MMP-2 as an early synovial biomarker for cranial cruciate ligament disease in dogs

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Introduction

Cranial cruciate ligament (CrCL) rupture is one of the most common causes of stifle osteoarthritis (OA) in dogs, and mostly occurs secondary to progressive, adaptive, or degenerative changes within the stifle joint (1, 2). It has been reported that contralateral CrCL rupture occurred in 37% of dogs 17 months after initial CrCL rupture diagnosis, and the risk of contralateral CrCL rupture increases to 60% with early radiographic changes, suggesting that dogs with unilateral CrCL rupture are predisposed to rupture of the contralateral CrCL within the following months (3).

The exact pathogenesis of CrCL disease leading to CrCL rupture and stifle OA remains unknown. It has been clearly demonstrated that experimental transection of the CrCL leads to synovitis and joint instability, but some studies suggest that synovitis and arthritis may precede development of CrCL rupture and joint instability (4-7). Stifle OA usually develops despite surgical treatment (1, 8). Numerous markers of OA have been evaluated in stifle joints with CrCL rupture to improve understanding of the early stage of canine cruciate ligament disease, but no single specific marker was found to be clinically relevant (9-13). In osteoarthritic joints, pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor (TNF)-a are synthesized by the synovium and potentiate the upregulation of biologically active substances, including matrix metalloproteinases (MMP), tartrate-resistant acid phosphatase and cathepsins, which participate in macrophage-mediated joint destruction (9, 14, 15).

Matrix metalloproteinase-2 and –9 have been previously correlated with CrCL rupture in dogs in a study that reported an up-regulation of MMP-2 and MMP-9 in CrCL tissues and in synovial fluid of dogs with CrCL rupture (16). They degrade collagens and proteoglycans in cartilage matrix and can degrade aggrecan (17). Matrix metalloproteinase-2 has been found to be produced by chondrocytes and synovial fibroblasts, and MMP-9 by chondrocytes, peripheral blood monocytes and neutrophils (18).

C-reactive protein (CRP) is an acute phase protein, the concentrations of which increase in response to inflammation or tissue destruction (19). Previous reports demonstrated that CRP concentration was demonstrated that CRP concentration was elevated in several conditions such as surgical trauma, pancreatitis, inflammatory bowel disease and autoimmune haemolytic
anaemia (20–23). One report mentioned CRP as a marker of interest in diagnosis and treatment monitoring of immune-mediated arthritis in humans (24).

As unilateral CrCL rupture appears to be a predisposing factor for contralateral CrCL rupture, increased MMP activity in a contralateral joint may be related to more advanced CrCL disease (3, 16, 25, 26). In order to further investigate the involvement of MMP and determine a potential biomarker of CrCL disease, we decided to study MMP-2 and MMP-9 activity in the synovial fluid of both stifle joints from dogs with unilateral CrCL rupture. The aim of the present prospective clinical study was to compare the MMP-2 and MMP-9 activity in synovial fluid of CrCL ruptured stifle joints to both contralateral clinically normal stifles of the same dogs and stifle joints of normal dogs without CrCL rupture. We hypothesized that the activity of MMP-2 and MMP-9 would be increased in the synovial fluid in both CrCL-ruptured joints and contralateral clinically normal joints from the same dogs compared to healthy stifle joints from normal dogs. We also hypothesized that serum CRP concentration would be increased in dogs with CrCL rupture compared to normal dogs.

Materials and methods

Study population

Client-owned large breed dogs that were presented with a suspected diagnosis of unilateral CrCL rupture were included in the study. Dogs had to exhibit clinical signs consistent with unilateral CrCL rupture. Exclusion criteria included any abnormality in the results of the complete blood count and serum biochemical profile analyses, as well as any history or evidence of previous joint surgery, hip disease, elbow disease, shoulder disease, inflammatory joint disease, congenital orthopaedic disease, neurologic disease, bilateral CrCL rupture or any local evidence of contralateral stifle joint lesion. Other exclusion criteria were other concurrent ligament lesions, patella luxation or concurrent meniscal lesion. Clinical evaluation of CrCL rupture was subjectively assessed by orthopaedic examination, and by the cranial drawer and cranial tibial thrust tests that were performed while the animal was under general anaesthesia. Age, weight, body condition score, gender and clinical history were recorded for each dog. As a control group, large breed client-owned dogs anaesthetized for elective neutering surgery were included. Inclusion in the control group required the absence of any abnormality detected during orthopaedic examination. The same exclusion criteria were applied to these normal dogs. All owners gave informed consent prior to enrolment and approval from the local Pays de Loire Animal Welfare committee was obtained.

Radiographs

Standard craniocaudal and mediolateral radiographic projections of both stifle joints were obtained under general anaesthesia for both CrCL-ruptured and normal dogs as described for dogs undergoing tibial plateau levelling osteotomy (TPLO) (27). The radiological degree of OA in stifle joints was graded according to a previously described scoring system (28). Three radiographic OA parameters were considered: (i) osteophytosis and enthesophytosis were scored (0–3) according to their size at 15 specific locations on the two radiographic projections, (ii) subchondral bone sclerosis was scored at nine locations, and (iii) joint effusion was also scored (0–3) on lateral radiographs, defining an OA score from 0 to 75. As synovial effusion could be an early feature related to CrCL disease, dogs with signs of synovial effusion in the control population as well as in the contralateral stifles of CrCL-ruptured dogs were also excluded.

Sample collection and surgical treatment

Dogs were premedicated with diazepam (0.2 mg/kg IV), and general anaesthesia was induced with propofol (6–8 mg/kg IV) and maintained after endotracheal intubation with isoflurane in 100% oxygen. Blood samples were collected by aseptic jugular vein puncture from all dogs without anticoagulant. After clotting for 30 minutes at room temperature, blood samples were centrifuged for 15 minutes at 2000 g. Synovial fluid samples were aseptically collected by percutaneous arthrocentesis from CrCL-ruptured stifle joints, contralateral clinically normal stifle joints, and healthy stifle joints of normal dogs. At least 0.5 ml was collected from CrCL-ruptured stifle joints, and about 0.2 to 0.3 ml from contralateral joints, and from stifle joints of normal dogs. Both synovial fluid and sera were immediately stored at –20°C. In all CrCL-deficient dogs, the CrCL rupture was confirmed by arthroscopy or medial arthrotomy. Joint examination included inspection and probing of the caudal cruciate ligament and menisci in order to detect any additional lesion. Arthroscopy portals or joint capsule were closed routinely and the TPLO procedure was performed on all CrCL-ruptured stifle joints. Stabilization of the osteotomy was performed with a 3.5 TPLO locking plate and postoperative radiographs were obtained. All procedures were performed by the same surgeon (BB).

Biochemical assays

Canine CRP was measured in serum using a solid phase sandwich enzyme-linked immunosorbent assay kits. Both MMP-2 and MMP-9 activities were measured using commercial capture antibody-based enzyme assay kits following manufacturer’s recommendations. Synovial fluids were thawed on ice, centrifuged to eliminate insoluble material (4000 g, 15 min, 4°C), and then diluted at 1:50 in provided buffers. Briefly, MMP present within the sample was captured by a specific antibody. After washing, any bound MMP-2 in its proform was activated using p-aminophenylmercuric acetate. The activated MMP cleaved a specific chromogenic peptide or a peptide between a fluorophore and a
quencher molecule. The colourimetric or fluorescent signal was proportional to the amount of active enzyme present in the sample. For each assay, a standard curve was done with canine CRP and human standard MMP provided by the manufacturers. All assays were performed in duplicate. For MMP activity assays, canine cross-reactivity was tested on synovial fluid samples from CrCL deficient dogs as well as dogs with normal stifle joints.

Statistical analysis
Data from sera and synovial fluids were reported as canine CRP and human MMP-2 and -9 ng/ml. Statistical analyses were performed using non-parametric Mann-Whitney U or Kruskal-Wallis tests with Dunn’s method for pairwise comparison, using software for data analysis8. For all comparisons, a p-value less than 0.05 was considered significant.

After initial statistical analysis, receiver operating characteristic (ROC) curve analysis was performed for MMP activity in synovial fluid from CrCL-ruptured stifle joints and contralateral clinically normal stifle joints of the same dogs compared to stifle joints of normal dogs without CrCL rupture. These curves were created according to the logistic regression model and area under the curve was calculated for assessment of diagnostic value of MMP activity. Correlation was also investigated for MMP activity in synovial fluid from CrCL ruptured joints and contralateral clinically normal stifle joints.

Follow-up
Outcome was assessed via repeat phone calls to the owners or to the referring veterinarian within the months following the primary surgical treatment to document the occurrence of contralateral CrCL rupture. Diagnosis of contralateral CrCL rupture was made by a veterinarian by orthopaedic examination of the animal while it was under general anaesthesia in all cases.

Figure 1 Comparison of matrix metallo-proteinase-2 (MMP2) activity in the synovial fluid of cranial cruciate ligament-ruptured joints, contralateral joints, and stifle joints from normal dogs (Controls). Graph bars represent the median ± interquartile range. *p <0.05; *** p <0.001.

Results
CrCL ruptured and normal dogs
Fourteen dogs with unilateral CrCL rupture were included in this study. All included dogs were presented for the complaint of acute lameness of five to 30 days duration. Of the 14 dogs, seven were spayed female, three were entire female, and four were entire male dogs. There were four Labrador Retrievers, three mixed breeds, and one individual of each of the following breeds: Rottweiler, Boxer, Bernese Mountain Dog, Leonberg, French Mastiff, Berger de Beauce, and Dogo Argentino. At the time of surgery, the mean ± standard deviation (SD) body weight was 38.2 ± 13.5 kg (range: 20–64 kg) and mean age ± SD was 63 ± 32.8 months (range: 14–112 months). Eleven large breed dogs were included in the control group: seven entire female and four entire male. The dogs were two German Shepherds, three mixed large breed dogs, and one individual of each of the following breeds: Boxer, Dogo Argentino, Bernese Mountain, Husky, Australian Shepherd, and Labrador Retriever. Both affected and unaffected dogs had a body condition score from 3 to 4. Mean ± SD body weight and age of the control group dogs were 30.6 ± 5.1 kg (range: 25–43 kg) and 38.5 ± 27.6 months (range: 12–107 months) respectively. Body weight and age did not differ significantly between CrCL-ruptured and normal dogs (p = 0.19 and p = 0.095 respectively).

Radiographic findings
Radiographic OA score (mean ± SD) was significantly increased in CrCL-ruptured stifle joints (14.4 ± 11) compared to contralateral joints (2.1 ± 1.4; p = 0.035) and to stifle joints from dogs of the control group (1.3 ± 0.9; p = 0.0007). However, there was no significant difference in the OA score between contralateral joints of CrCL-ruptured dogs and stifle joints of the normal dogs (p = 0.88).

Serum CRP and synovial MMP-2 and MMP-9 activity
The MMP-2 activity in synovial fluid was significantly greater in CrCL-ruptured joints (97.44 ± 46.69) compared to contralateral joints (48.77 ± 28.16) and to stifles from the normal dogs (25.05 ± 23.56) (p = 0.029 and p <0.0001 respectively). The MMP-2 activity was not significantly different between contralateral joints of CrCL-ruptured dogs and stifles joints of normal dogs (p = 0.17) (Figure 1). Compared to stifle joints from normal dogs, the fold changes with contralateral stifle joints and with CrCL-ruptured stifle joints were 2.21 and 5.52, respectively.

The ROC analysis for MMP-2 activity in synovial fluid from CrCL-ruptured joints and from normal dogs had an area under the curve of 94.9% (p <0.0001, IC95%=[87.9–100]). Furthermore, ROC analysis for MMP-2 activity in synovial fluid from CrCL-ruptured joints and from contralateral joints had an area under the curve of 83.2% (p = 0.0028, IC95%=[67.2–99.1]). For each comparison, we determined the optimal threshold as the point closest to the top-left part of the ROC plot with perfect sensitivity or specificity. This threshold was defined as 55.10 ng/ml equivalent of human MMP-2 for the comparison of CrCL-ruptured stifles and stifles from normal dogs (sensitivity and specificity of 85.7%) and 70.20 ng/ml equivalent of human MMP-2 for the comparison of CrCL-ruptured stifles and contralateral stifles (sensitivity and speci-
specificity of 78.6%) (Figure 2). A poor correlation between MMP-2 activity in synovial fluid from CrCL-ruptured joints and from contralateral joints of the same dogs (r = −0.01, p = 0.97) was also noted. The MMP-9 activity in synovial fluid did not significantly differ between CrCL-ruptured stifles joints, contralateral clinically normal stifles joints of the same dogs, and stifle joints of normal dogs (p = 0.059, Figure 3). Similarly, CRP serum measurements did not significantly differ between groups (p = 0.44, Figure 4).

Follow-up

During follow-up (25.8 ± 3 months; range: 19–29 months), one dog was euthanized for unrelated disease, seven dogs were free of clinical signs of contralateral CrCL, and six dogs (43%) developed a contralateral CrCL rupture at five, 12, 17, 18, 20 and 23 months respectively after the surgical stabilization of the first limb; a weak correlation between MMP-2 activity and time to rupture (r = 0.31, p = 0.56) was observed.

Discussion

In the present clinical study, we confirmed that MMP-2 activity was significantly increased in CrCL-ruptured stifle joints, a finding consistent with previous reports (16, 25, 26, 29, 30). Matrix metalloproteinases –2 appeared as a good discriminatory marker between CrCL-ruptured joints and stifle joints from normal dogs and an acceptable but poorer discriminatory marker between CrCL-ruptured joints and contralateral joints. Interestingly, neither MMP-2 nor MMP-9 was significantly increased in synovial fluid of contralateral stifles from CrCL-ruptured dogs compared to stifle joints of normal dogs.

Cranial cruciate ligament rupture is one of the most common causes of OA in the canine stifle joint (8, 31). Several studies have shown changes in the ligament tissue of ruptured CrCL compared to normal CrCL with loss of fibroblasts, increased amounts of chondroid metaplastic cells, and disruption of extra-cellular matrix in ruptured ligaments (1, 32). Development and progression of OA associated with CrCL rupture is expected, irrespective of surgical or medical treatments (1, 33, 34).

C-reactive protein is an acute phase protein produced in the liver and it has been widely investigated in human medicine as an inflammatory marker in rheumatoid arthritis (35, 36). In a previous study, serum CRP concentration was found to be elevated in dogs with acute induced stifle synovitis (37). However, in our study, serum CRP concentration was not significantly greater in dogs with CrCL rupture than in normal dogs. This finding is consistent with previous reports that did not demonstrate any significant difference in serum CRP level between osteoarthritic dogs and normal dogs (38, 39). A possible explanation for this result may be that joint inflammation changes related to CrCL rupture or progressing CrCL disease may not be sufficiently acute to provide significant serum CRP increase.

Matrix metalloproteinases have been found to be associated with OA and CrCL rupture. A previous study has shown that expression of MMP-2 and MMP-9 were increased in ruptured CrCL stifle tissues in dogs (16). Their activity has not been previously investigated in contralateral clinically normal stifle joints of dogs with CrCL rupture. Several markers of joint inflammation and OA have been identified in humans and dogs such as IL-1, IL-6, TNF, MMP, and nitric oxide (25, 40–45). Some of these reports suggested that MMP-2 may be specifically related to CrCL lesions. A human in vitro study demonstrated that after stretch injury, the CrCL fibroblasts di-
directly responded by increasing MMP-2 production (45). Expression of MMP-2 was found to be increased in canine ruptured CrCL stifle explants and more recently MMP-2 was detected in culture media from intact and partially torn CrCL explants (16, 26, 46). Matrix metalloproteinase-2 involvement as an effect or causal agent in CrCL disease has not yet been established. As recently suggested, MMP-2 accumulation may lead to an imbalance between MMP and tissue inhibitors of MMP, which may invert the balance of new tissue synthesis and damaged tissue degradation, leading to CrCL rupture (47). The composition of ruptured and intact CrCL was reported to be different, with a significantly higher concentration of pro-MMP-2 in ruptured CrCL as well as immature cross-links, glycosaminoglycan and water content. Moreover, the turnover of the collagen of ruptured CrCL was significantly higher than that of intact CrCL, with an increase of MMP-2 in ruptured CrCL (26).

The present study was conducted on synovial fluid samples and not on CrCL tissue in an attempt to provide a less invasive approach than CrCL tissue harvesting. We found a highly significant increase in MMP-2 activity in CrCL-ruptured stifles, confirming that MMP-2 is a significant marker for CrCL rupture, as already reported on synovial fluid in non-predisposed small breed dogs, and on CrCL tissues (16, 25, 26). We did not observe any significant difference in MMP-2 activity between stifle joints of controls dogs and contralateral joints, but there was a high dispersion level of the MMP-2 values in contralateral joints. This dispersion may be related to the heterogeneous status of contralateral CrCL, with a different extent of intra-articular changes, partial tears and synovitis, despite any instability or local signs of CrCL disease. This finding may be explained by the higher turnover of collagen in CrCL of predisposed breeds, which may promote CrCL disease and rupture (48).

Arthroscopic evidence of CrCL partial tear in the contralateral stifle joint has recently been reported in 75% of CrCL-ruptured dogs and correlated with severity of synovitis (6). Similarly, the variability of MMP-2 activity we observed in contralateral stifle joints may be related to the different degenerative and inflammatory conditions that could be observed in contralateral joints. Our data indicated that there was no significant difference in MMP-9 activity between joints with CrCL rupture, contralateral joints and joints from normal dogs. These results are in accordance with an in vitro study on canine CrCL explants that did not detect any significant difference between ruptured CrCL and intact CrCL stifles explants (26). However, another study measuring MMP-9 activity in synovial fluid revealed a significant difference between joints with CrCL rupture and joints from normal dogs (25). A possible explanation of the lack of a significant difference in our study may be related to the fact that our population of normal dogs were from breeds predisposed to CrCL rupture and not a population of non-predisposed small breed dogs. Similarly, a noticeable MMP-2 activity was detected in stifle joints from normal dogs, and such an activity in dogs from breeds predisposed to CrCL disease may be due to asymptomatic intra-articular early changes and could partially explain the absence of significant difference with contralateral joints of CrCL ruptured dogs.

Six of the 14 of the dogs with primary unilateral CrCL rupture developed contralateral CrCL rupture during follow-up but the correlation between MMP-2 activity and time to rupture in contralateral joints was poor. Our study population was probably not large enough to determine MMP-2 activity as a predictive factor for contralateral CrCL rupture and we could not document the precise extent of disease of the CrCL in these joints to relate it to the synovial fluid MMP-2 activity. Another limitation of our study was the absence of follow-up of contralateral joint until contralateral CrCL rupture. We could not repeat synovial fluid sampling on these joints and could not document any potential increase in MMP-2 activity related to the progressive articular changes until final CrCL rupture. In addition, as we used a kit with human MMP-2 standards, MMP-2 values depicted in this study are equivalent human MMP-2 values and cannot be considered as absolute canine values. However, based on our preliminary experiments, these quantifications are correlated to canine MMP-2, albeit exact values are only approximated. Another study with more samples and a canine-dedicated MMP-2 test would be necessary to assess the diagnostic power of MMP-2.

**Conclusion**

This study confirmed that MMP-2 activity was significantly increased in CrCL-ruptured joints and showed a wide range of MMP-2 activity values in contralateral stifle joints. Such a variability may account for the heterogeneous conditions of contralateral joints on predisposed breed dogs with ongoing CrCL disease. Further studies, such as repeated synovial fluid sampling procedures or advanced imaging modality examinations, will be necessary to explore the role of MMP-2 in progressing CrCL disease and to determine whether MMP-2 may help to identify dogs with a more severe contralateral CrCL disease and may help to propose more preventive approaches for contralateral joints.
Conflicts of interest
There are no conflicts of interest to report.

References

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