Evaluation of Serum Amyloid A and Melanoma Inhibitory Activity protein in relation to aseptic arthritis of the fetlock joint in horses.

A project for the Danish Certificate in Equine Diseases

Susanne Albæk Andersen

Faxe Animal Hospital, Industriparken 11, DK - 4640 Faxe

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Andersen, S.A.: Faxe Animal Hospital, Industriparken 11, DK- 4640 Faxe, Denmark.

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Sammendrag

Serum Amyloid A (SAA) er et akut fase protein og en potentiel markør for graden af inflammation. Melanoma Inbibitory Activity protein (MIA) frigives patologisk fra melanomer og fysiologisk fra chondrocytter og er en potentiel markør for brusksyntese og bruskregenerering. Dette studie har fokus på en evaluering af disse to ledvæskemarkører i relation til aseptisk artritis i kodeled på heste. Der blev udtaget ledvæske fra 46 kodeled fordelt på 35 heste. Diagnosticering blev foretaget via mønstring, longering på hård og blød bund, bøjeprøve, udførelse af intra-artikulær analgesi samt røntgenoptagelse af koden. Ud over heste med halthed relateret til kodeleddet blev der inkluderet 16 heste, hvor smerte i kodeleddet kun var relateret til en bøjeprøvereaktion og ikke til halthed ved mønstring og longering. Forsøgshestene gennemgik et behandlingsregime med ledbehandling med triamcinolonacetonid, boksro eller ophold på sygefold, skridt ved hånd og veterinær kontrol efter 2-3 uger. Hestene blev herefter opdelt i en ”succes” (rengående) og en ”ikke-succes” (fortsat halt eller positiv bøjeprøvereaktion) gruppe på baggrund af det kliniske respons på behandlingsregimet. Ledvæsken blev analyseret for MIA, SAA og Total Protein (TP). Statistisk fandtes ingen forskel på koncentrationen af SAA, MIA eller TP i de to grupper, ligesom der heller ikke fandtes forskel på parametrene i relation til røntgenforandringer eller halthed sammenlignet med bøjeprøvereaktion. Forsøget bekræfter ikke SAA`s og MIA`s potentiale som prognostiske markører i relation til aseptisk kodeledsartritis hos heste. Flere forsøgsfaktorer optræder dog som potentielle fejlkilder og kunne optimeres heriblandt laboratorietesten til kvantificering af MIA. Yderligere forskning på området er nødvendig for at drage endelige konklusioner på området.

Summary

Serum Amyloid A (SAA) is an Acute Phase Protein, which is a potential marker for inflammation. Melanoma Inhibitory Activity protein (MIA) is produced and secreted physiologically from chondrocytes, making MIA a potential marker for cartilage synthesis and regeneration. Pathologically MIA is secreted from malignant melanomas. The present study evaluates these two markers in relation to aseptic arthritis of the fetlock joint in horses. Synovia was collected from 46 fetlock joints from 35 horses. The diagnostic procedure included evaluation of the horse on a straight line, lunging on a hard and a soft surface, flexion test, intra-articular anaesthesia and radiographs. Horses with lameness associated to the fetlock joint were included in the study. As well as horses with pain in the fetlock joint only related to a flexion test, and not to lameness. The horses underwent a treatment regime with intra-articular injection with triamcinolone acetonide, box rest or turning out in a small paddock and hand walking until a veterinary lameness evaluation 2-3 weeks after treatment. The horses were divided into a "success" (sound) and a "non-success" (still lame) group according to the clinical response to the treatment regime. The synovia was analysed for MIA, SAA and Total Protein (TP). Statistically no difference in the concentration of SAA, MIA and TP was found between the two groups as well as no difference was found in the two groups in relation to radiographic changes or lameness compared to flexion test. The study does not confirm the potential of SAA and MIA as prognostic markers in relation to aseptic arthritis of the fetlock joint in horses. Several sources of errors can be identified in the study design, among these the high coefficient of variance in relation to the laboratory test for MIA. Further research is necessary to finally conclude on the subject.

Introduction

Lameness is the most common cause of temporary or permanent loss of training ability within horses and especially lameness related to joints. Fetlock arthritis is ranking high both within riding horses, leisure horses and thoroughbred racehorses (1-3). The response to intra-articular treatment of an arthritic joint depends on the status of the joint ranging from an acute synovitis to a chronic osteoarthritis, as the condition is reversible only in the early stages caused by the lack of ability by mature cartilage to repair defects (4). Therefore, arthritis has become of significant relevance for many research projects in relation to diagnostic and therapeutic solutions within horses. Research is continuously searching for parameters, which can make synovia or blood serve as a marker of the

pathological processes in the joint in order to better monitor joint conditions and thereby improve the ability to diagnose and prognosticate.

Serum Amyloid A

Equine Serum Amyloid A (SAA) is a 14 kDa protein (5) and a "major" Acute Phase Protein (APP) hence the serum concentration of SAA increases within hours with a 10 to 1000 fold above baseline during a systemic inflammatory response and quickly decreases (6-11). Hereby, SAA is a positive APP because of the increase during an inflammatory response whereas negative APPs decrease.

The physiological function of SAA is still not clear though inhibitory functions have been identified in relation to pyrexia in mice (12), the reaction of neutrophils on cytokines and bacteria in human blood (13), activation of thrombocytes in humans (14) and antigene production in mice (15). Synthesis of SAA involves cytokines such as Interleukin-1 (IL-1), IL-6 and IL-18 (6;16) and like other APPs SAA is predominantly a protein of hepatic origin (17).

In sound horses, the SAA concentration in synovia has been found to be less or equal to 0,7 mg/l (18). Experimentally LipoPolySaccharide (LPS) induced arthritis, has given a significant increase in SAA levels in serum and synovia within 8 -16 hours after induction, which confirms a local synthesis of SAA within inflamed joints (19;20). Concentrations of SAA in synovia have been found to be significantly elevated in horses with septic arthritis and tenosynovitis. In horses with osteoarthritis or osteochondrosis (n = 9), SAA in synovia did not vary significantly from healthy horses (18). In human *in vitro* studies, SAA messenger RNA (mRNA) was significantly elevated in synovial tissue from patients with rheumatoid arthritis and other inflammatory joint diseases (16;21-23).

It has been found that equine SAA in serum reflects the activity of a pathological condition (8;24), as well as a correlation between the level of equine SAA in synovia and Total Protein (TP) has been found (18).

Melanoma Inhibitory Activity protein

Originally MIA was identified as a small 11 kDa protein with no known homology to other proteins (25-28) which were secreted pathologically from malignant melanoma cells (29;30). Pathologically elevated serum levels of MIA are predominantly found in relation to metastatic melanomas and to some extent, the elevation of MIA in serum is correlated to the degree of metastasis (31;32) as MIA increases the ability of metastasis via regulation of cell attachment (33;34).

Melanoma Inhibitory Activity protein is also referred to as Cartilage-Derived Retinoic-Acid-Sensitive Protein (CD-RAP) due to a physiological production and secretion of MIA in chondrocytes, which is dependent on the differentiation status of the cells which includes retinoic acid (29;35).

Lately the use of MIA as a treatment for osteochondral defects has been suggested (36). The *in vitro* study showed that treatment with MIA in an osteochondral defect model, accelerated repair of the articular cartilage. The functional role of MIA is not fully understood, but MIA plays an important role in the maintenance of cartilage tissue by preventing ossification, which could be the reasons for the results of the study (36). In accordance to this, an earlier study using MIA deficient mice, showed that these mice had subtle cartilage defects indicating an essential role of MIA in relation to cartilage fibre architecture formation (37). An *in vivo* study using rabbits (n = 28) induced with a cartilage defect furthermore supported a potential therapeutic use of MIA by a significantly increased formation of new cartilage within MIA treated animals compared to the control group (38). A study with induced joint instability via an anterior cruciate ligament transection also in rabbits (n = 24) showed an effect of MIA treatments as well (39). The joints were treated intra-articularly every 10^{th} day for 13 weeks. A high dose of MIA gave a significantly reduced osteophyte formation and joint space narrowing. Further research is needed to draw final conclusions on the therapeutic potential of MIA.

Both rheumatoid arthritis and invasive growth of tumours are associated with destruction of tissue matrix (32) and research on human MIA in relation to rheumatoid arthritis and osteoarthritis have been made with interesting results (32;34;40;41). A study found that only patients with rheumatoid arthritis showed an elevation of MIA levels in serum comparable with the MIA levels of melanoma patients (32). Other rheumatic diseases only showed slight elevations of MIA levels, which could

make MIA a possible parameter to differentiate human rheumatoid arthritis from other joint diseases. The authors suggest that MIA is released from chondrocytes during cartilage destruction in these patients reflecting chondrocyte activity. Another study (40) compared the level of different marker proteins in serum and synovia from human patients with osteoarthritis or rheumatoid arthritis. This report showed a significantly higher level of MIA in synovia versus serum, though this was not significant for MIA in relation to patients with rheumatoid arthritis.

Another study on human rheumatoid arthritis and osteoarthritis showed a significantly higher level of MIA in synovia from patients with osteoarthritis compared to patients with rheumatoid arthritis (34), which is the opposite of a previous study on MIA serum levels (32). Furthermore, a significantly higher level of MIA in synovia was found in the mild cases of osteoarthritis and rheumatoid arthritis compared to moderate and severe cases, so that a decrease in MIA was seen with progression of the two diseases. Hence MIA seems to reflect the severity of the disease (34). The study by Müller-Ladner *et al.* (1999) (32) did not differentiate in relation to the severity of the diseases. The authors (34) suggest that the level of MIA reflects the restorative reaction of the chondrocytes hence the decrease with progression of the disease. A significant increase of MIA in serum was also found in runners after marathons, suggesting that MIA reflects aspects of the joint metabolism which are still not identified (42).

The equine MIA has been found to be 91% identical to the human MIA protein (43) and lately a study on equine MIA as a potential marker of chondrogenesis has indicated a potential marker within horses as well (44). In healthy horses, expression of MIA has been seen in chondrocytes and MIA could be detected in serum, synovia and culture medium from chondrocyte cultures (45). In healthy horses (n = 28) the MIA concentration in synovia has been found to range from 8,2 to 52,0 arbitrary units (ng/ml) (43). The results are relative (human equivalents) and not absolute caused by the use of a human and not an equine specific assay.

Total Protein

The concentration of Total Protein (TP) in synovia increases with joint inflammation to approach that of plasma. The estimation of TP is often used for routine analysis (46). The reference interval for TP in synovia in horses is 1,81 +/- 0,26 g/dl (47).

The present study aims to evaluate equine MIA and SAA under practical conditions in relation to horses with aseptic arthritis of the fetlock joint, which is a common diagnosis equine practice. The two parameters are compared to TP. It is hypothesized that the concentration of SAA in synovia will not vary significantly from healthy horses no matter the outcome of the treatment (18). Furthermore, it is hypothesized that the concentration of MIA in synovia will be significantly higher in the group of horses with a positive outcome of the treatment regime due to less progression of the joint disease (34).

Materials and methods

Study population

Fifty-seven fetlock joints were examined between March and October 2007 at Faxe Animal Hospital. The horses were available from the daily flow of patients at the clinic though two horses were examined at another equine clinic. Eleven horses were excluded from the study, as they did not fulfil the inclusion criteria for various reasons. Hereby the final study population was 46 fetlock joints distributed between 35 horses.

The horses were 35 privately owned horses (1 stallion, 14 geldings, 20 mares) and the population included various breeds both ponies, riding and pleasure horses and standardbreds. The age of the horses ranged from 3 to 14 years.

Study design

Horses responding to intra-articular anaesthesia of the fetlock joint by at least 75% reduction in lameness within 10-15 minutes were included in the study. Furthermore, horses with a positive flexion test of the toe and with a negative flexion test within 10-15 minutes after an intra-articular anaesthesia of the fetlock joint were included. Horses with lameness associated to other joints or structures were not excluded from the study.

Synovia (½ - 2 ml) was obtained from the fetlock joint into a 3 ml sterile syringe before performing intra-articular anaesthesia with 5-6 ml of 2% mepivacaine hydrochloride[1]. In two cases however 10 ml of 2% mepivacaine hydrochloride were used. The intra-articular anaesthesia of the fetlock joint was performed with the leg unloaded and via arthrocentesis, from a lateral approach using a 21G x 1,5" needle (48) (figure 1).

Figure 1: Arthrocentesis of the fetlock joint. Lateral approach.

Horses included in the study underwent the following clinical examination:

- Obtainment of history
- Examination by palpation
- Visual examination at rest
- Observation on a straight line walking and trotting before and after intra-articular anaesthesia of the fetlock joint
- Observation on a lunge, on a soft and a hard surface before and after intra-articular anaesthesia of the fetlock joint
- Flexion test of the toe for 45-60 seconds before and after intra-articular anaesthesia of the fetlock joint

Lameness was graded 0 to 5 on a straight line and on the lunge based on the American Association of Equine Practitioners (AAEP) grading system[2].

[1] Carbocain®

[2] 0 = No lameness, 1 = Lameness difficult to observe; not consistently apparent regardless of circumstances, 2 = Lameness difficult to observe at a walk or trot in a straight line; consistently apparent under some circumstances, 3 = Lameness consistently observable at a trot under all circumstances, 4 = Lameness obcious; marked nodding, hitching, and/or shortened stride, 5 = Lameness obvious; minimal weight bearing in motion or rest; inability to move (49).

Radiographic examination

All horses were examined radiographically using a standard set of 4 digital radiographs using lateromedial, dorsopalmar, oblique dorsolateral-palmaromedial/plantaromedial and oblique dorsomedial-palmarolateral/plantarolateral. Furthermore, one lateromedial projection was obtained of the contralateral fetlock joint. Radiographs were evaluated for Degenerative Joint Disease (DJD) of the fetlock joint and findings such as periarticular remodelling (osteophyte formation), narrowing of the joint space and subchondral sclerosis were noted as radiographic findings. Intra- or extra-articular fragments without the above mentioned osseous reactions and enthesiophyte formation in relation to the distal sesamoidean ligaments, were not considered as radiographic findings. All radiographs were evaluated by the same veterinarian.

Treatment

The study group underwent treatment of the affected fetlock joints with 3-10 mg of triamcinolone acetonide, with the above mentioned injection technique on the same day as the diagnostic work up. After injection a bandage was placed for 24-48 hours. When needed, recommendations were made in relation to shoeing and hoof balance, aiming for a straight hoof-pastern axis and a lateromedial hoof balance though different farriers were involved. A conventional bar shoe was used for this purpose. The horse was box rested or turned out in a small paddock (depending on temperament) and hand walked (15-20 minutes once or twice daily) until a veterinary follow up at 2-3 weeks after treatment. In most cases the same veterinarian who made the diagnostic work of the patient made the follow up. If sound, the horse gradually returned to full work and was turned out in a paddock. If not sound, the horse was re-treated or another treatment regime was initiated. Horses which had been treated in multiple joints and had no lameness associated to the fetlock joint, were evaluated via the flexion test which initially was the indication for treatment of the fetlock joint.

Laboratory tests

Synovia withdrawn from the fetlock joints was instantly transferred to an EDTA tube. No later than two hours after collection the synovia was centrifuged at 3500 rpm and the erythrocyte free synovia was transferred into two EDTA tubes and frozen until laboratory tests were made in October 2007.

The analyses of SAA[3] were made using a Turbidometric ImmunoAssay (TIA) analysis. This human TIA kit has previously been evaluated on equine blood (50).

The quantifications for MIA[4] were made with a human MIA photometric Enzyme-Linked Immuno Sorbent Assay kit (ELISA)[5] (51). All measurements were made in duplicates and a mean value was calculated. Also this kit has previously been evaluated on equine blood (43).

The analyses on TP were performed with an ADVIA^{TM} 1650 Chemistry System from Bayer.

Statistical analyses

The study group was divided into the two groups "successful" and "unsuccessful" in relation to treatment. The aim of the study was to show a possible difference in the level of SAA and MIA in the two groups. Hereby the following sample sizes were calculated (52): For SAA **n = 21** horses per group using: Z_{β} (power = 80%) = 0,84, $Z_{1-\alpha/2} = (Z_{0,975})^2$ at a 95% confidence interval = 1,96, σ^2 = variance = 58^2 (53), d = the estimated difference between the means in the two populations = 50 (own estimate). For MIA **n = 16** horses per group using: Z_{β} (power = 80%) = 0,84, $Z_{1-\alpha/2} = (Z_{0,975})^2$ at a 95% confidence interval = 1,96, σ^2 = variance = 50^2 (own estimate), d = the estimated difference between the means in the two populations = 50 (own estimate).

In this study the total group counted 46 fetlocks – 23 in the "successful" group and 23 in the "unsuccessful" group.

[3] Analyses were performed by the laboratory at the Faculty of Life Sciences at the University of Copenhagen
[4] Analyses were performed at the Faculty of Life Sciences at the University of Copenhagen
[5] Roche, Mannheim, Germany

The statistical analyses were carried out in Microsoft Excel using the Student's *t* test and χ^2 analysis. A *p* value < 0,05 was considered statistically significant.

Results

Melanoma Inhibitory Activity protein, Serum Amyloid A and Total Protein

Clinical and laboratory results are shown in table 1a and 1b.

As mentioned earlier two measurements were made for each MIA value making a Coefficient of Variance (CV) ranging from 0,2% to 23,2% with an average of 11,6%. The results of MIA are shown graphically in figure 2.

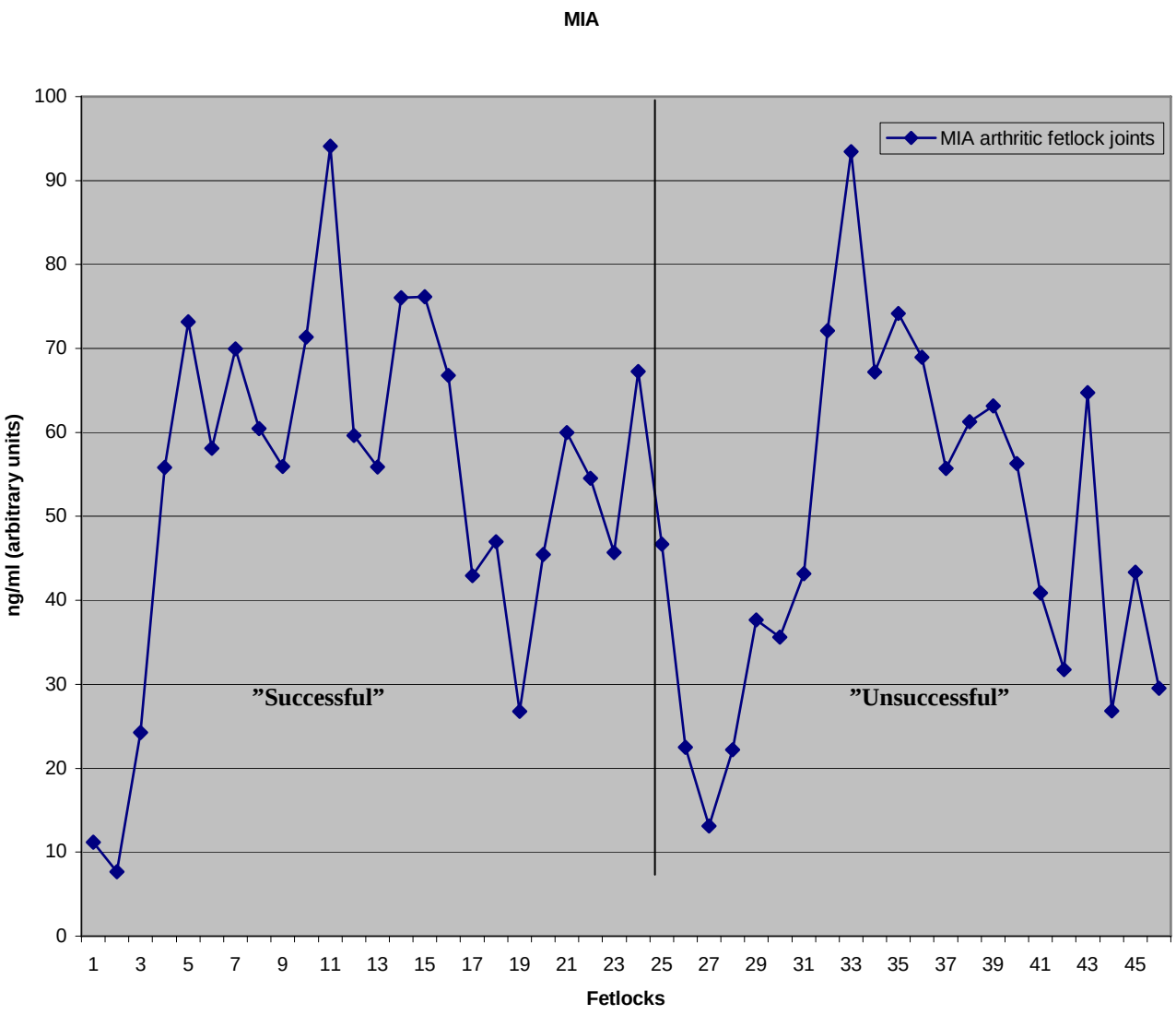

Figure 2: Melanoma Inhibitory Activity protein (MIA) concentrations in synovia in each fetlock joint. Fetlock joints nr. 1-23 is the “successful” group, whereas fetlock joints nr. 24-46 is the “unsuccessful” group.

Fetlock	Outcome	Fetlock	Radiographs	SAA (mg/l)	TP (g/l)	MIA (ng/ml)	CV (%)
1 (same horse as 2)	Sound	LF	Neg	0,8	12,72	11,22	12,6
2 (same horse as 1)	Sound	RF	Neg	0,9	19,14	7,65	15,3
3	Sound	LF	Pos	0,9	8,01	24,26	20,8
4	Sound	LF	Pos	0,6	9,34	55,83	15,4
5	Sound	LF	Neg	0,9	13,25	73,17	9,4
6	Sound	RF	Pos	0,6	6,81	58,13	15,1
7	Sound	LF	Neg	0,3	6,91	69,93	0,7
8	Sound	LF	Neg	0,6	17,46	60,46	1,0
9	Sound	RF	Neg	0,8	4,08	55,93	0,9
10	Sound	RF	Neg	0,8	9,33	71,33	5,0
11	Sound	RF	Neg	0,3	4,82	94,09	1,9
12 (same horse as 13)	Sound	RF	Pos	0,2	4,3	59,63	13,6
13 (same horse as 12)	Sound	LF	Neg	0,3	4,01	55,87	14,9
14	Sound	RF	Neg	0,6	13,5	76,04	14,2
15 (same horse as 16)	Sound	RF	Pos	0,8	6,19	76,15	12,5
16 (same horse as 15)	Sound	LF	Pos	0,8	6,84	66,80	2,4
17 (same horse as 18)	Sound	RF	Neg	1,0	4,89	42,92	8,1
18 (same horse as 17)	Sound	LF	Neg	0,7	4,23	46,98	6,1
19	Sound	RF	Neg	0,3	10,82	26,76	23,2
20	Sound	LF	Neg	0,7	15,76	45,45	12,4
21 (same horse as 22)	Sound	RF	Neg	0,9	9,1	60,00	2,9
22 (same horse as 21)	Sound	LF	Neg	1,3	13,19	54,56	21,7
23	Sound	LF	Neg	1,1	4,47	45,70	20,6
24	Not sound	RF	Neg	0,3	8,9	67,24	1,7
25	Not sound	RH	Neg	0,6	7,44	46,72	14,7
26	Not sound	RF	Pos	0,6	5,01	22,48	6,6
27	Not sound	LH	Neg	0,8	21,93	13,11	17,1
28 (same horse as 29)	Not sound	RF	Pos	0,7	21,02	22,20	7,6
29 (same horse as 28)	Not sound	LF	Pos	0,6	12,27	37,70	3,8
30	Not sound	RF	Neg	0,9	12,47	35,63	13,2
31	Not sound	LF	Neg	0,3	7,73	43,17	9,4
32	Not sound	LF	Neg	0,6	4,49	72,11	0,6
33	Not sound	RF	Neg	0,3	5,97	93,46	23,3
34 (same horse as 35)	Not sound	LF	Pos	0,8	5,14	67,20	16,8
35 (same horse as 34)	Not sound	RF	Pos	0,7	5,31	74,17	8,6
36	Not sound	LH	Neg	1,4	13,88	68,93	0,2
37	Not sound	LF	Neg	1,2	12,66	55,70	5,7
38	Not sound	LF	Pos	0,4	7,55	61,26	6,5
39 (same horse as 40)	Not sound	RF	Pos	0,6	4,34	63,15	1,4
40 (same horse as 39)	Not sound	LF	Pos	0,5	4,56	56,30	8,1
41	Not sound	LF	Neg	0,3	13,95	40,91	2,0
42	Not sound	LF	Pos	0,7	3,77	31,77	19,1
43	Not sound	RF	Neg	2,0	6,52	64,74	3,7
44	Not sound	LF	Neg	0,5	11,92	26,85	16,9
45	Not sound	RF	Neg	0,8	6,12	43,38	9,5
46	Not sound	RF	Neg	0,6	13,75	29,52	5,8

Table 1a: Analyses of synovia from horses with aseptic arthritis of the fetlock joint. Serum Amyloid A (SAA), Total Protein (TP), Melanoma Inhibitory Activity protein (MIA), Coefficient of Variance (CV), Left Front leg (LF), Right Front leg (RF), Left Hind leg (LH), Right Hind leg (RH).

Fetlock	Fetlock	Lameness (0-5)	Reaction to flexion test (0-5)	Lameness associated to	SAA (mg/l)	TP (g/l)	MIA (ng/ml)
1 (same horse as 2)	LF	1	1	Fetlock joint	0,8	12,72	11,22
2 (same horse as 1)	RF	1	1	Fetlock joint	0,9	19,14	7,65
3	LF	0	2	None	0,9	8,01	24,26
4	LF	1½	2 (sore)	Fetlock joint	0,6	9,34	55,83
5	LF	½	2 (sore)	Fetlock joint	0,9	13,25	73,17
6	RF	1	1	Fetlock joint	0,6	6,81	58,13
7	LF	1	1	Fetlock joint	0,3	6,91	69,93
8	LF	1	3 (sore)	Coffin joint	0,6	17,46	60,46
9	RF	1½	3 (sore)	Fetlock/coffin joint (50%/50%)	0,8	4,08	55,93
10	RF	1	1	Radiocarpal + intercarpal joints	0,8	9,33	71,33
11	RF	1	2 (sore)	Fetlock joint	0,3	4,82	94,09
12 (same horse as 13)	RF	1	2 (sore)	Fetlock joint	0,2	4,3	59,63
13 (same horse as 12)	LF	1	1 (sore)	Coffin joint	0,3	4,01	55,87
14	RF	½	1	Fetlock joint	0,6	13,5	76,04
15 (same horse as 16)	RF	1	2 (sore)	Palmar hoof region	0,8	6,19	76,15
16 (same horse as 15)	LF	1	2 (sore)	Coffin joint	0,8	6,84	66,80
17 (same horse as 18)	RF	1	2	Coffin joint	1,0	4,89	42,92
18 (same horse as 17)	LF	1	3	Coffin joint	0,7	4,23	46,98
19	RF	1	3	Intercarpal joint	0,3	10,82	26,76
20	LF	1	2	Fetlock joint	0,7	15,76	45,45
21 (same horse as 22)	RF	1	3 (sore)	Fetlock joint	0,9	9,1	60,00
22 (same horse as 21)	LF	1	3 (sore)	Fetlock joint	1,3	13,19	54,56
23	LF	1	1 (sore)	Coffin joint	1,1	4,47	45,70
24	RF	1	2	Coffin joint	0,3	8,9	67,24
25	RH	1½	Neg	Fetlock joint	0,6	7,44	46,72
26	RF	1	1	Fetlock joint	0,6	5,01	22,48
27	LH	1½	3	Fetlock joint	0,8	21,93	13,11
28 (same horse as 29)	RF	½	1	Fetlock joint	0,7	21,02	22,20
29 (same horse as 28)	LF	0	1	None	0,6	12,27	37,70
30	RF	1	1	Fetlock joint	0,9	12,47	35,63
31	LF	1	1	Fetlock joint	0,3	7,73	43,17
32	LF	0	2	None	0,6	4,49	72,11
33	RF	2	1 (sore)	Coffin joint	0,3	5,97	93,46
34 (same horse as 35)	LF	1½	2	Fetlock joint	0,8	5,14	67,20
35 (same horse as 34)	RF	1½	2	Fetlock joint	0,7	5,31	74,17
36	LH	1	1	Fetlock joint	1,4	13,88	68,93
37	LF	1	2 (sore)	Fetlock joint	1,2	12,66	55,70
38	LF	1	2 (sore)	Fetlock joint	0,4	7,55	61,26
39 (same horse as 40)	RF	1	3	Coffin joint	0,6	4,34	63,15
40 (same horse as 39)	LF	1	3	Fetlock joint	0,5	4,56	56,30
41	LF	1	2	Coffin joint	0,3	13,95	40,91
42	LF	1	3	Fetlock/coffin joint (50%/50%)	0,7	3,77	31,77
43	RF	1	1	Fetlock joint	2,0	6,52	64,74
44	LF	1	1	Fetlock joint	0,5	11,92	26,85
45	RF	1½	2	Fetlock/coffin joint (40%/60%)	0,8	6,12	43,38
46	RF	½	2	Fetlock joint	0,6	13,75	29,52

Table 1b: Analyses of synovia from horses with aseptic arthritis of the fetlock joint. Serum Amyloid A (SAA), Total Protein (TP), Melanoma Inhibitory Activity protein (MIA), Left Front leg (LF), Right Front leg (RF), Left Hind leg (LH), Right Hind leg (RH). Lameness is evaluated by the AAEP grading system (0-5). Degree of lameness is on a lunge on a soft surface. Reaction to flexion test is added lameness to the baseline lameness. Sore during the flexion test means that the horse intensely objected towards flexion of the toe.

Figure 3 shows the relationship between TP and MIA, which gives no evidence of correlation.

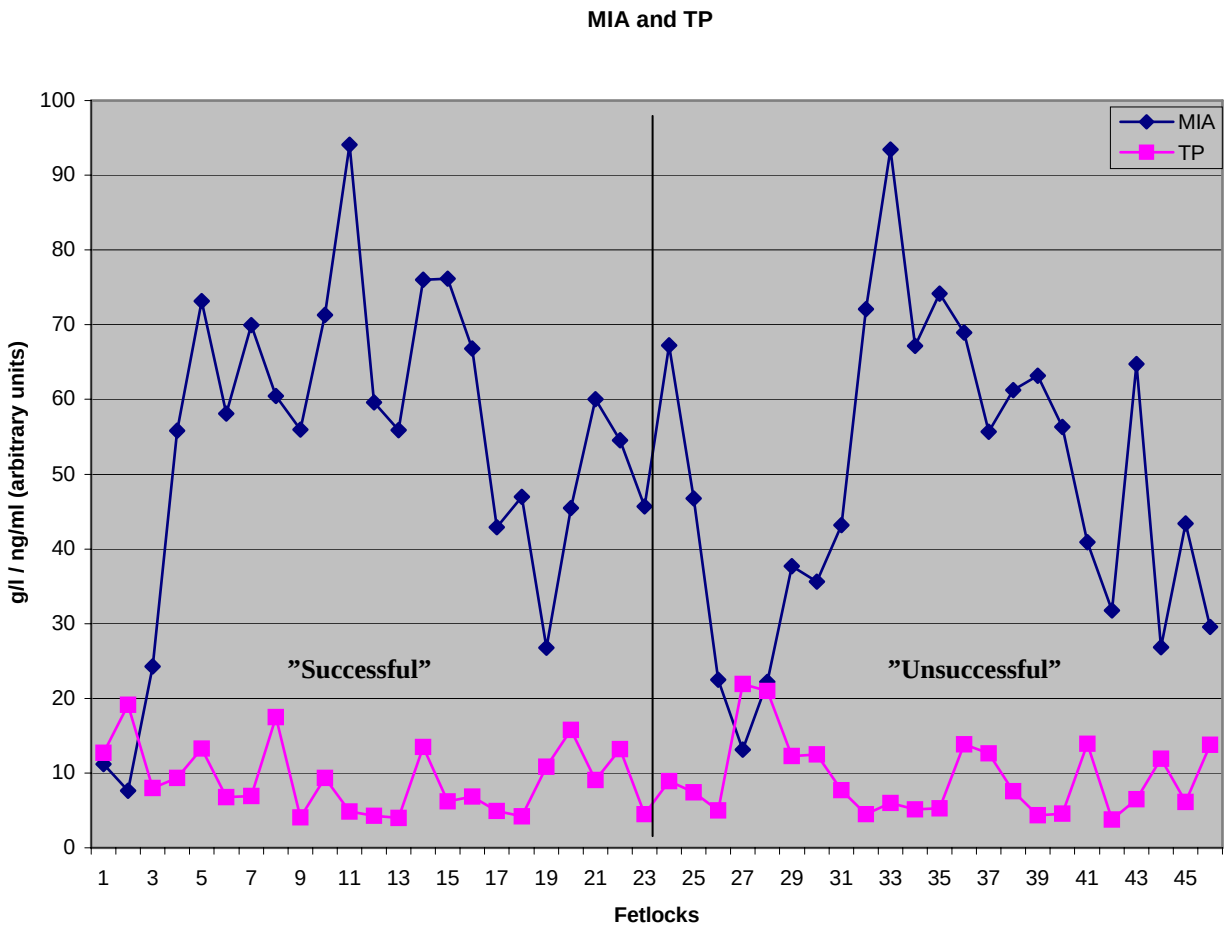

Figure 3: Melanoma Inhibitory Activity protein (MIA) and Total Protein (TP) concentrations in synovia in each fetlock joint. Fetlock joints nr. 1-23 is the “successful” group, whereas fetlock joints nr. 24-46 is the “unsuccessful” group.

Neither MIA, TP nor SAA could predict the response to the treatment regime. An equal number was found in the “successful” and the “unsuccessful” groups (2 x 23 horses) and *p*-values were all > 0,05 (see table 2).

Response to treatment	Parameter	n	Mean	Median	SD	*p*-value
Not lame	**MIA (ng/ml)**	23	53,9	55,9	21,0	0,48
Lame	**MIA (ng/ml)**	23	49,5	46,7	20,5	
Not lame	**TP (g/l)**	23	9,1	8,0	4,6	0,82
Lame	**TP (g/l)**	23	9,4	7,6	5,2	
Not lame	**SAA (mg/l)**	23	0,7	0,8	0,3	1,0
Lame	**SAA (mg/l)**	23	0,7	0,6	0,4	

Table 2: Response of fetlock joints to treatment in relation to Serum Amyloid A (SAA), Total Protein (TP), Melanoma Inhibitory Activity protein (MIA, Standard Deviation (SD).

None of the parameters TP, MIA and SAA were correlated to radiographic findings in the fetlock joints. The TP concentrations showed a slight tendency to present higher concentration levels in fetlock joints with radiographic findings with a *p*-value of 0,06 even though not statistically significant. Thirty-one fetlocks had no radiographic findings while 15 did show radiographic findings. All *p*-values within the three parameters were > 0,05 (see table 3).

Radiographic findings related to the fetlock joint and the success of treatment showed a *p*-value of 0,35 ($\chi 2 = 0,89$), thus horses without radiographic findings did not have a higher probability of going sound than horses with radiographic findings.

Radiographic status	Parameter	n	Mean	Median	SD	*p*-value
Negative	**MIA (ng/ml)**	31	51,6	54,6	21,7	0,98
Positive	**MIA (ng/ml)**	15	51,8	58,1	18,9	
Negative	**TP (g/l)**	31	10,2	9,3	4,8	0,06
Positive	**TP (g/l)**	15	7,4	6,2	4,4	
Negative	**SAA (mg/l)**	31	0,7	0,7	0,4	0,33
Positive	**SAA (mg/l)**	15	0,6	0,6	0,2	

Table 3: Radiographic status of fetlock joints in relation to Serum Amyloid A (SAA), Total Protein (TP), Melanoma Inhibitory Activity protein (MIA), Standard Deviation (SD).

Sixteen horses were included because of a flexion test associated to the fetlock joint and not because of lameness associated to the fetlock joint. The remaining 30 horses showed lameness associated to the fetlock joint. Again the three parameters did not show any difference in the two groups with all *p*-values > 0,05 (see table 4).

The ability to go sound after treatment for horses which were originally lame were not different from the horses which were originally not lame but showed a positive flexion test - *p*-value = 0,36 ($\chi 2 = 0,84$).

Lameness or flexion test	Parameter	n	Mean	Median	SD	*p*-value
Lameness	**MIA (ng/ml)**	30	49,3	55,8	21,4	0,30
Flexion test	**MIA (ng/ml)**	16	56,1	60,9	19,02	
Lameness	**TP (g/l)**	30	9,9	8,4	5,2	0,24
Flexion test	**TP (g/l)**	16	8,1	7,2	3,9	
Lameness	**SAA (mg/l)**	30	0,7	0,7	0,4	0,29
Flexion test	**SAA (mg/l)**	16	0,6	0,6	0,3	

Table 4: Fetlock arthritis causing lameness versus positive flexion test. Serum Amyloid A (SAA), Total Protein (TP), Melanoma Inhibitory Activity protein (MIA), Standard Deviation (SD).

Discussion

Studies in humans have shown significant elevations of SAA in synovial tissue from patients with rheumatoid arthritis and other inflammatory joint diseases (16;21-23). Within horses with low grade inflammatory joint diseases, these elevations of SAA in synovia have not been identified (18) which this study supports. Only septic and LPS induced arthritic joints and tenosynovitis have shown acute significant elevations of SAA in synovia (19;20). In this study the maximum level of SAA in synovia was 1,4 mg/l which is higher than the reference interval for SAA which is ≤ 0,7 mg/l (18) but bearing in mind that SAA is a "major" APP with a response of a 10 to 1000 fold, this modest increase cannot be concluded as a local inflammatory response which was also initially hypothesized. All synovia samples showed detectable levels of SAA which is also shown in an earlier study (18) but these did not vary significantly from healthy horses which the earlier study also showed (18).

Chrondrocytes release MIA during joint destruction hence MIA reflects chondrocyte activity (34). This study could not detect a difference in MIA concentrations in synovia in the two groups ("successful"/"unsuccessful") as hypothesized. Twenty-six out of 46 synovial samples had MIA values higher than the reference interval for healthy horses (43) but no relation to the two groups was seen. Elevations of MIA in synovia from humans have been found to be significantly higher in

cases of mild osteoarthritis and rheumatoid arthritis compared to moderate and severe cases giving a decrease in MIA with progression of the disease (34). This suggests that an increased level of MIA reflects a restorative reaction of chondrocytes in the joint and that chondrocytes loose their capacity for MIA synthesis with progression of a joint disease leaving MIA as a marker for lack of regenerative ability within the chondrocytes. Bearing this in mind, one would have expected the horses in our study which presented with the highest level of MIA would be the horses mostly prone to go sound after treatment, which we were not able to show.

Within humans, significantly increased levels of MIA have been found in runners after marathons (42). In horses this could mean that the activity level of the horse before the synovia is tested is highly relevant as MIA might reflect a change in joint metabolism after exhaustion, which is not necessarily pathological. This condition was not relevant in our study, as none of the horses had been worked the days before the clinical examination.

A recent study found no significant effect of age, different joints or gender on MIA concentrations in synovia in healthy horses (43). But the study found a variation between healthy individuals, which could be caused by an inter-individual variation and/or the effect of assay variation.

As well as being a marker for altered metabolism in joint cartilage (32), MIA has also been shown to change during fracture healing so that an increase in MIA was found in fracture cartilage and a decrease was found during the phase of endochondral ossification (54). In healthy horses it has been possible to detect MIA in synovial fluid from all samples indicating that MIA is always present as a part of maintenance of the cartilage (43).

In this study fetlock joints that showed no lameness but responded positively to a flexion test and had a positive response to intra-articular anaesthesia of the fetlock joint, were included as well as horses with lameness associated to the fetlock joint. Whether a positive flexion test reflects a pathological condition in the fetlock joint is questionable though as over 60% of sound horses (n = 100) in a study were found to have a positive response to a flexion test (55). In fact, the study (55) found that a slightly positive response to a flexion test performed with a force of 150 Newton for 60 seconds was more to be expected than a negative response. Furthermore, the response to a flexion test is highly dependable on horse-related factors such as age and gender (55), previous workload or rest at pasture (56) and inter-individual differences between examiners (57). It was found that the

odds for a positive flexion test in a mare were 2,2 times higher compared to a gelding as well as the tendency to respond positively to a flexion test increased with age (55;56). The present study included 20 mares, 14 geldings and 1 stallion aged from 3 to 14 years. Our study did not include a multi variance analysis for these possible confounders.

The reason for including horses which did not show lameness associated to the fetlock joint but showed a positive response to a flexion test, was that the flexion test could be eliminated with an intra-articular anaesthesia of the fetlock joint. The use of flexion tests in the present study was not strictly standardized as to force and time due to different examiners being involved. The recommendations for the force and time used for a flexion test vary from 100-300 Newton and 30-90 seconds (56;58-60). In our study a flexion test of 45-60 seconds was used with no quantification of the forces applied to the toe. The time span differed because of different clinicians. The same examiner performed the flexion test before and after intra-articular anaesthesia. This was done to standardize the clinical examination in particular the flexion test, which among experienced clinicians has been found to have a low CV (12%) when repeated by the same clinician (57). The group of examiners in the present study included three male and one female examiner which also includes a potential source of error, as a statistically significant difference has been identified between sexes as to the force applied during a flexion test as well as the variation between different clinicians has been identified to have a CV of 20% (57).

The horses included in this study with a positive flexion test only are not comparable to the earlier mentioned experiment with non-lame horses (55) as many of the horses included in our study were lame but with the lameness associated to another structure than the fetlock joint. Only 3 out of 16 horses were included with no lameness but a positive flexion test only. Thirteen horses were included because of a positive flexion test and lameness associated to other structures than the fetlock joint. This fact could potentially change the tendency to respond positively to a distal flexion test, as pain for instance associated to the coffin joint (9 cases) would also influence on the biomechanical use of the fetlock joint. This biomechanical stress factor on the fetlock joint could make the joint more prone to a positive outcome of a flexion test compared to horses with a non-compromised gait pattern. Whether this can be looked at as an arthritic joint or just a stressed joint is due to debate but in this present study these joints were included as it was concluded from a clinical point of view that treatment was necessary. The studies made on flexion tests have to the

author's knowledge all been made on the distal forelimb (55-57;59). This present study included 3 cases of hindlimb fetlock joints in which flexion tests were used equally to frontlimbs.

A volume of 5-6 ml of a 2% mepivacaine hydrochloride was used but in two cases of 500 kg horses a volume of 10 ml was used because of another veterinarian being involved. These conditions could potentially cause a difference in relation to diffusion (48;61). The recommendations for the volume of anaesthetic vary from 5-7 ml (49) to 10 ml (48). Furthermore, the horses in the present study were examined 10-15 minutes after local anaesthetic solution was injected, which is later than some of the recommendations on this subject which is 5-10 minutes (48;62).

The study design included four radiographic projections which is by some clinicians considered the standard views in relation to fetlock associated lameness (63;64), whereas others prefer to include a flexed lateromedial projection of the fetlock joint as a standard view as well (65). The flexed lateromedial fetlock joint projection permits a better evaluation of the dorsal sagittal ridge, which would be the argument for including this projection. The fetlock joints in this study were only evaluated clinically and radiographically which have certain limits as to conclude on the pathological conditions of the joints. An ultrasonographic examination of the cartilage in the fetlock joint and the soft tissue supporting the fetlock joint including the branches of the suspensory ligament could be a way to further evaluate the joint. The present study design might mistakenly have included diagnoses related to these structures because of diffusion of the local anaesthetic (48;61).

The horses were all treated intra-articularly with triamcinolone acetonide and box rest or turning out in a small paddock and hand walking for 2-3 weeks. The doses varied from 3 to10 mg/joint due to different sizes of horses, the individual degree of predisposure to laminitis (66) (i.e. subtle signs of Equine Metabolic Syndrome (EMS) or history of previous laminitis) and the amount of joints being treated attempting not to exceed a total of 18 mg triamcinolone acetonide (67). It would have been preferred that the horses all had their veterinary check the same amount of days after treatment but this was unfortunately not possible.

The CV for the MIA measurements is high ranging from 0,2% - 23,2% due to instability in the ELISA analyses on synovia which other authors have also experienced so that synovial markers in general represent the highest CVs compared to serum markers (41). The human ELISA kit used in

this study has shown both a high intra-assay CV ranging from 14,0% to 45,5% and a high inter-assay CV ranging from 14,2% to 20,7% (43) making the analytical process a potential source of error. The inter-assay CV might also reflect a biological variability not yet fully identified.

In the present study forelimb fetlocks (43 cases) clearly account for the majority of cases compared to hindlimb fetlocks (3 cases). This could be caused by the fact that the weight of a rider's mass affects the fore- and hindlimbs differently. During riding the forelimb fetlock joint shows more extension in the trot during the later part of the stance phase compared to the hindlimb and compared to trot in hand, which could make riding contribute to the aetiology of injuries more on forelimb fetlocks than hindlimb fetlocks (68).

Interestingly the ability to go sound after treatment was not related to lameness contra a positive flexion test or radiographic findings though the sample size is not sufficient for these calculations.

Conclusion

In conclusion, this study showed that quantification of MIA and SAA concentrations in synovia from horses with aseptic fetlock arthritis could not predict the response of the joint to a treatment regime with corticosteroids and rest. The concentration of SAA in synovia was invariably above the detection limit but no difference was found between the two groups. Several synovia samples showed MIA values above the reference interval for healthy horses but with no correlation to treatment outcome. The study does not support the use of SAA and MIA as prognostic markers in relation to aseptic arthritis of the fetlock joint**.** It was hypothesized that SAA would not vary between the two groups whereas MIA would be elevated in the "successful" group. The first hypothesis concerning SAA was confirmed while the second hypothesis concerning MIA was denied. Lately the focus on MIA points in direction of a therapeutic use, which may have more future relevance in relation to aseptic arthritis.

Several factors could be optimised in relation to the present study design such as a standardised protocol for the flexion test to make it a more objective and reproducible diagnostic tool, the use of just one examiner to minimize the variation between clinicians, focus on horse-related factors as

possible confounders such as gender, age, workload as well as a standardized use of the anaesthetic volume, treatment dose, period of rest and an improvement of the laboratory MIA ELISA test.

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"We've decided that Brownie has a foreign body in his paw. Doctor Grimshaw down on the end there is our radiologist and will be taking some pictures. Doctor Costello here is our pathologist and will want a blood sample. Doctor Kratz, our anesthesiologist, will prepare Brownie for surgery so that Doctor Evans, our surgeon can operate, with me assisting. Now, if you'd like his teeth cleaned at the same time, I can call Doctor . . . ."