Universiteit Utrecht

A TREATMENT BETWEEN PAINKILLERS AND SURGERY

The effect of intradiscal injection with celecoxib loaded microspheres in dogs suffering from lower back pain.

RESEARCH INTERNSHIP, MASTER VETERINARY MEDICINE OF COMPANION ANIMALS.
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Front page photo adapted from Dogopedia.[54]

Index

Abstract

The intervertebral disc (IVD) often displays degenerative changes and is a frequent cause of back problems recognized in non-chondrodystrophic dogs. Systemic NSAIDs have proven to be an insufficient treatment due to side effects and an inability to reach the IVD. Intradiscal injection of celecoxib (CXB) with a local release system such as polyesteramide microspheres (PEAM) could solve these challenges. The aim of this study was to investigate the regenerative and analgesic effects of this treatment in non- chondrodystrophic canines suffering from low back pain due to IVD degeneration and to evaluate the clinical efficacy. This research was conducted as a prospective, randomized, double-blinded, placebo- controlled clinical trial. Client owned dogs suffering from degenerative lumbosacral stenosis (DLSS) were divided into two groups. They were injected with either PEAM-CXB (n=20) or unloaded PEAM (placebo, n=10). This research was assessed after 6 and 12 weeks by clinical examination, MRI and owner questionnaires. Long term evaluation was done with owner questionnaires and telephone interviews. The study showed that intradiscal injection with PEAM-CXB had long lasting analgesic and anti-inflammatory effects and thus reduced the symptoms of IVD degeneration. Furthermore, this study demonstrated that this treatment is clinical efficient as it confirmed the safety of the treatment due to no indication of adverse effects short or long term. However, additional research has to be done to evaluate if intradiscal injection with PEAM-CXB results in the regeneration of the IVD.

Introduction

Dogs have 7 cervical, 13 thoracic, 7 lumbar, 3 sacral and circa 20 caudal vertebrae.[1] Between the vertebral bodies of C2-S1 and all the caudal vertebrae lie intervertebral discs (IVD).[1,2] Intervertebral discs are intended to bear compressive forces to which the spine is subjected and are involved in the flexibility of the spine. This is possible due to the morphology of the disc; it has a cushion-like centre and dense bundles of fibrous tissue surrounding it, the nucleus pulposus (NP) and the anulus fibrosus (AF), respectively.[1] The IVD often displays degenerative changes and are a frequent cause of back problems recognized in non- chondrodystrophic dogs.[1,3] Bergknut et al. (2013) reviewed research about IVD degeneration and describes it as:

> *An aberrant, cell-mediated response to progressive structural failure of the IVD and is associated with genetic predisposition, chronic physicomechanical overload and trauma, inadequate metabolite and nutrient transport to and from the cells within the IVD matrix, cell senescence and death, altered levels of enzyme activity, changes in matrix macromolecules, and changes in water content.*[2]

Although IVD degeneration is a common spinal disorder in dogs, there has not been merely as much research in canines as in humans. However, it has been proven that the canine IVD and the human IVD have a lot of similarities and that they can serve as a model for each other.[4]

Intervertebral disc degeneration

The IVD can be subjected to various loading forces, specifically axial compression, shear, tension, bending and torsion. The NP, AF, and endplates (EP) act as a team to oppose these forces. Trauma, chronic loading, genetic deposition and/or aging can instigate degeneration in these components which can ultimately lead to disease.[2]

Nucleus pulposus

The NP is located at the centre of the IVD and is a transparent and hydrated structure (Figure 1). The notochordal cell is the primary cell of the NP that regulates osmotic stresses and generates a formless basophilic matrix abundant with proteoglycans and collagen type II. These proteoglycans consist of a protein backbone with negatively charged glycosaminoglycan (GAG) side chains, which connect with

hyaluronic acid. These negatively charged complexes provide a strong osmotic gradient that attracts water into the NP. This creates a well-hydrated nucleus that attains a high intradiscal pressure, which can oppose the compressive stress that the disc has to endure. During degeneration the NP's cells change from notochordal to chondrocyte-like cells. This chondrification will result in an inability to produce the physiologic matrix composition.[2] This impairs the discs ability to attract water due to a decrease of proteoglycans and an increase of the coarser collagen type I.[2,5] The NP loses its function as a hydraulic cushion, and cannot properly withstand loading forces anymore (figure 1).[2]

Annulus fibrosus

The surrounding AF can keep the NP in place during compressive forces on the spine with its structured and strong fibrous lamellae. The AF also protects the NP against shearing caused by the applied burden and its own swelling pressure. Simultaneously, with the deterioration of the NP, the AF's collagen content increases and changes into a more rigid structure. The lamellar fibres become disorganised and more chondrocyte-like cells will form. The fibres that make the AF strong may rupture, which would result in fissures of the AF. The AF will be less capable in keeping the NP in place during loading and can cause additional stress on the NP which will aid in the degeneration process of the NP.[2]

End plates

The IVD is surrounded by the cartilaginous endplates (EP) on the adjacent vertebral bodies which contain the IVD. The EPs have capillary buds that supply the disc with nutrients and oxygen via diffusion and osmosis. Proteoglycans in the EP control the shipping of solutes in and out of the IVD which is crucial for preserving the integrity of the IVD.[2] Not much is known about the effect of IVD degeneration on the EP in dogs, though more research has been done on humans. The degeneration process in the EP of humans will cause a decrease in water, collagen and proteoglycan content and in later stages mineralization. This will result in obstruction of the capillaries and the transport of nutrients and oxygen to the disc which will impair any chance of regeneration and furthers degeneration. In later stages can degeneration lead to fractures in the EP.[2,6] Although the human IVD has shown to have a lot of similarities with canine IVDs, the human IVD is thicker, which might mean a difference in pathology.[4]

Inflammation

The NP is aneural and avascular due to the matrix with proteoglycan which prevents the ingrowth of nerves and capillaries.[7] The innervation and vascularization can mostly be found in the outer layers of the AF.[2] The nerve endings will extend further into the AF and NP during degeneration. This is the result of an increase of inflammatory mediators that promote the expression of nerve growth factors, and the destruction of the barrier of proteoglycans in the NP.[8] Stimulation of these nerve endings will lead to discogenic pain which contributes to the lower back pain that canines with IVD degeneration experience.[3] This stimulation is induced by inflammatory mediators, specifically prostaglandin E2 (PGE2), derived from cyclooxygenase-2 (COX-2). Several interleukins (IL) and tumour necrosis factor alfa (TNF-alfa) could also have an influence.[8-11]

The inflammatory mediators play an additional role in the catabolic process of IVD degeneration. They can induce the expression of matrix degrading enzymes and inhibit extracellular matrix synthesis. These matrix degrading enzymes or proteases have a physiological role in the destruction and the remodelling of the proteoglycans and collagen of the disc. These proteases include matrix metalloproteinases (MMP), disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and cathepsins. The proteases are upregulated during degeneration with help from the inflammatory mediators, resulting in catabolic effects.[10]

Further degeneration

The degeneration processes in the individual structures of the IVD will result in the decrease of the integrity of the disc as a whole. The dehydration and the breakdown of the disc causes a decrease in disc height, functionality, and flow of nutrients. The combination of an impairment of repair due to the avascular structure of an IVD along with physiological loading will result in a vicious cycle of no repair and more degeneration.[2] The weakened IVD can partially rupture and protrude dorsally. This herniation at the lumbosacral junction can lead to bulging of the dorsal longitudinal ligament on the cauda equina resulting in additional lower back pain. This compression of the nerves of the cauda equina can in severe cases result in lameness or paresis of the hind limbs, and faecal or urinary incontinence.[12] The tearing of the disc can result to more exposure of the neural endings and more inflammation which can contribute to the pain.[11,13] Instability at the lumbosacral junction as a consequence of the IVD degeneration can cause the EPs to proliferate. This will result in the development of osteophytes and ventral spondylosis. The proliferations will enhance the decrease of nutrient flow to the damaged IVD and further degeneration. These complications consisting of the Hansen type II herniation, cauda equina compression, osteoarthritic changes and bone sclerosis/ spondylosis, with the IVD degeneration at L7-S1 are a part of the degenerative lumbosacral stenosis (DLSS) syndrome.[2,3,12,14]

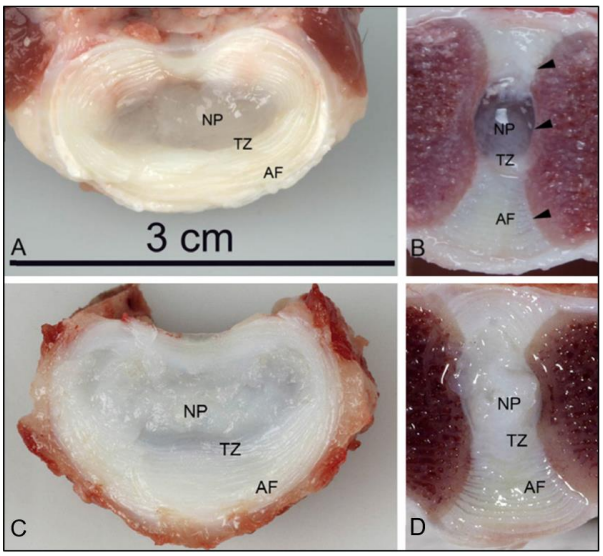

Figure 1: Top: Transverse (A) and sagittal (B) sections through a L5–L6 intervertebral disc of a mature non-chondrodystrophic dog, showing the nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF), and endplates (arrowheads). Bottom: Transverse (C) and sagittal (D) sections through the L5–L6 intervertebral disc of a 2-year-old chondrodystrophic dog, showing a dorsally located, drier nucleus pulposus (NP), a widened transition zone (TZ), and a normal annulus fibrosus (AF). This is similar to the IVD degeneration in non-chondrodystrophic dogs, but typically occurs later in life. Figure adapted from Bergknut et al. (2013) and Smolders et al. (2013).[2,3]

Non- chondrodystrophic dogs

Large, non- chondrodystrophic (NCD) dog breeds are specifically susceptible for IVD degeneration at the lumbosacral junction (L7-S1). This is particularly the case for the German Shepherd and Labrador retrievers.[3] Multiple characteristics of the L7-S1 junction of NCD breeds can contribute to a high workload and more wear and tear on the junction that could lead to IVD degeneration. The L7-S1 junction is for example more mobile compared to other junctions which can cause friction. The L7-S1 IVD also presents a low ventrodorsal shear stiffness, which can prompt a subluxation ventrodorsally. The L7-S1 facet joint has a slanted orientation compared with the neighbouring spinal segments which causes a disproportionally high workload on the L7-S1 IVD. Furthermore, there is an imbalance

between the dimensions of the L7-S1 IVD, facet joints and the bodyweight in large breeds compared to small breeds.[3]

Symptoms

Although IVD degeneration could be asymptomatic, some common symptoms can still arise. Dogs with IVD degeneration often have lower back pain.[12] This pain is thought to originate from the degenerating disc and can subsequently result from compression of the cauda equina seen with DLSS.[12,15] This pain can be evoked during clinical examination by applying pressure on the lumbosacral region. Other symptoms are lameness and weakness of the hind limbs, which can be recognised by a non-weight bearing hind limb or dragging of the toes.[12] These symptoms can worsen after exercise.[15] Dogs often have hypersensitive skin on the lower back and hind limbs, which can be observed by vocalisation and resistance from the dog when touched in this area or even self-mutilation. Additional symptoms that can be observed in the case of cauda equina compression are compromised tail movement or stance and urinary or faecal incontinence.[12,15]

Treatment

Current treatment of lower back pain in dogs due to IVD degeneration consists of a modified exercise regimen to reduce the loading on the disc and administration of analgesic drugs. The exercise regimen should consist of regular short walks and working dogs should have reduced work demands. If a dog with IVD degeneration is obese then weight reduction should also be implied to reduce the strain on the back. Drugs that are often used to reduce lower back pain are systemically administered nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids, which can be given locally in the epidural space as an injection. Surgery could be considered in case of severe DLSS and failure of conservative therapy, this most commonly involves a dorsal laminectomy.[12]

Several NSAIDs that are often used are specific cyclooxygenase-2 (COX-2) inhibitors which have fewer side effects than non-selective NSAIDs. These drugs inhibit COX-2 and thus the production of the inflammatory mediator PGE2. As discussed, inflammation has a significant role in IVD degeneration, which is why these anti- inflammatory drugs are used.[9,16-18] However, there are risks with administrating COX-2 inhibitors systemically. The drugs could cause several side-effects, have incompatibility with other drugs and have trouble penetrating into the avascular IVD, making it an insufficient treatment. This treatment does not intervene with the degeneration process, the preservation of the disc or induce possible regeneration of the IVD.[16,19-22]

Injection of a COX-2 inhibitor such as celecoxib (CXB), directly into the disc with a controlled release system could solve these challenges (figure 2).[9,16,22,23] A controlled release system for CXB is required to safely facilitate a higher dose in the disc, to release CXB over a prolonged period of time and to reduce the amount of re-injections and systemic side-effects. [16,22-24] previous research showed the effects of a release system with a poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-bpoly(ε-caprolactone-co-lactide) PCLA–PEG–PCLA hydrogel that released CXB. This proved to reduce clinical signs such as pain, though no regenerative effects were observed.[16]

The controlled release system used in this research are microspheres based on biodegradable polyester amides (PEAM). These microspheres degrade in a controlled manner and can therefore carefully release a loaded substance without depositing toxic by-products. These PEAM's degrade due to enzyme reactions, which means that this system is autoregulatory due to the enzymatic environment in a degenerating IVD.[9] This entails that when more inflammation develops in an IVD, more CXB will be released. This is a result of the upregulation of proteases through inflammatory mediators, consequently controlling the inflammation.[8,9] Locally controlled drug delivery with PEAMs has proved to be promising to this end. It is known that PEAM loaded with CXB (PEAM-CXB) could

decrease PGE2 production *in vitro* and *in vivo* and that it can suppress the effects of induced degeneration *in vivo* in a canine model of IVD degeneration.[9] These studies paved the way for this current research on a clinical level.

Intradiscal injection does come with risks such as an induce of degeneration with the puncture itself, a too large needle size or a injected volume that is too high.[23,25-27] Willems et al. (2017) found that a 40µl volume with a 26 or 27 G needle did not induce degeneration and that it is safe to inject into the discs of canines. The use of PEAMs for intradiscal injection was also concluded to be safe to use in degenerated IVDs of canines.[23]

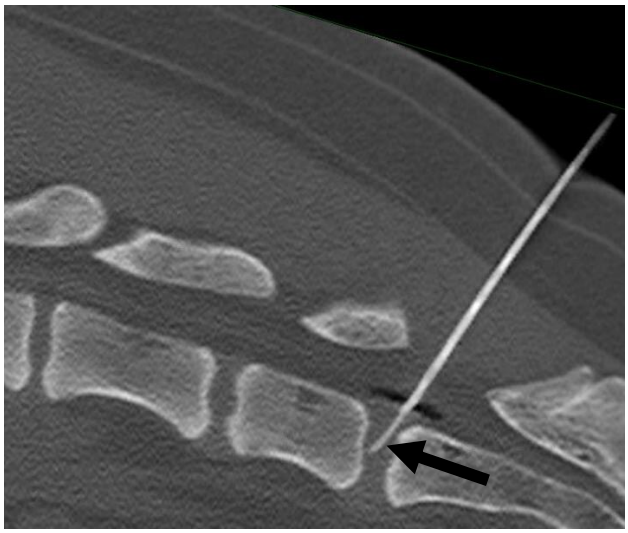

Figure 2: Laterolateral computed tomographic (CT) image obtained during intradiscal injection. Remark that the 20 G needle is advanced into the epidural space and that the 27 G needle (arrow) is positioned in the centre of the nucleus pulposus.

Aim of the study

The aim of this study is to investigate the regenerative and analgesic effects of intradiscal injection with celecoxib loaded microspheres in non- chondrodystrophic canines suffering from lower back pain due to IVD degeneration and to evaluate the clinical efficacy of this treatment. For this purpose, client owned dogs suffering from DLSS due to IVD degeneration were recruited with the owner's consent into a study with two arms: intradiscal injection of PEAM-CXB and unloaded PEAMs (placebo).

Material & methods

Study design

This research was conducted as a prospective, randomized, double-blinded, placebo- controlled clinical trial. The study was conducted upon approval of the Ethical Committee (trial number AVR 18-10, approval date 29-12-2017). Thirty dogs were included in the study with the inclusion and exclusion criteria and informed consent of the dog owners (table 1). The dogs were separated in a placebo and treatment group (PEAM-CXB). The allocation of the dogs was not known by the owners or the veterinarians until the 12 weeks follow-up. Twenty dogs were placed in the treatment group and were injected with polyesteramide microspheres loaded with celecoxib (23,3 mg/mL PEAMs with 20wt% celecoxib) in the IVD. The microspheres were synthesized according to previously reported protocols.[9,22] Ten dogs were included in the placebo group and were injected with microspheres only (23,3 mg/mL PEAMs). The dogs were distributed in weight groups to conclude injection volume: 15-30; 30-45;>45 kg 75 µL, 100 µL, 125 µL.[22] The study design with follow-up procedure is summarized in a flow chart (figure 4).

Table 1 : Inclusion and exclusion criteria for participation in the prospective clinical study. Table adapted from Tellegen et al., (2018). [22]

Inclusion criteria	Exclusion criteria
History of lower back pain for at least 6 weeks	Previously performed surgery on IVD
Refractory to oral pain medication for >4 weeks	Active discospondylitis or infection (i.e. pyoderma)
Side effects of oral pain medication	Lumbosacral fracture
Pfirrmann grade II-IV on T2-weighted MRI	Spinal neoplasia
Body weight > 12 kg	Severe extrusion of the IVD

The intradiscal injection

The procedure was carried out under general anaesthesia. Premedication consisted of butorphanol 0.2 mg/kg & dexmedetomidine 5 µl/kg, induction with intravenous propofol (1 mg/kg) and maintenance with isoflurane (2%) through a endotracheal tube.[22] The intradiscal injection was performed with a through the needle technique: with the patient in sternal recumbency and the hind limbs extended cranially, a 20 G epidural needle (4509757-13, Braun, Melsungen, Germany) was aseptically inserted. CT guidance (Siemens Somatom Definition AS, Siemens Healthcare) was used to check the depth and placement of the needle. The 20 G epidural needle was progressed through the ligamentum flavum until the dorsal AF was reached. The stylet was removed and a 12 cm long 27 G needle (7803-01 Hamilton, Bonaduz, Switzerland) was inserted through the epidural needle and pushed through the dorsal AF into the centre of the NP (figure 2). Positioning was again confirmed with CT. Lastly, a 100 µl gas tight syringe (7656-01 Hamilton, Bonaduz, Switzerland) was connected to the 27 G needle, and celecoxib-loaded microspheres or unloaded microspheres were gently injected.[16,22] After injection the needle was slowly retracted through the AF, letting the collagen fibres close behind the needle to prevent leakage of the injectate.[22]

Magnetic resonance imaging

The diagnosis of DLSS and IVD degeneration was based on the clinical picture including lower back pain and signs of DLSS on MRI scans. Therefore all dogs received a MRI scan at baseline (T = 0). To detect possible side effects and to observe injection effects on the degenerative process, the dogs that received the PEAM-CXB injection had a follow-up MRI after 3 months (T = 3). The treating veterinarian and dog owner were also informed on treatment allocation after 3 months. Follow-up was conducted on the 20 dogs that had received the PEAM-CXB treatment. Both the intervertebral discs at L6-L7 and L7-S1 were included to compare the treatment effect in L7-S1 to the adjacent disc. The MR images were taken with a high field 1.5T MRI unit (Ingenia, Philips, Best, The Netherlands). The MRI protocol included a sagittal T2-weighted Turbo Spin Echo (repetition time (TR) = 2500, echo time (TE) = 110 ms), a fat-suppressed T1-weighted Turbo Spin Echo using spectral presaturation (TR = 400 ms, TE = 8 ms), and a quantitative multiple spin-echo T2-mapping sequence for T2 mapping with a field of view (FOV) = 75 x 219 mm, acquisition matrix = 96 x 273, slice thickness = 3 mm, TR = 2000. Eight echoes were acquired with TE = 13 to 104 ms with 13 ms echo spacing. The scans were carried out with the patients under general anaesthesia according to standard protocols, positioned in dorsal recumbency with the pelvic limbs extended caudally.[9,22] All images were assessed by a board-certified veterinary radiologist. The grade of IVD degeneration was determined with the Pfirrmann grading system on the T2-weighted images (figure 3).[22,28] Mean T2 relaxation times were determined in an oval region of interest (ROI) in the L6-7 and L7-S1 IVDs on midsagittal T2-mapping images before the injection and after 3 months.[22]

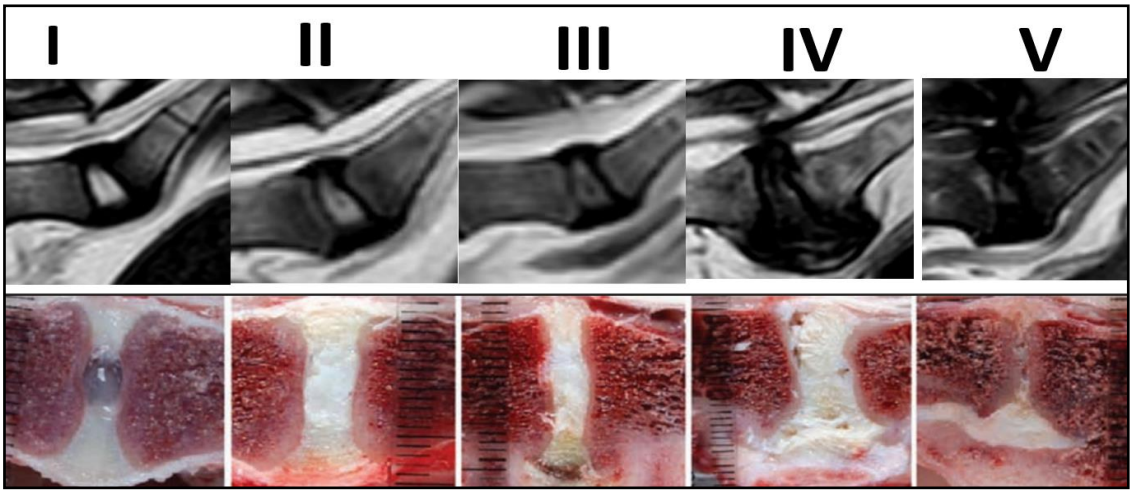

Figure 3: The top row depicts the Pfirrmann grading system with midsagittal high field MR images obtained from representative dogs. The bottom row shows the Thompson grading system with midsagittal photographs obtained from representative dogs. Grade 1 represents healthy IVDs to grade 5 that represents the end stage of IVD degeneration.. Figure adapted from Bergknut et al., 2011.[29]

Outcome

A questionnaire (scale 0-10 from worst to best; appendix 1) that gave more insight regarding behaviour and function of the dogs with lower back pain due to DLSS were supplied to the owners before treatment and after 6, 12 weeks and at long-term follow-up. The questionnaire has been validated and used in related studies.[16,30,31] Owners were allowed to administer pain medication that was given prior to inclusion if they observed an increase in lameness and incapacity to perform daily activities in their dogs for several consecutive days. If the owners needed guidance, they could call or email the study coordinators (A.R.T., T.W.) or arrange an extra outpatient visit.[22] Long-term follow-up of approximately 1-3 years involved a telephone interview and the same questionnaire, with the purpose of investigating if any long-term effects of the treatment and injection were observed. The interview consisted of questions about the wellbeing, activity, current medication, other treatments, and the opinion of the owner on the injection and the use of anaesthesia (appendix 2).

Statistical analysis

Descriptive statistics were calculated in terms of means and standard deviation (SD) for continuous variables including age, body weight and baseline questionnaire answers. Median and range were calculated for the discrete Pfirrmann grading variables. Statistical analysis was conducted on the results of the owner questionnaires and MRI data with the use of GraphPad. Group differences for the continuous variables were evaluated using independent sample *t*-tests ($P < 0.05$).[32] *P*-values < 0.05 were considered significant. *P*-values between 0.05 and 0.10 were considered to have a tendency of significance. The data from the T2-mapping, Pfirrmann grading and questionnaires was examined to determine if it was normally distributed. This was confirmed by assessing the Q-Q plots and Shapiro-Wilks tests. The T2 relaxation times and Pfirrmann scores at baseline were tested for correlation with a Pearson test and differences with a Mann-Whitney test to explain the use of T2 mapping. A Wilcoxon test was performed on the T2 relaxation times of the L7-S1 IVD injected with PEAM-CXB and of the L6-7 IVD to compare the data from baseline (T = 0) and after 3 months (T = 3). The difference in T2 relaxation times of L7-S1 IVD between baseline and T = 3 was compared to the baseline Pfirrmann grades with a Spearman correlation test. Wilcoxon tests and paired *t*-tests (if the data was normally distributed) were executed on the questionnaire data to compare baseline (T0) to the data after 6 weeks (T6), 12 weeks (T12) and long term (LT) per question.

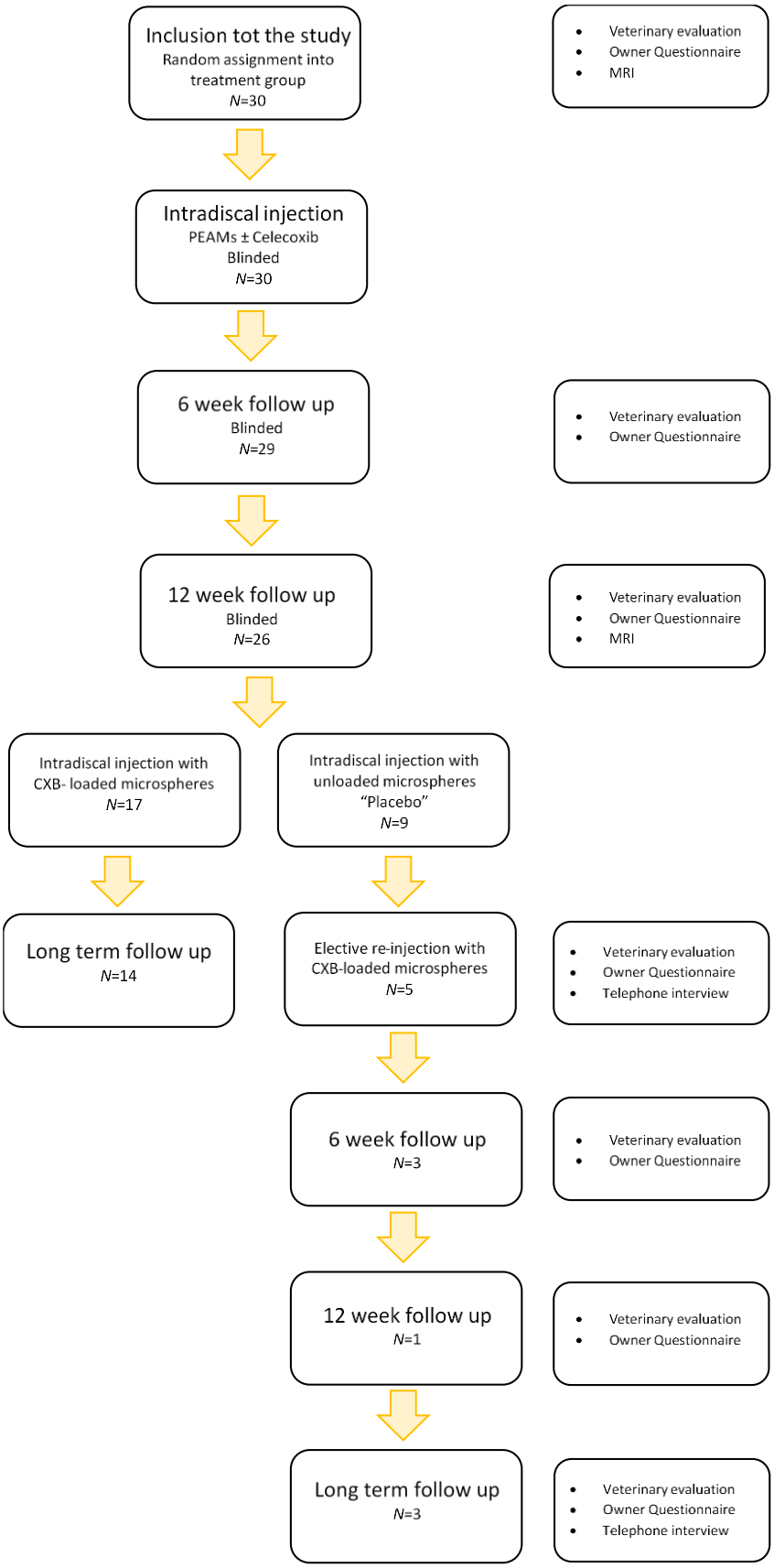

Figure 4: Flow chart of the prospective, randomized controlled study evaluating the clinical efficacy of intradiscal injection of celecoxib-loaded or unloaded polyesteramide microspheres.

Results

The study population at baseline

From 2017 to 2019 were several dogs assessed for lumbar back pain and 30 met the inclusion criteria (table 1). Twenty dogs were randomized into the PEAM-CXB group and 10 into the placebo group. The study population was analysed on patient details at baseline (table 2). Age and body-weight differences were not significant between the groups (P = 0.6 and P = 0.6, respectively). The male:female ratio in this study was 18:12. The median of the Pfirrmann grades determined at baseline were not different between the two groups. The distribution of the dogs over 19 different breeds (table 3) demonstrated that the retriever breed or mix was most abundant. Descriptive analysis was done for each question of the questionnaire at baseline (table 2). The means of the scores for 'hind limbs lameness' (P = 0.01), 'hind limbs weakness' (P = 0.04) and 'difficulty getting up' (P = 0.02) are significantly larger for the placebo group compared to the PEAM-CXB group. The mean of the scores for 'position of the tail' (P = .01) are significantly larger of the PEAM-CXB group compared to the placebo group. All dogs showed uneventful recovery after intradiscal injection.

Table 2: Descriptive statistics of the study population and the questionnaire subjects that were answered on a scale from 1-10 from worst to best. The population was divided in two groups: one that received an injection with polyesteramide microspheres loaded with celecoxib (PEAM-CXB) and one with only polyesteramide microspheres (Placebo). Analysis with independent sample t. tests were P<0.05 considered to be significant. Mean and SD or other if indicated. F: Female, FN: Female Neutered, M: Male, MN: Male Neutered.

Baseline characteristics	**PEAM-CXB (n = 20)**	**Placebo (n = 10)**	**P**
Age	5.0 ± 2.5	4.3 ± 2.4	0.466
Body weight	28.0 ± 7.0	29.0 ± 9.9	0.636
Sex (frequency)	F: - FN: 10 M: 3 MN: 7	F: 1 FN: 1 M: 4 MN: 4	
MRI Pfirrmann grade (median, range)	3, 4-2	3, 3-1	
Questionnaire			
Hind limb lameness	5.1 ± 2.2	7.3 ± 2.1	0.012
Hind limb weakness	6.3 ± 3.1	9.0 ± 2.4	0.039
Lower back pain	4.3 ± 2.3	5.3 ± 2.0	0.235
Difficulty getting up	5.7 ± 2.7	8.1 ± 1.9	0.016
Difficulty lying down	6.5 ± 2.6	8.0 ± 1.9	0.110
Hind legs muscle build-up	7.7 ± 2.2	7.7 ± 1,7	0.929
Position of the tail	8.8 ± 1.7	6.4 ± 3.2	0.012
Tail wagging	8.7 ± 1.9	7.9 ± 2.1	0.300
Urinary and faecal incontinence	8.9 ± 2.6	9.4 ± 1.1	0.523
Hypersensitivity lower back skin	6.2 ± 3.2	6.6 ± 2.9	0.741

Table 3: Frequency table of the patient's breeds. The population was divided in two groups: one that received an injection with polyesteramide microspheres loaded with celecoxib (PEAM-CXB) and one with only polyesteramide microspheres (Placebo).

Breed	PEAM-CXB	Placebo
Labrador Retriever	5	4
Labradoodle	2	-
Flatcoated Retriever	1	-
Cross breed Labrador	-	1
Cross breed Golden Retriever	2	1
Goldendoodle	1	-
Cross breed	1	-
Old English Bulldog	1	-
Weimaraner standing	1	-
Rottweiler	1	-
German Shepherd	1	-
Cross breed German Shepherd	1	-
Perro de agua Espanol	1	-
Small Münster Länder	1	-
English Cocker Spaniel	1	-
Rhodesian Ridgeback	-	1
Beagle	-	1
Border Collie	-	1
Basset Hound	-	1

MRI: Pfirrmann grading and T2 relaxation times

The T2 relaxation times had an indirect correlation with the Pfirrmann grades at baseline ($P < 0.0001$), which means that the Pfirrmann grades increase as the T2 relaxation times decrease (Figure 5A). At baseline T2 relaxation times between PEAM-CXB and placebo did not differ from each other (Mann-Whitney test $P = 0.6628$). The PEAM-CXB treated L7-S1 discs showed lower mean T2 relaxation times than the adjacent discs (L6-7) at baseline (Figure 5B).

The T2 relaxation times of the PEAM-CXB treated L7-S1 discs showed a tendency of a significant decrease ($P = 0.0656$) at T = 3 compared to baseline with a Wilcoxon test. The T2 relaxation times of the L6-7 adjacent discs at baseline and T = 3 were also compared with a Wilcoxon test and did not significantly change in time ($P = 0.7917$). The data point cloud of the L7-S1 discs at T = 3 appeared to be lower compared to baseline (Figure 5B). This was clarified with the difference in time of the T2 relaxation times of each disc, which showed that the T2 relaxation times of the PEAM-CXB treated L7-S1 discs indeed decreased after 3 months (Figure 6A). The difference in time also confirmed that the T2 relaxation times of the adjacent L6-7 discs did not decrease. The difference in time of the T2 relaxation times of the PEAM-CXB group was also compared to the Pfirrmann scores from baseline. The significant Spearman correlation of r = 0.5872 ($P = 0.0155$) showed that the difference in time of the T2 relaxation times were directly correlated with the Pfirrmann scores. This indicates that the decrease in T2 relaxation times between baseline and T = 3 was larger in discs graded with a lower Pfirrmann grade and that discs with a higher Pfirrmann grade increased in T2 relaxation times (Figure 6B).

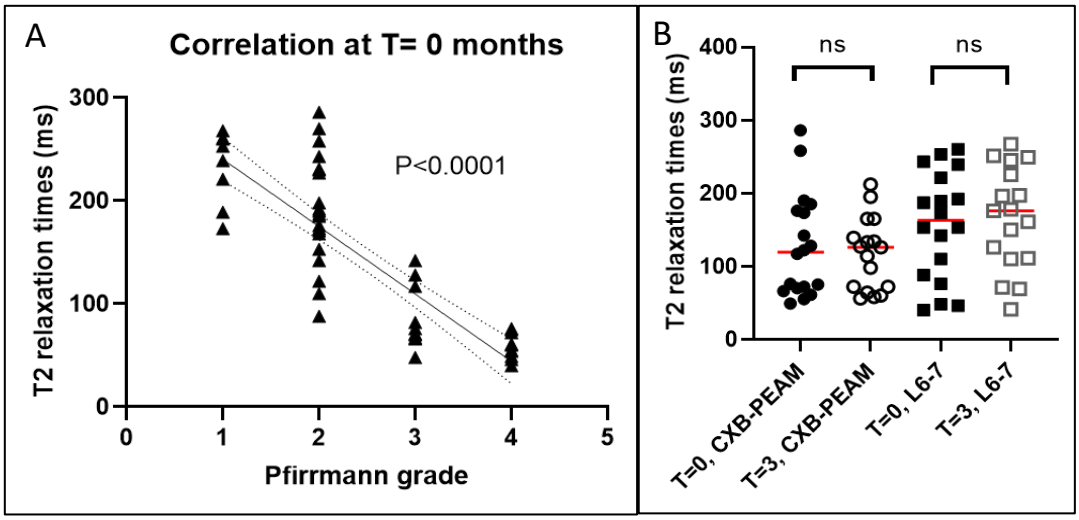

Figure 5: A: the correlation between T2 relaxation times and Pfirrmann grades at baseline (T = 0) of all L7-S1 discs. B: T2 relaxation times of the L7-S1 discs that received an injection with polyesteramide microspheres loaded with celecoxib (CXB-PEAM) and the adjacent L6-7 discs at baseline (T = 0) and at three months (T = 3).

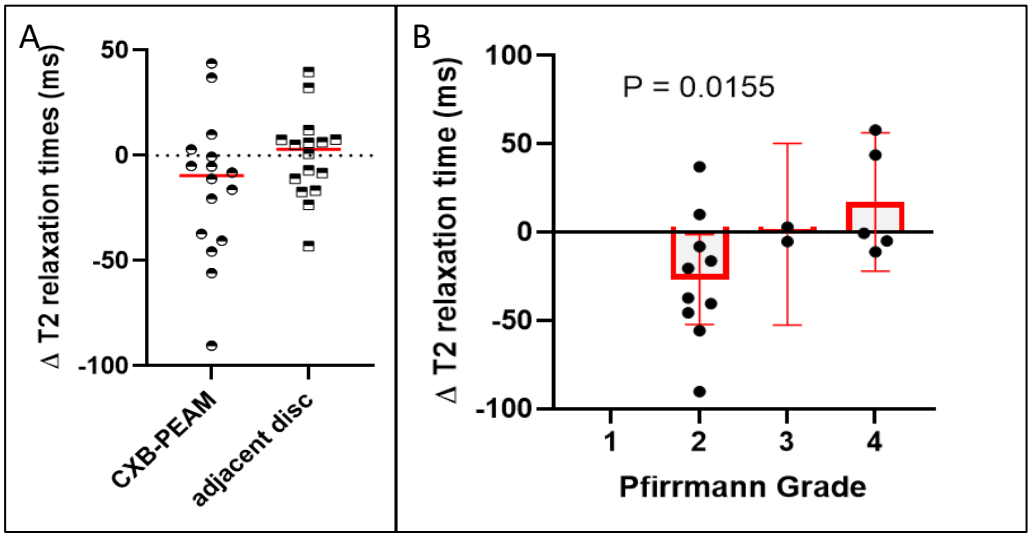

Figure 6: A: the difference in T2 relaxation times between baseline (T= 0) and three months (T = 3) of the L7-S1 discs that received an injection with polyesteramide microspheres loaded with celecoxib (CXB-PEAM) and L6-7 discs (adjacent disc). B: the difference in T2 relaxation times between baseline (T = 0) and three months (T = 3) of the L7-S1 discs that received an injection with polyesteramide microspheres loaded with celecoxib (CXB-PEAM) compared to the Pfirrmann grades of these discs at baseline (T = 0).

Questionnaire findings

The questionnaire data were not normally distributed. The questionnaire scores for the questions 'lameness' (T6: *P* = <0.0001, T12: *P* = 0.0004, LT: *P* = 0.002), 'lower back pain' (T6: *P* = 0.0037, T12: *P* = 0.0027, LT: *P* = 0.0422) and 'difficulty standing up' (T6: *P* = 0,0591, T12: *P* = 0.0366, LT: *P* = 0.0078) of the PEAM-CXB group at T6, T12 and LT showed a significant increase compared to baseline (T0). The questionnaire scores of the symptom 'hind limb weakness' increased significantly at T6 and T12 compared to baseline of the PEAM-CXB group (T6: *P* = 0.0159, T12: *P* = 0.0083). The questionnaire scores of the symptom 'difficulty lying down' increased significantly or had a tendency of significance at T12 and LT compared to baseline of the PEAM-CXB group (T12: *P* = 0.0703, LT: *P* = 0.0112). The questionnaire scores of the symptom 'hyperaesthesia/ hypersensitivity of the lower back skin' increased significantly or had a tendency of significance at T6 and T12 compared to baseline of the PEAM-CXB group (T6: *P* = 0.0237, T12: *P* = 0.0886) (figure 7 & table 4). The improvements can also be

seen in figures 9 - 13 and 18 in appendix 3. The symptoms hind legs muscle build- up, the position of the tail, tail wagging and urinary and faecal incontinence showed no significant change in questionnaire scores compared to baseline (figure 8 & table 4). The questionnaire scores of the placebo group showed no significant changes (table 5 & figures 9-18 in appendix 3).

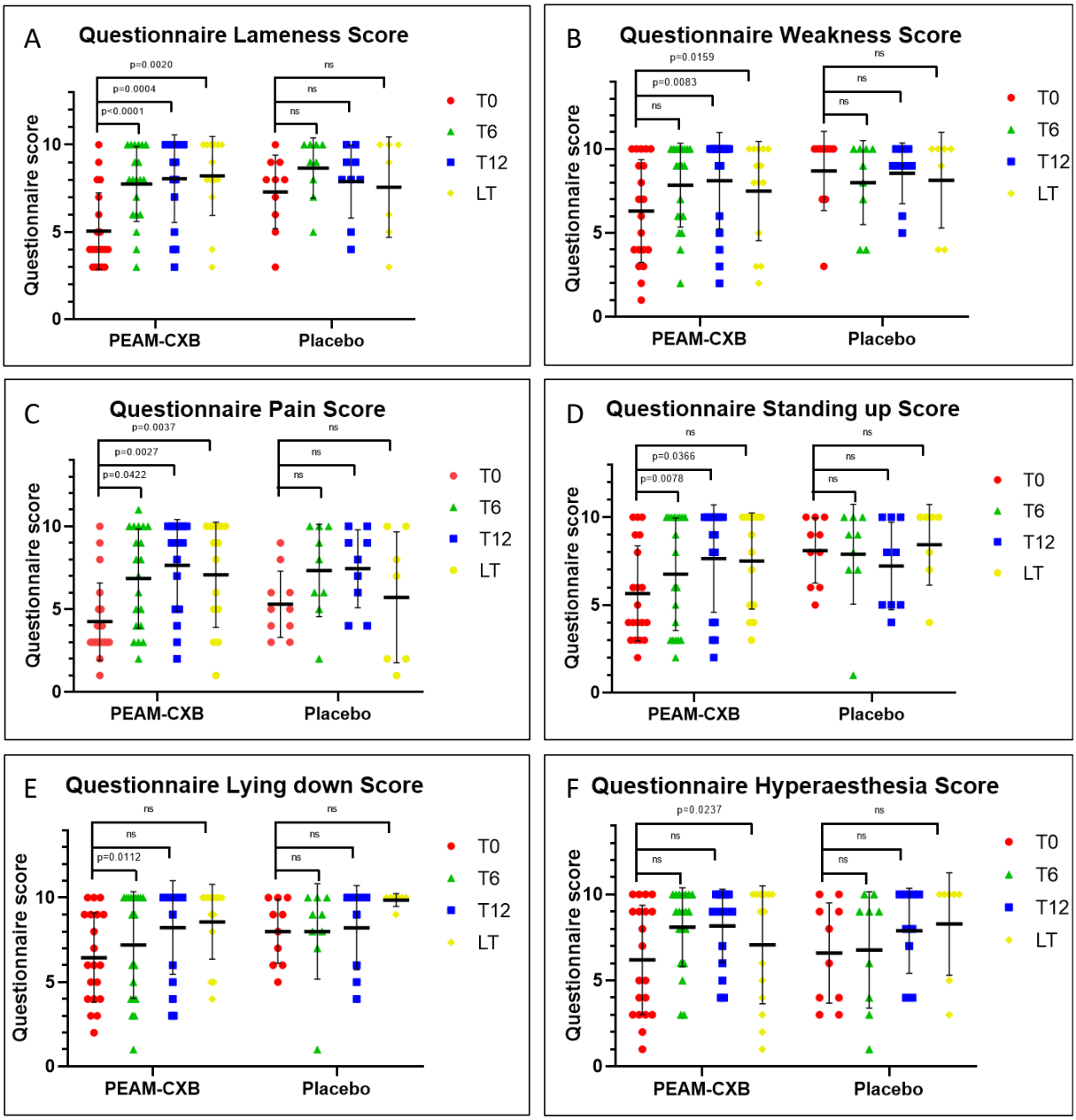

Figure 7: This figure consists of six graphs which show the questionnaire scores of questions from the questionnaire (see appendix 1) about hind limb lameness (A), hind limb weakness (B), lower back pain (C), difficulty standing up (D), difficulty lying down (E) and hypersensitivity of the lower back skin (F). The graphs show the answers of the owners from the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB) and the dogs injected with only polyesteramide microspheres (Placebo) at different points in time (T0 = baseline, T6 = after 6 weeks, T12 = after 12 weeks and LT = long term).

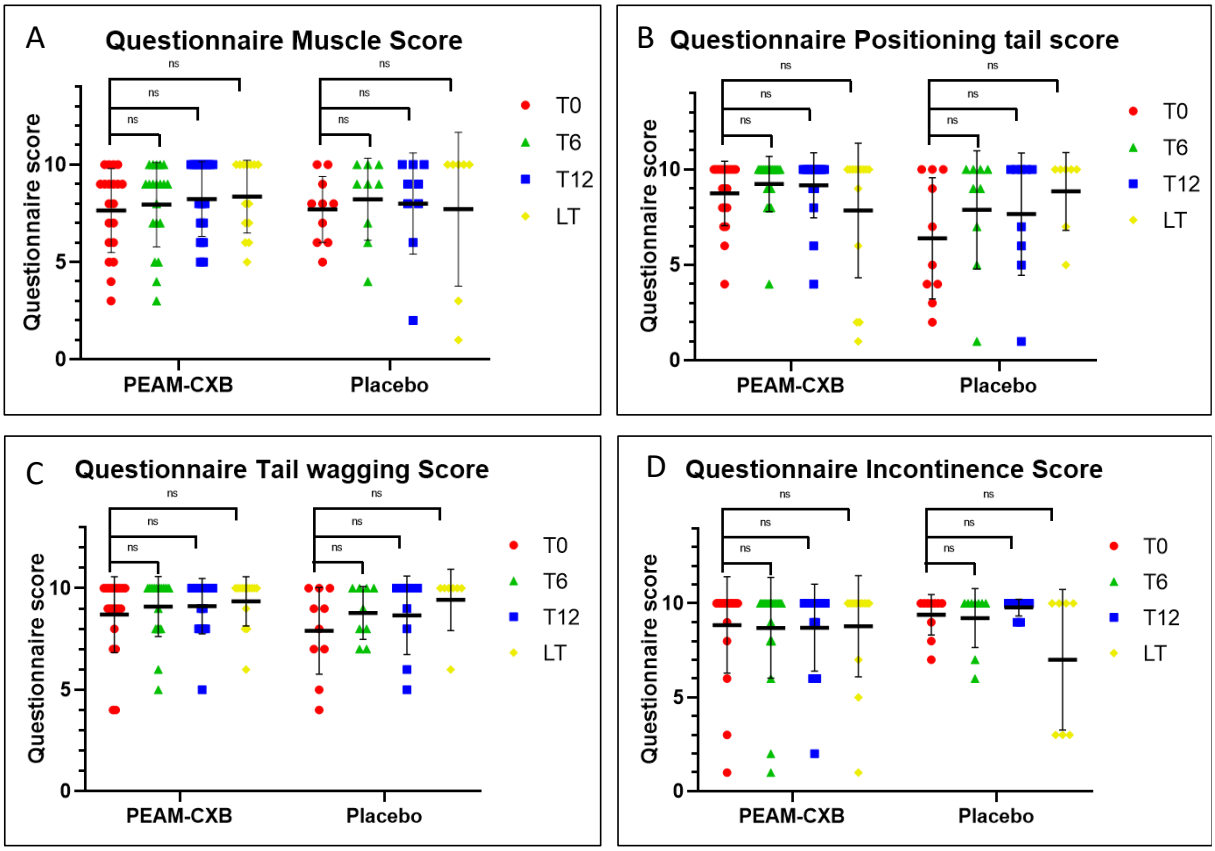

Figure 8: This figure consists of four graphs which show the questionnaire scores of questions from the questionnaire (see appendix 1) about hind legs muscle build-up (A), position of the tail (B), tail wagging (C) and urinary and faecal incontinence (D). The graphs show the answers of the owners from the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB) and the dogs injected with only polyesteramide microspheres (Placebo) at different points in time (T0 = baseline, T6 = after 6 weeks, T12 = after 12 weeks and LT = long term).

Table 4: P-values of the questionnaire answers from the owners of the PEAM-CXB group (red: significant (P< 0.5), yellow: tendency of significance (0,05<P<0,10), T0 = baseline, T6 = after 6 weeks, T12 = after 12 weeks and LT = long term).

Questionnaire subject (*P*<0.05)	**T0-T6**	**T0-T12**	**T0-LT**
Hind limbs lameness	<0.0001	0.0004	0.0020
Hind limbs weakness	0.0159	0.0083	0.1160
Lower back pain	0.0037	0.0027	0.0422
Difficulty standing up	0.0591	0.0366	0.0078
Difficulty lying down	0.2700	0.0703	0.0112
Hind legs muscle build-up	0.1748	0.2715	0.0791
Position of the tail	0.3007	0.2109	0.5000
Tail wagging	0.4600	0.4922	0.3906
Urinary and faecal incontinence	>0.9999	0.7813	0.8750
Hypersensitivity lower back skin	0.0237	0.0886	0.4346

Table 5: P-values of the questionnaire answers from the owners of the placebo group (red: significant (P < 0.5), T0 = baseline, T6 = after 6 weeks, T12 = after 12 weeks and LT = long term).

Questionnaire subject (*P*<0.05)	**T0-T6**	**T0-T12**	**T0-LT**
Hind limbs lameness	0.1250	0.5943	>0.9999
Hind limbs weakness	0.1250	0.3750	0.5625
Lower back pain	0.1475	0.0722	0.8788
Difficulty standing up	>0.9999	0.2188	0.9063
Difficulty lying down	>0.9999	>0.9999	0.0625
Hind legs muscle build-up	0.5000	0.6328	0.7500
Position of the tail	0.2500	0.1250	0.0625
Tail wagging	0.3750	0.4375	0.2188
Urinary and faecal incontinence	>0.9999	0.2500	0.3125
Hypersensitivity lower back skin	0.8961	0.3438	0.3125

Long term follow-up (telephone interview)

In addition to the questionnaire at the long-term follow-up, an interview with the owners over the phone was also conducted. This was done to give more insight in the long-term effects of the injection with PEAM-CXB or only PEAMs and to gather the owners opinion on the treatment and the need to use anaesthesia. Twenty one out of 30 (70%) owners were willing to contribute to long term follow-up. These could be divided into three groups of patients; 14 out of 20 (70%) owners of the dogs treated with PEAM-CXB were available, 4 out of 6 (66,6%) owners of the dogs from the placebo group that had received a reinjection with PEAM-CXB were available and 3 out of 4 (75%) owners of the dogs from the placebo group that did not receive a reinjection were successfully contacted. Five patients were lost to long follow-up due to unreachable owners. Three patients deceased in the period before the long-term follow-up, these dogs all had received an injection with the treatment. Of the 18 dogs that received treatment and were available during long-term follow-up, were 8 (44,4%) owners that reported their dog had some severity of lower back pain and were limited in certain movements and activities. nine out of 18 (50%) patients received medication for lower back pain, four of those owners reported that the medication caused a decrease in symptoms and pain in their dog. Five out of 18 (27,7%) patients received further treatment such as physiotherapy, treatment from a chiropractor, hydrotherapy and acupuncture. Three out of 18 (16,6%) patients had received a dorsal laminectomy. Eleven out of 18 (61,1%) owners would consider a re-injection for their dog if possible. Sixteen out of the total 21 (76%) owners had no problem with the use of anaesthesia for this treatment.

Discussion

This prospective, randomize, double-blinded, placebo- controlled clinical study was performed to investigate the regenerative and analgesic effects of intradiscal injection with celecoxib loaded microspheres in canines suffering from low back pain due to IVD degeneration and to evaluate the clinical efficacy of this treatment.

T2 mapping is an effective and objective measurement system for disc degeneration.

This study used MRI T2 mapping, a system that applies the quantification of water content, proteoglycan content and collagen sequence breakdown of the intervertebral disc as relaxation times.[33] Furthermore, this method allows quantitative evaluation of the T2 signal within the IVD tissue, thus enabling an objective assessment of the IVD degeneration as opposed to the subjective determination with the Pfirrmann grading system.[34] The significant indirect correlation between the Pfirrmann grades and the T2 relaxation times shown in the present study is in line with previous reports showing that T2 mapping is an effective and objective measurement system for disc degeneration.[33-

[36] T2 mapping was additionally used on the adjacent L6-7 disc to compare to the lumbosacral disc. This illustrated that the lumbosacral disc had lower T2 relaxation times. The comparison with the adjacent disc showed a confirmation of the IVD degeneration diagnosis of the L7-S1 discs.

The success of intradiscal treatment is correlated with the stage of degeneration.

Three months after intradiscal injection with PEAM-CXB, the T2 relaxation times were lower from the baseline values as can be seen in the negative difference in time of the T2 relaxation times of each disc. This seems to indicate that further degeneration occurred in the follow-up period. The observed decrease seemed unrelated to age, bodyweight or Pfirrmann grade as these were comparable between the PEAM-CXB and the placebo group. However, when the difference in T2 relaxation times between baseline and T = 3 was plotted against each Pfirrmann grade from baseline, it was evident that the decrease in T2 relaxation times was larger in discs graded with Pfirrmann grade 2 than grade 4. It thus seems that the healthier the disc is at baseline, the more degeneration occurs after the intradiscal injection. This negative correlation between the T2 relaxation times and the Pfirrmann grades could be explained by further degeneration caused by possible damage from the injection or a degeneration process that is natural. However, the placebo group was not followed-up by MRI, nor was data available from an unrelated patient population suffering from IVD degeneration to follow natural degeneration of the L7-S1 disc. Nonetheless, the adjacent disc (L6-7) was investigated with MRI and showed no significant change, which could suggest that the decrease in T2 relaxation times is not due to natural degeneration. This is not without prejudice as it is a different IVD and the stage of degeneration can differ between discs.[3] A ten year cohort study demonstrated alike to this research that discs with a lower Pfirrmann grade degenerated more as result of intradiscal injection.[37] Another study also concluded that the treatment for IVD degeneration was more effective for discs at a later stage of degeneration.[38] Pfirrmann grade 2 discs may not react properly to treatment but this current study shows signs that discs with Pfirrmann grade 4 do improve upon treatment. This can also be suggested by looking at other studies that researched treatments for IVD degeneration and utilize T2 mapping. These studies had lower T2 relaxation times at baseline, which means that these IVDs could have been in later stages of degeneration and thus had good results from the treatment.[9,16,39,40] It was excluded that the higher T2 relaxation times at baseline in this study could be explained by a difference in MRI techniques. There were no significant differences found between the MRI protocols from a similar study from Tellegen et al. (2017) and the current study.[16]

The increase in T2 relaxation times of the discs that were graded with Pfirrmann grade 4 could be suggestive of the anti-inflammatory and possible anti-degenerative effects of the intradiscal injection with PEAM-CXB. It has already been shown *in vitro* and in a canine model that PEAM-CXB can harness inflammation and can reduce pain mediators.[9] Another study also showed promising result using diclofenac, a different anti-inflammatory drug with a similar drug delivery system using biodegradable nanoparticles. It showed a decrease in pro-inflammatory mediators, a downregulation of metalloproteinases and also an upregulation of aggrecan and collagen in an organ culture model, suggesting repair of the degenerated IVD when used as intradiscal therapy.[41,42] Several studies have also been done in rats with other drugs that inhibit COX, such as Luteoloside, Icariin and Metformin. These therapies also showed a decrease on the progression of degeneration though the inhibition of COX.[43-45] These studies encourage the idea of using anti-inflammatory drugs, specifically COX-2 inhibitors, to stop IVD degeneration and possibly induce repairment of the disc.[46] This study showed the effect of intradiscal injection with PEAM-CXB on a clinical level, which the forementioned studies lack.[9,41-45]

Owners observed a reduction in symptoms seen with IVD degeneration

The results of the questionnaire showed an improvement of hind limb lameness, hind limb weakness, lower back pain, difficulty standing up, difficulty lying down and hypersensitivity of the lower back skin as result of the PEAM-CXB injection 3 months after injection. This is substantiated by regarding that the placebo group did not have any significant improvement. These improvements in questionnaire scores showed the long analgesic effect of the intradiscal injection with PEAM-CXB.

Although an improvement was seen with previous questionnaire subjects, no improvement was seen in hind legs muscle build-up, position of the tail, tail wagging and urinary and faecal incontinence. It seems that the PEAM-CXB injection had no effect on these clinical symptoms of IVD degeneration according to the questionnaire. However, the symptom urinary and faecal incontinence is often seen in more advanced stages of IVD degeneration, thus the lack of improvement was due to absence of this symptom in the patients, which could be confirmed with the high scores that were given by the owners.[12] The symptoms hind legs muscle build-up, position of the tail and tail wagging were scored very high by the owners as well. This could also be explained by an absence of these symptoms or the owners had trouble with evaluating these symptoms. Although no significant improvement was found regarding these symptoms, no decrease in questionnaire scores was found either, which could be due to intradiscal injection with PEAM-CXB.

Some differences were found between the PEAM-CXB and the placebo group at baseline. The mean scores for hind limbs lameness, hind limbs weakness and difficulty getting up were significantly larger for the placebo group. The placebo group did not significantly change over time, which could be explained by the discs being healthier at baseline regarding those symptoms. Other questionnaire subjects were not significantly larger and did not significantly change over time for the placebo group. Some *P*-values were not significant but showed a tendency, this evidence could be disputable. However, these *P*-values did show a trend compared to the *P*-values of the placebo group. Another point of discussion of these results is the reliability of the owners ability to assess the clinical symptoms of IVD degeneration with a questionnaire. It has been shown that the subjective assessment of lameness can differ between observers.[47] Although the results of the questionnaires seem very promising in this study it has to be looked at cautiously due to the varying perception of owners.

No adverse effects of injection and treatment were observed.

The long-term telephone interview was done to give more insight in the long-term effects of the injection with PEAM-CXB or only PEAMs and to gather the owner's opinion on the treatment and the need to use anaesthesia. Most owners would choose the injection again and no adverse effects or complications of the treatment were observed. The comparison of MRI findings after 3 months between the treated L7-S1 IVD and the L6-7 IVD additionally showed that the treatment did not cause detrimental effects for the L7-S1 disc except for the small decrease in T2 relaxation times for the L7-S1 IVDs that were graded lower. This is in line with previous research that proved the safety of intradiscal injection and PEAM-CXB.[9,23]

Limitations and future prospects

This study had several limitations that could indicate further research. The study group got smaller throughout the study due to unreachable owners or deceased patients. A larger study group could ensure more reliable results. It was not known if owners used additional medication, such as analgesics, after the injection. The owners were allowed to use analgesics if needed but this data was not available. It could have had influence on the results that could not be checked. As discussed before,

the questionnaire is not entirely reliable due to the owners varying perception that could have had an effect on the results of this study.

Other researches have used disc height and T2-weighted signal intensity parameters to evaluate the disc regeneration. These parameters were used to indirectly measure the water content of the disc, which signals regeneration when restored. Other studies also used staining for glycosaminoglycans and measured levels of gene-expression of proteoglycans using semi-quantitative RT-PCR to evaluate restoration of proteoglycans that indicate regeneration.[48,49] This study showed some increase in relaxation times (related to Pfirrmann) but no disc height data, staining or PCR was used, which could be considered for future research to gather more insight for the regenerative properties of PEAM-CXB.

Another prospect of intradiscal injection might be the combination of an anti-inflammatory drug like celecoxib and stem cells that could reduce inflammation, pain and degeneration but also distinctively repair the extracellular matrix of the disc. Mesenchymal stem cells (MSC) have been extensively researched as treatment for IVD degeneration. [14,38,40,49-52] MSCs can differentiate into cells similar as in the NP. MSCs have immunosuppressive properties and secrete growth factors that encourage regeneration of the disc's extracellular matrix.[14] These studies focus on regeneration which might have an enhanced effect when combined with the anti-inflammatory properties of an accompanying drug. The downsides of using stem cells as therapy for IVD degeneration are their high cost and the lack of research on a large clinical level.[52,53]

Conclusions

This study showed that intradiscal injection with celecoxib loaded microspheres in canines suffering from lower back pain due to IVD degeneration had analgesic and anti-inflammatory effects and thus reduced the symptoms of IVD degeneration. Furthermore, this study demonstrated that this treatment is clinical efficient as it confirmed that the treatment is safe and had no indication of adverse effects on short or long term. The study also concluded the importance of evaluating the stage of degeneration before treatment, due to the better reaction to treatment of IVDs that are in a later stage of degeneration. This research could in the future reduce the total cost of care and the burden of disease for patients and owners as it can result in a sufficient treatment option between the systemically administrated painkillers and elaborate surgery.

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Appendix

Appendix 1: Questionnaire

Questionnaire to the owners of dogs before, after 6 weeks, 12 weeks and long term after intradiscal application of polyesteramide microparticles with celecoxib. The questions could be answered on a 10-point scale.

1. Does your dog have pain in the pelvic limbs and shows lameness?
2. Does your dog show weakness in the pelvic limbs?
3. Does your dog have low back pain?
4. Does your dog have difficulty rising up?
5. Does your dog have difficulty lying down?
6. How would you rate muscle volume in the pelvic limbs of your dog?
7. How is your dog holding its tail?
8. Is your dog able to wag its tail?
9. Does your dog show loss of control of urination and defecation?
10. Does your dog show pain when you touch the lower back?

Appendix 2: Telephone interview

Open questions asked at the long term follow-up.

How is the wellbeing of your dog?
How is the movement and activity of your dog?
Does your dog currently receive any medication, if so which medication and what dosage?
Has your dog undergone any further treatment?
Given the effect, would you choose this specific treatment again?
How often per year would you like to have this treatment done if possible?
How do you feel about the fact that such a treatment requires anaesthesia?

Appendix 3: Questionnaire score graphs

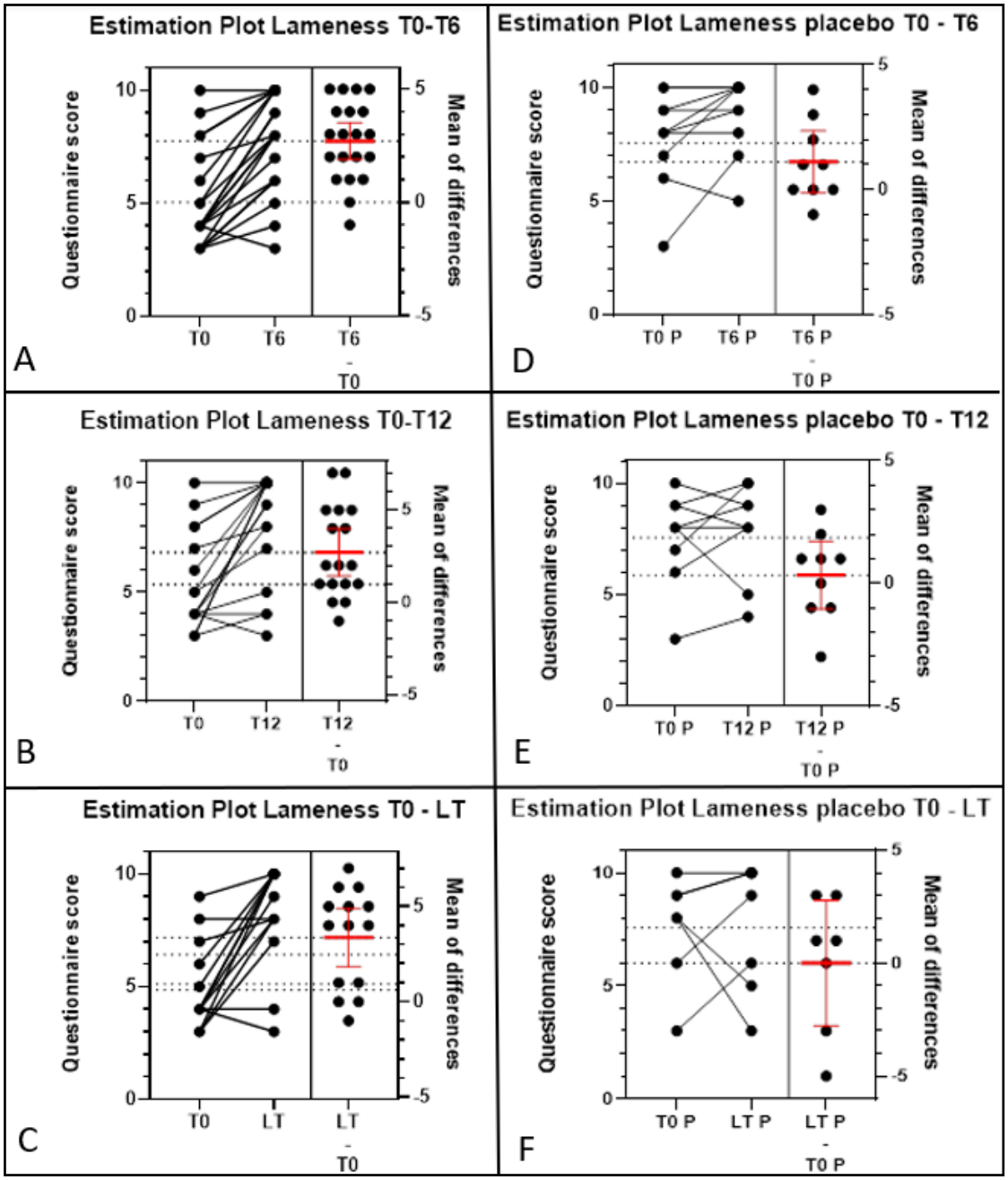

Figure 9: This figure contains six graphs showing the results from the question about hind limb lameness of the questionnaire (question 1 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

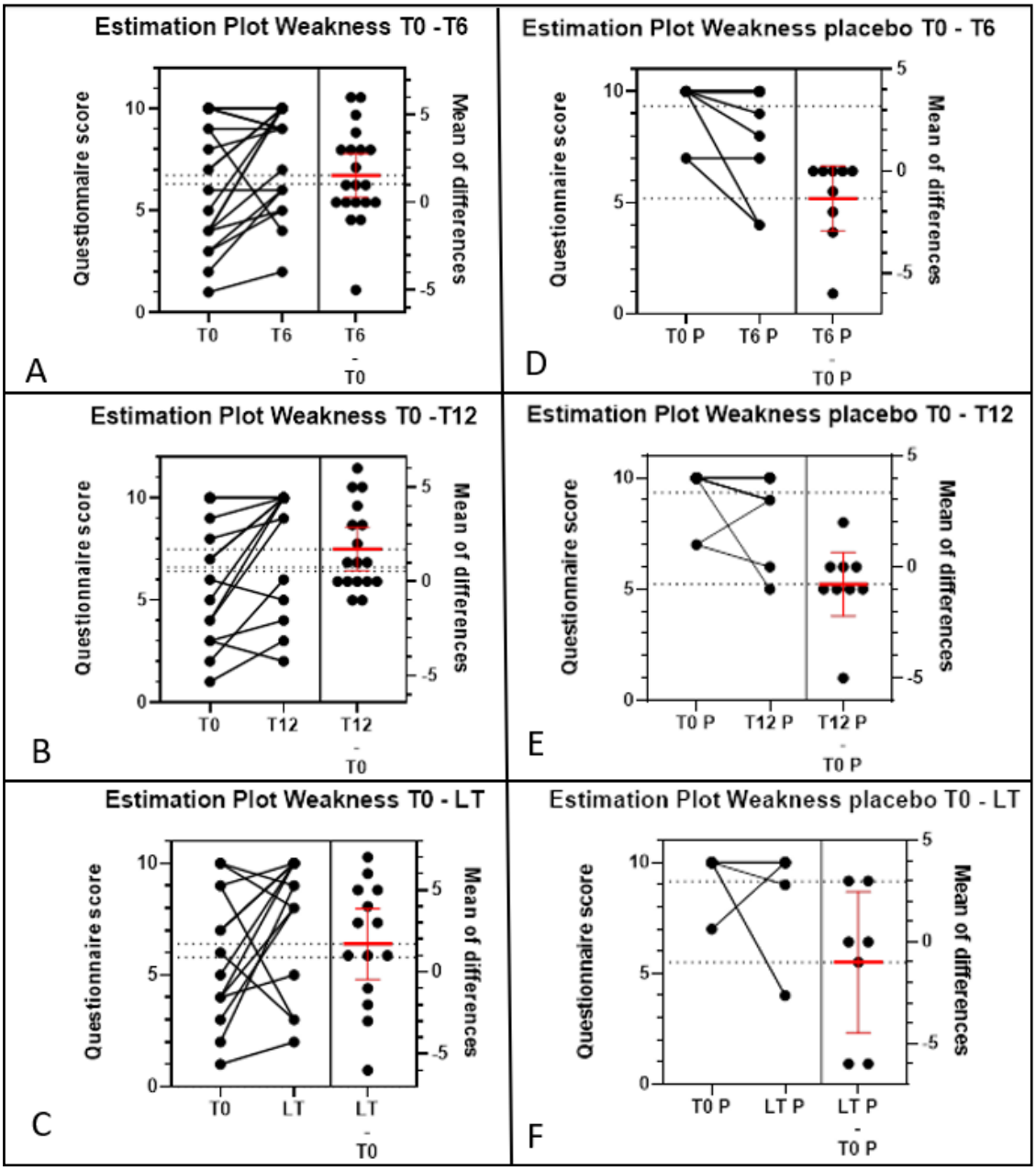

Figure 10: This figure contains six graphs showing the results from the question about hind limb weakness of the questionnaire (question 2 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

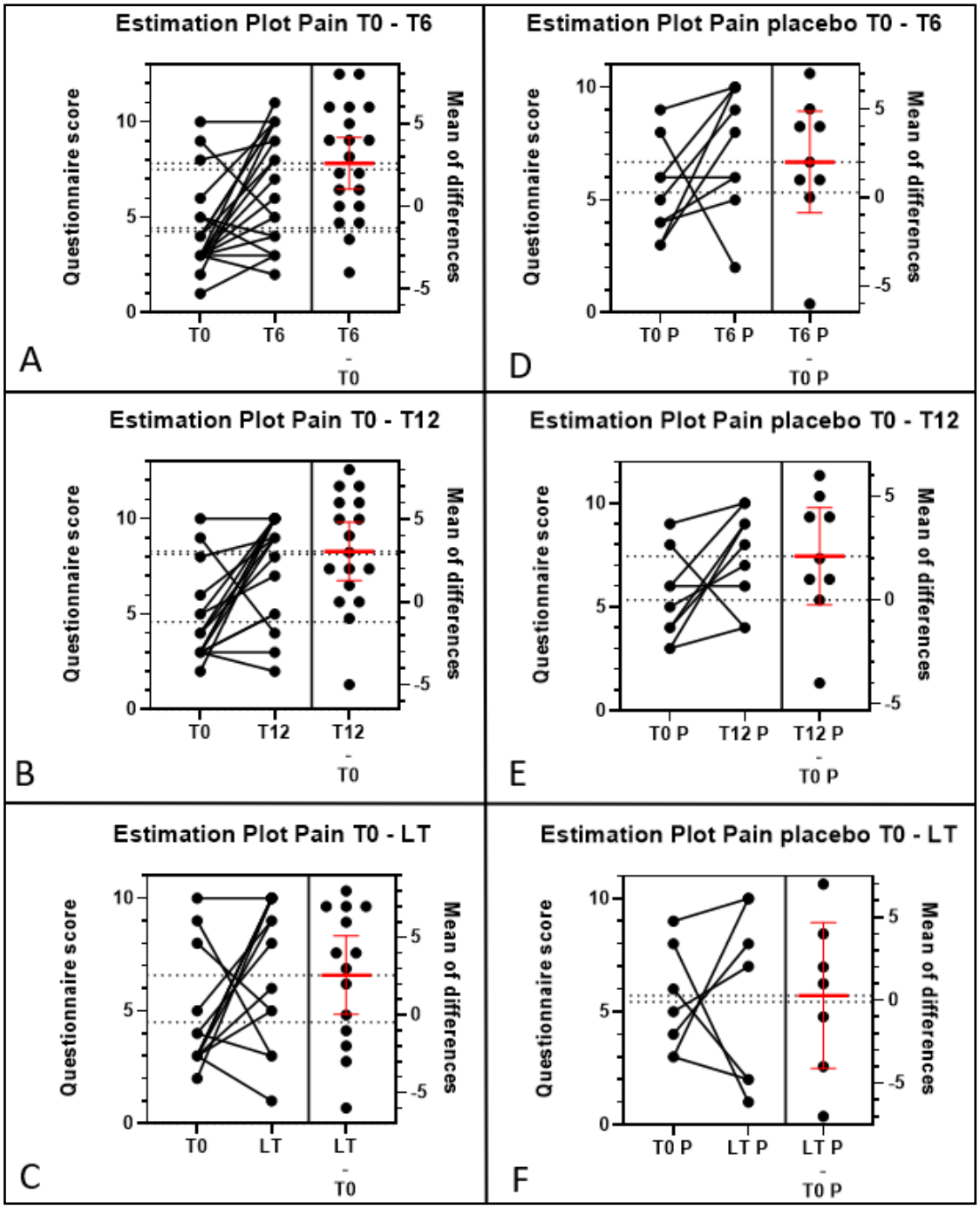

Figure 11: This figure contains six graphs showing the results from the question about lower back pain of the questionnaire (question 3 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

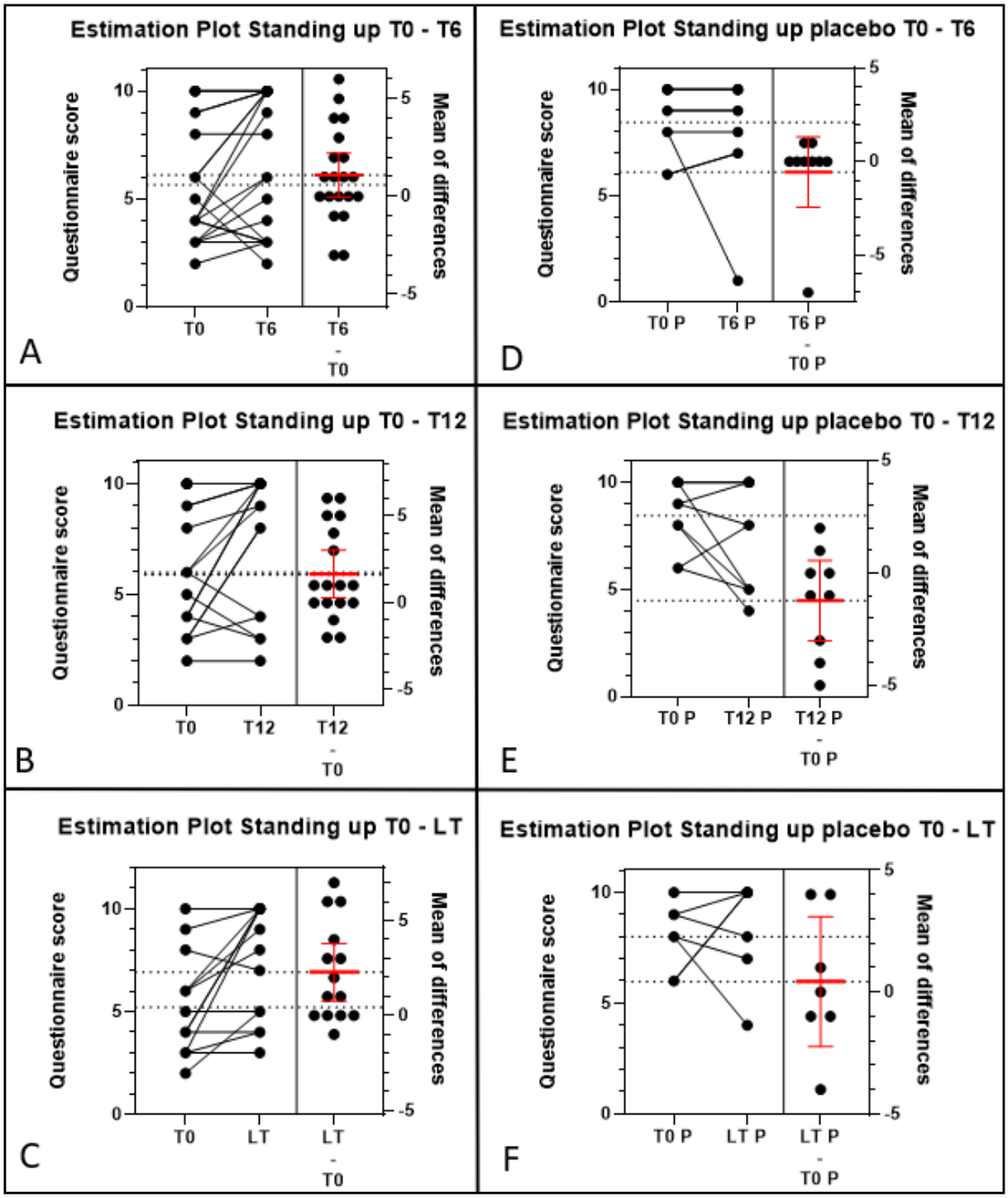

Figure 12: This figure contains six graphs showing the results from the question about difficulty standing up of the questionnaire (question 4 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

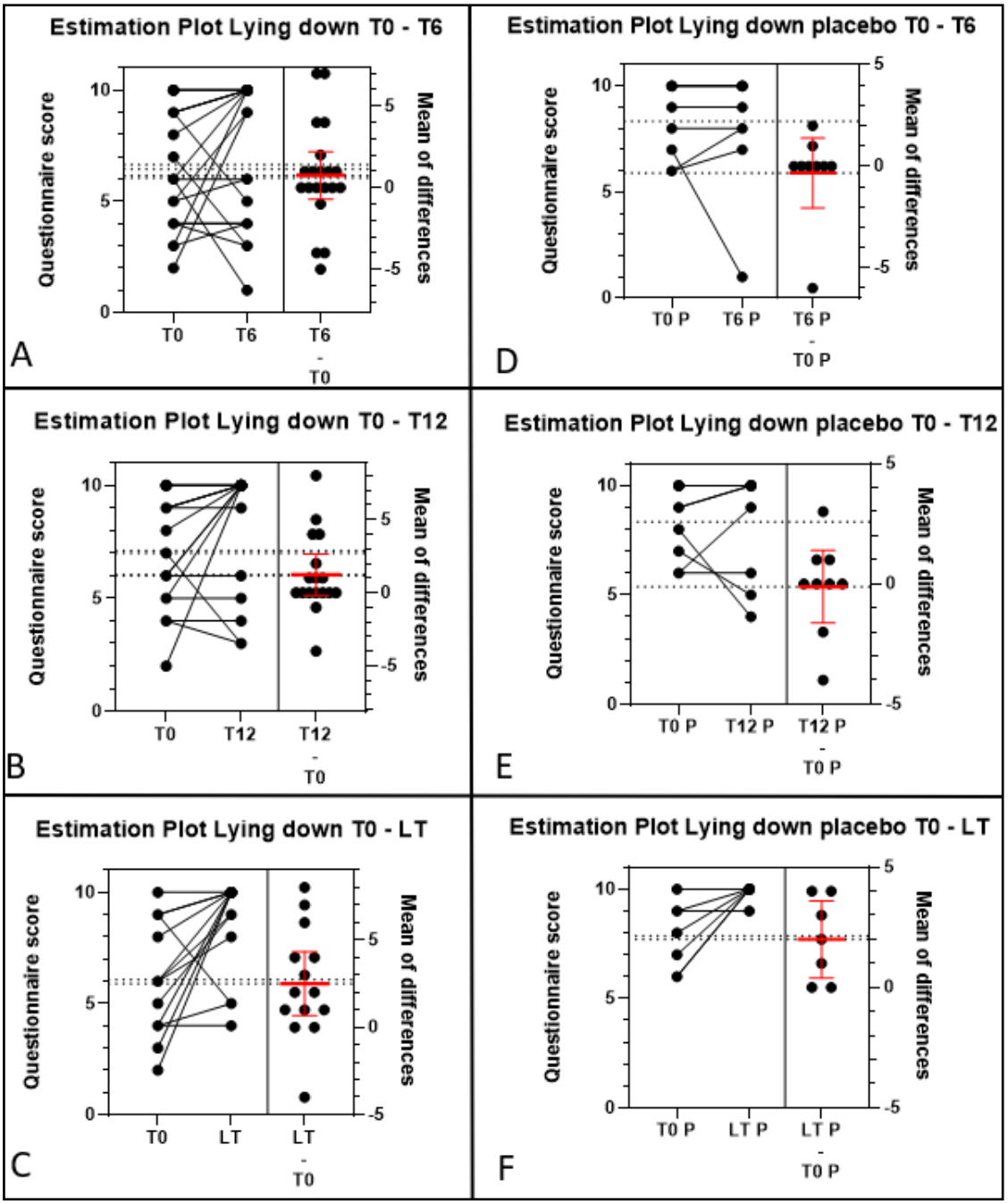

Figure 13: This figure contains six graphs showing the results from the question about difficulty lying down of the questionnaire (question 5 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

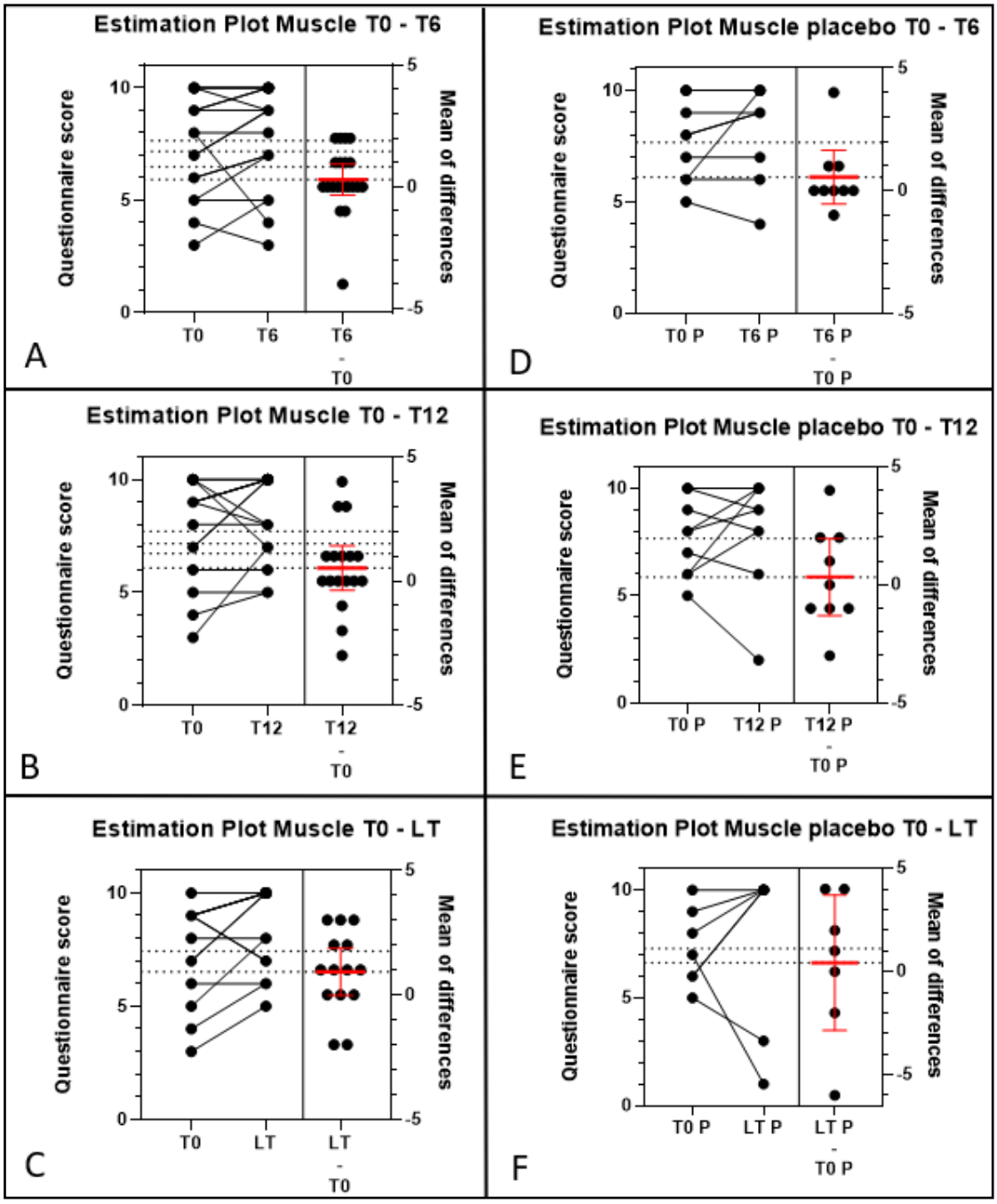

Figure 14: This figure contains six graphs showing the results from the question about hind legs muscle build-up of the questionnaire (question 6 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

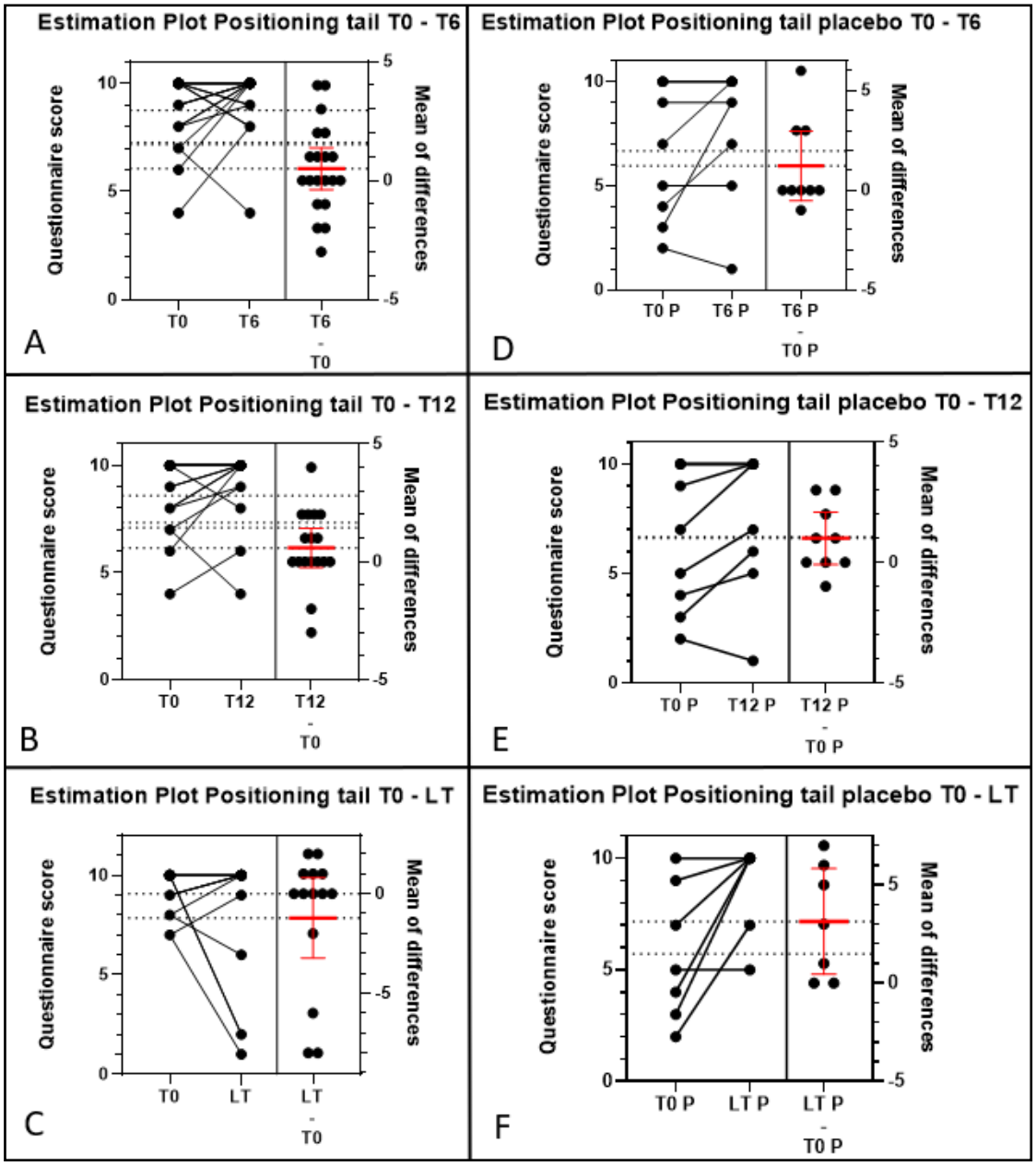

Figure 15: This figure contains six graphs showing the results from the question about the position of the tail of the questionnaire (question 7 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

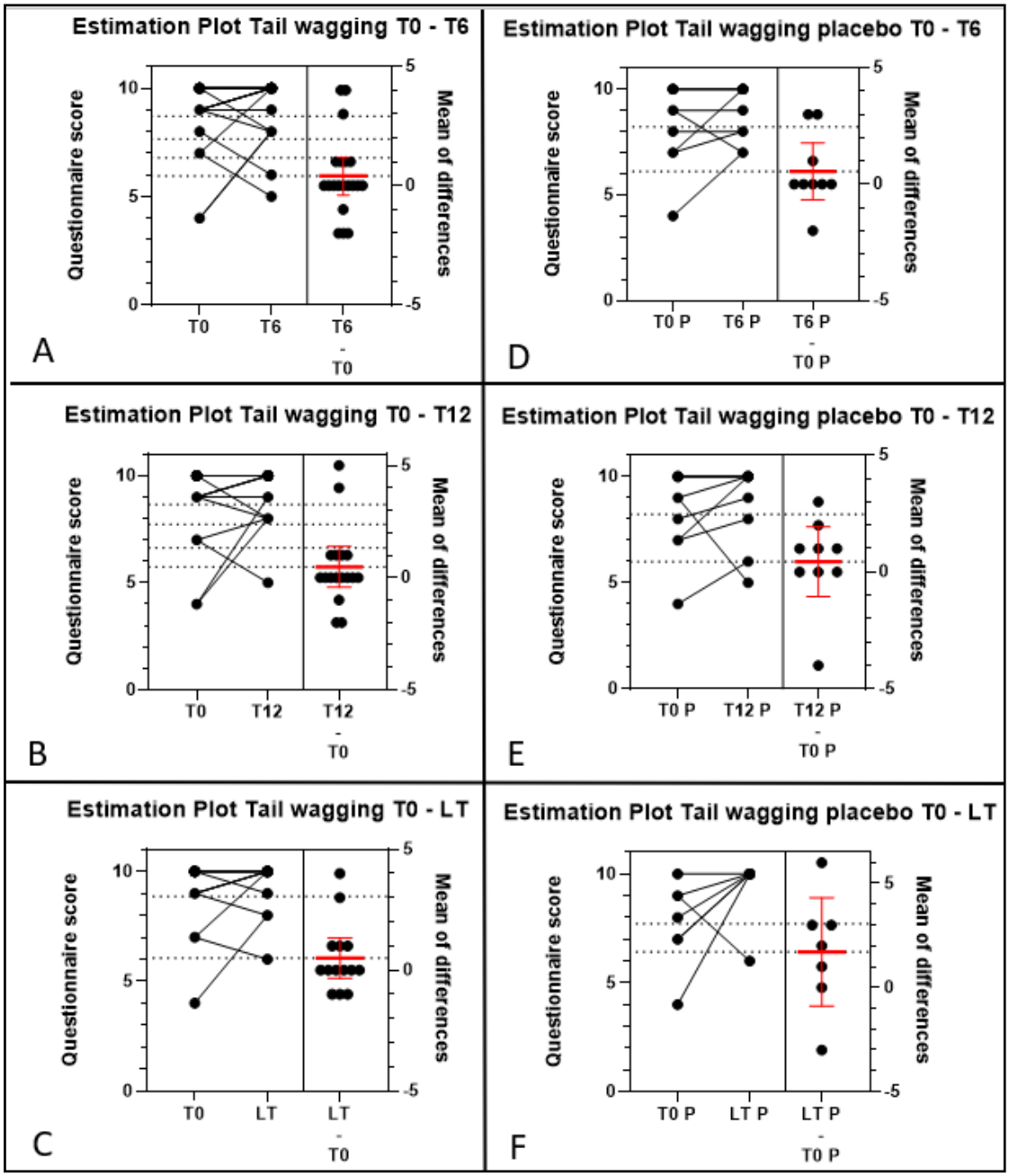

Figure 16: This figure contains six graphs showing the results from the question about tail wagging of the questionnaire (question 8 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

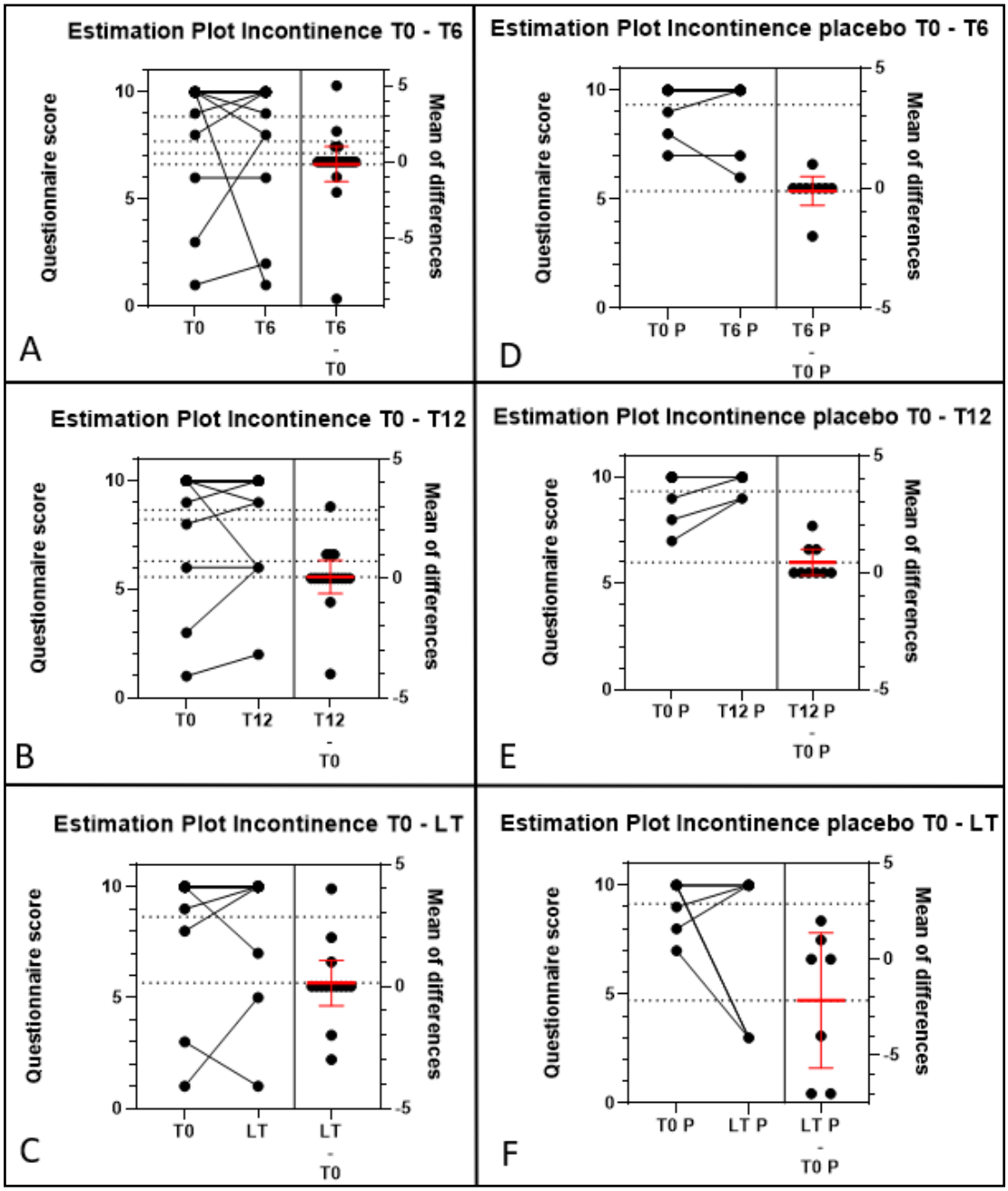

Figure 17: This figure contains six graphs showing the results from the question about urinary or faecal incontinence of the questionnaire (question 9 in appendix 1). Graphs A-C show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.

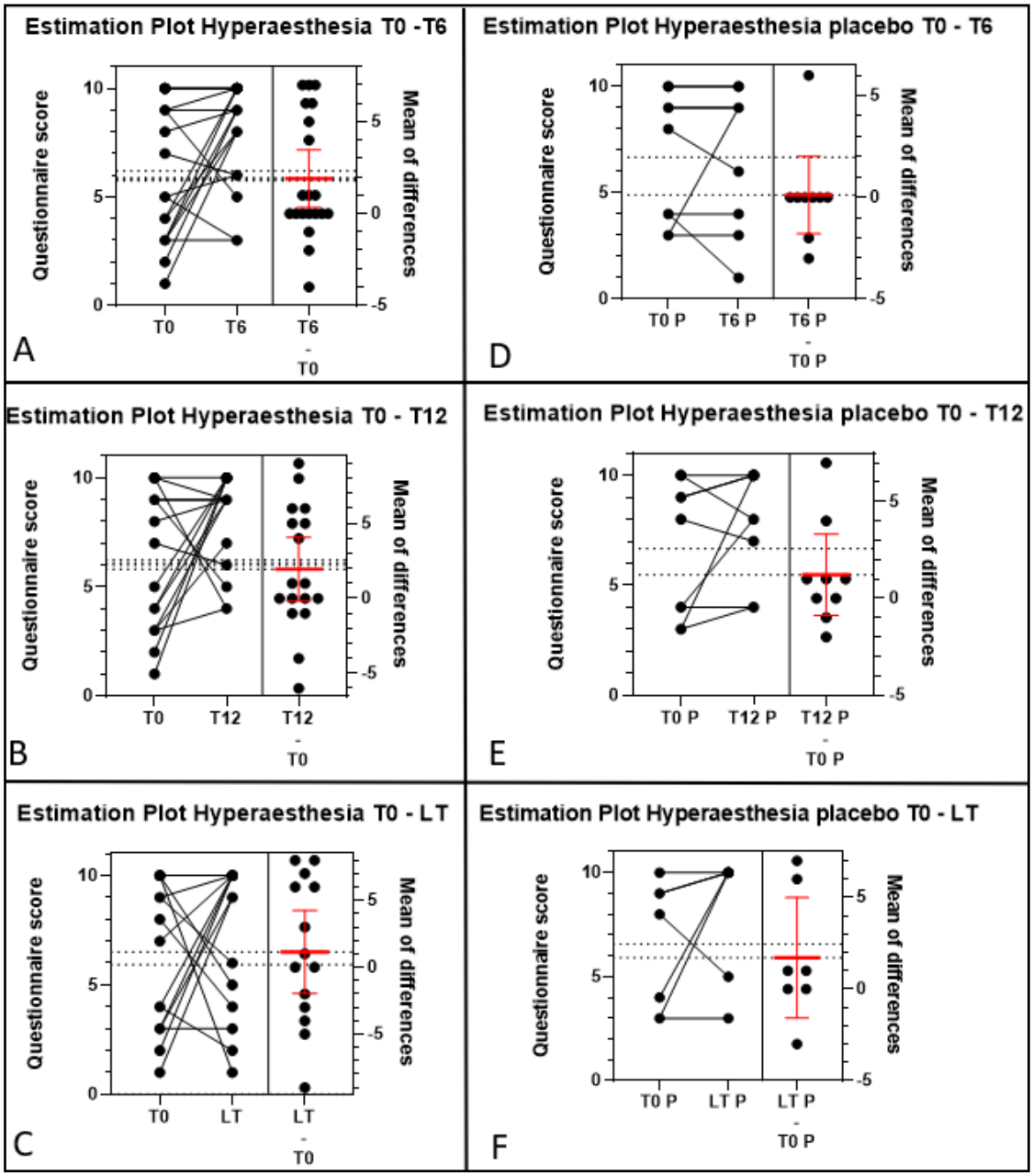

Figure 18: This figure contains six graphs showing the results from the question about hypersensitivity of the lower back skin of the questionnaire (question 10 in appendix 1). Graphs A-C left show the answers from the owners of the dogs injected with polyesteramide microspheres loaded with celecoxib (PEAM-CXB). Graphs D-F show the answers from the owners of the dogs injected with only polyesteramide microspheres (placebo). Each graph consists of the change in time on the left and the mean of differences on the right. A: change in score between baseline (T0) and 6 weeks (T6) of the PEAM-CXB group. B: change in score between baseline (T0) and 12 weeks (T12) of the PEAM-CXB group. C: change in score between baseline (T0) and long term (LT) of the PEAM-CXB group. D: change in score between baseline (T0) and 6 weeks (T6) of the placebo group. E: change in score between baseline (T0) and 12 weeks (T12) of the placebo group. F: change in score between baseline (T0) and long term (LT) of the placebo group.