg/kg, Pfizer) and a single dose of Augmentin. Subsequently, induction of anaesthesia was achieved with thiopental (6 mg/kg, Rhône Mérieux). Throughout the surgical procedures anaesthesia was maintained on midazolam 0.8 mg/kg, sufenta forte 0.007 mg/kg (Abbott Laboratories, ASTPharma, The Netherlands) and, when necessary, isoflurane or propofol (Abbott Laboratories).

All surgical procedures were carried out under aseptic conditions. A medial parapatellar approach, without dislocation of the patella, was chosen to open the knee and obtain exposure of the medial femoral condyle. After macroscopic inspection and scoring of the articular cartilage surface, as described by Mastbergen et al (Mastbergen et al 2006), a standardized chondral cylindrical cartilage defect was created into the subchondral bone, using a hand-operated drill (5 mm diameter), in the full-weight-bearing center of the medial femoral condyle. A sharp surgical spoon was used to create a stable rim surrounding the defect. The debrided cartilage was collected in a sterile 50 ml tube with DMEM, containing 100U/mg / 100 µg/ml penicillin/streptomycin (Invitrogen). Following this the cartilage defect was treated by microfracturing. For this the exposed subchondral bone at the bottom of the defect was perforated at 12, 8 and 4 o'clock using a 1.5 mm k-wire until blood appeared into the created defect. Subsequently the knee was closed in layers. In the meantime chondrons were isolated, using the rapid digestion protocol, from the debrided defect cartilage, and combined in the fibrinogen component of Beriplast® (Nycomed), with goat MSCs at a 10 % chondron 90 % MSC combination in a concentration of $1*10^6$ cells per milliliter. The goat MSCs were derived from iliac crest bone marrow aspiration in 2 Dutch milk goats which were assigned to another experiment under the same study protocol (DEC no. 2010.III.09.113). The bone marrow aspirate was cultured in αMEM supplemented with 15% foetal bovine serum (Hyclone, Thermo Fisher Scientific, the Netherlands), 100U/ml peninillin, 100 µg/ml streptomycin (Invitrogen) and 2mM L-glutamine (Glutamax, Gibco). Adhering cells were cultured for 2 passages and then stored in liquid nitrogen for later use. Next, the other knee was opened and a chondral lesion created as described above. The Beriplast® fibrinogen (with cells) and thrombin components (Nycomed) were prepared for surgical applica-

tion following the manufacturers' description. After this the Beriplast® (Nycomed) was injected into the defect and allowed to gelate for five minutes. The knee was flexed 10 times, to control for the stability of the Beriplast® construct within the defect, and closed in layers.

After surgery the animals were housed individually for a maximum of 24 hours and direct full-weight bearing was allowed. In case of signs of wound infection or pain, adequate antibiotics or pain medication were provided respectively, based on the judgement of the veterinarian. After 6 months the animals were euthanized by an overdose of pentobarbital (Euthesate®), treated legs were explanted and soft tissues removed. The macroscopic appearance of the articular cartilage of the medial and lateral femur and tibia and the articular cartilage that directly articulated with the created defect were scored by two observers (JEJB and MvR) using the Mastbergen score (Mastbergen et al 2006). The Mastbergen score uses a 0-4 points scale to describe the surface damage of the articular cartilage where an increasing score is related to increasing cartilage damage. Also the macroscopic appearance of the treated articular defect was scored (by JEJB and MvR) using the ICRS macroscopic evaluation of cartilage repair score which has recently been validated for cartilage regeneration (van den Borne et al 2007). This scoring system subdivides the quality of cartilage repair into a degree of defect repair, integration into the border zone and macroscopic appearance using a maximum score of 12 points. Here the higher the score the better the quality of defect repair is. For both scoring systems the scores of the two observers were averaged. In case the scores of two observers differed more than 2 points on subitems of the scoring systems, consensus was reached.

Full-thickness cartilage tissue samples were harvested from the anterior half of the defect and processed for GAG and DNA analysis (see protocol below). The posterior osteochondral half of the defect (including the cartilage surrounding this) as well as the articulating osteochondral medial tibia, and osteochondral lateral femur and tibia samples were harvested and fixed in 10% buffered formalin during 48 h. Subsequently all samples were decalcified using Luthra's solution (3.2% 11 M HCl, 10.0% formic acid in distilled water) and further processed for safranin-O histology (see protocol below). Stained sections were blinded and scored by two observers (JEJB and MvR) using the cartilage repair score, as described by O'Driscoll (O'Driscoll et al 1986; O'Driscoll et al 1988), for the osteochondral samples derived from the posterior defect and the Mankin score for the osteochondral samples from the lateral tibia and femur and from those having cartilage that articulated and surrounded the defect. For the O'Driscoll system a lower score is related to worse histological quality of the articular cartilage while for the Mankin score a higher score indicates worse outcome.

Biochemical analysis

All cartilage samples assigned to GAG en DNA analyses were digested overnight in papain (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 °C. GAG content was determined using a dimethylmethylene blue (DMMB) assay (Farndale et al 1986) where the complexation of GAGs with DMMB was measured spectophotometrically at 540 nm, using 595 nm as a reference. Chondroitin sulphate (shark; Sigma-Aldrich) was used as a standard.

Per sample, the DNA content was also determined from the papain digest using a picogreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Histological and immunohistochemical analysis

All fixed samples assigned for histological and immunohistochemical stainings were dehydrated by graded alcohol series, immersed in xylene and embedded in paraffin. For histology, 5 µm sections were stained with safranin-O (Merck, Germany) for GAG and nuclei and cytoplasm were counterstained with respectively Weigert's haematoxylin (Klinipath, the Netherlands) and 0.4 % fast green (Merck, Germany).

Immunohistochemical staining was performed on 5 µm sections. Antigen retrieval steps, 30 min pronase (Roche, the Netherlands, 1 mg/ml in PBS) followed by 30 min hyaluronidase (Sigma-Aldrich, 10 mg/ml in PBS) at 37 °C, were conducted for the collagen type I, II and VI staining followed by blocking of the sections using a 5 % PBS-BSA solution. The primary antibodies for collagen type I (Merck, 20 ug/mL in 5 % PBS/BSA), collagen type II (DSHB, USA, 1/100 in 5% PBS/BSA) and collagen type VI (1:10, 5C6, Developmental Studies Hybridoma Bank, Iowa City, IA) were incubated overnight at 4 °C. Following this the sections were washed in PBS-Tween and incubated with the secondary antibody for collagen type I (biotinylated secondary anti-mouse antibody, GE, Healthcare, UK; 1/200 in 5 % PBS/BSA) collagen type II (anti-mouse antibody conjugated with peroxidase, DAKO, the Netherlands, 5 µg/mL) and collagen type VI (anit-mouse-HRP, 1:100; Dako Glostrup, Denmark) for 1 h at room temperature. Binding of the secondary antibody was visualized using 3-diaminobenzidine (DAB, Sigma-Aldrich). Mayer's haematoxylin was used as a counterstaining.

Statistical analysis

All statistical analysis were performed using SPSS version 15.0. For both the *in vitro* and *in vivo* nude mice experiments comparing respectively chondronMSC to chondrocyteMSC and chondronMSC to chondron only differences in GAG and GAG/DNA were, per ratio, determined using a paired t-test. Differences in GAG and GAG/DNA between the chondronMSC ratios in the in vitro pellet cocultures were determined using a one-way ANOVA with posthoc Bonferroni test.

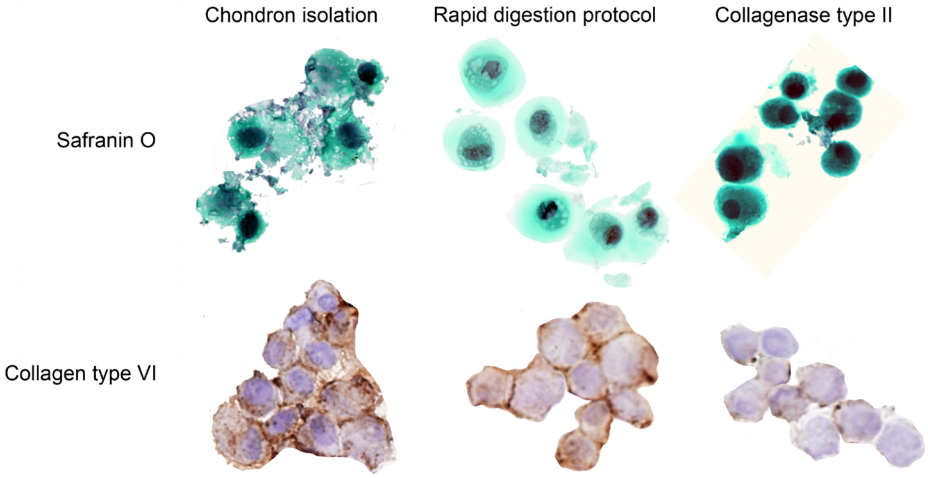
Regarding the *in vivo* goat experiments, the differences between the microfracture and chondronMSC treatments for the macroscopic, microscopic and biochemical analysis were tested using a paired t-test. For all analysis a difference of $p<0.05$ was considered statistically significant.

RESULTS

Donor material and cell isolation

All debrided cartilage used in this study was derived from patients with either a grade III cartilage lesion or OCD. On average 0.72±0.20 gr of debrided tissue was obtained from the focal lesions which resulted in $1.06\pm0.56*10^6$ chondrons after rapid digestion. The efficiency of the rapid digestion protocol was, thus, calculated at $1.37\pm0.50*10^6$ chondrons per gram of debrided tissue which was lower compared to the overnight chondrocyte isolation ($1.37\pm0.50*10^6$ chondrocytes per gram debrided tissue) however still sufficient when isolated chondrons are combined with another cell source. The collagen type VI and safranin-O staining shows that the rapid digestion protocol, indeed, results in isolated cells with preservation of their pericellular matrix which is comparable to the standard chondron isolation protocol and differs in pericellular matrix staining compared to the overnight chondrocyte isolation (Figure 1).

Figure 1 Collagen type VI and safranin-O staining comparing chondron isolation, rapid digestion and chondrocyte isolation.

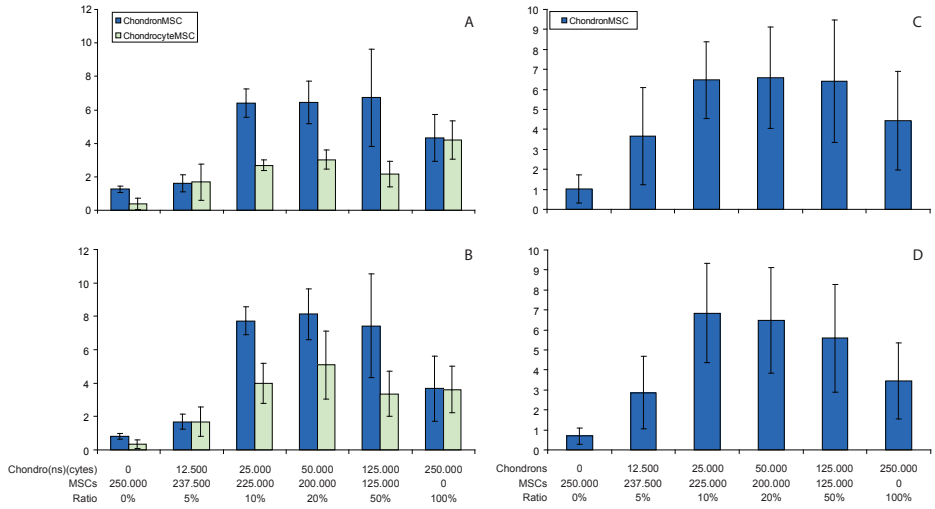


Collagen type VI and safranin-O staining show a pericellular matrix surrounding the chondrocytes after standard chondron isolation and rapid digestion but not after overnight chondrocyte isolation.

In vitro experiments

Partial replacement of chondrocytes by MSCs (10-50 % chondrocyteMSC cultures) resulted in a similar or higher amount of produced GAGs per DNA compared to 100 % chondrocyte cultures (Figure 2B). A dose dependent stimulation of cartilage specific matrix production of chondrons over chondrocytes was observed when combined with MSCs. For the coculture pellets containing 10-50 % chondrons the absolute GAG production was higher ($p \leq 0.03$) compared to the pellets having 10-5 0 % chondrocytes (Figure 2A). Also, partial replacement of the chondrons by MSCs resulted in a stimulation of absolute GAG production as the measured GAG in the 10-50 % chondronMSC pellets was higher ($p \leq 0.03$) compared to those in the 100% chondron pellets (Figure 2C). No differences ($p \geq 0.173$) in DNA were observed within and between the chondronMSC and chondrocyteMSC cultures (data not shown). GAG production per cell was also higher ($p \leq 0.03$) in the 10-50 % ratios of chondronMSC pellets compared to chondrocyteMSC pellets (Figure 2B). Also the 10-50 % chondronMSC pellets produced more ($p \leq 0.001$) GAG per cell compared to the 100 % chondron pellets (Figure 2D).

Figure 2 In vitro pellet co-cultures.



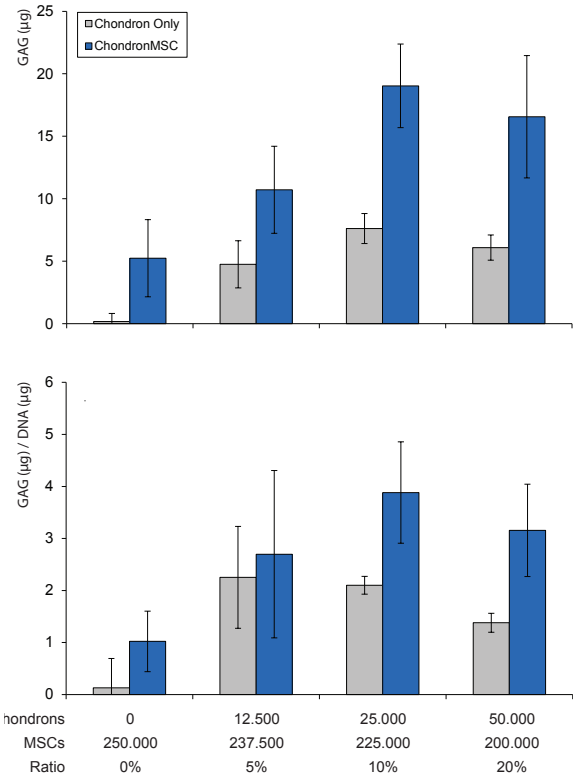
Direct comparison between chondrocytes and chondrons in coculture with MSCs from the same cartilage donors (n=3) and separate chondronMSC cultures (n=5). Absolute GAG and GAG/DNA content resulting from 4 weeks pellet culture. For the ratios 10-50 % the chondronMSC cultures show higher GAG and GAG/DNA compared to similar ratios between chondrocytes and MSC. Also, a replacement of 50-90 % of chondrons in culture by MSCs improves GAG production and GAG/DNA content. *p ≤ 0.003, # p ≤ 0.03, (n=5 donors).

Subcutaneous implantation in nude mice

After subcutaneous implantation the 5-20 % chondronMSC constructs produces more GAGs ($p \leq 0.04$) compared to similar amounts of chondrons alone, indicating a stimulating effect on cartilage-specific matrix production upon the addition of MSCs to chondrons (Figure 3). The DNA content did not differ ($p \geq 0.849$) between the chondronMSC ratios whereas the DNA content in the chondron only constructs showed a gradual decrease, according to the seeding density (data not shown). After correcting for the amount of DNA present after 4 weeks, still the 10-20 % chondronMSC constructs showed a higher ($p \leq 0.015$) GAG production per DNA compared to the related chondron only constructs (Figure 3).

Both the chondronMSC and chondron only constructs stained positive for collagen type II whereas the safranin-O staining was more intense in the chondronMSC compared to the chondron only constructs. Positive collagen type I staining was found surrounding

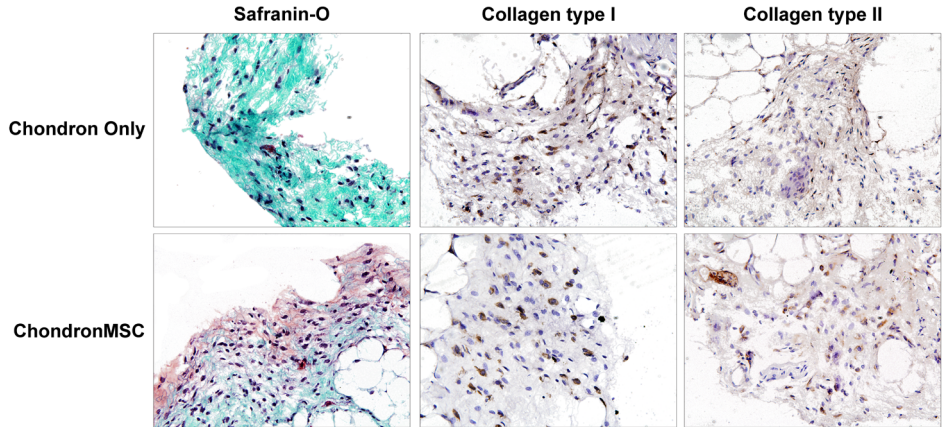
Figure 3 ChondronMSC vs chondron only after subcutaneous implantation.



GAG and GAG/DNA content after 4 weeks subcutaneous implantation of chondronMSC combinations and chondron only cultures all embedded in Beriplast®. For ratios 10-20 % the chondronMSC cultures produced more GAG and GAG/DNA compared to similar absolute numbers of chondrons only indicating an added value of adding MSCs. *$p \leq 0.04$, # $p \leq 0.015$, (n=1 donor).

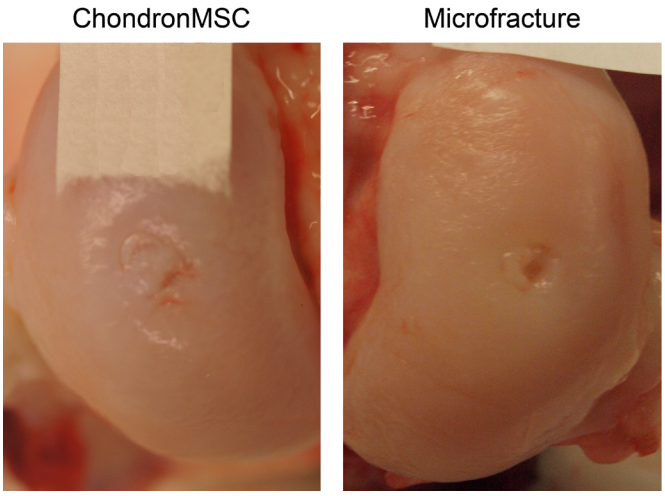
the cells in the chondronMSC constructs while for the chondron only constructs this was also present in the extracellular matrix (Figure 4).

Figure 4 Cartilage specific matrix staining after subcutaneous implantation.



ChondronMSC constructs show more safranin-O staining for GAGs compared to chondron only constructs while Collagen type I matrix staining is absent.

Figure 5 Macroscopic defect repair.



Best macroscopic defect repair for both the chondronMSC and microfracture treatment. ChondronMSC shows good defect fill with a relatively intact surface layer while after microfracture incomplete defect fill is present and the rim of the created defect is still visible.

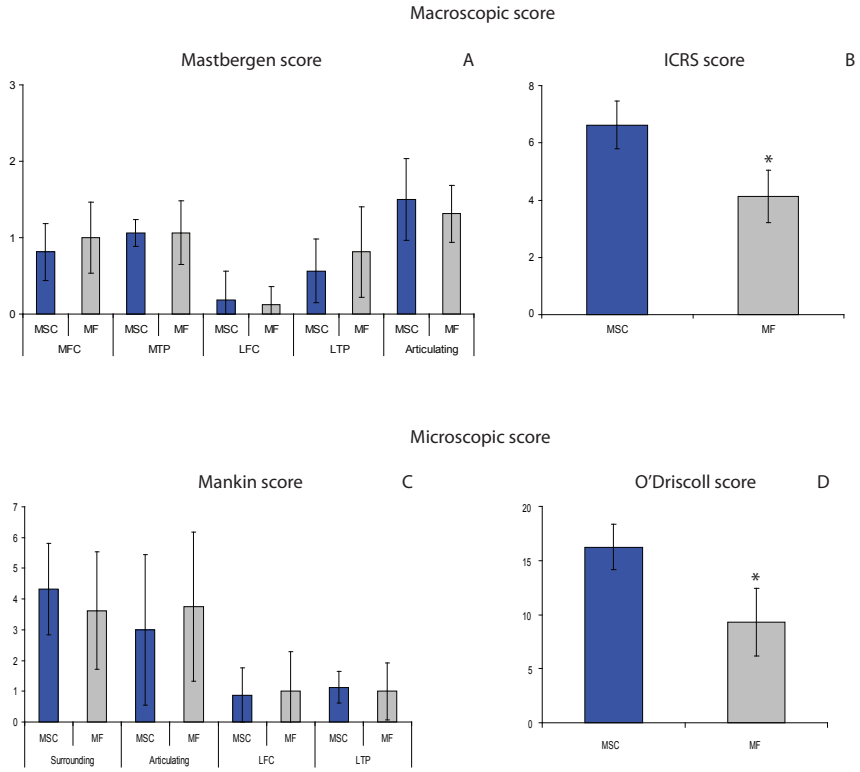
One-stage ChondronMSC therapy vs. microfracturing in goats

Surgery was performed without complications. All articular surfaces were free of signs of degeneration at first inspection during index surgery. After surgery all goats were

able to function with full-weight bearing within 4 hours and their maximum weight loss was 4.3 ± 1.4 % from their preoperative weight. During follow-up no signs of wound or intra-articular infections were observed.

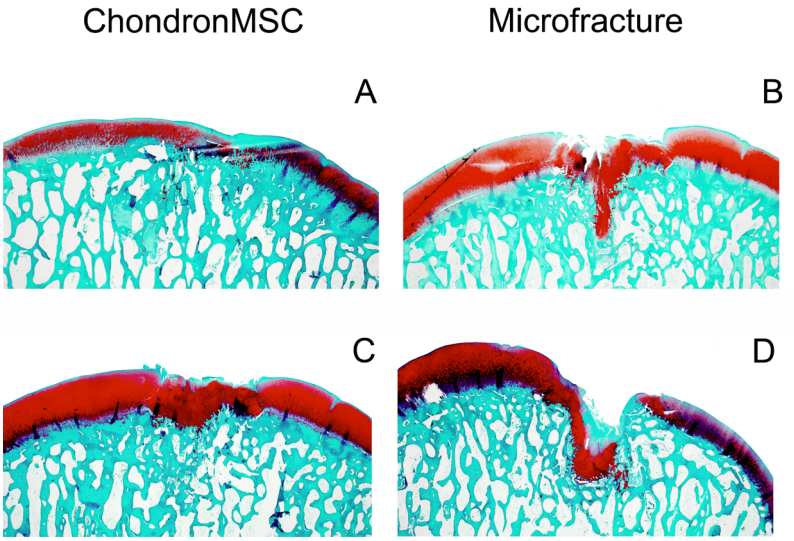
At 6 months after surgery the macroscopic appearance of the defects treated with microfracture showed an incomplete defect fill while those treated with the chondronMSC combination were almost completely filled with cartilage-like tissue (Figure 5). This was also confirmed by the ICRS macroscopic score for cartilage repair which was statistically significantly higher (p=0.002) in the chondronMSC (6.63±0.83) group compared to the microfracture group (4.13 ± 0.92) (Figure 6B). The macroscopic (Mastbergen) score of the other joint surfaces did not show a statistically significant difference (p≥0.17) between the two interventions (Figure 6A).

Figure 6 Macroscopic and microscopic scores.



Macro- and microscopic score of different anatomical locations of the goat knee 6 months after surgery. (MFC= medial femur condyle, MTP= medial tibia plateau, LFC = lateral femur condyle, LTP = lateral tibia plateau, Articulating = cartilage that directly articulates with the created defect, MSC = chondronMSC therapy, MF = microfracturimg, Surrounding = the cartilage that surrounds the created cartilage defect. *p≤0.002, (n=8).

Figure 7 Microscopic defect repair.



Slices were cut perpendicular through the middle of the created defect. For both the ChondronMSC and Microfracture treatment the best (A, B) and worst result (C, D) are shown. For both ChondronMSC (A,C) pictures complete to almost complete defect fill is achieved while for the microfracture pictures (B, D) the defect fill varied a lot between the different goats.

The histological quality of the regenerated tissue was statistically significantly ($p=0.001$) higher (as determined using the O'Driscoll score) in the ChondronMSC treatment group compared to the microfracture group (Figure 6D). When looking for histological signs of deterioration no statistically significant differences ($p \geq 0.48$) were identified between the two treatments on the other joint surfaces (Figure 6C). However for both treatments the histological Mankin scores were higher for the cartilage surrounding and articulating the treated defect compared to the cartilage in the lateral knee compartment (Figure 6C). Overall the histological result after ChondronMSC was consistently better compared to microfracture as the worst result still showed an acceptable level of defect fill and lateral integration with adequate Safranin-O staining while this was not the case after microfracture (Figure 7).

The absolute GAG production per gram regenerated tissue was highest ($p=0.022$) in the ChondronMSC (0.083 ± 0.037 mg GAG per gr tissue) compared to the microfracture (0.041 ± 0.013 mg GAG per gr tissue) treated defects. Also when corrected for DNA the articular cartilage in the ChondronMSC group (502 ± 129 µg GAG / µg DNA) contained statistically significant ($p=0.016$) more GAGs compared to the microfracture group (301 ± 94 µg GAG / µg DNA). Both the GAG per milligram tissue and the GAG per DNA did not show statistically significant differences between the ChondronMSC and microfracture groups for the other joint locations.

DISCUSSION

This study evaluated whether combining MSCs with chondrons is suitable for a one-stage cell-based regenerative treatment for focal articular cartilage lesions. We showed that cocultures of 10-20 % chondrons produced more GAG and GAG per DNA compared to chondrocyteMSC cocultures *in vitro* and chondron only cultures *in vivo*. In addition, a substitution of 80-90 % of the chondrons by MSCs led to higher absolute GAG and GAG per DNA content in vitro compared to the pellets where chondrons were not substituted with MSCs. Also, 6 months after treatment of a freshly created articular cartilage defect in goats, a combination of 10 % chondrons and 90 % MSCs in a Beriplast scaffold outperformed treatment with microfracture on macroscopic and microscopic level. Furthermore GAG production per gram regenerated tissue was higher in the chondronsMSC treatment compared to the microfracture treatment.

Stimulation of cartilage-specific matrix production by the combination of chondrocytes and MSCs has already been shown in several *in vitro* pellet and transwell cultures (Chen et al 2009; Tsuchiya et al 2004; Wu et al 2011). A substitution of 80 % chondrocytes by MSCs has, similar to our findings with chondrons, also shown to produce similar or more cartilage specific matrix proteins (Wu et al 2011). The mechanism by which this positive interaction works is still under debate. However, in pellet cultures using MSCs and chondrocytes from different species the MSCs disappeared over time as determined from PCR analysis (Wu et al 2011). This suggests that the MSCs stimulate chondrocyte proliferation and matrix production by trophic signaling instead of undergoing terminal chondrogenic differentiation themselves. Chondrocyte cultures under MSC conditioned medium enhanced cartilage specific matrix production but not to such an extent when MSCs were actually present during culture (Wu et al 2011). This suggests that, next to trophic factors, direct cell-cell contact also plays a role in the stimulatory environment of the chondrocyte MSC combination.

Preservation of the pericellular chondrocyte matrix, i.e. chondrons, in a 3D *in vitro* redifferentiation model showed higher Col2a1 and lower MMP13 and Col1a1 gene expression when compared to chondrocytes without a pericellular matrix (Vonk et al 2010). Also other studies showed a higher GAG production in chondron cultures compared to chondrocyte cultures (Larson et al 2002). In our cultures chondrons outperformed chondrocytes in terms of absolute GAG and GAG per DNA when both cells were combined with MSCs. The pericellular matrix that surrounds chondrocytes plays a vital role in the communication between the cell and its environment (Graff et al 2003). The added value of this surrounding could be beneficial for the chondrocyte when combined with MSCs as the pericellular matrix will aid in the cell-cell interaction and the presentation of trophic factors to the chondrocyte and thereby further enhance cartilage specific matrix production.

Approximately $1*10^6$ chondrocytes per cm^2 debrided defect are currently used to treat cartilage defects using ACI. In this study we were, on average, able to isolate $1.06\pm0.56*10^6$ viable chondrons from debrided defect cartilage within 45 minutes. When mixed with allogeneic MSCs a combination containing 10-20 % chondrons showed optimal cartilage formation in terms of quantitative matrix production and immunohistochemical collagen stainings. This indicates that, when a combination of chondrons and MSCs would be used, defects up to 10 cm^2 could be treated in a one-stage cell-based approach using the fast digestion protocol and allogeneic MSCs. This approach was tested in goats and compared to microfracture treatment. The microfracture technique is widely used in current clinical practice to treat small focal cartilage lesions. It is based on the stimulation and distribution of bone marrow derived cells into the joint space and finally is a one-stage procedure which shows acceptable results up to 2 years follow-up (Bekkers et al 2009; Steadman et al 2001). For these reasons we found microfracturing the reasonable reference treatment for our one-stage cell-based treatment using chondrons and MSCs.

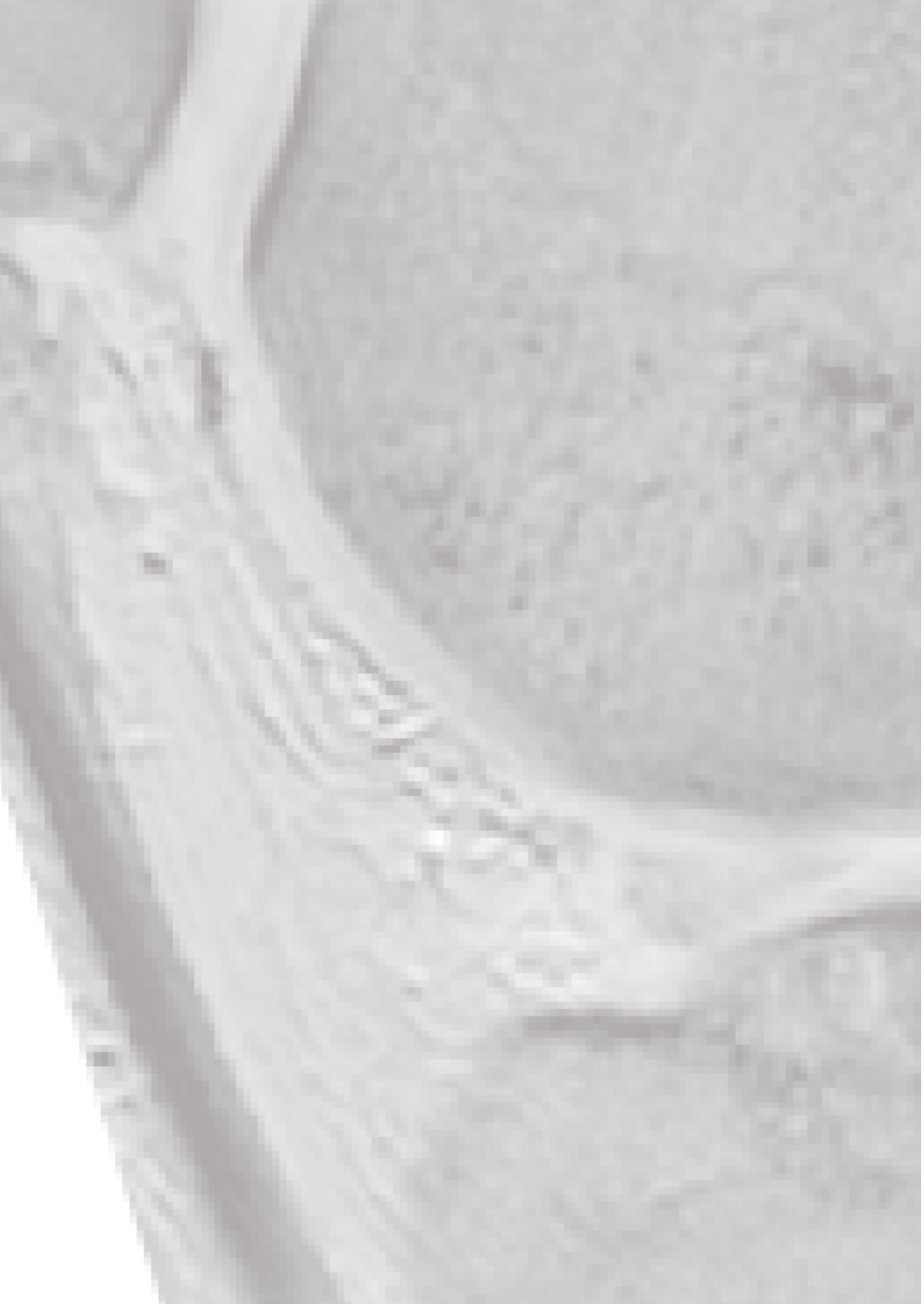
The model where two goat knees are treated simultaneously provides the possibility to directly compare two treatments without intra-animal variation. Also fewer animal are needed when this model is applied. The disadvantage however of such a model is the possible influence the one treatment has on the other when the goat is partially weight bearing one leg because of impaired healing. However, the goats in our study were directly able to fully weight bear their both knees without deterioration in mobility during follow-up.

In line with the *in vitro* and *in vivo* nude mice results the chondronMSC treatment in goats led to good cartilage formation at 6 months follow-up as quantified by macroscopic and microscopic scoring systems. Compared to microfracture the ChondronMSC treatment scored higher on macroscopic and microscopic defect regeneration and produced more GAG per gram regenerated tissue. The quality of tissue regeneration is believed to play a major role in treatment success (Nehrer et al 1999). Deterioration of clinical success, and eventually treatment failure, has been directly related to inadequate tissue regeneration (Nehrer et al 1999). Also, early differences in quality of tissue regeneration between characterized chondrocyte implantation and microfracture has shown to lead to relevant differences in clinical outcome at three years follow up (Saris et al 2008; Saris et al 2009).

Microfracture treatment of fresh cartilage defects has already been shown to introduce severe degeneration at the opposing cartilage in goats. Also, articular cartilage surrounding a focal lesion has been related to accelerated matrix damage due to the higher tendency to deform under direct axial strain (Braman et al 2005; Gratz et al 2008; Gratz et al 2009) In this study, for both treatments, the cartilage surrounding and directly articulating with the treated defects had macroscopic scores that resemble damaged

articular cartilage. Direct weight-bearing of the goats following surgery could have negatively contributed to the quality of the tissue that surrounded and opposed the cartilage defect.

In conclusion this chapter showed that the combination of chondrons and MSCs positively influences cartilage matrix formation and that this combination of cells can successfully be applied in a one-stage cell-based treatment of focal cartilage lesions which outperforms microfracture in a goat model.



Chapter 8

Diagnostic modalities for diseased articular cartilage; from defect to degeneration: a review

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ABSTRACT

The progression of cartilage matrix damage to generalized degeneration is associated with specific pathophysiological and clinical aspects. Reliable detection of stage-related characteristics of cartilage disease serves both a therapeutic and prognostic goal. Over the past years, several (pre)clinical diagnostic modalities for cartilage pathologies have been advocated. Each modality focuses on different aspects of the disease. Early diagnosis, before irreversible damage has occurred, opens up the possibility for better treatment and improves the patients' prognosis. This chapter will give an overview of the diagnostic modalities available for monitoring cartilage pathology and focuses on reliability, clinical value, current status and possible applications.

INTRODUCTION

Accurate diagnosis based on disease-related characteristics is a prerequisite for successful treatment and improves patients' prognosis. To determine the diagnostic value and future potential of a certain diagnostic tool, the applicability at various stages of a disease has to be determined. The process from cartilage matrix damage to generalized degeneration represents a disease continuum in which the reversibility of the inflicted damage varies depending on the stage of the disease. This process is thought to be initiated by changes in nutritional status due to sclerosis of the underlying bone and/or by microdamage as a consequence of biomechanical (over)load (Burr 2004). Related to this, repetitive low-impact injuries and single-event high impact injuries during sports accelerate the development of damage to the articular cartilage matrix (Lohmander et al 2007), putting active young adults at risk for early onset of cartilage degeneration and eventually osteoarthritis at middle age.

During the early stages following cartilage damage, the loss of proteoglycans and a disruption of the collagen network lead to impaired matrix biomechanics as characterized by tissue softening. Softened articular cartilage has a reduced capacity to resist and conduct impact forces during physiological loading, giving rise to surface fibrillation and fissures (Buckwalter et al 2005). Continued loading of this damaged cartilage matrix has a negative influence on disease progression, eventually leading to generalized cartilage degeneration as occurs during osteoarthritis.

Over the past decades, the spectrum of cartilage diagnostics has provided several options to recognize, visualize, quantify and analyze the events involved in the progression from a focal cartilage defect to generalized disease. Clinical signs and symptoms, radiographic analysis, arthroscopy and magnetic resonance imaging (MRI), and newer techniques, like ultrasound, delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC), optical coherence tomography and genetic profiling address different aspects of cartilage morphology and function. This chapter aims to provide an update and insight into cartilage diagnostics for clinical and research purposes, from early matrix damage and degeneration to generalized intra-articular disease with a focus on reliability, clinical value, current status and possible applications.

CLINICAL SYMPTOMS

The most severe cartilage degeneration is usually found in osteoarthritis. Presently, the most frequent clinically applied diagnostic modality for osteoarthritis is signs and symptoms as presented by the patient. Interestingly, the clinical symptoms of osteoarthritis are not related to cartilage degeneration but to other pathological events in

osteoarthritis. Pains, stiffness, functional impairment, crepitus, swelling and restricted movement are the clinical key characteristics of osteoarthritis. Of these, pain is the main reason for the patient to seek help. Although the exact mechanisms by which pain in osteoarthritis is generated remains unknown, nociceptive fibers are only found in the subchondral bone and joint capsule but not in cartilage, it is believed that intra-articular factors released from bone or synovium cause hypersensitivity of related structures like the periosteum, subchondral or marrow bone (Kidd 2006). Concomitant intra-articular hypertension and ischemia due to synovitis could be other sources of joint pain. Also subchondral venous obstruction, resulting in raised intraosseous pressure, is associated with severe degenerative changes in the joint and could be a source of pain at the end stage of the disease (Kidd 2006).

Another typical symptom in osteoarthritis is morning stiffness, which lasts less than 30 minutes, in contrast to inflammatory arthropathy, as defined by the diagnostic criteria of osteoarthritis by the American College of Rheumatology (Altman et al 1986). Osteophyte formation, subchondral bone remodeling and capsular thickening are biological changes that result in functional impairment and difficulties with activities of daily living (Brandt et al 2003).

Clinical characteristics of osteoarthritis are evaluated by several classifications and questionnaires, focusing on symptoms, daily functioning and quality of life, and help to report series of cases or describe the success rate of an intervention (Altman et al 1986; Roos and Lohmander 2003). However, these classifications are not suitable for the diagnosis of cartilage degeneration. This is indicated by the very low sensitivity (20-49 %) of major clinical signs, like pain and morning stiffness, when compared to radiographic scoring systems (Altman et al 1986; Hart et al 1991; Peat et al 2006). In general the clinical characteristics of osteoarthritis poorly reflect the actual degree of degeneration. Notwithstanding, in current daily practice they are the most important reason for a surgeon to decide for arthroplasty once radiographic osteoarthritis has been proven.

RADIOGRAPHIC ANALYSIS OF CARTILAGE

The combination of clinical and radiographic disease characteristics to diagnose end stage cartilage degeneration is commonly used in daily clinical practice. Although cartilage itself is invisible on plain radiography, it can be used to identify some disease-related characteristics. Kellgren and Lawrence introduced the first '*radiological assessment of osteo-arthrosis*'. They described several radiographic features, like osteophytes, periarticular ossicles, joint space narrowing, subchondral pseudocystic sclerotic areas and altered shape of bony ends (Kellgren and Lawrence 1957). Nowadays, these radiographic changes are generally accepted to be hallmarks of severe cartilage degenera-

tion and a representation of osteoarthritis. Despite the large inter- and intra-observer error (8-31 % observer bias), the Kellgren and Lawrence scale is frequently applied for the individual assessment of a patient's disease progression or effect measurement in a clinical tria (Kellgren and Lawrence 1957). More recently developed scoring systems for radiographic cartilage damage, by Altman and colleagues and Nagaosa and colleagues, provide a further sub-categorization of these individual radiographic features and show good intra- and inter-observer reproducibility (Altman and Gold 2007; Nagaosa et al 2000). Despite this, several studies show a poor to moderate correlation between the radiographic characteristics of degenerative cartilage and the actual degree of cartilage damage as determined by arthroscopy (Kijowski et al 2006; Kijowski et al 2006; Wada et al 1998).

Novel developments for the radiographic evaluation of ongoing cartilage degeneration based on computerized measurements of the generally accepted radiographic features might help to standardize measurement of these features and thus form a valuable tool to monitor disease progression or treatment effect in clinical trials (Conrozier et al 2004; Marijnissen et al 2008; Schmidt et al 2005). These computerized measurements show a good inter- and intra-class reliability and correlation (correlation scores varying from 0.50-0.99) to radiographic scoring systems. However, the position of the patient influences the shooting angle of the radiographic image and thus the computerized measurements of the radiographic features. Therefore these analytical algorithms may entail practical problems during the follow-up of patients.

Thus, although the assessment of radiographic characteristics for the diagnosis of osteoarthritis is still frequently applied in daily practice the actual extent of cartilage degeneration shows a poor correlation with these parameters.

ARTHROSCOPY

While clinical signs and radiography will only indirectly suggest cartilage damage and degeneration, arthroscopy introduced the advantage of direct visualization of the actual cartilage damage. Macroscopic signs of matrix damage, fibrillation and softening, can easily be assessed during arthroscopy by surface evaluation and cartilage probing. A disadvantage, however, is the subjective character of these observations. In an attempt to quantify and standardize the arthroscopic evaluation of cartilage damage, several scoring systems (e.g. the SFA and Outerbridge scales), based on size, grade and localization of cartilage damage, have been developed (Dougados et al 1994; Outerbridge 2001). When tested for accuracy, these systems show average to good inter-observer reproducibility (0.52-0.62) and intra-observer reliability (0.66-0.80) but tend to have higher agreement (81 % intra-observer agreement) for severe degenerative lesions

compared to intermediate and lower graded lesions (65 % intra-observer agreement) (Brismar et al 2002; Cameron et al 2003). This suggests that arthroscopic grading may not be suitable for quantitative assessment of early cartilage damage.
Alternative macroscopic scoring systems, like the ICRS- and OAS-score, have also been developed to provide a macroscopic evaluation of regenerative cartilage repair (van den Borne et al 2007). These systems showed good inter- and intra-observer reliability (0.62 and 0.56 ICRS and 0.73 and 0.65 OAS respectively) and can therefore be applied as outcome measure in clinical trials on cartilage regeneration.
Since macroscopic damage as visualized by arthroscopic evaluation, will most likely be irreversible, arthroscopy seems to be a good method of grading severe focal cartilage lesions but has inferior sensitivity for the diagnostic workup of early, matrix-related, cartilage damage.

PRECLINICAL ARTHROSCOPIC TOOLS FOR CARTILAGE EVALUATION

Ultrasonic evaluation of the articular cartilage is, primarily, based on the speed of sound in cartilage. The thickness of articular cartilage, as a representation of the tissue status, has been calculated from the speed and the so called 'time of flight' (Myers et al 1995). However, the reported discrepancy between the speed of sound in healthy (1658-1760 m/s) and degenerated (1567-1600 m/s) cartilage highly influences the thickness measurement (Myers et al 1995; Toyras et al 2002). In an attempt to provide a biomechanical quantification of the cartilage status, ultrasound measurements were combined with indentation tests (Laasanen et al 2002; Saarakkala et al 2004). However, given the variation of the speed of sound in cartilage according to the state of the tissue, resulting in large measurement errors on thickness and biomechanical moduli, the possible clinical application of this mechano-acoustic quantification of articular cartilage can be debated (Toyras et al 2003).
As articular cartilage matrix constituents influence the attenuation and (sub)surface reflections of high frequency ultrasound waves (Agemura et al 1990; Brown et al 2007) more detailed evaluation of ultrasound reflex echoes has been performed to describe the pathological changes during cartilage matrix damage and degeneration. However, the defined quantitative ultrasound parameters showed weak correlations to biochemical scoring (Hattori et al 2005) and were only able to distinguish healthy from severely degenerated samples (Kaleva et al 2008).
Analogous to ultrasound, Optical Coherence Tomography (OCT) departs from reflections of near infrared light instead of sound waves. The resolution of this technique for articular cartilage ranges from 10-15 μm. In addition, cross-sectional images can be derived from up to 2 mm deep into the tissue (Xie et al 2006). The histological fibrillation

index, a measure of surface fibrillation, was shown to correlate well to the OCT-derived fibrillation index (Xie et al 2006). However, even though OCT is able to show structural changes in (sub)surface collagen orientation and disorganization (Bear et al 2009), proteoglycan loss as part of early (traumatic) matrix damage (Huser and Davies 2006) is not likely to be detected. Another, more practical, limitation is the requirement of the OCT probe to be placed exactly perpendicular to the cartilage surface.
Thus both ultrasound and OCT allow for more objective measurements of cartilage quality than simple probing, however, the discriminative quality in the detection of various stages of de- or regeneration should be tested to really determine their additional clinical value.

MAGNETIC RESONANCE IMAGING

The broad spectrum of clinically available and recently developed MRI techniques, scoring systems and sequences allow for a sensitive analysis of cartilage from focal lesions to generalized disease. The fat-suppressed SPGR sequence produces a high cartilage signal and low signal from the adjacent joint fluid and is currently the standard for quantitative morphological imaging of cartilage (Eckstein et al 2006; Gold et al 2009). Semiquantitative measurements of cartilage volume, thickness and surface area, derived from various scanning sequences, show excellent inter- and intra-observer reliability and long term precision errors ranging from 1.4%-3.9%, which make these parameters attractive for longitudinal studies, patient follow-up and diagnostic procedures (Eckstein et al 2006). The availability of higher field strengths, up to 3 T, makes these measurements even more accurate with accuracy errors for 1.5 T field strengths ranging from 11-17 % and from 3-7 % for 3.0 T field strengths (Bauer et al 2006).
Several semiquantitative MRI scoring systems for osteoarthritis have been developed focusing on size and location of the lesions and subchondral, cartilaginous, bone, and meniscal abnormalities. The Knee Osteoarthritis Scoring System (KOSS) has a good overall reproducibility (ICC 0.77), however, a limited reproducibility for cartilaginous and subchondral tissue, ICC 0.64 and 0.63 respectively (Kornaat et al 2005). The inter-observer agreements of the Whole Organ Magnetic Resonance Imaging Score (WORMS) are good with an ICC for cartilage loss that is even greater than 0.90 (Peterfy et al 2004). Overall, these scoring systems provide good quality for evaluation of the osteoarthritic status of the joint. However, evaluation of a single case will take approximately 45 minutes and therefore limits the clinical implementation. The scoring system developed by Marlovits et al (Marlovits et al 2004), showed good inter-observer reliability (ICC > 0.80 for 8 out of 9 features) and significant correlations to subscales of the Knee injury and

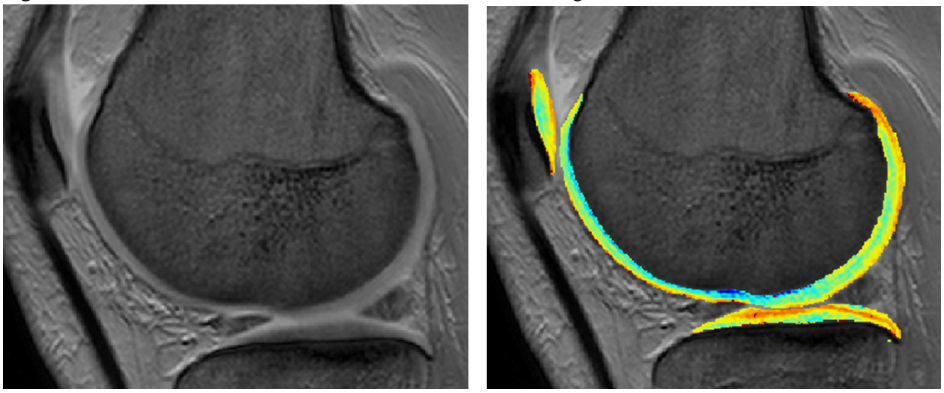
Osteoarthritis Outcome Score (KOOS), although the number of included patients was limited (Marlovits et al 2006).

Although current MRI sequences and scoring systems allow for good diagnostic accuracy for moderate to severe cartilage degeneration when compared to radiography, newer techniques have been developed to focus on imaging of cartilage constituents as possible tools in the detection of early cartilage damage. Degradation of the collagen matrix in cartilage enhances the mobility of water protons which can be sensitively detected *in vivo* by quantitative MRI T2 relaxation time (Liess et al 2002) Water proton mobility (measured by quantitative T2 mapping) also seems to reflect collagen architecture and density of articular cartilage (Fragonas et al 1998; Nieminen et al 2001).

An MRI-based technique that enables quantification of proteoglycans is delayed Gadolinium Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC), which is based on the negative, proteoglycan related, charge (also called fixed charged density (FCD)) in cartilage (Gray et al 2008). Intravenously administered diethylenetraminepentaacetic acid (Gd-DTPA^{2-}) is distributed at high concentrations in cartilage areas with low proteoglycan content and vice versa and therefore allows for mapping of proteoglycan distribution in articular cartilage (Figure 1). This technique shows good *in vivo* reproducibility (Multanen et al 2009) and shows good correlations (correlation scores 0.95-0.96) to the, biochemically determined, proteoglycan content *in vitro* (Bashir et al 1999; Xia et al 2008). A decrease of dGEMRIC signal has been observed after posterior cruciate ligament rupture when compared to the pre-trauma signal, indicating a disturbance of the cartilage matrix after knee trauma (Young et al 2005). In addition, significant correlations have also been described between the proteoglycan content in synovial fluid and T_{1GD} signal in the acute phase after anterior cruciate ligament rupture (Tiderius et al 2005). This illustrates the potential of dGEMRIC for early disease tracking and follow-up.

In addition to dGEMRIC, the $T_{1\rho}$ MRI technique also provides a quantitative map of the proteoglycan distribution in articular cartilage. This technique is based on water-

Figure 1 Conventional MRI and dGEMRIC scan of articular cartilage.



proteoglycan interactions and –content (Wheaton et al 2005). The advantage of the $T_{1\rho}$ MRI technique over dGEMRIC is that it does not need intravenous administration of contrast agents. *In vitro* studies show a strong correlation (correlation scores 0.92-0.98) between proteoglycan content and changes in $T_{1\rho}$ relaxation times (Akella et al 2001; Wheaton et al 2005).

Another technique that also uses the FCD to visualize proteoglycan loss from articular cartilage is sodium-MRI. The loss of negatively charged proteoglycans results in a lower FCD and induces a loss of positively charged sodium ions from the tissue which can be visualized by quantitative ^{23}NaMRI. This technique can only be performed at higher field strengths ($\geq$3 T) but is promising in detecting early proteoglycan loss from articular cartilage (Borthakur et al 2006; Shapiro et al 2002; Wheaton et al 2004).

Although conventional MRI sequences and scoring systems offer a good analysis of all the structures within the joints, they are only able to detect an articular cartilage defect when it is actually present, making them less suitable for the detection cartilage matrix disturbances as a disease stage preceding focal lesions. Newer experimental MRI techniques, like dGEMRIC, $T_{1\rho}$ MRI and sodium MRI, do provide a, validated, quantitative measurement of specific articular cartilage matrix constituents making them promising tools for the evaluation of early damage to articular cartilage.

MOLECULAR MARKERS OF DEGENERATION

Besides the development of imaging techniques and arthroscopic devices to quantify cartilage matrix damage and degeneration at different stages of the disease, molecular markers of damaged and degenerating articular cartilage have been studied in serum, urine and synovial fluid to provide for more sensitive hallmarks of degenerative cartilage disease (Lohmander 1994).

The irreversibility of articular cartilage damage is hypothesized to coincide with a phenotypic shift of articular chondrocytes. This shift may result in inappropriate expression of genes encoding for matrix constituents and eventually to decreased matrix stability (Stoker et al 2006). Elevated levels of keratan sulphate and COMP were found not only in serum of patients with radiographic OA, but also in serum of patients with recent joint trauma, such as anterior cruciate ligament rupture or medial meniscectomy (Pruksakorn et al 2009; Wakitani et al 2007). In joint trauma patients, KS was also shown to be elevated in the synovial fluid (Belcher et al 1997). Collagen neoepitopes have mainly been used as OA markers (Elsaid and Chichester 2006). In patients with knee pain, urine and serum levels of various collagen neoepitopes generated by protease cleavage, among which C-telopeptide of collagen type-II, collagen type-II cleavage neoepitope and collagen type-I and -II cleavage neoepitopes C1,2C, have been shown to correlate with the sever-

ity of radiographic OA (Cibere et al 2009). Inversely, high serum levels of propeptide collagen type-II were inversely correlated to OA (Cibere et al 2009).
Interestingly, not only cartilage degeneration may lead to changes in degradation parameters. Concentrations of MMP-1, MMP-3, procollagen type-I C-peptide (PICP), tissue inhibitor of metalloproteinase-1 (TIMP), PGs and deoxypyridinoline (DPD) showed a typical decrease in synovial fluid during the first year after ACI surgery (Schneider et al 2003), suggesting an inhibition of the degenerative process upon treatment. In addition to the extracellular matrix genes and degrading proteases, genes closely related to chondrocyte differentiation and chemokine and endothelin pathways have been related to early degenerative changes in human chondrocytes (Tchetina et al 2005). Also various cytokines have been implicated to be involved in cartilage degeneration during OA. However, synovial fluid levels of most infammatory cytokines are low or undetectable and it is not known to what extent serum cytokine levels are affected, which renders them unsuitable as diagnostic markers (Pratta et al 2006; Punzi et al 2005). In early joint degeneration, levels of interleukin-15 (IL-15) were found to be increased in the synovial fluids from patients with meniscus tears and cartilage thinning (Scanzello et al 2009). This area of research still needs expansion.
For a more extensive update on the ever growing field of molecular markers in cartilage disease, the author would like to further refer the reader to some excellent reviews (Abramson and Krasnokutsky 2006; Elsaid and Chichester 2006; Rousseau and Delmas 2007).

CONCLUSION

The various techniques available for the diagnosis of cartilage disease are based on imaging, biochemical and biomechanical characteristics of articular cartilage. Technical improvement and increasing knowledge of disease initiation and progression can be expected to positively influence current diagnostic modalities and form a basis for the development of new procedures. It has to be kept in mind however, that the capacity for sensitive diagnosis of cartilage status in itself will not improve treatment of early cartilage disease and even if new treatments can be developed, they may not be applied as long as a patient does not have any clinical signs and articular cartilage appears normal at regular arthroscopy. These aspects are important to consider in future development of diagnostic and therapeutic strategies in clinical practice.

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**Belangrijk: Probeer uitsluitend antwoord met betrek
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Symptomen

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Chapter 9

Validation of the Knee Injury and Osteoarthritis Outcome Score (KOOS) for the treatment of focal cartilage lesions

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ABSTRACT

This chapter aimed to validate the Knee Osteoarthritis Outcome Score (KOOS) for the treatment of focal cartilage lesions.

A total of 40 patients (mean age 35 ± 12 years) treated for a focal cartilage lesion in the knee were included. Test-retest data were collected with an intermediate period of 2 days. Patients were asked to complete the Dutch KOOS and complementary questionnaires (SF-36, Lysholm, EQ-5D) to evaluate the clinimetric properties of the KOOS in terms of internal consistency (Crohnbach's alpha), reliability (Intra-Class-Correlation (ICC) and Bland Altman plots), construct validity (Spearman's Rank correlation), floor and ceiling effects and responsiveness.

The Crohnbach's alpha of the KOOS subdomains and total score ranged from 0.74-0.96. The overall ICC of the KOOS was 0.97 while the subscales ranged from 0.87-0.95. The Bland Altman plots showed a small individual variance between the two assessments in time. Spearman's Rank correlations between the subscales of the KOOS and representative subscales of the SF-36, Lysholm and EQ-5D were high to moderate ranging from 0.43 to 0.70. We observed no floor effect while the largest observed ceiling effect was 10.3%. The responsiveness was moderate to large with the effect size ranging from 0.70 to 1.32 and the SRM 0.61 to 0.87.

This chapter illustrates the validity and reliability of the KOOS in measuring the clinical condition of patients after treatment of focal cartilage lesions and provides a basis for the use of the KOOS for future clinical research in cartilage repair.

INTRODUCTION

The growing activity in the field of regenerative cartilage therapy creates a need for validated outcome tools. Several instruments have been developed to measure the outcome of such treatment in both research and clinical setting. For example, the International Cartilage Repair Society (ICRS) Score and the Oswestry Arthroscopy Score (OAS) have shown to be useful tools for the macroscopic evaluation of cartilage repair (van den Borne et al 2007). However, patient-reported, self-administered questionnaires are preferred as instruments for the assessment of clinical outcome to prevent from observer administered bias (Roos et al 1998). The Western Ontario and McMaster Universities Index (WOMAC) is a frequently used disease-specific questionnaire to measure the treatment effect in patients with osteoarthritis (Bellamy et al 1988). However, the population presenting with focal cartilage lesions is generally younger and more active as compared to patients with osteoarthritis. Therefore, the Knee injury and Osteoarthritis Outcome score (KOOS) would fit this population better. The KOOS was developed as an extension of the WOMAC and designed to assess short-term and long-term symptoms and function in younger and/or more active patients with knee injuries, cartilage damage or different stages of osteoarthritis (OA) (Roos et al 1998). Validated language versions are available for use in Sweden, Germany, the United States, France, Singapore, Iran and the Netherlands (de Groot et al 2008; Ornetti et al 2008; Roos et al 1998; Salavati et al 2008; Xie et al 2006). The KOOS has been validated for several stages of osteoarthritis (Ornetti et al 2008; Salavati et al 2008; Xie et al 2006) and for orthopaedic interventions such as anterior cruciate ligament reconstruction (Roos et al 1998), meniscectomy (Roos et al 1998) and total knee replacement (Roos and Toksvig-Larsen 2003). Recently, short forms of the WOMAC and KOOS have also been validated for patients with different stages of OA (Perruccio et al 2008; Yang et al 2007).

Although already accepted and applied in several clinical trials to measure the outcome after treatment of focal cartilage lesions, the KOOS has not yet been validated for this patient population (Ossendorf et al 2007; Robertson et al 2007;Saris et al 2008). The aim of the present study was to evaluate the clinimetric properties of the KOOS for patients with focal cartilage defects, eligible for cartilage repair.

METHODS

Patients

Between February and April 2008 a total of 60 patients were invited by phone to participate in this study. All patients had been treated for a symptomatic focal cartilage lesion by either autologous chondrocyte implantation or microfracturing between February

2002 and July 2006 at the University Medical Center Utrecht, the Netherlands. The study was approved by and conducted according to the guidelines of the Ethics committee at the University Medical Center of Utrecht.

Study design and Questionnaires

Patients received two sets of questionnaires (marked as Day 1 and Day 3) by mail, each containing the Dutch KOOS and complementary questionnaires (Short Form-36, Lysholm, EuroQol-5D) which previously proved to measure similar constructs (Aaronson et al 1998; Rabin et al 2001; Smith et al 2008). Each patient was instructed to fill out the first set of questionnaires and immediately return them to the University Medical Center Utrecht using a pre-stamped envelope. Patients were asked to repeat the assessments with a two days interval (Marx et al 2003). Each patient was instructed by the investigator to open the second set of questionnaires two days after the first assessment. Scores which were not completed conform the set time-interval for the test-retest (both returned on the same day or with a > 4 day interval) or those with two or more missing items in any of the questionnaires were excluded from further analysis.

The Knee injury and Osteoarthritis Outcome Score (KOOS) is a patient-based, site specific, questionnaire that was developed to be used for short- and long-term follow-up of knee injury and knee osteoarthritis. The KOOS comprises five separately scored subdomains, based on 42 individual items. The subdomains are Symptoms (7 items), Pain (9 items), Activities of Daily Living (ADL) (17 items), Function in sport and recreation (5 items) and knee-related quality of life (QoL) (4 items). Each item is scored from 0 (least severe) to 4 (most severe). For each subdomain as well as the total KOOS score the score was normalized to a 0–100 scale with 100 being the best possible outcome, as previously described (Roos and Lohmander 2003).

The Short Form-36 (SF-36) is a widely used patient-based generic health-related quality-of-life questionnaire containing 36 items measuring health in eight domains. These include physical functioning, role limitations due to physical health problems, role limitations due to emotional problems, social functioning, vitality, mental health, bodily pain and general health perceptions. The Dutch version has been validated by Aaronson et al (Aaronson et al 1998).

The EuroQuol-5D (EQ-5D) is a questionnaire to measure health-related quality-of-life on the day of the assessment and contains five domains, namely, mobility, self-care, usual activities, pain/discomfort and anxiety/depression and a visual analogue scale (VAS) for overall health. The EQ-VAS is a vertical scale on which the subject rates their overall health from 0 to 100 (worst to best imaginable respectively) (Rabin et al 2001).

The Lysholm knee scoring scale is an eight-item questionnaire designed for the assessment of symptoms and functional disabilities resulting from a ligamentous injury. The items include pain, instability, locking, swelling, limping, walking stairs, squatting

and keeping support. Scores are calculated into one score from 0-100 (100 indicating normal knee function). Recently, the Lysholm knee scoring scale has been validated as an outcome measure for knee chondral damage (Smith et al 2008).

Evaluation of the clinimetric properties

Test-retest reliability of the KOOS subdomains and total score was determined with an interval of two days (Marx et al 2003), assuming the probability of a significant change in symptoms would be absent and the intermediate time too long for the patient to remember the exact previous answers. The test-retest reliability was measured with the intraclass-correlation-coefficient (ICC) with 95 % confidence interval, along with the smallest detectable difference (SDD). An ICC equal or superior to 0.70 is considered acceptable for test-retest reliability while an ICC of more than 0.80 represents excellent reliability (Ornetti et al 2008; Xie et al 2006). The SDD indicates the smallest change that can be distinguished from the measurement error (mean change ± 1.96 standard deviation change) (Ornetti et al 2008). In addition, the internal consistency was assessed and Bland and Altman plots were obtained. The internal consistency was measured by the Cronbach's alpha. A Cronbach's alpha coefficient equal or superior to 0.7 is generally considered to be acceptable (Cronbach and Warrington 1951). For the Bland and Altman plots the differences between the first and second assessment were plotted against the mean of the two assessments, describing the distribution of patients along the scoring scale within the 95 % limits of agreement (Bland and Altman 1986).

Construct validity was measured by comparing the subdomains of the KOOS with *a priori* hypothesized corresponding domains of the complementary questionnaires (SF-36, Lysholm, EQ-5D). For all *a priori* hypotheses the Spearman's rank correlation coefficients were obtained. Correlations of <0.35, 0.35-0.5 and >0.5 were considered as weak, moderate and strong respectively. *A priori* moderate-strong hypothesis of domains measuring similar constructs were generated according to theoretical hypothesis and the related literature (Roos et al 1998; Roos and Toksvig-Larsen 2003; Smith et al 2008): (1) KOOS symptoms with SF-36 physical functioning; (2) KOOS pain with SF-36 bodily pain and EQ-VAS; (3) KOOS ADL with the complete SF-36 questionnaire; (4) KOOS sport and recreation with the Lysholm knee scoring scale; (5) KOOS QoL with EQ-5D.

The feasibility was assessed by the floor and ceiling effects. Floor and ceiling effects were considered to be present if 15 % of patients scored the highest or lowest possible scores (Ornetti et al 2008).

The responsiveness was evaluated in another cohort of 36 patients of a recently published randomised trial comparing characterized chondrocyte implantation to microfracturing (Saris et al 2008). The included patients completed the KOOS and the Marx Activity Rating Scale (ARS) (Marx et al 2001) at baseline and 36 months follow-up. The standardized response mean (SRM) and Effect Size (ES) were calculated as a measure of

responsiveness. ES <0.50, <0.80 and >0.80 were respectively considered small, moderate and large.

The clinimetric properties were analysed with SPSS statistical software version 15.0 (SPSS inc. Chicago, IL). A p-value of $p<0.05$ was considered to represent a statistically significant difference.

RESULTS

Patients

Out of the initially 60 contacted patients a total of 46 (response 77%) was willing to participate. An additional 6 patients were excluded because of missing individual questionnaire items (n=4) and an insufficient response (n=2) conform the test-retest response characteristics. From the resulting 40 patients (mean age 35 ± 12 years, range 18-55; 70% men), 20 had been treated with autologous chondrocyte implantation while the other 20 had received microfracturing. The average postoperative time was 32 months and 87% of the patients had been treated between January 2005 and July 2006.

Reliability

Test-retest reliability for the KOOS as determined by the ICC was 0.97 for the total score whereas the ICC's for the subdomains ranged from 0.87-0.95 (Table 1). The SDD for the subdomains ranged from 4 to 12 points (Table 1). The KOOS internal consistency, as determined by the Crohnbach's alpha, was good for the individual subdomains with

Table 1 Mean KOOS scores and reliability of Dutch KOOS subdomain and total score.

KOOS Subdomain	Mean KOOS (SD) First assessment	Mean KOOS (SD) Second assessment	ICC (95% CI)	SDD
Symptoms	74 (17)	75 (17)	0.95 (0.90- 0.97)	5
Pain	77 (15)	77 (15)	0.92 (0.86- 0.96)	6
Function ADL	84 (14)	86 (12)	0.87 (0.77- 0.93)	7
Sport/ recreation	55 (26)	58 (25)	0.89 (0.81- 0.93)	12
QoL	49 (23)	53 (22)	0.95 (0.91- 0.97)	7
Total score	74 (15)	76 (14)	0.97 (0.93- 0.98)	4

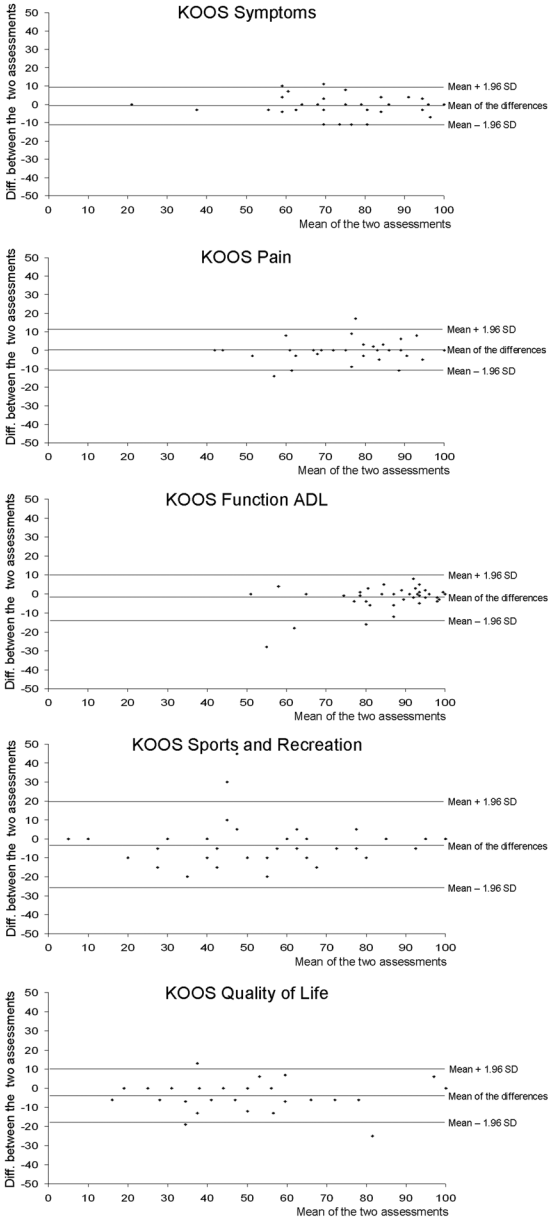
The ICC represents the IntraClassCorrelation whereas the SDD is the Standard Deviation of the Difference, (n=40).

Table 2 Internal consistency of the Dutch KOOS subdomains and total score, (n=40).

KOOS subdomains	Cronbach's alpha coefficient
Symptoms	0.74
Pain	0.88
Function ADL	0.95
Sport/ recreation	0.89
QoL	0.90
Total score	0.96

Cronbach's alpha ranging from 0.74-0.95 (Table 2). The Bland and Altman representations showed a small individual variance between the two assessments for each subdomain of the KOOS (Figure 1).

Figure 1 Bland and Altman plots

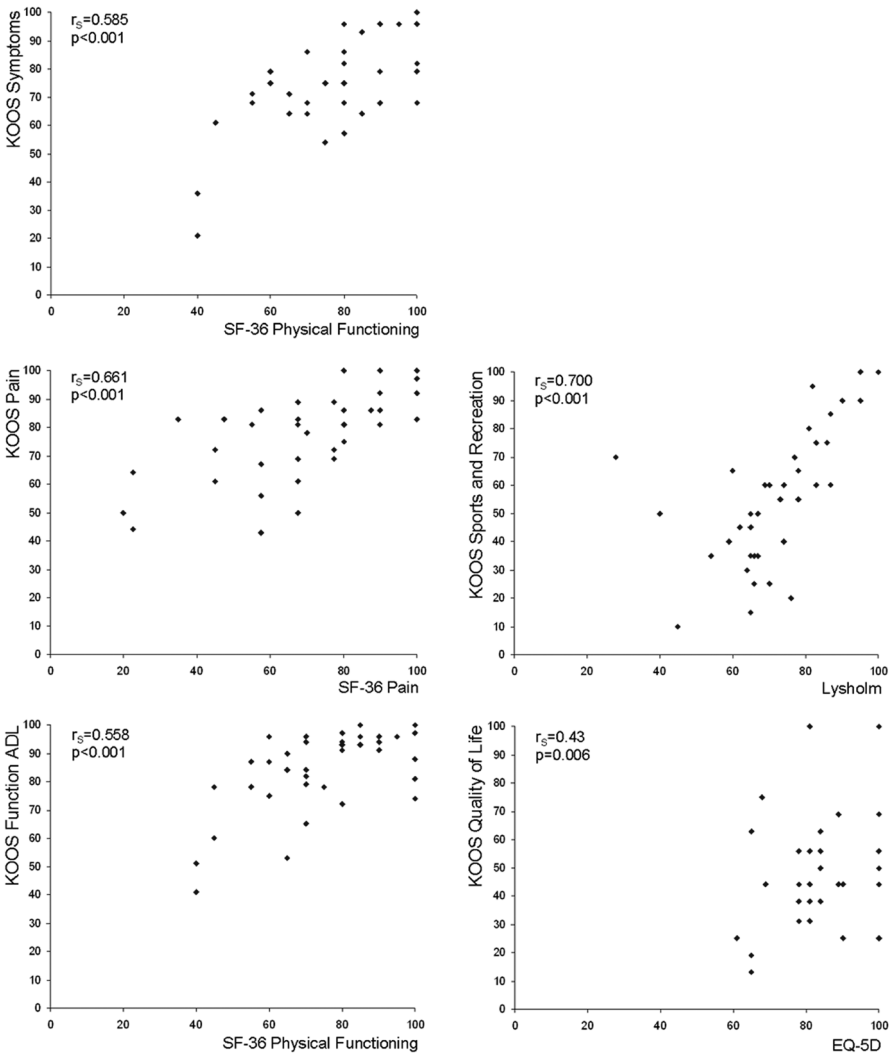


Bland and Altman representations showing a small individual variance between the two assessments for each subdomain of the KOOS (n=40).

Validity

Construct validity was moderate to high with Spearman's Rank correlations between the subdomains of the KOOS and representative subdomains of the SF-36, Lysholm and EQ-5D ranging from 0.43 to 0.70 (Figure 2). Moderate correlations were found for the

Figure 2 Spearman's Rank correlations



Spearman's Rank correlations for the *a priori* hypothesis between the KOOS subscales and similar other validated questionnaires showing acceptable correlations varying from 0.43-0.70 (n=40).

QoL subdomain compared to EQ-5D and VAS scores (r_s =0.43 and 0.44 respectively). The moderate correlations were statistically significant at the p=0.006 level (Figure 2). Strong correlations were observed for the *a priori* hypotheses; KOOS symptoms and SF-36 physical functioning (r_s = 0.585), KOOS pain and SF-36 pain (r_s = 0.661), KOOS ADL and SF-36 physical functioning (r_s = 0.558) and KOOS sport and Lysholm (r_s = 0.700). All strong correlations were statistically significant with p<0.001(Figure 2). No *a priori* unexpected weak correlations (r_s <0.5) were found. Floor and ceiling effects were absent (Table 3).

Table 3 Floor and ceiling effects of the Dutch KOOS subdomains and total score, (n=40).

KOOS Subdomains	Floor effects	Ceiling effects
Symptoms	0%	2.6%
Pain	0%	5.1%
Function ADL	0%	7.7%
Sports / recreation	0%	7.7%
QoL	0%	10.3%
Total score	0%	2.6%

Table 4 KOOS scores for microfracturing vs. ACI.

KOOS Subdomains	Mean KOOS (SD) Microfracturing	Mean KOOS (SD) ACI	p-value
Symptoms	74 (21)	74 (12)	0.96
Pain	78 (16)	76 (15)	0.69
Function ADL	85 (16)	83 (13)	0.76
Sport/ recreation	60 (27)	51 (25)	0.31
QoL	49 (23)	49 (23)	0.98
Total score	75 (17)	73 (13)	0.66

The p-value was calculated by an independent samples-t test, (n=40).

Table 5 KOOS responsiveness vs. ARS responsiveness.

Responsiveness	ES	SRM
KOOS Symptoms	0.72	0.61
KOOS Pain	0.82	0.71
KOOS Function / ADL	0.70	0.75
KOOS Sport / recreation	0.98	0.87
KOOS QoL	1.32	0.76
KOOS Total score	0.91	0.85
ARS	0.76	1.10

ES (effect size) represents the mean change in score from baseline to 36 months follow-up divided by the standard deviation of the pre-operative score. SRM (standardized response mean) indicates the mean change in score from baseline to 36months follow-up divided by the standard deviation of the mean change. An effect of <0.50, <0.80 and ≥0.80 were considered small, moderate and large respectively, (n=40).

The KOOS evaluation showed similar outcomes for both Autologous Chondrocyte Implantation and Microfracturing patients (Table 4).

Responsiveness

The responsiveness (Table 5) was moderate to large, with the ES ranging from 0.70 to 1.32 and the SRM ranging from 0.61 to 0.89, and showed a similar range as the ARS score (ES 0.76, SRM 1.10). The Activity in Daily Living subdomain showed the weakest responsiveness (moderate ES 0.70) while the Function in Sports and Recreation and QoL subdomains showed large responsiveness (ES 0.98 and 1.32 respectively).

DISCUSSION

This study evaluated the clinimetric properties of the KOOS for a cartilage repair population to validate the KOOS as an instrument to measure the clinical outcome after the treatment of a focal, symptomatic cartilage defect in the knee. This study clearly demonstrates the validity and reliability of the (Dutch) KOOS after the treatment of focal cartilage lesions, as shown by the good internal consistency, moderate to high construct validity and excellent test-retest reliability. Given the fact that language validated KOOS versions provide similar outcome for several patient populations we feel that these language versions of the KOOS are suitable instruments to measure clinical outcome after the treatment of focal cartilage lesions.

The results for the Spearman's Rank correlations supported the hypothesized good construct validity. Each subdomain of the KOOS showed strong correlations with corresponding domains, except for QoL, which only showed a moderate correlation to the EQ-5D. This is most likely due to a difference between the measured knee-related QoL (KOOS) and general health-related QoL (EQ-5D). This idea is supported by the overall higher scores obtained by the EQ5D. Although the Lysholm Knee Scoring Scale was originally designed to assess ligament injuries of the knee, it proved to demonstrate acceptable clinimetric performance for outcomes assessment of various chondral disorders of the knee (Smith et al 2008). This was supported by the strong correlation between the KOOS subdomain Function in Sports and Recreation and the Lysholm scale obtained in our study. However, suboptimal performance of some subdomains of the Lysholm scale for outcome assessment of various chondral disorders of the knee have been described as well (Kocher et al 2004).

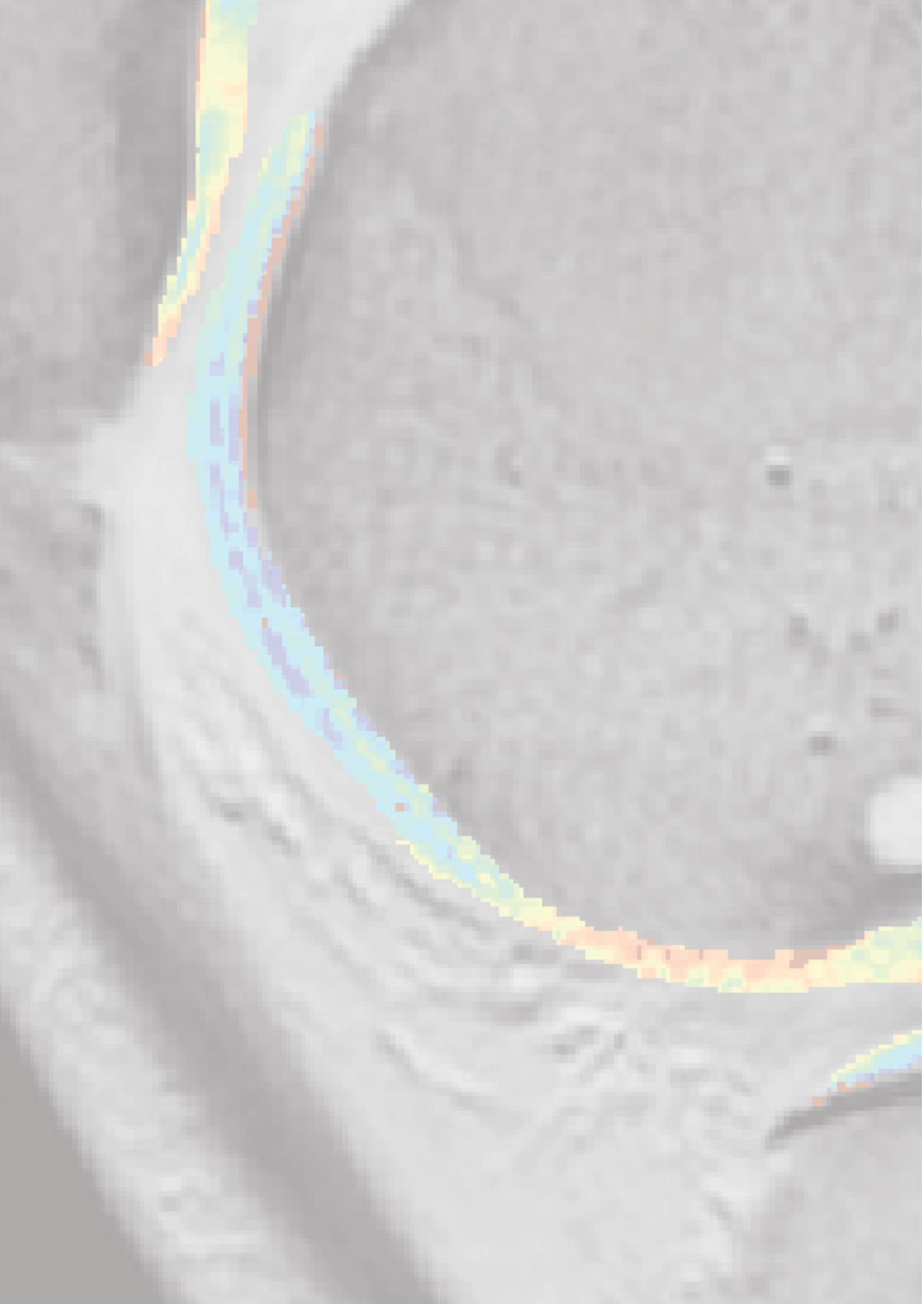
The KOOS has consistently shown acceptable responsiveness for different populations (Ornetti et al 2008; Roos et al 1998; Roos and Toksvig-Larsen 2003). In our study, we demonstrated relatively good responsiveness indicating the KOOS to be capable of measuring clinical improvement in patients who have been treated for a focal cartilage

lesion of the knee. The moderate ES score of the KOOS subdomains is most likely a characteristic of the treatment for focal cartilage lesions instead of moderate responsiveness as the ARS score showed a similar result.

Since there has been a steady increase in clinical research activity on the repair of focal cartilage lesions in the previous years, the field needs a reliable and detailed understanding of the clinical outcome. This will play an important role in assessing the effectiveness of the therapy, and facilitate its further development. The KOOS consistently proved to be a valid instrument in different languages, including Dutch, for the quantification of osteoarthritis or the success of specific orthopaedic interventions. Recently, the KOOS was compared to the International Knee Documentation Committee (IKDC) form to determine which instrument better reflected the symptoms and disabilities of the cartilage repair patient (Hambly and Griva 2008). However, they did not specify the studied cohort of articular cartilage repair patients and lacked a validation of the questionnaires used. To our knowledge this is the first study to validate the KOOS in a focal articular cartilage repair cohort. This can provide a worldwide instrument for the quantification of the clinical outcome for this patient population and increase possibilities for the comparison between (future) clinical trials.

Comparison of the KOOS scores in our study group to age matched population-based reference data (Paradowski et al 2006) shows a lower score for the cartilage repair group. This indicates that the instrument is capable to discriminate between healthy subjects and patients after cartilage therapy.

Based on the clinimetric properties presented in the present study we conclude that the KOOS questionnaire is a valid instrument to measure the clinical condition of patients undergoing treatment of a focal cartilage lesion. This study provides a basis for the use of the KOOS questionnaire in future clinical trials on cartilage repair and as a valid patient-reported, site-specific instrument in daily clinical practice. A further evaluation of the clinimetric properties in sub-groups, such as age and gender, would be of great value to provide self-administered questionnaires for patient specific sub-populations.



Chapter 10

Delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) can be effectively applied for longitudinal cohort evaluation of articular cartilage regeneration

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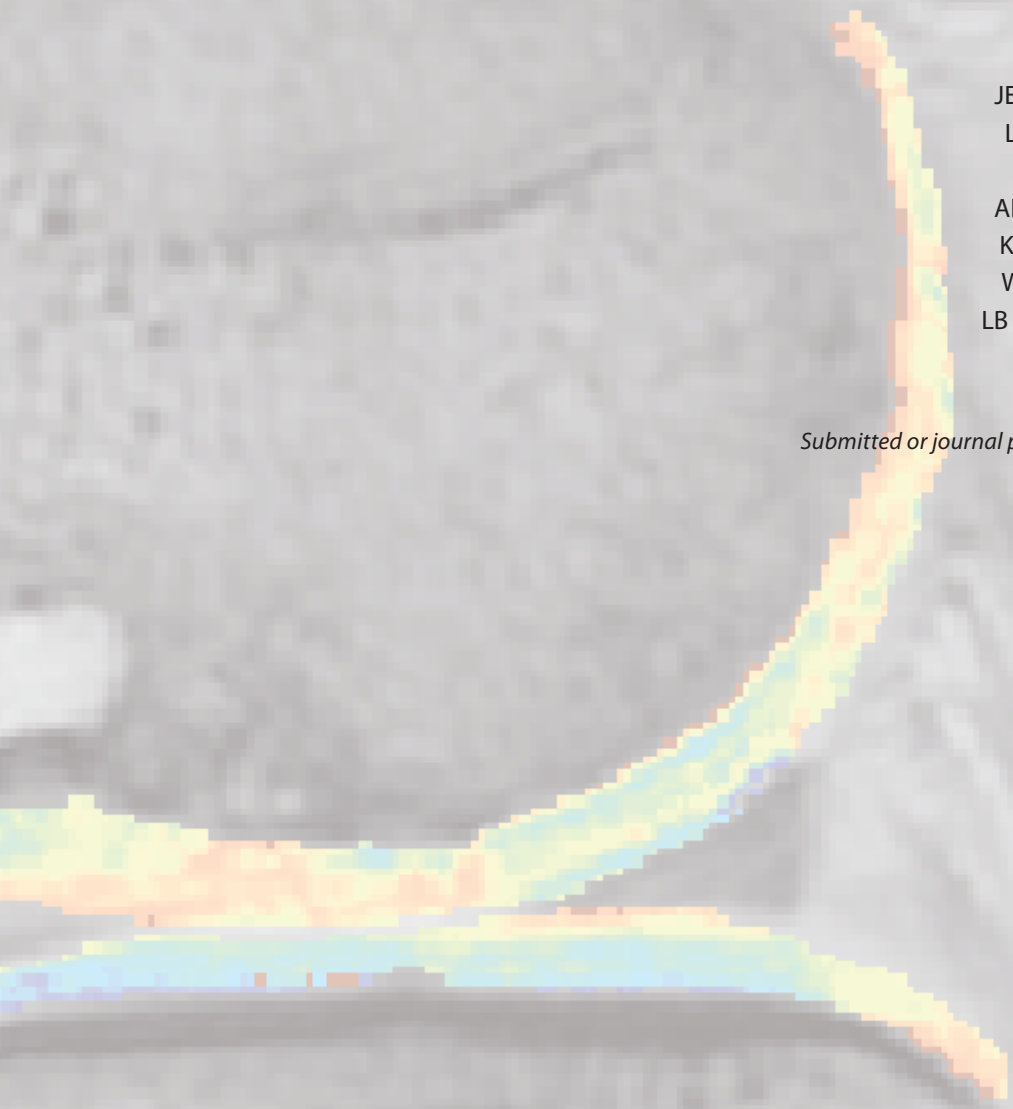


ABSTRACT

This chapter had two complementary aims; to examine the applicability of dGEMRIC for monitoring of the quality of tissue regeneration and joint degeneration when treating focal defects in the knee. Also, specific patient and defect characteristics were evaluated for their influence on tissue regeneration.

A total of 31 patients were examined before and after regenerative cartilage therapy for a focal cartilage lesion. DGEMRIC scans, KOOS and Lysholm questionnaires were obtained before surgery and at 3 and 12 months follow-up. Scanning took place on a 1.5-T MRI scanner ninety minutes after administration of Magnevist®. Six regions of interest (ROIs) were defined in which the average T1gd was determined after voxelwise fitting of the dGEMRIC signal equation to the data acquired at 5 inversion times. Changes in T1gd per ROI and questionnaire scores over time were tested using a Repeated-Measures ANOVA with posthoc Bonferroni test. Regression analysis was performed to evaluate the relation between changes in the T1gd at the treated defect and the T1gd in other joint compartments. Also, the relation between specific patient and defect characteristics and tissue regeneration was evaluated using multiple regression analysis.

Clinical scores and dGEMRIC indices showed a statistically significant improvement at 12 months follow-up. Most interestingly, a direct relationship ($p<0.007$) was found between improvement of T1gd at the 'defect' site and the T1gd at other locations in the joint (B-values 0.567-0.787). Patient age (<30 years) and defect size (< 3 cm^2) were shown to positively influence the T1gd of the cartilage in the whole joint.

This chapter showed that dGEMRIC is useful for the follow-up of cartilage regeneration. In addition, defect regeneration, patient age and defect size influenced the cartilage quality of the whole joint following defect treatment. These findings validate the use of dGEMRIC for noninvasive evaluation of the effects of cartilage regeneration.

INTRODUCTION

Focal articular cartilage lesions in the knee are frequently treated by microfracture or autologous chondrocyte implantation (ACI). Clinical outcome in cartilage surgery is partly determined by adequate tissue regeneration, as exemplified by the deterioration of clinical success, and eventually treatment failure, that is related to inadequate tissue regeneration (Nehrer et al 1999). Also, good structural repair at short-term follow-up showed to result in good clinical outcome at later time points (Saris et al 2008; Saris et al 2009).

In clinical trials, the success of cartilage regeneration is usually determined by histological evaluation of regenerated tissue obtained from an additional cartilage biopsy from the newly formed tissue. Histological evaluation mainly focuses on the tissue organization and the presence of articular cartilage matrix constituents such as collagen type II and glycosaminoglycans (GAGs). The disadvantages of a cartilage biopsy, and the main reasons for which it has not been introduced as a standard protocol in clinical practice, is the invasive nature of the procedure and the fact that it only provides local information. Therefore, a non-invasive method to determine tissue organization and to assess the distribution of relevant articular cartilage matrix proteins would be of great value in the evaluation of tissue regeneration.

The non-invasive MR imaging technique called dGEMRIC (delayed Gadolinium Enhanced MRI of Cartilage) can be used to visualize the distribution of GAGs in articular cartilage (Bashir et al 1999). This technique is based upon the fact that the negatively charged ions of the T1-shortening contrast agent gadolinium diethylene triamine pentaacetic acid (Gd-DTPA2-, Magnevist) distribute inversely proportional to the concentration of the also negatively charged GAGs in articular cartilage. As the local value of the longitudinal relaxation time after the administration of gadolinium-DTPA (T1gd) depends on the intrinsic T1 of the tissue and on the concentration of contrast agent, mapping T1 values in tissue allows a visualization of the contrast agent uptake, which also reflects the distribution of GAGs. T1gd maps are calculated from dGEMRIC scans acquired using 5 different inversion delay times using a curve fitting method (Bashir et al 1999; McKenzie et al 2006). In areas inside cartilage with high uptake of Gd-DTPA2-, and thus low amounts of GAGs, the resulting T1gd will be low, and vice versa. Using this, the dGEMRIC technique, by means of the T1gd, provides an indirect measure of the GAG content and distribution in articular cartilage. This is demonstrated by the good correlation that was found between biochemically determined GAG contents and the related T1gd times in *ex vivo* studies (Bashir et al 1999; Xia et al 2008). In addition, it was shown that the dGEMRIC technique can be used to evaluate the quality of articular cartilage after osteochondral autologous transplantation, high tibial osteotomy and matrix-assisted ACI (Domayer et al 2010; Parker et al 2011; Pinker et al 2008; Shirai et al 2011; Vasiliadis et al 2010). To

our knowledge, in only one study the T1gd values measured in a focal cartilage lesion were compared before and 1 year after matrix-associated ACI. No significant differences between the T1gd values obtained at both time points were found (Pinker et al 2008). However, the main outcome parameter of that study was to evaluate the zonal distribution of GAGs, using dGEMRIC, in normal and repair tissue. Therefore, the study may have been underpowered (n=15) to show statistically significant T1gd improvement between the preoperative and postoperative scans.

In addition to the availability of techniques evaluating the outcome of defect treatment, it is becoming increasingly evident that its success is directly dependent on patient characteristics (de Windt et al 2009). Factors such as age and gender of the patient and size, age and location of the focal lesion were shown to influence clinical outcome after regenerative cartilage therapy (de Windt et al 2009). However, it is not known to what extent these characteristics also affect the biological repair response. Further insight into patient characteristics influencing this process could help steering the therapeutic decisions for treatment of focal cartilage lesions towards optimal benefit for the individual patient.

Therefore, in this study we evaluated the applicability of dGEMRIC for monitoring of the quality of cartilage regeneration when treating focal defects. We also evaluated to what extent this therapy influences the cartilage quality in the whole knee. Also, specific patient and defect characteristics were evaluated for their influence on tissue regeneration.

MATERIAL AND METHODS

General study outline and patient population

This study was conducted with approval of the institutional ethical committee under protocol number 08-022/E. Patients with a focal articular cartilage lesion, confirmed by MRI, indicated for either microfracture, (M)ACI, ChondroCelect or Chondron treatment (Choi et al 2010) were eligible for inclusion in this study. Patients with general contraindications for MRI scanning, a known allergic reaction to gadolinium-containing contrast agents or with a history of kidney pathology were considered not eligible for inclusion. If, during surgical treatment, the treating physician found that the focal lesion or other cartilage surfaces were not suitable to receive any of the abovementioned treatments, the patient was excluded from the study. From April 2009 – March 2010 a total of 40 patients diagnosed with a symptomatic (osteo)chondral focal articular cartilage lesion met the inclusion criteria and were willing to participate in this study. The study procedures and risks were explained and, after a minimum of 14 days, informed consent was obtained by a physician not involved in the diagnostic and therapeutic process (JEJB).

Table 1 Baseline characteristics.

		Patients (n=31)
Gender		
	Male n(%)	23(74%)
	Female n(%)	8(26%)
Age mean(years) ± SD		36 ± 11
	<30y n(%)	12(39%)
	>30y n(%)	19(61%)
Type of treatment		
	MACI/CCI n(%)	12(39%)
	MF n(%)	12(39%)
	Chondron n(%)	7(22%)
Defect age* mean(months)±SD		24 ± 17
	<2y n(%)	12(50%)
	>2y n(%)	12(50%)
Defect size mean(cm^2) ± SD		4 ± 2
	<3 cm^2	12(39%)
	>3 cm^2	19(61%)

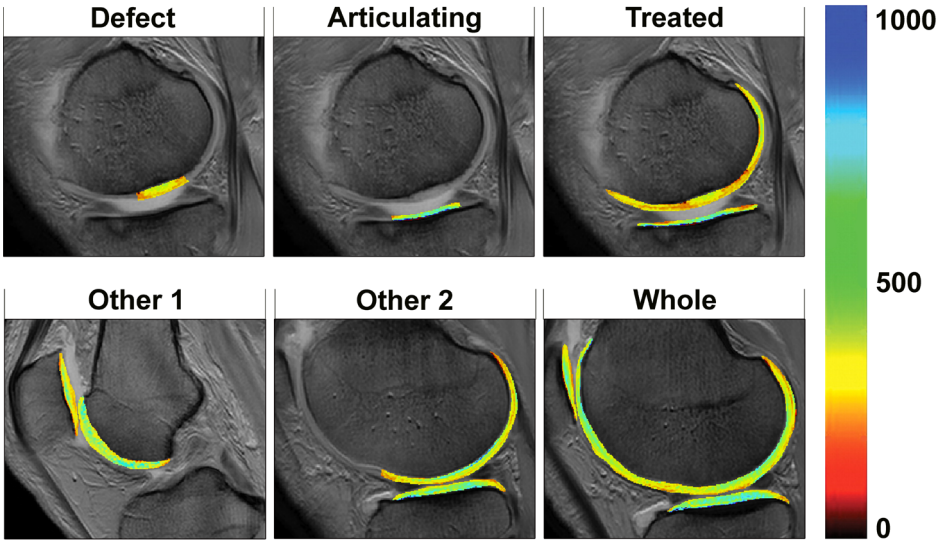
*This information was only available for the MACI/CCI and MF patients.

One patient was excluded when receiving her first study MRI because of MRI artefacts possibly resulting from previous anterior cruciate ligament reconstruction. In addition, 7 patients were excluded during surgery for two reasons; they either showed generalized cartilage degeneration (n=2) or the characteristics of the lesion were not suitable for abovementioned treatments (n=5). One patient was lost to follow-up at 12 months. The baseline characteristics of the 31 patients who were included and completed the study are provided in Table 1. All included patients were evaluated before surgery as well as 3 and 12 months after surgery by a dGEMRIC examination and clinical questionnaires.

Cartilage evaluation by dGEMRIC

All dGEMRIC scans were performed on a 1.5-T clinical MRI scanner (Achieva, Philips Healthcare, Best, The Netherlands) using a dedicated 8-element sense knee coil as a receiver (Philips Healthcare, Best, The Netherlands). Scanning took place 90 minutes after intravenous injection of Magnevist (Gd-DTPA2-, Bayer, Germany) at a dose of 0.2 mmol/kg body weight. After survey scans, a transient field echo (TFE) pulse sequence was used for dGEMRIC with 5 different inversion delay times (50, 150, 350, 650 and 1650 ms), as previously described by McKenzie et al (McKenzie et al 2006). A total of 36 partitions were obtained with a 256x232 in plane acquisition matrix resulting in a voxel size of 0.625 x 0.625 x 3 mm^3, using an echo time of 4.3 ms, a repetition time of 10 ms and a flip angle of 20 degrees. The average T1Gd per ROI was calculated after

Figure 1 Regions-Of-Interest.



Sagittal MRI slices of the scan with 350 ms inversion delay time showing example ROI segmentations as a color overlay. The color bar represents the calculated T1gd in milliseconds, where a high T1gd (1000 ms) is depicted as blue and a low T1gd (nearly 0 ms) as red.

voxelwise fitting of the dGEMRIC signal equation as a function of inversion time using the Levenberg-Marquardt non-linear least-squares method implemented in in-house developed software. On the images obtained in the dGEMRIC scan with an inversion delay time of 350 ms a total of 6 different ROIs (Figure 1) were drawn using a smartboard with projection on an interactive screen. The defect ROI was the region of the treated defect. In the articular cartilage directly opposing and articulating with the treated defect the articulating ROI was drawn. The three joint compartments, patellofemoral, lateral and medial tibiofemoral, were, depending on the site of the cartilage defect, separately identified as the treated ROI and two other ROIs. Finally a whole knee ROI was created that consisted of a segmentation of all the articular surfaces in the knee.

Evaluation of clinical outcome

The clinical treatment outcome was assessed using two different questionnaires both validated for the evaluation of the clinical status of patients treated for an articular cartilage lesion (Bekkers et al 2009; Smith et al 2008)

The Knee injury and Osteoarthritis Outcome Score (KOOS) was designed to evaluate the short- and long-term follow-up of treatment of knee injury and knee osteoarthritis. Recently this questionnaire was validated to measure the clinical condition in patients after regenerative cartilage surgery (Bekkers et al 2009). The KOOS consists of 5 subdomains; symptoms, pain, activities of daily living, function in sport and recreation and

knee-related quality-of-life. The KOOS score per subdomain (score 0-100) was calculated using the free available scoring sheet on the KOOS website (http://www.koos.nu/).
The Lysholm questionnaire was initially designed to evaluate the functional disabilities resulting from ligamentous injury. Recently, this questionnaire has also been validated to asses articular cartilage damage (Smith et al 2008). The questionnaire consists of 8 domains (pain, instability, locking, swelling, limping, walking stairs, squatting and keeping support) and translates to a score between 0 and 100 (normal knee function).

Statistical analysis

All statistical analysis was performed using SPSS statistical software version 15.0 (Chicago, USA). Absolute improvement from baseline at 3 and 12 months follow-up for (subdomains of) the clinical questionnaires and ROIs was tested using a repeated measures ANOVA with a repeated model fit and posthoc Bonferroni test.
Also regression analysis was performed to evaluate possible relations between our outcome variables. Before valid inclusion into the regression model, all variables were subjected to a normality test by the Kolmogorov-Smirnov coefficient, a test for intervariable correlation and multicollinearity (Pearson correlation coefficient and the variance inflation factor) and an assessment for autocorrelation (correlation within a single variable) with the Durbin-Watson coefficient. Also, in multiple regression analysis, the unstandardized residuals were evaluated for the absence of intercorrelation. A Kolmogorov-Smirnov coefficient with $p>0.05$ indicates normal distribution while a variance inflation factor close to 0 or >5 was considered indicative of multicollinearity. A Durbin-Watson coefficient close to 0 is related to strong negative autocorrelation, whereas a Durbin-Watson close to 4 suggests strong positive autocorrelation.
For each regression analysis, the B-coefficient, standard error of the B-coefficient, the 95% confidence interval (95%CI), the R2 and p-value of the model were obtained. The B-coefficient explains the relation between the predictor and dependent variable where an increase of 1 unit of the predictor results in an increase of the dependent variable by the value of B. This relation is statistically significant if the $p<0.05$ and causality counts for the percentage expressed by the R2.
Linear regression analysis was performed to evaluate whether local regeneration (expressed by the 12 months improvement in measured T1gd from baseline in the defect ROI) influences other joint compartments. For this, a single linear regression model was applied with the absolute improvement of measured T1gd in the defect ROI as a predictor variable and the absolute improvement of measured T1gd of the other ROIs (articulating, treated, other 1, other 2 and whole) as dependent variables.
Multiple linear regression with backward elimination was performed to test what patient characteristic were related to improvement in defect T1gd after 12 months. For all statistical analysis a p-value of $p<0.05$ was considered statistically significant.

RESULTS

DGEMRIC and clinical scores; improvement from baseline

At baseline, the T1gd ranged from 365-484 ms for the different ROIs (defect 365±46, articulating 484±125, treated 421±48, other1 422±60, other2 448±68, whole 432±54). The KOOS scores at baseline were lowest for the sports and quality of life subdomains (pain 59±19, activity of daily living 65±20, symptoms 62±18, sports 27±22, quality of life 24±15). The baseline Lysholm score was 48±21 points.

Except for the articulating ROI, the T1gd indices at 3 months after surgery were slightly, but statistically non-significantly, decreased compared to the baseline values (Table 2). After 12 months follow-up, the T1gd of the defect and the articulating ROI showed the largest, statistically significant ($p<0.01$) improvement from baseline, which was also clearly visible on the dGEMRIC images (Figure 2). In addition, the T1gd of the other ROIs (whole, treated and two other joint compartments) also showed a clear, and statistically significant ($p<0.01$), improvement from baseline.

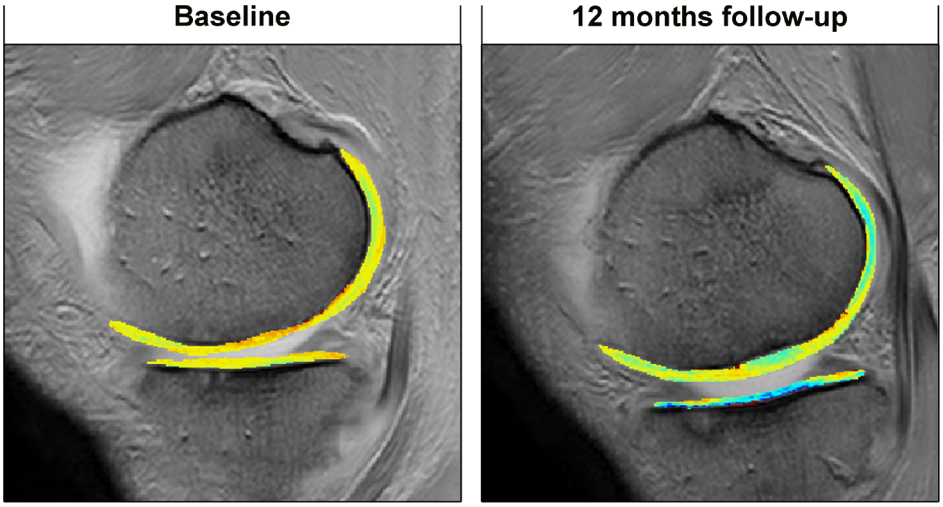
At 3 months after surgery, the clinical scores did not show a statistically significant change from baseline (Table 2). However, at 12 months follow-up all, but 3, patients showed clearly improved clinical scores. Improvement from baseline was noted on the Lysholm, the KOOS subdomains and the KOOS overall scores ($p<0.01$) (Table 2).

Table 2 Clinical outcome evaluation.

		Baseline – 3months	Baseline – 12 months
KOOS questionnaire			
	Pain	12±4 (5-20)*	21±4 (13-29)*
	Symptoms	4±4 (-4-12)	15±4 (7-23)*
	Activity	6±4 (-1-14)	20±4 (13-27)*
	Sport / recreation	-1±4 (-10-7)	29±5 (19-38)*
	QoL	5±3 (-1-11)	20±4 (13-28)*
	Overall KOOS	6±3 (0-13)	20±3 (14-27)*
Lysholm		9±4 (1-17)	28±3 (21-35)*
ROIs			
	Defect	-4±11 (-26-18)	103±13 (76-130)*
	Articulating	20±28 (-36-76)	158±46 (65-252)*
	Treated	-19±10 (-39-2)	49±18 (12-86)*
	Other 1	-11±10 (-30-9)	78±16 (44-111)*
	Other 2	-16±10 (-38-5)	44±14 (15-72)*
	Whole	-10±11 (-32-12)	51±15 (13-74)*

Improvement from baseline (mean ± SD) after 3 and 12 months (calculated by extracting the baseline values from the 3 and 12 month values) for both the clinical questionnaires and dGEMRIC ROIs. *$p<0.01$, ($n=31$).

Figure 2 Sagittal MRI of a representative baseline and 12 months follow-up dGEMRIC scan.



The blue pixels represent a high T1gd (1000 ms) while a low T1gd of 0 is labeled as red. At the preoperative situation a clear change in signal (from yellow to red) is visible at the site of the lesion when compared to the rest of the knee. At 12 months after surgery the overall signal in the knee is improved (more blue-green) with a clear signal improvement at the treated defect site.

Regression analysis; effect of defect treatment on distant cartilage quality

All variables in the regression analysis had a normal distribution (normality tests p>0.358) and no multicollinearity or autocorrelation were found (variance inflation factor, 1.000; Durbin-Watson range, 2.199–2.510).

The increase in T1gd after 12 months at the defect ROI was significantly related to the T1gd increase of the other ROIs in the joint (Table 3). The B-values ranged from 0.787-0.567 indicating that for each millisecond increase in T1gd at the treated defect after 12 months, the T1gd of the cartilage at another location in the joint increased with 0.787-0.567 ms.

Multiple regression analysis showed that the patient characteristics (gender, patient age, defect age and defect size,) did not influence (p>0.070) the improvement in T1gd after 12 months for the defect ROI. However, defect size and patient age were shown to influence the improvement in T1gd of the whole ROI at 12 months after surgery. A defect size >3 cm^2 was related to 58±24 less increase (p=0.024) in T1gd of the joint as a whole after 12 months compared to defects <3 cm^2 and in patients <30 years old a 152±47 stronger increase (p=0.005) in the T1gd was found compared to those >30 years old at 12 months after surgery.

Table 3 Defect treatment relates to overall cartilage improvement.

Dependent variable	B	S.E. B	p-value	95% CI Lower	95% CI upper	R^2
Treated T0T12	0.787	0.207	0.001	0.364	1.210	0.333
Other 1 T0T12	0.651	0.194	0.002	0.253	1.049	0.279
Other 2 T0T12	0.567	0.164	0.002	0.233	0.901	0.293
Whole	0.689	0.164	0.001	0.354	1.023	0.379

Linear regression analysis using the increase in T1gd from baseline to 12 months at the defect ROI as a predictor for the increase in T1gd from baseline to 12 months at other joint locations/ROIs. The B-value represents the increase in the dependent variable when the increase in the predictor is 1. The R2 represents the percentage for which the relation is causal, (n=31).

DISCUSSION

This study evaluated the feasibility of noninvasive monitoring by dGEMRIC of defect regeneration and general tissue integrity of cartilage in the joint after cartilage repair surgery. The dGEMRIC scanning technique was useful in detecting local cartilage regeneration in a focal defect one year after treatment, which was accompanied by clearly improved clinical scores. In addition, local regeneration was directly related to the improvement of cartilage quality in other joint compartments. Also, patient age and defect size influenced the treatment response of the articular cartilage in the whole knee.

The International Cartilage Research Society has recently published several guidelines for histological and MRI based evaluation of cartilage repair studies (Hoemann et al 2012; Trattnig et al 2012). Histological evaluation of newly formed cartilage provides information on the structural organization and can help to understand the biological success of tissue regeneration (Hoemann et al 2012). Disadvantages of histological evaluation are the time consuming processing and the small volume of tissue that can be analyzed. Moreover, the invasive nature of the necessary biopsy makes longitudinal follow-up less desirable from an ethical point of view. Contrast-enhanced MRI scanning protocols, such as dGEMRIC, are able to represent tissue structure and can be readily applied in a longitudinal follow-up. Moreover, with MRI the whole joint can be assessed instead of only small tissue volumes after biopsy. This is the first study to show that a dGEMRIC scanning protocol can be used to longitudinally show improvement in T1gd, as a representation of tissue GAG concentration, following regenerative cartilage treatment. Several other groups already used dGEMRIC to evaluate articular cartilage after ACI, but focussed on differences between repair and native tissue, the zonal organization of the newly formed tissue or only performed post-surgery dGEMRIC without baseline measurements (Domayer et al 2010; Pinker et al 2008; Trattnig et al 2007; Vasiliadis et al 2010). Considering the large variation in T1gd times between patients, it is difficult to define a consensus T1gd which represents acceptable or good quality cartilage after regeneration. Therefore, patient specific baseline measurements are essential when

cartilage quality following regenerative surgery is a relevant outcome in a longitudinal study.

During the different phases of cartilage regeneration the organization of matrix constituents and water content change continuously. These factors influence the T1 relaxation time of the newly formed tissue and most likely lead to differences of the measured T1 relaxation times in repair tissue compared to the reference healthy or degenerated cartilage. This should be taken into account when cartilage is being evaluated with the dGEMRIC technique. A direct comparison, using only post-contrast imaging, between repair tissue and other locations in the joint could, therefore, introduce erroneous interpretation of the data and does not represent the true GAG content in articular cartilage (Trattnig et al 2012). The delta relaxation rate (ΔR1 = 1/T1 precontrast – 1 / T1(Gd)) corrects for the differences in precontrast T1 and is preferred when different locations in the joint are being evaluated and compared in a cross-sectional study design (Trattnig et al 2012). However, per location in the joint (either repair or healthy reference tissue) the correlation between the T1gd and ΔR1 is high and separate interpretation of both outcome variables lead to similar conclusions (Trattnig et al 2009). The absence of pre-contrast imaging, in this study, combined with a longitudinal evaluation at predefined locations does, for abovementioned reason, not influence data interpretation nor change the final conclusions. In addition, patient comfort will decrease when also a precontrast MRI scan was performed as scanning time would be twice as long.

The clinical benefit following ACI and microfracturing is influenced by specific characteristics of the defect or patient (de Windt et al 2009; Filardo et al 2011; Knutsen et al 2007; Kon et al 2008; Saris et al 2009). Also, in specific cases one technique may performs better than the other one does (de Windt et al 2009; Gudas et al 2005; Knutsen et al 2007; Saris et al 2009). In this study, the size of the defect and age of the patient showed a direct relation to the overall improvement in T1gd of the articular cartilage in the knee at 12 months after surgery. This implies that specific biological characteristics of the defect and patient influence the response of the articular cartilage to regenerative surgery. The articular cartilage in the knee showed less improvement following cartilage surgery when a large defect (> 3 cm^2) had been present. Whether the size of the defect is positively correlated to the severity of disturbance in joint homeostasis remains to be seen, however, the presence of an articular cartilage defect has been shown to induce joint cartilage degeneration (Saris et al 2003). It has also been shown that larger defects, if left untreated, are related to an increased cartilage volume loss (Dell'Accio and Vincent 2010). Age influenced the improvement in T1gd following cartilage surgery in this study. Younger patients could be more sensitive for a regenerative response due to the senescence of cells and tissues related to the effects of aging (Martin and Buckwalter 2001).

Based on macroscopic and biochemical evaluation the treatment of an articular cartilage defect has been related to a decrease in degenerative characteristics at other joint

locations (Saris et al 2003). In this study we showed using regression analysis that defect treatment is related to the improvement of cartilage quality at other locations in the joint. These findings underline the importance of the concept of joint homeostasis and the role for early detection and intervention. The presence of an articular defect should be regarded as indicative of a joint disease rather than a local problem. Timely treatment has been shown to improve clinical outcome, i.e. timely restoration of the joint homeostasis improves the regenerative response of the whole joint (de Windt et al 2009; Saris et al 2009). Using dGEMRIC, such changes can be monitored thereby providing a reliable imaging tool for the evaluation of cartilage quality in the whole joint following cartilage repair.

In conclusion, this chapter showed that the dGEMRIC technique can be used to longitudinally measure local regeneration following cartilage repair surgery. Also, using dGEMRIC we showed that patient age and defect size influence the regenerative response of the joint cartilage to cartilage surgery and that local regeneration influences cartilage quality at distant locations in the joint. Taken together, these findings illustrate the value of dGEMRIC for the evaluation of the effects of cartilage regeneration and clearly indicate a role for early detection and intervention.

Chapter 11

Articular cartilage quality one year after TruFit implantation analyzed by delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC)

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Submitted for journal publication

ABSTRACT

This chapter aimed to evaluate defect rim degeneration, examine possible effects on the opposing cartilage surfaces and monitor defect region regeneration, using the dGEMRIC technique, one year after treatment of an osteochondral lesion by TruFit implantation.

A total of 13 patients (age 32 ± 8 years of age) were evaluated with the delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) one year after treatment of an osteochondral lesion by implantation of a TruFit BGS plug. The dGEMRIC scanning protocol was applied 90 min after intravenous Magnevist® (0.2 mmol/kg body weight) injection. Different regions-of-interest (ROIs) were defined; femur cartilage, cartilage directly surrounding the implanted TruFit plug, the TruFit plug, articulating and the non-articulating tibia cartilage. The average T1gd per ROI was calculated by a pixel-by-pixel curve fitting using the Levenberg-Marquardt method. Differences between the mean T1gd of the individual ROI for all patients were tested using an ANOVA with posthoc Bonferroni correction. A p-value < 0.05 was considered statistically significant.

The average T1gd of the Trufit ROI (385 ± 74 ms) was comparable ($p>0.339$) to those in the femur (409 ± 49 ms) and surrounding (392 ± 64 ms) ROIs. The average T1gd for the articulating and non-articulating ROIs were higher ($p<0.002$) compared to the femur, surrounding and TruFit ROIs while no difference ($p=0.160$) was observed between the tibia ROIs.

In conclusion, this chapter showed that implantation of a Trufit plug does not lead to cartilage defect rim degeneration nor initiate damage at the direct articulating surfaces. Also newly formed tissue inside the Trufit plug has similar T1 relaxation properties. Whether this suggests articular-cartilage-like tissue remains to be seen. Overall, the implantation of a Trufit plug for the treatment of small osteochondral lesions is safe for the directly related articular cartilage structures.

INTRODUCTION

Quantitative MRI of articular cartilage has rapidly developed in recent years and provides the clinician with a non-invasive tool to determine the biological consequence of an intervention. In this paper we examined the potential use of delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) for evaluation of cartilage quality one year after the implantation of a biodegradable scaffold in the treatment of osteochondral defects. Glycosaminoglycans (GAGs) and collagen type II, are the main components of the articular cartilage matrix and determine the compressive stiffness of the tissue. Throughout the process from healthy towards generalized cartilage degeneration GAGs are released from the matrix. This causes tissue softening and, under continued loading, breakdown of the collagen network. Therefore, a method for non-invasive, non-destructive quantitative evaluation of the concentration of GAGs in articular cartilage would be of great value for tracking disease progression and could assist in the diagnostic algorithm. Furthermore, such a technique could also be used to evaluate the success of a regenerative intervention and its influence on other cartilage surfaces in the knee.

Delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) has been introduced as a technique that enables a quantified analysis of the GAGs in articular cartilage (Bashir et al 1999). The negatively charged side chains in GAG molecules provide the articular cartilage matrix with a negative net charge resulting in a fixed charged density (FCD). The FCD in articular cartilage, which is thus a measure for the GAG distribution, can indirectly be determined following the administration of the negatively charged T1-shortening contrast agent gadolinium diethylene triamine pentaacetic acid ($Gd\text{-}DTPA^{2-}$, Magnevist®). Following intravenous injection of Magnevist® the $Gd\text{-}DTPA^{2-}$ anionic molecule will penetrate the cartilage and will distribute over the cartilage matrix inversely proportional to the GAG concentration. Thus, the uptake of $Gd\text{-}DTPA^{2-}$ will be higher in areas with low GAG content and vice versa. This technique has been shown to have good *in vivo* reproducibility and to represent the absolute and depth-dependent GAG content and organization in articular cartilage (McKenzie et al 2006; Multanen et al 2009; Xia et al 2008). Recent reports also showed the feasibility of the dGEMRIC technique to evaluate the success after cartilage repair (Trattnig et al 2007; Vasiliadis et al 2010).

Selection of the repair technique for the treatment of focal articular cartilage lesions in the knee is being directed by the size of the lesion, extension of the lesion towards or into the subchondral bone, duration of symptoms and the age and activity level of the patient (Bekkers et al 2009). The recently developed TruFit BGS plugs (Smith and Nephew, USA) are currently not included in these evidence-based treatment algorithms but could become an interesting option for small osteochondral lesions of the femoral condyle. The TruFit BGS plug is a bilayered cylindrical plug composed of PLG copolymer, calcium phosphate and PGA fibers. Cartilage regeneration inside TruFit plugs is most

likely instigated by the integration of cells, and growth factors derived from the bone marrow that infiltrates the plug. Recently, implantation in the medial femoral condyle and lateral trochlear groove showed good histological cartilage formation after 12 months in a goat model (Williams and Gamradt 2008). Also, small case series have shown clinical improvement from baseline 12 months after implantation (Saithna et al 2008; Williams and Gamradt 2008). However, when larger osteochondral lesions are being treated the bony incorporation of the plug can be delayed, leading to a treatment failure (Carmont et al 2009). Also, the cartilage surrounding and opposing an osteochondral graft could be damaged due to direct articulation or increased contact pressures caused by instability of the graft (Custers et al 2007; Tibesku et al 2004).
Therefore, this study aimed to evaluate defect rim degeneration, examine possible effects on the opposing cartilage surfaces and monitor defect region regeneration, using the dGEMRIC technique, one year after treatment of an osteochondral lesion by TruFit implantation.

MATERIAL AND METHODS

Patients and surgical technique

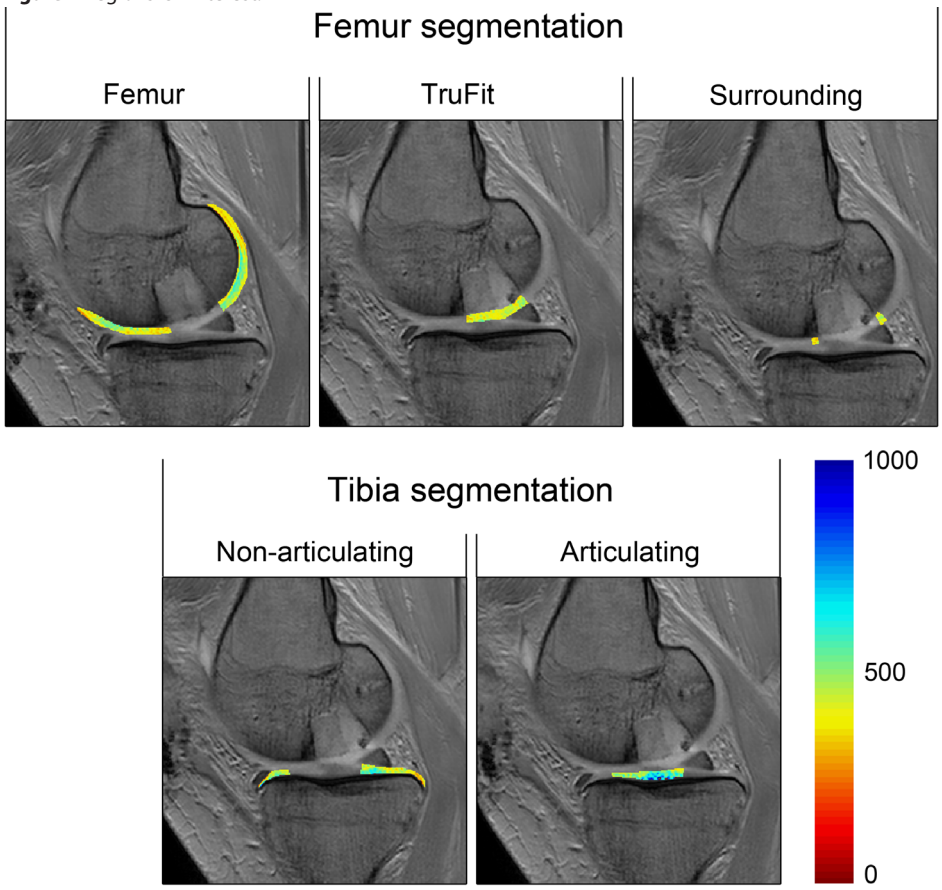
From August 2007-January 2010 a total of 13 patients (age 32 ± 8 years old) that had been treated with a TruFit implantation for their focal articular cartilage defect were willing to enter this study. General contraindications for MRI, a known allergic reaction to contrast agents and a history of, kidney disease were criteria for exclusion. All patients were informed about the risks associated with this study and contra-indications were evaluated. Patients were given a minimum of 3 days to consider participation after which consent was obtained. Evaluation of the treatment of focal cartilage lesions using dGEMRIC has been approved by the institutional ethical committee under protocol no. 08/022E.
All included patients suffered from a symptomatic osteochondral lesion of the knee. In general, the surgical procedure was started by a knee arthroscopy to evaluate all knee compartments for further pathology followed by the implantation of the TruFit BGS plug(s) (Smith and Nephew, USA) using a mini-arthrotomy. The focal lesion was sized and prepared for TruFit implantation by drilling cylindrical holes in the subchondral bone similar to the diameter of the TruFit plug. Debris from drilling was discarded and the bony wall of the drilled defect checked for stability. Following this the TruFit delivery device was used to determine the depth of the defect and the implant was trimmed accordingly. The TruFit plug was implanted flush with the articular surface and, again, stability was checked. The whole procedure took approximately 30 minutes and was, in all cases, performed by the same surgeon. After surgery patients were allowed partial-

weight-bearing for 6 weeks followed by a gradual increase in knee loading and function over a time span of 6 months under supervision of a physical therapist.
To evaluate the influence of Gadolinium on the T1 relaxation properties of the Trufit plug material, a total of 4 empty, unimplanted, Trufit plugs were scanned, while embedded in 2 % agarose, using the dGEMRIC protocol as described below, before and after overnight incubation in 1mM Magnevist® (Gd-DTPA^{2-}, Bayer, Germany).

Cartilage evaluation by MRI

One year after implantation of the TruFit plug(s) (Smith and Nephew) the articular cartilage quality was evaluated using an implementation of the dGEMRIC technique. For this, patients were intravenously injected with Magnevist® (Gd-DTPA^{2-}, Bayer, Germany) at 0.2 mmol/kg body weight and asked to walk for at least 15 minutes to facilitate uptake of the Magnevist by the articular cartilage. To standardize walking distance and joint loading all patients were guided through the same route by JEJB. MRI scanning on a clinical 1.5-T MRI scanner (Achieva, Philips Healthcare, Best, The Netherlands) took place 90 minutes after intravenous injection using a dedicated 8-element sense knee coil (Philips Healthcare, Best, the Netherlands) as a receive coil. The pulse sequence used was a 3D sagittal transient field echo (TFE) with 5 different inversion times (50, 150, 350, 650 and 1650 ms), resembling the protocol previously described by McKenzie et al (McKenzie et al 2006). The acquired voxel size was 0.625 x 0.625 x 3 mm^3, 36 partitions were acquired with an acquisition matrix of 256 x 232. The repetition time was 10 ms, the echo time was 4.3 ms and the flip angle was 20 degrees. The average T1Gd per region of interest (ROI) was calculated using pixel by pixel curve fitting with the Levenberg-Marquardt method using in-house developed software. All ROIs were located in the treated joint compartment.
Five different ROIs were manually drawn on the images obtained from the scanning sequence with a repetition time of 350 ms (Figure 1). At the femoral condyle the *femur, TruFit* and *surrounding* ROIs were created. The femur ROI was defined by the articular cartilage of the femur condyle at the TruFit implantation compartment, without the site of TruFit implantation (Figure 1). The *TruFit* ROI was defined as the cartilage site of the implanted Trufit plugs while the *surrounding* ROI described the 3mm ring of articular cartilage that surrounded the TruFit implantation (10pixels in x-y direction and 1 slice in both z directions). The *non-articulating* and *articulating* ROIs were created at the tibia cartilage in the TruFit-implanted knee compartment. The *non-articulating* ROI defined the tibia cartilage that did not articulate with the implanted TruFit plugs while the *articulating* ROI was described the tibia cartilage that directly articulated with the implanted TruFit plugs. The ROI segmentations were, with an interval of 2 months, repeated once by the same person (JEJB) for 5 patients to evaluate the internal consistency and reliability of the segmentation process.

Figure 1 Regions-Of-Interest.



Sagittal MRI of postoperative situation when an OCD lesion of approximately 1.5 cm was treated using two TruFit BGS plugs. The bone-plug interface is clearly visible and could be used to define the ROIs. The color bar represents the calculated T1gd, where a high T1gd (1000 ms) is depicted as blue and a low T1gd as red.

Statistics

Statistical analyses were performed using SPSS version 15.0. Differences between the mean T1gd of the individual ROIs for all patients were tested by a one-way ANOVA with posthoc Bonferroni correction. A p-value of $p<0.05$ was considered statistically significant. The internal consistency and reliability of the segmentation process was tested by calculating the Cronbach's alpha and intraclass correlation coefficient (ICC). For both the Cronbach's alpha and the ICC values of >0.70 were considered as acceptable while values >0.80 represent excellent internal consistency and reliability.

RESULTS

Defect characteristics and patient follow-up

All osteochondral lesions were located in the femoral condyles (7 medial and 6 lateral) and had an average lesion size of 1.9±0.7 cm^2. Three lesions were true osteochondritis dissecans (OCD) while 8 had another cause of osteochondral damage. The defects were filled with (a combination of) TruFit plugs with a diameter of 7, 9 or 11 mm, depending on the size of the lesion. No postoperative complications were seen. After a total follow-up of 12 ± 4 months 2 out of the 13 patients still had mild knee complaints which seemed not to be directly related to the implantation of the TruFit plug. One patient suffered from a chronic Bakers cyst while the other patient had developed a new condylar cartilage lesion not continuous with the TruFit plug implantation site. All other patients were pain free and had a full range-of-motion at maximum follow-up.

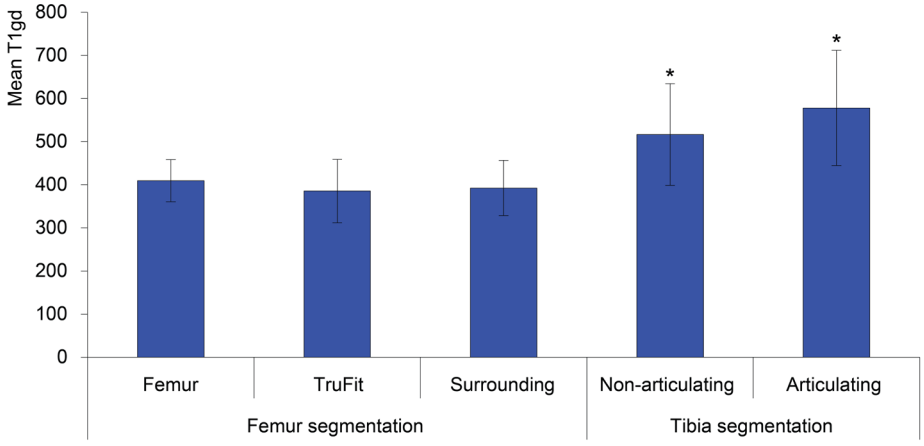
dGEMRIC analysis

The calculated T1 relaxation from the unimplanted TruFit plugs in agarose did not differ (p=0.605) between the pre- and post-contrast scans and ranged from 1224-1321 ms for the pre-contrast scans to 1196-1355 ms for the post-contrast scans.

The reliability and internal consistency of the segmentation process was good-excellent with a Cronbach's alpha of 0.963 and an ICC of 0.928.

After an average of 12 ± 4 months the bony part of the TruFit plugs was still visible on the T1-weighed MRI images, while the articular cartilage layer showed a homogenous T1gd

Figure 2 Average T1gd per ROI.



Average T1gd values for all cartilage ROIs from all patients showing values similar to that in cartilage tissue inside TruFit plugs without additional damage to the opposing, articulating, cartilage. (*p<0.002), (n=13).

value compared to the surrounding cartilage. The average T1gd of the surrounding ROI (392 ± 64 ms, 95%CI 346-418 ms) was similar ($p \geq 0.535$) compared to both the T1gd of the femur ROI (409 ± 49 ms) and the TruFit ROI (385 ± 74 ms). In addition, the average T1gd from the TruFit ROI (385 ± 74 ms, 95%CI 334-410 ms) did not differ significantly ($p=0.339$) from the T1gd values in the femur ROI (409 ± 49 ms, 95%CI 375-425 ms) (Figure 2).
The articulating ROI showed a similar ($p=0.160$) T1gd value (578 ± 133 ms, 95%CI 490-611 ms) compared to the non-articulating ROI (516 ± 118 ms, 95%CI 441-541 ms). Overall the tibia cartilage T1gd values were higher ($p<0.002$) compared to the femur T1gd values (Figure 2).

DISCUSSION

This imaging study evaluated the quality of articular cartilage, using a dGEMRIC scan protocol, of different anatomical locations in the knee one year after implantation of a Trufit plug and showed that the T1gd value of the articular cartilage directly surrounding the implanted Trufit plug does not differ from that in the femur cartilage. In addition, the T1gd values of the opposing cartilage that directly articulated with the Trufit plug did not differ from non-articulating cartilage at the tibia. These results indicate that an implanted Trufit plug does not damage the directly articulating cartilage nor enhance defect rim degeneration. The T1gd values in the cartilage part of the TruFit plug, one year after implantation, were similar compared to those in the femur cartilage. The T1 relaxation properties of the Trufit material was not influenced by the presence of Gadolinium *in vitro*. Therefore, the newly regenerated tissue inside the Trufit plug has similar T1 relaxation properties as femur cartilage.
The TruFit plug is designed to combine the effects of a direct bone and cartilage defect filler as well as structural support to allow regeneration from cells and stimulatory factors derived and absorbed from the bone marrow. Bone marrow stimulation techniques to restore articular cartilage surfaces, such as microfracture, have previously shown good clinical results (Bekkers et al 2009; Steadman et al 2003). However, frequently occurring intralesional osteophytes create inferior mechanical stability of the osteochondral tissue thereby reducing the durability of the regenerated cartilage tissue (Gomoll et al 2010). The TruFit plug consists of 2 layers that structurally separate the bone from the cartilage tissue thereby allowing tissue-dependent regeneration which possibly reduces the development of intralesional osteophytes while addressing the challenge of treating significant osteochondral defects. Therefore, the TruFit plug takes advantage of the regenerative capacity of the cells and proteins present in the bone marrow while intralesional osteophytes are not likely to develop.
The application of synthetic implants, with or without cells, for the treatment of focal cartilage lesions coincides with a potential concern on additional damage to the directly

opposing articulating surfaces. Particularly when such implants are not located flush with the articular surface they have been associated with increased cartilage erosion and contact pressures at the directly articulating cartilage surfaces (Becher et al 2008; Becher et al 2011; Custers et al 2007; Kirker-Head et al 2006). Also loose bodies from implanted material could initiate damage to articular structures in the knee or cause irritation of the synovial membrane eventually resulting in a synovitis. As presented, the femoral TruFit implantation has not been associated with additional damage to the directly articulating tibia cartilage. Also patients did not suffer from synovitis during our follow-up. This suggests that the implantation of the TruFit in limited amounts for small to median defects is safe and does not afflict any additional damage to the intra-articular environment.

Articular cartilage surrounding a focal lesion has previously been shown to be exposed to increased axial strain because of the higher tendency of the tissue to deform (Braman et al 2005; Gratz et al 2008; Gratz et al 2009). This may initiate accelerated matrix damage and tissue loss. Therefore increased tissue deformation could make a non-continous cartilage matrix prone to damage. This is also observed after autologous osteochondral transplantation as histological follow-up showed a failed lateral integration of the transplanted cartilage with concomitant severe signs of cartilage degeneration (Tibesku et al 2004). Also, bony cysts might occur when lateral integration fails which could, in the long run, lead to a decreased stability of the implanted autologous osteochondral plugs. Our data suggests good lateral integration of the articular part of the TruFit plug without any damage to the articular cartilage surrounding the TruFit plug. Therefore, the newly formed tissue inside the TruFit plug will be less susceptible for accelerated matrix damage owing to increased tissue deformation.

The T1gd index obtained from the newly formed tissue inside the TruFit plug (385 ± 74 ms) is lower compared to the T1 indices (range 400-700 ms) from literature after MACI or microfracturing (Trattnig et al 2007; Trattnig et al 2008; Vasiliadis et al 2010). However, the follow-up in these studies was at least 2 years while the follow-up presented in this study was 1 year. Further tissue maturation inside the TruFit plug could still take place after 1 year resulting in higher T1gd values at longer follow-up. Additionally, T1gd values in healthy asymptomatic volunteers also show a great variation (range 428-743 ms) when different dGEMRIC scanning protocols are applied (Mayerhoefer et al 2010; Tiderius et al 2005). Therefore, direct comparison of obtained T1gd values to reference data from literature should be interpreted with care as scanning protocols and post-processing methods differ.

In conclusion, this study showed that implantation of a Trufit plug does not lead to cartilage defect rim degeneration nor initiate damage at the direct articulating surfaces. Also, newly formed tissue inside the Trufit plug has similar T1 relaxation properties. Whether this suggests articular cartilage-like tissue remains to be seen. Overall, the implantation of a Trufit plug for the treatment of small osteochondral lesions is safe for the directly related articular cartilage structures.

Chapter 12

Patient profiling in cartilage regeneration: prognostic factors determining success of treatment for cartilage defects

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ABSTRACT

Cartilage therapy for focal articular lesions has been implemented for over a decade and is becoming increasingly available. However, specific analysis of patient characteristics that helps improve outcome or select patients for specific treatment is still lacking. This chapter analyses the prognostic value of patient age, defect size, defect age and defect location on the clinical outcome three years after cartilage therapy.

A total of 55 patients (mean age 35 ± 9 years), randomly selected from a prospective database, with a traumatic knee injury and treated for a focal cartilage lesion, were assessed with the Knee Injury and Osteoarthritis Outcome Score (KOOS). Patient characteristics (patient age, defect size, defect age and defect location) were tested for valid inclusion in the regression model. Multiple linear regression was used to determine which variables influenced the clinical improvement. Binary KOOS scores were generated based on age matched healthy subjects and assessed in a logistic regression analysis.

Normality tests confirmed normal distribution for each variable ($p<0.05$). Defect size did not influence clinical improvement ($p>0.05$). The clinical outcome of treatment of medial defects was better (10.38-25.26 points for the different KOOS subscales) compared to lateral defects ($p<0.05$). KOOS improvement from baseline was better (7.31-29.24 points for the different KOOS subscales) for patients <30 years compared to patients older than 30 years ($p<0.05$). Patients with defects aged <24 months were more likely ($p<0.05$, Odds Ratio: 1.8-4.0) to report the age-matched healthy reference KOOS score.

This chapter illustrates the influence of the patient age, defect location and defect age on the clinical outcome three years after treatment of a focal cartilage lesion in patients with a traumatic knee injury.

INTRODUCTION

Focal articular cartilage lesions can cause serious limitations in daily functioning and predispose to progressive degenerative changes leading to osteoarthritis (OA) in time. Currently, therapeutic strategies to repair focal lesions are primarily based on treatment history, the patient's physical demands and the preference of the surgeon (Getgood et al 2009; Williams III and Brophy 2008). The identification of individual patient characteristics that predict clinical outcome could be helpful in the development of patient-specific treatment strategies. In addition, it will help us in providing better information and more realistic expectations for various indications.

Several patient characteristics may be used to predict clinical outcome. Age is an example, as younger patients seem more likely to obtain better clinical results after cartilage therapy (Childers Jr. and Ellwood 1979; Knutsen et al 2007; Steadman et al 2003). Location of the defect is another important factor influencing clinical outcome. Patellar and trochlear cartilage lesions showed superior clinical and histological results when compared to femoral defects (Niemeyer et al 2008), but no information is available on how other defect locations may differ in terms of regeneration. In addition, it was suggested that smaller lesions show better cartilage repair when compared to larger defects and that various treatment methods may have a different effect depending on defect size (Cahill 1995). However, contrary to clinical intuition or common sense so far various studies have not shown a relation between defect size and the clinical outcome after articular cartilage therapy (Niemeyer et al 2008; Zaslav et al 2009). The activity level of the patient did seem to have a clear effect on clinical outcome (Knutsen et al 2007). Clinical outcomes might be better in active patients with goals when compared to more sedentary patients. Finally, attention has been focused on the age of the defect, which seems an important factor in determining clinical outcome. This either may be directly related to advanced degeneration of the bone and cartilage immediately surrounding the defect, or to a perturbation of joint homeostasis, negatively affecting the intra-articular environment and final clinical outcome (Saris et al 2003).

The aim of this chapter was to analyze the prognostic value, using regression analysis, of patient age, defect size, defect age and defect location on the clinical outcome three years after treatment of focal articular cartilage lesions.

MATERIALS AND METHODS

Patients

A total of sixty patients were randomly included from a prospective cartilage database (Socrates) based on the condition that all data needed, the defined study parameters

and a complete baseline and 36 months KOOS score, were present. All had been treated for a focal articular cartilage lesion with either autologous chondrocyte implantation (ACI), using 1st or 2nd generation ACI (Brittberg et al 1994) as described by Peterson and Brittberg, or microfracturing (MF), using the technique initially described by Steadman (Steadman et al 2001), between May 2003 and November 2005 in a specialized academic cartilage care clinic. In all patients the onset of symptoms was related to a knee trauma. Information on patient characteristics (patient age, defect size (cm^2), defect age (symptom to treatment delay) and defect location (medial or lateral)) were part of the database and verified against data extracted from the clinical files.
Reference data for healthy subjects were extracted from two hundred and ninety-two, age-matched (range 18-54), subjects who were randomly chosen from the Swedish National Population Record (Paradowski et al 2006).

Study design and measurement

Patients received the KOOS questionnaire before and three years after focal cartilage therapy. The KOOS is a patient-based, site-specific, questionnaire, developed to be used for short- and long-term follow-up of knee injury and knee OA (Roos and Lohmander 2003). Recently, the KOOS has been validated for the treatment of focal cartilage lesions as well (Bekkers et al 2009). For each subdomain as well as the total KOOS the score was normalized to a 0-100 scale, in which a value of 100 reflected the best possible outcome (Roos and Lohmander 2003). Patients with missing data in more than one assessed patient characteristic or more than two missing items of the KOOS were excluded from further analysis. A total of fifty-five patients were finally included in the analysis.

Statistical analysis

Linear regression

To test the underlying assumption (Chan 2004) that each patient characteristic could be validly included in the multiple linear regression model, they were subjected to a normality test by determining the Kolmogorov-Smirnov coefficient, a test for inter-variable correlation (correlation between variables) with the Pearson correlation coefficient and the variance inflation factor (VIF) and an assessment for autocorrelation (correlation within a single variable) with the Durbin Watson (DW) coefficient. A VIF close to zero is considered indicative for multicollinearity. A DW coefficient close to zero was considered indicative for strong negative autocorrelation while a DW close to four was considered indicative for strong positive autocorrelation respectively (Chan 2004; Durbin and Watson 1950). Patient characteristics not meeting the abovementioned underlying assumptions were not included in the multiple linear regression analysis but were assessed by single linear regression.

To check whether the influence of individual patient characteristics on the clinical outcome is independent of the type of cartilage surgery, an additional regression analysis was performed for the patients treated with MF and for those that received cell transplantation. In addition, we tested the homogeneity of the regression slopes to exclude a possible treatment-by-covariate effect between the treatment type and the included study parameters on the KOOS improvement after 36 months.
Multiple linear regression analysis with backward elimination was applied to determine which patient characteristic influenced clinical improvement three years after focal cartilage therapy. Patient age and defect age were dichotomized at thirty years and twenty-four months, respectively, based on previous suggestions in the literature (Gudas et al 2006; Knutsen et al 2004; Knutsen et al 2007; Saris et al 2009). For each variable the regression (B) and constant regression (B 'constant') coefficients along with the p-value and 95% confidence interval (95 % CI) were obtained. In this study the B coefficient indicates the mean KOOS improvement per cm^2 increase in defect size, for patients < 30 vs. ≥ 30 than years old, medial vs. lateral defects and defect age < 24 vs. ≥ 24 months. The B 'constant' coefficient is a hypothetical measure that indicates the absolute clinical improvement if an independent patient characteristic would be zero.

Logistic regression
Logistic regression analysis was used to determine separate odds ratios of each variable for the KOOS subdomains in comparison to healthy subjects (Paradowski et al 2006). Patients were categorized, based on their KOOS score three years after cartilage surgery, as lower or higher than the KOOS score of the reference data of the age matched healthy subjects (Paradowski et al 2006). Odds ratios (OR's) along with p-values and 95 % CI were obtained.
Linear and logistic regression analyses were assessed with SPSS statistical software version 15.0 (SPSS Inc. Chicago, IL). A p-value of $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

Patients

Out of the initial sixty patients, five were excluded due to missing data (n=2) or lost to follow-up (n=3). From the resulting 55 patients (mean age 35 ± 9 years, range 22-55; 75 % men), 30 had been treated with MF while the other 25 had received ACI. Thirty-six patients had participated in a randomized controlled clinical trial comparing MF to ACI. Mean follow up time from baseline was 35 ± 2 months. Mean defect size was 2.86 ± 1.90 cm^2. Twenty-five patients (45%) had a cartilage lesion on the medial femoral condyle.

The overall KOOS score at baseline was comparable between the patients with respective medial or lateral defects. (57±18 for medial and 67±19 for lateral defects). Forty-five patients (82%) had a sports-related knee trauma (soccer, tennis, judo etc.) while ten patients had a non-sports-related traumatic knee injury (misstep, car accident) (table 1). The homogeneity of the study parameters and KOOS scores (both baseline and after 36 months) for both treatment types (table 2) was good.

Table 1 Baseline characteristics.

Characteristic (n= 55)	**N (SD)**
Age (years)	35 (9)
Men (n)	41
Microfracturing (n)	30
ACI (n)	25
Defect size (cm^2)	2.86 (1.90)
Defect medial (n)	25
Defect lateral (n)	20
Follow up (months)	35 (2)
Defect age (months)	47.72 (48.76)
Sport related distortion (n)	45
Traumatic injury (not sport related) (n)	10
ACL rupture (n)	11
Age < 30 (n)	24
Age ≥ 30 (n)	31
Treatment < 24 months (n)	24
Treatment ≥ 24 months (n)	31

Table 2 Baseline characteristics per treatment type.

	ACI	**Microfracturing**
KOOS total score		
Baseline	59 (±17)	61 (±21)
36 months f/u	76 (±21)	66 (±35)
Defect size (cm^2)	3.25 (±2.30)	2.60 (±1.44)
Defect age		
< 24 months	10	13
>24 months	15	17
Patient age		
< 30 years old	11	13
>30 years old	14	17
Defect location		
Medial	16	19
Lateral	9	11

Linear regression

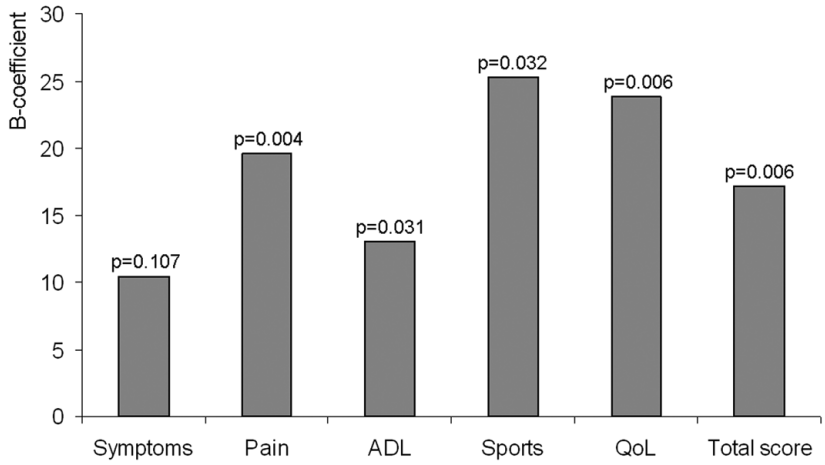
Normality tests, confirmed normal distribution, for each patient characteristic ($p<0.05$). No multicollinearity or autocorrelation was observed (VIF 1.112 and DW range 2.15-2.70). Linear regression analysis of each independent patient characteristic did not differ between the MF or ACI group. In addition, no treatment-by-covariate interaction

between treatment type and the included parameters was present as the Levene's tests of homogeneity of regression slopes was not statistically significant (p-values range 0.075-0.879). Patient age showed a high correlation to defect location and was, therefore, excluded from the multiple linear regression model.

The size of the defect did not influence clinical improvement at three years after surgery. Defect location and defect age proved to influence the KOOS improvement at three years after surgery. Medial defects were associated with a better KOOS improvement in all KOOS subdomains when compared to lateral defects (Figure 1). For example, the improvement in the KOOS Sports and Pain subdomains is respectively 25.26 (p=0.032) and 19.58 (p=0.004) points higher for the medial compared to the lateral defects. Defect age proved to influence the postoperative improvement as the 'B-constant' values were high (B 'constant' 11.96-21.12) indicating that, for example, patients without symptom-to-treatment delay would have a hypothetical 21.12 (p=0.021) points more improvement in function in sports and recreation subdomain when compared to patients with a significant treatment delay (Figure 2).

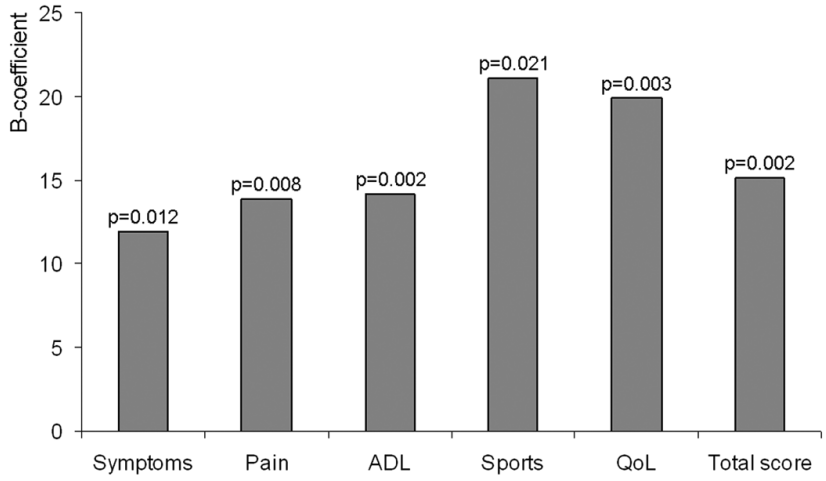
The single linear regression analysis of patient age showed that patients younger than thirty years old had a significantly higher clinical improvement on the KOOS subdomains than patients over thirty years of age (Figure 3).

Figure 1 Multiple linear regression analysis: defect location.



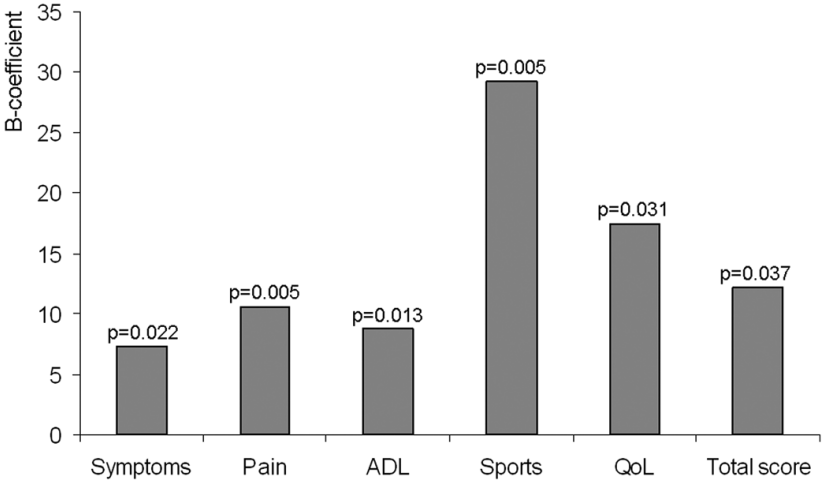
B-values of medial vs. lateral femoral defects three years after surgery.
Positive B-values show an overall higher post operative progression of KOOS scores for medial compared to lateral defects (n=55).

Figure 2 Multiple linear regression analysis: defect age.



B-(constant) values indicate absolute KOOS progression for KOOS total score and subdomains three years after surgery if defect age would be zero (n=55).

Figure 3 Single linear regression: Age < 30 or ≥ 30.



B- values show greater KOOS progression three years after surgery for patients younger than 30 years old compared to patients older than 30 years old (n=55).

Logistic regression

Defect age has a high relevance in predicting clinical outcome, as patients with a defect age < 24 months were more likely (OR range 1.8-4.0) to reach the age matched healthy reference KOOS values for almost all KOOS subdomains compared to those with a defect age ≥ 24 months (Table 3). For example, it is 4 times more likely (p=0.035) that patients with a defect age < 24 months reach the age-matched KOOS reference score of ≥ 88 points for the ADL subdomain three years after surgery, compared to patients with defects over 24 months old. Each dichotomized subdomain based on the reference scores for healthy subjects showed an adequate distribution, except for the QoL subdomain (Table 4).

Table 3 Logistic regression: defect age.

KOOS subdomain	OR	*P*	95% CI
Symptoms	2.10	0.020	0.15 - 0.490
Pain	2.48	0.070	0.12 - 1.089
ADL	4.00	0.035	0.07 - 0.904
Sports	1.80	0.037	0.18 - 0.683
QoL	1.71	0.488	0.13 - 2.653

OR's for defect age < 24 months vs. ≥ 24 months on KOOS subdomains three years after surgery, (n=55).

Table 4 KOOS subdomains dichotomized.

	KOOS subdomains									
	Symptoms		Pain		ADL		Sports		QoL	
Reference score*	87		6		8		6		77	
Binary score	*< 87*	*≥ 87*	*< 86*	*≥ 86*	*< 88*	*≥ 88*	*< 76*	*≥ 76*	*< 77*	*≥ 77*
Patients: n (%)	34 (61.8)	21 (38.2)	29 (52.7)	26 (47.3)	20 (36.4)	35 (63.6)	28 (50.9)	27 (49.1)	46 (83.6)	9 (16.4)

*Paradowski et al. BMC Muscluloskeletal Disorders 2006

KOOS subdomain scores were dichotomized based on scores of healthy subjects* and compared to patients of this study. All subdomains show good distribution except for the QoL subdomain, (n=55).

DISCUSSION

This study evaluated the effect of patient age, defect size, defect age and defect location on the clinical outcome, measured with the KOOS questionnaire, three years after treatment of a focal cartilage lesion in patients with a traumatic knee injury. To our knowledge, this is the first prospective clinical study that demonstrates the influence of individual patient characteristics, like patient age, defect location and defect age, on clinical outcome after cartilage repair surgery.

In the current study, single regression analysis of each independent variable did not differ for the MF vs. ACI cohort, hence analyses were carried out on both patient popula-

tions together. In this study population, defect size did not seem to predict the clinical outcome, which is in line with several other studies showing that clinical outcome in patients treated with ACI is independent of the size of the defect (Niemeyer et al 2008; Zaslav et al 2009). However, in one cohort of ACI patients, the clinical results were significantly better for lesions >3 cm^2 (Cahill 1995; Selmi et al 2008) while one study comparing MF with mosaic type osteochondral transplantation (OCT) showed that the clinical results for patients in the MF arm with a lesion larger than 2 cm^2, were worse compared to those with a lesion smaller than 2 cm^2. Interestingly, this was not found for patients in the OCT arm indicating a more stable regenerative product in larger lesions after OCT (Gudas et al 2006). Thus, defect size may predict clinical outcome for some treatments, but the conflicting data on the correlation in MF and ACI treatment in this study suggest that for the latter two treatment types, this is probably not the case, at least not at the three years follow-up studies here. This is highly relevant for treatment algorithms and rules in the application of microfracturing and cell-based therapies.

Defect location had a clear effect on clinical outcome, with defects at the medial femoral condyle showing a better KOOS improvement, three years after surgery, when compared to defects on the lateral femoral condyle. This is in accordance with a previous retrospective study where clinical outcome was better for microfracture treatment of femoral defects compared to either tibial, patellar or trochlear defects (Kreuz et al 2006). However, a recent animal study on the repair of osteochondral lesions was inconclusive regarding defect location (Jung et al 2009), which could suggest that the influence of defect location is treatment-specific as well.

In addition to defect location, defect age also had a clear effect on clinical outcome. Patient without symptom-to-treatment delay presented more improvement on the KOOS score at 3 years after surgery (Figure 2). This finding is underlined by patients with a defect age of less than 24 months who had up to 4 times more chance to reach the age matched healthy reference KOOS score compared to patients with a defect age >24 months (Table 3). These results are in line with the recent mid-term results of a RCT showing better clinical outcome for early vs. late treated cartilage defects (VanLauwe et al 2009). A possible reason for the influence of defect age on clinical outcome is the better environment of fresh defects, with limited or localized cartilage matrix disturbance or degeneration in the surrounding tissue (Saris et al 2003).

Lastly, patient age affected the clinical outcome, with better scores for patients younger than thirty years old. This may be in agreement with sub-analysis in previously published RCTs comparing either MF to ACI or MF vs. OAT, both showing better clinical outcome for patients younger than 30 years compared to those older than 30 years, unrelated to the type of treatment received (Gudas et al 2005; Knutsen et al 2007). These findings could be related to the effects of ageing on senescence of cells and tissues and thus the extent

of the regenerative response. It will be interesting to further analyze other age cut-off points within a similar patient group.

The studied population showed a homogenous etiology of the defect as the onset of symptoms could be related to a trauma in all patients. Although it seems likely that similar patient characteristics influence clinical outcome in the treatment of non-trauma-related articular cartilage lesions, it could be useful to investigate this relation for other defect aetiologies as well.

In conclusion, this study illustrates the influence of the patient age, defect location and defect age on the clinical outcome measured with the KOOS questionnaire three years after treatment of a focal cartilage lesion in patients with a traumatic knee injury. These results provide a basis for a more detailed estimation of the patients' prognosis and could be helpful in the development of a patient-specific treatment strategy.

Chapter 13

Treatment selection in articular cartilage lesions of the knee: a systematic review

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ABSTRACT

Several treatment options are available to repair articular cartilage lesions of the knee, however, evidence based, parameters for treatment selection are lacking. This chapter aims to identify parameters for valid treatment selection in the repair of articular cartilage lesions of the knee.

A systematic search was conducted in the databases EMBASE, MEDLINE and the Cochrane collaboration. The retrieved articles were screened for relevance on title and abstract followed by a full text study quality appraisal of the remaining articles. Eventually, a total of 4 randomized controlled trials were included.

Lesions size, activity level and age showed to be influencing parameters for the outcome of articular cartilage repair surgery. Lesions >2.5 cm^2 should be treated with sophisticated techniques, like autologous chondrocyte implantation (ACI) or osteochondral autologous transplantation (OAT), while microfracture is a good first line treatment option for smaller (<2.5cm2) lesions. More active patients show better results after ACI or OAT when compared to microfracture. Younger patients (<30 years) seem to benefit more from any form of cartilage repair surgery compared to those >30 years of age.

Lesion size, activity level and patient age are factors that should be considered in selecting treatment of articular cartilage lesions of the knee. In addition, these factors are a step towards evidence based, instead of surgeons preferred, treatment of articular cartilage lesions of the knee.

INTRODUCTION

Several surgical techniques have been proposed to treat chondral lesions of the knee (Alford and Cole 2005) Reparative methods aim to stimulate the formation of new fibrocartilage tissue by facilitating access to the vascular system and bringing new progenitor cells capable of chondrogenesis (e.g. microfracture procedure and drilling). Reconstructive methods aim to fill the defect with autologous, homologous or other tissue (e.g. autologous chondrocyte implantation (ACI) and osteochondral autologous transplantation (OAT)) (Detterline et al 2005). In contrast to reconstructive techniques, reparative methods are primarily done arthroscopically. However, newer reconstructive techniques are progressively being modernized towards arthroscopic application (Marlovits et al 2006).
Presently microfracture is a common first line treatment for patients with cartilage defects of the knee. The purpose of this technique is to create a clot in the cartilage lesion populated with platelets, growth factors and bone marrow derived stem cells (Steadman et al 2001). Several newer reconstructive techniques are presently used. In ACI autologous chondrocytes are taken by biopsy, expanded *ex vivo* and re-injected after expansion into patients' cartilage lesion. Successful repair of cartilage lesions by means of ACI was first described by Brittberg in 1994 (Brittberg et al 1994). Evolutions of this treatment, such as characterized chondrocyte implantation (CCI) and matrix-assisted-chondrocyte implantation (MACI) are currently being performed (Marlovits et al 2006; Saris et al 2008).
Besides ACI, OAT or mosaicplasty has also showed promising results for treatment of chondral lesions (Hangody and Fules 2003). In OAT osteochondral autografts (round cylinders of full-thickness cartilage attached to a plug of its underlying bone) are harvested arthroscopically from less-weight-bearing areas of the knee. The autograft plugs are than transplanted to the chondral defect (Hangody et al 2004).
With various surgical options for the treatment of articular cartilage defects in the knee the question arises which criteria should be used for treatment selection. This systematic review tries to identify valid, evidence-based factors that may be used to optimize treatment selection for the restoration of articular cartilage lesions of the knee.

METHODS

To identify relevant publications, a systematic review of the best available literature on the treatment of articular cartilage defects of the knee with microfracture, autologous chondrocyte implantation or autologous osteochondral transplantation was performed. Since there are a sufficient number of studies that apply similar interventions on a comparable patient population we were able to perform this meta-analysis according to the accepted guidelines. Given some diversity of outcome parameters with specific focus and discerning capabili-

ties between studies, an additional subsequent evaluation was done. In this the individual papers original data were not complied for meta-analysis but rather we searched for relevant indicators for treatment selection or patient profile characteristics. To obtain relevant articles for the research question a literature search was conducted on August 25th 2009 in the electronic databases of MEDLINE (http://www.pubmed.com), EMBASE (http://www.embase.com) and the Cochrane Collaboration (http://www.cochrane.org) with the following parameters: "mosaicplasty" OR "autologous osteochondral transplantation" OR "autologous osteochondral mosaicplasty" OR "AOTS" OR "osteochondral autologous transplantation" OR "microfracturing" OR "microfracture" OR "autologous chondrocyte implantation" OR "autologous chondrocyte transplantation" OR "characterized chondrocyte implantation" OR "MACI". The search in the MEDLINE database was limited by [TiAb] while the search in the database of the Cochrane Collaboration was performed for controlled trials.

The retrieved articles were initially screened for relevance by the title and abstract. Inclusion criteria were: prospective randomized (or quasi-randomized) comparative studies, comparison of at least two of the previously mentioned treatment options and treatment of focal cartilage lesions of the knee. A focal cartilage lesion was defined as an isolated (osteo)chondral articular cartilage lesion without degenerative characteristics of the surrounding or opposing cartilage. Exclusion criteria were prospective cohort studies, studies without abstracts and articles in languages other than English, French, German and Dutch. Following this, full text of the remaining articles was obtained and further appraised on quality, by two separate observers (JB, MI), based on the Cochrane Handbook for Systematic Reviews of Interventions (Higgins and Green 2008). To determine the possibility of bias we examined the selection procedure (selection bias) and homogeneity of the patient population and pre- and postoperative care (performance bias). Attrition bias was scored based on the percentage follow-up of the primary outcome parameter. Possible detection bias was quantified by blinding of observers, validity of outcome measures and the statistical analysis whereas reporting bias was assessed by differences in description of outcome parameters between the study groups. An additional quantification of the degree of possible bias was performed by the modified Coleman Methodology Score (Coleman et al 2000). Level of evidence (LOE) was determined according to the guidelines of the Oxford Center for Evidence-based Medicine (CEBM). Following this articles were selected, based on the risk of bias, Modified Coleman score and LOE, to answer our clinical research question.

Data was collected from the remaining, good-quality, articles. The extracted demographic data included: authors, year of publication, surgical procedures analyzed, number of patients, mean age, gender, main inclusion criteria, defect location, defect size and defect grade. Primary and secondary outcomes were extracted as well as follow-up information.

RESULTS

Due to the heterogeneity of outcome parameters between the selected studies meta-analysis was not performed and data is summarized and expressed in tables as presented in the original articles.

The literature search in MEDLINE resulted in a total of 865 articles. After screening for relevance on title and abstract 856 articles were rejected because of failure to meet the in- and exclusion criteria. From the resulting 9 articles (Bentley et al 2003; Dozin et al 2005; Gudas et al 2005; Gudas et al 2006; Horas et al 2003; Knutsen et al 2004; Knutsen et al 2007; Kon et al 2008; Saris et al 2008). Two reported on similar outcome parameters and follow-up of the same patient population in different journals (Gudas et al 2005; Gudas et al 2006). The first published article (Gudas et al 2005) was considered as the original article and included for further analysis. An additional 2 articles also described a similar patient population, however, with different follow-up (Knutsen et al 2004; Knutsen et al 2007). We extracted data from both these articles and present it as a part of their first publication (Knutsen et al 2004). The search in EMBASE and the Cochrane Collaboration databases resulted in 1232 and 62 articles respectively. After application of in- and exclusion criteria only the 7 previously selected articles from the MEDLINE database were identified (Bentley et al 2003; Dozin et al 2005; Gudas et al 2005; Horas et al 2003; Knutsen et al 2004; Knutsen et al 2007; Saris et al 2008).

The quality of the included studies was determined on the risk of bias, the Modified Coleman Score and LOE (Table 1). Selection bias could not be ruled out for most studies because of an insufficient description of the inclusion rate and the lack of explanation on the difference between study eligible patients versus those truly included in the treatment regime. In the studies by Saris et al (Saris et al 2008), in which 118 of 151 contacted patients were eventually randomized, and Bentley et al (Bentley et al 2003), which included 100 consecutive patients, the patient enrolment was clearly described. In the other studies the description of the patient selection process was scare or absent and thus difficult to value. Given the outcome in table 1, the risk of performance bias was limited indicating homogeneity in study groups and pre- and postoperative care. Attrition bias was absent in all studies except for the study by Dozin et al (Dozin et al 2005) and Horas et al (Horas et al 2003) since both reported a follow-up of <80%. Detection bias was possible for the studies by Dozin et al (Dozin et al 2005), Bentley et al (Bentley et al 2003) and Horas et al (Horas et al 2003) because they did not describe any blinding in the assessment of the main outcome parameters. The description of outcome parameters by the study of Dozin et al (Dozin et al 2005) was incomplete whereas Bentley et al (Bentley et al 2003) only selected one treatment group for their secondary histological outcome parameter. The methodological quality of the studies was further assessed by the Modified Coleman Score. Most LOE 1b studies presented a high Modified Coleman Score (>80) whereas the Coleman Score in the LOE 2b studies was <80. Based on the previously described quality assessment, four studies (Bentley et al 2003; Gudas et al

Table 1 Quality assessment.

Authors	Study design	Selection bias	Performance bias	Attrition bias	Detection bias	Reporting bias	Coleman	LOE
Kon et al.	PCS	-	+	+	-	+	67	2b
Saris et al.	RCT	+	+	+	+	+	94	1b
Knutsen et al.	RCT	±	+	+	+	+	89	1b
Dozin et al.	RCT	±	+	-	±	-	64	2b
Bentley et al.	RCT	+	+	+	±	±	74	1b
Horas et al.	RCT	±	+	-	±	+	79	2b
Gudas et al.	RCT	±	+	+	+	+	86	1b

LOE: Level of Evidence following the guidelines of the Oxford Center for Evidence-based Medicine, Coleman: Modified Coleman Methodology score (Coleman et al., 2000) assessed by authors JB and MI.

2005; Knutsen et al 2007; Saris et al 2008) were included for further analysis of evidence to answer our clinical research question. All included studies were LOE 1b and showed a moderate to high Modified Coleman Score with a moderate-low risk of possible bias.

Bentley et al (Bentley et al 2003) compared mosaicplasty to ACI for the repair of symptomatic cartilage defects with an average size of 4.66 cm^2 in 100 consecutive patients (Table 2 and 3). He evaluated the treatment success with the Modified Cincinnati score and ICRS macroscopic grading system with an average follow-up of 19 months but also reported on the 12 months follow-up results. One year after surgery 88% of the lesions of the medial femoral condyle treated by ACI had excellent to good clinical results, compared to 74 % treated by mosaicplasty ($p = 0.032$). However the statistically significance was not present when examining the results at 19 months ($p=0.277$), which was the average follow up of the complete group at the time the study was described (Table 4). At macroscopic evaluation 12 months after surgery the ACI technique showed superior ($p<0.01$) cartilage regeneration compared to mosaicplasty.

The study by Saris et al (Saris et al 2008) compared microfracture to CCI. They evaluated 118 patients with symptomatic lesions of the femoral condyles and an average lesion size of 2.5 cm^2 (Table 2 and 3). At 6, 12 and 18 months follow-up both treatments had similar clinical outcome as measured with the Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire and recorded a comparable incidence of adverse events (Table 4). Structural analysis of the quality of cartilage regeneration demonstrated a superior histomorphometric ($p=0.003$) and histological score ($p=0.012$) for CCI compared to microfracture.

Gudas et al (Gudas et al 2005) included 60 competitive or well-trained athletes with a symptomatic osteochondritis dissecans (OCD) or full-thickness articular cartilage lesion. All lesions were located on the weight bearing portion of the femoral condyles and had an average size of 2.8 cm^2 (Table 2 and 3). A total of 60 patients were randomized to receive either microfracture or OAT. Both techniques showed a significant improvement

Table 2 Study and patient characteristics.

Authors	Year	Procedures	No. of patients	Mean age of patients	Gender	Main inclusion criteria	Follow-up
Bentley et al.	2003	ACI Mosaicplasty	100	ACI: 30.9 y Mosaicplasty: 31.6 y	Overall: ♀43, ♂57	Symptomatic lesion Persisten pain and reduction in activities Lesion size >1 cm^2 Biomechanically normal joint	12 and 19 months reported
Saris et al.	2008	CCI Microfracturing	118	CCI: 33.9y Microfracturing: 33.9 y	Overall: ♀42, ♂76	Single symptomatic lesion Patient age 18-50 y Lesion size 1-5 cm^2 Location: femoral condyle	6, 12 and 18 months
Gudas et al.	2005	OAT Microfracturing	60*	OAT: 24.6y Microfracturing: 24.3 y	Overall: ♀22, ♂35	Single symptomatic OCD lesion Patient age <40 y Only well trained athletes Lesion size 1-4 cm^2 Weight bearing femur condyle	6, 12, 24, 36 months
Knutsen et al.	2007	ACI Microfracturing	80	ACI: 33.3y Microfracturing: 31.1 y	Overall: ♀12, ♂48	Single symptomatic lesion Patient age 18-45 y Lesion size 2-10 cm^2 Location: femur condyle or trochlea	2 and 5 years

*A total of 3 patients were excluded from analysis because of loss to follow-up. ACI: autologous chondrocyte implantation, CCI: characterized chondrocyte implantation, OAT: osteochondral autologous transplantation.

Table 3 Defect characteristics.

Authors	Defect size	Defect location	Defect grade
Bentley et al.	4.66 cm^2 (1-12.2 cm^2)	Medial femur 53 % Patella 25 % Lateral Femur 18 % Trochlea 3 % Lateral Tibia 1 %	Osteochondral or chondral defects (Outerbridge grade III or IV)
Saris et al.	CCI: 2.6±1.0 cm^2 MF: 2.4±1.2 cm^2	Femur condyles (not further defined)	CCI: 74 % Outerbridge grade IV MF: 82 % Outerbridge grade IV
Gudas et al.	OAT: 2.80±0.65 cm^2 MF: 2.77±0.68 cm^2	OAT: 89 % medial femur, 11 % lateral femur MF: 79 % medial femur, 21 % lateral femur	Outerbridge grade III and IV
Knutsen et al.	ACI: 5.1 cm^2 MF: 4.5 cm^2	Femur condyles (not further defined)	96 % Outerbridge grade III or IV 4 % Outerbridge grade II

CCI: characterized chondrocyte implantation, ACI: autologous chondrocyte implantation, MF: microfracture.

Table 4 Outcome parameters.

Authors	Clinical outcome	Macroscopic evaluation	Histological evaluation
Bentley et al.	Mean follow-up (19months): - Mod. Cincinatti good-excellent outcome (score>55) • Overall: ACI: 88%, Mosaicplasty: 69%, p=0.277 Follow-up after 12 months: - Mod. Cincinatti good-excellent outcome* • Medial femur: ACI (88%) Mosaicplasty (73%), p=0.032 • Lateral femur: ACI (92%) Mosaicplasty (40%), p=0.182 • Patella: (85%) Mosaicplasty (60%), p=0.076	ICRS macroscopic grading at 12 months: - Excellent-good ICRS grading: • ACI: 82 %, Mosaicplasty: 34 %, p<0.01	Only biopsies from ACI group Origin: 3 patella, 16 femur condyle Histological appearance: - 7 predominant hyaline - 7 mixture hyaline and fibrocartilage - 7 predominant fibrocartilage
Saris et al.	Overall KOOS score: - Baseline: • CCI: 56.30±13.61, MF: 59.53±14.95 - 6 months • CCI: 70.56±12.39, MF: 72.63±15.55 - 12 months • CCI: 73.26±14.66, MF: 73.10±16.01 - 18 months • CCI: 74.73±17.01, MF: 75.04±14.50	Not reported	Evaluation 12 months after surgery: - Histomorphometric score+: • Significantly higher score for the CCI groups as compared to the MF group, p=0.003 - Histology assessment score#: • Significantly higher score for the CCI groups as compared to the MF group, p=0.012
Gudas et al.	HSS clinical score: - Significant increase from baseline for both OAT (77.88±6.12 to 91.08±4.15, p<0.0001) and MF (77.22±8.12 to 80.60±4.55, p<0.05) - OAT significantly higher score after 12 (p<0.05), 24 (p<0.01) and 36 (p<0.01) months compared to MF ICRS questionnaire: - Significant increase from baseline for both OAT (50.67±4.05 to 85.88±4.69, p<0.001) and MF (50.84±4.07 to 75.59±4.64, p<0.05) - OAT significantly higher score after 12 (p<0.03), 24 (p<0.001) and 36 (p<0.001) months compared to MF	ICRS macroscopic grading at 12 months - Excellent-good ICRS grading • OAT: 78.6 %, MF: 34 % - Fair ICRS grading • OAT: 45 %, MF: 55 % Difference in ICRS grading was statistically significant higher (p=0.004) for OAT as compared to MF	Evaluation 12 months after surgery: - 11 biopsies from OAT group • Viable hyaline cartilage in all - 14 biopsies from MF group • 57 % fibrocartilage, 43 % fibroelastic tissue with different appearance than surrounding cartilage

Table 4 continued

Authors	Clinical outcome	Macroscopic evaluation	Histological evaluation
Knutsen et al.	Lysholm: - Significant improvement from baseline for both ACI and MF after 2 ($p<0.003$, $p<0.001$ resp.) and 5 years ($p<0.05$ both) VAS: - Significant reduction in pain from baseline for both ACI and MF after 2 ($p<0.001$ both) and 5 years ($p<0.05$ both) SF-36 physical functioning: - More improvement from baseline for MF compared to ACI after 2 years ($p=0.01$), however not after 5 years ($p=0.054$) Tegner score: - Improvement in activity level after 5 years for both ACI (p0.007) and MF ($p=0.002$)	ICRS macroscopic grading at 24 months: no significant difference between the ICRS macroscopic scores for ACI (8.1) and MF (9.1) was detected, $p=0.17$	Evaluation 24 months after surgery: - Total of 67 biopsies (ACI: 32, MF:35) • Hyaline 19 % ACI 11 % MF • Hyaline/fibrocart: ACI: 31 %, MF: 17 % • Fibrocartilage: ACI: 34 %, MF: 57 % • No tissue: ACI: 16 %, MF: 15 %

*Trochlea and lateral tibia not reported because of low number, Mod. Cincinatti: Modified Cincinatti score, ICRS macroscopic grading: International Cartilage Repair Society Score for the macroscopic evaluation of cartilage regeneration, KOOS: Knee injury and Osteoarthritis Outcome Score, CCI: characterized chondrocyte implantation, MF: microfracture, +Histomorphometric score: measure of proteoglycan and collagen II content which is considered as an objective measure of cartilage regeneration, #Histological assessment score is based on several histological components of the articular cartilage tissue, HSS: Hospital for Special Surgery clinical score, ICRS questionnaire: questionnaire from the International Cartilage Repair Society to measure the success of cartilage repair, OAT: osteochondral autologous transplantation, Lysholm: questionnaire to measure knee function, VAS: visual analogue scale, Tegner score: questionnaire to measure activity level of patients.

from baseline scores for the Hospital for Special Surgery (HSS) and International Cartilage Repair Society (ICRS) questionnaires (Table 4). In addition, the patients treated with OAT presented a statistically significant better clinical outcome compared to the microfracture group (Table 4). An additional analysis for age and lesion size showed a better outcome for patients < 30years, regardless of treatment, whereas the microfracture arm showed significant ($p<0.05$) worse results with a defect size > 2 cm^2. No association between lesion size and OAT could be found ($p<0.85$). Moreover, 93 % of the patients treated with OAT returned to their pre-injury sports level whereas only 52 % reached that level of activity in the microfracture group. The macroscopic appearance 12 months after surgery was better ($p=0.004$) for the OAT group compared to the microfracture group and histological evaluation showed more fibrocartilage in the microfracture group (Table 4).

The studies by Knutsen et al (Knutsen et al 2004; Knutsen et al 2007) compared ACI to microfracture in patients with symptomatic articular cartilage defects of the femoral condyles and reported on the 2 and 5 years follow-up (Table 2). The average defect size was 5.1 cm^2 for the ACI group and 4.5 cm^2 for the patients receiving microfracture (Table 3). After 2 and 5 years follow-up a significant improvement in Lysholm score and reduction in pain was recorded for both the ACI and microfracture arm. However, a statistically significant difference between the two treatment options could not be observed (Table 4). After 2 years the SF-36 physical functioning subscale showed a higher score for the microfracture group ($p=0.01$) compared to the ACI group, which was not present at 5 years follow-up ($p=0.054$) (Table 4). Age <30 years old resulted in a better clinical outcome after 2 ($p=0.007$) and 5 ($p=0.013$) years follow-up for both treatment options, whereas lesions <4 cm^2 showed better clinical results ($p<0.003$) in the microfracture group. The ACI group did not show a relation between lesion size and clinical outcome ($p>0.89$). Macroscopic and histological evaluation 2 years after surgery did not show a difference in outcome scores when comparing between treatments. However, the histological analysis of the biopsies from the ACI group tended to have ($p=0.08$) a more hyaline-cartilage-like appearance.

All four studies reported similarity in average patient's age of included patients (between 24 and 34 years old) and gender distribution (predominantly men) (Table 2). Lesions were classified as either Outerbridge grade III or IV, although a small proportion (4 %) of the lesions in the study by Knutsen et al (Knutsen et al 2004) were, despite their inclusion criteria (Table 3), graded as Outerbridge grade II. Typically, the lesions were located in the weight-bearing portion of the femoral condyles except for 29 % of the lesions in the study by Bentley et al (Bentley et al 2003) (Table 3).

The three excluded LOE 2b articles (Dozin et al 2005; Horas et al 2003; Kon et al 2008) show similar results when compared to the included studies (Bentley et al 2003; Gudas et al 2005; Knutsen et al 2004; Saris et al 2008) as ACI performed clinically better when compared to microfracture (Kon et al 2008) for defects ranging from 1-5 cm^2 and no clinically relevant difference could be observed between ACI and OAT for focal lesions

larger than 1 cm^2 (Dozin et al 2005; Horas et al 2003). In addition, patient age did show a significant correlation to the resumption of sport activity (Kon et al 2008).

DISCUSSION

Despite a careful analysis of patients' symptoms and additional information from MRI or arthroscopy, the treatment choice of articular cartilage defects in the knee is still based on the surgeon's preference instead of an evidence-based decision. Although some reports suggest an influence of defect size, localization and patients' age on clinical outcome (Kreuz et al 2006; Krishnan et al 2006; Niemeyer et al 2008), currently, the literature lacks a systematic analysis of factors which could be the basis for valid, evidence-based, treatment selection. This systematic review, of the best available literature, sought to identify factors influencing clinical outcome in cartilage repair surgery. A systematic search of the MEDLINE, EMBASE and Cochrane Collaboration databases resulted, after application of in- and exclusion criteria and quality assessment, in a total of four randomized controlled trials (LOE 1b). The overall quality of the included trials was good-moderate (Table 1) and the baseline patient characteristics, such as mean age, gender, defect location and Outerbridge grade, were comparable between the studied trials (Table 2 and 3). However, most of them failed to describe the percentage of available patients who met the inclusion criteria, and reported less than 80 % follow-up on secondary outcome parameters (Bentley et al 2003; Gudas et al 2005; Knutsen et al 2004; Knutsen et al 2007; Saris et al 2008). Another, more general problem, in randomized trials on the repair of articular cartilage is the limitation of blinding participants and surgeons, which was also recognized as a limitation in the trials reviewed. The limitations in methodological quality of cartilage repair studies is generally recognized and related to year of publication and the Oxford level of evidence (Jakobsen et al 2005). In addition, the use of various different outcome parameters makes comparison between trials troublesome. Ideally, clinically relevant focal defects (defect size up to 3 cm^2) should be treated in a trial setting and the patients should be and stratified based on BMI, defect location and post debridement defect size. In addition, the outcome should be reported after, at least, 2 years follow-up using biopsy, MRI and validated clinical outcome tools, such as the KOOS (Bekkers et al 2009) or the IKDC Subjective Knee Form (Hambly and Griva 2008), as outcome parameters.

All trials showed an improvement from clinical baseline scores, regardless of treatment. For defects >4 cm^2 a better clinical improvement was observed when treated with ACI compared to OAT (Bentley et al 2003). For medium sized cartilage defects (approximately 2.8 cm^2) treatment by means of OAT led to better clinical outcome in comparison to microfracture (Gudas et al 2005). In addition, Gudas et al (Gudas et al 2005) and Knutsen et al (Knutsen et al 2004; Knutsen et al 2007) reported a poorer

clinical outcome after microfracture for lesions larger than respectively 2 and 4 cm^2 while the clinical outcome after ACI or OAT was not influenced by defect size (Gudas et al 2005; Knutsen et al 2004; Knutsen et al 2007). This suggests that small to large lesions can be treated with sophisticated procedures, like OAT and ACI. For small osteochondral lesions, <1 cm^2, treatment by means of single-plug OAT is preferred, whereas for small chondral lesions microfracture is a good option.

The macroscopic appearance one year after surgery also provides a basis for the stratification of treatment based on the preoperatively measured defect size as can be concluded from the studies from Bentley et al. and Gudas et al (Bentley et al 2003; Gudas et al 2005) (Table 4). On the contrary, the study by Knutsen et al did not report any differences in clinical outcome or macroscopic appearance between ACI and microfracture, although none of the poor outcomes had good cartilage appearance histologically. It has previously been shown that early treatment of cartilage defects results in superior histological, biochemical and clinical outcome compared to late treatment (Saris et al 2003). Based on this knowledge and given the frequent chronic condition of the cartilage defect in the study by Knutsen, one would expect a larger proportion of poor outcomes. The study by Saris et al (Saris et al 2008). did not find any differences in KOOS score between the two treatment groups after one year follow-up. Here the shorter follow up and the longer duration of symptoms in the ACI arm of the study by Saris could, be reasons for similarity in clinical outcome. Literature suggests the difference between ACI and microfracture to be greater in those with a shorter period between defect origin and treatment and the difference to increase with the duration of follow up (VanLauwe et al 2009).

In a recent non-randomized study patients treated with ACI maintained high levels of activity while the activity level of patients treated with microfracture decreased with time (Kon et al 2008) Gudas et al (Gudas et al 2005) only selected well trained athletes for his trial and showed superiority in clinical outcome of OAT compared to microfracture (Gudas et al 2005). A sub-analysis in the study by Knutsen et al (Knutsen et al 2004) also illustrated better results in more active patients treated with ACI compared to microfracture (Knutsen et al 2004; Knutsen et al 2007). Based on these studies it seems reasonable that treatment of more active patients should be by means of ACI or OAT instead of microfracture. However, previous case series, reported an improvement in functional status and activity levels after microfracture treatment of articular cartilage lesions in a group of high impact athletes (Mithoefer et al 2006; Steadman et al 2003). Therefore, there is insufficient evidence to base treatment selection primarily on the activity level of the patient.

Besides defect size and activity level, age can also be an influencing factor on clinical outcome since all included studies demonstrated better clinical outcome in patients <30 years regardless of applied treatment strategy (Bentley et al 2003; Gudas et al 2005; Knutsen et al 2004; Saris et al 2008). Both microfracture repair and cellular regeneration seem to be more effective in the younger patient. Data currently does not allow us to

make strong selection choices based on age, however the successful regeneration and longevity of the tissue would have a greater impact in the young patient which could favor the cell therapy option in the young patient.

Although clinical outcome is the logical, most important, primary outcome parameter in cartilage repair trials, histological quality might provide valuable information about durability of the repair tissue. Repair tissues mainly composed of fibrocartilage have a higher likelihood of developing symptoms compared to repair tissues with predominantly hyaline cartilage (Henderson et al 2007). Even in an ACI cohort, hyaline morphology resulted in better performance compared to fibrocartilage morphology (Roberts et al 2003). Most of the studies in this review showed a statistically significant outcome (Bentley et al 2003; Saris et al 2008) or trend (Knutsen et al 2004; Knutsen et al 2007) towards superiority of the repair tissue after ACI in terms of histological and histomorphometric analysis. The study by Bentley et al (Bentley et al 2003) only evaluated the histological outcome after ACI which showed a predominant hyaline appearance. Whether the quality of the repair tissue in these studies really leads to improved clinical durability remains to be seen in future long term follow-up reports. In addition, previously published systematic reviews indicate that on a short term basis ACI is not cost-effective since it requires a two-staged procedure and the recovery of patients is relatively slow compared to microfracture (Clar et al 2005; Ruano-Ravina and Jato 2006; Wasiak et al 2006) However, economic modeling suggested that, on the long-term outcome, ACI would be cost-effective because it is more likely to produce durable hyaline cartilage (Clar et al 2005).

In conclusion, this systematic review is, to our knowledge, the first that primarily focuses on possible selection criteria for the treatment selection of Outerbridge grade III and IV articular cartilage lesions of the knee. Conclusive evidence to support treatment selection solely based on lesion size is currently not available. Based on the results of our systematic literature search we can conclude that small chondral and osteochondral lesions (<1 cm^2) should preferably be treated by microfracture or single-plug OAT. For larger lesions (>4 cm^2) microfracture has been associated with limited effectiveness. For larger lesions OAT and ACI are both good treatment options. In addition, it should be mentioned that estimation of the lesion size is extremely difficult (van den Borne et al 2007) and that use of the post-debridement lesion size is preferred. Activity level should also be assessed, using the Tegner activity rating scale and the KOOS ADL and Sports subdomains, or IKDC Subjective Knee Form since it can be used as an additional parameter, besides defect size, in the treatment selection of articular cartilage lesions. Results of treatment in active patients with large articular cartilage lesions are better after ACI or OAT compared to microfracture. Furthermore, patient's age influenced the clinical outcome. With the ongoing improvement of repair techniques, such as ACI, and the quality of randomized controlled trials the factors that should be considered in selecting treatment for articular cartilage lesions might change, however, currently this is the best available evidence.

Chapter 14

Summary, general discussion and clinical implications

Focal articular cartilage lesions impose a serious socioeconomic burden and challenge the injured. The inability to participate in sports or even being hampered in activities of daily living, due to knee pain, swelling and locking, are huge restrictions that do not fit the overall active lifestyle of the decennia in life (patients range from 20-40 years) at which a focal lesion usually clinically presents. Several treatments can be applied to manage the defect and restore the articular surface to improve knee function and reduce pain. Among those, microfracture, autologous chondrocyte implantation (ACI) and osteochondral autologous transfer (OAT) are most frequently applied. As each treatment is based on a different principle it is reasonable to assume that their performance differs per type of patient cohort. Therefore, increasing knowledge on what factors determine clinical success per treatment type is essential to take full advantage of the potential of a specific technique. In addition, the fact that a treatment is clinically successful does not mean that further evolution is impossible. ACI, for example, has some generally acknowledged shortcomings, which, if solved, could possibly even further improve the clinical results. This also goes for the evaluation of cartilage treatment. The introduction and use of validated outcome tools that provide an idea of the clinical success as well as the quality of structural regeneration would be ideal.
Therefore the aim of this thesis was to improve the clinical outcome of patients with a focal articular cartilage lesion treated with autologous chondrocyte implantation, by improvement of the surgical technique, the development of specific treatment algorithms and the evaluation and validation of suited outcome tools.

IMPROVEMENT OF AUTOLOGOUS CHONDROCYTE IMPLANTATION

The current autologous chondrocyte implantation procedure is limited by the amount of cartilage that can be biopsied during the first surgery. Therefore, insufficient cells can be obtained and *in vitro* expansion, which causes dedifferentiation of reimplanted cells, is still needed. Recent studies strongly suggest that the addition of another cell type to articular chondrocytes positively stimulates cartilage matrix formation. Cells from mesenchymal origin show the most consistent results regarding this interaction. As a consequence, a combination of cells would obviate expansion because enough cells will be available to fill the focal lesion within one surgery. *Chapter 2 and Chapter 7* describe the added value of mononuclear fraction cells (MNF) and mesenchymal stromal cells (MSC) when combined with cartilage cells. Both MNF cells and MSCs stimulate cartilage matrix production when a range of 10-20 % chondrocytes are combined with 90-80 % MNFs or MSCs. These results seem more outspoken when MSCs are used. Also when a combination of MSCs and chondrons was embedded in Beriplast® cartilage matrix formation was higher compared to the use of chondrons alone, as described in *Chapter*

7. Chapter 3 focuses on what scaffold best facilitates the cartilage matrix formation when a combination of chondrocytes and MNF cells is used. We showed that the combination of MNF cells and chondrocytes yield most cartilage matrix formation in Beriplast® as a scaffold. *Chapter 4* shows that the chondrogenic potential of the cells obtained from debrided defect cartilage is at least as good as of those from the current biopsy site used in ACI. Furthermore, the non-weight-bearing biopsy site showed to be chondropenic and its resident chondrocytes poorly capable of cartilage regeneration. The clinical handling and application of several fixation techniques for the fixation of a solid scaffold into a cartilage defect has been described in *Chapter 5*. Here we showed that optimal fixation cannot always be combined with preservation of scaffold integrity and that the interaction between material scientists and clinicians is mandatory to solve this problem. Finally, the concept of a one-stage cell-based regenerative cartilage treatment, based on the combination of cells has been tested in a relevant preclinical model. For this, two goat studies were designed where a fresh cartilage defect was treated using a either a combination of cartilage cells with MNF cells *(Chapter 6)* or with MSCs *(Chapter 7)* in Beriplast®, compared to microfracture treatment, focusing on safety, feasibility and treatment effect. The one-step chondrocyteMNF treatment showed better macroscopic cartilage regeneration compared to microfracture *(Chapter 6)*. However, incomplete defect fill and degenerative changes at distant locations in the joint were observed. On the other hand, the combination of chondrons and MSCs led to better macroscopic, microscopic and biochemical cartilage regeneration compared to microfracture *(Chapter 7)*.

EVALUATION AND VALIDATION OF OUTCOME TOOLS FOR CARTILAGE REGENERATION

The growing focus on treatment of cartilage defects using regenerative approaches creates a need for validated outcome tools that not only provide a quantitative measure of the clinical improvement but also informs on the quality of the regenerated articular cartilage and that at distant locations. *Chapter 8* provides an overview of the current diagnostic modalities that can be used to evaluate diseased and treated articular cartilage. *Chapter 9* illustrates the validity and reliability of the Knee Osteoarthritis Outcome Score (KOOS) in measuring the clinical condition of patients after treatment of focal cartilage lesions. The major question of *Chapter 10* and *Chapter 11* was whether the delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) can validly be applied to evaluate the effect of cartilage treatment on the articular surfaces in the knee. *Chapter 10* shows that dGEMRIC is able to longitudinally monitor local repair after regenerative cartilage surgery. Also *Chapter 10* illustrates the positive influence that local regeneration has on the quality of articular cartilage at distant locations in the knee as determined by

dGEMRIC. Finally, *Chapter 11* describes the use of dGEMRIC to show that implantation of a Trufit plug for the treatment of focal cartilage lesions is safe and does not introduce damage to the surrounding or articulating cartilage surfaces.

PATIENT ORIENTED TREATMENT SELECTION

Identification of specific patient and defect characteristics will aid in the development of personalized treatment plans and allow for a more detailed explanation of realistic expectations from surgery. *Chapter 12* illustrated that patient age, duration of symptoms and defect location are important prognostic factors that determine the clinical success of regenerative cartilage surgery. In addition, *Chapter 13* presents an evidence-based treatment algorithm for the treatment of focal cartilage lesions. This algorithm describes that small articular cartilage lesions should be treated using microfracture while larger lesions perform best when treated with ACI or OAT. The treatment selection of intermediate lesions depends on the activity level of the patient.

GENERAL DISCUSSION

Lesions of the articular cartilage are common. A review of 31,516 arthroscopies in symptomatic knees showed an incidence of focal articular cartilage lesions of 63 % (Curl et al 1997). In 37 % the symptoms were directly related to a single articular cartilage lesion while in the other cases also concomitant intra-articular pathologies, like ACL or meniscus tears, were present. Patients with knee symptoms usually present at the outpatient clinic with knee complaints that hinder them from sports participation or in general activities of daily living. In both cases, the quality of life of this generally young patient population (age 20-40 years) is affected. Treatment starts with defining the correct treatment indication, selection of the suited cartilage surgery and setting boundaries for realistic expectations from surgery based on the characteristics of the defect and patient. Newly developed technologies allow further evolution of current treatments to improve practical handling, create simpler treatment logistics and lead to higher quality tissue regeneration. In this thesis we showed that changing the cell source harbours a clear possibility to improve the procedure and outcome of ACI. Addition to MNF cells to chondrocytes in culture leads to improved cartilage formation *in vitro* but also after subcutaneous implantation in nude mice. This combination of cells, embedded in Beriplast® also showed better cartilage regeneration compared to microfracture in goats. Both *in vitro* and *in vivo* this effect was more outspoken when MSCs were combined with chondrocytes and even more when using chondrons. The basis for stimulation of cartilage matrix formation by MNFs and MSCs is most likely communication of cells by trophic factors from the added cell type and direct cell-cell contact (Wu et al 2011; Wu et al 2012). Although MSC and MNF cells disappear during *in vitro* culture, direct cell-cell contact at the beginning of culture is important as similar positive results on cartilage matrix production were not observed when MSC conditioned medium was used *in vitro* (Wu et al 2012). The proposed added value of the presence of another cell type is the continuously changing communication and adapted excretion of trophic factors by the communicating cells based on their needs. The stimulation of differentiation, cell replacement, tissue renewal, regeneration and wound repair by stimulating factors excreted by the tissue specific stem cells is a phenomenon that has been described for other tissues and organs as well. Bone marrow derived MSCs were able to initiate β-cell recovery in the pancreas of diabetic mice, via trophic factors (Milanesi et al 2012). Intramyocardial injection of cardiac stem cells in regions of infarction or stenosis resulted in either muscle regeneration or creation of large conductive vessels (Hosada 2012) indicating a role of the MSC in tissue repair and renewal. Also hepatic stem cells use soluble factors and growth factors to repair liver damage and initiate oval cell-mediated liver regeneration (Chen et al 2012). In skin wound repair, several reports suggested MSCs

leave their bone marrow niche and migrate to the damaged area to deposit paracrine factors that assist in wound repair (Fu and Li 2012).

The combination of MSCs or bone marrow cells obviate the need for expansion of chondrocytes and hence a second surgery in ACI. It appeared that the primary cartilage cells used also showed to be sensitive to stimulation from added cells and obtainable at sufficient amounts with good viability within the timeframe of one surgery using a newly develop digestion protocol. The advantage of this procedure is that it yields chondrons rather that chondrocytes, which appeared to result in optimal cartilage regeneration upon combination with MSCs. The microenvironment of the chondrocyte in its pericellular matrix previously showed to enhance chondrocyte extracellular matrix production compared to chondrocytes without a pericellular matrix (Larson et al 2002; Vonk et al 2010). In general the pericellular matrix stimulates cell-cell contact and is important for the communication of the cell with its environment. Direct binding of growth factors to pericellular matrix (PCM) proteins plays an important role here which would make the PCM more than just a provider of mechanical and structural support to the cell. Binding of growth factors to the surface PCM molecules facilitates signaling events which are crucial in processes such as cell proliferation, survival and differentiation (Brizzi et al 2012; Kim et al 2012). Integrins are known to play a major role in these signaling processes of the chondrocyte niche (Loeser 2012).

The only cells tested in coculture with chondrons and or chondrocytes in this thesis were MSCs and MNF cells. These cells were selected because of their mesenchymal origin and/or ease of harvest. Also other stem cells, capable of secretion of trophic factors, might be alternatives as well, for example adipose-derived stem cells, synovial stem cells, induced pluripotent stem cells (IPS) and embryonic stem cells. Stem cells from Hoffa's fat pad showed to have good chondrogenic potential, albeit *in vitro* (Jurgens et al 2009). Also adipose derived stem cells recently showed to improve cartilage specific gene expression when combined with chondrocytes (Hildner et al 2009). Synovium derived MSCs are discussed and presented as a potential viable source of MSCs with possibly even greater chondrogenic potential compared to bone marrow-derived MSCs (Shirasawa et al 2012; Suzuki et al 2012). Performance of these cells in animal models still needs to be evaluated. Moreover, harvesting synovium to obtain MSCs is painful and introduces a risk of joint infection. New innovations, such as the creation of the induced pluripotent stem (iPS) cell and the use of embryonic cells to culture cartilage in vitro are promising. However, before the latter two cell types can be applied in a one-stage clinical application to regenerate cartilage as a substitute of MSCs, hurdles dealing with safety (i.e. teratoma formation), ethics and *in vivo* performance have to be taken first.

As the use of MSCs in combination with non-expanded chondrons still would preclude a one-stage procedure, the option of using allogeneic cells was investigated. One-stage treatment of focal lesions in goats was performed using autologous chondrons and allogeneic MSCs. The latter cell might provide safety issues, based on immunological

grounds, when translation to a human phase one trial is conducted. However, MSCs lose their specific MHC expression during expansion which is only gradually increased when embedded in hydrogels (Griffin et al 2010; Mosna et al 2010; Yuan et al 2011). Moreover, several clinical trials, where allogeneic MSCs were intravenously injected, showed no side effects or major adverse events due to the use of allogeneic MSCs (Griffin et al 2010; Hare et al 2009; Koc et al 2002). Minor adverse events, like fever and mild allergic reaction, have been described following intravenous MSC treatment (Duijvenstein et al 2010; Karussis et al 2010). Moreover, as articular cartilage is avascular the contact between implanted allogeneic MSC and host rejection cells is unlikely. Therefore we propose a phase I trial where the safety of the treatment of focal cartilage lesions using allogeneic MSCs combined with chondrons embedded in Beriplast® could be tested as a logical next step to further develop this new treatment.

The additionally created cartilage damage to obtain cells for treatment of a focal cartilage lesion, as performed during the current biopsy in ACI, is illogical knowing that the debrided defect cartilage is discarded. Moreover, a small biopsy as currently performed in ACI would, although not tested in this thesis, most likely not provide enough primary cells for larger cartilage defects treated one-stage using a combination of chondrons and MSCs. Moreover, tissue from non-weight-bearing regions showed to be degenerative. This has previously been suggested based on mathematical modeling and local chondropenia in non-weight-bearing regions in overall healthy joints (Bullough et al 1973; Bullough and Walker 1976; Carter et al 2004). When taking the loading-directed development of articular cartilage during life into account it makes sense that low-loaded regions in the joint have other matrix properties compared to weight-bearing regions (Buckwalter and Mankin 1998). These results also urge a further re-consideration of transplanting autologous osteochondral plugs as currently being performed during the OAT procedure. Based on histological examination it seems that actually cartilage with a hypertrophic degenerative aspect is being transplanted. Also, signs of severe cartilage degeneration of the transplanted plugs have been described after OAT in sheep (Tibesku et al 2004). Recently introduced synthetic plugs, like the Trufit plug, might be a good alternative for the OAT procedure in small focal cartilage lesions.

Further development of cartilage treatments is important, however, without standardized and validated outcome measures the added value of such new developments would be hard to determine. For articular cartilage regeneration, macroscopic and microscopic measures have already been validated and used in several clinical trials (O'Driscoll et al 1986; O'Driscoll et al 1988; van den Borne et al 2007). Patient reported questionnaires, like the KOOS, are preferred when used as an instrument to assess clinical outcome to prevent from observer administered bias. Next to assessment of clinical success, the structural repair of the tissue is also essential. Previous reports show that deterioration of clinical success goes hand in hand with insufficient tissue repair (Nehrer et al 1999). The other way around, adequate tissue repair will eventually lead to bet-

ter clinical results (Saris et al 2008; Saris et al 2009). Newly developed MRI techniques representative for cartilage matrix components which are able to provide a mapping of the articular cartilage could provide additional information to determine the right indication for surgery and to provide detailed evaluation of treatment outcome (Bashir et al 1999; Fragonas et al 1998; Gray et al 2008; Liess et al 2002; Multanen et al 2009; Nieminen et al 2001; Xia et al 2008). In this thesis we showed that the dGEMRIC technique is able to show cartilage regeneration but also degeneration of articulating surfaces following cartilage surgery. However, the main disadvantage of dGEMRIC is from practical origin as an intravenous injection of gadolinium must be administered ninety minutes before scanning which is time consuming and logistically difficult to manage. For this reason other quantitative MRI techniques, like T2 mapping or T1ρ, could be interesting alternatives (Akella et al 2001; Fragonas et al 1998; Liess et al 2002; Nieminen et al 2001; Wheaton et al 2005; Wheaton et al 2005). So far prospective longitudinal studies with baseline measurements to show the potency of both MRI techniques to follow cartilage regeneration are lacking. Such studies could easily be included in the current clinical setting where patients receive MRI scans before and after cartilage surgery as both the T2 and T1ρ MRI scanning protocol can be applied without further patient preparations.
Selection of the surgical approach to repair an articular cartilage lesion is to a large extent based upon the preference and skills of the surgeon while possible influential factors play a minor role in this decision. A surgeon should be aware of the physiology of the selected treatment, have a clear idea on the eventual result the patient desires and his/her motivation to get there. These factors highly influence the type and quality of postoperative rehabilitation and thereby the clinical success. Evidence-based criteria that can be used as a guide to select the best treatment for a specific situation were compiled in a flow chart in this thesis (Bekkers et al 2009). In this flow chart treatment selection was primarily based upon post-debridement lesion size and activity level of the patient. However, other characteristics showed to influence clinical success after cartilage surgery as well. Location of the cartilage defect at the medial condyle has been associated with improved clinical outcome compared to those at the lateral condyle (Kreuz et al 2006; Windt de et al 2009). Patient age and duration of symptoms were both related to clinical success, independent of the treatment applied (Gudas et al 2005; Knutsen et al 2007; VanLauwe et al 2009; Windt de et al 2009). However, a recent 5 years follow-up comparing characterized chondrocyte implantation (CCI) to microfracture showed that defects with duration of symptoms less than 3 years resulted in statistically significant and clinically relevant better results after CCI compared to microfracture (Vanlauwe et al 2011). This difference was not seen in patients with symptom duration longer than 3 years until treatment. Apparently, late treatment, possibly due to an extended period of disturbed joint environment with ongoing cartilage matrix degeneration (Saris et al 2003), has more influence on CCI compared to microfracture. On the other hand a long

period of disturbed joint homeostasis could also disturb the intra-articular environment to such an extent that the environment itself causes a limitation of the maximum improvement following either cartilage surgery. Also, the activity level of the patient differentially affected the clinical outcome of the different treatments (Gudas et al 2005; Knutsen et al 2004; Knutsen et al 2007; Kon et al 2008). ACI and OAT were superior to microfracture in patients with a high activity level, but not in patients with average activity levels. Abovementioned characteristics are important as they provide the physician with the opportunity to create patient specific treatment plans instead of general, not evidence-based, guidelines. As an example, this means that a patient of 40 years old with a small symptomatic articular cartilage lesion since 1 year that would be happy with a reduction of pain during daily living should be treated differently compared to a patient of 25 years old with a similar lesion since 1 year wishing to go back to professional sports. This example shows the relevance of exploring the characteristics of the defect and the patient and its expectations from surgery. Without this, optimal benefit from cartilage surgery will not be achieved. In addition to that, most of the current studies that provide some evidence on factors that influence the clinical success of cartilage surgery are either retrospective or underpowered to do such subanalysis. Therefore new clinical trials that compare two or more cartilage repair procedures should be sufficiently powered to perform additional subanalysis that take the effects of treatment specific as well as patient specific characteristics on clinical outcome into account. Increasing knowledge on the clinical benefit of cartilage surgery at specific group of patients (i.e. with specific characteristics) would also make the QALY's (quality-adjusted life year) as a measure of disease burden easier to assess. Combining health related quality of life measures with the additional costs of this specific intervention allows the calculation of cost-utility ratios which are important, from both a health care as well as health insurance perspective, for the clinical implementation of new interventions.

CLINICAL IMPLICATIONS OF THIS THESIS

The discussion on the superiority of a specific cartilage surgery modality is a frequently started but less relevant discussion because all treatments differ based on their biological mechanism of action (stimulation, replacement, regeneration) to repair or regenerate articular cartilage defects. In short it is not important which treatment is better than which other alternative but rather we should have a scala of well understood treatments and biological regeneration mechanisms to fit each individual patient profile and how that may fit the desired outcome. The improvement of newly developed techniques should not focus and be commercialized as a new superior technique but be presented as a possible clinical improvement of a current treatment within the patient and defect-related boundaries of that improved therapy. Also, the clinical limitations for the application of the one-stage cell-based therapy, as preclinically developed in this thesis, are to be determined in upcoming clinical trials. In an animal model, this new treatment performed better compared to microfracture, which is the current, evidence-based, treatment for small lesions. Whether the use of a mix of chondrons and MSCs will lead to similar cartilage regeneration and clinical results as ACI and OAT in larger lesions is unclear, however, macroscopic and microscopic results *in vivo* are promising. After proven safety in a phase I trial, ideally, a phase III trial comparing the presented newly developed one-stage treatment to microfracture and ACI would be the logical next step to define the clinical field for this biosurgical innovation. Such trials should, ideally, be powered sufficiently to perform additional subanalysis on patient characteristics. Similar clinical results are to be expected from this one-stage treatment as have been reported for ACI while other factors will favour clinical use.

From a scientific point of view, further evaluation of what stimulating factors are essential in the communication with chondrons and direction towards improved cartilage matrix production would be valuable to improve understanding of cartilage cell and matrix biology. If identified and able to be managed and applied to such extend that it fits the need of the chondron depending on its cellular activity and matrix forming status addition of any cell type could be substituted by addition of stimulating factors. This situation would be ideal because safety issues related to allogeneic transplantation or implantation of stem cells would be solved.

Knowledge of the characteristics of a patient and their disease is, as shown in this thesis, essential to select the best-suited treatment approach and, thus, have optimal clinical benefit from surgery. For this reason treatment selection should change to evidence-based patient profiles instead of the preferred treatment of the surgeon. Also, globally accepted and validated outcome tools will ease direct comparison between trials. With similar outcome measures, meta-analysis can be performed which will create a stable, evidence-based, foundation for patient-profile-driven treatment selection. Developments in the field of quantitative MRI will provide information that leads to better substantiated indications for

surgery (for example no regenerative therapy in a degenerating knee) and allow for a more detailed evaluation of the effects of treatment, both locally and in the whole knee.
In conclusion, the treatment of focal articular cartilage lesions is not just performing that one surgery but a process where various modalities interact, support, influence and direct decision making and treatment evaluation. It all starts with the diagnosis of a lesion, selecting a suitable treatment, agree on expectations with your patient and ends with a proper rehabilitation and evaluation of treatment success. Along this path of decisions, treatments and clinical evaluation the constant focus should be pointed at optimal care for the patient and gaining knowledge to improve current standards. One-stage cell-based therapy is the next generation of cartilage repair and has opened a whole new and exciting field.

Chapter 15

References

Dutch summary / Nederlandse samenvatting

List of publications and presentations

Dankwoord

Curriculum vitae

Stellingen

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DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

Geïsoleerde defecten van het kniekraakbeen vormen een groot probleem voor de vaak jonge en actieve groep patiënten (20-40 jaar oud) die dit overkomt. Zwelling en pijn in de knie en het optreden van slotklachten beperken de patiënt in het beoefenen van sport, het professionele werk of normale dagelijkse activiteiten. Er zijn verschillende typen behandelingen voorhanden voor deze geïsoleerde defecten. Microfractuur, autologe chondrocyten implantatie (ACI) en autologe osteochondrale transplantatie (OAT) zijn de meest toegepaste vormen. Bij een microfractuur behandeling worden kleine gaatjes in het bot op de bodem van het kraakbeendefect gemaakt waardoor beenmerg het gat instroomt om aldaar de vorming van kraakbeen te stimuleren. ACI is een techniek waar tijdens een eerste operatie kraakbeencellen worden geoogst. Deze cellen worden vervolgens opgekweekt in een laboratorium en tijdens een tweede operatie teruggeplaatst in het schoongemaakte kraakbeendefect waar ze nieuw kraakbeen gaan vormen. Tijdens de OAT plastiek worden grote pluggen van bot en kraakbeen geoogst van onbelaste gedeelten in de knie die getransplanteerd worden naar het kraakbeendefect om dit te vullen.

Gezien iedere behandeling gebaseerd is op een ander werkingsmechanisme (stimulatie bij microfractuur, regeneratie bij ACI en transplantatie bij OAT) mag worden verwacht dat het succes van een specifieke behandeling afhankelijk is van bepaalde kenmerken van de patiënt. Identificeren van deze karakteristieken is essentieel om elke afzonderlijke patiënt te behandelen met de, voor hem of haar, juiste therapie om zodoende maximaal resultaat te behalen van het chirurgisch ingrijpen.
Daarnaast is het ook belangrijk de huidige therapieën niet te beschouwen als producten die af zijn maar als toepassingen die verder doorontwikkeld en verbeterd kunnen worden. Een mooi voorbeeld hiervan is ACI. Aanvankelijk werden de opgekweekte cellen teruggeplaatst in het defect dat vervolgens werd afgesloten door een stukje botvlies. Dankzij voortschrijdende kennis van materialen is het nu mogelijk de cellen op te lossen in een gel of ander biomateriaal waarmee het defect vervolgens wordt gevuld. Dit is met name vanuit chirurgisch technisch oogpunt een verbetering. Echter, ondanks deze vooruitgang moet de patiënt nog steeds twee keer geopereerd worden en krijgt hij minder gezonde, opgekweekte cellen teruggeplaatst die een mix van littekenweefsel en gezond kraakbeenweefsel vormen.

Ook voor de evaluatie van de behandeling van geïsoleerde kraakbeendefecten is er vooruitgang te boeken. Het gebrek aan gevalideerde vragenlijsten maakt het kwantificeren van klinisch behandelsucces onmogelijk. Daarnaast wordt op dit moment standaard MRI gebruikt om een indruk te krijgen van de mate van vulling van het defect als

maat voor chirurgisch effect van de behandeling. Dit zegt echter niets over de kwaliteit van het nieuw gevormde kraakbeen.

Ter verbetering van bovengenoemde problemen beoogt dit proefschrift de klinische uitkomst van patiënten met een kraakbeendefect behandeld met ACI te verbeteren door een aanpassing van de chirurgische behandeling, de ontwikkeling van patiëntspecifieke behandelalgoritmen en de validatie van geschikte uitkomstmaten.

Verbetering van autologe chondrocyten implantatie

De huidige ACI procedure wordt beperkt door de hoeveelheid kraakbeen dat kan worden geoogst uit de knie. Er kan simpelweg niet zoveel kraakbeen geoogst worden aangezien een nieuw defect wordt gecreëerd om het bestaande defect te behandelen. Om deze reden is de opbrengst van kraakbeencellen beperkt en is vermenigvuldiging in een kweeklaboratorium noodzakelijk. Dit veroorzaakt een soort van geheugenverlies bij de kraakbeencellen waardoor zij minder goed in staat zijn om gezond kraakbeen te vormen in vergelijking met cellen die niet vermenigvuldigd zijn in een kweekomgeving. Recente inzichten tonen aan dat het combineren van kraakbeencellen met ander typen (stam)cellen een positieve invloed heeft op de kraakbeenvorming. Cellen van mesenchymale origine lijken de meeste potentie te hebben om dit combinatie effect te stimuleren. De positieve bijkomstigheid van deze stimulerende interactie is dat vermenigvuldiging van cellen overbodig wordt aangezien er voldoende cellen voorhanden zijn om een kraakbeendefect in één operatie direct te vullen. Door cellen te combineren profiteer je van de interactie tussen kraakbeencellen en andere celtypen, het gebruik van gezondere, niet vermenigvuldigde kraakbeencellen en het gegeven dat de patiënt maar één operatie hoeft te ondergaan.

Hoofstuk 2 en 7 beschrijven de stimulerende werking op kraakbeenvorming van de interactie tussen kraakbeencellen en mononucleaire fractie (MNF) of mesenchymale stamcellen (MSC) uit beenmerg. Toevoegen van MNF cellen of MSCs aan kraakbeencellen leidt tot meer kraakbeenvorming dan gekweekte kraakbeencellen zonder toevoeging van andere cellen. Dit effect lijkt groter als MSCs worden toegevoegd aan kraakbeencellen in vergelijk met MNF cellen. In Hoofdstuk 3 wordt de combinatie van kraakbeen en MNF cellen gezaaid in verschillende matrices om te bekijken welke matrix het best de kraakbeenvorming stimuleert. Hieruit blijkt dat de hydrogel Beriplast® superieur is, zowel in een kweekmodel als na subcutane implantatie in naakte muizen. Hoofdstuk 4 toont aan dat kraakbeencellen verkregen uit restanten van kraakbeenweefsel in een kraakbeendefect meer potentie hebben tot kraakbeenvorming dan de cellen van het niet gewichtsdragende gedeelte in de knie dat nu wordt gebruikt voor ACI. Vervolgens laat hoofdstuk 5 zien dat de solide fixatie van een scaffold in een kraakbeendefect niet altijd gepaard gaat met behoud van integriteit van die scaffold. Om deze reden is verdere

ontwikkeling van fixatiemethoden en scaffolds noodzakelijk om goede fixatie zonder beschadiging van het implantaat te bereiken. Bovengenoemde studies hebben ertoe geleid dat een nieuwe kraakbeentherapie, gebaseerd op de combinatie van kraakbeen cellen en MNF (Hoofdstuk 6) en kraakbeen cellen en MSCs (Hoofdstuk 7) gemixed in Beriplast®, vergeleken werd met microfractuur behandeling van vers gecreëerde defecten in het kniekraakbeen van geiten. Hieruit is gebleken dat de combinatie van kraakbeencellen met MNF cellen leidt tot betere macroscopische kraakbeen reparatie terwijl de combinatie van kraakbeencellen met MSCs beter macroscopisch, microscopisch en biochemisch kraakbeenherstel geeft in vergelijking met microfractuur behandeling.

Evaluatie en validatie van uitkomstmaten voor kraakbeenregeneratie

De ontwikkeling van nieuwe regeneratieve therapieën voor de behandeling van geïsoleerde kraakbeendefecten vraagt tevens om gevalideerde uitkomstmaten die een kwantitatieve weerspiegeling zijn van de klinische vooruitgang en informeren over de kwaliteit van het nieuwgevormde weefsel. Hoofdstuk 8 geeft een overzicht van de huidige diagnostische mogelijkheden om kraakbeen en kraakbeenbehandeling te evalueren. Daarnaast beschrijft hoofdstuk 9 de validatie van de Knee Osteoarthritis Outcome Score (KOOS) als meetinstrument van de klinische verbetering na kraakbeenchirurgie. In Hoofdstuk 10 en 11 wordt de delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) techniek gebruikt om de chirurgische behandeling van geïsoleerde kraakbeendefecten te evalueren. Hoofdstuk 10 toont dat de dGEMRIC techniek valide gebruikt kan worden voor de evaluatie van kraakbeenregeneratie. Gebruik makend van de dGEMRIC techniek laat hoofdstuk 10 tevens zien dat lokale kraakbeenregeneratie een positieve invloed heeft op de algehele kraakbeenkwaliteit in de knie. Hoofdstuk 11 illustreert, met behulp van dGEMRIC, dat implantatie van een Trufit® plug in een klein kraakbeen defect veilig is en geen extra schade toebrengt aan het direct articulerende kraakbeen.

Patiëntgerichte behandelselectie

Iedere patiënt met een kraakbeendefect dient de behandeling te krijgen die het best past bij zijn situatie en leidt tot maximale ziektewinst. Identificatie van patiëntspecifieke en defectkarakteristieke factoren die de uitkomst van kraakbeenchirurgie beïnvloeden kunnen gebruikt worden om de best passende behandeling voor de individuele patiënt te selecteren. Daarnaast is het belangrijk dat deze behandeling goed aansluit op zijn of haar wensen en verwachtingen van chirurgie of deze eventueel bijstuurt. Hoofdstuk 12 toont aan dat factoren zoals leeftijd van de patiënt, duur van knieklachten en locatie van het defect in de knie belangrijke prognostische factoren zijn die het succes van kraakbeentherapie bepalen. Vervolgens worden in hoofdstuk 13 deze factoren gebruikt om zogenaamde evidence-based behandelalgoritmen te creëren die als leidraad gebruikt kunnen worden om de best passende behandeling voor de individuele patiënt te selec-

teren. Deze algoritmen beschrijven dat kleine kraakbeendefecten behandeld moeten worden met microfractuur terwijl grotere laesies, afhankelijk van het activiteitsniveau van de patient, behandeld moeten worden met ACI of OAT.

Conclusie

Samengevat hebben we in dit proefschrift aangetoond dat de combinatie van beenmerg en kraakbeencellen een toegevoegde waarde heeft op de kraakbeenvorming. Zodanig dat dit de basis is voor een nieuwe eenstaps kraakbeentherapie die in geiten betere resultaten geeft dan microfractuur behandeling. Tevens hebben we een nieuwe MRI techniek gevalideerd voor de evaluatie van de behandeling van kraakbeendefecten. Met deze techniek kan naast informatie over de mate van vulling van het defect na behandeling tevens een uitspraak gedaan worden over de kwaliteit van deze vulling. Deze techniek kan in de toekomst mogelijk een belangrijke rol gaan spelen in de indicatiestelling tot kraakbeenchirurgie en de evaluatie hiervan. Als laatste zijn verschillende factoren geïdentificeerd die een duidelijke relatie hebben met de klinische uitkomst na kraakbeenchirurgie. Deze factoren zijn vervolgens gebruikt om een behandelalgoritme te maken op basis waarvan de medisch professional zijn behandelbeslissing kan nemen. Met bovengenoemde onderzoeken draagt dit proefschrift bij aan de verbetering van het brede scala van behandeling en evaluatie van kraakbeendefecten.

Tenslotte kan worden gesteld dat de behandeling van een kraakbeen defect meer is dan het uitvoeren van de operatie. Het is een proces dat begint bij de eerste presentatie van de patient, beïnvloed wordt door verschillende factoren en mensen en eindigt met een uitgebreide evaluatie van de behandeling. Het begint met de diagnose van een defect, selectie van de correcte behandeling voor de specifieke patient, afstemmen en overeenkomen van de verwachtingen van de behandeling en gebruik maken van nuttige uitkomstmaten gedurende rehabilitatie onder leiding van gespecialiseerde profesisonals. Gedurende dit proces moet de focus gericht zijn op optimale kwaliteit in zorg voor de patient en verbreding van kennis omtrent genoemde problematiek om de huidige standaard te verbeteren. Eenstaps celtherapie is de volgende generatie van kraakbeenbehandeling en zal zorgen voor hernieuwde aandacht en inzichten in de behandeling van kraakbeen defecten.

LIST OF PUBLICATIONS AND PRESENTATIONS

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CURRICULUM VITAE

The author of this thesis was born on May 10th 1981, in Breda, the Netherlands. In 2000 he graduated from high school (VWO, Onze-Lieve Vrouwelyceum, Breda) and started to study health sciences at the Maastricht University. In 2001 he switched studies and started to study Medicine at the Utrecht University. In 2004 he started as a research student of the department of Orthopaedics at a project studying the influence of bisphosphonates on osteoblast differentiation (drs. MHM Wassen and dr. LB Creemers). In 2007 he was accepted as a student research fellow in the Biomechanics and Motion analysis lab of prof. K-N An at the Mayo Clinic in Rochester, America. Here he studied the ultrasonic and biomechanical properties of articular cartilage. Later that year he received his medical degree and started a PhD program entitled: 'Cartilage regeneration intervening to change the continuum' (prof. dr. DBF Saris, prof. dr. WJA Dhert and dr. LB Creemers). This project has resulted in several scientific publications and presentations at national and international meetings and eventually in this thesis. In 2011 he started his residency at the Department of Surgery at the Meander Medisch Centrum, Amersfoort the Netherlands (head: dr. AJ van Overbeeke), after which he will start his orthopaedic training in April 2013 at the UMC Utrecht, the Netherlands. In 2012 he was awarded the Stryker/ICRS clinical scientist travelling fellowship.

Joris lives together with Maaike Moman. They have one daughter, Jette.

STELLINGEN

Toevoegen van stamcellen uit beenmerg aan kraakbeencellen zorgt voor een stimulatie van de kraakbeenvorming in een kweekomgeving, in muizen en in geiten. *Dit proefschrift*

One-stage kraakbeen celtransplantatie gebaseerd op de combinatie van kraakbeen cellen en stamcellen is beter dan microfractuur behandeling in geiten. *Dit proefschrift*

De cellen uit een kraakbeendefect hebben een minstens zo goede potentie om kraakbeen te vormen dan de cellen die op dit moment gebruikt worden voor kraakbeen celtransplantatie. *Dit proefschrift*

Optimale fixatie gaat niet hand in hand met behoud van scaffold architectuur.
Dit proefschrift

De Knee Osteoarthritis Outcome Score (KOOS) is een valide vragenlijst om de klinische vooruitgang na kraakbeenchirurgie te meten. *Dit proefschrift*

De delayed Gadolinium enhanced MRI of Cartilage (dGEMRIC) is zeer geschikt om de regeneratie van kraakbeen in kaart te brengen. *Dit proefschrift*

Implantatie van een Trufit plug geeft geen schade aan het articulerende kraakbeen.
Dit proefschrift

Kijk verder dan de kenmerken van de kraakbeenlaesie, ook de patient daaromheen bepaalt het succes van je behandeling. *Dit proefschrift*

Bij de keuze van de juiste behandeling voor een kraakbeendefect zou evidence-based medicine in plaats van de kunde van de dokter doorslaggevend moeten zijn.
Dit proefschrift

Succes is de eigenschap om van de ene mislukking in de andere te gaan zonder je enthousiasme te verliezen. *Winston Churchill*

Ellende van ontwikkeling is dat je steeds meer beseft wat je niet begrijpt
Theo Maassen

Ambition without patience is a dangerous thing *Ferran Adria (chef El Bulli)*