Investigations on the quantitative and qualitative protein content in serum and synovial fluid of dogs with osteoarthritis

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Muhammad Shahid
Tierarzt aus Lahore, Pakistan

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OA	Osteoarthritis
CCL	Cranial cruciate ligament
CHD	Canine hip dysplasia
ECM	Extra-cellular matrix
COMP	Cartilage oilgo-matrix protein
ELISA	Enzyme-linked immune-sorbent assay
SF	Synovial fluid
MRI	Magnetic Resonance Imaging
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
MMP	Matrix metalloproteinase
mRNA	messenger Ribonucleic acid
IL-1β	Interlukin 1 Beta
CTX-II	Carboxy terminal cross linked type II
COL1α2-chain	Collagen type I Alpha 2 chain
COL3 α1-chain	Collagen type III Alpha 1 chain
FN	Fibronectin
ED-B+/ED-A	Extra domain B/Extra domain A
RA	Rheumatoid arthritis
HA	Hyaluronic acid
PHBP	Plasma hyaluronan binding protein
CS	Chondroitin sulphate
KS	Keratan sulphate
GAG	Glycosaminoglycan
TOF	Time of flight
MS/MS	Mass spectrometry
CRP	C-reactive protein
2-DE	2-dimensional electrophoresis
PAGE	Polyacrylamide gel electrophoresis
1-DE	1-dimensional electrophoresis
SDS	Sodium dodecyl sulphate

SF	Synovial fluid
SELDI	Surface enhanced laser desorption/ionization
LC-MS/MS	Liquid chromatography tandem MS
ESI	Electro-spray ionization
Apo AI	Apolipoprotein A-I
ACS	Autologous conditioned serum
ACLT	Anterior cruciate ligament Transection
MALDI TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry

1 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, poly-arthritis and cranial cruciate ligament (CCL) rupture together with medial meniscus are major risk factors of OA (Garner et al., 2013; Murakami et al., 2015). 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 with human OA research. The dog model is one of the frequently used animal models for OA exploration, since canine OA have anatomical, clinical and therapeutic similarities to human OA along with arthroscopy possibilities (Cook et al., 2010). Therefore, these features make the dog as an ideal species to study human OA. For this purpose, OA was developed through surgical induction of CCL rupture (Pond and Nuki, 1973) and meniscal transection (Luther et al., 2009) in dogs. Other surgical and chemical induction models were also delineated. The surgically induced methods included anterior cruciate transection, meniscectomy, abrasion, groove, valgus osteotomy, cartilage defects and reverse triple pelvic osteotomy, whilst chemically induced OA included the application of iodo-acetate, papain along with prednisone, chymopapain, calcium pyrophosphate crystals and oral quinolones (Cook et al., 2010). Due to the close resemblance of the dog model to human OA, the research focus of the present thesis was only on the dog OA model rather than other species, such as sheep, goat, rat and horse.

Biomarkers are commonly used to diagnose several diseases. However, reliable biomarkers for canine OA have yet to be discovered. Therefore, one of the goals of the on going studies in the field of veterinary and human orthopaedics 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.

Introduction

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 soluble' and 'wet soluble' biomarkers. Radiographs, magnetic resonance imaging (MRI), computed tomography scans (Spector et al., 1992) and ultrasound are dry soluble biomarkers, whereas genetic (DNA, RNA) and biochemical (protein, peptides, carbohydrate and lipid metabolites) molecules are considered to be 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 the pathogenesis of OA, catabolic mechanisms are increasing 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) (Oliviero et al., 2009). 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 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 (Mobasheri, 2012).

2 Literature Review

The literature provides a number of publications on the subject of serological assays for OA biomarker research in dogs. The current literature review summarized all of the canine OA biomarkers in Table 1 regarding protein and carbohydrate composition.

2.1 Collagen type II

Collagen type II is one of the major elements of the cartilage ECM, structurally composed of three identical collagen α1 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 synovial fluid (Oliviero et al., 2009) 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 MMP-9 was found to be elevated (Clements et al., 2009). Moreover, there was clear evidence that elevated canine MMP -2 and MMP-9 activities were present in CCL rupture SF (Boland et al., 2014; Rabillard et al., 2012). Different members of the MMP collagenases family (MMP-2, -3, -9 and -13) involved in initial collagen degradation (Hegemann et al., 2003; Settle et al., 2010). Therefore, MMP -2, -3 and -9 are potential biomarkers in canine OA SF.

Collagen type II neo-epitope is a product resulting from collagen type II breakage. In an anterior cruciate ligament transection (ACLT) model, collagen type II neo-epitope was increased in canine urine and its concentration was elevated in canine cartilage explant after IL-1β stimulation. Nevertheless, the collagenase inhibitors suppressed the elevation of collagen type II neo-epitope (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, ¾ and ¼ length fragments are released.

The ¾ 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 canine Pond-Nuki model was designed to check the role of oxidative stress in OA development. OA was experimentally induced by ACLT in 7 dogs. Analysis of preoperative and postoperative (interval of 30, 60 and 105 days) serum catalase displayed highest 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 marker 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 parameters, recorded in a naturally occurring diseased group. However, there was no significant correlation between C2C and clinical stifle joint OA. 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 extracellular collagen and glycosaminoglycan content (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 ($\pm$ SD) C2C concentration in MCD dogs was remarkably higher (112 ± 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 Coll2-1NO$_2$ 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 Coll2-1NO$_2$ 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 Coll2-1NO$_2$ 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).

The 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 (a non-steroidal anti-inflammatory drug) (Pelletier et al., 2004) and tiludronate (a 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 α2-chain (COL1A2) and collagen type III α1-chain (COL3A1) increased in OA cartilage relative to the control cartilage (Kevorkian et al., 2004). 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).

2.2 Glycoproteins

2.2.1 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. However, the SF COMP value did not show any difference between normal and abnormal MR imaging (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).

Strong physical activities, for example a marathon race, increased the concentration of COMP in serum of humans (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. An increased COMP level at its earlier stage was observed, which might be the result of cartilage injury. Therefore, COMP could be considered as a sensitive biomarker in articular cartilage injury (Qi and Changlin, 2006).

In one study, the value of COMP concentration was found to be significant higher in OA dog's serum compared to a control group. After intramuscular treatment with poly-sulfated glycosaminoglycan (GAG), COMP concentration was decreased in OA dogs in comparison with healthy dogs. The analysed 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 and synovial fluid compared to the control group after naturally occurring OA (Misumi et al., 2002). COMP value was also increased after experimentally induced OA by meniscectomy (Carlson et al., 2002; Lindhorst et al., 2000). 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).

2.2.2 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 peri-cellular 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. Immunohistochemistry indicated increased levels of FN and transforming growth factor β (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 isoforms of FN encoded by a single gene. There are two isoforms 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 isoform 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 $(V+C)^-$ isoform 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 isoform 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 isoform 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 analysed 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 signalling 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-1β, IL-6 or FN fragments) stimulate meniscus cells to produce more metallo-proteinases 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 chemokine's, cytokines and matrix degrading enzymes (Stone et al., 2014).

2.2.3 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.

2.2.4 Hyaluronan

Hyaluronic acid (HA) or hyaluronan, a polymer of molecular mass up to 10 kDa, 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 (2-DE) 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 (Przybysz et al., 2009; Steffey et al., 2004).

During canine orthopaedic 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 not promising biomarker neither in human RA nor in canine OA because its circulating level varies with physical activity and diurnal periods, reducing its effectiveness as a reliable clinical biomarker (Engströum-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).

2.2.5 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 $_kB$ (NF-$_kB$) (Iovu et al., 2008).

In canine hip dysplasia, the two isotopes (WF6 and 3B3) of CS were analysed to evaluate the process of OA. The results showed that CS epitope WF6 level was higher and 3B3 was lower 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).

2.2.6 Keratan sulfate

Keratin sulfate (KS) is an abundant element in aggrecan and thus much effort was made to develop a canine OA biomarker in the past (Stone et al., 2014). In humans, KS level in serum was not related to severity of 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 (Hegemann et al., 2002; Lindhorst et al., 2000). 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.

2.2.7 Aggrecan

Aggrecan is the substantial proteoglycan of cartilage tissues with a molecular weight of 220 kDa and is responsible for hydrodynamic functions, including weight bearing and elasticity. Furthermore, aggrecan 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 MMPs (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 (200-250 KDa) 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).

2.3 Concluding remarks about serological analysis of canine OA

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 SF, 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 for human 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 previously 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 analyse different proteins and peptides through matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF 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 dog's 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.

Table 1: Overview of studies reporting 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	Auto-antibodies	↑	↑ CCL		SF	82 dogs	ELISA	(De Rooster et al., 2000)
	C2C		↑ MCD		SF	19 disease + 8 control dogs	ELISA	(Prink et al., 2010)
	Col2-3/4C long mono and CTX-II	↑ in Stifle joint			SF	20 large mixed breeds	ELISA	(Matyas et al., 2004)
	Neo-epitope TIINE		↑ meniscect-omy		Urine		TIINE 45-mer assay	(Settle et al., 2010)
COMP	COMP	↑			Serum	16 OA + 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 + 22 control dogs	ELISA	(Steffey et al., 2004)

Biomarker Type	Fragment	Specificity			Sample Type	Number of animals	Method of detection	Reference
		OA	Diseased	Healthy				
Hyaluronan			↓ CCL		SF	Surgically induced OA in 6 + 21 control dogs	ELISA	(Venable et al., 2008)
		↓			SF	49 dogs	ELISA	(Plickert et al., 2013)
			↓ HD		Serum	25 disease + 98 control dogs	ELISA	Nganvongpanit et al., 2008)
			↓ induced OA			12 dogs	ELISA	(Budsberg et al., 2006)
Chondroitin sulfate	WF6		↑ HD		Serum	25 disease + 98 control dogs	ELISA	(Nganvongpanit et al., 2008)
	3B3		↓ HD		Serum	25 disease + 98 control dogs	ELISA	Nganvongpanit et al., 2008)
	3B3		↑CCL		SF	8 disease + 24 control dogs	ELISA	(Johnson et al., 2001; Johnson et al., 2002)
	7D4		↑CCL		SF		ELISA	(Johnson et al., 2001; Johnson et al., 2002)

Biomarker Type	Fragment	Specificity			Sample Type	Number of animals	Method of detection	Reference
		OA	Diseased	Healthy				
Keratan sulfate			↑ induced OA		Serum	12 dogs	ELISA	(Budsberg et al., 2006)
	5D4	↓			SF		ELISA	(Hegemann et al., 2002; Matyas et al., 2004)
Aggrecan	aggrecan 846 epitope		↑ CCL		Serum	20 mixed breed dogs	Radio-immno assay (RIA)	(Matyas et al., 2004)
	ARGN and AGEG peptides degraded fragments		↑ After canine menisc-ectomy		SF		Aggrecan neoepitope 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)

Biomarker Type	Fragment	Specificity			Sample Type	Number of animals	Method of detection	Reference
		OA	**Diseased**	**Healthy**				
MPO	Myeloper oxidase		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)

2.4 The use of proteomics in diagnosing osteoarthritis

Proteomics is an approach that helps to understand the expression, regulation and function of individual protein. So far, it is a very beneficial tool to develop new biomarkers in different orthopaedic diseases. Therefore, biomarkers are reliable to diagnose diseases at early stages. Proteins can be analysed in different body fluids such as SF (Oliviero et al., 2009), serum and plasma by employing two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE). It is a modern and accurate technology that had used to identify proteins' patterns in human rheumatoid arthritis (Smith et al., 2001) and equine orthopaedic diseases (Chiaradia et al., 2012). The segregation of different proteins can perform based on charge and mass ratio rather than posttranslational modifications that are evident in one dimensional gel electrophoresis.

The combination of 2-DE with silver staining technique gave better, constant and reproducible results in following samples' analysis such as synovial, serum and synovial tissue, collected from rheumatoid patients (Fritz et al., 1990). 2-DE is a puissant and far-flung applicable technique for proteomic analysis, which introduced in early 1970s. Proteins separated because of isoelectric focusing (IEF) in 1-DE whereas it upgraded in presence of sodium dodecyl sulphate (SDS) in 2-DE for segregation on basis of molecular weight. In both electrophoresis, poly-acrylamide gel matrix is being utilized for proteins' separation and resulting spots are being visualized through mass spectrometry (Beranova-Giorgianni, 2003).

Surface-enhanced laser desorption and ionization-time-of-flight mass spectrometry (SELDI-TOF-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and MALDI-TOF-MS techniques had successfully established to identify different proteins pattern in serum, plasma, SF and cartilage. Chromatographic technique has a better advantage over electrophoresis because there is no need of proteins extraction from gel, which enhances its sensitivity level. However, the combination of both is more competent to segregate proteins from complex sample mixtures. Mascot, an identification tool, is also being utilized to appraise the results which are created by MS (Ceciliani et al., 2014).

Electro-spray ionization (ESI) and MALDI are most reliable methods to generate protein ionization for mass spectrometric analyses. MALDI-TOF-MS is normally preferable to use in simple sample but liquid chromatography has its implementation in complex mixture analysis (Aebersold and Mann, 2003). It has been seen that de-staining procedure of 1-D or 2-D silver stain gel ameliorates the vehemence, eminence and sensitivity of mass peptides findings through MALDI-TOF-MS (Imai and Mische, 1999).

2.5 Canine and equine proteomics

Veterinary proteomics analysis was carried out on body fluids such as synovial, serum and cartilage in horses, and Mascot software package was used for analysing protein spots. The following proteins showed the augmented pattern in OA such as α-2 macroglobulin (α-2 MG), complement component C4A (C4A), carboxylesterase D1 (CE-D1), ceruloplasmin (CP), serotransferrin (ST), antithrombin III, vitamin D binding protein, inter-α-trypsin inhibitory heavy chain, apolipoprotein A-I isoforms (Apo AI) and serum albumin in SF. On the Contrary, proteins such as afamin, plasminogen, globulin gamma 1 heavy chain, immunoglobulin heavy constant gamma 5, transthyretin, haptoglobin and alpha 1B glycoprotein showed less appearance in SF. Autologous conditioned serum (ACS) treatment revealed the down regulation of following proteins such as ApO-A1, ST, CP, α-2 MG, C4A and CE-D1 (Chiaradia et al., 2012).

The role of interleukin (IL-1β) or its combination with or without anti-inflammatory drug (carprofen) tested on explants meta-carpophalangeal cartilage. The extracted peptides visualized by LC-MS/MS and protein profiling summarized, which further analysed by western blotting. The assay confirmed many proteins such as cartilage intermediate protein-1, clusterin (Clutterbuck et al., 2011), thrombospondin-1 (TSP-1), matrix metalloproteinases MMP1 and MMP3. The effect of interleukin (IL-1β) enhanced the level of matrix metalloproteinases, thrombospondin-1 but lower the level of clusterin confirmed through western blotting assay (Clutterbuck et al., 2011).

Cartilage oligomeric matrix protein (COMP) is a glycoprotein, which has an elementary role in collagen fiber stabilization and extracellular matrix (ECM)

maintenance. The equine tendon stimulated with IL-1β and prostaglandin E2 in order to induce COMP degradation and further assessed this feature through LC-MS/MS as well as multiple reaction monitoring. Interleukin (IL-1β) increased the proteolytic cleavage of COMP but prostaglandin E2 had no catabolic effect. At earlier stage of tendon injury, two cleavage fragments identified that could help in tendon disease neo-epitope assay (Dakin et al., 2014).

Cranial cruciate ligament rupture (CCLR) is a most common stifle joint orthopaedic disease in dogs. Therefore, LC-MS were selected to study serum and synovial fluid. No interesting results had found after serum analyses. Conversely, eleven different proteins had significantly expressed in SF. Out of these proteins; complement component 3 and factor I strongly expressed in SF. This was the first proteomic analysis suggested these proteins involved in OA (Garner et al., 2013).

In a different study, canine OA developed by the transaction of anterior cruciate ligament in eight dogs. A proteomic analysis of dog's serum has performed to understand patho-physiology through 2-D electrophoresis and MALDI-TOF-MS. Complement C3 and fetuin B proteins showed aggrandizement pattern but a number of proteins such as complement C1s, C4, inter-alpha-trypsin inhibitor H4 (ITIH4) and hyaluronan binding protein 2 express declined expression in serum between control and OA group. These investigations are important step towards biomarker development for earlier OA disease diagnosis in canine (Gharbi et al., 2013).

2.6 Concluding remarks on Proteomic analysis

Proteomics analysis proved truly a milestone to explore body fluid proteins in order to analyse regulatory patterns, up or down regulation of proteins in different orthopaedic diseases in human and veterinary medicine. Furthermore, human proteomics analysis is a more advance and onrush technique for discovery of biomarkers in OA diseases as compared to veterinary field. Indeed, it is a better guidance for veterinary researchers to explore pathology and develop biomarkers under the light of these outcomes. Although proteomics analysis in veterinary sciences had started a decade later,

but it has opened new horizons and channels to understand pathology as well as earlier diagnosis of OA.

3 Objectives of the study

The objectives of the present study, these are as follow:

1. Comparison of quantitative total protein concentration difference between SF from diseased dogs and healthy controls as a possible diagnostic tool.
2. To identify highly different expressed protein components in SF and serum of diseased dogs compared to healthy controls.
3. To develop new biomarkers through serological analyses that can be utilized as diagnostic tool in different canine orthopaedic diseases.

4 Materials and Methods

4.1 Patients and control dogs

All dogs for this study were presented at the Small Animal Clinic at the Freie Universität Berlin, Germany between April 2013 and October 2014. Because samples were collected from dogs as part of a routine clinical evaluation at the hospital or from dogs that were euthanized, approval of the university's animal care committee was not required. Client permission was obtained for use of the samples.

Each dog was recorded for age, weight, breed, and sex (Table 2). A total of 41 SF samples were collected from dogs, brought to the clinic for surgical treatment of cruciate ligament rupture with or without medial meniscus damage ($n = 16$), fragment coronoid process ($n = 3$), hip dysplasia ($n = 10$) and idiopathic polyarthritis ($n = 12$). First of all, orthopaedic examination was performed for each patient. The specialized orthopaedic examination diagnosed effected joint. OA was diagnosed by a specialized veterinary orthopaedic surgeon based on different criteria such as, degree of lameness, pain because of flexion and extension, joint effusion and crepitation (Hanson et al., 2006; Hazewinkel et al., 2008; Peterson and Keefe, 2004). The specialized radiologist further confirmed radiographic evidence of joint osteoarthritis including periarticular osteophytosis and bone sclerosis (Aragon and Budsberg, 2005; Hazewinkel et al., 2008). The specifics of the orthopaedic examinations are depicted in the Appendix of the thesis.

Table 2: General characteristics of dogs with OA in which SF samples (*n* = 41) were collected

Sample	Breed	Age (y)	Sex	Weight (Kg)	OA category	Disease Duration (Days)	Disease
1	Saint Bernard	0.5	Male	30	Degenerative	3	Polyarthritis
2	Saint Bernard	0.5	Male	30	Septic arthritis	3	Polyarthritis
3	Great Dane	1.1	Male	35	Degenerative	5	Hip dysplasia right
4	Jack Russell	2.5	Male	7	Degenerative	3	CCLR right
5	Mix	5	Female	25	Degenerative	15	CCLR right
6	Mix	6	Male	40	Degenerative	3	CCLR right
7	Beagle	7.5	Male castr.	23	Septic arthritis	4	CCLR right
8	Boxer	7.5	Female	25	Degenerative	28	CCLR left
9	Rottweiler	7	Male castr.	58	Degenerative	7	Polyarthritis
10	Rottweiler	7	Male castr.	58	Degenerative	7	Polyarthritis

Sample	Breed	Age (y)	Sex	Weight (Kg)	OA category	Disease Duration (Days)	Disease
12	Saint Bernard	0.5	Male	30	Degenerative	3	Polyarthritis
13	Saint Bernard	0.5	Male	30	Degenerative	3	Polyarthritis
14	Dogue de Bord.	1	Male	45	Degenerative	30	Hip dysplasia
15	German Shepherd	4	Male castr.	35	Degenerative	7	FPC
16	Mix	0.75	Male castr.	55	Degenerative	30	Hip dysplasia
17	Rottweiler	1	Male	39	Degenerative	60	Hip dysplasia
18	Rhod. Ridgeback	0.5	Male	16	Degenerative	30	CCLR right
19	Bobtail dog	9	Male	31	Degenerative	4	CCLR right
20	Mix	9	Male	9	Degenerative	60	CCLR right
21	Rottweiler	11	Female	45	Degenerative	90	Hip dysplasia
22	Bull dog	1	Female castr	25	Degenerative	14	Polyarthritis

Sample	Breed	Age (y)	Sex	Weight (Kg)	OA category	Disease Duration (Days)	Disease
23	Am. Staffordshire terrier	5.5	Male castr.	36	Degenerative	5	CCLR right
24	English bull dog	1	Male	21	Degenerative	30	FPC
25	Great Dane	1	Male	35	Degenerative	5	CCL right
26	Mix	4	Male	40	Degenerative	20	Hip dysplasia
27	Bull dog	1	Female castr.	25	Degenerative	14	Polyarthritis
28	Am. Staffordshire terrier	7	Male cast.	36	Degenerative	2	CCLR right
29	Boxer	10	Male castr.	39	Degenerative	2	CCLR left
30	Saint Bernard	6	Male	59	Degenerative	12	FPC
31	German Shepherd	1	Male	22	Degenerative	20	Hip dysplasia
32	German Shepherd	0.5	Male	20	Degenerative	3	CCLR right

Sample	Breed	Age (y)	Sex	Weight (Kg)	OA category	Disease Duration (Days)	Disease
33	German Shepherd	10	Male	32	Degenerative	10	CCLR right
34	German Shepherd	0.75	Male	32	Degenerative	36	Hip dysplasia
35	Jack Russel	13	Male castr.	8	Degenerative	4	CCLR left
36	German Shepherd	1	Male	48	Septic arthritis	60	Hip dysplasia
37	Golden retriever	10	Female	27	Degenerative	14	CCLR meniscus
38	Mix	10	Male castr.	40	Degenerative	90	Hip dysplasia
39	Labrador	1	Male	29	Degenerative	60	Polyarthritis
40	Labrador	1	Male	29	Degenerative	60	Polyarthritis
41	Saint Bernard	0.5	Male	30	Degenerative	3	Polyarthritis

The control group was comprised of 8 dogs with no clinical history of OA, which were euthanized other than orthopaedic and tumor diseases (Table 3).

Table 3: General characteristics of the control dogs (*n* = 8) in which SF samples (*n* = 17) were collected

Sample	Breed	Age (Years)	Sex	Weight (Kg)	Joint of SF collection
1	German shepherd	9	Male	33	Elbow right
2	German shepherd	9	Male	33	Elbow right
3	Rottweiler	11	Male	27	Elbow right
4	Rottweiler	11	Male	27	Knee right
5	Rottweiler	11	Male	27	Knee left
6	Rottweiler	12	Female	43	Knee right
7	Rottweiler	12	Female	43	Knee left
8	Bull dog	2	Male	27	Elbow right
9	Bull dog	2	Male	27	Elbow right
10	Mix	7.5	Male	40	Knee right
11	Mix	7.5	Male	40	Knee left
12	Cocker spaniel	2	Male	22	Knee left
13	Cocker spaniel	2	Male	22	Knee right
14	Dalmatian	7.5	Male	35	Knee right

Sample	Breed	Age (Years)	Sex	Weight (Kg)	Joint of SF collection
15	Dalmatian	7.5	Male	35	Knee right
16	Great Dane	2	Male	40	Knee right
17	Great Dane	2	Male	40	Knee left

4.2 Collection and preservation of the synovial fluid samples

Before surgery, orthopaedic surgeon collected a minimum of 1 ml SF from hip, stifle and elbow joints, which suffered from hip dysplasia, CCLR and elbow dysplasia respectively. The SF was also collected from canine idiopathic polyarthritis joints. Arthrocentesis procedure was performed by standard methods (De Camp and Schaefer, 2016). After SF collection, general cytology was immediately done including determination of colour, viscosity, total number of cells, protein refractometric readings and glass slide in order to evaluate cytology under higher power magnification microscope as shown in Table 4 (Brunnberg L et al., 2014), while remaining SF centrifuged at 4000 rpm at 4 °C for 4 minutes to remove any cells or particulate matter. After centrifugation, 10 µl of a protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany) was added in 0.5 ml SF and stored at -80 °C until further analysis.

To obtain samples from control dogs, a minimum of 0.5 ml SF was collected aseptically from joint within 1 hour after death by arthrocentesis procedure as described previously. The control SF was collected from 5 right elbow joints, 7 right knee joints and 5-left knee joints. After collecting SF, its cytology was studied and 10 µl of a protease inhibitor cocktail (Sigma-Aldrich) was added immediately to each sample, and the samples were stored at -80 °C until further analyses.

Table 4: Cytological examination of SF in order to differentiate normal to abnormal joints

	Normal joint	**Degenerative joint**	**Septic arthritis**	**Immune-mediated arthritis**
Quantity/Colour	Clear	Yellowish	Yellow-reddish	↑/ Yellowish
Transparency	Transparent	Transparent	Turbid/cloudy	Transparent to turbid
Viscosity	Very high	High	Very low	Very low
Nucleated cell number (/µl)	< 1000	1000 – 5000	>> 5000	1100 – 87000
Neutrophil (%)	< 5	< 10	> 90	15 - 95 (> 70)
Mononuclear cells (%)	> 95	> 90	> 10	5 – 85
Protein (g/l)	< 25	> 20	> 40	> 25 (> 40)

4.3 Blood sampling and serum collection

A blood sample (approximately 4 ml) was collected from 16 healthy dogs (table 5) and 13 dogs (table 6) with OA via cephalic vein in order to perform complete blood analysis and serum was prepared by centrifugation (2500 x g, 10 min). Each serum sample was then stored in an airtight container at –80 °C until further analysis.

Table 5: General characteristics of the healthy dogs (*n* = 16) in which control serum samples (*n* = 16) were collected

Sample	Breed	Age (Years)	Sex	Weight (Kg)
1	Terrier mix	9,2	Male cas	13
2	Shar pei	6	Female	18
3	Münsterländer	11,7	Male cas	29
4	Mix	8	Female	10
5	Fox terrier	11,33	Male	7
6	Mix	11,5	Male	11
7	Labrador	1,9	Male	27
8	Labrador	1,9	Female cas	29
9	Sonnenhund	12	Male	24
10	Golden Retriever	1,7	Male	29
11	Golden Retriever	1,7	Female	26
12	Mix	10,4	Female	12
13	Fox terrier	10,5	Female cas	11
14	Mix	6	Male	19
15	Cocker spaniel	12	Male	14
16	Cocker spaniel	12,5	Female	13

Table 6: General characteristics of the diseased dogs (*n* = 13) in which disease serum samples (*n* = 13) were collected

Sample	Breed	Age (Years)	Sex	Weight	Disease Duration (Days)	Disease
1	German Shepherd	1,2	Male	48	30	Hip dysplasia
2	Rottweiler	1	Male	39	50	Hip dysplasia
3	Jack Russel Terrier	2,5	Male	7	2	CCLR right
4	Bull dog	1,2	Female Cas	25	7	Polyarthritis
5	Boxer	7,7	Female	25	3	CCLR left
6	Boxer	9,8	Male Cas	39	5	CCLR left
7	Mix	7,2	Male Cas	24	14	Hip dysplasia
8	Mix	3	Male Cas	25	34	CCLR right
9	Bernese Mountain Dog	0,5	Male	30	4	Polyarthritis
10	Great Dane	1,1	Male	35	60	Hip dysplasia

Sample	Breed	Age (Years)	Sex	Weight	Disease Duration (Days)	Disease
11	Bernese Mountain dog	4,3	Male	48	7	Hip dysplasia
12	Mix	3,8	Male	40	24	Hip dysplasia
13	Mix	5,5	Male	36	3	CCLR Right

4.4 Protein quantification with Bradford Assay

The concentration of total protein in SF was measured using the Bradford method (Bradford, 1976). This method is based on the binding of protein to Coomassie brilliant blue G-250. The absorption maximum of the reagent solution increases to 595 nm when proteins are bound. Using spectrophotometric determination of absorbance allows measurement of the protein concentration in the solution.

The technique was used in this research that actually based on a reaction between Coomassie Briliant Blue G-250 (CBB) with basic amino acid residues in proteins. In fact, this method is very popular, sensitive, rapid and low inference by other substances. The results might vary in protein detection, therefore standard protein was used during Bradford assay. The binding of CBB with proteins involved an electrostatic interaction of the dye and positive charged amino acid (Arg and Lys) (Ku et al., 2013).

4.4.1 Equipment and Materials

- Microtiter plate (Greiner, Frickenhausen, Germany)
- Piston pipet and tips (Eppendorf, Wesseling-Berzdorf, Germany)
- Microtiter plate reader (Bio-Rad, Munich, Germany)
- Microplate manager software (Bio-Rad)
- Vortexer (Roth, Karlsruhe, Germany)
- 2 ml reaction tube (Roth, Karlsruhe, Germany)

4.2.2 Chemicals

- Albumin Bovine fraction pH 7.0 (Serva, Heidelberg, Germany)
- Bradford reagent
- 100 mg brilliant blue (Commassie blue) G250 in 50ml ethanol solution (Roth, Karlsruhe, Germany)
- Overnight stirred using a magnet mixer (Roth, Karlsruhe, Germany)
- Add 100 ml 85% phosphorus salt (Roth, Karlsruhe, Germany)
- To fill with 1 liter HPLC-H_2O (Roth, Karlsruhe, Germany)
- Filtration
- Siemens N Protein Stand SL (Siemens QQIM13, Marburg, Germany)

- HPLC H$_2$O

4.4.3 Procedure

1. The standard series were prepared from 2 mg/ml BSA stock solution. For this, 2 mg BSA was poured into a 2 ml tube with 1 ml HPLC water.

2. From the stock solution, 1:2 dilutions were prepared 8 times to give a total of 8 solutions ranging between 1 mg/ml (S1) and 0.007 mg/ml (S8).

3. 70 µL with 1:200 diluted serum or SF and 630 µL Bradford reagent were pipetted in 1.5 ml reaction tubes (doubled but equal in numbers).

4. The standard samples were prepared by taking 70 µl standard solution and 630 µl Bradford reagent. The blank had 70 µL of distillated HPLC water.

5. 150 µL of each sample, standard solution and blank was applied in triplicate to wells of a microtiter plate.

6. Absorbance was immediately evaluated at a wavelength of 595 nm in the spectrophotometer using the microplate manager software. The protein concentrations were calculated from the standard curve using the machine specific software.

4.5 Protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) allows the separation of proteins depending on their molecular weight (Laemmli, 1970). This method has been used as one dimensional gel electrophoresis under, either reducing or non-reducing conditions, due to their simplicity and high reproducibility. SDS denatures secondary and non-disulfide linked tertiary structures of proteins by adding negative charge to each protein in proportion to its mass. Thus, the motilities of negatively charged proteins are the linear function of their molecular weights, whereby smaller proteins move faster than lager proteins through the pores of the polyacrylamide gels during the electrophoretic run (Dunbar, 2012). This method and modifications of it are reliable methods for protein analysis and their characterization in basic research and clinical investigations as well.

Proteomics is an approach that helps to understand the expression, regulation and function of individual protein. Since, it is a very beneficial tool to develop new biomarkers in different orthopaedic diseases. Therefore, biomarkers are conducive to

diagnose diseases at early stages. Proteins can be analysed in different body fluids such as; synovial fluid (Oliviero et al.), serum and plasma by employing two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE). It is a modern and accurate technology that has been used to identify proteins' patterns in human rheumatoid arthritis (Smith et al., 2001) and equine orthopaedic diseases (Chiaradia et al., 2012). The segregation of different proteins can be performed based on charge and mass ratio rather than posttranslational modifications that are evident in 1-dimensional electrophoresis. The combination of two-dimensional electrophoresis with silver staining technique gave better, constant and reproducible results in following samples' analysis such as; synovial, serum and synovial tissue, collected from rheumatoid patients with statistical p value < 0.1 (Fritz et al., 1990). 2-DE is a puissant and far-flung applicable technique for proteomic analysis, which introduced in early 1970s. Proteins separated because of iso-electric focusing (IEF) in 1-DE whereas it upgraded in presence of sodium dodecyl sulphate (SDS) in 2-DE for segregation on basis of molecular weight and IEF. In both electrophoresis, poly-acrylamide gel matrix is being utilized for proteins' separation and resulting spots are being visualized through mass spectrometry (Beranova-Giorgianni, 2003). We utilized in our research 1dimensional SDS-PAGE technique to separate different proteins in SF diseased and controlled samples.

4.5.1 Equipment and materials

- Gel casting apparatus, Mini Protean 3 (Bio-Rad)
    - Casting stand
    - Spacer plate 0.75 mm
    - Short plates
    - Combs
    - Rubber seal
    - Glass plate fixer
    - Sample loading guide

- Electrophorese application, Mini protean 3 (Bio-Rad)
- Buffer reservoir, Mini protean 3 (Bio-Rad)

Materials and Methods

- Spinning machine
- Beaker glass
- Magnetic stirrer
- Piston pipet
- Reaction container
- Needle
- Water heater
- Plastic wrap

4.5.2 Chemicals

- Rotiphorese Gel 30 (Roth, 3029.1)
- TEMED (Roth, 2367.3)
- Low-molecular weight range standard (Bio-Rad)
- 10% Ammonium per sulphate (APS)
  - 1 g Ammonium per sulphate (Roth, A3678) + 9 g HPLC water (Roth, A511.2)
- Running buffer
  - 100 ml Tris glycine SDS buffer (Bio-Rad, 161-0772) + 900 ml aqua distillated water
- 0,5 M SDS/Tris/HCl pH 6.8
  - 30.29 g Trisbase (Roth, 5429.3, 121.14 g/mol)
  - 2 g SDS (Roth, 2326.1, 288.38 g/mol)
  - In HPLC water resolve (Roth, A511.2)
  - With 1M HCl pH-value adjusted
  - With HPLC water (Roth, A511.2) 500 ml to fill
- 1,5 M SDS/Tris/HCl pH 6.8
  - 90.87 g Trisbase (Roth, 5429.3, 121.14 g/mol)
  - 2 g SDS (Roth, 2326.1, 288.38 g/mol)
  - In HPLC water resolve (Roth, A511.2)
  - With 1M HCl pH-value adjusted
  - With HPLC water (Roth, A511.2) 500 ml to fill

➢ Sample buffer (SDS/Mercaptoethanol)

- 1.52 g Trisbase (Roth, 5429.3, 121.14 g/mol)
- 2 g SDS (Roth, 2326.1, 288.38 g/mol)
- 100 mg bromophenol blue (Sigma, B-6131)
- 20 ml glycerol (Roth, 3783.1, 92.10 g/mol)
- 2 ml mercaptoethanol (Roth, 4227.1)
- With concentrated HCl pH 6.8
- With HPLC water (Roth, A511.2) 100 ml to fill

4.6 Protein separation by gel electrophoresis

Prior to electrophoresis, glass plates were washed with distilled water. Precautionary measures were carried out to check either the glass plates were too tight or not. Therefore, water was filled with a pipette between the glass plates and removed with help of tissue paper. It was assured that there was no leakage of water after filling the glass plates. The gel material was poured between two glass plates. But, it was assured that gel was not poured so quickly and bubble formation was prevented.

The 12% sandwich gel was freshly prepared by mixing chemicals of stacking and separating gel and the gel was poured with the help of a piston pipet. The polymerization of the gel was initiated by TEMED and 10% APS. First, the separating gel was poured and overlaid with distilled water to remove air bubbles. One should be careful until the gel is not rigid. After that, the gel was left for one hour at room temperature (22-25 °C) for polymerization and the distilled water was rinsed and the stacking gel solution was layered on the top of the separating gel. The 10 or 15 well comb was carefully inserted into the stacking gel, before the gel was polymerized. The glass plates were wrapped with transparent film in order to prevent from dryness. The glass plate was properly labelled and stored overnight at 4°C. The serum or SF samples were diluted 1:50 with Laemmli sample buffer and were boiled for 5 minutes in a water bath. A needle was inserted on the lid of the reaction vessel because it prevented sample to come outside the vessel.

Before the electrophoresis procedure, the gel cassette was placed into the electrophoresis operation chamber and filled with running buffer. After removal of comb

from gel, it was carefully filled with sample by using a piston pipet. The 10µL/slot sample or low-molecular weight range standard was poured into each slot with pipet. Then, electrophoresis apparatus was connected properly with lid (red with red and black with black). After that, the gel was run with 15 mA/gel and ampere remained constant for 10 minutes. Subsequently, the gel was run with 30 mA/gel till the end of the electrophoresis procedure. The process ended when there was clear bromophenol blue bands formation and reached on the lower edge of the glass plates. The electrophoresis procedure duration depended on electricity voltage.

4.7 Coomassie brilliant blue staining

Coomassie brilliant Blue R (R250) is common used and makes staining bands, and this method is relatively straightforward. The staining of protein happens through the ionic interaction between the basic amino acids and the acidic dye. The secondary interactions occur because of hydrogen bonding, Vander Waals forces, and hydrophobic bonding between protein and dye as well as free dye and dye already attached with the protein. Different factors affect these interaction, including pH, ionic concentration, and solvents. So, most proteins bind with different amounts of dyes under different conditions (Wilson, 1983). Staining variations can also appear when sodium dodecyl sulfate (SDS) is bound to proteins. This is a specific problem if poor-quality SDS is used that comprises with impurities that may further affect both relative mobility of the proteins and intensity of the stain (Dunbar, 2012).

4.7.1 Equipment and Materials

- Magnetic stirrer
- Measuring cylinder
- Petri dishes
- Chemi Coc XRS Imager (Bio-Rad)
- Gel carrier
- Shaking incubator
- Tissue paper

4.7.2 Chemicals

- ➢ Decolorize 10% (Acetic Acid)
    - ○ 200 ml Acetic Acid (Roth, 3738.5)
    - ○ 1800 ml Aqua dist.
- ➢ Colour solution
    - • One tablet Phast Gel Blue R (GE Healthcare, 17-0518-01)
    - • Dissolve in 2 l 10% acetic acid overnight

4.7.3 Gel coloration and documentation

200 ml colour solution was required to stain the gel and it was incubated at 50 °C for 20 minutes. The excessive colour of Coomassie blue stain was removed with 10 % acetic acid. The absorbing tissue was placed inside the boundary of petri dish plate. Again, the gel was incubated at 50 °C for 20 minutes. Thereafter, the Coomassie blue stained gels were examined using the Chemi Coc XRS and the software Quantity One from Bio-Rad. The molecular mass of the proteins was evaluated by comparing with the low range molecular weight standard which included the following bands: 14.4 kDa, lysozyme; 21.5 kDa, trypsin inhibitor; 31 kDa, carbonic anhydrase; 45 kDa, ovalbumin; 66.2 kDa, serum albumin; 97.4 kDa, phosphorylase b.

4.8 Western blot analysis

Western blotting (WB) analysis is a technique, which is used to distinguish a specific protein from complicated mixture of proteins. Firstly, the different protein bands are separated on their molecular weight from a complicated mixture through gel electrophoresis. The interested protein bands are incubated with labelled antibodies to identify and measure the concentration of specific protein. WB is involved through different steps such as: preparation of sample, separation of protein band through gel electrophoresis, transferring the protein from the gel to the membrane, incubation with specific antibody, imaging and data analysis. Although the procedure of WB is simple but many problems could be raise during experimental procedure such as production of unusual bands, no bands, faint bands, and patchy spots on the blot. These problems produce unexpected results and should be handle carefully.

Materials and Methods

Western blotting (Protein blotting or immune-blotting) is an important procedure for proteins detection, particularly proteins that are of low abundance in a synovial fluid, serum or any complex protein mixture. This process involves the transfer of protein patterns from gel to Poly-vinyl-di-fluoride membrane. Actually, transfer of proteins to membranes was described in 1979 (Kurien and Scofield, 2015). The WB technique has certain advantages; a) it is quite easy to handle wet membrane, b) the proteins move on the membrane very quickly and different bands equally accessible, c) a small amount of reagents is needed for transfer analysis, d) it is also possible to make multiple replicates of a gel, d) prolong storage is also possible.

We performed western blotting procedure as described previously (Kurien and Scofield, 2015), in which protein bands were transfer from SDS-PAGE to an absorbent membrane. By using a thinner gels, transfer becomes more faster und complete. High molecular mass proteins blot poorly following SDS-PAGE, therefore low levels of detection on immune-blots. However, using heat, special buffers, and partial proteolysis digestion of the proteins could facilitate this problem and enhanced the efficiency of transfer.

PVDF, nitrocellulose, activated paper or activated nylon has been utilized to bind transferred proteins successfully. However, PVDF membranes have advantage due to its physical strength, chemical stability and high protein binding capacity. Therefore, we utilized PVDF membrane in our research. Moreover, nitrocellulose membranes cannot be stained with Coomassie brilliant blue. But PVDF membranes are flexible while staining with Coomassie brilliant blue. Activated paper binds with proteins through covalent binding that is disadvantageous because coupling method is incompatible with mostly gel electrophoresis system. Moreover, the paper is quite expensive and after the paper activation the reactive groups have a limited half-life. Therefore, we did not use activated paper and preferred PVDF membranes. Nylon has powerful mechanical strength but can only bind with a small amount of proteins, therefore it is not suitable for most applications (Kurien and Scofield, 2006). Therefore, PVDF has a better advantageous compare with other related options and became a best choice to utilize in this experimental procedure.

Proteins transferred from SDS-PAGE to PVDF membranes and were achieved through three different ways: a) simple diffusion b) vacuum-assisted solvent flow and c) western blotting. Simple diffusion procedure was developed originally in order to transfer proteins that separated by iso-electric focusing on thin gels to membranes. In this procedure, a membrane is placed on the surface of the gel and certain weight facilitated to diffusion process. After that, mass spectrometric performed to identify proteins. The transferred gel is stained with Coomassie brilliant blue. The major advantage of diffusion blotting compared to electro-blotting is that many transfers or imprints can be achieved from the same gel and further different antisera can be applied on identical prints. In vacuum blotting a suction pump attached with a gel dryer system and used to suck the different polypeptides from the gel to membrane. Electro-blotting is the most commonly used procedure in order to transfer proteins from a gel to membrane. Actually, it is a fast and accurate method compared to diffusion and vacuum blotting (Kurien and Scofield, 2006). We used wet transfer in which, the sandwich is placed in a buffer tank connected with electrodes.

4.8.1 Equipment and materials

- Tank blot apparatus (Bio-Rad)
  - Buffer reservoir
  - Ice pad
  - Western blot element
  - Blot cassette
  - Pads
  - Lid
- Power Pac 3000
- Magnetic mixer
- Filter papers
- Poly-vinyl-di-fluoride (PVDF) blot membrane (Millipore)
- Magnetic stirrer
- Petri dishes
- Chemi Coc XRS Imager

4.8.2 Chemicals

- ➢ 10x Botting transfer buffer
  - • 29.3 g Glycin (Roth, Karlsruhe, Germany)
  - • 58.1 g Tris base (Roth, Karlsruhe, Germany)
  - • 3.8 g SDS (Roth, Karlsruhe, Germany)
  - • With Aqua dist. 1000 ml to fill
- ➢ Blotting transfer buffer
  - • 100 ml 10x transfer buffer
  - • 700 ml Aqua dist.
  - • 200 ml methanol (Roth, Karlsruhe, Germany)
- ➢ 10 x TBS pH 7.5
  - • 60.55 g Tris base (Roth, Karlsruhe, Germany)
  - • 87.66 g NaCl (Roth, Karlsruhe, Germany)
  - • With Aqua dist. 1000 ml to fill
- ➢ 1 x TBS/0.1% Tween 20
  - • 100 ml 10x TBS pH 7.5
  - • 900 ml Aqua dist.
  - • 1 ml Tween 20 (Sigma)
- ➢ Blocking solution
  - • 10 g milk powder (Roth, Karlsruhe, Germany)
  - • Dissolved in 200 ml TBS/0.1% Tween 20
- ➢ Chemiluminescence solution
  - • 9 ml solution 1 (Roche) and 100 µl solution 2 (Roche, Mannheim, Germany)

4.8.3 Western blot procedure

Following 12% reducing SDS-PAGE electrophoresis, the gels were removed from the glass cassettes. The stacking gel was discarded and the resolving gel was incubated in 1 x transfer buffer for 15 min at 25 °C. During the gel incubation, six pieces of high-grade filter papers were cut to suitable size of the gel and soaked with two cassette sponges in 1xtransfer buffer for 15 min to equilibrate before blotting. An appropriate sized piece of PVDF membrane was cut to exact size of the gel and soaked for 5 min in 100% methanol.

The transfer apparatus was filled with 1 x transfer buffer and the transformation for two gels was undertaken at 100 V for 1 h at 4°C on ice. Afterwards, the PVDF membrane was washed and non-specific binding sites of the membrane were blocked with blocking buffer for 1 h. The solution was discarded and the membranes were incubated with the primary sheep anti-human polyclonal antibody (Gene Tex, Irvone, CA, USA) diluted 1:500 in TBS 0.1% Tween 20 overnight at 4°C. Coupling of the secondary antibodies was performed with horseradish conjugated polyclonal rabbit anti-sheep IgG (Dako) for 1 h at 25°C. Antibody binding was visualized using the Luminol reaction. Band intensity of Apo AI was read with an imager and analysed with the Quantity one software.

4.9 MALDI-TOF mass spectrometry and protein identification

Synovial proteins were separated by SDS-PAGE. Further, gels were stained with colloidal Coomassie brilliant blue staining (Candiano et al., 2004). Protein bands of interest were excised from gel, de-stained, reduced with TCEP (Tris- (2-carboxyethyl)-phosphin hydrochlorid, Roth, Karlsruhe, Germany), alkylated with iodacetamide (Sigma, Deisenhofen, Germany) and digested with trypsin (Serva, Heidelberg, Germany) at 37°C overnight. After digestion, the peptides were analysed with an Auto flex speed MALDI-TOF mass spectrometer (Auto flex speed, Bruker, Bruker Daltonik, Karlsruhe, Germany) as described elsewhere (Reeg et al., 2016). The mass spectra were transferred to BioTool software (BioTool, Bruker Daltonik, Bruker) for alignment with SwissProt database (http://www.uniprot.org/) by using the Mascot search engine (Matrix Science, London, UK). Search parameters were as follows: taxonomy was restricted to canines, Mass tolerance was 200 ppm, used enzyme was trypsin, missing cleavages ≤1 were accepted and variable modifications were defined as carbamidomethylation of cysteine. For reliable identification with Mascot a P values of < 0.05, corresponding to a protein score > 57, were expected. Additionally, results were accepted as potent candidates if sequence coverage was > 20%.

Surface-enhanced laser desorption and ionization-time-of-flight mass Spectrometry (SELDI-TOF-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) techniques had successfully established to identify different proteins pattern in serum, plasma, synovial fluid and cartilage. Chromatographic technique has a better advantage over

electrophoresis because there is no need of proteins extraction from gel, which enhances its sensitivity level. However, the combination of both is more competent to segregate proteins from complex sample mixtures. Mascot, an identification tool, is also being utilized to appraise the results which are created by MS (Ceciliani et al., 2014).

Electro-spray ionization (ESI) and MALDI are most reliable methods to generate protein ionization for mass spectrometric analyses. MALDI-MS is normally preferable to use in simple sample but liquid chromatography has its implementation in complex mixture analysis (Aebersold and Mann, 2003). It has been seen that de-staining procedure of 1-D or 2-D silver stain gel ameliorates the vehemence, eminence and sensitivity of mass peptides findings through MALDI-MS (Imai and Mische, 1999).

4.10 Quantification of Apolipoprotein A-I

A commercial ELISA for measurement of canine Apolipoprotein A-I (Apo AI) concentration in SF and serum was used in accordance with the manufacturer's instructions (Cusabio, College Park, MD, USA). The minimum detectable Apo AI concentration was 7 ng/ml. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively, according to the manufacturer user manual. The measurement of Apolipoprotein was also carried out through commercial available ELISA both in SF as well as in serum between control and diseased samples.

Briefly, the ELISA was performed by using a microtiter plate, the wells of which particularly precoated with Apo AI. The process of competitive inhibition was mediated between Apo AI in the wells of microtiter plate and unknown concentration of Apo AI in the SF samples or a standard protein (control). The samples in the wells were incubated along with a horseradish peroxidase-conjugated antibody that was specific for Apo AI. After that, color was produced by the addition of 3,3'5,5'-tetramethylbenzidin. The reaction was stopped by the addition of a stop solution, and the spectromphotometer was used to measure the light absorbance of each well at 450 nm.

4.11 Statistical analysis

Statistical analyses were done using SPSS version 23.0 (SPSS, version 23.0, Munich, Germany). The Kolmogorov-Smirnov-test was used to confirm that the data were not normally distributed. The differences between the groups were analysed the Mann-

Whitney U test for non-parametric. Differences were considered significant at a P value of less than 0.05.

5   Results

5.1 Evaluation of the patient group

The group with OA comprised of 41 dogs with a median age of 2.5 years (range: 0.42 to 13 years) of which there were 4 intact and 2 spayed bitches, 25 intact and 10 neutered males. The most frequently represented breeds were German shepherd ($n = 6$), St. Bernard ($n = 7$) and mix-breed ($n = 6$) and in total, other 13 different breeds were represented. Weights ranged from 7-59 kg with a median of 30 kg. Out of 41 dogs, 16 had cranial cruciate ligament rupture, 3 had elbow dysplasia, 10 had hip dysplasia and 12 had canine idiopathic polyarthritis. After cytological examination, these dogs were further categorized into degenerative ($n = 38$) and septic joints ($n = 3$).

The percentage of lameness, pain, joint effusion and crepitation was calculated from different joint as well as the radiological findings are summarized in Tables 7 and 8 respectively. Intra-operative findings were summarized in table 9. The following structure of joints including joint capsule, synovial membrane, bone cartilage and bone structure of knee joints ($n = 16$), elbow joints ($n = 3$) and hip joints ($n = 10$) were evaluated during surgery. The postoperative lameness, pain, joint effusion and crepitation were controlled after 8 and 16 weeks that were significantly reduced. The orthopaedic examination was really essential to diagnose CCLR, hip dysplasia, elbow joint dysplasia and poly-arthritis joints.

The diseased serum samples were collected from 13 dogs ($n = 13$) with a median age 3 years (range: 0.5 to 9.8 years) of which were 1 intact and 1 spayed bitches, 8 intact male and 3 neutered males. The most dominant was mix breed. Weights ranged from 7-48 with a median of 35 Kg.

Table 7: Summary of the presence of lameness, pain, joint effusions and crepitation in the patient group

Grade	Lameness		Pain		Joint effusion		Crepitation	
	Number of joints	% of joints	Number of joints	% of joints	Number of joints	% of joints)	Number of joints	% of joints
0	1	2.4	2	4.9	4	9.8	30	73.2
1	4	9.8	14	34.1	7	17.1	8	19.5
2	22	53.7	21	51.2	18	43.9	3	7.3
3	14	34.1	4	9.8	12	29.2	0	0
Total	41	100	41	100	41	100	41	100

Results

Table 8: Radiological examination from knee joints, elbow joints, hip joints and polyarthritis joints

Grade	Total Joints		Knee joint		Elbow Joint		Hip joint		Polyarthritis Joint	
	Joints	% of Joints	CCL Joints	% of Joints	Elbow Joints	% of Joints	HD Joints	% of Joints	PA Joints	% of Joints
0	4	9.8	4	25	0	0	0	0	0	0
1	16	39.0	8	50	2	66.7	3	30	3	25
2	14	34,1	2	12.5	1	33.3	5	50	6	50
3	7	17.1	2	12.5	0	0	2	20	3	25
Total	41	100	16	100	3	100	10	100	12	100

Table 9: Intra-operative findings of knee joints (*n* = 16), elbow joints (*n* =3) and hip joint (*n* = 10)

Grade	Joint capsule and synovial membrane		Bone cartilage		Bone	
	Joints	(%)	Joints	(%)	Joints	(%)
0	8	27.6	11	37.9	12	41.4
1	11	37,9	10	34.5	10	34.5
2	8	27.6	7	24.1	5	17.2
3	2	6.9	1	3.5	2	6.9
Total	29	100	29	100	29	100

5.2 Evaluation of the control group

The control SF group comprised 8 dogs with a median age of 7.5 years (range: 1.7 to 11.7 years). It included 1 intact female and 7 intact males. The median weight of the control dogs was 33 kg (range: 22 to 43 kg). While, the control serum group comprised of 16 dogs with a median age of 9.8 years (rang: 1.7 to 12.5 years), median weight was 16 kg (range: 7 to 29 kg) and were predominantly mixed breeds. Furthermore, it included 5 intact female and 2 spayed bitches, 7 intact male and 2 neutered males.

5.3 Quantitative and qualitative protein analysis

Quantitative protein analysis of synovial fluid (Figure 1) revealed that dogs with OA had significantly ($P < 0.001$) higher protein concentrations [median: 32.1 (range: 18.3-77.1) mg/ml] in comparison with healthy control dogs [19.8 (5.49-31.3) mg/ml].
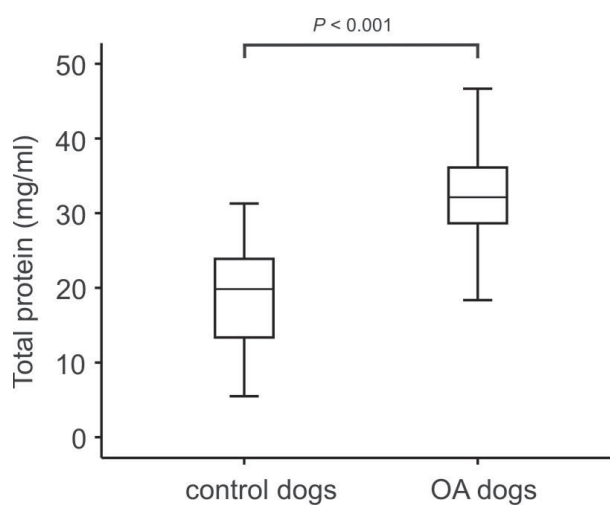
Figure 1: Comparison of synovial total protein concentration in control dogs and dogs with osteoarthritis (OA).

In order to characterize the synovial proteins qualitatively, the proteins were separated by one-dimensional SDS-PAGE according to their molecular weight as described in material and methods. Two representative gel images of the control dogs (Figure 2) and dogs with OA (Figure 3) are given.
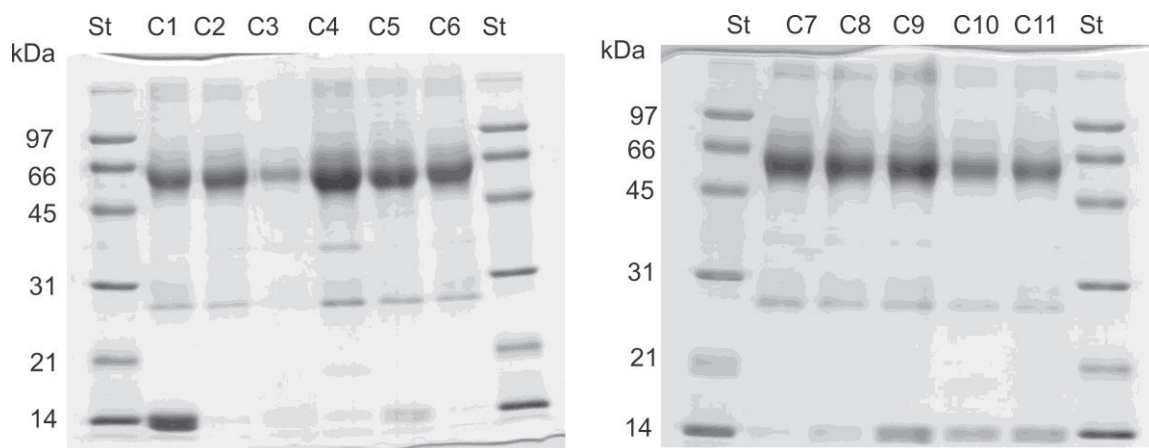
Figure 2: Representative SDS-PAGE profiles of proteins from synovial fluid from control dogs (C1-C11) stained with Coomassie brilliant blue. St, molecular weight standard proteins.
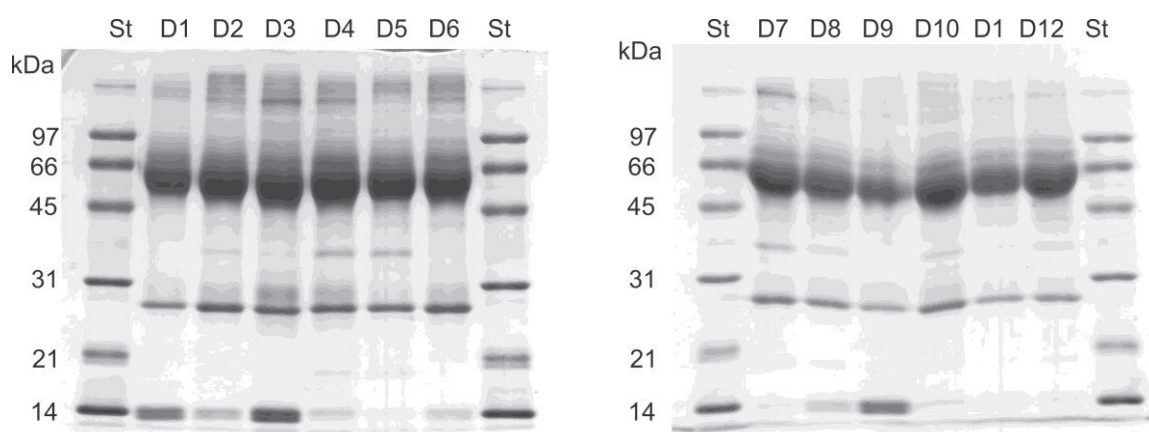
Figure 3: Representative SDS-PAGE profiles from synovial fluid from diseased dogs (D1-D12) stained with Coomassie brilliant blue. St, molecular weight standard proteins.

Results

The molecular weight ranges and the frequency of appearance of the protein bands in each molecular weight range are shown in Figure 4. Protein bands in the molecular weight ranges of 21-30 and 51-60 kDa dominated with the highest relative quantities in both, control and OA dogs (Figure 2 and 3). Analysis of the relative quantity of protein bands between control and OA dogs were significantly different in the molecular weight ranges of 21-30, 41-50, 51-60, 71-80, 81-90, 111-120, 131-140, 151-160 and > 180 kDa (Figure 4, Table 10).
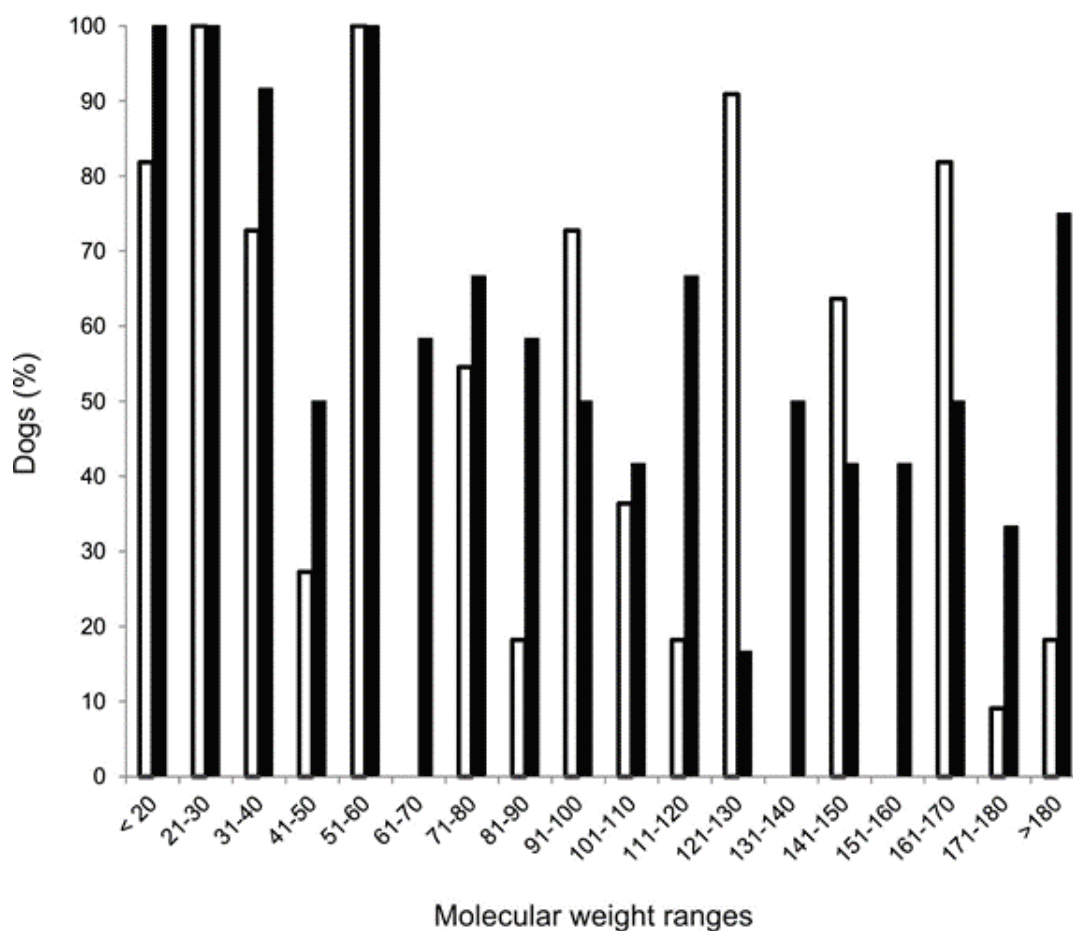
Figure 4: Frequency of dogs, which had bands of each molecular weight range, as detected by SDS-PAGE analysis. White bars indicate the control dogs and black bars indicate dogs with OA.

Table 10: Relative quantity of protein bands of each molecular weight range in SF of control dogs and dogs with OA

Molecular weight range (kDa)	Control dogs (%)	Dogs with OA (%)	P-value
< 20	7.41 ± 5.81	7.72 ± 4.57	0.89
21-30	9.19 ± 3.73	13.2 ± 2.59	0.007
31-40	2.98 ± 1.28	3.58 ± 2.79	0.52
41-50	0.38 ± 0.98	3.29 ± 2.94	0.005
51-60	65.8 ± 20.6	43.2 ± 4.44	0.001
61-70	5.40 ± 6.27	7.64 ± 6.38	0.42
71-80	0.31 ± 1.04	4.05 ± 3.80	0.005
81-90	1.02 ± 2.41	3.67 ± 3.03	0.035
91-100	1.09 ± 1.56	2.09 ± 2.67	0.29
101-110	0.50 ± 0.96	1.17 ± 1.40	0.203
111-120	0.26 ± 0.78	1.74 ± 1.40	0.006
121-130	1.33 ± 1.40	0.50 ± 1.10	0.13
131-140	0.09 ± 0.31	1.67 ± 1.52	0.003
141-150	0.77 ± 1.08	0.97 ± 1.67	0.74
151-160	Nd	1.75 ± 2.20	0.016
161-170	2.31 ± 2.20	1.91 ± 2.22	0.68
171-180	0.42 ± 1.38	1.22 ± 1.82	0.25
>180	0.80 ± 2.02	2.72 ± 1.74	0.031

5.4   Protein identification by MALDI-TOF-MS

The significantly different protein bands were excided from the gels and analysed by MALDI-TOF-MS after trypsin digestion. Among these proteins, serum albumin (66 kDa), haptoglobin (36.5 kDa), and Apolipoprotein A-I (Apo AI; 30.1 kDa) were identified using the Mascot search engine. However, only serum albumin and Apo AI revealed a significant Mascot probability value < 0.05 (Figure 5 and Table 11). Actually, 17 proteins were identified on the gel. In fact, a number of proteins were not recognized with available canine proteins data. However, another protein database of other species to identify these proteins in this research were not used.
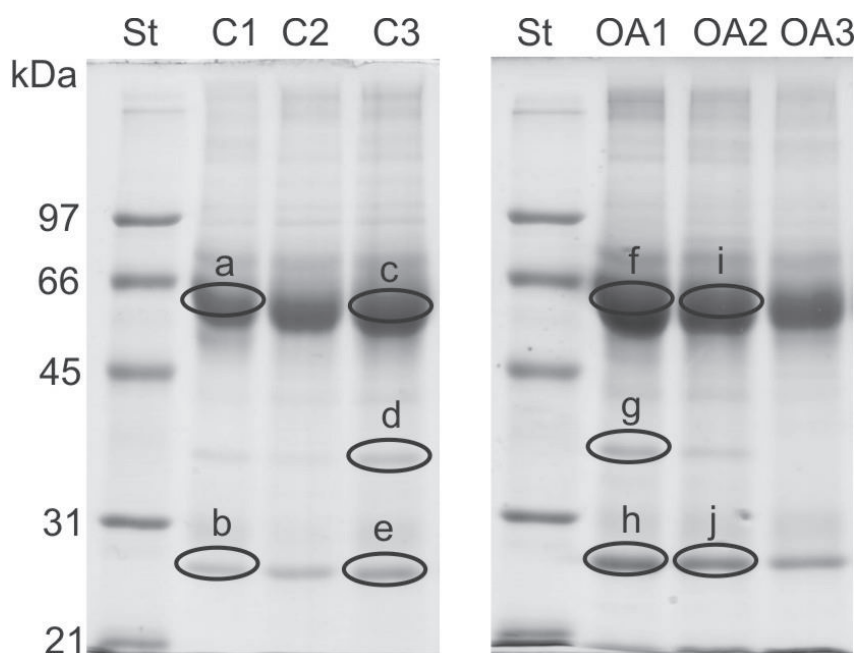
Figure 5: SDS-PAGE patterns of synovial proteins from healthy control dogs (C1-C6) and diseased dogs with OA (D1-D6). In boxes are enclosed the protein bands identified by MALDI-TOF-MS analysis as depicted in Table 9. St, molecular weight standard proteins.

Table 11: Proteins, which were identified in synovial fluid

Band Number	kDa	Protein name	Accession Number	Mascot Score	% Sequence coverage
A	68.6	Serum albumin	P49882	73*	28.9
B	30.1	Apolipoprotein A-I	P02648	92*	41.4
C	68.6	Serum albumin	P49882	81*	26.8
D	36.5	Haptoglobin	P19006	49	16.1
E	30.1	Apolipoprotein A-I	P02648	80*	29.3
F	68.6	Serum albumin	P49882	91*	29.1
G	36.5	Haptoglobin	P19006	49	13.4
H	30.1	Apolipoprotein A-I	P02648	96*	41.4
I	68.6	Serum albumin	P49882	149*	57.4
J	30.1	Apolipoprotein A-I	P02648	137*	50.8

* Mascot probability value <0.05

Furthermore, we measured the relative quantity of apolipoprotein A-I and albumin in SF. The relative quantity of albumin was higher in samples from control dogs compared to diseased samples due to unknown reason. However, the relative quantity of apolipoprotein A-I was higher in diseased samples compared to controlled samples (Table 10). So, the further validation of Apolipoprotein was performed through ELISA and western blotting analysis.

5.5   Quantification of Apoliporotein A-I by ELISA

A commercial available ELISA kit was used to quantify Apo AI concentrations in SF and serum from control dogs and dogs with OA. In synovial fluid, the concentrations of Apo AI were significantly higher ($P < 0.001$) in OA dogs [248 (39-488) μg/ml] compared to control dogs [0.0 (0-360) μg/ml] (Figure 6). Likewise, serum Apo AI concentrations were increased ($P < 0.001$) in the OA group [927 (631-1633) μg/ml] compared to the control group [239 (40-940) μg/ml] (Figure 7), although there was no difference in the concentration of total protein ($P = 0.19$; Figure 8). The detection range of ELISA was 28.12-1800 ng/ml. The SF and serum were diluted 1:1250 and 1:2500 folds (sample and sample diluent ratio) respectively. The ELISA kit was validated to determine Apo AI concentration in dog´s serum, plasma and tissue homogenates but it was not validated for SF.

Figure 6: Box plots of Apolipoprotein A-I (Apo AI) concentrations in synovial fluid of control dogs and dogs with osteoarthritis (OA). The box represents the interquartile range that is, 25-75% range; the horizontal bar represents the median value, and the T-bars represent the range of the data.
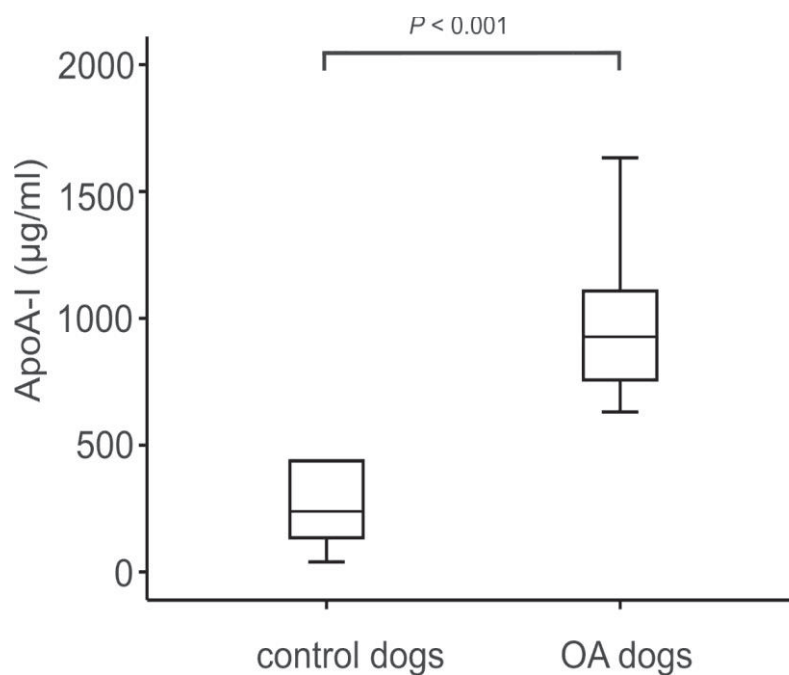
Figure 7: Box plots of Apolipoprotein A-I (Apo AI) concentration in serum of control dogs and dogs with osteoarthritis (OA). The box represents the interquartile range that is 25-75% range; the horizontal bar represents the median value, and the T-bars represent the range of the data.
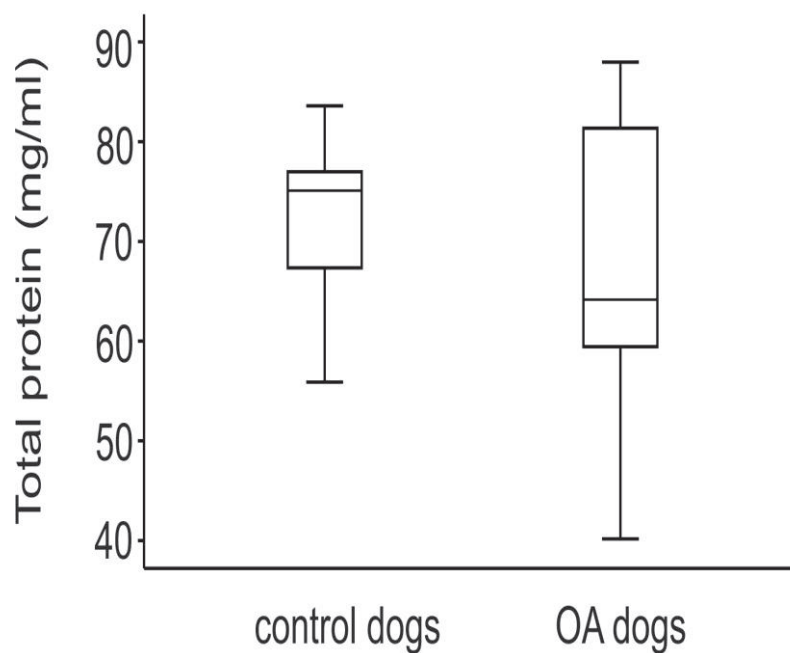
Figure 8: Box plot of total protein concentration in serum of control dogs and dogs with osteoarthritis (OA). The box represents the interquartile range that is 25-75% range; the horizontal bar represents the median value, and the T-bars represents the range of the data.

5.6 Quantification of Apoliporotein A-I through Western Blotting analysis

The concentrations of Apo AI was also measured in synovial fluid and serum from control dogs and dogs with OA through WB analysis. In SF, the concentrations of Apo AI were non-significantly in OA dogs [655704 (463561-755518) arbitrary units] compared to control dogs [669544 (534994-756925) arbitrary units] (Figure 9). However, serum Apo AI concentrations were increased ($P < 0.001$) in the OA group [386212 (334571-533605) arbitrary units] compared to the control group [280925 (255559-353235) arbitrary units] (Figure 10).
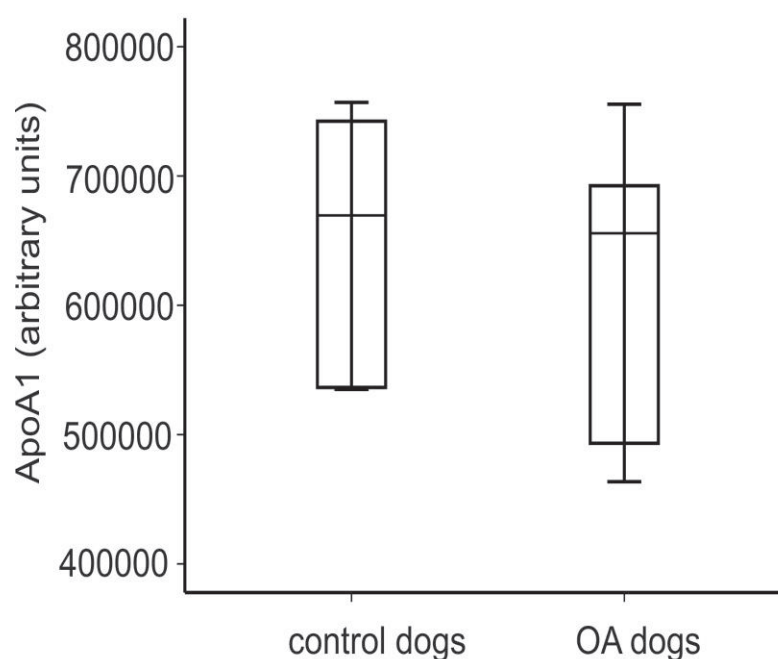
Figure 9: Box plots of Apolipoprotein A-I (Apo AI) concentrations in SF of control dogs and dogs with osteoarthritis (OA) using WB analysis. The box represents the interquartile range that is 25-75% range; the horizontal bar represents the median value, and the T-bars represents the range of the data.
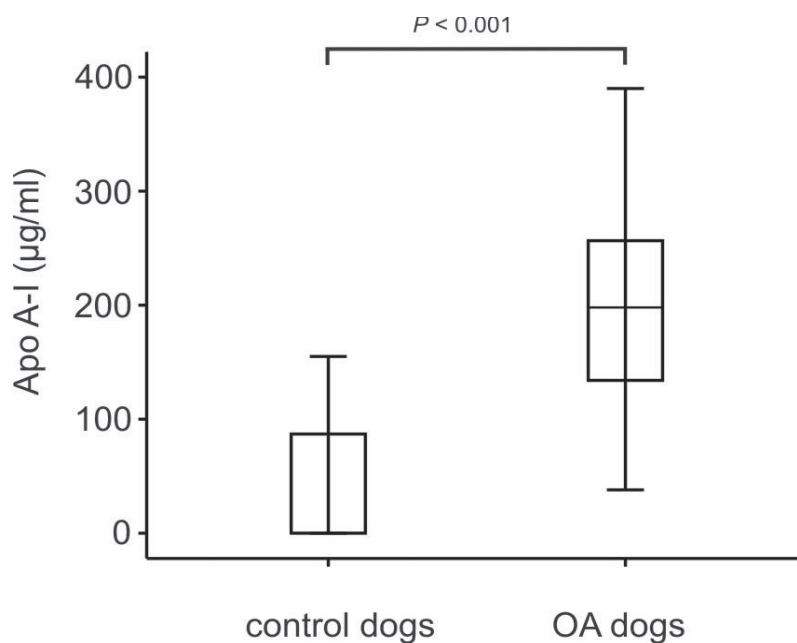
Figure 10: Box plots of Apolipoprotein A-I (Apo AI) concentrations in serum of control dogs and dogs with osteoarthritis (OA) using WB analysis. The box represents the interquartile range (ie, 25-75% range; the horizontal bar represents the median value, and the T-bars represent the range of the data.
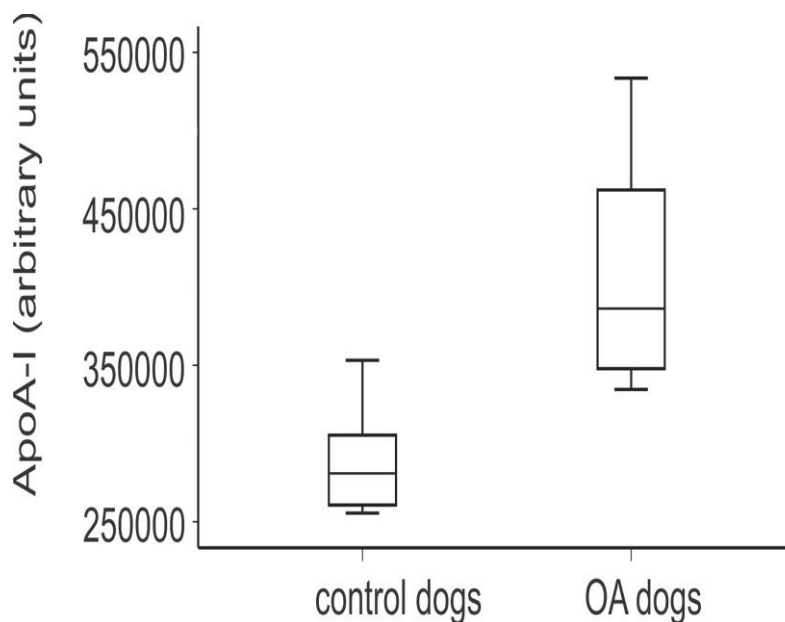
6 Discussion

In the present study, we collected 41 SF from different OA joints after naturally occurred orthopaedic diseases such as; CCLR, polyarthritis, hip and elbow dysplasia, which are further sub-divided into 16, 12, 10, and 3 joints respectively. On the other side, we collected controlled SF from 12 knees and 5 elbow joints. In the present study, firstly we measured total protein concentration in SF and it was significantly higher in OA dogs compared to the control group. We also compare total protein concentration difference between diseased and healthy knee joints. It was significant between CCLR and control joints. However, we did not compare the total protein concentration difference between diseased elbow, hip and polyarthritis joints with control samples because we did not have enough control SF samples from those joints, which is a limitation of the study.

Only a few studies measured total protein concentrations in canine SF. It was reported that the median protein concentration value was significantly higher in OA dogs compared to the control dogs. This finding is similar to the previously reported studies (Strøm et al., 1989; Walter et al., 2007). However, total protein concentration was also measured in canine cerebrospinal fluid as well (Riond et al., 2014). In another study, the determination of total protein concentration is a valuable diagnostic marker that is useful to diagnose canine idiopathic arthritis with a higher diagnostic accuracy than plasma C-reactive protein (cut off value of >2.54 g/dl with 58.3% sensitivity and 95.5% specificity) (Murakami et al., 2015). So, the measurement of total protein concentration in SF may be a useful parameter to diagnose canine OA because it can be assessed easily in a veterinary laboratory. Further clinical research is needed, whether the determination of total protein concentration is a simple test to support the diagnosis of OA in dogs.

1-DE on agarose gel is a commonly used diagnostic tool in canine diseases. However, little is known regarding the different proteins involvement in normal and diseased canine OA samples. One of the previous study applied both one and two-dimensional gel electrophoresis followed by tandem MS on canine serum proteomics. 1D-PAGE developed the normal serum ranges for albumin and globulin sub-fractions. 2-D PAGE analysed thirty-two distinct proteins between control and diseased samples. Out of these thirty-two proteins, twenty proteins belong to *Canis lupus familiaris* while the

Discussion

remaining 12 proteins belonging to other mammalian species. Possibly, proteins matching with other mammalian species were due to incomplete canine protein sequencing knowledge (Atherton et al., 2013). In the study, 1D-PAGE technique was applied and identified 17 different proteins and most of them were not matching with canine data.

Subsequent analysis of the qualitative protein patterns revealed proteins with relative high frequency in diseased SF in comparison with healthy control samples. A few studies have shown that alterations in synovial protein expression are related to the risk of developing canine OA and have been identified as promising biomarkers in the detection of the disease by implementing proteomic analysis (Garner et al., 2013; Gharbi et al., 2013). Therefore, different expressed protein bands were digested with trypsin and the resulting peptides analysed with MALDI-TOF mass spectrometry. Five proteins were identified but significant for albumin and Apolipoprotein A-I (Apo AI) due to Mascot probability vales.

The quantitative SF proteomics analysis was carried out in a previous study in order to elaborate the pathophysiological mechanism of canine stifle joint OA. This proteomics analysis utilized 1-gel electrophoresis and identified eleven different proteins (complement component 3 precursor, complement factor I precursor, Apolipoprotein B-100 precursor, serum paraoxonase, arylesterase 1, zinc alpha-2 glycoprotein precursor, serum amyloid A, transthyretin precursor, retinol binding protein 4 precursor, alpha-2 macroglobulin precursor, angiotensinogen precursor and fibronectin 1 isoform 1 preproprotein) in SF. The other animal species mass spectrometry entry database was matched with predicted canine proteins on the basis of homology (Garner et al., 2013). However, we use only canine mass spectrometry database to indicate and identify canine proteins. Indeed, we also implemented this technique to segregate different proteins in canine SF. But the major difference between our and previous research was depletion of abundant proteins. This research group used albumin and IgG depletion kit (Sigma-Aldrich, St Louis, Mo) to deplete abundant proteins. We did not use any depletion kit because removal of abundant proteins also removes less abundant proteins. So, it's better to deplete most abundant proteins from samples before running gel electrophoresis analysis in future research. Another difference between both studies was the identification

of proteins. We used MALDI-TOF-MS to identify proteins but previous study used Liquid chromatography-tandem mass spectrometry (LC-TMS). (Garner et al., 2013) also collected SF from stifle joint after CCLR surgery. It means that a large number of proteins expression changed significantly in SF after CCLR surgery secondary to OA development. But we collected diseased SF before surgery rather than after the surgery.

Another proteomics analysis identified different pattern of protein expression in canine osteoarthritis serum after CCLR (Gharbi et al., 2013). The serum proteomics analysis revealed the following over-expressed proteins fetuin B and complement C3. On the other hand, hyaluronan binding protein 2, inter-alpha-trypsin inhibitor H4, haptoglobin, complement C1s and C4 expression were decreased. In this study, canine OA was developed by the surgically transection of anterior cruciate ligament and serum samples were collected before and 12 weeks after surgery. It means that late OA samples were utilized to analyse protein expressions, which were more abruptly changed protein expression in dog's serum. (Gharbi et al., 2013) also removed most abundant proteins and used IgY-D11 dog specific column (Germany biotech company). It removed 11 abundant proteins in dog serum (Albumin, IgG, Apolipoprotein A-I, IgM, IgA, haptoglobin, α 2-Macroglobulin, α1-antitrpsin, α1-acid glycoprotein, Apo A-II, fibrinogen) (Gharbi et al., 2013). However, we did not remove abundant proteins by using any kit, therefore, we analysed the expression of most abundant proteins. The significant expression of albumin, haptoglobin and Apolipoprotein A-I were found in diseased and control samples. Therefore, abundant proteins can be used as diagnostic canine OA biomarker.

The previously implemented (Gharbi et al., 2013) methodology was more sensitive because 2-D gel electrophoresis and 2-D difference gel electrophoresis (2-D DIGE) were used, which can separate different proteins not only on their molecular weight but also on their charge as well. Three gels were used with labelling. Protein samples were labelled on lysine residues with Cy2 (Cydye DIGE fluor) for canine serum proteome mapping. The proteins of control samples were labelled with Cy5 (Cydye DIGE fluor) and proteins of OA samples were labelled with Cy3 (Cydye DIGE fluor). In comparison to this methodology, our analysis procedure was more simple, cheap and easily available in laboratory. We did not perform the labelling of proteins in disease and control samples.

Discussion

The acute phase response proteins can be divided into positive acute phase proteins (γ and β fibrinogen protein, α1- antitrypsin and haptoglobin family proteins) and negative acute phase proteins (albumin, Apolipoprotein A-I and transferrin) (Rosenkranz et al., 2010). Multiple bands of albumin (albumin, α1, α2, β2) and its precursor molecules were present in canine serum (Atherton et al., 2013; Gharbi et al., 2013) but further verification through serological analysis was not done. SF contains relative high abundance of Albumin and transferrin compared to fibrinogen, which are present in low concentration (Cretu et al., 2013). In juvenile idiopathic arthritis (JIA), different isoforms of the albumin in plasma (gi23307793, gi28592) and SF (gi119626069) were detected (Gibson et al., 2009) but serum albumin showed lower appearance in SF (Rosenkranz et al., 2010).

The identified Apo AI protein and the results were further verified through ELISA, revealing that a higher concentration of Apo AI in diseased SF and serum compared to control samples, which could be useful for canine OA diagnosis. Also utilized western bolting technique on diseased SF and serum compared to healthy samples. The analysis detected non-significant value difference between OA SF compared to control SF. It might be due the limited number of control SF samples. However, western blotting analysis revealed significant value in diseased serum compared to control serum samples. ELISA and western blotting techniques were used to validate a protein as a biomarker in canine OA. Both techniques are very essential to develop a biomarker. In previous research, researchers have identified a number of significant expressed proteins in diseased samples compared to healthy samples, but they did not implement ELISA and western blotting technique to validate these over expressed proteins to develop a biomarker. But, it is the advantage of this proteomics analysis that over expressed and significant proteins was validated through ELISA and western blotting analysis. So, Apo AI should be used as clinical diagnostic biomarkers in future perspective.

To the best of our knowledge Apo AI has not been reported yet in the context of canine OA. It is known that Apo AI is a negative acute phase protein, an essential part of high-density lipoprotein (Oliviero et al., 2009). It stimulates T-cells and binds together with macrophages, which enhances the permeability of endothelial barrier. It is also

interesting to know that it has inhibitory effect on the synthesis of TNF-α and interleukin-1β molecules. Therefore, Apo AI performs anti-inflammatory function in several orthopaedic diseases in human (Hyka et al., 2001).

In human rheumatoid arthritis, the primary extra-vascular inflammatory process considerably enhances HDL and Apo AI modulation both qualitatively as well as quantitatively (Terkeltaub, 2014). Additionally, Apo AI exhibits potential pro-inflammatory properties by inducing strong matrix metalloproteinase (MMP)-1 and MMP-3 expressions by chondrocytes and fibroblast-like synoviocytes, a pathophysiological mechanism involved in OA development (de Seny et al., 2015). Additionally, investigations in a rodent model of rheumatoid arthritis showed that Apo AI peptide hinders collagen-induced arthritis (Wu et al., 2014).

Similar to human rheumatoid arthritis, the high level of Apo AI protein in SF may modulate the process of disease development, inflammatory process and vasculitis (Trocmé et al., 2009). The high level of Apo AI has been measured in human rheumatoid arthritis SF (Ananth et al., 1993) as well as in juvenile idiopathic arthritis (Rosenkranz et al., 2010). Likewise, increased level of Apo AI has noticed in inflamed RA synovial tissues specifically in peri-vascular areas, which contains infiltrate of macrophages and T cells (Bresnihan et al., 2004). In equine OA, Apo AI showed increase level in SF. After treatment with autologous conditioned serum, this protein level had down regulated and joint showed better improvement in OA (Chiaradia et al., 2012).

Previous studies used the proteomic approach to identify various Apolipoprotein isoforms (Apolipoprotein C-I, Apolipoprotein H, Apolipoprotein A-IV, Apolipoprotein A-I, Apolipoprotein A-I precursor, Apolipoprotein A-II precursor, Apolipoprotein B-100 precursor, Apolipoprotein E precursor) in canine OA serum and SF (Atherton et al., 2013; Garner et al., 2013; Gharbi et al., 2013). However, these studies did not evaluate Apo AI in further clinical biomarker development. In the present study, quantitative ELISA measurement was carried out to evaluate whether Apo AI could be used as a diagnostic marker for canine OA. The data revealed that both, serum and SF Apo AI concentrations were significantly higher in the diseased group. These findings illustrate that Apo AI may be a novel and potential marker for the detection of canine OA. Even though, these results

are promising but further analyses should be performed on clinical cases to verify the usage of Apo AI as canine OA biomarker in serum and/or SF samples. In addition, it remains elusive, whether the depletion of abundant proteins will be a better strategy to explore complexity of proteins in canine orthopaedic diseases and whether such markers are reliable for clinical studies, because their identification and quantification further require sensitive procedure.

In conclusion, the results show that determination of total protein in SF may be helpful in the diagnostic of canine OA, because the present results showed a prominent total protein concentration difference between diseased SF compared to SF obtained from the control group. Moreover, higher Apo AI concentrations were shown in SF as well as in serum samples of the OA group. Therefore, Apo AI may be regarded as potential new biomarker to diagnose canine OA, which should be verified in further studies.

7 Future Perspective

Apolipoprotein use as a biomarker in episode schizophrenia (Song et al., 2014), hepatocellular carcinoma (Liu et al., 2014) and rheumatoid arthritis in human (Oliviero et al., 2009). According to our knowledge, Apo AI was not developed a biomarker in canine OA. So, we have developed Apolipoprotein a clinical biomarker in SF and serum in dog's OA after its confirmation through ELISA. On the other hand, we found a significant total protein concentration difference between diseased and healthy samples through Bradford analysis. Now, there is a substantial need to implant these parameters along with other diagnostic techniques (orthopaedic examination, radiography, computed tomography and general cytology) to diagnose OA in dog.

Proteomic analyses implemented recently in canine OA to detect general protein contents in biological fluids. It provides also a snapshot of the protein quantity change in reaction to pathological condition, disease development or medicinal treatment response. In this proteomic research, we used 1-D gel electrophoresis along with MALDI-TOF mass spectrometry. One dimensional gel electrophoresis is very simple technique, which separates protein contents on the basis of molecular weight. But in future research, it is important to use 2-D electrophoresis and automated multi-dimensional high-performance liquid chromatography (HPLC) in combination with mass spectrometry. These combinations of techniques are highly sensitive because 2-D electrophoresis separates proteins not only on their molecular weights but also on charges. The researcher will be in better position to explain and elucidate the patho-physiology of OA.

The collection of SF from healthy dogs was challenging. The SF was collected from dogs within 30 minutes after their death and did not have orthopaedic as well as tumor disease. We did not collect healthy SF from carpal, tarsal and hip joints that were the limitation of our research. Future researcher should collect healthy SF from carpal, tarsal and hip joints and should run quantitate protein analysis (Bradford analysis).

In recent research, we recognized most abundant proteins such as albumin, Apolipoprotein and hepatoglobulin in dog's SF and serum. These abundant proteins mask up and hide less abundant proteins. So, in future research it will very important to remove

these abundant proteins to explore less abundant protein in order to develop a clinical biomarker.

The depletion of abundant proteins carried out to expose less copious proteins in serum. Five different depletion columns such as; aurum serum protein mini-kit, multiple affinity removal columns, affinity depletion cartridges, albumin/ IgG removal kit, albumin and IgG removal kit exploited in order to remove massive proteins in human serum. Therefore, multiple affinity removal columns successfully removed albumin and IgG. At the same time, it was also appealing that multiple affinity removal columns removed four additional proteins (haptoglobin, transferring, IgA and α1-antitrpsin). After this procedure, depleted serum was further analysed through 2-D gel electrophoresis (2-DE) technique and results showed better emergence of low abundant proteins (Björhall et al., 2005). Human blood serum immuno-globulins also detached by using albumin/globulin proteins. That's why this procedure permits the manifestation of less abundant proteins such as human growth hormone, interleukin-12 and prostate-specific antigen (Adkins et al., 2002).

Proteomics analysis proved truly a milestone to explore body fluid proteins in order to analyse regulatory patterns, up or down regulation of proteins in different orthopaedic diseases in human and veterinary medicine. Furthermore, human proteomics analysis is a more advance and onrush technique for discovery of biomarkers in OA diseases as compared to veterinary field. Indeed, it is a better guidance for veterinary researchers to explore pathology and develop biomarkers under the light of these outcomes. Although proteomics analysis in veterinary sciences had started a decade later, but it has opened new horizons and channels to understand pathology as well as earlier diagnosis of OA.

8 Summary

Investigations on the quantitative and qualitative protein content in serum and synovial fluid of dogs with osteoarthritis

8.1 Background

The diagnosis of osteoarthritis in dogs remains a diagnostic challenge due to the lack of appropriate biomarkers. Therefore, this study was performed a proteomic approach to compare the protein patterns in dogs' synovial fluid and serum that might be helpful in elucidating pathways involved in joint damage and the identification of new diagnostic biomarkers.

8.2 Material and methods

SF was collected from dogs suffered from OA ($n = 41$) and healthy control dogs ($n = 8$), and serum was collected from OA dogs ($n = 13$) and healthy controls ($n = 16$). Protein concentrations were measured by Bradford analysis and were separated using SDS polyacrylamide gel electrophoresis. Proteins that were different between OA and healthy samples were digested by trypsin, analysed by MALDI-TOF mass spectrometry and compared with SwissProt database. A commercially available ELISA kit was used to evaluate Apo AI as diagnostic biomarker in SF and serum.

8.3 Results

Total protein concentrations were significantly higher ($P < 0.001$) in dogs with OA compared to control dogs. Serum albumin and Apolipoprotein A-I (Apo AI) were significantly identified in SF. Apo A-I was further confirmed in serum and SF by significantly higher ($P < 0.001$) concentration in dogs with OA compared to healthy controls.

8.4 Conclusion

The quantification of total protein and Apolipoprotein A-I concentrations in SF might be potential diagnostic biomarkers in canine OA.

9 Zusammenfassung

Untersuchungen zum quantitativen und qualitativen Proteingehalt im Serum und in der Gelenksflüssigkeit von Hunden mit Osteoarthritis

9.1 Hintergrund

Die Diagnose einer Osteoarhtitis bei Hunden bleibt aufgrund des Mangels an geeigneten Biomarkern eine diagnostische Herausforderung. Daher wurde in dieser Studie ein proteomischer Ansatz durchgeführt, um die Proteinmuster in Synovialflüssigkeit und Serum zu vergleichen, die bei der diagnostischen Aufklärung und Identifizierung neuer diagnostischer Biomarker bei Gelenkerkrankungen von Hunden hilfreich sein könnten.

9.2 Material und Methoden

Es wurde Synovialflüssigkeit von Hunden gesammelt, die an Osteoarthritis litten ($n = 41$) und gesunden Kontrollhunden ($n = 8$). Zusätzlich wurde Serum wurde von Osteoarthritis-Hunden ($n = 13$) und gesunden Kontrollen ($n = 16$) gesammelt. Die Proteinkonzentrationen wurden durch Bradford-Analyse gemessen und unter Verwendung von SDS-Polyacrylamid-Gelelektrophorese getrennt. Synovialproteine, die sich zwischen OA und gesunden Proben unterscheiden, wurden mit Trypsin verdaut, durch MALDI-TOF-Massenspektrometrie analysiert und mit der SwissProt-Datenbank verglichen. Ein kommerziell erhältlicher ELISA-Kit wurde verwendet, um Apo AI als diagnostischen Biomarker in SF und Serum zu evaluieren.

9.3 Ergebnisse

Die Gesamtproteinkonzentrationen waren bei Hunden mit Osteoarthritis im Vergleich zu Kontrollhunden signifikant höher (P <0,001). Es wurden Albumin und Apolipoprotein A-I (Apo AI) erfolgreich über die SwissProt-Datenbank identifiziert. Apo AI wurde im Serum und SF durch signifikant höhere (P <0,001) Konzentration bei Hunden mit Osteoarthritis im Vergleich zu gesunden Kontrollen weiter bestätigt.

9.4 Schlussfolgerung

Die Quantifizierung der Gesamtprotein- und Apo AI Konzentrationen in SF könnten potentielle diagnostische Biomarker in der Diagnostik der caninen Osteoarthritis sein.

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11 Appendix

11.1 Details on the orthopaedic examinations

11.1.1 Lameness

The degree of lameness is evaluated for every patient before surgery. The degree of lameness is divided into following grading system.

Grade 0: The patient has normal motion of effected limb and there is the no sign of lameness during walking and running accompanied with normal posture. The leg has full weight bearing capacity

Grade 1: There is the slight sign of lameness and the motion of leg is little bit restricted during movement. The normal posture of leg is little bit changed

Grade 2: There is significant lameness and change in posture; restricted movement of leg but it has slight weight bearing capacity

Grade 3: There is severe lameness and complete change in posture along with complete restricted movement and leg does not have weight bearing capacity

11.1.2 Pain

Grade 0: There is no sign of pain.

Grade 1: Pain during maximum flexion/extension redeemable

Grade 2: Pain during slight manipulation of the joint

Grade 3: Pain during touching of the joint

11.1.3 Joint effusion

The joint effusion of the hip joint is not remarkable during palpation. The knee joint effusion is tested during standing and laying position of the patient.

Grade 0: No joint effusion. The ligament patella is clearly in its boundary relative to their surrounding tissues

Grade 1: Slight noticeable joint effusion, ligament patella is on laterally and medially distinctly on its boundary. Joint capsule is appeared slightly bulged

Grade 2: There is moderate joint effusion and there is distinct swelling on both sides of ligament patella during palpation

Grade 3: There is significant joint effusion and ligament patella is clearly definable

The elbow joint is examined in standing position. The joint effusion is examined between humerus lateral epicondyle and olecranon.

Grade 0: There is no joint effusion. The bone points are relatively normal to the surrounding tissues and clearly differentiable

Grade 1: Slight noticeable joint effusion. The bone points are differentiable to the surrounding tissues. The joint swelling is examined between humerus lateral epicondyle and olecranon during palpation.

Grade2: There was moderate joint effusion. The bone points and joint capsule are contours with each other

Grade 3: There is significant joint effusion. Between the joint points, there is distinct protrusion of joint capsule during palpation

11.1.4 Crepitation

Grade 0: There is no crepitation

Grade 1: There is only slight crepitation but only noticeable during strong (hyper-flexion/hyperextension) palpation of the joint

Grade 2: There is moderate crepitation with moderate (hyper-flexion/hyperextension) palpation of the joint

Grade 3: There is significant crepitation with slight (hyper-flexion/hyperextension) palpation of the joint

11.2   Radiological Examination

The radiographic projections in two planes in knee joint, elbow joint and hip joint is obtained in the hospital. The knee joint (reference), elbow joint (reference) and hip joint are graded and evaluated.

11.2.1 Knee Joint

This grading score is also considered for polyarthritis joints because SF is collected from knee polyarthritis joints.

Grade 0: There is no OA in knee joint, tibial condyle sharply defined, eminence of the tibial inter-condylar elevated, sulcus extensor boundary clearly visible, femur condyle and ossa sesamoid sharp outlined, femur condyle sharply curved caudally, extensor fossa is not visible, patella sharply outlined, base and apex of patella very slight smooth and tapered

Grade 1: Low grade OA, tibial condyle blurred outlined, eminence of the tibial inter-condylar flattened, sulcus extensor boundary clearly visible, femur condyle and ossa sesamoid are not sharp outlined, extensor fossa visible, patella is not sharply outlined, base and apex of patella smooth and is not tapered

Grade 2: Medium grade OA, tibial condyle is not sharply defined, eminence of the tibial inter-condylar flattened, sulcus extensor boundary visible, femur condyle and ossa sesamoid are not sharp outlined, extensor fossa visible, lateral condyle in regard to medially noticeable, patella is not sharply outlined, base and apex of patella extracted

Grade 3: high grade OA, tibial condyle is not sharply defined, eminence of the tibial inter-condylar flattened, sulcus extensor boundary narrow and confined, tibial head caudally stretched, femur condyle and ossa sesamoid are not sharp outlined, extensor fossa enlarge, ossa sesamoid and trochlea ossis femoris detectable, femur condyle smooth, patella is not sharply outlined, base and apex of patella out stretched

11.2.2 Elbow joint
Grade 0: No sign of osteophytes and osteo-sclerosis

Grade 1: Low grade OA changes, osteophytes < 2 mm size one or multi-localization 1 to 8 or osteo-sclerosis

Grade 2: Medium grade OA, osteophytes 2-5 mm size one or multi-localization 1 to 8

Grade 3: high grade OA, osteophytes > 5 mm size one or multi-localization 1 to 8

11.2.3 Hip joint

Grade 0: There is no sign of OA, defined osteophytes and no sclerosis, especially no joint space narrowing, there is a deep seated ball (femoral head) and fit tightly into well formed socket

Grade 1: Low-grade OA changes, definite joint space narrowing, little bit osteophytes and sclerosis in the acetabular region

Grade 2: Medium grade OA, significant joint space narrowing, small osteophytes, there is sclerosis visible, deformity of femoral head and acetabulum

Grade 3: high grade OA, remarkable loss of joint space accompanied with osteophytes and sclerosis, increased deformity of the femoral head and acetabulum

11.3 Intra-operative macroscopic findings of the joints

This evaluation is not applied on poly-arthritis joints. During orthopaedic operation, the macroscopic findings of the joint including joint capsule along with synovial membrane, joint cartilage and bone structure was evaluated and graded in following grading system,

11.3.1 Joint capsule and synovial membrane

Grade 0: Joint capsule thin, elastic, synovial membrane moist, smooth, magnificent and bright

Grade 1: Joint capsule thin, elastic, synovial membrane red, low graded hypertrophy

Grade 2: joint capsule thick, rough elastic, synovial membrane red, medium graded hypertrophy

Grade 3: joint capsule fibrotic, synovial membrane more red, high graded hypertrophy

11.3.2 Joint cartilage

Grade 0: Joint cartilage uniform white, moist, smooth and bright

Grade 1: Joint cartilage partially changed in colour

Grade 2: Joint cartilage with spotted

Grade 3: Joint cartilage full or partially ulceration

11.3.3 Bone structure changes

Grade 0: Bones are smooth and no changes are remarkable

Grade 1: Little bit bone deform noticeable
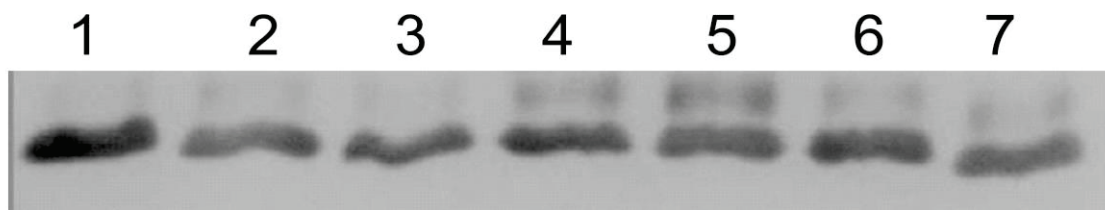
Grade 2: Exostoses formation noticeable

Grade 3: Significant exostoses observable

11.4 Raw data of Western Blotting analysis

The raw data of western blotting analysis is summarized from table 12 to 20.
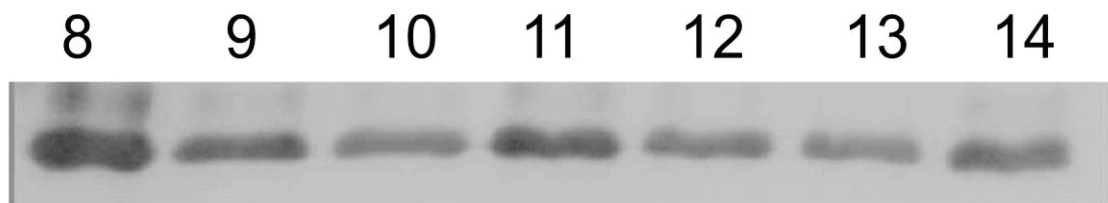
Table 12: The detail of diseased SF samples from 1 to 7 used in WB analysis

Gel name : 2015-10-23   06_2013 Kohorte 2   ApoA1-WB 01 bearbeitet (Raw 1-D Image)

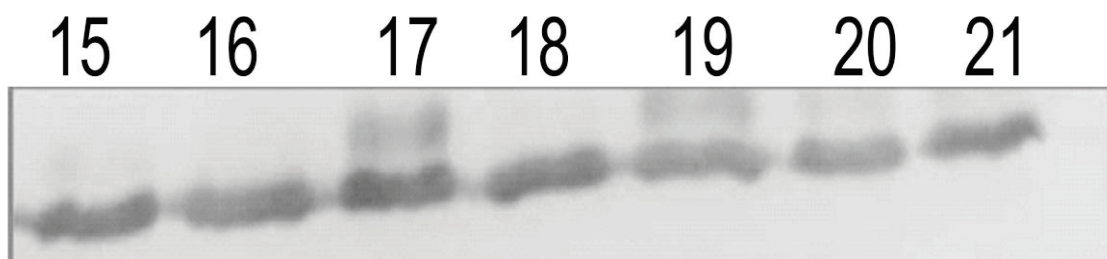| Index		Name	Volume INT*mm2	Adj. Vol. INT*mm2	Area mm2	Mean Value INT	Density INT/mm2
1	GELENKFLÜSSIGKEIT KRANK 02	U1	1901,818	318,376	2,771	686,237	531879,841
2	GELENKFLÜSSIGKEIT KRANK 03 KNIE LINKS	U2	1274,211	121,786	2,088	610,383	473087,580
3	GELENKFLÜSSIGKEIT KRANK 03 KNIE RECHTS	U3	1310,855	175,211	2,121	618,005	478995,843
4	GELENKFLÜSSIGKEIT KRANK 04 KNIE LINKS	U4	1523,256	231,779	2,393	636,456	493296,292
5	GELENKFLÜSSIGKEIT KRANK 04 KNIE RECHTS	U5	1387,965	139,084	2,240	619,681	480294,392
6	GELENKFLÜSSIGKEIT KRANK 07	U6	1640,883	237,822	2,605	629,913	488225,228
7	GELENKFLÜSSIGKEIT KRANK 08	U7	1497,029	188,968	2,503	598,092	463561,368

Table 13: The detail of diseased SF samples from 8 to 14 used in WB analysis

Gel name : 2015-10-23   06_2013 Kohorte 2   ApoA1-WB 02 bearbeitet (Raw 1-D Image)

Index		Name	Volume INT*mm2	Adj. Vol. INT*mm2	Area mm2	Mean Value INT	Density INT/mm2
8	GELENKFLÜSSIGKEIT KRANK 09 LUX	U1	2675,020	306,488	2,472	1082,109	838707,466
9	GELENKFLÜSSIGKEIT KRANK 09 TEP	U2	2140,022	399,913	2,249	951,613	737564,004
10	GELENKFLÜSSIGKEIT KRANK 10 ELLBOGEN LINKS	U3	1932,813	329,142	2,263	854,082	661971,186
11	GELENKFLÜSSIGKEIT KRANK 10 ELLBOGEN RECHTS	U4	2542,260	603,628	2,653	958,377	742806,722
12	GELENKFLÜSSIGKEIT KRANK 10 FUSS LINKS	U5	1261,436	119,752	1,395	904,438	701000,778
13	GELENKFLÜSSIGKEIT KRANK 10 FUSS RECHTS	U6	1794,805	315,798	2,227	805,965	624676,928
14	GELENKFLÜSSIGKEIT KRANK 10 HAND RECHTS	U7	1780,110	247,016	1,964	906,508	702604,704

Table 14: The detail of diseased SF samples from 15 to 21 used in WB analysis

Gel name : 2015-10-23   06_2013 Kohorte 2   ApoA1-WB 03 bearbeitet (Raw 1-D Image)

Index		Name	Volume INT*mm2	Adj. Vol. INT*mm2	Area mm2	Mean Value INT	Density INT/mm2
15	GELENKFLÜSSIGKEIT KRANK 11	U1	2282,820	365,478	2,591	881,145	682946,697
16	GELENKFLÜSSIGKEIT KRANK 12	U2	1886,084	163,612	2,128	886,502	687098,890
17	GELENKFLÜSSIGKEIT KRANK 13	U3	1924,380	216,904	2,048	939,839	728438,324
18	GELENKFLÜSSIGKEIT KRANK 14	U4	2157,100	323,407	2,441	883,667	684901,494
19	GELENKFLÜSSIGKEIT KRANK 15	U5	1311,805	84,420	1,575	832,708	645405,157
20	GELENKFLÜSSIGKEIT KRANK 16	U6	1238,193	107,466	1,543	802,411	621922,243
21	GELENKFLÜSSIGKEIT KRANK 17	U7	1366,574	175,228	1,615	845,996	655703,920

Table 15: The detail of diseased SF samples from 22 to 28 used in WB analysis
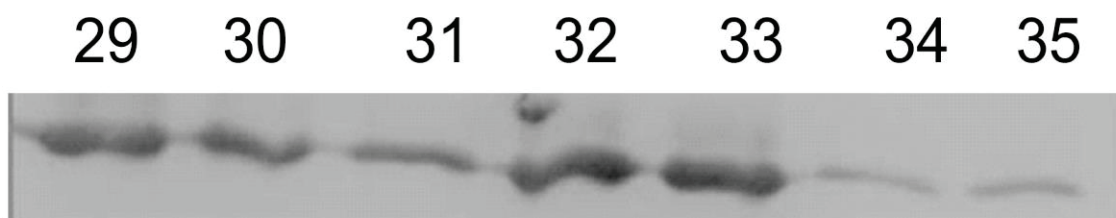

Gel name : 2015-10-23   06_2013 Kohorte 2   ApoA1-WB 04 bearbeitet (Raw 1-D Image)

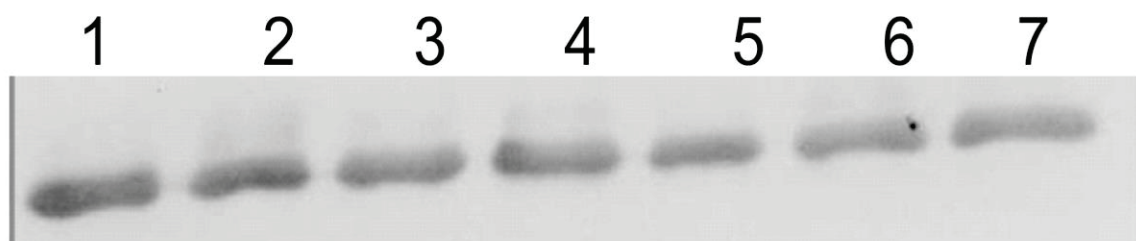| Index		Name	Volume INT*mm2	Adj. Vol. INT*mm2	Area mm2	Mean Value INT	Density INT/mm2
22	GELENKFLÜSSIGKEIT KRANK 18	U1	2057,727	365,239	2,291	898,016	696023,176
23	GELENKFLÜSSIGKEIT KRANK 19	U2	1275,275	66,045	1,308	974,777	755517,963
24	GELENKFLÜSSIGKEIT KRANK 20	U3	1125,002	80,398	1,259	893,393	692440,128
25	GELENKFLÜSSIGKEIT KRANK 21	U4	1215,239	54,043	1,267	959,157	743411,179
26	GELENKFLÜSSIGKEIT KRANK 22	U5	709,762	43,535	0,799	888,712	688812,035
27	GELENKFLÜSSIGKEIT KRANK 23	U6	1664,529	245,724	1,993	835,030	647204,852
28	GELENKFLÜSSIGKEIT KRANK 24	U7	1434,206	101,689	1,497	958,281	742732,384

Table 16: The detail of 29 diseased SF sample and healthy SF samples from 30 to 35 used in WB analysis

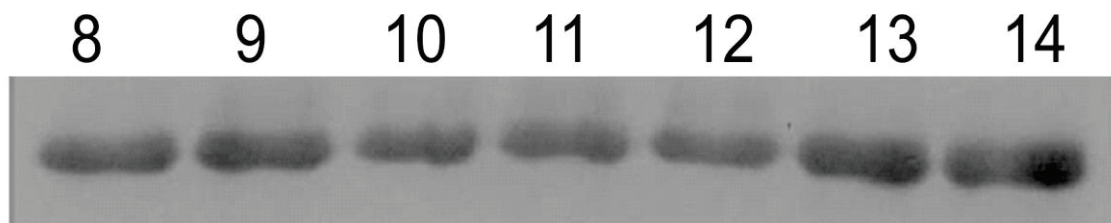Gel name : 2015-10-26   06_2013 Kohorte 2   ApoA1-WB 01 bearbeitet (Raw 1-D Image)

Index		Name	Volume INT*mm2	Adj. Vol. INT*mm2	Area mm2	Mean Value INT	Density INT/mm2
29	GELENKFLÜSSIGKEIT KRANK 25	U1	2457,108	350,032	2,695	911,644	706585,349
30	GELENKFLÜSSIGKEIT GESUND 1 KNIE LINKS	U2	2136,514	356,289	2,458	869,261	673735,705
31	GELENKFLÜSSIGKEIT GESUND 1 KNIE RECHTS	U3	1035,584	99,423	1,206	858,446	665353,427
32	GELENKFLÜSSIGKEIT GESUND 17 HAND LINKS	U4	2511,200	399,011	2,571	976,593	756924,983
33	GELENKFLÜSSIGKEIT GESUND 17 KNIE LINKS	U5	2771,722	540,968	2,894	957,767	742333,764
34	GELENKFLÜSSIGKEIT GESUND 732 HAND LINKS	U6	616,245	46,042	0,890	692,219	536516,197
35	GELENKFLÜSSIGKEIT GESUND 732 KNIE RECHTS	U7	594,903	41,875	0,862	690,254	534993,694

Table 17: The detail of healthy serum samples from 1 to 7 used in WB analysis

Gel name : 2015-10-26   06_2013 Kohorte 2   ApoA1-WB 02 bearbeitet (Raw 1-D Image)

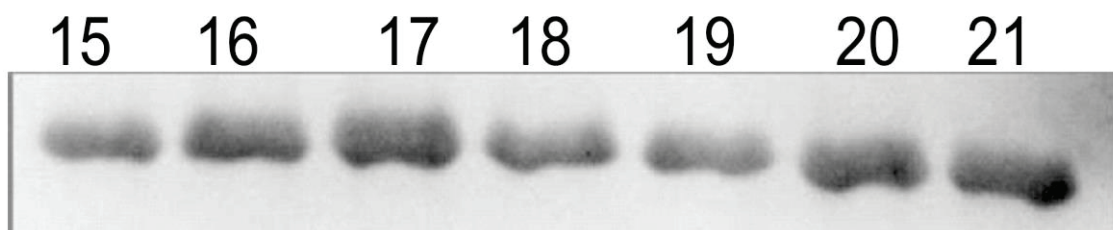| Index		Name	Volume	Adj. Vol.	Area	Mean Value	Density
			INT*mm2	INT*mm2	mm2	INT	INT/mm2
1	SERUM GESUND 01	U1	955,123	124,888	2,352	406,081	314739,893
2	SERUM GESUND 02	U2	920,916	179,624	2,411	381,900	295998,642
3	SERUM GESUND 03	U3	778,657	107,629	2,152	361,818	280433,167
4	SERUM GESUND 04	U4	803,876	116,359	2,214	363,087	281417,244
5	SERUM GESUND 05	U5	767,771	143,245	2,260	339,654	263254,853
6	SERUM GESUND 06	U6	904,855	154,460	2,744	329,724	255558,731
7	SERUM GESUND 07	U7	766,058	90,245	2,300	333,004	258100,947

Table 18: The detail of diseased serum samples from 8 to 14 used in WB analysis

Gel name : 2015-10-26   06_2013 Kohorte 2   ApoA1-WB 04 bearbeitet (Raw 1-D Image)

Index		Name	Volume	Adj. Vol.	Area	Mean Value	Density
			INT*mm2	INT*mm2	mm2	INT	INT/mm2
8	SERUM KRANK 07	U1	1180,157	133,747	2,734	431,666	334570,515
9	SERUM KRANK 08	U2	1012,397	72,624	2,268	446,346	345948,113
10	SERUM KRANK 09 TEP	U3	1231,346	142,039	2,747	448,274	347442,799
11	SERUM KRANK 10	U4	1187,901	131,869	2,404	494,205	383041,801
12	SERUM KRANK 11	U5	1004,759	94,605	1,934	519,517	402660,696
13	SERUM KRANK 12	U6	1331,320	186,011	2,966	448,831	347874,453
14	SERUM KRANK 13	U7	1383,745	202,694	3,068	451,008	349561,262

**Table 19: The detail of diseased serum samples from 15 to 21 used in WB analysis**

Gel name : 2015-10-26   06_2013 Kohorte 2   ApoA1-WB 05 bearbeitet (Raw 1-D Image)

Index		Name	Volume	Adj. Vol.	Area	Mean Value	Density
			INT*mm2	INT*mm2	mm2	INT	INT/mm2
15	SERUM KRANK 14	U1	1059,962	85,113	1,699	623,798	483485,510
16	SERUM KRANK 15	U2	1789,750	191,937	2,747	651,563	505005,008
17	SERUM KRANK 16	U3	2019,501	191,792	3,031	666,347	516463,799
18	SERUM KRANK 17	U4	1406,500	128,320	2,223	632,694	490380,597
19	SERUM KRANK 18	U5	1876,185	228,918	3,147	596,216	462107,320
21	SERUM KRANK 19	U6	2275,258	289,336	3,495	650,970	504545,699
21	SERUM KRANK 20	U7	1970,163	203,677	2,862	688,462	533604,548

Table 20: The detail of diseased serum samples from 22 to 26 used in WB analysis
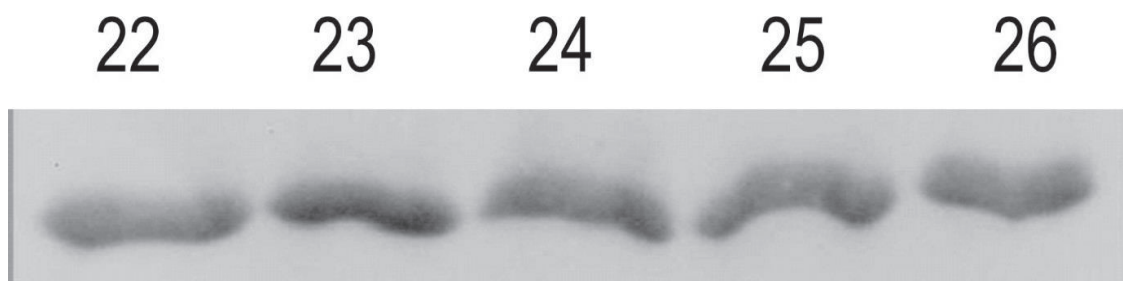
Gel name : 2015-10-26   06_2013 Kohorte 2   ApoA1-WB 06 bearbeitet (Raw 1-D Image)

Index		Name	Volume	Adj. Vol.	Area	Mean Value	Density
			INT*mm2	INT*mm2	mm2	INT	INT/mm2
22	SERUM KRANK 21	U1	1983,363	304,654	3,980	498,295	386212,188
23	SERUM KRANK 22	U2	1584,578	242,275	2,771	571,767	443157,773
24	SERUM KRANK 23	U3	1717,858	221,103	3,210	535,151	414778,188
25	SERUM KRANK 24	U4	1732,284	198,149	3,259	531,527	411969,185
26	SERUM KRANK 25	U5	1654,545	226,803	3,180	520,237	403218,672

12 Publication List

1. Use of proteomic analysis to determine the protein constituents of synovial fluid samples from the stifle joints of dogs with and without osteoarthritis secondary to cranial cruciate ligament rupture. Shahid M, Manchi G, Brunnberg L, Raila J. Am J vet Res. 2018 Apr; 79(4):397-403. doi: 10.2460/ajvr.79.4.397

2. A systemic review of existing serological possibilities to diagnose canine osteoarthritis with a particular focus on extracellular matrix proteoglycans and protein. Shahid, M.; Manchi, G.; Slunsky, P.; Naseer, O.; Fatima, A.; Brunnberg, L.; Raila, J. Polish Journal of Veterinary Sciences 2017 Vol.20 No.1 pp.189-201

3. World Congress on controversies, Debates and Consensus in Veterinary Medicine (COVET) in Prague, Czech Republic October, 2014, Poster Presentation on title "Protein pattern in synovial fluid as marker in the diagnosis of osteoarthritis in dogs"

4. The veterinary European physical therapy and rehabilitation association conference in Gdansk, Poland 19-20 September 2015, Oral presentation on title " Proteomics analyses of synovial fluid and serum in the diagnosis of OA in dogs"

13 Acknowledgments

I feel impelled to proffer my most unassuming and guileless gratitude to Almighty **Allah**, who is most compassionate, omnipotent, benign and whose munificent capacitated me to discern and strive for the higher ideas of life. I send blessing and salute with all respect to the Holy Prophet **"Muhammad" (**Peace and blessing of Allah be upon him), the intercessor, the eloquent and the illustrious, who ad infinitum remains a torch of guidance and knowledge for humanity as a whole.

I have no words for the true reflection of my feelings of gratitude for untiring efforts, keen interest and continued support of **Prof. Dr. Brunnberg Leo,** chairman, Clinic of Small Animal Medicine and Surgery, Freie Universität Berlin, Germany throughout my PhD studies. I always found him nice, noble and humble character adorned with enormous patience and sympathetic approach. I could never have achieved this milestone without his guidance, support and encouragement. His in-depth knowledge, fully devoted skills and well-balanced approach, and curiosity and inquisitiveness towards scientific matters make him a role model for me. I can proudly say that I am luck to have such a nice, visionary, learned, supportive, encouraging, humble and sympathetic person as my PhD supervisor. I don't know about others, but if I am given a chance to choose my advisor a hundred times, I will have only one choice, **Prof. Dr. Brnnberg Leo**, an epitome of goodness…

I feel momentous pleasure in transcribing my wholehearted thankfulness to my co-supervisor **Apl. -Prof. Dr. Jens Raila**, institute of Nutritional Science, Universität Potsdam, for sharing his enormous fund of knowledge and expertise with largess and acted as pillars of support in a time, which could by far have been difficult without his succor that made it seem so smooth and unproblematic.

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14 Declaration

I hereby declare that this dissertation is my own work and has not previously submitted anywhere for any award. Where other sources of information have been used they have been acknowledged.

Berlin 28.06.2018   Muhammad Shahid