Pferdeheilkunde – Equine Medicine 34 (2018) 6 (November/December) 563-573 DOI 10.21836/PEM20180608

Matrix metalloproteinase (MMP)-9 facilitates diagnostics in equine degenerative joint disease and may serve as osteoarthritis biomarker in horses

Julia Lerchbacher[1], Viola Häussler[2], Claudia Bräuning[1], Ina-Gabriele Richter[1], and Dirk Barnewitz[1]

[1] fzmb GmbH – Research Centre of Medical Technology and Biotechnology, 99947 Bad Langensalza, Germany
[2] Faculty for Veterinary Medicine, Freie Universität Berlin, 14195 Berlin, Germany

Summary: Due to limited possibilities to diagnose degenerative joint diseases (DJD) such as osteoarthritis at early stages, potential biomarkers are frequently investigated. The relationship of activity of matrix metalloproteinase (proMMP)-9 to clinical, arthroscopic and synovial fluid (SF) parameters, and changes therein after therapy were investigated in equine osteoarthritis. In the retrospective study, clinical data of 118 horses, 95 with lameness syndromes, were investigated. Affected joints were divided in 'group 1' without and 'group 2' with radiographic findings. SF samples were analysed for viscosity, protein content (PC), leukocyte count (LC) and proMMP-9 gelatinolytic activity. Some joints underwent arthroscopy to assess status of the cartilage and synovial membrane. After intra-articular therapy with corticosteroid-hyaluronic acid combinations or autologous conditioned serum, changes in proMMP-9 levels and lameness were analysed. Significant differences were found between control and affected joints in all parameters. Group 1 and 2 differed significantly in SF viscosity. ProMMP-9 was significantly higher in joints with aqueous compared to physiological SF in group 2. Significant correlations were found between proMMP-9 and PC, proMMP-9 and LC, and PC and LC. Examination of lameness, PC, LC, and viscosity identified only a marginal number of pathologically altered joints, in contrast to proMMP-9. The results indicate a correlation between strength of proMMP-9 gelatinolytic activity in SF and extent of joint disease. Analysis of proMMP-9 in SF seems more reliable than PC, LC, viscosity or lameness in equine DJD. This study shows that proMMP-9 is suitable as a biomarker in Osteoarthritis, providing information on joint condition, course of disease, and therapy response.

Keywords: lameness examination; synovial fluid; intra-articular therapy; radiographic findings; protein content; leucocyte count

Citation: Lerchbacher J., Häussler V., Bräuning C., Richter I.-G., Barnewitz D. (2018) Matrix metalloproteinase (MMP)-9 facilitates diagnostics in equine degenerative joint disease and may serve as osteoarthritis biomarker in horses. Pferdeheilkunde 34, 563-573; DOI 10.21836/PEM20180608

Correspondence: Dr. vet. med. Dirk Barnewitz, fzmb GmbH – Research Centre of Medical Technology & Biotechnology, Geranienweg 7, 99947 Bad Langensalza, Germany; dbarnewitz@fzmb.de

Introduction

In degenerative joint diseases (DJD) such as osteoarthritis (OA), changes of the articular cartilage are radiographically visible only in advanced stages of the disease and often not related to clinical signs (*Lohmander* et al. 1992, *Trotter* and *McIlwraith* 1996, *Kidd* et al. 2001). Arthroscopy – currently the gold standard for examination of joint diseases in horses – is invasive and risky, while magnetic resonance imaging (MRI) – the current gold standard in human medicine – is costly, not feasible in all equine joints and not yet extensively available for horses. In contrast, obtaining of synovial fluid (SF) is relatively easy from equine joints and considered as part of the standard examination procedure in lameness syndromes. Therefore, biomarkers in SF are appreciated more and more as a diagnostic method in DJD (*McIlwraith* 2005, *van den Boom* et al. 2005, *Abramson* and *Krasnokutsky* 2006, *Bauer* et al. 2006, *de Grauw* et al. 2009, *Barnewitz* et al. 2015). It is of great interest to find biomarkers, which vary with a specific diagnosis and normalize with successful therapy (*McIlwraith* 2005). Of particular interest are biomarkers, which comply to the five BIPED marker categories: Burden of disease, Investigative, Prognostic, Efficacy of intervention, and Diagnostic (*Bauer* et al. 2006).

Articular cartilage damages can be indirectly diagnosed through analysis of SF biomarkers, which emerge during cartilage matrix turnover (*Bauer* et al. 2006). Matrix metalloproteinases (MMP) are potential biomarkers, as they are associated with cartilage homeostasis and extracellular matrix turnover in both physiology and pathology (*Birkedal-Hansen* et al. 1993, *Murphy* et al. 2002, *Fietz* et al. 2008). The distribution of catabolic mediators such as MMPs plays a fundamental role in cartilage degradation (*McIlwraith* 1996). In the pathogenesis of OA, especially the gelatinases A MMP-2 (72 kDa type IV collagenase) and B MMP-9 (92 kDa type IV collagenase) play a significant role (*McIlwraith* 1996, *Clegg* et al. 1997a; *Clegg* et al. 1997b; *Clegg* et al. 1998; *Clegg* and *Carter* 1999, *Jouglin* et al. 2000, *Trumble* et al. 2001, *Fietz* et al. 2008, *Barnewitz* et al. 2015). They have a broad substrate specificity and can participate in the degradation of collagens, proteoglycans and other components of the basement membrane and connective tissue molecules of the extra cellular matrix (*Clegg* et al. 1998, *Murphy* et al. 2002). In addition, they contribute to DJD by activation of other MMPs (*Birkedal-Hansen* et al. 1993). Unlike MMP-2, MMP-9 is detectable in SF only under pathological joint conditions (*Mohtai* et al. 1993, *Clegg* et al. 1997b). Increased MMP-9 activity was found in SF during pathological conditions (*Clegg* et al. 1997b, *Jouglin* et al. 2000, *Trumble* et al. 2001, *Fietz* et al. 2008). In our previous study, increased proMMP-9 levels were detected over at least four weeks after creation of artificial defects on the hyaline cartilage of hocks (*Barnewitz* et al. 2015).

MMP-9 is involved in inflammatory diseases and thus predestined as biomarker for pathological joint changes (*Rorvik* and *Grondahl* 1995, *Clegg* et al. 1997b, *Jouglin* et al. 2000, *Fietz* et al. 2008, *Clutterbuck* et al. 2010). The present study contributes to the evaluation of MMP-9 as an SF biomarker for DJD. MMP-9 was analysed in SF of horses with naturally occurring osteoarthritis without or with radiographic findings. Possible relationships with clinical, arthroscopic and SF parameters and changes after intra-articular therapy were investigated.

Material and Methods

Study design and animals

Clinical data of 118 horses, which were presented to two horse clinics (Bargteheide and Kerken/Wachtendonk, Germany) between July 2008 and March 2011, have been evaluated retrospectively. The retrospective study did not involve animal experiments, all examinations were made with owners' consent and complied with the current laws for veterinary clinics of Germany.

Data from 95 horses were collected during standard-of-care treatment for joint-related lameness representing the 'patient group' (pat-grp) (n = 97 joints; two horses had two affected joints). Patients were categorized according to radiographic findings into 'group 1' (grp1) (n = 49 joints) without and 'group 2' (grp2) (n = 48 joints) with radiographically visible joint changes (one horse of each group had two affected joints). Grp1 joints were considered as "pre-radiological stage of OA", while grp2 joints were accounted as "pre-OA" and OA stage, according to the definitions of *Lohmander* et al. (1992). 17 patients, 6 of grp1 and 11 of grp2, were subjected to arthroscopy to examine the cartilage surface and synovial membrane (SM) macroscopically. Fifty-nine diseased joints, 29 of grp1 and 30 of grp2, were treated with intra-articular injections on the basis of decisions by the owner and veterinarian.

Twenty-three horses, representing the 'control group' (ctrl-grp) (n = 23 joints), had been admitted to clinics due to limb injuries, but did not show signs of lameness or systemic diseases. Joint involvement was excluded by radiographic examinations and SF analysis. 16 horses of the ctrl grp had to be euthanized for clinical reasons other than joint diseases and underwent arthroscopy post mortem.

120 SF samples were collected from 23 control and 97 patient joints (52 coffin joints (Articulatio interphalangea distalis), 46 fetlock joints (Art. metacarpophalangea), 10 carpal joints (Art. carpi), 4 tarsometatarsal joints (Art. tarsometatarseae), 3 hock joints (Art. tarsi), 3 pastern joints (Art. interphalangea proximalis), 2 stifle joints (Art. genus)). Arthrocentesis took place during clinical examination and in the pat-grp before administration of anaesthetics. After therapy, second SF samples were collected during follow-up examination (31 fetlock joints, 27 coffin joints, 1 pastern joint).

The horses were of different age (4–23 years, ∅ 11.18±4.66), sex (44 mares, 15 stallions, 59 geldings), and breeds (97 crossbred horses, 10 German riding ponies, 4 trotters, 3 Arabian horses, 3 Quarter horses, 1 Gypsy horse). These parameters did not differ significantly between groups (age: P = 0.316; sex: P = 0.920; breed: P = 0.246).

Clinical examination

Lameness duration (Ldur) was recorded. Lameness degree (Ldeg) was scored semi-quantitatively according to the „AAEP lameness scale"(Anon 1991). The joint causing lameness was identified by local nerve blocks and intra-articular anaesthesia according to usual standards. Radiographic examination was conducted according to usual standards. Operations for arthroscopy were performed under general anaesthesia in dorsal recumbency with operative approaches as described by *McIlwraith* (2005). Macroscopic evaluation of cartilage and SM followed a scheme modified according to *McIlwraith* et al. (2010) (Table 1).

Synovial fluid analysis

SF samples were analysed for MMP-9 gelatinolytic activity, protein content (PC), leukocyte count (LC) and viscosity. The latter was evaluated by means of a thread-pulling droplet and classified as physiological (0), reduced (I) and aqueous (II) (*McIlwraith* 1989a). MMP-9 analysis was done by gelatin

Table 1 Schemes to evaluate cartilage and synovial membrane (SM) findings. | *Schema zur Evaluierung von Korpel- und Synovialmembran (SM)-Befunden.*

	Score	Term	Description
Cartilage findings	0	none	No changes in the visible cartilage surface.
	I	low-grade	Very superficial erosion/fibrillation of the joint surface.
	II	moderate	Partial or total loss of cartilage.
	III	high-grad	Excessively deep cartilage erosions extending to the subchondral bone.
SM findings	0	none	No changes in the SM.
	I	acute	Joint capsule thin/elastic; isolated hyperaemia of the synovial villi.
	II	subacute	Joint capsule thin; extensive and distinct hyperaemia of the synovial villi; partially beginning hypertrophy of the synovial villi.
	III	chronic	Joint capsule appears thickened/rough, increased villus formation; villus thickening and/or appearance of abnormal synovial villi; partial hypertrophy of the synovial villi.

Modified according to McIlwraith et al. (2010). / *Modifiziert nach McIlwraith et al. (2010).*

zymography (*Clegg* et al. 1997a, *Barnewitz* et al. 2015). Before, all SF samples were centrifuged for 10 minutes at 5000 rpm and cell-free supernatant was transferred to Eppendorf tubes and stored at +4 °C for a maximum of 9 weeks until MMP-9 analysis. Classification into proMMP-9 grades corresponding to SF concentrations was elaborated based on comparison with equine-proMMP-9 standard protein samples with known concentration (Table 2).

Therapy

Intra articular injections contained either one of the corticosteroids (CS) betamethasone (Celestovet® 13,84 mg/ml; 40 mg/horse) or triamcinolone (Triamhexal® 10 mg/ml; 5 10 mg/joint) in combination with hyaluronic acid (HA) (Hy-50® Vet 17 mg/ml solution for injection ; 1–3 ml/joint) (grp1: 22; grp2: 20), or autologous conditioned serum (ACS) (Orthokine®vet Irap ; 2–4 ml/pastern joint, 4–6 ml/fetlock or coffin joint) (grp1: 7; grp2: 10). The horses were kept at box rest with occasional hand-walking until monitoring of disease progress three to four weeks after therapy. After intraarticular therapy, changes in proMMP-9 and lameness were assessed.

Data analysis

Statistical analysis was performed using Sigma Plot 11.0 . Test for normal distribution of data was done by Kolmogorov-Smirnov-Test. Changes in lameness and proMMP-9 after treatment were categorized into i) recovered, ii) decreased, iii) unchanged, and iv) increased and extent of change ( ) was calculated. Kruskal-Wallis One Way ANOVA was used to compare the values of each surveyed parameter between all three groups (grp1 vs. grp2 vs. ctrl-grp) and proMMP-9 between all categories of each parameter (e.g. SF viscosity: physiological vs. reduced vs. aqueous) and, if significant, post-hoc pairwise comparisons were done between two groups or two parameter categories using Mann-Whitney Rank Sum Test. proMMP-9 and Ldeg before and after therapy were compared using Wilcoxon Signed Rank Test. Correlation analysis was conducted using Pearson Product Moment Correlation Test (PC vs. LC) or Spearman Rank Order Correlation Test (MMP vs. PC, MMP vs. LC, MMP vs. Ldeg), giving the correlation coefficient r. Numbers of horses (per sex, breeds, change category after therapy) were compared between groups by Chi-square Test. Significance was considered when $P \leq 0.05$ (* 'significant') with further significance zones at $P \leq 0.01$ (** 'very significant') and $P \leq 0.001$ (*** 'highly significant').

Results

Findings before therapy

Gelatin zymography revealed no proMMP-9 gelatinolytic activity (grade 0) in all control joints and some joints of both pat-grp (6%/10%). Most joints of both pat-grp were assigned to proMMP-9 grade 0–I (39%/27%) or I (39%/33%). proMMP-9 grade IV was detected in no horse. proMMP-9 was not significantly different between grp1 and grp2 ($P = 0.354$), but significantly higher in both compared to ctrl-grp (each $P \leq 0.001$) (Table 3). In both pat-grp, proMMP-9 neither correlated with age ($r = -0.084$, $P = 0.562$/$r = -0.077$, $P = 0.599$), nor differed significantly between sex ($P = 0.958$/$P = 0.266$) or breeds ($P = 0.903$/$P = 0.928$).

SF viscosity was physiological in all joints of the ctrl-grp, more than half (52%) of grp2 and only 22% of grp1 (Table 4). SF viscosity was significantly more often reduced in grp1 compared to grp2 and in both groups compared to ctrl-grp (Table 3).

All control joints had values below the mean physiological value for PC of 2.5g/dl (*Trotter* and *McIlwraith* 1996) and the maximum physiological value for LC of 500 cells/ l (*de Grauw* 2011). In grp1 and grp2, the physiological values exceed regarding PC in 10% (8%/13%) and LC in 40% (37%/44%). PC and LC did not differ significantly between both pat-grp but were significantly elevated in both groups compared to ctrl grp (Table 3). Grp1 and grp2 differed significantly neither in Ldeg ($P = 0.932$) nor Ldur ($P = 0.446$) (Table 3).

Arthroscopy proved physiological cartilage and SM structures in all 16 control joints. All 17 arthroscoped patient joints had both findings of cartilage and SM changes of varying degrees, which did not differ significantly between pat-grp (cartilage: $P = 0.579$, SM: $P = 0.724$). In grp1, most joints showed superficial cartilage erosions (score I: 67%), while in grp2, more than a third had even deeper cartilage damage (score II: 36%) (Table 4). Half of grp1 had chronic (score III: 50%) and one-third sub-acute (score II: 33%) SM changes, while nearly half of grp2 had signs of subacute (score II: 45%) and more than one third of chronic (score III: 36%) SM findings (Table 4).

Relationships between proMMP-9 and other parameters

ProMMP-9 of the pooled pat-grp neither differed significantly between different Ldeg ($P = 0.420$) or different Ldur

Table 2 Scheme to categorize proMMP-9 gelatinolytic activity into proMMP-9 grades. | *Schema zur Kategorisierung von proMMP-9-gelatinolytischer Aktivität in proMMP-9-Klassen.*

Class	Concentration [ng/ml]*	Description
0	0-10	No increase of proMMP-9 (92 kDa)
I	10-40	Low increase of proMMP-9
II	41-99	Clear increase of proMMP-9
III	100-180	Extensive increase of proMMP-9
IV	>180	Most intensive increase of proMMP-9 and additionally active MMP-9 (82 kDa) activity

Range of concentration related to each MMP-9 grade. Range of concentrations were estimated in comparison to proMMP-9 standard protein with known concentration. Konzentrationsbereich für jede MMP-9-Klasse. Die Konzentrationsbereiche wurden im Vergleich zum proMMP-9-Standardprotein mit bekannter Konzentration geschätzt.

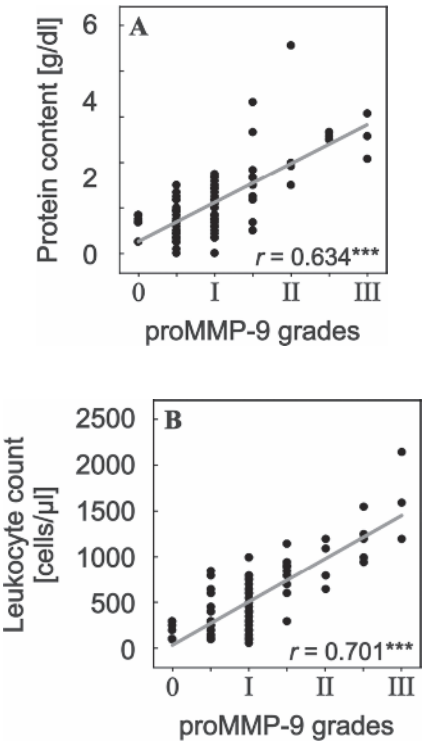


Fig. 1 XY scatter plots with correlations between synovial fluid paramters before therapy. Correlation between (A) proMMP-9 grade and PC, (B) proMMP-9 grade and LC, as well as (C) PC and LC with linear trend line and correlation coefficient r. (A-C) n = 97.
XY-Punktdiagramme mit Korrelationen zwischen Synovialflüssigkeits-Paramtern vor Therapie. Korrelation zwischen (A) proMMP-9-Klasse und PC, (B) proMMP-9-Klasse und LC, sowie (C) PC und LC mit linearer Trendlinie und Korrelationskoeffizient r. (A-C) n = 97

(P = 0.233), nor correlated significantly with Ldeg (r = 0.110, P = 0.283) or Ldur (r = 0.159, P = 0.120) (Table 4). There appears to be a non-significant, positive correlation of lower SF viscosity with higher proMMP-9 in both pat-grp (r = 0.011 P = 0.939/r = 0.053, P = 0.717) (Table 4). In grp2, proMMP-9 was significantly higher in joints with aqueous than with physiological SF (P ≤ 0.001).

A non-significant, negative correlation (r = - 0.431, P = 0.082) of lower proMMP-9 with increasing cartilage changes was observed in the pooled pat-grp (Table 4). Higher proMMP-9 levels (up to grade III) were found in cases of superficial cartilage defects (score I), whereas no or only weak proMMP-9 gelatinolytic activity (grade 0 or I) was found in joints with high-grade cartilage findings (score III). SM findings showed the opposite: a non-significant, positive correlation (r = 0.436, P = 0.078) of higher proMMP-9 with increasing SM damage (Table 4) where joints with acute SM findings (score II) had significantly higher proMMP-9 than with sub-

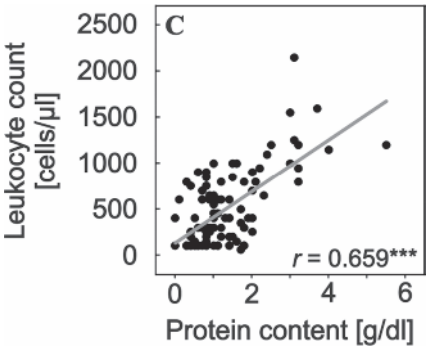


Table 3 Clinical, arthroscopic & synovial fluid parameters before therapy. | *Klinische, arthroskopische & Synovialflüssigkeits-Parameter vor Therapie*

	Group 1	Group 2	Control	P, diff.*	P, grp1 vs. grp2	P, grp1 vs. ctrl	P, grp2 vs. ctrl
	(n = 49)	(n = 48)	(n = 23)				
SF parameters							
proMMP-9 grade	0.92 ± 0.62 (0–3)	1.05 ± 0.75 (0–3)	0.00 ± 0.00 (0–0)	≤ 0.001***	0.354	≤ 0.001***	≤ 0.001***
SF viscosity	1.24 ± 0.80 (0–2)	0.67 ± 0.78 (0–2)	0.00 ± 0.00 (0–0)	≤ 0.001***	≤ 0.001***	≤ 0.001***	≤ 0.001***
PC [g/dl]	1.26 ± 1.01 (0.00–5.50)	1.36 ± 0.92 (0.00–4.00)	0.70 ± 0.48 (0.00–1.80)	0.004**	0.431	0.008**	≤ 0.001***
LC [cells/µl]	461 ± 380 (55–1600)	535 ± 443 (100–2150)	206 ± 123 (50–450)	0.004**	0.420	0.005**	0.002**
Clinical parameters							
Ldeg	2.73 ± 0.67 (1–4)	2.73 ± 0.57 (1–4)	0.00 ± 0.00 (0–0)		0.932	≤ 0.001***	≤ 0.001***
Ldur (median)	9-12 weeks	9-12 weeks	0 weeks		0.446	≤ 0.001***	≤ 0.001***
Arthroscopic paramters	(n = 6)	(n = 11)					
Cartilage score	1.50 ± 0.38 (1–3)	1.73 ± 0.79 (1–3)	0.00 ± 0.00 (0–0)		0.579	≤ 0.001***	≤ 0.001***
SM score	2.33 ± 0.82 (1–3)	2.18 ± 0.75 (1–3)	0.00 ± 0.00 (0–0)		0.724	≤ 0.001***	≤ 0.001***

Mean value ± standard deviation with range (min-max). Statistical significance denoted as ** very significant when P ≤ 0.01 and *** highly significant when P ≤ 0.001. | *Mittelwert ± Standardabweichung mit Bereich (Min-Max). Statistische Signifikanz angegeben als ** sehr signifikant wenn P ≤ 0,01 und *** hoch-signifikant wenn P ≤ 0,001.*

acute changes (score I) (P = 0.017). However, the sample size was too small to obtain statistically reliable statements.Highly significant (each P≤0.001) positive correlations were found between proMMP-9 and PC (r = 0.634), proMMP-9 and LC (r = 0.701), as well as PC and LC (r = 0.659) in the pooled pat-grp (Figure 1; Table 4).

Changes after therapy

After CS/HA therapy, proMMP-9 recovered in most joints (41%) and increased only in some joints (5%) of grp1, while in grp2 it remained mostly unchanged (55%) and recovered only in some joints (10%) (Table 5). After ACS therapy,

Table 4 Mean proMMP-9 grade per parameter before therapy. | *Mittlere proMMP-9-Klasse per Parameter vor Therapie*

	Group 1 (n = 49)	Group 2 (n = 48)	Group 1+2 (n = 97)
SF viscosity			
physiological	0.68 ± 0.40 (22%)	0.82 ± 0.50 (52%)	
reduced	0.83 ± 0.45 (31%)	1.00 ± 0.78 (29%)	
aqueous	1.09 ± 0.76 (47%)	1.89 ± 0.78 (19%)	
P (difference)	0.281	0.004**	
r; P (correlation)	0.011, 0.939	0.053, 0.717	
Ldeg			
0	(0%)	(0%)	(0%)
I	0.50 ± 0.00 (4%)	1.00 ± 0.00 (2%)	0.67 ± 0.29 (3%)
II	1.08 ± 0.84 (27%)	0.85 ± 0.75 (27%)	0.96 ± 0.79 (27%)
III	0.85 ± 0.53 (61%)	1.14 ± 0.78 (67%)	1.00 ± 0.68 (64%)
IV	1.13 ± 0.63 (8%)	1.50 ± 0.00 (4%)	1.25 ± 0.52 (6%)
V	(0%)	(0%)	(0%)
P, (difference)	0.360	0.416	0.420
r; P (correlation)			0.110, 0.283
Ldur			
< 4 weeks	0.75 ± 0.42 (12%)	1.10 ± 0.42 (10%)	0.91 ± 0.44 (11%)
5-8 weeks	0.94 ± 0.64 (37%)	1.18 ± 0.87 (29%)	1.05 ± 0.74 (33%)
9-12 weeks	1.00 ± 0.66 (33%)	1.08 ± 0.73 (40%)	1.04 ± 0.69 (36%)
4-6 months	0.8 ± 0.27 (10%)	0.60 ± 0.42 (10%)	0.70 ± 0.35 (10%)
7-12 months	1.00 ± 1.32 (6%)	1.83 ± 0.76 (6%)	1.42 ± 1.07 (6%)
> 12 months	0.50 ± 0.00 (2%)	0.25 ± 0.35 (4%)	0.33 ± 0.29 (3%)
P (difference)	0.919	0.120	0.233
r; P (correlation)			0.159, 0.120
	(n = 6)	(n = 11)	(n = 17)
Cartilage score			
0 (normal)	(0%)	(0%)	(0%)
I (low-grade)	1.25 ± 1.19 (67%)	1.90 ± 0.65 (45%)	1.61 ± 0.93 (53%)
II (moderate)	1.00 ± 0.00 (17%)	0.88 ± (0.63 (36%)	0.90 ± 0.55 (29%)
III (high-grade)	1.00 ± 0.00 (17%)	0.50 ± 0.71 (18%)	0.67 ± 0.58 (18%)
P (difference)	--	--	0.227
r; P (correlation)	--	--	-0.431, 0.082
SM score			
0 (normal)	(0%)	(0%)	(0%)
I (acute)	0.50 ± 0.00 (17%)	0.00 ± 0.00 (18%)	0.17 ± 0.29 (18%)
II (subacute)	0.75 ± 0.35 (33%)	1.80 ± 0.67 (45%)	1.50 ± 0.76 (41%)
III (chronic)	1.67 ± 1.15 (50%)	1.25 ± 0.50 (36%)	1.43 ± 0.79 (41%)
P (difference)	--	--	0.026*
r; P (correlation)	--	--	0.436, 0.078

Mean value ± standard deviation with relative number of horses (%). Statistical significance denoted as * significant when P ≤ 0.05 and ** very significant when P ≤ 0.01. | *Mittelwert ± Standardabweichung mit relativer Anzahl der Pferde (%). Statistische Signifikanz angegeben als * signifikant wenn P ≤ 0,05 und ** sehr signifikant wenn P ≤ 0,01.*

proMMP-9 remained unchanged in most joints of both pat-(57%/40%) and recovered only in some joints of grp1 (14%) (Table 5). An almost similar correlation was shown for change in lameness after CS/HA and ACS injection in both pat-grp (Table 5). After therapy, proMMP-9 and Ldeg differed significantly ($P \leq 0.001/P = 0.001$) between both pat-grp, in contrast to before therapy (Table 5).

After CS/HA therapy, Ldeg had changed significantly in both pat-grp (each $P \leq 0.001$), while proMMP-9 only changed significantly ($P \leq 0.001$) in grp1 (Table 5). ACS therapy did not evoke significant changes in proMMP-9 ($P = 0.750/P = 0.688$) or lameness ($P = 0.188/P = 0.438$) in both pat-grp (Table 5). When analysing the extent of change, after CS/HA therapy MMP and Ldeg were significantly higher in grp1 than in grp2 ($P = 0.013/P \leq 0.001$) (Figure 2), whereas after ACS treatment there was no significant difference between both pat-grp ($P = 0.961/P \leq 0.406$) (Table 5). Nevertheless, there was no significant difference in MMP or Ldeg between CS/HA and ACS therapy within both pat -grp (grp1: $P = 0.059/P = 0.139$;

Table 5 proMMP-9 grade and lameness degree after therapy. | *proMMP-9-Klasse und Lahmheitsgrad nach Therapie*

	Group 1	Group 2	P, grp1 vs. grp2
	(n = 29)	(n = 30)	
proMMP-9 grade change category; CS/HA | ACS[a.]			
recovered	41% | 14%	10% | 0%	0.064 | 0.466
decreased	27% | 14%	20% | 30%	
unchanged	27% | 57%	55% | 40%	
inreased	5% | 14%	15% | 30%	
proMMP-9 grade[b.]			
overall	0.41 ± 0.38 (0–1.5)	0.92 ± 0.54 (0–2)	≤ 0.001***
P, before vs. after	≤ 0.001***	0.277	
CS/HA (n = 42)	0.34 ± 0.32 (0–1)	0.80 ± 0.50 (0–2)	0.003**
P, before vs. after	≤ 0.001***	0.301	
ACS (n = 17)	0.64 ± 0.48 (0–1.5)	1.15 ± 0.58 (0.5–2)	0.096
P, before vs. after	0.750	0.688	
P, CS/HA vs. ACS	0.152	0.146	
ΔproMMP-9 grade[c.]			
overall	-0.55 ± 0.72 (-2.5–1)	-0.17 ± 0.75 (-2.5–1)	0.032*
CS/HA	-0.70 ± 0.70 (-2.5–0.5)	-0.15 ± 0.56 (-1.5–1)	0.013*
ACS	-0.07 ± 0.61 (-1–1)	-0.20 ± 1.06 (-2.5–1)	0.961
P, CS/HA vs. ACS	0.059	0.774	
Ldeg change category; CS/HA | ACS[a.]			
recovered	59% | 29%	15% | 10%	0.005** | 0.792
decreased	32% | 29%	40% | 30%	
unchanged	9% | 29%	45% | 40%	
increased	0% | 14%	0% | 20%	
Ldeg[b.]			
overall	0.97 ± 1.27 (0–4)	2.10 ± 1.12 (0–4)	= 0.001***
P, before vs. after	≤ 0.001***	= 0.001***	
CS/HA	0.68 ± 1.04 (0–4)	1.85 ± 1.09 (0–3)	0.002**
P, before vs. after	≤ 0.001***	≤ 0.001***	
ACS	1.86 ± 1.57 (0–4)	2.60 ± 1.07 (0–4)	0.378
P, before vs. after	0.188	0.438	
P, CS/HA vs. ACS	0.082	0.078	
ΔLdeg[c.]			
overall	-1.93 ± 1.39 (-4–1)	-0.67 ± 0.96 (-3–1)	≤ 0.001***
CS/HA	-2.18 ± 1.18 (-4–0)	-0.85 ± 0.93 (-3–0)	≤ 0.001***
ACS	-1.14 ± 1.77 (-4–1)	-0.30 ± 0.95 (-2–1)	0.406
P, CS/HA vs. ACS	0.139	0.209	

a. Relative number of horses (%). b.-c. Mean value ± standard deviation (SD) with range (min-max) (b.) in general and (c.) extent of change (**Δ**). Statistical significance denoted as ** very significant when $P \leq 0.01$ and *** highly significant when $P \leq 0.001$. | *a. Relative Anzahl der Pferde (%). b.-c. Mittelwert ± Standardabweichung (SD) mit Bereich (Min-Max) (b.) allgemein und (c.) Ausmaß der Änderung (**Δ**). Statistische Signifikanz angegeben als * signifikant wenn $P \leq 0,05$ und ** sehr signifikant wenn $P \leq 0,01$*

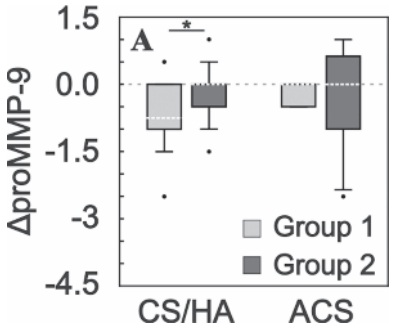
grp2: P = 0.774/P = 0.209) (Table 5). MMP and Ldeg correlated significantly in both pat-grp (r = 0.735, P ≤ 0.001/r = 0.397, P = 0.029) (Figure 3) and proMMP-9 correlated significantly with Ldeg after CS/HA treatment in grp1 (r = 0.686, P ≤ 0.001).

Considering the change in proMMP-9 and Ldeg after therapy simultaneously (Figure 4), most horses (59%) from grp1 showed recovered/reduced proMMP-9 and Ldeg, while most horses (50%) from grp2 showed unchanged/increased proMMP-9 and Ldeg. Noteworthy, no horse from both pat-grp had a recovered/reduced proMMP-9 with simultaneously unchanged/increased Ldeg, but a substantial amount of horses in both pat-grp (24%/20%) had an unchanged/increased proMMP-9 with simultaneously recovered/reduced Ldeg. After therapy, proMMP-9 was present despite recovered lameness in 17% of grp1 and 7% of grp2 (12% of pat-grp).

Discussion

Relationships between proMMP-9 and other parameters

ProMMP-9 was not found in control horses as expected (*Clegg* et al. 1997b, *Fietz* et al. 2008). This is due to the highly stable matrix of adult, healthy cartilage with its low level of matrix turnover (*van den Boom* et al. 2004) and the low participation of proMMP-9 in physiological metabolism in gadults (*Söder* et al. 2006). In contrast, almost all joints of grp1 and grp2 had increased proMMP-9 levels. It did not differ significantly between both pat-grp which was also found by *Fietz* et al. (2008) and suggests, that radiographically visible joint changes must not necessarily correlate with MMP-9



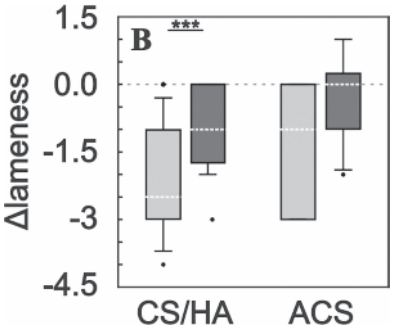
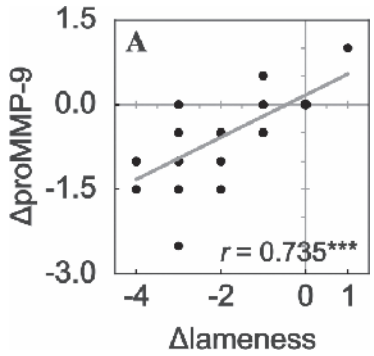


Fig. 2 Box plots with extent of change (Δ) in proMMP-9 grades and lameness degrees after therapy. Distribution of (A) ΔproMMP-9 and (B) Δlameness for CS/HA and ACS therapy. n: CS/HA: grp1 = 22, grp2 = 20; ACS: grp1 = 7, grp2 = 10.
*Boxplots mit Ausmaß der Änderung (Δ) von proMMP-9 Klassen und Lahmheitsgrade nach Therapie. Verteilung von (A) ΔproMMP-9 und (B) Δ*Lahmheit *für CS/HA- und ACS-Therapie. n: CS/HA: grp1 = 22, grp2 = 20; ACS: grp1 = 7, grp2 = 10*



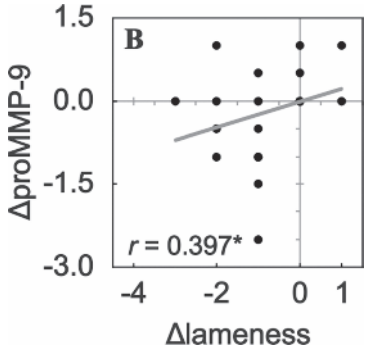


Fig. 3 XY scatter plots with correlation between extent of change (Δ) in proMMP-9 and lameness degrees after therapy. Correlation between Δ proMMP-9 grade and Δlameness for (A) grp1 and (B) grp2 including both therapies with linear trend line and correlation coefficient r. ngrp1 = 29, grp2 = 30.
*XY-Punktdiagramme mit Korrelationen zwischen Ausmaß der Änderung (Δ) von proMMP-9 und Lahmheitsgrade nach Therapie. Korrelation zwischen Δ proMMP-9 und Δ*Lahmheit *für (A) grp1 und (B) grp2 beide Therapien umfassend mit linearer Trendlinie und Korrelationskoeffizient r. n: grp1 = 29, grp2 = 30*

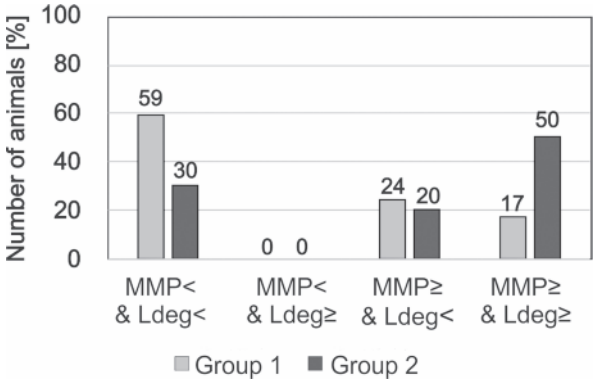


Fig. 4 Bar chart on the simultanous changes in proMMP-9 grade and Lameness degree after therapy. Relative number of horses with recovered/reduced (<) or unchanged/increased (≥) proMMP-9 grade (MMP) and Lameness degree (Ldeg). n: grp1 = 29, grp2 = 30.
Balkendiagramm über die simultane Änderung von proMMP-9-Klasse und Lahmheitsgrad nach Therapie. Relative Anzahl der Pferde (%) mit erholten/reduzierten (<) oder unveränderten/erhöhten (≥) proMMP-9-Klassen (MMP) und Lahmheitsgraden (Ldeg). n: grp1 = 29, grp2 = 30

activity in SF. Visible cartilage defects may be results of past morphological reconstruction processes with their underlying inflammation resolved (*Wenham* and *Conaghan* 2010, *de Grauw* 2011) and for this reason, MMP-9 levels may occur lower or depleted. But, inconspicuous radiographic findings cannot exclude the presence of massive cartilage damage, as it was partly seen in grp1, and thus do not exclude the presence of OA (*Lohmander* et al. 1992).

Despite radiographically unsuspicious joints in grp1, there were low- to high-grade cartilage changes as well as acute to chronic changes in SM and joints with subacute and chronic SM findings showed significantly higher proMMP-9. But, unlike other studies (*Jouglin* et al. 2000, *van den Boom* et al. 2005), no positive correlation was found between proMMP-9 and extent of cartilage damage. Rather a negative correlation was observed of decreasing proMMP-9 with increasing cartilage damage. Possible explanation could be, that cells responsible for secretion of catabolic mediators are unable or too few in number to fulfil this task properly due to the extent of cartilage erosion.

A significantly increased PC, as it was seen in both pat-grp, is considered as a sign for an inflammatory joint disease (*Trotter* and McIlwraith 1996) without providing a specific diagnosis (*McIlwraith* 1989b). The significant positive correlation between proMMP-9, PC, and LC underpins the theory that an increase in proMMP-9 occurs mainly through infiltrating neutrophil granulocytes during periods of advanced inflammatory processes (*Clegg* et al. 1997a, *Trumble* et al. 2001, *Fietz* et al. 2008). There are direct effects of inflammation mediators and enzymes on cartilage turnover and thus on the level of cartilage biomarkers in SF (*Poole* 1999, *Garnero* and *Delmas* 2003). In addition to the cumulative damage to the articular cartilage, local inflammation is one of the most important factors influencing the level of cartilage turnover markers in SF (*de Grauw* et al. 2009). Moreover, an increased volume of SF for example due to physical activity, acute joint inflammation, or other joint injuries (*Trotter* and *McIlwraith* 1996), may cause dilution effects of biological markers in SF (*Kraus* et al. 2002). These findings and differing values of proMMP-9 in patients most probably reflect different severities of joint inflammation.

Viscosity of SF is directly dependent on the hyaluronan content and is considered to be a measure for quantity, quality and degree of polymerization of HA (*McIlwraith* 1989b). In DJD, SF is often described as highly viscous, while in other inflammatory articular conditions, the viscosity is mostly expected to be decreased (*Rahn* 1999). The tendency of increasing proMMP-9 with decreasing viscosity of SF, as found in this study for both pat grp, as well as the significantly higher proMMP-9 in aqueous SF in grp2, suggests a correlation between proMMP-9 and inflammatory processes such as synovitis in the joint.

Impact of intra-articular therapy on proMMP-9 and lameness
In contrast to ACS, treatment with CS/HA significantly improved lameness in all patients. proMMP-9 only improved significantly in horses without radiographically visible joint changes after CS/HA injection. Therapy with CS and HA has shown to be effective in improving joint function and lessening pain caused by DJD, but in advanced stages of the disease with already existing joint damage it is considered insufficient (*Nizolek* and *White* 1981). An inhibitory effect of CS/HA on the production of proMMP-9 may be rejected at least for betamethasone and HA (*Clegg* et al. 1998). The poorer response to CS/HA therapy shown by the lack of significant improvement of proMMP-9 in grp2 may be attributable to the presumably greater impairment and more massive degenerative cartilage changes observed, and thus to the possible reduction of the response to conservative therapy.

Barnewitz et al. (2015) reports about the effect of treatment of acute traumatically induced joint defects with the autologous products platelet rich plasma (PRP) and mesenchymal stem cells. By recording proMMP-9 level over a period of seven weeks after artificial creation of the defect, increased proMMP-9 values of different degrees were detected over a period of at least four weeks. *Barnewitz* et al. (2015) concluded that differences in the proMMP-9 level as well as the duration of increased proMMP-9 presence may be interpreted as indications for different degrees of osteoarthritic processes in joints.

According to *Fietz* et al. (2008), MMP-9 is a particularly suitable biomarker in pathological articular changes such as septic arthritis caused by pathogens, while MMP-2 may be a suitable marker in both septic and non-septic joint diseases, for example OA and chronic arthritis. The results of this study are, however, in contrast to this but in agreement with other studies (*Clegg* et al. 1997b, *Jouglin* et al. 2000, *Trumble* et al. 2001), which also showed a significant increase of proMMP-9 in horses with non-septic joint diseases.

In DJD therapy, analysis of proMMP-9 in SF may help choosing the appropriate treatment strategy, determining the duration of treatment, and making orientating statements on the efficiency of the therapy. The presence of proMMP-9 in SF despite physiological PC, LC, and viscosity, as well as despite improvement of lameness after intra-articular treatment, signals that there might be a need for further resting, adaption of the motion management and treatment.

Conclusions

The present study confirms earlier studies showing MMP-9 is involved in cartilage degradation and can be seen as an indicator for catabolic processes in joint diseases. The findings confirm that proMMP-9 in SF is rather an indicator for the current status of a joint concerning pathological processes, than of the overall extent of cartilage damage that has accumulated over time (*van den Boom* et al. 2005).

In the light of these results, only 10%, 40% and 63% of joints were correctly identified as pathological based on analysis of PC, LC, and viscosity, respectively, and 12% of all patients would have been deemed cured due to recovered lameness. But proMMP-9 activity indicated that pathological conditions were persistent. The standard examination of SF and lameness is often limited by the circumstance that it sometimes cannot distinguish between physiological conditions (*Trotter* and *McIlwraith* 1996, *Mahaffey* 2002). Therefore, and due to the lack of MMP-9 in physiological SF, the identification of pathological joint states is more precise by analysis of MMP-9 than by PC, LC, viscosity, or lameness. Analysis of MMP-9 in

SF helps defining a targeted treatment strategy of DJD and monitoring disease progress as well as therapy response. Lameness and SF parameters such as PC, LC, and viscosity seem less reliable indicators in DJD in horses compared to proMMP-9.

In summary, the results of this study confirm that proMMP-9 analysis in SF of equine joints is a suitable diagnostic tool with great clinical value for DJD in horses. ProMMP-9 may be used as independent SF biomarker both in horses with septic and non-septic joint diseases. This study demonstrates that proMMP-9 can be used as biomarker for OA which provides information on joint status, disease progression, and response to therapy. Moreover, proMMP-9 may be considered a BIPED biomarker (*Bauer* et al. 2006), corresponding to all five marker categories: Burden of disease, Investigative, Prognostic, Efficacy of intervention, and Diagnostic.

Animal welfare statement

The study was carried out as retrospective study analysing clinical data from the years 2008 to 2011 preserved in horse clinics and did not involve animal experiments. Horses were neither (randomly) assigned to a specific group, nor chosen from a specific cohort, and no parameters were pre-defined. All examinations were made with owners' consent and complied with the current laws for veterinary clinics (Bundes-Tierärzteordnung, BTÄO) of the federal ministry for justice and consumer protection (Bundesministerium für Justiz und Verbraucherschutz, BMJV) of Germany.

Conflict of interest statemant

All authors have declared no competing interests.

Acknowledgement

We thank Fritz Seidl, M.A. Interpreting and Translating, for twofold proofreading and language editing the manuscript, and Stephan Henze (fzmb GmbH), Peter Föhr (Technical University of Munich), as well as Thomas Reuter (Institut Chemnitzer Maschinen- und Anlagenbau e.V.) for their statistical expertise.

References

Abramson S., Krasnokutsky S. (2006) "Biomarkers in Osteoarthritis." B NYU Hosp. Joint. Dis. 64, 77-81

Anon (1991) AAEP lameness scale. Definition and classification of lameness. Guide for veterinary service and judging of equestrian events. Lexington, American Association of Equine Pract.19

Barnewitz D., Karakine E., Richter I.-G., Lerchbacher J. (2015) "Zur Bedeutung der Matrix-Metalloproteinase (MMP)-9 bei der Lahmheitsdiagnostik." Prakt. Tierarzt. 96, 1124-1131

Bauer D. C., Hunter D. J., Abramson S. B., Attur M., Corr M., Felson D., Heinegård D., Jordan J. M., Kepler T. B., Lane N. E., Saxne T., Tyree B., Kraus V. B. (2006) "Classification of osteoarthritis biomarkers: a proposed approach." Osteoarth. Cartil. 14, 723-727

Birkedal-Hansen H., Moore W. G. I., Bodden M. K., Windsor L. J., Birkedal-Hansen B., Decarlo A., Engler J. A. (1993) "Matrix metalloproteinases; A Review." Crit. Rev. Oral Biol. Med. 4, 197-250

Clegg P. D., Burke R. M., Coughlan A. R., Riggs C. M., Carter S. D. (1997a) "Characterisation of equine matrix metalloproteinase 2 & 9 and identification of the cellular sources of these enzymes in joints." Equine Vet. J. 29, 335-342

Clegg P. D., Coughlan A. R., Riggs C. M., Carter S. D. (1997b) "Matrix metalloproteinases 2 & 9 in equine synovial fluid." Equine Vet. J. 29, 343-348.

Clegg P. D., Jones M. D., Carter S. D. (1998) "The effect of drugs commonly used in the treatment of equine articular disorders on the activity of equine matrix metalloproteinase-2 and 9." J. Vet. Pharmacol. Therap. 21, 406-4013

Clegg P. D., Carter S. D. (1999) "Matrix metalloproteinase-2 and -9 are activated in joint diseases." Equine Vet. J. 31, 324-330

Clutterbuck A. L., Harris P., Allaway D., Mobasheri A. (2010)"Matrix metalloproteinases in inflammatory pathologies of the horse." Vet. J. 183, 27-38

de Grauw J. C., van de Lest C. H. A., van Weeren P. R. (2009) "Inflammatory mediators and cartilage biomarkers in synovial fluid after a single inflammatory insult: a longitudinal experimental study." Arthritis Res. Ther. 11, R35

de Grauw J. C. (2011) "Molecular monitoring of equine joint homeostasis." Vet. Quart. 31, 77-86

Fietz S., Einspanier R., Hoppner S., Hertsch B., Bondzio A. (2008) "Determination of MMP-2 and 9 activities in synovial fluid of horses with osteoarthritic and arthritic joint diseases using gelatine zymography and immunocapture activity assays." Equine Vet. J. 40, 266-271

Garnero P., Delmas P. D. (2003) "Biomarkers in osteoarthritis." Curr. Opin. Rheumatol. 15, 641-646

Jouglin M., Robert C., Calette J.-P., Gavard F., Quintin-Colonna F., Denoix J.-M. (2000) "Metalloproteinases and tumor necrosis factor-alpha activities in synovial fluids of horses: correlation with articular cartilage alterations." Vet. Res. 31, 507-515

Kidd J. A., Fuller C., Barr A. R. S. (2001) "Osteoarthritis in the horse." Equine Vet. Educ. 13, 160-168

Kraus V. B., Huebner J. L., Fink C., King J. B., Brown S., Vail T. P., Guilak F. (2002) "Urea as a passive transport marker for arthritis biomarker studies." Arthritis Rheum. 46, 420-427

Lohmander L. S., Lark M. W., Dahlberg L., Walakovis L. A., Roos H. (1992) "Cartilage matrix metabolism in osteoarthritis: markers in synovial fluid, serum, and urine." Clin. Biochem. 25, 167-174

Mahaffey E. A. (2002) Synovial fluid. Diagnostic cytology and hematology of the horse. R. L. Cowell, R. D. Tyler. St. Louis, Mosby, 163-170

McIlwraith C. W. (1989a) Erkrankungen der Gelenke, Sehnen, Bänder sowie ihrer Hilfseinrichtungen. Adam's Lahmheit bei Pferden. T. S. Stashak. Alfeld Hannover, M. & H. Schaper, 339-485

McIlwraith C. W. (1989b) "Intraarticular medication for traumatic joint problems: do we understand the choices?" Comp. Cont. Educ. Prac. Vet. 11, 1287-1290, 1311

McIlwraith C. W. (1996) General pathobiology of the joint in response to injury. Joint disease in the horse. C. W. McIlwraith and G. W. Totter. Philadelphia, London, W.B. Saunders, 40-70

McIlwraith C. W. (2005) "Use of synovial fluid and serum biomarkers in equine bone and joint disease." Equine Vet. J. 37, 473-482

McIlwraith C. W., Frisbie D. D., Kawcak C. E., Fuller C. J., Hurtig M., Cruz A. (2010) "The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the horse." Osteoarth. Cartil. 18, 93-105

Mohtai M., Smith R. L., Schurman D. J., Tsuji Y., Torti F. M., Hutchinson N. I., Stetler-Stevenson W. G., Goldberg G. I. (1993) "Expression of 92-kD type IV collagenase/gelatinase (gelatinase B9) in osteoarthritic cartilage and its induction in normal human articular cartilage by interleukin-1." J. Clin. Invest. 92, 179-185

Murphy G., Knäuper V., Atkinson S., Butler G., English W., Hutton M., Stracke J., Clark I. (2002) "Matrix metalloproteinases in arthritic disease." Arthritis Res. 4 (Suppl. 3), 39-49

Nizolek D. J. H., White K. K. (1981) Corticosteroid and hyaluronic acid treatments in equine degenerative joint disease - A review. The Cornell Veterinarian. L. Krook. Ithaca, N.Y. 14853, Cornell Veterinarian, INC. 71, 355-375

Poole A. R. (1999) "An introduction to the pathophysiology of osteoarthritis." Front. Biosci. 4, D662-D670

Rahn B. A. (1999) Fracture biology, mechanics and healing. Equine surgery. J. A. Auer, J. A. Stick. Philadelphia, W. B. Saunders Company, 629-634

Rorvik A. M., Grondahl A. M. (1995) "Markers of osteoarthritis: A review of the literature." Vet. Surg. 24, 255-262

Söder S., Roach H. I., Oehler S., Bau B., Haag J., Aigner T. (2006) "MMP-9/gelatinase B is a gene product of human adult articular chondrocytes and increased in osteoarthritic cartilage." Clin. Exp. Rheumatol. 24, 302-304

Trotter G. W., McIlwraith C. W. (1996) Clinical features and diagnosis of equine joint disease. Joint disease in the horse. C. W. McIlwraith, G. W. Trotter. Philadelphia, W.B. Saunders, 120-145

Trumble T. N., Trotter G. W., Osford J. R., McIlwraith C. W., Cammarata S., Goodnight J. L., Billinghurst R. C., Frisbie D. D. (2001) "Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses." Am. J. Vet. Res. 62, 1467-1477

van den Boom R., Brama P. A., Kiers G. H., DeGroot J., Barneveld A., van Weeren R. R. (2004) "The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor activities in normal equine joints." Equine Vet. J. 36, 155-159

van den Boom R., van der Harst M. R., Brommer H., Brama P. A. J., Barneveld A., van Weeren P. R., de Groot J. (2005) "Relationship between synovial fluid levels of glycosaminoglycans, hydroxyproline and general MMP activity and the presence and severity of articular cartilage change on the proximal articular surface of P1" Equine Vet. J .37, 19-25; DOI 10.2746/0425164054406919

Wenham C. Y., Conaghan P. G. (2010) "The role of synovitis in osteoarthritis." Therap. Adv. Musculoscel. Dis. 2, 349-359