Havemeyer Foundation
Monograph Series No. 22

Proceedings of a Workshop on

EQUINE MUSCULOSKELETAL BIOMARKERS

30th October – 2nd November 2005
Colorado, USA

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Suites 3 & 4, 8 Kings Court, Willie Snaith Road, Newmarket, Suffolk CB8 7SG, UK

First published 2008

ISSN 1472-3158

Published by **R & W Communications**

Printed in Great Britain by Quality Print Services (Anglia) Limited

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EDITORS' FOREWORD

Over the past 10 years there has been considerable progress in the evaluation of synovial fluid and serum biomarkers and their usefulness in the management of musculoskeletal disease. Biomarkers have the following potential uses: 1. Improving our knowledge of the pathogenesis of equine joint disease, and associated pathological conditions of cartilage, bone and tendon; 2. Diagnosis of early disease in these tissues; and 3. The ability to monitor objectively responses to therapy both in experimental models and clinical cases. The state of knowledge in this area was originally assessed in a workshop on molecular markers of cartilage and bone metabolism in the horse in Northampton, UK in 2000. This workshop was organised by Joanna Price, Wayne McIlwraith, Stina Ekman and Leo Jeffcott. Pioneers of biomarkers in human orthopaedics, Robin Poole, Bruce Caterson, Mike Bayliss, Dick Heinegard, and Patrick Garnero also participated.

That workshop was sponsored by the Horseracing Betting Levy Board UK and Bayer Animal Health US, as well as the Research Committee of the Swedish Horse Racing Totalizator Board (ATG).

Since 2000, considerably more progress has been made and this symposium was organised to bring back key workers in this area to assess how progress had been made and where truly biomarkers fitted into our clinical armamentarium. This workshop was sponsored by the Dorothy Russell Havemeyer Foundation, with secondary assistance for travel by IDEXX. Considerably more work had been done by equine researchers and we were also fortunate to be able coax Professors Bruce Caterson and Dick Heinegard back to this symposium to correlate parallel advances of biomarkers in human orthopaedics. The willingness of the Havemeyer Foundation to support workshops of this nature is applauded by all concerned and the additional sponsoring of a monograph to ensure the information gained is disseminated to the widest possible audience is also appreciated. I express sincere thanks to the Foundation, in particular, to Mr Gene Pranzo, President of the Foundation whose continued enthusiasm and encouragement are much appreciated. Mr Pranzo was so impressed by the quality of the meeting, as well as the social programme that another symposium is planned in 4 years time.

Wayne McIlwraith
Workshop Organiser

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1982 **Second International Workshop on Lymphocyte Alloantigens of the Horse**
October - Cornell University, Ithaca, New York, USA
Organiser: Dr D. F. Antczak

1983 **Third International Workshop on Lymphocyte Alloantigens of the Horse**
April - New Bolton Center, University of Pennsylvania, USA
Organiser: Dr D. F. Antczak

1984 **First International Symposium on Equine Embryo Transfer**
October - Cornell University, Ithaca, New York, USA
Organisers: Drs D. F. Antczak and W. R. Allen

1985 **Fourth International Workshop on Lymphocyte Alloantigens of the Horse**
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1999

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Organiser: E. Squires

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October - Pardubice, Czech Republic
Organiser: J. and Z. Müller

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Organiser: M. Alvarenga

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USE OF SYNOVIAL FLUID AND SERUM BIOMARKERS IN EQUINE BONE AND JOINT DISEASE: A REVIEW

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SUMMARY

Considerable progress has been made in the use of synovial fluid in serum biomarkers in the diagnosis of equine bone and joint disease. This material is from a larger review article published in 2005 in the Equine Veterinary Journal, and presented as an initial lecture at a Havemeyer Symposium on Biomarkers in the Horse, sponsored by the Dorothy Russell Havemeyer Foundation, IDEXX and the Orthopaedic Research Centre at CSU.

INTRODUCTION

Significant economic losses due to a high prevalence of lameness in racehorses (the majority being associated with osteoarthritis (OA) are well recognised). Any improvement in prevention, diagnosis, treatment, and prognostication in this area is welcome and biomarkers are potentially useful for this purpose.

The osteoarthritic joint is characterised by damage to and loss of articular cartilage matrix components, along with reduced joint function. It is now well established that the natural disease in the horse is variable, but synovial membrane, fibrous joint capsule, articular cartilage, subchondral bone, and intra-articular ligaments can be involved in primary and/or secondary roles. The inflammatory reaction in the synovial membrane and capsule, any alteration in the dynamic equilibrium between the biosynthetic phase (chondrocytes synthesise and store extracellular matrix (ECM)) and degradation phase (proteolytic enzymes are activated) have been implicated in the pathogenesis of human OA (Sandell and Aigner 2001). Inflammatory proceses result in an increase in levels of inflammatory mediators, with release of micromolecules and their fragments into synovial fluid and serum following the anabolic and catabolic processes in the cartilage. Investigations at the ORC have emphasised a primary role for subchondral bone microdamage in the pathogenesis of traumatic joint disease and early detection of primary change in the subchondral bone is also desirable.

The terms 'biomarker', 'biochemical marker', and 'molecular marker' have all been used to describe either direct or indirect indicators of abnormal skeletal tissue turnover (Billinghurst 2001). These biomarkers are often molecules that are the normal products and byproducts of the molecular processes occurring within the skeletal tissue. In disease, alterations occur and the balance between the anabolic and the catabolic processes within the skeletal tissues and consequently, concentrations of biomarkers may increase or decrease. Most of the biomarkers we currently use in the horse are related to either the synthetic or degradative processes involving *type II* collagen and/or the proteoglycan molecules in the cartilage matrix. In addition, synthetic and degradative markers of *type I* collagen are used for early detection of bone problems. Biomarkers can potentially be used to: 1) clarify pathological processes in the joint; 2) differentiate diagnostically between affected and non-affected joints and distinguish the degree of degradation in articular cartilage; and 3) monitor the response to therapy.

According to the way they are detected, biomarkers can be subdivided into biochemical and immunological markers. A good example of a biochemical marker is the dimethyl methylene blue (DMMB) assay used for detecting

glycosaminoglycans (GAGs) in the synovial fluid and serum. Immunological markers provide a sensitive means to identify types and utilise monoclonal and polyclonal antibodies produced against various epitopes on fragments liberated both from normal and degenerating joint tissue. The term 'biomarkers' has more recently been applied to imaging techniques as well as genetic markers and microarrays. The review here addresses traditional biochemical and immunological markers that form the bulk of the literature.

Direct and indirect biomarkers

Direct biomarkers originate principally from cartilaginous or bony structures or are enzymes that are active only in these tissues. They provide specific information about alterations in cartilage matrix anabolism or catabolism. An example of a direct biomarker that detects collagen degradation is depicted in Figure 1.

Indirect biomarkers are not derived principally from the tissues that make up the joint, but have the potential to influence the metabolism of these tissues or the integrity. Indirect markers include cytokines such as IL-1, matrix metalloproteinases, eicosanoids (PGE_2 is a commonly used example in the equine synovial fluid), insulin-like growth factors, hyaluronan (HA), and C-reactive proteins (CRP).

Individual direct biomarkers of cartilage metabolism

Carboxypropeptide of type II collagen (CPII)

Biomarkers of anabolic processes include carboxypropeptide of *type II* collagen. Antibodies against CPII are a useful measure of *type II* collagen synthesis. Although CPII concentrations have been found to be not significantly higher in synovial fluid of joints with osteochondral fragmentation, the levels were significantly higher in the serum (Frisbie *et al.* 1999). It has also been shown that repeated use of IA methylprednisolone acetate leads to potentially harmful inhibition of CPII synthesis and also an increased release of degradation products of aggrecan from articular cartilage (Robion *et al.* 2001).

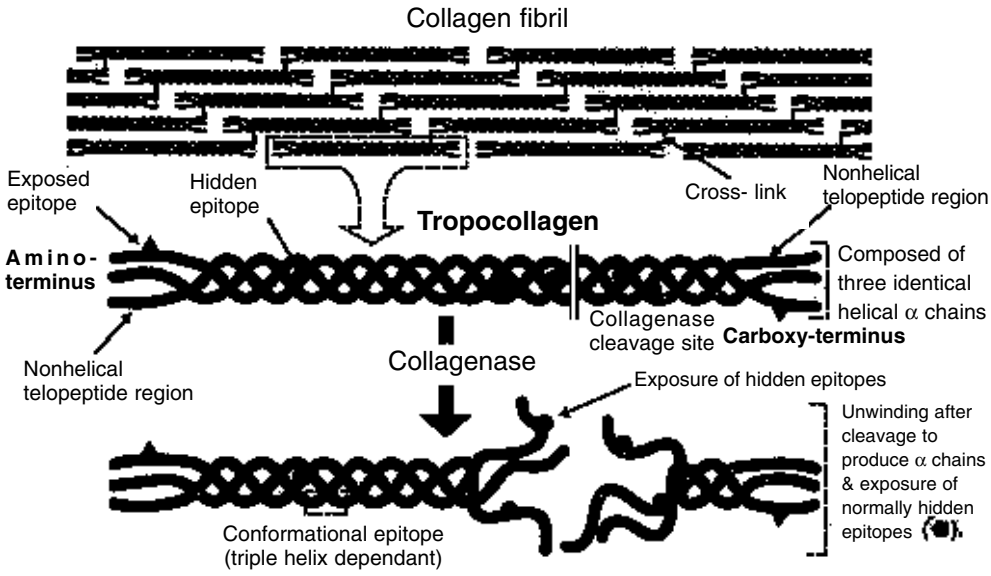

Fig 1: Structure of fibrillar type II collagen to show the composition of a collagen fibril with cross-links between the non-helical telopeptide regions of individual tropocollagen molecules and the helical regions of adjacent molecules. Each tropocollagen molecule is composed of a triple helix of three identical α chains. The cleavage site of collagenase is indicated. The cleaved triple helix unwinds to expose 'hidden' epitopes on α chains that are not detectable by antibodies in the native triple helix. Non-helical (telopeptide) and conformational (triple helical-dependent) epitopes are also indicated (Poole 1992).

Chondroitin sulphate (CS)

Chondroitin sulphate is a major GAG of aggrecan and has proven to be a useful biomarker for aggrecan synthesis. An epitope called CS-846 that is normally found in fetal and OA cartilage and is almost absent in healthy mature articular cartilage, has been measured in a number of species. In a study in the horse, CS-846 levels were significantly higher in joints with osteochondral fragmentation than in control joints, and serum levels were also significantly higher (Frisbie *et al.* 1999). Discriminate analysis using a combination of serum CS-846 and CPII concentrations allowed 79% of horses to be correctly classified as having osteochondral damage.

BIOMARKERS OF CATABOLIC PROCESSES

Type II collagen fragments

Biomarkers of catabolic processes include *type II* collagen fragments. Measuring the degradation of *type II* collagen with biomarkers has proven to be of benefit in monitoring OA as well as osteochondritis dissecans (OCD). Antibodies have been developed to identify *type II* collagen fragments that have been cleaved or denatured, exposing previously inaccessible regions (neoepitopes) of the molecule (Hollander *et al.* 1994; Fig 1). Using these antibodies, significant elevations in levels of degraded *type II* collagen have been demonstrated in synovial fluid and serum samples from horses, dogs, and rabbits with experimental OA (Billinghurst *et al.* 1997). Our equine options now includes the COL2-3/4C_{short} immunoassay for detecting collagenase-cleaved collagen fragments (*types I* and *II*) as well as more recently, a specific *type II* collagen degradation assay that is specific for the horse (Billinghurst 2001), which is designated as 234CEQ.

In a recent study of skeletal markers in OCD in foals (Billinghurst *et al.* 2004), a combination of significantly higher serum levels of CPII, higher levels of COL2-3/4C_{short} and lower levels of 234CEQ correlate with high osteochondrosis scores (radiographically). This study suggested there is an increased collagen turnover in OCD, but by measuring the serum levels of specific biomarkers of collagen metabolism, it is possible to identify foals with OCD and predict their clinical outcome (Billinghurst *et al.* 2004).

Glycosaminoglycans (GAGs)

The 1,9 DMMB assay for GAGs has been useful in a number of studies, and in recent work, is one of the markers that can distinguish osteoarthritis in an exercising horse from normal increases with exercise alone. Breakdown products of keratan sulphate have also been developed, but have limited usefulness in the horse. There are a series of monoclonal antibodies that have been developed to recognise epitopes in the chondroitin sulphate - GAG chains (Caterson *et al.* 1983 and 1999) and these are useful.

Cartilage oligomeric matrix protein (COMP)

Cartilage oligomeric matrix protein (COMP) is an abundance noncollagenous protein constituent of articular cartilage. A number of studies have been done with COMP in both joint disease and tendon disease. It is still uncertain where this product fits. Monoclonal antibody technologies have also been developed to recognise neoepitopes associated with breakdown of the aggrecan molecule by *aggrecanase*. Preliminary work in the horse shows high levels of aggrecanase activity in synovial fluid of diseased joints compared to levels of stromelysin and this has allowed characterisation of the mechanisms by which aggrecan is broken down in equine joint disease.

INDIVIDUAL DIRECT BIOMARKERS OF BONE METABOLISM

Biomarkers of anabolic processes

Carboxy and amino terminal propeptides (PICP and PINP): Biomarkers of anabolic processes include carboxy and amino terminal propeptides (PICP and PINP). During normal *type I* collagen synthesis, as with *type II*, cleavage of PICP and PINP off the procollagen molecule occurs and these cleaved propeptide fragments can be exploited as markers of bone formation. PICP has been measured and levels decrease significantly with age and increase with exercise when compared to non-exercised control horses (Price *et al.* 1995a, b). In a preliminary study, PICP was shown to have potential value as molecular marker for monitoring changes in matrix turnover following tendon injury (Jackson *et al.* 2003).

Osteocalcin: Osteocalcin is a small non-collagenous protein associated with bone assembly and turnover. A recent study in the horse, in which serum markers were used to differentiate concentrations of osteocalcin and CS846 concentrations of osteocalcin and CS846 provided the best correlation to the modified Mankin score and clinical degree of pain (Frisbie *et al.* 2003). Serum osteocalcin concentration was also affected by rate of growth in weanling horses of similar age, whereas there was no change with serum bone alkaline phosphatase (Petersen *et al.* 2001).

Biomarkers of catabolic processes

Type I collagen nonhelical telopeptide (ICTP): Biomarkers of catabolic processes include *type I* collagen nonhelical telopeptide. This fragment is used as a marker of bone resorption in human arthritis. However, levels of ICTP have been measured in the horse in relation to age, exercise, and breed differences, but have not been shown to be of value in detecting pathological processes. (Price *et al.* 1995a,b; Kawcak 1998).

Type I collagen C-telopeptides (CTX): *Type I* collage C-telopeptides have been proven to be a useful marker of specific bone resorption in humans. In a study in the horse done at the ORC, CTX was less useful than other cartilage biomarkers (CS846, CPII and GAG) in predicting whether serum was from a control exercised or an osteoarthritic horse (Frisbie *et al.* 2002).

Bone sialoprotein (BSP): Bone sialoprotein is elevated in human patients with clinical OA. Recent work in the horse established that there is an increase in BSP at the cartilage/bone interface when degenerative changes of the bone and cartilage are compared to the morphologically intact cartilage and bone. The challenge still is to measure BSP in the synovial fluid and serum of racehorses.

Conclusions

In conclusion, we are not yet at the stage of having a 'magic marker' to diagnose the degree of articular cartilage or bone disease in a single joint with 100% accuracy. However, much progress has been made. Various marker molecules have already been used successfully and an increasing number are coming onto the market in the form of ready-to-use kits. Although currently still used mainly in research settings, the time is not far away that these kits will be used widely in clinical practice. For a good assessment of the condition of the cartilage and other tissues of the joint, a combination of the determination of (repeatedly collected) selected markers with other diagnostic techniques, such as arthroscopy and/or MRI, seems more promising.

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PROTEOGLYCAN METABOLITES AS BIOMARKERS OF CARTILAGE DEGENERATION IN DEGENERATIVE JOINT DISEASES

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Over the past 25 years The authors' laboratory has pioneered the production and use of monoclonal antibody (mAb) technologies to study cartilage proteoglycan structure, function and metabolism in health and disease. Many of the mAbs produced by the authors' laboratory recognise structural (protein or carbohydrate) epitopes and enzyme-generated neoepitopes either present or generated, respectively in these cartilage proteoglycans. In past and recent studies it has now been realised that several of these mAbs can be used to recognise potential biomarkers (changes in cartilage proteoglycan biochemical composition and/or chondrocyte metabolism/phenotype) that can be used to monitor or diagnose metabolic changes that are occurring in the pathogenesis of degenerative joint diseases. This paper provides a summary of past and new studies identifying cartilage metabolic biomarkers that can be potentially used to diagnose or monitor the efficacy of therapeutic or surgical treatments of equine degenerative joint diseases.

STRUCTURAL BIOMARKERS

In the mid 1980s detection of cartilage aggrecan metabolites (keratan sulphate – KS) in the serum of arthritis patients was proposed as a potential biomarker of cartilage degeneration in degenerative joint diseases (Thonar *et al.* 1985). In the 1990s early studies investigated KS metabolites in equine synovial fluid and serum as potential biomarkers of degenerative joint disease (Alwan *et al.* 1990). Analyses of KS metabolites, in conjunction with other cartilage biomarkers, are still being used to evaluate the efficacy of pharmacological and nutraceutical treatments for horse joint pathology today (Caron *et al.* 2002; Orth *et al.* 2002; Celeste *et al.* 2005).

ANABOLIC NEOEPITOPE BIOMARKERS

In the mid- to late-1980s researchers from our laboratory first described the expression of anabolic neoepitopes, manifested as different chondroitin sulphate (CS) sulphation motifs on cartilage aggrecan glycosaminoglycans, as novel biomarkers indicative of anabolic (attempted repair) responses of chondrocyte metabolism in the pathogenesis of arthritic disease (Caterson *et al.* 1990; Visco *et al.* 1993; Slater *et al.* 1995). These mAb reagents have also been used to study anabolic/repair changes in equine cartilage metabolism (Todhunter *et al.* 1996; Dart *et al.* 2003). CS sulphation motif mAbs have also been produced by other laboratories and used in analyses of synovial fluid and serum for diagnosis of osteochondral fragmentation in horses (Frisbie *et al.* 1999).

CATABOLIC NEOEPITOPE BIOMARKERS

In the early 1990s researchers in our laboratory also pioneered the production and use of mAb technologies to detect catabolic neoepitopes generated by matrix protease cleavage of cartilage aggrecan in its interglobular domain (IGD) – see Caterson *et al.* (2000) for review. These mAb reagents were used by researchers at Dupont to discover the 'aggrecanases' (ADAMTS-4 and ADAMTS-5). One of these mAbs (BC-3) can be used to detect and now quantify aggrecan catabolites generated by aggrecanase catabolism of the IGD of cartilage aggrecan in the pathogenesis of arthritic disease. This mAb and other related monoclonal and polyclonal antibodies that recognise protease-generated neoepitopes in cartilage matrix macromolecules are showing increasing potential as a means of

identifying useful biomarkers for degradative processes involved in arthritic disease.

New potential biomarkers of degenerative joint disease

As the result of a 'side-project' emanating from recent studies aimed at generating new mAbs that recognise keratanase- or keratanase II- generated 'stub' neoepitopes, we also have produced new mAbs have also been produced that recognise core protein structural epitopes on 2 of the small leucine-rich proteoglycans (SLRPs) first found in corneal matrix stroma; ie keratocan and lumican. We have been aware for a long time that altered synthesis of matrix molecules and changes in the phenotypic expression of the hyaline articular cartilage chondrocytes are early signs of changes in the metabolism (and thus function) of articular cartilage with the onset of degenerative joint diseases. In addition, a recent study (Young *et al.* 2005) also described changes in gene and protein expression of biglycan, decorin, fibromodulin, and interestingly lumican in an ovine meniscectomy animal model of degenerative joint disease. We therefore evaluated the expression of keratocan and lumican as potential biomarkers of degenerative joint disease in human patients undergoing total hip or knee replacement surgery. Our studies have shown that analysis of gene (using qualitative RT-PCR) and protein (using Western blot analysis) expression of the SLRP keratocan is absent in 'normal' hyaline articular cartilage (from femoral head fractures) but upregulated in pathological cartilage obtained from patients with osteoarthritis (OA) of the knee or hip. In contrast, lumican gene expression appeared to be constitutive in both 'normal' and OA cartilage unlike that seen in the ovine animal model (Young *et al.* 2005). However, lumican expression at the protein level was absent in 'normal' cartilage and upregulated in OA cartilage samples similar to that seen in the ovine model of degenerative joint disease (Young *et al.* 2005). At present, it is not clear what is the function of altered SLRP (particularly keratocan and lumican) expression in the pathogenesis of degenerative joint disease. One possibility is that this unexpected expression of SLRPs signals a change in the chondrocyte phenotype whereby the cells attempt to produce a *type I* collagen, keratocan and lumican regulated, fibrillar scar, in response to the altered mechanical environment manifest with the onset of OA. Alternatively, changes in SLRP expression have been recently linked with the upregulation and occurrence of inflammation which is an important factor driving joint tissue destruction in arthritic diseases (Schaefer *et al.* 2005; Sjoberg *et al.* 2005). The potential for upregulation of keratocan, lumican and other SLRPs in equine joint pathology still needs to be investigated.

Monoclonal antibodies that recognise chondroitin sulphate (CS) sulphation motifs as potential biomarkers identify stem/progenitor cells in musculoskeletal tissues

In the mid 1980s (Caterson *et al.* 1990) researchers in our laboratory produced and characterised several mAbs that recognised linear glycosaminoglycan (GAG) sulphation epitope motifs in CS GAG present on connective tissue proteoglycans. Several of these have been used to detect anabolic/dedifferentiation changes in OA cartilage from humans and animal models (Caterson *et al.* 1990; Visco *et al.* 1993; Slater *et al.* 1995). In recent studies (Hayes *et al.* 2008) 3 of these mAbs [3-B-3(-); 4-C-3 and 7-D-4] have also been used to identify potential chondroprogenitor cells (and further differentiated cell subtypes) in the superficial zone/layer of hyaline articular cartilage where they are believed to reside (Dowthwaite *et al.* 2004). We have also used these mAbs to demonstrate their ability to identify potential stem/progenitor cell subpopulations in regions where they occur in developing intervertebral disc tissues and tendon. Preliminary studies, using chondrocytes from bovine cartilage, also indicate that we were potentially able to use FACS procedures to separate and isolate these stem/chondroprogenitor cells for their enrichment and use in tissue engineering technologies for musculoskeletal tissue repair and regeneration.

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PROTEOGLYCAN METABOLITES AS MARKERS OF CARTILAGE BREAKDOWN IN EQUINE JOINTS

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Progressive degradation of articular cartilage is a central feature of arthritis irrespective of the inciting cause, and is characterised by loss of aggrecan due to excessive degradation by aggrecanases followed by proteolysis of the collagen network. This latter event may signify the point of irreversible cartilage damage. Aggrecan loss from cartilage in joint disease is driven primarily by proteolysis by the A Disintegrin And Metalloproteinase with ThromboSpondin motif (ADAMTS) family of enzymes (Little *et al.* 2005). However, in late-stage disease, in concert with collagen proteolysis by the collagenolytic matrix metalloproteinses (MMPs), some MMP-cleaved aggrecan metabolites are observed. There is evidence that following aggrecan depletion but preceding, and possibly as a necessary pre-requisite for collagenolysis, other matrix components such as the small leucine rich repeat proteoglycans (SLRPs, decorin, biglycan and fibromodulin) that coat the collagen fibres are released from cartilage at least *in vitro* (Sztrolovics *et al.* 1999). Differentiating early from progressive and late degeneration of cartilage may therefore be possible by evaluating the sequential release of cartilage matrix constituents. We sought to use both *in vitro* models of progressive cartilage degeneration and synovial fluids from horses with arthroscopically-characterised joint disease, to determine whether different aggrecan and SLRP metabolites might serve as useful markers of progressive cartilage degeneration.

Articular cartilage was harvested from high and low weight-bearing regions of equine carpal joints with no morphological evidence of joint disease. Cartilage was cultured for 3 days in DMEM with 10% FCS and then washed and cultured for 28 days in serum free DMEM containing 10 ng/ml IL-1beta or 1ng/ml IL-1beta plus 50 ng/ml OSM, with media changes every 7 days (Little *et al.* 2005). Release of proteoglycan and collagen was quantitated using DMMB and hydroxyproline assays, respectively. Western blot analysis of ADAMTS- and MMP-generated aggrecan metabolites (BC-3 and BC-14 antibodies, respectively), MMP-generated type II collagen catabolites (9A4 antibody), and decorin, biglycan and fibromodulin (polyclonal antibodies to the C-terminus) in cartilage extracts and culture media at Days 7, 14, 21 and 28 was undertaken. Synovial fluids were harvested at the time of arthroscopic surgery by Professor Wayne McIlwraith and the cartilage damage and history for each animal recorded. Synovial fluids were subjected to associative CsCl density gradient ultracentrifugation, hyaluronidase digestion and Western blot analysis of aggrecan and SLRP metabolites.

It has previously been reported that stimulation of equine cartilage with both IL-1beta alone and IL-1/OSM induced significant ADAMTS- but not MMP-dependent aggrecan loss, with most release occurring over the first week of culture. The authors also observed increased link protein release from cartilage induced by IL-1 alone and IL-1/OSM, with no difference in the amount or fragmentation of the released link protein. Unlike other species, IL-1beta alone but not IL-1/OSM induced collagenloysis from Day 14 onwards in equine cartilage (Little *et al.* 2005). The collagen release was due to MMP activity and was associated with late stage C-terminal truncation, but not interglobular domain cleavage of aggrecan by MMPs. There was early (Day 7 and 14) and persistent (Day 21 and 28) release of decorin and fibromodulin from cartilage, but importantly this

only occurred in the cultures in which collagenolysis was subsequently induced (ie 10 ng/ml IL-1 alone but not IL-1/OSM). In contrast with other species evaluated, biglycan release was not induced from equine cartilage by either catabolic stimulus despite its presence in cartilage extracts. The pattern of aggrecan, collagen and SLRP release from cartilage was similar in high and low weight-bearing cartilage.

Analysis of synovial fluid from joints with varying pathology (chip fractures ± cartilage degeneration and ligament tearing) demonstrated many similarities and some differences from the *in vitro* cartilage degradation studies. Firstly, only ADAMTS- and not MMP-generated aggrecan metabolites were detected. The ADAMTS-generated fragments in joint fluids (BC-3 positive) ranged in size from 60 to >300kDa, with the largest metabolites not previously observed in the *in vitro* cartilage degradation studies. The overall (total) immunoreactivity with BC-3 did not correlate with glycosaminoglycan levels in the fluid. The pattern of BC-3 positive fragments differed markedly between horses, particularly with respect to the presence or absence of the very largest metabolite. Intact (45kDa) decorin core protein was detected in most synovial fluids, while minor catabolites were only rarely observed. Neither fibromodulin or biglycan core proteins were detected in any of the joint fluids examined, while MMP-cleaved collagen fragments were present in all joints.

Taken together, these results suggest that several cartilage matrix components may be useful markers of cartilage degradation in equine joint disease. Progressive degradation *in vitro* was associated with ADAMTS-driven aggrecan loss followed by decorin and fibromodulin but not biglycan release, and then collagenolysis by MMPs. The *in vivo* pattern of matrix component release is likely to be more complex as varying stages of cartilage degeneration may be simultaneously present in the one joint. Furthermore, changes in synthesis of aggrecan and SLRPs may occur *in vivo* which are not observed in the *in vitro* model where serum free conditions limit biosynthesis by the chondrocytes. In synovial fluids, BC-3 immunoreactivity was not just a surrogate marker of glycosaminoglycan levels, suggesting that it may be a useful biomarker of particular cartilage degenerative events. Furthermore, the pattern of BC-3 fragments differed markedly between joints in association with varying degrees of C-terminal truncation of the aggrecan molecules. Determining the C-terminal sequence of these fragments and generating new neoepitope antibodies may provide a better tool for monitoring their presence. Equine cartilage *in vitro* released decorin and fibromodulin prior to and in association with collagen breakdown, suggesting that these SLRPs may be useful to monitor *in vivo*. The reason that biglycan was not released from equine cartilage may be related to the fact that mature adult equine cartilage was used in the present studies. Unlike the *in vitro* situation, only decorin core protein was detected in synovial fluids of horses with joint disease and whether this arises from the cartilage or other joint tissues is presently unknown. While all of the synovial fluids in the present study were from joints evaluated arthroscopically, no control for parameters that may affect synovial biomarker levels (eg age, sex, exercise level, previous treatment etc) could be imposed limiting their utility to correlate biomarkers with the stage of cartilage degeneration. Future studies will evaluate changes in synovial fluid in an experimental model of equine joint disease where longitudinal analysis will allow progression of joint disease to be better correlated with changes in aggrecan (BC-3) and decorin metabolites in the joint fluid.

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PROSPECTIVE CLINICAL STUDY ASSESSING SERUM BIOMARKERS FOR MUSCULOSKELETAL DISEASE IN 2–3-YEAR-OLD RACING THOROUGHBREDS

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INTRODUCTION

The use of peripheral blood to assess musculoskeletal disease has been a goal researchers have been working towards for over a decade. There have been many studies to validate the usefulness of serum 'biomarkers' in the horse (McIlwraith 2004). To date, however, only one other study has assessed their usefulness in clinical cases (Jackson *et al.* 2005). This study showed significant differences in horses with dorsal metacarpal disease compared to controls but no difference in horses that went on to sustain a fracture in this region. The current study was designed to build on previous work by the investigators (Frisbie *et al.* 1999; 2002) that demonstrated promising results of biomarkers for the identification of intra-articular pathology in a controlled clinical or experimental setting. This study represents the real world application of 6 serum biomarkers for the detection of musculoskeletal injuries.

MATERIALS AND METHODS

Two- or 3-year-old Thoroughbred racehorses were entered into the study when they arrived at Thoroughbred race tracks in southern California. Each month a lameness examination was performed by a study veterinarian and peripheral blood collected and serum stored. Horses were removed from the study when (McIlwraith 2004) they were out of training for more than 30 days for any reason, or (Jackson *et al.* 2005) they were enrolled in the study for 10 months. Only horses that sustained a single musculoskeletal injury and had completed at least 2 months in the study were analysed. For the purposes of this study, a musculoskeletal injury was considered one of the following: intra-articular fragmentation (IAF), injury to a tendon or ligamentous structure (TL), incomplete or complete non-articular fracture (ICF) and periostitis (BS).

Serum samples were analysed for glycosaminoglycan (GAG), type I and II collagen (Col short), type II collagen synthesis (CPII), type II collagen content (Col CEQ), aggrecan synthesis (846), osteocalcin (OC) as a marker of bone formation and (CTX) as a marker of bone degradation as previously reported (Frisbie *et al.* 2002).

Statistical analysis was performed using the SAS statistical software package, version 8e. Non-parametric measures of assumption were tested using a Chi-squared analysis. All outcome variables that were concentrations were log transformed (natural log) to meet assumptions of normality. When direct comparisons were made a least squares means procedure was utilised and a p-value of <0.05 was considered significant. The presence of a musculoskeletal injury and type of injury were both assessed as main and interaction effects with the horse acting as a random effect.

RESULTS

Ninety-two of the 238 horses entering the study were not analysed because they did not complete at least 2 months in the study or were diagnosed with multiple lesions. Of the 146 horses, 75 (51%) were considered to sustain a musculoskeletal injury during the study, with the remaining 71 (49%) horses acting as control (CNT) horses. No significant difference in the proportions was observed in the control or injured horses by age, gender, or type of lesion sustained. Of the injured

horses, 23 (31%) sustained an intra-articular fragmentation (IAF), 18 (24%) a tendon or ligamentous injury (TL), 13 (17%) incomplete or complete non-articular fracture (ICF) and 21 (28%) were diagnosed with periostitis (BS).

When samples collected at the time of injury were compared to control horses the following observations were made: a significant drop in serum GAG levels were noted for the IAF and ICF horses. An increase in CTX was seen in TL horses. No significant changes were detected in BS horses, although significant increases in GAG, Col Short and decrease in osteocalcin were seen when baseline samples were compared to injured samples of the same BS horse.

Discussion

The results of this study indicated that biomarkers can be used as a diagnostic aid in clinical musculoskeletal injuries as well as provide insight to the pathogenesis of disease processes. Each of the injuries studied here had a unique biomarker pattern following injury, suggesting further study on predictability of injury is warranted. Furthermore the fact that the biomarkers were indeed significantly altered in clinical cases despite the confounding effects present in the 'real world' is promising.

Acknowledgments

This project was funded by the Grayson Jockey Club Research Foundation.

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DOES INFLAMMATION TRIGGER TISSUE DESTRUCTION OR IS TISSUE DESTRUCTION ACTIVATING INFLAMMATION IN JOINT DISEASE?

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Cartilage extracellular matrix consists of 2 major functional entities, the aggrecan molecules contributing an extreme charge density and therefore accomplishing an osmotic environment that retains water leading to a resistance to deformation. Collagen fibrillar networks provide resistance to tension and distribute load. There are a large number of molecules now known that interact both in the formation and in the maintenance of these macromolecular assemblies and also in concomitantly interacting with cell surface receptor relaying information to the cells on the condition of the matrix. Examples of altered catabolism, repair attempts and how matrix may interact with the cells were discussed.

There is a continuous turnover of these matrix molecules, necessary for the adaptation to load and repairing fatigue damage. In pathology there is both new synthesis in repair attempts and increased breakdown, putatively different in nature, of matrix constituents. Some of the fragments formed are no longer retained and diffuse into the synovial fluid. There is a potential for some of these bioactive fragments to affect, for example the inflammatory response. Any fragments activating inflammation have the potential to cause chronicity and progressive joint destruction by inducing production of catabolic cytokines. Examples of candidate molecules were discussed.

WHAT BIOMARKERS ARE TELLING US AND THE CHALLENGES AHEAD

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The measurement of cartilage and bone specific biomarkers (BM) of matrix synthesis and degradation in body fluids is not only potentially useful for the prediction, early diagnosis and staging of joint diseases, but also to define matrix processes, whether catabolic or anabolic. Furthermore, they may be used to define a response to medication and provide specific read-outs for targeted matrix therapy. A review of results from selected recently published and ongoing animal studies in our laboratories using BMs to assess cartilage and bone metabolism is provided.

Major alterations in cartilage aggrecan metabolism, including the release of markers of both synthesis (846) and turnover (KS), were recently detected by analyses of synovial fluid (SF) following repeated intra-articular injections of a clinically relevant dose of triamcinolone acetonide in normal horses (Celeste *et al.* 2005). These results were consistent with results of a prior study where methylprednisolone acetate injections were evaluated (Robion *et al.* 2001).

Non-steroidal anti-inflammatory drugs (NSAIDS), are also commonly used to treat the symptoms of equine osteoarthritis but have been reported to have adverse effects on both cartilage and bone metabolism. We chose to study skeletal metabolic effects of continuous oral administration of clinically used doses of NSAIDS by assessing a battery of markers of cartilage and bone synthesis and degradation sequentially in serum and SF. A significant increase in osteocalcin occurred in the sampled joints from treated horses and no other changes occurred in the BM levels over time. These findings are encouraging and suggest that, in horses at least, NSAID administration does not have adverse effects on cartilage and bone metabolism *in vivo* (Fradette *et al.* 2005).

The cartilage content of BMs may also be assessed in terminal animal studies to evaluate the effects of medication on cartilage matrix metabolism. No effects were detected of glucosamine on cartilage type II collagen metabolism, assessed by specific synthesis (CPII) and degradation markers (Col23/4c short- a marker of cleavage and Col2 3/4m a marker of degradation) in a rabbit model of OA, but a modest protection of GAG content was observed (Tiraloche *et al.* 2005). Combined, these various results provide support for the potential use of BM evaluation to assess therapeutic effects of drugs or toxicity.

Parallel to these studies, researchers have continued to explore what can be learned about osteochondrosis (OCD) from BM assessment. As a follow up to our earlier studies on BMs and OCD, we attempted to discriminate a population of yearlings with stifle OCD (marked joint effusion and a radiographic diagnosis) from a population without lesions based on serum BM analyses. Previously we reported that a differential alteration in aggrecan (decrease in 846 and KS epitopes) and type II collagen turnover (increase in CP II) was observed in young horses (Laverty *et al.* (2000) with tarsocrural OCD, based on SF analyses, and a selective increase in type II collagen cleavage by collagenase in femoropatellar OCD cartilage (Laverty *et al.* 2002). Despite results of these previous studies, we detected no difference in serum levels of the cartilage specific BMs analysed in this group of yearlings. We did however detect a significant increase in serum levels of bone alkaline phosphatase in the animals with OCD when compared to healthy animals (Laverty unpublished data). As it is difficult to obtain client compliance for synoviocentesis in these cases, it remains unanswered whether significant changes in the SF BM levels with stifle OCD occurs.

Many challenges remain ahead on our path to a better understanding of what molecules we are measuring and how they may be influenced.

Areas, amongst others, that need to be investigated include:

- Factors contributing to BM release (collagen versus GAG).
- Rates of formation, accumulation and clearance of BM from both normal and diseased joints.
- Routes of systemic clearance and sites of metabolism of BM.
- Influence of various degrees of joint inflammation on clearance.
- Genetic effects on individual variation in marker levels.
- The relationship between the amount of marker released and the cartilage content as the amount released may not only depend on cartilage quantity but also quality.

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INVOLVEMENT OF THE CARTILAGE/BONE JUNCTION IN EQUINE OSTEOARTHRITIS (OA) OF THE MIDDLE CARPAL JOINT

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The third carpal bone of the middle carpal joint is exposed to excessive load during the hyperextension phase of the racing stride in the standardbred trotter (STB; Johnston *et al.* 1995) and lameness due to osteoarthritis (OA) in this joint is common (Pool and Meagher 1990). Articular and bone lesions in the radial facet, such as superficial cartilage fraying, cartilage erosion, subchondral bone sclerosis, bone necrosis, osteocartilagenous interface collapse and incomplete fractures are often found at necropsy. The simultaneous changes in articular cartilage and underlying bone are of great importance in OA (Kawcak *et al.* 2001). Here, findings of a pilot and a published study (Ekman *et al.* 2005) are discussed, concentrating on the cartilage/bone interface in the middle carpal joint of trotters. The definition of equine OA may differ among clinicians, radiologists and pathologists and this is also discussed from a pathologist's point of view.

In a pilot study it was found that morphological changes at the radial facet from STBs differed with age (1-, 2-, 3- and 4-year-old) and training. A semilunar bonesclerosis developed together with cartilage matrix changes such as, reduced stainability, chondro and bone necrosis, chondrocyte proliferation (clusters) and collapse of the cartilage/bone interface. The lesions in the calcified articular cartilage and cartilage/bone interface could often be found without superficial fraying. Mild to moderate synovitis was often present and lameness had occasionally been seen in all of the horses with lesions, but was impossible to correlate to type of lesion.

Possible biomarkers for cartilage/bone changes, mirroring bone activity in the extracellular matrix could be found among the non-collagenous proteins forming 10% of the organic bone matrix. Candidates that have been implicated are; bone sialoprotein (BSP), osteocalcin, osteonectin, osteoadherin, osteopontin, biglycan, decorin. Many of these are, however, not bone-specific and many can not be used as markers for activity within the cartilage/bone interface.

BSP, a RGD-containing protein, (Franzen and Heinegård 1985) has been suggested as a marker for subchondral bone activity in OA (Wollheim 1999). It is a cell binding protein and binds strongly to hydroxyapatite (Oldberg *et al.* 1988). It is concentrated in the cartilage/bone interface early in experimental OA of guinea pigs and later in the deeper bone (deBrie *et. al.* 1997). An ultrastructural immunolocalisation of BSP in the osteocartilagenous interface of the equine third carpal bone showed a marked increase of BSP in the interface with degenerative changes compared to the intact cartilage/bone interface (Ekman *et al.* 2005). BSP immunolabelling was higher in the underlying intact bone of the normal areas compared to the fragmented bone of the degenerated areas. The changes found in these joints probably represent early OA and none of the horses had clinical evidence of lameness from the middle carpal joint at the time of euthanasia.

The findings suggest that BSP can be used as an *in vivo* biomarker for juxta articular bone activity in early OA.

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BONE BIOMARKERS IN HORSES: WHERE ARE WE NOW AND FUTURE PROSPECTS?

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The first papers describing the measurement of biochemical 'markers' of bone cell activity in horses were published in the early 1990s. The prospect of being able to non-invasively measure equine bone metabolism was very exciting and bone markers were considered to have great potential as both clinical and research tools. In the subsequent years many research groups have described the measurement of bone biomarkers in a variety of contexts and this is now an appropriate time to review this body of information and to attempt to assess the prospects for the future.

A number of bone markers have been measured in the horse, the majority using assays originally developed for human use and assessed for their equine cross-reactivity, although some equine-specific assays have been developed. Bone markers measured in horses include; osteocalcin, deoxypyridinoline (DpD), bone alkaline phosphatase (BALP), the carboxy-terminal propeptide of type I collagen (PICP), the carboxy-terminal telopeptide of type I collagen (CTX-MMP/ICTP), the carboxy-termial telopeptide of type I collagen (CTX-1) and the type I-collagen cross-linked N-terminal telopeptide of type I collagen (NTx). A number of studies have also demonstrated that bone marker concentrations are influenced by a number of controllable and uncontrollable variables that include age, breed, season, time of day and gender (although the influence of gender remains somewhat controversial). We have recently demonstrated that stage of oestrus can also influence bone marker concentrations.

Several studies have measured bone markers in order to monitor how different training regimens influence equine bone. These reports have described the measurement of a number of different bone markers in horses of different ages and exercised using a variety protocols. This makes it very difficult to directly compare and interpret the results of these studies however, what is clear is that exercise is an important variable that can influence bone marker concentrations. The most consistent data have been obtained from well designed experimental studies in which bone markers were compared in horses exposed to very different exercise regimens (eg no exercise compared to fairly intense exercise). Based on our early work in experimental treadmill studies, we then set out to establish whether bone markers could be used as a method for identifying those exercise regimens that may be osteogenic compared to those that may have detrimental effects on bone. We have now undertaken 2 studies in reasonably large cohorts of horses in commercial race training. Our results have lead us to conclude that 'in the field' bone markers have limited value for informing how training influences bone adaptation because so many variables can potentially influence their concentrations.

The other important question that needs to be addressed is whether bone markers can be used as diagnostic and/or prognostic markers? There have been a small number of reports which describe changes in bone marker concentrations in musculoskeletal diseases, including osteochondrosis and osteoarthritis. Because studies in humans have shown that bone markers are useful for identifying people at increased risk of osteoporotic fracture, one of our aims has been to test the hypothesis that bone markers could predict fracture in horses. To explore this hypothesis, we measured 4 bone markers in 409 2-year-olds and 365 3-year-olds at the start of their flat race training (OC, PICP, ICTP and CTX). Disappointingly, the conclusion of this study is that biochemical markers have no value for identifying

horses that are at risk of fracture in the subsequent training/racing season. Neither did we find that marker concentrations were different in fracture cases compared to controls when measured longitudinally. This study also showed that caution must be taken when interpreting data from small numbers of cases, since preliminary analysis of our data indicated that fracture was associated with a significant difference in specific bone marker concentrations. On a more positive note, we have shown that bone markers are potentially useful for the early identification of 2-year-old racehorses horses at increased risk of developing dorsal metacarpal disease (DMD), a fatigue damage injury associated with the introduction of high speed exercise. In man the other important application for bone markers is to monitor the effects of different treatments for metabolic bone disease. Bone markers could therefore prove to be extremely useful for monitoring the effects of different drugs or nutraceuticals on the equine skeleton, although very little work has been published in this area to date.

Conclusions

In the last 15 years research on equine bone markers has led to the development and validation of a variety of assays and the identification of a number of variables that affect bone markers (age, breed etc). There is no doubt that this research has helped contribute to our understanding of equine skeletal physiology and in experimental situations bone markers have also been shown to have value for monitoring the effects of exercise on bone cell activity. However, the jury remains out on whether bone markers have value as diagnostic or prognostic tools. Well designed clinical research studies are now required to address this issue. The use of bone markers in combination with genetic markers to assess risk of injury may also be possible in the future. An area that also deserves further study is the use of bone markers to objectively assess the effects on bone cell function of novel and established treatments for equine musculoskeletal disease.

ARE BONE MARKERS ALONE A USEFUL TOOL TO FOLLOW TREATMENT OR EXERCISE REGIMENS IN HORSES?

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INTRODUCTION

To finely tune bone equilibrium during growing of a foal, training or drug intervention, veterinarians need appropriate biomarkers and defined specific sites on weight-bearing structures to get information such as bone mineral density (BMD) or speed of sound (SOS) values (Lepage *et al.* 2001). This abstract summarises different studies performed to assess the effect of a non-aminobisphosphonates (Varela *et al.* 2002), and flat racing-exercise (Carstanjen *et al.* 2003a,b; Carstanjen *et al.* 2005) on different bone biomarkers.

MATERIALS AND METHODS

1) The effect of training was assessed on 2- to 4-year-old Thoroughbred horses subjected to flat racing-exercise according to 3 training intensities: slow gallop, canter and high speed work.

2) The effect of the non-aminobisphosphonates tiludronic acid (Tildren CEVA, France) via iv administration at 1 mg/kg during 30 min, was assessed on normal adult Standarbred horses.

Biochemical biomarkers

Biochemical biomarkers were investigated in serum using commercially available radio-immunoassays.

For bone formation: osteocalcin (OC:DSL-6900, DSL Inc, Webster, Tx, USA) and bone-ALP (Tandem-R Ostase, Immunotech, Belgium) were tested.

For bone resorption: carboxy-terminal cross-linked telopeptide of type I collagen generated by matrix metalloproteinases (CTX-MMP:Orion Diagnostica, Espoo, Finland); C-telopeptide of type I collagen cross-links (CTX-I: β Crosslaps, Elecsys, Roche Diagnostics, Switzerland) and Cathepsin K (Oxford Biosystems, England) were used. For Cathepsin K, levels obained were too low for good detection and it was concluded that this assay was not working in horses.

Bone imaging biomarkers

Speed of Sound (SOS; m/s) values were obtained with an ultrasound device using a probe with an axial transmission mode (Omnisense, Sunlight Ltd, Israel). Superficial dorsal, lateral and medial cortical bone of the third metacarpal bone (MC III) was assessed with this method. (Lepage *et al.* 1998; Carstanjen *et al.* 2003 a,b).

Bone mineral density (BMD; g/cm^2) and BMC (g) values were measured using a PIXI dual-energy X-ray mobile device (Lunar, France) and/or an Hologic device (Beford, USA). The mid-MCIII was chosen as an appropriate site to measure (Donabedian *et al.* 2005).

RESULTS AND DISCUSSION

Exercise follow-up

Two-year-old Thoroughbreds had significantly higher serum OC- and CTX-MMP-values as compared with 3-year-old horses. No difference in bone marker values was obtained between mares and stallions. Training intensity had no significant influence on serum OC and CTX-MMP-values. Two-year-old Thoroughbreds showed a significant increase in serum OC values between measurement cycles 2 and 3, as well as a significant decrease between cycles 4 and 5 and

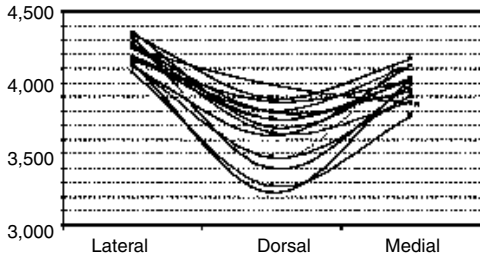

Fig 1: SOS profile obtained for each MCIII on its 3 different aspects. It is clear that the lateral and medial aspects have higher SOS values than the dorsal aspect of the bone. The only exception was on the left limb of a 2-year-old female with a higher SOS value on the medial side compared to the dorsal side (Bone 1998).

cycles 5 and 6. Sore shin formation had no influence on serum OC- and CTX-MMP-values. Corticosteroid caused a significant decrease in OC- and CTX-MMP-levels.

Speed of sound values of the dorsal aspect of MC III were significantly lower in comparison with values obtained at the lateral and the medial aspect of MC III (Fig 1). Mares had significantly higher SOS values at the dorsal aspect of MC III, when compared with corresponding values in stallions (Fig 2). SOS values decreased with age on the dorsal aspect of the bone and increased with age at the lateral and medial aspect of MC III. SOS values of dorsal MC III were significantly different between cycles, variations being different between 2-year-old and 3-year-old Thoroughbreds.

These results indicate that young exercising Thoroughbred racehorses have age-, gender-, and measurement-cycle-dependent variations in SOS values of MC III, which probably reflect adaptive variations in superficial cortical bone properties of MC III and also an adaptive process of bone metabolism, characterised by temporarily increased serum OC but unchanged CTX-MMP concentrations.

Treatment follow-up

Tiludronic acid therapy did not induce changes neither in bone formation markers, nor in CTX-MMP but 24 h post administration CTX-1 concentration was significantly decreased by an average of 72.4% (Table 1). In 2 different studies biochemical biomarkers [OC, bone-ALP, CTX-MMP, CTX-1] and [bone-ALP, CTX-1] respectively selected for bone metabolism

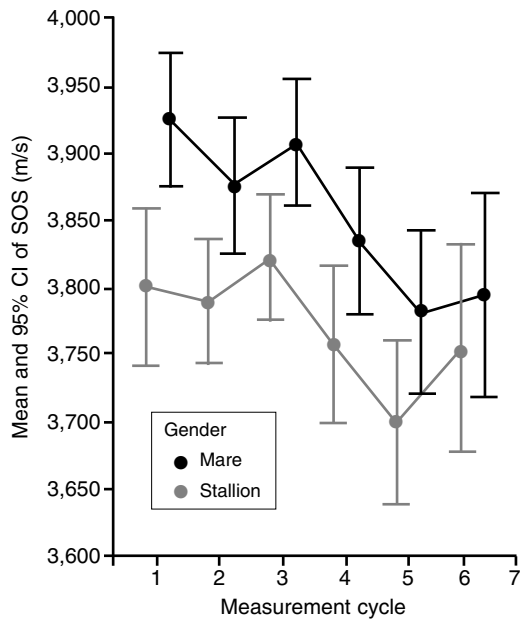

Fig 2: Mean and 95% CI of SOS values of Thoroughbred mares and stallions Thb horses (n=72) obtained at the dorsal aspect of MCIII during 6 measurement cycles (Bone 2003).

assessment in treated horses at rest for 4 months (Varela 2002) and during a 5 month period of cast immobilisation and training (Delguste *et al.* 2007), showed non-interpretable fluctuations except for the transient decrease in CTX-1 after each tiludronic acid treatment.

In the study after 4 months rest, *in vivo* QUS and *ex vivo* DXA results showed in treated animals a significant increase in SOS values on the dorsal MCIII cortex and an increase BMD. In the second study after 2 months of cast immobilisation and 3 months of remobilisation and exercise (Delguste *et al.* 2007), *in vivo* QUS and DXA results showed that treated horses had no significant variations in SOS values but a significant lower decrease in BMD compared to placebo (Delguste *et al.* 2007).

The decrease in serum CTX-1 indicates a possible antiresorptive efficacy of tiludronic acid. This type of acute, temporary and significant effect of drug administration on bone biochemical biomarkers was already described in horses for OC and CTX-MMP after corticosteroid administration (Lepage *et al.* 1993) or general anaesthesia, Grafenau *et al. (*1999). Non-significant differences between groups over a 4

TABLE 1: Bone biomarkers variations before and 24 h after IV administration of tiludronate at 1 mg/kg

	Before	+ 24 h	% variation	P
	m ± SD	m ± SD	m ± SD	
OC	6.4 ± 2.07	5.1 ± 1.62	-15.7 ± 26.92	0.125
bone-ALP	41.9 ± 2.7	46.5 ± 8.67	11.4 ± 22.74	0.156
CTX-MMP	7 ± 2.05	6.9 ± 1.33	2.7 ± 26.21	0.5
CTX-I	0.15 ± 0.102	0.048 ± 0.047	-72.4 ± 18.43*	0.0313*

m=mean; SD=standard deviation, % variation=percentage of variation between pre- and 24 h post treatment values, *significant decrease in CTX-1 concentration

month follow-up period for the selected biochemical biomarkers leads to the conclusion that bone metabolism of these treated horses was not impaired (Varela 2002). However, in the same experiment: MCIII bone properties obtained *in vivo* using QUS, MCIII and accessory carpal bone densities assessed *ex vivo* using DEXA have shown that tiludronic acid improves bone architecture and density on selected cortical and trabecular sites. This effect on bone density was confirmed by prevention of osteopenia in immobilised limb (Delguste *et al.* 2007).

Conclusion

It was becoming more and more evident from previous work that bone biochemical biomarkers because of their multiple and general variations, would not be able to resolve alone diagnostic situations, neither to give sufficient elements to follow training or treatment interventions in an equine sport population. These later works tend to confirm the hypothesis that for getting, at a specific period, a better interpretative *in vivo* image of bone adaptation to an external stress (training, drugs intervention), it is necessary to combine biochemical biomarkers reflecting the cellular level with imaging biomarkers reflecting the biomechanical level through bone composition, density and elasticity. However we need to find the appropriate biomarkers and appropriate measurement sites for clinical situations. These are still difficult points to address if we want this combination to lead the equine veterinarian to assess drug therapy fracture risk or an orthopaedic disease at an early stage.

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CARTILAGE OLIGOMERIC MATRIX PROTEIN (COMP) IN EQUINE MIDDLE CARPAL JOINT IN RELATION TO FRACTURES AND LOAD

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Lameness, as a result of repeated overload of the joint, leads to articular cartilage damage which may be accompanied by synovitis, ligament tear and subchondral bone sclerosis. Osteochondral fractures most commonly involving the dorsal, proximal joint margins of the carpal joint are also an important cause of training failure in the equine athlete.

In a recent study (Skiöldebrand *et al.* 2005) the authors found a high concentration of COMP in synovial fluid in Thoroughbreds (TB) with osteochondral fractures compared to Standardbred trotters (STB) with normal articular cartilage and articular cartilage with moderate lesions or osteochondral fractures

The articular cartilage of the unloaded osteochondral fragments expressed COMP mRNA, in contrast to the articular cartilage on the opposite side of the fracture, still subjected to load. Intact COMP was present in the joint with osteochondral fracture, which suggests an up-regulation of this protein where compressive loading of the fragment is low. A possible mechanism for the increased COMP could be that the dislocation of the osteochondral fragment results in a lower loading of the cartilage which could influence the synthetic activity of the chondrocytes. The chondrocytes in the cartilage specimen from the part of the fractured bone where load continues to be exerted did not express COMP mRNA. This is in agreement with a longitudinal study of young strenuously trained trotters, where lower COMP levels were correlated to total days of training and age (unpublished data).

Dynamic compression at high load and high frequency, as a result from strenuous training, has drastic effects on the metabolism of articular cartilage from the dorsal radial facet of the equine third carpal bones.

Development of new assays, quantifying fragments or detecting epitopes of matrix molecules specific for anabolic and catabolic processes will aid in the understanding of factors responsible for joint failure in the equine athletes. Also, studies focusing on cell responses, through integrins (receptors for specific proteins), during cyclic compression at different amplitudes and frequencies, can clarify how mechanical signals are transduced and how the chondrocyte will respond. Cell reaction will depend on the magnitude and rate of load as well as the duration and nature of the loading pattern (constant vs. intermittent).

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MOLECULAR MARKERS OF TENDON INJURY: CLINICAL AID OR RESEARCH TOOL?

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INTRODUCTION

While imaging modalities for evaluating tendons and ligaments have advanced considerably in recent years, they still have limitations. Some require general anaesthesia (eg MRI) which carries increased risk to the patient and increased cost, while the more established technique of ultrasonography can not be used as a screening procedure, often can not determine the ideal time to return to work, and frequently fails to detect the presence of intra-thecal tendon tears.

These shortcomings can be overcome with the use of molecular markers which may provide additional objective information on:

- tissue metabolism (eg effect of training)
- diagnois of injury (eg severity and stage)
- prognosis
- appropriate choice of treatment and monitoring its efficacy

One pre-requisite for a marker of a process in a particular tissue is a select or much enriched distribution to this compartment. Our studies into the matrix composition of tendon have provided several candidates for molecular markers of tendon disease, 2 of which are cartilage oligomeric matrix protein (COMP) and fibromodulin (FBM).

COMP is a non-collagenous extracellular matrix protein found predominantly in tissues whose function is mainly to resist load – cartilage, tendon, ligament, and meniscus. Studies in man have demonstrated that it may be used as a prognostic marker in rheumatoid arthritis and osteoarthritis. However, initial studies (reported in the previous molecular markers meeting) demonstrated that single serum levels of COMP where not predictive of tendon disease.

FBM is a member of the small leucine-rich repeat protein family (SLuRPs), predominantly associated with the collagen fibrillar network in soft tissues. It is especially abundant in tendon, where 'knock-out' experiments in mice have demonstrated it to have a functional role in tendon strength.

In this paper we report on new data which have extended our objective of developing a clinically useful assay for tendon injury.

RESULTS

Synovial fluid COMP levels as a predictor of intra-thecal tendon pathology

Analysis of synovial fluid (SF) from digital sheath synovial fluid in the presence of either tendon damage or sepsis revealed the presence of COMP fragments not present in normal digital sheath fluid. Recent clinical experience has suggested intra-thecal tendon tears are not only a common cause of tenosynovitis causing lameness but that pre-operative clinical examination and diagnostic ultrasonography do not allow the identification of these tears with confidence. It was therefore hypothesised that SF levels of COMP would be elevated in those cases with intra-thecal tendon pathology. SF samples were collected from 77 digital sheaths which were examined either tenoscopically (those with clinically significant pathologies; n=37) or at post mortem (to ensure an absence of pathology for controls; n=40). This has demonstrated significantly raised levels of COMP in cases of intra-thecal tendon disease compared to controls. This assay may therefore be very helpful

for the prediction of the presence of tendon damage pre-surgery.

Evaluation of a neo-epitope fibromodulin antiserum in tendon disease

A fibromodulin neo-epitope antiserum was evaluated against equine tendon extracts from young and aged tendon and also from 6 injured tendon extracts (from acute and chronic superficial digital flexor tendonitis). This neo-epitope was first identified in cartilage explants stimulated with IL-1. An antiserum, raised against the epitope, recognises the new N-terminus after MMP-13 cleavage of fibromodulin in bovine cartilage explants *in vitro*, and represents the portion of the molecule initially retained within the tissue. Although the N-terminal neo-epitope antiserum was developed to an *in vitro* fragment generated from IL-1 stimulation of bovine cartilage explants, it was found to label only extracts from tendons which had been recently injured and not extracts from chronic injury or aged tendons, thereby indicating a highly specific marker of acute tendon damage. Testing of synovial fluids with this antiserum confirmed that this epitope was not released into the synovial fluid in significant quantities.

Conclusion

The development of a serological assay for tendon injury is still the Holy Grail for the tendon clinician and, while much progress has been made in developing an assay which will recognise tendon injury from a blood sample in a sensitive and specific manner, this goal remains to be realised. However, in the process of developing these markers of released protein fragments, the accompanying information generated (such as identification of the complimentary epitope in the tissue-retained portion) and the use of other neo-epitope antisera developed in other species in different experimental models will potentially yield valuable information of understanding the disease process. One essential research tool that is still unavailable is the ability to detect the early stages of tendon degeneration (rather than clinical disease) and these reagents will potentially enable this to be done.

IMAGING BIOMARKERS: WHERE ARE WE GOING?

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Clinical imaging techniques for diagnosis of orthopaedic problems have advanced significantly in recent years with the advent of digital radiography and ultrasound, computed tomography and magnetic resonance imaging. These modalities are used routinely for diagnosis of disease, and even in the best of situations, are sometimes subject to scrutiny because of their inability in certain conditions to be accurate for diagnosis of the disease. The purpose of this paper is to provide evidence of how current imaging techniques have improved early diagnosis of orthopaedic diseases, demonstrate correlation of imaging results to pathological changes in tissues, and to describe current research efforts to improve diagnosis.

Radiography

Results of radiographs have been scrutinised because of the significant amount of disease needed to impart changes on the films. However, in recent years, digital technology has allowed clinicians to manipulate images in order to improve lesion detection. Yet despite these improvements, a 30–40% change in bone mineral density is still needed to detect a lesion radiographically, allowing for significant tissue changes to occur prior to detection (Greenfield 1986). In addition, it appears from recent research that subchondral bone from OA joints changes in opposing fashion – ie, the bone increases in mass but decreases in quality and density. Therefore, even with significant reduction in bone density, as occurs with OA, the resulting increase in bone tissue mass clouds the clinician's ability to detect bone changes.

Even though radiographs seem to be of low sensitivity in predicting or detecting early changes indicative of OA, in various human clinical studies radiographs have shown some benefit in assessing OA progression. Mazzuca *et al.* (2005) showed that in the human knee, OA progression (osteophytes and joint space narrowing) worsened with severity of the initial signs (Mazzuca *et al.* 2005). However, they also showed that the images can be influenced by several factors, including joint pain. (Mazzuca *et al.* 2002). Lanyon (1998), on the other hand, showed that osteophytes were correlated closely with joint pain, but that there was no consistent lower limit of joint space narrowing that was indicative of joint pain (Lanyon *et al.* 1998). Therefore, it appears overall that radiographs can help in monitoring joint disease, but that it is rather insensitive for early detection, or prediction of OA.

We continue to include radiographic assessment of joint disease in our studies and continue to find that when compared to treadmill-exercised horses, those with osteochondral fragments have significant worsening of radiographic lysis, proliferation and osteophyte formation at the fragment site at the end of the studies.

Nuclear scintigraphy

The reliability of nuclear scintigraphy to detect early OA in humans appears to be site-dependent. McCarthy *et al.* (1994) showed that it was predictive for hand OA, but Mazzuca showed it was inferior to baseline radiographs and clinical signs for predicting joint space narrowing (McCarthy *et al.* 1994; Mazzuca *et al.* 2006). However, Dieppe *et al.* (1993), showed that 88% of those individuals with severe nuclear scintigraphy changes initially went on to develop clinical OA, and that 100% of those without

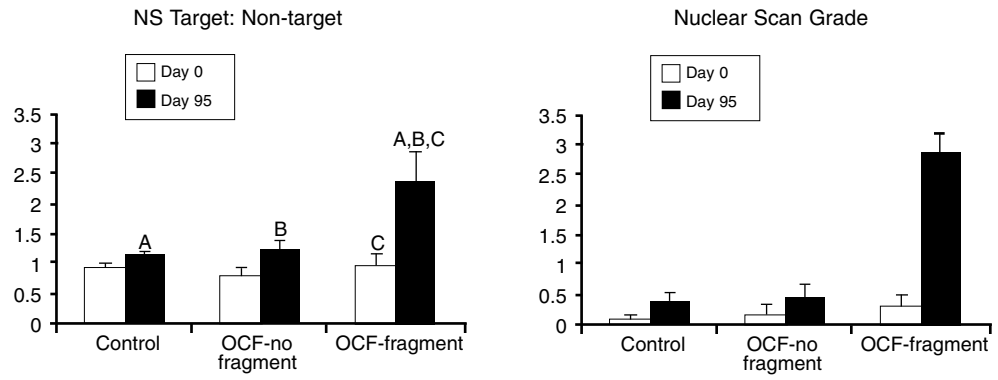

Fig 1: Nuclear scintigraphic results of the carpus from a study in which a group of horses (half with osteochondral fragments and half without) underwent treadmill exercise. The results were scored subjectively (left graph) and objectively in which the ratio of the uptake per pixel in the target area (carpus) was compared to a remote area. Those horses with osteochondral fragmentation were detectable beyond the background effects of exercise in this study. Same letters indicate significant differences.

severe changes did not develop clinical OA over a 5 year period. As for monitoring progression of OA, Balblanc *et al.* (1995) found that even though nuclear scintigraphy correlated well with radiograph severity, it was unreliable for monitoring progression of OA, since the nuclear scintigraphy results could increase or decrease with disease progression.

In a study in which the effects of 6 months of treadmill exercise on 2-year-old horses were evaluated, uptake of radiopharmaceutical was significantly higher in the metacarpophalangeal (MCP) and carpal joints of those that were exercised compared to age-matched non-exercised horses (Kawcak *et al.* 2000). The severity of uptake correlated with the degree of gross articular cartilage lesions in the MCP joint, but the significance of those lesions on future soundness were unknown. It was concluded from this study that exercise alone can induce a significant scintigraphic response. In a separate study though, nuclear scintigraphy was able to be used to separate those horses with osteochondral fragments from those that were only exercised (Fig 1). Although nuclear scintigraphy appears helpful in early diagnosis of disease, it lacks the specificity of being able to detect the exact lesion in the joint. However, it may be a useful screening and monitoring tool for OA.

Computed tomography

Computed tomography (CT) has had relatively little clinical use for diagnosis and monitoring of joint disease in any species. In most instances it has been used to quantify geometric parameters in joints, and changes in those parameters with disease (Holsworth *et al.* 2005; Matsui *et al.* 2005). Computed tomography has been used clinically to detect occult lesions in subchondral bone of joints and has played a limited role in presurgical planning.

Detection of subchondral bone density by CT has been performed, and in the authors' laboratory, Computed Tomographic Osteoabsorptiometry (CTO) patterns of density have been detected that appear to be common in cases of joint disease. For instance, in post mortem samples from racehorses, it is not uncommon to see vertical demineralisation patterns in the distal third metacarpal condyles in the area where condylar fractures commonly occur.

The limitation of clinical CT is that some consider the resolution to be too great in most instances to detect subtle changes in subchondral bone. As an example, Ekstein *et al.* (2000) showed that resolution limited detection of bone defects. Micro-CT had a close correlation to bone vascular pore size, but the others did not. MRI, CT and pQCT had resolution too large to see defects (59u, 350u and 330u respectively). We have seen something similar in that the bone formation response to disease and exercise in the subchondral bone plate is below the resolution of the typical clinical CT scanner. However, in the trabecular bone, the response seems great enough in most instances to detect a difference. As an example, in the osteochondral fragment/exercise

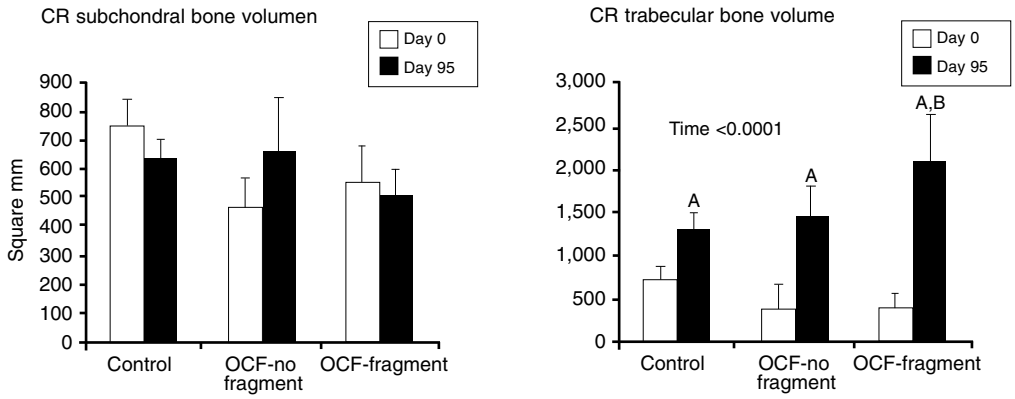

Fig 2: Results of CTO showing a significant increase in trabecular bone density in the radiocarpal bone of horses with osteochondral fragmentation vs those without. The same change in subchondral bone was not seen, and there actually appears to be a trend towards a decrease in density at that site. Same letters show significant differences.

study, CTO was performed during the study. It was shown that CT was not sensitive enough to show the modest drop in bone density in the subchondral bone, but was able to show the significant increase in the surrounding trabecular bone density (Fig 2). The pattern of subchondral bone mineralisation also appears to be indicative of changes in loading and pathologiacal conditions. As an example, the images in Figure 3 show the subchondral mineralisation patterns of a 3-year-old horse (right) and a 10-year-old horse (left) demonstrating the increase in surface area covered by high density bone as the animal ages. The 3-year-old horse shows a typical pattern that shows increased mineralisation at the proximal sesamoid bone (PSB) articulation with the third metacarpal condyle (A). The increase in density across the joint surface in older horses also appears in young exercised horses, which seems to either increase the density across the joint surface, or reduce the incongruency typical of normal joints. This alone may predispose the joint to abnormal loading.

In joints with lesions, the normal pattern of loading begins to change. In the condyle with gross signs of palmar arthrosis in Figure 4, the density at the site of PSB articulation, which should be greater than surrounding bone, shows reduced density. In Figure 5, a condyle with greater palmar arthrosis shows even greater reduction in subchondral density, and a saggital section shows that the demineralisation extends well into the trabecular bone. Therefore, it appears that CTO density pattern can be useful for characterising insidious disease processes, such as palmar arthrosis, which can lead to OA. Furthermore, the images, once acquired, can be cut into any plane and analysed. We have found this useful as linear, saggitally oriented demineralisation patterns have been seen in the palmar aspect of MCIIIs, which appear to occur in areas predisposed to condylar fractures (Fig 6).

Magnetic resonance imaging

Magnetic resonance imaging has revolutionised the detection of subtle joint diseases in all species, and in particular, the detection of soft tissue and articular cartilage lesions. However, as Ekstein *et al.* (2000) pointed out, its resolution is limited and subtle bone and joint lesions can sometimes be missed. Besides this though, the fact that various sequences can be used to highlight various tissue characteristics, and the addition of contrast agents that can be correlated to various biochemical reactions in tissues, MRI has the greatest ability to be used as a predictive marker of disease.

Magnetic resonance imaging is robust in its ability to be manipulated to address both geometric and biochemical changes in tissues. As a 3-dimensional imaging modality, image slices can be rendered into a composite image that can be manipulated. We have used MR images to form the basis for modelling studies of the carpus. The articular cartilage layer, meniscus and bone can be rendered and separated from the other tissues for study. Each layer can then be evaluated for physical characteristics, mechanical properties

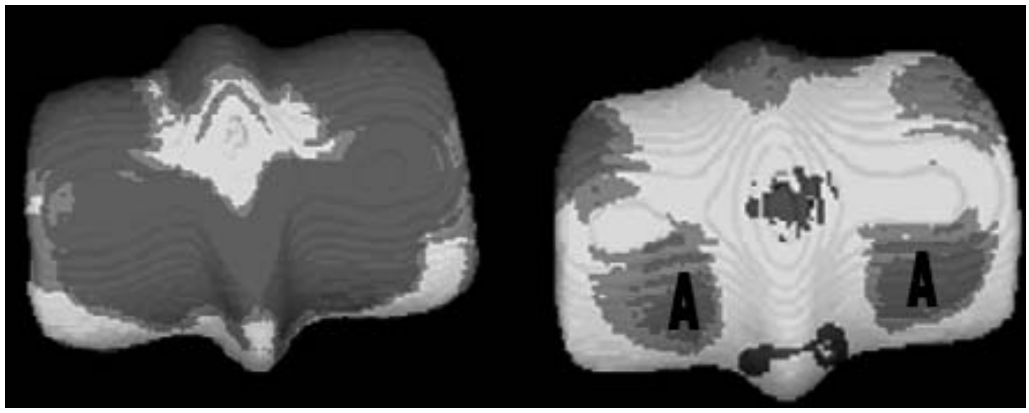

Fig 3: Three-dimensional surface renderings of computed tomography scans showing the distal third metacarpal condylar surface of a 10 year old horse (left) and a 3-year-old horse (right). Note the more highly mineralised subchondral bone in the 10 year old horse. In the 3-year-old horse, it is common to see a pattern of increased mineralisation where the proximal sesamoid bones articulate with the third metacarpal condyle (A).

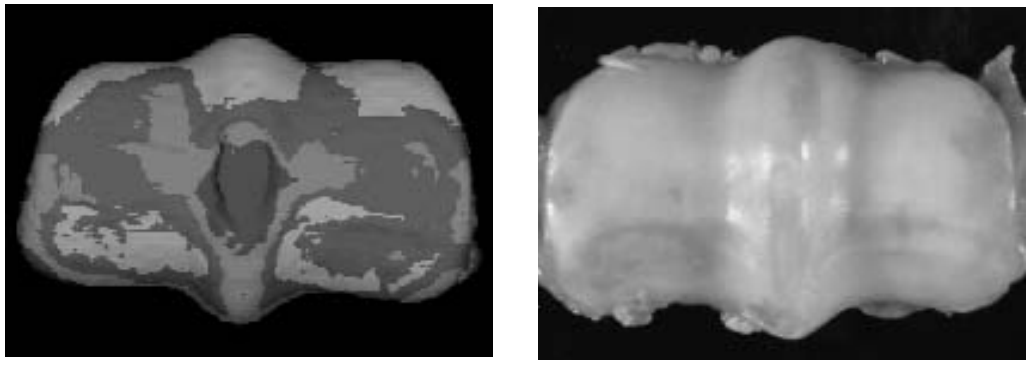

Fig 4: A 3-dimensional image of the third metacarpal condylar surface rendered from computed tomography scans (left) and the matching gross specimen (right). Note the reduced subchondral bone density in the area of proximal sesamoid bone articulation corresponding to an area of palmar arthrosis.

using finite element modelling or application of biochemical and material properties in a mapping configuration. Therefore, in one setting, through the manipulation of various sequence settings, a large amount of data can be acquired which can be used to assess everything from mechanical to biochemical status of tissues.

GAIT ANALYSIS

Although gait analysis is not an imaging modality, its application in clinical orthopaedics has been useful for early recognition of disease. Injury and pain often set off a change in limb loading which then can influence the other tissues. Initial decrease in weight-bearing has been shown to increase articular cartilage thickness and subchondral bone loss, which later in an OA model, resulted in articular cartilage loss and subchondral sclerosis (Braunstein *et al.* 1990). This change in weight bearing was thought to initiate these changes. In addition, it has been shown that people prone to knee OA have shown a significant increase in heel strike during gait, leading to the conclusion that this gait leads to propulsive loading within the knee that later causes OA (Radin *et al.* 1995) Although gait analysis will probably never stand on its own as a means of early diagnosis of bone and joint disease, it will be critical for biomechanical model validation and use in the future.

BIOMECHANICAL MODELLING

All imaging modalities to date focus on identifying tissue changes that result after the

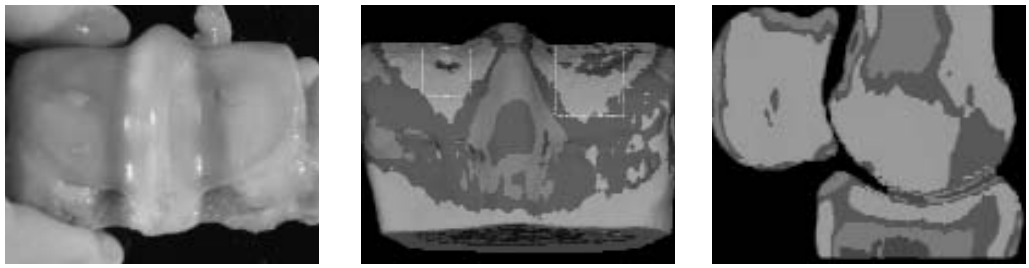

Fig 5: A gross specimen of the distal third metacarpal condyle (left) and corresponding 3-dimensional computed tomography scans (right). Note the significant reduction in subchondral bone density corresponding to the more severe palmar arthrosis lesions.

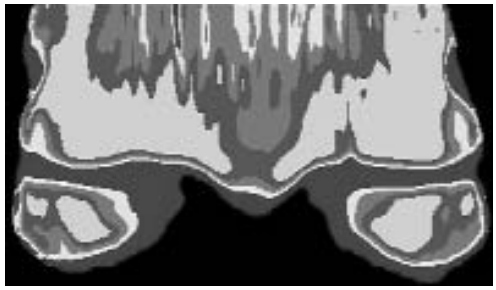

Fig 6: A frontal plane slice from a 3 dimensional computed tomography image showing an area of demineralization where condylar fractures are commonly seen.

initiating cause has occurred. As part of the paradigm of OA pathogenesis, it is known that several animal-specific parameters can lead to disease, including abnormal geometric configurations to joints, and abnormal limb use. Therefore, it would be most beneficial if joint characteristics that might lead to disease could be identified. Much like genetic markers, using biomechanical modelling to identify those animals with joints that are predisposed to disease would allow for modification of use and lifestyle in the hopes of preventing, or at least reducing the severity of disease.

Biomechanical modelling of limbs has occurred from 2 directions. The first is musculoskeletal modelling, in which the forces from limb loading and muscle forces are imparted on a geometric model of the limb, and moments about joints are calculated. These data give an impression of the loading on the joint as a whole. The second direction is finite element modelling of joint surfaces. With this technique, small nodes of data on the joint surface are identified and input parameters, such as that from musculoskeletal modelling data and materials testing of the tissues, are imparted into the model and the resulting joint surface stresses are calculated. Therefore, the joint surface geometry, tissue properties and loading are summed and the resultant joint surface stresses result in a 3 dimensional model.

As an example, we have approached modelling from the 2 directions. Using musculoskeletal modelling in collaboration with Marcus Pandy and Nick Brown, we have seen that the model is a good estimate of the moments about the fetlock joint (Brown *et al.* 2003a; Brown *et al.* 2003b; Brown *et al.* 2003c) however; work by Swanstrom *et al.* (2005), has shown that the muscle loading data may be different depending on the means of assessment (Swanstrom *et al.* 2005). In addition, we have performed finite element analysis in collaboration with the Orthopaedic Research Laboratory at Columbia University, and showed that the direction of loading on the joint surface can be determined (Koff *et al.* 2001). The key now is to combine the results from both musculoskeletal and finite element modeling to determine joint surface stresses on a patient specific basis.

Summary

It appears that imaging will always remain a key component of orthopaedic disease diagnosis, but at this time we need to continue pursuing greater sensitivity in its ability to be used to detect subtle joint damage and to predict those individuals that are predisposed to OA.

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INFRARED SPECTROSCOPY – A NEW TOOL FOR THE STUDY OF SYNOVIAL FLUID

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INTRODUCTION

Conventional synovial fluid (SF) analyses are not widely used for evaluation of non-infectious joint disease because they rarely provide clinicians with a specific diagnosis. Recently, ELISA and radioimmunoassay based evaluations of biomarkers within SF have been described. Complex multiple assays are required, individual testing using these techniques is expensive, and the relationships of the concentrations of the biomarkers to age, breed, gender and circadian rhythms are poorly understood. Early results show promise, but further study is required to determine their clinical usefulness for classifying osteoarthritis (OA). Currently we do not have the *in vivo* means to objectively identify the level of pathological progression in most cases of OA, primarily because no generally accepted objective standards exist. There is a real need for a rapid, economical, practical and reliable diagnostic test for objective evaluation of joint disease, as well as the unbiased monitoring of responses to treatment.

INFRARED SPECTROSCOPY

Infrared (IR) spectroscopy is rapidly emerging as a powerful diagnostic probe for biological molecules in man and animals. IR spectroscopy measures infrared absorption patterns of molecules when exposed to IR light. Molecules within a complex sample give rise to unique absorption spectra with characteristic absorption band patterns reflecting both structure and concentration. The absorption patterns within the IR spectra of biological samples may be viewed as biochemical fingerprints that correlate directly with the presence or absence of diseases. For example, IR spectroscopy has been used in the diagnosis of human diseases such as diabetes mellitus, Alzheimer's disease, breast tumours and arthritic disorders. The advantages of an IR spectroscopic approach in clinical diagnosis are that no reagents are required, and automated repetitive analyses can be carried out at very low cost. Moreover, since the IR spectrum of biological samples such as synovial fluid reflects the sum of all IR active components, the infrared spectra of such samples may carry infrared signatures of both known and unknown biomarkers rather than relying upon a few novel markers as indicators of disease.

APPLYING IR TECHNIQUES FOR THE STUDY OF JOINT DISEASE

It was hypothesised that joint disease leads to changes in equine synovial fluid composition, altering the IR absorption pattern of synovial fluid samples, and that these spectroscopic changes can be detected and used to differentiate the synovial fluid spectra of joints with arthritis from the spectra of control samples. The objective of our first study was to determine the feasibility and accuracy of using IR spectroscopic and pattern recognition techniques to differentiate synovial fluid samples from joints with disease from those of control samples. A severe from of joint disease, traumatic arthritis, was examined. Synovial fluid samples were collected from joints with osteochondral fracture and contralateral or ipsilateral control joints in 48 horses. An independent set of SF samples was collected from clinically and radiographically normal horses. Fourier transform infrared (FT-IR) spectra of synovial fluid were acquired and manipulated, and

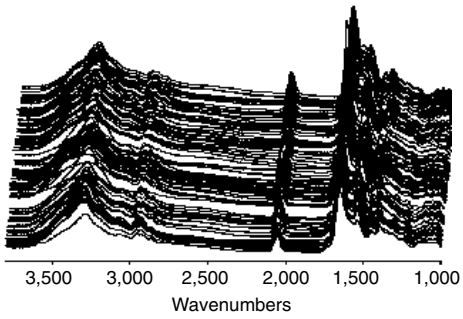

Fig 1: Acquisition of spectral data prior to signal averaging.

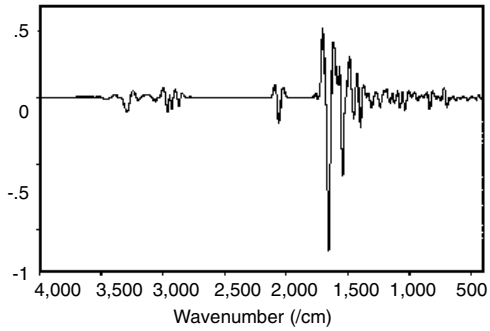

Fig 2: Second order differential of spectral average prior to linear discriminant analysis.

data from diseased joints were compared to controls to identify spectroscopic features that differentiated between the groups (Figures 1 and 2). A classification model using linear discriminant analysis (LDA) was developed based upon the spectral regions encompassing these features. Performance of the model was determined first using a validation dataset, and further by using an independent set of normal control data. Based upon these data, a classification model based upon 3 IR regions classified spectra from the calibration dataset with overall accuracy 97%. The same model produced an overall accuracy of 89% for the validation sample dataset, and 100% correct classification of the set of independent normal control joints. In conclusion, the IR spectroscopic patterns of SF from joints with traumatic arthritis differ significantly from the corresponding patterns for controls. This study also clearly demonstrated the potential for this classification algorithm to confirm the absence of disease in a 'normal' equine population. These alterations in IR absorption patterns may be exploited via an appropriate classification algorithm to differentiate the spectra of diseased joints from those of controls. The current results demonstrate the feasibility of a novel IR-based approach for the diagnosis of equine traumatic arthritis. Further recruitment of cases and normal control horses is anticipated to develop and expand the scope of applications, and is necessary to validate the clinical value and accuracy of this method for screening, diagnosing, and classifying equine joint disease.

WHERE ARE WE WITH POPULATION GENETICS IN THE HORSE: WHAT CAN BE LEARNED FROM STUDIES OF THE HUMAN GENOME AND WHERE CAN IT TAKE US?

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The collective interactions of the worldwide equine genetics community have resulted in advances in genetic research applied to improving the health of the domestic horse. Genetic markers and genetic mapping resources provided by many contributing groups have enabled genotyping efforts to collectively produce the genetic map of the horse. Many continuing and future studies in the horse will require either family or population based studies. Such studies require an accurate order of markers that can be derived from physical maps, such as a radiation hybrid panel. Furthermore, the horse linkage map, which provides recombination distances, has evolved from 2 recombination-based maps, the male-based half sib Horse Reference Families (HRF) and the 3-generation family produced at the Animal Health Trust. Equine researchers are starting to utilise these microsatellite markers and linkage maps to assist in identifying genes for heritable diseases in a variety of horse breeds. Some have also begun to use microsatellites near candidate genes, and allele association analyses, to investigate various heritable disorders.

Since such studies in the horse are in their infancy the experiences and technologies of those involved in the study of human disease are very important and much can be learned from them. We have recently seen the completion of the human genome sequence, the deposition of millions of SNPs into public databases, rapid improvements in SNP genotyping technology and the initiation of the International HapMap Project. This has allowed the progression from microsatellite analysis to the large-scale use of single nucleotide polymorphisms (SNPs). SNPs are the most abundant form of DNA variation in the human genome. It has been estimated that there are approx 7 million common SNPs with a minor allele frequency (MAF) of at least 5% across the entire human population. Most common SNPs are to be found in most major populations, although the frequency of any allele may vary considerably between populations. It is thought that an additional 4 million SNPs exist with a MAF between one and 5%. The stage is now set for SNP-based, genome-wide association studies, in which a dense set of SNPs across the genome is genotyped to survey the most common genetic variation for a role in disease or to identify the heritable quantitative traits that are risk factors for the disease.

One approach for identifying such genetic risk factors is the case-control association study, in which a group of individuals with disease is found to have an increased frequency of a particular genetic variant compared to a group of control individuals. A number of genetic risk factors for common disease have been identified by such association studies. Theses studies suggest that many different genes distributed throughout the human genome contribute to a total genetic variability of a complex trait, with any single gene accounting for no more than a few percent of the overall variability of the trait. Case-control study designs that include on the order of 1000 individuals can provide adequate power to identify genes accounting for only a few percent of the overall genetic variability of a complex trait, even when using the very stringent significance levels required when testing large numbers of common DNA variants. Studies have shown that variants in close proximity are often correlated. However, this correlation structure or linkage disequilibrium (LD) is complex and varies from one region to another, as well as between different populations. Selection of a maximally informative subset of common SNPs for use in association studies is necessary to provide sufficient power to assess the

causal role of common DNA variation in complex human traits. A large fraction of all common human SNPs are available in public databases, but there is still relatively little information concerning SNP allele frequencies. Therefore, more progress must be made before one can select an optimal subset.

Association studies that are genuinely genome-wide offer great promise to efficiently and comprehensively test common genetic variation across the genome for a role in common disease and complex traits in humans, as well as in a number of different animals including the horse. Following the success and advances that have been enabled from the completion of the human genome sequence, many other mammalian genomes, such as dogs, have recently been sequenced and the data deposited into public databases. It is hoped that in the near future we will witness sequencing of the equine genome and thus benefit from similar advances, especially in SNP technology. In the meantime we will learn from both SNP and microsatellite approaches across numerous genomes.

GENETIC MARKERS: USE OF MICROSATELLITE REPEATS IN EQUINE DISEASE ASSOCIATION STUDIES

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Equine microsatellite markers, short repeats of highly identifiable sequence such as $(CA)_{16}$, were first cloned in 1992 and found to be polymorphic. Their value in genome mapping, parentage and identity testing was acknowledged with the first Equine Gene Mapping Workshop being held in 1995. Microsatellite markers were also then recognised as potential tools for use in association studies to study the genetics of complex disease in the horse.

Whilst monogenic equine diseases have long been understood, little is known about the role of genes in complex disease in the horse. In man, through research into osteoporosis and stress fractures, many advances have been made in defining genotypes that predispose to fracture risk and influence bone mineral density and peak bone mass. Such research has been largely based on linkage studies. Linkage is the tendency for genes or other DNA sequences (such as microsatellite markers) at specific loci to be inherited together as a consequence of their physical proximity on a single chromosome. Families with multiple affected relatives are scanned with markers spaced across the genome; in theory, if enough markers have been typed, then at least one will lie sufficiently close to the disease gene that its inheritance pattern will match that for the disease gene. Thus the position of the disease gene is approximately known. When a mutation occurs at a particular locus, recombination in subsequent generations has the effect that the mutation will only remain close to the markers that were physically closest to it originally. These markers are in linkage disequilibrium (LD) with the mutation.

In complex conditions, such as fracture, there are problems with using linkage and positional cloning methods. Complex diseases are typically difficult to classify phenotypically because of their range of presentations, their aetiology is often just as variable in terms of biological mechanism and, lastly, they are likely to be caused by many genes contributing small effects, each with their own relative risk. These drawbacks have led genetic researchers towards the more statistically robust approach of association studies for characterisation of multigenic disease. A genetic association study investigates relationships between the frequency or severity of a trait (usually through cases and controls) and polymorphic DNA sequence. This variable sequence may be in the form of a microsatellite marker or single nucleotide polymorphism (SNP): the different possible variants, or alleles, in either instance are assessed for association with the disease. In a positive association, there are 3 conclusions: the polymorphism being tested affects the risk of disease directly, it is a marker in LD with some nearby genetic variant which affects the risk of disease or the association has occurred by chance.

In addition to statistical advantages, an association study has the added benefit that markers within (or in LD with) candidate genes can be used. Candidate genes are genes selected on their biological plausibility as being likely to influence the condition or disease. Regarding fracture, for example, many candidate genes with specific polymorphisms such as COL1A1 (collagen 1 alpha 1), IL6 (interleukin 6) and VDR (vitamin D receptor) are known to affect bone structure and function in man.

Since fracture remains a major cause of morbidity and mortality in Thoroughbred racehorses across the world, it seemed timely and appropriate to apply knowledge gained from, and approaches employed in, human studies to look at

genetic influences on fracture and tendonitis in the horse.

It was hypothesised that there was a genetic basis for fracture in the Thoroughbred horse. The aim of the study, supported jointly by the Royal Veterinary College and the Animal Health Trust, was to establish whether polymorphic genetic markers might be predictive of risk of fracture or tendonitis in Thoroughbred horses. The following objectives were identified:

- To identify polymorphic microsatellite markers linked with candidate genes known to affect bone and tendon structure and/or function
- To genotype these markers in DNA from cases of fracture and tendonitis and in matched control DNA
- To compare allele and genotype distribution statistically in order to identify associations of particular alleles or genotype with fracture and tendonitis.

Ten candidate genes were selected (COL1A1, COL1A2, TGFβ1, ACE, ESR1, IL6, IGF1, LRP5, IL1ra and COMP). Overgo probes were designed to each candidate gene and used to isolate clones containing all or part of that gene from an equine Bacterial Artificial Chromosome (BAC) library. Restriction fragments from these clones were size selected, purified, ligated into a pUC18 *Sma*I plasmid vector and transformed into *E. coli* TG1 cells. Transformed colonies containing the BAC insert were picked out in duplicate and cultured overnight. One set of these was probed using a α-5' ^{32}P-labelled poly-CA/poly-GT probe and any positive clones sequenced from the other set in order to locate microsatellite markers. Polymorphic markers were genotyped in case and control DNA extracted from blood samples from 1,240 horses in training in the UK.

Five informative markers were found for each of 5 candidate genes (COL1A1, IL6, IGF1, LRP5 and COMP) and were genotyped in 167 fracture cases from horses raced on the flat (334 controls), 74 fracture cases from jump horses (222 controls) and 133 cases of tendonitis (399 controls). Data analysis is ongoing but preliminary findings have indicated associations. For example, additive associations were identified between increased fracture risk and a IL6 marker in the jump horse group (with one copy of the 'risk allele', 1.6 x increased risk of fracture, 95% CI=1.08-2.49, p=0.02; with both copies as the 'risk allele', 3.2 x increased risk of fracture, 95% CI, 1.12-9.25, p=0.04). Early analysis of fracture type with respect to genotype has also yielded positive results, for example, a strong association between a COMP allele and condylar fracture (p=0.009). No associations have yet been found between genotype and tendonitis.

These data support the hypothesis that there is a genetic basis for risk of fracture in the Thoroughbred horse and show that a matched case control association approach may be used to study it. The results are biologically plausible (for example, COMP may be influential in endochondral ossification and subsequent condylar bone strength) and they are also consistent with findings in man (such as IL6 being known to influence human bone mass and remodelling). These early data highlight the need for future studies in this area. The Thoroughbred breed represents a closed group, 78% of the alleles in the current population being derived from just 30 founders. It is therefore a good population to use in such studies because it minimises problems of population stratification encountered in analogous human work. It also provides opportunity to study defined gene-gene or gene-environment interactions because aspects of the horses' environment, such as training, may be characterised. If the findings described here were corroborated and developed in such studies, then the aim of using genes as part of a 'risk assessment panel' in future strategies for reducing musculoskeletal disease in the horse could be achieved.

GENETIC MARKERS IN EQUINE ORTHOPAEDIC DISEASE

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Genetic markers can be used in a number of ways to both inform orthopaedic research to implicate future synovial fluid, urine and sera protein biomarkers as well as to act as markers for both disease itself or altered risk of susceptibility of disease. In this paper data will be presented from 4 different projects which have either used genetic markers to inform our knowledge of the pathological process in equine orthopaedic disease, or have used genetic markers to identify disease or altered risk to suffering from disease.

Gene expression in normal and pathological articular cartilage

Extensive studies have been performed to identify the phenotypic variation (as identified by quantifiable assay of gene expression) of equine chondrocytes in a variety of pathologies including acute and chronic OA, OCD and various stages of development in a variety of joints. We have quantified levels of expression of over 25 key matrix, proteinase and inflammatory genes in over 200 articular cartilage biopsies and identified those genes which are regulated in various stages of disease. The data has been analysed using a variety of statistical tests including mixed effects regression models and principal component analysis. Data will be presented on the variation in key matrix genes in various disease states and how this may inform the choice of candidate markers for identification of articular pathology.

Gene expression in normal and pathological superficial digital flexor tendon

Researchers have performed similar molecular phenotyping studies on equine tenocytes from both the tensional and compressional regions of the superficial digital flexor tendon in normal as well as acute and chronic tendonitis cases. Using similar statistical analyses we have identified some of the key phenotypic changes in equine tendon disease. Such data can be used to identify relevant biomarkers for diagnosis of such disease states.

Gene expression as a marker of articular disease in synovial fluid cytology

Whilst synovial fluid cytology is a well characterised diagnostic test in orthopaedic diagnosis, its use is often limited other than in the identification of orthopaedic sepsis. We have performed a preliminary study to investigate whether quantifiable assay for a variety of pro-inflammatory cytokine genes in synovial fluid cytology can be a useful marker in equine articular disease. Synovial fluid has been obtained from 10 clinically normal horses, 8 horses with acute joint trauma, 6 horses with chronic OA and 6 horses with clinical OCD. Immediately following sampling, 3 mls of synovial fluid are mixed with 3 mls of RNA later and transported to the laboratory. The cellular content of the fluid is pelleted by centrifugation, and then RNA is extracted using standard protocols. Due to the scant cellularity of many samples, RNA yield was low, and thus global amplification of the RNA was performed to achieve satisfactory levels of cDNA. The resultant cDNA libraries were quantified for gene expression levels for a variety of pro-inflammatory cytokines using qPCR. Significant differences in gene expression for a variety of pro-inflammatory cytokines and growth factors were identified

between both normal and diseased samples, as well as between the different disease states. In particular, levels of IL-1alpha, TNF-alpha, IL-1rap and TGFβ1 gene expression show particular promise as markers of joint pathology in synovial fluid cytology. Whilst this study is preliminary, the data indicates promise in this technique and we intend to apply this technique to a more clinically applicable cohort of animals, as well as expanding the number of potential genes to be quantified.

Single nucleotide polymorphisms (SNPs) in candidate genes for orthopaedic disease and their association with disease

In human medicine in recent years, there has been a considerable amount of work and interest into the role of complex genetic traits and the effect of such genetic variability on altered susceptibility to various diseases processes. In humans there is now substantial evidence that even minor variations (polymorphisms) in an individuals genetic code can lead to a substantial variation in an individual's susceptibility to a number of diseases. Hence, different individual's may show a widely different disease response due to only very small differences in genotype. These genetic differences are frequently as a result of single base pair changes, known as single nucleotide polymorphisms (SNPs). SNPs can lead to either transcription of qualitatively different proteins, if the variation is within the translated coding region, or may lead to quantitative differences in gene expression, as a result of promoter region SNPs leading to altered expression levels of proteins.

We have investigated SNPs in 2 equine genes, tumour necrosis factor-alpha (TNF-α) and cartilage oligomeric matrix protein (COMP). For the TNF gene we have used (d)HPLC wave analysis to screen a 600bp section of the promoter region in 150 horses of mixed breeds. From this analysis, we have identified 5 SNPs. We have then developed a Snapshot high throughput SNP typing assay and have genotyped over 1000 horses for these SNPs. From this analysis we have identified 10 different haplotypes, and demonstrated that haplotype frequency varies between different breeds of equidae. For the COMP gene we have investigated SNPs in 2 regions of the gene using similar analysis. Firstly, in the promoter region consisting of approximately 800bp in the 5' region directly preceding the start codon. Secondly in a portion of the gene which codes for the amino acids between amino acid 385 and 625. In the 5' UTR we have identified a total of 7 SNPs. In the coding region of the gene we have mapped the sites of the exons and introns within the gene and identified a further 7 SNPs. Somewhat surprisingly all the SNPs identified in this region were intronic, with no SNPs identified within exonic sequence and thus have an effect on the protein sequence. We have established high throughput genotyping assays for these SNPs, initially by using Taqman genotyping, and more recently using a sequenom mass–spec approach. We have genotyped a large cohort of Thoroughbred horses as well as native breed horses and established haplotype frequency for these SNPs and identified different haplotype frequency between breeds.

Researchers have had the opportunity to perform initial disease association studies to identify whether particular TNF-α and COMP haplotypes are associated with diseases such as carpal joint disease, bone spavin, lateral condylar fracture and superficial digital flexor tendonitis. So far we have failed to show any association between any of these haplotypes and any orthopaedic diseases. There may be no association between these genes and the particular diseases of interest. However, these studies were not of great power, and all had some deficiencies in study design, in particular the selection of control populations. If such genetic studies are to ever become a useful tool, the studies need to be expanded to include further candidate genes as well as recruitment of better cohorts (or case/controls) of animals at risk. In particular rigorous definitions of cases and controls, exemplary study design and a large enough study are all necessary in order to achieve a study of sufficient rigour and power to show an effect. Furthermore, it is vital that such studies involve the equine (breeding) industry as stakeholders as there appears to be some consternation by some individuals of what the possibilities of genetic testing could do to the "art" and commercial value of equine breeding.

Acknowledgements

This work was funded by the Wellcome Trust, The Waltham foundation, The Horserace Betting Levy Board and the Pet Plan Charitable Trust.

MOLECULAR MARKERS IN THE DIAGNOSIS OF EQUINE DISEASE

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INTRODUCTION

The fact that horses cannot easily communicate symptoms of disease or injury is especially serious for performance horses, for which forced exercise can seriously exacerbate existing injuries and disease – sometimes leading to catastrophic events. Thus, there is a clear unmet medical need for more convenient, faster and better clinical diagnostic tests for complex equine diseases. Veterinarians expect state-of-the-art technologies developed for human medicine to eventually make their way into veterinary practice. New genomic and proteomic technologies make it possible to develop highly multiplexed assays capable of providing the vet with more clinically relevant information from a single biological sample than is possible with conventional clinical laboratory tests. These molecular diagnostic (MDx) tests will significantly enhance the ability of the veterinarian to arrive at a definitive differential diagnosis for many indications that are currently difficult to diagnose.

Profiles of gene expression ('molecular biomarkers') in peripheral white blood cells can be used as surrogate markers of disease activity. Many human studies have demonstrated that molecular biomarkers are not only capable of diagnosing complex diseases, but can also provide information on the stage of disease, disease aggressiveness, disease prognosis and often aid in the selection of the most appropriate treatment. These advances are leading to the era of 'personalised medicine' in the human healthcare sector. In many cases, molecular biomarkers can diagnose disease and provide valuable prognostic information even before clear clinical symptoms are evident. This approach should work equally well in animals, including the horse.

Genetraks is leveraging technologies developed for the identification and application of molecular biomarkers in human medicine to develop molecular diagnostic and health monitoring tests for use in equine medicine. Currently a first-generation custom equine DNA microarray (Affymetrix, Inc., California, USA) is utilised to profile gene expression patterns in the blood of horses with confirmed diseases. Using sophisticated biostatistics to analyse equine blood samples obtained from controlled clinical studies, researchers have identified specific 'gene signatures' – panels of genes that are differentially expressed between diseased and unaffected control animals.

Here, state-of-the-art approaches available for the identification of molecular biomarkers for the diagnosis of equine disease will be discussed briefly. To illustrate how this works, the preliminary results of an ongoing study to identify a gene signature for equine osteoarthritis will be described. This study represents an ongoing collaboration between Genetraks and Drs Wayne McIlwraith and David Frisbie at the Orthopaedic Research Centre at CSU.

OSTEOARTHRITIS CASE STUDY

Osteoarthritis (OA) was induced unilaterally in the mid-carpal joint of 8 horses (2 groups of 4 each) via arthroscopic surgery on Day 0 of the study. Starting on Day 14, all horses were subjected to strenuous exercise 5 days per week for 8 weeks. The horses were assessed for lameness every 2 weeks using the AAEP grading scale.

Blood was collected from each horse on Days 0 (baseline), 14 (2 weeks post OA induction), 42 and 70 of the study. Total RNA was isolated,

amplified and analysed using Genetraks' custom equine GeneChip, which contains probes for approximately 3,100 unique equine genes derived from public databases and Genetraks' proprietary gene sequence database. Following hybridisation, arrays were imaged and analysed by standard algorithms using MAS5.0 and RMA. Median expression levels were calculated for each gene at each time point. Differential expression was determined for samples at each time point relative to baseline (Day 0). Data were subjected to principal components analysis and linear discriminant analysis. A p-value <0.05 was considered significant.

An initial OA gene signature containing 18 genes was identified using principal components analysis to interrogate the overall gene expression changes along the study timeline. Linear discriminant analysis demonstrated that gene expression profiles were statistically different between samples taken on Day 0 (baseline, pre-induction) and samples taken on days 42 (4 weeks of exercise) and 70 (8 weeks of exercise). A similar result was obtained when the serum protein biomarker data was analysed in a similar manner.

Further analysis utilising the in-depth disease knowledgebase developed by Dr's McIlwraith and Frisbie allowed us to reduce the initial gene signature to 6 genes that showed the strongest correlation with symptoms of disease and changes in serum protein markers. This gene signature provides the basis for the development of a clinical test for the diagnosis and monitoring of OA in horses. Further investigation using real-time polymerase chain reaction into the specific level of up and down regulation of these genes is currently underway. Testing on clinical field samples will also be performed to further validate this signature.

We are also developing gene signatures for other equine diseases including other causes of lameness, such as laminitis; infectious and inflammatory respiratory diseases; diseases of the GI tract and late-stage abortion.

Summary

Molecular biomarkers based on gene expression profiles in peripheral blood cells can be used to define gene signatures that provide useful information for the diagnosis and monitoring of complex equine diseases like osteoarthritis. Further work needs to be done to fully validate these gene signatures for widespread clinical use.

EQUINE GENE EXPRESSION ARRAY: APPLICATIONS

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The DNA microarrays are small, solid supports containing thousands of gene sequences that are immobilised or attached at fixed locations. This technological advance has permitted the simultaneous evaluation of hundreds of thousands of genes under identical conditions. Use of large-scale expression of patterns permits the classification of genes on the basis of biological function, contribution of patient data directly to research, and discovery of genes of unknown function on the basis of association with disease. Expression patterns can be diagnostic or prognostic and used for disease monitoring. Commercial mammalian DNA microarrays offer advantages of quality control and consistency, but are expensive.

Aggressive sequencing of animal genomes and expression libraries have expanded species specific databases of known sequences. Oligonucleotides can be specifically designed to attach to the unique target gene on the basis of the sequence information and permits the potential advantage of generation of comparison databases for longitudinal studies. We designed a first generation large-scale microarray (Equine Gene Chip, Affymetrix, Santa Cruz, California) of *in situ* synthesised oligonucleotides with the rigorous quality-control standards typical of these commercial arrays. See Gu and Bertone (2005), this array uses an automated microarray system of 11 25-oligomer probe pair sets; each probe pair consists of a perfect match and a single nucleotide mismatch for each gene sequence to be identified on the array. By use of a light-directed chemical synthesis process (photolithographic technique), highly dense glass oligoprobe array sets (>1,000,000 of the 25-oligomer probes) can be constructed in a small (approx 3 x 3 cm) plastic cartridge that serves as the hybridisation chamber. Light is emitted from the fluorescent reporter on the RNA only when it is bound to the probe. Intensity of the light emitted from the perfect match oligoprobe, compared with that emitted from the single nucleotide mismatched oligoprobe, is detected in a scanner, which in turn is analysed by use of a software program. The chip system provides a standard platform for array construction and data analysis that permits data comparisons among various experiments and laboratories.

For the array described here, 18,924 equine gene sequences were condensed to 3,098 equine 3 expressed sequence Tag (EST) sequences that were unique and annotated and met the inclusion criteria for an expression microarray. The equine oligonucleotide expression microarray that was designed and manufactured used 68,266 of the 25-oligomer probes to uniquely identify each gene. Most genes in the array (68%) were expressed in equine synoviocytes, mesenchymal stem cells and mononuclear cells, although mean % expressed genes will vary depending on tissue type. Importantly, this is not a tissue specific microarray. Repeatability of the array has been demonstrated to be high (r, >0.99). Using LPS the gene chip has been validated, specifically LPS upregulated >5-fold 84 genes many of which were inflammatory mediators, and downregulated 14 genes. An initial pattern of gene expression for effects of LPS on synoviocytes consisted of 102 genes. Il-beta, TNF and Col 1 have been validated by RT-PCR.

We chose to use a computer algorithm to curate the available equine sequence database to generate high-quality annotated species-specific gene sequences and probe sets for a gene expression oligomicroarray. These annotations by Blast N expanded the equine public database from 290 annotated genes to >3,000 provisionally annotated genes.

Applications

Experimental

Gene expression microarrays can be used to follow experimental challenges in cells, cultured tissues or tissues harvested from experimental animals. We have used the LPS model of synovial inflammation in our laboratory and have developed an LPS profile and signature.

Discovery

Signalling pathways for complex diseases, such as osteochondrosis or osteoarthritis, can be multiplexed using microarrays. Identification of many gene changes and the more significant gene changes as well as categorisation by biological function can be performed. Data on gene expression profiles for osteoarthritis in horses were presented as an example of identifying pathways in disease. These profiles can help direct therapeutic efforts as has been done with other models of OA in high throughput screening of OA molecular therapies. Discovery of genes or classes of genes that are involved in certain disease pathways can provide incite into developing biomarkers of disease or profiles that can assess severity or progression of disease.

Diagnostic

Tissues or cells that can be isolated in the live animal can be used to identify expression profiles that may correlate to disease. The accuracy of these signatures can be tedious to demonstrate due to overlapping profiles with confounding conditions of these diseases. Examples would include EPM or OCD or others. The methodology to produce these tests will be explained with summaries of data from studies.

Therapeutic

Therapies can be tested to demonstrate either normalisation of gene expression (such as for stress) or synovitis. Data were presented from an *in vitro* study that provided evidence that hyaluronic acid can provide protective gene expression profiles in LPS challenged synovial cells. The genes identified by microarray analysis were well-recognised mediators of inflammatory conditions of articular joints, particularly rheumatoid arthritis. Pre-treatment with either HA product resulted in decreased expression of inflammatory and catabolic genes and increased expression of anti-inflammatory and anabolic genes.

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