



Universiteit Utrecht

Pilot study to test attachment of repair tissue to a non-resorbable osteochondral implant with vertical channels



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Abstract

Restoring cartilage defects is a major challenge in humans and animals, including the horse. Most attempts of realizing cartilage regeneration results in fragile fibrous repair tissue and not in regenerative tissue. The repair tissue that forms naturally, eventually undergoes fibrillation and degeneration leading to further disruption of joint homeostasis. Lesions will therefore eventually lead to pain, swelling, and decreased mobility and will sometimes progress to osteoarthritis. Many therapies provide pain relief, but do not represent long-term solutions. As a result of a study from 2019, this pilot study is set up. In the study by Korthagen et al. (2019), osteochondral defects were made in the medial femoral trochlear ridge and filled with an implant. The implant was bi-layered which consists of polyetherketoneketone (PEKK) and a polyurethane elastomer. The outcome of this study was that cartilage did grow over the implant, but there was no adhesion to the underlying elastomer. In this pilot study, it was considered to solve this problem by micro-drilling vertical channels into the existing implant, hoping that cartilage would grow into the channels and this will ensure adhesion. The implants were placed at two locations, the medial femoral condyle and the medial femoral trochlear ridge in two Shetland ponies. The new implant with hand-drilled vertical channels and the control implant from the precedent study were placed randomly. After five weeks the ponies were euthanized and tissues were evaluated with the macroscopic view, optical coherence tomography, and histology.

The postoperative recovery was good in both ponies and minimal lameness was observed. The results of this pilot study were that cartilage grew over the implant location on the medial femoral condyle with an average thickness of 547,75 μm . The results at the trochlear ridge were better, as much more cartilage grew over it, with an average thickness of 843,50 μm . Once the implants were compared, the average thickness of the implants without channels was 578 μm and with channels was 812,25 μm . In histology, cartilage cells are found in the vertical channels which indicates better adhesion. The results are very promising, but long-term research with more ponies is needed.

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Introduction

Focal cartilage and osteochondral injuries can arise in various ways, for example, traumatic or degenerative (Buckwalter et al., 1994., Sanders et al. 2001). There are many reasons why cartilage reparability is very low, which results in a complex treatment. If the injury continues into the subchondral bone marrow, mesenchymal cells enter the defect from bone marrow and then fill the defect with a fibrous tissue, called fibrocartilage. The fibrocartilage does not have the same function and structure as the original articular cartilage. Lesions will lead to activity-related pain, decreased mobility, and joint effusion, which frequently are related to osteoarthritis. Also when the injuries are left untreated, the resulting chronic inflammation can lead to osteoarthritis (Fugazzola et al., 2020). Research shows that 60% of lameness in the horse is caused by osteoarthritis (Caron JP et al., 2003). Osteoarthritis is multifactorial and in many instances caused by injury (Hunziker., 2013). Once initiated, this lesioning process is progressive. The medical treatment of osteoarthritis in the horse is one of the most utilized therapeutic regimens in the equine practice, as well in the human medical field. In 2008 it was estimated that nearly 27 million adults in the US have clinical osteoarthritis (McIlwraith et al., 2012). Treating osteoarthritis in horses and humans is a long, complex, and difficult process. The pathophysiology of joint diseases is extremely complex and the multifactorial nature of the disease makes it challenging to investigate and determine an ideal therapy. This pilot study looks at a new therapy to treat focal cartilage lesions by using an implant. Before discussing the new treatment, first I look at the complex structure of cartilage and what the possible consequences could be of not treating a cartilage defect. Subsequently, it is also discussed which current therapies there are and why there is such a need for a new therapy.

Structural organization of hyaline cartilage

Hyaline cartilage, also named articular cartilage, consists of 95% of extracellular matrix, with sparsely distributed chondrocytes (Alford et al., 2005). The number of chondrocytes in this volume is quite low and varies in number, size, and shape according to the layer in which they are located. Each layer of hyaline cartilage has a different composition and architecture of chondrocytes, collagen, aggrecan, and fluid dynamics that relate directly to the function of that layer. Hyaline cartilage can be divided into four layers with different functions: superficial zone, middle or transitional zone, radial or deep zone, and the calcified layer (Figure 1).

The superficial zone is resistant to various forces tangentially to the surface. This layer also forms a kind of impermeable barrier against larger molecules from its synovial fluid. This layer has the highest concentration of water, the lowest concentration of proteoglycans, and the chondrocytes have an elongated shape. Type IX collagen is found in this layer between type II bundles that provide resistance to shear force. Another property of this layer is a low metabolic rate activity and thus a low regeneration capacity. Preservation of this layer is critical to protect the deeper zones (Alford et al., 2005; Martel-Pelletier et al., 2008). The middle zone or transitional zone is rather resistant against compression forces and has high concentrations of collagen, which are organized in random orientation. The shape of the chondrocytes in this layer is round (Alford et al., 2005).

The next zone is the radial or deep zone. This zone is resistant to compression and has a large diameter collagen fibrils oriented perpendicular to the surface. This layer has the highest concentration of proteoglycans, the lowest concentration of water, and the chondrocytes are organized in columns (Huber et al., 2000). Between the radial layer and the calcified layer, there is a thin layer, named the tide mark. The tide mark contains fibrils that the uncalcified layer allows to adhere to the calcified layer. It also contains pores through which nutrients can diffuse from the extracellular matrix. The calcified layer mainly acts as the anchor between the articular cartilage and subchondral bone through the tidemark (Alford et al., 2005). Injury to any part of these complex

systems can disrupt the normal biomechanical function of articular cartilage, leading to further degeneration.

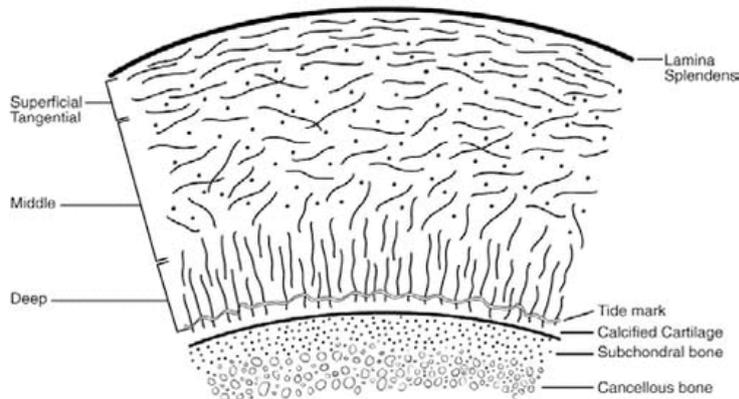


Figure 1: The different layers of the matrix (Alford et al., 2005).

Chondrocytes

Chondrocytes are a less than 10% part of its full matrix volume. As mentioned earlier, these chondrocytes differ in number, shape, and size according to the layer in which they are located. Chondrocytes have a mesenchymal stem cell origin and they have a function to synthesize the matrix (Alford et al., 2005). The sparse distribution and their low turnover rate allow for little cell-to-cell contact (Buckwalter et al., 1998). They also take care of the organization of the collagen, the proteoglycans, and the non-collagenous proteins in a well-ordered structure. Chondrocyte survival depends on the proper chemical and mechanical environment, including growth factors, mechanical loads, hydrostatic pressures, and piezoelectric forces (Alford et al., 2005). Chondrocytes generally obtain their nutrients from the synovial fluid. This causes chondrocytes to rise with low concentrations of oxygen and mainly use anaerobic metabolism (Buckwalter et al., 1998). They are controlled by multiple cytokines, such as interleukins, interferon, tumor necrosis factor, growth factor, and hormones produced through these chondrocytes have catabolic and anabolic effects on the cartilage. Many cytokines are produced by the chondrocytes themselves in response to stimuli and have an effect on the build-up or breakdown of the extracellular matrix. This is, for example, how mobilization of the joint leads to increased activity of the chondrocytes, while immobilization leads to decreased production of proteoglycans and loss of cartilage. Weight load leads within the chondrocytes to a change in composition, cell volume, acidity, and ion concentrations (Sun HB, 2010).

Extracellular matrix

The extracellular matrix (ECM) can be divided into a liquid component and the macromolecules. Examples of macromolecules are collagen, proteoglycans, and non-collagenous proteins. Similarly to the difference for the chondrocytes distribution, there is also a difference in the content and distribution of the ECM according to the location in the joint and the age of the patient (Huber et al., 2000; Alford et al., 2005). The matrix can be divided into a pericellular territorial and interterritorial region around each chondrocyte, going from close to further away from the chondrocyte. These regions are different in their specific composition with proteoglycans, compound proteins, and hyaluronic acid. They work together to form a hydro elastic suspension that protects against compression. The various components of the extracellular matrix are discussed later.

Collagen

75% of the dry weight from the articular cartilage consists of collagen. The extracellular matrix is for 80-90% composed of type II collagen, but types VI, IX, X, and XI are also present in smaller amounts (Alford et al., 2005). The largest quantitative differences in the composition of the articular cartilage arise from the difference in cartilage maturation. For example, finer fibrils can be found in young cartilage (composition > 10% collagen IX, > 10% XI and <80% collagen II), while in the mature cartilage, thicker and more varied fibrils (composition: 1% collagen IX, 3% collagen XI and >90% collagen II) will be found (Eyre D et al., 2002).

Type II collagen forms a heteropolymer together with type IX and type XI and is resistance to tension (Eyre DR et al., 1991). Type II, IX, and XI are cartilage-specific and cross-linked in a network that forms the extracellular skeleton and gives the skeleton its shape. In this way, it contributes to its firmness.

The orientation of the collagen fibrils varies greatly depending on the layer of the cartilage. In the deeper radial zone, the collagen fibrils have an orthogonal orientation on the joint surface. In the

intermediate layers, this orientation is more at random in different directions and towards its surface, this orientation is rather horizontal (Figure 2).

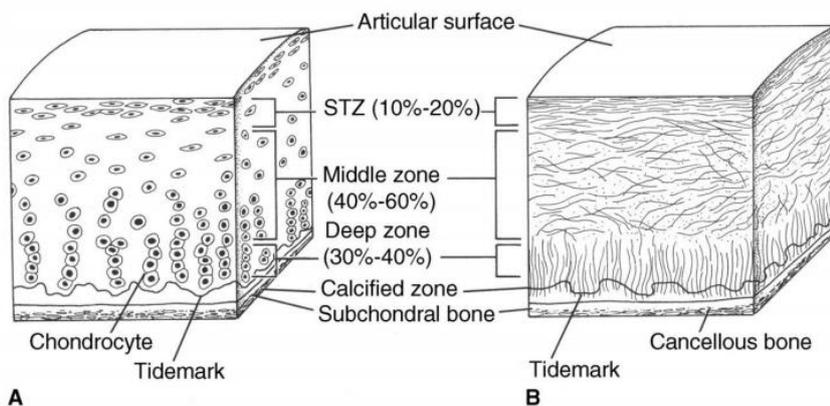


Figure 2: schematic overview of a cartilage joint with the shape of the chondrocytes and the organisation of the collagen (Buckwalter et al., 1994).

Proteoglycan

The proteoglycans constitute 12% of articular cartilage and are resistant to compression. The proteoglycans are the major non-fibrillary components of the cartilage tissue. Cartilage has two groups of proteoglycans, the large chains or aggrecans and the smaller chains, including decorin, biglycan, and fibromodulin (Buckwalter et al., 1998). The proteoglycans contain a protein core and one or more glycosaminoglycans, mostly represented by sulfated glycosaminoglycans (GAGs), chondroitin sulphate, and the keratan sulfate. The aggregate with hyaluronic acid, which is an unsulfated glycosaminoglycan, acts as a kind of backbone. It also ensures that the aggrecans remain trapped in the fibrous network of collagen (Alford et al., 2005).

Carboxyl and sulfate groups on the glycosaminoglycans have a negative charge. This negative charge creates a high affinity for water that helps cartilage resist compressive loads and causes aggrecans to repel one another, resulting in maximal volume expansion (Alford et al., 2005). The flow of water through charged areas of the proteoglycan-rich matrix generates piezoelectric charges that further modulate the rate of water flow contributing to the viscoelastic behaviour of articular cartilage (Vidal et al., 1988). When the pressure on the cartilage increases, the water pressure in the cartilage also increases, so that the water is driven away from the compressed region. At the same time, proteoglycans prevent this draining of water and macromolecules so that the loaded region is only slightly deformed (Alford et al., 2005).

Biomechanical function of articular cartilage

Cartilages have a biomechanical function, which consists of absorbing the same forces as the bone, but it must also be smooth so that two surfaces can slide over each other. The main function of the cartilage is to adapt to the pressure changes and the equal distributing this pressure load, while the

subchondral bone absorbs forces via compression. The cartilage thus acts as a force divider. This not only relies on the type of cells, but also on the architecture of the tissue.

The proteoglycans contained in the cartilage matrix are polyanionic. The negative charges of the aggregated proteoglycans push each other away, giving the molecule maximum volume. This volume increase is limited by the collagen network in which the aggrecans are located. However, when the cartilage is compressed, the repulsion forces between the negative groups increase. The result is a compressive stiffness of the cartilage. Also, the water bound to the aggrecans will be very difficult to leave the matrix. All this would not be as effective if the aggrecans were smaller and thus not caught in the collagen network, or if there would be collagen damage. The collagen fibres are also important in the resistance to shear forces on the cartilage. The fluid flow in the cartilage also strongly depends on the duration of the load. With a short impact, the cartilage behaves purely elastic and the water remains bound to the cartilage. When there is prolonged stress on the cartilage there will be a flow of water. This continues until there is a new equilibrium situation. So here the cartilage behaves rather viscoelastic and this is important for the function as impact distributor. The flow of water is also important for cartilage nutrition. Upon cartilage relaxation, this fluid returns along with new nutrients (Ranjan et al., 2006). This process is known as imbibition.

The finding that fluid flow and deformation are interdependent has led to a model of cartilage as a mixture of solid and liquid components, also known as the biphasic model of cartilage. The compressive strength of the cartilage is possible due to the proteoglycans and the flow of the interstitial fluid, while allowing the ability of the cartilage slide is due to the movement of collagen fibers and proteoglycans (Ranjan et al., 2006).

Cartilage repair

Articular cartilage in adults has no innervation, no lymph drainage, is avascular and the chondrocytes are fixed in the matrix. All these features ensure that the cartilage reparability is very low. Cartilage injury stimulates only a limited short-term response in the surrounding chondrocytes, which consists of proliferation and increased matrix turnover. If the injury continues subchondral into the bone marrow, mesenchymal cells enter the defect from bone marrow and then fill the defect with a fibrous regenerate, called fibrocartilage.

First, it is necessary to make a distinction between regeneration and repair. As soon as regeneration takes place, the damaged tissue is fully restored to its normal state with the original function. With replacement, the tissue is repaired by applying connective tissue with scar tissue. Most, if not all, attempts at realizing cartilage regeneration have so far resulted in cartilage repair (Krafts et al., 2010; Fugazzola et al., 2020). Certain tissues, such as the liver, has a capacity to proliferate with a self-renewing capacity. This is not the case with articular cartilage (Krafts et al., 2010).

The repair tissue, fibrocartilage, above the articular defect is composed primarily of type I collagen in the first stage (Amiel et al., 1985; Barr et al., 1994). The predominance of type II collagen in hyaline articular cartilage is considered to be the criterion that differentiates this tissue from repair fibrous tissue and fibrocartilage. New collagen is produced, but the body barely succeeds in incorporating it into the network structure. It is still unclear why the cartilage will not recover.

Pathophysiology of osteoarthritis

Currently, equine osteoarthritis may be considered as a group of disorders characterized by a common end-stage: progressive degeneration of the articular cartilage accompanied by changes in the bone and soft tissue of the joint (McIlwraith et al., 2012; Goodrich., 2006). The disease is a complex process which affects not only the cartilage but the entire joint. Ultimately the articular cartilage degenerates with fibrillation, fissures, ulceration, and full thickness loss of the joint surface. Also, osteophytes can develop at the edges of the joint due to osteoarthritis. Osteophytes are bone

spines that form as tissue repair and they are one of the features of osteoarthritis. The formation has the disadvantageous effect of limiting the mobility of the joint (Wong et al., 2016). The severity may be influenced by multiple factors.

The preservation of articular cartilage depends on keeping the cartilage architecture intact. By a minor injury, the complex structure and function of cartilage can be disrupted. The response to injury depends on the severity and depth of the injury.

The balance between catabolic and anabolic effects of the chondrocytes is disturbed by osteoarthritis. The chondrocytes are no longer able to maintain homeostasis between synthesis and degradation (Heijink et al., 2012). It is not known what causes this imbalance. This results in a change in the extracellular matrix, with increased water content and reduced proteoglycan content. Also, due to the disruption of homeostasis, there is a weakening of the collagen network as a result of reduced syntheses of type II collagen and an increased breakdown of collagen. In addition, there is an increased apoptosis of chondrocytes (Buckwalter et al., 2005). Proteoglycan loss, increased water content, decreased cartilage stiffness, and increased hydraulic permeability lead to increased force transmission to the underlying subchondral bone, which increases its stiffness and, in turn, causes impact loads to be more readily transmitted to the partially damaged cartilage. This vicious cycle is thought to contribute to the progression of articular cartilage injuries (Alford et al., 2005). Ultimately this created sclerosis and osteophytes.

It is generally accepted that IL-1 is the key cytokine at the early and late stages of osteoarthritis. IL-1 leads to the breakdown of the cartilage and it plays an important role in osteoarthritis, because it is responsible for the proliferation of the fibroblast.

Osteoarthritis can start in different ways, like disease in the synovial membrane, fibrous joint capsule, subchondral bone, ligaments, and articular cartilage. Also, a combination is possible (McIlwraith et al., 2012). The process can start at trauma causing a microfracture or inflammation causing a slight increase in enzymatic activity that may allow the formation of wear particles, which are then cleared by present macrophages (Wang et al., 2013). At some point, the production of the wear particles becomes too large and the system can no longer eliminate them and inflammatory mediators are formed. This causes chondrocytes to release degrading enzymes, which in turn leads to cytokines. These bind to chondrocyte receptors, leading to further release of metalloproteinase and inhibition of type II collagen production, thus further increasing cartilage decline. Circulation levels of TNF- α and IL-6 are associated with cartilage loss in humans knee with osteoarthritis (Stannus et al., 2010).

At the beginning of osteoarthritis compensatory mechanisms such as proliferation of chondrocytes and synthesis of matrix molecules are enough to maintain the integrity of the articular cartilage. In the end it is not enough to keep the integrity and there develop osteoarthritic changes. Ultimately, a domino effect is created because the subchondral bone is also affected. The bone contains more type I collagen, which leads to abnormal mineralization. This reduces the ability of the subchondral bone to absorb and dissipate its energy, increasing the forces transmitted through the joint and making the cartilage more susceptible (Neogi., 2012). A vicious circle arises because inflammation causes cartilage breakdown by cytokines which in turn created more inflammatory cells.

Surgical therapy

Currently, there are a number of surgical treatments to heal cartilage defects. There therapies are briefly discussed below.

Microfracture

This technique is used by humans and horses. This technique aims to support the natural regenerative capacity of stem cells from the bone marrow. This method, which involves making small

holes in the subchondral bone, ensures the migration of stem cells to the site of the (osteo) chondral defect to make repair tissue there (figure 3). This repair tissue that will be formed consists mainly of fibrocartilage.

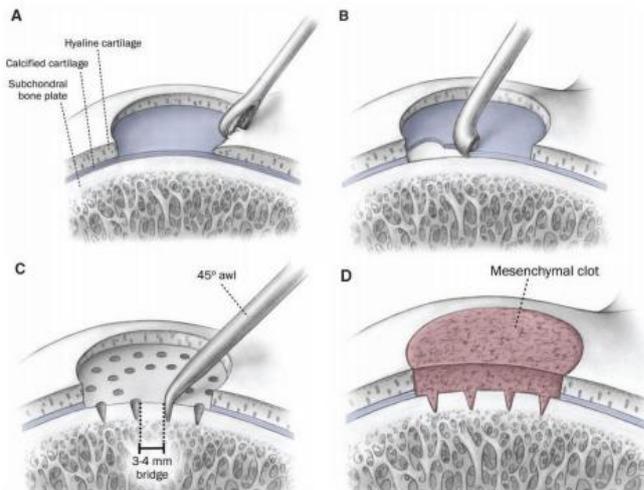


Figure 3: The four steps of microfracture. A: The debridement until a healthy cartilage edge is obtained. B: The removal of the calcified cartilage layer. C and D: The microfracture is done and the formation of the mesenchymal blood clot (Mithoefer K et al., 2009).

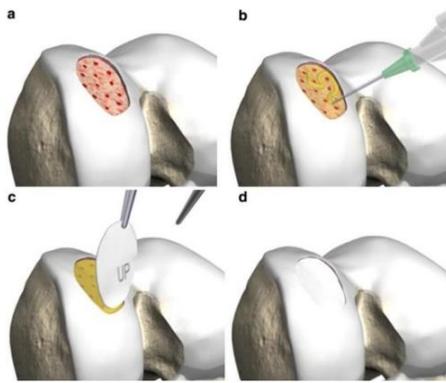
Various research studies on microfracture in the horse are done (Frisbie et al., 2006; Frisbie et al., 1999). Defects treated with microfracture have more repair tissue than defects without treatment. While there was no difference in the quality of the repair tissue. In humans, they have seen the short-term clinical effects of the treatment with microfracture in the human knee, but the long-term effects are still uncertain (Mithoefer., et al., 2009). Another adverse effect is the development of osteophytes, which may inhibit the repair reaction (Frisbie et al., 2006). Despite the unclear long-term benefit, this is a widely used technique in humans and horses, especially due to its economic and technical convenience.

Arthroscopic mosaic, arthroplasty or mosaicplasty

The principle of these technique is to create a surface nicely covered with intact hyaline cartilage (Hangody et al., 2008). One plug or several smaller plugs are used, which both consists of bone and cartilage. The plugs are used on the location of the knee where they carry less weight, usually the periphery of the femoral condyle. Hangody et al (2008) suggested that using multiple smaller plugs, can fill defects of 1 - 4 cm². By combining different plugs you can reduce the defect for 90 to 100 %. The disadvantages of this technique are the technical difficulties, failure of the plug, limited amounts of donor material, and morbidity at the donor site, which increases with the use of larger plugs. Studies in horses have shown that the results vary. In one study, osteochondral grafts were transplanted from the femoropatellar joint to the carpal bone. Nine months after surgery, fewer proteoglycans were present in the carpal bone. This made for softer cartilage that is less resistant (Hurtig et al., 2001). In another study, osteochondral plugs were extracted from the cranial part of the medial femoral trochlea and transplanted to the contralateral medial femoral condyle. After twelve months, 50% of the grafts were visible covered with hyaline cartilage, while the other half showed loss of glycosaminoglycans and transformation to fibrocartilage (Bodó, 2013).

Autologous matrix-induced chondrogenesis (AMIC)

This technique involves a combination of the microfracture technique with a collagen scaffold and fibrin glue (Gille et al., 2010). The first step is to remove the damaged cartilage and the second step is



to make microfracture perforations in the bone. A collagen type III/I membrane is then placed above the treated area. AMIC is especially useful for larger cartilage defects when chondrocyte implants are too expensive or not indicated (figure 4). The advantages of the technique are the relatively easy implementation, the low costs, and speed of the procedure (Gille et al., 2010).

Figure 4: AMIC technique. A: The damaged cartilage is removed. B and C: Microfracture perforations are made in the bone and the membrane is placed above it. D: The end result (Gille et al., 2010).

Autologous chondrocytes transplantation/implantation (ACI).

This is a two stages procedure. This means surgery takes place twice, which can be seen as a major disadvantage to this technique. The first step consists of harvesting the cartilage cells, usually from the femoral condyle. Venous blood is also taken and used together with the culture medium. In a second step, in vitro the cells must proliferate. A surgical procedure to place the chondrocytes in the defect is the last step. All fibrocartilage is removed to obtain healthy cartilage edges. Afterward the defect is filled with the cartilage cells. In a short follow up period, no significant difference can usually be demonstrated compared to microfracture (Knutsen et al., 2004). Matrix assisted chondrocyte implantation (MACI) is a second generation ACI technique. This technology offers some practical advantages. Some weaknesses of the ACI technique are: loss of cells, uneven distribution of the cartilage cells and the differentiation in a 2D environment. Some studies published results comparing MACI to ACI. Bartlett et al. published a randomized clinical trial in which 91 patients were randomized to treatment with ACI or MACI. However, no difference could be indicated in terms of clinical, histological or arthroscopic scores (Bartlett et al., 2005).

Once we look at the surgical techniques, there is no ideal yet. This is due to the fact that it is difficult to restore articular cartilage in the same structure and firmness. Therefore, this pilot study is looking at a different surgical option.

Future treatment options

Another possible cause that has not yet been discussed has to do with gene expression. Certain genes are switched on in the embryonic and postnatal phases. If it is known which ones, they could be activated and the body repairs the cartilage damage. Unfortunately, little is known about this at the moment and this is not a solution. Since there is no good treatment for osteoarthritis and no good surgical technique to cure cartilage defects, it is necessary to look for a new treatment.

The aim of this pilot study

In a study in 2019, they showed that it might be a solution to bring something foreign to the lesion in the joint, namely an osteochondral implant (Korthagen et al., 2019). In this study of 2019 they showed that repair tissue is formed on top of the elastomer within a few weeks. However, at the end of the study at 12 weeks, it was revealed the repair tissue will come loose and a long-term repair does not take place. Compared to the study in 2019, an attempt is made to improve the implant with vertical channels, because there was no adhesion (Korthagen et al., 2019). This pilot study aims to compare if the repair tissue attaches better to the elastomer with vertical channels, compared to the

one without channels. The hypotheses were: (1) The cartilage layer on the implant with channels is thicker; (2) The repair tissue attaches better to the non-resorbable implant with the channels in comparison with the one without channels. Based on the following tests; Optical coherence tomography, histology, and macroscopic view. The end goal is to place the implant in human knees with osteoarthritis or acute osteochondral lesions. The pony stifle joint is the most anatomically similar to the human knees.

Materials & methods

Experimental design

In this pilot study another osteochondral implant was implanted in two ponies. The implants used by Korthagen et al (2019) have a diameter of six mm, a length of seven mm with a medical-grade polyetherketoneketone (PEKK) base and a transparent elastomer top layer (figure 5). The top elastomer layer consists of polycarbonate loaded with collagen-mimetic peptides arginine-glycine-aspartic acid (RGD) and glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine (GFOGER) (Korthagen et al., 2019). The difference between those two implants were the small hand-drilled vertical channels (<1 mm) into the elastomer top layer of the new implant. The purpose of these channels was to create a better adhesion to the underlying elastomer, because the repair cartilage gets into the channels. During surgery, an osteochondral defect with the diameter of 6 mm in the right and left stifle joint, on the medial trochlear ridge and on the medial condyle of the femur was created through an arthrotomy and placed the implant. The different implants are placed randomly in each joint, one has channels and the other has no channels and will serve as a control. The control implant is the same as the implant in the study before this one (Korthagen et al., 2019).

After five weeks the ponies were euthanized and tissues were evaluated with the macroscopic view, optical coherence tomography, and histology.

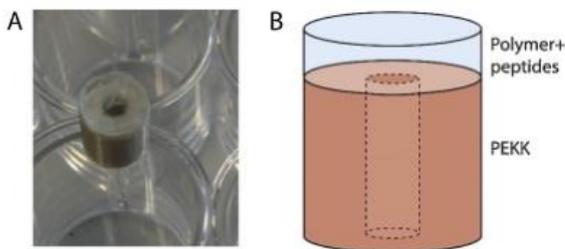


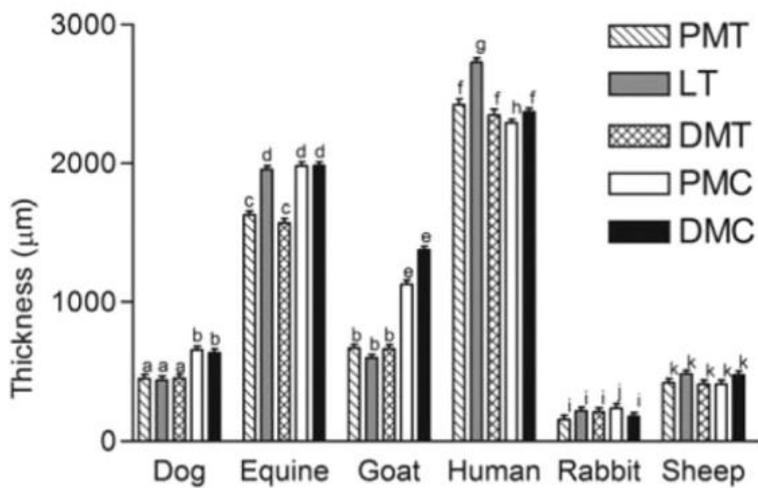
Figure 5: The implants of the study by Korthagen et al. (2019). This was the implant without the channels. A: picture of the implant. B: schematic representation of the implant (Korthagen et al., 2019).

Human knee and horse knee

Before going further about the method of this research, we first discuss why the horse is used as a model. Since it is originally intended that the treatment will be used in humans.

A defect in the equine medial femoral condyle is similar to the medial femoral condylar lesion in humans (McIlwraith et al., 2011). More advantages of the use of horses are the opportunity to use an arthroscope, the large size of the lesion with more tissue to evaluate, and the ability to have controlled exercise (McIlwraith et al., 2011). McIlwraith did research into the thickness of cartilage in different animal species. Samples were taken in five places with five animal species; human, horse, goat, dog, sheep and rabbit. The average articular cartilage thickness over 5 locations was 2.2 to 2.5 mm for humans, 0.3 mm for the rabbit, 0.4 to 0.5 mm for the sheep, 0.6 to 1.3 mm for the dog, 0.7 to 1.5 mm for the goat and 1.5 to 2.0 mm for the horse. The horse is the closest approximation to humans when looking at the articular cartilage thickness and subchondral bone thickness (figure 6) (McIlwraith et al., 2011; Frisbie et al., 2006). There is only a significant difference in cartilage thickness between the lateral and medial condyle in equine tissue, the reason for this is the larger loading of the medial condyle (Malda et al., 2012). In humans there is no difference in the thickness

of the medial condyle and the lateral condyle. The thickness of the cartilage varies per location, so there are other local loads (Shepherd et al., 1999). Therefore, it is investigated whether the thickness and load of the cartilage influences the adhesion of the cartilage of the implant. For this



pilot study, the horse's knee joint was used, because this joint was easily accessible for surgery, was similar to the knee in humans, and was one of the most common sites of osteoarthritis in humans. The joint of horses were very similar to the human knee. So it's easy to used horses for human research.

Figure 6: The thickness of the cartilage by different animals. The locations there are used are: proximal medial trochlea (PMT), lateral trochlea (LT), distal medial trochlea (DMT), proximal medial condyle (PMC) and the distal medial condyle (DMC) (Frisbie et al., 2006).

Animals

The pilot study with the osteochondral implant was performed in two Shetland ponies (2 mares), aged 4 and 8 years (mean 6 years) and between 140-160 kg (mean 150 kg). It would be unethical to immediately test this adapted implant in a large number of animals. Therefore, it was decided to conduct the pilot study of four weeks on two ponies. Two ponies were used, in case there are individual differences.

The animals were free of diseases and showed no lameness at the clinical examination before the surgery. The Shetland ponies were housed in individual stabled with straw that allowed visual contact. They receive hay a few times a day and they have unlimited water. After surgery, for those 4,5 weeks, the ponies get box rest. They only come out of there stable if the locomotion is assessed.

Human endpoint and discomfort

The human endpoint was reached as soon as there was septic arthritis, lameness more than 4/5, or lameness 4/5 that lasts longer than three days that does not improve despite maximum pain medication. The lameness grade was defined as follows:

- 0 = Not lame
- 1 = Intermittent lameness at trot
- 2 = Constant lameness at trot
- 3 = Intermittent lameness at walk/constant lameness at trot
- 4 = Constant lameness at walk and trot
- 5 = Not weightbearing

The discomfort during our experiment was moderate and mild. The discomfort was moderate during surgery, but mild during the rest of the experiment. See appendix I for the table.

Locomotion (Q horse, Equimoves)

Before and after surgery, lameness examination were performed on both ponies. One day prior to the operation our lameness examination was performed with an objective locomotion measuring

device, the Q horse and the Equimoves system. After 4 weeks the locomotion measurement will be repeated and compared to pre-operative measurements. By Q horse, an optical motion capture system (OMC), creates a 3D position by following the markers using different cameras. Parameters can reveal early signs of lameness have been defined and validated. Therefore, OMC systems were considered as the “gold standard” (Bolink et al., 2015). For this system a lot of cameras were needed and it is difficult to use it at another location, mostly only used in large clinics. An alternative for the optical motion capture system is Equimoves which was wireless and no cameras are needed but they used inertial measurement units (IMUs).

The symmetry parameters of the upper body can be calculated at several locations: poll, withers, sternum and sacrum. The vertical displacement was measured. At each stride, the upper body typically moved up and down two times for the successive left and right limb steps, so there were two peaks and two valleys in the signal, as shown in figure 7. When the horse moved symmetrically, these extrema are at the same level. The main vertical displacement parameters were max diff and min diff (Bosch et al., 2018). Markers were also used on the limbs to measure the following parameters: protraction, retraction, abduction and adduction. In a study they checked the agreement between Q-horse and Equimoves. The conclusion was that the agreement is good, except for the adduction and abduction. This is probably because the cameras at Q-horse have difficulty estimating depth. In contrast, Equimoves can therefore be an advantage. Another additional advantage was that the system can be used in a different location (Bosch et al., 2018). In this pilot study the vertical parameters will be checked and the measurements of Equimoves will be used.

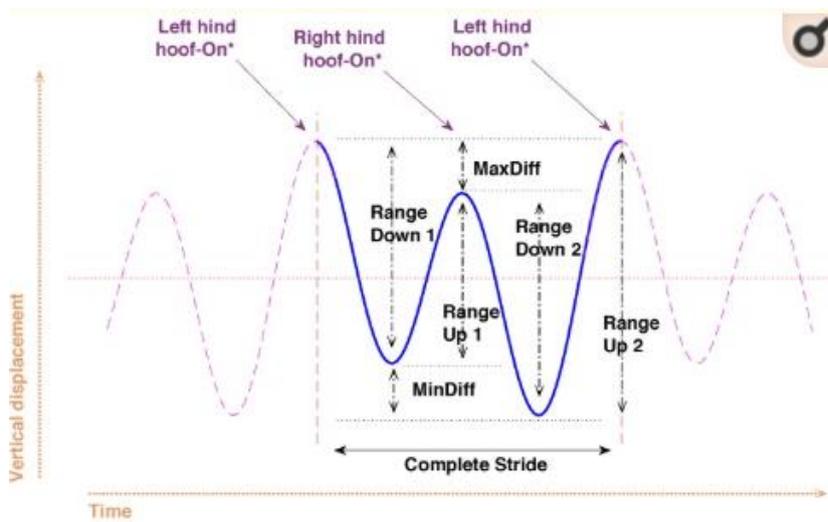


Figure 7: the parameters of the vertical measurement (Bosch et al., 2018).

Surgery

Through a previously placed 16 G jugular venous catheter, the ponies received premedication with detomidine (10 mg/kg IV) and morphine (0,1 mg/kg IV). An antibiotic prophylaxis is given with ampicillin (ampi-dry, 10 -15 mg/kg), procaine penicillin (procapen, 20 mg/kg IM) and gentamicin (1,5 – 7 mg/kg). Anaesthesia was induced with midazolam (0,06 mg/kg IV) and ketamine (2.2 mg/kg IV) and maintained with isoflurane in oxygen and continuous rate infusion (CRI) with detomidine and ketamine. The legs were positioned in an extended position. An arthrotomy of 5 – 6 cm into the femoropatellar joint was made between the middle and lateral patellar ligaments. An incision was created on the axial part of the medial trochlear ridge of the femur. A drill hole was created on this location with a drill of 5,9 mm. The implant was placed into the hole with the help of a hammer and mallet. Arthrotomy incision was closed in 5 layers: periarticular fat simple continuous vicryl-0, periarticular fascia simple interrupted vicryl-0, connective tissue simple continuous vicryl-0, subcutis

simple continuous vicryl2-0, skin interlocking suture monocryl 2-0. A Gauze roll stent was placed on top and fixated with x-stitches prolene-1.

To do the other location the legs were positioned in a flexed way. An arthrotomy into the medial femorotibial joint was made between the middle and medial patellar ligaments. An incision was created on the medial condyle surface. Making the hole, placing the implant and closing the wound is the same as discussed above. At the right side, the implants were used with the vertical channels and at the left side the implants without channels.

Pre-operative the ponies received meloxicam IV (0,6 mg/kg). Between the two operations x-ray were taken to check the locations of the implants. The days after the surgery the ponies gets tramadol (5 mg/kg) and meloxicam (0,6 mg/kg) per ox as long as necessary.

Follow up

In the time between the operation and the euthanasia the ponies were checked for swelling of the wound and perform a pain measurement with a composite pain scale (CPS) (Bussières et al., 2008). This is a complex scale and it is composed of behavioural, interaction, and physiological parameters. All parameters can be scored with digits 0 through 3, with 0 applied with no pain/normal situations, and 3 allowing with the most relevant pain expression for that parameter (Bussières et al., 2008). In a study by van der Loon (2010) they found that the CPS could potentially be a useful, reliable and easily applicable tool for assessing pain in horses. For full details of the CPS, see appendix II. The ponies were checked up daily for the first five days after surgery and once a week after the five days. The clinical evaluation was performed by two people each time. In the first 5 days after surgery an assessment of lameness and swelling of the joint was also done. For the locomotion score, the ponies were taken a few meters from the stable, so that they can walk a little bit. The wound was examined for: swelling, fluid flow, warmth and pain. Good attention should be paid to this since septic arthritis was one of the human endpoints. On the days when there was no check-up by us, there will be a check-up by the clinic's vets.

Euthanasia

The euthanasia of both ponies took place on a different day. The first pony on the first day and then all examinations immediately and the next day the other pony and examinations. Before the euthanasia the ponies were induced with midazolam (0,06 mg/kg IV) and ketamine (2.2 mg/kg IV). The ponies were placed in the supine position on the table with the legs tied, the ideal position to do all the examinations later. As soon as possible the pony were euthanized with Euthasol (0,25 ml/kg) IV. Then all examinations are carried out.

Tissue collection and macroscopic view

The hind legs were excised at the coxofermoral joints. The femoropatellar joints were opened and the following things were assessed: overall condition of the joint, colour of the repair tissue and adhesion of repair tissue to the surrounding tissue. Macroscopically, an indication could be given whether repair cartilage was present on the implant. As soon as cartilage was present, it could be assessed which aspect it had. Another important parameter to check is the extent to which the implant is covered with cartilage. After the macroscopic view, the thickness of cartilage on the implant could be assessed with OCT. Later on, an explanation will be given about this technique. All defects were examined manually with a standard arthroscopic hook probe and photographed. Biopsies (4 mm diameter) were taken on the edge of the defect area so that they contained both full thickness native cartilage tissue and repair tissue and were fixed in 4% formaldehyde solution.

Optical coherence tomography

OCT is a diagnostic technique based on the measurement of reflection and backscattering of light. In this technique, a probe with infra-red light was used which is held above the implant. It provides

cross-sectional images at resolutions comparable to that of low-power microscopy (figure 8) (te Moller et al., 2013). In a study with 36 metacarpophalangeal joints they look at different cartilage damage with OCT and arthroscopy. OCT results in a high resolution images of cartilage with a good indication of the thickness. OCT allowed for different lesions to be assessed and this technology allowed for sites not accessible with arthroscopy. Arthroscopy is used for detection of articular cartilage lesions as the golden standard by humans and horses (te Moller et al., 2013). Difficulties by the arthroscopy are: assessment is required by a surgeon. This makes it unreliable, since the interpretations can differ per surgeon (Oakley et al., 2003; Spahn et al., 2011). Furthermore it is difficult to make a distinguish between a deep and superficial cartilage defect, as well as the differentiation between intact and softened cartilage (Spahn et al., 2011). With arthroscopy it is easier to overlook a small lesion. In this study the conclusion is that OCT overcomes the most important limitations related to arthroscopy (te Moller et al., 2013).

Based on this study, it was decided to include OCT in this pilot study. After euthanasia and before removing the implants, OCT is used to assess the thickness of the cartilage above the implant.

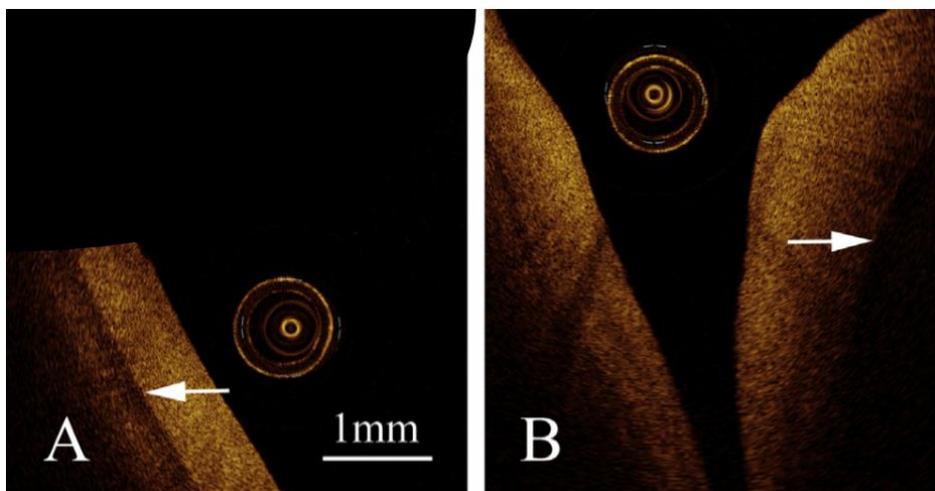


Figure 8: Normal cartilage view with the OCT. The interface between the cartilage and subchondral bone is visible with the white arrow (te Moller., 2013).

Histology

Histology was used to check whether cartilage has grown in the channels. It is also an important question to see whether cells are also present in the new tissue. This can give an indication of the degree of attachment. The histology is done by one independent person. First, the cartilage with the implant is fixed for one week in 4% formalin and then it is dehydrated in a series of ethanol. This series consists of 70%, 96% and 100% ethanol to extract the water from the tissue. Formalin fixed samples were embedded in methyl-methacrylate (MMA). This technique makes it possible to obtain semi-thin sections rich in detail from tissue with hard implants in it (Velde et al., 1977). The MMA is cured and a block of plastic is formed. The samples were cut with a Leica 4SP1600 Saw Microtome system in sections with a thickness of 30 – 40 μm (Vindas Bolaños., et al 2016). The sections were stained with methylene blue/basic fuchsine. All slides were evaluated under a Olympus BX51 microscope.

Results

The surgery

During the operation a hole was made in the bone with a drill and rinsed with NaCl to keep the area clean. Then the implant was inserted using tweezers and a hammer (figure 9). During the first surgery the implant in the left joint was not ideally placed. It was slightly above the surface. In the second surgery the implant was positioned in a better way.

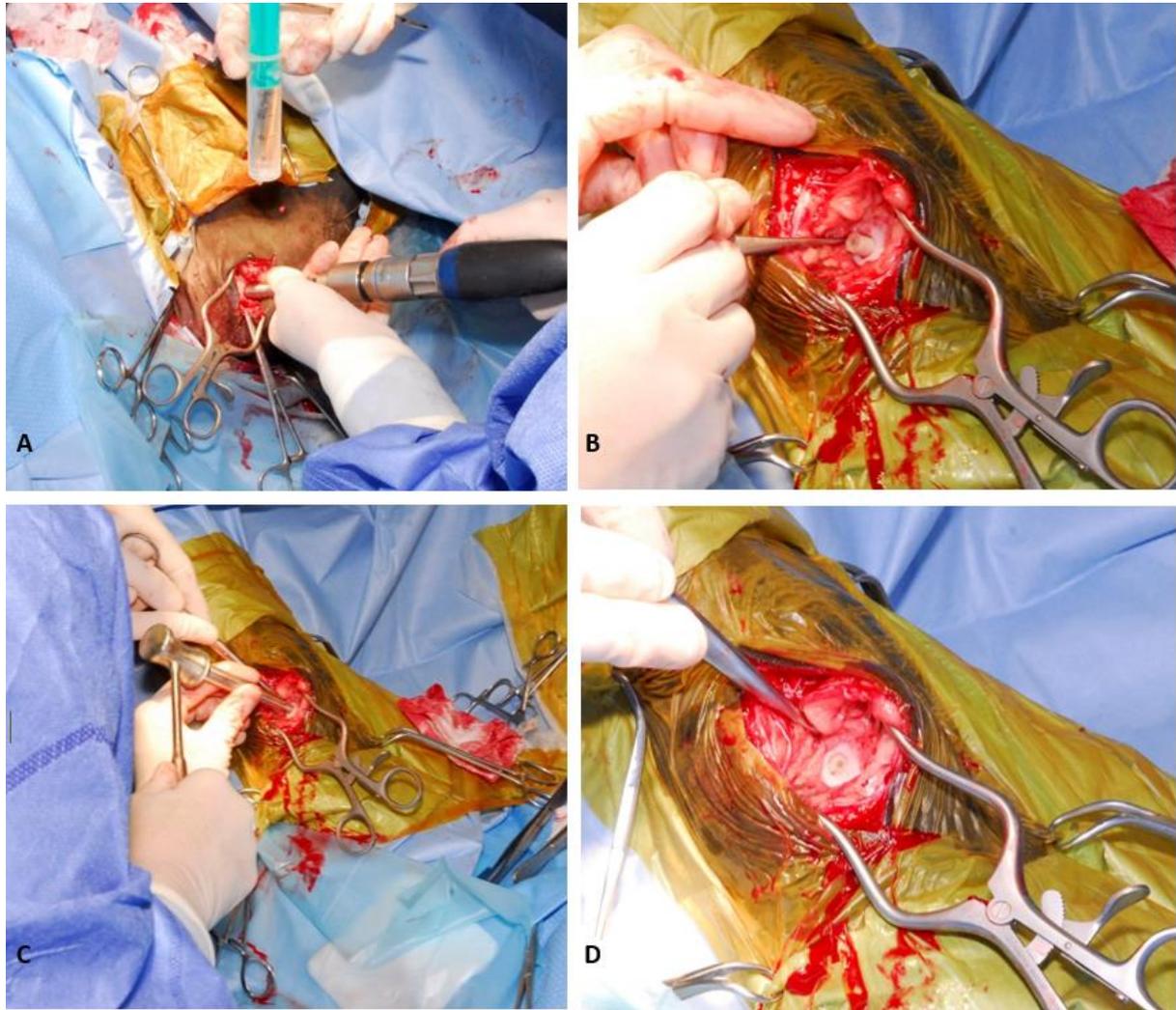


Figure 9: A: A hole was made in the bone with a drill and rinsed with NaCl to keep the area clean. B: The implant is placed in the hole with the help of a tweezers. C: The implant was carefully pushed into the hole using a hammer. D: The end result with the implant in the hole. Both in the medial trochlear ridge and on the medial condyle of the femur is done the same way.

X-rays

The image (figure 10) below shows the locations where the implants have been inserted. These are indicated by the circles. After the first operation, X-rays were taken immediately to be able to adjust the location for the next operation. The location in the joints for the second pony has been slightly adjusted after seeing the X-rays.

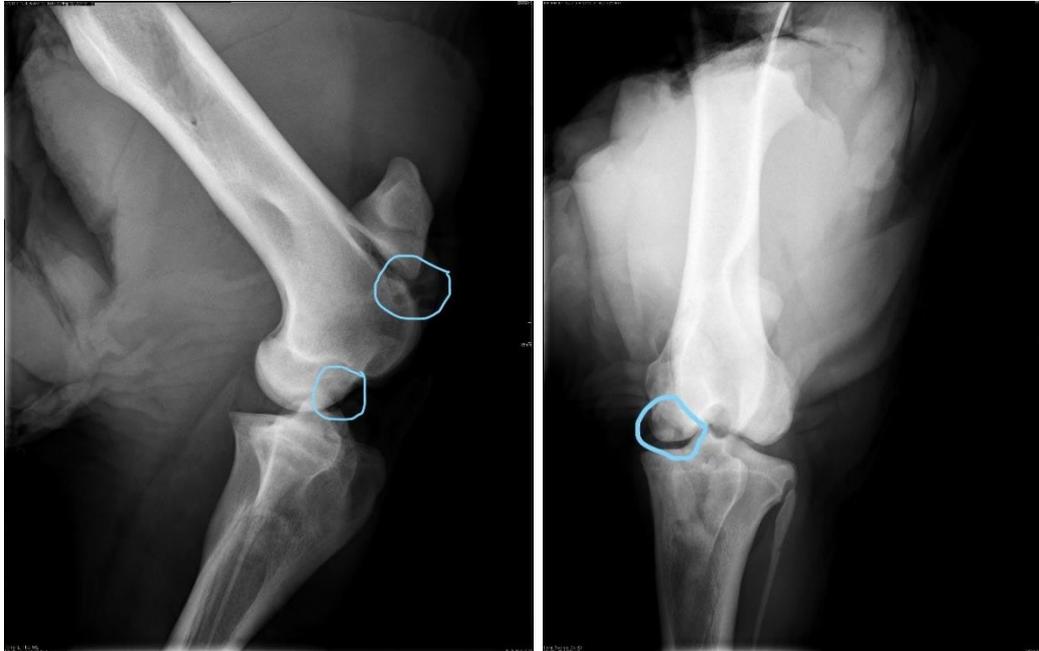


Figure 10: X-rays of pony 27 after the surgery.

Follow up of the pony's

In both abnormalities were seen in the lymph nodes, mucosae, or turgor. ponies, no During the experiment, the maximum lameness score the ponies received was 2/5 during the first days following the surgery. In the following period the score varied between 1/5, 0,5/5 or 0/5. Pony 29 had the first day a respiratory rate of 24/min and a heart rate of 48/min and showed mild signs of depression. Intermittent swelling of the stifle joints could also be seen. The swelling of the joint did not go away during the experiment, although clinical scores improved. Macroscopic assessment post-mortem confirmed the swelling to be a seroma.

Pony 27 had the first day after surgery a respiratory rate of 42/min and a heart rate of 56/min. The pony's lungs were auscultated and this revealed an enhanced lung sounds. For this reason, the pony gets wet hay. On day five the pony relieves her left leg before she gets the Metacam. She was laying in the stable, yawns a lot and eats badly. For this reason the pony got gastrogard. The next day she was much better and less depressed. Actually, the ponies would be examined every day for the first five days after surgery. Since the second pony was slightly depressed and the first pony had swelling of the joint, the ponies were examined for seven days. The seventh day was also the first day without Metacam. This was a great day to see if there was a visible difference between the days with and without Metacam. For a complete overview of the clinical data, see appendix III.

Locomotion before and after the operation

Both ponies showed no major lameness before and after surgery. As discussed above, the ponies were a little stiff for the first few days after surgery. There were also no major differences between the two measurement moments. Due to the corona, no access was possible to the data from Q horse and Equimoves. It was therefore been decided not to include these results in the report.

Macroscopic view and OCT

Pony 29 shows a layer of tissue on the left and the right trochlear ridge (figure 11 and 12). The aspect of the tissue above the implant looks different. The OCT images also show that a layer of tissue is growing above the implant. It is difficult to say how thick the layer is based on the images. As soon as the medial condyle is discussed, no tissue layer is visible on the right and the channels are clearly visible. The OCT images confirm this. On the left medial condyle no tissue is visible on the macroscopic view and on the OCT images only some ingrowth from the edges (figure 12).

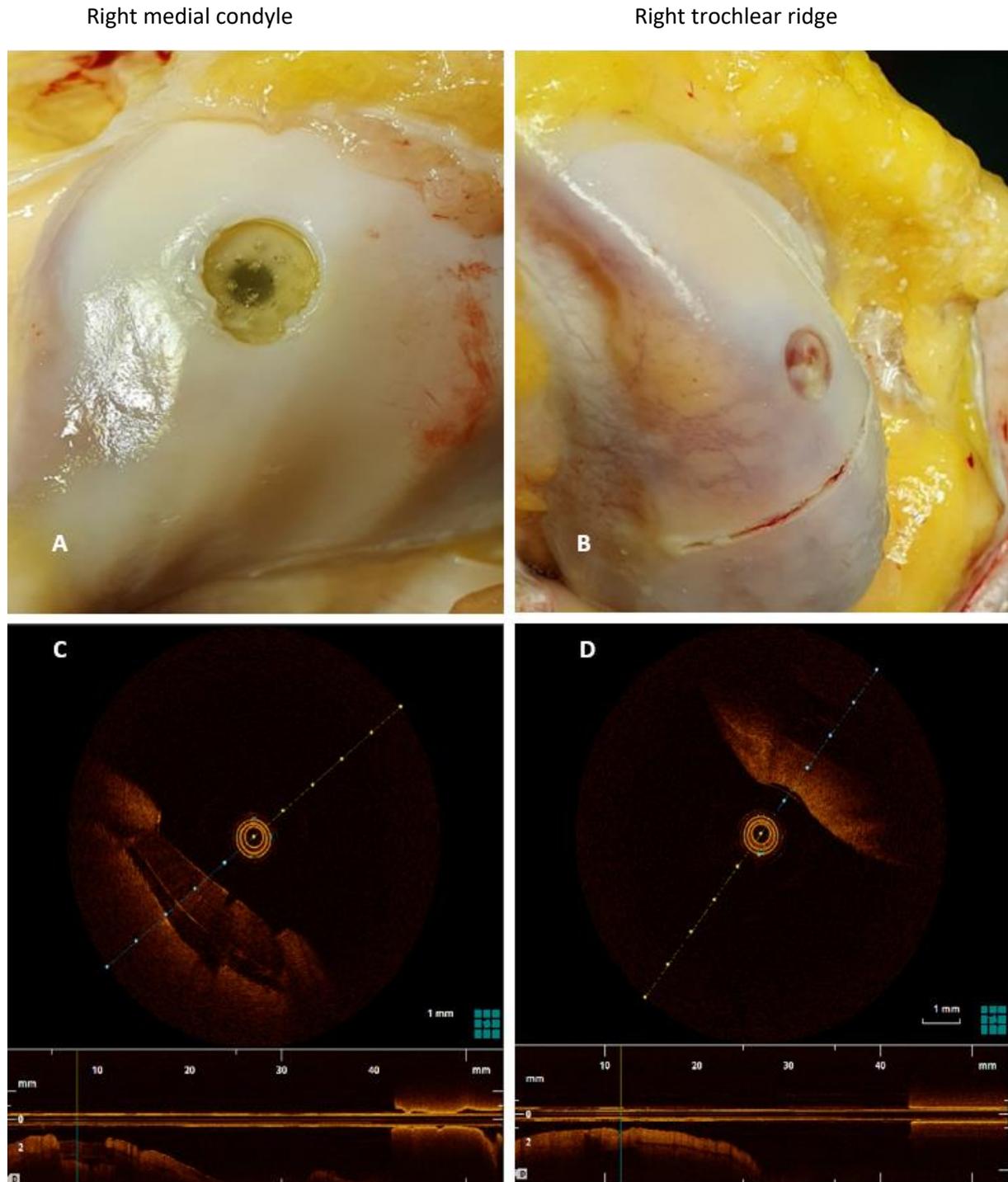


Figure 11: A: On the macroscopic view of the right medial condyle, the implant with the channels are visible and no cartilage has grown over it. C: The OCT image matches the images on the macroscopic view. B and C: The right trochlear ridge does have a cartilage layer over it. What can be seen on the macroscopic view and on the OCT.

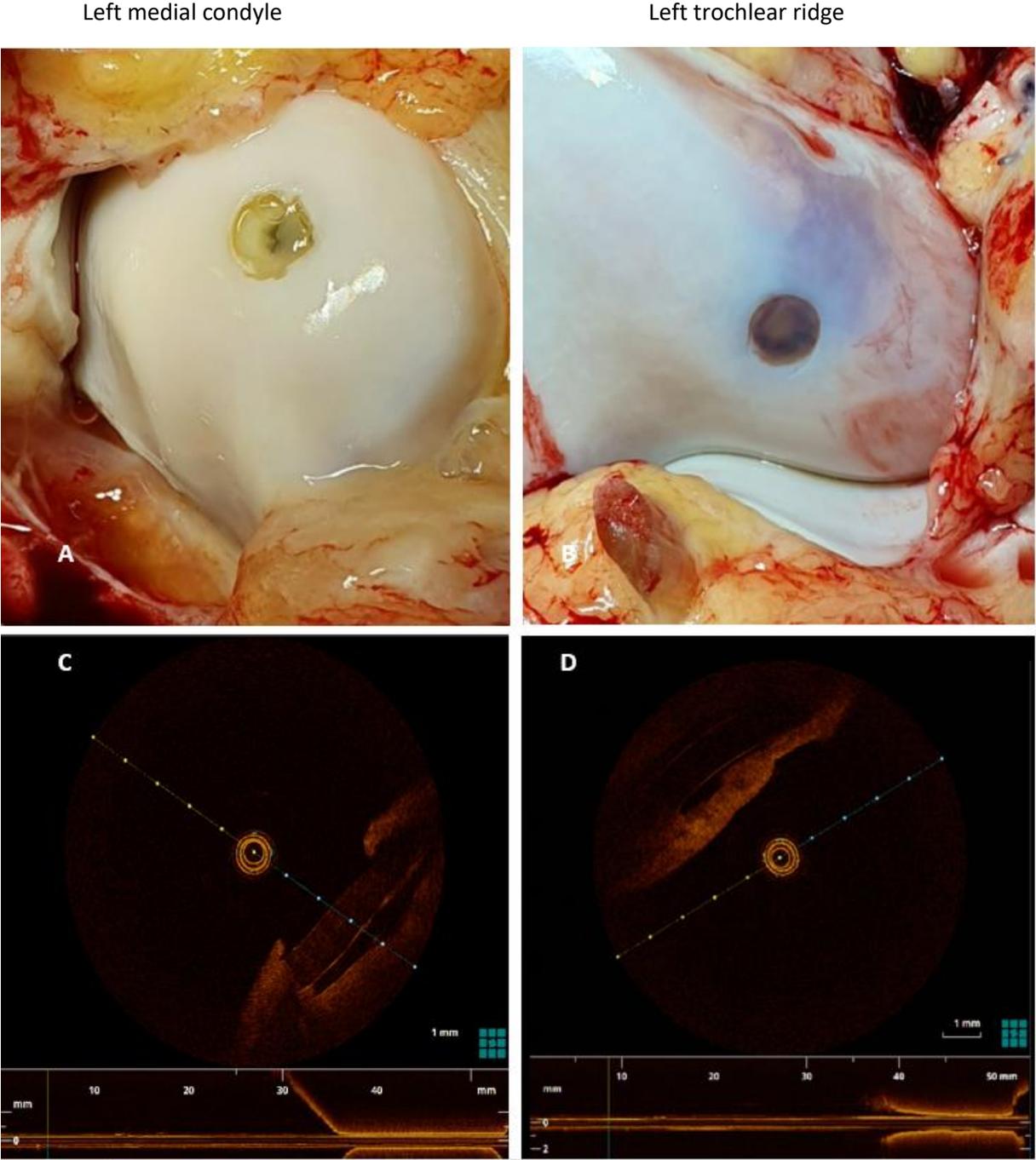


Figure 12: A: No cartilage is visible of the left medial condyle. C: The OCT images correspond to this, with only ingrowth of cartilage from the edges. B: In comparison with the left medial condyle there is a layer of cartilage over the left trochlear ridge. D: This is also clearly visible on the OCT images.

Pony 27: On the right medial trochlear ridge a nice even layer of tissue has grown over the implant (figure 13). This confirms the OCT. Compared to the left trochlear ridge it can be seen that on the macroscopic view it is difficult to see if there is a piece of tissue over it, but the OCT images indicate that there is a layer over the implant. The right and de left medial condyle both do not have a smooth layer of tissue and the edges were very messy. Only tissue is present at the edges on the right and the left medial condyle (figure 13 and 14).

Right medial condyle

Right medial trochlear ridge

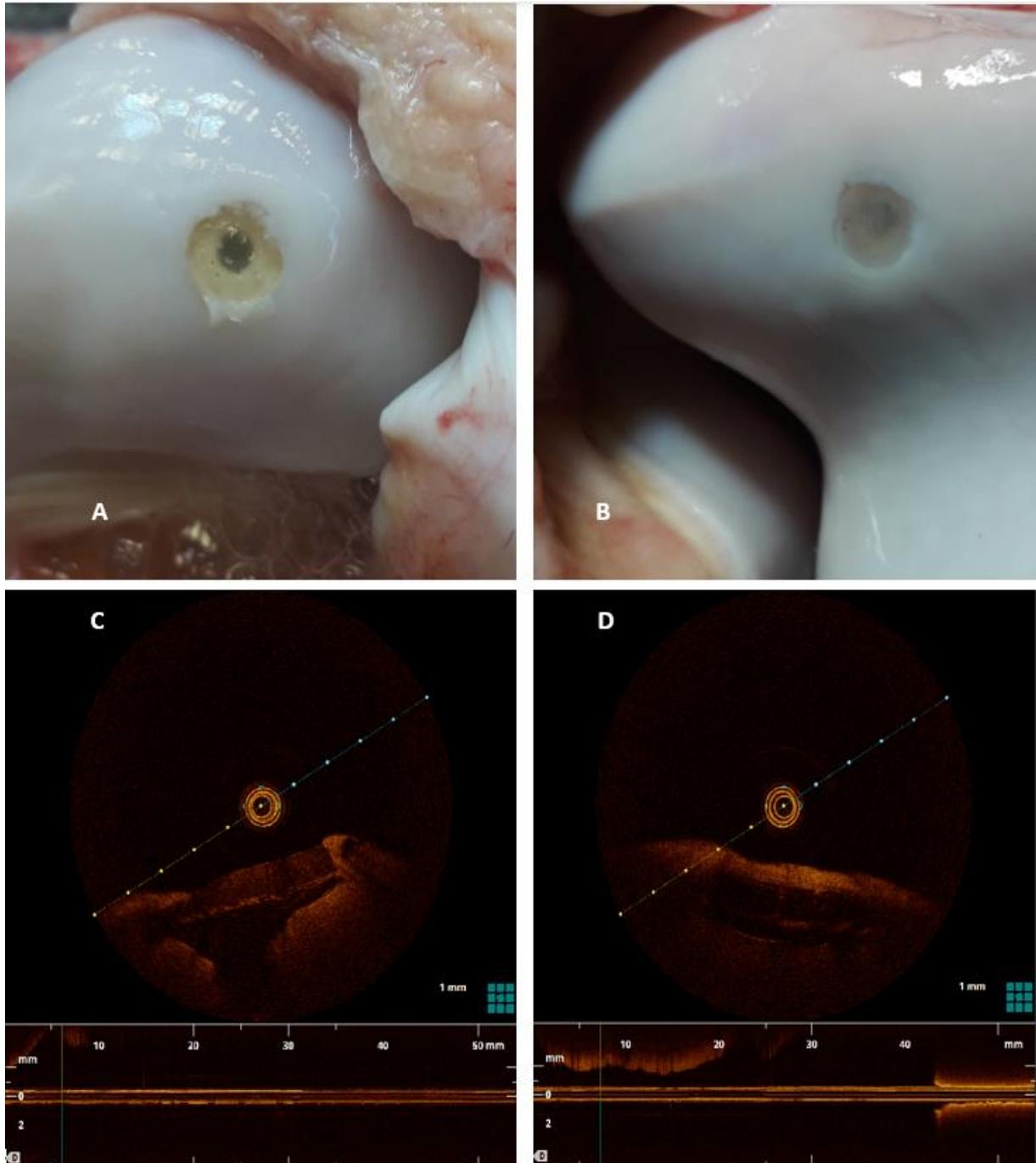
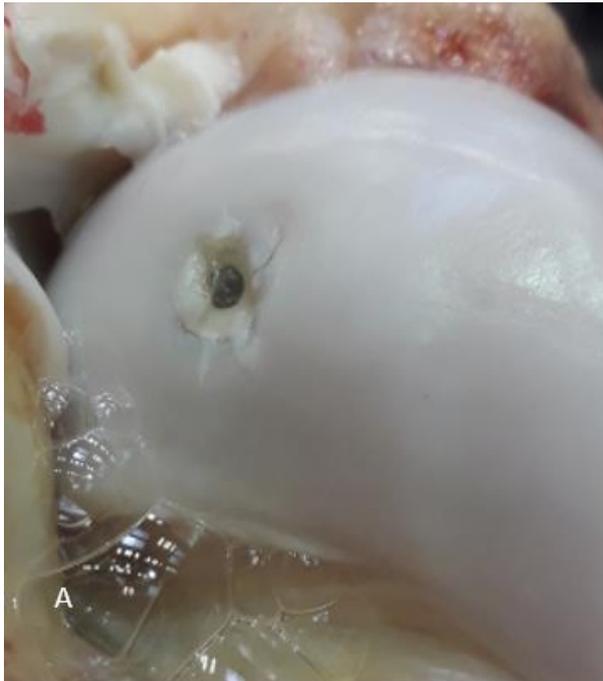


Figure 13: A: No cartilage is visible on the implant on the right medial condyle. The channels can still be seen. C: The OCT image also show that there is no cartilage over it. B: A nice thin layer of cartilage is visible on the right medial condyle. D: This correlates with the images from the OCT.

Left medial condyle



Left trochlear ridge

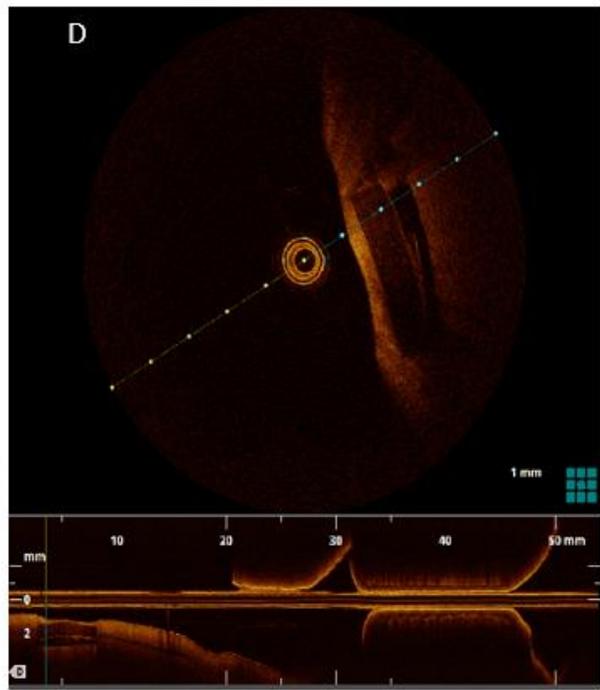
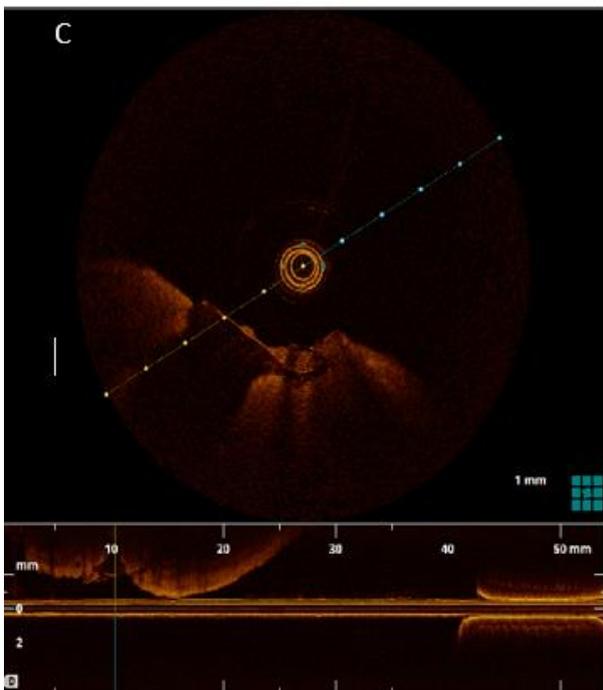


Figure 14: A: At the left medial condyle, the implant is still clearly visible. However, the corners are irregular. **C:** On the OCT image it is indeed visible that almost no cartilage grows over it.

B: The implant is still clearly visible and it is difficult to see in the photo. During the assessment of this implant, a piece of cartilage was certainly present above the implant. Only the adhesion was very poor. The piece of cartilage could easily be moved. **D:** Once the OCT images have been viewed, a layer of cartilage is certainly present.

Cartilage thickness

To answer the first hypothesis, the thickness of the cartilage is important. The thickness was measured in the OCT image in the point where the thickness was the maximum. A scale within the OCT image program was used. It was also examined whether the cartilage grows over the entire implant or whether it is only at the corners. Table 1 concluded that cartilage is present on the right and left medial condyle, but only present in the corners. The thickness is generally thinner than the cartilage of the trochlear ridge. In the trochlear ridge, a nice, even layer of cartilage has grown over the implant, which is on average thicker. There are no major differences between the two ponies. Only the cartilage on the trochlear ridge of pony 29 is thicker. Bases on these images, no statement could be made about the adhesion on the elastomer.

The main average thickness of the medial condyle was 562,50 μm by pony 27 and 531 μm by pony 29. The average of the trochlear ridge was 625 μm by pony 27 and 1062 by pony 29. Once both implants are compared, the following is noticeable: left implants, without channels, had an average thickness of 500 μm by pony 27 and 656 μm by pony 29. The right implants, with channels, had an average of 687,50 μm by pony 27 and 937 μm by pony 29. Thus more cartilage grows on the implants with channels compared to the implants without channels.

Pony 27	Right medial condyle (with channels)	Right trochlear ridge (with channels)	Average thickness implant with channels	Left medial condyle (without channels)	Left trochlear ridge (without channels)	Average thickness implant without channels
Cartilage present over the entire implant or only over small areas of corners	Only the corners	Over the entire implant	-	Only the corners	Over the entire implant	-
Thickness in μm	625 μm	750 μm	687 μm	500 μm	500 μm	500 μm
Pony 29						
Cartilage present over the entire implant or only over small areas of corners	Only the corners	Over the entire implant	-	Only the corners	Over the entire implant	-
Thickness in μm	687 μm	1187 μm	937 μm	375 μm	937 μm	656 μm

Table 1: Thickness and characteristic of growth of all implants.

Histology

Histology is our only valuable parameter to say something about the cartilage adhesion, namely hypothesis 2. Since it is practically very difficult to perform a force/loading test. These histology images show that repair tissue grows in the channels and cells are even present (figure 15). The repair tissue was well attached to the native cartilage and the elastomer but there was a clearly visible transition between the tissue. Only the implant on the medial trochlear ridge with channels had a good adhesion to the native cartilage and to the elastomer.

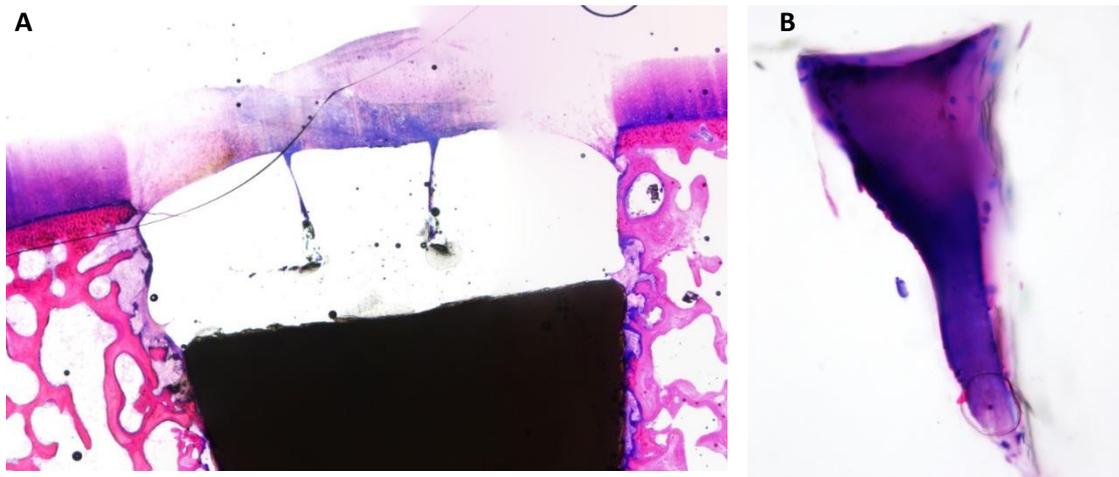


Figure 15 : A: An overview of the histology of the implant with the vertical channels. It was visible the repair tissue has grown over the implant, but also into the channels. B: A detailed picture of the channels. The channels were filled by repair tissue, but cells are also visible.

Discussion

The repair of focal cartilage defects remains a challenge, in humans and in animals. As the injury progresses and the cartilage defect extend into the subchondral bone, the blood supply in the bone starts the healing process which results in fragile fibrous repair tissue. This also increases the change of later cartilage damage in the future. Most attempts of realizing cartilage regeneration results in fragile fibrous repair tissue and not in regenerative tissue. The repair tissue that forms naturally, eventually undergoes fibrillation and degeneration leading to further disruption of joint homeostasis. An articular cartilage injury that is initially small can have a physical and chemical knock on effect on the surrounding normal articular cartilage. The regenerative interventions could delay the development of osteoarthritis in the case of cartilage lesions. The joint cartilage repair procedures provide the best results when the procedure is in the early stages and it is only a focal lesion. When the injury is left untreated, the resulting chronic inflammation can lead to osteoarthritis. Clinically there are different aims in cartilage repair. The first one is to restoration of joint function and homeostasis and the second one is the prevention or delay of the onset of osteoarthritis. The disease affects different levels: articular cartilage, the entire joint, subchondral bone, ligament, capsule, synovial membrane, and peri-articular tissue. This causes a domino effect of the inflammatory process into the secondary tissues which results in a release of inflammatory mediators. It is therefore necessary to stop the domino effect. The purpose of the implant is to ensure that the cartilage function is maintained and that no further damage or inflammation occurs

that ultimately leads to osteoarthritis.

A problem related to treating cartilage defects is that it is disastrous that cartilage damage is often not diagnosed until a later stage. It is often not noticed until the horse is limping and there is already a considerable inflammation. Because there are no nerves in cartilage, the body does not notice the damage to the cartilage. This only happens when the damage has reached the bone. Therefore, it would be ideal to diagnose the cartilage defect earlier at a time when the damage is still limited. This is more challenging in animals than in humans. Often when lameness is seen in horses, there is already osteoarthritis. People can indicate much sooner when their joint feels stiff or painful and in horses it is often not noticed until they are already lame. A possible solution would be to immediately switch to X-rays in case of a slight lameness. Or even take preventive X-rays. As soon as minor damage is visible, immediate action can be taken.

An additional disadvantage of this is that not every horse has to suffer from a minor damage. The degree of lameness is not always linked to the degree size of the defect. Some horses are already lame on a minor damage, where other horses do not seem to be in pain with a large damage with a possible inflammation. It would be ideal to diagnose cartilage defects earlier when the damage is still limited and there is no osteoarthritis yet.

The horse model was chosen for this pilot study because of equality with humans in terms of joint size, cartilage thickness and healing properties (Malda et al., 2012). Horses also suffer from comparative joint disease (Van Weeren et al., 2016). The use of ponies overrides that of horses because the joint size is even closer to that of humans and they are easier to house and they seem to recover faster from surgery. But there was one big difference between humans and horses: horses must be able to stand within half an hour after birth, while they are born with the same semi-developed cartilage as humans. How does their cartilage get the desired strength so quickly? Unfortunately, little is known about this in the literature.

Horses are at high risk for problems related to recovery and wound healing (McIlwraith et al., 2011; Husby et al., 2016). During the check-up of the ponies, it was also noticed that the ponies were not always easy to handle, which is not beneficial to recovery. The behaviour of the ponies mainly due to the short time of acclimatizing. There is certainly room for improvement for a possible subsequent study. To really practice on time with the ponies so that they can start doing certain things before the investigation begins.

The main problem in the first study analysing the effect of a biphasic osteochondral implant, was the non-attachment of cartilage to the implant (Korthagen et al., 2019).

In this pilot study the implant was placed just below the cartilage surface in relatively small osteochondral defects (6mm). In both stifle joints of two Shetland ponies were the implants placed in the medial femoral condyle and in the trochlear ridge during an operation. The implants were well tolerated and little to no lameness was visible during the pilot study. Repair tissue was observed, more on the medial trochlear ridge than at the medial condyle. Although the two ponies differed in the type of tissue macroscopically on the medial trochlear ridge.

On the medial condyle the percentage of cartilage above the implant was lower, the cartilage was thinner and only the edges were covered with tissue. The thickness is measured at the OCT image with the maximum thickness. With the OCT it was difficult to identify the implant in some locations. A possible explanation for this is that the channels are filled with cartilage, making it more difficult to distinguish the implant from the surrounding cartilage.

It seems as growth takes place from the edges, but the duration of the pilot study is too short. At the trochlear ridge you see a lot thicker cartilage and the entire surface is covered with cartilage. The main average thickness of the medial condyle is 562,50 µm by pony 27 and 531 µm by pony 29. The

average of the trochlear ridge is 625 μm by pony 27 and 1062 μm by pony 29. The reason for this significance difference lies in the difference between force distribution and load at both locations. In healthy horses, you can also see that the cartilage on the medial condyle of the femur is thinner than the cartilage on the medial trochlear ridge. The reason for this is the difference in loading (Malda et al., 2012; McIlwraith et al., 2011; Frisbie et al., 2006). This also gives a good explanation why this pilot study shows much better growth on the medial trochlear ridge. For the final application, this makes it easier in some places to repair focal cartilage defects with an implant than in other locations. The measurements of the average thickness of the cartilage is measured on the basis of the OCT image. This has been measured by means of a given scale in the program. It is not automatically done by the program, but calculated individually. As a result, it must be taken into account that the numbers may be less reliable and that there may be small deviations. The program is able to do this itself, but this was not successful for technical reasons.

Our first hypothesis was that the cartilage attach better to the implant with the channels. The only test that could be done was histology. Which is very nice to see that there are cells in the channels. So it gives an indication that there is some adhesion. It should be noted that tissue is visible on the elastomer and cells are present in the channels. In this pilot study no staining was done specifically for cartilage, so it cannot be said with 100% certainty what kind of tissue it is. For a follow-up study, it is therefore advisable to make paraffin sections, because more stains can be done on them. To have really hard evidence, it would be ideal to be able to perform a shear force test. This was tried during the pilot study. However, this was not successful because the implant was too small for the machine. There should be a solution to this for the follow-up study to see how the cartilage attaches to the implant. And with what forces you can pull those layers apart. To make a good decision on whether there is better adhesion, more tests should be done. Better adhesion of the cartilage to the implant is expected, because the channels form an anchor. Unfortunately, less to no literature can yet be found on implants with channels.

Another challenge during the research is the size of the channels. The optimal diameter of the vertically aligned punched channels was found to be 319 μm . Not only provides this diameter optimal circumstances for the cartilage to grow into, it also optimized anchoring potential. In the previous study it was shown that the chondrocytes of the horses have difficulty attaching to the elastomer. Several solutions have subsequently been devised: adding peptides to the material and making channels. The first solution is quite a challenge, so this pilot study looked at the channels. Beside compressive forces, articular cartilage experiences shear forces as well. The tissue layer on top of the implant should therefore be securely anchored to the elastomer resisting detachment by shear forces (Aken., 2019). Tissue ingrowth into the channels in the elastomer could provide this resistance. Subsequently, the ideal size of the channels was examined.

Seong et al. conducted a study looking at the ideal size of the channels. 270 μm diameter of the channels was ideal (Seong et al., 2017.). According to Lien et al., the optimum diameter of the channels is between 250 and 500 μm . The optimal diameter is best for the proliferation and production of extracellular matrix of chondrocytes (Lien et al., 2009). Another study from Jia et al., provided slightly more specific information, with cell proliferation in implants with bigger channels (450 μm) is higher than in implants with smaller (200 μm) channels (Jia et al., 2018). In another research they looked at three different diameters of channels, 188 μm , 319 μm and 741 μm . Ultimately 319 μm and 741 μm appear to have a significantly greater percentage of cell coverage than the 188 μm . Highest mean scores were found at 319 μm (Aken., 2019). This is consistent with the literature described above. Another option to get a better ingrowth and adhesion is to use the 3D printing. 3D printing provides the opportunity of creating a custom made porous structure that can

provide optimal pore properties for cartilage in growth and regeneration. The main benefit of this technique compared to making the channels is the possibility of creating interconnected pores which substantially improves nutrients flow and enhanced cellular adhesion and migration (Wang et al., 2015). Firstly, cells should be able to quickly fill the complete diameter and length of the hole to ensure proper anchoring. Secondly, more channels per surface area provides increased anchoring points leading to an improved distribution of anchor points. Unfortunately, the above diameters for the channels cannot be compared with this pilot study, because the diameter of the channels is unknown. For the follow-up study, this information can be included to check whether the diameter of the channels is ideal.

The second hypothesis was that the cartilage layer on the implant with channels is thicker. As soon as the implants were compared with and without channels then the left implants (without channels) have an average thickness of 500 μm by pony 27 and 656 μm by pony 29. The right implants have an average of 687,50 by pony 27 and 937 μm by pony 29. From this it can be concluded that more cartilage grows on the implants with channels compared to the implants without channels. This hypothesis is therefore confirmed on the basis of these averages. One reason for this better cartilage growth may lie in obtaining nutrients. By placing channels, the cartilage can obtain nutrients and grow better. Nutrients can diffuse from the synovial fluid into the cartilage up to 3.0 mm in depths (Williams et al., 1968). The total depth of our implant is 7mm. The question is whether this is a problem.

In a long term pilot study of 8 weeks with one healthy horse and a long term study with several horses, long-term outcomes were examined (Vindas et al., 2017). The results of this study show that incomplete filling of cartilage defects in a short term study has adverse effects in a long-term study (12-52 weeks). In the long term, this has caused severe degeneration of the surrounding cartilage and bone (Vindas et al., 2017). In our pilot study, the defects that are filled in the medial condyle are also not completely filled with tissue. This is one of the reasons why this pilot study also requires a long-term study with multiple ponies.

As indicated in the discussion above, there are a number of things that were not ideal in the study. But this does not have to be a problem as it is a pilot study. The first results with regard to the implant with the channels are hopeful. The next step is to do a long-term follow-up study with multiple ponies and an exercise regimen. If it is finally possible to fill in focal cartilage lesions with an implant, which leads to clinical improvement and the stagnation of the development of osteoarthritis, this would be a huge step. This would be an enormous improvement in functional treatment of osteochondral defects and degenerative joint disease. It can therefore have a huge social impact as so many people and animals worldwide suffer from these defects and diseases.

Conclusion

The aim of this pilot study was to check if the new implant with vertical channels had a better adhesion to the cartilage compared with the implant without the channels. With the following hypothesis: (1) the repair tissue attaches better to the non-resorbable implant with the channels in comparison with the one without channels; (2) The cartilage layer on the implant with channels is thicker.

To confirm hypothesis 1 only histology can be used, which does indicate nice ingrowth of cells. For this reason, the hypothesis is true. It is not yet hard evidence, because more tests should be done. The second hypothesis is also true. The left implants, without channels, had an average thickness of 578 μm and the right implants, with the channels, had an average of 812,25 μm .

On the basis of this pilot study, improvements have certainly been found compared to last year's study (Korthagen et al., 2019). With better adhesion and a thicker cartilage layer on the new implant with the vertical channels. Further long-term research is still needed to evaluate its potential for clinical use.

Acknowledgements

In addition, I would like to thank my supervisor, M. Fugazzola, for the help in planning, design of research and revising the manuscript. Also, I want to thank the participating veterinarians, J. De Grauw for help with the check of the pony's, N. te Moller for the OCT images, I. Smit for the locomotion tests and all of the other veterinarians.

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Appendix I

Table 1: An indication of the procedure and the associated discomfort.

Days	Description of procedure	Duration of procedure	Description of discomfort	Duration discomfort	Estimated level of discomfort
1	Surgery	1 – 3 hours	Discomfort due to surgical incision, due to induction and recovery	3-6 hours	Moderate
2 – 14	Monitoring of pain and providing pain medication	10 min	Very little discomfort	10 min	Mild
14	Euthanasia	15 min	Some stress from anaesthesia	10 min	mild

Appendix II

Table 2: The composite pain scale (CPS).

Interactive behavioural	Criteria	Score
	Responds attentively to noise/people Exaggerated response to sound Extreme to aggressive reaction to sound Stupor, hardly any reaction	0 1 2 3
Sight	Criteria	Score
	Clear, low head and ear posture, no reluctance to move Clear and alert, occasional head movements, no reluctance to move Restless, pricked ears, abnormal facial expressions, dilated pupils Excitement, constant body movements, abnormal facial expressions	0 1 2 3
Appetite	Criteria	Score
	Eat roughage quickly or have to fast Hesitates to eat roughage Eats little or shows little interest or chews and does not swallow Shows no interest	0 1 2 3
Body posture	Criteria	Score
	Stands or walks quietly Occasionally shifts to see weight or slight muscle tremors 1 or more legs do not bear weight or an abnormal weight distribution Analgesic (attempted to urinate) or prostration or muscle tremors	0 1 2 3
Kicks to belly	Criteria	Score
	Stands calmly, does not strike to stomach Hits occasionally (1-2x / 5 minutes) Hits more often (3-4 x/ 5 minutes) Hits extremely often (> 5x / 5 minutes) or tries to lie down and roll	0 1 2 3

Scratching/scraping	Criteria	Score
	Stands quietly, no scraping, no leg at rest	0
	Occasionally scrape, point or rest one leg (1-2 x /5 minutes)	1
	Often scrape, point or rest one leg (3-4 x /5 minutes)	2
	Scraping, pointing or resting leg extremely often (> 5x /5 minutes)	3
Head movements	Criteria	Score
	No signs of discomfort, head carried forward	0
	Interrupted head movements laterally and/or vertically or occasionally looking at flanks or flehms(1 -2 x /5 minutes)	1
	Interrupted, fast head movements laterally and/or vertically or often looking at flanks or flehms(3 -4 x/ 5 minutes)	2
	Constant head movements or looking at flanks of flehmen extremely often (>5 x /5 minutes)	3
To sweat	Criteria	Score
	Barely	0
	Feels clammy	1
	Feels wet or drops of sweat are visible	2
	Extreme sweating or drops of water run off the horse	3
Breathing	Criteria	Score
	8 -13 breaths/ minute	0
	14 – 16 breaths /minute	1
	17 – 18 breaths /minute	2
	More than 18 breaths /minute	3
Heartrate	Criteria	Score
	24 – 44 strokes/ minute	0
	45 – 52 strokes /minute	1
	53 – 60 stroke /minute	2
	More than 60 strokes /minute	3
Bowel sounds	Criteria	Score
	As expected with a healthy horse	0
	Reduced sounds	1
	No sounds	2
	Too much sounds	3
Temperature	Criteria	Score
	36,9 – 38,5	0
	36,4 – 36,9 or 38,5 – 39,0	1
	35,9 – 36,4 or 39,0 – 39,5	2
	35,4 – 35,9 or 39,5 – 40,0	3
Reaction on painful area	Criteria	Score
	No reaction	0
	Mild reaction	1
	Resists	2
	Aggressive reaction	3

Appendix III

Table 3: Overview of the check ups at the ponies.

Day	Pony 1 (Jessie)	Pony 2 (Nadia)
Day 1	Breathing: 24/minute Heartrate: 48/minute Temperature: 37,7 degrees Turgor, lymph nodes and mucosae are normal. Little bit swelling on the right stifle joint. 1/5 lame. Looks depressed.	Surgery
Day 2	Breathing: 36/minute Heartrate: 48/minute Temperature: 38 degrees Turgor, lymph nodes and mucosae are normal. Little swelling of the right stifle joint and she is more dragging with the leg. Less depressed than yesterday. Is alert and eat well. 0/5 lame.	Breathing: 42/minute Heartrate: 56/minute Temperature: It was not possible to temperature her. In the morning they measure 38 degrees. Turgor, lymph nodes and mucosae are normal. 1/5 lame, no swelling or warmth. Breathing is superficial and she is squeezing. Option is to make the hay a little wet
Day 3	Breathing: 11/minute Heartrate: 56/minute Temperature: 37,8 degrees Turgor, lymph nodes and mucosae are normal. Little swelling on the right stifle joint and she is more dragging with this leg. 1/5 lame.	Breathing: 40/minute Heartrate: 46/minute Temperature: 38 degrees Turgor, lymph nodes and mucosae are normal. No swelling of the joint. 1/5 lame.
Day 4	Breathing: 26/minute Heartrate: 36/minute Temperature: 37,3 degrees Turgor, lymph nodes and mucosae are normal. No longer dragging with her right leg. 0,5/5 lame. At the right joint a moderate swelling and on the left joint a mild swelling. It is more diffuse.	Breathing: 44/minute Heartrate: 44/minute Temperature: 38,1 degrees Turgor, lymph nodes and mucosae are normal. No swelling of the joint. 0,5/5 lame.
Day 5	Breathing: 18/minute Heartrate: 40/minute Temperature: 37,8 degrees Moderate overfilled joint. Both sides. Sensitive and painful to touch. Dragging with her right leg. 1/5 lame.	Breathing: 72/minute Heartrate: 46/minute Temperature: 38,2 degrees Seems a bit depressed. Twice laying in the stable. she yawns a lot and eats badly. Before

		she gets the Metacam she relieves her left hind leg. We gave her gastrogard. 2/5 lame.
Day 6	Breathing: 28/minute Heartrate: 48/minute Temperature: 37,2 degrees Left joint somewhat sensitive and warm. 0,5/5 lame. Same swelling as yesterday.	Breathing: 36/minute Heartrate: 40/minute Temperature: 37,6 degrees Pulls with her right hind leg. More alert than yesterday. No swelling. 1/5 lame.
Day 7 → first day without Metacam	Breathing: 38/minute Heartrate: 48/minute Temperature: 37,9 degrees Same swelling as yesterday. 0,5/5 lame	Breathing: 50/minute Heartrate: 56/minute Temperature: 37,5 degrees No swelling. 0/5 lameness score.
Day 8		
Day 9	Same swelling, eats good, lameness score: 0/5	No swelling, eats good, lameness score: 0/5
Day 10		
Day 11	0,5/5 lame, joint swelling marked, both locations, bilateral. Right less than left. Not overtly warm, painful. Incisions dry. Pony bright and alert.	0/5 lame. Bright and alert. No/minimal swelling of the joint.
Day 12	Breathing: 22/minute Heartrate: 48/minute Temperature: 37,6 degrees Lameness score: 1/5 → hind left leg	Breathing: 34/minute Heartrate: 60/minute Temperature: 38 degrees Lameness score: 1/5 → hind left leg. Minimal stifle joint swelling.
Day 13		
Day 14		
Day 15		
Day 16	Breathing: 28/minute Heartrate: 52/minute Temperature: 37,1 degrees Swelling is the same as the last days. Lameness score: 0,5/5	Breathing: 30/minute Heartrate: 40/minute Temperature: 38 degrees A little bit of swelling on the left hind leg. Walks fine. Lameness score: 0,5/5
Day 17		
Day 18		
Day 19		
Day 20	Breathing: 16/minute Heartrate: 48/minute Temperature: 38 degrees Bilateral swelling of the joint. No lameness: 0/5	Breathing: 50/minute Heartrate: 36/minute Temperature: 37,6 degrees

		Auscultation of the lungs: enhanced breath sounds. No ronchi. Lameness score: 0/5
Day 21		
Day 22		
Day 23		
Day 24		
Day 25		
Day 26		
Day 27	Breathing: 32/minute Heartrate: 48/minute Temperature: 37,4 degrees Not lame, Swelling is the same, not painful or warm.	Breathing: 50/minute Heartrate: 46/minute Temperature: 37,4 degrees Not lame
Day 28		
Day 29		
Day 30		
Day 31		
Day 32		
Day 33 → The day of the locomotion test with Q horse and Equimoves	Breathing: 32/minute Heartrate: 40/minute Temperature: 37,2 degrees	Breathing: 26/minute Heartrate: 48/minute Temperature: 37,8 degrees