The influence of exercise on joint homeostasis in the Shetland pony

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Table of contents

Summary

Reasons for preforming the study
Osteoarthritis is a very common problem in the horse, so the increase in its need for early diagnosis has led to the search for potential biomarkers in the joint. If the levels of such biomarkers are interpreted, it is important to know if they are influenced by exercise. Therefore, this study is preformed, to be able to compare these biomarker concentrations later with a study with induced osteoarthritis during the same exercise protocol.

Objective
Investigating the influence of exercise on joint homeostasis in the Shetland pony, reflected by catabolic, anabolic, inflammatory and cartilage breakdown biomarkers.

Method
Synovial fluid was collected from the left and right inter carpal joint of seven healthy adult Shetland ponies, which were free of lameness and had clinically normal carpal joints. Ponies were subjected to an exercise protocol that consisted of 5 weeks of treadmill training and 5 a week wash-out period. At certain time points, synovial fluid was collected and stored. After the last collection, catabolic markers (C2C and GAG), anabolic markers (CPII), inflammatory markers (CCL2 and PGE2) and cartilage breakdown markers (MMP) were evaluated.

Results
The exercise protocol caused a significant increase in CPII concentrations only. In MMP, CCL2 and PGE2 an effect of repeated arthrocentesis was detected.

Conclusions
CPII concentrations are affected by exercise and C2C, GAG, CCL-2, PGE2 and MMP concentrations are not. There is a significant increase in CPII concentration, indicating that the anabolic component in the joint homeostasis is increased after the exercise protocol. This study involved repeated arthrocentesis, which is visible in MMP, CCL2 and PGE2 concentrations.

Potential relevance
Exercise effects on these biomarker levels will presumably not interfere with effects caused by chondral defects in the up-coming defect-study, except for CPII. These biomarkers could help with the differentiation between the effects of exercise and the effects of exercise plus a defect. However, care should be taken to exclude outcomes affected by interventions such as repeated arthrocentesis.

Introduction

The horse has been domesticated decades ago. It was used not only as a source of food and clothes, but also as a mean of transport. The horse's locomotor capacities were perfect for military purpose, but also for the farmer and later for the athletic sports and leisure (Back & Clayton, 2013; Clayton, 2016).
The musculoskeletal system needs to deal with high weight and pressure, which sometimes leads to damage. Lameness, which is defined as "an alteration of normal gait, due to functional or structural disorder in the locomotor system" (Back & Clayton, 2013), is the most common health problem in horses (NEHS, 2016).

One of the main elements of the locomotor system of the horse is the synovial joint. The synovial joint connects two bony ends and consists of subchondral bone covered with articular cartilage (Figure 1). The inner lining of the joint capsule between those two bones is the synovial membrane, which contains synoviocytes. These cells make hyaluronan, which is, together with an ultra-filtrate of plasma, synovial fluid. Synovial fluid is a viscous liquid, which plays a large role in the communication between the elements of the joint. Furthermore, it assimilates forces during locomotion and nourishes the articular cartilage, which is avascular (Levick, 1995).
The subchondral bone also attenuates main forces. This type of bone lays, with a layer of calcified cartilage in between, under the cartilage (McIlwraith et al., 2016).

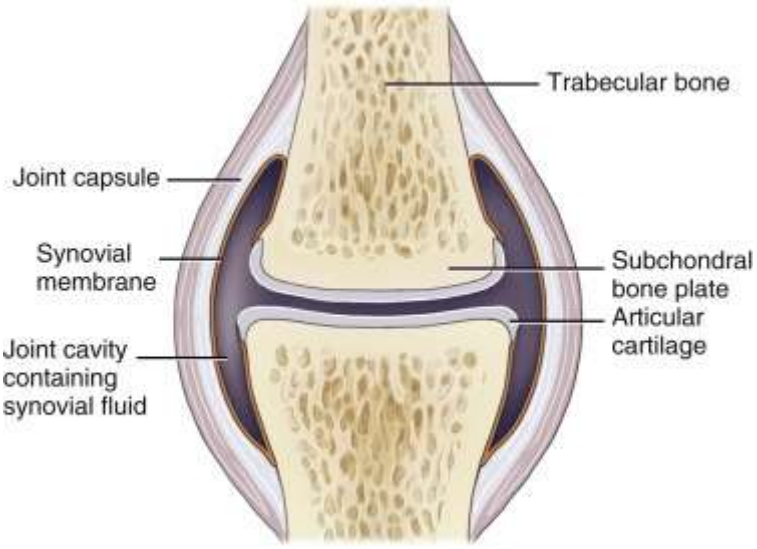

Figure 1: General overview of a synovial joint
From: De Grauw J.C., 2011. Molecular monitoring of equine joint homeostasis

Articular cartilage consists of extracellular matrix (ECM) - produced by chondrocytes - and water. ECM consists of mostly collagen, proteoglycans (such as glycoaminoglycans (GAGs)) and glycoprotein, minerals and lipids (de Grauw, 2011). The main type of collagen in the ECM is collagen type II. These molecules have cross-links within the same molecule, and between the adjacent molecules, whereby it fuses itself into fibrils (McIlwraith, 2010). These collagen fibrils can resist large mechanical forces. This enables the cartilage to function as a shock absorber during locomotion (de Grauw, 2011).

Homeostasis, meaning the dynamic balance between and within living cells to maintain internal stability, in the joint is necessary for a functional joint and thus a functional locomotor system. Locomotion on the other hand, also affects joint homeostasis (te Moller & van Weeren, 2017).

In human, there have been thoughts of a window wherein exercise have beneficial effects on the joint, but outside this window homeostasis is disturbed and the compensatory mechanisms cannot repair this (Hallett & Andrish, 1994). In horses, research found out that in flat race training the high-speed exercise (so the gallop) is beneficial in improving fracture resistance. When only big amounts of canter exercise have been done, highly risks to stress fracture occur. The balance between microdamage of the cartilage and the repair and adaptation of it will be in favour of the repair and adaptation when high-speed exercise is performed. For both speeds, long-distance exercise has negative effects on the joint and results in a higher risk of fracture (Verheyen et al., 2006).
On the other hand, lack of exercise also has negative effects on the joint. Joint immobilization followed by a gradual increase of load resulted in loss of bone mineral density and also the subchondral bone was not fully recovered after 8 weeks (van Weeren & Brama, 2016).
When the balance of catabolic and anabolic processes in the joint is disturbed, tissue damage will occur, which leads to loss of biomechanical resistance of the joint. Further damage could lead to joint disorders, for example osteoarthritis (OA) (McIlwraith et al., 2016).

Osteoarthritis is one of the main causes of lameness (Caron & Genovese, 2003). OA is defined as an end-stage of disorders in which the articular cartilage is deteriorated and the bone and soft tissues in the joint are changed (C. Wayne McIlwraith et al., 2016). It is characterized by the degeneration of articular cartilage, synovitis and changes in peri-articular and subchondral bone, caused by mechanical stress. Here the joint is seen as an organ, and any of the components of the organ could be the first to fail to repair the mechanical stress (Goldring & Goldring, 2007).

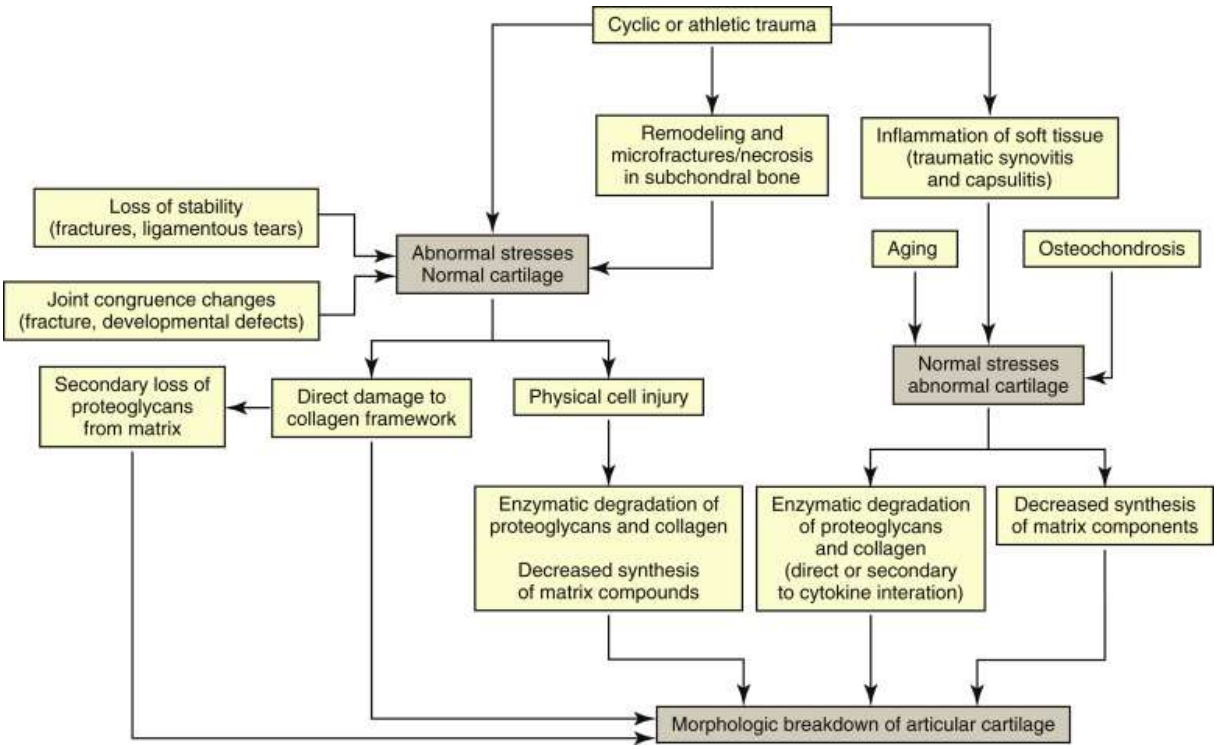

Figure 2: Diagram showing the possible pathways for degradation of articular cartilage
From: McIlwraith et al., 2016. Joint disease in the horse, Chapter 8.

The common mechanical factor of OA is the increase of stress in the joint, which becomes pathologic. As described above in Figure 2, this could be a result of a decrease in the bearing capacity of the joint or an increase in joint loading, for example during repetitive impulsive loading. Several studies in other species have investigated this overload could lead to the development in OA, for example in rats (Pap et al., 1998).

Acute traumatic injuries on the joint are a relatively uncommon cause of OA. More common injuries with OA as a result are chronic fatigue injuries, also known as repetitive impulsive loading injuries. These injuries are caused by repetitive loading of tissue below the threshold of tissue failure, but eventually exceed the capacity of tissue repair (McIlwraith et al., 2016).

Normally, the impulsive loads cause micro-injury of the articular cartilage and subchondral bone, but these have capacities to repair these injuries. The articular cartilage creates new cartilage – fibrocartilage - which is not comparable to normal (hyaline) cartilage, but could substitute the micro-injuries whereby normal joint function is continued and no further impairment is prevented (McIlwraith et al., 2016). Normal acting subchondral bone is viscoelastic and becomes stiffer when the joint loading increases, whereby the stress reduces. However, when loading is impulsive instead of gradually, the tissue cannot deform (Riggs, 2006).
When the impulsive loads exceed the capacity of repair, the articular cartilage becomes thinner and the subchondral bone becomes sclerotic and thicker. This results in the crawl up of the subchondral bone towards the cartilage (McIlwraith et al., 2016). Hereby, the calcified cartilage and the articular cartilage will shatter, which causes inflammation. Synovitis on itself also causes then a dysregulation of chondrocytes, whereby the remodelling of cartilage matrix is disrupted and further breakdown of the cartilage occurs (Goldring & Goldring, 2007). The sclerotic subchondral bone cannot resist normal loads, which causes a relative increase of joint loading (Brandt et al., 2008). In the end, all of this results in eburnated bone and synovitis, which cause pain and lameness (Carter et al., 2004).

To measure joint homeostasis and to detect osteoarthritis in an early stage, biomarkers could be used. Biomarkers are objectively measured indicators of normal biological processes, pathogenic processes or pharmacological responses to therapy. They are anatomic, physiologic, biochemical or molecular (de Grauw, 2011) and are classified following a BIPEDS classification. BIPEDS stand for Burden of disease, Investigative, Prognostic, Efficacy of intervention and Diagnostic (Bauer et al., 2006). Burden of disease biomarkers measures the severity of osteoarthritis in individuals. Prognostic biomarkers are used for the determination of risk in individuals without osteoarthritis or are used by the measure of clinical outcomes in individuals with symptoms of osteoarthritis. Efficacy of intervention biomarkers measures changes after treatments. Diagnostic biomarkers can distinguish between individuals with and without osteoarthritis and Investigative biomarkers are the markers which cannot be classified into one of the other categories (Lotz et al., 2014).

Molecular biomarkers could be subdivided into direct and indirect markers. Direct markers are markers that directly reflect catabolic and anabolic processes in the joint, for example fragment of cartilage ECM. Indirect markers are inflammation substances. When the homeostasis is out of balance, biomarkers differ from a healthy joint.
The best way to collect molecular biomarkers from a joint is by synovial fluid. Also bloodserum or urine could be used, but synovial fluid reflects the local environment of the joint directly, which bloodserum or urine could not. Synovial fluid is very sensitive to changes, because it is continuously produced and quickly cleared, so it is an accurate and real time measure of the local state of the joint (de Grauw, 2011).
Besides molecular biomarkers, there are other diagnostic techniques, which could detect joint damage and could provide information of the disease state. Magnetic Resonance Imaging (MRI) and arthroscopy are golden standard in human and equine OA (Oakley et al., 2005). The negative aspect of MRI is that it only could detect OA when the disease is well established, so when the joint is abnormal of structure. Therefore, this cannot be used for the early diagnose of OA. Arthroscopy gives a direct visualisation of the cartilage, but is an invasive procedure for the individual (Fuller et al., 2001). This is the reason why there is so much investigation to the biomarkers in synovial fluid, because this also could give a direct reflection on the joint status, and it is less invasive and time-consuming than diagnostic arthroscopy (de Grauw, 2011).

Various synovial fluid biomarkers have been distinguished. Direct catabolic markers, such as C2C, CTX-II, Col2-1 and Col2-1NO2, GAG and COMP, provide an indication of the activity of catabolic processes in the articular cartilage (de Grauw, 2011). Direct anabolic markers, such as CS, KS, ARGS and CPII, provide an indication of the activity of synthetic processes in the articular cartilage. Indirect markers, such as substance P, IGF, CCL2, MMP and PGE2, provide an indication of the occurrence of joint pain, lameness or ability to influence cartilage turnover.
C2C reflect the collagen type II cleavage/degradation, GAG reflects the breakdown of proteoglycans in cartilage, CPII reflects collagen type II synthesis, CCL2 and PGE2 reflect the size of inflammation in the joint and MMP reflects the activity of cleavage enzymes of collagen (de Grauw, 2011; C. Wayne McIlwraith et al., 2016). [1] This panel of biomarkers could help with the early diagnosis of disturbance in joint homeostasis (Frisbie et al., 2016).

Various studies have shown effects of exercise on biomarkers in OA-affected joints (Frisbie et al., 2008) but exercise on its own also affects joint homeostasis (te Moller & van Weeren, submitted to The Veterinary Journal, 2016). This is of importance for the correct appreciation of the influence of exercise on the onset and progression of joint pathology and/or the regeneration of damaged tissue. Exercise is a mechanical load on joints, and sometimes this becomes an overload.

Various studies have shown effects of exercise on joint homeostasis and biomarker concentrations in OA-affected joints (Frisbie et al., 2008; te Moller & van Weeren, 2017; van den Hoogen et al., 1998; van den Boom et al., 2004; Lamprecht & Williams, 2012). However, given the face that exercise influences joint homeostasis in itself, there should be differentiated between effects of exercise in healthy and in OA affected joints, before conclusions could be made about biomarker concentrations in OA affected joints. This is of importance for the correct appreciation of the influence of exercise on the onset and progression of joint pathology and/or the regeneration of damaged tissue. Exercise could cause an increase in CS846, CPII, GAG, Col CEQ, C1,2C, osteocalcin and Col I in synovial fluid (Frisbie et al., 2008).
Therefore, the aim of my research internship is to investigate the influence of exercise on joint homeostasis in the Shetland pony, reflected by a panel of biomarkers in the synovial fluid. The panel of biomarkers consists of catabolic markers (C2C and GAG), anabolic markers (CPII), inflammatory markers (CCL2 and PGE2) and cartilage breakdown markers (MMP).
The first hypothesis is that biomarker concentration will change under the influence of exercise. The second hypothesis is that these concentrations will return to baseline levels after discontinuation of exercise.

[1] *Abbreviations*
C2C: C-terminal propeptide of collagen type II
GAG: glucoaminoglycan
CPII: C-propeptide of procollagen type II
CCL-2: chemokine ligand 2
PGE2: prostaglandine E2
MMP: matrix metalloproteinase

Material and Methods

Experimental design
Eight adult Shetland ponies (male and female) were used in the study. All the ponies were free of lameness and had clinically normal carpal joints. Carpal joints have not been investigated radiographically, but were screened optically. One pony was excluded during the study due to lameness, so measurements have been done with seven ponies.
All ponies were assigned to the same group and had the same housing and feeding conditions when starting the protocol, because they were housed all together in the same stable and had a hayrack.

4 weeks before starting the protocol, the ponies were trained at low-intensity for 3 weeks on the treadmill so they got used to the training and a certain basis condition. After 2 weeks, an incremental exercise test was performed to determine the maximal heart rate for each pony. This was used to standardize the speed of the treadmill in the protocol. After this, the ponies got 2 weeks of rest, so the joints could recover from the test and start at basic levels when the protocol started. After 2 weeks of box rest, the exercise protocol started. This is further described in the schedule below (Table 1). The protocol has a build-up phase (BUP) of 1 week, followed by a constant/steady phase (SP) of 4 weeks. After Period 2 there was a wash-out period of 7 weeks. During the whole experimental period (pre-training phase, training phase and wash-out period), ponies were subjected to a daily basic exercise protocol (BEP), which consisted of 30 minutes walking in a horse walker.

Day	**Activity**				
	Walk (4.4 km/h)	*Trot (16 km/h)*	*Canter (21 km/h)*	*Trot (16 km/h)*	*Walk (4.4 km/h)*
1 (BUP)	5	4	2 walk	2	5
2 (BUP)	5	4	1	1	5
3 (BUP)	5	4	1	2	5
4 (BUP)	5	4	1	2	5
5 - 33 (SP)	5	4	2	2	5

Table 1: Schedule exercise protocol

At certain time points, synovial fluid was collected from the inter carpal joints (Figure 3).

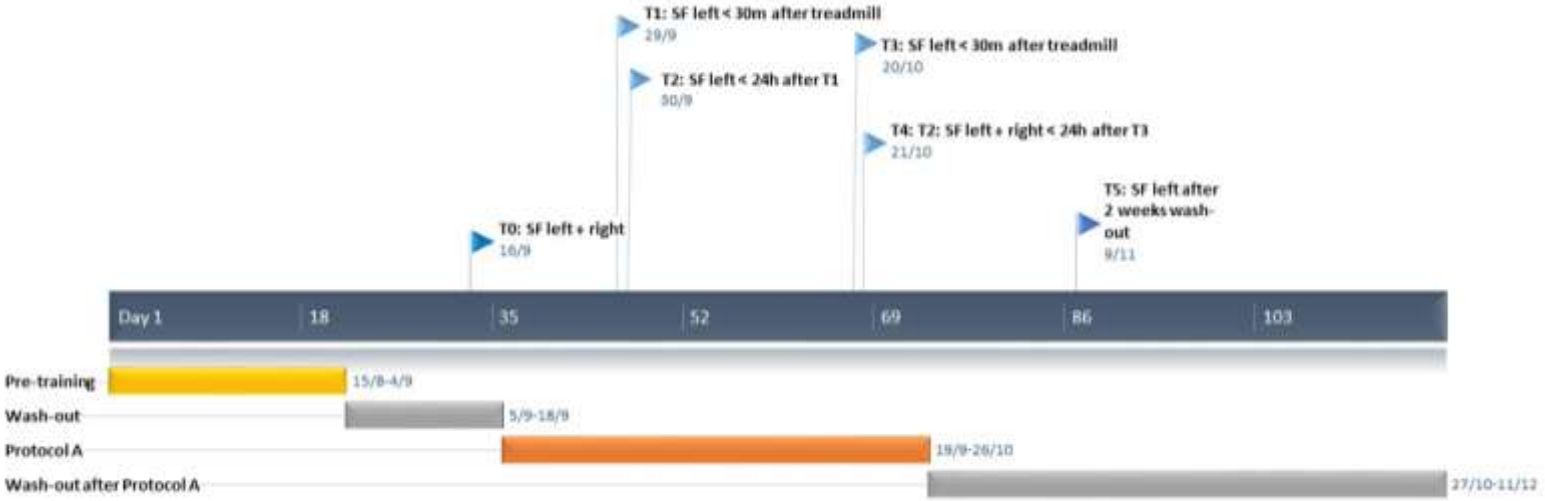

Figure 3: Timetable of collection of synovial fluid

Collection of synovial fluid
Firstly, ponies were lightly sedated by using detomidine (0.05mg/kg BWT, iv.) and butorphanol (0.01 mg/kg BWT, iv.) after taking a blood sample of 4 ml from the jugular vein. When needed, the ponies got a nose twitch. After shaving and disinfecting the carpi, synovial fluid (SF) samples were collected from the inter carpal joint using the dorsal approach. The amount of SF for each pony was approximately 1-1.5 ml at each time point. SF was transferred into plain tubes and centrifuged for 5 minutes at 5.000 rpm (2404 x g). After this, the SF was aliquoted and stored at -80 ºC until the assay was started. All of this happened within 30 minutes of collection. The blood samples were collected in EDTA tubes and rested for 15 minutes. After this, tubes were centrifuged for 10 minutes at 4.000 rpm (1538 x g) and blood serum was collected, aliquoted and stored at -80 ºC.

C2C, GAG, CPII, CCL2, PGE2 and MMP have been analysed. This panel of biomarkers is a selection of biomarkers, which give information on catabolic and anabolic processes, as well as the inflammatory status of the carpi.

C2C assay: commercially available ELISA from IBEX was used. 30µl SF sample, mouse C2C antibody and C2C standard stock for assay standard were used. After dilution 1:1 with Buffer III (30µl sample + 30µl Buffer III), 50µl of the diluted samples were pipetted to the mixing plate and 50µl of C2C antibody dilution (50µl C2C antibody + 6ml assay buffer) was added. The plate was pre-incubated on a high speed titre plate shaker for 30 minutes. After this, 80µl of each sample was transferred to the ELISA plate and this plate was incubated for 1 hour. After washing the plate 3 times, 100µl/well GAM-HRP conjugate diluted in Buffer III (22µl GAM-HRP + 11ml Buffer III) was added and this was incubated for 30 minutes. After washing the plate 6 times, 100µl/well TMB was added and this was incubated for 30 minutes. After this, reaction was stopped by adding 100µl/well Stop solution. The plate was read immediately after adding Stop solution at 450nm and 540nm at the Tecan Infinity 200 PRO. Second wavelength was a correction for optical imperfections in the plate. Results were expressed as ng C2C/ml SF.

GAG assay: the proteoglycan content of SF was estimated by measuring GAG-concentrations using DMMB (1,9-dimethylmethylen blue) metachromatic dye assay. SF was 1:1 digested in 100µl 1 mg/ml hyaluronidase (HAse, which is diluted in sodium acetate 25mM pH 6.5) by adding 20µl HAse to 20µl SF sample and incubated at 37ºC for 30 minutes. Then, HAse digested SF samples were diluted in PBS-EDTA 50 times by adding 245µl to 5µl sample. 100µl of this dilution was pipetted in duplo in the 96-wells plate and 200µl DMMB staining solution (to 1L demi water 16mg DMMB, 5ml 100% ethanol and 2.37g glycine were added and pH was set at 3.0 by adding HCl) was added to each well. The plate was measured immediately after adding DMMB staining solution at 525nm and 595nm. Equine chondroitin sulphate C 0.5mg/ml served as the assay standard. Results were expressed as µg GAG/ml SF.

CPII assay: commercially available ELISA from IBEX was used. 10µl SF sample, rabbit polyclonal CPII antibody and bovine CPII for assay standard were used. After dilution 1:10 with Buffer III (10µl sample + 90µl Buffer III), 50µl of the diluted samples were pipetted to the mixing plate and 50µl of CPII antibody dilution (50µl CPII antibody + 6ml assay buffer) was added. Plate was pre-incubated on a high speed titre plate shaker for 1 hour. After this, 80µl of each sample was transferred to the ELISA plate and this plate was incubated for 2 hours. After washing the plate 6 times, 100µl/well GAR-HRP conjugate diluted in Buffer III (50µl GAR-HRP + 11ml Buffer III) was added and this was incubated for 30 minutes. After washing the plate 6 times, 100µl/well TMB was added and this was incubated for 30 minutes. After this, reaction was stopped by adding 100µl/well Stop solution. The plate was read immediately after adding Stop solution at 450nm and 540nm at the Tecan Infinity 200 PRO. Second wavelength is a correction for optical imperfections in the plate. Results were expressed as ng CPII/ml SF.

CCL2 assay: commercially available ELISA from KingFisherBiotech was used. Before the day of assay, the 96-wells plate needed to be coated manually. This was performed by diluting coating polyclonal rabbit anti-horse CCL2 antibody 1:1000 in carbonate-bicarbonate buffer to get 1µg/ml concentration. 100µl per well of this diluted concentration was added to the 96-well plate. This plate needed to incubate overnight. On the day of assay, plate was blotted onto towels, rinsed 1 time in carbonate-bicarbonate coating buffer by adding 300µl/well of this coating buffer, and incubated with 300µl/well of blocking solution for 90 minutes. Then the normal procedure of the assay could start. 55µl SF sample, biotinylated rabbit CCL2 antibody and recombinant equine CCL2 1µg/ml for assay standard were used. After dilution 1:1 with NBB (nonspecific binding buffer, which is 100ml PBS,5.84g NaCl, 2ml Tween and 0.5g BSA, 55µl sample + 55µl NBB), 100µl of the diluted samples were pipetted to the mixing plate. The plate was incubated on a high-speed titre plate shaker for 90 minutes. After washing the plate 4 times, 100µl/well Detection AB solution was added and this was incubated for 60 minutes. After washing the plate 4 times, 100µl/well Streptavidin-HRP working solution was added and this was incubated for 30 minutes. After washing the plate 4 times, 100µl/well TMB substrate solution was added and this was incubated for 30 minutes in the dark. After this, reaction was stopped by adding 100µl/well Stop solution. The plate was read immediately after adding Stop solution at 450nm and 540nm at the Tecan Infinity 200 PRO. Second wavelength was a correction for optical imperfections in the plate. Results were expressed as pg CCL2/ml SF.

PGE2 assay: To 150µl SF, 50µl MQ was added and to this mix 100µl Internal Standard (IS) was added. IS is PGF2α (40pg/ml), which is necessary for determination of the different types of PG's during measurement. To each sample, 200µl 0.2M NH4-COOH (1.54g ammonium acetate + 100ml MQ and set at pH 3.3 by using formic acid) and 800µl ethyl acetate (200ml ethyl acetate + 40µl 10% butylated hydroxytoluene) was added. Samples were vortexed, centrifuged and placed into a -80°C freezer for 60 minutes. After this, an aqueous layer and a solid layer appeared. The aqueous layer contains PGE2, so this was pipetted and again 0.2M NH4-COOH and ethyl acetate was added and steps were repeated. After this, fluid was evaporated in the glass autosampler vials using a Speedvac system and 60µl 30% methanol was added to the vials. 30µl of this mix was transferred to a polypropylene microwell plate and analysis with LC/MS was done at the Biochemistry department. Results were expressed as pg PGE2/ml SF.

MMP assay: synovial fluid was diluted 1:10 in MMP buffer (consisting 6.05g TRIS, 2.92g NaCl, 0.74g CaCl2-H2O, 0.5g PEG-6000 and 1.25ml 20% Trition-100 solution, which is added up to 500ml with H2O and set at pH 7.5 using 6M HCl) by adding 180µl MMP buffer to 20µl SF sample. Then 35µl of each sample was pipetted in duplo in an 96-wells plate, one *positive* sample and one *negative*. Positive samples consisted of 35µl SF sample with 35µl substrate B solution (which is FS-6 diluted in MMP buffer with MQ added) and negative samples consisted of35µl SF sample with 35µl substrate C solution (which is FS-6 diluted in MMP buffer with EDTA 0.5M added). Afterwards, 20µl of each sample solution was transferred in triplo to a 384-wells plate. The plate was measured at the Biochemistry department with the CLARIOstar. EDTA blocked the MMP activity, so the negative samples were measured for excluding the background MMP activity. Results were expressed as RFU/s.

Statistical analysis

Statistical analysis was performed using the Prism statistical package from Graph Pad Software (version 5.2 for Windows). Normality was tested by using Shapiro-Wilk method. Differences between T0 and T4 for the right carpus, between T0 for the left and right carpi and between T4 for the left and right carpi were tested for significance using a paired two tailed t-test or a Wilcoxon signed rank test (nonparametric test) when data was found normally- or not normally distributed, respectively.

To determine whether repeated arthrocentesis lead to significant differences between time points T1 and T2 and/or T3 and T4 in the left carpus, a repeated measures ANOVA followed by Tukey's multiple comparisons post hoc test or - when data was not normally distributed - Friedman and Dunn's multiple comparison test was performed. Missing values precluded repeated measures ANOVA of CPII, C2C/CPII ratio and CCL2. An overview of tests used for the various comparisons are listed in Table 2. Differences were considered significant when $P<0.05$.

Time point **Biomarker**	**R0 vs R4 (effect of exercise)**	**R0 vs L0 (right vs left)**	**R4 vs L4 (effect of repeated arthrocentesis)**	**All time points of the left carpus**
C2C	Two tailed t-test	Two tailed t-test	Wilcoxon signed rank test	Friedman and Dunn's multiple comparison test
GAG	Two tailed t-test	Two tailed t-test	Two tailed t-test	Repeated measures ANOVA and Tukey's multiple comparison test
CPII	Two tailed t-test	Two tailed t-test	Two tailed t-test	No ANOVA, because of missing values
CCL2	Two tailed t-test	Two tailed t-test	Two tailed t-test	No ANOVA, because of missing values
PGE2	-	-	-	Friedman and Dunn's multiple comparison test
MMP	Two tailed t-test	Two tailed t-test	Two tailed t-test	Friedman and Dunn's multiple comparison test
C2C/CPII ratio	Wilcoxon signed rank test	Wilcoxon signed rank test	Wilcoxon signed rank test	No ANOVA, because of missing values

Table 2: Description of used statistical analysis
R0 vs R4 describes the difference of concentrations as a result of exercise during the different time points. Namely, R0 is the start of the exercise protocol and R4 is the end.
R0 vs L0 describes the difference of concentrations in the left and right carpus of the same pony at the start of the exercise protocol.
R4 vs L4 describes the difference of concentrations as a result of repeated arthrocentesis in the joint. Namely, in the left carpus repeated arthrocentesis within 24 hours was performed.

Results

This exercise protocol induced a significant difference in synovial fluid CPII concentrations between T0 and T4 of the right carpus. Only for the MMP, differences in biomarker activity were found between left and right carpi at T0 (Figure 4).
At T4 a significant difference between left and right carpi was found for MMP and CCL2. Additionally, an increase in MMP-activity and C2C-, CCL2- and PGE2&D2 levels due to repeated arthrocentesis were observed at both T2 and T4 compared to T1 and T3 respectively, in the left carpus, although this was significant for MMP-activity at T4 only. Similarly, CPII levels showed an increase at T4 compared to T3.

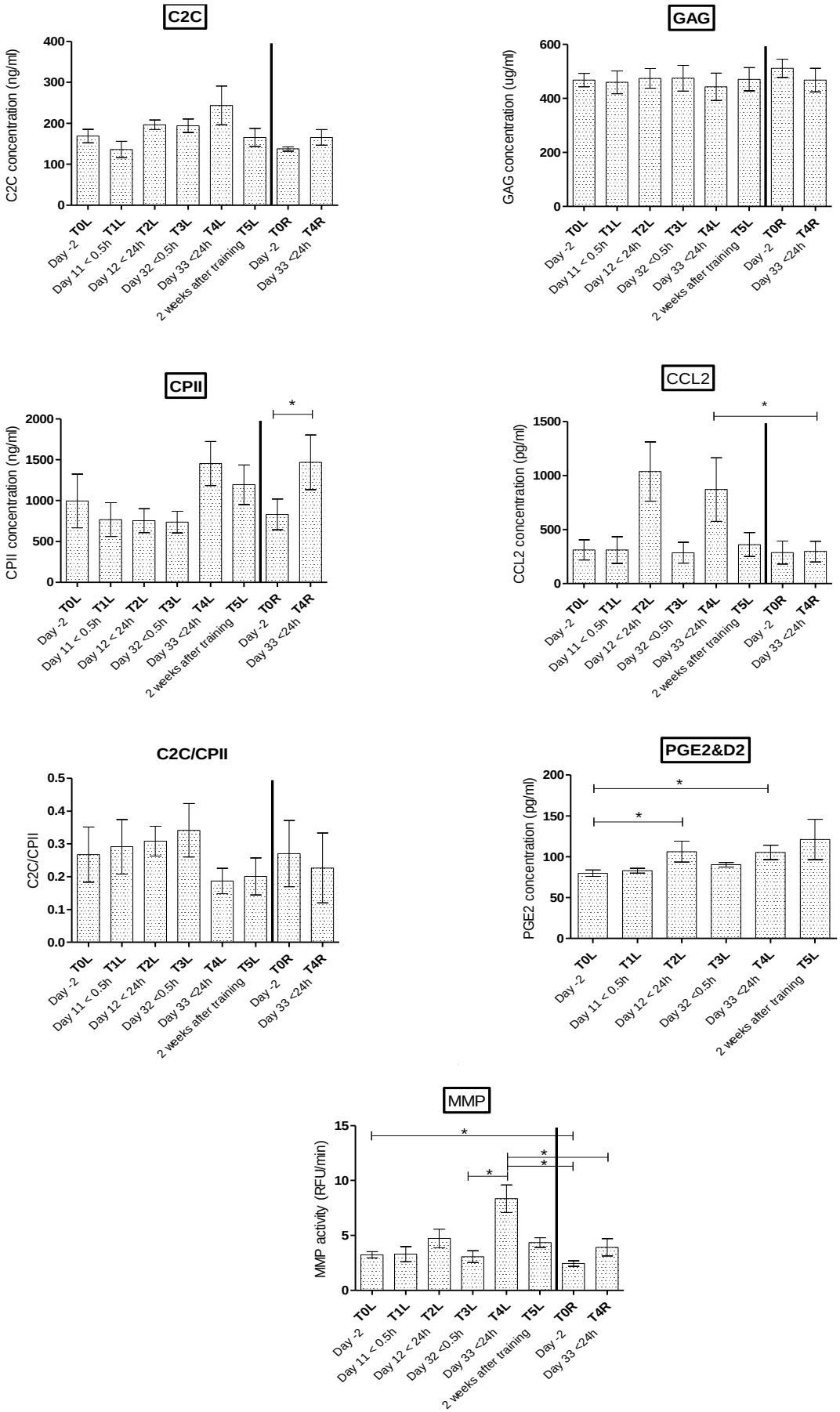

Figure 4: General overview of average synovial fluid C2C, GAG, CPII, CCL2, C2C/CPII ratio, PGE2&D2 and MMP concentrations ± SEM from the left and right carpal joints of seven ponies plotted by time points. Time points with "" symbol represent a significant change (P < 0.05) between time points.*

Discussion

The aim of this study was to investigate changes in synovial fluid from Shetland ponies as a result of an exercise protocol. It is the first study on synovial fluid biomarkers in Shetland ponies.
It was possible to identify several biomarkers, which reflect the effect of exercise, namely catabolic biomarkers C2C and GAG, anabolic biomarker CPII, inflammatory biomarkers CCL2 and PGE2 and cartilage breakdown biomarker MMP. When concentrations were significantly different between A0R and A4R, differences were considered the result of exercise. When concentrations were significant different between A0L and A0R, differences were due to left-right differences. In a healthy pony, this was not expected to be different. When concentrations were significantly different between A4L and A4R, differences were due to repeated arthrocentesis.

Major findings as seen in Figure 4 were the significant differences in CPII, CCL2, PGE2 and MMP concentrations.
CPII concentrations were significantly different between T0R and T4R, which can be explained as an effect of exercise. CCL2 and MMP concentrations were significantly different between T4L and T4R, and – although not always statistically significant – clear increasing trends in MMP-activity and C2C-, PGE2&D2- and CCL2- concentrations between T1 and T2 and/or T3 and T4 were observed, which are all due to repeated arthrocentesis. No further relevant significant differences were detected, besides a difference of baseline concentrations in the left and right MMP measurement, which can be explained by an inter-assay difference.

Several outcomes of our study agree with outcomes of other studies. The significant effect of exercise found in CPII concentrations agrees with the outcome of a similar study (Frisbie et al., 2008). However, those concentrations were much higher than ours (714000 µg compared to 1000µg at baseline). These differences may occur because of the use of young Warmblood horses in the other study instead of adult Shetland ponies in our study.
The significant effects of repeated arthrocentesis found in PGE2 and MMP concentrations agree with other studies (van den Boom et al., 2004; van den Boom et al., 2005). The authors explained that there should be a time window of 2 weeks between two subsequent joint punctures to exclude confounding effects of repeated arthrocentesis (van den Boom et al., 2004).
Also, the PGE2 concentrations agree with outcomes of other studies (Lamprecht & Williams; 2012; Frisbie et al., 2008; van den Boom et al., 2004; Baccarin et al., 2014) , the concentrations are all around 100pg/ml at baseline. However, in other studies signs of inflammation occurred within and after the exercise protocol, which did not occur in our study. PGE2 is a very sensitive indicator of changes within the joint. Perhaps, during our measurements, the clinical signs of inflammation were not visible yet, but the PGE2 was already increased. Perhaps it is better to use urea levels, like other authors do (Baccarin et al., 2014). Urea levels can be used to estimate the changes in synovial fluid volume due to joint effusion (for example due to inflammation).

Sometimes outcomes of our study did not agree with outcomes of other studies. For GAG concentrations, significant increases have been found during an exercise protocol (Frisbie et al., 2008; van den Boom et al., 2005; Van den Hoogen et al., 1998), whereas in our study no differences have been found. However, the baseline concentration in our study is around the same level as the significant different concentrations after exercise in the other studies. Perhaps our levels already were in an increased state when the protocol started, maybe due to the low-intensity training before the exercise protocol started or the basis exercise protocol the ponies have had. For a right interpretation of our GAG concentrations, the ratio between CS-846 and GAG should be used. CS-846 reflects the turnover of new created aggrecan molecules (Poole et al., 1994), so this ratio could give a better indication of GAG metabolism.

GAGs have been shown to be cleared from the joint within 24 hours (Antonas et al., 1973). Maybe this is the reason why we did not measure increased concentrations. Besides of this, GAG may not be released constantly to the SF. Maybe repeated measurements during one time point (for example every 2 hours) can be useful to detect any increase of the concentration, because we only measured within 30 minutes and 24 hours after exercise. However, as described above, the effect of repeated arthrocentesis may interfere with these outcomes.

Synovial fluid reflects the joint homeostasis, but it is highly influenced by the level of inflammation, disease, age and weight. These factors can lead to a disruption in the biomarker concentration, whereby this could lead to an under- or overestimation of the interpretation of this value.
Therefore, it is better to measure ratios of anabolic versus catabolic markers for specific cartilage matrix components. For example, the use of a CPII/C2C and a CS-846/GAG ratio. In our measurements, CPII was significantly different due to the exercise protocol, but by using the CPII/C2C-ratio, the significant difference disappeared. Perhaps the relatively values of these markers do not induce a significant difference, so the ratio prevent misinterpretations.

The biomarker concentrations we measured in our synovial fluid can be compared with blood serum levels. This can be of major value in screening clinical cases for osteoarthritis when the horses are in active training, because serum sampling is much less invasive than obtaining synovial fluid. Several studies also measured CPII, GAG and C2C serum concentrations (Alwan et al., 1991; Frisbie et al., 2008; Jackson et al., 2015; Nicholson et al., 2010) and these were in the same level as our synovial fluid measurements, except for the GAGs . Probably the GAG concentrations are in a large scatter, which also was seen in the SF concentrations of GAG in different studies. We also collected blood serum from our Shetland ponies. Analysis of biomarkers in these samples could be a good next step to obtain further insight in the correlation between synovial fluid and serum biomarkers.

The used biomarkers in this study give an indication of dynamics in the joint homeostasis during and after an exercise protocol. It was important to differentiate the effects of exercise from the effects of exercise plus a defect, so that resulted in the onset of this study. The outcomes of this study could be used later - when the ponies undergo a defect study and get this exercise protocol again - to differentiate osteoarthritis in an early stage from changes in biomarker levels due to exercise alone.

My second hypothesis - about concentrations returning to baseline after discontinuation of the exercise – could not be answered, because of the longer wash-out period after the exercise protocol. There was not enough time for T6L, this is why I cannot say anything about the long term effect of the exercise protocol.

Conclusion

To answer the research question of my study, the differences between T0R and T4R are the most important. I did not find any significant differences between those time points, except for CPII. This indicates that C2C, GAG, CCL-2, PGE2 and MMP concentrations are not affected by exercise, while CPII concentrations are affected by exercise.

There is a significant increase in CPII concentration. This indicates that exercise at this level of intensity may have a stimulating effect on anabolic processes, but does not have any significant effect on the catabolic or inflammatory processes in the carpal joint. However, this conclusion is only based on a relatively small panel of biomarkers.

An important finding is that for this panel of biomarkers, exercise will not interfere with the results of the defect-study later in time, except for CPII.

This study involved repeated arthrocentesis, which is visible in MMP-activity and C2C-, CCL2- and PGE2-concentration. This should be taken into account when the same protocol is followed again, for example in the defect-study later with these ponies.

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References

Alwan, W. H., Carter, S. D., Bennett, D., & Edwards, G. B. (1991). Glycosaminoglycans in horses with osteoarthritis. *Equine Veterinary Journal*, *23*(1), 44–47.

Antonas, K. N., Fraser, J. R., & Muirden, K. D. (1973). Distribution of biologically labelled radioactive hyaluronic acid injected into joints. *Annals of the Rheumatic Diseases*, *32*(2), 103–11.

Baccarin, R. Y. A., Rasera, L., Machado, T. S. L., & Michelacci, Y. M. (2014). Relevance of synovial fluid chondroitin sulphate as a biomarker to monitor polo pony joints. *Canadian Journal of Veterinary Research*, *78*(1), 50–60.

Back, W., & Clayton, H. . (2013). Equine Locomotion. Retrieved January 19, 2017.

Bauer, D. C., Hunter, D. J., Abramson, S. B., Attur, M., Corr, M., Felson, D., … For the Osteoarthritis Biomarkers Network, (V Kraus, Chair). (2006). Classification of osteoarthritis biomarkers: a proposed approach. *Osteoarthritis and Cartilage*, *14*(8), 723–727.

Brandt, K. D., Dieppe, P., & Radin, E. L. (2008). Etiopathogenesis of Osteoarthritis. *Rheumatic Disease Clinics of North America*, *34*(3), 531–559.

Caron, J., & Genovese, R. (2003). Principles and Practices of Joint Disease Treatment. In *Diagnosis and Management of Lameness in the Horse* (pp. 746–764). Elsevier.

Carter, D. R., Beaupré, G. S., Wong, M., Smith, R. L., Andriacchi, T. P., & Schurman, D. J. (2004). The mechanobiology of articular cartilage development and degeneration. *Clinical Orthopaedics and Related Research*, (427), S69–S77.

Clayton, H. M. (2016). Horse species symposium: Biomechanics of the exercising horse. *Journal of Animal Science*.

de Grauw, J. C. (2011). Molecular monitoring of equine joint homeostasis. *Veterinary Quarterly*, *31*(2), 77–86.

Frisbie, D. D., Al-Sobayil, F., Billinghurst, R. C., Kawcak, C. E., & McIlwraith, C. W. (2008). Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis and Cartilage*, *16*(10), 1196–1204.

Frisbie, D. D., McIlwraith, C. W., & de Grauw, J. C. (2016). Synovial Fluid and Serum Biomarkers. In *Joint Disease in the Horse* (pp. 179–191). Elsevier.

Fuller, C. J., Barr, A. R., Sharif, M., & Dieppe, P. A. (2001). Cross-sectional comparison of synovial fluid biochemical markers in equine osteoarthritis and the correlation of these markers with articular cartilage damage. *Osteoarthritis and Cartilage*, *9*(1), 49–55.

Goldring, M., & Goldring, S. (2007). Osteoarthritis. *J Cell Physiol.*, 213(3):626-34.

Hallett, M. B., & Andrish, J. T. (1994). Effects of Exercise on Articular Cartilage. *Sports Medicine and Arthroscopy Review*, *2.1*, 29–37.

Jackson, B. F., Reed, S. R., Price, J. S., & Verheyen, K. L. P. (2015). Relationship between serum biomarkers of cartilage and bone metabolism and joint injury in young Thoroughbred racehorses in training. *American Journal of Veterinary Research*, *76*(8), 679–687.

Lamprecht, E. D., & Williams, C. A. (2012). Biomarkers of antioxidant status, inflammation, and cartilage metabolism are affected by acute intense exercise but not superoxide dismutase supplementation in horses. *Oxidative Medicine and Cellular Longevity*, *2012*, 920932.

Levick, J. R. (1995). Microvascular Architecture and Exchange in Synovial Joints. *Microcirculation*, *2*(3), 217–233.

Lotz, M., Martel-Pelletier, J., Christiansen, C., Brandi, M. L., Bruyere, O., Chapurlat, R., … Reginster, J. Y. (2014). Republished: Value of biomarkers in osteoarthritis: current status and perspectives. *Postgrad Med J*, *90*(1061), 171–178.

McIlwraith, C. W. (2010). Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. *Equine Veterinary Journal*, *37*(5), 473–482.

McIlwraith, C. W., Frisbie, D. D., Kawcak, C. E., & Weeren, R. van. (2016). *Joint disease in the horse* (2nd ed.).

NEHS (2016). Lameness the most commonly reported health problem in horses. *The Veterinary Record*, *179*(15), 370.

Nicholson, A. M., Trumble, T. N., Merritt, K. A., & Brown, M. P. (2010). Associations of horse age, joint type, and osteochondral injury with serum and synovial fluid concentrations of type II collagen biomarkers in Thoroughbreds. *American Journal of Veterinary Research*, *71*(7), 741–749.

Oakley, S. P., Portek, I., Szomor, Z., Appleyard, R. C., Ghosh, P., Kirkham, B. W., … Lassere, M. N. (2005). Arthroscopy – a potential "gold standard" for the diagnosis of the chondropathy of early osteoarthritis. *Osteoarthritis and Cartilage*, *13*(5), 368–378.

Pap, G., Eberhardt, R., Stürmer, I., Machner, A., Schwarzberg, H., Roessner, A., & Neumann, W. (1998). Development of Osteoarthritis in the Knee Joints of Wistar Rats After Strenuous Running Exercise in a Running Wheel by Intracranial Self-Stimulation. *Pathology - Research and Practice*, *194*(1), 41–47.

Poole, A. R., Ionescu, M., Swan, A., & Dieppe, P. A. (1994). Changes in cartilage metabolism in arthritis are reflected by altered serum and synovial fluid levels of the cartilage proteoglycan aggrecan: Implications for pathogenesis. *Journal of Clinical Investigation*, *94*(1), 25–33.

Riggs, C. M. (2006). Osteochondral injury and joint disease in the athletic horse. *Equine Veterinary Education*, *18*, 100–112.

te Moller, N., & van Weeren, P. (2017). How exercise influences equine joint homeostasis. *The Veterinary Journal*, 222, 60-67.

van den Boom, R., Brama, P. A. J., Kiers, G. H., Degroot, J., Barneveld, A., & Weeren, P. R. (2004). The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor a activities in normal equine joints. *Equine Veterinary Journal*, *36*(2), 155–159.

van den Boom, R., van de Lest, C. H. a, Bull, S., Brama, R. a J., van Weeren, P. R., & Barneveld, a. (2005). Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E2 and glycosaminoglycans in healthy equine joints. *Equine Veterinary Journal*, *37*(3), 250–256.

van den Hoogen, B. M., van de Lest, C. H., van Weeren, P. R., Lafeber, F. P., Lopes-Cardozo, M., van Golde, L. M., & Barneveld, a. (1998). Loading-induced changes in synovial fluid affect cartilage metabolism. *British Journal of Rheumatology*, *37*(6), 671–6.

van Weeren, P. R., & Brama, P. A. J. (2016). Effects of Loading/Exercise on Articular Tissues and Developmental Aspects of Joints. In *Joint Disease in the Horse* (pp. 105–118). Elsevier.

Verheyen, K., Price, J., Lanyon, L., Wood, J., Brown, M., Wilson, W., & al., et. (2006). Exercise distance and speed affect the risk of fracture in racehorses. *Bone*, *39*(6), 1322–1330.