Synovial fluid as a mirror of equine joint (patho) physiology

Robin van den Boom

Utrecht - 2004

Cover: This picture was taken in the ‘poble Espagnol’ (Spanish village), Barcelona.

Synovial fluid as a mirror of equine joint (patho) physiology

synoviale vloeistof als spiegel van de (patho) fysiologie van het paardengewricht

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter I

General introduction

Introduction

Lameness is the most common reason for the (early) retirement of horses (Olivier *et al* 1997, Rossdale *et al* 1985, Todhunter and Lust 1990) and joint disease is the most frequent cause of lameness (Todhunter 1992). This indicates the importance of joint disease in the equine industry (Rossdale *et al* 1985). Currently, joint disease is usually diagnosed with the use of radiography but radiographic signs correlate poorly with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996) and extensive damage to the joint is present by the time changes become radiographically visible. Given the very limited repair capability of damaged articular cartilage, most of this damage is likely to be permanent. This has led to an intensive and ongoing search for markers of joint disease, which would enable earlier detection of joint disorders, when it might be possible to prevent further, permanent damage. To better understand joint disease the pathophysiological processes occurring within the joint itself need to be identified and synovial fluid will best reflect these. It is in direct contact with both the articular cartilage surface and the synovial membrane and its composition, to a certain extent, reflects joint metabolism. This means that synovial fluid markers potentially form powerful diagnostic tools.
In this introductory chapter several aspects of joint structure, physiology and pathology are discussed and a number of potential synovial fluid markers are introduced in order to provide the reader with a sound basis for the appreciation of the experimental work that is reported in the following chapters and of which a brief outline is given at the end of this introduction.

Joint structure and physiology

Synovial or diarthrodial joints are formed by the extremities of two (or more) bones, the articulating surfaces of which are covered by a thin layer of hyaline cartilage (Fig 1). The joint space contains synovial fluid (SF) and is enclosed by the fibrous joint capsule, lined on the inside by the synovial membrane. The congruency of the opposing joint surfaces (which is maintained by fibrous intra- and extracapsular ligaments), the hyaline cartilage and synovial fluid allow smooth, almost frictionless motion in one or multiple planes, depending on the architecture and function of the particular joint. Apart from allowing the articulation of two bones relative to each other, joints also absorb and dissipate loads applied to the limb. These loads can be quite extreme, especially given the weight of adult horses and the high speeds they are capable of reaching (Palmer and Bertone 1996), and have been estimated at 11.6 N/kg in the front limbs of trotting horses (Merkens *et al* 1993), 10,500 N in the galloping horse and 12,000 N on landing after jumping an 80 cm fence (Kingsbury *et al* 1978, Quddus *et al* 1978).

- *cartilage*

Articular cartilage is a highly specialised tissue consisting of a limited number of chondrocytes (<5%) imbedded in an extensive extracellular matrix (ECM). The chondrocytes are responsible for maintaining the ECM in which they lie and which is made up main-

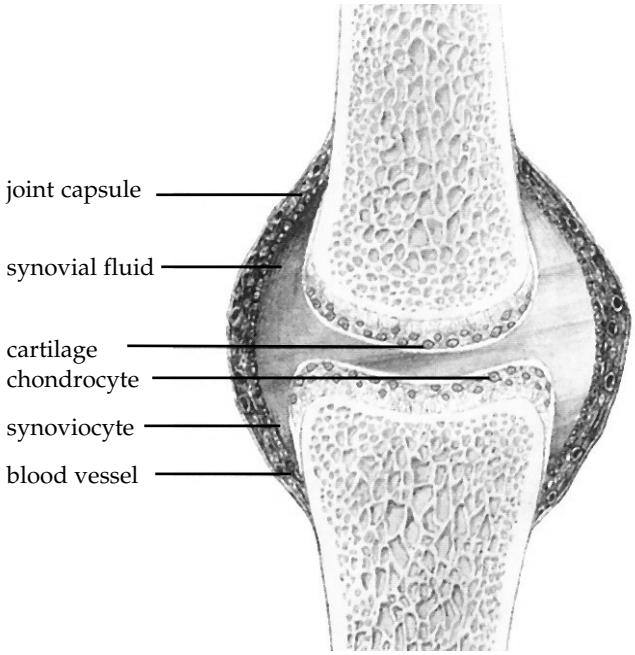

***Figure 1**: schematic representation of the structure of a synovial joint.*

ly of collagen, proteoglycans and water. On a dry weight basis collagen forms 50-80% of the cartilage matrix (Brama *et al* 2000b, Johnson *et al* 1980, Todhunter 1996, Vachon *et al* 1990). Most of the collagen in articular cartilage is type II collagen, with small amounts of type VI, IX, XI, XII and XIV. The functions of these other collagens are not completely understood but they are thought to be involved in stabilisation of the collagen fibres and regulation of fibril diameter (Todhunter 1996). Type II collagen is a fibrillar collagen and, like all fibrillar collagens, it is made up of multiple triple helices, each of which consists of three α chains. The amino acid sequence of the α chains is glycine-X-Y, where X and Y are often proline or hydroxyproline, and this pattern is characteristic of these collagens (Todhunter 1996). The small size of the glycine molecule at every third position allows tight turning of the α helix and hence the formation of collagen fibrils. The α chains of procollagen are formed on the rough endoplasmic reticulum of the chondrocyte and these are combined to form a triple helix in the Golgi apparatus. Following secretion into the extracellular space, the amino- and carboxy-terminal globular domains are removed enzymatically and the collagen molecules are arranged into fibrils. Crosslinks are formed between the fibrils, adding strength to the construction. The collagen fibres are arranged according to a specific architecture, crucial to the mechanical properties of articular cartilage. This pattern consists of 'arches' of collagen fibres, where the orientation is perpen-

dicular to the articular surface in the deep layers and parallel to it in superficial layers (Todhunter 1996).
Proteoglycans are less abundant in the ECM, representing 7.3-10% of the dry weight of the cartilage ECM (Vachon *et al* 1990). The majority (on a mass basis) of the proteoglycans are arranged in large aggregates known as aggrecan. The aggrecan monomer consists of a core protein attached to which are many glycosaminoglycan (GAG) side chains, mainly chondroitin sulphate (CS) and smaller amounts of keratan sulphate (KS) (Todhunter 1996). The protein core is formed in the rough endoplasmic reticulum of chondrocytes and CS and KS are attached in the Golgi apparatus. Most of the KS is attached to the core protein near the link protein, while the CS-rich domain is present nearer the carboxy-terminal end. After secretion aggregation occurs, with up to 100 monomers attached to a single hyaluronan (also known as hyaluronic acid, HA) chain by link proteins. In turn, hyaluronic acid molecules are connected to the collagen fibrils, creating a stable network. Apart from aggrecan, cartilage also contains several small proteoglycans including biglycan, decorin, fibromodulin, cartilage matrix protein, cartilage oligomeric protein, anchorin and chondrocalcin. As is the case for the minor collagens the functions of these less abundant proteoglycans is largely unknown, although some are presumed to play a structural role in the attachment of the chondrocyte to the surrounding extracellular matrix and/or the linking of the proteoglycans to the collagen network (Todhunter 1996). Therefore, some of these substances may be interesting potential markers of tissue integrity once more is known about their biological function.
Because they are negatively charged the proteoglycans attract water, causing a swelling pressure within the cartilage, which is counterbalanced by the collagen network. This biochemical composition of a collagen fibril network interspersed with strongly hydrophilic proteoglycans ensures the unique biomechanical properties of articular cartilage, combining compressive stiffness and resilience. These biomechanical characteristics allow articular cartilage to withstand the forces acting upon it during locomotion.

- synovial fluid

The synovial fluid (SF), which fills the joint space, and plays a role in lubrication, is an ultrafiltrate of plasma, with hyaluronan added to it. Because adult articular cartilage contains no blood vessels it depends to a large degree on SF for its nutrition and the removal of metabolites. The circulation of SF through the joint is driven by joint motion and the passage of water in and out of the cartilage matrix as a result of loading. As it is in direct contact with the articular cartilage, changes in cartilage composition are likely to be reflected in the composition of SF. In addition, SF contains mediators which influence cartilage metabolism, such as cytokines, eicosanoids, free radicals and hormones. Normally, SF contains few leucocytes (Todhunter 1996, Van Pelt 1974), but in the event of joint inflammation there is a rapid influx of white blood cells, and they and their products play an important role in that inflammatory process. This suggests that SF can provide useful information on processes occurring within the joint and the state of the articular cartilage. Because of the large size of the horse, with proportionally sized joints, SF is readily obtained from almost all joints.

- the synovial membrane

The synovial membrane is a thin layer, just several cell layers thick, which covers the connective tissue of the joint capsule, and lines the joint space. Because it is so thin, has no basement membrane and blood vessels pass within 5 to 10 µm of the surface there is an easy passage of many molecules from the circulation to the joint space, and vice versa. The synovial membrane is made up of two basic types of cells or synoviocytes: type A (macrophage-like) whose main function is phagocytosis and pinocytosis, and type B (fibroblastic), which primarily serve a secretory role (Barland *et al* 1962). A third type (C) of synoviocyte demonstrates characteristics intermediate between types A and B and it is thought that transformation of one cell-type to another is possible depending on biological demand (Krey *et al* 1971, Norton *et al* 1968, Roy *et al* 1966). The synoviocytes produce and secrete hyaluronan, as well as a whole array of macromolecules which mediate inflammation and the synthesis and degradation of cartilage components, such as cytokines, eicosanoids and proteases (Martel-Pelletier 2004, Spiers *et al* 1994a, Todhunter and Lust 1990). The synovial membrane, therefore, is far more than just the layer that encloses the joint and it is becoming increasingly obvious that it plays a critical role in joint homeostasis and pathology.

- growth, maturation and turnover

Cartilage is renowned for its poor regenerative capabilities and when damaged, little or no repair occurs and any new tissue formed is of inferior quality. This is especially true of adult cartilage and is largely a result of the extremely long turnover time of collagen, which has been estimated at more than 100 years in dogs and humans (Maroudas 1980, Maroudas et al 1992, Verzijl et al 2000). Compared to collagen, proteoglycans are replaced very quickly, with a half-life in articular cartilage of only 11-40 days (Jikko et al 1998). For this reason it is thought that damage to the collagen network represents the irreversible step during cartilage degradation (Martel-Pelletier 2004). The above figures apply to adult individuals in which turnover of the cartilage occurs at very low levels while in young, growing animals metabolism takes place at a much higher rate and considerable alterations in cartilage composition can occur (Lohmander et al 1992, Murphy and Knäuper 1997). The higher metabolic rate in foals enables the development of site differences in cartilage composition, which are absent at birth but have developed before reaching maturity (Brama et al 2000b). These differences are considered to be an adaptation to the different biomechanical demands placed on various areas of the joint during exercise (Brama et al 2000b). Once maturity has been reached cartilage metabolism slows dramatically and there is little or no change in equine cartilage composition (Brama et al 1999b, MacDonald et al 2002). It is important to be aware of these developmental effects, so as not to confuse them with changes which are the result of joint pathology. It also emphasises the importance of age-matched controls when investigating changes in cartilage or markers of disease. The higher metabolic rate in foals and young horses also implies that it may still be possible to influence cartilage composition in young animals, whereas once maturity is reached very little change is possible (especially in collagen).

Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease involving inflammation of the synovial membrane, i.e. synovitis, and degradation of articular cartilage. Periods of active inflammation are followed by periods of relative calm, resulting in varying presence of clinical signs: synovial effusion and lameness. Active synovitis leads to the release of factors mediating cartilage degradation and causes fibrosis of the synovial membrane and joint capsule, and advanced OA is paired with severe, irreversible cartilage damage and decreased joint mobility. The incidence of OA is high, both in man and in horses, and where it is almost exclusively a disease of the elderly in humans, young horses are also frequently afflicted. Despite extensive research the etiology of OA is still incompletely understood, although it is certainly multifactorial in origin, with mechanical, biochemical and genetic factors all being involved (Martel-Pelletier 2004). Not all joints are at an equal risk for the development of OA and in horses the metacarpophalangeal and carpal joints are most commonly affected (Pool 1996, Rossdale *et al* 1985). This would seem to indicate the importance of biomechanical factors, as a major difference between various joints is the pattern of loading to which they are subjected. The fact that OA does not develop symmetrically across an entire joint surface also suggests mechanical factors play an important role, as does the fact that the site at which cartilage degradation starts is specific for a particular joint. For example, in the equine metacarpophalangeal joint changes to the articular cartilage have been shown to develop more frequently and to be more severe at the dorsal edge of the proximal phalanx (Brommer *et al* 2003b, Cantley *et al* 1999).

Exercise is a source of mechanical (over)load on joints and has been shown to influence the development of OA. Strenuous running in rats led to the development of OA, but the running load must be extremely high for this to happen (Pap *et al* 1998), while moderate exercise seems to protect the articular cartilage (Kiviranta *et al* 1992).

In young, growing animals cartilage metabolism proceeds at a higher rate than in adults and results in net growth, while in healthy adult joints anabolism and catabolism are in balance, although small changes in cartilage composition are possible under the influence, for example, of exercise. In OA this balance is disturbed and degradation exceeds synthesis, resulting in cartilage breakdown. The first changes observed in OA are a loss of proteoglycans and swelling of the cartilage (Heinegard *et al* 1987, Westacott and Sharif 1996), which is thought to be caused by damage to the collagen network (Poole 1999). The loss of proteoglycans is probably reversible as they can be replaced (fairly quickly), but once the collagen network is disrupted the process is considered to be irreversible (Elliott and Cawston 2001, Martel-Pelletier 2004). In the early stage of cartilage degradation proteoglycan synthesis is increased (Uesaka *et al* 2002) in an attempt at repair, although not all newly formed proteoglycans are retained within the ECM.

Changes also occur in the synovial membrane, resulting in fibrosis, which is assumed to be the cause of restricted range of motion of affected joints (Pool 1996). In the later stages of OA, when cartilage alterations are well advanced (and largely irreversible) bony changes occur with the formation of osteophytes at the joint margins. These are thought to be formed in an attempt to stabilise the joint. The exact cause of lameness associated with OA is unclear but is thought to be due to pain associated with the synovial mem-

brane/joint capsule and subchondral bone and later with restriction of movement due to fibrosis (Simmons *et al* 1999b).
Although lameness is often observed in the early stages, other clinical signs, such as osteophyte formation and capsule fibrosis, appear late in the course of the OA process (Heinegard *et al* 1987). In the early stages of disease radiology is not useful for the detection of OA, as cartilage cannot be visualised directly using this technique and neither can the subtle changes in the subchondral bone plate that some think precede cartilage damage (Radin 1999). Later, as changes become more extensive they are detectable radiographically, but even then the changes seen on X-rays correlate poorly with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996). New imaging modalities, such as magnetic resonance imaging (MRI), are now becoming available in equine practice and these may reveal changes in articular cartilage architecture at an earlier stage. However, limited experience is currently available and the resolution of MRI equipment currently used in the clinical setting is not (yet) sufficient to detect wear and focal defects of articular cartilage (Yamada *et al* 1999).
This discrepancy between radiologic and clinical signs means that OA is often diagnosed in an advanced stage, at which time the joint is irreversibly damaged. This has led to an ongoing search for biological markers, enabling earlier detection OA, at which time further damage may be prevented by the implementation of appropriate therapeutic and management strategies.

Potential biomarkers

As described above, SF is in close contact with articular cartilage and changes in cartilage composition are likely to be reflected in SF. Also, inflammatory mediators formed by synoviocytes, chondrocytes or leukocytes are released into the joint space and potential markers present in SF can be divided into markers of inflammation and markers of degradation. Because SF can be readily obtained from equine joints, certainly the ones most commonly affected by disease, synovial markers are promising tools in the early diagnosis of these conditions. An additional advantage over serum markers is that SF markers provide information regarding specific joints. A disadvantage of synovial fluid markers is that arthrocentesis must be performed, which brings with it a (small) risk of introducing infection, is technically more difficult than collection of blood samples and may in itself affect joint homeostasis.

- inflammatory markers

Inflammation is a component of many joint diseases and cytokines orchestrate the inflammatory response, which means that their concentration is likely to be elevated in diseased joints. Two of the most widely studied pro-inflammatory cytokines are interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) and elevated levels of both have been found in cases of arthritides in horses (Hawkins *et al* 1993, Morris *et al* 1990, Todhunter *et al* 1996). Both of these cytokines have been shown to regulate many other substances, which play a role in joint disease, although TNF-α is less potent in many

respects than IL-1. They stimulate nitric oxide synthase (NOS), the production of matrix metalloproteinases (Scher *et al* 1996, Spiers *et al* 1994a) and prostaglandin E_2 (Hawkins *et al* 1993), inhibit proteoglycan synthesis and stimulate proteoglycan degradation, although higher concentrations are required to cause degradation (Bird *et al* 2000a). Because of its regulatory role in degradative processes TNF-α is considered a good predictor of joint disease (Bertone *et al* 2001).

Prostaglandins are members of the eicosanoid family and are released by synoviocytes and chondrocytes in response to cytokines or biomechanical stimuli. Cultured articular cells produced prostaglandin E_2 (PGE_2) upon stimulation with lipopolysaccharide (LPS) (Frean and Lees 2000) and IL-1 (May *et al* 1992). The prostaglandins, most notably PGE_2, are thought to play a role in the pain response in joint disease (Kirker-Head *et al* 2000) and they have been found in SF from a variety of arthritides (Chevalier 1997, Gibson *et al* 1996, von Rechenberg *et al* 2000). Prostaglandin E_2 is considered a sensitive indicator of joint disease in general, while prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) reflects chronic joint disease (Bertone *et al* 2001). The fact that prostaglandin concentrations were increased in all lame horses in a study conducted by May *et al* (1994) seems to confirm their role in pain perception. In the equine joint PGE_2 seems to be produced primarily by synoviocytes and therefore is thought to reflect synovitis (von Rechenberg *et al* 2000).

Nitric oxide (NO) is a molecule which has received a great deal of attention lately and which plays a role in many biological processes, often acting as a messenger molecule. Nitric oxide is produced by nitric oxide synthases (NOS) which exist in 2 forms: a constitutive form (cNOS) produces small amounts of NO almost continually while inducible forms (iNOS) liberate large amounts of NO when stimulated (MacDonald *et al* 1994, Scher *et al* 1996, Takafuji *et al* 2002, Tung *et al* 2002c). Recent studies have shown that NO influences articular cartilage metabolism, although its precise role in joint disease is still unclear at the present time. For example, it is considered to mediate the IL-1 induced inhibition of proteoglycan synthesis (Bird *et al* 2000a), plays a role in chondrocyte apoptosis (Kim *et al* 2003) and has been shown to activate MMPs (Murrell *et al* 1995). However, NOS inhibition led to the development of OA in one study (Fenton *et al* 2000) and in others NO slowed IL-1-induced proteoglycan degradation (Stefanovic-Racic *et al* 1994, 1995). In the equine joint chondrocytes are considered to be the major source of NO and elevated concentrations have been found in equine joint disease (von Rechenberg *et al* 2000).

Many of the effects of cytokines on cartilage are the result of protease activity and the matrix metalloproteinases (MMPs) are a group of enzymes involved in the physiological turnover of articular cartilage (Bluteau *et al* 2001, Chubinskaya *et al* 1999) but also in its pathological degradation (Hembry *et al* 1995, Lohmander *et al* 1994, Manicourt *et al* 1994). Cartilage destruction is thought to result from an imbalance between MMPs and their inhibitors (Lohmander *et al* 1993), which are various types of tissue inhibitors of metalloproteinases (TIMPs) and α-2-macroglobulin (α-2-M). The MMPs can be divided into four categories: collagenases, gelatinases, stromelysins and membrane bound MMPs

(Murphy *et al* 1990) and of these the collagenases break down the collagen triple helix and gelatinases act on unwound collagen molecules. Stromelysins degrade proteoglycans and, together with the membrane bound MMPs, play an important role in the activation of other MMPs. The MMPs are secreted as inactive zymogens (pro-MMPs), activated by enzymatic cleavage and most MMPs (> 95%) are present in an inactive state. Matrix metalloproteinases are regulated at several levels: gene expression, extracellular activation and by the formation of complexes with their inhibitors (Murphy *et al* 1990). Matrix metalloproteinases are also involved in equine joints, with general MMP and stromelysin activity being elevated in SF from foals and several classes of MMPs elevated in various types of joint disease, including OA (Brama *et al* 1998, 2000d, Clegg *et al* 1997a, 1997b, 1998b, Jouglin *et al* 2000).

- degradation markers

Finally, parts of the degraded cartilage matrix could function as biomarkers. As the extracellular matrix (ECM) of the articular cartilage is broken down its components are released into SF, reflecting cartilage destruction within the joint. In this way the glycosaminoglycan (GAG) concentration in SF may provide information on the proteoglycan content of articular cartilage. As the proteglycans in cartilage are broken down GAGs are released into SF. Previous studies have suggested that synovial GAG concentrations may be useful as predictors of joint disease (Heinegard *et al* 1987, Palmer *et al* 1995, Saxne *et al* 1985) and work by Chevalier (1997) even suggests that GAG concentrations reflect gross cartilage lesions. Myers (1999) however stated that this is not the case. Similarly, hydroxyproline, which is often used to estimate the amount of collagen in a certain tissue, may predict damage to the collagen network. In contrast to GAG levels little is known about hydroxyproline concentrations in SF from healthy and diseased equine joints (Maldonado *et al* 1983).

Factors influencing the level of potential biomarkers

- maturation and aging

As discussed above increased metabolism in articular cartilage is a feature of both normal turnover and disease, and a number of synovial fluid markers will be influenced by both. This emphasises again how important it is to be aware of the changes that occur in the processes of maturation and aging, in the absence of disease - so that the latter is not confused with the former. It also demonstrates the importance of using age-matched controls in biomarker research. Matrix metalloproteinase activity in SF has been shown to decrease dramatically with the development of the horse from foal to adult (Brama et al 1998, 2000d) and collagen and GAG content in cartilage have also been shown to be affected by age (Brama et al 1999b, 2000b, 2001, 2002). In adult horses however very little collagen metabolism is thought to occur and the composition of articular cartilage has been shown to be stable with respect to proteoglycan and collagen content (Brama et al 1999b, MacDonald et al 2002).

- *exercise*

Apart from maturation and aging a number of other factors may also influence marker levels in synovial fluid. Horses are kept almost exclusively for their athletic ability and the exercise they perform affects joint homeostasis. This is most obvious in neonates and growing horses, where exercise leads to the formation of site differences in cartilage composition (Brama *et al* 1999a, 2000b, 2002), but is also likely to play a role in mature horses. Exercise has been demonstrated to protect against proteoglycan loss in ponies (van den Hoogen *et al* 1998a) and leads to increased proteoglycan synthesis in equine joints (Bird *et al* 2000b, van de Lest *et al* 2000, van den Hoogen *et al* 1998a). In human athletes exercise caused a rising trend in SF levels of GAGs, stromelysin and TIMP (Roos *et al* 1995).

- *arthrocentesis*

Another factor that could alter marker levels in SF is the act of arthrocentesis. Although obviously necessary for the collection of SF, arthrocentesis is also performed in the course of lameness examinations (anaesthesia) and for the administration of intra-articular medication in diseased joints. It is possible that (repeated) arthrocentesis in itself may cause changes in marker concentrations. This has not been investigated specifically, although Billinghurst *et al* (1995) found that serial arthrocentesis did not have a significant effect on SF total protein, white blood cell count or TNF-α levels in the horse. Similarly, Lohmander *et al* (1998) reported that weekly aspiration of joint fluid (and lavage) did not present a confounding factor in longitudinal human studies investigating aggrecan fragments, MMP-1 and -3 and TIMP-1 levels in patients with knee pain and cartilage abnormalities.

Relationship between markers and disease

Apart from the recognition or exclusion of confounding factors in the interpretation of synovial biomarker concentrations the relationship with disease is crucial. One of the reasons for looking at the usefulness of biomarkers in the first place was the lack of such a correlation between radiology and clinical signs and severity of disease (Kidd *et al* 2001, Trotter and McIlwraith 1996). If biomarkers in SF are to be a valuable addition to our diagnostic repertoire they should accurately reflect the seriousness of the joint disease and/or the composition of the articular cartilage. When considering cartilage composition it is crucial to realise that this is not uniform across the entire joint surface. In the equine metacarpophalangeal joint for example collagen content is higher at the dorsal margin while GAGs are more abundant centrally (Brama *et al* 1999b, 2000c). Similar findings apply to the middle carpal joint (Murray *et al* 2001). The distribution of pathologically altered tissue is also not uniform across the joint surface and areas of diseased and normal cartilage can be present in one joint (Maroudas *et al* 1973). Therefore, in order to be able to relate marker levels in SF to the state of the articular cartilage, the damage to cartilage must be quantified in some way, taking into account these regional disparities.

For a number of our studies we used the cartilage degeneration index (CDI) developed by Brommer *et al* (2003a), which allows assessment of either the entire proximal joint surface of the proximal phalanx or specific regions thereof.

Aim and scope of the study

The aim of this thesis was to determine the value of a number of putative synovial fluid markers of joint disease in the horse, viewed in the context of physiological and external factors. Attention was focussed on the metacarpophalangeal joint, although in some cases other joints were also used to assess inter-articular differences. With the development and progression of joint disease (most notably osteoarthritis) inflammatory mediators, catabolic enzymes and breakdown products of articular cartilage are released into SF and all have been promoted as biomarkers. If they are to be reliably used as such it is vital to know if, and to what extent, they are influenced by factors other than joint disease. As pointed out above, among the most important factors that are not influenced by the disease itself are age (including stage of development), exercise and (repeated) arthrocentesis. This last factor is unique to SF markers and is particularly relevant as arthrocentesis of diseased joints is commonly performed to collect SF for analysis, perform local anaesthesia (in the course of a lameness examination) or for intra-articular medication. In the first part of this thesis (chapters II-V) the influence of the aforementioned factors on several synovial fluid markers is investigated. These potential markers included both inflammatory mediators and products of articular cartilage metabolism.
Once the influence of environmental factors has been established, the logical next step is to link marker levels to the actual disease status of the joint as the value of synovial markers is further dependent on how accurately they reflect joint pathology and the condition of the articular cartilage. So, next and partially overlapping, the relationship between a number of markers; the presence of disease and the severity of joint pathology was assessed (chapters V- VII). As a measure of joint pathology either the CDI, which can be seen as a reflection of accumulated cartilage damage, was used (chapters V-VI), or, in the case of infectious arthritis, the clinically widely used white blood cell count (chapter VII). Finally, a brief synopsis of the main results of the entire study is given (chapter VIII) in which the pitfalls and possibilities associated with the use of synovial fluid markers are reviewed and in which a likely scenario for future developments in this area is outlined.

Chapter II

The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor α activities in normal equine joints

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Summary

Reasons for performing the study: Matrix metalloproteinases (MMPs) and tumour necrosis factor α (TNF-α) may be useful as biomarkers of joint disease or inflammation. However, activity of both MMPs and TNF-α in synovial fluid (SF) may be influenced by non-pathological factors such as arthrocentesis or exercise.

Objective: To investigate the influence of repeated arthrocentesis and exercise on MMP and TNF-α activities in SF from normal equine joints.

Methods: SF was collected from the left metacarpophalangeal, radiocarpal and tarsocrural joints of 16 horses. Eight of these horses were subsequently subjected to an exercise programme on a treadmill and 8 were box-rested as controls. Arthrocentesis was repeated 14, 14.5, 17 and 24 days after the start of the exercise programme. General MMP and TNF-α activities were determined in SF.

Results: Repeated arthrocentesis caused a gradual increase but the exercise regimen no significant increase in MMP activity. There was a significant increase in TNF-α activity in SF collected from horses 2 h after cessation of the exercise programme.

Potential relevance: When using MMPs as biomarkers for joint disease, at least 14 days should elapse after previous arthrocentesis before subsequent SF collection. Moderate exercise does not increase MMP activity in SF from normal joints and it may be possible to ignore this as a source of error in evaluating MMP activity in diseased joints.

Introduction

Lameness is the most important cause of loss of use for horses (Olivier *et al* 1997, Rossdale *et al* 1985, Todhunter and Lust 1990) and joint disease is the most important cause of lameness (Todhunter 1992). This explains why osteoarthritis (OA), which in horses most commonly affects metacarpophalangeal and carpal joints, is one of the most economically important diseases in athletic and pleasure horses (Frisbie and McIlwraith 2000).
The radiological changes accompanying OA are visible only in advanced stages of disease and, furthermore, they are poorly correlated with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996). Marked articular cartilage degeneration can be present despite normal radiographic appearance of the joint (Lohmander *et al* 1992). This has led to a search for biomarkers to detect those early changes characteristic of joint disease.
Matrix metalloproteinases (MMPs) are a group of enzymes involved in the pathological degradation of articular cartilage and their presence in normal mature cartilage suggests they also play a role in homeostasis (Bluteau *et al* 2001, Chubinskaya *et al* 1999). The MMPs can be divided into 4 groups: 1) stromelysins (MMP-3, -10 and -11), degrading a wide range of substrates; 2) collagenases (MMP-1, -8 and -13), which cleave interstitial collagen triple helices; 3) gelatinases (MMP-2 and -9), digesting unwound collagen and gelatin; and 4) membrane-type MMPs (MMP-14, -15, -16 and -17) (Murphy *et al* 1990). MMPs are secreted as inactive zymogens (pro-MMPs) which are activated by enzymatic cleavage. Their effect depends on the ratio of active enzyme levels (which probably constitute less than 5% of total MMP concentrations) and tissue inhibitor of metalloproteinase (TIMP) levels.
The MMPs are considered to play a key role in the development of osteoarthritis (Hembry *et al* 1995, Lohmander *et al* 1994, Manicourt *et al* 1994). Synovial fluid concentrations of stromelysin (MMP-3) have been shown to be increased in the knees of human OA patients and in a dog model of OA (Lohmander *et al* 1993, Panula *et al* 1998) while collagenase levels in SF are also elevated in joint disorders, including OA in human knees (Lohmander *et al* 1993). In the equine metacarpophalangeal joint, MMP activity was increased in the presence of OA (Brama *et al* 1998). Finally, the gelatinases (MMP-2 and -9) are increased in synovial fluid from diseased equine joints (Clegg *et al* 1997b). Synovial fluid can be readily obtained in horses, and MMP activity in synovial fluid could therefore be a clinically useful marker for OA. It is possible, however, that factors other than joint disease affect the activity of MMPs in SF. Therefore, if they are to be used reliably as biomarkers, it is important to know how synovial fluid MMP activity is influenced by repeated arthrocentesis and exercise in the absence of disease. These are important external influences that OA-suspect horses are subjected to and that presumably might influence MMP activity. To our knowledge, there have been no studies to determine the effect of exercise and repeated arthrocentesis on equine synovial fluid MMP activity. There may also be variations in response based on the joint sampled, as the concentrations of a number of potential biomarkers have been shown to vary by joint sampled. For example, in one study, mean GAG, COMP and total protein levels were higher in the distal interphalangeal joint and navicular bursa than in the metacarpophalangeal joint, while MMP-2 activity was similar in all joints (Viitanen *et al* 2000).

Tumour necrosis factor α (TNF-α) is a key proinflammatory cytokine in arthritis (Haapala *et al* 2001) which is known to increase MMP synthesis (Richardson and Dodge 2000). Synovial fluid levels of TNF-α are increased in horse arthritides including acute joint disease and osteochondritis dissecans (Trumble *et al* 2001), and it is considered to be a good predictor of joint disease (Bertone *et al* 2001). Tumour necrosis factor α is formed as a 26-kDa, membrane-bound precursor which, to be released, must be specifically cleaved to its 19-kDa form by a TNF-α converting enzyme (Conway *et al* 2001). In horses with mild degenerative joint disease (DJD), racing leads to increased TNF-α levels in SF, peaking at 12 h post racing; while, in the same study, serial arthrocentesis had no effect on SF TNF levels (Billinghurst *et al* 1995). Intra-articular endotoxin administration causes TNF levels to peak after 2 h and values had returned to baseline levels by 8 h post injection (Hawkins *et al* 1995), demonstrating that TNF levels increase very rapidly. The present study was designed to test the hypothesis that exercise, repeated arthrocentesis and joint of origin do not influence MMP activity in synovial fluid and hence could be neglected as sources of error when investigating various MMPs as potential molecular markers. Therefore, overall MMP activity was assessed in synovial fluid obtained from 3 different normal joints (metacarpophalangeal, radiocarpal and tarsocrural) after repeated arthrocentesis and exercise. In addition, in order to determine whether the exercise regime or repeated arthrocentesis had any effect on joint inflammation, the activity of tumour necrosis factor α (TNF-α) was also determined.

Materials and methods

Collection of synovial fluid

Fourteen Dutch Warmblood horses and two Welsh ponies, 9 mares and 7 geldings, mean age 11.2 years (range 5-17 years) were used. The horses were assigned randomly to the exercise (8 horses) or control group (8 horses) with mares and geldings divided as evenly as possible over the two groups (exercise group, 4 geldings and 4 mares; control group, 3 geldings and 5 mares) in order to avoid a possible effect of gender. The age of the animals did not differ significantly between the 2 groups. All animals were clinically healthy and sound, kept stabled during the experimental period and fed a diet of commercial pellets and hay. No lameness was observed during the experiment.

After clipping, shaving and disinfection, arthrocentesis of the left metacarpophalangeal, radiocarpal and tarsocrural joints was carried out in a sterile manner in all horses and SF samples were collected in plain syringes. The mean ± s.e. volume of SF collected was 3.0 ± 0.2, 3.3 ± 0.2 and 4.4 ± 0.2 ml from the metacarpophangeal, radiocarpal and tarsocrural joints, respectively. The amount of SF collected for each horse was approximately the same at each timepoint. Synovial fluid was transferred into plain tubes and centrifuged for 10 minutes at 10,000 g, aliquoted and stored at -80ºC until further processing, within an hour of collection.

Horses in the exercise group were acclimated to the treadmill during the week before being subjected to a 5 day training regimen of increasing intensity. This consisted of 16 mins walking, 20 mins trotting and 4 mins canter on the treadmill followed by 30 mins

in the horse-walker, on the first day. The intensity was increased each day and the periods at a walk, trot and canter reached 14, 18 and 8 mins, respectively by Day 5, again followed by 30 mins in the horse-walker. The horses in the control group were confined to their stables with 5 mins of handwalking each day. SF was collected 14, 14.5, 17 and 24 days after the start of the experiment from the aforementioned joints in all horses (including the control group) as described above.
The study was approved by the University's Ethics Committee, in compliance with the Dutch Act on Animal Experiments.

MMP assay

Synovial fluid was diluted 12.5 times in a buffer consisting of 50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 5 mmol/l $CaCl_2$, 1 µmol/l $ZnCl_2$ and 0.01% Brij-35. MMP activity was determined using a slight modification of the fluorometric assay as described by DeGroot *et al* (2001b). Briefly, conversion of a 2.5 µmol/l concentration of the internally quenched fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) was measured in the presence of an EDTA-free general proteinase inhibitor cocktail solution (Complete, Roche Diagnostics) to prevent conversion of the substrate by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence and absence of MMP inhibitor BB94 (10 µmol/l). Since the substrate is not cleaved by aggrecanases, this approach detects only MMP-mediated substrate conversion and reflects the overall MMP activity in the SF samples. Incubations were performed in black Costar 384-well plates at 30ºC. Increase in fluorescence was followed in a thermostated fluometer (Cytofluor 4000, PerSeptive Biosystems, λ_{ex} = 485 nm, λ_{em} = 530 nm). The initial velocity of substrate turnover (linear increase in fluorescence over time) was used as a measure of enzyme activity.

TNF-α assay

The WEHI 164 clone-13 murine fibrosarcoma cell line was used in an in vitro cytotoxicity assay to measure TNF-α activity in the samples. The assay was conducted as described by Eskandari *et al* (1990) with some minor modifications. Briefly, the cells were maintained in Royal Park Memorial Institute 1640 (RPMI 1640) medium with 10% FCS, 50 u/ml penicillin (Gibco-BRL), 50 µg/ml streptomycin (Gibco-BRL), 5 x 10^{-2} mmol/l β-mercaptoethanol (Fluka AG) and 200 mmol/l L-glutamine (Gibco-BRL). The cells were cultured in bacterial grade petri dishes to prevent adherence. For the bioassay, the cells were brought into log phase. On the day of the assay, the cells were suspended in culture medium with a concentration of 5 x 10^3 cells/well and cultured for 24 h with 2-fold serial dilutions of the samples as a total volume of 100 µl in 96-well flat bottom microtitration plates. Cell survival/proliferation was measured using an alamar blue assay (Biosource International). The assay was performed as described by the manufacturer. Briefly, 10 µl alamar blue was added to all wells and cells were incubated for 3.5 h at 37°C in 5% CO_2. The optical density (OD) was measured with an ELISA reader (BioRad,

BioRad Laboratory) at 550 and 595 nm. A standard curve was constructed by linear regression of recombinant human (rh) TNF-α concentrations versus % difference in reduction of alamar blue (Equation 1).

Equation 1: Percent difference in reduction

$$\frac{OD_{550} - (OD_{595} \times R_0) \text{ for test well}}{OD_{550} - (OD_{595} \times R_0) \text{ for positive growth control}} \times 100$$

where R_0 correction factor = OD_{550}/OD_{595} of medium with alamar blue.
Plotting the dilution factor of the samples vs. % difference in reduction of alamar blue and using the linear part of this curve, TNF-α concentrations were determined from the standard curve. The mean cytokine concentration was calculated and used in further analysis.

Statistical analysis

Statistical analysis was performed using the Prism statistical package from Graph Pad Software. Normality was assessed using the Kolmogorov-Smirnov method and data were found to be normally distributed, allowing the use of parametric tests. Differences between the exercise and control groups (for MMP activity) and between joints (for TNF-α) were tested for significance using an unpaired t test. When there was no significant difference between groups, data were combined. A repeated measures ANOVA, followed by Dunnett's multiple comparisons test, was used to determine whether significant differences existed between timepoints. Differences were considered significant when $P<0.05$.

Results

MMP activity

There was no significant difference in general MMP activity in SF between the exercise and control groups at any timepoint, in any of the joints. This shows that the exercise programme provided did not affect general MMP activity in SF during the 10 days after its cessation; therefore, data were combined to determine the influence of repeated arthrocentesis. General MMP activity in SF of the metacarpophalangeal joint gradually increased with repeated arthrocentesis (Fig 1a). After an interval of 2.5 days (Days 14.5 to 17) and 7 days (Days 17 to 24), MMP activity was significantly increased compared to baseline values (Day 0). For the radiocarpal joint (Fig 1b), the pattern was shifted somewhat for significant timepoints; MMP activity was significantly increased compared to baseline when arthrocentesis was repeated after 12 h and 2.5 days (Days 14.5 and 17, respectively), but the increase was no longer significant after one week (Day 24). Finally, for the tarsocrural joint (Fig 1c), general MMP activity also increased after repeated arthrocentesis, but was only significantly higher than baseline when 2.5 days had passed

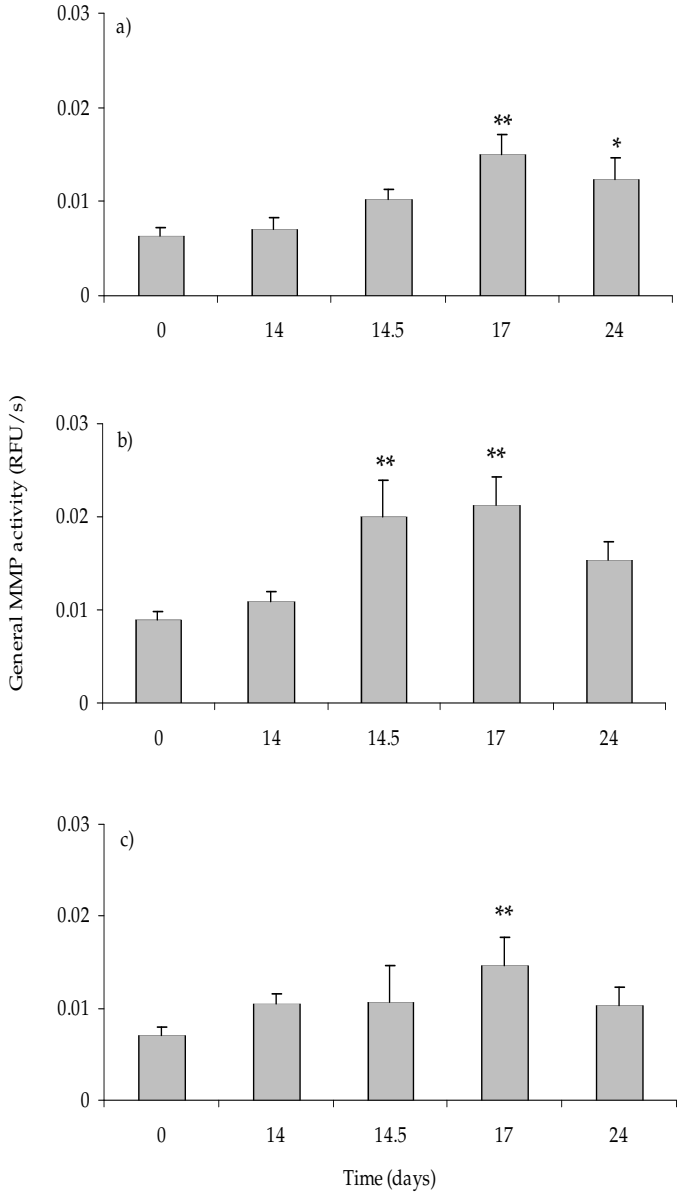

***Figure 1**: General MMP activity (RFU/s; mean ± s.e.) in synovial fluid from the left a) metacarpophalangeal, b) radiocarpal and c) tarsocrural joints of 16 horses plotted against time (days), showing the increase in MMP activity as a result of repeated arthrocentesis. * P<0.05 with respect to baseline values (day 0). ** P<0.001 with respect to baseline values (day 0).*

after previous arthrocentesis (Day 17). The increase in MMP activity was greater in the metacarpophalangeal and radiocarpal joints than in the tarsocrural joint, with the greatest activity being almost 2.5 times baseline in the former and 2 times baseline in the latter.

TNF-α bioassay

The activity of TNF-α in SF did not differ between the 3 joints studied and data were combined (Fig 2). In the SF collected from the exercised horses on Day 14 (2 h after completion of the exercise programme), TNF-α activity was roughly 2.5 times higher than the pre-exercise value (P<0.001). The activities measured on Days 14.5 and 17 (12 h and 3 days after completion of the exercise programme) were the same as before exercise. Repeated arthrocentesis alone did not cause a significant increase in TNF-α activity. Because of the large number of samples and the wish to determine TNF-α activity in one experiment (avoiding possible interassay variation), a selection of the samples was made. As alterations in TNF-α activity were expected to occur soon after the end of the exercise programme or after repeated arthrocentesis, Day 24 samples were not assayed for bioactivity.

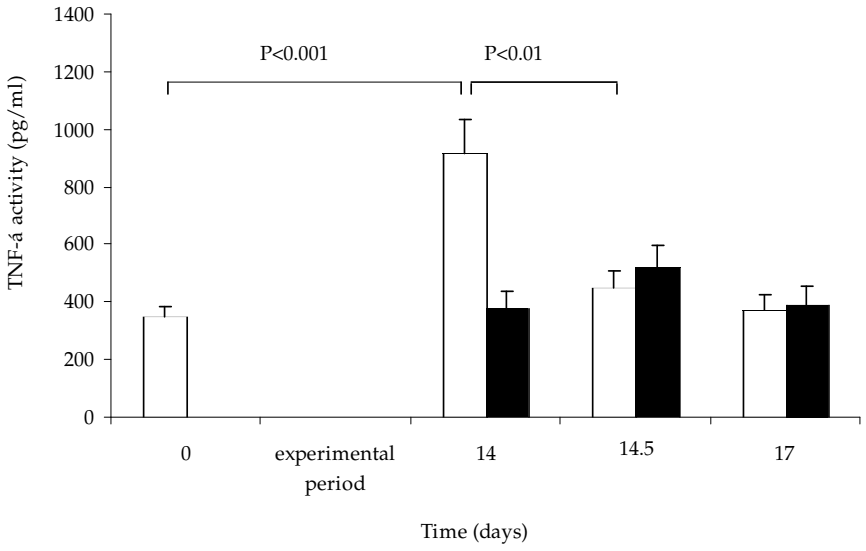

***Figure 2**: TNF-α activity (pg/ml; mean ± s.e.) in synovial fluid of horses in the exercise (open bars) and control (filled bars) groups vs. time, showing increased TNF-α activity in the exercise group shortly after completion of a treadmill exercise programme of increasing intensity. The exercise period is indicated in days and Days 14, 14.5 and 17 correspond to 2, 12 and 72 hours after the end of the last exercise session.*

Discussion

Juvenile horses have higher synovial fluid MMP activity than mature horses, as a result of increased metabolic activity and cartilage turnover (Brama *et al* 1998). All horses used in this study were older than 4 years, by which time MMP activity in the equine joint is considered to have reached stable mature levels. We used 2 ponies and 14 Warmblood horses in our study and it is possible that differences in MMP activity exist between horses and ponies. Breed differences for MMPs in SF have not been studied in equids, but Todhunter *et al* (1997) described such a breed difference for keratan sulphate concentrations in SF, so a difference may also exist for other markers. The data for the 2 ponies included in our study fitted inside the range found for the horses (data not shown). The mares and geldings used were divided as evenly as possible over the 2 groups in order to avoid a possible effect of gender, although this effect on MMP activity is unclear. In human studies, Lohmander *et al* (1993) found no influence of patient sex (or age) on MMP levels, while Manicourt *et al* (1994) stated that serum MMP-1 and TIMP-1 did not differ between males and females, while the serum concentration of MMP-3 was higher in males than in females in healthy adults but not different in OA patients. In a study involving mice, Chambers *et al* (2001) also found no influence of sex hormones on aggrecan degradation by MMPs.

The fluorogenic assay used in this study measures general MMP activity, rather than the concentrations of pro-MMPs. MMPs are activated by cleavage of a pro-MMP and active levels are considered to comprise less than 5% of total MMP levels, with the remainder being present as latent pro-MMPs or complexed to tissue inhibitors of metalloproteinases (TIMPs) and α-2-macroglobulin. The results in this study therefore reflect the true activity of MMPs (Birkedal-Hansen *et al* 1993, Lohmander *et al* 1992, Murphy *et al* 1990, Murphy and Knäuper 1997). Active MMPs are the most relevant as they cause the turnover and degradation of articular cartilage. Zymography is an insensitive technique which only measures gelatinase activity, and antibodies against the various MMPs are not available for horses at the present time. The substrate employed in this general MMP assay is converted mainly by gelatinases (MMP-2 and -9), by the collagenase MMP-13 and, to a lesser degree, by MMP-14. However, other MMPs such as MMP-1 and -3, which have a lower affinity for TNO211-F, can still be major contributors to substrate conversion if their concentrations are elevated.

Repeated arthrocentesis, 2.5 days after previous SF collection, leads to increased MMP levels in normal joints. This finding was unexpected but consistent, and cannot readily be explained. The third SF sample (taken 12 h after the experimental period) was often haemorrhagic, but the level of MMPs was not correlated with the degree of haemorrhage. Therefore, although peripheral blood neutrophils are known to be a source of MMPs (Spiers *et al* 1994a), it seems unlikely that they were the origin of increased MMP levels in this case. In articular sepsis, the inflammatory cell infiltrate is almost certainly the source of MMP-9 (Clegg *et al* 1997a). It is possible that arthrocentesis causes a mild reaction in the synovial membrane followed by an increased release or activation of MMPs, as these are also produced by synoviocytes (Clegg *et al* 1997a). This speculation might be supported by the fact that the increase in MMP activity was smallest in the tarsocrural

joint. As this is the largest of the 3 joints studied, an increase in MMP activity caused by a localised reaction of the synovial membrane will be most highly diluted. The effect of arthrocentesis appeared to be cumulative on a short-term basis, with the highest values being measured when arthrocentesis was performed 3 times within 3 days. One week later, MMP activity had declined but was still higher (significantly in the metacarpophalangeal joint) than the baseline values. When we consider the MMP activity in SF collected 2 weeks apart, there was no significant difference. From these data, it can be concluded that, at least for equine studies, when collecting SF for the determination of MMP levels (as biomarkers), 2 weeks should pass after previous arthrocentesis to be entirely certain that no 'arthrocentesis-effect' persists. This is longer than suggested by findings of Lohmander *et al* (1998), who reported that weekly aspiration of joint fluid (and lavage) did not present a confounding factor in longitudinal human patient studies.
Exercise did not lead to a significant increase in synovial fluid MMP activity. This finding is in accordance with those of Roos *et al* (1995), who saw no rise in MMP levels in man after 60 mins running or a 90 mins football match. The exercise to which the horses were subjected was similar in intensity to that which many horses perform on a daily basis. This implies that, when collecting SF to determine MMP activity as a measure of joint disease, exercise at moderate levels does not act as a confounding factor. This may be different when exercise becomes excessive or in diseased joints. Several weeks of intensive running wheel exercise in rats led to increased MMP-3 levels (Pap *et al* 1998, Roos *et al* 1995). It was interesting that the exercise level in this study did lead to a significant increase in TNF-α levels over baseline values (before the exercise programme started) shortly after the end of exercise. The timing of this rise was as might be expected, as intra-articular administration of endotoxin also caused TNF levels to peak at 2 h (Hawkins *et al* 1995). TNF values returned to baseline by 8 h in that study. In our study no sample was taken at 8 h, but at 12 h they were back to baseline level. Billinghurst *et al* (1995) found that TNF in synovial fluid of horses with mild degenerate joint disease (DJD) increased after racing with a peak at 12 h post racing. The peak at 12 h in that study may have been due to the increased level of exercise (racing) compared to that used in our study (treadmill exercise), and/or the presence of joint disease.
In conclusion, serial arthrocentesis leads to generalised increases in MMP activity in normal equine joints and this needs to be considered when using MMPs as biomarkers of joint disease or joint metabolism. The significant effect is absent when arthrocentesis is repeated after 2 weeks, despite some joint-related differences, and this would appear to be a safe period to avoid this repeated arthrocentesis effect. Moderate exercise does not seem to influence MMP activity in these normal joints, but cannot be discarded as a possible source of error when assessing the suitability of MMPs as potential molecular markers for joint disease until MMP activity is assessed in exercised diseased joints.

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Chapter III

Collagenase-1 (MMP-1) activity in equine synovial fluid: influence of age, joint pathology, exercise and repeated arthrocentesis

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Summary

Reasons for performing study: Matrix metalloproteinases (MMPs) are considered candidate biomarkers for both physiological and pathological tissue remodelling because of their key role in articular cartilage homeostasis. As disruption of the collagenous architecture is thought to be pivotal in chronic degenerative diseases such as osteoarthritis (OA), the collagenases form an interesting subset of the MMPs. The significance of any biomarker in synovial fluid (SF) can be assessed properly only when fluctuations in patterns induced by physiological processes such as development and growth, and by external influences and interventions such as exercise and repeated arthrocentesis, are known and taken into account.

Objectives: To investigate the activity of MMP-1 in equine SF at different stages of development and in joints affected by OA, and the influence of exercise and repeated arthrocentesis thereon.

Methods: MMP-1 activity was determined in SF of normal joints of fetal, juvenile and mature horses, and in SF of horses suffering from OA, using an internally quenched fluorogenic peptide substrate. MMP-1 activity was also measured in SF from horses subjected to an exercise regimen and those subjected to repeated arthrocentesis.

Results: An age- related decline in the SF levels of active MMP-1 was observed. MMP-1 activity was 15-fold higher in fetal than in juvenile animals, which showed significantly higher MMP-1 activity levels than mature horses. In SF of OA joints, MMP-1 activity was increased. Exercise did not affect MMP-1 activity in SF, but repeated arthrocentesis (within 60 h) increased MMP-1 activity significantly.

Conclusions: The high MMP-1 activity in SF of young individuals parallels the high metabolic activity occurring during rapid growth and differentiation at early age. The elevated MMP-1 activity in SF of OA joints probably reflects pathological matrix degradation, confirming the potential of MMP-1 to serve as a biochemical marker for early joint disease. Moderate exercise is not likely to influence the outcome of MMP-1 activity measurements in equine SF, but arthrocentesis should be taken into account as a possible confounding factor.

Potential relevance: Given the crucial role of the collagen matrix for tissue integrity, MMP-1 activity may be a useful tool in diagnostic, therapeutic or prognostic studies in horses suspected of OA. However, care should be taken to exclude fluctuations in MMP-1 activity induced by physiological processes such as development and growth, and by interventions such as repeated arthrocentesis.

Introduction

Cartilage is composed of a small number of chondrocytes within an extracellular matrix of collagen and proteoglycans. In articular cartilage, the proteoglycans are mainly responsible for compressive stiffness, whereas the collagen fibrils provide the tensile strength and maintain the integrity of the cartilage (Kempson 1980). Type II collagen is the principal component of the collagen network but small amounts of minor collagens such as types VI, IX, X and Xl are also observed (Mayne 1989).

The integrity of cartilage in normal, healthy adults is dependent on the steady state between the synthesis of the extracellular matrix by chondrocytes and the rate of its degradation. This extracellular matrix remodelling is a critical component of development and normal physiology (Vu and Werb 2000). Any change in this homeostatic steady state rapidly affects the healthy function of the cartilage and may lead to excessive degradation, which is characteristic of osteoarthritis (OA) (Tetlow *et al* 2001, Walter *et al* 1998). Failure of the collagen network is one of the first characteristics of OA (Bank *et al* 2000, Maroudas 1976) and leads to proteoglycan depletion and loss of biomechanical function. Whereas proteoglycans can be replaced relatively easily (Fell *et al* 1976, Jubb and Fell 1980), extensive damage to the collagen network is thought to be irreversible in mature individuals because of the extremely long turnover time of articular collagen (Maroudas 1980, Maroudas *et al* 1992, McAnulty and Laurent 1990, Verzijl *et al* 2000). This makes collagen damage into a critical event in the development of joint pathology, probably related to a point of no return in the development of the disease.

The mechanism of degradation of the collagen network of cartilage has been the subject of considerable interest because, in the native state, it is resistant to attack by most proteolytic enzymes (Matrisian 1990). Collagenases, enzymes of the matrix metalloproteinase (MMP) family, are among the relatively few enzymes capable of cleaving intact fibrillar collagen. MMPs with collagenase activity include MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14 (Aimes and Quigley 1995, Nagase and Woessner, Jr. 1999, Riley *et al* 2002). Cleavage occurs at a single locus in the collagen triple helix, creating 1/4 and 3/4 fragments which can then be further degraded by collagenase itself or by gelatinolytic enzymes such as gelatinase A and B, neutrophil elastase and plasmin at neutral pH, and cathepsins B, S and L at acidic pH. Recently, Kafienah *et al* (1998) showed that cathepsin K has the ability to cleave intact fibrillar collagen type II. Although collagenases clearly have an important role in the cleavage of intact fibrillar collagen, the identity of all proteinases involved therefore remains to be definitively established.

MMPs are considered candidate biomarkers for both physiological and pathological tissue remodelling because of their key role in articular cartilage homeostasis. They potentially reflect early cartilage damage, which is of specific interest in insidious degenerative processes such as OA in which the pathophysiological processes are known to be advanced by the time alterations can be detected by routine radiography (Lohmander *et al* 1992, 1993). In the horse, Clegg *et al* (1997a, 1997b) were the first to show increased MMP levels in relation to joint pathology. However, work on collagenases in the horse has been limited. Tung *et al* (2002a) were able to demonstrate MMP-1 and MMP-13 mRNA expression in an equine chondrocyte culture, but Trumble *et al* (2001) could not

detect MMP-1 mRNA in equine cartilage of normal and OA joints. In man collagenases are increased in the synovial fluid (SF) of patients with OA (Clark *et al* 1993, Lohmander *et al* 1993). Furthermore, synovial lining cells in human OA joints express increased MMP-1 and MMP-13 levels (Keyszer *et al* 1995). Chondrocytes from human OA cartilage express and secrete MMP-1 (Borden *et al* 1996, Reboul *et al* 1996, Wolfe *et al* 1993), MMP-8 (Chubinskaya *et al* 1996) and MMP-13 (Borden *et al* 1996, Mitchell *et al* 1996, Reboul *et al* 1996) at increased levels.

The use of collagenases, or MMPs in general, as biomarkers is not without pitfalls, as there are several ways to demonstrate the presence of collagenases which do not all yield comparable results. Collagenases have long been assayed using radiolabeled collagen fibrils or internally quenched fluorogenic peptides (Knight *et al* 1992). Unfortunately, these methods do not allow for the differentiation of the collagenases involved in the cleavage of these substrates. For this reason, other studies (Huebner *et al* 1998, Ishiguro *et al* 1999, Shlopov *et al* 1997) have focused on MMP gene expression levels (as determined by RT PCR) or on total amounts of MMPs including pro-MMPs, active MMPs and TIMP-inhibited MMPs; using immunological methods or zymography. The presence of (pro-)enzyme in body fluids and tissues, however, does not necessarily imply proteolytic activity. MMPs are activated by cleavage of a pro-MMP and levels of the active enzyme comprise less than 5% of total MMP levels, with the rest being present as latent pro-MMPs or as complexes with tissue inhibitors of metalloproteinases (TIMPs) or α–2-macroglobulins (Birkedal-Hansen *et al* 1993, Lohmander *et al* 1992, Murphy and Knäuper 1997). When assessing physiological or pathological processes, it may be more relevant to measure levels of active MMPs than to determine total protein levels that may reflect the potential for degradation rather than the damaging enzyme species itself.

The significance of any biomarker in SF can be assessed properly only when fluctuations in patterns induced by physiological processes, such as development and growth, and by external influences and interventions, such as exercise and repeated arthrocentesis, are known and taken into account. It is known that, whereas turnover is extremely slow in adult cartilage, metabolism in young individuals is maintained at a substantially higher level to allow for growth and remodelling (Lohmander *et al* 1992, Murphy *et al* 1990, Todhunter 1996). In the horse, we showed previously that general MMP activity and MMP-3 (stromelysin) activity is increased in immature individuals (Brama *et al* 1998, 2000d). However, data on MMP-1 are lacking.

In the horse, an important potential confounding factor in assessing biomarker levels is the effect of exercise. There is hardly any species that is so intricately linked with this factor as the horse, bred mainly for its exquisite locomotion system. In this respect, it is interesting to note that mechanical forces increase levels of MMP-1 mRNA in a vascular smooth muscle derived cell line (James *et al* 1993). Huebner *et al* (1998) found relatively higher levels of collagenase-1 mRNA at the medial tibial plateau compared to the lateral plateau in guinea pigs, an area that, in man, is known to bear more weight than the lateral side (Brown and Shaw 1984). Higher levels of MMP-1 mRNA may therefore be a constitutive response to mechanical loading.

Repeated arthrocentesis is necessary to obtain SF samples for the monitoring of disease progress. Recently, we showed an increase in general MMP activity due to serial arthro-

centesis (van den Boom *et al* 2004a) and speculated that this was a sequel to the local inflammatory response of the synovial membrane induced by the intervention. This finding makes repeated arthrocentesis a potentially confounding factor and warrants further investigations into the effect on specific MMPs. However, no data on the effect of the procedure on specific MMPs have so far been published.
In the present study, a selective fluorogenic assay was used to measure MMP-1 activity exclusively in SF of fetal, juvenile and mature horses, and in the SF of equine joints affected by OA. In addition, the influence of repeated arthrocentesis and exercise on MMP-1 activity was investigated.

Materials and methods

Animals

Material for the study of the effects of development and osteoarthritis came from horses that were subjected to euthanasia for reasons other than metacarpophalangeal joint disease. The influence of repeated arthrocentesis and exercise was studied in horses owned by the Department, that are used regularly for the training of undergraduate students in non-invasive procedures such as bandaging. The group consisted of 14 mature horses (mean age 9.6 years, range 5-17 years) which were free of lameness and joint effusion. The study was approved by the University's Ethics Comittee, in compliance with the Dutch Act on Animal Experiments.

Experimental design and sample collection

- stage of development and osteoarthritis

Synovial fluid (SF) was obtained from normal metacarpophalangeal joints of equine fetuses (n = 10), 5-month-old foals (n = 10), 11-month old foals (n = 10) and mature horses (n = 10, mean age 7.3 years, range 4-10 years). SF was also collected from 10 mature horses (mean age 8.2 years, range 4-11 years; age-matched with the above mentioned control animals; $P>0.05$) with evidence of OA of the metacarpophalangeal joint according to the criteria reported elsewhere (Brama *et al* 1998, 2000d). Synovial fluid samples were collected within 4 h after death, centrifuged (10,000 g for 10 mins) and the resulting cell free supernatant was frozen at -20°C until further analysis.

- exercise and repeated arthrocentesis

Arthrocentesis of the metacarpophalangeal joint was performed using the approach through the lateral sesamoidean ligament under standard aseptic conditions. After arthrocentesis, the horses were assigned randomly to the exercise (n = 8) or control (n = 6) group. The horses in the exercise group were accustomed to the treadmill for one week and subsequently subjected to a 5 day training regimen of increasing intensity. The first day's exercise consisted of 16 mins walking, 20 mins trotting and 4 mins of canter. The

exercise intensity gradually increased each day up to 14, 18 and 8 mins walk, trot and canter, respectively, by Day 5. Each day, the treadmill exercise was followed by 30 mins low intensity exercise in a horse-walker. The horses in the control group were confined to their stables with 5 mins hand walking each day. At Day 14, SF was collected from the metacarpophalangeal joints of all horses (2 h after the last training session of the exercise group). Subsequently, all horses were subjected to repeated arthrocentesis at 12, 72 and 240 h after the first intervention. SF samples were centrifuged for 10 mins at 10,000 g, and the resulting cell free supernatant was aliquoted and stored at -20°C until further processing.

MMP-1 activity assay

This study used a fluorogenic substrate to monitor net MMP activity. This type of substrate consists of a short amino acid sequence, recognised by MMPs, to which a fluorophore is attached. The fluorescence is quenched by an absorbing moiety (quencher), also coupled to the peptide. Upon cleavage, quenching is lost, and an increase in fluorescence is measured proportional to the amount of hydrolysed substrate (Beekman *et al* 1996, 1997, 1999).

- fluorogenic substrate; enzyme kinetics

The fluorogenic substrate TNO113-F used consists of the sequence Dabcyl-Gaba-Pro-Cha-Abu-Smc-His-Ala-Cys(Fluorescein)-Gly-Lys-NH_2 (TNO113-F; Dabcyl = 4-(4-dimethylaminophenylazo)benzoyl, Gaba = γ–amino-*n*-butyric acid, Cha = cyclohexylalanine, Abu = aminobutyric acid; Smc = S-methyl-cysteine). This peptide was synthesised on a 10 µmol scale by solid phase strategies on an automated peptide synthesiser as described previously (Drijfhout *et al* 1996). Fluorescein was coupled to the peptide via the cystein's thiol function using iodoacetamide fluorescein to increase sensitivity (Beekman *et al* 1999). Recombinant human MMPs (MMP-1, 2 nmol/1; MMP-2, 0.5 nmol/1; MMP-3, 2 nmol/1; MMP-8, 2 nmol/l; MMP-9, 0.5 nmol/1; MMP-13, 1 nmol/1; MMP-14, 1 nmol/1) were incubated with 125 nmol/1 TNO113-F at 25°C in 180 µl buffer A (50 mmol/1 Tris pH 7.5, 150 mmol/1 NaCl, 5 mmol/1 $CaC1_2$, 1 µmol/1 $ZnC1_2$, 0.01% (v/v) Brij-35). The catalytic efficiencies (K_{cat}/K_m in 10^3/mol/1/sec at 25°C), determined as described previously (Beekman *et al* 1996, 1997), for all available MMPs for TNO113-F are: MMP-1 = 13.0, MMP-2 = 30.8, MMP-3 = 2.6, MMP-8 = 63.6, MMP-9 = 98.2, MMP-13 = 203.1 and MMP-14 = 7.1. Further improvement of the assay specificity for MMP-1 was achieved by the addition of the MMP inhibitor RS102,223 (final concentration 1 µmol/1; a generous gift from Dr R. Martin, F. Hoffmann-La Roche, Ltd.). MMP-1 was not inhibited by RS102,223 (IC_{50}>700 nmol/1), while other MMPs are strongly inhibited at 1 µmol/l RS102,223 (IC_{50} values for MMP-3, -8, -9, -12, -13 and -14 are below 1 nmol/1; Beekman *et al* 1997, 1999).

MMP-1 activity in synovial fluid

A 25 µl aliquot of a 5-fold in buffer A (50 mmol/1 Tris pH 7.5, 150 mmol/1 NaCl, 5 mmol/1 $CaCl_2$, 1 µmol/1 $ZnC1_2$, 0.01% (v/v) Brij-35) diluted SF sample was mixed (650 g, 1 min) with 25 µl RS102,223 (1 µmol/1) and incubated for 30 mins to allow RS102,223 to inhibit all MMPs except MMP-1. An EDTA-free general proteinase inhibitor cocktail (Complete, Roche Diagnostics; 25 µl; one tablet in 12.5 ml buffer A) was then added, mixed (650 g, 1 min) and incubated for 30 mins, to prevent conversion of the substrate by proteinases other than MMPs. Finally, 25 µl of the substrate TNO113-F (5 µmol/1) was added and mixed (650 g, 1 min). All incubations were performed at 37°C in clear-bottom Costar 384-well plates (Corning Inc.). Increase in fluorescence at 37°C was followed for 2.5 h in a Cytofluor II (Applied Biosystems; λ_{ex} = 485 nm; λ_{em} = 530 nm) (DeGroot *et al* 2001a, Riley *et al* 2002). The initial velocity of substrate turnover (linear increase in fluorescence over time) was used as a measure of enzyme activity and expressed as an increase in fluorescence per minute (ΔFlu/min).

Statistical analysis

Data are presented as mean ± s.e. and expressed as MMP-1 activity normalised to baseline levels of control joints which were set at 1.00. When there was no significant difference between groups, data were combined. Differences between groups were tested by one way analysis of variance (ANOVA). Fisher's least significant difference multiple comparisons test of the means was applied as *post hoc* test. Differences were considered significant when $P<0.05$.

Results

Stage of development and osteoarthritis

Extremely high MMP-1 activity levels (15-fold higher than in normal juvenile and mature joints) were observed in SF of fetal metacarpophalangeal joints ($P<0.001$). After birth, MMP-1 activity declined gradually in SF from normal metacarpophalangeal joints (5 months > 11 months > mature; Fig 1 a). This decline coincides with the cessation of tissue turnover and growth in mature animals.
MMP-1 activity in SF of osteoarthritic metacarpophalangeal joints from mature horses was significantly higher than in the SF of age-matched healthy metacarpophalangeal joints (Fig 1 b).

Exercise and repeated arthrocentesis

There was no significant difference in MMP-1 activity between the exercise and control groups (Fig 2a). Therefore, data of both control and exercise groups were combined to determine the influence of repeated arthrocentesis. MMP-1 activity in SF of the metacarpophalangeal joint was increased considerably (1.8-fold; $P = 0.005$) 12 h after a previous

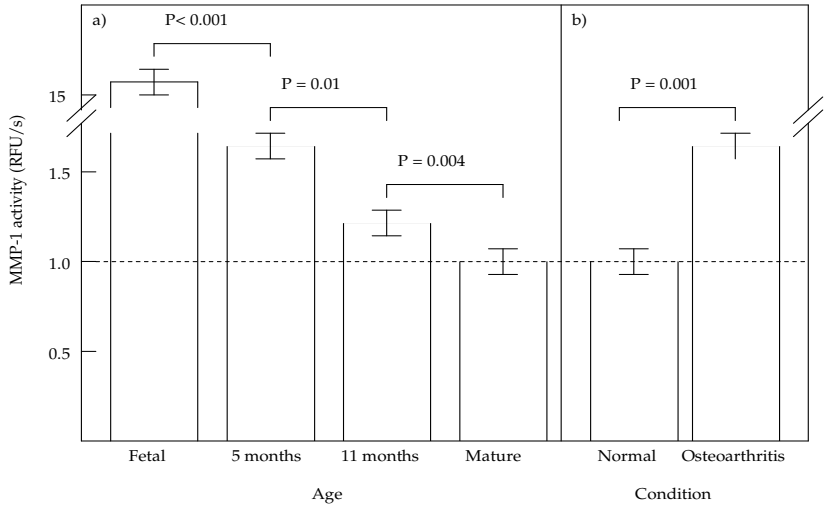

***Figure 1**: a) MMP-1 activity (set at 1.00 for normal mature; mean ± s.e.m.) in synovial fluid (SF) of normal fetal, juvenile (age 5 and 11 months) and mature (mean age 7.3 years, range 4-10 years) equine metacarpophalangeal joints. b) MMP-1 activity in SF of normal mature (mean age 7.3 years, range 4-10 years) and osteoarthritic mature (mean age 8.2 years, range 4-11 years; age-matched with the mentioned control animals; P>0.05) equine metacarpophalangeal joints.*

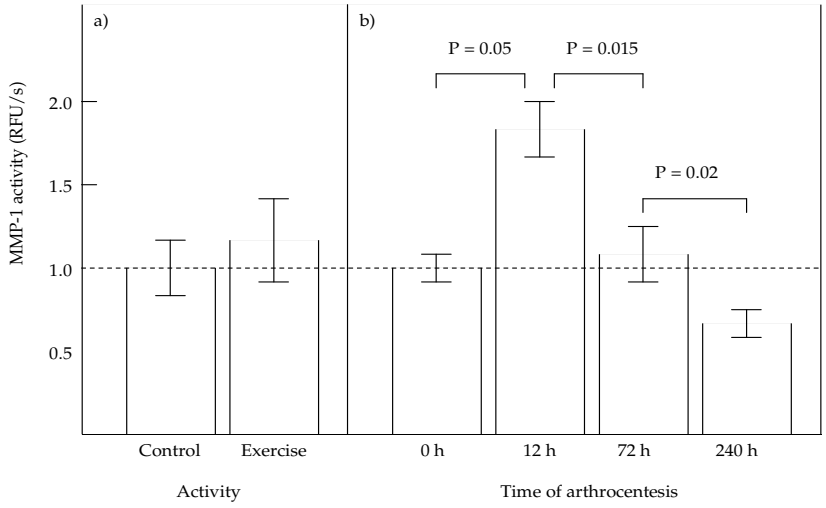

***Figure 2**: a) MMP-1 activity (set at 1.00 for nonexercised horses; mean ± s.e.m.) in synovial fluid (SF) of metacarpophalangeal joints of nonexercised (control group) and exercised horses. b) MMP-1 activity (mean ± s.e.m.) in SF of the metacarpophalangeal joint collected with arthrocentesis at 0 (control set at baseline 1.00), 12, 72 and 240 h.*

arthrocentesis of this joint (Fig 2b). When the interval of repetition was increased to 60 h, MMP-1 activity in SF had returned to baseline level. However, 7 days after the third arthrocentesis of the metacarpophalangeal joint, MMP-1 activity appeared to be decreased significantly compared to baseline levels (Fig 2b).

Discussion

Matrix metalloproteinases play an important role in articular cartilage extracellular matrix turnover and degradation. As it is the disruption of the collagenous architecture which is thought to result in the irreversible changes that are typical for chronic degenerative diseases such as OA, the collagenases form an interesting subset of the MMPs. Relatively little is known about the specific characteristics of the MMPs with collagenase activity that have been described so far (MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14). Of these, MMP-13 has been shown to be more potent in the degradation of soluble type II collagen than MMP-1 (Mitchell *et al* 1996, Reboul *et al* 1996), which has prompted much interest in this specific enzyme as a potential pathogenic factor in OA. Others have demonstrated enhanced MMP-1 over MMP-13 ratios in OA patients (Shlopov *et al* 1997), indicating that at this moment no definitive answer can be given as to the proportionate role of these enzymes in OA development.

It is important to keep in mind that, although data on gene expression and protein level are of importance, it is the amount of active enzyme that ultimately determines the destructive effects. Active MMP accounts for only 5% of total MMP (Birkedal-Hansen *et al* 1993, Lohmander *et al* 1992, Murphy and Knäuper 1997). By applying a new and specific assay for the activity of MMP-1 in SF, the role of MMP-1 in diarthrodial joints under physiological and pathological conditions may be further elucidated. Clinically, this may contribute to the development of a panel of specific markers of early joint disease before the disease becomes irreversible, and may help in the development of specific targets for therapeutical intervention.

The present study shows a gradual decrease in MMP-1 activity during development. In fetal joints, MMP activities are as much as 15-fold higher than in the mature horse, which is consistent with the rapid tissue turnover in the fetal joint, necessary for growth and the ongoing process of endochondral ossification. These data are in line with previously reported findings on general MMP activity and stromelysin (MMP-3) activity during development (Brama *et al* 1998, 2000d). The strong influence of developmental stage on MMP activity stresses the need for age-matched controls when studying pathological conditions, especially when growing individuals are concerned.

Early diagnosis of OA is a major problem, both in human and veterinary medicine (Balkman and Nixon 1998, Clegg *et al* 1997a, Lohmander *et al* 1992, 1993). Therefore, biochemical markers of early cartilage degeneration would provide clinicians with a useful tool to assess the current status of the cartilage. MMPs are prominent candidates for this role. In the horse, Clegg *et al* (1997b) showed that levels of the gelatinases MMP-2 and MMP-9 are significantly increased in both aseptic and septic joint diseases. Balkman and Nixon (1998) showed that MMP-3 mRNA expression is low in normal cartilage and synovial membrane, but increased in arthritic cartilage. We previously presented a 2-fold

higher overall MMP activity and a 4-fold higher MMP-3 activity in OA SF than in controls (Brama *et al* 1998, 2000d). The present study shows that MMP-1 activity is also increased in OA SF, although to a somewhat lesser extent (1.6-fold). The disparity in increase between MMP-1 and MMP-3 has also been reported in man (Lohmander *et al* 1993) and may reflect the different roles for these 2 enzymes in (patho)physiological tissue turnover. Given the proposed primary role of collagen damage in the process of OA (Bank *et al* 2000, Maroudas 1976), a moderate increase in collagenase activity may well expose enough proteoglycans to unleash a much higher increase in activity of stromelysin or the gelatinases.

In the equine study by Trumble *et al* (2001) on MMP mRNA expression in tissue samples from normal and diseased joints, only 3 of 59 synovial membrane samples showed detectable levels of MMP-1 expression and none of 45 cartilage samples. In combination with the present data that show a significant increase in MMP-1 activity, this underscores that mRNA expression levels do not necessarily reflect activity directly. Post-transcriptional regulation by activation of the pro-MMPs and inhibition of the active MMPs obscure the direct relation between mRNA and protease activity. In fact, it is likely that MMP-1 activity is determined more by these post-transcriptional processes than by changes in mRNA level.

The amount of exercise to which the horses were subjected in this study, similar in intensity to that which many horses perform on a daily basis, did not lead to a significant increase of MMP-1 activity in SF. This finding is in accordance with the report by Roos *et al* (1995), who saw no rise in MMP levels in man after 60 mins running or a 90 min football match. Apparently, the lack of any effect of moderate exercise is not restricted to MMP-1 alone, since van den Boom *et al* (2004a) could not show any such effect on general MMP activity. This implies that, when collecting SF to determine MMP activity, exercise at moderate levels will not act as a confounding factor. This may be different when exercise becomes excessive. It has been demonstrated that several weeks of intensive running wheel exercise in rats led to increased MMP-3 levels (Pap *et al* 1998).

Repeated arthrocentesis, 12 h after previous SF collection, leads to increased MMP-1 activity levels. It may be speculated that the mechanism is through the inflammatory response provoked in the synovial membrane by the arthrocentesis procedure. Synoviocytes are known to produce a whole array of MMPs, including MMP-1 (Firestein *et al* 1991, Keyszer *et al* 1995, Wolfe *et al* 1993). Also, an inflammatory response may result in the influx of leucocytes, which are known to be a source of MMPs, or of cytokines which may activate pro-MMPs. After 60 h, MMP-1 activity had returned to baseline levels. Lohmander *et al* (1998) reported that weekly aspiration of joint fluid (and lavage) did not present a confounding factor in longitudinal human patient studies. However, based on general MMP activities in equine SF, 2 weeks should pass after previous arthrocentesis (van den Boom *et al* 2004a). However, the present study indicated that a longer lasting effect may be present; MMP-1 activity levels were significantly lower after a third arthrocentesis 10 days after the first and 7 days after the last puncture. An explanation for this phenomenon might be that the molar ratio of TIMP-l:MMP-1 in resting human synoviocytes exceeds 10:1 (DiBattista *et al* 1995). If the rise in MMP-1 activity induces a (delayed) increase in TIMP-1, the decrease in MMP-1 activity at 10 days after the first

arthrocentesis may well represent some degree of overkill by TIMP-1. As long as the duration and pattern of the effect of repeated arthrocentesis has not been established definitively, it should be considered to be a potential confounding factor.

In conclusion, our data underscore the need for proper age-matching of subjects when assessing MMP activity levels. While moderate exercise does not seem to influence MMP-1 activity, arthrocentesis may have an effect, which should be taken into account. Given the crucial role of the collagen matrix for tissue integrity, MMP-1 activity may hence be a useful tool in diagnostic, therapeutic or prognostic studies in horses suspected of having OA. However, the importance of this enzyme cannot be fully appreciated until more is known about the roles and activities of the other collagenases. MMP-1 is expected to be of greater diagnostic and/or prognostic value as a member of a panel of markers, than as a stand alone biochemical marker.

Chapter IV

Influence of repeated arthrocentesis and exercise on synovial fluid concentration of nitric oxide, prostaglandin E2 and glycosaminoglycans in healthy equine joints

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Submitted

Summary

Reasons for performing the sudy: The importance of osteoarthritis (OA) in the horse and the difficulty in its early diagnosis have led to the search for potential biomarkers of joint disease. If the levels of such markers are to be accurately interpreted it is important to know if they are influenced by environmental factors and/or interventions such as exercise and repeated arthrocentesis.

Objective: To investigate the influence of repeated arthrocentesis and exercise on nitric oxide (NO), prostaglandin E_2 (PGE_2) and glycosaminoglycan (GAG) concentrations in synovial fluid (SF) from normal equine joints.

Methods: SF was collected from the left metacarpophalangeal (MCP), radiocarpal and tarsocrural joints of 16 horses. Half of the horses were exercised and arthrocentesis was repeated 14, 14.5, 17 and 24 days after the start of the exercise programme, in both exercised and control horses. Nitric oxide was determined in SF from the MCP joint only and PGE_2 and GAG concentrations were determined in SF from all joints.

Results: Repeated arthrocentesis caused an increase in NO concentration in the MCP joint on Day 14.5, in PGE_2 concentrations in the radiocarpal and tarsocrural joints on Day 14.5 and the release of GAGs into SF of the MCP and radiocarpal joints on Day 17. Exercise resulted in an increase in PGE_2 levels in all joints but did not influence the other parameters measured.

Potential relevance: Repeated arthrocentesis is a potential confounding factor for the use of synovial NO, PGE_2 and GAG concentrations as markers of joint disease. Based on this study such a confounding effect can be avoided if one week or more separates arthrocentesis. Moderate exercise causes a transient rise in PGE_2 in SF.

Introduction

Nearly all horses kept nowadays are used for riding, either for pleasure and/or in competition, and lameness is the most common reason for their retirement (Olivier *et al* 1997, Rossdale *et al* 1985, Todhunter and Lust 1990). The most important cause of lameness in horses is joint disease (Todhunter 1992) and this explains why osteoarthritis (OA) has been reported to be the most economically important disease in equine athletes and pleasure horses (Pool 1996). Osteoarthritis in the horse most often affects the metacarpophalangeal and carpal joints (Pool 1996, Rossdale *et al* 1985). The radiological changes that accompany OA become apparent only in the late stages of disease and correlate poorly with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996). This discrepancy between radiological signs and disease severity has led to a search for biological markers, to allow the earlier detection of joint disease, at which time rest and therapy may prevent further, permanent damage to the joint, and the articular cartilage in particular. If such biomarkers are to be reliably used for the detection of joint damage it is important to know or determine the levels in normal joints and how levels are influenced by common environmental influences such as exercise and repeated arthrocentesis.

Nitric oxide (NO) is a highly reactive, cytotoxic free radical, a by-product of the oxidation of L-arginine to citrulline, catalysed by NO synthases (NOS). The constitutive forms of this enzyme (cNOS) lead to continuous low levels of synthesis and inducible forms (iNOS) cause the generation of large amounts of NO when activated by endotoxins and cytokines (MacDonald *et al* 1994, Scher *et al* 1996, Takafuji *et al* 2002, Tung *et al* 2002c). Nitric oxide is considered to play a mediatory role in joint disease, although some controversy exists as to whether it serves an inflammatory or a protective function. Synovial fluid concentrations of NO were increased in OA (Farrell *et al* 1992), and von Rechenberg *et al* (2000) found elevated NO concentrations in the synovial membrane of equine joints with moderate OA. The creation of cartilage defects led to increased activity of iNOS (Tanaka *et al* 1998) and iNOS was also upregulated in OA chondrocytes (Amin *et al* 1995). Nitric oxide has been shown to catalyse the interleukin-1 (IL-1) induced inhibition of proteoglycan synthesis (Bird *et al* 1997), it plays a role in chondrocyte apoptosis (Kim *et al* 2003), and was demonstrated to activate matrix metalloproteinases (MMPs) (Murrell *et al* 1995). Experiments investigating NOS inhibition have proved inconclusive with NOS inhibitors leading to the development of OA in one study (Fenton *et al* 2000), while iNOS inhibition caused less severe OA and lower MMP levels in other studies (Connor *et al* 1995, Pelletier *et al* 1998, 1999, Stefanovic-Racic *et al* 1994, 1995). In another report a protective role for NO was also described, slowing proteoglycan degradation induced by IL-1 (Bird *et al* 2000a). The effect of NO may depend on the local concentration or the stage of disease, as a protective effect of NO on proteoglycan depletion was observed in early but not late OA cartilage explant culture (Stefanovic-Racic *et al* 1994, 1995).

Equine synoviocytes (from healthy joints) appear to have a low basal level of NO synthesis, which is not affected by lipopolysaccharide (LPS) or IL-1 (Frean *et al* 1997, Simmons *et al* 1999a), while equine chondrocytes were found to synthesise significant basal levels of NO (Wiseman *et al* 2003), and showed a dose dependent increase in NO synthesis when stimulated by LPS or IL-1 (Bird *et al* 1997, Fenton *et al* 2000, Frean *et al* 1997, Tung

et al 2002b). Von Rechenberg *et al* (2000) also found more NO in articular cartilage than in synovial membrane. In co-culture of articular chondrocytes and synovial cells mechanical stimulation induced intracellular Ca^{2+} release, which led to inhibited NOS expression (D'andrea *et al* 1998) and dynamic compression inhibited NO synthesis in cultured equine chondrocytes (Wiseman *et al* 2003), suggesting that physical factors, such as exercise, influence NO production within joints.

Prostaglandins, most notably prostaglandin E_2 (PGE_2), play an intimate role in articular inflammatory and nociceptive pathways (Kirker-Head *et al* 2000) and are readily released by both synovial cells and chondrocytes (Murakami *et al* 1998). Prostaglandin release is stimulated by joint inflammation or injury (Kirker-Head *et al* 2000, Murakami *et al* 1998), IL-1 (Dvorak *et al* 2002, Morris *et al* 1990), LPS (MacDonald *et al* 1994), cell damage, vascular distension or stress (Kirker-Head *et al* 2000) and equine chondrocytes in culture subjected to adverse conditions have also been shown to produce PGE_2 (May *et al* 1991). The rise in in PGE_2 concentrations is very rapid, peaking 2-9 hours after experimental induction (Gronblad *et al* 1988, Hardy *et al* 1998b, Hawkins *et al* 1993, Owens *et al* 1996).

Prostaglandin E_2 concentrations in SF have been shown to be elevated in most, if not all, joint disease, including OA, but concentrations were not correlated with radiological signs (Chevalier 1997, von Rechenberg *et al* 2000). In another study PGE_2 was found to be increased in SF from some horses suffering from a number of different arthritides, and elevated PGE_2 concentrations correlated well with the presence of lameness (May *et al* 1994). Prostaglandin E_2 is considered to be a sensitive predictor of joint disease (Bertone *et al* 2001), but the fact that more PGE_2 was found in equine synovial membrane than in cartilage (von Rechenberg *et al* 2000) supports the assumption that it is principally an indicator of active synovial inflammation (Kirker-Head *et al* 2000).

The extracellular matrix (ECM) of cartilage consists mainly of collagen, proteoglycans and water, produced by the limited number of chondrocytes which are imbedded within it. Proteoglycans are made up of a core protein attached to which are a large number of glycosaminoglycan (GAG) sidechains, mainly chondroitin sulphate and keratan sulphate (Todhunter 1996). On a dry weight basis GAGs form 7.3-10% of the cartilage ECM (Vachon *et al* 1990). As cartilage is broken down GAGs will be released into SF and concentrations are likely to increase. However, researchers have obtained conflicting results regarding the GAG content in SF in OA, with some finding elevated levels (Alwan *et al* 1990, 1991, Palmer *et al* 1995, Saxne *et al* 1985), others finding no difference (Fuller *et al* 2001, Little *et al* 1990, Myers *et al* 1996, van den Boom *et al* 2004b) and others still found lower levels in OA joints (Belcher *et al* 1997). However, GAGs in SF have been promoted by some as markers of cartilage turnover, both in human (Heinegard *et al* 1987, Saxne *et al* 1986) and equine studies (Palmer *et al* 1995).

We have previously shown that exercise affects TNF-α concentrations in equine joints and that repeated arthrocentesis leads to increased MMP activity in SF from healthy equine joints (van den Boom *et al* 2004a). It seems likely that other potential markers will also be influenced by these interventions and that changes in the concentration of cytokines and eicosanoids and MMP activity will influence cartilage metabolism, leading to the release of matrix components into SF.

The present study was designed to test the hypothesis that NO, PGE_2 and GAG concen-

trations in SF would be affected by exercise and repeated arthrocentesis. Therefore, prostaglandin and GAG concentrations were assessed in synovial fluid obtained from 3 different normal joints (metacarpophalangeal, radiocarpal and tarsocrural), and NO in SF from the metacarpophalangeal joint after repeated arthrocentesis and exercise.

Materials and methods

Collection of synovial fluid

Fourteen Dutch Warmblood horses and two Welsh ponies, 9 mares and 7 geldings, mean age 11.2 years (range 5-17 years) were used. The horses were assigned randomly to the exercise (8 horses) or control group (8 horses) with mares and geldings divided as evenly as possible over the two groups (exercise group, 4 geldings and 4 mares; control group, 3 geldings and 5 mares) in order to avoid a possible effect of gender. The age of the animals did not differ significantly between the 2 groups. All animals were clinically healthy and sound, kept stabled during the experimental period and fed a diet of commercial pellets and hay. No lameness was observed during the experiment.
After clipping, shaving and disinfection, arthrocentesis of the left metacarpophalangeal, radiocarpal and tarsocrural joints was carried out in a sterile manner in all horses and SF samples were collected in plain syringes. The mean (± s.e.) volume of SF collected was 3.0 ± 0.2, 3.3 ± 0.2 and 4.4 ± 0.2 ml from the metacarpophangeal, radiocarpal and tarsocrural joints, respectively. The amount of SF collected for each horse was approximately the same at each timepoint. Synovial fluid was transferred into plain tubes and centrifuged for 10 minutes at 10,000 g, aliquoted and stored at -80ºC until further processing, all within an hour of collection.
Horses in the exercise group were acclimated to the treadmill during the week before being subjected to a 5 day training regimen of increasing intensity. This consisted of 16 mins walking, 20 mins trotting and 4 mins canter on the treadmill followed by 30 mins in the horse-walker, on the first day. The intensity was increased each day and the periods at a walk, trot and canter reached 14, 18 and 8 mins, respectively by Day 5, again followed by 30 mins in the horse-walker. The horses in the control group were confined to their stables with 5 mins of handwalking each day. Synovial fluid was collected 14, 14.5, 17 and 24 days after the start of the experiment from the aforementioned joints in all horses (including the control group) as described above.
The study was approved by the University's Ethics Committee, in compliance with the Dutch Act on Animal Experiments.

Determination of nitric oxide production

Nitric oxide production was detected by measuring the amount of nitrite (NO^{2-}) formed according to the Griess reaction (Green *et al* 1982), with some modifications. In brief, samples were first treated with nitrate reductase and NADPH for 30 mins at room temperature, after which time they were deproteinised by the addition of methanol:diethylether (3:1 v/v) for 60 mins. Following centrifugation (10,000 rpm, 10 mins at 4ºC) acidified

sulphanilamide (1% w/v) was added to aliquots of cell supernatant, followed by addition of N-napthylethylenediamine dihydrochloride (1.4% w/v). The absorbance was measured at 540 nm using a Biorad 3550 microplate reader equipped with a spectrophotometer (Biorad Laboratory). The amount of nitrite formed was calculated according to the standard curve ($NaNO_2$) and NO expressed in µM.

Prostaglandin E_2 assay

Prostaglandin E_2 concentrations were determined using mass spectrometry (MS). Briefly, 700 µl SF and 10 µl internal standard (10 pmol/µl $PGF_{2\alpha}$) were incubated for 1 h at 37°C with 10 µl hyaluronidase (10 mg/ml), after which samples were centrifuged. The supernatant was applied to L1 Chrolut columns, which had been pretreated with 1ml aceton followed by 1 ml water, and flushed using 1 ml water, 1 ml 5% ethanol and 1 ml hexane successively. Prostaglandins were eluted from the column using 400 µl ethylacetate and reconstituted with 30 µl water. Prostaglandin concentrations were measured by MS using an API-365 triple quadrupole mass spectrometer (PE Biosystems), equipped with an electrospray ion source. Negative-mode MS was performed at a spray voltage of - 4.5 kV. Prostaglandins were separated at a flow rate of 200 µl/min on a Hypersil ODS column (Hewlett Packard) using a non-linear gradient from 2.5 mM ammonium acetate (pH 5.6) (eluate A), to acetonitrile : methanol (95:5 v/v), containing 2.5 mM ammonium acetate (eluate B). The column effluent was introduced into the mass spectrometer and for PGE_2 the transition between m/z 351 and m/z 271 was monitored. The PGE_2 concentration was expressed as pg/ml SF.

Glycosaminoglycan assay

The proteoglycan content of SF was estimated by measuring glycosaminoglycan (GAG) using the 1,9-dimethylmethylene blue (DMMB) metachromatic dye assay modified for use in microtitre plates. After papain digestion GAGs were precipitated and stained with 1,9-dimethyl-methylene blue (Blyscan kit, Biocolor Ltd.) and staining was quantified by measuring absorbance at 656 nm. Shark cartilage chondroitin sulphate (Sigma) served as standard. Results were expressed as µg GAG/ml SF.

Statistical analysis

Statistical analysis was performed using the SPSS software package (SPSS Inc., version 10.0). Normality was assessed using the Kolmogorov-Smirnov method. The GAG data were found to be normally distributed, but this was not the case for NO and these data were log transformed, to allow the use of parametric tests. Differences between groups and joints were tested for significance using an unpaired t-test. When no difference between exercise and control group was present data were combined to determine the influence of repeated arthrocentesis. A one-way ANOVA, followed by Bonferroni correction, was used to determine whether significant differences existed between timepoints. The level of significance was set at $P<0.05$.

Results

Nitric Oxide

There were no (significant) differences between the exercise and control groups and data were combined to investigate the result of repeated arthrocentesis. Nitric oxide in the metacarpophalangeal joint was significantly affected by arthrocentesis, with concentrations being significantly increased compared to baseline on Day 14.5, when arthrocentesis was repeated after an interval of 10 h (Fig 1). The NO concentration on Day 14.5 was higher in control horses than in the exercise group, but the difference was not statistically significant. When more than 10 h had passed following previous arthrocentesis NO levels were not increased.

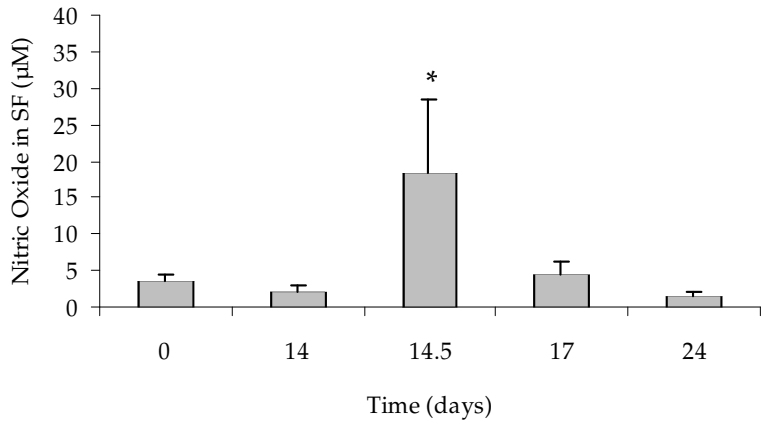

***figure 1**: Nitric oxide concentration (µM; mean ± s.e.m.) in SF from 16 horses (exercise and control groups combined), showing the increase as a result of repeated arthrocentesis. * P<0.05 with respect to baseline values (day 0).*

Prostaglandin E_2

Exercise caused a significant increase in PGE_2 levels in SF on Day 14, 2 h after the end of the last exercise session in all joints studied (Fig 2). Arthrocentesis also caused increased PGE_2 concentrations on Day 14.5, when arthrocentesis was performed with an interval of 10 h. This difference was statistically significant for the radiocarpal (Fig 2b) and tarsocrural (Fig 2c) joints.

Glycosaminoglycans

Glycosaminoglycan concentrations were not influenced by the exercise programme the horses were subjected to but repeated arthrocentis caused an increase in the GAG

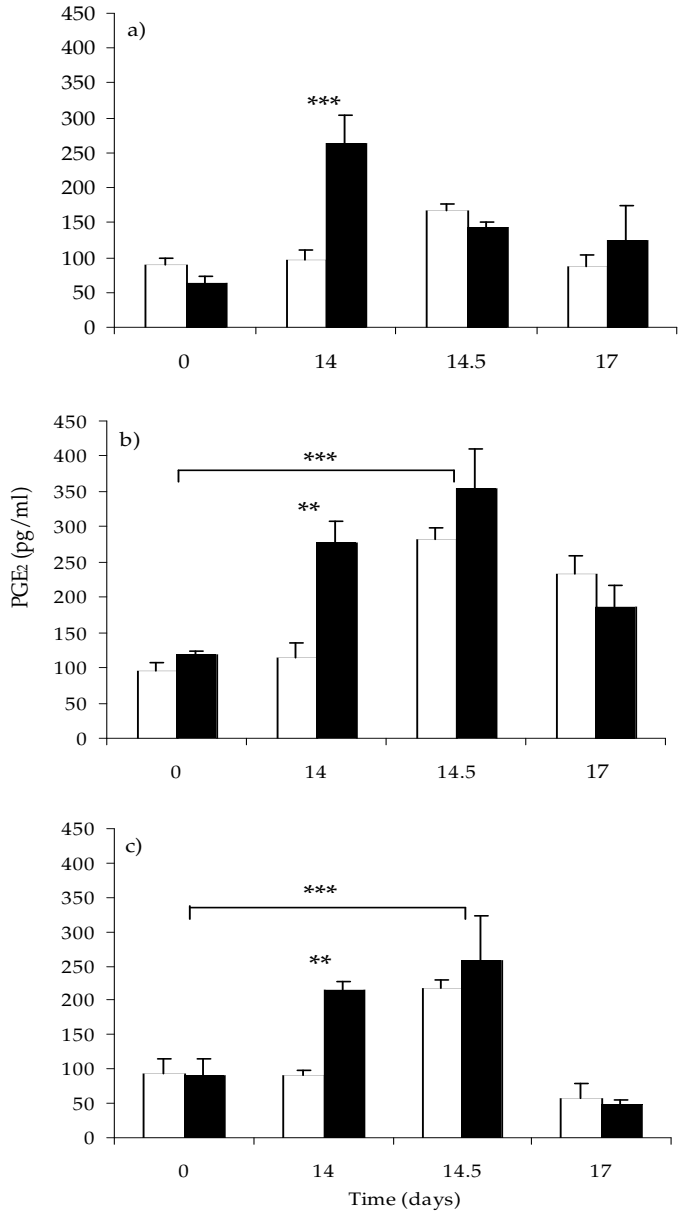

***Figure 2**: Prostaglandin E_2 (PGE_2) concentration (pg/ml; mean ± s.e.m.) in synovial fluid of horses in the exercise (solid bars) and control (open bars) group vs. time for a) metacarpophalangeal, b) radiocarpal and c) tarsocrural joints. The horses in the exercise group were subjected to a treadmill exercise programme between days 0 and 14. The influence of repeated arthrocentesis is reflected by the increased PGE_2 concentration on day 14.5. ** P<0.01 *** P<0.001*

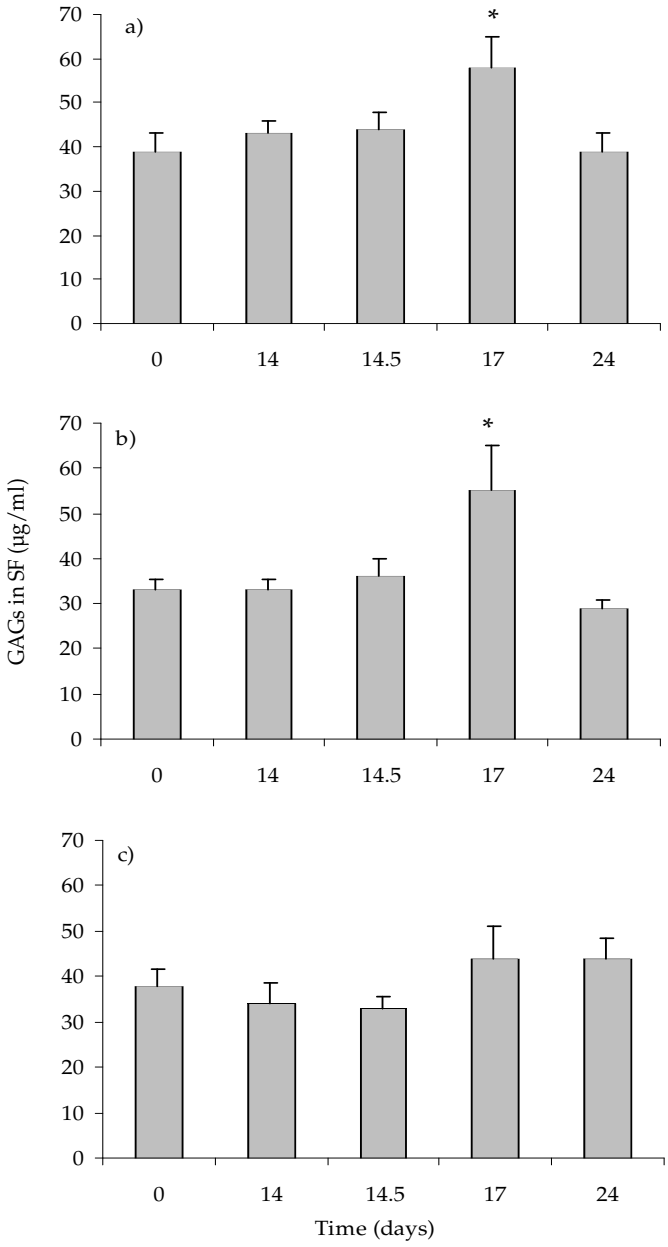

***Figure 3**: Glycosaminoglycan (GAG) concentration (µg/ml; mean ± s.e.m.) in synovial fluid from 16 horses (exercise and control groups combined) from the a) metacarpophalangeal, b) radiocarpal and c) tarsocrural joints, showing the increase as a result of repeated arthrocentesis. * P<0.05 with respect to baseline values (day 0).*

concentration in SF from the metacarpophalangeal (Fig 3a) and radiocarpal joint (Fig 3b) on Day 17, 60 h after previous arthrocentesis. This effect was not present in the tarsocrural joint (Fig 3c).

Discussion

There was a clear effect of repeated arthrocentesis on NO levels in SF from the MCP joint, similar to that previously demonstrated for MMP activity (van den Boom *et al* 2004a), although the timing was slightly different. Nitric oxide levels in the MCP joint peaked on Day 14.5 (10 h after previous arthrocentesis), while MMP activity reached the highest recorded activity on Day 17 (60 h after previous arthrocentesis). The effect was short-lived and NO levels were no longer elevated when arthrocentesis was performed after an interval of 60 h. Arthrocentesis involves only the synovial membrane (and not the articular cartilage) and it would seem that the NO is liberated by equine synoviocytes, upon stimulation. The nature of stimulation could be simply mechanical (the physical act of perforating the synovial membrane) or a localised inflammatory reaction caused by cellular disruption. It is also possible that NO is released by chondrocytes, following stimulation by a mediator derived from the synovial membrane. Exercise caused no additional NO release in the joints studied, and NO concentrations in control joints were actually higher than in exercised joints (although the difference was not statistically significant). This would suggest inflammatory rather than mechanical stimulation. Dynamic compressive strain inhibited NO synthesis in equine chondrocytes (Wiseman *et al* 2003) and mechanical stimulation also inhibited NO synthesis when chondrocytes and synoviocytes were co-cultured (D'andrea *et al* 1998). It is possible that exercise attenuated the NO production induced by arthrocentesis, although the effect of arthrocentesis was greater. The NO synthesised following repeated arthrocentesis probably leads to production and/or activation of other substances which may affect joint homeostasis. For example, NO has been shown to augment the expression and activation of MMPs (Murrell *et al* 1995) and thus NO could play a role in the MMP activation in SF from the MCP joint previously reported (van den Boom *et al* 2004a).

Both exercise and repeated arthrocentesis caused an elevation in PGE_2 concentration in SF, confirming that it is a very sensitive indicator of changes within the joint. Previous research has suggested that PGE_2 is released primarily by the synovial membrane and that it reflects synovial inflammation (Kirker-Head *et al* 2000, von Rechenberg *et al* 2000). Both exercise and arthrocentesis affect the synovial membrane, although the former will also have a mechanical influence on the articular cartilage. We are not aware of any study investigating the influence of exercise on prostaglandin concentrations in SF but Hardy *et al* (1998b) suggested that the wide variation found in PGE_2 in SF from the equine metacarpophalangeal joint in their study was caused by surgical manipulation. The results of this study and those of Hardy *et al* (1998b) indicate that movement of the joint is sufficient to cause increased PGE_2 concentrations in SF. The rise in PGE_2 seen after exercise could be the result of the last exercise session or the cumulative result of a week's exercise. Prostaglandin concentrations have been shown to rise very quickly after experimental induction, so it certainly seems possible that a single exercise session could induce

such a rise, although this cannot be ascertained with certainty from this study. The PGE_2 concentration was maximal on Day 14.5, but this increase was almost certainly the result of arthrocentesis performed on Day 14, and was still elevated when arthrocentesis was repeated after 60 h. We can therefore conclude with certainty that the effect of arthrocentesis on PGE_2 concentrations has passed after 2 weeks and concentrations have passed their peak 60 h after arthrocentesis.

The role of PGE_2 in joint disease is not entirely clear, with both protective and catabolic effects having been described, but Mehindate *et al* (1995) showed that PGE_2 plays a role in collagenase gene expression in human chondrocytes. This mechanism may also be responsible for the increased MMP activity observed in SF (van den Boom *et al* 2004a), which coincides with or follows the increase in PGE_2 concentration.

We found no difference in (baseline) GAG concentration between the various joints studied, while Fuller *et al* (1996) reported differences between metacarpophalangeal, distal and proximal interphalangeal joints. The GAG concentration may be related to joint size as the concentration increased with decreasing joint size (Fuller *et al* 1996). Repeated arthrocentesis caused a significant GAG release in the metacarpophalangeal and radiocarpal joints and is likely to have been caused, at least in part, by the increased MMP activity which also resulted from repeated arthrocentesis, as the GAG release occurred at the same time as maximal MMP activity. Aggrecanases are also likely to play a role in proteoglycan degradation, but these were not assayed in our studies. The GAG release was obvious and statistically significant in the metacarpophalangeal and radiocarpal joints, but not in the tarsocrural joints, which is similar to the findings regarding MMP activity (van den Boom *et al* 2004a). We speculate that the stimulus which initiates the cascade of events leading to GAG release involves the synovial membrane and because MCP and radiocarpal joints have a smaller volume than the tarsocrural joint the markers released will be further diluted in the latter and effects less obvious. The GAG release observed in control joints was greater than in those from the exercise group, although the difference was not statistically significant. Van den Hoogen *et al* (1998a) showed that in ponies exercise led to a decrease in GAG release compared to box-rest and this might have had a small effect on our results, but was certainly overshadowed by the influence of repeated arthrocentesis, as discussed above for NO.

The GAG concentration in SF is considered by some to be an indicator of joint disease, but as this study showed, repeated arthrocentesis also caused increased GAG concentrations in SF from healthy joints. The GAG concentration in SF was increased 1.5 times in the metacarpophalangeal joint and 1.7 times in the radiocarpal joints. The GAG levels had returned to normal one week later, showing that they are rapidly cleared from the joint. This confirms findings of Antonas *et al* (1973), who found that GAGs are cleared from the joint within 24 h, and according to Myers *et al* (1996) this clearance occurs even faster in inflamed joints. This illustrates a definite drawback to the use of GAG levels in SF for monitoring joint disease, as proteoglycan degradation has to be ongoing for GAG levels to be increased. Osteoarthritis in horses presents in an intermittent pattern, with periods of activity (lameness) and remission, and GAG concentrations might well be a more accurate marker of joint inflammation, rather than reflecting the overall OA status of the joint. The rapid clearance of GAGs from the joint space may also explain the

discrepancy between studies regarding the elevation of synovial GAG levels in joint disease. We have also shown that GAG concentrations in SF do not reflect the proteoglycan content of articular cartilage in the equine metacarpophalangeal joint (van den Boom *et al* 2004e).
Repeated arthrocentesis leads to the release and/or activation of several potential markers involved in the degradation of cartilage in normal joints and is a serious confounding factor for the assessment of biomarker levels in joint disease. The timing and duration of these 'athrocentesis effects' varies per biomarker but it would seem prudent to wait at least one week before repeating arthrocentesis (of the same joint) when studying markers of joint disease. Moderate exercise also caused a transient rise in PGE_2 concentrations in the radiocarpal and tarsocrural joints but did not influence the other parameters measured. The results of this study may help us in understanding the processes which occur within the equine joint and the complex interactions between various mediators of disease.

Acknowledgements

The authors would like to express their gratitude to Marjory Pollak and Andries Klarenbeek for their assistance during sample collection.

Chapter V

Assessment of the effects of age and joint disease on hydroxyproline and glycosaminoglycan concentrations in synovial fluid from the metacarpophalangeal joint of horses

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Summary

Objective: To assess the effects of age and joint disease on hydroxyproline and glycosaminoglycan (GAG) concentrations in synovial fluid from the metacarpophalangeal joint of horses, and evaluate the association of those concentrations with severity of osteoarthritis and general matrix metalloproteinase (MMP) activity.

Sample population: Synovial fluid was collected from the metacarpophalangeal joints of foals at birth (n = 10), 5-month-old foals (10), 11-month-old foals (5), and adult horses (73).

Procedure: Hydroxyproline and GAG concentrations were determined in synovial fluid samples. The severity of osteoarthritis in adult joints was quantified by use of a cartilage degeneration index (CDI) and assessment of general MMP activity via a fluorogenic assay.

Results: Hydroxyproline and GAG concentrations in synovial fluid were highest in neonates, and decreased with age. Concentrations reached a plateau in adults by 4 years, and remained constant in healthy joints. In synovial fluid from osteoarthritic joints, hydroxyproline and GAG concentrations were not increased compared with unaffected joints but hydroxyproline levels were significantly correlated with the CDI and with general MMP activity. There was no significant correlation between GAG concentration and CDI value or MMP activity.

Conclusions and Clinical Relevance: Changes in hydroxyproline concentration in synovial fluid appeared to indicate damage to collagen of the articular cartilage. In joints with osteoarthritis, the lack of high GAG concentration in synovial fluid and the absence of a significant correlation between GAG concentration and CDI values or MMP activity may severely limit the usefulness of this marker for monitoring equine joint disease.

Introduction

The articular surfaces of the bones that form diarthrodial joints are covered with a layer of hyaline cartilage, which consists of a limited number of chondrocytes in an abundant extracellular matrix made up mainly of collagen, proteoglycans, and water. On a dry weight basis, collagen (which is mainly type II) composes 50% to 80% of the cartilage matrix, (Brama *et al* 2000b, Todhunter 1996, Vachon *et al* 1990) whereas glycosaminoglycans (GAGs) compose 7.3% to 10% (Vachon *et al* 1990). Type-II collagen, like all fibrillar collagens, is made up of a triple helix consisting of 3 α chains; the amino acid sequence of an α chain is glycine-X-Y, where X and Y are often proline or hydroxyproline (Todhunter 1996). Hydroxyproline composes approximately 10% of the amino acids in collagen and because it is present only in very small quantities in other proteins it is often used to estimate the amount of collagen in tissues (Johnson *et al* 1980). The proteoglycans consist of core proteins to which large quantities of GAGs are attached, mainly chondroitin sulphate and keratan sulphate (Todhunter 1996). These GAGs contain negatively charged sulphate groups which strongly attract water. The core proteins of the proteoglycans are connected by link proteins to hyaluronic acid molecules that are attached to the collagen fibrils. This collagen fibril network interspersed with strongly hydrophilic proteoglycans ensures the unique biomechanical properties of articular cartilage, combining compressive stiffness and resilience.

In cartilage, matrix synthesis and degradation are balanced to achieve growth, remodelling, or equilibrium, depending on the age and disease status of the animal (Todhunter 1996). In mature rabbits and dogs, overall proteoglycan turnover time of articular cartilage is approximately 300 days (Maroudas 1980). However, compared with turnover of proteoglycans, collagen turnover times in adult dogs and humans are long, having been estimated at more than 100 years (Maroudas 1980, Maroudas *et al* 1992, Verzijl *et al* 2000). There are 2 conditions in which the metabolic rate of articular cartilage extracellular matrix is high: in young growing animals and in joints affected by disease, principally osteoarthritis. In equine neonates, there is a substantial and rapid increase in collagen content of the articular cartilage of the proximal phalanx between birth and 5 months, and a further increase in yearlings. During these same periods, the GAG content of the cartilage decreases (Brama *et al* 1999a, 2000b). Furthermore, differences in the collagen and GAG content of articular cartilage at various sites are detectable by 5 months, although these site differences are absent at birth (Brama *et al* 2002). These changes in collagen and GAG content and the development of site differences in foals and young horses indicate a much more rapid metabolism in articular cartilage, compared with that of adult horses. In osteoarthritis, there is a progressive loss of articular cartilage from the joint surface; as a consequence, the cartilaginous tissue increases extracellular matrix metabolism in an attempt to repair the damage. The extracellular matrix turnover that occurs physiologically in growing animals and the cartilage degradation associated with osteoarthritis are partially mediated by matrix metalloproteinases (MMPs). This group of zinc-dependent enzymes is composed of stromelysins, collagenases, gelatinases, and membrane-bound MMPs; of these enzymes, collagenases (MMP-1, -8, and -13) cleave the interstitial collagen triple helix, gelatinases (MMP-2 and -9) act on unwound collagen,

and stromelysins degrade proteoglycans (Murphy *et al* 1990). In synovial fluid from fetuses and young horses, MMP activities are much higher than those of adult horses, which suggests a role for these enzymes in the functional adaptation of articular cartilage (Brama *et al* 1998). Results of several studies in humans (Hembry *et al* 1995, Lohmander *et al* 1994, Manicourt *et al* 1994) and in horses (Brama *et al* 1998, 2000d, Clegg *et al* 1997b, Clegg and Carter 1999) have suggested that MMPs are involved in the breakdown of articular cartilage in degenerative joint disease.

Osteoarthritis is one of the most common causes of lameness in horses, and leads to the untimely retirement of many equine athletes (Rossdale *et al* 1985). To a large extent, this outcome is a consequence of the lack of a means of diagnosing the disorder in an early phase, when its development might be forestalled. The radiographic changes associated with osteoarthritis are only detected in advanced stages of disease; furthermore, those changes are poorly correlated with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996). Marked articular cartilage degeneration may be present in a joint despite its normal radiographic appearance (Lohmander *et al* 1992). This has led to a search for biomarkers that would be useful in the detection of early changes associated with joint disease. During physiologic extracellular matrix turnover (ie, growth and maturation) and pathologic degradation (ie, osteoarthritis) of articular cartilage, components of collagen molecules and proteoglycans (such as hydroxyproline and GAGs, respectively) are released into the synovial fluid. Hydroxyproline and GAG concentrations in synovial fluid are therefore potentially useful as biological markers of osteoarthritis. In fact, it has been suggested that concentrations of GAGs in synovial fluid are useful markers for the severity of joint disease (Alwan *et al* 1990, 1991). To date, the concentration of hydroxyproline in synovial fluid has not been investigated for the identification of diseased joints. The objectives of the study reported here were to assess the effects of age and joint disease on hydroxyproline and GAG concentrations in synovial fluid from the metacarpophalangeal joint of horses, and evaluate the association of those concentrations with severity of osteoarthritis and general MMP activity. In addition the potential value of hydroxyproline concentration in synovial fluid as an indicator of pathologic alterations in metabolic activity of the extracellular matrix of cartilage was evaluated.

Materials and Methods

Horses

Ten newborn foals, ten 5-month-old foals, five 11-month-old foals, and 73 adult horses were included in the study. The foals were part of a very large research project evaluating the influence of exercise on the development of the musculoskeletal system of foals up to age 11 months (van Weeren and Barneveld 1999), which was approved by the University's Ethics Committee and conducted in compliance with the Dutch Act on Animal Experiments. Specimens from adult horses were collected immediately after they were killed by normal slaughter (shot and desanguinated) at an abattoir. The age of the horses was estimated by examination of the lower incisors. No clinical data about the horses was available.

Collection of synovial fluid

After clipping, shaving, and disinfecting the skin of the metacarpophalangeal region, synovial fluid from both metacarpophalangeal joints of each foal was collected in 5-ml syringes following arthrocentesis performed in a sterile manner using a 40-mm, 21-gauge needle. If necessary foals were restrained with the use of a twitch or sedated with detomidine (Domosedan, Pfizer Animal Health). In the adult horses synovial fluid from both metacarpophalangeal joints was aspirated into 5-ml syringes, using a 40-mm, 18-gauge needle, within half an hour of death, following disinfection of the skin. After collection synovial fluid was centrifuged, aliquotted and stored in plain tubes at -80°C until analysis (approximately one year later). In addition to collection of synovial fluid specimens, the distal limbs of the 73 adult horses were isolated by disarticulation in the carpometacarpal joint and stored at -20°C (for approximately six months) until further processing. For this study only samples from the right forelimb (synovial fluid and distal limb) from each horse were used.

Hydroxyproline assay

Synovial fluid hydroxyproline concentration was determined by use of high performance liquid chromatography (HPLC) following derivitization with a fluorescent label of secondary amino acids (proline and hydroxyproline), as described (Bank *et al* 1997). In short, synovial fluid (2 to 4 µl) was hydrolyzed in 1 ml of 6M HCl (for 20 h at 110°C), dried overnight (approximately 17 hours) under vacuum (Speedvac, Thermo Savant) and dissolved in 100 µl of water (Milli Q®, Millipore Corp.). An aliquot was transferred to an HPLC insert and placed in the autoinjector of an HPLC system consisting of a high precision HPLC pump (model 480, Gynkotek), an autosampler (Sparks Holland Triathlon, Spark Holland), and a spectrofluorometric detector (model 821 FP, Jasco). Automated derivitization consisted of blocking of primary amino acids with o-phthaldialdehyde (Sigma), followed by labeling of secondary amino acids with 9-fluorenylmethyl chloroformate. Derivitized amino acids were injected onto a reversed phase HPLC column (TSK ODS 80™, 4.6 x 150 mm, Tosoh Biosep, Tosoh Corp.), and eluted with a tertiary gradient of citrate, acetonitrile, and methanol, as described (Bank *et al* 1996). Fluorescence was monitored at 254 nm (excitation) and 630 nm (emission) and data were recorded on-line by a computing integration system (Chromeleon™ version 4.30, Dionex). The amino acid standard for collagen hydrolysates (Sigma) served as a reference. The intra-assay variation was 3% and the interassay variation was 10%.

Glycosaminoglycan assay

Proteoglycan content of synovial fluid was estimated by measuring glycosaminoglycan concentration by use of the 1,9-dimethylmethylene blue (DMMB) metachromatic dye assay (modified for use in microtiter plates). After papain digestion, GAGs were precipitated and stained with DMMB (Blyscan kit, Biocolor Ltd.) and staining was quantified via measurement of absorbance at 656 nm. Shark cartilage chondroitin sulphate (Sigma)

served as standard. Results were expressed as µg of GAG per ml of synovial fluid.

General MMP activity assay

Matrix metalloproteinase activity was determined in the synovial fluid of adult horses by use of a slight modification of the fluorometric assay described by DeGroot *et al* (2001b). Briefly, conversion of the internally quenched fluorogenic substrate TNO211-F (2.5 µM; Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) was measured in the presence of an EDTA-free general proteinase inhibitor cocktail (Complete, Roche Diagnostics) to prevent conversion of the substrate by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence or absence of MMP inhibitor BB94 (10 µM). Because the substrate is not cleaved by aggrecanases, this approach detects only MMP-mediated substrate conversion and indicates the overall MMP activity in the synovial fluid samples.

Quantification of cartilage damage

In the metacarpophalangeal joints removed form the adult horses, the degree of cartilage damage (proteoglycan loss) was determined by use of a cartilage degeneration index (CDI) described by Brommer *et al* (2003a). Briefly, the limbs were thawed for two days at 7ºC, the metacarpophalangeal joints were opened and the proximal third of the proximal phalanx was isolated by transecting the proximal phalanx with a band saw. All surrounding soft tissues were trimmed away and the proximal articular surface of the proximal phalanx was stained with Indian ink. Digital images were obtained before and after staining, from which the mean grey pixel values were calculated by use of a validated algorithm. As Indian ink uptake is related to cartilage degradation, the increase in mean grey pixel value after staining is a measure of the relative amount of degenerated cartilage across the entire joint surface, represented as the CDI (%). These CDI values have been shown to have excellent correlation with a macroscopic grading system for osteoarthritis. For the purposes of this study, CDI values < 25% were considered indicative of unaffected (ie, healthy) cartilage, whereas CDI values ≥ 25% represented various degrees of osteoarthritis.

Statistical analyses

Differences in hydroxyproline and GAG concentrations between age groups were tested by use of a 1-way ANOVA. Correlations between hydroxyproline or GAG concentrations and MMP activity and CDI values were calculated by use of Pearson's correlation coefficient. Values of $P < 0.05$ were considered significant.

Results

Adult horses

Of the 73 adult horses used in the study, 13 were aged 1 to 4 years, 14 were aged 5 to 8 years, 15 were aged 9 to 12 years, 19 were aged 13 to 16 years, and 12 were > 16 years of age. Unfortunately, the origin of the horses was unknown and no clinical data was available.

Synovial fluid samples

The volume of synovial fluid (mean ± s.d.) collected from the joints of the neonates, 5-month-old foals, 11-month-old foals, and adult horses was 0.5 ± 0.1, 1.3 ± 0.3, 2.5 ± 0.5, and 4.6 ± 1.0 ml, respectively. Because the volume of synovial fluid collected per joint was variable there was not always enough for all assays to be performed for each animal and the number of samples measured is indicated in the figures. Because only a small amount of synovial fluid was obtained from the 11-month old foals and as it had previously been used for other assays there was only sufficient volume for determination of hydroxyproline concentration (but not for the GAG assay). The hydroxyproline and GAG assay were performed on 92 (67 adults + 25 foals) and 88 (68 adults + 20 foals) samples respectively, while the MMP assay was conducted on 71 adult samples.

Age effects

Of the unaffected joints, the highest hydroxyproline concentrations were detected in the synovial fluid of newly born foals (Fig 1), these being more than 11 times as great as those in mature horses ($P < 0.001$). The hydroxyproline concentrations decreased rapidly with age; the most notable decrease occurred during the first 5 months after birth. After the age of 4 years, no additional effects of aging were observed and hydroxyproline concentrations in unaffected joints remained stable throughout the entire life-span of the horses (Pearson correlation between hydroxyproline concentration and age of horses > 4 years: $r = 0.05$; $P > 0.05$). A similar pattern of change with age was detected for GAG concentrations in synovial fluid (Fig 2) with adult values being less than one-fourth of those in neonates.

Osteoarthritis

Of the 73 metacarpophalangeal joints examined from 73 adult horses, 56 were considered unaffected (CDI value, < 25%) and 17 had osteoarthritis (CDI value, ≥ 25%). Hydroxyproline data were available for 52 unaffected and 15 osteoarthritic joints and GAG data for 52 and 16 joints respectively. The mean ± s.e.m. age of horses from which unaffected joints and joints with osteoarthritis were obtained was 11.1 ± 0.7 and 12.8 ± 1.3 years, respectively; this age difference was not significant. The hydroxyproline concentration in synovial fluid obtained from unaffected joints and joints with osteoarthritis

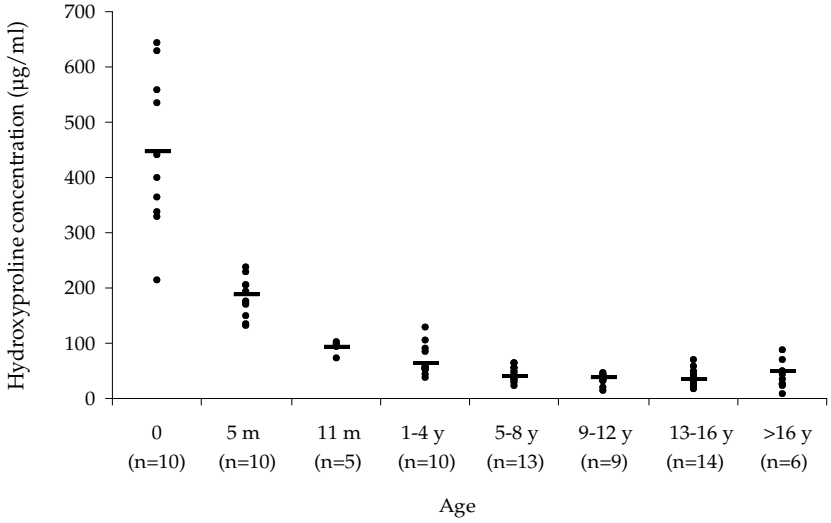

***Figure 1**: Hydroxyproline concentration in synovial fluid samples obtained from unaffected metacarpophalangeal joints of 77 horses, grouped by age (range, 0 to 23 years). Joints were classified as unaffected (ie, healthy) on the basis of a cartilage degeneration index (CDI) assessment (CDI value, < 25%). Horizontal bar represents mean concentration of group.*

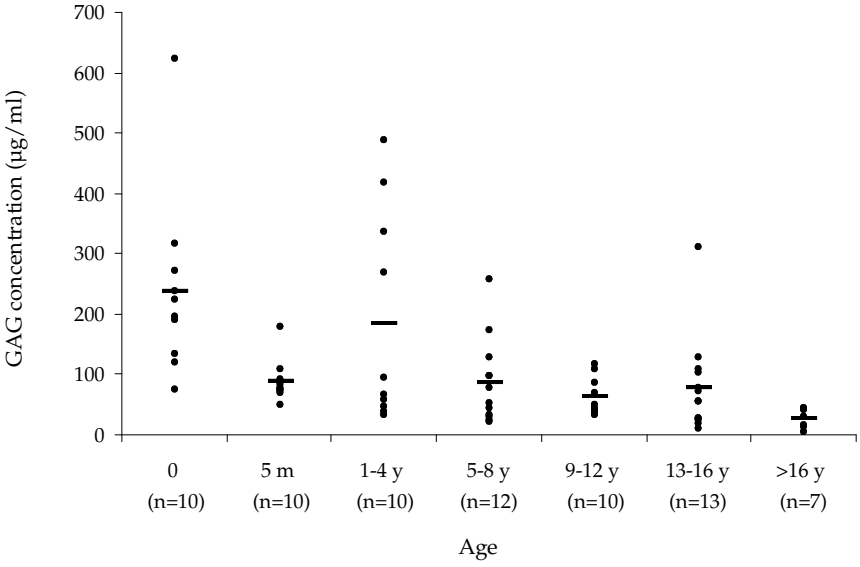

***Figure 2**: Glycosaminoglycan (GAG) concentration in synovial fluid samples from unaffected (CDI value, < 25%) metacarpophalangeal joints of 72 horses, grouped by age (range, 0 to 23 years). Horizontal bar represents mean concentration of group.*

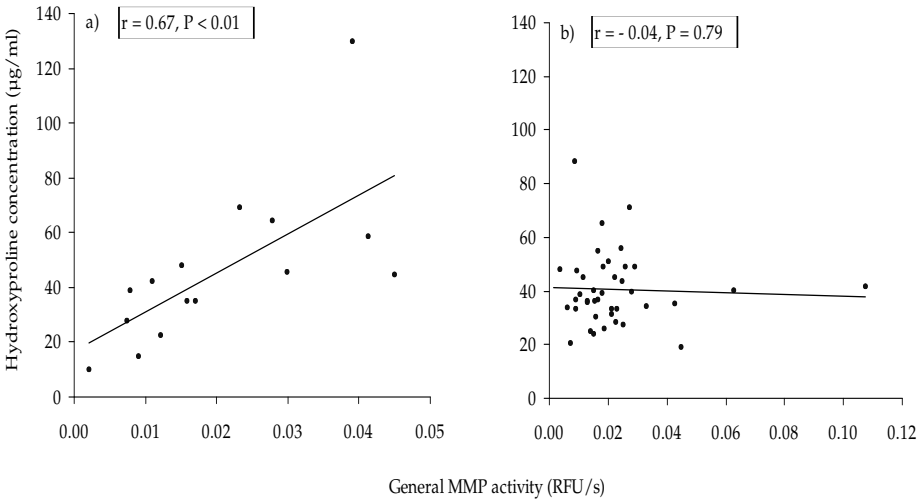

***Figure 3**: Hydroxyproline concentration in relation to general MMP activity (relative fluorescence units [RFU]/s) in synovial fluid samples from a) osteoarthritic (CDI value, ≥ 25%; n = 15) and b) unaffected (CDI value, < 25 %; 40) metacarpophalangeal joints in mature (> 4 years) horses. r = correlation coefficient (represented by the line).*

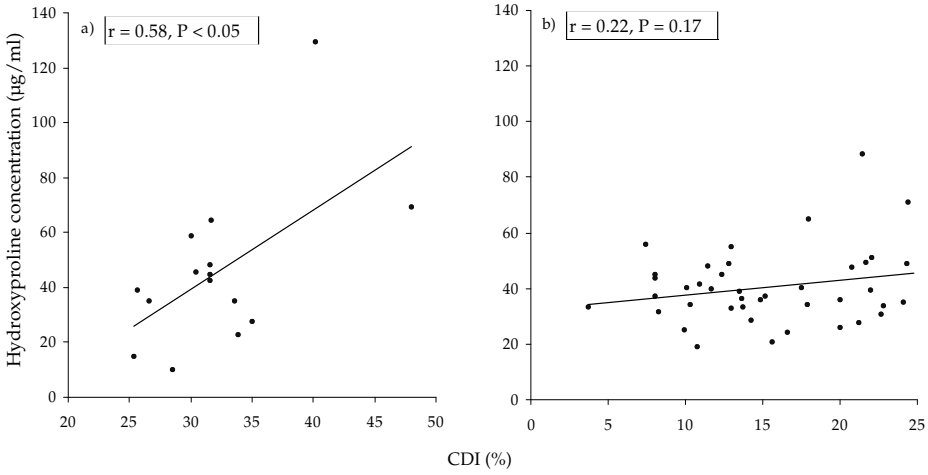

***Figure 4**: Hydroxyproline concentration in relation to the CDI in synovial fluid samples from a) osteoarthritic (CDI value, ≥ 25%; n = 15) and b) unaffected (CDI value, < 25%; 41) metacarpophalangeal joints in mature (> 4 years) horses. r = correlation coefficient (represented by the line).*

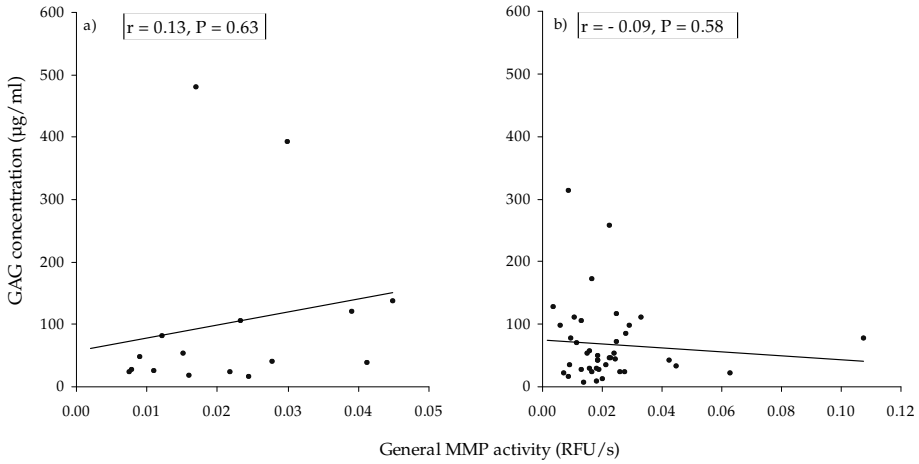

***Figure 5**: Glycosaminoglycan (GAG) concentration in relation to general MMP activity in synovial fluid samples from a) osteoarthritic (CDI value, ≥ 25%; n = 16) and b) unaffected (CDI value, < 25%; 41) metacarpophalangeal joints in mature (> 4 years) horses. r = correlation coefficient (represented by the line).*

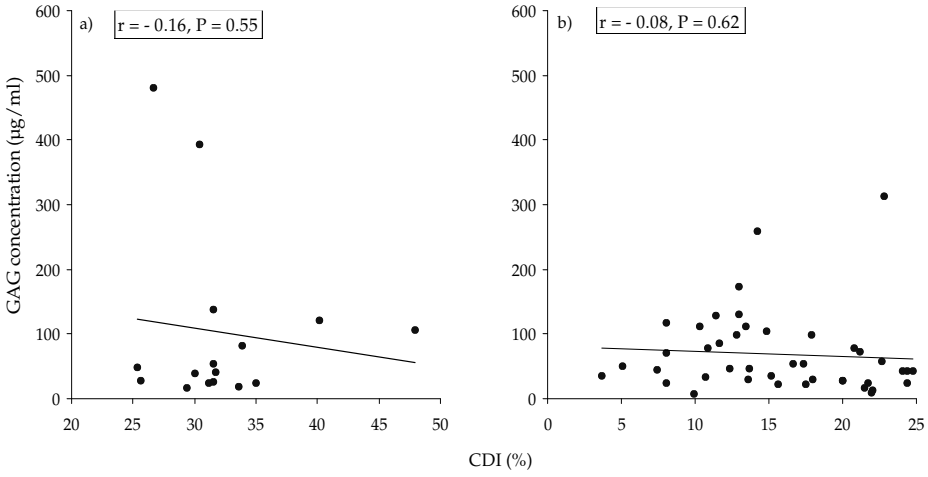

***Figure 6**: Glycosaminoglycan (GAG) concentration in relation to CDI in synovial fluid samples from a) osteoarthritic (CDI value, ≥ 25%; n = 16) and b) unaffected (CDI value, <25 %; 42) metacarpophalangeal joints in mature (> 4 years) horses. r = correlation coefficient (represented by the line).*

was not significantly different (41.1 ± 2.1 and 45.8 ± 7.4 μg/ml, respectively). The GAG concentration in the synovial fluid obtained from unaffected joints and joints with osteoarthritis was also not significantly different (84 ± 14 and 102 ± 34 μg/ml, respectively). In the group of horses with osteoarthritis (CDI value, ≥ 25%), there was a significant correlation ($r = 0.67$; $P < 0.01$) between hydroxyproline concentration and general MMP activity in synovial fluid (Fig 3a) and a slightly weaker but significant ($r = 0.58$; $P < 0.05$) correlation between hydroxyproline content and the CDI value (Fig 4a). These correlations were not present in unaffected joints from mature (> 4 years) horses (Fig 3b and 4b). There were also no significant correlations between GAG concentration and MMP activity or GAG concentration and CDI value for unaffected joints or joints with osteoarthritis (Fig 5 and 6).

Discussion

Results of the study reported here suggest that hydroxyproline and GAG concentrations in synovial fluid of horses are strongly related to age. The high concentrations detected in the synovial fluid of equine neonates indicate the rapid turnover of the articular cartilage matrix during the first months of life. In the synovial fluid of neonates, high hydroxyproline and GAG concentrations coincided with high MMP activity, which supports the suggestion that these enzymes are involved in growth-related cartilage turnover (Brama *et al* 1998, 2000d).

As horses mature, the concentrations of hydroxyproline and GAG in synovial fluid decrease; at maturity (ie, > 4 years of age), these concentrations have stabilized. Hydroxyproline concentration in synovial fluid of adult horses was approximately 9% of fetal concentration. The fact that hydroxyproline concentration was not correlated with age in adult horses is in accordance with the finding that the collagen content of articular cartilage does not change significantly with age in adult horses (Brama *et al* 1999b, MacDonald *et al* 2002); it also corresponds with the high turnover times for collagen in mature individuals. Articular cartilage GAG concentration also remains stable in horses after maturity has been reached (MacDonald *et al* 2002), as does GAG concentration in synovial fluid from clinically normal equine joints (Fuller *et al* 1996). The lower rate of cartilage metabolism in adults probably explains much of the limited repair capability of articular cartilage and lower MMP activity detected in unaffected joints of adults, compared with joints from juvenile horses. From our data, comparison of synovial fluid GAG and hydroxyproline concentrations in relation to age revealed that variation in GAG concentration was considerably higher than that of hydroxyproline concentration, although both putative markers had a comparable decrease in concentration with age.

As during physiologic cartilage turnover in foals, degradation products such as hydroxyproline and GAGs are released into the synovial fluid during the destruction of cartilage associated with development of osteoarthritis. However, in our study, neither hydroxyproline nor GAG concentrations in synovial fluid from joints with osteoarthritis were increased, compared with concentrations measured in unaffected joints. This is believed to be a result of the lower rate of cartilage turnover in joints with osteoarthritis, compared with that associated with joint remodelling during growth, and the apparent clearance of

hydroxyproline and GAGs from joints with osteoarthritis at a rate that prevents their accumulation to concentrations greater than those detected in unaffected joints.

Our finding regarding hydroxyproline concentration in synovial fluid is similar to that of Maldonado *et al* (1983) who were unable to detect hydroxyproline in synovial fluid samples obtained from horses with joint disease; however, our finding regarding GAG concentration in synovial fluid conflicts with that of Alwan *et al* (1990, 1991), who detected increased GAG concentration in synovial fluid obtained from joints with osteoarthritis compared to healthy joints. The definition of osteoarthritis which is used in a particular study and whether or not inflammation was present within the joint at the moment of arthrocentesis will affect the results obtained and may (in part) explain contradictory results presented by different authors. To classify metacarpophalangeal joints as affected by osteoarthritis or unaffected, we used an index method that has an excellent correlation with macroscopic scores of the gross extent of osteoarthritis in joints (Brommer *et al* 2003a); however, most of the joints that were classified as affected had a CDI value < 35%, which corresponds to mild disease that is usually not accompanied by changes that are detectable radiographically. Other investigators have mainly used radiographic criteria to assess the presence and extent of osteoarthritis, and such radiographic abnormalities are only visible in advanced stages of disease (Kidd *et al* 2001, Trotter and McIlwraith 1996). Furthermore, the development of osteoarthritis is characterized as intermittent, in that the disease becomes clinically manifest as alternating periods associated with many or few signs of joint pain. It is probable that both hydroxyproline and GAG concentrations in synovial fluid vary with time, depending on the degree of inflammation associated with development of osteoarthritis. However, because of the very limited repair capability of damaged adult cartilage, neither the radiographic nor macroscopic appearance will alter notably as the inflammatory component of the osteoarthritic process subsides (albeit temporarily). Interestingly, Ratcliffe *et al* (1988) detected high GAG concentration in synovial fluid samples in association with acute but not chronic joint disease in humans. Variable GAG release with time could explain conflicting results with respect to synovial fluid concentrations in unaffected joints and joints with osteoarthritis. It seems probable that more GAG would also be released into synovial fluid during active inflammation of the joint and it would be interesting to measure GAG concentration in joints with septic arthritis, for example. In our study, the history of the adult horses used was not known and therefore, we were unable to relate our findings to clinical signs. It would appear that obtaining a single measurement of the synovial fluid hydroxyproline or GAG concentration is not a suitable determinant of the presence or extent of osteoarthritis in a joint; repeated measurements may be useful in monitoring osteoarthritis-associated inflammation but this remains to be elucidated.

In the study of this report, the concentration of hydroxyproline in synovial fluid of osteoarthritic joints was significantly correlated with general MMP activity, whereas the concentration of GAG was not. This provides further evidence for the role of MMPs in collagen degradation in osteoarthritis and indicates that changes in synovial fluid hydroxyproline concentration may correspond with the extent of damage to collagen of the articular cartilage, which is an important aspect of osteoarthritis. The substrate (TNO211-F) employed in the general MMP assay used in our study is converted mainly

by gelatinases (MMP-2 and -9) and the collagenase MMP-13, and to a lesser degree by MMP-14. However, other MMPs such as MMP-1 and -3, which have a lower affinity for TNO211-F may be major contributors to substrate turnover if their concentration is high. Substrate conversion occurs only by active MMPs (< 5% of total MMPs) and not by pro-MMPs or MMPs inhibited by tissue inhibitors of metalloproteinase (TIMPs). In our study, the activity of pro-MMPs could have been estimated by remeasuring MMP activity after activation of pro-MMPs by p-aminophenylmercuric acetate but measurement of net enzyme activity was considered more appropriate for our purposes. The proteoglycans in articular cartilage are broken down not only by MMPs but also by aggrecanases, which do not cleave the substrate in the MMP assay used in our study. This could explain the lack of correlation between GAG concentration in synovial fluid and MMP activity, as a proportion of the GAGs in synovial fluid could have been released as a result of proteolysis by aggrecanase.

In osteoarthritic joints, there was a significant correlation between hydroxyproline concentration and the severity of cartilage damage (as determined by the CDI values); such a correlation was not established for unaffected joints. A similar correlation between GAG concentration and CDI value was not detected. Some of the proteoglycans lost from damaged cartilage can be replaced, whereas the damage to the collagen network is considered permanent because of its high turnover time. It could be argued that the CDI provides a measure of proteoglycan depletion rather than collagen damage because uptake of the Indian ink used in the CDI assessment is influenced by the proteoglycan content of the cartilage (Ficat and Maroudas 1975, Maroudas *et al* 1973); nevertheless, proteoglycan depletion and collagen damage can be assumed to be interrelated, with release of proteoglycans only when the collagen fibre network is disrupted.

There are a number of potential problems associated with synovial fluid sampling because various factors influence synovial fluid volume, which can cause dilution of biological markers. Evaluation of synovial fluid volume after exercise has resulted in contradictory findings; Miyaguchi *et al* (2003) reported that synovial fluid volume was reduced in joints of humans after exercise, compared with pre-exercise values, whereas Otterness *et al* (1998) detected less synovial fluid volume in joints of sedentary hamsters than joints of hamsters that had been exercised. Acute inflammation and most other joint injuries are associated with development of joint effusion (Kraus *et al* 2002, Trotter and McIlwraith 1996) but the latter might be attenuated by medication; for example, administration of phenylbutazone and ketoprofen reduces synovial fluid volume in joints of horses with induced synovitis (Owens *et al* 1994, Owens *et al* 1996). Similarly, intra-articular administration of corticosteroids has been reported to reduce synovial fluid volume in horses and cattle (Chunekamrai *et al* 1989, Marcoux 1977, Van Pelt 1963, 1967, Van Pelt *et al* 1970, 1971, 1972, Van Pelt and Riley, Jr. 1967, 1969) . In our study, we chose to investigate biomarker concentrations in synovial fluid obtained via aspiration, because factors influencing synovial fluid volume are difficult (if not impossible) to control and, in a clinical setting, aspiration is most practical.

In conclusion, hydroxyproline and GAG concentrations in synovial fluid from growing horses were much higher than concentrations detected in samples from joints of mature horses, which corresponded with a high rate of cartilage metabolism that accompanies

joint remodelling. As horses reached maturity, hydroxyproline and GAG concentrations in synovial fluid appeared to stabilize, which indicated a low level of collagen metabolism. With the development and progression of osteoarthritis, concentrations of hydroxyproline and GAGs in synovial fluid were not increased, compared with concentrations in unaffected joints, probably because of the intermittent character of the release of these substances which makes single measurements insufficient to accurately assess the degree of pathologic changes within a joint. The relationship that was found between the extent of cartilage degeneration (as quantified by the CDI value and MMP activity) and synovial fluid hydroxyproline concentration suggests that the latter may be an indicator of damage to collagen in articular cartilage (an important aspect of osteoarthritis), but this remains to be thoroughly investigated.

Chapter VI

Relationship between synovial fluid levels of glycosaminoglycans, hydroxyproline and general MMP activity and the presence and severity of articular cartilage change on the proximal articular surface of P1

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Summary

Reasons for performing study: Osteoarthritis (OA) is one of the most prevalent and disabling chronic conditions affecting horses and leads to degeneration of articular cartilage. Diagnosis is based on clinical symptoms in combination with radiography, which is relatively insensitive and provides only an indication of accumulated damage. Alternative methods, such as molecular markers, are therefore needed that can quantitatively, reliably, and sensitively detect osteoarthritic changes in the joints at an early stage of the disease. If such markers are to be used reliably it is important to know the relationship between marker concentration and cartilage composition.

Objectives: To study the relationship between cartilage composition, synovial fluid levels of glycosaminoglycans (GAGs), hydroxyproline (Hyp) and general matrix metalloproteinase (MMP) activity and the presence and severity of articular cartilage damage on the articular surface of P1.

Methods: Synovial fluid (SF) was collected from the metacarpophalangeal (MCP) joints of 60 adult horses. In SF levels of glycosaminoglycans (GAG), hydroxyproline and general MMP activity were determined. Further, GAG and denatured collagen content of the articular cartilage were determined at the dorsal articular margin of P1 (site 1) and at the central cavity (site 2). The presence and severity of cartilage change was quantified using the cartilage degeneration index (CDI), measured at the same two sites. Correlations between SF parameters, cartilage composition and degree of cartilage degeneration were sought using correlation analysis.

Results: There was no correlation between GAG or Hyp content of SF and the amount of GAGs or denatured collagen respectively in cartilage. In joints with moderate-severe cartilage damage the GAG content of site 1 was significantly lower than in joints with no-minimal cartilage change ($P=0.005$) and there was a negative correlation between the amount of denatured collagen and GAG content at site 1 in all joints ($r = -0.39$, $P=0.002$). Further, in joints with moderate-severe cartilage damage there was a significant positive correlation between MMP activity in SF and Hyp levels in SF ($r = 0.72$, $P<0.001$) and CDI at sites 1 ($r = 0.46$, $P=0.03$) and 2 ($r = 0.43$, $P=0.04$).

Conclusions: General MMP activity in joints with moderate-severe cartilage damage is related to the severity of those cartilage changes and to Hyp levels in SF. Glycosaminoglycan levels in SF are not directly related to MMP activity, GAG content of articular cartilage or severity of cartilage change.

Potential relevance: Glycosaminoglycan levels in SF are not helpful for the early detection of cartilage lesions. In damaged joints Hyp levels may give an indication of the severity of cartilage change as they are strongly related to MMP activity, but do not qualify as marker for the presence or absence of cartilage lesions.

Introduction

Horses are kept primarily for their athletic ability and their value drops considerably if they cannot be ridden or otherwise used for competition or pleasure. Lameness is the most important cause of wastage in the equine industry (Olivier *et al* 1997, Rossdale *et al* 1985, Todhunter and Lust 1990) and joint disease is the most prevalent cause of lameness (Todhunter 1992). This explains why osteoarthritis (OA) is one of the most economically important diseases in athletic and pleasure horses (Pool 1996). The distal limb joints in the horse are most at risk, with the metacarpophalangeal (MCP) joint being frequently affected (MacDonald *et al* 2002).

The first visible changes in OA involve increased water content of articular cartilage and a reduction in proteoglycan content, as well as changes in the structure of the proteoglycans, suggesting loss of the restraining capacity of the collagen network. In more advanced stages of disease the collagen network is clearly degraded, which leads to decreased collagen content and increased amounts of denatured collagen (Hollander *et al* 1994).

Macroscopically, cartilage degeneration is recognized by fibrillation, erosions and wear lines (McIlwraith 1996). The radiological changes accompanying OA are only visible in advanced stages of disease and, furthermore, they are poorly correlated with clinical signs (Kidd *et al* 2001, Trotter and McIlwraith 1996). Marked articular cartilage degeneration can be present despite a normal radiographic appearance of the joint (Lohmander *et al* 1992). Also, by the time radiographic changes become visible cartilage damage is well advanced and, given the very limited repair capability of adult articular cartilage, this means that therapeutic possibilities are limited. This has led to a search for biomarkers to detect those early changes characteristic of joint disease, as early detection may allow treatment of joint disease, or the implementation of measures to slow its progression (DeGroot *et al* 2002).

The layer of hyaline cartilage which covers the articular surface of the bones which form the diarthrodial joint allows load bearing, load distribution and shock absorption. It is able to fulfil these roles because of its unique biomechanical properties, combining compressive stiffness and resilience, and these characteristics are the result of its biochemical composition. The extracellular matrix (ECM) consists mainly of collagen, proteoglycans and water and is produced by a limited number of chondrocytes. Collagen (which is mainly type II collagen) is the most important component and, on a dry weight basis, forms 50-80% of the cartilage matrix (Brama *et al* 2000b, Todhunter 1996, Vachon *et al* 1990), while proteoglycans constitute 7.3-10% (Vachon *et al* 1990). Like all fibrillar collagens, type II collagen is made up of a triple helix consisting of three α chains, with an amino acid sequence of glycine-X-Y, where X and Y are often proline or hydroxyproline (Todhunter 1996). Hydroxyproline (Hyp) accounts for approximately 10% of the amino acids in collagen and, because it is present only in very small quantities in other proteins, it is often used to estimate the amount of collagen in a certain tissue (Johnson *et al* 1980). The proteoglycans are made up of core proteins to which large quantities of glycosaminoglycans (GAGs) are attached, mainly chondroitin sulphate and keratan sulphate (Todhunter 1996). These GAGs contain negatively charged sulphate groups which

strongly attract water. The core proteins of the proteoglycans are connected by link proteins to hyaluronic acid molecules that themselves are attached to the collagen fibrils.
Cartilage degradation, which plays an important role in disease processes such as OA, is likely to result in the release of biochemical components of the extracellular matrix. Therefore, SF levels of GAGs and hydroxyproline could reflect cartilage damage. Synovial fluid is relatively easy to obtain from equine joints and marker levels could be of great clinical value. There are, however, conflicting reports with respect to GAG levels in SF from OA joints, with some authors finding elevated levels (Alwan *et al* 1990, 1991, Palmer *et al* 1995, Saxne *et al* 1985), others finding no difference between normal and diseased joints (Fuller *et al* 2001, Little *et al* 1990, Myers *et al* 1996), while Belcher *et al* (1997) even found lower levels in OA. Despite this contradictory evidence synovial GAG levels have been promoted by some as markers of cartilage turnover, both in human (Heinegard *et al* 1987, Saxne *et al* 1986) and equine studies (Palmer *et al* 1995). Less information is available for synovial fluid levels of hydroxyproline, with Maldonado *et al* (1983) not finding an increase in individual pathological SF samples from horses.
Another group of biological markers is formed by enzymes which are involved in the pathological degradation of the cartilage matrix. The matrix metalloproteinases (MMPs) are a group of enzymes which have been implicated in the breakdown of articular cartilage in degenerative joint disease, both in man (Hembry *et al* 1995, Lohmander *et al* 1994, Manicourt *et al* 1994) and in horses (Brama *et al* 1998, 2000d, Clegg *et al* 1997b, 1999). This group of zinc-dependent enzymes can be divided into stromelysins, collagenases, gelatinases and membrane-bound MMPs of which the collagenases (MMP-1, MMP-8 and MMP-13) cleave the interstitial collagen triple helix, the gelatinases (MMP-2 and MMP-9) act on unwound collagen and the stromelysins degrade proteoglycans (Murphy *et al* 1990).
In order to be able to determine the value of SF markers in predicting joint disease it is important to know how the levels in SF relate to the composition of the articular cartilage and the presence and severity of cartilage damage. We hypothesized that SF levels of GAGs, Hyp and general MMP activity would be correlated with the composition of the articular cartilage and with the severity of cartilage lesions of P1. To test this SF was collected from the MCP joints of 60 adult horses and levels of GAGs, hydroxyproline and general MMP activity in synovial fluid were compared to GAG and denatured collagen concentration in two differently loaded cartilage sites and to the severity of cartilage damage, as quantified by the recently developed cartilage degeneration index (CDI) (Brommer *et al* 2003a).

Materials and methods

Sample collection

Immediately after slaughter synovial fluid was aspirated from the metacarpophalangeal joints of 60 horses, centrifuged, aliquotted and stored at -80ºC until analysis. The age of the horses was estimated by examination of the lower incisors and only samples from horses aged 5 years or older were used. The metacarpophalangeal joints of these horses

were isolated and stored at -20ºC until further processing. After determination of the cartilage degeneration index (CDI), cartilage was collected from two differently loaded sites of the articulating surface of P1. Site 1 was located at the medial dorsal articular margin of the proximal aspect of the P1 and site 2 in the midregion of the medial cavity of the joint surface (Brama *et al* 1999b). The cartilage samples were stored at -80ºC until determination of biochemical parameters.

Synovial fluid parameters

- hydroxyproline assay

Synovial fluid hydroxyproline levels were determined by HPLC analysis following derivatization with a fluorescent label of secondary amino acids (proline and hydroxyproline) as described previously (Bank *et al* 1997). In short, synovial fluid (2 to 4 µl) was hydrolysed in 1 ml 6 M HCl (20 hrs at 110°C), dried overnight under vacuum (SpeedVac, Thermo Savant) and dissolved in 100 µl water (Milli-Q, Millipore Corp.). An aliquot was transferred to an HPLC insert and placed in the autoinjector of an HPLC system consisting of a high precision HPLC pump (model 480, Gynkotek), an autosampler (Triathlon, Spark Holland), and a spectrofluorometric detector (model 821-FP, Jasco). Automated derivatization consisted of blocking of primary amino acids with o-phthaldialdehyde (Sigma), followed by labelling of secondary amino acids with 9-fluorenylmethyl chloroformate. Derivatized amino acids were injected onto a reversed phase HPLC column (Biosep TSK, Tosoh Corp., ODS-80^{TM}, 4.6 x 150 mm), and eluted using a tertiary gradient of citrate, acetonitrile and methanol, as described previously (Bank *et al* 1996). Fluorescence was monitored at 254 nm (excitation) and 630 nm (emission) and data were recorded on-line by a computing integration system (Chromeleon™ version 4.30, Dionex). The amino acid standard for collagen hydrolysates (Sigma) served as a reference. The intra-assay variation was 3% and the inter-assay variation was 10%. Hydroxyproline content was expressed as µg/ml SF.

- glycosaminoglycan assay

Proteoglycan content of SF was estimated by measuring glycosaminoglycan (GAG) using the 1,9-dimethylmethylene blue (DMMB) metachromatic dye assay modified for use in microtitre plates. After papain digestion GAGs were precipitated and stained with 1,9-dimethylmethylene blue (Blyscan kit, Biocolor Ltd.) and staining was quantified by measuring absorbance at 656 nm. Shark cartilage chondroitin sulphate (Sigma) served as standard. Results were expressed as µg GAG/ml SF.

- general MMP activity assay

Matrix metalloproteinase activity was determined using a slight modification of the fluorometric assay as described by DeGroot *et al* (2001b). Briefly, conversion of the internally quenched fluorogenic substrate TNO211-F (2.5 µM; Dabcyl-Gaba-Pro-Gln-Gly-Leu-

Cys[Fluorescein]-Ala-Lys-NH_2) was measured in the presence of the EDTA-free general proteinase inhibitor cocktail (Complete, Roche Diagnostics), to prevent conversion of the substrate by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence or absence of the MMP inhibitor BB94 (10 µM). General MMP activity was expressed as relative fluorescence units per second (RFU/s). Since the substrate is not cleaved by aggrecanases, this approach detects only MMP-mediated substrate conversion and reflects the overall MMP activity in the SF samples. Storage at -80ºC and several thaw cycles do not influence MMP activity (data not shown).

Quantification of cartilage damage

The degree of cartilage degeneration was determined using the Cartilage Degeneration Index (CDI) described by Brommer *et al* (2003a). Briefly, the proximal articular surface of P1 is stained using Indian ink, a digital image obtained and the mean grey pixel value calculated using a validated algorithm. Cartilage degeneration index values were calculated both for the entire joint surface and also separately for sites 1 and 2 (CDI-1 and CDI-2 respectively). Because cartilage damage to P1 develops first at the dorsal articular margin (site 1) (Brommer *et al* 2003b) joints were categorised as having moderate-severe or no-minimal cartilage damage based on the CDI-1, with values < 30% indicative of no-minimal cartilage change, and values ≥ 30% representing moderate-severe degeneration.

Cartilage parameters

- glycosaminoglycans

Cartilage samples were digested by papain in 200 µl of a 50 mM phosphate buffer, pH 6.5, containing 2 mM Na_2EDTA and 2 mM cysteine for approximately 16 hours at 65ºC. Proteoglycan content was determined using the DMMB assay as described for SF.

- denatured collagen

The amount of denatured collagen was determined by the assay described by Bank *et al* (1997), which is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the triple helix. Briefly, one piece of cartilage of each site was thawed and extracted with 4 M guanidinium hydrochloride in 0.1 M Tris HCl (pH 7.3) containing a cocktail of protease inhibitors. The denatured collagen in the cartilage matrix was then digested overnight at 37°C with 0.5 mg αCT (C-4129, Sigma) dissolved in 500 µl phosphate buffered saline (pH 7.4) containing 1 mM iodoacetamide and 1 mM EDTA. The supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen); both were hydrolysed with 6 M HCl at 110°C for 20 hours. The amount of the collagen-specific amino acid hydroxyproline was measured with reversed-phase high-performance liquid chromatography. The amount of denatured collagen was expressed as a percentage of total collagen.

Statistics

The joints were divided into those with moderate-severe cartilage degeneration and those with no-minimal cartilage degeneration, based on the value of CDI of site 1. Differences between these two groups were tested by an unpaired t-test. Normality was assessed using the Kolmogorov-Smirnov method and correlations were determined by parametric methods (Pearson's correlation analysis) when data were normally distributed, or non-parametric means (Spearman correlation analysis) when this was not the case. Correlations were sought between SF parameters, cartilage composition and the severity of cartilage change, both in the entire group, in joints with moderate-severe cartilage damage and joints with no-minimal cartilage damage. The level of significance was set at $P<0.05$. Because sufficient SF was not available for all assays the number of samples varied and is indicated in the figures.

Results

There were 24 joints with CDI-1 ≥ 30% (moderate-severe damage; average score 44.1 ± 2.3%) and 36 joints with CDI-1 < 30% (no-minimal cartilage damage; average score of 25.2 ± 1.1%). The joints with moderate-severe cartilage damage at the dorsal margin of P1 had an average total CDI of 27.1% (range: 13.6-48.0%), indicating that the cartilage change across the entire surface of P1 was never extreme. Results for the SF and cartilage parameters are presented in table 1 (mean ± s.e.m.).

	moderate-severe cartilage damage mean ± s.e.m.	no-minimal cartilage damage mean ± s.e.m.	significance
number of cases (n)	24	36	
age (years)	14.1 ± 0.9	11.6 ± 0.7	P=0.03
CDI-1 (%)	44.1 ± 2.3	25.2 ± 1.1	P<0.001
GAG SF (µg/ml)	89.7 ± 26.3	67.2 ± 9.4	NS
HYP SF (µg/ml)	39.9 ± 3.6	40.4 ± 2.4	NS
general MMP SF (RFU/sec)	0.017 ± 0.002	0.022 ± 0.003	NS
GAG cartilage site 1 (µg/mg dw)	39.4 ± 5.5	59.9 ± 4.5	P=0.005
GAG cartilage site 2 (µg/mg dw)	117.7 ± 6.4	117.3 ± 7.9	NS
denatured collagen site 1 (%)	17.45 ± 1.98	12.12 ± 1.16	NS
denatured collagen site 2 (%)	11.96 ± 1.15	10.34 ± 0.72	NS

***Table 1**: Measured parameters (mean ± s.e.m.) in synovial fluid (SF) from the metacarpophalangeal joint and cartilage from the articular surface of the proximal phalanx (sites 1 and 2). CDI-1 = cartilage degeneration index at site 1; GAG = glycosaminoglycans; HYP = hydroxyproline; MMP = matrix metalloproteinase; RFU = relative fluorescence units; dw = dry weight; s.e.m. = standard error of the mean.*

There was a small, but significant difference (P=0.03) in mean age between the group with moderate-severe cartilage damage (mean ± s.e.m.: 14.1 ± 0.9, range: 5-22 years) and joints with no-minimal damage (11.6 ± 0.7, 5-23 years). In joints with moderate-severe cartilage damage the percentage denatured collagen (17.5 ± 2.0%) was elevated compared to the joints with no-minimal cartilage change (12.1 ± 1.1%), although this difference was not statistically significant. The GAG content in moderate-severely damaged cartilage was decreased (39.4 ± 5.5 µg/mg) compared to minimally damaged cartilage (59.9 ± 4.5 µg/mg). The amount of denatured collagen in the entire group of adult horses was significantly higher at site 1 (15.7 ± 1.1%) than at site 2 (11.6 ± 0.6%, P=0.001) and there was a negative correlation between denatured collagen and GAG content in cartilage at site 1 (r = -0.39, P=0.002; Fig 1).

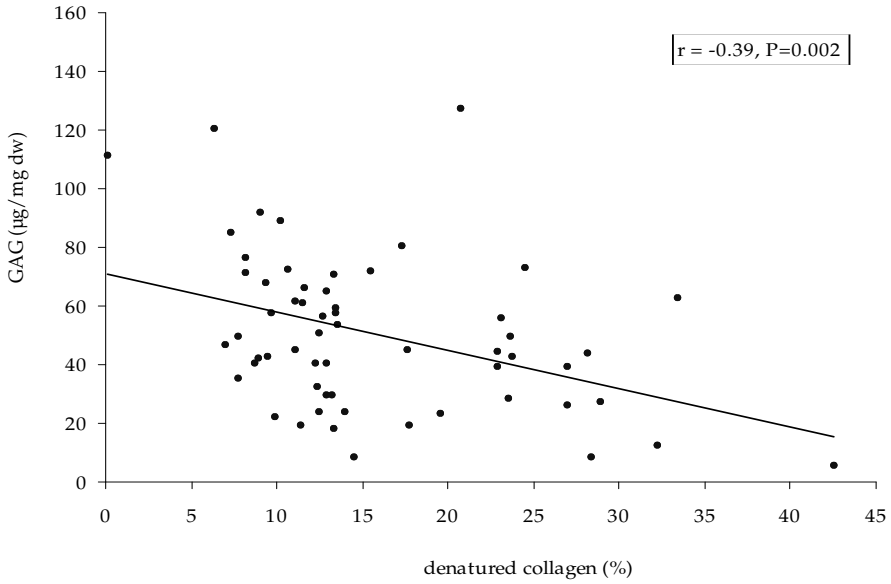

***Figure 1**: Glycosaminoglycan (µg/mg dw) content in relation to the relative amount of denatured collagen (%) of the articular cartilage of site 1 from the metacarpophalangeal joint (age range 5 to 23 years, n=59); r = correlation coefficient.*

There was no correlation between the GAG levels in SF and the GAG content of articular cartilage, neither in the entire group (site 1: r = 0.06, P=0.64; site 2: r = 0.13, P=0.35), the group with moderate-severe cartilage damage (site 1: r = 0.15, P=0.49; site 2: r = 0.07, P=0.76) nor in the group with no-minimal cartilage damage (site 1: r = -0.17, P=0.34; site 2: r = 0.06, P=0.74). Similarly, there was no correlation between Hyp levels in SF and the amount of denatured collagen in cartilage in the whole group (site 1: r = -0.13, P=0.36; site 2: r = -0.12, P=0.40), moderate-severely damaged joints (site 1: r = -0.12, P=0.63; site 2: r = -0.31, P=0.19), or minimally affected joints (site 1: r = - 0.30, P=0.08; site 2: r = -0.06, P=0.75).

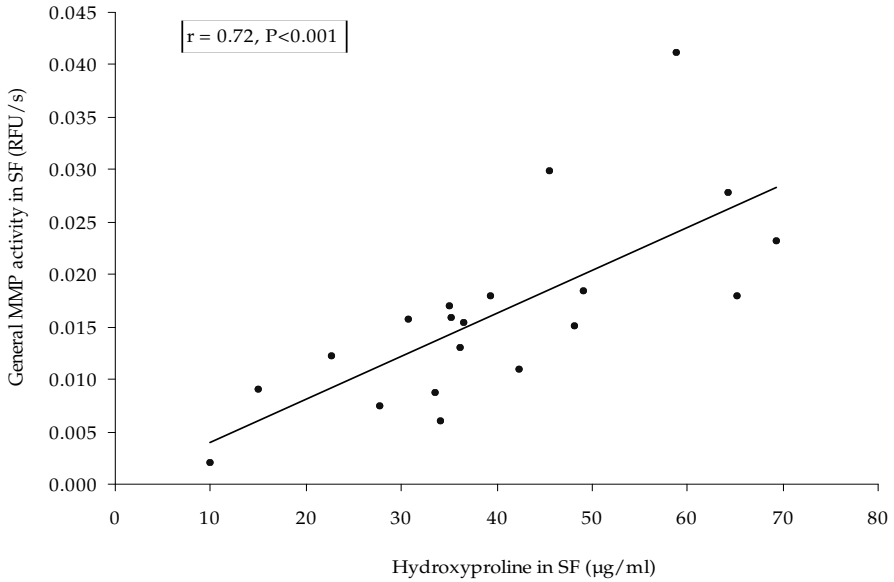

***Figure 2**: General MMP activity (RFU/sec) in relation to hydroxyproline (µg/ml) in synovial fluid (SF) from the metacarpophalangeal joints with moderate-severe cartilage damage (age range 5 to 22 years, n=20); r = correlation coefficient.*

In the joints with moderate-severe cartilage degeneration there was a strong, significant correlation between general MMP activity in SF and Hyp levels in SF ($r = 0.72$, $P<0.001$; Fig 2). There were weaker, but still significant, correlations between MMP activity and CDI at site 1 ($r = 0.46$, $P=0.03$; Fig 3a) and site 2 ($r = 0.43$, $P=0.04$; Fig 3b). These correlations were not present in joints with no-minimal cartilage damage.

Discussion

There was a small, but significant difference in the mean age between the group with moderate-severe cartilage damage and the group with no-minimal cartilage damage. Cantley *et al* (1999) demonstrated the presence of age-related changes to the articular cartilage of P1, which they suggested to be consistent with OA. Those authors postulated the presence in horses of an age-related osteoarthritic process, which may be accelerated by the stresses of racing and training. It is not clear whether the cartilage changes observed in the current study are the result of OA, aging or a combination of both, but there is no doubt that they were degenerative in character, thus allowing the evaluation of the relationship between synovial fluid levels of GAG, Hyp and general MMP activity and the severity of cartilage damage.

According to Brommer et al (2003b) the development of cartilage degeneration of P1 starts at the medial dorsal margin, and from there spreads to the rest of the articular surface. When comparing the group with moderate-severe cartilage damage to the group

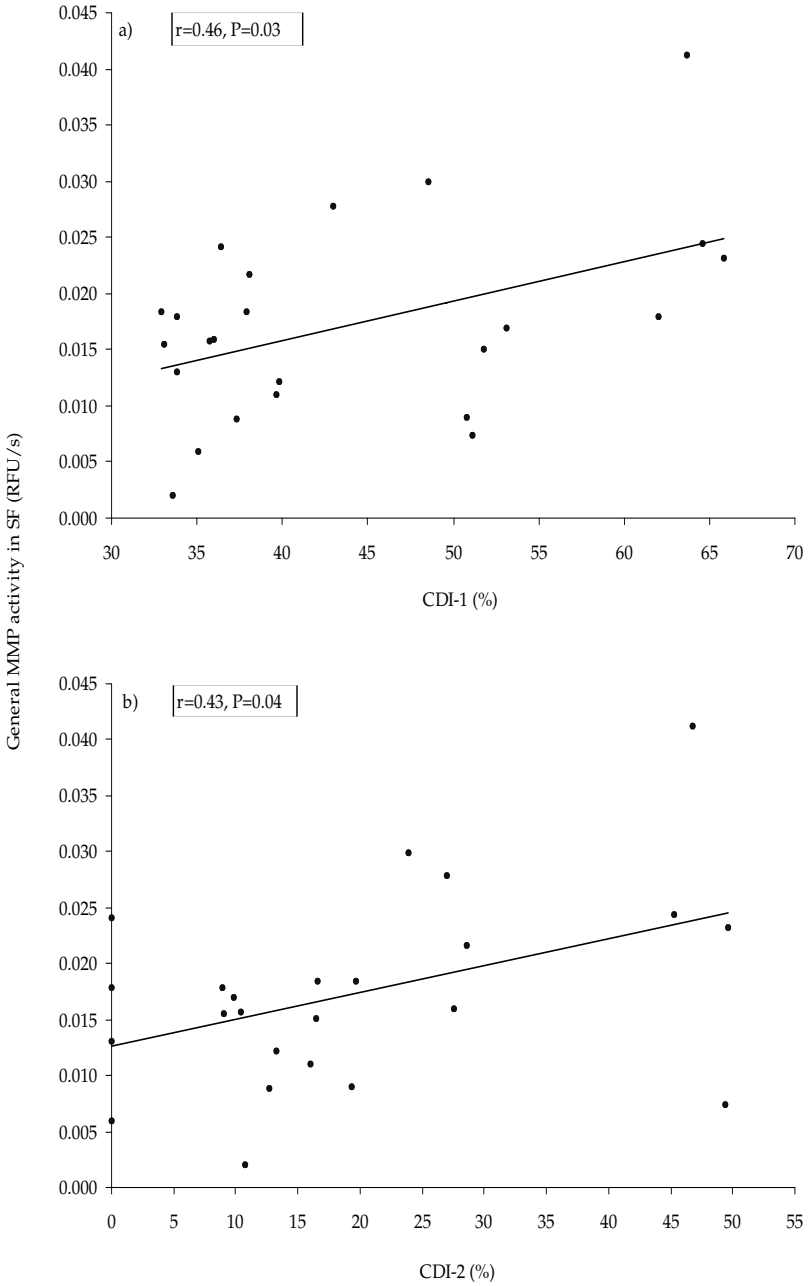

***Figure 3**: General MMP activity (RFU/sec) in synovial fluid (SF) from the metacarpophalangeal joint in relation to a) cartilage degeneration index at site 1 (CDI-1, %) and b) at site 2 (CDI-2, %) in joints with moderate-severe cartilage damage (age range 5 to 22 years, n=24); r = correlation coefficient.*

with no-minimal alterations the GAG concentration in the cartilage of site 1 (dorsal margin), but not at site 2, was found to be lower in the former, which confirms this distribution pattern. In OA, a loss of glycosaminoglycans from articular cartilage is one of the first biochemical changes seen (Sweet et al 1977, Westacott and Sharif 1996) and this suggests that either the changes seen in this study reflect OA or that cartilage degeneration starts with proteoglycan loss, irrespective of the cause.

As glycosaminoglycans are released from the extracellular matrix into synovial fluid their concentration in SF might be expected to be increased in joints with moderate-severe cartilage damage. This, however, was not the case and neither were GAG levels in SF correlated with GAG content of articular cartilage. There are a number of possible explanations for the lack of a correlation between SF GAG and cartilage damage. In the equine MCP joint, the area of the joint surface of P1 that is commonly affected by cartilage degeneration and hence suffers GAG loss (primarily the dorsal margin) is relatively small (Brommer et al 2003b) and increased GAG release from this small area is not likely to influence substantially the GAG concentration in SF. It is known that local changes in GAG concentration in articular cartilage, even if severe, do not affect the GAG concentration at other sites in the same joint (Maroudas et al 1973). Further, the other joint surfaces that make up the MCP joint (third metacarpal bone and the proximal sesamoid bones) were not taken into account, while these surfaces are known to become affected by cartilage breakdown as well.

Another explanation for the fact that synovial GAG levels were not elevated in joints with moderate-severe cartilage damage could be that GAG released into SF is subsequently cleared from the joint very rapidly and therefore levels do not accumulate to values greater than in normal joints. Glycosaminoglycans are released into SF and then pass via lymph vessels into the bloodstream (Okumura *et al* 2002, Worrall *et al* 1994) and they have been shown to leave the joint within a few hours (Antonas *et al* 1973). This process has been shown to occur even more rapidly in inflamed joints (Myers *et al* 1996). Glycosaminoglycan levels in SF might also be dependent on the stage of disease as Ratcliffe *et al* (1988) found increased GAG in SF in acute but not chronic joint disease. No clinical data were available from the animals in this study, but it can be assumed that most of the cartilage changes observed were chronic rather than acute. The results of several studies indicate that SF GAG reflects the degree of active cartilage proteoglycan degradation or turnover (Heinegard *et al* 1985, Saxne *et al* 1985, 1986). The lack of a history and clinical examination of the horses used precluded the study of a relationship between clinical signs, SF GAG levels and cartilage damage and composition. It would certainly be valuable to investigate synovial parameters in well documented clinical cases of OA. However, it is hardly feasible to obtain large numbers of cartilage samples from such horses.

There was no relationship between the amount of GAG in SF and the GAG content of the (remaining) articular cartilage. This may have been caused by increased GAG turnover in damaged cartilage, in an attempt to restore GAG levels to normal, or again by rapid clearance of GAGs from the joint. It is also possible that factors other than joint disease influence the amount of GAG in SF. Exercise has been shown to have such an effect, with serum keratan sulphate (KS) being elevated shortly post-exercise (Okumura *et al* 2002).

On the other hand, exercise has been shown to decrease GAG release from cartilage (van den Hoogen *et al* 1998a). Finally, it is possible that other tissues within the joint (primarily the synovial membrane) contribute to synovial GAG levels. Westacott and Sharif (1996) pointed out that, where most research has focussed on cartilage, the contribution of cells within other structures of the joint also warrants investigation. Osteoarthritis manifests not only as cartilage destruction but also as reactive synovitis (Chevalier 1997) and it is conceivable that the severity of this synovitis influences proteoglycan metabolism in the joint.

There was no difference in the amount of denatured collagen in articular cartilage between sites 1 and 2. It might be expected that the amount of denatured collagen would be higher at site 1, given the much higher occurrence of cartilage lesions at the dorsal articular margin. The finding that the amount of denatured collagen at site 1 is inversely related to the GAG content of cartilage is in accordance with findings by Hollander *et al* (1995) and demonstrates that damage to the collagen network leads to GAG loss, supporting the notion that the integrity of the collagen network is crucial for the retention of GAGs. Damage to type II collagen is considered to represent the start of irreversible cartilage degradation (Jubb and Fell 1980).

In the joints with moderate-severe cartilage damage Hyp levels in SF were not elevated compared to those with no-minimal damage. This is similar to findings by Maldonado *et al* (1983), who failed to detect increased Hyp levels in pathological SF from equine joints. Again, this could be due to rapid clearance from the joint, the limited surface area of affected cartilage in relation to the volume of synovial fluid or the influence of joint surfaces other than P1. Hydroxyproline levels in SF also failed to predict the relative amount of denatured collagen in the articular cartilage. However, Hyp levels in the SF of joints with moderate-severe cartilage damage were strongly and significantly correlated with general MMP activity and this was not the case in the group with no-minimal change. The MMPs are known to play an important role in matrix degradation and this strong correlation confirms the importance of MMP activity in collagen breakdown. The substrate used in the general MMP activity assay employed in this study is converted mainly by the gelatinases (MMP-2 and -9) and by the collagenase MMP-13. The relative importance of the various collagenases in collagen degradation is currently unclear. Studies have shown that MMP-13 is more potent than MMP-1 in the degradation of soluble type II collagen (Mitchell *et al* 1996, Reboul *et al* 1996) and Dahlberg *et al* (2000) suggested that the excessive cleavage of type II collagen in OA cartilage is mediated primarily by collagenases other than MMP-1. Others have demonstrated enhanced ratios of MMP-1 over MMP-13 in OA patients (Shlopov *et al* 1997). Our findings provide further evidence for the role of MMP-13 in collagen degradation in cartilage degeneration.

There was no correlation between MMP activity and GAG levels in SF. This could be due in part to the fact that proteoglycan degradation is not due exclusively to MMPs but also, to a large extent, to the aggrecanases. These two enzyme groups are differentially regulated in OA (Bluteau *et al* 2001) and recent evidence suggests that in OA aggregan degradation by aggrecanase occurs in the early stages and MMPs are responsible for proteoglycan breakdown in the later stages of disease (Caterson *et al* 2000).

In the group of joints with moderate-severe cartilage damage general MMP activity in SF

was significantly correlated with both CDI-1 and CDI-2. Dahlberg *et al* (2000) found no correlation between collagenase activity and Mankin grade, but type II collagen damage has been shown to correlate with disease severity as defined by histopathological score (Hollander *et al* 1994). Our findings imply that collagen degradation in cartilage degen-eration is mediated largely by the MMPs and that this collagen damage reflects the severity of the cartilage breakdown.

In previous studies MMP levels have been shown to be elevated in OA joints in horses (Brama *et al* 1998, 2000d, Clegg *et al* 1997b, 1999), but they were not increased in joints with moderate-severe cartilage damage in this study. However, the correlation with cartilage degradation seems to confirm the importance of these enzymes in cartilage breakdown. It is not clear why MMP activity was not elevated in joints with moderate-severe cartilage damage in this study. It is possible that MMP activity is more a measure of the actual activity status of a joint, while cartilage damage is the result of repeated insults to the articular cartilage and varying periods of both active degradation and attempted repair (Caterson *et al* 2000). Unfortunately, we knew very little of the history of the horses used in our study, but it is likely that most of the horses were not lame at the time of slaughter and that the process of cartilage degeneration had (temporarily) subsided. It would be interesting to measure MMP activity in joints suffering acute and chronic joint disease, or to be able to relate biochemical findings to clinical signs.

Another difficulty in evaluating the results of this study is the fact that several factors can influence synovial fluid volume and thus cause dilution of biological markers. For example exercise, acute joint inflammation and most other joint injuries have been shown to affect synovial fluid volume (Kraus *et al* 2002, Miyaguchi *et al* 2003, Otterness *et al* 1998, Trotter and McIlwraith 1996). However, these effects can be attenuated by medication, with both non-steroidal drugs (Owens *et al* 1996) and intra-articular corticosteroids (Chunekamrai *et al* 1989) having been demonstrated to reduce synovial effusion. In this study, it was decided to investigate biomarker concentrations in synovial fluid obtained via simple aspiration, because the factors influencing synovial fluid volume are difficult (if not impossible) to control and, in a clinical setting, aspiration is most practical.

In conclusion it can be stated that synovial GAG levels are neither related to the severity of cartilage change nor to GAG content of articular cartilage and can hence be discarded as reliable markers for cartilage damage. Hydroxyproline levels, while not related to the amount of denatured collagen in cartilage, are strongly related to MMP activity and may be useful as an indication of the actual disease status. General MMP activity is best related to the condition of the articular cartilage but also seems to be a better indicator of active processes than of the cartilage status in chronic cases. Future research should be aimed at investigating SF markers in well defined patient populations with clinically active OA.

Chapter VII

Assessment of Prostaglandin E2, matrix metalloproteinases, glycosaminoglycans, hydroxyproline and hyaluronan as synovial fluid markers in equine joints with infectious arthritis

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Summary

Objective: To assess prostaglandin E_2 (PGE_2), glycosaminoglycan (GAG), hydroxyproline and hyaluronan concentrations and matrix metalloproteinase (MMP) activity in synovial fluid from joints suffering infectious arthritis and to compare these with white blood cell count, which is the commonly used parameter for the assessment of infectious arthritis in the clinical setting. Additionally, the relationship between GAG, hydroxyproline and hyaluronan concentrations and PGE_2 levels and MMP activity was investigated.

Sample Population: Synovial fluid was collected from affected joints in eight horses suffering infectious arthritis and from eight healthy joints in eight control horses.

Procedure: PGE_2, GAG, hydroxyproline and hyaluronan concentrations, as well as MMP activity were measured in synovial fluid samples. Differences between infectious arthritis and healthy joints were tested and correlations between the different parameters were sought.

Results: PGE_2 and GAG concentrations, as well as general MMP activity, were significantly elevated in synovial fluid from joints with infectious arthritis, while hyaluronan concentration was decreased and there was no significant difference in the hydroxyproline concentration. In all samples taken together there was a significant positive correlation between PGE_2 and MMP activity ($r = 0.76$, $P = 0.001$) and between PGE_2 and GAG concentrations ($r = 0.53$, $P = 0.035$) and a negative correlation between PGE_2 and hyaluronan ($r = -0.67$, $P = 0.004$). In synovial fluid from infectious arthritis cases there was no correlation between white blood cell counts and any of the other parameters.

Conclusions and Clinical Relevance: Infectious arthritis caused increased PGE_2 concentration and MMP activity in synovial fluid and the correlations between PGE_2 and MMP activity, GAG release and decreased hyaluronan concentrations suggest PGE_2 may be a more reliable marker of infectious arthritis than the white blood cell count.

Introduction

Infectious arthritis is one of the most devastating joint diseases in horses, leading to rapid and extensive damage to the articular cartilage and which, if not treated in a timely and adequate manner, poses a career- and life-threatening problem. There are several possible etiologies: perforation of a joint space (either a wound, arthrocentesis or surgery), haematogenous spread, extension from a surrounding wound or idiopathic causes (Schneider *et al* 1992b). In foals the haematogenous route of infection occurs most frequently (Schneider *et al* 1992a), while in adult horses wounds and direct inoculation during arthrocentesis are usually to blame (Schneider *et al* 1992b). A wide variety of bacteria can be isolated from infectious arthritis cases and this depends to some extent on the cause of infection. *Enterobacteriaceae* are most frequently isolated in horses developing infection after a wound and joints infected after surgery or arthrocentesis are likely to contain *Staphylococci*, especially *Stahphylococcus aureus* (Bertone 1999).
The clinical signs accompanying infectious arthritis include localised heat, pain (lameness) and joint distension and diagnosis usually follows synovial fluid analysis. Joint infection is followed by a rapid influx of inflammatory cells, mainly neutrophils, and white blood cell (WBC) counts greater than 30,000 cells/µl and > 80% neutrophils are considered diagnostic (Bertone 1996). Currently, WBC counts (and bacteriology) are also used to follow disease progression and monitor the response to treatment. However, there reliability for this purpose can be questioned and other methods to assess disease severity are needed. In recent years much research has been performed on molecular markers, including those in synovial fluid, and these could prove useful in cases of infectious arthritis.
Both neutrophils and resident articular cells (synoviocytes and chondrocytes) produce a range of inflammatory mediators, such as cytokines and degradative enzymes, which exert their effects on the articular cartilage, and which may be used as disease markers. Experimental infection caused decreased proteoglycan synthesis and lower proteoglycan and collagen content in cartilage (Hardy *et al* 1998a, Smith *et al* 1987). These effects persisted when bacteria were filtered from media, showing that soluble factors act as messengers in these events. Many of the effects of bacteria are mediated by lipopolysaccharide (LPS, also known as endotoxin), either directly or indirectly via cytokines or eicosanoids. Lipopolysaccharide is a cell wall component of Gram-negative bacteria, liberated during rapid cell division or following cell death. Intra-articular LPS injection caused increases in white blood cell counts, total protein, tumour necrosis factor α (TNF-α) and prostaglandin E_2 (PGE_2) in SF (Hawkins *et al* 1993) and produced increased immunohistochemical staining for interleukin-1β (IL-1β) and TNF-α and its receptors in synovial membrane and cartilage (Todhunter *et al* 1996). Lipopolysaccharide also decreased proteoglycan synthesis and increased GAG and PGE_2 release in equine cartilage explants (MacDonald *et al* 1994). The cytokines, especially IL-1 and TNF-α are regarded as key cytokines in joint disease, also play a role in cartilage degradation (MacDonald *et al* 1994), an effect which is mediated (in part) through the activation of degradative enzymes.
Prostaglandins, especially prostaglandin E_2 (PGE_2), play an important role in inflamma-

tory and nociceptive pathways in joint disease (Kirker-Head *et al* 2000) and are released by both synoviocytes and chondrocytes in response to a variety of stimuli (Dvorak *et al* 2002, Kirker-Head *et al* 2000, MacDonald *et al* 1994, May *et al* 1991, Morris *et al* 1990, Murakami *et al* 1998). The concentration of PGE_2 is increased in nearly all inflamed joints, including cases of infectious arthritis (Bertone *et al* 2001), and levels appear to be related to the presence of lameness (May *et al* 1994) but not to radiological signs (Chevalier 1997, von Rechenberg *et al* 2000). It is considered to be a sensitive marker of joint disease (Bertone *et al* 2001) and seems to indicate active synovial inflammation, being released in greater quantities by equine synovial membrane compared to chondrocytes (Kirker-Head *et al* 2000, von Rechenberg *et al* 2000). Synovial fluid concentrations of PGE_2 were found to increase following endotoxin injection in horses (Hawkins *et al* 1993).

The matrix metalloproteinases (MMPs) are a group of enzymes involved in cartilage turnover, both during growth and maturation and in disease (Bluteau *et al* 2001, Chubinskaya *et al* 1999). They can be divided into four categories: collagenases, gelatinases, stromelysins and membrane type MMPs (MT-MMPs), and of these the collagenases cleave the collagen triple helix, gelatinases act on the unwound collagen molecule, stromelysins degrade proteoglycans and, together with MT-MMPs, activate other MMPs (Murphy *et al* 1990). The MMPs have been shown to play a role in numerous types of joint disease, in man, the horse and other species. In horses, Trumble *et al* (2001) found increased synovial fluid MMP in naturally occurring joint disease, including infectious arthritis. Similarly, increased gelatinase activity was measured in SF of cows with infectious arthritis (Arican *et al* 2000). Infectious arthritis also led to increased caseinase and collagenase activity, even when leukocyte counts had returned to normal (Spiers *et al* 1994b). The synovial membrane is considered to be an important source of MMPs in joint disease and this is supported by increased MMP activity in an experimental model of synovitis/capsulitis in the equine metacarpophalangeal joint (Cornelissen *et al* 1998). However, purified *Staphylococcal* medium also caused MMP secretion by human cartilage (Williams, III *et al* 1991).

The cytokines, eicosanoids and degradative enzymes together cause breakdown of the articular cartilage and inhibit the synthesis of components of the extracellular matrix (ECM). This leads to the release of ECM constituents into SF, where they can be determined following arthrocentesis. Cartilage is made up mainly of collagen, proteoglycans and water and its degeneration will liberate hydroxyproline and glycosaminoglycans (GAGs) as products of collagen and proteoglycan degradation respectively. In this way synovial hydroxyproline and GAG concentrations form biological markers, reflecting cartilage catabolism within the joint.

In synovial fluid both sulphated-GAG and chondroitin sulphate concentrations were increased in various groups of equine joint disease and were highest in severe, acute cases (Palmer *et al* 1995). Similarly, another study found increased GAG only in synovial fluid from acute joint disease in humans (Ratcliffe *et al* 1988). Plasma keratan sulphate concentrations were significantly increased and SF concentrations decreased in equine joints with infectious arthritis (Todhunter *et al* 1997). Equine cartilage explants exposed to LPS demonstrated decreased GAG synthesis and increased GAG release (MacDonald *et al* 1994) and when exposed to activated neutrophils GAG release was also increased

(MacDonald and Benton 1996). *Staphylococcus aureus* also induced GAG loss from viable human cartilage (Smith *et al* 1982).
While there are numerous reports on GAG release in infectious arthritis, less is known about collagen degradation, although experimental infection in rabbits led to collagen loss from articular cartilage (Daniel *et al* 1976, Smith *et al* 1987). Because the half-life of collagen in cartilage is much longer than that of proteoglycans it is possible that less release of collagen occurs in disease. Also, as collagens are hardly replaced in the extracellular matrix, collagen degradation is regarded as the start of irreversible cartilage damage in osteoarthritis (Billinghurst *et al* 2001), and the same is likely to apply for infectious arthritis. In this respect it was considered interesting to determine if hydroxyproline is released into synovial fluid.
Hyaluronan (or hyaluronic acid, HA) is an important component of synovial fluid, responsible for its viscosity and playing a role in joint lubrication. In articular cartilage long hyaluronan chains bind aggrecan monomers and thus help maintain normal cartilage structure. Bacterial infection was associated with decreased hyaluronan concentration in experiments with synovial membrane and cartilage explants (Hardy *et al* 1998a). Hyaluronan concentrations were also lower in synovial fluid from arthritic (acute and chronic, including osteoarthritis, taken together) joints of horses (Hilbert *et al* 1984) and in horses suffering infectious arthritis (Tulamo *et al* 1994). A study by Palmer *et al* (1995) however, showed no difference in hyaluronan concentrations in synovial fluid between various groups of joint disease and normal equine joints.
This study was conducted to assess the levels of a number of potential synovial fluid markers in cases of infectious arthritis. Therefore, PGE_2, GAG, hydroxyproline and hyaluronan concentrations, as well as general MMP activity, in synovial fluid in cases of equine infectious arthritis were determined, and compared to values in healthy joints. Additionally, the relationship between the various parameters was investigated.

Materials and methods

Horses

Synovial fluid was collected from eight horses presented to the department of Equine Sciences of Utrecht University suffering infectious arthritis, before the initiation of treatment. Diagnosis was based on clinical signs, positive bacterial culture and/or elevated WBC counts (> 30,000 cells/µl synovial fluid). The synovial fluid collected was divided into two portions: one part was submitted for bacterial culture and, following cell count, the remainder was centrifuged (10,000 g for 10 minutes) and stored at -80°C until biochemical analysis. Patient details and clinical history were recorded from patient records. Synovial fluid was also collected from clinically sound horses used in a previous study (van den Boom *et al* 2004a), which was approved by the University of Utrecht's Ethics Committee and conducted in compliance with the Dutch act on Animal Experiments.

Prostaglandin E_2 assay

Prostaglandin E_2 concentrations were determined using mass spectrometry (MS). Briefly, 700 µl SF and 10 µl internal standard (10 pmol/µl $PGF_{2\alpha}$) were incubated for 1 h at 37ºC with 10 µl hyaluronidase (10 mg/ml), after which samples were centrifuged. The supernatant was applied to L1 Chrolut columns, which had been pretreated with 1ml aceton followed by 1 ml water, and flushed using 1 ml water, 1 ml 5% ethanol and 1 ml hexane successively. Prostaglandins were eluted from the column using 400 µl ethylacetate and reconstituted with 30 µl water. Prostaglandin concentrations were measured by MS using an API-365 triple quadrupole mass spectrometer (PE Biosystems), equipped with an electrospray ion source. Negative-mode MS was performed at a spray voltage of - 4.5 kV. Prostaglandins were separated at a flow rate of 200 µl/min on a Hypersil ODS column (Hewlett Packard) using a non-linear gradient from 2.5 mM ammonium acetate (pH 5.6) (eluate A), to acetonitrile : methanol (95:5 v/v), containing 2.5 mM ammonium acetate (eluate B). The column effluent was introduced into the mass spectrometer and for PGE_2 the transition between m/z 351 and m/z 271 was monitored. The PGE_2 concentration was expressed as pg/ml SF.

MMP activity assay

Synovial fluid was diluted 12.5 times in a buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1µM $ZnCl_2$ and 0.01% Brij-35. MMP activity was determined using a slight modification of the fluorometric assay as described by DeGroot *et al* (2001b). Briefly, conversion of a 2.5 µM concentration of the internally quenched fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) was measured in the presence of an EDTA-free general proteinase inhibitor cocktail solution (Complete, Roche Diagnostics) to prevent conversion of the substrate by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in the presence and absence of MMP inhibitor BB94 (10 µM). Since the substrate is not cleaved by aggrecanases, this approach detects only MMP-mediated substrate conversion and reflects the overall MMP activity in the SF samples. Incubations were performed in black Costar 384-well plates at 30ºC. Increase in fluorescence was followed in a thermostated fluometer (Cytofluor 4000, Perseptive Biosystems, λ_{ex}= 485 nm, λ_{em}= 530 nm). The initial velocity of substrate turnover (linear increase in fluorescence over time) was used as a measure of enzyme activity, expressed as relative fluorescence units (RFU)/hour.

Glycosaminoglycan assay

Proteoglycan content in synovial fluid was determined by measuring the amount of polysulphated GAGs by a modification of the 1,9-dimethylmethylene blue (DMMB) assay described by Farndale *et al* (1986). To 5 µl of synovial fluid, 5 µl hyaluronidase (500 µg/ml in 50 mM sodium acetate (pH 5.2)) was added, followed after 30 minutes by 200 µl of reagent (46 µM DMMB, Sigma, 40 mM glycine and 42 mM NaCl adjusted to pH 3.0

with HCl) and after 15 min the absorbence at 525nm was measured. The assay was standardised with shark chondroitin sulphate (Sigma). Glycosaminoglycan concentrations were expressed as µg/ml synovial fluid.

Hydroxyproline assay

Synovial fluid hydroxyproline concentration was determined by use of high performance liquid chromatography (HPLC) following derivitization with a fluorescent label of secondary amino acids (proline and hydroxyproline), as described (Bank *et al* 1997). In short, synovial fluid (2 to 4 µl) was hydrolyzed in 1 ml of 6M HCl (for 20 h at 110°C), dried overnight (approximately 17 hours) under vacuum (Speedvac, Thermo Savant) and dissolved in 100 µl of water (MilliQ, Millipore Corp.). An aliquot was transferred to an HPLC insert and placed in the autoinjector of an HPLC system consisting of a high precision HPLC pump (model 480, Gynkotek), an autosampler (Sparks Holland Triathlon, Sparks Holland) and a spectrofluorometric detector (model 821 FB, Jasco). Automated derivitization consisted of blocking of primary amino acids with o-phthaldialdehyde (Sigma), followed by labeling of secondary amino acids with 9-fluorenylmethyl chloroformate. Derivitized amino acids were injected onto a reversed phase HPLC column (TSK ODS 80^{TM}, 4.6 x 150 mm, Tosoh Biosep, Tosoh Corp.), and eluted with a tertiary gradient of citrate, acetonitrile, and methanol, as described (Bank *et al* 1996). Fluorescence was monitored at 254 nm (excitation) and 630 nm (emission) and data were recorded online by a computing integration system (Chromeleon^{TM} version 4.30, Dionex). The amino acid standard for collagen hydrolysates (Sigma) served as a reference. The intra-assay variation was 3% and the interassay variation was 10% and hydroxyproline presented as µg/ml.

Hyaluronan assay

Uronic acid was determined in synovial fluid using a slight modification of the assay described by van den Hoogen *et al* (1998b). Briefly, 250 µl sulphuric acid (96% w/w, containing 120 mM sodium tetraborate) was added to 50 µl diluted synovial fluid (1:4 with water) and incubated for 1 hour at 80ºC. After cooling, background absorbance was measured at 540 nm. Subsequently, 5 µl m-hydroxydiphenyl reagent (5 µl m-hydroxydiphenyl in DMSO, 100 mg/ml, mixed with 1 ml 0.1 M NaOH just before use) was added and after 15 minutes absorbance was again measured at 540 nm. The change in absorbance reflects uronic acid and values were standardised with galacturonic acid. Hyaluronan content in synovial fluid was estimated by subtracting GAG from total uronic acid levels and expressed as µg/ml synovial fluid.

Statistical analysis

Differences in PGE_2, GAG, hydroxyproline and hyaluronan concentrations and MMP activity between synovial fluid from infectious arthritis patients and controls were tested using the Mann Whitney U test and correlations between parameters were sought by

use of Spearman's correlation coefficient (SPSS Inc.). The level of significance was set at $P<0.05$.

Results

Horses

Details of the infectious arthritis patients are shown in table 1. The mean age of the horses suffering infectious arthritis (5.8 ± 6.9 years) was lower than that of the control horses (10.3 ± 3.5 years) but the difference was not statistically significant ($P>0.05$).

	joint	age	Bacteriological culture
1	tarsocrural	1 year	*Staphylococci*
2	tarsocrural	5 days	*Cl. perfringens*
3	metacarpophalangeal	2.5 years	*Staphylococci*
4	humeroradial	2.5 years	negative
5	proximal interphalangeal	20 years	negative
6	tarsocrural	1 year	*Streptococci*
7	distal interphalangeal	9 years	*Pseudomonas*
8	metacarpophalangeal	10 years	*Streptococci*

***Table 1**: Details of infectious arthritis cases.*

Synovial fluid parameters

The white blood cell count of the infectious arthritis samples was 66,950 ± 73,790 cells/µl. Bacterial culture yielded positive results in 6/8 cases and the following bacteria were isolated: *Staphylococci* (2), *Streptococci* (2), *Clostridium perfringens* and *Pseudomonas*. Prostaglandin E_2 concentration was significantly higher in synovial fluid in cases of infectious arthritis (573.5 ± 314.0 pg/ml, mean ± s.d.) than in synovial fluid from unaffected joints (8.7 ± 1.6 pg/ml, Fig 1), as was MMP activity (2584.3 ± 4623.7 RFU/h vs. 5.7 ± 5.3 RFU/h, Fig 2) and the glycosaminoglycan concentration (55.0 ± 10.7 vs. 23.1 ± 2.1 µg/ml, Fig 3). Although the hydroxyproline concentration was higher in SF from infectious arthritis cases (168.2 ± 86.4 µg/ml, mean ± s.d.) compared to healthy joints (99.6 ± 59.0 µg/ml, Fig 4), the difference was not statistically significant. Hyaluronan concentrations however were significantly lower in synovial fluid from infectious arthritis joints (328.0 ± 28.7 vs. 497.1 ± 35.9 µg/ml, Fig 5).

When all samples were considered together there was a positive correlation between PGE_2 concentrations and MMP activity ($r = 0.76$, $P < 0.005$, Spearman correlation coefficient), between PGE_2 and GAG concentrations in SF ($r = 0.53$, $P < 0.05$, Spearman correlation coefficient) and a negative correlation between PGE_2 and hyaluronan ($r = -0.67$, $P < 0.01$, Spearman correlation coefficient). In the infectious arthritis group there was no correlation between WBC counts and any of the other parameters measured.

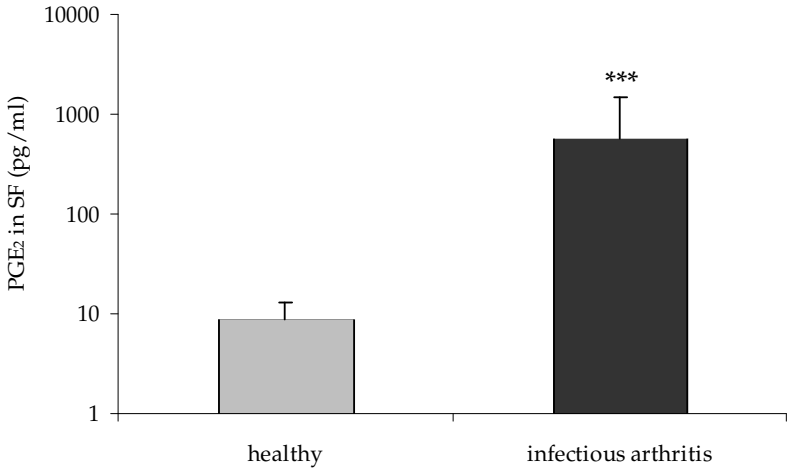


***Figure 1**: Prostaglandin E_2 (PGE_2) concentration (pg/ml; mean ± s.d., logarithmic scale) in synovial fluid (SF) from healthy joints and from joints with infectious arthritis. *** P<0.005.*

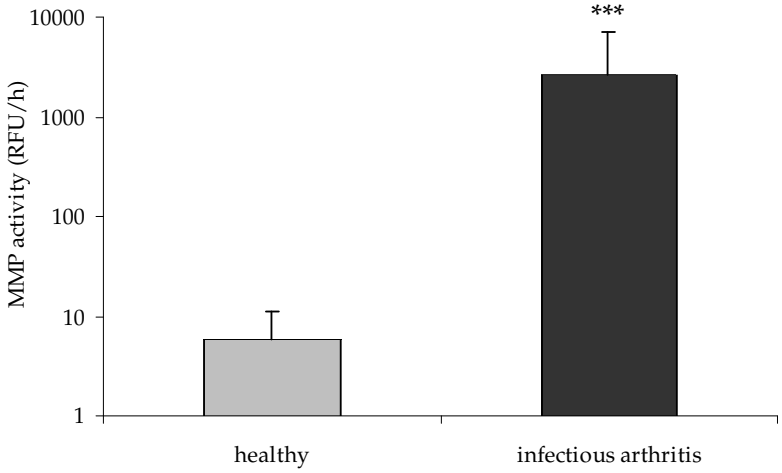


***Figure 2**: General matrix metalloproteinase (MMP) activity (relative fluorescence units (RFU)/hour; mean ± s.d., logarithmic scale) in synovial fluid (SF) from healthy joints and from joints with infectious arthritis. *** P<0.005.*

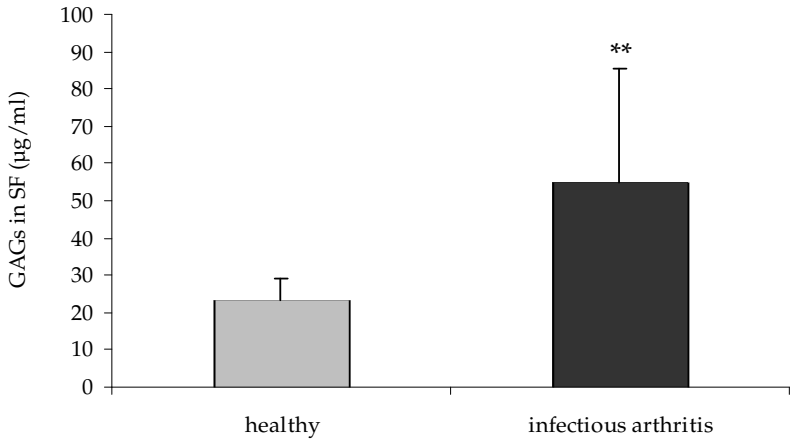


***Figure 3**: Glycosaminoglycan (GAG) concentration (*µg/ml; mean ± s.d.*) in synovial fluid (SF) from healthy joints and from joints with infectious arthritis. ** P<0.01.*

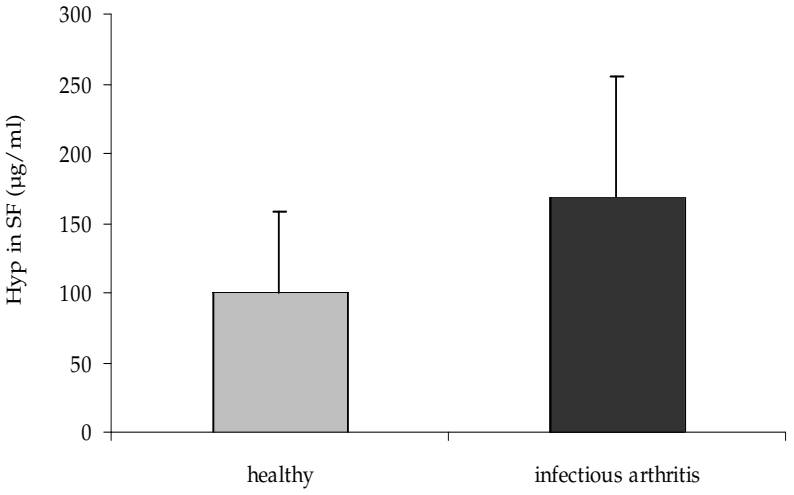


***Figure 4**: Hydroxyproline (Hyp) concentration (*µg/ml; mean ± s.d.*) in synovial fluid (SF) from healthy joints and from joints with infectious arthritis.*

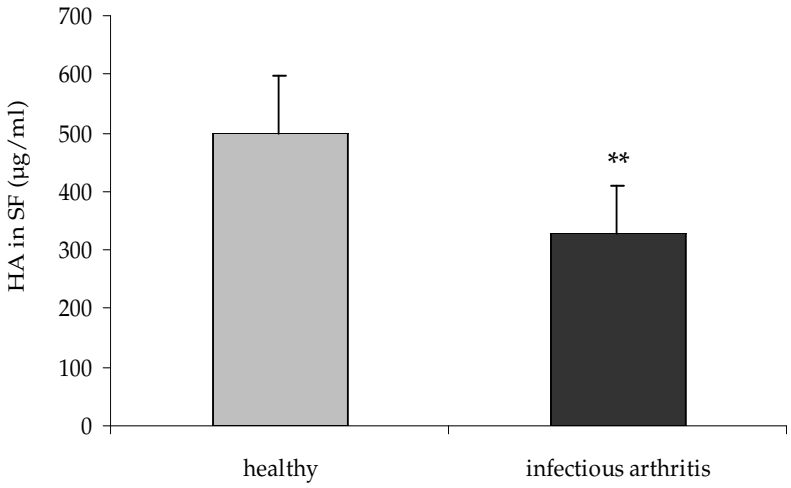


***Figure 5**: Hyaluronan (HA) concentration* (µg/ml; mean ± s.d.) *in synovial fluid (SF) from healthy joints and from joints with infectious arthritis. ** P<0.01.*

Discussion

The mean white blood cell count was high in most cases of infectious arthritis, although this was not the case in all samples. The increase in cell numbers is in accordance with other literature (Bertone 1996), where leukocyte counts >30,000/µl are regarded as diagnostic for infectious arthritis. On the other hand it has previously been demonstrated that caseinase and collagenase activity can be elevated in infectious arthritis even when cell counts have returned to normal (Spiers *et al* 1994b), demonstrating that cell count alone is not a reliable indicator of the presence or severity of disease. The lack of any correlation between WBC counts and other parameters in synovial fluid also suggests that the use of WBC counts in assessing disease severity has serious limitations.
Infectious arthritis was paired with dramatically increased PGE_2 concentrations in SF, as previously demonstrated for infectious arthritis (May *et al* 1994) and other types of equine joint disease (Bertone *et al* 2001, Gibson *et al* 1996, May *et al* 1994). This confirms its value as a marker of joint inflammation, elevated in nearly all types of joint disease (Bertone *et al* 2001). Levels were not correlated with white blood cell counts, which suggests that resident articular cells (chondrocytes and/or synoviocytes) are responsible for prostaglandin release. In the equine joint the synovial membrane is the most likely source of PGE_2 as it contained higher levels than the articular cartilage (von Rechenberg *et al* 2000) and this fact supports the notion that PGE_2 is principally an indicator of synovial inflammation (Kirker-Head *et al* 2000). Increased PGE_2 concentrations in synovial fluid from infectious arthritis cases may well play a role in lameness observed with this condition, as prostaglandins are involved in the nociceptive pathways within the joint

(Kirker-Head *et al* 2000, May *et al* 1994) although not as a direct mediator (Ferreira 1972). Further evidence for the involvement in pain perception is the finding that PGE_2 concentrations were elevated in most lame horses in a study performed by May *et al* (1994). Apart from a function in lameness PGE_2 also leads to cartilage damage. It decreases proteoglycan synthesis and increases GAG loss from chondrocytes (Fulkerson *et al* 1979, van de Loo and van den Berg 1990). As PGE_2 concentrations were correlated with MMP activity, it is tempting to assume that cartilage degradation in infectious arthritis is a result of PGE_2-induced MMP activation, but this may be too simplistic an approach. On the one hand PGE_2 was shown to be involved in MMP activation (Bunning and Russell 1989, Mehindate *et al* 1995), while in other studies exogenous PGE_2 reduced MMP activation (DiBattista *et al* 1994, Tung *et al* 2002a). These contradictory findings may well be related to differences in PGE_2 concentrations, as low concentrations increase and high concentrations decrease collagen synthesis (Di Battista *et al* 1996). The correlation between PGE_2 concentrations and other synovial parameters indicates that this eicosanoid plays a pivotal role in infectious arthritis.

Matrix metalloproteinase activity was also significantly higher in infecious arthritis cases than in healthy joints but was not related to white blood cell counts in synovial fluid. The elevated MMP activity is in accordance with findings by others who reported increased gelatinase and collagenase avtivity in horses suffering infectious arthritis (Clegg *et al* 1997b, Spiers *et al* 1994b, Trumble *et al* 2001). This cannot be considered surprising as both LPS (Arend *et al* 1985, Morales and Hascall 1989, Wilson *et al* 1987) and purified *staphylococcal* medium (Williams, III *et al* 1991) are known to stimulate the secretion of various metalloproteinases in cartilage explants. The MMPs are likely derived from both inflammatory cells and articular cells as correlations between cell numbers and MMP activity were present in some studies (Clegg *et al* 1997b, Trumble *et al* 2001) but not in another (Spiers *et al* 1994b). The presence of MMP-3 and -13 mRNA in synovial membrane and cartilage samples from diseased equine joints (Trumble *et al* 2001) clearly demonstrates that these tissues produce MMPs and synovial fibroblasts have also been shown to produce MMP-2 (but not MMP-9) (Clegg *et al* 1997a). The origin of enzyme activity also seems to depend on the (class) of MMP involved. The effects of MMPs depend on the balance between them and their inhibitors, tissue inhibitors of metalloproteinase (TIMPs) and α-2-macroglobulin (α-2-M). In normal joint homeostasis these are in equilibrium but in disease the balance shifts in favour of enzyme activity. Whereas TIMP activity was raised in aseptic joint disease it was minimal in septic arthritis (Clegg *et al* 1998a) and this imbalance could contribute to cartilage breakdown.

As might be expected the increase in MMP activity in infectious arthritis cases was paired by an increase in synovial GAG concentrations, whereas hydroxyproline levels were not significantly elevated. Between them the various MMPs are capable of degrading all components of the cartilage matrix and the increased MMP activity will contribute to cartilage damage in infectious arthritis. The substrate employed in the general MMP activity assay is converted mainly by the gelatinases (MMP-2 and -9) and by MMP-13 (collagenase-3), implicating these enzymes in infectious arthritis. The lack of a correlation between GAG concentrations and MMP activity might be explained (in part) by the fact the proteoglycans are broken down not only by MMPs, but also by aggrecanases, which

were not measured in our study. Palmer *et al* (1995) also found increased concentrations of GAGs and chondroitin sulphate in synovial fluid from diseased equine joints and stated that these could be derived from the synovial membrane, articular cartilage or both. Increased chondroitin sulphate concentrations in synovial fluid from joints without signs of synovitis suggest that cartilage damage alone can cause significant proteoglycan release (Palmer *et al* 1995). This is confirmed by increased release of GAGs from cartilage explants exposed to LPS (MacDonald *et al* 1994). Given the extensive cartilage destruction often seen in joints affected by infectious arthritis cartilage must be considered to be an important (if not the sole) source of GAGs in synovial fluid. The release of GAGs from cartilage can be the result of increased proteolysis of proteoglycans but may also result from damage to the collagen network, as has been demonstrated in damaged cartilage from the equine proximal phalanx (van den Boom *et al* 2004e). As the collagen network is broken down GAGs can escape and SF concentrations rise. Obviously, it would be extremely interesting to compare synovial GAG levels with cartilage composition in these cases, but fortunately most patients survived and cartilage was not available for analysis. Obtaining cartilage from large numbers of these cases, certainly at the time of active disease, would be very difficult. Cartilage may become available at a later date, following death from natural or other causes, and this might make it possible to determine what the long-term effects on cartilage are.

Hydroxyproline concentrations in synovial fluid from infectious arthritis cases were not elevated compared to synovial fluid from unaffected horses, indicating that damage to the cartilage collagen network is limited. Given the extremely high turnover time of collagen in adult articular cartilage this collagen damage is likely to represent an irreversible step in cartilage degradation in infectious arthritis. In analogy to osteoarthritis this collagen degradation is considered to mark a point of no return for cartilage breakdown (Billinghurst *et al* 2001). This may be slightly different for young animals as there is considerable collagen turnover in articular cartilage in foals (Brama *et al* 2000b), but in horses this development decreases (rapidly) with age and appears to have ceased by the age of 4 years (Brama *et al* 1999b). The decline in collagen metabolism in equine joints is paralleled by decreasing hydroxyproline concentrations in synovial fluid from foals to adult horses (van den Boom *et al* 2004b). This emphasises the importance of the rapid institution of therapy in infectious arthritis as both antibiotic treatment and joint lavage were able to delay or reduce collagen loss in experimental infectious arthritis (Daniel *et al* 1976, Smith *et al* 1987). The fact that hydroxyproline concentrations were not (yet) significantly higher in SF in infectious arthritits also suggests that (complete) recovery of articular cartilage may be possible.

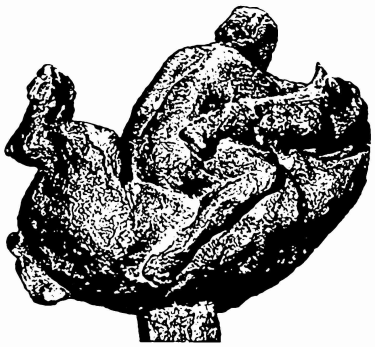
Similar to other studies (Hilbert *et al* 1984, Tulamo *et al* 1994) we also found decreased hyaluronan concentrations in synovial fluid from infectious arthritis cases. Synovial fluid dilution, resulting from joint effusion, has been suggested to be (partly) responsible for this effect (Hilbert *et al* 1984). However, the fact that bacterial infection led to decreased hyaluronan concentrations in media from synovium explants (Hardy *et al* 1998a) showed that synthesis is also decreased. Another cause of decreased hyaluronan levels could be degradation by hyaluronidases and free radicals (Palmer *et al* 1995). Hyaluronan is the major GAG produced by synoviocytes (Hamerman *et al* 1982), is important for joint

homeostasis (Laurent *et al* 1995) and is responsible for the viscosity of synovial fluid. Lowered hyaluronan concentrations result in the decreased viscosity usually observed in infectious arthritis. Exogenous hyaluronan is considered to have a favourable influence on cartilage metabolism and is frequently administered as intra-articular, intravenous or intramuscular medication in cases of joint disease. Hyaluronan increases proteoglycan synthesis in chondrocyte culture and explants (Frean *et al* 1999) and in osteoarthriris models (Han *et al* 1999, Kawcak *et al* 1997) and reduced proteoglycan loss in cartilage from the distal metacarpus (Frean *et al* 2000) The mode of action has not been fully elucidated but appears to involve anti-inflammatory mechanisms (Frean and Lees 2000, Kawcak *et al* 1997), including down-regulation of MMP-3 in synovium (Takahashi *et al* 1999). If this is indeed the case the lower hyaluronan concentrations found in infectious arthritis cases may make the articular cartilage even more susceptible to increased cytokine and MMP levels.

In conclusion, the results of this study appear to confirm the limited capability of WBC counts to accurately predict the seriousness of infectious arthritis. The extremely high PGE_2 concentration and MMP activity in synovial fluid from infectious arthritis cases, compared to healthy joints, suggests they are more reliable markers of inflammation. The correlations between PGE_2 and MMP activity and between PGE_2 and GAG and hyaluronan concentrations suggest that PGE_2 might be the most suitable marker of the actual state of the joint in these cases. The values in this study were obtained in synovial fluid collected before initiation of therapy and future research should be aimed at serial measurements of synovial fluid markers, to determine how they are affected by resolution of infection. Marker levels must also be related to the outcome of treatment to determine if they can be used to estimate the prognosis of infectious arthritis.

Chapter VIII

General Discussion



General Discussion

In October 2000 a workshop was held in Northampton (UK) to review the state of affairs regarding molecular markers of cartilage and bone metabolism in the horse, and to identify the most important issues relating to this field of research (Anon 2002). Although much work on biomarkers had already been performed at that time a number of crucial questions regarding internal and external factors influencing marker levels had not yet been (adequately) addressed. It was pointed out, for example, that the clinical value of markers could only be established if their inherent biological variability (due to factors such as age) is understood (and controlled for). Several speakers at that meeting also stressed the important effect of exercise, a vital management factor in the equine industry, on marker levels. An additional external influence, unique to synovial fluid (SF), is (repeated) arthrocentesis, an intervention which is obviously necessary for the collection of synovial fluid, but which in itself may alter its composition. This thesis addressed the relationships between a number of putative synovial fluid markers and age, exercise and repeated arthrocentesis (chapters II-V) and tried to relate levels of these markers to the severity of cartilage damage (chapters V-VII).

- aging

Age (from birth to maturation) had a profound effect on all of the markers studied, while it was not correlated with marker concentrations in adult horses. We found very high collagenase-1 (MMP-1) activity in synovial fluid (SF) of neonates, which declined with increasing age to reach steady adult levels by the age of approximately four years (Brama *et al* 2004 - chapter III). This is similar to what has previously been described for general MMP and stromelysin (MMP-3) activity in equine SF (Brama *et al* 1998, 2000d). We are not aware of any studies examining synovial MMP levels in babies or children, but Lohmander *et al* (1993) found no influence of age on MMP levels in SF in human adults. Apparently, MMP activity is high in SF from young individuals and declines with aging, reaching stable levels once skeletal maturity is reached.
The elevated MMP activity in foals was paralleled by high glycosaminoglycan (GAG) and hydroxyproline (Hyp) concentrations in synovial fluid. Both parameters were highest in SF from newly born foals and decreased with age (van den Boom *et al* 2004b - chapter V), with the biggest drop occurring during the first five months of life. By the age of approximately four years both GAG and Hyp levels had stabilized at adult values and were no longer correlated with age. This explains the lack of a correlation between SF GAGs and age in adult horses reported by Fuller *et al* (1996).

The biological interpretation of these findings seems obvious. When foals grow there is a high level of cartilage turnover/remodelling: cartilage is broken down and new cartilage is formed, and the MMPs play an important role in this process (Bluteau *et al* 2001, Chubinskaya *et al* 1999). This rapid cartilage metabolism leads to the release of matrix molecules (GAGs and hydroxyproline) into SF. It is during this same period that the heterogeneity in cartilage composition seen in adult horses begins to develop (Brama *et*

al 2000b). The high metabolic rate is in line with increased biosynthesis in chondrocyte culture and articular cartilage explants from foals and young horses, compared to those from adult horses (Dean *et al* 2003, Iqbal *et al* 2000). The increased MMP activity in these young horses does not result in net cartilage breakdown, but rather synthesis exceeds degradation resulting in growth. In other words, in foals and young horses, MMP activity and GAG and hydroxyproline concentrations are markers of normal physiology.
The rate of cartilage remodelling is gradually reduced and, in horses, appears to have stopped by the age of four years. In adult horses there is no significant correlation between age and water, collagen, GAG and hydroxylisine content or enzymatic crosslinking in cartilage (Brama *et al* 1999b, Vachon *et al* 1990). Similarly, in adult human cartilage GAG content also did not change with age (Maroudas *et al* 1973).

In a practical sense, it can be concluded that in adult horses (> 4 years) age is no longer a confounding factor for the use of MMPs, GAG or hydroxyproline as synovial markers, but these findings also imply that in young horses different reference values than those used for adult horses should be established and probably even subdivided for foals and juvenile horses. It may well be that changes in SF marker concentrations occurring with disease are insignificant compared with those accompanying this joint remodelling, making MMPs, GAG and hydroxyproline unsuitable as synovial fluid markers of pathological processes in young horses. This is especially relevant in breeds such as the thoroughbred and standardbred, which start their racing careers at two or three years of age.

- *exercise*

The vast majority of horses kept nowadays are used for a variety of equine sports and are exercised (almost) daily, either in training or in competition, and the exercise used in our studies influenced several of the markers studied. The moderate amount of exercise the horses were subjected to led to a transient rise in TNF-α levels in SF, which peaked shortly after the completion of exercise and had passed by 12 hours (van den Boom *et al* 2004a - chapter II). The same exercise programme did not lead to a significant increase in general MMP (van den Boom *et al* 2004a - chapter II) or collagenase-1 (MMP-1) activity (Brama *et al* 2004 - chapter III). This is in accordance with findings in ponies (van den Hoogen *et al* 1998a), where one weeks' exercise did not increase MMP activity, and with findings in human athletes, where 1-1½ hours of football or running also did not affect MMPs in synovial fluid (Roos *et al* 1995). Exercise did cause increased PGE_2 concentrations in SF (van den Boom *et al* 2004c - chapter IV), although the rise was considerably smaller than seen in infectious arthritis (van den Boom *et al* 2004d - chapter VII). In our experiments exercise did not affect nitric oxide concentrations or GAG release (van den Boom *et al* 2004c - chapter IV).
Tumour necrosis factor α (TNF-α) has been put forward as a marker of joint disease in horses (Bertone *et al* 2001), but apparently the timing of arthrocentesis is important for correct interpretation of synovial fluid levels, as collection of SF shortly after exercise (within 12 hours) could lead to misinterpretation of data. The same may well apply for other cytokines, although this was not investigated in our experiments. The increase in

TNF-α concentrations after exercise suggests that joint loading causes an 'inflammatory' reaction within the joint, the clinical relevance of which is hard to determine. Billinghurst *et al* (1995) found a similar rise in TNF activity in SF from thoroughbred horses with joint disease, and those authors attributed this phenomenon to an activation of the osteoarthritic process. The horses used in our study were clinically healthy; although it is possible that subclinical joint pathology might have been present. Billinghurst *et al* (1995) also found high levels of TNF in SF from normal joints and a possible explanation could be that these samples may have been taken shortly after exercise.
Tumour necrosis factor causes the production and/or release of other cytokines and degradative enzymes but it is impossible from our results to determine if this catabolic cascade is initiated every time a horse is exercised. Cartilage metabolism is regulated at many levels by the presence of inhibitors and it cannot be ruled out that such inhibitory processes are also stimulated by exercise, neutralising the increased cytokine levels. It is also conceivable that the increase in synovial TNF-α levels following exercise has a function in joint homeostasis. Articular cartilage composition is known to adapt in response to training (Brama *et al* 1999a, 2000a) and cytokines (including TNF-α) might well trigger this process. This means that TNF-α may be a marker of normal joint physiology, increased in response to (moderate) exercise. In this context it is interesting to note that the effects of TNF-α depend not only on its concentration but also on the number of receptors present and these have been shown to be more abundant in OA cartilage chondrocytes (Fernandes *et al* 2002, Kammermann *et al* 1996, Malemud and Goldberg 1999, Webb *et al* 1997, Westacott *et al* 1994), so a similar increase in TNF-α concentration is likely to cause stronger effects in OA joints than in healthy joints.

The lack of an exercise-effect on MMP activity might be considered surprising as TNF-α is known to induce MMP synthesis (Richardson and Dodge 2000) and activation (Okada *et al* 1992). The assay used in our studies measures only active MMPs, which make up no more than 5% of total MMP levels, and it could be that there was an increase in MMP production but that this was counterbalanced by raised levels of inhibitors, as levels of tissue inhibitor of metalloproteinase (TIMP)-1 in SF were increased in human athletes following exercise (Roos *et al* 1995).
The intensity of exercise may also be of importance and MMP activity might have been increased if the horses had followed a more intensive exercise programme. In general, strenuous exercise leads to degradative changes in articular cartilage in various species, including horses and humans (Brama *et al* 2000a, Helminen *et al* 2000) and extreme exercise intensity even led to the development of OA in mice and rats (Lapvetelainen *et al* 1995, Machner *et al* 2000, Pap *et al* 1998). The induction of OA by exercise is likely to be paired with increased MMP activity. Moderate exercise, on the other hand, has been demonstrated to be chondroprotective, increasing GAG synthesis and reducing GAG release in cartilage from horses and ponies (Bird *et al* 2000b, van de Lest *et al* 2000, van den Hoogen *et al* 1998a, 1999). In addition, a minimal amount of joint loading is necessary for the maintenance of normal cartilage as joint immobilisation or a sedentary lifestyle cause cartilage 'atrophy' and fibrillation (Fu *et al* 2001, Helminen *et al* 2000). The changes in cartilage metabolism and composition resulting from various exercise levels

are likely to be reflected in synovial fluid, and the fact that MMP activity was not increased in our studies indicates that the level of exercise employed (during one week) should not be considered detrimental to articular cartilage.
The exercise-induced increase in PGE_2 concentration could be related to the increase in TNF-α levels, as this cytokine stimulates PGE_2 release (Bunning and Russell 1989, Hawkins *et al* 1993). Prostaglandin E_2 is considered to be a very sensitive predictor of joint disease, being elevated in most types of arthropathy, while it is not present in most normal SF (Frisbie *et al* 2003, May *et al* 1994). It plays an important role in pain perception (May *et al* 1994), but is not related to the severity of clinical or arthroscopic signs of joint disease (Murakami *et al* 1998). As discussed above, moderate exercise can be considered beneficial for cartilage homeostasis and it is possible that PGE_2 plays a role in mediating physiological exercise-induced effects, as its actions on articular cartilage are not all detrimental. For example, exogenous PGE_{1+2} inhibited IL-1 induced proteoglycan degradation (DiBattista *et al* 1994). It is possible that in joints, in analogy to other organs, cyclooxygenase (COX)-1 induced PGE_2 production serves a homeostatic function, while PGE_2 released by COX-2 is involved in inflammatory processes.
The effects of exercise are likely to be mediated (at least in part) by mechanical stimulation of chondrocytes which causes upregulation of mRNA for aggrecan and downregulation for MMP-3 (Millward-Sadler *et al* 2000). Intermittent pressure led to increased cellularity, GAG content and collagen production in a chondrocyte culture system (Carver and Heath 1999a, 1999b), whereby proteoglycan synthesis also depends on the frequency of load application (Sauerland *et al* 2003). Greater loading however, resulted in proteoglycan loss and cell death in bovine cartilage explants and cyclic loading at more than one MPa for more than six hours increased MMP-3 levels (Lin *et al* 2004). In intact joints the type of stimulation determines the outcome, as stretching (as opposed to compression) of chondrocytes reduced proteoglycan synthesis (Tanaka *et al* 1998). The effects, however, are not always consistent, as mechanical stimulation induced nitric oxide (NO) and PGE_2 production in one study (Fermor *et al* 2002), while dynamic compression inhibited IL-1 induced NO and PGE_2 release in another (Chowdhury *et al* 2003). These in vitro experiments clearly show that mechanical factors influence joint homeostasis/metabolism and will lead to alterations in SF composition.

The amount of exercise performed by the horses in our studies is similar to that performed by many horses on a daily basis and should be considered to be of moderate intensity and thus would be expected to have a beneficial effect on joints in general and cartilage in particular. Therefore, the increases in TNF-α and PGE_2 concentrations may represent physiological processes. These effects are present shortly after exercise and appear to have passed by 12 hours, which seems to be a safe margin to avoid confounding effects by exercise on marker levels. This does not apply to all markers, as moderate exercise did not affect MMP activity, GAG concentration or NO concentration in synovial fluid from healthy equine joints. On the other hand, more strenuous exercise might have a longer lasting effect and/or influence other markers than the ones identified in this study.

- repeated arthrocentesis

Serial arthrocentesis did not lead to increased TNF-α levels (van den Boom *et al* 2004a - chapter II), although it is possible that each time arthrocentesis was performed a transient increase occurred which was not picked up in our study. This cannot be confirmed with absolute certainty based on our experimental findings as the shortest interval between two consecutive joint aspirations was ten hours and TNF-α concentrations can change very rapidly. The absence of an 'arthrocentesis effect' for TNF-α is in accordance with findings by Billinghurst *et al* (1995) and implies that timing of arthrocentesis is not critical when assessing TNF-α concentrations (as long as it is not performed within about 12 hours of exercise).
Repeated arthrocentesis (within several days) did cause an increase in general MMP activity (van den Boom *et al* 2004a - chapter II), and MMP-1 activity (Brama *et al* 2004 - chapter III), and obviously this is important when these substances are used as markers of joint disease. Arthrocentesis also caused increases in NO and PGE_2 concentrations in equine SF and led to the release of glycosaminoglycans (GAGs) (van den Boom *et al* 2004c - chapter IV).
There are several potential sources of increased MMP activity: the synovial membrane, articular cartilage, or leucocytes within the SF (Okada *et al* 1992, Spiers *et al* 1994a). The increase in MMP activity was more pronounced in the metacarpophalangeal and radiocarpal joints than in the tarsocrural joint and this could implicate the synovial membrane. The metacarpophalangeal and radiocarpal joints are smaller than the tarsocrural joints and the increased MMP activity resulting from 'stimulation' of a small part of the synovial membrane would be less diluted in the two smaller joints (which have approximately the same volume). Daily arthrocentesis caused mild-moderate haemorrhage and oedema of the synovial membrane in the tarsocrural joint (Lescun *et al* 2002) and might also lead to MMP production or activation. Transient synovitis, associated with partial meniscectomy in rabbits, also caused an increase in MMP-3 in chondrocytes and synovium (Mehraban *et al* 1998) although in this model TIMP levels in synovium were also elevated. Lu *et al* (2004) even propose synovectomy as a therapy for out of control synovitis cases to remove an important source of MMPs and thus lower MMP activity within the joint.
It seems likely that the elevated MMP activity in these joints is related to the increased NO and PGE_2 concentrations and GAG release, as there have been numerous studies showing that these factors influence each other. Nitric oxide is regarded as an important mediator in arthritis (Tung *et al* 2002c, Wiseman *et al* 2003), causing chondrocyte apoptosis and inhibiting chondrocyte proliferation (Blanco *et al* 1995, Blanco and Lotz 1995), making it a potential marker of joint disease. Elevated NO concentrations were found in culture media of equine OA chondrocytes in one study (von Rechenberg *et al* 2000) but SF levels were not increased in cases of equine joint disease in another (de la Calle *et al* 2002). The source of NO is likely to be cartilage as equine chondrocytes produce more NO than synoviocytes (Frean *et al* 1997, von Rechenberg *et al* 2000). Equine chondrocytes and cartilage explants both produce NO upon stimulation with IL-1β (Bird *et al* 1997, Tung *et al* 2002b) and it is possible that the chondrocytes produce NO in response to

another mediator released by the synovial membrane. Nitric oxide is involved in MMP activation (Murrell *et al* 1995) and in our experiments increased MMP activity coincided with or followed elevated NO levels. It may also stimulate COX-2 leading to the production of PGE_2 (Salvemini *et al* 1995) and in this way could contribute to the rise in PGE_2 seen after repeated arthrocentesis. The role of PGE_2 is not clear and contradictory actions have been described. On the one hand it is considered an inflammatory mediator involved in joint pain (Brenner *et al* 2004, Kirker-Head *et al* 2000, May *et al* 1994), elevated in almost all types of joint disease and involved in the activation of MMPs (Bunning and Russell 1989, Mehindate *et al* 1995), while in other studies exogenous PGE_2 was shown to limit MMP activation (DiBattista *et al* 1994, Tung *et al* 2002a). The effect on cartilage is likely to depend on local concentrations, and these may not be accurately reflected by synovial fluid concentrations. Although it is also produced by chondrocytes in response to a variety of stimuli (Frean and Lees 2000, May *et al* 1989) PGE_2 (in horses) is released mainly by the synovial membrane (von Rechenberg *et al* 2000) and reflects synovial inflammation. Experimentally induced synovitis also induced PGE_2 release (Owens *et al* 1996) and it seems probable that (repeated) arthrocentesis leads to a localised synovitis which causes the release of several potential markers, including PGE_2.
It can be assumed that the release and activation of MMPs resulting from repeated arthrocentesis caused the GAG release seen in the metacarpophalangeal and radiocarpal joints in both exercised and control horses (van den Boom *et al* 2004c - chapter IV). Matrix metalloproteinases can degrade all components of articular cartilage, with the stromelysins having the greatest affinity for proteoglycans. The collagenases, however, may also play a role as damage to the collagen network allows GAGs to escape. Aggrecanases, a recently identified group of enzymes, are also capable of proteoglycan digestion, but these were not measured in our studies. Repeated arthrocentesis is not a confounding factor for TNF-α concentrations in (adult) horses, but it does affect MMP activity, NO, PGE_2 and GAG concentrations.

In practical terms, it seems sufficient to wait one week following prior arthrocentesis when collecting SF for analysis of these potential markers.

- correlation of potential markers with disease status/cartilage composition

Osteoarthritis is characterised, among other things, by damage to the articular cartilage which starts with cartilage swelling, loss of proteoglycans and subsequent collagen depletion. While aggrecan loss is rapidly reversible (Bottomley *et al* 1997), collagen loss is considered to mark the irreversible stage of disease (Bottomley *et al* 1997, Caterson *et al* 2000, Curtis *et al* 2002). Another recommendation of the 2000 workshop was that the amount of cartilage damage in OA be identified (Anon 2002), to allow investigation of the relationship between cartilage status and marker levels. In a number of our experiments we used the cartilage degeneration index (CDI) to quantify cartilage damage and this score corresponds well with the macroscopic score of cartilage degradation (Brommer *et al* 2003a). Examination of cartilage from the medial dorsal articular margin of the proximal phalanx revealed lower GAG content in OA (high CDI) compared to healthy (low

CDI) joints (van den Boom *et al* 2004e - chapter VI), confirming that joints classified as OA using the CDI were indeed diseased.

A good biomarker will accurately predict the status of the articular cartilage, allowing timely diagnosis of joint disease and providing an opportunity for therapeutic intervention. This was one of the aspects studied in this thesis and the relationship between SF marker levels and cartilage damage and composition was investigated. Currently, it is only possible to determine the CDI for the proximal surface of the proximal phalanx and the other joint surfaces of the metacarpophalangeal joint were not evaluated. However, it is felt that (certainly in warmblood horses) the proximal articular surface of the proximal phalanx provides a representative indication of the condition of cartilage in the metacarpophalangeal joint.

Matrix metalloproteinases play an important role in cartilage destruction in OA. In human OA there is an upregulation of mRNA encoding for MMPs, increased MMP production and activity and all categories of MMPs have been implicated (Billinghurst *et al* 1997, Dahlberg *et al* 2000, Hembry *et al* 1995, Imai *et al* 1997, Okada *et al* 1992, Rørvik and Grondahl 1995, Shlopov *et al* 1997, Tetlow *et al* 2001). The increased MMP concentration and activity in diseased cartilage and synovium are reflected in increased MMP activity in SF (Beekman *et al* 1996). In horses, previous research has shown general MMP (Brama *et al* 1998) and stromelysin (Brama *et al* 2000d) activity to be higher than normal in OA joints and we demonstrated that MMP-1 activity is also increased in OA (Brama *et al* 2004 - chapter III). These findings would seem to support the use of MMPs as markers of OA. However, when investigating the relationship between general MMP activity and cartilage damage, MMP activity was not increased in joints with more cartilage degradation (van den Boom *et al* 2004e - chapter VI). Recent studies suggest that the MMPs are more involved in the later stages of OA (Caterson *et al* 2000, Little *et al* 1999, 2002) and the joints studied by us had fairly mild damage. MMP activity might have been elevated if damage had been more extensive/severe, although von Rechenberg *et al* (2000) found that explant cultures from joints with moderate OA released more MMPs than explants from severely arthritic joints. Apart from an upregulation of MMPs, TIMP-1 expression was also increased in SF form osteoarthritic joints and was correlated with MMP-1 and -3 expression (Ishiguro *et al* 1999). The increased TIMP levels could obviously neutralise MMP activity. Furthermore, the CDI can be seen as a measure of the total cumulative damage that has occurred to the joint in the course of disease, whereas MMP activity is a measure of actual proteolytic activity. Given the well-known intermittent nature of OA, damage accumulated over time and instantaneous activity of the disease process do not necessarily match.

Cartilage degradation leads to the release of extracellular matrix (ECM) components and proteoglycan levels in SF are elevated in (early) OA in man (Dahlberg *et al* 1992, Johnson *et al* 2002) and in acute and chronic joint disease, including OA, in horses (Alwan *et al* 1990, 1991, Palmer *et al* 1995). However, in our study we did not find increased GAG or hydroxyproline levels in SF from joints with more severe cartilage damage (van den Boom *et al* 2004b - chapter VI). Apparently the marker molecules are cleared from the joint space without accumulating to higher levels than in normal joints. Therefore, synovial fluid levels of these markers are not (directly) related to the degree of cartilage dam-

age, although the fact that hydroxyproline concentrations are related to MMP activity (van den Boom *et al* 2004b - chapter V) provides further evidence for the role of these enzymes in collagen breakdown.
In infectious arthritis PGE_2 concentrations and MMP activity in SF were very much higher than in SF from unaffected joints, confirming their potential as markers of inflammation. The increase in PGE_2 concentration and MMP activity were paired with elevated GAG and hydroxyproline concentrations, indicating ongoing cartilage damage, involving both proteoglycans and the collagen network. Raised GAG levels in SF have been previously demonstrated in human septic arthritis patients (Lohmander *et al* 1998), demonstrating that when active cartilage breakdown is taking place synovial GAG levels are increased. This provides support for the hypothesis that GAGs (and other markers) reflect the actual activity status of a joint, as do the findings that glycosaminoglycan release occurred in acute inflammatory joint disease (Ratcliffe *et al* 1988) and transient synovitis (Lohmander *et al* 1988).
These findings illustrate several important points. Cartilage destruction in OA does not occur at a continuous rate and is characterised clinically by alternating periods of lameness and soundness. During periods of active inflammation (of which infectious arthritis is an extreme example) inflammatory markers, such as MMP activity and PGE_2, are elevated, and the same may well be the case for NO and TNF-α. Similarly, these markers of inflammation may be increased only during periods of disease activity and could correspond with the presence of lameness in horses with joint disease. We did not know the clinical history of horses used in our study on cartilage composition and it would be extremely interesting to investigate PGE_2, NO and TNF-α concentrations, as well as MMP activity, in horses with OA presenting with lameness. In infectious arthritis increased levels of inflammatory markers coincided with elevated GAG concentrations in SF, which should be regarded as a marker of degradation, representing proteoglycan loss. It is conceivable that cartilage degradation in other forms of joint disease (including OA) is also (largely) restricted to periods of active inflammation, and this could explain why synovial fluid marker concentrations were not elevated in joints with (substantial) cartilage damage.

The division of markers into two categories may allow different therapeutic options, depending on whether the inflammatory or degradative component dominates. Cases with inflammation, without evidence of cartilage degradation, may benefit most from anti-inflammatory treatment, with NSAIDs or corticosteroids. If, on the other hand, there is mainly breakdown of the cartilage matrix, treatment aimed at optimising cartilage anabolism are more appropriate. Substances such as insulin-like growth factor I and II, transforming growth factor β, glucosamine, hyaluronic acid and link peptide have all been shown to promote proteoglycan and/or collagen synthesis in an experimental (Davenport-Goodall *et al* 2004, Dean *et al* 2003, Dodge and Jimenez 2003, Frean *et al* 1999, Frenkel *et al* 2000, Frisbie and Nixon 1997, Iqbal *et al* 2000, Möller *et al* 2000, Platt and Bayliss 1995, Thompson *et al* 2001, van Beuningen *et al* 2000) or clinical setting (Chevalier 1997, Reginster *et al* 2001).

Concluding remarks

This study illustrates the difficulty in interpreting synovial fluid biomarkers for the assessment of articular pathology. The potential markers that were investigated were influenced by physiological (age), management (exercise) and iatrogenic (repeated arthrocentesis) factors. These effects should be borne in mind and conditions at the time of synovial fluid sampling must be standardized with respect to these (and possibly other) confounding factors. Even when all of these causes of variation have been controlled for, a single marker is unlikely to provide sufficient information regarding the status of the investigated joint and the composition of a panel of markers would be a logical next step. Such a panel should include both markers of inflammation and degradation, with the second category possibly being subdivided into markers of proteoglycan and collagen catabolism. As collagen loss marks the start of irreversible cartilage damage, collagen degradation markers should be best related to prognosis. Using two categories of synovial fluid markers may also allow more specific treatment of joint disease, aimed either at reducing inflammation or improving cartilage composition.

Although ideally a single measurement of synovial markers would reveal both the condition of the joint and the prognosis, this seems unrealistic (at the present time), and serial analyses are required. This enables monitoring of disease progression, including the effects of treatment. After initiation of therapy, serial arthrocentesis (allowing sufficient time for arthrocentesis effects to wear off and standardizing exercise regimes) will reveal how different markers respond to medication. Multiple measurements in a single joint will also provide reference values for that particular joint, which might be different to those for other joints. In the (near) future global screening for abnormal joint metabolism may be possible with the use of serum markers (Billinghurst *et al* 2003, 2004, Frisbie *et al* 1999, Hultén *et al* 2002, Misumi *et al* 2002, Okumura *et al* 1997, Todhunter *et al* 1993) and when disease is detected by this method the joint(s) concerned can be identified by routine diagnostic techniques, and further evaluated using synovial fluid markers. The identification of potential markers has, until now, been guided by knowledge of their importance in either the inflammatory processes within the joint or in articular cartilage metabolism, and their numbers have been limited mainly by the availability of detection techniques. Advances in modern molecular biochemistry are rapidly changing this selective approach and allow a more overall assessment of joint metabolism using either molecular genetic techniques such as DNA micro-arrays (Weisong and Bertone 2004), or an approach utilising proteonomics. Such modalities should lead to an even better understanding of disease processes, allow detection of susceptible individuals (even before the presence of mild symptoms) and enable more precise targeting of therapeutic strategies.

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English summary

Osteoarthritis (OA) is a serious problem in the equine industry and an important cause of the (early) retirement of sport horses. Currently, the diagnosis is usually based on X-rays, but by the time changes become radiographically visible, extensive (often irreversible) joint damage is present. This has led to a search for substances, so-called markers, which accurately reflect the presence and severity of OA and allow its detection at an earlier stage. At that time rest and specific medication may prevent or limit permanent damage. Synovial (joint) fluid is in direct contact with the joint capsule and cartilage and its composition reflects the state of the joint. In this thesis the value of a number of synovial fluid markers is investigated.

A joint consists of the ends of two bones which move relative to each other (see Fig 1 in the general introduction). Both ends are covered by a thin layer of cartilage and the joint space is enclosed by the joint capsule. The joint space is filled with synovial fluid, a viscous fluid which feeds the cartilage (as this does not have its own blood supply) and contributes to smooth joint movement. Cartilage consists mainly of collagen, proteoglycans and water. Collagen is a protein which is present as fibres with great tensile strength and the fibres form a three-dimensional network. Within this network proteoglycans (a protein core with sugar sidechains) are trapped. Because the proteoglycans are negatively charged they attract water and cause a swelling pressure within the cartilage. This tissue swelling is restricted by the collagen fibres. This unique biochemical composition determines the mechanical properties of cartilage, combining stiffness and resilience.
Normally (in adults) a slow turnover of cartilage occurs, during which degradation and regeneration are balanced. It should be noted that proteoglycans can be replaced fairly quickly but collagen only very slowly. The halflife of collagen (the time in which half the collagen is replaced) in adults has been estimated at more than 100 years, although it is much shorter in growing individuals.
Osteoarthritis is a condition affecting both humans and horses and which leads to damage to the articular cartilage. First, proteoglycans are broken down and later the collagen network is also degraded. These changes reduce the strength of the cartilage and cartilage tears can develop. It is clear that damaged cartilage is less able to fulfil its tasks and with time cartilage of inferior quality will prohibit smooth joint movement. In the horse these changes cause pain and lameness. Osteoarthritis (eventually) also leads to bony changes at the joint margins and these are visible on X-rays (while cartilage itself cannot be seen on these images). However, by the time such changes become radiographically visible cartilage is severely damaged and (most of) the damage is permanent. Synovial fluid is in direct contact with cartilage and it seems likely that the composition of this fluid reflects the condition of the articular cartilage.
The concentration of inflammatory mediators, enzymes which play a role in cartilage degradation or constituents of cartilage in synovial fluid may reveal the status of a joint. The aforementioned substances form markers of OA (they mark the severity of the disease process). The aim of the research presented in this thesis was to investigate which other factors (apart from OA) influence these markers, and to determine which markers are useful for establishing the presence and severity of OA.

The matrix metalloproteinases (MMPs) form a group of enzymes involved in cartilage degradation and previous research has suggested that they may be indicators of OA. Apart from cartilage damage OA is also paired with inflammation and tumour necrosis factor α (TNF-α) reflects the degree of inflammation. To determine the value of these markers it is important to know if their concentrations are influenced by factors other than joint disease. The influence of exercise and repeated arthrocentesis (puncture of the joint capsule) on MMP activity and TNF-α concentration in the synovial fluid of healthy horses was investigated. Repeated arthrocentesis caused an increase in MMP activity but the training regimen used (two weeks of treadmill exercise) had no effect. The TNF-α concentration was elevated two hours after the last training session but was not influenced by repeated arthrocentesis. This means that when MMP activity and TNF-α concentration are used to predict the state of a joint previous exercise and arthrocentesis should be borne in mind.

The MMPs play an important role in cartilage degradation, both during growth and in joint disease. Because collagen can be replaced only very slowly collagen degradation marks the start of irreversible cartilage damage. Therefore, MMPs specifically involved in collagen degradation represent an interesting group of markers of OA. The MMPs can be divided into four categories and the collagenases are the only ones that can degrade the intact collagen fibres. One representative of this group is MMP-1 (collagenase-1). To determine the predictive value in case of joint disease it is important to know if MMP-1 activity in synovial fluid depends on the age of a horse, previous arthrocentesis and/or exercise performed by that horse. Further, it is essential to determine if MMP-1 activity is indeed increased in joints with OA. The MMP-1 activity was measured in synovial fluid of foals and horses of different ages, before and after a training programme, after repeated arthrocentesis and in synovial fluid from horses with severe OA. In the synovial fluid of young foals MMP-1 activity was much higher than in adult horses. This is related to growth/development of the joint, whereby existing cartilage is degraded but is replaced by newly formed cartilage. The training programme employed in this study did not influence MMP-1 activity but repeated arthrocentesis caused increased values. Severe OA was paired with higher MMP-1 activity in synovial fluid than seen in healthy joints. This study seems to confirm the value of MMP-1 as a marker of OA. When collecting synovial fluid for MMP-1 measurements previous arthrocentesis should be borne in mind and when interpreting values the age of the horse is important (especially for young horses).

Nitric oxide (NO), prostaglandin E_2 (PGE_2) and glycosaminoglycans (GAGs, part of the proteoglycans) are all potential synovial markers. These substances may also be influenced by external factors and the effects of exercise and repeated arthrocentesis were investigated. The two week training programme on the treadmill led to an increase in the PGE_2 concentration, two hours after the last exercise session, but did not affect the other parameters. Repeated arhtrocentesis caused increased NO and PGE_2 concentrations when arthrocentesis was repeated after 12 hours, and an increase in GAG levels after three joint taps performed with an interval of 12 and 60 hours. This study again shows

that when collecting synovial fluid for the assessment of synovial markers previous exercise and arthrocentesis may influence values.

Collagen consists of several components, so-called amino acids, and one of these is hydroxyproline, which accounts for approximately 10% of the amino acids in collagen. Within the collagen network proteoglycans are trapped. When cartilage is degraded it is likely that the levels of hydroxyproline and GAGs in synovial fluid are increased. The influence of age and severity of OA on hydroxyproline and GAG levels in synovial fluid were determined and levels were compared with MMP activity. Young foals had much higher hydroxyproline and GAG concentrations in synovial fluid than adult horses and values quickly decreased during the first years of life. After the age of four years levels remained constant. In the young, growing foal cartilage is remodelled: degraded and resynthesised, leading to high hydroxyproline and GAG levels in synovial fluid. In the adult horse this process occurs at a much lower rate and consequently hydroxyproline and GAG concentrations in synovial fluid are much lower. With the development of OA hydroxyproline and GAG concentrations are not significantly elevated but in horses with OA the hydroxyproline concentration is related to MMP activity (which is not the case in healthy joints). This is an extra indication that these enzymes are involved in collagen degradation in OA. There was no correlation between the GAG concentration in synovial fluid and MMP activity, possibly because other enzymes (the aggrecanases, not measured in our study) play a role in proteoglycan degradation.

A reliable marker will reflect the condition of the articular cartilage and the relationship between the hydroxyproline and GAG concentrations in synovial fluid and cartilage composition was investigated. Cartilage damage was quantified on one of the articular surfaces of the proximal phalanx and joints were divided into a group with severe cartilage damage and a group with little cartilage damage. One of the first changes associated with OA is a reduction in the proteoglycan content of cartilage. Also, OA does not develop uniformly across the joint surface but (in the fetlock joint) cartilage damage starts at the dorsal (front) margin, and later spreads to the central area of the joint. The GAG content in cartilage at the dorsal margin was lower in the group with severe cartilage damage. This confirms that joints considered abnormal were indeed diseased. The degradation of collagen results in the formation of denatured (damaged) collagen and its concentration in cartilage was higher in joints with severe cartilage damage, although the difference was not statistically significant. There was no correlation between the amount of GAGs or denatured collagen in cartilage and the GAG or hydroxyproline concentration in synovial fluid, respectively. A single measurement of these substances in synovial fluid does not reflect the state of the articular cartilage.

Infectious arthritis is a bacterial joint infection that leads to extensive cartilage damage and, if not treated adequately, can even become life threatening. Currently, the severity of the infection is established by counting the number of white blood cells in synovial fluid. In this study the concentrations of a number of synovial markers were investigated in the presence of infectious arthritis. All the affected joints had clearly elevated white

blood cell counts in the synovial fluid, but PGE_2 and GAG concentrations and MMP activity were also increased. The amount of hyaluronan (a normal constituent of synovial fluid, largely responsible for its lubricating properties) was lower in joints with infectious arthritis compared to healthy joints, while the hydroxyproline concentration did not differ significantly between affected and healthy joints. There was no correlation between the number of white blood cells and other parameters, but clear correlations between PGE_2 concentrations and GAG and hyaluronan concentrations and MMP activity. These results indicate that PGE_2, GAG, hyaluronan and MMP activity may be better markers for the severity of infectious arthritis in the horse than white blood cell counts.

This research demonstrates the difficulty in the use of markers to estimate cartilage damage. The markers that were investigated were influenced by physiological (age), management (exercise) and iatrogenic (arthrocentesis) factors. These factors must be borne in mind and conditions at the time of synovial fluid sampling standardized as far as possible with respect to these (and possibly other) influences. Even if all these factors are controlled for it is unlikely that a single marker will provide sufficient information regarding a particular joint, and a panel if markers should be investigated. Such a panel should include both inflammatory and degradation markers, with the latter being subdivided into markers of proteoglycan and collagen degradation. As collagen damage marks the start of irreversible damage collagen degradation markers are likely to be best related to the prognosis. The use of two categories of markers may also enable more targeted therapy, aimed at either the inflammation or at repair of damaged cartilage.
With the use of the ideal marker a single measurement would reveal the condition of the joint as well as the prognosis, but this seems unrealistic (at the present time), and multiple measurements are required (leaving sufficient time for the effects of previous arthrocentesis to have worn off). Repeated measurements also allow the progression of the disease process to be followed, including the effects of therapy employed, and will provide reference values for a particular joint, which may differ from those for another joint. In the (near) future a first indication of the presence of a joint disorder may be obtained from blood tests. Subsequently, the affected joint(s) can be detected using (current) diagnostic modalities and the severity further evaluated using markers in synovial fluid.

Nederlandse samenvatting

Gewrichtsslijtage (arthrose) is een ernstig probleem in de paardenbranche en een belangrijke oorzaak van (vervroegde) beëindiging van de carrière van sportpaarden. Vaak wordt de diagnose gesteld met behulp van röntgenfoto's, maar tegen de tijd dat afwijkingen zichtbaar worden op een röntgenfoto is er al uitgebreide (en vaak blijvende) schade aan het gewricht. Daarom wordt gezocht naar stoffen, zogenaamde markers (ze markeren de ernst van het proces), die de aanwezigheid en ernst van arthrose accuraat weerspiegelen en de detectie ervan in een vroeger stadium mogelijk maken. Op dat moment zouden rust en gerichte medicatie blijvende schade wellicht kunnen voorkomen of beperken. Gewrichtsvloeistof (synovia) staat in direct contact met de binnenbekleding van het gewricht en met het gewrichtskraakbeen. De samenstelling ervan geeft weer wat er in het gewricht gebeurt. In dit proefschrift wordt onderzocht welke synoviale markers (stoffen die worden gemeten in gewrichtsvloeistof) mogelijk geschikt zijn en welke factoren invloed hebben op hun concentraties.

Een gewricht bestaat uit twee botuiteinden die bewegen ten opzichte van elkaar. Beide uiteinden zijn bedekt met een dunne laag kraakbeen en het gewricht wordt begrensd door het gewrichtskapsel. De gewrichtsholte is gevuld met gewrichtsvloeistof, een viskeuze (stroperige) vloeistof die zorgt voor de voeding van het kraakbeen, omdat dat geen eigen bloedvoorziening heeft. De vloeistof draagt bovendien bij aan het soepel bewegen van het gewricht. Kraakbeen bestaat voornamelijk uit collageen, proteoglycanen en water. Collageen is een eiwit dat aanwezig is in de vorm van vezels met een grote treksterkte, en deze vezels vormen samen een driedimensionaal netwerk. In dat netwerk worden proteoglycanen gevangen, die zijn opgebouwd uit een eiwitkern met daaraan suikers. Doordat deze proteoglycanen een negatieve lading hebben trekken ze water aan en hierdoor heeft het kraakbeen de neiging om te zwellen. Dit zwellen wordt beperkt door de genoemde collageenvezels waardoor er in het weefsel een intrinsieke spanning ontstaat. De unieke biochemische samenstelling bepaalt zo de mechanische eigenschappen van het kraakbeen, waarin stijfheid wordt gecombineerd met veerkracht. Normaal (bij volwassen individuen) vindt er op beperkte schaal vernieuwing van het kraakbeen plaats, waarbij afbraak en opbouw met elkaar in evenwicht zijn. Hierbij dient opgemerkt te worden dat de proteoglycanen vrij snel vervangen kunnen worden, maar collageen slechts zeer langzaam. De halfwaarde tijd van collageen (de tijd waarin de helft van het aanwezige collageen vervangen wordt) bij volwassen dieren is geschat op meer dan 100 jaar, bij het nog groeiende dier is dat veel minder.
Arthrose, of (simpel gezegd) gewrichtsslijtage, is een aandoening die voorkomt bij zowel mensen als paarden en die gepaard gaat met schade aan het gewrichtskraakbeen. Eerst worden de proteoglycanen afgebroken en later wordt ook het collageen netwerk aangetast. Door deze veranderingen neemt de sterkte van het kraakbeen af en kunnen er scheuren ontstaan. Het zal duidelijk zijn dat het beschadigde kraakbeen minder goed in staat is zijn taken te vervullen en op den duur zal de inferieure kwaliteit van het kraakbeen leiden tot een minder soepele beweging van het gewricht. Bij het paard leiden al deze veranderingen tot pijn en kreupelheid.
Arthrose leidt (uiteindelijk) ook tot botnieuwvorming aan de randen van aangetaste gewrichten en dit is zichtbaar op röntgenfoto's (kraakbeen zelf is niet te zien op deze

foto's). Echter, tegen de tijd dat zulke veranderingen zichtbaar worden op foto's is het kraakbeen al ernstig aangetast. Omdat kraakbeen zeer slecht geneest, door de lange halfwaarde tijd van collageen, zal de (meeste) schade blijvend zijn. Gewrichtsvloeistof staat in direct contact met het kraakbeen en het ligt voor de hand dat de samenstelling van deze vloeistof de conditie van het kraakbeen weerspiegelt. Door de concentratie van ontstekingsmediatoren, van enzymen die een rol spelen bij de afbraak van kraakbeen of van bestanddelen van het kraakbeen in gewrichtsvloeistof te meten, zouden we mogelijk meer te weten kunnen komen over de gezondheidsstatus van het gewricht. De genoemde stoffen vormen zo markers voor arthrose. Het doel van dit proefschrift was om na te gaan welke andere factoren (naast arthrose) invloed hebben op deze markers, en ook welke markers bruikbaar zijn bij het stellen van de diagnose en het bepalen van de ernst van arthrose.

De matrix metalloproteinases (MMPs) vormen een groep enzymen die betrokken zijn bij de afbraak van kraakbeen en eerder onderzoek heeft aangetoond dat ze mogelijk een indicator voor arthrose zijn omdat ontstekingsprocessen een rol spelen bij het ontstaan en onderhouden van arthrose. Tumor necrosis factor α (TNF-α) is een stof die de mate van ontsteking weergeeft. Om de waarde van deze markerstoffen te bepalen is het belangrijk om te weten of de concentratie ervan in gewrichtsvloeistof ook beïnvloed wordt door andere factoren dan gewrichtsslijtage. De invloed van lichaamsbeweging en herhaalde gewrichtspunctie op MMP-activiteit en TNF-α-concentratie in de gewrichtsvloeistof van gezonde paarden werd onderzocht. Het herhaald aanprikken van een gewricht leidde tot een toename van de MMP-activiteit, maar het toegepaste trainingsregime (twee weken op een lopende band) had hier geen invloed op. De TNF-α–concentratie was twee uur na de laatste trainingssessie verhoogd om daarna weer te dalen tot hetzelfde niveau als voor de beweging; herhaald prikken had geen effect. Dit betekent dat wanneer wij MMP-activiteit en TNF-α-concentratie meten om iets over de toestand van het gewricht te kunnen zeggen, rekening dient te worden gehouden met voorafgaande beweging en gewrichtspuncties.

De MMPs spelen een belangrijke rol bij de afbraak van kraakbeen, zowel tijdens de groei als bij gewrichtsaandoeningen. Omdat collageen slechts zeer langzaam vervangen kan worden, markeert de afbraak van collageen het begin van onherstelbare kraakbeenschade. Om deze reden zijn MMPs die specifiek betrokken zijn bij de afbraak van collageen bijzonder interessant als marker van arthrose. De MMPs kunnen worden verdeeld in vier categorieën en de collagenases zijn de enige die het intact collageen kunnen afbreken. Eén van de vertegenwoordigers uit deze groep is MMP-1 (collagenase-1). Om de voorspellende waarde in geval van gewrichtsaandoeningen te kunnen inschatten, is het van belang om te weten of de MMP-1 activiteit in gewrichtsvloeistof afhankelijk is van de leeftijd van een paard, eerder uitgevoerde gewrichtspunctie(s) en/of lichaamsbeweging die het paard heeft gehad. Daarnaast is het essentieel om vast te stellen of MMP-1 activiteit inderdaad verhoogd is in gewrichten met arthrose. De MMP-1 activiteit is gemeten in gewrichtsvloeistof van veulens en paarden van verschillende leeftijden, voor en na een trainingsprogramma, na herhaaldelijke gewrichtspuncties en in

gewrichts-vloeistof van paarden met ernstige arthrose. In de gewrichtsvloeistof van jonge veulens was de MMP-1 activiteit veel hoger dan bij volwassen paarden. Dit heeft te maken met de groei van het gewricht, waarbij veel kraakbeen wordt afgebroken maar gelukkig ook weer wordt aangemaakt. Het bewegingsregime dat gebruikt werd in deze studie had geen invloed op MMP-1 activiteit, maar na herhaald aanprikken van een gewricht stegen de waardes. Ernstige arthrose ging ook gepaard met een hogere MMP-1 activiteit dan in gewrichtsvloeistof van gezonde gewrichten. Dit onderzoek lijkt de waarde van MMP-1 als marker van arthrose te bevestigen, maar bij verzameling van gewrichtsvloeistof dient in ieder geval rekening te worden gehouden met voorafgaande gewrichtspunctie(s) en bij de interpretatie moet rekening worden gehouden met de leeftijd van het paard.

Stikstof oxide (engels: nitric oxide, NO), prostaglandine E_2 (PGE_2) en glycosaminoglycanen (GAGs, een onderdeel van de proteoglycanen) zijn allemaal markers die in gewrichtsvloeistof voorkomen. Ook hiervoor geldt dat ze door externe factoren kunnen worden beïnvloed en het effect van lichaamsbeweging en herhaalde gewrichtspuncties is ook voor deze stoffen onderzocht. Het twee weken durende trainingsprogramma op de lopende band leidde tot een stijging van de PGE_2-concentratie in gewrichtsvloeistof, twee uur na de laatst verrichte arbeid, maar had geen invloed op de overige parameters. Herhaalde punctie van een gewricht veroorzaakte een toename van NO- en PGE_2-concentraties wanneer de punctie 12 uur na de voorgaande punctie uitgevoerd werd, en een stijging van het GAG gehalte in gewrichtsvloeistof na drie puncties met een interval van 12 en 60 uur. Deze studie toont wederom aan dat bij het verzamelen van gewrichtsvloeistof voor het meten van synoviale markers er rekening dient te worden gehouden met lichaamsbeweging en eerder uitgevoerde gewrichtspuncties.

Collageen is opgebouwd uit verschillende bouwstenen, zogenaamde aminozuren, en één daarvan is hydroxyproline, dat ongeveer 1/10 deel van de aminozuren uitmaakt. Tussen de collageenvezels zitten proteoglycanen. Bij de afbraak van kraakbeen ligt het voor de hand dat de hoeveelheden hydroxyproline en glycosaminoglycanen (GAGs) in gewrichtsvloeistof toenemen. De invloed van leeftijd en van verschillende stadia van arthrose (gewrichtsslijtage) op de hoeveelheid hydroxyproline en GAGs in gewrichtsvloeistof is onderzocht en de waardes zijn vergeleken met de MMP-activiteit. Jonge veulens hebben veel hogere hydroxyproline- en GAG-concentraties in hun gewrichtsvloeistof dan volwassen paarden en deze nemen snel af gedurende de eerste levensjaren. Na de leeftijd van vier jaar blijven de waardes constant. Bij het jonge, groeiende veulen wordt het kraakbeen opnieuw vormgegeven: afgebroken en weer opgebouwd en dat leidt tot hoge concentraties hydroxyproline en GAGs in gewrichts-vloeistof. Bij het volwassen paard gaat dit veel langzamer en zijn de hoeveelheden lager. De hydroxyproline- en GAG-concentraties nemen niet significant toe met het ontwikkelen van arthrose, maar bij paarden met arthrose is de hoeveelheid hydroxyproline gerelateerd aan MMP-activiteit (terwijl dit niet het geval is in gezonde gewrichten). Dit is een extra aanwijzing dat deze enzymen betrokken zijn bij de afbraak van collageen, die optreedt bij arthrose. Er is geen relatie tussen de hoeveelheid GAGs in gewrichtsvloeistof en MMP-activiteit,

mogelijk doordat ook andere enzymen (de aggrecanases, niet gemeten in onze studie) een rol spelen bij de afbraak van proteoglycanen.

Een betrouwbare marker weerspiegelt de toestand van het gewrichtskraakbeen en daarom is de relatie tussen de concentratie van hydroxyproline en glycosaminoglycanen (GAGs) in gewrichtsvloeistof en de samenstelling van het kraakbeen onderzocht.Van één van de gewrichtsoppervlakken van het kootbeen is de kraakbeenschade gekwantificeerd en de gewrichten zijn verdeeld in een groep met ernstige kraakbeenschade en een groep met weinig kraakbeenschade. Eén van de eerste veranderingen bij arthrose is dat de hoeveelheid proteoglycanen in het kraakbeen afneemt. Verder ontwikkelt arthrose zich niet gelijkmatig over het gewrichtsoppervlak maar worden de eerste veranderingen (van het gewrichtsvlak van het kootgewricht) waargenomen aan de voorste rand van het gewricht en pas later centraler. De hoeveelheid GAGs in het kraakbeen van de voorste rand van het gewricht was lager in de groep met ernstige kraakbeenschade. Dit bevestigt dat de groep die als afwijkend beschouwd wordt ook daadwerkelijk aangetast is. Bij de afbraak van collageen ontstaat eerst gedenatureerd (beschadigd) collageen. De hoeveelheid hiervan was hoger in gewrichten met ernstige kraakbeenschade, maar dit verschil was niet statistisch significant. Er bleek geen relatie tussen de hoeveelheid GAGs of gedenatureerd collageen in het kraakbeen en de hoeveelheid GAGs en hydroxyproline in gewrichtsvloeistof. Een enkele meting van deze stoffen in gewrichtsvloeistof zegt dus niets over de staat van het kraakbeen.

Septische arthritis is een door bacteriën veroorzaakte gewrichtsontsteking die leidt tot uitgebreide kraakbeenschade. Indien niet adequaat behandeld kan dit zelfs levensbedreigend worden. Momenteel wordt de ernst van de ontsteking grotendeels ingeschat aan de hand van het aantal witte bloedcellen in gewrichtsvloeistof. In de studie die beschreven wordt in dit proefschrift is onderzocht hoe de concentratie van een aantal synoviale markers verandert bij septische arthritis. Alle aangetaste gewrichten hadden inderdaad een duidelijk verhoogd aantal witte bloedcellen in de gewrichtsvloeistof. Tevens waren de PGE_2- en GAG-concentraties verhoogd, alsook de MMP-activiteit. De hoeveelheid hyaluronzuur (een normaal bestanddeel van gewrichtsvloeistof en belangrijk voor de smerende eigenschappen) was juist lager in geïnfecteerde gewrichten, vergeleken met gezonde exemplaren, terwijl de hydroxyproline concentratie niet significant verschilde. Er bleek geen relatie tussen het aantal witte bloedcellen en andere parameters, maar wel duidelijke relaties tussen PGE_2-concentraties en GAG- en hyaluronzuur-concentraties en MMP activiteit. Deze resultaten geven aan dat PGE_2, GAG, hyaluronzuur en MMP-activiteit mogelijk betere markers zijn dan witte bloedcellen voor de ernst van septische arthritis bij het paard.

Het in dit proefschrift beschreven onderzoek demonstreert hoe moeilijk het is om met behulp van markers in gewrichtsvloeistof kraakbeenaantasting te beoordelen. De onderzochte markers werden beïnvloed door fysiologische (leeftijd), gebruiks- (beweging) en diergeneeskundige (gewrichtspunctie) factoren. Met deze factoren dient rekening te worden gehouden en de omstandigheden op het moment van het verzamelen van gewrichts-

vloeistof moeten zo veel mogelijk gestandaardiseerd worden met betrekking tot deze (en mogelijk ook andere) invloeden. Zelfs in dat geval is het onwaarschijnlijk dat een enkele marker voldoende informatie verschaft over het betreffende gewricht en zal een combinatie (een pakket) van markers moeten worden onderzocht. Een dergelijk pakket zou zowel ontstekings- als degradatiemarkers moeten bevatten, waarbij deze laatste categorie nog verder verdeeld dient te worden in markers van proteoglycanen en collageen degradatie/afbraak. Aangezien collageenschade de start van onomkeerbare schade markeert, zijn collageen degradatiemarkers waarschijnlijk het best gerelateerd aan de prognose (vooruitzichten). Het gebruik van twee categorieën van markers zou wellicht ook een meer gerichte therapie mogelijk maken, gericht of tegen de ontsteking, of op herstel van de kraakbeen samenstelling.
Bij gebruik van de ideale marker zou een enkele meting de gezondheidsstatus van het gewricht alsook de prognose weergeven, maar dit lijkt (op dit moment, met de huidige stand van de wetenschap) niet realistisch, en meerdere metingen en gewrichtspuncties zijn vereist (met voldoende tijd tussen de puncties, zodat effecten van een voorgaande punctie verdwenen zijn). Meerdere meetpunten maken het ook mogelijk om de ontwikkeling van het ziekteproces te volgen, inclusief de effecten van een ingestelde therapie. Herhaalde metingen zullen ook referentiewaarden voor een bepaald gewricht opleveren, die zouden kunnen verschillen van die voor een ander gewricht. In de (nabije) toekomst zou een eerste aanwijzing voor de aanwezigheid van een gewrichtsaandoening verkregen kunnen worden uit bloedonderzoek. Vervolgens zou met behulp van (de huidige) diagnostische technieken en mogelijkheden het (de) betreffende gewricht(en) kunnen worden opgespoord, en vervolgens de ernst verder geëvalueerd met behulp van markers in gewrichtsvloeistof.

Dankwoord

Mensen die mij een beetje kennen zullen niet verbaasd zijn dat ik in dit dankwoord het voltooien van een promotie-onderzoek vergelijk met het lopen van een marathon. Er zijn nogal wat (meer of minder voor de hand - of zou dat moeten zijn voor de voet ? - liggende) overeenkomsten, waarvan één van de belangrijkste is dat het succes bij beiden grotendeels wordt bepaald door doorzettingsvermogen (te eigenwijs zijn om op te geven). Deze eigenschap kan, zeker wanneer deze wordt gekoppeld aan een zekere mate van discipline, een gebrek aan talent (grotendeels) compenseren. Voor beiden geldt ook dat je het in principe in je eentje doet, maar dat de hulp van een (groot) aantal mensen de prestatie mogelijk, danwel aangenamer maakt, en dit is de plaats om die mensen te bedanken.

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Naast de inzet van de marathonloper zelf is het van groot belang dat het 'thuisfront' achter het voornemen om een marathon te lopen staat, en hetzelfde geldt voor een promotieonderzoek. Het trainen voor een marathon en het doen van promotie-onderzoek vergen veel tijd (laat staan als je die twee probeert te combineren) en daarom is begrip van de 'partner' (om die verschrikkelijke term maar eens te gebruiken) vereist. Inge, jij bent al geruime tijd mijn thuisfront (da's positief bedoeld) en zonder jouw steun was mijn doorzettingsvermogen nog meer op de proef gesteld. Naast begrip uitte je soms ook kritiek (altijd opbouwend uiteraard - en recht voor zijn raap geserveerd) en deel je ook mijn enthousiasme voor sport in het algemeen en hardlopen in het bijzonder. Je weet wat je voor me betekent (al spreek ik het volgens jou niet vaak genoeg uit) en ik ga er van uit dat wij nog heel veel dingen samen zullen doen!

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soortgenoten in de toekomst kunnen profiteren van onze bevindingen.

De training zit erop, de koolhydraten zijn gestapeld, de wedstrijddag is daar en het startschot klinkt. Je kan niks meer veranderen en moet erop vertrouwen dat de geleverde (trainings-) arbeid volstaat voor een geslaagd resultaat. Maar, onderweg zijn er wel de onmisbare aanmoedigingen van de supporters, die je naar de finish schreeuwen. Hierbij wil ik al mijn supporters bedanken voor hun steun: (schoon) familie, vrienden, (ex-) collega's en kennissen. Eenieder doet dat op z'n eigen manier en de ene aanmoediging is daarom niet meer of minder waardevol dan een ander. Een goede aanmoediging komt vooral op het juiste moment.

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Curriculum Vitae

Robin van den Boom was born on 27th April 1970 in Wilrijk (Belgium). He attended Fullbrook Middle School and 6th form in New Haw (England) and in 1988 passed his A-levels. In that same year he started his studies of veterinary medicine at Utrecht University and graduated *with honours* in April 1996. On 1st May 1996 he started work at the Department of General and Large Animal Surgery, now the Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University. In October 2002 he was registered as Diplomate in Equine Surgery by the Royal Dutch Veterinary Association.

Robin van den Boom werd op 27 april 1970 geboren te Wilrijk (België). In 1988 behaalde hij het eindexamen aan Fullbrook middelbare school in New Haw (Engeland). In datzelfde jaar begon hij aan de studie diergeneeskunde aan de Universiteit Utrecht en in april 1996 legde hij het dierenartsexamen *met genoegen* af. Op 1 mei 1996 trad hij in dienst bij de vakgroep Algemene Heelkunde en Heelkunde der Grote Huisdieren, thans de Hoofdafdeling Gezondheidszorg Paard van de Faculteit der Diergeneeskunde, Universiteit Utrecht. In oktober 2002 werd hij geregistreerd als Specialist Chirurgie van het Paard door de Koninklijke Nederlandse Maatschappij voor Diergeneeskunde.