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Biomarkers of joint tissue metabolism in canine osteoarthritic and arthritic joint disorders

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Summary

Objective: To explore the levels of matrix metalloprotease-3 (MMP-3), tissue inhibitor of metalloproteases-1 (TIMP-1), 5D4 keratan sulfate, and two 3B3 chondroitin-sulfate epitopes in several canine osteoarthritic and inflammatory arthropathies.

Methods: Blood and synovial fluid were obtained from 103 dogs with rupture of the anterior cruciate ligament (ACLR), osteochondritis dissecans (OCD), fragmented coronoid process (FPC), patella luxation (PL), hip dysplasia (HD) or infectious arthritis. Dogs with non-musculoskeletal disorders were used as controls. The biomarkers were measured by immunoassays.

Results: Median levels of synovial MMP-3, TIMP-1 and molar ratios of MMP/TIMP-1 were significantly higher in the arthritis than in the control group. The release of 5D4 keratan sulfate epitope and serum 3B3 neoepitope was reduced in arthritis patients. Increases in synovial TIMP-1 in OA were less pronounced and the molar ratio of MMP-3/TIMP-1 remained far below 1.0, demonstrating a surplus of the protease inhibitor. In osteoarthritic patients median levels of synovial 5D4 keratan sulfate were up-regulated after ACLR and PL and were inversely correlated with increasing duration of lameness. Serum TIMP-1 levels were significantly reduced in the joint disorder group when compared with the control group.

Conclusion: Our observations present the TIMP-1 serum level as a potential marker for the detection of degenerative changes in cartilage and also indicate that in canine OA, the MMP-3 mediated matrix destruction is not of major importance. However MMP-3 seems to be a sensitive marker for the local inflammation in canine arthritis. © 2002 Osteoarthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: Osteoarthritis, Canine, Biomarkers, MMP-3.

Introduction

Osteoarthritis (OA) is a slow progressive disorder of synovial joints that affects about 20% of the canine population over 1 year of age. This joint disorder is characterized by a loss of balance between synthesis and degeneration of the articular cartilage constituents leading to subsequent erosion of joint cartilage, remodeling of the underlying bone, osteophyte formation and variable degrees of synovitis¹.

The most common causes of secondary OA seen in small animal practice are anterior cruciate ligament rupture (ACLR), osteochondritis dissecans (OCD), fragmented coronoid process (FPC) and hip dysplasia (HD). The majority of ACLRs are a result of chronic degeneration of the ligaments themselves caused by repeated stresses or by immune mediated synovitis. Experimental transection of the ACL (ACLT) in dogs results in similar pathological changes in the cartilage and serves as a model for the studies of OA. OCD is an enchondral ossification abnormality leading to formation of cartilage flaps in the shoulder,

elbow, stifle or tarsal joint. FPC is caused by asynchronous growth of radius and ulna and HD is characterized by joint laxity of the coxofemoral joint^{2,3}.

Early diagnosis of OA is a major problem both in veterinary and human medicine because the diagnosis is routinely established on the basis of the clinical and radiographic changes that occur only in the later stages of disease. Therefore biochemical markers of joint disease are of considerable interest as an expedient to distinguish pre-clinical diseases, to predict prognosis and to monitor the response to drug and surgical therapy.

This approach employs measurement of serum or synovial fluid levels of articular cartilage macromolecules, such as proteoglycans or glycosaminoglycans, and matrix-degrading enzymes.

The 5D4 keratan sulfate epitope has been widely used in studies of dogs with ACLR^{4,5} or the corresponding experimental OA model^{6–9}. Some other proposed markers of altered cartilage metabolism in OA are the two 3B3 epitopes on the chondroitin sulfate chains^{10,11}. Levels of these markers were shown to be elevated in osteoarthritic knee joints of dogs^{12,13}.

The increased breakdown of the extracellular cartilage matrix is considered to be catalysed by the matrix metalloproteases (MMPs) and the aggrecanases, members of the ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs) family¹⁴. The activities of MMPs are strictly regulated by endogenous tissue inhibitors of

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Table I
Data of the study population

Statistic	Diagnose							
	Control* (N=30)	ACLR (N=54)	FPC (N=14)	OCD (N=10)	PL (N=9)	HD (N=9)	Arthritis (N=7)	
Patient age	Median	6	5	1	1	7	4	4
	Range	<1–13	1–14	<1–6	<1–2	2–12	1–9	<1–10
Bodyweight (kg)	Median	22	34	41	34	30	38	34
	Range	7–70	8–73	22–60	25–62	10–62	30–55	30–40
Sex	Male	20	22	9	7	6	5	4
	Female	10	32	5	3	3	4	3

*Synovial fluid available for only four dogs (30 in total).

metalloproteases (TIMPs), which form inhibitory complexes with MMPs in a 1:1 stoichiometry¹⁵. Several studies have suggested that there may be a relative deficit of TIMPs in comparison to MMPs in the osteoarthritic joint^{16–18}. MMP-3 or stromelysin-1 is capable of degrading proteoglycan, laminin, fibronectin, or non-helical collagen, and is essential for the activation of other proMMPs¹⁹. Elevated levels of MMP-3 and TIMP-1 were demonstrated in cartilage and synovial fluid of human patients having OA or rheumatoid arthritis (RA)^{20,21}, as well as in studies of experimental canine OA^{22–24}.

The aim of this study was to explore the potential of a combination of biochemical markers for early diagnosis of canine OA. For the first time MMP-3, TIMP-1, 5D4 keratan sulfate epitope, 3B3 (–) and (+) chondroitin-sulfate epitope levels were measured together in the body fluids of dogs with spontaneous ACLR, FPC, OCD, HD, PL, infectious arthritis and in a control population by different ELISA systems. This approach allows to define reference ranges and to investigate the possible relationships between different biochemical markers and the duration of lameness following ACLR and the various diseases.

Materials and methods

SAMPLES

Joint disorder group

Dogs referred to the Clinic for Small Animals of the Free University of Berlin for treatment of ACLR, OCD, FPC, PL, HD or arthritis were studied. The arthritis group includes dogs with acute infectious (poly-) arthritis of the knees, carpi and tarsi caused by different bacteria or viruses. The diagnosis was based on clinical and radiological examination. Patient age, sex, and body weight and duration of clinical signs after ACLR were recorded (Table I). During surgery, synovial fluid from the affected knee and serum were collected.

Control group

Serum of dogs (N=26) presented at the clinic for non-musculoskeletal diseases were used as control samples. Lack of gross signs of OA was confirmed by visual inspection. Synovial fluid samples (N=4) were obtained from mature dogs that were euthanatized for non-orthopaedic reasons. In these dogs lack of signs of OA were confirmed by radiological examination of the synovial joints.

Sample processing

Serum and synovial fluid samples were centrifuged by 6500 × g for 10 minutes. Supernatants were stored at –80°C until assayed. The minimum sample volume for all determinations was 500 µl synovial fluid and 1.5 ml serum.

TIMP-1 ASSAY

The ELISA based on previously described protocols for human TIMP^{25,26}. ELISA plates were coated with 50 ng/well of an antidog TIMP-1 monoclonal antibody. Serum samples were diluted 1:10 and synovial fluid 1:100. Standards of recombinant canine TIMP-1 were prepared in a concentration range of 27.5–0.2 ng/ml and a rabbit anti-human TIMP-1 polyclonal antibody (Biotrend) was used at dilution of 1:1200. Incubation with a peroxidase conjugated goat antirabbit IgG (1:4000, Sigma) was followed and TMB (3,3', 5,5'-tetramethyl-benzidine) was added as peroxidase substrate.

STROMELYSIN-1 (MMP-3) ASSAY

An ELISA similar to the one described by Lark *et al.*²² and Walakovits *et al.*²⁷ was used. A mouse antidog-stromelysin-1 monoclonal antibody served as a trapping agent in a concentration of 100 ng/ml. Synovial samples were diluted 1:8 in saline. The second antibody, an antidog stromelysin-1 polyclonal antibody was added in a dilution of 1:6000. Recombinant canine stromelysin was used as standard in a concentration range of 63.0–0.49 ng/ml.

5D4 KERATAN SULFATE ASSAY

This ELISA was performed as described earlier²⁸.

3B3 CHONDROITIN SULFATE ASSAYS

The 3B3 epitopes were measured with an ELISA as described^{29,30} with following variation. CS standards were prepared from digested aggrecan (aggrecan monomer from bovine articular cartilage, Sigma) in the concentration range of 250–0.98 ng/ml. The fluids were considered to have a concentration of less than 10 ng/ml if the levels of chondroitin-sulfate were below the detection limit of the assay even at 1:5 dilutions.

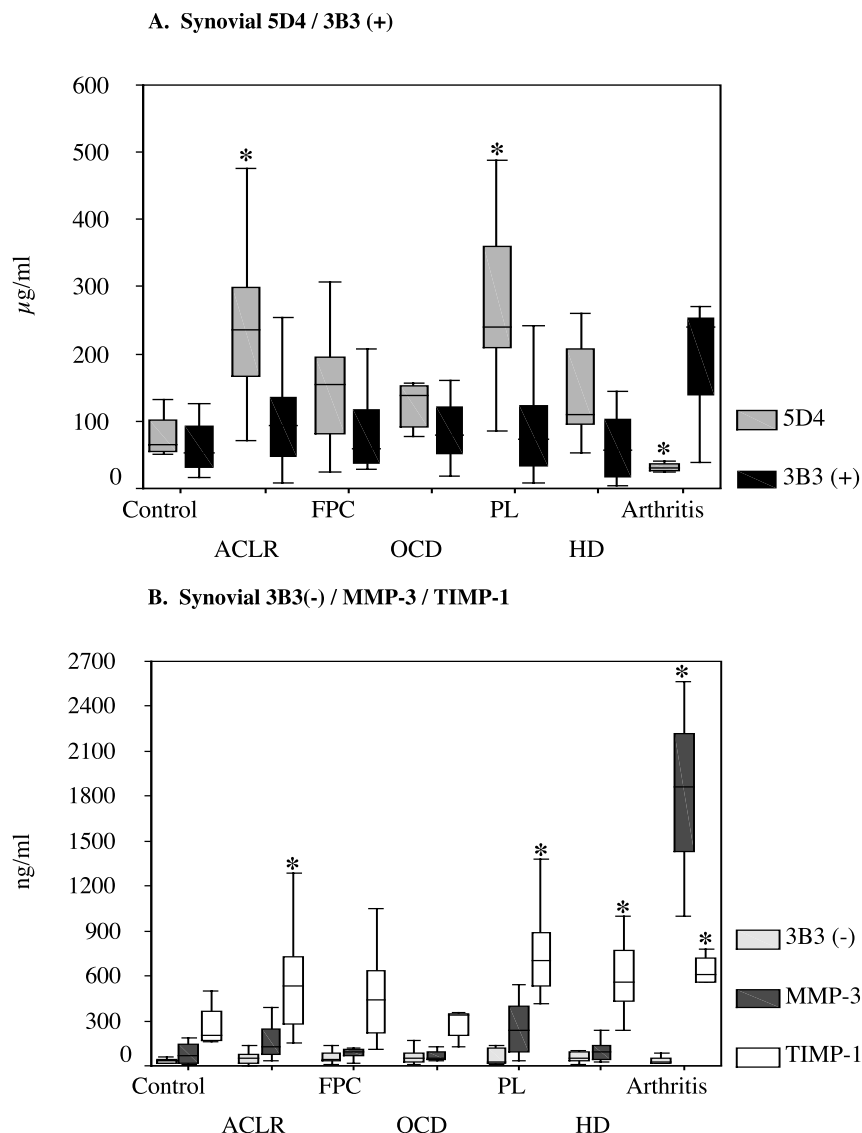


Fig. 1. Synovial concentrations of the biomarkers. Comparison of the values obtained in control dogs ($N=4$), dogs with osteoarthritic disorders [ACLR ($N=54$), FPC ($N=14$), OCD ($N=10$), HD ($N=9$) and PL ($N=9$)] and dogs with arthritis ($N=7$). Boxes represent the interquartile range, i.e. the middle 50% of the data, between the 25th and 75th quartile. Whiskers represent the largest and smallest observed scores that are less than 1.5 box lengths from the end of the box. (a) 5D4 keratan sulfate and 3B3(+) chondroitin-sulfate epitopes. (b) 3B3(-) chondroitin-sulfate epitope, MMP-3 and TIMP-1. *Statistically significant difference ($P<0.05$) relative to the median of the control animals.

STATISTICAL ANALYSIS

Quantitation was achieved by reference to a standard curve, using the cubic-spline method (BioTek Instruments). The concentrations of the marker substances in the samples were determined by using the absorbance values falling at the 40–50% inhibition of the standard curve. The ELISA data were analysed using the statistical program SPSS 10.0.

The Mann–Whitney U-test was used for comparison of different biomarkers. Spearman's rank correlation coefficient was used to compare the serum and synovial fluid values and also the biomarkers and the duration of clinical signs of ACLR. P -values less than 0.05 were considered significant.

Results

The concentrations of 5D4 keratan sulfate, 3B3(-) and 3B3(+) chondroitin-sulfate, MMP-3 and TIMP-1 in the synovial fluid and serum of control dogs and dogs suffering from different osteoarthritic or inflammatory arthropathies were measured. Figure 1 shows the comparison of the synovial biomarker concentrations and Fig. 2 those of the serum concentrations. The OA group is divided by primary aetiology. Statistically significant differences of the concentrations were calculated vs the corresponding control values.

In the analysed samples a moderate correlation became apparent between age and the synovial biomarkers 5D4 ($r=0.519$), and MMP-3 ($r=0.388$). There were no

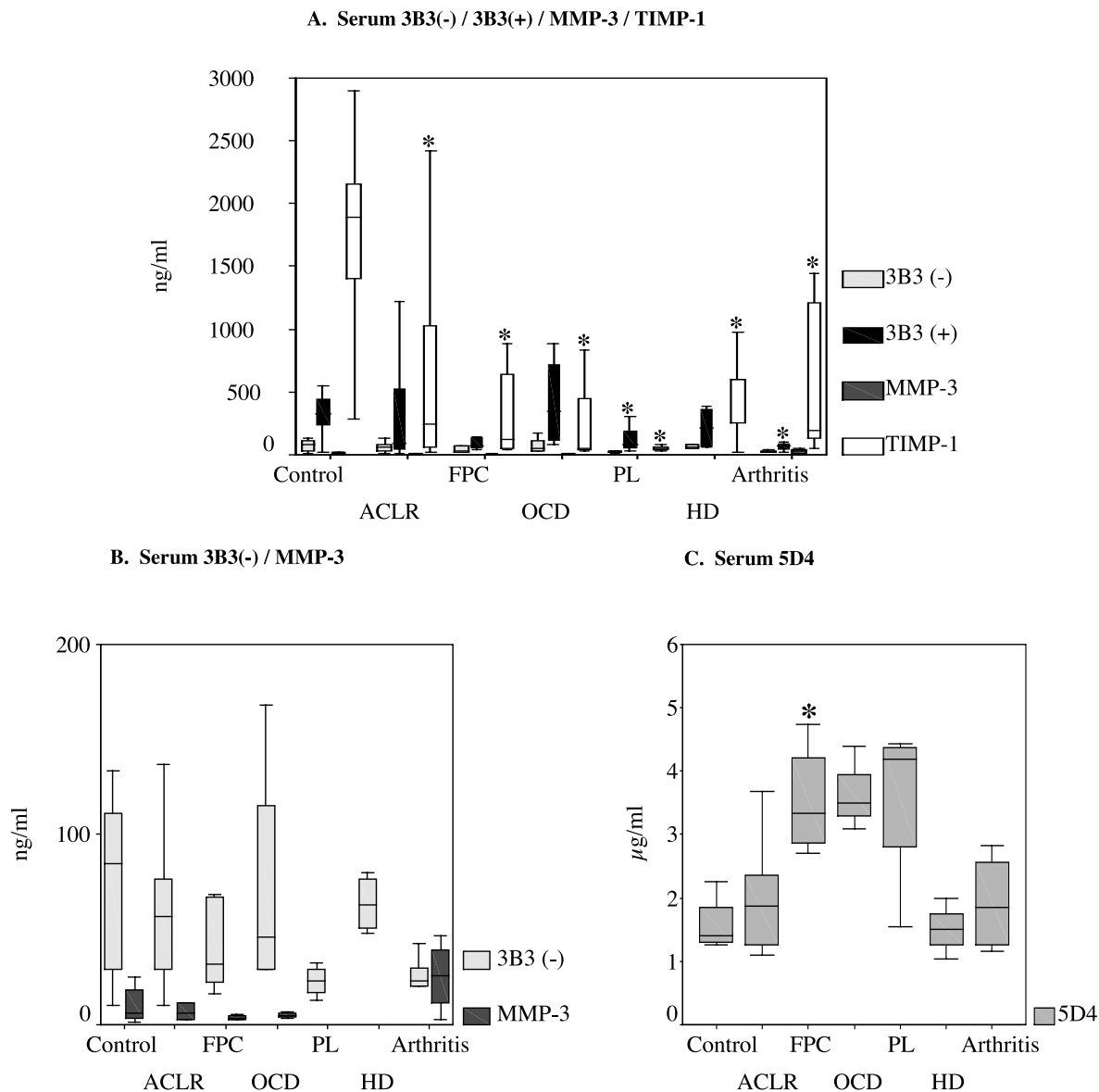


Fig. 2. Serum concentrations of the biomarkers. Comparison of the values obtained in control dogs ($N=20$), dogs with osteoarthritic disorders [ACLR ($N=54$), FPC ($N=14$), OCD ($N=10$), HD ($N=9$) and PL ($N=9$)] and dogs with arthritis ($N=7$). Boxes represent the interquartile range, i.e. the middle 50% of the data, between the 25th and 75th quartile. Whiskers represent the largest and smallest observed scores that are less than 1.5 box lengths from the end of the box. (a) 3B3 (-) and 3B3 (+) chondroitin sulfate epitopes, MMP-3 and TIMP-1. (b) Enlarged view of (a) (in the range 0 to 200 ng/ml). 3B3 (-) chondroitin-sulfate epitope and MMP-3. (c) 5D4 keratan sulfate. *Statistically significant difference ($P<0.05$) relative to the median of the control animals.

differences in the levels of the biomarkers between females and males. The TIMP-1, 5D4 and 3B3(-) levels in serum were not significantly correlated with those in the synovial fluid. In contrast MMP-3 ($r=0.567$, $P<0.05$) and 3B3(+)
($r=-0.594$, $P<0.05$) serum levels correlated with the corresponding synovial fluid levels.

ACLR-GROUP ($N=54$)

Synovial 5D4 and TIMP-1 were markedly increased in ACLR compared with the control patients. In contrast, serum TIMP-1 values were down-regulated ($P<0.05$). The highest 5D4 synovial fluid values (max. 1800 $\mu\text{g/ml}$) were observed in the first weeks after injury of the ACL, then

dropping to levels of about 100 $\mu\text{g/ml}$. Synovial fluid 5D4 ($r=-836$, $P<0.05$), 3B3(+)
($r=-536$, $P<0.05$), MMP-3 ($r=-288$, $P<0.05$), TIMP-1 ($r=-254$, $P<0.05$) are inversely correlated with the duration of clinical signs (Fig. 3).

However the composition of the synovial fluid altered after ACLR, reflected by a statistically significant decline of the 5D4/3B3(+)
ratio (-0.253 , $P<0.05$) and increase of the 3B3(-)/3B3(+)
ratio ($r=0.355$, $P<0.05$).

A strong correlation between the synovial MMP-3 and TIMP-1 values was not obvious ($r=0.183$, $P<0.05$). However, active MMP-3 is inhibited by stoichiometric amounts of TIMP-1. Assuming that canine MMP-3 has a molecular weight approximately 2.3 fold of canine TIMP-1 (M_r : 56 kDa; M_r : 23 kDa), a line showing where TIMP is

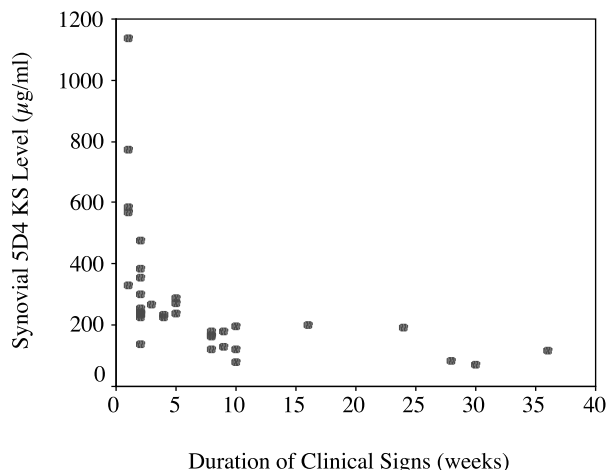


Fig. 3. Inverse correlation between the duration of clinical signs after ACLR and the synovial fluid 5D4 keratan sulfate levels. Each point represents one sample ($N=32$). The correlation was calculated using the Spearman's rank correlation coefficient ($r=-0.836$ and $P=0.005$).

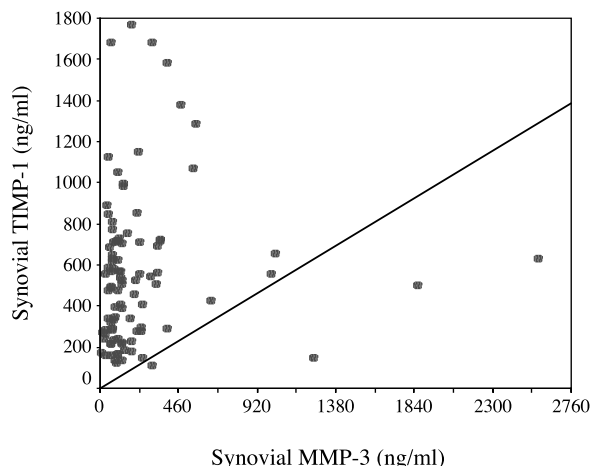


Fig. 4. Molar MMP-3/TIMP-1 ratio below 1.0 in the synovial fluid of dogs with osteoarthritic and inflammatory disorders. This figure shows the synovial MMP-3 levels vs the synovial TIMP-1 levels. The line represents the concentration where the enzyme and its inhibitor exist in equimolar proportions.

equimolar to MMP-3 could be drawn (see Fig. 4). This demonstrated that in molar terms all patients with ACLR had levels of TIMP-1 exceeding those of MMP-3. The molar ratio of MMP-3/TIMP-1 remained under concentrations of 1.0 during the first 48 weeks after injury of the cruciate ligament, too.

FPC-GROUP ($N=14$)

Concentrations of synovial biomarkers did not differ markedly from the control values but dogs with FPC had significant higher serum 5D4 and 10 fold lower serum TIMP-1 levels than the control animals ($P<0.05$).

OCD-GROUP ($N=10$)

The release of serum TIMP-1 was reduced about 37 times in the OCD vs the control group ($P<0.05$).

PATELLA LUXATION-GROUP ($N=9$)

Serum of dogs with patella luxation contained about 40 times less TIMP-1 than the corresponding control samples. Serum 3B3(+) was reduced too. In contrast, synovial 5D4 and TIMP-1 were up-regulated ($P<0.05$).

HIP DYSPLASIA-GROUP ($N=9$)

Synovial TIMP-1 levels were markedly increased ($P<0.05$), but in contrast the corresponding serum levels were down-regulated, demonstrating the missing correlation of these values.

ARTHRITIS-GROUP ($N=7$)

We also analysed serum and synovial fluid of seven dogs with diverse infectious joint disorders. Synovial fluid contained about 27 times more MMP-3 than the control samples. Increases in TIMP-1 were less pronounced. The molar ratios of MMP-3/TIMP-1 were also significantly up-regulated over 1.0 ($P<0.05$), indicating a surplus of the catabolic enzyme. Increases in serum MMP-3 were less pronounced, but the serum levels correlated with the corresponding synovial fluid levels ($r=0.567$, $P<0.05$). TIMP-1 serum levels were markedly down regulated. The release of the synovial 5D4 keratan sulfate epitope and serum 3B3 neopeptide was significantly reduced in these arthritis patients, too.

In summary, the median serum TIMP concentration was significantly reduced in the entire joint disorder group compared with the reference group of dogs affected by non-orthopaedic disorders ($P<0.001$). Concentrations of synovial MMP-3 were low both in the control and in the entire OA group. The arthritis group showed significantly increased concentrations of synovial MMP-3 compared to the OA ($P<0.005$) or to the control group ($P<0.05$).

Discussion

Cartilage destruction is a major characteristic of OA and is promoted by multiple matrix degrading enzymes, including the MMPs and aggrecanases. MMP-3 has been implicated as one key enzyme in the pathogenesis of OA, because it is able to damage proteoglycans and collagen and is crucial for activation of other MMPs.

This is the first study to characterize the stromelysin-1 (MMP-3) and TIMP-1 levels in dogs with naturally occurring OA and infectious arthritis. We could not detect any increase at all in synovial MMP-3 in dogs with various osteoarthritic joint disorders, whereas synovial TIMP-1 levels were increased in these dogs, demonstrating a molar excess of TIMP-1 over MMP-3 in the synovial fluid. Recently similar observations had been made in naturally occurring equine OA³¹.

Therefore, our data supports the hypothesis that the matrix degeneration in osteoarthritic joints could be promoted by other proteases like the recently identified aggrecanase-1 and -2¹⁴. Alternatively, the mild synovitis seen in canine OA may increase the clearance of the proteins from the canine joint and decrease the synovial fluid concentrations of the determined substances³².

Our observations vary from those obtained in a study of human OA induced by ACLR in which an elevation of synovial MMP-3 and a molar excess of MMP-3 over

TIMP-1 was recorded after the ligament rupture²⁰. In contrast, canine cartilage was shown to become hypertrophic for the first two years after injury of the ACL³³. Thus, assays of the above markers in dogs during the first two years after injury will not detect any loss of cartilage (e.g., imbalance of MMPs over inhibitors) as found in human OA.

At present there are only few other studies on canine MMP-3 or TIMP-1. In dogs with OA induced by tibial valgus osteotomy a moderate rise of synovial MMP-3 and TIMP-1 was found, but the molar ratio was not altered dramatically²³. The mild histological changes caused by this bone deformation differ from those caused by destabilization of the joint with ACLR³⁴. MMP-3 activity is increased significantly in cartilage of ACL deficient joints²⁴. During immobilization of the canine knee joint a decrease of synovial TIMP-1 was recorded whereas values for MMP-3 remained at the level of the control samples^{35,36}.

The most striking results of our study were the high MMP-3 and TIMP-1 concentrations in synovial fluid of dogs with infectious arthritis. Inflammatory synovial fluid contained about 27 times more MMP-3 than non-inflammatory synovial fluid. The molar ratios between MMP-3/TIMP-1 have raised over 1.0, confirming the hypothesis that MMP-3 is a sensitive marker for the local inflammation in this disease. This is in accordance with previous reports of high levels of MMP-3 in the synovial fluid and serum samples from human RA patients^{37–39}. Inflammatory stimuli like IL-1 and TNF- α were shown to stimulate the expression of MMP-3 in canine synovium and cartilage²².

The serum TIMP-1 levels found in our control samples were surprisingly high, showing up to a 40-fold increase compared to the arthropathies samples [see Fig. 2(a)]. This presents the TIMP-1 serum level as a potential marker for degenerative changes in cartilage. A recent study that examined the TIMP-1 serum level of human patients with HD found a strong correlation with progression of joint narrowing⁴⁰. Presently it is not clear whether unrelated diseases, which have not been diagnosed in our group of patients, cause also high TIMP-1 concentrations. This possibility cannot be excluded because the control samples were obtained from dogs submitted to the hospital for non-musculoskeletal diseases. In this context it is noteworthy that in a recent study Su *et al.*¹⁵ found that TIMP-1 mRNAs were inducible by different serum factors. A possible explanation for the wide range of all biomarker values demonstrated in this study could be the time lapse before initial examination (0.1–48 weeks), the presence of severe synovitis in some of the examined dogs and the different extent in joint damage (data not shown).

Furthermore, no correlation between the synovial and serum TIMP-1, 5D4 and 3B3 (-) values could be demonstrated, which is in line with data from previous reports on canine keratan sulfate and chondroitin-sulfate^{4,13}. The synovial inflammation present in osteoarthritic joints could affect the clearance rate of the measured markers from the joint and cause the different concentrations in corresponding blood and synovial fluid samples. Enhanced clearance from the canine joint has been reported for some proteins, e.g. albumin⁴¹.

It should be also emphasized that we found a significant decline of the synovial 5D4 keratan sulfate and the 5D4 keratan sulfate/3B3(+) chondroitin sulfate ratio with increasing duration of clinical signs after ACLR which is in contrast with the increase of the 3B3(-)/3B3(+) chondroitin-sulfate ratio and may reflect changes in the metabolism and composition of proteoglycans. Previous reports have shown that during the development of canine

OA newly synthesized proteoglycans lead to an increase in the ratios of chondroitin-sulfate to keratan sulfate and of chondroitin-4-sulfate to chondroitin-6-sulfate. This is believed to reflect the synthesis of proteoglycans characteristic of immature cartilage during OA^{42,43}. Our results are in agreement with those observed in dogs after rupture of the ACL⁶ and in the canine meniscectomy model¹³.

In conclusion, our results demonstrate that there is no significant rise of synovial MMP-3 in joints affected by naturally occurring OA, whereas inflammatory synovial fluid contains about 27 times more MMP-3 than non-inflammatory synovial fluid and serum levels will directly reflect the conditions occurring in the joint. Taken together, our data demonstrate that MMP-3 is more an indicator of the inflammatory process than of the matrix degeneration.

Assays of synovial fluid samples will more accurately reflect a derailed extracellular matrix metabolism in arthropathies, but MMP-3 and TIMP-1 serum concentrations could be of some value in detecting changes in metabolism of the canine cartilage as well.

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