# Autologous tissue transplantations for osteochondral repair

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# List of studies

Study 1: Christensen BB, Foldager CB, Olesen ML, Vingtoft L, Rölfing JHD, Ringgaard S, Lind M. Experimental articular cartilage repair in the Göttingen minipig: the influence of multiple defects per knee. J. Exp. Orthop. 2015;2(1):13.

Study 2: Christensen BB, Foldager CB, Jensen J, Jensen NC, Lind M. Poor osteochondral repair by a biomimetic collagen scaffold: 1- to 3-year clinical and radiological follow-up. Knee Surgery, Sport. Traumatol. Arthrosc. 18 February 2015.

Study 3: Christensen BB, Foldager CB, Jensen J, Lind M. Autologous Dual-Tissue Transplantation for Osteochondral Repair: Early Clinical and Radiological Results. Cartilage 2015 Jul;6(3):166-73:1-8.

Study 4: Christensen BB, Foldager CB, Olesen ML, Hede KC, Lind M. Implantation of autologous cartilage chips improves cartilage repair tissue quality in osteochondral defects: A study in Göttingen minipigs. Resubmitted for 2nd review for publication in The American Journal of Sports Medicine.

# **Chapter 1. Background**

Articular cartilage forms the articulating surface of synovial joints. Along with the synovial fluid it facilitates near frictionless movements in healthy joints. Furthermore, articular cartilage transmits the joint load to the underlying bone, and thereby acts as a shock absorber. Unfortunately, injuries to articular cartilage in the knee are frequent. In five different studies evaluating the articular cartilage in knee arthroscopies cartilage injuries were found in 60-66% of the patients[1]–[5]. Not only can focal cartilage injuries impair the quality of life as much as severe osteoarthritis[6], but they can lead to osteoarthritis and at least 12% (possibly upwards of 30-40%) of osteoarthritic cases are believed to be of posttraumatic origin[7]. Osteoarthritis is a growing problem estimated to affect 37% of people >60 years old[8]. Obesity is a major predictor[9] and with growing numbers of obese and elderly people, arthritis is expected to affect 25% of the entire adult population by 2030[10].

# Articular cartilage

Articular cartilage (or hyaline cartilage) is composed of only one cell type, the chondrocyte. The main responsibility of chondrocytes is to produce extracellular matrix. Chondrocytes make up only 2-5% of articular cartilage volume and since articular cartilage contains no blood vessels, nerves or lymphatic tissue, the chondrocyte is geared towards functioning at oxygen levels from 1% in the deep sections to 10% superficially[11], [12]. Due to the low oxygen tension, the main energy source is glycolysis rather than oxidative phosphorylation and the chondrocyte contains relatively few mitochondria. The cell must rely on diffusion of nutrients from the subchondral bone (>50%)[13], [14] and the synovial fluid[15].

The extracellular matrix of articular cartilage consists mainly of water (60-85%). The most common collagen type in articular cartilage is collagen type II, which makes up two-thirds of the dry weight of extracellular matrix. Additional collagen types include, but are not necessarily limited to, collagen type III, IV, VI, IX, X, XI, XII, XIV, XVIII and XXVII[16]-[18]. The structural organization of collagen fibrils and the zonal changes of collagen fibril orientation provide the mechanical stability and tensile strength of articular cartilage (Figure 1). The ability of the cartilage to resist compressive loads is mainly provided by the proteoglycan aggrecan. Aggrecan interacts with hyaluronic acid and creates an osmotic pressure that attracts water[11], [16], [19], [20]. When the joint is compressed, water is displaced from the extracellular membrane and the thickness of the cartilage is reduced with as much as 58%. Full recovery of articular cartilage thickness is seen within minutes to hours depending on the degree of compression[21], [22]. While the articular cartilage do participate in shock absorption, the peri-articular muscles are responsible for the majority of the absorption[23]. Other important factors contributing to the deformation are the subchondral bone, the menisci, the joint capsule/ligaments, the cortical bone and the synovial fluid[14], [24]. Articular cartilage can be divided into four separate zones as shown in Figure 1.



**Figure 1.** The four zones of articular cartilage. The superficial zone is the articulating tissue. Below is the transitional zone and the deep zone. The deepest zone consists of calcified cartilage. Between the deep zone and the calcified cartilage is the tidemark which is recognizable on histological sections. The subchondral bone is below the calcified zone and provides part of the nutrition through end arteries\*.

The superficial zone is the only articulating zone, and the tissue is in contact with the synovial fluid. The chondrocytes are flattened and the collagen fibrils are thin and aligned parallel to the surface. This zone is able to withstand the shear, tensile forces exerted by articulation[19], [25]. Furthermore, the chondrocytes in the superficial zone excrete the proteoglycan lubricin – originally termed superficial zone protein. Lubricin coats and lubricates the cartilage surface and prevents cell and protein adhesion[26]-[28]. In the transitional zone the collagen fibrils are slightly thicker than in the superficial zone and they appear more randomly arranged, with a slight preference of 45° orientation. The chondrocytes are more rounded in shape than in the superficial zone. In the deep zone the main function is to withstand compression forces. The collagen fibrils are woven into larger bundles of fibers that are arranged orthogonally to the surface. The collagen fiber bundles passes through the calcified cartilage and thereby anchors the cartilage to the subchondral bone[19], [25]. The chondrocytes are rounded and are arranged in columns. In the calcified zone the chondrocytes are larger in size. The calcified cartilage receives nutrients from end arteries in the subchondral bone (Figure 1\*)[14].

#### Injuries to cartilage and subchondral bone

Hyaline cartilage does not regenerate spontaneously when injured[25], [29]–[31] and cartilage injuries can cause pain, swelling, catching, limited mobility and lead to early osteoarthritis[7], [8], [32]. The most common causes of focal articular cartilage injuries are trauma, osteochondritis dissecans, and osteonecrosis.

Traumatic cartilage injuries are closely tied to knee ligament injuries. Cartilage damages have been reported in 23% of patients with acute anterior cruciate ligament (ACL) injuries, and in 54% of patients with ACL laxity[33]. Furthermore, osteochondral fractures have been reported in 40-50% of patients with patellar dislocations[34], [35]. The annual incidence of ACL ruptures in the general population is 61-77 per 100,000[36], [37], and with an estimated 5% annual ACL tear-rate in female soccer players, traumatic cartilage injuries are common[38].

Osteochondritis dissecans is a disease affecting the subchondral bone and the overlaying cartilage. Despite the suffix "-itis", osteochondritis dissecans is not believed to have an inflammatory component. The etiology is yet unknown, but it is thought to be either traumatic, through direct trauma or repetitive microtrauma causing transchondral fractures, ischemic, through compromised vascular supply to the subchondral bone, and/or genetic[39], [40]. The incidence of osteochondritis dissecans is a subject for debate. The peak incidence is found in the age group 10-20 years. Linden et al. found a maximum incidence of 19 per 100,000 in females and 29 per 100,000 in males in a Scandinavian population of 250,000[41]. In a mixed population (white, black, Hispanic and Asian) of 3.5 million Kessler et al. found a maximum incidence, in the same age group, of 3.9 per 100.000 females and 18 per 100,000 males[39]. Based on closure of the epiphyseal growth plate, osteochondritis dissecans is either termed juvenile or adult. Juvenile osteochondritis dissecans occurs in children and when managed conservatively heals spontaneously in 46-90% of cases[42]-[46]. In turn adult osteochondritis dissecans has a less positive prognosis. Jürgensen et al. treated 27 patients conservatively and found that only 14% of patients suffering from adult osteochondritis dissecans experienced any clinical improvements[47].

Osteochondritis dissecans lesions can be classified according to the International Cartilage Research Society (ICRS) osteochondritis dissecans classification (Figure 2).



**Figure 2.** The ICRS osteochondritis dissecans classification. ICRS osteochondritis dissecans grade I: The affected area is covered by intact cartilage. The cartilage is stable on probing but appears softer than healthy cartilage. ICRS osteochondritis dissecans grade II: The cartilage is stable on probing, but there is a partial discontinuity of the surface and the subchondral tissue. ICRS osteochondritis dissecans grade III: Complete discontinuity of the osteochondritis dissecans lesion, but it is not dislocated. ICRS osteochondritis dissecans grade IV: The fragment is dislocated and is loose within the defect or the joint[48]. (From www.cartilage.org)

When articular cartilage is injured, the morphology of the repair tissue is either fibrous, fibrocartilaginous or hyaline. Fibrous tissue consists of elongated fibrocytes in a loosely woven disorganized fibrous matrix (Figure 3A). Fibrocartilage consists of chondrocytes located in lacunas in a disorganized fibrous matrix (Figure 3B). Hyaline cartilage, also seen in Figure 1, consists of chondrocytes located in lacunas embedded in a highly organized hyaline matrix (Figure 3C). Fibrous tissue and fibrocartilage contains very little collagen type II and Glycosaminoglycan (GAG)-rich proteoglycans, and therefore lacks the elastic properties and long-term durability of hyaline cartilage.



Figure 3. A polarized light microscopy image of A) fibrous tissue, B) fibrocartilage and C) hyaline cartilage. Staining is H&E. Scale bar is 200  $\mu m.$ 

# The osteochondral unit

The subchondral bone plays a major role in articular cartilage pathology, and any attempt to repair a chondral lesion without sufficient support from an intact subchondral bone bed will likely result in failure[49], [50]. As previously mentioned, the articular cartilage is closely tied to the subchondral bone regarding biomechanics and it relies on the bone for nutrition. Consequently, the articular cartilage and the subchondral bone should be looked upon as an osteochondral unit. Pathological signals from the subchondral bone has been shown to induce phenotypic degenerative changes of bone marrow stem cells[51]. In addition, the activation of secondary centers of ossification can lead to thickening of the subchondral bone plate, with concomitant thinning of the articular cartilage[52], [53].

Treatment for cartilage lesions has also been shown to affect the subchondral bone. Saris et al. found subchondral bone thickening in 25% of patients treated with autologous chondrocyte implantation (ACI) and 51.5% of patients treated with microfracture (MFx)[54]. Furthermore, studies have found a 3-7 fold increase in ACI failure rate after penetration of the subchondral bone plate with MFx[55], [56].

# Articular cartilage treatment methods

Treatment of chondral and osteochondral injuries has been studied extensively during the last century, but so far, no treatment method has been established as gold standard. The difficulty of repairing articular cartilage was noted as early as 1743 by Hunter[31] and in 1851 by Paget[57]. In the early 1900's Lexer and Judet used allograft to repair damaged joints[58] and in the 1940's the first debridement procedures of damaged cartilage were performed[59].

# **Chondral defects**

Marrow stimulation techniques were introduced in 1959 with Pridie drilling[60]. In marrow stimulation techniques the subchondral bone is perforated to allow bone marrow to fill the chondral defect and facilitate a repair response. The technique was improved and labeled microfracture (MFx) by Steadman in 1992[61] (Figure 4A). In 1994 Mats Brittberg and Lars Peterson reported on the first cell based cartilage repair technique in humans, autologous chondrocyte implantation (ACI)[62]. In ACI (Figure 4B), an autologous cartilage biopsy is taken and the chondrocytes are cultured in a laboratory. The expanded chondrocytes are then implanted under a periosteal membrane (ACI-p, 1st Generation), under a synthetic membrane (ACI-c, 2nd Generation) or seeded onto a synthetic collagen scaffold (MACI<sup>®</sup> or ACI-m, 3rd Generation)[62], [63].



**Figure 4.** A schematic representation of A) Microfracture, B) Autologous chondrocyte implantation, C) An osteochondral scaffold and D) Particulated juvenile articular cartilage. 1; articular cartilage. 2; subchondral bone. 3; a microfracture hole. 4; a periosteal flap/synthetic membrane for ACI. 5; scaffold, e.g. the Trufit<sup>®</sup> or MaioRegen<sup>®</sup> scaffold. 6; cartilage chips embedded in fibrin glue.

The repair tissue produced by MFx is most often a mixture of fibrocartilage and fibrous tissue and hyaline repair tissue is rarely produced[64]–[69]. The consequence of low quality repair tissue is reduced durability compared with native hyaline cartilage. Studies have shown that treatment with MFx results in short term clinical improvements, especially in patients with smaller (<2 cm2) defects. After 2-5 years however, the clinical improvements deteriorate[67], [70], [71]. The repair tissue morphology of the MACI® treatment (3rd Generation ACI) is of slightly better structural quality. Authors have reported between 33-75% to be "hyaline-like" with the remaining repair tissue being a mixture of fibrocartilage and fibrous tissue[72]–[74]. In spite of the improved tissue quality, only one study has found short-term clinical, radiological, and histological improvements compared with MFx[75], and no studies have been able to show long-term clinical improvements compared with MFx[65], [66], [76]. No differences in clinical outcomes between 2nd generation and 3rd generation ACI has been found[72], [77]. Furthermore, ACI is limited by the high cost of two separate surgeries and commercial cell culturing.

#### Osteochondral defects

Various options are available for treatment of osteochondral pathology in the knee. Retrograde[78], [79] or antegrade[80] drilling can be used to promote reattachment of a stable osteochondritis dissecans lesion. Retro- and antegrade drilling is typically limited to ICRS grade 1 and 2 lesions in young patients with open physes and is not recommended for adult osteochondritis dissecans patients[81], [82]. Unstable fragments in ICRS grade 3 or 4 lesions can be fixed using metallic screws or biodegradable pins depending on fragment size[83], [84]. Pin fixation of the osteochondritis dissecans fragment provides favorable results in grade 3 and 4 lesions, especially in young patients[85]-[87]. In the absence of a viable fragment, osteochondral defects can be treated using autograft, allograft, or synthetic biomaterials. Treatment with autologous morselized cartilage/bone paste have been reported to result in long-term clinical improvements and fibrous/fibrocartilaginous repair tissue, but has not gained ground in the general clinical practice[88]-[91]. In the ACI sandwich technique, the bone defect is filled with autologous bone graft

and in the cartilage part cultured autologous chondrocytes are implanted between two synthetic scaffolds. The sandwich technique have resulted in >90% good or excellent results after 5.6 years, but the technique is limited by the high cost associated with the ACI part of the technique[92]. Fresh osteochondral allograft transplantation provides 5-year satisfaction of >86% and 15year satisfaction of >75%. The technique is limited by the donor availability and a very small risk of disease transmission. Since freezing or prolonged storage of the allograft is known to cause fissuring, delamination and chondrocyte death[93] the use of frozen allograft results in poor clinical outcome. Osteochondral autograft, or mosaicplasty, results in good long-term results for younger patients with moderate sized cartilage lesions[94], [95]. Mosaicplasty has been shown to be superior to MFx in randomized trials[96], [97]. Reports, however, are not unambiguous and others have failed to find any differences in clinical outcome when comparing mosaicplasty with MFx and ACI[98]. One study reported an increased failure rate and worse clinical outcome in the mosaicplasty group compared with an ACI group after 10 years[99]. In addition to the conflicting results, treatment with mosaicplasty is limited to small defects (<2-3 cm2) due to risk of donor site morbidity[95], [100], [101].

# Osteochondral scaffolds

Synthetic cell-free osteochondral scaffolds have been introduced to circumvent the lack of fresh donor tissue, donor site morbidity, the substantial costs of cell culturing, and multiple surgeries.

Osteochondral scaffolds are press-fitted into the osteochondral defect. They are believed, by principle, to rely on the migration of bone marrow derived mesenchymal stem cells (BMSCs) into the scaffold to facilitate osteochondral repair. The requirements of an ideal osteochondral scaffolds are biocompatibility, biodegradability, mechanical integrity, and a precise mechanical microenvironment. To be effective, a scaffold must not cause foreign body reactions and it must degrade in a predictable manner as the repair tissue replaces it. It must be durable enough to withstand the compressional forces of the joint and provide a platform for the regeneration of the native tissue. In vitro studies suggest that the stiffness of the microenvironment in scaffolds directs the BMSCs towards a certain cell lineage. A very soft microenvironment, mimicking brain tissue, will direct stem cells towards a neuronal phenotype. A stiffer microenvironment can direct stem cells towards a chondrogenic phenotype, and even stiffer environments results in an osteogenic phenotype[102], [103].

Numerous synthetic osteochondral scaffolds have been introduced, but only few are clinically available. The TruFit<sup>®</sup> (Smith & Nephew, Massachusetts) osteochondral scaffold is a cell-free, biphasic plug (Figure 4C). An early study reported very good shortterm results in 25 of 26 patients[104] and subsequent studies found similar positive results[105]–[108]. However, evidence of lack of/delayed scaffold incorporation and foreign body reactions was reported in 2009[109], [110]. In the following years, authors reported a complete lack of bone ingrowth[111] and no clinical improvements[112]. As a result the TruFit<sup>®</sup> scaffold was removed from the market in 2013.

The MaioRegen<sup>®</sup> scaffold is a multilayered bioresorbable scaffold (Figure 4C). It is designed to promote cartilage and bone regeneration and to be resorbed as the defect is repaired. The scaffold is multilayered to better mimic the different micro-environmental properties of each layer of the defect. The cartilage layer consists of 100% equine collagen type I. The tidemark consists of 60% equine collagen type I and 40% hydroxyapatite, while the subchondral layer consists of 30% collagen type I and 70% hydroxyapatite[113]–[115]. The results have been very promising. Ten clinical studies with up to 5 years follow-up have reported significantly improved clinical and radiological outcomes[116]–[125]. Short-term biopsies from the defect area revealed fibrocartilaginous repair tissue superficially, with trabecular bone in the subchondral area, and no traces of scaffold material[117]. MRI has shown relatively good defect filling, tissue integration and articular surface congruency, with hyaline like repair tissue[116], [117], [122], [123]. However, one study observed inhomogeneous repair tissue in the defects on MRI and one study have found changes in the subchondral lamina and subchondral bone [121], [125]. Prior to our study described in the present thesis, no study had investigated the subchondral bone after MaioRegen<sup>®</sup> implantation using CT.

Recent studies have introduced a new osteochondral plug – the Agili-C<sup>®</sup> implant. It is a biphasic implant made of marine coral exoskeletons in the bone part, and hyaluronic acid in the cartilage part. In a goat study, sponsored by the manufacturer, the implant showed promising results by being almost completely degraded after 6 months and leaving healthy bone and young hyaline cartilage in its stead[126]. A one-patient case report on the Agili-C implant has recently been published showing a restored articular surface after 24 months and clinical improvement including complete return to sports[127] (Figure 13). The Agili-C implant shows interesting potential, however the evidence is very limited and, as is the case with the MaioRegen<sup>®</sup> scaffold, no CT evaluation has been performed.

# Cartilage chips

The use of cartilage chips for chondral and osteochondral injuries are emerging as a cost-effective alternative to established methods. First mentioned in the literature in 1983 by Albrecht et al.[128], cartilage chips were highlighted as an alternative to cell culturing by Lu et al. in 2006. They found that chondrocytes from cartilage chips migrated from the extracellular matrix, both in vitro and in vivo in a goat model and the authors concluded that cartilage chips represented "... a simple, cost-effective treatment for cartilage repair..."[129]. The outgrowth of chondrocytes has since been confirmed in vitro in human articular cartilage chips[130]–[132], in human septal cartilage chips[133] and in vivo in rabbits[134], goats,[135], [136] and horses[137]. Furthermore, the use of juvenile cartilage chips have been shown to increase the production of extracellular matrix and result in increased GAG content, increased proliferation and outgrowth rate and increased proteoglycan and collagen type II content[130], [131], [138].

Prior to study 3 of the present thesis, clinical studies of two different methods involving cartilage chips had been published: 1) In a 2- year prospective randomized study cartilage autograft implantation system (CAIS) were compared with MFx[139]. CAIS uses autologous cartilage chips embedded in a polycaprolactone/polyglycolic acid scaffold with a polydioxane mesh sealed with fibrin glue. Patients treated with CAIS had a significantly better clinical outcome compared with patients treated with MFx[139], [140]. Unfortunately, CAIS was discontinued by the manufacturer since slow enrollment predicted a delayed return on investment[141]. 2) Particulated juvenile articular cartilage (PJAC) (DeNovo Natural Tissue Graft, Zimmer Biomet, Indiana, USA) utilizes the supposed increased migratory properties of juvenile articular cartilage (Figure 4D). Obtained from fresh cadaveric femoral condyles (donors under the age of 13), the cartilage is cut into 1-2 mm3 dices, and stored in blister packs containing nutrients[130], [142], [143]. This makes PJAC a readily

available, off the shelf product for chondral injuries. During surgery, the cartilage chips are embedded in fibrin glue and implanted in the chondral defect. So far eleven clinical studies on PJAC have been published. Five studies on 26 patients with talar osteochondral injuries[144]-[148] and six studies on 53 patients with chondral injuries in the knee[142], [143], [149]-[152] have been published. In general, clinical and radiological results have been very good, and only two clinical failures have been reported, one due to continued pain[149] and one due to delamination of the graft[150]. In addition, studies have demonstrated a technique for arthroscopic implantation of cartilage chips, thereby limiting the surgical trauma[144], [145]. So far, more than 7500 patients have been treated with DeNovo NT, and the DeNovo NT Longitudinal Data Collection Knee Study is expected to finish by 2021. The study will include approximately 250 patients, followed for 5 years, and it will shed light on the long-term effect of PJAC[153].

# Animal models for cartilage repair

Animal models are essential in developing and testing of new treatment methods in cartilage research. The available models for cartilage research can be divided into: Small animal models and large animal models. Small animal models include mice, rats and rabbits, while large animal models include of sheep, goats, dogs, pigs, minipigs, cattle, and horses. Small animal models are essential in "proof-of-concept" studies where theories are tested, and results obtained in vitro are applied in vivo. The smaller animals are easy to handle and house, they are inexpensive and a variety of genetic modifications are commercially available. Large animal models bridge the gap between "proof-of-concept" studies and clinical studies. A large animal model must resemble humans regarding body size, joint size, cartilage thickness, cartilage tissue characteristics and biological repair response. The animal must be affordable and easily handled. Finally, the animal must have reached skeletal maturity to limit spontaneous repair of defects and to resemble the clinical outcomes of the tested treatments. The cartilage thickness of each animal model and the age of skeletal maturity are shown in Table 1.

The Göttingen minipig is an attractive large animal model. The animal maintains a body weight of 30-50 kg throughout adulthood, the articular cartilage of the Göttingen minipig shares the same collagen arrangement as seen in humans[159] and the weight of the animal and the joint size resembles that of humans[154], [156]. Finally, the blood count, blood clotting properties and liver enzymes have values similar to those found in humans[160], [161]. The Göttingen minipig is discussed in detail in the methodological discussion chapter.

# Chapter 2. Aim

The aim of the current thesis was to:

1) Evaluate two different approaches for clinical osteochondral repair.

2) Develop a large animal model for testing autologous tissue transplantation for osteochondral repair.

The hypotheses of the four studies conducted can be found in the separate publications.

#### **Chapter 3. Summary of studies**

Study 1: Experimental articular cartilage repair in the Göttingen minipig: the influence of multiple defects per knee

We wanted to establish a clinically relevant, cost-effective, large animal model for chondral and osteochondral repair in the knee. We hypothesized that; a) the biological repair response of the applied treatments would be similar to what is found in a clinical setting, and b) that two defects per knee, rather than one, could be applied without affecting the repair outcome. We included sixteen skeletally mature Göttingen minipigs. The minipigs received bilateral trochlear osteochondral drill-hole defects or chondral defects ( $\emptyset$  6 mm), either one defect per knee (Figure 5, circle 1) or two defects per knee (Figure 5, circles 2 and 3). The chondral defects were treated with MACI, MFx or autologous cartilage chips. The osteochondral defects were treated with ADTT or autologous bone graft. Empty chondral and osteochondral defects were used as controls. MRI and CT were performed at 3 and 6 months, histology was performed 6 months postoperative. We found that the outcomes of the applied treatments were consistent with the outcomes in clinical studies. Furthermore, the use of two defects per knee did not have any significant effect on the repair response. The Göttingen minipig model was easy to handle, cost-effective and provided a predictable outcome.

Species	Mouse/Rat	Rabbits	Sheep	Minipig	Dog	Landrace pigs	Goat	Horse	Human
Cartilage thick- ness (mm)	0.1	0.3	0.4-0-5	0.3-0.8	0.6-1.3	1.5	0.7-1.5	1.5-2.0	2.2-2.5
Age at skeletal maturity (months)	Immature life long	5.5-8	17	18	12-24	18	48-36	60-72	180-204

Table 1. Cartilage thickness and age at skeletal maturity in animal models[29], [154]–[158].

The cartilage thickness of the horse makes it a very appealing animal model, however high acquisition costs and the need for specialized facilities limit the widespread use of the model. Goats and sheep are ruminant, which leads to concerns about the spreading of prion diseases and the use of dogs is limited due to their status as family pets.

Based on this study the use of two defects per knee, in male Göttingen minipigs is recommended. The model has been implemented as the standard animal model for cartilage research at the Orthopaedic Research Laboratory, Aarhus University Hospital.



**Figure 5.** Macroscopic images of a) a single defect knee, and b) a double defect knee. The defects have been marked with circles 1–3. The defects in these images have all been treated with ADTT. Remains of cartilage chips can be seen in all three defects as white areas in the defect. P=Proximal, D=Distal, M=Medial and L=Lateral.

Study 2: Poor osteochondral repair by a biomimetic collagen scaffold: 1- to 3-year clinical and radiological follow-up

In this study we aimed at evaluating the repair of osteochondral defects in patients treated with the MaioRegen® scaffold. The scaffold is cell-free and multi-layered, and is made of collagen type I and hydroxyapatite. Previous studies on the MaioRegen® scaffold has reported good clinical results. We included 10 patients suffering from osteochondral injuries. Six patients with injuries in the knee and four patients with injuries in the talus. We evaluated the with MRI and CT scans pre-operative, after 1 year and after 2.5 years. The bone and cartilage repair were evaluated semi-quantitatively using the MOCART score. The clinical outcome of knee patients were evaluated using the knee injury and osteoarthritis outcome (KOOS) score, the International Knee Documentation Committee (IKDC) score and the Tegner score. The clinical outcome of ankle patients were evaluated using the American Orthopaedic Foot and Ankle Society (AOFAS) Hindfoot score and the Tegner score. Of the 10 patients, two patients were re-operated due to treatment failure. They were excluded from the study. CT imaging revealed that 0/8 patients had complete regeneration of the subchondral bone. At 2.5 years, 6/8 patients had no or very limited (<10 %) bone formation in the defects (Figure 6b) and 2/8 had 50-75 % bone formation in the treated defect. MRI showed no improvement in the MOCART score at either 1 or 2.5 years. The IKDC score significantly improved from 41.3 to 80.7, and the KOOS pain subscale significantly improved from 63.8 to 90.8 at 2.5-years follow-up. We did not find improvements in the remaining KOOS subscales, the AOFAS Ankle-Hindfoot score or the Tegner score. Implantation of the MaioRegen® scaffold in osteochondral defects resulted in poor subchondral bone and cartilage repair at both 1- and 2.5-years follow-up. Improvements in clinical outcome scores were observed. The results of this study questions the biological repair potential of the MaioRegen® scaffold, and the use of the MaioRegen® scaffold has been discontinued in Denmark.



**Figure 6.** The MRI and CT scans of a representative patient treated with the MaioRegen scaffold. The cylindrical defect can clearly be seen in the 1 year CT (a), the 2.5 year CT (b), the 1 year MRI (c) and the 2.5 year MRI (d).

Study 3: Autologous dual-tissue transplantation for osteochondral repair: early clinical and radiological results

In this study we aimed to investigate early biological and clinical outcome of autologous dual-tissue transplantation. ADTT is a combined autologous bone and cartilage chips transplantation for treatment of osteochondral injuries. It is easily applicable and bypasses the need for costly cell culturing or synthetic material. In a prospective cohort we included eight patients with osteochondral injuries in the knee. In the ADTT treatment autologous bone is used to fill the osteochondral defect. The bone graft is applied to a level at the base of the adjacent cartilage. A cartilage biopsy from a low-weight-bearing site is cut into small chips and the chips are embedded in fibrin glue in the defect. MRI, CT and clinical outcome scores were applied preoperative and 1 year postoperative. The MRI MOCART score improved from 22.5 to 52.5 at one year (p < 0.01). All 8 patients had a bone filling of >80%. The IKDC score improved from 35.9 to 68.1, (p < 0.01), the Tegner score improved from 2.6 to 4.7, (p < 0.05), and all KOOS subscales, but one, improved significantly (p < 0.05) (Table 2). ADTT resulted in good subchondral bone repair and good cartilage repair. Significant clinical improvements were found after 1 year. The results of this study highlights ADTT as an effective, lowcost, treatment method for osteochondral injuries.

Score	Preoperative	SD	l vear	SD	Difference	95% CI	Р
	36.9	20	68 1	15.7	32.2	11.2-53.1	<0.001
KOOS	0017	20	00.1	1017	U LI L	1112 0011	
Pain	58.3	23.4	84.5	14.5	26.2	3.5-48.8	< 0.05
Symptoms	63.3	17.3	83.3	12.1	20.1	2.6-37.5	< 0.05
ADL	74.8	24.0	85.4	14.0	10.6	12.2-33.5	NS
Sport/recreation	24.3	17.4	57.2	22.7	33.0	9.4-56.4	< 0.01
QOL	27.7	13.9	62.7	8.1	35.0	21.8-48.3	< 0.01
Tegner	2.6	1.8	4.7	2.2	2.1	0.9-3.4	< 0.01
MOCART	22.5	8.5	52.5	14.4	30	17.3-42.7	<0.001

**Table 2.** The clinical outcome scores preoperative and one year postoperative.

 SD=standard deviation.
 CI=confidence interval.



**Figure 7.** MRI and CT of four representative patients. The first column is preoperative MRI, the second column is 1 year MRI and the third column is 1 year CT. Each row represents one patient.

Study 4: Implantation of autologous cartilage chips improves cartilage repair tissue quality in osteochondral defects: A study in Göttingen minipigs

The aim of this study was to investigate the biological role of cartilage chips. By comparing ADTT with autologous bone graft alone in the Göttingen minipig model, we isolated the effect of cartilage chips. The hypothesis was that the presence of cartilage chips would improve the quality of the repair tissue. We operated twelve Göttingen minipigs who received two osteochondral defects in each knee. The defects were treated with either ADTT or autologous bone graft alone (ABG). Six animals were euthanized at 6 months and 6 animals at 12 months. Follow-up consisted of histomorphometry, immunohistochemistry, semi-quantitative scoring and CT.

There was significantly more hyaline cartilage in the ADTT group (25.8%) compared to the ABG group (12.8%) at 6 months. At 12 months the fraction of hyaline cartilage in the ADTT group had not significantly changed, while in the ABG group the hyaline cartilage fraction had significantly decreased to 4.8%. At both 6 and 12 months there were significantly more fibrocartilage in the ADTT group (44% and 60.8%) compared with the ABG group (24.5% and 41%). The fraction of fibrous tissue was significantly lower in the ADTT group compared with the ABG group at both 6 and 12 months.

The presence of cartilage chips in an osteochondral defect facilitated the formation of fibrocartilage as opposed to fibrous tissue at both 6 and 12 months. This study substantiates the chondrogenic role of cartilage chips in osteochondral defects.



**Figure 8.** Histological image of an ADTT treated defect 12 months postoperative. The implanted cartilage chips (cc) are marked with lines, and between them fibrocartilaginous tissue (fibrocart.) is seen. Staining is HE.

# **Chapter 4. Methodological discussion**

To interpret the results of studies 1-4, one must be aware of the limitations and strengths of the applied materials and methods. The methods are described and discussed in this chapter.

# The Göttingen minipig

Before planning an animal study researchers have an obligation to consider animal ethics. Russell and Burch pioneered the field of animal ethics with the publication of "The principles of humane experimental technique" in 1959[162]. They introduced the principles of the three Rs: 1) Replacement, 2) Reduction and 3) Refinement. 1) Replacement is defined as the substitution of conscious living animal for insentient material. For example using tissue-, cell- or subcellular culture rather than animal models. 2) Reduction is the effort of reducing the number of animals needed to obtain a meaningful and statistically significant result. In short, using neither too few nor too many animals. 3) Refinement is an effort to reduce the suffering of the animals, by improving the anesthesia, surgical procedure, post-operative care, housing facilities etc.

The motivation for performing study 1 was to develop a costeffective large animal model with a predictable outcome by refining the surgical procedure and reducing the number of animals used.

In study 1 and study 4 of the present thesis, we used the Göttingen minipig as the large animal model. The Göttingen minipig is a member of the Sus scrofa domestica species, the same species as normal domesticated pigs. The Göttingen minipig is a breed characterized by proportional dwarfism where all body parts are proportionate, but reduced in size compared to normal land race pigs. It has been bred to be small in size throughout its lifetime, have a docile nature, a low in-breeding coefficient and large ear veins (for intravenous access). The animal is a crossing between the Minnesota minipig, the Vietnamese potbelly pig and the German landrace pig. They are bred and raised in specialized barrier facilities and are subjected to strict procedures regarding biosecurity. This results in reduced background pathology, reduced threat of secondary infections and increased safety for the technical staff[163]. The Göttingen minipig can only be acquired from one of the four available breeding facilities world wide

(Denmark, Germany, USA and Japan), and the cost of one adult minipig is €1305[164] (approximately 10-times the price of a normal Danish landrace pig for research purposes).

In study 1 and 4 we used skeletally mature animals. Skeletally immature animals have a higher potential for spontaneous repair of chondral and osteochondral injuries. Pigs are skeletally mature at 18 months and to prevent overestimation of the repair response, pigs used for cartilage research must have reached this age[156], [165]. By 18 months Danish landrace pigs weigh 200-250 kg, making handling of the animal unmanageable, whereas an 18 months old Göttingen minipig weighs 30-50 kg making handling and housing very easy.

In addition to the animal being skeletally mature, the studied defects must be of critical size to avoid spontaneous repair. Gotterbarm et al. investigated 180 chondral and osteochondral defects in the Göttingen minipig and found that defects with a diameter of 5.4 mm did not heal spontaneously. The same was seen with 6.3 mm defects and the authors concluded that both defects were critically sized[156]. The conclusions of Gotterbarm et al. were verified by the empty chondral and osteochondral defects of study 1, and it was decided that an empty control group was not needed in study 4.

# Surgical experiences on the Göttingen minipig

In the following, a step-by-step description of the anesthesia, surgery and post-surgical recovery is made. The description is based on the surgical experiences gained by performing knee operations in both hind legs of 64 Göttingen minipigs from 2012-2015.

# Pre-surgery

The animals should be fasting for at least 6 hours before the surgery. While the animal is in the pen it is pre-medicated with Azaperone (Stresnil, 0.1 mL/kg, Janssen Pharmaceutica, Belgium) and Midazolam (Dormicum, 0.1 mL/kg, Hoffmann-La Roche AG, Switzerland) administrated subdermally in the neck of the animal. With the animal sleeping, intravascular access is gained through an auricular vein. At the same time the animal is treated with prophylactic antibiotics (0.03 mL/kg, Penicillinprokain, Ceva Sante Animale, France). General anesthesia is established by an intravenous injection of etomidate (Hypnomidate, 0.25 mL/kg, Janssen Pharmaceutica, Belgium). With the animal in a prone position, tracheal intubation is performed using a standard laryngoscope and a size 5.5-6.5 tracheal tube. To ease the intubation process a dose of 10% lidocain spray can be administered endotracheally. General anesthesia is maintained with Sevoflurane® (3 %, AbbVie, Denmark) and fentanyl (0.175 mL/kg/h, Hameln pharmaceuticals, Germany). With the animal under general anesthesia, it is now placed in a supine position. Both knees are shaved, washed and prepared for the sterile procedure.

# Surgery

The surgical access to the knee joint, the creation of a chondral defect, and the closure of the wound is described in the legend for Figure 9, 1-12.

# Post-surgical recovery

After closure of the wound local analgesics are administrated to the skin and peri-articular tissue (Xylocain, 10 mL, 20 mg/mL, Astra Zeneca, Denmark). While closing the wound during the surgery, the Sevoflurane<sup>®</sup> and the fentanyl is discontinued. While monitoring the heart rate and the peripheral capillary oxygen saturation the ventilator can be removed. The oxygen saturation should be allowed to drop (not below 75%) since the resulting rise in carbon dioxide will drive the animal to start breathing. When the animal is breathing without the aid of the ventilator, the tracheal tube can be removed and the animal can be returned to the pen.

The animals are treated with Finadyne 5 % (Flunixin meglumin, 1.1 mg/kg, oral paste, Intervet, Denmark) for five days postoperative, and are allowed free cage activity immediately after the operation.

We gained additional surgical experience in a pilot study prior to study 1. A medial para-patellar approach was tested, which required the medial quadriceps vastus to be incised to gain access to the knee. This approach resulted in more postoperative pain as observed by limping on the operated leg in the animals.



Figure 9. A step-by-step description of the Göttingen minipig knee surgery. P=patella, tt=tibial tuberosity. 1) The patella ligament is found between the distal patella pole and the tibial tuberosity. 2) A 5 cm skin incision is made between the two landmarks. 3) A small Weitlaner selfretaining retractor is inserted and the patella ligament is exposed. 4) Using a scalpel, an incision (1 cm deep) is made into the ligament in the direction of the ligament fibers. Care must be taken to make the incision while the extremity is fully extended to make sure the trochlea is reached with at minimum of trauma to the knee. 5) Hoffa's body is penetrated and 6) the trochlea is exposed. 7) A 6 mm skin biopsy punch is used to outline the defect and 8) a curette is used to carefully remove the cartilage from the outlined defect. 9) A Ø 1 mm stainless steel stylus is used to clean the circumference of the defect. At this point the desired treatment is applied as described in study 1 and 4. If an osteochondral defect is required a 6 mm metal drill is used for manual drilling. 10) After the treatment has been applied the wound is closed in three layers. First, the patella ligament is closed with watertight continuous locking sutures. 11) Next the subdermal tissue is closed with continuous sutures and finally 12) the skin is closed with continuous intradermal sutures. All suture material is resorbable to avoid subjecting the animals to the stress of subsequent suture removal.

Translation from an experimental to a clinical setting

There are many pitfalls in the translation of results from an animal model into a clinical setting. The Göttingen minipig as an animal model for articular cartilage research has limitations. The size and weight of the animal is closer to humans than smaller animal models, however the Göttingen minipig is still significantly smaller than humans. In addition, four extremities rather than two carry the weight of 30-50 kg, making the load on each knee smaller than in humans.

# Cartilage thickness

The thickness of the articular cartilage of the Göttingen minipig model is 0.3-0.8 mm compared with 2.2-2.5 mm in humans (Table 1). This can be an issue when investigating chondral implants for cartilage repair, as the thickness of such an implant can exceed the thickness for the minipig cartilage. Furthermore, the relatively thin cartilage layer and the narrow field of operation makes execution of surgical procedures more difficult.

#### Acute vs. chronic defects

As described in Figure 9, the defects studied in most animal studies are made and treated by the surgeon during the same surgical session. Patients are rarely surgically treated for cartilage injuries immediately after the occurrence of the injury, so the acute injuries of animal studies differs from the defects treated in clinical studies, which are more chronic in nature. Chronic cartilage injuries will subject the joint to inflammatory stimuli and a changed biomechanical environment, and the healthy cartilage adjacent to the injury may suffer.

# Follow-up

The follow-up time in animal models are naturally significantly shorter than in a clinical setting. A follow-up period of 6 months in Göttingen minipigs is considered sufficient in the litterature[166]–[168] and was applied in study 1[169]. However, in study 4 we found an increase in the fibrocartilage fraction in the repair tissue from 6 to 12 months follow-up indicating that the repair process is not finished by 6 months. A significant development in outcome from 6 to 12 months has been shown in other studies[156], [170], and researcher should consider the follow-up period closely when planning new animal studies.

#### Post-operative rehabilitation

In a clinical setting, a patient treated for a cartilage defect will undergo post-operative rehabilitation. The patient will be fitted with a hinged brace and instructed to refrain from weight bearing for two weeks, followed by limited weight bearing for another 4 weeks. The Göttingen minipig does not accept leg braces for weight bearing limitations. This increases the strain on the repaired defects, and an underestimation of the effects of any applied treatment is expected compared with a clinical setting.

The most important end point for patients with knee injuries is to be able to perform the activities of daily living, to return to sports and recreational activities and to be free of pain[171]. When animal models are used for the development and testing of new treatment methods for cartilage injuries, none of these endpoints are measured. To compare the outcome found in animal models to what is found in humans, researchers must rely on evaluation methods available in both cases. The most common methods; macroscopic evaluation, histology and radiology will be described in the following.

# Macroscopic evaluation

Macroscopic scoring represents a useful first-line evaluation tool for cartilage repair. Several scoring systems exist, but only two scoring systems have been validated for clinical use[172], [173]; the ICRS macroscopic score[174], [175] and the Oswestry arthroscopic score[172]. The ICRS macroscopic score evaluates the defect fill, the integration to the adjacent tissue and the appearance of the surface, and a total score from 0 (severely abnormal) to 12 (normal) is obtained. The Oswestry arthroscopic score also evaluates defect fill, integration to adjacent tissue and appearance of the surface, however it differ from the ICRS macroscopic score by evaluating the color of the graft and the stiffness on probing. Furthermore, the "defect fill" can be scored as "hypertrophic" in the Oswestry score and not in the ICRS macroscopic score. In study 1 we chose the ICRS macroscopic score since we did not perform arthroscopic probing of the defects.

# Histological evaluation

#### Semi-quantitative scoring

Histology is the study of the microscopic anatomy of cells and tissues. It is commonly used in articular cartilage research to evaluate repair tissue in clinical studies, animal studies and in vitro studies. One of the most common evaluation methods is semi-quantitative scoring. Several different semi-quantitative scoring systems for articular cartilage have been proposed for cartilage repair tissue. The O'Driscoll score was the first semiquantitative score for cartilage repair[176]. It is comprehensive and designed for animal experiments. Pineda et al. later provided a much simpler score for general use[177], while the ICRS I and II scores are comprehensive scores utilizing a visual analog scale from 0-100 (VAS)[178], [179]. Common for the scores is that they all evaluate some, but not all, of the following: Repair tissue morphology, matrix staining, surface regularity, structural integrity, defect filling, osteochondral junction, adjacent bonding, basal integration, cellularity, clustering, adjacent cartilage, degeneration, mineral, blood vessels, subchondral bone, viability cell population, inflammation and cartilage plug quality.

The use of semi-quantitative scoring systems has limitations. The scores are mainly aimed at osteoarthritic joints or chondral defects, as only one of eighteen subcategories addresses the subchondral bone and none of them addresses the regeneration of the subchondral bone tissue. Furthermore, in the O'Driscoll and Pineda score, the scores of each category are summed to a total score. The rigid structure of the semi-quantitative scores means that two very different repair tissue compositions can obtain the same score. Finally, no study has been able to show that improved semi-quantitative histological scoring of cartilage translates into clinical improvements. Knutsen et al. compared ACI with microfracture in a 5-year randomized controlled study and found no correlation between histological findings and clinical outcome[76].

In part 1 of study 1 in the present thesis, the limited sample size only allowed us to evaluate qualitative histology according to cell morphology, glycosaminoglycan (GAG) staining and repair tissue surface. In part 2 of study 1 we used the ICRS II score to compare the repair outcome of one-defect knees with the outcome of twodefect knees. By the time study 4 was performed, we had implemented a method for quantitative histomorphometric evaluation of tissue types at the Orthopedic Research Laboratory[180]. This method was performed in addition to the ICRS II score.

# Histomorphometry

Histomorphometry is the quantitative evaluation of the morphology of the repair tissue. The quantification is done through the principles of stereology. Stereology allows us to extract quantitative information about an entire 3-dimensional osteochondral defect by analyzing a number of cross sections throughout the defect. The semi-quantitative scores are biased since the scoring of each subcategory relies on subjective interpretation, whereas histomorphometry reduces the risk of bias since the only prerequisite for reliable data is the ability of the blinded researcher to know one tissue type from another [180].

In study 4 we wanted to know the morphology of the repair tissue both in the superficial part of the defect (the cartilage part) and the deep part of the defect (the bone part). We made regions of interest (ROI) for both the cartilage part and the bone part (Figure 10a). To provide an estimate with a high precision, 40-50 viewing windows per ROI, per histological slide needs to be counted and furthermore, the counting grid of the 40-50 viewing windows should provide at least 100-200 counting points[180]. In a pilot study we determined that counting 50% of the cartilage ROI resulted in an average of 51 viewing windows and 380 counted points, and counting 30% of the bone ROI resulted in an average of 60 viewing windows and 830 counted points. Ideally sections through the entire defect should be analyzed, but in study 4 we wanted to reserve part of the defect to accommodate the possible need for further analysis and to have backup tissue in case of unforeseen complications in the embedding process. We halved the 6 mm defects at a random point (anywhere in the sagittal plane ±180°) and made the assumption that half of the defect was representative for the entire defect. Starting from a random point (approximately in the middle of the defect) sections of 7  $\mu$ m slices were cut. There were 14 sections per level and the distance between the levels was 350 µm. Cutting of sections was continued until the defect was no longer visible in the slices, resulting in 7-10 levels.

Using newCAST software (Visiopharm, Hørsholm, Denmark) a 5x5 counting grid was superimposed onto the sections using a 10x objective (Figure 10b). Each point in the counting grid was counted according to the tissue type. The tissue types present in the defects in study 4 were hyaline cartilage, fibrocartilage, fibrous tissue, bone, bone marrow and vascular tissue.

Histomorphometry is limited by the lack of descriptive qualitative information. This information was obtained by performing semiquantitative scoring (The ICRS II score). The use of both methods, as in study 4, provides a well rounded histological description of the treatment methods.

Another limitation of histomorphometry is that a prerequisite for the method is the ability of the observers to identify the tissue types correctly. However, Foldager et al. found that using polarized light microscopy to discriminate between tissue types, significantly limited the risk of bias[180].



**Figure 10.** a) The regions of interest drawn onto the histological slide. 1) is the cartilage part of the defect, while 2) is the bone part of the defect. Staining is H&E and the size bar is 1000  $\mu$ m. b) A 5x5 counting grid was superimposed onto the images at 10x objective. In this example, polarized light microscopy is used to distinguish between fibrocartilage and hyaline cartilage. To demonstrate how the counting was performed, each point in the grid has been labeled with "-" for no tissue, "h" for hyaline cartilage,

"fc" for fibrocartilage, "f" for fibrous tissue and "b" for bone. Staining is H&E and the size bar is 100  $\mu m.$ 

#### Immunohistochemistry

Immunohistochemistry is the process of detecting an antigen in tissue sections by visualizing the specific antibody-antigen bindings. In study 4 we applied immunohistochemistry to investigate the viability of the implanted cartilage chips. A study by Foldager et al. have shown that healthy hyaline cartilage stains positive for collagen type IV and laminin in the pericellular matrix of chondrocytes, whereas degenerative cartilage stains negative for laminin and faintly for collagen type IV[181]. Alternative methods for determining the cartilage viability include mechanical testing, testing for DNA content, GAG content and cellularity and confocal microscopy using LIVE/DEAD staining[182], [183]. Immunohistochemistry was chosen in study 4 since the staining could be performed on histological slides already in our possession. There were no need for additional technical equipment and there were no risk of causing damage to the specimen by doing mechanical testing.

The original immunohistochemic technique is a direct method. An enzyme-labeled primary antibody binds to the desired antigen. As this technique only involves one antibody the staining is very weak, and the direct method is rarely used. To amplify the staining signal a two-step indirect method has been developed: The unconjugated primary antibody binds to the antigen in the tissue and an enzyme-labeled antibody specific for the immunoglobulin of the primary antibody is then added. This amplifies the staining signal as several secondary antibodies can bind to the primary antibody.

In study 4 we used a three-step indirect method. After incubation with the primary antibody (polyclonal rabbit antibody specific for laminin or collagen type IV) the secondary antibody marked with biotin binds to immunoglobulins of the primary antibody. The signal is amplified by the tertiary antibody (streptavidin-horse radish peroxidase), which binds to the biotin of the secondary antibody. The reaction is developed by aminoethylcarbazol. Mayer's hematoxylin was used for counterstaining[184].

There are a number of pitfalls when using immunohistochemistry. The histological fixation process can cause epitope masking or loss of antigenicity, and some studies have even shown a progressive loss of antigenicity (in breast cancer markers) due to storing at room temperature[185]. By amplifying the signal, secondary and tertiary antibodies minimize the risk of a false negative due to weak staining. The risk of a false positive is reduced by always using negative staining controls[186], [187].

# Radiological evaluation

Magnetic Resonance Imaging is a non-invasive evaluation tool that offers information about the morphology of the repair tissue and the overall status of the joint. To be able to consistently and accurately evaluate cartilage repair procedures Marlovitz et al. developed the 2-dimensional MOCART score[188]. The MOCART score evaluates cartilage repair tissue using 9 subcategories: "Degree of defect repair and filling of the defect", "integration to border zone", "surface of the repair tissue", "structure of the repair tissue", "subchondral lamina", "subchondral bone", "adhesions" and "synovitis". In 2009 Welsch et al. proposed a modification of the 2D MOCART score by adding a TrueFISP 3-dimensional sequence and two additional subcategories; "bone interface" and "chondral osteo-phytes". The 3D MOCART score was used in study 2 and study 3

to quantify the changes seen on MRI. In study 3 we found a significant improvement in both the MOCART score and in the clinical scores, however in study 2 the significant clinical improvements were not accompanied by improvements in the MOCART score. This lack of correlation found in the present thesis has been a subject for debate previously. In a two-year follow-up study Marlovitz et al. found that the MOCART score was "a reliable, reproducible and accurate tool for assessing cartilage repair tissue", and that there was a statistically significant correlation between the clinical outcome score and the MOCART subcategories "filling of the defect," "structure of the repair tissue," "changes in the subchondral bone," and "signal intensities of the repair issue"[189]. However, de Windt et al. did a comprehensive literature review to evaluate the correlation between MRI and clinical outcome after cartilage repair. The authors included 32 studies in the review (including the Marlovitz study[189], [190]) and reported that only 9 of 32 studies (28%) found a correlation between the clinical outcome and the MOCART score. The authors concluded that: "Strong evidence to determine whether morphological MRI is reliable in predicting clinical outcome after cartilage repair is lacking"[190].

In study 1 we MRI scanned the animals preoperative and at 3 and 6 months, and in study 4 we MRI scanned the animals at 6 and 12 months. We planned on evaluating the images using the MOCART score, however when a trained radiologist evaluated the images, the spatial resolution was found to be too low to allow for a meaningful application of the MOCART score. Consequently the defects were evaluated only according to repair tissue surface. Due to the low spatial resolution of the images a decision was made to exclude MRI from study 4.

# Clinical outcome scores

Clinical outcome scores are important tools for monitoring the subjective progress of patients. A multitude of different clinical outcome scores exist. Benthien et al. performed a large study examining the clinical evidence of different surgical cartilage procedures. The authors found a low methodological quality in cartilage research in general, with few randomized controlled studies, and interestingly, in the 133 studies evaluated, the authors counted 27 different clinical outcome scores[191]. This makes inter-study comparisons very difficult since the outcome of one score cannot be compared to the outcome of another. In study 2 and 3 we employed three different scoring systems: The Tegner score was originally developed to evaluate activity level of ACL patients, but is widely used for other knee injuries. It is a onequestion score where the patients state their activity level on a scale from 0 to 10. The score "0" is defined as "on sick leave/disability" and the score "10" is defined as "participation in competitive sports such as soccer at a national or international elite level" (Appendix 1). The Tegner score has not been validated for patients with focal cartilage injuries.

The IKDC score is more comprehensive than the Tegner score. It assesses the patients' symptoms, sports function and daily activity level (Appendix 2). As the Tegner score, the IKDC score was originally developed for patients with ligament injuries, but it has been validated for knee injuries in general[192]. Hambly and Griva compared the IKDC score with the KOOS score and found that the IKDC score provided the most accurate short-term overall measure of symptoms and disabilities[171].

The KOOS score includes five subscales: symptoms, pain, activities of daily living, function in sport and recreation and knee-related quality-of-life (Appendix 3). The score was originally developed for knee osteoarthritis but has been validated for focal cartilage injuries[193]. While the output of Tegner and IKDC scores is a single numerical number, the KOOS score presents the scores of each subscale separately. This avoids the question of how much each subscale should affect the final outcome.

# Clinical outcome score selection

Clinical outcome scores are important follow-up tools, but for a researcher to deem one treatment superior to another, the applied score must be relevant to the type of injury and treatment studied. Tanner et al. compared 11 different clinical outcome scores based on how important the posed questions were to the patients[194]. The authors identified the KOOS and the IKDC score as the two best scores for general knee injury. This conclusion was based on how many patients had experienced the symptoms addressed in the scores and on how important the question was to the patient. Hambly et al. compared and analyzed the KOOS and IKDC scores. They concluded that the IKDC "provided the best overall measure of symptoms and disabilities that are most important to this population of postoperative articular cartilage repair patients" [171]. The conclusion of the study was later disputed by Roos et al. in a letter to the editor and a gold standard clinical outcome score for articular cartilage injuries is yet to be found[195]. Interestingly, Hambly et al. found that "knee-related quality of life" and "function in sport and recreation" were more important to the patients than "pain" and "symptoms". The authors reported that the most important question to the patients were "Modified lifestyle to avoid activities that are potentially damaging to the knee", whereas "can't straighten knee" were the least important question[171].

In study 2 and 3 of the present thesis the KOOS, IKDC, Tegner and AOFAS Hindfoot scores have been reported without comparison to a control group. This is a major limitation, and one that is shared with all clinical studies on the MaioRegen® scaffold and all but one on cartilage chips[139]. Of the nine clinical studies published on the MaioRegen® scaffold (one case report excluded[116]), all studies used the IKDC score, but none of them included a control group. Of the four clinical studies published on cartilage chips in the knee (case reports excluded[143]-[146], [149]), three used both the IKDC and KOOS score[139], [142], [150], one used the KOOS score[151] and only one included a control group. The importance of performing randomized clinical trials was highlighted in 2002 by Moseley et al.. The authors included a total of 180 patients with osteoarthritis of the knee. They randomized the patients to receive arthroscopic débridement, arthroscopic lavage or placebo sham surgery. The patients were followed for two years using five different self-reported clinical outcome scores. After two years the authors found no difference in clinical outcome in any score and they concluded that the clinical benefit of arthroscopic lavage and débridement could be attributed to the placebo effect[196].

The lack of a control group and randomized controlled trials means that authors risk promoting a new treatment method based on the placebo effect rather than actual clinical improvements.

# Chapter 5. General discussion

# **Replicability of Results**

It is well known in the field of research that scientists prioritize novelty over replication and that journals prioritize positive results over negative results. This creates bias as research results are subject to selective reporting, selective analysis and publication bias. In a controversial paper from 2005 Ioannidis makes the claim that "most published research findings are false" [197]. The paper has been widely discussed and a comment disputing the mathematical proof of the study was later published[198]. The loannidis study does however direct attention on the issue of reliability of research. In a recent open science collaboration study published in Science, the subject of the reliability of published research papers is discussed[199]. In the field of psychology 270 contributing authors replicated 100 studies. Ninety seven percent of the original studies had significant results (p<0.05), whereas only 36% of replicated studies had significant results. Furthermore, the authors subjectively rated only 39% of the studies to have successfully replicated the original results. Similar studies in the field of cell biology found replication success in 11% and 20-25% of the replicated studies [200], [201]. It is important to keep in mind that the mentioned studies are not discussing research fraud, but rather lack of reproducibility.

In study 2 we were not able to reproduce the favorable radiological findings of previous studies on the MaioRegen® scaffold. Several possible explanations exist. The authors of the original clinical studies on the MaioRegen® scaffold have observed a partial detachment of the scaffold in 13% of cases[124]. To address this problem, the authors conducted a study on the mechanical stability of the implant with and without fibrin glue. The authors found that fibrin glue improved the post-operative stability of the implant, and they went on to recommend the addition of fibrin glue when implanting the MaioRegen® scaffold[202]. In study 2 we did not use fibrin glue to fix the scaffold. As all clinical studies on the MaioRegen® scaffold to date, we followed the recommendation of the manufacturer, which is fixation by press fitting. This fixation technique worked well during the surgery and we did not observe any detachment of the implant at any time point.

An alternative explanation is differences in applied MRI sequences and the interpretation of the images by the radiologist. A radiologist trained in cartilage imaging evaluated all images. The radiologist was blinded as to what treatment was applied and the clinical outcome of the patient. We used both axial and coronal fast spin echo sequences, proton density-weighted, fat saturated and short tau inversion recovery. A variety of these sequences were also used in previous studies on the MaioRegen<sup>®</sup> scaffold[118], [124].

The possibility of selective reporting and selective analysis is controversial but must be considered. All but one of the previous clinical studies on the MaioRegen<sup>®</sup> scaffold have been published by affiliated authors from Italy, some of which are employed by the manufacturer of the scaffold (Finceramica Faenza SpA, Faenza, Italy). This affiliation has been disclosed in the publications and must be considered when interpreting the results. Verdonk et al. recently published the only study not originating from Italy. The authors treated 38 patients with the MaioRegen scaffold and found significant clinical improvements after two years. They also found significant improvements in the MOCART score, however the authors expressed concerns about the appearance of the subchondral bone. No CT evaluation was performed[125].

We, the authors of study 2, could also be subject to selective reporting and selective analysis, since the publication and presentation of controversial results attracts attention in the academic world. In both clinical studies (study 2 and 3) we aimed at providing full transparency of the results by presenting the radiology of each patient rather than a representative image. This gives the reader the best possible prerequisite for interpreting the results and for replicating the study.

In study 1 we successfully replicated the results of Gotterbarm et al[156]. We confirmed that 6 mm chondral and osteochondral

defects in fact are critical sized and that the surgical access is reproducible and provides a good insight to the trochlea. Furthermore, we improved the cost-effectiveness of the Göttingen minipig model by establishing that the use of two defects per knee does not affect the repair outcome.

# Osteochondral scaffolds

The idea of repairing osteochondral injuries with a cell-free offthe-shelf scaffold is appealing. A synthetic scaffold is often very easy to handle and implant, it eliminates donor site morbidity, surpasses problems with biological compatibility and only requires one surgery. A large number of different synthetic scaffolds exist, however most are aimed at full-thickness chondral injuries. Only few osteochondral scaffolds are available for clinical use.

The TruFit® BGS plug was originally developed for backfilling osteochondral autograft sites, but it has been used off-label as an osteochondral plug for articular cartilage repair. Two years after the first positive clinical report on the TruFit® plug, two studies reported the presence of foreign-body giant cells in the repair tissue[109], [203]. Several studies on delayed incorporation, graft failure and clinical outcome deterioration have since followed[110]–[112], [204]–[206], and Barber et al. have published CT images very similar to what was seen in study 2 of this thesis (Figure 11).



**Figure 11.** CT images from a) a patient treated with the TruFit<sup>®</sup> plug from Barber et al.[111] and b) a patient treated with the MaioRegen<sup>®</sup> scaffold from Christensen et al.[207]. A cylindrical cavity is seen at the site of the scaffold implantation in both images. Furthermore, a sclerotic edge is seen in the periphery of the cavity. Image "a" has been reprinted with permission from F. A. Barber.

Figure 11a and b shows the CT images approximately two years after the implantation of the  $\mathsf{TruFit}^{\circledast}$  plug (Figure 11a) and the MaioRegen® scaffold (Figure 11b). The similarities are evident in that a cylindrical cavity remains where bone was supposed to have regenerated. In both studies the implant seemed to impair ingrowth of bone instead of facilitating it, however the consequences of this impaired bone ingrowth are not known. No animal study has evaluated the outcome of the TruFit® or MaioRegen® scaffolds using CT, and in general, very few studies reporting a poor clinical outcome after osteochondral repair exists. This might partly be due to publication bias, but even the studies reporting a poor radiological outcome often have a good patientreported clinical outcome. Three studies on the MaioRegen® scaffold have found poor MRI results. Filardo et al. found the MRI results to be "less favorable" [121], Verdonk et al. found the subchondral lamina and bone changes seen on MRI a "concern"[125] and in study 2 of this thesis the CT imaging revealed a concerning lack of bone repair and MRI showed poor repair tissue quality[207]. However, in all three studies the poor radiological outcome did not correlate with poorer clinical outcome at the followup.

A well-known saving goes: "We treat the patient, not the MRI" and the lack of correlation raises the question if poor radiological results have any significance if the patient is doing well? Studies do point to the fact that focal cartilage injuries can progress to secondary osteoarthritis though: Linden et al. studied 48 patients with adult osteochondritis dissecans and found that after 33 years 79% had osteoarthritis of the knee and that the onset of the osteoarthritis came approximately 10 year earlier than secondary osteoarthritis (49 years old vs. 59 years old)[208]. Similarly, Messner and Maletius examined 28 young athletes suffering from cartilage defects, 14 years after the arthroscopic diagnosis. They found that even though the majority of the patients (22 patients) had good to excellent clinical results, 12 patients (43%) had significant joint space reduction in the compartment of the defect[32]. A possible explanation for the deterioration of unrepaired chondral/osteochondral defects is the pothole theory, explained in Figure 12a-d. When a pothole forms in a road you have to fill it to stop the pothole from expanding. If not repaired, each passing car will cause the defect to expand through increased rim stress (Figure 12a and d, black arrows). When the MaioRegen® scaffold is implanted the material filling the osteochondral defect, be it fibrous scar tissue or scaffold material, is less stiff than bone. An area of tissue with a low stiffness surrounded by tissue with a high stiffness will be compressed when loaded and act as a hole instead of an area with sufficient structural integrity (Figure 12d).

Kock et al. showed that when autologous osteochondral plugs are set just below the normal articular surface, the border contact pressure increased by 92%[209]. Peña et al. applied a finite element computer model and found that small defects are not significantly affected, however defects larger than 0.78 cm2 are subjected to increased rim stress[210]. The short/intermediate-term implications of increased rim stress were studied by Lefkoe et al. who found significantly decreased proteoglycan content in cartilage from the rim of 20-week-old defects in rabbits[211] and Jackson et al. found loss of contour of the femoral condyle one year after creating an osteochondral defect in a goat model[212].



Figure 12. A schematic drawing of the pothole theory. A) An osteochondral defect on the tibial plateau. The black arrows indicate the increased

rim stress. The grey arrows indicate joint loading. B) The diameter of the osteochondral defect has been increased due to the pothole effect. C) The MaioRegen scaffold implanted in an osteochondral defect. D) Due to the lower stiffness of the MaioRegen scaffold compared to the cartilage and bone, the scaffold is compressed during joint load causing the increased rim stress. The illustration has been modified from servier.com.

Should the long-term outcome of the MaioRegen<sup>®</sup> studies prove the pothole theory false, the lack of bone ingrowth and cartilage repair could be ignored if the clinical results were superior to other treatment methods. Figure 13 shows the 1-, 2- and 5-year IKDC score of patients treated with different treatment methods. It illustrates that the preoperative IKDC of the MaioRegen<sup>®</sup> studies are very similar to alternative treatment modalities and that the similarities continue two years post-operatively.



**Figure 13.** A comparison of the IKDC score of selected studies on osteochondral defects. CAIS and PJAC are on chondral defects and have been included for reference. The following treatment methods have been included: ADTT[207], Agili-C[127], CAIS[139], PJAC[150], Mosaicplasty[213]–[215], TruFit[104] and the MaioRegen scaffold[118]–[121], [123], [124], [216], [217]. The figure illustrates the similarities in clinical outcome regardless of the treatment modality.

Since the long-term consequences of the lack of bone and cartilage repair are unknown, long-term randomized controlled studies are much needed, and until they are available synthetic osteochondral scaffolds for clinical use cannot be recommended. Cartilage chips

The use of cartilage chips has shown great promise in articular cartilage repair. Three different approaches have now been tested in a clinical setting: The cartilage autograft implantation system (CAIS) and particulated juvenile articular cartilage (PJAC) applied to full thickness chondral defects, and autologous dualtissue transplantation (ADTT) for osteochondral defects. All three have shown a good safety profile and good clinical outcome.

	PJAC,	PJAC,	CAIS,	
	Farr et al.	Buckwalter et al.*	Cole et al.	ADTT, Christensen et al.
n	25	13	20	8
Age	38 ± 11	26 ± 12	33 ± 9	32 ± 7
Sex (ma-				
le:female)	18:7	3:10	16:6	5:3
Defect size, cm2	2.7 ± 0.8	2.3 ± 1.7	2.75 ± 1.5	3.1 ± 1.5
Defect location				
Condyle	18		14	6
Trochlea	11		10	2
Patella		13		
Talus				
Follow-up, years	2	0.7	2	2**

**Table 3.** A comparison of the patient demographics and defect characteristics in four clinical studies on cartilage chips. \*The Buckwalter et al. study followed up after eight months. \*\*Only clinical outcome at 2 years.

Cole et al. compared CAIS to MFx in a randomized controlled trial on 29 patients. After two years the patients treated with CAIS scored significantly higher in the IKDC and KOOS scores, whereas no difference was seen on MRI[139]. The CAIS study represents the only clinical randomized controlled trial on cartilage chips.

Five case reports[142]–[144], [146], [149] and four larger clinical studies[147], [150]–[152] have been published on PJAC. In general, the studies have found improvements in clinical and radio-logical outcome, however one case report on a 44-year-old woman has been published in which the PJAC treatment failed[149].

The ADTT treatment developed by Martin Lind was first described in study 3 of this thesis[218]. Clinical and radiological outcome scores significantly improved 1 year post-operative and the KOOS score was very similar to what was seen in the CAIS study and the PJAC study (Figure 14).

# Juvenile vs. adult cartilage

The three approaches have all been shown to result in clinical improvements in patients. The repair mechanism is believed to be chondrocyte outgrowth, but this has yet to be confirmed in vivo. Lu et al. were the first to report in vitro chondrocyte outgrowth from cartilage chips in 2006[129] and Sage et al. later found that collagenase treatment of the chips resulted in increased GAG and DNA content in the produced extra cellular matrix[133]. Since advanced age of articular cartilage is believed to cause increased calcification[219], decreased proteoglycan synthesis[220] and decreased chondrocyte response to growth factors[131], authors have investigated the use of juvenile cartilage allograft. Adkisson et al. studied juvenile and adult chondrocytes and found a dramatic age-related decline in the chondrogenic potential of chondrocytes, and showed that allogeneic juvenile chondrocytes did not stimulate an immunologic response[130]. Subsequent studies have found improved extra cellular matrix production and improved cartilage repair by combining juvenile and adult cartilage chips as compared with adult chips alone[131], [221].

Seeing that juvenile cartilage appears to have a greater chondrogenic repair potential, the clinical results of the PJAC treatment would be expected to surpass those of the CAIS and ADTT treatments, however this is not the case. Figure 14 compares the KOOS scores from the four clinical studies on cartilage chips.



**Figure 14.** A comparison of the KOOS scores of the four clinical studies on cartilage chips showing the similarities in clinical outcome regardless of treatment method. The follow-up time in the Buckwalter et al. study was eight months.

It shows that the baseline score and the 1 and 2 year postoperative score are very similar in the four studies. Table 3 compares the patient demographics and defect characteristics of the four studies. The table reveals similar defect sizes, defect locations, and age of patients across the four studies.

The four studies focus on the repair of chondral or osteochondral defects using cartilage chips. They include similar patients and show similar results at one and two years. This indicates that the improved chondrogenic potential of juvenile cartilage might not be responsible for the repair.

# Repair mechanism

In an in vitro study from 2013 Liu et al. offered a theory on how the chondrocytes migrate from the cartilage chips. The authors found several different matrix metalloproteinases (MMP) significantly up-regulated in the juvenile cartilage chips compared with the adult. They hypothesized that MMPs act by breaking down the extracellular matrix, thus allowing the chondrocyte to migrate through migrating channels (Figure 15a).



**Figure 15.** Migrating channel as shown by (a) Liu et al.[138] and (b) Skagen et al.[222]. a) Size bar is 100  $\mu$ m and staining is safranin-O. b) Size bar is 20  $\mu$ m and staining is H&E. (Permission to reprint by SAGE journals and Creative Commons)

The authors found migrating channels in juvenile and adult cartilage chips, but reported that the increased MMP levels in juvenile

cartilage allowed chondrocytes deeper in the chips to migrate out[138]. Skagen et al. found similar chondrocyte migration in 1year-old porcine cartilage explants. The authors found that the presence of fetal bovine serum resulted in increased migration and chondrocyte proliferation within the lacunae in the cartilage explants. They speculated that the chondrocyte migration was initiated by chemotactic factors entering the matrix from the culture medium. Furthermore, they reported that mechanical injury to the cartilage resulted in increased mitotic activity[222].

The migrating channels have not been spotted in vivo in any experimental or clinical study, and conflicting data have been found in other studies. Zingler et al. investigated the chondrocyte outgrowth potential of cartilage from eight donors aged 53-80 years. The patients were undergoing a total knee arthroplasty due to osteoarthritis and the authors obtained the cartilage biopsies from macroscopically normal appearing cartilage. After 4 weeks of culturing, no chondrocyte outgrowth was found and the authors concluded that no relevant outgrowth occurs from adult cartilage[223]. These results support the notion of decreased chondrogenic potential of adult cartilage. However, in an unpublished pilot study on juvenile porcine cartilage, we found results very similar to those of Zingler et al. In the pilot study we took cartilage biopsies from the knee of a 5 months old landrace pig and embedded the cartilage chips in fibrin glue. The explants were cultured in Dulbecco's modified Eagle's medium/F12, 10% fetal calf serum and 1% penicillin-streptomycin for two weeks. There were four groups: 1) No pre-treatment, no adjuvant therapy. 2) Collagenase pre-treatment for 15 minutes, no adjuvant therapy. 3) No pre-treatment, mesenchymal stem cells added to the fibrin glue clot. 4) Collagenase pre-treatment, mesenchymal stem cells added. After two weeks of culturing we found no evidence of any chondrocyte outgrowth in any group (Figure 16).



**Figure 16.** a) and b) No outgrowth of chondrocytes from cartilage chips after two weeks of culturing. b) shows intact pericellular membranes of chondrocytes indicating no migration. a) HE staining. b) Collagen type IV staining. Unpublished data

A possible explanation for the lack of chondrocyte outgrowth in the unpublished pilot study is the fact that no growth factors were added to the culture medium. The addition of growth factors could theoretically have stimulated the chondrocytes and resulted in chondrocyte outgrowth, however in the study by Zingler et al. the authors stimulated the cartilage using either cell lysate, High-Mobility-Group-Protein B1, Trefoil-factor 3, bone morphogenetic protein-2, transforming growth factor-ß1, or three-dimensional fibrin scaffolds. None of the above had any effect on the chondrocyte outgrowth[223].

In study 4 we determined that the presence of cartilage chips in an osteochondral defect in vivo results in an increased fraction of fibrocartilage, and this fraction increased from 6 to 12 months. While the fraction of fibrocartilage increased, the fraction of hyaline cartilage in the defect decreased from 25.8% to 20.1%. This was not statistically significant, but regardless of whether the hyaline tissue fraction decreased or stagnated, it is safe to say that it did not increase. These findings do not support the theory of chondrocyte outgrowth since the hyaline tissue fraction and not the fibrocartilage fraction would be expected to increase as chondrocytes multiply, migrate, and start producing hyaline extra cellular matrix.

Alternative theories on the repair mechanisms are 1) Paracrine stimulation of repair tissue or 2) Resorption of the implanted cartilage chip matrix, which will leave chondrocytes embedded in fibrous tissue instead of hyaline extracellular matrix. Stevens et al. found that DeNovo repair tissue in the vicinity of an autologous osteochondral plug maintained its hyaline nature better than more isolated DeNovo repair tissue. This could indicate that the presence of healthy hyaline cartilage has a positive effect on the repair tissue morphology through paracrine stimulation. In Figure 17 the results from study 4 of this thesis (Figure 17a) are compared with the results from Stevens et al. (Figure 17b)[149].



**Figure 17.** A comparison of the histological results of a) ADTT in a Göttingen minipig after 1 year and b) PJAC in a 44 year old patient after 2 years. In both images cartilage chips surrounded by fibrocartilage is seen. Both images are stained with H&E. Image "b" has been reprinted with permission from Hazel Stevens. cc; cartilage chips, fc; fibrocartilage

In both studies cartilage chips are seen surrounded by fibrocartilage. No evidence of chondrocyte outgrowth or migrating channels are seen in either the ADTT treatment (Figure 17a) or the DeNovo treatment (Figure 17b). The shape of the cartilage chips at the time of implantation is distinctive, with sharp straight edges, however in both images of Figure 17 the cartilage chip edges appear more rounded. In study 4 the hyaline tissue fraction decreased or stagnated. This indicates that the rounded shape of the cartilage chips is not due to chondrocyte outgrowth and matrix production, but rather partial resorption of the hyaline matrix, a process that possibly turns the hyaline cartilage into fibrocartilage. The data currently available is not sufficient to make a ruling on the repair mechanism(s) responsible for the clinical improvements observed, just as the presence of the placebo effect cannot be ruled out.

# Cartilage chips – Knowledge gaps

The limited clinical data on articular cartilage chips means that there are several gaps in our knowledge. So far the patients who have been treated with cartilage chips have been relatively young, with little comorbidity and with small defects (Table 3)[139], [150], [151], [218]. To maximize the benefit of the treatment we need additional knowledge of the following:

# Chip size

The size of the implanted cartilage chips varies from study to study, and little evidence exists regarding the optimal size for cartilage repair. In the CAIS and the PJAC treatments cartilage chips of 1 mm3 are used. Lu et al. found an inverse relationship between the size of the chip and the efficiency of outgrowth[129]. The same relationship was found when investigating outgrowth of meniscal fibrochondrocytes[224], and in a recent study Bonasia et al. found increased extra cellular matrix production when reducing the chip size from 2 mm3 to 1 mm3 and to <0.3 mm3 [225]. On the other hand one must consider if there is a lower limit of chip size. Damage to the cartilage tissue is significantly reduced by using sharp instrumentation compared with blunt instrumentation, but studies suggest that even sharp fragmentation of cartilage causes a zone of necrosis of 40-150 µm[222], [226], [227]. The clinical significance of the necrotic zone in cartilage chips is currently not known. Based on the data available we chose to use chips sized 0.25-0.5 mm3 in study 3 and 4, however no in vivo study has investigated the effect of the chip size.

# Chip coverage fraction

The density of cartilage chips in the defect is also a matter for discussion with little evidence to go by. Lu et al. reported, "1/10 of the defect-filling tissue was used to treat an entire defect" and stated that 2-300 mg of cartilage could cover a 10 cm2 defect[129]. Zimmer Biomet (Warsaw, IN, USA) states that one vial of DeNovo NT is designated to cover a 2.5 cm2 defect area. According to Zimmer Biomet this fills approximately 50% of the defect and is sufficient for repair[228], however Tompkins et al. reports the use of one vial per 1.3 cm2 of defect[152].

Cole et al. used approximately 200 mg of cartilage to cover a 2.7 cm2 defect[139]. In study 3 and 4 we did not weigh the cartilage biopsy. We harvested cartilage from non-weight bearing sites to cover approximately 70-80% of the defect[218].

Different surgeons use different amounts of cartilage chips in the defects, and no studies have been made to determine what amount yield the best results or if there is a minimum or a maximum amount of chips per cm2 for sufficient cartilage repair.

# Cartilage chip limitations

Treatment with cartilage chips has limitations. The defect must be contained with stable shoulders to avoid graft delamination, and the maximum defect size is limited by what is practically possible due to the cartilage surface curvature. Cartilage chip supply is limited by the potential concomitant donor site morbidity. As in ACI, the cartilage biopsy is taken from a low-weight-bearing site, and the donor site morbidity is believed to be negligible[229], [230]. In mosaicplasty the ratio between the area of the donor site and the area of the defect is 1:1. As previously mentioned the chip coverage fraction is not known, but an educated guess when using cartilage chips is perhaps a ratio of 1:1% - 1:2 or as Lu et al. reported 1:10 [129]. The implantation of cartilage chips is performed in an open procedure, which results in more postoperative pain and sequelae for the patient. However, an arthroscopic technique has been implemented for talar injuries[144], [145], [148] and a similar technique for knee injuries is set to follow.

# Conclusion

Through the studies of this thesis we successfully validated the Göttingen minipig as an animal model for cartilage repair. We found evidence that the MaioRegen® scaffold could impede bone regeneration and we introduced ADTT as a promising, cost-effective treatment for osteochondral injuries.

One aim of this thesis was to develop and test a large animal model for chondral and osteochondral repair. In study 1, the Göttingen minipig model was concluded to be a suitable animal model for cartilage repair studies. Furthermore we found that the animal model could be improved from an ethical and cost-effectiveness viewpoint, by applying two defects per knee. As a result of study 1, the Göttingen minipig model has been established as the standard animal model for cartilage research at the Orthopaedic Research Laboratory, Aarhus University Hospital.

Another aim of this thesis was to evaluate two different approaches for clinical osteochondral repair: The MaioRegen® scaffold and Autologous Dual-Tissue Transplantation. These two methods represent two fundamentally different approaches for tissue repair. The MaioRegen® is a commercially available, offthe-shelf synthetic scaffold, whereas ADTT is the transplantation of autologous bone and cartilage graft. In study 2, treatment of osteochondral defects with the MaioRegen® scaffold resulted in poor subchondral bone repair and incomplete cartilage repair as seen on CT and MRI. Even though significant clinical improvements were observed, the almost complete lack of bone ingrowth and cartilage repair raise serious concerns about the biological repair potential of the MaioRegen® scaffold. Based on the results of study 2, the use of the MaioRegen® scaffold in Denmark was discontinued.

In study 3 we reported the short-term clinical and radiological results of ADTT in eight patients. One year after the surgery we found significant improvements on MRI, CT and all clinical out-come scores. The fact that no cell culturing or scaffold is needed makes ADTT a promising, low-cost treatment method for oste-ochondral injuries.

In study 4 we applied the Göttingen minipig model (developed and validated in study 1) to evaluate the isolated effect of autologous cartilage chips in osteochondral injuries. The study confirmed the chondrogenic effect of cartilage chips, but the additional finding of increased fibrocartilage in the ADTT group combined with no change (or perhaps a decrease) in the hyaline fraction, questions the prevailing theory of the repair mechanism of cartilage chips.

#### Perspectives

The ever-increasing number of patients suffering from cartilage pathology, and the current and future financial pressure on the health care sector, calls for cost-effective treatment methods. Cartilage chips appears to be an effective, low-cost alternative to expensive cell- and scaffold-based treatment methods for osteochondral repair. Short-term results on ADTT are promising, and future long-term results will shed further light on the treatment method. This thesis has advised against the use of the MaioRegen® scaffold and promoted the use of ADTT. Just as important, it has led to new questions about the repair mechanism involved in cartilage repair using cartilage chips. Adjuvant therapy is the next logical step in treatment with cartilage chips, but before adjuvant therapy can be effectively applied we must expand the knowledge on the repair mechanisms. This is key in optimizing the treatment method, and future studies will investigate the basic science of cartilage chips and the use of adjuvant therapy.

The current literature on the field of cartilage chips in general is limited by the lack of randomized controlled trials. The 250 patient clinical trial on the PJAC treatment set to be published in 2021 will shed light on the long-term effect of juvenile cartilage chips, however this study will not have a control group, and chances are that the study will result in significant clinical and radiological improvements, just as the majority of studies on cartilage injuries (Figure 13 and Figure 14). To advance the knowledge on cartilage repair, large randomized controlled trials are needed. Until they are conducted we will be in the dark as to what treatment is the gold standard.

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#### Abstract

Articular cartilage forms the articulating surface of synovial joints. Along with the synovial fluid it facilitates near frictionless movement in healthy joints. Injuries to articular cartilage in the knee are frequent and can lead to severe osteoarthritis, which is expected to affect 25% of the adult population by 2030. Hyaline cartilage does not regenerate spontaneously when injured and the current clinical treatment methods suffer from high cost and relatively high failure rates. This calls for new treatment methods to be developed. The backbone of developing new treatment methods for cartilage injuries is a reliable, cost-effective, clinically relevant large animal model. Study 1 aimed at developing such a model.

We hypothesized that in the Göttingen minipig, the repair response of a selection of treatment methods would be similar to what is found in a clinical setting, and that two defects per knee, rather than one, could be applied without affecting the repair outcome. We found that the outcomes of the applied treatments were consistent with the outcomes in clinical studies. Furthermore, the use of two defects per knee did not have any significant effect on the repair response. The Göttingen minipig model was easy to handle, cost-effective, and provided a predictable repair response. Based on this study the use of two defects per knee, in male Göttingen minipigs is recommended. The model has been implemented as the standard animal model for cartilage research at the Orthopedic Research Laboratory, Aarhus University Hospital.

Synthetic osteochondral scaffolds represent an off-the-shelf, onestep treatment method, and preliminary clinical results have been promising. However, MRI investigations have shown issues related to subchondral healing. In study 2 we aimed at evaluating the osteochondral repair in 10 patients treated with the MaioRegen® synthetic scaffold. Of the 10 patients, two patients were reoperated due to treatment failure. CT imaging revealed that none of the eight remaining patients had complete regeneration of the subchondral bone. At 2.5 years, 6/8 patients had no or very limited (<10 %) bone formation in the defects and 2/8 had 50-75 % bone formation in the treated defect. MRI showed no improvement at either 1 or 2.5 years compared with baseline. Clinical outcome scores were improved at 2.5 years. These results raise serious concerns about the biological repair potential of the MaioRegen<sup>®</sup> scaffold. The use of the MaioRegen<sup>®</sup> scaffold has been discontinued in Denmark as a result of this study.

An alternative treatment approach for osteochondral lesions is combined transplantation of autologous bone graft and cartilage fragments, embedded in fibrin glue. In study 3 we investigated the early biological and clinical outcome of autologous dual-tissue transplantation (ADTT). ADTT is a combined autologous bone and cartilage chips transplantation for treatment of osteochondral injuries. It is easily applicable and bypasses the need for costly cell culturing or synthetic materials. After 1 year, all 8 patients had significant improvements on MRI, CT and all clinical outcome scores. This study establishes ADTT as a promising, low-cost, treatment option for osteochondral injuries in the knee.

To investigate the role of the implanted cartilage chips, we tested the isolated effect of the chips in the newly developed Göttingen minipig model. In study 4 we compared ADTT with autologous bone graft alone. The hypothesis was that the presence of cartilage chips would improve the quality of the repair tissue. Twelve Göttingen minipigs were included, and follow-up time was 6 and 12 months. Follow-up consisted of histomorphometry, immunohistochemistry, semi-quantitative scoring and CT. There was significantly more hyaline cartilage in the ADTT group compared with the autologous bone graft group at both 6 and 12 months. At both 6 and 12 months there were significantly more fibrocartilage in the ADTT group compared with the ABG group.

The presence of cartilage chips in an osteochondral defect facilitated the formation of fibrocartilage as opposed to fibrous tissue at both 6 and 12 months. This study substantiates the chondrogenic role of cartilage chips in osteochondral defects, but questions the widely accepted repair mechanism involved in cartilage chip treatment methods. Further studies on the repair mechanism(s) involved are needed to improve the clinical application of autologous cartilage chips.

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