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Biomarkers in equine synovial fluid and serum for the diagnosis of joint diseases

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1 ABBREVIATIONS

ABAH	4-aminobenzoic acid hydrazide
APMA	p-aminophenylmercuric acetate
APS	ammonium persulfate
approx.	approximately
BSA	bovine serum albumin
C	Celsius
Cm	castrated male
dist	distal
DMSO	dimethyl sulfoxide
DTPA	diethylene triamine pentaacetic acid
E	extinction
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
e.g.	*exempli gratia*
ELISA	enzyme linked immunosorbent assay
et al.	*et alii*
F	female
g	gram
h	hour
i.e.	*id est*
IGF	insulin-like growth factor
IL	interleukin
IQR	interquartile range
kDa	kilodalton
l	litre
M	molar
Ma	male
µl	microlitre
µM	micromolar
µm	micrometre
mA	milliampere

min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MT	membrane-type
n	number
NADPH	nicotinamide adenine dinucleotide phosphate
n.d.	not determined
NF-κB	nuclear factor kappa B
ng	nanogram
nm	nanometre
OA	osteoarthritis
OCD	osteochondrosis dissecans
PAGE	polyacrylamide gel electrophoresis
pg	picogram
PMN	polymorphonuclear neutrophil
PNPP	*p*-nitrophenyl phosphate
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
TEMED	tetramethylethylene diamine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
U	units
V	volt
WBC	white blood cell

2 INTRODUCTION

Lameness is the most common cause of impaired athletic performance in horses. Disorders of the musculoskeletal system, particularly joint diseases represent one of the major reasons for equine lameness (CARON and GENOVESE 2003; OLIVIER *et al.* 1997; TODHUNTER and LUST 1992). A recent survey revealed that equine joint diseases constituted at least 30% of insured events including permanent uselessness for riding and carting between 1997 and 2006 (JÄGER C, VTV VEREINIGTE TIERVERSICHERUNG, WIESBADEN, 2007- PERSONAL COMMUNICATION). According to this, joint diseases represent not only a welfare issue but also a source of economic loss due to veterinary expenses, limited performance, and early retirements.

The diagnosis of joint diseases is routinely established on the basis of clinical lameness examination, radiography, and conventional synovial fluid analysis. Accurate clinical and laboratory assessment of joint disease is essential to determine adequate treatment and prognosis for future soundness (DAGLEISH *et al.* 2003). However, definite diagnosis often remains difficult. Therefore, biomarkers in body fluids have received growing attention in an attempt to improve diagnosis, assessment of disease severity, and evaluation of therapeutic effects. Due to synovial fluid being restricted to a relatively inaccessible anatomic site, and the non-routine invasive nature of its sampling, recent measurements have focused on biomarkers in serum (ELSAID and CHICHESTER 2006). Of particular interest are those biomarkers that fluctuate with a specific diagnosis, but then normalize following successful therapy. Various marker molecules have already been used successfully in research settings, but to date none have been introduced and validated in everyday horse practice. For a good assessment of joint condition, a combination of selected markers seems to be most promising (MCILWRAITH 2005).

It is therefore the aim of this study to analyse the possible use of selective parameters in equine synovial fluid and serum as potential diagnostic markers of joint diseases. The results presented here may help to refine the diagnosis of distinct joint diseases based on a multi-parameter investigation which will lay the basis for accurate assessment of joint diseases.

3 LITERATURE REVIEW

3.1 Biology of synovial joint

3.1.1 Definition of synovial joint

A synovial (diarthrodial) joint is a freely moveable joint which is characterized by a joint gap between two opposing articular-cartilage-covered bone ends (KHAN *et al.* 2007). The joint cavity, which contains synovial fluid, is lined by a joint capsule and further stabilized by muscles, tendons, and/or ligaments (FRISBIE 2006; TODHUNTER 1996) (Figure 1).

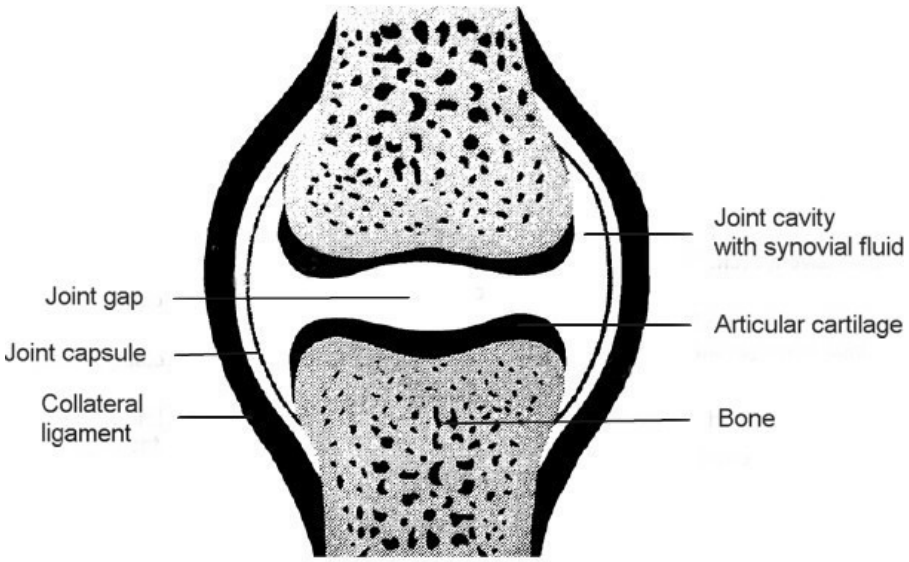

Figure 1: Schematic presentation of a synovial joint (adapted from BROMMER 2005).

3.1.2 Joint capsule

The joint capsule covers all articular surfaces with the exception of the articular cartilage and localized bone areas (CARON 1999). It is composed of an outside fibrous layer and a synovial membrane (synovium) that lines the inner surface of the joint (PALMER and BERTONE 1994).

The fibrous joint capsule comprises a dense connective tissue with low cellularity containing water and an extracellular matrix of collagen, predominantly type I, elastin, and proteoglycan. Its fundamental role is to provide mechanical stability to the joint (FRISBIE 2006).

The synovial membrane forms villi and plicae that differ in number, form, size, and distribution (LIEBICH and KÖNIG 2005) depending on their location within the joint. It is built of two layers, the intima and the subintima. The latter is adjacent to the fibrous joint capsule and contains a loose connective tissue of fibrous, areolar or adipose types in addition to blood vessels, lymphatics, and nerves (CARON 2003; PALMER and BERTONE 1994).

The innermost layer, the intima, consists of a cellular lining, one to four cells thick, including at least two morphologically and functionally different types of cells (synoviocytes): macrophagic cells (type A cells) and fibroblast-like cells (type B cells) (SERENA *et al.* 2005). Type A synoviocytes are non-fixed cells that possess the ability to phagocytose cell debris and metabolic waste products (IWANAGA *et al.* 2000). Type B synoviocytes extend dendritic processes towards the joint cavity to form a complex three-dimensional network in the luminal surface of the synovial membrane (KITAMURA *et al.* 1999). They are responsible for the production of specialized macromolecules including hyaluronan, collagens, and fibronectin for the intimal interstitium and synovial fluid (ABELES and PILLINGER 2006; CARON 2003). Several investigators have also described the presence of an intermediate cell type (type C cells) in various species which has common characteristics with both of the above cell types (FELL *et al.* 1976; NISHIJIMA 1981; WILKINSON *et al.* 1992) and may represent an undifferentiated synoviocyte capable of differentiating to either type depending on biological demand (FRISBIE 2006).

Synoviocytes form a discontinuous layer with no basement membrane in which cells are mostly separated by gaps of 1-2 mm in width (GARNERO *et al.* 2000).

Intercellular gaps are composed of an interstitial matrix including collagen types I, III, and V (ASHHURST *et al.* 1991), hyaluronan (WORRALL *et al.* 1991), chondroitin sulphate (WORRALL *et al.* 1994), fibronectin (POLI *et al.* 2004), and laminin (POLLOCK *et al.* 1990). The synovial membrane is richly supplied with blood. Its microcirculation exhibits the following significant architectural features: capillaries are closely located to the intimal surface (5 to 10 µm), and fenestrations are orientated towards the joint cavity (PALMER and BERTONE 1994; TODHUNTER 1996).

3.1.3 Synovial fluid

The synovial fluid is an ultafiltrat of plasma supplemented by hyaluronan and lubricin which are actively secreted by type B synoviocytes (BLEWIS *et al.* 2007; FRISBIE 2006; GARNERO *et al.* 2000).

It is formed by the passive ultrafiltration of plasma across the synovial capillary fenestrations into the synovial interstitium from which it then enters the joint cavity through the intercellular gaps between the synoviocytes (BRANNAN and JERRARD 2006; LEVICK 1995). The endothelium prevents large molecules such as fibrinogen and cells from leaving the capillaries (GARNERO *et al.* 2000). Filtration of molecules is maintained by the synovial permeability barrier consisting of the narrow space between synoviocytes and the composition of the interstitial matrix (TODHUNTER 1996); the latter also provides a sufficient outflow resistance to retain large solutes within the joint cavity (BLEWIS *et al.* 2007). Molecules less than 10 kDa including water, oxygen, carbon dioxide, tissue metabolites, and electrolytes readily equilibrate between the plasma and synovial fluid (FRISBIE 2006; GARNERO *et al.* 2000; SIMKIN 1995). The amount of electrolyte, glucose, and uric acid in normal synovial fluid is similar to that of plasma, whereas synovial protein constitutes approx. 25-35% of the plasma concentration of the same animal (BRANNAN and JERRARD 2006; LEVICK 1995; MCILWRAITH *et al.* 2001).

Hyaluronan is a glycosaminoglycan with an average mass of several thousand kDa (GARNERO *et al.* 2000; TODHUNTER 1996).

Together with the glycoprotein lubricin, it provides the synovial fluid with its characteristic viscosity responsible for the lubrication of joint structures which allows low-friction movement (BRANNAN and JERRARD 2006). In addition to that, synovial fluid acts as a convective and diffusional transport medium for nutrients supplying the articular cartilage with necessary nourishment as well as for waste products that exit the joint cavity through the subsynovial lymphatic system (LEVICK 1995).

3.1.4 Articular cartilage

The articular cartilage is of hyaline type. It covers the subchondral bone plate and provides a smooth gliding surface capable of withstanding compressive loads and maintaining normal joint environment (PALMER and BERTONE 1994). In adults, articular cartilage is characterized by a lack of vascularization, innervation, and lymphatic drainage (CARON 2003; FRISBIE 2006).

Joint cartilage is composed of three main components: a limited number of chondrocytes (< 5%), the extracellular matrix (20-30%), and interstitial water (60-80%) (BRAMA *et al.* 2000a; BRAMA *et al.* 2000b; HAYES *et al.* 2001).

3.1.4.1 Chondrocytes

The chondrocytes are the cellular component of the articular cartilage, capable of synthesizing extracellular matrix components. Articular chondrocytes are long-lived cells whose progeny ends after growth has ceased (MUIR 1995). They occur singly or in isogenous groups in lacunae representing a microenvironment (FRISBIE 2006; LIEBICH 2004). In the adult joint cartilage, chondrocytes derive their nourishment from the synovial fluid. Intra-articular transport of nutrients is derived by simple diffusion and convection, the latter is induced through intermittent loading of the cartilage by joint motion (CARON 2003; LEVICK 1995). The relationship between cell density and cartilage thickness appears to be inverse as diffusion limits the total number of cells that can be sustained in a given volume (MUIR 1995). Thus articular cartilage is limited to 4 mm of thickness for chondrocyte viability to be maintained (FRISBIE 2006).

3.1.4.2 Extracellular matrix

The extracellular matrix provides the articular cartilage with its strength, resistance to deformation, and ability to dissipate load and handle forces generated within the joint. On a dry weight basis it is made up mainly of collagens (50-80%) and proteoglycans (5-10%) (FRISBIE 2006).

Collagens are fibrous proteins composed of tropocollagen monomers. Each monomer contains three polypeptide left-handed alpha chains twisted to a right-handed superhelix (MUIR 1995; RAY *et al.* 1996). At least 16 different collagen types are expressed in mammalian articular cartilage. However, the most abundant collagen is collagen type II, comprising 85-95% of the total collagen content (EYRE *et al.* 2006; FRISBIE 2006). Type II collagen consists of three identical alpha chains. Its tropocollagen monomers are synthesised and secreted from the chondrocyte as procollagen molecules. Extracellular processing results in the conversion of procollagen to collagen (MCILWRAITH 2005).

Type II collagen is organized in form of fibrils which are composed of molecules aligned with quarter stagger. Cross-linking occurs within the same collagen molecule as well as between adjacent collagen molecules within the collagen fibril or from neighbouring fibrils (CARON 2003; ELSAID and CHICHESTER 2006; MCILWRAITH 2005). Significant degradation and resynthesis of fibrils occurs during growth; however, collagen turnover in adult articular cartilage is limited (EYRE 2002). Type II collagen fibrils form the three-dimensional meshwork of the cartilage matrix by copolymerising with other extracellular matrix molecules (MCILWRAITH 2005). It is this extensively cross-linked network together with the characteristic fibrillar organization that provides tensile strength to the cartilage.

Minor collagens such as types VI, IX, X, XI, XII, and XIV are present in modest amounts in articular cartilage and their specific roles in its structure and function have not been completely defined yet (CARON 2003; EYRE *et al.* 2006).

Proteoglycans are composite molecules consisting of both protein and glycosaminoglycan (polysaccharide) components. Aggrecan is the most abundant proteoglycan of articular cartilage comprising 85% of the proteoglycan content (FRISBIE 2006). The monomeric form of this molecule consists of a linear core protein to which glycosaminoglycans are covalently attached in a radial fashion. The three main glycosaminoglycans that make up aggrecan are chondroitin-4-sulphate, chondroitin-6-sulphate, and keratan sulphate (FRISBIE 2006). About 100 chondroitin sulfate molecules are found to preferentially attach to the carboxy end of the core protein, whereas approx. 30 keratan sulphate molecules are preferentially found in closer relation to its amino end (MCILWRAITH 2005; MUIR 1995).

Aggrecan monomers are non-covalently linked to a long filamentous hyaluronan molecule to form supramolecular aggregates which are stabilized by a link protein. These aggregates can be in the range of over 2 x 10^5 kDa in size, compromise over 100 aggrecan monomers, and are immobilised in the collagen network (TODHUNTER 1996). The negatively-charged glycosaminoglycans attract up to 50 times their weight in water, and it is this highly hydrated matrix that provides the cartilage with its compressive stiffness and ability to dissipate load (CHARNI-BEN TABASSI and GARNERO 2007; POOLE *et al.* 2002). This swelling tendency is restricted by the collagen network which is, even in unloaded cartilage, under constant tension (HAYES *et al.* 2001; MUIR 1995).

Other proteoglycans are present in relatively modest amounts in cartilage. They have been studied less but appear to function in the interaction with collagens and in the regulation of a variety of metabolic processes in cartilage (FRISBIE 2006).

3.1.4.3 Interstitial water

The space surrounding the macromolecules and chondrocytes is filled with water in which electrolytes, anabolites, and catabolites are dissolved.

This water is freely interchangeable with that in synovial fluid (CARON 2003). It plays an important role in chondrocyte metabolism as it is a transport medium for new matrix components and waste products (MOW *et al.* 1984). Water movement is thought to be important for the capacity of cartilage to absorb and distribute load and for its lubrication (CARON 2003).

3.1.4.4 Articular cartilage organisation

The articular cartilage can be divided into four contiguous zones representing a functional adaptation to the mechanical requirements of the different layers (FRISBIE 2006; TODHUNTER 1996). The superficial (tangential) zone has the highest cell density. Chondrocytes are elongated within collagen fibrils orientated parallel to the articular surface. The intermediate (transitional) zone shows larger and rounded chondrocytes, which are embedded, singly, or paired, in collagen fibrils in a randomly-organized meshwork. In the deep (radiate) zone, chondrocytes are larger and arranged in columns with their long axes vertical to the cartilage surface within collagen fibrils radially arranged around the articular surface (HAYES *et al.* 2001). In adults, these three upper zones are delineated from the most basal zone by an irregular line, called the tidemark (CARON 2003; FRISBIE *et al*. 1999b). The most basal layer is the zone of calcified or mineralised cartilage consisting of embedded chondrocytes dispersed in hydroxyapatite crystals either individually or in cell columns surrounded by a thin layer of non-mineralised matrix (PALMER and BERTONE 1994). Concentration of collagen is relatively low. Collagen fibrils are orientated radially to insert within the subchondral bone (CARON 2003).

3.1.5 Subchondral bone

The subchondral bone is a thin layer of bone which can be classified into the subchondral bone plate and the trabecular bone (KAWCAK *et al.* 2001). It provides stability and contour to the overlying cartilage (FRISBIE 2006). Subchondral bone is many times more compliant than cortical bone, allowing a degree of formation to absorb load and dissipate energy (CARON 2003; MANKIN AND RADIN 1993).

It is composed of a limited number of cells, namely osteoblasts, osteocytes, and osteoclasts (< 5% by volume), the extracellular matrix (70-75%), and interstitial water (20-25%). The extracellular matrix is composed of an inorganic component (65% of total bone matrix) of mineral salt [hydroxyapatite crystals: $Ca_{10}(PO_4)_6(OH)_2$] and an organic component (35% of total bone matrix) of predominantly type I collagen (TODHUNTER 1996). The inorganic phase provides rigidity and hardness, while the organic phase imparts resiliency and flexibility to the subchondral bone (PALMER and BERTONE 1996).

Vascular channels connect the trabecular bone with the calcified cartilage, thereby nourishing the deeper layers of the cartilage that are not nourished by synovial fluid (DUNCAN *et al.* 1987; MILZ and PUTZ 1994). These vascular channels also provide nourishment for osteocytes in the subchondral bone plate, whereas osteocytes in the trabecular bone receive their nourishment from marrow tissue (KAWCAK *et al.* 2001).

3.2 Pathobiology of synovial joint

The function of the synovial joint depends on the integrity of normal anatomy and cellular function of each of its components. Joint diseases in the horse are characterized by involvement of multiple joint structures, *e.g.* the synovial membrane, the articular cartilage, and the subchondral bone (CARON 2003; MCILWRAITH 2005; PALMER *et al*. 1996). The derangement of these structures in affected joints is a complex and dynamic process (FRISBIE 2006). The intensity of each tissue reaction depends on the degree of the disease (MCILWRAITH 1996b). A summary of interactions that may occur in an affected joint is presented in Figure 2.

3.2.1 Role of the synovial membrane

The synovial membrane has an integral role in influencing the joint environment, particularly on the composition of synovial fluid and thereby affecting the articular cartilage (PALMER *et al.* 1996).

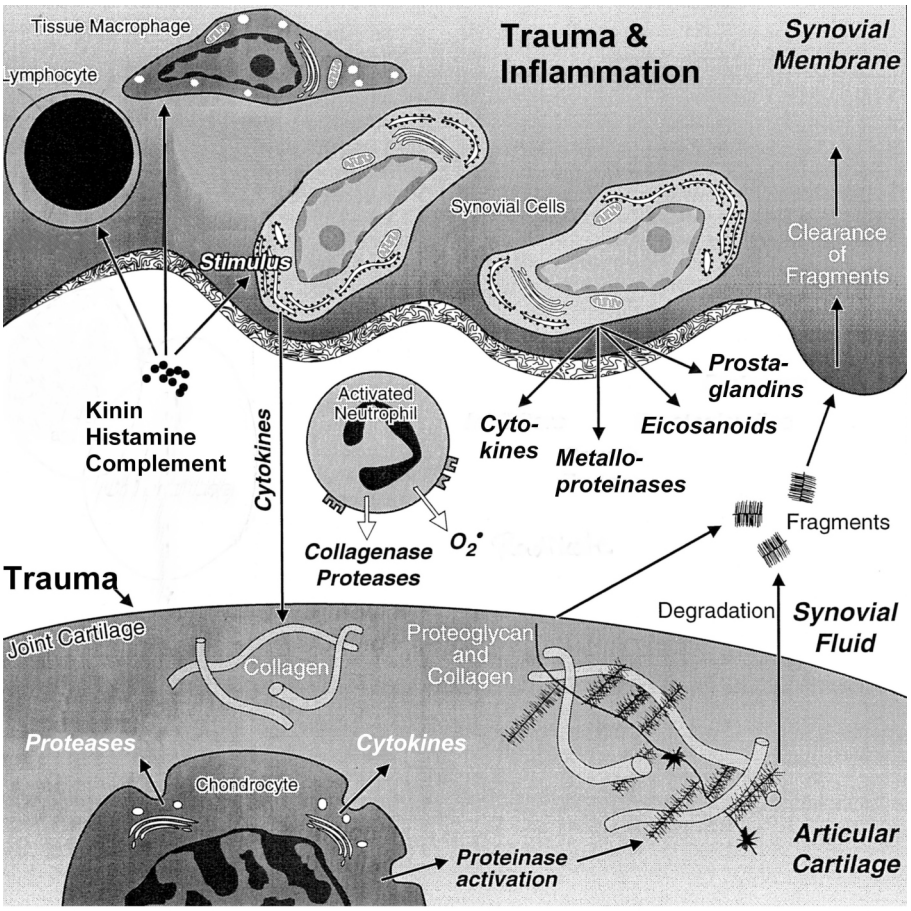

Figure 2: Summary of interactions potentially occurring in an affected joint (modified from BERTONE 1996).

Inflammation of the synovial membrane (synovitis) or the joint capsule (capsulitis) is present in the majority of joint diseases (MCILWRAITH *et al.* 2001). Primary synovitis or capsulitis is thought to result from traumatic injuries or chemical damage due to intra-articular injection of pharmaceuticals. Inflammation of the synovial membrane or joint capsule could also occur secondary to other damage within the joint such as free-moving cartilage degradation products (FRISBIE 2006; MCILWRAITH 1996b).

Inflammation of these tissues can be characterized by hypervascularity, oedema, hyperplasia of the intimal cell lining, villous hypertrophy, or infiltration of inflammatory cells (PALMER AND BERTONE 1994).

The increase in vascularity is associated with “gaps or leakiness” of the endothelial cell layer leading to an increase in protein concentration and white blood cell (WBC) count in the synovial fluid (FRISBIE 2006; GIBSON and ROONEY 2007).

This influx results in an elevated colloid osmotic pressure within the joint that promotes a net flow of water into the joint cavity leading to increased joint effusion (LEVICK 1995; WALLIS *et al.* 1987). In the initial state of inflammation, a number of non-specific mediators of inflammation enter the synovial fluid and attract leukocytes (MCILWRAITH 1996b). Those include kinin, histamine, products of the complement pathway as well as of the blood coagulation and fibrinolytic systems. Arriving immune cells infiltrate the joint and release a number of pre-formed mediators of inflammation including collagenases, proteinases, and oxygen-derived free radicals (PALMER and BERTONE 1994). Leukocyte-derived substances are capable of contributing to cartilage destruction and stimulation of macrophages, synoviocytes, and chondrocytes. These activated cells release induced mediators of inflammation such as cytokines, prostaglandins, eicosanoids, and matrix degrading enzymes such as metalloproteinases which are thought to play a major role in the destruction of the articular cartilage (ARMSTRONG and LEES 2002; MALONE 2002; MEYER *et al.* 2006).

3.2.2 Role of the chrondrocyte

The chondrocytes are responsible for maintaining their surrounding environment through a complex interaction between anabolic and catabolic mediators and mechanical stimuli (CARON 2003). In diseased joints, the catabolic processes are predominating. The most relevant catabolic mediators are cytokines such as IL-1 and TNF-α which can be secreted by synoviocytes and chondrocytes (FRISBIE 2006). Mechanical stress has shown to activate NF-κB in chondrocytes causing apoptosis in cell culture as well as an increase of IL-1 and TNF-α (AGARWAL *et al.* 2004; ISLAM *et al.* 2002).

An upregulation of pro-inflammatory cytokines results in the inhibition of aggrecan and collagen II synthesis, and moreover in the production of proteases by the chondrocyte (VERBRUGGEN *et al.* 2007). Indeed, these enzymes are thought to be the major mediators of matrix depletion of the articular cartilage resulting in a reduction in content as well as changes in the structure of matrix components (MCILWRAITH 1996b). Proteases include aspartic proteinases, cysteine proteinases, serin proteinases, and matrix metalloproteinases. (MEYER *et al.* 2006).

As a reparative attempt, matrix turnover rates of the chondrocytes may be unregulated in disease states. The cytokines that promote the anabolic cascade of cartilage metabolism are IGF-1 and TGF-β (CARON 2003). Both mediators have been found elevated in the chondrocyte from fibrillated cartilage (VERBRUGGEN *et al.* 2007). Proteoglycan turnover in dogs was estimated at 300 days, whereas turnover of collagen was found to be approx. 120 days (MCILWRAITH 2002). Collagen synthesis was reported to be accelerated up to 10-fold within two weeks after joint injury in dogs (EYRE 2002; EYRE *et al.* 2006). However, the activities of anabolic mediators and thus the rate of cartilage remodelling depend highly on the pathological condition (VERBRUGGEN *et al.* 2007).

A loss and/or change in composition of matrix components could affect the mechanical properties of the cartilage, making it less able to withstand normal loads. When perpetuating in the disease process, it will incite changes in other joint structures such as the synovial membrane and subchondral bone (FRISBIE 2006).

3.2.3 Role of the subchondral bone

A primary role for subchondral bone microdamage in the pathogenesis of equine joint disease has been emphasized (KAWCAK *et al.* 2001; MCILWRAITH 1996a). Changes in the subchondral bone have been described to precede those in articular cartilage in a range of different species (ANDERSON-MACKENZIE *et al.* 2005; CARLSON *et al.* 1996; MANSELL *et al.* 2007). The subchondral bone adapts to cyclic loading by appositional bone growth (FIRTH 2006; MCILWRAITH 1996b).

Examination of the subchondral bone in humans with osteoarthritis revealed a 20-fold increase in collagen turnover leading to a higher proportion of immature bone. This, together with a 25% reduction in mineralization, has shown to result in narrower and weaker bone collagen fibres (BAILEY *et al.* 2004; MANSELL *et al.* 2007). As a consequence, localized subchondral stiffening is thought to occur causing increased shear stress in the articular cartilage as well as cartilage breakdown and fibrillation (CARLSON *et al.* 1996; KAWCAK *et al.* 2001).

Moreover, it has been suggested that alterations in the phenotypic expression of the osteoblasts is associated with articular cartilage damage (BAILEY *et al.* 2004; HILAL *et al.* 1998; MANSELL *et al.* 2007). *In vitro* studies demonstrated that osteoblasts isolated from osteoarthritis-affected joints are capable of initiating degradation of cartilage proteoglycans (WESTACOTT *et al.* 1997). In this regard, it is assumed that degrading enzymes gain access to the articular cartilage via channels in the calcified cartilage and gaps in the tidemark (MANSELL *et al.* 2007; WESTACOTT 2002).

3.3 Joint diseases

Joint disease in the horse can be ascribed to the following etiologic factors: traumatic, developmental, and infectious (MCILWRAITH 1996b). Autoimmune joint diseases like rheumatoid arthritis as well as metabolic joint diseases such as gout are considered to play a minor role in the horse (CARTER *et al.* 1995; MAY 1996; OSBORNE *et al.* 1995). Any affliction of the equine joint can be described as arthritis which serves as a cover term for several disorders in the equine joint (MCILWRAITH 1996b).

3.3.1 Osteoarthritis

Osteoarthritis (OA) is considered to be one of the most important causes for lameness and impaired athletic performance in the horse (CARON 1992; CARON AND GENOVESE 2003). It represents a major part of the caseload for equine clinicians (FRISBIE 2005).

OA is characterized by a progressive degeneration of the articular cartilage accompanied by changes in the soft tissues and bone of the joint (MCILWRAITH 1996b). The aetiology of OA is considered to be multifactorial with several initiating causes (FRISBIE 2005; FRISBIE 2006). In the horse, three proposed pathogenetic mechanisms have been described (CARON 2003).

The first includes a defective articular cartilage such as the genetic type II collagen effect as defined in humans, leading to a failure of the cartilage under normal joint loading. The second involves abnormal change in the subchondral bone which is thought to respond to exercise by increasing its density to a pathologic level resulting in a less compliant cartilage-bone unit. The third and most common mechanism is based on normal cartilage that is exposed to abnormal forces resulting in metabolic alterations of chondrocytes in which the anabolic repair process is overwhelmed by the catabolic process. Particularly repeated microtrauma is considered to be the most common pathogenetic factor in equine OA (CARON 2003). However, it is also considered that OA can develop secondary to other joint diseases such as unresolved osteochondrosis dissecans or septic arthritis (FORTIER and NIXON 2005; MCILWRAITH 1996b; MCILWRAITH and VACHON 1988).

Matrix degeneration results in decreased content as well as changes in the structure of matrix components such as type II collagen and aggrecan (POOLE *et al.* 2002). It is manifested by surface fibrillation of the articular cartilage (Caron 2003) accounting for increased water content in cartilage lesions (CURTIN and REVILLE 1995). These alterations are accompanied by a reduced compressive stiffness of the cartilage unable to resist joint loading (PELLETIER *et al.* 1997) which in turn results in further cartilage degeneration leading to surface disruption, pitting, vertical clefts, ulceration, and a complete loss of cartilage at advanced disease stages (BRANDT and MANKIN 1994).

When clinically evident, OA is characterized by lameness and variable degrees of joint inflammation without systemic effects. Typical radiographic signs include joint space narrowing, subchondral sclerosis, periarticular marginal osteophytes, and periarticular enthesophytes (CARON 2003; PARK *et al.* 1996).

However, early diagnosis of articular cartilage depletion remains difficult as radiographic changes occur at later stages of the disease (CIBERE 2006).

3.3.2 Osteochondrosis dissecans

Osteochondrosis dissecans (OCD) is a major developmental joint disorder affecting young horses (AL-HIZAB *et al.* 2002; DIERKS *et al.* 2007). It can be defined as a disruption in the process of enchondral ossification taking place in the growing epiphyseal cartilage (DE GRAUW *et al*. 2006a; LAVERTY *et al*. 2002). OCD does not necessarily affect the performance as lameness is an inconsistent finding in horses with OCD (FORTIER and NIXON 2005; SCHENCK and GOODNIGHT 1996). However, this disorder can become clinically manifest after the horse has been subjected to athletic exposure (MCILWRAITH 1996a).

The disease appears to have a multifactorial origin including skeletal growth rates, nutrition, endocrinologic factors, exercise, biomechanics, and genetic effects (DIERKS *et al.* 2007; HURTIG and POOL 1996; JEFFCOTT 1991). Common characteristics of OCD include its occurrence during rapid growth and the development of multiple bilateral lesions at circumcript sites (LAVERTY *et al.* 2000). Abnormal chondrocyte differentiation is considered to lead to altered enchondral ossification resulting in irregular thickness of the cartilage (JEFFCOTT and HENSON 1998). Hypertrophic areas of cartilage represent a longer passage of diffusion for nutrients from the synovial fluid into the deeper layer of the cartilage. As a result of insufficient nourishment, necrosis of these layers can occur (DIERKS *et al.* 2007). Since areas of excessive thickness are more susceptible to shearing and compressive forces, secondary lesion of the cartilage such as fissures, detachment and fragmentation of cartilage, subchondral fractures and cysts, cartilage flaps, and joint mice can develop in the further course (HURTIG and POOL 1996; JEFFCOTT and HENSON 1998; TROTTER and MCILWRAITH 1981).

Clinical signs of OCD may include joint effusion and mild lameness (HERTSCH 1991), and can be found in the absence of radiological findings and vice versa (MCILWRAITH 1996a; MCILWRAITH 2002).

3.3.3 Septic arthritis

Septic (infectious) arthritis is a rapidly progressive highly destructive joint disease. It is characterized by the inoculation of the synovial membrane or synovial fluid with bacteria or other organisms such as fungi (BERTONE 2003) resulting in a massive inflammatory reaction (MORTON 2005). Infectious affection of the joint is a potentially life-threatening condition and has to be considered as an emergency (BERTONE 2003). The percentage of euthanasia due to septic arthritis has been reported to range from 15 to 20% in adult horses (KIDD *et al.* 2007). Moreover, the disease is often associated with a poor prognosis for a return to the previous level of performance (LAPOINTE *et al.* 1992; MEIJER *et al.* 2000; SCHNEIDER *et al.* 1992).

The common causes of septic arthritis in adult horses include traumatic wounds, joint injections, post-operative infections, and idiopathic causes (SCHNEIDER *et al.* 1992; WRIGHT *et al.* 2003). In foals hematogenous spread plays the major role in the disease development (BERTONE 2003). Predominant bacteria involved belong to the families of enterobacteriaceae, streptococci, and staphylococci (MOORE *et al.* 1992). Foals or horses with septic arthritis in consequence of wound infection mostly present multiple bacterial infections (BERTONE 2003).

Bacteria colonize the synovial membrane leading to activation of synoviocytes that produce inflammatory mediators. As a result inflammatory cells, mainly neutrophils, rapidly enter the joint to phagocytise the microorganisms and release degradative enzymes such as collagenases, free radicals, and inflammatory mediators including IL-1 and TNF-α (KIDD *et al.* 2007; MORTON 2005). Activation of the plasmin, kinin, and fibrinolytic systems occurs amplifiying the inflammation (BERTONE 1996). The massive amount of fibrin forms an intra-synovial fibrinocellular conglomerate, or pannus. The latter acts as a physical barrier to the synovium preventing membrane diffusion and thus joint nutrition (WRIGHT *et al.* 2003).

Rapid alteration in the articular cartilage composition of proteoglycan and collagen occurs leading to alterations in joint biomechanics. Irreversible joint destruction may result quickly, whereas extensive articular cartilage degradation is not found in most infected horses (BERTONE 1996).

Classic signs of septic arthritis are local heat, articular swelling, and rapidly progressive development of non-weight-bearing lameness. Occasionally fever, neutrophilic leukocytosis, and hyperproteinemia may be present as well. If pain is severe, heart and respiratory rates may be elevated (MORTON 2005). A diagnostic approach includes haematological examination, synovial fluid analysis including cytological and bacterial examination, and radiography (BERTONE 2003). Cases of septic arthritis have the highest WBC counts and protein levels. Positive synovial smears or cultures are useful to confirm the diagnosis of septic arthritis; however, a negative result does not exclude a joint infection (MCILWRAITH *et al.* 2001).

3.4 Synovial fluid analysis

Examination of the synovial fluid is important in the evaluation of equine joint diseases providing valuable information in addition to that obtained by clinical examination (MARXEN and SCHNEIDER 2003; MCILWRAITH *et al.* 2001). In this regard, gross examination including appearance and volume, measurement of protein concentration as well as cytological examination are thought to be the most important and useful parameters (TROTTER and MCILWRAITH 1996).

Synovial fluid from healthy joints is pale, yellow, and clear (GRABNER 2005; MAHAFFEY 2002). Streaks of blood in the aspirate are indicative of haemorrhage from needle puncture. Uniformly diffuse haemorrhage presents an acute traumatic situation, whereas dark yellow or pale amber samples are often associated with chronic traumatic arthritis (RAKICH and LATIMER 2003). The presence of opacity and flocculent material in the synovial fluid indicates synovitis. This change is marked in acute synovitis, however in general minimally in chronic degenerative joint disease and OCD (MCILWRAITH *et al.* 2001). The intense synovitis associated with septic arthritis results in a serofibrinous to fibrinopurulent sample (BERTONE 2003).

An increased synovial fluid volume is detected in most cases of active synovitis using clinical investigations (MCILWRAITH 1987). A decrease in volume is present in some cases of chronic degenerative joint disease (GRABNER 2005). In cases of OCD, volume increase is thought to be variable.

The volume of synovial fluid from septic arthritis is usually increased; however, this is dependent on the stage of the disease and the amount of fibrin present within the joint (MCILWRAITH *et al.* 2001).

Protein concentration in synovial fluid of horses from healthy joints has been documented as 18.1 ± 2.6 g/l (MCILWRAITH *et al.* 2001). Values increase with joint inflammation. Protein levels above 25 g/l are considered to indicate a pathologic fluid. A concentration above 40 g/l represents severe inflammation. Non-infected inflammatory conditions generally have protein concentrations below this level (TROTTER and MCILWRAITH 1996).

Cytological examination has been considered as the most important part of the synovial fluid analysis (RAKICH and LATIMER 2003). Erythrocytes are not normal constituents of synovial fluid (GRABNER 2005). Their presence in small numbers is usually attributed to contamination of the sample at the time of aspiration. The WBC count of synovial fluid from healthy joints has been reported as 167 ± 21 and 87 cells/µl respectively (MCILWRAITH *et al.* 2001), and the percentage of neutrophils is generally less than 10% (RAKICH and LATIMER 2003). OCD samples generally have WBC counts less than 1000 cells/µl. In traumatic and OA, the cell count may vary tremendously depending on the amount of active synovitis present. Cases of septic arthritis have the highest WBC counts. Cell counts greater than 30 x 10^3/µl are suggestive of infection and counts greater than 100 x 10^3/µl are pathognomonic (MCILWRAITH *et al.* 2001). Neutrophils are the predominant cells and account for more than 90% of differentiated cells (CARON 2003; FRISBIE 2006). Bacteria are not commonly seen on smear examinations of cases of septic arthritis. Up to 48% of samples suspected of having septic arthritis have reported to yield false negative results on Gram-stained smears (PILLE *et al.* 2004). Importantly, infected joints have been shown to present cell counts less than 30 x 10^3/µl (BERTONE 2003).

Indeed, it is considered that a 'grey zone' exists between traumatic arthritis with a high WBC count and infective arthritis with a low WBC count (MCILWRAITH *et al.* 2001), indicating that WBC count alone is not sufficiently sensitive to exclude or confirm septic arthritis (LI *et al.* 2007; MATHEWS *et al.* 2007).

At present, conventional synovial fluid analysis indicates the degree of synovitis and to some extent metabolic derangement within the joint (MCILWRAITH *et al.* 2001). However, it neither provides a specific diagnosis of joint disease nor information on articular cartilage damage. This, together with the limitations of routine clinical methods to diagnose early stages of OA, has led to the search for other biomarkers providing a more specific assessment of the stage of disease within the joint (FRISBIE 2003).

3.5 Biomarkers of joint disease

A biomarker is generally considered to be a characteristic that is objectively measured and evaluated as an indicator of a normal or pathogenic biological process, or pharmacologic response to therapy (WEIR and WALLEY 2006).

Biomarkers of joint disease have the potential to clarify a pathobiological process within a joint, discriminate between a healthy and diseased joint, characterize the degree of destruction of articular cartilage, and monitor the response to a therapeutic intervention (MCILWRAITH 2005). The ideal biomarker of OA should be exclusively produced in affected cartilage or any other joint tissue, must be validated by another form of measurement, and correlate with a 'gold standard' (FULLER *et al.* 2001); or relate to the nature of joint disease, the stage of degradation and/or ratio of its process, and must be measurable using established chemical or immunological methods (RØRVIK and GRØNDAHL 1995).

Marker molecules can be detected in body fluids including synovial fluid, serum, and urine (SCHER *et al.* 1996). Early work has focused on the analysis of synovial biomarkers which offers the advantage of being joint-specific. Moreover, synovial fluid measurement directly reflects joint metabolism due to its close proximity to joint structures (THONAR *et al.* 1993). On the other hand, aspiration of synovial fluid from arthritic joints is a non-routine invasive process which is associated with the risk of eliciting iatrogenic joint sepsis (DAGLEISH *et al.* 2003). Because synovial fluid is restricted to a relatively inaccessible anatomic site, it is difficult to obtain (PRINCE 2005). Thus recent investigations have focused on marker molecules in serum and urine (ELSAID and CHICHESTER 2006).

Biomarkers of joint disease can be classified into direct and indirect markers according to the tissue they derive from (THONAR *et al.* 1999). Direct biomarkers are defined as originating exclusively from articular cartilage or bone, whereas indirect biomarkers are principally produced by non-skeletal tissues but can influence the cartilage matrix anabolism or catabolism, or its integrity.

Indirect biomarkers consist of four major categories of substances: proteolytic enzymes and their inhibitors, pro-inflammatory cytokines, growth factors, and other molecules from nonskeletal tissue origin (THONAR *et al.* 1999). Examples include matrix metalloproteinases, aggrecanase, IGF-1, IL-1, IL-6 and TNF-α. Indirect biomarkers are described as useful in both research settings and experimental OA studies and are thought be of great value to understand and clarify the development of the disease process in a joint (MCILWRAITH 2005).

Direct biomarkers have been subdivided into markers of cartilage metabolism and those of bone metabolism (MCILWRAITH 2005). Markers reflecting articular cartilage metabolism are considered to be more informative about the assessment of articular cartilage damage (RAY *et al.* 1996) and include molecules of collagen turnover such as type II collagen fragments (ELSAID and CHICHESTER 2006), and markers of proteoglycan turnover involving glycosaminoglycans such as chondroitin and keratan sulphate (FULLER *et al.* 2001). Bone markers, which include fragments of type I collagen and non-collagenous proteins (GARNERO 2006), seem to be valuable in detecting the status of subchondral and epiphyseal bone disease as well as predicting fracture, and monitoring the significance of the bone in joint diseases (MCILWRAITH 2005).

A variety of marker molecules has been investigated in equine body fluids. Some have been successfully used in research settings and experimental models yielding promising results in indicating equine joint disease (FRISBIE 2003). However, there is no marker to diagnose the degree of joint disease in a single joint with 100% accuracy (MCILWRAITH 2005). To date, no biomarker has been introduced and validated in everyday horse practice.

3.5.1 Myeloperoxidase

Myeloperoxidase (MPO) is an iron-containing cationic protein present in high concentrations in azurophilic (primary) granules of neutrophils, and also in less quantity in monocytes and subpopulations of tissue macrophages (KLEBANOFF 1999).

Mature MPO has a molecular mass of 150 kDa and is composed of two identical subunits. Each MPO subunit consists of a glycosylated 59-kDa heavy chain and a nonglycosylated 13.5-kDa light chain bound by a single disulfide bridge (NAUSEEF 1990; NAUSEEF *et al.* 1995). The heavy chain is associated with a heme group which contains an iron bound by a protoporphyrin IX derivative (DUGAD *et al.* 1990; TAYLOR *et al.* 1995). Studies of MPO biosynthesis have reported that the protein is a product from a single gene (NAUSEEF *et al.* 1996). Following processing in the endoplasmic reticulum, where glycosylation, heme insertation, and proteolytic cleavage occur, the mature molecule is directed via Golgi apparatus into the primary granules and stored until neutrophil activation (NAUSEEF *et al.* 1998).

Neutrophils belong to the group of phagocytic cells playing a critical role in the host defence against infection (BABIOR 1999). When stimulated by a variety of agents, neutrophils consume molecular oxygen in a process known as respiratory burst. Catalyzed by membrane-associated NADPH oxidase, the oxygen molecule is reduced to superoxide anion, the majority of which is further converted to H_2O_2 by superoxide dismutase. Concomitant with respiratory burst, neutrophils release their primary granule content including MPO, elastase, lysozyme and other enzymes into the phagosome (BABIOR 1984; BABIOR 2000; HAMPTON *et al.* 1996).

The majority of H_2O_2 generated by the respiratory burst is used by MPO to oxidise halide ions (Cl^-, Br^-, I^-), as well as pseudohalide thiocyanate to their corresponding hypohalous acids (HAMPTON *et al.* 1998; NASKALSKI *et al.* 2002). Because of the high concentration of Cl^- in body fluids, HOCl is thought to be the principal product of the enzyme reaction (BABIOR 2000). Under acid conditions within the phagosome, HOCl predominates over its anion OCl^- and its reaction with excess chloride to form chlorine can occur (KLEBANOFF 1999).

The primary products of chloride oxidation (HOCl, OCl^-, Cl_2) are extremely powerful oxidants that react rapidly with neighbouring thiol, disulfide, and amino acid residues forming secondary products such as chloramines and aldehyds that contribute to the toxicity of the MPO system (HAZEN and HEINECKE 1997; HAZEN *et al.* 1996; KLEBANOFF 1999).

It is assumed that the primary function of MPO is to serve as a component of the microbicidal system within the phagosome. However, the MPO system can be discharged into the extracellular milieu by leakage prior to the closure of the phagosome, secretion after neutrophils bound to a complement- and/or antibody-coated surface, or by neutrophil lysis (KLEBANOFF 1999). Outside of the phagocytes, generation of oxidants can result in host damage to nearby cells and tissues.

Studies in humans have reported that products of the MPO/H_2O_2/Cl^- system are implicated in inflammatory diseases including glomerulonephritis (MALLE *et al.* 2003), cancer (KNAAPEN *et al.* 2006; TSURUTA *et al.* 1999), atherosclerosis (BONOMINI *et al.* 2008), vasculitis (RUTGERS *et al.* 2003), and arthritis (BASKOL *et al.* 2006; SCHILLER *et al.* 2000).

Several investigators have considered HOCl to play a prominent role in the degradation of the articular cartilage. *In vitro* studies have shown that HOCl may fragment collagen (DAVIES *et al.* 1993), degrade proteoglycans (KOWANKO *et al.* 1989; SCHILLER *et al.* 1996) and reduce the radius of collagen II aggregates (OLSZOWSKI *et al.* 2003). An indirect mechanism of cartilage degradation for HOCl has been proposed due to the activation of proteolytic enzymes such as MMPs (FU *et al.* 2001; WEISS *et al.* 1985). Products of the MPO/H_2O_2/Cl^- system have shown to depolymerise synovial fluid hyaluronic acid, thus potentially contributing to a reduction of viscosity in diseased joints (BAKER *et al.* 1989; GREEN *et al.* 1990; JAHN *et al.* 1999).

Elevated concentration and/or activity of MPO has been found in synovial fluid from joints with rheumatoid arthritis in humans (EDWARDS *et al.* 1988; HADLER *et al.* 1979; SCHILLER *et al.* 1996).

LAMMER (2001) and SPELLMEYER (2003) reported an increase of MPO activity in the synovial fluids of dogs from joints with OA compared to healthy controls suggesting synovial MPO as a suitable biomarker to indicate OA in dogs. In the horse, there are no studies to measure the MPO activity/content in synovial fluid available to date.

3.5.2 Matrix Metalloproteinases -2 and -9

Matrix Metalloproteinases (MMPs) are a group of zinc-containing and calcium-dependent proteolytic enzymes (MURPHY *et al.* 2002). To date, at least 25 different MMPs have been identified. According to their substrate specificity, primary structure and cellular localisation, the MMP family can be divided into five major subgroups, namely collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), membrane-type (MT-) MMPs (MMP-14, -15, -16, -17, 24, -25), and a diverse subgroup including MMP-7, -11, -12 (BURRAGE *et al.* 2006).

MMPs are multidomain proteins consisting of a proenzyme with an amino-terminal signal peptide and a propeptide domain, a catalytic domain, and a C-terminal domain (Figure 3). The signal peptide is required for directing the enzyme through the endoplasmatic reticulum and the Golgi apparatus, and for its secretion into the extracellular space while it is cleaved off.

The propeptide domain contains the sequence motif PRCGVPD ('cysteine switch' motif) which is highly conserved among MMPs and essential for maintaining enzyme latency (MURPHY *et al.* 2002). The catalytic domain contains the catalytic centre including a zinc-binding site responsible for substrate hydrolysis and inhibitor interactions, and three conserved histidine residues.

The C-terminal domain is thought to play a role in substrate binding and is connected to the catalytic domain via a proline-rich hinge region. MMP-2 and -9 have an extra insert of three fibronectin-like type II repeats in the catalytic domain that is involved in substrate recognition (MARTEL-PELLETIER *et al.* 2001).

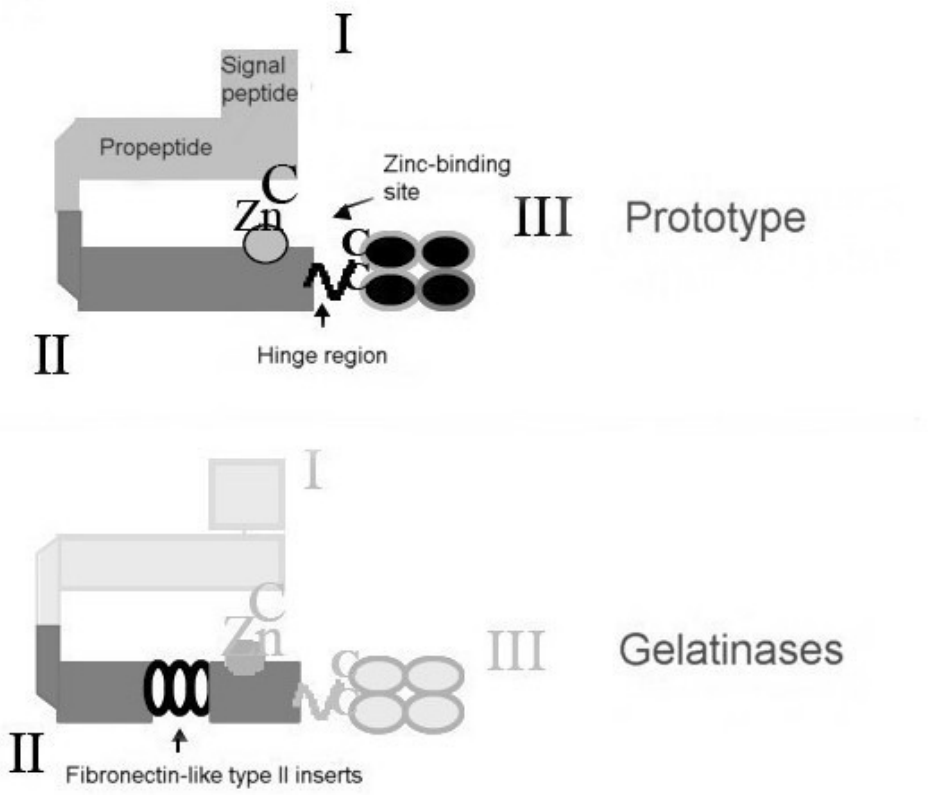

Figure 3: MMP domain structure. MMPs share three common domains: a proenzyme (I) with the 'cysteine switch' motif (C), a catalytic domain (II), and a C-terminal domain (III) (adapted from RAULO 2001).

MMPs are synthesized as pro-enzymes (zymogens, latent forms). In the pro-enzyme, the propeptide domain is positioned opposite the catalytic centre and coordinates the zinc ion with the thiol of the cysteine (MURPHY *et al.* 2002), thus excluding water which is required for peptide hydrolysis (NAGASE 1997). Most MMPs including MMP-2 and -9 are secreted from cells as pro-enzymes into the extracellular space immediately after their synthesis (WOESSNER 1991); however, inflammatory cells can store MMPs such as neutrophil collagenase and gelatinase in their primary granules (MANDAL *et al.* 2003).

MMPs are considered to be the major proteolytic enzymes involved in tissue remodelling in both physiological and pathological situations (NAGASE and WOESSNER 1999; VU and WERB 2000). They have the combined ability to degrade the major components of the extracellular matrix (BIRKEDAL-HANSEN 1995). In the horse, MMP-2 is secreted by a broad spectrum of mesenchymal cells including fibroblasts and chondrocytes, whereas MMP-9 is known to be mainly produced by monocytes, chondrocytes and PMNs (CLEGG *et al.* 1997b). Both gelatinases have a broad substrate spectrum. Apart from their preferential substrate gelatine, they interact with collagens IV, V, VII, X, elastin, fibronectin, and aggrecan (MENGSHOL *et al.* 2002; MURPHY *et al.* 2002), thus contributing to the degradation of fibrillar collagens, basement membrane components and stromal extracellular matrix molecules. Several studies have reported that MMPs are involved in a wide variety of pathological processes including cancer progression (NELSON *et al.* 2000), artherosclerosis (DOLLERY *et al.* 1995; PYO *et al.* 2000), pulmonary fibrosis (O'CONNOR and FITZGERALD 1994), emphysema (HAUTAMAKI *et al.* 1997), neuroinflammation (MANDAL *et al.* 2003), and joint disease (BURRAGE *et al.* 2006; MURPHY *et al.* 2002).

Regulation of MMP function occurs at the transcriptional as well as posttranscriptional levels. MMP mRNA expression is tightly regulated in a tissue-specific manner. Under physiological conditions, gene expression in the articular cartilage and the synovial membrane is low, but increases considerably in diseased joints in humans (BURRAGE *et al.* 2006). Their gene transcription is induced by several factors such as pro-inflammatory cytokines like IL-1β and TNF-α and growth factors including EGF (MARTEL-PELLETIER *et al.* 2001). After synthesis, the latent enzymes are secreted into the extracellular space where enzyme activation occurs. The major activation pathway *in vivo* is likely to be conducted by proteinases in a stepwise manner (NAGASE 1997).

A key step in the enzyme activation is the dissociation of the zinc and cysteine residue which allows the zinc to interact with water. This is followed by the removal of the propeptide resulting in a decrease of molecular weight (BIRKEDAL-HANSEN *et al.* 1993). MMP-9 has shown to be cleaved by enzymes such as MMP-1, -2, -3, and -7 (FRIDMAN *et al.* 1995; SANG *et al.* 1995), neutrophil elastase (FERRY *et al.* 1997), plasmin (OKADA *et al.* 1992), tissue kallikrein (MENASHI *et al.* 1994), and bacterial proteinases (OKAMOTO *et al.* 1997).

Activation of MMP-2 occurs at the cell surface potentially involving MT1-MMP and TIMP-2 (STRONGIN *et al.* 1995). Moreover, zymogens of most MMPs can be activated by non-proteolytic compounds including thiol group reactive agents such as APMA (FRISBIE *et al.* 1999a), HOCl, and denaturants like SDS. Once active, MMP activity is further regulated by several inhibitors, of which TIMPs are mainly involved (BREW *et al.* 2000). Indeed, an aberrant regulation, resulting in excess of MMPs over TIMPs, is thought to be a key event in the transition from physiological to pathological conditions (PUNZI *et al.* 2005).

In articular cartilage remodelling, MMP-2 and -9 are believed to be centrally involved. Elevated MMP-2 and -9 activities have been reported in synovial fluid from various joint diseases in humans (GAUDIN *et al.* 1997; MAKOWSKI and RAMSBY 2003; MAKOWSKI and RAMSBY 2005; MARINI *et al.* 2003; PEAKE *et al.* 2006; TAKEI *et al.* 1999) and other species (ARICAN *et al.* 2000; VOLK *et al.* 2003). However, in the horse, only few data on gelatinase activities are available (CLEGG *et al.* 1997b; JOUGLIN *et al.* 2000; KIDD *et al.* 2007; TRUMBLE *et al.* 2001). Moreover, to date specific MMP-2 and -9 activities have not been truly quantified in equine synovial fluid.

3.5.3 Markers of cartilage metabolism: C2C, CTXII, CPII, CS846

Under pathologic joint conditions, collagen II is disrupted leading to compromised stability of the collagen fibrils and cartilage erosion. Initial cleavage of collagen II is attributed to the collagenase subfamily of MMPs including MMP-1, -8, -13, and -14 (Figure 4).

Collagenases preferentially cleave intact fibrillar collagen at a single locus generating two fragments ¾ and ¼ the size of the collagen precursor. Following initial cleavage, the triple helix unwinds exposing the neoepitopes Col2-$^{3}/_{4}C_{long\ mono}$ (C2C) at the N-terminus and Col2 CTx (CTXII) at the C-terminus (ELSAID and CHICHESTER 2006). These fragments are formed only when collagen is digested, thus presenting promising markers for articular cartilage breakdown.

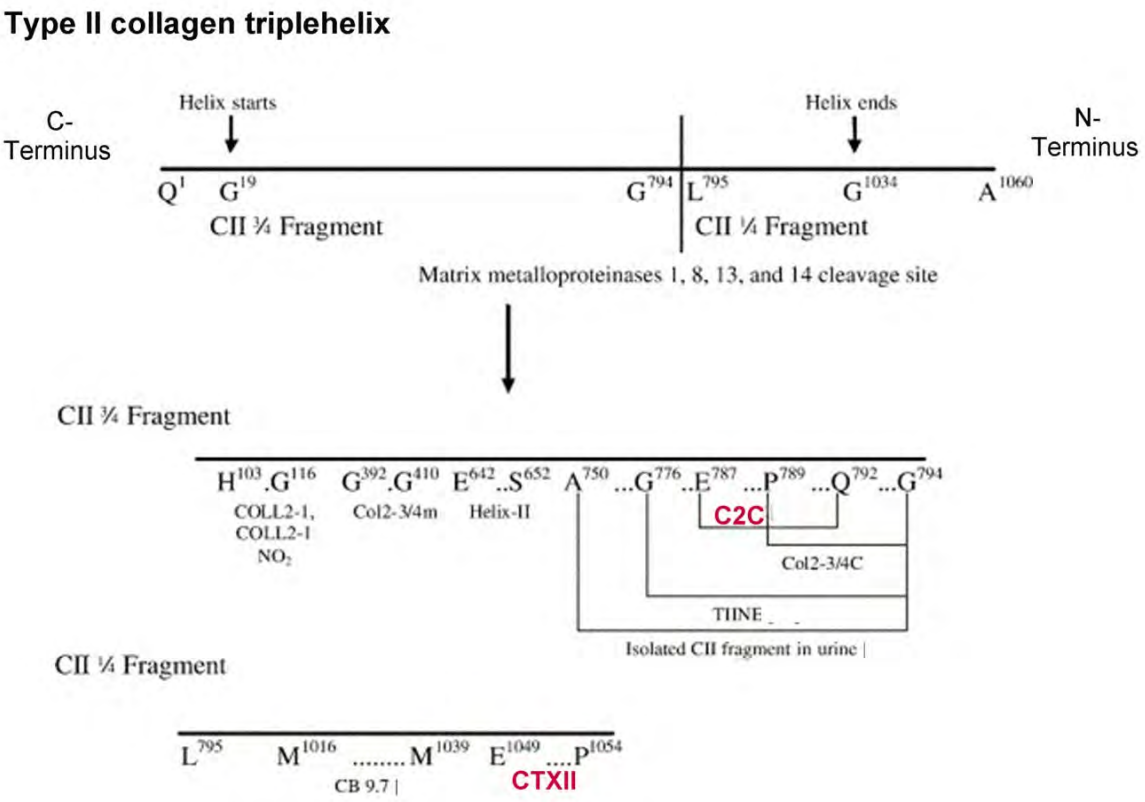

Figure 4: Generation of neoepitopes in the mature collagen type II through the initial cleavage at a single locus of the triplehelix (adapted from ELSAID and CHICHESTER 2006).

The potential of CTXII and/or C2C concentrations to indicate joint disease has been reported by previous studies in various species (CHEVALIER and CONROZIER 2005; CHU *et al.* 2002; ISHIGURO *et al.* 2001; LINDHORST *et al.* 2005; LOHMANDER *et al.* 2003; POOLE *et al.* 2004). Follow-up studies in dogs have demonstrated that both neoepitopes rise in synovial fluid and serum after experimentally induced joint injury (MATYAS *et al.* 2004), indicating that C2C and CTXII fragments show similar trends of release (ELSAID and CHICHESTER 2006).

Elevated C2C and/or CTXII concentrations have been detected in serum from different species affected with joint diseases (KONG *et al.* 2006; MATYAS *et al.* 2004; OESTERGAARD *et al.* 2006; SONG *et al.* 1999).

In the horse, a preliminary study revealed that CTXII fragments are increased in equine synovial fluid from osteochondrosis (TRUMBLE *et al.* 2006). To the author's knowledge, there are no data available supporting C2C level as an indicator for joint diseases in the horse.

Concurrently with the degradation of the extracellular matrix, chondrocytes may up-regulate the synthesis of extracellular collagen and proteoglycans in diseased joints in order to reduce cartilage damage. Type II collagen is produced as a procollagen which contains nonhelical propeptide extensions at the amino and carboxy ends. Both are removed extracellularly by proteolytic cleavage generating C- and N-terminal propeptides (ELSAID and CHICHESTER 2006). The peptide at the C-terminus, which is known as CPII, undergoes specific enzymatic cleavage. Its content has shown to be directly proportional to the rate of type II collagen synthesis in articular cartilage. Increased CPII levels were found in human OA cartilage compared to healthy cartilage (NELSON *et al.* 1998). Studies on human synovial fluid have demonstrated that CPII levels increase in joints from OA and rheumatoid arthritis (ISHIGURO *et al.* 2001; LOHMANDER *et al.* 1999; POOLE *et al.* 1994). Elevated CPII levels have also been detected in serum from patients affected with rheumatoid arthritis when compared to healthy controls (MANSSON *et al.* 1995).

The level of aggrecan synthesis can be evaluated by measuring the concentration of the epitope CS846 which is located on chondroitin sulfate chains (ROUSSEAU and DELMAS 2007). This epitope is normally found in foetal articular cartilage and almost absent from mature healthy cartilage, but its levels are significantly increased in OA cartilage from a number of species (MCILWRAITH 2005). Elevated CS846 levels have been detected in human cartilage and synovial fluid from OA (LOHMANDER *et al.* 1999; POOLE *et al.* 1994; RIZKALLA *et al.* 1992) and rheumatoid arthritis (ISHIGURO *et al.* 2001).

In an initial equine study, significantly increased CPII and CS846 concentrations were found in serum from osteochondral fragmentation compared to serum from healthy animals (FRISBIE *et al.* 1999a). Discriminant analysis using a combination of serum CS846 and CPII concentrations resulted in 79% accuracy for prediction of an osteochondral fragment.

A potential for synovial CPII and/or CP846 concentrations to indicate osteochondral injury has been suggested in horses (FRISBIE *et al.* 1999a; LAVERTY *et al.* 2000).

Work on markers of cartilage metabolism in synovial fluid and serum has yielded promising results in humans and other species, suggesting these molecules as good biomarkers for diagnosing and monitoring of changes that occur in the articular cartilage in diseased joints of horses.

4 OBJECTIVES

This thesis focuses on the analysis of biomarkers in equine synovial fluid and serum for the diagnosis of joint diseases subdivided into OA, chronic arthritis, septic arthritis, and OCD. Its main objectives are the following:

1. to establish a specifically modified MPO activity assay in the synovial fluid and serum of horses.

2. to evaluate MPO activity and MPO content in synovial fluid and serum of horses as markers of joint diseases.

3. to investigate the possible use of MMP-2 and -9 activities in synovial fluid and serum of horses as potential diagnostic markers of joint diseases.

4. to evaluate the content of markers of cartilage metabolism including C2C, CTXII, CPII and CS846 in synovial fluid and serum of horses as potential diagnostic markers of joint diseases.

5. to study MPO activity, MMP-2 and -9 activities, C2C, CTXII, CPII and CS846 concentrations in frequently collected synovial fluids of horses from septic arthritis to monitor the disease process during a time-course.

5 MATERIALS

5.1 Equine samples

A total of 109 synovial and 44 serum samples were obtained from 98 horses evaluated at the Clinic of Horses, General Surgery and Radiology of the Faculty of Veterinary Medicine of the Freie Universität Berlin between 2003 and 2006. Samples were aspirated by Anastasios Moschos or co-workers and kindly provided as part of a collaborative effort. In each case, only one of the horse's joints was punctured. General data of all animals, including age, sex, breed, and punctured joint, are listed in Tables 10-14 (Appendix I).

The control group consisted of 20 synovial and 11 serum samples. Control animals were selected according to MOSCHOS (2007):

1. The punctured horse showed no signs of general disease.
2. The punctured joint presented normal mobility.
3. No lameness could be attributed to the punctured joint.
4. No swelling, tenderness, or heat was noticed over the joint.
5. No thickness or lack of elasticity of the joint capsule was palpable.
6. No signs of synovial effusion were apparent in the punctured joint.
7. Synovial fluid showed normal appearance and physiological parameters after analysis.

The diseased group contained a total of 78 synovial and 33 serum samples and was subdivided into OA, chronic arthritis, septic arthritis, and OCD based on anamnesic data, clinical examination, radiography, and synovial fluid assessment (MOSCHOS 2007).

The OA group included 28 synovial and 11 serum samples and the chronic arthritis group consisted of 30 synovial and 11 serum samples. Both joint diseases were defined as having a history with a minimum duration of at least five weeks and characterized by lameness, positive response to flexion test, and perineural anaesthesia.

Joints from OA represented radiographic findings consistent with thinning of joint space and/or subchondral bone sclerosis and/or periarticular marginal osteophytes/enthesiophytes, whereas no radiographic changes were found in joints with chronic arthritis. The septic arthritis group contained 8 synovial and 4 serum samples. Septic arthritis was characterized by lameness, joint effusion, penetrating wound in the joint, and a WBC count of > 10,000 cells/µl synovial fluid. The OCD group consisted of 12 synovial and 7 serum samples. OCD was defined as having an osteochondritic fragment in convex articular surfaces in young horses. Punctured joints of the OCD group represented no osteoarthritic radiographic findings.

The follow-up group included 11 synovial fluid samples from 4 horses with septic arthritis. All horses were equally medicated. Treatment after initial examination included joint immobilization and medication including joint flushing by use of Ringer's solution and intravenous application of non-steroidal anti-inflammatory drugs. Progression of clinical signs was recorded from all patients on the basis of physical examination at the Clinic of Horses, General Surgery and Radiology of the Faculty of Veterinary Medicine of the Freie Universität Berlin. All patients showed improvement in joint function within a time-course of 8 weeks as indicated by body temperature, systemic and synovial WBC count, joint effusion, and lameness degree.

After aspiration, synovial fluid and serum samples were centrifuged immediately at 2500 *g* or 3000 *g* respectively for 10 min at 25°C; the cell-free supernatant was stored in aliquots at -20°C until analysis.

5.2 Chemicals

Name	Label	Source
ABAH	Myeloperoxidase Inhibitor 1	Calbiochem, Bad Soden
Acrylamid/Bis	Rotiphorese® Gel 30	Carl Roth GmbH & Co.KG, Karlsruhe
APS	Ammoniumpersulfat	Merck KGaA, Darmstadt
Brij35	Brij® 35	Sigma-Aldrich Chemie GmbH, Taufkirchen
Bromophenol Blue	Bromophenol Blue	Sigma-Aldrich Chemie GmbH, Taufkirchen
BSA	Albumin bovine Fraction V	Serva Electrophoresis GmbH, Heidelberg
C2C ELISA	Collagen Type II Cleavage ELISA	OSTEO*medical* GmbH, Bünde
CPII ELISA	Procollagen II C-Propeptide ELISA	OSTEO*medical* GmbH, Bünde
CS846 ELISA	Aggrecan Chondroitin Sulfate-846 Epitope ELISA	OSTEO*medical* GmbH, Bünde
CTXII ELISA	Serum Pre-Clinical Cartilaps® ELISA	OSTEO*medical* GmbH, Bünde
DMSO	Dimethylsulfoxid	Merck KGaA, Darmstadt
DTPA	Diethylenetriaminepentaacetic acid, ≥ 99%	Sigma-Aldrich Chemie GmbH, Taufkirchen
EDTA	Ethylenediaminetetraacetic acid 99.995%	Sigma-Aldrich Chemie GmbH, Taufkirchen
Gelatin	Gelatin from porcine skin for electrophoresis, Type A	Sigma-Aldrich Chemie GmbH, Taufkirchen
Glycerol	Glycerin, 87 Gew. %	Ferak Berlin GmbH, Berlin
Glycin	Glycin, ≥ 99%	Carl Roth GmbH & Co.KG, Karlsruhe
HCl	Hydrochloric acid, 1 mmol/l	Merck KGaA, Darmstadt
H_2O_2	Hydrogen peroxide, 30% wt.% sol. in water	Sigma-Aldrich Chemie GmbH, Taufkirchen
Hyaluronidase	Hyaluronidase	Sigma-Aldrich Chemie GmbH, Taufkirchen
Isopropanol	2-Propanol, Rotisolv®, ≥ 99.95%	Carl Roth GmbH & Co.KG, Karlsruhe

Table 1: Tabulation of chemicals used in this study.

Name	Label	Source
$MgCl_2$	Magnesiumchlorid-Hexahydrat, ≥ 98%	Carl Roth GmbH & Co.KG, Karlsruhe
MMP-2	Matrix Metalloproteinase 2 (MMP 2), Human 72 kDa	Invitek GmbH, Berlin
MMP-9	Matrix Metalloproteinase 9 (MMP 9), Human 92 kDa	Invitek GmbH, Berlin
MMP-2 Immunocapture Activity Assay	Matrix Metalloproteinase-2 (MMP-2) Biotrak Activity Assay System	Amersham Biosciences Europe GmbH, Freiburg
MMP-9 Immunocapture Activity Assay	Matrix Metalloproteinase-9 (MMP-9) Biotrak Activity Assay System	Amersham Biosciences Europe GmbH, Freiburg
MMP-2/MMP-9 Inhibitor III	MMP-2/MMP-9 Inhibitor III	Calbiochem, Bad Soden
MPO	Myeloperoxidase, Human Polymorphonuclear Leukocytes	Calbiochem, Bad Soden
MPO ELISA	Equine MPO ELISA	Biocode-Hycel, Liège, Belgium
Na-citrate-2-hydrate	Na-citrate-2-hydrat	Jenapharm GmbH & Co.KG, Jena
NaCl	Natriumchlorid	Carl Roth GmbH & Co.KG, Karlsruhe
NaH_2PO_4	Natriumdihydrogenphosphat1-hydrat	Merck KGaA, Darmstadt
Na_2HPO_4	*di*-Natriumhydrogenphosphat-2-hydrat	Merck KGaA, Darmstadt
o-Dianisidine	*o*-Dianisidine	Sigma-Aldrich Chemie GmbH, Taufkirchen
Protein Assay	Dye reagent concentrate	Bio-Rad Laboratories GmbH, Munich
Protein staining solution	PageBlue™ Protein Staining Solution	Fermentas GmbH, St. Leon-Rot
Protein molecular weigt marker	Precision Plus Protein™	BioRad Laboratories GmbH, Munich
SDS	Sodiumdodecylsulfat, ≥ 99%	Sigma-Aldrich Chemie GmbH, Taufkirchen
TEMED	N, N, N', N'-Tetramethylethylendiamin	Merck KGaA, Darmstadt
Tris/HCl	Tris (hydroxymethyl) aminomethane HCl	Serva Electrophoresis GmbH, Heidelberg
Triton X-100	Triton® X-100	Ferak Berlin GmbH, Berlin
Tyrosin	L-Tyrosin, ≥ 99%	Carl Roth GmbH & Co.KG, Karlsruhe

Continuation of Table 1.

5.3 Equipment and software

Name	Label	Source
Balance	MC1	Sartorius AG, Goettingen
Balance desiccator	Bp1200	Sartorius AG, Goettingen
Centrifuge	Biofuge fresco	Heraeus Instruments GmbH, Osterode
Data analysis software BiAS.	BiAS. for Windows (Version 8.3)	Epsilon-Verlag GbR, Darmstadt
Data analysis software Winlab	FL Winlab (Version 3.00)	PerkinElmer Optoelectronics GmbH, Wiesbaden
Data analysis software Microplate manager	Microplate Manager (Version 5.2)	BioRad Laboratories GmbH, Munich
Data analysis software SPSS	SPSS 15.0 for Windows	SPSS GmbH Software, Munich
Electrophoresis	Power Supply EPS 35500 XL	Amersham Pharmacia Biotech, Munich
Heating magnetic stirrer	Ikamag® Rct	Janke & Kunkel GmbH & CO.KG, Staufen
Imaging software MagicScan	MagicScan (Version 4.71)	Umax Systems GmbH, Willich
Incubator	Lab-Therm	Adolf Kühner AG, Basel
Luminescence Spectrometer	LS 50B	PerkinElmer Optoelectronics GmbH Wiesbaden
Microplate Shaker	IKA®-Schüttler MIS 4	Janke & Kunkel GmbH & CO.KG, Staufen
Microplate reader	Model 550	BioRad Laboratories GmbH, Munich
Mini gel apparatus	Mini-Protean® 3 Cell system	BioRad Laboratories GmbH, Munich
PH-Meter	Climatic 761	Knick Messgeräte GmbH & CO.KG, Berlin
Spectrophotometer	SmartSpec Plus	BioRad Laboratories GmbH, Munich
Scanner	PowerLook 1000	Umax Systems GmbH, Willich
Thermomixer	Compact	Eppendorf AG, Hamburg
Vortexer	Vibrofix VF1 Electronic	Janke & Kunkel GmbH & CO.KG, Staufen

Table 2 : Tabulation of equipment and software used in this study.

6 METHODS

6.1 Cytological examination

Cytological examination was assessed at the Clinic of Horses, General Surgery and Radiology of the Faculty of Veterinary Medicine of the Freie Universität Berlin. WBC counts were quantified using an automated cell counter (MOSCHOS 2007). Results were expressed in 10^6cells/ml. PMN content in synovial fluid samples was determined by manual differentiation of Pappenheim stained smears under light microscope. Results were expressed in % PMN content. All measurements were performed by Anastasios Moschos or co-workers and kindly provided as part of a collaborative effort.

6.2 Protein quantification

Protein quantification was performed using the Bio-Rad Protein Assay based on the method of BRADFORD (1976) in accordance with the manufacturer's protocol.

Synovial fluid and serum samples were diluted 1:5 in distilled water. A protein standard was prepared by diluting BSA in distilled water into different concentrations ranging from 0.2 to 1 mg/ml. Samples and standards were assayed in duplicate. Aliquots of 2.5 µl of each sample and standard dilution were dispensed into a test tube. Dye reagent was diluted 1:5 in distilled water, and a volume of 1 ml was added into each tube. After incubation at RT for 15 min the contents were mixed again and filled into glass cuvettes. Absorbance was read at 595 nm by spectrophotometer. Distilled water was used as blank value. A standard curve was prepared by plotting absorbance against standard protein concentration. Protein concentration of samples was expressed in mg/ml.

6.3 MPO analysis

6.3.1 MPO Photometric Activity Assay

MPO activity was measured using MPO photometric activity assay according to the method of KUMAR *et al.* (2002), with various modifications.

Synovial fluid and serum samples were diluted in citrate buffer (pH 5.5) to dilutions of 1:2 and 1:10, respectively. Aliquots of 60 µl were added to the assay mixture containing 0.1% Triton X-100, 0.65 mM *o*-dianisidine, and 0.08 M citrate buffer (pH 5.5). After adding 0.43 mM H_2O_2, a final volume of 300 µl was dispensed into a well of a 96-well microtiter plate. Absorbance was read at 450 nm on a microtiter plate reader every 5 min for up to 30 min. Graphs were prepared by plotting absorbance against time. Peroxidase activity was calculated according to the following equation:

$$\text{Peroxidase activity [U/ml]} = \frac{\Delta E \times V}{t \times \varepsilon \times d \times v}$$

ΔE $E_{30\ min} - E_{0\ min}$
V final volume
t time [min]
ε molar absorption coefficient for *o*-dianisidine at 450 nm [$mM^{-1}cm^{-1}$]
d light path [cm]
v sample volume

The molar absorption coefficient for *o*-dianisidine constituted 11.48 $mM^{-1}cm^{-1}$ (SPELLMEYER 2003).

Synovial fluid and serum samples were run additionally in the presence of 30 µM ABAH. Peroxidase activity was calculated and subtracted from that obtained in the absence of ABAH, and the resulting MPO activity was expressed as U/ml.

6.3.1.1 Inhibition study

The effect of different ABAH concentrations on the MPO activity was evaluated. For this purpose, ABAH concentrations ranging from 0.5 to 50 µM were added to 7 µM purified enzyme. MPO activity was calculated in the absence and presence of each ABAH concentration, and results were expressed as % inhibition of MPO activity. The smallest possible ABAH concentration that revealed a complete inhibition of 7 µM MPO was tested on increasing MPO concentrations of up to 15 µM. Moreover, this ABAH concentration was tested on 2 appropriate synovial fluid samples from different diagnostic groups. Absorbance was obtained in the absence and presence of ABAH, and results were graphically presented by plotting absorbance against time.

6.3.1.2 Influence of H_2O_2 concentration

To investigate the impact of different H_2O_2 concentrations on the kinetics of MPO, H_2O_2 concentrations ranging from 0.13 to 0.86 mM were added to 3 appropriate synovial fluid samples from different diagnostic groups. Absorbance obtained in the presence of ABAH was subtracted from that observed in the absence of ABAH, and results were graphically represented by plotting absorbance against time. The H_2O_2 concentration that revealed a linear kinetics of synovial MPO was additionally tested on 2 serum samples.

6.3.1.3 Influence of viscosity

The influence of sample viscosity on the MPO activity was assessed on 5 representative synovial fluid samples from different diagnostic groups with and without hyaluronidase treatment. For hyaluronidase treatment, samples were incubated with 60, 120, and 180 U/ml enzyme at 37°C for 30 min. Synovial fluids were centrifuged at 3000 *g* for 20 min at 25°C, and aliquots of each supernatant were used in the assay. Results were expressed as MPO activity obtained with and without hyaluronidase treatment.

6.3.1.4 Influence of sample dilution

The effect of different synovial fluid sample dilutions on the MPO activity was investigated using a control synovial fluid sample spiked with 3.5, 7, 14, or 21 µM purified MPO. Samples were then diluted to 1:8, 1:4, and 1:2 and MPO activity was determined. Results were graphically represented as MPO activity against sample dilution. An appropriate serum sample was additionally used in 3 dilutions ranging from 1:8 to 1:2, each spiked with known amounts of purified enzyme and MPO activity was measured. Moreover, MPO activities were determined in 4 different synovial fluids used at 1:2, 1:4, and 1:8 dilutions as well as in undiluted samples.

6.3.1.5 Reproducibility

Data reproducibility was investigated by using purified MPO diluted to concentrations of 3.5, 7, 14, and 21 µM, each measured in 5 parallel assays. Furthermore, 5 different synovial fluid samples were measured in parallel assays in triplicate. As an inter-assay control, 3.5 µM purified MPO was used in each run. Results were expressed as % variation.

6.3.2 MPO Fluorometric Activity Assay

MPO Fluorometric Activity Assay was performed in accordance with the method utilized by MARQUEZ AND DUNFORD (1995), with some modifications.

Purified human MPO in concentrations ranging from 0.1 to 0.5 µM was added to the assay mixture containing 1 mM tyrosin, 0.1 M DTPA and 0.9 M sodium phosphate buffer (pH 7.5). After addition of 0.2 mM H_2O_2, relative fluorescence intensity was recorded at 325 nm (excitation) and 405 nm (emission) on a luminescence spectrometer to obtain the time-zero value. Increase in relative fluorescence intensity was measured in a kinetic study (*i.e.* every 50 s) for up to 300 s. Graphs were prepared by plotting changes in relative fluorescence intensity against time.

Two synovial fluids from different diagnostic groups were diluted in sodium phosphate buffer (pH 7.5) to dilutions of 1:1000, 1:100, 1:10, and 1:2. Aliquots of 100 µl were added to the assay mixture and relative fluorescence intensity was recorded. Graphs were prepared by plotting changes in relative fluorescence intensity against time. Furthermore, 5 control synovial fluids were spiked with 0.2 µM purified MPO and relative fluorescence intensity was followed.

6.3.3 MPO ELISA

MPO concentration was measured using equine MPO ELISA according to the manufacture's instructions.

A MPO standard was prepared by diluting equine MPO in assay buffer to concentrations ranging from 1.5 to 48 ng/ml. Synovial fluid samples were used in dilutions of 1:3. Aliquots of 100 µl were dispensed into wells of 96-well microtiter plate pre-coated with rabbit polyclonal anti-MPO. The plate was covered with a sealing tape and incubated at 4℃ overnight. After washing, aliquots of 100 µl guinea pig polyclonal anti-equine MPO conjugated with alkaline phosphatase were added into each well, and the plate was incubated for 2 h at 37℃. Following another washing step, 100 µl of chromogen substrate PNPP were added into the wells after which the plate was incubated for 30 min RT. Aliquots of 100 µl stop solution were added and absorbance was read at 405 nm on a microtiter plate reader. A standard curve was prepared by plotting absorbance against standard MPO concentration. MPO concentrations were expressed in ng/ml.

Data reproducibility was investigated by using 3 different synovial fluid samples in triplicate during one experimental run. As an inter-assay control, 2 appropriate synovial fluid samples were used in each run.

6.4 MMP analysis

6.4.1 Gelatin zymography

Gelatinase activity was assessed using gelatin zymography according to the method utilized by LAEMMLI (1970), with some modifications.

SDS-PAGE was performed on a vertical mini gel apparatus. Gelatin was suspended in distilled water and incubated in a 50°C water bath until it was completely dissolved. A concentration of 2.5 mg/ml was added to the separating gel solution, containing 10% Acrylamid/Bis, 0.375 M Tris/HCl (pH 6.8), 0.1% SDS, 0.05% APS, and 0.1% TEMED. The liquid gel was placed into mini glass cassettes up to a height of 10 cm and 1 ml of isopropanol was added on top. After polymerisation, isopropanol was slowly removed using absorbent paper. The stacking gel solution including 4% Acrylamid/Bis in 0.125 M Tris/HCl (pH 6.8), 0.1% SDS, 0.05% APS, and 0.1% TEMED was added to the remaining space of the cassettes, and a 10-well comb was inserted. Synovial fluid samples were diluted to 1:10 in a 50 mM Tris/HCl sample buffer (pH 6.8), with 1.5% SDS, 5% glycerol, 0.005% Bromophenol Blue, and incubated at 37°C for 1 h prior to electrophoresis. Following polymerisation of stacking gel, combs were slowly removed, and aliquots of the 5 µl sample dilution or protein molecular weight marker were added into wells. Cassettes were inserted into an electrophoresis chamber filled with 250 ml of 12.5 mM Tris/HCl running buffer (pH 8.3) containing 96 mM glycine, and 0.1% SDS. Samples were electrophoresed at 50 V and 150 mA until the sample front reached the separating gel, followed by separation at 150 V and 150 mA for approx. 90 min. Gels were washed in 2.5% Triton X-100 for 1 h RT and rinsed twice in distilled water. After overnight incubation in a 50 mM Tris/HCl reactivation buffer (pH 7.6) containing 50 mM $CaCl_2$, 10 mM NaCl, and 0.05% Brij35 at 37°C, gels were washed three times in distilled water for 30 min and incubated in a protein staining solution for 1 h RT.

To investigate the effect of varying sample dilutions on the gelatinolytic activity, 2 appropriate synovial fluids were loaded in dilutions ranging from 1:30 to 1:2.

MMPs were identified by comparison to electrophoretic migration of 1 ng latent human MMP-2 and -9. Inhibition of gelatinase activity was investigated by adding 50 mM EDTA and 50 µM MMP2/MMP9 inhibitor III to the reactivation buffer. Activation of zymogens was demonstrated by including 1 mM APMA in the sample buffer. Digital images of the polyacrylamide gels were captured by scanning. Gels were analysed with the software Gene Tools. The optical assessment value of the sample band was compared with the value of 72 kDa gelatinolytic band of latent MMP-2 standard running on the same gel.

6.4.2 MMP-2 and -9 Immunocapture Activity Assays

MMP-2 and -9 activities were measured using Biotrak Activity Assay Systems in accordance with the manufacture's instructions.

MMP standard was prepared by diluting Pro-MMP-2 and -9 in assay buffer to concentrations ranging from 0.19 to 3 ng/ml and from 0.125 to 4 ng/ml, respectively. Aliquots of 100 µl synovial fluid and serum samples and standards were dispensed into a well of 96-well microtiter plate pre-coated with sheep polyclonal anti-MMP-2 or MMP-9. The plates were covered with a sealing tape and incubated overnight at 4°C. After washing, 50 µl APMA were added into standards and 50 µl of assay buffer were added into sample wells. Aliquots of 50 µl detection reagent containing modified pro-murokinase and specific chromogenic peptide substrate S-2444 were pipetted into all wells. Absorbance was read at 405 nm on a microtiter plate reader to obtain a time-zero value, and after incubation at 37°C for 2 or 6 h, respectively. MMP activity was represented by the change of absorbance over time. Values were compared with standard curves. MMP-2 and -9 activities were expressed in ng/ml.

Specificity of MMPs was investigated by adding 50 µM MMP2/MMP9 inhibitor III to each well of highest MMP-2 and -9 standard concentrations, and 2 control synovial fluid samples as well as 2 samples from septic arthritis.

Intra-assay variation was investigated using 3 different synovial fluid samples in triplicate during one experimental run. As an inter-assay control, 2 appropriate synovial fluids were used in each run.

6.5 Markers of cartilage metabolism

6.5.1 Cartilage degradation assays

Assays were used according to the manufacturer's protocols. Intra-assay variation was investigated by using 3 different synovial fluids in triplicate during one experimental run. As an inter-assay control, 2 synovial fluids were used in each run.

6.5.1.1 C2C ELISA

C2C standard was prepared by diluting C2C standard in assay buffer to concentrations ranging from 0 to 1 µg/ml. Aliquots of 50 µl samples and standards were dispensed into wells of a 96-well plate, and 50 µl mouse monoclonal antibody specific for C2C were added into each well. After the plate was incubated for 30 min on a titre plate shaker at 650 rpm, 80 µl of each well were transferred into the wells of a plate pre-coated with C2C neoepitope which was subsequently incubated for 30 min at 650 rpm, and washed. Aliquots of 100 µl goat anti-mouse horseradish peroxidase conjugate were added into the wells, the plate was incubated for 30 min at 650 rpm, washed, and 100 µl TMB were added into the wells. Following incubation for 30 min at 650 rpm, 100 µl stop solution were added and absorbance was read at 450 nm on a microtiter plate reader. Standard curve was prepared by plotting absorbance against C2C concentration. The C2C concentration was expressed in ng/ml.

6.5.1.2 CTXII ELISA

CTXII standard was prepared by diluting CTXII standard in assay buffer to concentrations ranging from 0 to 247.6 pg/ml.

Aliquots of 100 µl biotinylated monoclonal murine antibody specific for CTXII were added into wells of a 96-well plate pre-coated with streptavidin. After the plate was incubated on a titre shaker at 300 rpm for 30 min, and washed, 25 µl sample and standards were added into the wells. The plate was then incubated for 60 min at 300 rpm, washed, and 100 µl peroxidase conjugated murine monoclonal antibody specific for CTXII were added. Following incubation at 300 rpm for 60 min, the plate was washed, and 100 µl of TMB were added into the wells.

The plate was incubated at 300 rpm in total darkness, 100 µl stop solution were added, and absorbance was read at 450 nm with 650 nm reference on a microtiter plate reader. A standard curve was prepared by plotting absorbance against CTXII concentration. The CTXII concentration was expressed in pg/ml.

6.5.2 Cartilage synthesis assays

Assays were used according to the manufacture's instructions. Intra-assay variation was investigated by using 3 different synovial fluids in triplicate during one experimental run. As an inter-assay control, 2 synovial fluids were used in each run.

6.5.2.1 CPII ELISA

The 96-well plate was coated with 100 µl CPII neoepitope per well, incubated overnight at 4°C, and washed. CPII standard was prepared by diluting CPII standard in assay buffer to concentrations ranging from 0 to 2000 ng/ml. Aliquots of 50 µl sample and standard and 50 µl rabbit polyclonal antibody specific for CPII were dispensed into wells of 96-well plate. After the plate was incubated on a titre shaker at 650 rpm for 30 min, 80 µl from each well were transferred into wells of the plate pre-coated with CPII neoepitope after which it was incubated at 650 rpm for 2 h, and washed. Aliquots of 100 µl goat anti-rabbit horseradish peroxidase conjugate were added into the wells, and the plate was incubated at 650 rpm for 30 min at RT, washed again, and 100 µl TMB were added into the wells.

Following further incubation at 650 rpm for 30 min, 100 µl stop solution were added, and absorbance was read at 450 nm on a microtiter plate reader. A standard curve was prepared by blotting absorbance against CPII concentration. The CPII concentration was expressed in ng/ml.

6.5.2.2 CS846 ELISA

Synovial fluid and serum samples were diluted by a factor of 1:2 in assay buffer. A CS846 standard was prepared by diluting CS846 standard stock in assay buffer to concentrations ranging from 0 to 2000 ng/ml. Aliquots of 50 µl sample and standard were dispensed into wells of a 96-well ELISA plate pre-coated with goat anti-mouse antibody. After 50 µl of CS846 biotin and 50 µl of mouse monoclonal CS846 antibody were added, the plate was incubated on a titre shaker at 650 rpm for 2 h, washed, and 100 µl Streptavidin horseradish peroxidase conjugate were added into each well. The plate was incubated at 650 rpm for 30 min, washed, and 100 µl of TMB were added into the wells. Following further incubation at 650 rpm for 30 min, 100 µl stop solution were added, and absorbance was read at 450 nm on a microtiter plate reader and a standard curve was prepared by blotting absorbance against CS846 concentration. CS846 concentration was expressed in µg/ml.

6.6 Data presentation and statistical analysis

Data are graphically presented and statistically analysed using SPSS 15.0 and BiAS. Results are presented separately for the methodological analysis (chapter 7.1) and for the evaluation of distinct parameters in equine synovial fluid and serum (chapters 7.2 and 7.3).

Methodological data from MPO analysis are presented either in table form or in line diagrams. Results are expressed as mean and corresponding standard deviation. For the relationship between parameters, Spearman's rank coefficient of correlation was computed. *p* values below 0.05 were considered significant. Results from gelatin zymography are shown as images.

Single values of parameters evaluated in synovial fluid and serum are itemised in Tables 10-19 (Appendix I). Results for different diagnostic groups are presented in box plots. Boxes represent the interquartile range, *i.e.* the middle 50% of the data between the 25th and 75th percentile. The median is symbolised as a horizontal bar across the box. Whiskers indicate the largest and smallest scores observed that are less than 1.5 box lengths from the end of the box. Circles symbolize the largest scores between 1.5 and 3 box lengths from the end of the box, and stars represent values outside the latter range. Statistics including median, interquartile range (IQR), minimum, and maximum for each parameter were listed in Table 20 (Appendix II). Data were considered to be non-normally distributed values from independent sampling. For a statistical comparison between the diagnostic groups, the Kruskal-Wallis-test and Dunn's post hoc test were used. *p* values below 0.05 were considered significant. For all statistical tests, the *p*-values are listed in Table 21 (Appendix III). Only significant results are given in the text.

Relationships between selected single parameters are presented in scatter diagrams. For a statistical measure of correlation between synovial parameters, Spearman's rank coefficient of correlation was calculated (Table 22, Appendix III). Coefficients with values ≤ 0.2, ≤ 0.5, ≤ 0.7, ≤ 0.9, > 0.9 were considered to represent a very weak, weak, moderate, strong, or very strong correlation, respectively (BÜHL 2006). All coefficients of correlation were tested for being statistically significant (*p* value below 0.05). Single data of the follow-up group are graphically represented as a function of time in scatter diagrams.

Linear discriminant analysis was used on the basis of synovial fluid or serum parameters to classify horses as controls or as having a joint affected with OA, chronic arthritis, OCD, or septic arthritis. The cross-validated rate of correctly classified samples was used as a goodness of fit. Correct classification rates for single parameters and parameter combinations are presented in table form. For the combination of parameters that yielded the highest correct classification rate, relationships between original and anticipated diagnostic groups are stated in table form.

7 RESULTS

7.1 Methodological study

7.1.1 MPO Photometric Activity Assay

7.1.1.1 Inhibition study

Figure 5 shows the effect of different ABAH concentrations on the activity of 7 µM purified MPO. A 50% reduction of MPO activity was found at an ABAH concentration of 1.9 µM. The smallest possible ABAH concentration that revealed a complete inhibition of 7 µM MPO was found to be 30 µM (Figure 5). Increasing ABAH concentrations of up to 50 µM also resulted in a complete inhibition of 7 µM MPO. Addition of 30 µM ABAH to purified MPO concentrations of up to 15 µM resulted in a complete inhibition of MPO activity (Table 3).

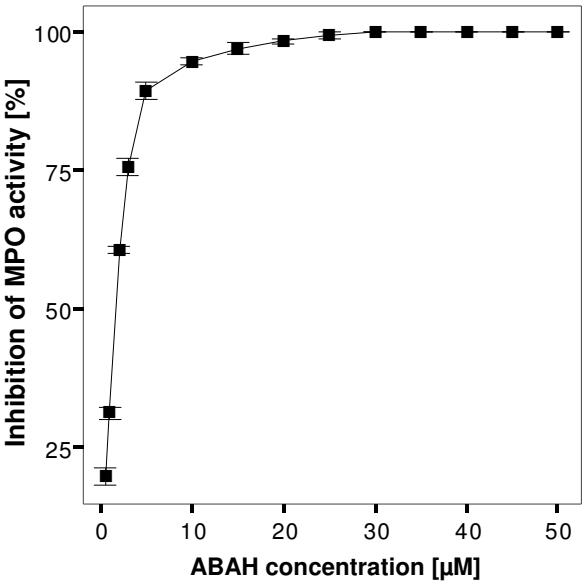

Figure 5: Effect of different ABAH concentrations on the activity of 7 µM purified MPO. This experiment was done in triplicate. Data shown are the mean of inhibition of MPO activity with corresponding standard deviation.

MPO concentration [µM]	MPO activity [U/ml]	
	No ABAH treatment	30 µM ABAH treatment
7	16.5 ± 0.001	0 ± 0
10	23.6 ± 0.003	0 ± 0
12	28.2 ± 0.004	0 ± 0
15	35.4 ± 0.001	0 ± 0

Table 3: Effect of 30 µM ABAH treatment on the MPO activity of different MPO concentrations. MPO activity was measured in 5 parallel assays. Data shown are the mean of MPO activity with corresponding standard deviation.

As shown in Figure 6, application of 30 µM ABAH to 2 representative synovial fluid samples of the control and septic arthritis group resulted in a linear reduction of absorbance of up to 30 min.

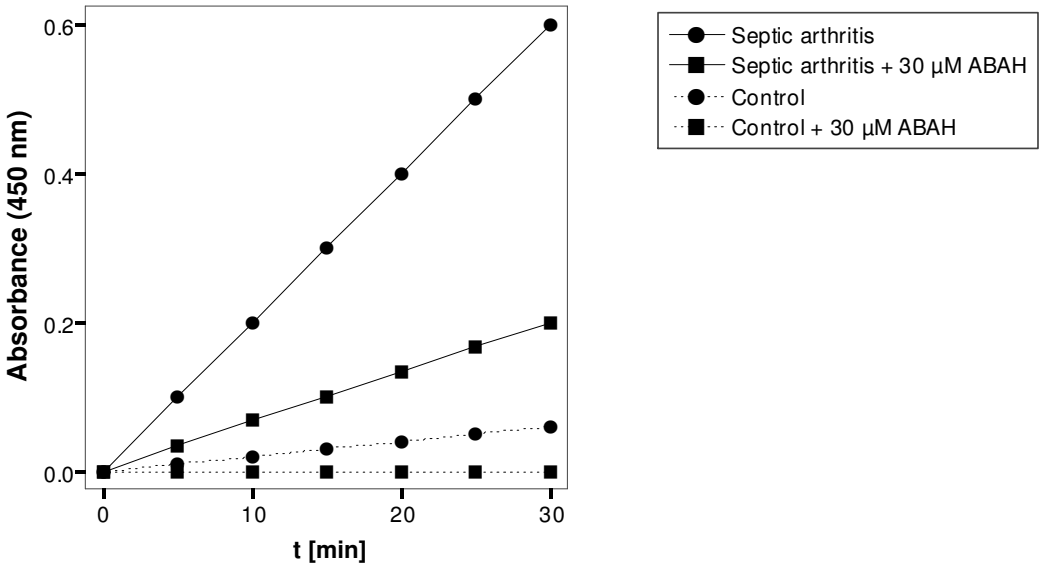

Figure 6: Effect of 30 µM ABAH on the absorbance of 2 synovial fluid samples. Data shown are the mean of absorbance. Corresponding standard deviations ranged from 0.001 to 0.003.

7.1.1.2 Influence of H_2O_2 concentration

Figure 7 shows the effect of different H_2O_2 concentrations on the kinetics of MPO of an appropriate synovial fluid sample. Addition of 0.43 mM H_2O_2 resulted in linear change of absorbance for up to 30 min, whereas no linear kinetic during the displayed time-course could be observed when using H_2O_2 in concentrations of 0.13, 0.28, 0.65, and 0.86 mM.

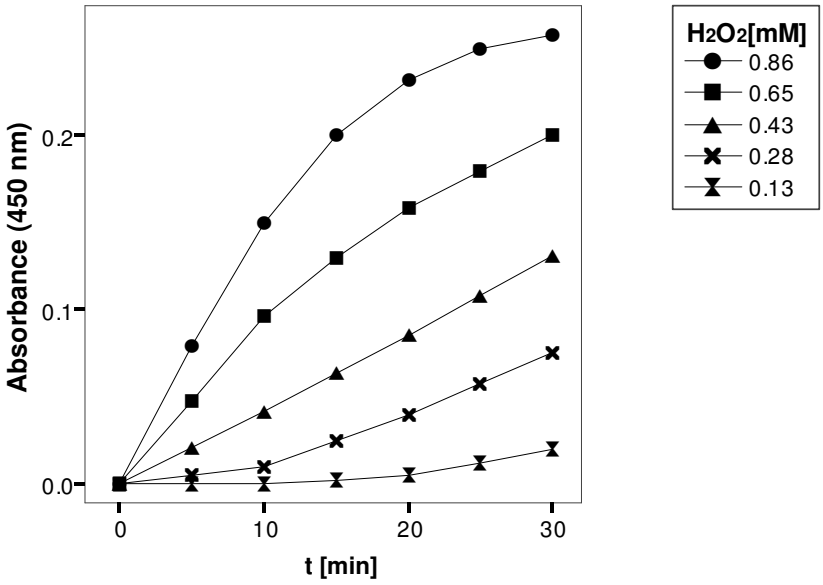

Figure 7: Effect of different H_2O_2 concentrations on the kinetics of MPO of a representative synovial fluid sample. This experiment was done in triplicate. Data shown are the mean of absorbance change. Standard deviations ranged from 0.001 to 0.003.

This experiment was repeated with 2 synovial fluid samples. As shown in Figure 8, addition of 0.43 mM H_2O_2 resulted in a linear kinetic of synovial MPO of up to 30 min. Moreover, 0.43 mM H_2O_2 to 2 appropriate serum samples also revealed a linear kinetic of MPO for up to 30 min (Figure 8).

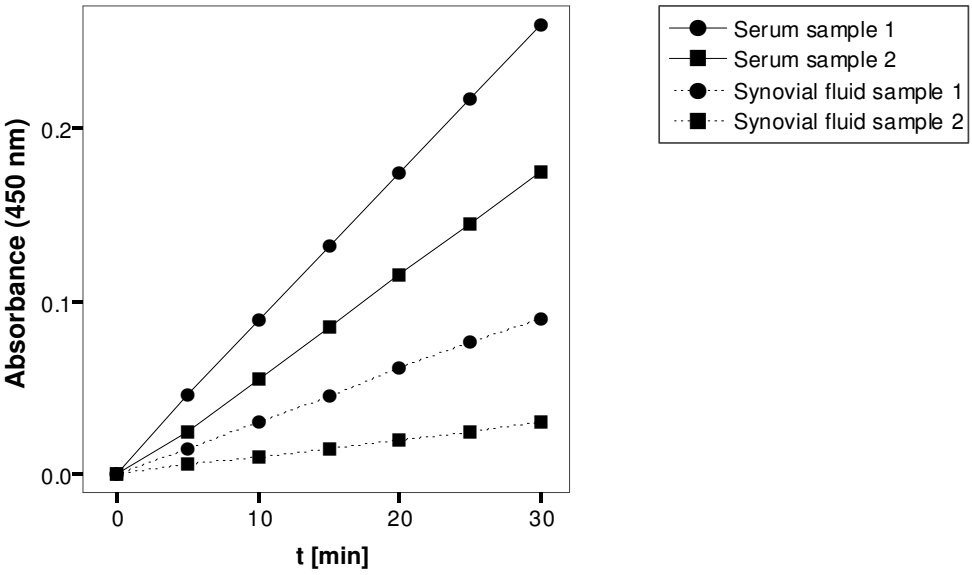

Figure 8: Effect of 0.43 mM H_2O_2 on the kinetics of MPO of synovial fluid and serum samples. This experiments were done in triplicate. Data shown are the mean of absorbance change. Standard deviations ranged from 0.001 to 0.005.

7.1.1.3 Influence of viscosity

As shown in Table 4, MPO activity in synovial fluids without hyaluronidase treatment did not differ markedly from values of samples treated with 60, 120, and 180 U hyaluronidase. In the majority of cases, hyaluronidase treatment resulted in a lower MPO activity. At least one decimal place was found decreased compared to corresponding values obtained in the absence of hyaluronidase treatment.

Synovial fluid sample	MPO activity [U/ml]			
	No hyaluronidase treatment	Hyaluronidase treatment		
		60 U/ml	120 U/ml	180 U/ml
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2	0.3 ± 0.001	0.3 ± 0.003	0.2 ± 0.002	0.2 ± 0.001
3	0.8 ± 0.002	0.6 ± 0.001	0.6 ± 0.002	0.7 ± 0.001
4	1.4 ± 0.002	1.4 ± 0.002	1.2 ± 0.002	1.3 ± 0.003
5	6.8 ± 0.003	6.5 ± 0.001	6.6 ± 0.001	6.8 ± 0.002

Table 4: Effect of hyaluronidase treatment on the MPO activity of synovial fluid samples. Each experiment was done in triplicate. Data shown are the mean of MPO activity with corresponding standard deviation.

7.1.1.4 Influence of sample dilution

Linearity for a control synovial fluid sample spiked with known amounts of purified MPO was demonstrated for different sample dilutions (Figure 9a). Moreover, 0.43 mM H_2O_2 to appropriate serum samples also revealed a linear kinetics of MPO for up to 30 min (Figure 9b). Recovery of purified enzyme was higher than 90% for individual sample dilution.

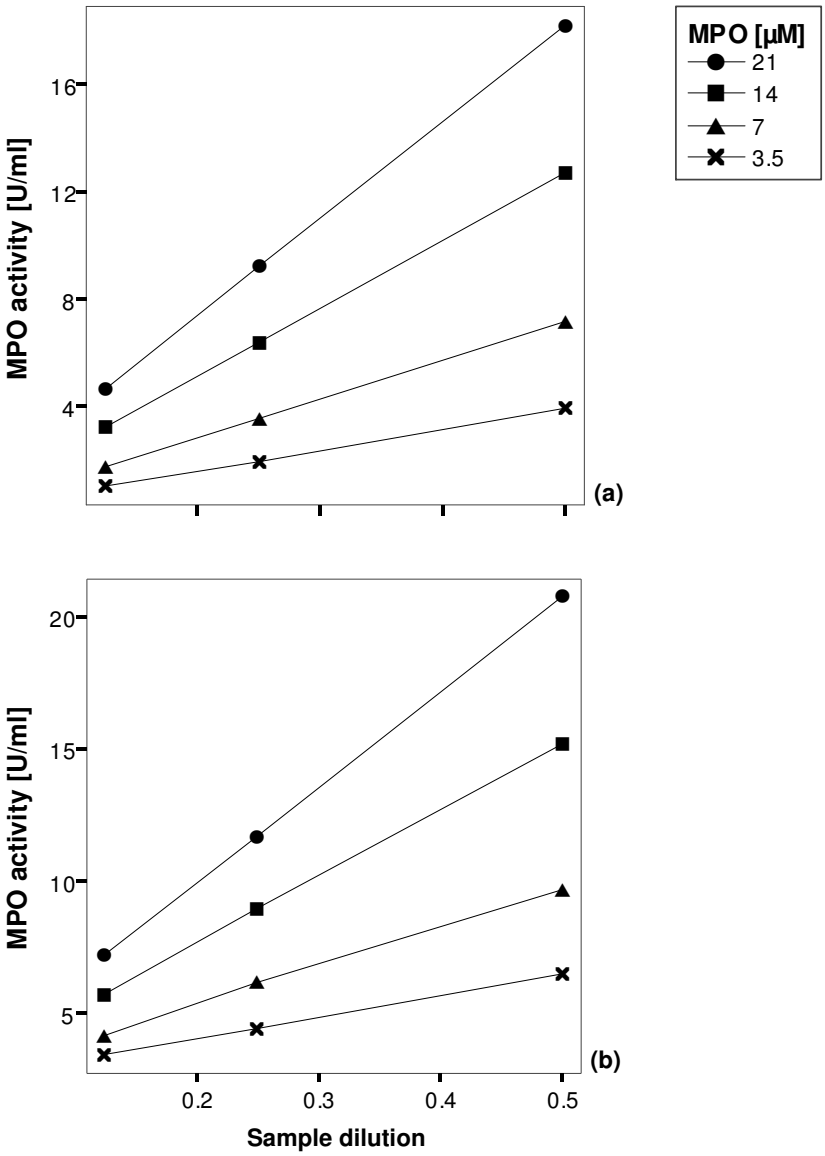

Figure 9: Effect of different dilutions of control synovial fluid (a) and serum sample (b) spiked with purified MPO. Sample dilutions shown as reciprocal values (1/dilution). Both experiments were done in triplicate. Data shown are the mean of MPO activity. Standard deviations ranged from 0.001 to 0.006.

Table 5 shows MPO activities of different synovial fluid sample dilutions. Samples which had no MPO at 1:2 dilution represented no MPO activity in undiluted samples. Low levels of MPO activity could be detected in undiluted samples as well as at 1:2 sample dilution, however not at dilutions of 1:4 and 1:8.

Synovial fluid sample	MPO activity [U/ml]			
	Sample dilution			
	1:1	1:2	1:4	1:8
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2	0.1 ± 0.001	0.1 ± 0.002	0 ± 0	0 ± 0
3	0.2 ± 0.001	0.2 ± 0.001	0 ± 0	0 ± 0
4	0.5 ± 0.002	0.4 ± 0.001	0.2 ± 0.002	0 ± 0

Table 5: Effect of different synovial fluid sample dilutions on the MPO activity. Each experiment was done in triplicate. Data shown are the mean of MPO activity with corresponding standard deviation.

7.1.1.5 Reproducibility

Data reproducibility was investigated at concentrations of 3.5, 7, 14, 21 µM purified MPO, each measured in 5 parallel assays. As shown in Figure 10, increase of absorbance was constant throughout consecutive assays and varying enzyme concentrations as indicated by Spearman's coefficient of correlation with a value of 0.97. Correlation between MPO concentration and absorbance increase was found to be significant ($p < 0.001$). For intra-assay variation 5 different synovial fluid samples were used in the assay, each measured in triplicate. As an inter-assay control, 3.5 µM purified MPO was used in each run. Variations of intra- and inter-assay control measurements were below 10%.

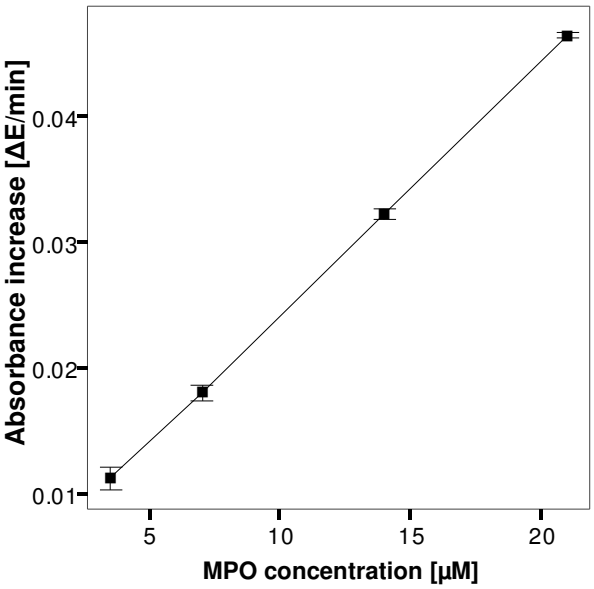

Figure 10: Effect of serial MPO dilutions ranging from 3.5 to 21 µM on the absorbance increase. This experiment was done in 5 parallel assays. Data shown are the mean of absorbance increase [ΔE/min] with corresponding standard deviation.

7.1.2 MPO Fluorometric Activity Assay

Figure 11 shows the effect of different MPO concentrations on the change of fluorescence intensity. All analysed MPO concentrations demonstrated linearity for up to 250 sec. The greatest increase in fluorescence intensity was observed for 0.4 µM MPO. For this concentration curve saturation was observed at 250 sec.

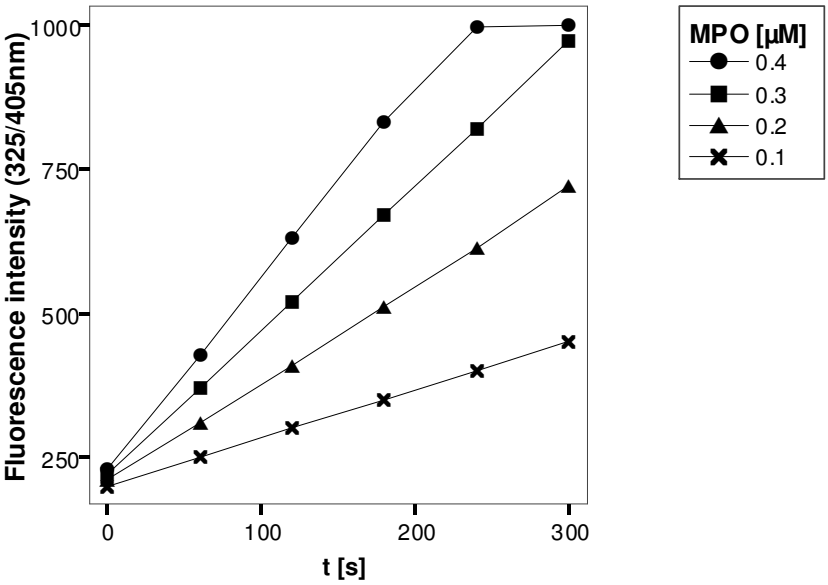

Figure 11: Effect of different MPO concentrations on the change of fluorescence intensity. This experiment was done in triplicate. Data shown are the mean of fluorescence intensity. Standard deviations ranged from 0.3 to 2.5.

Furthermore, change of fluorescence intensity was recorded for 2 different synovial fluid from different diagnostic groups dilutions ranging from 1:2 to 1:1000. As shown in Figure 12a, no constant increase in fluorescence intensity could be observed for any dilution over 300 sec. Moreover, 5 control synovial fluids in dilutions of 1:2, 1:10 and 1:1000 were spiked with 0.2 µM purified MPO and fluorescence intensity was recorded. No constant increase of fluorescence intensity could be observed in any of the sample dilutions over a time of 300 s (Figure 12b).

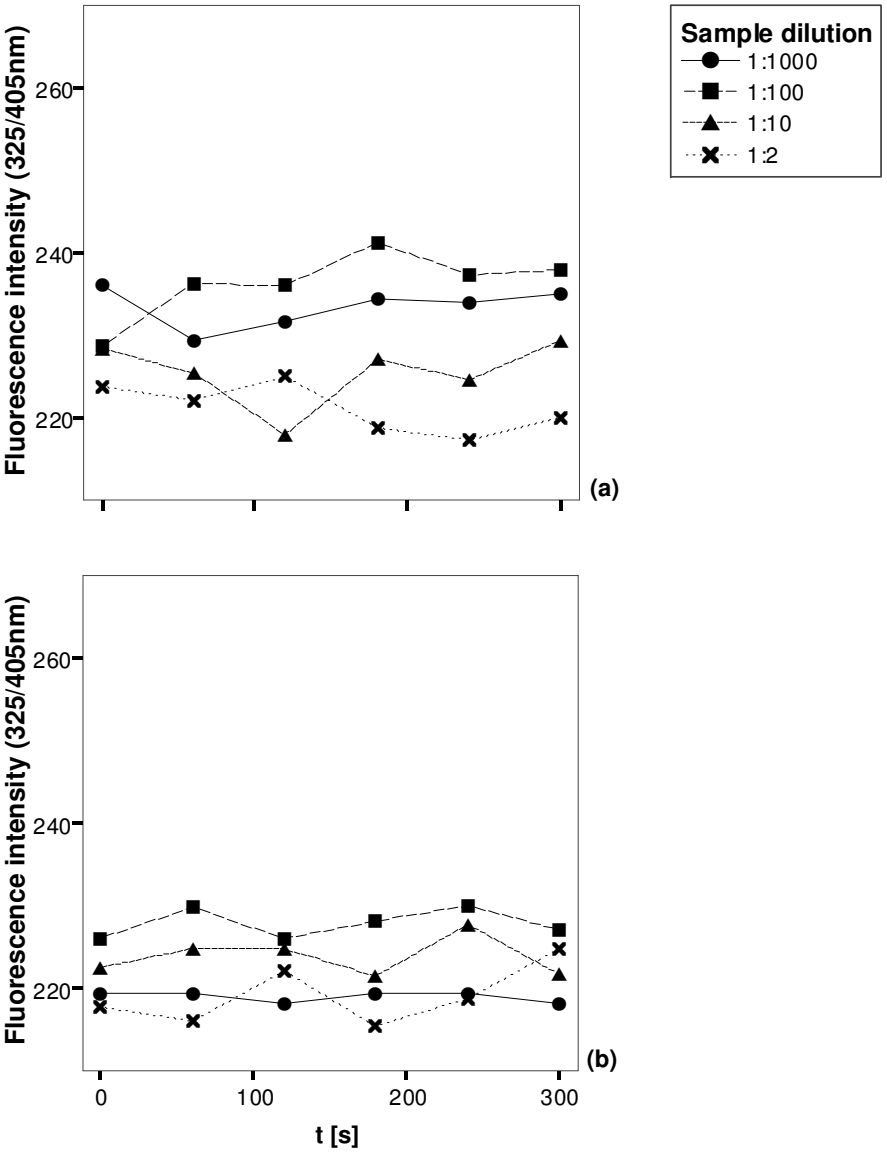

Figure 12: Effect of synovial fluid in varying sample dilutions alone (a) and spiked with 0.2 µM purified MPO (b) on fluorescence intensity. This experiment was done in triplicate. Data shown are the mean of fluorescence intensity. Standard deviations ranged from 5.5 to 17.1. Figure 12 shows a representative example.

7.1.3 Gelatin zymography

Figure 13 shows a gelatin zymogram of synovial fluid samples from the control and septic arthritis group, each loaded in sample dilutions ranging from 1:30 to 1:2. Gelatinolytic activities increased with decreasing sample dilution factor for both samples. The synovial fluid of the control group represented gelatinolytic activity at approx. 72 and 120 kDa (Figure 13a). The septic arthritis sample showed further gelatinolytic activity at approx. 45, 92, 120, and 250 kDa (Figure 13b). When the latter sample was loaded in dilutions of 1:5 and 1:2, individual gelatinolytic bands could not be clearly separated.

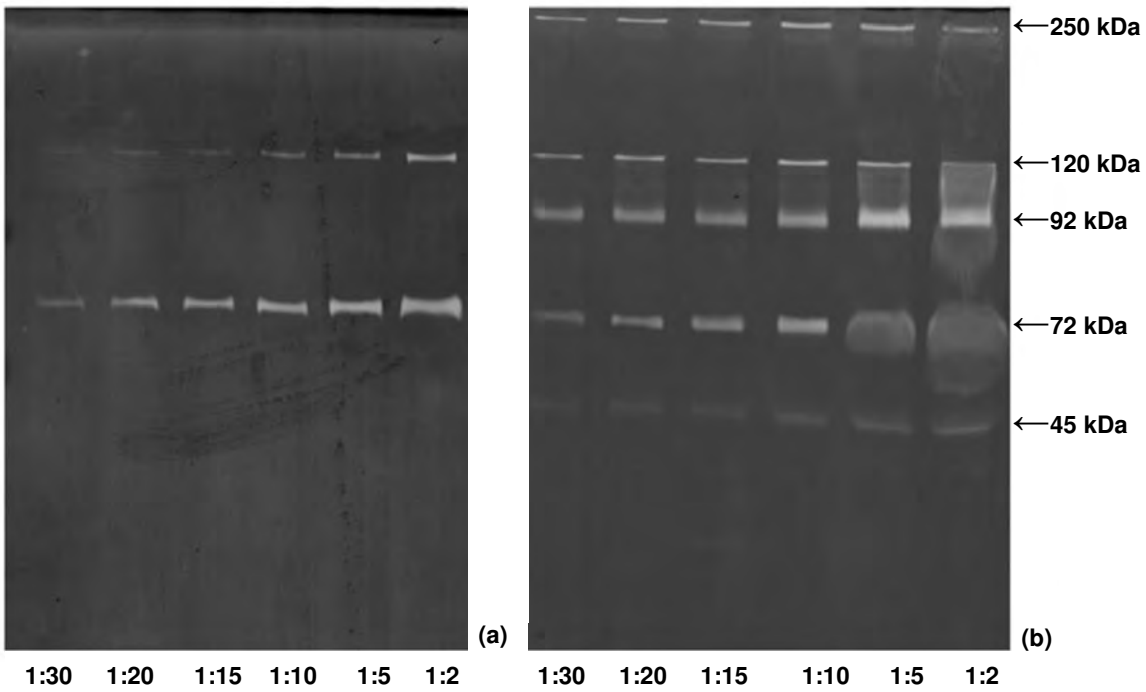

Figure 13: Gelatin zymogram of synovial fluid from controls (a) and septic arthritis (b). Varying sample dilutions were loaded on the gel as indicated.

As shown in Figure 14, overnight incubation of appropriate gels in a reactivation buffer containing 50 mM EDTA or 50 µM MMP2/MMP9 inhibitor III resulted in a complete inhibition of gelatinolytic activities in synovial fluid samples of the control and septic arthritis groups.

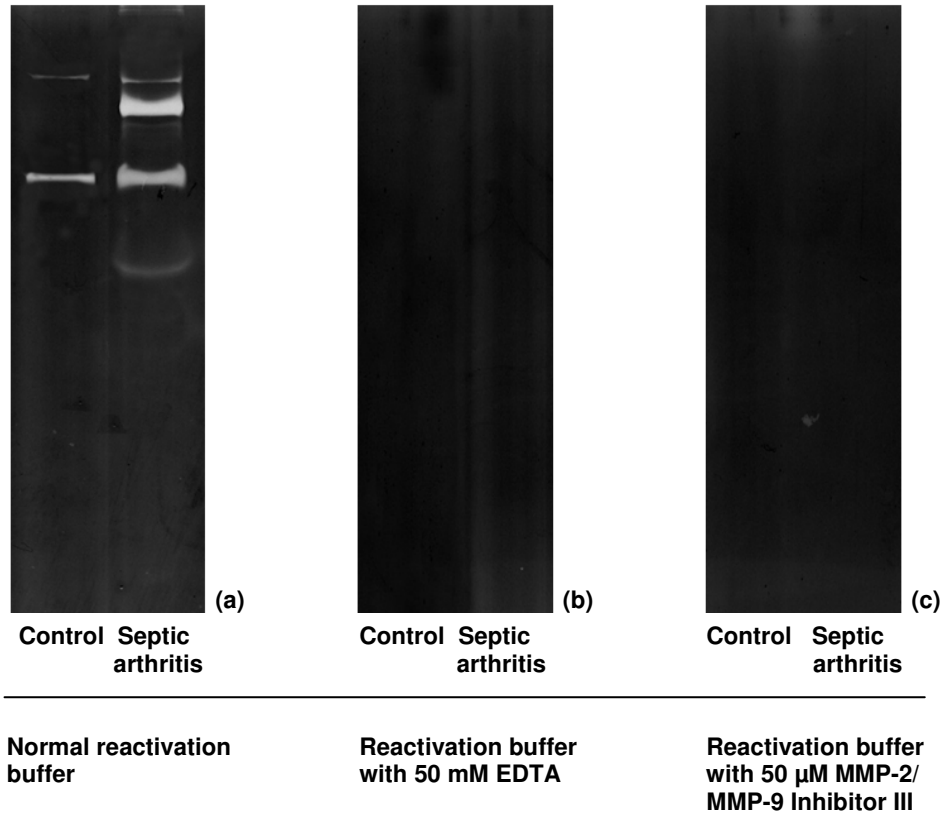

Figure 14: Gelatin zymogram of synovial fluids from controls and septic arthritis. Gels were incubated in normal reactivation buffer (a), reactivation buffer including 50 mM EDTA (b), and reactivation buffer with 50 µM MMP-2/MMP-9 Inhibitor III (c).

An appropriate synovial fluid sample from septic arthritis was loaded with human latent MMP-2 and -9 on the gel. As shown in Figure 15a, human latent MMP-2 and MMP-9 represented gelatinolytic activity at 72 kDa and at 92, 120, 250 kDa. Synovial fluid sample from septic arthritis showed gelatinolytic activity at similar kDa. The latter sample was additionally incubated with 1 mM APMA. APMA treatment resulted in conversion of the 92 kDa band to a triplet comprised of 92, 88, and 86 kDa bands, and the conversion of the 72 kDa band to a doublet of 72 and 68 kDa bands (Figure 15b).

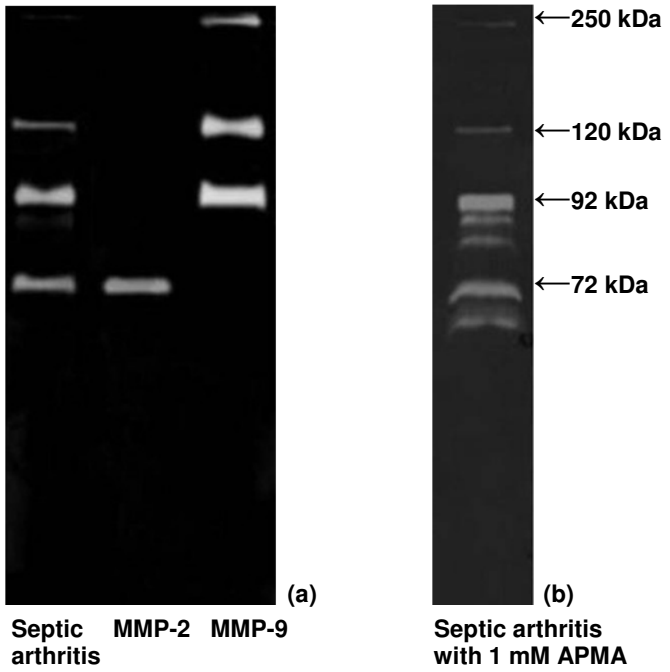

Figure 15: Gelatin zymogram of synovial fluid from septic arthritis, human latent MMP-2 and -9 (a), and synovial fluid from septic arthritis incubated in the presence of 1 mM APMA (b).

7.2 Evaluation of parameters in synovial fluid and serum

7.2.1 WBC count and protein concentration

Figure 16 shows the distribution of WBC count and protein concentrations in synovial fluid and serum samples. Raw data of WBC counts were adopted from MOSCHOS (2007). The greatest values for WBC count and protein concentration were observed in synovial fluid samples from septic arthritis. Median WBC count in the latter group was at least 400 times higher and median protein level was more than 3 times higher than corresponding values of controls and all other affected samples.

The median and overall range in WBC count and protein concentration in synovial fluid samples of controls did not differ markedly compared to those observed in groups of OA, chronic arthritis, and OCD. At least 75% of WBC values of the control, OA, chronic arthritis, and OCD groups remained below 0.1×10^6 cells/µl (Table 20, Appendix II). The control, OA, and chronic arthritis groups presented at most 3 outliers that were lower than 0.2×10^6 cells/µl. Protein levels of controls and groups of OA, chronic arthritis, and OCD were below 20 mg/ml. No values for WBC count and protein concentration of the latter groups overlapped with the range of data observed in the septic arthritis group. WBC count and protein concentration were significantly different in synovial fluid samples from septic arthritis compared to all other diagnostic groups (Table 21, Appendix III).

As shown in Figure 16, median and overall range in serum protein concentration did not differ markedly relative to controls and affected groups with OA, chronic arthritis, OCD, and septic arthritis. The majority of values for all affected groups overlapped with the range of data observed in controls. Protein values of all diagnostic groups were lower than 50 mg/ml (Table 20, Appendix II).

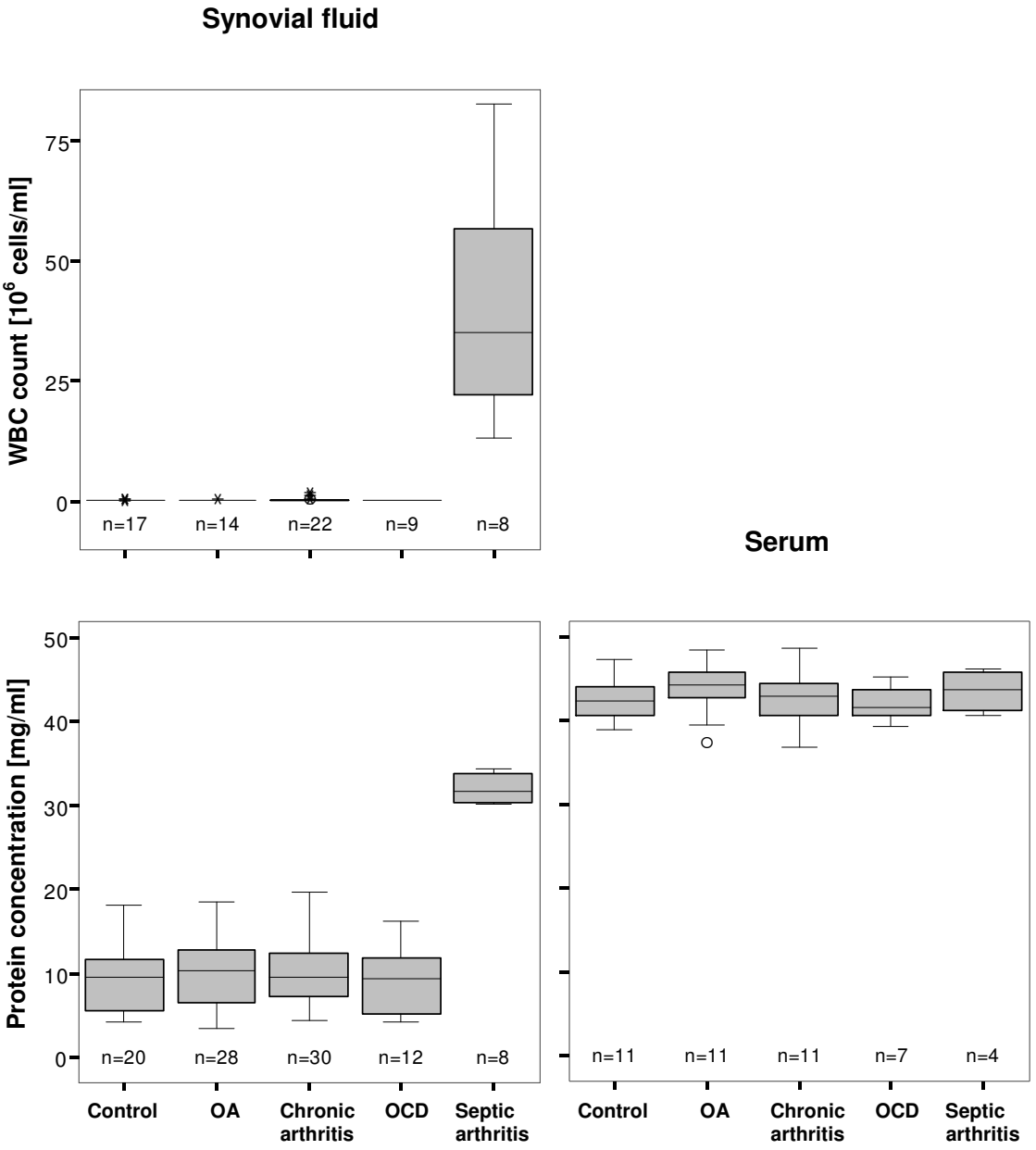

Figure 16: WBC count and protein concentration in synovial fluid and serum of indicated diagnostic groups.

WBC count and corresponding protein concentration were strongly correlated in synovial fluid samples as indicated by Spearman's coefficient of correlation with a value of 0.71. This correlation was found to be significant (Table 22, Appendix III).

Further cell differentiation was assessed in 6 synovial fluid samples from septic arthritis. Data were kindly provided by Anastasios Moschos or co-workers. All samples represented PMN content ≥ 90%.

7.2.2 MPO activity and MPO concentration

As shown in Figure 17, maximum levels of MPO activity and MPO concentration were observed in synovial fluid samples from septic arthritis. Median MPO activity and MPO concentration in the septic arthritis group were at least 11 times higher than corresponding values in all other groups.

The lowest median and overall range in MPO activity and MPO concentration was detected in control synovial fluids. At least 75% of control values for MPO activity were lower than medians of all other groups. More than half of the values for both parameters observed in affected groups with OA, chronic arthritis, and OCD fell within the overall range of data from controls. Synovial fluid samples from OA, chronic arthritis, and OCD presented several outliers of MPO activity which overlapped with values of the septic arthritis group. One value of MPO concentration from the chronic arthritis group fell within the range of data from septic arthritis samples. MPO activity and MPO concentration were significantly different in synovial fluid samples from septic arthritis compared to all other diagnostic groups (Table 21, Appendix III).

As shown in Figure 17, median MPO activity did not differ markedly in serum samples of controls and groups of OA, chronic arthritis, OCD, and septic arthritis. Overall data range in serum samples was markedly increased when compared to corresponding synovial fluids. The majority of serum values observed in all affected groups overlapped with the range of data from controls.

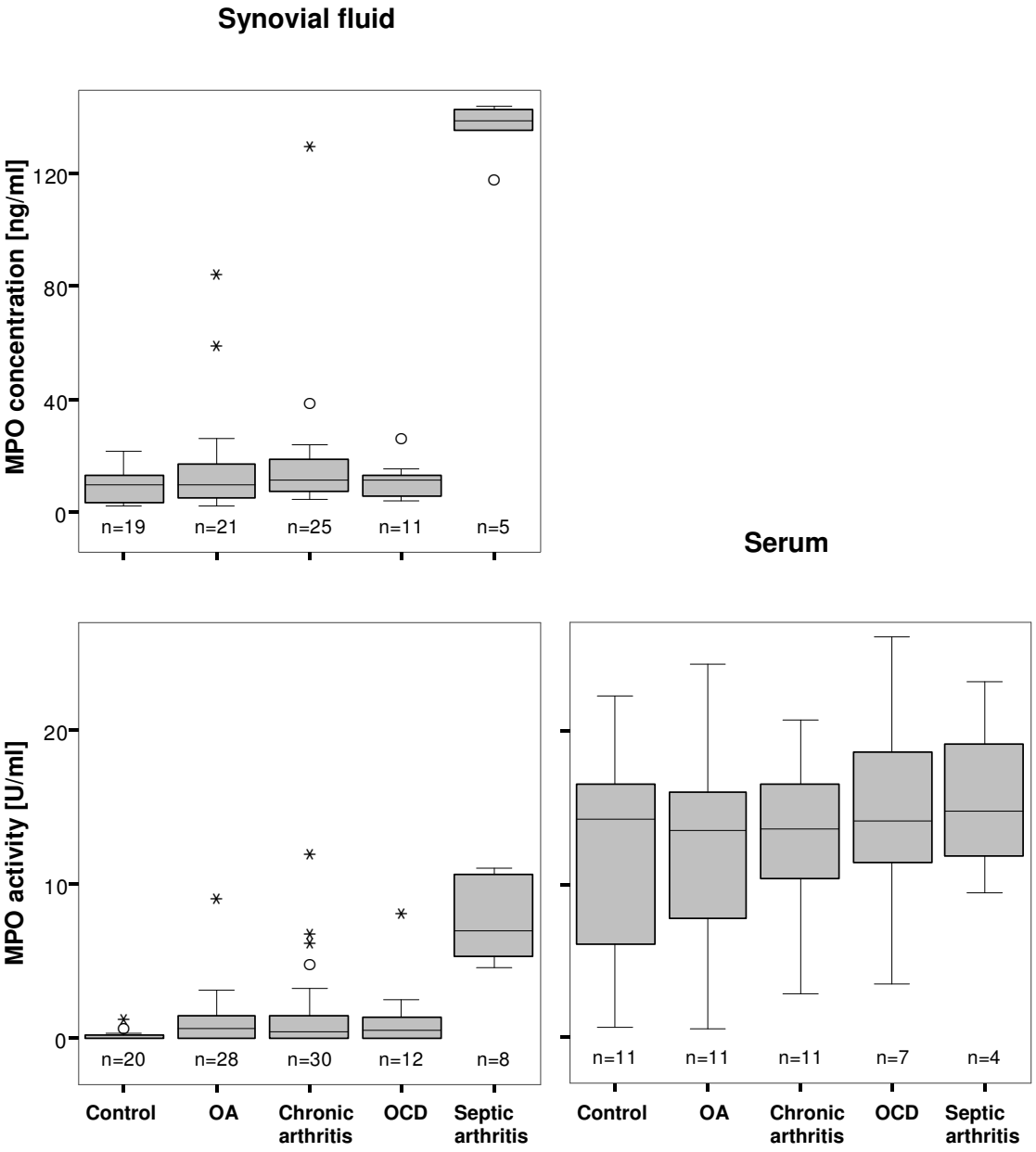

Figure 17: MPO activity and MPO concentration in synovial fluid and serum of indicated diagnostic groups. MPO activity was measured by MPO Photometric activity. MPO concentration was assessed in synovial fluids only; variations of intra- and inter-assay control measurements were below 5%.

Figure 18 shows the relationship between MPO activity and corresponding MPO concentration in synovial fluid samples irrespective of diagnostic groups. The relationship between both parameters was positive. MPO activity and MPO concentration were moderately correlated as indicated by Spearman's coefficient of correlation with a value of 0.61. This correlation was found to be significant (Table 22, Appendix III).

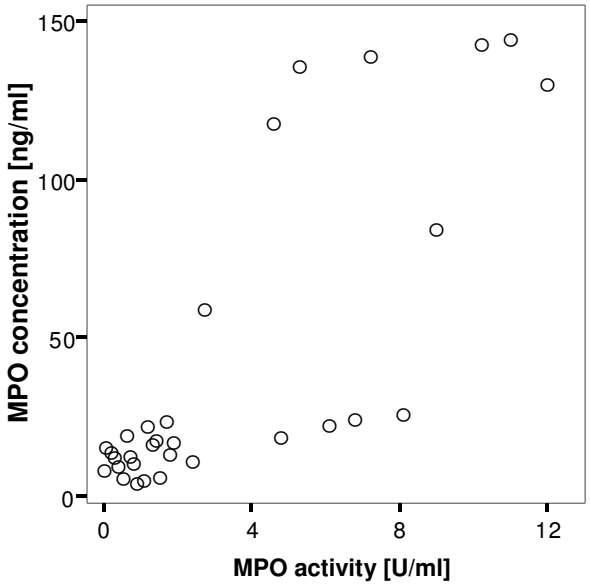

Figure 18: Relationship between MPO activity and –concentration in synovial fluid samples.

WBC count and corresponding values for MPO activity and MPO concentration were weakly correlated in synovial fluid samples; Spearman's coefficient of correlation was 0.42 and 0.38, respectively. Both correlations were found to be significant (Table 22, Appendix III).

7.2.3 MMP-2 and -9 activities

Activity values (AV) of MMP-2 and -9 were assessed by gelatin zymography in synovial fluids from the control (n=8), OA (n=8), chronic arthritis (n=8), septic arthritis (n=6), and OCD (n=8) groups (Tables 15-19, Appendix I). Gelatinolytic activity at 72 kDa increased in samples from OA, chronic arthritis, and septic arthritis compared to those from control and OCD (Figure 19). Septic arthritis samples contained marked activity at 92 kDa; however, this band was rarely detectable in samples from other diagnostic groups. Gelatinolytic activity at 120 kDa was found in greater intensity in septic arthritis samples compared to all other groups. Trace amounts of activity were detected at 45, 88, and 250 kDa in septic arthritis samples; however these bands were absent in samples from all other groups. AV at 72 kDa were significantly different in samples from OA, chronic arthritis, and septic arthritis compared to those from controls and OCD ($p < 0.001$). Septic arthritis samples showed a significant difference in AV at 88, 92, 120, and 250 kDa compared to all other diagnostic groups ($p < 0.01$).

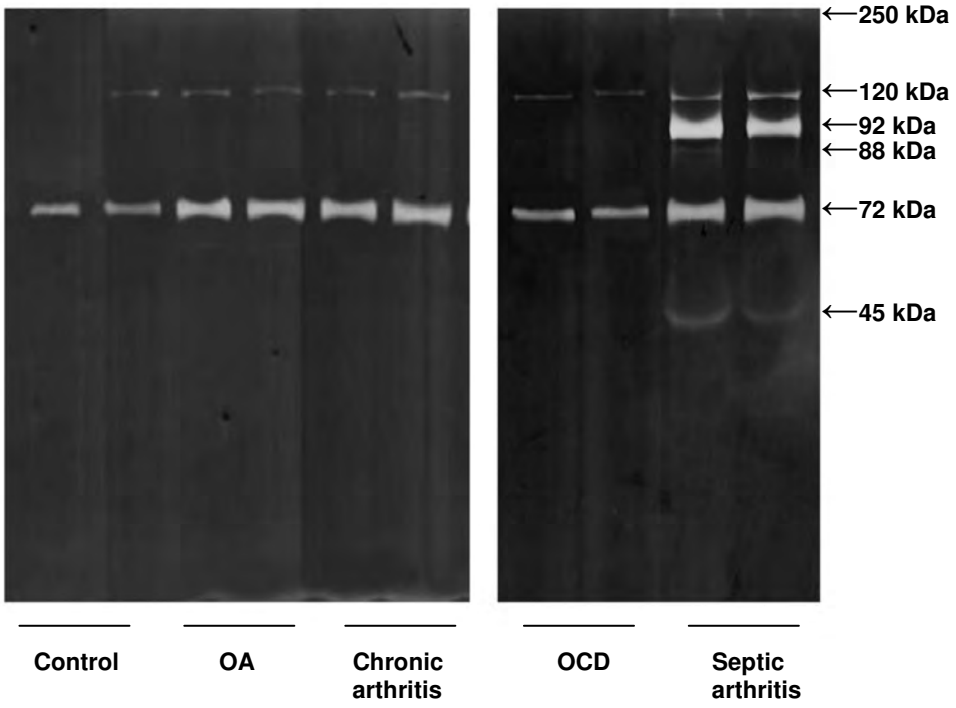

Figure 19: Gelatin zymogram of representative synovial fluids from indicated diagnostic groups.

Figure 20 shows the distribution of MMP-2 and -9 activities in synovial fluid and serum samples from different diagnostic groups as measured by MMP immunocapture activity assays. Synovial fluid samples from septic arthritis showed the highest medians in MMP-2 and -9 activities. No values for the latter group overlapped with the range of data observed in all other diagnostic groups.

The control group presented the lowest median and interquartile range in synovial MMP-2 and -9 activities. At least 75% of MMP-2 and -9 levels in controls did not exceed corresponding medians of all other diagnostic groups (Table 20, Appendix II). Maximum MMP-2 and -9 activities in OCD samples were no greater than those in the control group. Half of the MMP-9 values in groups of OA and chronic arthritis fell within the range of data from controls, whereas half of the MMP-2 values in the OA and chronic arthritis groups were at least as great as maximum control levels. There was a significant difference in MMP-2 and -9 activities in synovial fluid samples from septic arthritis compared to all other diagnostic groups. Moreover, MMP-2 activity was found significantly increased in synovial fluids from OA and chronic arthritis when compared to the control and OCD groups (Table 21, Appendix III).

As shown in Figure 20, median MMP-2 and -9 activities were similar in serum samples from controls and groups of OA, chronic arthritis, OCD, and septic arthritis. The majority of MMP-2 and -9 values detected in all affected groups overlapped with the range of data observed in the control group.

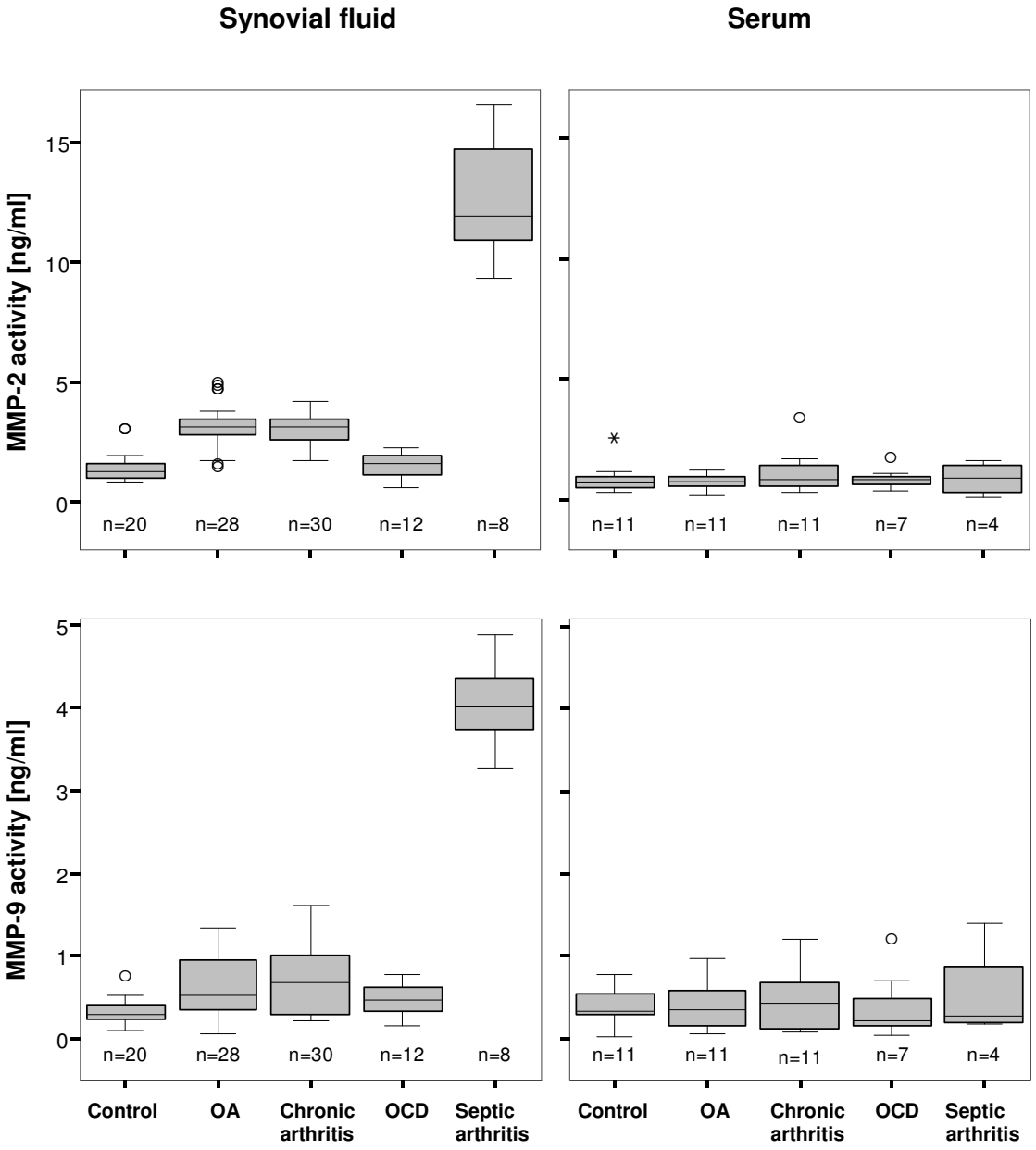

Figure 20: MMP-2 and -9 activities in synovial fluid and serum of indicated diagnostic groups. Variations of intra- and inter-assay control measurements were below 5%.

As shown in Figure 21, there was a positive relationship between MMP-2 and corresponding MMP-9 activity in synovial fluid samples irrespective of the diagnostic group. Both parameters were moderately correlated in synovial fluid samples as indicated by Spearman's coefficient of correlation with a value of 0.64. This correlation was found to be significant (Table 22, Appendix III).

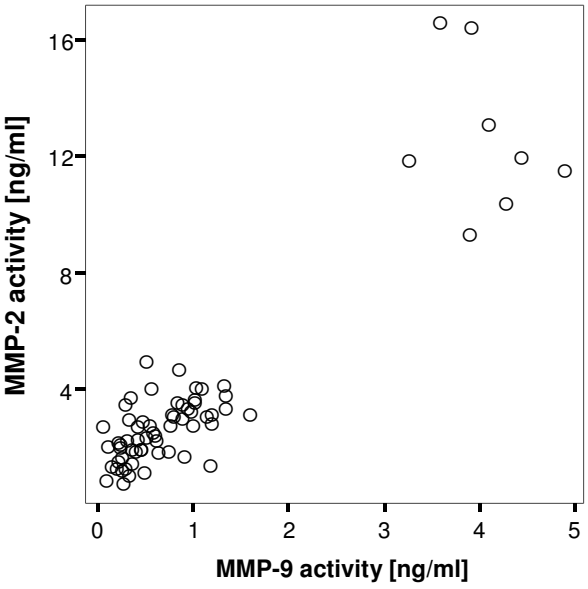

Figure 21: Relationship between MMP-2 and -9 activities in synovial fluid samples.

WBC count and corresponding values for MMP-2 activity were moderately correlated in synovial fluid samples as indicated by Spearman's coefficient of correlation with a value of 0.63. Correlation between WBC count and MMP-9 activity in synovial fluids was strong; Spearman's coefficient of correlation was 0.76. Both correlations were significant (Table 22, Appendix III).

Addition of 50 µM of MMP-2/MMP-9 Inhibitor III to highest MMP-2 and -9 standard concentrations and 2 synovial fluids of the control and septic arthritis groups resulted in 97% inhibition of MMP-2 and -9 activities, respectively.

7.2.4 C2C and CTXII concentrations

Figure 22 shows the distribution of C2C and CTX II concentrations in synovial fluid and serum samples from different diagnostic groups. The lowest median and overall range in C2C and CTXII concentrations were found in control synovial fluid samples. Control samples ranged between 74.2 and 185.4 ng/ml C2C concentration and between 71.3 and 196.4 pg/ml CTXII concentration (Table 20, Appendix II). Median C2C concentration did not differ markedly between groups of OA, chronic arthritis, OCD, and septic arthritis. More than half of the C2C levels from all affected groups was greater than the upper quartile of control values. At least half of the data observed in synovial fluids from OA and chronic arthritis were higher than the maximum CTXII concentration from controls. There was no marked difference in interquartile range in CTXII levels between the OCD and control groups (Table 20, Appendix II).

Maximum levels of CTXII concentration were observed in synovial fluid samples from septic arthritis (Figure 22). Median CTXII concentration in the latter group was at least 4 times higher than that of all other diagnostic groups. No values of the septic arthritis group overlapped with the range of data observed in all other groups. CTXII concentration significantly increased in the synovial fluid samples from septic arthritis compared to all other diagnostic groups. Moreover, there was a significant difference in CTXII concentration between synovial fluids of controls and groups of OA or chronic arthritis (Table 21, Appendix III).

As shown in Figure 22, the majority of C2C values observed in serum samples of the control group overlapped with the range of data from all affected groups. Similar medians and interquartile ranges in serum CTXII concentration were observed in controls and groups with OA, chronic arthritis, and OCD. CTXII concentration was found significantly elevated in serum samples from septic arthritis compared to controls (Table 21, Appendix III).

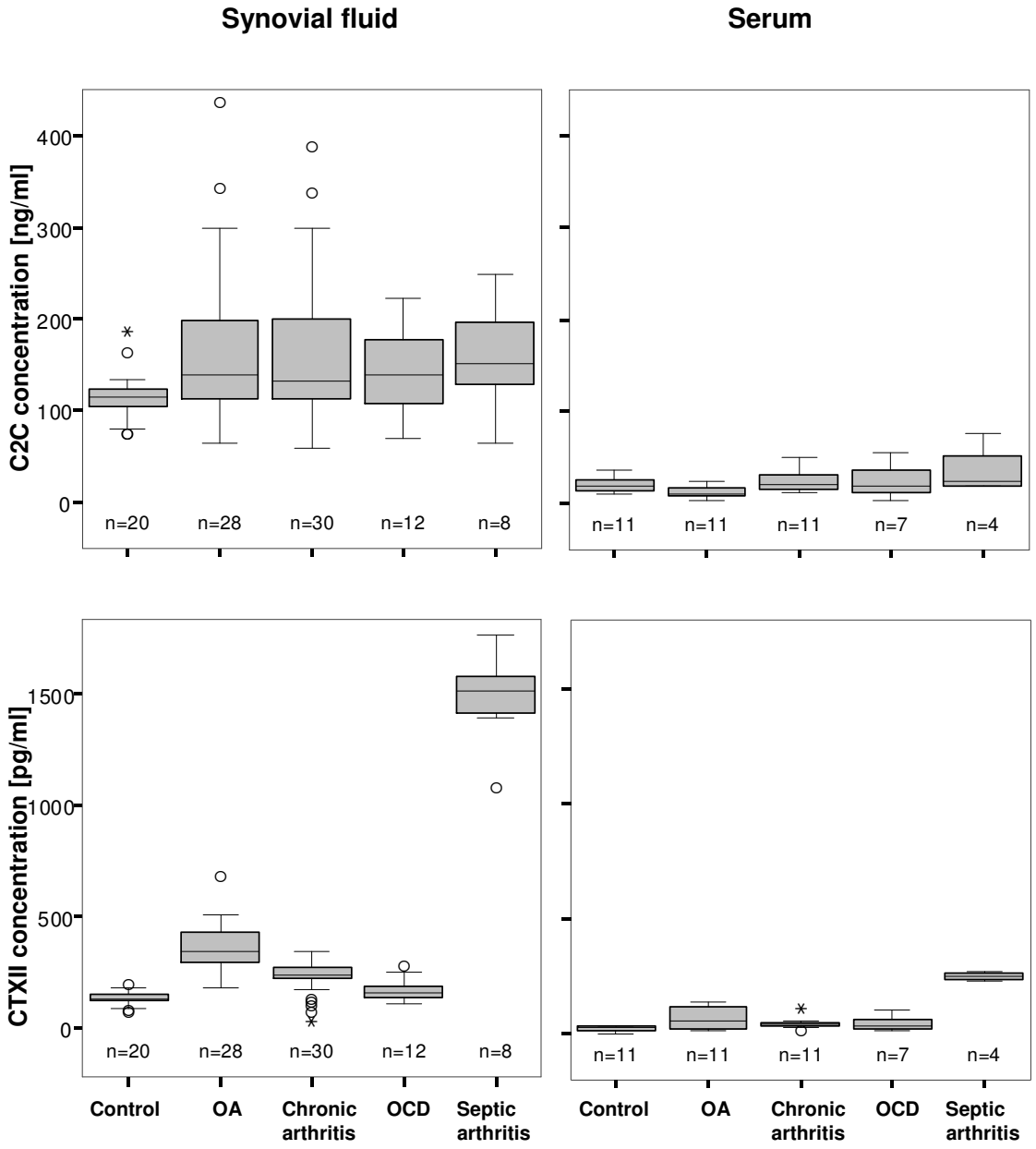

Figure 22: C2C and CTXII concentrations in synovial fluid and serum of indicated diagnostic groups. Variations of intra- and inter-assay control measurements were below 7%.

Figure 23 shows the relationship between C2C and CTXII concentrations in synovial fluid samples irrespective of the diagnostic groups. Both values were very weakly correlated; Spearman's coefficient of correlation was 0.14.

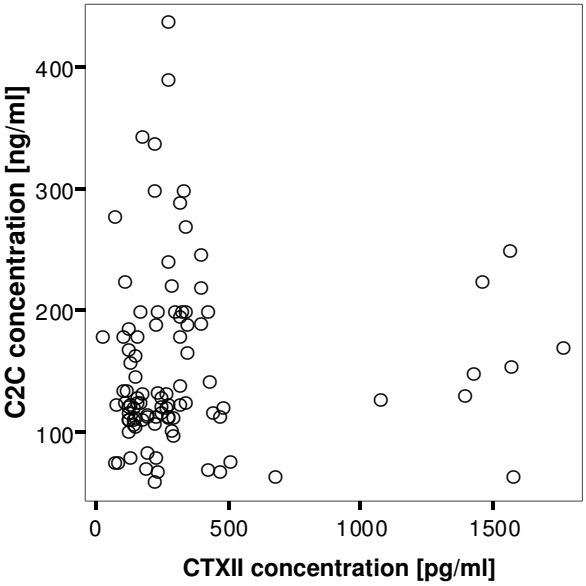

Figure 23: Relationship between C2C and CTXII concentrations in synovial fluid samples.

WBC count and corresponding values for C2C concentration were very weakly correlated in synovial fluid samples as indicated by Spearman's coefficients of correlation with value of 0.01. CTXII levels and WBC count were significantly weakly correlated; Spearman's coefficient of correlation was 0.38 (Table 22, Appendix III).

7.2.5 CPII and CS846 concentrations

As shown in Figure 24, the lowest median CPII and CS846 concentrations were observed in synovial fluid samples from OA, followed by the groups of chronic arthritis, controls, OCD, and septic arthritis. At least half of the values of the OA and chronic arthritis groups were less than the lower quartile of CPII and CS846 concentrations observed in the control and OCD groups. Moreover, the majority of CPII and CS846 values of the OCD group overlapped with the range of data observed in controls.

The greatest values for CPII and CS846 concentrations were observed in synovial fluid samples from septic arthritis. Several CS846 values and at least one CPII of the latter group fell within the range of data from the OCD group. CPII and CS846 concentrations were significantly different in synovial fluid samples from OA and chronic arthritis group compared to the control, OCD, or septic arthritis group (Table 21, Appendix III).

Median CPII and CS846 concentrations did not differ markedly in serum samples between controls and groups with OA, chronic arthritis, and OCD (Figure 24). The highest medians were observed in the septic arthritis group. No CS846 values of the septic arthritis group overlapped with the range of data observed in all other groups. CS846 concentration was significantly different in serum samples from septic arthritis group compared to all other diagnostic groups. Moreover, CPII concentration was significantly elevated in serum samples from septic arthritis samples relative to the chronic arthritis group (Table 21, Appendix III).

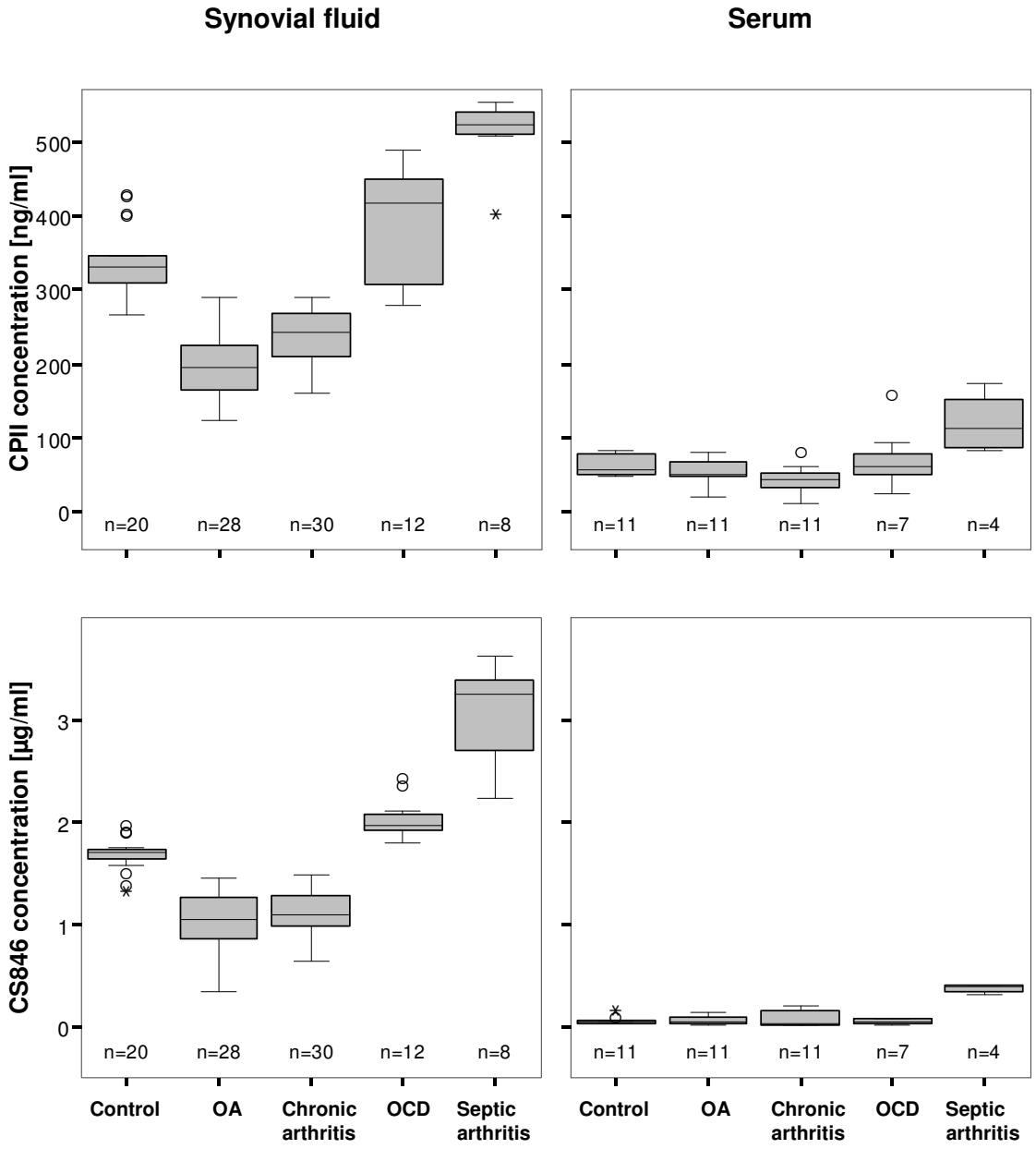

Figure 24: CPII and CS846 concentrations in synovial fluid and serum of indicated diagnostic groups. Variations of intra- and inter-assay control measurements were below 8%.

As shown in Figure 25, there was a positive relationship between CPII and corresponding CS846 concentrations in synovial fluid samples. Both parameters were strongly correlated; Spearman's coefficient of correlation was 0.77. This correlation was found to be significant (Table 22, Appendix III).

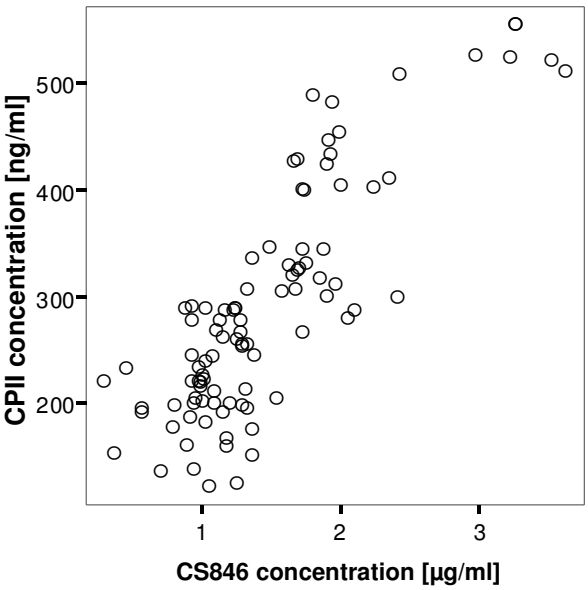

Figure 25: Relationship between CPII and CS846 concentrations in synovial fluid samples.

Correlations between WBC count and corresponding values for CPII and CS846 concentrations in synovial fluid samples were significantly weak as indicated by Spearman's coefficients of correlation with values of 0.28 and 0.32, respectively (Table 22, Appendix III).

7.2.6 Follow-up study

WBC count, protein concentration, MPO-, MMP-2 and -9 activities, CTXII, CPII, and CS846 concentrations were measured in synovial fluids from septic arthritis in the same horse during a time-course. As shown in Figure 26 and 27, values of WBC count and protein concentration observed in the first 3 weeks of the disease process exceeded those obtained on the day of initial examination. All WBC counts investigated from week 3 to 8 were lower than corresponding initial values, whereas protein levels did not fall below them until week 6 or 8.

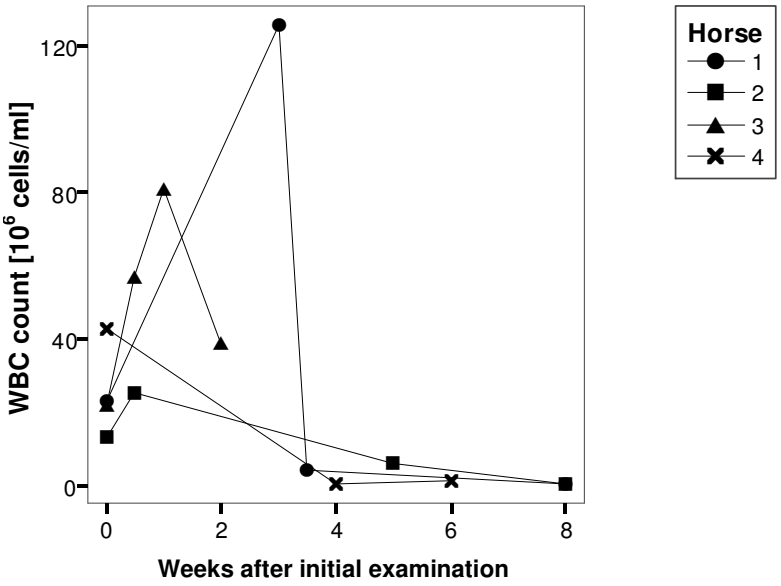

Figure 26: WBC count in repeated synovial fluids from septic arthritis.

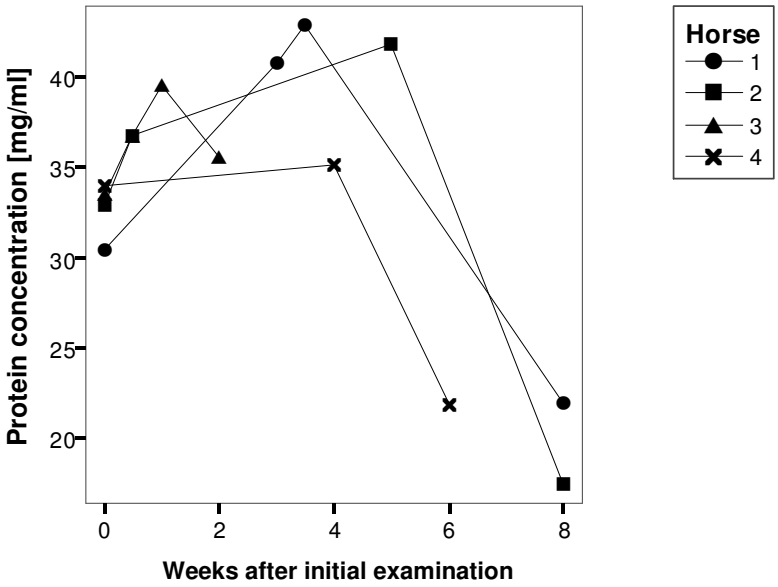

Figure 27: Protein concentration in repeated synovial fluids from septic arthritis.

No similar trends in the progression of MPO activity could be observed in the first 4 weeks after initial examination (Figure 28). MPO activity levels of horses 1 and 2 fell below the initial data, whereas at least one value of horses 3 and 4 exceeded those obtained on the day of initial examination. MPO activities of all investigated horses observed between week 5 and 8 were lower than initial levels.

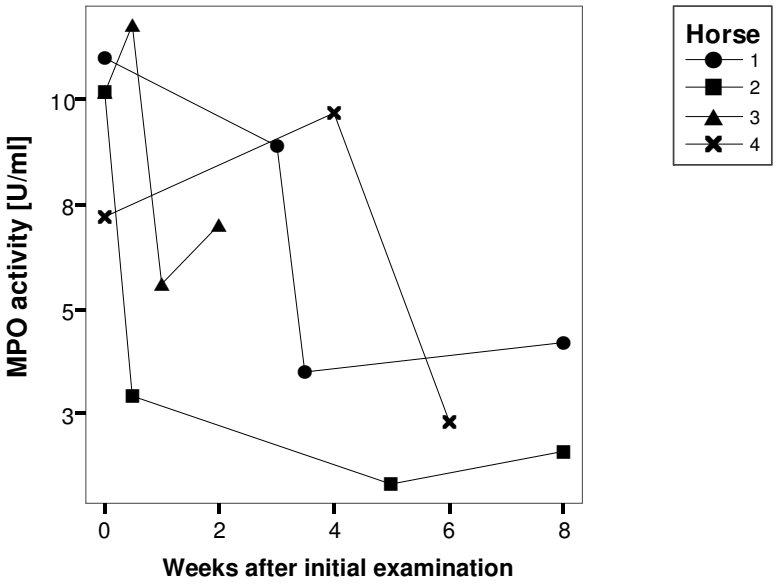

Figure 28: MPO activity in repeated synovial fluids from septic arthritis.

As shown in Figure 29, values of MMP-2 activity declined continuously during the displayed time-course. MMP-9 activities observed in the first 3 weeks of the disease process exceeded those obtained on the day of initial examination. All MMP-9 levels investigated between week 3 and 8 were lower than corresponding initial values.

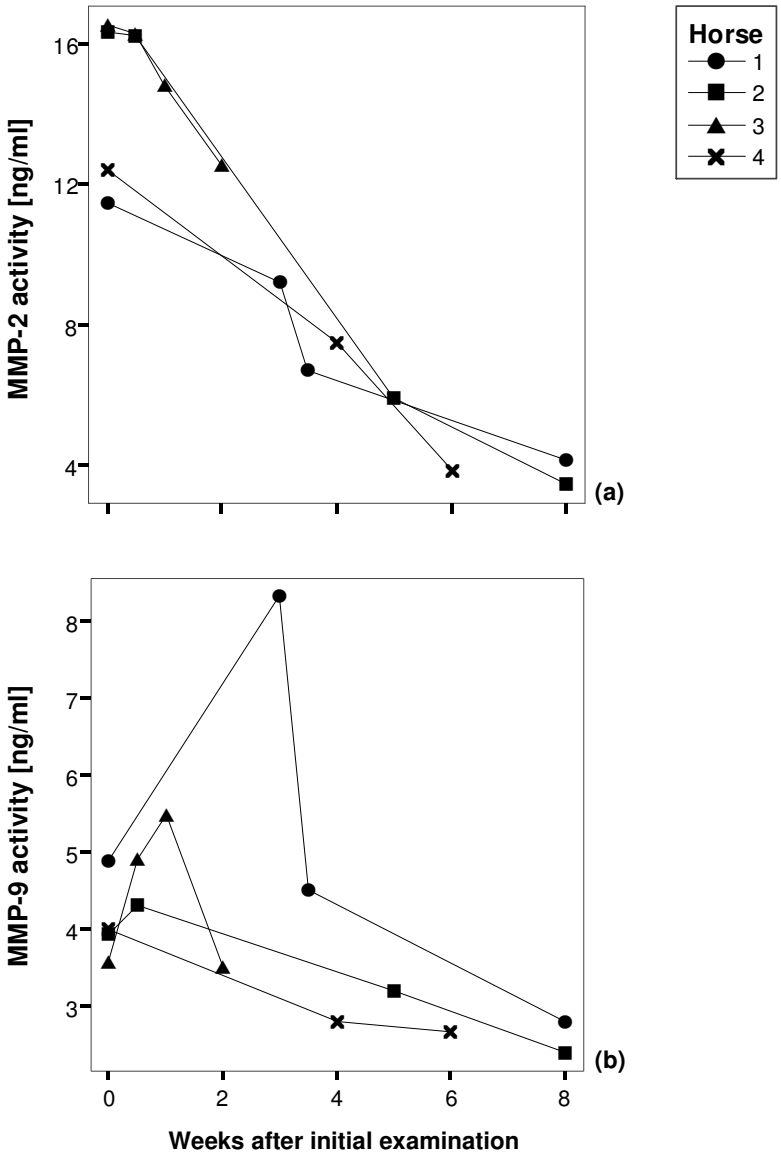

Figure 29: MMP-2 (a) and -9 activities (b) in repeated synovial fluid samples from septic arthritis.

CTXII concentrations continued to show a steady decrease in the same joints during the displayed time course (Figure 30).

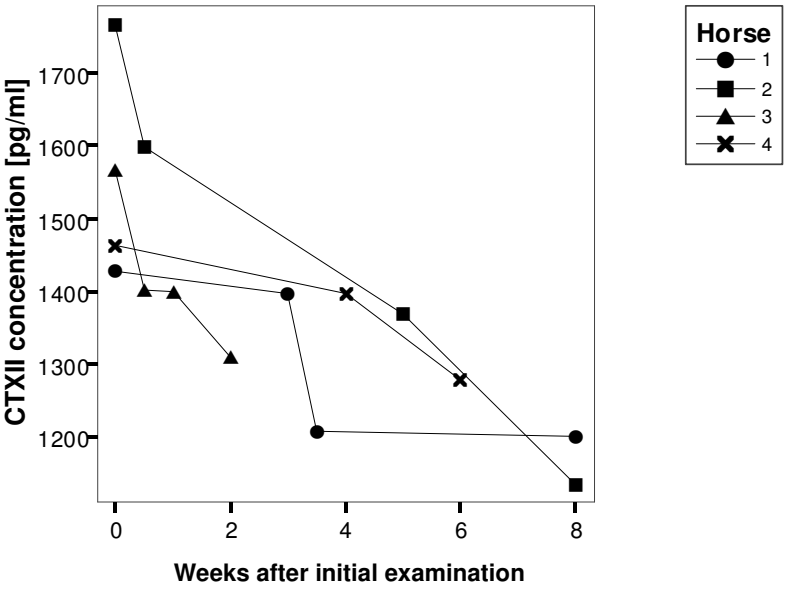

Figure 30: CTXII concentration in repeated synovial fluid samples from septic arthritis.

As shown in Figure 31, CPII and CS846 concentrations observed in the first 4 weeks of the disease process were lower than corresponding values obtained on the day of initial examination, whereas those obtained from week 4 to 8 exceeded corresponding initial values.

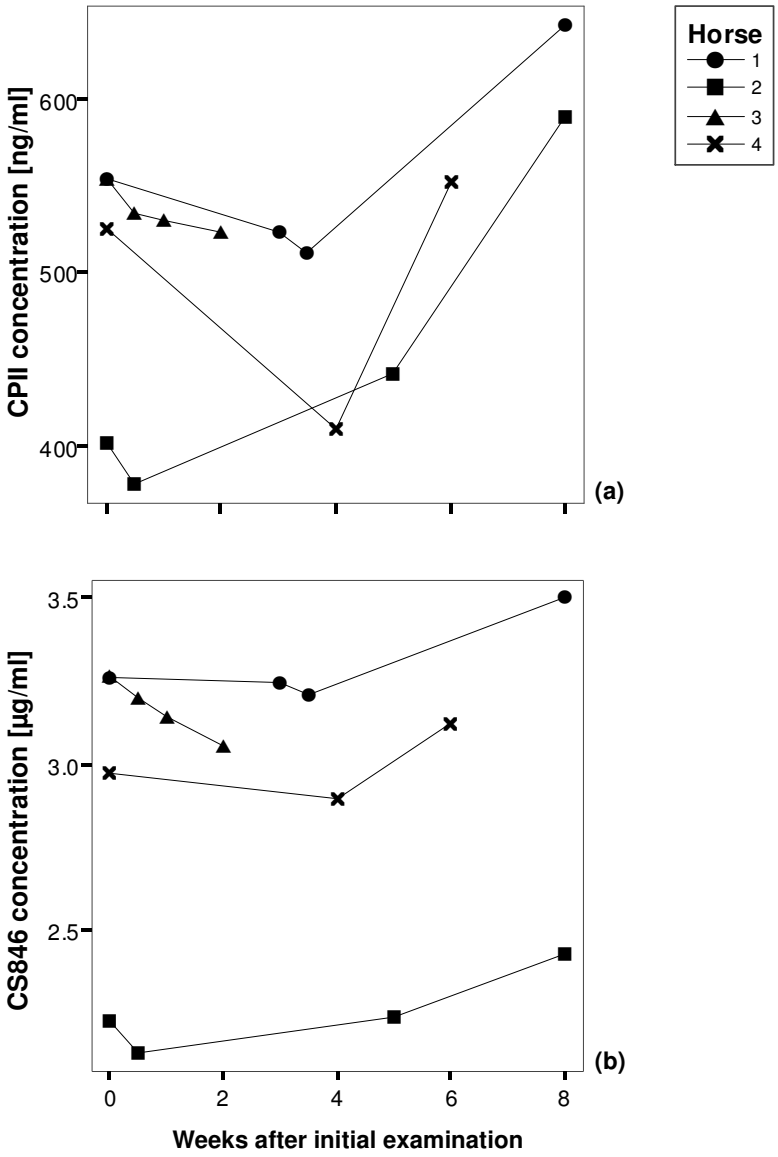

Figure 31: CPII (a) and CS846 concentrations (b) in repeated synovial fluid samples from septic arthritis.

7.3 Discriminant analysis

Samples were classified as controls or as deriving from an affected joint with OA, chronic arthritis, OCD, or septic arthritis on the basis of investigated parameters using discriminant analysis. Table 6 shows the percentage of correctly classified samples based on synovial parameters arranged according to increasing percentage of correct classification. Single parameters of MMP-2 activity, CS846-, CTXII-, and CPII concentrations revealed a correct classification rate of samples above 50%. A combination of the CTXII- and CPII concentrations revealed the highest correct classification percentage for a two-combination system of synovial parameters, namely 83.7%. A three-combination system of synovial CTXII, CPII and CS846 concentrations yielded the highest overall correct classification rate of 86.7%.

Synovial parameter	Correct classification [%]
Protein concentration	24.5
C2C concentration	27.6
WBC count	30.0
MPO activity	34.7
MPO concentration	39.5
MMP-9 activity	43.9
MMP-2 activity	51.0
CS846 concentration	62.2
CPII concentration	66.3
CTXII concentration	68.4
CTXII and CPII concentrations	83.7
CTXII, CPII, and CS846 concentrations	86.7

Table 6: Percentage of correctly classified samples based on indicated synovial parameters.

Table 7 represents the number and percentage of synovial fluid samples according to anticipated and original diagnostic groups based on CTXII, CPII, and CS846 concentrations. All septic arthritis samples were correctly classified. At least 75% of controls and samples from OA, chronic arthritis, and OCD were correctly classified.

Original diagnostic group		Anticipated diagnostic group					Total
		Control	OA	Chronic arthritis	OCD	Septic arthritis	
Number (%)	Control	16 (80.0)	0	0	4 (20.0)	0	20 (100.0)
	OA	0	25 (89.3)	3 (10.7)	0	0	28 (100.0)
	Chronic arthritis	0	3 (10.0)	27 (90.0)	0	0	30 (100.0)
	OCD	3 (25.0)	0	0	9 (75.0)	0	12 (100.0)
	Septic arthritis	0	0	0	0	8 (100.0)	8 (100.0)

Table 7: Number and percentage of synovial fluid samples according to anticipated and original diagnostic groups based on CTXII, CPII, and CS846 concentrations.

Table 8 shows the percentage of correctly classified samples based on serum parameters arranged according to increasing percentage of correct classification. CTXII concentration allowed the highest correct classification rate of 45.5%.

Serum parameter	Correct classification [%]
MMP-9 activity	4.5
MPO activity	11.4
C2C concentration	18.2
MMP-2 activity	25.0
Protein concentration	27.3
CS846 concentration	27.3
CPII concentration	29.5
CTXII concentration	45.5
CTXII and CPII concentrations	43.2
CTXII, CPII, and CS846 concentrations	43.2

Table 8: Percentage of correctly classified samples based on indicated serum parameters.

For the combination with the highest correct classification rate, number and percentage of serum samples were listed according to anticipated and original diagnostic groups in Table 9. All samples from septic arthritis were correctly classified. At least 80% of controls and more than 50% of samples from OA and chronic arthritis were correctly classified. However, more than 80% of OCD samples were misclassified as being controls or having OA or chronic arthritis.

Original diagnostic group		Anticipated diagnostic group					Total
		Control	OA	Chronic arthritis	OCD	Septic arthritis	
Number (%)	Control	9 (81.8)	0	2 (18.2)	0	0	11 (100.0)
	OA	5 (45.3)	6 (54.2)	0	0	0	11 (100.0)
	Chronic arthritis	3 (27.3)	2 (18.2)	6 (54.5)	0	0	11 (100.0)
	OCD	2 (28.6)	2 (18.2)	2 (28.6)	1 (14.3)	0	7 (100.0)
	Septic arthritis	0	0	0	0	4 (100.0)	4 (100.0)

Table 9: Number and percentage of serum samples according to anticipated and original diagnostic groups based on CTXII concentration.

8 DISCUSSION

The current study measures various parameters, namely MPO activity and MPO concentration, MMP-2 and -9 activities, C2C, CTXII, CPII and CS846 concentrations. The diagnostic potential of these factors was investigated in synovial fluid and/or serum of horses with joint diseases subdivided into OA, chronic arthritis, septic arthritis, and OCD. For this purpose, the applicability of selected assay systems has been evaluated in equine synovial fluid and serum. In the following, the results are discussed separately for the methodological study (chapter 8.1) and for the evaluation of distinct parameters in synovial fluid and serum of horses (chapters 8.2 – 8.4).

8.1 Methodological study

8.1.1 MPO Photometric Activity Assay

The MPO enzyme activity assay using *o*-dianisidine as a substrate has been applied earlier to synovial fluid and serum of various species (BASKOL *et al.* 2006; KUMAR *et al.* 2002; LAMMER 2001; LEFKOWITZ *et al.* 1999; MATHY-HARTERT *et al.* 1998; SPELLMEYER 2003; YARBROUGH *et al.* 1994). This; however, is the first study to adopt and evaluate MPO activity measurement in equine synovial fluid of horses.

The *o*-dianisidine-assay as described by KUMAR *et al.* (2002) is not specific for the MPO as many other peroxidases may interfere with the substrate (ISAAC and DAWSON 1999). To overcome this problem, ABAH was employed as a specific and potent inhibitor of the MPO. ABAH binds to the enzyme, converting it to its ferrous intermediate, thereby promoting an irreversible enzyme inactivation (BURNER *et al.* 1999). Benzoic acid hydrazides such as ABAH have been reported as excellent MPO inhibitors of the purified enzyme (KETTLE *et al.* 1995; WINTERBOURN *et al.* 2000). The results of the current study show that an ABAH concentration of 30 µM is effective to completely inhibit MPO activity in equine synovial fluid and serum, thus showing ABAH to be useful for the specific detection of MPO activity in equine samples.

In previous studies, the MPO enzyme reaction was stopped after a certain time period (BASKOL *et al.* 2006; KUMAR *et al.* 2002; LAMMER 2001; SPELLMEYER 2003). Thus only end-point colour development was quantified. Measurement of enzyme activity using a photometric assay highly requires absorbance linearity. Since end-point determinations only assume the linearity of reaction kinetics within a chosen system, a continuous kinetic photometric measurement was applied here. This study also demonstrates that the kinetics of MPO highly depends on the H_2O_2 concentration. As the application of different H_2O_2 concentrations on the *o*-dianisdine-assay has been reported in body fluids from various species (BASKOL *et al.* 2006; BROWN *et al.* 1999; KUEBLER *et al.* 1996; SPELLMEYER 2003; XIA and ZWEIER 1997), the H_2O_2 concentration for a linear kinetics of MPO over 30 min in equine samples was evaluated in this study and found to be 0.43 mM.

In view of the high viscosity of synovial fluid, it is recommended to treat highly viscous samples with hyaluronidase prior to analysis (GIBSON and ROONEY 2007; TERCIC and BOZIC 2001). Hyaluronidase splits hyaluronan polymers, thereby reducing fluid viscosity which potentially enables more accurate analysis. Pipetting error due to high synovial fluid viscosity has been reported to be 20-30% in humans (GREILING *et al.* 1995). Equine samples were treated with hyaluronidase under conditions similar to those reported for human fluid (DE JAGER *et al.* 2007; HAMERMAN *et al.* 1998; LETTESJO *et al.* 1998; SUGIUCHI *et al.* 2005). The marginally lower MPO activities obtained in the majority of hyaluronidase-treated samples might be at least partly due to the ongoing catabolic processes during their incubation with hyaluronidase at 37°C. This study reveals that hyaluronidase treatment has no or only a minor effect on MPO activity in equine synovial fluid, indicating that sample processing with hyaluronidase is not required for enabling accurate results.

Because synovial fluid is restricted to a relatively inaccessible site, it is usually difficult to obtain samples in larger quantities (MARXEN and SCHNEIDER 2003). Therefore, the degree of sample dilution sufficient for measuring MPO activity with appropriate sensivity was investigated in this study. Measurement of serial sample dilutions spiked with purified MPO demonstrated linearity of enzyme kinetics.

Synovial fluid samples which had no MPO activity at 1:2 dilution represented no MPO activity in undiluted samples. MPO activity of synovial fluid with low activity levels (< 1.0 U/ml) could be detected in undiluted samples as well as at 1:2 sample dilution; however, not at dilutions of 1:4 and 1:8, thus suggesting a synovial fluid dilution of 1:2 as suitable for MPO activity measurement in horses.

The *o*-dianisidine-assay introduced in the current study is characterized by a high percentage of MPO recovery. Measurement of intra- and inter-assay controls revealed excellent data reproducibility, thus confirming this assay as a reliable system for determination of MPO activity in equine synovial fluid and serum.

8.1.2 MPO Fluorometric Activity Assay

Since some synovial fluid samples showed very low or no MPO activities using the photometric activity assay, it cannot be excluded that these samples contained enzyme activities below the detection limit. Therefore, this study introduced for the first time a fluorometric activity assay for a potentially more sensitive determination of equine MPO activity. This assay contains tyrosine as a MPO substrate which is transformed into radicals. Two tyrosyl radicals can then interact forming the major fluorescent product dityrosine (KATO *et al.* 2003; MARQUEZ and DUNFORD 1995). Measurement of known amounts of purified MPO represented excellent linearity of relative fluorescence intensity over time, whereas no constant increase was found in synovial fluid samples used in different dilutions. Moreover, no recovery of purified MPO could be demonstrated in the samples, suggesting this assay to be less suitable for an application in equine synovial fluid.

It might be speculated whether synovial fluid compounds interfere with the tyrosine measurement. Interference with the emission signal from the reporter fluorophore by compound-quenching has been generally reported in fluorescence intensity measurements of enzyme assays (ALLEN *et al.* 2000; GRIBBON and SEWING 2003; TUREK-ETIENNE *et al.* 2003).

Free radicals are usually highly reactive species representing catalytic compounds of a variety of enzyme systems (STUBBE 1989). It could be assumed that tyrosine interaction with other reagents causes a reduced formation of fluorescent dityrosine in synovial fluid. Nitric oxide has shown to rapidly scavenge tyrosine radicals (EISERICH *et al.* 1995) and its presence in synovial fluid has been demonstrated (FUSELER *et al.* 1997). Therefore, it may be proposed that the synovial environment *e.g.* free radicals disturbes such radical-based fluorescent assays.

8.1.3 Gelatin zymography

Gelatin zymography is based on the hydrolysis of the preferential MMP-2 or -9 substrate. It has been widely used in body fluids of various species (ARICAN *et al.* 2000; KIDD *et al.* 2007; LOMBARD *et al.* 2005; VOLK *et al.* 2003). Various sample dilutions were investigated to find the optimum dilution for a comparison of gelatinolytic bands of different diagnostic groups. In this study, a synovial fluid dilution of 1:10 was found to be most suitable.

Given that other proteases such as MMP-1, -8, or -13 can lyse the same substrate (SNOEK-VAN BEURDEN and VON DEN HOFF 2005), this study employed two inhibitors to characterize the enzymes detected. All gelatinolytic bands were completely inhibited by EDTA, indicating that such enzymes were metalloenzymes. Application of MMP2/MMP9 Inhibitor III also resulted in a complete inhibition of gelatinolytic activities, confirming that the detected enzymes were MMP-2 or -9. MMP2/MMP9 Inhibitor III consists of a thiirane ring which selectively binds to the zinc ion of the active site of MMP-2 and -9, leading to a covalent enzyme modification (IKEJIRI *et al.* 2005). The latter study has shown this inhibitor to be suitable for an application in zymography.

Gelatin zymography allows measurement of total MMP activity since gel components activate the zymogens (BIRKEDAL-HANSEN and TAYLOR 1982; SNOEK-VAN BEURDEN and VON DEN HOFF 2005). It is known that SDS induces conformational changes in the latent enzyme causing the propeptide to fold back, thus exposing the catalytic centre (BIRKEDAL-HANSEN *et al.* 1993; NAGASE 1997).

Comparison to the migration of human latent MMP-2 and -9 indicated that activity at 72 kDa is due to MMP-2, and activities at 92, 120, and 250 kDa are due to MMP-9. In order to confirm that enzymes were present in latent forms, APMA was used in this study. APMA directly interacts with the sulfhydrylgroup of the propeptide leading to intramolecular cleavage upstream of the cysteine residue (BIRKEDAL-HANSEN *et al.* 1993; WOESSNER 1991). The resulting enzyme is characterised by a decrease in molecular weight. APMA treatment of synovial fluid samples resulted in the conversation of the 72 and 92 kDa enzyme bands, thus providing indication that gelatinolytic activities were due to latent MMPs.

The results of the current study support gelatin zymography as a suitable method for a more general detection of specific MMP-2 and -9 activities in equine synovial fluid. The identity of individual gelatinolytic bands detected using this system is further discussed in chapter 8.2.3 of this thesis.

8.2 Evaluation of parameters in synovial fluid

8.2.1 WBC count and protein concentration

WBC count and protein concentration increase in altered synovial fluid as a result of hypervascularity associated with synovitis or capsulitis (FRISBIE 2006; PALMER and BERTONE 1994). Both parameters are considered to be important in the examination of synovial fluid, providing an indication of the degree of joint inflammation (TROTTER and MCILWRAITH 1996).

The markedly increased WBC count and protein concentration in synovial fluid samples from septic arthritis are consistent with other studies in horses (CLEGG *et al.* 1997b; KIDD *et al.* 2007; TRUMBLE *et al.* 2001) and may represent a severe inflammation within the joint. WBC counts and protein levels decreased in synovial fluid of the follow-up group after the initiating phase of septic arthritis. Similar results were reported in a previous study demonstrating a decline in WBC counts in equine synovial fluid from septic arthritis as a result of successful treatment (KIDD *et al.* 2007).

It should be mentioned that although WBC count in this study allows a clear discrimination between septic arthritis samples and all other diagnostic groups, several authors have suggested WBC count alone as less sensitive to exclude or confirm septic arthritis (LI *et al.* 2007; MATHEWS *et al.* 2007).

Synovial fluid samples of the control, OA, chronic arthritis, and OCD groups presented WBC counts below $0.1x10^6$ cells/µl and protein levels below 20 mg/ml. These values are consistent with reported data for healthy fluids (TROTTER and MCILWRAITH 1996) and may indicate the absence of severe joint inflammation. It should be noted that these results reflect only the current status within the joint. Particularly in chronic joint diseases such as OA, WBC count can vary tremendously depending on the degree of active synovitis present (MCILWRAITH *et al.* 2001).

Automated cell counting in synovial fluid using the coulter counter is considered to be less accurate (DE JONGE *et al.* 2004) and less sensitive particularly within low cell ranges (MOSCHOS 2007). Manual counting of WBC counts, on the other hand, is thought to be highly subjective (GIBSON *et al.* 1999; SALINAS *et al.* 1997). Because of these technical limitations and the fact that synovial WBC count and total protein provide no information on articular cartilage damage (MCILWRAITH *et al.* 2001), the search for other biomarkers to indicate joint disease is clearly required.

8.2.2 MPO activity and MPO concentration

MPO is a highly specific enzyme of neutrophils with a strong oxidative activity (BABIOR 2000; KLEBANOFF 1999). Several investigators have reported MPO activity as a suitable estimate for neutrophil content (BRADLEY *et al.* 1982; KUEBLER *et al.* 1996; MCCONNICO *et al.* 1999). Accumulation of PMNs is a characteristic event in acute inflammation as present in septic arthritis (MCILWRAITH *et al.* 2001; MORTON 2005). Septic arthritis samples of the current study contained significantly increased MPO activities compared to all other diagnostic groups. The same samples showed markedly increased WBC counts and PMN contents greater than 90%, thus suggesting MPO activity as a marker for severe joint inflammation.

Lowest median and overall range of MPO activity were detected in controls, which is in line with results reported in dogs (LAMMER 2001; SPELLMEYER 2003). MPO activities of the OCD group did not differ significantly from controls. OCD has been classified as a non-inflammatory joint condition (MCILWRAITH *et al.* 2001) representing WBC and PMN counts in a similar range as controls (FRISBIE 2006). This is consistent with the data presented here and suggests MPO activity of less clinical value to indicate OCD.

In previous studies, significantly increased MPO activities were found in canine synovial fluids from OA (LAMMER 2001; SPELLMEYER 2003). According to the current results, a slight elevation of MPO activity could be detected in the OA group compared to controls; however, this difference was not significant. It should be mentioned that in these previous studies only an unspecific *o*-dianisidine-assay was used. While LAMMER (2001) employed no inhibitor, SPELLMEYER (2003) used sodium azide (NaN_3) as inhibitor, which is not highly specific for the MPO enzyme (DAVIES and EDWARDS 1989; JERLICH *et al.* 2000). Therefore, interpretation of these results might be limited due to measuring general peroxidase activities that may have interfered.

Synovial fluid samples from chronic arthritis showed no significant difference in MPO activity compared to controls. All horses from OA and chronic arthritis showed a chronic course of disease. Chronic joint diseases are cyclical processes with alternating periods of inflammatory activity and remission (MCILWRAITH *et al.* 2001; TRUMBLE *et al.* 2001). Since the results reflect only the current status within the joint, it cannot be excluded that MPO activities increase during advanced periods of inflammation. A remarkable number of MPO activity values was detected outside the 3 fold box lengths in the chronic arthritis group. It might be speculated that some recurring cases with a repeated episode of an acute inflammatory process may have interfered in these horses.

An extracelluar inactivation of the MPO enzyme has been reported by several authors (EDWARDS *et al.* 1987; KING *et al.* 1997; ORMROD *et al.* 1987), suggesting that MPO activity might not reflect the actual presence of MPO enzyme.

A previous study demonstrated that although few synovial fluid samples from rheumatoid arthritis showed no MPO activity, they still contained MPO enzyme (EDWARDS *et al.* 1988). In order to investigate whether MPO protein content is a more sensitive parameter for discriminating between joint diseases than MPO activity, this study used an equine MPO ELISA. MPO enzyme was present in all synovial fluid samples analysed; even so in a few samples no MPO activity could be detected. However, there was a significant difference between the septic arthritis and all other diagnostic groups for MPO concentration only, demonstrating that there is no difference in the discriminatory potential between MPO concentration and MPO activity.

Both parameters were moderately correlated, which is in line with a recently published study, where similar trends for MPO activities and MPO concentration in equine skin and laminar tissue could be shown over time (RIGGS *et al.* 2007). According to the results presented here, MPO activity as well as MPO concentration seems to indicate septic arthritis. However, the question whether the activity or concentration is a more suitable diagnostic marker cannot be answered until more is known about the role of MPO activity and MPO concentration in the progress of different joint diseases.

8.2.3 MMP-2 and -9 activities

MMP- 2 and -9 represent the gelatinase subgroup of the MMP family and might be centrally involved in articular cartilage remodelling. Degeneration of the articular cartilage is a major characteristic of OA, but it also occurs frequently in other joint diseases (MARINI *et al.* 2003).

Gelatin zymography provides information on the identity of individual MMPs and allows a relative comparison of the enzyme activity between samples (VOLK *et al.* 2003). Synovial fluids from OA, chronic arthritis, and septic arthritis contained significantly increased latent MMP-2 activity in zymography compared to controls and OCD. MMP-2 activity due to the active enzyme could not be detected in the current study.

These findings are consistent with previous observations in horses (CLEGG *et al.* 1997b; KIDD *et al.* 2007). Human PMN-derived MMP-9 is known to separate into 3 bands of activity, corresponding to the monomeric latent form (92 kDa), a high molecular weight dimeric form (> 180 kDa), and another form of 110 to 130 kDa (TRIEBEL *et al.* 1992). Septic arthritis samples of the current study showed significantly increased monomeric latent MMP-9 activity compared to all other affected joints and controls, which is in agreement with other reports in horses (CLEGG *et al.* 1997b; KIDD *et al.* 2007; TRUMBLE *et al.* 2001).

Synovial fluids from septic arthritis also contained significantly increased gelatinolytic activity at 120 kDa. The presence of the latter gelatinolytic band has previously been shown in synovial fluids from human and canine joints in which marked latent MMP-9 activity was detected (MAKOWSKI and RAMSBY 2003; PEAKE *et al.* 2006; VOLK *et al.* 2003). However, this is the first study to describe the presence of the 120 kDa gelatinolytic band in synovial fluid of horses. Previous studies reported a band of 120 kDa in human samples potentially representing complexed MMP-9 (GOLDBERG *et al.* 1992). It has been proposed that MMP-9 can associate with a microglobulin resulting in a band of approx. 125 kDa which is not dissociated in zymography (SNOEK-VAN BEURDEN and VON DEN HOFF 2005; TRIEBEL *et al.* 1992).

Gelatinolytic activity found at 250 kDa in few septic arthritis samples may represent the dimer of MMP-9. All samples in which the proposed dimer activity was detected showed marked latent MMP-9 activity as well as traces of the active MMP-9. Dimerisation of MMP-9 is thought to occur when MMP-9 is present in excess of TIMPs (GOLDBERG *et al.* 1992). This would imply that in samples where MMP-9 dimer is present MMP-9 is more likely to be present in its biologically active form. However, since inhibitors are dissociated from MMPs, gelatin zymography only gives an indication of the actual MMP activity within a sample (QUESADA *et al.* 1997).

The findings from zymographic analysis support the hypothesis that latent forms of MMP-2 and -9 are present in greater concentrations than active forms (CLEGG *et al.* 1997b; TRUMBLE *et al.* 2001). Moreover, these data confirm that synovial MMP-2 and -9 are elevated in equine joint diseases.

However, an absolute quantitative analysis of zymography appears to be critical (HUISMAN *et al.* 2004) due to “gel-to-gel” variability in sensitivity making it difficult to combine the data obtained from multiple gels (HASHIMOTO *et al.* 2005). For a quantitative determination of MMP-2 and -9 activities in equine samples, MMP-2 and -9 immunocapture activity assays were introduced in this study. These assays recognize enzymes in an active and uninhibited state, which might better reflect the current degradation process within a joint. Immunocapture activity assays have been shown to provide a simple, specific, and quantitative determination of active MMPs using samples from a range of animal species (CAPPER 1999a; CAPPER 1999b).

This study demonstrates that gelatinase activities increase considerably under pathological conditions. Samples from septic arthritis showed significantly higher MMP-2 and -9 activities as compared to controls, which agrees with a previous study in horses where total gelatinase activity was measured using an ELISA based on degradation of gelatin (CLEGG *et al.* 1997b). In the horse, MMP-9 is known to be produced by monocytes, chondrocytes, and PMNs; the latter are believed to be the main source of synovial MMP-9 in septic arthritis (CLEGG *et al.* 1997b). A strong correlation between MMP-9 activity and WBC count was identified in our study, which is in line with other observations (CLEGG *et al.* 1997b; TRUMBLE *et al.* 2001). Synovial fluids from septic arthritis contained PMN contents higher than 90%, indicating that increased MMP-9 activities were mainly released from infiltrating neutrophils.

No significant differences for MMP-2 and -9 activities could be detected between the control and OCD groups which is consistent with other results in horses (BRAMA *et al.* 1998; CLEGG *et al.* 1997b). However, a previous study in horses detected markedly increased gelatinolytic activities predominantly around chondrocytes in the deep zone of OCD cartilage by *in situ* gelatin zymography (AL-HIZAB *et al.* 2002). It could be assumed that levels of synovial enzyme activities might not necessarily reflect protein turnover present in the cartilage itself.

While MMP-2 activities of the current study were significantly increased in groups of OA and chronic arthritis as compared to controls, no significant differences could be detected for MMP-9 activity between these groups, thus demonstrating the importance to discriminate between MMP activities rather than measuring total gelatinase activity as performed by CLEGG *et al.* (1997b).

The elevated MMP-2 activity in the OA and chronic arthritis groups indicate that MMP-2 is involved in the progress of chronic joint disorders. In the horse, MMP-2 is secreted by connective tissue cells such as synovial fibroblasts and chondrocytes (CLEGG *et al.* 1997a). Production of MMPs within the joint is known to be induced by pro-inflammatory stimuli including IL-1 and TNF-α (BRINCKERHOFF and MATRISIAN 2002; MENGSHOL *et al.* 2002). Under chronic joint conditions as seen in OA, connective tissue cells within the joint are thought to be an important source of pro-inflammatory cytokines (BURRAGE *et al.* 2006). *In vitro* stimulation of equine articular cells and tissues with cytokines has shown to cause an increase of activated gelatinase enzyme released (CLEGG and CARTER 1999).

MMP-9 activity, on the other hand, was significantly increased in septic arthritis only, suggesting it to be more of an indicator for severe inflammatory joint conditions. As mentioned before, the results of the current study only reflect the current status within a joint. Since chronic diseases such as OA show variable degrees of inflammatory activity (MCILWRAITH *et al.* 2001; TRUMBLE *et al.* 2001), it could be assumed that MMP-9 activity in joints from OA and chronic arthritis increases during advanced periods of inflammation. Indication for that comes from the finding that MMP-9 activities together with WBC counts were found elevated in joints of the follow-up group in the early disease process of septic arthritis. MMP-2 and -9 activities decreased in the same joints in the course of successful therapy, which is in line with a previous study in horses (KIDD *et al.* 2007).

8.2.4 CTXII and C2C concentrations

Alteration in extracellular matrix composition appears to be due to abnormal matrix degradation and/or synthesis although the exact sequence of events in the progress of joint diseases remains unknown (FRISBIE *et al.* 1999a). This study used immunoassays to measure the epitopes C2C and CTXII which are generated after the initial cleavage of the collagen II triple-helix (ELSAID and CHICHESTER 2006).

To date, relatively few data on synovial CTXII levels in humans and other species have been published. Research on CTXII has focused on the fragment analysis in urine samples (ELSAID and CHICHESTER 2006; GARNERO 2006; ROUSSEAU and DELMAS 2007). A preliminary study in horses demonstrated the CTXII assay as a valid method to determine CTXII concentrations in synovial fluid (TRUMBLE *et al.* 2006).

The markedly increased CTXII concentrations in septic arthritis samples of the current study might reflect a rise in collagen II degradation in these joints. *In vitro* studies demonstrated that incubation of human cartilage explants with pro-inflammatory mediators such as IL-1 and TNF-α cause an increase in CTXII levels (ROY-BEAUDRY *et al.* 2003). Septic arthritis joints are known to contain the highest levels of pro-inflammatory cytokines (MORTON 2005). Data of the follow-up group demonstrate that CTXII levels consistently decreased in the septic arthritis joints in the course of successful therapy, suggesting its potential to indicate septic arthritis.

CTXII levels were found significantly increased in synovial fluids from chronic arthritis and OA as compared to controls. The potential of CTXII concentration to indicate OA has been confirmed by previous studies in humans (CHEVALIER and CONROZIER 2005; LOHMANDER *et al.* 2003), rodents (LINDHORST *et al.* 2005), and dogs (CHU *et al.* 2002; MATYAS *et al.* 2004). More recently, CTXII levels have shown to reflect the degree of cartilage degradation in a rat model of induced arthritis (OESTERGAARD *et al.* 2006). The current study reveals no significant difference in CTXII concentrations between the OA and chronic arthitis groups. While OA joints showed osteoarthritic radiographic findings, synovial fluid from chronic arthritis came from joints with clinical signs but no radiographic findings of disease.

It should be considered, that early pathological changes in the articular cartilalge not visible in the radiograph (CARON 2003; LOHMANDER *et al*. 1992) may have caused a misleading classification of at least some of these joints (MOSCHOS 2007), potentially accounting for the overlapping CTXII levels between both groups.

There was no significant difference in CTXII levels between the OCD and control groups of the current study. Preliminary data from naturally-occurring osteochondral injuries in the horse revealed elevated CTXII concentrations in synovial fluids from affected joints compared to healthy controls (TRUMBLE *et al.* 2006). Because of the relatively small number of OCD samples analysed in this study, further work is necessary to clearly evaluate the potential role of synovial CTXII concentration in the diagnosis of OCD.

The applicability of the C2C assay has been confirmed in various species including humans (ISHIGURO *et al*. 2001), rodents (KOJIMA *et al.* 2001; SONG *et al*. 1999), dogs (CHU *et al*. 2004), and horses (DE GRAUW et al. 2006b). The lowest median and overall ranges of C2C concentration in the current study were found in synovial fluids of the control group, which is consistent with reports in humans (ELSAID and CHICHESTER 2006). Surprisingly, no significant differences for C2C concentrations could be detected between any of the diagnostic groups. These results contrast with previous studies reporting the significance of C2C concentrations in the diagnosis of joint diseases, *e.g*. rheumatoid arthritis and OA in humans (ISHIGURO *et al.* 2001; POOLE *et al.* 2004), rodents (KOJIMA *et al.* 2001; SONG *et al.* 1999), and dogs (CHU *et al.* 2002). In the horse, a recently published preliminary study also failed to establish a relation between the synovial C2C content and the presence of clinically detectable joint pain (DE GRAUW *et al*. 2006b).

The marker concentration in synovial fluid can change depending on its clearance rate from the joint space (KRAUS 2006). Thus, it might be speculated that species differences in the joint clearance rate of the C2C neoepitope account at least partially for the contrary results. Studies on different clearance rates of selective molecules such as albumin between healthy and arthritis joints have been reported in dogs (MYERS *et al.* 1995).

Unfortunately, to the author's knowledge, no study regarding the kinetics of the release of C2C fragments has been published. According to the results presented here, synovial CTXII concentration seems to be more suitable to indicate joint disease than C2C concentration.

8.2.5 CPII and CS846 concentrations

In order to investigate potential changes in the synthesis of collagen type II and proteoglycan in diseased joints, this study used CPII and CS846 immunoassays (ELSAID and CHICHESTER 2006). The applicability of both assays in synovial fluid of horses was confirmed by the manufacturer.

Both CPII and CS846 levels of this study were slightly elevated in synovial fluids from OCD as compared to controls; however, this difference was not siginificant. These findings are consistent with previous data in the horse (DE GRAUW et al. 2006a). Particularly CS846 levels have been shown to increase in synovial fluid with increasing grade of osteochondral fragmentation, as seen by arthroscopy of horses (FRISBIE *et al.* 1999a). OCD horses of the current study were not classified according to the degree of disease severity. It would be of particular interest to investigate CPII and CS846 concentrations in horses with different severities of OCD in an extended study in the future.

Septic arthritis samples of this study contained markedly increased CPII and CS846 concentrations; however, when compared to controls this difference was not significant. An up-regulation of synovial CPII and CS846 concentrations has been detected in human synovial fluid from inflammatory joint conditions (ISHIGURO *et al.* 2001). The elevated CPII and CS846 levels may represent a reparative response of the chondrocytes to tissue damage, resulting from excessive activity of matrix-degrading enzymes (MORTON 2005). Indirect evidence for an increase in extracellular matrix degradation in septic arthritis joints of this study could be found by the significantly elevated synovial MMP-2 and -9 activities and CTX II concentrations.

CPII and CS846 concentrations were significantly decreased in samples from OA and chronic arthritis as compared to all other diagnostic groups. As a results of extracellular matrix disruption in the articular cartilage, chondrocytes become hypertrophic (POOLE *et al*. 2002). However, under chronic conditions, as seen in OA, chondrocytes can undergo apoptosis (POOLE *et al.* 2002), potentially leading to impaired synthesis of new matrix molecules. In the horse, percentage of chondrocyte apoptosis from OA cartilage was positively correlated with degree of cartilage degeneration (KIM *et al.* 2003). It could be assumed that the low CPII and CS846 concentrations, as found in this study for horses with OA and chronic arthritis, may indicate the inability of the cartilage to carry out reparative processes. Pro-inflammatory cytokines such as IL-1 and TNF-α have been reported to contribute to inhibition of extracellular matrix production by the chondrocyte (VERBRUGGEN *et al.* 2007). Recently, the role of cytokines in the pathogenesis of equine OA has been emphasized (SUTTON *et al.* 2007).

There was a strong correlation between CPII and CS846 concentrations in the current study. Values of both parameters showed similar trends in the follow-up group, suggesting that their synthesis is similarly regulated. A previous study in horses reported inverse changes of CPII and CS846 concentration in synoval fluid from OCD (LAVERTY *et al.* 2000). Therefore, it cannot be excluded that the regulation of their synthesis varies depending on the pathological joint condition. In human synovial fluid from OA, a positive correlation between CPII and CS846 concentrations has been detected (LOHMANDER *et al.* 1999). Both concentrations were also up-regulated in synovial fluid in patients from rheumatoid arthritis (ISHIGURO *et al.* 2001) and OA (LOHMANDER *et al.* 1996; POOLE *et al.* 1994). This study suggests that both CPII and CS846 concentration have the potential to indicate OA and chronic arthritis in equine affected joints.

8.3 Evaluation of distinct parameters in serum

Research on biomarkers in serum for the diagnosis of joint diseases has received growing attention (ELSAID and CHICHESTER 2006; MCILWRAITH 2005; THONAR *et al.* 1993). In the horse, relatively few studies on serum parameters for the diagnosis of joint diseases have been published to date.

In this study, MPO and MMP-2 and -9 activities did not differ markedly in serum samples between controls and affected groups, suggesting these parameters to be of less clinical value to indicate equine joint diseases. Previous observations in dogs reported serum MPO activity to be less suitable to reflect MPO levels of corresponding joints (SPELLMEYER 2003). However, elevated MPO and MMP-2 and -9 activities have been demonstrated in serum from humans with rheumatoid arthritis (BASKOL *et al.* 2006; TORSTEINSDOTTIR *et al.* 1999). Rheumatoid arthritis is a systemic disease often affecting extra-articular tissues (COMBE 2007; WALKER and RANATUNGA 2006). Therefore, measurements of serum MPO and MMP-2 and -9 activities might be more useful for identifying systemical effects not directly linked to specific joints disorders. In the horse, significantly enhanced MPO activities (YARBROUGH *et al.* 1994) and MMP-2 and -9 activities (AROSALO *et al.* 2007) have been reported in serum of patients with systemic intestinal pathologies.

The potential role of serum CTXII concentrations to indicate joint diseases has been demonstrated in various species including humans (KONG *et al*. 2006), rodents (OESTERGAARD *et al*. 2006; SONG *et al*. 1999), and dogs (MATYAS *et al.* 2004). In the current study, CTXII levels were significantly increased in serum from septic arthritis as compared to all other diagnostic groups, thus supporting serum CTXII concentration as an indicator for septic arthritis. However, no significant difference for serum CTXII concentration could be detected between OA or chronic arthritis and controls. Given that synovial fluid joints contain no more than 20% of the total mass of cartilage in the body, it is considered that the peripheral concentration of a marker molecule rises significantly only when its synthesis within the joint is pronounced (THONAR *et al.* 1993).

Another study has emphasized that the serum concentration of a biomarker can rise with increasing joint clearance of the marker molecule (KRAUS 2006) which in turn is associated with the grade of synovitis (MYERS *et al.* 1995). Thus, it might be speculated that the relatively low increase in CTXII concentration in synovial fluid from OA and chronic arthritis together with the absence of severe inflammation in these joints may at least partly account for the low CTXII levels in corresponding serum samples.

There was no significant difference for serum CPII and CS846 concentrations between the OA, chronic arthritis, OCD, and control groups in the current study. Since the synovial concentrations of both parameters were markedly decreased in joints from OA and chronic arthritis, this likely accounts for the low serum levels. In the horse, serum CPII and/or CS846 concentrations have been investigated in samples from osteochondral disorders and shown to increase with increasing severity of osteochondral injuries, as seen by arthroscopy (BILLINGHURST *et al.* 2004; FRISBIE *et al.* 1999a). As mentioned before, in the current study no attempt was made to classify OCD joints according to the degree of disease severity as this may have influenced the results. Serum CPII and CS846 concentrations of the current study were elevated in horses affected with septic arthritis. For CS846, this elevation was significant as compared to all other diagnostic groups. As shown for CTXII concentration, serum CS846 concentration may also be suitable to indicate septic arthritis.

8.4 Discriminant analysis

Discriminant analysis was used to classify equine samples into predefined diagnostic groups based on the parameters analysed in this study. The highest overall correct classification rate was found for the three-combination system of CTXII, CS846, and CPII concentrations, allowing 86.7% of horses to be correctly classified as either having or not having OA, chronic arthritis, septic arthritis, or OCD. To the author's knowledge, this is the highest percentage of correctly classified samples based on synovial parameters ever reported. Importantly, based on this combination, 100% of septic arthritis samples were correctly classified.

There was an overlap of diagnostic group classifications between the control and OCD groups, and the OA and chronic arthritis groups, yet again demonstrating the limited power of these parameters to discriminate between these joint diseases.

Single serum parameters as well as serum parameter combinations showed a hit ratio lower than 50%, indicating that serum parameters of this study are of smaller practical value for predictions on diagnostic group membership. This is a relatively low percentage compared to a previous study, reporting predictions on whether horses did or did not have osteochondral fragmentation based on serum CPII and CS846 concentrations to be correct in 79% of cases (FRISBIE *et al.* 1999a). It should be noted that this previous study only differentiated between healthy horses and horses with osteochondral fragmentation. In the current study, 100% of the septic arthritis samples of the current study were correctly classified based on CTXII concentration alone.

When predicting a diagnostic group membership of an unclassified equine sample based on the set of parameters presented here, it should be taken into account that analysis was performed on a limited population size with different diagnostic groups showing unequal sample numbers. Therefore the results of this study do not allow any general conclusions as to the whole equine population. However, these first data of this discriminant analysis provide a promising basis for further studies on equine biomarkers suitable for identifying joint diseases.

9 CONCLUSION

This study analysed the potential of various marker molecules, namely MPO activity and MPO concentration, MMP-2 and -9 activities, C2C, CTXII, CPII and CS846 concentrations in synovial and/or serum to indicate joint diseases in the horse.

For the determination of MPO activity in equine synovial fluid and serum, a specifically modified MPO activity assay was introduced and validated in this study. The results show this assay to be a reliable and feasible tool for a specific detection of MPO activity in horses. Synovial fluid parameter analysis reveals that MPO activity, MPO concentration, MMP-2 and -9 activities, and CTXII concentration have the potential to indicate septic arthritis. Furthermore, the results suggest MMP-2 and -9 activities as well as CTXII concentration as suitable markers for monitoring septic arthritis during a time-course.

Using discriminant analysis for the classification of samples into predefined diagnostic groups, it becomes evident that synovial CTXII concentration showed the greatest correct classification rate of 68.4% for a single parameter. The highest overall correct classification rate of 86.7% was found for a three-combination system of synovial CTXII, CS846, and CPII concentrations. These findings allow the conclusion that markers of cartilage metabolism, particularly CTXII, CS846, and CPII concentrations may have the greatest potential to discriminate between different joint diseases. Moreover, a combination of biomarkers seems to be most promising for an accurate assessment of equine joint condition in the future.

First data from serum parameter analysis suggest that the serum parameters investigated in this study are of less clinical value to indicate joint disease. Serum CTXII concentration showed the highest overall correct classification rate of 45.5%. Therefore, a desired peripheral sampling of body fluids would currently not provide significant diagnostic statements.

Taking all together, these observations provide new insights into the diagnostic potential of various biomarkers in equine joint diseases. Their significance has been appreciated and major findings have been published recently (FIETZ *et al.* 2007a and b). With this knowledge, early and adequate intervention in joint disease may be facilitated through a multi-parameter investigation of synovial fluid as an additional tool to clinical investigation.

10 Outlook

This study clearly reveals that for an accurate assessment of equine joint condition a combination of selected marker molecules in body fluids is most useful. However, first data from serum parameter analysis suggest that peripheral values of MPO, MMP-2 and -9 activities as well as C2C, CTXII, CPII, and CS846 concentrations are of less clinical value. Future studies with larger numbers of serum samples are required to confirm the very first results presented here.

The current study shows synovial MPO activity, MPO concentration, MMP-2 and -9 activities, CTXII, CPII, and CS846 concentrations to be beneficial in indicating joint disease in the horse. These promising findings call for further extended studies with a longer follow-up of animals. In this regard it is recommended to obtain synovial fluid sampling after defined intervals within a standardized protocol to establish reliable correlations between levels of marker molecules and disease activity or remission. Moreover, in order to improve the discriminatory potential of the parameters in equine joint diseases, the levels of marker molecules should be directly correlated with the degree of local cartilage degradation by use of high-sophisticated imaging techniques in future studies.

Positively evaluated parameters in synovial fluid may also provide candidate targets for pharmacological intervention, *e.g.* by inhibiting MPO, MMP-2 and -9 enzyme reaction, or represent appropriate monitoring markers for successful medical treatment. This calls for further research on the biomarkers investigated in this study.

11 SUMMARY

Biomarkers in equine synovial fluid and serum for the diagnosis of joint diseases

Joint disease has a major impact on the athletic performance in horses, thus representing not only a welfare issue but also a source of economic loss due to veterinary expenses, limited performance, and early retirements. Its diagnosis is routinely established on the basis of clinical lameness examination, radiography, and conventional synovial fluid analysis. Accurate assessment of joint disease is essential to determine adequate treatment and prognosis. However, definite diagnosis often remains difficult. Biomarkers in body fluids could offer clinicians a valuable tool to identify advanced stages of joint diseases more accurately. Various marker molecules have already been used successfully in research settings, but to date none has been introduced and validated in everyday horse practice.

The aim of this study was to evaluate the possible use of selective parameters, namely MPO activity and MPO concentration, MMP-2 and -9 activities, C2C, CTXII, CPII, and CS846 concentrations, in equine synovial fluid and serum as diagnostic markers of joint diseases. Equine samples were taken from horses affected with septic arthritis, osteoarthritis (OA), chronic arthritis, osteochondrosis dissecans (OCD), and from healthy controls. For the determination of MPO activity a specifically modified MPO assay was established in this study. The results show this assay to be a reliable and feasible tool for a specific detection of MPO activity in equine synovial fluid and serum. Furthermore, MPO concentration in synovial fluid was determined using an equine MPO immunoassay. MPO activity and MPO concentration were significantly increased in synovial fluids from septic arthritis compared to all other diagnostic groups.

Using gelatin zymography, this study demonstrates that MMP-2 and -9 activities increase considerably under pathological conditions. For a precise quantification of enzyme activities, MMP-2 and -9 immunocapture activity assays were introduced.

MMP-2 and -9 activities were significantly elevated in synovial fluids from septic arthritis compared to all other diagnostic groups, and MMP-2 activity was significantly increased in synovial fluids from OA and chronic arthritis compared to controls and OCD samples.

For the evaluation of markers of cartilage metabolism, C2C, CTXII, CPII, and CS846 immunoassays were applied on equine samples. CTXII concentration was significantly increased in synovial fluids from septic arthritis compared to all other diagnostic groups and in synovial fluids from OA and chronic arthritis compared to controls. Serum CTXII levels were significantly elevated in septic arthritis specimens compared to controls. No significant differences for C2C concentrations could be detected between any of the diagnostic groups. CPII and CS846 concentrations were significantly decreased in synovial fluids from OA and chronic arthritis compared to all other diagnostic groups. Serum CS846 concentration was significantly increased in septic arthritis specimens compared to all other diagnostic groups, and serum CPII concentration was significantly elevated in septic arthritis specimens relative to the chronic arthritis group.

Equine samples were further classified into predefined diagnostic groups based on certain parameter(s) using discriminant analysis. Serum parameters investigated in this study showed correct classification rates below 50% indicating that they are of less clinical value to indicate joint disease. Synovial CTXII concentration showed the greatest correct classification rate of 68.4% for a single parameter. A combination of biomarkers seems to be most promising for an accurate assessment of joint condition. The highest overall correct classification rate of 86.7% was found for a three-combination system of synovial CTXII, CS846, and CPII concentrations. It appears that synovial markers of cartilage metabolism, particularly CTXII, CS846, and CPII concentrations may have the greatest potential to discriminate between different joint diseases.

These observations provide new insights into the diagnostic potential of diverse biomarkers in equine joint diseases. The results presented here may help to refine the diagnosis of distinct joint diseases and provide a profound basis for further studies on biomarkers for equine joint disease.

12 ZUSAMMENFASSUNG

Biomarker in Synovia und Serum von Pferden für den Nachweis von Gelenkerkrankungen

Gelenkerkrankungen haben einen erheblichen Einfluss auf die sportliche Leistung von Pferden und verursachen zudem hohe wirtschaftliche Verluste durch tierärztliche Aufwendungen. Ihre Diagnose wird routinemäßig auf Grundlage der klinischen Lahmheitsuntersuchung, Röntgenbildauswertung und konventionellen Synoviaanalyse gestellt. Eine präzise Diagnose kann sich oft schwierig gestalten, ist jedoch für eine optimale Behandlung und Prognose von entscheidender Bedeutung. Die Analyse von Biomarkern in Körperflüssigkeiten kann dem Kliniker wertvolle Hinweise liefern, um Gelenkerkankungen präziser zu diagnostizieren. Verschiedene Markermoleküle wurden in der Forschung bereits erfolgreich getestet, jedoch bis zum heutigen Tag in der tierärztlichen Praxis noch nicht eingeführt und validiert.

Das Ziel dieser vorliegenden Arbeit war die Untersuchung selektiver Parameter, wie die der MPO-Aktivität und -Konzentration, der MMP-2 und -9-Aktivitäten, der C2C-, CTXII-, CPII- und CS846-Konzentrationen auf ihren potentiellen Einsatz als diagnostische Marker bei Gelenkerkrankungen des Pferdes. Die Proben wurden von an septischer Arthritis, Osteoarthritis (OA), chronischer Arthritis, und Osteochondrosis dissecans (OCD) erkrankten Pferden sowie von gesunden Kontrolltieren genommen. Für die Bestimmung der MPO-Aktivität wurde ein speziell modifizierter MPO-Assay etabliert und validiert. Die Ergebnisse zeigen, dass dieser für die spezifische Messung der MPO-Aktivität in der Synovia und im Serum von Pferden eine zuverlässige und praktikable Methode darstellt. Darüber hinaus wurde die MPO-Konzentration in der Synovia mit einem equinen MPO-Immunoassay ermittelt. Die MPO-Aktivität und MPO Konzentration waren in Gelenken mit septischer Arthritis im Vergleich zu Gelenken aller anderen diagnostischen Gruppen signifikant erhöht.

Mit Hilfe der Gelatine-Zymography wurden in den erkrankten Gelenken erhöhte MMP-2 und -9-Aktivitäten nachgewiesen. Für die genauere Quantifizierung wurden Aktivitäts-Immuno-Assays verwendet.

Signifikant erhöhte MMP-2 und -9-Aktivitäten wurden in der Synovia von Gelenken mit septischer Arthritis im Vergleich zu allen anderen diagnostischen Gruppen nachgewiesen. Zudem war die MMP-2-Aktivität in der Synovia von Gelenken mit OA und chronischer Arthritis signifikant höher, als die der Kontrollgruppe und der OCD-Proben.

Marker des Knorpelstoffwechsels wurden mittels C2C-, CTXII-, CPII-, und CS846-Immunoassays bestimmt. Die CTXII-Konzentration war bei septischer Arthritis im Vergleich zu allen anderen diagnostischen Gruppen signifikant erhöht. Erhöhte CTXII-Konzentrationen wiesen auch Gelenke von Pferden mit OA und chronischer Arthritis im Vergleich zu Kontrolltieren auf. Bezüglich der Serum CTXII- Konzentrationen waren die Werte bei septischer Arthritis signifikant höher verglichen zu den Kontrollen. Es konnten keine signifikanten Unterschiede zwischen den diagnostischen Gruppen bezüglich der C2C-Konzentrationen festgestellt werden. CPII- und CS846-Konzentrationen waren signifikant niedriger in Gelenken mit OA und chronischer Arthritis im Vergleich zu allen anderen diagnostischen Gruppen. Die Serum CS846-Konzentration war signifikant erhöht in Pferden mit septischer Arthritis im Vergleich zu allen anderen diagnostischen Gruppen. Ebenso konnte für die Serum CPII-Konzentration eine signifikante Erhöhung in Pferden mit septischer Arthritis, verglichen zu Pferden mit chronischer Arthritis, gezeigt werden.

Mit Hilfe der Diskriminanzanalyse auf der Basis der ermittelten Parameter, wurden die Proben in die klinisch vordefinierten diagnostischen Gruppen klassifiziert. Der Prozentsatz der korrekt klassifizierten Proben lag für die untersuchten Serum-Parameter unter 50%, was auf ein relativ geringes diagnostisches Potential schließen lässt. Die CTXII-Konzentration in der Synovia zeigte den größten Prozentsatz korrekt klassifizierter Proben (68,4%). Darüber hinaus scheint für eine präzise Diagnose der Gelenkerkrankung eine Kombination von Biomarkern am vielversprechendsten. Die höchste Gesamtrate korrekt klassifizierter Proben von 86,7% wurde für die Parameter-Kombination CTXII-, CS846- und CPII-Konzentrationen in der Synovia ermittelt.

Aus diesen Ergebnissen lässt sich schließen, dass die Marker des Knorpelstoffwechsels, insbesondere die CTXII, CS846 und CPII-Konzentrationen eine bestmögliche Differenzierung zwischen Gelenkerkrankungen erlauben.

Die vorliegenden Untersuchungen liefern neue Erkenntnisse hinsichtlich des diagnostischen Potentials ausgewählter Biomarker für Gelenkerkrankungen des Pferdes. Die hier vorgestellten Ergebnisse können die Diagnostik von Gelenkerkrankungen verbessern und bilden zudem eine solide Ausgangsbasis für weitere Studien.

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Berlin, den 29.04.2008

Simone Angelika Fietz

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
1	F	8	Warm-blood	Tarsocrural	**Synovia**	10.5	n.d.	0.0	3.5	0.9	0.5	109.2	176.0	320.6	1.7
					Serum	47.3	n.d.	0.6	n.d.	0.6	0.3	12.3	6.8	61.7	0.2
2	Cm	8	Warm-blood	Tarsocrural	**Synovia**	7.4	n.d.	0.0	3.1	1.7	0.4	124.6	158.2	307.6	1.7
					Serum	38.9	n.d.	2.5	n.d.	0.4	0.3	10.7	1.7	51.7	0.1
3	Cm	4	Through-bred	Carpal	**Synovia**	9.3	0.1	0.0	9.4	0.8	0.3	110.1	143.9	399.2	1.7
					Serum	41.1	n.d.	2.1	n.d.	0.4	0.6	24.9	35.9	50.2	0.2
4	Ma	3	Quarter	Carpal	**Synovia**	11.6	0.1	0.0	9.5	1.2	0.4	122.5	80.3	428.2	1.7
					Serum	42.2	n.d.	22.4	n.d.	0.5	0.5	35.5	32.0	53.0	0.4
5	Ma	3	Quarter	Tarsocrural	**Synovia**	10	0.1	0.0	3.5	1.2	0.3	185.4	127.5	426.8	1.7
					Serum	42.9	n.d.	18.4	n.d.	0.7	0.7	15.5	34.4	83.8	0.1
6	F	5	Standard-bred	Tarsocrural	**Synovia**	5.2	0.1	0.0	10.4	1.6	0.4	74.2	88.1	300.8	1.9
					Serum	40.1	n.d.	14.3	n.d.	2.6	0.3	22.7	26.2	57.2	0.2
7	F	6	Warm-blood	Tarsocrural	**Synovia**	17.7	0.4	0.0	3.4	3.1	0.5	110.1	125.6	266.2	1.7
					Serum	45.0	n.d.	16.9	n.d.	0.6	0.3	25.8	1.2	49.1	0.2
8	F	4	Warm-blood	Tarsocrural	**Synovia**	14.2	0.2	0.0	4.0	3.0	0.5	79.3	133.8	401.3	1.7
					Serum	42.4	n.d.	9.7	n.d.	0.8	0.4	19.7	31.1	79.2	0.6
9	Ma	2	Warm-blood	Carpal	**Synovia**	18.1	0.5	0.3	10.4	1.9	0.8	109.4	124.3	331.9	1.8
					Serum	43.3	n.d.	11.2	n.d.	1.2	0.1	12.6	29.0	49.9	0.1
10	Ma	14	Warm-blood	Tarsocrural	**Synovia**	7.4	0.1	0.3	12.0	1.3	0.2	99.4	128.3	306.5	1.6
					Serum	39.7	n.d.	14.3	n.d.	0.9	0.1	27.3	30.1	79.3	0.1

Table 10: Single data of the control group.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
11	Cm	13	Warm-blood	Tarsocrural	**Synovia** **Serum**	4.2 46.5	0.1 n.d.	0.3 16.2	15.8 n.d.	1.3 1.1	0.3 0.8	122.5 13.3	134.2 21.2	334.2 83.0	1.7 0.1
12	Cm	23	Warm-blood	Carpal	**Synovia**	11.3	0.1	0.0	13.8	1.5	0.2	116.2	125.9	312.4	2.0
13	Cm	1	Pony	Carpal	**Synovia**	9.9	0.1	0.6	19.4	1.1	0.2	74.7	71.3	325.7	1.7
14	F	2	Warm-blood	Carpal	**Synovia**	8.1	0.1	0.0	15.3	0.9	0.1	128.5	159.3	345.1	1.9
15	F	16	Through-bred	Tarsocrural	**Synovia**	5.2	0.1	0.0	6.8	1.1	0.2	120.1	144.1	329.6	1.6
16	Ma	3	Standard-bred	Carpal	**Synovia**	5.1	0.1	1.2	21.7	1.2	0.3	120.7	124.6	345.1	1.7
17	F	23	Standard-bred	Carpal	**Synovia**	12.3	0.1	0.0	5.0	1.1	0.3	162.8	154.2	320.8	1.7
18	Ma	22	Warm-blood	Carpal	**Synovia**	5.8	0.1	0.0	2.0	1.1	0.3	111.9	149.7	345.3	1.5
19	Cm	3	Warm-blood	Carpal	**Synovia**	11.8	0.1	0.0	2.6	0.9	0.1	82.6	196.4	336.0	1.4
20	F	6	Warm-blood	Dist. Inter-phalageal	**Synovia**	4.9	n.d.	0.0	n.d.	0.8	0.4	134.6	120.3	307.6	1.3

Continuation of Table 10.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
1	Cm	25	Warm-blood	Metacarpo-phalangeal	**Synovia**	10.2	0.1	0.8	17.3	2.9	0.2	114.4	194.6	153.3	0.4
					Serum	48.5	n.d.	3.4	n.d.	0.5	0.3	18.8	20.6	44.9	0.1
2	F	9	Standard-bred	Carpal	**Synovia**	9.3	n.d.	0.6	n.d.	2.8	0.5	115.4	445.8	217.4	1.0
					Serum	43.9	n.d.	6.3	n.d.	0.7	0.6	16.0	105.6	80.2	0.1
3	F	15	Pony	Femoro-patellar	**Synovia**	4.8	0.1	1.7	26.0	1.6	0.4	100.9	286.5	123.2	1.1
					Serum	41.8	n.d.	9.2	n.d.	1.0	0.7	24.7	15.1	51.3	0.1
4	Cm	11	Warm-blood	Tarsocrural	**Synovia**	13.3	0.1	0.7	11.7	2.7	0.1	246.4	398.8	182.1	1.0
					Serum	43.7	n.d.	0.5	n.d.	1.3	0.3	20.0	54.0	50.2	0.1
5	Cm	5	Warm-blood	Carpal	**Synovia**	11.0	0.1	1.9	17.1	3.3	0.9	194.4	321.2	139.9	1.0
					Serum	46.2	n.d.	14.5	n.d.	0.7	0.4	8.2	12.7	69.9	0.5
6	Cm	12	Warm-blood	Femoro-patellar	**Synovia**	13.4	n.d.	0.0	5.6	1.7	1.2	112.3	468.2	125.8	1.3
					Serum	37.5	n.d.	17.4	n.d.	0.8	0.6	7.1	56.2	60.9	0.3
7	Cm	15	Warm-blood	Tarsocrural	**Synovia**	16.2	n.d.	0.0	n.d.	3.1	0.6	76.4	508.0	195.8	1.3
					Serum	44.7	n.d.	24.5	n.d.	1.3	1.0	7.7	123.6	49.9	0.4
8	F	2	Warm-blood	Metatarso-phalangeal	**Synovia**	3.4	n.d.	3.1	n.d.	3.4	1.0	96.7	293.9	192.1	1.2
					Serum	46.3	n.d.	24.2	n.d.	0.9	0.1	2.6	126.0	39.9	0.3
9	F	2	Standard-bred	Carpal	**Synovia**	12.9	n.d.	2.6	n.d.	2.8	1.3	140.4	428.8	192.5	0.6
					Serum	44.2	n.d.	12.4	n.d.	0.2	0.1	10.0	140.6	19.8	0.2
10	Cm	14	Warm-blood	Tarsocrural	**Synovia**	10.3	n.d.	0.5	n.d.	3.8	0.5	220.7	286.9	162.3	0.9
					Serum	45.5	n.d.	14.7	n.d.	0.5	0.2	10.5	22.4	69.8	0.4

Table 11: Single data of the OA group.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
11	Cm	7	Warm-blood	Metatarso-phalangeal	**Synovia** **Serum**	9.4 39.6	n.d. n.d.	1.3 13.5	n.d. n.d.	5.0 1.0	0.3 0.1	68.1 10.0	469.2 32.5	221.7 66.3	0.3 0.1
12	F	1	Warmblood	Femoro-patellar	**Synovia**	8.3	0.1	0.0	8.8	1.7	0.3	112.4	276.7	199.4	0.8
13	Cm	7	Warm-blood	Metacarpo-phalangeal	**Synovia**	4.0	0.1	1.3	16.6	3.4	1.2	178.4	324.0	187.6	0.9
14	F	14	Arabian	Carpal	**Synovia**	18.5	0.4	0.2	17.5	1.5	0.3	198.6	329.9	178.6	0.8
15	F	2	Warm-blood	Femoro-patellar	**Synovia**	12.5	0.1	0.0	17.1	4.8	0.3	69.0	422.4	175.8	1.4
16	Ma	9	Warm-blood	Femoro-patellar	**Synovia**	14.1	0.1	1.1	5.2	3.2	0.3	198.3	427.6	255.9	1.3
17	Cm	18	Arabian	Carpal	**Synovia**	14.2	0.1	0.4	17.2	3.6	0.4	123.6	340.1	232.9	0.5
18	Cm	7	Warm-blood	Metacarpo-phalangeal	**Synovia**	3.6	n.d.	0.0	9.8	2.9	1.2	298.7	333.9	245.9	1.4
19	F	10	Warm-blood	Metacarpo-phalangeal	**Synovia**	5.2	n.d.	1.5	6.0	3.5	0.8	63.6	676.3	167.6	1.2
20	Cm	8	Warm-blood	Dist. Inter-phalangeal	**Synovia**	12.0	0.1	0.8	4.3	2.9	0.4	436.4	273.7	196.3	0.6

Continuation of Table 11.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
21	Cm	10	Warm-blood	Metacarpo-phalangeal	**Synovia**	4.5	0.1	9.0	84.2	2.6	0.3	219.1	397.6	291.0	0.9
22	F	10	Warm-blood	Metacarpo-phalangeal	**Synovia**	7.9	0.1	2.7	58.8	3.0	0.4	138.8	320.9	137.4	0.7
23	Cm	25	Pony	Tarsocrural	**Synovia**	3.6	0.1	0.0	3.4	1.8	0.4	122.8	278.0	151.4	1.4
24	F	12	Warm-blood	Metacarpo-Phalangeal	**Synovia**	8.4	n.d.	0.0	4.7	3.1	0.6	198.3	342.8	199.9	1.1
25	F	4	Warm-blood	Femoro-patellar	**Synovia**	7.9	n.d.	0.0	2.1	4.7	0.6	120.4	480.5	279.0	1.1
26	Ma	7	Warm-blood	Tarsocrural	**Synovia**	10.2	n.d.	0.6	n.d.	3.1	1.1	189.4	401.0	201.1	1.2
27	Cm	9	Warm-blood	Carpal	**Synovia**	11.4	n.d.	0.0	5.3	3.2	1.2	342.4	177.7	279.0	1.3
28	Cm	8	Warm-blood	Carpal	**Synovia**	12.4	n.d.	0.0	6.2	4.7	0.9	165.2	345.9	227.1	1.0

Continuation of Table 11.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
1	F	8	Warm-blood	Dist. Inter-phalangeal	**Synovia** **Serum**	4.4 36.8	n.d. n.d.	0.0 20.8	8.7 n.d.	3.5 3.4	0.3 0.1	111.9 18.2	293.4 45.7	206.0 61.3	1.0 0.8
2	F	6	Warm-blood	Carpal	**Synovia** **Serum**	5.3 43.2	0.1 n.d.	0.0 3.0	9.6 n.d.	2.3 1.6	0.5 0.8	121.4 15.4	248.8 56.0	261.9 50.3	1.2 0.3
3	F	10	Warm-blood	Carpal	**Synovia** **Serum**	9.2 42.9	0.1 n.d.	0.7 13.0	15.8 n.d.	1.9 0.7	0.2 0.1	107.0 25.9	225.8 10.8	260.3 46.5	1.3 0.7
4	Cm	11	Warm-Blood	Dist. Inter-phalangeal	**Synovia** **Serum**	10.3 44.7	0.2 n.d.	4.8 14.3	18.6 n.d.	3.0 0.6	0.9 0.1	133.5 50.8	103.1 23.7	160.5 44.4	1.2 0.8
5	Cm	11	Warm-blood	Tarsocrural	**Synovia** **Serum**	12.3 44.2	n.d. n.d.	2.4 2.8	11.2 n.d.	3.6 0.3	1.0 0.6	120.2 15.7	269.3 31.8	212.9 81.1	1.3 0.1
6	Cm	11	Warm-blood	Dist. Inter-phalangeal	**Synovia** **Serum**	12.7 39.8	0.1 n.d.	1.7 16.3	21.3 n.d.	2.6 1.3	0.6 1.2	112.4 35.8	196.7 39.3	223.1 54.7	1.0 0.1
7	F	6	Pony	Dist. Inter-phalangeal	**Synovia** **Serum**	10.0 42.6	0.4 n.d.	0.8 16.9	6.0 n.d.	3.4 0.6	1.4 0.9	115.5 13.7	248.7 34.9	211.2 41.3	1.1 0.5
8	Cm	5	Standard-bred	Metacarpo-phalangeal	**Synovia** **Serum**	5.1 45.2	0.1 n.d.	0.0 11.4	4.5 n.d.	2.7 1.7	0.2 0.1	113.3 12.0	228.9 45.2	206.2 11.8	1.5 0.1
9	F	8	Warm-blood	Tarsocrural	**Synovia** **Serum**	19.5 40.2	1.3 n.d.	0.0 9.3	38.7 n.d.	3.1 1.3	1.6 0.2	123.4 20.0	173.1 112.0	253.3 31.3	1.3 0.1
10	F	8	Pony	Carpal	**Synovia** **Serum**	11.3 41.1	0.1 n.d.	0.7 18.7	20.0 n.d.	3.1 0.9	0.8 0.4	276.3 23.3	71.3 32.8	234.7 37.2	1.0 0.1

Table 12: Single data of the chronic arthritis group.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
11	Cm	12	Warm-blood	Metacarpo-phalangeal	**Synovia** **Serum**	12.1 48.6	0.1 n.d.	0.0 13.6	5.5 n.d.	2.2 0.9	0.2 0.4	298.7 41.1	221.2 49.9	221.9 29.1	0.9 0.1
12	Cm	6	Arabian	Metacarpo-phalangeal	**Synovia**	5.1	0.1	0.0	7.3	2.6	0.2	123.9	114.0	198.8	1.3
13	Cm	11	Warm-blood	Metacarpo-phalangeal	**Synovia**	7.2	0.1	0.5	5.7	2.7	0.3	177.4	29.2	267.0	1.3
14	Cm	12	Warm-blood	Dist. Inter-phalangeal	**Synovia**	8.3	0.1	0.0	12.7	2.1	0.2	187.7	231.7	244.0	1.1
15	F	8	Warm-blood	Metacarpo-phalangeal	**Synovia**	13.6	0.1	0.4	5.3	1.7	0.3	122.7	321.0	289.8	1.0
16	Cm	6	Warm-blood	Dist. Inter-phalangeal	**Synovia**	18.2	0.6	0.0	18.9	3.7	1.0	337.0	224.8	289.9	1.2
17	Cm	11	Warm-blood	Dist. Inter-phalangeal	**Synovia**	16.2	0.2	12.0	129.6	3.3	1.0	268.1	343.2	256.0	1.3
18	F	8	Warm-blood	Dist. Inter-phalangeal	**Synovia**	10.8	0.1	0.4	5.2	1.9	0.4	287.6	318.1	245.1	0.9
19	Cm	12	Warm-blood	Dist. Inter-phalangeal	**Synovia**	7.5	0.1	6.8	24.0	3.3	0.4	198.7	234.9	288.3	0.9
20	F	6	Warm-blood	Dist. Inter-phalangeal	**Synovia**	7.3	0.1	0.2	10.1	2.6	0.4	87.5	213.4	287.5	1.2

Continuation of Table 12.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
21	F	13	Warm-blood	Dist. Inter-phalangeal	**Synovia**	15.2	0.2	0.0	12.0	4.1	1.0	131.2	267.6	235.8	1.0
22	Cm	8	Warm-blood	Dist. Inter-phalangeal	**Synovia**	4.3	0.1	0.0	10.4	3.6	0.5	199.6	298.5	221.9	1.0
23	Cm	7	Warm-blood	Dist. Inter-phalangeal	**Synovia**	8.3	0.2	6.1	22.3	4.0	1.1	187.5	345.8	289.7	1.2
24	F	6	Warm-blood	Dist. Inter-phalangeal	**Synovia**	19.6	1.8	0.7	10.4	3.2	1.6	68.7	231.7	201.7	1.0
25	F	8	Warm-blood	Metacarpo-phalangeal	**Synovia**	11.3	0.1	1.4	17.5	2.9	0.3	59.1	220.3	239.7	1.0
26	F	8	Pony	Dist. Inter-phalangeal	**Synovia**	8.9	n.d.	0.1	n.d.	3.1	0.8	167.0	127.6	288.2	1.2
27	F	5	Warm-blood	Metacarpo-Phalangeal	**Synovia**	5.9	n.d.	1.2	n.d.	3.4	1.0	133.1	237.7	200.0	0.9
28	F	8	Warm-blood	Dist. Inter-phalangeal	**Synovia**	8.1	n.d.	0.0	n.d.	4.1	1.3	67.6	237.9	267.9	1.1
29	F	9	Warm-blood	Tarsocrural	**Synovia**	9.9	n.d.	0.4	n.d.	3.5	0.9	388.6	274.1	279.0	0.9
30	F	4	Warm-blood	Metacarpo-phalangeal	**Synovia**	7.5	n.d.	3.2	n.d.	3.4	0.8	239.7	276.4	203.0	1.0

Continuation of Table 12.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
1	F	2	Standard-bred	Tarsocrural	**Synovia** **Serum**	12.3 39.4	n.d. n.d.	2.5 14.2	n.d. n.d.	1.8 0.9	0.6 0.2	105.6 3.0	143.9 15.6	446.0 23.8	1.9 0.2
2	Ma	1	Warm-blood	Metatarso-Phalangeal	**Synovia** **Serum**	16.2 39.9	n.d. n.d.	0.8 16.1	15.2 n.d.	2.3 1.1	0.6 0.7	69.6 8.4	193.0 37.9	481.3 157.2	1.9 0.1
3	F	3	Warm-blood	Metatarso-phalangeal	**Synovia** **Serum**	14.5 42.5	n.d. n.d.	8.1 26.2	25.9 n.d.	2.2 1.8	0.8 0.1	131.2 55.3	179.8 23.0	316.6 92.7	1.9 0.3
4	Cm	3	Warm-blood	Tarsocrural	**Synovia** **Serum**	5.3 41.6	0.1 n.d.	0.3 3.5	11.2 n.d.	1.7 0.9	0.5 0.2	178.4 46.0	160.1 30.8	433.0 56.2	1.9 0.1
5	Cm	3	Warm-blood	Tarsocrural	**Synovia** **Serum**	4.9 44.9	0.1 n.d.	0.0 21.3	11.7 n.d.	1.3 0.8	0.3 0.3	110.7 16.2	275.7 21.7	404.3 43.9	2.0 0.3
6	F	2	Warm-blood	Tarsocrural	**Synovia** **Serum**	8.2 41.5	0.1 n.d.	0.0 10.2	5.0 n.d.	1.2 0.4	0.5 0.1	178.0 25.9	104.3 87.4	287.7 61.9	2.1 0.1
7	F	2	Warm-blood	Metacarpo-phalangeal	**Synovia** **Serum**	11.2 45.3	0.1 n.d.	0.0 12.7	6.4 n.d.	2.0 0.5	0.5 1.2	199.6 18.6	173.1 99.7	454.0 64.0	2.0 0.3
8	Cm	3	Warm-blood	Metacarpo-phalangeal	**Synovia**	7.3	0.1	0.9	3.8	1.0	0.4	156.7	132.1	423.8	1.9
9	F	2	Warm-blood	Metacarpo-phalangeal	**Synovia**	4.7	0.1	0.0	13.0	1.4	0.2	222.8	115.1	487.7	1.8
10	Ma	1	Warm-blood	Tarsocrural	**Synovia**	10.5	0.1	1.8	13.2	1.8	0.6	128.9	251.1	279.2	2.1

Table 13: Single data of the OCD group.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
11	Ma	1	Warm-blood	Metacarpo-phalangeal	**Synovia**	4.2	0.1	0.0	5.3	1.1	0.3	145.7	153.0	299.8	2.4
12	Ma	1	Warm-Blood	Tarsocrural	**Synovia**	11.4	0.1	0.7	6.2	0.6	0.4	104.8	152.9	410.2	2.4

Continuation of Table 13.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
1	F	8	Warm-blood	Metacarpo-phalangeal	**Synovia** **Serum**	33.9 45.5	53.9 n.d.	4.6 14.3	142.4 n.d.	11.9 0.1	4.4 0.3	126.3 76.5	1079.3 238.5	511.3 92.3	3.6 1.6
2	F	0.5	Warm-blood	Carpal	**Synovia** **Serum**	33.5 41.8	59.9 n.d.	5.3 15.2	n.d. n.d.	10.4 1.7	4.3 0.2	63.4 19.2	1580.5 268.6	521.1 172.4	3.5 1.6
3	F	3	Warm-blood	Metacarpo-phalangeal	**Synovia** **Serum**	34.4 40.7	82.6 n.d.	5.3 23.3	144.0 n.d.	11.9 0.5	3.3 0.2	130.3 28.6	1395.3 260.0	524.6 82.3	3.2 1.5
4	Cm	4	Warm-blood	Carpal	**Synovia** **Serum**	30.3 46.2	27.5 n.d.	6.8 9.4	n.d. n.d.	9.3 1.3	3.9 1.4	153.6 18.6	1572.5 157.2	507.9 132.5	2.4 1.2

Table 14: Single data of the septic arthritis group.

Horse	Sex	Age [years]	Breed	Joint	Sample	Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [ng/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CTXII [pg/ml]	CPII [ng/ml]	CS846 [µg/ml]
5	Cm	18	Warm-blood	Carpal	**Synovia** 3 week 4 week 8 week	30.4 40.8 42.9 21.9	23.0 126.0 4.0 0.1	11.0 8.9 3.5 4.2	n.d. n.d. n.d. n.d.	11.5 9.2 6.7 4.2	4.9 8.3 4.5 2.8	148.3 n.d. n.d. n.d.	1427.0 1397.6 1206.5 1201.7	554.2 523.5 511.2 642.8	3.3 3.3 3.2 3.5
6	F	1	Warm-blood	Tarsocrural	**Synovia** 0.5 week 1 week 2 week	33.5 36.8 39.5 35.6	22.2 57.0 81.0 39.0	10.2 11.8 5.6 7.0	135.2 n.d. n.d. n.d.	16.6 16.3 14.8 12.5	3.6 4.9 5.5 3.5	248.3 n.d. n.d. n.d.	1567.1 1402.6 1398.8 1309.7	554.2 533.7 530.2 522.9	3.3 3.2 3.1 3.1
7	F	1	Warm-blood	Tarsocrural	**Synovia** 0.5 week 5 week 8 week	32.9 36.8 41.8 17.4	13.1 25.1 5.6 0.1	10.2 3.3 0.8 1.6	117.4 n.d. n.d. n.d.	16.3 16.3 5.9 3.4	3.9 4.3 3.2 2.4	169.0 n.d. n.d. n.d.	1766.4 1599.3 1367.9 1134.8	402.1 377.6 441.8 589.9	2.2 2.1 2.2 2.4
8	F	n.d	n.d.	Tarsocrural	**Synovia** 4 week 6 week	34.0 35.1 21.8	42.8 0.4 1.0	7.2 9.7 2.3	138.6 n.d. n.d.	12.4 7.5 3.8	4.1 2.8 2.7	223.4 n.d. n.d.	1464.0 1397.9 1278.4	524.9 409.4 551.7	3.0 3.0 3.1

Continuation of Table 14.

Horse	AV values of MMP-2 and -9 in synovial fluid					
	45 kDa	72 kDa	88 kDa	92 kDa	120 kDa	250 kDa
1	0.00	0.81	0.00	0.16	0.00	0.00
2	0.00	0.78	0.00	0.08	0.44	0.00
3	0.00	0.73	0.00	0.00	0.16	0.00
4	0.00	0.82	0.00	0.00	0.16	0.00
5	0.00	0.79	0.00	0.00	0.33	0.00
6	0.00	0.72	0.00	0.00	0.26	0.00
7	0.00	0.63	0.00	0.00	0.38	0.00
8	0.00	0.57	0.00	0.00	0.36	0.00

Table 15: AV values of MMP-2 and -9 assessed by gelatin zymography in synovial fluids of the control group. Human MMP-2 was used as a reference.

Horse	AV values of MMP-2 and -9 in synovial fluid					
	45 kDa	72 kDa	88 kDa	92 kDa	120 kDa	250 kDa
1	0.00	1.34	0.00	0.12	0.18	0.00
2	0.00	1.27	0.00	0.10	0.27	0.00
3	0.00	1.12	0.00	0.00	0.30	0.00
4	0.00	1.33	0.00	0.00	0.45	0.00
5	0.00	1.26	0.00	0.00	0.53	0.00
6	0.00	1.27	0.00	0.00	0.44	0.00
7	0.00	1.28	0.00	0.00	0.15	0.00
8	0.00	1.21	0.00	0.00	0.37	0.00

Table 16: AV values of MMP-2 and -9 assessed by gelatin zymography in synovial fluids of the OA group. Human MMP-2 was used as a reference.

Horse	AV values of MMP-2 and -9 in synovial fluid					
	45 kDa	72 kDa	88 kDa	92 kDa	120 kDa	250 kDa
1	0.00	1.27	0.00	0.18	0.41	0.00
2	0.00	1.21	0.00	0.06	0.08	0.00
3	0.00	1.61	0.00	0.05	0.49	0.00
4	0.00	1.26	0.00	0.08	0.13	0.00
5	0.00	1.35	0.00	0.00	0.32	0.00
6	0.00	1.31	0.00	0.00	0.07	0.00
7	0.00	1.32	0.00	0.00	0.08	0.00
8	0.00	1.42	0.00	0.00	0.16	0.00

Table 17: AV values of MMP-2 and -9 assessed by gelatin zymography in synovial fluids of the chronic arthritis group. Human MMP-2 was used as a reference.

Horse	AV values of MMP-2 and -9 in synovial fluid					
	45 kDa	72 kDa	88 kDa	92 kDa	120 kDa	250 kDa
1	0.00	0.83	0.00	0.02	0.17	0.00
2	0.00	0.79	0.00	0.00	0.21	0.00
3	0.00	0.12	0.00	0.00	0.27	0.00
4	0.00	0.84	0.00	0.00	0.15	0.00
5	0.00	0.78	0.00	0.00	0.38	0.00
6	0.00	0.97	0.00	0.00	0.40	0.00
7	0.00	0.68	0.00	0.00	0.32	0.00
8	0.00	1.06	0.00	0.00	0.25	0.00

Table 18: AV values of MMP-2 and -9 assessed by gelatin zymography in synovial fluids of the OCD group. Human MMP-2 was used as a reference.

Horse	AV values of MMP-2 and -9 in synovial fluid					
	45 kDa	72 kDa	88 kDa	92 kDa	120 kDa	250 kDa
1	0.28	1.25	0.12	1.61	0.71	0.21
2	0.19	1.24	0.09	1.58	0.62	0.24
3	0.27	1.38	0.00	0.73	0.65	0.27
4	0.00	1.28	0.14	1.49	0.63	0.00
5	0.00	1.30	0.00	1.07	0.61	0.26
6	0.00	1.36	0.00	0.86	0.65	0.00

Table 19: AV values of MMP-2 and -9 assessed by gelatin zymography in synovial fluids of the septic arthritis group. Human MMP-2 was used as a reference.

Parameter	Diagnosis	Synovia					Serum				
		n	Median	IQR	Minimum	Maximum	n	Median	IQR	Minimum	Maximum
WBC count [10^6 cells/ml]	Control	17	0.1	0.1 – 0.1	0.1	0.5					
	OA	14	0.1	0.1 – 0.1	0.1	0.4					
	Chronic arthritis	22	0.1	0.1 – 0.2	0.1	1.8					
	OCD	9	0.1	0.1 – 0.1	0.1	0.1					
	Septic arthritis	8	35.2	22.4 – 58.4	13.1	82.6					
Protein concentration [mg/ml]	Control	20	9.6	5.4 – 11.8	4.2	18.1	11	42.4	40.1 - 45	38.9	47.3
	OA	28	10.2	5.9 – 12.8	3.4	18.5	11	44.2	41.8 – 46.2	37.5	48.5
	Chronic arthritis	30	9.6	7.3 – 12.4	4.3	19.6	11	42.9	40.2 – 44.7	36.8	48.6
	OCD	12	9.4	5.0 – 12.1	4.2	16.2	7	41.6	39.9 – 44.9	39.4	45.3
	Septic arthritis	8	33.5	30.3 – 34.4	30.3	34.4	4	43.7	41 - 46	40.7	46.2
MPO concentration [ng/ml]	Control	19	9.4	3.5 – 13.8	2.0	21.7					
	OA	21	9.8	5.3 – 17.3	2.1	84.2					
	Chronic arthritis	25	11.2	6.7 – 19.5	4.5	129.6					
	OCD	11	11.2	5.3 – 13.2	3.8	25.9					
	Septic arthritis	5	138.6	126.3 – 143.2	117.4	144.0					
MPO activity [U/ml]	Control	20	0.0	0.0 – 0.2	0.0	1.2	11	14.3	2.5 – 16.9	0.6	22.4
	OA	28	0.6	0.0 – 1.5	0.0	9.0	11	13.5	6.3 – 17.4	0.5	24.5
	Chronic arthritis	30	0.4	0.0 – 1.5	0.0	12.0	11	13.6	9.3 – 16.9	2.8	20.8
	OCD	12	0.5	0.0 – 1.6	0.0	8.1	7	14.2	10.2 – 21.3	3.5	26.2
	Septic arthritis	8	7.0	5.3 – 10.8	4.6	11.0	4	14.8	10.6 – 21.3	9.4	23.3
MMP-2 activity [ng/ml]	Control	20	1.2	0.9 – 1.5	0.8	3.1	11	0.7	0.5 – 1.1	0.4	2.6
	OA	28	3.1	2.7 – 3.5	1.6	5	11	0.8	0.5 – 1.0	0.2	1.3
	Chronic arthritis	30	3.1	2.6 – 3.5	1.7	4.1	11	0.9	0.6 – 1.6	0.3	3.4
	OCD	12	1.5	1.1 – 1.9	0.6	2.3	7	0.9	0.5 – 1.1	0.4	0.9
	Septic arthritis	8	11.9	10.6 – 15.5	9.3	16.6	4	0.9	0.2 – 1.6	0.1	1.7

Table 20: Statistics of indicated parameters in synovial fluid and serum samples of different diagnostic groups.

Parameter	Diagnosis	Synovia					Serum				
		n	Median	IQR	Minimum	Maximum	n	Median	IQR	Minimum	Maximum
MMP-9 activity [ng/ml]	Control	20	0.3	0.2 – 0.4	0.1	0.8	11	0.3	0.3 – 0.6	0.0	0.8
	OA	28	0.5	0.3 – 1.0	0.1	1.3	11	0.3	0.1 – 0.6	0.1	1.0
	Chronic arthritis	30	0.7	0.3 – 1.0	0.2	1.6	11	0.4	0.1 – 0.8	0.1	1.2
	OCD	12	0.5	0.3 – 0.6	0.2	0.8	7	0.2	0.1 – 0.7	0.0	1.2
	Septic arthritis	8	4	3.7 – 4.4	3.3	4.9	4	0.3	0.2 – 0.1	0.2	1.4
C2C concentration [ng/ml]	Control	20	114.1	101.8 – 124.1	74.2	185.4	11	19.7	12.6 – 25.8	10.7	35.5
	OA	28	139.6	112.4 – 198.5	63.6	436.4	11	10.0	7.7 – 18.8	2.6	24.7
	Chronic arthritis	30	132.1	113.0 – 209.7	59.1	388.6	11	20.0	15.4 – 35.8	12.0	50.8
	OCD	12	138.5	106.9 – 178.3	69.6	222.8	7	18.6	8.4 – 46.0	3.0	55.3
	Septic arthritis	8	151.0	127.3 – 209.8	63.4	248.3	4	23.9	18.8 – 64.5	18.6	76.5
CTXII concentration [pg/ml]	Control	20	131.0	124.4 – 153.1	71.3	196.3	11	29.0	6.8 – 32.0	1.2	35.9
	OA	28	341.5	288.6 – 428.5	177.7	676.3	11	54.0	20.6 – 123.6	12.7	140.5
	Chronic arthritis	30	233.3	214.4 – 274.7	29.2	345.8	11	39.3	31.8 – 45.9	10.8	112.0
	OCD	12	156.5	135.1 – 189.7	104.3	275.7	7	30.8	21.7 – 87.4	15.6	99.7
	Septic arthritis	8	1513.2	1403.2 – 1566.8	1079.3	1766.4	4	249.2	231.0 – 266.4	228.5	268.6
CPII concentration [ng/ml]	Control	20	330.1	308.8 – 345.2	266.2	428.2	11	57.2	50.2 – 79.3	49.1	83.8
	OA	28	194.1	163.6 – 225.8	123.2	290.1	11	51.3	44.9 – 69.8	19.8	80.2
	Chronic arthritis	30	241.8	210.0 – 270.1	160.5	289.9	11	44.4	31.3 – 54.7	11.8	81.1
	OCD	12	417.0	304.0 – 452.0	279.2	487.7	7	61.9	43.9 – 92.7	23.8	157.2
	Septic arthritis	8	522.9	508.7 – 546.9	402.0	554.2	4	112.4	84.8 – 162.5	82.3	172.44
CS846 concentration [µg/ml]	Control	20	1.7	1.6 – 1.7	1.3	2.0	11	0.2	0.1 – 0.2	0.1	0.6
	OA	28	1.0	0.8 – 1.3	0.3	1.4	11	0.2	0.1 – 0.4	0.1	0.5
	Chronic arthritis	30	1.1	1.0 – 1.3	0.9	1.5	11	0.1	0.1 – 0.7	0.0	0.8
	OCD	12	1.9	1.8 – 2.1	1.8	2.4	7	0.2	0.1 – 0.3	0.1	0.3
	Septic arthritis	8	3.2	2.6 – 3.5	2.2	3.6	4	1.5	1.3 – 1.6	1.2	1.6

Continuation of Table 20.

p value		Synovial fluid					Serum				
		Control	OA	Chronic arthritis	OCD	Septic arthritis	Control	OA	Chronic arthritis	OCD	Septic arthritis
WBC count	Control	-	1.000	1.000	1.000	<0.005					
	OA	1.000	-	<1.000	1.000	<0.001					
	Chronic arthritis	1.000	<1.000	-	<1.000	<0.010					
	OCD	1.000	1.000	<1.000	-	<0.005					
	Septic arthritis	<0.005	<0.001	<0.010	<0.005	-					
Protein concentration	Control	-	1.000	1.000	1.000	<0.001	-	1.000	1.000	1.000	1.000
	OA	1.000	-	1.000	1.000	<0.001	1.000	-	1.000	1.000	1.000
	Chronic arthritis	1.000	1.000	-	1.000	<0.001	1.000	1.000	1.000	1.000	1.000
	OCD	1.000	1.000	1.000	-	<0.001	1.000	1.000	1.000	-	1.000
	Septic arthritis	<0.001	<0.001	<0.001	<0.001	-	1.000	1.000	1.000	1.000	-
MPO concentration	Control	-	<1.000	<1.000	<1.000	<0.001					
	OA	<1.000	-	1.000	1.000	<0.010					
	Chronic arthritis	<1.000	1.000	-	1.000	<0.050					
	OCD	1.000	1.000	1.000	-	<0.010					
	Septic arthritis	<0.001	<0.010	<0.050	<0.010	-					
MPO activity	Control	-	1.000	<1.000	1.000	<0.001	-	1.000	1.000	1.000	1.000
	OA	1.000	-	1.000	1.000	<0.010	1.000	-	1.000	1.000	1.000
	Chronic arthritis	<1.000	1.000	-	1.000	<0.010	1.000	1.000	1.000	1.000	1.000
	OCD	1.000	1.000	1.000	-	<0.050	1.000	1.000	1.000	-	1.000
	Septic arthritis	<0.001	<0.010	<0.010	<0.050	-	1.000	1.000	1.000	1.000	-
MMP-2 activity	Control	-	<0.001	<0.001	1.000	<0.001	-	1.000	1.000	1.000	1.000
	OA	<0.001	-	1.000	<0.005	<0.010	1.000	-	1.000	1.000	1.000
	Chronic arthritis	<0.001	1.000	-	<0.001	<0.010	1.000	1.000	1.000	1.000	1.000
	OCD	1.000	<0.005	<0.001	-	<0.001	1.000	1.000	1.000	-	1.000
	Septic arthritis	<0.001	<0.010	<0.010	<0.001	-	1.000	1.000	1.000	1.000	-

Table 21: *p* values of diagnostic group comparison of medians for indicated parameters in synovial fluid and serum.

p value		Synovial fluid					Serum				
		Control	OA	Chronic arthritis	OCD	Septic arthritis	Control	OA	Chronic arthritis	OCD	Septic arthritis
MMP-9 activity	Control	-	<1.000	<1.000	<1.000	<0.001	-	1.000	1.000	1.000	1.000
	OA	<1.000	-	1.000	1.000	<0.005	1.000	-	1.000	1.000	1.000
	Chronic arthritis	<1.000	1.000	-	1.000	<0.005	1.000	1.000	1.000	1.000	1.000
	OCD	<1.000	1.000	1.000	-	<0.001	1.000	1.000	1.000	-	1.000
	Septic arthritis	<0.001	<0.005	<0.005	<0.001	-	1.000	1.000	1.000	1.000	-
C2C concentration	Control	-	<1.000	<1.000	<1.000	<1.000	-	<1.000	1.000	1.000	1.000
	OA	<1.000	-	1.000	1.000	1.000	<1.000	-	<1.000	<1.000	<1.000
	Chronic arthritis	<1.000	1.000	-	1.000	1.000	1.000	<1.000	-	1.000	1.000
	OCD	<1.000	1.000	1.000	-	1.000	<1.000	1.000	1.000	-	1.000
	Septic arthritis	<1.000	1.000	1.000	1.000	-	1.000	<1.000	1.000	1.000	-
CTXII concentration	Control	-	<0.001	<0.010	<1.000	<0.001	-	<1.000	<1.000	1.000	<0.005
	OA	<0.001	-	<1.000	<1.000	<0.010	<1.000	-	1.000	1.000	<1.000
	Chronic arthritis	<0.010	<1.000	-	<1.000	<0.001	<1.000	1.000	-	1.000	<1.000
	OCD	<1.000	<1.000	<1.000	-	<0.001	1.000	1.000	1.000	-	<1.000
	Septic arthritis	<0.001	<0.010	<0.001	<0.001	-	<0.005	<1.000	<1.000	<1.000	-
CPII concentration	Control	-	<0.001	<0.001	<1.000	<1.000	-	1.000	<1.000	1.000	<1.000
	OA	<0.001	-	<1.000	<0.001	<0.001	1.000	-	<1.000	1.000	<1.000
	Chronic arthritis	<0.001	<1.000	-	<0.001	<0.001	<1.000	<1.000	-	<1.000	<0.005
	OCD	<1.000	<0.001	<0.001	-	<1.000	1.000	1.000	<1.000	-	<1.000
	Septic arthritis	<1.000	<0.001	<0.001	<1.000	-	<1.000	<1.000	<0.005	<1.000	-
CS846 concentration	Control	-	<0.001	<0.001	<1.000	<1.000	-	1.000	1.000	1.000	<0.050
	OA	<0.001	-	<1.000	<0.001	<0.001	1.000	-	1.000	1.000	<0.050
	Chronic arthritis	<0.001	<1.000	-	<0.001	<0.001	1.000	1.000	-	1.000	<0.050
	OCD	<1.000	<0.001	<0.001	-	<1.000	1.000	1.000	1.000	-	<0.050
	Septic arthritis	<1.000	<0.001	<0.001	<1.000	-	<0.050	<0.050	<0.050	<0.050	-

Continuation of Table 21.

Spearman's coefficient of correlation (*p* value)		Protein [mg/ml]	WBC [10^6 cells/ml]	MPO [U/ml]	MPO [pg/ml]	MMP-2 [ng/ml]	MMP-9 [ng/ml]	C2C [ng/ml]	CPII [ng/ml]	CS846 [µg/ml]	CTXII [pg/ml]
Synovial fluid	Protein [mg/ml]	-	0.71 (<0.001)	0.27 (<0.010)	0.24 (<0.050)	0.36 (<0.001)	0.48 (<0.001)	0.03 (<1.000)	0.19 (<1.000)	0.18 (<1.000)	0.29 (<0.010)
	WBC [10^6 cells/ml]	0.71 (<0.001)	-	0.42 (>0.010)	0.38 (>0.010)	0.63 (<0.001)	0.76 (<0.001)	0.01 (1.000)	0.28 (<0.050)	0.32 (<0.010)	0.38 (<0.010)
	MPO [U/ml]	0.27 (<0.010)	0.42 (>0.010)	-	0.61 (<0.001)	0.43 (<0.001)	0.36 (<0.001)	0.08 (<1.000)	0.01 (<1.000)	0.03 (<1.000)	0.40 (<0.001)
	MPO [ng/ml]	0.24 (<0.050)	0.38 (>0.010)	0.61 (<0.001)	-	0.36 (<0.001)	0.24 (<0.050)	0.13 (<1.000)	0.04 (<1.000)	-0.02 (<1.000)	0.25 (<0.050)
	MMP-2 [ng/ml]	0.36 (<0.001)	0.63 (<0.001)	0.43 (<0.001)	0.36 (<0.001)	-	0.64 (<0.001)	0.18 (<1.000)	-0.22 (<0.050)	-0.23 (<0.050)	0.65 (<0.001)
	MMP-9 [ng/ml]	0.48 (<0.001)	0.76 (<0.001)	0.24 (<0.050)	0.24 (<0.050)	0.64 (<0.001)	-	0.14 (<1.000)	0.02 (<1.000)	0.08 (<1.000)	0.46 (<0.001)
	C2C [ng/ml]	0.03 (<1.000)	0.01 (1.000)	0.08 (<1.000)	0.13 (<1.000)	0.18 (<1.000)	0.14 (<1.000)	-	-0.02 (<1.000)	-0.18 (<1.000)	0.14 (<1.000)
	CPII [ng/ml]	0.19 (<1.000)	0.28 (<0.050)	0.01 (<1.000)	0.04 (<1.000)	-0.22 (<0.050)	0.02 (<1.000)	-0.02 (<1.000)	-	0.77 (<0.001)	-0.25 (<0.050)
	CS846 [µg/ml]	0.18 (<1.000)	0.32 (<0.010)	0.03 (<1.000)	-0.02 (<1.000)	-0.23 (<0.050)	0.08 (<1.000)	-0.18 (<1.000)	0.77 (<0.001)	-	-0.22 (<0.050)
	CTXII [pg/ml]	0.29 (<0.010)	0.38 (<0.010)	0.40 (<0.001)	0.25 (<0.050)	0.65 (<0.001)	0.46 (<0.001)	0.14 (<1.000)	-0.25 (<0.050)	-0.22 (<0.050)	-

Table 22: Spearman's coefficient of correlation and *p* values for indicated parameters in synovial fluid.