Foreign body response to alginate beads injected in the equine intercarpal joint

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Abstract

Introduction - Osteoarthritis (OA) is the most important chronic musculoskeletal disorder in both humans and horses. OA is a major cause of early retirement in horses. During the whole process from initiation to the end stage of OA pain is experienced. Horses with OA can be treated systemically with anti-inflammatory and/or pain reducing pharmaceuticals. Intra-articular (IA) administration of corticosteroids, opioids and/or hyaluronic is used in modern-day equine veterinary practice. Treating horses IA repeatedly bears disadvantages and risks. The use of alginate beads as a slow-release carrier for pharmaceuticals or mesenchymal stem cells appears a promising new approach. This pilot study was designed to determine if alginate beads can be safely injected in an equine joint. The reaction along the study raised more questions on what happened in the joint as a reaction to the alginate beads.

Materials and Methods - Three separate horses were selected for a pilot study. The protocols were adjusted after each horse, according to the clinical reaction of the horses to the IA administration of solely alginate beads. Sampling of synovial fluid and synovium took place for further investigation on presence of biomarkers and histology. Enzyme-linked immunosorbent assays (ELISAs) against Interleukin-8 (IL-8), Chemokine Ligand 2 (CCL2), Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor alfa (TNFα) were performed on sampled synovial fluid. Haematoxylin Eosin staining was done on synovium samples. An attempt was made to design an *in vitro* predictor for the *in vivo* reaction to biomaterials like alginate beads using whole blood of 6 healthy blood donors. ELISA's against IL-8 en CCL2 were performed on the supernatant of the whole blood assay (WBA).

Results - After IA administration of alginate beads horses showed severe lameness. There were clear increasing values of all tested biomarkers in the synovial fluid sampled from the pilot study. Histology showed clear reaction to something present in the synovial fluid, without clear differentiation on what type of reaction was present. The WBA showed clear increasing values of IL-8 and CCL2 in reaction to the alginate beads that were used during the pilot study.

Discussion – Adjustments to the experimental design of this pilot study might have contributed to more valuable results. Knowing alginate itself is immunogenic could have predicted a reaction to alginate beads in horses, especially since it is known that horses seem particularly sensitive to a foreign body reaction in the joint. Horses often present with (transient) lameness and elevated white blood cell counts after injection of a biomaterial. Even materials that do not normally elicit an adaptive immune response and have been tested in other animals, can lead to swollen and painful joints in horses.

Conclusion - More research needs to be performed before extensive *in vivo* testing with alginate beads in horses can be done. The increase of biomarkers against Interleukin-8 (IL-8), Chemokine Ligand 2 (CCL2), Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor alfa (TNFα) suggest a foreign body response is active in the treated joints. An equine whole blood assay appears a promising *in vitro* test to predict an *in vivo* reaction to biomaterials.

Introduction

Osteoarthritis (OA), also referred to as degenerative joint disease or osteoarthrosis, is the most important chronic musculoskeletal disorder in both humans and horses. (7) OA is a major cause of early retirement in horses. (8–11) Along with other articular diseases OA constitutes the greatest single economic loss to the equine industry. (11) "Osteoarthritis can be described as the failed repair of damage that has been caused by excessive mechanical stress on joint tissues."(7,12) This definition of OA implies there are multiple factors that can lead to OA, but mechanical impact is central to all of these. (7) In horses synovitis is seen as an important primary event in the development of OA, and at the least as a factor that is repeatedly present in OA patients. (13) From a neutral perspective whether there are single or multiple primary factors in the onset of OA, it is generally agreed a vicious cycle is started off resulting in inflammation, structural damage, loss of resistance to loading and degradation of the joint. It is important to realise articular cartilage is incapable of complete self-repair. Damaged cartilage is replaced by fibrocartilage that is biomechanically less competent. In the articular cartilage progressing damage leads to softening, fibrillation, ulceration and eventually loss of cartilage. The synovial membrane and joint capsule become hypertrophic and fibrotic. There is sclerosis and eburnation of subchondral bone and formation of osteophytes and/or subchondral cysts. During the whole process from initiation to the end stage of OA pain is experienced. (7)

Apart from several systemic anti-inflammatory and/or pain treatments and other systemic treatments that claim to have a disease-modifying effects, there are different topical therapies for joint pain.(7) Intra-articular (IA) administration of corticosteroids is commonly used in the equine practice, whereas opioids and Hyaluronic Acid (HA) have also been used in experiments. Corticosteroids inhibit de nuclear factor (NF)-κB signalling pathway, and thus acts as a potent upstream inhibitor of inflammation. (7,14,15) There seems to be consent on when corticosteroids are used reasonably with limited frequency and dose intervals as well as dosage, then the advantages exceed the disadvantages and possible risks. (7,16)

Opioids are not commonly used in the equine practice and are unlikely to be used in horses with chronic joint pain in the future, because of its short duration of action and regulatory responsibilities. (7) Whereas hyaluronic acid has shown to have a substantial analgesic effect when administered in combination with corticosteroids. The dose of corticosteroids can be lowered when used in combination with HA, counteracting the possible deleterious effects of corticosteroid on the cartilage or due to the claimed 'chondroprotective' action of HA. (7,17) The combination of corticosteroids with HA is popular in the equine practice, even though there are limited controlled comparable data of IA administration of corticosteroids, HA and corticosteroid plus HA. (7)

The disadvantages and risks of repeatedly treating horses inside the joint ask for another approach. Ideally a one time treatment that is effective long-term, for example a slow release carrier of pharmaceuticals. Or even a more definitive treatment by which the affected cartilage can be repaired, for example with mesenchymal stem cells (MSC's). Alginate is seen as a possible carrier for these pharmaceuticals or MSC's. (26)

Prior to this pilot-study, alginate beads (without pharmaceuticals or MSC's) were injected in joint of rats. There was no indication of response to the alginate beads in rats. Since osteoarthrosis is such a big problem in horses, testing the beads in horses was a logical step before testing in the ultimate goal-patients; humans.

The initial aim of this study was to determine whether or not alginate beads are suitable for use in joints of horses. There was a plan to inject eight horses stationed in England with the beads in the near future.

For ethical reasons alginate beads were injected in initially one horse's intercarpal joints, these beads did not contain pharmaceuticals or MSC's and were the same beads used in rats. This horse had a severe reaction to the beads. Therefore is was decided the alginate beads needed to be tested on a second horse before trying it on the eight horses in England, and to determine what had caused this severe reaction. After testing on the

second horse, according to an adjusted protocol, a third horse was selected to be injected with alginate beads. Again the protocol was adjusted. All three horses showed severe reaction to the beads in the intercarpal joint(s). It was decided that before alginate beads would be used again in any horse, it had to be determined what had caused the reaction in these horses.

Alginate beads clearly caused a disturbance of the dynamic equilibrium in synovial joints of the horses used in this pilot study. This equilibrium is normally achieved by a natural balance between anabolic and catabolic processes. This can only be understood when the anatomy and physiology of the synovial joint is comprehended. (1) Synovial joints are also said to be the hinges of the vertebrate skeleton and enable movement and mobility. There are many different types of synovial joints. Despite the difference in anatomy the general structure is equivalent. The synovial joint connects long bones which ends are covered by a thin line of hyaline articular cartilage and supported by subchondral bone. The articular cartilage is in direct contact with synovial fluid (SF), which acts as a source nourishment for the cartilage itself as well as lubricant for the joint in motion. A fibrous joint capsule confines the synovial cavity, this specialized continuous one- to three-cell-thick layer forms this inner lining of the joint capsule and is known as synovial membrane or synovium. (1) The SM is equipped with a modest supply of nerve and autonomic fibres, believed to play an important role in joint effusion, nociception and synovial inflammation in joint disease. There is no basement membrane present. (2) Each joint disorder, either degenerative or inflammatory, is a disruption of intra-articular homeostasis, a disturbance of the dynamic equilibrium or 'steady state'.
The SF is produced by movement of fluid along the synovial capillary bed due to local hydrostatic and osmotic pressure changes. The lack of basement membrane allows great exchange of all sorts of components, except macromolecules, between plasma and SF. (3) Therefore the term ultra filtrate is in place. Normal SF is clear, very viscous and contains limited numbers of cells that are mostly mononuclear. Normal or healthy cell counts are less than 25 g/L, but can dramatically increase in case of acute joint inflammation. In that case neutrophils become the predominant cell type. Articular cartilage (AC) is aneural, avascular as well as alymphatic. The SF makes up for the lack of these components by serving as a source of nutrients and providing the possibility of disposing certain metabolites for the AC. The SF acts as a medium of communication between synovium and articular cartilage, in both physiological as well as pathological processes.(1) Biomarker measurement in SF is a method to study joint homeostasis *in vivo.* The term biomarker has been defined as 'a parameter that can be objectively measured and evaluated as an indicator of normal physiological or pathological processes, or the response to pharmacological intervention' by the Biomarkers Definitions Working Group. The SF provides an accurate reflection of the local microenvironment of the sampled joint at that timepoint, making SF preferable to blood and serum. (1,4) Crucial information on levels of inflammatory mediators, cytokines, growth-factors and catabolic enzyme activity can be provided by research done on SF. (1) Identification of parameters in synovial fluid (SF) is used to accurately assess intra-synovial inflammations in cases of joint disease and is still an active area of research. (5,6)

Natural alginate is an anionic polymer that in most cases is obtained from brown seaweed or *Phaeophyceae*. It has been broadly investigated and is being used for several biomedical applications. Alginate is highly biocompatible, low toxic, relatively low-priced and transforms into a gel by addition of a divalent cation like Ca^{2+}. There is structural similarity between alginate and extracellular matrices of living tissues which allow wide application such as in wound healing, slow-release of bioactive agents and even cell transplantation.(18)
It is also possible to biosynthesize alginate with more defined chemical structures and physical properties than alginate derived from seaweed. This is done by using the bacteria *Azotobacter* and *Pseudomonas*. In general the biosynthesis can be divided into (i) synthesis of precursor substrate, (ii) polymerization and cytoplasmic membrane transfer, (iii) periplasmic transfer and modification and (iv) export through the outer membrane. (18,20) The regulation of alginate biosynthesis in bacteria and the possibilities of modification empower production of tailor-made characteristics and therefore specific applications. The difference in the process of deriving or production can result in varying levels of purity of the alginate. (18)
Alginate is determined as a biomaterial that has great utility and potential in different biomedical applications.

Especially in wound healing, drug delivery, *in vitro* cell culture, and tissue engineering. Some of the most favourable features of alginate comprise biocompatibility, mild gelation conditions and the possibility to modify to acquire certain properties. Alginate has credentials in sage clinical use as wound dressing material and pharmaceutical and has been implanted safely in a variety of applications. (18)

Any biomaterial injected in a horse can be seen as foreign body by the immune system.
Foreign body responses are usually characterized by an acute and a chronic phase (29). The acute phase is characterized by neutrophil influx, probably mediated by chemo attractants such as Interleukin-8 (IL-8), and production of Interleukin-1 (IL-1β), Chemokine Ligand 2 (CCL2) and Tumor Necrosis Factor alpha (TNFα) by monocytes, macrophages, fibroblasts and endothelial cells (30,31). In the chronic phase there are mostly fibroblasts and macrophages that aim to either encapsulate or degrade the foreign body. (30) A foreign body reaction can occur when injecting biomaterials into the joint. (18) Cytokines such as Interleukin-8 (IL-8), Interleukin-1 (IL-1β), Chemokine Ligand 2 (CCL2) and Tumor Necrosis Factor alpha (TNFα) are produced by cells in the synovium or by infiltrating leukocytes and released into the SF. Then they can reach the articular cartilage and affect chondrocyte synthesis activities of cause direct cartilage breakdown. (41,42) IL-8 is a chemokine. Chemokines are mediators of inflammation that can induce chemotaxis in a variety of cells; neutrophils, monocytes, lymphocytes, eosinophils and fibroblasts. (43) IL-8 is one of the most potent chemo-attractants for polymorphonuclear neutrophils (PMNs), they stimulate their chemotaxis and generate reactive oxygen metabolites. It is synthesized by a variety of cells including monocytes/macrophages, chondrocytes, fibroblasts and osteoblasts. (44) IL-8 can boost release of inflammatory cytokines like IL-1β, CCL2 and TNFα in mononuclear cells. These may further modulate the inflammatory response. IL-8 can also stimulate neutrophil degranulation and adhere to endothelial cells by macrophage antigen-1. (45) Horses seem particularly sensitive to a foreign body reaction in the joint. (18,32)

During the pilot-study it became clear that the intra-articular administration of alginate beads caused a severe inflammation in the joint of horses. Therefore this pilot-study was ultimately aimed to investigate the factors involved in the response of equine joints to alginate beads. To determine the, possible species-associated, type of inflammation, presumably a foreign body response. Furthermore an attempt to develop an *in vitro* screening tool for this reaction was tested. (33)

Materials and Methods

One by one ultimately three horses were injected with the alginate beads in the intercarpal joint. These beads were provided by the Erasmus MC, University Medical Center Rotterdam. They were the same beads (composition and size) as used before in rats.

The clinical presentation of the first horse showed very severe lameness to the left and right frontlegs that were injected. There was a lot of production of synovial fluid (SF), which appeared cloudy when it was aspirated from the joints. Because of these findings the second horse was injected in just one front leg, left. The third horse was injected during a surgical practicum, and has therefore not walked past-injection as opposed to the other two horses. It was chosen to inject alginate beads in both front legs for this reason. This results in more limited clinical data collected from this third horse.

Alginate pilot-study

Selection of test animals and general design of the alginate pilot-study

The alginate pilot study was performed on three horses, at three different dates, following protocol that was adjusted while the pilot study was on the go. The first horse P1 (=WP9) and last horse P3 (=P1) were injected in both intercarpal joints, the second horse P2 (=WP12) was injected in only the left intercarpal joint. All horses were sampled of synovial fluid by arthrocentesis at timepoint 0 (T0) after which they were injected with a saline solution. This solution contained an unknown number of alginate beads and was injected by a veterinarian of the equine department of the Veterinary Faculty, University Utrecht. The timepoints at which synovial fluid (SF) was collected after the administration of the alginate beads differ per horse and leg due to the protocol that was followed and due to the time the horse was alive after the administration of the alginate beads in the joint(s). All synovium fluid samples were stored in eppendorf cups at a temperature of -80 degrees Celsius. After the euthanasia synovium was collected from all the injected intercarpal joints and embedded in paraffin.

Alginate pilot; Treatment + sample collection		P1		P2	P3	
		L	R	L	L	R
Alginate beads in saline injection (*1 ml, **3 ml) after SF collection at T0		+**	+**	+*	+*	+*
SF collection	T0	+	+		+	+
	T2	+			+	
	T4				+	
	T5,5				+	+
	T8	+	+	+		
	T24	+	+	+		
	T72	+				
	T168	+				
Synovium collection post-mortem		+	+	+	+	+

Figure 1 An overview of the treatment and sample collection protocol per horse and joint

P1

For the alginate pilot-experiment there was initially one horse selected; five years old New Forest pony stallion. This pony was in possession of the Reproduction Department, Veterinary Faculty, Utrecht University. This pony was selected for a chirurgical practicum, after which he would be euthanized. The reason for this selection was lameness to his right front leg due to osteochondrosis of the sagittal ridge of the right metacarpus and cartilage erosions of the distal metacarpal condylar cartilage. The intra-articular administration of alginate beads dissolved in saline (3 mL) was performed in both right and left intercarpal joints. There was no information about the condition of both intercarpal joint prior to the pilot-experiment. The pony was clinically monitored and samples of synovial fluid were taken at time points T2, T8, T24, T72 and T168 hours after injection with the beads in the left leg and time points T8 and T24 hours after injection with the beads in the right leg.

P2

The clinical information derived from the first pilot-experiment pony caused that the clinicians felt the need to

repeat the pilot with adjustments to the protocol. A second horse was selected. This horse was a general educational horse from the Veterinary Faculty, Utrecht University; a 19 year old KWPN mare. This horse was also selected for a chirurgical practicum, after which she would be euthanized. This horse was selected for this practicum for unknown reasons. There was synovial fluid taken from this joint prior to the administration of the alginate beads. The intra-articular administration of alginate beads in saline (1 mL) was done in solely the left intercarpal joint. There was no information about the condition of the left intercarpal joint prior to the pilot-experiment. The horse was clinically monitored and sampled from synovial fluid at time points T8 and T24. The mare was euthanized for reaching the humane end point 32 hours after administration of the beads in the joint. After euthanasia synovium was collected for histology.

P3

The clinical information derived from both prior pilot-experiments raised the need to repeat the administration of alginate beads in the intercarpal joint in a reconstructed protocol. The Veterinary Faculty selected a third horse for a chirurgical practicum; an 18 year old KWPN gelding. After this practicum the horse would be euthanized. There is no further information on the condition of the intercarpal joints of this horse prior to the pilot-experiment. Prior to the administration of the alginate beads synovial fluid was taken from the intercarpal joints. The intra articular administration of alginate beads in saline (1mL) was done at the day of the chirurgical practicum just prior to the anesthesia, in both intercarpal joints. The horse was sampled from synovial fluid at time points T2 and T4 hours during the course of the chirurgical practicum. After euthanasia at timepoint T5,5 hours after administration of the alginate beads synovial fluid was sampled from both left and right intercarpal joints and synovium was collected for histology.

Clinical evaluation

For P1 and P2 there has been a monitoring of clinical parameters. The level of lameness (1-5/5) was assessed by a veterinarian of the equine department of the Veterinary Faculty, University Utrecht, this was the same person that did the intra-articular administration of the alginate beads. This monitoring was done on a straight line in walk and trot on a hard surface and on a hard surfaced circle in walk and trot. In the second horse (P2) it was determined the humane end point was reached at T32 hours after administration of the beads; this was done by the same veterinarian that assessed the level of lameness. The third horse (P3) was under anesthesia in the hours post-injection, therefore no level of lameness was determined.
The assessment of the level of lameness of P1 was done on timepoints T8, T24 and T 48.
The assessment of the level of lameness of P2 was done on timepoints T4, T8, T24 and T30.

Histology

All available collected synovium samples (see figure 1 1) were embedded in paraffin and stored so they could be stained at a later time. There was no macroscopic evaluation performed on the appearance of the inside of the joint before the samples for microscopy were taken. For staining; synovium samples, after fixation in 10% formalin, were dehydrated by an ethanol-series and cleared in xylene, embedded in paraffin (3x 96% EtOH-3x 100% EtOH-3x xylene-paraffin) and sectioned (5µm) on a microtome (Microm). A Haematoxylin Eosin (HE) staining was done on the synovium tissue. To visualize cells the available cut and deparaffinized synovium sections were stained with haematoxylin (Merck 109249) and eosin (Merck 115935) following protocol designed by the Veterinary Laboratory (Appendix I). Available HE slides have been evaluated by Drs. W. Bergmann, veterinary pathologist at the Faculty of Veterinary Medicine, to acquire information about the meaning of distinctive cell types present in the tissues.

Synovial fluid sampling and processing

Synovial fluid (SF) sampling of live horses was done by means of arthrocentesis (disposable needle Sterican, size 2/green) from the intercarpal joint by a veterinarian from the Veterinary Faculty, University Utrecht. All SF samples are centrifuged at 5000 RPM for five minutes (Hettich EBA20 centrifuge). After which the supernatant was for the vast majority pipetted into eppendorf tubes and stored at -80°C. Some SF was directly transferred to the laboratory of the Veterinary Faculty, University Utrecht, for total protein (TP) measurement (most samples). The stored SF was thawed at room temperature before used for enzyme-linked immunosorbent assays (ELISAs) against IL-8, CCL2, IL-1β and TNFα.

ELISAs SF

An ELISA is a five-step procedure, after coating of the ELISA plate with antigen and blocking all unbound sites to prevent false positives, an antibody is added to the wells. Then an anti-goat IgG conjugated to an enzyme is

added. Reaction of a substrate with the enzyme to produce a coloured product indicating a positive reaction takes place. After this the colouring process is stopped and de plates get measured.
A total of four biomarker assays, were performed on (almost) each synovial sample. Some synovial samples ran out before all ELISA's were performed.
A commercially available Kingfisher Biotech do-it-yourself ELISA-kit (KFDIY0702E003) for Equine IL-8 was used for the quantitative determination of chemokine Interleukin-8 (IL-8) following protocol based on the instructions provided by the supplier (Appendix II). A commercially available Kingfisher Biotech do-it-yourself ELISA-kit for Equine CCL2 (KFDIY0694E003) was used for the quantitative determination of CCL2 following protocol based on instructions provided by the supplier (Appendix III). A commercially available Kingfisher Biotech do-it-yourself ELISA-kit for Equine IL-1β (KFDIY0699E003) was used for the quantitative determination of Interleukin-bèta (IL-1β) following protocol based on instructions provided by the supplier (Appendix IV).
A commercially available Thermo Scientific Equine TNFα ELISA reagent kit (product number ESS0017) was used for quantitative determination of tumor necrosis factor alpha (TNFα) following protocol provided by the supplier. (Appendix V) The plates were measured by the use of a TECAN plate reader.

Whole Blood Assay
In attempt to design an *in vitro* predictor for the *in vivo* intra-articular reaction to alginate a whole blood assay (WBA) was performed with equine blood.
Blood of 6 equine donors was taken with heparin (Greiner Bio-One, VACUETTE® lithium heparin tubes, 9mL, catalog number 95057-415) as anti-coagulant. The donors were clinically healthy research animals taking part in another study. No additional health testing was performed on the blood. The horses were not treated with medication at the time of the blood drawing or in the time before that, for that would have affected the experiment.

Different types of alginates were tested, but the beads of interest were specifically the 'oud kl g' (see results WBA) since these were the beads that were injected in the pilot study. These alginate beads were provided by the Erasmus University Rotterdam and were rinsed according to the provided protocol. The other types of alginate beads were of other composition and size, and were tested in this WBA for other purposes.
The different types of alginate beads were divided over the 48 wells plate (Greiner bio-one, Cellstar 48 Well Cell Culture Plate, Cat.-No. 677 180). After which 100 µl of donors blood diluted with medium (6x/1:5) was added. Other materials were added to the donor's blood in the WBA as a positive control; lipopolysaccharide (LPS) and polymethyl acrylate (PMA). In the negative control medium was added, instead of a biomaterial, to the blood to add up to an equal total volume of 330 µl per well.
The plate was incubated for 18 hours in at 37°C. The next day the medium with blood (and liquid materials) was pipetted into 1,5 mL eppendorf cups, and centrifuged at 1000G for 10 minutes. The supernatant was pipetted into 1,5 ml eppendorf tubes. Per material and donor 100 µl was pipetted onto the ELISA plates.
It was decided ELISA's on IL-8 and CCL2 should be performed on the supernatant of the WBA. A commercially available Kingfisher Biotech do-it-yourself ELISA-kit (KFDIY0702E003) for Equine IL-8 was used for the quantitative determination of chemokine ligand/Interleukin-8 (IL-8) following protocol based on the instructions provided by the supplier (Appendix II). A commercially available Kingfisher Biotech do-it-yourself ELISA-kit for Equine CCL2 (KFDIY0694E003) was used for the quantitative determination of CCL2 following protocol based on instructions provided by the supplier (Appendix III)

Statistical analysis
GraphPad Prism 7.04 was used to perform the statistical analyses over the results of the whole blood assay. To test normality a Shapiro-Wilk test was performed. When present an ordinary one-way ANOVA with a Bonferroni correction was performed to determine the significance of the results.

Results

Level of lameness

Two horses,P1 and P2, have been monitored on level of lameness at different time points. The third horse (P1) was under anesthesia in the hours post-injection, therefore this clinical parameter was impossible to monitor for this horse.

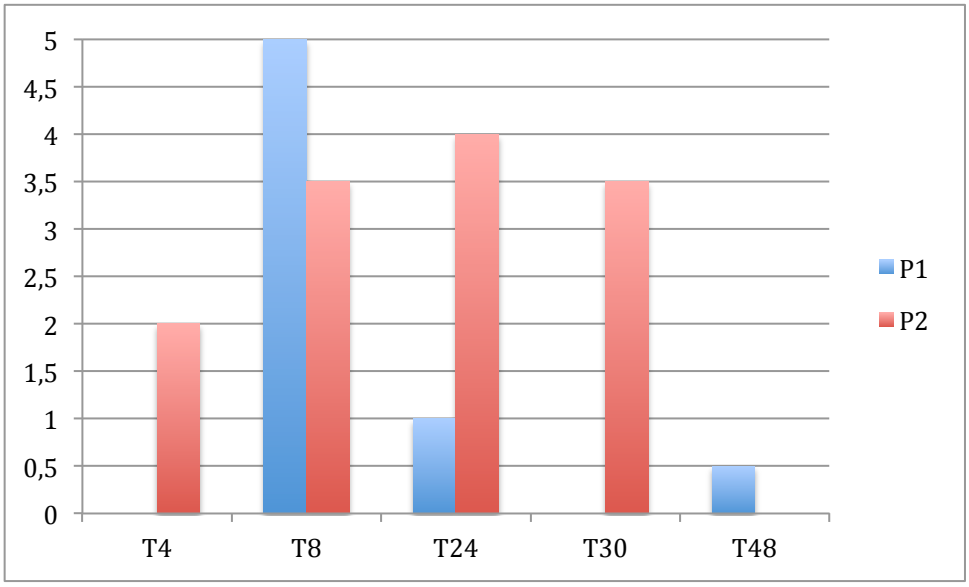


Figure 2 Level of lameness (1-5/5) of patiënts 1 and 2 at different timepoints after intra-articular administration with alginate beads.

For P1 there is a severe lameness of 5 out of 5 at timepoint 8, it was decided this horse needed pain medication; oral administration of tramadol and butorphanol. This was administered for the following period until the surgical practicum. At timepoint T24 the lameness decreased to 1 out of 5. The lameness at timepoint T48 was 0,5 out of 5.

For horse P2 the first lameness assessment was performed at timepoint 4 and resulted in a score of 2 out of 5. At timepoint 8 this lameness increased to a level of 3,5 out of 5. At that time tramadol (3000mg) and phenylbutazon (2g) was orally administered. The highest level of lameness was reached at timepoint 24; 4 out of 5. It was decided the horse needed intra-articular administration of morphine. At the last assessment, at timepoint 30, a level of lameness of 3,5 out of 5 was assigned. At timepoint 32 it was decided the horse had reached its humane endpoint despite the pain medication and was euthanized.

Synovial fluid

Directly after collection of synovial fluid a portion was used to measure the total protein, for most samples.

Total Protein	P1 left	P1 right	P2	P3 left	P3 right
T0	Not available	+		+	Not available
T2	Not available			+	
T4				+	
T5,5				+	+
T8	+	+	+		
T24	+	+	+		
T72	+				
T168	+				

Figure 3 An overview of the available SF samples for total protein measurement.

For some samples (P1 left T0 and T2, P3 right T0) there was not enough SF left after processing to measure the total protein. Figure 4 shows the mean and standarddeviation of the total protein (g/L). The total protein should be lower than 25 g/L. This is seen in all samples of timepoint 8 (n=3) and 24 (n=3).

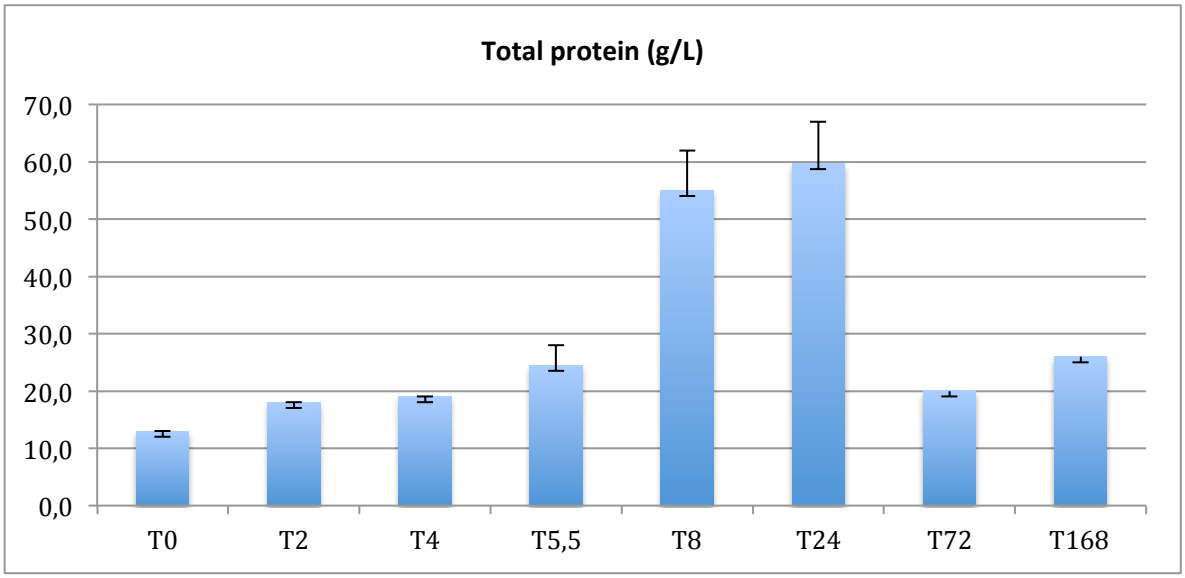


Figure 4 Total protein (g/L) in SF; mean and standarddeviation per timepoint

The Interleukin-8 (IL-8) ELISA was performed on the SF samples shown in Figure 5.

IL-8 ELISA	P1 left	P1 right	P2	P3 left	P3 right
T0	+	+		+	+
T2	+			+	
T4				+	
T5,5				+	+
T8	+	+	+		
T24	+	+	+		
T72	+				
T168	+				

Figure 5 An overview of the available SF samples for IL-8 ELISA

On all SF samples that were taken, an ELISA against IL-8 has been performed. Figure 6 shows the mean and standarddeviation of IL-8 (pg/ml). At timepoint 0 (before administration of the alginate beads), there was an IL-8 value close to zero. At timepoint 2 (n=2) the value increased to almost 300 pg/ml. For timepoint 4 there was only one sample (P3 left) that also showed a value close to 300 pg/ml. The highest value of IL-8 was measured at timepoint 5,5 (n=2), after which it seems to decrease in following time points 8, 24, 72 and 168.

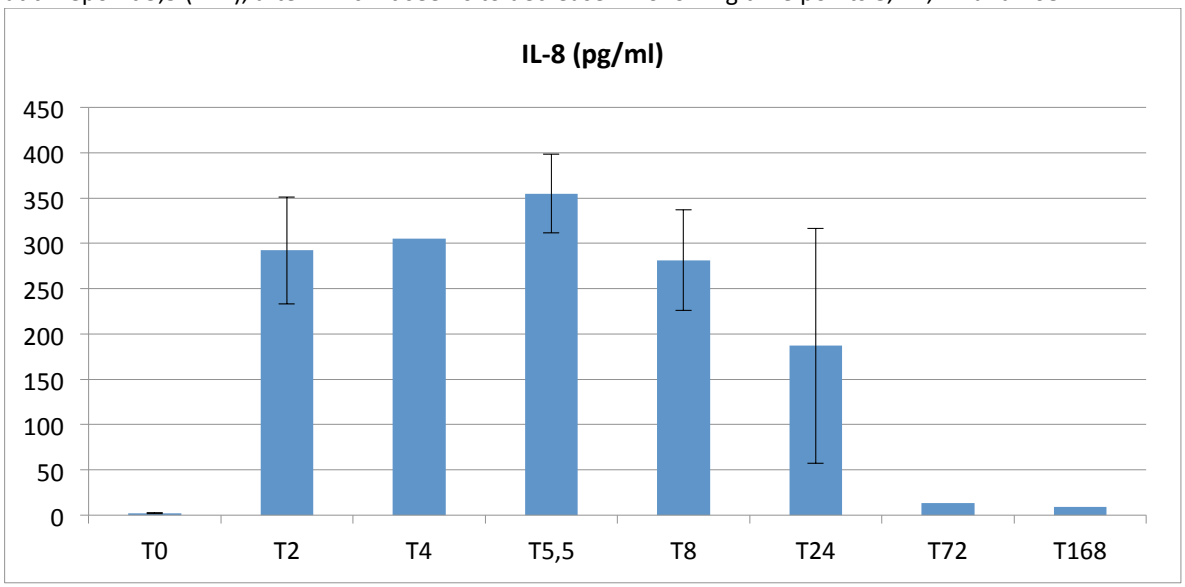


Figure 6 Interleukin-8 (pg/ml) in SF; mean and standarddeviation per timepoint

The Chemokine Ligand 2 (CCL2) ELISA was performed on the SF samples shown in Figure 7.

CCL2 ELISA	P1 left	P1 right	P2	P3 left	P3 right
T0	+	+		+	+
T2	+			+	
T4				+	
T5,5				+	+
T8	+	+	+		
T24	+	+	+		
T72	+				
T168	+				

Figure 7 An overview of the available SF samples for CCL2 ELISA

On all samples that were taken, an ELISA against CCL2 has been performed. Figure 8 shows the mean and standarddeviation of CCL2 (pg/ml). At time points 0 and 2 the CCL2 value was close to 0. From timepoint 4 to 8, there is an increase in value of CCL2. This decreases after timepoint 8.

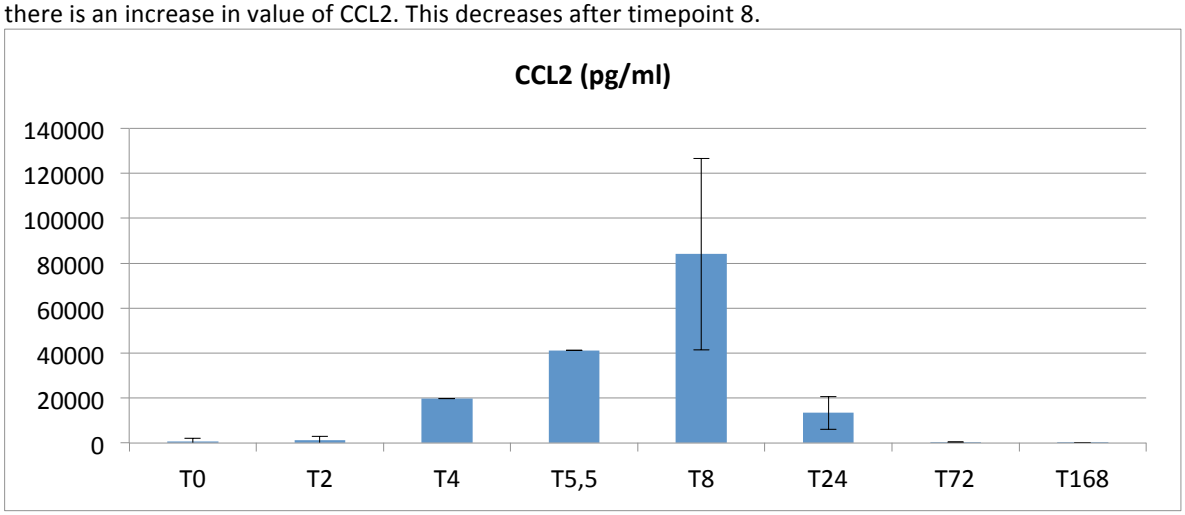


Figure 8 Chemokine Ligand 2 (pg/ml) in SF; mean and standarddeviation

The Interleuking-1β ELISA was performed on the SF samples shown in Figure 9.

IL-1β ELISA	P1 left	P1 right	P2	P3 left	P3 right
T0	+	+		+	+
T2	+			+	
T4				+	
T5,5				+	+
T8	+	+	+		
T24	+	+	+		
T72	+				
T168	+				

Figure 9 An overview of the available SF samples for IL-1β ELISA

On all SF samples that were taken, an ELISA against IL-1**β** has been performed. Figure 6 shows the mean and standarddeviation of IL-1**β** (pg/ml). At timepoint 8 (n=3) there is a clear increase in value of IL-1**β,** at this timepoint there is a large standarddeviation.

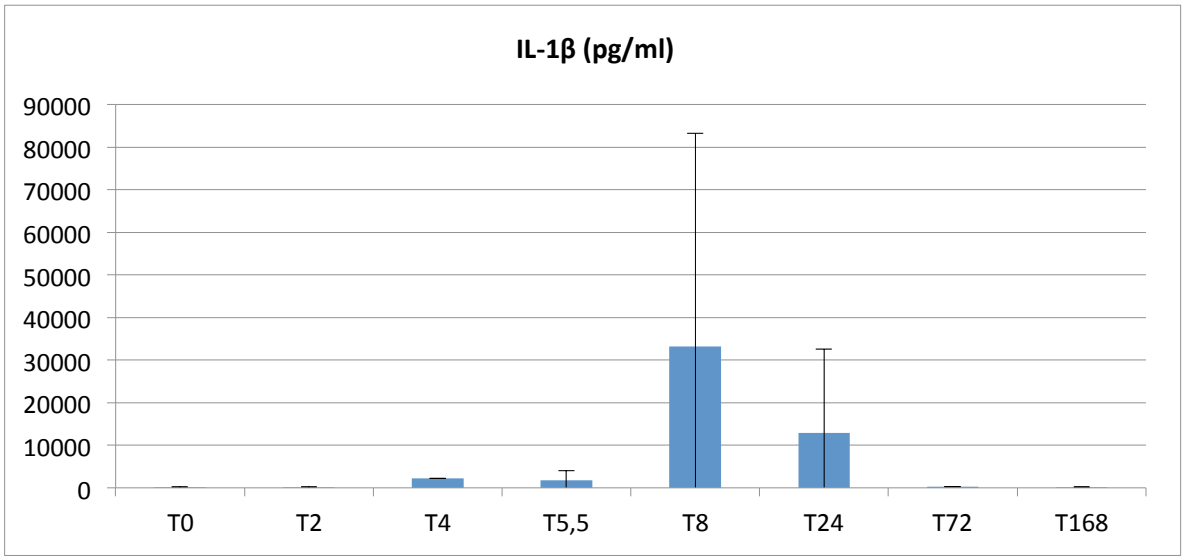


Figure 10 Interleukin-1β (pg/ml) in SF; mean and standarddeviation per timepoint

The Tumor Necrosis Factor-**α** ELISA was performed on the SF samples shown in Figure 9.

TNFα ELISA	P1 left	P1 right	P2	P3 left	P3 right
T0	+	+		Out of SF	+
T2	+			+	
T4				+	
T5,5				+	+
T8	+	+	+		
T24	+	+	+		
T72	+				
T168	+				

Figure 11 An overview of the available SF samples for TNFα ELISA

On all SF samples that were taken, an ELISA against TNF**α** has been performed. Figure 6 shows the mean and standarddeviation of TNF**α** (pg/ml). From timepoint 2 (n=2) there is a clear increase in value of TNF**α.** At timepoint 4 (n=1) the highest value of TNF**α** is measured. The value remains high until timepoint 8 and then decreases after that.

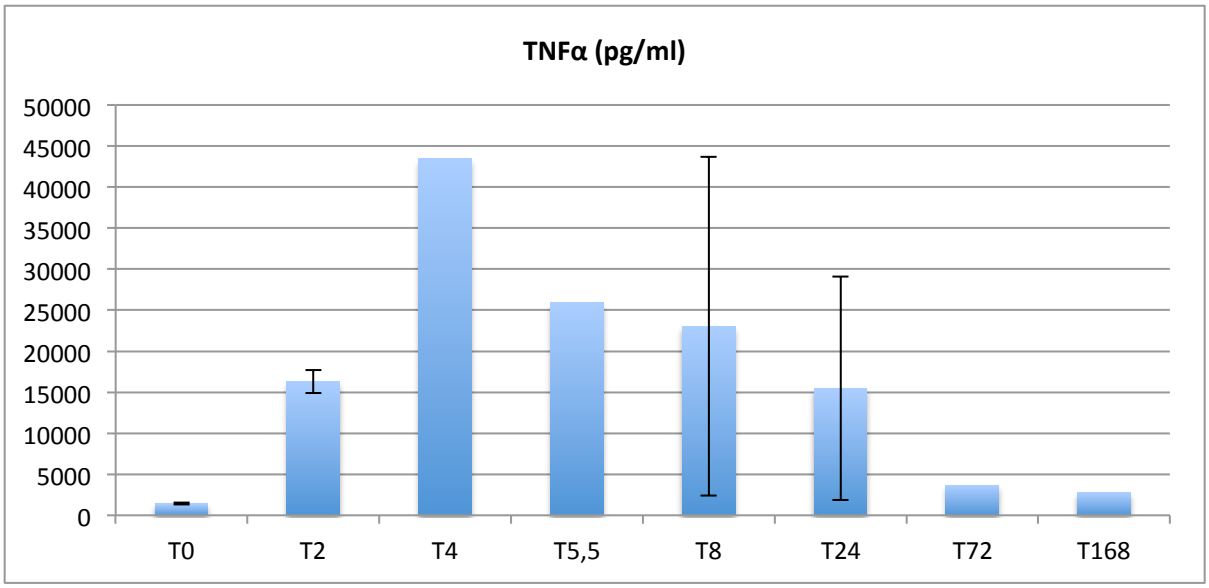
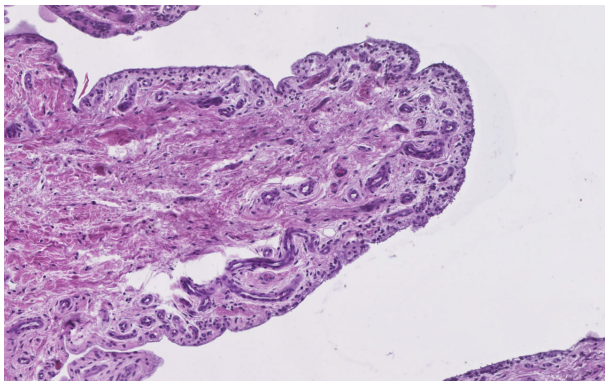


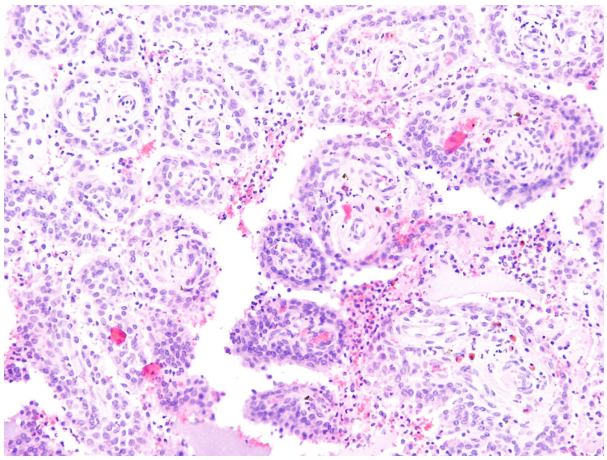
Figure 12 Tumor Necrosis Factor-α (pg/ml) in SF; mean and standarddeviation per timepoint

Histology

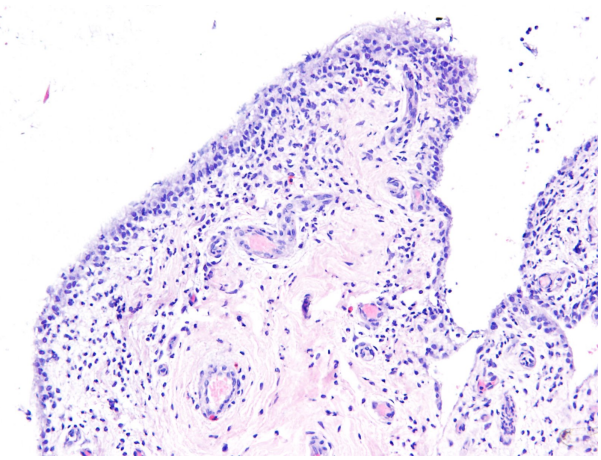
All joints treated with alginate beads have been samples on synovium for histology. All samples have been stained with the HE staining. The following pictures represent the best sample per joint. All histology samples have been evaluated by Drs. W. Bergmann, veterinary pathologist at the Faculty of Veterinary Medicine, and showed a lot of resemblance. In general the histology shows; neutrophil influx at the synovial borders that suggest reaction to something present in the synovial fluid. No particular celtype was identified that would indicate a specific foreign body response. The neutrophil influx could also occur in reaction to a microorganism in the synovial fluid according to Drs. W. Bergmann.



Picture 1 P1 left



Picture 2 P2



Picture 3 P3 left

Whole Blood Assay + ELISA

The ELISA against IL-8 was performed on the supernatant of all 6 blood-donors. LPS and PMA were used as positive controls, where in the negative control no substance was added in the wells.

IL-8 ELISA (pg/ml)	Negative control	LPS	PMA	Oud kl g	Oud gr G	Oud gr M
D1	37,15	165,3	1732,1	446,8	33,3	39,3
D2	49,65	277,75	2200,6	580,2	49,5	44
D3	89,05	219,85	1764,5	250,4	194,7	97,1
D4	128,35	404,3	774,4	1040,5	261	275,9
D5	48	144,4	812,7	2280,3	136,3	105,4
D6	88,9	500,85	1285,2	3137,45	189,6	322,9

Figure 13 Results of equine WBA and ELISA against IL-8 (pg/ml)

For all donors the negative control value measured lower than the value of the positive controls LPS and PMA and the value of 'Oud kl g'. Therefore both LPS and PMA functioned as positive controls. The IL-8 values in reaction to de alginate beads 'Oud kl g' (that were used in the pilot study) are high. As apposed to the values in reaction to the other types of alginate beads, that had other composition and size.

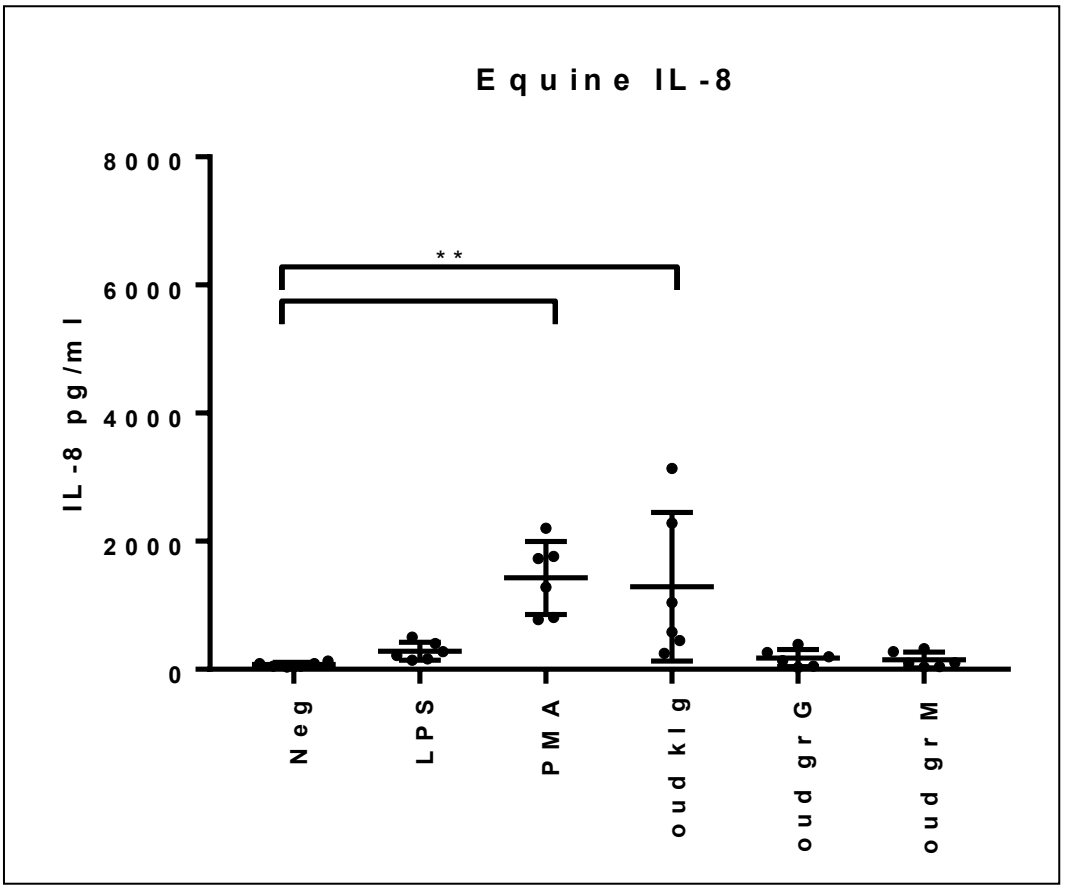


Figure 14 Mean and standarddeviation IL-8 (pg/ml)

Statistical analysis shows significant (P=0,0071) outcome when the alginate beads 'Oud kl g' are compared to the negative control. This was alto the case with positive control PMA, but not for LPS.

The ELISA against CCL2 was performed on the supernatant of all 6 blood-donors. LPS and PMA were used as positive controls, where in the negative control no substance was added in the wells.

CCL2 ELISA (pg/ml)	Negative control	LPS	PMA	Oud kl g	Oud gr G	Oud gr M
D1	17,65	2539,9	213,3	2511,2	303,9	117,5
D2	40,25	4064,15	530,7	5169,9	1448,3	1501
D3	253,65	6705,25	1164,8	6887,2	1705,7	1859,6
D4	0	3297	559,9	3168,1	0	0
D5	512,8	3561,85	986,7	3779,9	478,9	950,2
D6	244,9	1692,25	1099,2	2994,3	134,8	188,1

Figure 15 Results of equine WBA and ELISA against CCL2

For all donors the negative control value measured lower than the value of the positive controls LPS and PMA and the value of 'Oud kl g'. Therefore both LPS and PMA functioned as positive controls. The CCL2 values in reaction to de alginate beads 'Oud kl g' (that were used in the pilot study) are high. As apposed to the values in reaction to the other types of alginate beads, that had other composition and size.

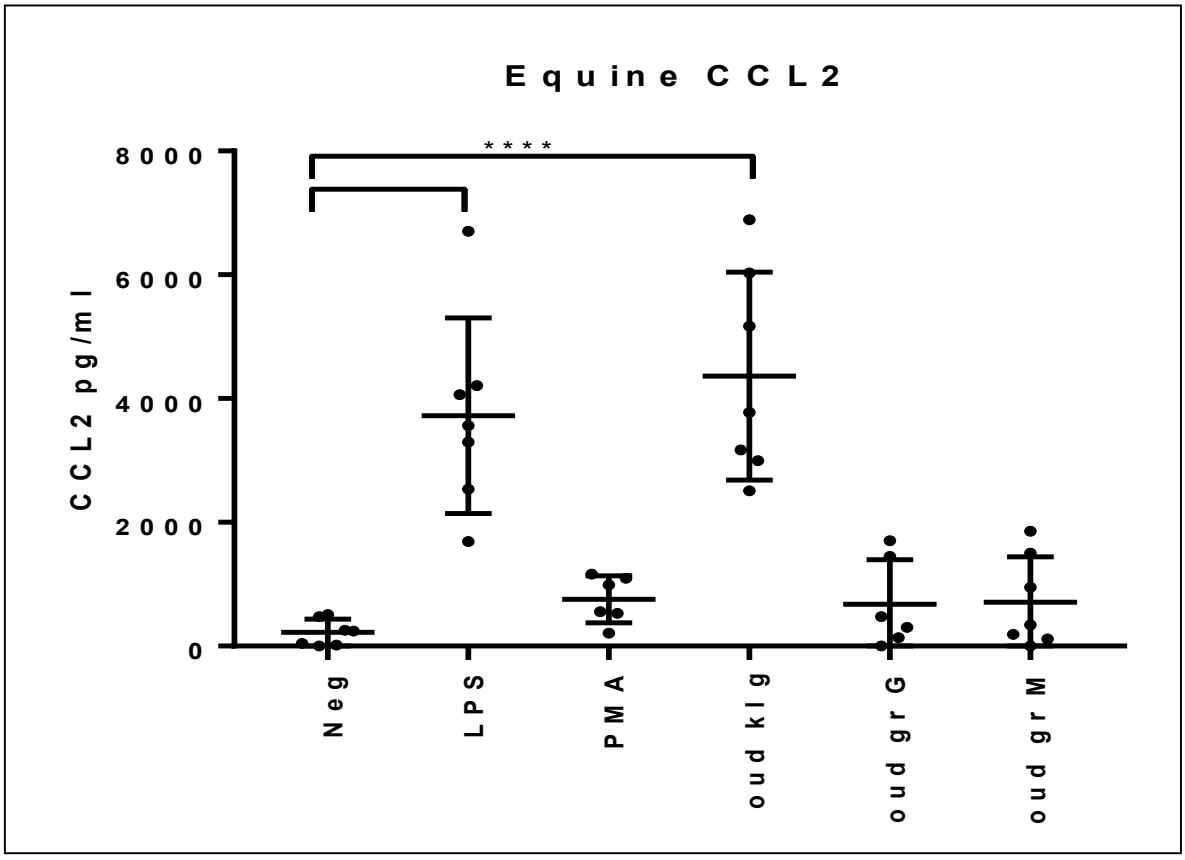


Figure 16 Mean and standarddeviation CCL2 (pg/ml)

Statistical analysis shows significant (P<0,0001) outcome when the alginate beads 'Oud kl g' are compared to the negative control. This was also the case with positive control LPS, but not for PMA.

Discussion

Pilot study

This pilot study was designed as a check to determine if the alginate beads that were used before in rats were 'safe' to use in horses, before a more broad testing would be performed in England. Therefore initially only one horse was selected to be treated in both intercarpal joints; P1. This horse had already been selected for the surgical practicum, which made it ethically responsible to use this horse for another scientific purpose. The first horse was a relatively young horse (5 years old), which suffered from lameness that originated in another joint. No evaluation on the state of the intercarpal joints was performed. This information on the state of the intercarpal joint could have lead to the decision this horse was not suitable for this experiment.
In hindsight we would preferably have treated only one intercarpal joint, instead of two at the same time, for ethical reasons. The lameness that developed was not expected upfront, making the decision on treating both intercarpal joint not a wrong call. In theory it is possible that despite extensive cleaning prior to injection a bacterium was taken in the joint at the injection-site. That this had happened in two joints would have been a great coincidence considering the experience of the people involved.
Arthrocentesis to collect synovial fluid was performed 6 times in the left leg, and 4 times in the right leg.

After the severe clinical signs of the first horse, a second horse was selected. This horse was much older (19 years), and also selected for the surgical practicum for unknown reasons. Again no evaluation on the state of the intercarpal joints was performed. Which would have been interesting since this horse was already 19 years old. It was decided, in reaction to the clinical evaluation of the first horse, only one intercarpal joint would be treated. This horse became severely lame already 4 hours after administration of the beads in the joint. Despite pain medication it reached it's human endpoint 30 hours after the administration of the beads, and was euthanized. Arthrocentesis in this horse was done on the 3 time points that seemed the most relevant in the first horse, T0, T8 en T24. This horse already appeared lame on timepoint T4, which would have been a good timepoint to also take SF. The horse showed clinical signs and it would have been interesting to see if the reaction could also be seen in the SF at this timepoint.

After the first two horses it was decided, for ethical reasons, no IA administration of the alginate beads would be repeated in a conscious horse before it was clear why the reaction was so severe in terms of lameness.
An alternative was to inject the horse just prior to the induction of anaesthesia of a surgical practicum. The next planned practicum was performed on a 18 year old horse. No evaluation of the state of the intercarpal joints was performed prior to the administration of alginate beads in both joints. A disadvantage was that no level of lameness could be monitored. This was justified by the ethical arguments. Also sampling of SF could only be done during the practicum. It was decided this would be done every two hours and at the end (T0, T2, T4 and T5,5). In hindsight the results of the ELISA's on different biomarkers were all increasing during this period T0-T5,5. The results of the ELISA's are corresponding to the results of the other two horses, by which we can conclude this horse would have also been lame if conscious.

SF sampling of the horses was not performed at equal timepoints. Due to that the results of the ELISA's are difficult to read, compare and judge. Statistical analyses on these results is not possible, due to too low numbers of testing. Since this experiment was performed as a pilot study, prior to a more extensive study in England, this is not wrong choice.

Literature reports high M content alginates to be immunogenic and approximately 10 times more potent in the onset of cytokine production in comparison with high G alginates. (21) Whilst others found little or no immune response. (22) Immunogenic responses might be caused by impurities in the alginate itself, as it is obtained from natural sources. Impurities like heavy metals, endotoxins, proteins and polyphenolic compounds could be present in the alginate. (18) There bacterial culture performed on the alginate beads prior to injection that turned out negative, so endotoxins were ruled out.
The exact content of M and G in the used alginate beads during this pilot study was not known. Knowing alginate itself is immunogenic could have predicted a reaction to alginate beads in horses, especially since it is known that horses seem particularly sensitive to a foreign body reaction in the joint. (18,32) Horses often present with (transient) lameness and elevated white blood cell counts after injection of a biomaterial. Even materials that do not normally elicit an adaptive immune response and have been tested in other animals, can lead to swollen and painful joints in horses. (18,32)
The increase of biomarkers against Interleukin-8 (IL-8), Chemokine Ligand 2 (CCL2), Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor alfa (TNFα) suggest a foreign body response is active in the treated joints.

Whole Blood Assay
The performed WBA was designed in attempt to predict the reaction to biomaterial *in vitro* before experimenting *in vivo.*
The results of the WBA show a significant increase in IL-8 and CCL2 in reaction to the alginate beads used in the pilot study. The reaction seen in the pilot study could, according to the results of the WBA, have been predicted by this *in vitro* test.

Also two other types of alginates were tested in this WBA, which show no increased levels of IL-8 and CCL2 in this WBA. Possibly there is a difference in composition causing this difference in response. There culture was performed on the alginate beads used in the pilot study that turned out negative; endotoxins were not present. Although a false negative result of this culture cannot be ruled out.
To determine whether this WBA is also a predictive test for the reaction to the other two types of alginate in equine joint, these types of alginates would have to be injected in the joints of horses. They would have to be clinically monitored en sampled from SF en synovium like the first two horses in the pilot study. For ethical reasons this is not done yet.

Conclusion

The initial aim of this study was to determine whether or not alginate beads are suitable for use in joints of horses. According to the results obtained, it is safe to say that the alginate beads used in this pilot study are not suitable for use in joints of horses. The used beads cause severe lameness en elevated biomarker levels in the SF.

During the pilot-study it became clear that the intra-articular administration of alginate beads caused a severe inflammation in the joint of horses. This pilot-study was ultimately aimed to investigate the factors involved in the response of equine joints to alginate beads and to determine the, possible species-associated, type of inflammation, presumably a foreign body response.
The results of the different biomarker levels in the SF of the pilot study suggest it is possible a foreign body response has taken place in the joints.

More research needs to be performed before extensive *in vivo* testing with alginate beads in horses can be done. An equine whole blood assay appears a promising *in vitro* test to predict an *in vivo* reaction to biomaterials.

References

1. de Grauw JC. Molecular monitoring of equine joint homeostasis. Vol. 31, Veterinary Quarterly. 2011. p. 77–86.
2. Hardy J, Bertone AL, Weisbrode SE, Muir WW, O'Dorisio TM, Masty J. Cell trafficking, mediator release, and articular metabolism in acute inflammation of innervated or denervated isolated equine joints. Am J Vet Res. 1998;59(1):88–100.
3. Lawry G V. Anatomy of Joints, General Considerations, and Principles of Joint Examination. In: Musculoskeletal Examination and Joint Injection Techniques. 2006. p. 1–6.
4. Rosenkranz ME, Wilson DC, Marinov AD, Decewicz A, Grof-Tisza P, Kirchner D, et al. Synovial fluid proteins differentiate between the subtypes of juvenile idiopathic arthritis. Arthritis Rheum. 2010;62(6):1813–23.
5. Ribera T, Monreal L, Delgado MA, Ríos J, Prades M. Synovial fluid D-dimer concentration in horses with osteochondritis dissecans and osteoarthritis. Vet Comp Orthop Traumatol. 2013;26(1):54–60.
6. Boland L, Danger R, Cabon Q, Rabillard M, Brouard S, Bouvy B, et al. MMP-2 as an early synovial biomarker for cranial cruciate ligament disease in dogs. Vet Comp Orthop Traumatol. 2014;27(3):210–5.
7. van Weeren PR, de Grauw JC. Pain in Osteoarthritis. Vol. 26, Veterinary Clinics of North America - Equine Practice. 2010. p. 619–42.
8. Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, Kwoh CK, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. Arthritis Rheum. 2008;58(1):15–25.
9. Wilsher S, Allen WR, Wood JLN. Factors associated with failure of thoroughbred horses to train and race. Equine Vet J. 2006;38(2):113–8.
10. Rossdale PD, Hopes R, Digby NJ, Offord K. Epidemiological study of wastage among racehorses 1982 and 1983. Vol. 116, The Veterinary record. 1985. p. 66–9.
11. Auer J, Stick J. Equine Surgery. Equine Surgery. 2012.
12. Brandt KD, Dieppe P, Radin E. Etiopathogenesis of Osteoarthritis. Vol. 93, Medical Clinics of North America. 2009. p. 1–24.
13. Brama PAJ. Joint Disease in the Horse [Internet]. Joint Disease in the Horse. 2016. 105-118 p. Available from: http://www.sciencedirect.com/science/article/pii/B9781455759699000085
14. Shalom-Barak T, Quach J, Lotz M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. J Biol Chem. 1998;273(42):27467–73.
15. Garvican ER, Vaughan-Thomas A, Redmond C, Gabriel N, Clegg PD. MMP-mediated collagen breakdown induced by activated protein C in equine cartilage is reduced by corticosteroids. J Orthop Res. 2010;28(3):370–8.
16. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - A review. Vol. 171, Veterinary Journal. 2006. p. 51–69.
17. Caron JP. Intra-articular injections for joint disease in horses. Vol. 21, Veterinary Clinics of North America - Equine Practice. 2005. p. 559–73.
18. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci [Internet]. 2012;37(1):106–26. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22125349%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3223967
19. Martinsen a, Skjåk-Braek G, Smidsrød O. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng. 1989;33(1):79–89.
20. Remminghorst U, Rehm BHA. Bacterial alginates: From biosynthesis to applications. Vol. 28, Biotechnology Letters. 2006. p. 1701–12.
21. Otterlei M, Ostgaard K, Skjåk-Braek G, Smidsrød O, Soon-Shiong P, Espevik T. Induction of cytokine production from human monocytes stimulated with alginate. J Immunother. 1991;10(4):286–91.
22. Zimmermann U, Klöck G, Federlin K, Hannig K, Kowalski M, Bretzel RG, et al. Production of mitogen-contamination free alginates with variable ratios of mannuronic acid to guluronic acid by free flow electrophoresis. Electrophoresis. 1992;13(1):269–74.
23. Orive G, Ponce S, Hernández RM, Gascón AR, Igartua M, Pedraz JL. Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates. Biomaterials. 2002;23(18):3825–31.
24. Lee J, Lee KY. Local and sustained vascular endothelial growth factor delivery for angiogenesis using an

injectable system. Pharm Res. 2009;26(7):1739–44.
25. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of Stem Cell Fate by Physical Interactions with the Extracellular Matrix. Cell Stem Cell. 2009;5(1):17–26.
26. Ma H-L, Hung S-C, Lin S-Y, Chen Y-L, Lo W-H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J Biomed Mater Res A [Internet]. 2003;64(2):273–81. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12522814&retmode=ref&cmd=prlinks%5Cnpapers2://publication/doi/10.1002/jbm.a.10370
27. Lee KY, Peters MC, Anderson KW, Mooney DJ. Controlled growth factor release from synthetic extracellular matrices. Nature. 2000;408(6815):998–1000.
28. Zhao X, Kim J, Cezar CA, Huebsch N, Lee K, Bouhadir K, et al. Active scaffolds for on-demand drug and cell delivery. Proc Natl Acad Sci [Internet]. 2011;108(1):67–72. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1007862108
29. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Vol. 20, Seminars in Immunology. 2008. p. 86–100.
30. Murphy KP, Travers P, Walport M, Janeway C. Janeway's Immuno Biology. Inc. New York and London. 2008.
31. McIlwraith CW, Frisbie DD, Kawcak CE, Fuller CJ, Hurtig M, Cruz A. The OARSI histopathology initiative–recommendations for histological assessments of osteoarthritis in the horse. Osteoarthr Cartil. 2010;18:S93–105.
32. Petit A, Redout EM, van de Lest CH, de Grauw JC, Müller B, Meyboom R, et al. Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses. Biomaterials. 2015;53:426–36.
33. Sokolov A, Hellerud BC, Lambris JD, Johannessen EA, Mollnes TE. Activation of polymorphonuclear leukocytes by candidate biomaterials for an implantable glucose sensor. J Diabetes Sci Technol [Internet]. 2011;5(6):1490–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22226271
34. Barrett AJ. Which proteinases degrade cartilage matrix ? Semin Arthritis Rheum. 1981;11(1 SUPPL. 1):52–6.
35. Brama P a, TeKoppele JM, Beekman B, van Weeren PR, Barneveld a. Matrix metalloproteinase activity in equine synovial fluid: influence of age, osteoarthritis, and osteochondrosis. Ann Rheum Dis. 1998;57(11):697–9.
36. Clegg PD, Coughlan a R, Riggs CM, Carter SD. Matrix metalloproteinases 2 and 9 in equine synovial fluids. Equine Vet J. 1997;29(5):343–8.
37. Chu SC, Yang SF, Lue KH, Hsieh YS, Li TJ, Lu KH. Naproxen, meloxicam and methylprednisolone inhibit urokinase plasminogen activator and inhibitor and gelatinases expression during the early stage of osteoarthritis. Clin Chim Acta. 2008;387(1–2):90–6.
38. May SA, Hooke RE, Lees P. Interleukin-1 stimulation of equine articular cells. Res Vet Sci. 1992;52(3):342–8.
39. Oke SL, Hurtig MB, Keates RA, Wright JR, Lumsden JH. Assessment of three variations of the 1,9-dimenthylmethylene blue assay for measurement of sulfated glycosaminoglycan concentrations in equine synovial fluid. Am J Vet Res. 2003;64(7):900–6.
40. GRAUW JC, LEST CHA, BRAMA PAJ, RAMBAGS BPB, WEEREN PR. In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. Equine Vet J [Internet]. 2009;41(7):693–9. Available from: http://doi.wiley.com/10.2746/042516409X436286
41. Martel-Pelletier J. Pathophysiology of osteoarthritis. Osteoarthritis Cartilage. 2004;12 Suppl A:S31–3.
42. Krasnokutsky S, Attur M, Palmer G, Samuels J, Abramson SB. Current concepts in the pathogenesis of osteoarthritis. Vol. 16, Osteoarthritis and Cartilage. 2008.
43. Proudfoot AEI. CHEMOKINE RECEPTORS: MULTIFACETED THERAPEUTIC TARGETS. Nat Rev Immunol [Internet]. 2002;2(2):106–15. Available from: http://www.nature.com/doifinder/10.1038/nri722
44. Lisignoli G, Toneguzzi S, Pozzi C, Piacentini A, Riccio M, Ferruzzi A, et al. Proinflammatory cytokines and chemokine production and expression by human osteoblasts isolated from patients with rheumatoid arthritis and osteoarthritis. J Rheumatol. 1999;26(4):791–9.
45. Pierzchala AW, Kusz DJ, Hajduk G. CXCL8 and CCL5 expression in synovial fluid and blood serum in patients with osteoarthritis of the knee. Arch Immunol Ther Exp (Warsz). 2011;59(2):151–5.
46. Bussières G, Jacques C, Lainay O, Beauchamp G, Leblond A, Cadoré JL, et al. Development of a composite orthopaedic pain scale in horses. Res Vet Sci. 2008;85(2):294–306.
47. Van Loon JPAM, Jonckheer-Sheehy VSM, Back W, René van Weeren P, Hellebrekers LJ. Monitoring

equine visceral pain with a composite pain scale score and correlation with survival after emergency gastrointestinal surgery. Vet J. 2014;200(1):109–15.
48. Krenn V, Morawietz L, Burmester GR, Kinne RW, Mueller-Ladner U, Muller B, et al. Synovitis score: Discrimination between chronic low-grade and high-grade synovitis. Histopathology. 2006;49(4):358–64.
49. Montes GS, Junqueira LC. The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Vol. 86 Suppl 3, Memorias do Instituto Oswaldo Cruz. 1991. p. 1–11.
50. Neumann U, Kubota H, Frei K, Ganu V, Leppert D. Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. Anal Biochem. 2004;328(2):166–73.
51. van den Boom R, Brama P a J, Kiers GH, de Groot J, van Weeren PR. Assessment of the effects of age and joint disease on hydroxyproline and glycosaminoglycan concentrations in synovial fluid from the metacarpophalangeal joint of horses. Am J Vet Res [Internet]. 2004;65(3):296–302. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15027675
52. de Grauw JC, Brama PA, Wiemer P, Brommer H, van de Lest CH, van Weeren PR. Cartilage-derived biomarkers and lipid mediators of inflammation in horses with osteochondritis dissecans of the distal intermediate ridge of the tibia. Am J Vet Res. 2006;67(7):1156–62.
53. Dey P, Saphos C a, McDonnell J, Moore VL. Studies on the quantification of proteoglycans by the dimethylmethylene blue dye-binding method. Specificity, quantitation in synovial lavage fluid, and automation. Connect Tissue Res [Internet]. 1992;28(4):317–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1284787
54. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. BBA - Gen Subj. 1986;883(2):173–7.

Appendix I - SOP002- Hematoxylin Eosin staining

(SOP002 UU-FVM-DGG-DGP Version 4, 26-07-2016)

1. Aim and Background

The most commonly used staining for looking at the morphology during histology is called hematoxylin and Eosin (HE). HE contains two dyes haematoxylin and eosin. Hematoxylin can be considered as a basic dye and stains the nuclei of cells blue and Eosin Y is an acid dye and colors eosinophilic structures in various shades of red, pink and orange and stains the cytoplasm.

2. Chemicals

- Acetic acid, (Boom 51830)
- Distilled water
- Eosin (Merck 115935, centrifuge room, safety cabinet)
- Ethanol 50% and 96%, See SOP00 buffers and solutions.
- Hydrochloric acid 37% (Merck 100317, Acid cabinet, fume hood)
- Graded alcohol solutions (60%, 70%, 80%, 96%, 100%)
- Mayers haematoxylin, (Merck 109249)(re-use multiple times)
- Depex (Merck 100579)
- Xylene (klinipath 4055-9005, safety cabinet)

Materials:

- Cover glass (VWR 631-0127/631-0146, IHC bench)
- Glassware for stainings

3. Preparing Solutions

- Eosin
  - Eosin Yellowish 0.2gram
  - Ethanol 50% 100ml
  - Acetic Acid 1 drop
- Hydrochloric acid- alcohol
  - Distilled water 400ml
  - Ethanol 96% 400ml
  - Hydrochloric acid 37% 4ml

4. Procedure

1. Deparaffinize slides by the following steps:
   Xylene-Xylene- 96% EtOH, 80% EtOH, 70% EtOH and 60% ETOH — 5min each step
2. Add slides to distilled water — 5min
3. Stain slides in haematoxylin solution (filter before use!) (re-use multiple times) — 10-20sec
4. Rinse the slides once with tap water and deposit this blue water as CA. III
5. Wash slides in running tap water — 10 min
6. Check the staining, if staining is too blue:
   De-stain slides with hydrochloric acid- alcohol (dip) and wash again for 10 min. in running tap water. If not, skip this step!
7. Stain the slides with eosin solution — 20sec
8. Dehydrate slides by the following steps:
   - - 1 time 70% EtOH quick in and out (eosin dissolves in 70% EtOH)
   - - 2 times 96% EtOH quick in and out
   - - 1 time 100% EtOH — 5min
   - - 2 times Xylene — 5min
9. Cover slides with Depex mounting medium.

5. Safety and Waste management

All the staining solutions and EtOH can be discarded in the cat. III chemical waste can (fluids) or bin (solids, e.g. filter paper).

Appendix II – SOP024 – ELISA against equine IL8 (SOP024 UU-FVM-DGG-DGP)

1. Aim and background

2. Chemicals and Materials

- Carbonate-bicarbonate coating buffer 100mM
  - 1L MQ
  - 3.03g Na_2CO_3
  - 6.0g $NaHCO_3$
  - Add to pH = 9,6 and filter sterile
- Coating Polyclonal Goat anti-horse IL8 antibody, 1mg/ml (King Fisher, PB0433E-100)
  - Dilute 1000x in carbonate bicarbonate buffer to get 1 ug/ml concentration
- Blocking buffer PBS 1% (w/v) BSA (50 ml needed for 1 plate)
  - 50 ml PBS
  - 0.5g BSA
- Wash buffer PBS 0.1% (v/v) Tween-20
  - 1L PBS
  - 1 ml Tween-20
- Dilution buffer PBS 1% (w/v) BSA + 0.1% (v/v) Tween-20
  - 50ml Wash Buffer
  - 0.5g BSA in 50 ml Wash Buffer
- Detection Biotinylated Goat anti-horse IL8 antibody 1.0mg/ml (King Fisher, PBB0434E-050)
  - Concentration: 0.02ug/ml
  - Dilute stock solution of IL8 biotinylated detection AB 50.000x in dilution buffer
    1. 1 ul IL8 Biotinylated detection AB in 499ul dilution buffer and vortex
    2. 100 ul out of dilution 1 in 9.9ml dilution buffer
- Recombinant equine IL8 1ug/ml (King Fisher, RP0312E-005)
  - Concentrations: 1000pg/ml
  - Dilute stock solution (1ug/ml) 1000x: 1ul stock + 999ul dilution buffer
- High sensitivity streptavidin-horseradish peroxidase, 10ug/ml (Thermo Scientific, 21134)
  - Concentration 20ng/ml
  - Dilute the streptavidin-HRP stock 500x in blocking buffer (needs to contain BSA, not Tween)
    - 10ml = 20ul HRP + 9980ul blocking buffer
- TMB substrate solution (Thermo Scientific, N600) ready to use
- Stop solution, 0.18M sulphuric acid
  - 100ml MQ
  - 1ml H_2SO_4

Materials

- EIA/RIA 96-well plates, Flatt bottom (Costar # 9018)
- Plate sealers
- Reagent reservoirs
- Multichannel
- 0,22 um filters (Merck Millipore)
- Syringes

3. Protocol

Day 1;

1. Filter the correct amount of carbonate bicarbonate buffer sterile
2. Dilute Coating Polyclonal goat anti-horse IL8 antibody (PB0433E-100) 1000x in carbonate-bicarbonate buffer to get 1 ug/ml concentration
3. Coat the 96-well plate with Coating Polyclonal goat anti-horse IL8 antibody (100ul/well)
4. Seal with plate sealer and incubate overnight at room temperature (RT)

Day 2;

1. Discard coating solution and blot blate onto paper towels
2. Rinse 1x in Carbonate-bicarbonate coating buffer (300ul/well, filter before use)

3. Blot plate onto paper towels
4. Incubate in Blocking buffer (filter) 90 min at RT (300ul/well, filter before use)
5. Prepare in Effendorf tubes a standard curve for Recombinant equine IL8 (RP0312E-005) by making 1:2 serial dilutions in Dilution buffer (filter) ranging from 1000 15pg/ml, see scheme
   Dilution of standard curve of IL8 Recombinant

Standard	**Pg/ml**	**IL8 Recombinant**		**Dilution buffer**
1	1000	1ul stock	+	999ul dilution buffer
2	500	300ul standard 1	+	300ul dilution buffer
3	250	300ul standard 2	+	300ul dilution buffer
4	125	300ul standard 3	+	300ul dilution buffer
5	62,5	300ul standard 4	+	300ul dilution buffer
6	31.25	300ul standard 5	+	300ul dilution buffer
7	15.62	300ul standard 6	+	300ul dilution buffer
8	0	0ul stock	+	300ul dilution buffer

6. Dilute synovial fluid samples 1:1 in dilution buffer
7. Discard Blocking solution and blot plate onto paper towels
8. Add 100ul of each standard (in duplo) or samples to each well. Cover plate with plate sealer and incubate for 90min at RT
9. Wash 4x in Wash buffer (300ul/well)
10. Incubate 60 min at RT in Detection Biotinylated goat anti-horse IL8 antibody (PBB0434E-050) (100ul/well)
11. Wash 4x in Wash buffer (300ul/well)
12. Incubate 30 min at RT in High sensitivity streptavidin-horseradish peroxidase (100ul/well)
13. Wash 4x in Wash buffer (300ul/well)
14. Incubate 30 min at RT in TMB substrate solution in the dark (100ul/well)
15. Stop the reaction by adding one volume of stop solution (100ul/well)
16. Clean plate bottom with EtOH if necessary
17. Measure absorbance at
    a. Wavelength 1 = 450 nm
    b. Wavelength 2 = 540 (or 570) nm
18. Final reading = measurement wavelength 1 – measurement 2 (this corrects for optical imperfections in the plate)
19. Plot mean absorbance reading on the y-axis versus log concentrations on the x-axis using a logistic equation (4 or 5 parameter fit). Note that the standard curve is sigmoidal and not linear.

Appendix III – SOP024-1 ELISA against equine CCL2 (MCP-1)

1. Aim and Background

This protocol is based on the do-it-yourself ElISA kit of KingFisherBiotech. This kit contains capture antibody, standard and detection antibody for development of an Equine CCL2 (MCP-1) ELISA.

This ELISA is recommended to measure Chemokine (C-C motif) ligand 2 (CCL2)**,** also known as monocyte chemotactic protein-1 (MCP-1). It is a small cytokine belonging to the CC chemokine family, it is characterized by two adjacent cysteines. CCL2 (MCP-1) recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection and is implicated in pathogeneses of several diseases characterized by monocytic infiltrates, such as psoriasis, rheumatoid arthritis and atherosclerosis.

2. Chemicals and Materials

Chemicals:

- **Carbonate-bicarbonate coating buffer** 100mM
  - 1LMQ,
  - 3.03g Sodium Carbonate (Merck 106392, *B27*)
  - 6.0g $NaHCO_3$ (Merck, *B66*)
  - Add to pH = 9.6 and filter sterile
- **Coating Polyclonal rabbit anti-horse CCL2 antibody** 1mg/ml (King Fisher PB0123E-100), *original vial 4*C*
  - dilute 1000x in carbonate-bicarbonate buffer to get 1ug/ml concentration
- **Blocking solution** PBS 1% (w/v) BSA (50 ml needed for 1 plate)
  - 50ml PBS (See SOP00 buffers and solutions)
  - 0.5g BSA (Sigma A3059, cold room)
- **Wash buffer** PBS 0.1% (v/v) Tween-20
  - 1L PBS (See SOP00 buffers and solutions)
  - 1ml Tween-20 (Boom 76021765)
- **Dilution buffer** PBS 1% (w/v) BSA + 0.1% (v/v) Tween-20
  - 50ml Wash Buffer
  - 0.5g BSA (Sigma A3059, cold room)
- **Detection Biotinylated rabbit anti-horse CCL2 antibody** 1.0mg/ml (King Fisher PBB0354E-050), *original vial 4*C*
  - Concentration: 0.1ug/ml
  - Dilute stock solution of CCL2 biotinylated detection AB 10000x in *dilution buffer*
    - 1µl CCL2 Biotinylated detection AB in 10 ml dilution buffer and vortex
- **Recombinant equine CCL2** 1ug/ml (King Fisher RP0054E-005), *aliquots -20C*
  - Concentration: 2500pg/ml
  - Dilute stock solution (1µg/ml) 400x: 2.5µl stock + 997.5µl *Dilution buffer*
- **High sensitivity streptavidin-horseradish peroxidase** 10ug/ml (Thermo scientific, 21134), original vial *4°C*
  - Concentration 20ng/ml
  - Dilute the streptavidin-HRP stock 500x in *Blocking solution (*needs to contain BSA, no Tween!)
    - 10ml = 20µl HRP + 9980µl Blocking solution
- **TMB substrate solution** (Thermo Scientific, N600), ready to use, *4°C.*
- **Stop solution**, 0.18M sulfuric acid , *4°C*
  - 100ml MQ
  - 1ml H_2SO_4 (Merck 320501, Acid cabinet, fume hood)

Materials:

- EIA/RIA 96-well plates, Flatt bottom (Costar # 9018)

- Plate sealers
- Reagent reservoirs
- Multichannel
- 0.22µm filters (Merck Millipore)
- Syringes **3. Protocol**

Day 1:

1. Filter the correct amount of carbonate bicarbonate buffer sterile
2. Dilute ***Coating Polyclonal rabbit anti-horse CCL2 antibody*** 1000x in carbonate-bicarbonate buffer to get 1ug/ml concentration
3. Coat the 96-well plate with ***Coating AB* solution** (100µl per well)
4. Seal with plate sealer and incubate overnight at room temperature (RT)

Day 2:

1. Discard coating solution and blot plate onto paper towels
2. Rinse 1 x in ***Carbonate-bicarbonate coating buffer*** (300µl/well, filter before use)
3. Incubate in ***Blocking solution*** 90 min at RT (300µl/well, filter before use)
4. Prepare in Eppendorf tubes a standard curve for ***Recombinant CCL2*** by making 1:2 serial dilutions in *Dilution buffer* ranging from 2500 to 39pg/ml, see scheme.

Dilution of standard curve of CCL2 Recombinant

Standard	Pg/ml	CCL2 Recombinant		Dilution buffer
1	2500	2.5µl stock	+	997.5µl dilution buffer
2	1250	300µl standard 1	+	300µl dilution buffer
3	625	300µl standard 2	+	300µl dilution buffer
4	312,5	300µl standard 3	+	300µl dilution buffer
5	156,25	300µl standard 4	+	300µl dilution buffer
6	78,125	300µl standard 5	+	300µl dilution buffer
7	39,062	300µl standard 6	+	300µl dilution buffer
8	0	0ul stock	+	300ul dilution buffer

5. Prepare dilution of samples in ***Dilution buffer*** if necessary (Filter Dilution buffer before use)
   a. Dilution for Serum, 1:1 in dilution buffer
   b. Dilution for medium, 1:10-1:50 in dilution buffer
   c. Dilution for synovial fluid, 1:1 in dilution buffer
6. Discard Blocking solution and blot plate onto paper towels
7. Incubate standards and samples in duplo in the plate for 90 min at RT (100µl/well)
8. Wash 4x in ***Wash buffer*** (300µl/well)
9. Incubate 60 min at RT in ***Detection AB solution*** (100µl/well)
10. Wash4xin***Washbuffer***(300µl/well)
11. Incubate 30 min at RT in ***Streptavidin-HRP working solution*** (100µl/well)
12. Wash4xin**Washbuffer**(300µl/well)
13. Incubate 30 min in ***TMB substrate solution*** *in the dark*! (100 µl/well)
14. Stop the reaction by adding one volume of ***Stop solution*** (100µl /well)
15. Clean plate bottom with EtOH if necessary
16. Measureabsorbanceat: wavelength 1 = 450 nm wavelength 2 = 540 (or 570 nm)
17. Final reading = measurement wavelength 1 – measurement wavelength 2 (this corrects for optical imperfections in the plate)
18. Plot mean absorbance readings on the y-axis versus log concentrations on the X-axis using a logistic equation (4 or 5 parameter fit). Note that the standard curve is sigmoidal and not linear.

Appendix IV - SOP024-2 Equine IL1β ELISA

1. Aim and Background

Based on protocol KingFisherBiotech do-it-yourself ELISA for Equine IL1β . This kit contains capture antibody, standard and detection antibody for development of an Equine IL1β.

Interleukin 1 beta (IL1β) also known as leukocytic pyrogen, mononuclear cell factor, lymphocyte activating factor. IL1β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a proprotein. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

2. Chemicals and Materials

Chemicals:

- Carbonate-bicarbonate coating buffer 100mM
  - 1LMQ,
  - 3.03g Na_2CO_3
  - 6.0g $NaHCO_3$
  - Add to pH = 9.6 and filter sterile
- Coating Polyclonal rabbit anti-horse IL1β antibody 1mg/ml (King Fisher KP1013E-100), original vial 4°C
  - Dilute 400x in carbonate-bicarbonate buffer to get 2.5ug/ml concentration
  - Blocking buffer PBS 1% (w/v) BSA (50ml needed for 1 plate) o 50ml PBS o 0.5g BSA
  - Wash buffer PBS 0.1% (v/v) Tween-20 o 1LPBS
  - 1ml Tween-20
- Dilution buffer PBS 1% (w/v) BSA + 0.1% (v/v) Tween-20
  - 50ml Wash Buffer
  - 0.5g BSA in 50 ml Wash Buffer
- Detection Biotinylated rabbit anti-horse IL1β antibody 1.0mg/ml (King Fisher KPB1014E-050), original vial 4°C
  - Concentration: 0.2ug/ml
  - Dilute stock solution of IL1b biotinylated detection AB 5000x in Blocking buffer
- Recombinant equine IL1β 1ug/ml (King Fisher RP0060E-005), aliquots -20°C
  - Concentration: 2500pg/ml
  - Dilute stock solution (1µg/ml) 400x: 2.5µl stock + 997.5µl Dilution buffer
- High sensitivity streptavidin-horseradish peroxidase 10ug/ml (Thermo Scientific, 21134), original vial 4°C
  - Concentration 10ng/ml Dilute the streptavidin-HRP stock 500x in Blocking buffer (needs to contain BSA, no Tween!)
    - 10ml = 20µl HRP + 9980µl Blocking buffer
- TMB substrate solution (Thermo Scientific, N600), ready to use, 4°C.
- Stop solution, 0.18M sulfuric acid , 4°C
  - 100ml MQ
  - 1ml H_2SO_4

Materials:

- EIA/RIA 96-well plates, Flatt bottom (Costar # 9018)
- Plate sealers
- Reagent reservoirs
- Multichannel
- 0.22µm filters (Merck Millipore)

- Syringes

3. Protocol

Day 1:

1) Filter the correct amount of carbonate bicarbonate buffer sterile

2) Dilute Coating Polyclonal rabbit anti-horse IL1β antibody 400x in carbonate-bicarbonate buffer to get 2.5ug/ml concentration

3) Coat the 96-well plate with Coating Polyclonal rabbit anti-horse IL1β antibody (100µl/well),

4) Seal with plate sealer and incubate overnight at room temperature (RT)

Day 2:

1. Discard coating solution and blot plate onto paper towels

2. Rinse 1 x in Carbonate-bicarbonate coating buffer (300µl/well, filter before use)

3. Blot plate onto paper towels

4. Incubate in Blocking buffer (filter) 90 min at RT (300µl/well, filter before use)

5. Prepare in Eppendorf tubes a standard curve for Recombinant equine IL1β by making 1:2 serial dilutions in Dilution buffer (filter) ranging from 2500 to 39pg/ml, see scheme Dilution of standard curve IL1β Recombinant:

Standard	pg/ml	IL1β Recombinant		Dilution buffer
1	2500	2.5µl stock	+	997.5µl
2	1250	300µl standard 1	+	300µl
3	625	300µl standard 2	+	300µl
4	312,5	300µl standard 3	+	300µl
5	156,25	300µl standard 4	+	300µl
6	78,125	300µl standard 5	+	300µl
7	39,062	300µl standard 6	+	300µl
8	0	0 µl	+	300µl

6. Dilute synovial fluid samples 1:1 in Dilution buffer

7. Discard Blocking solution and blot plate onto paper towels

8. Add 100µl of each standard (in duplo) or samples to each well. Cover plate with plate sealer and incubate for 90min at RT

9. Wash 4x in Wash buffer (300µl/well)

10.Incubate 60 min at RT in Detection Biotinylated rabbit anti-horse IL1β antibody (100µl/well)

11. Wash4xinWashbuffer(300µl/well)

12. Incubate 30 min at RT in High sensitivity streptavidin-horseradish peroxidase (100µl/well)

13. Wash4xinWashbuffer(300µl/well)

14.Incubate 30 min at RT in TMB substrate solution in the dark! (100µl/well)

15. Stop the reaction by adding one volume of Stop solution (100µl/well)

16. Clean plate bottom with EtOH if necessary.

17. Measure absorbance at

a) wavelength 1 = 450 nm
b) wavelength 2 = 540 (or 570 nm)

18. Final reading = measurement wavelength 1 – measurement wavelength 2 (this corrects for optical imperfections in the plate)

19. Plot mean absorbance readings on the y-axis versus log concentrations on the x-axis using a logistic equation (4 or 5 parameter fit). Note that the standard curve is sigmoidal and not linear.

Appendix V - SOP024-3 Equine TNFα ELISA

1. Aim and Background

This protocol describes the working of the Equine TNFα ELISA reagent kit of Thermo Scientific (product number ESS0017). This ELISA kit contains a pre-tittered coating and detection antibodies and recommended buffer and specific assay protocol optimized for the quantitative measurement of equine TNFα.

Tumor necrosis factor alpha (TNFα) is a cell signaling protein (cytokine) involved in systemic inflammation. The primary role of TNF is in the regulation of immune cells. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons.

2. Chemicals and materials

Chemicals:

- Carbonate-bicarbonate coating buffer 100mM
  - 1LmQ,
  - 3.03g Na_2CO_3
  - 6.0g $NaHCO_3$
  - Add to pH = 9.6 and filter sterile
- Blocking buffer PBS 1% (w/v) BSA
  - 50ml PBS
  - 0.5g BSA
- Wash buffer PBS 0.1% (v/v) Tween-20
  - 1LPBS
  - 1ml Tween-20
- Dilution buffer PBS 1% (w/v) BSA + 0.1% (v/v) Tween-20
  - 50ml Wash Buffer
  - 0.5g BSA in 50 ml Wash Buffer
- Anti-equine TNFα Coating antibody
  - Dilute 1:100 in carbonate-bicarbonate buffer
  - Add 110µl coating antibody to 10.89ml of carbonate-bicarbonate buffer
- Lyophilized recombinant Equine TNFα Standard (10ng/ml), see vial label
  - Dilute 1:10 in dilution buffer to get a concentration of 1000pg/ml
- Anti-equine TNFα Detection antibody o Dilute 1:100 in blocking buffer
  - Add 110µl detection antibody in 10.89ml of blocking buffer
- Streptavidin-HRP
  - Dilute 1:400 in blocking buffer
  - Add 30µl of Streptavidin-HRP to 12ml of blocking buffer
- TMB substrate solution (Thermo Scientific, N600), ready to use, 4°C.
- Stop solution, 0.18M sulfuric acid , 4°C
  - 100ml MQ
  - 1ml H_2SO_4

Note: Immediately upon receipt, aliquot and freeze the coating and detecting antibodies at -20°C. Avoid repeated freeze-thaw cycles.

Materials:

- EIA/RIA 96-well plates, Flatt bottom (Costar # 9018)
- Plate sealers for 96 well plates (15036)
- Reagent reservoir
- Multichannel

- 0.22µm filters (Merck Millipore)

- Syringes

3. Protocol

Day 1

1. Filter the correct amount of carbonate bicarbonate buffer sterile

2. Dilute Anti-equine TNFα Coating antibody 100 x in carbonate-bicarbonate buffer to get 1ug/ml

concentration

3. Coat the 96-well plate with Anti-equine TNFα Coating antibody (100µl/well)

4,Seal with plate sealer and incubate overnight at room temperature (RT)

Day 2

1. Aspirate Coating antibody and blot dry on paper towel
2. Add 300 µl of Blocking buffer to each well and incubate for 1hour at RT
3. Reconstitute Lyophilized recombinant Equine TNFα Standard (10ng/ml) with dilution buffer to prepare standard TNFα. Dilute reconstitute standard 1:10 in blocking buffer to prepare a concentration of 1000pg/ml
4. Make a 1:2 serial dilution according to scheme underneath

Prepare Standard TNFα:

standard	pg/ml	Standard	Dilution buffer
1	1000	100µl stock (10ng/ml)	900µl
2	500	250µl	250µl
3	250	250µl	250µl
4	125	250µl	250µl
5	62.5	250µl	250µl
6	31.25	250µl	250µl
7	15.62	250µl	250µl
8	0	0µl	250µl

5) Dilute synovial fluid samples 1:1 in dilution buffer

6. Add 100µl of each standard (in duplo) or samples to each well. Cover plate with plate sealer and incubate for 1hour at RT

7. Aspirate and wash 3 times with Wash buffer (300µl/well)

8. Dilute Anti-equine TNFα Detection antibody 1:100 in blocking buffer

9. Add 100µl per well and incubate for 1 hour at RT

10. Aspirate and wash 3 times with Wash buffer(300µl/well)

11. Dilute Streptavidin-HRP 1:500 in blocking buffer

12. Add 100µl per well and incubate for 30min at RT

13. Aspirate and wash 3 times with Wash buffer(300µl/well)

14. Add 100µl of Substrate Solution to each well and incubate for 30min in the dark!

15. Stop the reaction by adding 100µl of Stop Solution to each well

16. Measure absorbance at
    - wavelength 1 = 450
    - wavelength 2 = 550 nm as references.
    Measure plate within 30 minutes!

17. Final reading = measurement wavelength 1 – measurement wavelength 2 (this corrects for optical imperfections in the plate)

18. Plot mean absorbance readings on the y-axis versus log concentrations on the X-axis using a logistic equation (4 or 5 parameter fit). Note that the standard curve is sigmoidal and not linear