Dissertation

Preclinical trial to examine the efficacy and safety of the treatment with the autologous chondrocyte transplantation ovine test sample co.don chondrosphere® (ACT3D-S) compared to an untreated control group in subjects with cartilage defects of the knee in medial femoral condyle

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Preclinical trial to examine the efficacy and safety of the treatment with the autologous chondrocyte transplantation ovine test sample co.don chondrosphere® (ACT3D-S) compared to an untreated control group in subjects with cartilage defects of the knee in medial femoral condyle

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Abstract

The purpose of this GLP study was to show the efficacy and safety of the investigational product co.don chondrosphere® (ACT3D-S). ACT3D-S is a product for autologous chondrocyte transplantation that we used in an animal model, the merino land sheep. We compared the treatment of ACT3D-S (Group A: Investigational product) with an untreated control (Group B: Control Intervention) in a bilateral model, what means that by randomization one hind limb was chosen to be treated with ACT3D-S while the remaining hind limb was left without treatment. Therefore, the merino land sheep was used as an animal model and during two operations. In the beginning, a predefined cartilage defect was created in both hind limbs, and cartilage tissue was removed to cultivate cartilage spheroids which were implanted into a randomized defect 49 days later. Nine months after the implantation the animals were sacrificed, and the hind limbs were extracted to verify the efficacy using different visual, histological and MRI scores. Moreover, safety scores were collected during all study processes to verify the safety of our investigational product.

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III List of abbreviations

2D = two dimensional

3D = three dimensional

AA = Animal autopsy

ACI-C = Type I/III collagen covered autologous chondrocyte implantation

ACI-P = Periosteum covered autologous chondrocyte implantation

ACL = Anterior cruciate ligament

ACT/ACI = Autologous chondrocyte transplantation/implantation

ACT3D-S = autologous chondrocyte transplantation – 3D - sheep

ADR = Adverse Drug Reaction

AE = Adverse Event

ALT = Alanine aminotransferase

ANOVA = analysis of variance

AST = Aspartate aminotransferase

AT = Active treatment

ATMP = Advanced therapy medical products

BAI = Backscattered Amplitude Integral

Bands = Banded neutrophils

Baso = Basophilic granulocyte

Bili (dir) = Bilirubin (direct)

Bili (indir) = Bilirubin (indirect)

Bili (total) = Bilirubin (total)

BMI = Body mass index

BW = Bodyweight

CAE = Caprine Arthritis Encephalitis

CE = Council of Europe

CHOL = Cholesterol

chondrocyteMNF = Combined chondrocytes and mononuclear fraction cells

CI = Confidence interval

CRO = Contract research organisation

ECM = Extracellular matrix

Eos = Eosinophil granulocytes

ETN = (unique) ear tag number

EU = European Union

Fh-IZI = Fraunhofer-Institut für Zelltherapie und Immunologie

GAG(s) = Glycosaminoglycan(s)

GGT = Gamma-glutamyl transpeptidase

GLP = Good laboratory practice from the German "gute Laborpraxis"

GMP = Good manufacturing practices

hBM = human bone marrow

HCT = Hematocrit

HE = Hematoxylin and eosin

HGB = Hemoglobin

HIK = Histologie für Implantate und Knochen

HOSPITAL = Animal Hospital, Faculty of Veterinary Medicine, University of Leipzig

i.m. = intramuscular

i.v. = intravenous

ICRS = International Cartilage Repair Society

ICRS-MCAS = ICRS-Macroscopic cartilage Assessment Score

IKDC = International Knee Documentation Committee

IQR = Inter-quartile Range

ITT = Intended to treat / Intention-to-Treat

KOOS = Knee injury and osteoarthritis outcome score

LFC = Lateral femoral condyle

LTP = Lateral tibia plateau

LVG = Lehr- und Versuchsgut Oberholz

Lymph = Lymphocytes

MACT/MACI = matrix-associated/coupled autologous chondrocyte transplantation/implantation

Max = Maximal value

MCAS = Macroscopic knee (capsule) assessment score

MCH = Mean corpuscular hemoglobin

MCHC = Mean corpuscular hemoglobin concentration

MCV = Mean corpuscular volume

mean = Mean value

Median = Number separating the higher half of a data sample from the lower half

MEZ = Medizinisch Experimentelles Zentrum

MFC = Medial femoral condyle

Min = Minimal value

MOCART = Magnetic Resonance Observation of Cartilage Repair Tissue

Mono = Monocytes

MRI = Magnetic Resonance Imaging

MSC = Mesenchymal stem cell

MTP = Medial tibia plateau

NaCl = Sodium chloride (Natriumchlorid)

NIH40 = Name of the ultrasound transducer

OA = Osteoarthritis

OCD = Osteochondritis dissecans

p-value = probability

PBS = phosphate buffered saline

PEI = Paul-Ehrlich-Institut

PLT = Platelets, thrombocytes

PP = Per Protocol

pre-MSCs = pre-differentiated Mesenchymal stem cells

RBC = Red blood cell

SAE = Serious Adverse Events

SAF = Safety

SAP = Statistical Analysis Plan

SCID = Severe combined immunodeficiency

SD = Standard deviation

SE = Standard error

Segs = Segmented neutrophils

SmPC = Summary of Product Characteristics

SOP = Standard operating procedure

TG = Triglyceride

TGF-beta 3 = Transforming growth factor beta 3

TROCH = Trochlea

UBM = ultrasound microscope

un-MSC = undifferentiated mesenchymal stem cells

US = ultrasound (biomicroscopy)

VAS = Visual analogue scale

VETMED = Institute of Pathology, Faculty of Veterinary Medicine, University of Leipzig

WBC = White blood cell

1 Introduction

In the year 2010, the number of cases of patients with arthritis that had to be hospitalized was 418.350 (38.5% female, 62.5% male) in Germany (www.gbe-bund.de) whereby almost 80% of hospitalized patients with the diagnosis arthritis were undergoing an operation. [75]. For the arthritis of the knee, this resulted in 212.5 artificial knee joint operations per 100.000 inhabitants in the year 2009 in Germany, what made Germany and the USA to countries with the most significant amount of these operations. This led to direct costs of 7.62 billion Euros in the year 2009. These costs make this whole complex of arthritis, not just necessary to be investigated for the well-being of the older generation. Unfortunately, this leads to a financial burden on healthcare systems (www.gbe-bund.de). One way to reduce these costs is the further use and investigation of autologous chondrocyte transplantation (ACT, sometimes autologous chondrocyte implantation, ACI) which belongs to the advanced therapy medical products (ATMP) other cell-based procedures to regenerate cartilage tissue. These ATMP are defined as products that are "restoring, correcting or modifying physiological functions by exerting principally a pharmacological, immunological or metabolic action" (Regulation (EC) No 1394/2007). Chondrosphere® is such an ATMP consisting of autologous cartilage cells produced and cultured by the company co.don® in Teltow. However, to obtain an EU-wide approval further investigations especially about safety (SAF) and efficacy have to be done, to exclude side effects and risks of the therapy method.

In this "good laboratory praxis" (GLP) study, the efficacy and SAF of the analogous ovine spheroids were assessed in the treatment of chronic focal cartilage defects of knee joints in the femoral condyle. The three-dimensional ovine test sample ACT3D-S (Group A: Investigational Product/ Intervention) was compared to an untreated control group (Group B: Control Intervention) in a large animal model, the Merino land sheep.

1.1 Cartilage

The human cartilage is divided into three different subgroups, the elastic, articular and fibrocartilage, and is a supportive and connective tissue, whereby also muscles, nerves and epithelia tissue belong to this category. It develops as part of the mesenchyme, also called embryonic connective tissue.

The structure of elastic and hyaline cartilage is similar but differs in their properties. The main difference is, that elastic cartilage has a lot of yellow fibers [81] that obtain a very flexible structure that also can be bent several times without losing its form. The best example that demonstrates all characteristics of elastic cartilage is the outer ear whereby it also can be found in the eustachian tube and the epiglottis. [84]

The fibrocartilage is found between pubic symphysis, the *anulus fibrosus* of intervertebral discs, menisci and the temporomandibular joint. It is a tissue that has its characteristics between hyaline cartilage and dense connective tissue whereby it shows a lower mechanical resistance than hyaline cartilage.

The primary task of articular cartilage is to absorb any impact that occurs during walking, sports or working, in other words almost every movement [12, 81]. In cooperation with ligaments, muscles, synovial fluid and sometimes articular discs or menisci it reduces, although it is just 3-5mm thick, the pressure that affects the

bones and allows a frictionless movement [74]. Hyaline cartilage can be found in joints, the respiratory tract such as the nasal septum or the trachea and the costal cartilage. This type of cartilage has no supporting vessels as well as no own nervous innervation so that healing is hardly limited [21]. This is why the regeneration of hyaline cartilage is one of the leading problems in the treatment of diseases or injuries such as Osteoarthritis (OA) or anterior cruciate ligament (ACL) injuries [13]. One of its most important characteristics is the ability to be formable during pressure but to regain its structure as soon as the pressure vanishes.

For the hyaline cartilage, the general structure of cartilage will be explained. There are three essential types of cells: the chondrocyte, the chondroblast, and the chondroclast. The chondroblast is responsible for the production of all components of the intercellular matrix and they are part of the mesenchymal stem cell (MSC) system. Once the chondroblast stops the synthesis, it differentiates to the chondrocyte. The chondrocyte can be seen in histological stainings. They manifest themselves in little cell-islands. These cell-islands arise because the chondrocyte is still able to divide but inside the cartilage matrix, it is not possible for the cells to move anymore and can be seen in the right picture of **Figure 9**.

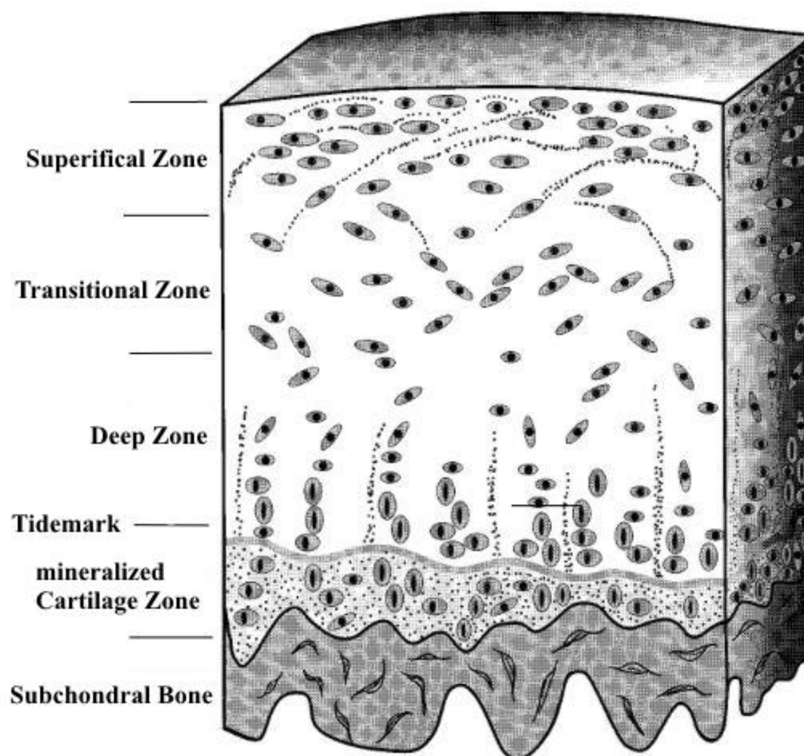
1.1.1 Anatomy

In general, cartilage is composed out of one single cell type, the chondrocyte. Chondrocytes are laying inside the extracellular matrix (ECM) which they are producing, and are just 1 to 10 % of the total cartilage volume [103]. They are alimented over diffusion from vessels that reach the perichondrium and because of this their metabolism is hypoxic. They produce and break down the ECM as well as proteins like type II collagen and aggrecan, also known as cartilage-specific proteoglycan [23]. The ECM can retain and keep a significant amount of water, whereby this is provided through the aggrecan structure, which is composed of many sulfated glycosaminoglycan (GAG) chains [23]. These negatively charged chains can bind water molecules and thus enable the viscoelasticity and flexibility of articular cartilage. Furthermore, it supports the transport of hydrophilic substances and thus the nutrition of the cells as well as aggravates the movement of bacteria.

Different cartilage layers with different amounts of water, cells and matrix proteins exist. These different layers as seen in **Figure 1** are the Superficial Zone, the transitional zone, the deep zone, the tidemark, the subchondral bone and the cancellous bone [103].

The superficial zone is forming the surface and is mainly constructed of type two collagen and fewer proteoglycans than the remaining zones. The chondrocytes show a spindle-shaped order inside this zone, what means, they lay approximately parallel to the surface. The chondrocytes in the transitional zone show a rather spherical character and the concentration of water and proteoglycans is raising. In the deep zone, the last zone before the tidemark, the highest concentration of proteoglycans and the lowest of water can be found. The chondrocytes form columns and are entirely spherical. The following tidemark builds a border between the actual cartilage and cartilage that seems to be a remnant of the endochondral ossification during the longitudinal growth in childhood. It is also separating the subchondral bone from the non-calcified area [103].

Figure 1: Histological cross-section - cartilage overview. Figure by [103].



1.1.2 Cartilage lesion - Classification

Different systems exist to classify cartilage lesions. Most common are the Outerbridge Scoring System [92] as well as the International Cartilage Repair Society (ICRS) Scores I [68] and II. [69] Furthermore Outerbridge and ICRS are roughly comparable. However, the scores that are used in this study will be explained in the section Methods and can be seen in Appendix I. Other classification systems made by Hungerford and Ficat, Bentley and much more can be seen in **Table 1** summarized by Noyes *et al.* [88].
After reviewing all these existing scores and after further investigations Noyes *et al.* made their classification system [88]. It includes four separate variables and can be seen in detail in **Table 1**.

- The description of the articular surface
- The extent (depth) of involvement
- The diameter of the lesion
- The location of the lesion

However, also Noyes concludes, that a visual scale by itself cannot be enough to describe or determine cartilage defects so that different existing classification systems have to be combined to compare it with other hospitals or studies.

Table 1: Review of previous classification symptoms of articular cartilage by Noyes *et al.* [76].

Review of previous classification symptoms of articular cartilage

AUTHOR	SURFACE DESCRIPTION OF ARTICULAR CARTILAGE	DIAMETER	LOCATION
Outerbridge	I - softening and swelling II - fragmentation and fissuring III - fragmentation and fissuring IV - erosion of cartilage down to bone	I - none II - < 1/2" III - > 1/2 " IV - none	Starts most frequently on medial facet of patella; later extends to lateral facet "mirror" lesion on intercondylar area of femoral condyles; upper border medial femoral condyle.
Hungerford and Ficat	I - Closed chondromalacia. Simple softening (small blister) macroscopically, surface is intact, varying degrees of severity from simple softening to "pitting edema", loss of elasticity. II. Open chondromalacia. a. Fissures - single or multiple, relatively superficial or extending down to subchondral bone. b. Ulceration - localized loss of cartilage substance, exposes dense subchondral bone. When extensive, bone has polished appearance (eburnated).	I - 1 cm^2 and then extends progressively in all directions. II - none	lateral facet - 2° excessive lateral pressure medial facet - 2° incongruence and combination of compression and shearing forces
	Chondrosclerosis - abnormally hard, not depressible.	Not localized but involves entire contact zone	
	Tuft formation - multiple deep fronds of cartilage separated from one another by deep clefts which extend to subchondral bone.		Centered on crest separating medial and odd facets.
	Superficial surface changes - surface fibrillation; longitudinal striations present in the axis of movement of the joint.		
Bentley	I - fibrillation or fissuring II - fibrillation or fissuring III - fibrillation or fissuring IV - fibrillation with or without exposure of subchondral bone	I - < .5 cm II - .5 - 1.0 cm III - 1.0 - 2.0 cm IV - > 2.0 cm	Most common at junction of medial and odd facets of patella
Casscells	I - superficial area of erosion II - deeper layers of cartilage involved III - cartilage is completely eroded and bone is exposed IV - articular cartilage completely destroyed	I - < or = 1 cm II - 1 - 2 cm III - 2 - 4 cm IV - "wide area"	Patella and anterior femoral surfaces.
Insall	I - swelling and softening of cartilage (closed chondromalacia) II - deep fissures extending to subchondral bone III - fibrillation IV - erosive changes and exposures of subchondral bone (osteoarthrosis)	None	I - IV: midpoint of patellar crest with extension equally onto medial and lateral patellar facets. IV: also involves opposite or mirror surface of femur. Upper and lower 1/3 nearly always spared (patella); femur never severe.
Goodfellow	Surface degeneration - surface flaking progressing to fibrillation. Basilar degeneration - fasciculation of collagen in the middle and deep zones, without at first, affecting the surface. - fasciculation (1) - articular surface is smoothly intact; spongy consistency; exhibits "pitting edema" - blister - fasciculation (11) - rupture of tangential surface fibers.	1 cm	"Odd" facet of patella Inferior part of central ridge separating medial and lateral facets.

Table 2: New Classification of articular cartilage lesions by Noyes *et al.* [74].

New classification of articular cartilage lesions

Surface description	Extent of involvement	Diameter (mm)	Location	Degree of knee flexion
1. Cartilage surface intact	A. Definite softening with some resilience remaining B. Extensive softening with loss of resilience (deformation)	<10 ≤15 ≤20 ≤25 >25	Patella A. Proximal ⅓ Middle ⅓ Distal ⅓ B. Odd facet Middle facet Lateral facet	Degree of knee flexion where the lesion is in weightbearing contact (e.g., 20–45 degrees)
2. Cartilage surface damaged: cracks, fissures, fibrillation, or fragmentation	A. <½ thickness B. ≥½ thickness		Trochlea Medial femoral condyle a. anterior ⅓ b. middle ⅓ c. posterior ⅓	
3. Bone exposed	A. Bone surface intact B. Bone surface cavitation		Lateral femoral condyle a. anterior ⅓ b. middle ⅓ c. posterior ⅓ Medial tibial condyle a. anterior ⅓ b. middle ⅓ c. posterior ⅓ Lateral tibial condyle a. anterior ⅓ b. middle ⅓ c. posterior ⅓	

1.2 Cartilage injuries

1.2.1 In general

There are three leading causes of cartilage injuries: acute trauma, inflammation, also known as arthritis in general, and circulatory disorders, whereby usually cartilage tissue cannot be restored and the best outcome would be the growth of fibrocartilage tissue inside the defect area [21]. With advancing age or high stress, as for obesity [40], the cartilage loses its shock absorption effect. At this moment a distinction is made for cartilage defects in knees. On the one hand, there are chronic false loading or stress [43] and on the other hand, acute injuries whereby especially a rupture of ligaments or menisci can cause post-traumatic cartilage defects [76]. Nevertheless, age and female sex are still the most critical risk factors for OA in patients with age above 50 [4, 35, 58, 63, 63].

Arthritis itself is an umbrella term for all inflammations in joints. Most common causes are the OA, traumatic injuries, gout, infection of the joint and age. Other forms are pseudo-gout, septic arthritis, rheumatic arthritis and much more. It can be seen, that this whole complex is a circle, with numerous endings and beginnings.

The acute lesion is typically a sports injury, especially sports with fast directional changes, high impact sports, contact sports and sports with a high risk of twisting ankle or knee, such as skiing, football, American football, volleyball, gymnastics or marathon. One of the most common sports injuries is the ACL rupture whereby for this injury, and summarized for every other knee ligamentous-capsular or meniscal injury, the risk to get a post-traumatic OA increases by the factor ten compared to uninjured patients [102]. Krych *et al.* [61] came to a similar conclusion for patients with dislocated knees, which often showed meniscal tear and cartilage damage that later often led to post-traumatic OA. The ALC rupture has almost no selfheal

ability and is frequently accompanied by articular cartilage damage, meniscal tears and an increased risk of knee OA [17, 66].

Anderson *et al.* [2] made a study to verify whether articular cartilage injuries in children and adolescents are correlated to the timing of ACL construction. Their results showed that not only the delay of reconstruction but also an existing instability as well as the return to sports before reconstruction resulted in a higher prevalence of cartilage and meniscal injuries. This resulted in recommending early treatment, especially for younger patients. Furthermore, especially football injuries are well studied because 200 million active players worldwide with an estimated injury-ratio of 10 to 35 per 1000 game hours and an estimated average cost of 150$ per treatment are a big load for every health care system [28]. S. Drawer *et al.* [26] worked out, using anonymous self-administered questionnaire, that most football players that retired from professional playing did that because of knee injuries (47%), followed by ankle injuries (21%). For the section of chronical diseases also knee was leading, adding that 32% of all respondents showed OA whereby the knee was the most frequently affected. Kujala *et al.* [62] examined former runners, soccer players, weightlifters, and shooters to determine a relationship between different physical loading conditions and findings of knee OA. Results showed tibiofemoral or patellofemoral OA in 3% of the shooters, 29% in soccer players, 31% in weightlifters and 14% in runners with significant differences. Whereby weightlifter showed the highest prevalence of patellofemoral OA, soccer players showed the highest prevalence of tibiofemoral OA. The results show that especially weightlifter and soccer players have an increased risk of premature OA.

Resumed these studies show that (1) acute injuries, also for young patients, can cause early onset cartilage defects, and (2) that also athletes that fulfilll competitive sports with chronical loads for individual joints have an increased risk to suffer from OA or cartilage degeneration effects in their later life.

Another disease that can cause arthritis is gout that more frequently is found in industrialized nations. It is defined as hyperuricemia above 390µmol/l with symptoms. These symptoms are divided into chronical symptoms such as cartilage destruction, reduced capacities, kidney stones and chronic kidney failure as well as acute symptoms with severe pain in joints especially for the great toe (podagra), fever and leucocytosis [112, 121]. The main problem lies in the surplus of uric acid (urate) and is followed by deposition of monosodium urate crystals inside articulations, peri-articulations and soft tissues as kidney for example. Causes are typical health problems of industrialized nations as obesity, purine-rich food and alcohol [15, 120, 121] whereby certainly also hereditary diseases exist that cause a surplus of uric acid. Recent studies say that gout arthritis is the most common arthritis form in males. Their prevalence of 1.4% exceeds the prevalence of rheumatoid arthritis that was stated around 0.5% to 1% [3, 78, 101]. A chronical surplus of uric acid can lead to the destruction of joints. The crystals precipitate inside the joints and slowly but continuously damage the cartilage surface followed by inflammation and swelling.

Osteochondritis dissecans (OCD), first described by König *et al.* (translated [59]) in 1887, is another common disease that can cause OA and appears in young [22] and adults. It is classified as “juvenile” or “adult” OCD. König concluded that loose bodies could occur after an acute osteochondral fracture or during

a low impact injury in a healthy joint. The spontaneous development of loose bodies without a trauma was stated as rare [29, 59]. Furthermore, he said that these pieces could lead to inflammation and necrosis inside the joint. However, König could not verify the exact pathological process, and it is still not 100% figured out and remains controversial [29, 118].

Most authors are still discussing different parts of the ethology whereas most agree that it is a multifactorial process in which trauma, ischemia, idiopathic and hereditary are stated as the most common causes. A treatment, especially for young people, is not standardized. In a review done by Edmonds *et al.* [29], outcomes between 50% and 94% were shown for non-operative treatments, whereby topic non-operative treatments included simple activity modification, bracing or even casting. On the other hand, there is an agreement in the literature that unstable OCD lesions and stable OCD lesions that fail the non-operative treatment should be operated as reviewed by Kocher *et al.* [57]. For the operative treatment are an amount of different methods as drilling [56, 65], bone grafting [52, 65, 65] , fixation [52, 55], alignment procedures [108] and debridement existing. For all methods, good to excellent results were stated. Last but not least is the ACT an excellent alternative for large osteochondral defects whereas 91% to 96% good to excellent results were made [37, 96]. X-Ray and MRI examples can be seen in **Figure 2**.

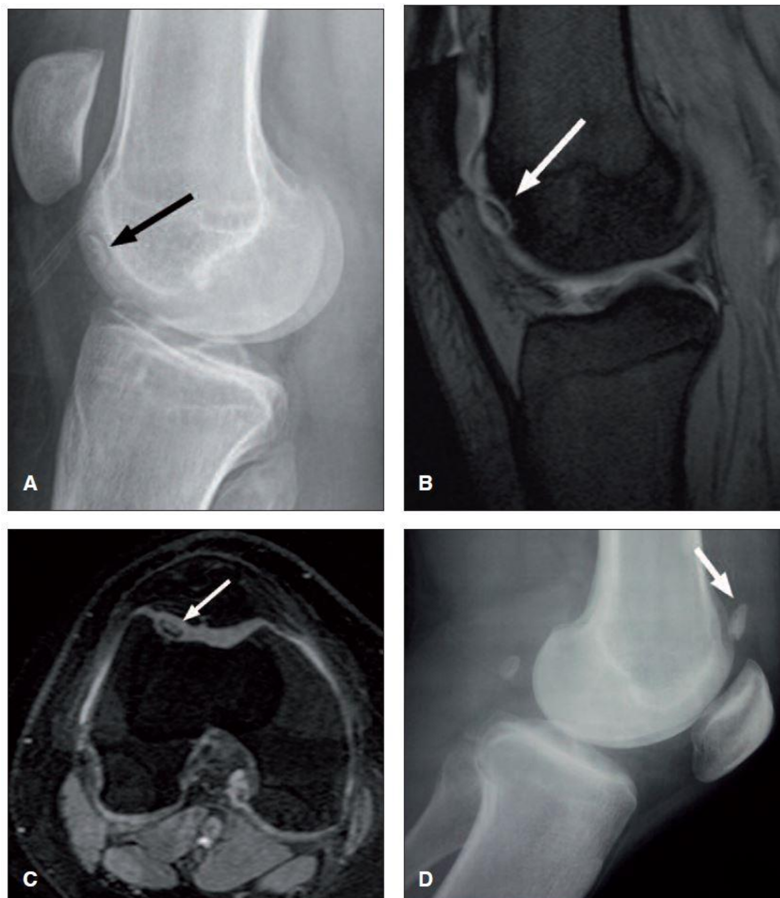


Figure 2: Osteochondral dissecans in X-Ray and MRI.
Lateral radiograph (**A**), saggital MRI (**B**) and axial MRI (**C**) of OD lesion of the trochlea (arrow). The lesion is demarcated from underlying subchondral bone with apparent anterior separation of the articular surface. **D**: Lateral radiograph showing a loose body (arrow). Illustration and figure caption from Zanon et al. [116].

However, several other diseases or injuries can lead to cartilage damage or defects, as well as several therapeutic approaches that try to fix this problem. The future lays in cell-based reconstruction whereas still more research is needed.

The standard diagnostic for OA is an X-ray in two or three planes. Typical signs are joint space narrowing, subchondral sclerosis, subchondral cyst formation and osteophytes [98]. Advantages for the Magnetic Resonance Imaging (MRI) are that it has a high efficacy, is not invasive and can state information about morphology, thickness, and volume to the surrounding cartilage [6]. Not all signs are needed to diagnose OA, and also radiographic signs without any clinical symptoms are possible.

In the end, OA is a combination of pathological changes inside the knee (e.g., on X-ray) including joint symptoms. These pathologies can differentiate a lot and result in a loss of articular cartilage, focal damage and joint destruction [4, 48]. Primary and secondary OA have to be distinguished. Although both have the same pathology the cause for primary OA is of hereditary nature. A higher prevalence for siblings was found as well as different genetic factors that increase the risk of getting OA under certain circumstances [109]. Secondary OA can be caused by several diseases like obesity and diabetes mellitus [100], congenital disorders, Marfan syndrome [1], Perthes' disease [45] and much more. Despite the name, OA is not a primary inflammatory disease but also not a disease primary relaying on mechanical stress. Berenbaum [10] concluded that the discussion, whether inflammation or mechanical processes should be seen as leading factor for OA should be ended because every mechanical or abnormal stress leads to intracellular signals that are eventually followed by different inflammatory mediators as prostaglandins, cytokines, and chemokines [104]. In the end, it is a complex sequence involving both, inflammation and mechanical stress.

1.2.2 Location of Cartilage Defects

Curl *et al.* [21] made one of the most prominent retrospective analysis of arthroscopies in the United States. 31.516 arthroscopies were reviewed whereby 53.569 hyaline cartilage defects were documented in 19.827 patients whereby in 63% of the arthroscopies cartilage lesions were found. The impressive results showed that more male (61.6%) than female (38.4%) had cartilage lesions and that 9.7% had grade I, 28.1% grade II, 41.0% grade III and 19.2% grade IV Outerbridge lesions whereby 72% of grade IV were found in patients older than 40 years. Companion injuries were found in 68% of the cases whereby the medial meniscus injury was the most common for patients older than 30 years, and ACL rupture was prevailing for younger patients (**Figure 3**).

A particular interest, referring to our study, lays in the occurrence and distribution for grade IV Outerbridge lesions. 26% of all patients with grade IV Outerbridge lesions had multiple ones. The most common localization was the medial femur condyle followed by patella, lateral femoral condyle (LFC) and trochlea (TROCH), as seen in **Figure 4**.

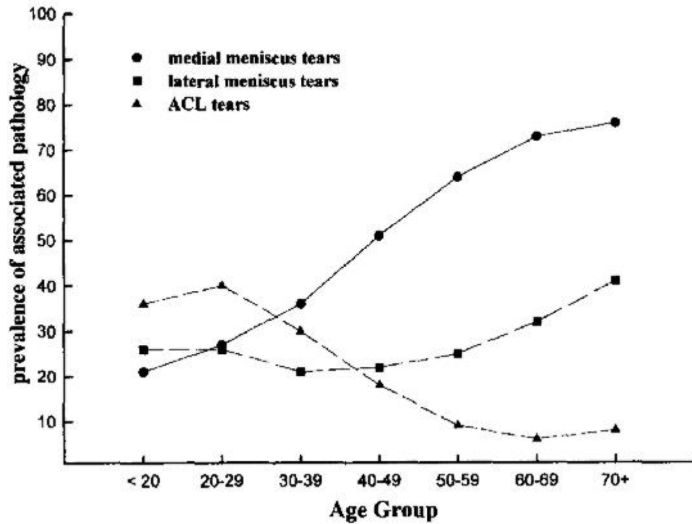


Figure 3: Percentage in each group with meniscal or ligament pathology. Ligament tears are reported for the ACLs only. Figure and illustration by Curl et al. [21].

A study done by Hjelle *et al.* [43] came to similar but not identical results. They found articular cartilage lesions in 61% of the patients undergoing arthroscopy but for scoring they used the ICRS score to verify the cartilage defects and found 14% grade I, 26% grade II, 55% grade III and 5% grade IV ICRS lesions. Furthermore, the most common location also was the medial femur condyle followed by patella, lateral tibia, LFC, and TROCH, whereby Hjelle *et al.* did not exclude grade I, II and III lesions for this statistic. Hjelle and his group focused on the cause and the pathological description of the defect. 38% of their patients remembered having an acute trauma, and the lesions were stated as OA in 44%, chondromalacia patellae in 23%, focal chondral lesions in 28%, OD in 2% and other types in 3% of all cases.

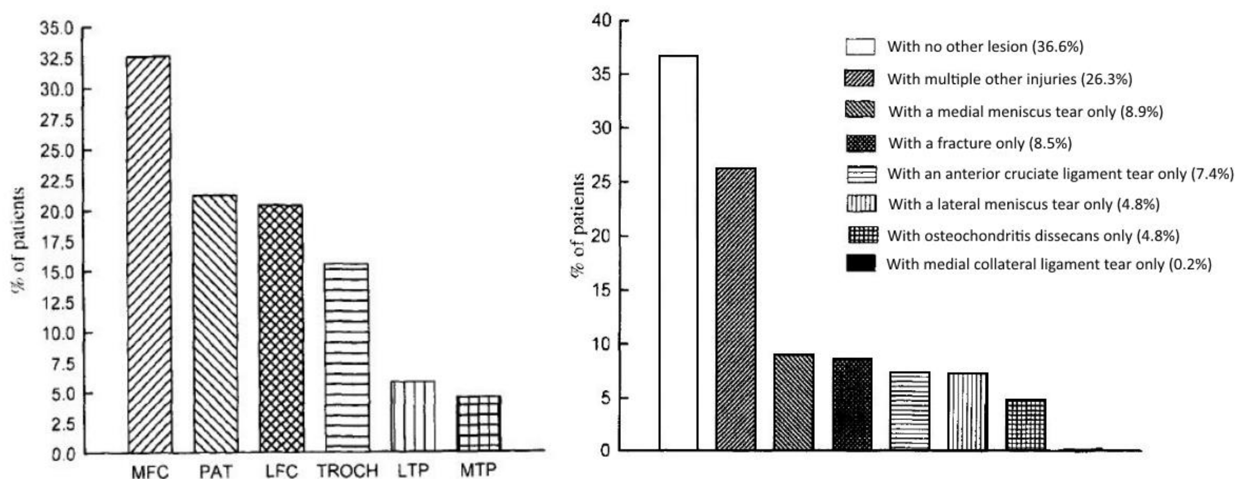


Figure 4: Most common localization and combined injuries.
Left: The Locations of single grade IV lesions in patients 40 years of age or younger. MFC, medial femoral condyle; PAT, patella; LFC, lateral femoral condyle; TROCH, trochlea; LTP, lateral tibia plateau; MTP, medial tibia plateau.
Right: A description of the knee lesions and injuries documented in patients under 40 years of age who had a single grade IV lesion. Figure and illustration by Curl et al. [21].

1.3 Cartilage treatment options

1.3.1 Oral therapy – Glucosamine and Chondroitin Sulphate

The following section gives a short overview of the two most frequently used oral supportive therapies for cartilage lesions and will mainly rely on the review done by Henrotin *et al.* [41]. The literature gives different statements to the efficacy of Glucosamine and Chondroitin Sulphate. A double-blind randomized placebo-controlled clinical study with a 2-year follow-up with 605 patients by Fransen *et al.* [31] came to the conclusion that the daily use of chondroitin sulphate and glucosamine for two years significantly decreased the joint space narrowing while reduced pain was found in all groups (chondroitin sulphate, glucosamine, both, and placebo). On the other hand, after the evaluation of more than 3800 patients, Wandel *et al.* [115] did not find any significant difference for reduced joint pain or joint space narrowing between placebo, chondroitin sulfate, glucosamine or the combination of both. Clegg *et al.* [18] had similar results. According to Wandel *et al.* they did not find any significant differences for knee pain reducing either, comparing placebo, glucosamine, chondroitin sulfate or the combination. The only significance was found for patients with initially moderate to severe pain comparing placebo and the combination of chondroitin sulfate and glucosamine. Considering all data, an ultimate recommendation cannot be given. Although more studies show an effect for pain reducing and reduced joint space narrowing than no effect, this effect seems not 100% provable and replicable. Finally, the choice is up to the doctor and his patients whether the doctor had successful experience with these medicaments and the patient is willing to take (more) medicaments and believes in the success.

1.3.2 Autologous Chondrocyte Implantation (ACI)

Right now three generations of ACI exist. The first generation uses chondrocytes laying under a membrane flap. The second generation, called matrix-associated autologous chondrocyte implantation (MACT), differs in using different scaffolds or substrates to support the cell division, matrix production, and integration into the chondral defect. The third and newest generation is characterized by utilization of allogenic tissue or autologous stem cells for cartilage regeneration. This avoids, even if rare, complications, like infections, caused by harvesting chondrocytes during the first arthroscopy. The first and second generation usually harvest Chondrocytes during a routine arthroscopy and cultivate them afterward.
Several studies were done for all generations. Mithöfer *et al.* [80] tested the potential of ACI to repair full-thickness knee articular cartilage lesions in soccer players and in how far they were able to return to soccer. 45 players were evaluated 41 ± 4 months and showed an outcome with 72% good to excellent results, 33% were able to return to soccer whereby 80% returned to the same skill level for 52 ± 8 months post-operatively. Saris *et al.* [105] compared characterized chondrocyte implantation with microfracture in 118 patients in a randomized multicentre trial. Results showed a better structural repair and better overall histologic evaluation for characterized chondrocyte implantation. Superior long-term outcomes for characterized chondrocyte implantation were expected and could be shown in a 3-year follow-up [106] with

greater clinical improvement for characterized chondrocyte implantation. Furthermore, better results were seen for early treatments and patients with higher characterization scores. Gooding *et al.* [36] compared periosteum covered and type I/III collagen covered autologous chondrocyte implantation (ACI-P and ACI-C) for osteochondral defects in 68 patients and found no significant difference with 67% for ACI-P and 74% ACI-C good to excellent results in a 2-year follow-up. The only difference was symptomatic hypertrophy in 36% of ACI-P patients with the need for shaving whereby 0 cases for ACI-C patients were found.

Studies of the third generation were done by Basad *et al.* [6] who compared microfracture with MACT. With a patients age between 18 and 50 and isolated chondral defects between 4 and 10cm², a follow-up was done with the last interval after 24 months. Significant better results were made for MACT in four scores (Lysholm ($p = 0.005$), Tegner ($p = 0.04$), ICRS patient ($p = 0.03$) and ICRS surgeon ($p = 0.02$) scores) and additionally no SAF issues were found.

Despite a small number of patients Crawford *et al.* [20] were able to reach significant lower pain scores as well as improved function and motion in comparison to baseline therapy with NeoCart® implant, which can be described as a proteoglycan- and GAG-rich, viable, and dynamic tissue-like implant [20]. Hyalograft C was tested by Nehrer *et al.* [85] and Marcacci *et al.* [70] and several others. Nehrer and his group had a prospective study with a follow-up between two and seven years. With a young patient cohort (mean age 32 ± 12) and a mean defect size of 4.4 ± 1.9cm², a highly significant increase in all knee scores was found whereby nine of eleven patients who had a secondary indication cases received a total knee arthroplasty because they showed resisting pain between two and five years. However, their conclusion was the recommendation of using Hyalograft C in young and healthy patients whereby results in patients with OA were poor. Marcacci and his group had a bigger cohort of 141 patients with a follow-up between two and five years. The patients' outcome was evaluated using the ICRS Functional status score, among other scores, whereby 71.4% reported they could do everything or almost everything they want with their knee whereby preoperative just 4.3% gave these answers. On the other hand, the group of patients who were restricted or very restricted in daily life was reduced from 95.7% preoperatively to 28.6% post-operatively. According to this, 95.7% of the knees were evaluated as normal or nearly normal by the objective International Knee Documentation Committee (IKDC) subjective evaluation of knee symptoms. All in all, their results agreed with the results of Nehrer´s group that Hyalograft C is a safe and effective therapeutic option for articular lesions. However, on 14th January 2013, Anika Therapeutics from Padua officially withdrew its application for a marketing authorization for Hyalograft C. This was confirmed by the European Medicines Agency whereby this withdrawal does not mean that Hyalograft C could not be relaunched after Anika Therapeutics submits new documents that remove the doubts of the Committee for Advanced Therapies. [49, 50]

Zeifang *et al.* [119] ran a randomized controlled trial to verify whether MACT or the original periosteal flap technology leads to better outcomes. They included 21 young patients with a mean age of 29.3 ± 9.1 years and a mean defect size of 4.1 ± 0.9cm². Several scores, as Lysholm and Gillquist score or Tegner

Activity score, were used. After one year no difference was found for efficacy between the outcome of ACI with periosteal flap technique or MACT. This result was confirmed in a follow-up one year later.
Ossendorf and his group [90] verified the efficacy of BioSeed®-C, a 3D bioresorbable two-component gel-polymer scaffold based on autologous chondrocytes, without using a control group. However, significant improvements were found for Lysholm score, modified Cincinnati Knee Rating system, the knee injury and OA Outcome Score (KOOS). In the viewpoint of the authors, BioSeed®-C is an effective alternative for patients with posttraumatic cartilage lesions or osteoarthritic defects in knees.
As mentioned above, the third generation uses an allogenic tissue or autologous stem cells. The idea behind this is to avoid two operations because the need to harvest chondrocytes for cultivation does not exist anymore and thus complications as infections can be avoided. Bekkers *et al.* [8] combined chondrocytes and mononuclear fraction cells (chondrocyte MNF) [8] and chondrons and MSC [9]. In their first work, they created freshly focal cartilage defects in knees of goats and compared the mononuclear fraction cells and chondrocytes with microfracture. The chondrocyte MNF treatment was convincing in macroscopic scores as well as in showing less degeneration post-operatively. On the other hand, no significance was found for microscopic scores and furthermore also not for GAG content so that further investigations are necessary. In his other study, he and his group wanted to avoid the two-staged surgical intervention by using chondrons and MSCs, as mentioned above, to directly combine the pre-gained MSC with chondrocytes. Different ratios were used to identify an optimal balance between both cell types. Chondrons and MSCs were cultured *in vitro* and implanted subcutaneously in nude mice and during a 1-step procedure into freshly created focal articular cartilage lesions in goats and compared with microfracture. Results showed significantly higher GAG production and an increased cartilage matrix formation. No remarkable SAF issues were found. Another group around Zak [117] published data for a MACT with a Novocart 3D scaffold that was based on bilayered collagen type I sponge. This procedure, also a 2-stage surgery, includes harvesting two or four full-depth cartilage cylinders as well as multiplying the isolated chondrocytes and finally combining them with the Novocart 3D scaffold. After a 2-year follow-up significant improvement was found for the results of Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score, 3D MOCART score, IKDC score, Noyes scale, Tegner activity scale, visual analogue scale (VAS) as well as KOOS subscales but without using the KOOS-symptoms subcategory. Another study based on Novocart 3D done by Niethammer *et al.* [86] dealt with the problem of hypertrophy when using ACI. The purpose was to investigate reasons and circumstances to understand post-operative hypertrophy in these cases better. With a collective of 41 patients, eleven cases with graft hypertrophy were found whereby especially NOVOCART 3D implantation in patients with acute events, such as acute injuries or OD, showed an increased risk of getting graft hypertrophy. The rest of the established criteria, such as body mass index (BMI), age, gender, showed no significance with the development of graft hypertrophy. However, problematic of some studies is that they always compared their, for example third generation, treatment with microfracture instead with second or first generation treatment to truly identify an improvement to newer investigated treatment methods.

1.3.3 Mesenchymal stem cell therapy

This cell-based therapy is a biological therapy that relays on the possibility of stem cells to differentiate along a pathway that leads to tissue as supportive and connective tissue, fat, muscles, ligaments and tendons. MSCs got a high potential for differentiation and proliferation as well as hold the potential to support the patient's selfheal abilities [72]. Furthermore, they are the basis for the hematopoiesis in bone marrow [93]. It is possible to gain MSCs out of many tissues as adipose tissue [38], successfully used because of its high share of MSCs, bone marrow, because of the well-known technic, umbilical cord blood and adult muscle [5]. The advantage of MSCs in adipose tissue is that with equal differentiation potential, a 300 times higher appearance of stem cells can be found. The full use of this potential lays in the future.

After gaining the stem cells, they are cultivated and either combined with a matrix/scaffold system or not. Especially the cultivation is essential to gain enough cells because, for example, the average concentration for MSCs in bone marrow is just 1 in 100.000 – 500.000 cells [14]. Furthermore more data, like the use of TGF-beta 3 (Transforming growth factor beta 3) for pre-differentiation [94] to chondrocytes has to be developed to improve the outcome of MSCs in chondral defects. Different studies exist, which deal with this problematic such as Marquass *et al.* [72] who compared implantation of matrix-associated pre-differentiated MSCs (pre-MSCs) versus articular chondrocytes. Their results showed that pre-MSC gels had a significantly better histological score than undifferentiated MSC (un-MSC) gels and an untreated control group. The repair tissue of the pre-MSC group had a significantly higher share of collagen type II than the other groups and showed radiological better bonding into the perilesional native cartilage.

However, other studies revealed that the use of MSCs could lead to immunosuppressive effects through modulation of cellular and immune pathways [53] whereby other studies add that in some pathological situations the total interaction between MSCs and human cells is still unknown and could lead to unexpected or unwanted symptoms [113]. Several other ways of treating or using MCSs seem to be possible but still need further investigation.

1.3.4 Osteochondral Autograft and Allograft

All grafts have in common that cartilage tissue is transplanted directly into the patients defect zone whereby osteochondral autograft or Autologous osteochondral transplantation uses patient's cartilage from a zone that is not subjected to be stressed or expected to be less stressed. Commonly used zones are the lateral or distal trochlea. A sort of osteochondral autograft was investigated by Wagner [114] in 1972 where he did an autogenous, orthotopic bone-cartilage transplantation. The results of that, confirmed by other surgeons of his time, were around 70% in two and five-year follow-up whereby the primary focus was on pain reduction and improved movement [116]. Because these procedures were much more rarely used in the last decade extensive studies as for microfracture, do not exist. A small study, done by Duany *et al.* [27], reported of nine patients with spontaneous Osteonecrosis of the knee who were treated with osteochondral autograft whereby eight patients showed a favorable clinical outcome by a mean follow-up of 42 years. The facts that it was a small study certainly has to be considered. Furthermore, Chow *et al.* [16] had a study

with 30 patients and a minimal follow-up of two years (mean follow-up 45.1 months, 24-63 months). An excellent or good outcome was stated for 83.3% of their patients while normal or near normal knees were stated in 86.7%. Furthermore increased the Lysholm score from a mean 43.6 points to 87.5 points. Interestingly they were able to find a significance between bad outcome and the length of preoperative symptoms like pain or knee swelling.

In comparison to autograft, a donor is needed for allograft transplantation. Allograft cartilage Transplantation can be combined with Meniscal allograft transplantation. Thus it can be very useful for combined injuries, as done by Getgood [34] whereby he combined meniscal and osteochondral allograft. The advantage of cartilage transplantation compared to other organs [32] is that cartilage triggers way less immune reactions [95]. Getgood's findings reviewed that many patients needed a reoperation but also showed a high overall graft survivorship. Furthermore, clinical improvements were found in all clinical cases with satisfaction. Towards failure rate, significance was found between higher failure rates and larger volume of transplanted tissue and a higher burden of other diseases. Horton *et al.* [44] tried to explore the outcome of revisions of osteochondral allograft transplantations. Their study revealed acceptable outcomes for old and patients with previous operations as well as for osteochondral revision transplantations. However, in the end, they rather recommended the revision osteochondral allograft transplantations for younger patients, which had a failed primary allograft operation, than for patients who undergo a progression of OA. For older patients with progressing OA, they less recommended it, because this was leading to higher failure rate.

Summarized advantages of these methods are that (1) they can be done in one surgery, also just with arthroscopy, (2) no intense immune reaction has to be expected for allograft transplantation [19, 32] and (3) low complications and low costs can be expected [16].

1.3.5 Microfracture

The aim of this method, which was developed by Steadman *et al.* [110], was to increase the joint cartilage regeneration and the development of replacement cartilage for patients with posttraumatic lesions of the knee that progressed to full-thickness chondral defects. His original method is adequately presented to provide its general approach. Like described in Steadman *et al.* [111], three portals are used for the arthroscopy – arthroscope, inflow cannula and working instruments. An overview is taken whereby other treatments usually are done before the microfracture is started. Important is to debride the defect as well as to remove calcified cartilage because this seems to interfere the integration into the existing matrix [47] and influences the attachment of repair tissue to the surrounding tissue [33]. The author [111] manually uses a small awl to drill many small holes inside the defects, whereby he begins at the edge and approaches to the center. These so-called microfractures have to be as close to each other as possible without reducing the stability of the nearby walls because the microfractures must not connect each other. The right depth can be assumed when fat drops or blood rises out of the bone marrow into the cartilage defect. The, by the author mentioned, exclusion criteria are axial malalignment, non-compliance or the apparent inability or

impossibility to follow the strict and essential post-operative procedure, partial-thickness defects as well as the impossibility, due to any other disease or accident, to bear most of the weight on the non-operated leg during the first post-operative time. Whereby subsequent studies used different protocols, and the efficacy of this post-operative treatment was not evaluated systematically yet [79]. Another mentioned relative exclusion criteria was the age above 60 years. This was examined by Kreuz *et al.* [60] in 2006 whereby they aimed to verify whether there is a significant difference between the outcome of patients aged 40 years or younger. They were able to show a significant difference in all groups for the preoperative scores, the scores at final follow-up as well as an excellent short-term efficacy. Furthermore, their study confirmed their hypothesis that younger patients with defects on the femoral, the tibia or patella had a significantly better outcome. Furthermore, better defect filling and better overall score in MRI 36 months after surgery were shown.

However, microfracture is the most frequently used, mostly first-line, therapy for cartilage defects in the knee [79] for the last decade. The well-known reasons for this are that several studies regard this method as safe and effective. Furthermore, this operation method is cheap, can be done in one operation and has a minimum amount of side effects or complications [111].

The overall problem of this method is that the growing tissue out of immigrating MSCs from bone marrow just differentiates to a mix of hyaline cartilage and fibrocartilage whereby the fibrotic part is the larger one. This leads to the problem that this mix is not as durable and resistant as normal hyaline cartilage [12] and sooner or later will again lead to problems like pain or reduced mobility. In the current literature also discussed is the question if microfracture is indeed superior to ordinary Debridement and if no, if it still should be used [11, 67].

1.4 Research and Development with animal models

The development of new treatments or the investigation to better understand pathology and physiology using animal models is more than 300 years old. For the almost same amount of time opponents and proponents for these methods exist. However, it is proven that many signs of progress in human medicine rely on animal tests. An example is the treatment of rachitis in early 1900 as well as passive immunization against tetanus and diphtheria. In the 1920s thyroid hormones were found as well as the resulting possibility to treat hypothyreosis. Just to mention one of the later and essential researches. In 2000 the first complete genome of drosophila, mice, rats, and humans was decoded. Much more examples could be named during this time, but that shall not be the task of this work.

Nonetheless, the question floating over animal research for almost all the time is, in how far results can be transferred to human medicine. A base existing since 1959, and now well known as the three R’s, are the aim of reduction, refinement, and replacement, published by William Russell and Rex Burch in the book “The Principles of Humane Experimental technique”. In the discussion about animal experiments, three major positions exist [87].

1) Any animal experiment that is wanted can be done
2) Not every animal experiment is done, but the existing have to depend on particular animal characteristics and particular circumstances
3) No animal experiment is done

Whereby the first and last position are slightly extreme positions, the second position stands for a realistic compromise. In most countries, the current existing way is the second one. Methodological problems detected by Pound *et al.* [99] are the variability in the selecting method, randomization, choice of comparison therapy and that outcome correlation in animals and humans do not have to correspond as well as that small differences between laboratories are neither reported nor recognized. In the end, Pound *et al.* conclude that more and rigorous evaluation is needed to show the transferability to human medicine whereby large systematic reviews should be used. The alternative is to replace animal procedures like in pharmaceutical, chemical and cosmetic areas. Great Britain decreased the number of animal experiments in the industries mentioned earlier significantly, while the number of procedures in universities and medical schools have risen by 52% [64].

It hardly seems that the future for investigation lays in experiments with *in vitro* and technical constructions and without animals. However, until today, the technical progress is not far enough and probably too expensive to provide results or lifelike procedures and therefore is (not yet) the perfect alternative.

For the development of chronological defects and treatment in knees, it is possible to investigate ways to improve the outcome of chondrocytes or MSCs *in vitro*. However, yet, no scientific model exists that could provide the underlying pathophysiology of movement and weight bearing that mainly affects the outcome of the treatment. Furthermore, previous studies by our group [42, 73] showed results leading to the possibility comparing human and merino land sheep cartilage as well as Hurtig *et al.* [47] who showed that the ovine knee is relatively similar to the human knee comparing structure and load areas.

2 Aim of the dissertation

The purpose of this GLP study was to show the efficacy and safety of the investigational product co.don chondrosphere® (ACT3D-S). ACT3D-S is a product for autologous chondrocyte transplantation that we used in an animal model, the merino land sheep. The principle of utilizing chondrosphere® is to implant spheroids into an existing cartilage defect. The spheroids are involving into the existing matrix and should refill the defect with new cartilage tissue.

We compared the treatment of ACT3D-S (Group A: Investigational product) with an untreated control (Group B: Control Intervention) in a bilateral model, what means that by randomization one hind limb was chosen to be treated with ACT3D-S while the remaining hind limb was left without treatment. All in all, with a total number of eleven merino land sheep, which fit our inclusion criteria, we had 22 knees to operate and score over time. The bilateral model was chosen to eliminate interindividual differences.

During the first operation, a predefined cylindric cartilage defect (7x2mm) was created in both hind limbs, and cartilage tissue was removed to cultivate cartilage spheroids. Therefore the cartilage cells were isolated, and the chondrocyte expansion took place inside a monolayer. At the end of the chondrocyte differentiation, the spheroids were placed in a special syringe and sent back for implantation into a randomized defect (left or right knee) 49 days after explantation. This duration was approximately the same time, the defect needed to chronify and to be comparable to human chronic cartilage defect zones.
Nine months after the implantation the animals were sacrificed, and the hind limbs were extracted to verify the efficacy using different visual, histological and MRI scores. Moreover, safety scores were collected during all study processes to verify the safety of the investigational product.

The primary objective was to show, that Group A, in comparison to Group B, would show an average of three more points for the O'Driscoll score (t-test: two-sided, alpha-level: 0.05) for the per protocol (PP) population.
Main categories were:

- Nature of predominant tissue
- Structural characteristics
- Freedom from cellular changes
- Freedom from degenerative changes in adjacent cartilage

Further efficacy scores were the ICRS (International Cartilage Repair Society) score I and II. The first one was a microscopic score with a maximum of 18 points and six different values as surface, matrix, cell distribution, cell population viability, subchondral bone and cartilage mineralization and was done after the explantation of the hind limbs after nine months. The second one, a microscopic score as well, evaluated the new cartilage tissue as well as the integration of it, had 14 different parameters and was done equally

after the explantation of the hind limbs and can be seen in detail in Appendix I: Scores and detailed Ultrasound Results. The ICRS-MCAS (macroscopic cartilage assessment score) was, like the name already tells, a macroscopic score and thus, was done during the first, second and after the last operation, and its values described the degree of defect repair, integration into border zone and macroscopic appearance of cartilage.

The 2-D and 3-D documentation of ultrasound biomicroscopy of the cartilage-bone architecture of the former lesion site were measured after the third surgery. It was planned to show a correlation between the nine quantitative categories of the ultrasound analysis and many other categories or items of other efficacy scores. The absolute ultrasound analysis (US) measures included six volumetric and three metric categories. The last efficacy score was the MRI based modified MOCART score that was done by making magnetic resonance images of the explanted limbs. It had nine variables that were used to verify the morphology and signal intensity of the repair tissue compared to the adjacent native cartilage.

To conclude, the ICRS-MCAS and the O'Driscoll score were the only two efficacy scores that were evaluated during the first and second operation. All other scores were done after the animals were euthanized and the hind limbs were explanted.

At least equally important was to show the safety of the investigational product co.don chondrosphere® (ACT3D-S). Therefore we used three different scores to verify the well being in general and each knee individually.

The systematic pain score was divided into two subcategories whereby one verified the general well being, and the other was directly referring to the operated knees. It had a maximum of 20 points and was done several times pre- and post-operatively. It included food and water uptake, behavior and facial expression, respiratory rate, movement behavior (general well being) as well as movement behavior, palpation of the wound and joint swelling/increased warmth/soft tissue swelling (knee specific).

The knee inflammation score was done several times during and between the operative periods. This score was individually for both hind limbs and included the typical five signs of inflammation *rubor, calor, dolor, tumor* und *functio laesa.*

Using these score, we were able to see how the animals reacted during the production phase and long-term husbandry and if there were any significances between treated and untreated control group.

The last safety score, the macroscopic knee assessment score, was done during the first and second operation and after the explantation of the hind limbs. This score described the health and condition of the defect and surrounding cartilage and included hypertrophy, ectopic cartilage production, delamination of transplant, kissing lesion, structural side defects, and synovial fluid.

3 General Information about the study

3.1 Task assignment

The different ways of somatic cell therapy for treating cartilage diseases or damages is hardly investigated these days [72, 82, 83]. Especially tissue engineered products are increasing. However, to develop these products, such as cartilage transplants, new methods and models are required to perform preclinical testing. For this purpose, the standard GLP testing methodology for SAF and efficacy of medications is not entirely appropriate. Particularly for the utilization of *in vitro* cultured and manipulated artificial tissue from somatic cells, a clear, structured and reasonable SAF testing under GLP conditions are necessary. The problem was that the primary parameters of non-clinical studies, like toxicity, carcinogenicity, rejection, and inflammation, were not precisely described within the existing GLP testing categories so that it was hard to classify it universally.

Chondrosphere® is such a tissue-engineered product, requiring these novel testing methods. The product is built of autologous cartilage cells (chondrocytes) that need to be taken from the patient several weeks before the actual treatment date. These cells are cultivated and rearranged in 3D fashioned spheroids. These spheroids are consisting out of thousands of cultivated chondrocytes and are representing the active substance of chondrosphere® for an ACT. The principle of utilizing chondrosphere® is to implant spheroids into an existing cartilage defect. The spheroids are involving into the existing matrix and should refill the defect with new cartilage tissue.

The primary purpose of this study was to verify the efficacy and safety of the analogous ovine spheroids. Herefor, a chronical focal cartilage defect of knee joints in the medial femoral condyle was used as a test sample. Since the Merino land sheep is quite comparable to human cartilage knee treatment [47], it was used to test the 3D ovine test sample (ACT3D-S). Two Groups, Group A: Investigational Product and Intervention Group B: untreated control group, were used whereby both hind limbs were randomly assigned to be treated or not. So every sheep had one site treated and one site untreated.

The GLP study was conducted as a multi-site study. The entire course of the study at several test sites was controlled and monitored by the directing GLP study facility, the Fraunhofer Institute for Cell Therapy and Immunology (Fh-IZI). Referring to previous preceding projects and own studies (Hepp *et al.*, 2009 [42]; Marquass *et al.*, 2010 [73]; Osterhoff *et al.*, 2011 [91]; Zscharnack *et al.* 2010 [122, 122]; Marquass *et al.*, 2011 [72]) and in consensus with the current “Recommendation from the International Cartilage Repair Society (ICRS) for Preclinical Studies for Cartilage Repair” [46] the entire GLP-compliant study process was designed and planned to fulfill the guidelines set forth in **Table 3**.

Due to the gained experience through these previous projects, the recommended species (merino land-sheep) was used once more for this pivotal study. Only adult animals were allowed as a biological test system, which had full mature hyaline cartilage structures in their joints. Through using a bilateral model, it was planned to minimize inter-individual variability. Making it comparable, equally sized full-thickness defects were created inside the medial femoral condyle (MFC) of the stifle and either be treated with the

test item ACT3D-S (Group A) or left untreated (Group B). At several time points (**Table 3**) the animals were inspected and several scores to verify safety and efficacy of the product were done. This study set-up fulfillls the recommendations as set by the ICRS and by the national authority Paul-Ehrlich-Institute (PEI). The basis of the scoring system were six well-accepted and well-acknowledged scores that consisted of three different histological assessments (E1-E3), one gross macroscopic scale (E4), one MRI score (E5) and an ultrasound biomicroscopy analysis (US). These scores were used to get qualitative and quantitative characterizations of the cartilage repair tissue before and after treatment as well as in comparison treated to untreated.

Besides these six efficacy scores also data for three SAF scores were collected. The main purpose of these scores were the overall health and well-being of the sheep (S1), signs of inflammation (S2) and treatment / cartilage-specific side effects in the stifle (S3). Besides that, also serum and whole blood (WBC) analysis, as well as several SAF examinations also during the long-term husbandry, were done. After the euthanasia of the animals, an extensive animal autopsy (AA) followed to exclude diseases that could interfere with the efficacy or SAF of the product. In the AA, attention was put to the major organs and their weights, the synovial membrane of the stifle joints as well as three major joints of the forelimbs and the whole musculoskeletal system.

However, also drawbacks are part of progress and development and have to be expected for the animal model as well as the one to one adaption from human medicinal product chondrosphere® (ACT3D) to the analogous ovine test sample ACT3D-S. **Figure 5** gives an overview of the principle sequence of this study.

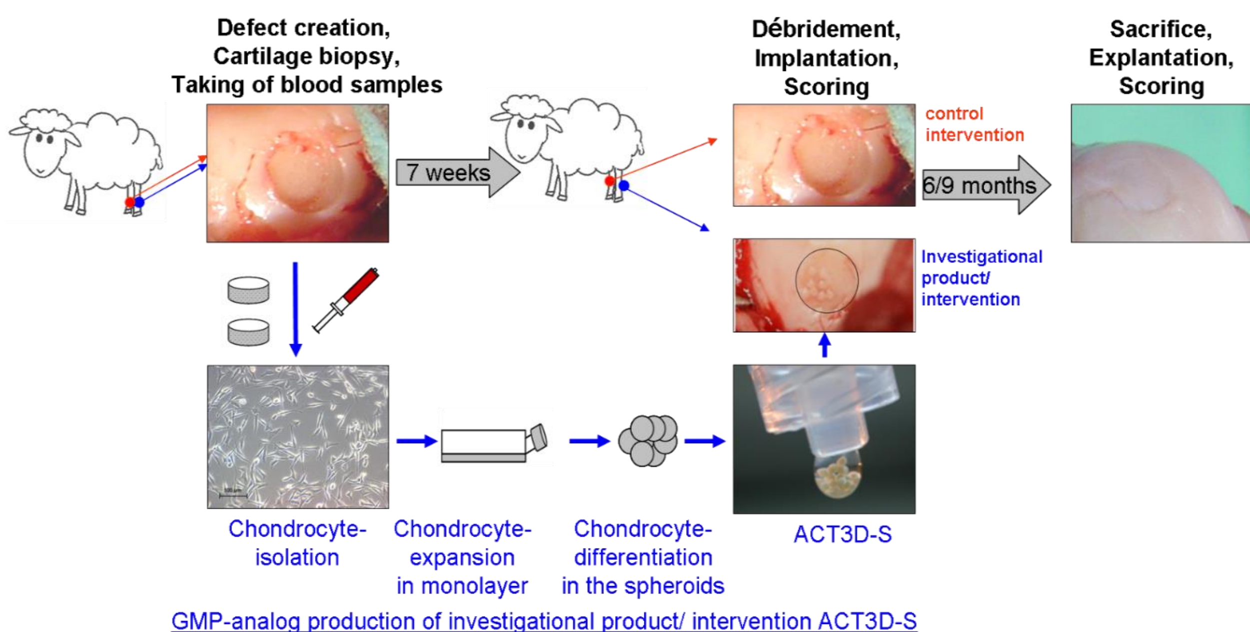


Figure 5: Overview of the design of the test study for all experimental animals.
The animals are treated in Group A (ACT3D-S; investigational product/intervention) and Group B (control intervention) on each femoral condyle during the implantation surgery. The procedure for treatment of a femoral condyle with the control intervention (Group B) is shown in red, while blue shows the more complicated procedure for treatment with the investigational product/intervention ACT3D-S (Group A) for the contra-LFC of all experimental animals.

3.2 Study design

This study was randomized, prospective, actively-controlled, blinded label for observers of MRI/histology/macroscopy/laboratory preclinical super trial with priority to efficacy and SAF. To avoid false data through inter-individual variances, a paired design was used. Two Groups were created – Active treatment (AT) and control treatment whereby both groups were parallel for each animal (bilateral mode). The objective of the study was to show that AT is significantly better compared to control treatment, whereby the effect should be better than a predefined superiority margin of three units on the related scale (Score E1).

At different time points different raw data for scores were obtained. An overview of scores and time points can be seen in **Table 3**.

Table 3: Assessment dates of all scores.

Assessment Dates of all Scores																										
Score	Date	Pre-OP	1st surgery			Degeneration				2nd surgery			Long-term husbandry											3rd surgery		
		Inclusion Exami-nation	-49d			-48d	-47d	-46d	-45d	0d			+1d	+2d	+3d	+4d	+1wk	+2wk	+3wk	+4wk	+3mo	+6mo	+9mo	+6mo/+9mo		
			V-2/I	V-2/II	V-2/III	V-1a	V-1b	V-1c	V-1d	V0/I	V0/II	V0/III	V1a	V1b	V1c	V1d	V2a	V2b	V2c	V2d	V3a	V3b	V3b	V4/I	V4/II	V4/III
Safety	S1	x	x			x	x	x	x	x			x	x	x	x										
	S2-*	x	x						x	x						x	x	x	x	x	x	x	x	x		
	S2-°	x	x						x	x						x	x	x	x	x	x	x	x	x		
	S3-*			x							x														x	
	S3-°			x							x														x	
Efficacy	AA																									x
	WBC																				x	x	x			
	E1-*			x							x															x
	E1-°			x							x															x
	E2-*																									x
	E2-°																									x
	E3-*																									x
	E3-°																									x
	E4-*			x	x						x	x														x
	E4-°			x	x						x	x														x
	E5-*																									x
	E5-°																									x
	US-*																									x
	US-°																									x
Photos	F-*			x	x						x	x“													x	
	F-°			x	x						x	x“													x	
Outerbridge	OB-*			x	x						x	x													x	
	OB-°			x	x						x	x													x	

Legend: d =day, wk = week(s), mo = month(s), V = visit/ward round, F = photographic documentation of medial femoral condyle, OB = Outerbridge classification
I = preoperative, II = before defect creation(V-2)/debridement(V0)/explantation(V4), III = after defect creation(V-2)/debridement(V0)/explantation(V4).
* = right knee. ° = left knee (randomised and blinded: group A and group B). ‘’ before and after application

S1 = Score S1 – systemic pain score
S2 = Score S2 – knee inflammation score
S3 = Score S3 – macroscopic knee (capsule) assessment score
AA = animal autopsy
E1 = Score E1 – O’Driscoll
E2 = Score E2 – ICRS-I
E3 = Score E2 – ICRS-II
WBC = whole blood count
E4 = Score E4 – ICRS-MCAS
E5 = Score E5 – modified MOCART
US = Ultrasonographic evaluation

3.3 Target Animal Population

The animals that were used for this study as biological test objects were merino land sheep, whereby inclusion criteria said, that they should be >50 kg, > two years and female. They were chosen because the organic/structural properties and the cellular mechanisms for tissue repair processes are similar in sheep and humans. This is in accordance with internationally recognized expert opinions, supported by numerous publications as Hurtig *et al.* in 2011 [46].

3.4 Dosage, Binding, and Randomization

13 (-1) Spheroids per ovine cartilage defect (Ø 7 mm, 38.5 mm^2) of ACT3D-S analog to mean administration dosage of 33 spheroids per cm² defect in human cartilage defect were planned. According to the summary of medicinal product characteristics of chondrosphere® the recommended dosage in a human patient is defined as a range from 10 to 70 spheroids per cm² cartilage lesion.

3.5 Blinding

Blinding of the implanting surgeons to the mode of administration is not applicable. Animals and explants were blinded to the explanting surgeon, radiologist, pathologist and lab personnel by using consecutive defect numeration without relation to treatment group.

3.6 Randomization

Each sheep was randomized to Group A or B. Through using a bilateral model, animal-to-animal variability could be left out of consideration. Cohort I was kept six months (182 ± 7 days) and cohort II nine months (273 ± 7 days) in the stable after the respective treatment. Random choice of animal allocation to one of the two respective cohorts (six vs. nine-month husbandry) is achieved by blinding of the test personnel. However, this work is just about the nine-month cohort. The hind limbs were allocated to treatment or no treatment by using a randomization list that was compiled prospectively by the statistician using a simple randomization scheme using 50 % probability for each side. One set of envelopes was prepared with a randomization number, readable for the investigator, and information about allocation to treatment or not. Sheep eligible for randomization were assigned a randomization number. The investigator assigned the randomization number in a sequential ascending order. The investigator selected the side of AT (and opposite side for control treatment) before treatment according to information provided in an envelope.

4 Material and Methods

4.1 Scores

In this study, we used five efficacy scores, three SAF scores, and an ultrasonographic evaluation. The five efficacy scores were the E1 – O'Driscoll Score [89], the E2 – International cartilage Society Score (ICRS) I [68], the E3 – International cartilage Society Score II [69], E4 – ICRS-MCAS [97] and E5 – modified MOCART [71]. The three SAF scores were the S1 – Systematic Pain Score, the S2 – Knee Inflammation Score and the S3 – Macroscopic Knee (capsule) assessment Score (MCAS).
In Appendix I (see chapter 8) a further inspection of parameters, variables, and subcategories can be done for all scores mentioned above.

4.2 Statistical Evaluation of test results

This chapter has all necessary information about the prospectively planned objectives, endpoints, and statistics of this study. The wide-ranged Statistical Analysis Plan (SAP) was made between the sponsor Co.don AG, the statistician from ACOMED statistics and the GLP test facility Fh-IZI on the ninth of August 2012.

4.2.1 Objectives

Primary objective

The primary objective was to prove, that the difference of overall Score E1 – O'Driscoll was larger than the superiority margin of three units between the product intervention with ACT3D-S (Group A) and the untreated control (Group B) at nine months after the implantation surgery.
If a difference in initial condition of the native cartilage (as measured by Score E1 at t=0, V-0/II) is found in analysis of covariates that strongly affects the results, the statistical analysis of the primary objective might include the initial condition of the native cartilage (as measured by Score E1 at t=0, V-0/II) as a covariate.

Secondary objectives

Concerning efficacy:

1. To assess a difference of Score E1 - O'Driscoll of ACT3D-S (Group A) and untreated control defect (Group B) nine months after the end of the respective treatment in an exploratory manner
2. To assess a difference of Score E2 - ICRS-I of ACT3D-S (Group A) and untreated control defect (Group B) after nine months after the end of the respective treatment in an exploratory manner
3. To assess a difference of Score E3 - ICRS- II of ACT3D-S (Group A) and untreated control defect (Group B) after nine months after the end of the respective treatment in an exploratory manner

4. To assess a difference in changes of Score E4 - ICRS-Macroscopic cartilage Assessment Score (ICRS-MCAS) from day 0 (= baseline of chronic defect before débridement) of all Groups A and B to nine months after the respective treatment in an exploratory manner
5. To assess a difference of Score E5 - modified MOCART of ACT3D-S (Group A) and untreated control defect (Group B) nine months after the end of the respective treatment in an exploratory manner
6. To assess ultrasound analysis to estimate neo-tissue formation volume, overfilling tissue volume, missing tissue volume, hypertrophic tissue volume, cyst volume, healthy tissue volume, healthy min thickness, healthy max thickness, healthy average thickness

Concerning SAF:

7. To assess SAF parameters for Group A and Group B including frequency and type of observed adverse events (AE) as well as Scores S1 – S3
8. Experimental analysis of whole blood cell count and serum chemistry in comparison with external reference values and within cohort
9. Experimental analysis of organ weights within cohort
10. Explorative analysis of synovial fluid and synovial tissues of knee joints of both hind limbs of ACT3D-S (Group A) and of untreated control defects (Group B) after nine months
11. Explorative analysis of synovial fluid and synovial tissues of three joints (shoulder, elbow, carpal joint) of both forelimbs after nine months
12. Exploratory analysis of musculoskeletal tissues after nine months

Additional analyses

13. To assess the influence of the initial condition of the native cartilage (as measured by Score E1 at t=0, (V-0/II)) as a covariate in comparison of overall Score E1 - O'Driscoll of ACT3D-S (Group A) and untreated control (Group B) at nine months after the end of the respective treatments
14. To assess all parameters are obtained from Group A and B with respect to flowchart (see **Table 3**)
15. To assess ultrasound analysis in comparison to Scores E1 - E5

4.2.2 Endpoints

Primary endpoint

The difference of overall Score E1 - O'Driscoll of ACT3D-S (Group A) and untreated control (Group B) at nine months after the end of the respective treatments.

Secondary endpoints

Note: Enumeration of secondary endpoints is related to enumeration of secondary objectives as defined above.

Concerning efficacy:

1. Score E2 - ICRS-I at nine months
2. Score E3 - ICRS-II at nine months

3. Absolute difference of Score E4 - ICRS-MCAS at nine months compared to baseline
4. Score E5 - modified MOCART at nine months
5. Ultrasound analysis measures at nine months (six volume measures and three metric measures)

Concerning SAF:

6. Score S1 – systemic pain score, Score S2 – knee inflammation score, Score S3 - ICRS-MCAS, as well as AE occurrences
7. Whole blood count and serum measurements (22 parameters: ALT (U/l), AST (U/l), Bili (total) (µmol/l), Bili (indir) (µmol/l), Bili (dir) (µmol/l), CHOL (mmol/l), GGT (U/l), TG (mmol/l), white blood cells (G/l), red blood cells (T/l), HGB (mmol/l), HCT (l/l), MCV (fl), MCH (fmol/l), MCHC (mmol/l), PLT (G/l), Baso (%), Eos (%), Bands (%), Segs (%), Lymph (%), Mono (%))
8. Organ weight measures (14 organs: lung, heart, brain, rumen (with contents), rumen (without contents), small intestine (with contents), large intestine (with contents), liver, kidneys, spleen, pancreas, udder, thyroid gland right, thyroid gland left, adrenal gland right, adrenal gland left, pituitary gland)
9. Synovial fluid and synovial tissue analyses of hind limbs (macroscopical and histological findings)
10. Synovial fluid and synovial tissue analyses of forelimbs (macroscopical and histological findings)
11. Musculoskeletal analyses (macroscopical and histological findings)

Additional analyses

12. Same as defined for the primary objective (Score E1)
13. Number of missing data on planned assessments
14. Correlation between ultrasound analysis and Efficacy Scores E1-E5

4.2.3 Statistics

Analysis populations

The following analysis populations are related to the nine-month assessment.

- Safety: All animals with treatment
- Intended to treat (ITT = SAF): All animals with treatment
- Per protocol (PP): all animals belonging to ITT and fulfillling inclusion criteria

Minor and major protocol violations are defined in a data review meeting before locking the database. As a result of the meeting, a list was provided allocating each animal to SAF, ITT, and PP.

Statistical methods for analysis

- Descriptive statistics: contingency tables (categorically-scaled variables by treatment: absolute + relative frequencies) and tables (mean value (mean), standard deviation (SD), standard error (SE), Median, inter-quartile range (IQR), minimal value (Min), maximal value (Max); by treatment) for continuously-scaled variables. However, not every single table, diagram or raw data is included in this work but is stored in our server.

<u>Primary objective</u>

- Comparison of Score E1 - O'Driscoll by treatment via t-test (two-sided, alpha-level: 0.05). Null hypothesis: ScoreE1AT (active treatment) = ScoreE1Control + 3
- Primary analysis is performed at PP population. Result for ITT population is presented in addition.

<u>Secondary objectives</u>

- Similar to the primary objective, but only for PP population, statistical tests are performed, but results are reported in an exploratory manner.
- No correction for multiplicity

<u>Additional analysis</u>

- analysis of covariates within the analysis of primary objective (Initial scores E1): analysis of variance (ANOVA) with repeated measurements
- Descriptive statistics
- Joined analysis of nine-month (cohort two) animals regarding primary and secondary objectives with reduced alpha level 0.025 (to adapt analysis for multiple testing)
- Evaluation of correlation between ultrasound analysis and Efficacy Scores E1-E5: Spearman Rank correlation or Regression methods/ANOVA.

<u>SAF</u>

- Descriptive statistics (SAF = ITT population). It should be noted, that most of the safety parameters are related to the animal and not to the knee. A group related assessment of SAF parameters will be performed with parameters describing individual knees.

<u>Dropouts/Missing values</u>

In preliminary experiments, no dropouts were observed. Because a production process is included using cartilage of animals, dropouts regarding missing substances for AT could occur. Approximately 15 % were accounted for both cohorts. Two animals were foreseen for such a purpose in the cohort two (n = 11).

Within statistical analysis of the primary objective, missing values were taken into account in the following way: The last observation carried forward rule is applied. Also, analyses including generalized linear mixed modeling as well as analysis using original data without imputation are performed regarding a sensitivity analysis. Results are discussed regarding robustness.

<u>Sample Size</u>

Concerning the primary endpoint (superiority margin 3):

The superiority of ACT3D-S (Group A) in comparison to untreated control defect (Group B) (superiority margin = minimal interesting difference three points) concerning Score E1 - O'Driscoll has to be shown. An effect of 5.5 points on Score E1 scale is assumed resulting in an effect of 2.5 above the predefined limit of 3. SD of differences is chosen to be 3.6 according to preliminary findings.

For a planned group size of nine animals each for the 9-month duration group (cohort two), the following powers are estimated when a two-sided paired t-test is applied (significance level of five %) and an effect

of 2.5, 3, 3.5 or four points is assumed: 50 %, 65 %, 78 % or 87 % respectively. Two animals were added to address possible dropouts.

Statistical software

Statistical analysis was performed using SAS software version 9.2 or later (SAS Institute, NC, USA).

4.3 Defined undesired events

4.3.1 Adverse Events

An adverse event (AE) was defined as any untoward veterinary occurrence in an animal during any preclinical trial phase (i.e., pre-transplantation period, treatment (ACT3D-S follow-up)). These occurrences do not necessarily have to have a causal relationship with the treatment. An AE can therefore be:

Any unfavorable and unintended sign:

- Bleeding into the defect at day of defect creation
- Bleeding into the defect at day of defect debridement and transplantation
- Reduced count by one spheroid (13-1 Spheroids)
- Severe capsule inflammation
- Symptom or disease temporally associated with the use of the treatment whether or not considered related to the treatment

AEs related to the surgical procedure or other aspects of the investigational product application process:

- Suspected or confirmed cases of infection
- Unexpected reactions (e.g., hypersensitivity, immunological, toxic)

Any other AE is, by definition, non-serious.

4.3.2 Serious Adverse Events

Definition of serious adverse events (SAEs) for subjects participating in the preclinical trial to examine the efficacy and SAF of the treatment with the autologous chondrocyte transplantation ovine test sample chondrosphere® (ACT3D-S) compared to untreated control group in subjects with cartilage defects of the knee in MFC:

An SAE was defined as an AE occurring during any preclinical trial phase (i.e., pre-transplantation period, treatment (ACT3D-S follow-up) that fulfillls one or more of the following criteria:

- results in death
- is life-threatening
- requires veterinary treatment by a veterinary surgeon
- results in persistent or significant disability/incapacity
- is an important veterinary event that may jeopardize the animal or may require veterinary medical intervention to prevent one or more of the outcomes listed above

Life-threatening

Life-threatening means that the animal was at immediate risk of death from the SAE as it occurred or it was suspected that use of the investigational product would result in the animals death. Life-threatening does not mean that an SAE occurred in a more severe form it might have caused death.

Veterinary treatment

Animal treatment by a veterinary surgeon is not in itself an SAE, although the reasons for it may be serious (e.g., bronchospasm, laryngeal edema).

Important veterinary event

Veterinary and scientific judgment should be exercised in deciding whether a case is serious in a situation where important veterinary events may not be immediately life-threatening or result in death, disability or incapacity but may jeopardize the animal or may require veterinary intervention to prevent one or more of the outcomes listed in the definition of an SAE. These should usually be considered as serious. Examples of such events are:

- Angioedema not severe enough to require intubation but requiring intravenous (i.v.) hydrocortisone treatment
- Hepatotoxicity caused by pain medication overdose requiring treatment
- Veterinary treatment for allergic bronchospasm
- Development of sepsis

4.4 Adverse Drug Reaction

An adverse drug reaction (ADR) is defined as any untoward veterinary occurrence in an animal during a preclinical trial phase (i.e., treatment (ACT3D-S follow-up) which does necessarily have to have a causal relationship with the treatment. An ADR can therefore be:

- An unfavorable and unintended sign
- Symptom or disease temporally associated with the use of the treatment very probably related to the treatment

4.5 Unexpected Adverse Event

An unexpected AE is an experience not previously reported (in nature, severity or incidence) during application if the investigational product.

4.6 Inclusion and Exclusion criteria

Inclusion criteria

1. Merino land sheep, female, Age: 2-3 years, weight: >50 kg (date of screening)
2. clinically healthy
3. intact cartilage structure in MFC

Exclusion criteria

1. Essential impairment of the animal’s general condition with observed behavioral changes
2. Internal parasites, Bluetongue, Caprine Arthritis Encephalitis (CAE)
3. Clinically proven illnesses of the musculoskeletal system
4. Failure of manufacturing ACT3D-S - no treatment PP possible
5. Knee inflammation with swelling, redness, pain, hyperthermia, impaired function
6. Capsule inflammation with turbidity of synovial fluid
7. Lack of defect surrounding cartilage structure

4.7 General study process

4.7.1 Acclimatization

The sheep flock, that was delivered from Schäferei Otto-Schölz (Pissen 17, 06237 Leuna) to the GLP test site LVG (teaching and testing farm) Oberholz in Großpösna (Veterinary Medicine Faculty of the University of Leipzig) near Leipzig, was comprised of 32 adult female merino land sheep (cohort one: 21 sheep, cohort two: eleven sheep). The Veterinary Medicine Faculty of the University of Leipzig documented the age of the sheep, their origin as well as the yellow and new white unique ear tag number (ETN). During the entrance examination, the S1/S2-Score Appendix I (see chapter 8) and veterinary inclusion examinations were implemented and documented by the veterinarian. When the veterinarian found no abnormalities, the animals were released for the study. Furthermore, the sheep obtained treatment against lungworms (Dectomax 10 mg/ml), and coccidiosis (Diclazuril 1 mg/kg of body weight) when clinical findings were present. At last, a hair cure was given after the shearing, to protect the animals against biting lice. The feeding was done with hay, silage, straw and green fodder out of LVG-internal production and was added by mineral and concentrated fodder. An acclimatization before being taken to the MEZ (medical-experimental center of the University of Leipzig) GLP test site, was enabled for at least one week, before beginning with further study procedures, including the first surgery. For this, the animals were randomly selected independently of age by the test personnel - blinded for this purpose - and sent to MEZ in special suitable trucks in groups of maximal 12 sheep. A random distribution was guaranteed through this first randomization. Before the first procedure began, the animals had at least one day of acclimatization in the MEZ Leipzig test site.

The first inspection of the animals was followed measuring their weight. Afterward, the animals were kept in stables with a maximum size of three animals per stable. Contact to other animals, which were kept in the MEZ, was prevented throughout the whole time. Cleaning was done daily if necessary. The sheep were held on an empty stomach one day before the first surgery (at least 24 hours) and the scores S1/S2 were repeated the day of the first surgery. After this, the animals were released for surgery.

4.7.2 First surgery – anesthesia and defect creation

After documentation of the ETN, the date of operation and weight of the animal, the premedication was given. It was a mix of 11 mg/kg BW Ursotamin, 1ml/100kg BW Rompun 2% and 1 ml Atropinsulphate (0.5 mg/ml) and was intramuscular (i.m.) injected in the *musculus semimembranosus*. An additional injection could be done when the first one was not sufficient enough. Before starting the first surgery, a complete blood draw in 24 Z gel monovettes took place and was marked with the ETN. The anesthesia was performed in the supine position. After fitting the permanent venous catheter, an infusion (NaCl (sodium chloride) 0.9%) up to 1000 ml was added. The narcosis was done using 1-2 mg/kg BW Narcofol intravenous until the swallowing reflex vanished. If necessary, 2-8 µg/kg BW Fentanyl i.v. and / or 5 mg/kg BW Ursotamin i.v. were added. For the intubation an endotracheal tube with a size between 7 and 8.5 was used, whereby the inhalation narcosis was maintained with Isoflurane (0.7-1.5% expiratory concentration) and a mix of oxygen and fresh air. Standard beginning parameters were 50% oxygen, 1-2 l/min fresh air, respiratory rate 12/min, tidal volume 8-10 ml/kg BW, positive end-expiratory pressure (PEEP) 5cm H_2O. By using a rumen tube and Vidisic eye gel, the risk of aspiration and dry eyes was decreased. Shortly before the operation began, a bolus of 2 µg/kg BW Fentanyl i.v. was given to reduce the peri- and post-operative pain and was supported by peri-operative 3 ml/50 kg BW Veracin. The Fentanyl bolus could also be used in demand during increasing heart rate, respiratory rate or movement. Shaving both hind limbs, positioning the intubated animal and adding a neutral electrode in the area of the right axilla, a sterile positioning of all surgical instruments on a movable table as well as cleaning (with Bactolin soap) and disinfection (betaisodona solution) were done. The standardized surgical hand disinfection, wearing sterile coats and gloves as well as the sterile covering of the operation area goes without saying. The last step before the first cut was another disinfection of the operation area with 0.5% Terralin Protect.

The incision was done para-patellar with a scalpel, and the step by step preparation with electrical anticoagulation followed until the articular capsule was seen. The capsule incision followed and the scoring of the visible cartilage (**Figure 6**), always done by one surgeon for both sides to prevent interpersonal differences, was done. The following scores were gathered: Outerbridge score, MCAS (S3), ICRS-MCAS (E4) as well as photo documentation was done. After this, the defect was marked with a 7 mm x 2 mm punch/stamp/die-cutter in the center of the MFC. A part of the cartilage was removed using a chondro-curette followed by drilling the defect and ended by flushing with sterile NaCl and a visual control for no bleeding. Another scoring using the Outerbridge score, ICRS-MCAS (E4) and photo documentation (**Figure 6**) followed. The removed cartilage was put into two different small tubes for each side and was marked with side and ETN. The more substantial part was put in biopsy tubes from co.don AG while a smaller part was put into tubes for histological preparation and analysis, was directly shock frosted with liquid nitrogen and sent to the GLP lab for future assessments. During all this, special attention was lying on sterility and documentation.

The operation ended with a wound closure including capsule, muscle, and skin stitches. The 24 serum monovettes were combined with the cartilage probe whereby the temperature inside the box was monitored

by TempMark®. Co.don was informed per telephone about delivery time because the biopsy had to be done within 48 hours. Until the uptake by Co.don, the biopsy had to be held between 15 and 25 degrees. After all this, the anesthesia was stopped by decreasing Isoflurane to 0% and increasing oxygen to 80-100% as well as increasing the fresh air flow to 5 l/min. After sufficient spontaneous breathing, the sheep were monitored for five more minutes until the extubation, with aspiration prevention, followed. The i.v. catheter was removed as well as covered with an elastic bandage. After return transport into the stable, the sheep were observed for ten minutes with attention for vigilance and excursions of the thorax.

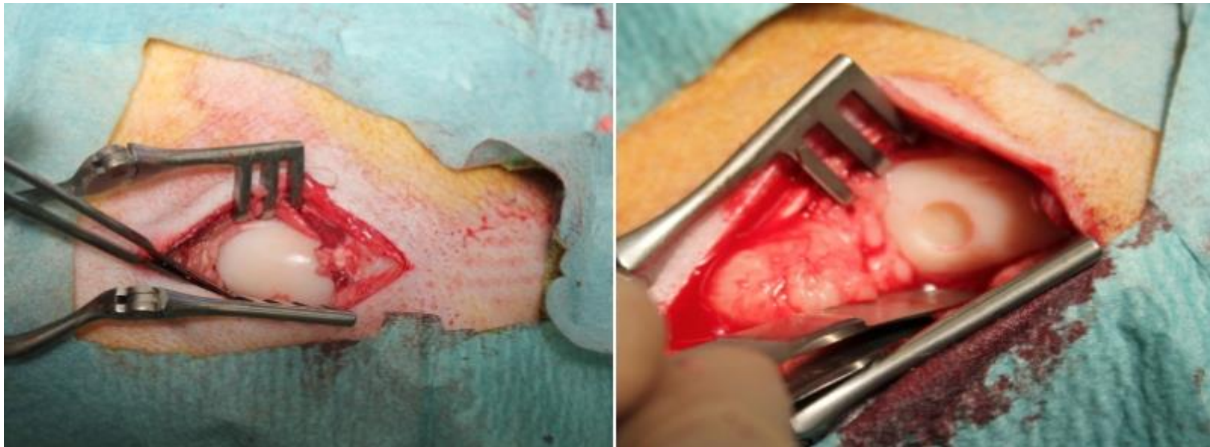


Figure 6: Intraoperative photos during the first surgery.**Left**: MFC after capsule incision and cleaning; **Right**: MFC after defect creation (7mm x 2 mm).

4.7.3 Production phase of ACT3D-S

The biopsy kit was received at co.don AG, and Good manufacturing practices (GMP)-analogue production and approval of the ACT3D-S investigational product/intervention was performed within 49 days based on the internal method standard operating procedure (SOP). The isolated primary ovine chondrocytes (of the biopsy) were cultured in several steps, as a monolayer in the beginning, and two weeks isolated in the end. The principle of ACT3D-S is based on the arthrotomic harvesting of the animal's chondrocytes isolated from healthy full-depth cartilage. They are cultivated *in vitro* in a GMP-analogue method to develop three-dimensional spheroids (ACT3D-S) that are subsequently transplanted into the chronic focal cartilage defect. After seven weeks they result in hyaline-like cartilage repair. Usually an untreated defect degenerates and is filled with fibrous tissue, whereby sometimes a fibrillation of surrounding cartilage and incurvation of native cartilage can be seen in the border zone.

4.7.4 Degeneration phase of the full-thickness cartilage defect

During the first hour after the operation, the animals were kept isolated or as couples inside the stable. The fact, that sheep are run and flee animals led to the point that immobilization was neither possible nor ethically correct. The first operation was followed by daily examinations and if necessary analgesia for four days. When symptoms of reduced food- and water-uptake or increased laying on the ground were found, it was possible to give 2.2 mg/kg BW Flunixin i.m. (50 mg Flunixin/ml Finadyne RPS) once a day until the symptoms were gone. The examination was based on the S1 Score, beside the last day when the S1 and S2 scores were taken and documented. Between the first and second operation, a maximum of 49 days was scheduled. This was enabled by transporting the animals from the MEZ back to LVG 4-7 days after the initial surgery where an observation and veterinary examinations were done before they would return to the MEZ for the second surgery. During this stay, a weekly examination by a veterinarian was done. After six weeks the return was performed in suitable trucks provided by the Veterinary Medicine Faculty of the University of Leipzig. An acclimatization was provided after being transferred to the MEZ Leipzig test site for at least one day whereby the requirement was to operate the animals exactly 50 days after their first surgery. The sheep were kept on an empty stomach one day before the second surgery began and S1 and S2 examinations, as well as the general treatment and organization, were repeated like during first surgery. After this, the animals were released for surgery. All observational data was saved by the test personnel and kept at the Fh-IZI test facility.

4.7.5 Second surgery - implantation of ACT3D-S

Before the second surgery started, a check of the "co.fix®" transplantation kit, provided by codon, had to be done to verify its completeness. The package consisted of the TempMark® temperature monitor, documentation from the pharmaceutical company, the retention samples as well as the investigational product/intervention ACT3D-S and was sent by courier within 36 hours. The anesthesia, as well as the preoperative management and disinfection, was done equally to the anesthesia of the first operation. The operation had the same sequences as the first operation until both femoral condyles were visualized and scored, whereby the old scar was used for the first cut. Old repair tissue was removed using biopsy punch or chondro curette. The taken cartilage repair tissue was put into small tubes and was flash frosted with liquid nitrogen. Both defects were equally prepared for possible implantation of the product by drilling a 7mm x 2mm defect like in the first surgery; a joint lavage with NaCl 0.9%, cleaning and drying with a sterile swap as well as visual control for bleeding inside the defect. To ensure the blinded randomization, the statistician made a randomization list by using a block randomization scheme with variable block lengths. When both Outerbridge grade four [92] defects were equally created, the list was used to decide whether to treat the one or the other side.

A biopsy for the separate histological preparation and evaluation that were based on E1 were taken from the cartilage tissue that was removed from both femoral condyles. To randomize, what site would receive treatment with ACT3D-S, a randomization list, which was prospectively created by the statistician using a black randomization scheme with variable block lengths, was used (**Figure 7**).

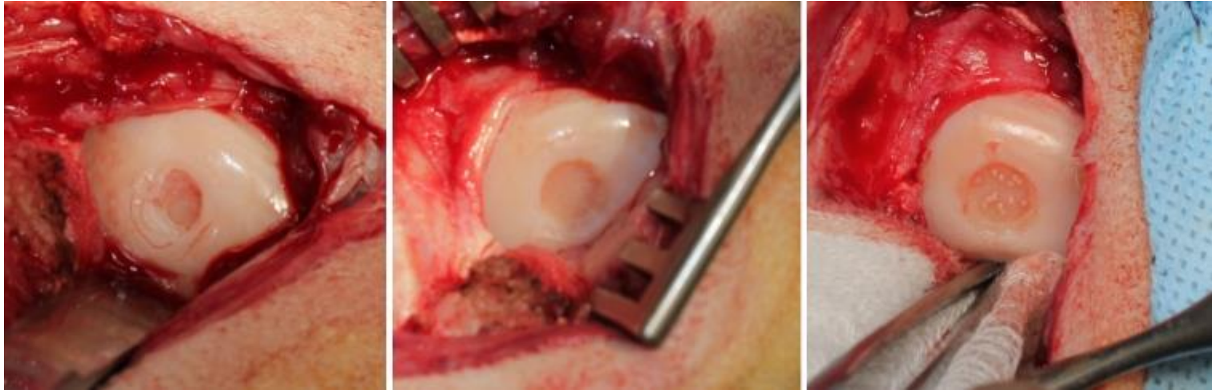


Figure 7: Intraoperative photos of the second surgery.
Left: MFC 49 days after defect creation; **Middle:** MFC after removing cartilage repair tissue and preparing for Implantation of the ACT3D-S test product; **Right:** MFC after Implantation of 13 ACT3D-S spheroids.

Afterward, the spheroids which were inside co.fix®, a unique syringe with a long and small plastic tube at the top for a more comfortable application of the spheroids inside the defect area, were implanted into the defect. As soon as 13, or at least 12, spheroids were positioned into the defect, the duration of 18 to 20 min, as prospectively planned, began. During this time the remaining liquid evaporated. It was additionally possible to use threads of swabs to dab away the remaining liquid. If a full swap was used, the capillarity of the swab would absorb the remaining water so fast that also spheroids would be pulled into the swab. The repositioning of the spheroids from the swab into the defect would have been a tough task.

It clearly evident that blinding of the implanting surgeons was not possible. The number of ACT3D-S spheroids per defect was 13 (or at least 12) for the randomized femoral condyle. As explained above, the procedure was kept similarly to the procedure in humans with 33 (or even 30 respectively) spheroids per cm² (Group A, investigational product/intervention). Photo documentation (**Figure 7**) followed after implanting the ACT3D-S in the femoral condyle. The remaining femoral condyle was used as an untreated control defect (Group B; control intervention). All observational data was saved by the test personnel and kept at the Fh-IZI test facility.

4.7.6 Long-term husbandry

It was done equally to the first operation. The sheep were kept isolated as couples inside the stable for one hour after the operation was done. The fact that sheep are run and flee animals led to the point that immobilization was not possible. The second operation was followed by daily examinations and if necessary analgesia for the next four days. The examination based on the S1 Score, whereby the S2 (for both stifles) score was added for the fourth day, were gathered and documented. When symptoms, like reduced food- and water-uptake or increased laying on the ground were found, it was possible to give 2.2 mg/kg BW Flunixin i.m. (50 mg Flunixin/ml Finadyne RPS) daily until the symptoms were gone. The examination based on the S1 Score, out of day four when the S1 and S2 (for both stifles) scores were taken and documented. After the second surgery, the return transport to LVG was made between four to seven days after the surgery. In LVG the animals were kept under veterinary medicine observation for nine months (273 ± 7 days, cohort two, n = 11) post-operatively. For the first four post-operative weeks, the S2 score

was weekly determined and was followed by three other data assessments after three, six and nine months (for animals of cohort two) of husbandry. Furthermore, blood was collected in one Z gel and one EDTA monovette, which was performed after three, six and nine months to perform an analysis with a focus on serum chemistry and whole blood cell count at the Contract research organization (CRO) HOSPITAL. Routinely after arriving at the LVG 0.2 mg/kg BW Dorametacin (Dectomax 10 mg/ml) was given to prevent lungworms. This treatment was repeated after half a year. Moreover, a treatment against coccidiosis with 1 mg/kg BW Diclazuril (Vecoxan 2.5 mg/ml) when toxicological findings indicated it as well as another shearing when necessary was done.
After nine months husbandry, the animals were transported back to the MEZ test site for the third and last operation. The transport was performed by the Veterinary Medicine Faculty of the University of Leipzig that used suitable trucks primarily designed for this purpose. Acclimatization was provided for at least one day after successful transport. The sheep were kept on an empty stomach one day before the third surgery began and S1 and S2 examinations were repeated like in the first and second surgery. After this, the animals were cleared for sacrifice. All observational data were saved by the test personnel and kept at the Fh-IZI test facility.

4.7.7 Third surgery - sacrifice / explantation

For the third and last surgery, both hind limbs were removed and marked. The anesthesia, as well as the preoperative management and disinfection, was done equally to the anesthesia of the first and second operation. T61, a medicament to euthanize animals, was intravenously injected with a minimal dose of 6 ml/50 kg BW. T61, on the hand, decouples the motor endplates in heart and respiratory musculature and on the other hand, causes a deep loss of consciousness as well as a depression of heart and respiratory rate, so that it results in a respiratory and cardiac arrest. During the application, the veterinarian frequently measured respiratory rate, heart rate and reflexes. The time of death was documented by the veterinarian. Afterward, the veterinarian cleared the animal for the explantation of both hind limbs. The explantation was done with special amputation scalpels and saws. For later transport, the extracted limbs were put into transparent plastic bags. For radiographic examination, these extremities were sent to the RADIO study location to undergo radiographic examination by using MRI to determine the E5 score. The S3 Score, Outerbridge scores, and photo documentation were done after the return of the hind limbs from RADIO study location.
To examine the synovial membrane, samples of both femoral condyles were taken and fixed in 4% formalin. The samples were sent to the CRO VETMED (Institute of Pathology, Faculty of Veterinary Medicine, University of Leipzig) for histological staining. The disarticulation followed, and the E4 score was determined. Afterward, the femoral condyles were used for the determination of the US analysis. All observational data were saved by the test personnel and kept at the Fh-IZI test facility. Both explanted femoral condyles including the investigational product/intervention and the control intervention (Groups A and B) were sent by courier to an external histological laboratory (HIK study site) for histological slide preparation.

For SAF data veterinary medical personnel of the CRO VETMED fulfillled a pathological examination of the sheep to search for any signs of tumor growth and was performed in accordance with the SAF recommendations of the ICRS. [46]

- autopsy of sheep under the guidance of a specialized veterinary pathologist with technical assistance
- autopsy and pathologic-anatomical findings with detailed diagnosis
- determination of organ weights (”major organs“)
- preservation of representative organ samples for histopathology (”all synovial tissues,“ ”major organs,“ “modified organs”) and, if necessary, for complementary etiological procedures
- synovial membrane of the shoulder, elbow, and carpal joints of the forelimbs on both sides was taken with a histological examination by hematoxylin and eosin stain
- synovial membrane of the knee joints of the hind limbs on both sides was taken with a histological examination by hematoxylin and eosin stain
- histopathological examination (overview staining, special stainings if necessary) of the synovial membrane (“all synovial tissues,“ “organs with anomalous pathologic-anatomical findings) and diagnosis
- sending samples to the State of Saxony's Health and Veterinary Research Institute for monitoring studies of TSE (transmissible spongiform encephalopathy) required by law (sheep age > 18 months)
- preparation of a detailed epicritic report on diagnostic findings and an overall assessment of the intended licensing procedure

The cadavers were stored in a cold environment until being collected. They were disposed of by an animal body disposal company (Zweckverband TBA Lenz, Staudaer Weg 1, 01561 Priestewitz). Apart from the animals that are further explained in section 5.3, no peculiar differences or pathological abnormalities were found. Therefore, no further explanation or data is taking place in this section.

4.7.8 Histology and immunohistochemistry of explants

The first step was to document the receipt of the explants at the HIK study side where they were separated into the investigational product/intervention ACT3D-S or the control group. After being embedded in Technovit plastic, samples were created with subsequent immunohistochemical (if required by the sponsor) and histochemical stains (including Safranin-O-lightgreen, Hematoxylin, and eosin) using standard techniques based on GLP documentation standards.

Hematoxylin and eosin (HE) stain

Deplastination:

The HE stain is the most common stain technique and colors all basophil structures blue while acidophil structures are marked in shades of red, pink or orange. The staining began with an incubation of the slices for 20 minutes with xylol and was followed by incubating twice with methoxyethyl acetate for 20 minutes,

respectively. A three-step carefully tipping followed beginning with 100% ethanol, then 96% ethanol and ending with 70% ethanol always until the solution ran down completely. The last step was the rinsing of the slices with demineralized water.

Staining:

The staining began by filtering approximately 180 ml of hematoxylin solution and using the filtered solution for ten-minute long staining. The slices were rinsed afterward by immersing them into tap water and were "blued" afterward by staying in tap water for ten minutes. This was followed by staining with 1% Eosin solution for 30 seconds and ended with thorough washing under tap water.

Dehydration:

Again a carefully tipping with 96% ethanol followed until the solution ran down completely. A twice incubation with 100% ethanol and twice incubation with xylol completed the whole process.

The stained slices were marked, stored at room temperature and finally sent to the test facility. Examples of our HE staining can be seen in **Figure 8**.

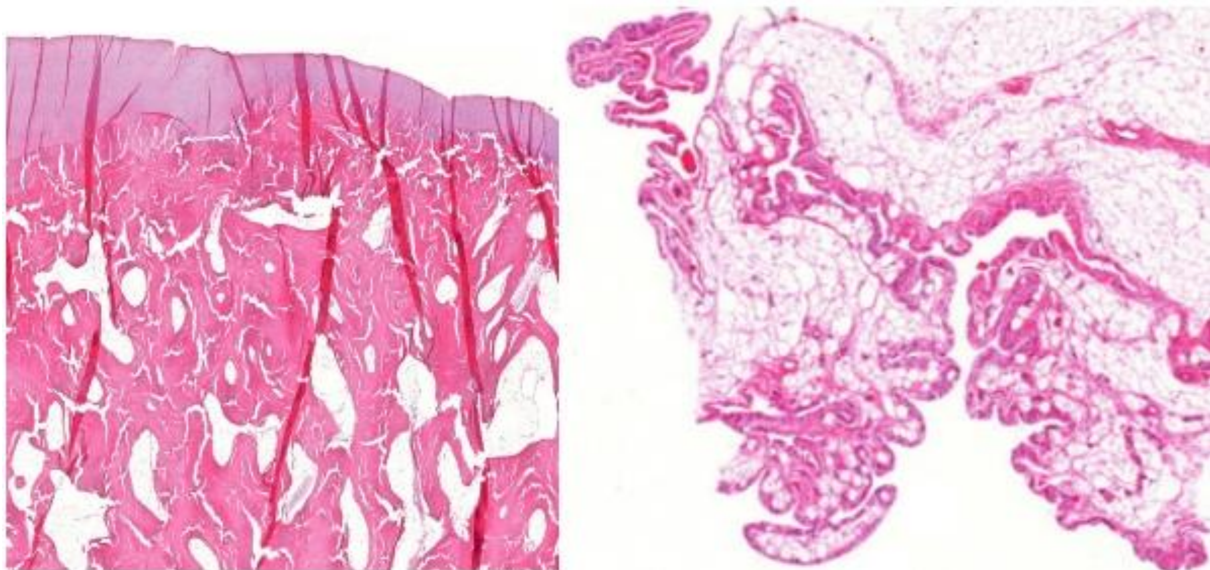


Figure 8: HE stains of cartilage and synovial tissue **Left:** HE staining of cartilage tissue; **Right:** HE staining of synovial tissue; hematoxylin stains basophilic substances as DNA/RNA dark blue or violet and eosin stains acidophilic substances as proteins pink or red.

Safranin stain

The Safranin stain is another ubiquitous way to color cells or tissue in human medicine. In the beginning, the slices were incubated for five minutes in xylol afterward a three-step carefully tipping followed, beginning with 100% ethanol, then 96% ethanol and ending with 70% ethanol always until the solution ran down completely. Next step was the incubation for ten minutes with hematoxylin, followed by rinsing the slices with demineralized water before they were immersed twice in 0.5% HCl alcohol. Thorough washing of the slices was followed by two incubations whereby a ten-minute tap water incubation was succeeded by a six min incubation with Fast Green (solution). Another washing with acetic acid 1% and incubation for four minutes in a Safranin-O solution was followed by rinsing the slices with 96% ethanol. The stain was finished by incubating twice with 100% ethanol and twice with xylol.

The stained slices were marked, stored at room temperature and finally sent to the test facility. Examples of our Safranin-O staining can be seen in **Figure 9.**

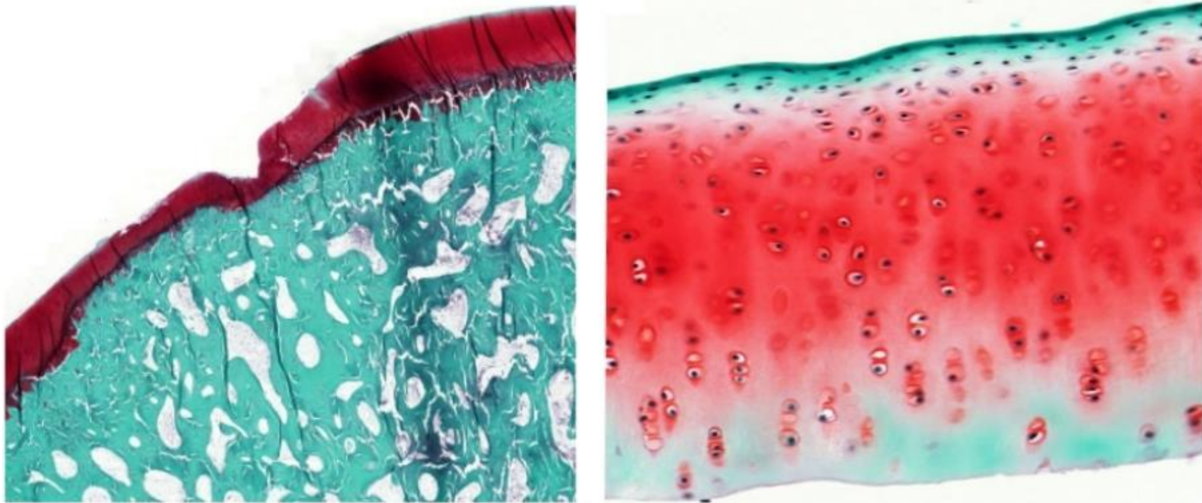


Figure 9: Safranin-O stain of cartilage tissue.**Left:** Safranin-O stain of cartilage tissue **Right:** Safranin-O stain of cartilage tissue in higher resolution so that the structure as mentioned in 1.1.1 can be seen; Safranin-O stains the nucleus black, cytoplasm grey-green and cartilage orange to red, depending on the amount of proteoglycans.

4.7.9 Cryosectioning and histology

First, protocols were documented for the native cartilage biopsies from both femoral condyles from the first surgery (healthy, native joint cartilage) and the second surgery (degenerated repair cartilage tissue) at the Fh-IZI test facility. The tissue was stored and embedded with sample production using cryosection technology and subsequent histochemical staining (including Safranin-O-lightgreen) with standard techniques based on GLP documentation standards. During the first operation, it was possible to gain healthy cartilage tissue whereby in the second operation just very little regenerated cartilage tissue could be received.

4.7.10 Scoring

Next step was the outstanding scorings including O'Driscoll score [89] (Score E1,), ICRS Visual Histological Assessment Scale I and II (Mainil-Varlet *et al.* 2003 [68], Mainil-Varlet *et al.*, 2010 [69] for Scores E2 and E3). To fulfilll the documentation of these, all explanted samples and slides that were planned to undergo immunohistochemical and histochemical staining had to be transported from the HIK study site to the Fh-IZI test facility. The histological scoring was done by an external pathologist, who was blinded by using recognized systems. Thi was equally done for the cartilage biopsies taken from the femoral condyles at the first and second surgery (Score E1). Photo documentation followed of all stained slides as be seen in **Figure 8** and **Figure 9**. All observational data were documented by the study personnel and stored at the Fh-IZI test facility. The last step was to decode the mostly blinded data what was done by the study director and statistician so that it was possible to perform statistical analysis with determination of significance (see 4.2). The O'Driscoll score was done with the Safranin-O staining and

checked with HE staining, whereby ten to twenty sections (each five µm thick) were cut at regular intervals from each block of tissue so that all areas of the graft could be examined and any error due to inadequate sampling could be minimized. Odd-numbered sections were stained with HE, and even numbered sections were stained with Safranin-O to detect GAGs in the matrix“ [89]. The points of all subcategories have to be summarized whereby the maximal result of 24 points shows an optimal cartilage regeneration and 0 points show no regeneration. (Appendix I, see chapter 8)

The ICRS Visual Histological Assessment Scale is done for two HE samples and furthermore two special staining as Safranin-O-Lightgreen-stain. In contrast to O'Driscoll the subcategories of ICRS I are not summarized but rather individually evaluated (Appendix I, see chapter 8). Mainil-Varlet *et al.* [68] described below:

"In order to facilitate comparisons of biopsy material collected at various centers, the ICRS Histological Endpoint Committee believes that it is important to standardize the fixation and staining techniques and to keep them as simple as possible. It is recommended that buffered formalin be used for fixation and that samples be routinely stained with HE (as is usually the case). Special stains should include one specific for proteoglycans and another for collagens (probably in conjunction with visualization under polarized-light conditions). In permanently mounted histological sections, Safranin-O binds to polyanions as an orthochromatic dye but without the development of metachromasia. Because of these properties, the staining intensity should correlate positively with the fixed-charge density in the cartilage matrix. The quantification of proteoglycans with use of Safranin-O requires a reproducible system."

Toluidine blue is also used as a proteoglycan stain in many laboratories, although its stoichiometric relationship is probably inferior to that of Safranin-O. Alcian blue is also frequently employed, but its staining pattern varies with pH and the concentration of the salt in the dye. Hence, the result is somewhat unpredictable. The ICRS Histological Endpoint Committee does not wish to recommend a particular proteoglycan stain as there is no clear evidence in the literature as to which is the optimal choice."

The Score E3 (ICRS II Score) is done with the same staining as the ICRS I and can be seen in detail in Appendix I (see chapter 8). Mainil-Varlet *et al.* [69] described it this way.

"The cartilage specimens were fixed in 4% formaldehyde before embedding in paraffin. Specimens were considered for assessment only if they could be oriented (i.e., the surface was identifiable) and included the subchondral bone plate. Sections were cut at up to 5-mm thickness and stained with HE, to evaluate general morphology, and Safranin-O or toluidine blue, to indicate proteoglycan content. Polarized light microscopy was used to assess collagen organization within the tissue. Cartilage sections were initially scored using the MODS and the ICRS I grading systems. Tissue assessment using the MODS grading system required scoring according to categorical numerical subscales, with respect to tissue morphology, matrix staining (with Safranin-O), structural integrity, chondrocyte clustering, formation of a tidemark, subchondral bone formation, architecture of the surface, extent of defect filling, lateral and basal integration, and inflammation."

It is self-explanatory that the individual items cannot be summarized and have to be seen individually.

4.7.11 Ultrasound biomicroscopy

Components

The portable ultrasound microscope (UBM) is composed of a computer with A/D-card (Gage CS14200, Gage, USA), a 2D scanner unit containing the ultrasound transducer (NIH40), a Pulser-Receiver (DPR500, JSR, USA) and a water tank with a temperature controller.

The samples, which were turned with the defect pointing upwards, were placed inside a beaker filled with 1 l of preheated PBS (phosphate buffered saline), before being placed inside the temperature controlled (measured with a digital thermometer) water tank. By positioning the scanner above the sample and aligning the scan area, it was provided that the defect area was included and the transducer-surface distance fit to the focus position of the transducer (~ 9mm). To avoid mistakes, the alignment was checked with cross-sectional b-mode scans and was followed by a C-scan with 24.4µm and 34.7µm scan increment in x- and y-direction, respectively. The received digitized pulse-echoes were saved for later processing. In the end, an area of 13.6 mm and 13.1 mm in x- and y-direction was scanned.

Signal processing

The evaluation of the pulse-echoes was done with custom-made software in MATLAB (MATLAB 2009b). The summation of each envelope curve created an overview image for each pulse-echo (Backscattered Amplitude Integral – BAI). Two steps were necessary to locate the surface position; first, for each pulse-echo signal, a threshold-based detection was performed on the envelope curves, providing a simple approximation of the surface location dth (x,y). In the second step, the surface map dth was smoothed by a custom LOWESS filter (Cleveland1979), modified for use on two-dimensional maps. This resulted in the deletion of outliers and in a surface map that followed smoothly the regions of the first incoming signals denoted as d (x,y). The threshold was set to -47dB and the span for the LOWESS filter to 0.68 mm.

The second part was to detect the cartilage-bone interface that was found by locating the maximum below the surface peak of the envelope curve averaged eight adjacent (in x-direction) pulse-echoes. A fixed speed of sound of 1660m/s was assumed for all samples for the conversion from the time of flight into the distance. Both, the surface map and the cartilage-bone interface map were reduced on <a rough grid with a grid size of 200 µm times 200 µm, to reduce the total number of points per grid to 4355.

Post-processing

Firstly, to fit optimal to the BAI image, the defect region was drawn in by adjusting a 7-mm circle. For further calculations, definitions were made. The inner part of the circle, excluding a fringe of 0.25 mm was defined as defect area whereby the outer part was defined as a healthy area. For optimal results, the actual surface and cartilage-bone interface positions of the rough grid were controlled manually and adjusted if necessary in corresponding b-mode images whereby unclear positions were excluded. A 2D polynomial fit fourth order excluding the third order terms was used to assume the intact surface and cartilage-bone

interface in the defect area to the positions of the healthy area. Some diagrams that were created out of this data can be seen in **Figure 10** with data of animal 43.

For the defect area different volumes were calculated:

- healthy tissue: between the assumed surface and assumed cartilage-bone interface
- neotissue: between assumed cartilage-bone interface and the actual surface
- overfilling tissue: tissue above the assumed surface
- missing tissue: empty volume between actual surface and assumed the surface
- hypertrophic bone: actual cartilage-bone interface above assumed cartilage-bone interface
- cyst volume: actual cartilage-bone interface below assumed cartilage-bone interface
- Additionally, the average, minimum and maximum cartilage thickness were calculated.

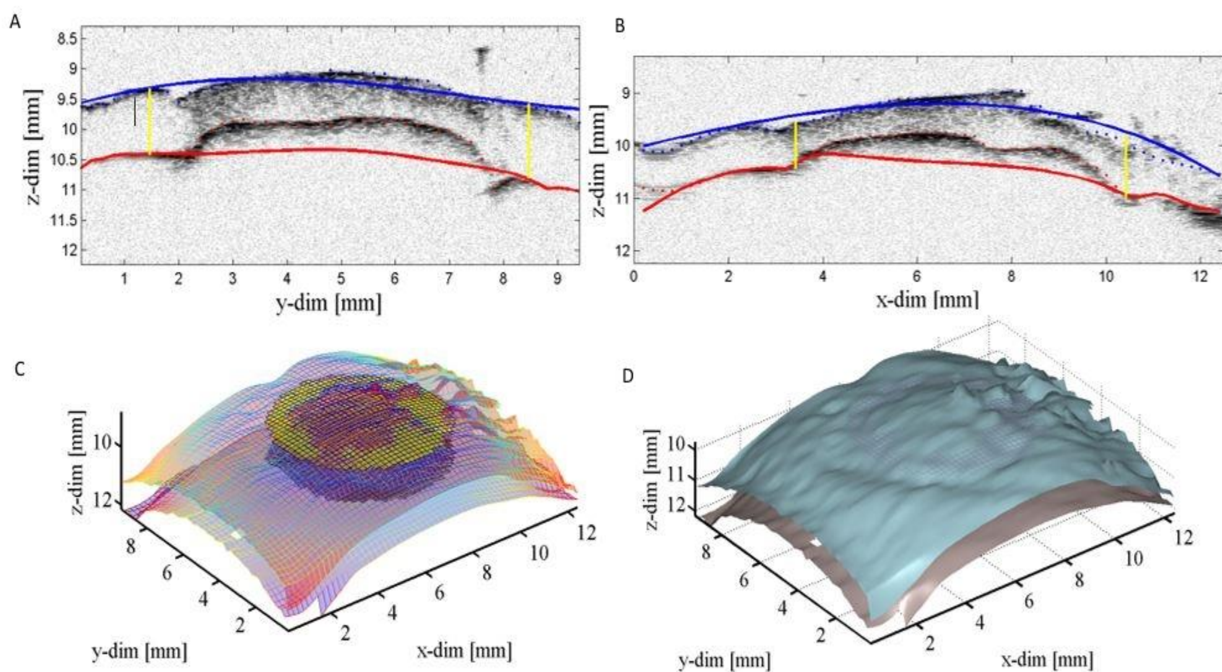


Figure 10: Example figures of cartilage ultrasound evaluation.
A: B-mode cross-section with selected defect border, surface, and subchondral bone interface – y-axis; **B:** B-mode cross-section with selected defect border, surface and subchondral bone interface – x-axis; **C:** Concurrent plot of selected planes and extrapolated model; **D:** cartilage surface plane with extrapolated cartilage surface at lesion area.

4.7.12 Specimen collection procedure

4.7.12.1 First surgery

Blood withdrawal

The biopsy kit that was sent by codon AG consisted of 24 serum monovettes with Z-gel coagulation activator. All of them were filled with whole blood by drawing blood from an anterior limb. This was done by using a tourniquet proximal to the vena cubiti media. The monovette was filled with low negative pressure and softly shaken afterward to prevent the sample from coagulating. To eliminate the possibility of confounding the samples were directly marked with their ETN. With this last step the

documentation was completed, and the monovettes were placed inside the transport box of the co.don AG biopsy.

It should be noted that the reference values described in the literature show significant variations. Therefore, the lowest of the minimal and the highest of the maximal limit values were combined and used from the three reference sources listed below. This decision was based on the fact that the reference ranges (or reference range collections) from universities typically are based on collected data from patients of the veterinary clinic and, therefore, only represent average values. Also, physiological differences can occur without clinical findings.

Our reference ranges were collected from the seventh edition of the book „*Klinische Labordiagnostik in der Tiermedizin*“ [7], the internet website of the Veterinary Faculty of the University of Leipzig [51] and the internet website of the University of Veterinary Medicine Vienna (http://wdk.vu-wien.ac.at, site no longer available online, Reference ranges were printed from the original site).

In general, it should be noted that the sheep were examined regularly monthly and did not show any clinical deviations at any time point during the 9-month study duration. For this reason, no further diagnostics were performed and variations in blood testing were considered coincidental findings. Insight into organ-specific changes can only be gained from pathology testing of the animals. It should be critically noted that initial values were not tested and, as a result, any physiological variations that might be caused by the treatment cannot be assessed, because the decision to take whole blood analysis was set after the beginning of the study.

Logically, several outliers in both directions were found for several blood parameters, but no change in a single parameter could be connected with symptoms of the animals. Due to this and the facts mentioned above it was decided to exclude the results as well as further discussion about the whole blood analysis from this thesis.

<u>Cartilage biopsy</u>

Before the 7 mm x 2 mm cartilage defect was cut out, a ring punch with the same size was used to predefine the proper place inside the MFC. After marking, the native cartilage tissue was removed with a chondro-curette to create an Outerbridge [92] grade IV cartialge lesion while leaving bone intact.

The cartilage tissue taken from each side with a 2 mm ring punch was placed in a separate, marked sterile cryotube, flash frozen in liquid nitrogen and sent to the Fh-IZI test facility.

The leftover of cartilage tissue was taken from both sides and put into an especially marked biopsy puffer tube from the biopsy kit. This tube was placed in the transport box of the co.don AG biopsy kit and all remaining documentation was completed.

4.7.12.2 Specimen collection in the second surgery (implantation)

Retention samples

The transport box of the transplantation kit including the "co.fix" application system from co.don AG included three reaction containers each containing one spheroid in NaCl solution, representing the individual retention samples of the test objects (ACT3D-S) for each experimental animal. After removing the NaCl from the cryotubes, the tubes were marked with an EG number, flash frozen in liquid nitrogen and archived in the gas phase of liquid nitrogen.

cartilage biopsy

After the defect zone of the degenerated repair cartilage tissue was marked with a ring punch in the central portion of the MFC, a chondro-curette was used to remove cartilage within the defect zone leaving bone intact.

The repair cartilage tissue taken from each side was placed into separate, marked, sterile cryotubes, flash frozen in liquid nitrogen and transported to the Fh-IZI test facility.

4.7.12.3 Specimen collection in the third surgery (sacrifice/explantation)

After disarticulation, radiography, and ultrasonography, the former defect regions on both femoral condyles were marked with a 25 mm ring punch, and the distal femurs were placed separately into anonymously marked transport containers with fixation solution within 30 minutes. The explants from the investigational product/intervention (Group A) and the explants for the control intervention (Group B) were sent to an external third-party histology laboratory at the HIK test site for explant histology. This specimen material, including the (non)-embedded explant parts and the anonymized, immunohistochemical and histochemical stained slides, underwent exit documentation and were sent to the Fh-IZI test facility.

After sacrificing and disarticulation, the body was stored at 4°C in a cooling chamber at the MEZ. The body of the euthanized sheep was then transferred by transporter to the CRO VETMED for AA as described in detail above. The animals were identifiable for the pathologists by their ETN.

Furthermore, the obtained specimens of the synovial membrane of both hind limbs were fixed in 4 % formalin and delivered in a 2 ml cryotube to the mentioned CRO for subsequent haematoxylin and eosin staining procedures. During the autopsy procedure, further specimens of the synovial membrane of the shoulder, elbow, and carpal joints of both forelimbs were taken, fixed in formalin, preserved, sliced and stained using hematoxylin and eosin routinely or in a random order depending on the macroscopic diagnosis.

4.7.12.4 Specimen collection during the long-term husbandry after three, six and nine months post-implantation

Blood withdrawal

A total of one serum monovette with the Z-gel coagulation activator and one EDTA monovette provided by the CRO HOSPITAL were filled with whole blood. Blood was drawn using an 18G needle set from the external jugular vein, and the monovettes were thoroughly shaken after adding the blood. Both monovettes were then marked with the consecutive, running ETN of the respective animal, the GLP documentation was completed, and the monovettes were placed into a transport box of the CRO HOSPITAL.

4.7.12.5 Transport of the specimens

Specimen material from the first surgery

Each experimental animal had an individual biopsy kit created by co.don AG. The transport box of the biopsy kit was used for specimen transport (whole blood, cartilage) from the GLP study site MEZ to co.don AG with the TempMark® temperature monitoring system. These collected specimens, which were needed for production of the investigational product/intervention, were transported by courier service at environmental temperatures within 48 hours. At the same time, both 2 mm cartilage biopsies were transported to the Fh-IZI GLP laboratory and stored in a -80°C freezer. The specimens were documented in the specimen receipt log at Fh-IZI.

Specimen material for the second surgery

The applicator with 13 spheroids containing the investigational product/intervention ACT3D-S (Group A) and the three cryotubes with one spheroid each in NaCl solution (retention samples) were transported from co.don AG to the GLP study site MEZ. The temperature was monitored with the TempMark® monitoring system in the transport box of the transplantation kit including the co.fix® application system. The ACT3D-S investigational product/intervention and the retention samples were transported in this transport box per courier service at environmental temperatures within 36 hours.

While the applicator with 13 spheroids was used for implantation as ACT3D-S, the retention samples of the investigational product (ACT3D-S) were transported from the GLP study site MEZ to the GLP laboratory at the Fh-IZI test facility, where they were documented in the receipt log for test and reference objects.

Specimen material from the second surgery

Before implanting the investigational product, the defect was examined for regenerated cartilage tissue. For most of the animals, no such tissue could be found. However, for the remaining animals, who expressed regenerated cartilage tissue, the tissue was removed and transported to the GLP laboratory at Fh-IZI and stored in a -80°C freezer for later use. The specimens were documented in the specimen receipt log at Fh-IZI.

Specimen material from the third surgery

Both femoral condyles of each animal – the femoral condyle treated with the investigational product/intervention and the femoral condyle with the control intervention – were sent to the external HIK site (third-party histology laboratory). The explanted distal femurs were sent in a fixation solution in a specimen container with anonymized markings to HIK at environmental temperatures along with the relevant documentation sheets. Receipt of specimens was documented. The femoral condyles treated with the investigational product/intervention and the control intervention underwent processing for histology, they were documented in an exit log, and all documentation, explant materials and the anonymized histologically-stained specimens on slides were returned to the Fh-IZI test facility. The specimens were documented in the specimen receipt log at Fh-IZI.

The dead bodies of the experimental animals were transported in a bin by a transporter from the MEZ to the CRO VETMED immediately after euthanasia and removal of both hind limbs. Subsequently, the bin was placed in a 4°C cooling chamber until routine AA was performed according to internal SOPs of the CRO. The cadavers were stored in a cold environment until disposal. They were disposed by an animal body disposal company (Zweckverband TBA Lenz, Staudaer Weg 1 in 01561 Priestewitz).

In parallel, the collected and 4 % formalin-fixed synovial membranes of the femoral condyles were transferred from the MEZ to the CRO VETMED immediately after sacrifice. Subsequently, these specimens were preserved, sectioned and stained with hematoxylin and eosin according to internal SOPs of the CRO. All specimens of the synovial membrane of the front and hind limbs will be stored at the CRO VETMED for two years.

The autopsy report was then reported to the GLP test facility Fh-IZI and the reports were attached to the form of the respective GLP documentation.

Specimen material from the long-term husbandry after three, six and nine months post-implantation

The marked monovettes were placed into the transport box supplied by CRO HOSPITAL. This transport box was used for specimen transport (whole blood) by transporter from the GLP study site LVG to CRO HOSPITAL. Subsequently, these specimens were analyzed according to internal SOPs of the CRO. All remaining specimens of the serum chemistry and whole blood count were stored at the CRO HOSPITAL for two years.

The diagnostic results were then reported to the GLP test facility Fh-IZI and the reports were attached to the form of the respective GLP documentation of the SOP ACT3D-S-05.

5 Results

5.1 Fulfillment of Inclusion/Exclusion criteria

5.1.1 Inclusion criteria

Ten of the eleven experimental animals fulfilled the three defined inclusion criteria completely on screening examination (4.6). Almost all animals had a weight larger than 50 kg, were clinically healthy and had an intact cartilage structure of the MFC. One sheep (T52) did not fit the criterion of weight > 50kg. T52 only weight 49 kg one the screening date. The discussion with the sponsor resulted in treating and monitoring T52 according to protocol because no replacement animal was available neither from the LVG animal contingent nor from O. Schölz' sheep farm. Later on, at the 15 August 2012, the sponsor decided at a data review meeting to include all data of animal 52 to the PP analysis.

5.1.2 Exclusion criteria

None of the eleven experimental animals were excluded from the study during the screening examination or during the remaining course of the study based on the defined seven criteria (4.6).

5.2 Recorded undesired events

In the cohort of eleven animals, no AEs were noted over the course of the entire active experimental phase based on the AE list (4.3.1). Undesired events such as reduced count of spheroid implantation, bleeding into the defect or joint inflammation were not observed.

5.3 Serious Adverse Events

In the cohort of eleven animals, two SAEs were noted over the course of the entire active experimental phase based on the SAE list (4.3.2).

SAE Animal 21:

The initial SAE report of the eighth of December 2011 stated an SAE diagnosis “results in death” for animal 21 and the investigator causality to the ACT3D-S treatment modality was reported as “no relationship”. There was no clear coincidence between the death of animal 21 to the administration of investigational product ACT3D-S. At this time point, the possible cause of the event “death” was stated as “while intubation too much aspiration of rumen juice because of unexpectedly long vomiting” by the principal investigator. After the standard premedication animal 21 was brought into the operating room. In preparation for the intubation Narcofol was injected. Directly after this, animal 21 began to vomit a lot whereby this inhibited the further intubation. Due to the vomit, the epiglottis was not visible and three more attempts followed until the fifth intubation succeeded. Unfortunately, the oxygen saturation sank to 35% during the failing attempts of intubation. Also with artificial respiration and increased inspiratory parameters the oxygen saturation never raised above 75% during the operation. Additional injections of Ursotamin (200 mg) and Narcofol (4 ml, 6 ml, 8 ml, 6 ml = 24 ml = 240 mg) followed to suppress the

spontaneous breathing totally. Also with a sufficient dosage of the muscle relaxant, it was impossible to completely suppress the spontaneous breathing, while spontaneous breathing on its own was insufficient. Aspirated rumen juice was suspected to decrease the pulmonary function. The implantation of the investigational product was done without complication.

Because of this large amount of narcotic medicaments, it took a lot longer for animal 21 to awake in comparison to the other animals. For the first half an hour back in the stable, the animal was unable to do anything besides breathing. After one hour it was able to eat slowly, to stand and to toddle a bit. It neither showed fear nor interest in its environment including humans, what actually would have been a normal reaction, also directly after an operation. Starting in the stable, animal 21 showed wheezing and hard breathing. At the end of 07.12.2011 the respiration remained constant bad, the reaction to the environment was still slow and lethargic, and the mucosa was cyanotic. In the morning of 08.12.2011, around 6:45 am, the animal was found dead in the stable. An external pathological examination by the Institute of Pathology, Faculty of Veterinary Medicine, University of Leipzig (VETMED) was directly initiated by the principal investigator after the hind limbs were extracted to examine the area of operation. The dissection report of animal 21 accompanied by the negative TSE- Screening finding was transmitted from the CRO VETMED to the principal investigator at the 15th of December 2011. Based on the pathological-anatomical and histopathological examination of the animal carcass, this SAE was probably caused by an agonal aspiration in the lung. Thus, the SAE Follow-up report for animal 21 of the 15th of December 2011 stated the diagnosis "agonal aspiration in the lung" as a possible cause for the event "death". The reported investigator causality to the ACT3D-S treatment modality was "no relationship" as there was no clear coincidence of the death of the animal to the administration of investigational product ACT3D-S.

In summary, the induction of anesthesia of animal 21 within the implantation surgery caused vomiting and aspiration of rumen contents. The entire pre-, peri- and post-operative procedures including the implantation of the investigational product ACT3D-S into the left stifle was accompanied with problematic anesthesia with an oxygen level never higher than 75%. Animal 21 was not able to recover from aspiration and the following hypoxemia, and died as a result of it during the following night.

SAE of animal 44

The implantation of the investigational product was done the 21.12.2011. The procedure went without complication. During the first three post-operative days, no difference between animal 44 and the rest was seen. The systematic pain score of day one was left three and right four points and was not significantly different from the rest (left ~2.78, right 2.84, $p>0.05$). Also day two with left three and right three (left ~2.78, right ~2.47) and day three with left three and right one (left ~2.44 and right ~ 2.03, $p>0.05$) showed no significant difference. This changed at the fourth post-operative day, when animal 44 showed acute severe lameness in the left and an acute mild lameness in the right hind limb, directly after the prospectively planned i.m. injection of Finadyne and Veracin resulting in a systematic pain score of left seven and right six points. Some minutes after this i.m. injection the animal was not interested any

more in its surrounding, showed a reduced appetite, had an increased respiratory rate (50% above normal) and showed a significant and increased pain in both hind limbs (**Figure 11**). Because of this, animal 44 received another injection of Finadyne and another examination was planned for 27.12.2011. During the next day, animal 44 received more injections of Fentanyl i.m (2.5 ml), Flunixin i.m (2.9 ml) and Veracin i.m (4.5 ml). The medicaments showed almost no effect, and the condition of animal 44 was stagnant. Animal 44 was laying on the ground and showed reduced appetite as well as an increased respiratory rate. The following findings were recorded: increased pain level in both hind limbs, hoof relief right, physical burden left just on the top of the hoof, mild lameness, low generation of heat for both sides and increased respiratory rate. At the 28.12.2011 Animal 44 got an injection of 2 ml Metamizole i.v. (Novaminsulfon – ratiopharm 1 g/2 ml). Almost no symptom changed for the better: significantly increased lameness left, severe lameness right, decreased water/food uptake, apart from low heat generation no pathological findings during palpation of the knee. Later after the Metamizole injection, a slight but also just temporary improvement followed. After discussion and consultation with the veterinarian, the animal was brought to the Veterinärmedizin Leipzig to continue the treatment and pain therapy. The following medicaments were given:

Date	Medication
29.12.11:	• 2 ml Metamizole i.v.
	• 3,5 ml Veracin i.m.
30.12.11:	• 4 ml Metamizole i.v. in the morning and afternoon, in the evening 8 ml Metamizol i.v.
	• 1,8 ml Finadyne i.v.
31.12.11:	• 8 ml Metamizol i.v. in the morning
	• 1,8 ml Finadyne i.v. in the evening
	• 3,5 ml Veracin i.m.
	• 200 ml Sterofundin G-5 Infusion
01.01.12	• 8 ml Metamizol i.v. in the morning and evening
	• 1,8 ml Finadyne i.v. in the evening
02.01.12	• 8 ml Metamizol i.v. in the morning
	• 0,4 ml Butorphanol i.v. (every 3-4 h)
03.01.12	• 0,4 ml Butorphanol i.v. (every 3-4 h)
	• 1,8 ml Finadyne i.v. in the midday
	• 8 ml Metamizol i.v. in the afternoon and evening
04.01.12	• 0,4 ml Butorphanol i.v. in the morning
	• 8 ml Metamizol i.v. in the morning

However, no improvement was found at no moment of therapy. After seven days of systemic pain medication at the “Chirurgische Tierklinik” of the Faculty of Veterinary Medicine, University of Leipzig, it was decided to euthanize animal 44. A discussion and consultation with the veterinarian and the PI resulted in the opinion, that after the slow but constant decreasing condition of animal 44, there was no therapy left. Animal 44 was barely able to move, nearly unable to stand, showed reduced water/food uptake and showed almost no fear nor interest in its environment. At fourth of January 2018 12am, the preparations for the euthanasia began. The animal was calmed down, and the responsible veterinarian joined the box. At 12:22 am the veterinarian injected 2,4 ml Ursotamin (250 mg) and 0,4 ml Xylazine (7.3 mg) for narcosis and anaesthesia. Three minutes later the injection of 8 ml T61 followed so that after further three minutes the time of death could be determined. An external pathological examination by the VETMED was initiated by the principal investigator. All this was reported by the principal investigator in the SAE Follow-up report of the fourth January 2012. This SAE report stated a SAE diagnosis “results in death” and the investigator causality to the ACT3D-S treatment modality was reported as “no relationship”. The cause of the death was reported as “still unknown,”. At this moment, there was no precise coincidence between the death of animal 44 and the investigational product ACT3D-S.

In the “Chirurgische Tierklinik” the veterinarians and the principal investigator decided to do a pathological-anatomical and histopathological examination of the animal carcass at the CROVETMED. They also added a bacterial and mycological examination of synovial fluid samples of both hind limbs to prove or exclude any infection. This was done in the “Institut für Bakteriologie und Mykologie” of the Faculty of Veterinary Medicine, University of Leipzig at the 04th January 2012. Both reports, of the fourth and sixth January 2012, reported a “high-grade staphylococcus aureus” in the synovial fluid samples for both hind limbs of animal 44.

At the 13th of January 2012, the dissection reported was sent from the CRO VETMED to the principal investigator and was added by the negative TSE-Screening. Through a pathological-anatomical and histopathological examination of the animal, carcass could be proved, that both hind limbs were infected by a chronic-active myositis. This could be caused by the frequently post-operative i.m. injections of analgesia and antibiotics. The lameness of both hind limbs can therefore be probably explained in the combination of the periathritis and the fibrinogen, partly necrotizing synovitis that was found as well during the analysis.

Additionally, in both synovial samples of animal 44 high-graded staphylococcus aureus was found. The path of infection, as well as the reason why only this one sheep was infected, stays unknown. The most probably possibilities are, that during the operation, the pathogen was brought into the knee. The other possibility is, that the pathogen was brought into the knee through the various i.m. injections after the operation. The fact, that the first three post-operative days animal 44 showed not significant difference to the other animals and that animal 44 showed an increased lameness directly after the i.m. injection at the fourth post-operative day, rather speaks for the second way. One the one hand this leads to the opinion that this option is more probable, but on the other hand, an infection would not show increased pain directly

after an injection. Another possibility is, that the needle of the injection injured a small nerve and so directly caused the pain and therefore the increased lameness. This would mean the injection and the infection would be chronological but not causally associated. In conclusion, it is still impossible to find the path of infection. It seems clearly, that the surgery itself caused the SAE "death" of animal 44. But the investigational product itself can be excluded as potential cause for the death of animal 44 because in both hind limbs, high-graded staphylococcus aureus was found, both hinds showed mild to severe lameness and only one side received the treatment. So it seems the whole procedure of a two-armed surgery, all peri- and post-operative procedure and the proved infection together caused the death of animal 44.
Based on the definitions of the analysis populations given in detail in the SAP and the Statistics chapter (4.2) both animals were part of the Intention-to-treat (ITT) analysis population. Further, based on the prospective definition "SAF = ITT population" the respective descriptive statistics on SAF were performed on eleven animals from cohort II.

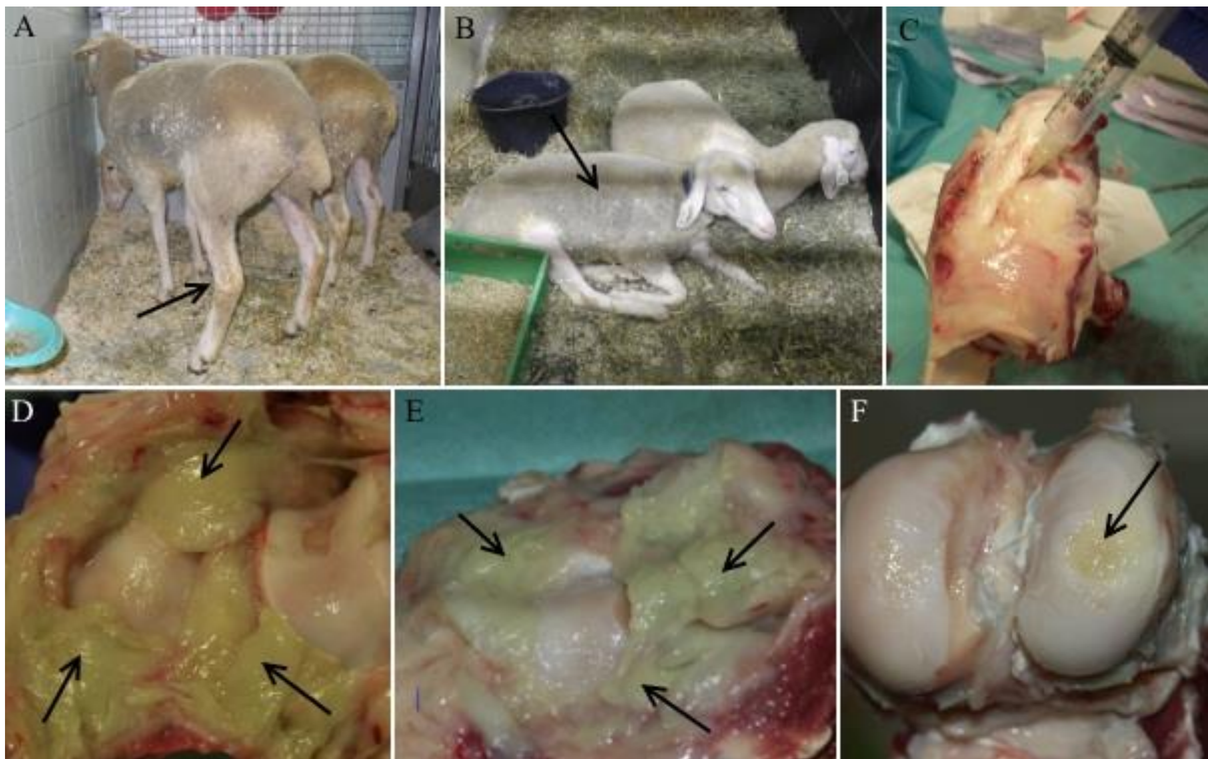


Figure 11: Photos of animal 44 in the stable of the Faculty of Veterinary medicine and the explanted knee joint.
A: Animal 44 at fourth post-operative day, 1 hour after the first i.m. injection that day. It can be seen, that the animal rather loads its front limbs and furthermore has its hind limbs(arrow) more bent than the animal in the back.; **B:** Animal 44 (arrow) inside the stable of "Chirurgische Tierklinik" of the Faculty of Veterinary Medicine, University of Leipzig with another animal due to the fact, that sheep are herd animals.; **C:** Gain of synovial fluid for bacterial and mycological examination. The color of this synovial fluid was a mix of moss and lime green, similar to the tissue seen in D and F.; **D** and **E:** The opened femoral condyles of the left (D) and right (E) leg. The inside (arrows) of the joint capsule is clearly colored lime green and moss. This was the first visible sign of a bacterial infection. **F:** Right femoral condyle after preparation. No visible sign of remaining spheroids (arrow) but similar coloring of the defect area as the synovial fluid and the joint capsule tissue.

5.4 Adverse Drug Reaction and Unexpected Adverse Event

Neither an ADR (4.4) nor an unexpected AE (4.5) was noted for any of the eleven experimental animals during the entire experimental phase.

5.5 Results of primary and secondary objectives and endpoints relating to the efficacy of ACT3D-S

To avoid false results the three histological assessments of the cartilage-specific scoring-systems E1, E2 and E3 were performed by Prof. Dr. Thomas Aigner himself. The assessment was done in a blinded fashion method based on HE-stained 5µm slices and Safranin-O-lightgreen stained 5 µm slices as well as 50 µm sections of one half femoral condyle. All slices were taken of one half femoral condyle as seen in **Figure 12**.

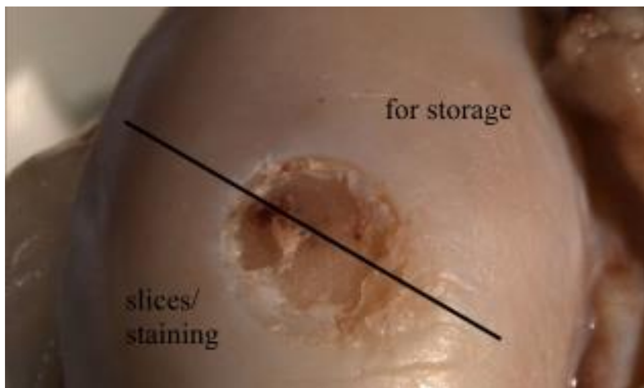


Figure 12: Cutting proposal for the femoral condyle of animal 21.
One half is kept for storage, and the other half is used for slices and staining.

A lot of raw data was unable to be obtained by Prof. Dr. Thomas Aigner because some observed histopathological characteristics were inapplicable for a constructive and useful evaluation according to single items or subcategories. In consequence, the sample size was reduced in both, the descriptive statistics and the performed statistical tests between both treatment modalities. Therefore, the particular sample size is indicated in the subsequent three chapters of the cartilage-specific scoring systems E1, E2, and E3.
These above mentioned histological staining methods allowed us to exclude the prospectively planned immunohistochemical staining (of aggrecan and collagen type 1 and 2) because the received scores were reproducible and almost no cartilage tissue was found.

5.5.1 Results of Score E1 - O'Driscoll score

The prospectively planned additional analysis no. 13 (as stated in section 4.2.1) for identification and consideration of potential differences in the initial condition of the native cartilage between both knee joints (between both treatment groups as a covariate) could not be performed as planned. It was impossible to isolate cartilaginous repair tissue out of the former lesion site in the majority of the knees at the time point V0/II. Consequently, measurements of E1 - O'Driscoll score were not possible and the planned analysis in

the statistical report on the so-called "baseline influence" was not possible. Thus, the following two subchapters report the respective analyses (ITT and PP) based on the absolute scores measured from samples of the cartilage repair tissue at sacrifice, the third surgery (V4/III). The E1 - O'Driscoll-Score has a total maximum score of 24 points and is divided into four categories with nine items. As mentioned above, a detailed list of all subcategories can be seen in Appendix I (see chapter 8). The E1 - O'Driscoll-Score is calculated by adding all these nine items of the four categories and was calculated at the time point V4/III (**Table 3**). The ITT analysis is only given for the overall E1 - score, whereas the PP analyses are given for the overall E1 – scores, and also for the four categories and the nine items.

5.5.2 Results of Score E1 - O'Driscoll score (Intention-to-Treat analysis)

Based on the Statistics definitions given in detail in the SAP and in the Statistics chapter (4.2.1and 4.2.3). All 15 secondary objectives – including the secondary objective no. 1 to "assess difference of Score E1 - O'Driscoll of ACT3D-S (Group A) and untreated control defect (Group B) nine month after the end of the respective treatment" – need to fulfill the statistical specifications.
Only for the PP population statistical tests were performed. Results of the ITT analysis population on E1 score were not presented.
However, no relevant statistical differences could be found for all efficacy parameters between the ITT and PP analyses populations because no efficacy related data could be collected for the two animals that were excluded from the study.

5.5.3 Results of Score E1 - O'Driscoll score (Per Protocol analysis)

The PP analysis was performed including nine animals from the 9-month animal cohort. As planned, all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis. The spaghetti plot, box plot and error bars showing ploratorythe E1 - score can be found in **Figure 13** below.

- The E1 - score (PP) showed no difference between the control and AT. The average and the range values were equal in both treatments.
- The E1 - score (PP) was calculated to the time point V4/III, based on nine animals from the 9-month animal cohort. The mean ± SD was 17.5 ± 1.7 (n=4) for Group A and 17.8 ± 2.6 (n=6) for Group B. There were no significant paired differences between the groups (n=3), with a p-value of 0.383.

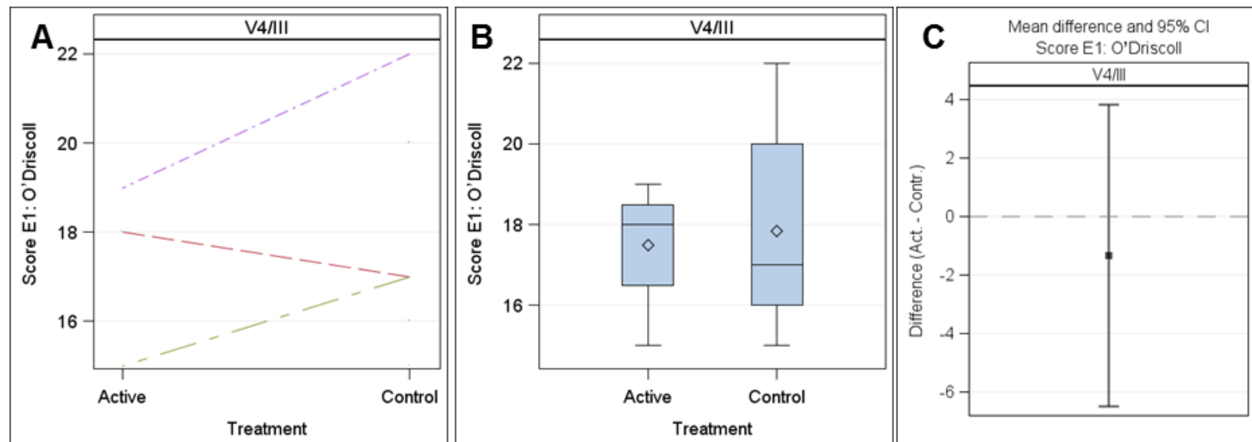


Figure 13: E1 - O'Driscoll score at V4/III, at sacrifice for control and treatment groups (PP).
(**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar. The spaghetti plot displays the E1 - score [theoretical range: 0-24] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E1 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E1 - score [theoretical range: 0-24] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E1 - score as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence interval (CI)s for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

5.5.4 Results of Score E2 - ICRS-I score

The E2 – ICRS-I – score, which was calculated at the time point V4/III (**Table 3**), has a maximum score of 18 points. As mentioned above, a detailed list of all subcategories can be seen in Appendix I (see chapter 8). The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis. The relative frequency of the six categories is graphically shown **Figure 14**.

A summary of the different E2 – ICRS-I – score values at the respective time points is listed below, comparing treatment group ("The investigational product/intervention ACT3D-S") and control group ("untreated defect as a control intervention"). The different values are indicated, including SD and statistical significance.

- The six categories of the E2 – showed no difference between the control and AT. The average and the range values were equal in both treatments.
- In the category "Surface" the Wilcoxon signed rank test result (S) was -1.50. There were no significant differences between the groups (n=9), with a p-value of 0.500.
- In the category "matrix" the Wilcoxon signed rank test result (S) was -3.00. There were no significant differences between the groups (n=9), with a p-value of 0.625.

- In the category “Cell distribution“ the Wilcoxon signed rank test result (S) was -2.50. There were no significant differences between the groups (n=9), with a p-value of 0.625.
- In the category “Cell population viability“ the Wilcoxon signed rank test was not applicable, since all groups were predominantly viable.
- In the category “Subchondral bone“ the Wilcoxon signed rank test result (S) was -0.50. There were no significant differences between the groups (n=9), with a p-value of 1.000.
- In the category “cartilage mineralization“ the Wilcoxon signed rank test was not applicable, since all groups were predominantly normal.

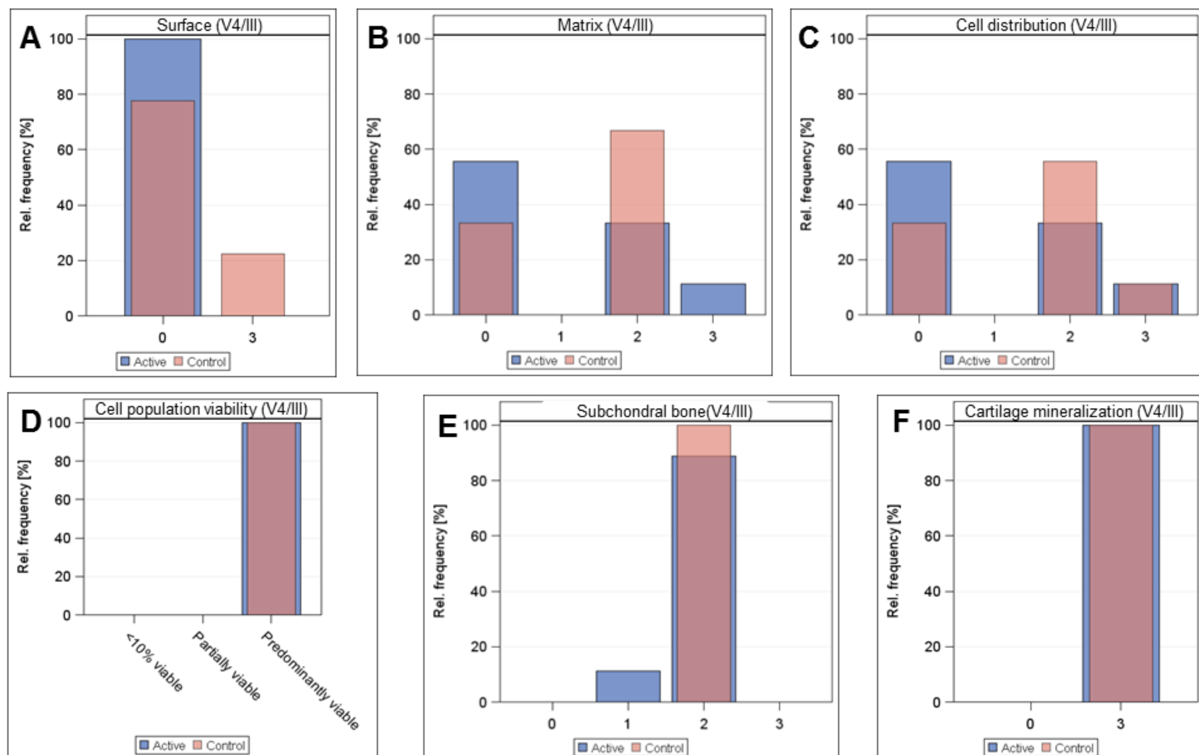


Figure 14: Frequency of E2 – ICRS-I – categories at V4/III, at sacrifice for control and treatment groups (PP). Bar Chart of the categories (**A**) “Surface“, (**B**) “matrix”, (**C**) “Cell distribution”, (**D**) “Cell population viability”, (**E**) “Subchondral bone” and (**F**) “cartilage mineralization (calcified cartilage)”. The bar chart displays the relative frequencies [%] of E2 – ICRS-I – categories [theoretical range: 0-100] assessments as determined at sacrifice (time point of explantation). The sum of all displayed categories for each treatment is 100. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery. The informative sum - score E2 was calculated at the time point V4/III based on nine animals from the 9-month animal cohort.

- The informative sum of score E2 did not show any difference between the control and AT. The average and the range values were similar in both treatments.
- In the informative sum - score E2 the Wilcoxon signed rank test result (S) was -1.50. There were no significant paired differences between the groups (n=3), with a p-value of 0.500.

The spaghetti plot, box plot and error bars shown for the informative sum score E2 can be found in **Figure 15**.

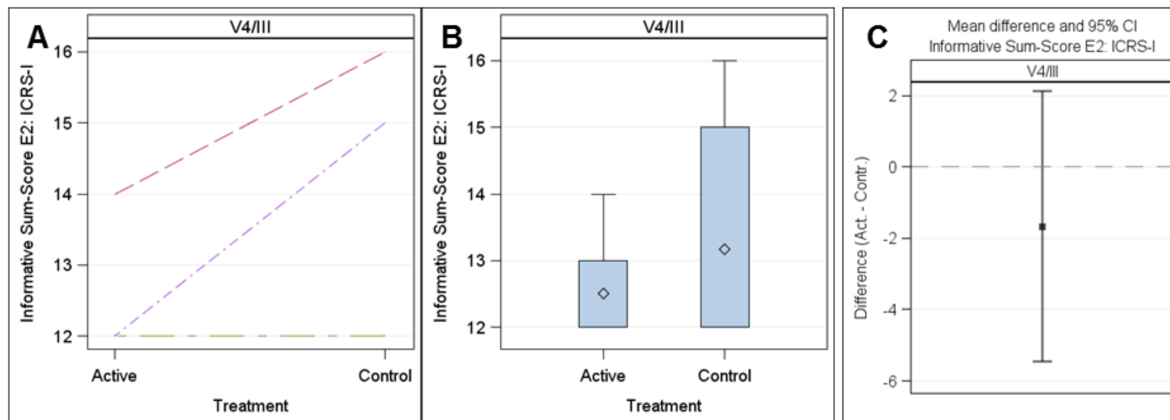


Figure 15: Informative sum score E2 at V4/III, at sacrifice for control and treatment groups (PP).(**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar.The spaghetti plot displays the informative Sum-Score E2 [theoretical range: 0-18] distribution as determined atsacrifice (time point of explantation). The lines represent a direct comparison of the informative Sum-Score E2 within one animal between control (untreated) and active (treated with ACT3D-S) treatment.The box plot displays the informative Sum-Score E2 [theoretical range: 0-18] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR (the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots.The error bar plot displays the mean paired difference between treatments (Active – Control) for the informative Sum-Score E2 as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

5.5.5 Results of Score E3 - ICRS II score

The E3 – score, that was calculated at the time point V4/III (**Table 3**) has a total mean maximum score of 100 points (total mean of all categories). As mentioned above, a detailed list of all subcategories can be seen in Appendix I (see chapter 8). The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

A summary of the different E3 – ICRS-II– score values at the respective time points is listed below, comparing treatment group (“The investigational product/intervention ACT3D-S”) and control group (“untreated defect as a control intervention”). The different values are indicated, including SD and statistical significance.

- The 14 categories of the E3 - score did not show any difference between the control and AT. The average and the range values were similar in both treatments.
- In the category “Tissue morphology (viewed under polarized light)” the mean ± SD was 36.7 ± 37.7 (n=9) for Group A and 56.3 ± 36.2 (n=8) for Group B. There were no significant differences between the groups (n=8), with a p-value of 0.345.
- In the category “matrix staining (metachromasia)” the mean ± SD was 41.1 ± 40.1 (n=9) for Group A and 65.0 ± 37.8 (n=8) for Group B. There were no significant differences between the groups (n=8), with a p-value of 0.308.

- In the category "Cell morphology" the mean ± SD was 47.8 ± 43.2 (n=9) for Group A and 70.0 ± 40.6 (n=8) for Group B. There were no significant differences between the groups (n=8), with a p-value of 0.397.
- In the category "Chondrocyte clustering (four or more grouped cells)" the mean ± SD was 87.5 ± 12.6 (n=4) for Group A and 96.7 ± 5.2 (n=6) for Group B. There were no significant differences between the groups (n=3), with a p-value of 0.225.
- In the category "Surface architecture" the mean ± SD was 67.5 ± 12.6 (n=4) for Group A and 81.7 ± 11.7 (n=6) for Group B. There were no significant differences between the groups (n=3), with a p-value of 0.074.
- In the category "Basal integration" the mean ± SD was 100.0 ± 0.0 (n=4) for Group A and 98.3 ± 4.1 (n=6) for Group B. There were no significant differences between the groups (n=3). Due to a SD of 0.0 the conduction of the T-test was not applicable.
- In the category "Formation of a tidemark" the mean ± SD was 80.0 ± 0.0 (n=4) for Group A and 86.7 ± 5.2 (n=6) for Group B. There were no significant differences between the groups (n=3). Due to a SD of 0.0 the conduction of the T-test was not applicable.
- In the category "Subchondral bone abnormalities/marrow fibrosis" the mean ± SD was 77.8 ± 19.2 (n=9) for Group A and 85.6 ± 7.3 (n=9) for Group B. There were no significant differences between the groups (n=9), with a p-value of 0.313.
- In the category "Inflammation" the mean ± SD was 100.0 ± 0.0 (n=9) for Group A and 100.0 ± 0.0 (n=9) for Group B. There were no significant differences between the groups (n=9). Due to a SD of 0.0 the conduction of the T-test was not applicable.
- In the category "Abnormal calcification/ossification" the mean ± SD was 100.0 ± 0.0 (n=4) for Group A and 100.0 ± 0.0 (n=6) for Group B. There were no significant differences between the groups (n=3). Due to a SD of 0.0 the conduction of the T-test was not applicable.
- In the category "Vascularization (within the repaired tissue)" the mean ± SD was 90.0 ± 0.0 (n=4) for Group A and 91.7 ± 4.1 (n=6) for Group B. There were no significant differences between the groups (n=3), with a p-value of 0.423.
- In the category "Surface/superficial assessment" the mean ± SD was 67.5 ± 18.9 (n=4) for Group A and 81.7 ± 7.5 (n=6) for Group B. There were no significant differences between the groups (n=3), with a p-value of 0.300.
- In the category "Mid/deep zone assessment" the mean ± SD was 43.3 ± 38.1 (n=9) for Group A and 62.5 ± 39.2 (n=8) for Group B. There were no significant differences between the groups (n=8), with a p-value of 0.416.
- In the category "Overall assessment" the mean ± SD was 37.8 ± 37.0 (n=9) for Group A and 65.0 ± 37.8 (n=8) for Group B. There were no significant differences between the groups (n=8), with a p-value of 0.260.

The total mean of the E3 - score was calculated at the time point V4/III, based on nine animals from the 9-month animal cohort.

- The total mean of the E3 - score did not show any difference between the control and AT. The average and the range values were similar in both treatments.
- The total mean ± SD was 84.5 ± 7.0 (n=4) for Group A and 88.8 ± 5.4 (n=6) for Group B. There were no significant paired differences between the groups (n=3), with a p-value of 0.185.

The spaghetti plot, box plot and error bars shown for the total mean E3 - score (PP) can be found in **Figure 16**.

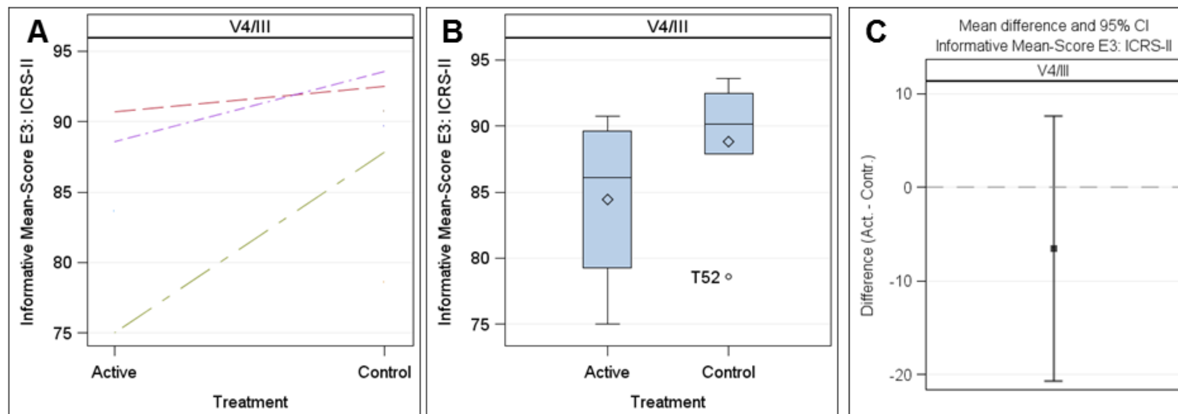


Figure 16: Total mean of the E3 - score at V4/III, at third surgery for control and treatment groups (PP).
(**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar.The spaghetti plot displays the total mean E3 – score [theoretical range: 0-100] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the total mean E3 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment.The box plot displays the total mean E3 – score [theoretical range: 0-100] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots.The error bar plot displays the mean paired difference between treatments (Active – Control) for the total mean E3 – score as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

5.5.6 Results of Score E4 - ICRS-MCAS score

The E4 – ICRS-MCAS-score, that was calculated between the time points V0/II and 4/III - score has a total maximum of twelve points. A detailed list of all subcategories can be seen in Appendix I (see chapter 8). The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis. The E4 – ICRS-MCAS-score did not show any differences between the control and AT. The average and the range values were similar in both treatments.

- The E4 – change score (change by treatment and paired treatment-related differences) (PP) was calculated between the time points V0/II and V4/III based on nine animals from the 9-month animal cohort. The mean ± SD was 6.4 ± 3.6 for Group A and 6.6 ± 4.1 for Group B. There were no significant differences between the groups, with a p-value of 0.927.

The spaghetti plot, box plot and error bars shown for the E4 - change scores (PP) can be found in **Figure 17**.

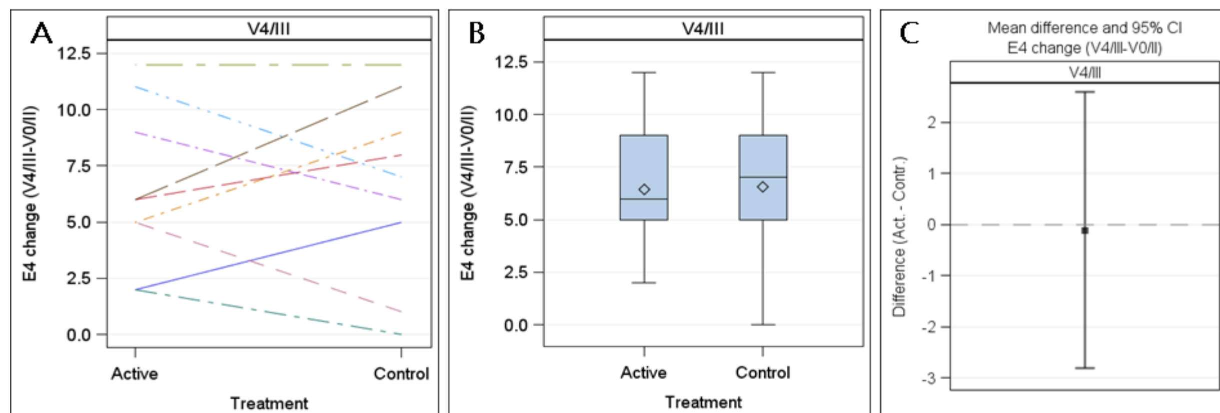


Figure 17: E4 – change score at V4/III-V0/II, at sacrifice minus implantation for control and treatment groups (PP). (**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar. The spaghetti plot displays the E4 change score [theoretical range: 0-12] distribution as determined at the V0/II, second surgery (time point of treatment) and sacrifice (time point of explantation). The lines represent a direct comparison of the individual E4 change scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E4 change score [theoretical range: 0-12] distribution as determined at the V0/II, second surgery (time point of treatment) and sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E4 change score as determined at the V0/II, second surgery (time point of treatment) and sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

5.5.7 Results of E5 Score - modified 2D-MOCART score

The E5-Modified 2D-MOCART – score, that was calculated at the time point V4/III, has a total maximum point score of 75. As mentioned above, a detailed list of all subcategories can be seen in Appendix I (see chapter 8). The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

- The E5 - score did not show any difference between the control and AT. The average and the range values were similar in both treatments.
- The E5 - score (PP) was calculated to the time point V4/III based on nine animals from the 9-month animal cohort. The mean ± SD was 26.1 ± 15.0 for Group A and 31.1 ± 16.5 for Group B. There were no significant differences between the groups, with a p-value of 0.559.

The spaghetti plot, box plot and error bars shown for the E5 - scores (PP) can be found in **Figure 18**.

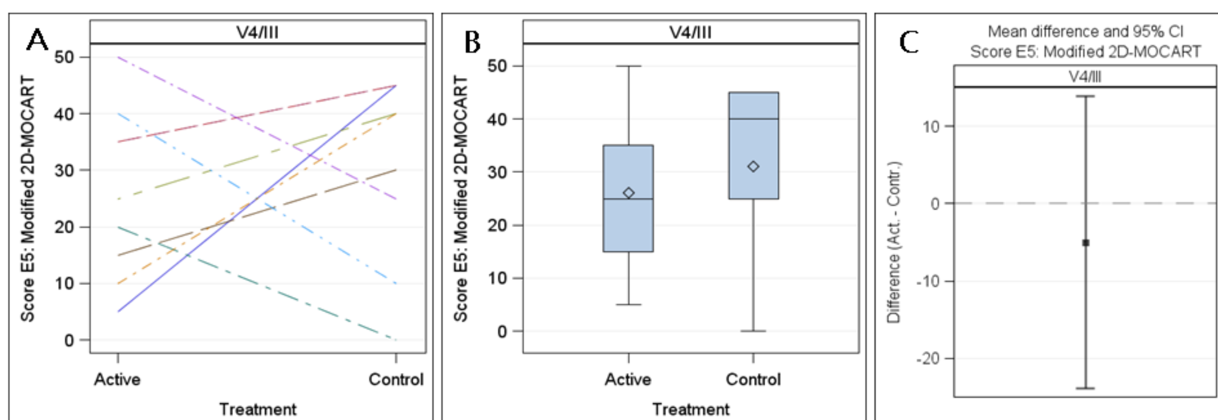


Figure 18: E5 - Modified 2D-MOCART at V4/III, at sacrifice between control and treatment groups (PP). (**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar. The spaghetti plot displays the E5 - score [theoretical range: 0-75] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E5 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E5 - score [theoretical range: 0-75] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E5 - score as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

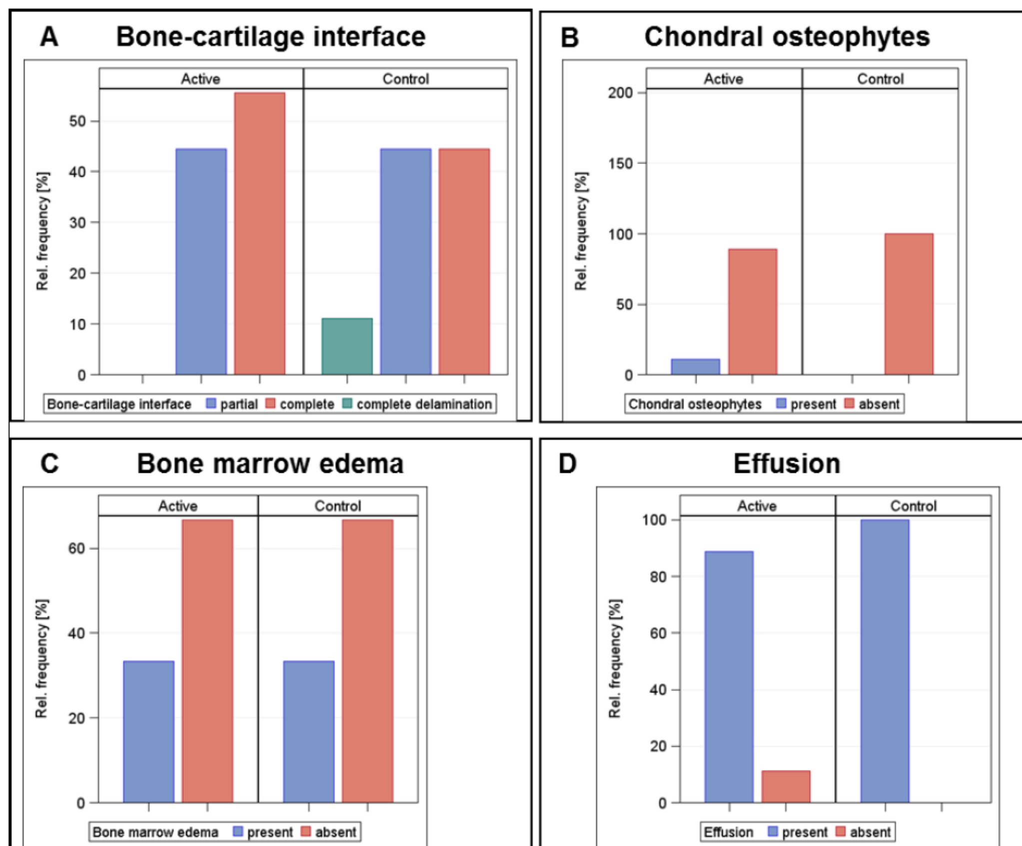


Figure 19: Frequency of additional parameters of the MRI analysis at V4/III, at sacrifice for control and treatment groups (PP).
Bar Chart of the categories (**A**) "Bone-cartilage interface", (**B**) "Chondral osteophytes", (**C**) "Bone marrow edema", (D) "Effusion".The bar chart displays the relative frequencies [%] of the MRI analysis [theoretical range: 0-100 %] assessments as determined at sacrifice (time point of explantation). The sum of all displayed categories for each treatment is 100. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

Additional information from MRI analysis, that was calculated at the time point V4/III (**Table 3**), includes the four parameters "Bone-cartilage interface", "Chondral osteophytes", "Bone marrow edema", and "Effusion". The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

The relative frequency of the four parameters of the additional MRI - diagnosis can be found in **Figure 19**.

- The additional MRI analysis did not show any notable difference between the control and AT. The relative frequencies of the four parameters were similar in both treatments.

5.5.8 Results of ultrasonographic evaluation

The 2-D and 3-D documentation of ultrasound biomicroscopy of the cartilage-bone architecture of the former lesion site was measured at the third surgery. Prospectively there was the plan to add analysis no. 15 (as stated in section 4.2) to show a correlation between the nine quantitative categories of the ultrasound analysis and many other categories or items of the E1 to E5 scores. However, this was not reported due to previous insignificant efficacy findings. The absolute ultrasound analysis (US) includes six volumetric [mm³] and three metric [mm] categories. As mentioned above, a detailed list of all subcategories can be seen in Appendix I (see chapter 8). The spaghetti plot, box plot and error bars shown for nine categories of the ultrasound analysis (PP) are graphically shown in the nine supplementary Figures in the section "Detailed Ultrasonographic Results" of Appendix I (see chapter 8).

The PP analysis was performed based on nine animals from the 9-month animal cohort at the time point V4/III (**Table 3**). The values obtained for animals 21 and 44 were not represented in this, as treatment of these animals was not compliant with the protocol. The whole calculation was based on nine samples of Group A and nine samples of Group B. Parametric and non-parametric testing was performed paired based on the nine animals. Overall seen, the nine categories of the ultrasound analysis did not show any difference between the control and AT. The average and the range values were similar in both treatments.

Retrospectively the items "Healthy average thickness" and the belonging intra-sheep deviation as well as "Neo-tissue formation volume" are sufficient to show the results of the ultrasonographic evaluation.

The analysis of the intra-sheep deviation [%] of the "Healthy tissue volume" [mm³] in relativity to sheep was not prospectively defined, but was added to find possible causes for the not finding an efficacy of the product and can be seen in **Figure 20**.

- The intra-sheep deviation [%] in the category "Healthy tissue volume" was calculated as (relative to mean value) difference between mean value and knee and was found to be 7.80 ± 6.10 (mean ± SD) [%] based on nine pairs of stifle joints measured at V4/III.

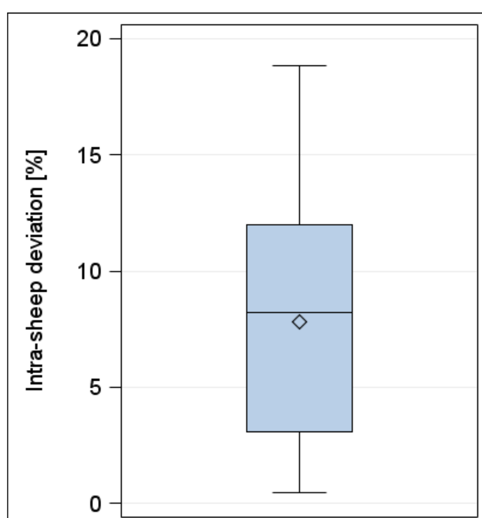


Figure 20: Intra-sheep deviation [%] relative to sheep specific mean value: Healthy tissue volume [mm^3] (PP).

This minor variance between both knee joints, rather said both treatment modalities might be interpreted as that an analogy or more likely a comparable bone-cartilage architecture at the former lesion sites in each of the animals can be found. This leads to the assumption that the found absolute ultrasound analysis measures are adequate for the comparison of the cartilage repair outcome.

The absolute and relative frequency of the category "Healthy average thickness [mm]" show a mean ± SD of 1.0 ± 0.2 for Group A and 1.1 ± 0.3 for Group B. There were no significant differences between the groups, with a p-value of 0.346. The spaghetti plot, box plot and error bars shown for the ultrasound analysis (PP) can be found in **Figure 21.**

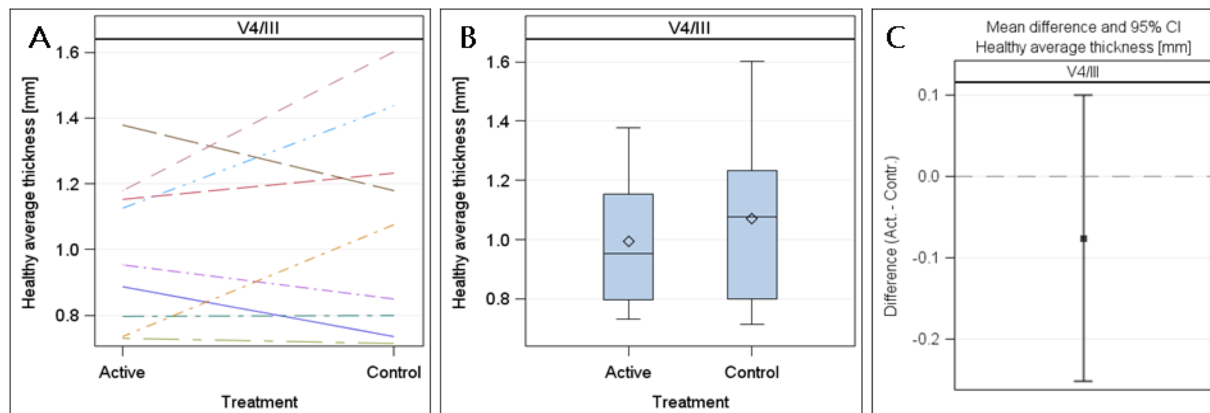


Figure 21: US - Healthy average thickness [mm] at V4/III, at sacrifice for control and treatment groups (PP). (**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar. The spaghetti plot displays the E6 - Healthy average thickness [mm] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Healthy average thickness [mm] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Healthy average thickness as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category "Neo-tissue formation volume [mm³]" show a mean ± SD of 21.7 ± 7.8 for Group A and 25.6 ± 8.8 for Group B. There were no significant differences between the groups, with a p-value of 0.212. The spaghetti plot, box plot and error bars shown for the ultrasound analysis (PP) can be found in **Figure 22**.

To summarize, no superiority could be found for the treatment in comparison with the untreated site. Although the boxplots in **Figure 21** and **Figure 22** show slight visual superiority no significance was found for these items. However, at least the ultrasound analysis measures seem to be quite suitable for the analysis of cartilage repair tissue and outcome.

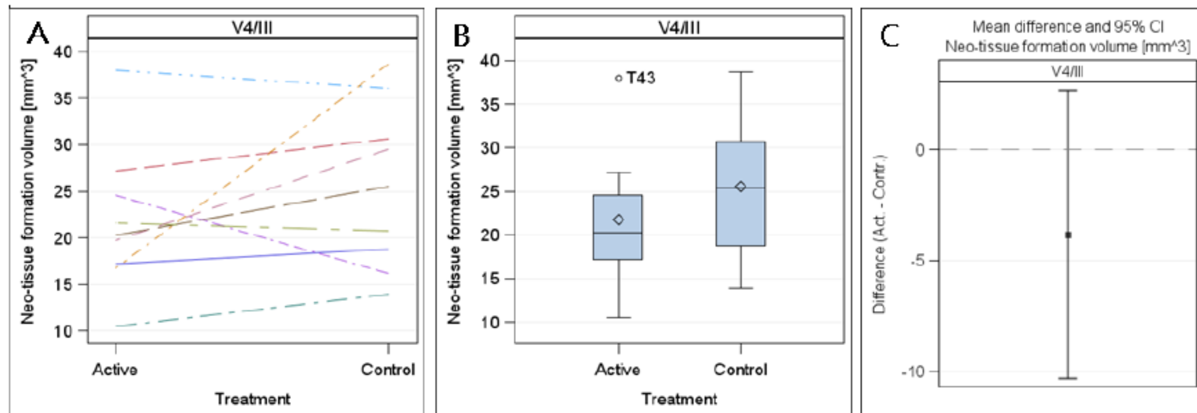


Figure 22: US - Neo-tissue formation volume [mm³] at V4/III, at sacrifice for control and treatment groups (PP).(**A**) Spaghetti plot, (**B**) Box plot, (**C**) Error Bar. The spaghetti plot displays the E6 – Neo-tissue formation volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 – Neo-tissue formation volume [mm³] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 – Neo- tissue formation volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % CI for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

5.5.9 Outerbridge classification

The last additional analysis for efficacy was the determination of the absolute and relative frequency of the Outerbridge classification (ITT), which has a total of four grades and can be seen in detail in Appendix I (see chapter 8). It was calculated at the time points V-2/II, V-2/III, V0/II, V0/III, V4/II (**Table 3**) and can be seen for V4/III in **Figure 23**.

- The Outerbridge classification (ITT) at sacrifice (time point of explantation) did not show strong difference between the control and AT. The relative and absolute frequencies were similar in both treatments.
- In the Outerbridge classification (ITT) the Wilcoxon signed rank test result (S) was 3.00. There were no significant differences between the groups, with a p-value of 0.250.

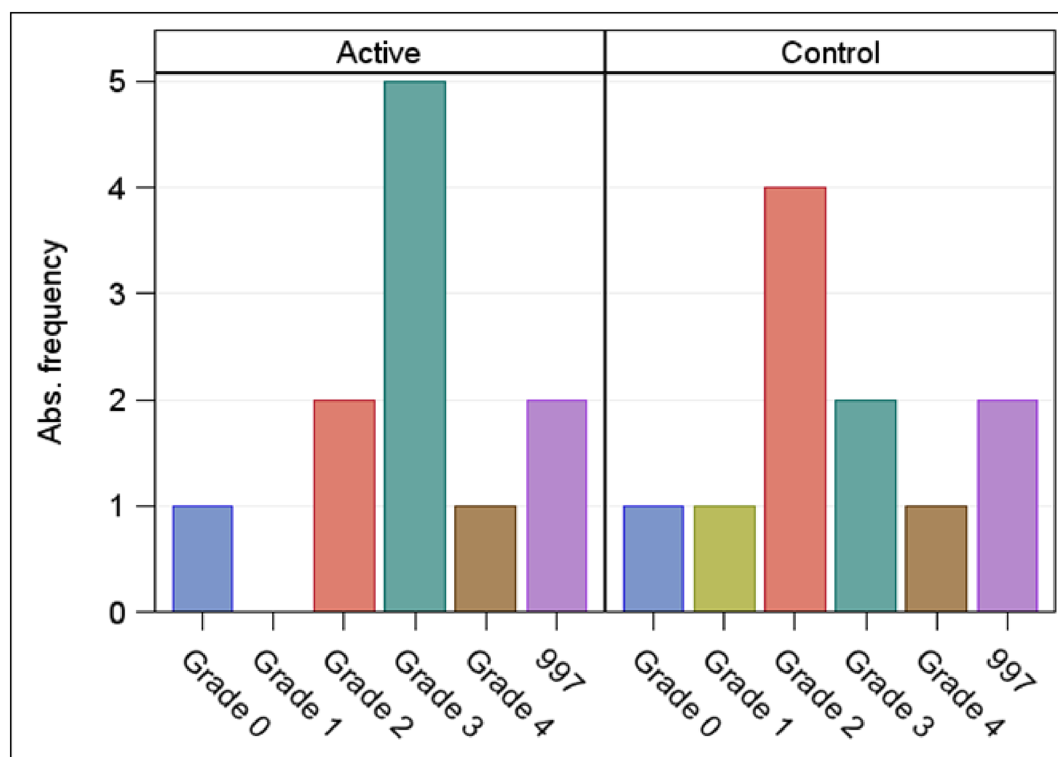


Figure 23: Frequency of Outerbridge classification grades at V4/II, at sacrifice for control and treatment groups (ITT). The bar chart displays the absolute frequencies [%] of the Outerbridge classification [theoretical range: 0-4] assessments as determined at sacrifice (time point of explantation). Missing values of animals 21 and 44 were indicated as 997. The sum of all displayed grades for each treatment is 11. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/II, sacrifice, third surgery.

5.6 Results of secondary objectives and endpoints related to the SAF of ACT3D-S

5.6.1 Results of Score S1- Systematic pain score

The S1 - systematic pain score, which was calculated at the following time points (**Table 3**), has a total maximum point score of 20 points and is divided into the subcategories S1A and S1B. The exact subcategories can be found in Appendix I (see chapter 8). The S1 score is calculated by adding both subcategories, resulting in a maximum score of 20, which would indicate a high pain score. The PP analysis was performed based on nine animals from the 9-month animal cohort. As planned, all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

The S1 systemic pain score of all animals is shown in **Figure 24** as box plots, also indicating the range of observed pain scores between the animals. The S1 systemic pain score at the different time points during the study are indicated below. In **Figure 25** the differences in S1 systemic pain score between the active (treated) group and control (untreated) group are indicated by error bars, with the control group set as 0. In the end, there were no statistical differences observed in the S1 systemic pain scores at the different time points between the treated and control group.

In summary, there were no statistical differences observed in the S1 - systematic pain score at the different time points between the treated and control groups. Apart from outliers, whereby some of them can be seen

in the boxplot of Figure 24, no significance could be found for any time point for the S1 - systematic pain score.

A summary of the different S1 - systematic pain score values at the respective time points is listed below, comparing treatment group ("The investigational product/intervention ACT3D-S") and control group ("untreated defect as a control intervention"). The different values are indicated, including SD and statistical significance.

- The mean ± SD of Group A and mean ± SD of Group B were 1.0 ± 0.0 at the time of inclusion. The pain score at the day of inclusion was, therefore, the same between control and treatment group, and no significant differences were observed between both groups.
- At V-2/I, the mean ± SD of Group A was 1.0 ± 0.0 and 1.0 ± 0.0 for Group B. There were no significant differences between the groups.
- The mean ± SD at V-1a was 2.0 ± 1.1 for Group A and 2.1 ± 1.2 for Group B. There were no significant differences between the groups, with a p-value of 1.000. The outlier with an elevated value in Group A was animal 47 and 52 as well as with a decreased value animal 27 and 28. Outlier in Group B was animal 27 with a decreased value.
- The mean ± SD at V-1b was 2.1 ± 1.1 for Group A and 1.9 ± 1.1 for Group B. There were no significant differences between the groups, with a p-value of 0.500. Although animal 47 was an outlier in Group B with an elevated value.
- The mean ± SD at V-1c was 1.9 ± 0.8 for Group A and 1.9 ± 1.1 for Group B. There were no significant differences between the groups, with a p-value of 1.000. Outlier with an elevated value in Group B was animal 27.
- The mean ± SD at V-1d was 1.1 ± 0.3 for Group A and 1.2 ± 0.4 for Group B. There were no significant differences between the groups, with a p-value of 1.000. Animal 26 was outlier with elevated values in Group A and B and animal 27 just had an elevated value in Group B.
- The mean ± SD at V0/I was 1.4 ± 0.5 for Group A and 1.1 ± 0.6 for Group B. There were no significant differences between the groups, with a p-value of 0.250. Group B had two outliers with elevated values (47, 52) and one with a decreased value (36).
- The mean ± SD at V1a was 2.8 ± 0.7 for Group A and 2.9 ± 0.8 for Group B. There were no significant differences between the groups, with a p-value of 1.000.
- The mean ± SD at V1b was 2.8 ± 0.7 for Group A and 2.6 ± 0.5 for Group B. There were no significant differences between the groups, with a p-value of 0.625.
- The mean ± SD at V1c was 2.2 ± 0.8 for Group A and 2.1 ± 0.6 for Group B. There were no significant differences between the groups, with a p-value of 1.000. Group B had two outliers with elevated values (30, 52) and one with a decreased value (36).
- The mean ± SD at V1d was 2.2 ± 1.0 for Group A and 1.8 ± 1.0 for Group B. There were no significant differences between the groups, with a p-value of 1.000. Group B had trough animal 30 one outlier with an elevated value.

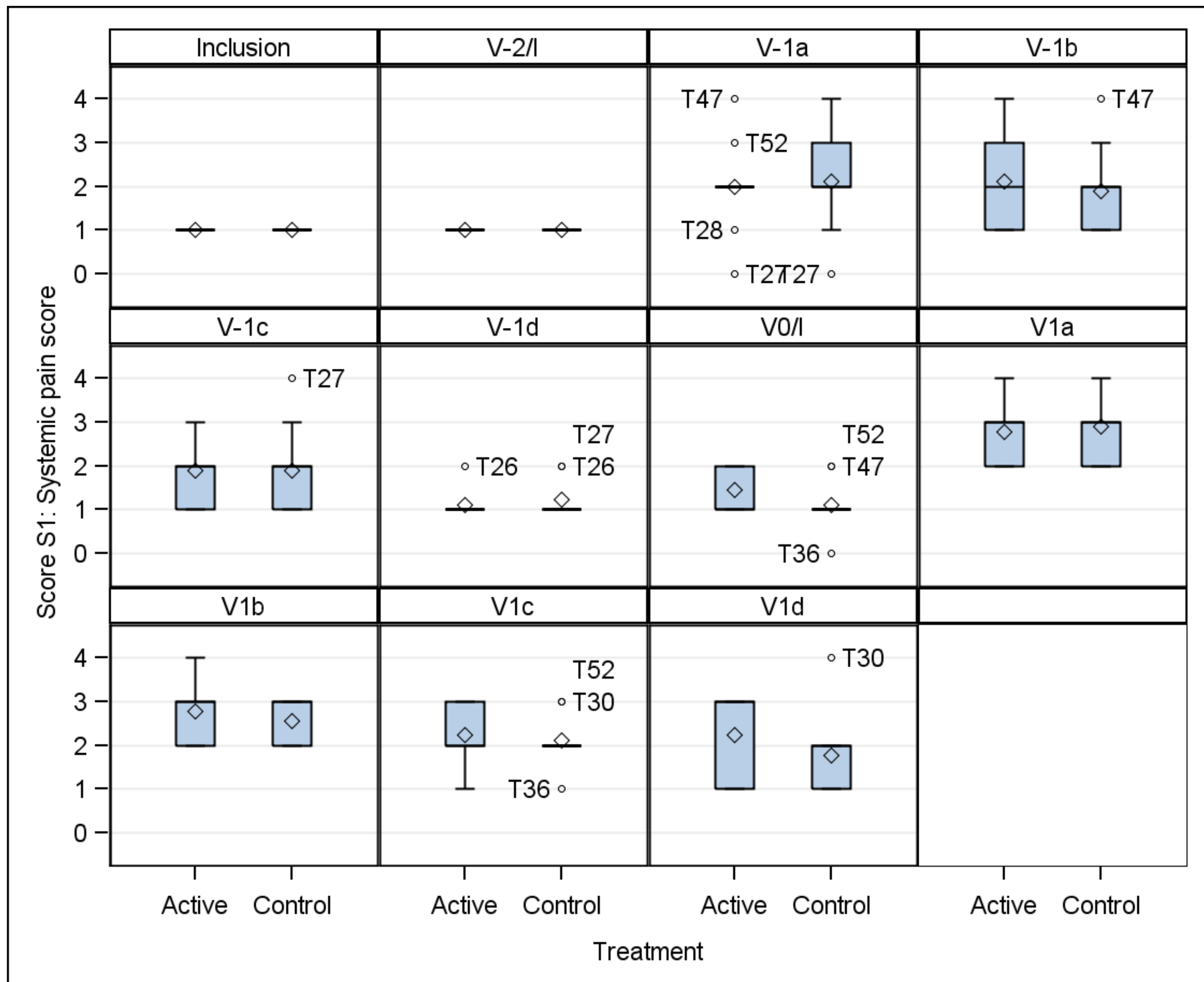


Figure 24: S1 - Systemic pain score assessed pre- and post-operatively after the first and second surgery (PP).The box plot displays the S1 - Systemic pain score [theoretical range: 0-20] distribution as determined at every visit, at inclusion and during the first four days post-operatively after the first and second surgery (time point of implantation). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond are related to the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1a, day 1 post-operatively; V-1b, day 2 post-operatively; V-1c, day 3 post-operatively; V-1d, day 4 post-operatively; V0/I, second surgery, day of implantation; V1a, day 1 post-operatively after second surgery; V1b, day 2 post-operatively after second surgery; V1c, day 3 post-operatively after second surgery; V1d, day 4 post-operatively after second surgery.

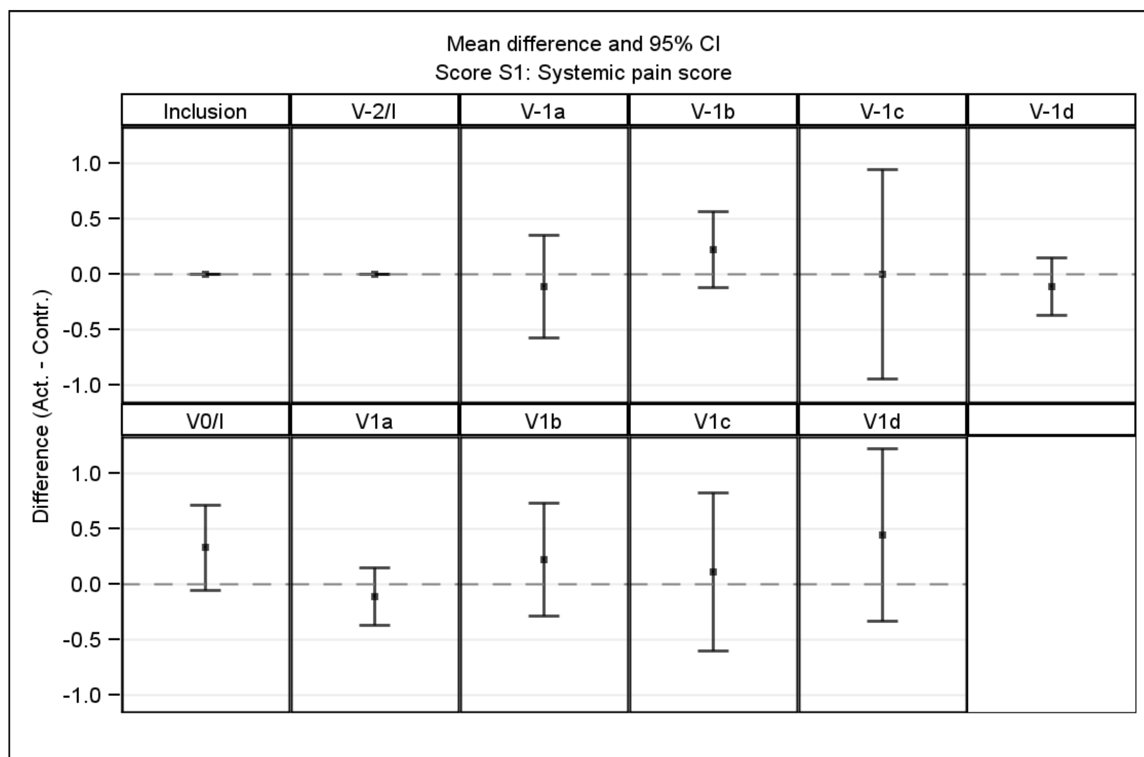


Figure 25: Differences in S1 - Systemic pain score between control and treatment group (PP).The Error Bar Plot displays the mean paired difference between treatments (Active – Control) for the S1 - Systemic pain score as determined at every visit, indicated by the filled squares. The dotted line at 0 represents a difference of 0 between the treatments. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective visit. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1a, day 1 post-operatively; V-1b, day 2 post-operatively; V-1c, day 3 post-operatively; V-1d, day 4 post-operatively; V0/I, second surgery, day of implantation; V1a, day 1 post-operatively after second surgery; V1b, day 2 post-operatively after second surgery; V1c, day 3 post-operatively after second surgery; V1d, day 4 post-operatively after second surgery.

5.6.2 Results of Score S2 – Knee inflammation score

The S2 – Knee inflammation score has a maximum point score of ten points (Appendix I, see chapter 8) and was collected at several time points (**Table 3**). It allows discrimination between the treatment (Group A) and control (Group B) group. This enables a determination of whether there is a difference between the untreated and treated knees concerning the S2 - knee inflammation score. It consists of five items, each of which is given zero to two points. These items are "*rubor, calor, dolor, tumor, functio laesa*". As planned, all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

The S2 - knee inflammation score of all animals is shown in **Figure 26** as box plots, also indicating the range of observed inflammation scores between the animals. The S2 knee inflammation pain score at the different time points during the study is indicated below. In **Figure 27**, the differences for the S2 systemic pain score between the active (treated) group and control (untreated) group are indicated by error bars, with the control group set as zero.

In summary, there were no statistical differences observed in the S2 - knee inflammation scores at the different time points between the treated and control group. Apart from outliers, whereby some of them can be seen in the boxplot of **Figure 26**, no significance could be found for any time point for the S2 – knee inflammation score.

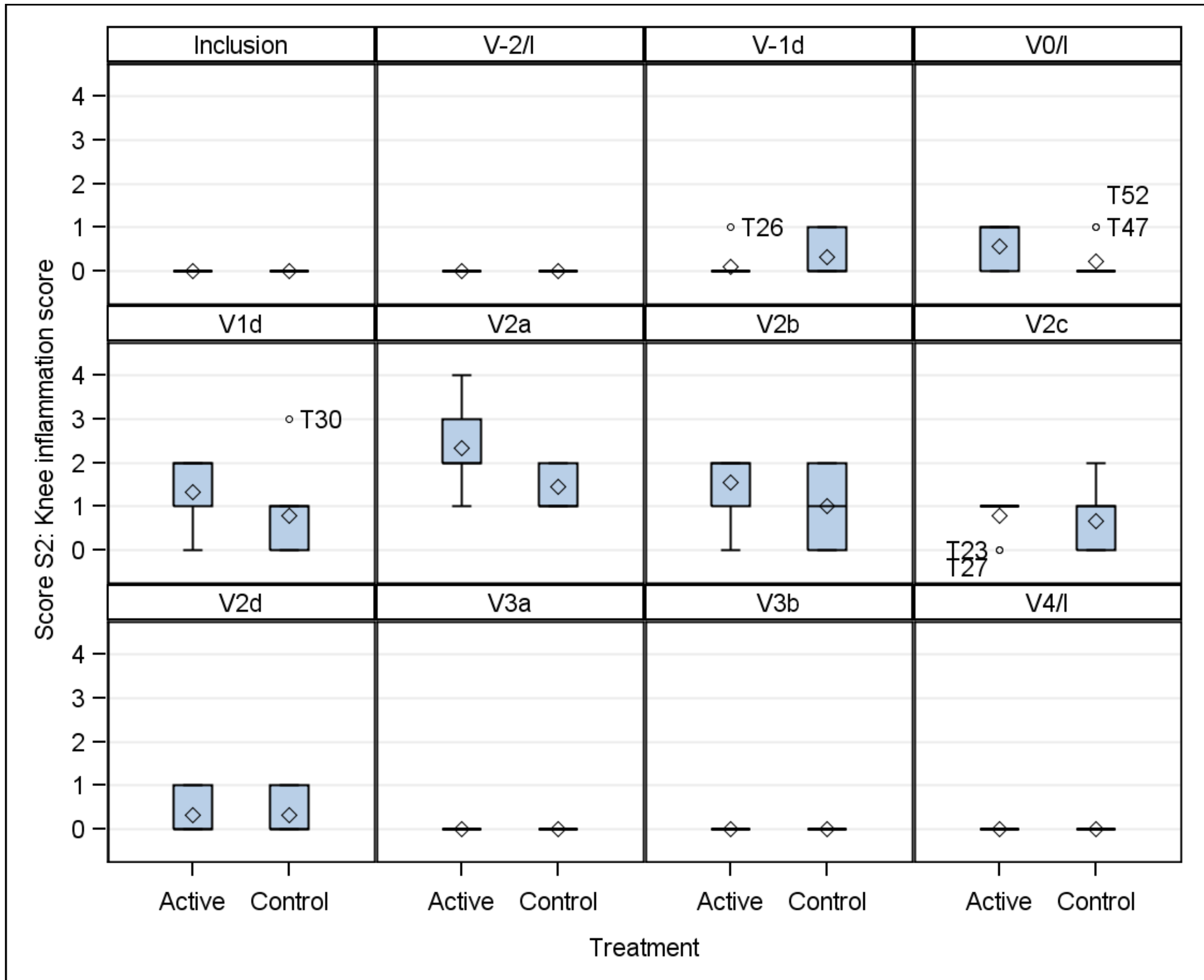


Figure 26: S2 - Knee inflammation score assessed pre- and post-operatively after the first and second surgery. The box plot displays the S2 - Knee inflammation score [theoretical range: 0-10] distribution as determined at every visit, at inclusion and during the first 4 days post-operatively after the first and second surgery (time point of implantation). The bottom and top edges of the box indicate IQR, the line inside the box indicates the median and the diamond are related to the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1d, day 4 post-operatively; V-1d, day 4 post-operatively; V0/I, second surgery, day of implantation; V1d, day 4 post-operatively after second surgery, V2a, 1 week after implantation; V2b, 2 week after implantation; V3c, 3 week after implantation; V2d, 4 week 1 after implantation, V3a, 3 month after implantation; V3b, six month after implantation, V4/I third operation.

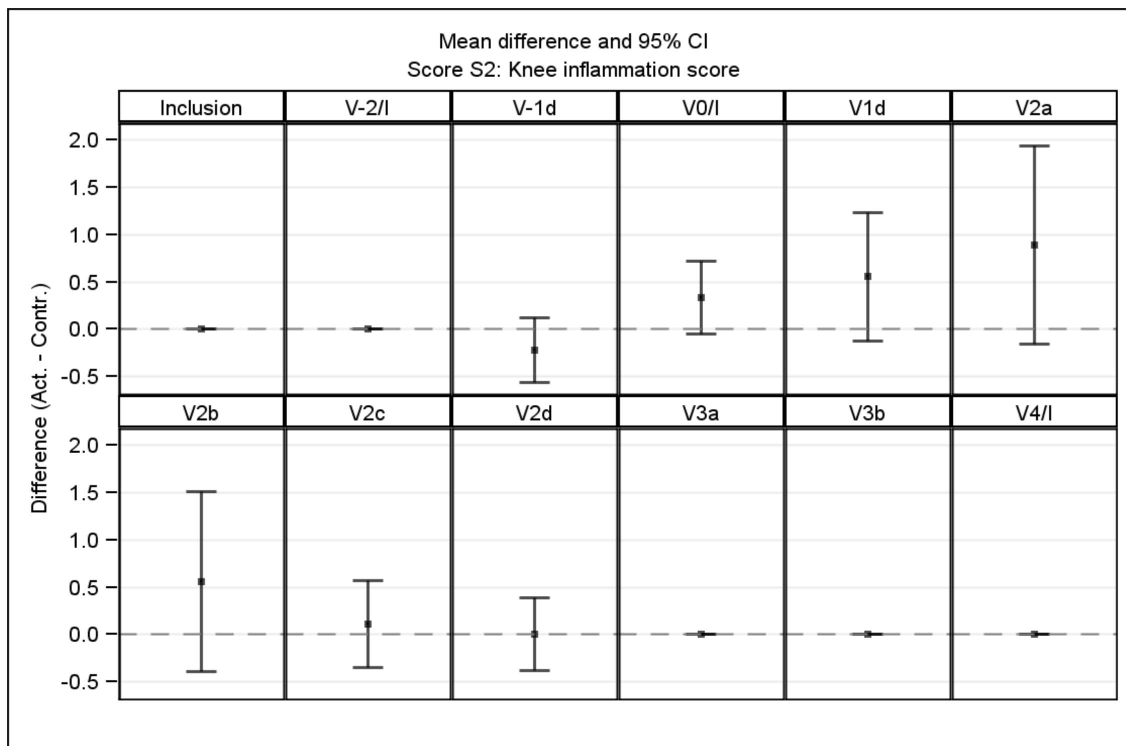


Figure 27: Differences in S2 - Knee inflammation score between control and treatment group. The Error Bar Plot displays the mean paired difference between treatments (Active – Control) for the S2 - Knee inflammation score as determined at every visit, indicated by the filled squares. The dotted line at 0 represents a difference of 0 between the treatments. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective visit. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1d, day 4 post-operatively; V-1d, day 4 post-operatively; V0/I, second surgery, day of implantation; V1d, day 4 post-operatively after second surgery, V2a, 1 week after implantation; V2b, 2 week after implantation; V3c, 3 week after implantation; V2d, 4 week 1 after implantation, V3a, 3 month after implantation; V3b, six month after implantation, V4/I third operation.

The mean standard deviation of the different S2 - knee inflammation score values at the respective time points is listed below, comparing treatment group (“The investigational product/intervention ACT3D-S”) and control group (“untreated defect as a control intervention”). The different values are indicated, including SD and statistical significance.

- The mean ± standard deviation (mean ± SD) of Group A (“The investigational product/intervention ACT3D-S”) and the mean ± SD of Group B (“untreated defect as a control intervention”) were 0.0 ± 0.0 at the time of inclusion. No significant differences were noted between the groups.
- At the V-2/I time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups.
- The mean ± SD at V-1d was 0.1 ± 0.3 for Group A and 0.3 ± 0.5 for Group B. There were no significant differences between the groups, with a p-value of 0.169. Animal 26 was an outlier in Group A with an elevated value.
- The mean ± SD at V0/I was 0.6 ± 0.5 for Group A and 0.2 ± 0.4 for Group B. There were no significant differences between the groups, with a p-value of 0.081. Animal 47 and 52 were outliers in Group B with elevated values.

- The mean ± SD at V1d was 1.3 ± 0.9 for Group A and 0.8 ± 1.0 for Group B. There were no significant differences between the groups, with a p-value of 0.095. Animal 30 was an outlier in Group B with an elevated value.
- The mean ± SD at V2a was 2.3 ± 1.0 for Group A and 1.4 ± 0.5 for Group B. There were no significant differences between the groups, with a p-value of 0.086.
- The mean ± SD at V2b was 1.6 ± 0.7 for Group A and 1.0 ± 0.9 for Group B. There were no significant differences between the groups, with a p-value of 0.214.
- The mean ± SD at V2c was 0.8 ± 0.4 for Group A and 0.7 ± 0.7 for Group B. There were no significant differences between the groups, with a p-value of 0.594. Animal 23 and 27 were outlier in Group A with reduced values.
- The mean ± SD at V2d was 0.3 ± 0.5 for Group A and 0.3 ± 0.5 for Group B. There were no significant differences between the groups, with a p-value of 1.000.
- At the V3a time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups
- At the V3b time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups.
- At the V4/I time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups.

5.6.3 Results of Score S3 – Macroscopic knee assessment score

The S3 - MCAS, which was collected at the intra-operative time points (**Table 3**), has a total maximum score of twelve points and can be seen in detail in Appendix I (see chapter 8). It enables the discrimination between the treatment (Group A) and control (Group B) group. This determines as to whether there is a difference between the untreated and treated knee concerning the S3 - MCAS possible. As planned, all values that were obtained for animal 21 and 44 were not represented in this, because their death resulted in being excluded from the PP analysis.

The S3 - MCAS of all animals is shown in **Figure 28** as box plots, also indicating the range of observed undesired effects in the stifle joints between the animals. The S3 - MCAS at the different time points during the study is indicated below. **Figure 29** shows the differences in MCASs between the active (treated) group and control (untreated) group are indicated by error bars, with the control group set as 0.

In summary, there were no statistical differences observed in the S3 - MCAS at the different time points between the treated and control groups. Apart from outliers, whereby some of them can be seen in the boxplot of **Figure 28**, no significance could be found for any time point for the S3 – MCAS.

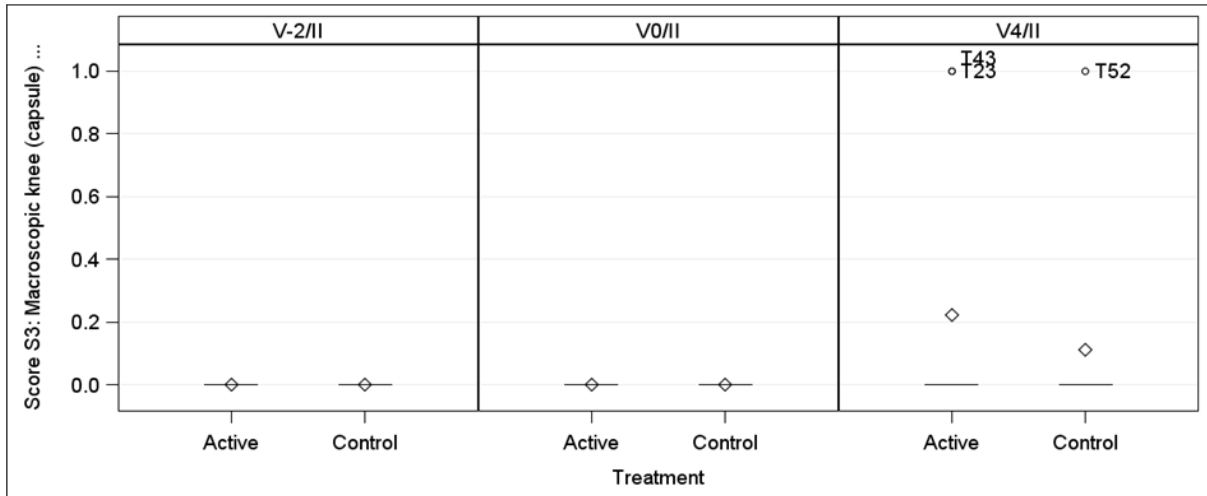


Figure 28: S3 - Macroscopic knee (capsule) assessment score assessed intra-operatively in the first, second, and third surgery (PP).
The box plot displays the S3 - Macroscopic knee (capsule) assessment score [theoretical range: 0-12] distribution as determined intra-operatively in the first, second, and third surgery. The bottom and top edges of the box indicate the inter-quartile range (IQR), the line inside the box indicates the median and the diamond are related to the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/II, first surgery; V0/II, second surgery, day of implantation; V4/II, third surgery.

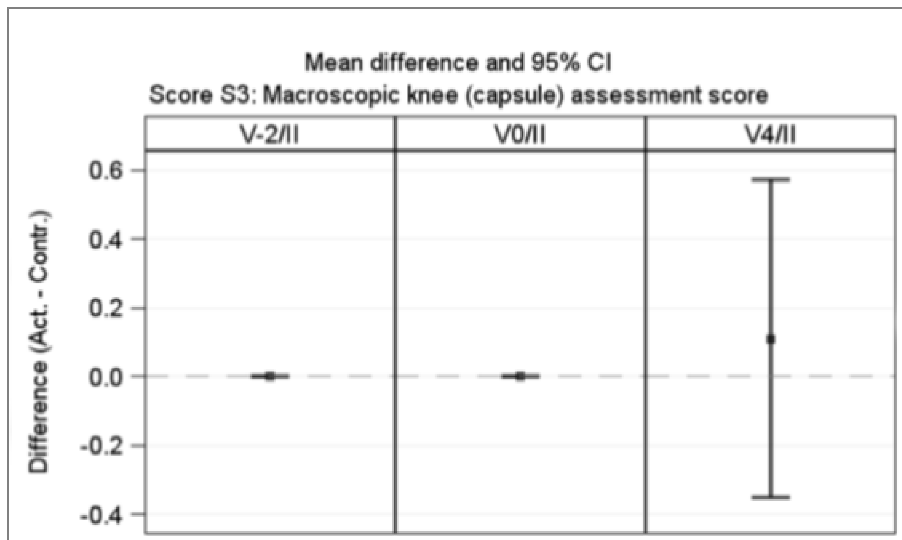


Figure 29: Differences in S3 – MCAS between control and treatment group (PP).The Error Bar Plot displays the mean paired difference between treatments (Active – Control) for the S3 - MCASas determined intra-operatively in the first, second, and third surgery, indicated by the filled squares. The dotted line at 0 represents a difference of 0 between the treatments. The error lines represent the 95 % CIs for the mean paired differences between the treatments. If the CI does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective visit. Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/II, first surgery; V0/II, second surgery, day of implantation; V4/II, third surgery.

A single analysis of the different S3 MCAS values at the respective time points is listed below, comparing treatment group ("The investigational product/intervention ACT3D-S") and control group ("untreated defect as a control intervention"). The different values are indicated, including SD and statistical significance.

- The item „Hypertrophy" was scored with 0 points for all animals at all time points, as no signs of hypertrophy were noted.
- The item „Ectopic cartilage formation" was scored with 0 points for all animals at all time points, as no signs of ectopic cartilage formation were noted.
- The item „Delamination of the transplant" was scored with 0 points for all animals at all time points, as no delamination of the transplanted tissue was noted at six months post-implantation.
- The item „kissing lesion" was scored with 0 points for all animals at V-2/II and V0/II with one exception. At the V4/II time point, animal 34 showed elevated values in Group A.
- The item „Structural side defects (e.g., osteophyte)" was scored with 0 points at the V-2/II und V0/II time points for all animals, as no osteophytes or structural defects were noted in the first two surgeries. At the time point of explantation / sacrifice (V4/II), seven animals were given 0 points in Group A and eight in Group B. Structural side defects or osteophytes were noted in two animals in Group A and in one animal in Group B, which were given one point each as a result.
- The item „Synovial fluid" was scored with 0 points for all animals at all time points, as the synovial fluid was unremarkable.
- At the V-2/II time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups.
- At the V0/II time point, the mean ± SD was 0.0 ± 0.0 for Group A and 0.0 ± 0.0 for Group B. There were no significant differences between the groups.
- The mean ± SD at V4/II was 0.2 ± 0.4 for Group A and 0.1 ± 0.3 for Group B. There were no significant differences between the groups, with a p-value of 0.594. Animals 23 and 43 were outliers in Group A, and animal 52 was an outlier in Group B, all animals showed elevated values.

6 Evaluation and Discussion of the test results

6.1 Evaluation and Discussion of secondary objectives and endpoints relating to the efficacy of ACT3D-S

In conclusion, after nine months and evaluating all results from the efficacy scores, no superior statistical significance could be found for the test object ACT3D-S between the treated and untreated control group for the prospectively defined secondary objectives number one to six in both the ITT and PP population analyses (4.2). It has to be added that the deadline for scientific studies that this work relates to was January the first of 2016. Therefore recent studies will not be part of this work.
These results were found for all obtained scores (PP) with focus on efficacy data. The following scores were part of this: the qualitative macroscopic evaluation of regenerated cartilage tissue based on the ICRS-MCAS E4 score (Appendix I, see chapter 8), the Outerbridge classification scheme (Appendix I), the imaging modalities including the radiographic modified MOCART E5 score with four parameters of the additional MRI (0) and the quantitative non-invasive ultrasound analysis (Appendix I) as well as the qualitative histopathological evaluation using the well-acknowledged O'Driscoll E1 score (Appendix I), ICRS-I E2 score (Appendix I) and ICRS-II E3 score (Appendix I). However, it is necessary to add that for the collected raw data as well as the resulting descriptive statistics in the efficacy scoring systems no significant difference was found between the two groups after nine months.
Regardless of the possible reasons for the obtained results, and with attention to the prospectively defined primary objectives (4.2). It is more than obvious that the null hypothesis, including the superiority of the AT with a superiority margin of three (out of 24 points; as measured by the overall E1 score - O'Driscoll), must be rejected at the given confidence level. Neither a superiority of the AT with ACT3D-S nor significant differences could be shown between the treated and controlled group.
Furthermore, it is important to note that the collected raw data and the resulting descriptive statistics in the efficacy scoring systems neither showed significant differences between the two treatment groups nor showed relevant differences between the means of the two groups in all subcategories or items within the efficacy assessment scales (see 5.5).
Receiving almost identical results neither fit the expectation of the pharmaceutical manufacturer co.don AG nor our group. Furthermore, these results could not be reconciled with prior published results from non-clinical studies and the clinical observations for the investigational product/intervention ACT3D-S.
As well as in the six months cohort, it is expected that the transplant got lost and therefore no superiority could be shown between the two groups. A more detailed analysis why the item could have failed will be seen in 6.1.1.. It seems that most likely the transplant got lost the first time the animal put weight on its legs, however, due to using a bilateral model an immobilization was not an option. Accordingly, the evaluation and discussion on the supposed failure of ACT3D-S is given in the chapters 6.1.1, 6.1.2 and 6.1.3. The aim of the AA was to identify possible anatomical changes that could be related to the

investigational product. Apart from the two dropouts, those are accurately described in section 5.3, the collected raw data showed no significant difference that was related to any finding during the whole study process. Therefore the detailed analysis of the animal autopsy data will not be part of this doctoral thesis.

6.1.1 Evaluation and discussion of indicators of potential loss of ACT3D-S

Four indicators were identified, that seem to be related to the fact, that the ovine test sample ACT3D-S was not present inside the focal full-thickness cartilage defect nine months after the implantation. Due to the expected loss of ACT3D-S, it was not possible to prove its effect and mode of action.

First of all, in none of our histological slides, neither stained with HE (**Figure 8**) nor with Safranin-O-lightgreen (**Figure 9**) showed the characteristic globules of former spheroids with high cellularity, what would be atypical for articular cartilage, including simultaneous positive staining for cartilage-specific ECM markers. This was already observed by Schubert *et al.* [107] and Meyer *et al.* [77] performing a small and large animal model study and a six and two months post-operative check for the efficacy of their treatments with spheroids respectively.

Both of these studies were non-clinical tries using cartilage spheroids similar to ACT3D-S. For these two, the high cellularity of the transplant was one of the hallmarks that made the identification of the former localization of the transplant possible. Schubert *et al.* used chondrocytes from human patients undergoing total knee arthroplasty. The gained chondrocytes were cultured, and their integrative capacity was tested by implanting them into severe combined immunodeficiency (SCID) mice [107].

The mice were sacrificed after four, twelve and 24 weeks to histologically investigate the morphological changes as well as to measure the concentration matrix proteins as collagen I and II. Furthermore, clearly higher cellularity compared to the remaining cartilage could be shown, and a hyaline-like character could be stated for the repair tissue. Concerning matrix proteins, a clear expression of GAGs, as well as a strong expression of collagen type II, was found. This small animal model confirmed the mode of action and suggested the efficacy of spheroids. However, to prove efficacy for such a method, studies in larger animal models are necessary.

Unlike Schubert *et al.*, Meyer *et al.* made a two-month follow-up using microsphere technology cultured autologous chondrocytes from mini-pigs. They created two acute full-thickness cartilage defects of 4mm diameter and placed them unilaterally in the minor load-bearing zone of the trochlear groove of one stifle joint. The unilateral defect creation, unlike in our study, made an immobilization for 24hours after the implantation possible. After a duration of two months, the animals were sacrificed. Meyer *et al.* found similar histological results as mentioned above for the mice study. Their stains showed high cellularity and accumulation of hyaline cartilage-specific markers like collagen type II or S100-protein [77].

However, there are points which limit the validity of this study. On the one hand, only three animals were treated, and, on the other hand, they received an overdose between 2.3 and 3.3 times that are normally recommended for treatment in humans, with a maximum dosage of 70 spheroids per cm². Although this

study was able to prove that it would be suitable for proof-of-principle models, it still is not recommended to use mini-pigs by the ICRS because Hurtig *et al.* stated this species as being tended to “Osteochondritis and cartilage growth deformities” above average [46].

A second indicator that proves that our pivotal study did not work out as expected is that unlike Schubert *et al.* [107] and Meyer *et al.* [77], our Safranin-O-lightgreen stained histological slides from the femoral condyle explants neither showed ECM accumulation nor spheroid-induced cartilage regeneration.

A third indicator seemed apparent when the final cartilage thickness and cartilage tissue filling data was reviewed. The depending scores with subcategories are the O’Driscoll score (“Thickness”), the E4 ICRS-MCAS score (“Degree of defect repair”), the E5 modified MOCART score (“Degree of defect repair and filling of the defect”), the ultrasound analysis (“Neo-tissue formation volume" and "Missing tissue volume”) and the “Outerbridge classification. None of our results can support the idea of defect filling by hyaline-like or fibrocartilaginous tissue forming it into healthy adjacent native cartilage with spheroids. In total contrast, a prospective clinical case series by Fickert *et al.* [30] treating 33 isolated cartilage defects in knee joints with ACT3D showed a complete filling in 94% (n=31) of the cases by using MRI imaging after twelve months.

The last indicator were scores that evaluate the bonding of regenerated tissue to the circumjacent cartilage tissue. It includes the E1 O'Driscoll score ("Bonding to the adjacent cartilage), the E4 ICRS-MCAS ("Integration to border zone") and the E5 modified MOCART score (“Integration to border zone"). These scores, which represent the bonding of ACT3D-S cartilage transplant into the border zone did not reveal any superior data to the untreated site. Moreover, comparing our data with the above mentioned prospective analysis from Fickert *et al.* [30], shows that their data did not reveal any detachment or delamination of the implanted tissue. This substantial difference to our study lays in a good integration and approximately complete bonding of the spheroids to the freshly debrided cartilage defect wall.

As mentioned above, four reasons could be gathered, to fulfill an objective evaluation of the observed non-superiority efficacy outcomes of the ACT3D-S test item compared to our untreated control group.

1. No elevated cellularity, 2. Missing ECM accumulation, 3. Low repair tissue thickness and 4. Missing bonding - indicates that the mechanism of action for the ACT3D-S was absent or impaired.

With regard to these four points, and based on the experiences of authors in the field of bilateral implantation of matrix-coupled ACT procedures in full-thickness cartilage defects, consequently the only probable explanation for the present data is a loss of the majority of the transplanted spheroids probably occurring within 24 hours after the implantation or directly the first time the animals bore weight, for the entire ACT3D-S treatment group. This is underpinned by the existing results that do not show any signs of remaining spheroids nor of repair tissue as found by other authors as mentioned above.

6.1.2 Evaluation and discussion of the impact of the animal study design on the loss of ACT3D-S

Concerning the chosen animal model, most of the methods and guidelines were used of “Recommendation from the International Cartilage Repair Society (ICRS) for Preclinical Studies for Cartilage Repair” by Hurtig *et al.*, 2011 [46]. Nevertheless, retrospectively we have to admit that these guidelines may be not 100% appropriate for a product like chondrosphere®.

Like mentioned before, another fact to argue about is the intra-operative self-adherence of the implanted spheroids. Although 20 minutes were recommended, we had the feeling, that this time was not enough to entirely bind the spheroids to the underlying cartilage in all cases. In a word, we think the majority of the spheroids got lost, the first time the animals bore weight. With this in mind, the spheroids could have been rinsed out by synovial fluid and blood or could have been pushed out by the pressure occurring during load-bearing. The combination of the characteristics of the bilateral animal model as well as of the spheroid product seem to support this. The model-related factor that could worsen loss of the transplants is the lack of partial or full joint immobilization immediately after implantation. However, as noted by ICRS, temporary immobilization of both stifles in a bilateral model is unethical and not possible [46].

A one-sided immobilization is well-tested during the last years in different large animal models, as by Mrugala *et al.* [83] with an ovine model as well as by using different immobilization techniques such as using a modified trapeze [54], a plaster cast [25], a monolateral external fixator [39] or a soft brace with limitation of flexion up to 30° (i.e. co.don non-clinical study cod 41/03). To summarize, no pain lasting for days or severe problems were detected for these methods. As stated by the ICRS, “unilateral models allow for less initial weight bearing on the defect limb, eliminate paraeffects, and are more amenable to partial- or full-joint immobilization”, while “bilateral models control for animal-to-animal variability in repair response“ [46].

What is the conclusion of this? Apparently, a combining of both is impossible, what directly leads to the conflict what strategy may be the superior one. Seen from a distance, we intended that the potential of intra-individual comparison of outcome, efficacy, and SAF be more valuable than the immobilization of a single hind limb. Furthermore, we wanted to avoid the problem of animal-to-animal variability in repair response. Retrospectively, the unilateral model appears to be clearly unsuitable for the skeletally mature merino land sheep, as the inter-individual variance of efficacy scores such as the E1 O’Driscoll score was very high in the control group (between one and 22 of 24 possible points). This shows high variability in the capacity for spontaneous healing of the critical-size cartilage defects, which could only have been accounted for a unilateral model with an extremely high number of subjects.

In contrast, the human post-operative treatment for patients treated with ACT3D (according to SmPC) is, logically, totally different and way more intensive than for our sheep, which had no special rehabilitation. First weeks of continuous passive motion exercises are followed by weeks of partial weight bearing. No need to mention that a treatment like this would not work for a bilateral model as well would be extremely

hard to fulfill for an animal-using model. This leads to the conclusion that the ability of the model reflect to human patients was reduced. The assumed detachment of spheroids from the defect bed due to the lack of knee immobilization is made yet more likely due to the differences in sheep joint architecture and cartilage height.

Osterhoff *et al.* [91] and other stated different facts that could underline the potential loss of ACT3D-S in an ovine model. On the one hand, a steeper curvature and a reduced size by a factor of three and on the other hand a cartilage depth in the defect site around only 1mm compared to 3mm in humans were mentioned.

Another point, we think was important, is the decreased protection that could be provided for the freshly adhered spheroids by the adjacent intact cartilage shoulder against high pressure, friction, shear forces and fluid flow for the main load-bearing zone of the tibiofemoral joint. This was intensified by a steeper curvature and reduced size by a factor three in the ovine stifle joint, as well as the difference of 2mm comparing the lower ovine cartilage thickness (1mm for the ovine model and 3mm for humans).

A possible way to avoid this is using another load-bearing zone as the trochlear groove for the critical-size defect [21, 43, 77]. However, it remains that cartilage replacement techniques are used more often in the tibiofemoral joint than in the patellofemoral joint, whereby the almost identical knee flexion angle of 178 degrees in the sheep results in a good adaptation of the human joint architecture and force effects.

6.1.3 Evaluation and discussion of the impact of the unit dosage form on the loss of ACT3D-S

In our view, according to the loss of the spheroids and the product-related factors, we make the selected standardized animal model and the product form as well as the applied dosage responsible.

The ACT3D spheroids are aggregates of autologous chondrocytes that are aligned in 3D shape. Spheroids are small cartilage transplants existing of autologous chondrocytes in a matrix-framework composed of their *in vitro* self-synthesized ECM. In our study 13 of these were implanted into the cartilage defect. Other MACTs products consist of only one entity that is implanted instead of several small entities that moreover were not externally fixated. Instead of using the self-adherence ability of chondrosphere® the other "normal" 3D matrix- or scaffold based MACT products use physical fixation by using fibrin glue, or by using autologous periosteal flaps as well as xenogenic synthetic membranes and fleeces for the little older first and second generation of ACP products [24]. Consequentially, these methods massively reduce the possible loss of transplant caused, i.e. by shear forces, pressure, friction and fluid flow.

On the one hand, our application form without a carrier is unique and matchless on the market for cartilage cell implantation, but on the other hand, this method failed in the relevant animal model with a 1:1 adaption of the human implantation technique. A modification of this procedure that would result in higher adherence, by using fibrin glue [Tissuecol®, Baxter, Germany] for a better and higher physical adherence

is not possible. Due to an unknown influence on the outcome, it cannot be used for efficacy testing in a large animal model.

This method was tested in a non-clinical study 41/03 of co.don AG, whereby also Merino land sheep were used for a pilot study. In this study, three defects with a 6mm diameter were created without damaging subchondral regions. Like in our study the creation was dried of body fluids afterward, and nine spheroids (dosage 30 spheroids per cm²) were applied using the co.fix® applicator, always from proximal to distal, and after this, the opened femoral condyle was left open for 20 minutes to enable adherence of the implanted spheroids. However, this pilot study was unilateral and therefore able to immobilize the treated joint. For this study, the spheroids were observed *in situ* after 8-9 days post-implantation and thus remained inside the joint. Because a bilateral model was chosen for this GLP study, like earlier mentioned, an immobilization was not possible.

During our study procedures, an investigation by Marquass *et al.* [72] was done in 2011. They were able to show that Merino land sheep are suitable for cartilage spheroid production, analogous to the production of chondrosphere®, as well as for investigations around the topic cartilage cell therapies.

For comparable and usable data, it is necessary, to use dosages similar to the human treatment. Prospectively we chose a dose of 33 spheroids per cm² (13 spheroids per 7mm diameter defect). This is within the range of 10-70 spheroids per cm² that is recommended in the SmPC. Our spheroids had a mean aggregate size of 507 ± 53 µm whereby, provided by co.don (internal information), the mean aggregate size of human spheroids was significantly higher with 631 ± 148 µm (n=1597). Out of this fact, we can conclude, that we had no secure evidence about the firmness, elasticity and most important about the adherence properties for the smaller spheroid size. However, a smaller size means a smaller connective area between spheroid and cartilage surface what results in lower self-adherence than the human ACT3D product. In our opinion, these decreased parameters should be compensated by going to the limit of 70 spheroids per cm² giving by the SmPC. This would lead to ~27 spheroids and could have resulted in better efficacy outcomes.

6.1.4 Evaluation and discussion of the Score S1 – Systematic pain score

All in all, the animals showed no severe pain what resulted in an overall low systematic pain score, whereby for both, the pre- and the post-operative scores, low results were found. Having a maximum result of 20 points the S1 score showed average values of two or three post-operatively (**Figure 30**, **Figure 31**) and a maximal value of four points. This leads to the assumption that neither the first operation with defect creation nor the second surgery with implantation of the test items or control treatment had any severe or negative long-term effects for the well-being of the animals. During almost all examinations most animals showed a slightly increased respiratory rate (one point), that was caused by the excitement of the animals when we entered the stable to carry out the examination. Low grade and isolated moderate changes were found for the items "Movement behaviour", "Palpation of the wound" and "Joint swelling, increased warmth, soft tissue swelling" before the last operation.

It can be concluded, that the general well-being was not hardly affected by the surgeries and that the use of ACT3D-S not resulted in any health impairment or negative long-term consequences.

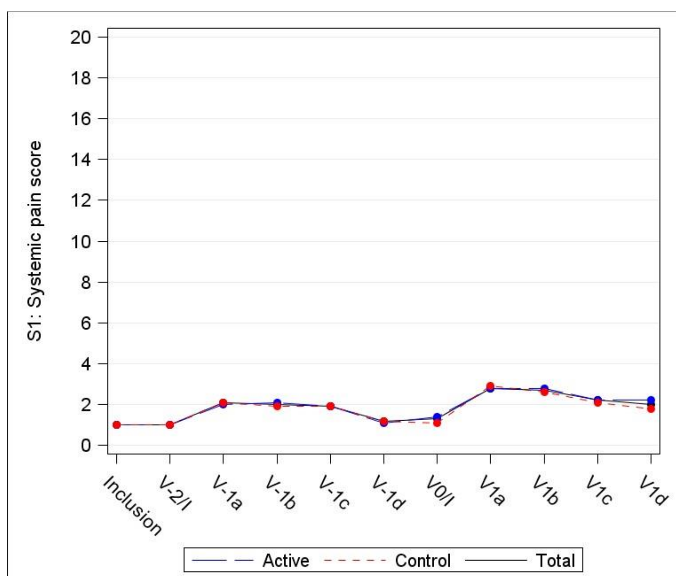


Figure 30: S1 - Systemic pain score over time in both control and treatment group (PP).The time course displays the mean values of the S1 - Systemic pain score as determined at each visit by treatment (blue dashed line: Active, red dashed line: Control, black solid line: Total mean) Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1a, day 1 post-operatively; V-1b, day 2 post-operatively; V-1c, day 3 post-operatively; V-1d, day 4 post- operatively; V0/I, second surgery, day of implantation; V1a, day 1 post-operatively after second surgery; V1b, day 2 post-operatively after second surgery; V1c, day 3 post-operatively after second surgery; V1d, day 4 post-operatively after second surgery.

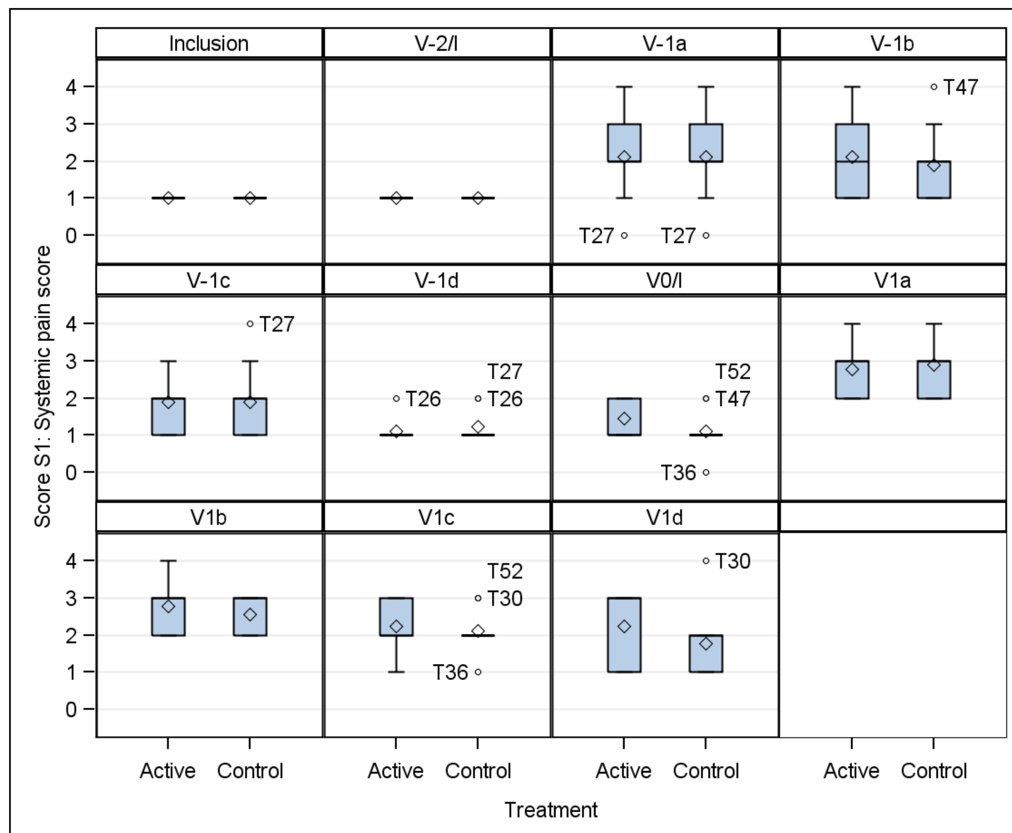


Figure 31: Box plot - Score S1: Systemic pain score in both control and treatment (PP). The bottom and top edges of the box indicate the IQR, the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. Time points of the scoring: Inclusion; V-2/I, first surgery; V-1a, day 1 post-operatively; V-1b, day 2 post-operatively; V-1c, day 3 post-operatively; V-1d, day 4 post- operatively; V0/I, second surgery, day of implantation; V1a, day 1 post-operatively after second surgery; V1b, day 2 post-operatively after second surgery; V1c, day 3 post-operatively after second surgery; V1d, day 4 post-operatively after second surgery.

6.1.5 Evaluation and discussion of the Score S2 - Knee inflammation score

The S2 Knee inflammation score, with a maximum score of ten points, had its highest average value one week after the implantation with 1.9 points (V2a, in **Figure 32**). This fell to an average of 0.3 points four weeks after surgery (V2d, in **Figure 32**) and was stated 0 points three months after the implantation (V3a, in **Figure 32**). The examination revealed that one point (“slight sign”) was mostly given for the items “*rubor*” and “*tumor*” whereby isolated findings were two points had to be given (“clear signs”) were also notified. Although it looks like Group A (AT / test item) has higher values than Group B (untreated control) no significant difference with p-values lower than 0.05 could be detected for any time point.

Summarized, no significant differences were found between Group A (test item) and Group B (untreated control) for the post-operative development of knee inflammation. This further indicates, as did the S1 Score, that the investigational product ACT3D-S does not seem to harm the animal or nor to support or maintain knee inflammation.

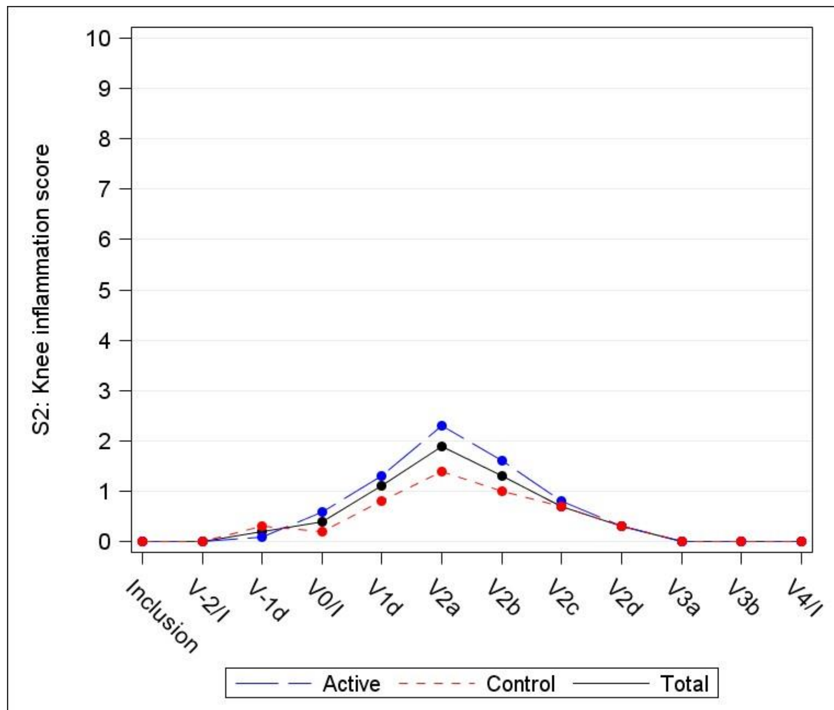


Figure 32: S2 - Knee inflammation score over time in both control and treatment group (PP).The time course displays the mean values of the S2 - Knee inflammation score as determined at each visit by treatment (blue dashed line: Active, red dashed line: Control, black solid line: Total mean).Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/I, first surgery;V-1d, day 4 post-operatively; V0/I, second surgery, day of implantation; V1d, day 4 post-operatively after second surgery; V2a week 1 post-operatively after second surgery, V2b week 2 post-operatively after second surgery, V2c week 3 post-operatively after second surgery, V2d week 4 post-operatively after second surgery, V3a month 3 post- operatively after second surgery, V3b month six post-operatively after second surgery, V4/I, third surgery.

6.1.6 Evaluation and discussion of the Score S3 - Macroscopic knee assessment score

The S3 - MCAS, with a maximum of twelve points, showed an average value of 0 points during first and second surgery (V-2/II, V0/II) whereby an average value of 0.2 points was found in both groups (**Figure 33**). Animal 23 and 43 of Group A (test item) and animal 52 of Group B (untreated control) showed "slight signs" (one point) for the item "Structural side defects (e.g. osteophytes)". The low occurrence as well as that no differences in absolute or relative frequency of these structural side effects could be found show that also for the S3 Score no negative effects can be observed that could be related to the treatment with ACT3D-S. The remaining items as "hypertrophy", "ectopic cartilage formation", "delamination of the transplant tissue" and "synovial fluid" showed no increased score at any time point. For the potentially existing change for "delamination" of the investigational product/intervention, it is possible that this change could not be detectable anymore for an observer nine months after surgery.

In summary, it should be stated that no significant differences were found between the post-operative courses of groups A and B. This indicates that the investigational product/intervention (ACT3D-S) does not appear to cause any additional danger regarding the six secondary findings listed above.

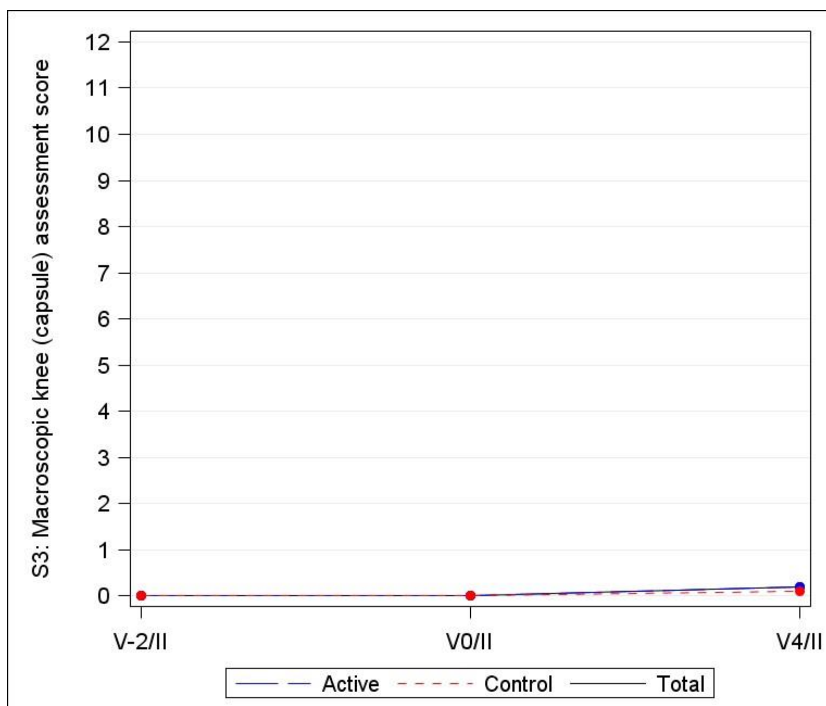


Figure 33: MCAS over time in both control and treatment group (PP).The time course displays the mean values of the S3 - MCASas determined intra-operatively in the first, second, and third surgery by treatment (blue dashed line: Active, red dashed line: Control, black solid line: Total mean).Active: ACT3D-S treatment; control: untreated control. Time points of the scoring: Inclusion; V-2/II, first surgery; V0/II, second surgery, day of implantation; V4/II, third surgery.

7 Conclusion

The purpose of this study was to show the efficacy and safety of the investigational product co.don chondrosphere® (ACT3D-S). ACT3D-S is a product for autologous chondrocyte transplantation that we used in an animal model, the merino land sheep. We compared the treatment of ACT3D-S (Group A: Investigational product) with an untreated control (Group B: Control Intervention) in a bilateral model, what means that by randomization one hind limb was chosen to be treated with ACT3D-S while the remaining hind limb was left without treatment. The study progress was divided into seven main phases.
The first phase was the Acclimatisation of the animals in the stable of the MEZ. It included the taking of the Scores S1 and S2 for preoperative safety assessment, as well as the preoperative management. The second phase was the defect creation whereby for each hind limb a cylindrical defect of 7 x 2 mm was created inside the medial femoral condyle and left without any treatment. In the following 49 days during the degeneration phase of the full-thickness cartilage. For four postoperative days, a daily examination was done and was followed by a weekly examination until the 49 days ended. The fourth phase was the implantation of ACT3D-S whereby both femoral condyles were prepared for the implantation. As soon as the preparation was completed, a sealed envelope including the randomized side was used, and the investigational product was inserted into the chosen defect side. This phase was followed by nine-month long-term husbandry. Again a 4-day daily examination was followed by four weekly examinations. Additionally, an examination after three, six and nine months was done. The sixth main phase was the sacrifice and explantation of both hind limbs. Therefore the animal was narcotized, and T61 was injected until the heart stopped and the veterinarian confirmed the death. The 7th phase included all data creation and analysis of histology slices, MRI data, ultrasound biomicroscopy and evaluation and interpretation of all scores.
To summarize, after nine months and evaluating all results from the efficacy scores, neither a superior statistical significance nor a significant difference could be found in for the test object ACT3D-S comparing the treated and untreated control group for the prospectively defined secondary objectives in both the ITT and PP population analyses.
Receiving almost identical results neither fit the expectation of the pharmaceutical manufacturer co.don AG nor our group. Furthermore, these results could not be reconciled with prior published results from non-clinical studies and the clinical observations for the investigational product/intervention ACT3D-S.
Three main causes were found to explain the efficacy results. First one is the potential loss of ACT3-DS. This theory of this loss was supported by finding no elevated cellularity, by missing ECM accumulation, low repair tissue thickness and missing bonding to the circumjacent cartilage tissue. The only probable explanation, why no sign of remaining spheroids nor repair tissue was found, is that the majority of the transplanted spheroids got lost during the first 24 hours or probably the first time the animals bore weight on their feet.

The second point is the chosen animal model. We intended that the potential of intra-individual comparison of outcome, efficacy, and SAF would be more valuable than the immobilization of a single hind limb. Furthermore, we wanted to avoid the problem of animal-to-animal variability in repair response so that we chose a bilateral animal model. Due to the fact, that immobilization of both hinds was not possible, the protection for our freshly adhered spheroid against high pressure, friction, shear forces and fluid flow, was simply not high enough. This theory was supported by a steeper curvature and reduced size comparing human and sheep cartilage as well as the point that human patients are not allowed to bear full weight on their foot for several weeks after the operation compared to our sheep that did that directly after the operation.

The third and last point is the influence of the unit dosage form. Other dosage forms normally consist of just one entity that is implanted using different kinds of fixation forms whereby we implanted several small entities just using the self-adherence capability. Due to an unknown influence on the outcome of efficacy testing in a large animal model, we were not able to use any fixation technique. However, on the one side, our application form without a carrier is unique and matchless on the market for cartilage cell implantation, but on the other side, it failed in the relevant animal model with a 1:1 adaption of the human implantation technique. On the other side the dosage we chose was 33 spheroids per cm² and so was within the range of 10-70 spheroids per cm². This means that to improve the efficacy of our product we could increase the dosage could be increased up to 70 spheroids per cm² without leaving the range of human treatment standards. However, previous studies of our group as from Marquass *et al.* showed the effective adhesion of our spheroids 8-9 days after the operation in merino land sheep, as well as other studies, showed elevated cellularity, ECM accumulation, high repair tissue thickness and good bonding to the circumjacent cartilage tissue.

In contrast to the efficacy data, the safety data consistently showed that the implantation of our product did not maintain knee inflammation parameters. Furthermore, all scores that were observing the feeling of pain and habits of our animals could not show any difference between the operated and control limb. This gathered data confirmed our previous assumption that the implantation of spheroids was no harm for the health of our animals and could satisfy all safety requirements.

To summarize in one section of text, we were unable to prove our primary endpoint that was defined as a difference of overall Score E1 - O'Driscoll of ACT3D-S (Group A) and untreated control (Group B) at nine months after the end of the respective treatments, whereby in my opinion the chosen bilateral model was the primary factor. But also the secondary endpoints regarding efficacy data were not able to confirm any superiority. On the other side the secondary endpoint data relating to the safety of the product were able to prove that our product did not harm the safety of the animal, whereby as explained above, the death of the two dropouts was not in relation to our investigational product. Reviewing previous studies of our group, it is evident that not the whole system of self-adherence-spheroids is a failure but instead the whole experimental model that was not suitable for our product. Existing studies with human patients showed far better results than our study, so that at least, the safety of our product could be proven.

8 Appendix I: Scores and detailed Ultrasound Results

E1	**O'Driscoll Score**	
Parameter	**Variable**	**Score**
Nature of predominant tissue		
Cellular morphology	hyaline articular cartilage	4
	incompletely differentiated mesenchyme	2
	fibrotic tissue or bone	0
Safranin-O staining of the matrix	normal or nearly normal	3
	moderate	2
	slight	1
	none	0
Structural characteristics		
surface regularity	smooth and intact	3
	superficial horizontal lamination	2
	fissures 25-100 per cent of the thickness	1
	severe disruption, including fibrillation	0
Structural integrity	normal	2
	slight disruption, including cysts	1
	severe disintegration	0
Thickness	100 per cent of normal adjacent cartilage	2
	50-100 per cent of normal cartilage	1
	0-50 per cent of normal cartilage	0
Bonding to the adjacent cartilage	bonded at both ends of graft	2
	bonded at one end, or partially at both ends	1
	not bonded	0
Freedom from cellular changes of degeneration		
Hypocellularity	normal cellularity	3
	slight cellularity	2
	moderate cellularity	1
	severe cellularity	0
Chondrocyte clustering	no clusters	2
	<25 per cent of the cells	1
	25-100 per cent of the cells	0
Freedom from degenerative changes in adjacent cartilage		
	normal cellularity, no clusters, normal staining	3
	normal cellularity, mild clusters, moderate staining	2
	mild or moderate hypocellularity, slight staining	1
	severe hypocellularity, poor or no staining	0
Maximum score		**24**

E2	**ICRS-I Score**	
Parameter	**Variable**	**Score**
Surface	smooth/continuous	3
	discontinuous/irregularities	0
Matrix	hyaline	3
	mixture: hyaline/fibrocartilage	2
	fibrocartilage	1
	fibrous tissue	0
Cell distribution	columnar	3
	mixed: columnar/clusters	2
	clusters	1
	individual cells/ disorganised	0
Cell population viability	predominantly viable	3
	partially viable	1
	<10% viable	0
Subchondral Bone	normal	3
	increased remodeling	2
	bone Necrosis/ granulation tissue	1
	detached/ fracture/ callus at base	0
Cartilage Mineralization	normal	3
(calcified cartilage)	abnormal/ inappropiate location	0
Maximum score		18

E3	**ICRS-II Score**	
Parameter	**Variable**	**Score**
1. Tissue morphology (viewed under polarized light)	100%	0-100%
	0%	
2. Matrix staining (metachromasia)	100%	0-100%
	0%	
3. Cell morphology	100%	0-100%
	0%	
4. Chondrocyte clustering (four or more grouped cells)	100%	0-100%
	0%	
5. Surface architecture	100%	0-100%
	0%	
6. Basal integration	100%	0-100%
	0%	
7. Formation of a tidemark	100%	0-100%
	0%	
8. Subchondral bone abnormalities/ marrow fibrosis	100%	0-100%
	0%	
9. Inflammation	100%	0-100%
	0%	
10. Abnormal calcification/ ossification	100%	0-100%
	0%	
11. Vascularization (within the repaired tissue)	100%	0-100%
	0%	
12. Surface/ superficial assessment	100%	0-100%
	0%	
13. Mid/ deep zone assessment	100%	0-100%
	0%	
14. Overall assessment	100%	0-100%
	0%	

E4	**ICRS-MCAS**	
Parameter	**Variable**	**Score**
Degree of defect repair	in level with surrounding cartilage	4
	75% repair of defect depth	3
	50% repair of defect depth	2
	25% repair of defect depth	1
	0% repair of defect depth	0
Integration to border zone	complete integration with surrounding cartilage demarcating border < 1mm	4
	75% of graft integrated, 25% with a notable border > 1mm width	3
	50% of graf integrated with surrounding cartilage, 50% with a notable border > 1mm	1
	from no contact to 25% of graft integrated with surrounding cartilage	0
Macroscopic appearance	intact smooth surface	4
	fibrillated surface	3
	small scattered fissures or cracks	2
	several small or few but large fissures	1
	total degeneration of grafted area	0
Maximum score		12

OB	**outerbridge score**
Parameter	**Variable**
Grad 0	normal cartilage
Grad 1	softening and swellin of the cartilage
Grad 2	fragmentation and fissuring in an area half an ich or less in diameter
Grad 3	fragmentation and fissuring in an area more than half an inch in diameter is involved
Grad 4	Erosion down to bone

E5	**modified 2D-MOCART score**	
Parameter	**Variable**	**Score**
Degree of defect repair and filling	complete (like sourrounding cartilage)	20
of the defect	hypertrophy (more than sourrounding cartilage)	15
Incomplete:	>50 per cent compared to adjacent cartilage	10
(less than sourrounding cartilage)	<50 per cent compared to adjacent cartilage	5
	subchondral bone exposed (complete delamination / dislocation	0
	free joint bodies)	
Integration to border zone	complete (to sourrounding cartilage)	15
	incomplete (to sourrounding cartilage)	10
Visible defect	<50 per cent of repair surface	5
	>50 per cent of repair surface	0
Surface of the repair tissue	Surface and lamina intact	10
Surface damaged (ulcera, fissures)	<50 per cent of repair tissue	5
	>50 per cent of repair tissue	0
Structure of the repair tissue	homogen	5
	inhomogen or fragmented cell formation	0
Signal intesity of the repair tissue	isointense	15
Dual T2 FSE	moderate hypointense	5
	strongly hypointense	0
Subchondral lamina	intact	5
	not intact	0
Subchondral bone	intact	5
	edema, granulation tissue, cysts, sclerosis	0
Maximum score		75

US **Ultrasonographic evaluation**		
Parameter	**Variable**	**Unit**
Evaluation criteria of regenerated tissue		
US_i_1	neo-tissue formation volume	[mm3]
US_i_2	overfilling tissue volume	[mm3]
US_i_3	missing tissue volume	[mm3]
US_i_4	hypertrophic tissue volume	[mm3]
US_i_5	cyst volume	[mm3]
US_i_6	healthy tissue volume	[mm3]
Evaluation of the healthy tissue		
US_i_7	healthy min thickness	[mm3]
US_i_8	healthy max thickness	[mm3]
US_i_9	healthy average thickness	

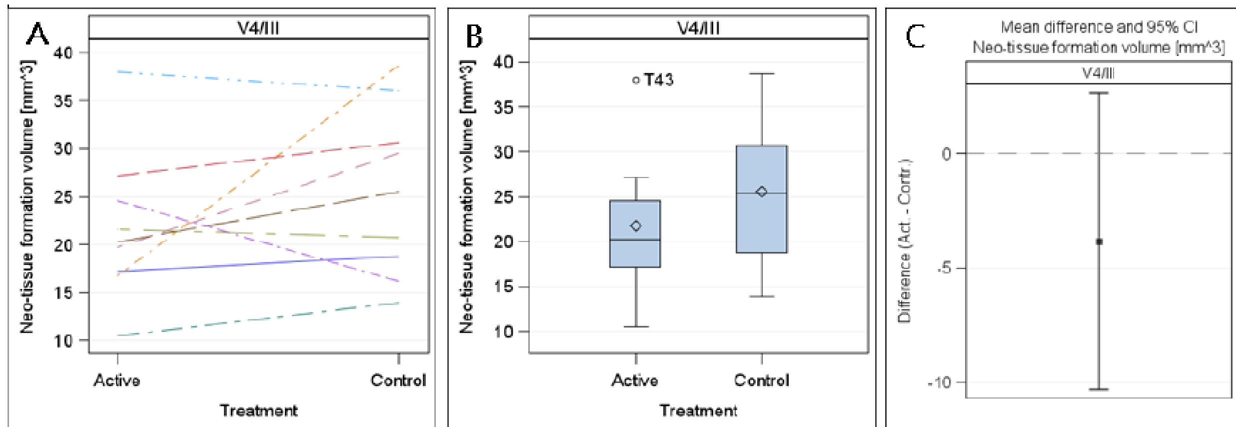
S1	**Systematic pain score**	
Parameter	**Variable**	**Score**
Food and water uptake	eats, drinks, chews the cud	0
	no chewing the cud, reduced appetite	1
	no drinking, no eating, no chewing the cud	2
Behavior and facial expression	awake, alert, lies or stands still	0
	awake, not interested in environment	1
	lethargic, hanging ears	2
	hanging head, very lethargic, eyes almost closed teeth grinding	3
	no reaction to environment, always laying, almost no reaction to external stimulation	4
Respiratory rate	20/min, normal	0
	25 to 50 per cent over normal	1
	> 50 per cent over normal	2
Movement behavior	normal movement, full load-bearing of all joints no lameness	0
	little lameness at operated limb, toe touches the ground every step	1
	lameness at operated limb, toe touches the ground from time to time	2
	lameness at operated limb, toe touches the ground just while walking with the flock	3
	severe lameness at operated limb, toe never touches the ground, neither while walking with the flock	4
Flock behavior	normal moving and acting in flock	0
	small changes, follows the flock	1
	moderate changes, follows the flock while being driven	2
	severe changes, always seperated from flock / no interest in flock	3
Palpation of the wound	no sign of pain	0
	slight signs of pain (skin twitching)	1
	moderate signs of pain (leg withdrawing)	2
	severe signs of pain (leg withdrawn, defensive movements)	3
Joint swelling, increased warmth, soft tissue swelling	no sign	0
	slight signs	1
	severe signs of pain (leg withdrawn, defensive movements)	2
Maximum score		20

S2	**macroscopic knee assessment score**	
Parameter	**Variable**	**Score**
Rubor	no sign	0
	slight signs	1
	severe signs	2
Calor	no sign	0
	slight signs	1
	severe signs	2
Dolor	no sign	0
	slight signs	1
	severe signs	2
Tumor	no sign	0
	slight signs	1
	severe signs	2
Functio laesa	normal movement, no restriction	0
	slight hobbling or slight restriction during stretching	1
	and bending	2
	lameness, no stretching or bending possible	
Maximum score		10

S3	**knee inflammation score**	
Parameter	**Variable**	**Score**
Hypertrophy	no sign	0
	slight signs	1
	severe signs	2
Ectopic cartilage formation	no sign	0
	slight signs	1
	severe signs	2
Delamination of the transplant	no sign	0
	slight signs	1
	severe signs	2
Kissing lesion	no sign	0
	slight signs	1
	severe signs	2
Structural side defects	no sign	0
(f.e. Osteophytes)	slight signs	1
	severe signs	2
Synovial fluid	no sign	0
	slight signs	1
	severe signs	2
Maximum score		10

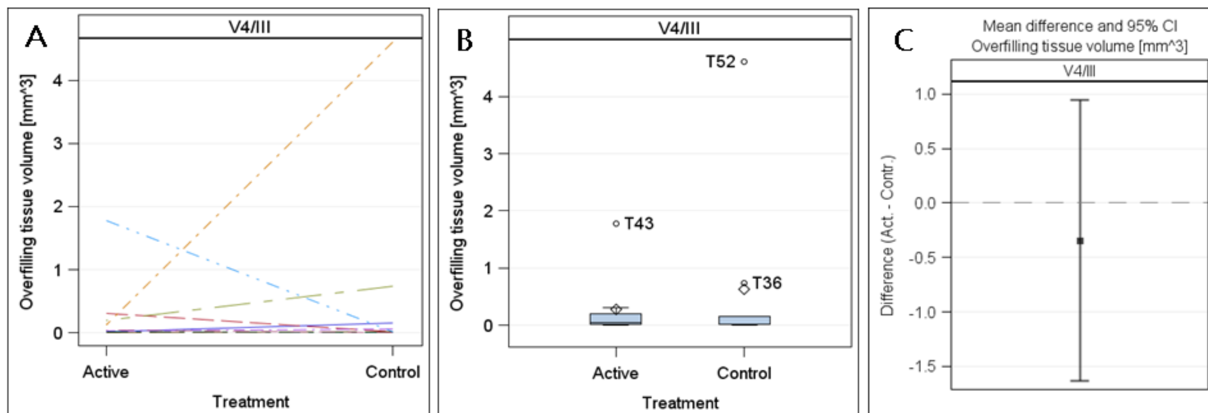
Detailed Ultrasonographic Results

The absolute and relative frequency of the category "Neo-tissue formation volume [mm^3]" are listed in Table 22 of Appendix XXI. The mean ± SD was 21.7 ± 7.8 for Group A and 25.6 ± 8.8 for Group B. There were no significant differences between the groups, with a p-value of 0.212.



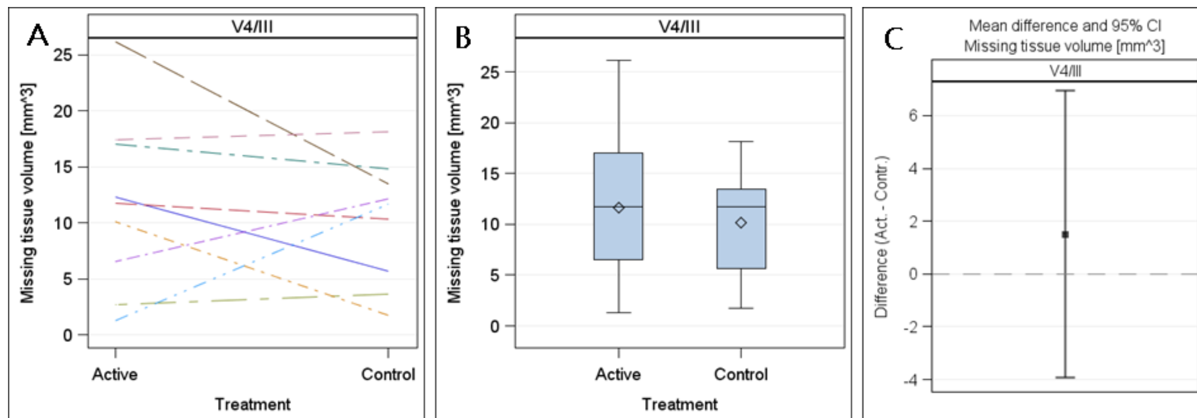
US - Neo-tissue formation volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 – Neo-tissue formation volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 – Neo-tissue formation volume [mm^3] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 – Neo- tissue formation volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0), then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Overfilling tissue volume [mm^3]” are listed in Table 23 of Appendix XXI. The mean ± SD was 0.3 ± 0.6 for Group A and 0.6 ± 1.5 for Group B. There were no significant differences between the groups, with a p-value of 0.556.



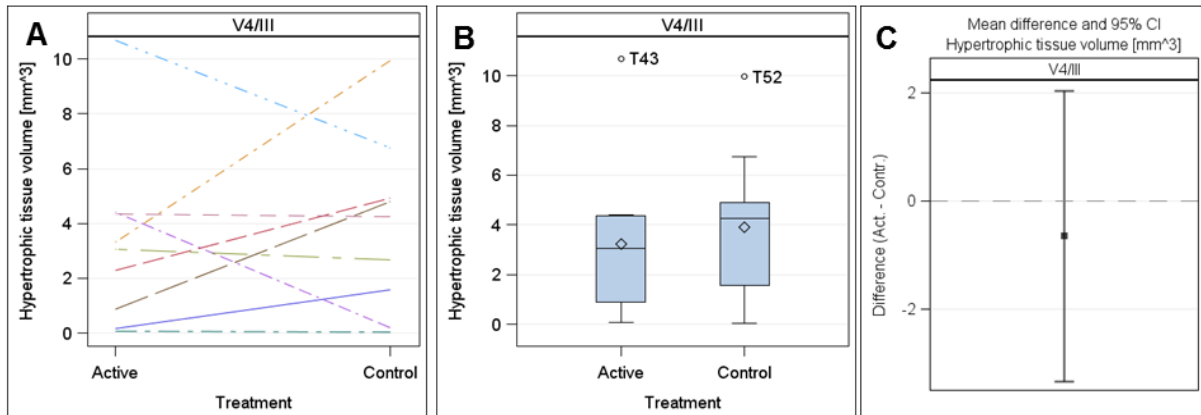
US - Overfilling tissue volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Overfilling tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - score scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Overfilling tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 – Overfilling tissue volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Missing tissue volume [mm^3]” are listed in Table 24 of Appendix XXI. The mean ± SD was 11.7 ± 7.8 for Group A and 10.2 ± 5.4 for Group B. There were no significant differences between the groups, with a p-value of 0.541.



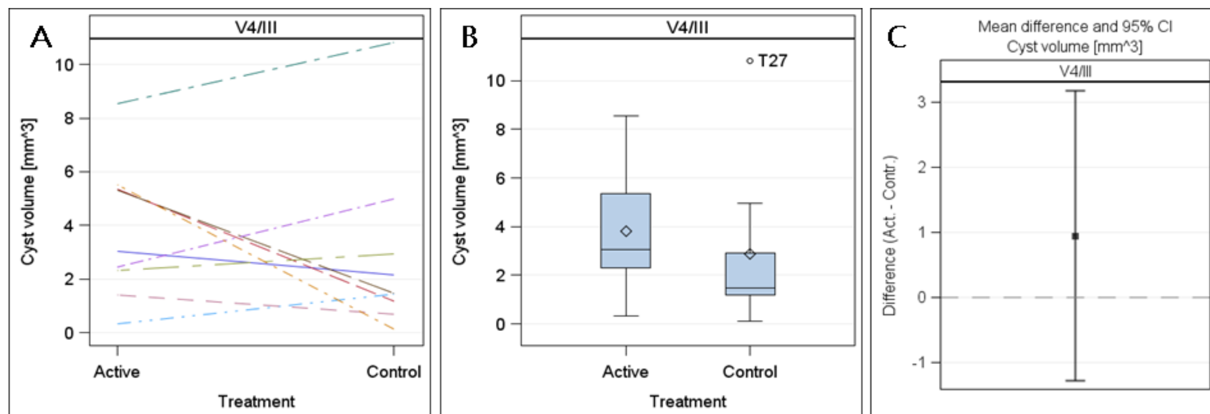
US - Missing tissue volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Missing tissue volume [mm^3 distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Missing tissue volume [mm^3 distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Missing tissue volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Hypertrophic tissue volume [mm^3]” are listed in Table 25 of Appendix XXI. The mean ± SD was 3.2 ± 3.2 for Group A and 3.9 ± 3.2 for Group B. There were no significant differences between the groups, with a p-value of 0.589.



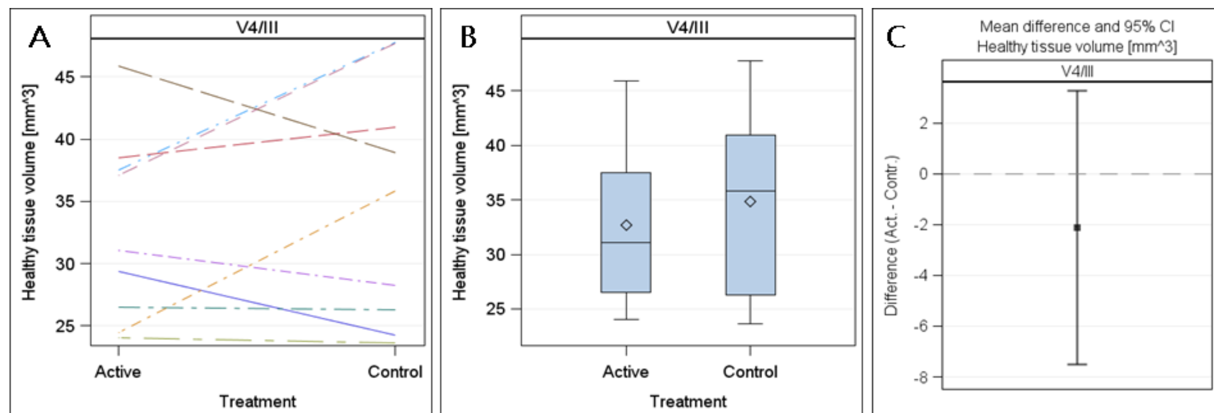
US - Hypertrophic tissue volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Hypertrophic tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Hypertrophic tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Hypertrophic tissue volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category "Cyst volume [mm^3]" are listed in Table 26 of Appendix XXI. The mean ± SD was 3.8 ± 2.6 for Group A and 2.9 ± 3.3 for Group B. There were no significant differences between the groups, with a p-value of 0.357.



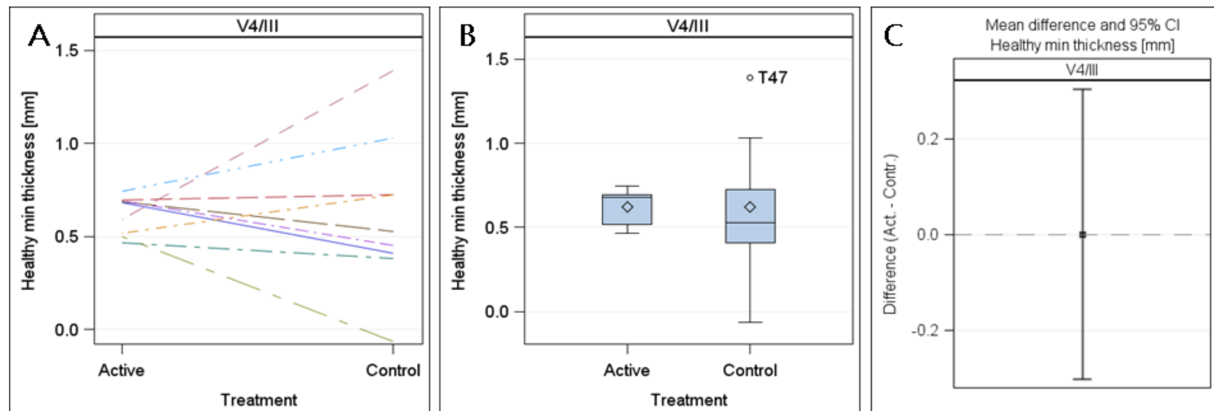
US - Cyst volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Cyst volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Cyst volume [mm^3] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 – Cyst volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Healthy tissue volume [mm^3]” are listed in Table 27 of Appendix XXI. The mean ± SD was 32.7 ± 7.5 for Group A and 34.8 ± 9.6 for Group B. There were no significant differences between the groups, with a p-value of 0.390.



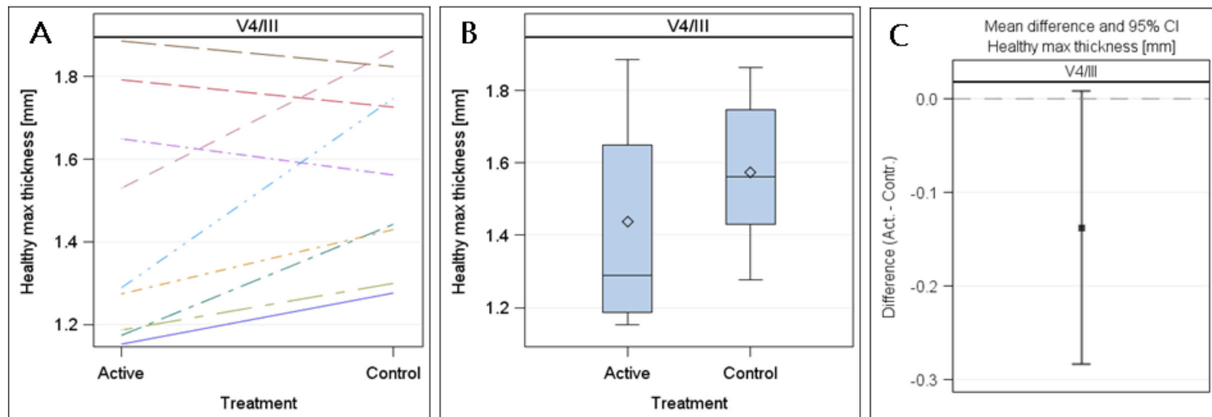
US - Healthy tissue volume [mm^3] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Healthy tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Healthy tissue volume [mm^3] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Healthy tissue volume as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Healthy min thickness [mm]” are listed in Table 28 of Appendix XXI. The mean ± SD was 0.6 ± 0.1 for Group A and 0.6 ± 0.4 for Group B. There were no significant differences between the groups, with a p-value of 0.999.



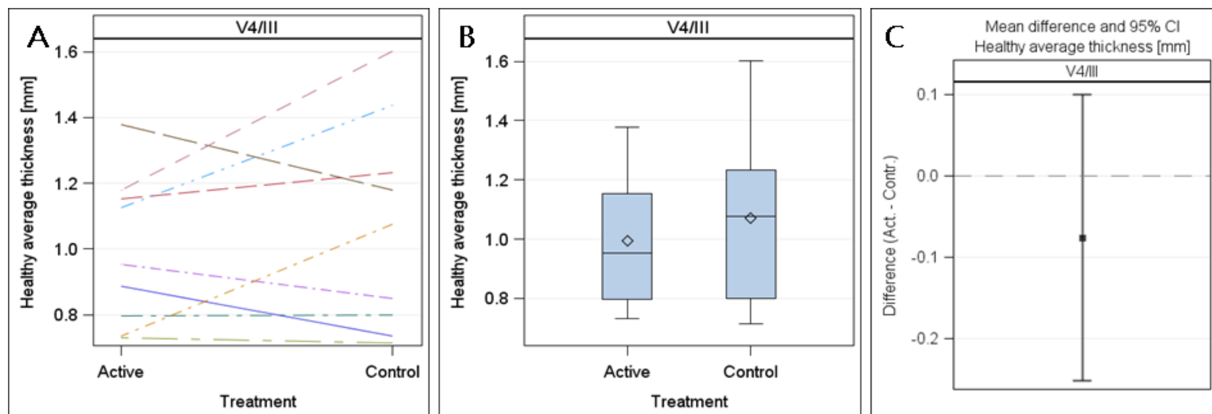
US - Healthy min thickness [mm] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Healthy min thickness [mm] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Healthy min thickness [mm] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Healthy min thickness as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given $\alpha=0.05$) of treatment-related difference is shown at the respective time point.Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category “Healthy max thickness [mm]” are listed in Table 29 of Appendix XXI. The mean ± SD was 1.4 ± 0.3 for Group A and 1.60 ± 0.2 for Group B. There were no significant differences between the groups, with a p-value of 0.062.



US - Healthy max thickness [mm] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Healthy max thickness [mm] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Healthy max thickness [mm] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Healthy max thickness as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

The absolute and relative frequency of the category "Healthy average thickness [mm]" are listed in Table 30 of Appendix XXI. The mean ± SD was 1.0 ± 0.2 for Group A and 1.1 ± 0.3 for Group B. There were no significant differences between the groups, with a p-value of 0.346.



US - Healthy average thickness [mm] at V4/III, at sacrifice for control and treatment groups (PP). (A) Spaghetti plot, (B) Box plot, (C) Error Bar. The spaghetti plot displays the E6 - Healthy average thickness [mm] distribution as determined at sacrifice (time point of explantation). The lines represent a direct comparison of the individual E6 - scores within one animal between control (untreated) and active (treated with ACT3D-S) treatment. The box plot displays the E6 - Healthy average thickness [mm] distribution as determined at sacrifice (time point of explantation). The bottom and top edges of the box indicate the intra-quartile range (IQR), the line inside the box indicates the median and the diamond shows the mean value. Whiskers extend to either the minimum/maximum value observed or a distance from the box of 1.5*IQR (whichever is nearest). Values that are a distance of more than 1.5*IQR from the box (outliers) are displayed as dots. The error bar plot displays the mean paired difference between treatments (Active – Control) for the E6 - Healthy average thickness as determined at sacrifice (time point of explantation). The dotted line at 0 represents the control (untreated) group, and the treatment group is expressed in comparison to this line. The error lines represent the 95 % confidence intervals for the mean paired differences between the treatments. If the confidence interval does not include the zero value (is completely below or above 0) then a statistical significance (given α=0.05) of treatment-related difference is shown at the respective time point. Active: ACT3D-S treatment; control: untreated control. Time point of scoring: V4/III, sacrifice, third surgery.

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11 Declaration

I formally declare that I have written the submitted dissertation independently and that I did not use any outside support except for the quoted literature and other sources mentioned in the paper. I herewith assure that no third parties have received either direct or indirect financial benefits from me for work connected with the submitted dissertation. The dissertation was not used in the same or in a similar version to achieve an academic grading and was not submitted to any other examination authority or published so far neither in Germany nor abroad. I have clearly identified and separately listed all of the literature which I employed for producing the dissertation, either literally or in content. All persons are named who were themselves directly involved or have contributed to the work. I also herewith declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current rules of the University of Leipzig [Vorlesung zur "Guten Wissenschaftlichen Praxis"].

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Date Signature

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