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*Review*

# A systemic review of existing serological possibilities to diagnose canine osteoarthritis with a particular focus on extracellular matrix proteoglycans and protein

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## Abstract

Extra-cellular matrix (ECM) components are important and their stabilization is significant in maintaining normal healthy joint environment. In osteoarthritis (OA), ECM components are altered and indicate disease progression. The joint ECM is composed of proteoglycans (aggrecan, perlecan, inter α-trypsin inhibitor), glycoproteins (fibronectin, lubricin, COMP) and collagen types (most abundantly collagen type II) which represent structural and functional transformation during disease advancement. ECM investigation revealed significant biomarkers of OA that could be used as a diagnostic and therapeutic tool in different canine orthopedic diseases. This review deliberates our current findings of how the components of ECM change at the molecular level during disease progression in canine OA.

**Key words**: extra-cellular matrix, canine osteoarthritis, biomarker, synovial fluid, proteomix analysis

## Introduction

Osteoarthritis (OA) is one of the most prevalent causes of joint degeneration, lameness, pain, and chronic physical disability in dogs despite advanced diagnostic approaches and modern therapies. Canine diseases, such as: elbow dysplasia, hip dysplasia (HD), poly-arthritis and cranial cruciate ligament (CCL) rupture together with medial meniscus are major risk factors of OA. OA requires intensive and long term treatment putting financial strain on pet owners. For canine CCL disease and stifle joint OA alone, the annual cost was estimated to be several billion dollars (Wilke et al. 2005).

Why are dogs important in OA research? Animal models were used in order to study human OA. In fact, these experimental models provide valuable advantages and significant information in comparison

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with human OA research. The dog model is one of the frequently used animal models for OA exploration, since dogs have anatomical, clinical and therapeutic similarities to human OA along with arthroscopy possibilities (Cook et al. 2010). Therefore, these features make the dog an ideal species to study human OA. For this purpose, OA was developed through surgical induction of CCL (Pond and Nuki 1973) and meniscal transection (Luther et al. 2009) in dogs. Other surgical and chemical induction models were also delineated (Cook et al. 2010). Due to the close resemblance of the dog model to human OA, we focused only on the dog OA model rather than other species, such as sheep, goat, rat and horse.

Biomarkers are commonly used to diagnose diseases. However, reliable biomarkers for canine OA have yet to be discovered. Therefore, one of the goals of ongoing studies in the field of veterinary and human orthopedics is to discover biomarkers for early diagnosis and therapy of OA. Biomarkers are measurable indicators for the specific biological state. Particularly, they reflect the presence, risk, or stage of a disease. In the clinic, biomarkers can be implemented as a diagnostic, prognostic and therapeutic tool (Rifai et al. 2006). Biomarkers point out the pharmacological response to therapeutic interventions. Biomarkers can be categorized into “dry” and “wet soluble” biomarkers. Radiographs, magnetic resonance imaging (MRI), computed tomography (Spector et al. 1992) and ultrasound are dry biomarkers, whereas genetic (DNA, RNA) and biochemical (protein, protein peptides, carbohydrate and metabolites) molecules are wet soluble biomarkers (Kraus et al. 2011). Radiography is usually used in diagnosing and monitoring of dog OA. MRI and CT scans are more sensitive than radiography. Nevertheless, their implementation is associated with high cost and the problem of availability. Likewise, Arthroscopy provides a magnificent internal view of articular cartilage but this is an invasive technique.

During OA, catabolic activities increase compared to anabolism in articular cartilage, resulting in significant loss of extra-cellular matrix (ECM) components. The major components of cartilage are proteoglycans, collagens, hyaluronan, and glycosaminoglycans along with non-collagen glycoprotein components such as: lubricin and cartilage oligomatrix protein (COMP). The loss of ECM is a main characteristic of cartilage destruction in OA. Hence, investigation of bio-chemical changes in ECM is believed to be an important factor in OA pathology. Due to these changes, a few discharged fragments could ultimately be assessed in urine, blood plasma, serum and synovial fluid (Oliviero et al. 2009). Multiple serological assays have been developed for the detection of OA. They permit the detection of fragments, including cytokines, proteoglycans, collagen and many others. These robust parameters represent disease severity and therapeutic interventions. Similarly, ELISA is also used to examine the biochemical marker, where antibodies react against different antigens in different biological fluids. The literature provides a number of publications on the subject of serological assays for OA biomarker research in dogs. The current review summarized all of the canine OA biomarkers in Table 1 regarding protein and carbohydrate.

## Collagen type II

Collagen type II is one of the major elements of the cartilage ECM, structurally composed of three identical collagen h1 chains in a triple helix, exceptionally N-, and C-telopeptides. The key function of this collagen is to safeguard the cartilage. This collagen is abnormally degraded in OA. One study measured SF antibody titers of collagen type I and II in stifle joint disease CCL rupture (partial or complete) accompanied with OA. The antibody titers of collagen type I and II were significantly increased in SF, especially in dogs with secondary OA as compared to the control dog group. Augmentations to collagen autoantibodies in SF were not precise for the kind of joint disorder. It was doubtful that anti-collagen antibodies had an initial dynamic role in CCL weakness (De Rooster et al. 2000).

Matrix metalloproteinase (MMP) family members play a pivotal role in collagen type II degradation (Chung et al. 2004). In dog OA cartilage, mRNA expression of MMP -2 and -9 was found to be elevated (Clements et al. 2009). Moreover, there was clear evidence that elevated canine MMP -2 and -9 activities were present in CCL rupture SF (Boland et al. 2014) (Rabillard et al. 2012). Different members of the MMP family (MMP collagenases -2, -3, -9 and -13) involved in initial collagen degradation (Settle et al. 2010) (Hegemann et al. 2003). Therefore, MMP -2, -3 and -9 are potential biomarkers in canine OA SF.

C2C is a concrete product resulting from collagen type II breakage. In an ACLT model, collagen type II neoepitope was increased in canine urine and its concentration was elevated in canine cartilage explant after IL-1f-stimulation. Nevertheless, the collagenase inhibitors suppressed the elevation of collagen type II neoepitope (Matsukawa et al. 2013). Consequently, this was shown to be a progressive step towards a therapeutic approach. The collagenases break collagen type II at approximately one quarter of the length of the molecule away from the C-terminus. As a consequence of this cleavage, l and L length

Table 1. Biomarkers used clinically in canine OA, including their specificity and method of detection.

| Biomarker type | Fragment | Specificity | | | Sample type | Number of animals | Method of detection | Reference |
|---|---|---|---|---|---|---|---|---|
| | | OA | Diseased | Healthy | | | | |
| Collagen Type II | Autoantibodies | ↑ | ↑ CCL | | SF | 82 dogs | ELISA | (De Rooster et al. 2000) |
| | C2C | | ↑ MCD | | SF | 19 diseased dogs + 8 control dogs | ELISA | (Prink et al. 2010) |
| | Col2-3/4C long mono and CTX-II | ↑ in Stifle joint injury | | | SF | 20 large mixed breeds | ELISA | (Matyas et al. 2004) |
| | Neo-epitope TIINE | | ↑ After canine menisce-ctomy | | Urine | | TIINE 45-mer assay | (Settle et al. 2010) |
| COMP | COMP | ↑ | | | Serum | 16 OA dogs + 5 control dogs | ELISA | (Fujiki et al. 2007) |
| | COMP | | | ↑ after intense exercise | SF Serum | | ELISA | (Qi and Changlin 2006) |
| Fibronectin | $(V+C)^-$ | ↑ | | | SF | 26 OA dogs + 22 control dogs | ELISA | (Steffey et al. 2004) |
| Hyaluronan | | | ↑ CCL | | SF | Surgically induced OA in 6 dogs + 21 control dogs | ELISA | (Venable et al. 2008) |
| | | ↓ | | | SF | 49 dogs | ELISA | (Plickert et al. 2013) |
| | | | ↓ HD | | Serum | 25 diseased dogs + 98 control dogs | ELISA | (Nganvongpanit et al. 2008) |
| | | | ↓ induced OA | | | 12 dogs | ELISA | (Budsberg et al. 2006) |
| Chondroitin sulfate | WF6 | | ↑ HD | | Serum | 25 diseased dogs + 98 control dogs | ELISA | (Nganvongpanit et al. 2008) |
| | 3B3 | | ↓ HD | | Serum | 25 diseased dogs + 98 control dogs | ELISA | (Nganvongpanit et al. 2008) |
| | 3B3 | | ↑CCL | | SF | 8 diseased dogs + 24 control dogs | ELISA | (Johnson et al. 2001) (Johnson et al. 2002) |
| | 7D4 | | ↑ CCL | | SF | | ELISA | (Johnson et al. 2001) (Johnson et al. 2002) |
| Keratan sulfate | | | ↑ induced OA | | Serum | 12 dogs | ELISA | (Budsberg et al. 2006) |
| | 5D4 | ↓ | | | SF | | ELISA | (Matyas et al. 2004) (Hegemann et al. 2002) |

cont. Table 1

| Biomarker type | Fragment | Specificity | | | Sample type | Number of animals | Method of detection | Reference |
|---|---|---|---|---|---|---|---|---|
| | | OA | Diseased | Healthy | | | | |
| Aggrecan | aggrecan 846 epitope | | ↑ CCL | | Serum | 20 mixed breed dogs | Radio immno assay (RIA) | (Matyas et al. 2004) |
| | ARGN and AGEG peptides degradation fragments | | ↑ After canine menisce-ctomy | | SF | | Aggrecan neo-epitope assays based on liquid chromato--graphy | (Settle et al. 2010) |
| | Aggrecan generated catabolites BC-3 BC-14 | | ↑ OA | | SF | Early and late OA dogs | Western blot analysis | (Innes et al. 2005) |
| YKL40 | Chitinase like molecules | | Anterior cruciate ligament | | Knee cartilage | | Trans--criptase PCR analysis | (Lorenz et al. 2005) |
| MPO | Myeloperoxidase | | Fragment medial coronoid process | | SF | | MPO assay | (Hurlbeck et al. 2014) |
| MMP-2 | | | CCL | | SF | 14 CCL in large breeds + 11 control dogs | ELISA | (Boland et al. 2014) |

fragments are released. The l fragment holds both C2C neo-epitope or Col 2-3/4C (long monomers) particular to collagen type II (Poole et al. 2004) and C1, 2C or Col2-3/4C (short monomers) that are present in both type I and type II collagen (Billinghurst et al. 1997). C2C is basically present in hyaline cartilage, intervertebral disc, and in minute quantities in other tissues (Poole et al. 2004).

This raises a relevant question whether OA pathogenesis is accompanied by oxidative stress. The Pond-Nuki model was designed to check the role of oxidative stress in OA development. OA was experimentally induced by anterior cruciate ligament transection in 7 dogs. Analysis of preoperative and postoperative (interval of 30, 60 and 105 days) serum catalase displayed climax activity on day 60. In contrast, malondialdehyde and C2C concentration were increased uninterruptedly throughout the experiment. This indicates to a possible relation between oxidative stress and cartilage obliteration (Goranov 2007).

There was no strong evidence that C2C could be applied as a diagnostic biomarker in canine serum and urine. A cross-sectional study was conducted to compare C2C concentration in canine serum, urine, and SF, between clinically developed stifle joint OA in CCL disease and a control group. Fragment correlation was checked with disease severity. C2C or Col 2-3/4C concentration was measured using a commercially available ELISA kit. Lameness, osteophytosis and joint effusion were important ($p<0.05$) parameters, recorded in a naturally occurring diseased group. However, there was no significant correlation between C2C and clinical stifle joint OA ($p>0.05$). C2C was not a cause of OA development, and therefore could not be used as a clinical biomarker (Hayashi et al. 2009). However, decreased levels of C2C and hyaluronic acid were better indicators of clinical disease improvement in canine serum after hip OA (Vilar et al. 2016). In a beagle OA model, platelet-rich plasma and adipose-derived mesenchymal stem cells played a substantial role in the improvement of ECM (collagen and glycosaminoglycan) (Yun et al. 2016).

In contrast to the above observations, another study noticed that C2C (Col 2-3/4C) concentration was increased in canine synovial fluid (Chu et al. 2002). A cross-sectional clinical study was conducted on canine elbow dysplasia with medial coronoid

disease (Valiyaveettil et al. 2005). The mean (+ SD) C2C concentration in MCD dogs was remarkably higher (112.3 + 24.8 ng/ml) than in the control group (76.1 + 16.9 ng/ml; P< 0.05). Therefore, C2C concentration in SF might be a potential biomarker for diagnosis of the degree of articular cartilage damage with MCD (Prink et al. 2010).

Coll2-1 and Coll2-1$NO_2$ are the degradation products of collagen type II that can indicate both disease succession and activity (Henrotin et al. 2007). A study was conducted to measure Coll2-1 and Coll2-1$NO_2$ during OA development after anterior cruciate ligament transection in dogs. Immunoassays depicted high serum concentrations with P values <0.001 and <0.05 respectively. The level of Coll2-1$NO_2$ showed a constant increase and reached its peak level after 6 and 8 weeks of surgery. It was also associated with osteophyte formation and reflected oxidative stress in OA (Henrotin et al. 2012).

The carboxy-terminal cross-linked fragments of collagen type II (CTX-II) showed an age dependent pattern. CTX-II was increased in SF (Hurlbeck et al. 2014) and serum (Schoenherr et al. 2010) of juvenile dogs. In a knee transection canine OA experimental model, CTX-II concentration in SF was remarkably higher in an affected joint compared to a contra-lateral control joint (Matyas et al. 2004).

Thiol-dependent enzyme cathepsin K reacts in a normal acidic pH environment that was evaluated to be produced by OA chondrocytes and was thought to play a major role in cartilage breakdown and aggrecans (Konttinen et al. 2002). Cathepsin K was involved in hyaline cartilage destruction as well as calcified cartilage and sub-chondral bone resorption at the earliest stage of dog OA (Pelletier et al. 2004). In OA cartilage, cathepsin K protein and its gene expression were remarkably increased in the superficial zone in comparison with normal cartilage (Pelletier et al. 2005). In the canine OA model, treatment with licofelone (non-steroidal anti-inflammatory drug) (Pelletier et al. 2004) and tiludronate (bisphosphonate) (Moreau et al. 2011) considerably decreased cathepsin K activity. Cathepsin K inhibitor (SB-553484) treatment reduced subjective gross and calculated degeneration scores by 29% and 46% respectively in dogs. Histo-pathologic analysis indicated that total tibial degeneration score decreased about 21%. In urine samples, biomarkers of collagen type I and II were decreased, which is a direct outcome of bone and cartilage degradation (Connor et al. 2009). These results appeared to show that cathepsin K played a key role in joint disability and lameness and its level decreased after treatment. It is generally believed that collagen fragmentation occurs during OA and these fragments were investigated as valuable diagnostic biomarkers. Hence, Cathepsin K, a less abundant component of the cartilage, can be a better diagnostic biomarker in relation to collagen type II fragments.

Collagen type I h2-chain (COL1A2) and collagen type III h1-chain (COL3A1) increased in OA cartilage relative to the control cartilage. In this study, elevated MMP-2, -9 and -13 gene expressions were assessed by reverse transcriptase polymerase chain reaction. Radio-graphically assessed OA severity could be correlated with cartilage gene expression (Clements et al. 2009). The relative increase in matrix metalloproteinase with collagens displayed its anabolic effect on collagens in canine OA cartilage. It is also interesting to know that MMP-13 was thought to be a major collagenase in OA cartilage and was basically responsible for collagen type II cleavage (Kevorkian et al. 2004).

## Glyco-proteins

### Cartilage oligomeric matrix protein (COMP)

COMP, also known as thrombospondin 5, is present abundantly in the synovium, tendon, cartilage, serum, and SF. COMP not only interacts with collagen types (I, II and IX) but also supports collagen types I and II in fibril formation. Therefore, it plays a fundamental role in the assembly, solidarity and safeguarding of the cartilage ECM (Chu et al. 2015).

Magnetic resonance imaging (MRI) is a useful supplementary tool accompanied with different practical biomarkers in order to detect articular cartilage degradation in dogs at its earlier stage. An elevated level of COMP was noticed in serum after intensive training indicating a potential relationship of COMP with knee cartilage degradation measured with MRI (P<0.01). However, the SF COMP value did not show any difference between normal and abnormal MR imaging (P>0.05) (Qi and Changlin 2007). In human OA, COMP correlation with disease severity was estimated by MRI (Hunter et al. 2007) but it did not exhibit any relationship with inflammatory biomarkers (Skoumal et al. 2006).

What is the effect of exercise on COMP? Strong physical activities, for example a marathon race, increased the concentration of COMP in serum (Andersson et al. 2006). Similarly, a higher COMP level was recorded in serum and SF after strenuous exercise, which ultimately reached its climax level after 4 and 6 weeks respectively in dogs. Meanwhile, changes in knee cartilage were evaluated with MRI examination (Qi and Changlin 2006). An increased COMP level at its earlier stage was observed, which might be the re-

sult of cartilage injury. Therefore, COMP could be considered as a sensitive biomarker in articular cartilage injury.

In one study, the value of COMP concentration was found to be significant (P=0.019) in OA dog;s serum compared to a control group. After intramuscular treatment with poly-sulfated glycosaminoglycan (PSGAG), COMP concentration was decreased (p<0.001) in OA dogs in comparison with healthy dogs. The analyzed improvement in lameness might be a response to therapy (Fujiki et al. 2007). Thus COMP could be used to monitor disease therapy and also as a diagnostic biomarker in OA dogs.

Canine COMP concentration was elevated in serum (44.9 ,g/ml) and synovial fluid (401.7,g/ml) compared to the control group (31.3 and 298.7 9 ,g/ml respectively) after naturally occurring OA (Misumi et al. 2002). COMP value was also increased after experimentally induced OA (meniscectomy) (Lindhorst et al. 2000) (Carlson et al. 2002). There was therefore clear evidence that COMP concentration was raised initially after meniscectomy in SF and remained the same during 12 weeks of follow up (Lindhorst et al. 2000). One study indicated decreased COMP concentration in SF after meniscal injury and correlation was observed between COMP and canine meniscal injury (Girling et al. 2006).

## Fibronectin

Fibronectin (FN), a higher molecular weight glycoprotein, is involved in a variety of cellular processes, including migration, adhesion, proliferation and differentiation. It is a major component of ECM, uses as a substrate for cell attachment (Bager et al. 2016). Chondrocytes are the main source of FN in OA cartilage. It was shown that total FN was increased directly with extra domain B (ED-B+) FN in OA cartilage. FN, together with collagen type VI, might perform a role in matrix-matrix cohesion and cell-matrix adhesion on agarose-cultured chondrocytes extracted from normal adult canine articular cartilage. Immunohistochemistry together with dual channel microscopy and digital image processing showed co-localization between FN and collagen type VI in the pericellular microenvironment regardless of a retaining mechanism in articular cartilage chondrocytes (Scanzello et al. 2015).

The subcuticular connective tissues are most sensitive in dogs. Matrix metalloproteinase inhibitors (MMPi) were administered in dogs, which ultimately became the cause of connective tissue alternation known as “fibrodysplasia”. Fibrodysplastic tissues showed significant activation and secretion of collagens (type III and I) after ultra-structural analysis. Immuno-histo-chemistry indicated increased levels of FN and transforming growth factor f (Westwood et al. 2009). Therefore, MMPi-induced fibrodysplasia is also a risk factor of musculoskeletal problems in dogs.

Fibronectin protein folds itself into a series of globular homologous repetitions of three different types I, II and III, comprises of 45, 60, and 90 amino acids respectively. Different cell types produce different multiple iso-forms of FN encoded by a single gene. There are two iso-forms of FN, one containing the V domain and another containing the ED-A domain employed in canine OA and human RA respectively. The $(V+C)^-$ is an iso-form of FN that lacks I-10, III-15 and domain V segments. Furthermore, it accounts for 55-80% of total FN tissue in articular cartilage (Stoffels et al. 2013). Although (V+C) – iso-form was present solely in cartilage, its presence in synovial fluid indicated its cartilage origin and proved it as a potential biomarker in order to observe canine OA. An elevated level of (V+C) – was noticed in canine SF in a contra-lateral knee suffering from CCL rupture. Thus, it might represent earlier changes in knee joint injury compared to a healthy joint. Nevertheless, there were alterable measurements between the control and diseased group. This was possible due to joint effusion in the affected knee joint that made it a less applicable clinical biomarker (Steffey et al. 2004). On the other hand, FN iso-form comprised with ED-A domain was more expressed after the stimulation of cytokines, hormones, growth factors and stress in different pathological diseases, including rheumatoid arthritis (RA) (Przybysz et al. 2009). This iso-form was particularly over expressed in SF, plasma and articular cartilage of RA patients in comparison to OA or fibrous RA. For this reason it is a suitable biomarker in RA disease (Miyamoto et al. 2002) (Przybysz et al. 2009). In RA patients, a direct correlation was noticed between ED-A and progressive joint destruction in SF. Therefore, ED-A in SF might be an indicator of joint destruction during RA (Przybysz et al. 2009).

Fibronectin fragments were not identified in canine SF and serum. However, numerous FN fragments (N-terminal FN) were identified in human OA and RA, which were produced by ADAM-8-mediated after FN cleavage at the Ala/Val site. The resulting FN fragments VYQP and VRAA neo-epitopes were therefore recommended as potential biomarkers. These neo-epitopes were further analyzed in OA cartilage and were co-localized in the area of aggrecan loss. VYQP neo-epitopes induced cartilage destruction (Zack et al. 2006) (Zack et al. 2009). Rac1 is needed for FN fragments to induce signaling and to increase chondrocyte MMP-13 production. Rac1 has the ability to stimulate MMP-13 production so that it

can perform an important function in OA cartilage destruction (Long et al. 2013). Furthermore, pro-inflammatory factors (IL-1f, IL-6 or FN fragments) stimulate meniscus cells to produce more metalloproteinases as well as catabolic gene expression. In fact, stimulation of the meniscus can enhance the OA development process after joint injury; there is an increased production of chemokines, cytokines and matrix degrading enzymes (Stone et al. 2014).

## Lubricin

Lubricin, a lubricating and superficial zone glycoprotein, is encoded by the PRG4 gene (Reesink et al. 2016). It has a central protective role in cartilage against friction-induced wear. Recent research has revealed its important role both in cell adhesion and proliferation. It has various functions in articular joints and tendons, such as surface protection and synovial cell growth (Szychlinska et al. 2016).

The role of lubricin is quite understood in human OA (Musumeci et al. 2014) and in other species, including the rabbit (Elsaid et al. 2005), rat (Musumeci et al. 2015), sheep (Young et al. 2006), equine (Reesink et al. 2016) and guinea pig (Wei et al. 2010). Lubricin is widely distributed in different structures; synovial fluid, articular cartilage, synovial fibroblasts, synoviocytes, meniscus, tendons and ligaments (Szychlinska et al. 2016).

The lubricating ability was evaluated by arthrotripsometer oscillating latex opposed to polished glass in in vitro analysis. In OA patients, the lubricating tendency of lubricin was decreased in synovial fluid compared to the healthy group (Jay et al. 2004). After a joint injury, lubricin synthesis was increased (Jones et al. 2009) in cartilage that was further isolated from the synovial fluid in OA and RA patients. However, liquid chromatography-MS analyses indicated that RA patients contained different sialylation compared to OA patients in which lubricin was enriched with mono-sialylated types (Estrella et al. 2010). The sialylation up-regulation indicated an inflammatory reaction during which sialyic acids residues gained the ability to increase lubrication.

In an animal model, treatment together with the combination of lubricin protected articular cartilage and prevented the process of OA development (Flannery et al. 2009); its potential bio-therapeutic implementation in OA is recommended (Bao et al. 2011). Lubricin played a significant role in reducing the gliding fraction by repairing the canine flexor digitorum prefunds tendon and maintaining tendon smoothness (Taguchi et al. 2009) (Zhao et al. 2014). However, its role in canine OA is yet to be evaluated.

## Hyaluronan

Hyaluronic acid (HA) or hyaluronan, a polymer of molecular mass up to 10 Daltons, is produced by synovial fibroblasts and is composed of repeating disaccharidic units of D- glucuronic acid and D-N-acetyl-glucosamine. HA is part of the normal cartilage matrix where it has a central role in ECM stabilization together with aggrecan interaction. Moreover, HA has hydrodynamic properties and performs fundamental functions in lubrication and osmotic stability (Nusgens 2010). In an experimentally induced OA, HA has a suppressive character in reducing chondrocyte apoptosis (Echigo et al. 2006).

Two-dimensional electrophoresis analysis revealed hyaluronan-binding protein 2 (also known as plasma hyaluronan binding protein, PHBP), which has the ability to link with hyaluronan. This protein was decreased in OA dog serum (Gharbi et al. 2013) and displayed a strong attraction to negatively charged substances, including hyaluronic acid, heparin and dextran sulfate. PHBP has the ability to interact with glycosaminoglycans and, as a result, cuts matrix proteins, such as fibrinogen and fibronectin (Choi-Miura et al. 2001). Therefore, PHBP has a catabolic effect on fibronectin and generates different iso-forms in ECM (Steffey et al. 2004) (Przybysz et al. 2009).

During canine orthopedic diseases, the level of HA was lowered in serum (Nganvongpanit et al. 2008) and synovial fluid (Venable et al. 2008). In contrast to these findings, elevated serum (Sasaki et al. 2013) and decreased SF levels of HA were noticed in human OA (Li et al. 2009). In RA patients, a decreased HA level was found in SF (Kosinska et al. 2015) and an elevated concentration was noticed in serum (Pothacharoen et al. 2006). In addition, HA molecular weight was reduced in canine OA (Venable et al. 2008) similar to RA patients (Kosinska et al. 2015).

HA is a promising biomarker neither in human RA nor in canine OA. The circulating HA level varies with physical activity and diurnal periods, reducing its effectiveness as a reliable clinical biomarker (Engström-Laurent and Hällgren 1987). Hyaluronan level decreased with the increase of disease severity and its concentrations were not so consistent in different OA stages; it is not therefore an ideal biomarker for diagnostic purpose (Plickert et al. 2013).

## Chondroitin Sulfate

Chondroitin sulfate (CS) binds covalently with aggrecan, leucine-rich proteoglycans, biglycan and decorin in ECM. CS contains different sequences of

N-acetyl D-galactosamine 4/6 sulphate and D-glucoronate residues, which are linked together (Šimáneka et al. 2005). CS is a vital element in the joint where it prevents space narrowing, decreases joint swelling and effusion. It has an anti-inflammatory role in chondrocytes and synovial fluid by inhibiting nuclear translocation of nuclear factors $_k$B (NF-$_k$B) (Iovu et al. 2008).

In canine hip dysplasia, the two isotopes (WF6 and 3B3) of CS were analyzed to evaluate the process of OA. The results showed that CS epitope WF6 level was higher ($p<0.01$) and 3B3 was lower ($p<0.05$) in serum compared to the control group (Nganvongpanit et al. 2008). The highest level of WF6 CS epitopes indicated the process of joint degradation in OA, whereas a decreased level of 3B3 showed less synthesis of this isotope. It appears that imbalance of these isotopes aggravates the disease process.

There was a noteworthy increase of 3B3 and 7D4 epitopes after naturally or experimentally induced CCL rupture compared to normal SF. However, their relationship to disease severity made their clinical usage limited (Johnson et al. 2002). These epitopes reached their peak levels after several months due to CCL transaction and indicated a linear relationship with disease progression regardless of CCL intra-articular or extra-capsular reconstruction (Johnson et al. 2001).

## Keratan Sulfate

Keratin sulfate (Stone et al. 2014) is an abundant element in aggrecan and thus much effort was made to develop a canine OA biomarker in the past. In humans, KS level in serum was not related to knee OA (Golightly et al. 2011). However, high serum KS level was observed in old knee trauma patients. Therefore, the serum level of KS in trauma patients represented articular cartilage damage (Wakitani et al. 2007). KS concentration fluctuated in canine SF due to severity of cartilage degradation while serum KS was increased after induced OA (Budsberg et al. 2006).

In SF, a lower level of KS 5D4 in canine OA was detected through ELISA and this indicated its inverse relationship with disease severity. The ratio of 5D4 KS/3B3 chondroitin sulfate was also decreased in SF in contrast with 3B3 (+/-) revealing metabolic changes in OA (Lindhorst et al. 2000) (Hegemann et al. 2002). Likewise, KS epitope 5D4 level was reduced in OA and RA in comparison with the healthy group (Spector et al. 1992). Tibial plateau osteotomy did not considerably change KS 5D4 expression, indicating that surgery had a minimum effect on proteoglycan metabolism (Girling et al. 2006). Tibial plateau osteotomy did not influence OA development. Current evidence shows that KS is not a clinically reliable biomarker due to its inverse relationship with disease progression and controversial research results.

## Aggrecan

Aggrecan is the substantial proteoglycan of cartilage tissues and is responsible for hydrodynamic functions, including weight bearing and elasticity. Furthermore, aggrecan (220 kDa) structure is made up of six domains: globular 1 (G1), inter-globular (IG), globular 2 (G2), KS, CS and globular 3 (G3) (Nia et al. 2015). Both canine knee fibro-cartilage and hyaline cartilage are dissimilar on a molecular basis, such as gene expression and spatial aggrecan distribution, and also on a concentration basis. These dissimilarities were analyzed using real time PCR, immuno-fluorescence microscopy and ELISA (anti-aggrecan G1 antibody) respectively (Valiyaveettil et al. 2005). In fact, aggrecan content decreased (40-50%) after OA development in contrast to other small proteoglycans (biglycan, fibromodulin and decorin), which increased in canine cartilage (Liu et al. 2003) regardless of age. Collagen type II and aggrecan mRNA ratios changed in cartilage after experimentally induced OA (Matyas et al. 2002).

Aggrecan 846 epitope is present on intact aggrecan molecules and is linked with CS at the level of the G3 domain in cartilage. After a joint injury, epitope concentration changed in SF and indicated degenerative changes (Matyas et al. 2004). On the other hand, the level of epitope in serum increased, while KS remained unchanged (Matyas et al. 2004). The increase of aggrecan 846 epitope in serum indicated earlier joint injury; it could therefore be used as a diagnostic biomarker.

Aggrecan degradation plays an important role in OA, in which newly formed C and N termini are produced after the cleavage of aggrecan by the reaction of matrix metalloproteinases (proteolytic enzymes) and aggrecanses respectively. C terminus containing GAG was released out of the matrix after the cleavage of the IG domain during aggrecan molecule breakdown. N terminus cleavage at the level of the Glu-Ala bond generated ARGN and AGEG peptides which were detected using polyclonal antibody (Gibson and Briggs 2016). ADAMTS -4 and -5, also known as aggrecanase 1 and 2 respectively, produced fragments of aggrecan at five different points which were recognized in diseased cartilage (Arner 2002) (Nagase and Kashiwagi 2003). MMPs were also responsible for aggrecan cleavage (Struglics et al. 2006). The resulting

products (ARGN and AGEG) were valuable degradation biomarkers only in canine SF. MMP-13 performed an active role in aggrecan degradation and its activity was reduced by using PF152 (MMP inhibitor) in dogs; this could ultimately decrease aggrecan peptides and cartilage lesions (Settle et al. 2010). The aggrecanases were actively involved in IGD cleavage at earlier stages in OA joints. BC-3 and BC-14 aggrecan metabolites (200-250Kd) were both able to differentiate between early and late stages of OA (Innes et al. 2005). However, MMPs mediated cartilage degradation at later stages of OA (Little et al. 2002).

## Conclusion

In the last two decades, efforts to discover a practicable solution for the diagnosis of human and canine OA have intensified. Biomarkers detect cartilage proteoglycan degradation and their resulting fragments, in synovial fluid, serum, plasma and urine and can be used to diagnose the disease, monitor its progression, and to evaluate therapeutic response. Therefore, efforts were focused on biomarker development. Dogs are considered an ideal animal in OA research because dog OA models provide significant information regarding OA diagnosis, pathogenesis and treatment. Although outstanding work has been done towards clinical biomarker development, the discovery of a reliable biomarker for OA remains elusive.

Different proteoglycan biomarkers are discussed in this review paper to assess their specificity and clinical use in canine OA. Researchers started to focus on biomarker development from fragments of protein in ECM, which restricted further research process on OA. There is an urgent need to study other proteoglycans, such as perlecan and inter alpha trypsin inhibitor and their possible involvement in canine OA. The role of perlecan has already been appraised in human OA (Tesche and Miosge 2004).

Researchers are now focusing on proteomic analysis in OA; this method of research leads to more clarification of cartilage ECM structure and degradation, and scrutinizes more efficiently the proteins in SF, serum and urine. Proteomics analysis has proven itself as a milestone in developing a biomarker in OA until now. Electrophoresis analysis is carried out to analyze different proteins and peptides through matrix-assisted laser desorption ionization-imaging mass spectrometry (MALDI-MS/MS). These abundant proteins can be purified as diagnostic biomarkers through ELISA and western blotting analyses.

Biomarkers are an indicator of disease severity and therapeutic response; for example, the role of poly-sulfated glycosaminoglycan treatment was studied in OA and its effect on different biomarkers (COMP, MMP-2, MMP-9 and CRP) was also investigated (Fujiki et al. 2007). Biomarkers are a valuable tool in diagnosing OA at earlier and later stages. Additionally, their implementation for the treatment of OA is another positive aspect. For the future investigation of the biomarkers of OA, integrating glycol-proteomics analysis of carbohydrate and protein structure should be included in combination rather than in isolation. Proteomics analysis was started earlier in humans than in dogs to resolve the OA problem; however, this analysis is very crucial in resolving OA in canines as well. Therefore, concerted efforts are required for proteomics analysis in canine OA.

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