PATIENT-CENTERED STRATEGIES TO PREVENT OSTEOARTHRITIS AFTER MENISCUS INJURY

CHELLA HAGMEIJER

Patient-centered strategies to prevent osteoarthritis after meniscus injury

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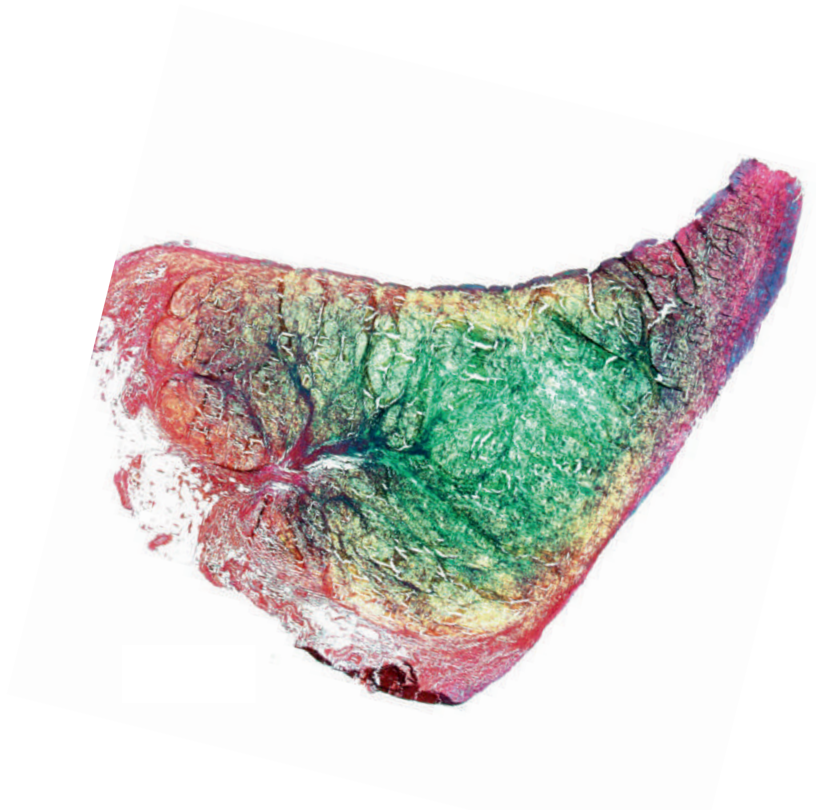
Chapter 1

General introduction, aims and thesis outline



Etiology of meniscus tears

Meniscus tears are extremely common in professional athletes performing high impact and full contact sports. However, a treatment for all different tear types to prevent osteoarthrosis at older age is not yet available. High-risk sports which are notable for increased contact and pivoting include soccer, basketball, and American football.[52] For professional athletes in particular, a fast recovery and return to sport is extremely important, and therefore rehabilitation programs with a goal of return to sports within 4-6 weeks after injury are not uncommon. Luis Suarez, a soccer player from Uruguay, tore his meniscus during training for the World Cup 2014. On May 21, 2014, Magnetic Resonance Imaging (MRI) demonstrated a meniscus tear, leading to partial meniscectomy. Suarez missed Uruguay's first World Cup game, but returned to the field in time for the second game on June 19, which they won 2-1. Suarez scored both goals. Within one month after surgery, he was successfully back on the field. Most (professional) athletes undergo partial meniscectomy for a fast recovery, providing good results at short-term follow-up because removal of the torn meniscus is an easily remedy for the existing symptoms. However, at long-term follow-up, meniscectomy disturbs the biomechanics of the knee, resulting in abnormal loading of the cartilage, possible malalignment and development of early osteoarthritis.

Meniscus tears are among the most frequently reported injuries in orthopedic literature and arthroscopic treatment for meniscus injury is one of the most common procedures in orthopedics.[1,31] Meniscus tears cause significant time loss in sports participation and productivity, affect quality of life and carry significant post-injury implications. [37,42,54]The mean annual incidence rate of meniscus injuries in the general population is 66-70 per 100,000 people in the United States.[26,28,43] The mean age of patients presenting with meniscus injury is 30 years with a peak incidence in females of 21-30 and 11-20 years in males.[16] The incidence of meniscus lesions in the pediatric/adolescent population is also increasing. This development might be correlated with the increase in participation in competitive sports activities at increasingly younger ages.[33,34,38]

Treatment of meniscus tears and anterior cruciate ligament injury

Shift in treatment

Strategies on how to treat meniscus tears have significantly changed over time. The meniscus was initially described as '*a functionless embryonic remnant*' by Sutton et al. in 1879. In 1967, Smillie et al. wrote "*If it is torn, take it out! Take it all out! Even if you just think it is torn, take it out!*"[48] They described a rapid recovery of function after (partial) meniscectomy, caused by removal of the mechanical obstruction and/or inflammation of the torn part of the meniscus.[48] However, there has been a recent and ongoing shift in the management of meniscus tears and modern orthopedic surgeons agree it is important to save as much meniscus tissue as possible.[7] This paradigm shift in treatment was prompted by insight into the important role of the meniscus in knee joint homeostasis, load transmission in the femorotibial joint, shock absorption, and mechanical joint stability, thereby protecting the articular cartilage.[22] The literature now shows that meniscus injury and (partial) meniscectomy are strongly correlated with the development of early osteoarthritis and should be avoided when possible.[19,40,54] With this new and evolving understanding of knee biomechanics, meniscus repair has become the gold standard of treatment when technically feasible, instead of partial meniscectomy.

Different meniscus tear types

Unfortunately, not all meniscus tears can be surgically repaired. Meniscus tears can be classified by tear type and tear location. Various tear types have been described in the literature and these include longitudinal-vertical, horizontal, radial, vertical flap, horizontal flap, bucket handle (an extension of a longitudinal-vertical tear) and complex tears (Figure 1).[23,26,56] Different tear types have differential healing potentials. Aside from tear type, tear location is also of importance for treatment options and clinical outcome. The meniscus can be divided into three different zones. (1) The red-red zone is the outer portion where vascularization is present. This zone mainly contains fibroblast-like cells. Repair is a good option in tears located in this vascularized part of the meniscus. (2) The red-

white zone is partially vascularized and contains a combination of fibroblast-like and chondrocyte-like cells. After meniscus repair, healing can occur in this zone. However, classically, the healing response is not as robust as that which is present in the red-red zone. (3) The white-white zone is the avascular part of the meniscus. This zone mainly contains chondrocyte-like cells and has no regenerative capacity. Thus, this zone cannot be surgically repaired (Figure 2).[23]

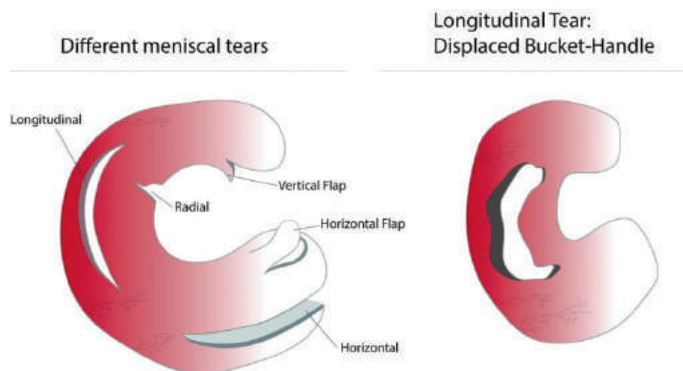


Figure 1: Different meniscal tear types.

With the goal of preserving meniscal tissue, repair of meniscus tears in an adult population has yielded good results, with immediate relief of mechanical symptoms such as locking and pain, along with objective and subjective clinical success at follow-up.[6,18] However, long-term data on clinical outcomes of meniscus repair in the pediatric and adolescent population is lacking. Obtaining this data is important because this young, active population often returns to pivoting sports which place stress on the already-damaged knee joint, and because incomplete or insufficient healing can lead to early onset of osteoarthritis.

Concomitant anterior cruciate ligament injury

Meniscus tears can present as isolated pathology after acute traumatic injuries of the knee.[32] However, acute meniscus tears in young patients are often associated with concomitant ligamentous injuries, with the anterior cruciate ligament (ACL) being most commonly involved.[21,51] Multiple studies support the idea that meniscus damage in an ACL-deficient knee alters knee kinematics, including anterior tibial load and anterior-to-posterior tibial translation.[2,3] Patients

with a torn ACL in combination with a meniscus tear show significantly better clinical results at short-term follow-up when the ACL reconstruction is combined with meniscus repair compared to meniscus resection. Patients undergoing meniscus repair at the same time as ACL reconstruction have demonstrated comparable clinical outcomes to patients with an isolated ACL tear undergoing reconstruction.[45] However, results of long-term follow-up on the fate of the meniscus in an ACL-deficient knee have yet to be described.

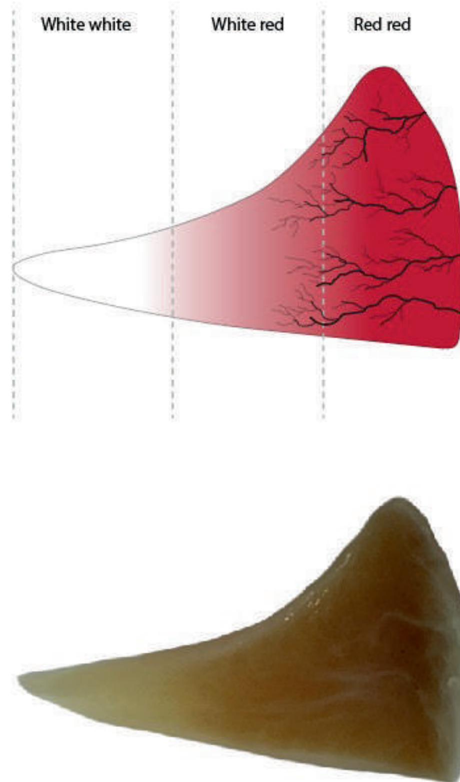


Figure 2: An illustrative cross section of the meniscus, combined with a macroscopic image of a human meniscus, demonstrating the three different zones. The three different zones have clinical relevance, since tears in the white-white zone cannot be surgically repaired.

Regenerative medicine for meniscus injury

Over the past two decades, regenerative medicine and tissue engineering have represented rapidly-growing fields. Research in these areas has been focused on the use of stem cells or progenitor cells for replacement or regeneration of damaged cells, tissues, and organs. The avascular structure and limited self-regenerating capacity of meniscus tissue, together with poor long-term outcomes after partial meniscectomy such as early osteoarthritis, make meniscus tears ideal candidates for treatment options using regenerative medicine.

Mesenchymal stem cells (MSCs) are multipotent adult cells harvested from bone marrow or adipose tissue. They have anti-inflammatory, anti-apoptotic and immunosuppressive properties, and are thus a potent source for cell therapy. The International Society for Cellular Therapy stated in 2006 that MSCs should match three minimum criteria; (1) be plastic-adherent, (2) express the surface markers CD73, CD90 and CD105, but not the hematopoietic markers CD45, CD34, CD14, CD11b, CD19, CD79a or HLA-DR, and (3) have tri-lineage mesenchymal differentiation capacity into osteoblasts, adipocytes, and chondrocytes. [17]
Four different mechanisms for the effective function of MSCs in the field of regenerative medicine have been proposed:

1) The general thought used to be that MSCs trans-differentiated into the required cell type, such as differentiation into chondrocytes for cartilage repair in isolated cartilage defects and osteoarthritis, or differentiation into cardiomyocytes after a myocardial infarction (Figure 3a).[62]

2) Another proposed mechanism is cell fusion (Figure 3b), where the MSCs fuse with another target cell to form a multinuclear cell known as syncytium, which can initiate a rapid differentiation process. However, this seems to occur too infrequently to account for meaningful improvement in tissue damage. [5,36]

3) Increasing evidence suggests that the therapeutic efficacy of MSCs depends on paracrine signaling (Figure 3c), stimulating the patient's resident cells to repair damaged tissue.[14,46,55] Paracrine signaling can be divided into extracellular vesicles and trophic factors such as growth factors and cytokines. Extracellular vesicles are membrane-enclosed structures excreted by MSCs to stimulate damaged cells. These extracellular vesicles are subdivided into exosomes (40-100 nm) which are formed by the invagination of the limiting membrane of the cellular endo-

lysosomal system and microvesicles (100-1000 nm) that sprout directly off the plasma membrane. These extracellular vesicles contain RNA and proteins which can be transferred between cells, playing an important role in intercellular communication and tissue repair.[24,55] Trophic factors excreted by MSCs have been demonstrated to be involved in angiogenesis and preventing cell apoptosis.[29] These paracrine factors can affect different pathways: immunomodulation, angiogenesis, proliferation and anti-apoptosis, which lead to different effects on the host cells.

4) The last pathway is direct cell-cell communication using gap junctions and nanotubes making mitochondrial transfer possible (Figure 3d). In cartilage repair, cell therapies have been extensively studied, ranging from in vitro experiments to clinical trials.[25] Co-cultures of MSCs and chondrocytes have demonstrated an enhanced effect of cartilage-like extracellular matrix (ECM) production and cell-cell communication.[8,57] De Windt et al. also showed an absence of allogeneic MSCs by DNA analysis after one year of local implantation in combination with chondrons in a human cartilage regeneration trial, confirming the paracrine effect of the MSCs.[58]

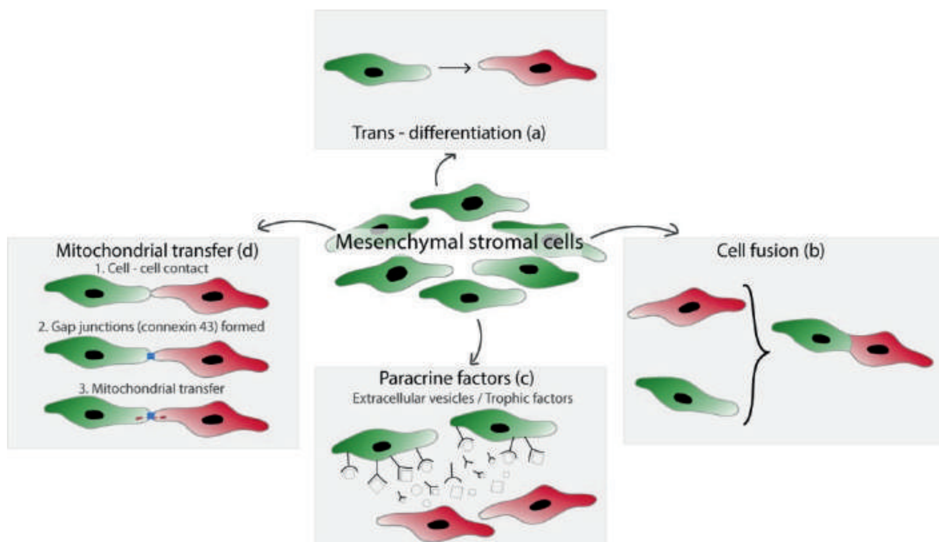


Figure 3: Four different mechanisms for the effective function of mesenchymal stromal cells in regenerative medicine. Trans-differentiation (a), cell fusion (b), excretion of paracrine factors (c) and mitochondrial transfer (d).

Both autologous and allogeneic MSCs are being used for different experimental treatments. Studies show potential benefits from the use of allogeneic MSCs instead of autologous for multiple reasons. Allogeneic cells are patient-friendly since the MSCs can be harvested from a donor at the time of another procedure or salvaged from waste tissue and no additional harvest surgery is necessary for the recipient. Following, they can easily be used in a one stage procedure or as an off-the-shelf product[59] because the MSCs can be precultured and stored until necessary. They are more cost-effective as they can be culture expanded and used for the treatment of multiple patients, dividing the costs of culture expansion and quality controls over multiple treatments. The last advantage is the possible pro-inflammatory effect triggered by the presence of allogeneic cells in the patient. Although HLA-DR expression by MSCs is extremely low, they might induce a low-grade inflammation which causes a boost in the regenerative effect. A superior effect of allogeneic MSCs compared to autologous hMSCs is seen in patients receiving transendocardial MSC injections for non-ischemic dilated cardiomyopathy, with improved endothelial function, greater suppression of TNF-α suggesting a shift towards a less inflammatory phenotype of the immune cells, and better clinical outcomes.[27]

In addition to MSCs, growth factors could play a role in regenerative medicine therapy. Growth factors are proteins or steroids which help regulate cell proliferation, differentiation, and migration. They also contribute to an increase in ECM production and therefore stimulation of tissue healing.[53,63] All growth factors target different receptors and pathways, and therefore invoke cell-specific responses. However, the endogenous concentration of growth factors in the knee joint is relatively low. Therefore, without any additional treatment, endogenous growth factors do not substantially influence the regeneration induced by new treatments.[30] When exogenous growth factors are injected in the knee joint, the effective half-life of such factors is often too short to contribute to regeneration. Hence, classically such factors have been of limited added value in tissue repair,[30] leading to an ongoing challenge in clinical growth and regeneration augmentation. Besides, administering growth factors in higher concentrations can have serious adverse events, as shown with rhBMP, which caused heterotopic bone formation in some cases.[4,9,44,61]

A more recent advancement in the field of regenerative medicine treatment is the use of scaffolds for meniscus defects, since a three-dimensional structure is needed to replace the damage meniscus. Two meniscus scaffolds are approved by the US Food and Drug Administration (FDA) for meniscus implantation: the Actifit® (Orteq® Sports Medicine Ltd New York, NY, US), and the Collagen Meniscus Implant® (CMI; Stryker® Kalamazoo, MI, US). Actifit is a polyurethane synthetic biodegradable meniscus scaffold, whereas the CMI, a type I collagen scaffold, is fabricated from minced bovine Achilles tendon, combined with glycosaminoglycans (both hyaluronic acid and chondroitin sulfate). After the collagen fibers are dehydrated inside a mold, they are chemically cross-linked using formaldehyde, resulting in a sponge-like porous structure in the shape of a meniscus.[12,47] A rim of the native meniscus needs to be present for suturing both types of scaffold into the knee joint. The porous structure of these scaffolds provides an environment for cell ingrowth by meniscus cells from the post-meniscectomy remnant and other cell types present in the synovial fluid of the knee joint. These cells engraft the implant and form new ECM while the scaffold slowly degrades. However, often the degradation rate of the scaffold is faster than the rate of engraftment and new tissue formation, leading to smaller menisci and altered knee mechanics.

Patient specific care

Shift towards subjective outcome parameters

As described previously, regenerative medicine is a growing field within orthopedic surgery and new treatments are developed rapidly. To be able to compare the outcomes of these new treatments, good outcome measures are necessary. Universal outcome parameters contribute to better outcome registration and follow-up after surgery, which makes it more reliable to compare different treatment strategies and their results. For the structural evaluation of cartilage defect repair, MRI is the gold standard, because of its non-invasive nature and detailed morphological evaluation.[39,60] However, there is no conclusive evidence on whether the objective outcome measurements of MRIs are correlated with clinical outcomes.[35,60] Most treatments in orthopedic surgery aim to reduce

symptoms such as pain and restricted function, which eventually leads to improved quality of life (QoL).[10] Since these elective surgeries aim to improve the QoL, the patient's subjective experience and goals after surgery are extremely relevant.[41] Therefore, a combination of objective radiographic parameters and subjective patient-reported outcome measures (PROMs) should be used to determine the relevant outcomes after treatment.

Patient centered care

According to the United States Food and Drug Administration (FDA), PROMs are defined as a report of the status of a patient's health condition that comes directly from the patient, without interpretation of the patient's response by a clinician.[20,13] The FDA emphasized the importance of patient input, next to literature and expert opinion, in the development of PROMs. However, most PROMs are designed by medical doctors without consulting patients.[13] The importance of involving patients in the development of PROMs is highlighted by studies proving that clinician assessment of patient in-hospital experience is not accurate.[11,15] For example, symptoms or concerns relevant in patients' opinions are often not reported in medical or nursing records.[49,50] This demonstrates the need for patients to be involved in the development of future PROMs.

Besides the lack of patient engagement in most currently used PROMS, the goal for each patient after knee surgery is different as well. Professional athletes want to return to sports as soon as possible, without missing too many games. Other patients want to participate in daily activities without experiencing pain. When reporting outcomes after knee surgery, these differences must be taken into account. A good outcome for one patient is not automatically satisfying for all patients. Nowadays, frequently-used outcome measures do not always take this variation into consideration, leading to a possible misrepresentation of subjectively reported outcomes. Thus, there is a need for a disease-specific, patient-approved questionnaire in orthopedic surgery, starting with sports-related knee injuries.

Aims of this thesis

The aim of this thesis is to improve long-term outcomes after meniscus surgery in young and active patients. We achieve this by demonstrating the long-term clinical results of current treatments (*part I*), showing *in vitro* results for improvements upon current treatments (*part II*), and focusing on patient-specific care to better measure outcomes after sports-related surgery of the knee (*part III*).

Outline of this thesis

Part I assessed consequences of meniscus tears and currently available treatments. *Chapter 2* provided an overview of risk factors for failure of meniscus repair surgeries in the pediatric population. In this way, outcomes after surgery could be better predicted and the treatment could be more patient specific, looking at more than just the injury. In *chapter 3*, the fate of new meniscus tears after ACL injury was described in a case series study of the population of Olmsted, Minnesota in the United States. This provides information on the prevalence of different meniscus tear types and their influence on the subsequent treatment and long-term outcomes.

In *part II*, we aimed to develop a one-stage cell-based therapy for meniscus regeneration in patients for which simple repair of the native meniscus is not an option. To develop a single-stage procedure, a meniscus scaffold and cells to induce regeneration are needed. We used autologous meniscus cells as the primary cell source; however, this isolation does not yield enough cells for seeding of the complete scaffold. Therefore, another cell source—or other factors to improve the cell migration, proliferation, and ECM production—are necessary to develop this procedure. *Chapter 4* demonstrated co-culture results of meniscus cells and MSCs to find the optimal ratio and communication potency between the two cell types. When using cell therapy for the meniscus, an implant is needed to provide an environment for cell ingrowth. Throughout this thesis, the Collagen Meniscus Implant (CMI) was used as the scaffold. Seeding of this scaffold with cells was assessed *in vitro* using two different methods: static seeding and seeding by injection (*chapter 4*). Those same two seeding methods were performed in a

cadaveric study, shown in *chapter 5*, to examine the influence of the complete arthroscopic procedure on cell survival, cell count, and cell distribution throughout the implanted CMI. In *chapter 6* we investigated a different way to stimulate migration and proliferation of meniscus cells by using a variety of growth factors in combination with the CMI to accelerate cell in-growth in the implant and promote regeneration.

Part III of this thesis focused on outcome measures after sports-related knee surgery. Health care is moving toward patient-centered care but most of the currently used patient-reported outcome measures (PROMs) were developed and approved by medical specialists. Therefore, in *chapter 7*, we developed the Patient Approved Knee Assessment (PAKA) in collaboration with patients to get a better idea of what they found to be relevant in recovery after knee surgery. Finally, we also sought to validate this measurement tool in the same patient category.

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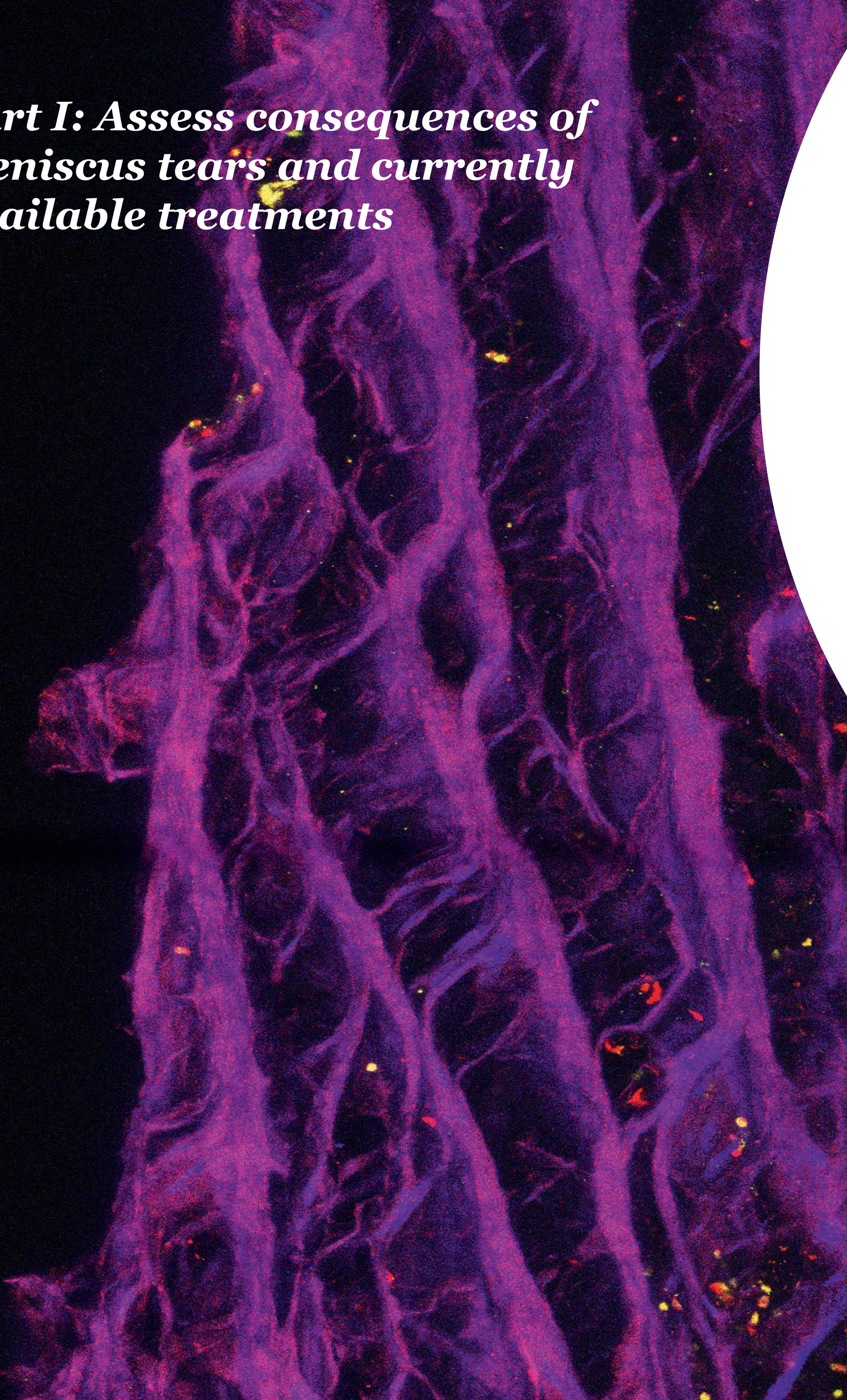
Part I: Assess consequences of meniscus tears and currently available treatments

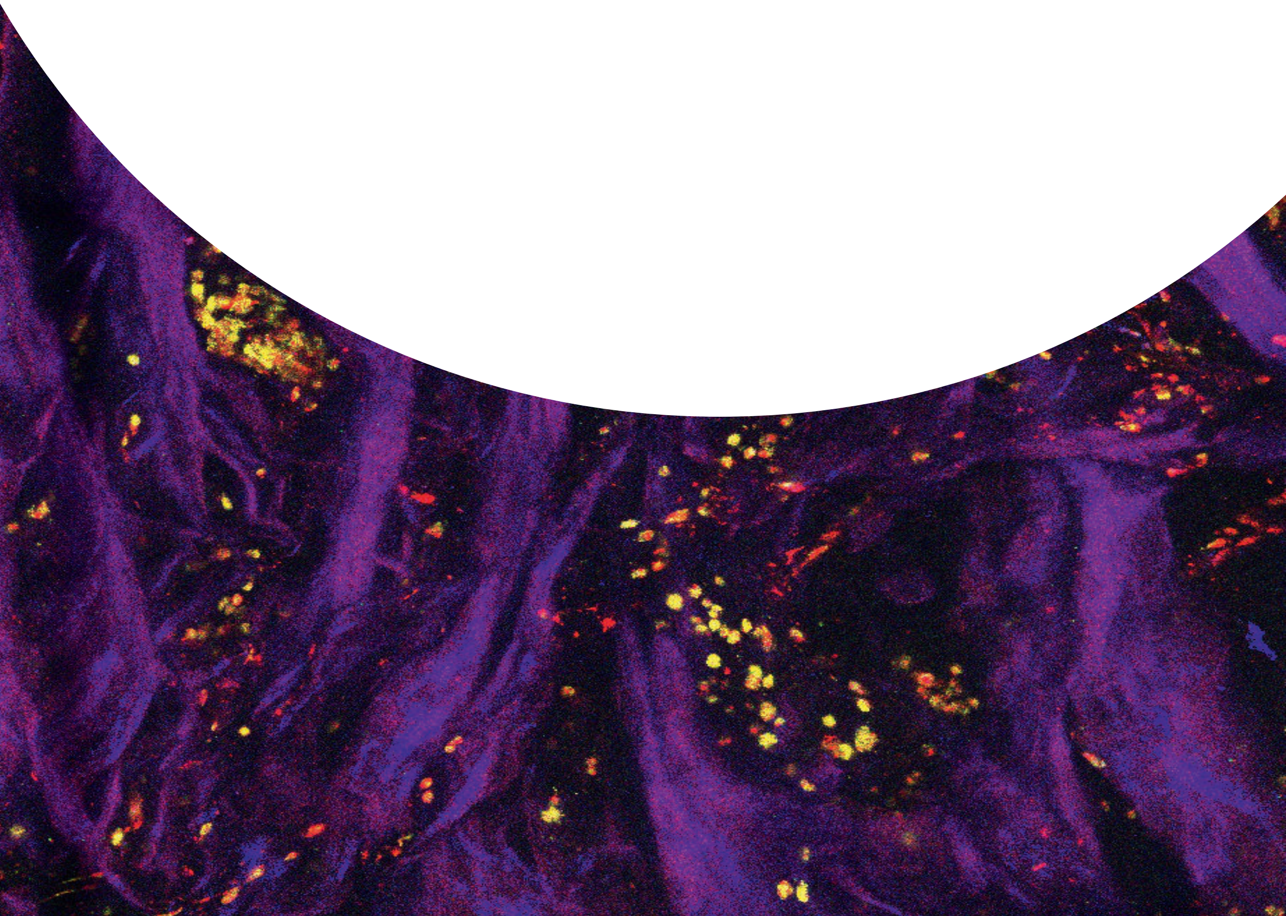


Chapter 2

Long-term Results After Repair of Isolated Meniscal Tears in Patients Aged 18 Years and Younger

Chapter 3

Secondary Meniscal Tears in Patients with Anterior Cruciate Ligament Injury



Chapter 2

Long-term Results After Repair of Isolated Meniscal Tears in Patients Aged 18 Years and Younger:
An 18-Year Follow-up Study

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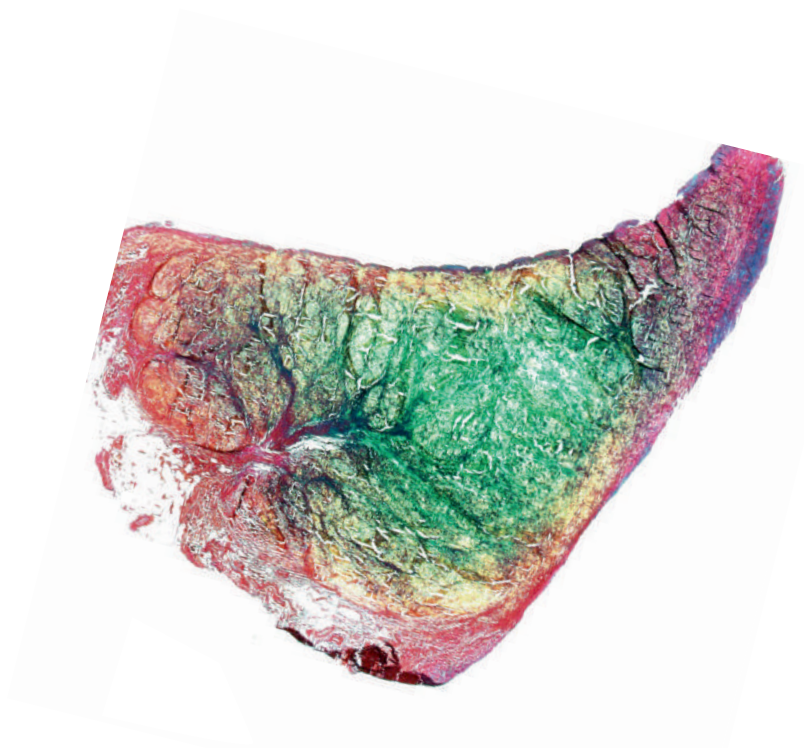

Abstract

Background: Meniscal repair is desirable over resection to prevent postmeniscectomy arthritis, especially among young and active patients. However, long-term data are currently lacking following isolated meniscal repair, particularly in the pediatric population.

Purpose: To report long-term follow-up of isolated meniscal tears treated by meniscal repair in a pediatric and adolescent population and to compare those results to previous midterm follow-up data reported. The authors hypothesized that these patients would have satisfactory function and reoperation rates at long-term follow-up.

Methods: Forty-four patients aged ≤ 18 years undergoing repair of an isolated meniscal tear (without concomitant anterior cruciate ligament injury) between 1990 and 2005 were included. At the time of final follow-up, recurrent tear, reoperations, and International Knee Documentation Committee (IKDC) and Tegner scores were determined. With logistic regression, the overall failure among tear types was calculated. Wilcoxon rank sum analysis were performed to calculate the differences in clinical outcome for different time points, and Spearman coefficients were calculated for Tegner and IKDC with different variables.

Results: At an average follow-up of 17.6 years (range, 13.1 - 26.0 years), 32 patients with 33 isolated meniscal repairs (29 male, 3 female) with a mean age of 16.1 (range, 9.9 – 18.7) were included in this study. At early follow-up, the overall failure rate was 14 of 33 (42%); complex tears (80%) and bucket handle tears (47%) had a higher overall failure rate compared with simple tears (18.2%), although only complex tears had significantly higher failure rate. However, no further failures occurred since midterm follow-up with any tear type. At final follow-up, the mean IKDC score was 92.3, which was significantly increased when compared with preoperative (65.3, $P < .0001$) and midterm (90.2, $P = .01$) scores. The mean Tegner score (6.5) was significantly lower than both preoperative (8.3, $P < .0001$) and midterm (8.4, $P < .0001$) scores. There was no difference in Tegner or IKDC score for patients with successful versus failed repair.

Conclusion: In conclusion, while there was a high early failure rate, this study demonstrates overall good to excellent long-term clinical outcomes after isolated meniscal repair in an adolescent population, even for those requiring reoperation. Early failure and reoperation rates were variable, depending on tear type, with complex multiplanar tears having more failures at short-term follow-up. However, at long-term follow-up, IKDC and Tegner scores were not significantly different for those with complex tears compared to other tear types.

Keywords: Meniscal tears; surgical repair; arthroscopy; pediatric patients; outcome; long-term follow-up; osteoarthritis

Introduction

Meniscal tears are very common orthopedic sports medicine injuries in the active adult population and are correlated with development of osteoarthritis.[7,17,28] With the goal of preserving meniscal tissue, meniscal repair for tears in an adult population has yielded good results in regards to immediate relief of mechanical symptoms, such as locking and pain. In addition, meniscal repair has demonstrated objective and subjective clinical success at follow-up.[2,6]

Despite the amount of data regarding meniscal repair outcomes among adults, few studies exist for meniscal repair in the pediatric and adolescent population. Current data demonstrate good results for clinical outcome and failure rate at short- to midterm follow up, but data on long-term follow-up after treatment are less well known.[10,11,14] Meniscal repair has become the treatment of choice because (partial) meniscectomy is strongly correlated with development of early osteoarthritis among children.[16,18] The isolated meniscal tear in pediatric and adolescent patients is less common but remains a well-recognized injury.[10,11,14] Meniscal tears accompanied by an anterior cruciate ligament (ACL) injury are more prevalent, better described in literature, and show a higher healing rate compared with isolated meniscal tears. [24,27] Therefore, the question of outcomes after repair of isolated meniscal tears in the pediatric and adolescent population remains of interest.[1,12,21] In addition, there is an increasing incidence of sports-related injuries of the knee, such as meniscal tears and ACL injuries in the pediatric population. This may be due to a higher engagement of children in competitive sports activities, or perhaps a better use of magnetic resonance imaging as a diagnostic tool, [8,26] making essential a good understanding of the course of treatment for these injuries.

The importance of meniscal preservation through repair is a well-understood, valid concept; however, there are limited long-term outcome data after meniscal repair. The goals of this retrospective study were to (1) obtain long-term follow-up of isolated meniscal tears in a pediatric and adolescent population treated by meniscal repair, (2) compare those results with previously reported midterm follow-up data, and 3) define the risk factors for failure of meniscal repair or worse outcome.

Methods

Patients

We utilized a patient group that was reported in a clinical study in 2008 by Krych et al.[11] Those patients met the criteria of inclusion by having an isolated meniscal repair procedure performed at the same institution between 1990 and 2005 and being ≤ 18 years old when the operation was performed. All patients were contacted retrospectively by phone, and information on reinjuries and reoperations was collected, as well as the International Knee Documentation Committee (IKDC) and Tegner activity scores.

Surgical Procedures

The inclusion criteria for this study, as described by Krych et al, [11] were full-thickness tears > 1 cm in length and within 6 mm of the meniscosynovial junction where stabilization of the lesion was possible. The meniscal tears were described during arthroscopy and categorized by simple (1 major tear component - longitudinal, horizontal/cleavage, or radial), bucket-handle (vertical tear with a displaced bucket-handle fragment), or complex tears (multi plane combination with ≥2 tear components).[11] The surgical techniques included (1) a vertical mattress inside-out technique with 2-0 Ethibond sutures (Ethicon Inc) with zone-specific cannulas[23], and (2) a hybrid technique with a combination of all-inside suture devices with 2-0 Ethibond via an inside-out technique. The postoperative protocol included protected weightbearing, limited range of motion (< 90°) for 3 to 4 weeks, and return to sports after 4 to 6 months based on clinical progress.

Outcome Measures

Failure was defined as reinjury of the previously repaired meniscus by clinical or radiographic examination, by reoperation on the same meniscus with repair or meniscectomy, or by any further treatment/care sought for injured meniscus. The reinjury rate after midterm follow-up was obtained by direct questioning of patients during a follow-up phone call. Patients were considered clinically successful when no reinjury occurred, no subsequent surgery was performed, and no further care was sought regarding the injured knee. We

considered clinically successful patients to be those who denied pain which was interfering with their activities. During this follow-up contact with the patients, outcome measures were collected by administering the Tegner activity and IKDC measures over the phone, by going through each question on the IKDC subjective scale and Tegner Activity Scale.

Statistical Analysis

A logistic regression model was used to determine the difference in overall failure of meniscal repair surgery among the simple, bucket handle and complex tear types. Because there was a nonnormal distribution of the data Wilcoxon rank sum analysis was utilized to compare mean IKDC and Tegner values between long-term follow-up and preoperative and midterm follow-up scores. Wilcoxin rank sum was also used to compare mean IKDC and Tegner values based on sex, laterality, surgical technique, and previous failure. Wilcoxon rank sum analysis was further used to compare mean time (in days) from injury to surgery, age at injury, and rim width. Kruskal-Wallis test was used to assess for differences in IKDC and Tegner scores among tear complexities (simple, bucket-handle, and complex). Last, Spearman analysis was done to test for correlations between IKDC and Tegner scores and rim width, time to repair, follow-up time, age, and age at injury. All statistical analysis was performed using JMP (v 13; SAS Institute Inc), and a *P* value of < .05 was considered significant.

Results

Patient Characteristics

Forty-seven patients who underwent 48 isolated meniscal repairs at a single orthopedic institute were initially included after application of the exclusion criteria per our institute's previous study, including ACL tear or reconstruction, grade II or III posterior cruciate ligament injury, full thickness (grade IV) osteochondral lesions, discoid lateral meniscal kiss tears, and periarticular fractures. That study included survival information from 45 meniscal repairs among 44 patients, as 3 patients were unable to be reached for follow-up. Of those 44 patients we were able to contact, 32 patients (3 female and 29 male) were

included in this study, with a mean age of 16.1 years (range, 9.9 to 18.7 years) at the time of meniscal repair, for a total of 33 isolated meniscal tears (1 male patient had bilateral meniscal repair surgery). In this long-term follow-up study, the mean follow-up of all 32 patients was 17.6 years (range, 13.1 to 26.0 years). Of the 32 patients, 20 had a meniscal tear of the left knee, 11 of the right knee and 1 bilateral, where 16 medial and 17 lateral menisci were affected. The incidence of bucket-handle tears was highest in our population (n = 17), followed by simple (n = 11) and then complex (n = 5) tears (Table 1).

Failure rate

We are unaware of the success or failure status of the 12 patients unavailable for long-term follow-up; therefore, these patients were excluded. Of the 33 knees included in the study, none had failed since last follow-up, in 2008 (Figure 1). However, there were 14 “failed” meniscal repairs reported between operative intervention and midterm follow-up. Of 5 patients with complex tear types, 4 (80.0%) were found to have failed between operative intervention and midterm follow-up. Conversely, 8 of 17 (47.1%) bucket-handle tears, and 2 of 11 (18.2%) simple tears failed in that same time period, for an overall failure rate of 14 of 33 (42%). Comparison of complex and simple meniscal tears, yielded an odds ratio of 18.000 (P = .034, 95% CI = 1.242 – 260.918) and for bucket-handle versus simple tears, 4.000 (P = .132, 95% CI = 0.659 – 24.297), meaning that simple tears are significantly less likely to fail after repair surgery compared to complex lesions but not compared to bucket-handle tears.

Reoperations

The original short-term follow-up described 17 failed repairs among the 47 patients,[11] where all 17 patients underwent repeat surgery. No new failures were presented at long-term follow-up (Figure 2). Two bucket-handle tears received revision repairs, whereas for the other 15 patients, partial meniscectomy was performed, with 20% to 70% meniscal removal. Of the 17 patients who needed a reoperation, 8 presented within the first 6 months.

Pt	Gender	Age at injury	Knee	Meniscus	Tear type	Repair technique	Rim width	Follow-up time	IKDC score	Tegner activity score
1	Male	16.1	L	Medial	Bucket handle	IO	2	20.9	96.6	7
2	Male	17	R	Lateral	Bucket handle	IO	2	18.7	98.9	7
3	Male	17.6	R	Lateral	Bucket handle	IO	2	17.9	89.7	5
4	Male	17.9	L	Medial	Bucket handle	AI	4	17.6	74.7	5
5	Male	17.3	L	Medial	Bucket handle	IO	1	17.4	89.7	7
6	Male	15.7	R	Medial	Bucket handle	IO	3	18.0	95.4	7
7	Male	13.9	R	Lateral	Bucket handle	IO	1	21.6	90.8	5
8	Male	17.7	L	Lateral	Bucket handle	IO	4	25.9	90.8	6
9	Male	14.4	L	Lateral	Bucket handle	AI	4	16.0	86.2	7
10	Male	17	L	Medial	Bucket handle	IO	6	12.2	87.4	5
11	Male	14.7	R	Lateral	Bucket handle	AI	2	15.7	97.7	7
12	Male	16.4	R	Medial	Bucket handle	IO	0	24.9	88.5	5
13	Male	14.3	R	Lateral	Bucket handle	IO	3	22.7	92	8
14	Male	16.5	L	Lateral	Bucket handle	AI	3	14.7	94.3	7
15	Male	15.2	L	Lateral	Bucket handle	AI	2	15.7	100	8
16	Male	18.4	L	Medial	Bucket handle	IO	5	14.8	94.3	5
17	Male	14.8	L	Medial	Bucket handle	IO	2	14.4	93.1	8
18	Male	16.8	L	Medial	Complex	IO	3	25.3	95.4	7
19	Female	15.8	L	Medial	Complex	IO	5	16.6	100	7
20	Male	17.7	L	Lateral	Complex	IO	4	14.2	87.4	7
21	Male	17.9	L	Lateral	Complex	AI	5	17.4	56.3	5
22	Male	17	R	Medial	Complex	IO	4	13.2	100	7
23	Male	17.5	R	Medial	Simple	AI	4	18.3	98.7	5
24	Male	14.7	L	Lateral	Simple	AI	2	18.9	94.3	7
25	Male	17.8	R	Medial	Simple	AI	3	14.9	100	7
26	Female	13.6	R	Medial	Simple	AI	5	16.7	98.9	7
27	Male	15.5	L	Medial	Simple	AI	2	14.6	98.9	7
28	Male	13.8	L	Medial	Simple	IO	3	15.0	98.9	9
29	Female	9.9	L	Lateral	Simple	IO	4	14.3	92	7
30	Male	14.1	L	Lateral	Simple	IO	4	21.9	92	8
31	Male	17.1	L	Lateral	Simple	IO	4	19.8	89.7	5
32	Male	18.7	L	Medial	Simple	AI	3	18.4	96.6	7
33	Male	16.2	R	Lateral	Simple	AI	2	13.1	98.9	7

Table 1; Patient characteristics. IO =Inside out; AI = All inside; IKDC = International Knee Documentation Committee

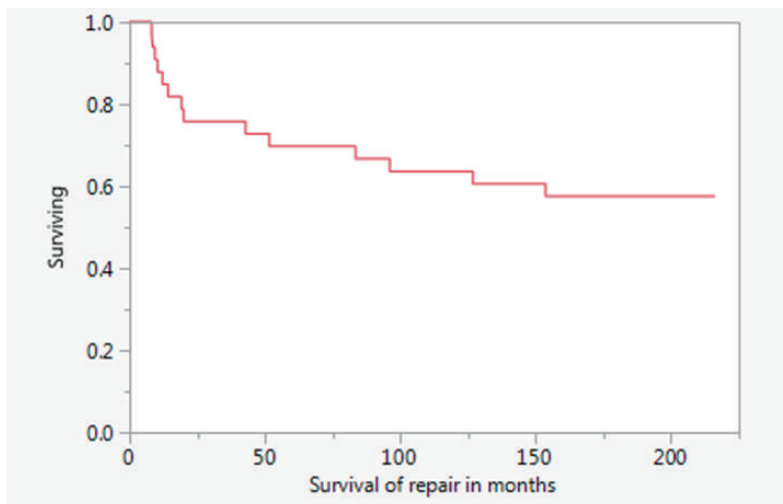

Figure 1: Kaplan Meier curve for the arthroscopic isolated meniscal repair for the failure free survival.

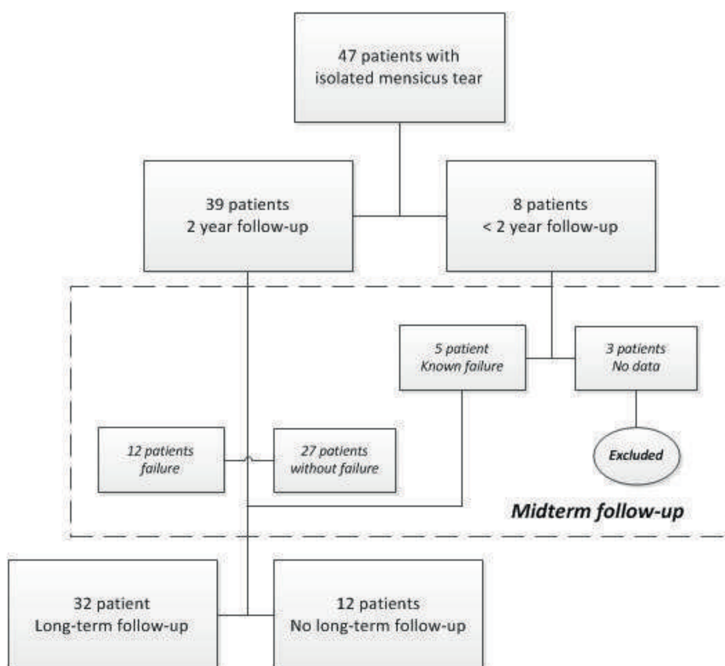

Figure 2: A total of 47 patients were included in the midterm follow-up study. Of those 47 patients, 39 were suitable for two year follow-up. Out of these 39 patients, 32 were contacted successfully for long-term follow-up.

Clinical outcomes and comparison to midterm outcome

The IKDC forms were administered and Tegner activity scores were determined at a mean postoperative follow-up of 17.6 years (range, 13.1 - 25.9 years). The mean IKDC score was 92.3 (range, 88.5 – 100.0), which was significantly increased when compared with both preoperative IKDC score (65.3, P < .0001) and midterm follow-up score (90.2, P = .01; mean, 5.8 years). After 17.6 years, the mean Tegner activity score was 6.53 (range, 5 – 9), which was significantly lower than both the preoperative (8.33, P < .0001) and the midterm score (8.39, P < .0001) (Figure 3).

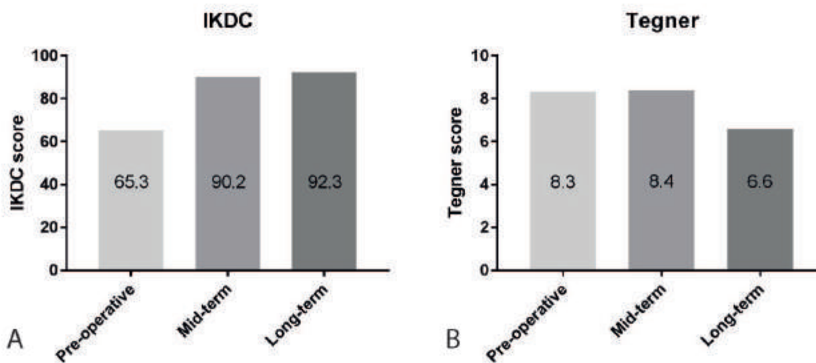

Figure 3: The figure demonstrates the IKDC (A) scores and Tegner Activity levels (B) for the three time points at which data was gathered, 1) Pre-operative, 2) Midterm follow-up (approximately 6 years), and 3) Long-term follow-up (approximately 18 years).

Risk factor analysis

Comparisons between failed repairs at any time during follow-up and successful repairs revealed that successful repairs had better IKDC scores failed repairs (94.2 vs 89.9, P = .10), although the difference was not statistically significant. Other comparisons were not significant between failed and successful groups: mean time from injury to repair (with injury, 50.7; without, 69.4; P = .86), age at injury (with injury, 16.5; without, 15.7; P = .43), rim width of the tear (with injury, 2.9; without, 3.1; P = .84), and Tegner score (with injury, 6.5; without, 6.7; P = .64).

We found no significant differences for IKDC scores when comparing laterality (left, 90.9; right, 95.0; P = .22), sex (female, 96.9; male, 91.9; P = .19), or medial versus lateral tears (medial, 94.0; lateral, 90.7; P = .20). Similarly, none of

these comparisons yielded a significant difference in Tegner activity levels: laterality (left, 6.7; right, 6.4; P = .49), sex (female, 7; male, 6.6; P = .63), and tear (medial, 6.6; lateral, 6.6; P = .84).

When tear types were compared, analysis of variance failed to show any significant differences in IKDC score (simple, 95.5; bucket, 91.8; complex, 87.8; P = .23) or Tegner activity level (simple, 6.9; bucket, 6.4; complex, 6.6; P = .53) (Figure 4).

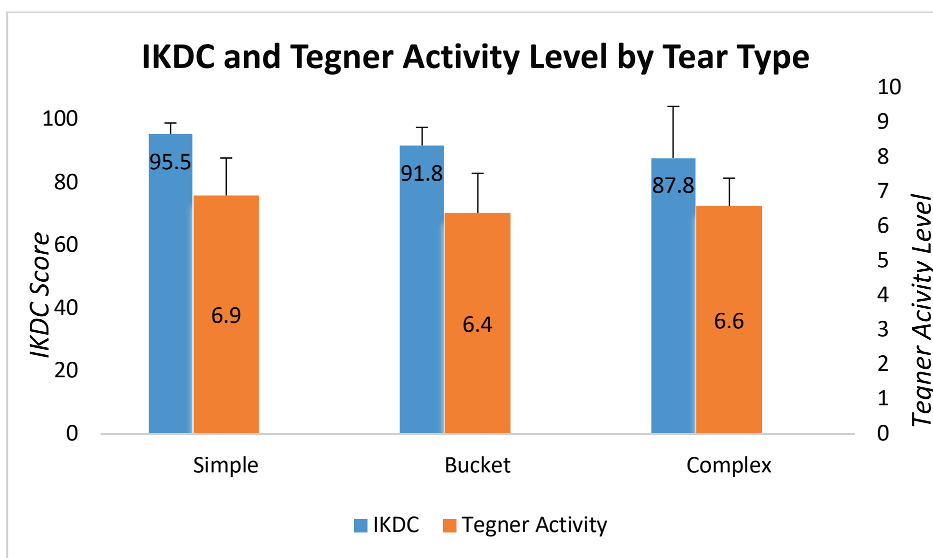

Figure 4: This figure demonstrates both IKDC and Tegner Activity level scores for all three meniscal tear types; simple, bucket handle, and complex. The left hand Y axis is the IKDC scores, and corresponds to the narrower, left hand side bar graph in each cluster. The right Y axis is the Tegner Activity Level score and corresponds to the wider, right hand side bar graph in each cluster. While complex tears seem to be associated with lower IKDC values, these were not shown to be significantly different from simple and bucket handle tears. Furthermore IKDC for all three tear types were not only acceptable, they were good to excellent.

When Spearman analysis was used to assess for correlation between different factors and outcome scores, older age (Spearman = 20.3528, P = .04) and older age at injury (Spearman = 20.5540, P = .0008) were each found to have a negative correlation with Tegner scores. Neither was found to have a significant correlation with IKDC scores (age: Spearman = 20.3066, P = .08; age at injury: Spearman = 20.2578, P = .15). Similarly, rim width, follow-up time, and time to

repair all failed to show significant correlation with IKDC or Tegner scores (Table 2).

Factors	Tegner score		IKDC	
	Spearman's correlation	*p-value*	*Spearman's correlation*	*p-value*
Older age	- 0.3528	0.04*	- 0.3066	0.08
Older age at injury	- 0.5540	0.0008*	- 0.2578	0.5
Rim width	- 0.0213	0.91	- 0.0620	0.74
Follow-up time	- 0.1568	0.38	- 0.2174	0.22
Time to repair	- 0.1288	0.48	0.0824	0.65

*Table 2: Spearman's analysis to assess for correlation between different factors and clinical outcome scores. * is p-value < 0.05*

Discussion

The most important finding of this study is that good to excellent clinical results can be obtained at long-term follow-up (mean, 17.6 years) for meniscal repair in a pediatric and adolescent population. Furthermore, our findings suggest that, except for tear complexity, other factors previously thought to confer poor or worse outcomes, such as rim width and lateral meniscal tears, had no significant influence at long-term outcome in the present cohort. This may be due to the relatively small size of our cohort; however, these outcomes are encouraging. Beyond meniscal tear characteristics, surgical technique, sex, laterality, and side all similarly failed to show any statistically significant differences in clinical success rate. One of the correlations to be demonstrated relevant was that between Tegner activity levels and older age as well as older age at injury. However, sports activity decreases with increasing age and thus may be an independent risk factor for lower Tegner scores and is not necessarily indicative of poorer surgical outcome leading to said lower score. Given all of our findings, we suggest meniscal repair in this

population can obtain good to excellent long-term results and tear size and location should not be a relative contraindication to attempt repair. In addition, tear complexity did not affect long-term clinical outcome, but the reoperation rate with subsequent partial meniscectomy in complex tear types was significantly higher as compared with the other types.

In the current study, the overall failure rate of arthroscopic repair of isolated meniscal tears among patients ≤ 18 years at midterm follow-up (mean, 5.8 years) was 38% (17 of 45 knees), as compared with an additional 0 failures at long-term follow-up (mean, 17.6 years).[11] The total percentage of failure at long-term follow-up is 42% (14 of 33 knees). This suggests that when a meniscal repair is successful in the first years after surgery, good long-term clinical outcome can be expected as well. In terms of best- and worst-case scenarios, is we take into account the patients who were lost to follow-up, the failure rate would be between 38% (17 failures out of 45 patients) and 64% (29 out of 45). A rim width >3 mm and complex meniscal lesions were previously described as risk factors for early failure of repair surgery.[10] Since those factors did not influence the long-term outcome, it could be possible that the original tears were not ideal candidates for meniscal repair, leading to early failure, likely the result of the tear not healing. Lucas et al similarly found no factors associated with failure after repairs.[14] In addition, early failures could be the result of initial healing with biomechanically inferior fibrous scar tissue, which is prone to retear in the early failures. Most early failures in our study population were caused by acute reinjury trauma within 1 year after repair surgery; therefore, early return to (competitive) sport could possibly be implicated as a risk factor. Shieh et al saw the same pattern in their study, where 80% of all the retears happened within the first 12 months.[25] Alternatively, if a repair fails early, it is possibly due to a lack of healing of the repair site, rather than reinjury. In the adult population, younger patients (<30 years) were at higher risk for revision of meniscal repair.[13,15] This could be due to the same effect of a higher engagement in competitive sportive activities among younger patients or the fact that early failures of meniscal repair might never have the change to heal. Currently, there are no studies performing second-look arthroscopy with biopsies after meniscal repair or

describing the actual healing process between the early failures and the successes after meniscal repair.

The IKDC is proven to be a valid method evaluating knee pain among children and adolescents [20,22] as well as adults [5,9] and is used in long-term follow-up of adolescent patients. At long-term follow-up, our patients scored very high on the IKDC (92.3), which is statistically though not clinically significantly higher than that at midterm follow-up (90.2). Schmitt et al [22] reported an IKDC score of 90.7 after 6.1 years of follow-up, which is comparable to our midterm results. Mintzer et al. [19] and Accadbled et al. [1] reported that 85% and 89% of the IKDC scores were > 75 at 5- and 3.1 year follow-up, respectively. The higher IKDC scores at long-term outcome are potentially affected by altered patient expectations because of the relative subjectivity of the IKDC. While many aspects of the IKDC are objective, there are slight subjective characteristics, and it is possible that patient expectations in an older population differ from those of the same population at a younger age, thereby causing a mild "inflation" of the scores. Finally, it is worth noting that those who underwent revision surgery for repair failure still obtained good to excellent IKDC scores that were neither statistically nor clinically significantly different than those among patients who did not require reoperation, (94.2 vs 89.9, P = .10).

Similarly, one of the correlations demonstrated in this study was a declining Tegner score with increasing age (6.6 at long-term follow-up). This again is most likely related to the natural tendency among people of older age to be less active and a decreasing involvement in competitive team sports.[3] Briggs et al[4] showed an inverse correlation between age Tegner activity level. The mean score in the general population for ages 18 to 30 was 6.5, where ages 31 to 45 years had a score of 5.9. The population of this study scored 8.3 at baseline (preoperatively) and 6.6 at long term follow-up, which is a greater difference as compared with the general population. However, at baseline, the study population was ≤18 years old and relatively active, since all the meniscal tears occurred during sports activities. Nevertheless, the long-term follow-up score of the study population was similar to that of the general population described by Briggs et al. This demonstrates no clinical difference in performance among our patients who underwent meniscal

repair at adolescent age as compared with the general population. At midterm follow-up (mean, 5.8 years), the Tegner score in our study (8.4) was higher than that in most studies on pediatric meniscal repair. Schmitt et al reported a Tegner score of 7.3 after 6.1 years, and Kraus et al showed an activity score of 7.0 after 2.3 years of follow-up.[10,22] An alternative explanation could be a decrease in activity level among patients attributed to knee symptoms related to their meniscal repair; however, given the good to excellent IKDC scores and lack of self-reported clinical symptoms we believe this to be less likely. In this study, no control group was included (ie, without meniscal injury/repair), which we believe would be a worthwhile comparison to make in future studies.

Revision meniscal surgery is relatively uncommon. [25] In this study, we could not identify any risk factors for failure of meniscal repair at long-term follow-up besides tear complexity. This could be due to the fact that we did not observe any additional failures at long-term follow-up in this small cohort study or because there are no other factors specifically contributing to long-term failure of meniscal repair. We also previously demonstrated complex tears to be a risk factor for failure of meniscal repair in the pediatric and adolescent population,[11] which was confirmed in this long-term follow-up study. However, no new failures were reported between midterm and long-term follow-up, suggesting failure is most likely to occur earlier after repair. With a mean follow-up of 40 months, Shieh et al[25] described skeletal immaturity and bucket-handle tears as risk factors for revision repair surgery for 23 of the 129 patients (18%) undergoing meniscal repair, thereby showing the highest failure rate within the first year after surgery. This confirms that tear complexity is a risk factor for early clinical failure, but not for poor long-term outcomes. It is also quite possible that tear complexity has an inverse relationship with time to meniscal healing and that more complex tears may take a much longer time to heal.

Several limitations of this study should be addressed. First, there are no magnetic resonance imaging data or radiograph images available at long-term follow-up; therefore, no data on radiographic progression of osteoarthritis could be described in our patients. Radiographic images were not obtained, as there were no

clinical symptoms to indicate our patients for imaging at long-term follow-up. However, future studies should certainly assess any development of arthritis. Second, the results of this study are the "best-case scenario," since the second-look imaging is absent; failures of repair without clinical presentation would be missed. Third, it is a retrospective study with a relatively small number of patients; however, isolated meniscal injuries in the pediatric population are uncommon, and to our best knowledge, this is the largest cohort describing the long-term clinical outcome of meniscal repair in pediatric patients. Unfortunately, there was a considerable percentage (27.3%) of patients lost to follow-up, possibly associated with the pediatric and adolescent population, which moves after high school to different states for college and work.

In conclusion, this study demonstrates good to excellent long-term clinical outcomes after isolated meniscal repair in a pediatric and adolescent population. Early failure and reoperation rates were variable depending on tear type, with complex multiplanar tears having more failures at short-term follow-up. At long-term follow-up, IKDC scores and Tegner scores were not significantly different for those with complex tears compared with other tear types.

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Chapter 3

Secondary Meniscal Tears in Patients with Anterior Cruciate Ligament Injury

Relationship Among Operative Management, Osteoarthritis, and Arthroplasty at 18 Year Mean Follow-Up

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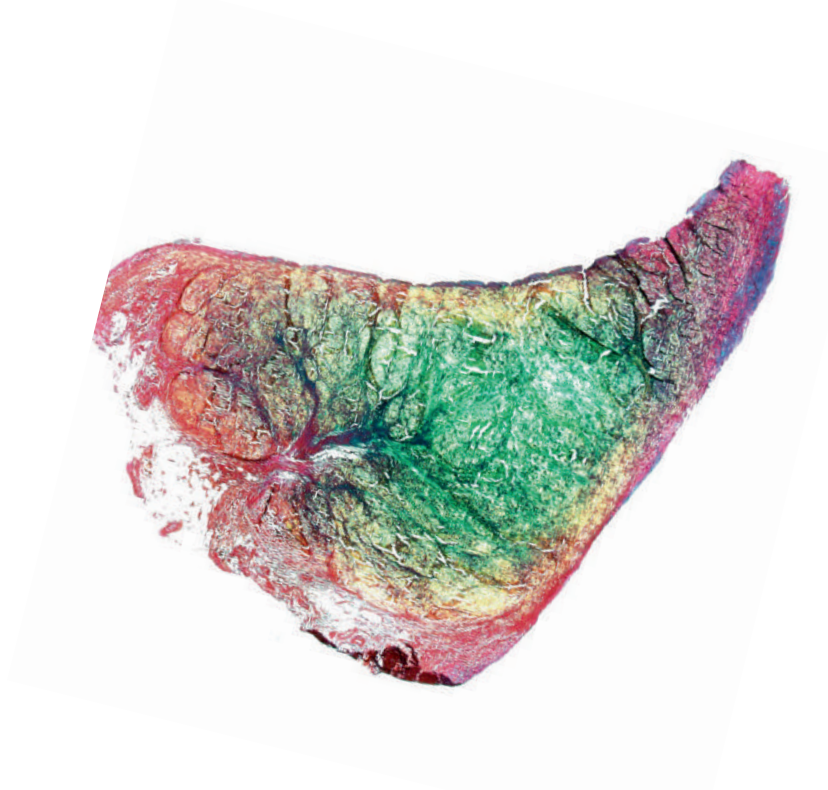

Abstract

Background: Anterior cruciate ligament (ACL) injury is one of the most frequent orthopedic injuries and reasons for time loss in sports and carries significant implications, including posttraumatic osteoarthritis (OA). Instability associated with ACL injury has been linked to the development of secondary meniscus tears (defined as tears that develop after the initial ACL injury). To date, no study has examined secondary meniscus tears after ACL injury and their effect on OA and arthroplasty risk.

Purpose: To describe the rates and natural history of secondary meniscus tears after ACL injury and to determine the effect of meniscus tear treatment on the development of OA and conversion to total knee arthroplasty (TKA).

Methods: A geographic database of > 500,000 patients was reviewed to identify patients with primary ACL injuries between January 1, 1990 and December 31, 2005. Information was collected with regard to ACL injury treatment, rates/ characteristics of the secondary meniscus tears, and outcomes, including development of OA and conversion to TKA. Kaplan-Meier and adjusted multivariate survival analyses were performed to test for the effect of meniscus treatment on survivorship free of OA and TKA.

Results: Of 1398 primary ACL injuries, the overall rate of secondary meniscus tears was 16%. Significantly lower rates of secondary meniscus tears were noted in patients undergoing acute ACL reconstruction within 6 months (7%) as compared to patients with delayed ACL reconstruction (33%, $P < .01$) and nonoperative ACL management (19%, $P < .01$). Of the 235 secondary meniscal tears identified (196 patients), 11.5% underwent repair, 73% partial meniscectomy, and 16% were treated nonoperatively. Tears were most often medial in location (77%) and complex in morphology (56% of medial tears, 54% of lateral tears). At the time of final follow-up, no patient undergoing repair of a secondary meniscus tear (0%) underwent TKA, as compared to 10.9% undergoing meniscectomy and 6.1% receiving nonoperative treatment ($P = .28$).

Conclusion: Secondary meniscus tears after ACL injury are most common among patients undergoing delayed surgical or nonoperative treatment of their primary ACL injuries. Secondary tears often present as complex tears of the medial meniscus and result in high rates of partial meniscectomy.

Key Terms: Meniscal tears; anterior cruciate ligament injury; long-term follow-up; osteoarthritis; total knee arthroplasty

Introduction

Anterior cruciate ligament (ACL) injury and meniscal tears are among the most frequently reported injuries in the orthopedic literature, cause significant time loss to sports participation and carry significant post-injury implications.[20] ACL injury has been strongly correlated with the development of secondary meniscus tears and early osteoarthritis (OA), especially in young and active patients.[7,16,27] Long-term outcomes following injury appear to be dependent on treatment strategy, such as acute reconstruction, delayed reconstruction, and non-operative management.[9,12,17,25] In a series of 964 patients, Sanders et al. demonstrated that patients treated with ACL reconstruction (ACLR) had a significantly lower risk of secondary meniscal tears, OA, and total knee arthroplasty (TKA) when compared to patients treated nonoperatively.[25]

Persistent knee instability after an ACL tear can lead to instability-related damage to the menisci, subchondral bone and articular cartilage with subsequent joint degeneration.[4] Secondary meniscal tears, defined as tears that develop after the initial ACL injury, represent another potential contributor to post-traumatic arthritis owing to the increased articular contact stresses associated with meniscal injury.[6,21] Meniscal tear type and complexity often influence repair potential and, subsequently, long-term outcome.[5,10] When tear configuration permits, repair is greatly preferred over meniscectomy for younger patients given the negative degenerative implications associated with loss of meniscal tissue and associated distribution of intra-articular forces.[5,10,11,32] Continued microtrauma in the setting of the ACL deficient knee can lead to meniscal degeneration to the extent that it can no longer be readily repaired.

The purposes of this study were (1) to define the rates of secondary meniscus tears after acute ACLR, delayed ACLR, and nonoperative management of initial ACL injuries with use of a large population-based database; (2) to determine the characteristics of these secondary meniscal tears; and (3) to determine the effect of tear treatment on the development of symptomatic OA and TKA at long-term follow-up. The hypotheses tested were that (1) acute operative management of ACL injury would be associated with a decreased rate of secondary meniscal tears, (2) secondary meniscal tears would frequently be complex in morphology and often require partial meniscectomy, and (3) meniscal repair would confer a protective

effect against OA and TKA as compared to meniscectomy and nonoperative management.

Materials and Methods

Study Population and Design

Patients with ACL injuries were identified with the Rochester Epidemiology Project (REP), a geographic database containing the medical records of > 500,000 patients of all ages residing in Olmsted Country, Minnesota, and neighboring areas.[24] Patients can authorize and choose not to participate in health record disclosure for external use through Minnesota Statute 144.295. The REP provides access to the complete medical records for all participating residents within its catchment area, regardless of the hospital where patients received care, which enables the determination of accurate incidence and natural history of diseases.[24,28,30,31]

The REP was used to identify all individuals with new ACL tears occurring between January 1, 1990 and December 31, 2005, based on physician-determined diagnostic codes. The database compiled complete diagnostic and procedural information from all medical centers Patients were subsequently followed in the database until December 31, 2017 in order to ensure sufficient follow-up to determine natural history after various ACL and meniscus interventions. All clinical notes, radiographic images, and operative notes related to the injury were manually reviewed in detail. Patients were included if they had a new-onset, full-thickness ACL injury, subsequent ipsilateral meniscus tear after ACL injury, and minimum 2-year follow-up. Exclusion criteria consisted of previous knee surgery, multiligamentous injury, unknown ACL treatment, unknown meniscal treatment, and unretrievable data from paper and/or electronic patient files. Subsequent meniscal tears were determined by reviewing the clinical notes, radiographic images, and operative notes after the primary ACL injury.

Of the 1398 subjects with an ACL injury retrieved from the REP database, 217 developed a secondary meniscal tear after an initial ACL injury. After manual review of medical records, data regarding the treatment of meniscus tears of 21 patients were not clearly reported, and these patients were excluded from further

analysis (Figure 1). For the remaining 196 patients, age at time of injury, sex, laterality, treatment of ACL injury, time from ACL injury to meniscal tear, meniscal tear type, location, and treatment modality were collected using standardized data collection forms. ACL treatment was divided into three categories: (1) acute ACLR within 6 months of the injury, (2) delayed ACLR > 6 months after injury, and (3) nonoperative management.[9] Meniscal tears were classified as simple (horizontal, vertical longitudinal, and small), bucket-handle, and complex (multiplane combinations, flap, oblique, parrot beak, and degenerative).[19] Tear locations were classified as posterior horn, anterior horn, and body, as reported in the pre-operative magnetic resonance imaging report and/or operation report. Meniscal tear treatment modalities included meniscal repair, partial meniscectomy, and nonoperative management. Patient records were reviewed to assess long-term outcomes including ACLR failure and development of symptomatic OA, defined as symptoms significant enough to warrant seeking care with a physician and requiring the presence of grade ≥2 Kellgren-Lawrence radiographic changes and conversion to TKA.

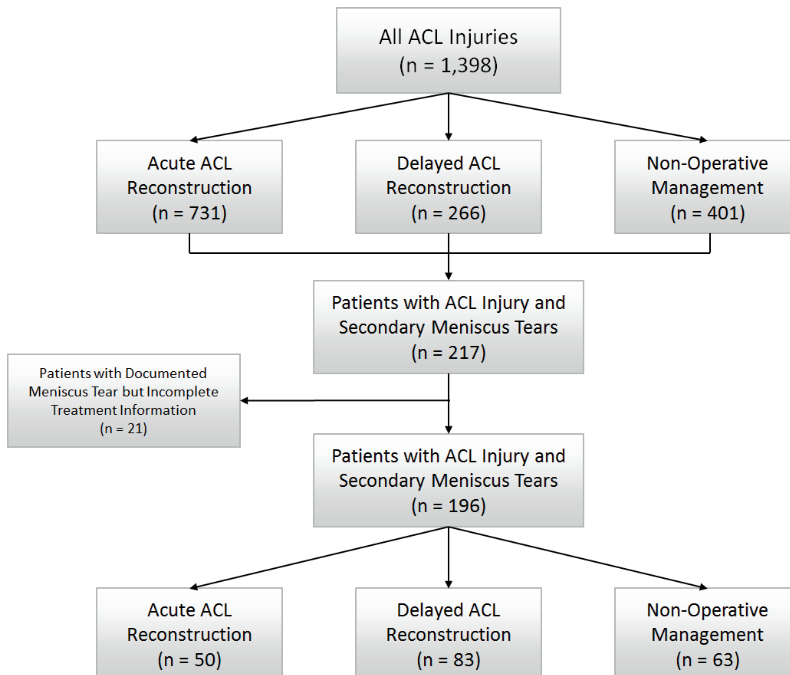

Figure 1: Diagram of the study population. ACL; anterior cruciate ligament

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Statistical Analysis

Patients variables and their associated SDs and percentages were reported for descriptive representation of the study cohorts. The operative and nonoperative groups were compared to evaluate for potential patients and lesion differences using Fisher exact test for proportions and the Kruskall-Wallis test for continuously distributed and ordinal variables. Cox proportional hazards regression was performed to determine risk factors for progression to symptomatic arthritis and TKA at the time of final follow-up. P values < .05 were considered significant. Analyses were conducted in R 3.4.3 (R Core Team, Vienna, Austria) and JMP Pro 13 (SAS Institute, Cary, NC).

Results

Rates of Secondary Meniscus Tears

The overall rate of secondary meniscus tears among patients with ACL injury was 16% (95% CI, 14% - 18%). Treatment-specific rates of secondary meniscal tears were 7% (95% CI, 5% - 9%) for acute ACLR within 6 months of the time of injury, 33% (95% CI, 28% - 39%) for delayed ACLR, and 19% (95% CI, 16% - 24%) for patients managed nonoperatively. Acute ACLR demonstrated a significantly lower rate of secondary meniscal tears than nonoperative management (P < .01), which had a significantly lower rate of tears as compared to delayed ACLR (P < .01).

Secondary Meniscus Tear Characteristics

Out of the 1398 ACL injuries analyzed, 820 patients (58.7%) had associated meniscal tears at the time of injury (435 medial, 259 lateral, 126 medial and lateral), of which 115 (14.0%) went on to primary repair. Of the 217 patients with secondary meniscal tears identified in the REP database, 196 patients (71 females, 125 males) with 235 meniscal tears (157 medial or lateral meniscus, 39 both) had comprehensive treatment documentation and were eligible for inclusion in subsequent analyses (Figure 1). Of the 196 analyzed cases with secondary meniscus tears, 12 (6%) represent retears in the same compartment. Patients had a mean ± SD age of 28.9 ± 9.6 years and were followed for a mean 17.5 years (range: 3.9 – 26.3).

Fifty patients (26%) were included in the acute ACLR group (mean follow-up, 14.9 years); 83 (42%) underwent ACLR > 6 months after the time of injury and were included in the delayed ACLR group (mean follow-up, 14.9 years); and 63 (32%) pursued non-operative management (mean follow-up, 19.1 years). Age at index ACL injury, meniscal laterality, medial meniscal tear treatment, and lateral meniscal differed among the 3 ACL treatment groups (Table 1). Mean age at injury for acute ACLR (25.2 years) and delayed ACLR (26.9 years) was significantly lower than for the nonoperative group (34.5 years, P < .01). The mean time to secondary meniscal tear was 8.4 ± 5.8 years for the acute ACLR group, 6.5 ± 4.4 years for the delayed ACLR group, and 9.5 ± 4.8 years for the nonoperative management group (P < .01). There was no significant difference in time to secondary tear between the early and late ACLR groups (P = .12). Nonoperative management resulted in a similar time to retear as early ACLR (P = .18) but significantly longer time to documented retear compared to delayed ACLR (P < .01). The mean overall follow-up was 7.9 ± 5.1 years (range, 3 months - 22 years 9 months).

In terms of secondary tear characteristics, the majority of tears were isolated to the medial meniscus (57%), whereas lateral meniscus and concurrent tears of both menisci were detected at similar, lower rates (23% and 20%, respectively) (Figure 2). The posterior horn was the most often affected meniscal location (64%). Overall, complex tears comprised a large percentage (55%) of secondary meniscal tears. When the proportion of complex tears was compared to other tear configurations in the medial meniscus, the complex tears were most common in the acute ACLR group (64%), followed by the nonoperative group (61%), and then delayed ACLR group (49%) (P = .048). Similarly, the proportion of complex lateral meniscal tears was highest in the acute ACLR group (60%) followed by the nonoperative (57%) and then delayed ACLR (47%) groups (P = .01).

Table 1: Patient and Meniscal Injury Characteristics for Patients with Secondary Meniscal Tears[a]

Variable	Acute ACLR (n = 50)	Delayed ACLR (n = 83)	Non-Operative (n = 63)	p-value
Demographics				
Age at ACL Injury	25.2 ± 10.1	26.9 ± 8.5	34.5 ± 8.1	**< 0.01**
Sex				
Female	17 (34.0%)	27 (32.5%)	27 (42.9%)	
Male	33 (66.0%)	56 (67.5%)	36 (57.1%)	0.42
Knee Laterality				
Left	29 (58.0%)	40 (48.2%)	30 (47.6%)	
Right	21 (42.0%)	43 (51.8%)	33 (52.4%)	0.47
Meniscus Laterality				
Medial	35 (70.0%)	51 (61.4%)	26 (41.3%)	
Lateral	5 (10.0%)	15 (18.1%)	25 (39.7%)	
Both	10 (20.0%)	17 (20.5%)	12 (19.0%)	**< 0.01**
Medial Meniscus Tear Characteristics				
# with Medial Tears	45 (90.0%)	68 (81.9%)	38 (60.3%)	**< 0.01**
Tear Locations				
Involved[b]	6 (13.3%)	2 (2.9%)	2 (5.3%)	
Anterior horn	10 (22.2%)	8 (9.6%)	5 (13.2%)	
Body	37 (82.2%)	50 (73.5%)	25 (65.8%)	
Posterior horn	5 (11.1%)	11 (16.2%)	10 (26.3%)	0.23
Unknown				
Tear Pattern				
Simple	3 (6.7%)	12 (17.6%)	4 (10.5%)	
Bucket-handle	9 (20.0%)	9 (13.2%)	6 (15.8%)	
Complex	29 (64.4%)	33 (48.5%)	23 (60.5%)	
Unknown	4 (8.9%)	14 (20.6%)	5 (13.2%)	0.30
Treatment				
Repair	7 (15.6%)	10 (14.7%)	0 (0.0%)	
Partial	33 (73.3%)	48 (70.6%)	27 (71.1%)	
meniscectomy				
Non-operative	5 (11.1%)	10 (14.7%)	11 (28.9%)	**0.02**
Lateral Meniscus Tear Characteristics				
# with Lateral Tears	15 (30.0%)	32 (38.6%)	37 (58.7%)	**< 0.01**
Tear Locations				
Involved*	5 (33.3%)	3 (9.4%)	8 (21.6%)	
Anterior horn	5 (33.3%)	4 (12.5%)	8 (21.6%)	
Body	5 (33.3%)	26 (81.3%)	23 (62.2%)	
Posterior horn	3 (20.0%)	3 (9.4%)	5 (13.5%)	0.09
Unknown				
Tear Pattern				
Simple	4 (26.7%)	15 (46.9%)	4 (10.8%)	
Bucket-handle	0 (0.0%)	1 (3.1%)	3 (8.1%)	
Complex	9 (60.0%)	15 (46.9%)	21 (56.8%)	
Unknown	2 (13.3%)	1 (3.1%)	9 (24.3%)	**< 0.01**
Treatment				
Repair	1 (6.7%)	5 (15.6%)	4 (10.8%)	
Partial	14 (93.3%)	23 (71.9%)	26 (70.3%)	
meniscectomy	0 (0.0%)	4 (12.5%)	7 (18.9%)	0.38
Non-operative				

[a] Values provided as mean ± SD or n (%). Bold indicates P < .05
[b] In total, 20 (13.2%) medial tears and 13 (15.5%) lateral tears extended into multiple locations

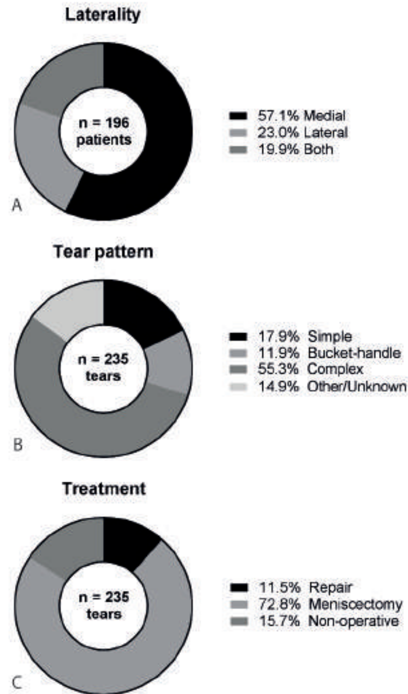

Figure 2: Characteristics of secondary meniscal tear (a) laterality, (B) pattern, and (C) treatment. A total of 235 meniscal tears (196 patients) were analyzed.

The most common overall treatment modality for secondary tears was partial meniscectomy, which occurred in at least 1 tear for 147 of 196 (75%) patients and 171 of 235 (73%) tears. Repair of secondary meniscus tears was most common in patients who elected for acute ACLR (12%) and delayed ACLR (10%), as compared to only 3% of those patients who elected for initial nonoperative ACL injury management ($P < .01$). A greater portion of patients who chose to pursue nonoperative therapy for their ACL injury also pursued nonoperative secondary meniscal tear management (25%), with the majority of the remaining patients undergoing simple partial meniscectomy (71%). In contrast, only 10.0% and 15% of the acute and delayed ACLR groups underwent nonoperative management of their meniscus tears ($P = .07$).

Treatment of Secondary Meniscus Tears and Symptomatic OA and TKA

Kaplan-Meier analysis was performed to investigate the relationship between meniscal tear management strategy and observed rates of symptomatic

OA and TKA. While no significant difference was found when OA and TKA rates were analyzed as a function of time since meniscal tear (Figure 3, A and B) or time since index ACL injury (Figure 3, C and D), patients who underwent repair demonstrated higher estimated survival rates as compared with patients undergoing meniscectomy or nonoperative management on all 4 analyses (P ≥ .15).

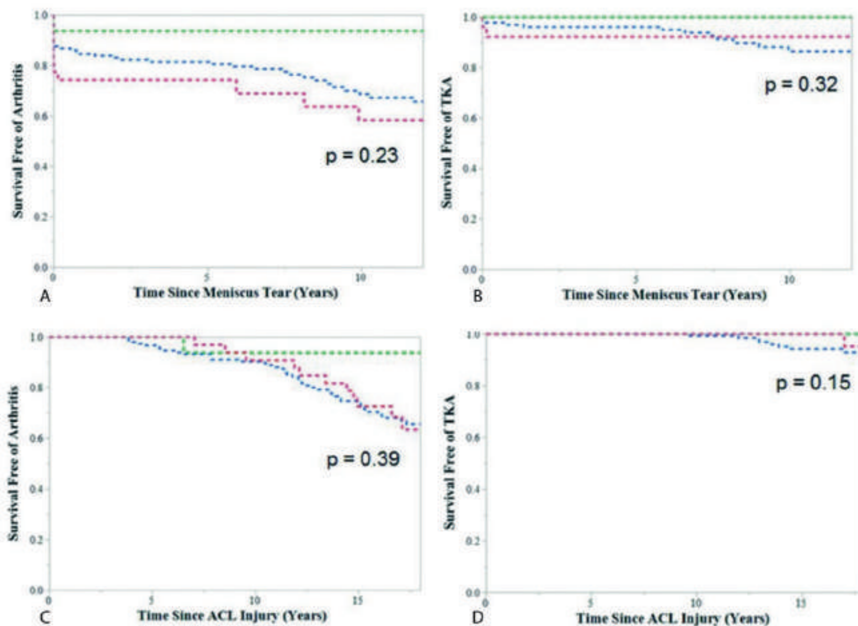

Figure 3: Kaplan Meier curves for patients who underwent secondary meniscal tear repair (green), meniscectomy (blue), and nonoperative treatment (red). Arthritis- and TKA-free survival is provided as a function of (A, B) time since meniscus tear and (C, D) time since index ACL injury.
ACL, anterior cruciate ligament; TKA, total knee arthroplasty.

Subsequently, age-adjusted multivariate survival analyses for OA and TKA were performed in light of the fact that patients treated nonoperatively significantly older than patients undergoing ACLR and meniscal repair (Table 2). While the observed hazard estimates for arthritis and TKA following meniscal repair and ACLR fell under 1.00, which may indicate a protective effect of repair / reconstruction (P ≥ .16), age was the only significant predictor of progression to OA and TKA at the time of final follow-up (P < .01). Of note, none of the 16 patients with meniscal tears who underwent repair progressed to TKA at a mean of 15.2 ±

4.9 years of follow-up. In comparison, 16 of 147 patients (11%) who underwent partial meniscectomy and 2 of 33 patients (6%) who elected for nonoperative management progressed to TKA (P = .28). Secondary tear morphology was found not to significantly affect rates of arthritis or subsequent TKA (P ≥ .33).

Table 2: Age-Adjusted Survival Free of Arthritis and TKA by Meniscal and ACL Management Strategy [a]

	Survival Free of Arthritis		Survival Free of TKA	
Variable	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Age, per year increase	1.06 (1.03 – 1.09)	< **0.01**	1.13 (1.08 – 1.19)	< **0.01**
Meniscus Management	Reference		Reference	
Non-operative	1.25 (0.70 – 2.22)	0.46	6.60 (0.67 – 64.82)	0.11
Meniscectomy	0.47 (0.10 – 2.36)	0.36		–
Repair			0.00 (0.00 – 0.00)[b]	
ACL Management				
Non-operative	Reference		Reference	
Acute Repair	0.97 (0.53 – 1.77)	0.91	0.21 (0.02 – 2.67)	0.23
Delayed Repair	0.66 (0.38 – 1.18)	0.16	0.62 (0.17 – 2.27)	0.47

[a] ACL, anterior cruciate ligament; HR, hazard ratio; TKA, total knee arthroplasty.
[b] All 16 meniscal tears that were repaired did not progress to TKA during follow-up. However, this is not powered for comparisons with the 16 of the 147 patients undergoing meniscectomy and 2 of the 33 patients with nonoperative treatment.

Discussion

ACL injury and associated meniscal tears are common in the orthopedic surgery practice and carry significant implications, including knee instability, OA, and subsequent conversion to TKA at long-term follow-up. The goals of this study were to define the rates and characteristics of secondary meniscal tears after ACL injury and to determine the effect of meniscal tear treatment on the development of symptomatic OA and TKA. The tested hypotheses were confirmed in that patients who underwent acute ACLR demonstrated the lowest rates of secondary meniscal tears and that observed secondary tears were most often complex in morphology. In the current study, meniscal repair consistently trended towards a protective effect against OA and against conversion to TKA in both the univariate and multivariate analyses; however, this was not found to be statistically significant.

Prior studies have described ACL injury and meniscal tears and presented outcomes of various management strategies, as well as the risk of subsequent OA.[26,27,32] However, the majority of the currently available literature concerns primary meniscal tears as opposed to secondary tears that develop over time.[11,22] The overall rates of secondary meniscal tears observed in this study were lowest for patients who underwent acute ACLR, which supports reconstruction of the ACL within 6 months for those pursuing operative management. While patients in the acute and delayed ACLR groups were similar in age, those who underwent sustained nonoperative management were nearly 10 years older on average than their operative counterparts. While likely multifactorial in nature, the lower age- and demand-associated activity level (ie, no cutting and pivoting sports) of older patients who elected for non-operative management may explain the lower rate of secondary meniscal tears observed in the non-operative management group as compared to patients with delayed ACLR, as well as the higher rate of complex meniscus tears in the acute ACLR group. In addition, patients initially treated nonoperatively for their ACL injury who developed a secondary meniscal tear were more likely to cross over from the non-operative treatment group to the delayed ACLR group to treat both the secondary meniscal tear and the ACL injury at the same time. Accordingly, patients who never underwent ACLR had a lower rate of secondary meniscal tears compared to those with delayed ACLR. Another influence could be detection bias, where symptomatic meniscal tears were more likely to be treated operatively in combination with ACLR, although data on decision making were not available for this study. The current data indicates that new meniscal tears lead to the decision to perform delayed ACLR. In approximately 70% of patients, the secondary meniscal tear occurred before the delayed ACLR, which may indicate that patients performed well after nonoperative management until they had a macroinstability episode that lead to a secondary meniscal tear. In addition, patients treated with delayed ACLR often undergo long-term postoperative imaging and follow-up, a potential source of increased detection of secondary meniscal tears in this group when compared to patients treated non-operatively.

Medial meniscual tears, especially in the posterior horn and midbody, are more common in knees with a chronic ACL injury, whereas lateral meniscal lesions

are classically associated with the acute trauma causing ACL injury.[13,15] These findings are mirrored in the current study, which demonstrated a high rate of medial meniscal pathology, with almost two-thirds of secondary tears located in the posterior horn of the meniscus. The association between chronic ACL deficiency and secondary tears of the medial meniscus is biomechanically most likely due to the role the medial meniscus plays in the stabilization of an ACL-deficient knee, as opposed to the more mobile and nonconstraining role of the lateral meniscus.[2] This is of clinical significance given that the shift in articular loading patterns associated with ACL and medial meniscal deficiency can both ultimately contribute to the development of OA.[2,6,13] In addition, secondary meniscal tears observed in this study were most often complex in morphology. In contrast, primary meniscal tears, such as those described in the study by Fox et al, are most often simple in morphology.[13] In this series, 60% of primary tears were simple, 10% were bucket-handle, and 30% were complex. These findings indicate that ACL deficiency leads to increased incidence of medial meniscal pathology as well as increased tear complexity when compared with primary meniscal tears. This is clinically significant given that partial meniscectomy, such as that performed in the setting of irreparable complex tears, has repeatedly demonstrated higher rates of OA and TKA compared with meniscal repair.[1,3,23,29]

In the current study, the statistically significantly parameter observed to influence the development of symptomatic OA was age at the time of initial ACL injury. This finding is consistent with previous studies that implicated age as a major risk factor for the development of meniscal injury as well as subsequent OA.[8,13] In the current study, multivariate analysis for risk of symptomatic OA showed that ACL and meniscal repair were potential protective factors, with estimated hazards ratios ranging from 0.47 – 0.97; however, this did not reach significance. In contrast, the estimated hazards ratios for partial meniscectomy were 1.25 for arthritis and 6.60 for survival free of TKA, which may indicate a trend towards increased OA and TKA risk. Of the 16 patients treated with meniscus repair, 0% went on to symptomatic OA by long-term follow-up, a further demonstration that significance may have been reached with increased power.

This study has some important limitations. First, there is likely a selective bias for lower-demand patients to elect for nonoperative management of ACL and

meniscal injuries. In parallel, the microinstability and trauma associated with such lower-demand activities may confound the subsequent observed rates of posttraumatic OA. Second, there is no clear literature definition for “delayed” ACLR, as studies cite “delay” that ranges from 10 weeks to up to 1 year after the time of initial injury.[14,18,26]. For the current study, the cut-off point of 6 months was set as a reasonable representation of the range of values reported as well as the senior author’s (A.J.K) clinical practice. Finally, the presented study includes only clinically diagnosed secondary meniscal tears or arthritis documented in the medical record. Therefore, data from patients who did not seek medical care or who moved from the studied geographical area could not be captured, and the results presented may underestimate the true incidence of secondary meniscal tears and OA following ACL injury. This could be an explanation for the lower follow-up time in the acute ACLR group and a possible cause for the lower OA rate in this group, since the age of these patient was lower at follow-up, which could mean the development of OA could occur at later age. In addition, future studies with larger sample sizes are needed to further elucidate the role of meniscal and ACLR repair in the risk and development of OA and TKA.

In conclusion, secondary meniscal tears after ACL injury are most common in patients who undergo delayed or nonoperative management of a primary ACL injury, and they often present as complex tears of the medial meniscus, which result in high rates of partial meniscectomy. Over the course of 18 years of mean follow-up, ACLR and meniscal repair demonstrated a consistent protective trend against symptomatic OA and subsequent TKA, although the results were not statistically significant. These results indicate that ACLR and secondary meniscal tear repair are important considerations in the optimization of outcomes following ACL injury. These outcomes can assist clinicians in counseling patients with regard to ACLR timing and its potential effect on subsequent meniscal tears and associated interventions.

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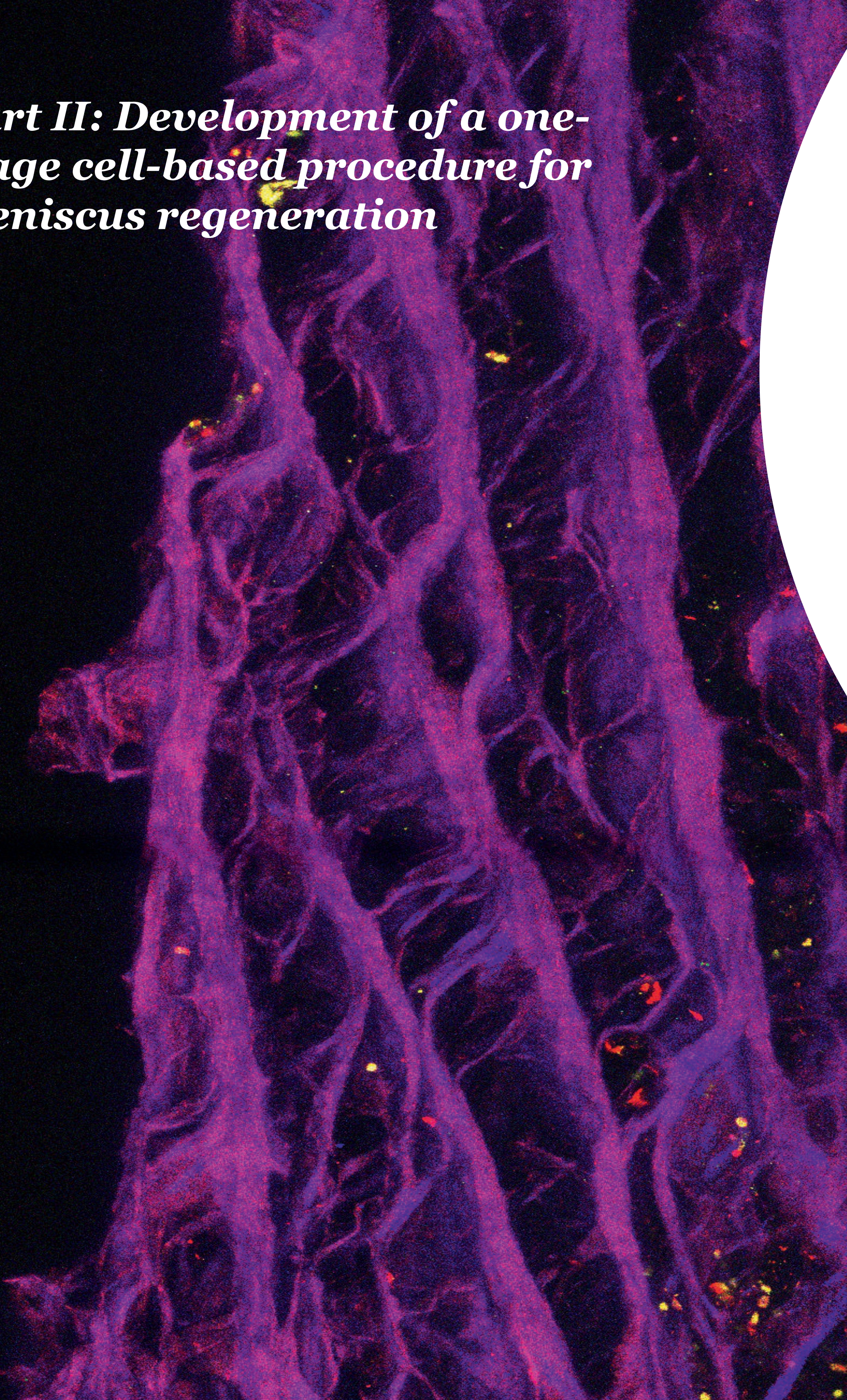
Part II: Development of a one-stage cell-based procedure for meniscus regeneration



Chapter 4

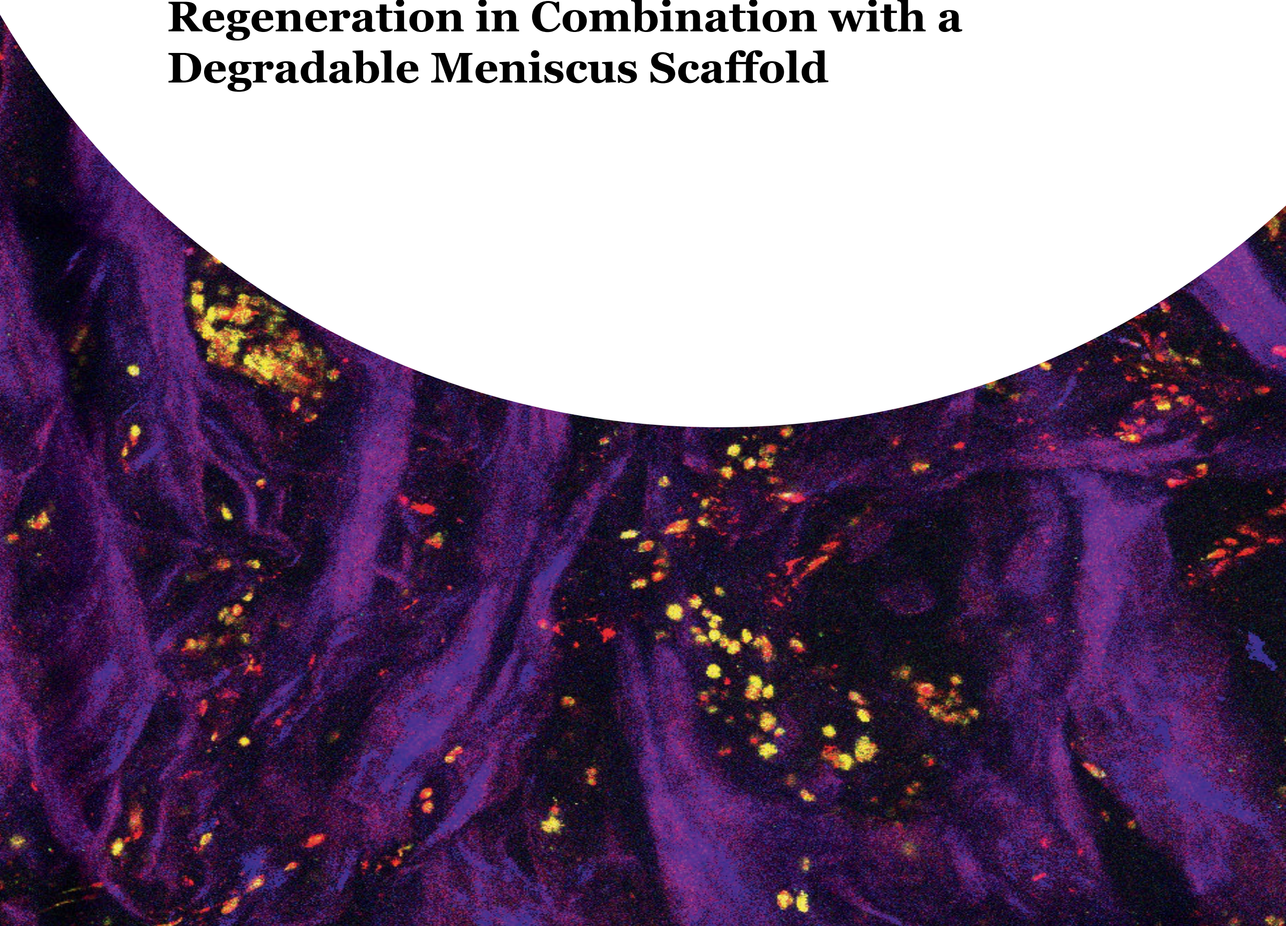
Meniscus Regeneration Combining Meniscus and Mesenchymal Stromal Cells in a Degradable Meniscus Implant

Chapter 5

Surgical Feasibility of a One-Stage Cell-Based Arthroscopic Procedure for Meniscus Regeneration

Chapter 6

Growth Factors Enhance Meniscus Regeneration in Combination with a Degradable Meniscus Scaffold



Chapter 4

Meniscus Regeneration Combining Meniscus and Mesenchymal Stromal Cells in a Degradable Meniscus Implant:
An *in vitro* Study

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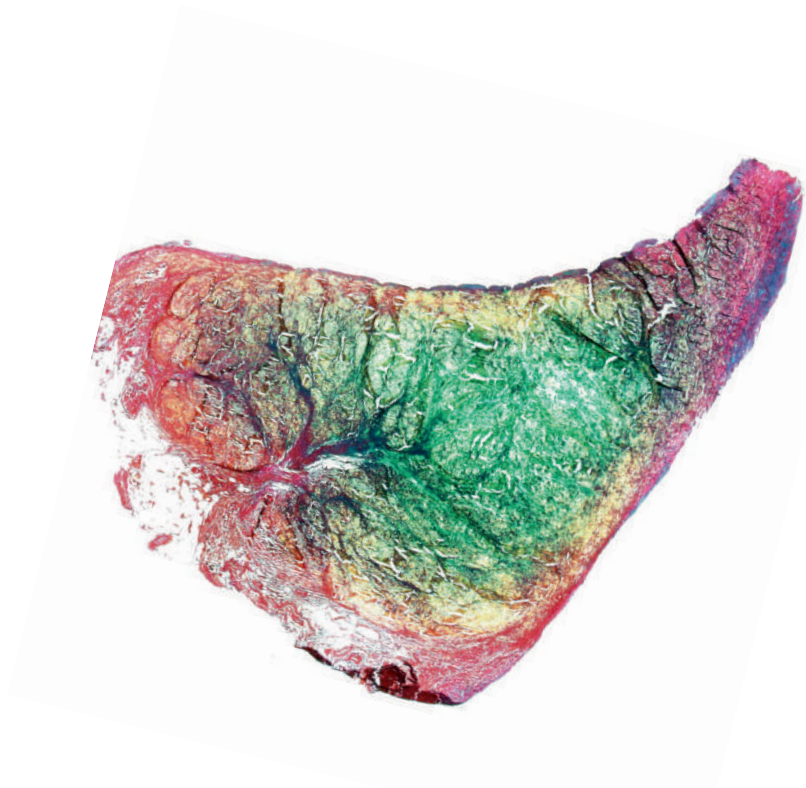

Abstract

Background: Meniscus regeneration is an unmet clinical need as damage to the meniscus is common and causes early osteoarthritis.

Purpose: The aim of the present study was to investigate the feasibility of a one-stage cell-based treatment for meniscus regeneration by augmenting a resorbable collagen-based implant with a combination of recycled meniscus cells and mesenchymal stromal cells (MSCs).

Methods: Cell communication and fate of the different cell types over time in coculture were evaluated by connexin 43 staining for gap junctions and polymerase chain reaction (PCR) to discriminate between meniscus cells and MSCs, based on a Y-chromosomal gene. To define optimal ratios, human meniscus cells and bone-marrow-derived MSCs were cultured in different ratios in cell pellets and type I collagen hydrogels. In addition, cells were seeded in the implant in fibrin glue by static seeding or injection.

Results: Cellular communication by gap junctions was shown in cocultures, and a decrease in amount of MSCs over time was demonstrated by PCR. 20:80 and 10:90 ratios showed significantly highest glycosaminoglycan and collagen content in collagen hydrogels. The same statistical trend was found in pellet cultures. Significantly more cells were present in the injected implant, and cell distribution was more homogenously as compared to the statically seeded implant.

Conclusion: The study demonstrated the feasibility of a new one-stage cell-based procedure for meniscus regeneration, using 20% meniscus cells and 80% MSCs seeded statically on the implant. In addition, the stimulatory effect of MSCs towards meniscus cells was demonstrated by communication trough gap junctions.

Keywords: Meniscus injury, meniscus regeneration, bone marrow mesenchymal stromal cells, meniscus cells, meniscus scaffold, collagen meniscus implant

Introduction

Meniscus tissue is characterized by low cell density and a dense extracellular matrix (ECM), which mainly consists of water, type I collagen, glycosaminoglycans (GAGs) and elastin.[10] With their semilunar wedge-shaped structure, the menisci play an important role in shock absorption, load transmission and stability of the knee.[11,18] Damage to the meniscus is a very common injury, which leads to loss of its chondroprotective role in the knee. Especially in young patients with high activity levels,[21,28] loss of meniscus function can lead to an increased risk of developing early osteoarthritis (OA).[7,18] (Partial) meniscectomy used to be the first choice in treatment for meniscus tears; however, due to the high risk of developing post-meniscectomy OA secondary to increased contact pressure on cartilage,[7,28] meniscus repair is becoming more popular. Meniscus repair is not suitable for all types of tears. Therefore, meniscus restoration using allograft transplantation or biodegradable meniscus scaffolds are of interest.[5,9]

Currently, the clinically-available acellular meniscus implant is the collagen meniscus implant (CMI®) (Stryker, Kalamazoo, MI, USA). This implant has a porous structure providing an environment for cell ingrowth. Clinical results after implantation of the CMI, evaluated by patient-reported outcome measures (PROMs), are promising, with a post-operative increase of the Lysholm score and Tegner activity scale and a decrease for visual analogue scale (VAS) pain up to ten years.[9,14,36] However, limited engraftment and neo-tissue formation by invading cells can lead to size reduction of the regenerated meniscus, consequently allowing opportunity for improvement of this treatment.[23] The present study proposed that replacing the deficient segment of a meniscus with a cell-seeded meniscus implant led to improved, more consistent, and better-distributed functional new meniscus-like tissue formation.

The number of meniscus cells recovered from the resected meniscus, even during an overnight digestion, are relatively low and not suitable for engraftment (±1.5*10^3 cells/mg meniscus[15]). It would be cost effective, causing lower patient burden and being logistically attractive to use these cells in a one-stage procedure for meniscus regeneration. Recently, a clinical study has shown the safety and feasibility of using a combination of recycled autologous chondrons with allogeneic

mesenchymal stromal cells (MSCs) for cartilage repair.[33,34] This and other studies suggest that allogeneic MSCs provide stimulatory and immunomodulatory factors for tissue repair and are able to positively stimulate a smaller number of meniscus cells, as an alternative to engraftment and differentiation.[3,24,25] For these reasons, allogeneic MSCs have even outperformed autologous MSCs in a comparative human study for the treatment of nonischemic dilated cardiomyopathy.[16]

The goal of this present *in vitro* study is to assess the conditions for a new one-stage treatment of meniscus regeneration. To achieve this goal, three main questions were analysed: (1) Do MSCs and meniscus cells communicate? (2) What ratio of MSCs and meniscus cells is optimal for production of native-like meniscus tissue? (3) What is the optimal method for delivering the cells uniformly into a clinically applicable scaffold?

Methods

Donors and cell isolation

Tissue from whole meniscus was obtained from redundant material of 11 patients that had undergone total knee replacement (mean age 65.9 (range 55 – 73) years, 4 male and 7 female). Collection of this patient material was performed according to the Medical Ethical 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.[6,8] Meniscus tissue was rinsed in phosphate buffered saline (PBS) with 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco) (1% pen/strep), cut into pieces of 2mm^3 and digested overnight at 37°C in 0.15% collagenase type 2 (CLS-2, Worthingtom, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibo) and 1% pen/strep. Meniscus cells were expanded for one passage in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USE) and 1% pen/strep[28] and used as passage 1 in all the experiments.

Human MSCs (hMSCs) were isolated from bone marrow biopsies from the iliac crest during total hip replacement from 6 patients after written informed consent was obtained (Medical Ethical Committee, University Medical Center Utrecht) as described previously.[12] Cells were expanded in α-MEM (minimal

essential medium, Gibco) supplemented with 10% FBS, 1% 20 mM l-ascorbic acid-2-phospate (1% ASAP; Sigma-Aldrich) and 1% pen/strep to be used at passage 3. Meniscus cells and MSCs from different donors were not pooled.

Fluorescent dye transfer

To assess gap-junction-mediated communication between hMSCs and meniscus cells, fluorescent dye transfer was used,(Asklund et al., 2003) 10 µM Vybrant CM-DiI (Molecular Probes) and 10 µM calcein-AM (Molecular Probes) were diluted in PBS and incubated with either meniscus cells or hMSCs for 1 h at 37°C. Afterwards, cells were washed with PBS and cocultured in a 50 : 50 ratio for 36 h as a monolayer in a 96-wells plate. Gap junctions were by fluorescence microscopy (EVOS Cell Imaging System, ThermoFIsher Scientific) after 24 and 26 h of culture through transfer of calcein-AM. 3 meniscus donors and 3 MSC donors were used for this experiment; all were combined and 3 technical replicates per condition were performed.

Cell pellet formation

Cells were counted with an automated cell counter (TC20TM Bio-Rad) at 1 : 1 dilution in trypan blue (Bio-Rad). Cell suspensions were prepared in the concentrations of 0%, 10%, 20%, 25%, 50%, 75% and 100% meniscus cells combined with hMSCs. In a U-bottom 96 wells plate (Greiner Bio-One, CELLSTAR®), a total of 250,000 cells per well and 200µL of differentiation medium (DMEM, supplemented with 1 % pen/strep, 2% 20 mM ASAP, 2% insulin-transferrin-selenium-X (ITSX, Invitrogen) and 2% human serum albumin (HSA; Sanquin, Utrecht, the Netherlands)) were centrifuged for 5 minutes at 300 xg to form pellets. Cell pellets were cultured for 28 d at 37°C with 5% CO_2; medium was changed 3 times per week and conditioned medium was stored at -20°C for biochemical analysis.

Type I collagen hydrogel preparation

Cell concentrations with 0%, 10%, 20%, 50% and 100% meniscus cells were prepared in suspension with hMSCs, using the same concentrations as for the cell pellets. Collagen gels were prepared from rat tail type I collagen (Corning) with

a final collagen concentration of 2 mg/ml per hydrogel; 2.5 μl of 5M NaOH were mixed with 800 μl of collagen solution (2.5 mg/ml in 0.02 N acetic acid). Cell suspensions were added, 100 μl of the combined solution was transferred to different wells of a 12-wells plate with a cell concentration of 250,000 cells in 200μL and incubated for 60 minutes at 37°C. Subsequently, 2 mL of differentiation medium was added. Hydrogels were cultured for 28 d, 1 mL of medium was changed 3 times per week, and stored at -20°C for future biochemical analysis.

Polymerase Chain Reaction (PCR)

Cell pellets, fibrin glue constructs, and collagen type I gel constructs of (1) monoculture meniscus cells and hMSCs and (2) coculture of 20% meniscus cells and 80% hMSCs were harvested at t = 0 d (4 constructs per condition), t = 14 d (4 constructs per condition), and t = 28 d (4 constructs per condition) for PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) as described by the manufacturer. Total RNA (500 ng) was reverse transcribed using the high-capacity cDNA Synthesis Kit (Applied Biosystems). PCRs was performed on 5x diluted cDNA using iTaq Universal SYBR Green Supermix (Bio-Rad) in a LightCycler 96 (Roche Diagnostics) according to the manufacturer's instructions.

In the cocultures used for PCR, all meniscus donors were female and all MSC donors were male, therefore during PCR, using primers for the genes on the Y chromosome, a distinction could be made between the different cell types. The housekeeping gene 18S was used and primers for lysine demethylase 5D (KDM5D) and ubiquitously transcribed tetratricopeptide-repeat-containing, Y-linked (UTY) were used to amplify the Y-chromosome (Table 1) and, therefore, the MSCs in the cocultures.

Biochemical analysis

After overnight digestion of the samples in papain buffer (250 μg/mL papain (Sigma-Aldrich), 0.2 M NaH2PO4, 0.1 M EDTA, 0.01 M cysteine) at 60°C, GAG content was determined by dimethylmethylene blue (DMMB) assay. Absorption ratio was set at 525 nm and 595 nm using chondroitin sulphate (Sigma-Aldrich) as a standard for calculating the GAG content. DNA content was determined by

Target gene		Oligonucleotide sequence	Annealing temperature (°C)
18S	Forward	5' GTAACCCGTTGAACCCCATT 3'	58
	Reverse	5' CCATCCAATCGGTAGTAGCG 3'	
KDM5D	Forward	5' TAACACACACCCGTTTGACAA 3'	60
	Reverse	5' GCTGCTGAACTTTGAAGGCTG 3'	
UTY	Forward	5' CACAAAGAAGTTGCTCAGGTACG 3'	60
	Reverse	5' TGTGGTTGTCGATTAGAGACAGA 3'	

Table 1; Primer sequence used for Real-time polymerase chain reactions (PCRs). Housekeeping gene 18S; Lysine Demethylase 5D (KDM5D); Ubiquitously transcribed tetratricopeptide repeat containing, Y-linked (UTY)

Picogreen DNA assay (Invitrogen), according to the manufacturer's instructions. Excitation and emission were set at 480nm and 520nm, respectively, and λDNA was used as a standard reference to calculate DNA content. Freeze-dried papain samples were used to determine collagen content of the constructs by hydroxyproline assay. 100uL of 1.4M citric acid (27490; Fluka) was added following overnight hydrolysis of the samples in 100uL of 4M NaOH (6498; Merck) at 108°C. Choramin-T reagent (2426; Merck) and dimethylaminobenzoaldehyde (3058; Merck) were added to the samples and hydroxyproline standard (104506.0010; Merck) was used to measure the absorption at 570nm. As 13.5% of collagen is composed of hydroxyproline, the amount of collagen was calculated from the hydroxyproline content.[22]

Histology and immunohistochemistry

Samples were fixed in 4% buffered formaldehyde, dehydrated in graded ethanol series, immersed in xylene, embedded in paraffin wax, cut in 5 µm thick sections and stained. Before performing staining and immunohistochemistry, sections were deparaffinized and in xylene and rehydrated in ethanol. To determine the cell distribution throughout the construct, sections were stained with Mayer's haematoxylin (Merck) and counterstained with eosin (Merck) (H&E staining). To evaluate proteoglycan content, 0.125% safranin O (Merck) counterstained with Weigert's haematoxylin (Klinipath, Duiven, the Netherlands) and 0.4% fast green (Merck) were used. Picrosirius red (Klinipath, Leuven, Belgium) / alcian blue

(Sigma-Aldrich) staining was used to visualize the collagen fiber orientation by polarized light microscopy.

After rehydration, sections for connexin 43, type I and II collagen immunohistochemistry were blocked for 10 min with 0.3% H_2O_2 solution and washed with PBS-0.1% Tween 20 (Sigma-Aldrich). Antigen retrieval was performed using 1 mg/mL pronase (Roche) in PBS and 10 mg/mL hyaluronidase (Sigma-Aldrich) in PBS, both for 30 minutes at 37°C. Sections were blocked with 5% PBS/bovine serum albumin (BSA) for 30 minutes at room temperature, followed by incubation with primary antibodies for either connexin 43 (GJA1, rabbit polyclonal antibody, 1 : 50 in PBS/5% BSA, Abcam), type I collagen (Col1, rabbit monoclonal antibody, 1 : 400 in PBS/5% BSA, Abcam) and type II collagen (II-II6B3, mouse monoclonal antibody, 1 : 100 PBS/5% BSA, DSHB, Merck). As negative controls, rabbit IgG (Dako) was used for connexine 43 (1 : 2000 in PBS/5% BSA) and type I collagen (1 : 10000 PBS/5% BSA) and mouse IgG (1 : 100 in PBS/5% BSA, Dako) for type II collagen. Antibodies were incubated overnight at 4°C and, subsequently, washed in 0.1% PBS-Tween 20 and incubated with the secondary antibody for connexin 43 (goat anti-rabbit – horseradish peroxidase (HRP) 1:100 in PBS/5% BSA; 3117332001; Roche), type I (EnVision+ System-HRP, anti-rabbit, K4003, Dako) and type II (goat anti-mouse IgG HRPm 1:100 PBS/5% BSA; P0447, Dako) collagen for 60, 30 and 60 min respectively at room temperature. Immunoreactivity, visualized with 3-diaminobenzidine (DAB, Sigma-Aldrich), was stopped using MilliQ water (Merck). Sections were counterstained with Mayer's haematoxylin diluted 1 : 1 in distilled water, dehydrated in different gradients of ethanol and mounted in Depex (Merck).

Seeding methods

CMI pieces (with a size of approximately 150mm^3) were seeded with 10% meniscus cells and 90% hMSCs, based on successful results using chondrons and MSCs in the same ratio.[2] Before seeding, the CMI was washed for 10 d in 100 mL PBS with 1% pen/strep. The fibrin glue (Beriplast, CSL Behring) used was diluted as described by Abbadessa et al[1] and all cells were mixed in the fibrinogen component of the fibrin glue. After seeding and incubation, scaffolds were moved to a new 24-wells plate (to exclude the cells not attached to the scaffold) for

subsequent calculation of matrix production and cell-count. Seeded constructs were cultured for 26 days in 1 mL of differentiation medium, which was changed 3 times per week and stored for biochemical analysis.

To mimic the clinical circumstances of *ex vivo* and *in vivo* seeding during arthroscopy, two different seeding techniques were used. Static surface seeding was performed on dry CMIs, resembling *ex vivo* seeding. 5 µL of cell suspension in fibrinogen, containing a total of 5.0 x 10^5 cells (5.0 x 10^4 meniscus cells and 4.5 x 10^5 hMSCs), were loaded on top of the CMI, immediately followed by 75 µL of thrombin and incubation for 15 minutes at 37°C. Seeding by injection was executed in wet CMIs, immersed in 1 mL of PBS in a 24-well plate, resembling *in vivo* seeding after arthroscopic implantation of the scaffold. Using a 1.0 mL syringe and a 23-gauge needle, 75 µL of cell suspension (5.0 x 10^5 cells, similar cell combination to static surface seeding) were injected into the CMI and incubated at 37°C for 15 minutes after injection of 75 µL of thrombin using a 23-gauge needle.

Cell distribution assessment using confocal microscopy

Accessing the cell distribution throughout the CMI after 26 d of culture using the different seeding methods was performed by creating three-dimensional (3D) images acquired by a Leica SP8 confocal microscope. Two pieces of CMI per seeding method were stained for 30 min with 0.5 µL/mL calcein AM (Molecular Probes) at RT and for 4 min with 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) followed by washing with PBS. A tile scan with z-stack was performed and the 3D images were merged using Image J.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA, USA). Differences in GAG and collagen per DNA for the different ratios and seeding methods were calculated by a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. The decrease in the KDM5D and UTY gene per culture condition at the different time points were calculated using a student's t-test. To determine whether there was a significant difference in relative decrease in amount of MSCs between monoculture of MSCs and coculture of MSCs and meniscus cells over time, the delta of the mean decrease

per condition was calculated and student's t-tests were performed. $P < 0.05$ was considered statistically significant.

Results

Communication and cell survival in cocultures

Immunohistochemistry for connexin 43 in the pellet cocultures showed staining for the monocultures of meniscus cells and hMSCs as well as for the different ratio of the cocultures (Figure 1a), indicating formation of gap junctions in both mono- and cocultures. When hMSCs were stained with calcein and meniscus cells with Vybrant CM-DiI, the dye transfer was shown most prominently by the yellow staining of the red meniscus cells, which also stained for the calcein transferred from the hMSCs (Figure 1b). When the hMSCs were incubated with the Vybrant CM-DiI and the meniscus cells with calcein, the dye transfer was less eminent. This suggests that there was active gap-junction-mediated communication, which was more active from hMSCs to meniscus cells than from meniscus cells to hMSCs.

PCR data of monoculture hMSCs and the coculture with meniscus cells, in either pellet, fibrin glue and type I collagen gel, showed a significant decrease in KDM5D and UTY over time for both mono and cocultures, and therefore a decrease in the amount of hMSCs over time (Figure 2). The decrease in cocultures was higher as compared to the decrease in hMSCs monocultures. In addition, in pellet culture, the decrease of hMSCs was significantly lower (KDM5D, $p = 0.013$; UTY, $p = 0.0006$) between $t = 0$ d and $t = 14$ d for monoculture of hMSCs as compared to coculture. Whereas between $t = 14$ d and $t = 28$ d, the mean decrease in hMSCs was higher in monoculture for cultures in fibrin glue and type I collagen (fibrin glue: KDM5D $p = 0.0427$, UTY $p = 0.4762$; type I collagen: KDM5D $p = 0.0448$, UTY $p = 0.0193$) (Figure 2).

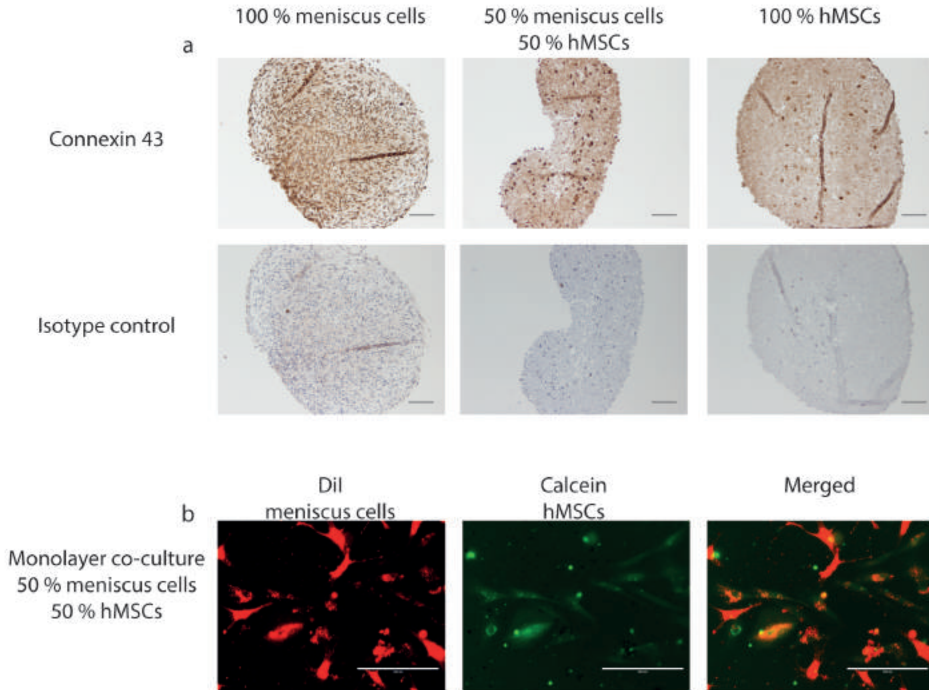

Figure 1: *Cell-cell communication*. Cell-cell communication by gap junctions between hMSCs and meniscus cells was determined by (a) the presence of connexin 43 in mono- and co-cultures in pellets after 28 d and (b) dye transfer : Vybrant CM-DiI (red), calcein (green) and an overlay of Vybrant CM-DiI and calcein (merged), where transfer of the calcein stained hMSCs to the meniscus cells stained with Vybrant CM-DiI is shown after 24 h. Scale bar: 200 µm.

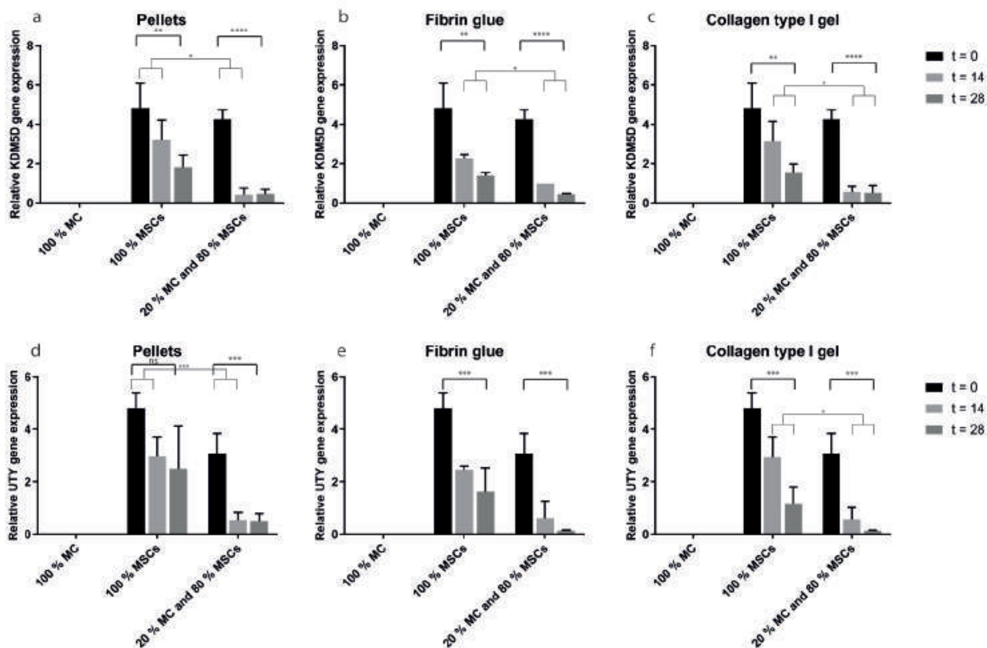

Figure 2: *PCR data after (co)culture of meniscus cells and MSCs.* (a-c) PCR data for both KDM5D and (df) UTY, representing the Y-chromosome genes in the male MSCs, showed a decrease in the amount of MSCs over time. Absolute difference between t = 0 d and t = 28 d was calculated for both monocultures of MSCs and co-culture of MSCs and meniscus cells (ratio 80 : 20). The delta of the mean decrease per culture condition was calculated and significant differences between monocultures of MSCs and co-cultures of MSCs with meniscus cells are marked with brackets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. MC: meniscus cells. ns: not significant.

Production of extracellular matrix in pellet cocultures

Biochemical analysis showed a significant decrease in DNA content of the cell pellets (n = 5 for biological replicates and n = 3 for technical replicates) after 4 weeks of culture for the ratios containing a percentage of hMSCs (Figure 3a). The larger the proportion of hMSCs, the fewer cells were present after 28 d of culture. The ratios with more than 50% hMSCs produced significantly more GAG content per DNA as compared to the 100% meniscus cells (Figure 3b), which indicated a stimulatory effect of hMSCs on meniscus cell GAG production, followed by hMSC apoptosis. The same assumption was demonstrated by the PCR. In assessment of total GAG content of the samples combined with the GAG in the medium, there were no differences observed for total GAG production. However, the cocultures with hMSCs seemed to perform better than the monoculture of meniscus cells

(Figure 3c). Also, a trend for a higher collagen content in the cell pellets was suggested when the proportion of hMSCs was higher. However, results were not statistically significant (Figure 3d).

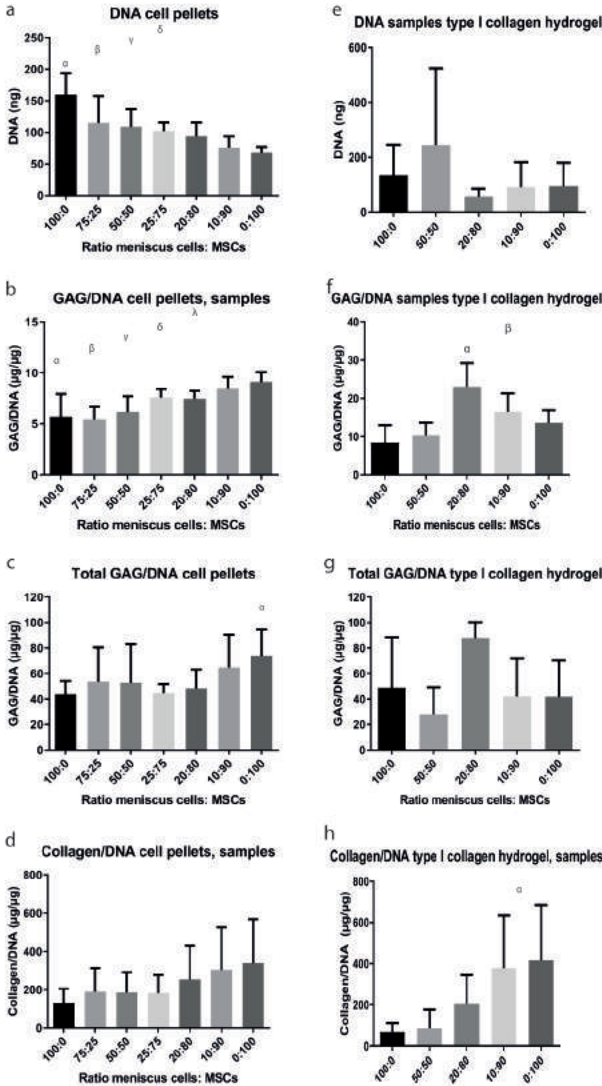

Figure 3: *Biochemical analysis after co-culture of meniscus cells and MSCs in different ratios*. (a,e) DNA content, (b,f) GAG content, (c,g) total GAG production and (d,h) collagen content, all corrected for DNA content, are shown for (a-d) cell pellets and (e-h) co-cultures in type I collagen hydrogel for the different ratios of meniscus cells and hMSCs after 28 d of culture. Data are shown as mean ± standard deviation; $p < 0.05$. (a) 100 % meniscus cells was statistically higher in DNA content than all the other conditions (α), 75 : 25 and 50 : 50 were significantly higher than 10 : 90 and 0 : 100 (β and γ) and 25 : 75 was higher than 0 : 100 (δ). (b) GAG/DNA in cell pellets was significantly higher in 100 : 0 (α) as compared to 75 : 25, but significantly lower as compared to 25 : 75, 10 : 90 and 100 : 0. 75 : 25 (β) was significantly lower than 25 : 75, 20 : 80, 10 : 90 and 0 : 100. 50 : 50 (γ) was significantly lower as compared to 25 : 75, 10 : 90 and 0 : 100. 100 : 0 was significantly higher as compared to 25 : 75 and 20 : 80 (δ and λ). (c) Total GAG/DNA in cell pellets was significantly higher in 0 : 100 (α) as compared to 100 : 0, 25 : 75 and 20 : 80. In both (d) collagen/DNA in cell pellets and (e) DNA content in type I collagen hydrogels, no significant differences were detected. (f) In the samples cultured in type I collagen hydrogels, 20 : 80 was significantly higher in GAG/DNA as compared to 100 : 0, 50 : 50 and 0 : 100 (α) and 10 : 90 was significantly higher than 100 : 0 (β). (g) No significant differences were found for total GAG/DNA in the co-cultures using collagen type I hydrogels. (h) Collagen content corrected for DNA in 10 : 90 and 0 : 100 was significantly higher as compared to 100 : 0 and 50 : 50 (α).

In H&E staining, pellets containing 50%, 80% and 100% meniscus cells had a higher cell density (Figure 4a), which is similar to the results of the DNA quantification (Figure 3). None of the ratios stained for GAG, indicating the amount of GAG was too low to be detected histologically (data not shown). IHC showed a more intense DAB staining for type I collagen as compared to type II collagen. These findings, i.e. a low amount of GAG and higher presence of type I as compared to type II collagen, were typical for native meniscus tissue (Figure 4b, c).

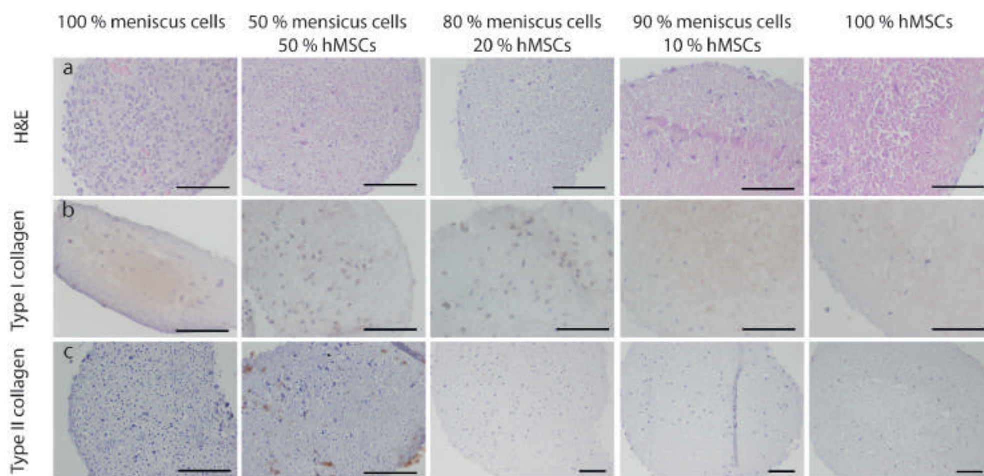

Figure 4: *Histological stainings of pellet cultures of different ratios of meniscus cells and MSCs, 20× magnification.* (a) H&E showed cell concentration in the different ratios. (b) Immunohistochemistry showed staining for type I collagen and (c) almost no staining for type II collagen in all ratios. Scale bar: 100 µm.

Production of extracellular matrix in collagen type I hydrogel

After 4 weeks of coculturing meniscus cells and hMSCS in type I collagen hydrogels (n = 3 for both biological and technical replicates), the DNA content was not statistically significant different among the different conditions (Figure 3e). GAG content and the total GAG production, both normalized for DNA content, were the highest ($p < 0.001$ and $p < 0.05$, respectively) in 20% meniscus cells and 80% hMSCs as compared to the other ratios (Figure 3f, g). A trend of more GAG production was observed in the hydrogels containing >50% hMSCs as compared to >50% meniscus cells, although not all results were statistically significant. Collagen

content, corrected for DNA, showed a significantly higher concentration in the conditions with 90% and 100% hMSCs (Figure 3h).

Histology showed an even distribution of cells throughout the different constructs; however, no proteoglycan content was detected. IHC showed a larger presence of type I collagen as compared to type II collagen (data not shown), similar to the pellet culture.

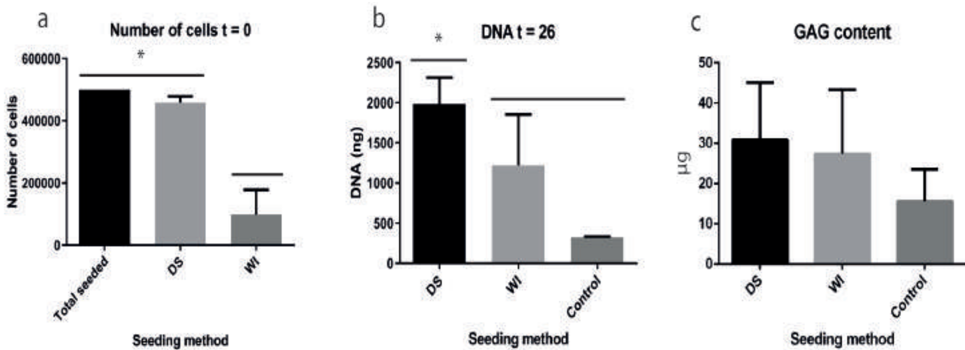

Figure 5: *Number of cells after two different seeding methods*. (a) Total number of cells at t = 0 d, (b) DNA content at t = 26 d and (c) GAG content for co-cultures of meniscus cells and hMSCs in a 10 : 90 ratio inside the CMI® for 26 d using different seeding methods (dry static and wet-injected, respectively). A CMI® without cells was used as the control group. Data are shown as mean ± standard deviation; * $p < 0.05$. DS = dry seeding; WI = wet seeding by injection.

Optimal in vitro seeding method

Immediately after seeding, the wet-injected CMI contained significantly fewer cells than the total amount of seeded cells ($p = 0.0070$) and the dry statically-seeded CMI ($p = 0.0096$). The amount of cells in the dry- and statically-seeded CMI were not statistically different from the total number of seeded cells (p= 0 .6899) (Figure 5a). After 26 d of culture (n = 3 for both biological and technical replicates), the CMIs seeded statically in a dry environment showed a significant higher DNA content as compared CMIs injected in a wet environment ($p = 0.0491$) (Figure 5b). GAG content appeared to be slightly higher in the first group although the data were not statistically significant ($p = 0.7249$) (Figure 5c). GAG release into the medium was significantly higher in the dry- and statically-seeded CMIs ($p = 0.0306$) (data not shown). Because the CMI is composed of bovine collagen, the

produced collagen content was determined using the ratio of collagen before and after culture corrected for an empty CMI. This resulted in no significant difference among the different seeding methods ($p = 0.3426$). Histological analyses showed a better cell distribution within the scaffold for the dry-seeded CMI as compared to the wet-seeded scaffolds. Figure 6 shows histology of the dry-seeded CMI, with a good cell distribution shown by H&E staining in Figure 6a. However, no proteoglycans were detected by histology (Figure 6b). Immunohistochemistry showed a high production of type I collagen and only minimal deposition of type II collagen (Figure 6c, 6d), which was similar to native meniscus tissue. 3D confocal images confirmed the homogenous distribution of cells throughout the whole CMI when the scaffold was seeded dry and statically (Figure 7a), whereas for the wet-injected CMI, there were only pockets of cells visible (Figure 7b).

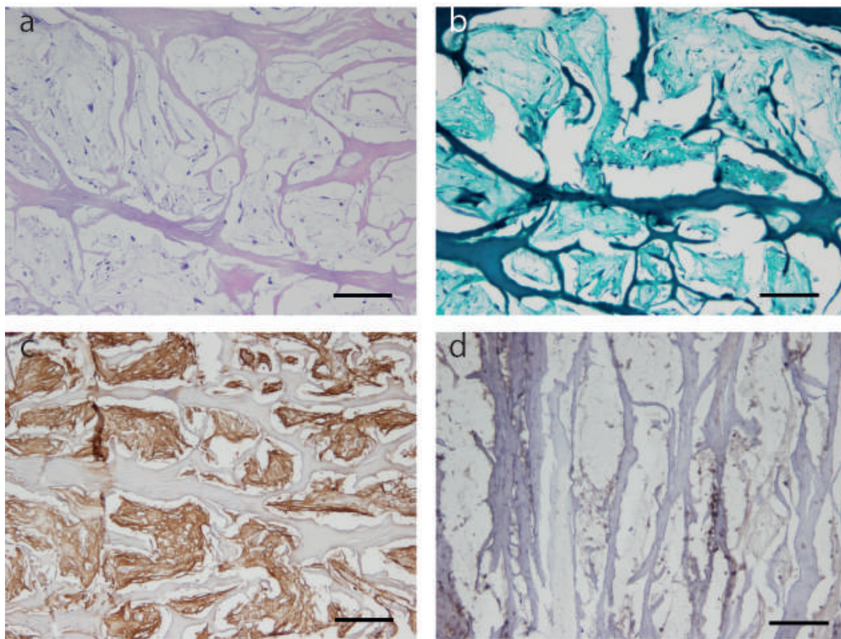

Figure 6: *Histological stainings*. (a) H&E, (b) safranin O/fast green and (c) immunohistochemistry for type I and (d) type II collagen of dry-seeded CMI® with a 10 : 90 ratio of meniscus cells and hMSCs, cultured for 26 d. Scale bar: 100 µm.

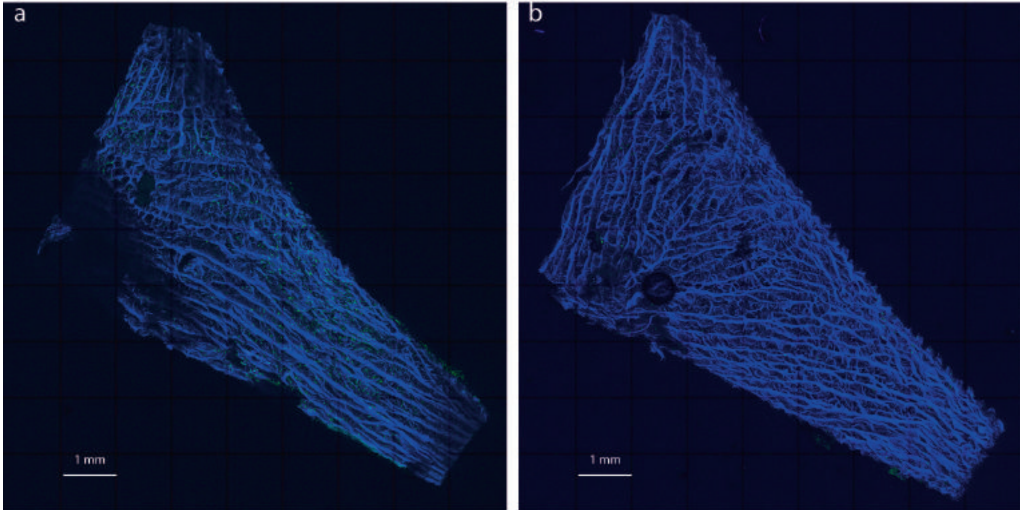

Figure 7: Cell distribution throughout the CMI for different seeding methods. Cells were stained with calcein AM (green) and the CMI with DAPI (blue). 3D images were taken using a confocal microscope (Leica) showing cell distribution throughout the CMI using (a) the dry static seeding method and (b) the wet-injected CMI (b).

4

Discussion

The goal of this *in vitro* study was to assess the conditions for a new one-stage cell-based procedure for meniscus regeneration. This study examined the interaction through gap junctions between hMSCs and meniscus cells and demonstrated a short survival period of hMSCs in cocultures, indicating a stimulating effect of hMSCs on meniscus cells. The optimal ratio for coculture of MSCs and meniscus cells was reported to be 80% hMSCs and 20% meniscus cells, where native-like meniscus tissue, type I collagen and a minimal amount of GAG were produced. Contiguously, the best seeding method for this cell combination into a clinically applicable scaffold was shown to be dry seeding. All these findings suggest that this new treatment method for meniscus regeneration was clinically applicable.

The immunohistochemistry for connexin 43, dye transfer experiments and PCR results demonstrated transfer of information from hMSCs to meniscus cells by gap junctions and a decrease in the number of hMSCs in time. The low amount of

male DNA after 4 weeks of culture, shown by PCR, indicated that MSCs disappeared after stimulating or transferring information to the meniscus cells. Liu (2019) has shown that hMSCs can transfer their functional mitochondria into injured endothelial cells after ischemic stroke in mice, protecting the endothelial cells from going into apoptosis.[17] After stimulating the meniscus cells, hMSCs seemed to disappear. Xu et al. (2004) have described the function of hMSCs by differentiation into the required cell type in e.g. isolated cartilage defects, osteoarthrosis, or after a myocardial infarction.[35] However, de Windt et al. (2015) have shown that the DNA of the newly formed cartilage tissue, in patients treated with a combination of allogeneic hMSCs and autologous chondrocytes, does not contain any DNA from the hMSC donor, only from the patient itself.[32] The present study showed that the decrease in cocultures was higher compared to the decrease in hMSCs monocultures. This, in combination with the results of de Windt et al. (2015), could indicate that, in coculture, hMSCs might have a more stimulatory effect on the production of GAG and collagen from the meniscus cells and contribute less to ECM production and replacement of avital native cells in damaged tissue; whereas, in monoculture, the ECM production might be regulated by the hMSCs themselves.

The willingness to use allogeneic hMSCs for future *in vivo* experiments is reinforced by the possible pro-inflammatory effect triggered by presence of allogeneic cells in the patient, which might cause a boost in the regenerative effect. Hare et al (2017), have shown a superior effect of allogeneic to autologous hMSCs in patients receiving transendocardial stem cell injections for non-ischemic dilated cardiomyopathy,. Such patients have an improved endothelial function, a greater suppression of tumour necrosis factor alpha (TNF-α; suggesting a shift towards a less inflammatory phenotype of the immune cells) and clinical better outcomes.[16]

The present study showed an increase in GAG and collagen production in cocultures compared to monocultures of meniscus cells. Cocultures with a higher percentage of hMSCs resulted in the highest ECM production. Similar results were previously described by Cui et al. (2012)[4] and Matthies et al.(2013)[19]. Coculture results were comparable to the results of cocultures of hMSCs and chondrons, as shown by Bekkers et al.(2013),[2] with the highest GAG/DNA production in 80% and 90% hMSCs, respectively. Nevertheless, the monoculture of hMSCs resulted in the

highest production of GAG and collagen per DNA, which was not described by others. A possible explanation could be that pellet culture might not be the optimal 3D culture method for ECM production by meniscus cells. In the native meniscus, the cells are dispersed throughout the ECM and there is very limited contact between cells. In pellet culture, the cells are aggregated together at high density without being surrounded by matrix, especially at the start of the culture. Consequently, results suggest that meniscus cells perform better in 3D hydrogels. This could partially explain the differences in ECM production between meniscus cells and hMSCs. However, Song et al. (2015)[26] showed less GAG and collagen production by MSCs compared to the cocultures and monocultures of meniscus cells, similarly to Cui et al. (2012) and Matthies et al. (2013)[4,19] Besides type of coculture, the type of MSCs could significantly influence the difference in outcome after *in vitro* coculture, as MSCs are a heterogeneous population of cells and their characteristics and regenerative potential is dependent on a variety of parameters, such as donor, location, harvest method, isolation method, expansion density, and the composition of expansion medium and culture medium. MSCs are often poorly characterized, making it challenging to compare the direct results of various studies. Synovium-derived mesenchymal stromal cells (SSC) were used by Song et al. (2015), differently from the marrow MSCs used in present study. In addition, Song et al. (2015) cocultured the pellets for a total of 2 weeks, half the time as compared to the current study. Therefore, it could be possible that MSCs started producing more ECM after the first 2 weeks of culture.[26]

Due to the possible negative effect of coculturing meniscus cells in pellets, the study included coculturing in a type I collagen hydrogel to closer mimic the native environment of the meniscus cells. Results showed a significantly higher production of GAG/DNA for the 80% and 90% hMSCs, and for the total GAG/DNA for the 80% hMSCs, with a lower production of GAGs in the hMSCs monoculture. However, collagen production is hard to determine due to the collagen already present in the hydrogel. Collagen content corrected for DNA showed a significantly higher concentration in the conditions with 90% and 100% hMSCs, which could either be the result of a higher collagen production by hMSCs and/or a higher break down of the type I collagen hydrogel by the meniscus cells. These findings

were different compared to the paper of McCorry et al. (2016),[20] who have shown the highest GAG production in the 50 : 50 ratio. However, McCorry et al. (2016) have used bovine cells, passage 4 MSCs (cultured with fibroblast growth factor) and passage 0 meniscus cells as compared to hMSCs passage 3 and human meniscus cells passage 1 in the current study. In addition, in the present study, coculture was harvested after 28 , compared to 15 dreported by McCorry et al. (2016)[20] Perhaps, the most importance difference is the fixed shape they used for the culturing the type I collagen hydrogels, so that the collagen gel could not contract during the culturing period, which also has an influence on the ECM production.[29,30]

The most frequently-described seeding methods reported in literature are static seeding, seeding by injection and centrifugal seeding.[13,27,31,37] Most studies are directed towards cell viability and distribution without considering the clinical applicability for a one-stage procedure where seeding of the scaffold has to be performed according to GMP-regulations. Zhang et al.(2015)[37] have reported the best cell distribution of MSCs and meniscus fibrochondrocytes using centrifugal seeding, although these results were not significantly better than static seeding. When static seeding was used, Thevenot et al. (2008)[27] have shown a high cell density in the top layer of the scaffold compared to the center and the bottom. This result does not compare with the present study results where a homogeneous distribution of cells throughout the whole scaffold in vertical direction was shown. Besides the seeding method, scaffold material could also influence cell number and cell distribution after seeding. Demineralised cancellous bone and poly(lactide-co-glycolide) (PLGA) scaffolds were used by Zhang et al. (2015)[37] and Thevenot et al. (2008)[27] respectively, having different material characteristics as compared to the CMI, including pore size. The CMI has a wide range in pore size (50 µm - 400 µm), whereas Thevonot et al. (2008)[27] (mean 212 µm, range 150 µm – 250 µm) and Zhang et al. (2015)[37] (268 µm) used a smaller pore size. The smaller pore sizes could possibly negatively influence the cell distribution after seeding. Moreover, the CMI has a sponge-like structure, absorbing fluids rapidly when seeded onto the scaffold, providing a good distribution of the cells when static seeding is used. Multiple injections (in a wet environment) into the CMI creates ‘pockets’ of cells

instead of a homogenous distribution. This result is not illustrated in the literature, since previous authors performed the injected seeding with only one injection.[27,31,37]

Conclusion

The present study demonstrated the *in vitro* feasibility of a new one-stage cell-based procedure for meniscus regeneration in young and active patients with non-repairable meniscus tears. In coculture hMSC stimulate meniscus cells to produce ECM by communication through gap junctions before going into apoptosis. The most optimal ratio for GAG and collagen production is 20% meniscus cells and 80% hMSCs. Static seeding resulted in a higher cell density and better cell distribution than wet seeding. The results of these *in vitro* experiments lay the foundation for clinical application of one-stage cell based meniscus regeneration procedures.

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Chapter 5

Surgical Feasibility of a One-Stage Cell-Based Arthroscopic Procedure for Meniscus Regeneration: A Cadaveric Study

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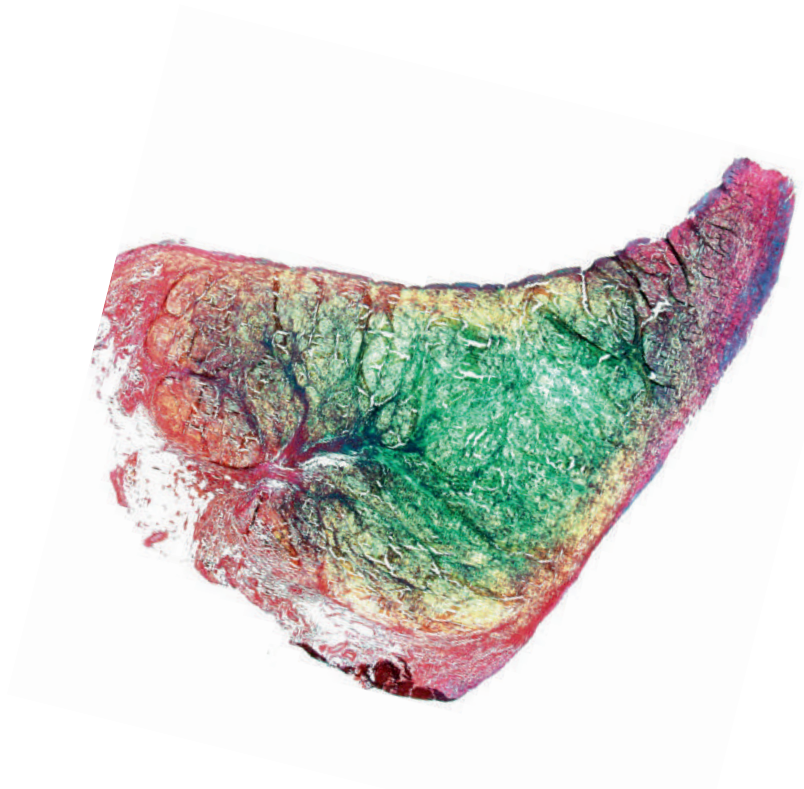

Abstract

Purpose: To test the technical aspects and feasibility of seeding a combination of meniscus cells isolated from a rapid digestion protocol and mesenchymal stromal cells (MSCs) (20: 80 ratio) into a meniscus scaffold for the development of a one-stage arthroscopic procedure for meniscus regeneration.

Methods: A cadaveric study was performed using nine fresh frozen human cadaveric knee joints. Two different arthroscopic cell-seeding methods were applied to the Collagen Meniscus Implant (CMI®) as carrier scaffold: either (1) seeding before arthroscopic surgical implantation of the scaffold or (2) after implantation of the scaffold. The cells were injected inside the scaffold, using fast green-stained fibrin glue as carrier, to macroscopically visualize the amount of fibrin glue. Macroscopic pictures and confocal microscopy analyses were used to determine cell distribution and viability. In addition, the DNA content in the cell-seeded scaffold was determined. In addition, different concentrations of Liberase were examined to find the optimal concentration for rapid digestion of meniscus tissue.

Results: Macroscopically, seeding before implantation showed a better distribution of fast green-stained fibrin glue carrier than seeding the scaffold before surgical implantation. In addition, it resulted in significantly more cells and a better cell distribution compared with seeding the scaffold after arthroscopic implantation. Both seeding methods did not affect cell viability. After rapid digestion, 0.0125% Liberase resulted in the highest cell isolation efficiency.

Conclusions: This study demonstrates that living human meniscus cells can be isolated efficiently, combined with MSCs in 20: 80 ratio, and uniformly delivered into a currently available meniscus scaffold. This scaffold can then be arthroscopically implanted, creating a one-stage solution for partial meniscal deficiency.

Keywords: Meniscus; Arthroscopy; Mesenchymal stromal cells; Collagen Meniscus Implant; Cadaveric study

Introduction

Meniscus injuries are very common, especially in young and active patients. Approximately 15% of all knee injuries involve a tear of the meniscus.[25] The main functions of the menisci are load transmission, shock absorption and stability of the knee.[13] These native functions are impaired when an injury occurs causing a meniscus tear. This meniscus deficiency can result in excessive forces and abnormal loading of the articular cartilage, leading to osteoarthritis (OA).[2,3] Meniscus tears in the nonvascularized zone have limited healing capacity, and currently the most frequently used surgical treatment involves (partial) removal of the damaged meniscus,[13] contributing even further to the early development of OA.[3,8,9,22] To potentially prevent postmeniscectomy arthritis and treat postmeniscectomy syndrome, options such as implanting a porous meniscus scaffold have been introduced. Currently, a commercially available scaffold fabricated from bovine type I collagen will degrade within 1-2 years after implantation.[6] These porous scaffolds have shown promising clinical results up to 12 years follow-up, by demonstrating less pain, higher subjective scores using patient reported outcome measures, and a higher activity level than a partial meniscectomy control group.[26,33] Ideally, healing may occur by surrounding resident meniscus cells and cells from synovial lining engraft the CMI, thereby forming meniscus-like tissue, while the biomaterial slowly degrades. However, this process is very slow, and long term MRI demonstrates that <10% of the patients have a meniscus regenerate size similar to the native meniscus.[32]

Therefore, this process potentially could be accelerated and improved by seeding the porous artificial meniscus with cells, as shown by Martinek et al in a goat study.[20] Specifically, a combination of autologous meniscus cells and allogeneic mesenchymal stromal cells (MSCs) is promising, as these cell types could be seeded during a one-stage surgical procedure, thereby avoiding a costly two-stage autologous cell expansion. In addition, allogeneic MSCs have been proven to be safe and a viable cell source for implantation with autologous chondrons for regeneration of knee cartilage.[4,29,30] Finally, biochemical and histological analysis of *in vitro* co-culturing of MSCs and meniscus cells demonstrated excellent matrix production of glycosaminoglycans (GAGs) and type I collagen, especially in the ratios where a higher percentage of MSCs (up to 90%) was used.[15]

For this one-stage surgical procedure to become clinically feasible, meniscus tissue digestion and uniform delivery of the cells into a meniscus scaffold need to be optimized. Specifically, a rapid digestion protocol[4] for meniscus tissue is needed to harvest sufficient meniscus cells to combine with allogeneic MSCs within the time frame of one surgery. These cells would then be delivered to a commercially available meniscus biodegradable scaffold. For optimization of seeding the meniscus scaffold with cells, two seeding methods are possible in clinical practice: (1) seeding *before* surgical implantation of the scaffold, or 'dry seeding' (as the meniscus scaffold is still dry outside the knee at the time of seeding) or (2) seeding *after* surgical implantation in a fluid arthroscopic knee environment, or 'wet seeding' (as fluids present in the knee at the time of implantation are already taken up by the scaffold). In both methods, the cells are injected inside the scaffold, using fibrin glue as a carrier for the cells to ensure local delivery. Therefore, the purpose of this study was to test the technical aspects and feasibility of seeding a combination of meniscus cells and MSCs (20: 80 ratio) into a meniscus scaffold, using a rapid digestion protocol to isolate meniscus cells, for the development of a one-stage arthroscopic procedure for meniscus regeneration.

Methods

Study outline

To evaluate the feasibility of combining a commercially available meniscus scaffold (Collagen Meniscus Implant, (CMI®); Stryker, Kalamazoo, MI) with autologous meniscus cells and allogeneic MSCs in a 20: 80 ratio[15] during a one-stage arthroscopic procedure, a laboratory and cadaveric study on human knee specimens were performed.

Donors

Meniscus tissue was obtained as redundant material from four patients undergoing total knee arthroplasty. The anonymous use and collection of this material was performed according to the Medical Ethical 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.[7,12] For the

MSCs, bone marrow biopsies were obtained from the iliac crest during total hip replacement after written informed consent was obtained (Medical Ethical Committee, University Medical Center Utrecht).

Nine post mortem fresh frozen human legs were provided by the anatomy department of the University Medical Center Utrecht. These were graciously donated by people who signed written informed consent during lifetime for postmortem donation of their entire body for educational and research purposes.

Cell isolation

For a one-stage surgical application, it would be desirable to complete the isolation of autologous meniscus cells from the debrided meniscus tissue within 40 minutes, yielding enough cells for 20% meniscus cells (for a 2.5 cm defect a total of 2,000,000 cells is used[30]) of the total seeded amount. Therefore, different concentrations of Liberase (Roche, Germany) were examined to find the optimal protocol for rapid digestion of meniscus cells.

For the cadaveric study, the entire meniscus of four donors was cut in pieces of 2 x 2 mm, washed in phosphate buffered saline (PBS) and digested in 0.15% collagenase type II (Worthington, Lakewood, NJ) dissolved in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Bleiswijk, The Netherlands) with 1% penicillin/streptomycin (1% pen/strep; 100 U/mL / 100 µg/mL; Invitrogen, Life Technologies) overnight at 37°C on a shaker plate.

In addition, meniscus cells were acquired from four different donors using adapted versions of the rapid digestion protocol from Bekkers et al.,[4] where concentrations of 0.05% 0.025% and 0.0125% of Good Manufacturer Practice (GMP)-grade Liberase (Roche, Germany) in DMEM with 1% pen/strep were used for the digestion of 100 mg of meniscus tissue with duration of 40 minutes at 40°C on a shaker plate. Afterward, for both digestion methods, the suspension was filtered through a 100 µm cell strainer (BD Biosciences), spun by centrifugation for 5 minutes at 300 g, resuspended in DMEM supplemented with 1% pen/strep and 10% fetal bovine serum (FBS; HyClone, Logan, UT), counted with an automated cell counter 1 : 1 diluted in trypan blue (Biorad) to detect dead cells (TC20™ Bio-Rad, CA). The meniscus cells for the cadaveric study were cultured up to passage 3 or 4, whereas for evaluation of the rapid digestion protocol, the total cell count and

5

viability were corrected for the weight of the tissue. To exclude the erythrocytes from the count, only cells larger than 6 μm were considered meniscus cells.
Human MSCs (hMSCs) were isolated from bone marrow biopsies as described previously[14] and were expanded in α-minimal essential medium supplemented with 10% FBS, 1% l-ascorbic acid-2-phospate (ASAP; Sigma-Aldrich) and 1% pen/strep to be used at passage 5 or 6.

Preparation of the fibrin glue containing cells

To seed and seal the meniscus cells and hMSCs inside of the meniscus scaffold, a commercially available, clinical grade fibrin glue kit (Tisseel, Baxter) was used, consisting of two components: fibrinogen and thrombin used 1:1. When fibrinogen or thrombin is mentioned, it concerns specifically one of the two components, whereas fibrin glue refers to the complete product. Fastgreen (Merck, Germany) was dissolved in PBS with a concentration of 0.4mg/mL and filter sterilized. Cells were trypsinized, spun by centrifugation for 5 minutes at 300 g and resuspended in this solution in combination with the fibrinogen and stored in a syringe with a final cell concentration of 3.0×10^6 cells per 1 mL of fibrin glue, meaning 6.0×10^5 meniscus cells and 2.4×10^6 MSCs per mL.

The 20% meniscus cells and 80% MSCs was chosen based on previous co-cultures of meniscus cells and MSCs.[15] Production of tissue, most comparable to native meniscus tissue, was shown using the combination of meniscus cells and MSCs in this ratio. Besides, 20% meniscus cells is a clinically feasible percentage for performing a one-stage procedure.

Surgical Procedure

Arthroscopy of nine fresh frozen knee joints was performed by two orthopedic surgeons, using standard instrumentation and technique with routine anterolateral and anteromedial portals, creating a full thickness meniscus defect both on the medial and lateral side with a defect size between 2.5 and 3 cm. A rim of the meniscus, the anterior, and posterior roots were preserved for attachment of the meniscus scaffold during implantation. The meniscus scaffold were fixed to the rim of the meniscus using two inside-out 2-0 FiberWire® Meniscus Repair Needles (Arthrex, Naples, FL). In each knee joint the two different cell-seeding procedures

were performed, meaning that both procedures were executed four times on both lateral and medial sides.

Two methods of cell seeding were applied in this study:

1. Dry seeding: after measuring the defect size, the meniscus scaffold was trimmed to the appropriate size and the first 0.5 mL of the fibrinogen (containing the cells as described previously) component was injected into the scaffold followed by injection of the thrombin component, both using a 23-gauge needle. Afterward, the seeded meniscus scaffold was transferred to the portal, by using a clamp and sutured to the meniscus rim, without stopping the saline flow (Fig. 1A-C).
2. Wet seeding: for this procedure the meniscus scaffold was first surgically implanted and sutured as described above, and the cells were injected afterward. To prevent the cells from washing out, the flow of saline from the arthroscopy was stopped during the injection of the cells. Three different locations (to distribute the cells throughout the complete implant) were chosen to inject the cells with a 18 gauge spine needle (6.00IN 1.2mm x 152mm; Becton, Dickinson and Company, Frankline Lakes, NJ) through the contralateral portal of the meniscus defect, where the 0.5 mL cell suspension in fibrinogen was injected, the needle was left at the final injection location and the syringe was changed to one containing 0.5 mL of thrombin, which was also injected at the three locations (Fig. 1D-F).

Assessment of macroscopic pictures

After the two procedures were performed, the knee was opened and macroscopic pictures of the joint with the implants were taken. Because the fibrinogen component of the fibrin glue was stained with fast green, it was possible to macroscopically visualize the amount of leakage and distribution of the fibrin glue.

Cell distribution and viability

Visualization of the cell distribution throughout the meniscus scaffold and measurement of cell viability between different seeding methods was performed by creating three-dimensional (3D) images acquired from a Leica SP8 confocal microscope. For three scaffolds per seeding method, the scaffold was cut in the

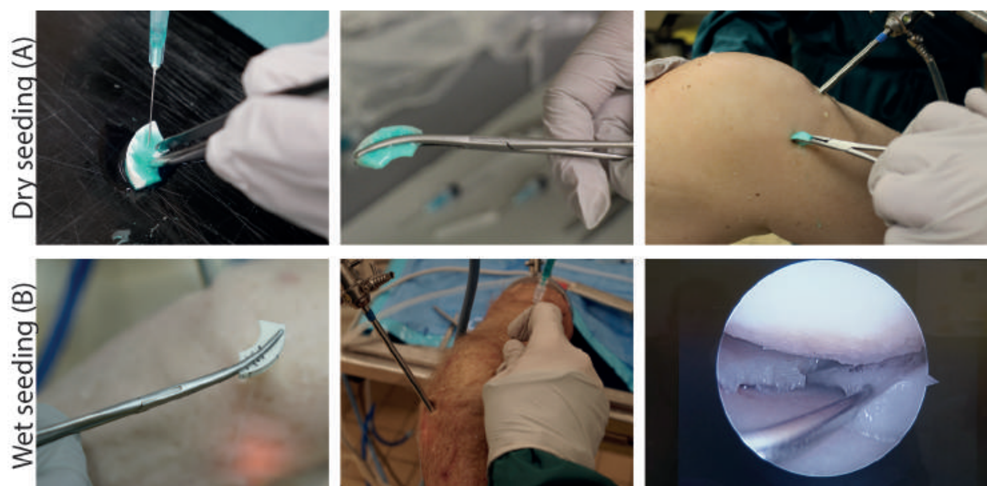

Figure 1: Different seeding methods. *Dry seeding* was performed by injection of the fibrinogen, which contained the cells, and was stained with fast green (A) followed by the thrombin component. Afterwards a mosquito forceps was used (B) to put the seeded scaffold through the portal (C) and sutured into the meniscus defect. During *wet seeding*, the meniscus scaffold first implanted into the knee joint using a mosquito forces (D) and sutures. Afterwards the fibrinogen, containing the cells, followed by thrombin were injected into the scaffold using a 18 gauge spine needle through the portal of the arthroscopy (E), into the implanted meniscus scaffold (F)

sagittal plane in six pieces and imaged with the microscope, to include both the core of the scaffold, as well as the superficial zone. LIVE/DEAD assay was then performed to stain the cells present in the scaffold after the arthroscopic procedures. The six even pieces were incubated for 30 minutes with 0.5 µL/mL Calcein AM (Molecular Probes) and 1 µL/mL Ethidium homodimer-1 (Molecular Probes) in PBS. After washing with PBS, the pieces of meniscus scaffold were stained for 4 minutes with 100ng/mL DAPI and washed again in PBS. Merged 3D images from the Leica confocal microscope (with a mean depth of 178 µm (range 86.7 – 238.7) were analyzed using ImageJ to calculate the ratio of live and dead cells per image.

Assessment of cell amount after seeding

After confocal imaging the meniscus scaffold pieces were digested overnight at 60°C in papain. Prior to the picogreen DNA assay (Invitrogen), ethanol (EtOH) precipitation of DNA was performed on the papain samples to

wash out the fast green. And then 25 µL 3 M sodium acetate and 725 µL 100% EtOH was added to 250 µL papain digestion and stored at -20°C overnight. The samples were then centrifuged for 30 minutes, the EtOH was removed, and the pellets were washed two times in 75% EtOH. Picogreen DNA assay was used according to the manufacturer's instructions to determine the DNA content of the meniscus scaffold after implantation into the knee. λDNA was used as a standard reference to calculate the DNA content at an excitation measured at 480nm and emission set at 520nm.

Surgical implantation evaluation

Feedback of the surgeons on their experience and the feasibility of the different procedures was reported after each surgical implantation.

Statistical analysis

The sample size, based on preliminary *in vitro* results of the different seeding methods, was calculated with nQuery Advisor® Version 7.0. When using the mean difference and a significance level of 5% and a power of 80%, the required sample size was five procedures per seeding method. A paired Student's t-test was performed on the picogreen assay to compare the amount of DNA in the meniscus scaffold after the two different seeding methods. A one-way analysis of variance was used to calculate the amount of cells after digestion with different concentrations of Liberase. A p-value of < 0.05 was considered significant.

Results

Optimal rapid digestion protocol for isolation of meniscus cells

After comparison of the three different concentrations of Liberase for digestion of meniscus tissue within 40 minutes at 40 °C, there was no significant difference between the total amount of cells after digestion for the four different donors (mean 2.18×10^6 cells per gram tissue, range 2.17×10^5 - 5.23×10^6). The 0.05% Liberase group resulted in significantly more living cells than 0.025% ($p = 0.0179$). However, when compared to 0.0125%, there was no significant difference (Fig. 2).

The percentage of living cells was the largest for the 0.0125% group, with no difference in cell number.

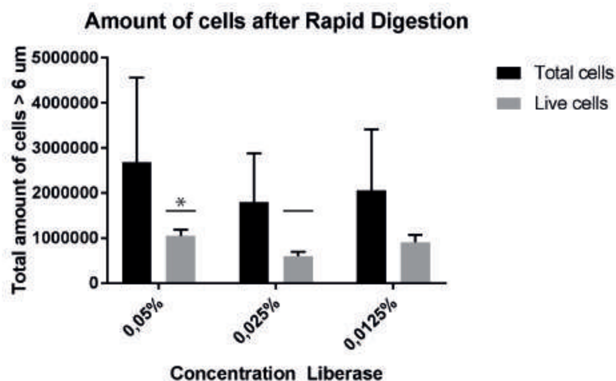

Figure 2: Cell counting after rapid digestion of meniscus tissue using 0.05%, 0.025% and 0.0125% Liberase showed a significant difference in living cells between 0.05% and 0.025% Liberase, but not compared to 0.0125%. A significant difference with a p-value <0.05 is shown with *.

Best seeding method for MSCs and meniscus cells in a Collagen Meniscus Implant

In the macroscopic images of the human knees, the amount of fibrin glue, containing the cell suspension of 20% meniscus cells and 80% MSCs, inside the meniscus scaffold is shown by the amount of fast green staining (Fig. 3). Dry seeding shows a brighter staining of the fast green in the meniscus scaffold and a more homogenous distribution (Fig. 3 A, C) compared to the meniscus scaffolds where wet seeding was used (Fig. 3 B, D). After injection of the fibrin glue in the wet seeding method, Figure 3 D shows leakage of the fibrin glue into the joint instead of the meniscus scaffold. A clear difference in fast green staining was observed in the sagittal plains of the meniscus scaffolds for dry (E) and wet (F) seeding. Macroscopically the dry seeding method demonstrated a higher amount of fibrin glue throughout the entire construct.

After EtOH precipitation of DNA on the papain-digested tissue, the picogreen assay (as quantification for the amount of cells) showed a significant difference between the amounts of DNA in the two seeding methods ($p = 0.0096$), in favor of dry seeding (mean 4800 ng, standard deviation (SD) 1935) compared to wet seeding after implantation (mean 2928 ng, SD 1223) (Fig. 4).

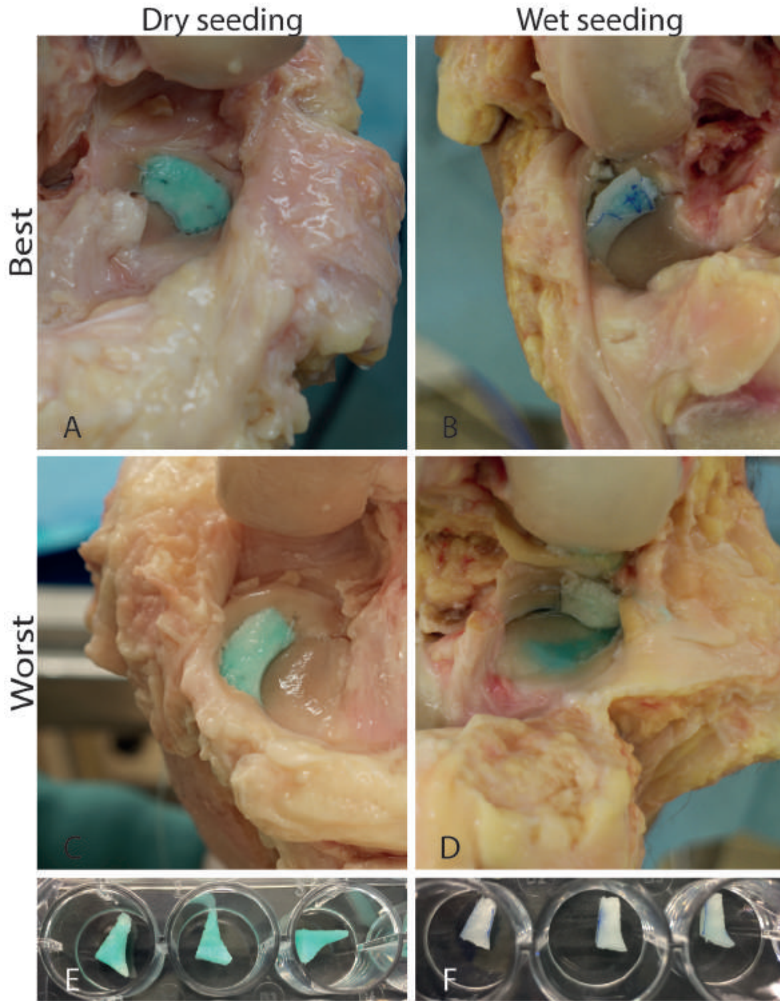

Figure 3: Pictures showing a meniscus defect replaced with the Collagen Meniscus Implant in a human cadaveric knee with the most efficient seeding after both dry (A) and wet (B) seeding. The worst results for both seeding methods are shown in C and D. Sagittal plains of the seeded CMI are shown in E (dry) and F (wet).

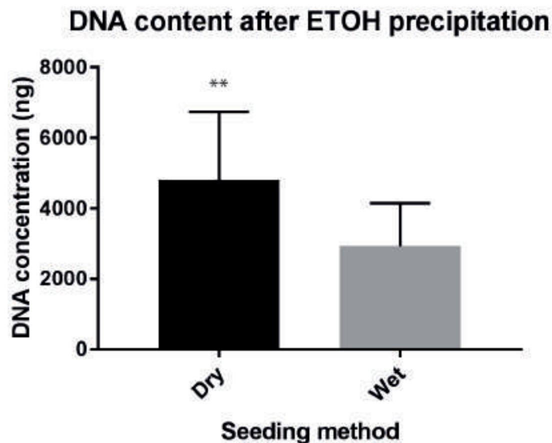

Figure 4: Picogreen assay showed a significant higher DNA content in the pre-seeded Collagen Meniscus Implants (CMIs) compared to the CMIs seeded after implantation after ethanol precipitation ($p = 0.0096$).

Confocal imaging of the meniscus scaffolds after implantation showed even distribution of the cells throughout the meniscus scaffold when dry seeding was used (Fig. 5 A). Some areas contained fewer cells than other, but the overall distribution was acceptable (Fig. 5 B – E). Around the meniscus scaffold, a layer of fibrin glue containing cells was formed. In contrast, the cell distribution was less homogenous for wet seeding (Fig. 6 A, B, D, E), and damage of the meniscus scaffold was observed at the location of needle injection (Fig. 6 C). When processing the confocal images with ImageJ (z-stacks of 270μm), dry seeding demonstrated 305.8% more cells compared to the wet seeding, with 99.3% and 99.5% living cells, respectively.

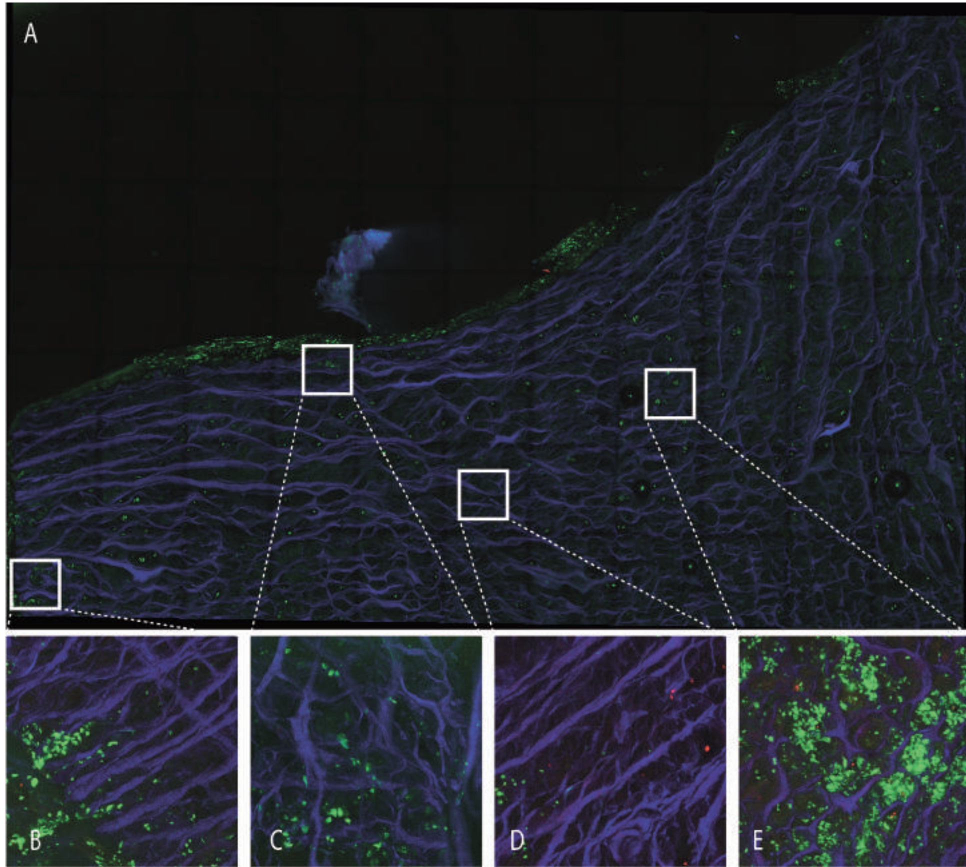

Figure 5: Merged confocal image after pre-seeding of the Collagen Meniscus Implant (CMI). The CMI is stained with DAPI (blue) and the cells for live/dead assay, showing the cell distribution throughout the CMI (A) with close-ups of different areas (B – E) to demonstrate the cell distribution throughout the complete scaffold. Viability of the cells was shown by the green Calcein staining.

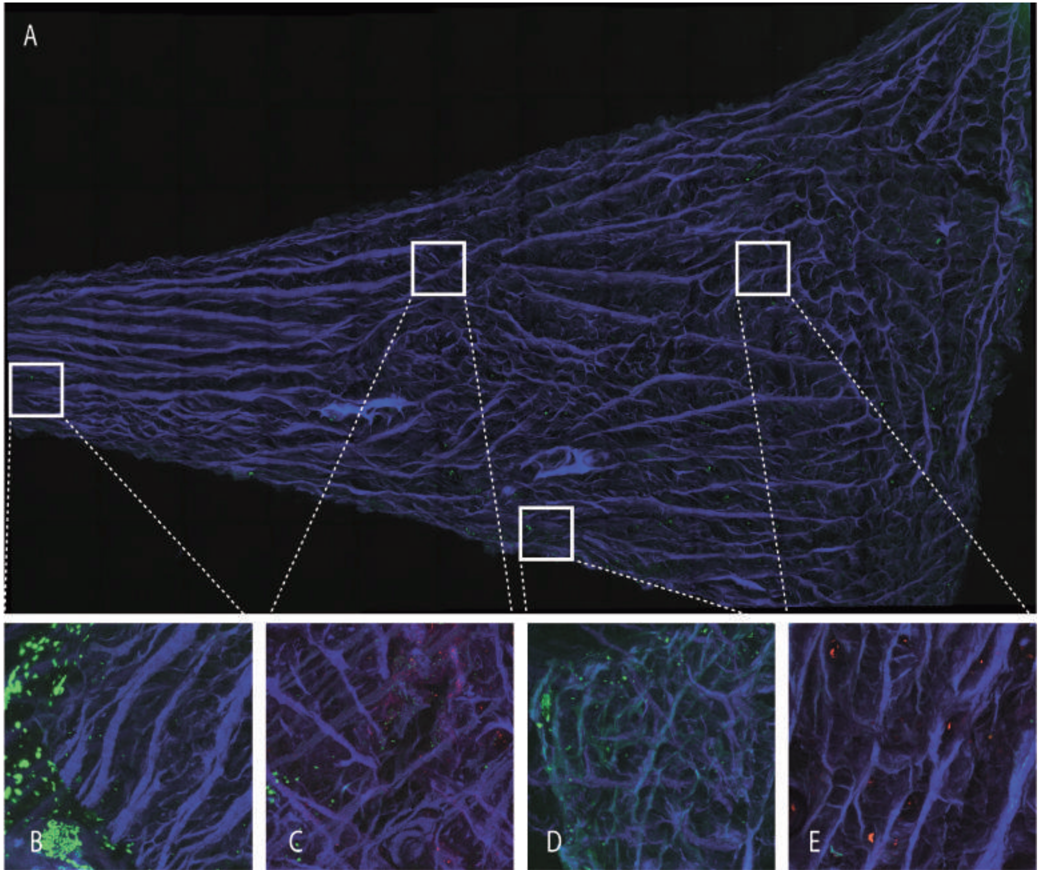

Figure 6: Merged confocal image of the Collagen Meniscus Implant (CMI) seeded after implantation. The CMI is stained with DAPI (blue) and the cells for live/dead assay, showing the cell distribution throughout the CMI (A) with close-ups of different areas (B – E) to demonstrate the cell distribution throughout the complete scaffold. Viability of the cells was shown by the green Calcein staining. Figure E demonstrates damage at the injection spot of the scaffold, which is not visible in the dry seeded scaffold.

Discussion

This study demonstrated the feasibility of combining recycled autologous meniscus cells and allogeneic MSCs in a meniscus scaffold during an arthroscopic one-stage procedure for meniscus regeneration. Rapid digestion of meniscus tissue with 0.0125% Liberase resulted in sufficient living cells for a 20: 80 ratio with allogeneic MSCs during a one-stage procedure. Both seeding methods did not affect cell viability, but dry preseeding of the meniscus scaffold resulted in more cells, and

a better distribution of the cells in the scaffold, compared to wet seeding after arthroscopic implantation.

Feedback of the surgeons suggested the surgical procedure of dry seeding to be more feasible in clinical practice. The structure of the meniscus scaffold does not seem to change after seeding; therefore, the implantation of a seeded meniscus scaffold is not different from the implantation of an unseeded scaffold. Moreover, it is relatively easy to seed the cells inside of the meniscus scaffold outside of the knee in a dry environment, and no additional damage is administered to the scaffold during the dry seeding procedure, compared with the wet seeding.

However, logistically, for a single-stage procedure, dry seeding is less efficient than wet seeding. After performing the partial meniscectomy, the tissue will be subjected to enzymatic digestion and washing steps to isolate the meniscus cells and mix them with 'off-the-shelf' allogeneic MSCs in fibrin glue, which takes about 90 min in total, 60 min between start of the operation and seeding of the cells In the interim, the surgeon can implant the meniscus scaffold in the defect and seed afterwards, when using wet seeding, which will lead to a time of 50 min between start of the procedure and injection of the cells. In contrast, when applying dry seeding, the surgeon must wait until the cell mixture is prepared before continuing the surgery.

However, a disadvantage of seeding the scaffold after implantation is the saline present in the knee joint, preventing a good distribution of cells throughout the scaffold because of its sponge-like character. It is possible to completely drain the knee from saline to inject the meniscus scaffold in a nonaqueous environment, but the visibility for the surgeon will be suboptimal, making it less suitable to inject the cells reproducibly. Even when the flow is paused to prevent washing out the cells, it is difficult to determine whether the needle is inside of the scaffold, or already in the outer rim of the meniscus.

To date, an optimal cell density for regenerative medicine is not yet described. The total amount of cells seeded into the meniscus scaffold was based on the IMPACT trial (NCT02037204)[29] and in Autologous Chondrocyte Transplantation (ACI) for cartilage defects,[5] where approximately 2.0×10^6 cells/cm^2 are used. Both ACI and the IMPACT trial have demonstrated good results for cartilage regeneration using this cell concentration of either chondrocytes, or the

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combination of chondrons and hMSCs.[5,29,30] Other cell-based treatments for meniscus regeneration show a wide variance in number of cells used, depending on the strategy to accomplish meniscus regeneration.[19] In small and large animal models, 0.5 to 5 million and 15 to 150 million cells were used, respectively. However, most of these methods included intra-articular injections, which seem to require a larger number of cells than compared to local application with seeding a scaffold with the cells.

In animal models, meniscus regeneration methods in which a scaffold was used, cell concentrations ranged between $1.0x10^6$ to $10.0x10^6$/mL.[10,18,28,31] A CMI seeded with meniscus cells and cultured *in vitro* for 3 weeks was implanted in sheep by Martinek et al.,[20] with a seeding density of $10.0x10^6$ cells per 3.25 cm CMI. Baker et al. used a cell concentration of $5.0x10^6$/mL for seeding a poly(ε-caprolactone) (PCL) scaffold with human meniscus cells *in vitro*.[2] Thus, even for the application of meniscus cells combined with a scaffold, cell concentrations vary widely and there is no consensus on the effect of a higher number of seeded cells. Furthermore, a higher cell concentration may not translate to a higher cell density after seeding. Equally high seeding efficiencies were shown by Weinand et al. for cell concentrations of $1.0x10^6$ and $2.0x10^6$/mL compared to $5.0x10^6$/mL when oscillating seeding of the Vicryl scaffold was used.[27]

Nevertheless, this study showed a significant difference in seeding efficiency between the two different seeding methods, using the same cell concentrations, resulting in 99% living cells inside of the scaffold. A 20:80 ratio of meniscus cells and MSCs were used, which is clinically feasible as enough autologous cells can be isolated. For a defect of 2.5 cm, 0.65 grams of tissue can be harvested (data not shown) resulting in approximately 600,000 meniscus cells. A 2.5 cm defect is comparable with 30% of the meniscus scaffold, meaning $2.0x10^6$ cells in total and 400,000 meniscus cells are needed. Overall, this indicates that the current cell yield after rapid digestion is sufficient for this procedure. The combination of the 20% autologous meniscus cells with 80% allogeneic MSCs makes this procedure suitable for a one-stage operation. First, there is no additional operation needed to harvest and expand autologous MSCs from bone marrow of the patients, making it a two-step procedure and increase costs considerably.[24] Second, we think that using allogeneic MSCs instead of autologous

will lead to better tissue regeneration by the secretion of trophic pro-regenerative factors and anti-inflammatory factors.[16,23,30]

For this study, a standard fluid arthroscopy of the knee was performed. Carbon dioxide (CO_2)-insufflated arthroscopy is also described for the knee, and could be used during the seeding procedure after implantation of a meniscus scaffold.[17,21] Vascellari et al. described the combination of a standard arthroscopy for the debridement of the cartilage defect, and preparation of the graft for matrix-induced autologous chondrocyte implant (MACI) followed by a CO_2-insufflated arthroscopy for implantation of the graft using fibrin glue.[21] No adverse events or complications related to the surgery were observed during the surgical procedure and follow-up period. In addition, the visualization during CO_2 arthroscopy is similar compared to arthroscopy with saline inflow. This indicates CO_2 insufflated arthroscopy could be safe and might be an option for seeding cells after fixation of the meniscus scaffold. However, during injection of the scaffold after implantation, it was not only difficult to inject the meniscus scaffold with cells due to the sponge-like character after soaking in saline, it was also difficult to determine whether the needle was in the scaffold or already in the outer rim of the meniscus. This problem would not be solved by using CO_2-insufflated arthroscopy.

Rapid enzymatic digestion of meniscus tissue is performed on manually minced pieces of meniscus with an approximate size of 1 to 2 mm^2. Automated mincing of the meniscus tissue is faster, could possibly lead to a higher cell yield, and could shorten digestion time because the smaller pieces would allow the Liberase to further penetrate the each fragment of tissue and improve the digestion effectivity.[11] Therefore, automated mincing could potentially accelerate the time needed for the one-stage procedure for meniscus regeneration. To the best of our knowledge, there is nothing known on combining automated mincing of tissue with enzymatic digestion for cell isolation.

Limitations

Several limitations to this study could be identified. First, the number of cells after rapid digestion was calculated per 1 gram of meniscus tissue. However, the amount of tissue retrieved from partial meniscectomy was not standardized. Nevertheless, when 2.0×10^6 cells/cm^2 were used, like during ACI, 1.2×10^6 meniscus

cells are needed to fill a defect comprising an entire meniscus scaffold. The amount of fibrin glue and number of cells are proportional to the percentage of scaffold used for the defect. This target should be reached, even when accounting for the lowest amount of received tissue during partial meniscectomy. Likewise, there was a broad range in the number of isolated cells within the same concentration of Liberase. This could be due to quality of meniscus tissue, which we received from patients undergoing a total knee arthroplasty.

Second, the fibrinogen and thrombin for this study were added consecutively instead of simultaneously through the DuoSet device, which is advised for the usage of fibrin glue. When injecting the fibrin glue inside of the meniscus scaffold, more pressure is needed compared to injecting it on a surface. This is especially relevant for the 18-gauge spine needle, as when the two components are mixed inside of the needle, it will clog, making it impossible to inject the two components at the same time. To be able to compare the different seeding methods, dry seeding was performed using the two components consecutively as well. Moreover, in *in vitro* studies this method of cross-linking is often used and validated.[1] Therefore, we assume this injection method of fibrin glue will not affect the crosslinking process of the fibrin glue inside of the meniscus scaffold.

Finally, in this study, only the procedure of implanting and seeding a meniscus scaffold was examined, but the knees were not stressed with mechanical loads or motion. Therefore, we have no information on the (sheer) force in the fibrin glue containing the cells in the scaffold. However, since the treatment after closure of the knee was the same in both seeding methods, this was not relevant to the results of this initial study.

In conclusion, this study demonstrates that living human meniscus cells can be isolated efficiently, combined with MSCs in 20:80 ratio, and uniformly delivered into a currently available meniscus scaffold. This scaffold can then be arthroscopically implanted, creating a one-stage solution for partial meniscal deficiency.

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Chapter 6

Growth Factors Enhance Meniscus Regeneration in Combination with a Degradable Meniscus Scaffold

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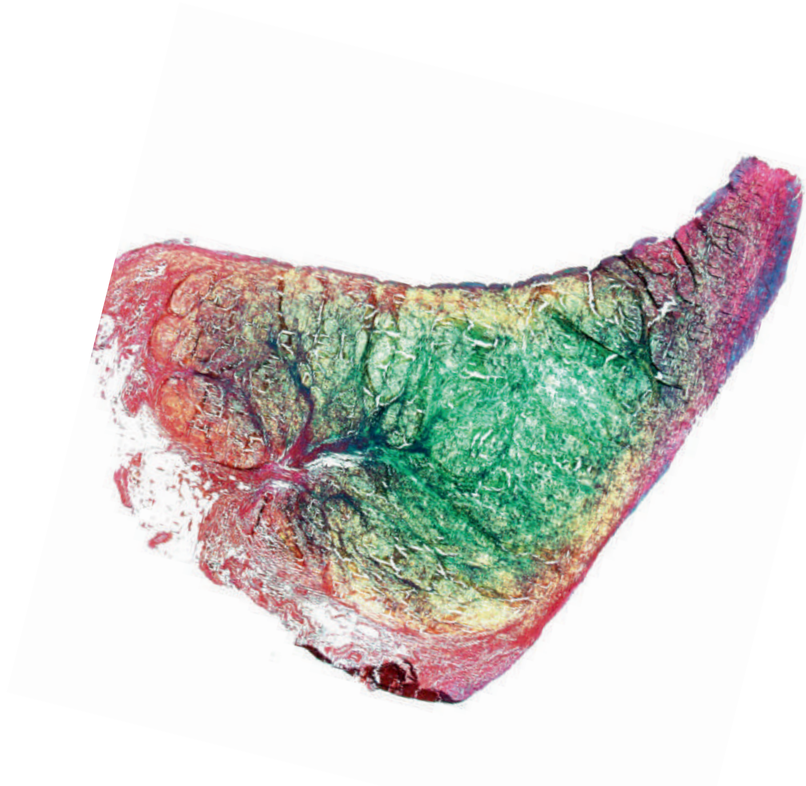

Abstract

Background: Meniscus injury and osteoarthritis are strongly correlated. Meniscus regeneration could be enhanced by targeting meniscus cells and mesenchymal stromal cells (MSCs) with growth factors (GFs) in combination with the Collagen Meniscus Implant (CMI®) to accelerate cell ingrowth into the implant.

Purpose: The goal of this study was to examine the GFs most beneficial for migration, proliferation, and extracellular matrix (ECM) production of meniscus cells and MSCs, and use GF binding peptides to functionalize the CMI to improve meniscus regeneration after partial meniscectomy.

Methods: Migration of meniscus cells and MSCs under influence of IGF-1, PDGF, VEGF, TGF-β1, FGF and platelet lysate (PL) was quantified in a transwell assay. Proliferation and migration were studied in a micro-wound assay. Subsequently, meniscus cells were cultured for 28 days in presence of the different GFs and PL to determine proteoglycan and collagen production. The CMI was functionalized with a VEGF binding peptide by reacting its amines with the carboxylic acid groups of the collagen through NHS/EDC chemistry. Immunohistochemistry against VEGF was performed on the CMIs and the fluorescence intensity quantified using ImageJ.

Results: Cell migration was significantly enhanced by PL and PDGF, whereas PL and TGF-β1 significantly increased the proliferation of meniscus cells and MSCs. TGF-β1 also enhanced the ECM production in meniscus cells. (Fig. 1) A higher fluorescence signal was observed when the CMIs displayed VEGF binding peptides meaning that more VEGF was captured and immobilized on these matrices. Accordingly, the CMI can be functionalized with GF binding peptide in order to capture and deliver GFs to the surrounding cells. (Fig. 2)

Conclusion: This study shows PDGF, TGF-β1 and PL stimulate migration, proliferation and/or ECM production of meniscus cells and MSCs. Therefore, the possibility of functionalizing the CMI with GF binding peptides could enhance meniscus regeneration after partial meniscectomy.

Keywords: Growth factors; Meniscus; Mesenchymal stromal cells; Platelet rich plasma; Regeneration; Scaffold

Introduction

The meniscus is a c-shaped, fibrocartilage structure in the femorotibial joint. It is essential for load transmission from the femur to the tibia, stability of the knee joint, and articular surface protection.[19,34] Meniscus injury is the most common indication for orthopaedic surgery and is strongly correlated with development of early osteoarthritis (OA).[14,37,51] Partial meniscectomy can contribute to the development of OA by increasing the contact pressure on the articular cartilage up to 235%, which eventually may lead to degenerative changes in the cartilage.[3,33,36] To prevent these increased contact pressures and decreased contact area, repair or replacement of the meniscus is of great importance. [4,13,15] The ability to self-repair in meniscus tissue is limited to the vascularized region, and this healing potential decreases with age.[35,42,50] Moreover, healing from repair is only successful in the vascularized outer zone of the meniscus of young patients.

Implantation of a scaffold after partial meniscectomy could overcome this problem and has been studied previously.[38,45,54] The use of a cell-seeded scaffold increases tissue formation and leads to more organized tissue compared to the use of an empty scaffold.[29] A scaffold could be seeded with autologous multipotent mesenchymal stromal cells (MSCs), which has shown promising results *in vivo* in various animal models.[11,23,28] However, this would be a great burden on the patient as it would require two procedures. Moreover, harvesting and culturing of autologous cells is a costly and time-consuming process and it would be highly preferable to develop a single-stage procedure. Nevertheless, it is challenging to obtain a sufficient amount of cells in a single arthroscopic procedure without cell expansion. This could be overcome by combining allogeneic MSCs with autologous cells from the meniscectomized tissue[22,52,53] or by incorporating growth factors within the scaffold to increase migration of cells into the scaffold, thereby stimulating proliferation and extracellular matrix (ECM) production.

Growth factors could attract the patient's resident meniscus cells and MSCs present in the synovium and the meniscus, towards the scaffold.[39,47,48] Besides single growth factors, platelet rich plasma (PRP) and platelet lysate (PL; containing the platelet released growth factors) were shown to have a positive effect on migration and proliferation of meniscus cells and MSCs.[7,20] To date, the effect of growth factors on migration of meniscus cells and MSCs remains to be elucidated.

Furthermore, *in vivo* lifespan of growth factors is too short to sustain biological activity,[26] and subsequently a method to attract endogenous growth factors from the knee joint/synovium to the meniscus scaffold is necessary to secure an ongoing stimulating effect of the growth factor. Crispim et al. 2017 showed immobilization of TGF-β1 on polycaprolactone (PCL) using a functionalization process for a growth factor binding peptide to the PCL, resulting in presentation of the targeted growth factor to the cells inducing a cellular response.[10]

Therefore, the purpose of the present study is 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 ECM production of meniscus cells and MSCs. We hypothesized these growth factors and PL can accelerate meniscus regeneration by targeting the mechanisms mentioned above. Additionally, we hypothesized that by functionalizing the Collagen Meniscus Implant (CMI®; Stryker, Michigan, USA) with a growth factor binding peptide, a continued effect of the targeted growth factor could be achieved.

Materials and methods

Donors and cell isolation

Human meniscus cells were isolated from redundant degenerated 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. [12,17] 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^{0}C overnight. Meniscus cells were expanded in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% pen/strep. Meniscus cells were expanded and used at passage 2.

MSCs were isolated from bone marrow biopsies from the iliac crest during total hip replacement after written informed consent was obtained (Medical Ethical Committee, University Medical Center Utrecht), and characterized as described previously. [21] They were expanded in a-MEM supplemented with 10% FBS (HyClone), 0.2 mM l-ascorbic acid-2-phosphate (2% ASAP, Sigma-Aldrich), and 1% pen/strep and cultured upon use at passage 3.

Whole blood for platelet rich plasma (PRP) and platelet lysate (PL) was obtained from anonymous donors at the in hospital mini donor service of the University Medical Center Utrecht.

Different growth factors and Platelet lysate medium

Growth factors 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% human serum albumin (HSA; Sanquin), 2% ASAP, 2% Insulin-Transferrin-Selenium-X (ITSX, Invitrogen), and 1% pen/strep.

Whole blood with anticoagulant (Na3Citrate) was received from donors at the University Medical Center Utrecht, and centrifuged at 250 G for 10 minutes. The top layer, consisting of the plasma, was centrifuged at 750 G for an additional 10 minutes, the supernatant was collected, and the pellet was suspended in 1/3 of the supernatant, creating platelet rich plasma (PRP). Platelet lysate was formed by freeze thawing the suspension for 3 cycles (-80 ^{0}C to 37 ^{0}C) and afterwards centrifuged at 8000 G for 10 minutes. Upon use, the platelet lysate was diluted at 1% and 10% in DMEM supplemented with 2% HSA, 2% ASAP, 2% ITSX, 1% pen/strep and 3.3 U/ml heparin.

Micro-wound assay

Both meniscus cells and MSCs (n=3) were seeded in monolayer and expanded up to 80% confluency in a 12-wells 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 of PBS. Growth factor

and PL were dissolved in different concentrations in the medium (as mentioned above) with 10µM 5-ethylnyl-2'-deoxyuridine (EdU; Click-iT™ EdU Alexa Fluor® 488 Imaging Kit; Invitrogen) and added to the wells. At t=0, t=24, and t=48 six pictures were taken along the micro-wound using an inverted light microscope. Using Photoshop CS6 software (Adobe Systems), the pictures were automatically 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 t=0 image.

After 48 hours of culturing, 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% HSA, 2% ASAP, 2% ITSX, and 1% pen/strep in a concentration of 500.000 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 wells plate. 450 µL of growth factor, PL, or control medium was added to the wells of the 24 wells plate. The plates were incubated for 4 hours at 37^0C, 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 membrane was cut out of the insert, mounted on a microscope slide, and migrated cells were counted using an upright light microscope.

Pellet culture in fibrin glue

Meniscus cells were trypsinized, dissolved in 50 µL diluted fibrinogen (Tisseel, Baxter international Inc., IL USA, 1:15 in PBS) and combined with 50 µL diluted thrombin (Tisseel, Baxter international Inc., IL USA, 1:50 in PBS) in a 96 wells plate.[1] The fibrin constructs consisting of 250.000 cells were crosslinked for 15 minutes at 37^0C. Afterwards, the constructs were put in a 48 wells plate with 250 µL growth factor, PL, or control medium. The cell-seeded fibrin constructs were cultured for 28 days at 37°C with 5% CO_2, medium was changed 3 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 and KGSWWSSSH were synthesized following Fmoc solid peptide synthesis procedures, 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 [32].

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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 protein, 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 used for cell culture or blocked for one hour with PBS containing 1% (w/v) bovine serum albumin (BSA) followed by the same washing steps for imaging. Next, the CMIs were incubated with a primary antibody (2 µg/mL; rabbit polyclonal anti-human VEGF, PeproTech) in the blocking solution

during 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 scanning electron 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 centrifuged from whole blood described earlier was used to coat the CMI. First, 40μL of PRP was added to the CMI slices, continuously 20μL of $CaCl_2$ and 20μL of thrombin (Tisseel, Baxter) were added to the CMI the constructs were incubated for 15 min in 37°C to solidify the PRP gels in the CMI structures.

Cell migration into the functionalized Collagen Meniscus Implant

For both meniscus cells and MSCs, fibrin glue constructs with 250.000 cells were formed, which were attached to outer rim of the CMI functionalized for VEGF, the CMI functionalized with the scrambled peptide, a non-treated CMI which were all incubated with 1 μg/mL of VEGF (PeproTech) in PBST 0.5% for one hour and a CMI coated with PRP. After trypsinization of the cells and before creating the constructs, both cell types were stained by incubating them for 1 hour at 37^0C in 1mmol Vibrant CM-DiI. The cells were dissolved in fibrinogen 1:15 diluted in PBS and 50 μL was added to a 96 wells plate. Afterwards 50 μL of thrombin, 1:50 diluted in PBS was added, and the constructs were crosslinked in the incubator in 15 minutes at 37^0C. Subsequently the constructs were taken out with a spoon, transferred onto the CMIs in a 48 wells plate and 1 mL of differentiation medium was added. The constructs were cultured for 7 days, and medium was changed every other day. After 7 days of culture the samples were harvested, the CMI was counterstained with DAPI for 4 minutes and a tile scan with the confocal microscope was taken at 20x magnification with an area of 5.2 mm^2 and 50μm Z-stack. 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. Afterwards the samples were digested in papain and picogreen assay was performed to quantify the amount of cells inside of the CMI.

Biochemical analyses

Biochemical analyses were performed for both pellet culture in fibrin glue and the migration assay into the functionalized CMI. 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). Picogreen assay was used according to the manufacturer's instructions to determine the DNA content of the constructs. Excitation and emission were set at 480nm and 520nm respectively 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 for determining the GAG content with an absorption rate set at 525 nm and 595 nm.
Papain samples were both freeze-dried and hydrolyzed overnight at 108°C for determining the collagen content using hydroxyproline assay. Chloramine-T (Merck) and Dimethylaminobenzoaldehyde (Merck 3058) was added, and hydroxyproline (Merck 104506.0010) was used as standard to measure the hydroxyproline content at 570 nm. The final collagen content was calculated from the hydroxyproline, since 13.5% of collagen is composed of hydroxyproline.[41]

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 scramble 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.

Results

Increased migration of meniscus cells and MSCs using PDGF and Platelet Lysate

Micro-wound

The micro-wound filling by meniscus cells for three different concentrations (10.0, 1.0, and 0.1 ng/mL) of growth factors and two concentrations of PL (10% and 1%) were compared at t=24 and t=48. At t=24 only the wound filling in the 10% PL conditions was significantly higher ($p<0.0001$) compared to the control group (data not shown). However, at t=48, 1% PL and 1 ng/mL PDGF showed a significantly increased wound filling with a covered area of 36.7% (SD 16.1) and 31.1% (SD 29.2) respectively (Fig. 1A). VEGF and FGF also increased scratch filling at concentrations of 1 ng/ml (wound filling of 21.1% (SD 4.6) and 22.1% (SD 10.9)), however these differences were not statistically significant (Fig. 1A). In general, at a concentration of 1 ng/ml the presence of growth factors led to an increase in wound filling, which was more compared to the other concentrations at 48 hours (data not shown). Therefore all other experiments were continued with a growth factor concentration of 1 ng/mL and 1% PL.

In general, MSCs showed increased wound filling compared to the meniscus cells. 1% PL gave a significantly increased wound filling compared to the control (57.2% (SD 21.6) compared to 24.0% (SD 2.7)) at t=24. At t=48, both 1% PL and PDGF showed an increased filling of the scratch with 79.7% (SD 8.8, $p<0.001$) and 62.9% (SD 6.4, $p< 0.01$) respectively, compared to 41.5% (SD 1.6%) for the control. However, TGF-β1 showed a decreased wound filling compared to the control at 48 hours, showing only 15.7% (SD 7.3, $p<0.05$) coverage of the scratch. Other GFs showed no increase or decrease in the wound filling (Fig. 1B).

Transwell

PL significantly increased migration of both meniscus cells and MSCs in the transwell migration assay. For meniscus cells 1% PL increased the amount of migrated cells from 12 (SD 9) to 111 (SD 46), with a p-value <0.01 (Fig. 1C). For MSCs the amount of migrated cells in the control group was 10 (SD 4) compared to 346 (SD 137) in the 1% PL group (p-value <0.01) (Fig. 1D). PDGF and FGF showed a slight increase in meniscus cell migration, without a significant difference. This was the same for VEGF and FGF in MSCs.

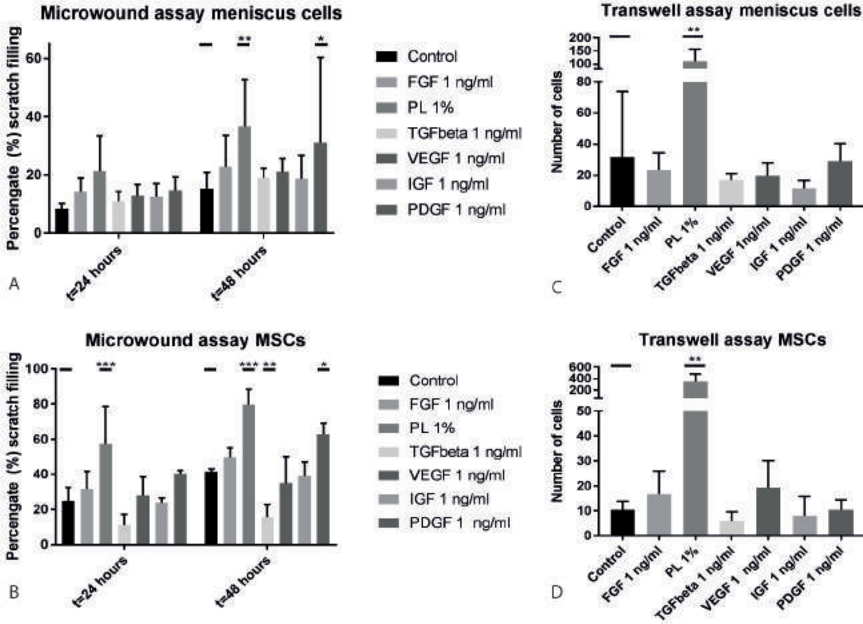

Figure 1: Migration of meniscus cells and mesenchymal stroma cells (MSCs) in the microwound assay (A and B) and the transwell assay (C and D) using fibroblast growth factor (FGF), platelet lysate (PL), transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and platelet-derived growth factor (PDGF). No significant difference was shown in scratch filling after 24 hours for meniscus cells, and after 48 hours migration in PL ($p<0.001$) and PDGF ($p<0.05$) medium was significantly higher than the control (A). MSCs after 24 and 48 hours in PL medium showed a significantly increased scratch filling ($p<0.001$) and PDGF showed an increase of migration after 48 hours ($p<0.05$) (B). Besides, TGF-β1medium resulted in a significant decrease in scratch filling compared to the control after 48 hours ($p<0.01$) (B). Both meniscus cells (C) and MSCs (D) showed a significant increase ($p< 0.01$) in migration through the transwell membrane after 4 hours using PL medium.

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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 could be calculated (Fig. 2A and B). For meniscus cells, the control group showed a ratio of 0.41 (SD 0.14), which was significantly lower than the 0.71 (SD 0.14) ratio of PL (p-value 0.0095) and 0.68 (SD 14) in TGF-β1 (p-value 0.0213). An increase in proliferation was also demonstrated for PDGF (0.54, SD 0.19), but this was not significant compared to the control group (p-value 0.4151) (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).

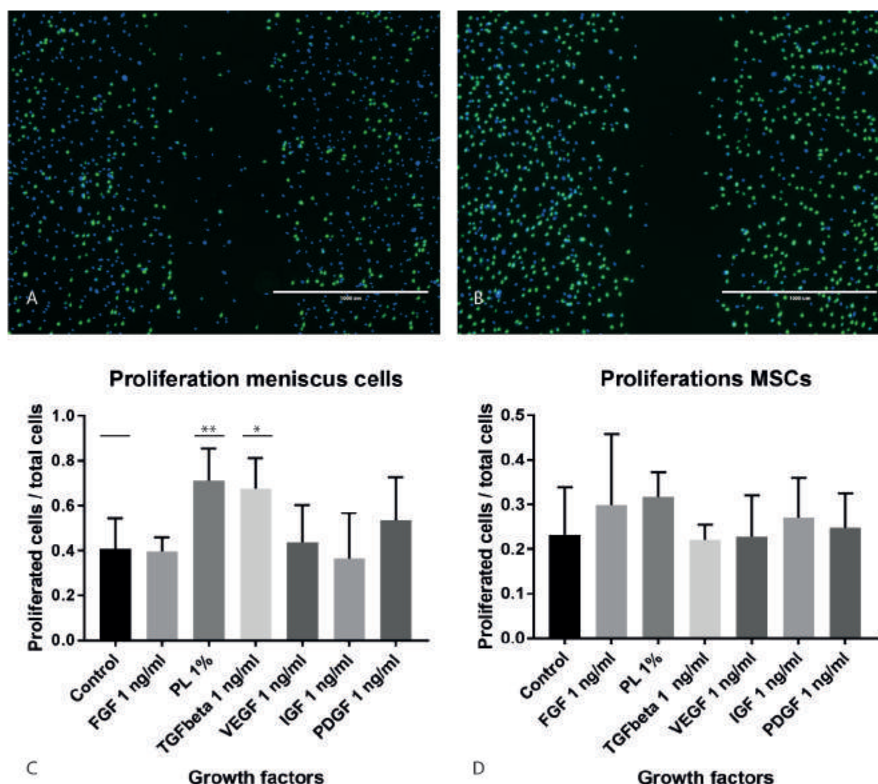

Figure 2: Proliferation of meniscus cells and mesenchymal stromal cells (MSCs) using different growth factors in the micro wound assay, demonstrated by 5-ethynyl-2'-deoxyuridine (EdU) assay. Proliferated cells shown in green by the EdU staining and the non-proliferated cells are stained blue using Hoechst. The control sample (A) compared to the proliferation in TGF-β1 medium after 48 hours (B). Significant differences in proliferation for meniscus cells were detected in PL (p= 0.0095) and TGF-β1 (p= 0.0213) as shown in figure C. Figure D showed no significant proliferations ratios for MSCs.

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). Only TGF-β1 significantly increased formation of GAGs in the fibrin glue constructs (p <0.0001) (Fig. 3B). There was no significant effect of any of the GF or PL on the production of collagen (Fig. 3C).

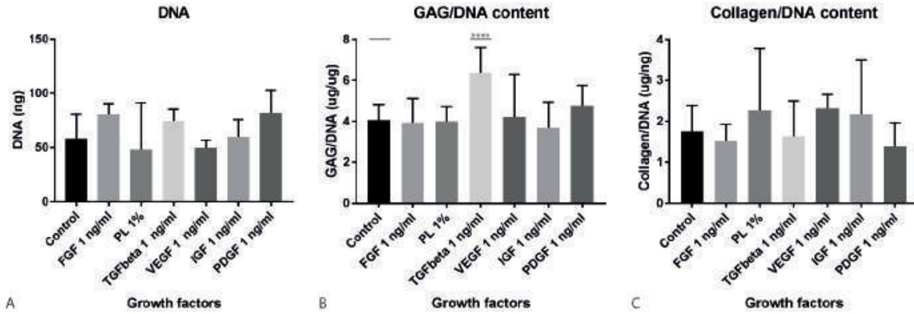

Figure 3: Biochemical analysis after 28 days of culturing meniscus cells in fibrin glue constructs adding different growth factors and PL in the medium. No significant difference was shown for DNA content in the samples cultured with the different growth factors (A). TGF-β1 demonstrated a significant ($p<0.0001$) increase in glycosaminoglycans (GAGs) production corrected for DNA compared to the control (B). For collagen production per DNA there were no significant differences shown (C).

Functionalization of the Collagen Meniscus Implant increases the cell ingrowth

The CMI was successfully functionalized with VEGF binding peptide. Figure 4 shows scanning electron microscopy images of the 5 different groups of functionalized CMI, where the VEGF bound to the CMI was fluorescently labeled. The CMI functionalized with the VEGF binding peptide (Fig. 4F) showed significantly ($p<0.001$) higher fluorescence intensity units compared to the 4 other groups (Fig. 4B – E).

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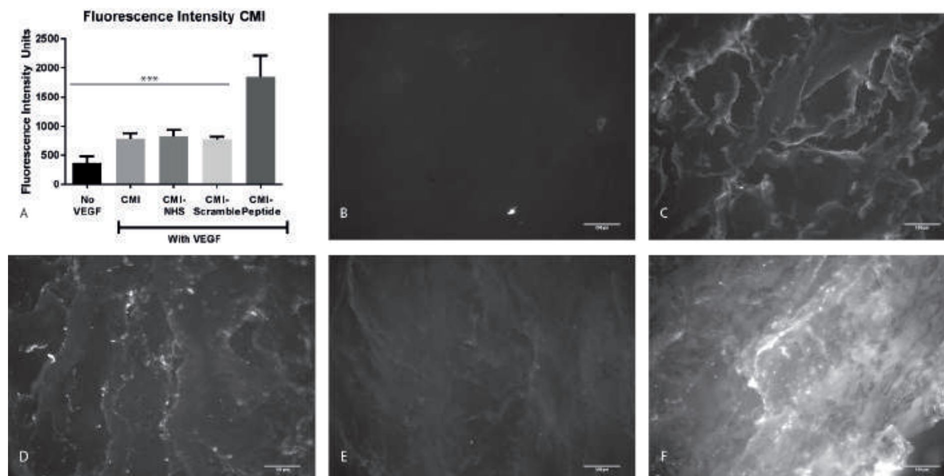


Figure 4: Immobilization of vascular endothelial growth factor (VEGF) on functionalized Collagen Meniscus Implants (CMI) using a peptide binding protein for VEGF (n=8). Visualization of the VEGF with fluorescence microscopy and measuring the intensity per picture, quantified using ImageJ, showed a significant ($p< 0.001$) higher amount of immobilized VEGF in the CMIs functionalized for the VEGF growth factor binding peptide compared to the controls (A). The controls were CMI with MES buffer and without VEGF (B), CMI with MES buffer and with VEGF (C), CMI with EDC/NHS, MES buffer and VEGF (D), CMI with EDC/NHS, a scramble VEGF peptide and VEGF (E), and CMI with EDC/NHS, VEGF binding peptide and VEGF (F).

After processing and analyzing the confocal images of the seven-day migration assay with meniscus cells in fibrin glue constructs attached to the CMI, there were significantly more cells present in the CMIs functionalized for VEGF and coated with PRP. 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 (Fig. 5B), compared to round cells not aligned along the fibers in the scrambled and negative control group (Fig. 5C and 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 were shown in Fig. 5E and 6E respectively. A significant difference was 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 experiments. There was slightly higher density of MSCs in the

PRP-functionalized group compared to VEGF-functionalized group; however this was not statistically significant.

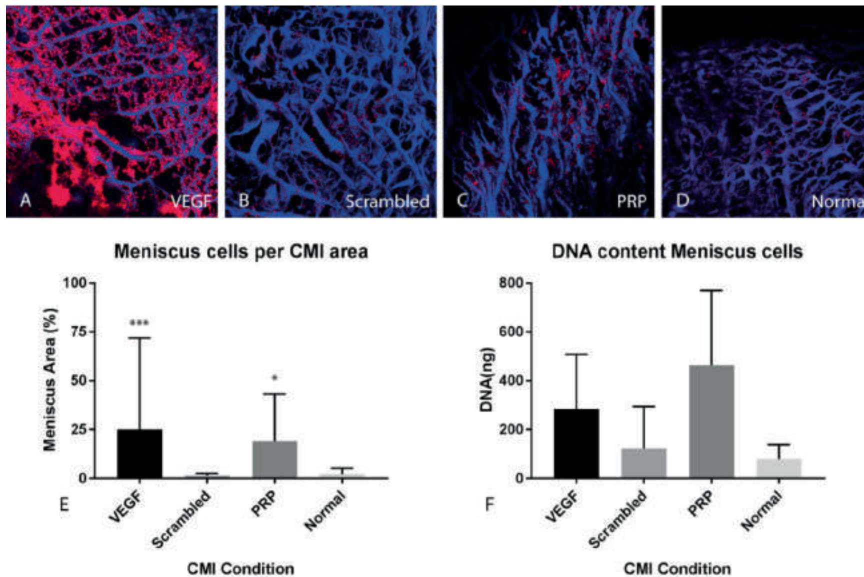


Figure 5: Cell migration of meniscus cells into the Collagen Meniscus Implant (CMI) (n=6). The CMI is stained with DAPI (blue) and meniscus cells with DiI (red) (A – D). Figure E corresponds with A – D, and showed significant more cells in the CMI functionalized for VEGF ($p< 0.001$) and the PRP group ($p< 0.05$). Quantifying the DNA in the whole constructs showed more cells in the VEGF and PRP group, however this was not statistically significant (F).

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). However these differences were not statistically significant. CMI constructs with VEGF-functionalization and PRP-coated CMI constructs without cells were tested for initial DNA background value. The values were negligible when comparing to other values of CMI containing cells (data not shown).

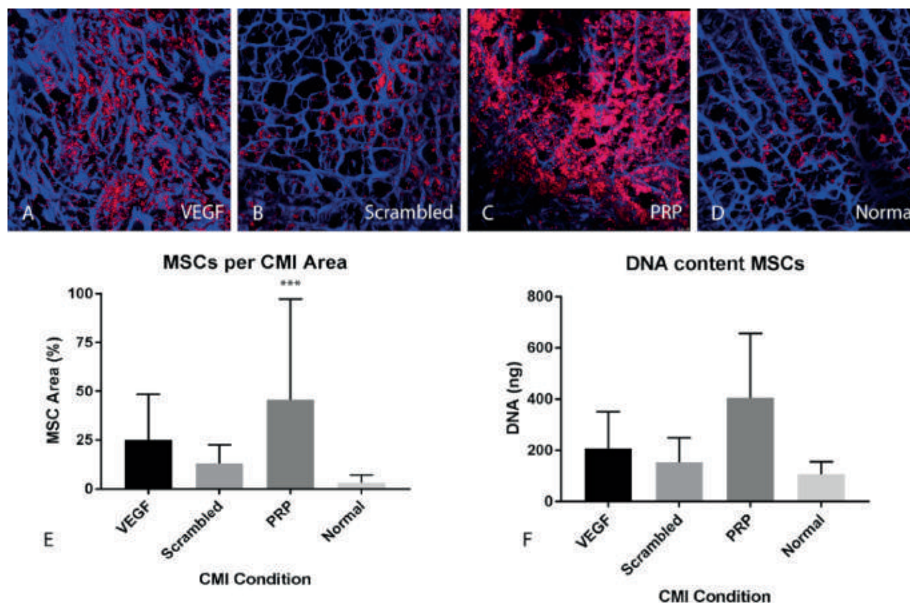


Figure 6: Cell migration of mesenchymal stromal cells (MSCs) into the Collagen Meniscus Implant (CMI) (n=6). The CMI is stained with DAPI (blue) and MSCs with DiI (red) (A – D). Figure E corresponds with A – D, and showed significant more cells in the CMI functionalized with PRP ($p< 0.001$). Quantifying the DNA in the whole constructs showed more cells in the PRP group, however this was not statistically significant (F).

Discussion

This *in vitro* study showed the effect of IGF-1, PDGF, FGF, TGF-β1, VEGF and Platelet Lysate on migration, proliferation and ECM production of meniscus cells and MSCs. Additionally, the feasibility of functionalizing the Collagen Meniscus Implant with a growth factor binding peptide for VEGF or coating with PRP was explored to potentially translate these *in vitro* results to a clinical application. This study demonstrated that migration of meniscus cells and MSCs was increased by both PL and PDGF, and proliferation of meniscus cells was enhanced by PL and TGF-β1. TGF-β1 also increased the GAG production of meniscus cells after 4 weeks of culture. However, none of the growth factors had a stimulating effect on the proliferation of MSCs. Cell ingrowth of both meniscus cells and MSCs into either the CMI functionalized for VEGF and the CMI coated with PRP resulted in a higher cell density inside of the CMI after seven days of

culture compared to the negative control, which indicates we can attract endogenous growth factors present in the knee joint without injecting additional growth factors.

The choice of growth factors and concentrations was based on previous literature on meniscus cells, cartilage and chondrocytes.[5,9,18,43] Recently, PL and PRP are gaining increasingly more attention, due to the positive effect on tissue regeneration.[8,26,30] As a wide range of dose-dependent concentrations of growth factors is given in literature,[5,25] we first performed a pilot study with concentrations of 0.1 ng/mL, 1 ng/mL, and 10 ng/mL for all different growth factors, and concentrations 0.1%, 1%, and 10% for PL. There were no significant differences between 1 ng/mL and 10 ng/mL, but both showed an enhanced effect compared to 0.1 ng/ml. As overdosing with growth factors is a general concern for regenerative therapies,[27] the concentration of 1 ng/mL for all the growth factors and 1% PL was chosen for the subsequent *in vitro* experiments (supplemental data).

We found a positive effect of TGF-β1 on the ECM production and proliferation of meniscus cells. This is in contrast with the findings of Riera et al., who reported that TGF-β1 had no significant effect on proliferation of meniscus cells.[44] The effect of TGF-β1 should be further elucidated, especially since it decreased wound filling by MSCs in our experiments. TGF-β1 is however used for chondrogenic differentiation of MSCs; therefore it could be possible that an increase in differentiation lead to a decrease in proliferation. In addition, the experiments by Riera et al. were performed with pig cells, which might respond different to human recombinant TGF compared to human cells.[44]
VEGF is an important angiogenic growth factor that has been hypothesized to increase microvasculature in the avascular zone, hereby potentiating repair. However, results of previously published research are inconsistent and the role of VEGF remains to be elucidated.[16,43,56] In our study, VEGF did not have a positive effect on scratch filling, migration or proliferation of either meniscus cells or MSCs.
FGF is a powerful mitogen in chondrogenesis. It plays an important role in the positive effect of co-cultures of meniscus cells and MSCs on proliferation and collagen synthesis, as this could be counteracted by eliminating FGF.[49] However,

we found no statistically significant increase of proliferation or collagen production of meniscus cells by adding FGF to the medium. The effect on co-cultures could be different than what our results suggest, as it is hypothesized that MSCs and meniscus cells communicate via the release of a pool of cytokines and growth factors and not just through one single growth factor.
IGF-1 is an essential anabolic growth factor in cartilage tissue, but its effect is highly concentration dependent.[46] However, in this study we found no effect on migration or proliferation of MSCs or meniscus cells. Moreover, we demonstrated that IGF-1 in the concentration of 1 ng/ml had no effects on collagen or proteoglycan production.

Crispim et al. 2017 showed good results for functionalization of PCL and this study demonstrated the possibility of functionalizing the Collagen Meniscus Implant with growth factor binding peptides to attract endogenous growth factors present in the knee joint.[10] Peptides for VEGF binding were used to demonstrate the variety of growth factors which can be used for functionalization. The migration assays exhibited the best results for PDGF amongst the single growth factors. PDGF has three different isoforms (AA, AB and BB) affecting different PDGF receptors,[31] which makes it more difficult to use PDGF in this proof-of-principle study. Since PDGF and VEGF family members are closely related, functionalization with a VEGF capturing peptide was chosen for this proof-of-principle study.[2]

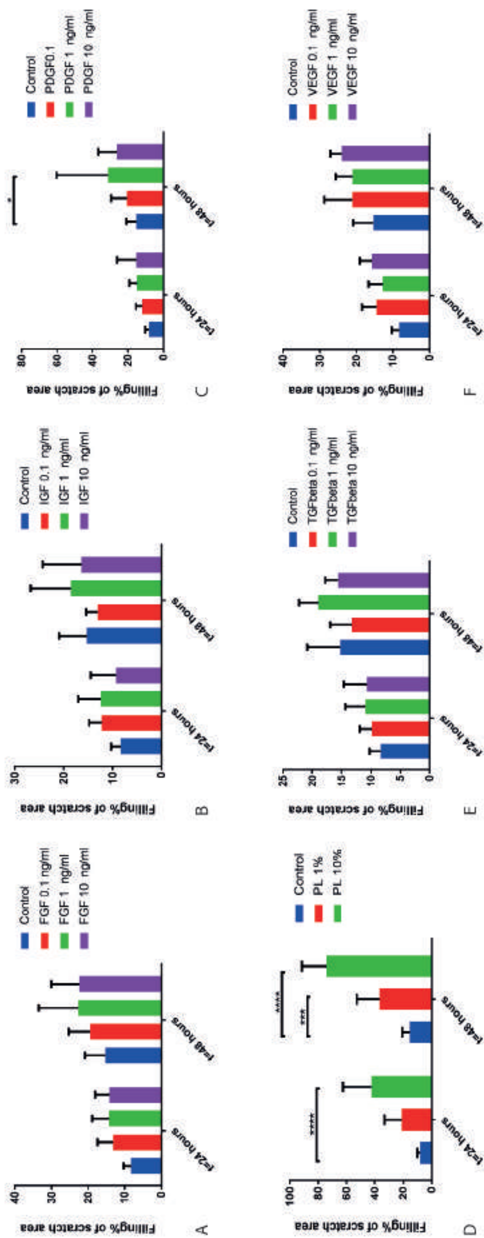
In addition, PRP was included in the same assay of migration of cells into the CMI as PL showed increased migration and micro-wound repair. An increase in cell migration, proliferation and ECM production is seen in the *in vitro* cultures of this study with meniscus cells and MSCs using PL. Migration of cells into the CMI while coating it with PRP gel was increased within 7 days of culture. Currently, clinical trials using PRP injections for early cartilage degeneration to delay the placement of a total knee arthroplasty show good results so far, and is implemented as experimental treatment. PRP can be made from autologous peripheral blood avoiding the possibilities of an immune reaction. However, PRP can lead to formation of unstructured and more fibrous ECM [20] and the study of Zellner et al[55] showed no effect of PRP on regeneration in a punch defect of a meniscus using a hyaluronan collagen composite matrix. Nevertheless, there is no consensus and

standardization in the preparation, storage and application method of PRP, which might explain the opposite results found for PRP treatment.[6,40] Overall it is considered a safe and easy-to-use autologous minimally invasive and minimally manipulated cell product with high regenerative potential. Also this study showed that injecting CMI with PRP might enhance cell ingrowth and new tissue formation, opening possibilities towards new approaches with PRP.

There are limitations of the current study design. Only the effect of single growth factors was examined in this study, but a combination of multiple growth factors could be beneficial, targeting migration, proliferation and ECM production at the same time by different growth factors. In addition, a combination of growth factors could work as a catalyst or simultaneously. In a study by Hoben et al,[24] adding PDGF to TGF-β1 led to a 3-fold increase in collagen production compared to the use of TGF-β1 alone. Functionalization of the CMI with multiple growth factor binging peptides is also an option, and therefore combinations of two or more growth factors should be further investigated.

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 protein and PRP which led to increased meniscus cell and MSC migration into the meniscus implant. Therefore, the results of this study provide the possibility that functionalizing the CMI with growth factor binding peptides could enhance meniscus regeneration after partial meniscectomy.

Supplemental data



Supplemental data: Scratch filling after 24 and 48 hours of the microwound assay using different concentrations of fibroblast growth factor (FGF; A) insulin-like growth factor-1 (IGF-1; B), platelet-derived growth factor (PDGF; C), platelet lysate (PL; D), transforming growth factor beta 1 (TGF-β1; E), and vascular endothelial growth factor (VEGF). For the growth factors concentrations of 0.1 ng/ml, 1 ng/ml and 10 ng/ml, and for PL 1% and 10% were used. * $p< 0.05$, *** $p< 0.001$, **** $p< 0.0001$

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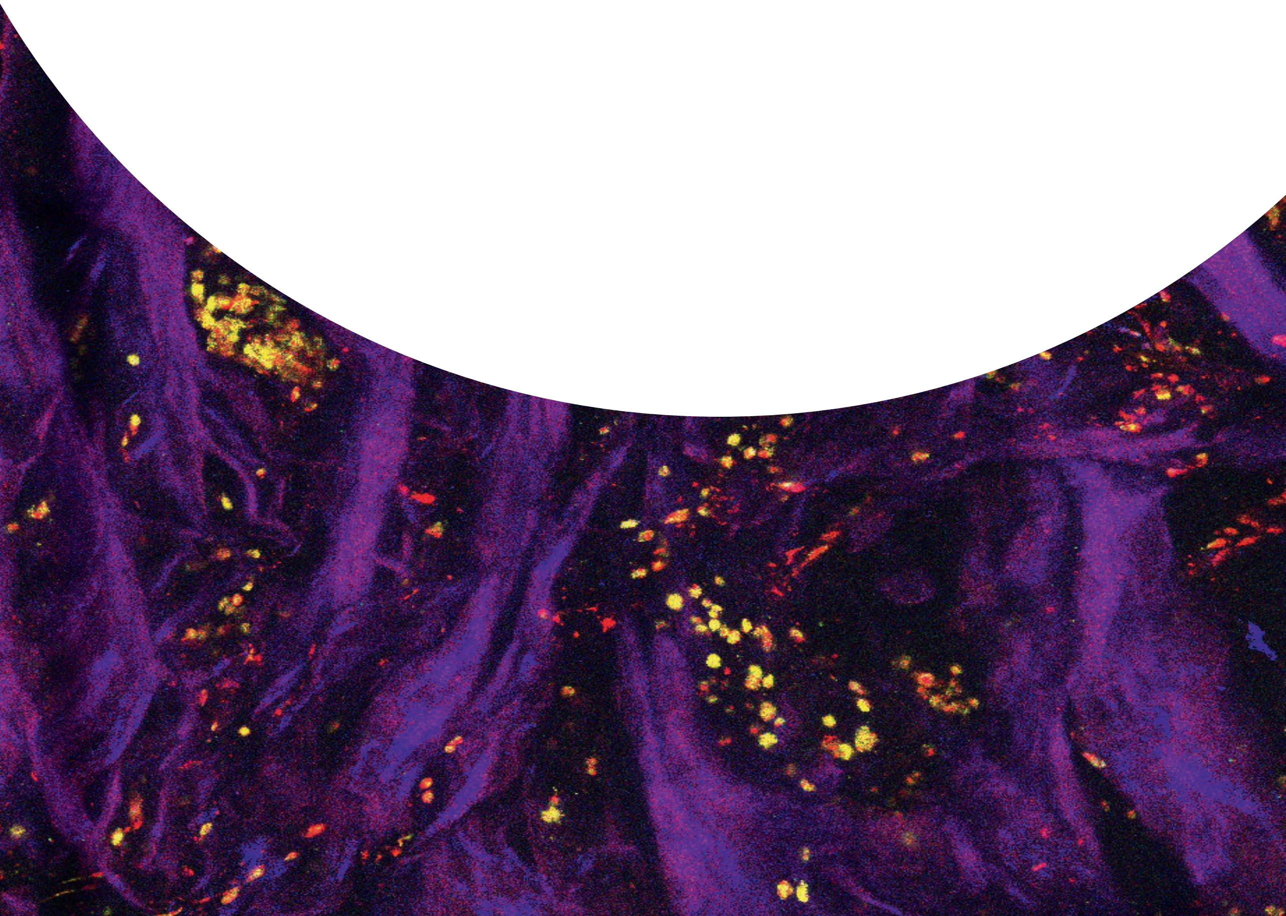
Part III: Development and Validation of a new PROM for Sports Related Knee Injuries

Chapter 7

Development and Validation of the Patient Approved Knee Assessment for Sport Related Knee Injuries



Chapter 7

Development and Validation of the Patient Approved Knee Assessment for Sport Related Knee Injuries

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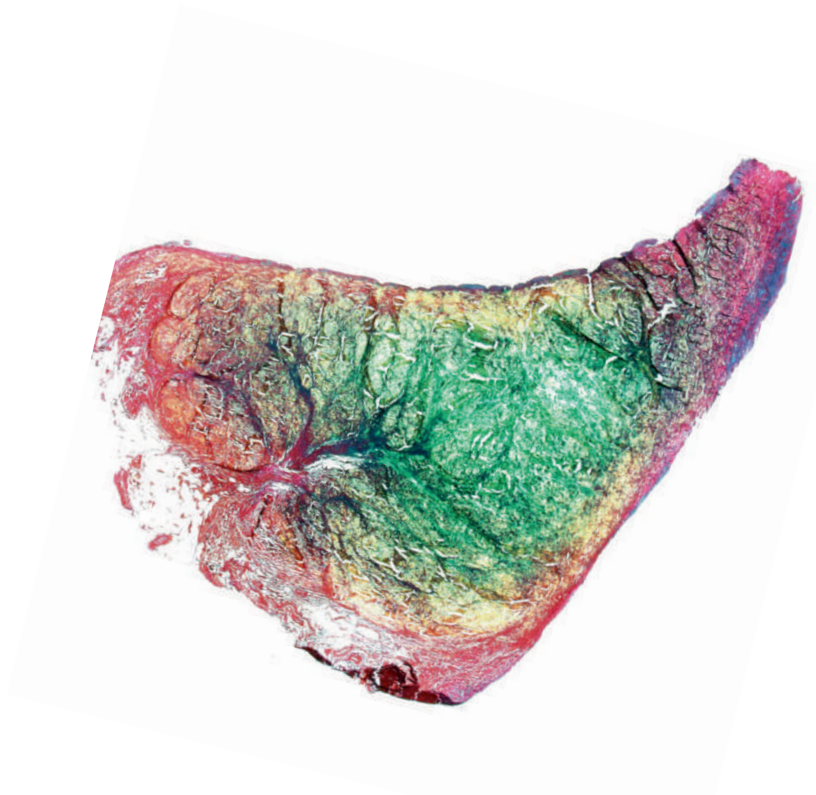

Abstract

Background: Patient reported outcome measures (PROMs) are widely used in the field of orthopedic surgery to measure clinical outcomes after treatment. However, patients are often not involved in the development of these questionnaires and doctors tend to misinterpret symptoms and side effects relevant for patients.

Purpose: To develop and validate a new PROM, the Patient Approved Knee Assessment (PAKA), for patients with sports related knee injury. The development will be in collaboration with patients in this particular injury category to incorporate patient opinions on what is important in outcomes after surgery.

Methods: For the development of the PAKA, patients from a single center database for knee cartilage repair scored all questions used in existing PROMs for relevance using a visual analogue scale (VAS). They also reported if there were questions missing that were relevant to determining their function. The PAKA was developed by selecting questions with a VAS score higher than 9 combined with questions reported as desirable by patients. The new questionnaire was sent out to the patients again to measure their satisfaction. For validation, patients with sports related injury of the knee were included from 2 hospitals. They were asked to fill out the PAKA and other existing PROMs with a 2-day interval in-between. The data was used to determine the internal consistency, reliability and construct validity of the PAKA.

Results: A total of 319 patients evaluated existing PROMs. The 15 best scoring questions were combined to create the PAKA. The PAKA was evaluated by 119 of the 319 patients (37.3%) and the overall satisfaction rate was 94.2%. A total of 49 patients were included for the validation, and 29 for the test-retest reliability. The internal consistency of the subdomains and total score, using the Cronbach's alpha coefficient, ranged from 0.852 – 0.962. Test-retest reliability, measured by the intraclass correlation coefficient, ranged from 0.922 – 0.976, and the construct validity was demonstrated by a Spearman's rank correlation coefficient, ranging from 0.36 – 0.81.

Conclusion: This study presented the development and validation of the Dutch version of the PAKA for patients with sports related knee injury. The PAKA showed an excellent satisfaction in patients with sports related injuries of the knee. It generated excellent internal consistency, excellent test-retest reliability, and strong construct validity. Therefore, the PAKA can be used in clinical practice to increase patient compliance in follow-up and to improve the quality of clinical outcome measures in patient centered care.

Keywords: Sports related knee injuries; Patient reported outcome measures (PROM); Patient centered care; PAKA; Validation; Reliability

Introduction

In the modern healthcare system, patient-centered care is the gold standard, and patients want to be involved in the decision-making regarding treatment strategy.[18] Shared decision-making and patient engagement is growing due to the availability of information on the Internet and changes in society where individuals increasingly desire an active role in the fate of their own lives.[2,10,13] In orthopedic surgery, many of the surgical treatments are elective, based on reducing symptoms such as pain and restricted range of motion to eventually improve the quality of life (QoL).[4] Therefore, shared decision-making and patient-centered care are very important in this medical specialty.

In addition to decision-making, outcome measurements need to be patient-centered as well, to determine the success after surgery.[20] Radiography and Magnetic Resonance Imaging (MRI) are often used as outcome measurements after surgery, however, there is no conclusive evidence on whether these objective outcome measures correlate with the clinical results.[19,27] Therefore, Patient Reported Outcome Measures (PROMs) have been increasingly adopted into the field of orthopedic surgery, to include patients' opinions and measure subjective outcomes after surgery.[21] Numerous different PROMs are used in the orthopedic specialty, varying from general health to disease-specific PROMs.[16,21,22] This is a good step forward in patient-centered care, however, most PROMs are developed by healthcare professionals without the engagement or approval of patients.[7] The importance of involving patients in the development of PROMs is highlighted by studies proving that clinician assessment of patient in-hospital experience is not accurate.[6,8,25] For example, symptoms or concerns relevant to patient opinion are often not reported in medical or nursing records,[23,24] implying that patients should be involved in the development of a new PROM.

The purpose of this study was (1) to develop a new patient-centered PROM for sports related knee injuries, in collaboration with patients, the Patient Approved Knee Assessment (PAKA), to better understand patient's goals and important outcomes after sports related knee surgery, and (2) to validate this new PROM in Dutch to contribute to the ongoing movement towards patient-centered care and outcome measures. We hypothesize the PAKA to be a relatively short PROM containing only questions patients consider relevant, which will increase the

compliance and participation of follow-up questionnaires after sport related knee surgery.

Methods

Approval of the medical ethical committee of the University Medical Center Utrecht (UMCU) was obtained. The study adhered to all the tenets stated in the Declaration of Helsinki. The study was divided into three phases. In the inventory phase, patients ranked the categories and questions of existing PROMs for relevance to their situation as a knee patient and submitted any missing questions or categories. A new questionnaire, the PAKA, was created from the questions with highest relevance and the most frequently submitted questions. In the evaluation phase, patients were offered the opportunity to review and appraise the new questionnaire, and adjustments could be made when needed. In the validation phase, a different cohort of patients was asked to complete several existing PROMs and the PAKA. Statistical analysis was then performed to determine the validity of the new PAKA.

Inventory

Patients from a single center database for knee cartilage repair were contacted for participation in this study. Patients were approached by e-mail, and if no e-mail address was provided, patients were contacted by phone and asked for participation and functional e-mail address. Any patient not responding after three phone calls was excluded from participation.

Questions were compiled from seven already existing PROMs: the Lysholm Scoring Scale, the Knee injury and Osteoarthritis Outcome Score (KOOS), International Knee Documentation Committee (IKDC) Subjective Knee Form, Western Ontario & McMaster Universities Osteoarthritis Index (WOMAC), the Tegner Activity Level Scale, Hospital for Special Surgery Knee Score (HSS Knee Score), and Modified Cincinnati Rating System Questionnaire for the Knee. These were combined into one online survey (InterActive Studios, Rosmalen, the Netherlands), duplicate questions were removed, and all questions were accessible using a personal hyperlink sent to the participants by email.

In total, 45 different questions were divided among the following categories: Pain, Daily Functioning, and Symptoms. Participants scored every question and category for relevance to their situation using a slider on a Visual Analogue Scale (VAS) without numbers, ranging from completely irrelevant (0) to very relevant (10). Three separate free text-boxes were added to answer the following questions: “Were you able to express all your feelings and thoughts about your knee in this questionnaire?”, “Did you miss any categories?”, and “Did you miss any questions?”. Participants were asked to complete the questionnaire twice, to have a more objective outcome, with a two-day interval assuming the probability of the participants remembering their exact previous answers highly unlikely.

Evaluation

Participants who responded to the compiled ‘inventory’ questionnaire were asked to rate the newly developed PAKA via the online survey program. A personal hyperlink was sent to the participants, containing the evaluation questionnaire, which assessed length of the PAKA, completeness, patient satisfaction, and missing or redundant questions.

Validation

Between March and July 2016, a total of 120 patients received a letter from their surgeon asking for participation in the validation. Again, between April and July 2018, patients were contacted at the outpatient clinic at the University Medical Center Utrecht (UMCU) and Diakonessenhuis, Utrecht asking for informed consent to participate in the study. Inclusion criteria for the validation of the PAKA were: (1) age older than 18 years; (2) sport related knee surgery at the UMCU or the Diakonessenhuis within the past 12 months; (3) possession of a functional e-mail address. Sports-related knee surgeries included autologous chondrocyte implantation (ACI) or micro-fracturing to treat non-degenerative cartilage injury, removal of loose bodies, anterior and/or posterior cruciate ligament (ACL and PCL, respectively) reconstruction, patellar surgery, medial or lateral meniscus surgery, and other knee ligament procedures.

The participants received a personal hyperlink on ‘day 1’ (test), leading them to an online survey containing the SF-36, Lysholm, KOOS and the PAKA.

They received the same survey two days later (retest). Again, an interval of two days was chosen assuming the probability of the participants remembering their exact previous answers highly unlikely, while also assuming no significant change in symptomatology. Any non-responders were contacted by phone and asked for their participation.

Statistical analysis

Clinimetric qualities of the PAKA were analyzed using SPSS version 22.0 (IBM, New York, USA). Internal consistency was quantified using Cronbach's alpha and Bland and Altman plots. An alpha of 0.7 is deemed acceptable, whereas an alpha >0.8 represents good and >0.9 represents excellent internal consistency.[11] Internal consistency was reported for all subdomains and the total score.

The test-retest reliability for the total scores and separate subcategories was assessed with the intra-class correlation coefficient (ICC). For the ICC, a value of 0.40-0.59 represents fair reliability, 0.60-0.74 is deemed as good, and 0.75-1.00 is excellent test-retest reliability.[9]

Construct validity was measured using Spearman's rho, comparing the different categories and total score of the PAKA with corresponding subdomains of the Lysholm, KOOS and SF-36 questionnaires. Values for Spearman's rho of <0.35 were considered weak, 0.35 - 0.5 moderate, and >0.5 strong.[3]

Feasibility was assessed by checking for floor and ceiling effects, defined as at least 15% of the participants scoring within a 10% margin of the lowest or highest score possible for a subdomain. If floor or ceiling effects are present, it is likely extreme items are missing in the questionnaire.

Results

Inventory

The single center database contained 714 patients. After contact by e-mail or phone, 520 patients with a mean age of 37.05 years (range 16-66, SD±11.16) agreed to participate. Response rates for the test (day 1) and retest (day 3) were 50.8% (n=264) and 40.8% (n=212), respectively.

Of the 45 questions in the compiled questionnaire, any question scoring < 5% under the question category average (Pain 9.00, Daily Functioning 8.77 and Complaints & Symptoms 8.75) was included in the new questionnaire. Of the original 9 questions concerning Pain, 24 concerning Daily Functioning and 11 concerning Complaints and Symptoms, 7 (77.8%), 17 (70.8%) and 8 (72.7%) questions were used in the PAKA, respectively.

A total of 83/266 (31%) participants stated they felt question categories were missing in the compiled questionnaire. Frequently suggested new categories were: Quality of Life (QoL) and Mental Impact (n = 20), Sports and Work (n = 16) and Aftercare (n = 5). Categories for QoL and Sports and Work were added. A special category for aftercare was not added; this category was only submitted by patients demanding imaging of their knee, which, in clinical practice, is only granted after consulting a physician. Furthermore, a demand for MR scans does not quantify functioning after surgery.

Of the 266, 79 patients (29.7%) stated that they felt single questions were missing in the compiled questionnaire. Questions submitted more than 5% and thus included in the PAKA (supplemental data 1).

Evaluation

The response rate was 122 out of 319 (38.2%). Average rating of the PAKA was 7.8/10. 94.2% of patients were satisfied with the PAKA. 97.6% felt the PAKA contained no redundant questions. 91.1% of patients felt the number and length of questions in the PAKA was adequate. 85.7% of patients stated the PAKA addresses all of their thoughts and feelings concerning their knee. 85.5% of patients stated no questions were missing. Six patients reported missing an open field for additional notes, which was added to the PAKA after this evaluation (Figure 1). (See supplemental data 2 for the complete Dutch version of the PAKA).

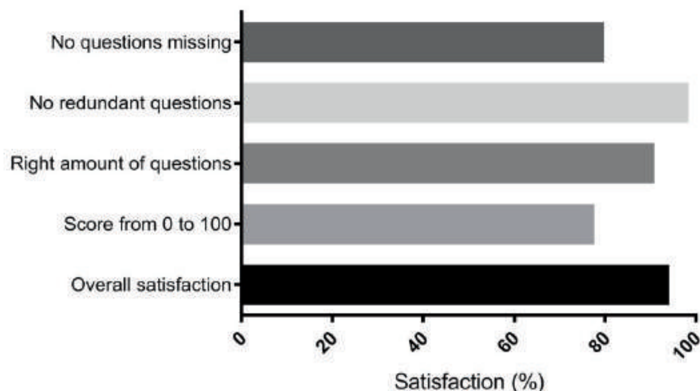

Figure 1; The satisfaction rate of the developed Patient Approved Knee Assessment (PAKA) after feedback from patients was included.

Validation

<u>Subjects</u>

Out of the 142 initially contacted patients, 72 (50.1%) were included for validation, of which 49 (34.5%) completed at least one of the two sets of questionnaires. Mean age was 36.2 years (range 18 – 62) and 23 (47%) patients were male (Table 1). Both pre-operative and post-operative patients were included in this study. The number and type of injury for each patient, together with the surgery they underwent, are reported in table 1. Twenty patients did not complete the second set of questionnaires in time. The mean test-retest time was 6.3 days (range 3 – 45 days) for all patients who completed both the test and retest. After removal of patients who were ineligible for test-retest evaluation, the mean test-retest time was 2.1 days. This resulted in a total of 29 inclusions available for test retest reliability.

<u>Internal consistency</u>

Cronbach's alpha for the PAKA subdomains ranged from 0.854 to 0.951, and for the total score an internal consistency of 0.962 was reported. This indicated a good to excellent internal consistency of the PAKA (Table 2).

Table 1; Patient demographics	**N = 49**
Mean age (years)	36.2 (18 – 62)
Sex	
Male	23 (47%)
Female	26 (53%)
Number of injuries	
1	40 (82%)
2	7 (14%)
≥3	2 (4%)
Injury	
Anterior cruciate ligament injury	11 (22%)
Meniscal tear	12 (24%)
(Osteo)chondral defect	
Medial femoral condyle	13 (27%)
Lateral femoral condyle	7 (14%)
Patella/trochlea	7 (14%)
Posterior cruciate ligament injury	1 (2%)
Patellar subluxation	2 (4%)
Collateral ligament injury	0 (0%)
Surgery	
Anterior cruciate ligament reconstruction	11 (22%)
Meniscectomy	11 (22%)
Meniscal repair	1 (2%)
Cartilage repair	
Autologous chondrocyte	13 (27%)
implantation	9 (18%)
Microfracture	1 (2%)
MaioRegen	
Posterior cruciate ligament reconstruction	1 (2%)
Tibial tuberosity transposition	2 (4%)

Table 2; Internal consistency	
PAKA subdomain	**Cronbach's alpha coefficient**
Pain	0.951
Function	0.913
Work and Sport	0.912
Quality of life	0.854
Total score	0.962

PAKA = Patient approved knee assessment

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Test-retest reliability

The ICC of the PAKA was 0.968 (95% IC 0.937 – 0.983) for the total score and ranged between 0.922 to 0.976 for the subdomains, indicating excellent test-retest reliability (Figure 2 and Table 3).

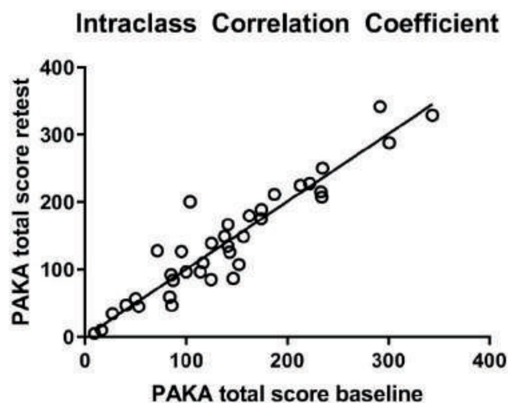

Figure 2; Test-retest reliability was determined by the intraclass correlation coefficient (ICC). The different outcomes per patient for the test and retest of the total score were plotted, resulting in an ICC of 0.968 (as seen in Table 3).

Table 3; Test-retest reliability	
PAKA subdomain	**ICC (95% CI)**
Pain	0.976 (0953 – 0.990)
Function	0.930 (0.887 – 0.962)
Work and Sport	0.933 (0.895 – 0.961)
Quality of life	0.922 (0.873 – 0.956)
Total score	0.968 (0.937 – 0.983)

PAKA = Patient approved knee assessment; ICC = Intraclass correlation coefficient; CI = Confidence interval

Construct validity

Construct validity was measured by Spearman's rank correlation coefficient, comparing PAKA subdomains to the Lysholm and relevant subdomains from the KOOS and SF36. The construct validity varied between 0.36 (Function

and SF-36 Physical Functioning) and 0.81 (Pain compared to KOOS Pain) (Table 4). This indicates a strong construct validity for the PAKA.

Table 4; Construct validity

PAKA subdomain	Corresponding subdomain	Spearman's rank correlation coefficient
Pain	KOOS Pain	0.81 ***
	SF-36 Bodily Pain	0.72 ***
Function	KOOS Symptoms	0.57 ***
	KOOS Activities of daily living	0.66 ***
	SF-36 Physical Functioning	0.36 *
Work and Sport	KOOS sport and recreation	0.79***
	Lysholm total score	0.50**
Quality of life	KOOS quality of life	0.74***

PAKA = Patient approved knee assessment; KOOS = Knee injury and Osteoarthritis Outcome Score; SF-36 = Short Form Health Survey
* $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$

Floor and ceiling effects

Ceiling effects were absent for the PAKA. Only for the subdomain Work and Sport, a floor effect was detected. For the total score, no floor or ceiling effects were present (Table 5).

Table 5; Floor and ceiling effects

PAKA subdomains	Ceiling effect (% of patients with lowest score + 10%)	Floor effect (% of patients with highest score - 10%)
Pain	13.5%	0%
Function	13,5%	0%
Work and Sport	13.5%	19.1%*
Quality of life	9.1%	5.4%
Total score	*0%*	*0%*

PAKA = Patient approved knee assessment
* = Floor effect

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Discussion

PROMs are widely used in the field of orthopedic surgery to measure clinical outcomes after treatment. However, patients are often not involved in the development of these questionnaires and doctors tend to misinterpret symptoms and side effects relevant for patients. Therefore, this study presented the development and validation of a new PROM, the Patient Approved Knee Assessment (PAKA) for patients with sports related knee injuries. This PROM is developed in collaboration with patients in this particular injury category, allowing doctors to better understand the important outcome parameters after surgery from a patient perspective, and improving the patient compliance of follow-up questionnaires. Our hypothesis was confirmed that the PAKA will be a relatively short questionnaire with a high patient satisfaction on the quality and importance of this PROM. In addition, the PAKA showed an excellent internal consistency, an excellent test-retest reliability, a strong construct validity, and no floor and ceiling effect in the total score.

The excellent internal consistency was shown by high Cronbach's alpha scores. The PAKA is a short questionnaire and the excellent Cronbach's alpha scores might indicate there are still redundant questions present since all the questions in one category are scored equally by one patient at a given time point. However, all the questions were rated by patients with sports related knee injuries and scored as relevant. Therefore, the number of questions is good, and the excellent internal consistency increases the power of the clinical outcomes of the PAKA. In addition, the results for the Spearman's rank correlations support the presence of a good construct validity. Each subdomain of the PAKA showed a strong correlation with corresponding domains of other questionnaires, except for the PAKA subdomains Daily Functioning and Work and Sports, which only showed a moderate correlation with the SF-36 physical Functioning, and the Lysholm score, respectively. The KOOS subdomain sport and recreation showed a good correlation with the Lysholm score in other studies.[3,5] However, when comparing this subdomain of the KOOS to the Lysholm outcomes in our population, similar correlations are shown as for the PAKA Work and Sports subdomain compared

with the Lysholm. Therefore, the PAKA is not shown to be inferior to the KOOS for the subdomain sports (and work) in patients with sports related injury of the knee.

Since patient-centered care and clinical outcome measures are both increasing fields of interest within orthopedic surgery and research[1,10,15], the need for a questionnaire developed in collaboration with patients is growing. New treatments for patients with sports related knee injuries are developed rapidly, especially with the growing field of regenerative medicine.[12,26] To correctly evaluate clinical outcomes after new treatments and compare them to outcomes after existing surgical treatments or non-operative therapy, an instrument which can exactly measure the items important for recovery from a patient's perspective is needed. This questionnaire has proven to satisfy patients' need to report their outcomes after surgery. Besides, using one PROM in clinical practice and for all clinical studies makes it easier and more reliable to compare different treatments and different studies. This could contribute to better clinical practice in orthopedic surgery.

Several limitations of this study should be addressed. First, the population size of this study is relatively small. Only 50.1% of the patients approached for this study signed the informed consent form and could be included in the study. In addition, 20% did not fill out the retest, and another 20% did not fill out the retest within the mandatory time limit, resulting in 29 patients for the test-retest reliability and 49 for the other clinimetric properties. This number of participants is low compared to other validation studies.[3,14] Second, the evaluation of the newly developed PAKA was done within the same group of patients who were involved in the development process. Therefore, the outcome of satisfaction with the PAKA could be biased. Nevertheless, in total, 300 patients with sports related injury of the knee were contacted for the inventory and evaluation process, which is a good representation of the patients for which the PAKA will be used in clinical practice. Third, this study did not account for responsiveness, defined as the ability to detect any clinically important changes over time.[17] Therefore it is not yet known whether the PAKA can detect these changes after patients undergo surgery. To report the responsiveness, long-term follow-up of at least six months to one year is necessary.

However, the long-term follow-up is already ongoing, and these data can be presented within the next year.

In conclusion, this study presented the development and the validation of the Dutch version of the Patient Approved Knee Assessment (PAKA) for patients with sports related knee injury. The PAKA was developed in collaboration with patients in this particular injury category to incorporate patients' opinions on what is important in outcomes after surgery. The PAKA showed an excellent satisfaction rate for patients. In the validation process, the PAKA presented with an excellent internal consistency, excellent test-retest reliability, and a strong construct validity. Therefore, the PAKA can be used in clinical practice to increase patient compliance in follow-up, and to improve the quality of clinical outcome measures in patient-centered care.

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Supplemental data 1

Question	Times submitted (%)
How is the other, healthy knee?	6 (7,6%)
Do you use pain medication?	9 (11,4%)
Do you see a physiotherapist? If yes, what is the result of treatment?	24 (30,4%)
What would you like to be able to do with the affected knee?	8 (10,1%)
How often are you confronted with your knee?	6 (7,6%)
Would you say surgery was successful?	6 (7,6%)
Are you able to kneel?	6 (7,6%)
To which degree can you rely on your knee?	4 (5,1%)

Supplemental data 2

Patient Approved Knee Assessment (Dutch)

Deze vragenlijst heeft betrekking op de klachten van uw <u>*aangedane/geopereerde*</u> *knie gedurende de afgelopen 4 weken.*

1. Hoe vaak ervaart u pijn van uw knie?
   Nooit -- Altijd

2. Als u pijn heeft aan uw knie, hoe ernstig is deze dan?
   Geen pijn --Ergst voorstelbare pijn

3. Wanneer ervaart u pijn aan uw knie?
   Tijdens sport/zware belasting ----------------------- In rust *0 NVT*

4. Hoe vaak gebruikt u pijnstilling voor de pijn van uw knie?
   Nooit --- Altijd *0 NVT*

5. Dagelijkse bezigheden.
Hoe **pijnlijk** zijn de volgende activiteiten voor u en wat is de **moeilijkheidsgraad** die u ervaart door uw knieklachten.

Activiteiten	**Pijnlijk**	**Moeilijkheidsgraad**
1. Slapen/liggen in bed	Niet -----------Extreem	Niet -----------Extreem
2. Opstaan vanuit bed	Niet -----------Extreem	Niet -----------Extreem
3. Zitten	Niet -----------Extreem	Niet -----------Extreem
4. Gaan zitten en opstaan van toilet	Niet -----------Extreem	Niet -----------Extreem
5. In en uit de auto stappen	Niet -----------Extreem	Niet -----------Extreem
6. Staan	Niet -----------Extreem	Niet -----------Extreem
7. Lopen op een vlak oppervlak	Niet -----------Extreem	Niet -----------Extreem
8. Traplopen	Niet -----------Extreem	Niet -----------Extreem
9. Knielen/op de knieën zitten	Niet -----------Extreem	Niet -----------Extreem
10. Huishoudelijke activiteiten	Niet -----------Extreem	Niet -----------Extreem
11. Winkelen	Niet -----------Extreem	Niet -----------Extreem

6. Hoeveel hinder ervaart u in het algemeen door uw knie?
   Geen -- Erg veel

7. Hoe beoordeelt u de functie van uw knie?
   a) Functie vóór het letsel aan uw knie
      Goed -- *Slecht*
   b) Huidige functie van uw knie
      Goed -- *Slecht*
   c) Functie van uw niet aangedane knie
      Goed -- *Slecht*

8. Bewegelijkheid van de knie.
   a) Kunt u de knie helemaal strekken?
      Volledig -- *Niet*
   b) Kunt u de knie helemaal buigen?
      Volledig -- *Niet*

9. Hoe vaak heeft u een instabiel gevoel van de knie?
   Nooit -- *Altijd*

10. In welke mate kunt u op uw knie vertrouwen?
   Volledig -- *Niet*

11. In hoeverre wordt u beperkt in uw werk/hobby's door de klachten aan uw knie?
   Niet -- *Volledig*

12. Heeft de fysiotherapie een goed effect op uw herstel?
   Volledig--- *Niet*   *O NVT*

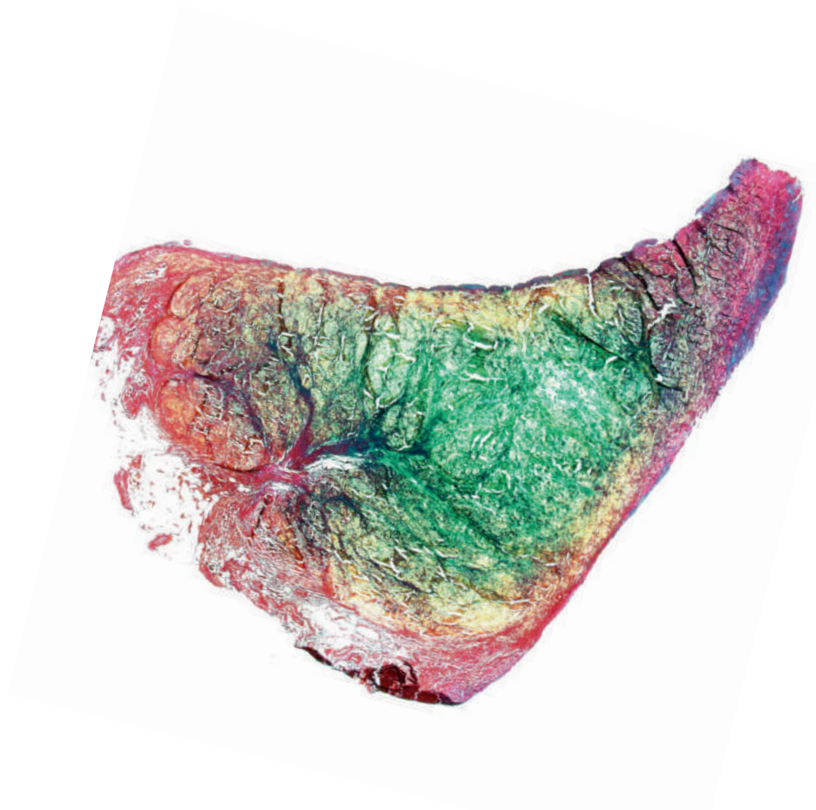
13. Lichamelijke gesteldheid tijdens recreatieve/sportieve activiteiten. Hoe **pijnlijk** zijn de volgende activiteiten voor u en wat is de **moeilijkheidsgraad** die u ervaart door uw knieklachten.

Sportieve activiteiten	**Pijnlijk**	**Moeilijkheidsgraad**
1. Op uw hurken zitten	Niet -----------Extreem	Niet -----------Extreem
2. Springen	Niet -----------Extreem	Niet -----------Extreem
3. Draaien/roteren van de knie	Niet -----------Extreem	Niet -----------Extreem
4. Hardlopen/joggen	Niet -----------Extreem	Niet -----------Extreem

14. Wat is het niveau van functioneren wat u na de operatie wil bereiken?
   a) ....
   ....(*ruimte voor patiënt om zelf iets in te vullen)*
   b) Kies een van de onderstaande opties
   o Erg inspannende activiteiten, zoals springen of draaibewegingen zoals in basketbal of voetbal.
   o Inspannende activiteiten zoals zwaar lichamelijk werk, skiën of tennis.
   o Matige activiteiten, zoals matig lichamelijk werk, rennen of joggen.
   o Lichte activiteiten zoals lopen, huishoudelijk werk of werken in de tuin.

Chapter 8

Summary, general discussion and future perspectives



Summary

This thesis has contributed to the understanding of meniscus tissue, the development of meniscus tears, and the long-term results of currently available treatments, while demonstrating different options to improve meniscus regeneration. In addition, this thesis showed the importance of patient-centered care in outcome measures after sports related knee surgery.

Finding the optimal treatment for meniscus tears to reduce symptoms in the acute setting and prevent the development of early osteoarthritis (OA) at long-term follow-up remains a challenge for orthopedic surgeons. This thesis provided evidence to reduce acute symptoms of meniscus lesions using meniscus repair and demonstrated the *in vitro* possibilities for a one-stage cell-based meniscus repair. The results obtained in the *in vitro* studies can be translated to a clinical trial to restore the native meniscus tissue and function after acute tears of the meniscus in young and active patients.

Part I

In chapter 2 we showed good to excellent clinical outcomes following meniscus repair in a pediatric and adolescent population with an isolated meniscus tear after 18 years of follow-up. At short-term follow-up, early failure and reoperation rates of the meniscus repair were reported, mainly in complex multiplanar tear types. However, at long-term follow-up, no additional reoperations or clinical failures were reported. Different risk factors for early failure of meniscus repair were assessed based on the Tegner and International Knee Documentation Committee scores. Only older age at time of follow-up showed significantly lower outcomes in Tegner score. Older age at time of injury, tear complexity, rim width of the meniscus tear and follow-up time did not make a difference in clinical outcome.

Chapter 3 demonstrated secondary meniscus tears after anterior cruciate ligament (ACL) injury are most common in patients who undergo delayed or non-operative management of a primary ACL injury. They often present as complex tears of the medial meniscus which are less amenable to repair and result in partial meniscectomy in 73% of cases. After 18 year follow-up, a possible preventive effect

of both ACL reconstruction and meniscus repair in the development of OA was reported for patients with a secondary meniscus tear after ACL injury.

Part II

The *in vitro* feasibility of a new one-stage cell-based procedure for meniscus regeneration was demonstrated in chapter 4. An increase in extracellular matrix (ECM) production was shown using a combination of meniscus cells and mesenchymal stromal cells (MSCs) in a 20:80 ratio. It appeared the MSCs stimulated the meniscus cells to produce ECM using communication via gap junctions before disappearing from the coculture. These two cell types can be combined with a meniscus scaffold, the Collagen Meniscus Implant (CMI®), to provide an environment for tissue production. This study investigated different *in vitro* methods for seeding the cells into the meniscus scaffold, where static seeding onto a dry scaffold showed the highest cell density and the best cell distribution compared to seeding by injection into a wet scaffold. The results of these *in vitro* experiments laid the foundation for clinical applications of a one-stage cell-based meniscus regeneration procedure.

The *in vitro* results of the different seeding methods were assessed for their clinical applicability in a cadaveric study, described in chapter 5, where a one-stage arthroscopic procedure for meniscus regeneration was mimicked. First, rapid digestion of meniscus tissue with 0.0125% Liberase resulted in sufficient living meniscus cells for a 20:80 ratio with allogeneic MSCs during a one-stage procedure. Second, this study showed a high cell count inside of the scaffold implanted *in vivo*, a good cell distribution throughout the complete scaffold, and no effect on cell viability when the cells were statically seeded onto a dry CMI, which was arthroscopically implanted afterwards. First implanting the CMI and then injecting the cells into the wet scaffold showed significantly worse results for all three different outcome parameters.

Besides MSCs, growth factors show great potential for meniscus regeneration, especially platelet derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), and a combination of growth factors found in platelet lysate (PL) as shown in chapter 6. These growth factors showed stimulation of migration, proliferation, and/or ECM production for both meniscus cells and

MSCs. To implement the effect of growth factors in a clinical setting and improve the current treatment of meniscus regeneration using a CMI, we showed a method to functionalize the CMI for growth factor binding peptides. Cell ingrowth of both meniscus cells and MSCs into either the CMI functionalized for VEGF or the CMI coated with PRP resulted in a higher cell density inside of the CMI compared to the negative control, which indicates we can attract endogenous growth factors present in the knee joint without injecting recombinant exogenous growth factors.

Part III

In chapter 7 we developed and validated a new Patient Reported Outcome Measure (PROM), the Patient Approved Knee Assessment (PAKA), for patients undergoing sports related knee surgery. The PAKA was developed in collaboration with patients to better understand and report patient needs and outcomes after knee surgery, contributing to the movement towards patient-centered care. To measure the differences in outcome after newly developed treatment options, uniform outcome measures are needed. Besides objective radiographic outcome measures, subjective PROMs are widely used in orthopedic surgery, showing the increasing interest in patient involvement during treatment and rehabilitation. However, most of these PROMs are developed by medical physicians, who consider different symptoms and risk factors to be important compared to patients. Therefore, a patient centered PROM was developed in collaboration with patients to account for all their needs after surgical treatment.

General discussion

Good alignment of the knee can prevent osteoarthritis

The anterior cruciate ligament (ACL) prevents anterior translation of the tibia and plays an important role in providing rotational stability of the knee.[12] After ACL injury, these stabilizing functions should be adopted by the menisci, femoral and tibial condylar geometry, and active muscle control.[12] The medial meniscus especially serves as a secondary stabilizer of the knee because of its firm attachment to the tibia, preventing anterior tibial translation. However, in chronic knee injuries, tears of the medial meniscus are commonly seen, possibly leading to an increased instability of the knee joint, whereas lateral meniscus tears are often generated after an acute trauma of the knee.

Our study on the development of secondary meniscus tears after ACL injury showed a significantly lower rate of new meniscus tears in patients undergoing acute ACL reconstruction. This could indicate that by providing new stability to the knee using ACL reconstruction, there is a lower burden on the other stabilizers of the knee, preventing chronic damage. With ACL reconstruction preventing anterior tibial translation, and preserved meniscus function (load transmission, shock absorbance, and stability), there is no increased load on the articular cartilage which would decrease the chance of developing early osteoarthritis after traumatic injury of the knee.

This theory could especially be important in young and active patients since their rate of return to (rotating) sport is higher than that of the older population. However, our data demonstrated no differences in the frequency of secondary medial versus lateral meniscus tears for patients treated non-operatively for their ACL tears. On the other hand, the frequency of lateral meniscus tears was significantly higher in patients treated non-operatively compared to the patients treated with ACL reconstruction (ACLR), both acute and delayed. This high rate of lateral meniscus tears could be caused by a high percentage of new acute traumas in ACL deficient knees, even though it is less likely these older patients return to high demand sports. Thus, even in patients who are not performing contact and pivoting sports, an ACL reconstruction should be considered. This could prevent (new) meniscus tears and possible cartilage damage, which occur due to instability

of the ACL-deficient knee. In addition, Sanders et al. 2017 showed a high incidence of symptomatic arthritis in patients with ACL tears treated non-operatively compared to matched peers without ACL injury, and a significantly higher risk for secondary meniscus tears, which supports our results and ideas on the importance of good alignment of the knee to prevent an unstable joint and thereby the development of early osteoarthrosis.[21]

Non-operatively treated patients had a higher percentage of lateral meniscus injuries compared to the ACLR groups. This could mean new trauma had occurred due to instability of the knee, since lateral meniscus tears often present after acute trauma of the knee. Both ACLR groups had a higher percentage of medial meniscus tears, which could indicate that chronic inflammation and/or damage of the knee already began immediately after the acute trauma, although the stability of the knee was restored afterwards.

Taking the ACL injury out of the equation, multiple studies showed good objective and subjective clinical success after meniscus repair for both short-term and long-term follow-up in adult patients.[3,9] Our study on isolated meniscus repair was the first study to describe long-term follow up in a pediatric and adolescent population. We described an overall failure rate of arthroscopic repair of isolated meniscus tears of 42% after 18 years of follow-up (14 of the 33 knees), where all the failures were reported before midterm follow-up (5.8 years). Complex tear types and a rim width of 0.3 mm were found to be risk factors for the (early) failure of the meniscal repair. At long-term follow-up, no new failures were reported.

Meniscus repair attempts to restore the native shape of the meniscus, improving alignment of the knee and healing of the torn meniscus tissue. However, the exact biological mechanism of meniscus repair is unknown. Bansal et al 2017 showed data on meniscus repair at short term follow-up, but data on follow-up after one year in animal studies is very limited, and does not provide information on the primary healing process.[2] Accordingly, information about the healing process after meniscus repair, type of formed tissue, and influence on the articular cartilage is not well known. In clinical trials, second-look arthroscopies after meniscus repair are uncommon as well, making it difficult to obtain evidence for complete healing of the meniscus tissue. One may hypothesize that just replacing

the torn meniscus in its original position provides good alignment of the knee, even without complete biological healing.

Our results showed failure of meniscus repair only in the early stages. This could be the result of a re-tear caused by an acute re-injury of the biologically inferior fibrous scar tissue formed in the first phase after repair. Possible changes to rehabilitation programs could lead to better long-term outcomes. In addition, most failed repairs were complex tear types, indicating these tear types might not be suitable for repair. Coming up with good indications for meniscus repair can increase the success at long-term follow-up.

Based on the results of our studies, we recommend performing ACL reconstruction in the early stages after injury, and meniscus repair for simple and bucket-handle meniscus tears. These interventions can restore good alignment of the knee, prevent instability, and optimize loading of the articular cartilage. This could contribute to prevention of early-onset osteoarthritis after traumatic injury of the knee. For complex tear types, partial meniscectomy combined with a replacement of the meniscus using regenerative medicine might be a good option. To conclude, good alignment of the knee is necessary for all different treatment options. Without good alignment, repairing or regenerating the meniscus is not advantageous, because in these cases the instability of the knee joint is predictive for new damage to the meniscus and the development of osteoarthritis.

There is added value of regenerative medicine therapy in meniscus injury

The incidence of concomitant meniscus tears in ACL injuries is high; approximately 55-65% is reported in literature.[17,22–24] In addition, it is well-known that the healing rate of meniscus repair is higher when this procedure is combined with an ACL reconstruction.[11] The influence of bone marrow in the knee joint—due to the drilling of holes in ACL reconstruction—can play a role in the increased healing rate. Also, a higher level of growth factors, specifically platelet-derived growth factor (PDGF), was detected in the synovial fluid directly after ACL reconstruction compared to meniscectomy alone, because the drilling of the

femoral and tibial tunnels may release bone marrow, creating an biologically advantageous environment.[26,27] These two factors provide evidence for a place in regenerative medicine therapy in meniscus repair, using both MSCs and growth factors.

This thesis showed an increase in GAG and collagen production in cocultures of meniscus cells combined with MSCs, compared to monocultures of meniscus cells. Cocultures with a higher percentage of MSCs resulted in the highest production of ECM. Other studies for both meniscus tissue and articular cartilage[4,7,18] showed similar results, indicating MSCs stimulate ECM production in combination with other cell types. Different pathways are described by which MSCs interact with other cell types and promote EMC production and proliferation. However, the exact mechanism of the role of MSCs in regenerative therapies is still unknown.

Although it was originally believed that MSCs would differentiate into the cell type they were cultured with, recent studies show that MSCs stimulate other cells to proliferate and produce new ECM, while they disappear.[6,10,20,28,30] We have shown the same mechanism for MSCs in coculture with meniscal cells. By using male MSCs and real time PCR for a Y chromosomal gene, we showed that the male cells disappeared from the cocultures, while the total amount of cells remained approximately the same, suggesting the meniscus cells proliferated. Positive connexin-43 staining and exchange of cytosolic labelling suggest the formation of active gap junctions. Although not directly shown in this thesis, it is likely that other methods of communication were also used by the cells. It is known that MSCs can also communicate in an indirect way, paracrine signalling, by the secretion of trophic factors and extracellular vesicles. The direct contact by the cells likely just enhanced the communication potential by adding direct transfer of signalling factors in the cytosol by gap junctions.

In the literature, there is discussion about whether autologous or allogeneic MSCs should be used for regenerative therapy. The safety of allogeneic stem cells has been proven in several clinical trials.[13,30] In addition, many studies assign the effect of MSCs primarily to their trophic cell properties; they produce extracellular vesicles, cytokines and growth factors that suppress the immune response by inhibiting B- and T-cell proliferation and monocyte maturation, and by promoting

generation of regulatory T cells and M2 macrophages.[1] Only one paper has made a direct comparison between autologous and allogeneic MSCs in favor of allogeneic MSCs.[13] Other benefits are that allogeneic MSCs can be made as an 'off-the-shelf' product to use in a one-stage procedure, can thus also be administered in acute or emergency situations, are more cost-effective and less time-consuming.[16] Therefore we want to use allogeneic MSCs for our future *in vivo* experiments. In patients with a meniscus tear, allogenic MSCs can potentially boost the regenerative effect in combination with autologous meniscus cells, producing a regenerated meniscus very similar to the native tissue, and restoring joint homeostasis by producing immunomodulatory signalling factors.

This thesis demonstrated that not only MSCs but also growth factors can play a part in regenerative medicine treatments. Both platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), and platelet lysate (PL) increased proliferation and/or ECM production of meniscus cells and MSCs. The concept of functionalizing the CMI with growth factor binding peptide was demonstrated with vascular endothelial growth factor (VEGF). To optimize this procedure for meniscus regeneration, functionalization for other growth factor binding peptides is necessary. A gradient of different growth factor binding peptides throughout the meniscus would be ideal to stimulate the cells to different functions. Since PDGF promoted migration of both meniscus cells and MSCs, it would be convenient to functionalize the outer rim of the meniscus implant with growth factor binding peptides that would allow PDGF to attract cells from the native meniscus into the scaffold to promote new tissue formation. TGF-β1 also increases proliferation and ECM production of meniscus cells, which is of great use throughout the complete scaffold. Therefore, functionalization of the CMI with more than one growth factor to target multiple pathways can increase the regenerative capacity of the treatment.

Besides figuring out how to functionalize the meniscus implant with different growth factor binding peptides, the ideal concentration of growth factors is difficult to determine. There needs to be a balance between the right combination of growth factors (bound to the peptides) and paracrine factors secreted by MSCs to create a regenerative microenvironment. A wide range of dose-dependent

concentrations of different growth factors is given in the literature.[5,15] To use the growth factor binding peptides in clinics, we want to capture the endogenous growth factors of the knee joint and increase their effectiveness because of the higher local concentration at sites where that specific growth factor is needed. This biological effect can be partially regulated by adjusting the amount of growth factor binding peptides used for functionalization, but is also partially dependent on the amount of growth factors present in the knee joint. An animal study, using growth factor binding peptides for different growth factors conducive to meniscus regeneration, should be performed. Hevesi et al 2019 showed a model for ACLR in rabbits, using an ACL sleeve, that is useful to study functionalized biomaterial and determine the biological availablity of growth factors present in the knee joint.[14]

Based on the studies and statements addressed in this discussion, we have proven regenerative medicine therapies can be of great added value for meniscus injuries. This is especially true in the more complex tear types, where the current treatment is not optimal. We can apply for a first-in-man clinical trial, using allogeneic MSCs in combination with autologous meniscus cells and a meniscus scaffold. Functionalization of a meniscus scaffold with growth factor binding peptides still needs to be further investigated *in vitro* before introducing it into clinical practice.

Patient-centered care is important

Nowadays patients are more and more up-to-date on symptoms of disease, treatments and experimental therapies. They want to be involved in their treatment process, leading to the importance of shared decision making. To guide patients through the different treatment options, and together decide what the best treatment is for that particular patient, the medical doctor needs to know what is important for patients in outcomes after surgery. Currently, the success of a treatment is based on clinical outcome measures reported by different PROMs, developed by medical doctors. This will not provide the best clinical outcome measures. A PROM developed in collaboration with patients can highlight the specific items that patients indicate as relevant for good rehabilitation and a successful treatment. The questionnaire developed in this thesis can provide this

information in patients with sports related knee injuries. In addition, using one PROM for all patients within the same injury category allows for better comparison of clinical outcomes after surgery versus non-operative treatment. This will again contribute to better clinical data to inform patients and achieve transparent shared decision-making and patient-centered care.

Conclusions and future perspectives

This thesis demonstrated a strong role for meniscal repair after meniscus injury, provided that the indication is established correctly for patients. We showed that a stable knee joint with an intact ACL is less likely to (re)tear the meniscus. In addition, the pediatric and adolescent population showed an early and higher failure rate after meniscal repair in complex tear types with a rim width > 3mm. These findings indicate that meniscus repair shows better results in a stable knee joint (with an intact ACL). Besides, early failure of meniscus repair happens more often than late failure, which might be due to (too) early return to sport. Lastly, complex tear types might not be suitable for meniscus repair.

Solid guidelines can contribute to improved clinical outcomes after treatment for meniscus injury. Our recommendation for these guidelines include (1) combining ACL reconstruction with meniscus treatment; (2) using meniscus repair only in simple and bucket-handle tear types, with a rim width ≤2mm; (3) creating a conservative rehabilitation program after meniscus repair; (4) introducing a new intervention, meniscus regeneration, for complex tear types to prevent OA after partial meniscectomy.

This thesis reported good to excellent clinical outcomes after meniscus repair. However, meniscus repair is not suitable for all tear types. Based on the promising *in vitro* results of this thesis, a first-in-man clinical trial for meniscus regeneration should be performed. Regenerative medicine for meniscus tears can improve the clinical outcome for tear types in which repair is not an option. The Collagen Meniscus Implant (CMI) is FDA approved for clinical use, and allogeneic MSCs are proven to be safe for the use of cartilage regeneration.[29]

The cell ratio of 20% allogenic MSCs and 80% autologous meniscus cells is proven to produce the highest amount of ECM, most comparable with native meniscus tissue. The amount of autologous meniscus needed for this ratio is clinically feasible to yield from harvested meniscus tissue, using Liberase. By statically seeding this cell combination onto the CMI, with fibrin glue as a carrier, good distribution and cell survival can be achieved, after which the scaffold can be arthroscopically implanted into the knee joint. A conservative rehabilitation program should be developed, patient progress should be monitored by using PROMs, MRI data should be obtained to monitor the degeneration of the scaffold and new tissue formation by augmented cells, and a second-look arthroscopy combined with a biopsy should be performed after one year. These outcome measures need to be combined to report the effectiveness of this new one-stage procedure for meniscus regeneration in young and active patients with an acute meniscus tear.

Personalized medicine is a growing field of orthopedic care and research. In regenerative medicine, porous materials are necessary to manufacture different type of scaffolds that provide mechanical stability and allow cell ingrowth to stimulate new tissue formation.[8] It has already been shown that combining preoperative imaging with 3D printing of orthopedic implants (personalized medicine), results in improvement of surgical accuracy and clinical outcomes.[19,25] In regenerative medicine, personally designed and 3D printed scaffolds based on preoperative imaging, can contribute to better clinical outcomes after surgery. This could lead to a shift in orthopedic surgery, from treating end-stage diseases such as osteoarthritis, to preventing end-stage diseases through treatments (i.e. meniscus and cartilage repair) using regenerative medicine.

How will this thesis change clinical practice?

1. We provided clear guidelines for when to use meniscal repair in the pediatric and adolescent population to prevent failure of treatment.
2. We demonstrated stability of the knee is of great importance. Therefore, in young and active patients with ACL injury (with or without concomitant meniscal tear), we recommend ACL reconstruction to prevent (secondary) meniscal tears and osteoarthritis.
3. We reported the *in vitro* and pre-clinical data for a new treatment in patients with a meniscal tear, meniscus regeneration. These data provide enough encouraging results to start a first-in-man clinical trial.
4. We developed and validated the Patient Approved Knee Assessment (PAKA) for patients with sports related knee injuries. This PROM can contribute to obtaining more relevant clinical outcome measures, better comparisons of different treatments, and moving towards patient-centered care.

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Appendices

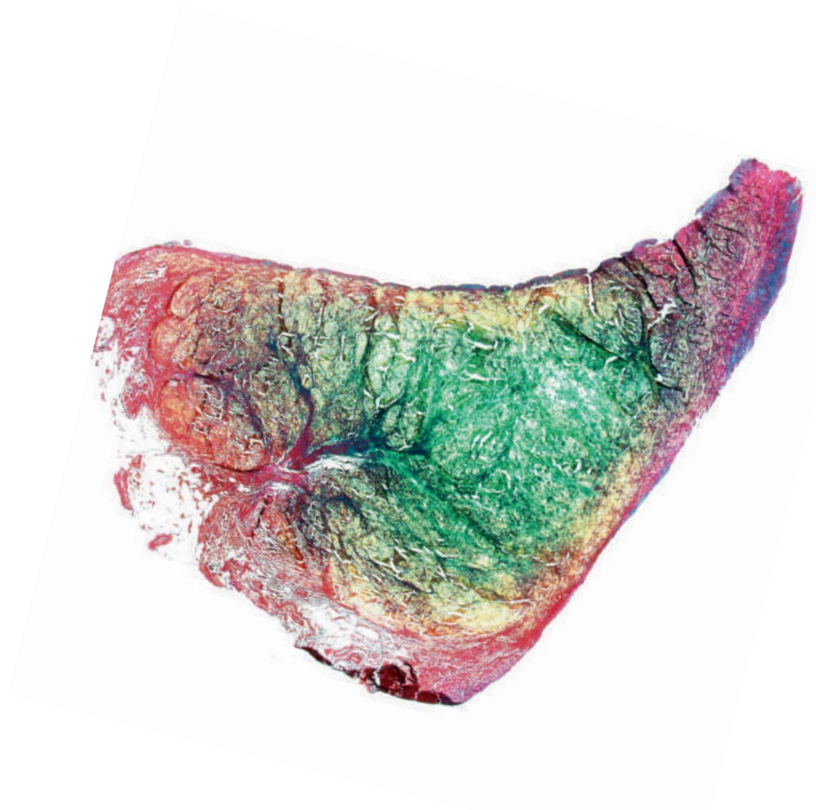
Dutch summary / Nederlandse samenvatting

List of abbreviations

List of publications

Acknowledgements / Dankwoord

Curriculum Vitae



Dutch summary

Nederlandse samenvatting

Meniscus letsel is een van de meest voorkomende blessures binnen de orthopedie en de artroscopische behandeling ervan een van de vaakst uitgevoerde operaties. De meniscus is een half maanvormige, kraakbeenachtige structuur welke zich bevindt in de knie. De belangrijkste functies van de meniscus zijn shock absorptie, bijdragen aan de mechanische stabiliteit van het kniegewricht en daarmee het gewrichtskraakbeen beschermen. Om deze reden is er een sterke correlatie tussen meniscus letsels en het ontwikkelen van vroegtijdige artrose van de knie. De gouden standaard voor het behandelen van meniscus letsel was lange tijd partiele meniscectomie (het verwijderen van het gescheurde gedeelte van de meniscus). Echter, hierdoor wordt op langere termijn het gewrichtskraakbeen ook aangetast, waardoor deze behandeling ook artrose kan induceren. Daarom wordt er gezocht naar een nieuwe behandeling voor meniscus letsel, om de functie van de meniscus intact te houden.

Dit proefschrift heeft bijgedragen aan het verder begrijpen van meniscus weefsel, het oplopen van meniscus letsels en de lange termijn resultaten van de verschillende behandelingen voor meniscus scheuren. Ook laten we verschillende manieren en technieken zien die de huidige behandelingen kunnen verbeteren. Tenslotte richt dit proefschrift zich op het belang van patiënt gerichte zorg, in uitkomstmaten na sport gerelateerde letsels van de knie.

Het blijft tot nu toe een uitdaging voor orthopedisch chirurgen om de optimale behandeling voor meniscus letsels te vinden. De optimale behandeling vermindert symptomen als pijn, zwelling en mechanische blokkade in het acute stadium en voorkomt ontwikkeling van artrose van de knie op lange termijn. De verschillende studies in dit proefschrift laten zien dat het hechten van de meniscus de symptomen in het acute moment kan verminderen. De resultaten van de *in vitro* studies tonen aan dat er mogelijkheden zijn voor het ontwikkelen van een één-staps procedure om, met behulp van verschillende type cellen, meniscus regeneratie te

bevorderen. De *in vitro* resultaten kunnen vertaald worden naar een klinische trial waarbij het meniscusweefsel geregenereerd kan worden. Hiermee kan uiteindelijk de functie van de meniscus behouden blijven na acuut meniscus letsel. Dit is vooral van belang in de jonge en actieve patiënten populatie.

Deel I

Hoofdstuk 2 laat goede tot uitstekende klinische resultaten zien, 18 jaar na meniscopexie van geïsoleerd meniscus letsel bij kinderen en adolescenten. Tijdens korte termijn follow-up, vroegtijdig falen van de behandeling en re-operaties werden gerapporteerd, met name complexe scheuren. Daarentegen werden bij de lange termijn follow-up geen aanvullende re-operatie of falende behandelingen gevonden. Risicofactoren voor het falen van de meniscopexie waren geëvalueerd door middel van de Tegner score en de IKDC scores. Alleen oudere leeftijd tijdens de follow-up liet significant lagere Tegner scores zien. Oudere leeftijd tijdens het oplopen van meniscus letsel, complexiteit van de scheur en follow-up tijd, maakte geen verschil in de klinische uitkomsten.

Hoofdstuk 3 laat zien dat secundaire meniscus scheuren na voorste kruisband (VKB) letsel het meeste voorkomen bij patiënten die in een later stadium een VKB reconstructie krijgen en bij patiënten die conservatief behandeld worden in vergelijking met patiënten die binnen 6 maanden na VKB letsel een reconstructie krijgen. Deze secundaire meniscus letsels zijn vaak complexe scheuren van de mediale meniscus welke slecht geschikt zijn voor meniscopexie (het hechten van de meniscus) waardoor ze in 73% van de gevallen tot partiele meniscectomie leidden. Deze 18 jaar follow-up studie toont aan dat zowel VKB reconstructie als meniscopexie kunnen leiden tot het voorkomen van de ontwikkeling van artrose in patiënten met secundaire meniscus scheuren na VKB letsel.

Deel II

Hoofstuk 4 laat met *in vitro* experimenten zien dat het haalbaar is om een één-staps procedure, met behulp van cellen, te ontwikkelen voor meniscus regeneratie. We toonden aan dat wanneer er een combinatie van 20% meniscus cellen en 80% mesenchymale stromale cellen (MSCs) werd gebruikt, er een toename was van de productie van extracellulaire matrix (ECM). Het is

aannemelijk dat de MSCs de meniscus cellen stimuleren om meer ECM te produceren. Deze communicatie lijkt te gaan via gap junctions, waarna de MSCs verdwijnen. Wanneer deze twee celtypes gecombineerd worden met een meniscus implantaat, de Collagen Mensicus Implant (CMI®), zorgt dit voor een 3D structuur waarin de cellen zich kunnen verplaatsen en ECM kunnen produceren. Deze studie onderzocht verschillende *in vitro* methodes om de twee celtypes te zaaien in het meniscus implantaat. Statisch zaaien op een droog implantaat liet de hoogste cel dichtheid en de beste cel verdeling door het gehele implantaat zien in vergelijking met injectie van cellen in een nat implantaat. De resultaten van deze *in vitro* studie leggen de basis voor het klinisch toepassen van een één-staps procedure met cellen voor meniscus regeneratie.

De *in vitro* resultaten uit hoofdstuk 4, werden in hoofdstuk 5 getest voor hun klinische toepasbaarheid. Hier is middels een kadaverstudie een één-staps artroscopische procedure voor meniscus regeneratie nagebootst. Als eerste leverde snelle digestie van meniscus weefsel met 0.0125% Liberase genoeg levende meniscus cellen op om te combineren met allogene MSCs in een 20:80 ratio tijdens één procedure. Vervolgens liet deze studie voor het statisch zaaien op een droge CMI een hoge cel dichtheid in het *in vivo* geïmplanteerde implantaat zien. De cellen waren goed verdeeld over het gehele implantaat en het artroscopische implanteren van de met cellen gezaaide CMI had geen effect gehad op de levensvatbaarheid van de cellen. Significant slechtere resultaten, voor alle drie de uitkomtparameters, werden gezien wanneer de CMI eerst geïmplanteerd werd waarna de cellen artroscopisch geïnjecteerd werden.

Zoals in eerdere hoofdstukken beschreven, laten MSCs grote potentie zien voor een bijdrage in meniscus regeneratie. Naast de MSCs kunnen verschillende groeifactoren ook meniscus regeneratie bevorderen. Met name platelet derived growth factor (PDGF), transforming growth factor β1 (TGF-β1), en een combinatie van groeifactoren welke gevonden wordt in platelet lysate (PL) en platelet rich plasma (PRP), laten in hoofdstuk 6 veelbelovende resultaten zien. Deze groeifactoren stimuleren de migratie, proliferatie en / of de ECM productie voor zowel meniscus cellen als voor MSCs. Om het effect van groeifactoren te implementeren in een klinische setting, en daarmee de huidige behandeling voor meniscus regeneratie middels de CMI te verbeteren, hebben wij een methode laten

zien waarbij de CMI gefunctionaliseerd wordt voor groeifactor bindende eiwitten. De CMIs gefunctionaliseerd voor een groeifactor of gecoat met PRP laat een betere cel ingroei en hogere cel dichtheid zien van zowel meniscus cellen als MSCs. Dit kan betekenen dat *in vivo*, de CMI gefunctionaliseerd voor groeifactor bindende eiwitten, endogene groeifactoren uit de knie kan aantrekken en daardoor de migratie van cellen richting de CMI en de ECM productie van deze cellen kan bevorderen.

Deel III

Hoofdstuk 7 laat de ontwikkeling en validatie van een nieuwe Patient Reported Outcome Measure (PROM), de Patient Approved Knee Assessment (PAKA), zien. Dit is een vragenlijst, gemaakt voor patiënten met sport gerelateerde blessures van de knie, ontwikkeld in samenwerking met patiënten met deze blessures om beter te begrijpen en te rapporteren wat voor patiënten belangrijk is als uitkomst van hun operatie. Als we weten wat voor de patiënten belangrijk is, en dit goed kunnen objectiveren, dan kunnen we werken naar meer patiënt gerichte zorg en behandelingen.

Om verschillen te meten tussen nieuwe en bestaande behandelingen, zijn uniforme uitkomstmaten nodig. Naast objectieve radiologische uitkomsten worden subjectieve PROMs veelvuldig gebruikt binnen de orthopedie. Dit laat zien dat er in toenemende mate interesse is voor de betrokkenheid van patiënten gedurende de behandeling en het herstel. Toch is het grootste gedeelte van de PROMs ontwikkeld door artsen en andere zorgverleners. Uit onderzoek blijkt dat zij symptomen en risico's die voor patiënten belangrijk zijn anders inschatten, waardoor de bestaande vragenlijsten mogelijk niet de beste informatie weergeven. Daarom is de PAKA, een PROM ontwikkeld in samenwerking met patiënten, waardevol om alle aspecten die voor patiënten van belang zijn rond de operatie en tijdens de revalidatie duidelijk in kaart te brengen en te evalueren.

List of abbreviations

3D	Three-dimensional
α-MEM	Minimal Essential medium
ACI	Autologous Chondrocyte Implantation
ACL	Anterior cruciate ligament
ACLR	Anterior cruciate ligament reconstruction
ANOVA	One-way analysis of variance
ASAP	l-ascorbic acid-2-phospate
BSA	Bovine serum albumin
CMI	Collagen Meniscus Implant
CO2	Carbon dioxide
DAB	3-diaminobenzidine
DAPI	4’6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2’-deoxyuridine
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GF	Growth factor
H&E	Haematoxyline and eosin
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSS	Hospital for Special Surgery

IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IKDC	International Knee Documentation Committee
ITSX	Insulin-Transferrin-Selenium-X
KDM5D	Lysine demethylase 5D
KOOS	Knee injury and Osteoarthritis Outcome Score
LCL	Lateral collateral ligament
MCL	Medial collateral ligament
MACI	matrix-induced autologous chondrocyte implant
MES	2-(N-morpholino)ethanesulfonic acid
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal stromal cells
NHS/EDC	N-hydroxysuccinimide/ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
OA	Osteoarthritis
PAKA	Patient approved knee assessment
PBS	Phosphate buffered saline
PCL	Poly(ε-caprolactone)
PCL	Posterior cruciate ligament
PCR	Polymerase chain reaction
pen/strep	penicillin and streptomycin
PDGF	Platelet-derived growth factor
PL	Platelet lysate
PLGA	poly(lactade-co-glycolide)
PROM	Patient reported outcome measure
PRP	Platelet rich plasma
QoL	Quality of life
REP	Rochester Epidemiology Project
SD	Standard deviation
SF-36	Short Form Health Survey
SSC	Synovium-derived mesenchymal stromal cells
TGF-β1	Transforming growth factor beta 1
TKA	Total knee arthroplasty

UMCU	University Medical Center Utrecht
UTY	Ubiquitously transcribed tetratricopeptide-repeat-containing, Y-linked
VAS	Visual Analogue Scale
VEGF	Vascular endothelial growth factor
WOMAC	Western Ontario & McMaster Universities Osteoarthritis Index

List of publications

Cell-based meniscus repair and regeneration: At the brink of clinical translation? Korpershoek JV, de Windt TS, Hagmeijer MH, Vonk LA, Saris DBF. *Orthop J Sports Med. 2017 Feb 21;5(2)*

Surgical Feasibility of a One-Stage Cell-Based Arthroscopic Procedure for Meniscus Regeneration: A Cadaveric Study. Hagmeijer MH, Vonk LA, Kouwenhoven JW, Custers RJH, Bleys RL, Krych AJ, Saris DBF. *Tissue Eng Part C Methods. 2018 Dec;24(12):688-696*

Long-term Results After Repair of Isolated Meniscal Tears Among Patients Aged 18 Years and Younger; An 18-Year Follow-up Study. Michella H. Hagmeijer, Nicholas I. Kennedy, Adam J. Tagliero, Bruce A. Levy, Michael J. Stuart, Daniel B. F. Saris, Diane L. Dahm, and Aaron J. Krych. *Am J Sports Med. 2019 Mar;47(4):799-806*

Secondary Meniscus Tears in Patients 1 with Anterior Cruciate Ligament Injury: Relationship between Operative Management, Osteoarthritis, and Arthroplasty at 18 Year Mean Follow-Up. Michella H. Hagmeijer, Mario Hevesi, Vishal S. Desai, Thomas L. Sanders, Bruce A. Levy, Michael J. Stuart, Daniel B.F. Saris, Aaron J. Krych. *AM J Sports Med. 2019 Jun;47(7):1583-1590*

Meniscus regeneration combining meniscus and mesenchymal stromal cells in a degradable meniscus implant; an in vitro study. MH Hagmeijer, LA Vonk, M Fenu, Y van Keep, AJ Krych, DBF Saris. *Eur Cell Mater. 2019 Aug 12;38:51-62*

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Growth Factors Enhance Meniscus Regeneration in Combination with a Degradable Meniscus Scaffold. MH Hagmeijer, JV Korpershoek, L Chen, J Crispim, P Jonkheijm, AJ Krych, DBF Saris, LA Vonk [*Under revision at J Tissue Eng Regen Med*]

Development and Validation of the Patient Approved Knee Assessment for Sport Related Knee Injuries. MH Hagmeijer, JG Steverink, TAC van Meel, LA Vonk, A Goud, JEJ Bekkers, DBF Saris [*Manuscript in preparation*]

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Curriculum vitae

Michella (Chella) Hendrika Hagmeijer was born on the 7th of July 1989 in Utrecht, The Netherlands. She graduated from high school (St. Bonifatius college, Utrecht) in 2007 and subsequently started medical school at the University of Utrecht.

During her study and rotations she became interested in the musculoskeletal system. In her last year of medical school she did a research internship at the Sports Medicine department at the University Medical Center Utrecht, and two clinical rotations at the Orthopaedic department of both the University Medical Center Utrecht, and Sint Antonius Hospital in Leidsche Rijn.

After graduation from medical school, in June 2015, she started her PhD project at the department of Orthopaedic surgery at the University Medical Center Utrecht under the supervision of prof. dr. D.B.F. Saris and dr. L.A. Vonk. The main goal of this project was to develop a new one-stage cell-based arthroscopic treatment for meniscus regeneration. In November 2017, as part of her PhD, she moved to Rochester (MN, USA) for half a year to work as an exchange PhD student at the Mayo Clinic, under the supervision of prof. A.J. Krych. This research resulted in several oral- and poster presentations at (inter)national conferences.

After working as a full-time PhD student for 3 years, she worked as a non-training resident in orthopaedics at the Onze Lieve Vrouwen Gasthuis in Amsterdam for one year. She is currently working as a non-training resident in general surgery at the Diakonessenhuis in Utrecht.