Do cartilage and subchondral bone act together in development and disease?

Mark R. van der Harst

Utrecht – 2005

Cover: “Spirit”

Do cartilage and subchondral bone act together in development and disease?

Hoe is de interactie tussen kraakbeen en subchondraal bot gedurende ontwikkeling en gewrichtspathologie?

(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 10 februari 2005
des middags te 4.15 uur

door

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geboren op 08 juni 1969, te Laren (NH)

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CIP-gegevens Koninklijke Bibliotheek, Den Haag

Harst, Mark Robert van der

Do cartilage and subchondral bone act together in development and disease?
Mark Robert van der Harst
Utrecht: Universiteit Utrecht, Faculteit Diergeneeskunde
Proefschrift Universiteit Utrecht. - With ref. - With summary in Dutch.
ISBN: 90-393-3926-0

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Layout and cover by Multimedia Centrum Diergeneeskunde
Cover photograph by G. Veenstra; CT scan by Y. ter Steege
Printed by Ridderprint Offsetdrukkerij BV, Ridderkerk, The Netherlands, 2005

Wie nooit van mening is veranderd,
heeft zelden iets geleerd.

Contents

Chapter I

General introduction

From Hippocrates down to the present age….

A synovial or diarthrodial joint can be defined as a structure that allows movement and the transfer of load between bones (Todhunter 1996a). It is this dualistic function, permitting movement that is as frictionless as possible, and providing enough strength to transmit the considerable forces generated by locomotion, that poses such severe challenges to the joint and makes failure, *i.e.* the development of pathological conditions, a common phenomenon. It has long been recognised that joint failure, especially damage to the cartilage layer, is a serious condition for which no real cure exists. In 1743 William Hunter noted in his lecture for the Royal Society of London that: *"If we consult the standard Chirurgical Writers from Hippocrates down to the present Age, we shall find, that an ulcerated Cartilage is universally allowed to be a very troublesome Disease; that it admits of a Cure with more Difficulty than a carious Bone; and that, when destroyed, it is never recovered"* (Hunter 1743). One hundred years later Sir James Paget stated: *'There are, I believe, no instances in which a lost portion of cartilage has been restored, or a wounded portion repaired with new and well-formed permanent cartilage, in the human subject'* (Paget 1853). Hunter's "ulcerated cartilage" and Paget's "wounded cartilage" would now be called cartilage affected by osteoarthritis (OA).
Much has changed since the days of Hunter and Paget, but joint disorders (of which OA is by far the most predominant) remain among the most invalidating diseases in man and several other species of animals. In the latter category, the impact of OA is most severe in the horse as this species is usually kept for its locomotor capacities. Osteoarthritis is among the most prevalent disorders in the elderly human population, with up to 70% of the population over the age of 65 being affected (Sowers *et al.* 2003). The yearly costs for OA in the US alone have been estimated at $ 15.5 billion (Fenton *et al.* 2000). In equine athletes joint pain and loss of mobility caused by OA are common causes of poor performance and early retirement (Olivier *et al.* 1997, Rossdale *et al.* 1985). In a mixed breed referral hospital population, joint disorders, principally OA, accounted for 54% of diagnoses in orthopaedic patients (Todhunter and Lust 1992). These figures in man and horse indicate that, as opposed to some other medical conditions, adequate preventive measures for joint-related problems in general and OA in particular, have not yet been found. This is largely due to our insufficiently detailed knowledge of the complex functioning of the joint and, directly related to this, the exact pathogenesis of chronic degenerative disorders such as OA.
Scientific knowledge in general has increased exponentially since the days of Hunter and Paget, let alone Hippocrates, and progress in the biomedical field has been particularly rapid in the last few decades. This thesis uses present-day, state-of-the-art techniques to analyse the extracellular matrix (ECM) of various tissues that are important in load transfer through the joint and that might play a role in the pathogenesis of OA. Through the detailed analysis of samples from normal mature individuals, juvenile individuals in which there still is development and growth, and

individuals affected by (early) OA, this thesis hopes to contribute some knowledge, be it only a modest amount, which may help our understanding of joint physiology and pathophysiology. Subject of study is the horse, because of the impact of joint-related disorders on the functioning and welfare of the species itself, and because of the important parallels to the situation in man. In this introductory chapter an overview of normal joint structure and function is given as well as current views regarding on the pathogenesis of OA. In the concluding paragraph the conceptual thoughts behind the thesis, that have driven the research presented in the chapters II-VI and which form the main body of the work, are explained.

The joint: pivotal to motion

A synovial joint consists of the articular surfaces of at least two bones covered with a thin layer of hyaline cartilage. The synovial joints facilitate predictable and energy-efficient movement, but at the same time form part of the musculoskeletal system that gives structure to the body and transmit the often considerable loads that are generated during weight bearing and locomotion. These combined functions of frictionless movement, weight bearing and load transmission cannot be realised by the thin cartilage layer alone. The underlying subchondral bone and the joint capsule provide strength, while ligaments and the synovial fluid help stabilise the joint (Fig. 1). Each of these components will be briefly discussed.

Articular cartilage
The articular cartilage forms a smooth, gliding surface allowing articulation of a joint. Cartilage has a water content of approximately 60-85 % in mature horses (Brama *et al.* 1999a, Todhunter 1996a), which, in combination with the fine network of collagen fibrils, gives it a translucent, glasslike or hyaline appearance. On dry weight basis cartilage contains about 50% collagen, 35% proteoglycans, 10% glycoproteins, 3% minerals and 1% lipids and 1 to 12% chondrocytes (by volume). The thickness of the articular cartilage layer differs between joints, weight bearing and non-weight bearing areas within a joint, and between young and adult animals.

- Collagen
The main constituent of cartilage on dry-weight basis is collagen. Most of the collagen in articular cartilage is type II (85 to 90%), but small amounts of types VI, IX, XI, XII and XIV are also present. Type II collagen is generally considered to provide the tensile strength of articular cartilage. The functional unit of fibrillar collagens consists of a so-called triple helix formed by three α-chains of about 1000 amino acids each and these are arranged as a framework in which the collagen fibrils are stabilised by covalent intermolecular cross-links (Eyre *et al.* 1984a, Eyre *et al.* 1991, Eyre and Wu 1995). Collagen biosynthesis involves several unique post-translational modifications. After translation of the procollagen

α-chain, specific proline and lysine residues are hydroxylated by prolyl and lysyl hydroxylase, respectively. Hydroxyproline is needed for the formation and stabilisation of the triple helix by hydrogen bonds with proline residues.

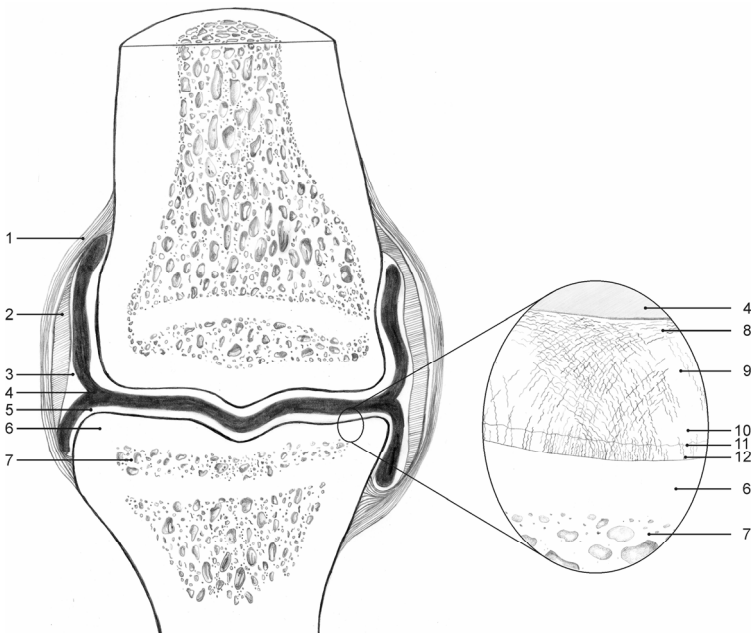

Fig. 1: Schematic representation of the structure of a synovial joint.
1. collateral ligament; 2. joint capsule; 3. synovial membrane; 4. synovial fluid; 5. cartilage; 6. subchondral bone; 7. trabecular bone; 8. superficial cartilage layer; 9. middle cartilage layer; 10. deep cartilage layer; 11. tidemark; 12. calcified cartilage layer.

Hydroxylysine residues serve as sites for enzymatically-regulated O-linked glycosylation and cross-linking of collagen. After fibril formation, intermolecular pyridinoline cross-links are formed: hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links. The second mechanism of intermolecular cross-linking of collagen is via a non-enzymatic reaction with glucose, also called non-enzymatic glycation (NEG). This process occurs in a random fashion and is time-dependent. A well-identified NEG-product is pentosidine, which is derived from lysine, arginine and ribose moieties. Its presence has been demonstrated in numerous collagenous and non-collagenous tissues with a slow turnover in which its concentration has been shown to increase linearly with age (Sell and Monnier 1989). It is therefore more important in proteins that have a long biological half-life,

i.e. collagen, as in such molecules this type of cross-link can accumulate and thus alter the properties of the tissue (Verzijl *et al.* 2000, Verzijl *et al.* 2002). The turnover time of type II collagen in mature individuals is known to be extremely long and may be more than 100 years (Maroudas *et al.* 1992, Verzijl *et al.* 2000).
The collagen fibrils are arranged according to a specific architecture, which is crucial for the mechanical properties of articular cartilage. This pattern consists of 'arches' of collagen fibres that have a perpendicular orientation to the articular surface in the deep layers, but a parallel one in the superficial layers (Fig. 1).

- Proteoglycans
Proteoglycans, which form the other major solid component of the articular cartilage matrix, are defined as any molecule that have a core protein to which at least one glycosaminoglycan (GAG) chain is covalently bound (Hascall and Kimura 1982). These GAGs are linear repeating disaccharide chains, which are highly negatively charged by carboxylate- and sulphate groups. Glycosaminoglycans found in cartilage include chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid, which is the only GAG that is not sulphated and which is not found as a side chain of a protein core. Proteoglycans play an important role in the resistance to compressive forces as they are interspersed between the collagen fibrils and through their hydrophilic character, generate a swelling pressure that provides the collagen network with an intrinsic tension. Proteoglycans and collagen fibrils together account for both the compressive stiffness and resilience of articular cartilage.

Subchondral and trabecular bone
Bone is a composite material, made up of an organic matrix and mineral deposits. It is commonly divided into compact and trabecular bone based on its structure. The compact bone provides a dense cortical layer around all bones, thereby giving support to the internal trabecular network and at the same time enclosing the bone marrow. Compact bone gives the long bones the strength that is needed to support the body during the cyclic dynamic loading that takes place during locomotion and other physical activities (Zoetis *et al.* 2003). In the joint, the subchondral bone plate is the layer of compact bone immediately beneath the articular cartilage. Cartilage and subchondral bone act in concert when performing their mechanical duties, with the former acting as the surface layer receiving primary impact and the latter as a structural girder and shock absorber (Layton *et al.* 1988). Besides its role in shock absorption and partial transmission of load to the deeper layers of trabecular bone, the subchondral bone influences, to a certain extent, the nutrition of the adjacent cartilage. Although most nutrition of the cartilage will come from diffusion of synovial fluid (Coimbra *et al.* 2004), it has been suggested that certain parts of the deeper layers of the cartilage are metabolically influenced by subchondral capillaries (Duncan *et al.* 1985). Deeper underneath the joint surface the subchondral plate converts gradually from compact to trabecular or cancellous

bone. Trabecular bone is composed of a network of fine, interlacing partitions, called trabeculae, with a diameter ranging from 10-400 µm enclosing cavities that contain either haematopoietic or fatty marrow. Trabecular bone is remodelled much more rapidly (5-10 times as fast) than compact or cortical bone (Mosekilde 1993, Parfitt 1988). Compact and trabecular bone not only differ in structure, but also in the extent of collagen post-translational modifications and chemistry of their mineral phases (Bigi *et al.* 1997).
Bone tissue in general consists of approximately 80% minerals or inorganic material and only 20% organic material (Zoetis *et al.* 2003). The interrelationship between the two components gives the tissue its characteristic mechanical properties that make it capable of withstanding compressive, bending and torsional forces.

- Organic component
Approximately 95% of the organic matrix is formed by collagen, with type I collagen being the predominant type found in bone. Type I and II collagen molecules are rather similar in structure. However, they are not identical. Collagen type II is made up of three identical α_1 chains, and collagen type I of two α_1 chains and one α_2 chain that has a slightly different amino acid composition (Todhunter 1996a). Further, type I collagen has a lower degree of hydroxylation of lysine residues and less glycosylation compared to type II collagen. Pyridinoline cross-links between fibrils are formed in both types. Compact bone contains higher hydroxylysine and pyridinoline cross-link concentrations, while trabecular bone displays a higher degree of glycosylation of hydroxylysine (Suarez *et al.* 1996). These differences will affect the mechanical strength of the two bone types and also have an effect on the nucleation sites for mineral deposition, as there is strong evidence that the cross-link profile has a regulatory function in mineralisation (Wassen *et al.* 2000).
The remaining 5% of organic matrix consists of proteoglycans and glycosaminoglycans. In bone, proteoglycans do not form the large aggregates that predominate in cartilage. Glycosaminoglycans serve as a cementing substance between layers of mineralised collagen fibres.

- Inorganic component
The mineral portion of bone consists primarily of calcium and phosphate, mainly in the form of calcium-hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$), but also as amorphous calcium phosphate (Boskey 1981). In addition to calcium and phosphorus, bone mineral contains carbonate, magnesium, fluoride, and citrate in variable amounts. Magnesium plays a key role in the process of crystallisation. The amount of magnesium decreases as the process of calcification becomes less intense in the maturing animal (Bigi *et al.* 1992, Bigi *et al.* 1997).
The interaction of the inorganic and organic matrix of bone is somewhat ambiguous. As alluded to earlier, collagen cross-links have been reported to regulate fibril mineralisation (Wassen *et al.* 2000). However, once fibrils are

mineralised, it appears that chemical interactions between collagen molecules producing HP and LP residues are inhibited. The mineralisation immobilises the collagen molecules, replacing water and stopping telopeptide activation necessary for cross-linking (Eyre *et al.* 1984b).

Synovial fluid
In healthy joints a small volume of synovial fluid, a colourless or pale-yellow viscous liquid, occupies the intra-articular space. The synovial fluid is an ultra-filtrate of plasma, with the addition of hyaluronan and plays a role in lubrication during joint movement. The periodical loading and unloading of the joint surface induced by locomotion maintains a flow of synovial fluid through the cartilage matrix, which is essential for nutrition of the tissue and for the removal of metabolites as articular cartilage is not vascularised in the mature animal. Normal intra-synovial pressure is sub-atmospheric (–2 to – 6 cm H_2O), which may assist in stabilising the joint. Because synovial fluid is in direct contact with articular cartilage, changes in articular cartilage composition and/or metabolism are likely to be reflected in the composition of synovial fluid. Synovial fluid contains inflammatory mediators such as cytokines, eicosanoids, free radicals, and growth factors and metabolites or tissue components that have been described as the "mirror" of joint (patho)physiology (van den Boom 2004). In fact, synovial fluid is the principal source of candidate molecules in the ongoing search for biomarkers of (early) joint disease.

Joint capsule
The joint capsule consists of a thick fibrous portion, which is lined by a thin subsynovium (lamina propria) and synovium (synovial membrane). The latter structure is in direct contact with the synovial fluid. The major blood vessels, which branch in the fibrous joint capsule and ramify in the subsynovium are accompanied by lymphatic vessels and nerves, which means that unlike in cartilage, all features necessary for the development of a classical inflammatory reaction are present. The extracellular matrix of the joint capsule consists predominantly of collagen (type I), proteoglycans, non-collagenous proteins and water.
The synovial membrane or intima is an incomplete layer, one to four synoviocytes thick, with no basal membrane. This facilitates the efflux of capillary exudate into the synovial cavity. Synoviocytes are cells that have either phagocytic (type A cells) or secretory (type B cells) functions (Palmer and Bertone 1994). A third type (C) of synoviocyte demonstrates characteristics intermediate between types A and B and is thought to be capable transforming into either depending on demand (Krey *et al.* 1971, Norton *et al.* 1968). The synoviocytes produce and secrete hyaluronan, as well as a whole array of macromolecules that influence the synthesis and degradation of cartilage components and/or are inflammatory mediators, such as proteinases, cytokines, and eicosanoids (Martel-Pelletier 2004, Spiers *et al.* 1994, Todhunter and Lust 1990). The synovial membrane, therefore,

is far more than just a layer that lines the joint cavity and it is becoming increasingly clear that it plays an important role in joint homeostasis and pathology.

Joint metabolism and homeostasis

Articular cartilage

The chondrocytes are responsible for maintaining the structural integrity of cartilage under the prevailing loading conditions through a delicate balance between synthesis and breakdown of extracellular matrix (ECM) macromolecules. Within limits, connective tissue cells respond to mechanical forces and may adapt and remodel the ECM by altering this balance (Murray *et al.* 2001a). They are incited to do so by external signals, which include cytokines, growth factors, nitric oxide (NO), vitamins, and possibly specific matrix degradation products.

As mentioned earlier, collagen turnover time is extremely slow in mature individuals, but the presence of type II collagen pro-peptides in adult articular cartilage shows that remodelling does take place, albeit slowly. Matrix metalloproteinases (MMPs) are involved in tissue turnover and MMPs with collagenase activity (MMP-1,-2,-8,-13,and -14) cleave the intact collagen molecule, which results in unwinding of the helical structure, making further degradation by other proteinases possible (Murphy *et al.* 1987, Murphy & Reynolds 2002).

Proteoglycan metabolism is a much more active process, also in mature individuals. The enzymatic degradation is mediated by MMPs (such as stromelysin or MMP-3 and the gelatinases, MMP-2 and -9) and aggrecanases (Poole *et al.* 1995). Proteoglycans are replaced very quickly, with a half-life in articular cartilage of only 11-40 days (Jikko *et al.* 1998).

Bone

Of the two types of bone, compact and trabecular, the latter is considered to be more metabolically active (Kiebzak 1991). In contrast to the relative inertness of the collagen network of mature articular cartilage, bone undergoes perpetual change in a continuous process of remodelling that involves not only replacement of old bone by new bone but also a reshaping of the architecture in response to changing biomechanical loading conditions. This process is most obvious in the trabecular bone and occurs according to Wolff's law since the phenomenon was first described by Julius Wolff in the late 19th century (Wolff 1892). Bone matrix is formed in two stages, the deposition of the organic matrix and subsequent mineralisation. During matrix deposition, osteoblasts secrete the initial matrix called osteoid, which is made up of type I collagen, various proteins, and sulphated glycosaminoglycans. During mineralisation, calcium phosphates and carbonates that were stored previously in intracellular vesicles are released into the matrix where they are deposited onto collagen fibrils with the help of glycoproteins (Buckwalter *et al.* 1996, Fawcett 1994). Remodelling involves the recruitment and

activation of the bone resorbing cells (osteoclasts) and the bone forming cells (osteoblasts) (Parfitt 1988). The mechanism regulating this remodelling process is largely unknown, but undoubtedly involves local regulatory factors that are released in response to changes in biomechanical loading.

Osteoarthritis: Conflicting concepts

Advanced OA can be described as a disorder characterised by deterioration of articular cartilage and the formation of new bone at the joint margins of articulating surfaces (Hough 1993). Unfortunately, these advanced stages of OA are of little scientific and practical interest. They have little scientific value as they represent mainly secondary changes and are not likely to provide any insight into or further elucidation of the exact pathogenesis of the disease. They have no practical value as the statements by Hunter and Paget still hold today and the remnants of articular cartilage in advanced cases of OA can be considered beyond repair.
The initial stages of OA are harder to identify and there are conflicting opinions about where OA develops first and which layers are involved in the very first stages of the disease. Cartilage degeneration in the form of fibrillation, erosions and wear lines is present long before bony changes can be visualised by conventional radiographic techniques (Todhunter 1996a). Biochemically, in the early stages of the disease, articular cartilage becomes depleted of proteoglycans and the water content increases (Dieppe 1999, Kiviranta *et al.* 1988, Maroudas *et al.* 1973). In a slightly more advanced stage changes in the collagen network can be detected (Hollander *et al.* 1995). Signs of cartilage degradation, without any signs of pathological changes in the underlying subchondral bone (Dedrick *et al.* 1993, Donohue *et al.* 1983, Yamada *et al.* 2002), suggest that the disease begins in the articular cartilage layer. This onset may be prompted by inflammatory mediators that induce primary biochemical changes (Lee *et al.* 1974, Mankin and Brandt 1992, Mow *et al.* 1992, Stephens *et al.* 1979, Wei *et al.* 2001), which are now recognised as being essential elements of this disease which was once qualified as merely degenerative and non-inflammatory in character (Attur *et al.* 2002). In this respect, NO is an interesting substance as it acts as a mediator that is up-regulated in various physiological and pathophysiological processes in the body in which tissue metabolism is increased. The effects that are exerted by NO in osteoarthritic cartilage have been studied extensively (Fernandes *et al.* 2002; Hashimoto *et al.* 1999; Jang *et al.* 1998; Kim *et al.* 2003; Mazzetti *et al.* 2001; Studer *et al.* 1999), but most of the studies on NO production in OA cartilage have been carried out using chondrocyte cultures and less is known about what happens at the tissue level.
At the other end of the spectrum, Radin and co-workers suggested that changes in the subchondral bone plate might play a much more prominent role than previously thought (Radin *et al.* 1970, Radin and Paul 1971, Radin and Rose 1986, Radin

1999). Indeed, some studies report OA-related changes in the subchondral structures in the presence of an apparently intact cartilage layer (Oettmeier *et al.* 1989a, Stougard 1974). It is hypothesised that changes in the relative density and architecture of the underlying subchondral bone may have a profound effect on both the initiation and progression of cartilage damage. Stiffer bone adjacent to the overlaying cartilage layer and specifically a steep stiffness gradient enhances the risk of cartilage damage by acting as potent stress raisers in certain areas (Murray *et al.* 2001b, Radin and Rose 1986, Radin *et al.* 1991, Radin 1999). However, although stiffening of the subchondral bone would suggest an increase in mineralisation, and sclerosis is a hallmark of at least the more advanced stage of OA, there are conflicting reports regarding mineralisation levels in relation to OA. In some cases an increase in mineralisation in OA joints is reported (Belmonte-Serrano *et al.* 1993, Hart *et al.* 1994, Madsen *et al.* 1994, Nevitt *et al.* 1995), but a number of other studies did not detect an association between mineralisation and OA (Burr 1998, Ficat and Maroudas 1975, Healey *et al.* 1985, Reid *et al.* 1984).
The question whether subchondral bone change occurs before cartilage deterioration or subsequent to it has thus not yet been resolved (Adolphson *et al.* 1994, Bailey and Mansell 1997, Burr and Schaffler 1997, Muehleman *et al.* 2002, Oegema Jr *et al.* 1997) and the truth may even be more in the proverbial middle than ever thought. In recent work it has been suggested that repetitive high-impact loads may induce micro-cracks in subchondral bone. These micro-cracks will then stimulate remodelling and increase the mineralisation and thickness of the subchondral bone layer (Burr and Radin 2003). The process of remodelling produces a cascade of growth factors that can diffuse from bone into the cartilage layer and cause apoptosis of chondrocytes, resulting in loss of cartilage integrity (Fernandes *et al.* 2002, Massicotte *et al.* 2002). It is not exactly clear if direct damage to the cartilage may also occur during the high loading episodes. This concept emphasises how the articular cartilage and subchondral bone layers are inextricably linked and function together to form the load-bearing surface of the joint. To date, such a comprehensive approach where cartilage and bone are considered as a functional entity is not common practice in OA research.

Aim and scope of the thesis

The main aim of this thesis was to further elaborate on the concept of the joint as a functional entity. In other words, the principal goal of this work was to test the hypothesis that the joint should not be viewed as a composite structure consisting of a number of different and distinct connective tissue layers, but rather as an “organ” that is made up of functionally related tissues that share many characteristics and reaction patterns. A second aim, which was derived from the same concept but was more practical in character, was to identify pathology-driven changes in this entity of functionally related tissue layers and in synovial fluid that

might be classified as early signs of OA. This in the hope of making a meaningful contribution to the discussion regarding the pathogenesis of OA, which has been briefly outlined previously.
The equine metacarpophalangeal joint was selected as the joint of interest because of the large body of detailed knowledge that has been built up over the last decade with respect to this joint within our Department's research group, in a combined effort with collaborating institutions (Brama 1999b, van den Boom 2004). Within the same framework of this research continuum with respect to equine joint physiology and pathology, it was decided to focus on biochemical characteristics of the tissue layers under scrutiny. The existing extensive knowledge on a large number of biochemical characteristics, especially with regard to collagen and proteoglycan parameters in cartilage but to a certain extent also in subchondral bone, formed an excellent basis for continued research and for venturing into as yet unexplored areas.
To realise the main goals of this thesis, *i.e.* to determine whether there is indeed a functional connection between the three constituting elements of the load bearing surface of the joint (articular cartilage, subchondral and trabecular bone), an extensive comparative biochemical analysis was performed to assess the normal biochemical composition of the separate layers in a group of mature animals without signs of disease. An additional goal of this project was to provide a reference database with which the outcome of following studies could be compared. The majority of these exhaustive analyses was formed by collagen and proteoglycan biochemistry, but the deliberate decision to include the two bony layers (subchondral and trabecular bone) necessitated the additional study of a large number of mineral parameters (Chapter II).
The strong influence of biomechanical loading on local tissue characteristics led to the study of two differently loaded sites in the work described in Chapter II. This aspect became more important still in Chapter III, where a similar analysis as in the preceding chapter is performed, but this time in a group of juvenile, hence still immature horses. Earlier investigations in cartilage only had shown that the biochemical characteristics of this tissue are heavily influenced by loading in the early juvenile period, leading to the so-called concept of functional adaptation that seems to have a wider validity than the horse alone (Brama *et al.* 2000a, Helminen *et al.* 2000). The main question in Chapter III is to what extent this functional adaptation is a generalised phenomenon across all layers in the joint and also whether differences exist in the age at which site differences develop between the organic and inorganic (mineral) components of subchondral or trabecular bone. This area has not been previously explored, but is of great interest in the equine industry given the high incidence of bone-related problems in horses that are strenuously trained at a young age.
Chapter IV uses the database generated in Chapter II to compare normal tissue specimens with specimens from horses showing signs of (early) OA. Here, the contentious issue on the origins of OA is touched on. Does early cartilage damage,

quantified using a well-validated technique that was developed by another member of the team working on joint physiology and pathology (Brommer *et al.* 2003a), relate only to changes in the cartilage layer itself, or to changes in the other layers as well? If the latter is the case, are these changes more evident or more extensive in one layer than in the other? Without the pretension of providing the definitive answer, it could be expected that, if such differences were to exist, they might at last give an indication on the origin of OA.
In Chapter V the matter of early OA is pursued further, and the same technique is used to quantify cartilage damage, but attention is now shifted towards synovial fluid as a potentially rich source of substances that might indicate early pathologic events. This chapter can be seen as complimentary to the other four given the role of synovial fluid as a reflection of what happens at tissue level.
Chapter VI returns to the tissue level, but uses new and so far unexplored parameters for the detection of pathological processes in the three layers of tissue that make up the load bearing surface of the joint. In this chapter attention is focused on nitrite as a measure for the highly volatile NO, and on nitrotyrosine, a metabolite of NO. Nitric oxide is a somewhat ambiguous substance that has a physiologic role in tissue homeostasis, but may show increased levels under pathologic conditions. Nitrotyrosine is regarded as a marker of tissue damage. The combination of the two may give information on both metabolic and disease status of tissues.
In Chapter VII the key word is load and the crucial question regards the way the horse, or more specifically the equine musculoskeletal system, copes with load. The significance of the horse to mankind has always been based on its capacity to sustain load, either directly when used as a beast of burden or for haulage, or indirectly when used for transport or athletic performance. In all cases the musculoskeletal system is loaded and it is the discrepancy between the magnitude of loading and the capacity to sustain this load that often lies at the basis of musculoskeletal injury. As the last chapter of this thesis, Chapter VII places the main findings of the work in this perspective. The chapter further tries to relate these findings to other recent work in the horse and in other species, as the general concepts of articular (patho)biology have a much wider application than a single species.

CHAPTER II

AN INTEGRAL BIOCHEMICAL ANALYSIS OF THE MAIN CONSTITUENTS OF ARTICULAR CARTILAGE, SUBCHONDRAL AND TRABECULAR BONE

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(Osteoarthritis & Cartilage: 2004; 12: 752-761)

Summary

Objective: In articular joints, the forces generated by locomotion are absorbed by the whole of cartilage, subchondral bone and underlying trabecular bone. The objective of this study is to test the hypothesis that regional differences in joint loading are related to clear and interrelated differences in the composition of the extracellular matrix (ECM) of all 3 weight-bearing constituents.
Method: Cartilage, subchondral- and trabecular bone samples from two differently loaded sites (site 1, dorsal joint margin; site 2, central area) of the proximal articular surface of 30 macroscopically normal equine first phalanxes were collected. Collagen content, cross-linking (pentosidine, hydroxylysylpyridinoline (HP), lysylpyridinoline (LP)) hydroxylation, and denaturation, as well as glycosaminoglycan (GAG) and DNA content were measured in all three tissues. In addition, bone mineral density (BMD), the percentage of ash and the mineral composition (calcium, magnesium and phosphorus) were determined in the bony samples.
Results: For pentosidine cross-links there was an expected correlation with age. Denatured collagen content was significantly higher in cartilage at site 1 than at site 2 and was higher in trabecular bone compared to subchondral bone, with no site differences. There were significant site differences in hydroxylysine (Hyl) concentration and HP cross-links in cartilage that were paralleled in one or both of the bony layers. In subchondral bone there was a positive correlation between total (HP+LP) cross-links and Ca content. For Ca and other minerals there were corresponding site differences in both bony layers.
Conclusions: It is concluded that there are distinct differences in distribution of the major biochemical components over both sites in all three layers. These differences show similar patterns in cartilage, subchondral bone and trabecular bone, stressing the functional unity of these tissues. Overall, differences could be interpreted as adaptations to a considerably higher cumulative loading over time at site 2, requiring stiffer tissue. Turnover is higher in trabecular bone than in subchondral bone. In cartilage, the dorsal site 1 appears to suffer more tissue damage.

Introduction

A joint consists of one or more opposing congruent osseous structures covered by a highly specialised connective tissue layer of hyaline articular cartilage. The main functional requirement of the articular cartilage is withstanding compressive and shear forces and transferring these forces to the underlying bone (Muir 1995). This bone consists of a subchondral plate, a thin layer of bone just beneath the articular cartilage that has energy absorbing properties, and a supporting three-dimensional lattice work of platelets and struts, the so-called trabecular or cancellous bone of

the metaphysis (Radin and Rose 1986). The trabecular sheets are organised in such a way that they transmit the stresses from the overlying layers of articular cartilage, calcified cartilage, and subchondral plate to the metaphyseal cortices (Radin and Rose 1986). It can thus be said that one of the main functions of a joint, apart from enabling articulation through smooth sliding of the articular surfaces, is the dissipation of the considerable forces that are generated by locomotion.

Biomechanical behaviour of the tissue is determined by both ultra-structure and biochemical composition of the extracellular matrix (ECM). This insight has prompted extensive research into the molecular composition of the ECM of articular cartilage in the equine metacarpophalangeal joint in recent years. From this research it emerged that there is a distinct topographical variation in many biochemical parameters, matching with the variation in biomechanical loading as generated by locomotion (Brama *et al.* 2000b, Brama *et al.* 2001a). This topographical heterogeneity has been shown to be non-existent in the newborn animal and its formation is thought to be directed by biomechanical forces in the early postnatal period through the so-called process of functional adaptation (Brama *et al.* 2002a). Cartilage, subchondral bone plate and underlying trabecular bone together form a functional entity. There is a clear relationship between biochemical composition of the cartilage ECM and susceptibility to osteoarthritis (OA) (Brama *et al.* 1999a). There is also growing awareness that in the pathophysiology of diseases such as OA, osteochondritis dissecans, and rheumatoid arthritis, subchondral bone changes play an important role (Bailey and Mansell 1997). Further, it has been stated earlier that changes in the relative density and architecture of the underlying subchondral bone may have a profound effect on both the initiation and progression of cartilage damage (Radin and Rose 1986). Although a topographical variation in composition of the subchondral bone plate in relation to loading has been demonstrated in several species, it is surprising that no comprehensive studies have been conducted aiming at the simultaneous analysis of the matrix composition of cartilage and the underlying subchondral and trabecular bone layers and their interrelationship (Armstrong *et al.* 1995, Kiviranta *et al.* 1987, Oetmeier *et al.* 1992).

In this study the hypothesis is tested that regional differences in loading within a joint are related to clear and interrelated differences in the composition of the ECM of all three weight-bearing constituents. To this end, the study aimed at the quantification and analysis of the major structure and strength determining components of the ECM at two differently loaded sites of all three tissue layers in healthy normal joints. Knowledge of site- and tissue-related biochemical characteristics will help understanding joint function and is indispensable for a correct interpretation of aberrations found in joint pathologies such as OA.

Materials and methods

Joints
Thirty right metacarpophalangeal joints from adult Warmblood slaughter horses (mean age 10 years, range 5-23 years) were harvested immediately after death and stored at -20 °C until processing. One day before the measurements, the joints were thawed and opened. The proximal two-thirds of the first phalanx were isolated from the rest of the limb and the surrounding tissue was dissected. All joints were normal at macroscopic inspection and there was no evidence of any pathology related to the area. To corroborate this, articular cartilage degeneration was quantified with help of the Cartilage Degeneration Index (CDI) (Brommer *et al.* 2003a). Briefly, the amount of Indian ink uptake across the entire cartilage surface was quantified by digital imaging of the native and the Indian ink stained articular cartilage surfaces. The increase in mean grey level of the articular surface is the basis for calculation of the CDI (range from 0 to 100%). A CDI score of < 25% is seen as indicative of none to minor degenerative changes (Brommer *et al.* 2004). In this study only joints were included with a CDI score of < 25%.

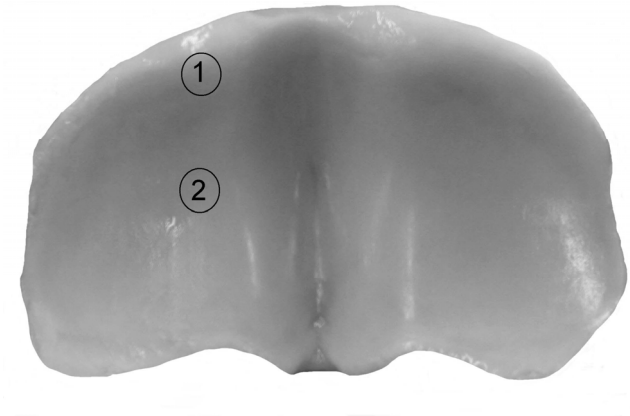

Fig. 1. Sample sites on the proximal articular surface of the first phalanx.
(1 = mediodorsal articular margin; 2 = central fovea)

Sampling procedure
After establishing the CDI, a 6 mm wide slice of phalanx I, containing cartilage, subchondral bone and trabecular bone was cut in dorsopalmar direction, perpendicular to the articular surface and through the centre of the medial fovea, using a band saw. These slices contained two differently loaded sites: site 1 located close to the dorsal articular margin, and site 2 at the central fovea (Fig. 1).

A slice of 1-2 mm thickness was cut off, placed in Burckhardt's fixative for 4 days and thereafter kept in 100% ethanol until measurement of bone mineral density (BMD). From the remaining sample the cartilage was taken off with a scalpel and the subchondral and trabecular bone were separated with a milling cutter. Each bone and cartilage sample was further divided into 4 pieces and stored at - 80 °C until further analysis.

Determination of Bone Mineral Density
BMD was measured with a peripheral quantitative computed tomography (pQCT) machine (Stratec XCT 960A) adapted for measuring small bones. Two 360˚ X-ray scans were taken with a thickness of 1 mm and a resolution of 0.148 mm X 0.148 mm. The pQCT machine was calibrated with a standard of hydroxyapatite embedded in acrylic plastic. The scans were performed in two horizontal planes: at 2 mm under the cartilage layer through the subchondral plate and at a depth of 8 mm through the trabecular bone. BMD was expressed as mg/cm^3.

Mineral analysis
One piece of subchondral- and trabecular bone of each site was thawed and defatted by placing in ether for 1 week. After drying for 1 h at 105 °C dry weight was determined. The drying time of 1 h proved to be sufficient in a test series where various samples were dried and subsequently weighed after drying times up to 24 h (data not shown). The fat-free, dry samples were ashed (540 °C for 6 h), weighed and then dissolved in 15 ml 4 M hydrochloric acid. Calcium and magnesium content were determined by atomic absorption spectrophotometry (Perkin Elmer 3300). Phosphorus content was determined according to the method of Quinlan and DeSesa (1955). The amount of minerals was expressed as mmol per gram dry bone weight. The ash concentration was expressed as percent of dry bone weight.

Crosslink and amino acid analysis
One piece of subchondral and trabecular bone and cartilage of each site was thawed and bone samples were demineralised for three weeks in buffered 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 7.4). The bone and cartilage samples were hydrolysed (110 °C, 20-24 h) with 800 μL 6 M HCl, dried in a Speed Vac (Savant SC 110) and dissolved in 500 μL (bone) or 200 μL (cartilage) water containing 10 μM pyridoxine (internal standard for the cross-links analysis (Sigma)) and 2.4 mM homoarginine (internal standard for the amino acid analysis (Sigma)). Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid (HFBA (Fluka)) in 10 % (vol/vol) acetonitrile (Rathburn Chemicals) for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M borate buffer pH 8.0 (0.1 M boric acid adjusted to pH 8.0 with 5 M sodium hydroxide) for amino acid analysis.

Reversed-phase high-performance liquid chromatography of cross-links (100 μL of the 5-fold diluted sample) was performed on a micropak ODS-80 TM column (TOSOH biosep) as described elsewhere (Bank *et al.* 1996, Bank *et al.* 1998). HP and LP purified from adult human bone and calibrated vs the PYD/DPD HPLC calibrator (Metra) and pentosidine (calibrated by mass spectroscopy) served as a standard (Bank *et al.* 1997a).
For amino acid analysis, 200 μL of the 250-fold diluted sample was derivatised at room temperature with 200 μL acetone containing 6 mM 9-fluorenylmethyl-chloroformate (FMOC-Cl) (Fluka). Termination of the derivatisation reaction, removal of excess reagent, and chromatography were performed as described elsewhere (Bank *et al.* 1996). Calibration was performed with an amino acid standard for collagen hydrolysates (Bank *et al.* 1996, Bank *et al.* 1998). The quantities of the cross-links HP, LP and pentosidine as well as the hydroxylysine (Hyl) levels were expressed as number of residues per collagen molecule, assuming 300 hydroxyproline (Hyp) residues per triple helix (mol/mol collagen).

Analysis of denatured collagen
The amount of denatured collagen was determined by the assay described by Bank *et al.* (1997b), which is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the triple helix. Briefly, one piece of cartilage, subchondral and trabecular bone of each site was thawed and bone samples were demineralised for 3 weeks in buffered 0.5 M EDTA (pH 7.4). Bone and cartilage were 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 bone and 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 h. The amount of the collagen-specific amino acid Hyp was measured with reversed-phase high-performance liquid chromatography. The amount of denatured collagen was expressed as a percentage of total collagen.

Glycosaminoglycans
Trabecular bone and cartilage samples were digested by papain (P3125 (Sigma)) in 200 µl of a 50 mM phosphate buffer, pH 6.5, containing 2 mM Na_2EDTA and 2 mM cysteine for approximately 16 h at 65 ºC.
Proteoglycan content was determined by measuring the amount of polysulphated glycosaminoglycans (GAG's) in the papain digest of the bone samples by a modification of the 1,9-dimethylmethylene blue (DMMB) assay described by Farndale *et al.* (1986). To 10 µl diluted sample from papain digest, 10 µl 1 % (w/v) bovine serum albumin (Sigma) and 200 µl of reagent (46 µM DMMB (Sigma)) 40 mM glycine and 42 mM NaCl adjusted to pH 3.0 with HCl were added and after 30

min the absorbency at 525 nm was measured. The assay was standardised with shark chondroitin sulphate (Sigma). GAG amount was expressed as µg/mg dw sample of bone.

DNA analysis
A 10 µl aliquot of the papain digest was used to determine DNA content as a measure for the amount of cells using the fluorescent dye Hoechst 33528 as described by Kim *et al.* (1988). In short: dye solution was added to the papain digest of the bone samples and measured immediately after mixing using a LS-2b (excitation at 365 nm and emission at 460 nm). Calf thymus DNA (D-4764 (Sigma)) was used as reference. Results were expressed as µg DNA/mg dw.

Statistical analysis
All measured parameters were expressed as mean ± SD. Statistical analysis of the data was performed with help of the software package SPSS version 10 for Windows (SPSS Inc.). Differences between respectively sites and the different bone layers were tested by use of ANOVA (factors: site and layer). Correlations were tested using a Pearson's product moment correlation analysis. The level of significance was set at $p<0.05$.

Results

No age relationship was found for any of the parameters, except for pentosidine in cartilage, subchondral and trabecular bone. For this reason, all parameters except for pentosidine were expressed as mean with standard deviation (SD).
In all three tissues, collagen content (measured as Hyp per proline ratios) was slightly higher (1-5%) in site 2 than in site 1, reaching statistical significance for the subchondral and trabecular bone (Table 1). The fractions of denatured collagen were of the same order in the three layers investigated and oscillated between 10 and 15%. In cartilage there was more denatured collagen in site 1 than in site 2 suggesting early degenerative changes in site 1. Further there was significantly less denatured collagen (~1-3%) in subchondral bone compared to trabecular bone with no significant site differences (Table 1, Fig. 2), which may reflect differences in turnover rate between those two types of bone.
Hydroxylysine content in cartilage was double to triple the content in bone (Table 1, Fig. 3), thus reflecting the presence of highly hydroxylated type II collagen in cartilage (on average 45 Hyl residues per triple helix) vs type I collagen in bone (~15 Hyl per collagen). In cartilage and subchondral bone, site 2 had a higher lysyl hydroxylation than site 1, but the difference in the latter tissue was considerably less. There was no site difference in trabecular bone, but overall content at this level was about 25-35% less than in subchondral bone.

Table 1 - The measured collagen and mineral parameters of different sites (joint margin and central fovea) in cartilage, subchondral bone and trabecular bone (mean ± SD) of normal equine proximal first phalanx. n = number of samples.

	CARTILAGE		SUBCHONDRAL BONE		TRABECULAR BONE	
	Site 1 (mean ± SD) (n)	**Site 2 (mean ± SD) (n)**	**Site 1 (mean ± SD) (n)**	**Site 2 (mean ± SD) (n)**	**Site 1 (mean ± SD) (n)**	**Site 2 (mean ± SD) (n)**
Hydroxylysine/collagen (mol/mol)	40.76 ± 13.02$^{\#\#\#}$ (30)	52.73 ± 5.37 (27)	17.74 ± 3.55$^{\#\#***}$ (30)	20.08 ± 2.39*** (30)	12.89 ± 2.17 (30)	12.96 ± 1.74 (30)
Hydroxyproline/proline (mol/mol)	0.67 ± 0.08 (30)	0.68 ± 0.07 (27)	0.64 ± 0.03$^{\#\#\#***}$ (30)	0.67 ± 0.02*** (30)	0.67 ± 0.02$^{\#\#\#}$ (30)	0.69 ± 0.02 (30)
HP/collagen (mol/mol)	0.64 ± 0.27$^{\#\#\#}$ (30)	0.85 ± 0.11 (27)	0.20 ± 0.07$^{\#\#\#**}$ (23)	0.32 ± 0.06*** (29)	0.12 ± 0.02$^{\#\#\#}$ (30)	0.15 ± 0.03 (30)
LP/collagen (mol/mol)	0.03 ± 0.02$^{\#\#\#}$ (30)	0.01 ± 0.003 (23)	0.07 ± 0.04$^{\#***}$ (20)	0.05 ± 0.01*** (29)	0.09 ± 0.02 (29)	0.09 ± 0.03 (30)
Denatured collagen (%)	15.28 ± 6.66$^{\#}$ (30)	11.73 ± 4.24 (27)	11.38 ± 2.5* (30)	11.87 ± 2.58*** (30)	12.65 ± 3.14 (30)	14.04 ± 4.05 (30)
GAG (µg/mg dw)	59.27 ± 26.91$^{\#\#\#}$ (30)	124.05 ± 39.92 (30)	--	--	1.72 ± 0.40 (30)	1.69 ± 0.44 (29)
DNA (µg/mg dw)	2.39 ± 0.90$^{\#\#\#}$ (30)	1.63 ± 0.49 (29)	--	--	0.95 ± 0.20 (30)	1.03 ± 0.15 (29)
GAG/DNA (µg/ µg DNA)	28.31 ± 15.82$^{\#\#\#}$ (30)	72.80 ± 17.10 (29)	--	--	1.84 ± 0.39 (30)	1.66 ± 0.39 (29)
BMD (mg/cm^3)	--	--	990 ± 75$^{\#\#\#***}$ (30)	1072 ± 59*** (29)	875 ± 135$^{\#\#\#}$ (30)	707 ± 149 (29)
Ash (%)	--	--	58.17 ± 1.42$^{\#\#\#***}$ (28)	60.29 ± 2.42*** (29)	61.67 ± 1.31$^{\#\#}$ (28)	62.55 ± 1.48 (29)
Calcium (mmol/g dw)	--	--	5.58 ± 0.20$^{\#\#\#***}$ (29)	5.80 ± 0.21*** (29)	5.93 ± 0.16$^{\#}$ (29)	6.07 ± 0.37 (30)
Phosphorus (mmol/g dw)	--	--	2.97 ± 0.08$^{\#\#\#***}$ (28)	3.12 ± 0.11*** (29)	3.17 ± 0.12$^{\#}$ (30)	3.29 ± 0.21 (30)
Magnesium (mmol/g dw)	--	--	0.07 ± 0.005$^{\#\#***}$ (29)	0.08 ± 0.006*** (29)	0.08 ± 0.006$^{\#\#\#}$ (30)	0.09 ± 0.008 (30)

= Significant difference between the joint margin (site 1) and the central fovea (site 2) ($^{\#}$ P< 0.05; $^{\#\#}$ P < 0.01; $^{\#\#\#}$ P < 0.001).
* = Significant difference between the subchondral and trabecular bone within the same site (* P< 0.05; ** P < 0.01; *** P < 0.001)

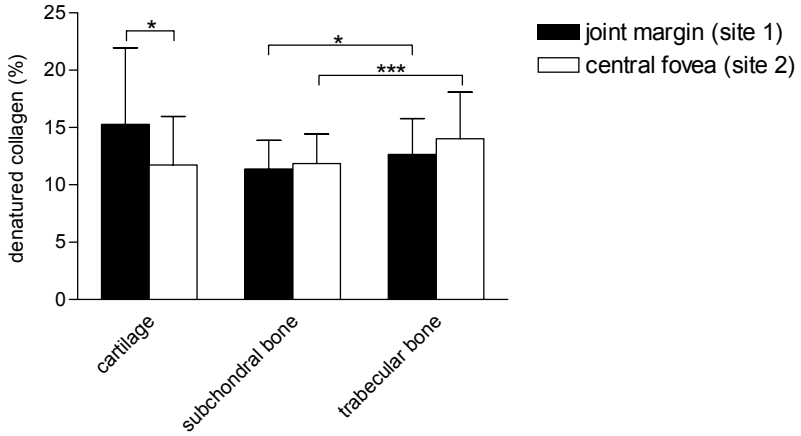

Fig. 2. Percentage denatured collagen (mean ± SD) in cartilage, subchondral- and trabecular bone at the joint margin, site 1 (solid bars) and the central fovea, site 2 (open bars) of the proximal first phalanx. Asterisks denote significant difference between the two different sites and subchondral bone versus trabecular bone; * P < 0.05, ** P < 0.01, *** P < 0.001.

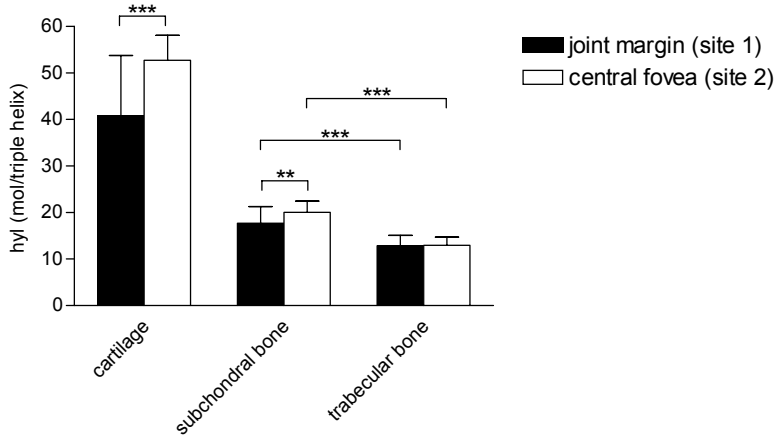

Fig. 3. Hydroxylysine content (mol/triple helix; mean ± SD) of cartilage, subchondral- and trabecular bone at the joint margin, site 1 (solid bars) and the central fovea, site 2 (open bars) of the proximal first phalanx. Hyl = hydroxylysine. For further explanation see caption of Fig. 2.

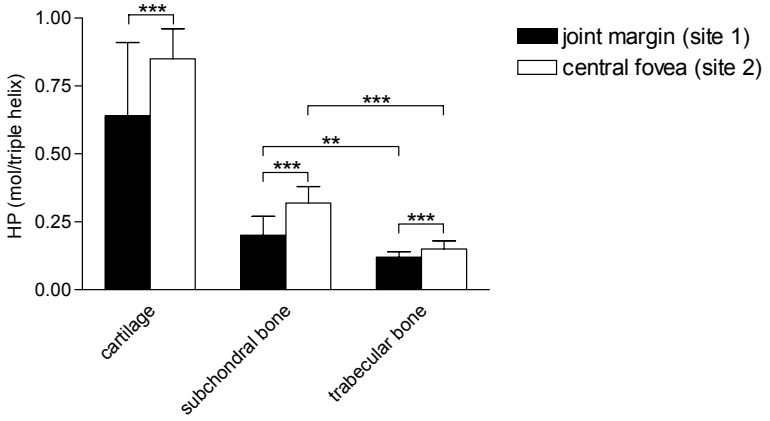

Fig. 4. Hydroxylysylpyridinoline crosslinking (mol/mol triple helix collagen; mean ± SD) of cartilage, subchondral- and trabecular bone at the joint margin, site 1 (solid bars) and the central fovea, site 2 (open bars) of the proximal first phalanx. HP = Hydroxylysylpyridinoline. For further explanation see caption of Fig. 2.

Since lysyl hydroxylation has been related to the thickness of the collagen fibres, these differences may reflect a functional difference between these two types of bone.

Since collagen cross-linking is important in the determination of tissue strength, the level of pyridinoline cross-linking was measured. As expected, hydroxylysylpyridinoline (HP) cross-links are much more abundant in cartilage than in the underlying bone (Table 1, Fig. 4); the opposite, though to a smaller extent, is true for lysylpyridinoline (LP) cross-links (Table 1). In bone, HP cross-links are better represented at site 2. In case of subchondral bone, levels at this site even reach a 50% higher concentration than at site 1 (Fig. 4). Further, levels in subchondral bone are higher than in trabecular bone. In cartilage levels were found to be higher at site 2 also. At site 1 in this tissue there was a large variation, reflected in the high SD. This may be caused by the early degenerative changes that are apparently present (based on collagen damage, see above). For LP cross-links significant site differences are seen in cartilage and subchondral bone. In bone, levels in trabecular bone are significantly higher than in subchondral bone at both sites (Table 1). In addition to the enzymatically-regulated cross-links HP and LP, the advanced glycation cross-link pentosidine was measured. In cartilage, subchondral and trabecular bone the pentosidine level increases with age. This increase is significant in all three tissues at both sites, except for site 1 in subchondral bone (Fig. 5a,b,c).

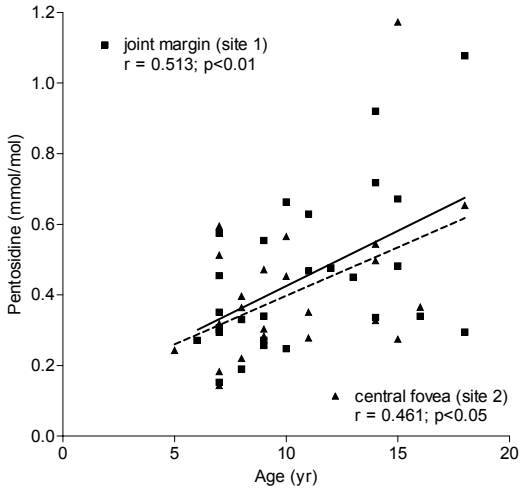

Fig. 5a. Number of pentosidine cross-links (mmol/mol triple helix collagen) in equine cartilage in relation to age at the joint margin, site 1 and the central fovea, site 2 of the first phalanx. P = significance level; r = correlation coefficient.

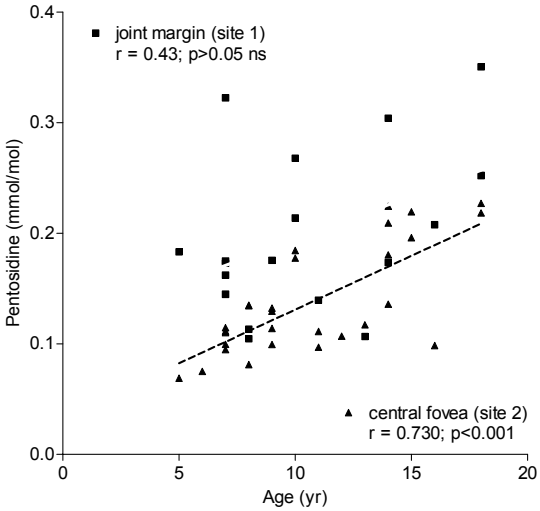

Fig. 5b. Number of pentosidine cross-links (mmol/mol triple helix collagen) in subchondral bone in relation to age at the central fovea, site 2 of the first phalanx. P = significance level; r = correlation coefficient.

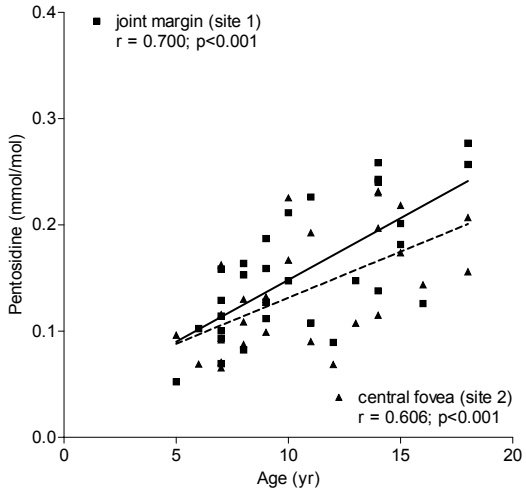

Fig. 5c. Number of pentosidine cross-links (mmol/mol triple helix collagen) in equine trabecular bone in relation to age at the joint margin, site 1 and the central fovea, site 2 of the first phalanx. P = significance level; r = correlation coefficient.

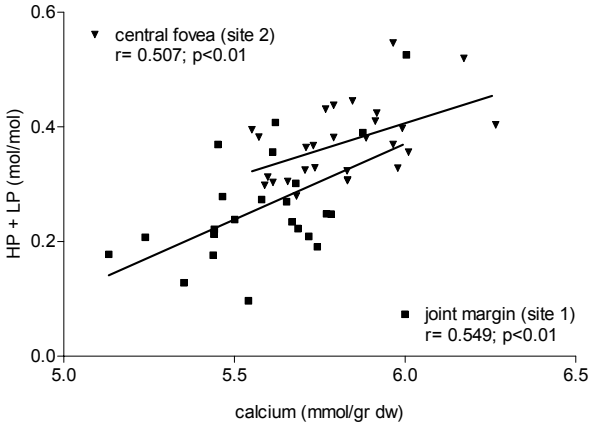

Fig. 6a. Correlation between calcium content (mmol/g dw) and total amount of pyridinoline cross-links (HP plus LP in mol/mol triple helix) at the joint margin, site 1 and the central fovea, site 2 of subchondral bone samples of the proximal first phalanx. HP = hydroxylysylpyridinoline; LP = lysylpyridinoline; dw = dry weight; P = significance level; r = correlation coefficient.

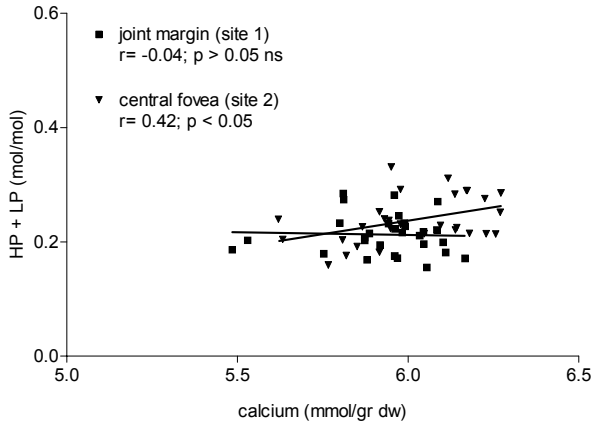

Fig. 6b. Correlation between calcium content (mmol/g dw) and total amount of pyridinoline cross-links (HP plus LP in mol/mol triple helix) at the joint margin, site 1 and the central fovea, site 2 of trabecular bone samples of the proximal first phalanx. HP = hydroxylysylpyridinoline; LP = lysylpyridinoline; dw = dry weight; P = significance level; r = correlation coefficient.

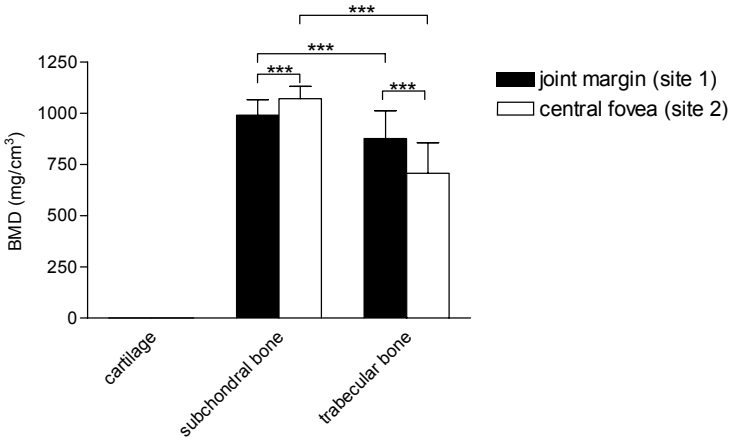

Fig. 7. BMD (mg/cm^3; mean ± SD) measured 2 mm under the cartilage layer through the subchondral bone plate and at a depth of 8 mm through the trabecular bone at the joint margin, site 1 (solid bars) and the central fovea, site 2 (open bars) of the first phalanx. BMD = Bone mineral density. For further explanation see caption of Fig. 2.

GAGs and DNA levels were determined in cartilage and trabecular bone samples only as the risk for contamination with cartilage of the subchondral bone samples was deemed too high. A slight contamination with cartilage will hardly affect collagen related parameters as these are in the same order of magnitude, but would affect measurements of GAGs and DNA considerably because of the important inherent differences in normal tissue levels. In cartilage, GAG levels at site 2 were more than double those at site 1 (Table 1). For DNA, the picture was reversed (Table 1). In trabecular bone GAG levels were 1.4-3% of those in cartilage, DNA content was about half the content in cartilage. In trabecular bone there was no site difference in any of these parameters.
The overall pattern for all mineral parameters (Ca, Mg, P and ash content) was similar. In all cases content of these elements was higher at site 2 than at site 1, and content was higher in trabecular bone compared to subchondral bone (Table 1). Fig. 6 (a, b) shows that there is a concomitant increase in the total amount of cross-links (HP and LP) with the increase in calcium content. This relationship is significant in both sites of the bone layers, except for trabecular bone at site 1.
Bone mineral density (BMD) showed a more complex pattern. BMD was consistently higher in subchondral bone than in trabecular bone, but there were reverse site differences with higher BMD at site 2 in subchondral bone, but a lower level at the same site in trabecular bone (Table 1, Fig. 7).

Discussion

The cartilage covered epiphysis of a bone can be considered as a multi-composite structure consisting of different layers. These layers have different material properties but act together as a functional unit to fulfil properly the functions of the joint, *i.e.* weight-bearing, articulation and dissipating the forces generated by locomotion. The analysis of a single element of this multi-composite structure may yield useful information, but is necessarily of a limited scope as no information about the other constituting elements is obtained. For this reason, the present study focused on the simultaneous analysis of the three major layers that can be discerned: cartilage, subchondral bone and trabecular bone. It is recognised that this approach is limited too. A finer division including for instance the layer of calcified cartilage and the transitional zone of the subchondral and trabecular bone would have been preferable. However, this was impossible due to economic and logistic constraints.
It was a deliberate choice to analyse tissue layers at two sites that are very differently loaded. The site near the dorsal articular margin (site 1) has been shown to be loaded only at high speeds, or during heavy athletic activity (Brama *et al.* 2001a). Then, the area is very heavily loaded, but during low intensity activities such as standing or walking this is a non-contact area. The central fovea of the metacarpophalangeal joint is always loaded as long as the horse is on its feet,

which is almost 24 h per day in mature animals. This makes the area a constantly loaded site with, however, considerably lower peak loads (Brama *et al.* 2001a). Although it can be assumed that differences will be largest in compressive loads, it is probable that there will be differences in tensile and shear loading as well at these sites, which may contribute to differences in behaviour of the tissue.

As expected, large differences in biochemical composition were found between cartilage and bone. However, there were also substantial differences between subchondral and trabecular bone as well as parallels between cartilage and bone, especially with respect to the differences between the two investigated sites. The interaction of the hydrated GAGs and the cross-linked collagen network determine the unique biomechanical properties of cartilage (Kempson 1980). The mechanical properties of subchondral bone are dependent on the fractions of collagen and minerals (Evans *et al.* 1992, Knott *et al.* 1995). Both tissues will in some stage of their development respond, albeit in some cases differently, to the biomechanical challenges they are subjected to. In this respect it should be realised that, due to the spatial arrangement of the tissue layers, the impact of loading will always be higher on the cartilage layer and become more attenuated towards the trabecular bone. Site differences can, therefore, be expected to be more marked in the more superficial layers.

Denatured collagen is not only a measure for mechanically induced damage, but may indicate turnover caused by proteinase-mediated tissue remodelling as well. The latter may especially be the case in bone where the turnover times of collagen type I are much shorter than those of collagen type II in cartilage, which have been reported to be extremely long (Maroudas *et al.* 1992, Verzijl *et al.* 2000). Denatured collagen levels were higher in trabecular bone than in subchondral bone and, in cartilage only, higher at site 1 than at site 2. In bone this difference is probably caused by differences in remodelling rate, which can be supposed to be related to functional differences in handling by the tissue of the forces generated by locomotion. The thin and compact subchondral bone plate will most likely to a large extent transmit these forces, whereas most dissipation will take place in the trabecular layer underneath, inducing remodelling activity. In cartilage, the much higher level at site 1 is likely to be indicative of real damage, as remodelling activity is extremely low by definition in mature individuals. This is in line with the common occurrence of degenerative ailments such as OA at this site (Brama *et al.* 1999a, Pool 1996).

Hydroxylysine levels are significantly higher at site 2 in cartilage, which is in line with earlier findings by Brama *et al.* (2000b). The difference diminishes with depth from the joint surface: a small difference in subchondral bone, none in trabecular bone. As low Hyl levels have been reported to correspond with higher biomechanical strength in bone, this distribution pattern might reflect the dissipation of force throughout the bony layers underneath the articular cartilage (Knott *et al.* 1995, Yang *et al.* 1993).

Hydroxyproline expressed per proline is a measure for the relative amount of collagen within the protein pool. The levels in subchondral bone are fractionally, but constantly and significantly higher at site 2 compared to site 1. Although this variable is not equal to collagen content, the observation is in agreement with findings by Brama *et al.* (2001b), who found higher collagen levels at site 2 in a study on the effect of exercise on subchondral bone composition. The same distribution holds for HP cross-links where differences in subchondral bone are considerable. Both collagen in itself and cross-linking add to overall strength and these differences may be explained by the fact that, although peak loading is less, total loading over time will be more at site 2, which might require a stronger structure. The higher HP level in subchondral bone compared to trabecular bone can be explained by differences in remodelling rate, resulting in a lower state of maturation in the trabecular bone and hence a lower HP content (Eyre *et al.* 1984b, Eyre *et al.* 1988).
Collagen molecules have an exceptional long lifetime once they have been incorporated into the ECM. This makes them susceptible to the accumulation of advanced glycation end products (AGEs) via the so-called Maillard reaction (Monnier 1989). The process results in increased cross-linking, such as pentosidine formation from lysine, sugar and arginine moieties. Pentosidine is one of the few Maillard cross-links of which the structure has been elucidated, and is used as sensitive marker for the entire process of AGE formation (Vlassara *et al.* 1995). The accumulation of AGEs depends on the turnover rate of a protein or tissue and thus the metabolic activity of the tissue (Verzijl *et al.* 2000). In human supraspinatus tendon, a structure that is often subjected to micro trauma and is frequently involved in the so-called rotator cuff syndrome, the remodelling rate is high and no increase in pentosidine levels is seen. In contrast, there is a linear increase with age of these products in the nearby biceps brachii tendon, which is infrequently, if ever, involved in pathology (Bank *et al.* 1999). The lack of a significant increase with age in site 1 of subchondral bone only might be indicative of a high incidence of microtrauma at this site too. Chip fractures at this site are well known and frequently described clinical findings (Birkeland 1972, Kawcak and McIlwraith 1994, Petterson and Reyden 1982, Whitton and Kannegieter 1994).
GAG levels are 35- to 70-fold higher in cartilage than in trabecular bone, illustrating the inherent differences in functional composition of these tissues in which the collagen network is supported by GAGs to create resilience (cartilage), or by minerals to give strength (bone). The huge site difference in cartilage is in line with earlier reports, and is thought to be a response of the tissue to the large differences in loading pattern (Brama *et al.* 2001a). Similar findings were reported earlier by Kiviranta *et al.* (1987), who found a significantly lower GAG content at the proximal joint margin in the femur of young Beagles, a site that was only loaded during severe overextension. DNA levels do not show site differences in trabecular bone, but in cartilage levels are higher at site 1. These differences may be illustrative for the rather harsh conditions at this site where collagen damage is

common and the need for repair may be high, but where cells are probably less vital. An indication of the latter may be that GAG levels, when expressed per DNA and hence indicative for cellular productivity, are more than 2.5-fold lower at site 1.
The mineral in bone that provides rigidity is hydroxyapatite. This inorganic compound of bone accounts for approximately 65% of the total bone matrix. The remaining 35% consist of water, collagen, proteoglycans and non-collageneous proteins (Boskey 1981, Todhunter *et al.* 1996b). A higher ash percentage means a lower collagen and/or water volume and thus stiffer bone (Dequeker *et al.* 1997). The stiffness of the bone enables it to support relatively high loads without substantial deformation (Radin and Rose 1986). It has been shown that in areas of excessive load concentration, bone formation activity and bone stiffness were high (Matsui *et al.* 1997). They demonstrated more bone formation and denser bone in the superficial layer of the subchondral plate. This loading-driven interaction between cartilage and subchondral bone may cause changes in structure, which will be not uniform across the joint, and are related to the depth from the joint surface (Burr and Schaffler 1997, Matsui *et al.* 1997, Oegema *et al.* 1997). Stiff bone adjacent to the layer of articular cartilage is also thought to be a risk factor for cartilage damage and a potential cause of OA (Murray *et al.* 2001b, Radin and Rose 1986, Radin *et al.* 1991, Radin 1999). In this study we found higher ash levels in trabecular bone than in subchondral bone and consistently higher levels at site 2, resulting in a somewhat more elastic tissue at site 1 (Fratzl *et al.* 1996). The fact that ash levels in trabecular bone are higher than in subchondral bone does not mean the former is the stiffer layer, as the tissue properties are not only determined by the material properties, but by the structural peculiarities as well. Trabecular bone has a much more open structure than subchondral bone and hence is a considerably less stiff tissue type. In this study this is reflected by the BMD data. BMD, when determined by pQCT, is a measure of mineral content in a unit volume of bone. This is an apparent mineral density, influenced by both porosity and true mineral density (McCalden *et al.* 1993, Wang *et al.* 2000). Given the fractional differences in ash content, the differences in BMD in this study are largely caused by differences in porosity. Subchondral bone is the stiffer structure with both less porosity and stiffer material at site 2. This site difference is in agreement with the findings by Murray *et al.* (2001b), who observed in equine carpal bones that subchondral bone at heavily loaded dorsal sites was thicker and materially stiffer than at less loaded palmar locations of the same bone. Trabecular bone is less stiff, will therefore yield more and thus be more incited to remodel, which is a consistent finding throughout this study. The reverse site 1 – site 2 differences can be explained by the differences in architecture of the trabecular bone that becomes denser from the centre towards the cortical bone, thus towards site 1.
The individual minerals follow the general pattern of ash content with higher levels in trabecular bone and at site 2 compared to site 1, probably reflecting overall metabolism. Magnesium levels are about 10-15% higher in trabecular bone

compared to subchondral bone. In calcified tissues the amount of Mg associated to the apatitic phase is known to be higher at the beginning of the calcification process (Bigi *et al.* 1992, Bigi *et al.* 1997). Therefore, the higher Mg levels probably reflect the overall younger age of this tissue because of the higher remodelling rate.
An interesting functional link between mineralised and non-mineralised ECM is exemplified by the positive correlation between total (HP+LP) cross-links and calcium content. The correlation is present at both sites in subchondral bone, in trabecular bone at site 2 only [Fig. 6(b)]. Whereas Ca content stands for stiffness of bone, cross-links provide stiffness and strength to collagen. This correlation is thought to illustrate well the differences in loading and the resulting response of the tissue at these sites and to be a fine example of the functional resemblance and interrelationship of the various layers that make up the joint.
It is concluded that there are distinct and significant differences in the biochemical composition of articular cartilage, subchondral bone and trabecular bone at differently loaded sites. These differences, although they are not always similar in the different layers, do not stand apart. In general, the bony layers in the central area of the joint (site 2) are characterised by stronger and stiffer tissue with higher mineral contents and a denser cross-link distribution. Towards the joint margin (site 1) stiffness diminishes and more elasticity can be expected based on the biochemical composition. Here, also more damage can be expected caused by high intensity, low frequency and irregular loading. This is especially evident in the cartilage layer (that will inevitably sustain the heaviest impact), where denatured collagen levels are relatively high. In the bony parts the trabecular layer is metabolically most active with the greater part of remodelling taking place in the central area (underneath site 2). This overall pattern can, most likely, be explained by load distribution and by the dissipation of forces by the joint surface and underlying tissues. Total load (cumulative force over time) will be higher in the central area. Dissipation of forces is accomplished principally by the trabecular layer. Of this layer the central area is, again, most challenged. The topographical variation in biochemical composition seems, therefore, to be largely loading induced and most of it can be explained via the same mechanisms. Knowledge of these topographical differences in the various layers and of their interrelationship seems indispensable for the study of pathological processes that take place at and underneath the joint surface.

Acknowledgements

The authors would like to express their gratitude to Ms M.I.J.A. Pollak, Mr A. Klarenbeek and H.S. Wouterse for their help with the preparation and processing of the samples. We also would like to thank dr. A.G.H. Ederveen and Mr C. Spanjers of the Department of Pharmacology of N.V. Organon (Oss, the Netherlands) for their indispensable help in measuring BMD.

CHAPTER III

STUDY OF CARTILAGE AND BONE LAYERS OF THE BEARING SURFACE OF THE EQUINE METACARPOPHALANGEAL JOINT RELATIVE TO DIFFERENT TIMESCALES OF MATURITY

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(Equine Veterinary Journal: Accepted)

Summary

Reasons for performing study: A detailed and comprehensive insight in the normal maturation process of the different tissues that make up functional units of the locomotor system such as joints is necessary as input in the debate about the influence of early training on musculoskeletal tissues.
Objectives: To simultaneously study the maturation process in the entire composite structure that makes up the bearing surface of a joint (cartilage, subchondral and trabecular bone) in terms of biochemical changes in the tissues of juvenile horses (6 months – 4 years) at 2 differently loaded sites of the metacarpophalangeal joint, compared to a group of mature horses (> 4 years).
Methods: Age-related changes in biochemical characteristics of the collagen part of the extracellular matrix (hydroxylysine, hydroxyproline, hydroxypyridinum cross-links) of articular cartilage and of the underlying subchondral and trabecular bone were determined in a group of juvenile horses (n=13) and compared to a group of mature horses (n=30). In both bony layers bone mineral density, ash content and levels of individual minerals were determined as well.
Results: In cartilage, subchondral bone and trabecular bone virtually all collagen parameters in juvenile horses already were at a similar (stable) level as in mature horses. In both bony layers bone mineral density, ash- and calcium content were also stable in the mature horses, but continued to increase in the juvenile group. For magnesium there was a decrease in the juvenile animals, followed by a steady state in the mature horses.
Conclusions: In juvenile horses of 6 months to 4 years of age the collagen network of all three layers within the joint has already attained a mature biochemical composition, but the mineral composition of both subchondral and trabecular bone continues to develop until approximately age 4 years.
Potential relevance: The disparity in maturation of the various extracellular matrix components of a joint can be assumed to have consequences for the capacity to sustain load and should hence be taken into account when training or racing young animals.

Introduction

Disorders of the musculoskeletal system contribute more than three times as much to economic losses in the equine industry than any other organ system does and thus form the biggest threat to the equine athlete. Among these disorders joint problems take the most prominent place and are the principal reason for wastage, as has been observed in racehorses (Olivier *et al.* 1997, Rossdale *et al.* 1985) and in more mixed equine populations (Todhunter and Lust 1990).
The bearing surface of the joint is composed of a layer of articular cartilage that rests on the subchondral plate, which itself is supported by the underlying

trabecular bone. These tissues have been studied separately to a varying extent, but it is only recently that the joint is envisaged as an organ in which these three layers together form a functional entity. In the horse it has been shown that these layers have certain biochemical characteristics and reaction patterns in common, but there are layer-specific differences as well (Harst *et al.* 2004). The biomechanical characteristics of these tissue layers, and hence the resistance to injury, are to a large extent determined by the biochemical composition of their extracellular matrices (ECM).

The ECM of articular cartilage consists primarily of water, proteoglycans and collagen (type II). The proteoglycan molecules possess large amounts of polyanionic glycosaminoglycan (GAG) side-chains that attract water and thus create a hydrodynamic force that is resisted by the tension of the three-dimensional collagen fibrils (Rosenberg and Buckwalter 1986, Todhunter 1996a), which are characterised by high breaking strengths due to the presence of covalent hydroxypyridinum cross-links (Eyre *et al.* 1984b). This results in a tissue with unique biomechanical properties that provides the shear-resistant and weight bearing surface essential for normal joint function (Kempson 1980).

In bone, the mineral component that is present in the form of hydroxyapatite makes up approximately 65% of the total bone matrix. The mineral component will therefore be the principal determinant of biomechanical strength (Dequeker *et al.* 1997), but is not the sole actor as water, collagen (type I), proteoglycans and non-collageneous proteins that account for the remaining 35% play a role as well (Boskey 1981). Of the non-mineral components the collagen skeleton of the ECM is of special importance as it has been documented that it is this structure that has a regulating role in the apposition of mineral components (Wassen *et al.* 2000).

At birth the development of the musculoskeletal system is far from completed, but will go on, at diminishing speed, during the first years of life. There is growing insight that in mammalian species the amount and intensity of exercise during this period of postnatal growth has a steering effect on the development of the musculoskeletal tissues and may influence later tissue quality in terms of injury resistance and proneness to develop chronic degenerative diseases such as osteoarthritis (OA) (Helminen *et al.* 2000). Knowledge of the influence of exercise on the development of the musculoskeletal system is essential for the equine industry, especially in those branches of equestrian activity in which training starts at an age when the animal is still growing, such as Thoroughbred and Standardbred racing. However, although it is clear that lack of exercise may lead to a retardation of development that may even become irrevocable under certain conditions, too much exercise or the wrong type of exercise will have deleterious effects too (Barneveld and van Weeren 1999, Brama *et al.* 2000c, Brama *et al.* 2002a). There seems to be a delicate balance representing an optimum that has not been well established yet. This situation makes it so far impossible to answer the question whether training should start earlier rather than later, and forms the drive behind training experiments in young animals that seek to condition the

musculoskeletal system by exercise and aim at increasing injury resistance (McIlwraith 2000, Smith *et al.* 1999). The effect of exercise on the musculoskeletal system in young individuals can only be assessed if sufficient knowledge exists about normal, not manipulated development, *i.e.* situations where there is no imposed additional exercise, or any suppression of exercise.
Studies on the normal age-related development of articular cartilage in the horse have been numerous in recent years (see van Weeren and Brama 2003 for review), but work on the bony layers of the bearing surface of the joint have been limited to the subchondral bone plate only and were limited in both number of biochemical parameters and age range as they did not include animals older than one year of age (Brama *et al.* 2001b, 2002b). In cartilage it has been shown that no major changes occur to the collagen network after that age (Brama *et al.* 2002a), but the bony skeleton is known not to be mature before the age of approximately four years when the last growth plates of the appendicular skeleton close (Kainer 2002). The present study therefore aims at the determination of changes in the biochemical composition of the extracellular matrices of all three constituents of the bearing surface of the joint, *i.e.* articular cartilage, subchondral bone and trabecular bone, in the age range 6 months – 4 years. The hypothesis tested was that in all layers developmental changes can be found, but that they may follow a different timescale. Attention was focused on those elements of the ECM that are known to contribute directly to the biomechanical strength of the tissues, *i.e.* collagen (cartilage and bone), and minerals (bone only). To this end, the absolute content and the rates of change in a number of collagen variables, including cross-links, in all three layers and in the two bone layers also bone mineral density and the mineral components, were determined at two differently loaded sites in a group of horses belonging to the age category of 6 months to 4 years and compared to those from mature horses (4 years and older).

Materials and methods

Joints
Forty-three right metacarpophalangeal joints from Warmblood slaughter horses were used from a larger batch of joints that had been harvested immediately after death and stored at -20 °C until processing. One day before the measurements, the joints were thawed at room temperature and opened. The proximal two thirds of the first phalanx were isolated from the rest of the limb and the surrounding tissue was dissected. For this study joints were used that were normal at macroscopic inspection and had no evidence of any pathology related to the area. Articular cartilage degeneration was quantified with help of the Cartilage Degeneration Index (CDI) (Brommer *et al.* 2003a) and only joints with a CDI score of < 25% were included. The joints were divided into two groups, one including animals younger than 4 years (n=13; mean age 2.4 years; range 6 months- 4

years), the second animals older than 4 years (n=30; mean age 10.5 years; range 5-18 years).

Sampling procedure
After establishing the CDI, a 6 mm wide slice of phalanx I, containing cartilage, subchondral bone and trabecular bone was cut in dorsopalmar direction, perpendicular to the articular surface and through the centre of the medial fovea, using a band saw. These slices contained two differently loaded sites: site 1 located close to the dorsal articular margin, and site 2 at the central fovea (Fig. 1). A slice of 1-2 mm thickness was cut off, placed in Burckhardt's fixative for 4 days and thereafter kept in 100% ethanol until measurement of bone mineral density (BMD). From the remaining sample the cartilage was taken off with a scalpel and the subchondral and trabecular bone were separated with a milling cutter. Each bone and cartilage sample was further divided into 4 pieces and stored at - 80 °C until further analysis.

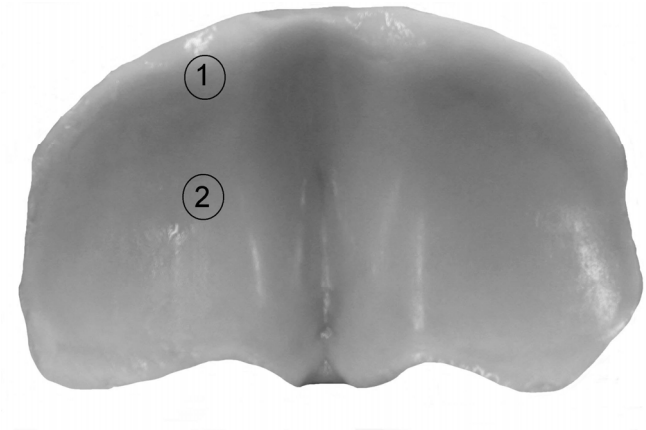

Fig 1: Sample sites on the proximal articular surface of the first phalanx. (1 = mediodorsal articular margin; 2 = central fovea)

Determination of Bone Mineral Density
Bone Mineral Density (BMD) was measured with a peripheral quantitative computed tomography (pQCT) machine (Stratec XCT 960A). Two 360 degrees X-ray scans were taken with a thickness of 1 mm. The pQCT machine was calibrated with a standard of hydroxyapatite embedded in acrylic plastic.
The scans were performed in two horizontal planes: at 2 mm under the cartilage layer through the subchondral plate and at a depth of 8 mm through the trabecular bone. BMD was expressed as mg/cm^3.

Mineral analysis
One piece of subchondral- and trabecular bone of each site was thawed and defatted by placing in ether for 1 week. After drying for 1 hour at 105 °C dry weight was determined. The drying time of 1 hour proved to be sufficient in a test series where various samples were dried and subsequently weighed after drying times up to 24 hours (data not shown). The fat-free, dry samples were ashed (540 °C for 6 hours), weighed and then dissolved in 15 ml 4 M hydrochloric acid. Calcium and magnesium content were determined by atomic absorption spectrophotometry (Perkin Elmer 3300). Phosphorus content was determined according to the method of Quinlan and Desesa (1955). The amount of minerals was expressed as mmol per gram dry bone weight. The ash concentration was expressed as percent of dry bone weight.

Crosslink and amino acid analysis
One piece of subchondral and trabecular bone and cartilage of each site was thawed and bone samples were demineralised for three weeks in buffered 0.5 M ethylene diamino tetra-acetic acid (EDTA, pH 7.4). The bone and cartilage samples (100-200 μg dw) were hydrolysed (110 °C, 20-24 h) with 800 μL 6 M HCl, dried in a Speed Vac (Savant SC 110) and dissolved in 500 μL (bone) or 200 μL (cartilage) water containing 10 μM pyridoxine (internal standard for the cross-links analysis (Sigma)) and 2.4 mM homoarginine (internal standard for the amino acid analysis (Sigma)). Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid (HFBA (Fluka)) in 10 % (vol/vol) acetonitrile (Rathburn Chemicals) for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M borate buffer pH 8.0 (0.1 M boric acid adjusted to pH 8.0 with 5 M sodium hydroxide) for amino acid analysis.
Reversed-phase high-performance liquid chromatography of cross-links (100 μL of the 5-fold diluted sample) was performed on a Micropak ODS-80 TM column (TOSOH biosep). The elution conditions were as described elsewhere (Bank *et al.* 1996, Bank *et al.* 1998). HP and LP purified from adult human bone and calibrated versus the PYD/DPD HPLC calibrator and pentosidine (calibrated by mass spectroscopy) served as a standard (Bank *et al.* 1997a).
For amino acid analysis 200 μL of the 250-fold diluted sample was derivatised at room temperature with 200 μL acetone containing 6 μM 9-fluorenylmethyl-chloroformate (FMOC-Cl (Fluka)). Termination of the derivatisation reaction, removal of excess reagent, and chromatography were performed as described elsewhere (Bank *et al.* 1996). Calibration was performed with an amino acid standard for collagen hydrolysates (Bank *et al.* 1996, Bank *et al.* 1998). The quantities of cross-links as well as hydroxylysine (Hyl) were expressed as number of residues per collagen molecule, assuming 300 hydroxyproline (Hyp) residues per triple helix (mol/mol collagen).

Analysis of denatured collagen
The amount of denatured collagen was determined by the assay described by Bank *et al.* (1997b), which is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the triple helix. Briefly, one piece of cartilage, subchondral and trabecular bone of each site was thawed and bone samples were demineralised for three weeks in buffered 0.5 M EDTA (pH 7.4). Bone and cartilage were 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 bone and 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 as a measure for total collagen. The amount of denatured collagen was expressed as a percentage of total collagen.

Statistical analysis
For each site in the two age groups the mean and the standard error of the mean (s.e.m.) of all parameters were calculated. Since age effects within each age group could be expected, differences between the groups were tested non-parametrically using the Kruskal-Wallis test. When a statistical difference in a parameter was found between the age groups, the linear regression curve of each age group was calculated and compared to each other using F-statistics. Correlation was tested using a Pearson's product moment correlation analysis. Statistical significance was considered when $P<0.05$. Statistical analysis of the data was performed with help of the software package Graph Pad Prism (GraphPad Software, Inc.).

Results

For the collagen parameters of the cartilage layer there are few differences between horses less than 4 years of age compared with those older than 4 years (Table 1). There is a significant increase in LP cross-links at site 1 only, all other parameters do not show a change except for pentosidine levels at both sites. The latter increase was expected, as pentosidine cross-links are a product of the process of non-enzymatic glycation that is merely time-dependent and goes on regardless of the age of the animal. Pentosidine cross-link levels also increased at both sites in the subchondral bone, and in trabecular bone (Table 2). In both bony layers the vast majority of the collagen parameters do not show any change too, as in cartilage (Table 2). In subchondral bone the only change is an increase in

denatured collagen at site 2. In trabecular bone hydroxylysine levels fall at site 1, as do hydroxyproline levels at site 2.

Table 1 - Collagen parameters of two differently loaded sites (joint margin; site 1 and central fovea; site 2) in the articular cartilage layer (mean ± s.e.m.). Values are given for respectively the age category 6 months - 4 years (≤ 4 years) and 4 years and older (> 4 years). n = number of samples. Hyl= hydroxylysine; Hyp= hydroxyproline; LP= lysylpyridinoline cross-links; HP= hydroxylysylpyridinoline cross-links; pent= pentosidine cross-links; den= denatured; coll= collagen; ns= not significant.

	CARTILAGE		
Collagen parameters variable and site	**≤ 4 years mean ± s.e.m. (n)**	**> 4 years mean ± s.e.m. (n)**	**p-level**
Hyl (mol/mol coll) (site1)	35.58 ± 2.66 (13)	40.76 ± 2.38 (30)	ns
Hyl (mol/mol coll) (site 2)	53.00 ± 1.23 (11)	52.73 ± 1.03 (27)	ns
Hyp (mol/mol) (site 1)	0.70 ± 0.02 (13)	0.67± 0.01 (30)	ns
Hyp (mol/mol) (site 2)	0.66 ± 0.03 (11)	0.68 ± 0.01 (27)	ns
HP (mmol/mol coll) (site1)	606.30 ± 69.18 (11)	637.58 ± 49.58 (30)	ns
HP (mmol/mol coll) (site 2)	967.04 ± 61.77 (11)	853.58 ± 20.42 (27)	ns
LP (mmol/mol coll) (site1)	10.78 ± 1.63 (12)	26.80 ± 3.40 (30)	**<0.001**
LP (mmol/mol coll) (site 2)	12.23 ± 1.64 (11)	12.39 ± 0.63 (23)	ns
Pent (mmol/mol) (site1)	0.23 ± 0.03 (12)	0.49 ± 0.05 (29)	**<0.001**
Pent (mmol/mol) (site 2)	0.25 ± 0.04 (11)	0.42 ± 0.04 (26)	**<0.05**
Den collagen (%) (site1)	16.86 ± 2.93 (13)	15.28 ± 1.22 (30)	ns
Den collagen (%) (site 2)	11.42 ± 1.46 (10)	11.73 ± 0.82 (27)	ns

For the mineral components of both subchondral bone and trabecular bone the situation is entirely different. Bone mineral density, ash and calcium content are all significantly higher in the mature group compared to the juvenile horse group. The opposite is seen for magnesium, which is significantly higher in the juvenile horse group (except for site 2 of trabecular bone). Phosphorus content is only significantly different in trabecular bone (Table 2).

Table 2 - Collagen and mineral parameters of two differently loaded sites (joint margin; site 1 and central fovea; site 2) in the layers of subchondral bone and trabecular bone (mean ± s.e.m.). Values are given for respectively the age category 6 months - 4 years (**≤** 4 years) and 4 years and older (> 4 years). n = number of samples. Hyl= hydroxylysine; Hyp= hydroxyproline; LP= lysylpyridinoline cross-links; HP= hydroxylysylpyridinoline cross-links; pent= pentosidine cross-links; den= denatured; coll= collagen; BMD= bone mineral density; ns= not significant.

	SUBCHONDRAL BONE			**TRABECULAR BONE**		
Variable and site	**≤ 4 years mean ± s.e.m. (n)**	**> 4 years mean ± s.e.m. (n)**	**p-level**	**≤ 4 years mean ± s.e.m. (n)**	**> 4 years mean ± s.e.m. (n)**	**p-level**
Collagen parameters						
Hyl (mol/mol coll) (site1)	16.31 ± 0.47 (13)	17.74 ± 0.65 (30)	ns	14.24 ± 0.38 (13)	12.89 ± 0.40 (30)	**<0.05**
Hyl (mol/mol coll) (site 2)	17.99 ± 1.16 (13)	20.08 ± 0.44 (30)	ns	12.44 ± 0.39 (13)	12.96 ± 0.32 (30)	ns
Hyp (mol/mol) (site 1)	0.64 ± 0.006 (13)	0.64 ± 0.005 (30)	ns	0.67 ± 0.008 (13)	0.67 ± 0.004 (30)	ns
Hyp (mol/mol) (site 2)	0.68 ± 0.02 (13)	0.67 ± 0.004 (30)	ns	0.71 ± 0.006 (13)	0.69 ± 0.003 (30)	**<0.01**
HP (mmol/mol coll) (site1)	159.53 ± 11.15 (12)	201.61 ± 15.54 (23)	ns	134.16 ± 7.11 (13)	123.62 ± 3.88 (30)	ns
HP (mmol/mol coll) (site 2)	277.87 ± 18.01 (13)	322.06 ± 10.61 (29)	ns	150.41 ± 7.19 (13)	148.59 ± 5.14 (30)	ns
LP (mmol/mol coll) (site1)	82.79 ± 7.88 (9)	67.31 ± 8.75 (20)	ns	104.04 ± 8.14 (13)	90.44 ± 3.49 (29)	ns
LP (mmol/mol coll) (site 2)	39.51 ± 2.34 (12)	47.83 ± 2.53 (29)	ns	98.81 ± 7.33 (13)	64.65 ± 5.50 (30)	ns
Pent (mmol/mol) (site1)	0.11 ± 0.03 (9)	0.21 ± 0.02 (19)	**<0.01**	0.08 ± 0.01 (13)	0.16 ± 0.01 (30)	**<0.001**
Pent (mmol/mol) (site 2)	0.08 ± 0.01 (13)	0.14 ± 0.01 (29)	**<0.001**	0.07 ± 0.01 (12)	0.14 ± 0.01 (30)	**<0.001**
Den collagen (%) (site1)	10.06 ± 0.66 (13)	11.38 ± 0.46 (30)	ns	12.31 ± 0.64 (12)	12.65 ± 0.57 (30)	ns
Den collagen (%) (site 2)	9.88 ± 0.34 (13)	11.87 ± 0.47 (30)	**<0.01**	11.94 ± 0.54 (12)	14.04 ± 0.74 (30)	ns
Mineral parameters						
BMD (mg/cm^3) (site 1)	829 ± 43 (11)	990 ± 14 (30)	**<0.001**	733 ± 47 (11)	875 ± 25 (30)	**<0.05**
BMD (mg/cm^3) (site 2)	888 ± 44 (11)	1072 ± 11 (29)	**<0.001**	581 ± 34 (11)	707 ± 28 (29)	**<0.05**
Ash (%) (site 1)	54.89 ± 0.71 (13)	58.17 ± 0.27 (28)	**<0.001**	58.84 ± 0.51 (13)	61.67 ± 0.25 (28)	**<0.001**
Ash (%) (site 2)	57.88 ± 0.57 (13)	60.29 ± 0.45 (29)	**<0.01**	60.28 ± 0.58 (13)	62.55 ± 0.28 (29)	**<0.01**
Calcium (mmol/gr dw) (site 1)	5.33 ± 0.07 (13)	5.58 ± 0.04 (29)	**<0.01**	5.60 ± 0.08 (13)	5.93 ± 0.03 (29)	**<0.001**
Calcium (mmol/gr dw) (site 2)	5.59 ± 0.05 (13)	5.80 ± 0.04 (29)	**<0.01**	5.67 ± 0.09 (13)	6.07 ± 0.07 (30)	**<0.001**
Magnesium (mmol/gr dw) (site 1)	0.09 ± 0.002 (13)	0.07 ± 0.001 (29)	**<0.001**	0.09 ± 0.002 (13)	0.08 ± 0.001 (30)	**<0.001**
Magnesium (mmol/gr dw) (site 2)	0.09 ± 0.002 (13)	0.08 ± 0.001 (29)	**<0.001**	0.09 ± 0.002 (13)	0.09 ± 0.002 (30)	ns
Phosphorus (mmol/gr dw) (site 1)	2.94 ± 0.03 (13)	2.97 ± 0.01 (28)	ns	3.04 ± 0.02 (12)	3.17 ± 0.02 (30)	**<0.001**
Phosphorus (mmol/gr dw) (site 2)	3.06 ± 0.02 (13)	3.12 ± 0.02 (29)	ns	3.18 ± 0.03 (13)	3.29 ± 0.04 (30)	**<0.01**

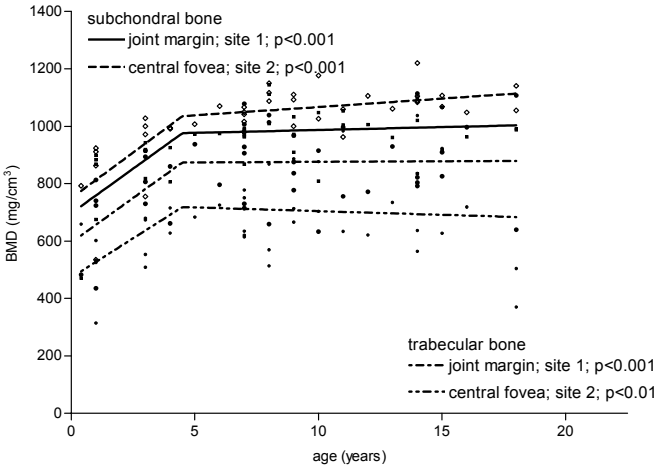

Fig. 2: Bone mineral density (BMD; in mg/cm^3) in relation to age at sites 1 and 2 for subchondral bone and trabecular bone. The p-value relates to the difference in slope (*i.e.* rate of change) of the curves for the juvenile (≤ 4 years) and mature (> 4 years) horses.

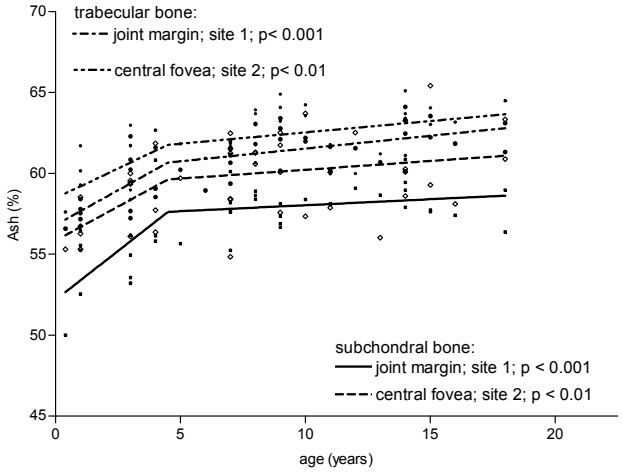

Fig. 3: Ash (%) in relation to age at sites 1 and 2 for subchondral bone and trabecular bone. The p-value relates to the difference in slope (*i.e.* rate of change) of the curves for the juvenile (≤ 4 years) and mature (> 4 years) horses.

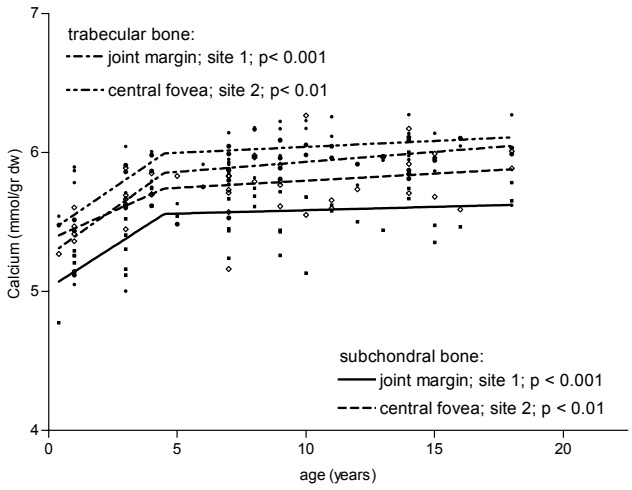

Fig. 4: Calcium content (mmol/gr dw) in relation to age at sites 1 and 2 for subchondral bone and trabecular bone. The p-value relates to the difference in slope (*i.e.* rate of change) of the curves for the juvenile (≤ 4 years) and mature (> 4 years) horses. dw = dry weight.

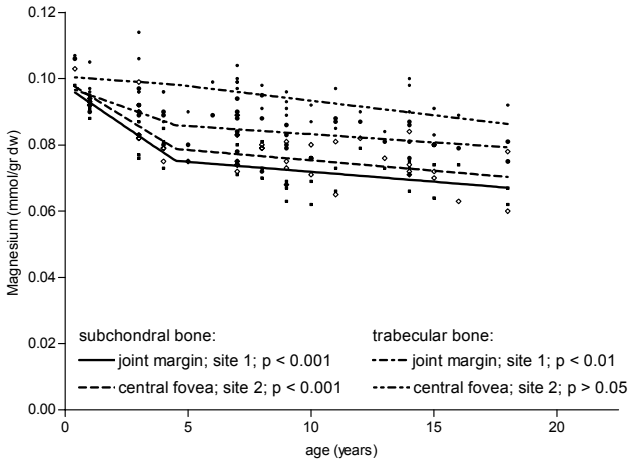

Fig. 5: Magnesium content (mmol/gr dw) in relation to age at sites 1 and 2 for subchondral bone and trabecular bone. The p-value relates to the difference in slope (*i.e.* rate of change) of the curves for the juvenile (≤ 4 years) and mature (> 4 years) horses. dw = dry weight.

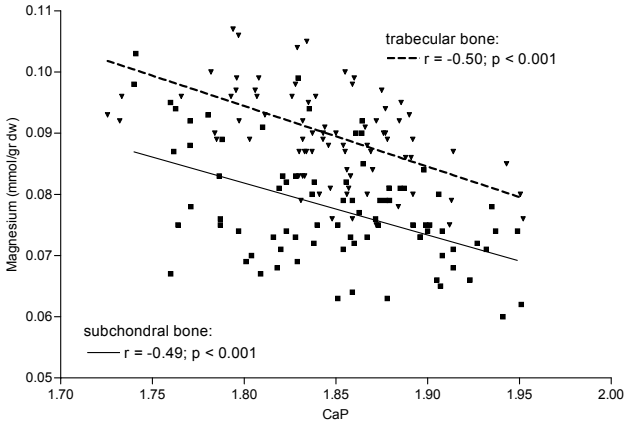

Fig. 6: Correlation between magnesium content (mmol/gr dw) and the calcium-phosphorus ratio (CaP) using data from both age categories and both sample sites combined in subchondral bone and trabecular bone. dw = dry weight; p = significance level; r = correlation coefficient.

Figures 2-5 visualise the results graphically by comparing the individual curves of each age group to a curve of the combined data using F-statistics. No consistent curve differences (juvenile compared to adult group) were seen in all collagen-related parameters of cartilage, subchondral and trabecular bone (data not shown), but the mineral parameters of the bone layers show significantly different slopes for the juvenile group compared to the mature animals. This is true for BMD in subchondral and trabecular bone (Fig. 2), but also for ash content and for the individual mineral parameter calcium, which is the main mineral component (Figs. 3-4). The patterns are basically the same for both sites 1 and 2. Magnesium shows an inverse pattern with a rather sharp decrease in the juvenile horses, followed by a significantly less rapid decrease in the mature horses (Fig. 5). For phosphorus, there is a significant difference in rate of change of the content only at site 2 in trabecular bone, where there is a change from an increase in the juvenile animals to a steady state in the older ones. This, however, is not the case at the other site or in subchondral bone (Table 2).
When the Ca/P ratio is plotted against the magnesium content using data from both age categories and from sites 1 and 2, significant and almost identical correlations emerge between both variables in both subchondral and trabecular bone (Fig. 6).

Discussion

Collagen parameters of the extracellular matrix (ECM) of the cartilage layer showed no differences between the juvenile and the mature horses. This finding was not unexpected as it confirms earlier work in which it was shown that the typical topographic heterogeneity in biochemical composition of the ECM, as can be found in mature individuals for the larger part originates in the first 6 months of life (Brama *et al.* 2000a, Brama *et al.* 2002a). Only HP cross-links developed a little later, but by the end of the first year the collagen network has been largely formed in the so-called process of functional adaptation (Brama *et al.* 2002a). This process is supposed to be initiated and maintained by biomechanical loading and can thus to a certain extent be compared to the well-known law of Wolff as first formulated for trabecular bone (Wolff 1892). Of the animals in the young age category, one was 6 months old, all others were one year of age or older and no major changes to the collagen network of articular cartilage could therefore be expected. Indeed, the topographical differences that were found in the mature animals were consistent with those reported earlier (Brama *et al.* 2000b) and were present in the juvenile animals as well, except for a small difference in LP cross-links, a parameter that had not been studied *in extenso* before.
It is interesting to note that apparently the same holds for most of the measured parameters of the collagen skeleton of the subchondral and trabecular bone. In subchondral bone there is only an increase in denatured collagen in the older age class in site 2. It is the question, however, to what extent this can be seen as part of a continuing maturation process, or as a sign of increased degenerative changes in the older animals. Although none of the animals had visible cartilage damage and CDI levels were below the threshold level of 25% in all cases, some increase in degenerative changes at molecular level with increasing age is not improbable. All other collagen-related parameters, including the hydroxypyridinum cross-links, remained at a similar level. In trabecular bone there were differences in hydroxylysine level at site 1 only, and in hydroxyproline at site 2 only, but here too all other collagen parameters did not change, including the cross-links. Although it is evident that there are major differences between the collagen networks of cartilage and bone with respect to collagen type and structural arrangement, the conclusion seems therefore justified that also in bone no major changes in the composition of the collagen (with respect to the parameters that were examined) take place in the young-adult animal. It seems that in the horse the collagen networks of all three constituents of the bearing surface of the joint have attained a mature composition at an age of approximately one year. Some restrictions must be made, however. The results of this study concern biochemical composition only and no statement can be made about ultrastructural aspects, that may be of critical importance for tissue strength as well. Further, the similarity between the tissues leaves alone the difference that the collagen part of the ECM of articular cartilage, once formed, is known to have a very limited turnover rate that is the main obstacle

for repair, whereas bone retains a good remodelling capacity throughout life, thus permitting complete functional and anatomical fracture healing (Cornell and Lane 1992, Maroudas *et al.* 1992, Verzijl *et al.* 2000).
Maturity in tissue composition is not yet reached in the young age category with respect to mineral contents. Mineralisation is clearly still ongoing in the animals aged 4 years and under, which is exemplified by the large increase in BMD and the concomitant and related increase in ash content and Ca content and the decrease in Mg content. Of magnesium it is known that in calcified tissues the amount associated to the apatitic phase is higher at the beginning of the calcification process, and then gradually decreases (Bigi *et al.* 1992, Bigi *et al.* 1997). This latter process is also illustrated by the negative correlation in both subchondral bone and trabecular bone between Ca/P ratio and magnesium content in the young age group.
The disparity in development rate of the two main constituents of the ECM of bone, with collagen preceding mineralisation, is in line with the initiating and regulating role that the collagen network has been reported to have with respect to the process of mineralisation (Knott and Bailey 1998, Wassen *et al.* 2000). It has been speculated that the post-translational modifications of the collagen network play a key role in the regulation of mineralisation (Knott and Bailey 1998).
The two sites were chosen because they are known to have very different loading patterns. The site at the dorsal articular margin (site 1) of the joint is subject to intermittent peak loads while the central fovea (site 2) is continually loaded as long as the animal is on its feet, but to a lesser extent (Brama *el al.* 2001a). It has been shown in earlier work that these loading differences are not only related to differences in cartilage extracellular matrix composition (Brama *et* al. 2000b), but to differences in the underlying bony layers as well, thus stressing the concept of the bearing surface of the joint as a functional entity (Harst *et al.* 2004). It is interesting to note in this study that the differences in time frame of the maturation processes of the collagen part of the ECM and of the mineral components were largely the same for both sites that were investigated. This leads to the conclusion that, although at differently loaded sites the end result of the process of functional adaptation of extracellular matrix components to biomechanical influences is different, the speed at which this process takes place apparently is the same.
The mineral content and the collagen network are both important determinants of the mechanical properties of the tissue (Knott *et al.* 1995, Oxlund *et al.* 1995). Mature bone consists for more than 50% of minerals and these are evidently of great importance for ultimate strength. It can therefore be argued that horses do not reach full skeletal maturity before the age of approximately 4 years, *i.e.* around the time the last growth plates of the appendicular skeleton close (Kainer 2002). This means that in those branches of equestrian sports where (very) early training is the rule, tissues will be loaded that have less than optimal strength due to differences in the levels of maturity of their matrix constituents. It can be speculated that the impact of (heavy) loading on these structures and the potential

for provoking damage are different too. Although this study did not include samples of the cortices of long bones, it is possible that similar differences in bone maturation exist there also, which might give an explanation why early heavy training in young Thoroughbred almost invariably results in a high incidence of sore shins, whereas sever cartilage damage in this age category is seen much less frequently (Mason and Bourke 1973, More 1999).
The somewhat unexpected finding that, as in cartilage, the composition of the collagen matrix in bone does not alter after the age of one year raises the question to what extent this is a general developmental phenomenon that could apply to other structures as well that are made up of collagen type I, such as tendons and ligaments. The significance of such a mechanism would be greater for those structures where the remodelling capacity is known to be limited due to low metabolic rates in adult animals, or unfavourable conditions for healing like in flexor tendons. The answer to this question is beyond the scope of this study, which was limited in type of samples (bearing surface joints only) and age classes (no animals in the 0-6 months age category).
It is concluded that great similarities exist in the time path of maturation of the collagen part of the matrix of all three layers that make up the bearing surface of the joint: articular cartilage, subchondral bone and trabecular bone. No essential alterations in post-translational modifications of collagen will take place after the age of approximately one year. However, the mineralisation process of both the subchondral bone and the underlying trabecular bone does not reach its final, mature state before the age of approximately 4 years. The disparity in maturity of the various ECM components can be assumed to have consequences for the loading capacity of the tissue and hence for the impact of training and racing.

Acknowledgements

The authors would like to express their gratitude to Ms M.I.J.A. Pollak, Mr A. Klarenbeek and H.S. Wouterse for their help with the preparation and processing of the samples. We also would like to thank dr. A.G.H. Ederveen and Mr C. Spanjers of the Department of Pharmacology of N.V. Organon (Oss, the Netherlands) for their indispensable help in measuring BMD.

CHAPTER IV

AN INTEGRAL BIOCHEMICAL ANALYSIS OF ARTICULAR CARTILAGE, SUBCHONDRAL- AND TRABECULAR BONE OF THE EQUINE METACARPOPHALANGEAL JOINT WITH EARLY OSTEOARTHRITIS

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(American Journal Veterinary Research: Accepted)

Abstract

Objective: To test the hypothesis that in early osteoarthritis (OA) changes can be found in all 3 layers (cartilage, subchondral- and trabecular bone) that constitute the bearing surface of the joint.
Sample population: Right metacarpophalangeal joints from 59 mature Warmblood horses.
Procedure: From two differently loaded sites of phalanx I cartilage, subchondral- and trabecular bone samples were collected. Biochemical parameters (crosslink content, amino acid content, denatured collagen, glycosaminoglycans (GAG), DNA, bone mineral density, ash content and mineral composition) were determined.
Results: In cartilage GAG was lower in the OA-group and there was a loss of site differences in cellularity and LP crosslinks. In subchondral bone cross-links were lower in the OA-group, with also a loss of site differences in LP crosslinks. Subchondral ash content was higher in the OA-group. In trabecular bone hydroxyproline was higher in OA joints. In all three layers and at both sites the linear increase with age of the pentosidine cross-links had diminished or was lost in the OA group.
Conclusions: In early OA effects can be seen in all 3 layers, but are of a distinct character. In the bony layers the collagen network is principally affected with only minor changes in the mineralisation parameters. The dissimilarity in response of the different tissues and between the sites that are affected may be related to differences in biomechanical loading and transmission and dissipation of force.
Clinical relevance: The concept of functional interrelationship of the tissue layers that make up the bearing surface of a joint is supported. The observation that in early OA besides in cartilage also changes were found in the bone layers might give a clue as to where OA-related changes start.

Introduction

The capacity of the horse to perform successfully depends on its inherent athletic ability and on the capacity to keep free from diseases and conditions that interfere with soundness. Lameness is the most important cause for wastage in various populations of horses (Olivier *et al.* 1997, Rossdale *et al.* 1982). Joint diseases are the most important single cause of lameness and osteoarthritis (OA) is in the horse, as in man, by far the most important joint ailment in mature individuals (Burstein *et al.* 1975, Lee *et al.* 1974, Todhunter 1996a).
Osteoarthritis is classically considered an inherently non-inflammatory disorder of movable joints characterised by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins (Hough 1993). Although recent debate strongly questions the non-inflammatory character of the disease,

there is no doubt that the disorder affects both cartilage and underlying bone, at least in its later stages (Attur *et al.* 2002). There is less consensus about the initial phase of the disease. Several reports claim that sclerosis of the subchondral bone plate precedes articular cartilage damage (Burr 1998, Carlson *et al.* 1994, Dequeker *et al.* 1995, Radin *et al.* 1984, Radin and Rose 1986, Radin *et al.* 1991). In this scenario, repetitive impulse loading leads to repeated micro-trauma and consequently to sclerosis and thus stiffening of the subchondral bone, followed by cartilage thinning and degeneration (Burr and Schaffler 1997). In this same line, Mansell and Bailey (1998) reported that bone collagen metabolism was increased in osteoarthritic femoral heads with greatest changes in the subchondral bone. However, others report degradation of articular cartilage typical of early OA without any signs of pathological changes in the underlying subchondral bone (Dedrick *et al.* 1993, Donohue *et al.* 1983, Yamada *et al.* 2002). This observation led to the opposing view that the disease begins in the articular cartilage, possibly prompted by inflammatory mediators that induce primary biochemical changes (Lee *et al.* 1974, Mankin and Brandt 1992, Mow *et al.* 1992, Stephens *et al.* 1979, Wei *et al.* 2001). The question whether subchondral bone change occurs before cartilage deterioration or subsequent to it has hence not yet been resolved (Adolphson *et al.* 1994, Bailey and Mansell 1997, Burr and Schaffler 1997, Muehleman *et al* 2002, Oegema *et al.* 1997). Indications for an answer to this question can only be obtained from studies focusing at the very early stages of OA.

In recent years, much research has been performed on the molecular composition of the extracellular matrix (ECM) of articular cartilage in the equine metacarpophalangeal joint. From this research it emerged that there are distinct site differences in biochemical composition, matching with the variation in biomechanical loading as generated by locomotion (Brama *et al.* 2000b, Brama *et al.* 2001a). Similar research into the composition of the subchondral bone plate in relation to loading in other species has shown comparable site differences (Armstrong *et al.* 1995, Kiviranta *et al.* 1987, Oettmeier *et al.* 1992). In the horse, research has been limited so far. Brama and co-workers (2001b, 2002b) demonstrated that there were site differences in the subchondral bone plate of the proximal articular surface of the equine first phalanx in a limited panel of matrix constituents (collagen, water and calcium content). A relationship between biochemical composition of cartilage and subchondral bone and proneness to injury was suggested (Brama *et al.* 2001b). Recently, a first report was presented of a comprehensive study that simultaneously analysed the composition of articular cartilage and underlying subchondral and trabecular bone at the proximal articular surface of the equine first phalanx from sound animals (Harst *et al.* 2004). From that study, that envisaged bone and cartilage as a single functional entity, it became clear that there are distinct site-related differences in the distribution of the major biochemical components in all 3 layers (cartilage, subchondral bone and trabecular bone), and that these differences showed to a certain extent similar patterns in all 3 layers.

In the present study the hypothesis is tested that in early OA changes will be found in the biochemical composition and the site differences of all 3 layers, but not in an identical fashion. In line with this, an additional purpose of this study was to verify if the character and severity of the pathological changes in these layers might give a clue as to where the primary damage originates. To this end, a comprehensive biochemical analysis was carried out of samples of the cartilage, subchondral bone and trabecular bone layers from 2 differently loaded sites in a series of mildly osteoarthritic joints and the outcome was compared to data collected earlier in normal joints.

Materials and methods

Joints
Right metacarpophalangeal joints from fifty-nine adult Warmblood slaughter horses (mean age 12.6 years, range 5-23 years) were harvested immediately after death and stored at -20 °C until processing. One day before the measurements, the joints were thawed and opened. The proximal two thirds of the first phalanx were isolated from the rest of the limb and the surrounding tissue was dissected. Articular cartilage degeneration was quantified with help of the Cartilage Degeneration Index (CDI) (Brommer *et al.* 2003a, Brommer *et al.* 2003b). Briefly, the amount of Indian ink uptake across the entire cartilage surface was quantified by digital imaging of the native and the Indian ink stained articular cartilage surfaces. The increase in mean grey level of the articular surface is the basis for calculation of the CDI (range from 0 to 100%). This procedure was performed for the entire joint, resulting in a general CDI, and for two specific differently loaded regions of interest: site 1 located close to the dorsal articular margin and site 2 at the central fovea, indicated as CDI_1 and CDI_2 respectively (Fig. 1). The joints were divided into two groups. The first group (n=30) had a CDI_1 <25%, which is indicative of none to minor degenerative changes, and has been used to generate normal values that have been published earlier (Harst *et al.* 2004). These data are used again in the present study and serve as controls. The samples from the second group (n=29) were analyzed for this study. These animals had CDI_1 values > 25%, with most of them in the lower ranges, making that the entire group could be classified as mildly osteoarthritic.

Sampling procedure
After establishing the CDI, a 6 mm wide slice of phalanx I, containing cartilage, subchondral bone and trabecular bone was cut in dorsopalmar direction, perpendicular to the articular surface and through the centre of the medial fovea, using a band saw. These slices contained both sites 1 and 2. A slice of 1-2 mm thickness was cut off, placed in Burckhardt's fixative for 4 days and thereafter kept in 100% ethanol until measurement of bone mineral density (BMD).

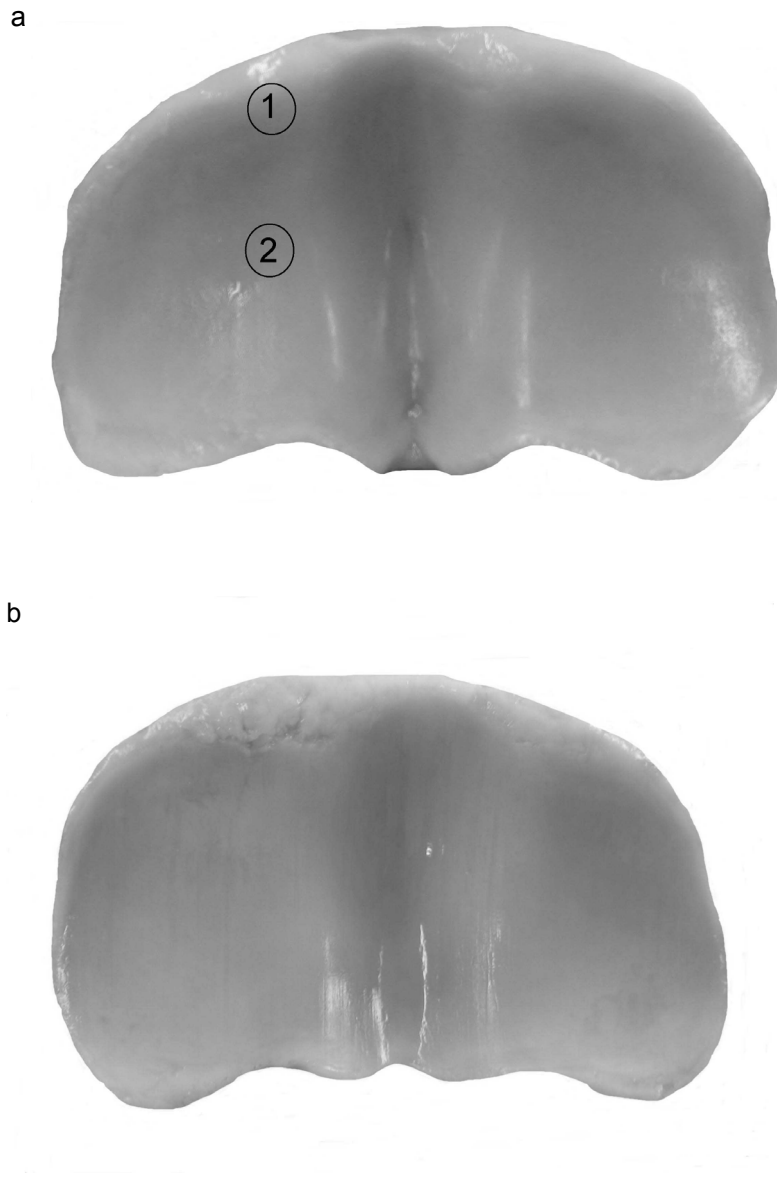

Fig. 1: Sample sites on the proximal articular surface of the first phalanx. (1 = mediodorsal articular margin; 2 = central fovea). Figure 1a articular surface without osteoarthritis (CDI: 8.8%; CDI_1: 19.5%); figure 1b articular surface with early osteoarthritis (CDI: 18.8%; CDI_1: 42.5 %), principally located at the joint margin (site 1).

From the remaining sample the cartilage was taken off with a scalpel and the subchondral and trabecular bone were separated with a milling cutter. Each bone and cartilage sample was further divided into 4 pieces and stored at - 80 °C until further analysis.

Determination of Bone Mineral Density
Bone Mineral Density (BMD) was measured with a peripheral quantitative computed tomography (pQCT) machine (Stratec XCT 960A) adapted for measuring small bones. Two 360 degrees X-ray scans were taken with a thickness of 1 mm and a resolution of 0.148 mm x 0.148 mm. The pQCT machine was calibrated with a standard of hydroxyapatite embedded in acrylic plastic. The scans were performed in two horizontal planes: at 2 mm under the cartilage layer through the subchondral plate and at a depth of 8 mm through the trabecular bone. BMD was expressed as mg/cm^3.

Mineral analysis
One piece of subchondral- and trabecular bone of each site was thawed and defatted by placing in ether for 1 week. After drying for 1 hour at 105 °C dry weight was determined. The drying time of 1 hour proved to be sufficient in a test series where various samples were dried and subsequently weighed after drying times up to 24 hours (data not shown). The fat-free, dry samples were ashed (540 °C for 6 hours), weighed and then dissolved in 15 ml 4 M hydrochloric acid. Calcium and magnesium content were determined by atomic absorption spectrophotometry (Perkin Elmer 3300). Phosphorus content was determined according to the method of Quinlan and DeSesa (1955). The amount of minerals was expressed as mmol per gram dry bone weight. The ash concentration was expressed as percent of dry bone weight.

Cross-link and amino acid analysis
One piece of subchondral and trabecular bone and cartilage of each site was thawed and bone samples were demineralised for three weeks in buffered 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 7.4). The bone and cartilage samples were hydrolysed (110 °C, 20-24 h) with 800 μL 6 M HCl, dried in a Speed Vac (Savant SC 110) and re-dissolved in 500 μL (bone) or 200 μL (cartilage) water containing 10 μM pyridoxine (internal standard for the cross-links analysis (Sigma)) and 2.4 mM homoarginine (internal standard for the amino acid analysis (Sigma)). Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid (HFBA (Fluka)) in 10% (vol/vol) acetonitrile (Rathburn Chemicals) for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M borate buffer pH 8.0 (0.1 M boric acid adjusted to pH 8.0 with 5 M sodium hydroxide) for amino acid analysis.
Reversed-phase high-performance liquid chromatography of cross-links (100 μL of the 5-fold diluted sample) was performed as described elsewhere (Bank *et al.*

1996, Bank *et al.* 1998). HP and LP purified from adult human bone and calibrated versus the PYD/DPD HPLC calibrator and pentosidine (calibrated by mass spectroscopy) served as a standard (Bank et al. 1997a).
For amino acid analysis, 200 μL of the 250-fold diluted sample was derivatised at room temperature with 200 μL acetone containing 6 μM 9-fluorenylmethyl-chloroformate (FMOC-Cl (Fluka)). Termination of the derivatisation reaction, removal of excess reagent, and HPLC analysis were performed as described elsewhere (Bank *et al.* 1996). Fluorescence was monitored at excitation 254nm and emission 630nm. Calibration was performed with an amino acid standard for collagen hydrolysates (Bank *et al.* 1996, Bank *et al.* 1998). The quantities of cross-links as well as hydroxylysine (Hyl) were expressed as number of residues per collagen molecule, assuming 300 hydroxyproline (Hyp) residues per triple helix.

Analysis of denatured collagen
The amount of denatured collagen was determined by the assay described by Bank *et al.*, which is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the triple helix (Bank *et al.* 1997b). Briefly, one piece of cartilage, subchondral and trabecular bone of each site was thawed and bone samples were demineralised for three weeks in a buffered 0.5 M EDTA (pH 7.4) solution. Bone and cartilage were 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 bone and 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.

Glycosaminoglycan analysis
Trabecular bone and cartilage samples were digested by papain (P3125 (Sigma)) 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 by measuring the amount of polysulphated GAG's in the papain digest of the samples by a modification of the 1,9-dimethylmethylene blue (DMMB (Sigma)) assay described by Farndale *et al.* (1986). To 10 µl diluted sample from papain digest, 10 µl 1% (w/v) bovine serum albumin (Sigma) and 200 µl of reagent (46 µM DMMB, 40 mM glycine and 42 mM NaCl adjusted to pH 3.0 with HCl) were added and after 30 min the absorbency at 525nm was measured. The assay was standardised with shark chondroitin sulphate (Sigma). GAG amount was expressed as µg/mg dw sample of cartilage respectively bone.

DNA analysis
A 10 µl aliquot of the papain digest was used to determine DNA content as a measure for the amount of cells using the fluorescent dye Hoechst 33528 as described by Kim *et al.* (1988). In short: dye solution was added to the papain digest of the bone and cartilage samples and measured immediately after mixing using a LS-2b (excitation at 365nm and emission at 460 nm). Calf thymus DNA (D-4764 (Sigma)) was used as reference. Results were expressed as µg DNA/mg dw.

Statistical analysis
All measured parameters were expressed as mean ± sd. Statistical analysis of the data was performed with help of the software package SPSS version 10 for Windows (SPSS Inc.). Differences between the specific sites or the OA and non-OA group were tested by use of a multivariate ANOVA (factors: age, site and cartilage degeneration index). When there was an interaction present between OA and site, a Tukey multiple group comparison was performed between each combination of sites (i.e. 1 and 2) and OA status (i.e. OA and non-OA), to identify the nature of this interaction. Correlation was tested using a Pearson's product moment correlation analysis. The level of significance was set at $p<0.05$.

Results

All collected data for articular cartilage, subchondral bone plate and trabecular bone are given in tables 1-3 as mean ± standard deviation (sd) for the animals that were classified as mildly osteoarthritic (designated as "osteoarthritic joints"), and for the reference animals that were judged to be free of OA ("non-osteoarthritic joints"). In the articular cartilage significant differences between the OA and the non-OA group were observed only for the GAG content, which was considerably less in the OA group than in the non-OA group (Table 1). If all animals are taken into account (with and without OA), there is a significant negative relationship between Cartilage Degeneration Index and GAG-content of site 1 (Fig. 2). There is a significant difference in the percentage of denatured collagen between sites 1 and 2 in both non-OA and OA groups, with a higher level at site 1 (Table 1). This difference increases, but this increase is not statistically significant. All site differences that existed between sites 1 and 2 in normal cartilage are also found in osteoarthritic cartilage, except for LP cross-links and DNA content. For these parameters there is an interaction with the occurrence of OA, resulting in a loss of site differences (Table 1). The linear increase with age of the pentosidine cross-links found in healthy cartilage could not be demonstrated in OA-affected joints. At both sites 1 and 2 this relationship has lost statistical significance (Table 4).
In the subchondral bone plate, osteoarthritic subchondral bone has lower hydroxylysine content. It also appears to have less HP cross-links per triple helix collagen than normal subchondral bone.

Table 1 - The measured collagen and mineral parameters of different sites in cartilage (mean ± sd) of normal and osteoarthritic equine proximal first phalanx. n = number of samples. P-values are not given for pentosidine as this parameter is influenced by age and age distributions in the OA and non-OA groups were not identical.

	CARTILAGE						
	Non-osteoarthritic joints		Osteoarthritic joints				
	Site 1	Site 2	Site 1	Site 2	Main statistical analysis		
	(mean ± sd) (n)	(mean ± sd) (n)	(mean ± sd) (n)	(mean ± sd) (n)	Site effect p-value	OA effect p-value	OA-site interaction
Hydroxylysine/collagen (mol/mol)	40.76 ± 13.02 (30)	52.73 ± 5.37 (27)	36.67 ± 11.90 (27)	50.46 ± 7.98 (28)	**<0.001**	ns	--
Hydroxyproline/proline (mol/mol)	0.67 ± 0.08 (30)	0.68 ± 0.07 (27)	0.68 ± 0.08 (28)	0.69 ± 0.09 (28)	ns	ns	--
HP/collagen (mmol/mol)	637.58 ± 271.57(30)	853.58 ± 106.08(27)	641.87 ± 295.99 (29)	815.38 ± 130.13 (27)	**<0.001**	ns	--
LP/collagen (mmol/mol)	26.80 ± 18.64 (30) *	12.39 ± 3.02 (23) #	26.63 ± 18.45 (29)	22.22 ± 13.77 (28)	n.a.	n.a.	**yes**
Denatured collagen (%)	15.28 ± 6.66 (30)	11.73 ± 4.24 (27)	16.94 ± 9.14 (27)	11.60 ± 5.16 (27)	**<0.05**	ns	--
GAG (µg/mg dw)	59.27 ± 26.91 (30)	124.05 ± 39.92 (30)	44.30 ± 29.17 (29)	113.32 ± 41.30 (29)	**<0.001**	**<0.05**	--
DNA (µg/mg dw)	2.39 ± 0.90 (30) §¶	1.63 ± 0.49 (29)	1.99 ± 0.83 (29)	1.73 ± 0.37 (29)	n.a.	n.a.	**yes**
GAG/DNA (µg/µg DNA)	28.31 ± 15.82 (30)	72.80 ± 17.10 (29)	26.23 ± 20.03 (29)	67.00 ± 22.50 (29)	**<0.001**	ns	--
Pentosidine/collagen (mmol/mol)	0.49 ± 0.28 (29)	0.42 ± 0.22 (26)	0.70 ± 0.49 (28)	0.47 ± 0.25 (28)	--	--	--

* = significant difference of LP between site-1 non-OA and site-2 non-OA (P<0.01)
# = significant difference of LP between site-2 non-OA and site-1 non-OA (P<0.01)
§ = significant difference of DNA between site-1 non-OA and site-2 non-OA (P<0.001)
¶ = significant difference of DNA between site-1 non-OA and site-2 OA (P<0.01)

Table 2 - The measured collagen and mineral parameters of different sites in subchondral bone (mean ± sd) of normal and osteoarthritic equine proximal first phalanx. n = number of samples. P-values are not given for pentosidine as this parameter is influenced by age and age distributions in the OA and non-OA groups were not identical.

	SUBCHONDRAL BONE						
	Non-osteoarthritic joints		**Osteoarthritic joints**				
	Site 1	**Site 2**	**Site 1**	**Site 2**	**Main statistical analysis**		
	(mean ± sd) (n)	**(mean ± sd) (n)**	**(mean ± sd) (n)**	**(mean ± sd) (n)**	**Site effect p-value**	**OA effect p-value**	**OA-site interaction**
Hydroxylysine/collagen (mol/mol)	17.74 ± 3.55 (30)	20.08 ± 2.39 (30)	16.20 ± 2.97 (29)	19.07 ± 3.10 (29)	**<0.001**	**<0.05**	--
Hydroxyproline/proline (mol/mol)	0.64 ± 0.03 (30)	0.67 ± 0.02 (30)	0.64 ± 0.03 (29)	0.67 ± 0.02 (29)	**<0.001**	ns	--
HP/collagen (mmol/mol)	201.61 ± 74.52 (23)	322.06 ± 57.16 (29)	159.79 ± 51.62 (24)	262.44 ± 95.09 (29)	**<0.001**	**<0.01**	--
LP/collagen (mmol/mol)	67.31 ± 39.14 (20)*	47.83 ± 13.63 (29)	49.53 ± 28.10 (23)	56.67 ± 24.81 (29)	n.a.	n.a.	**yes**
Denatured collagen (%)	11.38 ± 2.54 (30)	11.87 ± 2.58 (30)	11.34 ± 2.57 (29)	11.41 ± 1.92 (29)	ns	ns	--
BMD (mg/cm^3)	990 ± 75 (30)	1072 ± 59 (29)	992 ± 55 (28)	1066 ± 63 (28)	**<0.001**	ns	--
Ash (%)	58.17 ± 1.42 (28)	60.29 ± 2.42 (29)	59.43 ± 2.62 (29)	61.58 ± 1.83 (28)	**<0.001**	**<0.01**	--
Calcium (mmol/gr dw)	5.58 ± 0.20 (29)	5.80 ± 0.21 (29)	5.60 ± 0.29 (29)	5.83 ± 0.22 (29)	**<0.001**	ns	--
Phosphorus (mmol/gr dw)	2.97 ± 0.08 (28)	3.12 ± 0.11 (29)	3.02 ± 0.11 (28)	3.16 ± 0.12 (28)	**<0.001**	ns	--
Magnesium (mmol/gr dw)	0.07 ± 0.005 (29)	0.08 ± 0.006 (29)	0.07 ± 0.005 (29)	0.08 ± 0.007 (29)	**<0.001**	ns	--
Pentosidine/collagen (mmol/mol)	0.21 ± 0.10 (19)	0.14 ± 0.05 (29)	0.24 ± 0.09 (21)	0.17 ± 0.07 (29)	--	--	--

* = significant difference of LP between site-1 non-OA and site-2 non-OA ($P<0.05$)

Table 3 - The measured collagen and mineral parameters of different sites in trabecular bone (mean ± sd) of normal and osteoarthritic equine proximal first phalanx. n = number of samples. P-values are not given for pentosidine as this parameter is influenced by age and age distributions in the OA and non-OA groups were not identical.

	TRABECULAR BONE						
	Non-osteoarthritic joints		**Osteoarthritic joints**				
	Site 1	**Site 2**	**Site 1**	**Site 2**	**Main statistical analysis**		
	(mean ± sd) (n)	**(mean ± sd) (n)**	**(mean ± sd) (n)**	**(mean ± sd) (n)**	**Site effect p-value**	**OA effect p-value**	**OA-site interaction**
Hydroxylysine/collagen (mol/mol)	12.89 ± 2.17 (30)	12.96 ± 1.74 (30)	12.86 ± 1.61 (28)	11.83 ± 1.67 (28)	ns	ns	--
Hydroxyproline/proline (mol/mol)	0.67 ± 0.02 (30) *¶	0.69 ± 0.02 (30)	0.66 ± 0.02 (29) #	0.71 ± 0.03 (29)	n.a.	n.a.	**yes**
HP/collagen (mmol/mol)	123.62 ± 21.27 (30)	148.59 ± 28.16 (30)	120.88 ± 29.90 (29)	134.70 ± 30.90 (29)	**<0.001**	ns	**--**
LP/collagen (mmol/mol)	90.44 ± 18.77 (29)	64.65 ± 30.13 (30)	85.20 ± 26.47 (29)	87.26 ± 26.66 (29)	ns	ns	**--**
Denatured collagen (%)	12.65 ± 3.14 (30)	14.04 ± 4.05 (30)	12.93 ± 3.41 (27)	14.43 ± 2.88 (29)	**<0.01**	ns	**--**
GAG (µg/mg dw)	1.72 ± 0.40 (30)	1.69 ± 0.44 (29)	1.73 ± 0.35 (28)	1.61 ± 0.36 (29)	ns	ns	**--**
DNA (µg/mg dw)	0.95 ± 0.20 (30)	1.03 ± 0.15 (29)	1.03 ± 0.17 (28)	1.07 ± 0.18 (29)	ns	ns	**--**
GAG/DNA (µg/µg DNA)	1.84 ± 0.39 (30)	1.66 ± 0.39 (29)	1.72 ± 0.44 (28)	1.52 ± 0.33 (29)	**<0.05**	ns	**--**
BMD (mg/cm^3)	875 ± 135 (30)	707 ± 149 (29)	865 ± 164 (28)	703 ± 116 (28)	**<0.001**	ns	**--**
Ash (%)	61.67 ± 1.31 (28)	62.55 ± 1.48 (29)	61.67 ± 1.43 (28)	62.99 ± 1.51 (29)	**<0.001**	ns	**--**
Calcium (mmol/gr dw)	5.93 ± 0.16 (29)	6.07 ± 0.37 (30)	5.95 ± 0.12 (28)	6.07 ± 0.15 (29)	**<0.01**	ns	**--**
Phosphorus (mmol/gr dw)	3.17 ± 0.12 (30)	3.29 ± 0.21 (30)	3.19 ± 0.10 (29)	3.27 ± 0.07 (29)	**<0.001**	ns	**--**
Magnesium (mmol/gr dw)	0.08 ± 0.006 (30)	0.09 ± 0.008 (30)	0.08 ± 0.007 (29)	0.09 ± 0.006 (29)	**<0.001**	ns	**--**
Pentosidine/collagen (mmol/mol)	0.16 ± 0.06 (30)	0.14 ± 0.05 (30)	0.19 ± 0.08 (28)	0.15 ± 0.05 (29)	--	ns	--

* = significant difference of hydroxyproline between site-1 non-OA and site-2 non-OA (P<0.01)
¶ = significant difference of hydroxyproline between site-1 non-OA and site-2 OA (P<0.001)
# = significant difference of hydroxyproline between site-1 OA and site-2 OA (P<0.001)

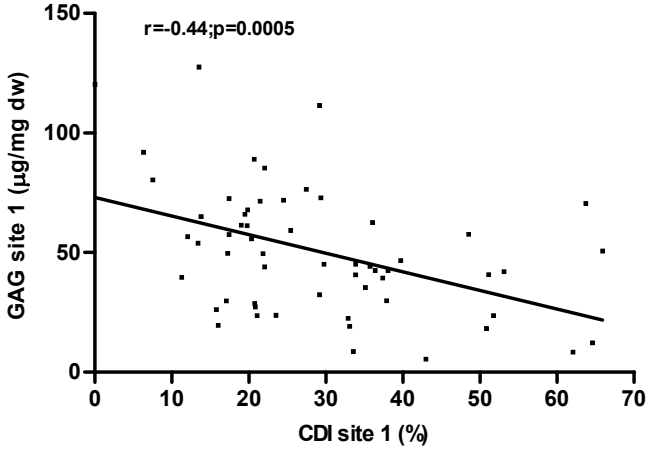

Fig. 2: Glycosaminoglycan content (GAG; µg/mg dw) of equine cartilage in relation to cartilage degeneration index (CDI) of joint margin, site 1. P = significance level; r = correlation coefficient. dw= dry weight.

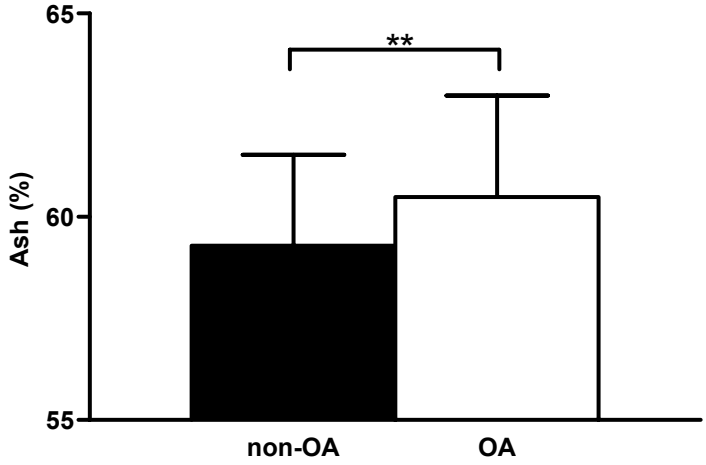

Fig. 3: Ash percentage (mean ± sd) of subchondral bone in samples from the non-osteoarthritic (non-OA) and osteoarthritic (OA) proximal first phalanx. As there was no significant interaction between site and occurrence of OA, sites 1 and 2 have been taken together. Asterisks denote significant difference between the non-OA and OA group; ** = P<0.01.

Table 4 - Pearson correlation coefficients (r) with age of pentosidine content in the three examined layers at both sites 1 and 2. There is a significant linear correlation with age at almost all sites in the normal individuals. In the OA-affected animals this correlation has decreased and has lost statistical significance in most cases, indicating a higher metabolic level.

Tissue type and site	Non-OA (r)	p-value	OA (r)	p-value
Cartilage site 1	**0.51**	**<0.01**	0.07	ns
Cartilage site 2	**0.46**	**<0.05**	0.31	ns
Subchondral bone site 1	0.43	ns	0.42	ns
Subchondral bone site 2	**0.73**	**<0.001**	0.4	**<0.05**
Trabecular bone site 1	**0.7**	**<0.001**	0.51	**<0.01**
Trabecular bone site 2	**0.6**	**<0.001**	0.19	ns

This applies to both sites 1 and 2 (Table 2). In this layer site differences (between site 1 and 2) have disappeared with respect to LP cross-links due to OA. The change in pentosidine content in relationship to age is not as clear as in the cartilage layer, but the increase with age at site 2 tends to be less in the OA-group than in the healthy animals (Table 4). There are no significant differences in any of the individual mineral-related parameters and BMD. However, ash content is significantly higher in the subchondral bone plate underlying cartilage affected by OA than in subchondral bone of normal cartilage (Table 2, Fig. 3).
In trabecular bone, there is an interaction between OA and site for hydroxyproline, causing a significant increase at site 2 for this parameter (Table 3). No other OA related changes are present in trabecular bone in neither the mineral nor the non-mineral parameters, except for pentosidine. The increase of pentosidine cross-links with age has become less strong at site 1 and has disappeared at site 2 (Table 4).

Discussion

Osteoarthritis is typically a chronic and insidious disease that may have affected large parts of the joint before becoming clinically manifest. For a better understanding of the pathogenesis of OA these well-established forms of the disease are of little value as secondary processes will have obscured or effaced any initial signs. In this study, a deliberate selection was made of joints that could be classified as very early osteoarthritic by setting the benchmark at CDI_1>25%. This meant a much lower CDI for the entire joint and a specific CDI for site 2 that was only slightly higher than zero in most cases, as in the horse OA is known to

start at site 1 and then spread gradually over the joint (Brommer *et al.* 2003b). No clinical data were available from this group of slaughter horses, but it can be assumed that most of them will not have shown clinical signs that could have been attributed to the joints examined.
Differences between OA and non-OA joints were expectedly few because of the very mild form that was opted for. Differences were, however, found in all 3 layers. In the cartilage layer there was a significant reduction in GAG-content in the osteoarthritic specimens and a loss of site differences for LP cross-links and DNA due to the effect of OA. In subchondral bone HP cross-links were considerably and significantly reduced at both sites 1 and 2 in OA joints and here the significant site 1/2 difference for LP cross-links disappeared too. In subchondral bone a reduced concentration of hydroxylysine is present in the OA group. Lower hydroxylysine content has been shown to be related to thicker and closer packing of the collagen fibrils (Miles *et al.* 2002, Mizuno *et al.* 2001, Notbohm *et al.* 1999). In another study, such closer packing was related to a higher degree of mineralisation (Li and Aspden 1997). In total this will lead to a denser, and probably more brittle tissue. As for every HP cross-link three hydroxylysine residues are needed, the lower hydroxylysine content may be related to lower degree of cross-linking as found in this study (Knott and Bailey 1998).
In trabecular bone there was a significant OA-related increase of the hydroxyproline to proline ratio at site 2. There were thus differences at both sites in the cartilage layer, at both sites in the subchondral bone layer and at site 2 only in the trabecular bone layer. It can be speculated that this somewhat different reaction pattern has to do with the differences in handling of the biomechanical loading that is generated by locomotion. It is the cartilage layer that sustains the initial impact, which is known to be highest at the intermittently loaded site 1 (Brama *et al*. 2001a). Total cumulative load over time is higher at the continuously loaded site 2, which might have more effect on the trabecular layer of which it has been stated earlier that at this level site 2 will have to take a larger portion of the burden of dissipating forces than site 1 (Harst *et al.* 2004).
The differences that were found in the cartilage layer were minimal, but consistent with other reports concerning (early) OA. There was a significant correlation between CDI and GAG-content. Proteoglycan loss is known to be one of the early signs of OA. Indian ink particles are prevented from entering intact cartilage surface with an unaffected proteoglycan-rich matrix, but have a high affinity for articular cartilage with surface fibrillation and proteoglycan depletion (Brommer *et al.* 2003a, Chang *et al.* 1997, Madsen *et al.* 1994, Maroudas *et al.* 1973). Another early indication of cartilage damage may be the loss of site differences in DNA content. Severe OA is known to result in an almost complete loss of site differences of many biochemical parameters, and loss of the superficial cartilage layer, where cell density is highest, has been reported as a sign of early OA (Brama *et al.* 2000c, Mitchell *et al.* 1992). In the present study the cell density at site 1 diminished and became of the same level as in site 2.

In pentosidine levels also indication of changes consistent with early OA could be found in the form of alterations in the relationship of pentosidine cross-link content with the age of the animals. In healthy animals there is a linear increase of the non-enzymatic glycation product with age and it is known that this accumulation of this advanced glycation end product, resulting from the spontaneous reaction of sugars with proteins, is a physiological process in many tissues. The level of pentosidine is largely determined by the turnover rate of the extracellular matrix (Verzijl *et al.* 2000). Pentosidine can therefore be used to assess the remodelling rate of the collagen network in a wide range of tissues. In tendons it has been shown that repeated micro-damage, as occurs in the supraspinatus tendon in the so-called rotator cuff syndrome, will halt the physiological linear increase through the increase of tissue turnover that leads to removal of the glycation product (Bank *et al.* 1999). In the present study there were clear changes in the relationship of the pentosidine cross-links with age. Not only in the cartilage layer, but in all layers and at all sites, except for site 1 in subchondral bone where no relationship could be found in healthy tissue either, there is either a complete loss of the relationship of pentosidine levels with age, or the increase has become less and of less significance.

In the subchondral bone plate an interesting phenomenon is observed. There is a significant decrease of HP levels at both sites 1 and 2 in the OA group compared to the non-OA group. This difference is not seen in the overlying articular cartilage, which is in agreement with earlier findings (Bank *et al.* 2000, Takahashi *et al.* 1996). HP and LP cross-links are mature cross-links. A lower level of these may hence indicate a higher turnover, resulting in what could be called a less mature bone matrix. Brama *et al.* (2001a, 2001b) found higher cross-link levels at site 2 in a series of normal specimens and related this to the loading pattern determined in another study. They conjectured that the intermittent high peak loading at site 1 would lead to a higher remodelling rate and hence lower cross-link levels. In the present study site 1 – site 2 differences in the non-OA group are similar to those found by Brama *et al.* (2001b). In the OA group this difference remains, but overall levels at both sites are lower. This may indicate a higher remodelling rate compared to the non-OA group. The higher remodelling rate might possibly be interpreted as a very early sign of OA in the subchondral bone plate. This is a potentially interesting finding as cross-links influence the structure and mechanical strength of the collagen network (Knott *et al.* 1995, Oxlund *et al.* 1995). Apart from this direct influence on material properties, there is an indirect influence as well as the collagen network has been demonstrated to be leading in the process of mineralisation (Brown *et al.* 2002, Wassen *et al.* 2000). On the other hand mineralisation has been shown to have an effect on the packing fraction of collagen fibrils. Increased mineralisation will lead to a closer packing of collagen molecules and reduced water content, and thus to stiffer bone (Dequeker *et al.* 1997, Li and Aspden 1997). Increasing stiffness of the subchondral bone plate has been incriminated as one of the major initiating factors of OA (Burr 1998, Radin

and Rose 1986). In the present study there are some indications that, probably very early, changes in mineral composition are taking place. There are small, but significant, differences in ash content at sites 1 and 2 between the animals with minimal OA and the normal individuals. But, the changed collagen network may probably precede these changes in the ash content.
There were no differences in BMD between both groups. In literature, there are no consistent results when BMD is measured in OA and non-OA joints. In some cases an increase of BMD in OA joints is described (Belmonte-Serrano *et al.* 1993, Hart *et al.* 1994, Madsen *et al.* 1994, Nevitt *et al.* 1995). However, a number of other studies did not report an association between BMD and OA (Burr 1998, Healey *et al.* 1985, Price *et al.* 1987, Reid *et al.* 1984). An explanation might be that BMD measured using quantitative computed tomography is a measure of mineral content in a unit volume of bone, which is not a true mineral density, but an apparent bone mineral density (Wang *et al.* 2000). This may make the technique not sensitive enough to detect early and relatively mild changes in mineral content in early OA.
In the trabecular bone again only collagen parameters show (minimal) changes. The increase in hydroxyproline to proline ratio indicates an increase in collagen over non-collageneous protein, which may represent a response to increased loading. The increased collagen amount may be indicative of an increase in tensile strength as adaptation to loading (Knott *et al.* 1995, Oxlund *et al.* 1995).
It is concluded that in the very early and mild stages of OA that were studied changes at the molecular level take place in all 3 layers that together constitute the bearing surface of the joint. However, changes take place at different sites in the different layers. Changes include the proteoglycan component of the cartilage layer and characteristics of the collagen network in both bony layers. Changes in any of the mineral-related parameters were seen in subchondral bone only and were relatively minimal, suggesting that these develop at a later stage of the disease.
It is acknowledged that the question about the determination of the order in the events that take place during the development of early OA can only be definitively answered based on studies in which developments are monitored during some time, or in which sequential samples are taken. Further, more aspects than molecular composition alone, such as for example tissue architecture, should be taken into account. Nevertheless, the fact that most changes are detected in the subchondral bone plate, and less in cartilage and trabecular bone may perhaps provide an indication of the sequence in time of these changes.

Acknowledgements

The authors would like to express their gratitude to Ms M.I.J.A. Pollak, Mr A. Klarenbeek and H.S. Wouterse for their help with the preparation and processing of the samples. We also would like to thank dr. A.G.H. Ederveen and Mr C. Spanjers of the Department of Pharmacology of N.V. Organon (Oss, the Netherlands) for their indispensable help in measuring BMD.

CHAPTER V

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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(Equine Veterinary Journal: 2005; 37: 19-25)

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 and general 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 (Hyp) and general matrix metalloproteinase (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 1996a). 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.* 2000a, Todhunter 1996a, 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 1996a). Hydroxyproline 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 1996a). 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, Alwan *et al.* 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, Brama *et al.* 2000d, Clegg *et al.* 1997, Clegg and Carter 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 -80º C 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 -20° C 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.* 1999a). The cartilage samples were stored at -80º C 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.* 1997b). 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 in a Speed Vac (Savant SC 110) and dissolved in 100 µl water. 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), 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^{TM} version 4.30, Dionex). The amino acid standard for collagen hydrolysates[6] 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-dimethyl-methylene blue (Blyscan kit, Biocolor Ltd.) and

staining was quantified by measuring absorbance at 656 nm. Shark cartilage chondroitin sulfate (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.* (2001). 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.* (1997b), 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 $\geq$ 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 ± sem). There was a small, but significant difference ($P=0.03$) in mean age between the group with moderate-severe cartilage damage (mean ± sem: 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).

Table 1 - Measured parameters (mean ± sem) in synovial fluid (SF) from the metacarpophalangeal joint and cartilage from the articulating 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; sem = standard error of the mean.

	moderate-severe cartilage damage	**no-minimal cartilage damage**	
	mean ± sem	**mean ± sem**	**p-level**
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 activity 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

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).

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.

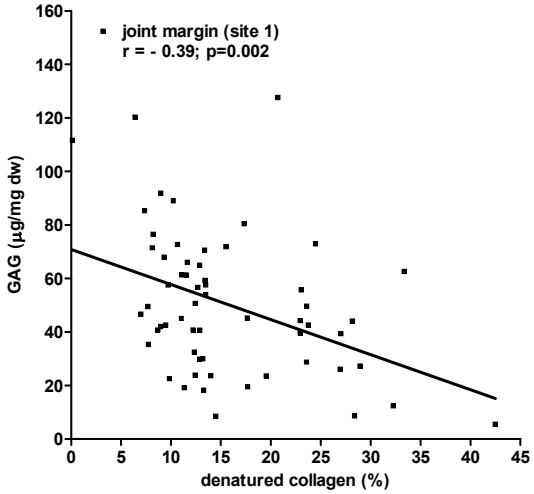

Fig. 1: Glycosaminoglycan (µg/mg dw) content in relation to the relative amount of denatured collagen (%) of the articular cartilage of site 1 from the metacarpophalangeal joint (age range 5 to 23 years, n=59); r = correlation coefficient.

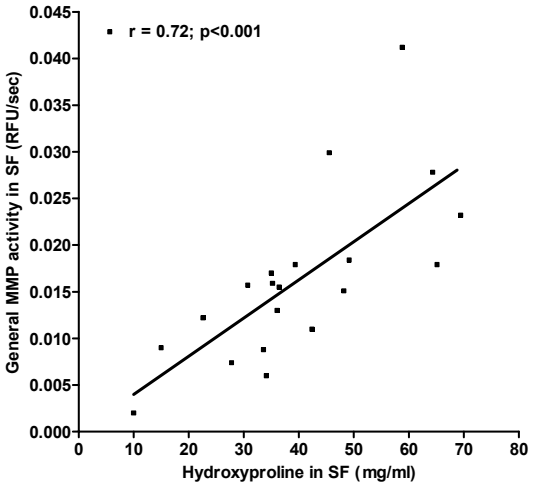

Fig. 2: General MMP activity (RFU/sec) in relation to hydroxyproline (µg/ml) in synovial fluid (SF) from the metacarpophalangeal joints with moderate-severe cartilage damage (age range 5 to 22 years, n=20); r = correlation coefficient.

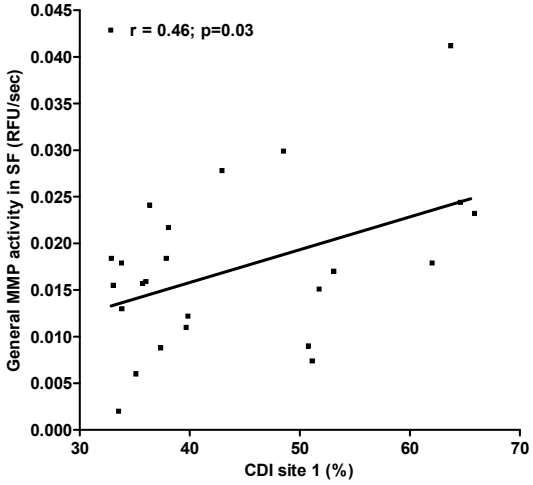

Fig. 3a: General MMP activity (RFU/sec) in synovial fluid (SF) from the metacarpophalangeal joint in relation to cartilage degeneration index (CDI) at site 1 (%) in joints with moderate-severe cartilage damage (age range 5 to 22 years, n=24); r = correlation coefficient.

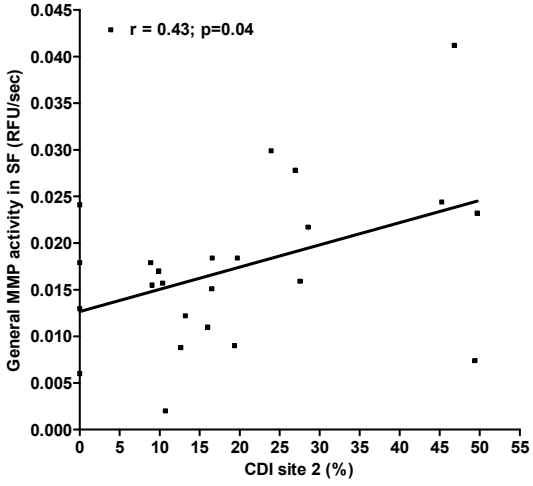

Fig. 3b: General MMP activity (RFU/sec) in synovial fluid (SF) from the metacarpophalangeal joint in relation to cartilage degeneration index (CDI) at site 2 (%) in joints with moderate-severe cartilage damage (age range 5 to 22 years, n=24); r = correlation coefficient.

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, ageing 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 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, Saxne *et al.* 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.* 1998). 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 hydroxyproline 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 degeneration 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, Brama *et al.* 2000d, Clegg *et al.* 1997, Clegg and Carter 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 *et al.* 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 VI

NITRITE AND NITROTYROSINE CONCENTRATIONS IN ARTICULAR CARTILAGE AND SUBCHONDRAL BONE AS MEASURES FOR METABOLIC LEVEL AND OXIDATIVE STRESS IN NORMAL, JUVENILE AND OSTEOARTHRITIC EQUINE METACARPOPHALANGEAL JOINTS

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(Submitted)

Summary

Objective: Osteoarthritis (OA) is a chronic debilitating joint disorder in which the importance of the role of inflammatory processes has been increasingly recognised. In advanced cases, both the articular cartilage and the underlying bony layers are affected, but the exact sequence of events and their localisation in the initial phase of the pathogenesis remain uncertain. The objective of this study was to measure products of inflammatory processes to help define the early stages of OA.
Methods: Nitric oxide (NO) and nitrotyrosine were measured in articular cartilage and subchondral and trabecular bone layers of the dorsal articular joint margin of horses affected by OA, unaffected (control) mature horses and unaffected juvenile horses. Data were correlated with cartilage damage as quantified by the Cartilage Degeneration Index (CDI).
Results: In all three layers the NO concentration was higher in OA-affected joints. In addition, the concentration of NO was significantly higher in cartilage and subchondral bone of juvenile horses compared with normal horses.
Nitrotyrosine was significantly higher in subchondral bone of OA-affected horses compared with healthy controls. Significantly lower nitrotyrosine levels were measured in trabecular bone of the juvenile horses.
Conclusions: Similarities were observed over the three tissue layers, especially with respect to the cartilage and subchondral bone layers, which stresses the importance of the concept of the bearing surface of the joint as a functional entity. Nitric oxide concentration appears to be a useful indicator of increased tissue metabolism, but cannot discriminate between physiological and pathological processes. The increased nitrotyrosine levels in the subchondral bone suggests that this layer is important in early or moderate OA, and implies a role of oxidative stress in the development of the disease. Insights into the mechanisms involved in the development of OA may help in the establishment of adequate preventive measures.

Introduction

Osteoarthritis (OA) is a debilitating condition that may affect a large number of joints in both man and horses (Pool 1996, Sharma 2001). It has a multi-factorial aetiology and there is large individual variation in the speed with which the disease progresses. Age, genetics, trauma and more general biomechanical influences are among the factors that contribute to the osteoarthritic process (Sharma 2001). Classically, OA has been viewed as a mainly degenerative disorder of mobile joints, characterised by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins (Bareither *et al.* 1998, Muehleman *et al.*

2002). However, recent debate strongly questions the non-inflammatory character of the disease (Attur *et al.* 2002).

Another contentious issue is the site where the disease initiates. Although it is well known that synovial membrane, subchondral bone and ligaments, as well as cartilage may be implicated, the major focus for OA research has remained primarily on the articular cartilage. Both cartilage degeneration and associated pathological bone changes have been well documented in end-stage disease of synovial joints (Nevitt *et al.* 1995). Several reports claim that sclerosis of the subchondral bone plate precedes articular cartilage damage (Burr and Schaffler 1997, Burr 1998, Carlson *et al.* 1994, Dequeker *et al.* 1995, Mansell and Bailey 1998, Radin *et al.* 1984, Radin and Rose 1986, Radin *et al.* 1991). However, others report degradation of articular cartilage typical of OA without any signs of pathological changes in the underlying subchondral bone (Dedrick *et al.* 1993, Donohue *et al.* 1983, Yamada *et al.* 2002). This observation led to the opposing view that the disease begins in the articular cartilage (Lee *et al.* 1974, Mankin and Brandt 1992, Mow *et al.* 1992, Stephens *et al.* 1979, Wei *et al.* 2001).

Nitric oxide (NO) is a substance that fulfils a physiological role in tissue homeostasis where it has a regulatory function, but is also up-regulated under inflammatory conditions, exerting cytotoxic effects through the creation of oxidative stress (Vuolteenaho *et al.* 2001). Nitric oxide induces apoptosis or programmed cell death following various insults (Blanco and Lotz 1995). However, in the case of tissue damage, it appears to play a dualistic role. On the one hand NO and its byproducts, reactive oxygen species (ROS), induce the inflammatory component of OA. On the other hand it is capable of inducing tissue damage and tissue destruction, as well as inhibiting the formation of repair tissue and new extracellular matrix components (Blanco and Lotz 1995, Cao *et al.* 1997, Kobayashi *et al.* 2001).

Nitric oxide is a highly volatile substance that is rapidly degraded into nitrite. Consequently, nitrite is commonly used as a marker for NO synthesis (van't Hof and Ralston 2001). As nitrite is continually cleared from the body by renal excretion, the nitrite concentrations that are measured in tissue can be considered representative for ongoing NO production (ter Steege *et al.* 1998).

Nitrotyrosine is formed following the reaction of NO with superoxide producing peroxinitrite, which (among others) nitrosylates tyrosine rings in proteins, thereby producing nitrotyrosine (Diwan *et al.* 2000). It has been identified as an additional marker of inflammation and enhanced NO production and the presence of nitrotyrosine has been suggested to represent oxidative damage of cellular proteins. Furthermore, it correlates with diseases, such as OA and neurological disorders (Beal 2002, Carlo and Loeser 2003). Nitrotyrosine is not cleared continually, and may thus accumulate.

Recently, the concept that the bearing surface of the joint should be comprehensively envisaged as a functional entity has been more widely accepted. In addition, simultaneous investigation of the constituting elements (cartilage,

subchondral bone and trabecular bone) and the relationship between each layer is preferable than considering them as disparate entities (Hayami *et al.* 2004, Muehleman *et al.* 2002, Radin and Paul 1971, Radin and Rose 1986). In the horse, this concept has been applied to the metacarpophalangeal joint in mature and juvenile horses to monitor changes related to development (Harst *et al.* 2004, Harst *et al.* (accepted EVJ)). Furthermore, horses showing early signs of OA exhibited changes in extracellular matrix components in all layers, which were most evident in the subchondral bone (Harst *et al*. accepted AJVR). In the present study, a similar comprehensive approach is used to assess the presence and levels of nitrite and nitrotyrosine, to investigate the potentially important role of NO in the initial phases of OA and taking nitrotyrosine as a more definitive indicator of tissue damage. Nitrite and nitrotyrosine levels were determined in the three layers that constitute the bearing surface of the joint, *i.e.* cartilage, subchondral bone and trabecular bone, in specimens from normal, osteoarthritic, and juvenile equine metacarpophalangeal joints.

Materials and methods

Joints

Forty-three right metacarpophalangeal joints from Warmblood horses were harvested immediately after death and stored at -20 °C until processing. Articular cartilage degeneration was quantified with help of the Cartilage Degeneration Index (CDI) (Brommer *et al.* 2003a). In this method, the amount of Indian ink uptake across the entire cartilage surface is quantified by digital imaging of the native and stained articular cartilage surfaces and the increase in mean grey level of the articular surface is used as basis for calculation of the CDI (range from 0 to 100%). This procedure was performed for the entire joint, resulting in a general CDI, and for a specific area of interest (CDI_1) located close to the dorsal articular margin. This site is where cartilage degeneration is known to start and from where it gradually spreads over the joint (Brommer *et al.* 2003b).

In this study, the cohort was divided into three groups. The first group (n=15) (mean age 10.2 years, range 5-18 years) that was classified as normal, had a CDI_1 <25%, which is indicative of none to minor degenerative changes (Brommer *et al.* 2004). The second (n=15) (mean age 14.7 years, range 8-22 years) had CDI_1 values > 25%, indicative of mild to severe cartilage changes, and the third group (n= 13; mean age 2.4 years, range 6 months-4 years) contained juvenile horses. These horses all had a CDI_1 < 25%.

Sampling procedure

After establishing the CDI, a 6 mm slice of phalanx I, containing cartilage and bone was cut in dorsopalmar direction, perpendicular to the articular surface and through the centre of the medial fovea, using a band saw. The cartilage of the area of

interest was removed with a scalpel and the subchondral and trabecular bone layers were separated with a milling cutter. Each bone and cartilage sample was homogenized in a microdismembrator (Braun) with a stainless steel ball-mill under liquid nitrogen. After suspension in distilled water, samples were stored at - 80 °C until further analysis.

Protein measurement
The protein concentration was measured by using a modified method of Lowry (Flores 1978). All chemicals were obtained from Sigma unless specified otherwise. Briefly, 200 µl reagent was added to 20 µl sample for 20 minutes, after which time 20 µl folin's (Merck) reagent was added. After an additional 45 minutes incubation period at room temperature samples were measured spectrophotometrically at 750 nm (Biorad 3550). Bovine serum albumin (BSA) was used as a standard to calculate the protein content as µg/ml.

Nitrite assay
Nitrite, a stable end product of nitric oxide, was measured in cartilage and subchondral bone samples by using the spectrophotometric Griess reaction (Green *et al.* 1982). Twenty microliters of sulphanilamide (1 % w/v) in phosphoric acid (5 %) (Merck) was added to 200 µl of the sample and incubated for 10 minutes at room temperature before adding 20 µl naphtyl ethylenediamide (1.4 %). Samples were incubated for 2 minutes and centrifuged for 5 minutes at 13000 rpm. Seventy-five microliters of supernatant was transferred to a 96 well plate and the optical density was measured at 540 nm (Biorad 3550). Nitrite concentrations were calculated by comparison with the optical density of standard solutions of sodium nitrite (Merck). The nitrite concentration was expressed as µmol/mg protein. All samples were analysed in triplicate.

Nitrotyrosine ELIFA
An enzyme-linked immunoflow assay, or ELIFA, was used in order to quantify the amount of adsorbed nitrotyrosine by microplate filtration in which aqueous samples are drawn past a nitrocellulose membrane (Easy-Titer ELIFA microplate filtration device, Pierce). Samples were diluted with Tris-buffered saline (TBS, 150 mM NaCl, 10 mM Tris, pH 7.5) and samples and standards were drawn through the nitrocellulose membrane for a period of 5 min (flowrate, 40 µl/min/well) using a Microperpex S peristaltic pump (LKB). Washing buffer (200 µl/well) TBS-T (TBS with 0.05% Tween-20) was subsequently drawn through 3 times at the same flow rate. The nitrotyrosine was detected by drawing 50µl mouse anti-nitrotyrosine (Zymed) (TBS-T, 0.1% BSA, and 0.5µg/ml antibody) through the membrane. After washing 3 times, 50 µl secondary rabbit anti-mouse (Ig) antibody conjugated with horseradish peroxidase diluted 5000× in 0.1% BSA in TBS-T, was drawn through. The membrane was washed for another 3 times. Bound horseradish peroxidase was detected by the substrate, 3,3,5,5-tetramethylbenzidine (TMB), which was

drawn past the membrane and collected in a 96-well polystyrene plate containing 50 µl 2 M H_2SO_4 per well. The optical density of the wells was measured at 450 nm with a microplate reader (Biorad 3550), and the nitrotyrosine contents were calculated and expressed relative to the standard (arbitrary units). All samples of each layer were analyses simultaneously, and in triplicate.

Statistical analysis
All data are expressed as mean ± standard error of the mean (sem). Differences between the OA, non-OA and the juvenile horses were tested by using the non-parametric Kruskal-Wallis test, completed with the post-hoc Dunn's multiple comparison test. Correlations between different parameters were tested using the non-parametric Spearman's correlation coefficient test. Data were considered to be statistically significant when $p<0.05$. Statistical analysis of the data was performed with help of the software package GraphPad Prism (GraphPad Software, Inc.).

Results

Nitrite in cartilage and bone
The NO concentration in cartilage, as indicated by the nitrite level, was significantly higher in the group with cartilage damage (OA) compared with those with healthy cartilage (Fig. 1a). Similarly, the nitrite concentrations in the subchondral and trabecular bone were significantly higher in horses with cartilage lesions compared with controls (Figs. 1b-c).
In the juvenile horses, a significantly higher nitrite concentration was measured in cartilage and subchondral bone compared with the mature horses classified as having normal joints. In the trabecular bone the levels were comparable with those in normal, mature horses (Figs. 1a-c).

Nitrotyrosine in cartilage and bone
There was a significantly higher nitrotyrosine level in the subchondral bone of the animals classified as having cartilage damage compared with the normal animals (Fig. 2b). However, this was not the case in either the cartilage and trabecular bone (Figs. 2a,c).
In the juvenile age class nitrotyrosine levels in both cartilage and subchondral bone were similar to those found in mature horses. In contrast, in trabecular bone, the nitrotyrosine level was significantly lower than in normal, mature animals (Figs. 2a-c).

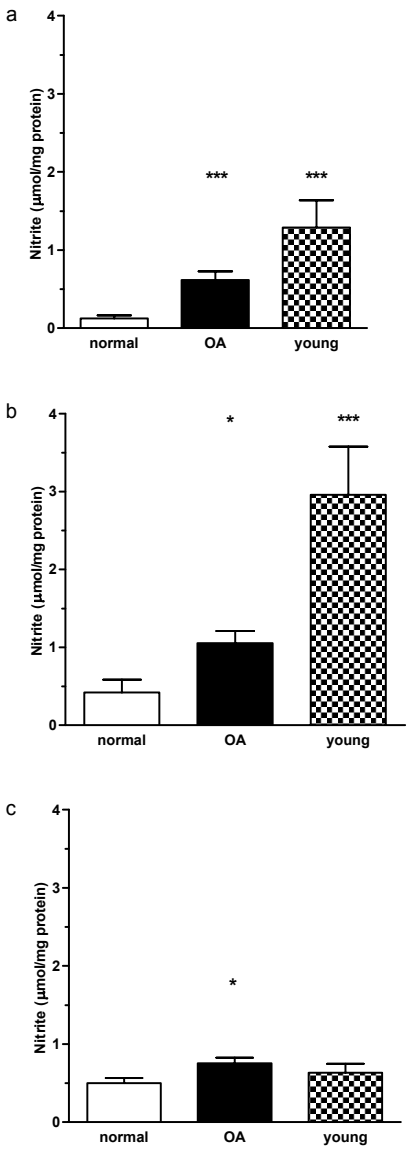

Fig. 1a-c: Nitrite content (µmol/mg protein; mean ± sem) in cartilage (a), subchondral bone (b) and trabecular bone (c) of samples collected from the proximal first phalanx of normal mature joints (n=15), joints affected by osteoarthritis (n=15) and normal immature joints (n=13). Asterisks denote a significant difference with the normal mature joints; * = P<0.05, *** = P < 0.001.

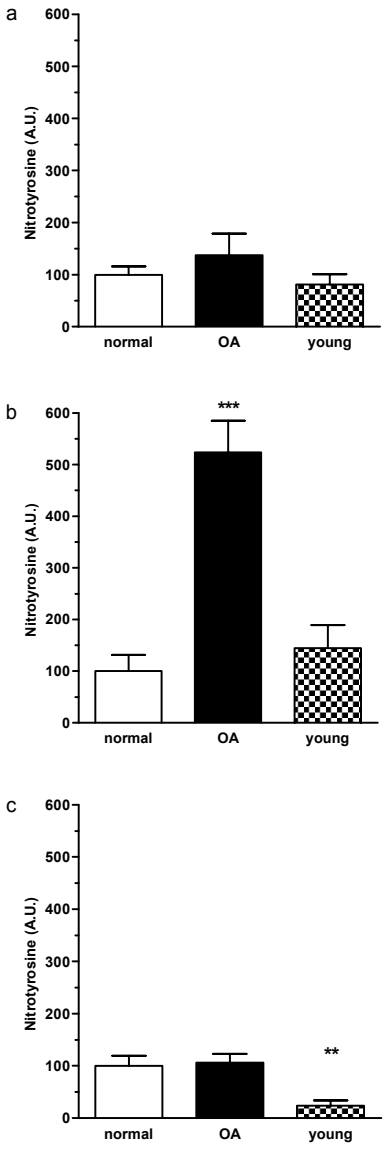

Fig. 2a-c: Nitrotyrosine content (A.U.; mean ± sem) in cartilage (a), subchondral bone (b) and trabecular bone (c) of samples collected from the proximal first phalanx of normal mature joints (n=15), joints affected by osteoarthritis (n=15) and normal immature joints (n=13). A.U. = arbitrary units. Asterisks denote a significant difference with the normal mature joints; ** = $P < 0.01$, *** = $P < 0.001$.

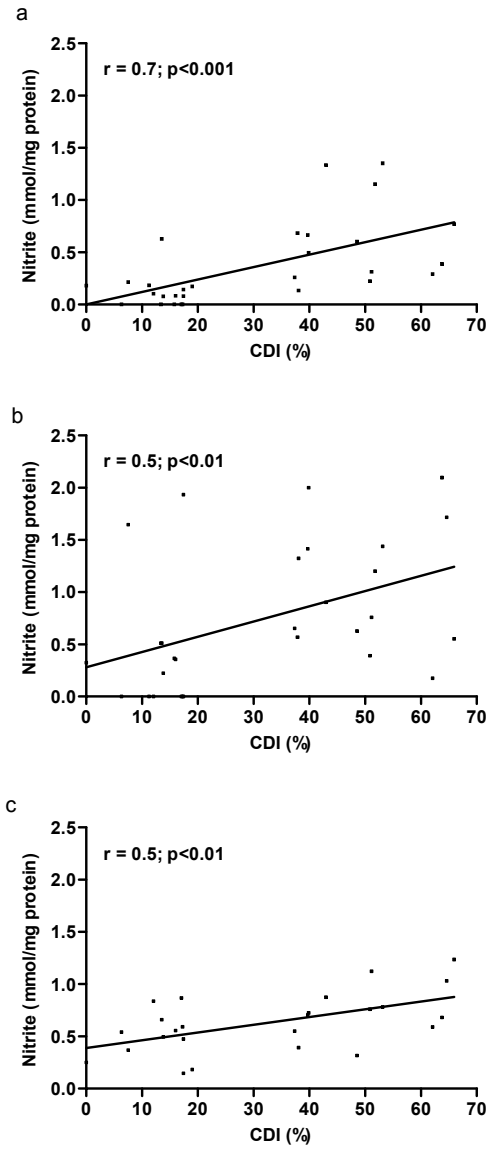

Fig. 3a-c: Correlation between nitrite content (µmol/mg protein) and cartilage degeneration index (%) in cartilage (a), subchondral bone (b) and trabecular bone (c) of samples collected from the equine proximal first phalanx. CDI = cartilage degeneration index; P = significance level; r = correlation coefficient.

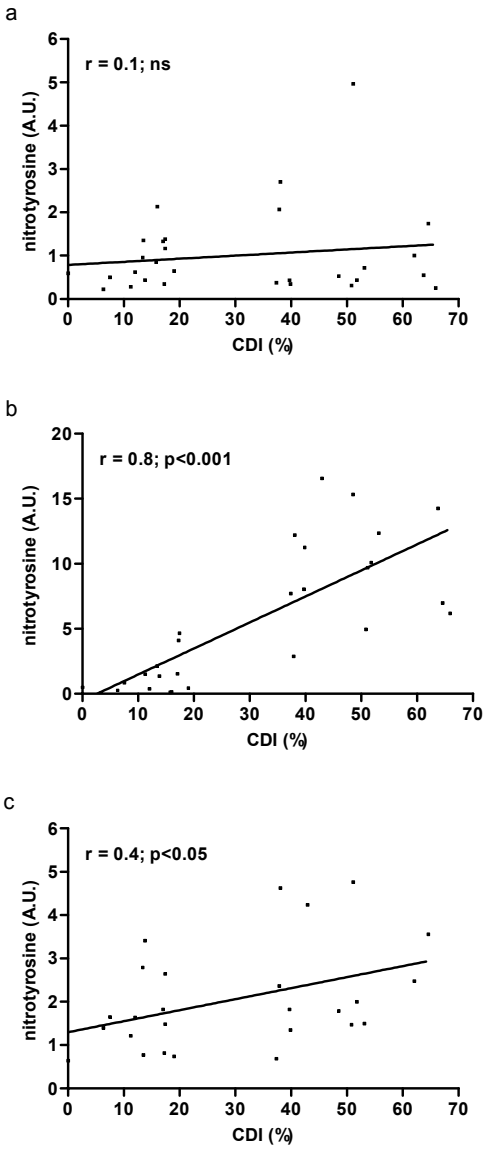

Fig. 4a-c: Correlation between nitrotyrosine content (A.U.) and cartilage degeneration index (%) in cartilage (a), subchondral bone (b) and trabecular bone (c) of samples collected from the equine proximal first phalanx. CDI = cartilage degeneration index; A.U.= arbitrary units; P = significance level; r = correlation coefficient.

Relationship of nitrite and nitrotyrosine levels with CDI

Figs. 3a-c and 4a-c present the correlations between the CDI and the levels of nitrite and nitrotyrosine for the combined group of mature animals (normal animals and animals with a CDI_1>25%) in all 3 layers. There is a positive and significant correlation of the nitrite level with the CDI in all layers, which is stronger in cartilage (Fig. 3a; r=0.7) than in the other two layers (Fig 3b-c; r=0.5 in both layers).

With respect to nitrotyrosine, there was no significant correlation with CDI in articular cartilage (Fig. 4a, r=0.1), a strong correlation in subchondral bone (Fig. 4b, r=0.8), and a considerably less strong correlation in the trabecular bone layer (Fig. 4b, r=0.4).

Discussion

Nitric oxide acts as a mediator that is up-regulated in various physiological and pathophysiological processes in the body in which tissue metabolism is increased. Growth is such a process, as NO content in cartilage has been shown to be increased in cartilage during development and maturation (Hukkanen *et al.* 1999). Nitric oxide is also up-regulated in fracture repair, which can be seen as a physiological response to a pathologic event (Diwan *et al.* 2000, Zhu *et al.* 2002). In inflammatory reactions NO has both regulatory and cytotoxic effects (Diwan *et al.* 2000, Werners *et al.* 2004).

In the present study nitrite levels in normal, mature horses were similar in both the subchondral and trabecular bone. These levels may be considered representative for steady state, undisturbed tissue metabolism. In the cartilage layer the level was lower in terms of µmol per mg protein, but will be of more or less the same absolute level in the bony layers, as the protein content of cartilage is much higher than of bone. The lower levels of NO in relation to protein content are in agreement with the substantially lower metabolic activity of articular cartilage compared to bone in mature individuals, resulting in extremely long turnover times for collagen (Maroudas *et al.* 1992, Verzijl *et al.* 2000). Nitrite levels were higher in the cartilage and subchondral bone layers of the juvenile animals, which may be indicative of the higher metabolic activity in this age class where development and growth are ongoing processes. It is not entirely clear why the NO in the trabecular layer was not increased, but remained the same level as in the mature animals. It may be speculated that the direct mechanical challenge to the tissues in this vulnerable period of development and growth plays a role. The cartilage and subchondral bone layers sustain the impact peaks generated during physical activity more directly than the trabecular bone through which the force is more gradually dissipated.

The effects that are exerted by NO in osteoarthritic cartilage have been studied extensively (Fernandes *et al.* 2002, Hashimoto *et al.* 1999, Jang *et al.* 1998, Kim *et al.* 2003, Mazzetti *et al.* 2001, Studer *et al.* 1999). Increased NO concentrations

have been measured in chondrocyte cultures from OA joints (Amin *et al.* 1995, Frean *et al.* 1997, Hashimoto *et al.* 1998). In addition, an increase of NO in synovial fluid collected from diseased joints has been reported (Jang *et al.* 1998). Nitric oxide promotes a number of catabolic effects on chondrocytes and this could result in matrix loss. Reported effects of NO on chondrocytes include: inhibition of matrix synthesis; modulation of metalloproteinases; increased susceptibility to injury by other oxidants and induction of apoptosis (Cake *et al.* 2003, Takahashi *et al.* 2000). Most of the studies on the NO production in OA cartilage have been carried out using chondrocyte cultures. In cell cultures, however, the phenotype of isolated chondrocytes alters rapidly resulting in an increase in proliferation (Vuolteenaho *et al.* 2001). Therefore, data from studies with cultured chondrocytes are not directly applicable to intact osteoarthritic cartilage. In the current study, we measured NO concentrations from cartilage and bone samples *ex vivo*. To the authors' knowledge, no results have been previously presented regarding NO in equine cartilage and bone from the same joint. Nitrite, the end product of NO degradation, was significantly elevated in samples from animals affected by OA in comparison to the normal, mature animals. The relative increase of nitrite was highest in the cartilage layer, and least in the trabecular bone layer. These results support earlier findings that showed that extracellular matrix composition in equine metacarpophalangeal joint specimens affected by (early) OA showed changes in all three layers (Harst *et al.* accepted AJVR). The increase in NO over all layers and the positive correlation of NO level with CDI in all these layers suggest that in (early or moderate) OA, increased metabolic activity is present throughout the entire bearing surface of the joint, and not only in the articular cartilage layer. This stresses the importance of the adjacent bone layers and is in line with other work on this subject (Burr and Radin 2003, Dequeker *et al.* 1997, Radin and Rose 1986).

Whereas nitrite is only an indicator for NO production, protein associated nitrotyrosine is a more suitable marker for damage induced by reactive nitrogen intermediates derived from NO. It is metabolised in the process of protein turnover, but, as many extracellular matrix proteins of connective tissues have a relatively long to even very long half-life, nitrotyrosine can, to a certain extent, be seen as a measure for accumulated damage. In the present study nitrotyrosine levels in mature animals are thought to represent normal steady state values. Levels in the juvenile group were at a similar level as in the mature individuals in the cartilage and subchondral bone layers. This means that although NO levels were significantly higher in those layers in the juvenile animals, resulting in nitrosylation occuring at a higher rate, the higher protein turnover rate compensates for this. In the trabecular bone of such juvenile horses, nitrotyrosine was significantly lower. This may be caused by the fact that in this layer the NO concentration was similar to the concentration in mature horses (whereas levels were much higher in cartilage and subchondral bone), combined with the high rate of protein metabolism in juvenile animals, leading to a high turn-over rate of nitrotyrosine.

In joints with osteoarthritic changes nitrotyrosine levels are only significantly increased in the subchondral bone layer. This can be interpreted as a significant increase in oxidative stress in this layer. In addition, the correlation of nitrotyrosine with the CDI index of the cartilage layer is highest in the subchondral bone layer. These are interesting findings as it suggests that there may be a relation between the cartilage degeneration and the production of nitrotyrosine in the underlying subchondral bone plate. During peak loading the cartilage and subchondral bone layers are most directly and strongly affected and it is not exactly known which layer is initially disrupted, if loading is excessive and damage occurs. The subchondral bone plate is in direct contact with the cartilage and could influence its degradation and remodelling. Bone is a rich source of growth factors that play key roles in the regulation of the formation and resorption of this tissue (Massicotte *et al.* 2002). Nitric oxide has a biphasic effect on bone metabolism. It has been shown that a slow and moderate release of nitric oxide stimulates the replication of primary rat osteoblasts, while a rapid release and high concentration of NO inhibits proliferation and induces apoptosis (Broulik *et al.* 2003, Mancini *et al.* 2000). It is not impossible that loading-induced micro-fractures in the subchondral bone layer may stimulate the production of cytokines, growth factors and prostaglandins and other inflammatory mediators including NO, which may migrate through the bone-cartilage interface and stimulate cartilage breakdown. There is indeed evidence of the presence of channels and fissures in the interface between cartilage and bone that provide a route for biological signals between these compartments (Massicotte *et al.* 2002).

It is concluded that the patterns of nitrite and nitrotyrosine concentrations in the tissue layers that make up the bearing surface of the joint in juvenile, normal mature and OA-affected horses show great similarities. In the cartilage and subchondral bone layers these patterns are almost identical, which stresses the importance of envisaging the joint as a functional entity. Nitrite levels are increased in most layers in both the juvenile and OA-affected group, suggesting that this parameter can be used as a measure for the intensity of metabolic activity, but not to discriminate between physiological and pathological events. The fact that nitrotyrosine levels, of which it is presumed that these give information about pathological processes, were increased in the subchondral layer of OA-affected animals may indicate that increased oxidative stress in this tissue layer plays a role in (early or moderate) osteoarthritis.

Acknowledgements

The authors would like to express their gratitude to Mr A. Klarenbeek for his assistance during sample collection and to Ms M.I.J.A. Pollak for her tireless efforts during the sample measurements and for critically reviewing experimental work.

Chapter VII

General discussion

Designed to be loaded

For millennia, the horse has played an important role in the history of mankind as virtually the only means of rapid transport. Due to the mechanical revolution after World War I, the significance of the horse as a working animal decreased. However, from a utilitarian working animal, the horse has converted into an equine athlete. The rise in popularity of equestrian sports now provides an alternative function for the animal. This change in use has to a certain extent changed the horse, as horses have always been bred to serve their intended use. However, both the "old" and the "new" horses have one thing in common: the use that mankind makes of the horse means a severe challenge to the musculoskeletal system. It is all about (heavy) loading and the (large) capacity to sustain load. Injury is on the brink of the two.
The musculoskeletal system of the horse has adapted to biomechanical challenge, *i.e.* loading, in many ways and at many levels. An example is the function of the equine tendons. Tendons, with their elastic capacity, act as energy saving 'springs' during standing and walking and thus obviate the need for heavy musculature that would affect the agility of the animal. In fact, the muscles attached to the long flexor tendons are very small relatively speaking and perform an amount of minimal work. Their principal function is to dampen the vibration by the muscles fibres, not to generate limb movement. This mechanism protects the bone and tendon from fatigue failure (Wilson *et al.* 2001).
Adaptations to obtain an optimal biomechanical function can also be found at the tissue level. Recent work by Brama *et al.* showed that the biochemical composition of the extracellular matrix (ECM) of articular cartilage adapts to the locally differing loading conditions. In other words, the biochemical make-up differs across the joint surface as an adaptation to the varying biomechanical forces generated during physical activity, giving rise to a so-called "topographical heterogeneity" in tissue composition (Brama *et al.* 2000a, Brama *et al.* 2001a).

Load-driven development

The German scientist and first director of the famous Charité orthopaedic centre in Berlin Julius Wolff (1836-1902) was one of the first to realise that tissue may respond to loading. According to his theory, which has become known as Wolff's law, the activity of osteoclasts and osteoblasts in trabecular bone is influenced by mechanical stress, resulting in a denser structure at locations where stress influence is highest (Wolff 1892). This process is valid for healthy individuals in all age classes, but is of special importance in the juvenile period of development and growth.
The insight that a similar load-driven development takes place in the collagen network of articular cartilage is more recent. Brama and colleagues showed that

the biochemical composition of the ECM of articular cartilage in a newborn foal is identical over the entire joint surface and therefore does not show the topographical heterogeneity that is characteristic for the mature animal. This condition, present at birth, has been called the "blank joint" (Brama *et al.* 2000a). Immediately after birth, with the foal's first steps, biomechanical forces will be applied to the cartilage layer and the initially blank surface develops until the load-adapted topographical heterogeneity, characteristic for the mature joint, is reached. There is one essential difference between the developmental processes in bone and articular cartilage. The process in cartilage is restricted to a limited period of time, which in the horse is basically the first year of life, because of the quick decline in metabolism that leads to extremely long turnover times for collagen in mature animals. This difference is of importance for the repair capacity of the tissue after injury. In bone the biochemical composition and the biomechanical properties after fracture healing are comparable to those in bones that never sustained a fracture. In contrast, severely damaged cartilage lacks the capacity for adequate repair in mature individuals, as already noted by Hunter (1743). It is this incapacity for (complete) repair, *i.e.* the inability to restore the balance between biomechanical loading and the capacity to sustain load, that is the quintessence of osteoarthritis (OA). A solution for this disorder, which is a major cause of disability in humans and of early retirement from athletic activity in horses, can only be found through the comprehensive and in-depth study of joint physiology and pathology.

More than two layers of cartilage: an in-depth approach of joint function

The function of the synovial joint is to allow movement and transfer load between bones. The cartilage layer cannot perform the latter task on its own, without the support of the surrounding tissue, *i.e.* the adjacent bone layer, the joint capsule and the collateral ligaments (Radin *et al.* 1970, Radin and Rose 1986). Absorption of load by the cartilage is limited and most will be passed on to the subchondral bone layer and then further to the trabecular bone beneath. Due to the spatial arrangement of these tissue layers, the impact of loading will be greatest in the cartilage layer and the tightly connected subchondral bone, to become more attenuated towards the trabecular bone. Given this close functional relationship of the various layers, it may be presumed that there will also be similarities in composition and reaction patterns. Further, changes in one of the layers can be expected to influence the others. This leads to the concept that the load-bearing surface of the joint should be considered as a functional entity and should be studied as such, rather than as separate layers.
In Chapter II of this thesis this concept is applied to the load-bearing surface of the equine proximal phalanx in healthy, mature individuals. The biochemical composition of cartilage, subchondral bone and trabecular bone was analysed in detail at two different sites that had previously been shown to have very different

loading patterns (Brama *et al.* 2001a). Unlike in cartilage, in the bony layers much attention was paid to the mineral components, as these form the major fraction of the ECM of bone and, together with the collagen component, provide the structure and strength of bone. Clear site differences could be demonstrated for virtually all biochemical parameters. This was true for both the collagenous and non-collagenous components of the organic ECM, but also for the mineral component, which showed a comparable heterogeneity in relation to differences in sustained load. When comparing the bony layers, site differences were more outspoken in the subchondral bone than in the trabecular bone. Further, of the significant site differences found in the collagen parameters in the cartilage layer (hydroxylysine content, hydroxylysylpyridinoline (HP) cross-links, lysylpyridinoline (LP) cross-links, and the relative amount of denatured collagen), similar differences were encountered in the subchondral bone layer in three of these parameters (hydroxylysine and the two cross-links), but only in one (HP cross-links) in the trabecular layer. This might indicate a closer relationship between cartilage and subchondral bone than between trabecular bone and the other two layers.
The next question to be addressed was the developmental aspect. In the horse, the specific site differences in cartilage develop during the first year of life, under the influence of weight-bearing and articulation (Brama *et al.* 2000a). After one year the collagen skeleton of the cartilage layer is more or less mature and, because the collagen turnover is extremely low in cartilage, any further development will be limited (Brama *et al.* 2002a, Maroudas *et al.* 1992, Verzijl *et al.* 2000). Chapter III investigates whether this same time frame also applies to the bony components of the joint. It could indeed be demonstrated that for the collagen parameters in subchondral and trabecular bone a similar time frame applies. Collagen parameters in all layers that make up the load-bearing surface of the joint appear to have reached maturity by one year of age and after that do not change significantly, again stressing the close relationship between these layers. However, the situation is different with respect to the inorganic component. Here, development has not yet ceased by one year of age, but continues until the age of four. Bone mineral density, ash content and calcium content all continued to increase until the age of 4 years and remained steady beyond that age. Magnesium, of which it is known that the amount associated with the apatitic phase is higher at the beginning of the calcification process and then gradually decreases (Bigi *et al.* 1992, Bigi *et al.* 1997), followed an inverse pattern: a decrease until the age of 4 years followed by stable levels.
The finding that the development of the collagen network precedes mineralisation is in line with other studies that reported collagen to have an initiating and regulating role in the mineralisation process (Knott and Bailey 1998, Wassen *et al.* 2000). However, because it is the combination of the collagen and minerals that gives bone its characteristic mechanical properties, it must be concluded that before the age of four years the tissue is not yet fully adapted to face maximal biomechanical challenges. Given the intricate functional relationship between bone

and the overlying cartilage, too much loading before the joint has reached complete maturity may result in damage to both cartilage and bone. This is of more than only academic importance. The long maturation process of bone may be related to the high incidence of bucked shins and slab and chip fractures in the carpal and fetlock joints in Thoroughbreds and Standardbreds, two breeds in which training (and racing) starts at a very early age (McIlwraith 1996b and 1996c, Nunamaker 1996). Whereas the primary bone failure in these cases may heal completely, the secondary cartilage damage increases the risk of the development of OA in these joints. It seems justified to conclude that, while a certain amount of exercise is necessary for the proper development of the equine joint, excessive loading before the age of 4 years should be considered detrimental.

Inseparable in function and failure

From Chapters II and III it is clear that the tissue layers that constitute the load-bearing surface of the joint act as a functional entity in healthy mature horses and during joint development. It seems logical to presume that this may also be the case in pathologic processes like OA. Indeed, several studies have suggested that there is a close interaction between the progression of joint cartilage degeneration and remodelling of subchondral bone (Murray *et al.* 2001b, Radin and Rose 1986, Radin 1999). However, there is still debate about the site of the primary event in OA. Osteoarthritis in its final stages is characterised by a progressive deterioration of the cartilage layer combined with changes in the bone layer and the surrounding soft tissue, but there are conflicting reports about the initial phase of the disease. Many studies have focused on the alterations in the articular cartilage in OA; less on changes in subchondral bone, because these have been considered secondary. Bone changes in advanced OA, such as sclerosis, cyst and osteophyte formation, are well defined (Anastasiou *et al.* 2003, Burr 1998, Goker *et al.* 2000, Lane and Nevitt 2002, Murray *et al.* 2001b, Oettmeier *et al.* 1989a, Oettmeier and Abendroth 1989b), but at that stage it is impossible to conclude if cartilage changes initiate the bone changes or *vice versa*. We deliberately analysed all 3 layers simultaneously in samples from animals with no more than early or mild OA and found biochemical changes in all of them (Chapter IV). They were, however, most common in subchondral bone and articular cartilage. The trabecular bone showed only minor changes. This may be explained by the fact that impact loads are absorbed by cartilage, subchondral bone and the surrounding soft tissue before being transmitted to the underlying trabecular bone (Radin *et al.* 1970). Another explanation why OA-related changes in trabecular bone are less evident than in subchondral bone may be the overall higher remodelling rate of this layer in OA and non-OA joints (Layton *et al.* 1988, Mosekilde 1993, Zoetis *et al.* 2003). In subchondral bone OA-affected specimens had a higher hydroxylysine content, a substantially lower number of HP cross-links (almost 20% in the central area of the

joint surface); in cartilage with signs of early OA there was only a lower glycosaminoglycan (GAG) content. Therefore, without giving a definitive answer to the question where OA starts, our data suggest that the subchondral bone layer may be more important than previously thought by many researchers. A possible mechanism may involve subchondral bone sclerosis, leading to the bone layer losing its elastic properties. This could result in progressive cartilage erosion and ulceration, because loads are transferred back from the bone layer to the cartilage (Dedrick *et al.* 1993, Norrdin *et al.* 1998, Norrdin *et al.* 1999).

Another contentious issue in the pathogenesis of OA is the relative importance of inflammatory processes. Classically, OA has been viewed as a mainly degenerative disorder characterised by deterioration of articular cartilage, but recent insights in the molecular mechanisms involved strongly question the non-inflammatory character of the disease (Attur *et al.* 2002). Further, it is known that specific inflammatory products from the synovial fluid induce cartilage degeneration (Hooiveld *et al.* 2004, Nakamura *et al.* 1999) and that repetitive loading can induce chronic synovitis. These considerations led us to examine inflammatory mediators and products of ECM metabolism in synovial fluid from joints affected by OA (Chapter V). There was a fair correlation between general matrix metalloproteinase (MMP) activity and the degree of cartilage degeneration ($r=0.46$, $p=0.03$) and a good correlation between MMP activity and the collagen degradation product hydroxyproline ($r=0.72$, $p<0.001$). This finding suggests general MMP activity is a good biomarker for OA. However, it must be realised that MMP activity is more indicative of the actual activity status of a joint, whereas cartilage damage is the accumulated result of repeated insults to the articular cartilage (Caterson *et al.* 2000). The origin of the MMPs is not clear. They may originate from the synovial membrane, as well as from the chondrocytes and then diffuse to the synovial fluid. A similar process has been described for cytokines, growth factors, prostaglandins and other inflammatory mediators that are produced in bone tissue and which can migrate into the cartilage layer (Massicotte *et al.* 2002).

Nitric oxide (NO) is an indicator of tissue metabolism and the nitrosylation product nitrotyrosine provides a more direct indication of tissue damage induced by oxidative stress, and thus of pathology. Levels of NO were elevated in all tissue layers of juvenile horses, indicating the high tissue turnover in this category of animals (Chapter VI). The Nitrotyrosine content showed a five-fold increase in the subchondral bone layer of OA-affected joints compared to tissue from normal, mature joints, which is a further indication of the important role of this layer in (early) OA.

Chapters V and VI both support the vision that OA has a substantial inflammatory component and is more than a merely degenerative disorder. Further, it can be concluded from Chapters IV-VI that, although there is no doubt that damage to the cartilage layer plays an essential part in the pathogenesis of OA, it is important to take the surrounding tissues into account. Particularly the cartilage and subchondral bone layers are so tightly connected that the question can be posed

whether it will ever be possible to make a clear distinction between events in these layers and to pinpoint unequivocally one of the two as the site where OA starts. Perhaps these layers are as inseparable as Siamese twins and the initial damage can originate in either of them, but only proceed when the other layer is somehow also affected. In fact, the question where OA starts becomes a semantic issue when the joint is regarded as a functional entity.

From the present age to a future world…..

The title of the first paragraph of this thesis *"From Hippocrates to the present age"* is a citation from the well-known lecture on cartilage that William Hunter gave in June 1743 for the Royal Society of London. Hippocrates (460-367 BC) lived not too long after the onset of modern scientific thinking that originated in the port towns of Asia Minor and nearby islands in the 7th century BC (Dunlop and Williams 1996). The great Greek philosophers observed nature in its broadest sense and compiled theories that served for discussion afterwards. They did not dedicate themselves to experimental testing: natural phenomena were black boxes with a visible input and a visible output from which general rules and laws could be deduced. The actual processes going on in the black boxes remained obscure. Modern Western science is characterised by an emphasis on experimental testing, and is thus forced to use a reductionist approach, as the reality is too complex for controlled and repeatable experimentation. In this way we have gathered a tremendous amount of information with regard to a myriad of natural phenomena in the past few centuries. Modern technology helps us to study natural processes down to the molecular and even sub-molecular level and we now know that there are stunning amounts of events going on in the black boxes. The new challenge is to put the pieces together and go back to the original subject of study. Reductionism may be a hallmark of modern science; nature knows only the holistic concept. In order to appreciate this detailed knowledge of local phenomena is necessary and even indispensable, but should be interrelated and put together to understand general reaction patterns of organs or organisms.
This thesis is a step, admittedly crude and modest, in that direction. The thesis has confirmed that the joint should be viewed as a functional entity. It has also shown that the functional relationship between some layers is tighter than between others. From the work in this thesis it has become clear that the subchondral bone and cartilage layers are so tightly related in healthy mature, and in developing and diseased animals that probably every approach in which these layers are separately analysed should be considered as artificial. However, it should be recognised that considering only these two layers as a functional entity is probably also insufficient, as reaction patterns from other relevant tissues, especially the synovial membrane, also have to be taken into account. The road from the present age to a future world, in which we will know in detail the processes occurring in the

various tissues based on state of the art molecular biology and genomics and will be able to integrate this information to the level of the entire joint using the advances in information technology, may still seem long. However, essential fundamental obstacles no longer exist and this road is well worth travelling as the outcome will be of great benefit in the treatment, but above all in the prevention, of joint disease in man and horse.

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English summary

Introduction

The role of the horse in society has changed considerably in recent times from a utilitarian working animal to an equine athlete. However, regardless of this change, lameness in general and osteoarthritis (OA) in particular have remained predominant causes of early retirement of these animals. Adequate preventive measures for joint-related problems such as OA have not yet been found. This is largely due to the lack of detailed knowledge of the complex functioning of the joint and consequently of the exact pathogenesis of chronic degenerative disorders like OA. The horse is of specific interest in the area of joint disease because of the impact of joint-related disorders on the functioning and welfare of the species itself, and because of the important parallels to the human situation.
The synovial joints facilitate predictable and energy-efficient movement and form part of the musculoskeletal system that gives structure to the body and transmits the, often considerable, loads that are generated during weight bearing and locomotion. These complex functions cannot be performed by the cartilage layer alone, but require that this layer acts together with the bone layer; the former acting as the surface layer receiving primary impact and the latter as a structural girder and shock absorber. These considerations lead to the theoretical concept that the load-bearing surface of the joint should be seen as a functional entity and therefore should be studied integrally and not as separate layers, as is conventionally done.

The fetlock joint

The main goal of this thesis was to determine whether this concept holds, *i.e.* whether there is indeed a functional connection between the three constituting elements of the load-bearing surface of the joint (articular cartilage, subchondral and trabecular bone). To this end, first an extensive simultaneous biochemical analysis was performed of these layers to assess the normal biochemical composition in a group of mature animals without signs of disease. It was a deliberate choice to analyse tissue layers at two sites that were known to be very differently loaded. The first site (site 1) has been shown to be loaded only at high speeds, or during heavy athletic activity. The second site is continuously loaded in the standing horse. The equine fetlock joint was selected as the joint of interest because of the large body of detailed knowledge that has been built up over the last decade with respect to this joint within our Department's research group, in a combined effort with collaborating institutions. After the detailed analysis of samples from normal mature individuals, juvenile individuals in which there still is development and growth, and individuals affected by (early) OA were to be studied in the hope to contribute to our understanding of joint physiology and pathophysiology.

From the study in sound, mature animals (Chapter II) it emerged that there was a distinct topographical variation, matching with the variation in biomechanical loading as generated by locomotion, in many biochemical parameters of articular cartilage, subchondral bone and trabecular bone at differently loaded sites. In general, site 2 (the continually loaded area) was characterised by stronger and stiffer tissue with higher mineral contents and a denser cross-link distribution. Towards the joint margin (site 1) stiffness diminished and more elasticity could be expected based on the biochemical composition. Knowledge of these topographical differences in the various layers and of their interrelationship is indispensable for the study of pathological processes that take place at and underneath the joint surface.

Joint development

In chapter III, which focuses on young, growing animals, we hypothesized that developmental changes would be found in all layers, but that they might follow a different timescale. Attention was focused on those elements of the extracellular matrix (ECM) that are known to contribute directly to the biomechanical strength of the tissues, *i.e.* collagen and minerals. We concluded that great similarities exist in the time path of maturation of the collagen part of the matrix of all three layers that make up the bearing surface of the joint: articular cartilage, subchondral bone and trabecular bone. No essential alterations in post-translational modifications of collagen took place after the age of approximately one year. However, the mineralization process of both the subchondral bone and the underlying trabecular bone does not reach its final, mature state before the age of approximately 4 years. The disparity in maturity of the various ECM components can be assumed to have consequences for the loading capacity of the tissue and hence for the impact of training and racing. Knowledge of the influence of exercise on the development of the musculoskeletal system is essential for the equine industry, especially in those branches of equestrian activity in which training starts at an age when the animal is still growing, such as Thoroughbred and Standardbred racing. There seems to be a delicate balance representing an optimum that has not yet been well established.

Normal joints versus osteoarthritic joints

When comparing normal joints with those showing early and mild stages of OA (Chapter IV), we demonstrated that there were changes in all three layers that together constitute the bearing surface of the joint. Most changes could be found at different sites in the different layers and included the proteoglycan component of the cartilage layer and characteristics of the collagen network in both bony layers. Alterations in any of the mineral-related parameters were only observed in

subchondral bone and were relatively minimal, suggesting that these develop at a later stage of the disease. The fact that most changes were detected in the subchondral bone plate and to a lesser extent in cartilage and trabecular bone may perhaps be indicative of the sequence in which changes occur.

Synovial fluid

Classically, OA has been viewed as a mainly degenerative disorder characterised by deterioration of articular cartilage, but recent insights into the molecular mechanisms that are involved strongly question the non-inflammatory character of the disease. These considerations led us to examine inflammatory mediators and products of ECM metabolism in synovial fluid from joints affected by OA (chapter V). Synovial fluid is relatively easy to obtain from equine joints and marker levels are thus potentially of great clinical value. Synovial fluid contains inflammatory mediators such as cytokines, eicosanoids, free radicals, and growth factors and metabolites or tissue components, and is thus the principal source of candidate molecules in the ongoing search for biomarkers of (early) joint disease. Cartilage degradation is likely to result in the release of biochemical components of the extracellular matrix into the synovial fluid. If markers are to be used reliably, it is therefore important to know the relationship between marker concentration and the disease status of the cartilage. Based on the results from this study it can be stated that synovial glycosaminoglycan (GAG) levels are related neither to the severity of cartilage change, nor to GAG content of articular cartilage. They can hence be discarded as reliable markers for cartilage damage. However, hydroxyproline levels, while not related to the amount of denatured collagen in cartilage, are strongly related to MMP-activity and thus may be useful as an indication of the actual disease status. General MMP-activity appeared to be best related to the condition of the articular cartilage of all potential markers tested, but still seems to be a better indicator of the activity of the process than of the cartilage status in chronic cases.

Nitrite and nitrotyrosine in cartilage and bone

In chapter VI we returned to the tissue level, and used new, as yet unexplored parameters in the form of metabolites of nitric oxide (NO) for the detection of pathological processes in the three layers of the joint surface. Interesting results were obtained from which it was concluded that the patterns of nitrite and nitrotyrosine concentrations in the three tissue layers show great similarities. In the cartilage and subchondral bone layers these patterns were almost identical, which stresses the importance of envisaging the joint as a functional entity. Nitrite levels were increased in most layers in both the juvenile and OA-affected group,

suggesting that this parameter can be used as a measure for the intensity of metabolic activity, but not to discriminate between physiological and pathological events. The fact that nitrotyrosine levels, of which it is presumed that these give information about pathological processes, were increased in the subchondral layer of OA-affected animals may indicate the important role of this layer in (early) OA. The combination of NO and nitrotyrosine data may give information on both metabolic and disease status of tissues.

Interdependence

It can be concluded that the cartilage, subchondral bone and trabecular bone layers of the joint surface indeed have many things in common in sound and mature animals with respect to their biochemical make-up. They also share common reaction patterns and act together in development and disease. Furthermore, changes in one of the layers can be expected to influence the others. This confirms the concept that the load-bearing surface of the joint should be considered as a functional entity and should be studied as such, rather than as separate layers.

Nederlandse samenvatting

Inleiding

De functie van het paard in de maatschappij is in de loop van de tijd veranderd. Het paard werd vroeger gebruikt als transportmiddel of voor het bewerken van het land. Tegenwoordig wordt in de westerse maatschappij het paard hoofdzakelijk gebruikt als recreatie- of sportpaard. Onafhankelijk van de periode waarin het paard leefde betekenden kreupelheden in het algemeen en de gewrichtsaandoening artrose in het bijzonder, vaak het einde van de carrière van het paard. Voor gewrichtsaandoeningen zoals artrose zijn nog geen goede behandelingen bekend, om nog maar niet te spreken over een goede leidraad voor de preventie ervan. Dit komt onder andere omdat onvoldoende bekend is over het functioneren van een gewricht en de ontstaanswijze van artrose.
Het gewricht heeft naast een scharnierende ook een dragende functie. Een gewricht brengt bij belasting en beweging de krachten van het ene skeletdeel over op het aangrenzende deel. Hierbij zorgt de relatief dunne laag gewrichtskraakbeen voor het makkelijk over elkaar glijden van de skeletdelen. Om de krachten die op een gewricht komen te staan op te kunnen vangen, is het kraakbeen afhankelijk van het onderliggende bot. Dat bot heeft dus een zeer belangrijke ondersteunende functie.

Kootgewricht

Voor het onderzoek, beschreven in dit proefschrift, is gekozen om het kootgewricht van het paard nader te onderzoeken in relatie tot de aandoening artrose. De reden dat voor dit gewricht is gekozen is tweeledig. Ten eerste is dit gewricht vaak door artrose aangedaan en ten tweede is door onderzoek in het verleden binnen ons instituut reeds veel bekend geworden over het kootgewricht en zijn aandoeningen. De resultaten van dit onderzoek kunnen op zichzelf bekeken worden maar fungeren bovendien als een schakel tussen onderzoek uit het verleden en onderzoek in de toekomst. Dit promotieonderzoek zal hopelijk een bijdrage leveren aan het ophelderen van vragen in het kader van gewrichtsfysiologie en -pathologie. Centraal staat de vraag, of er aanwijzingen zijn die duiden op een samenhang tussen de lagen kraakbeen, subchondraal bot, en trabeculair bot. Hiervoor zijn diverse biochemische bepalingen uitgevoerd op bot- en kraakbeenmonsters van paarden die nog in de groei waren, volwassen gezonde paarden, maar ook van paarden met kenmerken van artrose. Als leidraad fungeerde de samenstelling van kraakbeen en bot bij gezonde, of in elk geval artrose-vrije, volwassen paarden (hoofdstuk II). Op deze wijze ontstond er een soort blauwdruk van de drie lagen die het gewricht de samenstelling geven waardoor het de complexe functie kan vervullen. Er is voor gekozen om van het gewricht twee locaties te nemen die verschillend belast worden en deze onderling te vergelijken. De eerste locatie (site 1) ligt aan de voorrand van het kootbeen en hiervan is ondermeer gebleken dat

deze niet wordt belast tijdens rust of lichte arbeid maar alleen gedurende zware inspanning. Daarnaast is van deze locatie bekend dat artrose in het algemeen hier aanvangt. Daarentegen is van de tweede locatie (site 2), centraal in gewrichtsvlak gelegen, bekend dat deze (als het paard staat) continu wordt belast en dat dit punt als laatste wordt aangetast door artrose. Uit het onderzoek beschreven in hoofdstuk II bleek dat er een duidelijke variatie in biochemische samenstelling bestaat tussen de twee onderzochte locaties. Mogelijk zijn deze verschillen in samenstelling te verklaren als een aanpassing op de belastingsverschillen tussen de twee locaties. Het is uit eerder onderzoek bekend dat verschillende locaties van kraakbeen en subchondraal bot bij de geboorte nog een identieke biochemische samenstelling hebben maar zich na de geboorte verschillend ontwikkelen, mogelijk door andere biomechanische eigenschappen. Deze waarschijnlijk door belasting geïnduceerde biochemische aanpassing is niet alleen in het kraakbeen aanwezig maar ook in het eronder gelegen subchondrale en trabeculaire bot. De continue belaste locatie (site 2) heeft in bot een hoger mineraal gehalte met meer verbindingen tussen de collageenmoleculen dan site 1. Door deze samenstelling zal site 2 stevig genoeg zijn om zo de continue belasting te weerstaan. Daarentegen heeft site 1 in alle lagen een dusdanige biochemische samenstelling dat deze elastischer is, waardoor het de hoge piekbelastingen kan opvangen.

Ontwikkeling gewrichtslagen

In hoofdstuk III zijn de resultaten van de kraakbeen- en botsamenstelling van gezonde volwassen paarden vergeleken met niet volgroeide en artrosevrije paardengewrichten. De hypothese van dit onderzoek was dat in de gewrichtslagen van niet volgroeide dieren nog een zekere ontwikkeling gaande is en dat deze ontwikkeling in de verschillende lagen niet op hetzelfde tijdstip eindigt. Ook werd in dit onderzoek gekeken naar de hoofdbestanddelen van het weefsel, namelijk de collageensamenstelling en in bot ook naar de samenstelling wat betreft mineralen. Opvallend in de uitkomst was dat in alle drie de lagen het collageenskelet na het eerste levensjaar niet meer essentieel veranderde. Geconcludeerd kan worden dat in deze lagen de ontwikkeling van collageen eenzelfde tijdspad doorloopt. Maar de samenstelling qua mineralen is na één jaar zeker nog niet volledig ontwikkeld. Deze ontwikkeling zet zich voort tot ongeveer de leeftijd van vier jaar. Hierdoor zal het botweefsel zeker tot de leeftijd van vier jaar nog niet de sterkte hebben zoals die aanwezig is bij de volwassen paarden. Het bot kan nog niet een extreme belasting die op het kraakbeen wordt uitgeoefend, ondersteunen. Een hoge belasting tijdens inspanning op een gewricht voordat het volledig is volgroeid, kan tot gevolg hebben dat hierbij (mogelijk onherstelbare) beschadigingen optreden. Dit gegeven is met name van belang bij diverse takken van paardensport waarbij het gebruikelijk is dat paarden al op jonge leeftijd onderworpen worden aan hoge belasting.

Gezond versus artrotisch

In een vervolgonderzoek (hoofdstuk IV) is op dezelfde locaties en in dezelfde drie lagen gekeken naar de verschillen tussen gezonde en artrotische gewrichten. Bij de artrotische gewrichten waren in alle lagen verschillen waar te nemen. In kraakbeen was de hoeveelheid proteoglycanen verminderd en in de twee botlagen waren collageenwijzigingen gevonden. Bij de mineralen was alleen in het subchondrale bot minimale verandering te zien. Dit duidt erop dat in eerste instantie bij artrose alleen het collageen wordt aangetast en mogelijk in een later stadium de mineraal component. Het feit dat bij beginnende artrose in alle lagen verschuivingen optreden, maar dat alleen bij subchondraal bot ook veranderingen (weliswaar minimaal) op mineraal niveau waarneembaar zijn kan duiden op een belangrijke rol van deze laag in het kader van artrose.

Onderzoek gewrichtsvloeistof

Oorspronkelijk werd artrose gezien als een degeneratieve aandoening maar steeds vaker wijzen onderzoeken erop dat ontsteking een belangrijke rol speelt. Het is zeer goed voorstelbaar dat vanuit het kraakbeen degeneratieproducten danwel ontstekingsproducten in de gewrichtsvloeistof komen. Indien het mogelijk zou zijn om deze producten in gewrichtsvloeistof te meten, zou wellicht in de praktijk artrose in een vroeg stadium aangetoond kunnen worden. Dit uitgangspunt is gebruikt in hoofdstuk V. Het is bekend dat gewrichtsvloeistof verschillende stoffen kan bevatten zoals bijvoorbeeld cytokinen, vrije radicalen, groeifactoren en bestanddelen van het kraakbeen. Gewrichtsvloeistof is zeker bij paarden betrekkelijk eenvoudig en in relatief grote hoeveelheden te verzamelen. Door het meten van de concentratie van kraakbeenbestanddelen in de gewrichtsvloeistof of van enzymen die van belang zijn bij de kraakbeenafbraak, is het misschien mogelijk om een indruk te krijgen van de gezondheidsstatus van het gewricht. Met de resultaten uit dit hoofdstuk kan geconcludeerd worden dat, alhoewel in gewrichten met artrose een daling van de hoeveelheid glycosaminoglycanen van kraakbeen optreedt (hoofdstuk IV) dit niet meetbaar is in gewrichtsvloeistof met een verandering in de hoeveelheid glycosaminoglycanen. Dit wijst erop dat de concentratie van glycosaminoglycanen in de gewrichtsvloeistof geen goede afspiegeling is van de hoeveelheid in het kraakbeen en dus ook niet als indicator voor de mate van artrose gebruikt kan worden. Daarnaast is in de artrosegroep in tegenstelling tot in de artrosevrije groep de concentratie van hydroxyproline in de gewrichtsvloeistof goed gecorreleerd aan de matrix metalloproteinasen (MMP) activiteit. Uit deze resultaten kan geconcludeerd dat MMP een belangrijke rol speelt bij de degeneratie van collageen.

Stikstofoxide en nitrotyrosine in kraakbeen en bot

Ook op weefselniveau zijn er aanwijzingen dat er bij artrose sprake is van een ontsteking. In hoofdstuk VI is gekeken naar de hoeveelheid stikstofoxide (NO) in de verschillende gewrichtslagen. Stikstofoxide is een product dat vrijkomt in fysiologische en pathologische processen. Bij de artrose groep is dit product in alle lagen duidelijk hoger, echter het feit dat bij groeiende paarden ook een verhoging te zien is, geeft aan dat NO gezien moet worden als aanwijzing voor verhoogd metabolisme. Nitrotyrosine is een metaboliet die gevormd wordt onder invloed van NO uit het aminozuur tyrosine en het is bekend dat nitrotyrosine een goede maat is voor pathologische processen. De resultaten van dit onderzoek laten zien dat nitrotyrosine is verhoogd in het subchondrale bot van de artrosegroep. Dit suggereert andermaal de betrokkenheid van deze laag in het kader van artrose. Hieruit kan geconcludeerd worden dat de combinatie van NO en nitrotyrosine een goede maat is voor de metabole activiteit en voor het ziektestadium van het gewricht.

Interdependentie

Tenslotte kan geconcludeerd worden dat bij gezonde volwassen paarden het kraakbeen, subchondraal bot en trabeculair bot van een gewricht veel overeenkomsten vertonen in hun biochemische samenstelling. Daarnaast vertonen de drie betreffende lagen een overeenkomstig reactiepatroon tijdens groei en ziekte. We kunnen veronderstellen dat een verandering in één van de gewrichtslagen van invloed is op de overige lagen. Deze bevindingen ondersteunen het concept dat een gewricht gevormd wordt door een nauwe interactie of zo men wil interdependentie van de verschillende lagen. Bij gewrichtsonderzoek dient men hiermee rekening te houden en het verdient dan ook de aanbeveling dat deze lagen in plaats van afzonderlijk als geheel bekeken worden.

Dankwoord

Vaak wordt gekscherend gezegd dat het dankwoord één van de meest gelezen onderdelen van een proefschrift is. Op mij rust dus een zekere druk en ik zal proberen met woorden aan te geven hoezeer ieders bijdrage is gewaardeerd en van belang is geweest bij de totstandkoming van dit werk. Kan ik in enkele woorden iets opschrijven waarin de meeste mensen zich terugvinden op een herkenbare wijze en de juiste plaats? Ik moet toegeven dat deze vraag mij met enige schroom vervulde om aan dit dankwoord te beginnen en ik wil mij excuseren voor het geval iemand zich niet of onvoldoende herkent.

Laten we beginnen bij het begin. Mijn ouders wil ik bedanken omdat zij voor een groot deel hebben bijgedragen aan de vorming van de persoon die ik nu ben. Daar ben ik dankbaar voor en vooral zeer trots op. Ik dank hen voor de nimmer aflatende steun bij al mijn keuzes door de jaren heen.

Hooggeleerde Professor Barneveld, beste Ab, ik wil je danken voor de afgelopen acht jaar. Op jouw afdeling, nu geheten Hoofdafdeling Gezondheidszorg Paard, ben ik in eerste instantie begonnen als specialist in opleiding chirurgie paard. Aan het einde van de specialistenopleiding heb je mij de mogelijkheid geboden om dit promotietraject in te gaan. Het resultaat hiervan ligt nu voor je. Ik wil je bedanken voor je goede adviezen en betrokkenheid bij mijn onderzoek. Onze besprekingen gedurende het onderzoek samen met de copromotoren hebben mijn werk gestroomlijnd en de voortgang zeker gesteld.

Copromotoren van het eerste uur, Pieter en René, ook aan jullie ben ik veel dank verschuldigd. Pieter, je weet dat ik het af en toe benauwd kreeg van al je enthousiaste ideeën. Ik begon aan het ene en even later had je weer een ander, volgens jou briljant, idee. Uiteindelijk is mijn onderzoek dan af en ik kijk voor mezelf op de afgelopen vier jaar terug als een boeiend leerproces. René, mag ik jou hartelijk danken voor je onbeschrijfelijke inzet in het leesbaar maken van de manuscripten. Je vermogen om de zaken zo op papier te zetten zoals ik het zou willen, is groot. Ik wens jullie beiden succes met het onderzoek voor de toekomst.
Chris, derde copromotor, het is me een eer dat je de vraag om mijn copromotor te worden hebt aanvaard. Ik herinner me nog het eerste overleg bij jou op de kamer over een aantal biochemische bepalingen die ik wilde gaan uitvoeren. Aan het einde van de bespreking keerde ik terug met onder de ene arm mijn ordner en onder de andere een fles zelf gebrouwen bier. Wauw, wat was dat een lekker maar heftig flesje! Dit begin was kenmerkend voor de rest van de samenwerking. Ik kwam op jouw afdeling met een ordner of mijn monsters en ging meestal terug met mooie resultaten. Als de bepalingen andere uitkomsten gaven dan verwacht, bleef jij altijd onbegrijpelijk rustig. Op dat soort momenten wilde ik het liefst het roer omgooien en direct verder met een ander onderdeel. Meestal suggereerde je dan eerst een bak koffie te nemen, en gedurende de koffie werd het liefst over andere

zaken gesproken. Vervolgens maakte je een kleine modificatie aan het protocol en warempel weer was er een stapje in de goede richting gemaakt.

TNO Preventie en Gezondheid, divisie biomedisch onderzoek in Leiden heeft een zeer groot deel van de bepalingen voor mijn proefschrift mogelijk gemaakt. Gedurende enkele maanden heb ik daar gebruik mogen maken van de aanwezige kennis en apparatuur en heb ik kunnen zien hoe professioneel deze divisie met wetenschappelijk onderzoek omgaat. Ik wil iedereen van TNO bedanken voor hun gastvrijheid maar een aantal personen wil ik graag apart noemen. Jeroen, als coördinator van alle bepalingen die ik daar heb uitgevoerd. Ik waardeer het zeer dat je ondanks je drukke werkzaamheden toch zoveel tijd hebt gestopt in het begeleiden van mijn onderzoek. Geesje en Benno, bedankt voor jullie grandioze hulp in het verwerken van die enorme lading monsters.
Ik wil de afdeling Farmacologie van N.V. Organon in Oss bedanken voor het verlenen van alle medewerking bij het meten van het mineraal gehalte in de verschillende botmonsters.

Op onze eigen afdeling zijn ook een aantal mensen die ik wil noemen. Marjory en Andries, jullie bijdrage aan dit onderzoek was fantastisch. Jullie hebben mij geholpen vanaf het allereerste moment. Het begon met het afzakken naar België om daar ondervoeten te “verzamelen”. Een tikje macaber was het wel; we zullen maar zeggen dat het een goed doel diende. Andries, onze volgende stap was het verzagen van de verzamelde benen tot hapklare brokjes. Dit was een riskante bezigheid zeker als je bedenkt dat ik mijn vingers in de toekomst nog wilde gebruiken als paardenchirurg. Ik zal de stapels literatuur die jij altijd hebt verzorgd gaan missen. Marjory, jij weet nu in ieder geval wat een microdismembrator is! Jouw inbreng gedurende het hele onderzoek is bewonderenswaardig, dank voor je inzet bij alle bepalingen. Jij hebt het onder andere mogelijk gemaakt dat ik in Leiden bij TNO bezig was, terwijl de bepalingen binnen de Hoofdafdeling door konden gaan. Zonder jouw hulp was de op papier zo ideale combinatie van kliniek en onderzoek het tegenovergestelde. Ik voel me een bevoorrecht mens dat ik met jullie beiden mocht samenwerken.

Hugo, door de mogelijkheid te krijgen om van jouw laboratorium en expertise gebruik te maken zijn de resultaten van de mineralen tot stand gekomen. De nauwkeurige manier van werken die je Marjory en mij hebt opgelegd, heeft zijn vruchten afgeworpen. Ik vond het één van de leukste en spannendste bepalingen en was onder de indruk van de ‘knowhow’ die jij hebt op het gebied van de mineraalhuishouding in skeletweefsel. Ik hoop dat in de toekomst deze kennis meer gebruikt gaat worden.
Harold, jij hebt de meetmethode van de kraakbeen degeneratie factor (CDI) ontwikkeld en uitgevoerd. Deze CDI heeft onder andere voor mijn onderzoek de mogelijkheid gecreëerd om de verschillende groepen van kraakbeendegeneratie te

formeren. Het was voor mij een mooie ervaring om samen met Robin en jou het promotietraject te doorlopen. Succes met je onderzoek en klinische werkzaamheden bij de Hoofdafdeling in de toekomst en met de laatste loodjes van je formidabele promotieonderzoek in het bijzonder.
Sarah, onze samenwerking begon al bij het “gentamicin almost never ending project”. Deze artikelen zijn nu een feit en jouw inzet heeft in ieder geval bijgedragen tot de “best research presentation” van het ECVS congres in 2001. Bij het uitdenken en uitvoeren van het stikstofoxide- en nitrotyrosineonderzoek is je inzet super geweest! Dit hoofdstuk is van grote waarde voor mijn proefschrift en ik denk dat in de toekomst uit dit onderwerp nog veel mooie resultaten kunnen komen.

Barbara, Eelke, Jos, Robin en Sarah mag ik jullie hartelijk danken voor het kritisch commentaar bij het doorlezen van verschillende delen van mijn proefschrift. Madelon en Martin dank voor jullie grafische bijdragen aan de figuren in mijn proefschrift. Ook dank voor de diverse lekkere en gezellige etentjes onder het motto ‘we moeten wat bespreken voor het proefschrift’. Yvette van de afdeling Diagnostische Beeldvorming, ik ben blij met je hulp bij het vervaardigen van de mooie CT-scan voor de omslag. Multimedia Centrum Diergeneeskunde jullie hebben mij enorm ontlast door de lay-out en de omslag te verzorgen. Ik hoop dat de meesten het net zo geslaagd vinden als ik zelf.
Robin, studiemaat, roeimaat, zeer fijne collega-maat, kamermaat, promovendusmaat maar vooral een goede maat. Ik waardeer onze vriendschap en alhoewel we beiden op een nieuwe en verschillende werkplek zullen zitten, hoop ik dat de vriendschap nog lang voortduurt.

Piet, Greet en Peter; het verlaten van Bunnik valt me om meerdere redenen zwaar. Eén van de redenen is dat het zomaar bij jullie aanwaaien in de toekomst iets minder gemakkelijk zal gaan. Het was altijd weer heerlijk om na een dag werken op de Uithof bij jullie langs te kunnen komen en me thuis te voelen. Hoewel ik iets verder weg kom te wonen zal ik proberen de tuigpaarden en jullie prestaties op de hoef te blijven volgen.
Ik wil mijn paranimfen, Barbara en Marjory, bedanken voor hun inzet en steun tijdens de laatste fase van mijn promotieonderzoek. Jullie hebben een enorme bijdrage geleverd in dit voor mij feestelijke moment. Ik hoop dat jullie hier net zo van genieten als ik.
Alle collega’s van de Hoofdafdeling Gezondheidszorg Paard wil ik bedanken. Terwijl jullie de kliniek draaiende hielden, heb ik de unieke mogelijkheid gekregen om veel tijd aan mijn onderzoek te kunnen besteden. Voor de toekomst hoop ik dat ondanks alle veranderingen binnen de Hoofdafdeling, de huidige en toekomstige onderzoeker ook de mogelijkheid krijgt om op professionele wijze onderzoek uit te voeren. Ik besef terdege dat zonder jullie hulp dit proefschrift nooit binnen vier jaar af had kunnen zijn. Ik ben dankbaar voor jullie grote collegialiteit en in het bijzonder

wil ik jullie bedanken voor de flexibiliteit die er was om diensten over en weer op te vangen. Last but not least wil ik Thea, Marloes en Birgit hartelijk bedanken voor hun steun en toeverlaat. Het was een eer om met jullie samen te werken. Tevens heb ik van jullie geleerd om tijdens de spoeddiensten het hoofd koel te houden. Ik zal de weekevaluaties op vrijdagmiddag missen! Meiden jullie zijn "the best!". Ik wens alle collega's succes en sterkte voor de toekomst en hopelijk betekent mijn vertrek niet 'uit het oog uit het hart'!

De afgelopen jaren is veel tijd in mijn werk gaan zitten. Dit zal wel niet veranderen in de toekomst, want nieuwe uitdagingen hebben zich al aangediend. Op mijn nieuwe werkplek in "Dierenhospitaal Visdonk" is mij het afgelopen jaar alle medewerking verleend om eerst mijn promotie te kunnen afronden, alvorens definitief naar Roosendaal te vertrekken. Ik wil mijn nieuwe collega's bedanken voor hun geduld. Ik verheug me op het feit dat ik me nu wel volledig kan gaan storten op mijn werk in West-Brabant en omstreken.

Vrienden, familieleden en bekenden, mijn nieuwe werk zal veel tijd gaan kosten maar diegenen die mij kennen, weten dat ik dit werk zo graag doe. Wel zal ik streven naar een gezonde balans tussen werk en privé. Indien dit in de toekomst volgens jullie niet lijkt te gaan lukken, roep dan tegen mij: "Was je maar ambtenaar gebleven". Ik zal je zeggen, die hint komt dan wel door!

Curriculum Vitae

Mark van der Harst werd op 8 juni 1969 geboren te Laren (NH). In 1988 behaalde hij het VWO diploma aan het Willem de Zwijger College te Bussum. In 1988 begon hij de studie Diergeneeskunde aan de Universiteit Utrecht. Tijdens zijn studie Diergeneeskunde is hij in 1992 begonnen met de studie Rechtsgeleerdheid aan de Universiteit Utrecht en heeft dit in 1993 afgesloten met het behalen van het propadeutisch examen. Zijn dierenartsexamen werd behaald in 1996. Sinds 1997 is hij werkzaam als Specialist in Opleiding, Chirurgie Paard op de Hoofddafdeling Gezondheidszorg Paard (HGP) van de Faculteit der Diergeneeskunde, Universiteit Utrecht. In 2001 is hij begonnen met een promotie onderzoek bij betreffende Hoofdafdeling Gezondheidszorg Paard. Dit onderzoek werd verricht onder begeleiding van Prof. dr. A. Barneveld; dr. P.R. van Weeren; dr. P.A.J. Brama en dr. C.H.A. van de Lest en de resultaten zijn beschreven in dit proefschrift. Sinds 2003 is de auteur van dit proefschrift geregistreerd als Specialist Chirurgie van het Paard bij de Koninklijke Nederlandse maatschappij voor Diergeneeskunde.

Mark van der Harst was born on 8th June in Laren (NH). In 1988 he passed his final examination at the Grammar School “Willem de Zwijger College” in Bussum. In that same year he started his studies of Veterinary Medicine at Utrecht University. During this study he also studied law for one year, passing his examination in 1993. In 1996 he graduated in Veterinary Medicine and since 1997 has worked at the Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University. In 2001 he commenced his PhD study at the same Department supervised by Prof. dr. A. Barneveld, dr. P.R. van Weeren, dr. P.A.J. Brama and dr. C.H.A. van de Lest, the results of which are described in this PhD book. Since 2003 he has been registered as a Diplomate in Equine Surgery by the Royal Dutch Veterinary Association.