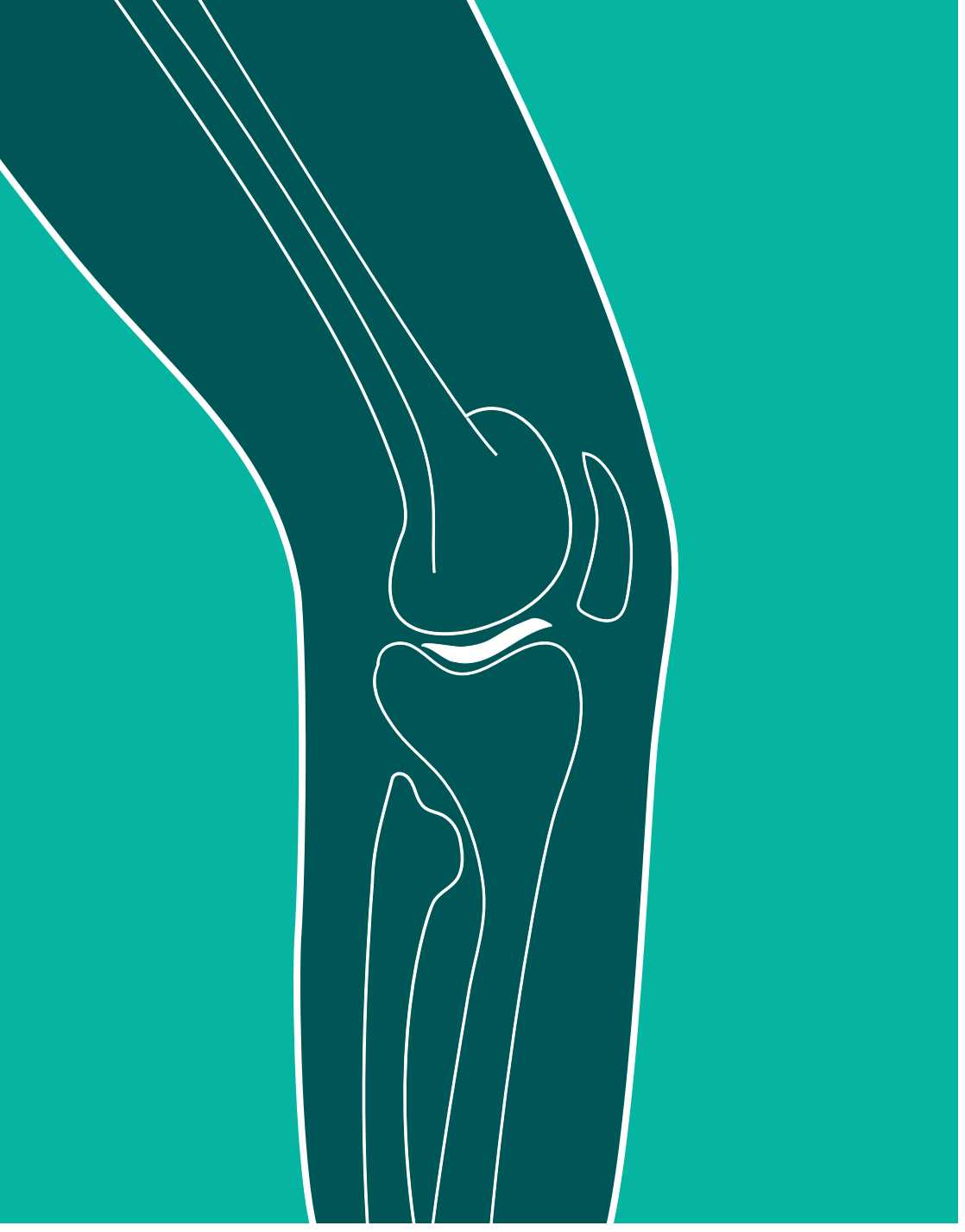

Knee joint preservation

Strategies for tissue repair and regeneration

Jasmijn Korpershoek

Knee joint preservation

Strategies for tissue repair
and regeneration

Jasmijn Vivian Korpershoek

Knee joint preservation - Strategies for tissue repair and regeneration
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Knee joint preservation

Strategies for tissue repair and regeneration

Behoud van het kniegewricht
Strategieën voor weefselherstel en regeneratie

(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. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op 8 juni 2022
des middags te 12.15 uur

door

Jasmijn Vivian Korpershoek

geboren op 30 april 1992
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Dr. L.A. Vonk

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Addenda

01

General introduction and thesis outline

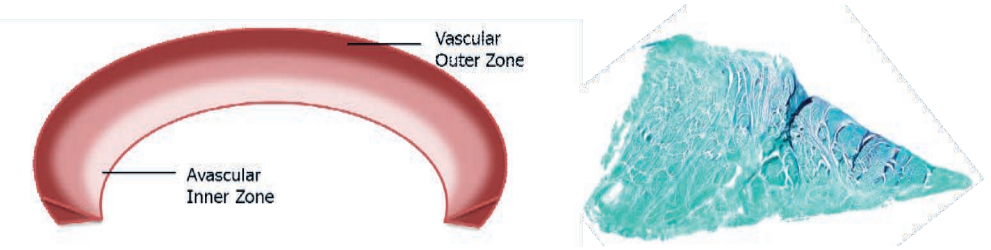
Introduction

The knee joint is one of the most complex joints in the human body[1]. The knee is considered an organ consisting of highly specialized structures such as synovium, synovial fluid, (subchondral) bone, articular cartilage, menisci, muscles, tendons and ligaments. All these structures together maintain joint homeostasis in physiological conditions, meaning that there is a dynamically regulated metabolic equilibrium, regulated by cytokines, inflammatory mediators and growth factors[2]. The joint homeostasis can be disrupted by mechanical stress, aging, infection, and inflammation, which ultimately might lead to pain and disability[2,3]. Proper functioning of the different structures in the knee allows for maintaining joint homeostasis and is of paramount importance in the preservation of the joint. The research presented in this thesis aims at creating clinically applicable treatment options and improved understanding of knee joint preservation. First, clinically available treatments are evaluated. The role of stability for protection of the menisci is assessed and the potential of platelet-rich plasma (PRP) for knee preservation in osteoarthritis (OA) is evaluated. Then, one-stage strategies for repair and regeneration of cartilage and menisci are investigated. The potential of different cell types for repair and regeneration of menisci and cartilage is studied and the endogenous progenitor cells of these tissues are characterized. The potency of cell communication was assessed in co-cultures of cartilage or meniscus cells with mesenchymal stromal cells (MSCs) and the effect of different growth factors on meniscus regeneration is evaluated.

Meniscus

The knee menisci are c-shaped fibrocartilage structures in the tibiofemoral joint[4]. They play a crucial role in shock absorption and load transmission, as well as smooth articulation and stability of the knee joint[5]. Menisci are composed of type I collagen in radial and circumferential fibers[6]. The circumferential fibers convert compressive forces into circular traction, which reduces compressive stress on the tibia[6]. The meniscus contains of small amounts of proteoglycans that attract water due to their negative charge and therefore aid in shock absorption. The inner zone has a hyaline cartilage-like phenotype and cells, and the outer rim is composed of fibrocartilage tissue (Figure 1)[4]. The regenerative potential in the meniscus is limited to the vascularized outer zone and diminishes with age[7]. Meniscus injury occurs in young and active patients as a result of instability or mechanical stress[8] or as a result of OA in the more elderly patient[9]. Meniscus injury can lead to pain, swelling, and locking if (part of) the meniscus is torn or displaced. Surgical repair of meniscus tears is more successful in the biologically viable tissue of the outer zone in young patients, which is limited to certain cases[10]. Partial meniscectomy,

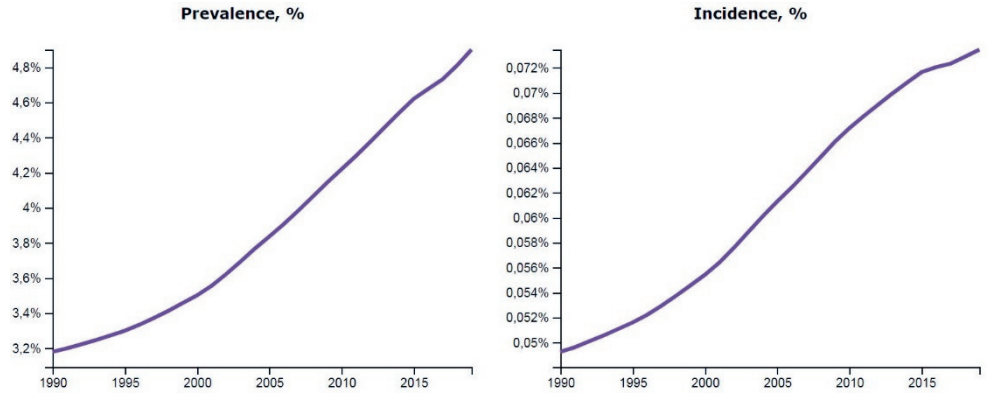
the excision of the injured tissue, is the only treatment option for many. Meniscectomy relieves the symptoms in the short term, but leads to loss of contact area and increased contact pressures, thereby predisposing the knee to OA[11]. Preserving the meniscus function by replacing meniscectomized tissue could potentially prevent these pathological changes in the knee joint. Different substrates for meniscus replacement are currently available for clinical application and the options differ between countries. Meniscus allograft transplantation was introduced as a logical method to restore the meniscus function after (sub)total meniscectomy with the aim of delaying or preventing OA. Although short-term results are promising and midterm graft survival is 85-90%, long-term survival of meniscus allografts decreases to 50-70%[12]. In addition, availability of allografts is limited and allograft transplantation requires complex logistics and is heavily regulated[13]. Moreover, the allografts are used fresh-frozen or freeze-dried, resulting in cell death. The cell death in allografts may lead to shrinkage of the tissue[12] and result in a suboptimal fit. Alternatively, a meniscus scaffold can be used for meniscus replacement. Currently, two scaffolds are approved for clinical use in the European Union and United States: the Actifit® and the collagen meniscus implant (CMI®). The Actifit® is a polyurethane scaffold, whereas the CMI® is composed of bovine collagen. Both scaffolds are to be sutured to the meniscus outer rim after meniscectomy of the inner zone. The scaffolds are designed to slowly resorb while cells from the outer rim populate the implant and form new meniscus tissue. However, in practice, the implants are prone to shrinkage and do not offer long-term solution[14,15]. Different additive manufacturing techniques for meniscus replacement are currently under evaluation[16] but none have reached a position in regular clinical practice.



| Figure 1. Schematic representation of the meniscus with inner zone consisting of hyaline-like cartilage and vascular outer zone that consists of fibrocartilage (A), and section of healthy human meniscus showing limited staining for Safranin-O (red, proteoglycans) and abundant staining of fast green (green, collagens) (B).

Articular cartilage

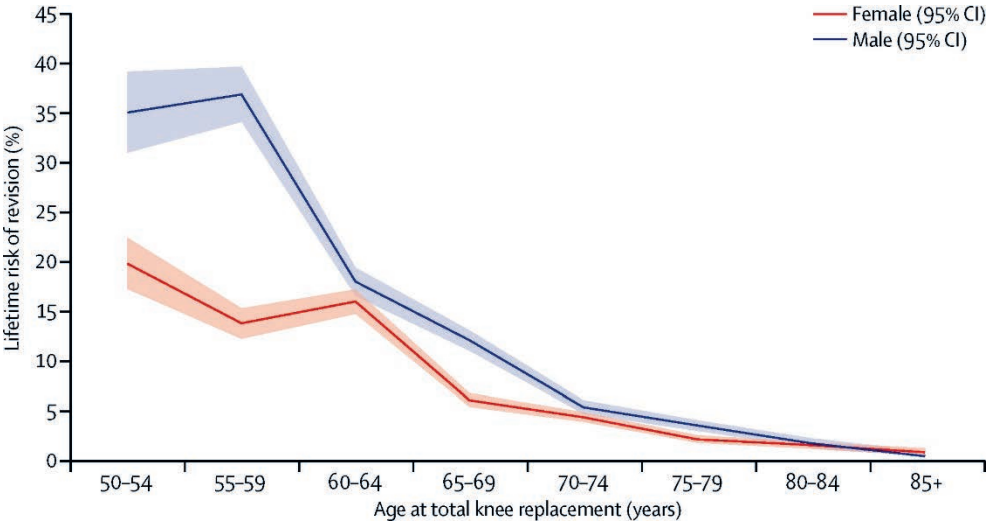
Hyaline cartilage is an aneural, avascular tissue composed of organized collagen fibers (mainly type II collagen) and proteoglycans. Few chondrocytes maintain the extracellular matrix under normal, low-turnover conditions. Focal cartilage defects, occur in young and active patients and are not repaired spontaneously[17]. Symptoms of cartilage defects include pain, swelling, and sanding sensations. These symptoms cause major disability and have a large impact on quality of life. In fact, patients scheduled for cartilage repair surgery report similar quality of life as patients scheduled for total knee replacement[18]. Defects under 2 cm^2 of surface are generally treated with microfracture[19]. With microfracture, the subchondral bone is punctured, which allows bone marrow components to reach the defect and form fibrocartilaginous repair tissue[20]. It is generally accepted that defects measuring over 2 cm^2 require more advanced techniques. Again, the availability of these advanced therapies for large cartilage defects are heavily dependent on location. In the Netherlands, large cartilage defects are treated with autologous chondrocyte implantation (ACI). In a first arthroscopic surgery, a cartilage biopsy is obtained from the defect rim or a non-weight bearing location in the knee, from which chondrocytes are isolated and culture-expanded over a 6- to 8-week period[21,22]. The culture-expanded chondrocytes are implanted in the cartilage defect in a second surgical procedure. ACI has been clinically applied for three decades during which several improvements have been made[23]. The most important drawbacks of this treatment, cell number and dedifferentiation as well as the necessity for two surgeries along with the costs related to autologous cell expansion, remain challenging. Furthermore, the availability of ACI relies heavily on the manufacturer's commercial strategies and ACI is not included in standard health care reimbursement in many countries[24,25].



| Figure 2. The global burden of knee osteoarthritis. Prevalence and incidence in percent from 1990 to 2019

Osteoarthritis

Osteoarthritis is defined by the Osteoarthritis Research Society International (OARSI) as 'a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. OA manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, an/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness'[26]. In short, this definition describes that OA develops when the joint homeostasis cannot be maintained and is characterized by a vicious circle of derangements. The prevalence of radiographically confirmed symptomatic knee OA is 5% of the world population[27] and this number is increasing (Figure 2). Aging of the population and increased prevalence of obesity[28] are often proposed as explanation of this increase. However, after correcting for increased life expectancy and obesity, OA still increased a two-fold between the early industrial (1905-1940) to post-industrial era (1976-2015) in Western countries[29]. Equally alarming are the increasing rates of total knee arthroplasty, especially in younger patients[30]. Although this procedure has a high patient satisfaction rate in elderly patients[31], younger patients have lower patient satisfaction rates[32] and a high risk of complications and (early) revision (Figure 3)[31,33–35]. Therefore, OA is a largely unaddressed world-wide medical challenge, and preservation of the joint of utmost importance. Current OA treatment consists of symptom reduction and ultimately the only option is total knee arthroplasty. No successful disease modifying treatments are available to date. Early intervention may prevent the vicious circle leading to symptomatic OA, for example by regeneration of cartilage defects or meniscal damage. Once OA has become symptomatic, the attention should shift to joint preservation by slowing the progress of OA, symptom relief, or restoring normal joint functions.



| Figure 3. Lifetime risk of revision after total knee replacement. Plot showing estimates of lifetime risk of total knee replacement revision against age at the time of primary total knee replacement surgery (in 5-year age bands) and stratified by sex (results adjusted for lost and censored population)

Regeneration of meniscus and cartilage

Regenerative medicine is defined as the field aiming at the replacement or regeneration of cells, tissues or organs to restore normal function. Unfortunately, the regenerative potential in the knee is limited as William Hunter already stated in 1743[36]. In the past decades, an increasing body of evidence has revealed possibilities for functional tissue regeneration in the knee. The following paragraphs will describe the advances that have been made resulting in the research described in this thesis.

Cell based regeneration

MSCs were first named Mesenchymal Stem Cell in 1991[37] due to their ability to differentiate towards bone, cartilage, and fat tissue. The 'stemness' of these cells was soon the object of debate, resulting in renaming the MSCs Multipotent Mesenchymal Stromal cells as proposed by the International Society for Cellular Therapy (ISCT) in 2005[38]. In addition, criteria were set to characterize MSCs[39], mainly by the distinction from hematopoietic cells also found in bone marrow. In 2017, the name Medicinal Signaling Cells was proposed, based on the secretory function of MSCs and the idea that MSCs are derived from pericytes[40]. Regardless of this debate on the right nomenclature, the many functions and importance of these cells is generally recognized. Since the discovery of MSCs, their anti-inflammatory properties, multilineage differentiation, and ability to excrete trophic factors and extracellular vesicles have led to a wide variety of

applications. Recently, even the shuttle of entire organelles such as lysosomes and mitochondria has been added to the toolbox of MSCs[41,42].

By using allogeneic MSCs, cell therapies consisting of autologous meniscus cells or chondrocytes can be applied in single-stage surgeries. Autologous chondrons are combined with allogeneic MSCs (Instant MSC Product Accompanying Autologous Chondron Transplantation, IMPACT) for treatment of articular cartilage defects[43,44]. With IMPACT, chondrons are isolated intraoperatively from debrided cartilage from the defect rim. These chondrons are mixed in a 10:90 ratio with allogeneic bone marrow MSCs and applied in the cartilage defect. A phase I/II study shows feasibility, initial safety and efficacy of this treatment up to 5 years[45]. No autosomal DNA of the MSC donor is present in the repaired defects one year after surgery[43,44], indicating that autologous cartilage formation is stimulated by allogeneic MSCs, and MSCs do not differentiate. Direct communication by contact via gap junctions[46], as well as communication over larger distances using extracellular vescicles[47] or growth factors[48] play a role in this chondroinductive effect. Similarly, co-cultures of meniscus cells and MSCs demonstrate meniscus extracellular matrix formation and could thus be applied for meniscus regeneration when applied together with the CMI®[49]. However, as described previously, there are still unresolved drawbacks to the usage of the CMI®.

Due to the broad definition of MSCs, MSCs or progenitors of many different origins including cartilage, adipose tissue, synovial membrane, and meniscus have been identified and evaluated for regenerative purposes. The intra-articular sources of progenitors are thought to have a lower tendency for hypertrophy than bone marrow MSCs[50]. Moreover, MSCs derived from the specific tissue of interest might be primed for differentiation in the direction of this specific tissue. Although the presence of progenitor populations has been demonstrated in healthy as well as osteoarthritic tissue, the role of these progenitors in the pathophysiology of OA remains to be elucidated.

Biologics for osteoarthritis

Orthobiologics are a collection of substances that are suggested to be able to influence musculoskeletal tissues and regeneration. Hyaluronic acid emerged as one of the first biologics, with the aim of substituting one of the main components of the hyaline cartilage. The pain relief after hyaluronic acid treatment is moderate to zero, but due to the lack of good alternatives, it is still broadly used in clinical practice[51,52]. Corticosteroids are another broadly used alternative that offers some short-lived pain relief, but long term treatment could worsen joint destruction and atrophy[53].

Platelet-rich plasma (PRP) later emerged as autologous product. PRP was first described in the 1970s as ‘plasma with a platelet count above that of peripheral blood’ by

hematologists that used it to treat patients suffering from thrombocytopenia[54]. PRP is obtained from whole blood by centrifugation, after discarding the sediment containing erythrocytes. Platelets play an important role in hemostasis and wound healing. Upon activation, platelets release alpha-granules full of cytokines and growth factors. *In vitro,* these factors work anti-inflammatory, and promote cell migration, proliferation and formation of extracellular matrix[55,56], which presents the clinical potential. The lack of regulatory limitations in the use of autologous PRP led to a quick implementation in the clinic. To date, there is no evidence indicating structural improvements after treatment with PRP (such as increased cartilage volume or quality)[57,58]. Moreover, there is no consensus on the effectiveness of PRP for pain/symptom relief[59], even though PRP has been clinically used for two decades. This lack of consensus is caused by substantial differences in reported outcomes[57,60,61], a large variation in the composition and preparation strategy for PRP and the overall poor quality of research[62]. In the last years, guidelines for the reporting on PRP and the design of the studies were proposed, aiming to improve quality and comparability of research and to move the field forward[63].
More recently, stem-cell based biologics for OA have gained great interest. Especially autologous, minimally manipulated products that do not require culture expansion are the subject of investigation, due to their potential in one-stage treatments and lower regulatory burden. Stromal vascular fraction is a product from lipoaspirate, that is obtained by enzymatic digestion or mechanical dissociation. Apart from 1-15% MSCs, various other (potentially regenerative or anti-inflammatory) cells are present[64]. Another example of is bone marrow concentrate. Bone marrow concentrate contains only 0.001 to 0.01% autologous MSCs, but density gradient centrifugation might increase this number. Furthermore, the various growth factors present in bone marrow concentrate might stimulate cartilage regeneration[65]. So far, initial results of these treatments indicate symptom modification and pain relief, but no structural improvement of OA.

Aim of this thesis:

The research in this thesis aims at creating clinically applicable treatment options and improved understanding of knee joint preservation. Studies in the full range of translational research, from basic science to clinically available treatments are included. The research can be divided into three sub-aims:

1. The evaluation and optimization of currently clinical available treatments aiming at knee preservation
2. Evaluation of different cell types for one-stage tissue repair and regeneration
3. Promoting tissue repair and regeneration using trophic effects and cell communication

Contents of this thesis

For the first aim, we evaluate two knee preservation treatments that are clinically available and explore potential for optimization of current treatments. First, the role of knee joint stability for maintaining proper meniscus function and protection of the meniscus (repair) is investigated. Then, we assess the effect of a commercially available PRP product (Autologous Conditioned Plasma®, ACP®) in the treatment of knee OA. Here, we aim to identify predictors of better clinical outcomes and to study the effect of variations in ACP® composition. These factors could improve patient selection or product optimization, thereby improving treatment outcomes.

Second, this thesis aims at evaluating different cell types for tissue repair and regeneration. Using a systematic review of the pre-clinical literature, different cell types are assessed for application in meniscus repair and regeneration. Moreover, the added value of using cells is studied by comparison of cell-free and cell-based treatments. Additionally, we isolate meniscus and cartilage progenitor cells and characterize these cells to study if they hold potential for meniscus and cartilage cell therapy or tissue engineering. Therefore, gene-expression profiles, proliferative capacities and extracellular matrix formation of progenitor cells are compared to cartilage or meniscus cells.

Lastly, we assess whether trophic effects and cell-communication can be employed for regeneration in one-stage treatments. Here, we aim to identify growth factors that promote meniscus cell migration and extracellular matrix formation and employ these in a functionalized CMI® for meniscus replacement. Furthermore, we investigate the stimulatory effect of MSCs on matrix formation in meniscus and cartilage co-cultures in both *in vitro* and *in vivo* set-ups. We study the potential of MSCs combined with meniscus cells for meniscus replacement *in vitro*. In addition, we design a randomized controlled trial to study the effectiveness of MSCs combined with chondrons for treatment of cartilage defects. Furthermore, we aim to identify the causes of the powerful effect of MSCs by studying the transport of mitochondria between MSCs and chondrocytes. Lastly, we evaluate the effect of this transport on cartilage formation.

02

Does anterior cruciate ligament reconstruction protect the meniscus and its repair? A systematic review

Korpershoek JV, de Windt TS, Vonk LA, Krych AJ , Saris DBF.
Does anterior cruciate ligament reconstruction protect
the meniscus and its repair? A systematic review.
Orthop J Sports Med. 2020 Jul 28;8(7)

Abstract

Background

ACL tear and meniscal injury often co-occur. The protective effect of (early) ACL reconstruction on meniscal injury and its repair is not clear. The purpose of this systematic review is to assess the protective effect of ACLR on the meniscus and provide clinical guidelines for managing ACL tears and subsequent meniscal injury. We aim to answer the following questions 1). Does ACL reconstruction protect the meniscus from subsequent injury? 2). Does early ACL reconstruction reduce secondary meniscal injury compared with delayed ACL reconstruction? 3). Does ACL reconstruction protect the repaired meniscus?

Methods

A systematic review was performed using MEDLINE and EMBASE electronic databases according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Search terms included ACL, reconstruction, and meniscus. Studies describing primary ACL reconstruction and nonoperative treatment in adult patients were included, as well as studies indicating timing of ACL reconstruction. The included articles are assessed individually for risk of bias using the modified Cochrane Risk of Bias Tool and Methodological index for non-randomized studies.

Results

One level 2 randomized controlled trial, as well as several level 3 and 4 studies, indicate a protective effect of ACL reconstruction on meniscal injury compared with nonoperative treatment. There is weak (level 3) evidence of the protective effect of early ACL reconstruction on the meniscus. Meniscal repair failure is less frequent in ACL reconstructed than ACL deficient patients (level IV).

Conclusion

The evidence collected in this review is suggestive of a protective effect of ACL reconstruction for subsequent meniscal injury (level 2 evidence). ACL reconstruction should be performed within three months of injury (level 3 evidence). Meniscal injury requiring surgical repair in the ACL deficient knee should be treated with repair accompanied by ACL reconstruction (level 3 evidence). The paucity of level 2 studies prevents the formation of guidelines based on level I evidence. There is a strong clinical need for randomized or prospective trials to provide guidelines on (timing of) ACL reconstruction and meniscal repair.

Introduction

Anterior Cruciate Ligament (ACL) tears affect more than 120.000 patients per year in the United States and in 48-65% of patients these tears are associated with meniscal injury[66-68]. Similarly, in a cohort of more than 9000 meniscal repairs, 40.5% of the repairs were performed together with ACL reconstruction (ACLR)[69]. ACL tears are associated with a high risk of osteoarthritis, with an osteoarthritis incidence of 50% at 10-20 years after the ACL tear[70,71]. According to a meta-analysis, the most important predictor of osteoarthritis after ACLR was meniscectomy (odds ratio 3.45)[72].

ACLR is thought to restore stability in the knee, thereby protecting the knee from further (meniscal) damage. This is in contrast to findings of a randomized controlled trial (RCT) comparing early ACLR with nonsurgical rehabilitation, in which a similar number of meniscal surgeries was found in both groups[73]. It is therefore unclear what effect ACL tears and their reconstruction have on the meniscus. Likewise, no consensus is available regarding the success of meniscal repair in ACL deficient versus ACL reconstructed knees. Although retrospective studies[69,74] have reported higher success rates of meniscal repair concomitant with ACLR compared with meniscus repair in ACL deficient knees, these studies are of low methodological quality and prone to bias.

Several studies investigating the effect of ACLR are currently under way. In a prospective cohort including 7 sites in Sweden, patients receive either ACLR or nonoperative care with possible delayed ACLR. Patients of 1 center will undergo diagnostic magnetic resonance imaging (MRIs) at several time points during follow up, in order to assess secondary meniscal injury[75]. An RCT comparing ACLR versus nonoperative care was recently completed in the Netherlands[76], but data are not yet available in the literature. Therefore, treatment of ACL tear is currently based on expert opinion and personal experience rather than high level evidence. Critical literature review can provide evidence that supports or changes clinical strategies and can identify gaps in the available evidence.

The purpose of this systematic review was to assess the protective effect of ACLR on the meniscus and provide clinical guidelines for handling ACL tear and subsequent meniscal injury. We aimed to answer the following questions 1). Does ACLR protect the meniscus from subsequent injury? 2). Does early ACLR reduce secondary meniscal injury compared with delayed ACLR? 3). Does ACL reconstruction protect the repaired meniscus? We used the PICOS (participants, interventions, comparisons, outcomes, study designs) process to address the research questions, as described in Table 1.

Methods

A systematic review of the literature was performed according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines[77]. A search was conducted on 7 November 2019 in the electronic databases of MEDLINE and Embase using the following search strategy: ("anterior cruciate ligament"[mesh] OR (Anterior OR cranial) AND cruciate AND ligament*) AND reconstruction AND "meniscus [mesh] OR Menisc*). In Embase, conference abstracts, conference papers, conference reviews and reviews were excluded from the search.

We chose our inclusion criteria carefully in order to minimize risk of bias. Studies were included based on the PICOS listed in table 1, regardless of whether an item was a primary or secondary outcome. The first author (JVK) assessed eligibility by screening of the titles and abstracts. The decision rules and extended inclusion and exclusion criteria are described in Appendix 1. For each included study the following data were extracted: the study design, number of patients, in- and exclusion criteria of the study, patient age, presence and type of ACL injury, presence and type of meniscal injury, treatment methods and timing, follow-up duration, outcome of the specific research question, and level of evidence[78]. The included RCTs were assessed individually for risk of bias using the modified Cochrane Risk of Bias Tool (table 2)[79]. Non-randomized studies were evaluated using the methodological index for non-randomized studies (MINORS) criteria (table 3)[80]. If outcomes of different studies conflicted, conclusions were based on level 2 studies. If level 2 studies were not available, level 3 or 4 studies with higher MINORS scores and lower risk of bias as discussed in the final part of each paragraph were weighted heavier in the conclusions.

Table 1. PICOS (Participants, Interventions, Comparisons, Outcomes, Study Designs) for the research questions

	Does ACLR protect the meniscus from subsequent injury?	Does early ACLR reduce secondary meniscal injury compared with delayed ACLR?	Does ACL reconstruction protect the repaired meniscus?
Participants	Adults	Adults	Adults with concomitant ACL tear and meniscal injury
Interventions	Primary ACLR	Primary ACLR (acute)	Primary ACLR + meniscal repair
Comparisons	Nonoperative treatment	Primary ACLR (delayed)	Nonoperative treatment for ACL + meniscal repair
Outcomes	(secondary) meniscal injury	(secondary) meniscal injury	Healing, re-operation, re-tear, or tear worsening
Study Designs	All study designs	• All prospective studies • Retrospective studies describing a total cohort of ACL tears (including a nonoperative group) • Retrospective studies that report assessment of meniscal injury at two time points	All study designs

Abbreviations: ACL(R), anterior cruciate ligament (reconstruction)

Table 2. Critical appraisal randomized studies

Author	Level of evidence	Random sequence generation	Allocation concealment	Selective reporting	Other sources of bias	Blinding (participants and personnel)	Blinding (outcome assessment)	Incomplete outcome data
Frobell[73], Snoeker[81]†	2	Low	Low	Low	High	High	High	?
Meunier[82], Andersson[83], Andersson[84], Odesten[85]†	3	High	High	?	?	High	High	Low
Fithian[86]	3	High	High	?	?	High	High	High
Raviraj[87]	3	Low	Low	?	?	High	High	Low
Bottoni[88]	3	Low	Low	?	High*	High	High	Low

† These publications are combined as these studies describe data obtained from the same patient group and clinical trial.

*Delayed group not permitted to return to army duty/recreational sport. A question mark indicates that the item was not described in sufficient detail; thus, the risk of bias is unclear

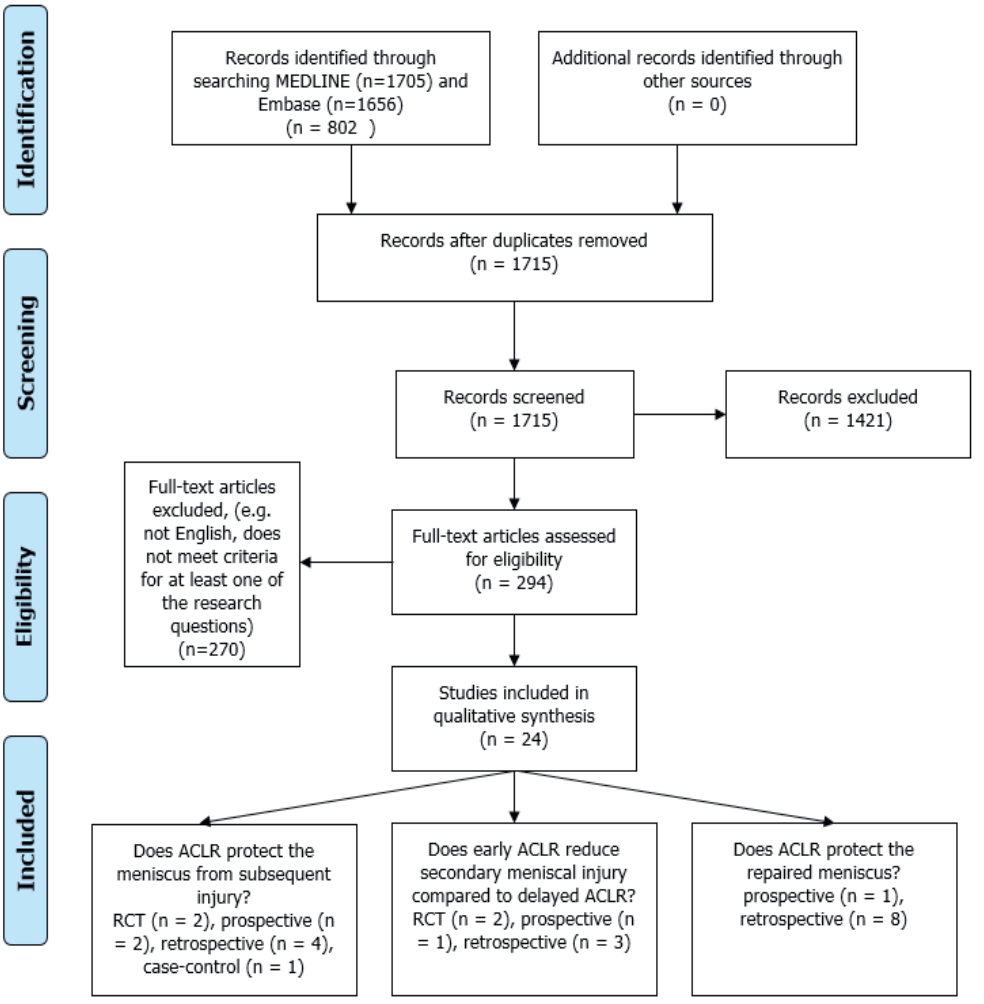
| Table 3. Critical appraisal non-randomized studies

Author	Level of evidence	Methodological index for non-randomized studies (MINORS)
Dunn[89]	3	21
Kessler[90]	4	15
Meuffels[91]	4	12
Daniel[92]	3	14
Sanders[93]	3	14
Hagmeijer[94]	3	15
Herbst[95]	3	16
Yoo[96]	4	13
de Roeck[97]	4	13
Foster[98]	4	13
Van der Wal[99]	4	12
Majeed[100]	4	11
Gallacher[101]	4	9
Albrecht-Olsen[102]	4	14
Plasschaert[103]	4	10
Kimura[104]	4	10
Valen[105]	4	9
Jensen[106]	4	10
Austin[107]	4	10

MINORS (Methodological Index for Nonrandomized Studies) scores were out of a possible ideal of 16 for noncomparative studies and 24 for comparative studies.

Results

The literature search yielded 1705 articles in MEDLINE and 1656 in Embase (figure 1). The extracted data can be found in the supplementary data.



| Figure 1. Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) Flow diagram: summary of the literature search. Abbreviations: ACL(R), anterior cruciate ligament (reconstruction); RCT, randomized controlled trial

Does ACL reconstruction prevent subsequent meniscal injury?

In 1 RCT, treatment with early ACLR was compared with rehabilitation with the possibility of delayed ACLR[73]. In the early ACLR group, a lower number of meniscal surgeries (5 meniscectomies and 1 repair) were reported during 2-year follow up ($p<0.001$) than in the nonoperative group (26 meniscectomies and 3 repairs). At baseline, more meniscal surgeries were performed in the early ACLR group (24 partial meniscectomies and 10 repairs) than in the nonoperative group (15 meniscectomies and 6 repairs) and therefore the total amount of meniscal surgeries did not differ statistically between both groups ($p=0.20$). At 5- years follow-up, half of the patients in the rehabilitation group underwent delayed ACLR. New or worsening meniscal injury occurred in 45% of patients who underwent early ACLR and 53% of patients in the rehabilitation group[81]. Patients who did not undergo early ACLR had a relative risk of 2.1 (95% CI 1.1-3.9) for medial meniscal injury and 1.0 (95% CI 0.6-1.5) for lateral meniscal injury.

In the trial by Odensten et al.[85], surgical treatment was compared with nonoperative treatment[82–85]. Meniscal injury and treatment were comparable between the groups at baseline. During follow-up there were 5 cases of secondary meniscal injury in the surgical group (total n = 42) and 18 cases in the nonoperative group (n =52, $p=0.015$). In this trial, 30% of patients allocated to receive nonoperative treatment later underwent ACL repair[82].

In the prospective cohort studied by Fithian et al.[86] 207 patients were assigned a risk level based on preinjury sports participation and degree of laxity. Patients with moderate risk were assigned to receive ACLR within 3 months or nonoperative treatment based on day of presentation. The number of baseline meniscal surgeries was comparable between both groups. During follow-up, 10 meniscectomies (22%) and 3 meniscal repairs (7%) were performed in the nonoperative group, whereas only 1 repair (4%) was performed in the early ACLR group, indicating that ACLR prevents late-phase meniscal injury and that nonoperative therapy might impair reparability of the meniscus[86].

Similar results were found in several cohort studies[89–94]. For instance, ACLR within 6 weeks of trauma decreased secondary meniscal surgery by 56% in the lateral meniscus and by 42% in the medial meniscus ($p<0.0001$) in an US army active-duty population[89]. In the cohort studied by Sanders et al.[93], 37.4% of patients treated nonoperatively for ACL tears were later diagnosed with meniscal injury, compared with 19.8% of patients treated with delayed ACLR and 6.1% treated with early ACLR[93].

Some important confounding effects in the abovementioned studies should be considered. Early meniscal surgery reduces the risk of late-phase meniscal surgery. Subsequently, more aggressive treatment of meniscal injury in the early ACLR group could result in an overestimation of the protective effect of ACLR compared with

nonoperative treatment. Such a difference in initial meniscal treatment between patients treated with early ACLR or nonoperative treatment was shown in 2 studies[73,91], whereas comparable rates of meniscal surgery in the early-phase were reported in other studies[82,86,92]. In addition, nonrandomized studies entail a high risk of selection bias based on activity level. In practice, more active patients are advised to undergo ACLR, whereas other patients are recommended nonoperative treatment. The risk of meniscal injury increases in active patients, and in 2 studies[88,92], nonoperatively treated patients were advised not to participate in cutting or jumping sports, or return to military duty. These treatment biases result in an underestimation of the protective effect of ACLR. Contrarily, it could also be hypothesized that nonoperatively treated patients have a higher risk of early re-injury due to a faster return to sport. Additionally, high-level athletes might not enroll in a RCT with a nonoperative group, and therefore be underrepresented in these studies.

Does early ACLR reduce secondary meniscal injury compared with delayed ACLR?

The number of meniscal injuries after early and delayed ACLR was compared in 2 RCTs and 2 large cohort studies. In 1 RCT[87], no differences in number of medial or lateral meniscal injury were found when patients underwent ACLR within 2 weeks after injury (7 days on average) versus within 4 to 6 weeks after injury (32 days on average). In the study of Bottoni *et al.*[88], the number of medial meniscal tears was comparable between patients who underwent ACLR within 3 weeks (41.2%) or patients who underwent ACLR later than 6 weeks (85 days on average) after injury (42.9%). Importantly, 57.1% of medial tears in the acute group were repairable, whereas only 26.7% of menisci in the delayed group could be repaired[88]. Postoperative stiffness, range of motion, and clinical outcomes did not differ between early and delayed ACLR in either trials[87,88]. In a cohort containing all inhabitants of Olmsted County (Minnesota, US), the risk of secondary meniscal injury was higher in patients who underwent delayed (>1 year) ACLR compared with patients who did not have ACL injury (hazard ratio 4.6). The hazard ratio decreased to 1.6 when patients underwent ACLR within a year after injury[93]. In a similar cohort, 19% of patients treated nonoperatively for ACL rupture were found to have a concomitant meniscal injury. Meniscal injury was less prevalent (7%) in the group that was treated with ACLR within six months after injury, than in the group that underwent delayed ACLR (33%) ($p<0.01$)[94].

The fate of the meniscus in ACL deficient knees was studied in 4 case series. The number of meniscal tears increased between 2 preoperative MRIs[96], or between diagnostic arthroscopy and ACLR[97,98]. Moreover, existing tears deteriorated[96].

Meniscal repair failures and reparability were not different in a prospective cohort of patients with both meniscal and ACL injuries treated within 48 hours or after 3 months[95]. The comparability of these studies is limited by the use of different cut-off periods for early or delayed ACLR. The only RCTs comparing early and delayed ACLR used a cut-off of 4 and 6 weeks for delayed ACLR, although the average time of ACLR in these studies would still be considered early ACLR in the cohort studies. It can be expected that most patients do not return to sports in this time frame, making it difficult to evaluate the protective effect of ACLR. A major confounding effect in the retrospective case series[96–98] is that only patients who underwent ACLR are included; patients with ACL deficiency who received nonoperative treatment were not included. Meniscal injury could be the reason that patients return to the clinic, whereas the incidence of meniscal injury might be low in the group that remains nonoperative. This is reported in several studies[82,86,92,94].

Does ACL reconstruction protect the repaired meniscus?

The results of meniscal repair combined with ACLR versus meniscal repair in ACL deficient knees have been described in different studies, although no RCT has been published. Failure of meniscal repair was compared between patients who underwent simultaneous or delayed (> 6 weeks) ACLR in a cohort study by Majeed et al.[100]. Meniscal repair failed in 14.5% of patients who underwent concomitant ACLR (or within 6 weeks), and in 27% of patients who underwent delayed reconstruction ($p<0.05$)[100]. Similar results are reported by Gallacher et al.[101] and several other researchers[99,103,106,107], although in these studies results were not statistically significant or no statistical analysis was reported. Only 1 study reports no detrimental effect of ACL deficiency on survival of meniscal repair, although no specific data were provided[105].

In 2 studies healing rates of meniscal repair were assessed with second-look arthroscopy. In the first study, a residual tear was visible in 18 out of 19 menisci in patients who underwent concomitant ACLR, whereas this was the case in 12 out of 19 ACL deficient knees[102]. In the second study, healing was reported in all knees that had concomitant ACLR, whereas in the ACL deficient 4 tears remained unhealed ($p<0.005$)[104].

Many of these studies were prone to detection bias, because the success of meniscal repair in patients who underwent concomitant ACLR was not evaluated at later stage, whereas during a delayed ACLR a repair failure could be detected. Moreover, the 2 retrospective studies both had a high risk of selection bias, because Plasschaert et al[103] treated only patients with minor instability and no significant pivot shift nonoperative and Albrecht-Olsen et al[102] advised ACLR in all high-demand athletes, which could lead to an underestimation of the positive effect of ACLR on healing of meniscal repairs[102,103].

Discussion

An overall decrease in meniscal injury after ACLR was found in RCTs as well as prospective studies and large cohorts. This is suggestive of a protective effect of ACLR on the meniscus. Nonetheless, the risk of subsequent meniscal injury remains 3.73 times higher compared with the contralateral knee, as demonstrated in a cohort of 4087 patients with no meniscal injury detected during ACLR[108]. This cohort study did not assess the timing of ACLR; therefore the risk of meniscal injury after early ACLR could be smaller. Patient factors such as age and activity levels were not considered in the current review, but should be taken into account in the decision making process. Moreover, cost-effectiveness of ACLR has not determined been, but will be analyzed using data from a clinical trial that was recently completed[76].

In 2 RCTs[87,88], meniscal injury did not increase between 3 and 6 weeks and between 2 and 4-6 weeks after injury, respectively. ACLR provides a protective effect if performed within 6 to 12 months after injury compared with delayed ACLR, based on large geographical cohort studies (level 3 evidence)[93,94]. However, these cohort studies did not consider the selection bias to undergo delayed ACLR (patients with symptoms of meniscal injury) and result should be interpreted with care. Return to sports before ACLR was an independent risk factor for lateral meniscal tears in a systematic review evaluating the effect of surgical timing on meniscal injury in adolescents[109]. Return to a normal activity level cannot be expected within 4-6 weeks, which is the time frame investigated in the abovementioned RCTs.

In all except 1 study[105], failure and healing rates indicated a beneficial effect of ACLR on meniscal repair (level 4 evidence). Therefore, the increased risk of repair failure in unstable knees should be considered and performing meniscal repair in combination with an ACLR is recommended. A stable environment was also shown to benefit meniscal repair in a recent study, in which repair failure of medial meniscal tears decreased when rotatory laxity and residual pivot shift were resolved by reconstruction of the anterolateral ligament in addition to ACLR[110]. Interestingly, higher success rates have been reported for meniscal repair in combination with ACLR than for meniscal repair in stable knees[69,74]. Different causes for this observation can be hypothesized. The type of meniscal injury that occurs in combination with ACLR could be more amenable to repair. Alternatively, the healing could be improved owing to biological factors released from the drill-holes at ACLR[111,112]. Moreover, rehabilitation after ACLR could be more effective and protective of the knee compared with the rehabilitation after isolated meniscal repair. The importance of knee stability has been underlined in recent studies that showed comparable healing rates of repaired and untreated stable ramp lesions when ACLR was performed[113,114]. Interestingly, all unhealed menisci had a remaining instability

of more than 3mm side-to-side difference after ACLR[114]. Moreover, a recent meta-analysis emphasized the role of the meniscus in knee stability, reporting that ACLR with concomitant meniscal repair reduced anterior knee joint laxity compared with ACLR with meniscectomy[115].

Limitations

The best available literature has considerable methodological limitations. Because of the lack of standardized and randomized studies and the abundance of low-quality studies there were several choices to be made. To minimize the risk of bias, we chose to exclude retrospective studies correlating the incidence of meniscal injury to the timing of ACLR. The possible bias in these studies is confirmed by studies that report meniscal injury in patients undergoing early, delayed or no ACLR[86,92], in which the incidence of meniscal injury is higher in the delayed group than in the nonoperative group. Moreover, if the follow-up duration after surgery is limited, the follow-up times are different between the early and delayed group and some of the patients undergoing early ACLR might develop meniscal lesions after this follow-up, which was also shown in the literature[116–119].

The difference in initial meniscal treatment between patients treated with (early) ACLR compared with nonoperatively treated patients is an important confounder to consider. Increased early-phase meniscal treatment (during ACLR) was shown to decrease late-phase meniscal surgery in 2 studies[73,91]. However, few studies distinguish between early-phase and late-phase meniscal treatment, making it difficult to estimate the effect of this confounder. In an ongoing prospective cohort study[75] a subgroup of patients will receive multiple MRIs during a two year follow-up period. Results of this study will show the effect of ACLR on meniscal injury in time.

We chose to include all different techniques for ACLR and meniscal repair and did not differentiate between different types of meniscal injury. This could limit the comparability of the studies and explain contradicting findings. However, by using this inclusive approach we were able evaluate a broad section of the existing literature. Unfortunately, based on the available literature it was not possible to perform a meta-analysis. Furthermore, the literature comparing early and delayed ACLR was limited and susceptible to bias.

Although subject to substantial limitations, this is the first comprehensive review that accounts for bias in this field of research. Even though we were not able to draw firm conclusions on all topics, by thoroughly reviewing the current literature we were able to identify the most important questions for future research.

Conclusions and implications

ACLR provides protective effects for subsequent meniscal injury (level 2 evidence). This should be confirmed in an RCT comparing ACLR versus nonoperative treatment. Such an RCT should monitor and report meniscal injury and treatment (meniscal repair or meniscectomy) at the early-phase as well as during follow-up in order to differentiate between pre-existing and subsequent meniscal injury. Additionally, meniscal treatment should be standardized in trials comparing ACLR and nonoperative treatment, to prevent differences in meniscal treatment at baseline. These trials should attempt to include a good representation of the patient population, including competitive athletes. If ACLR is performed, it is recommended that this take place within 3 months of injury (level 3). Because of the limited and low-quality of current evidence, the effect of timing of ACLR on subsequent meniscal injury should be investigated in a randomized study. It is important to investigate a clinically relevant delay, in which patients are likely to return to normal activity levels. Patients in both treatment groups should undergo the same postoperative rehabilitation and should be comparable in terms of activity levels and preoperative laxity.

Meniscal repair failure is higher in ACL-deficient knees (level 3 evidence), and surgeons and patients should be aware of this risk. The effect of ACLR without meniscal repair should be investigated for stable meniscal injury.

| Appendix 1. Screening Criteria

Title/abstract screening

Exclusion criteria:

- Language other than English
- No primary ACLR
- Case reports describing a single patient
- No adult patients
- Cadaver studies
- Finite element analysis

Full-text screening

Inclusion criteria:

- Language other than English
- No primary ACLR
- Case reports describing a single patient
- No adult patients
- Cadaver studies
- Finite element analysis

Exclusion criteria:

- Articles describing both nonoperative treatment and ACLR for ACL rupture; AND
    - o Reporting rates of meniscal injury: OR
    - o Reporting rates of healing, re-operation, re-rupture or tear worsening of meniscal tears
- Articles describing different timepoints of ACLR: AND
    - o Reporting rates of meniscal injury; AND
        - ☐ Prospective study; OR
        - ☐ Retrospective including nonoperative group; OR
        - ☐ Retrospective that assess meniscal injury at two timepoints

Data extraction tables

| Table A1. Does ACLR protect the meniscus from subsequent injury?

Author	n	Study design	ACL treatment	Meniscal injury	Meniscal treatment	Timing of treatment (range)	Follow-up duration (range)	Nonoperative treatment	ACLR
Frobell[73], **Snoeker**[1]	121	RCT	BPB; 4S-HT (G+ST)	NS	Partial resection or fixation	<10 wk	2 y (Frobell); 5 y (Snoeker)	Baseline: 15 partial resection, 6 fixation 2-y follow-up: 26 partial resection, 3 fixation 5-y follow-up: new or worsening meniscal damage, 53%; delayed ACLR, 51%	Baseline: 24 partial resection, 10 fixation 2-y follow-up: 5 partial resection, 1 fixation 5-y follow-up: new or worsening meniscal damage 45%
Meunier[82], **Andersson**[83], **Andersson**[84], **Odesten**[85]	94	RCT	Nonsurgical, repair; augmented repair	NS	Meniscectomy or repair	NS	14-19 y	18/52 meniscal injury; 42% meniscectomy (intention to treat); 50% delayed ACLR	5/42 meniscal injury; 29% meniscectomy (intention to treat)
Dunn[89]	6576	Retrosp	ACLR, nonoperative	Medial, lateral or any meniscal injury	NS	<6 wk or >6 wk (initial nonsurgical)	Median 29.5 mo		56% reduction lateral meniscal reoperation; 42% medial meniscal reoperation
Kessler[90]	50	Retrosp	BPB, nonoperative	NS	NS	NS	11.1 y (7.5-16.3)	18/68 nonoperative reoperations for meniscal injury	7/68 reoperations for meniscal injury
Meuffels[91]	209	Case-Control	BPB, nonoperative	NS	Meniscectomy	>6 mo (2-258)	10 y	80% meniscectomy, 40% during late-phase	68% meniscectomy; 12% meniscectomy post-ACLR

Author	n	Study design	ACL treatment	Meniscal injury	Meniscal treatment	Timing of treatment (range)	Follow-up duration (range)	Nonoperative treatment	ACLR
Fithian[86]	209	Prosp	Early ACLR (midthird patella tendon autograft) or nonoperative	NS	Repair or partial meniscectomy	<3 mo	6.6 y	Early-phase: 5/46 meniscectomy and 2/46 repair Late-phase: 1 meniscectomy 16 delayed ACLR → 9/16 meniscectomy, 3/16 meniscal repair	3/26 repair; 4/26 meniscectomy; 1 repair and 1 revision ACLR
Daniel[92]	236	Prosp	ACLR (not further specified), nonoperative	NS	Early repair recommended	<90 d or >90 d	46-113 mo	38/191 (20%) meniscal surgery; 44/191 late ACLR (27/44 meniscal injury)	Early ACLR: 2/45 (4%) late-phase meniscal surgery
Sanders[93]	1928	Retrosp	BPB autograft, HT autograft, allograft, nonoperative	New meniscal tear	meniscectomy or repair	<1 y vs >1 y	13.7 y (2 mo - 25 y)	37.4% secondary meniscal injury	Early ACLR: 6.1% secondary meniscal injury Delayed ACLR: 19.8% secondary meniscal injury
Hagmeijer[94]	1398	Retrosp	ACLR (not further specified), nonoperative	Subsequent meniscal injury	meniscectomy, repair, nonoperative	<6 mo or >6 mo	2 y minimal	19% meniscal injury	Early ACLR: 7% Delayed ACLR: 33%

Abbreviations: 4S-HT, four-strand hamstring tendon; ACLR, anterior cruciate ligament reconstruction; BPB, bone-patellar tendon-bone; G, gracilis; HT, hamstring tendon; NS, not specified; Prosp, prospective; Retrosp, retrospective; ST, semitendinosus tendon.

| Table A2. Does early ACLR reduce secondary a injury compared with delayed ACLR?

Author	n	Study design	ACL treatment	Meniscal injury	Meniscal treatment	Timing of treatment	Follow-up duration	Early ACLR	Delayed ACLR
Bottoni[88]	69	RCT	4S-HT (G+ST)	medial, lateral	repair (in-side-out) or debridement	<3 wk or >6 wk	165-869 d	Medial: 23.5% repair; 17.7% meniscectomy Lateral: 52.9% meniscectomy	Medial: 11.4% repair; 31.5% meniscectomy Lateral: 0.1% repair; 25.6 meniscectomy
Raviraj[87]	105	RCT	4S-HT (G+ST)	medial, lateral	debridement, partial excision	<2 wk or 4-6 wk	26 mo	Medial: 18 meniscal injuries Lateral: 20 meniscal injuries	Medial: 13 meniscal injuries Lateral: 22 meniscal injuries
Herbst[95]	206	Prosp	anatomical single bundle withautologous HT graft	NS	meniscal repair, meniscectomy	<48 h vs after 3 mo rehabilitation	24 mo	23.3% meniscal repair failure	16.7% meniscal repair failure
Foster[98]	75	Retrosp	4S-HT	NS	partial or total meniscectomy, repair	<6 mo or >6 mo between arthro-scopy and ACLR	Until surgery	16% deterioration; 62.9% of deteriorations were meniscal tears	63% deterioration
Yoo[96]	31	Retrosp	Autologous BPB or 4S-HT	Medial meniscal injury	arthroscopic all-inside suture	>12 mo after injury	mean 49.9 mo		1st MRI: 55% meniscal tear 2nd MRI (average 36 mo later): 84% meniscal tear. 42% deterioration of tears (no tear → tear; → longitudinal bucket handle)
de Roeck[97]	68	Retrosp	PT autograft, HT	NS	NS	Mean injury to arthroscopy: 10.1 mo; mean time to ACLR: 13.2 mo	Until surgery		Diagnostic arthroscopy: 63.2% tear During ACLR: 73.5%

Abbreviations: 4S-HT, four-strand hamstring tendon; ACLR, anterior cruciate ligament reconstruction; BPB, bone-patellar tendon-bone; G, gracilis; HT, hamstring tendon; NS, not specified; PT, patellar tendon; Prosp, prospective; RCT, randomized controlled trial; Retrosp, retrospective ST, semitendinosus tendon.

Table A3. Does ACLR protect the repaired meniscus?

Part 1

Author	n	Study design	ACL treatment	Meniscal injury	Meniscal treatment	Timing of treatment	Follow-up duration (range)	ACL-Deficient	ACLR
Van der Wal[99]	238	Retrosp	Nonoperative, concomitant ACLR, postponed ACLR. Autologous 4S-HT (G+ST).	NS	repair	<2 wk, 2-12 wk and >12 wk	median 41 mo	100% failure of meniscal repair (3/3)	Concomitant: 20.4% failure Postponed ACLR: 33.3% failure Intact ACL: 31.7% failure
Majeed[100]	136	Retrosp	Nonoperative, concomitant ACLR, postponed ACLR.	Medial, lateral	All-inside with FasT-Fix anchors	repair <6 wk of injury; ACLR concomitant/ delayed >6 wk	mean 9 mo (1-26)		Concomitant: 14.5% failure of repair Postponed ACLR: 27% failure of meniscal repair
Gallacher[101]	172	Retrosp	delayed ACLR, concomitant ACLR, nonoperative. HT and PT.	NS	all inside (clear fix, or FasT-fix, or ultrafast fix)	delayed group ACLR 2.8 mo after meniscus repair	Delayed: mean 6.1 y Concomitant: 5.4 y	50% success rate	72% success rate
Albrecht-Olsen[102]	68	RCT	NS	longitudinal vertical meniscal tears (bucket handle, displaced or in situ)	Inside-out vs meniscus arrow (all inside)	<2 mo to >1 y	Re-arthroscopy after 3-4 mo	12/19 healed	18/19 healed
Plasschaert[103]	51	Retrosp	concomitant ACLR, delayed, non-operative. BPB	meniscal lesion in conjunction with a tear of the ACL	Outside-in	<14 d or 14 d - 1 y	3.5 y	1/5 failure	1/8 failure

Author	n	Study design	ACL treatment	Meniscal injury	Meniscal treatment	Timing of treatment	Follow-up duration (range)	ACL-Deficient	ACLR
Kimura[104]	46	Retrosp	ACLR, nonoperative.	vertical tear (longitudinal part shorter than 2 cm)	Suture, peripheral vascular stimulation, synovial pedicle flap	mean 15 mo	Re-arthroscopy 2-30 mo after surgery	1/5 healed	26/26 healed ACL intact: 11/15 healed
Valen[105]	57	Retrosp	Concomitant ACLR, earlier ACLR, delayed ACLR. BPB.	medial, lateral, Displaced bucket handle, posterior	Outside-in	NS	mean 2 y	No detrimental effect of ACL deficiency	
Jensen[106]	49	Retrosp	ACLR, nonoperative.	Vertical unstable tears in outer third	arthroscopic repair (inside out)	<14 d or >14 d	average 4.5 y (1-6.3)	46% retear	0% retear ACL intact: 11% re-tear
Austin[107]	101	Retrosp	ACLR (PT allograft or autograft), Nonoper-ative	longitudinal tears within 5 mm of the meniscosynovial junction >1cm/ symptoms on provocation and <1 cm. lateral, medial	inside out (body and bucket handle) and outside-in (posterior tears)	>3 wk after injury	NS	2 repair failure	unclear

Abbreviations: 4S-HT, four-strand hamstring tendon; ACLR, anterior cruciate ligament reconstruction; BPB, bone-patellar tendon-bone; G, gracilis; HT, hamstring tendon; NS, not specified; PT, patellar tendon; Prosp, prospective; RCT, randomized controlled trial; Retrosp, retrospective ST, semitendinosus tendon.

03

Intra-articular injection with Autologous Conditioned Plasma does not lead to a clinically relevant improvement of knee osteoarthritis: a prospective case series of 140 patients with 1 year follow-up

Korpershoek JV, Vonk LA, De Windt TS, Admiraal J, Kester EC, Van Egmond N, Saris DBF, Custers RJH. Intra-articular injection with Autologous Conditioned Plasma does not lead to a clinically relevant improvement of knee osteoarthritis: a prospective case series of 140 patients with 1-year follow-up. Acta Orthop. 2020 Dec;91(6)

Abstract

Background

Platelet-rich plasma (PRP) is broadly used in the treatment of knee osteoarthritis, but clinical outcomes are highly variable. We evaluated the effectiveness of intra-articular injections with Autologous Conditioned Plasma (ACP®), a commercially available form of platelet-rich plasma, in a tertiary referral center. Secondly, we aimed to identify which patient factors are associated with clinical outcome.

Methods

140 patients (158 knees) with knee osteoarthritis (Kellgren and Lawrence grade 0-4) were treated with 3 intra-articular injections of ACP®. The Knee Injury and Osteoarthritis Outcome Score (KOOS), pain (Numeric Rating Scale; NRS), and general health (EuroQol 5 Dimensions; EQ5D) were assessed at baseline and 3, 6, and 12 months follow-up. The effect of sex, age, BMI, Kellgren and Lawrence grade, history of knee trauma, and baseline KOOS on clinical outcome at 6 and 12 months was determined using linear regression.

Results

Mean KOOS increased from 37 at baseline to 44 at 3 months, 45 at 6 months, and 43 at 12 months follow-up. Mean NRS-pain decreased from 6.2 at baseline to 5.3 at 3 months, 5.2 at 6 months, and 5.3 at 12 months. EQ5D did not change significantly. There were no predictors of clinical outcome.

Conclusion

ACP® does not lead to a clinically relevant improvement (exceeding the minimal clinically important difference) in patients suffering from knee osteoarthritis. None of the investigated factors predicts clinical outcome.

Introduction

Platelet-rich plasma (PRP) is an autologous blood product with a platelet concentration above the concentration of peripheral blood, which is obtained after separating the different blood components by centrifugation. Due to the important function of platelets in the physiology of hemostasis, inflammation, and tissue repair[120,121], PRP emerged as a potential treatment for osteoarthritis (OA). High levels of growth factors and cytokines present in platelets stimulate production of cartilage extracellular matrix, proliferation of chondrocytes, and migration of chondrocytes *in vitro*[55,56]. The potential beneficial effect of PRP in OA, together with the lack of regulatory restrictions in the use of these minimally manipulated autologous products, has rushed the field forward. The efficacy of PRP for the treatment of OA in clinical trials varies between no clinically relevant effect and a strong analgesic effect[61,122–127].

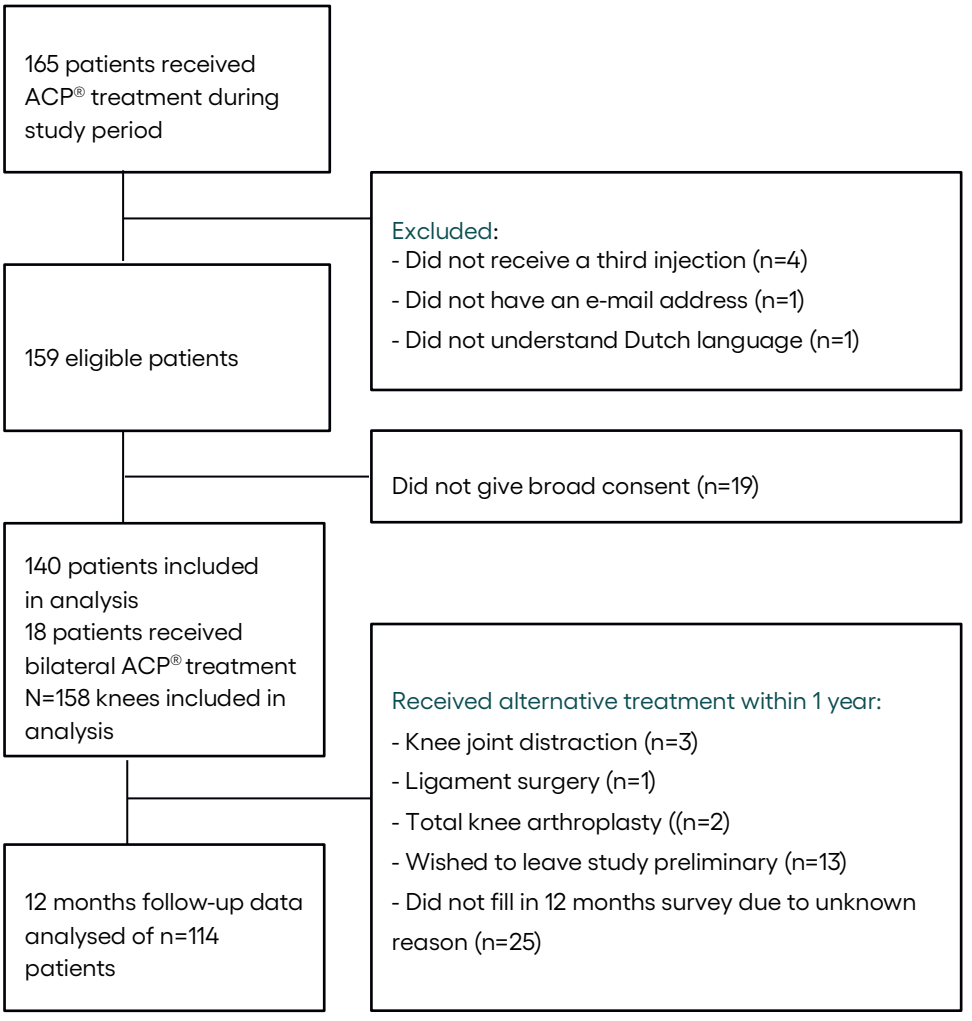
The efficacy of a commercially available PRP, Autologous Conditioned Plasma (ACP®, Arthrex GmbH, Munich, Germany) has been proven in the setting of RCTs[123,128,129], but effectiveness has not been investigated in daily clinical practice. Moreover, the effect of different patient factors on the clinical outcome after ACP® treatment is unknown.

This prospective case series aims to assess the effectiveness of ACP® in clinical practice and to investigate the effect of sex, age, BMI, radiographic OA grade (Kellgren and Lawrence), history of knee trauma, and baseline Knee Injury and Osteoarthritis Outcome Score (KOOS) on clinical outcome. Since there is no consensus on whether PRP is more effective in mild or advanced OA[130–132], we included patients with symptomatic OA of all grades. We hypothesize that treatment with ACP® leads to clinically relevant improvement in $KOOS_5$ and that clinical outcome can be predicted with any of the investigated patient factors.

Methods

Study design and setting

This prospective case series includes patients treated with ACP® in an academic hospital (University Medical Center Utrecht, The Netherlands) between March 2017 and October 2018. A minimal follow-up of 1 year was chosen, because the effect of ACP® reaches its maximum between 6 and 12 months [123,129,133]. Inclusion criteria were: first series of ACP®, symptomatic OA (Kellgren and Lawrence grade 0 to 4), sufficient understanding of Dutch language to fill in the questionnaires and written informed consent. Exclusion criteria were: less than 3 ACP® injections and earlier treatment with ACP®.



| Figure 1. Flow diagram of patient recruitment. Abbreviations: ACP®, Autologous Conditioned Plasm

Patients

140 patients (158 knees) could be included (Figure 1). 43 patients received 1 of the 3 injections with a 2-week interval (due to public holidays and other scheduling issues), all others received 3 consecutive injections with a 1-week interval. Sex, age, and BMI were collected from the patient records. History of knee trauma was defined as having a previous diagnosis of traumatic meniscus tear, cartilage defect or cruciate ligament tear. Baseline data were complete for all patient factors except BMI (35% missing) (Table 1). We did not monitor or correct for the use of other medications during the study period.

Table 1. Baseline characteristics of 140 included patients (158 knees)

Characteristic	N= 158
Age, mean (SD)	49 (10)
Female sex, n (%)	80 (51)
BMI, mean (SD)	28 (4.1)
History of traumatic injury, meniscus, anterior cruciate ligament, cartilage defect, n(%)	79 (50)
Baseline $KOOS_5$, mean (SD)	37 (14)
Baseline NRS-pain, mean (SD)	62 (1.9)
Baseline EQ5D, mean (SD)	63 (19)
Bilateral treatment, n	18
Kellgren and Lawrence Grade, n (%)	
0	8 (5.1)
1	40 (25)
2	55 (35)
3	43 (27)
4	12 (7.6)

Abbreviations: BMI, body mass index; EQ5d, EuroQol 5 dimensions; $KOOS_5$, average of the 5 subscales of the Knee Injury and Osteoarthritis Outcome Score (KOOS); NRS-pain, numeric rating scale; SD, Standard Deviation

Radiographic assessment

Patients underwent anteroposterior and lateral view radiographies prior to treatment. Kellgren and Lawrence grade was assessed by 3 blinded observers. In case 1 observer rated the radiograph with 1 grade lower or higher than the others, the grade of the 2 observers was accepted. If the grades of 2 observers were 2 or more apart, agreement was reached in a consensus meeting. Interobserver reliability was assessed using a 2-way random intraclass correlation coefficient. The internal consistency of the Kellgren and Lawrence grade was good with a Cronbach's alpha of 0.89.

ACP® preparation

The Arthrex ACP® Double-Syringe System (Arthrex GmbH, Munich) was used for preparation of ACP®. 15 mL of peripheral blood was drawn and centrifuged at 360G for 5 minutes to separate the blood components. Approximately 3-6 mL ACP® was drawn into

the inner syringe and injected into the knee joint using a superolateral approach with the patient in supine position.

ACP® composition

Using the CELL-DYN Emerald hematology analyzer (Abbott B.V., Abbott Park, Illinois), 28 random samples of leftover material from ACP® syringes were analyzed anonymously in order to characterize the administered PRP. Platelet, erythrocyte, and leucocyte concentration were measured in duplicate. The volume of injected material was documented.

Patient Reported Outcome Measures

Patients completed all questionnaires using an online survey tool (OnlinePROMS, InterActive Studios, Rosmalen, the Netherlands) at baseline and at 3, 6, and 12 months follow-up. Possible scores ranged from 0-100 (worst-best) for KOOS and EuroQol 5 Dimensions (EQ5D), and 0-10 (best-worst) for Numeric Rating Scale for pain (NRS-pain). Dutch translations of KOOS[134], EQ5D[135], and NRS-pain[136] were used. In case of bilateral treatment, patients filled in 2 separate surveys. Patients received a reminder after 5 and 10 days, and were contacted by telephone after 2 weeks in order to increase compliance. 89% of the patients filled out the survey at baseline, 87% at 3 months, 76% at 6 months and 75% at 12 months follow-up. Of patients that were lost to follow-up, data collected up to that point were included in the analyses.

Data processing and statistics

Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) (version 15.0.0.2, Chicago, Illinois). Baseline patient factors are reported by means and standard deviation (SD) or number of patients and percentages. Outcomes are shown as average and 95% confidence intervals (CI). Missing data were not imputed; patients with missing outcome variables were not included in the analysis of those specific variables. P-values <0.05 were considered significant.

The primary outcome, the effectiveness of ACP® at 1 year, was evaluated using the change from baseline to 1 year follow-up in the average score on the 5 subscales of the KOOS (pain, symptoms, activities of daily living, sport and recreation, and knee related quality of life). Change from baseline ($\Delta KOOS_5$) was estimated as an average population change using generalized estimating equations (GEE). $\Delta KOOS_5$ was compared to the minimal clinically important difference (MCID) recommended for KOOS [137] using the CI. Since a MCID for conservative OA treatment has not been defined and the MCID is

highly variable based on calculation method and subscale of KOOS[138], we compare our data with the MCID of 8-10 recommended by the developers of the KOOS[137].
In order to address selective loss to follow-up, using a subgroup-analysis, patients lost to follow-up at 12 months were compared to the group that completed the follow-up. In another subgroup-analysis, patients that returned for a second series of ACP® injections after more than 1 year were compared to patients that did not undergo second ACP® treatment. Baseline factors were compared between subgroups using t-tests for continuous variables and Pearson Chi-square for quantitative variables.
Correlation between the $\Delta KOOS_5$ and sex, age, BMI, Kellgren and Lawrence grade, history of knee trauma, and baseline $KOOS_5$ was assessed using GEE. As a rule of thumb, minimal sample size for a linear model is 10 patients per factor included in the model, therefore a minimum of 120 patients was included. Collinearity was assessed using correlation matrices, linearity using a scatterplot. Variables reaching a p-value lower than 0.2 in the univariate regression were entered in a multivariate regression model. Variables were removed from the multivariate model in order of p-value (highest first). Variables reaching a $p<0.05$ in the multivariate model were retained.

Ethics, funding, data-sharing, and potential conflicts of interest

This study was submitted to the institutional ethical review board of the University Medical Center Utrecht (METC 19-242, 03-04-2019; METC 17-005, 10-01-2017) and was conducted according to the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all individual participants included in the study. This research was supported by the Dutch Arthritis Foundation (LLP-12). The study dataset is available from the corresponding author upon reasonable request. The authors declare that they have no competing interests.

Results

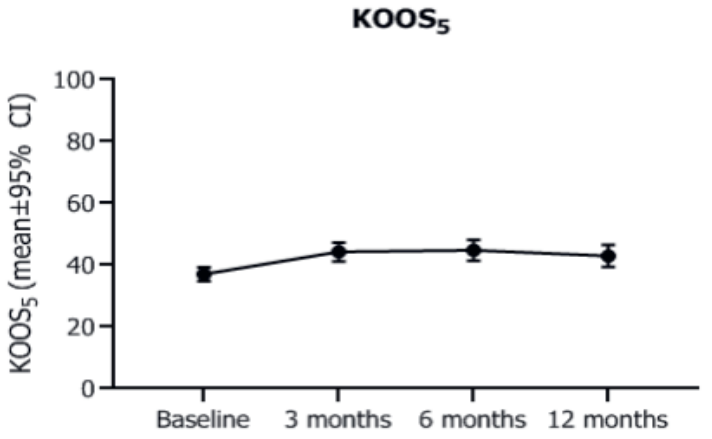
Patients

A flow diagram of patient recruitment can be found in Figure 1. Of all patients, 89% filled out the survey at baseline, 87% at 3 months, 76% at 6 months and 75% at 12 months follow-up.

ACP® composition

Platelet concentration of 28 random anonymous samples of 18 patients was 513 (184) $\times 10^9$/L, leucocyte concentration was 6.0 (10) $\times 10^9$/L, and erythrocyte concentration was

0.07 (0.08) ×10^9/L. The average volume of the injected ACP® of which these 28 samples were derived from was 4.4 (0.8) mL.



| Figure 2. $KOOS_5$ after autologous conditioned plasma treatment. $KOOS_5$ is the average of the 5 subscales of the Knee Injury and Osteoarthritis Outcome Score (KOOS). Image shows mean and 95% Confidence Interval (CI) at baseline, 3, 6, and 12 months after treatment with Autologous Conditioned Plasma.

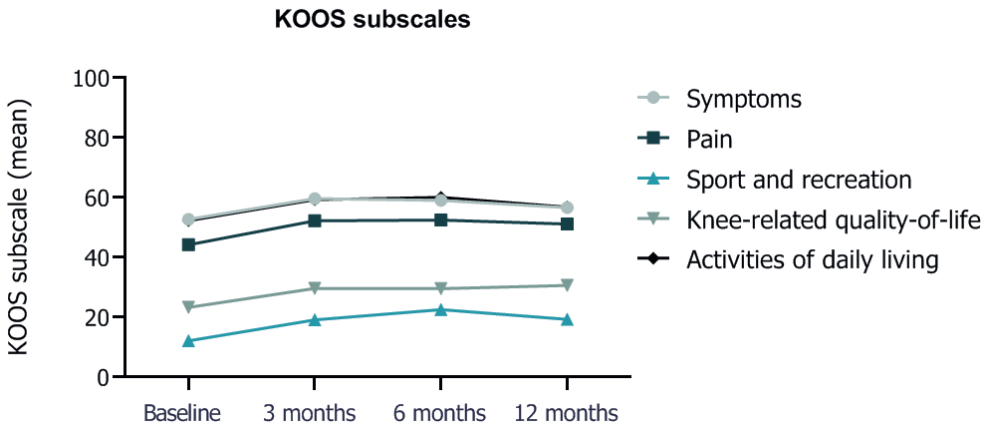
Patient reported outcomes at 3, 6, and 12 months follow-up

Compared to baseline, $KOOS_5$ increased at 3, 6, and 12 months after treatment (all $p<0.05$; Figure 2). There were no statistically significant improvements between the follow-up-assessments. $\Delta KOOS_5$ partially overlapped with the MCID of 8-10 at 3 months (CI, 4.9-9.5), 6 months (CI, 4.7-11), and 12 months (CI, 2.8-9.0) after treatment. At 6 months, 28% of patients reached the MCID of 8 or higher, 23% reached the MCID at 12 months. The change from baseline was comparable and statistically significant in all KOOS-subscales (Figure 3). Pain (NRS) decreased from baseline to 3, 6, and 12 months after treatment, but did not improve statistically significant between follow-up assessments. EQ5D was similar in all of the assessments (Table 2).

| Table 2. Patient reported outcomes after treatment with autologous conditioned plasma.

	Baseline	3 months	6 months	12 months
$KOOS_5$	37 (35-39)	44 (41-47)	45 (41-48)	43 (39-47)
EQ5D	63 (60-66)	64 (61-68)	67 (63-70)	66 (62-70)
NRS	6.2 (5.8-6.5)	5.3 (4.9-5.7)	5.2 (4.8-5.6)	5.3 (4.8-5.7)

Table shows average (confidence interval)



| Figure 3. KOOS-subscales. Average KOOS in the subscales pain, symptoms, function in activities of daily living, function in sport and recreation, and knee related quality of life at baseline, 3, 6, and 12 months after treatment with Autologous Conditioned Plasma. *Abbreviations: KOOS, Knee Injury and Osteoarthritis Outcome Score*

Loss to follow-up

At baseline, age, BMI, history of knee trauma, Kellgren and Lawrence grade, and $KOOS_5$ of patients that were lost to follow-up at 12 months did not differ from patients that completed the follow-up. The group that was lost to follow-up consisted of more men (64%). At 3 months, patients that were lost to follow-up at 12 months had a $\Delta KOOS_5$ of 6.8 (CI, 1.9-12), and patients that completed the follow-up had a $\Delta KOOS_5$ of 7.2 (CI, 4.6-9.8). The missing values in $KOOS_5$ at 12 months were imputed using the values of $KOOS_5$ at 3 months in order to assess the effect of this loss to follow-up. The $KOOS_5$ of the complete dataset, including the imputed data, is 43 (CI, 40-46).

Second series of ACP® injections

After more than a year, a second series of ACP® injections was given to 31 patients (34 knees). At baseline, these 31 patients did not differ from the others in sex, age, BMI, Kellgren and Lawrence grade, history of knee trauma, and $KOOS_5$. At 6 months, the patients that later returned for a second series had a $\Delta KOOS_5$ of 15 (CI, 9.4-21), whereas the patients that did not return for a second series of ACP® injections had a $\Delta KOOS_5$ of 5.4 (CI, 2.2-8.6). At 12 months, the patients that returned for a second series of injections had a $\Delta KOOS_5$ of 9.5 (CI, 4.2-15), and the others had a $\Delta KOOS_5$ of 4.7 (CI, 0.1-8.2).

Linear regression

Sex, age, BMI, Kellgren and Lawrence grade, history of knee trauma, and baseline $KOOS_5$ were not associated with clinical outcome ($KOOS_5$) (Table 3). The variables sex, history of knee trauma, baseline $KOOS_5$, and BMI were entered in a multivariate model, but not retained due to a p-value higher than 0.05.

| Table 3. Univariate linear regression with coefficients of several factors in the prediction of $KOOS_5$

Generalized Estimating Equations		
Factor	**b**	**p-value**
Age	-0.1(-0.3-0.1)	0.4
Sex (male)	-4(-8.6-0.7)	0.1
BMI	0.6(-0.2-1.1)	0.1
History of traumatic injury*	-0.5(-5.1-4.1)	0.8
$KOOS_5$ at baseline	-0.1(-0.3-0)	0.1
Kellgren and Lawrence Grade		0.1
0	Reference category	Reference category
1	-8.2(-18.5-2.1)	0.1
2	-1.5(-11.5-8.7)	0.8
3	-4.5(-14.8-5.9)	0.4
4	0.4(-12.5-13.1)	0.9

*Meniscus injury, anterior cruciate ligament rupture, cartilage defect.
Abbreviations: BMI, body mass index; CI, Confidence Interval; $KOOS_5$, average of the 5 subscales of the Knee Injury and Osteoarthritis Outcome Score (KOOS).

Discussion

In this prospective case series, treatment with intra-articular ACP® for knee OA led to a statistically significant, but not clinically relevant, improvement of the $KOOS_5$ after 3, 6, and 12 months follow-up. None of the investigated patient factors predicted clinical outcome, in contrast to our hypothesis. The highest change from baseline ($\Delta KOOS_5$) was observed at 6 months and did not exceed the MCID for KOOS [137]. In patients that returned for a second series of ACP® injections after 1 year, the $\Delta KOOS_5$ exceeded the MCID at 6 months, but decreased at 12 months. 79% of patients did not return for a

second series, due to a longer-lasting improvement, or based on the low $\Delta KOOS_5$ in these patients at 6 months, more likely due to an insufficient improvement.
Poor clinical results were described previously in a RCT using a different PRP composition[139]. After treatment with PRP, no superior clinical improvement was found compared to hyaluronic acid and the improvement in IKDC-score (International Knee Documentation Committee) did not reach the MCID[140]. However, reported results of PRP treatment are predominantly good[141,142] and we expected a higher $\Delta KOOS_5$ after treatment.
An important source of variation and possible explanation for our findings is the different settings in which studies are executed. In a RCT, the efficacy of PRP is investigated under controlled circumstances. The participants are selected in order to minimize co-morbidity and the protocol is designed to reach maximal patient and caregiver compliance. In this prospective case series, the effectiveness of PRP was investigated in the setting of daily clinical practice[143,144] and our real-world data show that $\Delta KOOS_5$ does not exceed the MCID. Moreover, the observed improvement might be largely attributable to a placebo-effect, as a recent meta-analysis showed that placebo injections can lead to a clinical improvement above the MCID in RCTs[145]. The placebo-effect in clinical practice might be even larger[146]. Additionally, regression to the mean might contribute to the observed effect in our study, especially since the population is highly selected by inclusion from a tertiary referral center. Furthermore, difficulty of publication of negative results, especially of non-randomized studies, might lead to publication bias, which is not considered in recently published meta-analyses[141,142]. Lastly, differences in rehabilitation protocols, number of injections, varying composition between different preparations[147], and administration-intervals might influence clinical outcome. This remains a black box for PRP and hampers comparability of studies.
The poor results cannot be attributed to the composition of ACP®, as the current composition is similar to that reported by Cole *et al.* (2017) and the manufacturer[148], with approximately twice the platelet concentration of peripheral blood[149] and a leucocyte concentration classified as minimal[150]. However, we found a notable variability in platelet and leucocyte concentration. In addition, we did not measure concentrations of cytokines and growth factors, which could provide useful information on the bioactivity of ACP®.
Notable differences in patient populations do exist between our study and other ACP® studies. We included patients in a tertiary referral center for joint preservation, with severe complaints and almost 10 points lower baseline KOOS compared to another ACP® study[133]. This could mean that ACP® is not effective in patients with severe complaints, even though our regression analysis indicated that baseline KOOS does not predict

clinical outcome. Secondly, our patient age (mean 49 years) was lower than that in other ACP® studies (mean 55-59 years)[123,129,133], but we found no effect of age on clinical outcome, similar to the results of Cole *et al.*[123] In 2 studies[133,151], younger age was even associated with a better outcome. Thirdly, patients with post-traumatic OA were included in our series, while other studies have excluded patients with a history of knee surgery [129] or treatment for a cartilage defect[128], but in our case series history of knee trauma did not predict clinical outcome. Lastly, we included 18 patients with bilateral complaints, whereas these patients were excluded in other studies[123,128]. Patients with bilateral complaints have a lower physical function and lower probability of improvement than patients with unilateral OA, and PROMs are influenced by contralateral knee pain[152,153]. To summarize, notable differences exist in patient population, but based on the results of our regression analysis and the small number of patients with bilateral complaints, these differences cannot fully explain our poor clinical outcome.

Limitations

First, this is a prospective case series, thus lacking a control group. Since previous RCTs showed efficacy of ACP® under ideal circumstances, we explicitly chose to investigate effectiveness in clinical practice. As a result, 43 patients received 1 of the intra-articular injections with a 2-week interval, while the others received all injections with a 1-week interval. This might result in variation in effectiveness, which is also a drawback for implementation of PRP in daily practice and could explain the differences between outcomes in RCTs and our clinical data. Secondly, within this heterogeneous patient population, various patient factors could influence clinical outcome, but limiting our exclusion criteria allowed us to study a population representative for the (heterogeneous) population in our clinical practice and to evaluate the influence of patient factors on treatment outcome. At the same time, the small number of included patients with Kellgren and Lawrence grade 0 and 4 limits generalizations in these groups. Effectiveness will need to be investigated in a larger cohort of patients with early (non-radiographic) or end-stage (grade 4) OA. Thirdly, a relatively large patient group was lost to follow-up. However, the average $KOOS_5$ did not change substantially when missing data at 12 months was imputed using data of 3 months. We therefore estimate the effect of this loss to follow-up to be small. Lastly, the MCID recommended for KOOS is 8-10[137], but the MCID in OA patients can actually range between 1.5 and 21 depending on calculation method and KOOS-subscales[138], and does not account for the invasiveness of the treatment or its placebo effect. A MCID for non-invasive OA therapy should be established in order to determine whether the demonstrated effectiveness reaches a meaningful level for patients.

Conclusions and implications

There was no clinically relevant improvement in the majority of patients, nor did most patients return for additional ACP® treatment. No predictors of improved clinical outcome were identified. In the limited number of patients that reached the MCID, the effect of ACP® decreased between 6 and 12 months, necessitating a second series of treatment after 1 year. In our view, ACP® should not be used in daily clinical practice in the current form and population. Future research should aim at improving the clinical outcome of this treatment by optimization of the composition of PRP and/or patient selection, before implementation in daily practice. This study demonstrates the gap between efficacy in RCTs and effectiveness in clinical practice, which underlines the importance of evaluating effectiveness after market-approval.

04

Autologous Conditioned Plasma injections do not result in a clinically relevant improvement in the majority of patients with knee osteoarthritis: better outcomes in middle-aged patients with non-traumatic etiology

Korpershoek JV, Vonk LA, Filardo G, De Windt TS, Admiraal J, Kester EC, Van Egmond N, Saris DBF, Custers RJH. Autologous Conditioned Plasma injections do not result in a clinically relevant improvement in the majority of patients with knee osteoarthritis: better outcomes in middle-aged patients with non-traumatic etiology. *Submitted*

Abstract

Background

Autologous Conditioned Plasma (ACP, Arthrex) is a commercially available platelet concentrate with promising results from early clinical trials. We investigated the clinical effect after 3 injections with ACP for the treatment of knee osteoarthritis (OA) in a tertiary referral center and studied the influence of ACP composition and of different patient factors as predictors of treatment effect.

Methods

This prospective series included 261 patients (308 knees) receiving ACP treatment for knee OA. The average patient age was 51 years. The improvement up to 12 months follow-up was measured using the Knee injury and Osteoarthritis Outcome Score (KOOS). ACP composition was analyzed for 100 patients. The predictive value of age, sex, history of knee trauma, Kellgren and Lawrence grade, body mass index, and composition of ACP were evaluated.

Results

KOOS improved from 38±14 at baseline to 45±18 at 3 months, 45±18 at 6 months, and 43±18 at 12 months; 40% of patients reached an improvement above the minimal clinically important difference (MCID) after 6 months. Variation in ACP composition did not correlate with KOOS. Older age led to a higher clinical benefit. The lack of history of traumatic knee injury also predicted better outcomes. No other factor significantly predicted outcomes.

Conclusion

The improvement in KOOS after treatment with ACP does not reach the MCID in the majority of patients. Middle-aged patients with non-traumatic knee OA may present better outcomes. The composition of ACP varies between patients but does not predict outcomes within the evaluated range.

Introduction

Worldwide, knee osteoarthritis (OA) affects 5% of the population [27]. There is a need for knee preserving treatments that aim at reducing symptoms, regenerating the damaged tissues, or at least restoring joint homeostasis and slowing down OA progression. Platelet-rich plasma (PRP) is a concentrate of autologous platelets, which has emerged as treatment for OA due to the high concentrations of growth factors and cytokines [154–156]. Although the first applications of PRP for knee OA reached the clinic 2 decades ago, the efficacy of this treatment is still subject of debate. Randomized controlled trials (RCTs) showed results varying between strong effects on pain and symptoms [157] to no beneficial effect compared to viscosupplementation [139] or placebo [57]. The composition of PRP differs between different preparation methods or manufacturers [154–156], and an inter- and intra-patient variability exist because of variation in composition of whole blood [149]. Autologous conditioned plasma (ACP), a commercially available single-spin leucocyte poor PRP, is widely used in the clinical practice for the treatment of knee OA. In our previous case series [158], injections with ACP did not lead to a clinically relevant improvement in patient reported outcome measures (PROMS). Clinical outcomes of ACP or PRP treatment could improve by optimizing patient selection or product composition. Using real-world data gathered using a prospective registry of patients visiting a tertiary referral center, the clinical effect of ACP for treatment of knee OA was studied. This study included a large number of patients and aimed to identify predictors of clinical outcome. Additionally, we analyzed product composition in a subgroup of 100 patients to identify inter- and intra-patient variability and predictive value of product composition for outcomes. We hypothesized that patient factors like age and body mass index (BMI) and higher platelet concentrations predict better treatment outcomes and could be used to optimize PRP treatment for OA.

Methods

Study design

This prospective case study included patients treated with ACP at the University Medical Center Utrecht, Utrecht, the Netherlands, between March 2017 and April 2020 to ensure 1-year follow-up. This study was performed according to the World Medical Association Declaration of Helsinki. The study was approved by the institutional ethical review board (METC 19-242, 03-04-2019; METC 17-005, 10-01-2017) and the institutional review committee for Biobanking (TCBio 18-633, 02-10-2018). All patients provided written informed consent for participation in the study. The patients included in the analysis of ACP composition provided additional consent for analysis of their material.

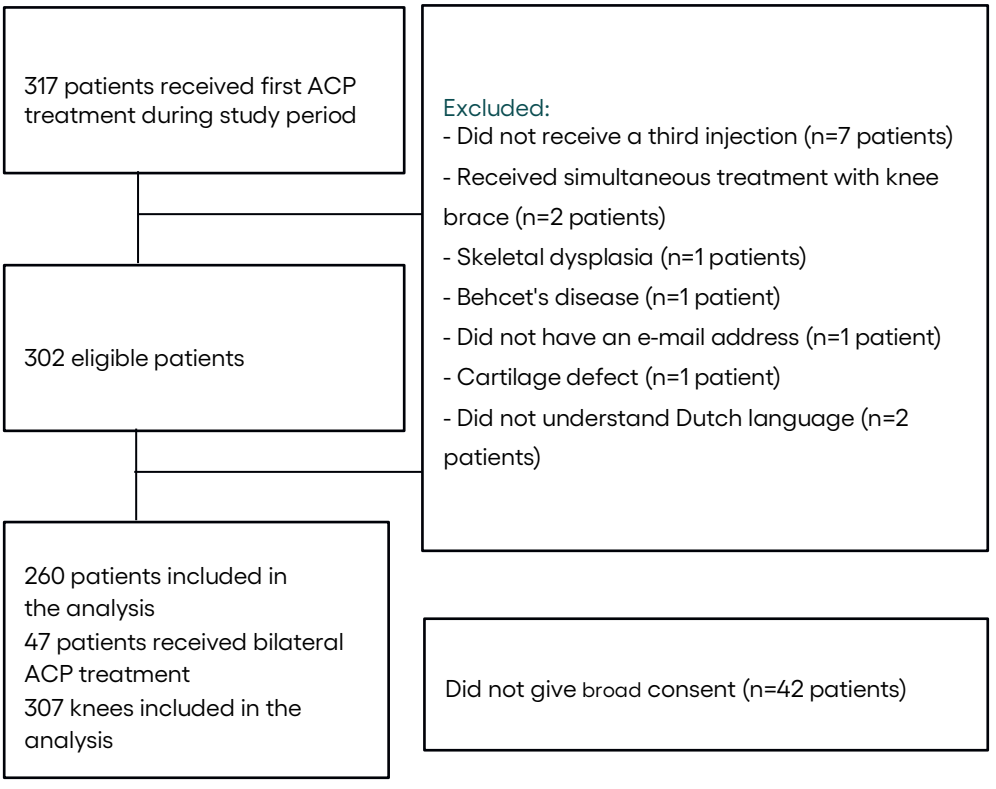
The study is reported according to the Strengthening in the Reporting of OBservational studies in Epidemiology (STROBE) guidelines (supplemental 1) for reporting cohort studies[159].

Participants

In order to select a representative population for our clinic, broad inclusion criteria and a small selection of exclusion criteria were used. Inclusion criteria were: symptomatic OA, no concomitant treatment, sufficient understanding of Dutch language. Exclusion criteria were: prior treatment with ACP, less than 3 ACP injections received, and other musculoskeletal pathologies affecting the knee.

Funding

This research was supported by the Dutch Arthritis Foundation (LLP-12).



| Figure 1. Flow diagram of patient inclusion

Data collection

An overview of patient inclusion can be found in Figure 1. Age, sex, history (meniscus injury, anterior cruciate ligament rupture, cartilage defect, tibia plateau fracture), and body mass index, were collected from the electronic patient database for all patients. Data were complete except for BMI (24% missing). Data on comorbidities were not collected, except for inflammatory conditions that were a contra-indication for participation.

Kellgren and Lawrence grading

Anterior-posterior knee radiographs were taken of all patients before undergoing treatment. Kellgren and Lawrence grades were determined by 2 experienced orthopedic surgeons and 1 clinical researcher. If the difference in grades was 2 or more between 2 graders, consensus was reached in a meeting. If a grader valued the Kellgren and Lawrence 1 grade lower or higher than the other 2 graders, the value that was agreed on by 2 graders was used. The inter-grader reliability was estimated by an intraclass correlation coefficient (ICC) using a 2-way mixed model with absolute agreement. There was excellent agreement between graders with an ICC of 0.93.

Description of ACP administration

ACP was prepared according to the manufacturers (Arthrex GmbH, Munich, Germany) recommendations: 15 mL autologous peripheral blood was drawn and centrifuged at 360 G for 5 minutes. This resulted in a variable volume of ACP in the top layer that was aspirated into the inner syringe of the double syringe system; the bottom layer with erythrocytes was discarded. The ACP was immediately injected into the knee joint using a posterolateral approach with the patient in supine position. No platelet activation was performed. Time between blood draw and injection was a maximum of 30 minutes, during which the product was kept at room temperature. Of 100 patients, approximately 200 µL residual ACP was stored in an Eppendorf with 30 µL citrate to prevent clotting and used for composition analysis. ACP composition was analyzed using a CELL-DYN Emerald hematology analyzer (Abbott B.V., Abbott Park, IL, USA). Platelet, erythrocyte, and leucocyte concentrations were measured in duplicate. The volume of injected ACP was documented. Analyses of whole blood was not performed, therefore no platelet recovery rates could be calculated. No rehabilitation protocol or immobilization were advised.

Patient reported outcome measures

PROMS were recorded before treatment (baseline), and at 3, 6, and 12 months after treatment using an online survey tool (OnlinePROMS, Interactive Studios, Rosmalen, the Netherlands). Questionnaires included Knee Injury and Osteoarthritis Outcome Score (KOOS), EuroQol 5 Dimensions 5 levels (EQ5D-5L), and Numeric Rating Scale for pain (NRS-pain) during activity and in rest. Scores ranged between 0 (worst) to 100 (best) for KOOS and EQ5D-5L, and from 0 (best) to 10 (worst) for NRS-pain. Patients filled in separate questionnaires in case of bilateral treatment. For patients that underwent other treatment before completing the follow-up, the last values were carried forward to address selective loss to follow-up. Response rates for the PROMS were 89% at baseline, 88% at 3 months, 80% at 6 months and 74% at 12 months.

Data-analysis and statistics

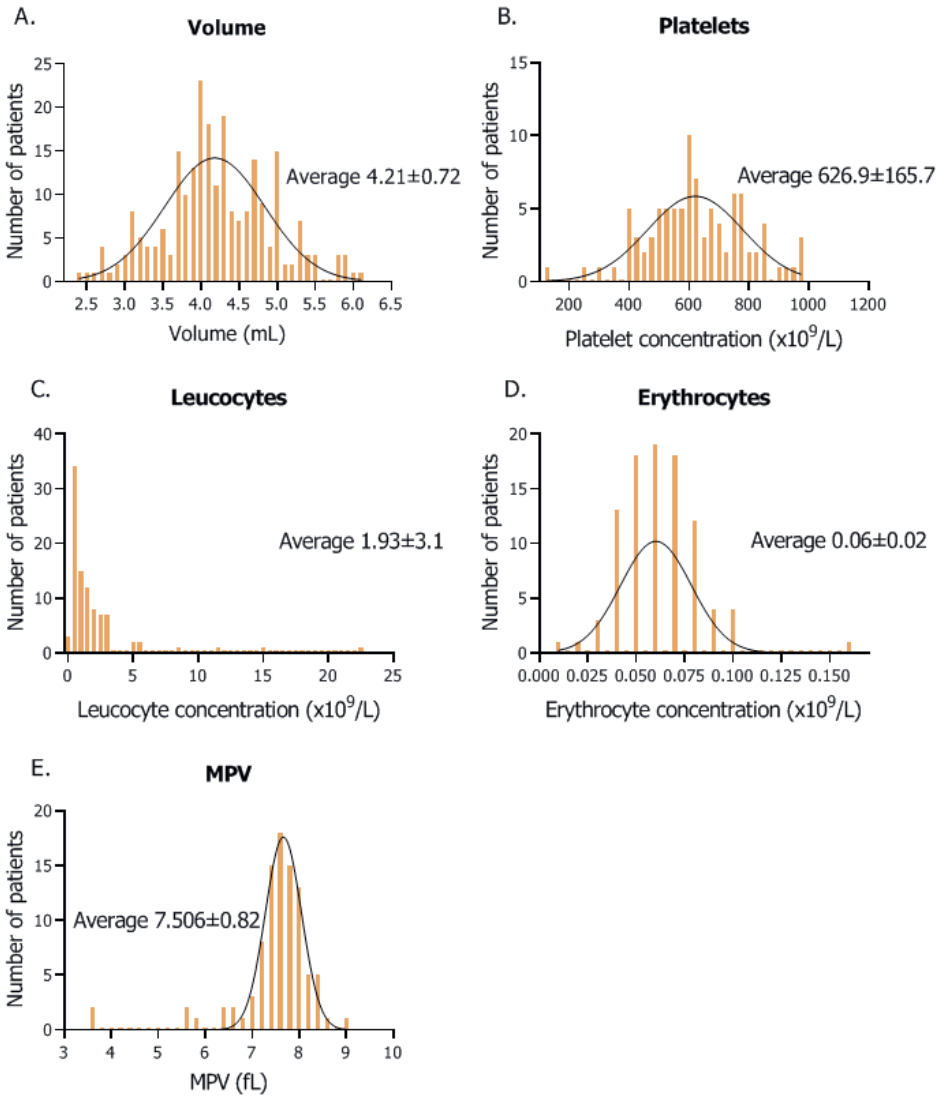
Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) (version 26.0.0.1, IBM Corp, Armonk, NY, USA). Baseline characteristics are reported as mean ± standard deviation (SD) or number of patients and percentage of total. Outcomes are shown as average and 95% confidence intervals (CI). Missing data were not imputed except for patients that underwent alternative treatment. With Generalized Estimating Equations (GEE), all available data of all patients are included in the model. P-values below 0.05 were considered statistically significant. The improvement in the average score on the 5 subscales (pain, symptoms, activities of daily living, sport and recreation, and knee-related quality of life) of the KOOS ($KOOS_5$) was assessed using GEE with multiple measurements, taking into account the possible correlation of data of bilaterally treated patients. Change in $KOOS_5$ was compared to the minimal clinically important difference (MCID) recommended for KOOS of 8-10[37,160]. For NRS-pain, both the scores for pain in rest and in activity were used.

Predictive value of age, sex, history (meniscus injury, anterior cruciate ligament rupture, cartilage defect, tibia plateau fracture), Kellgren and Lawrence grade, BMI, and composition of ACP were analyzed using GEE. The sample size of 216 knees translates to sufficient power to evaluate the 12 predictors, because as a rule of thumb 10 patients should be included per predictor. Collinearity was assessed using a correlation matrix and linearity using a scatterplot. For Kellgren and Lawrence grade, repeated contrast coding was used to compare grade 0 to grade 1, grade 1 to grade 2 and so on. To assess the influence of loss to follow-up, characteristics of patients that completed the 12 months survey were compared to patients that did not complete the follow-up. Correlations among ACP compositions in the 3 injections were assessed using Pearson correlations.

| Table 1. Baseline characteristics of included patients

Baseline Characteristics	N=307 knees
Age, mean (SD)	51 (10)
Female sex	183 (59%)
BMI, mean (SD)	29 (4.8)
History of traumatic injury (meniscus, anterior cruciate ligament, cartilage defect, tibia plateau fracture)	132 (43%)
$KOOS_5$, mean (SD)	37 (13)
Sport and recreation	13 (16)
Pain	43 (16)
Symptoms	51 (18)
Activities of daily living	52 (18)
Knee-related quality of life	24 (15)
NRS-pain in rest, mean (SD)	5.0 (2.3)
NRS-pain in activity, mean (SD)	7.1 (1.8)
EQ5D, mean (SD)	65 (19)
Other knee received ACP treatment	94 (31%)
Kellgren and Lawrence Grade	
0*	9 (2.9%)
1	60 (20%)
2	107 (35%)
3	96 (31%)
4	36 (12%)

* MRI or arthroscopically diagnosed osteoarthritis, BMI, body mass index; EQ5D-5L, EuroQol 5 Dimensions 5 levels; $KOOS_5$, the average score on the 5 subscales (pain, symptoms, activities of daily living, sport and recreation, and knee-related quality of life) of the Knee injury and Osteoarthritis Outcome Score (KOOS); NRS, numeric rating scale; SD, standard deviation.



| Figure 2. Average and distribution of ACP characteristics in 100 patients. *Abbreviations*: *MPV, mean platelet volume*

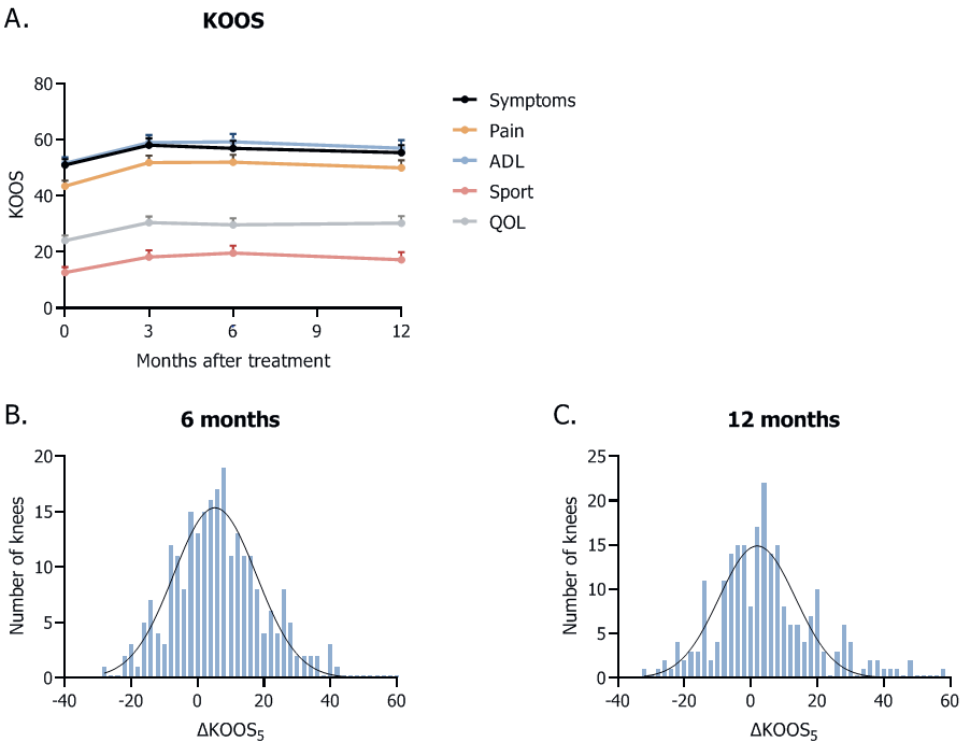
Results

Baseline characteristics and ACP composition

Baseline characteristics are shown in table 1. A total of 17 knees underwent alternative treatment before completing follow-up of 12 months. Alternative treatments were: knee joint distraction (6 knees), ligament surgery (1 knee) removal of a loose body (1 knee), genicular nerve block (1 knee), treatment elsewhere (2 knees), knee brace (1 knee), total knee replacement (2 knees), new series of ACP (2 knees), and a program provided by rehabilitation medicine (1 knee). Average injected ACP volume and platelet concentration, leucocyte concentration, erythrocyte concentration and mean platelet volume (MPV) were variable among patients (Figure 2). Based on the estimated population platelet concentration in whole blood [149], the estimated concentration was 2-3 times with a platelet recovery of 70%. Platelet concentration, leucocyte concentration, erythrocyte concentration, and MPV of the 3 consecutive injections that patients received were not significantly correlated, although intra-patient variation was smaller than interpatient variation. For example, the average of standard deviations of the platelet concentration of 3 injections in 1 patient was 162, whereas the standard deviation between all measurements in the population was 215. Injected volumes of the consecutive injections were correlated with an average Pearson correlation of 0.6. Characterization of the ACP according to Kon et al. [63] can be found in annex 1.

Effectiveness of ACP

$KOOS_5$ improved significantly from baseline to all time points. The improvements were comparable in all subscales (Figure 3). Of the available data, an improvement above the minimal clinically important difference (MCID) was reached in 45% of patients at 3 months, 40% of patients at 6 months and 33% of patients at 12 months. The change in KOOS from baseline was normally distributed around the average improvement. The patients that did not fill out 12 months follow-up (for a reason other than they received alternative treatment), did not have a different $KOOS_5$ at baseline, 3 months follow-up, or 6 months follow-up. The patients that did not complete the questionnaires at 12 months follow-up had a BMI of 1.6 points higher than the patients that did complete follow up (P=0.03), the other baseline characteristics were not different between the groups. NRS-pain in rest and in activity improved from baseline to all time points. EQ5D did not change (table 2).



| Figure 3. Mean (95% confidence interval) KOOS at baseline and after Autologous Conditioned Plasma treatment (A), change in $KOOS_5$ at 6 (B), and 12 months after treatment (C) showing distribution in the study population**.** *Abbreviations: ΔKOOS5, the change in average score on the 5 subscales (pain, symptoms, activities of daily living, sport and recreation, and knee-related quality of life) of the Knee injury and Osteoarthritis Outcome Score (KOOS).*

| Table 2. Outcomes and 95% confidence intervals after ACP injections.

	Baseline	3 months	6 months	12 months
$KOOS_5$	38 (36-40)	45 (42-48)	45 (42-48)	43 (40-45)
NRS-pain in rest	5.0 (4.7-5.3)	3.9 (3.5-4.3)	3.8 (3.5-4.2)	4.0 (3.6-4.4)
NRS-pain in activity	7.0 (6.8-7.3)	6.1 (5.7-6.4)	6.2 (5.9-6.5)	6.2 (5.9-6.6)
EQ5D-5L	66 (64-69)	66 (64-69)	67 (65-70)	67 (64-70)

Abbreviations EQ5D-5L, EuroQol 5 Dimensions 5 levels; $KOOS_5$, the average score on the 5 subscales (pain, symptoms, activities of daily living, sport and recreation, and knee-related quality of life) of the Knee injury and Osteoarthritis Outcome Score (KOOS); NRS, numeric rating scale.

Among the evaluated factors, younger patients presented less improvement, while older age led to a higher clinical benefit (B=0.2, p=0.03). The lack of history of traumatic knee injury such as cartilage defects, meniscus injury, injury to the cruciate ligaments or tibia plateau fracture also predicted better outcomes (B=4.0, p=0.03). These predicting factors correlated with a Pearson Correlation of 0.4; therefore, these factors were not entered into the multifactorial model. No other factors significantly predicted outcomes in this series (Table 3). Of all included patients, 50 patients (60 knees) returned for a second series of 3 ACP injections. At baseline, there were no differences between patients that returned for a second series or patients that did not return for a second series. At 6 months, patients that later underwent a second series of ACP had a $\Delta KOOS_5$ of 12 (CI 7-18) whereas other patients had a $\Delta KOOS_5$ of 6 (CI 4-8). At 12 months patients that later returned for a second series of ACP had a $\Delta KOOS_5$ of 7 (CI 2-10) and other patients had a $\Delta KOOS_5$ of 4 (2-7).

| Table 3. Linear regression with coefficients of factors in the prediction of $KOOS_5$

	Generalized Estimating Equations	
Variable	**B (CI)**	**P-value**
History of knee trauma	-4.1 (-0.4--7.6)	0.0
Age	0.2 (0.0-0.4) 0.0	0.0
Male Sex	1.8 (-2.0-5.7)	0.4
Bilateral treatment	-0.6 (-4.6-3.4)	0.8
Radiographic osteoarthritis grade (Kellgren and Lawrence)*		
1	-14 (-26- -1.7)	0.0
2	-3.1 (-7.9 – 1.8)	0.2
3	1.5 (-2.7-5.7)	0.5
4	0.3 (-5.2-5.8)	1
BMI	-0.2 (-0.6-0.2)	0.3
Mean platelet concentration	0.0 (0.0-0.0)	0.7
Mean leucocyte concentration	-0.2 (-0.7-0.4)	0.6
Mean erythrocyte concentration	-130 (-294-33.0)	0.1
Mean injected volume	-2.0 (-4.7-0.8)	0.2
Mean platelet volume	-1.0 (-6.3-4.1)	0.7

*grade compared to former grade using repeated contrast coding, e.g. grade 1 is 1 vs 0, grade 2 is grade 2 versus 1.

Discussion

In the majority of patients of this large series, intra-articular injections with ACP did not result in an improvement of KOOS above the MCID. The limited number of patients that reports a clinically relevant improvement in the current study is also illustrated by the small number of patients that returned for a second series of treatment. The ACP composition varied between patients, but this variation did not predict clinical outcomes. Older age and non-posttraumatic OA predicted better outcomes of ACP treatment for knee OA and could be used to improve treatment indications and outcomes.

The low clinical benefit of ACP injections for the treatment of knee OA is in agreement with our previous report [158] and a recently published RCT comparing PRP to placebo injections [57]. Both studies used a low concentration of platelets, with the platelet dosage administered being within the low range of blood derived treatments considered within PRP products in the field. While it is possible that higher doses could lead to different outcomes, a wide range of results has been reported also by previous reports on the same product. ACP was previously investigated by other authors for the treatment of knee OA, with heterogeneous findings. Using the same evaluation tool, Cerza et al. [129] and Smith et al. [128] reported highly satisfactory results at 6 months, with a total WOMAC score improvement of 43 in 60 patients (67 years old) and of 36 points in 15 patients (54 years old), respectively, while recently Sun et al. [161] documented with the same score at the same follow-up a much lower score improvement of 15 points in 38 patients (58 years old) treated with ACP for knee OA. In this light, the larger series documented in our study adds important information to the debate on ACP effectiveness. The low percentage of patients reaching a MCID warrants caution in offering this treatment solution to patients, which should be aware of the results and have proper expectations. In addition, the wide range of results in the literature in different settings with different patient populations could be explained by different factors, which should be identified, aiming at understanding the real potential and indications for this biological treatment approach.

This study aimed to improve treatment indications and clinical outcomes after ACP treatment by identifying patient or ACP factors that predict better outcomes. BMI and Kellgren and Lawrence grade did not affect clinical outcomes in our study, like in a recent large RCT [57]. However, the BMI of patients that did not fill in the 12 months surveys was higher at baseline than the BMI of the remaining patients, suggesting selective loss to follow-up. In our cohort, older age and non-post traumatic OA predicted better outcomes. Age is a controversial factor, patient age did not affect outcomes in a retrospective cohort study focused on identifying predictors of effectiveness of PRP therapy for knee OA [162], while other authors showed that older age increased the odds for treatment failure [163]. However, the average patient age in these studies was 60-70 years

whereas the average age in our cohort was 50 years. Thus, optimal patient age could lie between 50 and 70 years. The young age of the patients representing our cohort might be attributed to the fact that the patients were included in a tertiary referral center. The high incidence of posttraumatic OA and low subscales for sports and recreation indicated that our patient population was young and more active, and the knee complaints posed a heavy burden on the quality of life. While most of the literature reported good results in older patients [164], lower results have been reported for younger and active patients seeking to return to sport [165], with a more disappointing outcome in line with the findings of this study. Active patients affected by knee OA have less satisfactory results since only half can achieve the same sport level as before the onset of symptoms, and they should be made aware of their low chances of benefit from this treatment. Older patients, often less aspiring of an active lifestyle might benefit more from ACP treatment. The activities that patients wish to perform should be taken into account in future studies. Similar to our findings, in a RCT of PRP-treatment in a sports clinic, patients around 50 years of age with around 50% incidence of post-traumatic OA were included [166], and comparable improvements were reported in the PRP arm of the study. Patients between 50 and 70 years old, and patients with no history of traumatic knee injury might benefit more from PRP treatment.

The ACP composition of 100 patients was measured and correlation to treatment outcomes was assessed. Similar to earlier reports [63,167], platelet concentration did not correlate with treatment outcomes. The naturally occurring variation in ACP composition did not predict clinical outcomes. The MPV correlates to the content of platelets [63], thus there could be an effect of MPV on clinical outcomes. However, the small variations in MPV did not predict outcomes in our study. Injection of a single dose of 10 billion platelets resulted in reasonable improvements in Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score in the study by Bansal et al [58]. The absolute number of platelets per injection was on average 4 times lower in our study. Platelet number in ACP could be too low, and these results might favor administration of a single high dose of platelets instead of 3 injections with lower concentrations. As long as no direct dosing studies are performed, firm conclusions on optimal composition cannot be drawn due to the heterogeneity in the studied products and patient populations. Dosing studies on platelet concentration, leucocyte concentration, and MPV could lead to a more bioactive PRP, but large patient numbers would need to be enrolled in such studies to sort out all of the contributing factors. In the study by Zahir et al [167], an *in vitro* inflammatory co-culture model was established to predict which patients benefit more of treatment, as BMI, age, sex, and platelet concentration did not predict outcomes. Patients that did not respond well to PRP treatment also lacked the inhibition of pro-inflammatory cytokine Tumor

Necrosis Factor Alpha (TNFα) in the *in vitro* model. Thus, certain factors in PRP resulted in differences in bioactivity between patients, but at this time, it is unclear which factors are responsible. Identifying these factors could improve patient selection or development of a synthetic or allogeneic PRP with a higher bioactivity.

Strengths and limitations

This is a non-controlled study; therefore, the size of a placebo effect in the current study is unclear. The placebo effect is likely considerable, as injections with saline lead to an improvement exceeding the MCID in half of the patients [145], similar to our findings with ACP injections. However, this study shows the results of ACP treatment in a real-world setting, and the large number of patients included in this series and broad inclusion and exclusion criteria are strengths for generalizability of the study. Furthermore, the large number of patients included in this series allowed us to identify factors that predict better outcomes and could optimize patient selection. Another limitation of this study is that we did not analyze whole blood samples and we cannot relate the ACP composition to these values. Therefore, we do not have any information of the variations in the ACP preparation process between patients or injections. The analysis of the predictive value for ACP composition in a large group of patients is a strength of this study.

Conclusions and implications

Intra-articular injections with ACP do not lead to a clinically relevant improvement in the majority of patients with knee OA, therefore ACP should not be used as a routine treatment in the clinical setting. Instead, patients that might benefit more of treatment should be selected. This study identified the lack of a history of traumatic knee injury and older age as predictors for better outcome. As the patients in the current cohort were relatively young, the optimal age for PRP treatment might lie between 50 and 70 years. Other predicting patient factors should be identified. The naturally occurring variation in blood derivatives composition did not predict clinical outcomes in this series treated with ACP and optimizing the composition of PRP products should be the focus in follow-up research, preferably using large dosing studies.

05

Cell-based meniscus repair and regeneration: At the brink of clinical translation? A systematic review of preclinical studies

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Abstract

Background

Although preclinical research focusing on augmentation of meniscal tear repair and regeneration after meniscectomy is encouraging, clinical translation remains difficult. The purpose of this study is to systematically evaluate the literature on in vivo meniscus regeneration and explore the optimal cell sources and conditions for clinical translation. We aimed at thorough evaluation of current evidence as well as clarifying the challenges for future preclinical and clinical studies.

Methods

A search was conducted using the electronic databases of MEDLINE, Embase, and the Cochrane Collaboration. Search terms included *meniscus, regeneration,* and *cell-based*.

Results

After screening 81 articles based on title and abstract, 51 articles on in vivo meniscus regeneration could be included; 2 additional articles were identified from the references. Repair and regeneration of the meniscus has been described by intra-articular injection of multipotent mesenchymal stromal (stem) cells from adipose tissue, bone marrow, synovium, or meniscus or the use of these cell types in combination with implantable or injectable scaffolds. The use of fibrochondrocytes, chondrocytes, and transfected myoblasts for meniscus repair and regeneration is limited to the combination with different scaffolds. The comparative in vitro and in vivo studies indicate that the use of allogeneic cells is as successful as the use of autologous cells. The implantation or injection of cell-seeded scaffolds increased tissue regeneration and led to better structural organization compared to cell free methods. None of the studies mentioned in this review compare the effectiveness of different (cell seeded) scaffolds.

Conclusion

There is heterogeneity in animal models, cell types, and scaffolds used, and limited comparative studies are available. The comparative in vivo research that is currently available is insufficient to draw strong conclusions as to which cell type is the most promising. However, there is a vast amount of in vivo research on the use of different types of multipotent mesenchymal stromal (stem) cells in different experimental settings, and good results are reported in terms of tissue formation. None of these studies compare the effectiveness of different cell-scaffold combinations, making it hard to conclude which scaffold has the greatest potential.

Introduction

The meniscus is essential for shock absorption, stability of the knee joint, and articular surface protection.[11] Meniscus damage is one of the most common injuries seen by orthopaedic surgeons, with an annual incidence of 66 to 70 per 100,000 people. Meniscal tears can be caused by trauma or degenerative disease. Traumatic meniscus injury is frequent among high school athletes, with an incidence of 5.1 per 100,000 in the United States[11,168]. Meniscus injury is an essential predictor of development of degenerative joint disease and is strongly correlated with the incidence of subsequent osteoarthritis[11,169]. Thus, retaining, repairing, or even replacing the meniscus receives increasingly more attention. The proper prevention and treatment of meniscal damage is an important and large-volume unmet medical need.

The ability of the torn meniscus to self-repair is limited[7,11,170]. Hypovascularity, hypocellularity, high density of the extracellular matrix, presence of inflammatory cytokines, and mechanical stress all contribute to low or absent self-repair, particularly in the avascular zone[7,171–173]. Current treatment strategies are primarily aimed at pain relief and improvement of joint function. Meniscectomy leads to loss of contact area, which eventually may lead to degenerative changes and osteoarthritis[169]. The incidence of osteoarthritis (both radiographic and symptomatic) has been shown to increase up to 7 fold after total meniscectomy in a 16-year follow-up cohort study[174]. The amount of resected tissue was a predictor of osteoarthritis[175]. Although partial meniscectomy showed to increase radiographic signs of osteoarthritis, it did not significantly increase symptoms at 8- to 16-year follow-up[176]. This is a drawback of this frequently used therapy, particularly in young, athletic patients. On the other hand, 2 recent randomized trials showed that physical therapy performs equally to partial meniscectomy in terms of pain reduction and functional improvement[177,178]. However, in the study by Katz et al[177]. 30% of the patients allocated to physical therapy still received a meniscectomy within 2 months, thus limiting the advantage of conservative treatment. Although the advancement of arthroscopic surgical procedures and increased attention to osteoarthritis have led to numerous new methods for meniscal repair, these are mainly indicated in young patients with a traumatic tear within, or close to, the vascular zone, and reported failure rates are high (20%-24%)[169,179]. Different biomaterials, such as collagen and glycosaminoglycan scaffolds, allow for ingrowth and differentiation of cells, potentiating repair after meniscectomy. Although encouraging clinical results are achieved by scaffold implantation, a failure rate of up to 10% is reported, and data on long-term outcomes are lacking[169,180].

The use of cells for regeneration or augmentation of repair holds great potential, as cells can adapt to the new environment and respond to signals sent by the damaged

meniscus[181] or differentiate and actively contribute to the repair[182]. In this way, cell therapy may improve long-term outcomes of partial and total meniscectomy and allow for a broader indication of repair of both for traumatic as well as degenerative meniscus tears.

Many studies describe the use of multipotent mesenchymal stromal (stem) cells (MSCs) because they are easy to isolate and expand and have the capacity to differentiate into the chondrogeneic lineage[183]. In the first human trial, Vangsness et al[184] studied intra-articular injection of MSCs in patients from 18 to 60 years of age awaiting partial medial meniscectomy based on magnetic resonance imaging (MRI) findings. Included patients received intra-articular MSCs 7 to 10 days after partial meniscectomy. Safety data, meniscal volume, and clinical outcomes were evaluated during a 2-year follow-up period. Significant improvement with greater than 15% increased meniscal volume was reported in 24% of patients. Two case reports describe intra-articular injections of MSCs and report significant clinical improvement[185] and increased meniscal volume[186]

Fibrochondrocytes[172,187–189], chondrocytes[190–193], and myoblasts[194,195] have also been used for regeneration of the meniscus in animal models. While many preclinical studies have been performed, the abovementioned reports highlight the lack of evidence for cell-based meniscal repair augmentation and regeneration and warrant a thorough evaluation of the studies performed. Our systematic literature review was aimed at unraveling the most promising cell types or culture conditions described in vivo. Moreover, we will evaluate whether a cell carrier or scaffold could increase effectiveness of cell-based treatments, as it could support cellularity and tissue ingrowth while providing mechanical support to the meniscus[173]. The purpose of this review is to assess potential targets for optimization of cell-based meniscus repair augmentation and regeneration after partial and total meniscectomy. We included all in vivo models for meniscal tears in which repair was augmented by cell-based therapy, as well as total and partial meniscectomy models in which regeneration (formation of neo-menisci) was targeted.

Methods

A systematic review of literature aimed at cell-based systems for meniscal regeneration was performed according to the PRISMA guidelines. A search was conducted on May 18, 2016 in the electronic databases of MEDLINE, Embase, and the Cochrane Collaboration using the following search strategy: (Regenerative medicine'/exp OR regeneration OR regenerative OR regenerating OR repair OR repairing OR reparation OR replace OR replacement OR replacing OR augment OR augmentation OR augmenting OR restore OR restoring OR restoration OR 'tissue engineering' OR regenerate) **AND** ('knee

meniscus'/exp OR meniscus OR menisci) **AND** ('cells'/exp OR cell OR cells OR cellular). The articles were screened by title and abstract using the following inclusion criterion: papers describing a cell-based system for meniscus repair tested in vivo. Papers describing in vitro experiments were excluded, as were articles looking solely at femoral or tibial cartilage regeneration. Reviews, case reports, missing full texts, and papers in languages other than English were also excluded.

Results

The literature search yielded 525 articles in MEDLINE, 640 articles in Embase, and 2 articles in the Cochrane Collaboration. After removing 313 duplicates and screening titles and abstracts, the full text of 81 articles was screened, after which 51 articles could be included; 2 additional articles were identified from the references. Included articles and the different strategies for meniscus regeneration can be found in Table 1. Table 2 demonstrates the different outcomes measured in the in vivo experiments of all articles.

Table 1. Strategies for Meniscus Regeneration **Part 1**

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Desando et al[196]	Sheep	Unilateral medial meniscectomy	Bone marrow con-centrate or BMSCs in hyaluronan	Iliac crest BMSCs; autologous	Bone marrow aspirate 39 x 10^6 (6 to 53); 6 x 10^6 MSCs	Hyaluronan	12 weeks
Kondo et al[197]	12- to 13-year-old primates	Partial meniscecto-my (ante-rior half of medial meniscus)	Aggregates	SMSCs	14 x 0.25x10^6	No aggregates	16 weeks
Qi et al[198]	Rabbits	Partial meniscecto-my (ante-rior half of medial meniscus)	Targeted cell deliv-ery; intra-articular injection of super-paramagnetic iron oxide–labeled cells	ATMSCs	2 x 10^6	Unlabeled cells, saline	12 weeks
Julke et al[190]	Goats	Meniscal tear	Wrapping with por-cine collagen mem-brane with or with-out expanded chon-drocytes	Porcine articular chondrocytes	15 x 10^6	Inside-out suturing	6 months
Ozeki et al[199]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Implantation of au-tolo-gous Achilles tendon with alloge-neic MSCs	Rat synovial MSCs	1 x 10^6	Achilles tendon graft	8 weeks
Nakagawa et al[200]	Micromini-pigs	Meniscal tear	Suturing + injection of MSC suspension	Allogeneic SMSCs	20 x 10^6	Suture + suspen-sion	12 weeks
Ferris et al[201]	Horses	Meniscal tears	Surgery + injection of BMSCs	Autologous BMSCs	15 to 20 x 10^6	Surgery (previous data)	24 months
Hatsushika et al[202]	Pigs	Partial meniscecto-my (ante-rior half of medial meniscus)	3-time injection of allogeneic synovial MSCs with 2-week intervals	Allogeneic SMSCs	50 x 10^6	PBS	16 weeks

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Okuno et al[203]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injec-tion of syngeneic MSCs, minor mis-match, major mis-match injection	SMSCs	5 x 10^6	PBS	4 weeks
Shen et al[204]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injec-tion of hMeSPCs 1 and 2 weeks after meniscectomy	Human meniscus stem/progenitor cells	6 x 10^6	PBS	12 weeks
Zhu et al[195]	Dog	Meniscal tear	Implantation of a PLA/PGA scaffold with myoblasts ex-pressing hCDMP-2	Myoblasts	-	Suture only; PLA/ PGA scaffold with myoblasts carrying an empty vector; PLA/PGA scaffold with addition of recombinant hCDMP-2	12 weeks
Esposito et al[187]	Rabbits	Medial meniscecto-my	Implantation of a PLDLA/ PCL-T scaf-fold with chon-dro-cytes	Allogeneic me-niscus fibrochon-drocytes	1 x 10^6 /mL	Cell-free scaffold/ meniscectomy	24 weeks
Hatsushika et al[205]	Rabbits	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injec-tion of SMSCs	SMSCs	10 x 10^5	Meniscectomy	6 months
Katagiri et al[206]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Implantation of ag-gregates	SMSCs	0.25x10^6	Injection of 5 x 106 cell suspension and 25,000 cell suspension	12 weeks

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Osawa et al[172]	Athymic rats	Meniscal tear	Intra-capsular injec-tion of fetal menis-cus cells	Fetal meniscus cells	0.5 x 10^6	PBS	4 weeks
Shen et al[207]	Rabbits	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injection of MMSCs	Allogeneic MMSCs	6 x 10^6	PBS	12 weeks
Moriguchi et al[208]	Pigs	4-mm cylindrical de-fect	Implantation of a tissue-engineered construct	Allogeneic SMSCs	0.2 x 10^6 cells + 3-week culture	Empty defect	6 months
Zellner et al[209]	Rabbits	4-mm longitudinal meniscal tear	Implantation of a hya-lu-ronan/collagen matrix with precultured BMSCs	Autologous BMSCs	0.1 x 10^6	Suture; empty matrix; matrix with BMSCs; matrix with PRP	12 weeks
Horie et al[181]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injec-tion of BMSCs	Rat or human BMSCs	2 x 10^6	PBS	8 weeks
Gu et al[194]	Dogs	Partial meniscecto-my (anterior horn of medial meniscus)	Implantation of PLGA scaffold with myoblasts	Autologous my-oblasts	15 x 10^6 + 3-week culture	Empty defect; empty scaffold	12 weeks
Horie et al[182]	Rabbits	1.5-mm cylindrical defect	Injection of SMSCs	Allogeneic SMSCs	2 x 10^6	PBS	24 weeks
Kon et al[192]	Sheep	Medial meniscecto-my	Implantation HA PCL scaffold with ex-panded chondro-cytes	Autologous chondro-cytes	40 x 10^6 cells/ scaffold + 14-day spinner flask culture	Cell-free scaffold/ meniscectomy	12 months
Hong et al[210]	Rabbits	Meniscal tear at an-terior tibial attach-ment	Pull-out repair + im-plan-tation of scaf-fold and hBMSCs	Human BMSCs	2 x 10^6	Pull-out repair	8 weeks

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Ruiz-Iban et al[211]	Rabbits	Meniscal tear	Suture + injection of ATMSCs in Matrigel	Allogeneic ATMSCs	0.1 x 10^6	Suture	12 weeks
Zellner et al[212]	Rabbits	Meniscal punch	Matrices + BMSCs, PRP, BM aspirate	Autologous BMSCs	1.5 x 10^6	Cell-free hyal-uronan collagen matrix	12 weeks
Dutton et al[213]	Pigs	Meniscal tear	Suturing + injection of BMSCs in fibrin glue	Autologous BMSCs	1-2 x 10^6	Suture; suture + injection of fibrin glue	8 weeks
Zhang et al[214]	Goats	Meniscal tear	Injection of BMSCs transfected with hIGF-1 in a calcium alginate gel	Autologous BMSCs	30 x 10^6 cell/ml	Empty defect; injection of BMSCs in calcium alginate gel; injection of cal-cium alginate gel	16 weeks
Horie et al[215]	Rats	Partial meniscecto-my (ante-rior half of medial meniscus)	Intra-articular injec-tion of SMSCs and BMSCs	Allogeneic SMSCs + BMSCs	5 x 10^6	PBS	12 weeks
Kon et al[191]	Sheep	Medial meniscecto-my	Implantation of HA PCL scaffold with expanded chondro-cytes	Autologous chondro-cytes (cartilage)	40 x 10^6 cells + 14-day spinner flask culture	Empty defect; empty scaffold	4 months
Angele et al[216]	Rabbits	Partial meniscecto-my (middle third of the medial meniscus)	Implantation of hyluronan/ gelatin scaffold with BMSCs	Autologous BMSCs	2.5 x 10^6 + 14-day culture	Empty defect; empty scaffold	12 weeks

Part 5

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Weinand et al[193]	Pigs	Meniscal tear	Implantation of a vicryl mesh scaffold (polyglactin) with chondrocytes	Allogeneic vs au-tol-ogous chon-drocytes (from knee joint or ear)	2.1 x 10^6	Empty defect; suture; empty scaffold	12 weeks
Martinek et al[188]	Sheep	Subtotal meniscec-tomy (medial menis-cus resected leaving 2-mm peripheral me-niscal ridge)	Implantation of CMI with fibrochondro-cytes	Autologous fibro-chon-drocytes	10 x 10^6 + 3-week culture	Empty defect; empty scaffold	3 months
Weinand et al[217]	Pigs	Meniscal tear	Implantation of vicryl mesh scaffold (polyglactin) with chondrocytes	Articular, auricular, costal alloge-neic chondro-cytes	Scaffold in suspension of 5 x 10^6	Empty defect; suture; empty scaffold	12 weeks
Kang et al[189]	Rabbits	Medial meniscecto-my	Implantation of PLGA with fibro-chondrocytes	Allogeneic me-niscal fibrochon-drocytes	2 x 10^6 on scaffold → 1-week culture	-	36 weeks
Abdel-Hamid et al[218]	Dogs	Meniscal tear	Injection of BM aspi-rate	Autologous cen-trifuged BM aspi-rate	2 ml of 4 ml aspirate	Empty defect; not centrifuged BM aspirate	12 weeks
Peretti et al[219]	Pigs	Meniscal tear	Implantation of a devitalized allogene-ic meniscal slice with chondrocytes	Autologous chon-drocytes (articular carti-lage)	Cultured in 2 x 10^6 chon-drocytes	Empty defect; suture; empty scaffold	9 weeks
Walsch et al[220]	Rabbits	Partial meniscecto-my (ante-rior half of medial meniscus)	Implantation of type I colla-gen sponge with autologous BMSCs	BMSCs	0.5 x 10^6 /ml, seeded 8-12 hours	Periosteal autograft; empty scaffold	24 weeks
Ishimura et al[221]	Rabbits	Meniscal tear	Injection of fibrin glue with BM aspi-rate	BMSCs	+/- 1 x 10^6 cells (0.1 mL bone marrow aspi-rate)	Empty defect; empty scaffold	12 weeks
Port et al[222]	Goats	Meniscal tear	Implantation of fi-brin clot with BM aspirate	Autologous BM aspirate	Not specified	Empty defect; suture; empty scaffold	4 months

Subcutaneous models

Part 6

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Ding et al[223]	Rabbit cells in rat	-	Injection of cells in Matrigel	MMSCs vs BMSCs	0.3 x 10^6	-	3 weeks
Schwartz et al[224]	Bovine me-nis-ci in athymic rats	Bucket-handle tear	MSCs on collagen scaffold; collagen gel; hyaluronic acid	Meniscus cells vs synovi-al cells vs adipocytes	x 10^6 in colla-gen scaf-fold, 0.1x10^6 in col-lagen gel, 2 to 3 x 10^6 cells alone, 100.000 in HA	Injection of MSCs without scaffold	9 weeks
Gu et al. (2013) [225]	Dog myoblasts in nude mice	NA	Implantation PLGA scaffold with my-oblasts	Myoblasts in-duced with CDMP-2 and TGF-beta1	15 x 10^6	PLGA scaffold with uninduced cells	12 weeks
Ferris et al[226]	Horse menis-ci in nude mice	Meniscal sections	Fibrin glue and BMSCs	Allogeneic BMSCs	0.2 x 10^6	Fibrin glue	4 weeks
Yoo et al[227]	Swine me-nis-cal disks in nude mice	Meniscal disks	PLGA scaffold with chondrocytes	Articular chon-drocytes (dynam-ic oscillating cul-turing)	1.68 x 10^6 ± 0.25 x 10^6	Empty scaffold; static culturing	12 weeks
Schoenfeld et al[228]	Human me-niscal tis-sue in athymic mice	Meniscal cells	Implantation of PGA polymer scaffold with fibrochondro-cytes	Human fibro-chondrocytes	1 x 10^6 + 1-week incubation	Empty scaffold	12 weeks
Scotti et al[229]	Swine me-nis-cal slices in nude mice	Meniscal slices	Articular fibrochon-drocytes in fibrin glue + hydrogel	Swine meniscus fibrochondro-cytes	2.4 x 10^6	Fibrin glue + hydrogel	4 weeks
Marsano et al[230]	Human cells in nude mice	NA	Different cell types on non-woven hyalu-ronan meshes	Human meniscus, fat pad and syno-vial membrane cells	4 x 10^6	NA	6 weeks
Peretti et al[231]	Lamb me-nis-cus in nude mice model	Meniscal tear	Devitalized meniscal tissue with cultured chondrocytes, fibrin glue around the construct	Chondrocytes	Cultured in 12 x 10^6 chon-drocytes	Empty defect; suture; empty scaffold	14 weeks

OA models

Part 7

Study	Animal Model	Defect (Tear/ Meniscectomy)	Strategy	Cell Source	Amount of Cells	Control	Duration of Follow-up
Ude et al[232]	Sheep	Medial meniscectomy (and excision of anterior cruciate ligament)	Intra-articular injection of chondrogenically induced cells	ATMSC vs BMSC autologous	**20 x 10^6**	Medium	6 weeks
Caminal et al[233]	Sheep	Meniscal tear (full-thickness articular cartilage defect)	Intra-articular injection of BMSCs	BMSCs	**11 x 10^6 or 12 x 10^6**	Medium	6-12 months
Al Faqeh et al[234]	Sheep	Medial meniscectomy (and excision of anterior cruciate ligament)	Intra-articular injection of BMSCs	Autologous BMSCs	**10 x 10^6**	Basal medium	6 weeks
Agung et al[235]	Rats	Meniscal tear (and anterior cruciate ligament tear, articular cartilage defect)	Intra-articular injection of BMSCs	Allogeneic GFP+ BMSCs	**1 x 10^6 vs 10 x 10^6**	Sham operation; saline	4 weeks
Murphy et al[236]	Goats	Medial meniscectomy (and excision of anterior cruciate ligament)	Intra-articular injection of sodium hyaluronan with cells	Autologous BMSCs expressing eGFP	**10 x 10^6**	Sodium hyaluronan	26 weeks

Abbreviations: ATMSCs, adipose tissue–derived mesenchymal stem cells; BM, bone marrow; BMSCs, bone marrow–derived mesenchymal cells; CDMP-2, cartilage–derived morphogenetic protein-2; CMI, collagen meniscus implant; GFP, green fluorescent protein; HA/PCL, hydroxyapatite/poly(epsilon-caprolactone); hCDMP-2, human cartilage–derived morphogenetic protein-2; hIGF-1, human insulin-like growth factor-1; hMeSPC, human meniscus stem/progenitor cell; MSC, mesenchymal stem cell; MMSCs, meniscal mesenchymal stem cells; NA, not applicable; OA, osteoarthritis; PBS, phosphate-buffered saline; PLA/PGA, poly-lactic acid/poly-glycolic acid; PLDLA/PCL-T, poly(L-co-D,L-lactic acid)/poly(caprolactone-triol); PLGA, polylactic-co-glycolic acid; PRP, platelet-rich plasma; SMSC, synovial mesenchymal stem cell; TGF-beta1, transforming growth factor-beta1.

Table 2. Outcome Measures **Part 1**

Article	Biomechanic	Macroscopic Evaluation	Histology	OARSI	mmunohisto chemistry	Cell Tracking	MRI	EM
Desando et al[196]		x	X		x			
Kondo et al[197]		x	X				x	
Qi et al[198]		x	X		x	x	x	
Jülke et al[190]		x	X					
Ozeki et al[199]		x	X		x	x		
Nakagawa et al[200]	x	x	X			x	x	
Ferris et al[201]								
Hatsushika et al[202]		x	X	x	x		x	
Okuno et al[203]		x	X		x	x		
Shen et al[204]		x	X		x	x		x
Zhu et al[195]		x	X		x			
Esposito et al[187]		x	X					
Hatsushika et al[205]		x	X	x		x		
Katagiri et al[206]		x	X		x	x		
Osawa et al[172]			X		x	x		
Shen et al[207]	x	x	X		x	x		x
Moriguchi et al[208]		x	X					
Zellner et al[209]	x	x	X					
Horie et al[181]		x	X	x	x	x		
Gu et al[194]		x	X		x			
Horie et al[182]		x	X		x	x		
Kon et al[192]		x	X	x				
Hong et al[210]		x	X					
Ruiz-Iban et al[211]		x	X		x	x		
Zellner et al[212]		x	X		x	x		x
Dutton et al[213]	x	x	X			x		
Zhang et al[214]		x	X		x		x	x
Horie et al[215]		x	x		x	x		x
Kon et al[191]		x	x		x	x		
Angele et al[216]		x	x		x			x
Weinand et al[193]		x	x			x		

Part 2

Article	Biomechanic	Macroscopic Evaluation	Histology	OARSI	mmunohisto chemistry	Cell Tracking	MRI	EM
Martinek et al[188]		x	x					
Weinand et al[217]	x	x	x					
Kang et al[189]	x	x	x		x			x
Abdel-Hamid et al[218]		x	x		x			
Peretti et al[219]		x	x		x			
Walsch et al[220]		x	x		x	x		
Ishimura et al[221]		x	x		x			
Port et al[222]	x	x	x		x			
Subcutaneous models								
Ding et al[223]			x		x			
Schwartz et al[224]		x	x					
Gu et al. (2013) [225]		x	x		x			
Ferris et al[226]		x	x					
Yoo et al[227]		x	x		x	x		
Schoenfeld et al[228]		x	x		x			
Scotti et al[229]		x	x					x
Marsano et al[230]			x		x			
Peretti et al[231]		x	x		x			
OA models								
Ude et al[232]		x	x		x			
Caminal et al[233]		x	x				x	
Al Faqeh et al[234]		x	x					
Agung et al[235]			x		x	x		
Murphy et al[236]		x	x		x	x		

Abbreviations: EM, electron microscopy; MRI, magnetic resonance imaging; OARSI, Osteoarthritis Research Society International

Cellular Meniscal Augmentation

Cell Types

MSCs isolated from different tissues are frequently used for regenerative purposes of the meniscus. MSCs are defined by their ability to form colonies and adhere to plastic, their expression of specific surface markers, and their trilineage potential[39]. MSCs from adipose tissue, bone marrow, synovium, and meniscus are used for repair augmentation of meniscal tears and regeneration after (partial) meniscectomy. The use of other cell types, including fibrochondrocytes[187–189], chondrocytes[192,193,217], and transfected myoblasts[195], is in currently available literature limited to the combination with different scaffolds. In the following paragraphs, we will focus on literature on in vivo augmentation of meniscal tear repair and regeneration after meniscectomy. We will elaborate on the effectiveness of MSCs of different sources and cell numbers needed for an optimal effect.

Synovial versus Bone Marrow MSCs

Synovial MSCs (SMSCs) can be extracted from the synovium during a simple arthroscopic procedure, but the synovium contains only a small amount of multipotent colony-forming cells[237]. Bone marrow MSCs (BMSCs) are frequently used in regenerative medicine and can be expanded from bone marrow in non-weight bearing areas[217]. In vitro, BMSCs form less colonies but show higher cell numbers per colony than SMSCs. SMSCs and BMSCs both have high chondrogenic potential, as shown by the formation of pellets with a cartilaginous matrix. In a comparison of the effectiveness of intra-articular injection of SMSCs and BMSCs at passage 3 in a partial meniscectomy model in rats, both groups formed neomenisci. No notable differences were found between the BMSC injection group and the SMSC injection group at macroscopic evaluation. Histologically, both experimental groups showed healing and formation of type II collagen. The injected cells were still present at 12 weeks, as shown by LacZ tracking. Hierarchical clustering analysis showed that the gene expression profile of meniscal cells is closer to that of SMSCs than BMSCs[215].

Bone Marrow vs Meniscal MSCs

Meniscal MSCs (MMSCs) can be isolated from meniscal tissue resected at meniscectomy. In a study comparing BMSCs and MMSCs, both were demonstrated to have trilineage potential and to express markers indicative of stem cells (e.g. Nanog, CD44 and CD90). BMSCs formed larger colonies and grew faster than MMSCs. However, when the MMSCs seeded in Matrigel were implanted subcutaneously in rats, MMSCs had

a larger tendency toward the chondrogeneic lineage, whereas BMSCs had a greater tendency toward the osteogenic lineage[223]. This in vivo experiment, however, does not take into account the effect of load-bearing mechanics and the articular joint environment, limiting the relevancy of these findings.

Meniscus Cells, Adipocytes, Synovial Cells, and Chondrocytes

In the model used by Schwartz et al[224], a bovine meniscus with bucket-handle tear was treated with adipocytes, meniscal cells, or synovial cells and placed on the dorsum of rats. The tears treated with meniscus cells had higher histological scores for integration than menisci treated with adipocytes or synovial cells. Marsano et al[230] demonstrated greater proliferation rates of chondrocytes than of meniscus, fat pad, and synovial cells in vitro. Moreover, chondrocytes placed on a hyaluronan scaffold formed meniscus-like tissue when placed subcutaneously in nude mice, whereas meniscus, fat pad, and synovial cells formed a tissue consisting mostly of fibroblastic cells with minimal extracellular matrix and no detectable glycosaminoglycans.

Cell Number

The majority of studies have used 15 to 50 million cells in large animal models (goats, dogs, pigs)[188,190,192,194,202,232] and around 0.5 to 5 million in small animals (rabbits, rats, mice)[172,181,182,187,199,203,210,215]. Desando et al[196] stated that menisci of sheep injected with bone marrow concentrate after partial meniscectomy with greater cell numbers have higher regenerative ability, although this study was not sufficiently powered to find statistically significant evidence, and individual data were not shown. Agung et al[235] demonstrated that migration was only directed toward the anterior cruciate ligament (ACL) 4 weeks after injection of 1 million green fluorescent protein (GFP)–positive BMSCs in rats with ACL, meniscus, and femoral condyle injury. When 10 million cells were injected, migration was directed toward the ACL as well as the meniscus. Moreover, toluidine blue was observed around GFP-positive cells, indicating tissue regeneration by the BMSCs or embedding of the MSCs in existing extracellular matrix. These findings indicate that injection of a higher cell number is required to potentiate regeneration of the meniscus in case of more complex knee injury. However, converting these quantities to the same cell number per body weight in humans translates to the use of over 500 million cells to reach the same effect. Because this is not feasible in clinical practice, the effect of aggregates of 25 000 synovial MSCs was tested in a partial meniscectomy model. The implantation of 5 aggregates of 5,000 cells and 50 aggregates of 500 cells increased chondrogenesis and led to longer cell survival. Moreover, more MSCs were found to be attached to the site of damage when compared with a cell suspension of 25 000 cells[206]. The authors

thus hypothesized that the use of aggregates increased the effect of stem cells and decreased the quantity of cells needed. In another study performed by the same group[197], the regenerative effect of placement of aggregates of SMSCs after partial meniscectomy was studied in aged primates. The aggregates led to formation of bigger neomenisci and higher histological scores (modified Pauli score). However, the control group (no treatment) also showed regeneration of the meniscus, indicating the intrinsic regenerative capacity of menisci in cynomolgus macaques. Another approach is the use of tissue-engineered constructs (TECs) for augmentation of meniscus repair, which has been recently reported by Moriguchi et al[208], In this study, 0.2 million SMSCs were cultured for 3 weeks in a high-density suspension culture with ascorbic acid to develop 3-dimensional constructs of SMSCs and extracellular matrix. Implantation of this TEC into a 4-mm cylindrical defect led to complete healing; the defect was filled with fibrocartilage after 6 months.

In conclusion, there is no consensus on the effect of injection of different cell numbers, as limited research is available and results are conflicting. Moreover, the number of cells needed for repair augmentation might be different from the amount needed for regeneration after (partial) meniscectomy. The use of aggregates or TECs could increase the success of injecting MSCs in terms of regeneration. These techniques should be compared with injection of different cell numbers to prove their efficiency.

Cell Tracking/Mechanism of Action

In a variety of studies, DiI labeling or GFP was used to track cells and observe the fates of these cells. Horie et al[182] concluded that injected SMSCs were recruited toward the defect and that these SMSCs contribute actively to tissue repair by forming extracellular matrix. Other experiments, conducted by the same group, indicated that injected BMSCs act as trophic mediators by increasing type II collagen expression by meniscal cells and hereby stimulating meniscus regeneration[181]. Hatsushika et al[205], however, stated that intra-articularly injected SMSCs after partial meniscectomy induced formation of synovial tissue, which in turn differentiated into meniscal tissue. SMSCs did not differentiate into meniscal cells directly. In experiments by Desando et al[196], intra-articular injection of bone marrow concentrate in hyaluronan was compared with injection of BMSCs in hyaluronan after partial meniscectomy. Here it was hypothesized that growth factors excreted by bone marrow concentrate have beneficial effects on meniscus regeneration. However, both treatments led to meniscus formation with good cell density and proteoglycan content, and both treatment types contributed to protection against progression of osteoarthritis, as measured by decreased fibrillations of cartilage surface, decreased

proteoglycan loss, and decreased subchondral bone thickness compared with the control group.

Thus, MSCs may be recruited to the site of meniscal damage and may serve both as trophic mediators as well as differentiating meniscus cells, although strong evidence for these suggestions is lacking.

Allogeneic vs Autologous

Use of autologous cells is safe since disease transmission is not possible, and immunological rejection is not of concern. Clinical translation is difficult, as harvested cells need to be expanded, leading to longer treatment delay and rendering single-step procedures impossible. Moreover, cell expansion in good manufacturing procedures (GMPs)–licensed laboratories generate high healthcare costs. The use of allografts or allogeneic cells may therefore be preferable. After partial meniscectomy, injection of autologous SMSCs and SMSCs matched based on major histocompatibility complex (MHC) class I genes gave similar modified Pauli scores for histology, whereas MHC class I mismatched SMSCs led to altered scores[203]. In a comparison between allogeneic and autologous chondrocytes on a vicryl scaffold in swine, no differences in healing of the meniscal tear was noted[193]. The mentioned studies could indicate that the use of allogeneic cells is as successful as the use of autologous cells. Moreover, the first trials for meniscus or cartilage defects show safe use of allogeneic cells, with no serious treatment-related adverse events[44,184].

Cell Delivery

Ideally, scaffolds provide mechanical strength, deliver cells to the appropriate site, allow cellular organization, and provide stimuli for the growth and formation of meniscus tissue[238–240]. Implantable scaffolds provide stability and facilitate ingrowth of cells, but they have to be surgically implanted and (pre)seeded with cells. Application of injectable scaffolds is less invasive. Moreover, these scaffolds are flexible enough to attach and adapt to irregular defects. However, fast degradation and poor biomechanical properties are a great drawback[194]. Tissue-extracted scaffolds provide natural structure and stability and facilitate ingrowth but have limited availability and need to be surgically implanted and (pre)seeded. Schwartz et al[224] reported that MSCs lead to superior repair when they are injected in suspension compared with injection in a collagen scaffold, collagen gel, or hyaluronic acid. However, no quantitative data were provided, and these results were obtained in a subcutaneous model, which, since biomechanics are not involved, underestimates the positive effect of scaffolds on joint stability and cell organization. Moreover, the number of cells seeded in the scaffolds was

lower than the number of cells injected. To date, no other studies have compared the use of cells-scaffold combinations to the use of cells alone.

Implantable Scaffolds

The most commonly used scaffolds are polylactic acid (PLA), polyglycolic acid (PGA), and a combination of these (PLGA).[240] Hyaluronic acid is a natural polymeric hydrogel and could be added to these scaffolds. The use of cell-seeded polymer scaffolds to facilitate regeneration has yielded promising results. Angele et al[216] implanted a hyaluronan/gelatin scaffold seeded with BMSCs after removal of the middle third of the medial meniscus. This implantation led to formation of meniscus-like cartilage and integration into host tissue in rabbits. Similar results were reported by Zellner et al[209], who found that hyaluronan/collagen scaffolds seeded with BMSC medium initiated fibrocartilage-like repair tissue in a 4-mm longitudinal meniscal tear, with good integration and biomechanical properties. Moreover, both studies demonstrate the superiority of the use of cell-seeded scaffolds compared with empty scaffolds, in terms of macroscopic signs of healing and extracellular matrix organization.

Although MSCs are frequently used for tissue regeneration, the use of different cell sources has also been described. Gu et al[225] induced chondrogeneic differentiation of dog myoblasts by culturing them in a CDMP-2 and TGF-beta1–enriched medium. After lentiviral transfection of hCDMP-2 into dog myoblasts, the myoblasts on a PLGA scaffold induced regeneration in meniscal tears in dog menisci[195].

The use of fibrochondrocytes for meniscal repair seems obvious, but few studies have used this approach. This could be because the cells can only be found in the meniscus, and availability is limited. However, combinations of polymeric scaffolds with fibrochondrocytes have been demonstrated to offer potential benefit[187,189,228].

In summary, the majority of these studies show increased tissue regeneration and better organization in the cells-scaffold combinations compared with empty scaffolds[187,209,216]. None of these studies compare the effectiveness of different scaffolds, making it hard to conclude which scaffold has the highest potential.

Injectable Scaffolds

The first reports of injectable scaffolds as delivery vectors for stem cell application were in meniscal tears and used fibrin. Port et al[222] showed no effect of either the addition of a fibrin clot (formed by stirring autologous blood) or both a fibrin clot and BMSCs before suturing the meniscal defect. Ishimura et al[221] used fibrin glue (consisting of fibrinogen, aprotinin, factor XIII, thrombin, and $CaCl_2$) in combination with BMSCs and reported complete filling of the tear with fibrocartilaginous tissue, whereas the meniscal tears

treated with fibrin glue alone were filled with immature cartilaginous tissue. Dutton et al[213] examined the addition of BMSCs in fibrin glue after suturing an avascular tear and found macroscopic complete healing in 75% of the experimentally treated pig menisci, whereas none of the untreated menisci showed complete healing. Moreover, BMSC-treated menisci had a significantly greater Young's modulus than the non–cell-treated menisci, although achieving only 25% of the stiffness of the normal meniscus.

Murphy et al[236] induced osteoarthritis by excision of the medial meniscus and resection of the ACL in goats. After the injection of BMSCs in a sodium hyaluronan gel, macroscopic evaluation showed the formation of neomenisci. The meniscal tissue that was formed in the cell-treated group consisted of fibroblast-like cells surrounded by type I collagen and rounded cells surrounded by type II collagen, whereas the control group did not show this kind of tissue. Moreover, when compared with injection of hyaluronan gel, injection of BMSCs in hyaluronan gel reduced the progression of osteoarthritic degradation processes such as erosion of articular cartilage, osteophyte formation, and changes in subchondral bone. Ruiz-Iban et al[211] induced macroscopically complete healing by injection of adipose tissue–derived MSCs (ATMSCs)-Matrigel combination after suturing a meniscal defect in a rabbit model. Moreover, meniscal fibrochondrocytes were present at the repaired area.

Thus, there have been different successful approaches in the use of various MSCs combined with injectable scaffolds[211,213,214,236]. However, more research is required, comparing the use of different injectable scaffolds to implantable scaffolds seeded with or without cells such as MSCs.

Tissue-Extracted Scaffolds

Ozeki et al[199] used autologous Achilles tendon grafts in rat knees after resection of the anterior part of the medial meniscus. Grafts placed in a suspension of allogeneic SMSCs for 10 minutes prior to implantation had better histological scores compared with grafts alone, and the SMSCs could be tracked up to 8 weeks around the graft and native meniscus. Martinek et al[188] used a collagen meniscus implant that was fabricated from bovine Achilles tendon and type I collagen and seeded with autologous fibrochondrocytes. After implantation in the peripheral rim of a meniscus after subtotal meniscectomy, formation of meniscal tissue, enhanced vascularization, scaffold remodeling, and extracellular matrix production was reported. Peretti et al[219,231] implanted lamb meniscal slices seeded with chondrocytes in a subcutaneous nude mice model. After 14 weeks, in 7 of 8 meniscal slices, both sides of the incision were connected at gross inspection. Histological analysis showed integration of the scaffold in the meniscal samples and filling of the defect with chondrocytes. Jülke et al[190] used a porcine collagen

membrane to wrap chondrocytes around a meniscal tear in goats and reported that the addition of chondrocytes led to more connecting tissue formation and tear margin contact.

Although the use of tissue-extracted scaffolds has potential benefits for meniscus repair augmentation and meniscus regeneration, the translation of these methods to clinical practice is complicated by the low availability of the biomaterials as well as the clinical applicability of cell-based treatments.

Discussion

For repair and regeneration of meniscal damage, several cell-based approaches have been described in this review. The available literature indicates that cell-based therapies can stimulate formation of meniscus-like tissue with extracellular matrix, including types I and II collagen. Moreover, several studies show increased biomechanical properties of cell-treated menisci compared with non–cell-treated controls, although reported mechanical strengths are only 25% to 50% of the strength of native meniscus[200,209]. Several approaches are described, such as intra-articular injection of MSCs, implantation of TECs or aggregates of MSCs, injection of MSCs in an implantable scaffold, and implantation of chondrocytes and fibrochondrocytes on various scaffolds.

The majority of studies describe the use of MSCs and illustrate the value of these cells for tissue engineering. However, the question remains as to what limits clinical translation? One reason could be that few comparative studies have been conducted, making it hard to draw a conclusion as to which cell type yields the most promising results. Harvesting SMSCs can be achieved during an arthroscopic procedure. These cells have high proliferation rates, and the gene profile of SMSCs is similar to that of meniscal fibrochondrocytes. For both ATMSCs and SMSCs, the drawback of limited availability could be overcome by using allogeneic cells. BMSCs can be harvested from non-weight bearing articular surfaces[217]. The downside of using these cells is that they may have a greater tendency toward osteogenesis[223]. MMSCs improved healing in the meniscus[204,207], and several studies have shown the safety and effectiveness of using allogeneic MMSCs[202,203]. Allogeneic MMSCs can be harvested from excised menisci or cadavers, making them a valuable tool in meniscus regeneration. Only 2 studies have compared the use of allogeneic cells to autologous cells, and the limited effect of allogeneic MSCs in the clinical trial by Vangsness et al[184] could stimulate studies comparing allogeneic and autologous MSCs.

In this review, the use of various scaffolds is discussed. Although the presented studies are heterogeneous in the use of scaffolds and cell types, nearly all studies indicate a superior role of cell-scaffold combinations compared with the use of scaffolds

alone[187,209,216]. However, no research comparing different types of scaffolds (with cells) is available. Tissue-extracted scaffolds have proven their value in diverse in vivo studies, but their application is limited by their clinical (GMP) applicability. Injectable scaffolds can easily be injected and have the ability to mold to irregular defects. Cell-seeded implantable scaffolds also hold potential, since scaffolds seeded with fibrochondrocytes, chondrocytes, and even myoblasts have shown to increase regeneration of the meniscus. However, more research should be conducted comparing the use of cell-scaffold combinations (either injectable, implantable, or tissue-extracted scaffolds) to the injection of cells alone. In addition, the need for preseeding scaffolds has its limitations because it requires cell expansion and the availability of a GMP-approved cell therapy facility.

This systematic review included meniscal tear models, meniscectomy models, and osteoarthritis models. The mechanisms of the cell-based therapy might be different in these models, as this includes augmentation of repair of traumatic or degenerative tears or formation of new tissue in empty volume after meniscectomy. However, regenerated tissue was often seen in repair augmentation of meniscal tears, as cell-based treatment led to organized meniscus tissue and not fibrotic scar tissue[209,221,236].

Although results of the in vivo experiments discussed in this review look promising, only 1 clinical trial has been performed to date. The translation of in vivo studies to clinical practice has proven to be difficult. Firstly, it is challenging to model meniscal pathology as seen in humans. In experimental settings, the treatment is given immediately or within 2 weeks after the meniscal injury. In clinical practice, treatment is usually provided months after trauma due to patient and/or doctor delay. In clinical practice, there remains an unmet clinical need for effective meniscal repair/regeneration. The regeneration rate is different in acute defects compared with chronic meniscal degeneration. Indeed, Ruiz-Iban et al[211] demonstrated delay of treatment for 3 weeks leads to a decrease in the beneficial effect of ATSCs on meniscal damage. Chronic meniscus damage models provide a better reflection of the human pathology and could provide further insight in the potency of different regenerative medicine approaches.

Another limitation is the use of animal models that have innate regenerative capacity. In rat menisci, spontaneous regeneration occurs, which limits the translational value of these models[181,204,215]. This is particularly complicated in the use of biodegradable scaffolds, as these will be subjected to higher biomechanical strain. Therefore, as an animal model, rabbits may be preferred due to their limited regenerative capacity[182]. However, the rabbit meniscus has higher vascularization than the human meniscus and is relatively small, making it difficult to place the defect in the avascular zone[241]. Ghadially et al[242] demonstrated that menisci in rabbits, dogs, pigs, and sheep do not have innate

regenerative capacity, which makes them suitable animal models. However, because the vascularity and cellularity of the meniscus decrease upon maturation, the animals used have to be skeletally mature[4,171]. Moreover, the gait of these animals is different than that of humans, leading to a different pattern of mechanical load on the meniscus. A more suitable animal model was used by Kondo et al[197], who studied meniscus regeneration in cynomolgus macaques. These primates are known to stand and walk mainly on their hind legs and are genetically closer to humans than non-primates. The implantation of aggregates of 250,000 cells led to increased meniscal volume on MRI and macroscopically and better quality of the regenerated tissue, as measured by a higher modified Pauli histological score. Moreover, the control group showed minimal increase in meniscal volume after 16 weeks. This is comparable to the human situation, as Vangsness et al[184] did not observe an increase in meniscal volume greater than 15% in any patients in their control group. This indicates that these macaques, like humans, have no intrinsic regeneration.

Additionally, the currently available studies are limited by the lack of universal outcome measurements, making it difficult to compare different strategies. Moreover, few studies report mechanical testing of the regenerated menisci[189,200,207,209,213,222]. Although results look promising compared with suture alone or empty scaffolds, reported biomechanical strengths are still only 25% to 50% of the native meniscus. However, studies with a follow-up of 6 months or longer show ongoing proliferation, maturation, and organization of the regenerated tissue[182,187,189,190,192,205,208,233]. These findings suggest that biomechanics could improve up to 1 year after treatment, thus requiring longer follow-up periods to determine the effect.

Regulations on the use of minimally manipulated autologous cells are less strict, which allows for easier and faster clinical translation. However, the use of minimally manipulated autologous cells has not been described, as all included studies use precultured cells.

Although no research has been conducted to investigate the value of cocultures in cell-based meniscus regeneration in vivo, it could hold great potential because it exploits the benefit of MSCs as trophic mediators. Indeed, there have been promising results in articular cartilage repair in diverse in vivo models[243] as well as in a recent clinical trial (NCT02037204). For meniscal regeneration, cocultures of human BMSCs and fibrochondrocytes have also been tested in vitro, showing formation of neomenisci with enhanced extracellular matrix production compared with MSCs or fibrochondrocytes alone[244]. The use of cocultures could be of value in meniscus tissue engineering because it would allow a single-stage procedure. Autologous SMCs could be harvested arthroscopically and combined with allogeneic precultured fibrochondrocytes in an

intra-articular injection. This way, the low MSC availability would no longer limit the clinical translation.

Conclusions and implications

The studies included in this systematic review are heterogeneous in animal models, cell types, and scaffolds used, and there are limited comparative studies available. Although it is hard to conclude which strategy holds the greatest potential based on our findings, cell-based meniscus repair is promising. The use of cell-scaffold combinations was found to be superior to the use of empty scaffolds. Different cell-based therapies stimulated formation of meniscus-like tissue with organized extracellular matrix. Cell-based meniscus repair augmentation and regeneration is closer to translation than currently thought, and the first human clinical studies are now being performed**.** Minimally invasive and readily available strategies such as intra-articular injection of SMSCs or MMSCs hold potential as they can be used without need for preseeding of the scaffolds. Although strict regulations on the use allogeneic cells limit clinical translation, an advantage of these cells is the possibility of preculturing them without the need for an extra harvesting and expansion procedure. The success of the first clinical trial by Vangsness et al[184] might be enhanced by injecting the cells in an injectable scaffold or onto an implantable scaffold, giving multiple injections, or combining MSCs with different cell types. Future research should aim at the efficiency of these regenerative procedures in large animals without innate regenerative capacity and in chronic damage models. Efficiency should be measured by a universal method of biomechanical testing, preferably with a minimum 6-month follow-up. In addition, early phase clinical trials are needed to bridge the gap between preclinical and clinical meniscus repair.

06

Selection of highly proliferative and multipotent meniscus progenitors through differential adhesion to fibronectin: a novel approach in meniscus tissue engineering

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Abstract

Background

Meniscus injuries can be highly debilitating and lead to knee osteoarthritis. Progenitor cells from the meniscus could be a superior cell type for meniscus repair and tissue-engineering. The purpose of this study is to characterize meniscus progenitor cells isolated by differential adhesion to fibronectin (FN-prog).

Methods

Human osteoarthritic menisci were digested and FN-prog were selected by differential adhesion to fibronectin. Multilineage differentiation, population doubling time, colony formation and MSC surface markers were assessed in the FN-prog and the total meniscus population (Men). Colony formation was compared between outer and inner zone meniscus digest. Chondrogenic pellet cultures were performed for redifferentiation.

Results

FN-prog demonstrated multipotency. The outer zone FN-prog formed more colonies than the inner zone FN-prog. FN-prog displayed more colony formation and a higher proliferation rate than Men. FN-prog redifferentiated in pellet culture and mostly adhered to the MSC surface marker profile, except for HLA-DR receptor expression.

Conclusion

This is the first study that demonstrates differential adhesion to fibronectin for isolation of a progenitor-like population from the meniscus. The high proliferation rates and ability to form meniscus extracellular matrix upon redifferentiation together with the broad availability of osteoarthritis meniscus tissue, makes FN-prog a promising cell type for clinical translation in meniscus tissue-engineering.

Introduction

The meniscus is a fibrocartilage structure in the knee that is predominantly composed of circumferentially orientated type I collagen fibres and low amounts of glycosaminoglycans (GAGs) surrounded by water. It plays an important role in shock absorption, load transmission and stability of the knee. Meniscus injuries can lead to knee pain, locking and swelling and are highly disabling. The treatment of a meniscus injury is dependent on the location of the tissue damage, as the ability to heal differs between the inner and outer zone. The avascular inner zone is composed of chondrocyte-like cells and does not heal, while the vascularized outer zone has a fibrocartilage phenotype and some healing potential[4,245,246]. Therefore, meniscus tears in the outer zone of young patients can be successfully repaired, but overall 66% of meniscus tears remain irreparable[7,10,171]. Meniscus tears unsuitable for repair are treated using (partial) meniscectomy with a 7-fold increase in the odds of developing osteoarthritis[174,247]. Currently, approaches for (stem) cell based therapies for meniscus repair and regeneration are emerging[248,249]. These therapies often employ multipotent mesenchymal stromal cells (MSC), but hypertrophy and osteogenesis are common drawbacks of these stem-cell like or signaling cells[223,250]. Results of the first clinical trial employing MSCs after meniscectomy are suboptimal[184] and there seems to be a paradigm shift towards the use of specific progenitor cells40. In the last decades, the existence of a progenitor cell population in healthy as well as osteoarthritic cartilage has been suggested[251–253]. Cartilage progenitor cells can be isolated by employing their differential adhesion to fibronectin (DAF) based on the high affinity for the fibronectin receptor[253]. Cartilage progenitor cells have high proliferative and multipotent capacity and increased chondrogenic differentiation potential compared to bone marrow MSCs[254,255], with a lower tendency for terminal hypertrophic differentiation[256]. Recently, the presence of meniscus progenitor cells has been suggested in rabbits[223,257] and humans[258–260]. Meniscus progenitor cells might be a therapeutic target for meniscus preservation and a promising cell type for meniscus tissue-engineering, especially due to the high availability of osteoarthritic tissue. However, meniscus progenitor cells are not thoroughly characterized, and unlike for articular cartilage, the DAF protocol has not been explored for isolation of meniscus progenitors.

Therefore, the purpose of this study is to isolate progenitor cells from osteoarthritic menisci using DAF. To test the ability of DAF to select a progenitor population, the acquired cells (FN-prog) were compared to the total meniscus population (Men). Men and FN-prog were characterized according to the MSC guidelines by the International Society for Cellular Therapy (ISCT)[39]. Moreover, other progenitor-characteristics like clonogenicity and proliferation were assessed. Lastly, the potential of FN-prog for

meniscus tissue engineering was compared to Men by assessing redifferentiation in pellet culture using gene expression, release and deposition of GAGs and deposition of collagen.

Methods

Cell Isolation and Culture

Collection of meniscus tissue was performed according to the Medical Ethics regulations of the University Medical Center Utrecht and the guideline "Human Tissue and Medical Research: Code of Conduct for responsible use" of the Dutch Federation of Medical Research Societies[261,262]. Meniscus tissue was obtained from patients with Kellgren and Lawrence grade III and IV osteoarthritis undergoing total knee arthroplasty (n = 11, age 52–84). Both female and male donors were used and both donor genders were balanced within experiments. No individual grading was performed on the tissue. Lateral and medial menisci were pooled and cut into cubical pieces of approximately 2 mm^3 and digested for 2 h in Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies Europe B.V., Bleiswijk, The Netherlands) with 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco) (1% p/s) and 0.2% pronase (Roche Diagnostics GmbH, Mannheim, Germany) under continuous movement (20 rpm) at 37°C, followed by digestion in DMEM with 1% p/s, 5% heat-inactivated Fetal Bovine Serum (FBS; Biowest, Nuaillé, France) and 0.075% collagenase II (Wortington Biochemical Corporation, Lakewood, NJ, USA). For the total meniscus population (men), the digest was plated on culture flasks and cultured in meniscus expansion medium (DMEM with 10% FBS and 1% p/s) up to passage 2. For the isolation of FN-prog, culture flasks were coated with 10 ng/mL fibronectin (Sigma-Aldrich, Saint Louis, MO, USA) in PBS at 37°C for 2 h. A total of 500 cells/cm^2 were plated on the coated flasks and non-adherent cells were removed after 20 min. FN-prog were cultured up to passage 4 in progenitor expansion medium (αMEM (minimal essential medium, Gibco) with 10% FBS, 20 mM l-ascorbic acid-2-phospate (1% ASAP; Sigma-Aldrich) and 5 ng/mL basic fibroblast growth factor (bFGF; Peprotech, London, UK) at 37°C and 5% CO_2. For comparison between Men and FN-prog, Men cells were switched to progenitor expansion medium after the first passage and cultured up to passage 4 in progenitor expansion medium. Cells were passaged upon reaching 90% confluency. Population doublings per day were calculated by dividing the number of harvested cells by the number of seeded cells and the number of days. For comparison between inner and outer zones, the outer third and inner third of 5 menisci were digested separately and colony formation was assessed as described below.

Colony Formation

To assess colony formation and affinity for fibronectin, cells were seeded on fibronectin coated wells in a density of 500 cells/cm^2 (passage 0), 222 cells/cm^2 (Men passage 2), 111 cells/cm^2 (Men passage 4), or 22 cells/cm^2 (FN-prog passage 4). After 20 min, non-adherent cells were removed, and progenitor expansion medium was added. After 3 days, medium was renewed, and after 7 days the cells were fixed and colonies visualized using 0.05% Crystal Violet (Sigma-Aldrich) in Milli-Q water. To assess colony formation in absence of a prior fibronectin adhesion step, 11 cells/cm^2 (Men passage 2, FN-prog passage 4) and 6 cells/cm^2 (FN-prog passage 4).

Multilineage Differentiation

For osteogenic differentiation, cells were cultured in monolayer until 50–70% confluent and differentiated for 3 weeks in osteogenic medium (αMEM with 10% FBS, 1% ASAP, 1% p/s, 10 mM β-glycerolphosphate, and 10 nM dexamethasone). For adipogenic differentiation, cells were cultured until confluent and differentiated for 3 weeks in adipogenic medium (αMEM with 10% FBS, 1% p/s, 1µM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), and 1.72 µM insulin (Sigma-Aldrich)). For chondrogenic differentiation, 250,000 cells were pelleted and cultured for 3 weeks in chondrogenic medium (DMEM, 1% ASAP, 1% p/s, 1% Insulin-Transferrin-Selenium+ Premix (Corning, Corning, NY, USA), 0.1 µM dexamethasone, and 10 ng/mL TGF-β1 (Peprotech)). For hypertrophic differentiation, pellets were cultured for chondrogenic differentiation followed by a 1-week culture in hypertrophic medium (DMEM, 1% ASAP, 1% p/s, 0.2 mM dexamethasone, 10 mM β-glycerolphosphate, and 1 nM triiodothyronine (Sigma-Aldrich)). Following osteogenic differentiation, cells were fixed in 70% ethanol and stained with 40 mM Alizarin Red S (pH 4.1; Sigma-Aldrich) for 5 min. Following adipogenic differentiation, cells were fixed in 4% buffered formaldehyde solution and stained with 0.3% Oil Red O (Sigma-Aldrich) in isopropanol for 30 min. Following chondrogenic and hypertrophic differentiation, pellets were fixated in a 4% buffered formaldehyde solution and further processed as described in 'histology and immunohistochemistry'.

Expression of MSC Markers

Cells were labeled with antibodies against CD105, CD73 (R&D Systems, Minneapolis, USA), CD90, CD34, CD79A, HLA-DR (Miltenyi Biotec Bergisch Gladbach, Germany), CD11b, and CD45 (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. Cells were mixed with the antibodies in FACS buffer (0.5% bovine serum albumin, 2 mM EDTA in PBS) and incubated in the dark at room temperature for 30 min.

Samples were analyzed on a FACS Canto II and LSR Fortessa X20 (BD Biosciences, Allschwil, Switzerland). Stainings with single antibodies and fluorescence minus one were used as controls.

Chondropermissive Cultures/ Redifferentiation

For the analysis of redifferentiation after expansion, 250,000 cells were pelleted and cultured at 37°C and 5% CO_2 for 28 days in chondropermissive medium (DMEM, 1% ASAP, 1% p/s, 2% Albuman (Human Serum Albumin, 200 g/L; Sanquin Blood Supply Foundation, Amsterdam, the Netherlands), 2% insulin-transferrin-selenium-ethanolamine (ITS-X; Gibco)) in the absence and presence of 10 ng/mL TGF-β1. Per group, 5 donors were used. Medium was changed twice per week and collected for analysis.

Gene Expression

Gene expression was assessed at the end of the expansion phase and after 28 days of redifferentiation culture. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Then, 200–500 ng RNA was reverse-transcribed using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reactions (RT-PCR) were performed using an iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a LightCycler 96 (Roche Diagnostics) according to the manufacturer's recommendations. RNA levels were quantified relative to levels of housekeeping gene 18S. Primer sequences can be found in Table 1.

Release and Deposition of Glycosaminoglycans and Collagen

Pellets were harvested after 28 days and digested using a papain digestion buffer (250 µg/mL papain; Sigma-Aldrich, 0.2 M NaH_2PO_4, 0.1 M ethylenediaminetetraacetic acid [EDTA], 0.01 M cysteine, pH 6) at 60°C overnight. GAG content in the digests (deposition) and medium (release) was assessed using a dimethylmethylene blue (DMMB; pH 3) assay to quantify sulphated GAGs. The absorbance was measured at 525 and 595 nm using a spectrophotometer and the ratio at 525/595 nm calculated. Chondroitin-6-sulfate (Sigma-Aldrich) was used as a standard.

For the analysis of collagen deposition, digests were lyophilized followed by a hydrolyzation in 4 M NaOH overnight at 108 °C. Samples were neutralized using 1.4 M citric acid. Then, 50 mM chloramine-T (Merck, Darmstadt, Germany) in oxidation buffer was added. After 20 min incubation, dimethylaminobenzoaldehyde (Merck) in 25% (*w/v*) perchloric acid in 2-propanol was added. Absorbance was measured at 570 nm after 20

min incubation at 60°C. Hydroxyproline (Merck) was used a standard since 13.5% of collagen is composed of hydroxyproline. DNA content of digests was measured using a Quant-iT PicoGreen dsDNA assay (Invitrogen) and was used to normalize collagen and GAG content.

Table 1. Primer sequences for quantitative real-time PCR.

Gene name	Oligonucleotide sequence (5'to 3')	Product size (bp)
18S	Fw: GTAACCCGTTGAACCCCATT Rv: CCATCCAATCGGTAGTAGCG	151
ACAN	Fw: CAACTACCCGGCCATCC Rv: GATGGCTCTGTAATGGAACAC	160
COL2A1	Fw: AGGGCCAGGATGTCCGGCA Rv: GGGTCCCAGGTTCTCCATCT	195
COL1A1	Fw: TCCAACGAGATCGAGATCC Rv: AAGCCGAATTCCTGGTCT	191
DNER	Fw: AAGGCTATGAAGGTCCCAACT Rv: CTGAGAGCGAGGCAGGATTT	137
MCAM	Fw: AGCTCCGCGTCTACAAAGC Rv: CTACACAGGTAGCGACCTCC	102
CDK1	Fw: AAACTACAGGTCAAGTGGTAGCC Rv: TCCTGCATAAGCACATCCTGA	148

Abbreviations: ACAN, aggrecan; bp, base pair; COL1A1, collagen type I alpha 1 chain; COL2A1, collagen type II alpha 1 chain; CDK1, Cyclin-dependent kinase 1; DNER, Delta and Notch-like epidermal growth factor-related receptor; Fw, forward; MCAM, Melanoma cell adhesion molecule; Rv, Reverse.

Histology and Immunohistochemistry

Pellets were harvested after 28 days and fixated in a 4% buffered formaldehyde solution. After embedding in paraffin, 5 µm sections were cut. After deparaffinization, sections were stained with 0.4% Fast Green (Merck) followed by 0.125% Safranin-O (Merck) and Weigerts hematoxylin (Clin-Tech, Surrey, UK). Immunohistochemistry for type I and II was performed as following. Antigen were retrieved with 1 mg/mL pronase (Sigma-Aldrich) for 20 min at 37°C, followed by 10 mg/mL hyaluronidase (Sigma-Aldrich) 20 min at 37°C. Sections were blocked with a 5% bovine serum albumin (BSA) in PBS solution for 30 min. Samples were incubated with the primary antibody (type I collagen, rabbit monoclonal 1/400 or type II collagen, mouse monoclonal 1/100 in PBS/BSA 5%) overnight at 4 °C.

Sections were washed and incubated with horseradish peroxidase-conjugated anti-rabbit or mouse secondary antibody (Dako, Glostrup, Denmark) for 30 min at room temperature. For type X collagen, antigen were retrieved using 1 mg/mL pepsin (Sigma-Aldrich) in 0.5M acetic acid for 2 h at 37°C, followed by 10 mg/mL hyaluronidase for 30 min at 37°C. Sections were blocked with 5% BSA in PBS for 30 min. Samples were incubated with the primary antibody (type X collagen, mouse monoclonal 1/20 in PBS/BSA 5%) overnight at 4 °C. Sections were washed and incubated with biotin-conjugated anti-mouse secondary antibody (GE healthcare, Little Chalfont, UK) for 60 min at room temperature, followed by an enhancement step with streptavidin—peroxidase (Beckman Coulter, Woerden, The Netherlands) for 60 min at room temperature. Immunoreactivity was visualized using diaminobenzidine peroxidase substrate solution (DAB, Sigma-Aldrich). Mayer's hematoxylin was used as counterstaining.

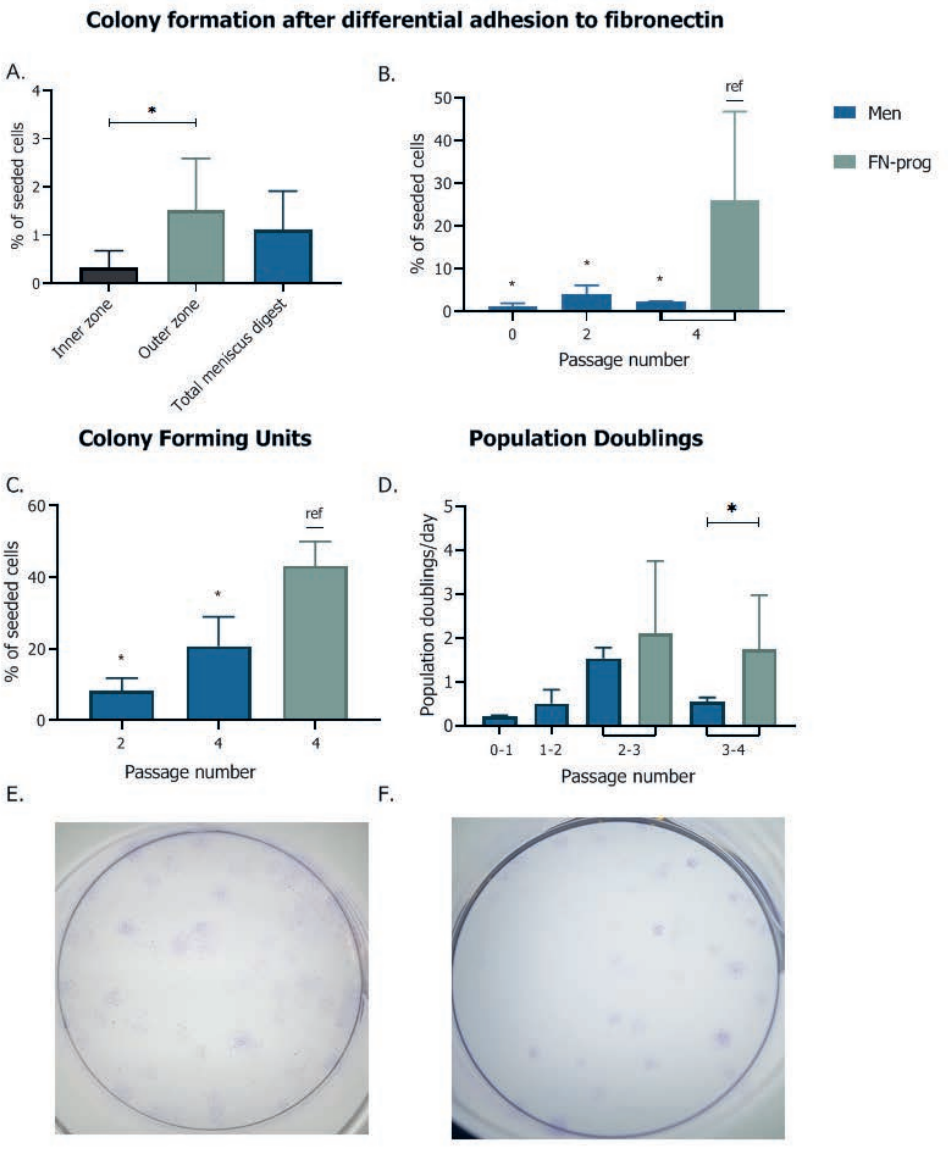
Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8.3 (GraphPad Software, Inc., La Jolla, CA). Data are presented as mean ± standard deviation (SD). Colony formation was compared between inner and outer zone of the same donor using a two-tailed paired t-test (Figure 1a). To test for differences in colony formation and population doubling between FN-prog and Men, a one-way ANOVA with Dunnett's multiple comparisons correction was performed (Figures 1b-d). Relative gene expression of FN-prog at passage 4 was compared to the expression Men at passages 2 and 4 using a one-way ANOVA with Dunnett's multiple comparisons correction (Figure 3a,b). To assess differences in CD318 and MCAM surface marker expression between Men and FN-prog of the same donors, a two-tailed paired t-test was used (Figure 3C). Relative gene expression and matrix formation of Men and FN-prog were compared in the presence and in absence of TGF-β1 using a one-way ANOVA with Dunnett's multiple comparisons correction (Figure 4a,b). *P* values below 0.05 were considered significant.

Results

Selection of a clonogenic population from the meniscus inner and outer zone

Colony formation of the total meniscus, outer and inner zone digest was assessed after DAF. Of the total meniscus digest, 1.1±0.8% of cells were clonogenic with affinity for fibronectin. 0.3±0.4% of the inner zone cells formed colonies, whereas 1.5±1.1% of the outer zone cells formed colonies (Figure 1a). Fibronectin affinity of different passages of FN-prog and Men was assessed after 20 minutes adhesion to fibronectin (Figure 1b). Colony



| Figure 1. Colony formation after fibronectin selection of (a) inner zone, outer zone and total meniscus digest at passage 0 and (b) different passages of the total meniscus population (Men) and fibronectin selected cells (FN-prog), comparison between different passage numbers of the total meniscus population (Men) and fibronectin selected cells (FN-prog) in (c) colony forming units on culture dishes without fibronectin coating and (d) population doublings per day. Representative pictures of colonies formed after seeding (e) Men at 11 cells/cm2 or (f) FN-prog at 6 cells/cm2 both at passage 4, stained with crystal violet blue.*, $p<0.05$; ref, reference category.

formation of FN-prog at passage 4 was higher than the formation of Men. To assess colony formation regardless of fibronectin affinity, colony formation on culture dishes

was also assessed. Again, colony formation of FN-prog at passage 4 was higher than that of Men (Figure 1c). Moreover, FN-prog retained their proliferative capacities, while the proliferation rate of Men diminished after the third passage (Figure 1d). Representative pictures of the colonies are shown in Figure 1e and 1f.

Expression of MSC markers

Over 98% of FN-prog and Men expressed the surface markers CD90 and CD105. CD73 was expressed in 74±15% of Men and 85±7.0% of FN-prog. Negative markers CD45, CD34, CD79α, and CD11b were negative (<2% positive) in both FN-prog and Men. In 4 out of 5 donors 11-53% of FN-prog expressed the HLA-DR receptor and therefore did not meet the ISCT criteria [39]. In 1 out of 5 Men donors the HLA-DR receptor was expressed in 17% of the cells (Figure 2a).

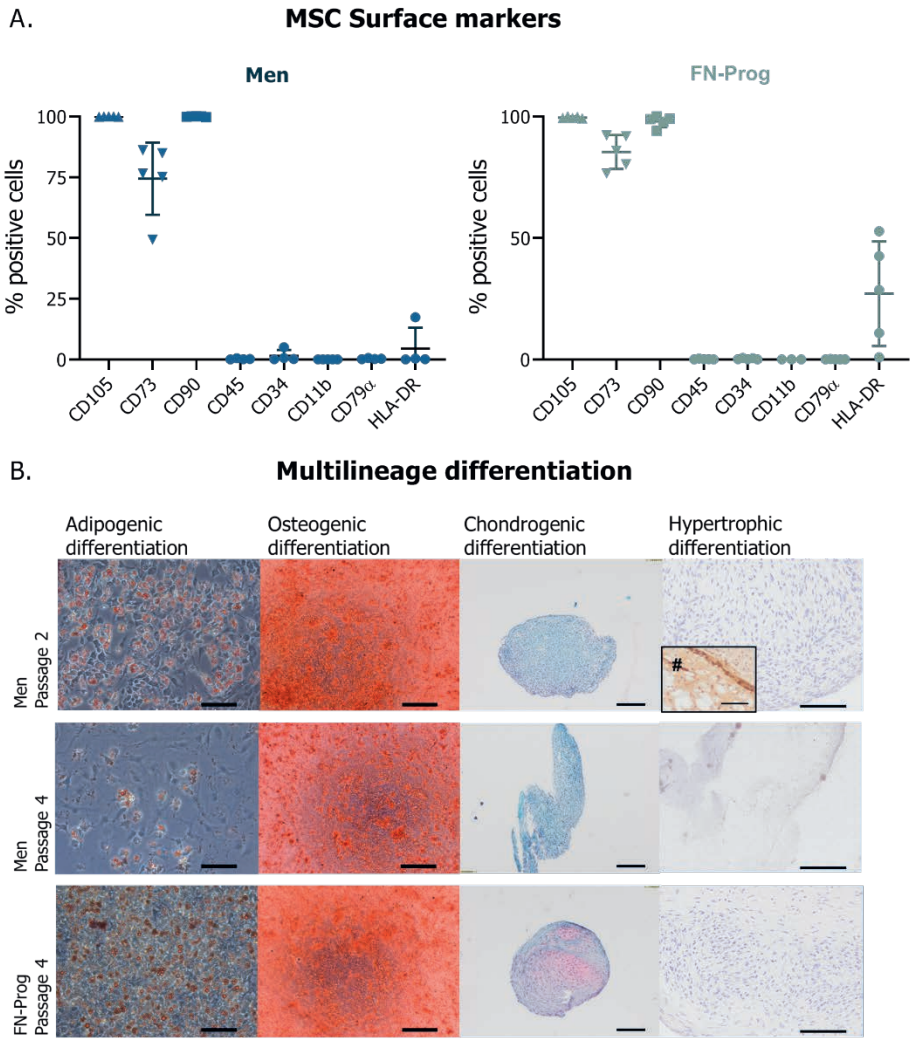
Multilineage potential

All cell populations showed oil Red O staining after 3 weeks of culture indicative of adipogenic differentiation. Similarly, all populations produced mineralized matrix upon osteogenic induction confirmed by Alizarin Red staining. After 3 weeks of pellet culture in chondrogenic medium, all 5 FN-prog donors showed GAG deposition as indicated by Safranin-O staining, whereas none of the donors showed GAG deposition in the total meniscus population at passage 2. Cells of the total meniscus population at passage 4 were not able to form or maintain a firm pellet up to 4 weeks and did not show GAG deposition. None of the populations showed hypertrophic differentiation when subjected to hypertrophic media as assessed by type X collagen deposition, while the positive control of bone marrow derived MSCs was positive for type X collagen staining (Figure 2b).

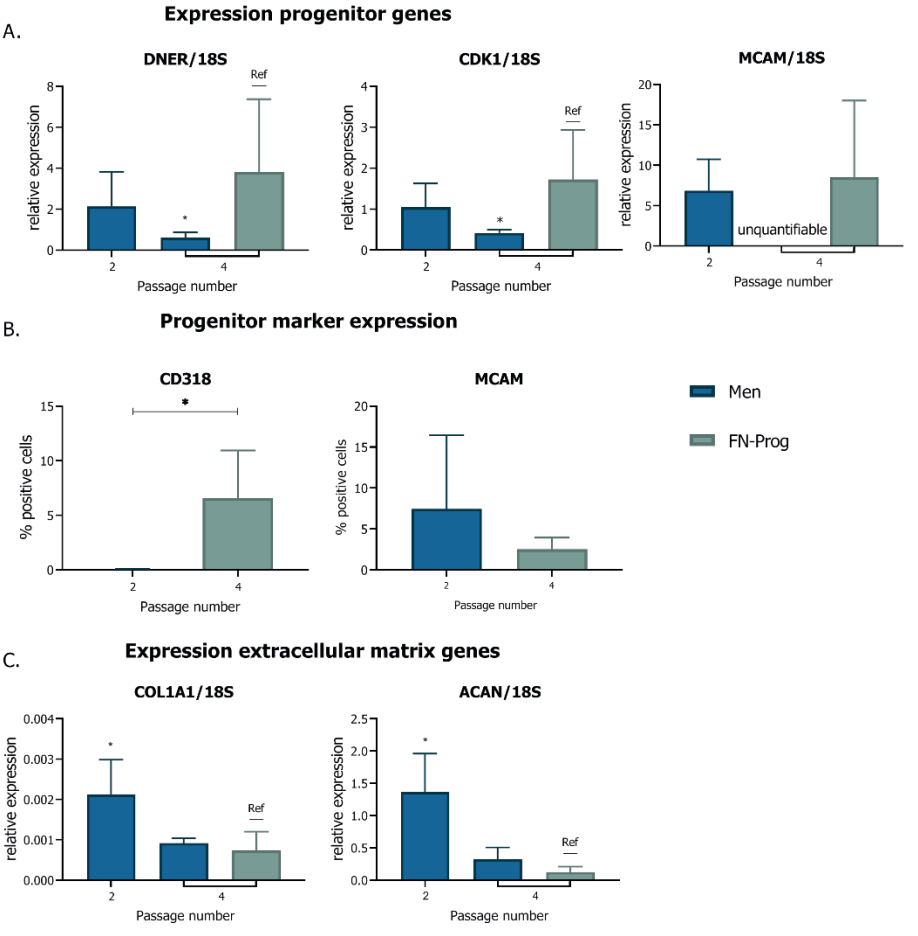
Expression profile after monolayer expansion

Genes associated with a degenerative (Delta and Notch-like epidermal growth factor-related receptor (*DNER*)) or cartilage (Cyclin-dependent kinase 1 (*CDK1*)) progenitor fate[258] were expressed relatively higher in FN-prog than Men at passage 4 (Figure 3a). CD318, a marker associated with degenerative meniscus progenitor cells, was expressed higher in FN-prog than Men (0.05±0.06 vs 6.5±4.4) (Figure 3b). A meniscus progenitor fate[258] can be assessed using the pericyte marker Melanoma Cell Adhesion Molecule (*MCAM*)[263] (also known as CD146). Gene-expression of *MCAM* did not differ significantly between FN-prog at passage 4 and Men at passage 2 (Figure 3a). Expression of *MCAM* in Men passage 4 was below the limit for quantification using qPCR. Surface marker expression of MCAM as assessed by flow cytometry did not differ significantly between Men and FN-prog

(7.5±9.0 vs 2.5±1.5 % positive cells) (Figure 3b). Expression of extracellular matrix genes collagen type I α1 chain (*COL1A1*) and aggrecan (*ACAN*) were lower in FN-prog than in Men (Figure 3c). Expression of collagen type II α1 chain (*COL2A1*) was detectable but not quantifiable.



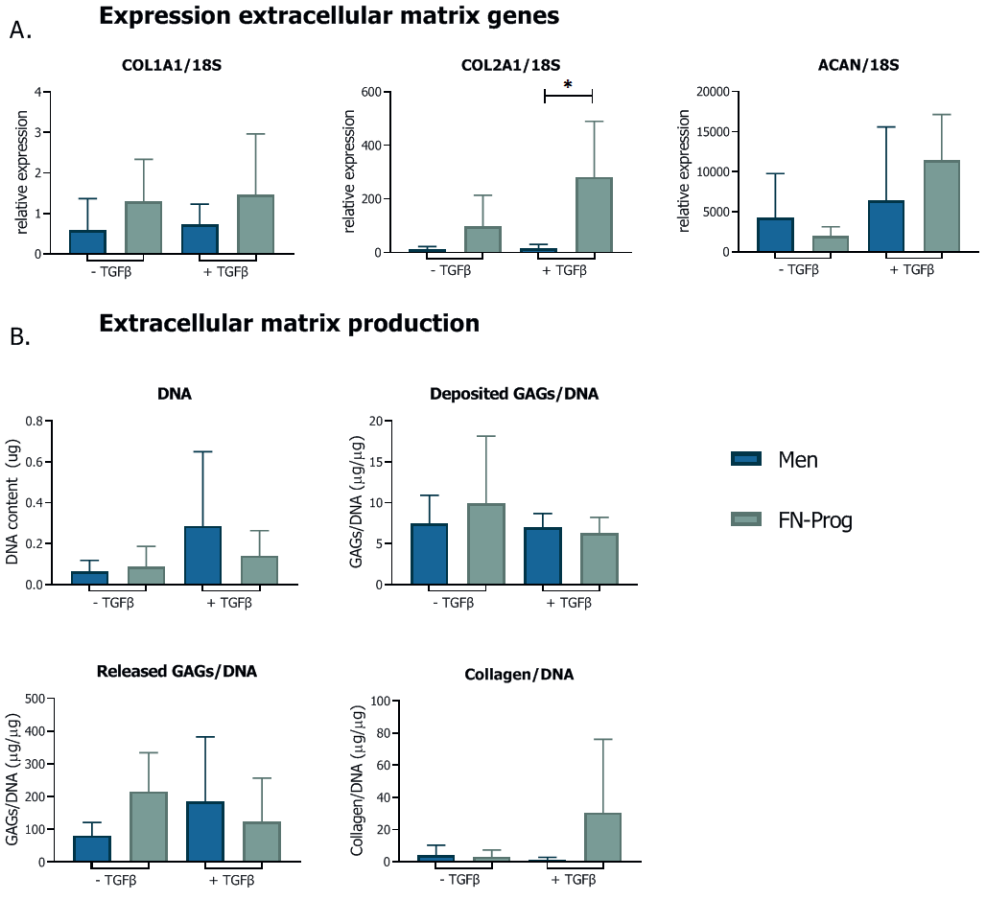
| Figure 2. Characterization according to the MSC guidelines of the International Society for Cell & Gene Therapy39for (a) surface marker expression and (b) adipogenic (oil Red O staining), osteogenic (Alizarin Red staining), chondrogenic (Safranin-O/ Fast Green staining), hypertrophic (type X collagen immunohistochemistry) differentiation. N=5 donors per group per condition. Donor-matched samples were used to culture the total meniscus population (Men) and fibronectin selected cells (FN-prog). #, bone marrow mesenchymal stromal cells passage 4 that were hypertrophically differentiated were used as positive control for type X collagen immunohistochemistry. Scale bars represent 100µm.



| Figure 3. Expression of (a) genes associated with meniscus extracellular matrix production and (b) meniscus progenitor phenotype as measured by quantitative real-time PCR and (c) progenitor marker expression measured using flow-cytometry after monolayer expansion. N=5 donors per group per condition. Abbreviations: ACAN, aggrecan; COL1A1, collagen type I alpha 1 chain; CDK1, Cyclin-dependent kinase 1; DNER, Delta and Notch-like epidermal growth factor-related receptor; FN-prog, fibronectin selected cells; MCAM, Melanoma cell adhesion molecule; Men, total meniscus population*, $p<0.05$; ref, reference category.

Chondropermissive/Redifferentiation culture

After culturing both cell populations in pellets in chondropermissive medium for 28 days, gene expression of meniscus matrix genes, GAG production and collagen and GAG stainings were analysed. *COL1A1*, *COL2A1* and *ACAN* expression of FN-prog did not differ from Men. Upon addition of TGF-β1 to the chondropermissive medium, only *COL2A1* expression was higher in FN-prog compared to Men (Figure 4a). DNA content did not differ between groups. Similarly, total production of GAGs (deposition and release) was comparable. Collagen deposition in FN-prog seemed higher than Men but this did not

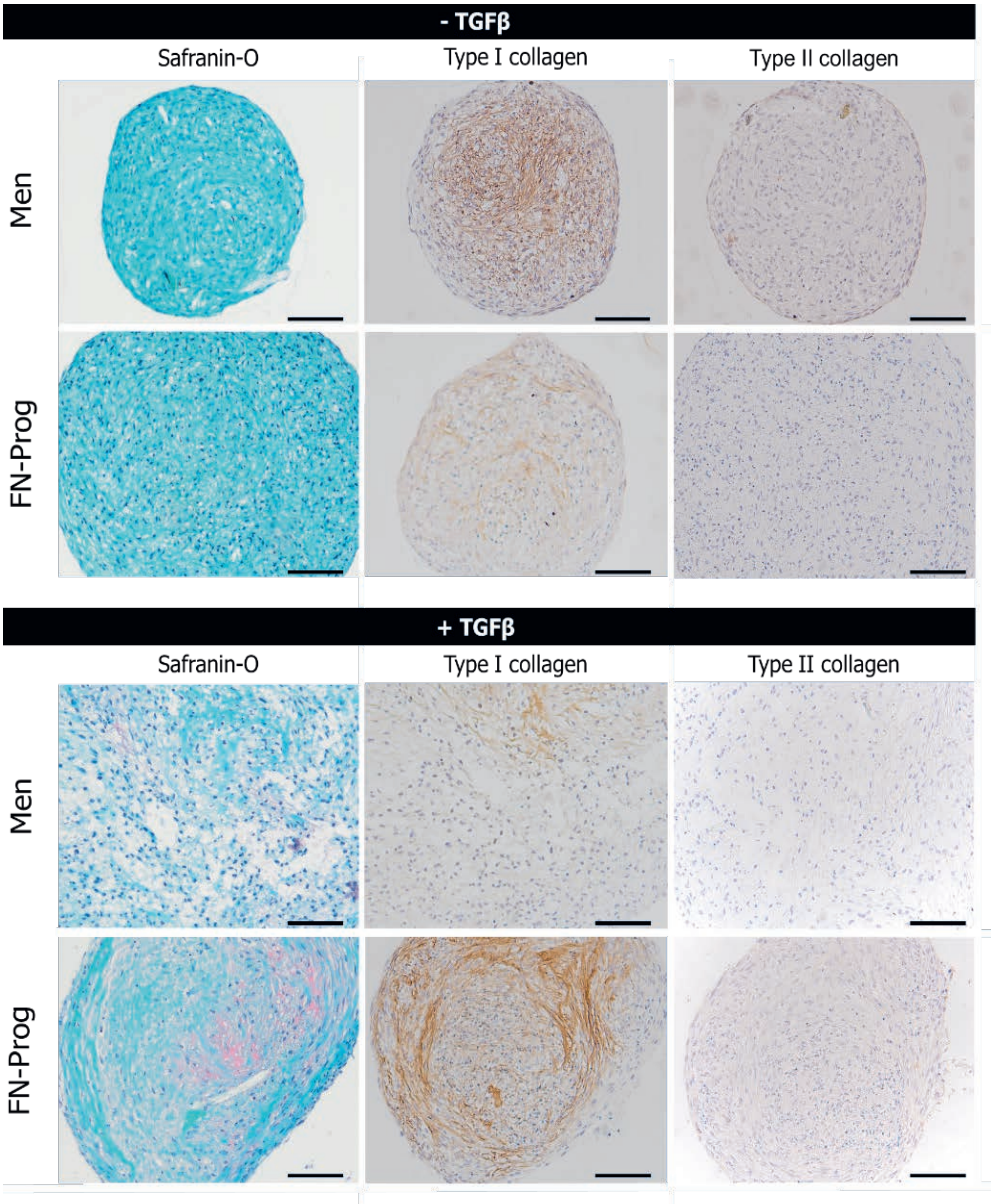


| Figure 4. Expression of genes associated with meniscus extracellular matrix production (a), and DNA content, glycosaminoglycan deposition and release normalized for DNA content and collagen deposition measured by hydroxyproline assay, normalized for DNA content after 28 days of pellet culture in chondropermissive medium in absence or presence of TGF-β1 (b). N=5 donors per group per condition. *Abbreviations: ACAN, aggrecan; COL1A1, collagen type I α1 chain; COL2A1, collagen type II α1 chain; GAG, glycosaminoglycan; TGFβ, transforming growth factor beta 1; *, p<0.05.*

reach statistical significance (p=0.16) (Figure 4b). In absence of TGF-β1, safranin-O staining indicative of glycosaminoglycan deposition was absent in all pellets. The deposition of type I collagen was more pronounced by pellets of Men cells compared to FN-prog. In the Men pellets, type I collagen was mainly found in the inner regions of the pellets, while for FN- prog it was distributed throughout the pellets, but in lower amounts. In the pellets that were cultured in presence of TGF-β1, safranin-O staining was positive in one donor of Men and all donors of the FN-prog. There was a low deposition of type I collagen by Men in some areas of the pellet, while FN-prog had deposited more type I collagen, that was located mostly towards the outer regions of the pellet. Type II collagen staining was absent or low in all groups (Figure 5).

Discussion

This is the first study to isolate meniscus progenitor cells through DAF and to characterize the obtained cell population according to the MSC criteria of the ISCT. We confirmed selection of a distinctive cell population which differs from the total meniscus population in terms of colony formation, proliferation, chondrogenic differentiation, and gene-expression. The advantages of this population in terms of expansion and redifferentiation potential make this a promising cell type for meniscus tissue engineering.
DAF has been used for isolation of progenitor cells from cartilage for almost two decades[253], but was never used for isolation of meniscus progenitors. Here, isolated FN-Prog showed affinity for fibronectin up to at least passage 4. Interestingly, FN-prog from both inner and outer zone meniscus digest formed colonies. The inner zone of the meniscus is regarded as unable to regenerate, but the presence of progenitor-like cells in inner zone meniscus has been suggested previously to contain CD146+ cells[172], clonogenic cells[257], and migrating cells[260,264] with progenitor-like properties. The reason for aberrant regeneration in the inner zone in presence of progenitor cells is unclear, but the lack of contact with blood-derived stimulating factors might prevent the progenitor cells to respond to injury. In agreement with our findings, the number of progenitor-like cells in the inner zone was lower compared to the outer zone. Therefore, FN-prog from the outer zone are presumably overrepresented in the FN-prog population. Nevertheless, the entire meniscus could be used as a cell source to obtain FN-prog. This facilitates easy isolation and increases the amount of available tissue. Moreover, the presence of FN-prog or progenitor-like cells in the inner meniscus creates potential for repair or regeneration in



| Figure 5. Representative sections of pellets cultured for 28 days in differentiation medium in absence of TGF-β1 or presence of TGF-β1. N=5 donors per group per condition. Scale bars represent 100µm.

the inner zone, for example by enhancing the activity or density of FN-prog. This could change the current dogma of the inability of inner zone meniscus to regenerate.
The ability to grow clonally is one of the characteristics of progenitor cells. In the current study, FN-prog formed more colonies and proliferated faster than Men. Likewise, a larger colony number and size were previously reported in progenitor-like cells compared to

meniscus cells[257,264]. The possibilities for fast and extensive culture expansion creates potential for the use of FN-prog for clinical tissue engineering purposes. The prolonged and fast proliferation might indicate a more progenitor-like state/stemness of this population.

Another indicator of the progenitor-like phenotype of FN-prog is the multilineage potential. Multilineage potential of meniscus progenitor cells was previously reported in progenitor populations selected based on colony formation[204,257,265], or migration from the meniscus[260,264,266]. Here, FN-prog differentiated towards the adipogenic, osteogenic, and chondrogenic lineage, contrary to meniscus cells that did not display chondrogenic differentiation. Here, in contrast to bone-marrow derived MSCs, and similar to results shown for cartilage progenitor cells [256], a lack of hypertrophic differentiation was demonstrated in FN-prog. Again, these characteristics support the use of FN-prog over MSCs for meniscus tissue engineering[250,267].

COL1A1 and *ACAN* expression of FN-prog were low during expansion and normalized upon redifferentiation, a phenomenon also seen in culture expanded chondrocytes[254,268]. Notably, upon addition of TGF-β to the chondropermissive medium, the *COL2A1* expression was higher in FN-prog than in Men upon culture, although the expression was too low to translate into an abundant deposition of type II collagen on histology. Together with the GAG deposition, this indicates a progenitor-like state with chondrogenic tendency and makes FN-prog a feasible cell type for cartilage tissue engineering. However, only a limited amount of GAGs is found in the healthy native meniscus[269]. Therefore, GAG deposition might be a suboptimal outcome to assess meniscus extracellular matrix formation, and type I collagen deposition could be used instead. Both FN-prog and Men showed type I collagen deposition.

The surface marker profile of FN-prog corresponded largely to the profile for MSC marker expression as defined by the ISCT. High expression of CD90 and CD105 are also found in populations of both progenitor cells and fibrochondrocytes as shown by single cell RNA sequencing at passage 0[258]. However, apart from the differences in CD73 expression, the markers do not discriminate between Men and FN-prog. Additionally, MSC marker expression increases after culture expansion[259]. The inability to discriminate between cell populations based on immunophenotype is a known drawback in MSC research[270]. To verify the existence of a true and pure meniscus progenitor population, specific markers are currently lacking. More specific markers might increase the purity of this population or demonstrate the physiological or pathological role in the meniscus.

Furthermore, HLA-DR expression was positive in 4 out of 5 FN-prog donors. Although MSCs are explicitly defined by negativity for HLA-DR[39], HLA-DR expression is found even in clinical batches of bone marrow MSCs from 2 different Good Manufacturing Practice

facilities and should not be used as a strict criterion for release of MSC[271,272]. HLA-DR expression of bone marrow MSCs is upregulated in an inflammatory environment, e.g. by contact with interferon γ[271,273]. Similarly, the HLA-DR expression in meniscus progenitors might be upregulated due to the osteoarthritic inflammatory environment. However, HLA-DR positive and negative MSCs do not differ in morphological, differentiation and immunomodulatory characteristics, thus HLA-DR expression might not be relevant for the MSC function or even improve the anti-inflammatory properties of MSCs[271,273]. Furthermore, expression might be decreased by expansion beyond confluence[274], which might be applied to FN-prog for allogeneic use to decrease the risk of immune reactions. The effect of HLA-DR expression on FN-prog behaviour and the effect of increasing culture time on HLA-DR expression of FN-prog remain to be investigated.
Finally, the progenitor cells were selected by differential adhesion to fibronectin, which has been extensively used for the selection of progenitor cells from articular cartilage[252,253,275,276] . At passage 4, the FN-prog still had more affinity for fibronectin compared to Men. This does imply that expansion of the total meniscus population does not selectively increase the population of FN-adherent cells and that the FN-prog is a distinctive population. For articular cartilage progenitors, the fibronectin receptor CD49e is responsible for the increased fibronectin adhesion capacity[252]. For these cells it was shown that expression of CD49e did not change between passage 0 and 10[277], but the expression of the fibronectin receptor is dynamic as it increased in culture[278]. In the meniscus, fibronectin is located in the cell membrane of fibrochondrocytes and in the territorial matrix throughout the meniscus[279]. To our knowledge, it is unknown how osteoarthritis influences this distribution. In our current study, fibronectin is only used for the initial selection directly after isolation of the cells from the meniscus. Subsequently, the cells are transferred and passaged on tissue culture plastic without fibronectin coating. Therefore, it is unlikely that the fibronectin adhesion has an influence on the differentiation of the selected cells at passage 4.

Limitations

The cell populations compared in this study are both isolated from OA meniscus, which draws into question the applicability of these cell types for tissue engineering of healthy meniscus. The inflammatory environment of an OA joint might activate degenerative pathways, like the interleukin 1β induced activation of degenerated meniscus progenitor cells[258]. The use of healthy meniscus cells is not a practical alternative, due to the limited availability. Moreover, the degenerative state of meniscus progenitors might be reversible as a shift from degenerative meniscus progenitors to meniscus progenitors was seen upon TGF-β treatment[258]. This identifies TGF-β as a possible treatment target. The use of

meniscus fragments obtained during meniscectomy of traumatic meniscus injury might hold potential over the use of osteoarthritic injury. Indeed, in chondrocytes from cartilage defect rims performed better than chondrocytes from healthy (non-weight bearing) regions[280]. However, caution should be exercised until a comparison with populations from healthy meniscus has been made.

Conclusions and implications

The currently isolated progenitor population is an attractive option for tissue engineering purposes. The availability of FN-prog is almost unlimited as the entire OA meniscus can be used for isolation which is often discarded as redundant material after total knee replacement. Fast expansion and continued *in vitro* redifferentiation as indicated by type I deposition and proteoglycan production enables large scale (off the shelf) usage. Further research should elucidate the role of FN-prog in healthy and osteoarthritic tissue in order to employ these cells as therapeutic target or increase the defective endogenous regeneration and at clinical translation of this cell population.

07

Progenitor cells in healthy and osteoarthritic human cartilage have extensive culture expansion capacity while retaining chondrogenic properties

Rikkers M, Korpershoek JV, Levato R, Malda J, Vonk LA. Progenitor cells in healthy and osteoarthritic human cartilage have extensive culture expansion capacity while retaining chondrogenic properties. Cartilage. 2021 Dec;13(2_suppl)

Abstract

Background

Articular cartilage-derived progenitor cells (ACPCs) are a potential new cell source for cartilage repair. This study aims to characterize endogenous ACPCs from healthy and osteoarthritic (OA) cartilage, evaluate their potential for cartilage regeneration, and compare this to cartilage formation by chondrocytes.

Methods

ACPCs were isolated from full-thickness healthy and OA human cartilage and separated from the total cell population by clonal growth after differential adhesion to fibronectin. ACPCs were characterized by growth kinetics, multilineage differentiation, and surface marker expression. Chondrogenic redifferentiation of ACPCs was compared to chondrocytes in pellet cultures. Pellets were assessed for cartilage-like matrix production by (immuno)histochemistry, quantitative analyses for glycosaminoglycans and DNA content, and expression of chondrogenic and hypertrophic genes.

Results

Healthy and OA ACPCs were successfully differentiated towards the adipogenic and chondrogenic lineage, but failed to produce calcified matrix when exposed to osteogenic induction media. Both ACPC populations met the criteria for cell surface marker expression of MSCs. Healthy ACPCs cultured in pellets deposited extracellular matrix containing proteoglycans and type II collagen, devoid of type I collagen. Gene expression of hypertrophic marker type X collagen was lower in healthy ACPC pellets compared to OA pellets.

Conclusion

This study provides further insight into the ACPC population in healthy and OA human articular cartilage. ACPCs show similarities to MSCs, yet do not produce calcified matrix under well-established osteogenic culture conditions. Due to extensive proliferative potential and chondrogenic capacity, ACPCs show potential for cartilage regeneration and possibly for clinical application, as a promising alternative to MSCs or chondrocytes.

Introduction

Cartilage defects larger than 2 cm^2 are currently treated by transplantation of autologous culture-expanded chondrocytes[281,282]. As chondrocytes are the resident cell type in cartilage, this cell type is the prime candidate for cartilage repair. Although long term results are promising with good patient reported outcomes and radiographic signs of cartilage formation[281,283], drawbacks of the treatment remain. Extensive culture of chondrocytes for autologous administration induces dedifferentiation and loss of phenotype[284]. Additionally, graft hypertrophy can lead to continued ailments and may necessitate further revision surgery[285]. These drawbacks can potentially be resolved by other cell sources. Mesenchymal stromal cells (MSCs) are frequently used[286] due to the relatively non-invasive methods for isolation, extensive culture-expansion potential[287,288], and efficient *in vitro* differentiation into chondrocytes producing cartilaginous tissue[289,290]. However, the associated risk of MSCs differentiating into hypertrophic chondrocytes and subsequent endochondral ossification poses a challenge[291]. While chondrocytes and MSCs are impacting the way cartilage defects are treated, different cell sources overcoming potential limitations may further advance the quality of repair tissue, and hence possibly clinical outcomes, leaving a gap for improvement in cell-based cartilage tissue engineering. Initially, a small portion of isolated articular chondrocytes was described to grow clonally and differentiate into several lineages[254]. Next, a distinct progenitor cell with stem cell-like characteristics was identified in the superficial zone, first in bovine[253] and later also in human cartilage[252]. This endogenous progenitor population is referred to as cartilage stem cells, mesenchymal or chondrogenic progenitor cells, or articular cartilage-derived progenitor cells (ACPCs).

Besides extensive culture-expansion[292], ACPCs are successful at producing neo-cartilage *in vitro*[293,294] and *in vivo*[252,255]. Several reports indicate that ACPCs have no tendency to differentiate into hypertrophic chondrocytes, unlike MSCs[256,295]. These combined features give ACPCs a preference over chondrocytes and MSCs for cartilage regeneration. Furthermore, similar to chondrocytes[296], pathological origin could influence ACPC performance. Indeed, OA-derived ACPCs were shown to possess chondrogenic properties, like healthy cartilage-derived ACPCs[297]. However, direct comparisons of chondrogenic potential of ACPCs from healthy and OA cartilage are limited[251,297]. Direct comparison can provide insight in the pathophysiology of OA and the potential role of ACPCs in health and disease.

The current study aims to characterize and compare fibronectin-selected ACPCs from healthy and OA human cartilage. By direct comparison of ACPC populations to full-depth cartilage cell populations derived from the same donors, their potential for cartilage regeneration is investigated.

Methods

Tissue collection

Macroscopically healthy cartilage (n = 6, age 46 - 49) was isolated post-mortem from full-weight bearing and non-weight bearing locations of the knee (Department of Pathology, University Medical Center Utrecht). Osteoarthritic (OA) cartilage was obtained from redundant material from patients (n = 6, age 55 - 79) undergoing total knee arthroplasty. Anonymous collection of this tissue was performed according to medical ethics regulations of the University Medical Center Utrecht and the guideline "good use of redundant tissue for research" of the Dutch Federation of Medical Research Societies[298]. Human MSCs (n = 6, age 30 – 66) were derived from bone marrow from the iliac crest of patients receiving spondylodesis or total hip arthroplasty surgery after their informed consent and according to a protocol approved by the local medical ethical committee.

Cell isolation and expansion

Cartilage from all parts of the joint (weight bearing and non-weight bearing) was pooled for each donor. Cartilage pieces were digested in 0.2% (w/v) pronase (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 1% (v/v) penicillin/streptomycin (pen/strep; 100 U/mL, 100 µg/mL; Gibco) for two hours at 37°C, followed by overnight digestion in 0.075% (w/v) type II collagenase (Worthington, Lakewood, NJ) in DMEM with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biowest) and 1% pen/strep under agitation. The population from here on referred to as "chondrocytes" is the total cell population isolated from cartilage, without any purification or selection[252]. Chondrocytes were expanded using chondrocyte expansion medium (DMEM, 10% FBS, 1% pen/strep). To isolate ACPCs, the digest was seeded at 500 cells/cm^2 at 37°C on culture plastic pre-coated for one hour with fibronectin (1 µg/mL in PBS containing $MgCl_2$ and $CaCl_2$; Sigma-Aldrich) using serum-free medium (DMEM with 1% pen/strep). After 20 minutes, non-adhered cells were washed away and ACPC expansion medium was added (DMEM, 10% FBS, 1% pen/strep, 200 µM l-ascorbic acid 2-phosphate [ASAP; Sigma-Aldrich], and 5 ng/mL basic fibroblast growth factor [bFGF; PeproTech]). On day 6, colonies (>32 cells) were isolated using sterile glass cloning cylinders (Sigma-Aldrich). Collected colonies were pooled and ACPCs were further expanded on conventional tissue culture plastic with ACPC expansion medium.

The mononuclear fraction of bone marrow was separated by centrifugation using a Ficoll-Paque density gradient (GE Healthcare, The Netherlands) and plated using MSC expansion medium (Minimum Essential Media [αMEM; Gibco, The Netherlands], 10% FBS,

1% pen/strep, 200 µM ASAP, and 1 ng/mL bFGF). MSCs were expanded up to passage four before use in differentiation assays.

Flow cytometry

Cells were washed in buffer (0.5% w/v bovine serum albumin [BSA], 2mM EDTA in PBS) and incubated with antibodies against CD49e, CD146, CD166 (Miltenyi Biotec), CD105 (Abcam), CD90, CD73, or a cocktail of markers (CD45, CD34, CD11b, CD79A, HLA-DR; all R&D Systems) according to the manufacturers' instructions. Labelled cells were analysed using a BD FACSCanto II or BD LSRFortessa (BD Biosciences, USA). Dead cells were excluded using 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma) or 1 µg/mL 7-Aminoactinomycin D (7AAD; Molecular Probes). Results were analysed using FlowJo V10 data analysis software package (TreeStar, USA).

Colony Forming Unit Fibroblast Assay

Chondrocytes directly after isolation (passage 0) and ACPCs and chondrocytes at passages two and four were seeded at 50 and 100 cells per 6-well plate well in duplicates. Cells were cultured with corresponding expansion medium. After seven days, colonies were fixed with 10% formalin and stained for 45 minutes using 0.05% (v/v) Crystal Violet (Sigma-Aldrich) in Milli-Q water and counted.

Multilineage Differentiation

For chondrogenic and hypertrophic differentiation, 2.5 x 10^5 ACPCs (passage four) or chondrocytes (passage two) were pelleted in 15 mL Falcon tubes by centrifugation at 320 *g* for five minutes. Pellets were cultured in chondrogenic medium (DMEM with 1% pen/strep, 2% (v/v) human serum albumin [HSA; Albuman, Sanquin Blood Supply Foundation], 1% insulin-transferrin-selenium-ethanolamine [ITS-X; Gibco], 0.1 µM dexamethasone, 0.2 mM ASAP, and 10 ng/mL TGF-β1 [PeproTech]). After 21 days of chondrogenic differentiation, half of the pellets was fixed using 10% buffered formalin. The other half of the pellets was exposed to hypertrophic medium (DMEM with 1% pen/strep, 1% ITS-X, 1 nM dexamethasone, 200 µM ASAP, 10 mM β-glycerophosphate [BGP; Sigma-Aldrich], and 1 nM 3,3′,5-Triiodo-L-thyronine [Sigma-Aldrich]) for seven additional days. After a total of 28 days, the hypertrophic -treated pellets were fixed with 10% formalin and processed for histology.

For osteogenic and adipogenic differentiation, ACPCs (passage four) and chondrocytes (passage two) were seeded in 24-well plate wells using corresponding expansion media. Upon subconfluency, monolayers were treated with osteogenic medium (αMEM with 10% FBS, 1% pen/strep, 200 µM ASAP, 10 mM BGP, and 10 nM dexamethasone [Sigma-Aldrich])

or adipogenic medium (αMEM with 10% FBS, 1% pen/strep, 1 µM dexamethasone, 0.5 mM 3-Isobutyl-1-methylxanthine [IBMX; Sigma-Aldrich], 0.2 mM indomethacin [Sigma-Aldrich], and 1.72 µM insulin [Sigma-Aldrich]) for 21 days. After 21 days, monolayers were fixed with 10% formalin and stained for calcium deposits by 40 mM alizarin red S in demineralized water (pH 4.1; Sigma-Aldrich) or intracellular lipid vacuoles by 7.3 mM oil red O (Sigma-Aldrich) in 60% isopropanol. For all assays, MSCs were ran in parallel as positive controls. Additional osteogenic differentiation was performed by expanding and differentiating ACPCs using several batches of FBS (Biowest and Gibco) and platelet lysate[299] (Sanquin Blood Supply Foundation). Also, monolayers were treated with osteogenic medium with 100 ng/mL recombinant human Bone Morphogenetic Protein-2[300] (InductOS) and cell pellets[252] were stimulated with osteogenic medium, both for 21 days. Results are shown in Supplemental Figure S1.

Pellet redifferentiation culture

For the redifferentiation cultures, 2.5 x 10^5 cells were pelleted in ultra-low attachment 96-well plate wells by centrifugation at 320 *g* for five minutes. Pellets were cultured for 28 days in redifferentiation medium (DMEM with 1% pen/strep, 2% HSA, 2% ITS-X, and 1% ASAP). Half of the pellets was supplemented with 10 ng/mL TGF-β1. Used medium was stored at -20°C for further analysis. Pellets were processed for biochemical analyses, gene expression, and (immuno)histochemistry.

Biochemical analysis of pellets

Pellets were digested overnight with papain (250 µg/mL papain [Sigma-Aldrich], 0.2 M NaH_2PO_4, 0.1M EDTA, 0.01M cysteine, pH 6) at 60°C. Deposition of sulphated glycosaminoglycans (GAGs) and release into the medium was measured by a dimethylmethylene blue assay (pH 3). The 525 / 595 nm absorbance ratio was measured using chondroitin-6-sulfate (Sigma-Aldrich) as a reference. DNA was quantified using a Quant-iT™ PicoGreen assay (Invitrogen) according the manufacturer's instructions.

Histological analysis of pellets

Pellets were processed for histology by dehydration through graded ethanol steps, clearing in xylene, and embedding in paraffin. Five µm-sections were stained for proteoglycan production with 0.125% safranin-O (Merck) counterstained with 0.4% fast green (Sigma-Aldrich) and Weigert's hematoxylin (Clin-Tech). Collagen deposition was visualized by immunohistochemistry. Sections were blocked in 0.3% (v/v) hydrogen peroxide. Antigen retrieval for type I and II collagen was performed with 1 mg/mL pronase (Sigma-Aldrich) and 10 mg/mL hyaluronidase (Sigma-Aldrich) and for type X

collagen with 1 mg/mL pepsin (Sigma-Aldrich) and 10 mg/mL hyaluronidase for 30 minutes at 37°C. Sections were blocked with 5% (w/v) BSA in PBS for one hour at room temperature and incubated with primary antibodies for type I collagen (EPR7785 [BioConnect], 1:400 in 5% PBS/BSA), type II collagen (II-II6B3 [DHSB], 1:100 in 5% PBS/BSA) and type X collagen (X53 [Quartett], 1:20 in 5% PBS/BSA) overnight at 4°C. Appropriate IgGs were used as isotype controls. Next, type I collagen sections were incubated with BrightVision Poly-HRP-Anti Rabbit (ImmunoLogic) and type II collagen sections were incubated with goat-anti-mouse IgG HRP-conjugated (DAKO, P0447; 1:100 in 5% PBS/BSA), both for one hour at room temperature. Type X collagen sections were incubated with biotinylated sheep-anti-mouse IgG (RPN1001V [GE Healthcare]) for one hour at room temperature, then with streptavidin-HRP for one hour at room temperature (DAKO, P0397; 1:1000 in 5% PBS/BSA). Next, all stainings were developed using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). Cell nuclei were counterstained with Mayer's hematoxylin (Klinipath).

Gene expression

Gene expression analysis was performed by real-time polymerase chain reaction (PCR). RNA was isolated from cells and pellets using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (200-500 ng) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCRs were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in the LightCycler 96 (Roche Diagnostics) according to the manufacturer's instructions. Primers (Invitrogen) are listed in Table S1. Relative gene expression was calculated using 18S as a housekeeping gene. Amplified PCR fragments extended over at least one exon border (except for 18S). The primer for detection of two splice variants of COL2A1 extended across exon 2 of the gene and results in amplification of splice variants IIa and IIb. PCR products were separated on 2% (w/v) agarose gel electrophoresis and stained with SYBR Safe (Invitrogen).

Statistical analysis

Experiments for flow cytometry, colony-forming efficiency, and multilineage differentiation were performed with cells from six healthy and six OA cartilage donors, unless stated otherwise. Pellet redifferentiation culture was performed with cells from three donors and three technical replicates per donor. All data are expressed as mean±standard deviation (SD). Data were analyzed using the GraphPad Prism 8 software package (GraphPad Software, United States). Normality was confirmed with a Shapiro-Wilk test ($p>0.05$). Groups were compared using an unpaired *t*-test, one- or two-

way analysis of variance (ANOVA) with Bonferroni post-hoc test. Gene expression data was not normally distributed and therefore analysed with a Mann-Whitney or Kruskall-Wallis test with Dunn's post-hoc test. A value of $p<0.05$ was considered statistically significant.

Results

Cells isolated from human cartilage have a chondrogenic profile

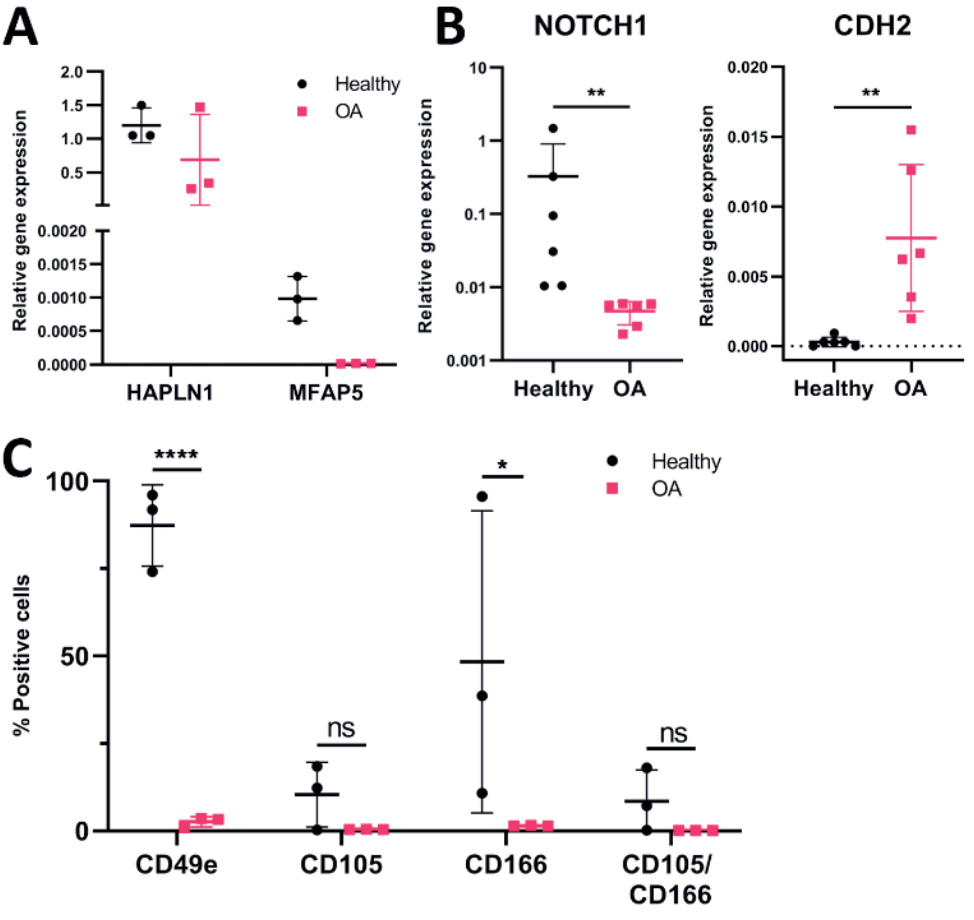
All samples consistently contained cells highly expressing the cartilage-specific marker cartilage link protein (HAPLN1), while having low expression of the synovial-specific marker microfibril-associated glycoprotein-2[301] (MFAP5; Figure 1A). Expression of HAPLN1 was comparable between cells directly after digestion of healthy versus OA cartilage (1.199±0.259 vs 0.687±0.678). Likewise, expression of MFAP5 was low in cells from both disease states (0.0010±0.0003 vs 0.000016±0.000001), confirming successful isolation of cartilage cells without contamination of synoviocytes from the tissues.

Expression of progenitor-specific markers in freshly isolated chondrocytes

Gene expression of freshly isolated chondrocytes revealed a significant difference in Notch receptor 1 (NOTCH1) between healthy and OA-derived cells (0.3274±0.5821 vs 0.0047±0.0016; $p=0.0022$; Figure 1B). Notably, expression of n-cadherin (CDH2) was significantly higher in cells derived from OA cartilage compared to healthy (0.0003±0.0003 vs 0.0077±0.0053; $p=0.0061$; Figure 1B). Cells positive for cell surface marker CD49e (integrin-α5) were significantly decreased in OA-derived cells compared to healthy (87.3%±11.6% vs 2.6%±1.4%; $p<0.0001$; Figure 1C). Likewise, expression of CD166 was decreased in OA-derived cells (48.4%±43.2% vs 1.4%±0.1%; $p=0.0122$). Marker expression of CD105 as well as co-expression of CD105 and CD166 did not differ between the groups.

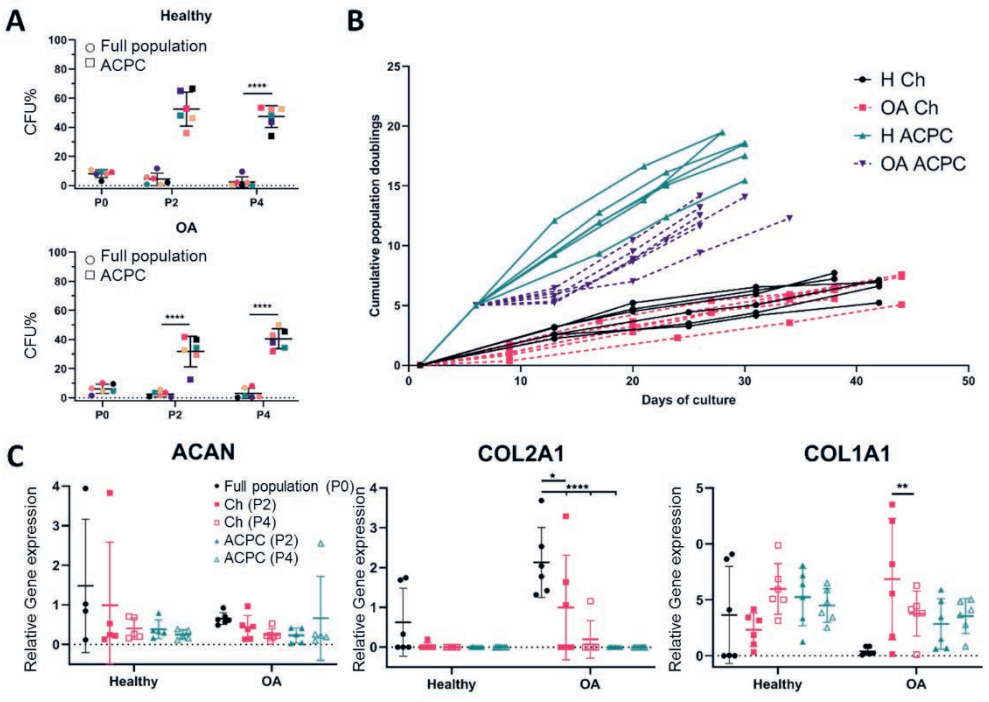
Selection by adhesion to fibronectin results in a population with high clonogenicity and proliferative capacity

Colony-forming efficiency of ACPCs at passage two and four was higher than of the full population ($p<0.0001$ for all groups; Figure 2A). Morphology of representative Crystal Violet-stained colonies can be seen in Supplemental Figure S2. Healthy and OA ACPCs underwent 18.1±1.5 and 13.0±1.0 population doublings respectively until reaching the fourth passage after 29.3±1.0 and 28.0±3.3 days, healthy and OA chondrocytes had 6.8±0.9 and 6.4±1.0 population doublings respectively until reaching passage four after 40.7±2.1 and 41.0±3.3 days (Figure 2B). Expansion of chondrocytes and ACPCs in ACPC-expansion medium resulted in similar population doublings per passage and similar



| Figure 1. Characterization of full-depth cartilage cell populations (A) Expression of cartilage- and synovial-specific genes in freshly isolated chondrocytes (n = 3 for both). Gene expression of hyaluronan and proteoglycan link protein 1 (HALPN1) is consistently high in chondrocytes isolated from healthy and osteoarthritic (OA) cartilage, while microfibril associated protein 5 (MFAP5) is low in cells from both tissues. (B) Gene expression of Notch receptor 1 (NOTCH1) was higher in healthy compared to OA cartilage. Expression of n-cadherin (CDH2) was significantly increased in OA cartilage-derived cells (n = 6 for both). (C) Surface marker expression of CD49e and CD166 were decreased in OA-derived cells compared to healthy cartilage-derived cells (n = 3 for all). **p<0.05 **p<0.01 ****p<0.0001. Three technical replicates per donor, each data point represents data of one donor.*

passage length (Figure S3A and B), while expansion in chondrocyte-expansion medium resulted in a less population doublings by chondrocytes while their culture time per passage decreased (Figure S3C and D). OA ACPCs and chondrocytes lost expression of type II collagen (COL2A1) during expansion (Figure 2C). Expression of type II collagen splice variants IIa and IIb did not reveal any distinct differences between chondrocytes and ACPCs or between passage numbers (Figure S4).

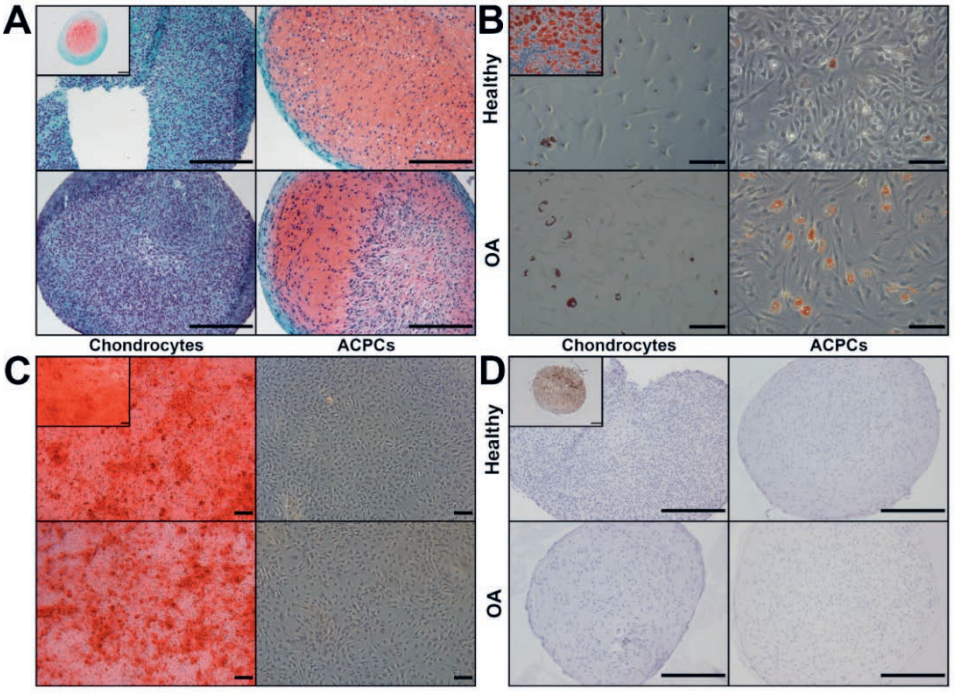


| Figure 2. Culture-expansion of articular cartilage-derived progenitor cells versus non-selected chondrocytes. (A) Colony-forming units (CFU) at increasing cell passages (P0, P2, P4). Articular cartilage-derived progenitor cells (ACPCs) form significantly more colonies at passage 2 and 4, compared to chondrocytes. Colours within the graphs represent different donors (n = 6 for all). (B) Cumulative population doublings of ACPCs and chondrocytes. Healthy and osteoarthritic (OA) ACPCs underwent 18.1 ± 1.5 and 13.0 ± 1.0 population doublings respectively until reaching the fourth passage, healthy and OA chondrocytes underwent 6.8 ± 0.9 and 6.4 ± 1.0 population doublings respectively until reaching passage 4 (n = 6 for all). (C) Expression of type II collagen (collagen type I alpha 1 chain; COL2A1) decreased in OA chondrocytes and ACPCs during expansion. Expression of type I collagen (collagen type II alpha 1 chain; COL1A1) was increased in passage 2 chondrocytes (n = 6 for all). **p<0.05 **p<0.01 ****p<0.0001. Three technical replicates per donor, each data point represents data of one donor.*

ACPCs fail to produce mineralized matrix upon osteogenic and hypertrophic induction

All healthy and OA ACPCs differentiated into the chondrogenic and adipogenic lineage, indicated by safranin-O and oil red O stainings (Figure 3A and 3B). Chondrocyte pellets stained less for proteoglycans than ACPC pellets (Figure 3A, left panels). Osteogenic differentiation was evident in chondrocytes (Figure 3C, left panels), while ACPCs stained negative for mineralized matrix by alizarin red (Figure 3C, right panels). Osteogenic

differentiation was also unsuccessful when ACPCs were expanded with different batches of FBS and platelet lysate, and when the osteogenic differentiation medium was supplemented with 100 ng/mL BMP-2 or when ACPC pellets were stimulated with osteogenic medium (Supplemental Figure S1). All chondrocytes and ACPCs were unable to differentiate into hypertrophic chondrocytes, indicated by negative staining for type X collagen (Figure 3D).

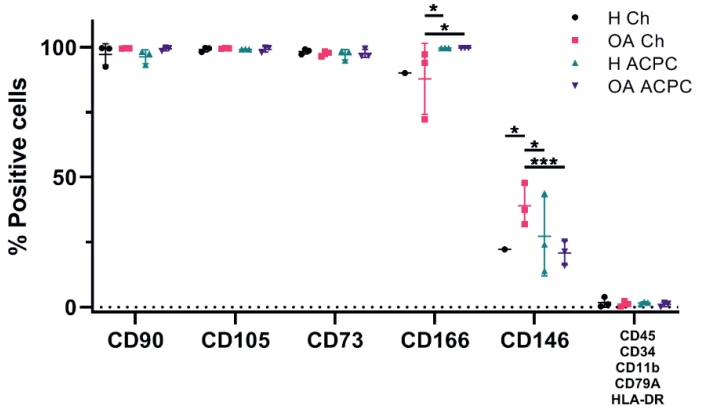


| Figure 3. Differentiation of chondrocytes and articular cartilage-derived progenitor cells into four lineages. (A) Chondrogenic differentiation, indicated by staining of proteoglycans by safranin-O. (B) Adipogenic differentiation, indicated by staining of lipid droplets by oil red O. (C) Osteogenic differentiation, shown by staining of the mineralized matrix by alizarin red. (D) Hypertrophic differentiation, figure shows type X collagen immunohistochemnistry. Inserts show bone marrow-derived mesenchymal stromal cells (MSCs) differentiated in parallel as positive controls. N = 6 for all cell types, a representative image per cell type was selected. All scale bars = 200 µm.

Expression of bone marrow-derived MSC surface markers in culture-expanded ACPCs

Expression of cell surface markers defined for bone marrow-derived MSCs in monolayer culture[39], CD90, CD105, and CD73, was >95% in all populations. CD166-expression was >99% in all ACPC donors, but lower in OA chondrocytes (87.8%±13.6) compared to healthy ACPCs (99.7%±0.1; p=0.0322) and OA ACPCs (99.8%±0.1; p=0.0315). Expression of CD146 was higher in OA chondrocytes (39.0%±8.1) compared to healthy chondrocytes

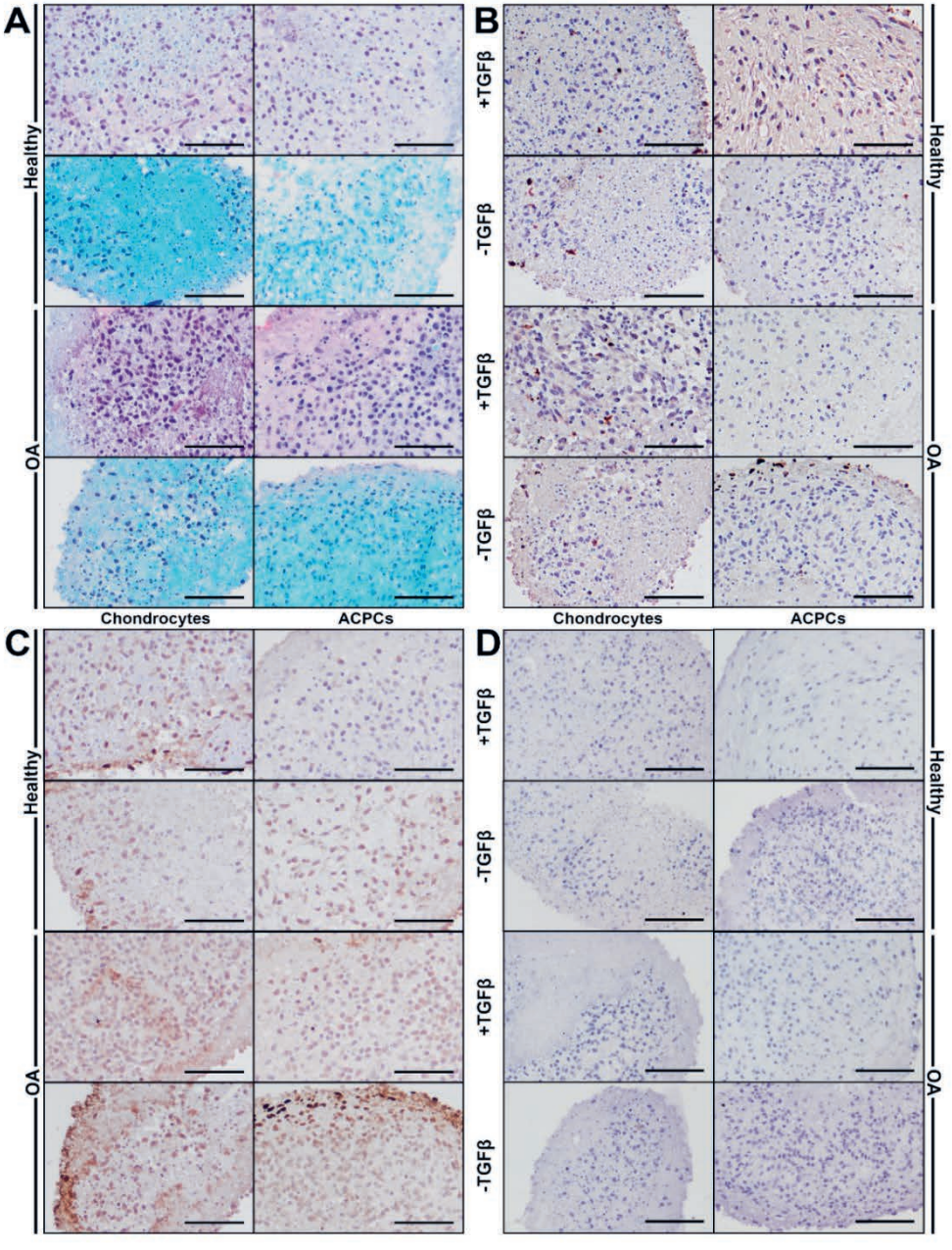
(22.2%±0.0; *p*=0.0322), healthy ACPCs (27.3%±15.2; *p*=0.0352), and OA ACPCs (20.9%±4.6; *p*=0.0003). Expression of several markers was tested using a cocktail containing antibodies against CD45, CD34, CD11b, CD79A, and HLA-DR. All cell types were <2% positive for this cocktail of markers (Figure 4).



| Figure 4. Cell surface marker expression by flow cytometry. Expression of CD90, CD105, CD73 was >95% in all donors (n = 3). CD166 expression was lower in osteoarthritic (OA) chondrocytes compared to healthy and OA articular cartilage-derived progenitor cells (ACPCs). CD146 expression was higher in OA chondrocytes compared to the other cell types. All cell types were <2% positive for CD45, CD34, CD11b, CD79A, and HLA-DR. *p<0.05 ***p<0.001. Three technical replicates per donor, each data point represents data of one donor.

ACPC pellets produce proteoglycans and type II collagen *in vitro*

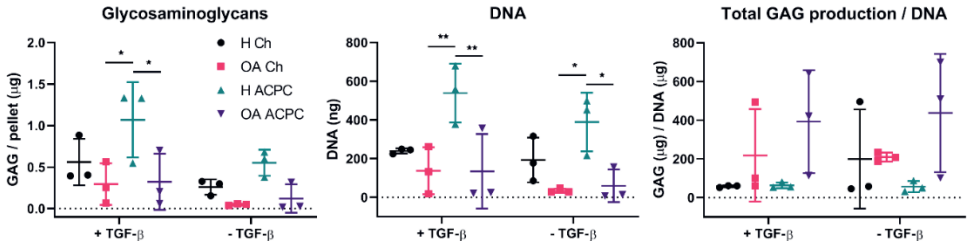
Chondrocytes and ACPC pellets stained positive for proteoglycans when stimulated with 10 ng/mL TGF-β1. When the redifferentiation medium was not supplemented with TGF-β1, pellets stained negative for proteoglycans (Figure 5A). Type II collagen production was only found in healthy ACPC pellets cultured in the presence of TGF-β1. All other conditions showed no type II collagen-positive matrix (Figure 5B). Additionally, healthy ACPC pellets stimulated with TGF-β1 were the only condition negative for type I collagen (Figure 5C). All cultures were negative for X collagen (Figure 5D). Healthy ACPC pellets cultured with TGF-β1 contained more GAGs compared to OA chondrocyte and OA ACPC pellets (1.1±0.4 µg vs. 0.3±0.3 µg and 0.3±0.3 µg, *p*=0.0115 and *p*=0.0152, respectively; Figure 6, left panel). Similarly, DNA content was higher in healthy ACPC pellets, independent of TGF-β1 (with TGF-β1: 539.0±152.0 ng vs. 137.4±121.8 ng and 134.2±192.8 ng, *p*=0.0058 and *p*=0.0054, respectively, without TGF-β1: 389.4±151.5 ng vs. 34.0±10.7 ng and 59.7±84.5 ng, *p*=0.0153 and *p*=0.0264, respectively (Figure 6, middle panel)). Production of GAG corrected for the amount of DNA was not different between the groups (Figure 6, right panel).



| Figure 5. Histological staining of redifferentiated pellets. (A) Chondrocyte and articular cartilage-derived progenitor cells (ACPC) donor-matching pellets stained positive for proteoglycans by safranin-O when stimulated with TGF-β1 for four weeks. (B) TGF-β1-stimulated healthy ACPC pellets were positive for type II collagen and (C) negative for type I collagen. (D) None of the groups stained positive for type X collagen (D). N = 3 for all cell types, a representative image per cell type was selected. Scale bars = 100 µm.

Reduced expression of hypertrophic marker type X collagen

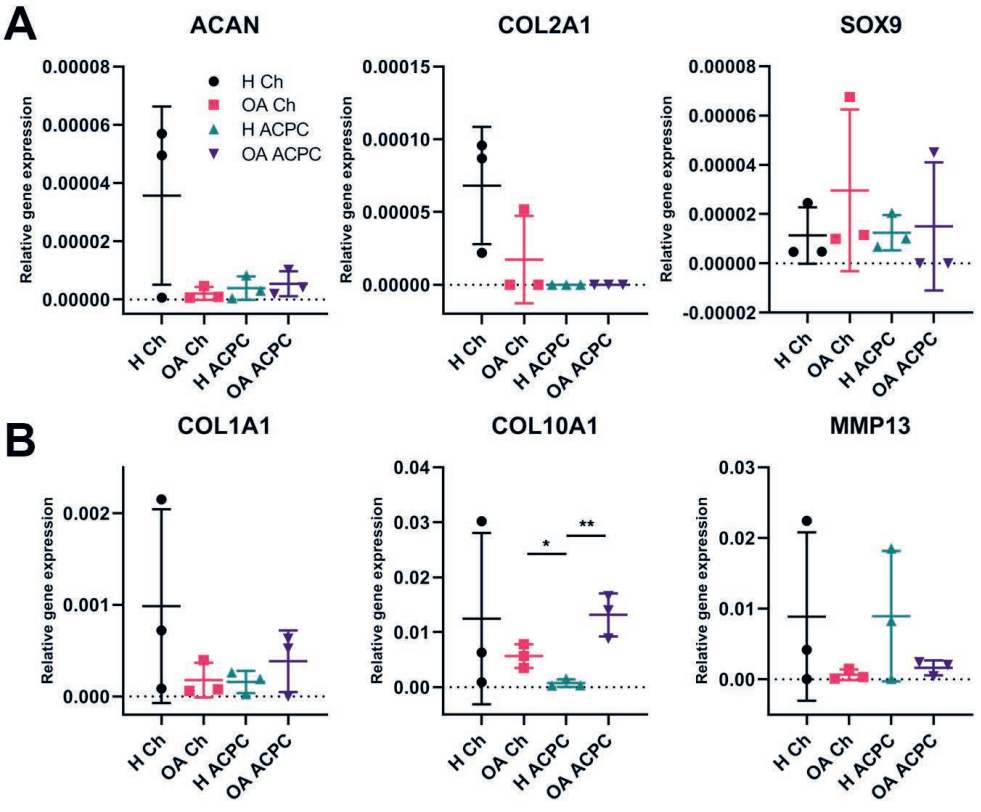
Gene expression analysis was only performed on TGF-β1-treated pellets, as insufficient amounts of RNA could be isolated from non-TGF-β1-treated pellets. No difference was found between expression of chondrogenic genes aggrecan (ACAN), type II collagen (COL2A1), and SRY-box transcription factor 9 (SOX9) between the experimental groups (Figure 7A). Noteworthy, COL2A1 expression in ACPC pellets was close to zero. Expression of the hypertrophic marker type X collagen (COL10A1) was lower in healthy ACPC pellets compared to OA ACPC pellets (0.00074±0.00071 vs. 0.01315±0.00393; p=0.0028) and compared to OA chondrocyte pellets (0.005664±0.002154; p=0.0296). Expression of type I collagen (COL1A1) and matrix metallopeptidase 13 (MMP13) was not different between the groups (Figure 7B).



| Figure 6. Glycosaminoglycan and DNA content in redifferentiated pellets after four weeks. Quantification of glycosaminoglycans (GAGs) shows significantly more GAGs produced in healthy articular cartilage-derived progenitor cells (ACPC) pellets cultured in the presence of TGF-β1 (left panel). DNA quantification shows significantly more DNA present in healthy ACPC pellets, suggesting bigger pellet sizes (middle panel). Total GAG production corrected for DNA content reveals no differences between the experimental groups (right panel). N = 3 for all cell types. *p<0.05 **p<0.01. Three technical replicates per donor, each data point represents data of one donor.

Discussion

The current study aimed to characterize human ACPCs from healthy and OA cartilage and determine their potential for cartilage regeneration. While fibronectin-selected progenitor populations have been described in healthy[252] and OA human cartilage[297,302], this study is the first to directly compare chondrogenic functionality of ACPCs from healthy and OA cartilage to chondrocytes derived from matching donors. The results confirm the presence of an ACPC population in human articular cartilage[251,252,297,302]. Differential adhesion to fibronectin resulted in a cell population that was capable of clonal growth, extensive culture-expansion, multilineage differentiation, and had a limited tendency to produce mineralized matrix and terminally differentiate into hypertrophic



| Figure 7. Gene expression of redifferentiated pellets. (A) Expression of chondrogenic genes aggrecan (ACAN), type II collagen (collagen type I alpha 1 chain; COL2A1), and SRY-box transcription factor 9 (SOX9) was not different between the groups. (B) Type X collagen expression was lower in healthy articular cartilage-derived progenitor cells (ACPC) pellets compared to osteoarthritic (OA) ACPC pellets. Expression of type I collagen (collagen type II alpha 1 chain; COL1A1) and matrix metallopeptidase 13 (MMP13) was not different between the groups. N = 3 for all cell types. *p<0.05 **p<0.01. Three technical replicates per donor, each data point represents data of one donor.

chondrocytes. As chondrogenic potential of chondrocytes can be dependent on the disease state of cartilage[296], ACPCs derived from healthy and OA cartilage might provide as good candidates for cartilage repair. The current side-by-side comparison of healthy- with OA-derived ACPCs and donor-matched chondrocytes provides an overview of these cells' potential for cartilage regeneration.

We investigated full-depth healthy and OA cell populations attempting to find correlations between previously reported progenitor markers and ACPC quantity. Expression of NOTCH1 was found to be higher in healthy cells compared to OA, confirming previous findings[303]. At the same time, CDH2 was significantly upregulated in OA cells. The cell-cell adhesion molecule N-cadherin is related to cellular condensation in

early chondrogenesis during development and absent in differentiated cartilage[304,305]. This might be a result of chondrocyte clustering in OA[306,307] and the cells potentially obtaining a more premature chondrogenic phenotype. CDH2 in culture-expanded ACPCs was previously found to be higher in non-OA ACPCs compared to OA ACPCs[308], indicating that this difference is lost upon selection for progenitors and culture expansion. Therefore, NOTCH1 and CDH2 might be used as markers to distinguish between healthy- and OA-derived cells.

Expression of surface markers CD49e (integrin-α5, part of the key fibronectin receptor) and CD166 was lost in the total population of OA cells compared to healthy cells and no difference in CD105/CD166-double positive cells was found. These findings are in contrast to previous ones[251], where a higher percentage of double positive cells was found in OA tissue. However, others found similar amounts[309] or more CD105/CD166-double positive cells in healthy cartilage versus OA[310]. The OA cartilage in the current study was obtained from end-stage OA patients and was not scored on OA severity. Severely degraded OA cartilage has lost most of its superficial layer and would subsequently also have lost superficial zone cells, which express CD49e[253] and CD166[309]. Separation based on CD49e-expression could lead to a population with improved chondrogenic potential, like healthy cartilage-derived ACPCs.

Our results show separation based on differential adhesion to fibronectin results in a population with enriched colony-forming efficiency and increased proliferative potential. Fibronectin-selected ACPCs were previously found to maintain telomerase activity and telomere length up to at least 22 population doublings[252,311], which is more than the number of population doublings reached in our study. When using the same expansion media to expand both cell types, population doublings in chondrocytes were limited compared to ACPCs with chondrocyte-expansion medium, supporting the findings of higher cell yields of ACPCs. To add, culture time of chondrocytes decreased over passaging, indicating increasing cell size and possible dedifferentiation. On the contrary, OA-derived ACPCs lost mRNA expression of type II collagen upon culture expansion. Articular chondrocytes are known to dedifferentiate in monolayer expansion[284], but regain their phenotype when exposed to appropriate culture conditions[254,312]. Similarly, our ACPCs regained their differentiation potential and especially healthy cartilage-derived ACPCs were successful in producing type II collagen- and proteoglycan-rich matrix *in vitro*, while chondrocytes were less effective. Gene expression and protein deposition after the culture period of four weeks did not correspond, for which type II collagen is the most evident example. While H-ACPCs pellets cultured with TGF-β1 did not express the gene corresponding for type II collagen, immunohistochemistry revealed a slightly positive staining in these pellets. While discrepancies between protein and gene

expression are regularly seen[313], the chondrogenic response of ACPCs in this case might have been earlier than that of chondrocytes, resulting in differences in gene expression between the cell types at the four-week evaluation point. Evaluation of gene expression throughout the culture period would give insight into the timing of the response. Furthermore, evaluation of individual clones of ACPCs would shed more light on cell performance and would allow for selection of populations with optimal characteristics, as was investigated before[252,311]. Yet, for the purpose of the current study it would considerably delay expansion time to obtain sufficient amounts of cells for tissue engineering and limit clinical application.

Since there is a need for identification of unique markers for selecting ACPCs, we specifically looked into gene expression of type II collagen splice variants in order to investigate whether this marker could be used for discerning ACPCs and chondrocytes. Splice variant IIa is an established marker for juvenile chondrocytes or mesenchymal cells, while variant IIb is expressed by mature chondrocytes[314,315]. Although ACPCs would be chondrogenic precursors and were expected to express the immature variant of type II collagen, no differences were found here between the cell types or passage numbers, a possible result of the cells being in the expansion phase rather than in redifferentiation and are not actively producing extracellular matrix.

ACPCs are generally referred to as MSC-like as they, besides holding multilineage differentiation potential, meet the surface marker criteria to identify MSCs[39]. In addition, there are some indications that ACPCs have similar anti-inflammatory properties as MSCs[316]. More than 95% of the ACPCs described here expressed MSC-markers CD90, CD105, CD73, and CD166, and expression of a panel of negative markers is <2%. Noteworthy, ACPCs were negative for HLA-DR, making these populations potentially interesting for allogeneic applications. Culture-expanded chondrocytes exhibit a similar pattern of surface marker expression. While the expression pattern of the ACPC populations investigated here are in line with previous reports[252,293,317], caution should be taken when drawing conclusions. Evaluating expressed surface markers straight after ACPC-selection from the total pool of cells is the only way to directly compare cell populations and avoid the effect of culture expansion on the expression profile.

Both ACPC populations were unable to produce mineralized matrix upon stimulation with various osteogenic differentiation media and protocols. While osteogenesis is generally confirmed in human ACPCs[252,297,302], indications of reduced osteogenic potential exist. Interestingly, consistent results have been reported on decreased or absent expression of hypertrophic chondrocyte marker type X collagen[256,293,295,318] or early osteogenic marker alkaline phosphatase[294]. The differences in osteogenic differentiation potential between the populations investigated here and fibronectin-isolated ACPC

populations described by others are remarkable. As ACPCs originate from the cartilage, the cells might be more primed towards the chondrogenic lineage rather than to differentiate into osteoblasts or continue towards terminal hypertrophic differentiation. Since others do report on osteogenic differentiation of ACPCs, minor differences in culture media composition might explain the discrepancies. Isolation and culture protocols should be conducted side-by-side to elucidate differences between ACPC populations. Bone marrow-MSCs are associated with the risk of hypertrophic cartilage formation, when cells either differentiate or deposited matrix is remodelled into bone[250,256]. Because hypertrophy in autologous chondrocyte implantation (ACI) continues to be a challenge[285], the reduced osteogenic drift of ACPCs holds great promise for these cells.

Healthy cartilage-derived ACPCs produce cartilage ECM *in vitro* containing proteoglycans and type II collagen, and are devoid of type I collagen. In addition, these healthy ACPC pellets had low expression of type X collagen mRNA. Cartilage harvest site and tissue quality can be important for eventual cartilage production. To obtain healthy ACPCs, we combined all load-bearing and non-load bearing cartilage from healthy knee joints, while chondrocytes isolated for ACI procedures are generally from non-load bearing areas. However, chondrocytes from macroscopically healthy, full-weight bearing cartilage were shown to produce more proteoglycans and type II collagen *in vitro*[296]. Separating sub-groups of ACPC populations based on the degree of weight bearing might provide further insights into the physiological role of progenitors in cartilage homeostasis. While we have not investigated it in the current study, several studies report on an increased number of ACPCs in OA cartilage[251,297,319,320] and numbers of ACPCs increasing after mechanical stimulation[321,322].

ACPCs were used in a caprine model and had good lateral integration with the native cartilage[252], showing potential for use in a two-step cartilage repair procedure. Furthermore, a pilot study with 15 patients employing matrix-assisted autologous chondrocyte transplantation reported satisfactory histological and pain scoring one year after surgery[255]. ACPCs were expanded for a maximum of three weeks, substantially shorter than the expansion time needed for chondrocytes, which is generally four to eight weeks[21,323,324], depending on growth speed and defect size. In spite of these promising early clinical results, direct comparisons between chondrocytes and ACPCs are necessary to identify advantages in length of culture expansion and quality of the repair tissue.

Conclusion and implications

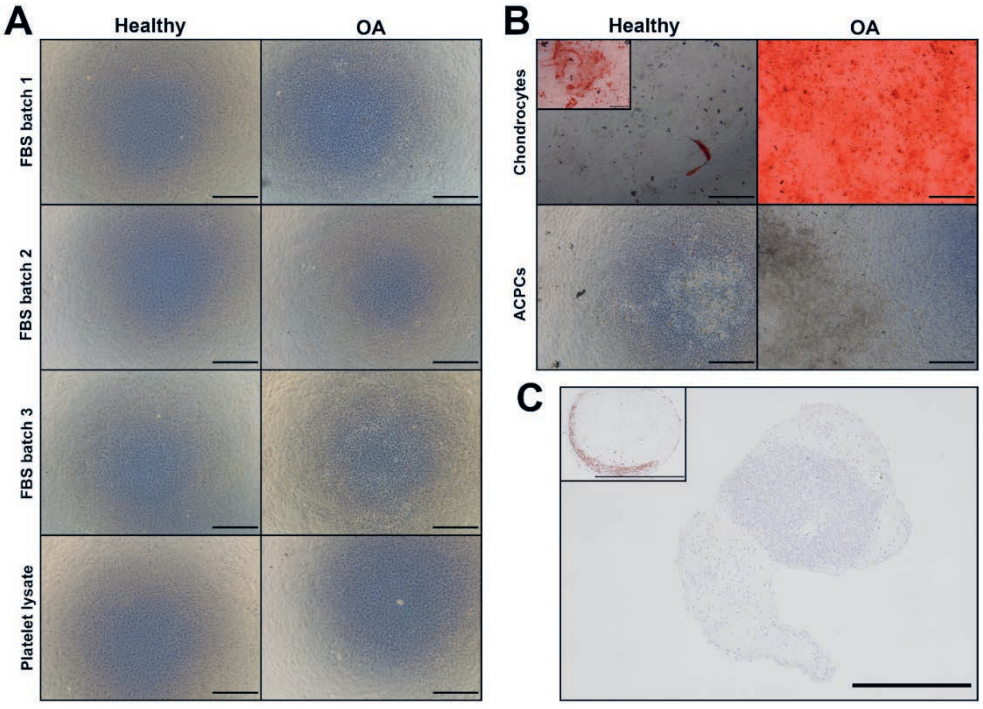
To conclude, ACPCs isolated here show potential for cartilage regeneration, possibly in an autologous approach replacing chondrocytes. The limited potential of these ACPC populations to produce mineralized matrix and absence of type X collagen protein and mRNA expression in healthy cartilage-derived ACPCs is promising. These observations combined with extensive *in* vitro expansion potential of ACPCs can have major implications for future cartilage repair treatments.

Appendix

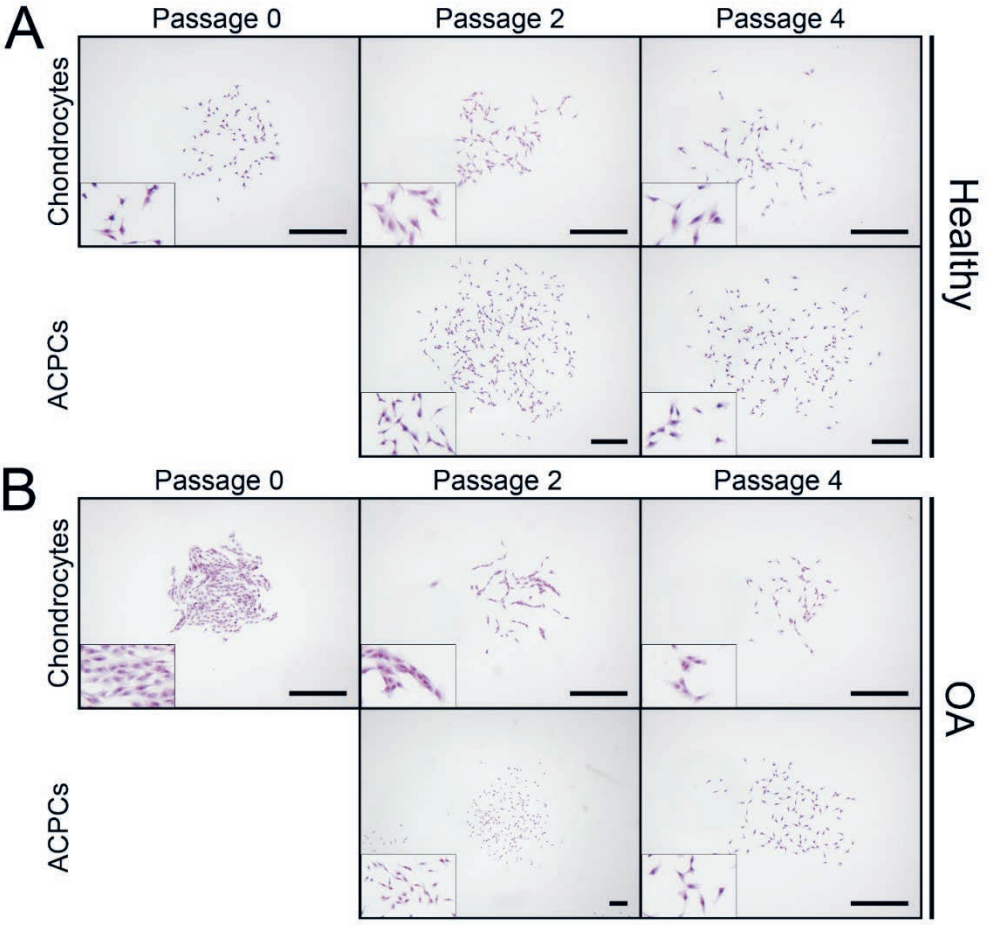
Table S1. Primer sequences for quantitative real-time PCR

Gene name	Oligonucleotide sequence (5' to 3')	Annealing temperature (ºC)	Product size (bp)
18S	Fw: GTAACCCGTTGAACCCCATT Rv: CCATCCAATCGGTAGTAGCG	57	151
ACAN	Fw: CAACTACCCGGCCATCC Rv: GATGGCTCTGTAATGGAACAC	56	160
CDH2	Fw: GCGTCTGTAGAGGCTTCTGG Rv: GCCACTTGCCACTTTTCCTG	60	293
COL10A1	Fw: CACTACCCAACACCAAGACA Rv: CTGGTTTCCCTACAGCTGAT	56	225
COL1A1	Fw: TCCAACGAGATCGAGATCC Rv: AAGCCGAATTCCTGGTCT	57	191
COL2A1	Fw: AGGGCCAGGATGTCCGGCA Rv: GGGTCCCAGGTTCTCCATCT	57	195
COL2A1 (spanning exon 1 – 5)	Fw: CCGCGGTGAGCCATGATTCG Rv: AGGCCCAGGAGGTCCTTTGGG	54	385 (IIa) 178 (IIb)
HAPLN1	Fw: TGAAGGATTAGAAGATGATACTGTTGTG Rv: GCCCCAGTCGTGGAAAGTAA	59	80
MFAP5	Fw: CGAGGAGACGATGTGACTCAAG Rv: AGCGGGATCATTCACCAGAT	59	72
MMP13	Fw: GGAGCATGGCGACTTCTAC Rv: GAGTGCTCCAGGGTCCTT	56	208
NOTCH1	Fw: AAGCTGCATCCAGAGGCAACC Rv: TGGCATACACACTCCGAGAACAC	60	172
SOX9	Fw: CCCAACGCCATCTTCAAGG	60	242

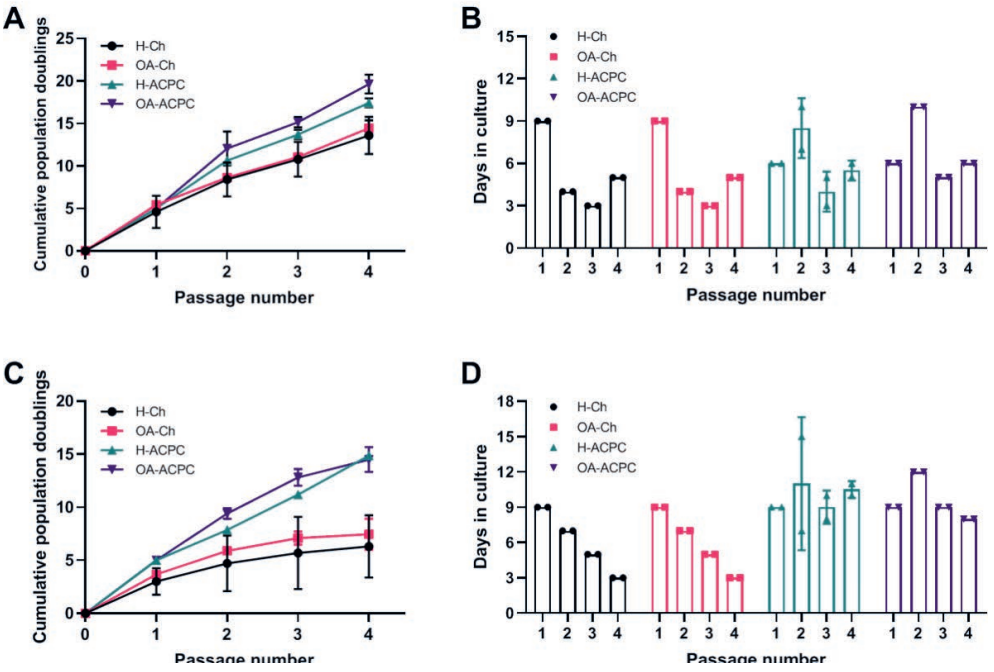
Forward (Fw) and reverse (Rv) primers. ACAN, aggrecan; CDH2, cadherin 2; COL10A1, collagen type X alpha 1 chain; COL1A1, collagen type I alpha 1 chain; COL2A1, collagen type II alpha 1 chain; HAPLN1, hyaluronan and proteoglycan link protein 1; MFAP5, microfibril associated protein 5; MMP13, matrix metallopeptidase 13; NOTCH1, Notch receptor 1; SOX9, SRY-box transcription factor 9



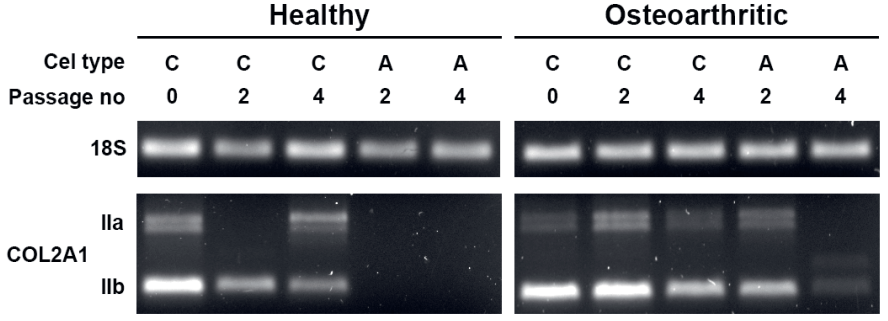
| Figure S1. Osteogenic differentiation of articular cartilage-derived progenitor cells. (A) Articular cartilage-derived progenitor cells (ACPCs) were expanded and differentiated for three weeks using expansion medium and osteogenic differentiation medium supplemented with three batches of fetal bovine serum (FBS) or platelet lysate, after which the monolayers were stained with alizarin red. (B) Chondrocyte and ACPC monolayers were differentiated for three weeks using osteogenic differentiation medium supplemented with 100 ng/mL recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) and stained with alizarin red. (C) A representative ACPC pellet stimulated for three weeks with osteogenic differentiation medium and stained for detection of calcium deposit by von Kossa stain. Insert shows a pellet of bone marrow-derived MSCs cultured in parallel. N = 3 for all. All scale bars = 200 µm.



| Figure S2. Morphology of colonies. Gross morphology of colonies from (A) chondrocytes and articular cartilage-derived progenitor cells (ACPCs) derived from healthy cartilage at passage 0, 2, and 4 and colonies from (B) chondrocytes and ACPCs derived from osteoarthritic (OA) cartilage stained with 0.05% Crystal Violet. The inserts show a 12X magnifications of the original image. N = 6 for all cell types. All scale bars = 200 µm. Two technical replicates per donor.



| Figure S3. Expansion of articular cartilage-derived progenitor cells versus chondrocytes in two distinct media. (A) Cumulative population doublings of articular cartilage-derived progenitor cells (ACPCs) and chondrocytes in ACPC expansion medium at each consecutive passage. (B) Culture time of ACPCs and chondrocytes per passage in ACPC expansion medium. (C) Cumulative population doublings of ACPCs and chondrocytes in chondrocyte expansion medium and (D) culture time per passage in chondrocyte expansion medium. N = 2 for all experiments, ACPC and chondrocyte donors are matching.



| Figure S4. Gene expression of type II collagen splice variants. Polymerase chain reaction (PCR) product analysis of type II collagen splice variants IIa and IIb in chondrocytes after isolation (passage 0) and chondrocytes and articular cartilage-derived progenitor cells (ACPCs) at passage 2 and 4. Products were separated on an agarose gel and visualized with SYBR Safe. For both pathological states, 18S and COL2A1 PCR products were ran on the same gel. A, ACPC; C, chondrocyte; COL2A1, collagen type II alpha 1 chain; IIa, splice variant IIa; IIb, splice variant IIb.

08

The regenerative effect of different growth factors and platelet lysate on meniscus cells and mesenchymal stromal cells and proof-of-concept with a functionalized meniscus implant

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Jonkheijm P, Krych AJ, Saris DBF, Vonk LA.
The regenerative effect of different growth factors and platelet
lysate on meniscus cells and mesenchymal stromal cells
and proof of concept with a functionalized meniscus implant.
J Tissue Eng Regen Med. 2021 Jul;15(7)

Abstract

Background

Meniscus regeneration could be enhanced by targeting meniscus cells and mesenchymal stromal cells (MSCs) with the right growth factors. Combining these growth factors with the Collagen Meniscus Implant (CMI®) could accelerate cell ingrowth and tissue formation in the implant and thereby improve clinical outcomes.

Methods

Using a transwell migration assay and a micro-wound assay, the effect of insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-β1), fibroblast growth factor (FGF) and platelet lysate (PL) on migration and proliferation of meniscus cells and MSCs was assessed. The formation of extracellular matrix under influence of the abovementioned growth factors was assessed after 28 days of culture of both MSCs and meniscus cells. As a proof-of-concept, the CMI® was functionalized with a VEGF binding peptide and coated with platelet rich plasma (PRP) for clinical application.

Results

Our results demonstrate that PDGF, TGF-β1 and PL stimulate migration, proliferation and/or extracellular matrix production of meniscus cells and MSCs. Additionally, the CMI® was successfully functionalized with a VEGF binding peptide and PRP which increased migration of meniscus cell and MSC into the implant.

Conclusion

This study demonstrates proof-of-concept of functionalizing the CMI® with growth factor binding peptides. A CMI® functionalized with the right growth factors holds great potential for meniscus replacement after partial meniscectomy.

Introduction

The meniscus is a c-shaped, structure in the tibiofemoral joint composed of fibrocartilage. It is essential for load transmission, stability, and articular surface protection in the knee joint[325,326]. Meniscus injury is common and strongly correlates with the development of early osteoarthritis (OA)[327–329]. Repair of meniscus injury is only successful in the vascularized region of the meniscus of young patients, where some regenerative capacity is present. Regeneration does not occur in the inner zone and in older patients [171,173,330]. Therefore, treatment often consists of (partial) meniscectomy, which increases the contact pressure in the articular cartilage, eventually leading to degeneration[11,331]. Meniscus replacement or stimulation of meniscus regeneration could potentially prevent or delay the onset of OA[332]. A clinically available implant for partial meniscus replacement is the Collagen Meniscus Implant (CMI®; Stryker, Michigan, USA). The CMI® improves short-term outcomes, but tissue deposition is limited and (partial) resorption occurs in several years[14,333]. In order to improve quality of the tissue and durability, a scaffold or implant could be seeded with cells [49]. Implantation of a scaffold seeded with autologous multipotent mesenchymal stromal cells (MSCs) has shown promising results *in vivo,* but has a high patient burden due to the necessity of two procedures[196,197,202]. Moreover, harvesting and culturing of autologous cells is costly and time-consuming, therefore single-stage procedure is highly preferable. In order to obtain a sufficient amount of cells in a single arthroscopic procedure without cell expansion, autologous meniscus cells from the meniscectomized tissue could be complemented with allogeneic MSCs[43,49]. Alternatively, incorporating growth factors within the scaffold could attract the patient's resident meniscus cells and MSCs present in the synovium and the meniscus[265,334,335]. The combination of growth factors present in platelet-rich plasma (PRP) and platelet lysate (PL) were shown to have a positive effect on migration and proliferation of meniscus cells and MSCs[336]. To date, the effect of the different growth factors on migration of meniscus cells and MSCs remains to be elucidated. Furthermore, the *in vivo* lifespan of growth factors is too short to sustain biological activity[337]. Thus, a method to secure an ongoing effect is necessary. Such method has recently been described by Crispim *et al.,* who immobilized growth factors on polycaprolactone (PCL) using a functionalization process for a growth factor binding peptide[338,339]. However, it is still unknown whether this functionalization can be used to attract meniscus cells and MSCs to the CMI®.

Therefore, this study aims to assess the effect of the anabolic growth factors 1) insulin-like growth factor-1 (IGF-1), 2) platelet-derived growth factor (PDGF), 3) vascular endothelial growth factor (VEGF), 4) transforming growth factor beta 1 (TGF-β1), 5) fibroblast growth factor (FGF) and PL on migration, proliferation, and extracellular matrix (ECM) production

of meniscus cells and MSCs. Moreover, it shows proof-of-concept of the functionalization of the CMI® with a growth factor binding peptide and assesses the effect of this functionalization. We hypothesize that these growth factors and PL accelerate meniscus regeneration by targeting the mechanisms mentioned above. Additionally, we hypothesize that by functionalization of the CMI® with a growth factor binding peptide, a continued effect of the targeted growth factor could be achieved.

Methods

Cell isolation

Human meniscus cells were isolated from menisci from patients who had undergone total knee arthroplasty. Collection of meniscus tissue was performed according to the Medical Ethics regulations of the University Medical Center Utrecht and the guideline "Human Tissue and Medical Research: Code of Conduct for responsible use" of the Dutch Federation of Medical Research Societies[261,262]. The menisci were washed in phosphate buffered saline (PBS) twice and manually cut into pieces of 2 mm. The tissue was digested in 0.15% collagenase type II (CLS-2; Worthington) in DMEM (Gibco, Life Technologies) with penicillin (100 U/mL; Gibco, Life Technologies) and streptomycin (100mg/mL; Gibco, Life Technologies) (1% pen/strep), at 37°C overnight. Meniscus cells were expanded in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% pen/strep and used at passage 2.
The use of human MSCs was approved by the institutional ethical review board (TCBio 08-001 and 18/739). MSCs were isolated from bone marrow aspirates obtained from three donors who provided written informed consent. MSCs were isolated and characterized as described previously[340], and expanded in αMEM supplemented with 10% FBS (HyClone), 0.2 mM l-ascorbic acid-2-phosphate (2% ASAP, Sigma-Aldrich), and 1% pen/strep for use at passage 3.

Growth factors and Platelet lysate medium

Human recombinant IGF-I (Sigma-Aldrich), human PDGF (Sigma-Aldrich), human recombinant FGF-basic (R&D Systems), human recombinant TGF-β1 (R&D systems), and human recombinant VEGF (Novus Biologicals) were diluted in concentrations of 10 ng/mL, 1 ng/mL, and 0.1 ng/mL in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) supplemented with 2% Albuman (human serum albumin 200 g/l; Sanquin), 2% ASAP, 2% Insulin-Transferrin-Selenium-X (ITSX, Invitrogen), and 1% pen/strep. For the preparation of PRP and PL, blood was obtained through the Mini Donor Service, a blood donation facility for research purposes approved by the medical ethics committee

of the University Medical Center Utrecht, Netherlands. All donors have provided written informed consent, in accordance with the declaration of Helsinki. Whole blood was collected in 3.2% sodium citrate-containing tubes and centrifuged at 250 G for 10 minutes. The pelleted erythrocytes were discarded and the top layer containing the platelets was centrifuged at 750 G for 10 minutes. The supernatant (plasma) was collected, and the pellet containing the platelets was suspended in 1/3 of the supernatant. PL was formed by freeze-thawing the suspension for three cycles (-80 ^{0}C to 37°C) in order to release the growth factors from the platelets, and centrifuged at 8000 G for 10 minutes. Upon use, the PL was diluted at 1% and 10% in DMEM supplemented with 2% Albuman, 2% ASAP, 2% ITSX, 1% pen/strep and 3.3 U/ml heparin. Using the CELL-DYN Emerald hematology analyzer (Abbott B.V., Abbott Park, Illinois), PRP of nine donors was characterized in terms of platelet, erythrocyte, and leucocyte concentration. Pooled PRP of at least three donors was used in the experiments.

Micro-wound assay

Both meniscus cells and MSCs (n=3) were seeded in monolayer and expanded up to 80% confluency in a 12-well plate. Cells were washed with PBS, a micro-wound was made by scratching over the cell monolayer with a 200µL pipette tip, and cell debris was aspirated after an additional wash with PBS. Growth factors and PL were diluted as mentioned above and supplemented with 10µM 5-ethylnyl-2'-deoxyuridine (EdU; Click-iT™ EdU Alexa Fluor® 488 Imaging Kit; Invitrogen) and added to the wells. At 0, 24 and 48 hours after scratching, six pictures were taken along the micro-wound using an inverted light microscope. Using Photoshop CS6 software (Adobe Systems), the pictures were merged, and an area of 17.708 by 48.697 pixels was cropped out at the same spot for every time point, and analyzed in ImageJ. The cells in the scratch were identified using color thresholding, and were calculated as percentage of the image at 0 hours after scratching. After 48 hours cells were washed with PBS, fixated in formaldehyde 4% (Klinipath), and permeabilized with PBS-Tween (PBST) 0.1%. Proliferated (EdU) and total cells (Hoechst) were visualized using the manufacturer's protocol using excitation and emission of 495/519 nm and 392/440 nm respectively (Thermo Fisher Scientific, Waltham, MA, USA). Three pictures were taken at different locations along length of the micro-wound using an EVOS FLoid™ Cell Imaging microscope, and analyzed via color thresholding and 'analyze particles' in ImageJ.

Transwell migration assay

Meniscus cells and MSCs were trypsinized and suspended in DMEM supplemented with 2% Albuman, 2% ASAP, 2% ITSX, and 1% pen/strep in a concentration of $5*10^{5}$ cells/mL.

450 µL of this cell suspension was added to the cell culture inserts (12 mm, polycarbonate, 8.0 µm; Merck Millipore) which were placed in a 24-well plate. 450 µL of growth factor, PL, or control medium was added to the wells of the 24-well plate. The plates were incubated for 4 hours at 37°C, before washing with PBS, and cleaning the upside of the polycarbonate membrane with a Q-tip to remove the remaining cells. Cells that were migrated through the membrane were fixated in formaldehyde 4%, and stained using Mayer's Hematoxylin. The polycarbonate membrane was cut out of the insert, mounted on a microscope slide, and migrated cells were counted using a light microscope.

Extracellular matrix formation

Meniscus cells were resuspended in a 1:15 diluted fibrinogen component of Tisseel fibrin glue (Baxter international Inc., IL USA) and combined with 1:50 diluted thrombin (Tisseel, Baxter international Inc., IL USA). The fibrin constructs consisting of $2.5*10^5$ cells in 100 µL were allowed to gelate for 15 minutes at 37°C. Afterwards, the constructs were put in a 48-well plate with 250 µL growth factor, PL, or control medium. The fibrin constructs were cultured for 28 days at 37°C with 5% CO_2, medium was changed three times per week and conditioned medium was stored at -20°C for analysis.

Functionalization of the Collagen Meniscus Implant for different growth factors

For functionalization of the CMI®, peptides with the sequence KGSWWAPFH (VEGF binding peptide) and KGSWWSSSH (scrambled peptide) were synthesized following Fmoc solid peptide synthesis procedures as described previously [338], purified and characterized with High-performance liquid chromatography (HPLC) and mass spectrometry. The CMIs® were incubated in 1mL 50mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH=5.2) containing 1mM of peptide during 1 hour at room temperature. After 1 hour, 1mL of MES containing 50mM of N-hydroxysuccinimide/ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (NHS/EDC) was added to the CMIs®. The reaction was carried out for 24 hours at room temperature. The functionalized CMIs® were washed three times with PBST (0.5%), and afterwards rinsed three times with PBS [341]. Using this method, five different conditions of functionalization were created to determine the quality of the functionalization. 1) CMI® + MES buffer, without VEGF, 2) CMI® + MES buffer, with VEGF, 3) CMI® + EDC/NHS and MES buffer and VEGF, 4) CMI® + EDC/NHS + Scrambled VEGF Peptide and VEGF, and 5) CMI® + EDC/NHS + VEGF binding peptide and VEGF.

After functionalizing the CMIs® for the VEGF peptide binding peptide, they were incubated with 1 µg/mL of VEGF (PeproTech) in PBST 0.5% for one hour with gentle agitation. Afterwards, the CMIs® were washed three times for 10 min with PBST 0.5% and PBS, and blocked for one hour with PBS containing 1% (w/v) bovine serum albumin (BSA) followed by the same washing steps. For imaging, CMIs® were incubated with a primary antibody (2 µg/mL; rabbit polyclonal anti-human VEGF, PeproTech) in the blocking solution for one hour with agitation. The CMIs® were washed as mentioned above and incubated with a secondary antibody (8 µg/mL; goat anti-rabbit Alexa Fluor 594, Invitrogen) in PBS containing 1% w/v BSA for one hour with gentle agitation. Before image acquisition with a fluorescence microscope, the CMIs were washed three times for 10 min with PBST and rinsed three times with PBS. Fluorescence intensity was quantified using ImageJ. PRP was used to coat the CMI®. First, 40µL of PRP was added to the CMI® slices, followed by 20µL of $CaCl_2$ and 20µL of thrombin (Tisseel, Baxter). The constructs were incubated for 15 minutes at 37°C to allow gelation of the PRP gels in the CMI®.

Cell migration into the functionalized Collagen Meniscus Implant

Cell migration into CMI® assays were conducted in four groups, 1) VEGF-functionalized; 2) scrambled peptide; 3) PRP-functionalized; and 4) control (non-functionalized). Meniscus cells and MSCs were trypsinized incubated for 1 hour at 37°C in lipophilic live cell membrane stain Vibrant CM-DiI. Fibrin constructs with $2.5*10^5$ cells (meniscus or MSCs) were formed as described above, which were seeded onto the outer rim of the cross-section of the four groups of CMIs®. The constructs were cultured for 7 days in DMEM supplemented with 2% Albuman, 2% ASAP, 2% ITSX, Invitrogen, and 1% pen/strep and medium was changed every other day.

Confocal microscopy imaging

After 7 days of culture, the CMIs® were washed three times for 10 min with PBST and rinsed three times with PBS to remove the unattached cells. The CMI® was counterstained with DAPI for 4 minutes the CMIs® were imaged using confocal microscopy (Leica SP8X) using excitation at 358nm and 549nm. Images of the two channels were merged using ImageJ and color threshold was applied to select cell area and collagen area. Selected cell area and CMI® collagen fibers area were used to calculate the total area with cells per collagen fibers.

VEGF retention

For analyzation of VEGF retention by the CMI® and release into the medium, CMIs® were incubated with 100ng VEGF (Novus Biologicals), washed six times in PBST and PBS and

subsequently cultured for seven days in DMEM supplemented with 2% Albuman, 2% ASAP, 2% ITSX, Invitrogen, and 1% pen/strep. Culture medium was changed every other day. VEGF concentrations in the media and washing fluid were determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Duoset ELISA kit, R&D Systems).

Biochemical analyses

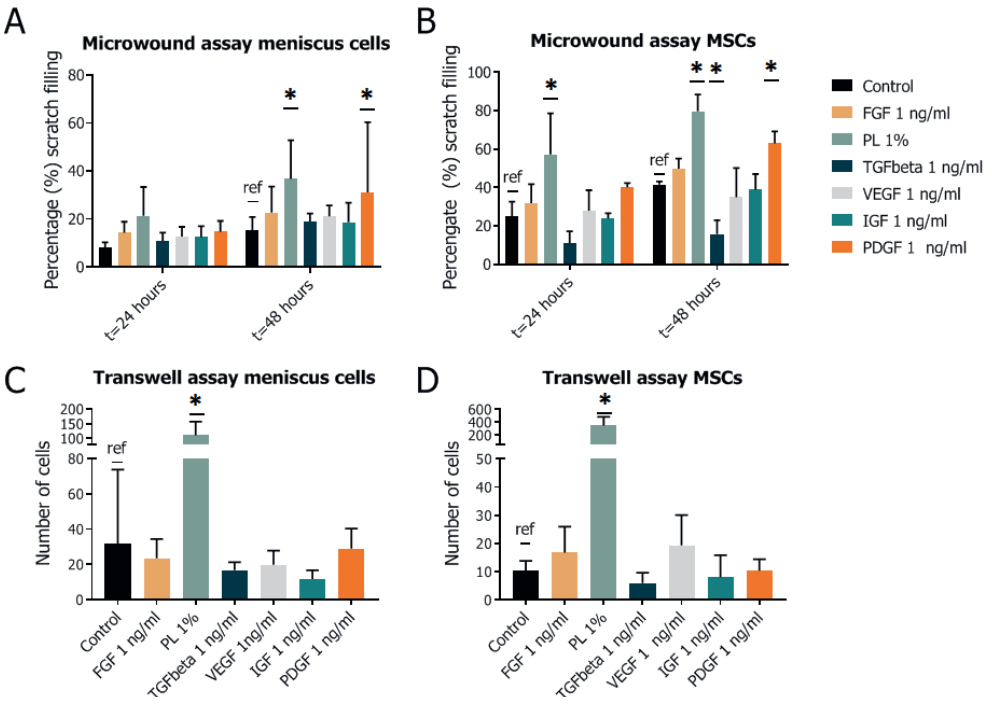
Biochemical analyses were performed on fibrin constructs cultured for assessment of ECM formation and the functionalized CMIs®. After culturing, the fibrin constructs and functionalized CMIs® were digested at 60^{0}C overnight in papain buffer (250 µg/mL papain (Sigma-Aldrich), 0.2 M Na2EDTA, 0.1 M NaAc, and 0.01 M cysteine). The PicoGreen® dsDNA quantitation assay was used according to the manufacturer's instructions to determine the DNA content of the constructs. Excitation was set at 480nm, emission 520nm and λDNA was used as a standard reference. Glycosaminoglycan (GAG) content was determined using dimethylmethylene blue (DMMB) assay. Chondroitin sulphate (Sigma-Aldrich) was used as standard and absorbance measured at 525 nm and 595 nm.

Papain samples were both freeze-dried and hydrolyzed overnight at 108°C for determining collagen content using a hydroxyproline assay. Chloramine-T (Merck) and Dimethylaminobenzoaldehyde (Merck 3058) were added, and hydroxyproline (Merck 104506.0010) was used as standard to measure the hydroxyproline content at 570 nm. Collagen content was calculated from the hydroxyproline content, since 13.5% of collagen is composed of hydroxyproline[342].

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Data are presented as mean±SD. A two-way analysis of variance (ANOVA) and the Dunnett post hoc test were performed to determine significant differences between all growth factor or PL groups and the control, and the interactive effect of donor variability was taken into account.

Confocal images for cell ingrowth into the CMI® were analyzed using ImageJ. Student T-test was used to assess the significance level of difference between VEGF-functionalized groups and scrambled peptide groups; and PRP-functionalized group with non-functionalized group. ANOVA was used to assess the difference between time points. P-values < 0.05 were considered statistically significant.



| Figure 1. Migration of meniscus cells and mesenchymal stroma cells (MSCs) in the micro-wound assay (A and B) and the transwell assay (C and D) using fibroblast growth factor (FGF), platelet lysate (PL), transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF). *, p<0.05

Results

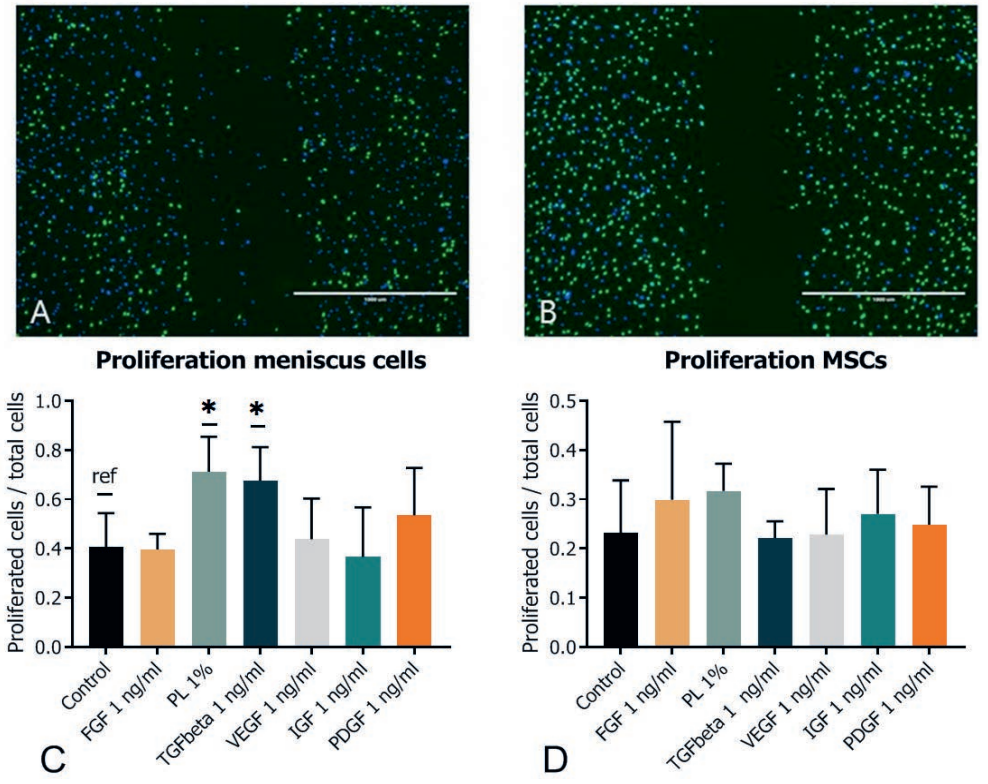
PRP characterization

Compared to whole blood, PRP contained 1.5 times concentrated platelets (298.5±166.3*10^9/L). PRP contained 1.2±0.9*10^9/L leucocytes and 23.3±12.2*10^9/L erythrocytes.

PDGF and Platelet Lysate increase migration of meniscus cells and MSCs

Micro-wound

Filling of the micro-wound by meniscus cells in medium supplemented with 10.0, 1.0, and 0.1 ng/mL of growth factors or 1 or 10% of PL was evaluated at 24 and 48 hours. At 24 hours the wound filling in the 10% PL conditions was significantly higher (p<0.0001) compared to the control group (Supplementary figure 1). At 48 hours, 1% PL (36.7±16.1% filling) and 1 ng/mL PDGF (31.1±29.2% filling) significantly increased wound filling



| Figure 2. Proliferation of meniscus cells and mesenchymal stromal cells (MSCs) using fibroblast growth factor (FGF), platelet lysate (PL), transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF) in the micro-wound assay, demonstrated by 5-ethynyl-2'-deoxyuridine (EdU) assay. Proliferated cells shown in green by the EdU staining and the non-proliferated cells are stained blue using Hoechst. An example from the control (A) compared to TGF-β1 group after 48 hours (B). Proliferated cells/total cells for meniscus cells (C) and MSCs (D). **, p<0.05*

compared to the control (15.3±5.6%). Wound filling of VEGF and FGF did not reach statistical significance (Fig. 1A). In general, at 48 hours the presence of growth factors at a concentration of 1 ng/ml led to more wound filling compared to the other concentrations (supplementary figure 1). Therefore, all other experiments were continued with a growth factor concentration of 1 ng/mL and 1% PL. Compared to the meniscus cells, wound filling was more extensive in the MSCs. 1% PL significantly increased wound filling of MSCs compared to the control (57.2±21.6% compared to 24.0±2.7%) at 24 hours. At 48 hours, both 1% PL and PDGF showed an increased filling of the scratch with 79.7±8.8%, and 62.9±6.4%, compared to 41.5±1.6% for the control. TGF-β1 decreased wound filling

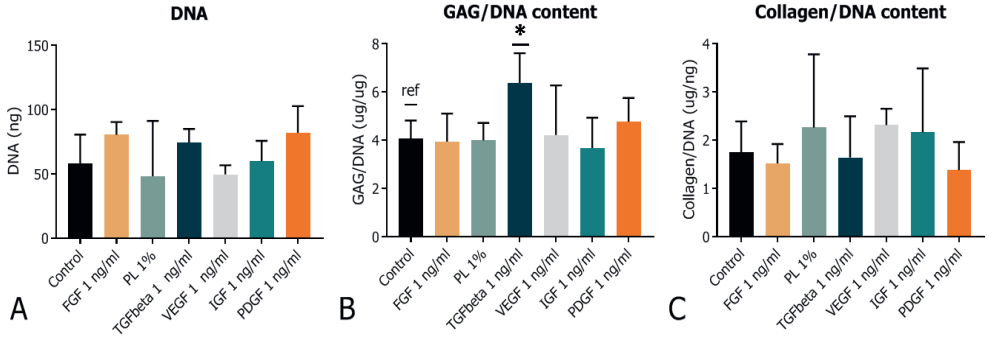
compared to the control at 48 hours, with only 15.7±7.3% coverage of the scratch. Other GFs did not increase or decrease wound filling (Fig. 1B).

Transwell migration

PL significantly increased migration of both meniscus cells and MSCs in the transwell migration assay. For meniscus cells 1% PL significantly increased the number of migrated cells from 12±9 to 111±46 (Fig. 1 C). For MSCs the number of migrated cells in the control group was 10±4 compared to 346±137 in the 1% PL group (p-value <0.01) (Fig. 1D). PDGF and FGF did not significantly increase meniscus cell migration and VEGF and FGF did not increase migration in MSCs.

TGF-β1 and Platelet Lysate increased proliferation of meniscus cells

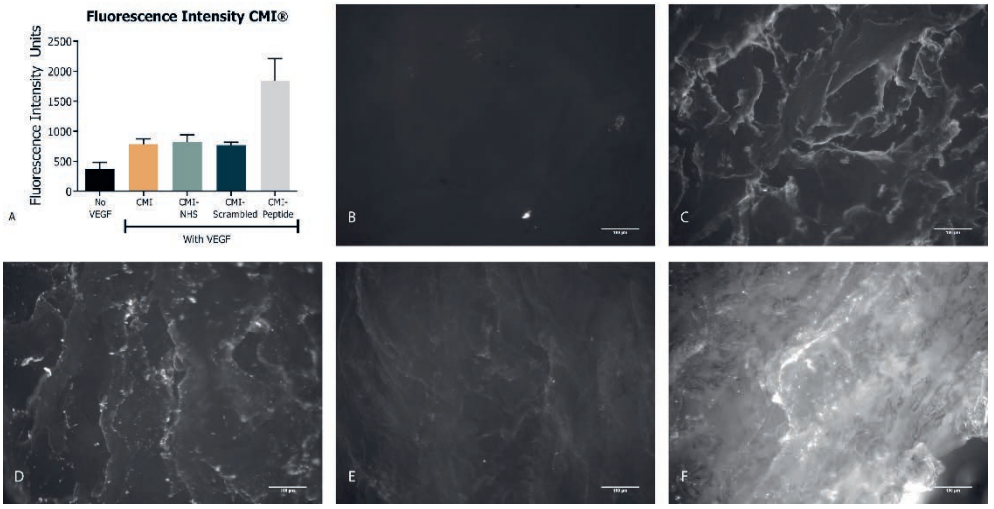
By labelling the proliferated cells with EdU in the micro-wound assay, the ratio of proliferated cells/total amount of cells (green/blue ratio) at 48 hours was calculated (Fig. 2A and B). For meniscus cells, the control group showed a ratio of 0.41±0.14, which was significantly lower than the 0.71± 0.14 ratio of PL and 0.68±14 in TGF-β1. The proliferation was not significantly higher in the PDGF-group (0.54±0.19) compared to the control group (Fig. 2C). Overall, MSCs showed a lower proliferation ratio compared to the meniscus cells. Besides, none of the growth factors or PL significantly increased the proliferation of MSCs after 48 hours (Fig. 2D).



| Figure 3. Biochemical analysis after 28 days of culturing meniscus cells in fibrin glue constructs with the addition of fibroblast growth factor (FGF), platelet lysate (PL), transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF). Figure shows DNA content (A), glycosaminoglycan content normalized for DNA (GAG/DNA) (B), and collagen content normalized for DNA (C). *, p<0.05

TGF-β1 stimulates production of extracellular matrix of meniscus cells

The DNA content of the different constructs did not differ significantly after 28 days of culture, however there was a trend towards a higher DNA content in FGF, TGF-β1, and PDGF compared to the control group (Fig. 3A). TGF-β1 significantly increased formation of GAGs in the fibrin glue constructs (Fig. 3B). There was no significant effect of any of the growth factors or PL on the production of collagen (Fig. 3C).

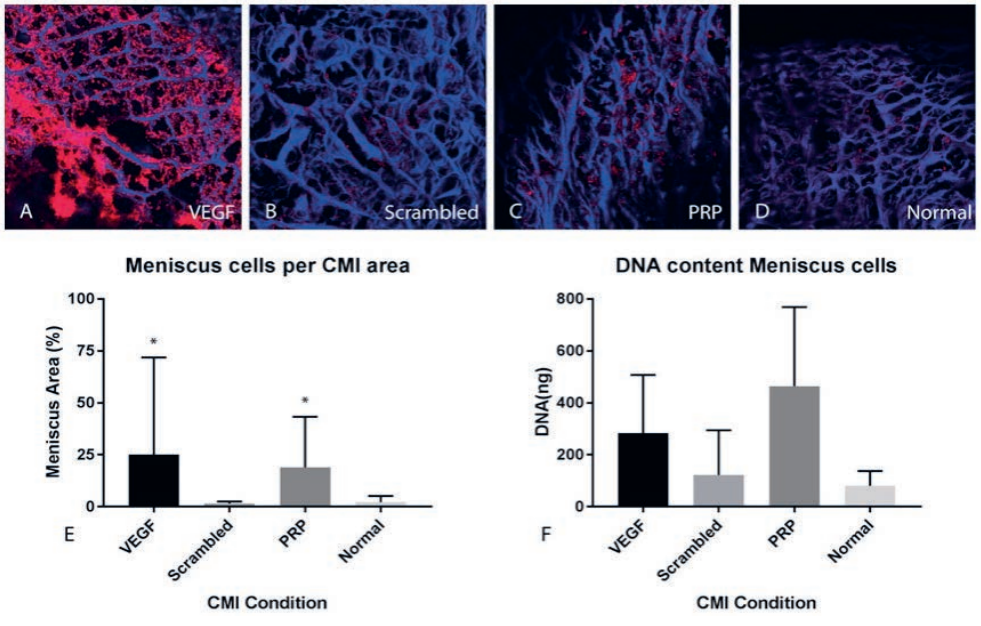


| Figure 4. Immobilization of vascular endothelial growth factor (VEGF) on functionalized Collagen Meniscus Implants (CMI®) using a VEGF binding peptide (n=8). Quantified fluorescence intensity (A). Example images of: CMI® with MES buffer without VEGF (B), CMI® with MES buffer and VEGF (C), CMI® with EDC/NHS, MES buffer and VEGF (D), CMI® with EDC/NHS, a scrambled VEGF peptide and VEGF (E), and CMI® with EDC/NHS, VEGF binding peptide and VEGF (F).

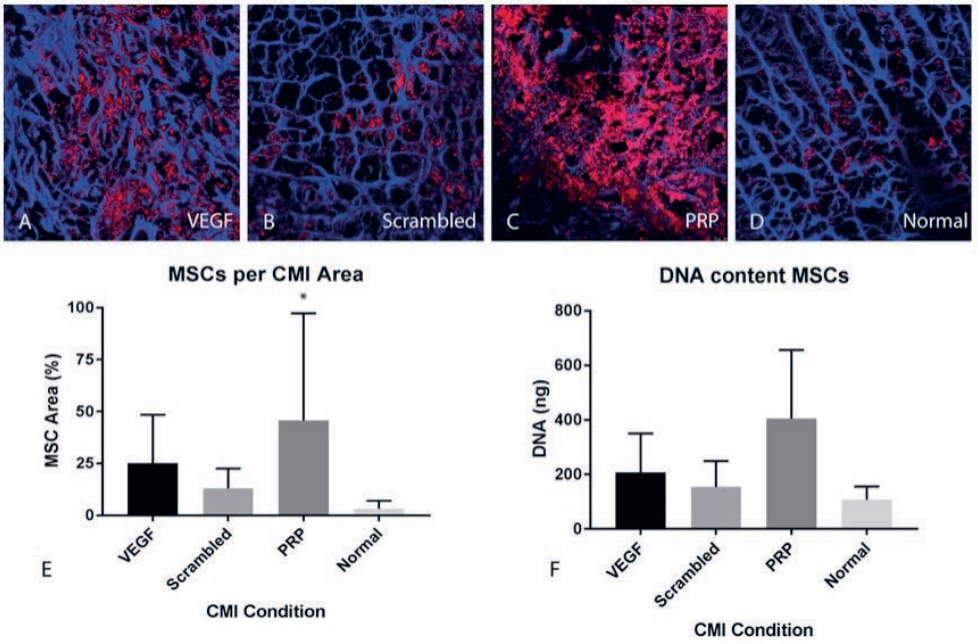
Functionalization of the Collagen Meniscus Implant increases the cell ingrowth

The CMI® was successfully functionalized with VEGF binding peptide. Figure 4 shows fluorescence microscopy images of the five different groups of functionalized CMI, with fluorescently labeled VEGF bound to the CMI®. Compared to the four other groups (Fig. 4B – E), the CMI® functionalized with the VEGF binding peptide (Fig. 4F and supplementary figure 2) showed significantly higher fluorescence intensity units. Moreover, VEGF binding was 94.8±1.4% in the CMI® functionalized for VEGF, compared to almost no binding in the CMI® functionalized for scrambled peptide (supplementary figure 3A). VEGF release in medium was lower in the CMI® functionalized for VEGF, than the CMI® functionalized for scrambled peptide and plain CMI® (supplementary figure 3B). In the migration assay with constructs of meniscus cells in fibrin glue, significantly

more meniscus cells were present in the CMIs® functionalized for VEGF and coated with PRP after seven days. In VEGF functionalized groups, the meniscus cells were aligned well along the CMI® fibers, and showed cell aggregates in the higher cell density areas (Fig. 5A). In PRP groups, cells were situated along fibers and the space between fibers filled with PRP gels, compared to round cells not aligned along the fibers in the scrambled and negative control group (Fig. 5B-D). Similar effects were seen for MSCs (Fig. 6A-D). Comparison of the area of meniscus cells and MSCs standardized for area of CMI® collagen fibers between conditions are shown in Fig. 5E and 6E. A significant difference can be observed between VEGF-functionalized group and scrambled-peptide functionalized group for meniscus cells migration. A significant difference between PRP-coated group and negative control was also observed in both meniscus cells and MSCs migration. The density of MSCs in the PRP-functionalized group did not differ significantly compared to VEGF-functionalized group. DNA quantification after papain digestion showed results in accordance with the confocal pictures and analysis of the images. The highest cell amounts were seen in the PRP and VEGF group, followed by the scrambled peptide and the negative control (Fig. 5F and 6F), although the differences did not reach statistical significance.



| Figure 5. Cell migration of meniscus cells into the Collagen Meniscus Implant (CMI®) (n=6). The CMI® is stained with DAPI (blue) and meniscus cells with DiI (red). The figure shows CMI® with EDC/NHS, VEGF peptide and VEGF (A), and CMI® with EDC/NHS, a scrambled VEGF binding peptide and VEGF (B), CMI® coated with PRP (C) and CMI® (D). E shows percentage of meniscus cells per CMI® area. F shows DNA quantification in the whole constructs. *, $p<0.05$



| Figure 6. Cell migration of mesenchymal stromal cells (MSCs) into the Collagen Meniscus Implant (CMI®) (n=6). The CMI® is stained with DAPI (blue) and MSCs with DiI (red) (A – D). The figure shows CMI® with EDC/NHS, VEGF peptide and VEGF (A), and CMI® with EDC/NHS, a scrambled VEGF binding peptide and VEGF (B), CMI® coated with PRP (C) and CMI® (D). E shows percentage of meniscus cells per CMI® area. F shows DNA quantification in the whole constructs. *, $p<0.05$

Discussion

Regeneration or replacement of the meniscus can potentially prevent or delay onset of osteoarthritis after meniscectomy. In the current study, we explored the potential of several growth factors and PRP/PL for stimulating the migration, proliferation and ECM formation of meniscus cells and MSCs. Moreover, we demonstrated proof-of-concept of a technique to capture and immobilize growth factors to a clinically available meniscus implant for the purpose of increased regeneration after meniscus replacement.

We based the choice of growth factors and concentrations on previous literature on meniscus cells, cartilage and chondrocytes[343–345]. As a wide range of dose-dependent concentrations of growth factors is given in literature[343,346], the concentration we used was based on results of a pilot study (supplementary figure 1) with concentrations of 0.1 ng/mL, 1 ng/mL, and 10 ng/mL for the growth factors, and concentrations 0.1%, 1%, and 10% for PL. We were reluctant to use high growth factor concentrations, as overdosing is a general concern for regenerative therapies[347].

In the transwell and micro-wound assay, migration of both meniscus cells and MSCs was increased by PL and PDGF. The effect of PL on migration of meniscus cell was similar to

the effect described by Ishida *et al.*, who demonstrated that migration of meniscus cells and synovial MSCs into a biodegradable gelatin hydrogel increased by addition of PRP[337]. Similarly, migration into a decellularized meniscus increased by PDGF-BB bound to the scaffold [348] and transwell migration of murine MSCs increased by PDGF-AA[349]. Here, migration of MSCs in the transwell assay decreased by TGF-β1. This decreased migration could be attributed to the stimulatory effect of TGF-β1 for chondrogenic differentiation of MSCs, which might in turn inhibit migration and proliferation. However, migration in a transwell assay in murine MSCs did increase by TGF-β1[350] at a concentration of 100ng/mL, which might indicate a dose dependent effect. Additionally, in the microwound assay, proliferation of meniscus cells increased by PL and TGF-β1. However, TGF-β1 had no significant effect on proliferation in the study by Riera et al.[351], which could be explained by the fact that they used pig cells, which might respond different to human recombinant TGF-β1 than the human cells that were used here. The effect of TGF-β1 should be further elucidated in order to clear up these controversies. Interestingly, proliferation of MSCs was not stimulated by any of the growth factors, although VEGF, PDGF, TGF-β1 and FGF were previously reported to increase proliferation[352]. These differences could indicate a dose-dependent effect of the growth factors. In our study, the proliferation rate of MSCs was lower compared to meniscus cells, as assessed by the proportion of cells that have proliferated at least once. It has been described that in MSCs a small proportion of the cells divides rapidly and is responsible for the high proliferation rate. ECM formation significantly increased by the addition of TGF-β1 in the medium of meniscus cells. Indeed, increased proteoglycan synthesis of meniscus cells on scaffolds in presence of TGF-β1 and FBS have been described earlier[353]. Matrix formation was not stimulated by PL in our cultures, similar to the deleterious effect of PL addition during the redifferentiation phase in cartilage[354]. The stimulatory effects of PL, PDGF and TGF-β1 on migration, proliferation and/or ECM formation, identify these growth factors (substrates) as potential target for meniscus regeneration. Targeting migration, proliferation and ECM production at the same time by different growth factor holds great potential, as this could work as a catalyst. Indeed, the addition of PDGF to TGF-β1 led to a 3-fold increase in collagen production compared to the use of TGF-β1 alone[355]. The combined effect of these growth factors should be further investigated.

In the current research, proof-of-concept of functionalization of the CMI® with growth factor binding peptides was demonstrated. Functionalization with a PDGF binding peptide holds promise as shown in the migration and proliferation assays. However, PDGF has three different isoforms (AA, AB and BB) affecting different PDGF receptors[356], which makes it unfavorable to use PDGF in this proof-of-concept study. PDGF and VEGF family members are closely related[357], therefore functionalization with a VEGF capturing

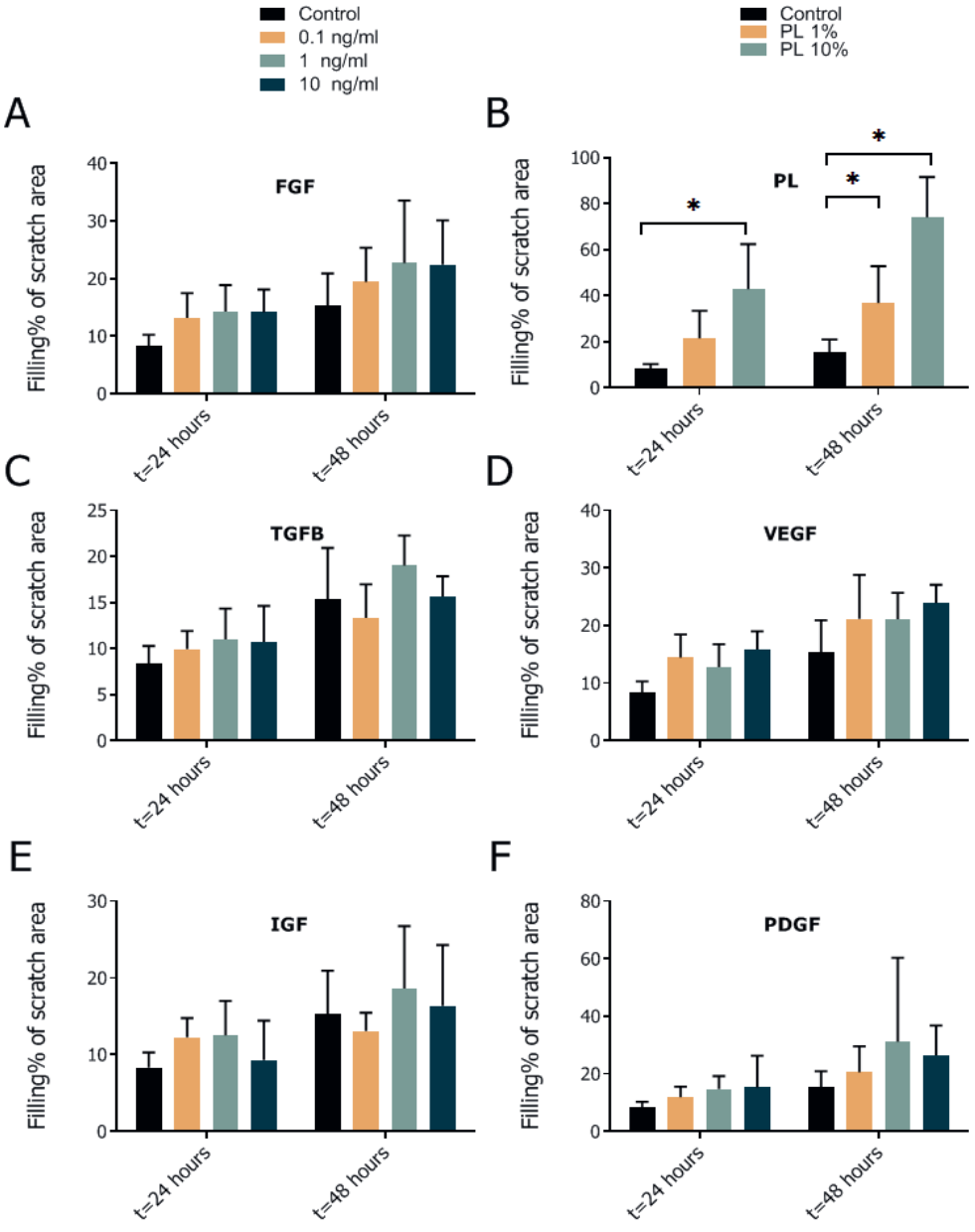
peptide was chosen for this proof-of-principle study. Peptides for VEGF binding demonstrate that a variety of growth factors can be used for functionalization. Due to the stimulatory effect of PL on migration and proliferation, a PRP-coated CMI® was also as a proof-of-concept. Both the CMI® functionalized for VEGF and the CMI® coated with PRP resulted in a higher cell density inside the CMI® after seven days of culture compared to the negative control. The role of PRP for regenerative strategies in cartilaginous tissues remains controversial, as PRP seems to primarily increase proliferation, while at the same time decreasing differentiation[354]. *In vivo*, PRP could provide an initial boost for proliferation, and after the rapid decline in growth factors concentration due to the short half-life, redifferentiation and ECM formation could occur. The CMI® functionalized for VEGF and the CMI® coated with PRP show high potential for clinical translation by the attraction of endogenous growth factors present in the knee joint without injecting additional growth factors.

There are limitations of the current study design. First, the effect of single growth factors was examined in this study, whereas functionalization of the CMI® with multiple growth factor binding peptides is possible, and therefore combinations of two or more growth factors should be further investigated. Based on the results in this study, the potential effect of PDGF in combination with TGF-β1 should be further explored. Secondly, PL and PRP contain a mix of growth factors, and the effect of PRP and PL can therefore be attributed to an additional effect of the growth factors present. More insight into the effects of PRP and PL could in the future be obtained by testing a 'synthetic PRP' using a combination of growth factors in their concentrations as present in the PRP. Thirdly, the effect of the functionalization on mechanical properties of the CMI® was not investigated. However, we do not expect functionalization to have a major impact on the mechanical properties in the short term, as the collagen network in the CMI® is not disrupted by the functionalization process. In the long term, we expect of positive effect of the functionalization due to improved tissue formation, which should be confirmed *in vivo*. Moreover, expect no decrease in quality of functionalization upon implantation as there was no negative effect of mechanical stress on the functionalization of bioactive tape or polycaprolactone for other growth factors *in vivo*[338,339]. Additionally, the stability of the functionalization is based on highly stable amide bond[358] and amidation and acetylation of the peptides which makes them resistant to proteolytic and enzymatic degradation[359]. Lastly, a disadvantage of the CMI® is that does not restore the exact composition, morphology and mechanical characteristics of the native meniscus, but it is one of the few implants that is currently available in the clinic. Functionalization of the CMI® might lead to formation of native meniscus tissue and overcome these disadvantages.

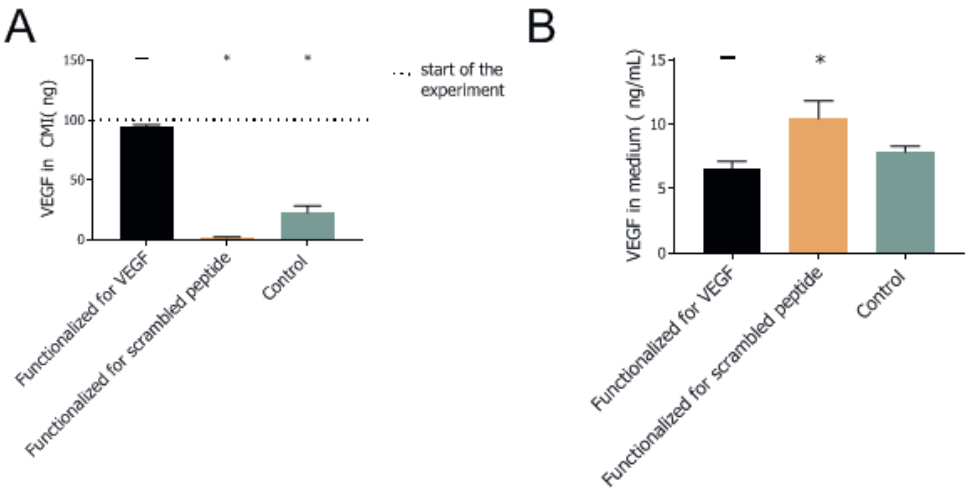
Conclusion and implications

In conclusion, this study demonstrated stimulation of migration, proliferation and/or ECM production for meniscus cells and MSCs using PDGF, TGF-β1 and PL. Additionally, the CMI® was successfully functionalized with a VEGF binding peptide and PRP which led to increased meniscus cell and MSC migration into the meniscus implant. Therefore, the results of this study demonstrate feasibility of functionalization of the CMI® with growth factor binding peptides or PRP for enhancement meniscus regeneration after partial meniscectomy.

Appendix



| Figure S1. Migration of meniscus cells in the microwound assay with (A) fibroblast growth factor (FGF), (B) platelet lysate (PL), (C) transforming growth factor beta 1 (TGFB), (D) vascular endothelial growth factor (VEGF), (E) insulin-like growth factor-1 (IGF), and (F) platelet-derived growth factor (PDGF). *, $p<0.05$



| Figure S2. Vascular Endothelial Growth Factor (VEGF) binding in the collagen meniscus implant (CMI®), after incubating the CMI® with 100ng VEGF (A). VEGF released into the medium in 7 days culture (B).

09

Potential of melt electrowritten scaffolds seeded with meniscus cells and mesenchymal stromal cells

Korpershoek JV, Ruijter M, Terhaard BF, Hagmeijer MH, Saris DBF, Castilho M, Malda J, Vonk LA. Potential of melt electrowritten scaffolds seeded with meniscus cells and mesenchymal stromal cells. Int J Mol Sci. 2021 Oct 18;22(20)

Abstract

Background

Meniscus injury and meniscectomy are strongly related to osteoarthritis, thus there is a clinical need for meniscus replacement. The purpose of this study is to create a meniscus scaffold with micro-scale circumferential and radial fibres suitable for a one-stage cell-based treatment.

Methods

Poly-caprolactone-based scaffolds with three different architectures were made using melt electrowriting (MEW) technology and their in vitro performance was compared with scaffolds made using fused-deposition modelling (FDM) and with the clinically used Collagen Meniscus Implants® (CMI®). The scaffolds were seeded with meniscus and mesenchymal stromal cells (MSCs) in fibrin gel and cultured for 28 days.

Results

A basal level of proteoglycan production was demonstrated in MEW scaffolds, the CMI®, and fibrin gel control, yet within the FDM scaffolds less proteoglycan production was observed. Compressive properties were assessed under uni-axial confined compression after 1 and 28 days of culture. The MEW scaffolds showed a higher Young's modulus when compared to the CMI® scaffolds and a higher yield point compared to FDM scaffolds.

Conclusion

This study demonstrates the feasibility of creating a wedge-shaped meniscus scaffold with MEW using medical-grade materials, and seeding the scaffold with a clinically-feasible cell number and -type for potential translation as a one-stage treatment.

Introduction

The human meniscus is a fibrocartilaginous tissue in the knee that shows a distinct architecture with an inner zone, composed of hyaline cartilage-like tissue, and an outer zone with a more fibrous phenotype[4,360]. It plays a crucial role in load transmission in the knee due to an organized network of circumferential and radial collagen fibres[6,360,361]. Meniscus injury is highly disabling and affects young, active patients, as well as the elderly. The regenerative capacity of the meniscus is limited to the vascular zone and declines with aging. Therefore, successful surgical repair of meniscal tears is limited to the vascularized region and to young patients[7,171]. Because roughly 66% of all meniscus tears are irreparable[10], treatment often involves meniscectomy, *i.e.*, the removal of the damaged part of the meniscus. Meniscectomy relieves symptoms in the short-term, but is related to a high risk of developing osteoarthritis due to loss of contact area between the long bones and altered load bearing[11,169,174]. Current strategies for replacement of the meniscus have important drawbacks. Transplantation of meniscus allografts is costly and highly regulated in the European Union[13]. It requires complex logistics as donor availability is limited, and high-level evidence on long-term effectiveness is lacking[362,363]. The Collagen Meniscus Implant (CMI®; Stryker, Kalamazoo, USA), a clinically available implant composed of bovine type 1 collagen, offers short term clinical improvement, yet tissue deposition is limited in the long-term[14]. Moreover, the CMI® does not account for the zonal organization and direction of collagen fibres in the meniscus. The clinical need for a mechanically competent meniscus implant is therefore unmet. Ideally, such implant should allow for sufficient dampening and load transfer while being able to remodel to the joint *in vivo*. In order to improve performance in the long-term and reactiveness to the joint environment, it should be biocompatible with an optimal pore size and pore interconnectivity to achieve cell infiltration and tissue ingrowth[364]. Pre-seeding a scaffold with cells could stimulate tissue formation and thereby enhance the long-term performance of a meniscus scaffold.

A potential solution to the limited mechanical properties of current implants, such as the CMI® or ACTIfit (Orteq® Sports Medicine Ltd New York, NY, USA), could lie in mimicking the collagen fibre architecture of native meniscus tissue. Recent developments on additive manufacturing technologies, or more specifically, fibre deposition technologies seem promising for mimicking the complexity at native tissue resolution. Recent applications of such technologies for the fabrication of meniscus scaffolds focus on achieving a strong fused deposition modelling (FDM) polymeric framework that can be combined with previously proven hydrogel biomaterials and cells and other bioactive moieties[365,366]. Polymeric fibres that are produced by FDM (fibre diameter within the hundreds of micrometer scale) are generally stiffer as compared to thinner sub-

micrometer scale fibres produced by other fibre deposition technologies such as melt electrowriting (MEW) [367]. Limitations of using a stiff supporting framework with large fibre diameters, such as done with FDM, include limited load transfer to seeded cells which consequently compromise their mechano-regulated differentiation and neo-tissue deposition . Additionally, the polymeric fibres resulting from FDM may lead to damage on the opposing articulating cartilage surfaces due their size and stiffness. Solution electrospinning can mimic the (nano-sized) fibres of native meniscus tissue, but this technology generally uses toxic solvents and does not allow controlled fibre deposition, necessitating the addition of thicker FDM-based support fibres to obtain the aligned fibre architecture[368]. For other tissues such as articular cartilage and heart muscle, controlled and aligned fibres were previously deposited using MEW to mechanically reinforce cell-laden hydrogels[367,369]. Next to this reinforcing effect, MEW fibres are made using of medical grade polymers, can be deposited with high reproducibility, allow for sufficient pore interconnectivity, and have a less rough and / or stiff surface as compared to FDM fibres due to the micro-scale of the fibres. Therefore, MEW provides potential to recapitulate the fibre architecture of native meniscus tissue, while allowing space for the cells to produce meniscus-like tissue. Such MEW scaffolds can then be used for testing or potentially implantation purposes.

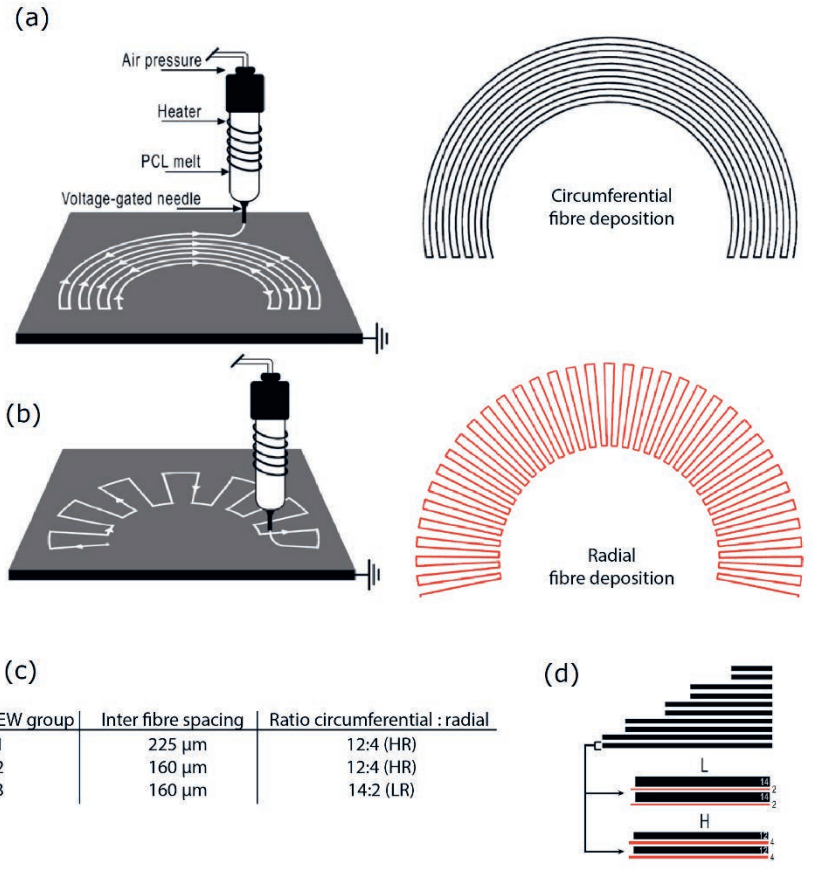
In order to facilitate clinical translation of a pre-seeded scaffold, the number of autologous cells should not exceed the number that can be harvested during a single surgical procedure. A sufficient number of cells/stimuli for tissue formation can be achieved by combining recycled autologous meniscus cells isolated from the meniscectomized tissue with allogeneic mesenchymal stromal cells (MSCs). The feasibility of using these cell combinations was already shown in a human clinical trial for cartilage defects[43], and in an *in vitro* experiment for the meniscus[49]. Using a combination of off-the-shelf allogeneic MSC and autologous meniscus cells that are harvested during the surgery allows for implantation in a one-stage procedure, thus limiting patient burden and costs of treatment[370,371].

In this study, we demonstrate feasibility of fabrication of a wedge-shaped meniscus scaffold with circumferential and radial fibres, made from medical grade materials with MEW. We used a combination of meniscus cells and MSCs to seed the scaffold with a clinically feasible cell-source and number for one-stage meniscus replacement. Compressive properties were assessed under confined uniaxial loading and proteoglycan production was assessed after 28 days of culture.

Methods

Scaffold design and printing

Scaffold design was based on native meniscus fibre architecture using micro-meter scale fibres in a circumferential (Figure 1A) and radial (Figure 1B) direction using MEW. These two different layers were deposited with a programmed inter fibre spacing of 225 µm or 160 µm (Figure 1C) and the ratio of circumferential : radial fibres was 14 : 2 (low radial, LR) or 12 : 4 (high radial, HR) (Figure 6C,D). As for high throughput testing, the meniscus scaffolds were scaled down a factor 4 to fit into 24 well culture plates.
MEW was performed with polycaprolactone (PCL, PURASORB, Corbion, The Netherlands) at 90°C, a collector distance of 5 mm, collector velocity of 10 mm/s, voltage of 9 kV, at a pressure of 0,118 MPa (3D Discovery, regenHU, Switzerland). Printability was assessed by measuring the fibre diameter and inter fibre spacing along the circumferential and radial lengths of the prints. These measurements were performed on images taken with scanning electron microscope (SEM, Phenom Pro Desktop SEM, Thermo Fischer Scientific, USA) by using Fiji software (ImageJ, version 2.0.0-rc-54/1.51h). SEM was performed with an accelerating voltage of 10 kV to image the MEW fibres. Prior to imaging, samples were coated with 2 nm of gold to improve imaging quality. Homogeneity of the fibre diameter was assessed by the standard deviation and the measured inter fibre spacing was compared to the programmed inter fibre spacing. To assess if the ratio of circumferential : radial fibres was achieved, SEM imaging was used with the same parameters as for the fibre measurements. FDM scaffolds were made from PCL with screw-driven extrusion at 3 rev/min, an air pressure of 0.125 MPa, and a collector velocity of 2mm/s at a temperature of 80°C (3D Discovery, regenHU, Switzerland).



MEW group	Inter fibre spacing	Ratio circumferential : radial
1	225 µm	12:4 (HR)
2	160 µm	12:4 (HR)
3	160 µm	14:2 (LR)

| Figure 1. Scaffold design inspired by native fibre architecture. (**a**) Printhead trajectory of circumferential fibres. (**b**) Printhead trajectory of radial fibres. (**c**) Variables in design include variety in inter fibre spacing and in the ratio between the circumferential and radial fibres. (**d**) Illustration of variety in design of the ratio of circumferential and radial fibres. *Abbreviations: HR: high ratio of radial fibres, LR: low ratio of radial fibres, PCL: polycaprolactone*

Cell isolation and culturing

Primary human meniscus cells were isolated from osteoarthritic menisci obtained after total knee arthroplasty from 3 female donors (62-81 years old). The tissue was handled anonymously according to the guidelines of the Federation of Dutch Medical Scientific Societies[261] and as approved by the ethical review board of the University Medical Center Utrecht. Briefly, menisci were cut into 1-2 mm cubical pieces and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco, The Netherlands) with 0.2% pronase (Roche Diagnostics GmbH, Mannheim, Germany), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco) (1% p/s) at 37°C for 2 hours followed by a digestion in DMEM with

0.075% collagenase type 2 (Wortington Biochemical Corporation, Lakewood, NJ, USA), 1% p/s, and 10% heat-inactivated Fetal Bovine Serum (FBS; Biowest) at 37°C. The digested tissue was run over a 70 µm strainer (Greiner Bio-One International GmbH) to remove debris, after which meniscus cells were cultured up to passage 2 in DMEM with 1% p/s and 10% FBS. The use of human MSCs was approved by the institutional ethical review board (TCBio 08-001 and 18/739). MSCs were obtained from bone marrow aspirates from 4 patients (male and female, age 35-71) undergoing hip replacement or spinal surgery after written informed consent was obtained. Briefly, bone marrow aspirate was Ficoll separated and MSCs were expanded up to passage 4-5 in αMEM (minimal essential medium, Gibco) with 10% FBS, 1% 20 mM l-ascorbic acid-2-phospate (1% ASAP; Sigma-Aldrich), and 1% p/s.

Scaffold and CMI® preparation

CMIs® were reduced to the same dimensions of the MEW scaffolds using a cutting guide. Prior to seeding the downscaled CMIs® with cells, they were treated with 1% p/s and 50µg/ml gentamicin in PBS for 7 days and dried overnight. Scaffolds were treated with 1M NaOH in H_2O to increase hydrophilicity and improve immersion of the scaffolds with fibrin gel.

Seeding of the scaffolds

Tisseel fibrin gel (Tisseel, Baxter BV) was used in a 1:50 dilution of the thrombin component (=10 IU thrombin/mL with 8 µmol/mL calcium chloride) and a 1:15 dilution of the fibrinogen component (= 5-8 mg fibrinogen, 1-3 IU/mL factor XIII with 20o KIU/mL aprotinin). Interconnectivity of the fibrin glue fibers[372] was not measured in the current study. MSCs and meniscus cells were mixed in a 20:80 ratio in the of fibrinogen in PBS. The CMIs® and MEW scaffolds were placed in a seeding mold, after which 30 µl fibrinogen solution containing a total of $1.5x10^5$ cells was added. Thrombin was added and the fibrin gel was allowed to gelate for 20 minutes at 37°C. The seeded scaffolds were cultured at 37°C/5% CO_2 for 28 days in DMEM with 1% p/s, 2% Albuman (Human Serum Albumin, 200g/l; Sanquin Blood Supply Foundation, the Netherlands), 2% insulin-transferrin-selenium-ethanolamine (ITS-X; Gibco), and 1% ASAP, with medium changes twice a week. Low attachment plates suspension plates (Greiner Bio-One) were used to prevent attachment of released cells to the bottom. Therefore, the amount of DNA released in the culture medium could be used as a measure of cell retention in the scaffold. DNA content after seeding and DNA release in the first week after seeding was quantified using the Quant-iT PicoGreen kit (Invitrogen) according to the manufacturer's protocol, with excitation at 485 nm and emission at 535 using the Fluoroskan Ascent

(Thermo Scientific) for 3 technical replicates (1 donor combination). Cell distribution in the scaffold was visualized on a thunder microscope (Leica) after staining the cells with 10 µM calcein-AM (Molecular Probes) for 30 minutes at 37°C.

Mechanical analysis

The mechanical properties were analysed using confined compression of the scaffolds with an aluminium custom-made loading head in the shape of the scaffolds on a Dynamical Mechanical Analyser (DMA, Q800, T.A. Instruments, New Castle, DE, USA) (Supplementary Figure 1). A preload of 0.001N was applied after which the scaffolds were compressed until 30% of the original height with 20% compression per minute. Compressive Young's modulus was calculated from the slope of the stress-strain curves. To determine the yield point, yield strength and ultimate strength, a force ramp of 1.5N/min to 18N was performed. Mechanical properties were assessed for 5 technical replicates (1 donor combination) at day 1 and 3 technical replicates per donor combination (3 donor combinations) at day 28.

Computed Tomography

To analyse scaffold shape after seeding and culture, scaffolds were imaged through µCT scanning using a Quantum FX µCT scanner (voxel size = 29.29µm^3 µm^3, 90 kV tube voltage, 200 µA tube current, and 26 s of scan time, Perkin Elmer, USA) after 1 and 28 days of culture. Using ImageJ, 3 dimensional images were assembled and the landmarks function was used to measure the scaffolds.

Extracellular matrix formation

After 28 days of culture, scaffolds were digested at 60°C overnight in papain solution (50 µg/mL papain; Sigma-Aldrich, 0.2 M NaH_2PO_4, 0.1M EDTA, 0.01M cysteine, pH 6). Proteoglycan content of the scaffold and proteoglycan release into the culture medium were assessed using the Dimethylmethylene Blue (DMMB; pH 3) assay to quantify sulphated GAGs. Chondroitin-6-sulfate (Sigma-Aldrich) was used as a standard. Absorbance was measured at 525 and 596 nm. Proteoglycan production was normalized for DNA, which was quantified as indicated above. Matrix formation was assessed for 3 technical replicates per donor combination (3 donor combinations).

Histology

Scaffolds were fixed and embedded in paraffin in two orientations and cut into 5 µm sections. Cell morphology and distribution were assessed using Haematoxylin and Eosin staining and RGB staining[373]. For RGB staining, sections were stained with 1% alcian blue

in 3% aqueous acetic acid (pH 2.5) for 20 minutes and rinsed in tap water. Following this, sections were stained with 0.04% fast green in distilled water for 20 minutes and rinsed in tap water for 5 minutes. Lastly, sections were stained with 0.1% picrosirius red for 30 minutes, followed by 2 changes of 5 minutes in 1% acidic acid in tap water. For type I and II collagen immunohistochemistry, antigen were retrieved using 1 mg/mL pronase (Sigma-Aldrich) followed by blocking with 5% bovine serum albumin (BSA) in PBS for 30 minutes. Samples were incubated with the primary antibody (type I collagen, rabbit monoclonal 1/400 in PBS/BSA 5% or type II collagen, mouse monoclonal 1/100 in PBS/BSA 5%) overnight. Sections were incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (DAKO) for 30 minutes after washing. Immunoreactivity was visualized using diaminobenzidine peroxidase substrate solution (DAB, Sigma-Aldrich) and sections were counterstained with Mayer's hematoxylin.

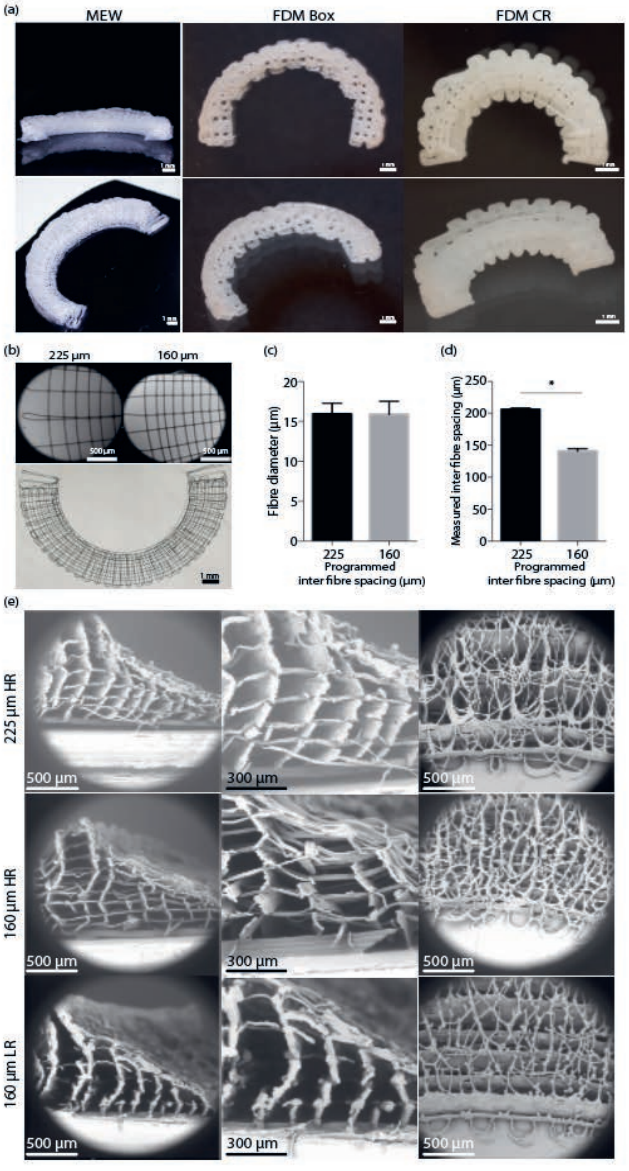
Statistics

Data were analyzed using Prism GraphPad (version 8.3, San Diego, CA, USA). Data are shown as mean ± standard deviation (SD). A student's t-test was used to compare measured inter fibre spacing. Young's modulus and proteoglycan production was compared between MEW groups and all other groups using Welch ANOVA with a Dunnett's T3 correction for multiple comparisons since variances were not equal. Similarly, the DNA content was compared between the fibrin control and different scaffolds using Welch ANOVA with a Dunnett's T3 correction. DNA release in medium was regarded illustrative data and no statistics were performed on this data. Yield points, yield strength and ultimate strength were compared using ordinary ANOVA with a Sidak correction. Assumptions were checked visually using residual, homoscedasticity and QQ plots. P-values below 0.05 were assumed significant and indicated by *.

Results

Scaffold fabrication

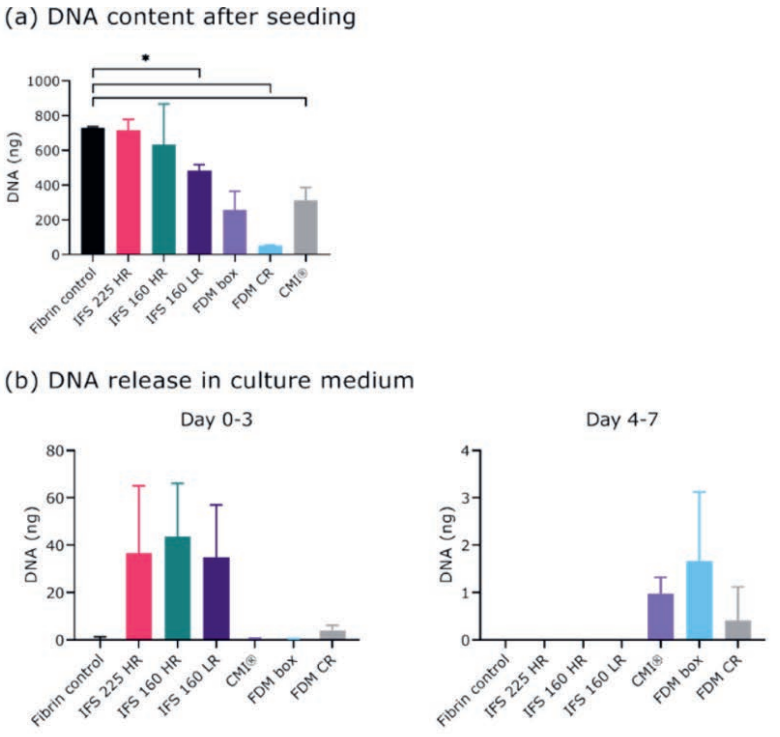
MEW scaffolds macroscopically showed the native meniscus wedge-like shape (Figure 2A). The two different FDM scaffolds, boxes-shaped (Box) and circumferential/radial-shaped (CR), macroscopically showed the wedge architecture, yet in a lower resolution (Figure 2A). A distinction between the circumferential and radial fibres was observed upon alternating these layers for both 225 µm and 160 µm inter fibre spacings (Figure 2B). An average fibre diameter of 15.9 ± 1.6 µm was found for the 225 µm programmed inter fibre spacing and an average fibre diameter of 15.8 ± 1.6 µm was found for the 160 µm spacing (Figure 2B). Additionally, the measured inter fibre spacing was close to the programmed line spacing and showed a high reproducibility (Figure 2C,D). On a microscopic level, the wedge shape could be observed for both the 225 µm and 160 µm fibre spacing (Figure 2E). Furthermore, the difference in circumferential and radial fibres and the variation in ratio (HR or LR) of these radial fibres is shown (Figure 2E).

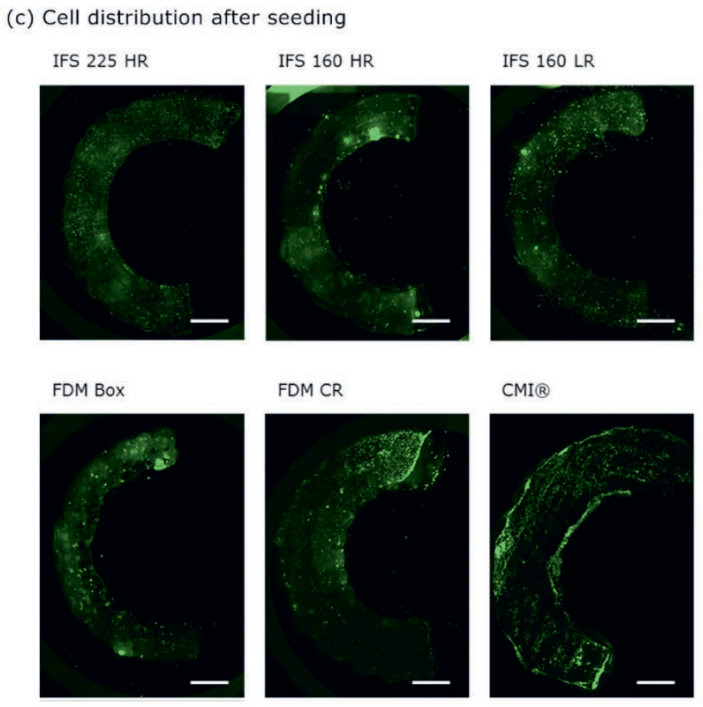


| Figure 2. Printability of circumferential and radial melt electrowritten fibres to achieve a wedge shaped meniscus. (a) Macroscopic image of Melt electrowritten (MEW) scaffold, Fused deposition Modelling (FDM) scaffold with an inner boxed-shaped (Box) architecture, and FDM scaffold with an inner circumferential/radial (CR) architecture. (b) Top view of a single layer of circumferential and radial fibres. (c) Fibre diameter of fibres for both inter fibre spacings (n=3 per group). (d) Measured inter fibre spacing for both programmed inter fibre spacings (n=3 per group). (e) Scanning electron microscopy images of scaffolds with both inter fibre spacings and the different ratios of circumferential and radial fibres. * = $p < 0.05$. *Abbreviations; HR: high ratio of radial fibres, LR: low ratio of radial fibres.*

Seeding and culture of scaffolds

The DNA content of seeded scaffolds was comparable between the HR MEW-scaffolds and a fibrin gel control, indicating successful seeding of these scaffolds (Figure 3A). The FDM CR and CMI® contained significantly less DNA after seeding than the fibrin control. No significant differences were found between the different scaffold geometries. DNA release into the medium was minimal compared to the DNA content after seeding, indicating good retention of the cells in the scaffolds (Figure 3B). All scaffolds showed homogeneous distribution of live cells in the scaffold as shown by calcein AM staining (Figure 3C).



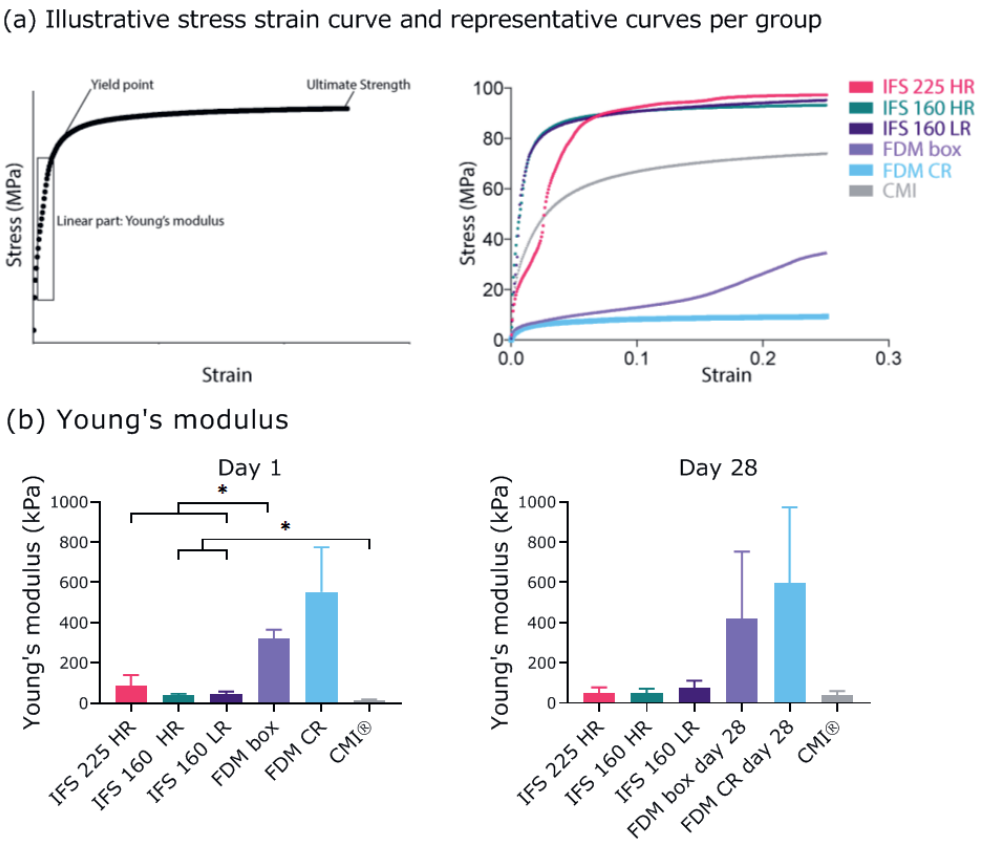


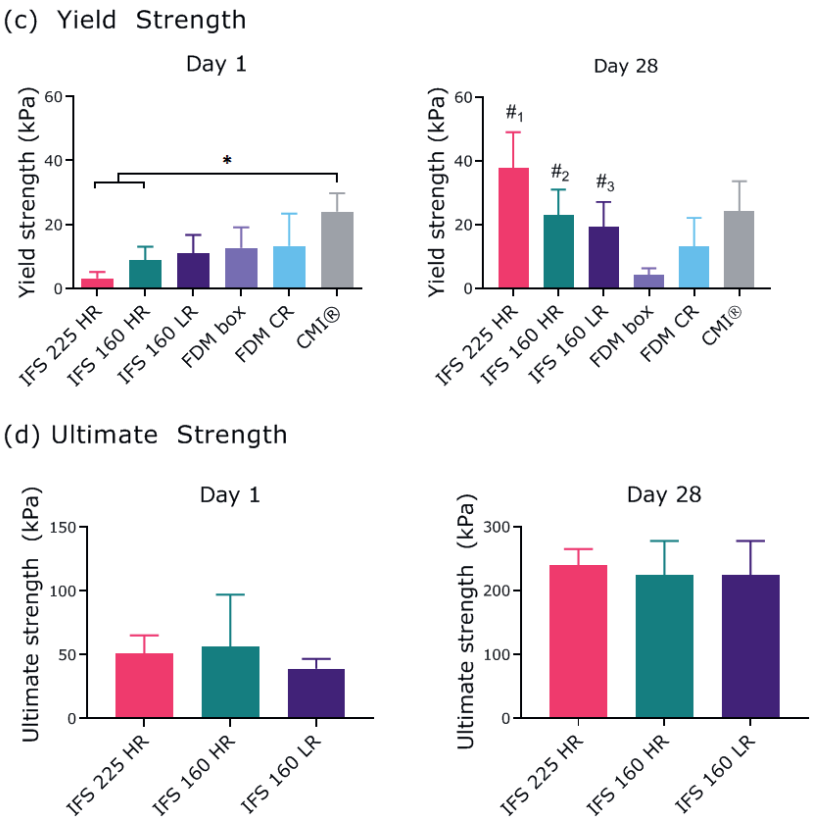
| Figure 3. Cell seeding and DNA release into the culture medium. (a) DNA content of seeded scaffolds and fibrin gel controls. (b) DNA release in culture medium as indirect measure of cell retention in the scaffold in the first week after seeding (n=3 technical replicates, 1 donor). (c) Cell distribution 1 day after seeding, green fluorescent dye is Calcein AM. *, $p<0.05$. Abbreviations; 160: 160 µm, 225: 225 µm, CMI®: Collagen Meniscus Implant®, IFS: inter fibre spacing, HR: high ratio of radial fibres, LR: low ratio of radial fibres, FDM: fused deposition modelling, CR: circumferential and radial fibres, Box: box-structure. Scale bar is 2 mm.

Mechanical properties of in vitro cultured scaffolds

Mechanical properties were assessed after 1 and 28 days of culture. The stress strain curves of different groups show similar behaviour upon compressive loading, yet at different strain values (Figure 3A). At day 1, MEW scaffolds with an inter fibre spacing of 160µm (40 ± 7 and 46 ± 11 kPa for HR and LR, respectively) showed a significantly higher compressive Young's modulus than the CMI® scaffolds (13 ± 5 kPa) (Figure 4B). The FDM Box scaffold had a significantly higher Young's modulus than the CMI® and all MEW scaffolds (Figure 4B). At day 1, the yield strength was achieved at a higher strain for the MEW scaffolds (73 ± 8 and 70 ± 11 for the IFS 160 HR and IFS 160 LR, respectively) as compared to the FDM scaffolds (23 ± 2 and 11 ± 5, for the Box and CR, respectively), indicating a larger elastic region for the MEW scaffolds (Supplementary Figure 2). At day

28, all MEW groups showed the yield point at a higher strain as compared to the FDM groups. Yield strength was comparable between the MEW and FDM scaffolds at day 1, whereas the CMI® (24 ± 5 kPa) had a higher yield strength than the IFS 225 HR (3 ± 2 kPa) and IFS 160 HR (8 ± 4 kPa) (Figure 4C). At day 28, yield strength in MEW groups had increased (Figure 4C). Ultimate strength of CMI® and FDM groups was not above the higher limits of the testing set-up. Ultimate strength of MEW groups increased between day 1 and day 28 and did not differ significantly between groups (Figure 4D).





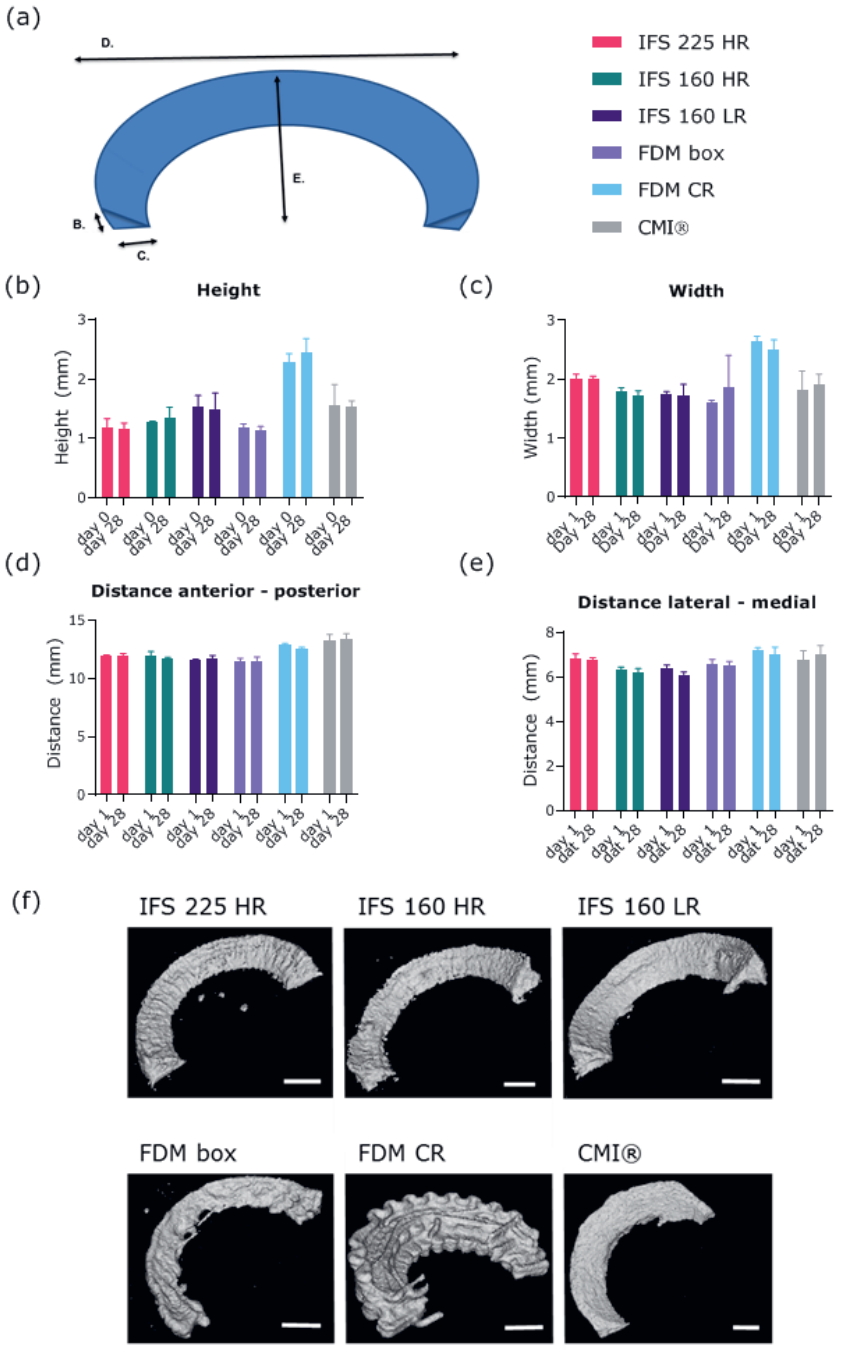
| Figure 4. Mechanical characteristics of scaffolds seeded with co-cultured mesenchymal stromal cells and meniscus cells (80:20) in fibrin gels 1 day after seeding and after 28 days of culture (n=3 donors per group, 3 technical replicates per donor). (a) Illustrative stress strain curve and representative stress strain curves of measured groups. (b) Young's Modulus (c) Yield Strength (d) Ultimate strength. **, p<0.05; #$_1$ p<0.05 compared to all groups except IFS 160 HR; #$_2$, p<0.05 compared to all groups; #$_3$, p<0.05 compared to all groups except CMI® and FDM CR; #$_4$, p<0.05 compared to FDM box and IFS 225 HR. Abbreviations; 160: 160 µm, 225: 225 µm, CMI®: Collagen Meniscus Implant®, IFS: inter fibre spacing, HR: high ratio of radial fibres, LR: low ratio of radial fibres, FDM: fused deposition modelling, CR: circumferential and radial fibres, Box: box-structure.*

Scaffold shape fidelity

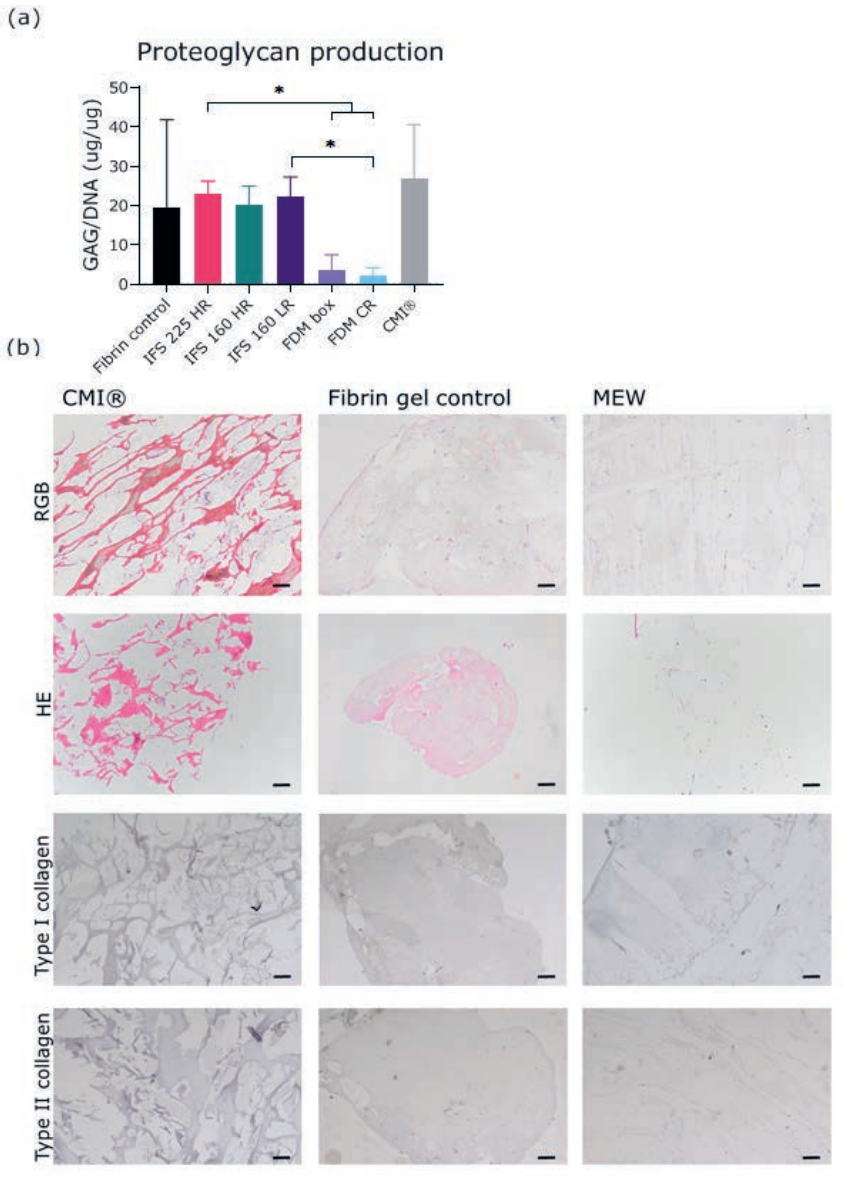
One of the main functions of the scaffold is to provide a framework and cells might affect the shape of this scaffold during culture. Therefore, shape fidelity was assessed using the dimensions of the scaffolds over the 28-culture period as a measure (Figure 5A). Irrespective of fibre reinforcing tactic or internal fibre structure, scaffolds retained shape over time in height, width, anterior-to-posterior distance, and lateral-to-medial distance (Figure 5B-F).

Extracellular matrix formation during 28 days of culture

After 28 days of culture, glycosaminoglycan (GAG) production, normalized per DNA, was similar between MEW scaffolds and the fibrin control (Figure 6A). IFS 225 HR had a significantly higher GAG production than both FDM groups (23.0 ± 3.2 vs 3.5 ± 3.9 and 2.2 ± 2.0). GAG production by IFS 160 LR was significantly higher than FDM CR, but not than FDM box ($p=0.05$). GAG production by IFS 160 HR was not significantly different from FDM groups ($p=0.05$ and $p=0.06$). Cells were found throughout the scaffolds on the sections in that were taken at different locations and in two directions (Figure 6B-D). Picrosirius red and alcian blue staining were observed in fibrin gel controls and MEW scaffolds, but low indicating deposition of small amounts of collagens and proteoglycans. There was minimal deposition of type I collagen in the scaffolds. Collagen type II staining was negative in all scaffolds (Figure 6B).



| Figure 5. (**a**) Schematic overview of measured dimensions (**b**) height of the scaffold, (**c**) width of the scaffold, (**d**) anterior - posterior distance, (**e**) lateral - medial distance, (**f**) representative µCT images per group after 28 days of culture. N=3 donors, 1-2 technical replicates per donor. Abbreviations; 160: 160 µm, 225: 225 µm, CMI®: Collagen Meniscus Implant®, IFS: inter fibre spacing, HR: high ratio of radial fibres, LR: low ratio of radial fibres, FDM: fused deposition modelling, CR: circumferential and radial fibres, Box: box-structure. Scale bar is 2 mm.



| Figure 6. Cell distribution and extracellular matrix formation after 28 days of co-culture. (**a**) GAG production normalized for DNA content of scaffolds (n= 3 donors, 3 technical replicates per donor). (**b**) Picrosirius Red, Fast Green and Alcian Blue (RGB), Hematoxylin and Eosin (HE), type I collagen and type II collagen stained sections showing cell distribution and tissue deposition in collagen meniscus implant (CMI), fibrin gel control, and melt electrowriting (MEW) scaffold after 28 days of culture(n= 3 donors, 2 technical replicates per donor. *, $p<0.05$; 160: 160 µm, 225: 225 µm, Box: box-structure, CR: circumferential and radial fibres, FDM: fused deposition modelling, HR: high ratio of radial fibres, IFS: inter fibre spacing, LR: low ratio of radial fibres.

Discussion

In this study, a scaled-down meniscus-like scaffold was fabricated from medical grade materials using MEW and seeded with a combination of meniscus cells and MSCs. As the natural architecture of the native meniscus is imperative for its function in load transmission, the fabrication of such a shape was an important aspect of this study. On a macro-scale level, the meniscus consists of a round-rim in the x-y plane and a wedge-shape in the out-of-plane direction. Especially for the MEW scaffolds, this scaled down version did still encompass the macroscopic wedge-shaped structure. With the relatively low resolution of FDM fibre deposition, the wedge shape was less smooth as compared to the MEW scaffolds. The seeded cells remained viable in the scaffold during 28 days culture and produced a basal level of GAGs. During 28 days of culture, the cell-seeded scaffolds increased in yield strength and ultimate strength. MEW scaffolds showed higher strains as compared to FDM scaffolds, suggesting that the MEW scaffolds have a larger elastic region as compared to the FDM ones.

MEW was used with the aim to replicate the intricate fibre architecture that includes both circumferential and radial orientated fibres. MEW scaffolds can be created from medical grade materials with high precision and reproducibility, which is imperative for clinical translation[374–377]. To create live-sized scaffolds that reflect natures architecture, the inter fibre distances could be decreased further. The inter fibre spacing achieved in this study (160 and 225 µm) were chosen because of deposition reproducibility in current printing path with the machine used. To improve mechanical properties, the inter fibre spacing could be decreased to increase the overall fibrous content, yet, cell infiltration and migration should then be re-evaluated. A recent study on the limits of inter fibre distances in MEW-based scaffolds reported around 60 µm inter fibre distances, which shows feasibility of decreasing fibre distances[378]. Recently, possibilities in scaffold design are increasing by the fabrication of out-of-plane fibres[379], incorporated spanning fibre sheets[380], and micro-scale layer shifting[381]. The latter uses an offset printing trajectory to overcome the electrostatic autofocussing effect and therefore allows nonlinear geometries[381]. Using an offset printing trajectory, a rounded-rim, wedge shape geometry was made for the first time using MEW technology, which showcases the potential use of MEW for more intricate geometries.

In this study, we explicitly chose to use clinical grade materials and cell types and a cell number that can be achieved within a single surgery. The cell density used here was based on the cell concentration used in the treatment of articular cartilage defects[43,282]. This concentration cannot be obtained with autologous meniscus cells without culture expansion[370], therefore a combination of MSCs and fibrochondrocytes[49,382] was used in contrast to previously reported approaches that have used only meniscus cells[383,384].

Although this cell concentration, without the addition of growth factors, does not lead to extensive tissue formation *in vitro*[49], good results are obtained *in vivo* using this cell concentration for cartilage defects[43,282] and it is a feasible cell number for use in one-stage treatment. We successfully seeded the scaffolds with this cell combination and showed good cell retention during 28 days culture, which might be attributed to the micro fibre size and small pores. After 28 days of culture, HE staining showed a homogenous distribution of the cells throughout the scaffold. The shape of the scaffolds was stable during the 28 days of culture, although this does not guarantee that the scaffolds will retain their shape *in vivo* upon mechanical loading. The yield stress and ultimate strength of MEW scaffolds seemed to increase between day 1 and day 28, which indicates tissue formation in the scaffolds. Moreover, formation of a basal level of GAGs (comparable to the fibrin gel control group) was demonstrated. Deposition of collagen and proteoglycans (as indicated by picrosirius red and alcian blue staining) were low in all scaffolds. The aim of this research was to investigate whether any ECM deposition could take place in our scaffolds and compare this to the FDM and CMI® controls. As the aim was not to produce large amounts of extracellular matrix, static culture conditions were used without the supplementation of growth factors. *In vivo*, the seeded cells will be provided with the stimulating mechanical cues and growth factors in the joint, which might further enhance matrix formation and mechanical properties. Interestingly, FDM scaffolds had a lower GAG production, which could be explained by a lower seeded cell number or the presence of large fibres which both might impair cell communication and EMC production. For clinical translation, the scaffolds should not be subject to fast resorption *in vivo*. PCL fibres are still present 6 months after implantation in and equine joint after extensive loading. This suggests suitability of the PCL scaffolds for clinical usage[385].

Limitations

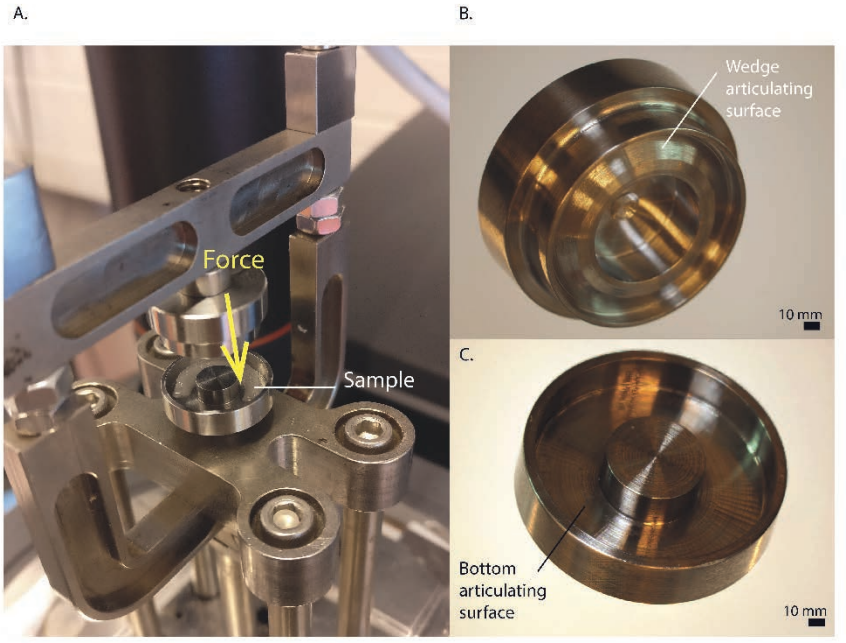
This study shows that it is feasible to obtain similar compressive properties with medical grade cell-laden materials and microscale MEW fibres as compared to the CMI®. Although promising, it should be noted that a scaled down model of the meniscus rather than a full size meniscus was used here in order to enable high throughput screening *in vitro*. Even though the fabrication of live-sized scaffolds for clinical use should be possible[386], the mechanical properties of such a full-scale scaffold should be re-evaluated. Additionally, the compressive properties of these scaffolds are not within the range of human native meniscus yet, as native meniscus has a Young's modulus in the megapascal range[387]. However, the improvement in yield strength and ultimate stress within 28 days of static culture demonstrate the potential of this approach using cells. In

the current approach for mechanical testing, fibres undergo tensile forces while stretching from compressive loading. However, this does not include compression under different angles of sliding motion, or pull-out testing. By using a custom made compression head, the complete wedge underwent compression. Surface roughness was not assessed in this study, but previous in vivo studies using fibres of comparable thickness deposited using MEW showed no damage to the opposing structures in the joint[385]. Lastly, overall tissue formation was limited in this study, which might be attributed to the low cell numbers, static culture conditions and absence of growth factor stimulation. In the current approach, we explicitly choose to use clinically feasible cell numbers in order to facilitate clinical translation as one-stage treatment, in which tissue formation by the seeded cells will be guided by the joint environment after implantation. Nonetheless, we did not compare formation of more meniscus specific extracellular matrix (e.g. type I collagen), as immunohistochemistry is not sensitive enough for such small amounts of formed tissue. Instead, we used GAG production to assess tissue formation, which is commonly used in meniscus research even though the GAG content in a healthy meniscus is generally relatively low[269].

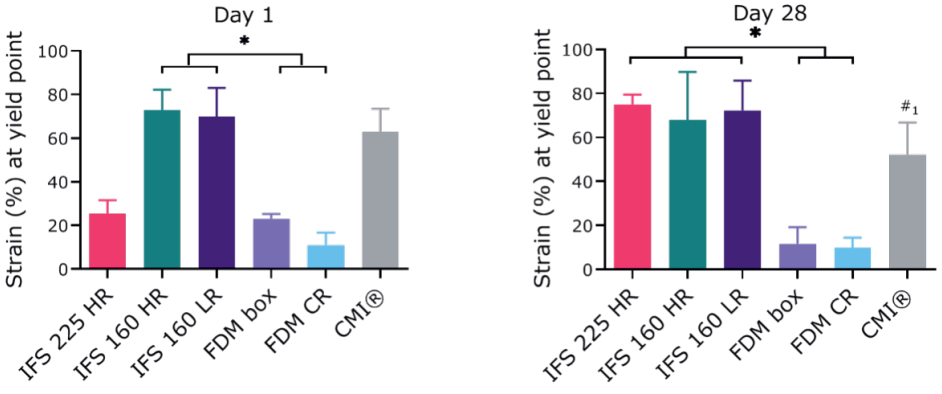
Conclusion and implications

This study demonstrates feasibility of creating wedge-shaped MEW scaffolds seeded with clinically feasible cell numbers and types for potential translation as one-stage treatment. The efficacy of these scaffolds for meniscus replacement should be further evaluated *in vivo*.

Appendix



| Figure S1. Mechanical characterization of scaffolds. (a) Compressive testing system, (b) Custom made scaffold holder for compression of the wedge surface (top) and (c) Custom made scaffold holder for compression (bottom).



| Figure S2. Strain (%) at yield point for all scaffolds at day 1 and day 28. . *, $p<0.05$; $\#_1$ $p<0.05$ compared to all groups except IFS 160 HR. Abbreviations; 160: 160 µm, 225: 225 µm, CMI®: Collagen Meniscus Implant®, IFS: inter fibre spacing, HR: high ratio of radial fibres, LR: low ratio of radial fibres, FDM: fused deposition modelling, CR: circumferential and radial fibres, Box: box-structure.

10

Efficacy of one-stage cartilage repair using allogeneic mesenchymal stromal cells and autologous chondron transplantation (IMPACT) compared to nonsurgical treatment for focal articular cartilage lesions of the knee: study protocol for a crossover randomized controlled trial

Korpershoek JV, Vonk LA, Kester EC,
Creemers LB, de Windt TS, Kip MMA, Saris DBF, Custers RJH.
Efficacy of one-stage cartilage repair using allogeneic
mesenchymal stromal cells and autologous chondron transplantation (IMPACT)
compared to nonsurgical treatment for focal articular cartilage lesions of the knee:
study protocol for a crossover randomized controlled trial. Trials. 2020 Oct 9;21(1)

Abstract

Background

Articular cartilage defects in the knee have poor intrinsic healing capacity and may lead to functional disability and osteoarthritis (OA). 'Instant MSC Product accompanying Autologous Chondron Transplantation' (IMPACT) combines rapidly isolated recycled autologous chondrons with allogeneic MSCs in a one-stage surgery. IMPACT was successfully executed in a first-in-man investigator-driven phase I/II clinical trial in 35 patients. The purpose of this study is to compare efficacy of IMPACT to nonsurgical treatment for treatment of large (2-8cm^2) articular cartilage defects in the knee.

Methods

Sixty patients will be randomized to receive nonsurgical care or IMPACT. After nine months of nonsurgical care, patients in the control group are allowed to cross over receive IMPACT surgery. The Knee Injury and Osteoarthritis Outcome Score (KOOS), pain (numeric rating scale, NRS), and EuroQol five dimensions five levels (EQ5D-5L) will be used to compare outcomes at baseline and three, six, nine, 12, and 18 months after inclusion. Cartilage formation will be assessed at baseline, and six and 18 months after inclusion using MRI. An independent rheumatologist will monitor the onset of a potential inflammatory response. (Severe) adverse events will be recorded. Lastly, the difference between IMPACT and nonsurgical care in terms of societal costs will be assessed by monitoring healthcare resource use and productivity losses during the study period. A health economic model will be developed to estimate the incremental cost-effectiveness ratio of IMPACT vs. nonsurgical treatment in terms of costs per quality adjusted life year over a five year time horizon.

Discussion

This study is designed to evaluate the efficacy of IMPACT compared to nonsurgical care. Additionally, safety of IMPACT will be assessed in 30 to 60 patients. Lastly, this study will evaluate the cost-effectiveness of IMPACT compared to nonsurgical care.

Introduction

Background and rationale

Cartilage defects are common knee injuries that lead to a deterioration of sports performance, increased work leave and limitations in daily activities. Cartilage defects may eventually lead to osteoarthritis (OA) due to the limited healing capacity of cartilage [17,388]. Treatment aims at obtaining a pain free joint function by achieving structural tissue repair. Small cartilage defects are successfully treated using microfracture, but treatment of large defects (2-8 cm^2) requires more advanced techniques. The application of fresh allografts for large or deep defects is limited by the high costs and poor availability[389]. Synthetic implants are easy to use and short-term results look promising, but the quality of the repair tissue is poor[390,391]. Autologous chondrocyte implantation (ACI) is a two-stage treatment, in which a biopsy of healthy cartilage from a non-weight bearing location in the knee is taken during an initial knee arthroscopy. Approximately 180.000-455.000 chondrocytes are isolated from such a biopsy[282], while millions of cells per mL of defect filling are needed[392,393]. In order to obtain sufficient chondrocytes to repair the cartilage defect, a period of cell expansion is required[282]. After approximately 4-13 weeks, the cultured autologous chondrocytes are re-implanted into the cartilage defect in a second surgical procedure. Although ACI procedures showed good mid-term and long-term results[394,395], chondrocyte expansion leads to a decrease in type II collagen and increase in type I collagen gene expression, which are both signs of dedifferentiation[284,396]. Furthermore, ACI is a costly procedure due to the requirement of cell culture[371] and it has been unavailable in many European countries after different products have been withdrawn from the European market[25,397]. Due to this limited availability, nonsurgical care consisting of physiotherapy and pain medication remains the treatment of choice.

Both *in vitro* and *in vivo*, cartilage formation has been shown to improve by direct contact between multipotent mesenchymal stromal cells (MSCs) and articular chondrocytes[243,398–401]. This stimulatory effect on cartilage matrix formation further increases when MSCs are combined with chondrons (chondrocytes with their pericellular matrix)[401]. Using this combination of cells allows for a one-stage application, as autologous cells can be used without expansion. 'Instant MSC Product accompanying Autologous Chondron Transplantation' (IMPACT) combines 10% autologous chondrons with 90% allogeneic MSCs in a single surgery for the treatment of cartilage defects. Compared to the two-stage ACI procedure, IMPACT decreases the patient burden and significantly reduces the costs of treatment[371]. Safety, feasibility, initial efficacy, and structural tissue repair of IMPACT was shown in a cohort of 35 patients with cartilage lesions (3.2 ± 0.7 cm)(NCT02037204)[43,44].

Objectives

The current phase III randomized controlled trial explores the efficacy of IMPACT compared to nonsurgical care in 60 patients with large (2-8cm^2) articular cartilage defects of the knee. We allow patients in the nonsurgical group to cross-over to the IMPACT group after nine months nonsurgical care. Follow-up will be at least 18 months after IMPACT. The primary objective is to compare the Knee Injury and Osteoarthritis Outcome Score (KOOS) at three, six, and nine months follow-up. The secondary objective of this study is to examine morphology and proteoglycan content of repair tissue six and 18 months after treatment. Safety endpoints will be determined by the number of (treatment-related) adverse events. In addition, the incremental cost-effectiveness ratio (ICER) of IMPACT vs. nonsurgical care and vs. delayed surgical intervention will be calculated. More specifically, the effect of IMPACT compared with nonsurgical care and delayed surgical intervention in terms of healthcare resource use, productivity losses and accompanying costs during the study period will be determined and extrapolated to a five year time horizon.

Additional consent provisions for collection and use of participant data and biological specimens

A subgroup of 15 patients will be asked to participate in an optional study, in which the structure and composition (glycosaminoglycan content) of the regenerated tissue are studied using high resolution imaging. These patients will undergo additional MRI-scans using a 7-Tesla MRI-scanner at baseline, six, and 18 months. Regardless of treatment allocation, patients can volunteer for this part of the trial by indication on the informed consent form.

Trial design

In this phase III randomized controlled clinical trial, IMPACT is compared to nonsurgical treatment. The patient allocation ratio is 1:1. Patients in the nonsurgical group are allowed to cross over to the treatment group after nine months follow-up.

Methods

Study setting

The IMPACT-trial will be performed in a tertiary referral hospital (University Medical Center Utrecht (UMC Utrecht)) in the Netherlands that is specialized in treatment of cartilage defects of the knee. Patients are recruited at the Mobility Clinic, which includes the outpatient clinic of orthopedics, sports medicine and rheumatology, and is part of

the UMC Utrecht. Patients are considered for inclusion if they meet the criteria as defined below.

Eligibility criteria

Primary inclusion criteria (at the outpatient clinic)

- The patient provides written informed consent, is able to understand the content of the study, understands the requirements for follow-up visits and is willing to complete the questionnaires and provide the required information at follow-up visits.
- Symptomatic articular cartilage defect of the knee (femoral condyles or trochlea) 2-8 cm^2.
- Age $\geq$18 and $\leq$45 years old.

Primary exclusion criteria (at the outpatient clinic)

- Malalignment of >5 degrees (correctional osteotomy is allowed during the trial).
- (History of) OA, defined as Kellgren-Lawrence grade $\geq$3 as determined from appropriate radiography.
- Joint instability (ligament reconstruction is allowed during the trial).
- Concomitant inflammatory disease that affects the joint (rheumatoid arthritis, metabolic bone disease, psoriasis, gout, symptomatic chondrocalcinosis).
- (History of) Septic arthritis.
- (History of) Total meniscectomy in the target knee joint.
- Any surgery in the index knee joint six months prior to study inclusion.
- Risk groups for MRI due to the magnetic field such as patients with pacemakers, nerve stimulators, metal particles, stents, clips or implants, (possible) pregnancy or breast feeding.

Definitive eligibility is assessed during surgery based on the criteria below.

Definitive inclusion criteria (during surgery)

- Modified Outerbridge Grade III or IV isolated cartilage lesion of the knee.
- A post-debridement size of the cartilage lesion $\geq$ 2cm^2 and $\leq$ 8 cm^2.
- At least 50% of functional meniscus remaining. Meniscal repair or resection is allowed during the IMPACT surgery provided that the surgeon is able to confirm that at least 50% of functional meniscus remains.
- Stable knee ligaments (i.e. anterior and posterior cruciate ligaments).

Definitive exclusion criteria (during surgery)

- Patients with lesions > 8 cm^2.
- Patients with osteoarthritic lesions Kellgren-Lawrence grade ≥3 not diagnosed before surgery.

Who will take informed consent?

Patients with an MRI- or previous arthroscopically confirmed isolated articular cartilage lesion will be screened for eligibility to participate in this study based on the abovementioned criteria. After the patient has been assessed as eligible by the treating orthopedic surgeon, he/she will receive initial study information. After at least two weeks of reflection, patients are invited to meet with the research physician to discuss any remaining questions and sign the informed consent.

Interventions

Intervention description

IMPACT is a one-stage cell-based regenerative therapy for isolated articular cartilage lesions. The investigational product consists of 10% autologous chondrons recycled from the debrided defect tissue and 90% allogeneic MSCs in Tisseel® tissue glue (Baxter B.V, Utrecht, the Netherlands) which will act as a cell carrier for implantation. The MSCs are obtained from bone marrow of healthy non-HLA matched donors in the GMP-licensed Cell Therapy Facility (Department of Clinical Pharmacy, University Medical Center Utrecht) and cultured and characterized as described previously[44]. To summarize, the bone marrow aspirate is density-separated and MSCs are isolated using plastic adherence. The MSCs are expanded up to passage three after which they are cryopreserved. MSCs are characterized by the expression of CD73, CD105, and CD90 and the absence of expression of CD45, and CD3.
IMPACT surgery[43,44] consists of a mini-arthrotomy, during which the cartilage defect is debrided and stable borders are created. The debrided cartilage tissue is transported to the Cell Therapy Facility, where chondrons are isolated from the tissue using a rapid digestion protocol; minced cartilage is digested in 40 minutes in Liberase MNP-S GMP Grade (Roche, Mannheim, Germany). The autologous chondron-suspension is run over a 100µm strainer (Corning Inc., New York, USA) to remove the undigested cartilage matrix. The chondrons are washed twice to remove the enzyme and counted using 3% acetic acid with methylene blue (STEMCELL Technologies Germany GmbH, Köln, Germany). Allogeneic cryopreserved MSC are thawed and the chondrons and MSCs are mixed at a 10:90 ratio in the fibrinogen component of Tisseel®. The fibrinogen and thrombin

component of Tisseel® are mixed upon application, which causes the product to gelate. Two million cells per ml are implanted in the defect. The rehabilitation protocol we use is equal to that after ACI. Briefly, during rehabilitation patients are allowed 10% weight bearing the first three weeks, after which the load is increased gradually up to 50% at six weeks and 100% at eight to 12 weeks. From five months onward, the rehabilitation protocol aims at improving coordination, increasing muscle strength and becoming functional in moderately intensive activities. Patients can return to low-impact sports after nine months and to high-impact sports at the earliest after 12 months. Patients treated for cartilage defects in the trochlea are allowed weight-baring in the first six weeks after surgery, but will use an extension brace in order to limit the flexion while weight baring.

Explanation for the choice of comparators

The control group receives standard care, which is nonsurgical. Due to the varying availability of ACI in the Netherlands, this is the comparator of choice for cartilage defects of 2-8 cm^2. The control group is allowed the option to take pain medication at their own discretion as well as physical therapy by their own physical therapist.

Criteria for discontinuing or modifying allocated interventions

Patients can leave the study at any time for any reason if they wish to do so without any consequences. The patient's participation in this study can also be ended by the investigator if the patient is uncooperative and/ or does not attend study visits. The patient data that have been collected up to that moment will be included in the analysis. In case too many data are missing (e.g. missing baseline or all of the follow-up patient-reported outcome measures (PROMs), study visits or MRI-scans), the patient will be replaced by a new patient. This study will be prematurely ended in case of any abundance in adverse events or procedure / compound-related complications or if the independent rheumatologist advises this termination. Criteria for study termination include: any Suspected Unexpected Serious Adverse Reaction (SUSAR) or Serious Adverse Event (SAE) based on an allergic reaction and clear allergic or iatrogenic effects in two or more patients including patients which report back to the hospital with serious iatrogenic complaints. In case of premature ending, all included patients will be informed by their treating orthopedic surgeon. In case of illness, patients will be asked to contact the primary investigator. Patients that are discovered during surgery not to meet the criteria will not receive IMPACT and will be treated according to the standard of care, based on the findings during surgery. These patients will be removed from the study. Patient data included up to that moment will be included in the analysis.

Out of Specification Product

An Out of Specification (OOS)-product is a product that cannot be made according to the criteria, for example when an insufficient number of chondrons is isolated. In this case, a risk-benefit assessment of implantation of the OOS-product will be done by the treating orthopedic surgeon and Qualified Person. They will consider alternative treatment options for the patient, whether manufacturing can be (partly) repeated, and the ratio of the cells restored. The OOS-product will contain a higher percentage of MSCs in order to compensate for the missing chondrons, and two million cells per ml will be implanted. Patients that receive an OOS-product will not be included in efficacy analyses, but AE, SAE, SUSARs will still be reported and the patient will be included in safety and cost-effectiveness analyses. Patients will be informed of this OOS-procedure both during the screening visit and through the patient information letter, before signing the informed consent form. The researchers will report implantation of an OOS-product to the Central Committee on Research Involving Human Subjects (CCMO) and Health and Youth Care Inspectorate (IGJ), according to the CCMO guidelines.

Treatment algorithm in case of foreign body response.

If there is suspicion of an acute (within 48 hours) foreign body response after surgery, a consultation by an independent rheumatologist will be requested immediately. Depending on the severity of the reaction, initial treatment will consist of NSAIDs, anti-histamines, or immunosuppressants. If no improvement occurs within 48 hours, treating specialists will consider a diagnostic knee aspiration (in case of signs of infection), anaphylaxis protocol, or debridement and lavage. In case of a late immunological response (after 72 hours), infection will be excluded as a cause of the reaction, prior to starting the algorithm as mentioned above.

Strategies to improve adherence to interventions

The nonsurgical protocol consists of physiotherapy and/or pain medication and can be adjusted to the individual patient's needs. The adherence to this protocol will be high as it does not consist of strict guidelines. Adherence to the rehabilitation protocol after IMPACT will be monitored by the specialized physiotherapists in our center. They are in close contact with the treating physiotherapists and monitor progression during study visits.

Relevant concomitant care permitted or prohibited during the trial

Concomitant surgery such as anterior cruciate ligament reconstruction or alignment correction is permitted during the trial but will be registered. Injections into the index

knee are not permitted six months pre- and twelve month post-operatively.

Provisions for post-trial care

The sponsor has insurance, which is in accordance with the legal requirements in the Netherlands (Article 7 Medical Research Involving Human Subjects Act (WMO)). This insurance provides coverage for damage to research subjects through injury or death caused by any activities of the study. The insurance applies to the damage that becomes apparent during the study or within four years after the end of the study.

Outcomes

The primary outcome is the comparison in total KOOS between patients with cartilage defects that are treated with IMPACT and patients treated with standard care (nonsurgical treatment) until nine months after randomization. Total KOOS is an average of the scores in the five subscales of KOOS[137]. Other outcomes of interest are: outcomes in the five subscales of KOOS, pain (numeric rating scale, NRS), general health (EuroQol five dimensions five levels, EQ5D-5L), and structural repair (MRI). After nine months, patients in the nonsurgical group are allowed to undergo IMPACT surgery, this will be regarded as failed nonsurgical treatment and the time of crossover will be recorded. Change from baseline assessed with the KOOS of the total group of patients treated with IMPACT at three, six, nine, 12 and 18 months after treatment will be evaluated (per protocol). The potential effect of time until surgical treatment will be assessed. Clinical safety will be determined by active tracing of the adverse event rate observed after IMPACT and nonsurgical therapy. Additionally, emergence of an immune response will be assessed by screening for anti-HLA antibodies in peripheral blood pre-operatively and four weeks postoperatively. The HLA phenotype of MSC donors will be compared to newly formed anti-HLA antibodies. Societal costs will be assessed by monitoring the costs related to the IMPACT procedure and the accompanying rehabilitation period, as well as costs related to nonsurgical (or delayed) treatment. In this analysis, two scenarios will be compared: (I) IMPACT vs. nonsurgical treatment, and (II) IMPACT vs. delayed surgical treatment. In the first scenario, it is assumed that patients randomized to nonsurgical treatment will not undergo IMPACT in the next five years, whereas the second scenario includes patients who were randomized to nonsurgical care and opt for IMPACT after nine months follow-up. In both scenarios, costs of other healthcare related resource use (including physiotherapy, home care, medication use, and costs related to adverse events), as well as costs attributable to health-related work leave (i.e. productivity losses) will be collected over a follow-up period of (at least) nine months. For nonsurgical treatment, health outcomes (from the EQ-5D-5L) and costs within the first nine months

will be extrapolated to calculate costs/QALY over a five year time horizon. Similarly, health outcomes and costs for (delayed) IMPACT will also be extrapolated to a five year time horizon, using results from the completed phase I/II study (unpublished results).

Table 1. Participant timeline

Outpatient Clinic: Screening (MRI) Study information provided in writing Baseline visit: Informed consent Baseline study MRI Randomization Baseline PROMs	
Group A: IMPACT	**Group B: Control (nonsurgical)**
Preoperative screening Anti-HLA antibodies	3 months after inclusion PROMs Check-up study physician (by telephone)
Surgery/1 day postoperative Blood chemistry (CRP, ESR, Leucocytes) Visit physical therapist Check-up rheumatologist Check-up study physician	6 months after inclusion PROMs Check-up study physician MRI
1 week postoperative Blood chemistry (CRP, ESR, Leucocytes) Check-up rheumatologist Check-up study physician	9 months after inclusion PROMs
4 weeks postoperative Blood chemistry (CRP, ESR, Leucocytes, anti-HLA antibodies) Check-up rheumatologist Check-up study physician	In case of cross-over to IMPACT: follow group A In case nonsurgical treatment is continued: 12 months after inclusion: PROMs
3 months postoperative Check-up study physician (by telephone) PROMs	18 months after inclusion: PROMs MRI
6 months postoperative Check-up study physician PROMs MRI	
9 months postoperative PROMs	
12 months postoperative Check-up study physician PROMs	
18 months postoperative Check-up study physician PROMs MRI	

Abbreviations: : CRP, C-reactive protein; ESR, Erythrocyte sedimentation rat; HLA, human leukocyte antigens; IMPACT, Instant MSC Product accompanying Autologous Chondron Transplantation; MRI, Magnetic resonance imaging; PROMs, patient-reported outcome measures

Sample size

The sample size was calculated for the primary objective (treatment effect up to nine months postoperatively) based on the Hotelling-Lawley trace[402,403]. Based on a standard deviation of 15[137], correlation of the repeated measures of 0.7 (data from our phase I trial[43]), and with a power of 0.8 and alpha of 0.05, a minimum of 44 patients should be included to detect a minimal clinical relevant treatment effect of 10 for KOOS[137]. To account for potential loss to follow-up, and uncertainties in the correlation pattern and standard deviation for nonsurgical treatment, this was rounded up to 60 patients in total.

Recruitment

Patients will be recruited at the Mobility Clinic (which includes the outpatient clinic of the department of Orthopedics) of the University Medical Center Utrecht. We carry out over 250 surgical procedures for cartilage defects in our center annually.

Sequence generation

Patients will be randomized into variable block sizes of two and four, stratified by defect size (<4 or ≥4cm^2), using Castor EDC[404].

Concealment mechanism

Allocation is not concealed and will be revealed to both the patient and the researcher upon randomization.

Implementation

After signing the informed consent forms, the researchers will use Castor EDC[404] to allocate the patient to one of the study arms. The study group will be revealed at the same time to both the patient and researcher.

Who will be blinded

Patients, researchers and surgeons will not be blinded, since this is impossible due to the major difference between the two groups (surgical versus nonsurgical).

Procedure for unblinding if needed

The trial design is open label, therefore there is no unblinding procedure.

Data collection and management

Plans for assessment and collection of outcomes

Data will be derived from electronic patient records and collected with an electronic Case Report Form (eCRF) using Castor EDC (Good Clinical Practice (GCP) Compliant)[404]. Patients will use an online survey (OnlinePROMS, InterActive Studios, Rosmalen, the Netherlands) to answer questionnaires. Laboratory tests are performed by the central diagnostic laboratory and MRIs will be made at the department of Radiology of the UMC Utrecht. All radiographic data acquired during the study will be anonymized and saved in a study folder on our protected research server. Only the study team has access to this specific study folder. For the cost-effectiveness analysis, the resources required for the different procedures (i.e. IMPACT or nonsurgical treatment), as well as for the IMPACT product that is used (materials, operation theatre etc.), and the duration of the accompanying hospitalization, will be derived from the electronic patient records). In addition, the results of the iMTA Medical Consumption Questionnaire will be used to collect data on resource use (including physiotherapist visits, home care, medication use, etc.) and the results of the iMTA Productivity cost questionnaire will be used to collect data on productivity losses[405]. All resource use will be multiplied with cost prizes, which will be obtained from the Dutch Healthcare Authority[406], from UMC Utrecht hospital tariffs or from the Dutch manual for performing health economic evaluations[407], to calculate total societal costs. These costs will be combined with the QoL outcome measures (EQ-5D-5L), to calculate the incremental cost-effectiveness ratio of IMPACT compared to nonsurgical therapy in terms of cost per QALY over a five-year time horizon.

Plans to promote participant retention and complete follow-up

The patients will receive extensive information about the study set-up and requirements during the recruitment. The importance of completion of the follow-up will be stressed. Patients are allowed to stop at any time during the study and are not obliged to give a reason to discontinue. If possible, the patient will be asked to complete the online survey at nine months after inclusion. Questionnaires are completed using an online survey, and therefore patients can do this at any convenient moment. All patients are reminded throughout the study to fill out the questionnaires during study visits. Throughout the follow-up period, the researchers will check responses and if necessary contact patients for completion of their follow-up.

Data management

Patient data will be collected with a GCP compliant eCRF (Castor EDC)[404].
Questionnaires will be answered online and output will be stored in SPSS. Back-ups in the study folder on the protected research server will be made regularly(once per three months). Informed consent and end-of trial dates will be recorded in the electronic patient dossier and signed paper forms will be stored within our hospital in a locked room. (S)AEs will be recorded in the eCRF. To be able to reproduce study results and to help future users to understand and reuse data, all changes made to the raw data and all steps taken in the analysis will be documented in the eCRF and IBM SPSS (version 15.0.0.2, Chicago Illinois). Source data will remain available in electronic patient record and OnlinePROMS. All research data, including patient material will be archived for 30 years after the study has ended according to the guidelines for Advanced Therapy Medicinal Products (ATMPs).

Confidentiality

Research data will be stored using a study identification code for each participant. The key to the identification code list will only be available to the research team during the study and will be documented and safeguarded by the principal investigator according to research guidelines after completion of the study. No patient identification details will be reported in publications. Plans for collection, laboratory evaluation and storage of biological specimens for genetic or molecular analysis in this trial/future use
In case of leftover material, this will be stored within the Cell Therapy Facility according to the ATMP legislation.

Statistical methods

Statistical methods for primary and secondary outcomes

Data will be analyzed using IBM SPSS (version 15.0.0.2, Chicago Illinois). Data from the primary objective (KOOS, EQ-5D-5L) will be presented as continuous variables. To compare between the two treatment groups, a mixed model analysis will be performed, with treatment group and assessment date of the PROMs (e.g. baseline, three months, six months) as fixed factors. Differences will be considered statistically significant for the fixed effect of treatment groups if $p<0.05$[408]. The 95% confidence interval of the fixed effect size will be used to assess whether treatment difference reaches the minimally clinical important difference.
T1rho-scores will be calculated from the biochemical MRI scans, differences in T1rho-scores will be compared between the time points t=0 (at inclusion before IMPACT or

nonsurgical treatment) and t=6 and 18 months after inclusion or surgery. Differences will be compared using student's t-test. The differences in T1rho-score will be tested for normality using Q–Q plots. P-values less than 0.05 will be considered significant. We will calculate the ICERs of IMPACT vs. nonsurgical treatment and vs. delayed surgical treatment in terms of cost/QALY over a five year time horizon. The effect of uncertainty in input parameters on the ICERs will be calculated by means of one-way sensitivity analysis as well as probabilistic sensitivity analyses using Monte Carlo simulation.
All (S)AE(I)s will be summarized and recorded including the nature, date and time of onset, date of resolution, determination of seriousness, severity, action taken, outcome and possible causality to study treatment. SAE data will be presented in a descriptive manner.

Interim analyses

There are no interim analyses planned.

Methods for additional analyses (e.g. subgroup analyses)

There are no subgroup analyses planned.

Methods in analysis to handle protocol non-adherence and any statistical methods to handle missing data

The primary outcome will be assessed using an intention-to-treat analysis. Missing data will be reduced to a minimum by using the appropriate measures described above. Mixed models do not require imputations for missing data. If any statistical method is needed to account for missing data in the secondary outcomes, multiple imputation will be used.

Plans to give access to the full protocol, participant level-data and statistical code

The datasets used and/or analyzed during the current study can be made available by the corresponding author upon reasonable request, and in agreement with the research collaboration and data transfer guidelines of the UMC Utrecht.

Oversight and monitoring

Composition of the coordinating center and trial steering committee
This is a monocenter study designed, performed and coordinated in the UMC Utrecht. Day to day support for the trial is provided by: Principle investigator: takes supervision of the trial and medical responsibility of the patients.

Data manager: organizes data capture, safeguards quality and data.
Study coordinator: trial registration, coordinates study visits, annual safety reports.
Study physician: identifies potential recruits, takes informed consent, ensures follow-up according to protocol.The study team meets biweekly. There is no trial steering committee or stakeholder and public involvement group.

Composition of the data monitoring committee, its role and reporting structure

In agreement with the advice from the central Data Safety Monitoring Board (DSMB) committee of the UMC Utrecht, a DSMB has not been appointed for this study. The decision was based on the lack of SAEs in the phase I/II trial. Moreover, since this is not a blinded study, there is no DMSB required to protect blinding of the researchers and physicians. Lastly, due to the expected rapid inclusion and treatment of patients, interim assessments of a DSMB will not add value to the safety in this study. A rheumatologist knowledgeable in the field of allergic/immunologic reactions, will be assigned as safety officer for this study. In case of SAEs, this safety officer will be contacted within 48 hours. The safety officer will assess if the SAE is (definitely or possibly) related to treatment. In case of (possible) treatment relation, further safety measures will be taken on advice of the safety officer.

Adverse event reporting and harms

All adverse events reported by the subject or observed by the investigators will be recorded. The causality to the study treatment event will be recorded. Several complications are considered as AEs of Interest (AEIs) based on information from the previous trial and theory of the study procedures: arthralgia, swelling or crepitation other/ longer than may be expected and resulting in alteration in medical care, synovitis, surgical site infection, migration or dislocation of the graft, knee locking, haemarthrosis, arthrofibrosis, chondropathy (including a new cartilage lesion in the same knee or a secondary lesion), general surgery related disorders (pneumonia, deep vein thrombosis, pulmonary embolism, disorders resulting from general or local anesthesia, tissue hypertrophy. SAEs will be reported to the CCMO following the CCMO guidelines.

Frequency and plans for auditing trial conduct

An independent study monitor, Julius Clinical Research B.V (Zeist, The Netherlands) was appointed according to the guidelines of the Dutch Federation of University Medical Centers (NFU 2.0) (October 2012) for study specific auditing. Based on these guidelines, the estimated risk for this study is considered moderate. The independent monitor makes

two on site visits per year and checks presence and completeness of the investigation file. Moreover, the monitor checks the following data for 25% randomly picked patients: informed consents, in- and exclusion criteria, source data, missing and reporting for (S)AEs/SUSARs. For more information, the monitoring plan can be consulted. Auditing can also take place by national or international health authorities, like the Dutch Health and Youth Care Inspectorate (IGJ).

Plans for communicating important protocol amendments to relevant parties (e.g. trial participants, ethical committees)

A 'substantial amendment' is defined as an amendment to the terms of the CCMO application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree: the safety or physical or mental integrity of the subjects of the trial; the scientific value of the trial; the conduct or management of the trial; or the quality or safety of any intervention used in the trial.
All substantial amendments will be notified to the CCMO and to the competent authority. Non-substantial amendments will be recorded and filed. In case amendments concern or affect participants in any way, they are informed about the changes. If needed, additional consent will be requested and registered. Online trial registries will be updated accordingly.

Dissemination plans

Results of this research will be disclosed completely in international peer-reviewed journals. Both positive as well as negative results will be reported. Patients will receive a laymen summary of the results in case they opted-in to receive outcomes on a study level.

Discussion

This randomized controlled trial is designed to investigate efficacy of IMPACT compared to nonsurgical care. Safety of IMPACT one-stage surgery for articular cartilage defects will be monitored in 30 to 60 patients. Also, cost-effectiveness of IMPACT will be compared to nonsurgical care.

Limitations

There are several limitations to consider. First, cartilage defects may lead to major disability with a high patient burden, therefore patients are likely to request immediate surgical treatment. The possible delay of surgical treatment by allocation to the nonsurgical control group might impair patient inclusion or increase drop-out in the

control group. Risk of drop-out will be minimized by properly informing the patients of the study set-up and goals. Secondly, we compare IMPACT surgery to nonsurgical therapy instead of ACI surgery. A nonsurgical control group was explicitly chosen due to the limited availability of ACI in Europe in the last decade. A comparison with ACI can be made retrospectively using our prospective registry, which includes data on safety, efficacy, and treatment and societal costs of the patients treated with ACI at our center. In addition, to the best of our knowledge, no randomized controlled trial has been performed previously that compares cell therapy to conservative treatment. Lastly, the follow-up period after surgery is relatively short (1.5 years) and long-term efficacy remains to be investigated. We ask permission of all patients to contact them after the study period in order to investigate long term follow-up and aim to include all patients in our prospective registry.

Conclusion and implications

This clinical trial will provide insight into the efficacy of IMPACT compared to nonsurgical care. By the use of nonsurgical therapy as a comparator group, we will gain insight into the natural course of disease of nonsurgically treated patients. Moreover, we will establish a control group that can be used universally and independent of availability of (different types of) cell therapy for cartilage defects. Lastly, emergence of an immune response will be assessed by screening for anti-HLA antibodies in peripheral blood. This will provide useful insights in the *in vivo* behavior of MSCs, which can be transferred to other applications of MSCs, for example in regenerative medicine.

11

Mitochondrial transport from mesenchymal stromal cells to chondrocytes increases DNA content and proteoglycan deposition *in vitro* in 3D cultures

Korpershoek JV, Rikkers M, Wallis FSA, Dijkstra K, te Raa M, de Knijff P, Saris DBF, Vonk LA. Mitochondrial transport from mesenchymal stromal cells to chondrocytes increases DNA content and proteoglycan deposition in vitro in 3D cultures. *Submitted*

Abstract

Background

Allogeneic mesenchymal stromal cells (MSCs) are used in the one-stage treatment of articular cartilage defects. Recently, a role for mitochondrial transfer in the treatment effect of MSCs has been suggested in several regenerative treatments. The aim of this study is to investigate whether transport of mitochondria exist between chondrocytes and MSC and to investigate if the transfer of mitochondria to chondrocytes contributes to the mechanism of action of MSCs.

Methods

Chondrocytes and MSCs were stained with MitoTracker, and CellTrace was used to distinguish between cell types. After 4 to 24 hours of co-culture, the uptake of fluorescent mitochondria was measured using flow cytometry. Transport was visualized using fluorescence microscopy. Microvesicles were isolated using ultracentrifugation and presence of mitochondria was assessed using flow cytometry. Mitochondria were isolated from MSCs and transferred to chondrocytes using MitoCeption. Pellets of 100.000 chondrocytes, chondrocytes with transferred MSC mitochondria, and co-cultures (chondrocyte:MSC; 10:90) were cultured for 28 days. DNA content was measured using qubit fluorometric quantification and proteoglycan content using a Dimethylmethylene Blue Assay. Mitochondrial DNA of cultured pellets and of repair tissue of patients that underwent treatment with autologous chondrons and allogeneic MSCs was quantified using single nucleotide polymorphisms genotyping to assess the fate of transferred mitochondria over time.

Results

Mitochondrial transfer occurred bidirectionally within the first 4 hours until 16 hours of coculture. Transport took place via tunnelling nanotubes, direct cell-cell contact, and via extracellular vesicles After 28 days of pellet culture, DNA content and proteoglycan deposition were higher in chondrocyte pellets to which MSC mitochondria were transferred than the control groups. No donor mitochondrial DNA was traceable in the biopsies, whereas an increase in MSC mitochondrial DNA was seen in the pellets.

Conclusion

These results suggest that mitochondrial transport plays a role in the chondroinductive effect of MSCs on chondrocytes *in vitro*. However, *in vivo* no transferred mitochondria could be traced back after one year.

Introduction

Multipotent mesenchymal stromal (stem) cells (MSCs) can be isolated from bone marrow, adipose tissue, synovial membrane, and other tissues[409]. Due to their multilineage differentiation potential[39], anti-inflammatory properties[410], and signalling through trophic factors[411] and extracellular vesicles[412], MSCs are used in a wide spectrum of regenerative treatments. One of the treatments employing MSCs is IMPACT (Instant MSC Product accompanying Autologous Chondron Transplantation). IMPACT is a new treatment for articular cartilage defects of the knee and combines 10% recycled autologous chondrons with 90% off-the-shelf available allogeneic MSCs[43–45]. Results of a phase I/II trial using IMPACT for treatment of articular cartilage defects showed safety and feasibility of this procedure[43,44], and 5-year clinical outcomes were promising [45]. The repaired cartilage defect site did not contain autosomal DNA of the MSC donors, suggesting that the MSCs do not differentiate, but rather act as signalling cells[40,413], possibly through secretion of chondroinductive[46,48] and anti-inflammatory agents[414]. The transfer or organelles, such as mitochondria, might also contribute to the stimulatory effect of MSCs on chondrogenesis. MSC-derived mitochondria enhanced phagocytic capacity of alveolar macrophages and ameliorated lung injury by improving mitochondrial function and ATP turnover in a murine model[415,416]. Furthermore, transplanted MSC mitochondria restored mitochondrial function and decreased apoptosis in rabbit cardiomyocytes postischemia[417], and intra-myocardial injection of autologous mitochondria improved ventricular function in patients with ischemic injury[418]. While the occurrence of mitochondrial transfer from equine, mice, and rat MSCs towards chondrocytes has been described[419–421], it has not been demonstrated in human cells before. Moreover, it is unclear whether transport takes place from chondrocyte to MSC as well. As shown in other tissues than cartilage, transfer of mitochondria can play a role in tissue repair, but its role in MSC-stimulated chondrogenesis is unknown. Chondrocytes need adenosine triphosphate (ATP) for production of the main components of cartilage glycosaminoglycans (GAGs) and type II collagen[422], which is provided normally by anaerobic glycolysis[423]. Under glucose-deprived conditions or glycolysis inhibition, chondrocytes switch to oxidative phosphorylation to maintain ATP production[424]. Thus, the presence of functional mitochondria in chondrocytes is of paramount importance for their prolonged survival. Mitochondrial dysfunction can develop after pathological mechanical loading[425], and is one of the hallmarks in the development of osteoarthritis[426]. Transfer of functional mitochondria could prevent or resolve this mitochondrial dysfunction. Therefore, the aim of this study is to investigate whether mitochondrial transfer takes place between human chondrocytes and MSCs. We study the timing of mitochondrial transfer as well as different modes of transport *in vitro*. Additionally, we

investigate the effect of inflammation and senescence on mitochondrial transfer by pre-incubating with tumor necrosis factor α (TNF-α) and mitomycin C. Using MitoCeption[427], we analyse the effect of transferring MSC-derived mitochondria to chondrocytes on DNA content and proteoglycan deposition in 3D cultures. Lastly, in order to study mitochondrial transfer *in vivo,* we isolate DNA from cartilage biopsies of six patients treated with IMPACT[43,44] and used single nucleotide polymorphisms (SNP) genotyping to determine the presence of MSC donor mitochondrial DNA.

Methods

Donors and cell isolation

Human MSCs were isolated from bone marrow of healthy donors in the GMP-licensed Cell Therapy Facility (Department of Clinical Pharmacy, University Medical Center Utrecht) as approved by the Dutch central Committee on Research Involving Human Subjects (CCMO, Bio-banking bone marrow for MSC expansion, NL41015.041.12). The parent or legal guardian of the donor signed the informed consent approved by the CCMO (n = 5, age range = 2 – 12). In brief, the mononuclear fraction was separated, MSCs were isolated by plastic adherence, and expanded for three passages in Minimum Essential Media (αMEM, Macopharma, Utrecht, The Netherlands) with 5% (v/v) platelet lysate and 3.3 IU/mL heparin and cryopreserved. Subsequently, MSCs were culture-expanded for two or three additional passages in MSC expansion medium (αMEM (Gibco, Bleijswijk, The Netherlands), 10% (v/v) fetal bovine serum (FBS; Biowest, Nuaillé, France), 1% penicillin/streptomycin (pen/strep; 100 U/mL, 100 µg/mL; Gibco), 200 µM l-ascorbic acid 2-phosphate (ASAP; Sigma-Aldrich), and 1 ng/mL basic fibroblast growth factor (bFGF; PeproTech, London, UK)). Cartilage was obtained after debridement of focal cartilage lesions from patients undergoing autologous chondrocyte implantation (ACI), and is considered medical waste or redundant material (n = 5, age range = 18 – 38). The tissue collection was performed according to the Medical Ethics regulations of the University Medical Center Utrecht and the guideline “Human Tissue and Medical Research: Code of Conduct for responsible use” of the Dutch Federation of Medical Research Societies [261,262]. Chondrocytes were isolated from the debrided cartilage by digestion in 0.2% (w/v) pronase (Sigma-Aldrich, Saint-Louis, MO, USA) in Dulbecco’s Modified Eagle’s medium (DMEM, 31966; Gibco) with 1% pen/strep for two hours, followed by overnight digestion in 0.075% (w/v) collagenase II (CLS-2, Worthington, Lakewood, NJ, USA) in DMEM supplemented with 10% FBS and 1% pen/strep). Isolated chondrocytes were culture-expanded to passage two in chondrocyte expansion medium (DMEM, 10% FBS, 1% pen/strep).

Quantification of monolayer mitochondrial transfer

To enable identification of the different donor and receiving cell type in culture, the donor cell type was labelled with CellTrace Violet (Invitrogen, Carlsbad, CA, USA) and MitoTracker Red CMXRos (Molecular Probes, Invitrogen) according to the manufacturer's instructions. Receiving cells were unlabelled. Cells were stained one day prior to initiation of the coculture. Additionally, cells were pre-treated with 0.02 µg/mL mitomycin C (Substipharm, Paris, France) for six days to induce senescence [428] or with 10 ng/mL tumor necrosis factor α (TNF-α, R&D Systems, Minneapolis, MN, USA) for 24 hours to mimic an *in vitro* inflammatory environment [429].

MSCs (passage 5 or 6) and chondrocytes (passage 2) were seeded in 6-well plates at a density of 100,000 cells per well in a 1:1 ratio. Dual-stained donor cells were plated 24 hours before initiation of the coculture. Unstained receiving cells were added to the pre-seeded donor cells and cocultures were maintained for 24 hours in chondrocyte expansion medium. After 0, 4, 8, 16, and 24 hours, cocultures were trypsinized, washed, and resuspended in phosphate-buffered saline (PBS) supplemented with 0.4% (v/v) human serum albumin (HSA; Albuman, Sanquin, Amsterdam, The Netherlands). Samples were analysed using a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA). For each condition, 20,000 events were recorded. Flow cytometry results were extracted and analysed using RStudio (R Core Team, Vienna, Austria) and FlowJo V10 data analysis software package (Tree Star Inc, Ashland, OR, USA).

Imaging

To enable identification of the different donor and receiving cell type in culture, the donor cell mitochondria were labelled with MitoTracker Red CMXRos (Molecular Probes, Invitrogen) and the chondrocytes (or half of the cells in CH → CH) were stained with CellTrace Violet (Invitrogen) according to the manufacturer's instructions. To visualize tunneling nanotubes (TNT), the donor cell type was stained with DiD (Vybrant™ Multicolor Cell-Labeling Kit, Invitrogen) in co-cultures. Additionally, the actin skeleton of all cells in all cultures was stained using 100 nM SiR-Actin (Spirochrome AG, Tebu Bio, Heerhugowaard, The Netherlands). Monolayers were imaged using a THUNDER fluorescence microscope and LASX acquisition software (both Leica microsystems, Wetzlar, Germany). TNTs were imaged using a Leica SP8X Laser Scanning Confocal Microscope (Leica microsystems) and LASX acquisition software.

Extracellular vesicle isolation

To evaluate presence of mitochondria in extracellular vesicles (EV) and changes in EV secretion initiated by coculture, donor cells were dual stained using CellTrace Violet and

MitoTracker Red CMXRos as described in 'Quantification of monolayer mitochondrial transfer' or left unstained. Cells were cultured in monocultures or cocultures in 1:1 ratio for 24 hours in vesicle-deprived chondrocyte expansion medium, after which the conditioned medium was collected for processing. Cell debris were removed from conditioned medium by centrifugation for 5 minutes at 320 *g*, followed by 15 minutes at 1,500 *g*. Subsequently, the medium was centrifuged at 16,000 *g* for 1 hour to pellet EVs[430,431]. After discarding the supernatant, EVs were washed, resuspended in buffer (PBS with 0.5% (w/v) bovine serum albumin (Roche Diagnostics GmbH, Mannheim, Germany) and 2mM ethylenediaminetetraaceticacid (EDTA)) and then analysed using a BD LSR Fortessa flow cytometer (BD Biosciences, Allschwil, Switzerland) and FlowJo V10 data analysis software package (Tree Star Inc). For each condition, 10,000 events were recorded.

Delivery of MSC mitochondria to chondrocytes in monolayer

To investigate the effect of MSC-derived mitochondria on chondrocytes, mitochondria isolated from MSCs (pre-stained with MitoTracker Red CMXRos) were directly transferred into chondrocytes. MSCs were culture-expanded and half of the cells were treated with mitomycin C to induce senescence (sMSC). Mitochondria were isolated using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Mitochondria were transferred into chondrocytes as previously described [427]. Briefly, mitochondria were added to monolayers of chondrocytes and subjected to two consecutive centrifugation steps with an interval of 2 hours. The moment after the first centrifugation cycle was considered T0. Efficiency of MitoCeption on pre-seeded chondrocyte monolayers was measured using increasing concentrations of mitochondria. Then, isolated mitochondria of 9 x 10^5 MSCs or senescent MSCs were used for MitoCeption on a monolayer of 1 x 10^5 pre-cultured chondrocytes (CH) to mimic a CH:MSC ratio of 10:90 as used in IMPACT[43,401]. Intra-cellular location of the mitochondria was confirmed 1 day after MitoCeption with fluorescence microscopy and effect of different dosages of mitochondria was assessed using flow cytometry.

Metabolic activity of chondrocytes after mitochondrial transfer

Metabolic activity of the MitoCepted chondrocyte monolayers was determined directly after MitoCeption (T=2h), after 26h, and 44h using the conversion of resazurin to resorufin (44 mM; Alfa Aesar, Thermo Scientific) by measuring fluorescent intensity at 560 nm excitation and 590 nm emission.

Gene expression of chondrocytes after mitochondrial transfer

Total RNA of chondrocyte monolayers was isolated at T=2h, T=6h, T=26, and T=46h after MitoCeption using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCRs were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in the LightCycler 96 (Roche Diagnostics GmbH) according to the manufacturer's instructions. Primers (Invitrogen) are listed in Table 1. Relative gene expression was calculated using 18S as a housekeeping gene and normalized for gene expression of that donor before MitoCeption. Amplified PCR fragments extended over at least one exon border (except for 18S).

Delivery of MSC mitochondria to chondrocytes in 3D chondropermissive culture

To investigate whether transfer of MSC-mitochondria into chondrocytes affects chondrogenesis, isolated mitochondria were transferred into chondrocytes during the pellet formation. Mitochondria of 9 x 10^5 MSCs or sMSCs were isolated as described in 'Direct mitochondrial transfer through MitoCeption in monolayer' and added to 1 x 10^5 chondrocytes in suspension. Pellets of 1 x 10^5 chondrocytes were formed by centrifugation at 320 *g* for five minutes in 15 mL Falcon tubes. MitoCeption on monolayers was performed in parallel to compare efficiency of MitoCeption in pellets and in monolayers. Pellets were cultured for 28 days in chondropermissive medium (DMEM, 2% HSA, 2% (v/v) insulin-transferrin-selenium-ethanolamine (ITS-X; Gibco), 200 µM ASAP, and 1% pen/strep). Control pellets consisted of chondrocytes alone and CH:MSC cocultures in a 10:90 ratio (both 1 x 10^5 total). Medium was changed twice per week and collected for analysis. After one and two weeks of culture, MitoCeption was repeated on a subset of pellets. Control pellets were also subjected to centrifugation at these time points. Results are displayed in Fig. S2.

Release and deposition of glycosaminoglycans

Pellets were harvested after 28 days of culture, digested in a papain digestion buffer (250 µg/mL papain (Sigma-Aldrich), 0.2 M NaH_2PO_4, 0.1M EDTA, 0.01M cysteine, pH 6.0) at 60°C overnight. Deposition of sulphated glycosaminoglycans (GAG) in the pellet digests and release into the culture medium was measured using a dimethylmethylene blue assay (DMMB; pH 3.0). Absorbance was measured at 525 / 595 nm using chondroitin-6-sulfate (Sigma-Aldrich) as a standard. DNA content of digests was quantified using Qubit dsDNA HS Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

Histological analyses

Pellets were processed for histology by fixation in a 4% buffered formaldehyde solution, followed by dehydration through graded ethanol steps, clearing in xylene, and embedding in paraffin. Sections of 5 µm were cut, stained with 0.125% safranin-O (Merck, Darmstadt, Germany), and counterstained with 0.4% fast green (Sigma-Aldrich) and Weigert's hematoxylin (Clin-Tech, Glasgow, UK). Type I and II collagen deposition was visualized by immunohistochemistry. Sections were blocked in 0.3% (v/v) hydrogen peroxide, followed by antigen retrieval with 1 mg/mL pronase (Sigma-Aldrich) and 10 mg/mL hyaluronidase (Sigma-Aldrich), both for 30 minutes at 37°C. Sections were blocked with 5% (w/v) BSA in PBS for one hour at room temperature and incubated with primary antibodies for type I collagen (EPR7785 (BioConnect, Huissen, The Netherlands), 1:400 in 5% PBS/BSA) and type II collagen (II-II6B3 (DHSB, Iowa City, IA, USA), 1:100 in 5% PBS/BSA) overnight at 4°C. For type I collagen, rabbit IgG (DAKO, Glostrup, Denmark; X0903) was used as isotype control and for type II collagen, mouse IgG (DAKO X0931) was used. Next, type I collagen sections were incubated with BrightVision Poly-HRP-Anti Rabbit (VWR, Radnor, PA, USA) and type II collagen sections were incubated with goat-anti-mouse IgG HRP-conjugated (DAKO, P0447; 1:100 in 5% PBS/BSA) for one hour at room temperature. Immunoreactivity was visualized using diaminobenzidine peroxidase substrate solution (DAB, Sigma-Aldrich). Mayer's hematoxylin (Klinipath, Olen, Belgium) was used as counterstaining.

DNA analysis

DNA was isolated from digest of cartilage pellets at 0, 1, 2, and 4 weeks after culture and from the cartilage biopsies of six patients, taken one year after treatment with IMPACT [43,44] and from corresponding MSCs. Biopsies were compared to donor MSCs.

Extraction

DNA was extracted using the Qiamp DNA mini and blood mini kit according to the manufacturers protocol "DNA purification from blood or body fluids (spin protocol)". 100µL of digested cells were added to 100µL PBS to acquire the appropriate volume. Elution was performed in 200µL nuclease free water.

Mitochondrial DNA analysis

A PCR was performed on 42 mitochondrial DNA SNP's (SNP's, primers and input in primermix can be found in S3). PCR's were performed in a total volume of 12.5µL with a mix containing 1.25µL GeneAmp 10x PCR-bufferI (Applied Biosystems), 1.25µL 10x dNTP's, 1.25µL MT-DNA primermix, 1µL MgCl2 (25mM, Applied Biosystems), 0.5µL Tag gold (5U/µL,

Applied Biosystems) and 7.25µL of DNA extract. All PCRs were performed on a GeneAmp® PCR System 9700 using the following program: 94°C for 10 minutes, 27 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72◦C for 30 seconds and final extension of 5 minutes at 72◦C. A Qiaxcel run was performed to verify the amplification success.
To prepare Illumina sequencing libraries from all PCR products, barcoded adapters were ligated to the PCR products using the KAPA library preparation kit® (KAPA biosystems). An end-repair reaction was performed with 2.5µl of PCR product in a total volume of 35µl for 30 min at 20°C. The A-tailing and adapter ligation were performed in a total volume of 25µl for 30 min at 30°C and 20°C respectively. For adapter ligation, barcoded adapters were used in a final concentration of 60nM. During the preparation of the libraries, no additional amplification occurred.
Prepared libraries were quantified and subsequently pooled equimolar. Sequencing was performed on the MiSeq® Sequencer (Illumina) with 5% PhiX control library. Sequencing was performed according to the manufacturer's protocol using v3 sequencing reagents. The MiSeq sequencing data were analyzed using a home-made pipeline that starts with FLASH[432], followed by TSSV[433] and FDStools[434]. FLASH was used to align paired-end reads and obtain a consensus sequence of higher quality. When paired-end reads differed more than 33%, they were discarded. When a difference less than 33% occurred between the two reads, bases with the highest quality score were incorporated in the consensus sequence. By providing TSSV with the primer sequences, the reads containing the region surrounding the SNP were recognized and counted. FDStools is a software tool package for the analysis of massive parallel sequencing data. It has the capability of recognizing and correcting noise from PCR or sequencing artefacts. FDStools was used to compare the sequence with the SNP to revised camREF, resulting in an HTML-file that can be viewed and analyzed (see figure). All sequences with a percentage <5% of the most frequent sequence were filtered out. This cut-off is based on validation results that showed background noise is hardly ever higher than 5%. The MT-DNA results of the cultured samples were compared to the result of the MSC and CH MT-DNA to determine whether a mixture was present.

Autosomal DNA analysis

A VeriFiler™ Plus (Thermofisher Scientific) PCR was performed using 2.5µL mastermix and 1.25µL primermix in a total volume of 12.5µL. The amount of input DNA was 0.5ng, when possible. Otherwise 8.75µL of DNA extract was added. PCR conditions were according to protocol. Capillary electrophoresis was performed on the AB3500xL according to manufacturer's protocol. Results were analyzed using GeneMarker HID V2.9.5. The

autosomal DNA results of the cultured samples were compared to the result of the MSC and CH autosomal DNA to determine whether a mixture was present.

Statistical analyses

Data were analysed using GraphPad Prism version 8.3.0 (GraphPad Software, San Diego, CA, USA). Data are shown as mean ± standard deviation (SD) unless stated otherwise. P-values below 0.05 were considered statistically significant. ANOVA was used to test for significant differences in fluorescence between consecutive time-points (Fig. 1). As a follow-up, SIDAK correction for multiple comparisons was used. Two-way repeated measures ANOVA was used to test for differences between senescent and inflammatory conditions and control condition (Fig. 3), chondrocyte control and MitoCeption groups (Fig. 4), and MitoCeption groups and chondrocyte/coculture controls (Fig. 5), taking into account donor variability. Here, a Dunnet's post hoc test was performed to account for multiple comparisons.

| Table 1. Primer sequences for quantitative real-time PCR.
Forward (Fw) and reverse (Rv) primers.

Gene name	Oligonucleotide sequence (5' to 3')	Annealing temperature (ºC)	Product size (bp)
18S	Fw: GTAACCCGTTGAACCCCATT Rv: CCATCCAATCGGTAGTAGCG	57	151
ACAN	Fw: CAACTACCCGGCCATCC Rv: GATGGCTCTGTAATGGAACAC	56	160
BCL2	Fw: GCGTCTGTAGAGGCTTCTGG Rv: GCCACTTGCCACTTTTCCTG	60	293
COL2A1	Fw: AGGGCCAGGATGTCCGGCA Rv: GGGTCCCAGGTTCTCCATCT	57	195

Abbreviations: ACAN, aggrecan; BCL2, B cell lymphoma 2; COL2A1, collagen type II alpha 1 chain.

Results

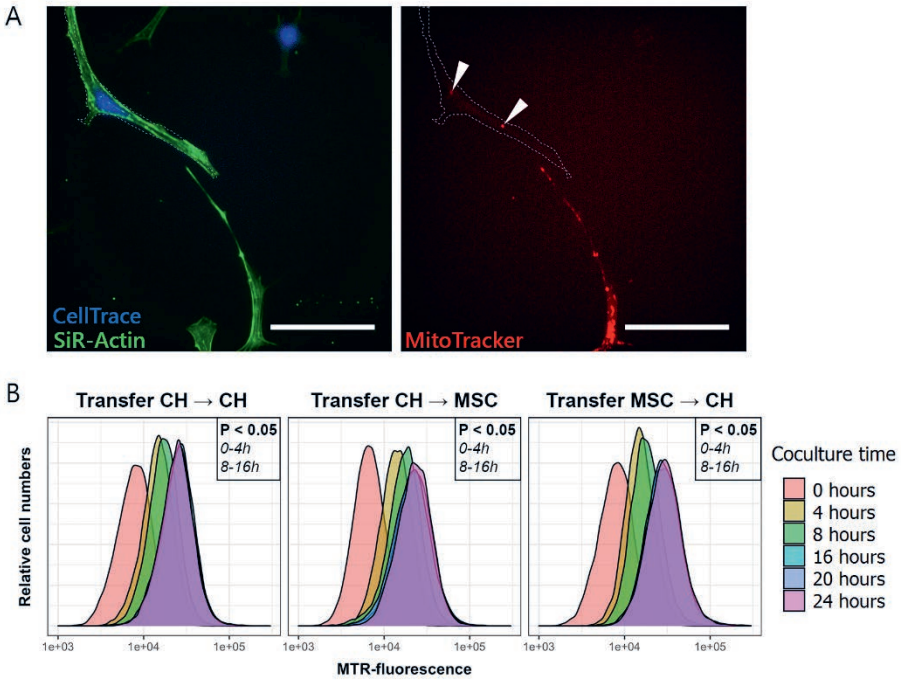
Mitochondrial transfer takes place between chondrocytes and mesenchymal stromal cells

Cells stained with CellTrace (receiving cells) gained fluorescent mitochondria from donor cells that were stained with MitoTracker (Fig. 1A, indicated by white arrows). Stained mitochondria were transferred among chondrocytes, and between chondrocytes and MSCs. Using flow cytometry, mitochondrial transfer was quantified by measuring

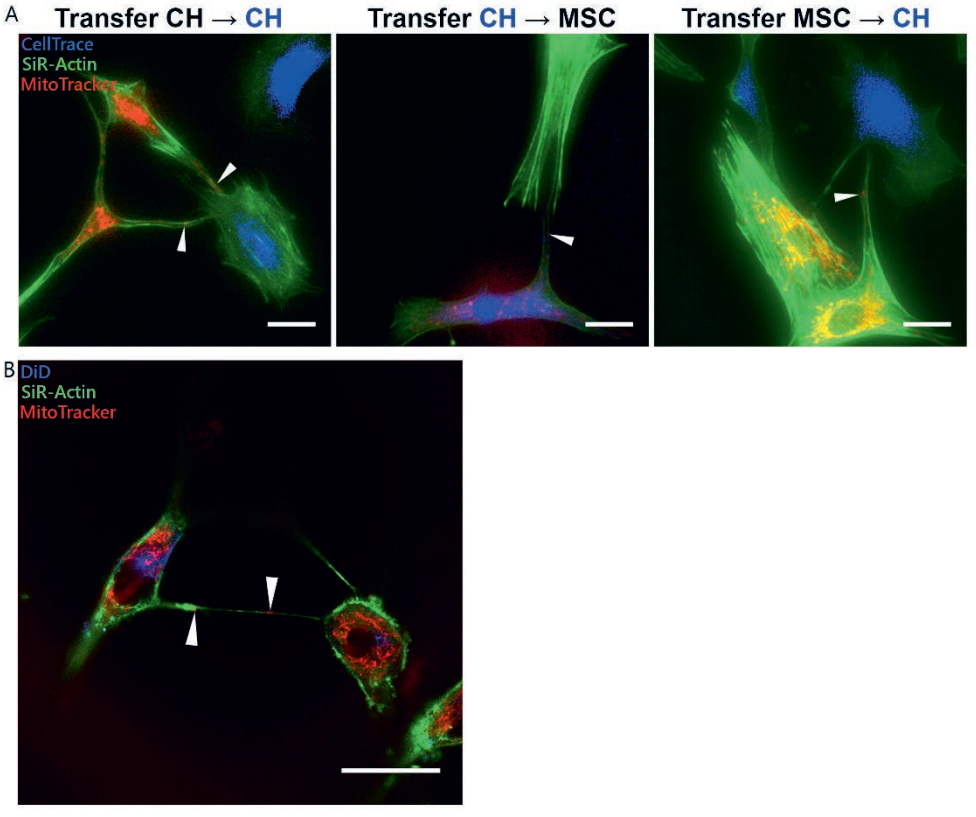
increase in fluorescence in receiving cells. Increase in fluorescence was significant from 0 to 4 hours and from 8 to 16 hours in all three coculture conditions. No further increase in fluorescence was found after 16 hours in any of the conditions (Fig. 1B).

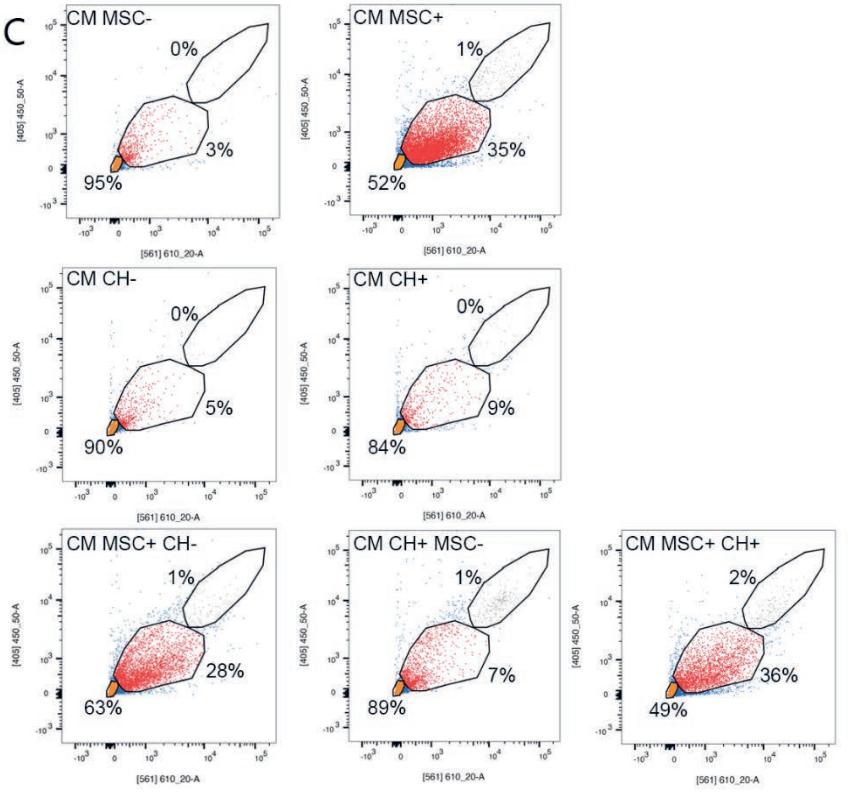
Mitochondrial transfer takes place through direct cell-cell contact, tunnelling nanotubes, and extracellular vesicles

Transfer of mitochondria occurred through direct cell-cell contact (Fig. 2A), as mitochondria (MitoTracker, in red) were seen in broad actin-containing (SiR-Actin, in green) cell protrusions between two cell types (indicated by white arrows). Additionally, transfer took place over larger distances as mitochondria were detected in tunnelling nanotubes (TNTs) between both cell types (Fig. 2B). A mitochondrion in a TNT is indicated by the white arrow. Traces of DiD (in blue) are found in the receiving chondrocyte, suggesting transfer of the cytosolic dye from the stained MSCs. In conditioned medium of stained MSCs or CH, a population stained with MitoTracker as well as CellTrace (Fig. 2C, in red) was identified as extracellular vesicles (EVs) containing mitochondria. In conditioned medium of unstained cells, this population overlapped with the population identified as background noise (in orange). In conditioned medium of MSC monocultures, 35% of events were marked as mitochondria-containing EVs, whereas in conditioned medium of CH 9% of events were marked as mitochondria-containing EVs. In co-cultures where only MSCs were stained, 28% of events were marked as mitochondria-containing EVs, whereas 7% of events were marked as mitochondria-containing EV in the co-cultures where only CH were stained. In co-cultures where both CH and MSC were dual stained, 36% of the events were mitochondria-containing EVs, suggesting that MSCs are stimulated to excrete EVs containing mitochondria in presence of CH, while this is not the case for CH in presence of MSC.



| Figure 1. Transfer of mitochondria between chondrocytes and mesenchymal stromal cells. (A) Stained mitochondria (MitoTracker, in red) are transferred from a donating mesenchymal stromal cell (MSC) to a receiving chondrocyte (CH) stained with celltrace (in blue). Sir-Actin stains F-actin in all cells (in green). Scale bar = 50 µm. (B) Quantification of transfer of mitochondria from donor to receiving cell, measured with flow cytometry. Mitochondrial transfer between all cell combinations (CH → CH, CH → MSC, and MSC → CH) occurred predominantly in the first four to eight hours after initiation of the coculture. In all cases, 20.000 events were recorded. *Abbreviation: MTR: mitotracker*


A
Transfer CH → CH
Transfer CH → MSC
Transfer MSC → CH
CellTrace
SiR-Actin
MitoTracker
B
DiD
SiR-Actin
MitoTracker



| Figure 2. Mechanisms mediating transfer of mitochondria between cells. (A) Visualization of mitochondrial transfer between among chondrocytes (CH) and CH, between CH and mesenchymal stromal cells (MSC), and vice-versa. Donating cells were stained with MitoTracker (in red), CH were stained with CellTrace (in blue), and F-actin of all cells was stained with SiR-Actin (in green). Mitochondria transported between two cell types are indicated by the white arrows. Scale bar = 25 µm. (B) Transport of mitochondria through a tunnelling nanotube between MSC and CH. Donating MSCs were stained with DiD (in blue) and MitoTracker (in red). F-actin of all cells was stained with SiR-Actin (in green). Image taken after 16 hours of co-culture. Scale bar = 25 µm. (C) Flow cytometry analysis of (co)culture conditioned media (CM) for small particles including mitochondria-containing microvesicles (in red). Noise and particles negative for both dyes are depicted in orange, cells (upper gate) are depicted in grey. Intensity of MitoTracker (561/610 nm) is depicted on the x axis, intensity of CellTrace (405/450 nm) is depicted on the y axis. MSC- and CH- are unstained. MSC+ and CH+ are dual stained for MitoTracker and CellTrace. In unidirectional cocultures (lower panels, left and middle), the first cell type is dual stained while the other is unstained. In the bidirectional coculture (lower panel, right), both cell types are dual stained. In all cases, 10.000 events were recorded.

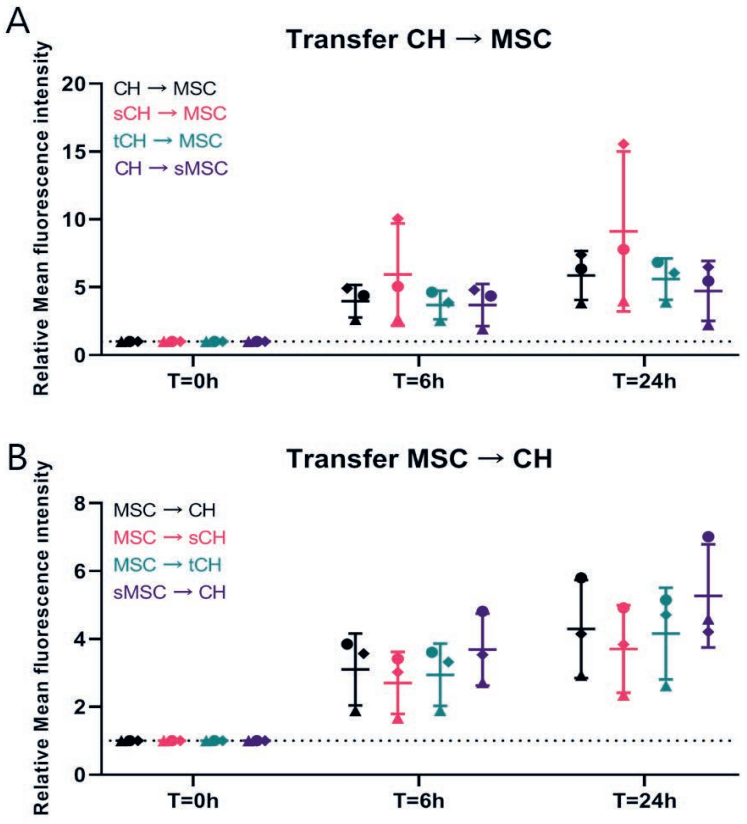
Cell stress does not affect mitochondrial transfer

The effect of inflammation and senescence on mitochondrial transfer were investigated among chondrocytes and between chondrocytes and MSCs. Cells were pre-treated with TNF-α or mitomycin C to mimic cell stress. There was no significant difference in transfer between any of the groups and the control condition, although over time the fluorescence intensity increased (Fig. 3).

Uptake of MSC mitochondria increases gene expression of aggrecan and B-cell lymphoma 2 in chondrocytes

To assess the effects of MSC-derived mitochondria on chondrocytes in chondropermissive culture, mitochondria were transferred into chondrocytes by MitoCeption[427,435]. 24 hours after transfer, mitochondria (in red) were detected intracellularly in chondrocyte monolayers (Fig. 4A). The number of transferred mitochondria was dose-dependent as confirmed by flow cytometry (presented dose as equivalent to number of MSCs used for isolation).

For further experiments, mitochondria of 900,000 MSCs were transferred onto 100,000 chondrocytes, in order to mimic a cell ratio of 90:10, which is optimal for chondroinduction[43,401]. When mitochondria of 900,000 MSCs were transferred on 100,000 chondrocytes, 74% ± 1.6 of the chondrocytes were positive for MitoTracker (Fig. 2B). Mitochondria derived from senescent MSCs were included to investigate whether these would exert similar effects as mitochondria from normal (proliferating) MSCs. MitoCeption of mitochondria and senescent mitochondria did not alter metabolic activity in chondrocyte monolayers at 24 and 42 hours of coculture (Fig. 4C). At T=2h, mRNA expression of aggrecan (ACAN) was significantly up-regulated in CH that received mitochondria compared to CH controls and CH that received senescent mitochondria. Expression of type II collagen (COL2A1) at 26 hours after MitoCeption with mitochondria was higher in two donor combinations, but not consistently among all donor combinations ($p<0.1$). ACAN and COL2A1 expression declined at 46 hours in all groups. Additionally, mRNA expression of B-cell lymphoma (BCL2), a marker for cell survival 436, was significantly higher 26 hours after MitoCeption with mitochondria, but not with senescent mitochondria (Fig. 4D). Individual values are shown in Figure S1.

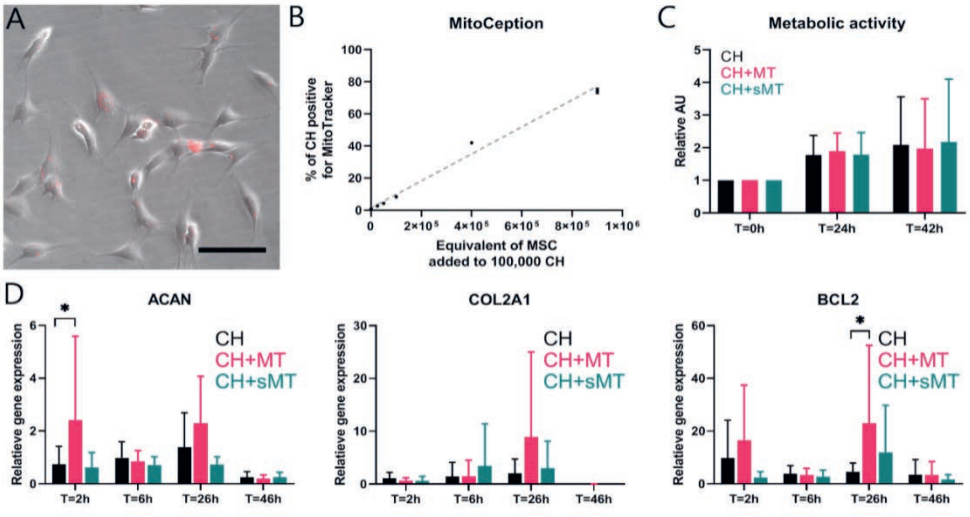


| Figure 3. Effect of inflammation and senescence on mitochondrial transfer. (A) Mitochondrial transfer from chondrocytes (CH), chondrocytes pretreated with tumor necrosis factor α (TNF-α) to induce inflammation (tCH), and chondrocytes pretreated with mitomycin C to induce senescence (sCH), to mesenchymal stromal cells (MSC) and MSCs pretreated with mitomycin C to induce senescence (sMSC). tends to increase when CH are senescent (sCH). (B) Mitochondrial transfer from sMSCs and MSC to CH tends to be increased in case of sMSC and sCH. Simulating an inflammatory environment using TNF-α in CH using did not influence speed and magnitude of mitochondrial transfer. Inflammation and senescence did not significantly change mitochondrial transfer.

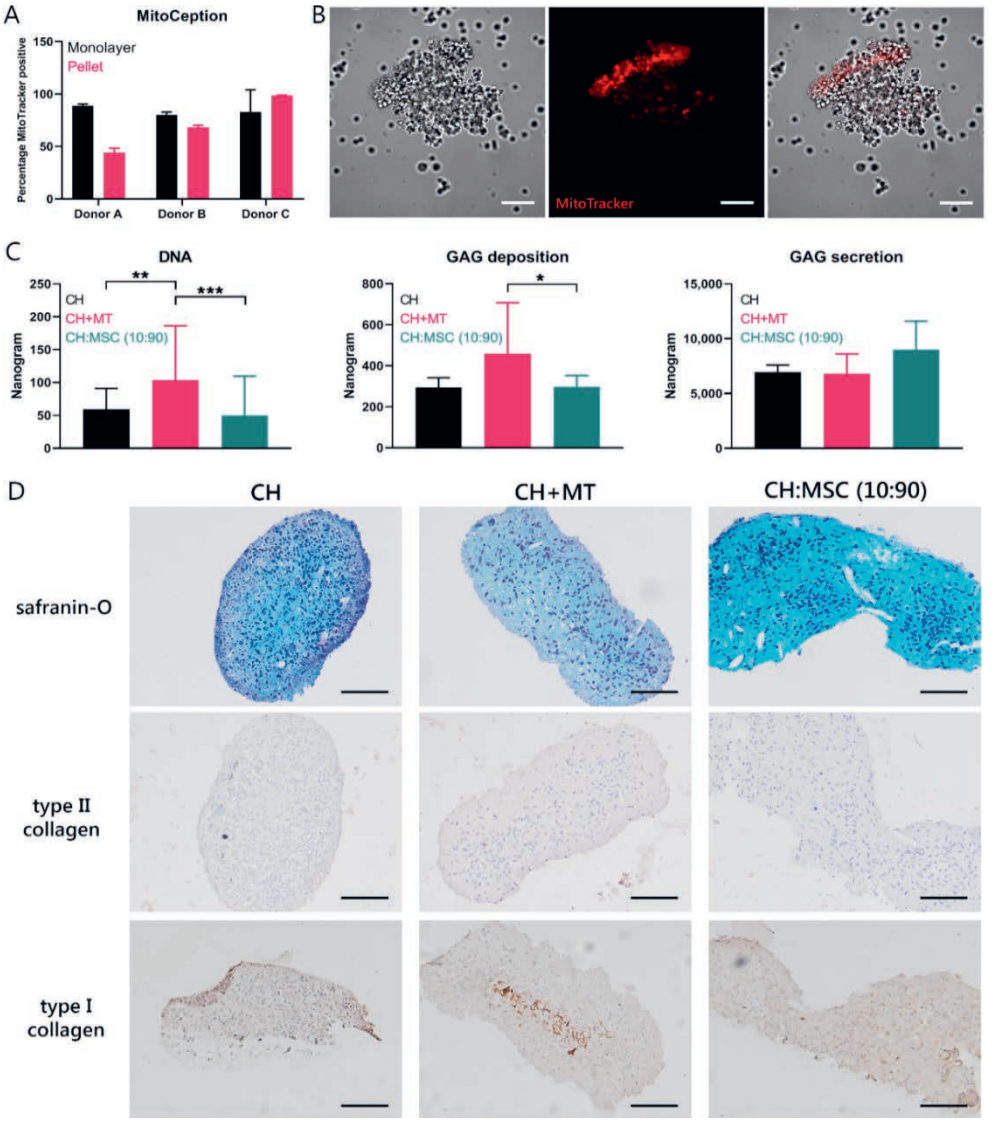
Transferred mitochondria exert a chondrogenic effect in chondropermissive culture

To investigate the effect of transferred mitochondria on cartilage extracellular matrix production *in vitro*, isolated mitochondria from MSCs were transferred into chondrocytes using MitoCeption during formation of cell pellets at initiation of the culture. Efficiency of the MitoCeption protocol in pellets was compared to the efficiency monolayers (Fig. 5A). Efficiency in pellets was comparable to monolayers in two donors, and lower in one donor (donor A, 89% ± 1.5 vs. 44% ± 4.4). Transferred mitochondria (in red) are detected in

chondrocyte pellets one day after initiation of the culture (Fig. 5B). Brightness of MitoTracker was higher in one side of the pellet, where more cells were stacked on top of each other. Stained mitochondria were found throughout the entire pellet. After 28 days of chondropermissive culture, the amount of DNA was higher in pellets that received mitochondria (CH+MT) compared to control chondrocyte pellets (CH) and chondrocyte and MSC cocultures (CH:MSC (10:90)) (Fig. 5C, left panel). Similarly, the amount of GAGs deposited in the pellets was higher compared to the chondrocyte and MSC coculture (Fig. 5C, middle panel). Secretion of GAGs into the culture medium was not different between the three groups (Fig. 5C, right panel). GAG deposition was insufficient to result in positive safranin-O staining in all groups. The type II collagen staining was negative in all pellets. There was a slight staining positive for type I collagen, especially in the centre of the chondrocyte with MSC mitochondria pellet (Fig. 5D).



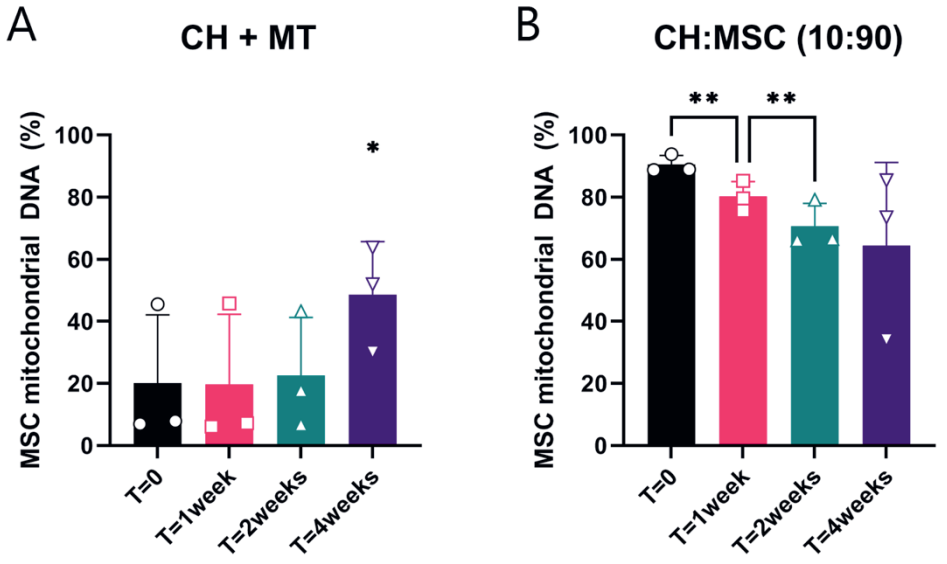
| Figure 4. Direct mitochondrial transfer through MitoCeption. Mitochondria (MT) of 900,000 mesenchymal stromal cells (MSCs) were isolated and transferred into chondrocytes (CH) via MitoCeption. (A) MSC-derived mitochondria, stained with MitoTracker (in red), localized intracellularly in chondrocyte monolayers. Scale bar = 100 µm. (B) Dose dependent effect of MitoCeption using increasing concentrations of MT transferred into monolayers of 100,000 chondrocytes. Symbols depict averages of two measurements ± standard deviation and the grey line shows linear regression. (C) Metabolic activity of chondrocyte monolayers as indicated by the conversion of resazurin to resorufin (ex: 560 nm, em: 590 nm) at 24 and 42 hours after MitoCeption with MT and senescent MTs (sMT), both derived from 900,000 MSCs. N = 3 donor combinations. (D) mRNA expression of aggrecan (ACAN), type II collagen (COL2A1)), and B-cell lymphoma 2 in chondrocyte monolayers at 2, 6, 26, and 46 hours after MitoCeption with MT and sMT derived from 900,000 MSCs. N = 3 donor combinations, 2 technical replicates per donor. *p<0.05



| Figure 5. Chondrogenic effect of direct transfer of mitochondria. (A) Efficiency of transfer of MSC-derived mitochondria (MT) and senescent MSC-derived mitochondria (sMT) into chondrocyte (CH) pellets compared to MitoCeption on CH monolayers depicted for the three donor combinations. (B) Mitochondria stained with MitoTracker (in red) are localized in chondrocyte after simultaneous pelleting of cells and mitochondria. Scale bar = 100 µm. (C) Quantification of DNA and glycosaminoglycan (GAG) deposition and secretion of CH pellets after 28 days of chondropermissive culture in pellets. Control groups consisted of CH only and CH and MSC in coculture (CH:MSC, ratio 10:90). *p<0.05, **p<0.01, ***p<0.001. (D) Histological analysis for proteoglycans (safranin-O), type II collagen, and type I collagen. Scale bar = 100 µm.

Mitochondrial and autosomal DNA quantification

Using a mitochondrial DNA SNP assay, 42 amplicons of the mitochondrial DNA were analysed to assess the contribution of CH and MSC DNA in pellets after 0, 1, 2, and 4 weeks of culture. In the chondrocytes that received mitochondria, the relative amount of mitochondrial MSC DNA increased between 0 and 4 weeks. In the CH and MSC cocultures, the relative amount of mitochondrial MSC DNA decreased from 0 to 1 week and from 1 to 2 weeks (Figure 6). At 4 weeks, the mitochondrial MSC DNA was 64% of the total DNA, whereas the autosomal DNA of the CH was the highest contribution at this time point (data not quantifiable). The mitochondrial DNA of cartilage biopsies of six patients, taken one year after treatment with 90% allogeneic MSCs and 10% autologous chondrons were also analysed from presence of mitochondrial DNA of the MSC donors. No donor mitochondrial DNA could be detected in the biopsies. Absence of autosomal MSC DNA was already determined before.



| Figure 6. Mitochondrial DNA from mesenchymal stromal cell (MSC) donors present in cultured pellets. (A) Chondrocytes (CH) cultured with mitochondria (MT) of MSCs. (B) Chondrocyte and MSC cocultures in 10:90 ratio. **, p<0.001

Discussion

In this study, we demonstrated bidirectional transport of mitochondria between chondrocytes and MSCs for the first time. Additionally, we identified three mechanisms responsible for mitochondrial transport, which are direct cell-cell contact, TNTs, and EVs. Finally, we showed compelling evidence of a chondrogenic effect of transferring MSC-derived mitochondria to chondrocytes through MitoCeption, indicating that

mitochondrial transfer might be one of the underlying mechanisms of MSC-induced chondrogenesis.

Mitochondrial transfer could have an important role in the prevention or treatment of this mitochondrial dysfunction. Transfer of mitochondria is initiated in the first hours of coculture and reaches an equilibrium after sixteen hours. The timing of mitochondrial transfer was not explicitly researched before, but others have found indications of mitochondrial transfer at 10-12 hours from MSCs to chondrocytes[419,420] and at 4 hours between MSCs and macrophages[416]. Interestingly, the transport of mitochondria occurs not only from MSCs to chondrocytes, but chondrocytes also transfer mitochondria to MSCs. Different explanations of this transfer could be hypothesized. The transfer of defective mitochondria from chondrocytes towards MSCs might be a damage signal, as transfer by cardiomyocytes and endothelial cells induced the anti-apoptotic function of MSCs and secretion of cytoprotective enzymes[437]. Moreover, defective mitochondria could be excreted by chondrocytes for degradation by MSCs, a process known as transmitophagy[438]. Lastly, depolarized mitochondria might be recycled by fusion with recipient cell mitochondria, increasing the metabolic state of the recipient[439]. To summarize, uptake of healthy MSC mitochondria by chondrocytes would benefit the metabolic state, while clearance of defective mitochondria could prevent the damage caused by oxidative stress.

Direct cell-cell contact, TNTs, and EVs are all mechanisms for mitochondrial transfer. The importance of direct cell-cell contact between MSCs and chondrocytes for *in vitro* chondroinduction has been shown earlier[440]. In direct cocultures, expression of gap junction protein connexin 43 was up-regulated[46]. Although mitochondria cannot physically pass gap junctions, connexin 43 is a mediator of mitochondrial transport [441]. In fact, connexin 43 was reported to be essential in EV-mediated mitochondrial transfer between MSCs and alveolar cells[442]. MSC-derived extracellular vesicles enhance chondrogenesis of osteoarthritic chondrocytes *in vitro*[47]. Here, mitochondria containing EVs were identified, indicating that mitochondria might play a role in the chondrogenic effect of MSC-derived extracellular vesicles. Mitochondrial transport through TNT is another frequent mechanism for transport of mitochondria[443], and it has been described to occur between human MSCs and renal tubular cells[444], cardiomyocytes[445], vascular smooth muscle cells[446], and endothelial cells[447]. TNTs likely play a pivotal role in the transport between MSCs and chondrocytes, which is shown for the first-time in the current study. Next to mitochondrial transfer, TNTs allow transfer of various cellular components, including proteins, lysosomes and RNA [443], which was not studied here but could provide other explanations of the MSC-chondrocyte coculture mechanism.

Upon addition of MSC mitochondria to chondrocytes, DNA content and proteoglycan deposition increased, thus mitochondrial transfer might play an important role in the chondrogenic effect of MSCs. Gene expression showed increased ACAN and BCL2 expression, indicating a possible chondroinductive effect as well as increased survival. Similarly, a higher expression of type II collagen and proteoglycans was described [421] in osteoarthritic chondrocytes that had taken up MSC mitochondria. In the current study, an increase in type II collagen deposition could not be demonstrated with immunohistochemistry. Overall, the deposition of type I and II collagen was low, and the GAGs present after pellet culture were not abundant enough to result in red Safranin-O staining. This could be attributed to the fact that no growth factors were added in the chondropermissive culture. In the study by Wang *et al*[421], increased chondrogenesis might be attributed to extracellular vesicles or trophic factors as well, as it was studied in co-culture. The increased chondrogenesis in chondrocytes with MSC mitochondria might be at least partially explained by promoting cell survival or proliferation in chondrocytes by restoring the energy balance[448], since matrix production per cell did not increase in chondropermissive cultures. Another effect of mitochondrial transfer might be the regulation of autophagy[449], since autophagy is activated under hypoxic stress conditions[450] and protects against mitochondrial dysfunction. This interaction could be the focus of follow-up research.

In vitro, the contribution of DNA of transferred MSC mitochondria increased between 2 and 4 weeks, indicating that there is a sort of selective advantage of MSC mitochondria above CH mitochondria in culture, Moreover, the contribution of MSC mitochondrial DNA in co-cultures exceeded the contribution of MSC autosomal DNA in these cultures. This could indicate that this positive selection for MSC mitochondria also takes place in co-cultures. However, the fate of transferred mitochondria and the occurrence of mitochondrial transfer *in vivo* remain unknown, as we could not detect mitochondrial DNA of donor MSCs in cartilage biopsies taken one year after cell therapy with autologous chondrons and allogeneic MSCs. Earlier studies have shown presence of human mitochondrial DNA up to 28 days in murine macrophages[439]. Similarly, the autosomal MSC DNA decreases in 28 days of coculture[46], and no autosomal MSC DNA can be detected *in vivo* after one year [43]. The possibility that mitochondrial transfer occurs solely *in vitro* cannot be excluded, but mitochondrial transfer has been shown between MSCs and cardiac[437] or alveolar cells[442] *in vivo*. More likely, donor mitochondria are not retained in receiving chondrocytes over a prolonged period.

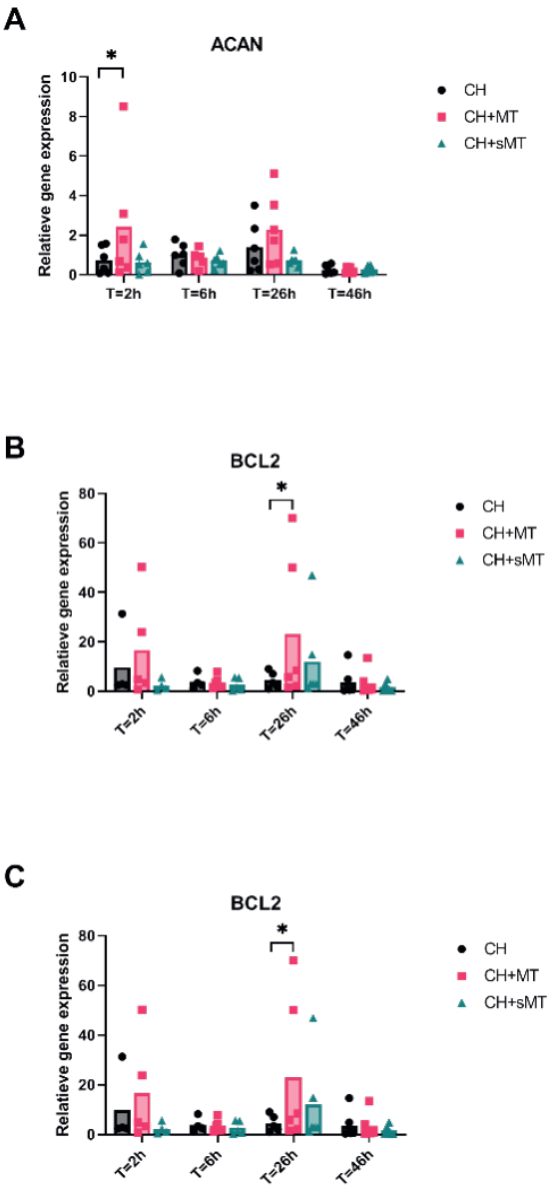
Limitations

In contrast to our hypothesis, mimicking cell stress conditions using induction of inflammation or senescence did not significantly alter mitochondrial transfer. Similarly, inflammation induction by IL-1β treatment did not alter total transfer during 10 hours of coculture of chondrocytes and MSCs as described by Bennett *et al*[451]. Inflammation might not play an important role in mitochondrial transfer, or the inflammatory phenotype resulting from these treatments are not well retained *in vitro* after removing the factors. Likewise, senescence did not change mitochondrial transfer significantly. *In vivo,* senescence is induced by mechanical stress in the rim of cartilage defects[452] and drives aging and related pathologies. In osteoarthritis, senescent cells excrete catabolic factors causing cartilage degradation. Here, senescence induction by mitomycin C did not alter total mitochondrial transport. Senescence and the resulting formation of reactive oxygen species might compromise the quality and number of mitochondria, but this was not investigated here. The generalizations of this study are limited by the *in vitro* character of the experiments. However, primary human cartilage defect chondrocytes were used together with MSCs from our Good Manufacturing Practices (GMP) certified cell therapy facility in order to closely mimic the clinical situation.

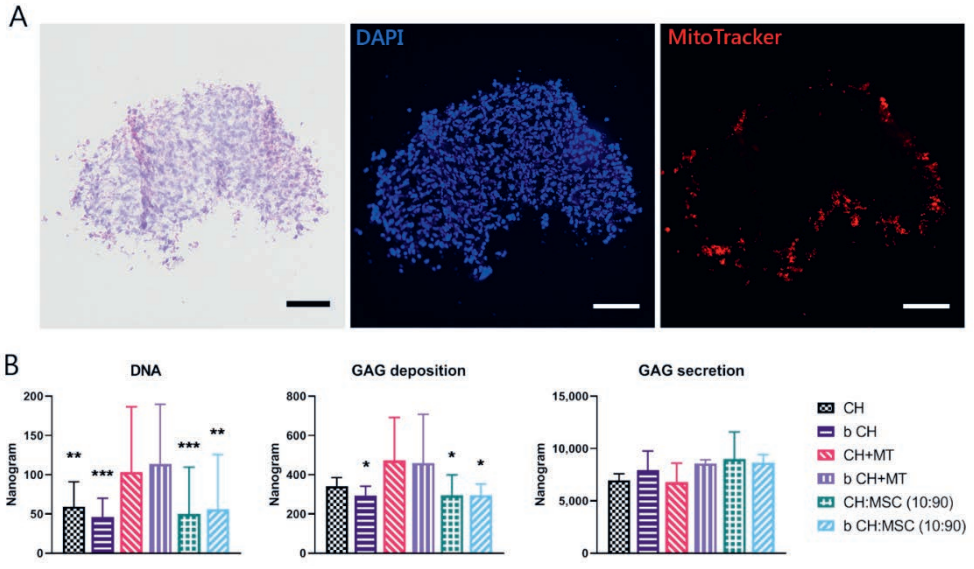
Conclusion and implications

The presented results demonstrate the role of mitochondrial transport in the chondroinductive effect of MSCs on chondrocytes. Treatment with MSCs or mitochondria in the acute phase of cartilage injury might prevent or treat mitochondrial dysfunction and subsequent ROS accumulation, and therefore counteract one of the first steps towards development of osteoarthritis[425]. Moreover, pre-selection of MSCs for their capacity to donate functional mitochondria or take up damaged mitochondria for degradation could enhance the effect of MSCs in cocultures. Eventually, potential of MSC-derived mitochondria as a method for cell-free therapies could be explored. Cell-free therapies have advantages including lower safety profiles, and homogenization of treatment. However, limiting treatment to mitochondria disregards other possible functions of MSCs such as transmitophagy of defective mitochondria and reactivity to damage signals with trophic factors, extracellular vesicles, or TNT communications. Additionally, efficient long-term storage of mitochondria should be investigated and chondroinductive potency upon thawing should be confirmed[453].

Appendix



| Figure S1. Direct mitochondrial transfer through MitoCeption. Mitochondria (MT) of 900,000 mesenchymal stromal cells (MSCs) were isolated and transferred into chondrocytes (CH) via MitoCeption. mRNA expression of aggrecan (ACAN), type II collagen (COL2A1; both markers for chondrogenesis), and B-cell lymphoma 2 (BCL2; marker for cell survival) in chondrocyte monolayers at 2, 6, 26, and 46 hours after MitoCeption with MT and sMT derived from 900,000 MSCs. ACAN expression was increased in CH+MT compared to CH right after MitoCeption (T=2h), BCL2 was increased in CH+MT 26 hours after MitoCeption (T=26h). N = 3 donor combinations, 2 technical replicates per donor. *p<0.05



| Figure S2. Chondrogenic effect of periodically repeating MitoCeption. A subset of chondrocyte (CH) pellets received additional doses of mitochondria (MT) at 7 and 14 days during chondropermissive culture. (A) Haematoxylin and eosin staining of re-MitoCepted pellet at 14 days (left panel), 4',6-diamidino-2-phenylindole (DAPI, middle panel), and MitoTracker (right panel) of the same pellet show detection of transferred mitochondria in the perimeter of the pellet. Scale bar = 100 µm. (B) Quantification of DNA and glycosaminoglycan (GAG) deposition and secretion of CH pellets after 28 days of chondropermissive culture in pellets. Group bCH+MT received additional doses of mitochondria (boost) at 7 and 14 days in culture. Additional control groups bCH and bCH:MSC (10:90) were also subjected to centrifugation at these time points. *p<0.05, **p<0.01, ***p<0.001.

Table S3. SNP's, primers and input in primermix **Part 1**

Marker	Forward primer	Reverse Primer	µl (f+r mix 50 pmol/ µL)
mt239(234-247)	tgtaaaacgacggccagtGTGTTAAT-TAATTAATGCTTgTrrGACAT	caggaaacagctatgaccGAAAGTG-GcTGTGCAGACrTT	15,6
mt456+462+477(455-478)	tgtaaaacgacggccagtcAC-CCCcCAACTAACACATTATTT	caggaaacagctatgacccGgGG-gTTGTaTTGrTGrGAT	14,4
mt930(927-931)	tgtaaaacgacggccagtCACACGAT-TAACCCAAGyCAATA	caggaaacagctatgaccTGATcTA-AAACACTCTTTACGCCG	4,4
mt1018(1017-1031)	tgtaaaacgacggccagtAAAAACTC-CAGtTGACACAAAAT	caggaaacagctatgaccGCTATTGT-GTgTTCAGAtATGTTAAAG	4,4
mt1189(1188-1194)	tgtaaaacgacggccagtGACCTGGC-GGTGCTTCAT	caggaaacagctatgaccCGATTA-CAGAACAGGCTCCTCTA	5,0
mt1438(1431-1446)	tgtaaaacgacggccagtGGTCGAAG-GTGGATTTAGCA	caggaaacagctatgaccGGCCcT-GTTCAACTAAGCAC	4,1
mt1738(1734-1748)	tgtaaaacgacggccagtAACCTTAg-CcAAACCATTTACC	caggaaacagctatgaccCGCCAG-GTTTCAATTTCTATCG	3,8
mt2706(2698-2708)	tgtaaaacgacggccagtATTGACCT-GCCCGTGAAGAG	caggaaacagctatgaccGG-GTCTTCTCGTCTTGCTGT	7,5
mt3010(3006-3013)	tgtaaaacgacggccagtCTCGAT-GTTGGATCAGGACA	caggaaacagctatgaccACCTTTA-ATAGCGGCTGCAC	5,6
mt3333(3330-3337)	tgtaaaacgacggccagtCCATGgC-CAACCTCCTACT	caggaaacagctatgaccCCATTGC-GATtAGAATGGGTA	6,5
mt3423(3421-3441)	tgtaaaacgacggccagtCTACG-CAAAGGCCCCAAC	caggaaacagctatgaccCGTCAGC-GAAgGGyTGTAG	15,0
mt3516(3512-3519)	tgtaaaacgacggccagtAAACCCgc-cACATCTrCCATCA	caggaaacagctatgaccAAGGTcGG-GGCGGTGAT	6,3
mt3796(3796-3801)	tgtaaaacgacggccagtAGTG-GCTCCTTTAACCTCTCC	caggaaacagctatgaccGAGGT-GTTCTTGTGTTGTGAT	6,3
mt4793(4792-4795)	tgtaaaacgacggccagtCATAaTaGC-TATAGCAATAAAACTAGGAA	caggaaacagctatgaccGGGACT-CAGAAGTGAAAGGGG	5,0
mt5004(4998-5010)	tgtaaaacgacggccagtTAAAC-CAAACCCArCTACGC	caggaaacagctatgaccCCTATgTG-GGTAATTGAGGAGT	3,8
mt6371(6364-6376)	tgtaaaacgacggc-cagtTCTTCTCCTTACACCTAGCAG	caggaaacagctatgaccTGAT-GAAaTTGATGGCCCCTAAG	4,4
mt6776(6771-6785)	tgtaaaacgacggccagtCCTaGG-gTTTATCGTGTGAGCA	caggaaacagctatgaccCGTGTGTC-TACGTCTATTCCTAC	3,5
mt7768(7762-7780)	tgtaaaacgacggccagtATACTAA-CATCTCAGACGCTCA	caggaaacagctatgaccTGATGGCG-GGcAGGATAGT	3,8
mt8448(8445-8460)	tgtaaaacgacggccagtACTATTcCT-CATCACCCAACTAA	caggaaacagctatgaccGTGAGG-GaGGTAgGTGGTAG	6,9
mt8697(8694-8705)	tgtaaaacgacggccagtTGACTAAT-CAAACTAAcCTCAAAACA	caggaaacagctatgaccGGTTC-GTCCTTTAGTGTTGTGT	3,8
mt9716(9715-9724)	tgtaaaacgacggccagtGCACTGCT-tATTACAATTTTACTGG	caggaaacagctatgaccTCTGAG-GCTTGTAGGAGGGTA	3,8
mt10034(10028-10035)	tgtaaaacgacggccagtGTATA-AATAGTACCGTTAACTTCCAATT	caggaaacagctatgaccGTTTAT-TACTCTTTTTTGAAtGTTGTCAA	4,4

Part 2

Marker	Forward primer	Reverse Primer	µl (f+r mix 50 pmol/ µL)
mt10211(10198-10212)	tgtaaaacgacggccagtCTTcGAC-CCTATATyCCCCG	caggaaacagctatgaccGGTaATAGC-TACTAAGAAGAATTTTATGG	5,0
mt10873(10872-10876)	tgtaaaacgacggccagtCCACAG-CCTAATTATTAGCATCATCC	caggaaacagctatgaccAG-GTTGtTGTtGATTTGGTTA AAAAATAG	4,4
mt11176(11164-11186)	tgtaaaacgacggccagtACcTTggc-TATCATCACCCG	caggaaacagctatgaccGAaGTATGTg-CCTGCGTTCA	3,1
mt11251(11250-11260)	tgtaaaacgacggccagtCCTTC-CCCTACTcATCGCAC	caggaaacagctatgaccAGTGAG-CCTAGgGTGTTGTG	5,0
mt11332(11330-11340)	tgtaaaacgacggccagtC-CCAAGAaCTATCAAACTCCTGA	caggaaacagctatgaccAAGCTATTGT-GTAaGCTAGTCATATT	3,8
mt11467+11485(11466-11490)	tgtaaaacgacggccagtAGTACTTG-CCGCAGTACTCT	caggaaacagctatgaccTGAGAAT-GAGTGTGAGGCGT	4,4
mt11719(11709-11725)	tgtaaaacgacggccagtCGGCG-CAgTCATtCTCATAA	caggaaacagctatgaccGCTAGG-CAGAATAGTArTGAGGA	4,4
mt11812(11810-11830)	tgtaaaacgacggccagtTCCTCTCT-CAAGGaCTTCAAACT	caggaaacagctatgaccAG-GCTTGCTAgAAGTCATCA	4,4
mt11947(11941-11951)	tgtaaaacgacggccagtTCTCCT-GATCAAaTATCACTCTCCT	caggaaacagctatgaccAGGGcTGT-GAcTAGtATGTTGA	3,8
mt12633(12629-12641)	tgtaaaacgacggccagtCCCtGTAG-CATTgTTCGTTACAT	caggaaacagctatgaccGgTCT-GAGTTTATATATCACAGTGAGAAT	3,8
mt12705(12693-12705)	tgtaaaacgacggccagtGAcCCAAA-CATTAATCAGTTCTTCAA	caggaaacagctatgaccGTaACTA-AGATTAGtATGGTaATTAGGAA	5,0
mt13617(13613-13623)	tgtaaaacgacggccagtAAGCG-CCTATAGCACTCGAA	caggaaacagctatgaccCGAGGTt-GaCCTGTTAGGGT	5,6
mt13789(13789-13791)	tgtaaaacgacggccagtCTTCCAAA-CAACAaTCCCCCTC	caggaaacagctatgaccGCGAGGGCt-GTGAGTTTTAG	7,5
mt14470(14461-14471)	tgtaaaacgacggccagtTACTCCT-CAATAGCCATCGC	caggaaacagctatgaccGGGGaATGaT-GGTTGTyTTTG	5,3
mt14766(14764-14788)	tgtaaaacgacggccagtCCAATGAC-CCCaATACGCAA	caggaaacagctatgaccAGGTCGAT-GAaTGAGyGGTT	15,6
mt14783+14793+14798 (14780-14801)	tgtaaaacgacggccagtACG-CAAAAcTAaCCCCCTAATA	caggaaacagctatgaccATGGGGTGG-GGAGGTCGA	11,3
mt15257(15252-15262)	tgtaaaacgacggccagtTCAAT-GaaTCTGAGGaGGCTACT	caggaaacagctatgaccAAGAATCGT-GTGAGGGTGGG	4,4
mt15775(15774-15776)	tgtaaaacgacggccagtCTGAaTCG-GAGGaCAACCAG	caggaaacagctatgaccCCAATGATG-GTAAAAGGGTAGC	6,6
mt15904+15907(15896-15908)	tgtaaaacgacggccagtTCAAATGgg-CCTGTCCTTGT	caggaaacagctatgaccTccGGtTTA-CAAGACTGGTGT	4,3
mt16162(16153-16176)	tgtaaaacgacggccagtCgGTAC-CAtAAAtACTtgrCyACCT	caggaaacagctatgaccATGGGGAGG-GGGTkTtGAT	8,8
Nuclease free water	-	-	58,6

12

General discussion and summary

Knee joint preservation is an unmet medical need and tissue repair and regeneration can provide great benefit to patients, both by symptom relief in the short term as well as maintained function and mobility in the long term. In this thesis I described our explorations for such patient centered innovations. The following paragraphs summarize the main findings.

Summary

We **first aimed** to optimize currently available treatments for knee preservation and addressed this aim by reviewing existing literature and studying a large patient cohort at the outpatient Mobility Clinic of the University Medical Center Utrecht. We found that ACL reconstruction protects the meniscus from secondary injury, as indicated by level II evidence. Moreover, failure of meniscus repair is more frequent in ACL deficient knees (chapter 2). This illustrates the importance of the function of the ACL in maintaining stability, which is a prerequisite for successful repair and for prevention of secondary damage. We were also interested in the effect of intra-articular injections with autologous conditioned plasma (ACP®) for treatment of knee osteoarthritis (OA), and possible targets for optimization of patient selection or product composition. Intra-articular ACP® injections led to a statistically significant, but not clinically relevant improvement in patient reported outcomes scores (chapters 3 and 4). As the patient reported improvements were comparable to placebo effects reported elsewhere, there is a strong need for optimization of ACP® treatment. The composition of the injected ACP® in 100 patients showed variations in platelet, leucocyte and erythrocyte concentrations. This variable content did not correlate with changes in individual outcomes. A largely different product composition might be necessary to improve outcomes, or other factors than those measured here determine the potency of ACP®. Patient selection might improve success rates, as younger patient age and posttraumatic OA predicted lower improvement in patient reported outcome measures. Overall, ACP® treatment for knee OA should be optimized to obtain satisfactory outcomes.

The **second sub-aim** of this thesis was to evaluate the potential of different cell types for regeneration and tissue repair. Cell-based meniscus regeneration or repair was evaluated using a systematic literature review of preclinical research, in order to identify optimal cell types (chapter 5). Different sources of mesenchymal stromal cells (MSCs) improved repair and regeneration, among which MSCs from adipose tissue, bone marrow, synovium, and meniscus. Autologous as well as allogeneic cells showed potential for clinical translation and both have their own advantages. Furthermore, cell-based approaches led to better structural organization of transplants or repair tissue

when compared to cell-free methods, regardless of which cell types were used. Therefore, cell-based approaches should be preferred for tissue repair or regeneration in the meniscus, which further stresses the importance of the biological processes in joint preservation. We isolated and characterized progenitors from cartilage or meniscus, and evaluated the potential of these cells for cell therapy or tissue engineering purposes. The presence of progenitor cells in the meniscus inner zone shows promise as this part of the meniscus is often regarded incapable of regeneration (chapter 6). Meniscus progenitors showed multilineage differentiation with an increased chondrogenic differentiation compared to meniscus cells. Cartilage progenitors from OA and healthy tissue did not form calcified matrix and cultures were negative for type X collagen (chapter 7). Thus, cartilage progenitors have a low tendency for hypertrophic differentiation, as opposed to bone marrow MSCs. All progenitor populations had a high proliferation rate and colony forming ability. Importantly, in addition to these advantages in cell expansion, the progenitors were capable of cartilage and meniscus matrix formation upon redifferentiation. The ease of isolation, high proliferative capacities, and continued redifferentiation ability make progenitors interesting cell types for tissue-engineering purposes. Furthermore, progenitors are a promising therapeutic target to enhance endogenous repair mechanisms.

The **final sub-aim** of this thesis was to promote regeneration using trophic effects and cell communication. We first assessed if growth factors and platelet lysate promote migration of meniscus cells and MSCs (chapter 8). platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1) and platelet lysate were shown to enhance migration of meniscus cells and MSCs. Furthermore, we were able to capture growth factors on the Collagen Meniscus Implant (CMI®) in order to lure the cells present in the joint towards the implant. This approach could be used as a cell-free method to attract cells to the implant and aid tissue formation. Ideally, a combination or gradient of growth factors could be used. A growth factor that stimulates migration, such as PDGF, could attract cells, after which another growth factor could stimulate the cells to form extracellular matrix. This shows proof-of-principle of an innovative off-the-shelf solution for meniscus replacement. Next, we aimed to improve the meniscus scaffold mechanical characteristics by using melt-electrowriting to mimic native architecture. Furthermore, to improve biological adaptability the chondroinductive effect of MSCs in combination with meniscus cells was employed (chapter 9). The scaffold showed good compressive properties *in vitro*, and a basal level of meniscus matrix formation by the seeded cells. As we designed this scaffold with materials approved for clinical use with a clinically feasible cell number and type for one-stage treatment, this show promise for clinical translation.

The chondroinductive effect of MSCs is also employed in IMPACT for cartilage cell transplantation. IMPACT is a one-stage treatment combining autologous chondrons and allogeneic bone marrow MSCs for the treatment of cartilage defects. In order to bring the IMPACT procedure a step further to clinical practice, we designed a study protocol for a phase III randomized controlled trial (RCT). Efficacy and cost-effectiveness of IMPACT versus nonsurgical care will be assessed in a crossover RCT (chapter 10). Moreover, safety of IMPACT will be evaluated based on surgery of 30 to 60 patients. The data gathered in this trial could aid in the clinical translation of this regenerative treatment but also provide additional safety and efficiency data on the use of allogeneic MSCs in the joint. This is valuable information that could also be useful for applications in other tissues such as the meniscus. Lastly, we aimed to further unravel the mechanisms behind the effect of MSC co-cultures by studying the communication between MSCs and chondrocytes using mitochondrial transfer (chapter 11). The bidirectional transfer of mitochondria between MSCs and chondrocytes takes place via tunneling nanotubes, more broad cell contact and extracellular vesicles. The exact function of this transport is currently unknown, but literature suggest that chondrocytes could excrete defective mitochondria as a damage signal for MSCs. In turn, MSCs can transfer mitochondria to chondrocytes as a means of cell rejuvenation. In our study, the transfer of MSC mitochondria to chondrocytes increases the amount of DNA and proteoglycan deposition in the cultures. The transfer of mitochondria is one of the methods of communication between MSCs and chondrocytes that contributes to the chondroinductive effect of MSCs. This could further enhance MSC based chondrogenesis by improving MSC donor selection.

Discussion

The research described in this thesis has added to our understanding of knee joint preservation. Some of the applications described here show potential for clinical translation that could improve patient care. For these approaches the next steps should be undertaken to move from bench to bedside. In addition, we have learned which approaches did not work or require further optimization, which is also of great value. The following paragraphs discus the lessons learned and identify remaining challenges for knee joint preservation.

Setting the stage for knee preservation

We need to set the stage for successful regeneration or tissue repair, by addressing underlying causes of cartilage or meniscus injury. One of such underlying causes is instability, which results in pathological loading caused by excessive or abnormal translocation or rotation and causes damage to cartilage and meniscus[454,455]. Stability is maintained primarily by the cruciate and collateral ligaments and secondary by the menisci and the muscles around the knee joint. The ACL is one of the most frequently injured structures in the knee[456], which prevents anterior translation of the tibia and provides rotational stability. As demonstrated in chapter 2, ACL deficiency increases the odds of failure of meniscus repair and the incidence of secondary meniscus injury. The necessity of knee stability for protection of the meniscus and its repair is not a new concept per se, as knee stability is also a prerequisite for cartilage repair[457]. In fact, the incidence of cartilage defects is almost 80% in chronic ACL deficient knees[458]. Interestingly, the meniscus functions as a secondary stabilizer in ACL deficient knees, and partial resection of the posterior horn of the medial meniscus increases instability[459]. Additionally, meniscectomy increases anterior knee laxity compared to meniscus repair in the ACL reconstructed knee[115]. This all underlines the importance of maintaining stability by preservation of ACL and meniscus function. Secondly, limb alignment should be considered. In physiological alignment of the leg, approximately 75% of the body weight is transferred through the medial side of the knee[460]. Varus malalignment further adds to this pressure on the medial compartment and negatively impacts the repair in cartilage defects of the medial femoral condyle[460]. Therefore, malalignment of over 5 degrees should be corrected when treating cartilage or meniscus injury, and correction should be considered in cases between 1 and 5 degrees varus[461]. Lastly, the chronicity of the pathology is an important factor in the success of regenerative treatments. Cartilage transplantations 10 weeks after injury have inferior outcomes compared to immediate transplantations[462,463]. Similarly, a 3 week delay of meniscus repair decreases tear healing[211]. The reduction in healing of older injury could be attributed to the cascade of

pathological derangements that occur after trauma. Progression of cartilage or meniscus injury to OA causes the failure of regenerative treatments. Regenerative treatments address a single factor, but OA is a more complex disease that needs a different approach. Identifying the signs that mark this 'point of no return' could improve outcomes of regenerative treatments. After this, focus should shift to joint preservation by symptom management or slowing disease progression. Alternatively, all involved factors should be addressed for a curative approach.

PRP was proposed as such joint preserving treatment for OA due to the presence of anti-inflammatory cytokines and abundance of growth factors in platelets. In an *in vitro* model of OA, treatment with PRP decreased inflammation, increased gene-expression of cartilage matrix genes, and decreased expression of genes associated with cartilage degradation[464]. In our clinical practice, ACP® injections do not lead to a clinically relevant symptom relief in knee OA. In addition, evidence of structural improvements after PRP treatment is reported in many animal studies[465], but there are no indications of disease modifications in humans. Often, the product composition of PRP is blamed for dissatisfactory findings. The large variations in product composition make it difficult to interpret and compare contradicting studies and provide an easy explanation for potential treatment failures. The presence of too little platelets[63], too little growth factors[466], or too much leucocytes[467] is blamed for treatment failure. The varying outcomes after PRP treatment might be better explained by the complexity of OA. PRP treatment might be suitable for symptom relieve in some of the many phenotypes of OA. For example, PRP preparations pre-selected for anti-inflammatory capacities or pain modulation might improve clinical outcomes. As there is no clinical evidence of structural improvement after PRP treatment, PRP selection should not focus on regenerative capacities. Some of the components of PRP, such as VEGF[468] or pro-inflammatory cytokines[469] could negatively impact the joint. Therefore, removal of these factors might improve outcomes. In order to be able to overturn the cascade of events in OA, a drastically other approach to the use of PRP should be considered. Allogeneic PRP was safe in a pilot study including 60 patients[470], allogeneic PRP acquired from young patients could potentially improve outcomes in OA treatment compared to the autologous PRP of middle-aged or elderly patients. Namely, when the bloodstreams of young and aged mice are connected, the muscle progenitors of old mice rejuvenate, while those of young mice age[471]. This indicates that the plasma contains factors that can modulate aging and could potentially improve PRP treatment as it is a more regenerative approach. Lyophilizing this allogeneic (young) PRP could increase shelf-life and standardization[472], but the effect of lyophilizing on these modulating factors should be investigated. Additionally, platelets are known to excrete vesicles that contain intact

mitochondria[473]. Selecting PRP or even loading PRP with high amounts of mitochondria might improve outcomes of PRP treatment, as mitochondrial dysfunction and oxidative stress are both hallmarks in the pathophysiology of OA. Although further research into these optimized forms of PRP could improve clinical outcomes, it remains debatable whether PRP will meet its promise. Pre-manipulating PRP or using selected allogeneic PRP will increase the regulatory burden together with the price of treatment. The substantially increased costs should be justified by a large improvement in treatment outcomes. The quick release and short half-life of the growth factors in PRP are another challenge to be tackled. Altogether, it is likely that the complexity of OA warrants a more complex solution then proposed so far.

The potency of cells

Until we obtain sufficient understanding of the biological processes that underlie aging and regeneration, cells are needed to facilitate regeneration. The presence of viable cells enables adaptation to the joint environment by reactivity to other cells, mechanical stimuli, growth factors, and cytokines. As described in chapter 5, cell-based treatments outperformed cell-free treatments in the case of meniscus repair and regeneration. The shrinkage in acellular approaches for meniscus replacement like the CMI®[14,15] or cryopreserved meniscus allografts[12] further underline the importance of viable cells for maintaining the mechanical function in the joint. Similarly, impaired cell viability is an important predictor of inferior long-term outcomes of osteochondral allograft transplantation[474]. Therefore, storage conditions of living transplants are now optimized for maximal cell viability[475,476]. The crosstalk between the cells present in the joint is still largely unknown to us. Due to the complexity of these interactions, replacing the cells by individual growth factors or stimuli is likely not successful in the short term.
One of the remaining questions is how many cells are needed. For cartilage cell transplantations, a cell number of 1-2 million cells per cm^2 is used[392], based on satisfactory results in the first case series of autologous chondrocyte implantation (ACI)[282]. Using a higher cell number might improve outcomes, as this mimics the 10 million cells per mL present in articular cartilage[477] or the density during embryonic cartilage development[478]. However, harvesting more cartilage for cell isolation results in increased donor site morbidity. In addition, prolonged culture expansion of chondrocytes increases costs, leads to dedifferentiation, and increases genetic instability[479]. On the other hand, using a lower cell number would reduce culture expansion time and therefore treatment costs, but effects on treatment outcomes are unknown. To date, the effect of different dosages for cartilage cell transplantations is only reported once. In this study of spheroid-based ACI, a factor 10 difference in dose did not lead to differences in safety and

efficacy[480]. The minimal or optimal number of cells required for cartilage cell transplantations is therefore unknown. For meniscus cell therapy, no studies comparing different cell densities in a cell-seeded scaffold exist. In chapter 9, 2.5 million cells per mL were used, based on the good results in cartilage transplantation and the feasibility to obtain sufficient cells for one-stage treatment. The cellularity of the native meniscus ranges from 10 million cells per mL in the avascular zone to 28 million cells per mL in the vascular zone[481]. As this is a higher density than in articular cartilage, a higher cell density might be beneficial for meniscus regeneration. Alternatively, different densities could be used in the vascularized and avascular zone, mimicking the native gradient of densities. The effect of different cell densities should be compared *in vivo*.

One stage treatment

A cartilage biopsy results in approximately 180,000 – 455,000 cells[282], and 1-2 million cells per mL are currently used for cartilage cell transplantations. Therefore, culture expansion and two surgical procedures are often required to obtain sufficient cells for transplantation. However, due to the high patient burden, difficult logistics and high treatment costs of two-stage, different one-stage approaches are emerging.
In chapter 10, a study protocol for a phase III RCT comparing IMPACT to nonsurgical care is presented. In this one-stage treatment, 90% allogeneic MSCs supplement 10% autologous chondrons. The MSCs function as medicinal cells, as the MSC donor DNA is not present in the repair tissue after 1 year[43]. Instead of differentiation of MSCs, chondroinductive effect of MSCs contributes to the success of this treatment. In the last two decades, the functions of MSCs have been extensively studied. MSCs are known to have anti-inflammatory properties[410], and signal through trophic factors[411], and extracellular vesicles[412]. All these functions of MSCs likely contribute to the chondroinductive effect of MSCs. Moreover, in chapter 11 the role of mitochondrial transfer between MSCs and chondrocytes has been added to this list. Although the exact meaning of this transport is unclear, the transfer of MSC mitochondria to chondrocytes increases the amount of DNA and total proteoglycans in redifferentiation cultures. This suggests that the transfer of mitochondria from MSC to chondrocyte increases proliferation or cell survival, rather than increasing the proteoglycan production per cell. The reaction of MSC to danger signals by releasing anti-apoptotic factors and relieving oxidative stress through mitochondrial transfer[437], could indicate that MSCs play a role in physiological repair situations. The transfer of defective mitochondria from chondrocytes to MSCs could be a form of transmitophagy[41], in which the MSCs are used for the breakdown of defective mitochondria. Further understanding of the functions of MSCs might improve current one-stage treatments like IMPACT by better MSC selection.

MSCs could be selected for their ability to donate healthy mitochondria or take up defective mitochondria. Eventually, MSCs might even be 'enhanced' for their functions, for example by loading them with high amounts of healthy mitochondria, or modifying them to excrete high amounts of certain growth factors.
The use of allogeneic cells for cartilage cell transplantation could also facilitate one-stage treatment. As described in chapter 7, progenitors can be isolated from cartilage tissue using fibronectin selection. Cartilage progenitors exhibit many of the MSC-criteria[39] such as the expression of MSC surface-markers and plastic adherence, although they do not form mineralized tissue *in vitro*. Cartilage progenitors might also demonstrate immunomodulatory responses[316]. The lack of specific progenitor markers and the lack of specificity of the MSC markers make it hard to fully characterize and identify the progenitor populations[482]. The existence of a (heterogenous) cartilage progenitor population does have potential for cartilage transplantations and tissue engineering. Cartilage progenitors can be extensively culture expanded at high proliferation rates, while maintaining the chondrogenic redifferentiation potential and low tendency for hypertrophic differentiation. Therefore, the clinical use of cartilage progenitors is not limited by the poor availability of cartilage tissue. In addition, cartilage progenitors do not express Human Leukocyte Antigen – DR isotype (HLA-DR) and are therefore an attractive cell population for allogeneic cartilage transplantations. However, when autologous and allogeneic cartilage progenitors were compared for the treatment of cartilage defects in an equine model[483], autologous cells improved quality of the repair tissue on histologic and macroscopic evaluation compared to allogeneic progenitors. If this warrants the use of autologous cells in humans, progenitors could not be used in a one-stage approach, which would be a great drawback of such application. Alternatively, the chondroinductive and anti-inflammatory capacities of the cartilage progenitors could be further evaluated to explore the possibility to replace MSCs with the progenitor cells for applications in combination with autologous chondrons.
Minced cartilage in combination with fibrin gels, collagen membrane[484], or PRP[485] is increasingly used in the treatment of cartilage defects as a one-stage treatment. To date, a limited amount of evidence on the outcomes of minced cartilage treatment is available and histological confirmation of hyaline cartilage formation is lacking. If indeed cartilage defects could regenerate after applying minced cartilage, this is an encouraging single-stage treatment with easy logistics and minimal manipulation. Cartilage fragments have a low cellularity, therefore success of minced cartilage treatment could indicate that only a small number of cells is necessary for cartilage regeneration instead of the 1-2 million cells per cm^2 that are currently used. This knowledge could facilitate improvements or simplifications in other treatments such as

IMPACT and ACI. Direct comparisons between cell therapies and minced cartilage approaches are currently lacking, as well as thorough histological evaluation of treatment outcomes of minced cartilage, therefore caution with these techniques is warranted.

No cell therapies for meniscus replacement are currently available in the clinic. In this thesis, different one-stage meniscus replacement strategies were described. For a cell-based approach, a combination of MSCs and meniscus cells was used. The combination of 80% allogeneic MSCs and 20% autologous meniscus cells increases meniscus extracellular matrix formation and is a clinically feasible cell number for one-stage treatment[49]. For meniscus replacement, the cells are ideally applied in a 3D scaffold that will provide immediate mechanical support, until the scaffold slowly dissolves and is replaced by newly formed meniscus tissue. The scaffolds that are currently available clinically (Actifit and the CMI®) are prone to shrinkage, while meniscus tissue formation is limited. In addition, the Actifit and the CMI® do not take the architecture of native collagen fiber into account. In chapter 9, the MSCs and meniscus cells were applied in a melt electrowritten scaffold that was based on the native tissue architecture. Redifferentiation culture led to a basal level of meniscus matrix formation in the scaffolds, and the scaffolds had a higher Young's modulus compared to the CMI®. Following up on this study, meniscus regeneration and durability of the scaffolds should be confirmed *in vivo*. Promising results *in vivo* could result in rapid clinical translation, as the safety of allogeneic MSCs has been extensively demonstrated and clinically approved materials were used for electrowriting of the scaffolds.

Employing the endogenous cells in the joint

Further understanding of the biologic processes of tissue repair and regeneration could eventually lead to cell-free treatments. Cell-free treatments have advantages like lower safety profiles, lower costs, and higher standardization or homogenization of treatment. For instance, cell free treatments could be based on the chondroinductive effects of MSCs. Further understanding of the communication between MSCs and chondrocytes or meniscus cells might enable the development of these cell-free methods. Instead of providing allogeneic MSCs, the signals that MSCs excrete could be provided. However, cell-free methods are often based on only one mechanism of action, for example by administration of certain growth factors, anti-inflammatory agents, or mitochondria. This way, the complexity of the MSC functions and the crosstalk between cells are underestimated. The use of viable MSCs will likely outperform cell-free methods, since the communication with cartilage or meniscus cells induces a tailor-made reaction of the MSCs.

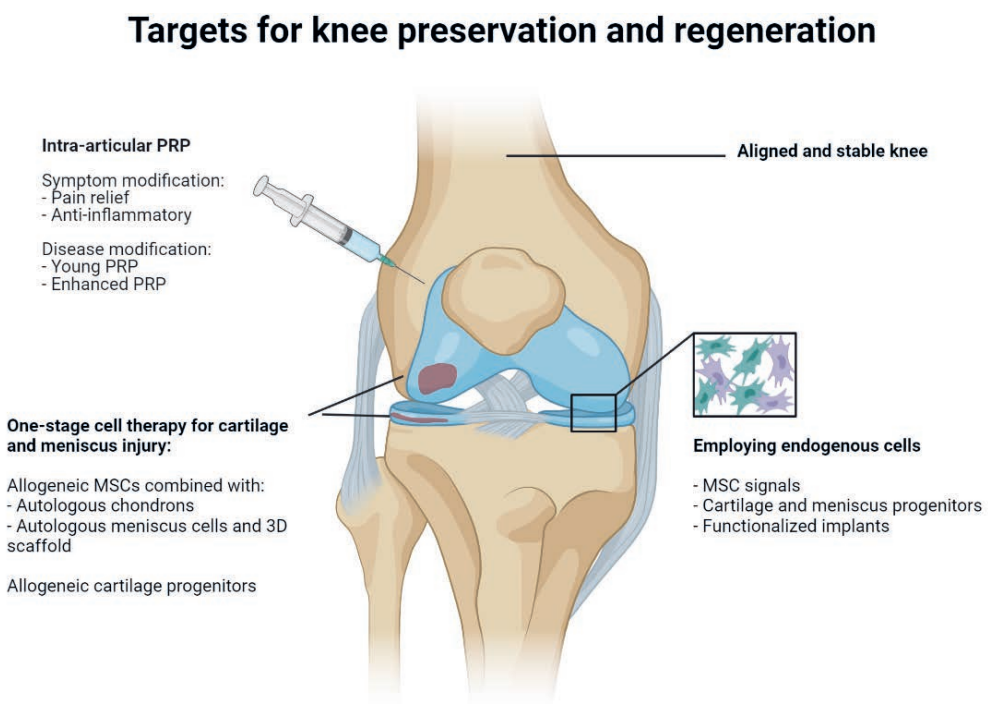
Another approach for a cell-free method is addressing the endogenous progenitors present in the knee. Progenitors are present in both cartilage and meniscus and have the ability to produce extracellular matrix *in vitro*. The exact function and role of progenitors in (patho)fysiology is unknown. Migrating progenitors of advanced OA cartilage have enhanced osteogenic and adipogenic capacities and lower chondrogenic differentiation compared to progenitors from early OA cartilage[319] and the amount of progenitors is higher in OA cartilage than in healthy cartilage[251,319]. The OA progenitors might produce aberrant or scar like tissue and therefore contribute to the pathological remodeling in end-stage OA. If progenitor cells are one of the factors driving OA, selective inhibition of progenitors might slow the disease. Similarly, senescent cells create a pathological joint environment by secreting catabolic and pro-inflammatory cytokines and selectively killing senescent cells inhibits the development of OA[426]. On the other hand, progenitors migrate towards the injury location after cartilage or meniscus injury[264,321], indicating that progenitors could also be an internal repair mechanism that fails to succeed due to the detrimental influence of the synovial inflammation on joint homeostasis in OA. In this case, activating or stimulating the progenitors with the right growth factors or anti-inflammatory cytokines could restore this innate repair mechanism. Correcting the imbalance between catabolism and anabolism by activating the progenitors might reverse some of the tissue destruction. For example, a switch from degenerative progenitor to meniscus progenitor phenotype was induced by treatment with TGF-β[258]. Further understanding of the role of progenitor cells could open the door for cell-free approaches for knee preservation.

Finally, smart implants that release growth factors or capture growth factors from the synovial fluid, could attract cells into the implants and stimulate matrix formation. These smart implants would provide off-the-shelf solutions at lower prices. In chapter 8, the effect of various growth factors on meniscus cells and MSCs was studied. Platelet lysate and Platelet-derived growth factor (PDGF) increased migration of meniscus cells and MSCs, while Transforming growth factor beta (TGF-β) increased proliferation and formation of meniscus extracellular matrix by meniscus cells. As a proof-of-concept, a system to functionalize the CMI® with a Vascular endothelial growth factor (VEGF) binding peptide. This functionalization of the CMI® led to increased migration of meniscus cells and MSCs into the implant. For further optimization of this technique, the use of a gradient of growth factors could be explored. For example, VEGF and PDGF could be captured in the meniscus outer zone to attract cells and stimulate angiogenesis[56]. In the inner zone, TGF-β could be captured to stimulate chondrogenic differentiation and formation of proteoglycans. Platelet lysate increased proliferation and migration of meniscus cells and MSCs, but did not increase matrix formation. Pre-

treating an implant with platelet lysate could initially boost the cell number in the implant. The cells could form extracellular matrix after this initial migration and proliferation boost, resulting in increased meniscus tissue formation overall. Moreover, the technique to functionalize scaffolds with growth factor binding peptides could be expanded to melt electrowritten scaffolds, as the functionalization of polycaprolactone was also described[338,339]. The improved mechanical properties of the melt electrowritten scaffolds compared to the CMI® favor the use of these scaffolds.
The future will learn whether the techniques that address the endogenous cell populations can match the effect of cell therapy. The approaches described here focus on single or a small number of factors and mechanisms, which could be an oversimplification and limitation of these treatments. However, further insight into the biology of repair mechanisms and regeneration could result in more complex solutions and tailor these solutions to specific stages of disease or disease phenotypes.

Conclusions

The research in this thesis aimed at creating clinically applicable treatment options and improved understanding of knee joint preservation. Some of the findings described in this thesis could have a direct impact on patient care, such as the identification of targets for patient selection for PRP treatment and the importance of stability of the ACL for protection of the meniscus. Other studies provide a base for follow-up research that could benefit patients in the future. We have demonstrated the potency of cartilage and meniscus progenitors for tissue engineering and possibly in future cell therapies. We have shown different approaches for meniscus replacement and started an RCT on one-stage cartilage transplantation that will bring us a step closer to providing this regenerative treatment in clinical practice. Lastly, we have unraveled another piece in the puzzle of MSC-induced chondrogenesis, that can improve MSC based treatments and increases our understanding of the communication in the joint.



| Figure 1. Schematic summary of targets for knee preservation

Samenvatting in het Nederlands

Behoud van het kniegewricht
Strategieën voor weefselherstel en regeneratie

De knie is een van meest complexe gewrichten in het menselijk lichaam. Het gewricht wordt onderhouden door een nauwe samenwerking in de verschillende weefsels die allen een belangrijke rol spelen. Voor het behoud van een gezond kniegewricht is het functioneren van al deze weefsels belangrijk. Wanneer een of meerdere van deze weefsels niet functioneren, leidt dit uiteindelijk tot artrose. Op dit moment leidt 5% van de wereldpopulatie aan knieartrose, en dit percentage zal de komende jaren fors doorstijgen. Door pijnklachten en verminderde mobiliteit heeft artrose een flinke impact op het leven van de patiënt, en zorgt voor een grote maatschappelijke last. Een knieprothese geeft goede resultaten bij oudere patiënten, maar de tevredenheid van patiënten van middelbare leeftijd is een stuk lager, met hogere risico's op complicaties. Daarom is het van groot belang het gewricht zo lang mogelijk gezond te houden en gewrichtsherstellende in plaats van -vervangende behandelingen te introduceren.
Het onderzoek in dit proefschrift richt zich op het creëren van klinisch toepasbare behandelopties en het verbeteren van het begrip van gewrichtsbehoud. Eerst hebben wij de momenteel klinisch beschikbare behandelingen geëvalueerd en mogelijkheden voor optimalisatie onderzocht. Vervolgens hebben we verschillende strategieën voor herstel en regeneratie van kraakbeen en menisci onderzocht. De potentie van verschillende celtypen werd onderzocht en we karakteriseerden twee progenitor (voorloper) cel-populaties. Als laatste werd de kracht van cel-communicatie onderzocht in co-kweken van Mesenchymale Stromale Cellen (MSC) en kraakbeen of meniscus cellen.
Voor de optimalisatie van momenteel beschikbare behandelingen voor kniebehoud hebben wij de bestaande literatuur bestudeerd en data geanalyseerd van een groot patiëntencohort op de Mobility Clinic van het Universitair Medisch Centrum Utrecht. Uit literatuuronderzoek bleek dat reconstructie van de voorste kruisband de meniscus beschermt tegen secundair letsel. Bovendien is falen van meniscusherstel frequenter in patiënten zonder functionele voorste kruisband (hoofdstuk 2). Dit illustreert het belang van de functie van de voorste kruisband voor stabiliteit, wat een voorwaarde is voor succesvol herstel en voor het voorkomen van secundaire schade.
Wij waren ook geïnteresseerd in het effect van intra-articulaire injecties met autoloog geconditioneerd plasma (ACP®) voor de behandeling van gonartrose, en mogelijkheden voor optimalisatie van patiëntselectie of productsamenstelling. Intra-articulaire ACP® injecties leidden tot een statistisch significante, maar niet klinisch relevante verbetering in patiënt gerapporteerde uitkomsten scores (hoofdstuk 3 en 4). Aangezien de door de

patiënt gerapporteerde verbeteringen vergelijkbaar waren met elders gerapporteerde placebo-effecten, is er een sterke behoefte aan optimalisatie van de ACP®-behandeling. De samenstelling van het geïnjecteerde ACP® bij 100 patiënten vertoonde variaties in de concentraties bloedplaatjes, leukocyten en erytrocyten. Dit variabele gehalte correleerde niet met veranderingen in individuele uitkomsten. Mogelijk is een sterk afwijkende productsamenstelling nodig om de uitkomsten te verbeteren, of bepalen andere factoren dan die hier zijn gemeten de potentie van ACP®. De selectie van patiënten zou de succespercentages kunnen verbeteren, aangezien een jongere leeftijd van de patiënt en posttraumatische OA een lagere verbetering van de door de patiënt gerapporteerde uitkomstmaten voorspelden. In het algemeen zou de ACP® behandeling voor knie-OA geoptimaliseerd moeten worden om bevredigende resultaten te verkrijgen.
Het tweede subdoel van dit proefschrift was het evalueren van verschillende celtypes voor regeneratie en weefselherstel. Meniscusregeneratie of meniscusherstel door middel van cellen werd bestudeerd in een systematisch literatuuronderzoek van preklinisch onderzoek, om optimale celtypen te identificeren (hoofdstuk 5). Verschillende bronnen van mesenchymale stromale cellen (MSCs) verbeterden het herstel en de regeneratie, waaronder MSCs uit vetweefsel, beenmerg, synovium, en de meniscus. Zowel autologe als allogene cellen bleken potentieel te hebben voor klinische toepassing en beide hebben hun eigen voordelen. Bovendien leidden celtherapieën tot een betere structurele organisatie van transplantaten of herstelweefsel in vergelijking met celvrije methoden, ongeacht welke celtypes werden gebruikt. Daarom zouden celtherapieën de voorkeur moeten krijgen voor weefselherstel of -regeneratie in de meniscus. Dit benadrukt ook hoe belangrijk het is de biologische processen te begrijpen voor in gewrichtsbehoud.
Daarnaast isoleerden en karakteriseerden we voorlopercellen uit articulair kraakbeen en de meniscus, en evalueerden het potentieel van deze cellen voor celtherapie of tissue engineering. De aanwezigheid van voorlopercellen in de binnenste zone van de meniscus is veelbelovend, omdat dit deel van de meniscus vaak wordt beschouwd als niet in staat tot regeneratie (hoofdstuk 6). Meniscus voorlopercellen vertoonden differentiatie naar vet, kraakbeen en bot met een verhoogde chondrogene (kraakbeen) differentiatie in vergelijking met meniscuscellen. Kraakbeen voorlopercellen van artrotisch en gezond weefsel vormden geen botmatrix en de kweken waren negatief voor type X collageen (hoofdstuk 7). Kraakbeen voorlopercellen hebben dus een lage neiging tot hypertrofische differentiatie, in tegenstelling tot beenmerg MSCs. Alle voorlopercellen hadden een hoge proliferatie en kolonievormend vermogen. Belangrijk is dat, naast deze voordelen bij cel expansie, de voorlopercellen in staat waren om kraakbeen- en meniscusmatrix te vormen bij redifferentiatie. Het gemak waarmee ze geïsoleerd kunnen worden, hun hoge proliferatieve eigenschappen en hun vermogen om te

herdifferentiëren zelf na vele delingen maken voorlopercellen interessante celtypes voor weefsel-engineering doeleinden. Bovendien zijn voorlopercellen een veelbelovend therapeutisch doelwit om endogene herstelmechanismen te versterken.
Het laatste subdoel van dit proefschrift was het bevorderen van regeneratie door gebruik te maken van trofische effecten en cel-communicatie. Eerst hebben we onderzocht of groeifactoren en plaatjeslysaat de migratie van meniscuscellen en MSCs bevorderen (hoofdstuk 8). Aangetoond is dat platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1) en plaatjeslysaat de migratie van meniscuscellen en MSCs bevorderen. Bovendien waren we in staat om groeifactoren te binden aan een Collageen Meniscus Implantaat (CMI®) om zo de cellen uit het gewricht naar het implantaat te lokken. Deze benadering zou kunnen worden gebruikt als een celvrije methode om cellen naar een implantaat te lokken en weefselvorming te bevorderen. Idealiter zou een combinatie of gradiënt van groeifactoren kunnen worden gebruikt. Een groeifactor die de migratie stimuleert (zoals PDGF) zou cellen kunnen aantrekken, waarna een andere groeifactor de cellen zou kunnen stimuleren tot de vorming van extracellulaire matrix. Hieruit blijkt proof-of-principle van een innovatieve kant-en-klare oplossing voor meniscusvervanging.
Vervolgens probeerden we de mechanische eigenschappen van een meniscusimplantaat te verbeteren door gebruik te maken van melt electrowriting om de natuurlijke collageen architectuur van een meniscus na te bootsen. Om de biologie te verbeteren werd gebruik gemaakt van het chondro-inductieve effect van MSCs in combinatie met meniscuscellen (hoofdstuk 9). Het implantaat vertoonde goede mechanische eigenschappen *in vitro*, en we zagen een basaal niveau van meniscusmatrixvorming door de gezaaide cellen. We hebben dit implantaat ontworpen met materialen die goedgekeurd zijn voor klinisch gebruik en met een klinisch toepasbaar aantal en type cellen voor éénstapsbehandeling.
Het chondro-inductieve effect van MSCs wordt ook gebruikt in IMPACT voor kraakbeenceltransplantatie. IMPACT is een éénstapsbehandeling waarbij autologe kraakbeencellen met hun omliggende matrix en allogene MSCs uit beenmerg worden gecombineerd voor de behandeling van kraakbeendefecten. We ontwierpen een studieprotocol voor een fase III gerandomiseerde gecontroleerde studie (RCT). De effectiviteit en kosteneffectiviteit van IMPACT ten opzichte van niet-chirurgische zorg wordt onderzocht in een cross-over RCT (hoofdstuk 10). Bovendien zal de veiligheid van IMPACT worden geëvalueerd op basis van gegevens van 30 tot 60 patiënten. De gegevens die in deze studie worden verzameld, kunnen helpen bij de implementatie van deze regeneratieve behandeling in de kliniek, maar leveren ook aanvullende gegevens op over de veiligheid en efficiëntie van het gebruik van allogene MSCs in het gewricht. Dit

is waardevolle informatie die ook nuttig kan zijn voor toepassingen in andere weefsels, zoals de meniscus. Ten slotte bestudeerden we de mechanismen achter het effect van MSC co-kweken verder door de communicatie met behulp van mitochondriale transfer tussen MSCs en kraakbeencellen te evalueren (hoofdstuk 11). De bidirectionele overdracht van mitochondriën tussen MSCs en kraakbeencellen vindt plaats via tunneling nanotubes, celcontact via bredere contactpunten en extracellulaire blaasjes. De precieze functie van dit transport is momenteel onbekend, maar in de literatuur wordt gesuggereerd dat kraakbeencellen defecte mitochondriën zouden kunnen uitscheiden als een noodsignaal dat de MSCs waarnemen. Daarop kunnen MSCs mitochondriën overdragen aan kraakbeencellen als trigger voor cel-verjonging. Uit onze studie blijkt dat de overdracht van mitochondriën van MSC naar kraakbeencellen leidt tot een toename van de hoeveelheid DNA en proteoglycaanafzetting in de kweken. De overdracht van mitochondriën is een van de methoden van communicatie tussen MSCs en kraakbeencellen die bijdraagt aan het chondro-inductieve effect van MSCs. Dit zou de op MSC gebaseerde chondrogenese verder kunnen verbeteren door de MSC donorselectie te verbeteren.

Conclusie

Het onderzoek in dit proefschrift was gericht op het creëren van klinisch toepasbare behandelopties en het vergroten van het begrip van behoud van het kniegewricht. Sommige van de bevindingen kunnen een directe impact hebben op de patiëntenzorg, zoals de identificatie van targets voor patiëntselectie voor PRP behandeling en het belang van stabiliteit van de voorste kruisband voor bescherming van de meniscus. Andere studies bieden een basis voor vervolgonderzoek waar de patiënt van de toekomst baat bij heeft. We lieten zien welke voordelen kraakbeen- en meniscus voorlopercellen bieden voor tissue engineering en in mogelijke toekomstige celtherapieën. We hebben verschillende benaderingen voor meniscusvervanging gepresenteerd en zijn gestart met een RCT voor éénstap-kraakbeenceltransplantatie die ons dichterbij het aanbieden van deze regeneratieve behandeling in de klinische praktijk brengt. Tenslotte hebben we een stukje ontrafeld in de puzzel van MSC-geïnduceerde chondrogenese, dat MSC-gebaseerde behandelingen kan verbeteren en ons begrip van de communicatie in het gewricht vergroot.

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Curriculum Vitae

Jasmijn Korpershoek was born in Roosendaal, the Netherlands on the 30th of April 1992. After graduating high school in 2010 (Gertrudiscollege, Roosendaal), she studied Biomedical Sciences at Utrecht University in the Netherlands. During this study, she spent a semester at Lund University in Sweden for an exchange.

After obtaining her bachelor degree, she was accepted to the four-year Selective Utrecht Medical Master (SUMMA) program at Utrecht University to become a medical doctor and clinical researcher. During this time she developed an interest in orthopedics, which led to her research on meniscus regeneration. She performed her clinical rotations at Gelre Ziekenhuizen in Apeldoorn, the Netherlands, University Medical Center Utrecht in Utrecht, the Netherlands and Hospital Hilario Sánchez in Masaya, Nicaragua.

After graduating in 2017, she continued her research with a PhD in Regenerative Medicine at Utrecht University under supervision of prof. dr. Daniel Saris, dr. Lucienne Vonk and dr. Roel Custers. In 2019, she was awarded an education grant to visit the International Cartilage Regeneration and Joint Preservation Society (ICRS) world congress in Vancouver, Canada. In 2021, she won the prof. ir. Dr. Rik Huiskes award for best basic science abstract at the Dutch Orthopedic Society (NOV) annual meeting. In 2022, Jasmijn was selected for the Orthoregeneration/ICRS Research Fellowship. After completing her PhD thesis in 2022, she continued her research in joint regeneration as a Postdoctoral research fellow at the Mayo Clinic in Rochester, Minnesota, USA.