Universitat Autònoma de Barcelona

Meniscal scaffold for partial meniscal defect: clinical and and laboratory results

Francesco Poggioli

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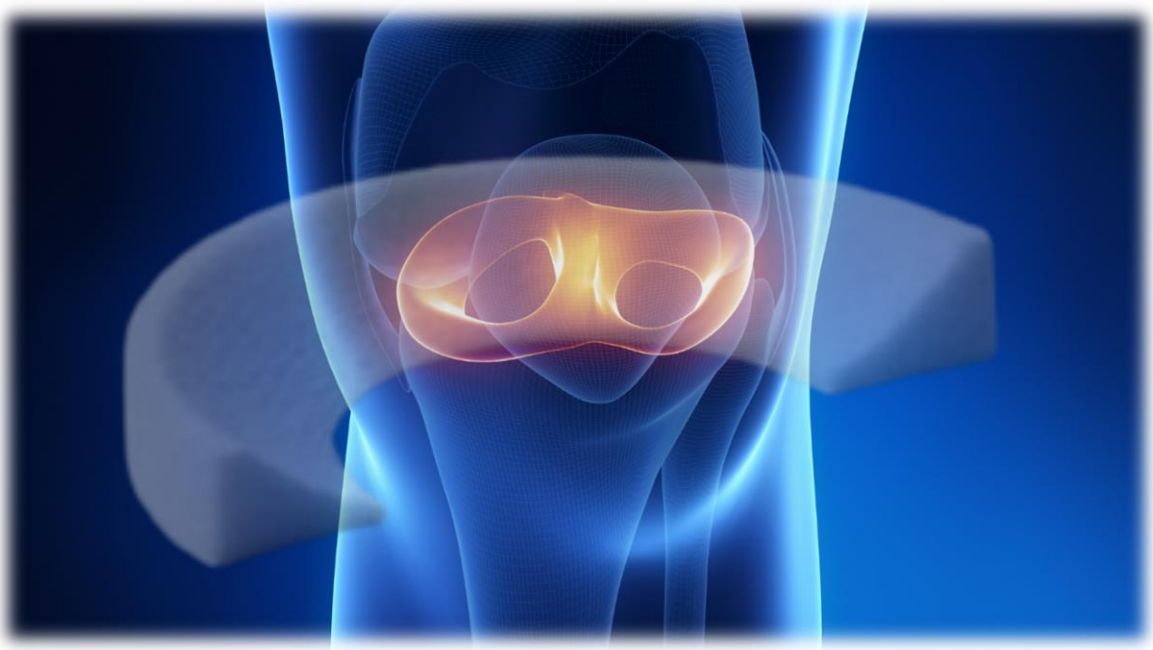

Doctoral thesis

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July 2021

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Acknowledgements

To my wife Ilaria who showed me the true meaning of happiness.

To Joan Carles Monllau because, with her daily actions, she showed me the difference between being a great surgeon and being both a great surgeon and a great person.

To myself because, after all, beautiful people attract beautiful people....

Index

Introduction

The menisci, once considered as vestigial remnants[1] are nowadays known to provide crucial knee functions including proprioception, shock absorption, weight transmission, enhancement of articular conformity and joint stability[2].

In the 1970s, the prevailing thinking and knowledge about menisci was not yet what it is today; in fact, the best and recommended treatment for a meniscal tear was to remove as much as possible of what was formerly considered as a useless structure[3].

Today the situation has changed a lot until a global consensus was found around the concept: "Save the meniscus"[4] that is, always try to preserve, repair, or replace the meniscus[5]. This consensus derives from several publications that have increasingly highlighted the importance of these fibrocartilaginous structures capable of maintaining a healthy knee joint[6–8].

However, when repair is not viable, meniscal replacement (partial or total) seems to be the most adequate method, whenever possible[9]. In order to replace previously removed or damaged meniscus many solutions have been evaluated, including synthetic materials, autogenous tissue, and allograft tissue[10–15]. Among these solutions, two are those currently used: MAT (meniscal allograft transplantation) and meniscal scaffold implantation (subject matter of this dissertation).

These surgical treatments have specific and different indications. MAT is indicated when it is necessary to restore entirely or almost entirely the meniscus while implantation of a scaffold is to be considered for partial meniscus replacement and need for integrity of the meniscal roots and peripheral rim remain.

An important topic for the understanding the logic underlying the sequence of articles forming this thesis is meniscal extrusion. The term “meniscal extrusion (ME)” refers to the situation in which the meniscal tissue is at least 25% displaced outside the tibial margin[16]. It has been suggested[17] that this "denudation" of the tibial surface plays a negative role

against the normal biomechanical functioning of the meniscus. This concept, carried over into the context of scaffolds, could diminish their effectiveness.

Artificial meniscal scaffolds has become popular in the last decades due to promising clinical results[10]. The two most studied scaffolds are the Collagen Meniscal Implant (CMI, ReGen Biologics, Franklin Lakes, NJ) and a biodegradable and synthetic acellular scaffold composed of aliphatic polyurethane (Actifit®; Orteq Ltd., London, UK).

The rationale about the use of a meniscal scaffold is twofold:

1. increase the meniscal surface thus improving patient symptoms resulting from post-meniscectomy syndrome and,
2. regenerate the meniscus to preserve joint integrity.

Although our studies showed results in line with the literature regarding improvement in symptomatology, the meniscal regeneration part gave exactly opposite results. Subsequent studies have focused precisely on this second part demonstrating how by enhancing a scaffold with mesenchymal cells and using appropriate laboratory techniques can be obtained, in vitro, the repopulation of meniscal tissue.

The goal of this dissertation and the articles of which it is composed was to answer the following four questions:

1. What is clinical and MRI results of the meniscal scaffold for partial meniscal defect?
2. Does Preoperative Remnant Meniscal Extrusion have an influence on postoperative extrusion and knee function?
3. With laboratory techniques could increase biocompatibility of the scaffold?
4. What's the difference between the regenerated meniscal tissue of a scaffold loaded with mesenchymal stromal cell in comparison with a cell-free scaffold.

Therefore, below I show what papers have been featured along the way. Two have already been published while the last two are in the process of submitting and I add them to complete the thesis.

A. Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up. *Monllau JC, Poggioli F, Erquicia J, Ramírez E, Pelfort X, Gelber P, Torres-Claramunt R*. Arthroscopy. 2018 May;34(5):1621-1627. doi: 10.1016/j.arthro.2017.12.019. Epub 2018 Feb 23.
B. Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function? *Gelber PE, Torres-Claramunt R, Poggioli F, Pérez-Prieto D, Monllau JC*. J Knee Surg. 2020 May 25. doi: 10.1055/s-0040-1710377. Online ahead of print.
C. Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold. *Raquel Arredondo, Francesco Poggioli, Santos Martínez, María Piera, Raúl Torres, Laura Tío, JC Monllau*.
D. In vitro evaluation regenerated meniscal tissue of a a polyurethane scaffold loaded with mesenchymal stromal cell in comparison with a cell-free scaffold.

General contents

Anatomy and biomechanics

Introduction

The Latin word meniscus comes from the Greek word mēnískos, meaning “crescent,” diminutive of mēnē, meaning “moon”[18]. Originally described as a vestigial structure, the menisci are now known to be essential for the normal functioning and longevity of the knee joint. The primary function of the meniscus is to transmit load across the tibiofemoral joint by increasing congruency, thereby decreasing the resultant stress placed on the articular cartilage. To achieve this, the menisci form a mobile containment on the tibial plateau adapting to the rolling gliding and rotating movement of the femoral condyles. The menisci also play a secondary role in shock absorption, stability, lubrication, nutrition, and proprioception to the knee joint. Understanding the anatomy and biomechanics of the meniscus is essential for the next step: to treat related pathologies.

Embryology and development

The menisci arise from a condensation of the intermediate layer of mesenchymal tissue surrounding the joint capsule. The characteristic shape of the lateral and medial menisci is achieved between the 8th and 10th week of gestation. The developing menisci are highly cellular and vascular, with a blood supply extending the entire width and length of the menisci. As the fetus continues to develop, there is an increase in collagen content in a circumferential arrangement with a concomitant decrease in cellularity. Weight-bearing and joint motion during development are important factors in determining the orientation of the collagen fibers. By adulthood, only the peripheral 10 to 30% are vascular. Despite

these histological changes, the proportion of tibial plateau covered by the corresponding meniscus is relatively constant throughout fetal development, with the medial and lateral menisci covering approximately 51–74% and 75–93% of the surface areas, respectively[19].

Anatomy

Medial meniscus and medial compartment

The medial meniscus is C-shaped and occupies approximately 60% of the articular contact area of the medial compartment. The medial compartment is tightly fixed between the two strongest ligaments, the posterior cruciate ligament (PCL) and the medial collateral ligament system, including the posterior oblique ligament (POL) with the meniscus. Therefore, there is less rotation excursion on this side and the axis of internal–external rotation stays in this medial compartment. The medial knee compartment can be called the stable knee compartment. The medial collateral ligament (MCL) is tight in extension and loose in flexion. It is also tightened in external rotation and loose in internal rotation. During flexion, the posterior part of the MCL is folded under the anterior part of the MCL. A complex system of progressive fibers recruitment towards extension when more force for the resistance against valgus is necessary can be observed. The most important and strongest protector against valgus and external rotation is the semimembranosus with its insertion close to the POL on the tibia and with its five tendon arms, two to the tibia, one to the POL - medial meniscus, one as oblique popliteal ligament to the fabella and the fifth into the aponeurosis of the popliteus muscle. It controls the posteromedial stability from an ideal position in all directions *(fig.1)*. In extension, the pars directa goes to the posterior tibial crest stabilizing against valgus. In flexion, the pars reflexa passing under the MCL is the most important restraint of internal rotation [19,20].

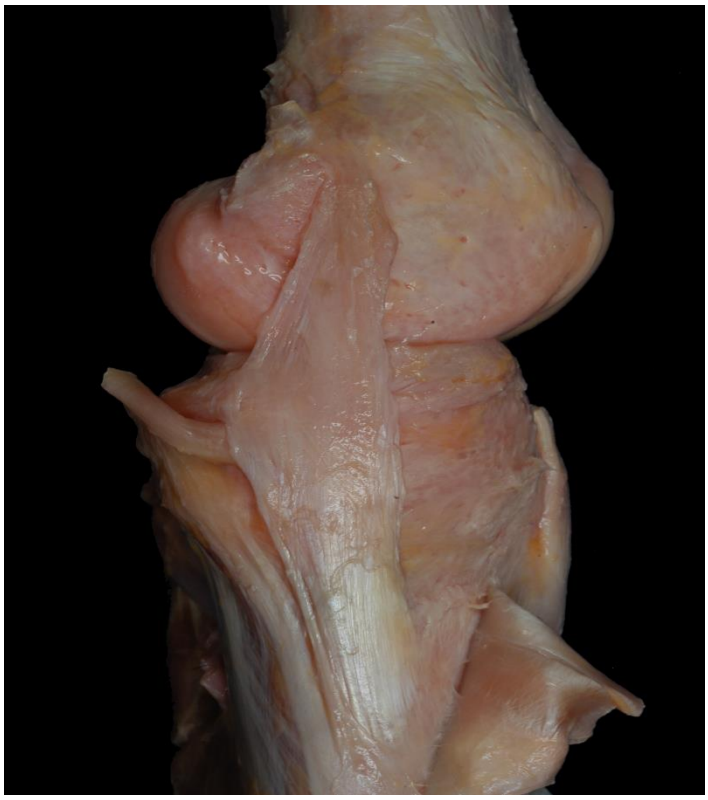


Fig 1. Dissection of the medial aspect of theknee at the level of layer II, showing anterior-ly the longitudinal fibers of the superficial medial collateral ligament (MCL) and, posterior to it, the Posterior Oblique Ligament (POL). More posterior, the direct attachment of the semimembranous tendon can be seen below the joint line.

Lateral meniscus and lateral compartment

The lateral meniscus is almost uniformly circular and in contrast to the medial meniscus, it is smaller and considerably more mobile. It also occupies a greater portion of the articular surface (80% vs. 60%). In contrast to the medial knee compartment, the lateral one can be called the mobile knee compartment. This is because the axis of rotation is based in the medial compartment. Consequently, the lateral compartment has no distinct ligament, which directly connects the tibia and the femur, as this would imply too much length change during extension–flexion and external–internal rotation. It is now back to be popular the anterolateral ligament (ALL) that should be better considered, as we will see onward, like a thickening of the lateral capsule. The lateral collateral ligament (LCL) runs from the femur to the fibula, as the proximal tibiofibular joint allows the necessary adaption for the needed length change during flexion–extension. The biceps tendon

tightens the LCL by its course around the ligament. The LCL represents a good example of a dynamised ligament, meaning that by contraction of the biceps tendon, the LCL is actively tightened. The popliteus system is dynamically stabilizing the lateral knee compartment. It has three tendon arms and acts as a primary static stabilizer to external rotation. The first tendon arm, which consists of synovial reflections above and below the meniscus, also known as popliteomeniscal fascicles, is directed towards the posterior wall of the lateral meniscus. The second one represents the popliteofibular ligament, which connects the fibular head and the popliteus tendon. It is the thickest part of the popliteus system. The third tendon arm runs underneath the LCL to its insertion, which is slightly ventral and distal to the femoral LCL insertion. A torn popliteofibular ligament leads to increased rotational freedom of the popliteus tendon. In such a case, the popliteus tendon is unconstrained from the popliteofibular ligament restraints and approximately 1 cm longer allowing more tibial rotation. The popliteus muscle belly lies on the medial backside of the proximal tibia. With its tendon, it is an important internal rotator and an important secondary restraint to the PCL. The popliteal aponeurosis is closely interwoven with tendon fibers of the semimembranosus muscle, which creates a direct link between the posteromedial and the posterolateral structures. The iliotibial tract (ITT) attaches at Gerdy's tubercle and functions as anterolateral stabilizer of the knee joint. The Kaplan fibers, which connect the ITT with the distal lateral femoral condyle, represent a dynamic ligamentous junction. It diverts the strong forces of the tensor fasciae latae and gluteus maximus muscles *(fig.3)*. The posterior fibers of the ITT are almost isometric at flexion angles between 0° and 50°. Between 50° and 90° flexion, the posterior fibers of ITT decrease in length. The anterior fibers of the ITT increase in length between 0° and 40° of flexion and were then almost isometric from 40° to 90°. Recently, the importance of the ALL has been rediscovered. Segond was the first to mention a capsular avulsion at the lateral tibia. In addition, in early textbooks and publications, it has been already mentioned as capsule-ligamentous thickening of the anterolateral capsule or the mid-third capsular ligament. However, it clear function and anatomical description have been vague and inconsistent. The origin of the ALL is on the lateral femoral epicondyle proximal and posterior to the popliteus tendon insertion. It inserts on the lateral meniscus and tibia 5

mm distal to the tibiofemoral joint and posterior to Gerdy's tubercle. The ALL is most tight during combined flexion and internal tibial rotation. Hence it serves as stabilizer for internal rotation.

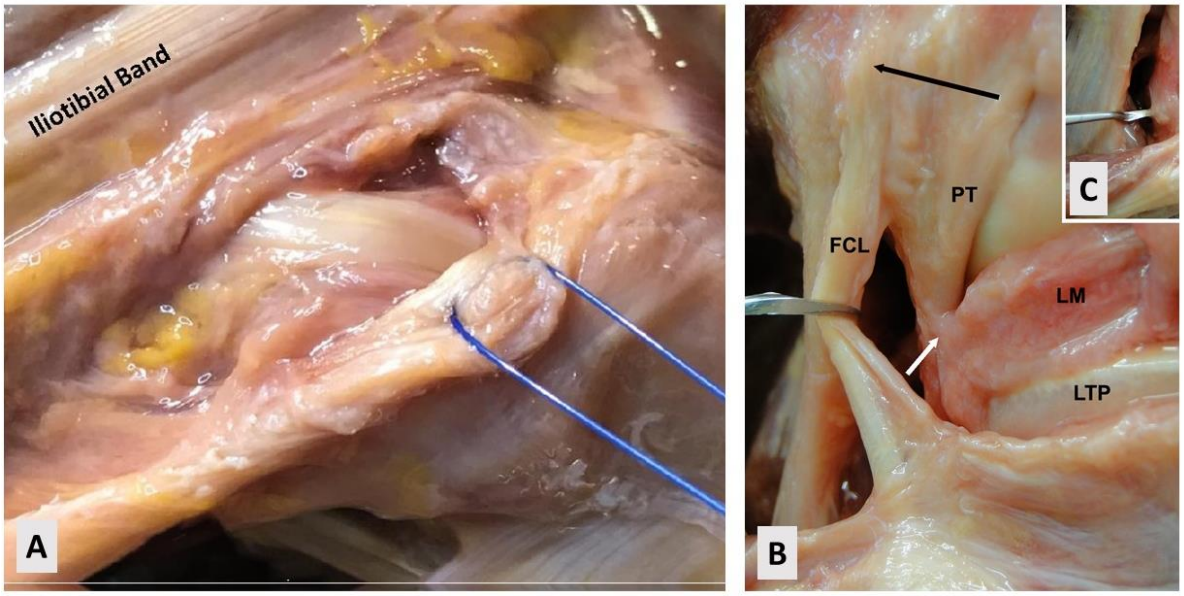


Fig 2 A Cadaveric dissection of the Lateral Side of the knee. View of the FCL (in layer II), after opening and retracting the FL. B Cadaveric dissection of the Lateral Side of the knee. Note the femoral collateral ligament (FCL) the popliteus tendon (PT), the lateral meniscus (LM), the lateral tibial plateau (LTP)

Biochemistry of the meniscus

The meniscus is composed of a dense extracellular matrix (ECM) composed of primarily of water (72%) and collagen (22%), interposed with cells. Other constituents include glucosaminoglycans (17%), DNA (2%), adhesion glycoproteins (<1%), and elastin (<1%). These proportions vary according to age, injury, or pathological condition. Collagen is the main fibrillar component of the meniscus and varies in amount depending on region within the meniscus. Collagens are primarily responsible for the tensile strength of the meniscus, contributing up to 75% of the dry weight of the ECM. In the red zone, type I collagen is predominant (80% composition by dry weight), with other collagen variants (e.g., type II, III, IV, VI, and XVIII) present in less than 1%. Type 1 collagen fibers are oriented circumferentially, in the deeper layers of the meniscus, parallel to the peripheral border. In

the most superficial region of the menisci, type 1 fibers are oriented in a more radial orientation. Radially positioned “tie” fibers are also present in the deep zone and woven between the circumferential fibers to provide structural integrity. In the white zone, collagen (70% by dry weight) is composed of only two types of collagen - types II (60%) and I (40%). The collagen fibers are heavily cross-linked and are ideal for transferring vertical compressive load into “hoop stresses”. Classification of meniscal cells is controversial, with no uniform characterization accepted in the literature; histological examination of the inner white zone of the menisci reveals rounded cells, that behave similarly to fibrochondrocytes or chondrocyte-like cells. In contrast, the cells of the outer red zone have an oval or fusiform appearance and are classified as fibroblast. A third cell population has been identified in the superficial zone of the meniscus. These cells are flattened and fusiform and lack cell extensions. Although the exact purpose of these cells is unknown, it has been suggested that they might be specific progenitor cells with a regenerative capacity.

Vascular anatomy

Blood vessels and lymphatics can be found throughout the menisci from the time of birth to a child's first birthday. Shortly after the menisci become weight-bearing structures (18 months), the blood and lymph supply is reduced to the outer 25% to 33% of the body of the menisci. The inner portion of these fibrocartilaginous wedges becomes avascular. Another portion of the menisci that is relatively avascular is the posterolateral aspect of the lateral meniscus next to the popliteal tendon. Nutrition is supplied to the menisci of the adult through blood vessels in the peripheral portion and by diffusion from the synovial fluid for the central portions of the menisci. Research has suggested that the diffusion of nutrients from the synovial fluid requires the intermittent loading and release of stress on the menisci via body weight and muscular force. During the first year of an infant's life, the menisci do not experience a significant amount of weight-bearing or muscular force, and therefore the inner portion of the menisci cannot rely on diffusion from the synovial fluid. A direct blood supply to the entire meniscus is therefore necessary

prior to the erect standing and walking capabilities of the human infant. Once an infant does master a bipedal gait pattern, the resultant stress from body weight and muscular forces is thought to be too much for the blood vessels in the inner portion of the menisci, and this region then become avascular. Beyond the age of 50, the blood and lymph supply to the outer portion of the menisci is reduced to the outer 10% to 33%. Age changes in the menisci include an increase in keratan sulfate and an increase in hyaluronic acid, both of which may play a role in the reduction of the peripheral blood supply to the menisci. The former is suspected of interfering with the nutrition of the cells and the latter is thought to inhibit the movement of water within the menisci. The anterior and posterior horns of the menisci remain highly vascularized. This may be necessary due to the high concentration of nerves in this region and it may be possible since the meniscal horns are not subjected to weight-bearing forces. The joint capsule and knee synovial receive their blood supply from both the inferior and superior medial and lateral genicular arteries. Branches from these blood vessels give rise to a peri-meniscal capillary plexus within the synovial and capsular tissues of the knee. This capillary plexus supplies the anterior and posterior horns as well as the peripheral portion of the menisci. A peripheral, vascular, synovial fringe extends a short distance over both the femoral and tibia surfaces of the menisci. The middle geniculate artery, along with terminal branches of the medial and lateral genicular arteries, also supplies blood vessels to the menisci through a vascular synovial covering of the antrior and posterior horn [20–22].

Load transmission

As you will read more in the dedicated chapter of this work, studies with long-term follow-up of meniscectomized knees have shown the importance of the meniscus in the functioning of the knee. Fairbank was first to describe the direct load-bearing function of the meniscus by describing the degenerative changes in meniscectomized knees. Fairbank described narrowing of the joint space, flattening of the femoral condyle, and the

formation of osteophytes and attributed these changes to the loss of the meniscus. Since then, several animal and clinical studies have confirmed Fairbank's thesis that the meniscus is an important protective, load-bearing structure. Biomechanical studies have demonstrated that approximately 40–60% of load acting on the extended knee joint is transmitted to the meniscus (65–70% lateral and 40–50% medial). In flexion, this increases up to 90%. During weight bearing, axial forces compress the menisci, resulting in "hoop" (circumferential) stresses. Hoop stresses rely on the conversion of axial force into tensile strain through the circumferential collagen fibers of the meniscus. The lateral meniscus is displaced more than the medial meniscus during compression, but because of the semilunar anatomy, load is transmitted away from the center of the femoral condyles resulting in tensile stress toward the tibial plateau. When standing, the meniscus absorbs most of the load; however, when the knee is in gait or stair climbing, variations in contact stresses occur. A recent cadaveric study by Gilbert et al. found that during gait, peak contact stresses of the medial plateau occurred in areas of cartilage–cartilage contact, while on the lateral meniscus peak contact stresses occurred under the meniscus [23]. During stair climb, peak contact stresses of the medial meniscus were located in the posterior aspect of the plateau, under the meniscus. While in the lateral meniscus, during the late phase of stair climb, peak contact stresses were reported in the zone of cartilage–cartilage contact. Several studies have demonstrated that load is well distributed when the meniscus is intact, however, its removal results in a significant reduction in femoral condyle contact area and a significant increase in contact stress. Several studies have reported that total lateral meniscectomy results in a 40–50% decrease in contact area and an increase in contact stress in the lateral component (200–300% of what is considered normal), which significantly increases the load per unit area and may contribute to accelerated articular cartilage damage and degeneration [20,22].

Shock absorption

The shock absorbing capacity of the menisci has been demonstrated by studies measuring the vibrations in the proximal tibia resulting from gait. From this, it has been shown that shock absorption is approximately 20% less in knees without menisci. This function of the menisci is associated with their viscoelastic properties, the main component of which is the water content of the tissue. Therefore, on impact, shock is absorbed by frictional drag forces, which occur as the fluid escapes the tissue.

Stability

The incongruous articulation between the convex femoral condyles and flat tibial plateau is improved by the concave-shaped superior surface of each meniscus. The firm attachment of the medial meniscus to the tibia contributes to anterior stability of the knee, and is more frequently torn (particularly in ACL-deficient knees) because it is less mobile. The intact meniscus limits excess motion in all directions, contributing to the stability of the knee joint. Although the exact function of the meniscofemoral ligaments (Wrisberg and Humphrey) remains unknown, it is believed that in flexion and internal rotation, the popliteal tendon retracts the posterior horn, thus reducing entrapment of the lateral meniscus between the femur and tibia. Joint stability is further facilitated by the soft tissue structures of the knee joint capsule. The role that the menisci play in joint stability can best be demonstrated in studies investigating laxity in ACL-deficient, meniscectomized or meniscus-torn knees. Findings include greater anterior tibial translation in knees with a sectioned ACL and medial meniscectomy as compared with knees with only ACL sectioning. However, ACL sectioning and lateral meniscectomy did not cause an increase in anterior translation in contrast to medial meniscectomy. Shoemaker and Markolf stated that the posterior horn of the medial meniscus is the most important structure resisting anterior tibial force in the ACL-deficient knee [24]. Allen et al. showed that the resultant force in the medial meniscus of the ACL- deficient knee increased by 52% in

full extension and by 197% at 60 degrees of flexion under a 134-N anterior tibial load. Musahl et al. reported that the lateral meniscus plays a major role in the pivot-shift maneuver as lateral meniscectomy increases translation and rotation and increases the pivot shift. All of these studies show significant changes in kinematics in the ACL-deficient knee and confirm the important role of the menisci in knee stability [19].

Joint lubrication and nutrition

The menisci may also play a role in the lubrication and nutrition of the knee joint. In a series of studies, MacConaill, reported that the coefficient of friction of the knee joint is increased by 20% following meniscectomy [25]. The precise mechanism by which lubrication occurs remains unknown; however, some authors believe that when the knee is loaded, the menisci compress and circulate synovial fluid into the articular cartilage, reducing the frictional forces during weight-bearing and providing joint nutrition. The system of microcanals within the meniscus that is located close to the blood vessels communicates with the synovial cavity. It is believed that these may provide fluid transport for lubrication and nutrition.

Proprioception

The menisci may serve a proprioceptive role as suggested by the presence of mechanoreceptors in the anterior and posterior horns of the menisci. Quick-adapting mechanoreceptors (e.g. Pacini corpuscles) are thought to mediate the sensation of joint motion, while slow-adapting receptors (e.g. Ruffini endings and Golgi tendon organs) are believed to mediate the sensation of joint position. The identification of these neural elements, located mostly in the middle and outer third of the meniscus, indicates that the meniscus can detect proprioceptive information, thus playing an important afferent role in the sensory feedback mechanism of the knee [26,27].

Meniscus lesions: Diagnosis and Classification

Introduction

Injuries of the menisci are one of the most prevalent injuries in the human body. The prevalence of an acute meniscal injury has been estimated of being 60 out of 10^5 patients. Given the magnitude of the problem, it is important for clinicians to diagnose meniscal tears accurately. Although imaging techniques have a role to play in confirming the diagnosis, it is inappropriate to order imaging tests for every patient with a knee injury. This is because every test has false positive and false negative results, and if an imaging test shows a meniscal tear in a patient with no signs of a meniscal tear, it likely represents a false positive test and should not be operated on. An accurate clinical diagnosis of a meniscal tear can be difficult, especially when there is other concomitant intra-articular pathology[28].

Clinical examination

Many signs and symptoms associated with meniscal tears have been described in the literature with variable reported rates of diagnostic accuracy. No single test is pathognomonic for a torn meniscus, and so, in addition to a careful history, the physician must rely on a collection of physical findings derived from a variety of reliable tests. The physician then combines the results of physical and historical findings with other diagnostic information to render a diagnosis and formulate a treatment plan. Before the physician performs a physical examination, it is essential that he obtains a focused full history and the chief complaint should be elicited. History-taking should be performed carefully. Good history-taking is the

most important and significant medical procedure, highly related to the capability and the experience of the physician. A history is mostly indicative of the disease itself and thus essential to lead to the final diagnosis. The age of the patient and the time that has passed since the onset of symptoms should be noted, while a traumatic painful knee in a young patient should be distinguished from a non-traumatic chronic knee pain in a patient over 40 years of age. Physician should also recognize the timing of pain: patients with a sudden onset of pain without reporting an antecedent trauma may have underlying articular cartilage degeneration, a degenerative meniscus lesion, or other pathology. The presence of chronic recurrent pain and swelling after exercising could be indicative of a meniscal tear irritating the joint. The clinical examination is the most important part of patient's assessment, and the indication for MRI should be given after that and when additional information is required for the treatment. Use an algorithm that includes known steps such as: inspection, palpation, joint movement, and the joint specific test is essential.

Imaging

After clinical examination, radiological assessment will determine and confirm the diagnosis of a meniscus tear. Radiographs are considered the most appropriate first imaging modality in the workup of traumatic and especially non-traumatic knee pain. The projections to be used differ depending on what we suspected during the clinical examination. Magnetic resonance imaging (MRI) is the gold standard imaging method for the assessment of meniscal lesions [29,30]. With the help of MRI, many of the essential characteristics of meniscal tears that are critical to management, such as their location, shape, length, and depth, can be described. MRI can be used to identify other injuries, such as cartilage injury and ligament tears, especially ACL tears, the presence of which may also influence the decision whether to perform surgery [29,31,32]. A healthy meniscus is triangular and prismatic in shape, producing a low-intensity signal in all sequences, with a homogeneous and weaker signal than that of cartilage. Meniscal tears appear as linear areas of high signal intensity located within the

normal low-intensity zones on both T1- and T2-weighted images (*Fig.4*). Degenerative changes related to the presence of local mucoid degeneration are areas of high signal intensity on T1- and particularly T2-weighted scans. A displaced bucket handle meniscus tear can occur in the sagittal MRI with the “double PCL sign” (meniscus within the notch) and in the coronal view with the meniscus displaced in the notch.

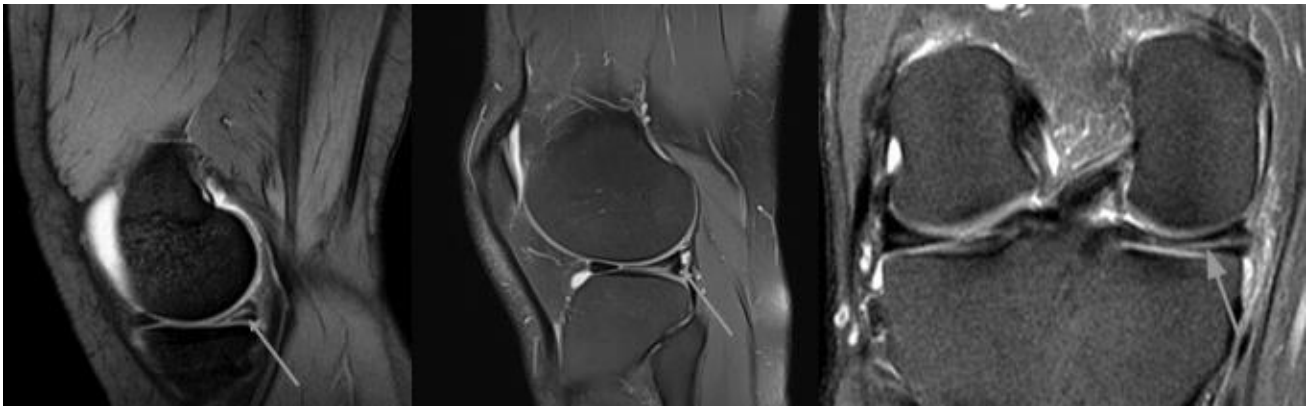


Fig 4 Sagittal and coronal magnetic resonance images showing abnormal (high) signal intensity (arrows) into the meniscus extending to the articular surface.

There are several meniscal classifications, with regard to MRI, the following stratification (*tab.1*) was proposed [33]:

Grade 1	a small focal area of hyperintensity, no extension to the articular surface
Grade 2	linear areas of hyperintensity, no extension to the articular surface
2a	linear abnormal hyperintensity with no extension to the articular surface
2b	abnormal hyperintensity reaches the articular surface on a single image
2c	globular wedge-shaped abnormal hyperintensity with no extension to the articular surface
Grade 3	abnormal hyperintensity extends to at least one articular surface (superior or inferior) and is referred as a definite meniscal tear

Tab 1 Stratification of meniscal lesions according to magnetic resonance imaging

In this classification, some conditions are not considered like anatomic variants and pitfalls that can mimic a tear, including discoid meniscus, meniscal flounce, a meniscal ossicle, and chondrocalcinosis.

Classification of meniscal injuries

The International Society of Arthroscopy, Knee Surgery and ISAKOS Knee Committee formed a Meniscal Documentation Subcommittee in 2006 with the objective of developing a reliable, international meniscal evaluation and documentation system to facilitate outcome assessment [28,34]. The ISAKOS classification of meniscal tears provides sufficient intra-observer reliability for pooling of data from international clinical trials designed to evaluate the outcomes of treatment for meniscal tears [35].

Tear Depth

The partial tear extends through either the superior or inferior surface of the meniscus. A horizontal tear may also be a partial tear. The complete tear extends through both the superior and inferior surface of the meniscus [33].

Rim Width

In the zone classification, tears may involve more than one zone. The tears should be graded based on how far the tear extends into the meniscus. For example, a complete radial tear that extends through zones 3, 2, and 1 should be graded as a zone 1 tear [28,36].

Zone 1 tears have a rim width of less than 3 mm.

Zone 2 tears have a rim width of 3–5 mm.

Zone 3 tears have a rim width of more than 5 mm.

Radial Location

Grade location of the tear with two formats:

(a) Indicate whether the tear is posterior, midbody, or anterior in location. Tears should be graded according to all the zones in which they are located. For example, a complete bucket-handle medial meniscus tear might be in the posterior, mid body, and anterior zones [37].

(b) The posterior-anterior classification is demonstrated on the diagram (*Fig.9*). Indicate where tear is: anterior, posterior, or both. A radial tear in the middle lateral meniscus from anterior to posterior should be marked as radial tear mid body [38].

Tear Pattern

The tear should be graded according to the following patterns (*Fig.5*) [39]:

(a) Longitudinal-vertical: extension is a bucket-handle tear

(b) Horizontal

(c) Radial

(d) Horizontal flap(e) Vertical flap

(f) Complex

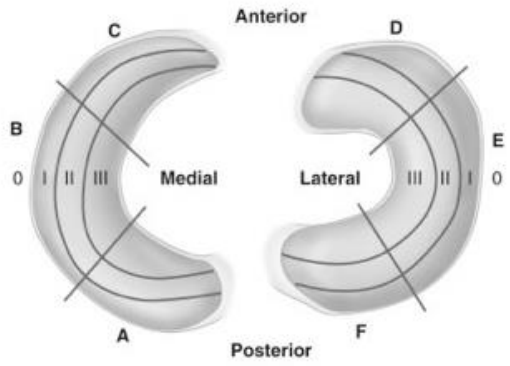


Fig 5 A. Zone classification of meniscus (modified from Cooper et al.). Most anterior zone of medial meniscus is labeled C, whereas most anterior zone of lateral meniscus is labeledD . 0 is meniscosynovial junction; I is outer third, II is middle third, and III is inner third of each meniscus. B. Four basic patterns of meniscal tears: I, longitudinal; II, horizontal; III, oblique; and IV, radial.[Campbell's Operative Orthopaedics. Phillips, Barry B.; Mihalko, Marc J.. Published January 1, 2017. Pages 2486-2566.e7. © 2017]

The lesions should be classified according to the predominant pattern.

Complex tears include two or more tears patterns. A tear in the lateral meniscus that extends partially or completely in front of the popliteal hiatus should be graded as central to the popliteal hiatus [28].

Meniscus lesions: treatment options

Introduction

“If it is torn, take it out, take it all out. Even if you just think it's torn, take it out”. This sentence written in 1967 by Smillie [40] implies that the meniscus was considered useless. Since then, advances in diagnostic methods, management techniques, and outcome assessment have improved our understanding of meniscal function and pathophysiology. Now we know that meniscus should be preserved whenever is possible [41]. Consequently, from the total meniscectomy, first open and then arthroscopic, orthopedic surgeons moved, when it is possible, to the partial meniscectomy. Various meniscal repair techniques have also been added to this option, which will be described later. Menisci can get injured by traumatic event or degenerate over time, the two situations, often involving different ages, require a different approach and have different solutions.

Traumatic meniscus tears

Traumatic meniscus tear is defined as a tear that is in general associated with an adequate knee injury. Among the different types of tears, vertical tears such as longitudinal (including bucket handle tears) and radial tears belong to this group [42]. Flap tears can belong to it, too, if they are secondary to a vertical longitudinal tear and not a horizontal cleavage. The location is the first feature to be evaluated for treatment decisions. Vascularity of the meniscus has important implications regarding possibility of healing process and thus indications [36]. It is thus very important to exactly locate the tear, according on one hand to the periphery of the

meniscus and on the other hand to the segment of the meniscus. Cooper et al. [37] described one of the most commonly used classification systems (*fig.5*).

In the zone classification, tears may involve more than one zone. The tears should be graded based on how far the tear extends into the meniscus. Tears located at the peripheral attachment sites (menisco-femoral and menisco-tibial), or zone 1, are also commonly referred to as outer third, or red-red (R/R), tears. Tears located in the middle third (zone 2) are classified as red-white (R/W) tears, and tears in the inner third (zone 3) are termed white-white (W/W) tears[43].

The second feature to be evaluated for treatment decisions is stability. In unstable meniscus tears, the central part of the lesion can be dislocated into the joint space until the center of the femoral condyle thus evoking locking and sudden pain or it engages or is able to engage between the tibia plateau and the MCL or in to the notch. A typical example is a longitudinal tear that temporarily changes to a bucket handle tear as well as a flap tear that engages between the femoral condyle and tibial plateau [44]. In terms of partial or very short meniscus tears, a stable tear is defined as a tear that is not displaceable with the probe [45]. Radial tears are in general defined as unstable[46].

Treatment of traumatic meniscus tears

The goal is to resect the part of the meniscus which is torn and not to extend the meniscectomy to the whole meniscus. This is usually easy in a traumatic tear where the fissure is well defined. In a stable knee, menicectomy is a gesture to be reserved as a last resort when other possibilities have been discarded. Pujol et al. [28] did a review of the literature, including long-term (more than 8 years) outcomes on stable knees in young patients (less than 40 years) with traumatic tears. Eleven studies (level IV) have been identified. At a mean 11.8-year follow-up, functional outcomes of medial meniscectomy are

good or very good in 84–95 % of the cases, but a joint narrowing is present on X-rays in 19–60 % of cases. Lateral meniscectomies provide good or very good results in 58–95%, and joint narrowing is present in 33–65 % of cases. Factors of bad prognosis are:

- Side: medial meniscectomies have better outcomes than lateral ones [28,47–49]. Rate of recurring procedure is higher. Rate of osteoarthritis is much higher [47]. Rapid lateral chondrolysis can be observed after lateral meniscectomy even in traumatic tears [48].
- Amount of resection: the incidence of arthrosis is less important after partial meniscectomy [37,45,46].
- Status of the cartilage at the time of surgery.

Techniques such as meniscal repair, whatever it is, should be encouraged considering that the literature already highlights the benefits. In fact some authors demonstrated better outcomes in a repair group than in a meniscectomy group at 10.6-year follow-up [28]. All procedures were proposed for vertical longitudinal tears in zone 1 or 2. All sub items of the KOOS score were better in the repair group, except quality of life. The risk of secondary osteoarthritis was significantly reduced in the repair group.

Degenerative meniscus tears

Degenerative meniscus lesions typically comprise a slow progression of symptoms (asymptomatic most of the time), and they can be associated to cavitations, several tear patterns, softened meniscal tissue, fibrillation, and/or other degenerative changes [50,51]. Typically, a degenerative meniscus comprises signal changes observed in MRI with a horizontal cleavage in the knee of a middle-aged or older person. Intra-meniscal linear signal changes are often reported, sometimes communicating with the inferior meniscal surface. Progressive mucoid degeneration and weakening of the meniscus ultrastructure are often

described [52]. Degenerative meniscal matrix changes are possibly related to early stage osteoarthritis. Such changes, in combination with progressive malalignment and overload on the affected compartment, could thus lead to meniscal fatigue, rupture, and extrusion [50,53,54].

Treatment of degenerative meniscus tears

There are no evidence-based guidelines for the best surgical approach concerning meniscectomy of an irreparable degenerative meniscus tears. Meniscectomy can always be considered for irreparable complex tears, but it is currently considered as a “last option” given the awareness of the deleterious long-term consequences [55]. Moreover, the amount of resected tissue seems to be implicated in the consequences of meniscectomy [56]. In some cases it can be combined to partially resect the unstable part of the meniscus but still preserve or even repair the remaining [57]. For this reason, it would be advisable limited resection of any meniscal tears to the unstable component, and whenever possible, try to repair and preserve meniscus suture. The French Arthroscopy Society Group has reported favorable outcome irrespective on the type of meniscectomy [58]. Identified risk factors of poor results included the presence of degenerative cartilage lesions (OR 2.8), resection of the meniscal wall (OR 2.2), and age >35 (OR 5.0). In summary, meniscectomy is thus proposed when mechanical symptoms are present and fail to respond to conservative treatment (non-operative treatment is a reasonable first line strategy) and a meniscal tear is identified on MRI which is suitable for improvement by standard arthroscopic two- portals approach.

Focus on problem and solutions

Post-meniscectomy syndrome

In 1948, Fairbanks described the changes that occur in the knee following meniscectomy, including ridge formation, narrowing of the joint space, and attending of the femoral condyles [59]. These changes lead to alterations in the biomechanics of the knee joint. Cox et al. have studied the effects of partial and total meniscectomy in dogs [60].

Partial meniscectomy is a relatively simple surgical gesture with an excellent immediate outcome and a lower re-operation rate [61,62] but led to less severe degenerative changes, with the degree of degeneration directly related to the amount of meniscus resected. In dogs which have been submitted a total meniscectomy, the degree of degenerative change was directly related to the amount of missing fibrocartilage. It was concluded that the knee menisci function to protect the articular cartilage from degenerative damages.

Meniscectomy significantly increases contact pressures of the tibiofemoral joint [63], which has been demonstrated in several studies, especially in patients with preexisting chondral damage [64,65]. It was also associated with poorer postoperative outcomes when considering knee function, Lysholm Scores, Tegner activity level and instability. The lateral compartment is less conforming than the medial compartment; loss of the meniscus on the lateral side may lead to an increased amount of instability and resultant force transmission to the articular cartilage, leading to increased degeneration and potentially the poor outcomes observed [66,67].

In medium and long term, degenerative changes often appear in the meniscectomized compartment, which are sometimes very symptomatic and require treatment.

The onset of pain in the compartment where meniscectomy has been performed is a relatively frequent and that is way the set of symptoms and signs that appear in the patient

after this procedure has been called post-meniscectomy syndrome. When this pain appears, and the conservative treatment does not work, different surgical options can be considered. The idea of meniscal replacement has developed over the last twenty years. The use of allogeneic meniscal transplants (MAT) has been popularized among surgeons to treat patients with complete meniscal defects. More recently, different meniscal implants have refined the concept of meniscal replacement in symptomatic partial defects. Thus, at present, the therapeutic management of the post-meniscectomy syndrome should be directed primarily to three objectives: 1) correction of the limb load axis if there is a deformity; 2) treatment of associated chondral lesions and 3) replacement of partial or total meniscal defect. Consequently, the ideal candidate must reflect certain features: middle-aged/young patients with symptomatic painful knees following lateral or medial meniscectomy (usually several years before) with normal lower limb alignment, without knee instability and usually without severe cartilage defects (Outerbridge I–II) [68–70].

Allogenic meniscus and scaffolds

Allograft:

Several characteristics of the meniscus make it optimal tissue to transplant. First, meniscal tissue produce a minimal immune response. Immune reactions have been described in 1.3% of transplants reported in the literature [71]. Consequently, the immune response to the meniscal allograft does not seem to affect the clinical outcome of the transplantation. The primary functions of the meniscus can be accomplished, even if the structure is lacking live cells. The meniscus is for the most part acellular, and most of its function is derived from its structure. The fate of the meniscal cells that accompany the meniscal allograft is unknown. Arnoczky et al. have studied the cellular repopulation of deep-frozen meniscal allografts. The menisci appeared to be repopulated with cells that originated from the adjacent synovium; however, the central core of the meniscus remained acellular. There was also loss of collagen orientation in the superficial layer of the meniscus [72].

The optimal method for meniscal graft preservation has yet to be determined. Currently, there are four primary preservation methods, including fresh, cryopreserved, fresh-frozen (deep-frozen), and freeze-dried.

Scaffolds:

Meniscal scaffolds may be derived from extracellular matrix (ECM) (eg, collagen, GAG, hyaluronan) or synthetic materials. These acellular, porous scaffolds are implanted within in the knee joint without cells with the intention of being populated by matrix-generating cells from the peripheral meniscal rim, vasculature, and/or synovium. A scaffold has become popular to treat symptomatic partial meniscal defects, providing pain relief and restoring the function of the meniscal tissue. Two scaffolds have been marked until now.

Collagen meniscal implant (CMI)

The Collagen Meniscal Implant (CMI, ReGen Biologics, Franklin Lakes, New Jersey) was the first to market with good results in series that exceed 10-years of follow-up [73]. CMI is manufactured from type I collagen harvested from bovine Achilles tendon. The harvested tendon is washed, the collagen fibers are isolated and purified using sequential chemical treatments and organic solvents, and the purified collagen fibers are swollen in the presence of equal quantities of hyaluronic acid and chondroitin sulfate. GAGs are added and the resulting compound is co-precipitated by the addition of ammonium hydroxide. The collagen fibers and associated extracellular components are then dehydrated and manually oriented in a mold. The resulting structure is lyophilized and sterilized by gamma irradiation. The end product is an acellular scaffold intended to support cell migration and de novo tissue growth from existing meniscal tissue, the synovium, and synovial fluid [74]. The CMI can be trimmed to match the specific dimensions of a patient's meniscal defect. The resulting implant has the tensile strength to support attachment of the implant to a rim of the native

meniscus with sutures and immediately withstand the sheer and compression forces within the knee joint, while maintaining a porous matrix to allow tissue regeneration [75].

Polyurethane scaffold (Actifit®)

Posteriorly it was designed a biodegradable, synthetic and acellular scaffold that was composed by an aliphatic polyurethane (Actifit®; Orteq Ltd). The ultrastructure of this scaffold is characterized by 80% porosity and 20% low reabsorption rate polymer (*Fig.6*). Within the polymer there are softer polycaprolactone segments that constitute 80% of the polymer, and the rest of the 20% is a more rigid urethane. Degradation starts with hydrolysis of polycaprolactone segments that lasts up to 5 years, the polyurethane segments are removed by macrophages and giant cells while the scaffold is replaced by cells coming from the surrounding tissues [76,77]. Its highly porous structure should facilitate cellular migration optimally leading to regeneration of meniscal tissue and restoration of meniscal function [78].

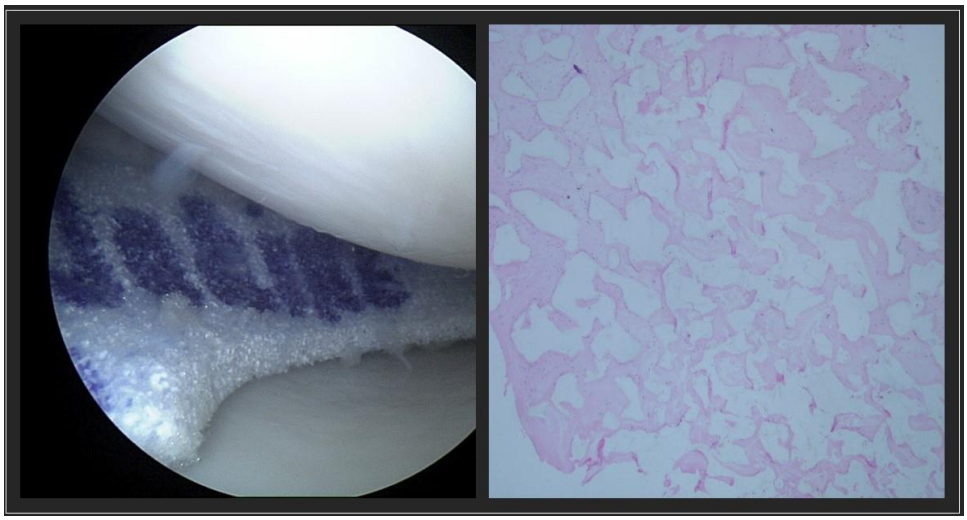


Fig 6 (A) The Actifit® implant. (B) Histological image of Actifit (1 year post-implantation) hematoxylin/eosin x100

Actifit®: when and how?

Clinical Indications

Actifit® is indicated for use in individuals who have symptomatic medial meniscus deficiency. This may be offered in the acute setting for an irreparable meniscus tear following partial or subtotal meniscectomy, or in the subacute or chronic setting for patients with symptomatic post-meniscectomy syndrome. Medial and lateral Actifit® implantation has been successfully achieved in Europe for both acute and chronic lesions. Meniscus specific indications include an intact peripheral rim to prevent meniscal extrusion and stable root attachments to allow for secure time-zero scaffold fixation. Correction of knee malalignment, instability, and treatment of focal cartilage lesions should be performed concomitantly (ie, realignment osteotomy, anterior cruciate ligament (ACL) reconstruction, cartilage restoration, respectively) or as part of a staged treatment [75].

Contraindications

Contraindications to Actifit® include uncorrected knee alignment, ligamentous instability, untreated focal high grade ipsilateral compartment cartilage lesion, total or subtotal meniscectomy (devoid of peripheral rim and/or stable meniscal roots), X-ray evidence of moderate to severe joint space narrowing, global arthritis, and increased body mass index (BMI).

Surgical Technique

The meniscal defect is evaluated and the rest of the meniscus is debrided to the vascular zone and, using a customized measuring device (*fig.7*), an implant is chosen for the specific

defect and cut on the surgical table using a scalpel. Standard inside-out, outside-in, and all-inside suturing techniques have been described to secure the implant (*fig.8-9*). The implant is introduced via the ipsilateral working portal using a specially designed delivery cannula and subsequently guided by the initial suture, which serves to lasso and temporarily secure the meniscal implant. Vertical mattress sutures are placed to secure the implant to the rim of the existing meniscus. The implant is secured to the anterior and posterior horn of the existing meniscus via horizontal mattress sutures. Use of 2-0 non-absorbable sutures is recommended. After the implant is properly secured to the existing meniscal tissue, the initial temporary suture is removed and the newly placed meniscal implant is checked for fixation integrity.

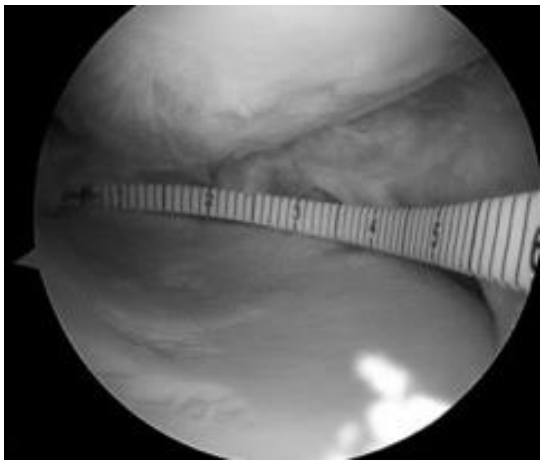


Fig 7 Once the meniscal defect was regularized, its sizing was performed with a specially designed flexible rod. [The magnetic resonance aspect of a polyurethane meniscal scaffold is worse in advanced cartilage defects without deterioration of clinical outcomes after a minimum two-year follow-up Pablo Eduardo Gelber et al. The Knee, The, 2015-10-01 Copyright © 2015 Elsevier B.V]

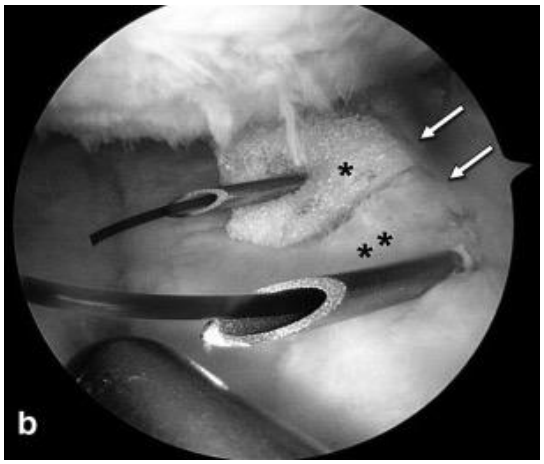


Fig 8 A horizontal outside–inside suture is being added to a previously horizontally placed all-inside suture (arrows) to fix the anterior end of the implant (*) to the host meniscal tissue (**). [Gelber et al. The Knee, The, 2015-10-01]

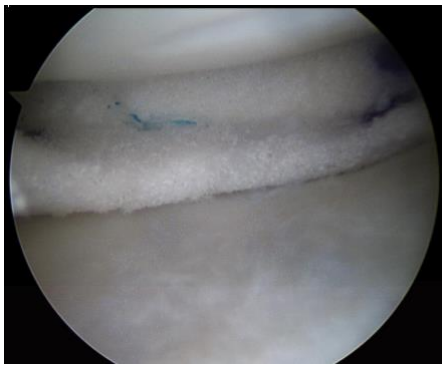


Fig 9 Implant in place.

Postoperative rehabilitation protocol

The postoperative rehabilitation protocol is designed to limit excessive loads on the implant prior to tissue ingrowth. Immediately following implantation, the knee is placed in a knee brace locked in full extension and kept non-weight-bearing for 6 weeks. Knee motion is started immediately postoperatively with flexion limited to 60 degrees for the rest 4 weeks and 90 degrees for the following 2 weeks. After 6 weeks, the brace is unlocked, the patient can be fully weight bearing, and physical therapy starts and continues until 6 months postoperatively, at which point the patient resumes normal activities. These are general guidelines; the protocol and timeframes for progression of activities will vary based on the individual patient's progression [79].

Clinical outcomes

Verdonk et al. reported the first prospective, single-arm, multi-center, proof-of-principle study to determine the clinical efficacy, safety, and performance of the implant in 52 patients at 2 years' minimum follow-up. The irreparable partial meniscal defects were in either compartment (34 medial and 18 lateral), with 88% of patients having undergone 1 to 3 previous surgeries on the index meniscus [75,80].

They observed tissue growth into the scaffold in 36 of 42 subjects using dynamic contrast-enhanced MRI (DCE-MRI) at 6 months. At 12 months, second-look arthroscopy

demonstrated tissue ingrowth in all subjects, and in 10 of 33 subjects, the meniscal lesion was filled. Biopsies showed that the regenerative tissue was composed of type I collagen, fibroblasts, and fibrochondroblast-like cells. Importantly, no evidence of articular cartilage damage related to the presence of the implant was found, and stable or improved International Cartilage Repair Society (ICRS) cartilage grades were observed in 92.5% of patients between baseline and 24 months. The 2-year re-operation rate was 17% in this study and was mainly attributed to the procedure by the authors. Re-operation was more common on the lateral side, and 7% of failures were attributed to the scaffold where procedural deficits were not involved, presenting with knee pain and effusion. A smaller study of 10 patients by Efe et al. reported similar findings, with general clinical improvement and lack of serious adverse side effects, synovitis, or signs of joint injury/inflammation in the operated compartment at 1 year [81]. Similarly, Kon et al. described improvement in pain and functional symptoms in a similar cohort of 18 patients at 2 years without adverse side effects [82]. Bulgheroni et al. reported similar clinical outcomes at 2 years, and arthroscopic biopsies revealed a bifringent scaffold with an amorphous, heterogeneous matrix with spindle like fibroblasts and bulging fibrochondrocyte-like cells at 4 months [83]. Later biopsies demonstrated more organized tissue, with some biopsies demonstrating chondrocyte-like arrangement, all coincident with grade 2 Genovese MRI signal intensity. Regarding concomitant procedures, Gelber et al. investigated the use of medial Actifit® implantation during opening wedge HTO for medial meniscus deficient varus knees in a prospective comparative study (40 men and 20 women, median age of 51 years). At a mean follow-up of 31.2 months, patients treated with realignment osteotomy and meniscectomy demonstrated superior improvement in functional scores (Western Ontario Meniscal Evaluation Tool [WOMET], IKDC, and VAS) compared to patients with concomitant implantation of a medial Actifit® [73]. Patients were satisfied ed equally with both procedures. Bouyarmane et al. reported a multi-center study focused on the use of Actift® in chronic, symptomatic post-partial meniscectomy lateral compartments [84]. Fifty-four patients (37 males/17 female, mean age 28 years) were followed, and significant improvements in VAS, IKDC, and all Knee Injury and Osteoarthritis Outcome Score (KOOS) subscores were demonstrated at 2 years. Three patients (5.5%)

underwent reoperation for pain; all three had varying degrees of scaffold tears, of which two responded to partial debridement. Finally, Gelber et al. evaluated the influence of articular chondral injury on the Actifit® MRI, using the Genovese criteria in 54 patients at a mean follow-up of 39 months [85]. The presence of an increased degree of chondral injury using the ICRS cartilage score was associated with worse morphological MR characteristics and smaller size of the scaffold, likely because of the unfavorable biomechanical environment in the setting of chondral lesions; neither MR signal intensity (all Genovese type 2) nor short-term functional outcomes were affected by the degree of chondral injury. It is also worth noting that concomitant procedures were warranted in 69.5% of patients in this study, including ACL reconstruction and HTO, PCL reconstruction (one case), and microfractures.

Meniscal Extrusion - what it means?

Meniscal extrusion is defined as a condition in which a variable proportion of meniscal tissue is dislocated outside the articular surface of the tibia[86] (*fig.10-11)*. It has been reported in the literature that a certain degree of meniscal extrusion is normal within the degenerative process of the knee[87], but it also has been identified as a risk factor in the development of knee osteoarthritis. The lack of cartilage coverage by meniscal tissue affects load distribution capacities. This leads to the loss of cartilage and to subsequent knee osteoarthritis.

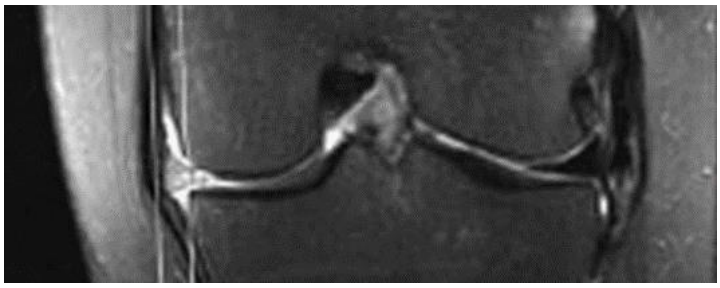


Figure 10: Magnetic resonance imaging magnetic resonance image extrusion of the graft beyond graft beyond the limit of the of the tibial plateau (arrow)

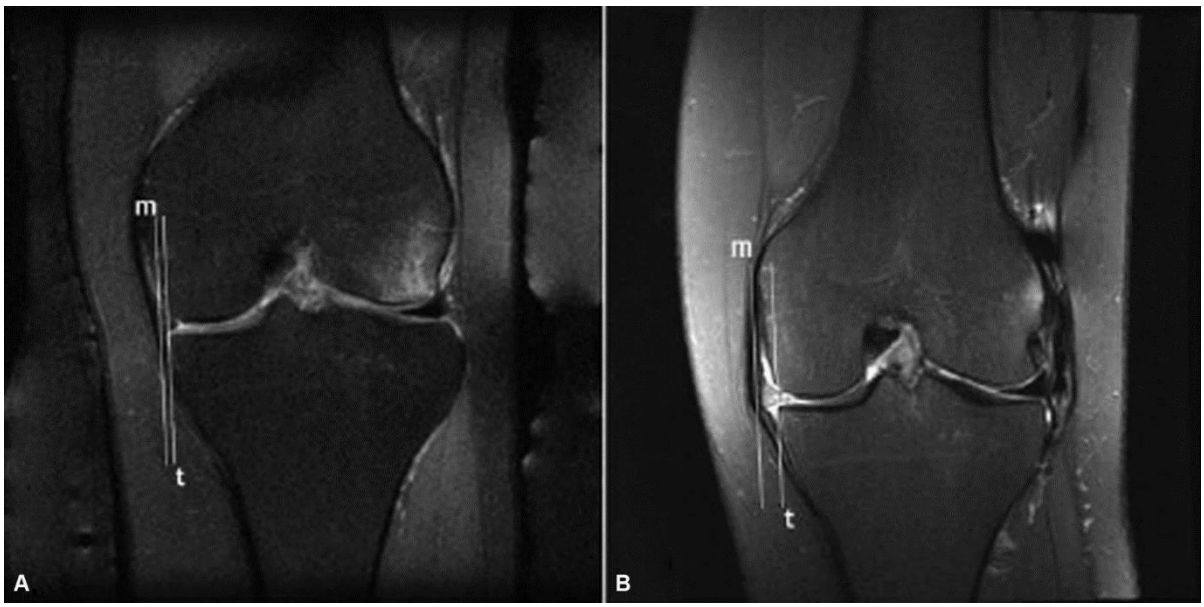


Fig.11 MRI of a left knee. (A) Preoperatively, meniscal remnant extrusion was 2 mm (minor extrusion) in this case. (B) The postoperative MRI used to calculate scaffold extrusion showed that the scaffold was 5 mm (major extrusion) beyond the tibial margin. m, meniscal remnant or scaffold border; MRI, magnetic resonance imaging; t, tibial margin.

De Coninck et al and Faivre et al[88,89] studied the impact of preoperative meniscal remnant extrusion (MRE) on the clinical outcomes of polyurethane scaffold implantation and on postoperative scaffold extrusion (SE). They viewed that preoperative and postoperative ME were not correlated with functional outcomes at the 2-year follow-up. Furthermore, they also observed that extrusion increased from the preoperative period to the postoperative follow-up. Both studies included a limited number of patients.

The second article in this thesis evaluated whether the preoperative MRE was correlated with the postoperative SE in a large group of patients. The secondary aim was to assess whether SE has an influence on the clinical outcomes at the 2- year follow-up.

Workflow and correlations

This thesis consists of two articles already puibblished and two submitted in process of being published. The first two papers examine the clinical side of scaffolds. Examining, the first, the mid-term clinical and radiographic outcomes (five years), and the second the potential correlation with meniscal extrusion.

The third article, more laboratory-based, evaluated how the use of a fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a scaffold. Finally, the fourth objective was qualitatively evaluate, in vitro, the regenerated meniscal tissue of a scaffold loaded with mesenchymal stromal cell in comparison with a cell-free scaffold.

Hypothesis

1. Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up. *Monllau JC, Poggioli F, Erquicia J, Ramírez E, Pelfort X, Gelber P, Torres-Claramunt R*. Arthroscopy. 2018. The main hypothesis of this study was that the scaffold would be able to improve pain and knee function as well as be replaced by new meniscus-like tissue as the MR imaging shows it.
2. Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function? *Gelber PE, Torres-Claramunt R, Poggioli F, Pérez-Prieto D, Monllau JC*. J Knee Surg. 2020 May 25. The main hypothesis of this study was that preoperative extrusion of the peripheral rimwould not correlate with postoperative SE. It was also hypothesized that the degree of extrusion would have no relationship to the functional outcomes.
3. Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold. *Raquel Arredondo, Francesco Poggioli, Santos Martínez, María Piera, Raúl Torres, Laura Tío, JC Monllau*. The main hypothesis of this study was that fibronectin improved the capacity of MSCs to adhere to the scaffold, and did not impair their ability to differentiate into chondrocytes and produce ECM.

4. Regeneration of partial meniscus defect using mesenchymal stromal cells (MSC) and a polyurethane meniscal scaffold in a rabbit model. The hypothesis of this article is that there are no tissue quality differences between scaffold loaded with mesenchymal stromal cell in comparison with a cell-free in a rabbit model.

Materials and Methods

The material and method section of this doctoral dissertation corresponds to that reported in each of the research papers that make up the thesis.

Paper 1 (2018)

Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up. *Monllau JC, Poggioli F, Erquicia J, Ramírez E, Pelfort X, Gelber P, Torres-Claramunt R*. Arthroscopy. 2018.

A series of 32 patients that underwent an Actifit® implantation between 2008 and 2011 by the same surgical team, were prospectively studied. There were 25 males and 7 females with median age of 41.3 ± 11.1 years at the time of index surgery. The procedure was performed only on patients with either persistent medial or lateral joint line compartmental pain due to a previous partial meniscus resection. The presence of anterior and posterior meniscus remnants as well as an intact outer rim of the meniscus was a necessary condition for the procedure. An anterior cruciate ligament (ACL) deficient knee was not considered a contraindication if the ligament was reconstructed at the same time as the polyurethane scaffold implantation. Similarly, varus knees were not a contraindication if the malalignment was addressed previously or concomitantly with the meniscal substitution. Exclusion criteria were the complete loss of the corresponding meniscus, advanced kissing chondral lesions, untreated instability, untreated varus or valgus malalignment greater than five degrees, inflammatory arthritis, polyurethane allergies, autoimmune disease and pregnancy. The study was approved by the clinical research ethics committee of our institution (Dex-Actifit®). All the patients signed informed consent to participate in the study as well as for the evaluation and publication of the results.

Functional evaluation

The clinical evaluation was performed by using established patient-reported outcome scores: the Knee injury and Osteoarthritis Outcomes Score (KOOS), the International Knee Documentation Committee (IKDC), the Lysholm Score and the Tegner Score. All these questionnaires were administered preoperatively and in the last follow-up visit. Patient satisfaction was evaluated with a subjective score and graded as very satisfied (four points), satisfied (three points), neutral (two points), somewhat dissatisfied (one point) and not satisfied at all (0 points).

MRI evaluation

A T2 Mapping MRI was performed at baseline and at a 5-years follow-up with a 1.9-Tesla MRI device (Prestige 2T; Elscint, Haifa, Israel) using gradient-echo T2-weighted, spin-echo T1-weighted, fat saturation fast spin-echo, and T2-weighted sequences in coronal, sagittal, and transverse slice orientations. The scaffold morphology was evaluated according to the method described by Genovese et al[85]. This score evaluates the morphology and size of the scaffold: type I, means totally resorbed scaffold; type II, small scaffold with regular (a) and/or irregular (b) morphology; and, finally, type III, scaffold with the same size and shape to the normal meniscus. The method also assesses the signal intensity of the scaffold: type I, markedly hyper-intense, type II, slightly hyper-intense and type III, iso-intense when is compared with the normal meniscus. Meniscus extrusion was measured on coronal view as described by De Coninck et al[90].

To further refine the meniscal regrowth assessment, a total meniscal volume (TMV) was calculated with features integrated into OsiriX v7.0.4 lite software so-called "ROI segmentation". The overall volume of each meniscus was calculated by using all the coronal MRI slices of the meniscus. Initially it was selected the appropriate range of image signal intensities based on the gray-level index values of the pixels within each meniscus. After this, a coloured marker images boundaries the limits of the meniscus. Overall volume (cm3) of the menisci was calculated using the total surface areas obtaining a 3D image for each meniscus.

Statistical analysis

Statistical analyses were performed using STATA/SE 12.1 (StataCorp 4905 Lakeway Dr College Station, TX 77845 USA). Categorical variables are expressed as percentages and frequencies. Mean and standard deviations as well as medians, minimums, and maximums were calculated for each continuous variable. The results were statistically analysed and compared using a Student t-test for parametric data with normal distribution. The level of significance was set at < 0.05.

Paper 2 (2020)

Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function? *Gelber PE, Torres-Claramunt R, Poggioli F, Pérez-Prieto D, Monllau JC*. J Knee Surg. 2020 May 25.

A retrospective study was conducted to assess all the patients who had had a medial polyurethane scaffold implanted for a post-meniscectomy syndrome. The study was approved by the clinical research ethics committee of the institution. A minimum follow-up of 2 years was required. All patients were operated on by the same surgical team (four surgeons). The same technique and similar postoperative protocols were used. Those patients with a malalignment of >5 degrees as well as patients with untreated knee instability were excluded. If the malalignment or the instability was corrected in the same surgical procedure, it was not considered exclusion criteria. Rheumatic diseases, polyurethane allergies, and pregnancy were other exclusion criteria.

Radiological and Functional Measurements

An MRI was performed preoperatively and 2 years postoperatively and extrusion was then compared. All examinations were performed in the study hospitals, but measurements were made by the same experienced musculoskeletal radiologist who was blinded for the study purposes. Extrusion (in mm) of the meniscal remnant was calculated in a coronal view, preoperatively. At the last follow-up MRI, the same measurements were performed

to calculate SE. The chosen coronal view used to make measurements was defined as the single slice presenting the greatest area of the medial spine. If this was difficult to differentiate, the image which showed the greatest width of the tibia plateau was chosen. The medial–lateral meniscal coronal width and meniscal body extrusion to the closest 0.1mm were measured. Regarding the definition of major and minor extrusion those patients with preoperative extrusion of <3mm were included in Group 1, whereas those patients with a preoperative extrusion equal to or greater than 3mm were included in Group 2. Functional outcomes were analyzed by means of the Western Ontario Meniscal Evaluation Tool (WOMET), International Knee Documentation Committee, the Kujala and Tegner scores, as well as visual analog scale. They were assessed preoperatively and at the last follow-up visit. Satisfaction was assessed at the last follow-up. Patients were asked to rate their satisfaction with the end result of the surgery on a scale ranging from 0 to 4 (with 4 being the best score).

Statistical Analysis

Categorical variables were presented as frequencies and percentages. The mean and standard deviation (SD) were calculated for each continuous variable. The comparison between MRE and SE was assessed using the paired t-test. The chi-square and the Wilcoxon's tests, depending on the case, were used to compare the pre and postoperative results of the different knee tests. Correlation coefficients between extrusion and the different scores and the differences between the post and preoperative periods were calculated using Spearman's rankcorrelation coefficient. Thestatisticalanalysis was performed using the SPSS 19 (SPSS Inc.; Chicago, IL) statistical package. The significance level was set at $p< 0.05$.

Paper 3 (2021 | Accepted (11/2021 - Regenerative Therapy)

Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold. *Raquel Arredondo, Francesco Poggioli, Santos Martínez, María Piera, Raúl Torres, Laura Tío, JC Monllau*

Isolation and characterization of rBM-MSCs

To harvest MSCs, we used two skeletally mature New Zealand white rabbits. They were fully sedated by intra-muscular injection of Ketamine (35 mg/kg) and Xylazine (5 mg/kg), followed by sevoflurane inhalation (2%, rate 2 litres/min). Then, we performed a medial parapatellar approach on the right knee of the animals. After dislocating the kneecap, we made a puncture on the medial femoral condyle with an 18G hypodermic needle. We aspirated the rabbit bone marrow (rBM) while making rotational needle movements. Finally, we collected the rBM in a syringe with citrate to avoid coagulation. The protocol including all animal care and experimental procedures was approved by the Ethical Committee of Animal Experimentation of our institution (CEEA-PRBB) and by the competing regional authorities.

We purified rBM mononuclear cells (MNCs) following the SepMate™ isolation tubes protocol. Briefly, after aspiration, rBM was diluted with an equal volume of PBS + 2% FBS and gently mixed. Such cell dilution was pulled on the SepMate™ tube previously filled with Ficoll. The tube was then centrifuged at 1200 g for 10 min at room temperature (RT). The enriched MNCs fraction was washed twice with 10% PBS + 2% FBS and centrifuged for 10 min at 400g at RT. The obtained cells were then seeded at a density of 2×105/cm2 and their media replaced every 3-4 days, until the cells reached an 80%-90% confluence (8-10 days). At this point, only rBM-MSCs were surviving and growing. Therefore, rBM-MSCs were trypsinised and seeded in a 75cm2 flask at the concentration of 5·104 cells/cm2. To expand them, rBM-MSCs were cultured with DMEM supplemented with 10% FBS for 14 days. Alternatively, they were cultured with StemPro® MSC SFM XenoFree (Gibco, life technologies) for 3 days and then with MesenPRO RS™ Medium (Gibco, life technologies)

for 11 days. Cell media were replaced every 3 to 4 days. For our experiments, we only used cells from the first and the second passages.

The MSC multipotency test was performed in triplicate using commercial kits: “StemPro® Osteogenesis Differentiation Kit”, “StemPro™ Chondrogenesis Differentiation Kit”, and “StemPro™ Adipogenesis Differentiation Kit” (Gibco).

Cell count was performed with Neubauer Chamber using Trypan Blue exclusion and Flow Cytometry. For flow cytometry, we used an internal microsphere CountBright™ counting standard (Thermo Fischer Scientific), with settling properties similar to lymphocytes. We carried out each quantification in triplicate.

Establishment of rabbit chondrocyte (rCHs) culture

Cells were thawed from frozen stocks obtained from previous works (14). rCHs were seeded in a 75 cm2 flask with a density of 5×104 cells/cm2 in DMEM medium supplemented with 10% FBS and 50 µg/ml Ascorbic Acid (AA). They were grown for 14 days at 37ºC with 5% CO2 and 60% of relative humidity. The medium was replaced twice per week. For our experiments, we only used cells from the first and the second passages.

Scaffold preparation, cell seeding and culture

We cut a cylindrical piece with a diameter of 4 mm and a height of 2 mm from a commercial Actifit®® structure. We sterilized the scaffolds by increasing ethanol concentration batch (50%. 70%, absolute ethanol). Afterwards, they were washed three times in PBS, and finally immersed in DMEM medium for 24 h. For the scaffolds to be coated with FN, the DMEM medium included 1% FN. Scaffolds were then let dry for 24 h.

Culture cells (rCHs or rBM-MSCs) reaching a confluence of 80-90%, were harvested and resuspended in DMEM at a concentration of 1x106 cells/µl. Afterwards, cells were seeded on sterilized scaffolds at a concentration of 5x107 cells/cm3 and cultured in wells in a non-adherent 48-well plate. rBM-MSCs were cultured with chondrocyte differentiation medium. Such medium consisted in DMEM supplemented with 10 µl/ml Insulin Transferrin Selenium (ITS), 50 µg/ml ascorbic acid, 10-7M Dexamethasone, and 10 ng/ml TGF-β. For

chondrocytes cultures on scaffolds, the culture medium used consisted in DMEM supplemented with 10%FBS and 50 µg/ml ascorbic. Cells were cultured for 3 weeks and medium was changed 3 times per week. A total of six scaffolds were cultured for each condition.

Evaluation of cell proliferation

To assess cell viability and proliferation on each scaffold, we performed an MTS assay (Abcam). Briefly, 10% MTS reagent was added to cell culture media and incubated for 3 hours in standard culture conditions. Then, we briefly shook the plate and measured the absorbance at 490 nm. We evaluated each sample in triplicate.

Scaffold colonization and evaluation of ECM production

To evaluate the diffusion of the cells through the scaffold and their ECM production, we histologically evaluated the slices from 3 scaffolds per condition (FN-coated+rBM-MSCs, non-coated+ rBM-MSCs, and non-coated +rCHs). Samples were fixed overnight with 10% formalin, and then put in a 15% sucrose (in PBS) bath, for 6 hours. Finally, they were kept in a 30% sucrose bath for 18 h. Afterwards, scaffolds were embedded in OCT, cooled down in a bath of dry ice and isopropanol, and frozen at -20ºC.

From the OCT blocks, we obtained 4 µm transversal sections with Cryostat (Leica CM3050 S). These sections were then stuck to SuperFrostPlus® slides. Stains were performed in horizontal racks to avoid losing material because of the low adherence of the scaffold to the slide glass. We finally performed the following evaluations:

• Scaffold colonization. We evaluated cell spreading along the Scaffold through Haematoxylin – Eosin and DAPI staining. Sections were stained in Mayer's haematoxylin solution (30%) (Sigma Aldrich diagnostics®) for 15 sec to 1 min. Afterwards, they were washed in running tap water for 10 min, soaked three times in 80% ethanol + 0.15% Hydrochloric Acid (HCl), and three times in 0.3% ammonia water. Then the samples were rinsed in distilled water and washed 5 min in 95% ethanol before counterstaining in 0.5% eosin Y alcoholic (Bio-Optica ref. 05-10003/L) solution for 15 to 30 sec. Finally, slides were dehydrated and mounted with Dibutylphalate Polystyrene Xylene (DPX) new medium. For

DAPI, the slides were washed with TPBS, mounted with an aqueous mounting agent containing 4 '6-diamino-2-fenilindol (DAPI) (1:200).

• Collagen stain. Sections were stained in Weighert's haematoxylin solution (Sigma-Aldrich) for 15 sec to 1 min and washed in running tap water for 10 min. Then, they were stained for 1 hour in picrosirius red (PSR) solution (Sirius red F3B Sigma-Aldrich "Direct Red 80") in saturated aqueous picric acid (Sigma Aldrich), pH 2. Afterwards, the sections were washed twice in acidified water (0.1 N HCl), and three times in absolute ethanol (5 min each). Finally, they were cleared in xylene for 5 min and mounted with DPX new medium.

• Proteoglycan stain. Sections were stained 5 min in 1% alcian blue (Merck Millipore) in 3% acetic acid. Then, they were soaked in 0.5% aqueous periodic acid solution for 5 min and, finally, they were bathed for 15 min in Schiff's reagent (Merck Millipore). At every step, the slides were washed with tap water for 3 min and rinsed in distilled water. Finally, they were stained with haematoxylin solution modified according to Gill III (Merck Millipore) for 20 sec and washed for 3 min with tap water. After dehydration, samples were mounted with DPX new medium.

To observe the samples, we used an Automated Upright Microscope BX61 in bright light and took pictures with an Olympus digital camera using the software cell Sens Standard. We also observed the samples under an Epifluorescence Eclipse Ni-E Microscope. For PSR staining observation, we used 3 filters: DAPI, FITC, and TxRed. Pictures were captured at 20x and 40x magnifications with a Nikon digital camera and processed with the photo program software Nikon NIS-E Advanced Research.

Results

The results section of this doctoral dissertation corresponds to that reported in each of the research papers that make up the thesis.

Paper 1 (2018)

Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up. *Monllau JC, Poggioli F, Erquicia J, Ramírez E, Pelfort X, Gelber P, Torres-Claramunt R*. Arthroscopy. 2018.

All the patients were followed for a median of 70.17 ± 7.49 (range 63-93) months.

There were 18 left and 14 right knees implanted with scaffolds, 21 of which were medial and 11 lateral. The average length of the implant was 45 ± 7.6 mm. Fixation of the Actifit® implant required a mean of 3.5 ± 0.7 all-inside sutures (posterior horn and meniscal body) and 0.8 ± 0.7 outside-in sutures (anterior horn). There were no complications related to the Actifit® implantation in any patient. Additional or combined procedures were performed in all but 5 patients, being high tibial valgus osteotomy (HTO) the most frequent. The preoperative mechanical axis in those patients who underwent HTO realignment, averaged 6.04 ± 5.01° of varus. Functional scores were summarized in following table.

	Preoperative	**Final follow-up**	**p value**
KOOS	48.56 ± 4,27	79.34 ± 2,48	p < 0,00
IKDC	41.68 ± 3.65	68.76 ± 3.30	p < 0,00
Lysholm	40.71 ± 3.92	78.06 ± 3.43	p < 0,00
Tegner	5.07 ± 0.53	5.66 ± 0.39	n.s.

Either KOOS, IKDC and Lysholm scores significantly improved at last follow-up. However, Tegner score showed no differences at this moment. The satisfaction expressed by the patients about knee function in the special questionnaire at the end of the process was scored as 3.3 points

MRI examination at baseline and at 60 months of follow-up was performed in 19 out of 32 patients (60%) included in the study. The rest of patients weren't able or did not consent to have an MRI at this moment. Sixteen out of 19 postoperative MRI showed a mean of 2.4 ± 1.28 mm extrusion of the scaffold, after 5 years follow-up. A complete re-absorption of

the meniscal scaffold was observed in the other 3 cases. With regard to the MRI scaffold shape and morphology, these 3 cases were classified as Genovese type I. The other 16 cases were classified as type IIb. With regard to the MRI signal intensity, 5 patients showed iso-intensity (Genovese type III), 2 cases markedly hyper-intensity (type I) and the remnant 13 patients presented a slight hyper-intensity of the scaffold. The TMV estimate was 1.14 cm3 (SD 0.17) before surgery while at the last follow-up was 1.61 cm3 (SD 0.18) (n.s.).

Paper 2 (2020)

Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function? *Gelber PE, Torres-Claramunt R, Poggioli F, Pérez-Prieto D, Monllau JC*. J Knee Surg. 2020 May 25.

During the period under review, a total of 98 polyurethane scaffolds were implanted. Four of them were not implanted in a post-meniscectomy syndrome and were thus excluded.

Three patients with untreated concurrent knee instability were also excluded. In 29 patients, postoperative MRI was not available. A total of 62 patients (46 men and 16 women) were included. The median follow-up was 45 months(range,25–69). The patients had a median age of 41.3 years (range, 17–58years). Four patients who had been previously operated with ahigh tibial osteotomy underwent a plate removal procedure in the follow-up period. None of these patients needed a new surgery to remove the scaffold during this period. The mean preoperative MRE was 2.8 mm1.2 and the mean postoperative SE was 3.8 mm 1.8 (p¼0.00). All functional scores improved postoperatively (Tab1). Tab.2 shows the correlation (Spearman's rho) between the differences in extrusion between the pre- and postoperative periods and their correlation with the different scores studied. An important correlation was observed in the WOMET (rho 0.61, p¼0.02). Regarding the differences between the two groups, preop-erativeMREinGroup1was1.85 mm (SD0.83), whereasGroup2 had a mean preoperative MRE of 3.7 mm (SD 2.2) ($p<0.01$). Patients from Group 1 had an SE at 2 years postoperatively of3.86 mm (SD 0.7), whereas it was 3.98 mm (SD 1) in Group 2(p¼0.8). No differences in the evaluated functional outcomes in either group in both periods under study (Tab.3) were found. Similarly, no

differences were observed when the preoperative MRE (Group 1 vs. Group 2) was compared with the differences in the assessed scores.

Table 1 Mean and standard deviation of the different scores in the pre and postoperative periods

	Preoperative	**Postoperative**	***p*-Value**
WOMET	37.9 (SD 11.2)	67.6 (SD 20.4)	<0.01
IKDC	34.1 (SD 17)	70.4 (SD 16.8)	<0.01
Tegner	5.1 (SD 1.8)	4 (SD 1.8)	<0.01
Kujala	48.4 (SD 14.4)	83.3 (SD 16)	<0.01
VAS	7.22 (SD 1.2)	2.67 (SD 2.1)	<0.01

Table 2 Correlation (rho-Spearman) between the ME differences from the postoperative period and the preoperative period when comparing with the same differences in the functional scores

	ME difference	**p-Value**
WOMET differences	0.61	0.02
IKDC differences	0.08	0.79
Tegner differences	-0.1	0.72
Kujala differences	-0.06	0.84
VAS differences	-0.19	0.53

Table 3 Values for different scores assessed for Groups 1 and 2

	Group 1			**Group 2**		
	Preop	**Postop**	**Rho/*p*-Value**	**Preop**	**Postop**	**Rho/*p*-Value**
WOMET	40.55 (11.9)	67.6 (15)	-0.37/0.46	32.4 (13.8)	56 (25.5)	0.57/0.18
IKDC	35 (20.8)	68.7 (13.7)	-0.44/0.38	28.7 (16.8)	61.3 (20.9)	0.67/0.09
Tegner	5.7 (1.7)	4 (1.7)	0.64/0.16	5.3 (1.7)	3.7 (1.9)	-0.03/0.9
Kujala	48.7 (15.6)	81.75 (14.8)	-0.54/0.26	43 (14.6)	76.2 (20)	0.39/0.38
VAS	7.3 (0.8)	3.5 (1.9)	0.52/0.28	7 (0.7)	3.3 (1.7)	-067/0.09
Ahlbäck	1.5 (0.8)	1.5 (0.8)	-0.13/0.8	1.8 (0.9)	1.6 (0.9)	0.00/1.0
Satisfaction		2.9 (0.8)	-059/0.21		2.4 (1.1)	0.41/0.35

Paper 3 (2021 | Submitted)

Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold. *Raquel Arredondo, Francesco Poggioli, Santos Martínez, María Piera, Raúl Torres, Laura Tío, JC Monllau*

Isolation of rBM-MSCs and multilineage differentiation

MNCs obtained from the rBMs and cultured, showed the expected morphological features under light microscopy (Fig. 1). Surprisingly, while progressing to MSCs, cell morphology gradually became non-fibroblastic (Fig. 2A-D). To discard the effect of possible differentiation inductors in the serum, we tested three different batches of FBS in the culture media. We did not observe any differences in cell morphology so differentiation inductors, if any, where shared by the three sera. To avoid the effect of FBS, we checked a culture protocol with low FBS concentration. rBM-MSCs were cultured in both cell culture conditions. Flow cytometry analysis showed that cells cultured in medium supplemented with FBS were fewer (approximately 25%), although cultures were at similar confluence conditions, and have larger size than cells cultured with low FBS concentration (Fig. 3). Furthermore, these last cells achieved the expected fusiform morphology with digital expansions typical for MSCs (Fig. 2E-H). We used the culture protocol with low FBS concentration for the subsequent isolation of rBM-MSCs.

Before seeding the rBM-MSCs on the scaffolds, we performed multipotency test and achieved differentiation to the three linages (chondrocytes, osteocytes, and adipocytes) (Suppl. Fig. 1).

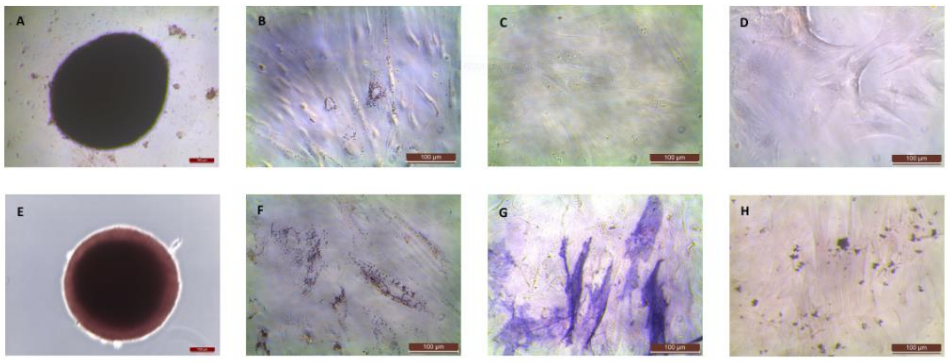
Adhesion and proliferation of rBM-MSCs on non-coated and FN-coated scaffolds

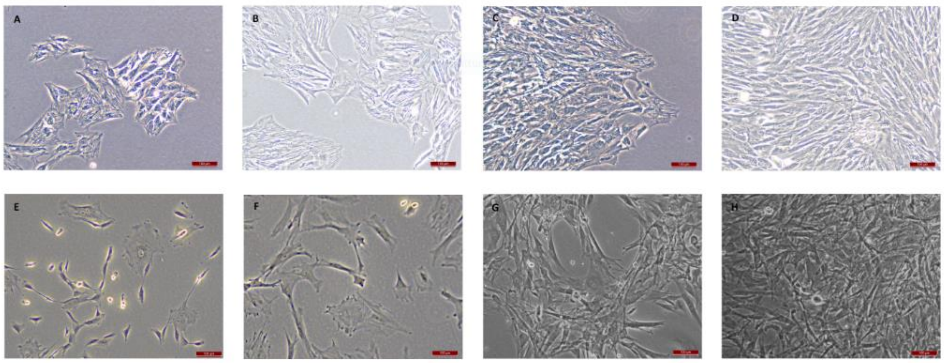
Although rBM-MSCs were able to attach and proliferate both on non-coated and FN-coated scaffolds, these features were improved on the FN-coated scaffold. 14 days after cell seeding, the number of proliferating cells present on FN-coated scaffolds were 145% (95% CI 107%-182%) higher than the proliferating cells grown on non-coated scaffolds.

Evaluation of rBM-MSCs cell spreading and ECM production

The protocol followed to perform the histology studies caused loss of material from the slide. The surface properties of the scaffold prevented to perform a suitable bond to the surface, although the slide had a pre-treatment to improve its adhesion properties. This fall of material avoided evaluating the cell migration along the complete sheet. We could not observe any cell attached to the untreated scaffold seeded with rBM-MSCs (Fig. 4A; E), while the other two condition (FN-coated+rBM-MSCs, and non-coated+rCHs) present similar cell staining (Fig. 4F; J; K; O).

The production of both proteoglycans and collagen could be evaluated as ECM presented good adhesion to the slide, and therefore could be observed in the areas where it has been deposited (Fig. 4). As expected, rBM-MSCs seeded on untreated scaffolds exhibited no ECM formation (Fig. 4B-D), as no cells were observed. Big areas of tissue formation and high ECM production were obtained from rCHs seeded on untreated scaffolds (Fig. 4L-N), while rBM-MSCs seeded on FN-coated scaffolds also produced ECM but in less extension (Fig. 4G-I). Although rCHs produced more ECM than rBM-MSCs, the quality of the ECM produced was comparable. Indeed, both showed similar content of acidic and neutral mucins (Fig. 4G and 4L).




A
B
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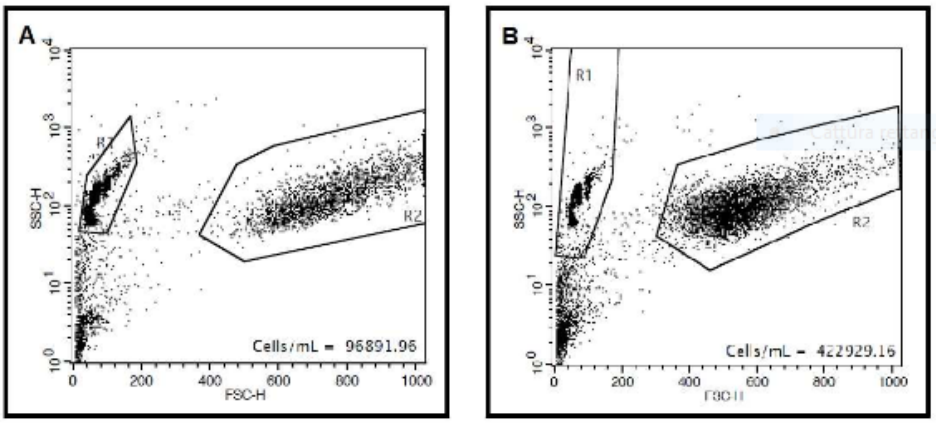
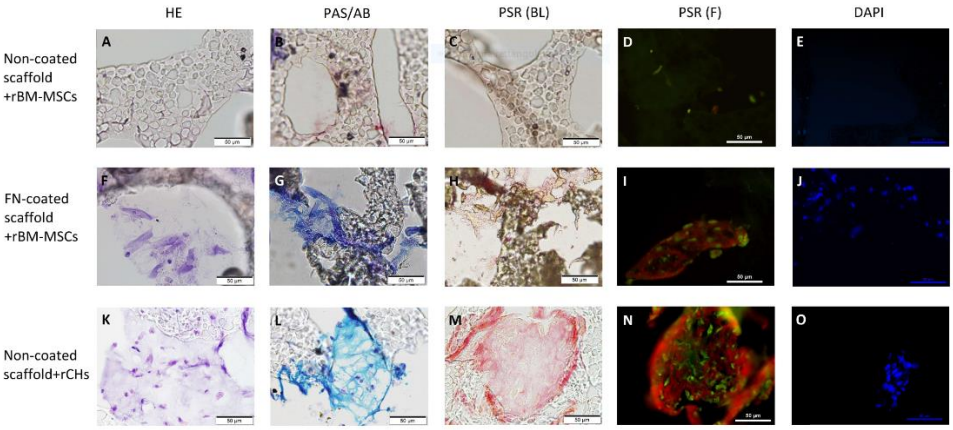

A
SSC-H
FSC-H
R1
R2
Cells/mL = 96891.96
B
SSC-H
R1
R2
Cells/mL = 422929.16


HE
PAS/AB
PSR (BL)
PSR (F)
DAPI
Non-coated scaffold +rBM-MSCs
FN-coated scaffold +rBM-MSCs
Non-coated scaffold+rCHs
A
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Discussion

The discussion is divided into two parts: the first part is about the clinical results.

First article

The first article, as previously described, examines imaging and Functional Outcomes After a Actifit® (Polyurethane Meniscal Scaffold).

The most important finding of this investigation was the good functional outcomes of the Actifit® scaffold at a minimum of 5-years of follow-up. That finding confirmed the main hypothesis of the study. A second and surprising finding of the present study was the small increase in meniscal tissue (in terms of volume) and the incomplete in-growth of new meniscus-like tissue, like the native one, promoted by the scaffold (as measured by the Genovese score). These last data refused our second hypothesis. Dhollander et al.[91] have investigated a large series of patients that received an Actifit® scaffold for a symptomatic partial meniscus defect at a minimum of 5-year follow-up. Similar to our results, the clinical outcomes were rated as good. In that series, the greatest clinical improvement obtained takes place during the first 2 years postoperatively[92] and most of the scores do not improve further, instead they keep on stable. Schüttler et al.[78] also reported the results of a series of 18 medial Actifit® at 2 years of follow-up. They found statistically significant improvements in functional outcomes compared to baseline, in all but one patient. Similarly, the activity level did not improve beyond 2 years after index surgery in this series. Some other authors have also shown great clinical improvement with this scaffold in the short-term follow-up[93–95] even in cases of advanced cartilage injuries[96]. All these results were quite similar to those reported for the CMI scaffold at similar follow-up periods[97,98]. Additionally, as the CMI has been used for a longer period, good functional outcomes results have been also shown at 10-year follow-up[99,100].

The scaffold morphology and the MRI appearance have been also assessed in the present investigation by using the Genovese score[101]. In all but 3 cases, the shape of the new meniscal tissue presented a decreased volume and irregular shape at a 5-year period. In the remnant 3 cases, the scaffold was simply re-absorbed. These results are similar to

those encountered by Dhollander et al. and, more recently, by Leroy et al. using the Actifit® scaffold[102,103] and those observed in a systematic review of the CMI scaffold at 5- and 10-years of follow-up[104]. In all these series' most of the scaffolds were classified as Genovese type IIb meaning scaffolds that have a decreased and irregular shape. However, this type of grading would include a wide range of meniscus–like tissue. To further refine these common findings, in the present series a volumetric study of the new meniscal tissue has been introduced. This type of MRI measurement was first introduced by Narvy et al.[105], they aim to categorize the meniscus size in non-cadaveric knees. The TMV obtained (scaffold plus remnant meniscus) at the final follow-up was not significantly superior to the one observed prior to the scaffold implantation. This data objectively defines that the type IIb meniscus observed in the present series was closer to a situation of re-absorbed scaffold rather than a normal meniscus.The signal intensity of the new meniscal tissue was also studied with the Genovese score. While in the series of Dhollander et al[91] 60% of cases presented a markedly hyper-intensity of the scaffold (Type I), in our series, most of the scaffolds were rated as type II (slightly hyper-intense) and only 2 cases, showed a markedly hyper-intensity of the signal. In the systematic review of the MRI evaluation of CMI series, Zaffagnini et al.[104] observed that 55.6% of scaffolds were rated as type II, 11.1% as type I and only 33.3%, presented a similar MRI-intensity (type III) than a normal meniscus, at 5-years after index surgery. However, at 10-years follow-up, the number of CMI scaffolds showing a normal Genovese type III (iso-intense) signal decreased to 11.1%. Therefore, it seems that the collagen scaffold had a slow maturation and remodelling process that ends up at 5-years follow-up. However, the 10-years results showed a worsening of those results suggesting a possible degeneration process of the scaffold beyond this period of time. In the case of the polyurethane scaffold Actifit®, the maturation process lasts around 5-years, due to the policaprolactone long resorption process. Therefore, in theory the quality and amount of meniscal tissue observed at this moment should be definitive. Further studies with longer follow-up will response this question. The importance of the menisci extrusion on the knee function has raised some concern in previous literature. The use of scaffolds has also been related to extrusion although some published series do not investigate this phenomenon[95,102]. Therefore, the amount of available information in this

respect is scarce. Faivré et al[106] observed that the preoperative menisci extrusion had a direct correlation with the Actifit® extrusion at 2-years after the implantation. They reported a mean extrusion of 4 mm and 3.4 mm at 1- and 2-years of follow-up, respectively. In the current series, the mean extrusion at 5-years of follow-up was found to be 2.78 mm. This figure is considered no or minor extrusion as it is below the widely accepted extrusion threshold (3 mm)[107]. This finding suggests that the new meniscal tissue was doing its function. However, the still not normal aspect of these new meniscus 5-years after implantation causes concern. Finally, the amount of tissue needed to maintain the compartment healthy and without pain remains a mystery this paper cannot solve. The present investigation has several limitations. First of all, the small sample size, although similar to previously published series. Secondly, although prospective the lack of a control group for comparison should be acknowledge as a limitation. Thirdly, and probably one of the most important, the impossibility to assess all the series at the final follow-up with a MRI. However, we were able to scan more than 50% of our patients at a period of 5 years after surgery.

Second article

In this second article has been evaluated the preoperative meniscal remnant extrusion and its correlation with the postoperative scaffold extrusion in a large group of patients. The secondary aim was to assess whether scaffold extrusion has an influence on the clinical outcomes at the 2- year follow-up. The main finding of this study was that, independently of the preoperative MRE, all the patients had major SE at 2-year follow-up. Secondly, the WOMET score showed a high correlation with the increment of the SE observed over the studied period. When it comes to comparing preoperative and postoperative extrusion after a polyurethane scaffold implantation, De Cornick et al[90] observed preoperative MRE of 2.17 mm (SD 0.84) in a series of 26 patients. This extrusion increased to 4.45 mm (SD 0.89) at 3 months postoperatively and remained steady afterward. Similarly, Faivre et al[106] observed preoperative MRE of 2.7 mm that increased to 4 mm at the 1- year follow-up and it decreased to 3.4 mm at the 2-year follow-up. Conversely, in the current investigation with a considerably higher number of cases, that finding is not confirmed. In the series presented here, postoperative ME does not depend on preoperative MRE. Surprisingly,

those patients with minor MRE in the preoperative period achieve SE similar to those patients with major preoperative MRE. It is possible that once the extrusion process has been initiated, it only settles down when it reaches around 4 mm. Again, this was not only observed in the current study but also in the series by Faivre et al and De Cornick et al.[90,106]. These results are like those observed with meniscal allograft transplantation (MAT) at short follow-up.[108] They also observed around 30% of ME at 3-year follow-up after MAT. However, the indications for meniscal scaffolds and meniscal allografts are different. While a polyurethane scaffold can only be implanted in a meniscal tissue remnant, the MAT procedure is done in a previous complete meniscectomized compartment. Thus, an assessment of prior MRE in patients with performed MATs is not possible. From the clinical standpoint, all the patients showed a similar improvement in terms of the functional scores evaluated at the 2-year follow-up. This is also in agreement with those previously reported series[90,109]. In series with a longer follow-up[78,110]. Functional improvements were also observed. However, as the MRI aspect worsened and the size considerably decreased, its potential chondroprotective effect was questioned. In the current series, it was observed that the mean WOMET score and the degree of extrusion had a positive correlation between the pre- and postoperative evaluations. The WOMET score is a meniscus-specific evaluation tool that was not used in those two referenced studies[90,106]. In the same way as in other more generic knee scores were used. ME is a risk factor for cartilage loss and knee osteoarthritis. For this reason, it is a challenge for surgeons to prevent or diminish graft or implant extrusion in patients undergoing meniscal substitutions. Different methods have been described to prevent extrusion in MAT. A small reduction in the size of the allograft,[111] osteophyte excision[112], or a concomitant caspulodesis[112] has been shown to decrease allograft extrusion. However, no technical recommendation has yet been described to control SE. Although this is the largest series reporting on extrusion of meniscal scaffolds, it has some weaknesses. The patients were studied retrospectively and followed up for only 2 years. While the later can be obviously questioned from a functional point of view, the extrusion process is known to end a few months after the meniscal substitution. The fact that the extrusion was only studied in MRI coronal views with a single slice extrusion assessment performed in the supine position

can also be questioned. Other authors have described alternative methods to study the extrusion on the sagittal and axial planes[112]. However, a recent study concluded that the method used in the current investigation correlates most closely with the true perpendicular extrusion measurements obtained from manually segmented models[112]. In this study, cartilage pathology or osteoarthritis progression was not checked since the follow-up was too short to analyse these parameters. However, it would be interesting to follow these patients longer to check the possible effect SE has on the progression of cartilage degeneration. In conclusion, the SE observed at the 2-year follow-up after the implantation of a polyurethane scaffold did not depend on preoperative MRE (major or minor extrusion). The WOMET score, which was the only meniscal-specific functional scored used, detected some inferior results in the most extruded meniscal scaffolds. This is basically a radiological study that has attempted to analyse the effect previous MRE may have on postoperative SC. The clinical differences observed at the 2- year follow-up that were assessed with a specific-meniscus score should be taken with caution and might be analysed again with a longer follow-up study to confirm that the SE could be implied with the final clinical outcomes.

Third article

The third article (Acepted 11/2021 | Regenerative Therapy) and the fourth (in submission) addendum to this thesis concern the laboratory part.

Regarding the third article we know, from previous literature (and our first article), clinical improvement and tissue formation in the short term. In contrast, in long-term studies, the new tissue decreased in volume and assumed an irregular shape. Moreover, in some cases, the scaffold was totally reabsorbed, without new tissue formation. Mesenchymal stem cells (MSCs) are a promising tool for tissue regeneration because of their multipotency. In this sense, MSCs can be combined with scaffolds that display an optimal degradation/reabsorption rate and bioactivity. This combination represents a promising approach for meniscus repair. In this work, we aimed to study the behaviour of MSCs on Actifit® in vitro, in comparison to chondrocytes. We also wanted to investigate the effect of a fibronectin (FN) coating on this behaviour. We seeded rabbit bone marrow

mesenchymal stem cells (rBM-MSCs) and rabbit chondrocytes (rCHs) over non-coated and FN -coated scaffolds-

We describe a protocol for rBM-MSCs culture (that is independent of the effect of FBS batches), proliferation, and differentiation into chondrocytes. We seeded rBM-MSCs on a polycaprolactone-polyurethane scaffold coated with FN and cultured them in a chondrocyte differentiation medium. In these conditions, they were able to attach, proliferate, and differentiate, producing an ECM that resembles the one produced by chondrocytes. It is well-known that synthetic polymers have higher lifespan than biological ones, but they present the disadvantage of displaying lower biocompatibility[112]. However, several works demonstrated that chondrocytes and fibrochondrocytes adhere and proliferate over synthetic polymers, producing an ECM that resembles the meniscal one, both in animal models[113,114], and in humans[115,116]. We observed similar results *in vitro* with chondrocytes. Nevertheless, we also showed that the bioactivity of the scaffolds for rBM-MSCs is much lower. In particular, rCHs proliferated over non-coated scaffolds forming colonies and synthetizing ECM, whereas rBM-MSCs proliferated much less and did not produce ECM. This discrepancy between *in vivo* and *in vitro* data could indicate that the cells colonizing the scaffold *in vivo* are differentiated cells migrating from adjacent areas of the meniscus, rather than MSCs from the synovial fluid. MSCs presence in synovial fluid increases after some knee pathologies or surgical procedures[117,118] and their presence is suggested to play a role in the healing of defects such as meniscal tears. However, it is still not clear, whether the effect is a direct action of their proliferating and differentiating capacity or is rather mediated by secretion of trophic and immunomodulation factors[119]. Our results suggest that in surgeries for Actifit® implantation, the benefit of MSC presence will not be related with their direct scaffold colonization and regeneration capacity, as their ability to adhere to Actifit® is low, but rather to a paracrine effect. Integrins are adhesion receptors mediating cell-cell and cell-matrix interactions. They are not only implicated in cell binding but also in intracellular signalling. MSCs lack several integrins that are present in chondrocytes (13). This could prevent MSCs to adhere and proliferate over non-coated scaffolds. However, fibronectin receptor (integrin alpha5beta1) is expressed by undifferentiated MSCs[119], allowing their interaction with FN-coated scaffolds, that will

exhibit an improved bioactivity in terms of attachment and/or proliferation. Achatz et al. tested MSCs behaviour on Actifit®[119] and they observed excellent cell distribution though the polyurethane scaffold, with more than 75% of pores being cell-populated, extensive production of proteoglycans and collagen type II, and moderate production of collagen type I. The different protocol for cell seeding could explain the differences between their results and ours. While we worked on natural cell diffusion throughout the scaffold, Achatz et al. loaded MSCs using a rotary valve vacuum pump, and therefore forcing the cells to occupy pores in the centre of the scaffold. This way, MSCs located in pores and not adhered to the material were not removed when culture media was changed, as it happened instead with our protocol. Once the embedded MSCs differentiate to chondrocytes, they could proliferate all along the scaffold and produce ECM. We selected this loading protocol to evaluate the capacity of rBM-MSC to migrate and invade all the material. Unfortunately, this could not be evaluated because material was lost along the histology protocol. The current work presents several limitations. First, we could not evaluate the colonization of the scaffold because it did not bind properly to the slide glass, so it was hardly observed in the histology preparations. Longer cultured time that allows rBM-MSC migration, cell differentiation, and ECM production could improve adherence of the material. Furthermore, other histology techniques, such as Methyl-methacrylate[119], used for hardness materials, could increase integrity of the sections obtained. Second, the experiments have been performed with rBM-MSCs harvested from a low number of donors (only two individuals). Nevertheless, the results are consistent between both cell lines. Finally, we used an animal model that is distant from humans on the phylogenetic scale. However, the expression of integrins in human MSCs has been extensively studied and fibronectin receptors are present in these cells. In conclusion, in orthopaedic surgery of meniscal injuries, we hypothesized that scaffolds are colonized by differentiated cells (fibrochondrocytes and chondrocytes) migrating from adjacent areas of the meniscus. However, chondrocytes have low proliferative capacity and, furthermore, hypertrophic chondrocytes have altered protein expression, producing aberrant ECM that finally leads to the apoptosis of the cells[119]. This phenomenon might be behind the failure of the scaffold at long run[110]. The use of FN-coated scaffold allows MSC to attach to Actifit® *in*

vitro. This finally leads to MSC differentiation into new cells producing ECM like the original cells. Therefore, our results could have crucial implications in the clinical use of the scaffolds.

Conclusions

First article

Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up. *Monllau JC, Poggioli F, Erquicia J, Ramírez E, Pelfort X, Gelber P, Torres-Claramunt R*. Arthroscopy. 2018.

The main conclusion of this study was that the use of polyurethane meniscal scaffold in patients with symptomatic meniscus deficit leads to a good functional outcome at 5-years after surgery. However, the MR-imaging aspect of the new meniscal tissue is far from a native meniscal tissue and the volume of the new tissue is far less than expected.

Second article

Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function? *Gelber PE, Torres-Claramunt R, Poggioli F, Pérez-Prieto D, Monllau JC*. J Knee Surg. 2020 May 25.

The SE observed at the 2-year follow-up after the implantation of a polyurethane scaffold did not depend on the pre-operative MRE (major or minor extrusion). The WOMET score, which was the only meniscal-specific functional scored used, detected some inferior results in those most extruded meniscal scaffolds

Third article

Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold. *Raquel Arredondo, Francesco Poggioli, Santos Martínez, María Piera, Raúl Torres, Laura Tío, JC Monllau*

In orthopaedic surgery of meniscal injuries, we hypothesized that scaffolds are colonized by differentiated cells (fibrochondrocytes and chondrocytes) migrating from adjacent areas of the meniscus. However, chondrocytes have low proliferative capacity and, furthermore,

hypertrophic chondrocytes have altered protein expression, producing aberrant ECM that finally leads to the apoptosis of the cells. This phenomenon might be behind the failure of the scaffold at long run. The use of FN-coated scaffold allows MSCs to attach to Actifit® in vitro. This finally leads to MSC differentiation into new cells producing ECM similar to the ones produced by chondrocytes. Therefore, our results could have crucial implications in the design of scaffolds to improve their clinical use in tissue regeneration and functionality following orthopaedic surgery.

Copy of the papers

Magnetic Resonance Imaging and Functional Outcomes After a Polyurethane Meniscal Scaffold Implantation: Minimum 5-Year Follow-up

Joan C. Monllau, Ph.D., Francesco Poggioli, M.D., Juan Erquicia, M.D., Eduardo Ramírez, M.D., Xavier Pelfort, Ph.D., Pablo Gelber, Ph.D., and Raúl Torres-Claramunt, Ph.D.

Purpose: To report the magnetic resonance imaging (MRI) and clinical outcomes at a minimum 5-year follow-up in a series of patients with postmeniscectomy syndrome and treated with a polyurethane scaffold. **Methods:** All consecutive patients operated on from September 2008 to February 2011 for either persistent medial or lateral joint line compartmental pain receiving a polyurethane scaffold due to a previous partial meniscus resection with a minimum 5-year follow-up were included. Functional scores (Knee Injury and Osteoarthritis Outcomes Score, International Knee Documentation Committee, Lysholm, and Tegner) were assessed preoperatively and at the last follow-up. The state of the scaffold as well as postoperative scaffold extrusion and the total remaining meniscal volume was also evaluated in MRI. **Results:** Thirty-two patients were included. The mean follow-up was 70.8 ± 7.5 months. The functionality of the knees improved in all the scores used ($P < .001$) except for the Tegner score that stayed steady. Most of meniscal implants showed extrusion of 2.4 mm (95% confidence interval [CI], 1.1-3.7) were smaller and a hyperintensity signal was seen in the MRI. Three scaffolds were resorbed at the last follow-up. The meniscal volume, determined by MRI, was 1.14 cm^3 (95% CI, 0.96-1.31) preoperatively and 1.61 cm^3 (95% CI, 1.43-1.7) at the last follow-up. No differences were presented. **Conclusions:** The use of a polyurethane meniscal scaffold in patients with a symptomatic meniscus deficit had a good functional outcome at 5 years after surgery. However, the implanted scaffolds did not present normal meniscal tissue with MRI, and the implant volume was considerably less than expected. The fact that most of patients included received different concomitant procedures during scaffold implantation introduces a degree of performance bias into the results. **Level of Evidence:** Level IV, case series.

See commentary on page 1628

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The authors report the following potential conflicts of interest or sources of funding: J.C.M. receives consultancy fees from ConMed-Linvatec and Surgival and receives payment for lectures including service on speakers bureaus from ConMed-Linvatec and Smith & Nephew. P.G. receives consultancy fees from ConMed; is employed in Hospital Sant Pau; receives payment for lectures including service on speakers bureaus from ConMed; and receives travel/accommodations/meeting expenses from CONMED. Full ICMJE author disclosure forms are available for this article online, as supplementary material.

Received June 22, 2017; accepted December 15, 2017.

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0749-8063/17776/$36.00

https://doi.org/10.1016/j.arthro.2017.12.019

Postmeniscectomy syndrome is defined as pain occurring in a previously meniscectomized knee compartment. It is believed that the pain is the result of overload due to meniscal tissue loss.[1-7] Although several treatments can be considered, the etiologic approach seems to be the restitution of the lost meniscal tissue. This can be accomplished with either meniscal allograft transplantation or a meniscal scaffold, depending on whether the meniscus defect is complete or partial.

In the last decade, meniscal scaffolds have been shown to successfully treat symptomatic partial meniscal defects, providing pain relief and restoring the function of the knee. Two scaffolds have been marketed until now. The Collagen Meniscal Implant (CMI, ReGen Biologics, Franklin Lakes, NJ), a bovine collagen scaffold, was the first to market with good results in series that has more than 10 years of follow-up.[8,9] More recently, a biodegradable and synthetic acellular scaffold composed

of aliphatic polyurethane has been introduced (Actifit; Orteq Ltd., London, UK). This new scaffold aims to improve some of the limitations of the bovine scaffold. These limitations were its rapid degradation, being of bovine origin, and difficulty in its handling.[10] The ultrastructure of this scaffold is characterized by its 80% porosity and 20% low reabsorption rate polymer. Within the polymer, there are softer polycaprolactone segments that constitute 80% of the polymer and the remaining 20% is a more rigid urethane. Degradation starts with hydrolysis of the polycaprolactone segments that lasts up to 5 years. The polyurethane segments are removed by macrophages and giant cells, whereas the scaffold is replaced by cells coming from the surrounding tissues.[10-12] Dhollander et al.[13] have recently published the first polyurethane scaffold implantation series with a minimum 5-year follow-up. They reported improved knee joint function and pain relief. However, they questioned its theoretical chondroprotective effect and observed that almost 40% of the implants failed. These data agree with those reported by Schüttler et al.[14] with a 4-year follow-up. Although these 2 studies reported good functional outcomes at mid-term follow-up, they do not confirm the good imaging aspect and low failure rate reported in the short follow-up studies.[15-18] To date, only these 2 series on a polyurethane meniscal scaffold with mid-term results have been published. It is important that more clinical and radiological data with at least a 5-year follow-up be published to better understand the true effect of the scaffold as a meniscal substitute.

The aim of this study was to report the magnetic resonance imaging (MRI) and clinical outcomes at a minimum 5-year follow-up in a series of patients with postmeniscectomy syndrome and treated with a polyurethane scaffold. Total meniscal volume (TMV), measured in MRI, was considered the primary variable. It was hypothesized that the scaffold would be able to improve on pain relief and knee function as well as be replaced by new meniscus-like tissue according to MRI.

Methods

This is a retrospective study that included all consecutive patients who were operated on from September 2008 to February 2011 for either persistent medial or lateral joint line compartmental pain receiving a polyurethane meniscal scaffold due to a previous partial meniscus resection. Those patients with a complete loss of the corresponding meniscus, symptomatic grade III or IV chondral injury in whatever knee compartment, untreated instability, untreated varus or valgus malalignment greater than 5°, inflammatory arthritis, polyurethane allergies, autoimmune disease, and pregnancy were excluded. All the patients who were finally included in the study were called up for clinical and MRI evaluation. The presence of anterior and posterior meniscus remnants as well as an intact outer rim of the meniscus was the necessary condition for the procedure. An anterior cruciate ligament–deficient knee was not considered a contraindication if the ligament was reconstructed at the same time as the polyurethane scaffold implantation. Similarly, varus knees were not a contraindication if the malalignment was addressed previously or concomitantly with meniscal substitution.

The study was approved by the clinical research ethics committee of our institution (Dex-Actifit). All the patients signed informed consent to participate in the study as well as for the evaluation and publication of the results.

Surgical Technique

The implantation of the meniscal scaffold was performed with a fully arthroscopic technique through standard anterolateral and anteromedial portals. The remaining meniscus was trimmed and trephinated by using an intramuscular needle from inside-out to the joint capsule to create multiple bleeding areas. Radiofrequency was also used at the synovial junction to promote a healing response.[19] The meniscal defect was measured with a ruler and the scaffold was oversized by 10% to better fit in the defect, as recommended. The implant was fixed with nonabsorbable sutures, either with an all-inside suture in the posterior horn or with an outside-in suture at the body and anterior horn, when necessary. Data on scaffold length and the number of all-inside and outside-in stitches needed were collected.

Subsequently, concomitant surgical procedures were performed when called for (Table 1).

Postoperative Protocol

Passive and active range of motion were started on the first postoperative day. Flexion was limited to 60° the first 3 weeks and progressed to 90° until the sixth postoperative week. From that moment on, unrestricted range of motion was allowed. A locked brace was used in all cases until correct muscle control had been acquired. Partial weight bearing was allowed from the fourth postoperative week and full weight bearing

Table 1. Concomitant Surgical Procedures

Surgical Technique	n
Isolated polyurethane scaffold implantation	7
Microfractures	3
ACL-R	6
ACL-R + microfractures	2
HTO	3
HTO + microfractures	9
PCL-R	1
ACL-R + HTO	1

ACL-R, anterior cruciate ligament reconstruction; HTO, high tibial osteotomy; PCL-R, posterior cruciate ligament reconstruction.

the eighth week after the surgery. Unrestricted physical activity and sports were allowed after the sixth postoperative month. This protocol was modified and adapted in case of concomitant surgical procedures.

Functional Evaluation

The patients included in the study were evaluated preoperatively and at the last follow-up. For this, the patients included in the study were called up for a clinical evaluation. This evaluation was performed with patient-reported outcome scores: the Knee Injury and Osteoarthritis Outcomes Score (KOOS), the International Knee Documentation Committee (IKDC), the Lysholm score, and the Tegner score.[20-22] Patient satisfaction was evaluated with a subjective score and graded as very satisfied (4 points), satisfied (3 points), neutral (2 points), somewhat dissatisfied (1 point), and not satisfied at all (0 points).

MRI Evaluation

A T2 mapping MRI was performed preoperatively and at the 5-year follow-up with a 1.9-Tesla MRI device using gradient-echo T2-weighted, spin-echo T1-weighted, fat saturation fast spin-echo, and T2-weighted sequences in coronal, sagittal, and transverse slice orientations. Scaffold morphology was evaluated based on the method described by Genovese et al.[23] This score evaluates the morphology and size of the scaffold. A type I means totally resorbed scaffold, a type II is a small scaffold with regular (a) and/or irregular (b) morphology, and a type III scaffold with the same size and shape as the normal meniscus. The method also assesses the signal intensity of the scaffold. Type I is markedly hyperintense, type II is slightly hyperintense, and type III is isointense when compared with the normal meniscus. Meniscus extrusion was measured on the coronal view as described by De Coninck et al.[24] Further assessment of remnant meniscal regrowth was performed by calculating the TMV. This was accomplished with the "ROI segmentation" feature integrated into the OsiriX v7.0.4 Lite software. The overall volume of each meniscus was calculated by using all the coronal MRI slices of the meniscus (Fig 1A). Initially, the appropriate range of image signal intensities based on the gray-level index values of the pixels within each meniscus was selected. After that, a colored marker image was used to demarcate the boundaries of the meniscus. The overall volume (cm^3) of the menisci was calculated using the total surface areas by obtaining a 3D image for each meniscus (Fig 1B). This method was previously used by Narvy et al.[25]

Statistical Analysis

Statistical analyses were performed using STATA/SE 12.1 (Stata, College Station, TX). Categorical variables are expressed as percentages and frequencies. Mean and standard deviations as well as medians, minimums, and maximums were used for each item of descriptive data. Mean and 95% confidence intervals (CIs) were used for each continuous variable. Mean and standard deviations as well as medians, minimums, and maximums were calculated for each continuous variable. The results were statistically analyzed and compared using the Student *t*-test for parametric data with normal distribution. The level of significance was set at $<.05$.

Results

No patient was lost to follow-up. There were 25 males and 7 females with a mean age of 41.3 ± 11.1 (range 23-60) years at the time of index surgery. The mean follow-up was of 70.2 ± 7.5 (range 63-93) months. Considering the TMV as the main variable, a post hoc power analysis was performed. With an alpha error probability of .05 and with an effect size of 0.8, a power of 100% was obtained.

There were 18 left and 14 right knees. Of those, 21 were medial and 11 lateral. The average length of the

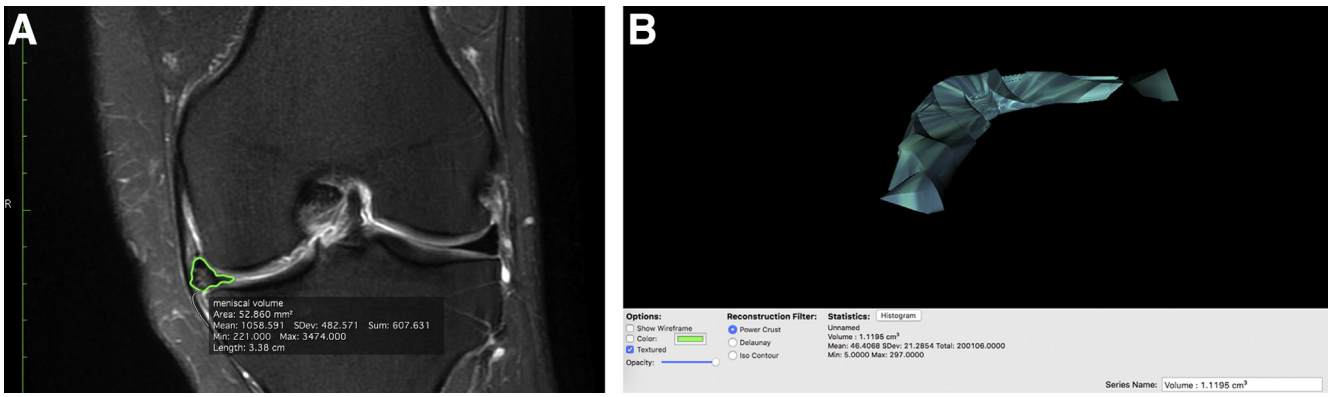

Fig 1. An example of a left knee medial meniscus supplemented with a polyurethane scaffold. (A) Total meniscal volume was calculated using coronal magnetic resonance imaging slices. The meniscus tissue was marked in green. (B) The 3D image obtained in one of the menisci studied.

Table 2. Functional Outcomes and Satisfaction

	Preoperative	Final Follow-up	*P* Value
KOOS	48.6 (95% CI, 44.3-53)	79.4 (95% CI, 76.9-82)	$P < .001$
IKDC	41.7 (95% CI, 38.1-45.4)	68.7 (95% CI, 65.4-72.1)	$P < .001$
Lysholm	40.7 (95% CI, 36.8-44.7)	78.1 (95% CI, 74.7-81.6)	$P < .001$
Tegner	5.1 (95% CI, 4.6-5.6)	5.7 (95% CI, 5.3-6.2)	$P = .23$

CI, confidence interval; IKDC, International Knee Documentation Committee; KOOS, Knee injury and Osteoarthritis Outcomes Score.

implant was 45 mm ± 7.6 mm. Fixation of the polyurethane scaffold implant required a mean of 3.5 ± 0.7 all-inside sutures and 0.8 ± 0.7 outside-in sutures. No patients suffered any complication during and/or after scaffold implantation. There were no complications related to scaffold implantation in any patient. Additional or combined procedures were performed in all but 7 patients (Table 1), high tibial valgus osteotomy (HTO) being the most frequent. The preoperative mechanical axis in those patients who underwent HTO realignment averaged 6° ± 5° of varus. After the surgery, the mechanical axis in the 12 patients who required the HTO was 0.5° ± 2° of varus. Eight patients were operated during the follow-up period. Five patients who had been previously operated with an HTO underwent a plate removal procedure in the follow-up period. Three patients needed a new surgery to remove the scaffold. Those 3 scaffolds presented no signs of meniscal tissue in-growth.

Functional scores are summarized in Table 2. The KOOS, IKDC, and Lysholm scores significantly improved at the last follow-up. However, the Tegner score showed no differences at that time. Patient satisfaction with the procedure scored a mean of 3.3 (95% CI, 3.13-3.47) (range 2-4).

MRI at baseline and at the last of follow-up was performed on 19 of the 32 patients (60%) included in the study. The remaining patients either rejected or did not consent to having an MRI at that moment. Sixteen of the 19 postoperative MRIs showed a mean 2.4 mm (95% CI, 1.12-3.68) of scaffold extrusion after 5 years of follow-up. Complete reabsorption of the meniscal scaffold was observed in the other 3 cases. With regard to the MRI scaffold shape and morphology, these 3 cases were classified as Genovese type I. The other 16 cases were classified as type IIb. Relative to MRI signal intensity, 4 patients showed isointensity (Genovese type III), 2 cases markedly hyperintensity (type I), and the remaining 10 patients presented a slight hyperintensity (type II) of the scaffold.

The TMV estimate was 1.14 cm^3 (95% CI, 0.96-1.31) before surgery (meniscal remnant), whereas it was 1.61 cm^3 (95% CI, 1.43-1.79) at the last follow-up (n.s.). Figure 2 shows an arthroscopic image of the meniscal tissue observed 4 years after scaffold implantation. It is possible to observe the incomplete in-growth of new meniscus-like tissue in the polyurethane scaffold.

Discussion

The main finding of this study was the degree polyurethane meniscal scaffold resorption observed at a minimum 5-year follow-up. The small, statistically nonsignificant increase in meniscal tissue (in terms of volume) and the incomplete in-growth of new meniscus-like tissue promoted by the scaffold (as measured by the Genovese score) affirmed that finding. Therefore, these data do not support our main hypothesis. Secondarily, good functional outcomes were observed after implantation of a polyurethane scaffold at a minimum of 5 years of follow-up. That finding confirms the second hypothesis of the study.

Dhollander et al.[13] investigated a large series of patients who had a polyurethane scaffold implanted due to a symptomatic partial meniscus defect at a minimum 5-year follow-up. Similar to our results, the clinical outcomes were rated as good. In that series, the greatest improvement was achieved during the first 2 years postoperatively[24] and most of the scores stayed at the same level afterward. Schüttler et al.[14] also reported the results of a series of 18 medial polyurethane scaffolds with a minimum of 2 years of follow-up. They found improvements in functional outcomes, compared with baseline, in all but 1 patient. Similarly, the activity level did not improve beyond 2 years after the index surgery. Some other authors have also reported great clinical improvement with this scaffold in the short-term follow-up,[16,18,26] even in cases of advanced cartilage injuries.[17]

Interestingly enough, these results were comparable to those reported for the CMI scaffold at similar follow-up periods.[27-29] Although the polyurethane implant has been available for a shorter period of time, there are already long-term follow-up studies reporting favorable outcomes with the use of the CMI.[8,9,30]

Scaffold morphology and the MRI aspect were assessed with the Genovese score.[23] In all but 3 cases, the shape of the new meniscal tissue showed decreased volume and an irregular shape at the 5-year follow-up. In the remaining 3 cases, the scaffold was completely reabsorbed. These results are similar to those found in the Dhollander et al. study, in the Leroy et al. polyurethane scaffold studies,[13,31] as well as in those observed in a systematic review of the CMI scaffold at 5- and 10-year follow-ups.[32] In all these series, most of the scaffolds were classified as Genovese type IIb, which

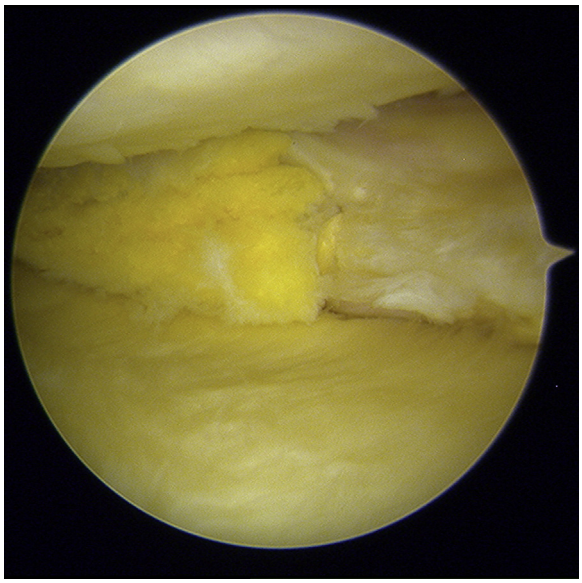

Fig 2. An arthroscopic image of a new meniscus tissue obtained 4 years after polyurethane scaffold implantation. The image corresponds to a medial meniscus of a left knee viewed from the anterolateral portal.

correspond to scaffolds with a decrease in size and with an irregular shape. However, this type of grading would include a wide range of meniscus-like tissue. In an effort to further refine these common findings, a volumetric assessment of the new meniscal tissue was also performed. This type of MRI measurement was first introduced by Narvy et al.[25] They aimed to categorize the meniscus size in noncadaveric knees. The TMV obtained (scaffold plus remnant meniscus) at the final follow-up was not significantly superior to the one observed before scaffold implantation. These data objectively define that the type IIb meniscus observed in the present series was closer to a situation of reabsorbed scaffold rather than a normal meniscus, which also questions the utility of the Genovese score to grade meniscal shape and size. This scale has also been questioned by a previous study of the CMI scaffold.[33]

The Genovese score was also used to assess the signal intensity of the new meniscal tissue. Dhollander et al.[13] observed that 60% of the cases showed a hyperintensity of the scaffold (type I). In our series, most of the scaffolds were rated as type II (slightly hyperintense) and only 2 cases showed marked signal hyperintensity. In the systematic review of the MRI evaluation of CMI series at 5 years after index surgery, Zaffagnini et al.[34] observed that 55.6% of the scaffolds were rated as type II, 11.1% as type I, and only 33.3% had a similar MRI intensity (type III) to a normal meniscus. However, at 10-year follow-up, the number of CMI scaffolds showing a normal Genovese type III (isointense) signal decreased to 11.1%. Although the collagen scaffold did not mature rapidly and the remodeling process was slow in the period up to the 5-year follow-up, the 10-year results showing a worse MRI aspect suggest a possible degenerative process of the scaffold at work. In the case of the polyurethane scaffold, the maturation process lasts around 5 years. It is due to the long policaprolactone resorption process.[12] Thus, the quality and amount of meniscal tissue observed at this moment should be definitive. To sum up, the MRI study of these scaffolds at the minimum 5-year follow-up showed a small increment in the amount of meniscal tissue. Furthermore, this scaffold is partially extruded and its radiological appearance is not as it initially seems.

Limitations

The present investigation had several limitations. There was no control group and the sample size was small even though it is comparable to previously published series. The fact that just 60% of patients consented to an MRI at the last follow-up is another limitation. An important limitation is that the series included patients who underwent concomitant procedures to address not only their meniscal problems but also concurrent deficiencies. This is an obvious and important limitation that makes a more accurate assessment of the scaffold implantation difficult because combined procedures introduce a degree of performance bias into the results. Finally, the fact of not studying cartilage status with the MRI and the fact of not including different observers to test inter-rater reliability in the MRI analysis can also be considered limitations.

Conclusions

The use of a polyurethane meniscal scaffold in patients with a symptomatic meniscus deficit leads to a good functional outcome at 5 years after surgery. However, implanted scaffolds do not show normal meniscal tissue with MRI and the implant volume is considerably less than expected. The fact that most of patients included received different concomitant procedures during scaffold implantation introduces a degree of performance bias into the results.

Acknowledgment

The authors thank Eric Goode for his help in correcting the manuscript.

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Polyurethane Meniscal Scaffold: Does Preoperative Remnant Meniscal Extrusion Have an Influence on Postoperative Extrusion and Knee Function?

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J Knee Surg

Abstract

Meniscal extrusion (ME) has been identified as a risk factor in the development of knee osteoarthritis. The relevance of this finding when a meniscal scaffold is used has not been extensively studied. The objective of this study was to determine whether preoperative meniscal remnant extrusion (MRE) was correlated with postoperative scaffold extrusion (SE) or with functional outcomes at the 2-year follow-up. Retrospective study included all polyurethane scaffolds implanted with a minimum 2-year follow-up. A magnetic resonance imaging (MRI) was performed preoperatively and postoperatively at 2 years. Extrusion was measured in millimeters in a coronal view. Patients were assigned to either group 1 or 2 depending on the preoperative MRE being either <3 mm (minor extrusion) or 3 mm (major extrusion). Functional outcomes were analyzed by means of the Western Ontario Meniscal Evaluation Tool (WOMET), International Knee Documentation Committee, Kujala and Tegner scores, as well as visual analog scale. Satisfaction was also documented. Sixty-two out of 98 patients were available to undergo an MRI at final follow-up. The mean age was 41.3 years (range, 17–58) and the mean follow-up was 45 months (range, 25–69). The mean preoperative MRE was 2.8 mm (standard deviation [SD] 1.2) and the mean postoperative SE was 3.8 mm (SD 1.8) ($p < 0.01$). All functional scores improved during the study period. When the correlation (Spearman's rho) between the difference in extrusion between the pre 26 and postoperative periods and their correlation with the different scores was assessed, correlation was only observed in the WOMET (rho 0.61, $p = 0.02$). The preoperative MRE in Group 1 was 1.85 mm (SD 0.83) and 3.7 mm (SD 2.2) in Group 2 ($p < 0.01$). At final follow-up, SE was 3.86 mm (SD 0.7) in Group 1, whereas it was 3.98 mm (SD 1) in Group 2 ($p = 0.81$). No differences were observed in the scores used for these two groups. The SE observed at the 2-year follow-up after the implantation of a polyurethane scaffold did not depend on preoperative MRE (major or minor extrusion). The WOMET score, which was the only meniscal-specific functional scored used, showed some inferior results in the most extruded meniscal scaffolds. This is a retrospective case series. Level of evidence is 4.

Keywords
- polyurethane scaffold
- Actifit
- meniscal extrusion
- meniscus
- knee

received
November 9, 2019
accepted
March 21, 2020

DOI https://doi.org/10.1055/s-0040-1710377.
ISSN 1538-8506.

The current trend in meniscal tear management is to preserve as much meniscal tissue as possible. However, this is not always possible. When pain appears in the meniscectomized compartment, restitution of the lost tissue is an option.[1] Meniscal scaffolds prove to be a valid alternative when there is a considerable loss of meniscal tissue but still there is some tissue remnant, specifically its peripheral rim.[2]

To date, two different meniscal scaffolds have been marketed for partial meniscal defects. One is the Collagen Meniscal Implant (ReGen Biologics; Franklin Lakes, NJ) and the other is the Actifit polyurethane scaffold (Orteq Ltd., UK). While good clinical results have been observed at the mid- and long-term follow-ups,[3–7] magnetic resonance imaging (MRI) assessment showed abnormal findings such as diminished morphology, signal intensity alteration, or cartilage degeneration progression.

Meniscal extrusion (ME) has been identified as a risk factor in the development of knee osteoarthritis. The lack of cartilage coverage by meniscal tissue affects load distribution capacities. This leads to the loss of cartilage and to subsequent knee osteoarthritis. De Coninck et al and Faivre et al[8,9] studied the impact of preoperative meniscal remnant extrusion (MRE) on the clinical outcomes of polyurethane scaffold implantation and on postoperative scaffold extrusion (SE). They viewed that preoperative and postoperative ME were not correlated with functional outcomes at the 2-year follow-up. Furthermore, they also observed that extrusion increased from the preoperative period to the postoperative follow-up. Both studies included a limited number of patients.

The main objective of this study was to evaluate whether the preoperative MRE was correlated with the postoperative SE in a large group of patients. The secondary aim was to assess whether SE has an influence on the clinical outcomes at the 2-year follow-up. The main hypothesis of this study was that preoperative extrusion of the peripheral rim would not correlate with postoperative SE. It was also hypothesized that the degree of extrusion would have no relationship to the functional outcomes.

Materials and Methods

A retrospective study was conducted to assess all the patients who had had a medial polyurethane scaffold implanted for a postmeniscectomy syndrome. The study was approved by the clinical research ethics committee of the institution.

A minimum follow-up of 2 years was required. All patients were operated on by the same surgical team (four surgeons). The same technique and similar postoperative protocols were used. Those patients with a malalignment of >5 degrees as well as patients with untreated knee instability were excluded. If the malalignment or the instability was corrected in the same surgical procedure, it was not considered exclusion criteria. Rheumatic diseases, polyurethane allergies, and pregnancy were other exclusion criteria.

Surgical Technique

The whole procedure was performed arthroscopically. A release of the medial collateral ligament was performed with a pie-crusting technique when necessary. The meniscus defect was trimmed to fit the scaffold. The polyurethane scaffold was prepared with an extra length of 5 to 10 mm of the measured defect to compensate for the effect of the horizontal sutures, which partially shrinks the polyurethane scaffold. Once in place, it was fixed to the posterior horn of the meniscal remnant using all-inside sutures. For the meniscal body or those corresponding to the anterior horn, an outside-in repair technique was used.

Radiological and Functional Measurements

An MRI was performed preoperatively and 2 years postoperatively and extrusion was then compared. All examinations were performed in the study hospitals, but measurements were made by the same experienced musculoskeletal radiologist who was blinded for the study purposes.

Extrusion (in mm) of the meniscal remnant was calculated in a coronal view, preoperatively (►**Fig. 1**). At the last follow-up MRI, the same measurements were performed to calculate SE. The chosen coronal view used to make measurements was defined as the single slice presenting the greatest area of the medial spine. If this was difficult to differentiate, the image which showed the greatest width of the tibia plateau was chosen. The medial–lateral meniscal coronal width and meniscal body extrusion to the closest 0.1 mm were measured. Regarding the definition of major and minor extrusion,[10,11] those patients with preoperative extrusion of <3 mm were included in Group 1, whereas those patients with a preoperative extrusion equal to or greater than 3 mm were included in Group 2.

Functional outcomes were analyzed by means of the Western Ontario Meniscal Evaluation Tool (WOMET), International Knee Documentation Committee, the Kujala and Tegner scores, as well as visual analog scale. They were assessed preoperatively and at the last follow-up visit. Satisfaction was assessed at the last follow-up. Patients were asked to rate their satisfaction with the end result of the surgery on a scale ranging from 0 to 4 (with 4 being the best score).

Statistical Analysis

Categorical variables were presented as frequencies and percentages. The mean and standard deviation (SD) were calculated for each continuous variable. The comparison between MRE and SE was assessed using the paired *t*-test.

The chi-square and the Wilcoxon's tests, depending on the case, were used to compare the pre and postoperative results of the different knee tests. Correlation coefficients between extrusion and the different scores and the differences between the post and preoperative periods were calculated using Spearman's rank correlation coefficient. The statistical analysis was performed using the SPSS 19 (SPSS Inc.; Chicago, IL) statistical package. The significance level was set at $p < 0.05$.

Results

During the period under review, a total of 98 polyurethane scaffolds were implanted. Four of them were not implanted in a postmeniscectomy syndrome and were thus excluded.

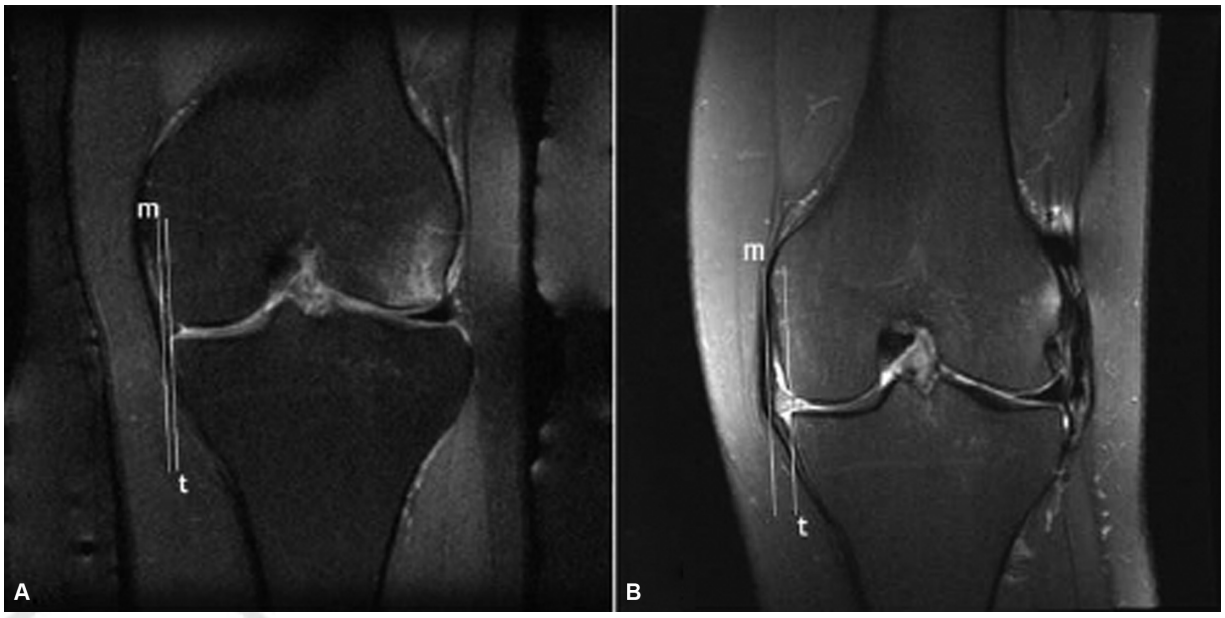

Fig. 1 MRI of a left knee. (**A**) Preoperatively, meniscal remnant extrusion was 2 mm (minor extrusion) in this case. (**B**) The postoperative MRI used to calculate scaffold extrusion showed that the scaffold was 5 mm (major extrusion) beyond the tibial margin. m, meniscal remnant or scaffold border; MRI, magnetic resonance imaging; t, tibial margin.

Table 1 Mean and standard deviation of the different scores in the pre and postoperative periods

	Preoperative	Postoperative	*p*-Value
WOMET	37.9 (SD 11.2)	67.6 (SD 20.4)	<0.01
IKDC	34.1 (SD 17)	70.4 (SD 16.8)	<0.01
Tegner	5.1 (SD 1.8)	4 (SD 1.8)	<0.01
Kujala	48.4 (SD 14.4)	83.3 (SD 16)	<0.01
VAS	7.22 (SD 1.2)	2.67 (SD 2.1)	<0.01

Abbreviations: IKDC, International Knee Documentation Committee; SD, standard deviation; VAS, visual analog scale; WOMET, Western Ontario Meniscal Evaluation Tool.

Table 2 Correlation (Spearman's rho) between the ME differences from the postoperative and preoperative periods when compared with the same differences in the functional scores

	ME difference	*p*-Value
WOMET differences	0.61	0.02
IKDC differences	0.08	0.79
Tegner differences	−0.1	0.72
Kujala differences	−0.06	0.84
VAS differences	−0.19	0.53

Abbreviations: IKDC, International Knee Documentation Committee; ME, meniscal extrusion; VAS, visual analog scale; WOMET, Western Ontario Meniscal Evaluation Tool.

Three patients with untreated concurrent knee instability were also excluded. In 29 patients, postoperative MRI was not available.

A total of 62 patients (46 men and 16 women) were then included. The median follow-up was 45 months (range, 25–69). The patients had a median age of 41.3 years (range, 17–58 years). Four patients who had been previously operated with a high tibial osteotomy underwent a plate removal procedure in the follow-up period. None of these patients needed a new surgery to remove the scaffold during this period.

The mean preoperative MRE was 2.8 mm ± 1.2 and the mean postoperative SE was 3.8 mm ± 1.8 ($p = 0.00$). All functional scores improved postoperatively (►**Table 1**). ►**Table 2** shows the correlation (Spearman's rho) between the differences in extrusion between the pre- and postoperative periods and their correlation with the different scores studied. An important correlation was observed in the WOMET (rho 0.61, $p = 0.02$).

Regarding the differences between the two groups, preoperative MRE in Group 1 was 1.85 mm (SD 0.83), whereas Group 2 had a mean preoperative MRE of 3.7 mm (SD 2.2) ($p < 0.01$). Patients from Group 1 had an SE at 2 years postoperatively of 3.86 mm (SD 0.7), whereas it was 3.98 mm (SD 1) in Group 2 ($p = 0.8$). No differences in the evaluated functional outcomes in either group in both periods under study (►**Table 3**) were found. Similarly, no differences were observed when the preoperative MRE (Group 1 vs. Group 2) was compared with the differences in the assessed scores.

Discussion

The main finding of this study was that, independently of the preoperative MRE, all the patients had major SE at 2-year follow-up. Secondly, the WOMET score showed a high correlation with the increment of the SE observed over the studied period.

When it comes to comparing preoperative and postoperative extrusion after a polyurethane scaffold implantation, De Cornick et al[8] observed preoperative MRE of 2.17 mm (SD

Table 3 Values for different scores assessed for Groups 1 and 2

	Group 1			Group 2		
	Preop	Postop	Rho/*p*-Value	Preop	Postop	Rho/*p*-Value
WOMET	40.55 (11.9)	67.6 (15)	−0.37/0.46	32.4 (13.8)	56 (25.5)	0.57/0.18
IKDC	35 (20.8)	68.7 (13.7)	−0.44/0.38	28.7 (16.8)	61.3 (20.9)	0.67/0.09
Tegner	5.7 (1.7)	4 (1.7)	0.64/0.16	5.3 (1.7)	3.7 (1.9)	−0.03/0.9
Kujala	48.7 (15.6)	81.75 (14.8)	−0.54/0.26	43 (14.6)	76.2 (20)	0.39/0.38
VAS	7.3 (0.8)	3.5 (1.9)	0.52/0.28	7 (0.7)	3.3 (1.7)	−067/0.09
Ahlbäck	1.5 (0.8)	1.5 (0.8)	−0.13/0.8	1.8 (0.9)	1.6 (0.9)	0.00/1.0
Satisfaction		2.9 (0.8)	−059/0.21		2.4 (1.1)	0.41/0.35

Abbreviations: IKDC, International Knee Documentation Committee; Preop, preoperative; Postop, postoperative; VAS, visual analog scale; WOMET, Western Ontario Meniscal Evaluation Tool.
Note: Mean and standard deviation. The *p*-value studied the correlation between the differences in the meniscal extrusion and the differences for each score studied.

0.84) in a series of 26 patients. This extrusion increased to 4.45 mm (SD 0.89) at 3 months postoperatively and remained steady afterward. Similarly, Faivre et al[9] observed preoperative MRE of 2.7 mm that increased to 4 mm at the 1-year follow-up and it decreased to 3.4 mm at the 2-year follow-up. Conversely, in the current investigation with a considerably higher number of cases, that finding is not confirmed. In the series presented here, postoperative ME does not depend on preoperative MRE. Surprisingly, those patients with minor MRE in the preoperative period achieve SE similar to those patients with major preoperative MRE. It is possible that once the extrusion process has been initiated, it only settles down when it reaches around 4 mm. Again, this was not only observed in the current study but also in the series by Faivre et al and De Cornick et al.[8,9] These results are like those observed with meniscal allograft transplantation (MAT) at short follow-up.[10] Abat et al[10] observed around 30% of ME at 3-year follow-up after MAT. However, the indications for meniscal scaffolds and meniscal allografts are different. While a polyurethane scaffold can only be implanted in a meniscal tissue remnant, the MAT procedure is done in a previous complete meniscectomized compartment. Thus, an assessment of prior MRE in patients with performed MATs is not possible.

From the clinical standpoint, all the patients showed a similar improvement in terms of the functional scores evaluated at the 2-year follow-up. This is also in agreement with those previously reported series.[8–14] In series with a longer follow-up,[6,7] functional improvements were also observed. However, as the MRI aspect worsened and the size considerably decreased, its potential chondroprotective effect was questioned. In the current series, it was observed that the mean WOMET score and the degree of extrusion had a positive correlation between the pre- and postoperative evaluations. The WOMET score is a meniscus-specific evaluation tool that was not used in those two referenced studies[8,9] in the same way as in other more generic knee scores were used.

ME is a risk factor for cartilage loss and knee osteoarthritis.[15–17] For this reason, it is a challenge for surgeons to prevent or diminish graft or implant extrusion in patients undergoing meniscal substitutions. Different methods have been described to prevent extrusion in MAT. A small reduction in the size of the allograft,[18] osteophyte excision,[19] or a concomitant caspulodesis[20] has been shown to decrease allograft extrusion. However, no technical recommendation has yet been described to control SE.

Although this is the largest series reporting on extrusion of meniscal scaffolds, it has some weaknesses. The patients were studied retrospectively and followed up for only 2 years. While the later can be obviously questioned from a functional point of view, the extrusion process is known to end a few months after the meniscal substitution.[8,21] The fact that the extrusion was only studied in MRI coronal views with a single slice extrusion assessment performed in the supine position can also be questioned. Other authors have described alternative methods to study the extrusion on the sagittal and axial planes.[22] However, a recent study concluded that the method used in the current investigation correlates most closely with the true perpendicular extrusion measurements obtained from manually segmented models.[22] In this study, cartilage pathology or osteoarthritis progression was not checked since the follow-up was too short to analyze these parameters. However, it would be interesting to follow these patients longer to check the possible effect SE has on the progression of cartilage degeneration.

In conclusion, the SE observed at the 2-year follow-up after the implantation of a polyurethane scaffold did not depend on preoperative MRE (major or minor extrusion). The WOMET score, which was the only meniscal-specific functional scored used, detected some inferior results in the most extruded meniscal scaffolds. This is basically a radiological study that has attempted to analyze the effect previous MRE may have on postoperative SC. The clinical differences observed at the 2-year follow-up that were assessed with a specific-meniscus score should be taken with caution and might be analyzed again with a longer follow-up study to confirm that the SE could be implied with the final clinical outcomes.

Conflict of Interest
None declared.

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Addendum

Fibronectin-coating enhances attachment and proliferation of mesenchymal stem cells on a polyurethane meniscal scaffold

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Abstract

The most common surgical strategies for meniscal injuries are meniscal suture and partial meniscectomy. Patients undergoing meniscectomy could complain from knee pain due to an overload in the ablated compartment. In these cases, implantation of tissue engineering scaffold could be indicated. Follow-up assessments of the two commercial scaffolds available for meniscus repair showed clinical improvement and tissue formation in the short term. But in long-term studies, the new tissue has an aberrant structure.

Mesenchymal stem cells (MSCs) are a promising tool for tissue regeneration because of their multipotency and self-renewal. Therefore, MSCs can be combined with scaffolds becoming a promising approach for treating meniscal defects.

We aimed to study the behaviour of MSCs on a polycaprolactone-polyurethane (PCL-PU) scaffold *in vitro*. MSCs express fibronectin (FN) receptor so we also investigate the effect of FN coating on the bioactivity of the scaffold. We seeded rabbit bone marrow MSCs (rBM-MSCs) and rabbit chondrocytes (rCHs) over non-coated (NC) and FN-coated scaffolds. We evaluated cell functionality and differentiation, in terms of proliferation and production of extracellular matrix (ECM), in three different conditions: rBM-MSCs+FN-scaffold, rBM-MSCs+NC-scaffold, and rCHs+NC-scaffold.

rBM-MSCs+FN-scaffolds showed more cells on proliferation (145%; 95% CI: 107%-182%) compared with rBM-MSCs+NC-scaffolds. rCHs+NC-scaffold displayed the highest production of ECM, followed by rBM-MSCs+FN-scaffolds.

These results suggest that MSCs have low capacity attachment to commercial PCL-PU scaffolds and a FN-coating is necessary to improve the scaffold bioactivity. These results could be applied in the design of scaffolds, and might have important clinical implications in orthopaedic surgery of meniscal injuries.

Keywords: Meniscal injuries, post-meniscectomy syndrome, tissue engineering, scaffolds, fibronectin, Mesenchymal stem cell

Introduction

Meniscal injuries are one of the most repeatedly treated damage in orthopaedic surgery. Meniscal tissue has limited ability to heal because of its low cellularity, dense ECM, and poor vascularization. In case of meniscal tear, meniscal repair is the preferred treatment to preserve a healthy joint. However, the torn tissue is often removed to ameliorate symptoms and some knees do not tolerate meniscectomy, resulting in the so-called post-meniscectomy syndrome (unicompartmental pain without significant articular cartilage wear) [1]. Therefore, several approaches have been proposed to substitute the missing tissue, including allograft meniscus transplantation (AMT) and tissue engineering.

While AMT is a reliable therapeutic option, its limited source and specific issues related to tissue banking policies make this treatment difficult in some countries. As for tissue engineering, several materials have been tested to construct meniscal scaffolds, which are mainly made of ceramic, biological, or synthetic polymers. All have specific advantages and disadvantages; therefore, new strategies consist in using composite scaffolds comprising different materials [2]. To enhance their biocompatibility, synthetic polymers are often covered with proteins (such as collagen, gelatine, fibronectin, and laminins), and/or peptides containing pro-adhesive sequences. These modifications enhance their physicochemical, mechanical, and degradability properties [3].

Currently, only two commercial scaffolds are available for meniscus repair: a collagen and glycosaminoglycan scaffold (Collagen Meniscal Implant (CMI®) and a polycaprolactone-polyurethane (PCL-PU) scaffold (Actifit®). Both have bioresorbable and biocompatible characteristics, showing easy reabsorption and allowing adherence and proliferation of fibroblast and chondrocytes. CMI® is more biocompatible than Actifit®, but it also

exhibits faster reabsorbing rate, leading to the disappearance of the scaffold before new tissue formation. In both cases, short term follow-up assessments showed a clinical improvement with meniscal-like tissue formation and presence of fibrochondrocytes [4-7]. Cohort studies with 5 years or longer follow-up demonstrated that clinical improvement was maintained. However, the repaired meniscus area did not show a healthy morphology. Indeed, it showed a decrease in tissue volume, an irregular shape, and in some cases total reabsorption of the scaffold without new tissue formation [8-10]. Therefore, it might be assumed that scaffolds delay the clinical worsening, but the adverse effects at long term will be similar to those observed in partial meniscectomies.

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into various cell lineages deriving from the mesoderm. This characteristic, together with the fact that MSCs, in contrast to some differentiate cells, can be expanded *in vitro*, makes them a good resource for new tissue regenerative therapies. According to the U.S. National Institute of Health, MSCs are being used in more than 800 clinical trials for various conditions, including bone and cartilage defects [11]. However, no legislative authority has yet approved their use for the treatment of any disease. MSCs are a promising therapy for meniscal repair because they are able to differentiate into the corresponding cells, and to produce growth factors that induce tissue repair. Preclinical trials showed that the use of MSCs enhanced the repair of meniscal defects. These studies used fibrin clots, scaffold-free engineered meniscal tissue, and cell-seeded scaffolds, in combination with MSCs. The latter seem to be the most useful tool to ensure support long-term effect of the MSCs at the site of the defect [12].

A promising approach for meniscus repair could be to use a combination of MSCs and scaffolds with optimal degradation/reabsorption rate and bioactivity. MSCs lack several receptors already present in differentiated cells, limiting the binding to some surfaces. However, expression of fibronectin (FN) receptor have been reported in MSCs [13]. Therefore, the use of FN-coated scaffolds could increase their biocompatibility. The aim of the current work was to investigate *in vitro* the ability of rabbit MSCs to proliferate and differentiate into functional chondrocytes on a FN-coated PCL-PU scaffold. First, we assessed the multipotency of rabbit bone marrow mesenchymal stem cells (rBM-MSCs). Then, we evaluated the bioactivity of the modified scaffold by investigating the capability of MSCs to adhere to the surface and proliferate, differentiate, and produce an ECM that mimics the meniscal tissue. We hypothesized that FN improved the capacity of MSCs to adhere to the scaffold and did not impair their ability to differentiate into chondrocytes and produce ECM.

Materials and Methods

Isolation and characterization of rBM-MSCs

To harvest MSCs, we used two skeletally mature New Zealand white rabbits. They were fully sedated by intra-muscular injection of Ketamine (35 mg/kg) and Xylazine (5 mg/kg), followed by sevoflurane inhalation (2%, rate 2 litres/min). Then, we performed a medial parapatellar approach on the right knee of the animals. After dislocating the kneecap, we made a puncture on the medial femoral condyle with an 18 G hypodermic needle. We aspirated the rabbit bone marrow (rBM) while making rotational needle movements. Finally, we collected the rBM in a syringe with citrate to avoid coagulation. The protocol including all animal care and experimental procedures was approved by the Ethical

Committee of Animal Experimentation of our institution (CEEA-PRBB) and by the competing regional authorities.

We purified rBM mononuclear cells (MNCs) following the SepMate™ isolation tubes protocol. Briefly, after aspiration, rBM was diluted with an equal volume of PBS + 2% FBS and gently mixed. Such cell dilution was pulled on the SepMate™ tube previously filled with Ficoll. The tube was then centrifuged at 1200 g for 10 min at room temperature (RT). The enriched MNCs fraction was washed twice with 10% PBS + 2% FBS and centrifuged for 10 min at 400 g at RT. The obtained cells were then seeded at a density of $2\times10^5/cm^2$ and their media replaced every 3-4 days, until the cells reached an 80%-90% confluence (8-10 days). At this point, only rBM-MSCs were surviving and growing. Therefore, rBM-MSCs were trypsinised and seeded in a $75cm^2$ flask at a concentration of $5\cdot10^4$ cells/cm^2. To expand them, rBM-MSCs were cultured with DMEM supplemented with 10% FBS for 14 days. Alternatively, they were cultured with StemPro® MSC SFM XenoFree (Gibco, life technologies) for 3 days and then with MesenPRO RS™ Medium (Gibco, life technologies) for 11 days. Cell media were replaced every 3 to 4 days. For our experiments, we only used cells from the first and the second passages.

The MSC multipotency test was performed in triplicate using commercial kits: "StemPro® Osteogenesis Differentiation Kit", "StemPro™ Chondrogenesis Differentiation Kit", and "StemPro™ Adipogenesis Differentiation Kit" (Gibco).

Cell count was performed with Neubauer Chamber using Trypan Blue exclusion and Flow Cytometry. For flow cytometry, we used an internal microsphere CountBright™ counting standard (Thermo Fischer Scientific), with settling properties similar to lymphocytes. We carried out each quantification in triplicate.

Establishment of rabbit chondrocyte (rCHs) culture

Cells were thawed from frozen stocks obtained from previous works [14]. rCHs were seeded in a 75 cm^2 flask with a density of 5×10^4 cells/cm^2 in DMEM medium supplemented with 10% FBS and 50 μg/ml Ascorbic Acid. They were grown for 14 days at 37ºC with 5% CO_2 and 60% of relative humidity. The medium was replaced twice per week. For our experiments, we only used cells from the first and the second passages.

Scaffold preparation, cell seeding and culture

We cut a cylindrical piece with a diameter of 4 mm and a height of 2 mm from a commercial Actifit® structure. We sterilized the scaffolds by increasing ethanol concentration batch (50%, 70%, and absolute ethanol). Afterwards, they were washed three times in PBS, and finally immersed in DMEM medium for 24 h. For the scaffolds to be coated with FN, the DMEM medium included 1% FN. Scaffolds were then let dry for 24 h.

Cultured cells (rCHs or rBM-MSCs) reaching a confluence of 80-90%, were harvested and resuspended in DMEM at a concentration of $1x10^6$ cells/μl. Afterwards, cells were seeded on sterilized scaffolds at a concentration of $5x10^7$ cells/cm^3 and cultured in wells of a non-adherent 48-well plate. rBM-MSCs were cultured with chondrocyte differentiation medium. Such medium consisted in DMEM supplemented with 10 μl/ml Insulin Transferrin Selenium (ITS), 50 μg/ml ascorbic acid, 10^{-7}M Dexamethasone, and 10 ng/ml TGF-β. For chondrocytes cultures on scaffolds, the culture medium used consisted in DMEM supplemented with 10% FBS and 50 μg/ml ascorbic acid. Cells were cultured for 3 weeks and medium was changed 3 times per week. A total of six scaffolds were cultured for each condition.

Evaluation of cell proliferation

To assess cell viability and proliferation on each scaffold, we performed an MTS assay (Abcam). Briefly, 10% MTS reagent was added to cell culture media and incubated for 3 hours in standard culture conditions. Then, we briefly shook the plate and measured the absorbance at 490 nm. We evaluated each sample in triplicate.

Scaffold colonization and evaluation of ECM production

To evaluate the diffusion of the cells through the scaffold and their ECM production, we histologically evaluated the slices from 3 scaffolds per condition (FN-coated+rBM-MSCs, non-coated+ rBM-MSCs, and non-coated +rCHs). Samples were fixed overnight with 10% formalin, and then put in a 15% sucrose (in PBS) bath, for 6 hours. Finally, they were kept in a 30% sucrose bath for 18 h. Afterwards, scaffolds were embedded in OCT, cooled down in a bath of dry ice and isopropanol, and frozen at -20ºC.

From the OCT blocks, we obtained 4 μm transversal sections with Cryostat (Leica CM3050 S). These sections were then stuck to SuperFrostPlus® slides. Stains were performed in horizontal racks to avoid losing material because of the low adherence of the scaffold to the slide glass. We finally performed the following evaluations:

- **Scaffold colonization.** We evaluated cell spreading along the scaffold through Haematoxylin – Eosin and DAPI staining. Sections were stained in Mayer's haematoxylin solution (30%) (Sigma Aldrich diagnostics®) for 15 sec to 1 min. Afterwards, they were washed in running tap water for 10 min, soaked three times in 80% ethanol + 0.15% Hydrochloric Acid (HCl), and three times in 0.3% ammonia water. Then the samples were rinsed in distilled water and washed 5 min in 95% ethanol before counterstaining in 0.5% eosin Y alcoholic (Bio-Optica) solution for 15 to 30 sec. Finally, slides were dehydrated and mounted with

Dibutylphalate Polystyrene Xylene (DPX) new medium. For DAPI, the slides were washed with TPBS, mounted with an aqueous mounting agent containing DAPI (1:200).

- **Collagen stain.** Sections were stained in Weighert's haematoxylin solution (Sigma-Aldrich) for 15 sec to 1 min and washed in running tap water for 10 min. Then, they were stained for 1 hour in picrosirius red (PSR) solution (Sirius red F3B Sigma-Aldrich "Direct Red 80") in saturated aqueous picric acid (Sigma Aldrich), pH 2. Afterwards, the sections were washed twice in acidified water (0.1 N HCl), and three times in absolute ethanol (5 min each). Finally, they were cleared in xylene for 5 min and mounted with DPX new medium.
- **Proteoglycan stain.** Sections were stained 5 min in 1% alcian blue (Merck Millipore) in 3% acetic acid. Then, they were soaked in 0.5% aqueous periodic acid solution for 5 min and, finally, they were bathed for 15 min in Schiff's reagent (Merck Millipore). At every step, the slides were washed with tap water for 3 min and rinsed in distilled water. Finally, they were stained with haematoxylin solution modified according to Gill III (Merck Millipore) for 20 sec and washed for 3 min with tap water. After dehydration, samples were mounted with DPX new medium.

To observe the samples, we used an Automated Upright Microscope BX61 in bright light and took pictures with an Olympus digital camera using the software cell Sens Standard. We also observed the samples under an Epifluorescence Eclipse Ni-E Microscope. For PSR staining observation, we used 2 filters: FITC, and TxRed. Pictures were captured at 20x and 40x magnifications with a Nikon digital camera and processed with the photo program software *Nikon NIS-E Advanced Research*.

Results

Isolation of rBM-MSCs and multilineage differentiation

MNCs were obtained from the rBMs and cultured, showing the expected morphological features under light microscopy (Fig. 1). Surprisingly, while progressing to MSCs, cell morphology gradually became non-fibroblastic one (Fig. 2A-D). To discard the effect of possible differentiation inductors in the serum, we tested three different batches of FBS in the culture media. We did not observe any differences in cell morphology so differentiation inductors, if any, were shared by the three sera. To avoid the effect of FBS, we checked a culture protocol with low FBS concentration. rBM-MSCs were cultured in both cell culture conditions. Cells cultured in medium supplemented with FBS were fewer (approximately 25%) and had a larger size than cells cultured with low FBS concentration, although cultures were at similar confluence conditions (Fig. 2E-H and Fig. 3). Furthermore, cells cultured with low FBS concentration showed the fusiform morphology and digital expansions typical for MSCs (Fig. 2E-H). Finally, we used the culture protocol with low FBS concentration for the subsequent isolation of rBM-MSCs.

Before seeding the rBM-MSCs on the scaffolds, we assess their stemness by multipotency test and achieved differentiation to the three linages (chondrocytes, osteocytes, and adipocytes) (Suppl. Fig. 1).

Adhesion and proliferation of rBM-MSCs on non-coated and FN-coated scaffolds

Although rBM-MSCs were able to attach and proliferate both on non-coated and FN-coated scaffolds, these features were improved on the FN-coated scaffold. 14 days after cell seeding, the number of proliferating cells present on FN-coated scaffolds were 145% (95% CI 107%-182%) higher than the proliferating cells grown on non-coated scaffolds.

Evaluation of rBM-MSCs differentiation potential and functionality *cell spreading and ECM production*

The protocol followed to perform the histology studies caused loss of material from the slide. The surface properties of the scaffold prevented to perform a suitable bond to the surface, although the slide had a pre-treatment to improve its adhesion properties. This impeded to evaluate the cell migration along the complete sheet. Nevertheless, we could not observe any cell attached to the untreated scaffold seeded with rBM-MSCs (Fig. 4A-E); whereas the other two condition (FN-coated+rBM-MSCs, and non-coated+rCHs) present similar cell attachment and staining (Fig. 4F-J and K-O).

The production of both proteoglycans and collagen could be evaluated as ECM presented good adhesion to the slide; therefore, it could be observed in the areas where it had been deposited (Fig. 4). As expected, rBM-MSCs seeded on untreated scaffolds exhibited no ECM formation (Fig. 4B-D), as no cells were observed. Big areas of tissue formation and high ECM production were obtained from rCHs seeded on untreated scaffolds (Fig. 4L-N); rBM-MSCs seeded on FN-coated scaffolds also produced ECM, but display a lower amount (Fig. 4G-I). Although rCHs produced more ECM than rBM-MSCs, the quality of the ECM produced was comparable. Indeed, both showed similar content of acidic and neutral mucins (Fig. 4G and 4L).

Discussion

In this study, we describe a protocol for rBM-MSCs culture (that is independent of the effect of FBS batches), proliferation, and differentiation into chondrocytes. We seeded rBM-MSCs on PCL-PU scaffold coated with FN and cultured them in a chondrocyte

differentiation medium. In these conditions, they were able to attach, proliferate, and differentiate, producing an ECM that resembles the one produced by chondrocytes.

Repairment is the first option for injured meniscus. However, a partial or total meniscectomy is sometimes required to ameliorate pain and function, occasionally resulting in the post-meniscectomy syndrome. In order to avoid it, AMT and tissue engineering have been proposed to substitute the missing tissue. Regarding tissue engineering, several materials have been tested to produce meniscal scaffolds. It is well-known that synthetic polymers have higher lifespan than biological ones, but they present the disadvantage of displaying lower biocompatibility [2]. However, several works demonstrated that chondrocytes and fibrochondrocytes adhere and proliferate over synthetic polymers, producing an ECM that resembles the meniscal one, both in animal models [15, 16], and in humans [7, 17]. We observed similar results *in vitro* with chondrocytes. Nevertheless, we also showed that the bioactivity of the scaffolds for rBM-MSCs is much lower. In particular, rCHs proliferated over non-coated scaffolds forming colonies and synthetizing ECM, whereas rBM-MSCs proliferated much less and did not produce ECM. This difference between rBM-MSCs and rCHs could indicate that the cells colonizing the scaffold *in vivo* are differentiated cells migrating from adjacent areas of the meniscus, rather than MSCs from the synovial fluid. MSCs presence in synovial fluid increases after some knee pathologies or surgical procedures [18-20] and their presence is suggested to play a role in the healing of defects such as meniscal tears. However, it is still not clear whether this effect is a direct action of their proliferating and differentiating capacity or is rather mediated by secretion of trophic and immunomodulation factors [21]. Our results suggest that in surgeries for Actifit® implantation, the benefit of native MSCs presence in

synovial fluid will not be related with their direct scaffold colonization and regeneration capacity, as their ability to adhere to Actifit® is low, but rather to a paracrine effect.

Integrins are adhesion receptors mediating cell-cell and cell-matrix interactions. They are not only implicated in cell binding but also in intracellular signalling. MSCs lack several integrins that are present in chondrocytes [13]. This could prevent MSCs to adhere and proliferate over non-coated scaffolds. However, fibronectin receptor (integrin alpha5beta1) is expressed by undifferentiated MSCs [13], allowing their interaction with FN-coated scaffolds, that will exhibit an improved bioactivity in terms of attachment and/or proliferation.

Achatz et al. tested MSCs behaviour on Actifit [22] and they observed excellent cell distribution though the polyurethane scaffold, with more than 75% of pores being cell-populated, extensive production of proteoglycans and collagen type II, and moderate production of collagen type I. The different protocol for cell seeding could explain the differences between their results and ours. While we worked on natural cell diffusion throughout the scaffold, Achatz et al. loaded MSCs using a rotary valve vacuum pump, and therefore forcing the cells to occupy pores in the centre of the scaffold. This way, MSCs located in pores and not adhered to the material were not removed when culture media was changed, as it happened instead with our protocol. Once the embedded MSCs differentiate to chondrocytes, they could proliferate all along the scaffold and produce ECM. We selected this loading protocol to evaluate the capacity of rBM-MSC to migrate and invade all the material. Unfortunately, this could not be evaluated because material was lost along the histology protocol.

The current work presents several limitations. First, we could not evaluate the colonization of the scaffold because it did not bind properly to the slide glass, so it was hardly observed in the histology preparations. Longer cultured time that allows rBM-MSC migration, cell differentiation, and ECM production could improve adherence of the material. Furthermore, other histology techniques, such as Methyl-methacrylate [23], used for hardness materials, could increase integrity of the sections obtained. Second, the experiments have been performed with rBM-MSCs harvested from a low number of donors (only two individuals). Nevertheless, the results are consistent between both cell lines. Finally, we used an animal model that is distant from humans on the phylogenetic scale. However, the expression of integrins in human MSCs has been extensively studied and fibronectin receptors are present in these cells.

In conclusion, in orthopaedic surgery of meniscal injuries, we hypothesized that scaffolds are colonized by differentiated cells (fibrochondrocytes and chondrocytes) migrating from adjacent areas of the meniscus. However, chondrocytes have low proliferative capacity and, furthermore, hypertrophic chondrocytes have altered protein expression, producing aberrant ECM that finally leads to the apoptosis of the cells [24]. This phenomenon might be behind the failure of the scaffold at long run [25]. The use of FN-coated scaffold allows MSCs to attach to Actifit® *in vitro*. This finally leads to MSC differentiation into new cells producing ECM similar to the ones produced by chondrocytes. Therefore, our results could have crucial implications in the design of scaffolds to improve their clinical use in tissue regeneration and functionality following orthopaedic surgery.

Acknowledgments

The authors would like to thank JF Sánchez for his assistance in rabbit surgeries and Laura Triginer for her assistance in histology techniques, and photography and image analysis. Authors also acknowledge Daniel Vivas and Joaquin Vives from Banc de Sang i Teixits de Catalunya for their support in the isolation and culture of rBM-MSCs. Finally, we want to thank ThePaperMill for the assistance in manuscript editing and English language support.

This work had been carried out in the Surgery and Morphological Sciences Doctorate framework of Universitat Autònoma de Barcelona.

RT and SM have received funding from MBA for research projects in meniscal repair. The company is not financing the manuscript.

There are no other competing interests.

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Figure legends

Figure 1.- MNCs' morphology observed at 4 (A), 7 (B), 9 (C), and 11 (D) days after BM aspiration. Culture media was DMEM+10% FBS. Magnification bar: 100 μm.

Figure 2.- rBM-MSCs' morphology observed at 4 (A, E), 7 (B, F), 9 (C, G), and 11 (D, H) days after mononuclear cell culture trypsinization. Culture media was DMEM+10%FBS in the upper row images (A-D), and low-FBS media (see Materials and Methods) in the lower row images (E-H). Magnification bar: 100 μm.

Figure 3.- Cytometry results and cell count of rBM-MSCs cultured in DMEM+10%FBS (A) and low-FBS media (B) after 11 days of culture.

Figure 4.- Representative histological images of uncoated scaffolds seeded with rCHs (A-E) and rBM-MSCs (F-J), and FN-coated scaffolds seeded with rBM-MSC (K-O), after 21 days of *in vitro* chondrogenesis. Haematoxylin-Eosin (HE) (A, F, K), PAS- Alcian blue (B, G, L), Picrosirius red (PRS) (C-D, H-I, M-N), and DAPI (E, J, O) stainings, observed in bright field or under fluorescence. Magnification bar: 50 μm.

Supplementary Figure 1.- rBM-MSC differentiation towards chondrogenic (E), adipogenic (F), and osteogenic (G, H) lineages. Staining of control cells (undifferentiated) is also shown (A-D). Magnification bar: 100 μm.

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