Modulating soluble mediators to restore joint homeostasis

ANIKA IRIS TSUCHIDA

Promotoren:

prof. dr. D.B.F. Saris
prof. dr. W.J.A. Dhert

Co-promotor:

dr. L.B. Creemers

The printing of this thesis was financially supported by:
Nederlandse Orthopaedische Vereniging, Nederlandse Vereniging voor Matrix Biology, Reumafonds, Annafonds. Chipsoft.

The research described in this thesis was financially supported by Smith and Nephew and by the Biomedical Materials Institute program, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

ISBN: 978-94-6169-440-9

Layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands.
Photograph on cover: Mattie van Rijen.

MODULATING SOLUBLE MEDIATORS TO RESTORE JOINT HOMEOSTASIS

Modulatie van oplosbare factoren om de gewrichtshomeostase te herstellen (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus,

prof.dr. G.J. van der Zwaan,

ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op donderdag 14 november 2013 des middags te 4.15 uur

door

Anika Iris Tsuchida

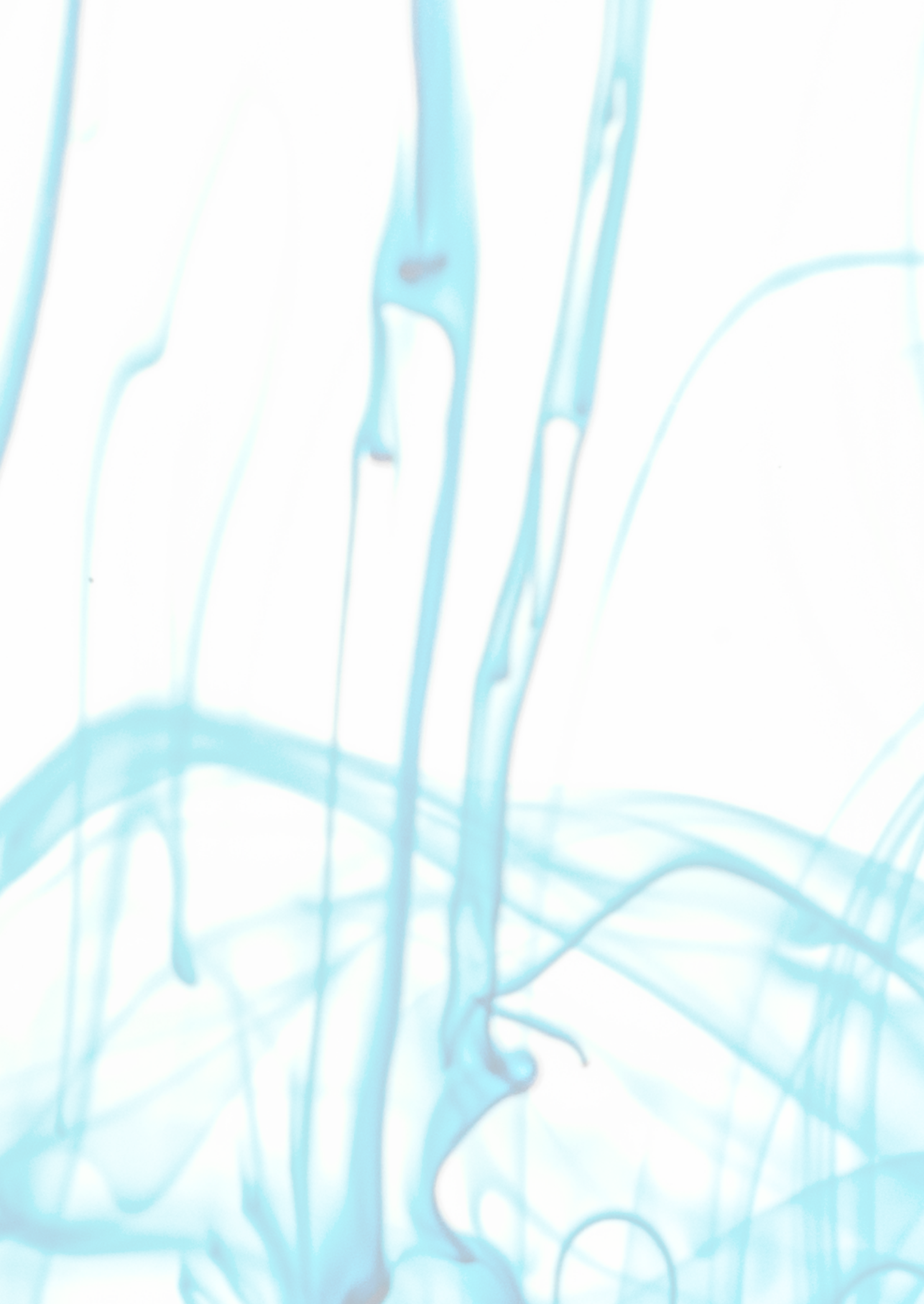
geboren op 4 juni 1985
te Panama, Panama

CONTENTS

LIST OF ABBREVIATIONS

ACAN	Aggrecan
ACI	Autologous chondrocyte implantation
ACL	Anterior cruciate ligament
ADAMTs	A disintegrins and metalloproteinases with thrombospondin motifs
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
CD	Cartilage defect
COL	Collagen
COX-2	Cyclooxygenase-2
CS	Chondroitin sulphate
DDR2	Discoidin domain receptor 2
DMEM	Dulbecco's modified Eagle's medium
DMMB	Dimethylmethylene blue
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
HGF	Hepatocyte growth factor
HIF-2α	Hypoxia- inducible factor-2α
IFNγ	Interferon γ
IGF	Insuline-like growth factor
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
ITS-X	Insuline-transferrine-selenium-X
KS	Keratan sulphate
LDH	Lactate dehydrogenase
MACI	Matrix-assisted autologous chondrocyte implantation
MAPK	Mitogen activated protein kinases
MCP	Monocyte chemotactic factor
MMP	Metalloproteinases
MRI	Magnetic resonance imaging
MSC	Mesenchymal stromal cell
NF-κB	Nuclear factor κB
NGF	Neural growth factor
OA	Osteoarthritis
OAT	Osteochondral autologous transplantation

OPG	Osteoprotegerin
OSM	Oncostatin M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEGDA	Polyethylene-glycol-diacrylate
PGE_2	Prostaglandin E_2
PEOT/PBT	Poly(ethylene oxide terephthalate)/poly(butylene terephthalate)
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
SOX9	(Sex determing region Y)- box9
SSP1	Secreted phosphoprotein 1
TIMP	Tissue inhibitor of metalloproteinases
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
VEGF	Vascular growth factor
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide



Chapter 1

General introduction and aims

CASE PRESENTATION

A 34-year-old male patient presents with a painful right knee. He had a partial medial meniscectomy of the same knee 5 years ago, which initially allowed for renewed participation in sports. However, in the last year he experienced progressive symptoms of that knee: at first he only had pain and effusion after running and soccer, but lately the medial pain is almost constantly present, severely limiting sports activities and also affecting normal activities of daily life such as prolonged walking and squatting. There are no complaints of giving way or catching and locking of the knee.

On physical examination, there is a moderate effusion of the right knee, range of motion is 130 degrees of flexion, full extension, and palpation of the medial joint line is painful. The knee ligaments are intact and during the McMurray test for meniscal tears the patient experiences pain, but no "click" is felt. MRI of the knee reveals an isolated full thickness articular cartilage defect at the load-bearing region of the medial femoral condyle. The suggested procedure is arthroscopy with intra-operative evaluation of the defect and, depending on the size of the lesion, either microfracture or cartilage biopsy for subsequent autologous chondrocyte implantation. After debridement, the defect measures 1.5 by 2.5 cm and therefore a biopsy is taken. Eight weeks later, the cartilage cells are reimplanted using a carrier scaffold.

CARTILAGE DEFECTS

This case illustrates some of the typical problems encountered in patients with symptomatic cartilage defects of the knee. The exact etiology of cartilage defects is largely unknown, but a relationship with joint trauma has been suggested[1, 2]. Also, sports participation, especially high-risk sports such as soccer and skiing, have been associated with increased incidence of cartilage defects[2]. Furthermore, concomitant injuries such as anterior cruciate ligament and meniscal tears, are often observed[1] and may contribute to the development of cartilage defects[3, 4]. Symptomatic articular cartilage defects mostly affect the young to middle aged population and cause symptoms such as pain, joint effusion, and loss of knee function thereby reducing the ability to perform activities of daily living and participate in sports. For diagnosing cartilage defects, arthroscopy is still the golden standard[5]. Although the specificity of MRI to diagnose cartilage defects is excellent, its sensitivity is not and MRI systematically underestimates the dimensions of a cartilage defect[6].

Cartilage defects are classified according to macroscopic appearance into grades I to IV, with an increasing grade reflecting an increased severity of the cartilage damage[7]. Treatment of articular cartilage defects is important to restore function and reduce

symptoms such as pain, but also because focal defects may progress to osteoarthritis (OA) if left untreated[8-10]. Only grade III and IV cartilage defects are treated surgically and determination of the most suitable treatment is partly dictated by lesion size[11]. However, the success of cartilage repair on an individual basis is difficult to predict, repair tissue is far from identical to native tissue[12-15] and it remains to be determined whether timely treatment of defects really delays or prevents the development of OA.

TREATMENT OPTIONS

The three main surgical treatment options for symptomatic focal cartilage defects are: bone marrow stimulating techniques, of which currently microfracture[16] is most common, tissue replacements such as osteochondral autologous transplantation (OAT)[17], and cell-based therapies such as autologous chondrocyte implantation (ACI)[18] and subsequent newer generations of this concept.

Microfracture is an arthroscopic technique in which, after debridement of the cartilage defect, small holes are created in the subchondral bone with a surgical awl. Bone marrow cells can enter the joint and are thought to attach to the base of the defect in the blood clot that is formed and eventually remodel into cartilage[19]. It is a relatively simple technique with good outcomes on the short term, but it has been suggested that clinical results obtained with this technique are less durable, possibly due to the more fibrous repair tissue[20].

During the OAT procedure, an osteochondral plug is harvested from a healthy, non weight-bearing location in the knee and transplanted to fill the defect. This technique is limited by donor site availability and therefore is not recommended for lesions larger than 4 cm^2 and may lead to pain at the donor site[21].

Autologous chondrocyte implantation was first described in 1994 by Brittberg *et al*[18]. Since then, this technique and its various modifications have shown clinical effectiveness in case-series with mid- to long-term follow-up[22-26], nonetheless, only a handful randomized controlled trials have been performed[14, 27-30]. In these trials, both superiority as well as similarity of ACI to other techniques at various time-points after surgery have been demonstrated[13, 14, 27-33]. Comparing ACI to OAT, more failures with OAT (55%) than with ACI (17%) were recently reported at 10 years follow-up[33]. Two studies reported that overall there were no differences between ACI and microfracture[13, 31] at five years follow-up, although more stringent selection of patients for autologous chondrocyte implantation may result in better outcomes, as autologous cells were more effective in defects with an onset < 3 years[34]. All in all, to date, there is no general consensus on which surgical technique is best[35]. *Large randomized clinical trials including several out-*

come parameters in addition to clinical outcome, such as histology and MRI, would provide a more definite answer, as shown in the current thesis.

IMPROVING CARTILAGE REGENERATION

The repair tissue obtained with cartilage repair procedures is hyaline-like at best but still far from identical to native tissue[13-15, 36-38]. It lacks the layered structure, has inferior mechanical properties to native cartilage and integration with adjacent cartilage is sub-optimal. In order to truly advance cell-based cartilage repair procedures into becoming cartilage regeneration procedures, the quality of cartilage repair tissue will need to be improved. To optimize cartilage regeneration, briefly three factors play an important role: the (phenotype of) cells used, the cells' microenvironment, including biomaterials used as carrier matrices and paracrine signaling, and the host joint environment. Classical ACI is performed with autologous chondrocytes obtained from a non-weight bearing location in the knee that are expanded prior to reimplantation. However, the numbers of cells that can be obtained are small, which necessitates expansion, leading to dedifferentiation, and inferior cartilage matrix production[39, 40]. An additional drawback is the donor site morbidity inflicted by taking a cartilage biopsy. Hence, there is an increasing interest in using non-expanded and therefore non-dedifferentiated cells, or cell types other than chondrocytes, such as mesenchymal stromal cells, or a combination of the two[41]. Mesenchymal stromal cells have the advantage that they can be obtained with relatively minimal additional damage and expanded to obtain great cell numbers. However, their main drawback is their tendency to hypertrophy[42]. *For these reasons, we focused on the potential of non-expanded cells, in particular by exploring appropriate biomaterials and the role of endogenous soluble mediator production to enhance redifferentiation and thus stimulate cartilage matrix production.*

In the last decade, there have been many advances in biomaterial sciences and many different biomaterials have been explored for cartilage tissue engineering. Biomaterials produced from natural polymers such as collagen, fibrin, chitosan, silk and hyaluronic acid have the advantage of containing natural binding sites for cells and other biomimetic signals[43]. Binding of natural substrates through, for example, integrin receptors, can regulate gene expression and chondrocyte behaviour[44]. However, the properties of biomaterials based on synthetic polymers are much easier to tailor, offering virtually endless possibilities without, for instance, problems related to batch-to-batch variations. Until now, the focus of biomaterial-based research on cartilage regeneration has been mainly based on the investigation of single biomaterials, with only a few comparative studies. Thus, insight as to what material would be preferable in terms of stimulation of

regeneration is lacking. *In particular, the role of biomaterials in influencing the expression and secretion of soluble mediators and thus to regulate regeneration is unknown.*

Finally, cartilage regeneration is also greatly dependent on patient and defect characteristics. Whilst some characteristics such as age, defect location, defect age and activity level have been identified as prognostic factors[45], there are still various unknown factors that determine the outcome of regenerative treatments. Many of these factors are likely to concern the host joint environment in which the repair has to take place. Whilst in experimental models the defect is often created and treated at once, in patients, defects have often existed for a longer period prior to treatment, causing a disturbance in joint homeostasis[46].

KNEE JOINT HOMEOSTASIS

One of the reasons why current treatments for cartilage defects sometimes fail clinically and/ or histologically may lie in the fact that current therapies focus exclusively on the cartilage defect, while all tissues of the knee joint together are likely to determine its characteristics and hence affect the regenerative response. In healthy joints, the interactions between cartilage, synovium, subchondral bone, menisci and ligaments create a stable environment with a balanced metabolism. Communication between the various tissues of the knee mostly occurs through the release of soluble mediators into the synovial fluid. Anabolic and catabolic factors, inflammatory and anti-inflammatory components are all exchanged between these structures, affecting each other. The concept of joint homeostasis is used to describe this delicate equilibrium[32, 47], in which joint tissues are capable of responding adequately to changes that fall within physiological limits and hence maintain a steady state without net changes.

Contrarily, in joints with cartilage defects and OA, the balance between matrix deposition and degradation is disturbed at the expense of cartilage integrity. Various factors probably contribute to a disturbance in joint homeostasis: joint (impact) trauma, obesity, altered mechanical loading and joint instability; these are just a few of the components recognized so far[48-51]. Joint impact trauma can cause chondrocyte cell death and apoptosis and induces in surviving chondrocytes the production of inflammatory mediators such as interleukin (IL)-1β, tumor necrosis factor (TNF)α, nitric oxide and prostaglandin E_2 and proteases including matrix-metalloproteinases (MMPs) and disintegrins and metalloproteinases with thrombospondin motifs (ADAMTs)[52-55]. Impact trauma may also lead to the leakage of blood into the joint, which can directly affect the cartilage by causing chondrocyte apoptosis and decreasing proteoglycan synthesis as well as cause synovial inflammation[56]. Localized mild synovial inflammation is regularly observed in patients with cartilage defects[57] and some form of synovitis is common in OA and plays

an important role in OA pathogenesis[58-60]. Activation of synovial fibroblasts and cells of both the adaptive and innate immune system, including macrophages, B-cells and T-cells, are thought to lead to the production of a broad spectrum of cytokines and chemokines[61]. In addition, recent evidence pointed at cartilage chondrocytes as potential sources for cytokine production[62], which may also further potentiate the degenerative processes in an auto-and paracrine manner. *In this thesis, we further explore the presence and production of several factors in native tissue, and in in vitro culture.*

The classic inflammatory and catabolic cytokines suggested to play a role in OA are IL-1β and TNFα[63-65]. Although their negative effects on cartilage integrity have been extensively described *in vitro*, high, supra-physiological concentrations have usually been used, while actual concentrations measured in synovial fluid are ten- to thousand-fold lower[66-68]. Furthermore, results from clinical trials using IL-1 inhibitors have been far from satisfactory[69]. Therefore, it seems likely that other soluble mediators are important. Other inflammatory cytokines implicated in cartilage degeneration include IL-1α, oncostatin M (OSM), IL-6 and interferon-γ (IFNγ)[63, 70, 71]. Also, chemokines, including CXCL8 (IL-8), RANTES, MCP-1 and many more CC and CXC chemokines, have been implicated in cartilage degeneration, chondrocyte apoptosis and chondrocyte hypertrophy[72, 73]. In conjunction with obesity, a state of low-grade inflammation is present and inflammatory factors called adipokines are produced, which are thought to be instrumental in the pathogenesis of OA in obese patients[74]. All these cytokines in turn can mediate extracellular matrix (ECM) degradation by chondrocytes through the induction of MMPs and their inhibitors (TIMPs) and aggrecanases and further amplify inflammation by inducing cytokine production by chondrocytes[75-77]. In addition, matrix fragments released from the cartilage surface into the synovial fluid can trigger the innate immune system through activation of complement and activation of pattern recognition receptors called Toll-like receptors[78-80]. Chondrocytes and synovial cells express these receptors and binding of damage-associated molecular patterns (DAMPS) leads to activation of transcription factors of which nuclear factor-κB (NF-κB) has been recognized to be very important[58]. NF-κB activation in turn leads to the transcription of more inflammatory cytokines, chemokines and inducible enzymes such as iNOS and cyclooxygenase (COX)-2, many of which are again capable of inducing NF-κB. Ultimately, this may result in a vicious circle in which inflammation is continuously propagated and matrix degradation increased, resulting in more inflammation. In addition, many of the secreted cytokines inhibit matrix synthesis. This is illustrated by the finding that synovial fluid from injured joints decreases chondrogenesis[70, 81, 82]. *However, inflammation has also been postulated to be required for tissue regeneration, including cartilage and bone[83] and until now, very few studies have been carried out evaluating the actual effects of inhibiting inflammation on cartilage production and maintenance, which was addressed in this thesis by using celecoxib, a COX-2 inhibitor.*

Even if general inhibition of inflammation may not result in a protective effect on joint cartilage, targeting specific factors may be a feasible option, as synovial fluid is known to contain a mix of inhibitory and stimulatory factors[84]. Mapping the synovial fluid profile in healthy and pathological joints may provide clues as to which factors may play a role. Although the presence of soluble mediators in the synovial fluid of patients with OA has been reasonably well described[66, 84-86], very little is known about which factors are present in the synovial fluid of patients with symptomatic cartilage defects. This is remarkable since timely and proper recognition of patients at risk for impaired cartilage repair or progressive cartilage loss may also provide a window of opportunity to delay or prevent the development of OA. Characterizing these soluble mediators and exploring their effects on cartilage regeneration is crucial for optimal cartilage repair by the application/development of disease-modifying approaches that can be applied prior to or during cartilage repair techniques. *We have evaluated a comprehensive panel of soluble mediators and compared their presence in patients with symptomatic defects to patients with OA and controls without joint pathology, and for some of these investigated their actual role in cartilage matrix metabolism.*

Selective targeting of soluble mediators and/or the use of small molecules to influence the knee joint environment could be of great value to improve the outcomes of cartilage regeneration and prevent further cartilage loss.

AIMS OF THIS THESIS

The studies presented in this thesis are directed at optimizing outcomes after cartilage repair by identifying the optimal surgical treatment and characterizing and modulating soluble mediators present during regeneration. To this end, several specific aims were defined.

Identifying the best surgical treatment

1. To compare clinical, histological and MRI outcome parameters of MACI versus microfracture for repair of cartilage defects in the knee.

Characterizing and modulating soluble mediators to improve cartilage regeneration

2. To characterize the presence of soluble mediators present in the joint and produced during regeneration; identify possible differences between healthy donors and donors with cartilage damage and explore a possible relationship with the quality of cartilage regeneration

3. To compare non-expanded and expanded chondrocytes in terms of cartilage matrix and cytokine production during regeneration in relation to commonly used biomaterials.
4. To study the feasibility of targeting CXCL8, IL-6 and COX-2 activity during cartilage regeneration

OUTLINE OF THIS THESIS

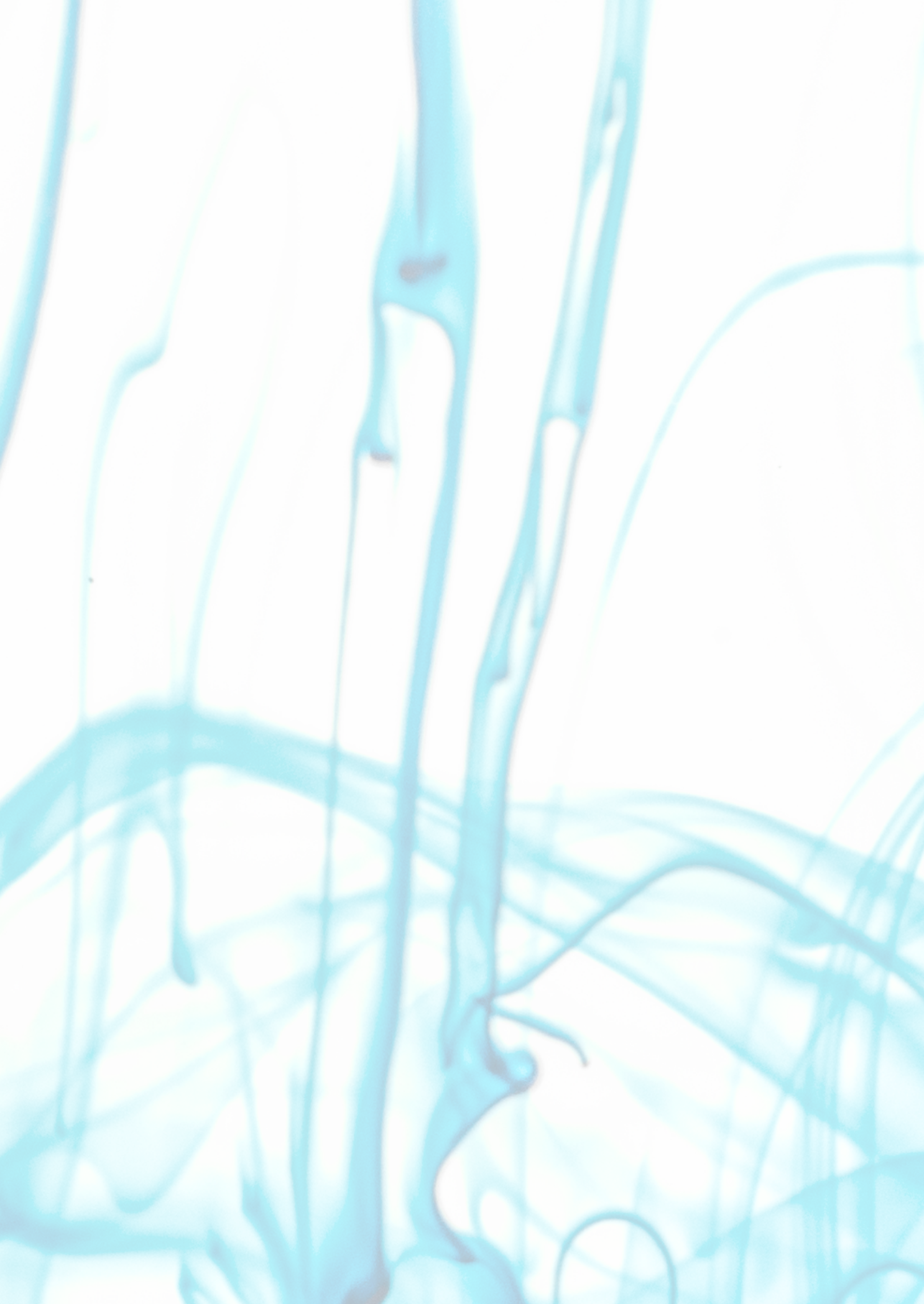
Current treatments for focal cartilage defects include matrix assisted autologous chondrocytes implantation (such as MACI) and microfracture. Both provide good mid- to long-term outcomes, but there is no general consensus on which treatment is better. In *Chapter 2,* we present a large, open-label, multi-center, pan-European, randomized controlled clinical trial comparing MACI to microfracture.

Clinical cartilage repair is unfortunately not always successful and histological cartilage repair is almost never as good as observed in experimental models. Most likely, this discrepancy can be explained by the disturbed joint environment in which regeneration has to take place. In *Chapter 3,* we characterize the diseased joint environment by measuring soluble mediators present in the synovial fluid, cartilage and those produced by chondrocytes during regeneration, of patients with cartilage defects compared to donors without joint pathology and patients with OA. As the use of more differentiated cells has been suggested to result in better cartilage repair, we also evaluated differential expression of cytokines and growth factors in non-expanded and expanded cells and related these to regeneration capacity.

In *Chapter 4,* we continue to explore the differences between non-expanded and expanded cells in terms of cartilage matrix and cytokine production, in response to four commonly used biomaterials for ACI.

In *Chapters 5 and 6,* we studied the role of two of the cytokines found to be present in the joint and previously suggested to have negative effects on chondrocytes. The roles of CXCL8 (also known as IL-8) and IL-6 were evaluated during regeneration and in an osteoarthritic environment.

The outcomes of cartilage repair procedures in OA heretofore have been disappointing, likely because the osteoarthritic environment is particularly challenging for cartilage regeneration. Celecoxib is a specific COX-2 inhibitor suggested to have positive effects on tissue integrity by reducing inflammation and possibly also enhance repair. In *Chapter 7,* we evaluate the effects of celecoxib on inflammatory mediators and cartilage regeneration in several OA models.



Chapter 2

Matrix applied characterized autologous cultured chondrocytes (MACI) implant versus microfracture: two-year follow up from a prospective, randomized trial

Daniel B.F. Saris
Andrew Price
Wojciech Widuchowski
Marion Bertrand-Marchand
Jacob Caron
Jon Olav Drogset
Pieter Emans
Libor Paša
Aleš Podškubka
Konrad Slynarski
Tomas Trc
Anika I. Tsuchida
Mauritz Bezuidenhoudt
Bernd-Jan Sanson
David Levine
Mats Brittberg

Accepted for publication in Am J Sports Med

ABSTRACT

Background

Evidence for the efficacy and safety of matrix applied characterized autologous cultured chondrocytes (MACI) implant versus microfracture for the treatment of cartilage defects was limited.

Purpose

To compare clinical efficacy and evaluate the safety profile of the MACI implant versus microfracture in the treatment of patients with symptomatic articular cartilage defects of the knee.

Study Design

Randomized, controlled clinical trial; Level of evidence, 1.

Methods

Patients enrolled in the SUMMIT (Demonstrate the Superiority of MACI implant to Microfracture Treatment in patients with symptomatic articular cartilage defects in the knee) trial had ≥1 symptomatic focal articular cartilage defect (Outerbridge grade III or IV; ≥3 cm^2) of the femoral condyles and/or trochlea, with a baseline Knee Injury and Osteoarthritis Outcome Score (KOOS) pain score of <55. The co-primary efficacy endpoint was the change from baseline to 2 years for the KOOS pain and function (sports and recreational activities) subscales. Histological evaluation and MRI assessments of structural repair tissue, treatment failure, and the remaining 3 KOOS subscales were also assessed.

Results

Of the 144 patients who were treated, 95% completed the 2-year assessment. Patients had a mean age of 33.8 years and a mean lesion size of 4.8 cm^2. The co-primary endpoint, the KOOS pain and function subscales, was significantly better (pain LS mean: 11.76; function LS mean: 11.41; p=0.001) for the MACI implant versus microfracture groups. Significantly better scores for the KOOS activities of daily living (p<0.001), quality of life (p=0.029) and other symptoms (p<0.001) were also observed for patients treated with the MACI implant than with the microfracture procedure. Repair tissue quality was good as assessed by histology/MRI but a difference could not be shown between treatments. A low number of treatment failures and no unexpected safety findings were reported.

Conclusions

Cartilage defect treatment using the MACI implant was clinically and significantly better than the microfracture group, with similar structural repair and safety, when used to treat symptomatic cartilage knee defects in this large, randomized controlled trial.

Clinical Relevance

The MACI implant procedure offers a more efficacious alternative than microfracture with a similar safety profile for the treatment of symptomatic articular cartilage defects of the knee.

INTRODUCTION

Cell therapy has been an integral part of the technovolution[87] of methods in cartilage repair, utilizing autologous chondrocytes to generate an effective repair tissue. Treating cartilage lesions is important as cartilage injury is prevalent and can lead to significant pain and reduced function. In Europe and the US, the incidence of chondral defects has been shown to be approximately 60% in those undergoing arthroscopy[2, 88-90]. Articular cartilage has limited self-healing capacity due to its lack of access to blood stream[91] and dense matrix. If left untreated, cartilage lesions can become symptomatic and may progress to osteoarthritis[92-95].

The first autologous chondrocyte implantation (ACI) for cartilage repair was performed 25 years ago[18]. Over time, the procedure has advanced to collagen-covered ACI (CACI; second-generation technology)[96-98] and then to the matrix applied characterized autologous cultured chondrocytes (MACI; Genzyme Biosurgery, Cambridge, MA, USA) implant, a third-generation technology. Progression from the first- to second- to third-generation technology resulted in added benefits to the patients; these include shorter procedure time, smaller incision size, more consistent cell seeding, less pain from periosteal hypertrophy, and less adverse events (AEs) overall[99-103]. For the MACI implant, chondrocytes are cultured in monolayer and seeded on a collagen membrane, which is then implanted directly in the defect. Culturing cells on the membrane allows for their proliferation and redifferentiation to a more chondrogenic phenotype after monolayer culture, and the cells are better fixed and distributed in the defect[99, 104-106]. Physical properties of the type I/III collagen membrane (ACI-Maix, Matricel GmbH, Germany) make it tear resistant and durable, but not self-adherent, and also permit the implant to be easily trimmed and handled[99, 104, 105]. Overall, good clinical outcomes and repair tissue have been shown with the MACI implant with a good safety profile, especially less periosteal hypertrophy than with the ACI procedure[99-101, 107, 108].

Microfracture (MFX), a bone marrow stimulating procedure developed before cell therapies[109], is frequently used to repair specific cartilage injuries. While MFX provides good clinical outcomes, these are not always sustained[20, 110, 111]. Previous studies show that patients with smaller lesions have better clinical outcomes with MFX than patients with larger lesions[112], whereas lesions on the trochlea do not improve as well as those on the femoral condyle[110]. Repair tissue with MFX has been shown to be fibrous in nature,[41] compared with more hyaline-like repair tissue reported with the MACI implant[99]. In addition, intralesional osteophytes may result from MFX and could compromise any successful clinical outcomes with the procedure. Microfracture may also negatively affect outcome of subsequent cell-based cartilage repair treatment[113, 114].

Conducting randomized controlled trials (RCTs) of surgical interventions in orthopedics is challenging. Most striking is the unethical nature of a control "placebo" or sham

surgery. Establishing appropriate controls is difficult as different surgical procedures may create different-sized incisions or require a different number of steps, such that blinding is impossible. Another challenge is the large placebo effect seen with sham surgeries. Unblinded healthcare providers must also maintain clinical equipoise so patients will not perceive or have expectations around receiving a beneficial treatment. In our study, we attempt to address these issues in the largest, Good-Clinical-Practice, randomized, controlled trial with the highest power to date in cartilage repair, as per the guidance of regulatory agencies.

Compared with most previous randomized trials of cell therapy versus MFX, this study uses a third generation technology, has higher statistical power with more patients, and includes larger lesion sizes. Although MFX is traditionally used for the treatment of smaller lesions, clinicians also treat larger defects with MFX[32], since there are few alternative treatment options. The primary objective of our study was to compare clinical efficacy and safety of the MACI implant with microfracture in the treatment of patients with symptomatic articular cartilage defects.

MATERIALS AND METHODS

Study Design

The SUMMIT (Demonstrate the Superiority of MACI implant to Microfracture Treatment [SUMMIT] in patients with symptomatic articular cartilage defects in the knee) trial was a Good Clinical Practices (GCP), prospective, randomized, open-label, parallel-group, multicenter study conducted at 16 European sites (NCT00719576), with enrollment beginning in May 2008. Articular cartilage defects of the medial femoral condyle (MFC), lateral femoral condyle (LFC) and/or trochlea were treated with the MACI implant procedure or arthroscopic MFX. The study protocol and informed consent form were approved by appropriate national or local ethics committee at each study site. The study was conducted according to GCP and principles of the Declaration of Helsinki.

Patient Population

Male and female patients aged 18 to 55 years old with ≥1 symptomatic cartilage defect and a baseline moderate to severe Knee Injury and Osteoarthritis Outcome Score (KOOS) pain score (<55) were included. Index defects were Outerbridge Grade III or IV focal cartilage defects on the MFC, LFC, and/or trochlea and were at least 3 cm^2 in size. Osteochondritis dissecans (OCD) lesions were allowed if no bone graft was required. A stable knee was required; ligament repair or reconstruction procedures were allowed before or concurrently with study treatment. An intact or partial meniscus (≥50%) was also required; meniscal repair or resection was allowed before or concurrently with the

cartilage repair procedure if ≥50% of functional meniscus was retained. All patients provided written informed consent before participating in the study.

Major exclusion criteria included any knee joint surgery within 6 months prior to screening (not including diagnostic arthroscopy); modified Outerbridge Grade III or IV defect(s) on the patella or tibia; symptomatic musculoskeletal condition in the lower limbs that could impede efficacy measures in the target knee joint; total meniscectomy, meniscal allograft, or bucket handle tear or displaced tear requiring >50% removal of the meniscus in the target knee; malalignment requiring an osteotomy to correct tibial-femoral or patella-femoral alignment; Kellgren-Lawrence grade 3 or 4 osteoarthritis; inflammatory disease or other condition affecting the joints; or septic arthritis within 1 year prior to screening.

Surgical Procedures

The control selected for efficacy comparison was MFX as recommended by the FDA and EMA for this protocol and in their guidances[115, 116]. MFX is also universally considered a standard first-line therapy for cartilage repair, is widely available, and is used clinically in larger lesions, thus, reflecting "real-world" experience.

At baseline arthroscopy (performed within 8 weeks of screening) to assess cartilage lesion and surrounding cartilage, biopsies were taken from all patients.Following biopsies, eligible patients were intra-operatively randomized, using an interactive voice response system and computer-generated 1:1 randomization scheme, to the MACI implant or arthroscopic microfracture. The same surgical procedure was performed on any additional Outerbridge grade III and IV lesions in the target knee; lesions grade I or II were not treated or debrided only.

For the MACI implant procedure, a cartilage biopsy (approximately 200 mg) was aseptically harvested from a minor or non-weightbearing, healthy area of the femoral condyle. The biopsy was sent to Genzyme Biosurgery (Cambridge, MA, USA) where autologous chondrocytes were isolated, cultured and seeded onto a purified, resorbable, porcine-derived collagen type I/III membrane (ACI-Maix, Matricel GmbH, Germany). The final MACI product was a 20 cm^2 (5 x 4 cm) membrane seeded with 500,000 to 1 million cells per cm^2.

The MACI implant procedure was performed via mini-arthrotomy 4 to 8 weeks after baseline arthroscopy. Briefly, the lesions were debrided back to a vertical rim of stable, healthy cartilage without breaching the subchondral bone. The shape and size of the lesion(s) were assessed and a template for each lesion was created. The MACI implant was trimmed to the correct size and shape of the defect, and placed down into the debrided base of the defect with the cells facing the subchondral bone. The implant was then secured in place using a thin layer of fibrin sealant on the base and edges of the

defect, and stability of the implant checked while fully extending and flexing the knee a number of times.

Microfracture was performed at the time of arthroscopy strictly according to the technique described by Steadman et al[109]. In brief, the lesion was debrided back to stable, healthy cartilage avoiding damage to the subchondral bone. Multiple fracture holes were made in the subchondral bone with a sharp surgical awl so that the centers of the holes were 3 to 4 mm apart and 4 mm deep. All surgeons were trained on all surgical procedures, which we standardized for the study.

Second-look arthroscopy was used to assess the knee joint according to the International Cartilage Repair Society (ICRS) macroscopic evaluation criteria and obtain a biopsy of repair tissue at year 2 to be scored independently and blinded to treatment origin using the ICRS II histological outcome score.

Rehabilitation

The 4-phase, standardized rehabilitation program was the same for both treatment groups, based on a report by Steadman et al.[117], but was individualized for each patient. Patients progressed through the program at different rates based on lesion size, lesion location, pre-operative duration of symptoms, physical condition, patient motivation, and the expected course of healing for the procedure employed. Only when certain goals were reached at the end of each rehabilitation stage were the patients allowed to progress to the next stage.

The 4 rehabilitation phases were designed to avoid deleterious forces to the repair site and to promote a gradual and safe return to function and activity. The focus of the early protection phase (Phase I: weeks 0-6) was to protect the new repair tissue and to restore joint homeostasis using restricted weight-bearing and range of motion exercises. Patients moved on to the transition phase (Phase II: weeks 6-12) when they attained full passive knee extension and knee flexion to 120° with minimal pain and swelling, and were able to perform quadriceps set exercises with good contraction and no lag. The focus of this second phase was to restore full range of knee motion and to begin to work on muscle strength using exercises that gradually increased in weight-bearing and range of motion.

The remodeling phase (Phase III: weeks 12-26) was started when patients attained a full range of motion with minimal pain and swelling, and when they reached 20% hamstring strength and 30% quadriceps strength of the contralateral leg, as well as being within 30% of balance testing of the contralateral leg. Patients also had to be able to walk 1-2 miles or ride a bike for 30 min. The focus of the remodeling phase was to improve muscle strength and endurance and to reintroduce activities, while monitoring the increase in activity. Finally, patients with full range of motion, achieving strength 80-90% of contralateral leg, and balance 75-80% of contralateral leg with no pain, in-

flammation or swelling moved on to the maturation phase (Phase IV: weeks 26-52). In this last phase, the patients were allowed to participate in full unrestricted activity by developing programs based on the patient needs and type of activity (low impact sports: 4-6 months; moderate impact sports: 8 months; high impact sports: 12-18 months).

Study Endpoints

The primary efficacy analysis was based on a co-primary endpoint of change from baseline to year 2 for the patient's KOOS pain and function (sports and recreational activities) scores. A response rate based on KOOS pain and function scores at year 2 and earlier time points was one of the secondary endpoints (responder defined as a ≥10-point improvement in both the KOOS Pain and Function subscales).

Other predefined secondary endpoints included the histological evaluation of structural repair biopsies harvested from the core of the index lesion (year 2), as measured by the macroscopic ICRS overall assessment; MRI assessments of structural repair parameters at baseline, and at years 1 and 2, and change from baseline to years 1 and 2 for degree of defect fill, degree of repair tissue integration with adjacent native cartilage, and signal intensity of the repair tissue relative to adjacent native cartilage as measured using the Whole Organ MRI (WORMS) score[118] (the Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) scoring system[119] was not available at the time of study design); treatment failure rate at year 2 and earlier time points; change from baseline in KOOS pain and function at earlier time points; and change from baseline in KOOS activities of daily living (ADLs), knee-related quality of life (QOL), and other symptoms at year 2. Both histology and MRI measures were evaluated in a blinded fashion. Patients were defined as a treatment failure if, at any time after week 24, they had a patient and physician global assessment the same or worse than baseline, a <10% improvement in KOOS pain, physician-diagnosed failure ruling out all other potential etiologies, *and* the physician decided that surgical re-treatment was needed.

Other study endpoints included change from baseline in KOOS pain, symptoms, knee-related QoL, ADLs subscales at earlier time points (weeks 24, 36, 52, and 78); change from baseline to years 1 and 2 in the overall knee condition using the Modified Cincinnati Knee Rating System[120], International Knee Documentation (IKDC)[121], 12-Item Short Form Health Survey (SF-12)[122], and European Quality of Life (EuroQOL) 5 dimensions questionnaire (EQ-5D); and Macroscopic ICRS cartilage repair assessment score in patients having a biopsy taken via arthroscopy at year 2.

Patients were evaluated for adverse events (AEs) at each study visit. An AE was defined as any undesirable physical, psychological or behavioral effect experienced by a patient during the study period, independent of treatment relatedness. Adverse events were fully described and recorded by severity, duration, and relationship to treatment, and were considered treatment emergent if the AE began or worsened after treatment.

All AEs were categorized with the Medical Dictionary for Regulatory Activities (MeDRA). Adverse events were considered serious if they were life threatening, required in-patient hospitalization, resulted in significant disability, or might otherwise jeopardize the patient. Subsequent surgical procedures (SSPs) were those performed on the target knee during the study; SSPs were not considered treatment failure but were classified as a serious AE.

Statistical Analysis

To power the study at 85% to detect a difference between groups, a sample size was estimated at a total of 144 patients (72 patients per arm) based on the change from baseline to year 2 in the co-primary efficacy endpoints of KOOS pain and function with an alpha of 0.05 (and accounting for patient study discontinuation), assuming a difference of 12 points each for KOOS pain and function with SDs of 20 and 30, respectively, and a correlation coefficient of 0.56 between the co-primary endpoints.

All patients who were randomized and treated were analyzed. The co-primary endpoint was analyzed with SAS® (Cary, NC, USA) using a multivariate analysis of variance (MANOVA) model and the last observation carried forward (LOCF) approach.The final MANOVA model included treatment, study site, and baseline KOOS scores. All other changes in the KOOS subscales at all other time points were analyzed using analysis of variance (ANOVA) and LOCF. Differences between groups were tested by MANOVA and the Cochran-Mantel-Haenszel χ^2 test for histology, and by Cochran-Mantel-Haenszel χ^2 test for the proportions of responders and defect fill. The Cochran-Mantel-Haenszel χ^2 was also used to analyze differences in response rates between groups by lesion size (>4 cm^2, >5 cm^2), lesion location (MFC/LFC/trochlea), and osteochondritis dissecans (OCD) etiology (yes/no).

Predictor variables were also tested *post hoc* on the co-primary endpoint changes from baseline using multivariate analysis of covariance (MANCOVA) with treatment and center as fixed effects and baseline KOOS pain and function, age, total defect size, occurrence of previous surgery, duration of symptoms and index lesion location as covariates. Only significant covariates at a 0.05 level were included in the final model. The Wilk's Lambda test statistic and associated *p*-value were used to test the statistical significance for the co-primary endpoints between MACI and microfracture.

Differences between treatment groups for treatment failure were not tested because of the low number of failures.

RESULTS

Patient and Lesion Characteristics

A total of 144 patients were enrolled in the study and treated with the MACI implant (n=72) or microfracture (n=72; Figure 1). Most of the patients (95%; 137/144) completed a full 2 years of the study (MACI implant [n=70], microfracture [n=67]). No patients treated with the MACI implant discontinued due to lack of efficacy compared with 3 patients treated with MFX. Patients had a mean age of 33.8 years and a mean BMI of 26 kg/m^2, and 65% were male and all were of Caucasian origin (Table 1). The onset of symptoms before current treatment was a mean of almost 6 years for the MACI implant group and almost 4 years for the MFX group. More patients in the MFX group (37.5%) than in the MACI implant group (23.6%) had a sports activity level rated as highly competitive prior to onset of symptoms; slightly more patients in the MACI implant group (55.6%) were at a recreational activity level than in the MFX group (44.4%). Mean baseline scores

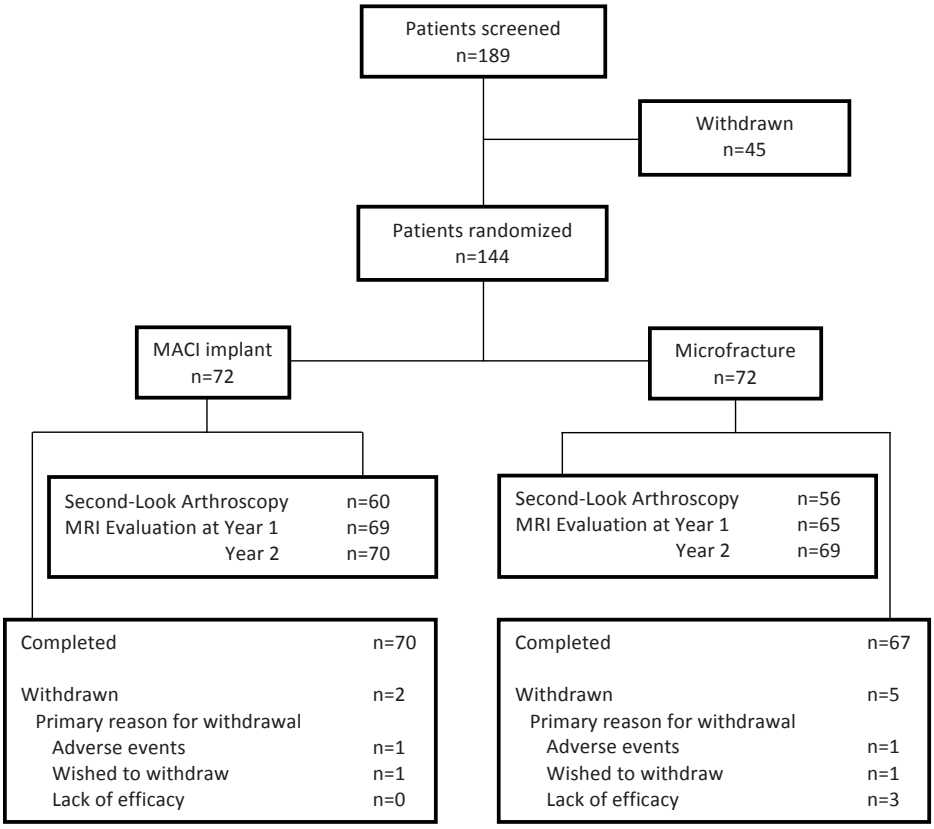


Figure 1: Patient Disposition

for KOOS pain and KOOS function were 37.0 and 14.9 in the MACI implant arm, and 35.5 and 12.6 in the microfracture arm, respectively.

Lesions had a mean size of 4.8 cm^2, and most were located on the MFC or LFC as opposed to the trochlea (Table 1); the majority of lesions (66.7%) were completely contained (Table 1). Acute trauma was the most common underlying etiology for the lesions (54.2%) followed by chronic degeneration and osteochondritis dissecans.

Table 1: Patient and Lesion Characteristics

	MACI implant (n=72)	Microfracture (n=72)
Patients		
Age (years), mean ± SD	34.8 ± 9.2	32.9 ± 8.8
Gender, % male	62.5	66.7
Race, % White	100	100
BMI (kg/m^2), mean ± SD	26.2 ± 4.3	26.4 ± 4.0
Duration of symptoms (years)		
Mean (range)	5.8 (0.05 –28.0)	3.7 (0.1 – 15.4)
Baseline KOOS pain	37.0 ± 13.5	35.5 ± 12.1
Baseline KOOS function	14.9 ± 14.7	12.6 ± 16.7
Lesions		
Index lesion size (cm^2), mean ± SD	4.9 ± 2.8	4.7 ± 1.8
Total defect surface area (cm^2), mean ± SD	5.8 ± 5.1	5.3 ± 2.5
Location, n (%)		
MFC	54 (75.0)	53 (73.6)
LFC	13 (18.1)	15 (20.8)
Trochlea	5 (6.9)	4 (5.6)
Etiology, n (%)		
Acute trauma	33 (45.8)	45 (62.5)
Chronic degeneration	18 (25.0)	9 (12.5)
Osteocondritis dissecans	8 (11.1)	12 (16.7)
Unknown	9 (12.5)	6 (8.3)
Other	4 (5.6)	0
Outerbridge grade, n (%)		
III	21 (29.2)	15 (20.8)
IV	51 (70.8)	57 (79.2)
Lesion Containment, n (%)		
Completely contained	50 (69.4)	46 (63.9)
Partially contained	22 (30.6)	26 (36.1)

KOOS: Knee Injury and Osteoarthritis Outcome Score; LFC: lateral femoral condyle; MACI: matrix applied characterised autologous cultured chondrocytes; MFC: medial femoral condyle; SD: standard deviation.

Prior and concurrent procedures are listed in Table 2. The most common prior surgical procedures were diagnostic arthroscopy, debridement of cartilage lesion, microfracture, and loose body removal. The most common concomitant procedures were loose body removal, partial medial meniscectomy, and graft reconstruction during cartilage biopsy or implantation; and loose body removal and synovectomy/synovial plica excision during the core biopsy at year 2.

KOOS Pain and Function

Two years after treatment, the improvement with the MACI implant over MFX in the co-primary endpoint was clinically and statistically significant (p=0.001), with the KOOS pain being 11.76 (LS mean) and KOOS function (sports and recreation) being 11.41 (Table 3). Changes in KOOS pain and function at year 2 are shown in Figure 2. The significant improvement for the MACI implant over MFX was observed for KOOS pain and function as early as 36 weeks (p<0.03), and was maintained at 52 weeks (p<0.025; Figure 3) and out to 104 weeks.

The percentage of patients who responded to treatment at year 2, with at least a 10-point improvement from baseline in both KOOS pain and function scores (Figure 4), was significantly greater (p=.016) for the MACI implant group (87.5%) than the MFX group (68.1%). The trend towards a significantly better response rate for the MACI implant compared with MFX was also evident at 78 weeks (p=0.098).

Predictors' subanalysis of the response rates were performed using patient and lesion characteristics (Table 4). When response rates were analyzed by patient characteristics, significantly more patients responded with the MACI implant than with MFX when patients were male, younger than 34.5 years (median age), only had 1 lesion, had lesions resulting from acute trauma, had 1 prior surgery, or had duration of symptoms lasting more than 3 years. Response rates were not significantly different between patients with or without prior cartilage surgeries. When analyzed by lesions characteristics, significantly more patients responded with the MACI implant compared with MFX when their lesions were >4 cm^2, on the MFC, and not of OCD origin.

Analysis of lesion size and etiology as response predictors based on mean change from baseline of the co-primary endpoint showed significant improvements with the MACI implant versus MFX in patients with lesions 3 to ≤6 cm^2 (p=0.002), with acute trauma (p=0.046), with no chronic degeneration (p=0.007), with no concurrent surgery (p=0.004), and with longer symptom duration (p=0.018), while improvements were not significantly different in patients with larger lesions (6-10 cm^2), without acute trauma, with chronic degeneration, with a concurrent surgery, or with shorter symptom duration. No difference in response predictors was observed when analyzed by days since last surgery between the treatments.

Table 2: Most Frequent Prior and Concomitant Procedures

	MACI implant (n=72)	Microfracture (n=72)
Patients with prior procedure, n (%)	65 (90.3)	60 (83.3)
Prior procedures		
Diagnostic arthroscopy	35 (53.8)	28 (46.7)
Debridement	20 (30.8)	13 (21.7)
Partial meniscectomy, medial	17 (26.2)	7 (11.7)
Loose body removal	16 (24.6)	13 (21.7)
Microfracture	12 (18.5)	18 (30.0)
Shaving	12 (18.5)	12 (20.0)
Other	11 (16.9)	10 (16.7)
ACL repair	9 (13.8)	5 (8.3)
Lavage	7 (10.8)	1 (1.7)
Subchondral drilling	7 (10.8)	4 (6.7)
Synovectomy/synovial plica excision	5 (7.7)	4 (6.7)
Partial meniscectomy, lateral	4 (6.2)	7 (11.7)
Fixation of OCD fragment	4 (6.2)	2 (3.3)
Hardware removal	4 (6.2)	3 (5.0)
Abrasion arthroplasty	2 (3.1)	0
Lateral release of patella retinaculum	2 (3.1)	2 (3.3)
Osteochondral autograft	2 (3.1)	0
Biopsy harvest for ACI	1 (1.5)	0
Bone graft	1 (1.5)	0
Lateral meniscal repair	1 (1.5)	0
Patella tracking	0	1 (1.7)
Medial meniscal repair	0	1 (1.7)
Medial collateral ligament repair	0	1 (1.7)
Concomitant procedures		
During index biopsy or implantation	26 (36.1)	22 (30.6)
Loose body removal	7 (26.9)	9 (40.9)
Synovectomy/synovial plica excision	6 (23.1)	3 (13.6)
Partial medial meniscectomy	6 (23.1)	4 (18.2)
Graft reconstruction	4 (15.4)	6 (27.3)
Other	4 (15.4)	3 (13.6)
Partial lateral meniscectomy	2 (7.7)	4 (18.2)
Lateral release of patella retinaculum	0	1 (4.5)
During week 104 biopsy	19 (26.4)	17 (23.6)
Other	13 (68.4)	13 (76.5)
Loose body removal	6 (31.6)	5 (29.4)
Synovectomy/synovial plica excision	2 (10.5)	3 (17.6)
Partial medial meniscectomy	1 (5.3)	0
Partial lateral menisectomy	0	1 (5.9)

ACI: autologous chondrocyte implantation; ACL: anterior cruciate ligament; MACI: matrix applied characterised autologous chondrocytes; OCD: osteochondritis dissecans.

Other Clinical Outcomes

Year 2 mean improvements from baseline in the other KOOS subscales (ADL, QOL, and other symptoms) were significantly better for patients treated with the MACI implant versus MFX ($p<0.001$, $p=0.029$, and $p<0.001$ respectively; Figure 3). At 24 weeks, mean improvement for the other symptoms subscale was already significantly better for the MACI implant group compared with the MFX group ($p=0.031$) and a trend towards significantly greater improvement ($p=0.078$) was seen for ADL. At 36 weeks, significant improvements from baseline were observed for ADL and other symptoms but not for

Table 3: Mean Scores (± SD) for Patient-Reported Outcomes with the MACI Implant and Microfracture at Baseline and Year 2

	MACI implant				Microfracture					
	Baseline	**n**	**Year 2**	**n**	**Baseline**	**n**	**Year 2**	**n**	**LS Mean Difference**	***p*-Value***
KOOS subscales										
Pain	37.0 ± 13.5	72	82.5 ± 16.2	72	35.5 ± 12.1	71	70.9 ± 24.2	70	11.76	0.001†
Function	14.9 ± 14.7	72	60.9 ± 27.8	72	12.8 ± 16.7	71	48.7 ± 30.3	70	11.41	
Activities of Daily Living	43.5 ± 18.2	72	87.2 ± 16.5	72	42.6 ± 19.6	72	75.8 ± 24.2	71	12.01	<0.001
Knee Quality of Life	18.8 ± 14.7	72	56.2 ± 23.9	72	17.2 ± 14.1	72	47.3 ± 27.0	71	8.98	0.029
Other Symptoms	48.3 ± 16.9	72	83.7 ± 14.0	72	44.4 ± 18.6	72	72.2 ± 19.5	71	11.61	<0.001
Modified Cincinnati Knee Rating System	3.0 ± 1.2	72	6.4 ± 2.1	72	3.0 ± 1.2	72	5.4 ± 2.2	71	1.05	0.002
IKDC Subjective Knee Evaluation	32.9 ± 13.3	71	65.7 ± 18.5	72	29.3 ± 13.4	72	58.8 ± 22.3	71	5.94	0.069
SF-12 Physical Component Score	-1.77 ± 0.86	72	-0.32 ± 0.89	72	-1.93 ± 0.82	69	-0.82 ± 1.12	71	0.51	0.001
SF-12 Mental Component Score	0.04 ± 1.2	72	0.45 ± 0.9	72	-0.17 ± 1.3	69	0.49 ± 1.0	71	-0.09	0.523
EQ-5D Visual Analogue Scale Score	60.8 ± 20.9	72	77.5 ± 15.3	72	56.2 ± 22.1	72	73.4 ± 18.4	70	3.75	0.148

*p-value for difference between treatments in LS means for change from baseline to year 2.

†p-value for co-primary endpoint (KOOS pain and function) for difference between treatments in LS means for change from baseline to year 2.

EQ-5D: European Quality of Life (EuroQOL) 5 dimensions questionnaire; IKDC: International Knee Documentation Committee, KOOS: Knee Injury and Osteoarthritis Outcome Score; MACI: matrix applied characterised autologous cultured chondrocytes; SF-12: 12-Item Short-Form Health Survey

QOL. At 52 and 78 weeks, mean improvements were significantly better for all KOOS subscales for the MACI implant group compared with the MFX group.

The improvements in the modified Cincinnati score from baseline to year 1 and 2 were significantly better with the MACI implant (p=0.018 and p=0.002, respectively) than with MFX (Table 3). Similarly, the change from baseline in IKDC scores was significantly better in patients treated with the MACI implant at year 1 (p=0.009) than patients treated with MFX, although this difference was no longer significant at year 2 (p=0.069).

Significantly better SF-12 physical health scores from baseline to year 1 and 2 were observed for the MACI implant group (p=0.029 and p=0.001, respectively) compared with the MFX group, but no significant difference (p=0.209 and p=0.523, respectively) in mental health scores were observed between the 2 groups (Table 3). Increases in EQ-5D VAS scores from baseline to year 2 were similar for both groups. There was no significant difference in the mean improvement in overall health status at year 1 (p=0.335) or at year 2 (p=0.148) from baseline.

No analyses were conducted with regard to treatment failure rates and time to treatment failure between the 2 treatment groups because of the small number of treatment failure cases. Only 2 patients in the MFX group were deemed treatment failures and no patients in the MACI implant group were considered treatment failures.

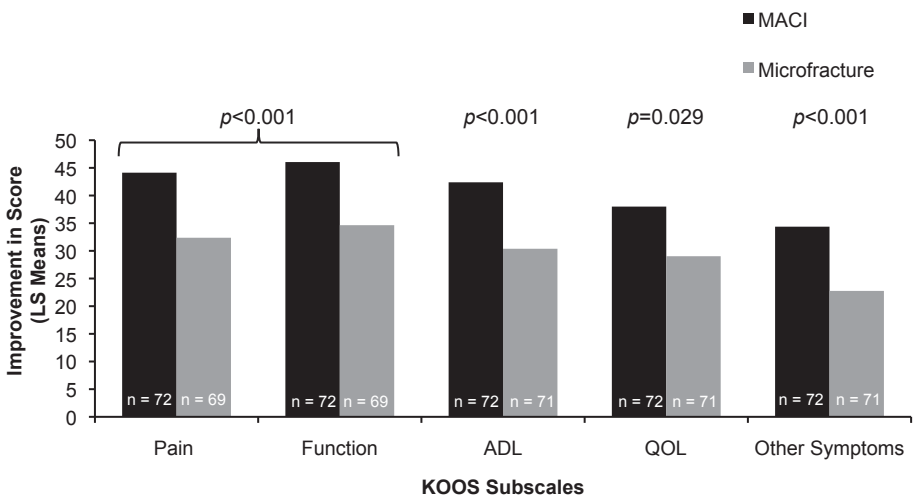
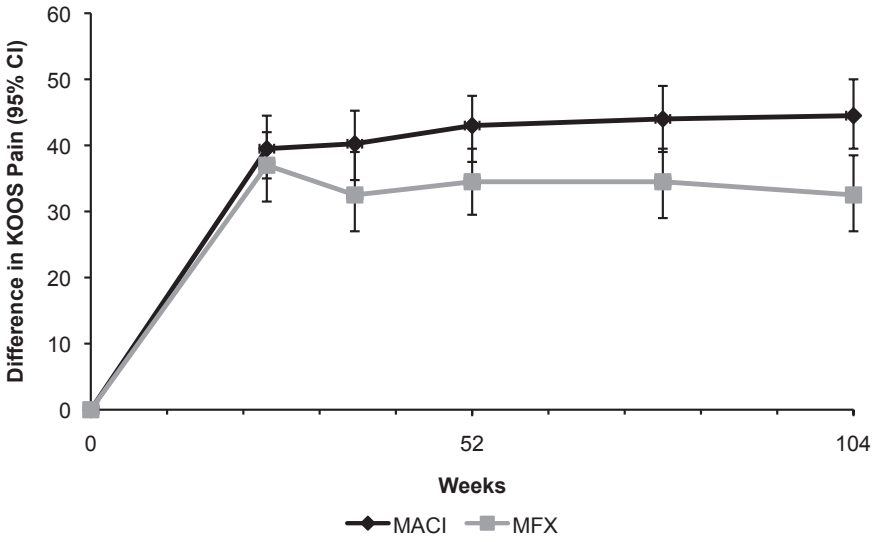


Figure 2: Changes from baseline to Year 2 in all Knee Injury and Osteoarthritis Outcome Score (KOOS) subscales for patients treated with the matrix applied characterised autologous cultured chondrocytes (MACI) implant or microfracture

A. Pain



B. Function

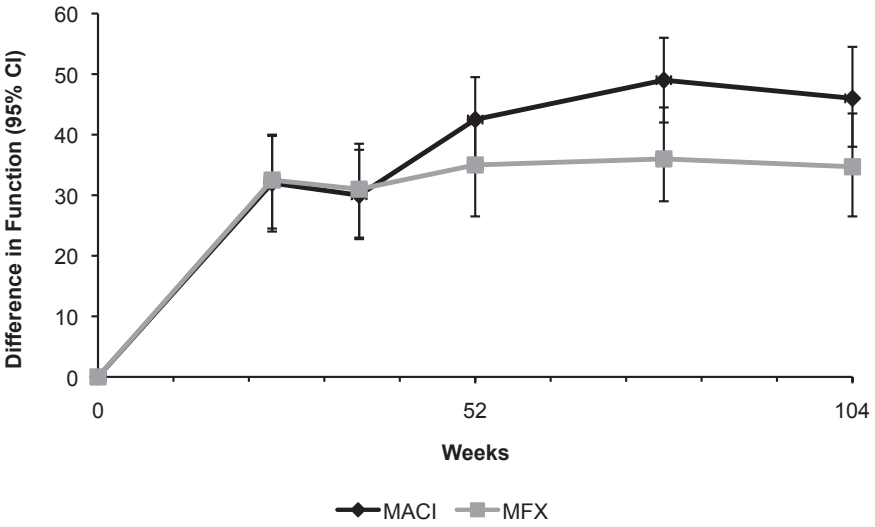


Figure 3: Mean (95% CI) Knee Injury and Osteoarthritis Outcome Score (KOOS) pain (A) and function (B) improvement over time for patients treated with the matrix applied characterised autologous cultured chondrocytes (MACI) implant or microfracture. A significant improvement ($p<0.030$) was observed for the MACI group compared with the MFX group for the KOOS Pain and Function at year 1, which was maintained to year 2 ($p<0.025$).

Repair Tissue Assessment

One hundred sixteen patients (MACI implant n=60; MFX n=56) had a second-look arthroscopy and biopsy (Figure 1). Overall, structural repair tissue was very good; however, the mean microscopic ICRS II overall assessment score between the 2 groups (63.8 versus 62.3; LS mean difference 1.52) was not significantly different (p=0.717). However, greater ICRS II overall assessment scores were observed at year 2 for patients treated with the MACI implant versus MFX if they had had >1 prior cartilage repair surgery, whereas patients treated with MFX versus the MACI implant did better if they had lesions located at the trochlea, osteochondritis dissecans lesions, and no prior cartilage repair surgery.

Repair tissue assessment at year 2 with the macroscopic ICRS II cartilage repair scores showed similar scores between the 2 groups, with no significant difference in overall repair assessment, degree of defect repair, graft integration to border zones, and macroscopic appearance (Table 5). Approximately 76% of patients in the MACI implant group had nearly normal (Grade II) or normal (Grade I) for the overall repair assessment versus 60% in the MFX group. Conversely, 22.2% of patients in the MFX group had an abnormal (Grade III) or severely abnormal (Grade IV) assessment compared with 12.5% in the MACI implant group, although the MFX group has more missing (18% versus 11%). The majority of patients had a degree of defect repair that was in line with the surrounding cartilage, showed graft integration to border zones that was either complete or with a smaller than 1 mm demarcating border, and had repair tissue with an intact smooth or fibrillated surface.

MRI evaluation of structural repair was performed in 134 patients at year 1 and in 139 patients at year 2 (Figure 1). MRI evaluation of structural repair at year 1 and 2 showed improvement in defect filling for both treatment groups but with no statistically significant differences. Two years after treatment, 83% of patients in the MACI group and 77%

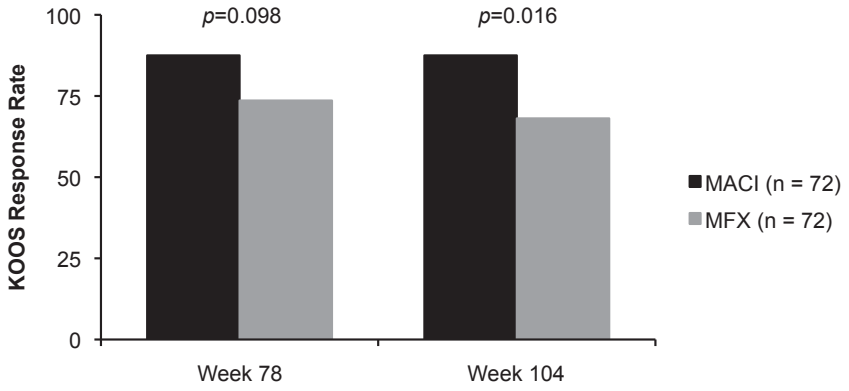


Figure 4: Percentage of patients who responded (≥10-point improvement in Knee Injury and Osteoarthritis Outcome Score (KOOS) pain and function at year 2)

of patients in the MFX group showed a degree of defect fill that was more than 50% of the defect depth.

Table 4: Response Rates* with MACI Implant and Microfracture at Year 2

		Response rates, % patients (n/N)		
		MACI implant	**MFX**	***p*-value**
Overall		87.5 (63/72)	68.1 (49/72)	0.016
Patient Characteristics				
Gender	Male	91.1 (41/45)	68.8 (33/48)	0.012
	Female	81.5 (22/27)	66.7 (16/24)	0.465
Median Age	<34.5 years	91.2 (31/34)	68.4 (26/38)	0.027
	≥34.5 years	84.2 (32/38)	71.9 (23/34)	0.210
Acute Trauma	Yes	87.9 (29/33)	55.8 (24/45)	0.004
	No	87.2 (34/39)	92.6 (25/27)	0.483
No. of Lesions	1	90.0 (45/50)	65.5 (36/55)	0.006
	>1	81.8 (18/22)	76.5 (13/17)	0.964
Prior Cartilage Surgeries	No	90.0 (27/30)	74.2 (23/31)	0.386
	1	87.0 (20/23)	67.9 (19/28)	0.110
	>1	84.2 (16/19)	53.9 (7/13)	0.061
Prior Surgeries	No	100 (11/11)	92.3 (12/13)	0.347
	1	100 (12/12)	62.5 (10/16)	0.027
	>1	81.6 (40/49)	62.8 (27/43)	0.087
Symptoms Duration	≤3 years	81.8 (27/33)	68.9 (21/45)	0.323
	>3 years	92.1 (35/38)	66.7 (18/27)	0.017
Lesion characteristics				
Size	>4 cm^2	97.1 (34/35)	77.4 (24/31)	0.014
	≤4 cm^2	78.4 (29/37)	60.9 (25/41)	0.225
	>5 cm^2	95.2 (20/21)	76.2 (16/21)	0.078
	≤5 cm^2	84.3 (43/51)	64.7 (33/41)	0.067
	>6 cm^2	100.0 (9/9)	86.7 (13/15)	0.253
	≤6 cm^2	85.7 (54/63)	63.2 (36/57)	0.015
Location	MFC	87.0 (47/54)	66.0 (35/53)	0.034
	LFC	92.3 (12/13)	73.3 (11/15)	0.191
	Trochlea	80.0 (4/5)	75.0 (3/4)	0.858
OCD	Yes	87.5 (7/8)	91.7 (11/12)	0.761
	No	87.5 (56/64)	63.3 (38/60)	0.006

*Patients were considered responders if they had ≥10-point improvement over baseline in KOOS pain and function scores.

Safety

No unexpected safety events were reported. Treatment-emergent AEs (TEAEs) were observed in 55 patients (76.4%) in the MACI group and 60 patients (83.3%) in the MFX group. Most treatment-emergent AEs were of moderate or mild intensity. The most common TEAEs (Table 6) were arthralgia (57.6%), headache (23.6%) and nasopharyngitis (11.8%). The incidence of TEAEs considered related to study treatment was comparable between the 2 treatment groups (MACI: 34.7% and MFX: 38.9%). The most common

Table 5: Macroscopic ICRS Cartilage Repair Assessment Scores

n (%)	MACI implant (n=72)	Microfracture (n=72)
Overall Repair Assessment		
Grade I (Normal)	14 (19.4)	8 (11.1)
Grade II (Nearly normal)	41 (56.9)	35 (48.6)
Grade III (Abnormal)	4 (5.6)	12 (16.7)
Grade IV (Severely abnormal)	5 (6.9)	4 (5.6)
Missing	8 (11.1)	13 (18.1)
Degree of Defect Repair		
In Line With Surrounding Cartilage	45 (62.5)	45 (62.5)
75% Repair of Defect Depth	10 (13.9)	10 (13.9)
50% Repair of Defect Depth	4 (5.6)	4 (5.6)
25% Repair of Defect Depth	4 (5.6)	4 (5.6)
0% Repair of Defect Depth	1 (1.4)	1 (1.4)
Missing	8 (11.1)	8 (11.1)
Graft Integration to Border Zones		
Complete Integration	21 (29.2)	15 (20.8)
Demarcating Border <1 mm	20 (27.8)	20 (27.8)
¾ Integrated, ¼ With Border >1 mm	14 (19.4)	13 (18.1)
½ Integrated, ½ With Border >1 mm	3 (4.2)	7 (9.7)
No Contact to ¼ Integrated	6 (8.3)	4 (5.6)
Missing	8 (11.1)	13 (18.1)
Macroscopic Appearance		
Intact Smooth Surface	25 (34.7)	16 (22.2)
Fibrillated Surface	21 (29.2)	22 (30.6)
Small, Scattered Fissures	13 (18.1)	13 (18.1)
Several Small or Few but Large Fissures	3 (4.2)	5 (6.9)
Total Degeneration of Grafted Areas	2 (2.8)	3 (4.2)
Missing	8 (11.1)	13 (18.1)

ICRS: International Cartilage Repair Society; MACI: matrix applied characterised autologous cultured chondrocytes

Table 6: Most Frequently Reported (>5%) Treatment-Emergent Adverse Events

Adverse event n (%)	MACI implant n=72	Microfracture n=72
Any TEAE	55 (76.4)	60 (83.3)
Arthralgia	37 (51.4)	46 (63.9)
Headache	13 (18.1)	21 (29.2)
Nasopharyngitis	10 (13.9)	7 (9.7)
Back pain	8 (11.1)	7 (9.7)
Joint swelling	7 (9.7)	4 (5.6)
Joint effusion	5 (6.9)	4 (5.6)
Influenza	4 (5.6)	5 (6.9)
Pyrexia	4 (5.6)	2 (2.8)
Cartilage injury	3 (4.2)	9 (12.5)
Procedural pain	3 (4.2)	4 (5.6)
Ligament sprain	2 (2.8)	4 (5.6)
Abdominal pain	0 (0.0)	5 (6.9)

TEAE: treatment-emergent adverse event

related TEAEs were treatment failure, arthralgia, and joint swelling. In each group, 1 patient (1.4%) discontinued the study prematurely because of treatment-emergent AEs.

Treatment-emergent serious AEs were reported more frequently in the MFX group (26.4%) than in the MACI group (15.3%), which was attributed to treatment failure, cartilage injury, and arthralgia in the MFX group. No deaths occurred in this study.

The number of patients with at least 1 SSP was not significantly different (p=.0427) between the MACI group (8.3%) and the MFX group (9.7%). Two SSPs were experienced by 2 MFX patients, but by no MACI patient. Increasing age significantly decreased the likelihood of at least 1 SSP occurring (p=0.038).

DISCUSSION

Our study demonstrates that cartilage defect treatment using the MACI implant is clinically and statistically significantly better than MFX for treating symptomatic cartilage defects of the knee, meeting our study's predefined co-primary endpoint. Overall, patients treated with the MACI implant had superior KOOS scores covering all 5 subscales (Pain, Function, ADL, QoL, and Other Symptoms) than patients treated with MFX after 2 years. Additionally, significantly more patients in the MACI implant group had a 10-point improvement or more in both their KOOS pain and function scores versus those in the MFX group. More patients responded when treated with the MACI implant than with

MFX if they were male, young, had symptoms for more than 3 years, had lesions resulting from trauma, larger than >4 cm^2, located on the MFC, and not of OCD in origin.

Scores for the modified Cincinnati Knee Rating System, and SF-12 physical component score also improved significantly more with the MACI implant than with MFX. In addition, no treatment failures were reported for the MACI group compared with 2 in the MFX group as per the failure protocol definition. Further, repair tissue with the MACI implant also showed good structural outcomes, although not statistically different than with MFX. Finally, the safety profile was similar between the groups and no unexpected safety issues were encountered.

Our better outcomes with the MACI implant versus MFX are consistent with the results from the recent smaller randomized trial of Basad et al[123]. In their study (N=60) treating single, isolated, symptomatic chondral defects of the femoral condyle or patella, the Lysholm, Tegner, and patient and surgeon ICRS scores improved significantly more with the MACI implant than with MFX after 2 years[123]. In a case series of 34 patients, the Lysholm-Gillquist score improved by more points with the MACI implant than with MFX (48 versus 29)[124]. Unlike these comparator studies, ours is a more rigorous, multicenter, pragmatic study with MRI and histology endpoints that may help change cartilage repair treatment policy.

Historical series and cohort studies have also shown good outcomes with cartilage cell therapy. However in randomized comparisons, patient-reported outcomes have been shown for the most part to be similar between earlier generation ACI technology and MFX[13, 31]. Vanlauwe and colleagues, found no statistical difference in a combined KOOS score for the overall group of patients who were treated with characterized chondrocyte implantation (CCI) or microfracture after 5 years[31]. Similarly, in a study reported by Knutsen and colleagues, clinical outcomes were similar between ACI and MFX at 5 years; but at 2 years, the SF-36 physical component was better with MFX than with ACI[13]. While previous studies with MFX show good clinical outcomes[125, 126], some reports show that such improvements are not always sustained[110, 111, 127-129]. In 3-year studies by Kreuz et al., clinical results were shown to deteriorate between the 18- and 36-month time points, which depended on the age of the patient and/or location of the lesion[110, 128]. In a 48-month study, Mithoefer and colleagues found that while various clinical outcomes improved up to 24 months after MFX, not all were maintained after 24 to 36 months[111].

Good clinical outcomes reported with the MACI implant in our study are also similar to those reported in previous MACI implant case series. Marlovits and colleagues[101] reported good clinical outcomes with few complications, and a low rate of treatment failure in a 5-year follow-up study of patients treated with the MACI implant procedure. Consistent with our study, the patients had significant improvement from baseline on all KOOS subscales, modified Cincinnati, and IKDC, as well as significant improvements in the Tegner-Lysholm scores as early as 1 year after treatment[101]. In another 5-year follow

up of MACI-implant–treated knee chondral defects[100], patients demonstrated significant improvements from baseline 2 years later, which were maintained to 5 years in all 5 KOOS subscales, both mental and physical component scores of SF-36, ROM knee extension, and a 6-minute walk test. Good clinical outcomes after MACI were also demonstrated in a 2-year study, also reported by Ebert and colleagues[107], where patients who followed a traditional or an accelerated rehabilitation program had similar improvements from baseline in KOOS scores, SF-36, and VAS scores at 2 years, but significantly less pain over time was seen for those in the accelerated versus the more traditional rehabilitation program. In a follow-up study of these patients, significant improvements from baseline in the reported KOOS subscales (sports and recreation, and quality of life) were still evident at 5 years[130].

Of the previous studies described above that reported safety, the MACI implant provided a good safety profile, similar to our study[100, 101, 123]. In one study, typical postoperative swelling and effusion was observed in patients, but resolved within 4 weeks of the MACI procedure[101]. In another study, 2 patients developed a deep vein thrombosis early after treatment while 1 patient developed a postoperative hematoma; all patients recovered without sequelae[100]. In all of the studies, no deaths occurred[100, 101, 123].

Beneficial results with MFX here are also consistent with previous MFX studies showing good clinical outcomes[125, 126]; however, some reports show that such improvements with MFX are not always sustained past 18-24 months, unlike what we found[110, 127, 128].

Our analysis of predictors by response rate showed that more patients with longer duration of symptoms (>3 years) or younger age (< 34.5 median age) improved with MACI vs. MFX. However, Vanlauwe and colleagues found that patients with less time since symptom onset (<3 years versus ≥3 years) did better with CCI than with MFX, while cell therapy in older defects did not seem to have an added benefit[31]. Furthermore, no discernable difference was observed between younger (<35 years) and older (≥35 years) patients[31]. In another study, younger patients (<30 years) had better clinical outcome than older patients but regardless of treatment with MACI or MFX[13]. The reasons for the inconsistencies in our results compared with these previous cell therapy studies are unknown but may pertain to patient population or technique differences.

Structural endpoints assessed by MRI and repair tissue histology assessed by the ICRS II score demonstrated good quality repair tissue with the MACI implant. However, the good quality repair tissue with the MACI implant was not numerically different than that found with MFX, even given the clinical results favoring the MACI implant. These findings were unexpected in that MFX performed better than anticipated, as previous studies show better repair tissue with autologous cell therapies than with MFX. In a study by Bachmann and colleagues, MRI-evaluated repair tissue was of better quality with the MACI implant than that with MFX[124]. For the 27 patients who received the MACI implant, defect fill was more consistent than with MFX (n=7), and 78% of patients' repair

tissues was integrated with adjacent cartilage[124]. Further, the MRI signal indicated that the signal intensity of the repair tissue with the MACI implant was close to that of the surrounding native cartilage, but was not homogenous with a signal intensity different than that of adjacent normal cartilage with MFX[124].

Other studies show better repair tissue with other cell-therapy technologies than with MFX. One year after characterized chondrocyte implantation (CCI), structural repair tissue was better than with MFX[14], as shown by better mean histology assessment (blinded) score (p=0.012), particularly in chondrocyte phenotype (p<0.01) and tissue structure (p<0.05) in CCI-treated patients than in MFX-treated patients[14]. Safranin O and collagen II stainings were also more intense as detected by the mean histomorphometry score with CCI than with MFX (p=0.03)[14]. However, MRI assessment showed similar repair tissue after 3 years[32], with no report on repair tissue at year 5[31].

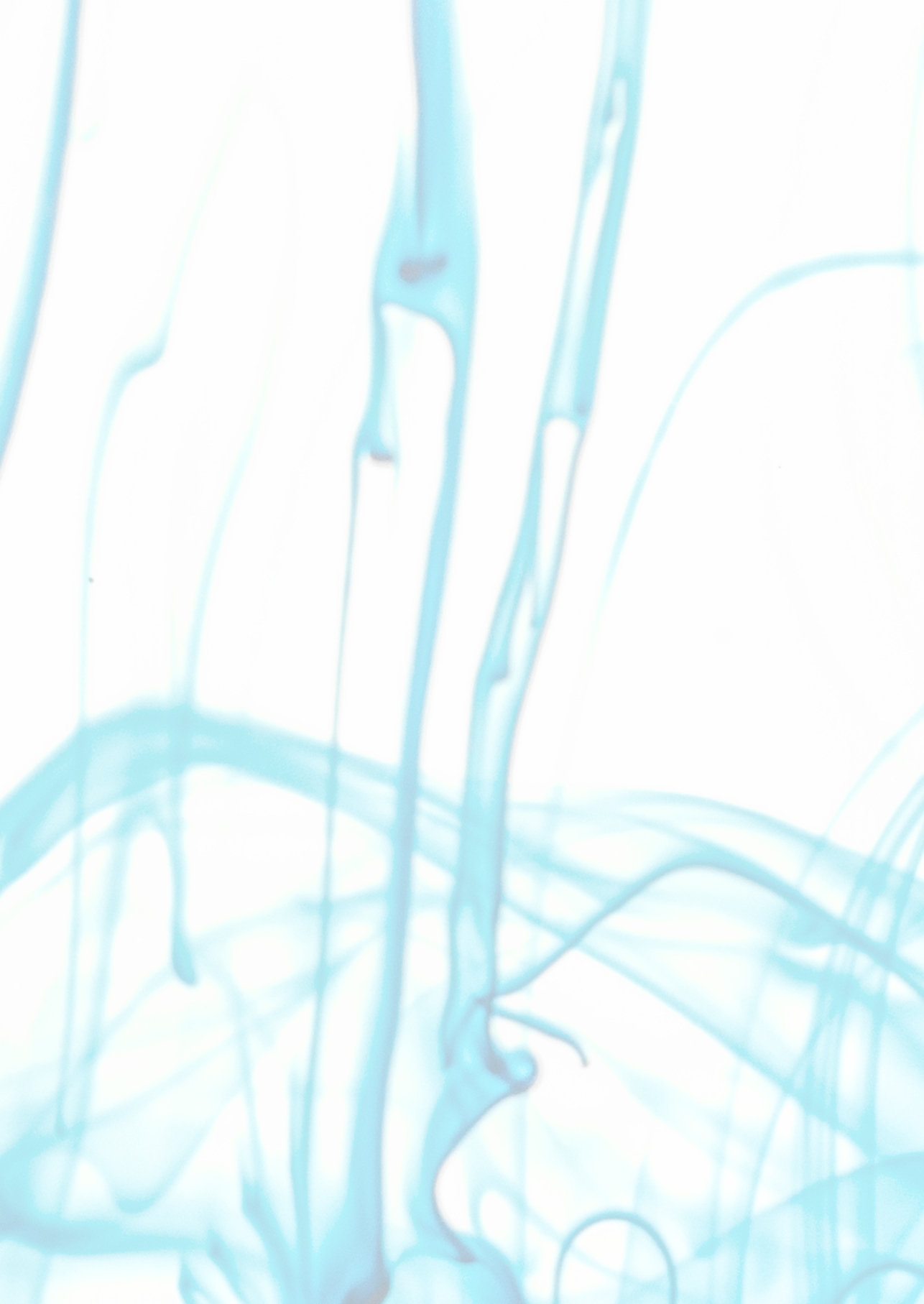
The reasons for our unanticipated similar results in repair tissue between the MACI implant and MFX are unknown. The clinical relevance and applicability to long-term clinical outcome of the ICRS II, a recently developed grading system for cartilage repair, especially in terms of histology still needs to be established[131]. Further, one cannot ensure that biopsies taken were the best representative sample of the total repair tissue especially since the samples were taken by individual surgeons and not by one dedicated sampling person, although this would apply equally to both groups[132]. Evidence for the "overperformance" of MFX in the present study can be found in a study comparing MFX with CCI, as our overall ICRS II score with MFX (62.3) was numerically higher than that in the MFX-CCI comparison (approximately 44)[14]. Finally, an error in the protocol that directed pre-operative MRI reads to be scored as post-operative reads may have contributed to these unexpected results. Further investigation of this scoring issue is currently underway.

Additional longer-term comparative studies are needed to further understand the relationship between clinical outcomes and integrity of the structural cartilage tissue. A systematic review and meta-analysis reported by de Windt et al. found that the majority of articular cartilage repair of the knee studies show limited or no correlation between clinical outcomes and MRI parameters; only 28% of studies (9 of 32) showed a correlation between clinical outcome and MOCART or Henderson scores[133]. This is in line with guidance from regulatory agencies (EMA and FDA) that suggests MRI data, as well as histology data, are not predictive of outcome, and that clinical outcomes assessing pain and function are the most important parameters in determining the efficacy of cell-based therapies[115, 116]. Nevertheless, an extension of our study is currently underway, where 3- and 5-year outcomes will be assessed, which may reveal a difference in MRI structural outcome between MACI and MFX treatments. Lastly, we did not examine biochemical properties of the repair tissue, which may help explain the better clinical outcome with the MACI implant.

Some of the limitations of this study include the fact that it was a multicenter study and therefore procedures were performed by many surgeons, and that it was not a blinded study. Procedures being performed by more than one physician may introduce investigator variability; although all surgeons were trained on standardized surgical procedures and their training was audited by the sponsor. In addition, given that the surgical techniques for the MACI implant (2 surgeries required) and microfracture (1 surgery) are different, the study could not be blinded; however, histological and MRI evaluations were assessor blinded. Comparing 2- and 1-step surgeries is also a limitation, but the similar safety profile between the two demonstrates that the 2-step procedure is not more risky than the 1-step procedure, also reflected in our clinical experience. Due to the inherent heterogeneity of cartilage repair tissue, one limitation of the histological evaluation is the inability to ensure that the biopsy acquired was representative of the total cartilage repair tissue[132]. Also, it is possible that the favorable results observed for patients in both treatment groups could have been positively influenced by the rigorous patient education and follow-up inherent in the study protocol.

Our SUMMIT clinical trial is one of the very few Good Clinical Practice-conducted, prospective, multi-center, randomized, controlled study of cell-based cartilage repair to date. The study included stringent inclusion and exclusion criteria, standardized surgical and rehabilitation procedures, and ensured a comprehensive patient follow up. Other strengths of the study included the use of validated clinical outcomes and histology and MRI assessments.

Overall, improvements in clinically relevant endpoints such as pain and function, as opposed to those of structural repair, remain the more important endpoints for the study of cartilage defects with regard to patient care[132]. This trial demonstrated that the MACI implant provides more pain relief and functional improvement when compared with MFX, with a similar safety profile, when treating symptomatic articular cartilage defects of the knee based on significantly better outcomes for various patient-reported efficacy endpoints.



Chapter 3

Synovial fluid cytokine profiles depend on cartilage pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes

Anika I. Tsuchida
Michiel Beekhuizen
Marieke C. 't Hart
Timothy R.D.J. Radstake
Wouter J. A. Dhert
Daniel B. F. Saris
Gerjo J.V.M. van Osch
Laura B. Creemers

Submitted for publication

ABSTRACT

Objective

To evaluate whether profiles of several soluble mediators in synovial fluid and cartilage tissue are pathology-dependent and how their production is related to *in vitro* tissue regeneration by chondrocytes from diseased and healthy tissue.

Methods

Samples were obtained from donors without joint pathology (n=39), with focal defects (n=65) and osteoarthritis (n=61). A multiplex bead assay (Luminex) was performed measuring up tot 21 cytokines: IL-1α, IL-1β, IL-1RA, IL-4, IL-6, IL-6Rα, IL-7, IL-8, IL-10, IL-13, TNFα, IFNγ, OSM, LIF, adiponectin, leptin, MCP1, RANTES, bFGF, HGF, VEGF.

Results

In synovial fluid of patients with cartilage pathology, IL-6, IL-13, IFNγ and OSM levels were higher than in donors without joint pathology ($p \leq 0.001$). In cartilage tissue from debrided defects, VEGF was higher than in non-pathological or osteoarthritic joints ($p \leq 0.001$). IL-1α, IL-6, TNFα and OSM concentrations (in ng/ml) were markedly higher in cartilage tissue than in synovial fluid ($p < 0.01$). Culture of chondrocytes generally led to a massive induction of most cytokines ($p < 0.001$). Although the release of inflammatory cytokines was also here dependent on the pathological condition ($p < 0.001$) the actual profiles were different from tissue or synovial fluid. Cartilage regeneration was lower by healthy unexpanded chondrocytes than by osteoarthritic or defect chondrocytes.

Conclusions

Several pro-inflammatory, pro-angiogenic and pro-repair cytokines were elevated in donors with symptomatic cartilage defects and osteoarthritis, although different cytokines were elevated in synovial fluid, tissue and cells. However, these alterations did not affect *in vitro* regeneration with these chondrocytes, as this was at least as effective or even better compared to healthy chondrocytes.

INTRODUCTION

Soluble mediators, such as cytokines, chemokines, adipokines and growth factors are key regulators of cartilage metabolism. Under homeostatic conditions the production of these mediators is tightly regulated. In many joint diseases, including osteoarthritis (OA) and joint trauma, elevated levels of inflammatory cytokines are present[134] but their pathophysiological role is not always well established. In inflammatory diseases such as rheumatoid arthritis, the synovial cells produce large quantities of inflammatory cytokines and degradative enzymes[135], which reach the cartilage through the synovial fluid. Although less outspoken, synovial inflammation is also present in OA[58, 136] and sometimes in joints with cartilage defects[57], and the balance between anabolic and catabolic mediators has been suggested to be in favour of catabolic factors leading to a net breakdown of cartilage. In addition to their possible role in the pathophysiology of joint disease, soluble mediators present in the joint may also affect cartilage repair, both in osteoarthritis and in cartilage defect treatment[81, 82].

These mediators are not only produced by the synovial lining cells, but also by resident chondrocytes in response to both biological and mechanical stimuli[134]. In fact, in OA, certain cytokines appeared to be predominantly produced by cartilage rather than synovial tissue[62]. Furthermore, some mediators were shown to bind to extracellular matrix components, such as glycosaminoglycans (GAGs), in cartilage[137]. Therefore local concentrations in the microenvironment of the chondrocyte might differ from those in the synovial fluid. Evaluating the presence of mediators in the cartilage tissue in addition to the synovial fluid might provide more insight into the possible mechanisms in cartilage regeneration and degeneration.

In addition to the cytokines in synovial fluid and cartilage extracellular matrix, studying the cytokine profile during *in vitro* cartilage regeneration may provide essential clues as to which mediators are important for tissue engineering of cartilage and to what extent cytokine production by chondrocytes may affect the outcome of cartilage repair procedures. Few studies have directly compared healthy chondrocytes to chondrocytes obtained from cartilage defects and/or osteoarthritic chondrocytes in terms of regenerative capacity[138] and a comprehensive secretory profile is lacking. Characterizing the mediators produced by both healthy and diseased chondrocytes may allow for more specific inhibition of mediators in order to improve cartilage regeneration, especially by dedifferentiated or diseased chondrocytes.

Therefore in this study we compared the presence of soluble mediators commonly suggested to play a role in joint pathology in synovial fluid and cartilage tissue for donors with healthy cartilage, patients with symptomatic cartilage defects and patients with end-stage osteoarthritis. In addition, the production of these and additional cytokines by isolated chondrocytes during *in vitro* cartilage regeneration was investigated using

both expanded and non-expanded chondrocytes obtained from donors with different joint pathology.

MATERIALS AND METHODS

Synovial fluid and cartilage sample collection and cell isolation

Collection of all patient material was done according to the Medical Ethical regulations of the University Medical Centre Utrecht and according to the guideline 'good use of redundant tissue for clinical research' constructed by the Dutch Federation of Medical Research Societies on collection of redundant tissue for research[139]. This study does not meet the definition of human subjects research or require informed consent. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[140].

Macroscopically healthy articular cartilage and synovial fluid was obtained from donors within 24 hours post mortem (see Table 1 for details). Defect cartilage and synovial fluid was obtained from donors undergoing either microfracture or autologous chondrocyte implantation for focal grade III and IV cartilage defects. During those procedures, the cartilage defect is debrided to remove all cartilage remnants down to the subchondral bone and create a stable cartilage rim. The debrided cartilage was used. Of the 22 patients with symptomatic cartilage defects, one had associated anterior cruciate ligament (ACL) injury and a history of partial meniscectomy; another three had received previous partial meniscectomies and one an ACL reconstruction. OA cartilage and synovial fluid was obtained from donors undergoing total knee arthroplasty. Synovial fluid was spun down at 300 g to remove debris, and stored at -80 °C until use or analysis.

Table 1: Donor characteristics

Donortype	Synovial fluid	Tissue	Non-expanded chondrocytes	Expanded chondrocytes
Healthy	n=20	n= 9	n= 8	n= 15
age, average (range)	40 (25-47)	69 (54-81)	66 (54-75)	67 (54-81)
% male	unknown	80	50	60
Cartilage defect	n= 31	n= 16	n= 10	n= 13
age, average (range)	33 (20-48)	33 (16-45)	32 (18-44)	24 (19-36)
% male	unknown	47	70	85
Osteoarthritis	n=27	n= 27	n= 12	n= 13
age, average (range)	70 (53-81)	67 (46-90)	69 (49-83)	70 (46-83)
% male	unknown	23	38	38

Cartilage samples were rinsed in phosphate buffered saline (PBS), and either snap frozen in liquid nitrogen and stored at -80 °C until later protein extraction or cut into small pieces and enzymatically digested overnight at 37 °C in a 0.15% collagenase type II (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Bleiswijk, The Netherlands) with penicillin/streptomycin (100 U/mL/100 µg/mL; Invitrogen, Life Technologies). After digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Diego CA, USA), and the chondrocytes were spun down by 10 minutes centrifugation at 300 g.

Protein extraction

The frozen cartilage samples (Table 1) were cut in 7 µm sections with a cryomicrotome. The sections were collected and carefully weighed. Approximately 100 mg of cartilage tissue was collected per sample and lysed in 5 µl lysis buffer per mg tissue with complete protease inhibitor (Complete Lysis M; #04719964001, Roche Diagnostics, Almere, The Netherlands). The samples were cooled on ice and homogenized using 1.4 mm ceramic beads (VWR, Amsterdam, The Netherlands) in a bead beater. Subsequently, the samples were centrifuged at 4 °C for 4 minutes at 13000 rpm and supernatants collected. Supernatants were then filtered by centrifuging through a polypropylene tube containing a 0.22 µm nylon membrane (Spin-X column; Corning, Amsterdam, The Netherlands) prior to measurements of the cytokine levels.

Measurement of cytokine levels

To determine the cytokine levels in the synovial fluids and tissue extracts of healthy, defect and OA donors and in the conditioned media (day 7) of healthy, defect and OA chondrocytes during regeneration, a multiplex bead-assay (Luminex) was performed as previously described [141, 142]. A panel of a total 21 cytokines was available (IL-1α, IL-1β, IL-1RA, IL-4, IL-6, IL-6Rα, IL-7, IL-8, IL-10, IL-13, TNFα, IFNγ, OSM, LIF, adiponectin, leptin, MCP1, RANTES, bFGF, HGF, VEGF) of which 11 were measured in the synovial fluid, 19 in tissue extracts and all 21 in conditioned media. Briefly, specific antibodies were coupled to carboxylated beads (Luminex Corporation, Austin TX, USA). The levels of IL-6 and IL-8 present in the synovial fluid have previously been published[143, 144]. Recombinant cytokines were used to make a standard curve. Synovial fluid was first treated with hyaluronidase (type IV-S, Sigma-Aldrich, Zwijndrecht, The Netherlands) at a concentration of 20 U/ml for 30 minutes at 37°C and then filtered by centrifuging through a Spin-X column. Subsequently, the synovial fluid samples were diluted 1:2 with HPE-0.1375% Tween (Sanquin, Amsterdam, The Netherlands). To block possible interfering antibodies present in the synovial fluid, the samples were diluted with an equal volume of 10% (v/v) normal rat and mouse serum (Rockland Immunochemicals Inc., Gilbertsville, PA, USA). Tissue extracts and medium samples were directly incubated with the coupled beads.

After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin–phycoerythrin (BD Biosciences) for 10 minutes. After washing, the samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules CA, USA) with Bio-Plex Manager software, version 3.0. The concentration of cytokines was expressed as pg/mL using the standard curves. Results of specific ELISAs for determination of cytokine levels have previously been shown to be comparable to multiplex bead assay (Luminex)[62].

Regeneration culture

Isolated chondrocytes from healthy, defect and OA cartilage were used either directly after isolation or after expansion to passage two (see table 1 for more details). Expansion was performed in monolayer at 37 °C and 5% CO_2 at a seeding density of 5000 cells per cm^2 in expansion medium consisting of DMEM, 10% fetal bovine serum (Hyclone, Thermo Scientific, Etten-Leur, the Netherlands), penicillin/streptomycin (100 U/mL/100µg/mL) and 10 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA). Unexpanded chondrocytes and expanded chondrocytes were seeded on collagen type II-coated (Chicken sternal cartilage; Sigma-Aldrich, #C9301) Millicell filters (Millipore Co., Bedford MA, USA), at $1.6x10^6$ cells per cm^2 and redifferentiated during 28 days in redifferentiation medium consisting of DMEM, 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), 2% insulin-transferrin-selenium (ITS)-X (Invitrogen) and 5 ng/mL transforming growth factor-β_2 (TGF-β_2; R&D systems).

Glycosaminoglycan and DNA analysis

After culture, the regenerated tissue was digested overnight in a 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) assay[145]. 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. Supernatants were also collected and analyzed for GAGs released into the medium.

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

Statistical analysis

All statistical analyses were performed using SPSS 18.0. (SPSS Inc., Chicago, IL, USA) Results are displayed as mean ± standard deviation (SD). Differences in cytokine levels between healthy, cartilage defect and OA donors were determined using the Kruskall-Wallis test with *post hoc* Mann-Whitney *U*-test. Differences in cytokine levels between synovial fluid and tissue were determined using Mann-Whitney *U*-test and between tissue, unexpanded and

expanded cells with the Kruskall-Wallis test. Principal component analysis was performed to identify clusters of related cytokines in synovial fluid, cartilage tissue and production by both non-expanded and expanded cells. Separate principal component analysis of healthy, cartilage defect and OA samples was not possible due to the limited number of subjects per disease condition. Only cytokines with communalities > 0.5 were included. Loading factors were maximized using Varimax rotation with Kaiser Normalisation. The number of clusters was decided based on the scree-plot and eigenvalues (> 1.0). Cytokines were categorized per cluster when their loading scores were > 0.5. A Crohnbach's α was performed on each cluster to verify consistency. Differences in GAG and DNA content between H, CD and OA donors were determined using ANOVA with *post hoc t*-test and differences between unexpanded and expanded chondrocytes by *t*-test. Multiple linear regression analysis with backward elimination was performed to identify cytokines that best predict cartilage regeneration as measured by GAG/DNA.

RESULTS

Elevated levels of IL-6, IL-13, IFNγ, OSM and VEGF in donors with symptomatic focal cartilage defects

In total 11 soluble mediators were measured in the synovial fluid of healthy donors, donors with symptomatic cartilage defects and osteoarthritic donors. Four of them were present at significantly different concentrations, namely IL-6, IL-13, IFNγ and OSM ($p \leq 0.001$; Table 2); The concentrations of IL-6 and OSM were significantly lower in synovial fluid of donors without known joint pathology than in synovial fluid of patients with cartilage defects or OA (in line with previous data[143]). For IL-13 and IFNγ the concentrations were highest in synovial fluid of patients with a cartilage defect.

A total of 20 soluble mediators were measured in cartilage tissue extracts of healthy cartilage, cartilage debrided from defects and osteoarthritic cartilage (Table 3). Only VEGF concentrations were statistically significantly different, with the highest concentrations in cartilage debrided from defects and the lowest concentrations in healthy cartilage ($p < 0.001$; Tables 2 and 3).

To be able to compare the levels in synovial fluid and cartilage tissue, the wet weight of tissue was taken to represent to its volume according to an average of 1.05 g/ml density for tissues devoid of inorganic constituents[146] such as bone. This would underestimate values by 5% when expressed per volume unit. Concentrations of IL-1α, IL-1β, IL-6, TNFα, IFNγ and OSM were different between synovial fluid and cartilage tissue ($p<0.01$). Especially in 1 ml cartilage debrided from defects and OA cartilage, some tissue explants revealed concentrations of IL-1α, IL-6, TNFα and OSM that were at least ten to a hundred fold higher than in 1 ml synovial fluid (Table 2).

Table 2: Cytokines in the synovial fluid and cartilage tissue

Synovial fluid					Cartilage				
Cytokines (per ml SF)	**Healthy (n=20)**	**Defect (n=31)**	**Osteoarthritis (n= 27)**	**p-value***	**Cytokines per g tissue**	**Healthy (n=9)**	**Defect (n=16)**	**Osteoarthritis (n=27)**	**p-value***
IL-1α, pg/ml	16 ± 10 (100%)	14 ± 8 (100%)	15 ± 22 (89%)	0.085	IL-1α, pg/g	5 ± 13 (22%)	366 ± 706 (25%)	183 ± 391 (26%)	0.470
IL-1β, pg/ml	1 ± 2 (25%)	15 ± 18 (55%)	8 ± 16 (41%)	0.032	IL-1β, pg/g	0 ± 0 (0%)	23 ± 63 (13%)	0 ± 1 (4%)	0.336
IL-4, pg/ml	0 ± 0 (0%)	2 ± 3 (32%)	1 ± 4 (15%)	0.026	IL-4, pg/g	0 ± 0 (0%)	1 ± 2 (13%)	0 ± 1 (7%)	0.261
§IL-6, pg/ml	64 ± 120 (40%)	261 ± 385 (96%)	396 ± 508 (82%)	<0.001*	IL-6, pg/g	1064 ± 3193 (11%)	1473 ± 3162 (19%)	935 ± 2739 (19%)	0.752
IL-7, pg/ml	0 ± 0 (0%)	0 ± 0 (0%)	5 ± 28 (4%)	0.459					
§IL-8, pg/ml	25 ± 29 (75%)	27 ± 33 (59%)	52 ± 95 (85%)	0.352	IL-8, ng/g	12 ± 20 (67%)	10 ± 30 (31%)	15 ± 44 (48%)	0.442
IL-10, pg/ml	1 ± 6 (5%)	0 ± 0 (0%)	9 ± 35 (15%)	0.134	IL-10, pg/g	0 ± 0 (0%)	812 ± 2113 (13%)	578 ± 1650 (15%)	0.400
IL-13, pg/ml	1 ± 2 (5%)	38 ± 41 (64%)	18 ± 40 (33%)	<0.001*	IL-13, pg/g	0 ± 0 (0%)	799 ± 1641 (25%)	250 ± 648 (15%)	0.121
TNFα, pg/ml	0 ± 0 (0%)	2 ± 8 (9%)	4 ± 20 (11%)	0.324	TNFα, pg/g	522 ± 1084 (22%)	3069 ± 4453 (25%)	1733 ± 3212 (30%)	0.467
IFNγ, pg/ml	47 ± 17 (100%)	68 ± 38 (100%)	51 ± 69 (93%)	0.001*	IFNγ, pg/g	0 ± 0 (0%)	226 ± 848 (13%)	0 ± 0 (0%)	0.101
OSM, pg/ml	2 ± 7 (5%)	22 ± 46 (64%)	38 ± 121 (22%)	0.001*	OSM, pg/g	66 ± 108 (44%)	170 ± 241 (38%)	220 ± 424 (37%)	0.786
					LIF, pg/g	0 ± 0 (0%)	2797 ± 6515 (13%)	3529 ± 10164 (22%)	0.279
					Adiponectin, ug/g	12 ± 10 (100%)	57 ± 62 (88%)	19 ± 29 (100%)	0.002
					Leptin, ng/g	15 ± 29 (33%)	120 ± 169 (63%)	112 ± 136 (83%)	0.026
					MCP1, pg/g	182 ± 423 (22%)	1336 ± 1711 (50%)	1249 ± 1250 (68%)	0.044
					RANTES, pg/g	0 ± 0 (0%)	1225 ± 2163 (31%)	510 ± 1121 (22%)	0.056
					bFGF, ng/g	129 ± 335 (44%)	439 ± 691 (69%)	615 ± 719 (74%)	0.055
					HGF, pg/g	3171 ± 6757 (56%)	5824 ± 5110 (69%)	2986 ± 3452 (74%)	0.113
					VEGF, ng/g	19 ± 11 (100%)	212 ± 149 (88%)	49 ± 28 (100%)	<0.001*
					IL-1RA, pg/g	495 ± 1017 (22%)	1856 ± 3172 (25%)	2504 ± 10792 (33%)	0.573

The presence of cytokines was measured in synovial fluid (SF) and cartilage tissue extracts from healthy donors and donors with symptomatic cartilage defects and osteoarthritic donors. Results are represented as average ± standard deviation (% of samples in which the cytokine was present), differences in cytokine levels between healthy, cartilage defect and OA donors were determined using the Kruskall-Wallis test (*p*-values shown in table) with *post hoc* Mann-Whitney *U*-test;*$p<0.001$. §Data published or submitted for publication previously[16,17].

Post hoc Man-Whitney *U*-tests: Synovial fluid: IL-6 H VS. CD $p<0.001$, H vs. OA $p=0.001$; IL-13 H vs. CD $p<0.001$, H vs. OA $p=0.018$, CD vs. OA $p=0.029$; IFNγ Hv s. CD $p=0.008$, H vs. OA $p=0.05$, CD vs. OA $p=0.001$; OSM H vs. CD $p<0.001$, CD vs. OA $p= 0.030$. Tissue: VEGF H vs. CD $p<0.001$, H vs. OA $p=0.003$, CD vs. OA $p<0.001$.

Cytokine production by isolated and cultured chondrocytes during regeneration

Soluble mediator production by isolated but unexpanded chondrocytes during regeneration was very similar between healthy, cartilage defect and OA chondrocytes (Table 3). Only IL-1β was produced at significantly higher amounts by chondrocytes from debrided cartilage from defects compared to healthy and OA chondrocytes ($p<0.001$; Table 3). Total GAG production per DNA was not different between healthy, cartilage defect and OA regeneration cultures (Figure 1C). However most of the GAG produced during regeneration was not retained in the newly formed tissue but released in the medium (Figure 1B). Remarkably, unexpanded chondrocytes from defect cartilage (GAG/DNA 19.6 ± 15.1 μg/μg) and OA chondrocytes (GAG/DNA 22.8 ±17.1 μg/μg) retained more of the produced GAG in the matrix than isolated unexpanded chondrocytes from healthy cartilage (GAG/DNA 9.4 ± 4.4 μg/μg; $p<0.001$; Figure 1A).

Expansion in monolayer for two passages had a major effect on cytokine secretion. The secretion of most cytokines was reduced at least by half after expansion, but the expression of IL4, IFNγ, HGF and, to a lesser degree also IL-1α and IL-1β, was clearly increased by expansion of chondrocytes (Table 3). Although before expansion only IL-1β was differentially released between chondrocytes from different joint pathologies, culture-expanded chondrocytes from OA cartilage showed a higher production of IL-6, IL-8 and LIF during regeneration culture compared to chondrocytes from healthy cartilage and/ or cartilage debrided from defects ($p<0.001$; Table 3).

The GAG content per DNA of the newly formed cartilage by culture-expanded chondrocytes after four weeks of culture was lower than by unexpanded chondrocytes and not different between chondrocytes isolated from healthy cartilage, cartilage defect or OA. In contrast, total GAG production per DNA was highest by expanded chondrocytes from cartilage debrided from defects (Figure 1C) which was associated with a higher GAG release by these cells (Figure 1B).

When comparing cytokine production per mg of DNA present in native tissue or in regeneration cultures, cytokine production by chondrocytes in cell culture was generally much higher than by chondrocytes in their native matrix ($p<0.001$ for all cytokines except OSM, $p=0.043$; Table 3). Exceptions were TNFα, adiponectin, bFGF and HGF, which were lower ($p<0.001$ for all cytokines) in cultured cells, and leptin which was similar. IL-1β, IL-4, IL-10, IL-13, IFNγ, LIF and RANTES were not present in healthy cartilage tissue, but were induced during regeneration culture.

Cluster analysis

Synovial fluid

Principal component analysis revealed three clusters of correlated cytokines (Figure 2). Cluster 1 consisted of IL-1α, IL-7, IL-8, IL-10, TNFα and OSM and explained 53% of variance

Table 3: Cytokines produced by chondrocytes in native tissue and in culture per cell

	Tissue				Unexpanded chondrocytes				Expanded chondrocytes			
Cytokine /mg DNA	**Healthy (n= 9)**	**Defect (n= 16)**	**Osteoarthritis (n= 27)**	**p-value***	**Healthy (n= 8)**	**Defect (n= 10)**	**Osteoarthritis (n= 12)**	**P-value***	**Healthy (n=15)**	**Defect (n= 13)**	**Osteo-arthritis (n=13)**	**p-value***
IL-1α, pg/mg	5 ± 12	198 ± 630	408 ± 1062	0.844	439 ± 268	993 ± 743	460 ± 271	0.006	1718 ± 2689	1978 ± 1464	2569 ± 2528	0.006
IL-1β, pg/mg	0 ± 0	14 ± 41	0 ± 2	0.336	14 ± 33	816 ± 968	125 ± 151	<0.001*	283 ± 368	369 ± 270	459 ± 408	0.010
IL-4, pg/mg	0 ± 0	0 ± 1	0 ± 1	0.536	18 ± 49	18 ± 40	40 ± 76	0.691	148 ± 224	200 ± 163	263 ± 259	0.002
IL-6, pg/mg	4813 ± 14438	678 ± 1867	1840 ± 6389	0.928	1715 ± 397	1739 ± 437	1918 ± 77	0.037	423 ± 414	136 ± 268	721 ± 319	<0.001*
IL-7, pg/mg					1288 ± 378	1120 ± 547	1304 ± 619	0.457	300 ± 401	411± 343	545± 555	0.008
IL-8, ng/mg	39 ± 94	7 ± 23	28 ± 102	0.192	1070 ± 572	786 ± 625	784 ± 399	0.036	28 ± 36	36 ± 43	113 ± 85	<0.00*
IL-10, pg/mg	0 ± 0	252 ± 783	969 ± 3344	0.483	3021± 2455	16529 ± 20669	4925 ± 3429	0.015	5200 ± 6788	6524 ± 4806	9471 ± 9333	0.029
IL-13, ng/mg	0 ± 0	378 ± 783	545 ± 1531	0.296	2027 ± 597	3249 ± 1155	2936 ± 1220	0.002	4486 ± 6088	5614 ± 3730	6787 ± 5935	0.006
TNFα, pg/mg	499 ± 1020	2054 ± 4520	3567 ± 8065	0.714	253 ± 184	414 ± 346	426 ± 452	0.319	183 ± 221	217 ± 148	310 ± 315	0.154
IFNγ, pg/mg	0 ± 0	175 ± 677	0 ± 0	0.101	50 ± 214	54 ± 245	173 ± 636	0.615	538 ± 866	720 ± 767	1036 ± 1287	0.079
OSM, pg/mg	156 ± 279	135 ± 239	394 ± 776	0.963	111 ± 164	2736 ± 3990	369 ± 640	0.138	26 ± 37	26 ± 23	46 ± 55	0.135
LIF, ng/mg	0 ± 0	1 ± 4	5 ± 13	0.238	76 ± 50	194 ± 127	97 ± 56	0.005	38 ± 37	23 ± 16	48 ± 24	0.000*
Adipon, ng/mg	15700 ± 12550	60900 ± 74720	20000 ± 26960	0.238	1582 ± 903	3689 ± 5136	1777 ± 1220	0.723	20 ± 31	22 ± 21	36± 41	0.022
Leptin, ng/mg	14 ± 29	75 ± 151	161 ± 199	0.009	22 ± 7	26 ± 7	22 ± 9	0.125	22 ± 24	29 ± 18	35 ± 27	0.014
MCP1, n=pg/mg	672 ± 1890	806 ± 1067	2216 ± 3780	0.068	376 ± 148	626 ± 474	331 ± 219	0.065	43 ± 14	38 ± 20	53 ± 49	0.176
RANTES, pg/mg	0 ± 0	986 ± 2687	1074 ± 2852	0.208	129 ± 149	63 ± 80	106 ± 89	0.057	3 ± 4	1 ± 1	4 ± 4	0.034
bFGF, ng/mg	196 ± 527	514 ± 762	760 ± 923	0.089	12 ± 15	39 ± 68	11 ± 18	0.427	20 ± 17	23 ± 14	34 ± 26	0.021
HGF, pg/mg	3262 ± 6163	5763 ± 10551	3911 ± 4661	0.565	151 ± 243	431 ± 470	353± 534	0.118	4243 ± 6533	5314 ± 4061	7242 ± 7595	0.004
VEGF, ng/mg	33 ± 22	173 ± 133	66 ± 53	<0.001*	638 ± 468	828 ± 522	742 ± 298	0.333	126 ± 23	133 ± 29	124 ± 25	0.610
IL-1RA, pg/mg	475 ± 960	990 ± 1938	1871 ± 7209	0.868	2611 ± 2167	20259 ± 23520	11005 ± 17284	0.002	349 ± 260	433 ± 260	579 ± 442	0.031
IL-6Rα, pg/mg					8217 ± 2190	5397 ± 5195	4666 ± 5094	0.037	3276 ± 10387	593 ± 324	456 ± 333	0.064

The production of cytokines by chondrocytes in their native tissue (tissue extracts) and during culture (conditioned media day 7 both non-expanded and expanded chondrocytes) per DNA was measured in samples from healthy, debrided cartilage from defects and osteoarthritic cartilage. Results are represented as median (inter-quartile range), differences in cytokine levels between healthy, cartilage defect and OA donors were determined using the Kruskall-Wallis test (*p*-values shown in table) with *post hoc* Mann-Whitney *U*-test; **P*<0.001.

Post hoc Man-Whitney *U*-tests: Tissue: VEGF H vs. CD $p=0.002$, H vs. OA $p=0.024$, CD vs. OA $p=0.002$. Unexpanded chondrocytes: IL-1β H vs. CD $p<0.001$, H vs. OA $p=0.002$, CD vs. OA $p=0.013$. Expanded chondrocytes: IL-6 H vs. CD $p<0.001$, H vs. OA $p<0.001$, CD vs. OA $p<0.001$; IL-8 H vs. OA $p<0.001$, CD vs. OA $p<0.001$; H vs. OA $p=0.008$, CD vs. OA $p<0.001$.

with an eigenvalue of 5.9. Cluster 2 contained IL-1β, IL-4 and IL-13 (variance explained 27%, eigenvalue 2.9) and cluster 3 contained IL-6 and IFNγ (variance explained 10%, eigenvalue 1.1).

Cartilage tissue

A total of five clusters were identified which respectively explained 33%, 14%, 11%, 11% and 7% of variance (eigenvalues 6.2, 2.7, 2.1, 2.0 and 1.3 respectively; Figure 2). Although some of the cytokines found in cluster 1 (IL-1α, IL-10, IL-13, TNFα, OSM, LIF, MCP1, RANTES) showed some overlap with cluster 1 identified in synovial fluid, mostly clusters were

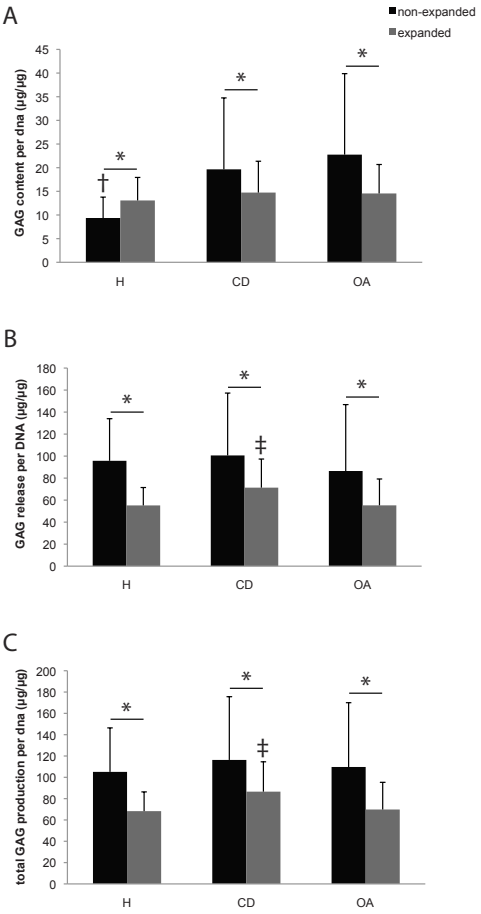


Figure 1. Cartilage matrix production by healthy, cartilage defect and OA chondrocytes. Cartilage regeneration cultures of three healthy (H), three cartilage defect (CD) and three osteoarthritic (OA) donors, with either non-expanded or expanded chondrocytes. (A) GAG content per DNA, (B) cumulative GAG release per DNA (C) total GAG production (GAG content + cumulative GAG release) per DNA after 28 days of culture; *$p<0.05$, †$p<0.05$ compared to CD and OA, ‡$p<0.05$ compared to H and OA.

different (Figure 2). Components of cluster 2 were: adiponectin, bFGF, HGF, VEGF; cluster 3: IL-1β, IFNγ; cluster 4: IL-6, IL-8; cluster 5: IL-4, leptin, IL-1RA.

Chondrocytes during regeneration cell culture

Principal component analysis of the cytokines produced by non-expanded cells revealed five clusters which respectively explained 34%, 19%, 12%, 8% and 5% of variance (eigenvalues 7.1, 3.9, 2.5, 1.6 and 1.1 respectively; Figure 2). Cluster 1 contained: IL-1α, IL-1β, IL-10, OSM, MCP1, IL-1RA; cluster 2: IL-4, IL-7, IL-13, IFNγ, leptin, HGF; cluster 3: IL-6, IL-8, RANTES; cluster 4: LIF, adiponectin, bFGF; cluster 5: LIF, VEGF, IL-6Rα. Cytokines produced by expanded cells clustered in only four clusters (66%, 11%, 6% and 5% of variance explained with eigenvalues of 13.8, 2.4, 1.2 and 1.0 respectively). Cluster 1 consisted of IL-1α, IL-1β, IL-1RA, IL-4, IL-7, IL-10, IL-13, TNFα, IFNγ, OSM, adiponectin, leptin, bFGF, HGF; cluster 2: IL-6, IL-8, LIF, MCP1, RANTES; cluster 3: VEGF; cluster 4: IL-6Rα.

None of the cytokines or clusters were related to GAG/DNA production.

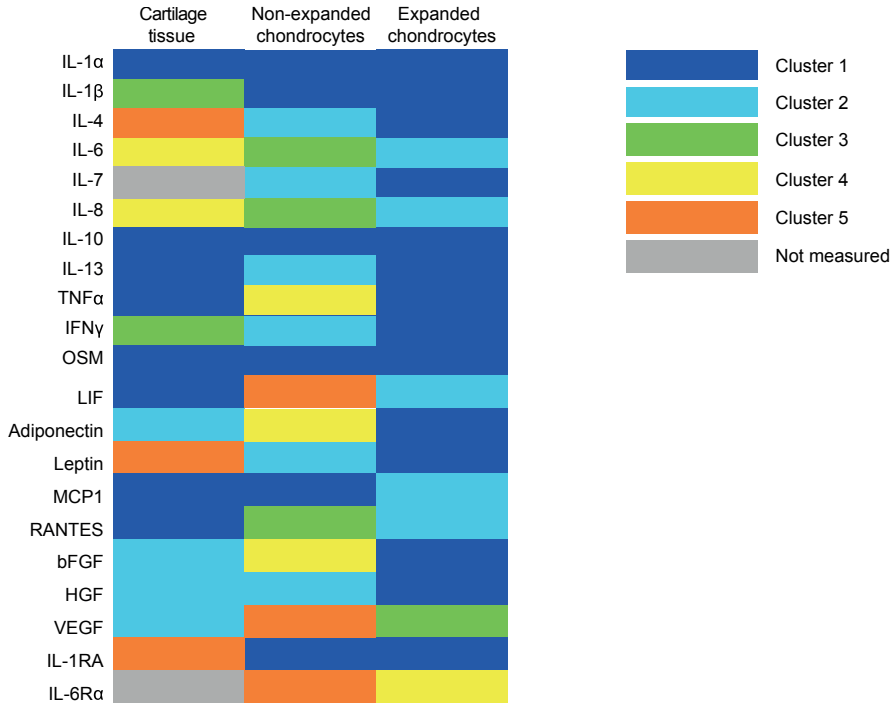


Figure 2: Principle component analyses of cytokines in cartilage tissue and cells. Principal component analysis of the measured cytokines in cartilage tissue and conditioned media (day 7) of non-expanded and expanded chondrocytes during regeneration. The different clusters are indicated by different colours.

DISCUSSION

To the best of our knowledge, this is the first report directly comparing cytokine presence in synovial fluid and in cartilage and cytokine production by isolated chondrocytes from donors without joint pathology, patients with symptomatic cartilage defects and patients with osteoarthritis. Several pro-inflammatory, pro-angiogenic and pro-repair cytokines were elevated in donors with symptomatic cartilage defects and OA. Interestingly, different cytokines were upregulated in synovial fluid, cartilage tissue and isolated chondrocytes. Especially in diseased joints, local concentrations of inflammatory cytokines present in the cartilage tissues were markedly higher than in synovial fluids.

We observed high concentrations of inflammatory (IFNγ, OSM, IL-6) and pro-repair (IL-13) cytokines in synovial fluid, both in cartilage defects and in OA. The increased presence of these cytokines may be one of the reasons for the inferior clinical results observed for autologous chondrocyte implantation in patients with longer existing degenerative defects and OA[31, 147]. Elevated levels of inflammatory mediators have previously been demonstrated in the synovial fluid of patients with joint trauma and OA, and synovial fluids from these patients have been shown to hamper cartilage regeneration[70, 82]. However, the composition of the synovial fluid in patients with symptomatic cartilage defects had hardly been characterized until now.

Generally, inflammatory mediators are thought to negatively affect cartilage integrity, but this may be a too simplistic view. OSM present in the synovial fluid is indeed known to inhibit *in vitro* cartilage regeneration[70]. However, IFNγ can also inhibit IL-1 induced MMP-13 expression in healthy and osteoarthritic chondrocytes[148 149] and we have recently shown that IL-6 can under specific conditions also stimulate cartilage matrix formation[150]. Therefore effects of cytokines may not only depend on the presence of other cytokines with opposite effects, but also on the disease status, and effects on chondrocytes may be different than on other cell types. The elevated presence of IL-13 in cartilage defects had not been reported until now. It is a Th2 cytokine that mediates alternative macrophage activation, a process that is now recognized to play a role not only in immunity, but also in tissue homeostasis and repair[151]. IL-13 is classically Th2 derived, but it can also be produced by various other cells and mediates fibrotic aspects of innate immune activation through both TGFβ-dependent and -independent pathways in pulmonary, hepatic, renal, dermal and gastrointestinal inflammation and fibrosis[152]. The direct effects of IL-13 on chondrocytes are not well known, but it has been shown to prevent collagen release induced by IL-1α and OSM in bovine nasal cartilage[153]. The effects on inflammation and cartilage integrity have been evaluated in various murine arthritis models, but not in osteoarthritis models or defect models. Decreased cartilage destruction and chondrocyte apoptosis were found with local overexpression of IL-13, but both decreased[154] as well as increased inflammation[155] were reported depending

on the model employed. Furthermore, microarray analysis of peripheral blood cells of patients with early OA (limited damage to the cartilage surface at arthroscopy, traumatic chondral defects excluded) identified IL-13 receptor as one of the six genes significantly downregulated[156]. It may be interesting to explore whether IL-13 receptor down-regulation also occurs in patients with focal cartilage defects and whether this is predictive for outcome.

We also observed that the concentrations of the inflammatory cytokines IL-1α, IL-1β, IL-6, TNFα, IFNγ and OSM in diseased cartilage tissue were distinctly elevated compared to those found in synovial fluid. This may indicate that their role in cartilage turnover in these conditions may be more substantial than can be deduced from only evaluating synovial fluid concentrations. All of these cytokines can have catabolic effects on cartilage and chondrocytes[157]. This also indicates that although the synovial membrane has been commonly implied in governing the inflammatory process leading to cartilage destruction, it appears that rather the cartilage in itself may guide this.

In cartilage tissue an increased presence of VEGF was found in cartilage debrided from defects and to a lesser extent also in OA cartilage compared to healthy cartilage. Additional to its stimulatory effects on angiogenesis, VEGF may also directly influence cartilage. Increased presence of VEGF and its receptors have been previously been found in OA cartilage tissue[158]. VEGF has been implicated in OA pathogenesis since injection of VEGF into mice knees induced OA[159] and VEGF immunolocalization correlated with cartilage destruction in several experimental animal models[160]. More recently it has not only been detected in late OA but also in early stages of OA[161]. VEGF has also been implicated to play a role in regeneration as it was shown to inhibit aggrecan and collagen II synthesis[162] and inhibition of VEGF using a monoclonal antibody improved cartilage regeneration[163]. Our results also support a role for VEGF in cartilage defects, although small quantities were also found in healthy tissue.

Culture of chondrocytes generally led to a massive induction of cytokines released. The cytokine profiles were also distinct from those observed in cartilage tissue. Non-expanded chondrocytes obtained from debrided defect cartilage produced more IL-1β than healthy and OA chondrocytes and IL-6, IL-8 and LIF were differentially produced by expanded chondrocytes. IL-6 and IL-8 are two key cytokines known to be secreted by senescent cells, something known as the senescence-associated secretory phenotype (SASP)[164, 165]. Both replication as well as stresses such as oxidative stress, DNA damage reagents and several cytokines, including IL-6 and IL-8[164, 165], can induce DNA damage and telomere shortening. A stress-induced rather than replication induced senescence-like phenotype has been reported in OA chondrocytes, especially near osteoarthritic lesions[166, 167]. Expansion of the OA chondrocytes may have amplified the already present DNA damage thereby increasing the senescence-associated secretion of cytokines. Also

differences in age between donors with cartilage defects and OA in particular may have contributed to differences in cytokine levels[168].

Chondrocyte cell cultures are frequently used to study inflammatory mechanisms involved in cartilage metabolism and OA. However, cytokine profiles of healthy and diseased cartilage were distinctly different between native tissue and cartilage cells in culture, even when the cells were not expanded in culture and had a differentiated chondrogenic phenotype. The different clustering of cytokines released by chondrocytes in culture compared to in native tissue further underline this and may indicate a different biological process and a general response to culture. The role of this generalized upregulation of cytokine production in cell culture is not clear, nor what mechanism is involved. In cartilage, like in general wound healing, a temporary inflammatory activation has been suggested to be crucial for chondrogenic differentiation[83]. This discrepancy between cytokine production in cell culture and in their native environment calls for caution with extrapolation of the results of chondrocyte culture to clinical OA.

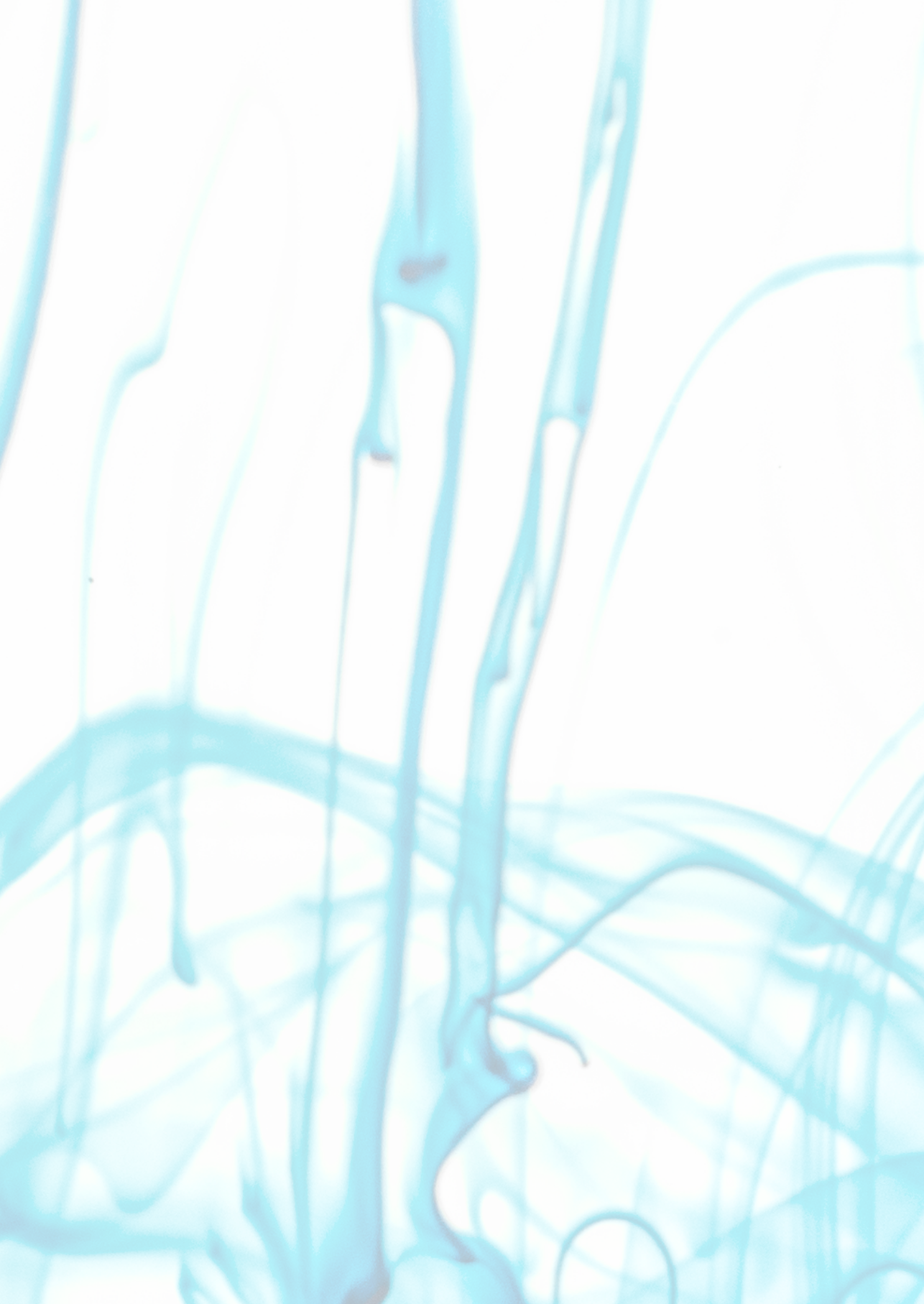
Despite the increased release of inflammatory cytokines by chondrocytes obtained from cartilage debrided from defects and OA cartilage, *in vitro* regeneration with these chondrocytes was at least as good or even superior to regeneration with healthy chondrocytes in terms of GAG production, confirming other studies[169-172]. In fact, glycosaminoglycan production is increased in mild to moderate severity OA cartilage[173, 174].

Regression analysis did not reveal any cytokines or clusters of cytokines that correlated to cartilage matrix production. Cytokines could have dual or opposing effects. Perhaps, other, here unmeasured mediators, are important for regeneration, or cytokine release during cell culture more reflects a general response to culture rather than a pathophysiological process. Further research is needed to clarify what, if any, is the role of cytokines during regeneration.

Limitations of our study include the fact that donors were not age- or gender-matched. However, patients with symptomatic cartilage defects are young and predominantly male[93], whilst OA generally affects older patients and so these differences between the populations are inherent to the affliction. Secondly the collection of tissue from donors without joint pathology was performed post-mortem as opposed to the intra-operative collection of OA and cartilage defect synovial fluid. However, tissue was always collected as soon as possible post mortem, usually several hours and up to a maximum of 24 hours postmortem. Cartilage is not vascularized and remains very much unaltered the first 24 hours[175] and previous work from our group showed no difference in viability between freshly isolated chondrocytes from healthy, post-mortem obtained, and grade III cartilage defect tissue[138].

Taken together, we showed that several pro-inflammatory, pro-angiogenic and pro-repair cytokines were elevated in patients with symptomatic cartilage defects and OA, and that different cytokines were upregulated in synovial fluid, cartilage tissue and

isolated cartilage cells. Research into the mechanisms governing this differential release may shed a light on the actual role of these factors in degeneration and regeneration of cartilage tissue.



Chapter 4

Pronounced biomaterial dependency in cartilage regeneration using non-expanded compared with expanded chondrocytes

Anika I Tsuchida
Joris EJ Bekkers
Michiel Beekhuizen
Lucienne A Vonk
Wouter JA Dhert
Daniel BF Saris
Laura B Creemers

Regen Med. 2013 Sep;8(5):583-95

ABSTRACT

Aims

We aimed to investigate freshly isolated compared with culture-expanded chondrocytes with respect to early regenerative response, cytokine production and cartilage formation in response to four commonly used biomaterials

Materials & Methods

Chondrocytes were both directly and after expansion to passage two, incorporated into four biomaterials: Polyactive™, Beriplast®, Hystem®, and a type II collagen gel. Early cartilage matrix gene expression, cytokine production and glycosaminoglycan (GAG) and DNA content in response to these biomaterials were evaluated.

Results

Hystem induced more GAG production, compared to all other biomaterials ($p \leq 0.001$). Non-expanded cells did not always produce more GAGs than expanded chondrocytes, as this was biomaterial-dependent. Cytokine production and early gene expression were not predictive for final regeneration.

Conclusions

For chondrocyte-based cartilage treatments, the biomaterial best supporting cartilage matrix production will depend on the chondrocyte differentiation state and cannot be predicted from early gene-expression or cytokine profile.

INTRODUCTION

Matrix assisted Autologous Cartilage Implantation (MACI) is gaining popularity as treatment for focal cartilage lesions of the knee. For this procedure several different matrices of both natural and synthetic composition and in various forms such as hydrogels, sponges and membranes, are employed. In particular hydrogels are attractive, because they can be injected arthroscopically to fill a defect and provide a 3D microenvironment for chondrocytes, either recruited from the neighboring cartilage or added exogenously[176]. Hydrogels of natural materials such as hyaluronic acid, fibrin and collagen have the additional advantage of containing natural binding sites for chondrocytes and other biomimetic signals[43]. As they are often very soft and degrade rapidly, recent approaches have included the combination of natural hydrogels with porous polymer scaffolds to provide initial mechanical strength whilst still maintaining optimal cell retention and chondrogenic differentiation[177, 178].

Although generally the clinical outcomes of MACI procedures are satisfactory, suboptimal cartilage quality is often observed. Partly this has been ascribed to the use of expanded and thus dedifferentiated chondrocytes[39, 40], which has incited research into approaches using non-expanded chondrocytes, whether or not co-implanted with other cell types such as mesenchymal stromal cells (MSCs). However, it is not clear to what extent the matrices used for implantation are optimal for cartilage regeneration. Clinical studies comparing various matrices are virtually non-existent[179] and also *in vivo* and *in vitro* comparisons are scarce[180, 181]. Thus the role of the biomaterials used *in vivo* for cartilage repair remains to be elucidated, in particular the influence of the biomaterials on chondrocyte behavior. It has been suggested that the early phenotypical response of chondrocytes to a biomaterial is more important than the prolonged presence of the biomaterial and biological cues it holds[178, 182]. Early cartilage matrix gene expression in response to a specific biomaterial if indicative of final cartilage matrix formation, may aid in the identification of suitable biomaterials for MACI.

In addition to regulating matrix production by cell-matrix interaction and subsequent modulation of matrix gene expression[44], matrices can also affect cell behaviour by the induction of soluble factors such as pro- and anti-inflammatory cytokines. Binding of ligands in natural hydrogels has been shown to induce the production of cytokines by chondrocytes[183]. Several of these factors are known to affect regeneration[150, 184] and to be endogenously produced by chondrocytes[150, 185], but still limited knowledge is available on endogenous cytokine production of chondrocytes cultured in biomaterials.

Therefore, in the current study, cartilage regeneration by non-expanded and expanded chondrocytes in four commonly used biomaterials for MACI was investigated in relation to the release of growth factors and cytokines and the very early expression of cartilage matrix specific genes. The four biomaterials studied were Beriplast®, which is a

commercially available fibrin glue[186], which was either or not reinforced with a PEOT/PBT copolymer network, Hystem®, which is a hyaluronan based gel[187], and a type II collagen gel.

MATERIALS AND METHODS

Chondrocyte isolation and expansion

Macroscopically healthy articular cartilage from the femoral condyles of six donors (n=6, age 53 to 82, average 61) was obtained within 24 hours post-mortem from donors without any history of major joint trauma, osteoarthritis or inflammatory joint disease, and absence of cartilage defects or synovial inflammation upon inspection during autopsy at the department of Pathology according to the Medical Ethical regulations of the University Medical Center Utrecht and approved by the institutional review board (protocol number 01/163).

For cell isolation, the cartilage samples were rinsed in phosphate buffered saline (PBS), cut into small pieces and enzymatically digested overnight at 37 °C in a 0.15% collagenase type II solution (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Life Technologies, Bleiswijk, The Netherlands) with penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen, Life Technologies). After digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Diego, CA, USA), and the chondrocytes were spun down by centrifugation at 300g. Chondrocytes were then washed in PBS and counted and assessed for viability using Trypan Blue (Sigma-Aldrich, Zwijndrecht, the Netherlands).

Expansion was performed in monolayer at 37 °C and 5% CO_2 at a seeding density of 5000 cells per cm^2 in expansion medium consisting of DMEM, 10% fetal bovine serum (Hyclone, Thermo Scientific, Etten-Leur, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA).

Preparation of cell-seeded scaffolds

Four different biomaterials, Polyactive, Beriplast® (Nycomed, Hoofddorp, the Netherlands), Hystem® (Glycosan, Alameda, CA, USA) and collagen type II hydrogel, were prepared in 16 wells chamber slides (Nunc, Langenselbold, Germany) to form cylindrical scaffolds with a diameter of 5 mm and a height of 5 mm. Chondrocytes were, either directly after isolation or after expansion to passage 2, incorporated into the various biomaterials at a cell seeding density of 875 cells per mm^3. Expanded chondrocytes were from the same donors as non-expanded chondrocytes. Per condition a minimum of five scaffolds were used.

The Polyactive scaffold was prepared as described before[188]. In brief, PEOT/PBT copolymer granules (PoroGen B.V., Bilthoven, the Netherlands) with a 1000/70/30 composition were printed using a 3D fiber deposition technique[189] with the BioScaffolder system (SysEng, Hünxe, Germany). Fibers were printed in a 0º - 90º orientation with a fiber spacing of 600 µm consisting of 14 layers (2 mm thickness after o/n swelling in phosphate buffered saline). Cylindrical scaffolds with a diameter of 5 mm were punched out and sterilized by 2 h immersion in 70% alcohol followed by overnight drying at 1 h UV-light exposure. Cells were seeded by immersion in the fibrinogen component of Beriplast and injected into the pores of the Polyactive scaffold directly followed by intraporous injection of the trombin component of the Beriplast ® and allowed to set for 30 min.

Per Beriplast® scaffold the cells were resuspended in the fibrinogen component of the gel, combined with the trombin component and allowed to set for 10 min at room temperature.

Hystem® hydrogels were prepared following the manufacturer's instructions. The carboxymethyl hyaluronic acid-DTPH (Hystem component, Glycosan) was dissolved in 1 ml of degassed, de-ionized water (DG water, Glycosan). The cells were resuspended in the Hystem component and combined with the extralink solution (polyethyleneglycol-diacrylate (PEGDA) powder dissolved in 0.5 ml DG water, Glycosan) at a 4:1 ratio. Gelation was allowed during 2 h at room temperature.

A collagen type II hydrogel was prepared by combining 800 µl of collagen type II solution (Bovine, AteloCell, Koken, Tokyo, Japan) with 100 µl 10x DMEM (Sigma-Aldrich), 10 µl PBS, 40 µl demineralised water and 50 µl 1M $NaHCO_3$, on ice. Cells were resuspended in the combined solution and incubated at 37 °C and 5% CO2 for 3 h.

Culture of seeded scaffolds:

Cell-seeded scaffolds were cultured for 24 h (n=3 donors) or three weeks (n=3 donors) in a 16 wells chamber slide in chondrocyte redifferentiation medium consisting of DMEM, 0.2 mM l-ascorbic acid-2-phosphate (AsAp; Sigma-Aldrich), 2% human serum albumin (Sanquin, Amsterdam, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml), 2% insulin-transferrin-selenium (ITS)-X (Invitrogen) and 5 ng/ml transforming growth factor (TGF)β (R&D Systems). Culture medium was changed twice a week and culture supernatant was either directly analyzed for cytotoxicity (see below) or stored at -80 °C until later analysis.

Cytotoxicity

Lactate dehydrogenase (LDH) activity, as a measure of cytotoxicity, was analyzed in the conditioned culture medium at day 1, 2, 9, 16 and 21, using a commercially available LDH kit (Roche Diagnostics, Almere, the Netherlands) following the manufacturers' instructions. Culture medium was used as a blank to correct for background signal.

Real-time PCR

To study the very early response of chondrocytes to the biomaterial, seeded scaffolds (n=8 per biomaterial, per donor) were harvested after 24 hours and gene expression of aggrecan (*ACAN*), type II collagen (*COL2A1*), type I collagen (*COL1A1*), runt-related transcription factor 2 (*RUNX2*), osteopontin (secreted phosphoprotein 1, *SPP1*), (sex determining region Y)-box 9 (*SOX9*) and type X collagen (*COLX*) was investigated by real-time PCR and also compared to non-cultured chondrocytes.

Scaffolds were first snap frozen in liquid nitrogen, ground with pestle and mortar and sonicated for 30 min. Total RNA was isolated from the scaffolds with Trizol (Invitrogen) as described by the manufacturer. Total RNA (500 ng) was reverse transcribed using iScript cDNA Synthesis Kit (Biorad Laboratories, Hercules CA, USA) in a total volume of 20 µl at 25 ˚C for 25 min and 42 ˚C for 30 min, followed by inactivation of the enzyme at 80 ˚C for 5 min.

Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics). The LightCycler reactions were prepared in 20 µl total volume with 7 µl PCR-H_2O, 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 10 µl LightCycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics), to which 2 µl of 5 times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table 1. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) and 18S were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation $\sqrt{}$(Ywhaz x 18S). With the Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by Light Cycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

Multiplex enzyme-linked immunosorbent assay (ELISA)

To determine cytokine levels in the conditioned medium, a multiplex ELISA was performed as previously described[141, 142]. The cytokines measured were interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-6 receptor (IL-6R), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-13 (IL-13), tumor necrosis factor- α (TNF-α), interferon-γ (IFN-γ), oncostatin M (OSM), osteprotegerin (OPG), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), hepatocyte growth factor (HGF). Briefly, specific antibodies were coupled to carboxylated beads (Luminex Corporation, Austin TX, USA). Conditioned media collected at day 5 were incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with strepta-

vidin–phycoerythrin (BD Biosciences) for 10 minutes. After washing, the samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories) with Bio-Plex Manager software, version 3.0. The concentrations of cytokines in the conditioned medium were calculated using the standard curves, with results expressed as pg/ml. Results of specific ELISAs have previously been shown to be comparable to the Luminex ELISA.[62]

GAG and DNA analysis

After 21 days culture, the samples (n=4 scaffolds per biomaterial, per donor) were digested overnight in a papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 ºC, followed by determination of the glycosaminoglycan (GAG) content using the dimethylmethylene blue (DMMB) assay[145]. 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. For the Hystem scaffold, which may show a signal due to the presence of hyaluronic acid, values were corrected by using

Table 1. Primer sequences used for real time PCR

Target gene		Oligonucleotide sequence	Annealing temperature (°C)
18S	Forward	5′ GTAACCCGTTGAACCCCATT 3′	57
	Reverse	5′ CCATCCAATCGGTAGTAGCG 3′	
YWHAZ	Forward	5′ GATGAAGCCATTGCTGAACTTG 3′	56
	Reverse	5′ CTATTTGTGGGACAGCATGGA 3′	
ACAN	Forward	5′ CAACTACCCGGCCATCC 3′	57
	Reverse	5′ GATGGCTCTGTAATGGAACAC 3′	
COL1A1	Forward	5′TCCAACGAGATCGAGATCC 3′	57
	Reverse	5′ AAGCCGAATTCCTGGTCT 3′	
COL2A1	Forward	5′ AGGGCCAGGATGTCCGGCA 3′	56
	Reverse	5′ GGGTCCCAGGTTCTCCATCT 3′	
RUNX2	Forward	5′ ATGCTTCATTCGCCTCAC 3′	56
	Reverse	5′ ACTGCTTGCAGCCTTAAAT 3′	
SPP1	Forward	5′ CATCTCAGAAGCAGAATCTCC 3′	56
	Reverse	5′ CCATAAACCACACTATCACCTC 3′	
COLX	Forward	5′ CACTACCCAACACCAAGACA 3′	56
	Reverse	5′ CTGGTTTCCCTACAGCTGAT 3′	
SOX9	Forward	5′ CCCAACGCCATCTTCAAGG 3′	60
	Reverse	5′ CTGCTCAGCTCGCCGATGT 3′	

YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; ACAN, aggrecan; COL1A1, α1(I)procollagen; COL2A1, α1(II)procollagen; RUNX2, runt-related transcription factor 2; SPP1, secreted phosphoprotein 1, osteopontin, COLX, type X collagen; SOX9, (sex determining region Y)-box 9.

the unseeded scaffolds as blank. Supernatant was also collected and analysed for GAGs released into the medium.

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

Histological evaluation

Scaffolds were fixed in 4% buffered formaldehyde, dehydrated in alcohol, rinsed in xylene and infiltrated and embedded with paraffin. For histology, 5 µm sections were deparaffinized in xylene, rehydrated in alcohol and 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. The deposition of type II collagen was evaluated by immunohistochemistry. Antigen retrieval was performed by 1 mg/ml pronase (Sigma-Aldrich) for 30 min at 37 °C followed by hyaluronidase incubation 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 primary antibody against human type II collagen (II-II6B3 1/100 in 5% PBS/BSA, Hybridomabank, USA). 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. Antibody binding was visualized using 3-diaminobenzidine (DAB, Sigma-Aldrich). All immunohistochemical sections were counterstained using Mayer's haematoxylin.

Statistical analysis

All statistical analyses were performed using SPSS 18.0. Differences between the effects on LDH release, matrix production and gene expression were determined by ANOVA with *post hoc t*-test with Bonferroni correction. For the cytokine measurements significance was set at $p = 0.05/16 = 0.003125$ to correct for the 16 cytokines measured. Differences between various time points were determined by paired *t*-tests and differences between non-expanded and expanded cells by paired *t*-tests.

RESULTS

Cytotoxicity

LDH release as a measure of cytotoxicity was evaluated at several time points. With the use of non-expanded cells, early cytotoxicity at day 2 was lowest for Beriplast ($p<0.03$ compared to Hystem and collagen gel; Figure 1). At day 16, LDH activity was highest for Polyactive ($p<0.05$ compared to all other biomaterials; Figure 1). In expanded cells, the LDH release profile was slightly different as the lowest release was for Polyactive at day

2 (p<0.02 compared to Hystem and collagen gel; Figure 1) and for collagen gel at day 16 (p<0.03 compared to Polyactive and Beriplast; Figure 1). Non-expanded cells generally showed a higher LDH release than expanded cells, especially at day 2 (p<0.04; Figure 1).

Early gene expression

Biomaterial-based induction of tissue specific genes was evaluated in non- expanded and expanded cells after 24 h of culture in the scaffolds and compared to gene expression prior to seeding. In non-expanded cells, induction of expression of cartilage matrix genes was highest in the collagen scaffold (p<0.001; Figure 2). However, also *COL1A1* gene expression was high in this biomaterial (p≤0.001; Figure 2). No induction of *SPP1* was found. In expanded cells, *ACAN* gene expression was higher in the fibrin containing scaffolds (p<0.003 compared to Hystem and collagen gel; Figure 2) paralleled by a high *SOX9* gene expression in Polyactive (p<0.01 compared to Hystem and collagen gel). None of the biomaterials induced expression of type II collagen in expanded cells. Furthermore, in contrast with non –expanded cells, *SPP1* was significantly upregulated in expanded cells in all biomaterials except for in Hystem (p<0.005; Figure 2). *COLX* gene expression was below detection levels in all conditions and *RUNX2* gene expression was below detection levels in all but the expanded cells incorporated in type II collagen gel, with a thirtyfold induction compared to expression prior to incorporation.

Cartilage matrix formation

For non-expanded cells, after the 21 days culture period, pronounced differences were found in extracellular matrix protein production and release in the different biomaterial scaffolds. Hystem induced the highest cartilage matrix production, as both GAG content and GAG per DNA were highest in this biomaterial (p<0.005; Figures 3A,D). GAG release

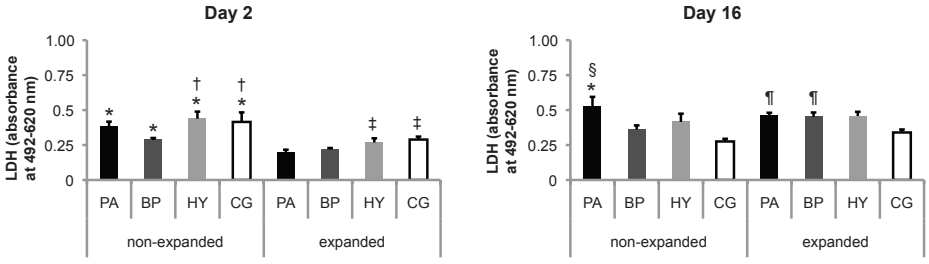


Figure 1. LDH release at days 2 and 16 of culturing of seeded scaffolds. Differences in LDH release between the various biomaterials within either the non-expanded or expanded groups are demonstrated. Results are displayed as mean ± standard error of the mean. *Differences in LDH release between non-expanded and expanded cells within a specific biomaterial: p<0.05 (n = 3 donors; five scaffolds per biomaterial per passage). †p<0.05 compared with BP; ‡p<0.05 compared with PA; §p<0.05 compared with BP, HY and CG; ¶p<0.05 compared with CG. BP: Beriplast®; CG: Type II collagen gel; HY: HyStem®; PA: Polyactive™.

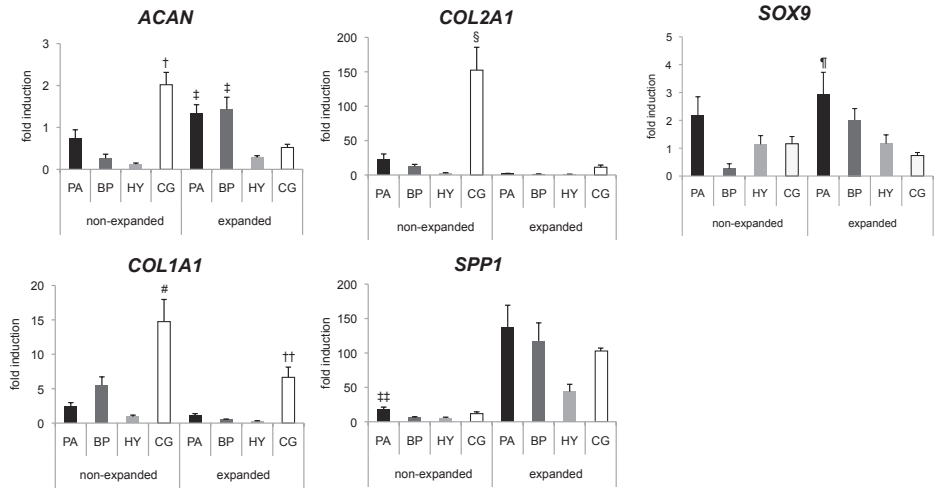


Figure 2. Early biomaterial-specific induction of (fibro)cartilage matrix genes. Induction of specific genes by the biomaterials as evaluated at day 1 by comparing non-expanded cells directly after isolation or expanded cells directly after trypsinization. COLX and RUNX2 gene expression are not shown here as COLX was below detection limits in all biomaterials and RUNX2 was only detected in expanded cells incorporated in CG. Significant differences in gene expression between the various biomaterials with the non-expanded or expanded group are demonstrated. Results are displayed as mean ± standard error of the mean.
†$p<0.001$ compared with PA, BP and HY; ‡$p<0.01$ compared with HY and CG; §$p<0.001$ compared with PA, BP and HY; ¶$p<0.01$ compared with HY and CG; #$p\leq0.001$ compared with PA and HY; ††$p<0.001$ compared with PA, BP and HY; ‡‡$p<0.03$ compared with HY (n = 3 donors, seven scaffolds per biomaterial per passage). BP: Beriplast®; CG: Type II collagen gel; HY: HyStem®; PA: Polyactive™.

was at least two-fold higher in the collagen gel compared to the other biomaterials at both time-points ($p<0.003$; Figure 3B). The scaffolds with fibrin contained less DNA after three weeks of culture than the Hystem and collagen gel ($p<0.006$; Figure 3C).

In scaffolds containing expanded cells, final cartilage matrix production per cell expressed as GAG per DNA was not affected by the biomaterial used (Figure 3D). GAG content was slightly increased and GAG release obviously increased in the Beriplast and Polyactive scaffolds compared to Hystem and/or collagen gel ($p<0.01$; Figures 3A,B).

Comparing expanded to non-expanded cells, matrix production (GAG content and GAG/DNA) was higher in both fibrin-based scaffolds ($p<0.01$) and lower in Hystem gel for the former cell type ($p<0.05$). No difference was found between the two cell populations in the collagen gels. There was no difference in DNA content between non-expanded and expanded in the fibrin scaffolds, whilst in Hystem and collagen gel, DNA content was higher with non-expanded cells ($p\leq0.003$).

Histological evaluation of cartilage matrix formation after three weeks generally showed if any, very little proteoglycans in the fibrin and collagen gels. The Hystem scaffold itself stained brightly red with safranin-O making it impossible to distinguish

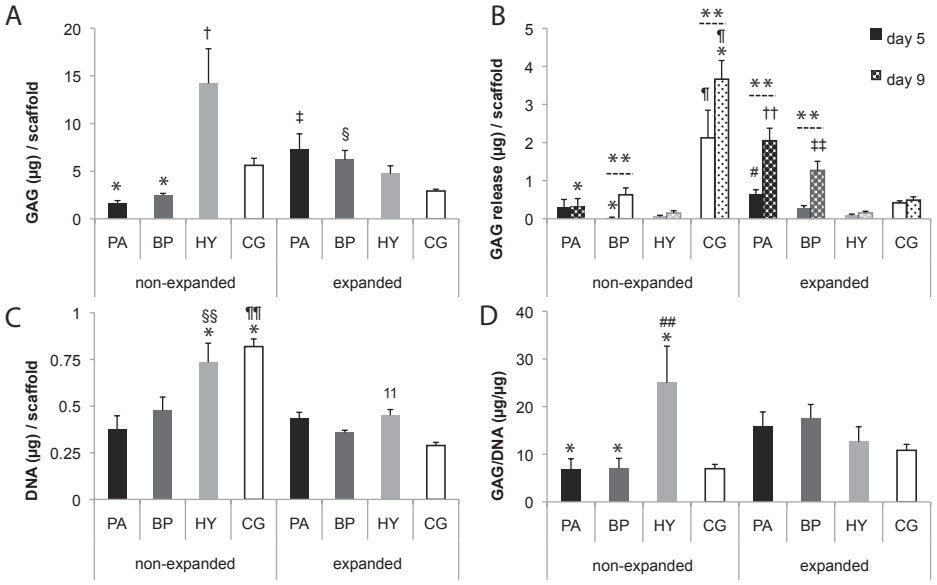


Figure 3. Cartilage regeneration by nonexpanded and expanded cells. Non-expanded and passage 2-expanded chondrocytes were combined with either PA, BP, HY or CG and evaluated after 21 days of culture. (A) GAG content per scaffold; (B) GAG release at day 5 and 9 per scaffold; (C) DNA content per scaffold; and (D) GAG:DNA. Significant differences between the various biomaterials within the non-expanded or expanded groups are demonstrated. Results are displayed as mean ± standard error of the mean. *Significant differences between non-expanded and expanded cells: $p<0.05$; **Difference between day 5 and 9: $p<0.05$ ($n = 3$ donors, four scaffolds per biomaterial per passage).
†$p\leq0.001$ compared with PA, BP and CG; ‡$p<0.05$ compared with HY and CG; §$p<0.01$ compared with CG; ¶$p<0.003$ compared with PA, BP and HY; #$p<0.001$ compared with BP and HY; ††$p\leq0.001$ compared with BP, HY and CG; ‡‡$p<0.001$ compared with HY and CG; §§$p<0.006$ compared with PA and BP; ¶¶$p<0.001$ compared with PA; ##$p<0.05$ compared with BP and CG; and †††$p<0.005$ compared with PA, BP and CG.
BP: Beriplast®; CG: Type II collagen gel; GAG: Glycosaminoglycan; HY: HyStem®; PA: Polyactive™.

between the scaffold material and newly formed proteoglycans. Immunohistochemical evaluation of type II collagen deposition showed scattered pericellular positive staining in the fibrin-based scaffolds and Hystem. In the type II collagen gel it was not possible to discern between the scaffold material and newly deposited collagen. No obvious differences were observed between non-expanded and expanded cells (Figure 4).

Cytokine profile

When using non-expanded cells, robust biomaterial-dependent differences in cytokine and growth factor production were measured, except for IL-6 and NGF (Figure 5). For most factors measured, their production tended to be lowest in Polyactive and highest in Beriplast and collagen gel. There was no association between the secretion of cytokines and cartilage matrix production. High production of catabolic cytokines such

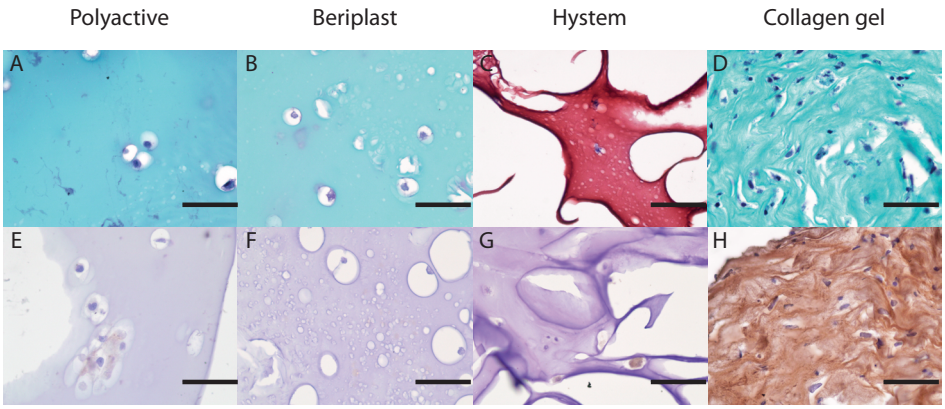


Figure 4. Histological evaluation of scaffolds after 3 weeks of culture. (A–D) Safranin-O (Merck, Germany) staining of expanded chondrocyte-seeded scaffolds after 3 weeks of culture. Note that HyStem® itself stains bright red due to the presence of hyaluronic acid. (E–H) Type II collagen immunohistochemical staining. Positive staining of the bovine type II collagen present in the collagen gel is demonstrated. Scale bars: 50 µm.

as IL-1 and OSM in Beriplast and collagen gel was accompanied by high production of growth factors such as bFGF and HGF.

When using expanded cells, generally differences in cytokine and growth factor production between the biomaterials were much smaller. Only IL-6 and OPG production was differentially regulated ($p<0.02$; Figure 6). IL-6 production was lowest in collagen gel ($p<0.02$ compared to Polyactive and Hystem) and OPG was lowest in Hystem and collagen gel ($p<0.001$ compared with Polyactive and Beriplast).

Cytokine and growth factor production was generally much higher when using non-expanded, compared to expanded cells (note the difference in y-axis between Figures 5 and 6), especially in Beriplast, Hystem and collagen gel ($p<0.05$; Figure 5).

Cytokine and growth factor production at day 9 was generally lower than at day 5 (data not shown).

DISCUSSION

In this study, we show that cartilage matrix production by incorporated chondrocytes depended on the biomaterials used, moreover, that this effect was more pronounced in non-expanded chondrocytes. Although also cytokine production by non-expanded cells varied extensively depending on the biomaterials used, this was not related to matrix production, at an early nor at a late time-point in culture. Likewise, early cartilage matrix gene expression was a poor predictor of eventual cartilage matrix formation for both cell types. Cartilage matrix formation by non-expanded cells was highest in the

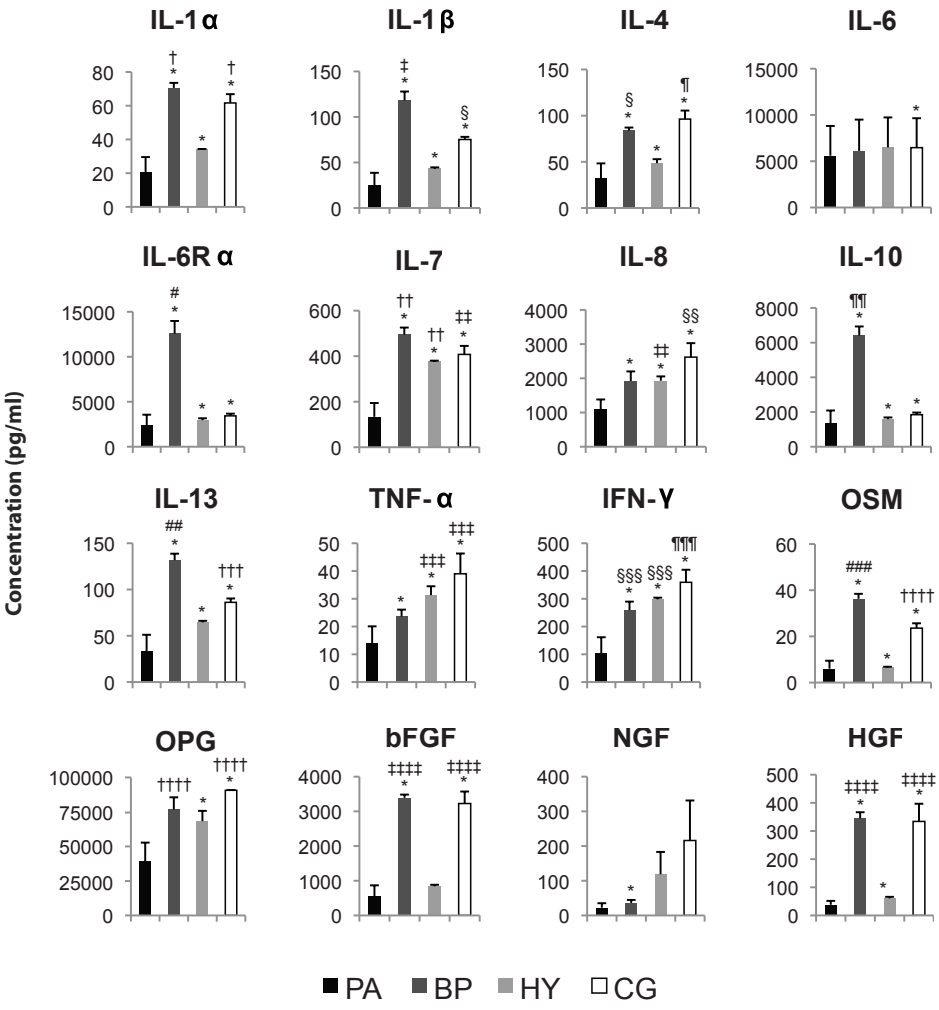


Figure 5. Cytokine and growth factor release by nonexpanded cell-seeded scaffolds. A panel of 16 cytokines and growth factors was measured in the culture supernatant at day 5 with multiplex ELISA. Note the different y-axes for the various factors. Differences between the biomaterials (PA, BP, HY and CG) are demonstrated. Results are displayed as mean ± standard error of the mean.

*Differences between nonexpanded and expanded cells within a biomaterial: $p<0.05$ (see Figure 6 for the cytokine and growth factor release from expanded cell-seeded scaffolds; n = 3 donors, three scaffolds per biomaterial). †$p<0.05$ compared with PA and HY; ‡$p<0.05$ compared with PA, HY and CG; §$p<0.01$ compared with PA; ¶$p<0.05$ compared with PA and HY; #$p<0.001$ compared with PA, BP and HY; ††p 0.01 compared with PA; ‡‡$p\leq0.001$ compared with PA; §§$p<0.05$ compared with PA and BP; ¶¶$p<0.001$ compared with PA, BP and HY; ##$p<0.05$ compared with PA, HY and CG; †††$p<0.05$ compared with PA; ‡‡‡$p<0.05$ compared with PA; §§§$p<0.01$ compared with PA; ¶¶¶$p<0.001$ compared with PA; ###$p<0.05$ compared with PA, HY and CG; ††††$p<0.05$ compared with PA; ‡‡‡‡$p<0.001$ compared with PA and HY.

BP: Beriplast®; CG: Type II collagen gel; HY: HyStem®; PA: Polyactive™.

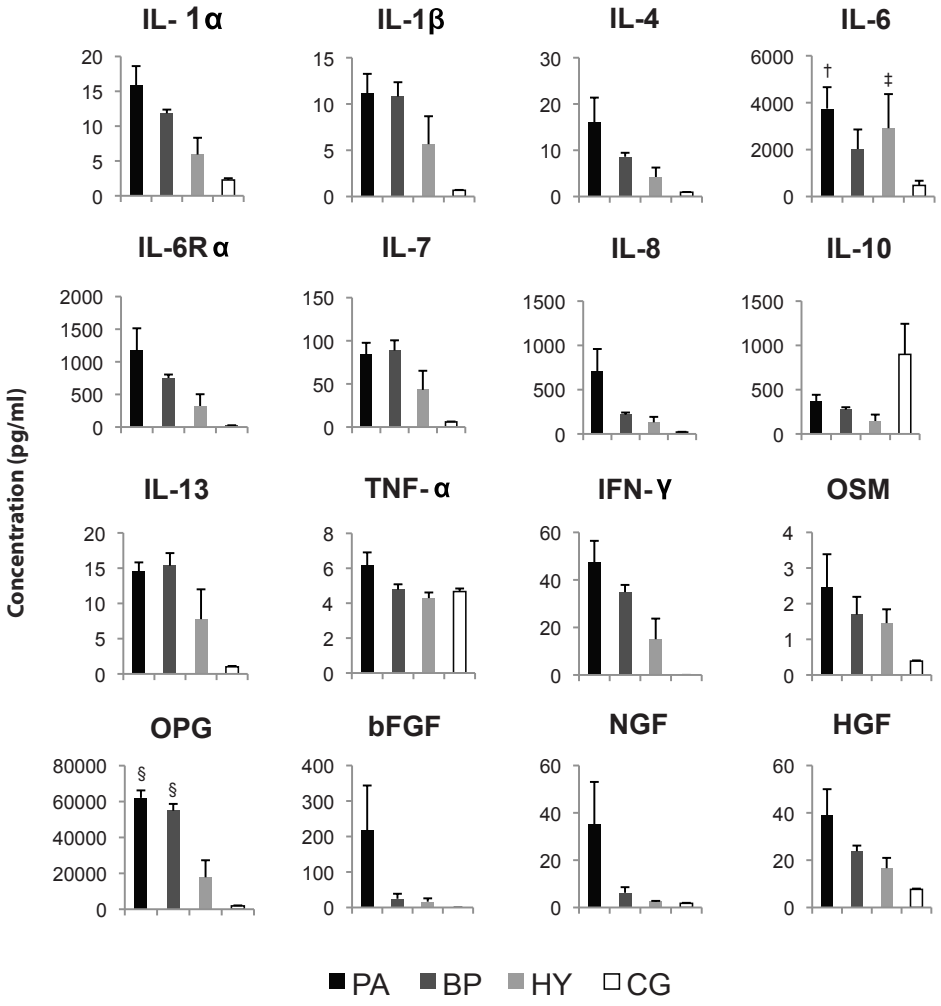


Figure 6. Cytokine and growth factor release by scaffolds seeded with expanded cells. A panel of 16 cytokines and growth factors was measured in the culture supernatants at day 5 with multiplex ELISA. Note the different y-axes for the various factors. Differences between the biomaterials (PA, BP, HY and CG) are demonstrated. Results are displayed as mean ± standard error of the mean. †p = 0.001 compared with CG, ‡p<0.02 compared with CG, §p<0.001 compared with HY and CG (n = 3 donors, three scaffolds per biomaterial).
BP: Beriplast®; CG: Type II collagen gel; HY: HyStem®; PA: Polyactive™.

hyaluronic acid-based Hystem scaffold, whilst no differences were found between the biomaterials when expanded cells were used.

The striking effect of biomaterial use on non-expanded cells suggests that the more differentiated phenotype allows for a more specific interaction with the environment

than dedifferentiated chondrocytes. Although not the interaction with the biomaterials itself was studied here, differences in the expression of surface receptors, including those regulating cell-matrix interaction such as CD44 and integrins, may explain the differences in cytokine production and early cartilage matrix gene expression[190]. Binding of these receptors leads to intracellular signaling resulting in changes in proliferation and gene expression and it has previously been shown that expression of many of those cell surface receptors is modulated during expansion and dedifferentiation[190]. Non-expanded cells therefore behave differently than expanded chondrocytes, especially when brought into contact with natural substrates. Previously it was shown that passaged chondrocytes did not show a differential response to different natural 2D substrates such as fibronectin, collagens or decellularized extracellular matrix formed by cells grown in monolayer[191, 192]. Apparently, the cell-biomaterial interactions of non-expanded cells are probably also responsible for the modulation of cytokine and growth factor production, given the pronounced differences in secretory profile depending on the biomaterial used. Integrin signaling has been shown to induce inflammatory cytokines[193, 194] and in various cell types actin cytoskeletal remodeling has been shown to regulate cytokine secretion[195] and potentiate nuclear factor kB signalling[196]. Although this has not been shown for chondrocytes, inflammatory cytokines have been shown to modulate the chondrocyte actin cytoskeleton [197], and possibly cytoskeletal remodeling in turn also affects cytokine production and/or secretion in this cell type.

For cartilage regeneration using non-expanded chondrocytes, the Hystem scaffold seemed most optimal. GAG content was highest in this scaffold, although like in the fibrin-based scaffolds, type II collagen production was limited, possibly due to the relatively short culture period. Hystem consists of hyaluronic acid (HA), a naturally occurring polysaccharide in cartilage with many pro-chondrogenic functions[176], which makes it a promising biomaterial for cartilage engineering. Indeed, addition of HA to other biomaterials such as alginate, chitosan and fibrin has been shown to enhance extracellular matrix formation[198]. Depending on the degree of polymerization, HA has been shown to have either inflammatory or anti-inflammatory effects[79]. Low molecular weight HA (6-20 kDa) has been shown to induce inflammatory cytokines through NF-κB activation, whilst high molecular weight (>10^6 Da) has anti-inflammatory effects and also has been shown to inhibit matrix degradation by aggrecanases and metalloproteinases[199-201]. In the current study, the HA-based hydrogel generally induced a minimal production of cytokines. Although the molecular weight of Hystem is not provided, it is synthesized of HA-DTPH of 158kDa which is then crosslinked with PEGDA[202], which will result in a high degree of polymerization and thus high molecular weight.

Non-expanded chondrocytes proliferated more readily in collagen gels than in other biomaterials, which could be explained by the concomitantly high levels of FGF, a cytokine known to stimulate proliferation. No data are available on the possible induction of FGF by

collagen binding, but interference with integrin binding may reveal whether this relevant mechanism is involved. Type II collagen has been shown to induce inflammatory cytokines such as IL6 and IL8 and the matrix degrading MMP-13 through binding of discoidin domain receptor 2 (DDR2)[203]. Both cytokines were indeed produced at higher levels if chondrocytes were cultured in the collagen gel scaffold. This may have been in line with the observation that GAG release was significantly higher in the collagen gel than in the other biomaterials. No differences were observed between the biomaterials with respect to GAG content. Possibly small differences between the biomaterials were masked by the use of TGFβ in the culture medium. However, this factor is required for regeneration and its omission would most likely have resulted in no detectable matrix production at all.

Fibrin is naturally involved in wound healing and is therefore also believed to be an attractive natural polymer for regenerative medicine. Fibrin and fibrin degradation products can contribute to inflammation[204], but can also attenuate TNFα, IL-6 and IL-8 production of the chondrocyte-like nucleus pulposus cells in response to IL-1α[205]. We observed a cytokine production that was relatively high in comparison with the other biomaterials for most of the cytokines measured, but indeed not for TNFα, IL-6 and IL-8. Furthermore fibrin is a suitable biomaterial for chondrogenesis of mesenchymal stromal cells, especially in combination with TGF[206]. Possibly this is due to its capability to act as a reservoir for various growth factors such as TGF and bFGF[207].

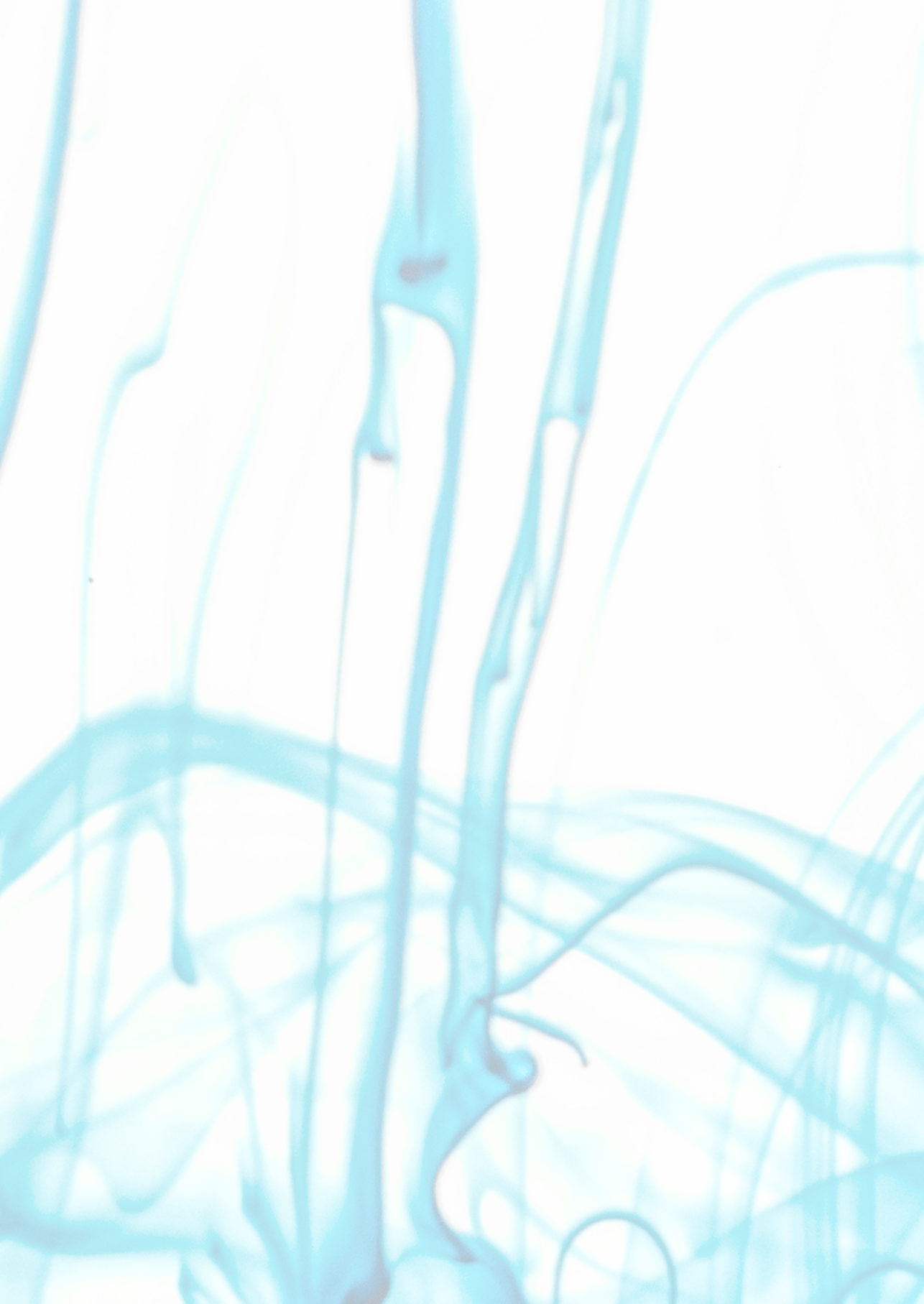
The production of growth factors and cytokines specific to a certain biomaterial is likely to be important for cartilage regeneration, because various cytokines and growth factors have been shown to affect cartilage matrix synthesis[184]. We chose to measure a broad panel of cytokines chosen on our previous studies on synovial fluid composition[84, 208, 209]. However, the release of the cytokines measured did not correlate to final cartilage matrix production. This may suggest that proinflammatory and anti-inflammatory factors counteracted each other or that other soluble factors and/or degradative enzymes played a role. The generally lower release of cytokines by expanded cells may be caused by a general loss of gene expression observed with dedifferentiation of chondrocytes in 2D monolayer culture[210]. However, GAG production did not differ between non-expanded and expanded cells in most biomaterials, most likely due to the 3D culture and/or the redifferentiation medium. Whether cytokine production during regeneration has a functional role at all needs to be investigated.

Not only cytokine production, but also early gene expression patterns seemed unrelated to final matrix production. In particular, aggrecan gene expression was lowest in the Hystem scaffold whilst GAG content after three weeks was highest in this biomaterial. Although we do not know the mechanism responsible for this observation, we hypothesize that this may be related to the chondrocyte's capacity to 'sense' its HA rich environment through for example the HA receptor CD44[211, 212]. The positive effects of high molecular weight HA may be mediated by creating a favorable environment for regeneration by re-

ducing degradative enzymes, rather than by having a direct stimulatory effect on cartilage matrix synthesis. Lack of a similar effect on expanded chondrocytes may be explained by the altered expression of CD44 by expansion[213]. It may also be possible that the high amount of sulphated GAGs measured by the dmmb-assay, which is generally thought to represent aggrecan production by chondrocytes, may at least partly be explained by the production of other proteoglycans. Furthermore, posttranscriptional, translational and protein degradation regulation have been shown to account for approximately 60 percent of the variation in protein concentration, and thus less than half of the final matrix production can be explained by changes in RNA[214]. In addition, upon implantation of cell-biomaterial constructs *in vivo*, cell and construct behavior may also deviate from the results shown *in vitro*. This was demonstrated before for cartilage matrix gene expression by expanded versus non-expanded cells, which was shown to be lower for expanded cells regenerating *in vitro*[215], in 2D and in 3D culture, whereas *in vivo*, no difference between non-expanded cells and expanded cells was found upon seeding on PLGA plugs and insertion into cartilage defects in pigs[216]. In contrast to these data, the results of current *in vitro* study did not reveal a decreased GAG production of expanded compared to the non-expanded cells, except for in the Hystem scaffold. However, we analyzed ECM production at the protein rather than the mRNA level and used human cells instead of porcine chondrocytes.

CONCLUSIONS AND FUTURE PERSPECTIVES

Altogether, the current study shows that interactions between chondrocytes and biomaterials depend on expansion of chondrocytes. The finding that cartilage regeneration by non-expanded cells differed depending on the biomaterial used could have important implications for the use of non-expanded chondrocytes for MACI. Further research should be directed at verifying whether the differences found are also maintained *in vivo* upon implantation of the scaffolds. In addition, unraveling the biological functional functions of cytokines during regeneration may provide insight into general mechanisms of cartilage tissue regeneration.



Chapter 5

CXCL8 (interleukin-8) in synovial fluid and autocrine production by chondrocytes; association with cartilage pathology, but no effect on regeneration or terminal differentiation

Anika I Tsuchida
Daniel BF Saris
Marijn Rutgers
Michiel Beekhuizen
Joris EJ Bekkers
Bernd Geurts
Angela Kragten
Mattie van Rijen
Wouter JA Dhert
Laura B Creemers

Submitted for publication

ABSTRACT

Introduction

This study aimed to evaluate the possible role of the inflammatory chemokine CXCL8 (interleukin-8) in cartilage regeneration in joint pathology. To this end its presence in the synovial fluid and its production by chondrocytes of patients with symptomatic cartilage defects was compared to healthy and osteoarthritic donors. Secondly, the effect of CXCL8 on cartilage metabolism and hypertrophic differentiation was evaluated.

Methods

CXCL8 concentrations were determined by ELISA in the synovial fluid and conditioned media of regenerating chondrocytes from healthy, cartilage defect and osteoarthritic donors. The effect of CXCL8 on cartilage regeneration was evaluated by both inhibition of endogenously produced CXCL8 and by addition of exogenous CXCL8, with a particular emphasis on previously reported induction of hypertrophic differentiation reflected by type X collagen up-regulation. The effect of CXCL8 on cartilage matrix integrity was evaluated by culturing osteoarthritic explants in the presence of exogenously added CXCL8.

Results

CXCL8 was present in the synovial fluid of healthy, cartilage defect and osteoarthritic donors without apparent differences. However, CXCL8 production by chondrocytes during regeneration was dependent on the origin of the tissue, osteoarthritic chondrocytes producing more CXCL8 than chondrocytes obtained from a defect, which in turn produced more than healthy chondrocytes ($p<0.001$). Inhibition of endogenous CXCL8 produced by chondrocytes resulted in a statistically significant but minor reduction in glycosaminoglycan (GAG) release ($p=0.043$). Conversely, exogenously added CXCL8 increased GAG release slighlty ($p=0.027$). No induction of hypertrophic differentation was found. Addition of CXCL8 to osteoarthritic explants did not affect cartilage matrix turnover.

Conclusion

CXCL8 is not differentially regulated *in vivo*, but its production by regenerating chondrocytes is dependent on the original disease status of the joint. However, its effects on cartilage regeneration are minor and it does not induce hypertrophy as suggested previously.

INTRODUCTION

Focal cartilage lesions, if left untreated can lead to progressive pain and symptoms and ultimately the development of osteoarthritis (OA). Current treatments such as autologous chondrocyte implantation (ACI) employ tissue-engineering techniques to repair the cartilage defect. Despite many developments in culture conditions, biomaterials and cell selection, the final repair tissue is hyaline-like at best and therefore suboptimal. Currently, only chondrocytes obtained from healthy cartilage are used for ACI, but ideally also chondrocytes from the cartilage that is debrided from the rims of the defect during the procedure may be used to avoid further damage. In addition, extension of the indication for ACI may be a promising approach for treatment of early OA.

Besides the chondrocytes used, also the environment in which the cells are reimplanted greatly affects the cartilage regeneration. Clinical outcomes of ACI in patients with OA are disappointing[147] and *in vivo* cartilage repair in old defects is more challenging than in fresh defects[34, 46]. The synovial fluid of OA and traumatized joints contains soluble factors that negatively affect cartilage repair[81, 82]. Modulating the joint environment by targeting soluble mediators known to inhibit regeneration may provide a cue to further improve regenerative treatments for cartilage pathology.

Catabolic factors known to be present in the synovial fluid of traumatized and OA joints such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) have an established negative role in cartilage turnover. However, their presence in the synovial fluid is limited[48, 66, 217] and inhibition of these factors has only partially been able to counteract the inhibition of cartilage matrix formation in an osteoarthritic environment[218]. Therefore it is likely that other factors in the synovial fluid contribute to the observed impaired cartilage regeneration.

One of the other inflammatory factors postulated to play a role in cartilage destruction is CXCL8. It is present in joints with rheumatoid arthritis and its levels were correlated to clinical signs of disease[219]. Also in anterior cruciate ligament injury[220, 221] and OA[220, 222] the presence of CXCL8 has been described, but whether or not the levels are elevated is controversial, because comparative data on healthy joints were scarce till now. Also it is unknown whether in joints with symptomatic cartilage defects CXCL8 is present. CXCL8 was found to be produced by cells of various joint tissues such as monocytes/macrophages, fibroblasts, chondrocytes, osteoblasts and fat pad cells[220, 223-225]. It induced chemotaxis of neutrophils and neutrophil degranulation and could enhance the release of inflammatory cytokines by mononuclear cells[220].

In addition to being proinflammatory, CXCL8 has been reported to induce chondrocyte hypertrophy as reflected by the induction of type X collagen production[73, 226, 227], chondrocyte apoptosis[228, 229] and production of catabolic enzymes such as lysosomal glycosidases and matrix metalloproteinase (MMP)-3[230]. Hypertrophy is a hallmark fea-

ture of OA cartilage, and inadvertent endochondral ossification is thought to lead to intralesional osteophytes after cartilage repair surgeries[231]. The presence of CXCL8 in synovial fluid could play a role here. However, despite the inflammatory response induced by CXCL8, no data are available on the actual effect of CXCL8 on cartilage regeneration, nor is it clear whether joint pathology is accompanied by upregulation of CXCL8. This study measures the presence of CXCL8 in the synovial fluid of patients with symptomatic cartilage defects in comparison to healthy controls and OA patients and its production by isolated cells during regeneration is measured. Furthermore the role of CXCL8 is evaluated in *in vitro* cartilage regeneration by articular chondrocytes harvested from cartilage defect areas and in repair by OA explants.

MATERIALS AND METHODS

Synovial fluid and cartilage sample collection

All use of patient material in this study was approved by the local ethics committee. Healthy cartilage (n=13) and synovial fluid (n=20, age 25 to 47, average 39.6 years) was obtained during autopsy within 24 hours post-mortem from donors with no documented history of joint disease and macroscopically intact and healthy appearing cartilage and surrounding knee joint tissues. Debrided defect cartilage (n=13) and synovial fluid (n=22, age 20 to 48, average 33.0 years) was obtained from donors undergoing ACI. During this procedure the cartilage defect (Outerbridge grade III) was debrided to remove all cartilage remnants down to the subchondral bone and create a stable cartilage rim. OA cartilage (n=24) and synovial fluid (n=27, age 53 to 81, average 69.5 years) was obtained from donors undergoing total knee arthroplasty for end-stage OA (Outerbridge grade IV). Synovial fluids were aspirated using a small syringe. Defect and OA cartilage were placed in DMEM after removal and processed directly postoperatively, within one hour after removal.

Cell isolation

Cartilage tissue was rinsed in phosphate buffered saline (PBS), cut into small pieces and enzymatically 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, Life Technologies, Bleiswijk, The Netherlands) with penicillin/streptomycin (100 U/ml/100 µg/ml, Invitrogen, Life Technologies). After digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Diego, USA), and the chondrocytes were spun down by centrifugation at 300 g. Chondrocytes were then washed in PBS and counted and assessed for viability using Trypan Blue (Sigma-Aldrich, Zwijndrecht, the Netherlands) and a Burker-Turk haemocytometer. Cells were seeded at

a density of 5000 cells per cm^2 in monolayer at 37 °C and 5% CO_2 in expansion medium consisting of DMEM, 10% fetal bovine serum (Hyclone, Thermo Scientific, Etten-Leur, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA) and used at passage 2.

Regeneration culture

After two passages, the chondrocytes were redifferentiated on collagen type II coated (chicken sternal cartilage, #C9301, Sigma-Aldrich) Millicell filters (Millipore Co., Bedford, USA), at a seeding density of $1.6x10^6$ cells per cm^2 [232, 233]. Chondrocytes were cultured during 28 days in redifferentiation medium consisting of DMEM, 0.2 mM l-ascorbic acid-2-phosphate (AsAp, Sigma-Aldrich), 2% human serum albumin (Sanquin, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml), 2% insulin-transferrin-selenium (ITS)-X (Invitrogen) and 5 ng/mL TGF-β_2 (R&D systems). The effect of exogenous CXCL8 was investigated by the addition of rhCXCL8 (#208-IL; R&D Systems) to both healthy (n=3) and OA (n=3) chondrocytes. A concentration of 10 ng/ml CXCL8 was chosen based on previously reported induction of hypertrophic differentiation of chondrocytes with this concentration[73, 227]. Since cartilage defect and OA chondrocytes turned out to produce high levels of CXCL8 during redifferentiation, we also studied the role of CXCL8 in tissue regeneration by cartilage defect chondrocytes (n=3) and OA chondrocytes (n=3) through blockage of CXCL8 with an activity-inhibiting antibody. To this end, the medium was supplemented with either 1 µg/ml anti-hCXCL8 (purified mouse monoclonal IgG_1; #MAB208, R&D Systems) or IgG_1 isotype control (#MAB002; R&D Systems). The dose of 1 µg/ml anti-hCXCL8 was chosen based on its ability to completely neutralize chemotaxis of the CXCR2-transfected BaF3 mouse cell line in response to 20 ng/ml CXCL8 (data provided by the manufacturer), a concentration that far exceeds the maximum concentration reached in the regeneration cultures. Medium was changed three times a week. Per condition, six filters were seeded, five for biochemical analysis and one for histological evaluation.

Osteoarthritic explant culture

OA cartilage from eight donors was cut into explants of ~1 mm by 1 mm with mean SD wet weight of 11.9 ± 3.7 mg per cartilage explant, at three to eight explants per condition. The explants were pre-cultured for 24 hours in culture medium, consisting of DMEM, penicillin/streptomycin (100 U/100 µg/ml), 1% ITS-X, 0.1 mM AsAp and 0.2% proline (Sigma-Aldrich), after which either or not recombinant human CXCL8 (10 ng/ml, R&D Systems) was added. In a limited set of experiments (n=6 donors), IL-1β (Gibco, Life Technologies) was added at 100 pg/ml or 10 ng/ml to check for a possible synergistic effect with CXCL8 on cartilage matrix breakdown. Explants were cultured for an additional

10 days with medium renewal every other day and the supernatant was collected and stored at -20 °C until later analysis.

Measurement of IL-8 levels

To determine the CXCL8 levels in the synovial fluids of healthy, cartilage defect and OA donors and in conditioned media of healthy, cartilage defect and OA chondrocytes during regeneration, a multiplex ELISA was used as previously described[141, 142]. Briefly, specific antibodies were coupled to carboxylated beads (Luminex Corperation, Austin, USA). Recombinant CXCL8 was used to make a standard curve. Synovial fluid samples were first treated with hyaluronidase (10 mg/ml, Sigma-Aldrich), for 30 min at 37 °C. Conditioned media samples were directly incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin–phycoerythrin (BD Biosciences) for 10 minutes. After washing, the samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules, USA) with Bio-Plex Manager software, version 3.0. The concentration of CXCL8 in the media and synovial fluid was calculated using the standard curves, with results expressed as pg/ml. Results of specific ELISAs for determination of CXCL8 have previously been shown to be comparable to Luminex analysis[62].

Glycosaminoglycan and DNA analysis

After culture, the samples were digested overnight in a papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine, pH 6.0) at 56 ºC, followed by determination of the glycosaminoglycan (GAG) content using the dimethylmethylene blue (DMMB) assay[145]. 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.

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

MMP activity assay

An MMP activity assay was performed as previously described[234]. Briefly, 100 µl of 10 µM FS-6 (Bachem, Bubendorf Switzerland) in buffer (0.1 M Tris, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Triton X-100, 0.1% (w/v) PEG6000, pH 7.5) was added to 100 µl of conditioned medium of CXCL8 supplemented cultures (day 7 and day 14). Conditioned medium of osteoarthritic chondrocytes stimulated with 10 ng/ml TNFα (R&D Systems) was taken along as a positive control. Development of the fluorescent signal was monitored over 24 hours. The linear slope (RFU/h) was calculated as a measure of general MMP activity.

Histological evaluation

Neocartilage on filters was fixed in 4% buffered formaldehyde, dehydrated in alcohol, rinsed in xylene and infiltrated and embedded with paraffin. For histology, 5 µm sections were deparaffinized in xylene, rehydrated in alcohol and 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. The deposition of type II collagen was evaluated by immunohistochemistry as described below.

To evaluate hypertrophic differentiation, the highly specific marker collagen type X collagen and two other markers of hypertrophy, namely osteopontin and osteonectin were evaluated by immunohistochemistry[235]. For type X collagen immunohistochemistry, antigen retrieval was performed on rehydrated paraffin sections 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. For osteopontin, osteonectin and type II collagen antigen retrieval was performed by 1 mg/ml pronase (Sigma-Aldrich) for 30 min at 37 °C followed by hyaluronidase incubation 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 primary antibody against human collagen type X, clone 53 (Quartett, Germany, 1/20 in 5% PBS/BSA), primary antibody against human osteopontin (MPIIIB10-1 1/100 in 5% PBS/BSA, Hybridomabank, USA), primary antibody against human osteonectin (AON-1 1/100 in 5% PBS/BSA, Hybridomabank, USA) or primary antibody against human type II collagen (II-II6B3 1/100 in 5% PBS/BSA, Hybridomabank, USA). 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. Antibody binding was visualized using 3-diaminobenzidine (DAB, Sigma-Aldrich). All immunohistochemical sections were counterstained using Mayer's haematoxylin. A minimum of three sections per donor per condition were stained. Human tibial growth plate was used as a positive control for the hypertrophic differentiation markers and human healthy cartilage for type II collagen and mouse IgG_1 (Dako, Heverlee, Belgium) for isotype control.

Statistical analysis

All statistical analyses were performed using SPSS 18.0. Results are displayed as mean ± standard deviation (SD). Differences in CXCL8 concentration were determined by ANOVA for the synovial fluids, and by nested ANOVA for the conditioned media, both with *post hoc t*-test with Bonferroni correction. Differences between control and test samples (either addition or inhibition of CXCL8) in cartilage matrix formation and MMP activity were determined by univariate analysis of variance with randomized block design to correct for interdonor variability. Two-way ANOVA was used to evaluate both

individual and interaction effects of IL-1β and CXCL8 on OA cartilage explants. Normal distribution of the residuals was verified by Kolmogorov-Smirnov test and homogeneity of variances by Levene's test.

RESULTS

CXCL8 in synovial fluid

The synovial fluid of cartilage defect donors contained 27 ± 7 pg/ml CXCL8, which was comparable to that of healthy donors (mean 29 ±7 pg/ml), and slightly lower than that of OA donors (mean 52 ±18 pg/ml), although this difference did not reach statistical significance (Figure 1A).

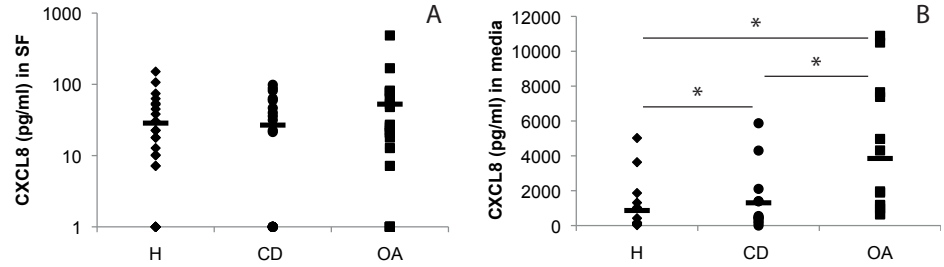


Figure 1. Concentration of CXCL8 in the synovial fluid (1A) and conditioned media (1B) of chondrocytes during regeneration from healthy (H), cartilage defect (CD) and osteoarthritic (OA) donors. 1A: Concentration CXCL8 in synovial fluid (SF) of healthy (H), cartilage defect (CD) and osteoarthritic (OA) donors. Note the logarithmic scale on the y-axis. 1B: Passage 2 expanded chondrocytes from healthy (H), cartilage defect (CD) and osteoarthritic (OA) cartilage were seeded at high density on type II collagen-coated filters and cultured during 28 days. Concentration of CXCL8 in conditioned media of chondrocytes during regeneration on day 7; *p<0.001.

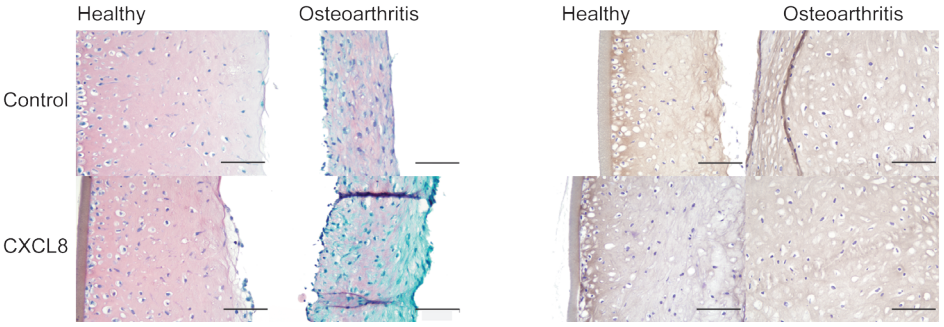


Figure 2. Cartilage matrix formation. Representative safranin-O and type II collagen staining of healthy and osteoarthritic chondrocytes redifferentiated on filters during 28 days with or without addition of 10 ng/ml CXCL8. Scalebars indicate 100 µm.

CXCL8 production in regeneration culture

Chondrocytes during regeneration released high concentrations of CXCL8 into the culture media (Figure 1B). OA chondrocytes (3927 ± 3592 pg/ml) produced significantly more CXCL8 than cartilage defect chondrocytes (1306 ± 1703 pg/ml) and healthy chondrocytes (865 ± 1430 pg/ml, $p<0.001$). Cartilage defect chondrocytes, in turn, produced significantly more CXCL8 than healthy chondrocytes ($p<0.001$).

GAG turnover and DNA content in regeneration culture

Overall regeneration (GAG/DNA between 10-20 μg/μg) was similar tor previously reported for this regeneration model[150, 236]. Safranin-O staining showed moderate to abundant presence of GAGs and mild to moderate staining for type II collagen without apparent differences between controls and CXCL8-supplemented cultures (Figure 2). Addition of 10 ng/ml exogenous CXCL8 during chondrocyte redifferentiation showed small differential effects at the biochemical level in healthy and osteoarthritic chondrocytes. CXCL8 increased GAG release into the medium in healthy, but not in OA chondrocytes (471 ± 110 μg versus 420 ± 106 μg for healthy chondrocytes, 358 ±151 μg versus 380 ± 153 μg for OA chondrocytes; Figure 3). However, this increased GAG release was very limited and not explained by differences in MMP activity as this was generally low and not different between controls and CXCL8 supplemented cultures, both at day 7 (Figure 4) and day 14 (data not shown).

Similarly, inhibition of CXCL8 activity during cartilage defect chondrocyte redifferentiation marginally decreased GAG release into the media (340 ± 96 μg versus 365 ± 96 μg, $p=0.01$; Figure 5). In osteoarthritic chondrocytes, inhibition of CXCL8 slightly decreased DNA content (6.8 ± 1.7 μg versus 7.5 ± 1.7 μg, $p=0.001$).

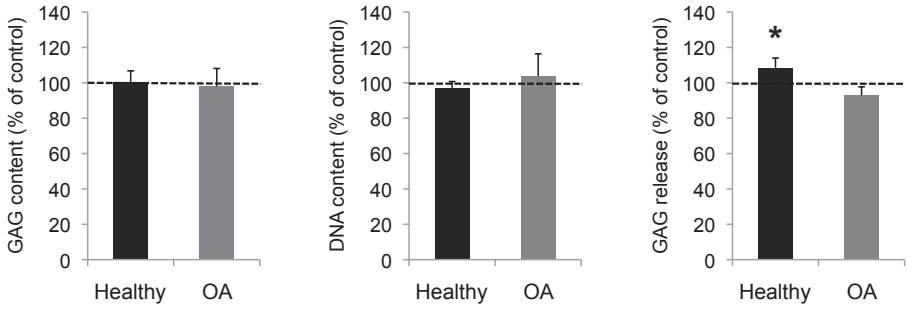


Figure 3. Cartilage regeneration in the presence of CXCL8. GAG content, DNA and GAG release by healthy and osteoarthritic (OA) chondrocytes redifferentiated on filters during 28 days with or without 10 ng/ml exogenous CXCL8 (mean ± SD in μg). Values are presented as percentage of the controls without CLCX8.; *$p=0.03$.

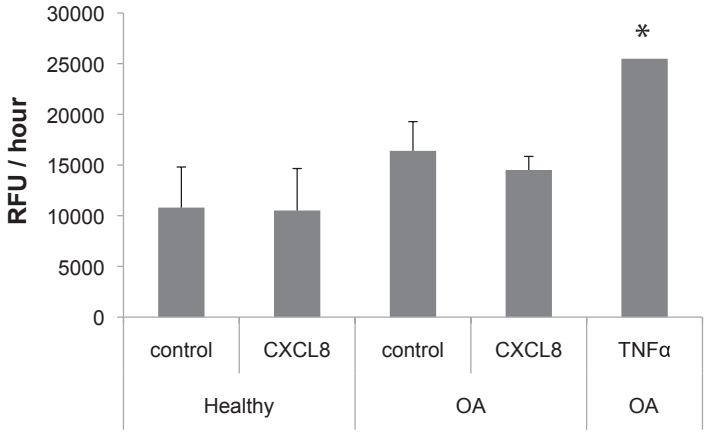


Figure 4. MMP activity during regeneration. Relative fluorescent units per hour (RFU/hour) were calculated as a measure of general MMP activity of healthy and OA chondrocytes with or without 10 ng/ml exogenous CXCL8 (mean ± SD) at day 7 of regeneration culture. Conditioned medium of osteoarthritic chondrocytes stimulated with 10 ng/ml TNFα were taken along as positive control; *p<0.05.

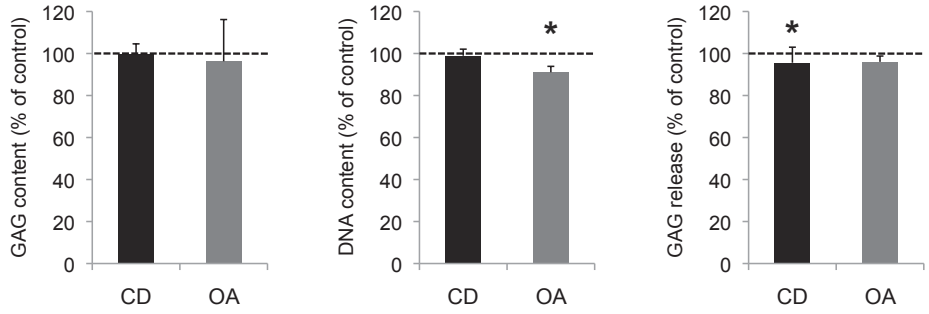


Figure 5. Cartilage regeneration after inhibition of endogenously produced CXCL8. GAG content, DNA and GAG release in cartilage defect (CD) and osteoarthritic (OA) chondrocytes redifferentiated on filters during 28 days with or without inhibition of CXCL8 (mean ± SD in μg). Values are presented as percentage of the controls cultured in the presence of isotype antibody; *p≤0.02.

GAG turnover and DNA content in osteoarthritic explant culture

To study the effect of the presence of CXCL8 on existing cartilage tissue, we performed cartilage explant studies in the presence of CXLC8. As the availability of explants from defect cartilage was too limited, only OA cartilage explants were used. Addition of CXCL8 to osteoarthritic explants did not alter cartilage matrix metabolism as measured by GAG and DNA content at the end of culture. Also GAG release from the explants was not modulated by CXCL8 (Figure 6). Although IL-1β addition did result in an increase in GAG release, the presence of CXCL8 did not enhance this effect (Figure 7).

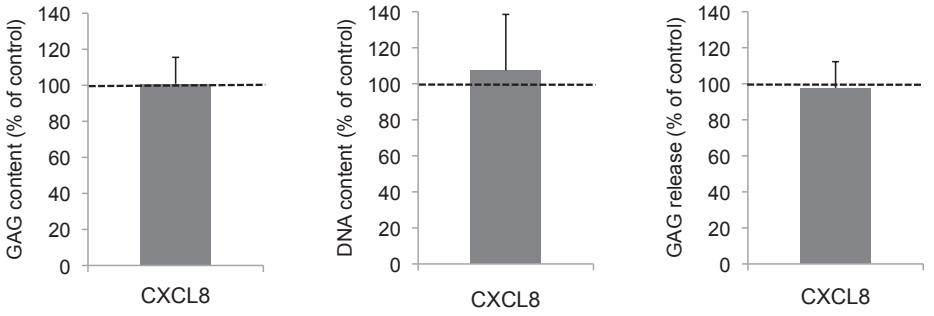


Figure 6. OA cartilage explant culture with or without exogenous CXCL8. GAG content, DNA and GAG release in osteoarthritic explants from eight donors either or not supplemented with 10 ng/ml recombinant CXCL8 (mean ± SD in μg). Values are presented as percentage of the controls without CLCX8.

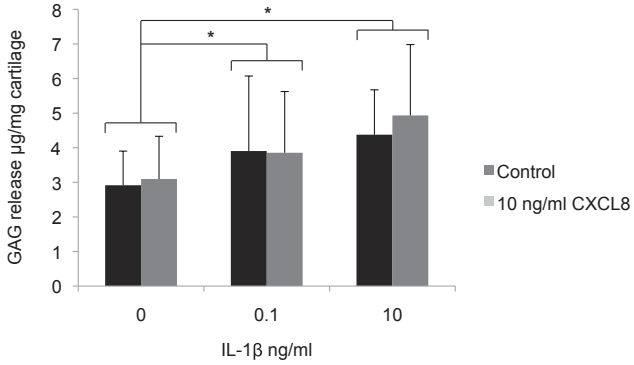


Figure 7. OA cartilage explant culture with IL-1β and CXCL8. GAG release in osteoarthritic explants from six donors either or not supplemented with 10 ng/ml CXCL8 and/or 0, 0.1 or 10 ng/ml IL-1β (mean ± SD in μg/mg cartilage). IL-1β significantly increased GAG release (p=0.031, *p<0.05), but there was no additive effect of CXCL8 (p=0.657).

Immunohistochemical evaluation of hypertrophic markers

Overall, very little collagen type X was formed during cartilage regeneration. Occasionally some positive staining was observed in the cell cytoplasm in both healthy and OA chondrocytes, but even exogenous addition of high concentrations of recombinant CXCL8 to chondrocyte regeneration culture did not increase type X collagen deposition (Figure 8). Furthermore, no staining for osteopontin and osteonectin was observed, regardless of CXCL8 addition or inhibition (data not shown).

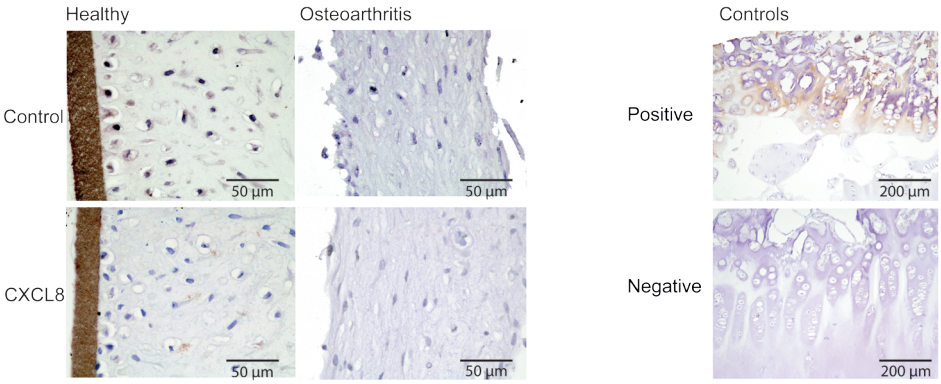


Figure 8. Type X collagen production in neocartilage formed in the presence of CXCL8. Type X collagen in healthy and osteoarthritic chondrocytes regenerating on filters with or without exogenous CXCL8. Occasionally some positive staining for type X collagen was found in the cell cytoplasm, but not in the matrix and independent of CXCL8 activity. Human tibial growth plate was used as a positive control and mouse IgG1 for isotype control.

DISCUSSION

This study demonstrates the presence of the proinflammatory chemokine CXCL8 in the synovial fluid of healthy, cartilage defect and OA donors, without any apparent differences. However, during regeneration of chondrocytes, high levels of CXCL8 are produced that varied with the disease status of the cartilage the cells were obtained from. OA chondrocytes produced more CXCL8 than healthy and cartilage defect chondrocytes and cartilage defect chondrocytes produced more than healthy chondrocytes. Although the inhibition of CXCL8 activity during regeneration of cartilage by healthy and cartilage defect chondrocytes resulted in a significant decrease of GAG release, this effect was very small and in OA chondrocytes only a decreased DNA content was found. Also in OA cartilage explants, no effect of CXCL8 on cartilage turnover was found. Importantly, in none of the regeneration cultures nor in the explant model an effect of CXLC8 on type X collagen or other markers of hypertrophic differentiation was noted.

CXCL8 has been demonstrated in the synovial fluid of patients with knee osteoarthritis or other knee pathology, but whether or not its levels were increased as opposed to healthy joints was uncertain because generally healthy controls were lacking in these studies[66, 220-222, 237]. We found no significant differences in the concentrations of CXCL8 between healthy and OA donors, which is in line with a recent study in which for the first time levels of CXCL8 in synovial fluids collected from healthy donors within 24 hours post-mortem, were compared to synovial fluid of an OA population of a wide range of disease severity, including also mild OA[66]. In the current study, the variation in CXCL8 levels in the synovial fluid of all three types of donors was large, therefore a larger sample size may have still resulted in differences, although these would likely have been

small. For joints with cartilage defects CXCL8 levels in the synovial fluid had not been compared to healthy joints until now. No significant difference in CXCL8 concentration existed when compared to both healthy and osteoarthritic synovial fluids. This is in contrast to previous data on CXCL8 levels measured after ACL rupture, which were shown to be high immediately after injury and subsequently decrease in the course of three weeks[221], suggesting an acute and a more chronic phase in its production. In the current study, synovial fluid sampling in the symptomatic cartilage defect patient group was performed per-operatively, with at least several weeks between the time of injury and treatment. Therefore we cannot exclude that CXCL8 levels might have been higher in the more acute stage.

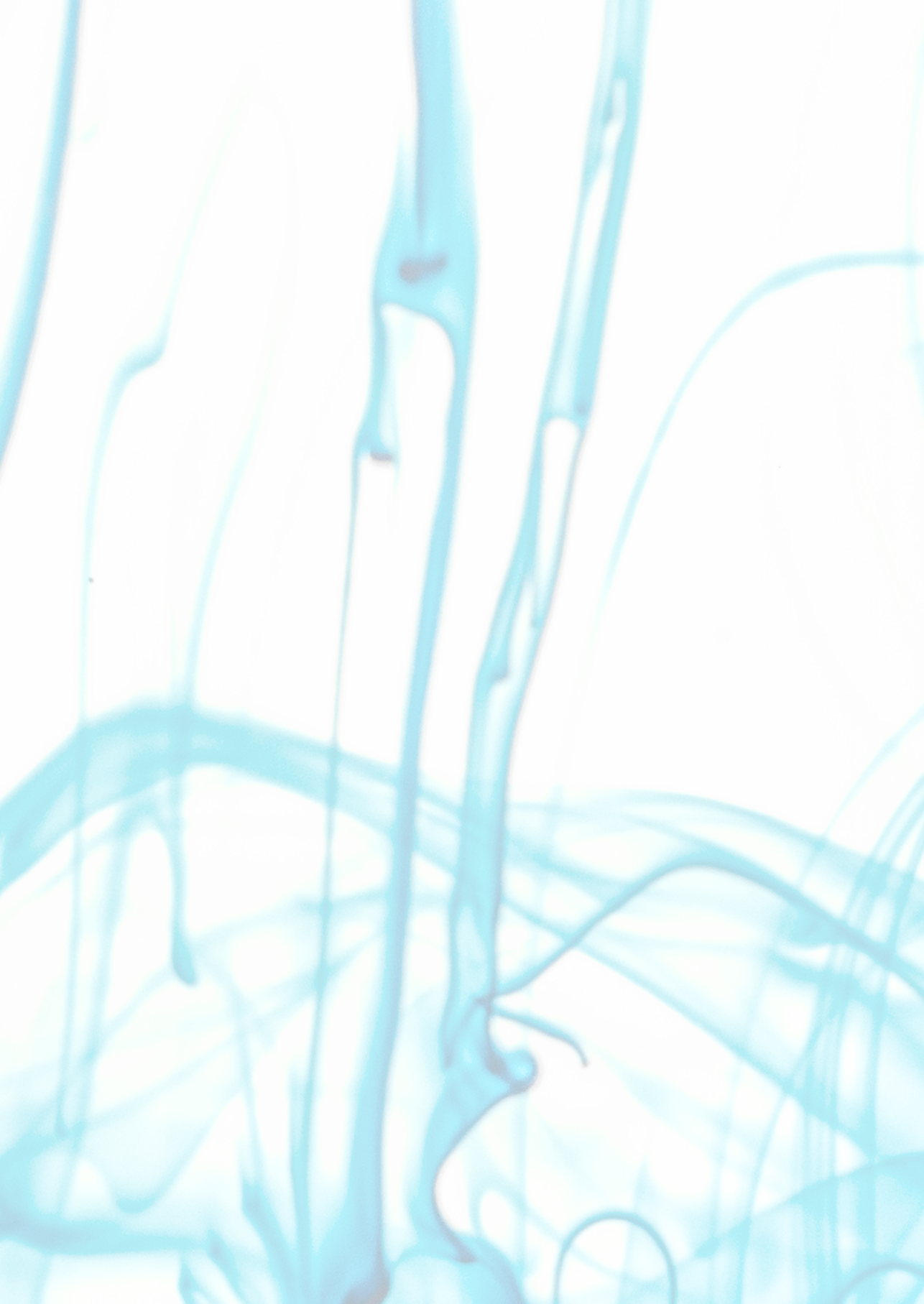
Despite the absence of clear differences in synovial fluid CLCX8 levels, CXCL8 production during regeneration was elevated by chondrocytes from cartilage debrided from the rims of defects and OA cartilage, even after expansion and concurrent dedifferentiation. A major mode of action suggested previously for CXCL8 is the induction of chondrocyte hypertrophy, as reflected by type X collagen production[73]. This is a characteristic of OA and also found in repair tissue after treatment for cartilage defects and the high levels produced by cells from diseased joints may therefore explain this phenomenon. However, in the current study only occasionally some positive staining for type X collagen was demonstrated in the cell cytoplasm, but not in the matrix and irrespective of CXCL8 activity, suggesting hypertrophic differentiation is not induced by this factor. This was also supported by the absence of osteopontin and osteonectin. The discrepancy with previously found upregulation of type X collagen production, may be explained by the fact that these studies were based on chondrocytes in suspension culture, which is quite uncommon and does not represent physiological repair.

CXCL8 is also known as a key cytokine known to be secreted by senescent cells, as part of the senescence-associated secretory phenotype (SASP)[164, 165]. The senescence-like phenotype has been reported in OA chondrocytes, especially near osteoarthritic lesions[166, 167]. It is tempting to speculate that also chondrocytes debrided from the rims of defects may have become more senescent by the incurrence of trauma, albeit not to the same extent as in OA chondrocytes. A higher degree of senescence in OA chondrocytes may additionally also be explained by the higher age of OA donors. Autocrine or paracrine stimulation may then further enhance senescence as CXCL8 can reinforce it[164], especially in OA cartilage where CXCL8 and its receptors CXCR1 and CXCR2 appeared to be upregulated[230, 238]. Whether senescence would be relevant for the application of these cells in cartilage regeneration in the course of ACI is not clear. Although regeneration achieved with OA and defect chondrocytes was not different from cells expressing lower levels of IL-8, it cannot be excluded that on the long run *in vivo* the tissue may have been more unstable.

Besides inducing senescence, CXCL8 is also a known mitogen in other diseases such as the hyperplasia of the airway smooth muscle mass in asthma[239] and various human cancers[240]. We found a small decrease in DNA content after four weeks of regeneration by OA chondrocytes and further research should be directed at evaluating whether CXCL8 also plays a role in the chondrocyte proliferation observed in OA.

CXCL8 stimulation of passage 2 expanded healthy chondrocytes, the cells employed in ACI, very marginally increased the release of proteoglycans into the medium and likewise its inhibition slightly decreased release by healthy and cartilage defect chondrocytes. CXCL8 has been shown capable of induction of N-acetyl-β-D-glucosaminidase, a lysosomal glycosidase, which is able to degrade glycosaminoglycans and hyaluronic acid[230]. Cultured chondrocytes produce various glycosidases, which in turn have been shown to increase GAG release[241]. However, it is unclear to what extent these mechanisms are also relevant *in vivo*, especially in the presence of other cytokines that may enhance or counteract the effects of CXL8. No effects on matrix production were observed in OA chondrocytes, during regeneration nor explant culture, also not when co-stimulated with IL-1, a pivotal cytokine in OA that enhances the effects of many other inflammatory cytokines[242]. This may be related to the observation that OA chondrocytes have been shown to be less responsive to both anabolic and catabolic stimuli[243, 244], which may be at least partly caused by the higher age of OA donors[244]. In addition, epigenetic regulation, shown to occur in osteoarthritic chondrocytes[245] may play a role in altering their response.

In conclusion we show that the proinflammatory cytokine CXCL8 is also present in the synovial fluid of patients with symptomatic cartilage defects, although it does not seem to be differentially regulated *in vivo*. *In vitro,* the effects on cartilage regeneration were negligible and hypertrophic differentiation was not induced. However, the increased release of CXCL8 by defect and OA chondrocytes, possibly indicating higher senescence, may require more attention, especially as pre-clinical research is exploring the option of defect and OA chondrocytes for ACI.



Chapter 6

Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model

Anika I Tsuchida
Michiel Beekhuizen
Marijn Rutgers
Gerjo JVM van Osch
Joris EJ Bekkers
Arjan GJ Bot
Bernd Geurts
Wouter JA Dhert
Daniel BF Saris
Laura B Creemers

Arthritis Res Ther. 2012 Dec 3;14(6):R262.

ABSTRACT

Introduction

This study aimed to determine whether, as in osteoarthritis, increased levels of interleukin-6 (IL-6) are present in the synovial fluid of patients with symptomatic cartilage defects and whether this IL-6 affects cartilage regeneration as well as the cartilage in the degenerated knee.

Methods

IL-6 concentrations were determined by ELISA in synovial fluid and in conditioned media of chondrocytes regenerating cartilage. Chondrocytes were obtained from donors with symptomatic cartilage defects, healthy and osteoarthritic donors. The effect of IL-6 on cartilage regeneration and on metabolism of the resident cartilage in the knee was studied by both inhibition of endogenous IL-6 and addition of IL-6, in a regeneration model and in osteoarthritic explants in the presence of synovial fluid, respectively. Readout parameters were DNA and glycosaminoglycan (GAG) content and release. Differences between controls and IL-6 blocked or supplemented samples were determined by univariate analysis of variance using a randomized block design.

Results

Synovial fluid of patients with symptomatic cartilage defects contained more IL-6 than synovial fluid of healthy donors ($p = 0.001$) and did not differ from osteoarthritic donors. IL-6 production of osteoarthritic chondrocytes during cartilage regeneration was higher than that of healthy and defect chondrocytes ($p < 0.001$). Adding IL-6 increased GAG production by healthy chondrocytes and decreased GAG release by osteoarthritic chondrocytes ($p < 0.05$). Inhibition of IL-6 present in osteoarthritic synovial fluid showed a trend towards decreased GAG content of the explants ($p = 0.06$).

Conclusions

Our results support a modest anabolic role for IL-6 in cartilage matrix production. Targeting multiple cytokines, including IL-6, may be effective in improving cartilage repair in symptomatic cartilage defects and osteoarthritis.

INTRODUCTION

Cytokines are thought to play an important role in articular cartilage degeneration[246]. In rheumatoid arthritis (RA), the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are known to have pivotal roles in its pathophysiology[135]. In addition to IL-1 and TNF-α, interleukin-6 (IL-6) has been demonstrated to play a role in cartilage degeneration in RA. In mice models of RA, cartilage destruction was shown to be dependent on IL-6[247, 248]. Furthermore, tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, now has an established role in the treatment of RA[249]. Besides efficacy in the amelioration of clinical signs and symptoms, tocilizumab has also demonstrated to reduce joint space narrowing and levels of cartilage degradation biomarkers[250-252].

Although not as pronounced as in RA, mild and intermittent inflammation is frequently observed in symptomatic focal cartilage lesions, a condition thought to predispose to the development of osteoarthritis (OA), and in OA. Elevated concentrations of inflammatory mediators, including IL-6, have been found in the serum and synovial fluid of OA patients[66, 85, 217, 253-257] and correlated to radiographic knee OA[258, 259]. However, the presence of IL-6 in joints with symptomatic cartilage defects has not been evaluated until now. In other joint injuries known to predispose to OA, such as anterior cruciate ligament (ACL) injuries[50, 67, 68] and meniscal tears[253, 260], increased levels of IL-6 have been detected in the synovial fluid. High levels of intra-articular inflammatory cytokines may, in addition to causing degeneration, also hamper tissue regeneration as cartilage repair is affected by the composition of the synovial fluid[34, 81, 82].

In OA most of the IL-6 present in the knee originates from the synovium[62]. However, chondrocytes in culture are capable of producing IL-6, albeit at low levels under most conditions[261-263]. Various stimuli, such as inflammatory molecules[264, 265] and binding of (fragmented) matrix components, which bind through discoidin domain receptor 2 (DDR2)[203, 266] have been reported to induce IL-6 synthesis, and these mechanisms are also proposed to play a role in OA. Chondrocytes can be stimulated by IL-6 either by binding directly to the gp80 receptor, or more commonly through trans-signaling, in which IL-6 binds first to the soluble IL-6 receptor α (IL-6Rα) in the synovial fluid, and then forms a heterodimeric association with the membrane-bound gp130 receptor[267].

Despite its possible role in OA, studies investigating the role of IL-6 in OA models have provided inconsistent results. *In vitro* stimulation of chondrocytes with IL-6 has revealed anabolic effects, such as up-regulation of tissue inhibitor of metalloproteinases-1 (TIMP-1)[268] and type II collagen[269], as well as catabolic effects, such as down-regulation of cartilage matrix genes[270, 271], inhibition of proteoglycan synthesis[272] and stimulation of aggrecanase production[71, 273]. *In vivo* models have also revealed both chondroprotective and chondrodegenerative properties of IL-6. A protective role of IL-6 in a spontaneous

OA model was reported in ageing male mice[274], but through both mechanically induced OA and OA induced by hypoxia-inducible factor-2α (HIF-2α), IL-6 was identified as the mediator of cartilage degradation[275]. However, many other studies have failed to demonstrate a direct effect of IL-6 on cartilage matrix metabolism[276-278]. One of the explanations for this lack of effect may lie in the simplified set up of many studies in which IL-6 has been added, since the action of IL-6 may depend on other factors in the joint, in particular in the synovial fluid.

This study evaluated the presence of IL-6 in the synovial fluid of patients with symptomatic cartilage lesions and patients with late stage OA, its production by chondrocytes isolated from these patients, and its role in cartilage regeneration. In addition, to evaluate the possible effects of high levels of IL-6 in the synovial fluid on cartilage in the knee, we cultured OA cartilage explants in the presence of OA synovial fluid in which IL-6 was selectively inhibited.

MATERIALS AND METHODS

Synovial fluid and cartilage sample collection and cell isolation

Collection of all patient material was done according to the Medical Ethical regulations of the University Medical Centre Utrecht and according to the guideline 'good use of redundant tissue for clinical research' constructed by the Dutch Federation of Medical Research Societies on collection of redundant tissue for research[139]. This study does not meet the definition of human subjects research or require informed consent and anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital[140].

Macroscopically healthy articular cartilage (n=6) and synovial fluid (n=20, age 25 to 47, average 40 years) were obtained from donors without any history of major joint trauma, osteoarthritis or inflammatory joint disease and absence of cartilage defects and synovial inflammation upon inspection of the knee within 24 hours post-mortem. Consistently only one knee per donor was used. Defect cartilage (n=3) and synovial fluid (n=22, age 20 to 48, average 33 years) were obtained from donors undergoing either microfracture or autologous chondrocyte implantation (ACI) for focal grade III and IV cartilage defects (only grade III cartilage was used for chondrocyte isolation). During those procedures, the cartilage defect was debrided to remove all cartilage remnants down to the subchondral bone and create a stable cartilage rim. The debrided cartilage was used for chondrocyte isolation. Chondrocytes from this location were recently shown to have good regenerative capacities compared to cells harvested from non-weight bearing cartilage normally used for ACI[138]. Of the 22 patients with symptomatic cartilage defects, one had an associated ACL injury and a history of partial meniscectomy, another

three had previously received partial meniscectomies and one had undergone an ACL reconstruction. OA cartilage (n=12) and synovial fluid (n=27, age 53 to 81, average 70 years) were obtained from donors undergoing total knee arthroplasty. Synovial fluid was centrifuged at 13000 g for two minutes to remove debris, and stored at -80 °C until use or analysis. Glucocorticoids affecting cytokine production are not prescribed at our institution for patients with focal cartilage lesions and patients with end-stage OA eligible for total knee replacement.

Cartilage samples were rinsed in phosphate buffered saline (PBS), cut into small pieces and enzymatically digested overnight at 37 °C in a 0.15% collagenase type II (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Bleiswijk, The Netherlands) with penicillin/streptomycin (100 U/mL/100 µg/mL; Invitrogen, Life Technologies). After digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Diego CA, USA), and the chondrocytes were spun down by 10 minutes centrifugation at 300 g.

Measurement of IL-6 levels

To determine the IL-6 levels in the synovial fluids of healthy, defect and OA donors and in the conditioned media of healthy, defect and OA chondrocytes during regeneration, a multiplex ELISA was performed as previously described[141, 142]. A total of 12 cytokines were measured of which IL-6 was most differentially regulated by healthy, defect and OA donors and hence chosen for further investigation. Briefly, specific antibodies (i.a. rat anti-human IL-6, MQ2-13A5; BD Biosciences) were coupled to carboxylated beads (Luminex Corporation, Austin TX, USA). Recombinant human IL-6 (BD Biosciences, #550071) was used to make a standard curve. Synovial fluid samples were first treated with hyaluronidase (type IV-S, Sigma-Aldrich, Zwijndrecht, The Netherlands) at a concentration of 20 U/ml for 30 minutes at 37 °C and then filtered by centrifuging through a polypropylene tube containing a 0.22 µm nylon membrane (Spin-X column; Corning, Amsterdam, The Netherlands). Subsequently the synovial fluid samples were diluted 1:2 with HPE-0.1375% Tween (Sanquin, Amsterdam, The Netherlands). To block possible interfering antibodies present in the synovial fluid the samples were diluted with an equal volume of 10% (v/v) normal rat and mouse serum (Rockland, Gilbertsville PA, USA). Medium samples were directly incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies (i.a. biotinylated rat anti-human IL-6, MQ2-39C3; BD Biosciences), samples were thoroughly washed and incubated with streptavidin–phycoerythrin (BD Biosciences) for 10 minutes. After washing, the samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules CA, USA) with Bio-Plex Manager software, version 3.0. The concentration of IL-6 in the media and synovial fluid was expressed as pg/mL using the standard curves. Results of specific

ELISAs for determination of IL-6 levels have previously been shown to be comparable to multiplex ELISA for conditioned medium, plasma and knee lavage samples[62, 141, 260].

Regeneration culture

Isolated chondrocytes from healthy, defect and OA cartilage were expanded in monolayer at 37 °C and 5% CO_2 at a seeding density of 5000 cells per cm^2 in expansion medium consisting of DMEM, 10% fetal bovine serum (Hyclone, Thermo Scientific, Etten-Leur, the Netherlands), penicillin/streptomycin (100 U/mL/100µg/mL) and 10 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA). After two passages (P2), the chondrocytes were seeded on collagen type II-coated (Chicken sternal cartilage; Sigma-Aldrich, #C9301) Millicell filters (Millipore Co., Bedford MA, USA), at $1.6x10^6$ cells per cm^2. Chondrocytes were redifferentiated for 28 days in redifferentiation medium consisting of DMEM, 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), 2% insuline-transferrine-selenium (ITS)-X (Invitrogen) and 5 ng/mL transforming growth factor-β2 (TGF-β2; R&D systems). Since fibrillar type II collagen was previously shown to induce IL-6 release from chondrocytes[203, 266], we also measured the release of IL-6 from P2 chondrocytes (n=3 healthy donors) seeded at a density of $1.6x10^6$ cells per cm^2 on filters coated with type I collagen (rat tail; BD Biosciences, #354249), and denatured type I and II collagen. Collagen was denatured by heating for 45 minutes at 70 °C. Levels of IL-6 in the conditioned media were determined by specific ELISA for IL-6 (Cytoset; Invitrogen) according to the manufacturer's instructions.

Endogenous IL-6 production of defect chondrocytes proved to be not significantly different from healthy chondrocytes, which were both much lower than that of OA chondrocytes. Therefore the role of IL-6 endogenously produced by defect chondrocytes (n=3) and OA chondrocytes (n=3) was studied through blockage of IL-6 with an activity-inhibiting antibody. To this end, the medium was supplemented with either 1 or 4 µg/mL anti-hIL-6 (purified mouse monoclonal IgG_1; R&D systems, #MAB206) or IgG_1 isotype control (R&D systems, #MAB002). The dose of anti-hIL-6 was chosen based on an IL-6-dependent murine plasmacytoma proliferation assay, as described earlier[279]. Furthermore, IL-6 activity was blocked with 100 ng/mL tocilizumab (RoActemra®, Roche, Woerden, the Netherlands), a humanized monoclonal antibody directed against the IL-6 receptor. The concentration of tocilizumab was chosen based on previously observed average concentrations of IL-6 receptor in the synovial fluid of patients with OA of approximately 10-40 ng/mL[256, 257]. Since the effects of IL-6 inhibition were limited in expanded cells, we also verified the effects of IL-6 inhibition in freshly isolated OA cells (P0; n=3).

Endogenous IL-6 production was relatively low in healthy chondrocytes, so the possible effects of high concentrations of IL-6 were further investigated by the addition of

10 ng/mL rhIL-6 with 25 ng/mL rhIL-6Rα (R&D Systems, #206-IL, #227-SR) to both healthy (n=3) and OA (n=3) chondrocytes. Medium was changed three times a week and supernatants were collected and stored at -80 °C until later analysis. Per condition, six filters were seeded with chondrocytes, five for biochemical analyses and one for histological evaluation.

Osteoarthritic cartilage explant culture

OA cartilage from three donors was cut into explants of ≈1 mm by 1 mm with a mean wet weight (± SD) of 7.8 ± 2.8 mg. Cartilage explants were cultured for 14 days in explant medium consisting of DMEM, penicillin/streptomycin (100 U/mL/100 µg/mL), 1% ITS-X, 0.1 mM AsAp and 0.2% proline (Sigma-Aldrich), which was supplemented with either 0 or 25% (v/v) pooled OA synovial fluid from eight donors. Six explants per condition were used, five for biochemical analyses and one for histological evaluation. To study the role of IL-6 present in the synovial fluid, 4 ug/mL anti-hIL-6 or IgG_1 isotype control and/or 100 ng/mL tocilizumab were added to the medium. Medium was changed three times per week and collected and stored at -20 °C until later analysis.

The role of IL-6 was also studied by the addition of IL-6 to culture medium. Explants from 8 OA donors (11.3 ± 3.8 mg, minimum of 3 explants per condition) were pre-cultured for 24 hours in culture medium after which rhIL-6 (50 ng/mL) with IL-6 receptor (rhIL-6Rα; 200 ng/mL)[242] was added to experimental groups, but not to control groups. Explants were cultured for an additional 10 days with medium renewal every other day and the conditioned medium was collected and stored at -20 °C until later analysis.

Glycosaminoglycan and DNA analysis

After culture, the explants and the regenerated tissue were digested overnight in a papain buffer (250 µg/mL papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56 °C, followed by quantification of the glycosaminoglycans (GAGs) content using the dimethylmethylene blue (DMMB) assay[145]. The ratio of absorption at 540 nm to 595 nm was used to calculate the GAG content, using chondroitin-6-sulphate (shark; Sigma-Aldrich) as a standard. The GAG content in conditioned medium was also measured.

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

Histological evaluation

Both regenerated tissue and explants 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, Darmstadt, Germany) for GAG and counterstained with Weigert's haematoxylin (Klinipath, Duiven, the Netherlands) and 0.4% fast green (Merck) for nuclei and cytoplasm, respectively.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Results are displayed as mean ± standard deviation (SD). Differences between controls and IL-6 blocked samples and differences between controls and IL-6 supplemented samples were determined by univariate analysis of variance using a randomized block design and *post hoc LSD*-test when four or more conditions were compared to each other. Differences in IL-6 concentration were determined by the Kruskal-Wallis test, using *post hoc* Mann-Whitney *U*-test and Bonferroni correction for synovial fluids, and using nested ANOVA with *post hoc t*-test and Bonferroni correction for conditioned media. Differences between the various collagen coatings were determined by univariate analysis of variance with *post hoc t*-test with Bonferroni correction.

RESULTS

IL-6 in synovial fluid

The synovial fluid of donors with symptomatic cartilage lesions contained significantly more IL-6 than that of healthy donors (261 ± 385 pg/mL versus 64 ± 120 pg/mL, p=0.001), and was slightly lower but not significantly different from OA patients (396 ± 508 pg/mL; Figure 1). IL-6 levels in the five patients with symptomatic focal cartilage defects and associated or previous ACL injury and/or partial meniscectomy were not significantly different from the group as a whole (154 ± 70 pg/mL, p=0.6).

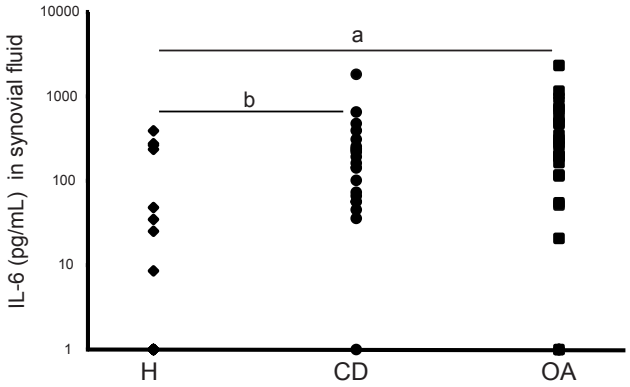


Figure 1. Increased concentration of IL-6 in the synovial fluid of patients with cartilage damage. Concentration of IL-6 in the synovial fluid of healthy (H), symptomatic cartilage defect (CD) and osteoarthritic (OA) donors. Note the logarithmic scale of the Y-axis; a = p <0.001, b = p = 0.001.

IL6 production in cell culture

In regeneration cultures, chondrocytes of the various origins produced IL-6 reaching concentrations that were at least tenfold higher than the concentrations present in the synovial fluid of the corresponding donor category (Figure 2). OA chondrocytes (9368 ± 3284 pg/mL) produced significantly more IL-6 than both healthy (2814 ± 995 pg/mL) and defect chondrocytes (3246 ± 2089 pg/mL, $p<0.001$). There was no significant difference in IL-6 production between healthy and defect chondrocytes.

To verify whether IL-6 production during regeneration was induced by the fibrillar type II collagen used for coating the filters in this model, we measured IL-6 production of regenerating chondrocytes on filters coated with various collagens. There was no difference in IL-6 production between type I and II collagen-coated filters and also not between native or denatured collagen-coated filters (Table 1). GAG and DNA content were also similar between the various coatings.

Table 1. Effect of collagen coating on IL-6 and cartilage matrix production

	Col I	Col II	Col ID	Col IID	*P*-value
IL-6 (pg/ml)	4,294 ± 2,152	4,604 ± 1,661	4,268 ± 1,446	5,463 ± 1,991	0.543
GAG (µg)	80 ± 24	85 ± 30	77 ± 25	85 ± 34	0.361
GAG release (µg/ml)	408 ± 29	410 ± 35	400 ± 38	498 ± 157	0.412
DNA (µg)	10 ± 1	9 ± 1	10 ± 2	10 ± 1	0.818

No significant differences were observed between type I collagen (Col I), type II collagen (Col II), denatured type I collagen (Col ID) and denatured type II collagen (Col IID) coating on IL-6 production at day 7, GAG content after 28 days of culture, cumulative GAG release during 28 days of culture and DNA content after 28 days of culture. Results are displayed as mean ± SD.

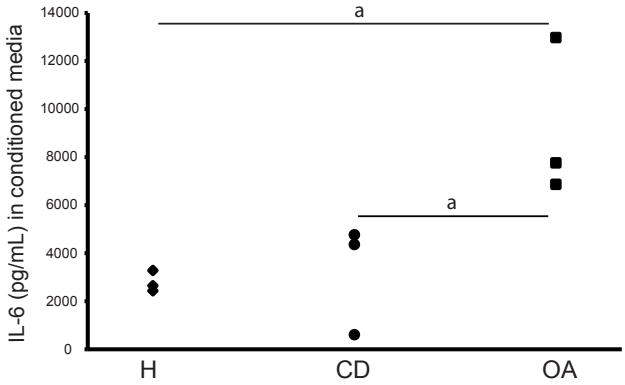


Figure 2. Cartilage regeneration culture: IL-6 production. Passage 2 expanded chondrocytes from healthy (H), defect (CD) and osteoarthritic (OA) cartilage were seeded at high density on type II collagen-coated filters and cultured during 28 days. Concentrations of IL-6 in conditioned media of chondrocytes during regeneration on day 7 is shown; a = $p<0.001$.

Regeneration culture

To evaluate whether the high levels of IL-6 produced by the chondrocytes during regeneration play a direct role in cartilage regeneration, IL-6 was inhibited using an activity-inhibiting antibody during regeneration of P2-expanded defect- and OA chondrocytes. As no difference was found in IL-6 production between healthy and defect chondrocytes, only defect and osteoarthritic chondrocytes were studied. No effects were found on cartilage matrix production, although an increase in DNA content was found in OA chondrocytes (p=0.009, Figure 3). Verification of these results using non-expanded osteoarthritic chondrocytes similarly showed no effect on cartilage matrix production and also the effect on DNA was no longer found (data not shown). Antagonism of the IL-6 receptor with tocilizumab in osteoarthritic chondrocytes failed to influence GAG and DNA content (data not shown).

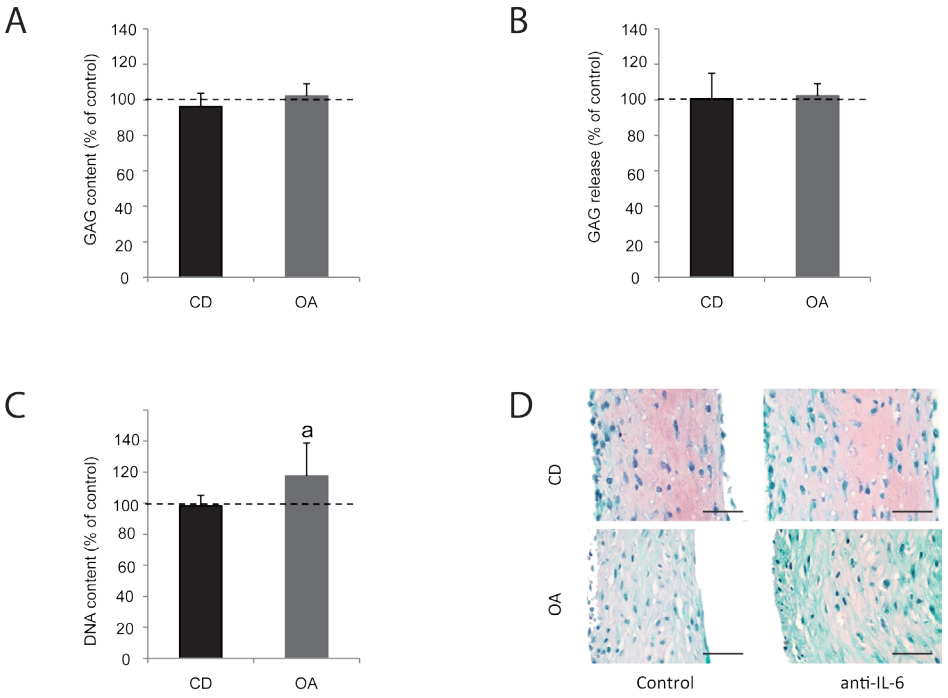


Figure 3. Cartilage regeneration after inhibition of endogenously produced IL-6. Cartilage regeneration cultures of three defect (CD) and three osteoarthritic (OA) donors with blockage of endogenous IL-6 with an activity-inhibiting antibody. (A-C) GAG content, GAG release and DNA content after 28 days of culture in IL-6 blocked samples depicted as percent of control samples (mean ± SD); a = p=0.009. (D) Safranin-O staining of representative CD and OA chondrocyte donors regenerating either without (control; IgG isotype) or with inhibition (anti-IL-6) of endogenous IL-6. Scale bars indicate 50 µm.

In healthy and defect chondrocytes endogenous IL-6 production was much lower than in OA chondrocytes. We therefore hypothesized that these cells could be more responsive to stimulation with exogenous IL-6 than OA chondrocytes. To examine whether exogenously added IL-6 could affect regeneration, 10 ng/mL rhIL-6 with 25 ng/mL rhIL-6Rα was added during regeneration culture of healthy and OA chondrocytes. In healthy chondrocytes, exogenous rhIL-6 increased GAG production in the neocartilage and a higher GAG/DNA ratio was found (p=0.002; Figures 4A and 4D). In OA chondrocytes, IL-6 decreased GAG release (p<0.001; Figure 4B) without affecting final GAG content in the neocartilage. DNA content was not modified by the addition of IL-6 (Figure 4C).

Osteoarthritic explant culture

To study the effect of high levels of IL-6 present in the synovial fluid on resident cartilage in the knee, we performed OA cartilage explant studies in the presence of OA synovial

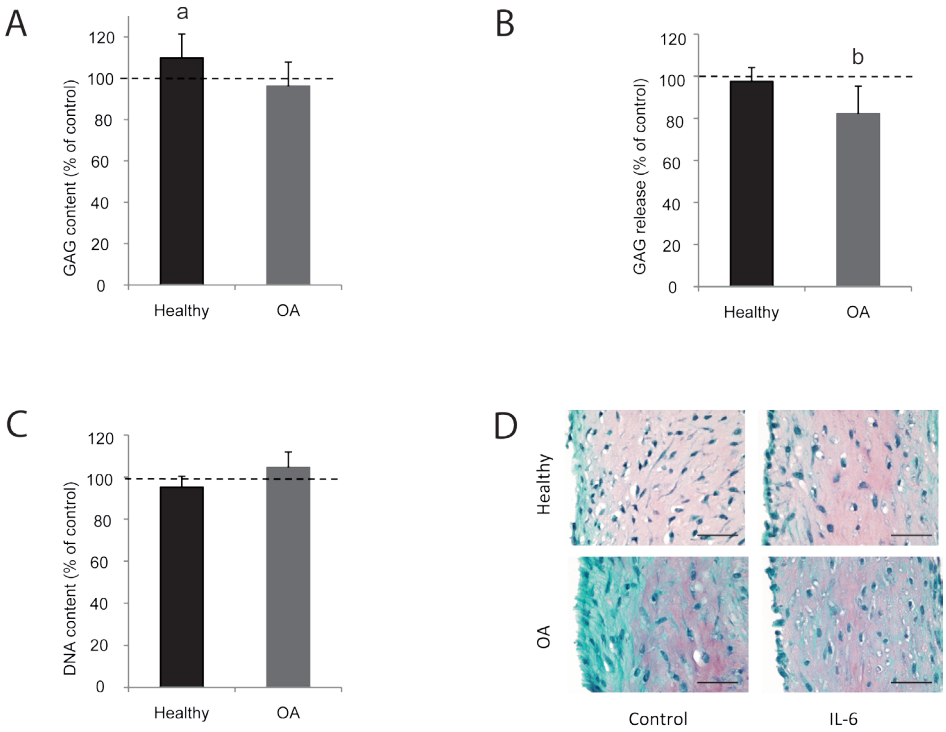


Figure 4. Cartilage regeneration with addition of IL-6. Cartilage regeneration cultures of three healthy (H) and three osteoarthritic (OA) donors with addition of rhIL-6 (10 ng/mL) and rhIL-6Rα (25 ng/mL). (A-C) GAG content, GAG release and DNA content of IL-6 supplemented samples depicted as percentage of control samples (mean ± SD); a = p=0.009, b = p<0.001. (D) Safranin-O staining of neocartilage generated by chondrocytes from healthy and osteoarthritic donors without or with addition of rhIL-6. Scale bars indicate 50 µm.

fluid in which IL-6 was inhibited. Ideally we would have also liked to perform these experiments using synovial fluid and cartilage explants from patients with chondral defects, but due to the very limited amount of material that can be obtained from these patients this was not feasible. In the absence of synovial fluid, inhibition of IL-6 did not alter the GAG and DNA content of the cartilage explants, nor was GAG release affected (data

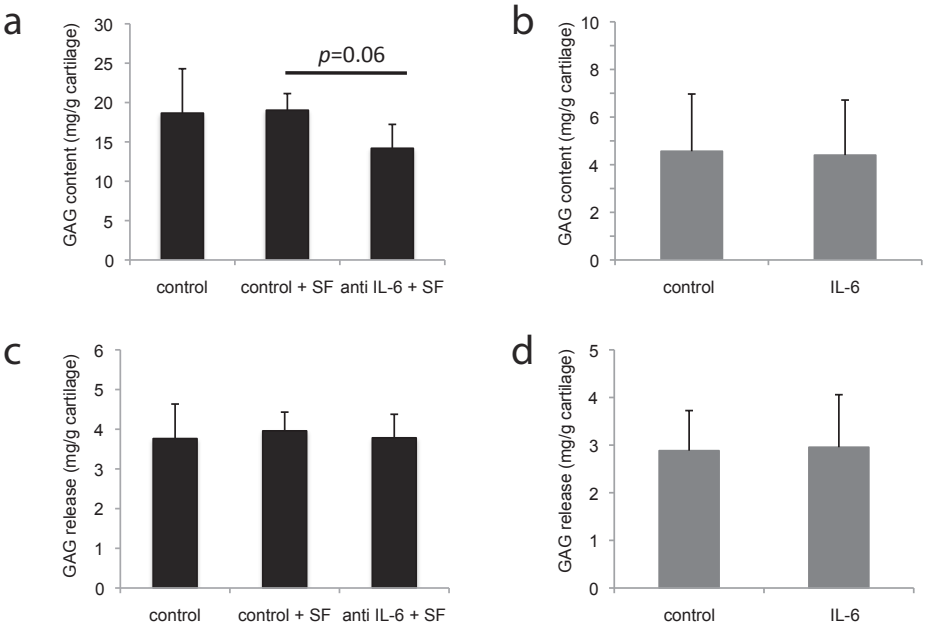


Figure 5. OA cartilage explant culture with inhibition of synovial fluid IL-6 or addition of exogenous IL-6. (A,C) GAG content and GAG release in OA cartilage explants from three donors cultured either in medium only, medium supplemented with 25% OA synovial fluid (SF) or medium with 25% OA synovial fluid in which IL-6 is blocked with an activity-inhibiting antibody (mean ± SD in mg/g); p=0.06. (B,D) GAG content and GAG release in OA cartilage explants from eight donors cultured in the presence or absence of rhIL-6 (50 ng/mL) with rhIL-6Rα (200 ng/mL).

not shown). However, when IL-6 was inhibited in the presence of synovial fluid a trend towards a decreased GAG content of the explants was observed (p=0.06; Figure 5A). In the absence of IL-6 inhibitors, the addition of synovial fluid increased the DNA content of explants (54.0 ± 28.0 µg/g cartilage versus 33.6 ± 20.9 µg/gr cartilage, p=0.002), and this effect was abolished by blocking IL-6 (42 ± 20 versus 54 ± 28 µg/g cartilage). GAG release was neither affected by the addition of synovial fluid nor by inhibition of IL-6 (Figure 5C).

Exogenous IL-6 in combination with soluble IL-6 receptor in the absence of synovial fluid did not alter the GAG (Figure 5B) or DNA content of the explants and also did not modulate GAG release (Figure 5D).

DISCUSSION

In this study we show increased IL-6 levels in the synovial fluid of patients with symptomatic cartilage defects compared to normal subjects. The IL-6 levels in patients with symptomatic cartilage defects were comparable to levels in patients with OA. Furthermore, we demonstrated for the first time that chondrocytes, especially OA chondrocytes, produce high concentrations of IL-6 during regeneration. Inhibition of this endogenously produced IL-6 did not affect cartilage matrix turnover, but addition of extra IL-6 increased the GAG content of neocartilage formed by healthy chondrocytes and decreased GAG release by osteoarthritic chondrocytes in an *in vitro* regeneration model. Furthermore, inhibition of IL-6 present in the synovial fluid showed a trend towards decreased matrix production in OA explants. Collectively, these results point towards an anabolic role of IL-6 in cartilage repair, albeit with limited effects.

Inflammatory mediators secreted by synovium and present in the synovial fluid have been demonstrated to affect cartilage regeneration *in vitro*[34, 62, 81, 82, 280]. Therefore, it is essential to characterize the mediators present in the synovial fluid of symptomatic cartilage defects and osteoarthritic joints and to determine their role in cartilage metabolism, in order to verify whether the outcomes of cartilage repair procedures such as ACI could potentially be enhanced by modulating the intra-articular environment. Levels of IL-6 comparable to those reported here were previously shown in the synovial fluid from healthy[66] and OA[66, 253, 281] joints, however, only limited data were available on IL-6 levels in joints with symptomatic focal cartilage defects. These are typically the joints that will be treated to stimulate regeneration of cartilage with techniques such as ACI, and therefore of particular importance for regenerative medicine strategies. Only one study has reported on the levels of IL-6 in cartilage lesions of variable depth, but did not specify whether the damage was focal or whether more generalized OA-like cartilage degeneration was present in the knee[281], which is an important selection criterion for the indication of ACI. However, IL-6 levels seemed to correlate with the grade of cartilage damage as the synovial fluid concentration of IL-6 increased with lesion severity. In our study, only symptomatic focal grade III and IV cartilage lesions in otherwise healthy knees were included. This is more clinically relevant for cartilage regeneration, although no distinction was made between grade III and grade IV defects.

Most likely, IL-6 present in the synovial fluid originates from the cells in the synovial membrane[256]. In addition, adipose tissue, including that of the fat pad in the knee, is an important source of inflammatory mediators, including IL-6[282, 283], at least partly explaining the association of OA with obesity. Although we did not collect information regarding the BMI of the donors, it is possible that the OA donors were more obese, which could account at least partly for the higher levels of IL-6 found in OA synovial fluid. Furthermore chondrocytes can produce IL-6, although chondrocytes embedded

in their original matrix produce very little IL-6[62]. However, during regeneration, chondrocytes produced high levels of IL-6, which, if they were then implanted intra-articularly, in theory, could cause high local concentrations and affect cartilage regeneration. The production of IL-6 was not a result of the type II collagen used in this model, as filters coated with type I collagen, which is not capable of inducing IL-6[266], resulted in the same IL-6 production. Possibly IL-6 production was induced by the TGF-β included in the current *in vitro* regeneration model, as was reported previously[263]. However, those data were obtained using non-expanded chondrocytes in short term, rather than during regeneration culture. In addition, the differences in IL-6 production between various cell types cannot be explained by the addition of TGF-β, as this was the same for all cell types.

Little is known on the role of IL-6 during regeneration, but inhibition of IL-6 did not influence GAG or DNA content of the newly formed cartilage. Possibly the effect of IL-6 inhibition on cartilage formation was partly masked by the regenerative effect of TGF-β. However, addition of IL-6 did decrease GAG release by OA chondrocytes. In addition, in explant culture, no TGF-β was present and no clear effect was found here.

The results found here are partly in contrast to the previously described reduction of cartilage matrix gene expression and inhibition of proteoglycan synthesis[270-272, 284]. Although the latter two effects of IL-6 can be seen as inhibitory, inhibition of synthesis can still be accompanied by a lack of net change in proteoglycan content if its deposition is increased. Indeed we observed less GAG release into the medium without affecting final GAG content upon addition of IL-6 to OA chondrocytes, suggesting that although total synthesis is reduced, final regeneration is the same. Unfortunately this aspect of cartilage regeneration is usually not addressed. Other differences between the current and previous studies evaluating the effect of IL-6 on chondrocyte metabolism may be based on the cells used, which were mainly bovine, porcine or rabbit chondrocytes. When human OA chondrocytes were used, actually no effect of IL-6 was seen on aggrecan production[276]. In the current study, addition of IL-6 to healthy chondrocytes, which produce much less IL-6, resulted in increased GAGs being deposited into the newly formed matrix. This is in line with previous studies using healthy human chondrocytes, revealing anabolic mechanisms upon addition of IL-6 such as up-regulation of TIMP-1 and bone morphogenetic protein-7 (BMP-7)[269, 285]. Furthermore, in osteoarthritic explant culture, a trend towards decreased matrix production upon inhibition of IL-6 was observed. Exogenous IL-6 in combination with soluble IL-6 receptor in the absence of synovial fluid did not alter cartilage turnover, suggesting that IL-6 in the synovial fluid exerts its effects via interaction with other factors. The limited availability of synovial fluid restricted its presence in culture to 25% and therefore higher percentages may have yielded more pronounced effects. The limited effect of IL-6 inhibition on cartilage matrix turnover is unlikely to be due to a lack of inhibitory activity of the antibody, as this and other

similar antibodies have demonstrated effectiveness in inhibiting IL-6 bioactivity in other models, including models with chondrocytes as target cells[272, 286]. Diffusion limitations in the cartilage explants may have prevented complete penetration of the antibodies to inhibit locally produced IL-6, thus still allowing for paracrine signaling. However, this is probably limited as chondrocytes in their native extracellular matrix hardly produce IL-6[62]. Penetration of tocilizumab, required for the inhibition of IL-6 signaling, into the newly formed cartilage during regeneration may have been suboptimal, allowing IL-6 signaling through the membrane-bound IL-6 receptor. This could explain the lack of effect that tocilizumab had during osteoarthritic chondrocyte regeneration.

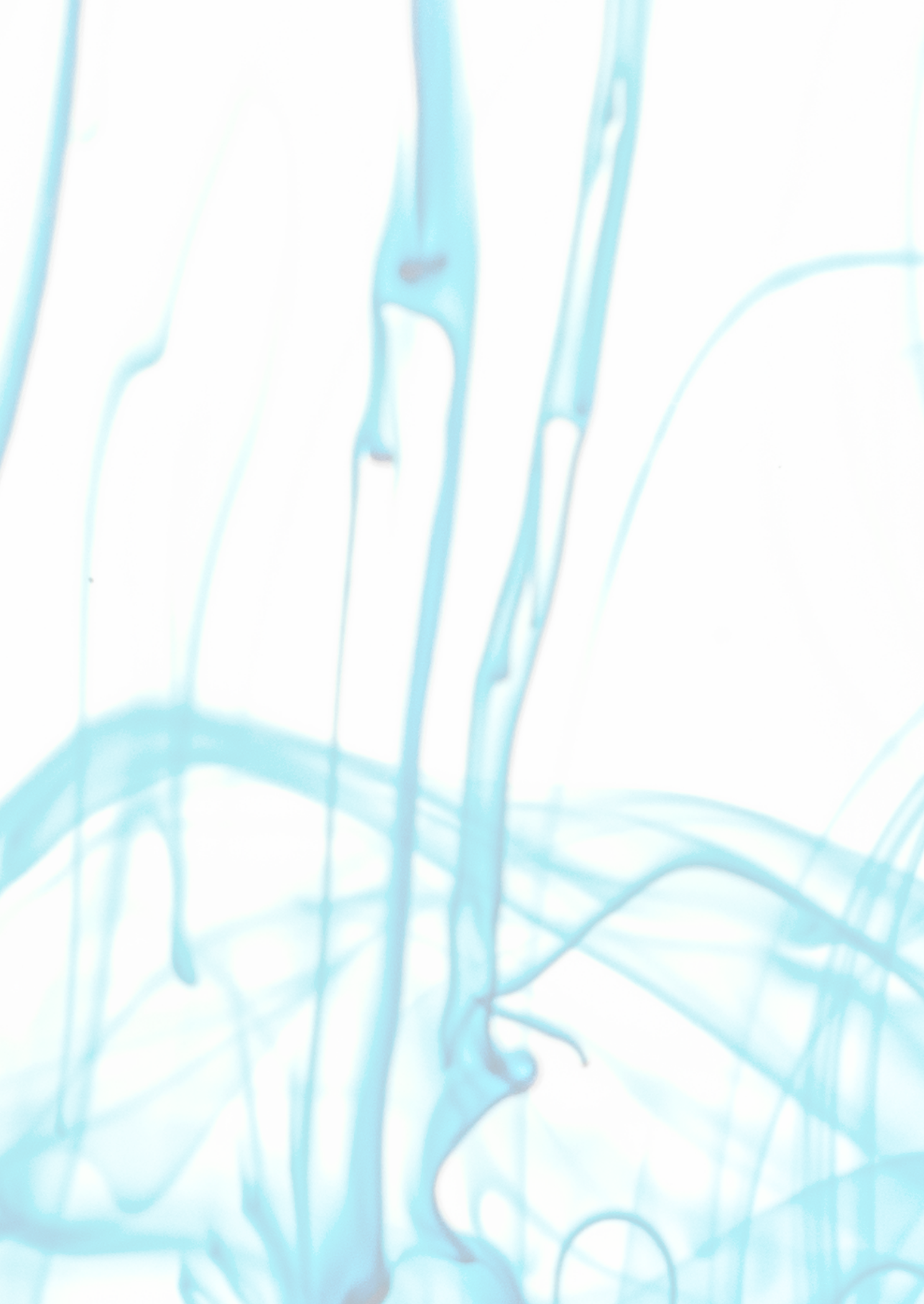
The role of IL-6 in cartilage metabolism has been the subject of much debate. IL-6 is often described as a modulatory factor, because it can induce both anabolic and catabolic mechanisms. Recently, IL-6 injection into the knee joint of mice was described to cause cartilage destruction, but in that study, like in many others, supra physiological concentrations of IL-6 were used[275]. Concentrations of IL-6 similar to those found in the synovial fluid usually do not have effects on the expression of cartilage matrix proteins in cartilage[276-278]. To our knowledge, this is the first study to demonstrate an effect of physiological concentrations of IL-6 on cartilage matrix production during regeneration, albeit modestly. IL-6 is known to be induced by various catabolic stimuli present in OA such as IL-1β[264], prostaglandin E_2 (PGE_2)[265], increased shear stress[287] and extracellular matrix components such as hyaluronan fragments[288] and matrillin-3[266]. IL-6 in turn, is capable of inducing factors such as metalloproteinases (MMPs)[289], TGFβ[290], vascular endothelial growth factor (VEGF)[291], and many others which are important for tissue remodeling. In bone IL-6 also induces remodeling through increased osteoclastogenesis[292], which is thought to be important in the observed inhibition of radiographic disease progression in RA patients treated with tocilizumab[250, 293]. IL-6 has also been shown to have anabolic effects on cartilage, both indirectly through the up-regulation of factors such as TIMP-1[285], BMP-7[269] and TGFβ[290], as well as directly through the up-regulation of cartilage matrix proteins[269]. In another study, injection of IL6 into the joint cavity of mice stimulated proteoglycan synthesis in cartilage[294], while IL-6 knockout mice showed more extensive naturally occurring cartilage loss[274] and reduced proteoglycan synthesis[274, 295]. The current study indicates that IL-6 has a mainly anabolic role in *in vitro* cartilage regeneration, although the effects are not strong, with increased GAG production in healthy chondrocytes and decreased GAG release in OA chondrocytes. Possibly the IL-6 in the synovial fluid of patients with symptomatic cartilage defects is induced in the course of regeneration and plays a role in tissue regeneration after cartilage damage.

Although cartilage pathology seemed to clearly affect IL-6 production and at least part of the response to interference with this factor, several other donor-related factors may have additionally influenced the results. To start with, there is the typical age difference found between OA donors (average 70 years[296]) and patients with cartilage defects,

which usually present around 30 years of age[93]. However, IL-6 levels were not found to correlate with age[297]. The use of post-mortem collection of healthy synovial fluid and cells as opposed to the intra-operative collection of OA and cartilage defect synovial fluid and cells is less likely to have affected the results. Previous work from our group showed that there was no difference in viability between freshly isolated chondrocytes from healthy (post-mortem obtained) and grade III cartilage defect tissue[138] which is in line with the observation that viability and cartilage matrix content is very much unaltered within the first 24 hours[175, 298, 299]. Synovial fluid is contained in a relatively isolated compartment[300, 301] and when kept at 4 °C, levels of IL-6 have been shown to be stable for more than six hours[302].

CONCLUSIONS

This study indicates that the level of IL-6 is increased in joints with symptomatic cartilage defects or OA compared to healthy joints. Moreover, the elevated levels of IL-6 appear to promote anabolic metabolism of the resident chondrocytes, and seems beneficial for formation of neocartilage during *in vitro* regeneration. Further research is necessary to evaluate whether targeting multiple cytokines or pathways, including IL-6, may be an effective means to improve cartilage matrix production during ACI or microfracturing in symptomatic cartilage defects or OA.



Chapter 7

Suppression of inflammation by celecoxib does not support cartilage repair in osteoarthritic cartilage explants and fails to reduce inflammation during regeneration

Anika I Tsuchida
Michiel Beekhuizen
Laura B Creemers
Daniel BF Saris
Wouter JA Dhert
Tim JM Welting
Gerjo JVM van Osch

Submitted for publication

ABSTRACT

Aims

This study aims to elucidate whether suppression of inflammation by COX-2 affects cartilage repair and regeneration using different culture models.

Methods

The productions of various inflammatory mediators, including prostaglandin E_2, were determined by (multiplex) ELISA. Glycosaminoglycan production and release were determined in a model of osteoarthritic cartilage explants and a co-culture model of osteoarthritic cartilage explants with synovium and a tissue regeneration culture by isolated chondrocytes.

Results

Celecoxib decreased the production of multiple inflammatory factors, such as IL-6, oncostatin M, IL-1 and prostaglandin E_2 by osteoarthritic joint tissues. However, it did not affect glycosaminoglycan turnover. Inflammation during chondrocyte regeneration culture was largely COX-2 independent.

Conclusions

We found no indications for an effect of celecoxib on cartilage repair, despite an effect on several inflammatory factors in addition to prostaglandin E_2. Inhibition of inflammation in osteoarthritis may not be required for tissue repair, although still useful for pain reduction.

INTRODUCTION

Osteoarthritis (OA) is a chronic joint disorder characterized by joint pain and disabilities. Key features of OA are degeneration of the cartilage, changes of the bone and synovial inflammation[303]. Inflammation is assumed to play a crucial role in osteoarthritic cartilage degeneration by the induction of various extracellular matrix degrading enzymes and inhibition of matrix protein production. Celecoxib, a specific cyclooxygenase (COX)-2 inhibitor, is commonly used in the treatment of pain in OA and has fewer gastro-intestinal side effects than the commonly used non-steroidal anti-inflammatory drugs (NSAIDS)[304]. COX-2 expression can be induced by several cytokines in inflammatory conditions such as (rheumatoid) arthritis or acute tissue damage[305, 306]. In turn COX-2 inhibition was postulated to positively affect tissue integrity through inhibition of inflammation, thereby reducing ensuing breakdown of tissue and repressive effects on repair and regeneration[307-310]. Inhibition of inflammation by COX-inhibitors has been suggested to be mediated through both COX-dependent and independent pathways[311].

Nonetheless, the described effects of celecoxib on cartilage regeneration and repair are conflicting[312]. Recent studies in COX-2 knockout mice show no protective effect on cartilage degeneration in a surgically induced model of OA[313]. Furthermore, chondrogenesis studied in different chondrogenic models did not show an effect of celecoxib on proteoglycan synthesis[314] or collagen type II production[315]. However, positive effects of celecoxib include inhibition of chondrocyte hypertrophic differentiation[315] and chondroprotection in OA in an *in vivo/ex vivo* study with human patients[309]. In addition, celecoxib decreased cartilage degeneration in an OA cartilage explant model[307, 308]. However, all of these studies measured short-term effects, not the result of longer-term exposure on final tissue integrity, which may explain the conflicting data found. In addition, despite the role of COX-2 in inflammation and its postulated effect on cartilage matrix breakdown, until now little attention has been paid to the effect of celecoxib on the production of inflammatory mediators[314] and to what extent this is related to changes in cartilage metabolism.

In the current study we investigated the effect of celecoxib on the production of inflammatory factors and on cartilage repair and regeneration using a range of *in vitro* models with osteoarthritic cartilage and synovial tissue and human chondrocytes, focussing on long-term effects and measuring glycosaminoglycans (GAGs) as chondrogenic readout.

MATERIALS AND METHODS

Collection of cartilage and synovial tissue

Healthy cartilage (macroscopically intact cartilage, from a knee without signs of cartilage degeneraton or synovial inflammation) was collected from post-mortem donors and osteoarthritic cartilage and synovial tissue was collected from end-stage OA patients undergoing total knee arthroplasty. Cartilage explants were harvested from the femoral condyles, cut into square pieces of approximately one by one mm and the wet weight was determined (mean 12 +/- 5 mg). Synovial tissue was cut into small pieces and wet weight was determined (mean 21 +/- 9 mg). From each donor, six cartilage explants and one synovial tissue explant were fixed immediately in 10% buffered formalin for (immuno)histological evaluation and six cartilage explants were stored at -20 °C for biochemical analysis at t=0. The rest of the cartilage and synovial tissue explants were used for culturing. This study does not meet the definition of human subjects' research or require informed consent, as anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our university hospital. The material was used according to the national guidelines 'code of conduct for the proper secondary use of human tissue'[140, 316] and approved by the institutional review board (protocol number 01/163).

Culture model with osteoarthritic cartilage

Osteoarthritic cartilage explants were cultured in the presence or absence of celecoxib (Biovision) dissolved in DMSO. Celecoxib was added at different concentrations (0.1 μM, 1.0 μM and 10 μM) with as control condition 0.1% DMSO. Explants were cultured in a total of 1 ml of culture medium. Culture medium (DMEM, 2% Human Serum Albumin, 1% penicillin/streptomycin, 1% ITS-X, 0.4 mM ascorbic acid) was renewed three times a week and stored at -80 °C for further analysis. After 21 days of culture the explants were stored at -20 °C for biochemical analysis. In total 6 different OA cartilage donors were used for the cultures (4 explants per condition).

Co-culture model with osteoarthritic cartilage and synovial tissue

A co-culture of cartilage and synovial tissue was used to study the effect of celecoxib in a particularly inflammatory osteoarthritic environment, as previous described[62]. In short, osteoarthritic cartilage explants were co-cultured with osteoarthritic synovial tissue in the presence or absence of celecoxib. Just as in the cartilage explants alone, a total of 1 ml of culture medium was used and a dose response was performed (0.1 μM, 1.0 μM and 10 μM). Total culture period was 21 days and medium was renewed three times a week and stored at -80 °C for further analysis. In total 6 OA cartilage donors with 4 cartilage/synovium explants per condition were used for the cultures.

Tissue regeneration culture by isolated chondrocytes

For the regeneration culture both healthy and osteoarthritic articular cartilage was cut and digested in collagenase 0.15% (w/v) (Worthington, Lakewood, USA) in Dulbecco's Modified Eagle Medium (DMEM) overnight at 37 °C. Subsequently, the chondrocytes were seeded in a monolayer at a cell density of 5000 cells/cm^2 and cultured in expansion medium containing DMEM, 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (100 U/100 µg/ml) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA). Chondrocytes were cultured until passage 2 and then used in a cartilage regeneration model as described previously[82, 232]. In short, 720.000 cells were seeded at high density on collagen type II (chicken sternal cartilage, #C9301, Sigma-Aldrich, St louis, MO, USA) coated Millicell filters (Millipore Co., Bedford, MA, USA) and cultured in redifferentiation medium containing DMEM, 2% Human Serum Albumin, 1% penicillin/streptomycin, 1% ITS-X (Gibco; #51500, Bleiswijk, The Netherlands), 0.4 mM ascorbic acid and 5 ng/ml TGF-β2 (R&D Systems, Minneapolis, USA). The constructs were cultured in a total of 1 ml of culture medium and in the presence or absence of 1.0 µM celecoxib (Biovision, Milpitas CA, USA) dissolved in DMSO (final concentration of DMSO for all cultures was 0.1%). Control conditions included the same concentration of DMSO as the celecoxib cultures. The cultures were incubated at 37 °C in 5% CO_2. Culture medium was renewed three times a week for total culture period of 28 days. Conditioned culture medium was stored in -80 °C for further analysis. In total 6 OA and 6 healthy chondrocyte donors with 5 constructs per condition were used for the cultures.

(Immuno)histochemistry

For (immuno)histochemistry, fresh cartilage and synovial tissue explants and filters after 28 days were fixed overnight in 10% buffered formalin, embedded in paraffin and cut into 5 µm sections. To show the cartilage degeneration in cartilage explants and neocartilage formation in the filters, a safranin-O/fast-green staining was used[317]. For COX-2 immunolocalization, the sections were blocked in 0.3% H_2O_2 in methanol for 20 minutes. For antigen retrieval, sections were boiled in citrate buffer (0.01 M; pH 6.0) for 10 minutes and placed in TBS-BSA for 60 minutes, after which the sections were incubated with mouse COX-2 antibody (Cayman chemical; CX229; 1:200; Michigan, USA[318]) overnight. The samples were washed with TBS-BSA and incubated with biotinylated goat anti-mouse IgG (Dako, Denmark, 1:200 in TBS-BSA) for 1 hour. Samples were washed and incubated with Streptavidin (Dako, 1:500 in TBS-BSA) for also 1 hour. Finally, samples were developed with DAB or Powervision (Leica Microsystems, Wetzlar, Germany). As an isotype-specific negative control mouse IgG1 (Dako, 1:200 in TBS-BSA) was used in the same concentration as the COX-2 antibody.

COX-2 activity

To determine COX-2 activity in tissue and cell-conditioned medium, a prostaglandin E_2 (PGE_2) ELISA was performed (KGE004B, R&D; Minneapolis, USA) according to the manufacturer's protocol. COX-2 activity was expressed as PGE_2 concentration in pg/ml.

Multiplex ELISA

The presence of interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, oncostatin M (OSM), monocyte chemotactic protein (MCP)-1, adiponectin, leptin and neurotrophic growth factor (NGF) in conditioned medium was determined using multiplex ELISA as described previously[141, 142]. Validation of the Multiplex ELISA was previously done by de Jager *et al*., showing excellent sensitivity and specificity[142]. The samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad laboratories). The concentration of all measured mediators in the conditioned medium was calculated using the standard curves and are expressed as pg/ml. Only the conditioned media of day 7 and day 28 were measured.

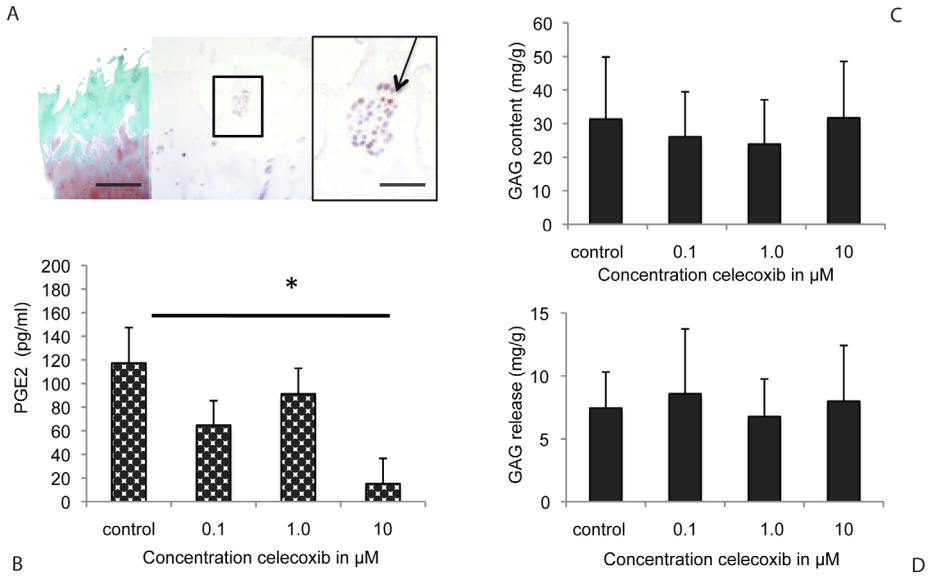


Figure 1. Effect of celecoxib on OA cartilage explants. The safranin-O staining showed osteoarthritic cartilage changes (A). The presence of COX-2 in OA cartilage explants is visible with IHC (A; pink staining, positive cell indicated by the arrow in magnification). Celecoxib in a concentration of 10 µM decreases PGE_2 production by OA cartilage explants (B; day = 4; mean +/- SD of 3 donors, 4 explants per condition; *p<0.001). In cartilage explants cultured alone no effect of celecoxib was seen on GAG content (C) or total GAG release (D) during the entire culture (mean +/- SD of 6 donors, 4 explants per condition).

Glycosaminoglycan (GAG) content and GAG release

GAG release and total GAG content was quantified using the dimethylmethylene blue (DMMB) spectrophotometric analysis described by Farndale *et al*[319]. Cartilage explants and filters were digested in 400 µl 2% papain (Sigma-Aldrich) in 50 mM phosphate buffer, 2 mM N-acetylcysteine, and 2 mM Na_2-EDTA (pH 6.5) at 60 °C overnight. Two hundred microliter DMMB solution and 100 µl of medium sample or papain digest were mixed and absorbance read at 540 nm and 595 nm using a spectrophotometer (Bio-Rad Laboratories, Hercules CA, USA). As reference, chondroitin sulfate C (Sigma-Aldrich) was used. GAG release and GAG content for the explants are given in mg GAG/g cartilage and for the filters in µg GAG/µg DNA.

DNA assay

The DNA content per sample was determined from the papain digest using a Picogreen DNA assay (Invitrogen, Life Technologies, USA) in accordance with the manufacturer's instructions

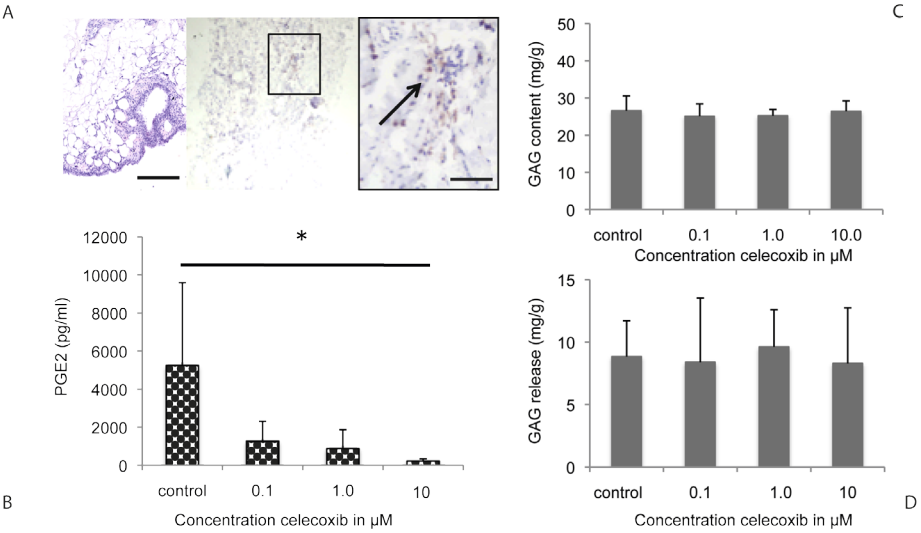


Figure 2. Effect of celecoxib on OA cartilage explants co-cultured with OA synovial tissue. The H&E staining showed osteoarthritic synovial changes (A). IHC showed the presence of COX-2 in OA synovial tissue explants (red staining, indicated by arrow in magnification). Celecoxib decreases PGE_2 production in a dose dependent manner (B; day = 4; mean +/- SD of 3 donors, 4 cartilage/synovium explants per condition; *p<0.001). In cartilage explants cultured with synovial tissue no effect of celecoxib was seen on GAG content (C) after 21 days of culture or total GAG release (D; mean +/- SD of 6 donors; 4 cartilage/synovium explants per condition).

Statistical analysis

Data are expressed as mean with standard deviation (SD). SPSS 16.0 software (SPSS Inc. Chicago, Illinois, USA) was used for the statistical analysis. Univariate analysis of variance (ANOVA), with a randomized block design and a *post hoc* Bonferroni was used to test for differences between different conditions. Normal distribution of the residuals was verified by Kolmogorov-Smirnov test and homogeneity of variances by Levene's test. Differences with a *p*-value less than 0.05 were considered statistically significant. No *post hoc* correction for multiple testing was determined in the multiplex ELISA data.

RESULTS

Suppression of inflammation in osteoarthritic explant model

To study if COX-2 inhibition could support the repair of an osteoarthritic cartilage matrix, OA cartilage explants were cultured in the presence of celecoxib. Immunohistochemistry for COX-2 confirmed the presence of the COX-2 enzyme in osteoarthritic cartilage (Figure 1A). Osteoarthritic cartilage explants produced low amounts of PGE_2

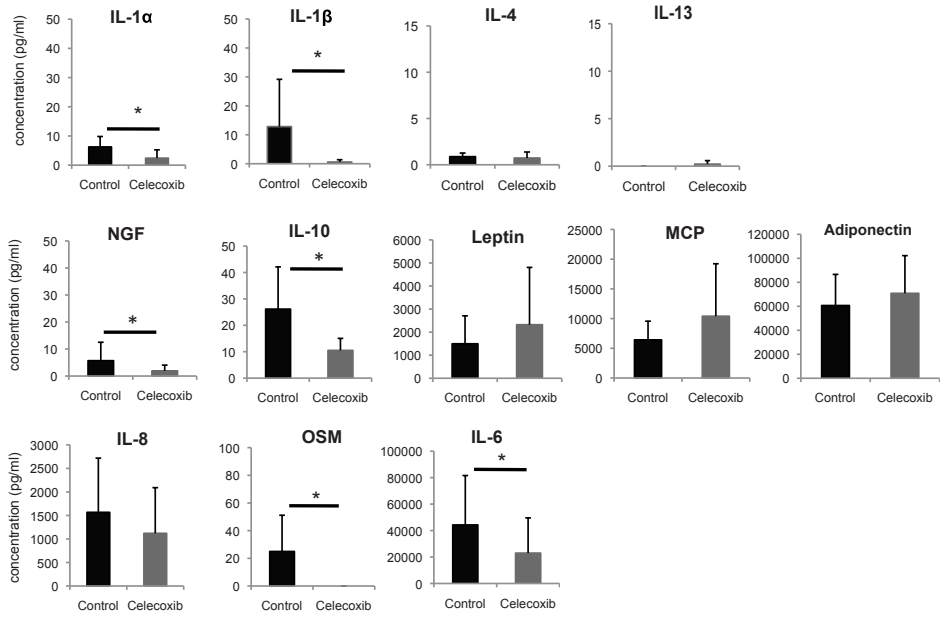


Figure 3. Production of mediators in the co-culture model at day 7. Celecoxib at 10 μM decreases production of IL-1α, IL-1β, IL-6, IL-10, OSM and NGF. No effect is seen on the production of leptin, adiponectin, IL-8 and MCP-1 (mean +/- SD of 3 donors, 4 cartilage/synovium explants per condition; **p*=0.05; no *post hoc* correction for multiple testing).

and celecoxib decreased the production of PGE_2 (p=0.01; Figure 1B). However, celecoxib had no effect on GAG content or GAG release (Figures 1C/D).

Suppression of inflammation in coculture model of osteoarthritic cartilage and synovial tissue

To better approach the heightened inflammatory conditions in OA, cartilage explants were co-cultured with osteoarthritic synovial tissue. Immunohistochemistry for COX-2 showed the presence of the COX-2 enzyme in both osteoarthritic cartilage and osteoarthritic synovial tissue (Figure 2A) and a clear production of PGE_2 in the co-culture (Figure 2B). In addition, IL-1α, IL-1β, NGF, IL-10, leptin, MCP, adiponectin, IL-8, OSM and IL-6 were produced (Figure 3). IL-1α and IL-1β were previously shown to be produced by cartilage explants, IL-8, IL-6 and OSM exclusively by synovial tissue and IL-10 by both cartilage and synovial tissue[62]. IL-4 and IL-13 were not detectable in medium conditioned by osteoarthritic cartilage and synovial tissue. Celecoxib dose-dependently decreased PGE_2 production in co-culture (*p<0.001; Figure 2B). Addition of 10 μM of celecoxib

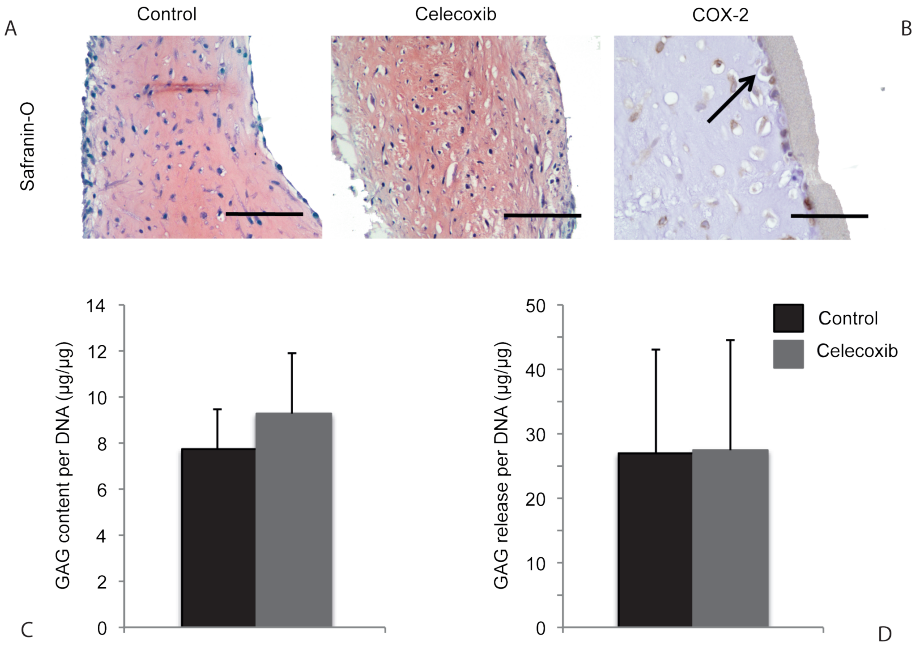


Figure 4. Effect of celecoxib on healthy chondrocytes. Cartilage formation after 28 days of culture is visible as safranin-O positive staining for both cultures with and without celecoxib (A). The presence of COX-2 in healthy chondrocytes is demonstrated with immunohistochemistry (B) (day = 28, brown staining; indicated by the arrow, bar represents 100 μm). Celecoxib had no effect on total GAG content at day 28 (C) or release during the 28 days of culture (D; mean +/- SD of 6 donors, 5 constructs per condition).

also decreased the production of the inflammatory cytokines IL-1α, IL-1β, IL-6 and OSM, but also of IL-10 and NGF (*p<0.05; Figure 3). No effect of celecoxib was seen on the production of leptin, adiponectin, IL-8 and MCP-1. At day 28 the same mediators were produced, albeit at lower levels than at day 7, with the same effects of celecoxib (data not shown). However, celecoxib, irrespective of the concentration used, did not have an effect on GAG content or release in cartilage explants cultured with synovial tissue (Figures 2C/D).

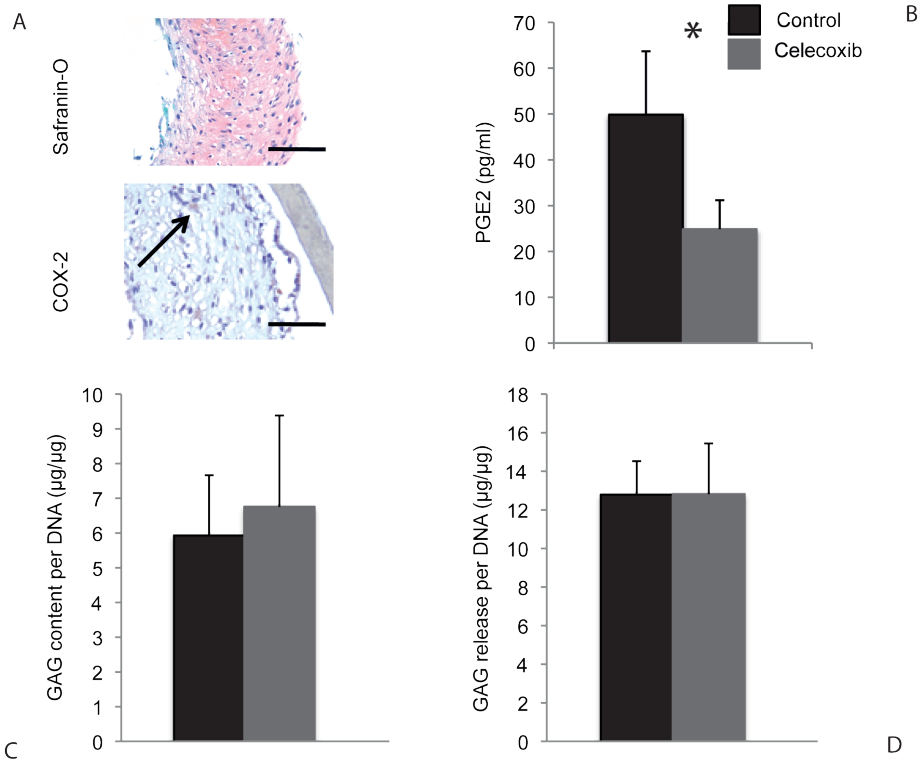


Figure 5. Effect of celecoxib on OA chondrocytes. Neo-cartilage formation after 28 days of culture is visible as safranin-O positive staining (A). The presence of COX-2 in OA chondrocytes is demonstrated with immunohistochemistry for COX-2 (B; brown staining; indicated by the arrow, scalebar represents 100 μm). Celecoxib decreased PGE_2 production by chondrocytes (day =10), however, still some PGE_2 production was present (*p=0.03; mean +/- SD, average of 3 donors 4 constructs per condition). Celecoxib had no effect on total GAG content at day 28 (C) or release during the 28 days of culture (D; mean +/- SD of 6 donors, 5 constructs per condition).

COX-2 independent inflammation during tissue regeneration culture by isolated chondrocytes

We then used isolated healthy and osteoarthritic chondrocytes in a cartilage regeneration model to investigate the effect of celecoxib on cartilage repair since this might be a more sensitive model to study tissue generation. Safranin-O staining showed the formation of neocartilage by the chondrocytes after 28 days of culture (Figures 4A and 5A). Immunohistochemistry for COX-2 confirmed the presence of COX-2 enzyme in healthy and osteoarthritic chondrocytes (Figures 4B and 5A). PGE_2 release by osteoarthritic chondrocytes was higher than by healthy chondrocytes (below detection limit, data not shown), indicating increased inflammatory reactions (Figure 5B). PGE_2 production could be inhibited by celecoxib, albeit not completely (Figure 5B; p=0.03). In addition, celecoxib was unable to decrease the high amounts of IL-6, IL-8, MCP-1 and NGF at day 7 of high-density culture (Figure 6). At day 28 the same mediators were produced, albeit lower than at day 7, with a similar lack of effect of celecoxib, (data not shown). Addition

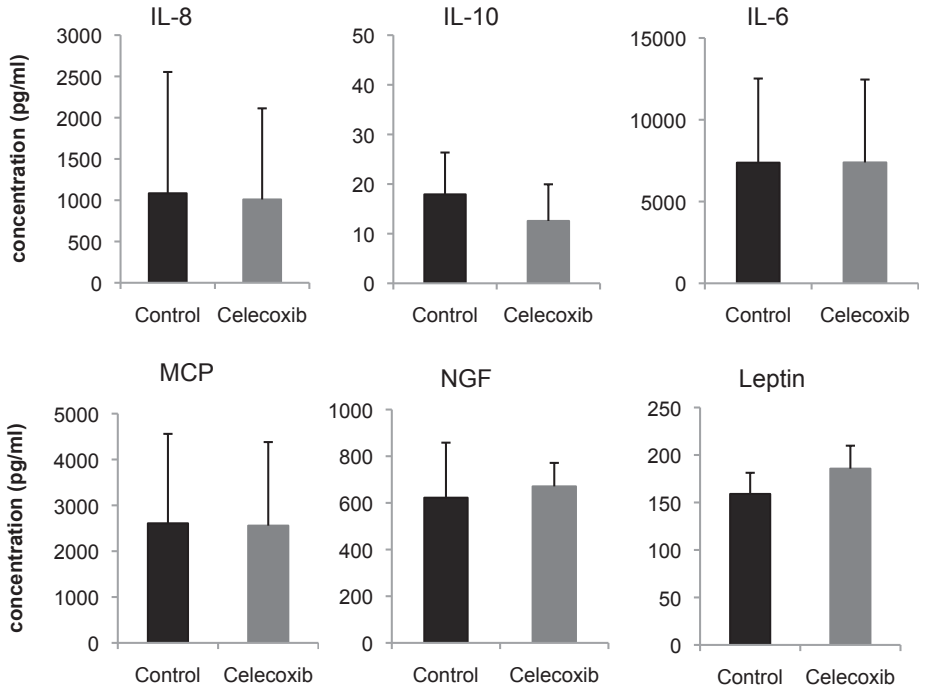


Figure 6. Production of mediators by OA chondrocytes. OA chondrocytes produced IL-6, IL-8, IL-10, MCP-1, NGF and leptin at day 7 of regeneration culture. There was no production of IL-1α, IL-1β, IL-4, IL-13, OSM and adiponectin by the OA chondrocytes (not shown). Celecoxib had no effect on the production of the produced mediators (mean +/- SD of 3 donors, 4 constructs per condition; no *post hoc* correction for multiple testing).

of celecoxib failed to affect GAG content (Figures 4C and 5C), GAG release (Figures 4D and 5D) or DNA (data not shown) of chondrocytes in regeneration culture.

Summarizing, celecoxib had no effect on cartilage matrix turnover in osteoarthritic cartilage explants nor in isolated chondrocytes, although in isolated chondrocytes PGE2 production was only partially inhibited and other inflammatory mediators were unaffected.

DISCUSSION

In the current study, celecoxib was found not to affect cartilage repair in osteoarthritic explants or a co-culture of osteoarthritic synovial tissue with osteoarthritic cartilage, despite the clearly decreased secretion of many inflammatory mediators including PGE_2. Inflammation during tissue regeneration culture by chondrocytes was mostly COX-2 independent and celecoxib did not affect cartilage regeneration.

In previous studies where cartilage explants were cultured in the presence of a pro-inflammatory stimulus, e.g. IL-1 or tumor necrosis factor (TNF), celecoxib did inhibit the induced degeneration[320]. Recently, we showed that factors produced by OA synovial tissue can decrease cartilage matrix content. However, this effect consisted of a decrease in matrix production rather than an increase of degradation[62], in apparent contrast to the abovementioned cytokine-induced degeneration models. The lack of effect on cartilage metabolism could be related to the type of OA tissues studied. In this study, cartilage and synovial tissue were obtained from patients with end-stage OA undergoing total knee replacement. This 'late' OA tissue may have a less 'inflammatory' character than early OA tissue[60, 321]. COX-2 expression was found to be significantly lower in synovial tissue obtained from joints with greater OA severity than with lower OA severity[322]. Furthermore, levels of inflammatory mediators secreted by the synovium in end-stage osteoarthritis were not different to those from healthy synovium and less responsive to stimulation with IL-1α, indicating that in end-stage OA inflammation may play a subordinate role[323]. Another explanation for the lack of effect of COX-2 inhibition on cartilage repair may be a concomitant decrease in anti-inflammatory factors, such as IL-10, which may have counteracted the effects of inhibiting inflammatory mediator production. Moreover, inhibition of the COX-2 enzyme will not only inhibit the production of PGE_2, but also of other prostaglandin mediators, e.g. $PGF_{2\alpha}$, PGD_2 and thromboxane. Where PGE_2 has been associated with cartilage destruction in a cartilage explant model[324], other prostaglandins have been associated with enhanced cartilage production. In particular PGD_2 and $PGF_{2\alpha}$ have been shown to increase collagen type II synthesis and aggrecan synthesis[325, 326].

Alternatively, the repressive effects of inflammatory mediators on cartilage matrix production may be somewhat overestimated. We recently showed that IL-6, a classic inflammatory cytokine, in fact modestly stimulated cartilage matrix production during tissue regeneration culture and its inhibition in OA synovial fluid cocultured with OA explants decreased GAG content[143]. In addition, despite the increase production of PGE_2 and other inflammatory mediators, GAG production by osteoarthritic chondrocytes is in fact increased[173]. Recent evidence suggests inflammation may, in addition to degeneration, also have a role in regeneration[327]. Transient activation of NF-κB was shown to facilitate chondrogenesis through the induction of SOX9[83, 328, 329]. Our data also supports a role of inflammation on regeneration, as high concentrations of inflammatory mediators were found to be released during tissue regeneration culture by isolated chondrocytes. However, contrary to the production of inflammatory mediators by osteoarthritic cartilage explants, production by chondrocytes during regeneration culture was not COX-2 dependent. Various other mechanisms are involved in the production of inflammatory cytokines, including NFκB activation and STATs pathway, which were not studied here[330].

Also PGE_2 production in regeneration culture was only partially decreased by COX-2 inhibition, possible indicating involvement of COX-1. This may implicate that strategies to improve cartilage regeneration by cell-based procedures such as autologous chondrocyte implantation, may require a very different approach than strategies to develop disease-modifying treatments for OA.

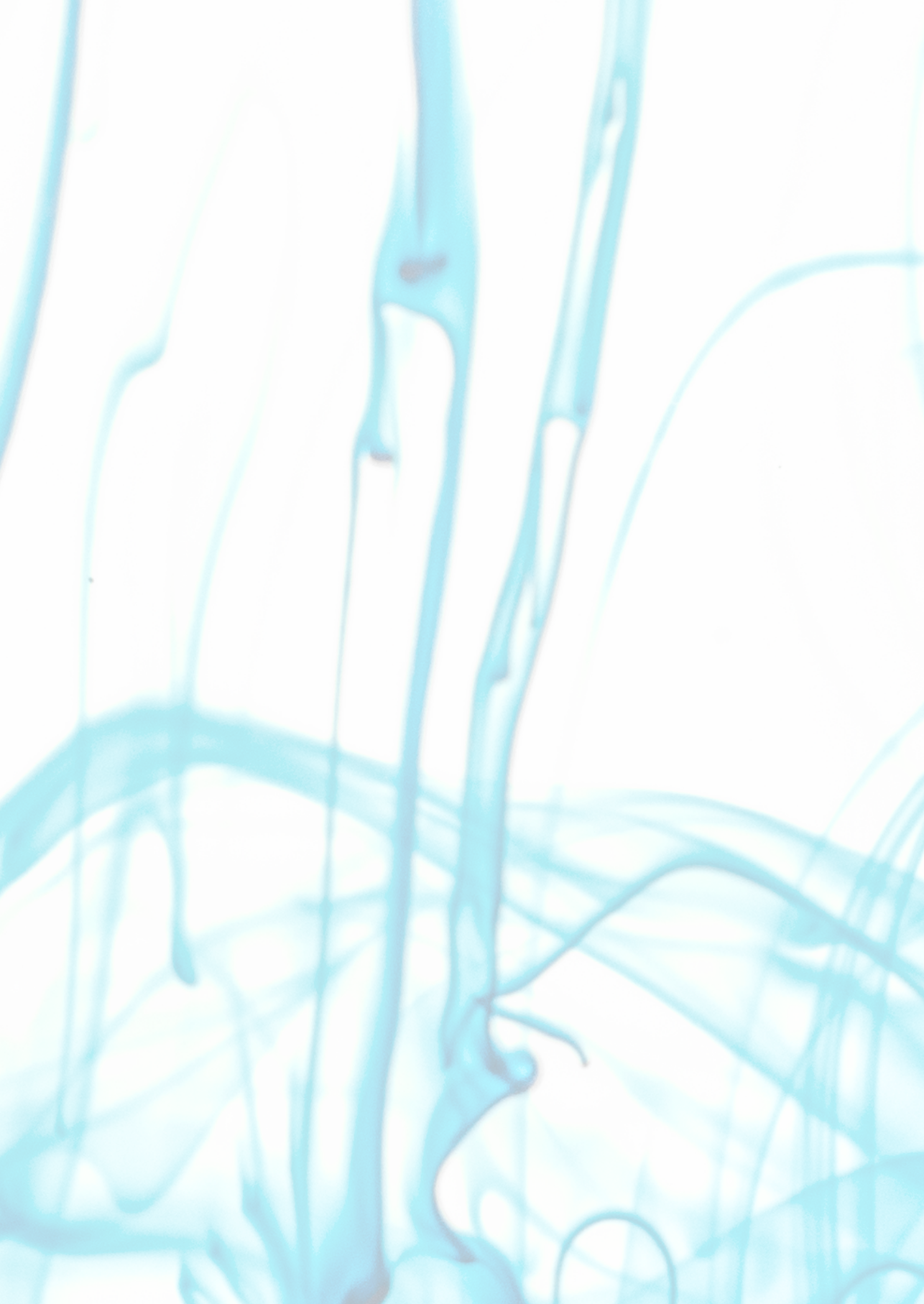
Celecoxib has been suggested to have both COX-dependent and independent potential as a disease-modifying drug[311, 331], but we found no decrease in inflammatory mediators and no effect on matrix production in the regeneration cultures. Although we found no effects of celecoxib on GAG content, we cannot exclude an effect on collagen synthesis. However, as this would be expected to lead to higher proteoglycan retention and hence content in the tissue[332], this probably did not occur. Another possible effect on neocartilage tissue we did not study, is the inhibition of chondrocyte hypertrophy found previously[315]. However, these effects were noted in a different *in vitro* model using prehypertrophic cells, whereas in the current regeneration model collagen X protein was shown previously to be undetectable[236].

Our study is the first to evaluate the effect of celecoxib on inflammation measuring a broad panel of cytokines, chemokines and growth factors produced by osteoarthritic cartilage and synovial tissue. In previous studies only IL-6 and IL-8 production were evaluated[314]. Although addition of celecoxib resulted in a clear reduction of inflammatory mediators, such as PGE_2, IL-6, NGF and IL-1 in the co-culture model, it did not alter GAG production or GAG release. Synovial inflammation and the produced inflammatory mediators are associated with pain sensation in OA[333, 334] and this could explain the pain reduction achieved by treatment with celecoxib[335]. When looking at all the donors separately for GAG metabolism, clear differences in the response to celecoxib between

individual donors could be observed. Although the number of donors used for the current study is too low to draw any conclusion, we cannot exclude the possibility that patients may be divided in responder and non-responder categories. This could also explain why in multiple studies using OA cartilage and chondrocytes in *in vitro* models, differential effects on cartilage metabolism were seen.

CONCLUSIONS AND FUTURE PERSPECTIVES

To study the effect of inhibition of inflammation on cartilage, we have used a variety of different long-term culture models with human tissues and cells in the presence of the COX-2 inhibitor celecoxib. So far no studies compared multiple culture models, focusing on cartilage repair in cartilage explants and regeneration in chondrocytes. We found indication that inflammation is regulated differently in osteoarthritic tissues and cells. A true potential of celecoxib as a disease modifying osteoarthritic drug did not emerge from this study, although of course its application as pain treatment is still important.



Chapter 8

Summary and general discussion

CASE FOLLOW-UP

Following surgical treatment of the cartilage defect of the medial femoral condyle by matrix-assisted autologous chondrocyte implantation, the patient, as described in the introduction at the beginning of this thesis, followed a stepwise, phased rehabilitation protocol, guided by a trained physical therapist. Individualized rehabilitation, with criteria-based rather than time-based progression to the next phase of rehabilitation, is very important for the success of cartilage repair surgery. In the first several weeks after surgery, no weight bearing is allowed in order to protect the implant with the cells. In this stage of rehabilitation, focus lies on regaining neuromuscular control of, in particular, the quadriceps function, isometric strength training and restoring full range of motion. In the weeks thereafter, weight bearing is gradually increased until full weight bearing is achieved at approximately week 6-12. After that, intensity and impact of the exercises are gradually increased and propriocepsis is improved. After about six to nine months, sport-specific and on-field training can commence. All this time, pain and joint effusion are important parameters to monitor. Guided physical therapy is usually ceased after a year, but exercising the knee remains important as the repair tissue likely remodels during the subsequent years.

For an orthopedic surgeon treating patients with symptomatic cartilage defects, it is very important to prepare the patient for this extensive rehabilitation period, properly managing the patient's expectations. Depending on the patient's profession, working might not be possible for at least several months and retraining may be wise. Furthermore, return to sports takes about a year on average, and is influenced by factors such as age psychosocial factors and pre-injury activity level. Good teamwork with the physical therapist, to educate and encourage the patient, is vital to the success of cartilage repair procedures.

One year after autologous chondrocyte implantation, the patient was almost completely pain free when performing normal activities of daily living and sports activities such as jogging. MRI showed good filling of the defect. However, he did not return to playing soccer out of fear of re-injury.

Regeneration rather than repair of articular cartilage, is the holy grail in the treatment of focal cartilage defects because it has been suggested that treatment failures may be related to a more fibro-cartilaginous repair tissue[110, 336, 337]. Furthermore, timely and adequate treatment of focal cartilage defects may also prevent the progression to osteoarthritis, a disease that poses a particularly large socioeconomic burden to society[338], and so far knows no disease-modifying treatment.

ACI is the first cell-based treatment that holds the potential of truly regenerating rather than repairing the articular cartilage surface. Pioneer studies investigating the

nature of the repair tissue obtained with ACI, discovered that rather than producing a fibrous-like scar tissue, a hyaline-like cartilage repair tissue is formed[18, 339]. This repair tissue was observed to be increasingly hyaline-like with increasing time after the initial surgery, indicating that the tissue continues to mature for at least several years after treatment[337]. Moreover, the repair tissue was shown to be subject to enzymatic activity and undergoing remodeling[340]. This has led to the hypothesis that joint homeostasis influences cartilage repair and that given the right circumstances, true regeneration of articular cartilage is an attainable goal. Anatomical and functional regeneration of cartilage, fully restoring form and function, will require an understanding of the complexity of joint homeostasis and application of an integrative approach that addresses various factors in the knee joint environment, including the soluble mediators affecting chondrocytes.

The aim of this thesis is to improve the outcomes of cartilage regeneration, by identifying the best surgical treatment for a specific patient population, and by finding and targeting those factors that are instrumental to cartilage regeneration, with a particular focus on soluble mediators.

IDENTIFYING THE BEST SURGICAL TREATMENT

Several surgical techniques are available for the treatment of patients with symptomatic cartilage defects. Taking the population with symptomatic cartilage defects as a whole, the patient-reported outcome scores after ACI are good and there are no great statistical differences between the various cartilage repair techniques in randomized comparison[13, 34, 35, 123, 341]. However, specific patient populations may benefit more from ACI than from microfracture, while osteochondral solutions may be preferable for others. For example, characterized chondrocyte implantation (CCI) was significantly better than microfracture in patients with defects that existed less than 3 years, but not in defects that existed for over 3 years[34]. Duration of symptoms was previously shown to also be a predictor of clinical outcome in patients treated with MACI[342]. We showed that also in larger defects (> 4 cm^2) MACI leads to significantly better clinical outcome than microfracture (*Chapter 2*). The reason for this has not been definitely established, but we speculate that the limited biomechanical challenge posed by the inferior repair tissue obtained with microfracture in a small area can be compensated by the healthy cartilage surrounding it, but this mechanism fails when the surface area is too large or is in a particularly demanding location such as the patellofemoral area[343].

Many other patient-related[11, 344-347] and defect-related[11, 113, 344, 345] predictors of outcome after cartilage repair have been identified, such as patient age, the etiology of the cartilage defect or the increased inflammatory status in obesity. The increased presence

of inflammatory mediators has been linked to the increased risk of complicated wound healing[348] but may also negatively influence cell-based cartilage therapy. Although not compared to other cartilage repair procedures, clinical outcomes were severely compromised in obese patients two years after ACI[346]. Patient age and the etiology of the defect are also likely to give rise to biological changes in the joint and a different profile of soluble mediators regulating tissue turnover.

Unfortunately, very few of these predictors have been evaluated in relation to a specific cartilage repair procedure. This is because generally, studies were insufficiently powered to allow subgroup analysis. Evidence-based, selective indication for cell-based therapy is important, because accurate prediction of clinical outcome after ACI on an individual level remains challenging. Furthermore, strict indications based on proven clinical merit for these types of expensive treatments are necessary to keep health care affordable and thereby accessible. To this end, randomized controlled clinical trials with sufficient numbers of patients to allow subgroup analyses are necessary.

Additionally to patient-reported clinical outcomes, evaluation of the cartilage repair tissue by MRI and histology are important in the evaluation of cartilage repair procedures. Interestingly, we observed no differences in MRI and/or histological outcomes of MACI versus microfracture at two years follow-up (*Chapter 2*). MRI and histological analysis may not have been sensitive enough to detect differences; additional biochemical evaluation of the repair tissue might be. Furthermore, two years follow-up may have been insufficient as the failures with microfracture usually occur after two years[110] and the remodeling of the repair tissue is notoriously slow[349]. Therefore, structural assessment in combination with clinical outcomes will remain essential, also in the long-term evaluation of new autologous chondrocyte implantation products and procedures.

The execution of well-designed, sufficiently powered randomized controlled clinical trials prior to widespread use in patients will result in high-quality evidence regarding the optimal surgical treatment of focal cartilage lesions in specific patient populations that is lacking to date. However, the technovolution[87] of ACI in the past 20 years may be decelerated because the application for market registration is a costly and timely procedure that is difficult to realize without commercial funding. This would be unfortunate since the technique still has various limitations that could be improved, such as logistics, costs, and particularly the quality of the repair tissue.

IMPROVING THE CARTILAGE REPAIR TISSUE BY ADDRESSING CHONDROCYTE PHENOTYPE

To date, the repair tissue formed after ACI is reported to be hyaline or hyaline-like in variable percentages ranging from 27 to 73%[18, 36, 337, 350-354]. It must be noted that these

are very heterogeneous studies including various versions of ACI, different histological scoring systems, biopsies at various lengths of follow-up and quite a few of these studies are biased by limited numbers of patients that were willing to undergo a second-look arthroscopy with biopsy. Nonetheless, two more recent studies evaluating repair tissue after MACI and CCI, both using the ICRS II scoring system, showed that the mean overall histology score was below 60 (out of 100) for both cell therapies[14, 37]. Even the more hyaline-like repair tissue is different from native cartilage in several aspects: it lacks the layered structure, contains type I collagen normally absent, and the type II collagen fibers are organized randomly rather than in arches[350, 355]. The lack of collagen organization and lower GAG content are likely to contribute to the observed lower mechanical properties of the repair tissue[350, 356, 357]. Additionally to decreased amounts of GAGs, the types of GAGs present in the repair tissue were also altered. The amounts of chondroitin sulphate (CS) and keratan sulphate (KS) chains were shown to be reduced two-fold, while the amount of HA was markedly increased[357]. Repair tissue was also shown to contain much higher amounts of procollagen IIA[354]. Procollagen IIA is produced by chondroprogenitor cells and normally is not present in adult articular cartilage, but it is re-expressed in the middle layers of OA cartilage[358]. Both the production of procollagen IIA and aberrant GAGs, have been suggested to be caused by an altered chondrocyte phenotype[354, 357, 358]. For example, monolayer expansion has been shown to reduce the production of KS chains by chondrocytes[359].

Classical ACI is likely to be hampered in its ability to result in true regeneration of cartilage tissue because it is performed with culture-expanded and therefore dedifferentiated[39, 40] autologous chondrocytes. In addition, implanting cells with a high chondrogenic phenotype is essential because it has been shown to result in better clinical outcome[14, 360, 361]. Addressing chondrocyte phenotype will be particularly important when considering alternative sources of chondrocytes for cell-based regenerative procedures. Alternative sources of chondrocytes, such as those obtained from the rims of the defect during the debridement as standard part of the procedure, will reduce the damage inflicted by ACI. However, before these cells can be used for ACI, more insight into their behavior in the sense of their secretory profile and regenerative potential in comparison to healthy chondrocytes is necessary. This may be a first step in also exploring cell-based therapies for OA, which are considered unrealistic as of yet.

Clinical results of ACI in osteoarthritic patients are inferior to those with focal lesions[147]. Although there are many aspects in which OA chondrocytes are different from healthy chondrocytes, the osteoarthritic joint environment may negatively affect the final outcome to a greater extent than the actual regenerative capacity of OA chondrocytes. We showed that GAG production is in fact higher by OA chondrocytes and chondrocytes from cartilage debrided from defects than healthy chondrocytes (*Chapter 3*). The reason for this phenomenon is not clear, but the observation is almost as old as

OA cartilage research[173] itself and has recently received renewed attention[362, 363]. From a clinical point of view the possibility of using OA chondrocytes is attractive, as there are many more patients with OA than there are with focal defects, and so far no disease-modifying treatments exist. Although speculative, this may be due to the removal of these cells from a hostile joint environment.

SOLUBLE MEDIATORS IN THE JOINT ENVIRONMENT

In addition to chondrocyte phenotype, the joint environment in which the cells are reimplanted is very important to the success of cartilage regeneration. In particular soluble mediators present in joint space are likely to have a profound effect on chondrogenesis and repair. These factors, derived from several joint tissues, are found in the synovial fluid of the patient with cartilage pathology, but can also be produced by the chondrocytes themselves in response to various stimuli.

Synovial fluid mediators

Soluble factors produced by subchondral bone, (inflamed) synovial tissue, (damaged) menisci and ligaments and cartilage itself, are released into the synovial fluid, which is in direct contact with all of the knee structures.

The composition of the synovial fluid greatly affects cartilage regeneration. Both osteoarthritic synovial fluid and synovial fluid of injured joints has been shown to inhibit cartilage regeneration *in vitro*[81, 82]. Also *in vivo*, synovial fluid levels of MMP-3 and IGF levels were still elevated compared to controls one year after ACI[364], indicating ongoing remodeling and possibly explaining the observed insufficient quality of repair tissue. It is therefore important to characterize the synovial fluid of patients with symptomatic cartilage defects and identify which factors negatively affect cartilage regeneration. Although several inflammatory mediators have been identified in the synovial fluid of patients with OA and joint trauma, it is not known whether these same mediators are also present in the synovial fluid of patients with symptomatic cartilage defects. Not all symptomatic cartilage defects may be caused by joint trauma, as only about half of all patients can relate the onset of their symptoms to previous knee trauma[1, 2, 88]. Particularly in those patients, other soluble mediators may be present. Incidentally, the presence of a few factors in synovial fluid of patients undergoing cartilage repair procedures has been described[365, 366], but direct comparisons to healthy and OA synovial fluid were lacking until now. We indeed confirmed the existence of a distinct cytokine profile in patients with symptomatic cartilage defects characterized by elevated levels of several pro-inflammatory and pro-repair cytokines (*Chapter 3*).

Cytokines and growth factors can bind to components of the extracellular matrix[137, 367], thereby creating local concentrations. A novel finding of this thesis is that in fact concentrations of several inflammatory cytokines are distinctly higher in cartilage tissue than in a similar volume synovial fluid, in particular in cartilage debrided from defects and OA cartilage. This may indicate that the role of specific cytokines may be greater than can be deduced from their presence in synovial fluid alone.

Endogenous production of soluble mediators by chondrocytes

Soluble mediators such as cytokines and growth factors can also regulate the chondrocyte's own behavior in an auto- and paracrine manner. Endogenous production of cytokines can be induced by several stimuli. Chondrocytes reimplanted into a focal defect are particularly vulnerable to mechanical stresses as well as biochemical stimuli due to the lack of an extracellular matrix surrounding them. Chondrocytes possess membrane proteins such as integrins, G-proteins and strain sensitive ion channels, which translate mechanical forces into biological signals. Activation of these mechanoreceptors leads to intracellular signaling, resulting in activation of transcription factors and altered gene expression[368]. Subsequently, matrix synthesis can be increased[369], but excessive mechanical loading can induce catabolic pathways and is thought to contribute to OA pathogenesis. Also the production of inflammatory mediators, such as IL-6, CXCL8 (IL-8) and COX-2 is mechanosensitive[287, 370, 371]. Additionally, damage to the intact cartilage surface was shown to cause release of mediators normally immobilized in the extracellular matrix[367] and induce various inflammatory pathways, including the mitogen activated protein kinases, Src kinases and NF-kB[372].

Chondrocytes were also found to produce particularly high concentrations of soluble mediators by mere isolation and culture. Whether this is caused by a removal from their native matrix or is an artificial culture-induced effect that would disappear upon implantation *in vivo* is unknown. In addition, in *Chapter 4,* we show that this production may be modulated by the biomaterial used as a carrier matrix for ACI. Biomaterials from natural polymers can bind to integrins and other specific cell receptors, thereby activating downstream signaling pathways and transcription factors and increasing cytokine release[43, 183, 203]. For example, collagen-based biomaterials may interact with DDR2 and activate various downstream pathways such as mitogen-activated protein kinase, Janus kinase, protein kinase C and NF-κB pathways[183, 373]. In chondrocytes, a DDR2-dependent increased release of IL-6 has been reported[203]. In other cell types, many more cytokines such as IL-12, TNFα, IFNγ, and TGFβ are regulated by DDR2 binding[374, 375] and it is likely that also in chondrocytes more cytokines are regulated by DDR2. Using the same type II collagen-coated filter model as described in *Chapters 3, 4, 5 and 6*, it was previously reported that DDR2 and its downstream target MMP-13 were not induced at day 28 after culture[236]. It was suggested that the 2D nature of the system limited contact of the cells

with collagen and the effects may have been more pronounced in a 3D collagen environment. We indeed observed a high release by chondrocytes encapsulated in a type II collagen gel of most mediators measured, but only when non-expanded cells were employed. In fact, when culture-expanded cells were incorporated into the collagen gel, the release of most mediators was low, in line with the reduction in cytokine production we found in regeneration culture (*Chapter 3*). Expansion of cells alters the expression of cell surface receptors[190] which may explain the altered chondrocyte behavior, including cytokine release. Altered receptor expression may also explain why unpassaged chondrocytes showed a much more versatile cytokine release in response to the different biomaterials. Likewise, there were distinct differences in regeneration potential between unexpanded chondrocytes from donors without joint pathology, with symptomatic defects and OA, but not in regeneration potential by culture expanded chondrocytes (*Chapter 3*). However, both in biomaterial-based and filter-based regeneration, we were unable to pinpoint specific cytokines associated with regeneration. The observation that the secretory profile of non-expanded chondrocytes was hardly different between healthy, defect and OA chondrocytes further suggests that defect and OA cells may be equally suitable to regenerate cartilage (*Chapter 3*). However, the increased secretion of senescence-associated cytokines after expansion by these two cell types suggests that the same may not be true when chondrocytes are expanded prior to reimplantation.

Interestingly, the postulated added value of using differentiated, unpassaged, cells was not as outspoken in all of the biomaterials tested as it is in redifferentiation models such as filter or pellet cultures (*see also Chapter 3*). In particular, in fibrin-based hydrogels, cartilage matrix production by expanded cells was not necessarily inferior to that by non-expanded cells. Fibrin-based hydrogels are known to be beneficial to the chondrogenic differentiation of MSCs[376, 377], likely due to their ability to bind endogenously produced growth factors such as TGF[376, 378, 379]. A similar mechanism may also be responsible for the observed enhanced matrix production by expanded chondrocytes.

The ability of specific biomaterials to bind certain cytokines or growth factors is attractive because it can be used both for controlled delivery of incorporated growth factors as well as binding of endogenously produced growth factors thereby enhancing cartilage formation[380]. In contrast, the ability of a biomaterial to shield the implanted chondrocytes from the inflammatory signals from the environment may also have advantages[381]. The ideal biomaterial should regulate soluble signaling in such a way that the differentiating chondrocytes are shielded from the catabolic cues from the host environment and endogenous production of anabolic cues is stimulated and immobilized in the biomaterial in a biomimetic manner to optimize regeneration.

MODULATING SOLUBLE MEDIATORS ACTIVITY TO ENHANCE REGENERATION

Deciphering the role of each of these factors in cartilage repair and regeneration has proven not to be easy. The experiments performed in *Chapters 4 and 5* prove that up-regulation of an inflammatory cytokine does not necessarily implicate a causal relation to increased cartilage degeneration or impaired regeneration. IL-6, an inflammatory cytokine often suggested to be instrumental to cartilage degeneration, and found in increased concentrations in the synovial fluid of patients with symptomatic cartilage defects and OA, even had a mild stimulatory effect on *in vitro* regeneration (*Chapter 4*). Furthermore, inhibition of IL-6 in OA synovial fluid added to OA explant culture showed a trend towards decreased glycosaminoglycan content of the explants. This is in sharp contrast to the known catabolic role of IL-6 in rheumatoid arthritis[135]. Also, a more general reduction of inflammation with a small inhibitory molecule such as celecoxib, a COX-2 inhibitor, did not enhance cartilage matrix production despite reducing the release of several inflammatory cytokines (*Chapter 6*). The findings of *Chapters 4, 5 and 6* question the well-established dogma that reducing inflammation is beneficial to joint integrity. It seems that at least regeneration is not enhanced by a reduction of inflammation and even the opposite may be true. Many, if not all repair processes in the human body start with an inflammatory phase, and such observations are in line with work by others that suggest that inflammation may be necessary for regeneration as well. Several authors have shown that early, transient NF-κB activation is essential to chondrogenesis by inducing SOX9[83, 328, 329]. Furthermore, recent work evaluating pathways involved in embryonic development of joints and articular cartilage identified many genes associated with various inflammatory pathways to be upregulated[382]. Our work indicates that there are distinct differences in the way inflammatory mediators are regulated between chondrocytes in cartilage tissue and isolated chondrocytes in a tissue regeneration culture. In general, cytokine release was massively increased in regeneration cultures compared to cartilage tissue. Several inflammatory cytokines that were not present in the cartilage were induced during regeneration culture. Furthermore, differences in cytokine levels between healthy cartilage, cartilage debrided from defects and OA cartilage were not the same in tissue and regeneration culture. The production of inflammatory mediators by OA cartilage explants and OA cells in regeneration culture was also differentially regulated. The production of inflammatory mediators was COX-2 dependent in the former, but not in the latter (*Chapter 6*). This different mechanism regulating the production of soluble mediators during regeneration culture may also explain why the profile of these mediators was not related to cartilage matrix formation (*Chapters 3 and 4*). However, it is also possible that the role of soluble mediators in regeneration tends to be overstated.

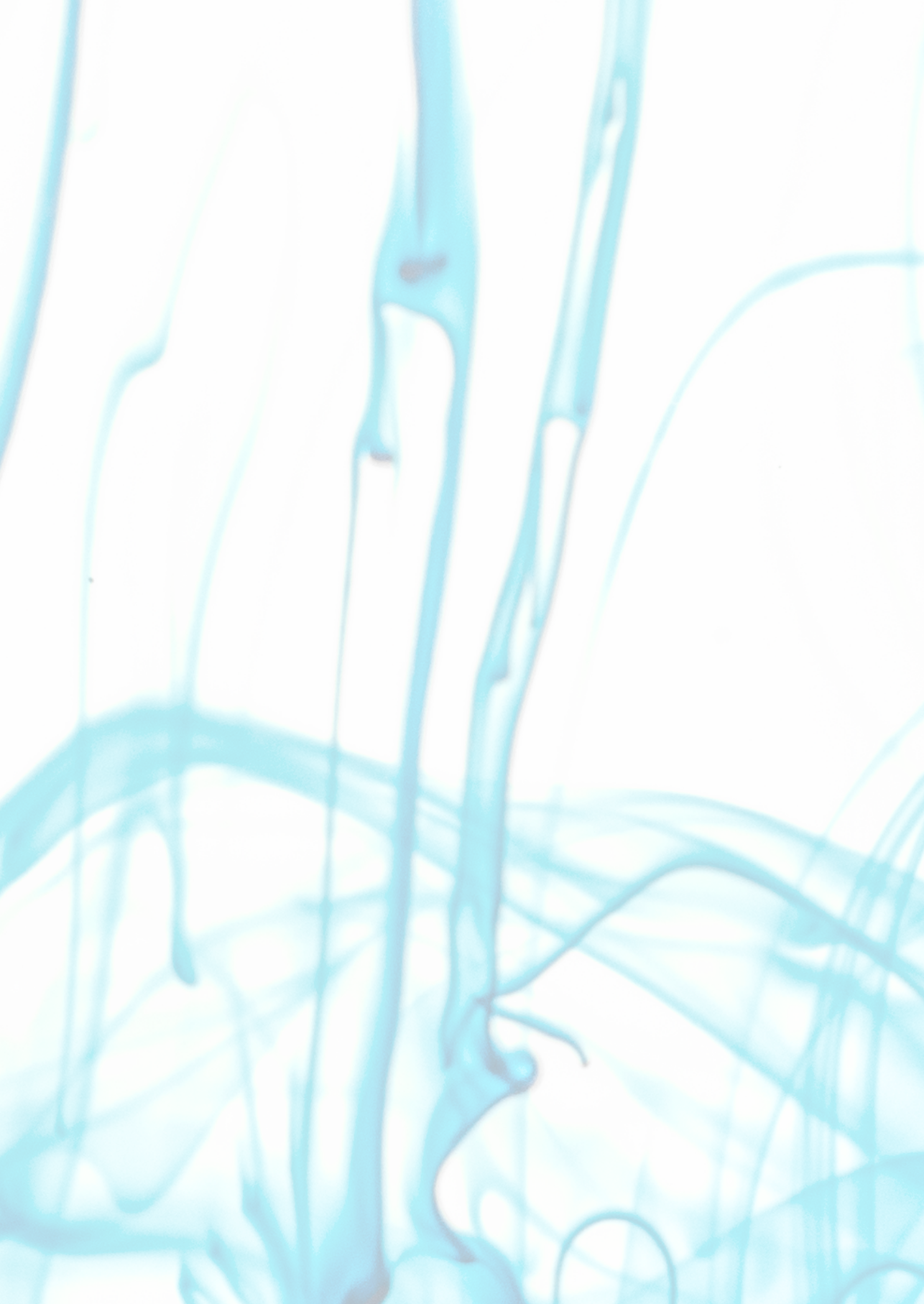
Cytokines may have various effects depending on the knee joint environment and context. The knee joint environment comprises many factors interacting with each other

in a complex system. Cytokines are well known to stimulate each other's production. In particular, IL-1β is known to induce many other cytokines and *in vivo* inhibition of IL-1β reduced the production of many inflammatory mediators[76]. Soluble mediators may act synergistically but may also counteract each other. For example, different combinations of IGF-I, TGFβ-1, FGF-2, BMP-2 and BMP-7 can, depending on the combination, either enhance or reduce cell proliferation and matrix production[383]. TGFβ and IL-1β, two key players in cartilage anabolism and catabolism respectively, are known for their elaborate interplay[384, 385]. Also, IGF-I can counteract the effects of IL-1β[386]. Redundancy in the system may explain why interference with one factor only has a limited overall effect. In addition, by modulating an inflammatory factor, the concurrent changes found in the levels of anti-inflammatory factors may lead to no net effect in regenerative pathways. Furthermore, differences in cell phenotype may entail a differential response to biochemical cues. For example, CXCL8 has been shown to induce hypertrophic differentiation in monolayer-expanded chondrocytes under non-adherent conditions, but this was not observed in chondrocytes exposed to regenerative stimuli (*Chapter 5*). Also, it has been suggested that OA chondrocytes are less responsive to inflammatory stimuli[243, 387]. Exploring whether in symptomatic cartilage defects and OA, the same cytokines and mechanisms are responsible for the regulation of enzyme systems that regulate cartilage turnover, will be very important. The roles of inflammatory mediators in joint disease and cartilage integrity can vary extensively depending on the knee joint environment and disease. This is illustrated by the overt discrepancy in the obtained success by treatment with biologicals between rheumatoid arthritis and osteoarthritis[388]. Also, in our own data we clearly observed that modulating IL-6 and CXCL8 signaling had differential effects on chondrocytes from healthy, debrided defect and OA cartilage.

Further investigation, for example through micro-array analysis of cartilage debrided from defects, may reveal whether similar pathways are active in this cartilage as in OA cartilage or perhaps are more similar to cartilage disrupted by trauma. Furthermore, more attention to the other tissues of the knee joint and their contribution and response to soluble mediators will be important. A specific set of genes in degenerated menisci was correlated to chondral defects[389]. Similarly, the role of synovial inflammation needs to be further elucidated as it is often present in patients with symptomatic cartilage defects and is instrumental in the pathogenesis of OA[58]. The success of cartilage regeneration may also largely depend on bone-specific factors[390]. Ultimately, to improve cartilage regeneration, especially in challenging conditions such as with the use of non-healthy cells or severely disturbed joint homeostasis, at least several mediators need to be investigated for the effect on regeneration, not limited to those present in synovial fluid but also targeting endogenous production by chondrocytes and addressing the chondrocyte microenvironment. High-throughput technologies would be of great value here, at least for initial screening purposes.

CONCLUSIONS

Regenerative cartilage therapy meets a previously unmet medical need in current clinical care. Autologous chondrocyte implantation results in significantly better clinical outcome than microfracture in patients with defects > 4 cm^2 at two years follow-up. This information is of great value for the evidence-based, selective indication of ACI. In addition to the need to select patients most likely to benefit from cell-based therapies, there still is an unmet medical need to improve this treatment. The synovial fluid of patients with symptomatic cartilage defects and OA are each characterized by a distinct profile of soluble mediators, in which several pro-inflammatory but also pro-repair cytokines are elevated compared to donors without joint pathology that may both affect cartilage regeneration. Despite the increased presence of inflammatory mediators, inhibition of individual inflammatory mediators or enzymes did not have large effects on cartilage regeneration, possibly due to counteracting factors. It is therefore not easy to identify which factors are contributing most to the effects on cartilage regeneration. In order to improve cell-based regenerative therapies, modulation of at least several soluble mediators will likely be needed. We here describe chondrocytes to be potent producers of cytokines that, in addition to the other tissues of the knee joint, contribute greatly to the presence of these soluble factors in the synovial fluid. Additionally, local concentrations of cytokines in cartilage tissue were sometimes many times higher than in a similar volume of synovial fluid. Therefore, locally acting soluble mediators may be very different from those in the synovial fluid and affect chondrocytes in an auto- or paracrine manner. To improve the quality of repair tissue and clinical outcome, an integrative approach addressing multiple soluble mediators, not limited to those present in the synovial fluid but also targeting endogenous production by chondrocytes and addressing the chondrocyte micro-environment will be necessary.



Addendum

REFERENCES

1. Hjelle K, Solheim E, Strand T, Muri R, Brittberg M. Articular cartilage defects in 1,000 knee arthroscopies. *Arthroscopy.* Sep 2002;18(7):730-734.
2. Widuchowski W, Widuchowski J, Trzaska T. Articular cartilage defects: study of 25,124 knee arthroscopies. *Knee.* Jun 2007;14(3):177-182.
3. Slauterbeck JR, Kousa P, Clifton BC, et al. Geographic mapping of meniscus and cartilage lesions associated with anterior cruciate ligament injuries. *J Bone Joint Surg Am.* Sep 2009;91(9):2094-2103.
4. Maffulli N, Binfield PM, King JB. Articular cartilage lesions in the symptomatic anterior cruciate ligament-deficient knee. *Arthroscopy.* Sep 2003;19(7):685-690.
5. Bekkers JEJ, Tsuchida AI, Saris DBF. *Cartilage Injury in Evidence Based Orthopedics*. Oxford, UK: Wiley Blackford; 2011.
6. Campbell AB, Knopp MV, Kolovich GP, et al. Preoperative MRI underestimates articular cartilage defect size compared with findings at arthroscopic knee surgery. *Am J Sports Med.* Mar 2013;41(3):590-595.
7. Outerbridge RE. The etiology of chondromalacia patellae. 1961. *Clin Orthop Relat Res.* Aug 2001(389):5-8.
8. Cibere J, Sayre EC, Guermazi A, et al. Natural history of cartilage damage and osteoarthritis progression on magnetic resonance imaging in a population-based cohort with knee pain. *Osteoarthritis Cartilage.* Jun 2011;19(6):683-688.
9. Carnes J, Stannus O, Cicuttini F, Ding C, Jones G. Knee cartilage defects in a sample of older adults: natural history, clinical significance and factors influencing change over 2.9 years. *Osteoarthritis Cartilage.* Dec 2012;20(12):1541-1547.
10. Loken S, Heir S, Holme I, Engebretsen L, Aroen A. 6-year follow-up of 84 patients with cartilage defects in the knee. Knee scores improved but recovery was incomplete. *Acta Orthop.* Oct 2010;81(5):611-618.
11. Bekkers JE, Inklaar M, Saris DB. Treatment selection in articular cartilage lesions of the knee: a systematic review. *Am J Sports Med.* Nov 2009;37 Suppl 1:148S-155S.
12. Hanifi A, Richardson JB, Kuiper JH, Roberts S, Pleshko N. Clinical outcome of autologous chondrocyte implantation is correlated with infrared spectroscopic imaging-derived parameters. *Osteoarthritis Cartilage.* Sep 2012;20(9):988-996.
13. Knutsen G, Drogset JO, Engebretsen L, et al. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *J Bone Joint Surg Am.* Oct 2007;89(10):2105-2112.
14. Saris DB, Vanlauwe J, Victor J, et al. Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. *Am J Sports Med.* Feb 2008;36(2):235-246.
15. Gikas PD, Bayliss L, Bentley G, Briggs TW. An overview of autologous chondrocyte implantation. *J Bone Joint Surg Br.* Aug 2009;91(8):997-1006.
16. Steadman JR, Rodkey WG, Rodrigo JJ. Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clin Orthop Relat Res.* Oct 2001(391 Suppl):S362-369.

17. Hangody L, Kish G, Karpati Z, Udvarhelyi I, Szigeti I, Bely M. Mosaicplasty for the treatment of articular cartilage defects: application in clinical practice. *Orthopedics.* Jul 1998;21(7):751-756.

18. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med.* Oct 6 1994;331(14):889-895.

19. Williams RJ, 3rd, Harnly HW. Microfracture: indications, technique, and results. *Instr Course Lect.* 2007;56:419-428.

20. Mithoefer K, McAdams T, Williams RJ, Kreuz PC, Mandelbaum BR. Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: an evidence-based systematic analysis. *Am J Sports Med.* Oct 2009;37(10):2053-2063.

21. Hangody L, Fules P. Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: ten years of experimental and clinical experience. *J Bone Joint Surg Am*. 2003;85-A Suppl 2:25-32.

22. Schneider U, Rackwitz L, Andereya S, et al. A prospective multicenter study on the outcome of type I collagen hydrogel-based autologous chondrocyte implantation (CaReS) for the repair of articular cartilage defects in the knee. *Am J Sports Med.* Dec 2011;39(12):2558-2565.

23. Moradi B, Schonit E, Nierhoff C, et al. First-generation autologous chondrocyte implantation in patients with cartilage defects of the knee: 7 to 14 years' clinical and magnetic resonance imaging follow-up evaluation. *Arthroscopy.* Dec 2012;28(12):1851-1861.

24. Filardo G, Kon E, Di Martino A, Iacono F, Marcacci M. Arthroscopic second-generation autologous chondrocyte implantation: a prospective 7-year follow-up study. *Am J Sports Med.* Oct 2011;39(10):2153-2160.

25. Moseley JB, Jr., Anderson AF, Browne JE, et al. Long-term durability of autologous chondrocyte implantation: a multicenter, observational study in US patients. *Am J Sports Med.* Feb 2010;38(2):238-246.

26. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med.* Jun 2010;38(6):1117-1124.

27. Knutsen G, Engebretsen L, Ludvigsen TC, et al. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. *J Bone Joint Surg Am*. Mar 2004;86-A(3):455-464.

28. Dozin B, Malpeli M, Cancedda R, et al. Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial. *Clin J Sport Med.* Jul 2005;15(4):220-226.

29. Bentley G, Biant LC, Carrington RW, et al. A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee. *J Bone Joint Surg Br.* Mar 2003;85(2):223-230.

30. Horas U, Pelinkovic D, Herr G, Aigner T, Schnettler R. Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. *J Bone Joint Surg Am*. Feb 2003;85-A(2):185-192.

31. Vanlauwe J, Saris DB, Victor J, Almqvist KF, Bellemans J, Luyten FP. Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. *Am J Sports Med.* Dec 2011;39(12):2566-2574.

32. Saris DB, Vanlauwe J, Victor J, et al. Treatment of symptomatic cartilage defects of the knee: characterized chondrocyte implantation results in better clinical outcome at 36 months in a randomized trial compared to microfracture. *Am J Sports Med.* Nov 2009;37 Suppl 1:10S-19S.

33. Bentley G, Biant LC, Vijayan S, Macmull S, Skinner JA, Carrington RW. Minimum ten-year results of a prospective randomised study of autologous chondrocyte implantation versus mosaicplasty for symptomatic articular cartilage lesions of the knee. *J Bone Joint Surg Br.* Apr 2012;94(4):504-509.

34. Vanlauwe J, Saris DB, Victor J, Almqvist KF, Bellemans J, Luyten FP. Five-Year Outcome of Characterized Chondrocyte Implantation Versus Microfracture for Symptomatic Cartilage Defects of the Knee: Early Treatment Matters. *Am J Sports Med.* Sep 9 2011.

35. Vasiliadis HS, Wasiak J. Autologous chondrocyte implantation for full thickness articular cartilage defects of the knee. *Cochrane Database Syst Rev.* 2010(10):CD003323.

36. Briggs TW, Mahroof S, David LA, Flannelly J, Pringle J, Bayliss M. Histological evaluation of chondral defects after autologous chondrocyte implantation of the knee. *J Bone Joint Surg Br.* Sep 2003;85(7):1077-1083.

37. Enea D, Cecconi S, Busilacchi A, Manzotti S, Gesuita R, Gigante A. Matrix-induced autologous chondrocyte implantation (MACI) in the knee. *Knee Surg Sports Traumatol Arthrosc.* May 2012;20(5):862-869.

38. Marcacci M, Berruto M, Brocchetta D, et al. Articular cartilage engineering with Hyalograft C: 3-year clinical results. *Clin Orthop Relat Res.* Jun 2005(435):96-105.

39. Kang SW, Yoo SP, Kim BS. Effect of chondrocyte passage number on histological aspects of tissue-engineered cartilage. *Biomed Mater Eng.* 2007;17(5):269-276.

40. Salzmann GM, Sauerschnig M, Berninger MT, et al. The dependence of autologous chondrocyte transplantation on varying cellular passage, yield and culture duration. *Biomaterials.* Sep 2011;32(25):5810-5818.

41. Bekkers JE, Tsuchida AI, van Rijen MHP, et al. One-stage cell-based cartilage regeneration using a combination of chondrons and mesenchymal stromal cells; comparison to microfracture. *Accepted for publication in American Journal of Sports Medicine.* 2013.

42. Pelttari K, Winter A, Steck E, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum.* Oct 2006;54(10):3254-3266.

43. Toh WS, Spector M, Lee EH, Cao T. Biomaterial-mediated delivery of microenvironmental cues for repair and regeneration of articular cartilage. *Mol Pharm.* Aug 1 2011;8(4):994-1001.

44. Woods A, Wang G, Beier F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. *J Cell Physiol.* Oct 2007;213(1):1-8.

45. de Windt TS, Bekkers JE, Creemers LB, Dhert WJ, Saris DB. Patient profiling in cartilage regeneration: prognostic factors determining success of treatment for cartilage defects. *Am J Sports Med.* Nov 2009;37 Suppl 1:58S-62S.

46. Saris DB, Dhert WJ, Verbout AJ. Joint homeostasis. The discrepancy between old and fresh defects in cartilage repair. *J Bone Joint Surg Br.* Sep 2003;85(7):1067-1076.

47. Hui AY, McCarty WJ, Masuda K, Firestein GS, Sah RL. A systems biology approach to synovial joint lubrication in health, injury, and disease. *Wiley Interdiscip Rev Syst Biol Med.* Aug 8 2011.

48. Irie K, Uchiyama E, Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. *Knee.* Mar 2003;10(1):93-96.

49. Laberge MA, Baum T, Virayavanich W, et al. Obesity increases the prevalence and severity of focal knee abnormalities diagnosed using 3T MRI in middle-aged subjects--data from the Osteoarthritis Initiative. *Skeletal Radiol.* Jun 2012;41(6):633-641.

50. Elsaid KA, Fleming BC, Oksendahl HL, et al. Decreased lubricin concentrations and markers of joint inflammation in the synovial fluid of patients with anterior cruciate ligament injury. *Arthritis Rheum.* Jun 2008;58(6):1707-1715.

51. Guilak F. Biomechanical factors in osteoarthritis. *Best Pract Res Clin Rheumatol.* Dec 2011;25(6):815-823.

52. Stolberg-Stolberg JA, Furman BD, William Garrigues N, et al. Effects of Cartilage Impact with and without Fracture on Chondrocyte Viability and the Release of Inflammatory Markers. *J Orthop Res.* Apr 25 2013.

53. Lee JH, Fitzgerald JB, Dimicco MA, Grodzinsky AJ. Mechanical injury of cartilage explants causes specific time-dependent changes in chondrocyte gene expression. *Arthritis Rheum.* Aug 2005;52(8):2386-2395.

54. Fitzgerald JB, Jin M, Dean D, Wood DJ, Zheng MH, Grodzinsky AJ. Mechanical compression of cartilage explants induces multiple time-dependent gene expression patterns and involves intracellular calcium and cyclic AMP. *J Biol Chem.* May 7 2004;279(19):19502-19511.

55. Stevens AL, Wishnok JS, White FM, Grodzinsky AJ, Tannenbaum SR. Mechanical injury and cytokines cause loss of cartilage integrity and upregulate proteins associated with catabolism, immunity, inflammation, and repair. *Mol Cell Proteomics.* Jul 2009;8(7):1475-1489.

56. van Meegeren ME, Roosendaal G, van Veghel K, Mastbergen SC, Lafeber FP. A short time window to profit from protection of blood-induced cartilage damage by IL-4 plus IL-10. *Rheumatology (Oxford).* Feb 14 2013.

57. Loeuille D, Chary-Valckenaere I, Champigneulle J, et al. Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. *Arthritis Rheum.* Nov 2005;52(11):3492-3501.

58. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. *Bone.* Aug 2012;51(2):249-257.

59. Wenham CY, Conaghan PG. The role of synovitis in osteoarthritis. *Ther Adv Musculoskelet Dis.* Dec 2010;2(6):349-359.

60. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat Rev Rheumatol.* Nov 2010;6(11):625-635.

61. Haseeb A, Haqqi TM. Immunopathogenesis of osteoarthritis. *Clin Immunol.* Mar 2013;146(3):185-196.

62. Beekhuizen M, Bastiaansen-Jenniskens YM, Koevoet W, et al. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: Establishment and characterization of a long-term cartilage-synovium coculture. *Arthritis Rheum.* Jul 2011;63(7):1918-1927.

63. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol.* Jan 2011;7(1):33-42.

64. Blom AB, van der Kraan PM, van den Berg WB. Cytokine targeting in osteoarthritis. *Curr Drug Targets.* Feb 2007;8(2):283-292.

65. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin Orthop Relat Res.* Oct 2004(427 Suppl):S27-36.

66. Kokebie R, Aggarwal R, Lidder S, et al. The role of synovial fluid markers of catabolism and anabolism in osteoarthritis, rheumatoid arthritis and asymptomatic organ donors. *Arthritis Res Ther.* Mar 24 2011;13(2):R50.

67. Cuellar VG, Cuellar JM, Golish SR, Yeomans DC, Scuderi GJ. Cytokine profiling in acute anterior cruciate ligament injury. *Arthroscopy.* Oct 2010;26(10):1296-1301.

68. Catterall JB, Stabler TV, Flannery CR, Kraus VB. Changes in serum and synovial fluid biomarkers after acute injury (NCT00332254). *Arthritis Res Ther.* 2010;12(6):R229.

69. Jotanovic Z, Mihelic R, Sestan B, Dembic Z. Role of interleukin-1 inhibitors in osteoarthritis: an evidence-based review. *Drugs Aging.* May 1 2012;29(5):343-358.

70. Beekhuizen M, Osch Gv, Bot A, et al. Inhibition of oncostatin M in osteoarthritic synovial fluid increases GAG production in osteoarthritic cartilage repair Submitted.

71. Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. *Matrix Biol.* Nov 2000;19(6):549-553.

72. Borzi RM, Mazzetti I, Marcu KB, Facchini A. Chemokines in cartilage degradation. *Clin Orthop Relat Res.* Oct 2004(427 Suppl):S53-61.

73. Merz D, Liu R, Johnson K, Terkeltaub R. IL-8/CXCL8 and growth-related oncogene alpha/CXCL1 induce chondrocyte hypertrophic differentiation. *J Immunol.* Oct 15 2003;171(8):4406-4415.

74. Berry PA, Jones SW, Cicuttini FM, Wluka AE, Maciewicz RA. Temporal relationship between serum adipokines, biomarkers of bone and cartilage turnover, and cartilage volume loss in a population with clinical knee osteoarthritis. *Arthritis Rheum.* Mar 2011;63(3):700-707.

75. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum.* Jun 2012;64(6):1697-1707.

76. Santangelo KS, Nuovo GJ, Bertone AL. In vivo reduction or blockade of interleukin-1beta in primary osteoarthritis influences expression of mediators implicated in pathogenesis. *Osteoarthritis Cartilage.* Dec 2012;20(12):1610-1618.

77. Sylvester J, Ahmad R, Zafarullah M. Role of Sp1 transcription factor in Interleukin-1-induced ADAMTS-4 (aggrecanase-1) gene expression in human articular chondrocytes. *Rheumatol Int.* Feb 2013;33(2):517-522.

78. Sofat N, Robertson SD, Wait R. Fibronectin III 13-14 domains induce joint damage via Toll-like receptor 4 activation and synergize with interleukin-1 and tumour necrosis factor. *J Innate Immun.* 2012;4(1):69-79.

79. Campo GM, Avenoso A, D'Ascola A, et al. Hyaluronan differently modulates TLR-4 and the inflammatory response in mouse chondrocytes. *Biofactors.* Jan-Feb 2012;38(1):69-76.

80. Happonen KE, Heinegard D, Saxne T, Blom AM. Interactions of the complement system with molecules of extracellular matrix: relevance for joint diseases. *Immunobiology.* Nov 2012;217(11):1088-1096.

81. Rodrigo JJ, Steadman JR, Syftestad G, Benton H, Silliman J. Effects of human knee synovial fluid on chondrogenesis in vitro. *Am J Knee Surg.* Fall 1995;8(4):124-129.

82. Yang KG, Saris DB, Verbout AJ, Creemers LB, Dhert WJ. The effect of synovial fluid from injured knee joints on in vitro chondrogenesis. *Tissue Eng.* Oct 2006;12(10):2957-2964.

83. Caron MM, Emans PJ, Surtel DA, et al. Activation of NF-kappaB/p65 facilitates early chondrogenic differentiation during endochondral ossification. *PLoS One.* 2012;7(3):e33467.

84. Beekhuizen M, Gierman LM, van Spil WE, et al. An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid. *Osteoarthritis Cartilage.* Apr 15 2013.

85. Gobezie R, Kho A, Krastins B, et al. High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis Res Ther.* 2007;9(2):R36.

86. Ritter SY, Subbaiah R, Bebek G, et al. Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis Rheum.* Apr 2013;65(4):981-992.

87. Mastbergen SC, Saris DB, Lafeber FP. Functional articular cartilage repair: here, near, or is the best approach not yet clear? *Nat Rev Rheumatol.* May 2013;9(5):277-290.

88. Aroen A, Loken S, Heir S, et al. Articular cartilage lesions in 993 consecutive knee arthroscopies. *Am J Sports Med.* Jan-Feb 2004;32(1):211-215.

89. Heyworth BE, Lee JH, Kim PD, Lipton CB, Strauch RJ, Rosenwasser MP. Hylan versus corticosteroid versus placebo for treatment of basal joint arthritis: a prospective, randomized, double-blinded clinical trial. *J Hand Surg Am.* Jan 2008;33(1):40-48.

90. Widuchowski W, Lukasik P, Kwiatkowski G, et al. Isolated full thickness chondral injuries. Prevalance and outcome of treatment. A retrospective study of 5233 knee arthroscopies. *Acta Chir Orthop Traumatol Cech.* Oct 2008;75(5):382-386.

91. Vasiliadis HS, Wasiak J, Salanti G. Autologous chondrocyte implantation for the treatment of cartilage lesions of the knee: a systematic review of randomized studies. *Knee Surg Sports Traumatol Arthrosc.* Dec 2010;18(12):1645-1655.

92. Drogset JO, Grontvedt T. Anterior cruciate ligament reconstruction with and without a ligament augmentation device : results at 8-Year follow-up. *Am J Sports Med.* Nov-Dec 2002;30(6):851-856.

93. Heir S, Nerhus TK, Rotterud JH, et al. Focal cartilage defects in the knee impair quality of life as much as severe osteoarthritis: a comparison of knee injury and osteoarthritis outcome score in 4 patient categories scheduled for knee surgery. *Am J Sports Med.* Feb 2010;38(2):231-237.

94. Messner K, Maletius W. The long-term prognosis for severe damage to weight-bearing cartilage in the knee: a 14-year clinical and radiographic follow-up in 28 young athletes. *Acta Orthop Scand.* Apr 1996;67(2):165-168.

95. Widuchowski W, Widuchowski J, Faltus R, et al. Long-term clinical and radiological assessment of untreated severe cartilage damage in the knee: a natural history study. *Scand J Med Sci Sports.* Feb 2011;21(1):106-110.

96. Gomoll AH, Probst C, Farr J, Cole BJ, Minas T. Use of a type I/III bilayer collagen membrane decreases reoperation rates for symptomatic hypertrophy after autologous chondrocyte implantation. *Am J Sports Med.* Nov 2009;37 Suppl 1:20S-23S.

97. Gooding CR, Bartlett W, Bentley G, Skinner JA, Carrington R, Flanagan A. A prospective, randomised study comparing two techniques of autologous chondrocyte implantation for osteo-

chondral defects in the knee: Periosteum covered versus type I/III collagen covered. *Knee.* Jun 2006;13(3):203-210.

98. Harris JD, Siston RA, Brophy RH, Lattermann C, Carey JL, Flanigan DC. Failures, re-operations, and complications after autologous chondrocyte implantation--a systematic review. *Osteoarthritis Cartilage.* Jul 2011;19(7):779-791.

99. Brittberg M. Cell carriers as the next generation of cell therapy for cartilage repair: a review of the matrix-induced autologous chondrocyte implantation procedure. *Am J Sports Med.* Jun 2010;38(6):1259-1271.

100. Ebert JR, Robertson WB, Woodhouse J, et al. Clinical and magnetic resonance imaging-based outcomes to 5 years after matrix-induced autologous chondrocyte implantation to address articular cartilage defects in the knee. *Am J Sports Med.* Apr 2011;39(4):753-763.

101. Marlovits S, Aldrian S, Wondrasch B, et al. Clinical and radiological outcomes 5 years after matrix-induced autologous chondrocyte implantation in patients with symptomatic, traumatic chondral defects. *Am J Sports Med.* Oct 2012;40(10):2273-2280.

102. Sohn DH, Lottman LM, Lum LY, et al. Effect of gravity on localization of chondrocytes implanted in cartilage defects. *Clin Orthop Relat Res.* Jan 2002(394):254-262.

103. Vijayan S, Bartlett W, Bentley G, et al. Autologous chondrocyte implantation for osteochondral lesions in the knee using a bilayer collagen membrane and bone graft: a two- to eight-year follow-up study. *J Bone Joint Surg Br.* Apr 2012;94(4):488-492.

104. Gavenis K, Schmidt-Rohlfing B, Mueller-Rath R, Andereya S, Schneider U. In vitro comparison of six different matrix systems for the cultivation of human chondrocytes. *In Vitro Cell Dev Biol Anim.* May-Jun 2006;42(5-6):159-167.

105. Gigante A, Bevilacqua C, Ricevuto A, Mattioli-Belmonte M, Greco F. Membrane-seeded autologous chondrocytes: cell viability and characterization at surgery. *Knee Surg Sports Traumatol Arthrosc.* Jan 2007;15(1):88-92.

106. Zheng MH, Willers C, Kirilak L, et al. Matrix-induced autologous chondrocyte implantation (MACI): biological and histological assessment. *Tissue Eng.* Apr 2007;13(4):737-746.

107. Ebert JR, Robertson WB, Lloyd DG, Zheng MH, Wood DJ, Auckland TA. A prospective, randomized comparison of traditional and accelerated approaches to postoperative rehabilitation following autologous chondrocyte implantation. 2-year clinical outcomes. . *Cartilage.* 2010(1):180-187.

108. Salzmann GM, Paul J, Bauer JS, et al. T2 assessment and clinical outcome following autologous matrix-assisted chondrocyte and osteochondral autograft transplantation. *Osteoarthritis Cartilage.* Dec 2009;17(12):1576-1582.

109. Steadman JR, Rodkey WG, Singleton SB, Briggs KK. Microfracture technique for full-thickness chondral defects: technique and clinical results. *Oper Tech Orthop.* 1997;7:300-304.

110. Kreuz PC, Steinwachs MR, Erggelet C, et al. Results after microfracture of full-thickness chondral defects in different compartments in the knee. *Osteoarthritis Cartilage.* Nov 2006;14(11):1119-1125.

111. Mithoefer K, Williams RJ, 3rd, Warren RF, et al. The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study. *J Bone Joint Surg Am.* Sep 2005;87(9):1911-1920.

112. Miller BS, Joseph TA, Barry EM, Rich VJ, Sterett WI. Patient satisfaction after medial opening high tibial osteotomy and microfracture. *J Knee Surg.* Apr 2007;20(2):129-133.

113. Minas T, Gomoll AH, Rosenberger R, Royce RO, Bryant T. Increased failure rate of autologous chondrocyte implantation after previous treatment with marrow stimulation techniques. *Am J Sports Med.* May 2009;37(5):902-908.

114. Pestka JM, Bode G, Salzmann G, Sudkamp NP, Niemeyer P. Clinical outcome of autologous chondrocyte implantation for failed microfracture treatment of full-thickness cartilage defects of the knee joint. *Am J Sports Med.* Feb 2012;40(2):325-331.

115. European Medicines Agency. Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee.08 April 2010. 2010.

116. U.S. Department of Health & Human Servides FDA. Guides for Industry. Preparation of IDEs and INDs for Products intended for Repair or Replace Knee Cartilage. . December 2011. 2011.

117. Steadman JR, Rodkey WG, Briggs KK. Microfracture to treat full-thickness chondral defects: surgical technique, rehabilitation, and outcomes. *J Knee Surg.* Summer 2002;15(3):170-176.

118. Peterfy CG, Guermazi A, Zaim S, et al. Whole-Organ Magnetic Resonance Imaging Score (WORMS) of the knee in osteoarthritis. *Osteoarthritis Cartilage.* Mar 2004;12(3):177-190.

119. Marlovits S, Striessnig G, Resinger CT, et al. Definition of pertinent parameters for the evaluation of articular cartilage repair tissue with high-resolution magnetic resonance imaging. *Eur J Radiol.* Dec 2004;52(3):310-319.

120. Noyes FR, Barber SD, Mooar LA. A rationale for assessing sports activity levels and limitations in knee disorders. *Clin Orthop Relat Res.* Sep 1989(246):238-249.

121. Irrgang JJ, Anderson AF, Boland AL, et al. Development and validation of the international knee documentation committee subjective knee form. *Am J Sports Med.* Sep-Oct 2001;29(5):600-613.

122. Ware J, Jr., Kosinski M, Keller SD. A 12-Item Short-Form Health Survey: construction of scales and preliminary tests of reliability and validity. *Med Care.* Mar 1996;34(3):220-233.

123. Basad E, Ishaque B, Bachmann G, Sturz H, Steinmeyer J. Matrix-induced autologous chondrocyte implantation versus microfracture in the treatment of cartilage defects of the knee: a 2-year randomised study. *Knee Surg Sports Traumatol Arthrosc.* Apr 2010;18(4):519-527.

124. Bachmann G, Basad E, Lommel D, Steinmeyer J. [MRI in the follow-up of matrix-supported autologous chondrocyte transplantation (MACI) and microfracture]. *Radiologe.* Aug 2004;44(8):773-782.

125. Gill TJ. The treatment of articular cartilage defects using microfracture and debridement. *Am J Knee Surg.* Winter 2000;13(1):33-40.

126. Steadman JR, Briggs KK, Rodrigo JJ, Kocher MS, Gill TJ, Rodkey WG. Outcomes of microfracture for traumatic chondral defects of the knee: average 11-year follow-up. *Arthroscopy.* May-Jun 2003;19(5):477-484.

127. Gudas R, Kalesinskas RJ, Kimtys V, et al. A prospective randomized clinical study of mosaic osteochondral autologous transplantation versus microfracture for the treatment of osteochondral defects in the knee joint in young athletes. *Arthroscopy.* Sep 2005;21(9):1066-1075.

128. Kreuz PC, Erggelet C, Steinwachs MR, et al. Is microfracture of chondral defects in the knee associated with different results in patients aged 40 years or younger? *Arthroscopy.* Nov 2006;22(11):1180-1186.

129. Mithoefer K, Williams RJ, 3rd, Warren RF, Wickiewicz TL, Marx RG. High-impact athletics after knee articular cartilage repair: a prospective evaluation of the microfracture technique. *Am J Sports Med.* Sep 2006;34(9):1413-1418.

130. Ebert JR, Smith A, Edwards PK, Hambly K, Wood DJ, Ackland TR. Factors predictive of outcome 5 years after matrix-induced autologous chondrocyte implantation in the tibiofemoral joint. *Am J Sports Med.* Jun 2013;41(6):1245-1254.

131. Mainil-Varlet P, Van Damme B, Nesic D, Knutsen G, Kandel R, Roberts S. A new histology scoring system for the assessment of the quality of human cartilage repair: ICRS II. *Am J Sports Med.* May 2010;38(5):880-890.

132. Mithoefer K, Saris DBF, Farr J, et al. Guidelines for the design and conduct of clinical studies in knee articular cartilage repair: International Cartilage Repair Society recommendations based on current scientific evidence and standards of clinical care. *Cartilage.* 2011;2(2):100-121.

133. de Windt TS, Welsch GH, Brittberg M, et al. Is Magnetic Resonance Imaging Reliable in Predicting Clinical Outcome After Articular Cartilage Repair of the Knee?: A Systematic Review and Meta-analysis. *Am J Sports Med.* Jan 30 2013.

134. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther.* 2009;11(3):224.

135. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med.* Dec 8 2011;365(23):2205-2219.

136. de Lange-Brokaar BJ, Ioan-Facsinay A, van Osch GJ, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. *Osteoarthritis Cartilage.* Dec 2012;20(12):1484-1499.

137. Gandhi NS, Mancera RL. The structure of glycosaminoglycans and their interactions with proteins. *Chem Biol Drug Des.* Dec 2008;72(6):455-482.

138. Bekkers JE SD, Tsuchida AI, van Rijen M, Dhert WJ, Creemers LB. Chondrogenic potential of articular chondrocytes depends on their original location. *Submitted for publication.*

139. www.federa.org.

140. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For. *BMJ.* Sep 21 2002;325(7365):648-651.

141. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J Immunol Methods.* May 2005;300(1-2):124-135.

142. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol.* Jan 2003;10(1):133-139.

143. Tsuchida AI, Beekhuizen M, Rutgers M, et al. Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model. *Arthritis Res Ther.* Dec 3 2012;14(6):R262.

144. Tsuchida A, Saris D, Rutgers M, et al. CXCL8 (interleukin-8) in synovial fluid and autocrine production by chondrocytes; association with cartilage pathology, but no effect on regeneration or terminal differentiation. Submitted.

145. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res.* 1982;9(4):247-248.

146. Nadeshdin WA. Zur Untersuchung der Minderwertigkeit der Organe an Leichen. *Deutsche Zeitschrift für die Gesamte Gerichthiche Medizine.* 1932;18:426–431.

147. Filardo G, Vannini F, Marcacci M, et al. Matrix-assisted autologous chondrocyte transplantation for cartilage regeneration in osteoarthritic knees: results and failures at midterm follow-up. *Am J Sports Med.* Jan 2013;41(1):95-100.

148. Ahmad R, Qureshi HY, El Mabrouk M, Sylvester J, Ahmad M, Zafarullah M. Inhibition of interleukin 1-induced matrix metalloproteinase 13 expression in human chondrocytes by interferon gamma. *Ann Rheum Dis.* Jun 2007;66(6):782-789.

149. Ahmad R, El Mabrouk M, Sylvester J, Zafarullah M. Human osteoarthritic chondrocytes are impaired in matrix metalloproteinase-13 inhibition by IFN-gamma due to reduced IFN-gamma receptor levels. *Osteoarthritis Cartilage.* Aug 2009;17(8):1049-1055.

150. Tsuchida AI, Beekhuizen M, Rutgers M, et al. Interleukin 6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model. *Arthritis Res Ther.* Dec 3 2012;14(6):R262.

151. Van Dyken SJ, Locksley RM. Interleukin-4- and Interleukin-13-Mediated Alternatively Activated Macrophages: Roles in Homeostasis and Disease. *Annu Rev Immunol.* Jan 3 2013.

152. Lafyatis R, Farina A. New insights into the mechanisms of innate immune receptor signalling in fibrosis. *Open Rheumatol J.* 2012;6:72-79.

153. Cleaver CS, Rowan AD, Cawston TE. Interleukin 13 blocks the release of collagen from bovine nasal cartilage treated with proinflammatory cytokines. *Ann Rheum Dis.* Feb 2001;60(2):150-157.

154. Woods JM, Amin MA, Katschke KJ, Jr., et al. Interleukin-13 gene therapy reduces inflammation, vascularization, and bony destruction in rat adjuvant-induced arthritis. *Hum Gene Ther.* Feb 10 2002;13(3):381-393.

155. Nabbe KC, van Lent PL, Holthuysen AE, et al. Local IL-13 gene transfer prior to immune-complex arthritis inhibits chondrocyte death and matrix-metalloproteinase-mediated cartilage matrix degradation despite enhanced joint inflammation. *Arthritis Res Ther.* 2005;7(2):R392-401.

156. Marshall KW, Zhang H, Yager TD, et al. Blood-based biomarkers for detecting mild osteoarthritis in the human knee. *Osteoarthritis Cartilage.* Oct 2005;13(10):861-871.

157. Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y. Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann Rheum Dis.* Dec 2008;67 Suppl 3:iii75-82.

158. Pfander D, Kortje D, Zimmermann R, et al. Vascular endothelial growth factor in articular cartilage of healthy and osteoarthritic human knee joints. *Ann Rheum Dis.* Nov 2001;60(11):1070-1073.

159. Ludin A, Sela JJ, Schroeder A, Samuni Y, Nitzan DW, Amir G. Injection of vascular endothelial growth factor into knee joints induces osteoarthritis in mice. *Osteoarthritis Cartilage.* Dec 19 2012.

160. Yamairi F, Utsumi H, Ono Y, Komorita N, Tanaka M, Fukunari A. Expression of vascular endothelial growth factor (VEGF) associated with histopathological changes in rodent models of osteoarthritis. *J Toxicol Pathol.* Jun 2011;24(2):137-142.

161. Jansen H, Meffert RH, Birkenfeld F, Petersen W, Pufe T. Detection of vascular endothelial growth factor (VEGF) in moderate osteoarthritis in a rabbit model. *Ann Anat.* Sep 2012;194(5):452-456.

162. Chen XY, Hao YR, Wang Z, Zhou JL, Jia QX, Qiu B. The effect of vascular endothelial growth factor on aggrecan and type II collagen expression in rat articular chondrocytes. *Rheumatol Int.* Nov 2012;32(11):3359-3364.

163. Nagai T, Sato M, Kutsuna T, et al. Intravenous administration of anti-vascular endothelial growth factor humanized monoclonal antibody bevacizumab improves articular cartilage repair. *Arthritis Res Ther.* 2010;12(5):R178.

164. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell.* Jun 13 2008;133(6):1006-1018.

165. Kojima H, Kunimoto H, Inoue T, Nakajima K. The STAT3-IGFBP5 axis is critical for IL-6/gp130-induced premature senescence in human fibroblasts. *Cell Cycle.* Feb 15 2012;11(4):730-739.

166. Rose J, Soder S, Skhirtladze C, et al. DNA damage, discoordinated gene expression and cellular senescence in osteoarthritic chondrocytes. *Osteoarthritis Cartilage.* Sep 2012;20(9):1020-1028.

167. Harbo M, Bendix L, Bay-Jensen AC, et al. The distribution pattern of critically short telomeres in human osteoarthritic knees. *Arthritis Res Ther.* 2012;14(1):R12.

168. Leong DJ, Sun HB. Events in articular chondrocytes with aging. *Curr Osteoporos Rep.* Dec 2011;9(4):196-201.

169. Hollander AP, Dickinson SC, Sims TJ, et al. Maturation of tissue engineered cartilage implanted in injured and osteoarthritic human knees. *Tissue Eng.* Jul 2006;12(7):1787-1798.

170. Tallheden T, Bengtsson C, Brantsing C, et al. Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. *Arthritis Res Ther.* 2005;7(3):R560-568.

171. Stoop R, Albrecht D, Gaissmaier C, et al. Comparison of marker gene expression in chondrocytes from patients receiving autologous chondrocyte transplantation versus osteoarthritis patients. *Arthritis Res Ther.* 2007;9(3):R60.

172. Cavallo C, Desando G, Facchini A, Grigolo B. Chondrocytes from patients with osteoarthritis express typical extracellular matrix molecules once grown onto a three-dimensional hyaluronan-based scaffold. *J Biomed Mater Res A.* Apr 2010;93(1):86-95.

173. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am.* Apr 1971;53(3):523-537.

174. Bulstra SK, Buurman WA, Walenkamp GH, Van der Linden AJ. Metabolic characteristics of in vitro cultured human chondrocytes in relation to the histopathologic grade of osteoarthritis. *Clin Orthop Relat Res.* May 1989(242):294-302.

175. Lasczkowski GE, Aigner T, Gamerdinger U, Weiler G, Bratzke H. Visualization of postmortem chondrocyte damage by vital staining and confocal laser scanning 3D microscopy. *J Forensic Sci.* May 2002;47(3):663-666.

176. Kim IL, Mauck RL, Burdick JA. Hydrogel design for cartilage tissue engineering: a case study with hyaluronic acid. *Biomaterials.* Dec 2011;32(34):8771-8782.

177. Schuurman W, Khristov V, Pot MW, van Weeren PR, Dhert WJ, Malda J. Bioprinting of hybrid tissue constructs with tailorable mechanical properties. *Biofabrication.* Jun 2011;3(2):021001.

178. Schagemann JC, Chung HW, Mrosek EH, et al. Poly-epsilon-caprolactone/gel hybrid scaffolds for cartilage tissue engineering. *J Biomed Mater Res A.* May 2010;93(2):454-463.

179. Welsch GH, Mamisch TC, Zak L, et al. Evaluation of cartilage repair tissue after matrix-associated autologous chondrocyte transplantation using a hyaluronic-based or a collagen-based scaffold with morphological MOCART scoring and biochemical T2 mapping: preliminary results. *Am J Sports Med.* May 2010;38(5):934-942.

180. Schlegel W, Nurnberger S, Hombauer M, Albrecht C, Vecsei V, Marlovits S. Scaffold-dependent differentiation of human articular chondrocytes. *Int J Mol Med.* Nov 2008;22(5):691-699.

181. Albrecht C, Tichy B, Nurnberger S, et al. Gene expression and cell differentiation in matrix-associated chondrocyte transplantation grafts: a comparative study. *Osteoarthritis Cartilage.* Oct 2011;19(10):1219-1227.

182. Schagemann JC, Kurz H, Casper ME, et al. The effect of scaffold composition on the early structural characteristics of chondrocytes and expression of adhesion molecules. *Biomaterials.* Apr 2010;31(10):2798-2805.

183. Vonk LA, Doulabi BZ, Huang C, Helder MN, Everts V, Bank RA. Collagen-induced expression of collagenase-3 by primary chondrocytes is mediated by integrin α1 and discoidin domain receptor 2: a protein kinase C-dependent pathway. *Rheumatology (Oxford).* Mar 2011;50(3):463-472.

184. Goldring MB, Otero M, Plumb DA, et al. Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur Cell Mater.* 2011;21:202-220.

185. Rohner E, Matziolis G, Perka C, et al. Inflammatory synovial fluid microenvironment drives primary human chondrocytes to actively take part in inflammatory joint diseases. *Immunol Res.* Jun 2012;52(3):169-175.

186. Malicev E, Radosavljevic D, Velikonja NK. Fibrin gel improved the spatial uniformity and phenotype of human chondrocytes seeded on collagen scaffolds. *Biotechnol Bioeng.* Feb 1 2007;96(2):364-370.

187. Liu Y, Shu XZ, Prestwich GD. Osteochondral defect repair with autologous bone marrow-derived mesenchymal stem cells in an injectable, in situ, cross-linked synthetic extracellular matrix. *Tissue Eng.* Dec 2006;12(12):3405-3416.

188. Bekkers JE, Tsuchida AI, Malda J, et al. Quality of scaffold fixation in a human cadaver knee model. *Osteoarthritis Cartilage.* Feb 2010;18(2):266-272.

189. Woodfield TB, Malda J, de Wijn J, Peters F, Riesle J, van Blitterswijk CA. Design of porous scaffolds for cartilage tissue engineering using a three-dimensional fiber-deposition technique. *Biomaterials.* Aug 2004;25(18):4149-4161.

190. Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture. *J Cell Physiol.* Mar 2005;202(3):731-742.

191. Brodkin KR, Garcia AJ, Levenston ME. Chondrocyte phenotypes on different extracellular matrix monolayers. *Biomaterials.* Dec 2004;25(28):5929-5938.

192. Hoshiba T, Yamada T, Lu H, Kawazoe N, Chen G. Maintenance of cartilaginous gene expression on extracellular matrix derived from serially passaged chondrocytes during in vitro chondrocyte expansion. *J Biomed Mater Res A.* Mar 2012;100(3):694-702.

193. Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR. Functional genomic analysis in arthritis-affected cartilage: yin-yang regulation of inflammatory mediators by alpha 5 beta 1 and alpha V beta 3 integrins. *J Immunol.* Mar 1 2000;164(5):2684-2691.

194. Lowin T, Straub RH. Integrins and their ligands in rheumatoid arthritis. *Arthritis Res Ther.* 2011;13(5):244.

195. Chemin K, Bohineust A, Dogniaux S, et al. Cytokine Secretion by CD4+ T Cells at the Immunological Synapse Requires Cdc42-Dependent Local Actin Remodeling but Not Microtubule Organizing Center Polarity. *J Immunol.* Sep 1 2012;189(5):2159-2168.

196. Kustermans G, El Mjiyad N, Horion J, Jacobs N, Piette J, Legrand-Poels S. Actin cytoskeleton differentially modulates NF-kappaB-mediated IL-8 expression in myelomonocytic cells. *Biochem Pharmacol.* Nov 15 2008;76(10):1214-1228.

197. Novakofski KD, Torre CJ, Fortier LA. Interleukin-1alpha, -6, and -8 decrease Cdc42 activity resulting in loss of articular chondrocyte phenotype. *J Orthop Res.* Feb 2012;30(2):246-251.

198. Coates EE, Riggin CN, Fisher JP. Matrix molecule influence on chondrocyte phenotype and proteoglycan 4 expression by alginate-embedded zonal chondrocytes and mesenchymal stem cells. *J Orthop Res.* Jun 4 2012.

199. Julovi SM, Ito H, Nishitani K, Jackson CJ, Nakamura T. Hyaluronan inhibits matrix metalloproteinase-13 in human arthritic chondrocytes via CD44 and P38. *J Orthop Res.* Feb 2011;29(2):258-264.

200. Yasuda T. Nuclear factor-kappaB activation by type II collagen peptide in articular chondrocytes: its inhibition by hyaluronan via the receptors. *Mod Rheumatol.* Dec 7 2012.

201. Yatabe T, Mochizuki S, Takizawa M, et al. Hyaluronan inhibits expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic chondrocytes. *Ann Rheum Dis.* Jun 2009;68(6):1051-1058.

202. Zheng Shu X, Liu Y, Palumbo FS, Luo Y, Prestwich GD. In situ crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials.* Mar-Apr 2004;25(7-8):1339-1348.

203. Klatt AR, Zech D, Kuhn G, et al. Discoidin domain receptor 2 mediates the collagen II-dependent release of interleukin-6 in primary human chondrocytes. *J Pathol.* Jun 2009;218(2):241-247.

204. Jennewein C, Tran N, Paulus P, Ellinghaus P, Eble JA, Zacharowski K. Novel aspects of fibrin(ogen) fragments during inflammation. *Mol Med.* May-Jun 2011;17(5-6):568-573.

205. Buser Z, Liu J, Thorne KJ, Coughlin D, Lotz JC. Inflammatory response of intervertebral disc cells is reduced by fibrin sealant scaffold in vitro. *J Tissue Eng Regen Med.* May 18 2012.

206. Park JS, Shim MS, Shim SH, et al. Chondrogenic potential of stem cells derived from amniotic fluid, adipose tissue, or bone marrow encapsulated in fibrin gels containing TGF-beta3. *Biomaterials.* Nov 2011;32(32):8139-8149.

207. Spicer PP, Mikos AG. Fibrin glue as a drug delivery system. *J Control Release.* Nov 20 2010;148(1):49-55.

208. Rutgers M, Saris DB, Dhert WJ, Creemers LB. Cytokine profile of autologous conditioned serum for treatment of osteoarthritis, in vitro effects on cartilage metabolism and intra-articular levels after injection. *Arthritis Res Ther.* 2010;12(3):R114.

209. Rutgers M, Saris DB, Yang KG, Dhert WJ, Creemers LB. Joint injury and osteoarthritis: soluble mediators in the course and treatment of cartilage pathology. *Immunotherapy.* May 2009;1(3):435-445.

210. Ma B, Leijten JC, Wu L, et al. Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture. *Osteoarthritis Cartilage.* Apr 2013;21(4):599-603.

211. Knudson W, Casey B, Nishida Y, Eger W, Kuettner KE, Knudson CB. Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. *Arthritis Rheum.* May 2000;43(5):1165-1174.

212. Takahashi N, Knudson CB, Thankamony S, et al. Induction of CD44 cleavage in articular chondrocytes. *Arthritis Rheum*. May 2010;62(5):1338-1348.

213. Albrecht C, Schlegel W, Eckl P, et al. Alterations in CD44 isoforms and HAS expression in human articular chondrocytes during the de- and re-differentiation processes. *Int J Mol Med.* Feb 2009;23(2):253-259.

214. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* Apr 2012;13(4):227-232.

215. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res.* Mar 2005;23(2):425-432.

216. Chiang H, Liao CJ, Wang YH, et al. Comparison of articular cartilage repair by autologous chondrocytes with and without in vitro cultivation. *Tissue Eng Part C Methods.* Apr 2010;16(2):291-300.

217. Scanzello CR, Umoh E, Pessler F, et al. Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. *Osteoarthritis Cartilage.* Aug 2009;17(8):1040-1048.

218. Heldens GT, Blaney Davidson EN, Vitters EL, et al. Catabolic factors and osteoarthritis-conditioned medium inhibit chondrogenesis of human mesenchymal stem cells. *Tissue Eng Part A.* Jan 2012;18(1-2):45-54.

219. Kraan MC, Patel DD, Haringman JJ, et al. The development of clinical signs of rheumatoid synovial inflammation is associated with increased synthesis of the chemokine CXCL8 (interleukin-8). *Arthritis Res.* 2001;3(1):65-71.

220. Pierzchala AW, Kusz DJ, Hajduk G. CXCL8 and CCL5 expression in synovial fluid and blood serum in patients with osteoarthritis of the knee. *Arch Immunol Ther Exp (Warsz).* Apr 2011;59(2):151-155.

221. Cameron ML, Fu FH, Paessler HH, Schneider M, Evans CH. Synovial fluid cytokine concentrations as possible prognostic indicators in the ACL-deficient knee. *Knee Surg Sports Traumatol Arthrosc.* 1994;2(1):38-44.

222. Verburgh CA, Hart MH, Aarden LA, Swaak AJ. Interleukin-8 (IL-8) in synovial fluid of rheumatoid and nonrheumatoid joint effusions. *Clin Rheumatol.* Dec 1993;12(4):494-499.

223. Lisignoli G, Toneguzzi S, Pozzi C, et al. Proinflammatory cytokines and chemokine production and expression by human osteoblasts isolated from patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol.* Apr 1999;26(4):791-799.

224. Clockaerts S, Bastiaansen-Jenniskens YM, Feijt C, et al. Cytokine production by infrapatellar fat pad can be stimulated by interleukin 1beta and inhibited by peroxisome proliferator activated receptor alpha agonist. *Ann Rheum Dis.* Jun 2012;71(6):1012-1018.

225. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.

226. Johnson KA, van Etten D, Nanda N, Graham RM, Terkeltaub RA. Distinct transglutaminase 2-independent and transglutaminase 2-dependent pathways mediate articular chondrocyte hypertrophy. *J Biol Chem.* May 23 2003;278(21):18824-18832.

227. Cecil DL, Rose DM, Terkeltaub R, Liu-Bryan R. Role of interleukin-8 in PiT-1 expression and CXCR1-mediated inorganic phosphate uptake in chondrocytes. *Arthritis Rheum.* Jan 2005;52(1):144-154.

228. Olivotto E, Vitellozzi R, Fernandez P, et al. Chondrocyte hypertrophy and apoptosis induced by GROalpha require three-dimensional interaction with the extracellular matrix and a co-receptor role of chondroitin sulfate and are associated with the mitochondrial splicing variant of cathepsin B. *J Cell Physiol.* Feb 2007;210(2):417-427.

229. Battistelli M, Borzi RM, Olivotto E, et al. Cell and matrix morpho-functional analysis in chondrocyte micromasses. *Microsc Res Tech.* Aug 15 2005;67(6):286-295.

230. Borzi RM, Mazzetti I, Cattini L, Uguccioni M, Baggiolini M, Facchini A. Human chondrocytes express functional chemokine receptors and release matrix-degrading enzymes in response to C-X-C and C-C chemokines. *Arthritis Rheum.* Aug 2000;43(8):1734-1741.

231. Klinger P, Surmann-Schmitt C, Brem M, et al. Chondromodulin 1 stabilizes the chondrocyte phenotype and inhibits endochondral ossification of porcine cartilage repair tissue. *Arthritis Rheum.* Sep 2011;63(9):2721-2731.

232. Kandel RA, Boyle J, Gibson G, Cruz T, Speagle M. In vitro formation of mineralized cartilagenous tissue by articular chondrocytes. *In Vitro Cell Dev Biol Anim.* Mar 1997;33(3):174-181.

233. Yang KG, Saris DB, Geuze RE, et al. Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. *Tissue Eng.* Sep 2006;12(9):2435-2447.

234. Neumann U, Kubota H, Frei K, Ganu V, Leppert D. Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. *Anal Biochem.* May 15 2004;328(2):166-173.

235. Gawlitta D, Farrell E, Malda J, Creemers LB, Alblas J, Dhert WJ. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. *Tissue Eng Part B Rev.* Aug 2010;16(4):385-395.

236. Rutgers M, Saris DB, Vonk LA, et al. Effect of Collagen Type I or Type II on Chondrogenesis by Cultured Human Articular Chondrocytes. *Tissue Eng Part A.* Jan 2013;19(1-2):59-65.

237. Koch AE, Kunkel SL, Burrows JC, et al. Synovial tissue macrophage as a source of the chemotactic cytokine IL-8. *J Immunol.* Oct 1 1991;147(7):2187-2195.

238. Borzi RM, Mazzetti I, Macor S, et al. Flow cytometric analysis of intracellular chemokines in chondrocytes in vivo: constitutive expression and enhancement in osteoarthritis and rheumatoid arthritis. *FEBS Lett.* Jul 23 1999;455(3):238-242.

239. Halwani R, Al-Abri J, Beland M, et al. CC and CXC chemokines induce airway smooth muscle proliferation and survival. *J Immunol.* Apr 1 2011;186(7):4156-4163.

240. Zhu YM, Woll PJ. Mitogenic effects of interleukin-8/CXCL8 on cancer cells. *Future Oncol.* Oct 2005;1(5):699-704.

241. Liu J, Shikhman AR, Lotz MK, Wong CH. Hexosaminidase inhibitors as new drug candidates for the therapy of osteoarthritis. *Chem Biol.* Jul 2001;8(7):701-711.

242. Rowan AD, Koshy PJ, Shingleton WD, et al. Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. *Arthritis Rheum.* Jul 2001;44(7):1620-1632.

243. Fan Z, Bau B, Yang H, Soeder S, Aigner T. Freshly isolated osteoarthritic chondrocytes are catabolically more active than normal chondrocytes, but less responsive to catabolic stimulation with interleukin-1beta. *Arthritis Rheum.* Jan 2005;52(1):136-143.

244. Fortier LA, Miller BJ. Signaling through the small G-protein Cdc42 is involved in insulin-like growth factor-I resistance in aging articular chondrocytes. *J Orthop Res.* Aug 2006;24(8):1765-1772.

245. Roach HI, Yamada N, Cheung KS, et al. Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. *Arthritis Rheum.* Oct 2005;52(10):3110-3124.

246. Goldring MB, Otero M. Inflammation in osteoarthritis. *Curr Opin Rheumatol.* Sep 2011;23(5):471-478.

247. Liang B, Song Z, Wu B, et al. Evaluation of anti-IL-6 monoclonal antibody therapy using murine type II collagen-induced arthritis. *J Inflamm (Lond).* 2009;6:10.

248. Jazayeri JA, Carroll GJ, Vernallis AB. Interleukin-6 subfamily cytokines and rheumatoid arthritis: role of antagonists. *Int Immunopharmacol.* Jan 2010;10(1):1-8.

249. Singh JA, Furst DE, Bharat A, et al. 2012 update of the 2008 american college of rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. *Arthritis Care Res (Hoboken).* May 2012;64(5):625-639.

250. Kremer JM, Blanco R, Brzosko M, et al. Tocilizumab inhibits structural joint damage in rheumatoid arthritis patients with inadequate responses to methotrexate: results from the double-blind treatment phase of a randomized placebo-controlled trial of tocilizumab safety and prevention of structural joint damage at one year. *Arthritis Rheum.* Mar 2011;63(3):609-621.

251. Hashimoto J, Garnero P, van der Heijde D, et al. Humanized anti-interleukin-6-receptor antibody (tocilizumab) monotherapy is more effective in slowing radiographic progression in patients with rheumatoid arthritis at high baseline risk for structural damage evaluated with levels of biomarkers, radiography, and BMI: data from the SAMURAI study. *Mod Rheumatol.* Feb 2011;21(1):10-15.

252. Garnero P, Thompson E, Woodworth T, Smolen JS. Rapid and sustained improvement in bone and cartilage turnover markers with the anti-interleukin-6 receptor inhibitor tocilizumab plus methotrexate in rheumatoid arthritis patients with an inadequate response to methotrexate: results from a substudy of the multicenter double-blind, placebo-controlled trial of tocilizumab in inadequate responders to methotrexate alone. *Arthritis Rheum.* Jan 2010;62(1):33-43.

253. Regan EA, Bowler RP, Crapo JD. Joint fluid antioxidants are decreased in osteoarthritic joints compared to joints with macroscopically intact cartilage and subacute injury. *Osteoarthritis Cartilage.* Apr 2008;16(4):515-521.

254. Doss F, Menard J, Hauschild M, et al. Elevated IL-6 levels in the synovial fluid of osteoarthritis patients stem from plasma cells. *Scand J Rheumatol.* Mar-Apr 2007;36(2):136-139.

255. Ushiyama T, Chano T, Inoue K, Matsusue Y. Cytokine production in the infrapatellar fat pad: another source of cytokines in knee synovial fluids. *Ann Rheum Dis.* Feb 2003;62(2):108-112.

256. Desgeorges A, Gabay C, Silacci P, et al. Concentrations and origins of soluble interleukin 6 receptor-alpha in serum and synovial fluid. *J Rheumatol.* Aug 1997;24(8):1510-1516.

257. Kotake S, Sato K, Kim KJ, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res.* Jan 1996;11(1):88-95.

258. Stannus O, Jones G, Cicuttini F, et al. Circulating levels of IL-6 and TNF-alpha are associated with knee radiographic osteoarthritis and knee cartilage loss in older adults. *Osteoarthritis Cartilage.* Nov 2010;18(11):1441-1447.

259. Livshits G, Zhai G, Hart DJ, et al. Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study. *Arthritis Rheum.* Jul 2009;60(7):2037-2045.

260. Cuellar JM, Scuderi GJ, Cuellar VG, Golish SR, Yeomans DC. Diagnostic utility of cytokine biomarkers in the evaluation of acute knee pain. *J Bone Joint Surg Am.* Oct 2009;91(10):2313-2320.

261. Calamia V, Rocha B, Mateos J, Fernandez-Puente P, Ruiz-Romero C, Blanco FJ. Metabolic Labeling of Chondrocytes for the Quantitative Analysis of the Interleukin-1-beta-mediated Modulation of Their Intracellular and Extracellular Proteomes. *J Proteome Res.* Aug 5 2011;10(8):3701-3711.

262. Henrotin YE, De Groote DD, Labasse AH, et al. Effects of exogenous IL-1 beta, TNF alpha, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes. *Osteoarthritis Cartilage.* Sep 1996;4(3):163-173.

263. Guerne PA, Carson DA, Lotz M. IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. *J Immunol.* Jan 15 1990;144(2):499-505.

264. Fan Z, Bau B, Yang H, Aigner T. IL-1beta induction of IL-6 and LIF in normal articular human chondrocytes involves the ERK, p38 and NFkappaB signaling pathways. *Cytokine.* Oct 7 2004;28(1):17-24.

265. Wang P, Zhu F, Konstantopoulos K. Prostaglandin E2 induces interleukin-6 expression in human chondrocytes via cAMP/protein kinase A- and phosphatidylinositol 3-kinase-dependent NF-kappaB activation. *Am J Physiol Cell Physiol.* Jun 2010;298(6):C1445-1456.

266. Klatt AR, Paul-Klausch B, Klinger G, et al. A critical role for collagen II in cartilage matrix degradation: collagen II induces pro-inflammatory cytokines and MMPs in primary human chondrocytes. *J Orthop Res.* Jan 2009;27(1):65-70.

267. Sims NA, Walsh NC. GP130 cytokines and bone remodelling in health and disease. *BMB Rep.* Aug 2010;43(8):513-523.

268. Schaible HG, von Banchet GS, Boettger MK, et al. The role of proinflammatory cytokines in the generation and maintenance of joint pain. *Ann N Y Acad Sci.* Apr 2010;1193:60-69.

269. Namba A, Aida Y, Suzuki N, et al. Effects of IL-6 and soluble IL-6 receptor on the expression of cartilage matrix proteins in human chondrocytes. *Connect Tissue Res.* 2007;48(5):263-270.

270. Legendre F, Dudhia J, Pujol JP, Bogdanowicz P. JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of Type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression. *J Biol Chem.* Jan 31 2003;278(5):2903-2912.

271. Poree B, Kypriotou M, Chadjichristos C, et al. Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1.Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter. *J Biol Chem.* Feb 22 2008;283(8):4850-4865.

272. Guerne PA, Desgeorges A, Jaspar JM, et al. Effects of IL-6 and its soluble receptor on proteoglycan synthesis and NO release by human articular chondrocytes: comparison with IL-1. Modulation by dexamethasone. *Matrix Biol.* Jun 1999;18(3):253-260.

273. Rogerson FM, Chung YM, Deutscher ME, Last K, Fosang AJ. Cytokine-induced increases in ADAMTS-4 messenger RNA expression do not lead to increased aggrecanase activity in ADAMTS-5-deficient mice. *Arthritis Rheum.* Nov 2010;62(11):3365-3373.

274. de Hooge AS, van de Loo FA, Bennink MB, Arntz OJ, de Hooge P, van den Berg WB. Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. *Osteoarthritis Cartilage.* Jan 2005;13(1):66-73.

275. Ryu JH, Yang S, Shin Y, Rhee J, Chun CH, Chun JS. Interleukin-6 plays an essential role in hypoxia-inducible factor 2alpha-induced experimental osteoarthritic cartilage destruction in mice. *Arthritis Rheum.* Sep 2011;63(9):2732-2743.

276. Sanchez C, Deberg MA, Burton S, Devel P, Reginster JY, Henrotin YE. Differential regulation of chondrocyte metabolism by oncostatin M and interleukin-6. *Osteoarthritis Cartilage.* Oct 2004;12(10):801-810.

277. Ley C, Svala E, Nilton A, et al. Effects of high mobility group box protein-1, interleukin-1?, and interleukin-6 on cartilage matrix metabolism in three-dimensional equine chondrocyte cultures. *Connect Tissue Res.* 2011;52(4):290-300.

278. McNulty AL, Miller MR, O'Connor SK, Guilak F. The Effects of Adipokines on Cartilage and Meniscus Catabolism. *Connect Tissue Res.* Jul 25 2011.

279. Nordan RP, Potter M. A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. *Science.* Aug 1 1986;233(4763):566-569.

280. Heldens GT, Davidson EN, Vitters EL, et al. Catabolic factors and osteoarthritis-conditioned medium inhibit chondrogenesis of human mesenchymal stem cells. *Tissue Eng Part A.* Jan 2012;18(1-2):45-54.

281. Maiotti M, Monteleone G, Tarantino U, Fasciglione GF, Marini S, Coletta M. Correlation between osteoarthritic cartilage damage and levels of proteinases and proteinase inhibitors in synovial fluid from the knee joint. *Arthroscopy.* Jul-Aug 2000;16(5):522-526.

282. Klein-Wieringa IR, Kloppenburg M, Bastiaansen-Jenniskens YM, et al. The infrapatellar fat pad of patients with osteoarthritis has an inflammatory phenotype. *Ann Rheum Dis.* May 2011;70(5):851-857.

283. Distel E, Cadoudal T, Durant S, Poignard A, Chevalier X, Benelli C. The infrapatellar fat pad in knee osteoarthritis: an important source of interleukin-6 and its soluble receptor. *Arthritis Rheum.* Nov 2009;60(11):3374-3377.

284. Hui W, Bell M, Carroll G. Oncostatin M (OSM) stimulates resorption and inhibits synthesis of proteoglycan in porcine articular cartilage explants. *Cytokine.* Jun 1996;8(6):495-500.

285. Silacci P, Dayer JM, Desgeorges A, Peter R, Manueddu C, Guerne PA. Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. *J Biol Chem.* May 29 1998;273(22):13625-13629.

286. Long D, Blake S, Song XY, Lark M, Loeser RF. Human articular chondrocytes produce IL-7 and respond to IL-7 with increased production of matrix metalloproteinase-13. *Arthritis Res Ther.* 2008;10(1):R23.

287. Wang P, Zhu F, Lee NH, Konstantopoulos K. Shear-induced interleukin-6 synthesis in chondrocytes: roles of E prostanoid (EP) 2 and EP3 in cAMP/protein kinase A- and PI3-K/Akt-dependent NF-kappaB activation. *J Biol Chem.* Aug 6 2010;285(32):24793-24804.

288. Pulai JI, Chen H, Im HJ, et al. NF-kappa B mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. *J Immunol.* May 1 2005;174(9):5781-5788.

289. Hashizume M, Mihara M. Desirable effect of combination therapy with high molecular weight hyaluronate and NSAIDs on MMP production. *Osteoarthritis Cartilage.* Nov 2009;17(11):1513-1518.

290. Villiger PM, Kusari AB, ten Dijke P, Lotz M. IL-1 beta and IL-6 selectively induce transforming growth factor-beta isoforms in human articular chondrocytes. *J Immunol.* Sep 15 1993;151(6):3337-3344.

291. Hashizume M, Hayakawa N, Suzuki M, Mihara M. IL-6/sIL-6R trans-signalling, but not TNF-alpha induced angiogenesis in a HUVEC and synovial cell co-culture system. *Rheumatol Int.* Oct 2009;29(12):1449-1454.

292. De Benedetti F. The impact of chronic inflammation on the growing skeleton: lessons from interleukin-6 transgenic mice. *Horm Res.* Nov 2009;72 Suppl 1:26-29.

293. Le Goff B, Blanchard F, Berthelot JM, Heymann D, Maugars Y. Role for interleukin-6 in structural joint damage and systemic bone loss in rheumatoid arthritis. *Joint Bone Spine.* May 2010;77(3):201-205.

294. van de Loo FA, Joosten LA, van Lent PL, Arntz OJ, van den Berg WB. Role of interleukin-1, tumor necrosis factor alpha, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of in situ blocking in murine antigen- and zymosan-induced arthritis. *Arthritis Rheum.* Feb 1995;38(2):164-172.

295. van de Loo FA, Kuiper S, van Enckevort FH, Arntz OJ, van den Berg WB. Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Am J Pathol.* Jul 1997;151(1):177-191.

296. Klop C, de Vries F, Lalmohamed A, et al. COX-2-Selective NSAIDs and Risk of Hip or Knee Replacements: A Population-Based Case-Control Study. *Calcif Tissue Int.* Oct 9 2012.

297. Rubenhagen R, Schuttrumpf JP, Sturmer KM, Frosch KH. Interleukin-7 levels in synovial fluid increase with age and MMP-1 levels decrease with progression of osteoarthritis. *Acta Orthop.* Feb 2012;83(1):59-64.

298. Pallante AL, Bae WC, Chen AC, Gortz S, Bugbee WD, Sah RL. Chondrocyte viability is higher after prolonged storage at 37 degrees C than at 4 degrees C for osteochondral grafts. *Am J Sports Med.* Nov 2009;37 Suppl 1:24S-32S.

299. Drobnic M, Mars T, Alibegovic A, et al. Viability of human chondrocytes in an ex vivo model in relation to temperature and cartilage depth. *Folia Biol (Praha).* 2005;51(4):103-108.

300. Madea B, Kreuser C, Banaschak S. Postmortem biochemical examination of synovial fluid--a preliminary study. *Forensic Sci Int.* Apr 15 2001;118(1):29-35.

301. Tumram NK, Bardale RV, Dongre AP. Postmortem analysis of synovial fluid and vitreous humour for determination of death interval: A comparative study. *Forensic Sci Int.* Jan 30 2011;204(1-3):186-190.

302. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. *Cytokine.* Nov 2000;12(11):1712-1716.

303. Felson DT. Clinical practice. Osteoarthritis of the knee. *N Engl J Med.* Feb 23 2006;354(8):841-848.

304. Frampton JE, Keating GM. Celecoxib: a review of its use in the management of arthritis and acute pain. *Drugs.* 2007;67(16):2433-2472.

305. Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum.* Dec 2003;33(3):155-167.

306. Geng Y, Blanco FJ, Cornelisson M, Lotz M. Regulation of cyclooxygenase-2 expression in normal human articular chondrocytes. *J Immunol.* Jul 15 1995;155(2):796-801.

307. Mastbergen SC, Bijlsma JW, Lafeber FP. Selective COX-2 inhibition is favorable to human early and late-stage osteoarthritic cartilage: a human in vitro study. *Osteoarthritis Cartilage.* Jun 2005;13(6):519-526.

308. Mastbergen SC, Marijnissen AC, Vianen ME, et al. Inhibition of COX-2 by celecoxib in the canine groove model of osteoarthritis. *Rheumatology (Oxford).* Apr 2006;45(4):405-413.

309. de Boer TN, Huisman AM, Polak AA, et al. The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: ex vivo evaluation of human cartilage tissue after in vivo treatment. *Osteoarthritis Cartilage.* Apr 2009;17(4):482-488.

310. El Hajjaji H, Marcelis A, Devogelaer JP, Manicourt DH. Celecoxib has a positive effect on the overall metabolism of hyaluronan and proteoglycans in human osteoarthritic cartilage. *J Rheumatol.* Nov 2003;30(11):2444-2451.

311. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* Oct 2001;15(12):2057-2072.

312. Zweers MC, de Boer TN, van Roon J, Bijlsma JW, Lafeber FP, Mastbergen SC. Celecoxib: considerations regarding its potential disease-modifying properties in osteoarthritis. *Arthritis Res Ther.* 2011;13(5):239.

313. Fukai A, Kamekura S, Chikazu D, et al. Lack of a chondroprotective effect of cyclooxygenase 2 inhibition in a surgically induced model of osteoarthritis in mice. *Arthritis Rheum.* Jan 2012;64(1):198-203.

314. Sanchez C, Mateus MM, Defresne MP, Crielaard JM, Reginster JY, Henrotin YE. Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1beta and nonsteroidal antiinflammatory drugs. *J Rheumatol.* Apr 2002;29(4):772-782.

315. Welting TJ, Caron MM, Emans PJ, et al. Inhibition of cyclooxygenase-2 impacts chondrocyte hypertrophic differentiation during endochondral ossification. *Eur Cell Mater.* 2011;22:420-436; discussion 436-427.

316. [www.federa.org] DfoBSS.

317. Rosenberg L. Chemical basis for the histological use of safranin O in the study of articular cartilage. *J Bone Joint Surg Am.* Jan 1971;53(1):69-82.

318. Prins MJ, Verhage RJ, ten Kate FJ, van Hillegersberg R. Cyclooxygenase isoenzyme-2 and vascular endothelial growth factor are associated with poor prognosis in esophageal adenocarcinoma. *J Gastrointest Surg.* May 2012;16(5):956-966.

319. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* Sep 4 1986;883(2):173-177.

320. Mastbergen SC, Lafeber FP, Bijlsma JW. Selective COX-2 inhibition prevents proinflammatory cytokine-induced cartilage damage. *Rheumatology (Oxford).* Jul 2002;41(7):801-808.

321. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis.* Sep 2005;64(9):1263-1267.

322. Ning L, Ishijima M, Kaneko H, et al. Correlations between both the expression levels of inflammatory mediators and growth factor in medial perimeniscal synovial tissue and the severity of medial knee osteoarthritis. *Int Orthop.* Jun 2011;35(6):831-838.

323. Gierman LM, van El B, van der Ham F, et al. Profiling the secretion of soluble mediators by end stage osteoarthritis synovial tissue explants reveals a reduced responsiveness to an inflammatory trigger. *PLoS One.* 2013;8(5):e62634.

324. Hardy MM, Seibert K, Manning PT, et al. Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. *Arthritis Rheum.* Jul 2002;46(7):1789-1803.

325. Jakob M, Demarteau O, Suetterlin R, Heberer M, Martin I. Chondrogenesis of expanded adult human articular chondrocytes is enhanced by specific prostaglandins. *Rheumatology (Oxford).* Jul 2004;43(7):852-857.

326. Laufer S. Role of eicosanoids in structural degradation in osteoarthritis. *Curr Opin Rheumatol.* Sep 2003;15(5):623-627.

327. Mastellos D, Germenis AE, Lambris JD. Complement: an inflammatory pathway fulfilling multiple roles at the interface of innate immunity and development. *Curr Drug Targets Inflamm Allergy.* Feb 2005;4(1):125-127.

328. Park M, Yong Y, Choi SW, Kim JH, Lee JE, Kim DW. Constitutive RelA activation mediated by Nkx3.2 controls chondrocyte viability. *Nat Cell Biol.* Mar 2007;9(3):287-298.

329. Ushita M, Saito T, Ikeda T, et al. Transcriptional induction of SOX9 by NF-kappaB family member RelA in chondrogenic cells. *Osteoarthritis Cartilage.* Aug 2009;17(8):1065-1075.

330. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev.* Aug-Oct 2002;13(4-5):413-421.

331. Dave M, Amin AR. Yin-Yang regulation of prostaglandins and nitric oxide by PGD2 in human arthritis: Reversal by celecoxib. *Immunol Lett.* Apr 2013;152(1):47-54.

332. Lavietes BB. Kinetics of matrix synthesis in cartilage cell cultures. *Exp Cell Res.* Sep 1971;68(1):43-48.

333. Sofat N, Ejindu V, Kiely P. What makes osteoarthritis painful? The evidence for local and central pain processing. *Rheumatology (Oxford).* Dec 2011;50(12):2157-2165.

334. Dray A, Read SJ. Arthritis and pain. Future targets to control osteoarthritis pain. *Arthritis Res Ther.* 2007;9(3):212.

335. Bianchi M, Broggini M. A randomised, double-blind, clinical trial comparing the efficacy of nimesulide, celecoxib and rofecoxib in osteoarthritis of the knee. *Drugs.* 2003;63 Suppl 1:37-46.

336. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J Bone Joint Surg Am.* Apr 1993;75(4):532-553.

337. Brun P, Dickinson SC, Zavan B, Cortivo R, Hollander AP, Abatangelo G. Characteristics of repair tissue in second-look and third-look biopsies from patients treated with engineered cartilage: relationship to symptomatology and time after implantation. *Arthritis Res Ther.* 2008;10(6):R132.

338. Vos T, Flaxman AD, Naghavi M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* Dec 15 2012;380(9859):2163-2196.

339. Richardson JB, Caterson B, Evans EH, Ashton BA, Roberts S. Repair of human articular cartilage after implantation of autologous chondrocytes. *J Bone Joint Surg Br.* Nov 1999;81(6):1064-1068.

340. Roberts S, Hollander AP, Caterson B, Menage J, Richardson JB. Matrix turnover in human cartilage repair tissue in autologous chondrocyte implantation. *Arthritis Rheum.* Nov 2001;44(11):2586-2598.

341. Harris JD, Siston RA, Pan X, Flanigan DC. Autologous chondrocyte implantation: a systematic review. *J Bone Joint Surg Am.* Sep 15 2010;92(12):2220-2233.

342. Ebert JR, Smith A, Edwards PK, Hambly K, Wood DJ, Ackland TR. Factors Predictive of Outcome 5 Years After Matrix-Induced Autologous Chondrocyte Implantation in the Tibiofemoral Joint. *Am J Sports Med.* Apr 25 2013.

343. Noyes FR, Barber-Westin SD. Advanced Patellofemoral Cartilage Lesions in Patients Younger Than 50 Years of Age: Is There an Ideal Operative Option? *Arthroscopy.* May 24 2013.

344. Niemeyer P, Salzmann GM, Hirschmuller A, Sudkamp NP. [Factors that influence clinical outcome following autologous chondrocyte implantation for cartilage defects of the knee]. *Z Orthop Unfall.* Feb 2012;150(1):83-88.

345. Krishnan SP, Skinner JA, Bartlett W, et al. Who is the ideal candidate for autologous chondrocyte implantation? *J Bone Joint Surg Br.* Jan 2006;88(1):61-64.

346. Jaiswal PK, Bentley G, Carrington RW, Skinner JA, Briggs TW. The adverse effect of elevated body mass index on outcome after autologous chondrocyte implantation. *J Bone Joint Surg Br.* Oct 2012;94(10):1377-1381.

347. Jaiswal PK, Macmull S, Bentley G, Carrington RW, Skinner JA, Briggs TW. Does smoking influence outcome after autologous chondrocyte implantation?: A case-controlled study. *J Bone Joint Surg Br.* Dec 2009;91(12):1575-1578.

348. Cheung KP, Taylor KR, Jameson JM. Immunomodulation at epithelial sites by obesity and metabolic disease. *Immunol Res.* Jun 2012;52(3):182-199.

349. Bayliss MT, Howat S, Davidson C, Dudhia J. The organization of aggrecan in human articular cartilage. Evidence for age-related changes in the rate of aggregation of newly synthesized molecules. *J Biol Chem.* Mar 3 2000;275(9):6321-6327.

350. Peterson L, Brittberg M, Kiviranta I, Akerlund EL, Lindahl A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. *Am J Sports Med.* Jan-Feb 2002;30(1):2-12.

351. Henderson IJ, Tuy B, Connell D, Oakes B, Hettwer WH. Prospective clinical study of autologous chondrocyte implantation and correlation with MRI at three and 12 months. *J Bone Joint Surg Br.* Sep 2003;85(7):1060-1066.

352. Tins BJ, McCall IW, Takahashi T, et al. Autologous chondrocyte implantation in knee joint: MR imaging and histologic features at 1-year follow-up. *Radiology.* Feb 2005;234(2):501-508.

353. Loken S, Ludvigsen TC, Hoysveen T, Holm I, Engebretsen L, Reinholt FP. Autologous chondrocyte implantation to repair knee cartilage injury: ultrastructural evaluation at 2 years and long-term follow-up including muscle strength measurements. *Knee Surg Sports Traumatol Arthrosc.* Nov 2009;17(11):1278-1288.

354. Roberts S, Menage J, Sandell LJ, Evans EH, Richardson JB. Immunohistochemical study of collagen types I and II and procollagen IIA in human cartilage repair tissue following autologous chondrocyte implantation. *Knee.* Oct 2009;16(5):398-404.

355. Roberts S, McCall IW, Darby AJ, et al. Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology. *Arthritis Res Ther.* 2003;5(1):R60-73.

356. Vasara AI, Nieminen MT, Jurvelin JS, Peterson L, Lindahl A, Kiviranta I. Indentation stiffness of repair tissue after autologous chondrocyte transplantation. *Clin Orthop Relat Res.* Apr 2005(433):233-242.

357. Sharma A, Wood LD, Richardson JB, Roberts S, Kuiper NJ. Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage. *Arthritis Res Ther.* 2007;9(4):R79.

358. Aigner T, Zhu Y, Chansky HH, Matsen FA, 3rd, Maloney WJ, Sandell LJ. Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. *Arthritis Rheum.* Jul 1999;42(7):1443-1450.

359. Funderburgh JL, Funderburgh ML, Mann MM, Conrad GW. Physical and biological properties of keratan sulphate proteoglycan. *Biochem Soc Trans.* Nov 1991;19(4):871-876.

360. Niemeyer P, Pestka JM, Salzmann GM, Sudkamp NP, Schmal H. Influence of cell quality on clinical outcome after autologous chondrocyte implantation. *Am J Sports Med.* Mar 2012;40(3):556-561.

361. Pietschmann MF, Horng A, Niethammer T, et al. Cell quality affects clinical outcome after MACI procedure for cartilage injury of the knee. *Knee Surg Sports Traumatol Arthrosc.* Nov 2009;17(11):1305-1311.

362. Lahm A, Kasch R, Mrosek E, et al. Semiquantitative analysis of ECM molecules in the different cartilage layers in early and advanced osteoarthritis of the knee joint. *Histol Histopathol.* May 2012;27(5):609-615.

363. Ono Y, Sakai T, Hiraiwa H, et al. Chondrogenic capacity and alterations in hyaluronan synthesis of cultured human osteoarthritic chondrocytes. *Biochem Biophys Res Commun.* May 20 2013.

364. Vasara AI, Konttinen YT, Peterson L, Lindahl A, Kiviranta I. Persisting high levels of synovial fluid markers after cartilage repair: a pilot study. *Clin Orthop Relat Res.* Jan 2009;467(1):267-272.

365. Marini S, Fasciglione GF, Monteleone G, Maiotti M, Tarantino U, Coletta M. A correlation between knee cartilage degradation observed by arthroscopy and synovial proteinases activities. *Clin Biochem.* Jun 2003;36(4):295-304.

366. Schmal H, Niemeyer P, Sudkamp NP, Gerlach U, Dovi-Akue D, Mehlhorn AT. Pain perception in knees with circumscribed cartilage lesions is associated with intra-articular IGF-1 expression. *Am J Sports Med.* Sep 2011;39(9):1989-1996.

367. Vincent TL, McLean CJ, Full LE, Peston D, Saklatvala J. FGF-2 is bound to perlecan in the pericellular matrix of articular cartilage, where it acts as a chondrocyte mechanotransducer. *Osteoarthritis Cartilage.* Jul 2007;15(7):752-763.

368. Wang JH, Thampatty BP. An introductory review of cell mechanobiology. *Biomech Model Mechanobiol.* Mar 2006;5(1):1-16.

369. Khoshgoftar M, Wilson W, Ito K, van Donkelaar R. The effects of matrix inhomogeneities on the cellular mechanical environment in tissue-engineered cartilage: an in silico investigation. *Tissue Eng Part C Methods.* May 16 2013.

370. Healy ZR, Zhu F, Stull JD, Konstantopoulos K. Elucidation of the signaling network of COX-2 induction in sheared chondrocytes: COX-2 is induced via a Rac/MEKK1/MKK7/JNK2/c-Jun-C/EBPbeta-dependent pathway. *Am J Physiol Cell Physiol.* May 2008;294(5):C1146-1157.

371. Chauffier K, Laiguillon MC, Bougault C, et al. Induction of the chemokine il-8/kc by the articular cartilage: possible influence on osteoarthritis. *Joint Bone Spine.* Dec 2012;79(6):604-609.

372. Watt FE, Ismail HM, Didangelos A, et al. Src and fibroblast growth factor 2 independently regulate signaling and gene expression induced by experimental injury to intact articular cartilage. *Arthritis Rheum.* Feb 2013;65(2):397-407.

373. Carafoli F, Hohenester E. Collagen recognition and transmembrane signalling by discoidin domain receptors. *Biochim Biophys Acta.* Nov 2 2012.

374. Poudel B, Ki HH, Lee YM, Kim DK. Induction of IL-12 production by the activation of discoidin domain receptor 2 via NF-kappaB and JNK pathway. *Biochem Biophys Res Commun.* May 10 2013;434(3):584-588.

375. Poudel B, Yoon DS, Lee JH, Lee YM, Kim DK. Collagen I enhances functional activities of human monocyte-derived dendritic cells via discoidin domain receptor 2. *Cell Immunol.* Jul-Aug 2012;278(1-2):95-102.

376. Diederichs S, Baral K, Tanner M, Richter W. Interplay between local versus soluble transforming growth factor-beta and fibrin scaffolds: role of cells and impact on human mesenchymal stem cell chondrogenesis. *Tissue Eng Part A.* Jun 2012;18(11-12):1140-1150.

377. Ha H, Kim I, Lee SK, Yoon JI, Kim DE, Kim M. Fibrin glue improves the therapeutic effect of MSCs by sustaining survival and paracrine function. *Tissue Eng Part A.* May 24 2013.

378. Liang MS, Andreadis ST. Engineering fibrin-binding TGF-beta1 for sustained signaling and contractile function of MSC based vascular constructs. *Biomaterials.* Nov 2011;32(33):8684-8693.

379. Anitua E, Prado R, Orive G. Endogenous morphogens and fibrin bioscaffolds for stem cell therapeutics. *Trends Biotechnol.* Jun 2013;31(6):364-374.

380. Kim M, Kim SE, Kang SS, Kim YH, Tae G. The use of de-differentiated chondrocytes delivered by a heparin-based hydrogel to regenerate cartilage in partial-thickness defects. *Biomaterials.* Nov 2011;32(31):7883-7896.

381. Kwon H, Sun L, Cairns DM, et al. The influence of scaffold material on chondrocytes under inflammatory conditions. *Acta Biomater.* May 2013;9(5):6563-6575.

382. Jenner F. *On the genesis of articular cartilage. Embryonic joint development and gene expression - implications for tissue engineering*. Utrecht: Faculty of Veterinary Medicine, Utrecht University; 2013.

383. Shi S, Mercer S, Eckert GJ, Trippel SB. Growth factor transgenes interactively regulate articular chondrocytes. *J Cell Biochem.* Apr 2013;114(4):908-919.

384. Pujol JP, Chadjichristos C, Legendre F, et al. Interleukin-1 and transforming growth factor-beta 1 as crucial factors in osteoarthritic cartilage metabolism. *Connect Tissue Res.* 2008;49(3):293-297.

385. Bauge C, Girard N, Leclercq S, Galera P, Boumediene K. Regulatory mechanism of transforming growth factor beta receptor type II degradation by interleukin-1 in primary chondrocytes. *Biochim Biophys Acta.* May 2012;1823(5):983-986.

386. Montaseri A, Busch F, Mobasheri A, et al. IGF-1 and PDGF-bb suppress IL-1beta-induced cartilage degradation through down-regulation of NF-kappaB signaling: involvement of Src/PI-3K/AKT pathway. *PLoS One.* 2011;6(12):e28663.

387. Thomas B, Thirion S, Humbert L, et al. Differentiation regulates interleukin-1beta-induced cyclo-oxygenase-2 in human articular chondrocytes: role of p38 mitogen-activated protein kinase. *Biochem J*. Mar 1 2002;362(Pt 2):367-373.

388. Smelter E, Hochberg MC. New treatments for osteoarthritis. *Curr Opin Rheumatol*. May 2013;25(3):310-316.

389. Rai MF, Patra D, Sandell LJ, Brophy RH. Transcriptome analysis of human injured meniscus reveals a distinct phenotype of meniscus degeneration with aging. *Arthritis Rheum*. May 8 2013:NA.

390. Funck-Brentano T, Cohen-Solal M. Crosstalk between cartilage and bone: when bone cytokines matter. *Cytokine Growth Factor Rev*. Apr 2011;22(2):91-97.

NEDERLANDSE SAMENVATTING

Regeneratie van het gewrichtskraakbeen is het ultieme doel in de behandeling van focale kraakbeendefecten van de knie. Herstel van het gewrichtskraakbeen met fibreus kraakbeenweefsel is waarschijnlijk gerelateerd aan het falen van de behandeling. Bovendien kan adequate en tijdige behandeling van een focaal kraakbeendefect mogelijk de ontwikkeling van artrose voorkomen. Artrose is een groot probleem voor de samenleving, met name omdat er nog geen behandeling bestaat die het verloop van de ziekte kan beïnvloeden.

Autologe chondrocytenimplantatie is de eerste, op cellen gebaseerde, behandeling die de potentie heeft om werkelijk regeneratie, en niet reparatie, van het kraakbeen te bewerkstelligen. Uit vroege studies die de samenstelling van het herstelweefsel onderzochten, is gebleken dat het herstellende kraakbeen wordt beinvloed van buitenaf, zoals bijvoorbeeld enzymen in de gewrichtsvloeistof. Hierdoor is het idee ontstaan dat gewrichtshomeostase het kraakbeenherstel beïnvloedt en dat, onder de juiste omstandigheden, regeneratie van het gewrichtskraakbeen mogelijk is. Om zowel een anatomisch als functioneel normaal kraakbeen te regenereren, is het noodzakelijk dat we de complexiteit van de gewrichtshomeostase beter begrijpen en alle aspecten van het gewrichtsmilieu, inclusief de oplosbare factoren die in de knie een rol spelen, adresseren.

Identificatie van de beste chirurgische behandeling

Er bestaan een aantal chirurgische behandelingsmogelijkheden voor symptomatische kraakbeendefecten van de knie. Gerandomiseerde klinische onderzoeken hebben tot nu toe geen grote verschillen laten zien tussen de verschillende behandelingen voor de algemene patiëntenpopulatie met een kraakbeendefect. Echter, wij laten zien dat voor patiënten met een groot kraakbeendefect (> 4 cm^2), autologe chondrocytenimplantatie resulteert in een betere klinische uitkomst na twee jaar dan microfractuur (*Hoofdstuk 2*). Histologische analyse van het herstelweefsel en MRI analyse lieten echter geen verschillen zien tussen de twee behandelingen *(Hoofdstuk 2*). Gerandomiseerde klinische onderzoeken met voldoende patiëntenaantallen zijn noodzakelijk voor de wetenschappelijke onderbouwing van de klinische indicatiestelling voor specifieke patiëntgroepen met focale kraakbeendefecten van de knie. Het is echter ook belangrijk dat de innovatie van autologe chondrocytenimplantatie doorgaat, omdat deze behandeling nog verscheidene beperkingen kent; met name de kwaliteit van het herstelweefsel zou verbeterd kunnen worden.

Verbetering van het herstelweefsel door het fenotype van de chondrocyten te adresseren

Het herstelweefsel dat wordt gevormd na autologe chondrocytenimplantatie is op zijn best hyalien-achtig en verschilt in verscheidene opzichten van het oorspronkelijke kraakbeen: het bevat type I collageen, de type II collageen fibrillen hebben niet de juiste karakteristieke organisatie en de hoeveelheid en de samenstelling van de types glycosaminoglycanen is afwijkend. Dit wordt waarschijnlijk onder andere veroorzaakt doordat de chondrocyten die worden geïmplanteerd in de knie, zijn gededifferentieerd doordat ze eerst in monolaag zijn geëxpandeerd. Onderzoek naar de mechanismes waardoor het fenotype van de chondrocyten het herstelweefsel beïnvloedt, is ook van belang wanneer, behalve de nu gebruikte chondrocyten uit gezond kraakbeen, ook chondrocyten verkregen uit de randen van het defect of uit artrotisch kraakbeen gebruikt gaan worden. Deze laatste twee bronnen van kraakbeen zijn interessant om zo de additionele schade die wordt toegebracht aan de knie te reduceren en celtherapie ook voor artrosepatiënten mogelijk te maken. De klinische resultaten van autologe chondrocytenimplantatie bij artrosepatiënten zijn nog niet heel goed. Echter het regeneratiepotentiaal van deze chondrocyten is minstens net zo goed als van gezonde chondrocyten (*Hoofdstuk 3*). Waarschijnlijk worden de inferieure resultaten voor een groot deel veroorzaakt door de verstoring van de gewrichtshomeostase.

Oplosbare mediatoren in het gewrichtsmilieu

Behalve het fenotype van de chondrocyten, is ook het gewrichtsmilieu, inclusief de oplosbare mediatoren aanwezig in de knie, van belang voor regeneratie van het gewrichtskraakbeen.

De synoviale vloeistof bevat veel van dit soort oplosbare factoren en eerder is gebleken dat de samenstelling van deze vloeistof verandert na gewrichtstrauma en bij artrose, en dan belemmerend is voor kraakbeenregeneratie. Ook bij patiënten met symptomatische kraakbeendefecten bleek de samenstelling van de synoviale vloeistof anders te zijn dan bij donoren zonder kraakbeenschade of patiënten met artrose, en verhoogde hoeveelheden pro-inflammatoire en pro-herstel cytokinen te bevatten (*Hoofdstuk 3*). Behoudens de synoviale vloeistof, bevinden zich ook oplosbare mediatoren in het kraakbeen zelf (*Hoofdstuk 3*). Bovendien waren de concentraties van verscheidene inflammatoire cytokines in kraakbeenweefsel vele malen hoger dan in de synoviale vloeistof. Dit suggereert dat de mediatoren door de chondrocyten zelf worden geproduceerd, auto- en of paracrien werken en mogelijk een belangrijkere rol spelen dan afgeleid zou kunnen worden uit hun aanwezigheid in de synoviale vloeistof.

Wij vonden een hoge productie van cytokines gedurende *in vitro* regeneratie (*Hoofdstukken 3 en 4*). Bovendien werd deze productie gemoduleerd door het biomateriaal gebruikt voor het opnemen van de chondrocyten (*Hoofdstuk 4*). Met name niet-geëx-

pandeerde chondrocyten lieten een specifiek afgiftepatroon van mediatoren zien in reactie op het biomateriaal. Bovendien was kraakbeenvorming door niet geëxpandeerde chondrocyten in veel grotere mate afhankelijk van het gebruikte biomateriaal dan het geval was bij het gebruik van geëxpandeerde chondrocyten. Echter, er was geen relatie tussen de productie van specifieke mediatoren en kraakbeenvorming (*Hoofdstukken 3 en 4*). Het afgifteprofiel van oplosbare mediatoren was nauwelijks verschillend tussen niet-geëxpandeerde chondrocyten uit gezond kraakbeen, kraakbeen uit de randen van een defect, en artrosekraakbeen, terwijl er wel verschillen waren in regeneratiepotentiaal (*Hoofdstuk 3*). Geëxpandeerde chondrocyten van defecten en artrosekraakbeen lieten met name verhoogde productie van met veroudering geassocieerde cytokines zien, terwijl ze evenveel kraakbeenmatrix vormden als chondrocyten uit gezond kraakbeen (*Hoofdstuk 3*).

Moduleren van oplosbare mediatoren om kraakbeenregeneratie te verbeteren

Het ontcijferen van de werking en functie van al deze mediatoren zal niet eenvoudig zijn. Verhoogde productie van specifieke factoren is niet indicatief voor een rol in kraakbeendegeneratie of -regeneratie (*Hoofdstukken 3, 4, 5 en 6*). IL-6, een inflammatoire cytokine, bleek in verhoogde mate aanwezig te zijn in de synoviale vloeistof van patiënten met symptomatische kraakbeendefecten en artrose (*Hoofdstuk 6*). Echter IL-6 had hier geen katabole rol zoals bij patiënten met reumatoïde artritis, maar juist een licht anabole functie gedurende *in vitro* regeneratie en herstel van artrotisch kraakbeen. Ook de aanwezigheid van CXCL8 (IL-8), een cytokine waarvan eerder gesuggereerd is dat het hypertrofie van chondrocyten zou veroorzaken had maar zeer minimale effecten op kraakbeenmatrixproductie, en induceerde geen hypertrofie gedurende regeneratie, noch in kweek met artrotisch kraakbeen (*Hoofdstuk 5*). Ook het remmen van inflammatie met de COX-2 remmer celecoxib had geen effect op kraakbeenproductie, alhoewel het wel de vorming van inflammatoire mediatoren remde (*Hoofdstuk 7*). Ons werk trekt de algemeen aangenomen veronderstelling dat inflammatie nadelig is voor de integriteit van kraakbeen, in twijfel.

Conclusies

Regeneratieve behandelingen beantwoorden een tot nu toe onbevredigde klinische behoefte. Autologe chondrocytenimplantatie resulteert in betere klinische uitkomst na 2 jaar bij patiënten met defecten groter dan 4 cm^2 en heeft de potentie om in regeneratie van het gewrichtskraakbeen te resulteren, maar daarvoor zal herstel van de gewrichtshomeostase van groot belang zijn. Patiënten met symptomatische kraakbeendefecten hebben een verhoogde aanwezigheid van een aantal pro-inflammatoire en pro-herstel mediatoren in de knie die de kraakbeenregeneratie zouden kunnen beïnvloeden. Modulatie van een selectie individuele oplosbare mediatoren had geen

grote invloed op de vorming van kraakbeen, mogelijk doordat er tegenovergesteld werkende mediatoren aanwezig waren, maar ook omdat deze een geheel andere rol kunnen spelen. Bovendien bleken chondrocyten zelf grote hoeveelheden van deze oplosbare mediatoren te kunnen produceren en waren lokale concentraties van inflammatoire cytokines in kraakbeen soms vele malen hoger dan in synoviale vloeistof. Om de kwaliteit van het herstelkraakbeen en klinische uitkomsten te optimaliseren, zal een gecombineerde aanpak van belang zijn, door het interfereren met meerdere mediatoren, zowel in synoviale vloeistof als endogeen geproduceerd door chondrocyten.

ACKNOWLEDGEMENTS

Prof. Dr. Saris, beste Daan,
Dit boekje is er gekomen door jouw visie. Jij leidt je team met bijna helderziende vooruitstrevendheid die ik enorm bewonder. Alleen jij kent het volledige masterplan, maar op de momenten dat het ertoe doet weet ik dat ik jou in mijn team heb. Ik ben je erg dankbaar voor de kansen die jij hebt gecreëerd.

Prof. Dr. Dhert, beste Wouter,
Alhoewel jij niet mijn eerste promotor bent, is talloze malen gebleken hoe betrokken je bent bij mijn ontwikkeling. Ik waardeer het heel erg dat je mij regelmatig vroeg om even bij je langs te lopen. Je hebt altijd oog voor wat er speelt op de afdeling, ook onder de promovendi. Ik denk dat veel van de goede team spirit op onze afdeling te maken heeft met jouw leiding , integerheid en inzet.

Dr. Creemers, beste Laura,
Van jou heb ik echt geleerd wat wetenschap inhoudt. Bedankt dat je altijd enthousiast bleef ook al wilde ik zelf soms mijn data in de prullenbak gooien. Ik heb je betrokkenheid en inlevingsvermogen altijd gevoeld en ik zal het bloemetje voor mijn eerste paper nooit vergeten.

Papa, mama en Kenji,
Pap, mam, het klinkt zo cliché, maar het is echt waar: jullie hebben me altijd vrij gelaten om mijn eigen keuzes te maken. Mam, mijn leergierigheid en doorzettingsvermogen komen zeker weten van jou. Dad, you have taught me that being deliberate and modest goes a long way. Thank you for your chanting. Kenji, mijn niet meer zo kleine broertje, ik vind het knap van je dat je je niet uit het veld laat slaan.

Opa, bedankt dat ik altijd bij jullie mocht logeren, en dat de sjoelbak altijd klaar stond als ik terug kwam van mijn coschap.
Oma, mijn favoriete oma. Van jou heb ik zoveel geleerd, ik bedoel, wie van mijn leeftijd kan er nu sokken breien? De belangrijkste lessen zijn echter niet zomaar in woorden uit te drukken. Jij bent supernuchter, weet van aanpakken, zeurt nooit, wil zelfs nu op verjaardagen mij nog op de goede stoel laten zitten omdat die 'oude billen eerst op moeten' en laat je door niemand de les lezen. Deze kerst zit ik weer naast jou.

Paranimfen Kim en Michiel,
Kim, my sista from another mista, zo voelt het soms echt.

Beekie, de sportieve held. Zonder jou was dit een heel dun boekje geworden. Ik hoop dat we nog heel veel samen gaan werken en er nog vele weekendjes Amsterdam mogen volgen.

Joris, ik zou jou toch niet zomaar onder de collega's kunnen scharen. Jij bent toch een beetje mijn grote voorbeeld. Jij hebt me laten zien hoe leuk orthopaedie is en hebt mij het zelfvertrouwen gegeven om ervoor te gaan.

Claire, we zien elkaar weliswaar niet meer letterlijk elke dag zoals vroeger, maar voor mij is er niets veranderd.

Hanneke, fijn dat je er altijd voor me bent. Marrit, bedankt voor je verfrissende blik en relativeringsvermogen. Hanna, jammer dat we voorlopig niet meer samen naar cursus kunnen, maar dat komt goed.

Rhandy, uberregelneef, keukenprinses en touroperator. Met jou is het altijd heerlijk chillen, we hebben nog maar een paar W-hotels te gaan.

Mich, zullen we nog een keer gaan shoppen in Houston? En of je aub niet meer wilt springen in de lift.

Zeergeleerde Fiona, rasoptimist, fijn hè dat we dit achter de rug hebben.

Hsiao-yin, thank you for the gossip!

Marijn: ik had het van te voren nooit gedacht, maar ik heb de laatste tijd steeds vaker teruggedacht aan de adviezen die je mij aan het begin van mijn promotie gaf. Dankjewel.

De boys: Tom, (toch handiger hè zo'n digitale agenda), Tommy,(ouwe, jij komt toch wel naar het diak? Kunnen we samen Caribische muziekjes luisteren), Loek en Jetze. Mattie, nog steeds een beetje een van de boys, ik mis je nu al in het diak.

Alle andere collega's: Linda (we gaan nog heel veel wijntjes drinken samen toch?), Willemijn, Anita, Saskia, Angela, Yvonne, Debby, Jos (dank dat je er vanaf het begin vertrouwen in had), Jacqueline, Lucienne, Wouter, Ruud, Mechteld, Peter, Croes, Janssen en Ruth (fijn om jou weer als collega te hebben).

SUMMIT study team: I feel extremely fortunate to have been part of such a wonderful team. Veel dank ook aan Jessica, Janet en alle polidames. Danny, ik vind je fantastisch. Ik

hoop dat ik een klein beetje heb opgepikt van jouw talent om patiënten te motiveren. Ook aan iedereen van de fysio CMH, hartelijk dank voor de koffie en gezelligheid.

Prof. Dr. van Osch, beste Gerjo, ik ben je heel erg dankbaar voor je enorme enthousiasme, inzet en talent om de belangrijkste boodschap nog veel krachtiger te brengen. Ik heb heel veel van je geleerd.

Prof. Dr. Lafeber, beste Floris, ik ken weinig professoren die ik zaterdagochtend om acht uur mag bellen om in huis te komen voor kraakbeen. Veel dank hiervoor.

Prof. Dr. Radstake en Dr. Welting, hartelijk dank voor jullie waardevolle input.

Veel dank aan alle studenten die mij de afgelopen jaren hebben geholpen: Bernd, Jan Willem, Laura en in het bijzonder Marieke.

AB, Atie, Eef, Steef, Nien, Tas, Maik en Claire: bedankt voor al 10 jaar gezellige jaarclub-etentjes.

Esther, Lieselotte, Laura, Gerdien, Marloes en Sorina: fijn dat ik bij jullie af en toe lekker toe kan geven aan die beroepsdeformatie.

Thomas, you are the one person that knows me better than anyone. It is hard to find the words that will do justice to your endless support and what you mean to me. Tu déchires, toujours toujours.

CURRICULUM VITAE

Anika Iris Tsuchida was born on June 4th, 1985 in Panama, Panama. After graduating at the Wolfert van Borselen in Rotterdam she started her medical study at Utrecht University. In 2008 she first started a research project at the Department of Orthopaedics at the University Medical Center Utrecht, investigating methods of scaffold fixation in the knee (supervised by Dr. J.E.J. Bekkers, Dr. L.B. Creemers and Prof. Dr. D.B.F. Saris). After completing medical school at the end of 2009 she commenced a PhD project titled "Modulating soluble mediators to restore joint homeostasis", supervised by Prof. Dr. D.B.F. Saris, Prof. Dr. W.J.A. Dhert and Dr. L.B. Creemers. The research described in this thesis has resulted in several publications and presentations at (inter)national conferences. In 2013 she was awarded the Eikelaar award for best research project and presentation at the annual Dutch Artroscopy Society meeting.

Currently she is working as a surgical resident at the Department of Surgery at the Diakonessenhuis in Utrecht, under the supervision of Dr. T. van Dalen. She will commence her orthopaedic training in January 2015.

LIST OF PUBLICATIONS

DBF Saris, A Price, W Widuchowski, M Bertrand-Marchand, J Caron, JO Drogset, P Emans, L Paša, A Podskubka, K Slynarski, T Trc, AI Tsuchida, M Bezuidenhoudt, BJ Sanson, D Levine, M Brittberg. Matrix Applied Characterised Autologous Cultured Chondrocytes (MACI) Implant versus Microfracture: Two-Year Follow Up from a Prospective, Randomized Trial. *Accepted for publication in Am J Sports Med.*

AI Tsuchida, M Beekhuizen, MC 't Hart, TRDJ Radstake, WJA Dhert, DBF Saris, GJVM van Osch, LB Creemers . Synovial fluid cytokine profiles depend on cartilage pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. *Submitted*

AI Tsuchida, JEJ Bekkers, M Beekhuizen, L Vonk, WJA Dhert, DBF Saris, LB Creemers. Pronounced biomaterial dependency in cartilage regeneration by non-expanded compared to expanded chondrocytes. *Regen Med. 2013 Sep;8(5):583-95*

AI Tsuchida, DBF Saris, M Rutgers, M Beekhuizen, JEJ Bekkers, B Geurts, A Kragten, WJA Dhert, LB Creemers. CXCL8 (interleukin-8) in synovial fluid and autocrine production by chondrocytes; association with cartilage pathology, but no effect on regeneration or terminal differentiation. *Submitted*

AI Tsuchida, M Beekhuizen, M Rutgers, GJVM van Osch, JEJ Bekkers, AGJ Bot, B Geurts, WJA Dhert, DBF Saris, LB Creemers. Interleukin 6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model. *Arthritis Res Ther. 2012 Dec 3;14(6)*

AI Tsuchida, M Beekhuizen, LB Creemers, DBF Saris, WJA Dhert, TJM Welting, GJVM van Osch. Suppression of inflammation by celecoxib does not support cartilage repair in osteoarthritic cartilage explants and fails to reduce inflammation during regeneration. *Submitted*

M Rutgers, LW Bartels, AI Tsuchida, RM Castelein, WJA Dhert, KL Vincken, RJ van Heerwaarden, DBF Saris. dGEMRIC as a tool for measuring changes in cartilage quality following high tibial osteotomy: a feasibility study. *Osteoarthritis Cartilage 2012 Oct;20(10):1134-41*

JEJ Bekkers, AI Tsuchida, MHP van Rijen, LA Vonk, WJA Dhert, LB Creemers, DBF Saris. Single-stage cell-based cartilage regeneration using a combination of chondrons and

mesenchymal stromal cells: comparison with microfracture. *Am J Sports Med. 2013 Sep;41(9):2158-66*

JEJ Bekkers, LB Creemers, AI Tsuchida, MHP van Rijen, RJH Custers, WJA Dhert, DBF Saris. One-stage focal cartilage defect treatment with bone marrow mononuclear cells and chondrocytes leads to better macroscopic cartilage regeneration compared to microfracture in goats. *Osteoarthritis Cartilage. 2013 Jul;21(7):950-6*

JEJ Bekkers, LW Bartels, RJ Benink, AI Tsuchida, KL Vincken, WJA Dhert, LB Creemers, DBF Saris. Delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC) can be effectively applied for longitudinal cohort evaluation of articular cartilage regeneration. *Osteoarthritis Cartilage. 2013 Jul;21(7):943-9*

JEJ Bekkers, AI Tsuchida, DBF Saris. (2011) Cartilage Injury, in Evidence-Based Orthopedics (ed M. Bhandari), *Wiley-Blackwell, Oxford, UK. doi: 10.1002/9781444345100.ch99*

ThS de Windt, JEJ Bekkers, AI Tsuchida, J Ouwerkerk, DBF Saris. De technovolutie van kraakbeenregeneratie. *Nederlands Tijdschrift voor Fysiotherapie. 2011 Juni;121(2):11-7*

JEJ Bekkers, AI Tsuchida, J Malda, LB Creemers, RJM Castelein, DBF Saris, WJA Dhert. Quality of scaffold fixation in a human cadaver knee model. *Osteoarthritis and Cartilage 2010 Feb; 18(2):266-72*

JEJ Bekkers, AI Tsuchida, A Kolk, WJA Dhert, DBF Saris, LB Creemers. Scaffold and cell composition influence chondrogenesis in 3D coculture of chondrocytes with mononuclear fraction cells. *Submitted*

JEJ Bekkers, DBF Saris, AI Tsuchida, MHP van Rijen, WJA Dhert, LB Creemers. Chondrogenic potential of cells for articular chondrocyte transplantation depends on their original Location. *Tissue Eng part A. 2013 Sep 24*