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Introduction
This paper is in follow-up to the 2009 Dorothy Russell Havemeyer Foundation workshop on biomarkers. The end goal of this effort is to develop a validated biomarker platform that could be used practically for diagnosis and prognosis of disease, and response to therapy, as well as disease prediction and study of disease of the joint organ, bone, tendon and ligamentous structures. This paper reviews current knowledge and future needs as well as gaps in knowledge.

State of knowledge in equine musculoskeletal biomarkers was reviewed by McIlwraith in 2005. Since that time specific reviews have been made by Garvican et al (2010a, 2010b) and Mobasheri et al (2010) as part of a collection of papers on biomarkers in veterinary medicine published in the Veterinary Journal. More recently the use of serum biomarker levels for predicting severe musculoskeletal injury has been reported (Frisbie et al 2010).

The need and potential benefit of biomarkers of disease in horses is quite comparable to that of humans and workshops and working groups have similarly been used to identify the needs and strategies for biomarker development (Kraus et al 2010, Kraus et al 2011). It has been said that a disease starts when detected by the best biomarker available to define it (Kraus et al 2011). Those authors proposed that this usually requires the presence of a clinical symptom, which often occurs well into the progression of an illness or disease. There is significant evidence that there are often early, pre-symptomatic biomarkers of illness and disease which if detected may allow for earlier treatment. This forms the basis for the power and importance of implying biomarkers to osteoarthritis (OA), a disease often characterized by a prolonged, asymptomatic molecular phase, a pre-radiographic phase and a recalcitrant later radiographic stage with evident structural joint changes, frequent pain and loss of function (Kraus et al 2011).

The equine athlete may also present somewhat unique applications for biomarkers of musculoskeletal health that may not be as relevant in humans. One of the issues in people is for example who gets monitored for OA biomarkers or in which groups will it be of most cost benefit? The “low hanging fruit” in human medicine is likely to be in predicting patients that will have rapid progression of existing joint disease and thus may benefit most from available therapies, monitoring response to therapy for existing disease, and identifying which patients will and which will not progress to OA following injury such as ACL tear. These applications would also apply to the horse with existing disease or following injury, and thus biomarker research can inform in both directions between the species. However in horses, a greater need may be in monitoring currently healthy athletes to predict and thereby hopefully prevent both catastrophic injuries such as long-bone fractures or tendon/ligament tears, as well as impending joint disease. This use of biomarkers will often fall outside the various classifications described in the BIPED system. Advances in this area would be extremely useful in equine medicine, not only from the perspective of preventing disease and maximizing performance, but also in managing the
increasing ethical issue of racing-associated injuries. There is little research in biomarker monitoring of human athletes, and in this area the equine community could lead the way and provide novel insights for translation to humans.

Participants in the 2009 Havemeyer workshop were Wayne McIlwraith, David Frisbie, Chris Little, Troy Trumble, Peter Clegg, Roger Smith, Jack Quinn, Chris Kawcak, Jo Price, Elwyn Firth, Mark Vaudin, Sheila Laverty, Eva Skioldebrand, Stina Ekman, Chris Riley and John Kisiday as well as prominent experts from the human biomarker research field Robin Poole, Dick Heinegård, Bruce Caterson and Virginia Byers Kraus. The help of the last four participants has been critical in guidance and, in particular, the recent papers of Kraus et al (2010, 2011) may be used as templates for how we have moved forward in parallel fashion.

This paper represents the currently available biomarker applications and a plan for the way forward by the authors.

Definitions
A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). This is in contrast to a clinical endpoint that is a marker or variable that measures how a patient feels, functions or survives. A biomarker becomes a surrogate endpoint when it is appropriately qualified to substitute for a clinical endpoint. The evolution in molecular biology has led to the expansion of the notion of what constitutes a potential biomarker to include, not only proteins and protein fragments, but also metabolites, carbohydrate biomarkers, genomic biomarkers (RNA and DNA), cellular biomarkers (that may be captured for instance in a cell pellet extracted from body fluids), and imaging biomarkers (Kraus et al 2011). Biomarkers have also been classified, based upon their characteristics, into two major groups: the so called soluble or “wet” biomarkers (fluid biomarkers) and “dry biomarkers” which consist of visual analog scales (VAS), questionnaires, performed tasks or imaging (Kraus et al 2011).

In a recent review on biomarkers in OA, it was noted that the OA disease process is increasingly being considered a continuum, beginning with an inciting event, such as genetic variation or injury, progressing through molecular, pre-radiographic and radiographic stages culminating in end-stage disease (Kraus 2010). Based on this reclassification of the disease as a continuum of a series of stages, it is proposed that biomarkers could play a pivotal role in disease detection and monitoring, particularly during the critical, early molecular stages when other tools are not readily able to identify nascent OA (Kraus 2011). In human medicine such efforts are occurring in parallel with efforts to harness biomarkers for applications in a variety of diseases and to standardize the regulatory process for biomarker qualification in validating new therapies.

The terms ‘qualification’ and ‘validation’ have also been used in the context of biomarkers. Qualification is a process applied to a particular biomarker to support its use as a surrogate endpoint in drug discovery, development or post-approval and, where appropriate, in regulatory decision making (Biomarkers Definitions Working Group 2001). In contrast, validation of a biomarker is much broader and can relate to verification of analytical performance characteristics
(such as precision, accuracy, or stability) as well as clinical correlation of a biomarker with a biological process or clinical outcome.

Further information can be gathered from the summary of the 2009 OA Biomarkers Workshop (the first workshop as part of the Osteoarthritis Research Society International [OARSI] OA biomarkers global initiative) (Kraus et al 2010) and the Osteoarthritis Research Society International Federal Drug Administration (OARSI-FDA) Biomarkers Working Group (Kraus et al 2011). An earlier 2006 meeting produced the BIPED classification system (Bauer et al 2006) which organized biomarkers into five categories depending on their ability (BIPEDS – burden of disease, investigative, prognostic, efficacy of intervention, diagnosis of a disease and safety of interventions). A potential model for tabulating available biomarker tests in horses is the method of van Spil et al (2010) (the detailed tables have been supplied by Virginia Kraus).

Development of a biomarker sample bank (Dr.’s Sheila Laverty, Christopher Riley and Peter Clegg)

It was agreed by the working group that this should be a major priority. While Biobanking is emerging as an important research tool in the human field, it is now also gaining momentum in veterinary medicine (Schrohl et al 2008; Castelhano et al 2009). A Biobank is a repository of biological material that has been collected and stored in a standardized fashion and whose phenotype, origin, date of collection and location can be easily determined (Schrohl et al 2008). These specimens can then be distributed to the Biobank users based on preset guidelines. Specimens may be stored at one site or several sites. However, the overarching feature is a powerful informatics program that permits efficient and reliable management of all the Biobank’s specimens (Amin et al 2010). A key element of a Biobank is that all necessary legal and ethical permissions are in place to allow appropriate use of materials for research purposes. This is obviously complicated in the case of an international Biobank where different legislative frameworks and cultural issues may have an impact (Blow et al 2009). An additional important objective is to harmonize not only sample collection and storage methods but also data recording (quality, completeness, consistency) relating to samples at different storage sites (Founti et al 2009; Amin et al 2010).

The reasons to create an Equine Joint Disease Biobank are various and include: maximization of use of fluids and tissues; reduction in the number of horses used in clinical research; fostering global collaborative studies by facilitating access to samples; the development of standard operating procedures for archiving, collection, storage, and treatment to generate reliable results; avoidance of a wealth of tissue being underutilized or discarded; provision of a larger database and enhances the number of cases included in studies (improved statistical power) as equine research is expensive. In the case of naturally occurring disease (OA and osteochondrosis) some sites may have access to abattoirs, slaughter houses and clinical necropsy facilities where specimens may be harvested and banked to facilitate collaboration with others. Furthermore, because investigative technologies are becoming increasingly expensive and specialized it will facilitate multicenter studies and synergize equine research in the field.
Summaries of equine biomarker technologies currently used and available
It was agreed at the time of the 2009 meeting that a table would be prepared of biomarkers to include their state of qualification and validation as well as sensitivity, specificity and other specific details. Based on evaluation of these techniques it has been chosen to summarize biomarkers technologies below and a workshop to place all techniques into tabulated form with specifics of qualification, validation, sensitivity and specificity is planned in 2013.

Equine inflammatory biomarkers in synovial fluid and serum (Dr. Stina Ekman and Dr. Eva Skjöldebrand)
Osteoarthritis (OA) of synovial joints always involves a low grade inflammation with proinflammatory cytokines; such as IL-1, IL-6 (also modulatory) and TNF and anti-inflammatory factors; IL-1 receptor antagonist (ra), IL-10, IL-4 (Goldring and Otero, 2011; Kapoor et al 2011) and interferon-gamma (IFN-γ) (Kelchtermans et al 2008). Other inflammatory factors are; HMGB-1 (Hou et al 2011), prostaglandins (DeGrauw et al 2009), acute phase protein as serum amyloid A (SAA) (Hulten et al 1999), complement components (Osborne et al. 1995) and metalloproteinases( MMP:s) (for review see: Abigail et al 2012; Heinegård and Saxne, 2011).

It is proposed that more than 90% homology of amino acid sequences between equine and other species is a minimal requirement for the antibody to cross react. To prove the specificity of a non-equine antibody on specific equine epitopes, a gel electrophoresis and a Western Blot has to be performed, and a single band of the appropriate molecular weight should be identified. Also the protein (usually fractionated on a gel) should ideally be analyzed with mass spectrometry to fully identify the protein. Descriptions of the antibodies based on review of publications as well as experience of the authors are summarized here. In many publications the specificity of the non-equine antibody is not clearly stated; hence the validation of the assays used could not be described.

Interleukin-1 (IL-1) is a general name for two distinct proteins, IL-1 alpha and IL-1 beta. They bind to the same receptors and exert identical biological effects. Due to the low homology of IL-1 amino acid sequences between equine and other species (65-75%) (Kato et al 1997), equine specific antibodies/assays are needed. Non-specific immune assays were initially used in equine research (Bertone et al 2001) and a bioassay in synovial fluid measured by phytohaemagglutinin induced proliferation of C3H/HeJ mouse thymocytes has been reported (Alwan et al 1991). The use of an equine specific ELISA from R&D has been described by Hraha et al (2011) and an IL-1 beta monoclonal antibody has been used in synovial fluid and is available from R&D (Kamm et al 2010). This ELISA did not detect concentrations above the lower limit of sensitivity. A commercial ELISA (Kingfisher Biotech NQSA) has been tested on synovial fluid, serum and cell culture medium by Ekman and Skjöldebrand 2011 (unpublished data). The concentration range measured was 2.34 ng/ml – 150 ng/ml and up to 430 ng/ml in synovial fluid from OA joints was found.

Equine IL-1 receptor antagonist (eq IL-1ra): this assay is now available through R&D Systems and is a sandwich ELISA. Due to the low homology of IL-1ra between equine and other species, equine specific assays must be used. Validation of the ELISA is not described in the commercial kit. Use of this ELISA has been reported by Hraha et al (2011). Previously a human IL-1ra
ELISA was useful in looking at gene therapy with an adenoviral vector (Frisbie et al 2002) but cross reactivity was not possible in another study with autologous conditioned serum (Frisbie et al 2007). In the latter study a mouse ELISA resulted in some degree of cross reactivity.

**Interleukin-6 (IL-6):** a sandwich ELISA using polyclonal goat anti-equine IL-6 antibody for encoding, with recombinant equine IL-6 as a standard in biotinylated goat anti-equine IL-6 has been developed and used in serum (Burton et al 2009, Ley et al 2007).

**Tumor necrosis factor (TNF) alpha and beta** bind to the same receptors and they have an amino acid identity of 28%. TNF-L is the proteolytic ligand of the TNF super family. Equine TNF-L shares 69%-89% amino acid sequence identity with bovine, canine, rat, feline, human (87%), mouse, porcine and rat (Su et al 1991). Equine specific ELISA from Thermo-Scientific has been used by Hraha et al 2011.

**IL-10** equine specific ELISA (R&D Systems) has been used by Hraha et al 2011. Validation data for the ELISA is not provided in the commercial kit. Other equine inflammatory markers that have been successfully assayed include High-ability group box 1 protein (HMGB-1) (Brown et al 2009, Ley C doctoral thesis 2010) and prostaglandins E2 (PGE2) (Kawcak et al 1997). Other ELISA assays available include equine IL-4 and IL-2 with antibodies available from R&D Systems as well as interferon-gamma (IFN-gamma) also from R&D, substance P (DeGrauw et al 2009), nitric oxide (Simmons et al 1999) and acute phase protein serum amyloid A (SAA) (Hulten et al 1999) and metalloproteinases (van den Boom et al 2004).

**Markers of collagen degradation and synthesis in cartilage (Dr.’s David Frisbie and Peter Clegg)**

Type II collagen is a major component of articular cartilage and its breakdown is a key feature of osteoarthritis. The products of cartilage collagen metabolism can be detected in the, synovial fluid, blood and urine. Several biomarker assays are available that can be used to measure the synthesis and degradation of collagen and therefore provide information regarding cartilage turnover (Garvican et al 2010a).

**PIICP:** The cleaved C-propeptide of type II collagen is detected by the procollagen type II C-propeptide (PIICP) assay (also referred to variously as CPII) and is released as part of the secretion of newly synthesized type II collagen from the cell. Immunoassays for PIICP have been developed. The ELISA detects the presence of three kDa C-propeptides, connected by disulphate links which are released into the circulation following cleavage by C-propeptide. The half life of the cleaved propeptide is relatively short (in cartilage 16 hours) (Nelson et al 1998) (in serum 18 hours) (Poole 2000) and is therefore a useful indicator of recent synthesis. Samples of PIICP were increased in both synovial fluid and serum of clinical cases of osteochondral fragmentation in the carpus (Frisbie et al 1999), and showed increases with both exercise and experimentally induced OA in a controlled equine study (Frisbie et al 2008). In another study synovial fluid PIICP levels were not predictive of lameness located in the metacarpophalangeal joint (deGrauw et al 2006), CPII was also increased in synovial fluid and serum of young horses with OCD (Laverty 2000; Billinghamurst et al 2004; Billinghamurst et al 2003) Baseline concentrations of PIICP are reportedly higher in the serum of dogs and horses than humans; as a
result the greater systemic PIICP production may mask any upregulation which occurs from a single damaged joint (Robion et al 2001). However, work with a single joint equine OA model has caused consistent change in equine serum analysis (Frisbie et al 2008). Caution should be exercised when comparing CPII results across studies: the earliest CPII studies, prior to the development of commercial ELISAs, were performed using radioimmunoassays (Frisbie et al 1999; Laverty 2000; Robion 2011; Celeste 2005) and absolute differences in serum levels detected could be attributed to differences in techniques.

**PIIAN/PIINP**: The N-propeptide of type II collagen includes a 69 amino acid, cysteine-rich domain (this is termed type IIA collagen or PIIAN for the first of two isoforms seen in immature chondrocytes). The other isoform (type IIB or PIINP) excluding this globular domain is produced by mature adult chondrocytes (Sandell 1991). The N-propeptides are cleaved and released as part of collagen secretion and therefore may be indicative of collagen synthesis. Use of PIIAN/PIINP has provided some information in human OA but appear to show most promise when combined with other biomarker assays (Garvican et al 2010a).

**CTX-II** is a type II collagen C-telopeptide fragment (CTX-II) ELISA that uses a monoclonal antibody specific for a six amino acid sequence present exclusively in the C-terminal telopeptide of type II collagen. Release of this telopeptide occurs through proteolysis of collagen within cartilage extracellular matrix by both MMPs (Karsdal et al 2008) and cathepsin K (McDougall JJ et al 2010). Increase in CTX-II fragment levels has been associated with OA outcome parameters in humans. In human OA patients concentrations of urinary CTX-II (uCTX-II) were found to be 1.53 fold higher than the healthy controls and a trend towards an association between joint space narrowing and uCTX-II levels were noted (Christgau et al 2001). Increased amounts of urine and synovial fluid CTX-II have been detected soon after knee injury with higher concentrations correlated with more rapid progression of destruction (Christgau et al 2001, Lohmander et al 2003, Garnero 2000). Increases in synovial fluid CTX-II have also been associated with osteochondral fragmentation in adult horses when compared to a control population (Nicholson et al 2010). While a significant decrease in the serum levels of CTX-II were seen in a similar comparison. It should be noted that normal yearlings also have an increased synovial fluid concentration compared to normal adults and differences based on the anatomic location of the joint was also demonstrated with this biomarker.

A C2C/Col CEQ assay exists for the detection of the neoepitope generated at the C-terminal end of the three-quarter length fragment by the action of collagenolytic MMP at a specific site in the collagen II triple helix. Thus the C2C antibody recognizes the majority of collagenase cleaved type II collagen. In most studies the C2C assay has shown increased levels associated with OA. In the horse C2C concentrations were elevated in synovial fluid following experimental joint inflammation (de Grauw et al 2009) and in both synovial fluid and serum in early experimental OA (Frisbie et al 2008).

The Col CEQ assay was developed to deal with species nuances and identifies the C-terminus neoepitope produced by collagenase digestion specifically of equine type II collagen. This neoepitope has been shown to increase in equine OA (Billinghurst et al 2001). Concentrations of synovial fluid and serum Col CEQ have also been shown to significantly increase in joints with experimental OA and also it arises as a result of exercise (Frisbie et al 2008).
C1.2C antibody (Col II-3/4Cshort) detects collagenase-cleaved equine type II collagen but also reacts with fragments of type I collagen in human, equine and bovine synovial fluid samples (Billinghurst et al 1997, 2000). It is possible that type I collagen from sources such as bone, tendon, ligament and skin have distorted results from previous studies utilizing the C1.2C assay (Garvican et al 2010a). This biomarker has also been shown to increase with exercise and early experimental OA (Frisbie et al 2008).

Helix-II (urinary type II collagen helical peptide) is a newer biochemical marker of cartilage degradation in human OA (Charni et al 2005; Eyre and Weis 2009) but has not been tested in the horse at this stage.

Cathepsin K (cat-k) cleavage of type II collagen. There is a neoeptitope antibody marker for cat-K cleavage of type II collagen in articular cartilage (Dejica et al 2008) and also evidence to suggest that based on using this antibody that cat-K degrades articular cartilage in naturally occurring equine osteoarthritis (Vinardell et al 2009).

**Bone biomarkers (Dr. Joanna Price)**

Changes in bone mass and structure associated with disease can be assessed using quantitative ultrasound (QUS), dual energy x-ray absorptiometry (DEXA) or quantitative computerized tomography (pQCT) (Drum et al 2007). However, it may take months for changes in bone mass and architecture to be of sufficient magnitude that they can be detected using these methods. Furthermore, QCT and DEXA are not straightforward to use in horses in the field and thus their use is currently predominantly restricted to research studies. In contrast, bone biomarkers measure dynamic changes in bone cell activity and can be measured in body fluids using fairly straightforward methods. They can also be repeated at frequent intervals and so are convenient to use in clinical case-based studies. Although bone biomarkers remain predominantly research tools, a considerable amount of work has been undertaken in recent years on their potential clinical applications including identification of horses at risk of fracture and other injuries (Frisbie et al 2011).

Bone biomarkers are generally classified as markers of bone formation or markers of bone resorption/degradation, although some reflect changes in both processes. In general they are enzymes expressed by osteoblasts or osteoclasts or are organic components released during the synthesis and resorption of bone matrix (Price 2011). Equine bone biomarker research has been informed by the extensive use of bone turnover markers for evaluating human metabolic bone disease processes, osteoporosis in particular. A small number of equine-specific assays have been developed and many equine studies have utilized markers that were originally developed for use in man. Formation biomarkers include the following:

1. *Bone alkaline phosphatase* (ALP) human immunoassays have now been validated for use in the horse (Price et al 1995).
2. *Osteocalcin (OC or BGP)* which is otherwise known as ‘bone gla-protein’ (BGP) is the most abundant noncollagenous protein in bone matrix and a small fraction is released into the circulation following synthesis by osteoblasts. Equine specific assays have been
developed in recent years and a competitive human immunoassay has been validated for equine use (Hoyt and Siciliano 1999).

3. **The carboxyterminal propeptide of type I collagen (PICP)** type I collagen is the most abundant collagen in bone and the procollagen molecule contains both amino (PINP) and carboxyterminal (PICP) extension domains that are enzymatically cleaved off before fibril formation and are released into the circulation. These propeptides provide quantitative measures of newly synthesized type I collagen and in humans serum levels reflect the rate of bone formation. PICP is measured in horses by a human RIA (Price et al 1995). However, because type I collagen is not ‘bone specific’ synthesis in other collagen type I containing soft tissues (e.g. skin, tendon) may contribute to PICP concentrations in serum. Human PINP assays that have been tested to date do not appear to show equine cross reactivity.

Biomarkers that measure changes in bone resorption are usually a reflection of osteoclastic resorption of bone matrix with degradation of products of type I collagen released into the circulation. The most recently developed resorption biomarkers can be measured in serum and this has increased the repertoire that can be used in horses.

1. **Cross-linked collagen telopeptides**, cross-links are associated amino (N-) and carboxy (C-) termini in the type I collagen molecule and a number of peptide assays have been developed for measuring telopeptides generated during osteoclastic resorption. The first of these was an RIA for the carboxy-terminal telopeptide of type I collagen (ICTP) and this assay works well in horses (Price et al 1995). The C-terminal telopeptide recognized by the ICTP assay is specifically generated by the action of MMPs rather than cathepsin K which releases a separately identified fragment (CTX-1, see below) (Garnero et al 2003). The ICTP assay is therefore often abbreviated CTX-MMP. The ICTP assay was used quite extensively in early bone biomarker studies in the horse (Price 2011). As noted above for formation markers, degradation products recognized by ICTP can arise from any tissue containing this collagen, and elevated levels have been reported in humans with myocardial infarction (Manhenke et al 2011).

2. More recently an immunoassay (CTX-1) was developed that recognized the C-terminal telopeptide of type I collagen released by cathepsin K, and this has been used in the horse (Frisbie et al 2008). Although not exclusively expressed by osteoclasts, cathepsin K appears to be the predominant enzyme responsible for proteolysis of collagen in bone associated with the activity of these cells (Fuller et al 2007) so this biomarker may be more specific for turnover of bone that other collagen I catabolites. (JO COMMENT?)

3. Other markers of bone resorption include hydroxyproline (HYP), hydroxyllysine glycosides and pyridinium cross links of collagen. Pyridinoline (PYR) or hydroxylysol Pyridinoline (HP) is found in cartilage, bone ligaments and vessels whereas dioxypyridinoline (DPD) or lysolpyridinoline (LP) is found most exclusively in bone and dentine. In osteoclastic bone resorption the cross-links are cleaved and the components released into the circulation. High performance liquid chromatography (HPLC) provides a reliable method for measuring total urinary DPD and PYD concentrations in the horse but the method is cumbersome and although it is possible to measure DPD and PYD in horse serum, this assay at the moment can only be used in horses less than two years of age since serum concentrations in mature horses are below the limit of detection.
Because various factors can influence biomarkers in horses including time of day, diet, season, age, gender, pregnancy, lactation, stage of estrus, breed, and inter-current disease, these should therefore be controlled for in any study (e.g. samples should be collected at the same time of day).

There is accumulating evidence that bone biomarkers particularly, if measured serially in the same animal, have potential for identifying horses at risk of injury and as objective measures for monitoring treatment. They also have been used for monitoring the effects of exercise on the equine skeleton (Price et al 2003). Although one study measuring bone biomarkers at the start of the training season (ICTP, PICP, CTX-1 and osteocalcin) to predict fracture showed them not to be of value, another study showed that longitudinal (monthly) sampling was useful (osteocalcin and CTX-1) in predicting injury (Frisbie et al 2011). Osteocalcin and ICTP were significantly higher in horses that subsequently developed dorsal metacarpal disease.

**Protein Biomarkers (Dr. Roger Smith)**

The development of a specific assay for a protein biomarker of tendon injury relies on identifying a protein which either is specific for tendon tissue or with at least a restricted distribution, and/or whose fragmentation with injury is specific for injury process. In addition, the biomarker must be released from tendon with damage and reach the general circulation with no or limited removal on route to blood. It must be detectable in easily accessed body fluids with have low natural levels to enable easy differentiation between normal and injury. Our studies into the matrix composition of tendon have provided several candidates for molecular markers of tendon disease, two 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 both rheumatoid arthritis and osteoarthritis (Saxne and Heinegard 1992; Lohmander et al 1994). COMP is especially enriched in mid-metacarpal region of superficial digital flexor tendon in young adult horse (Smith et al 1997), which is the most common site of injury, and has been found to be released after injury with limited removal by the lymphatics prior to entry into the blood.

FBM is a member of the small leucine-rich repeat protein family (SLRPs), predominantly associated with the collagen fibrillar network in tissues. It is especially abundant in tendon, where ‘knock-out’ experiments in mice have demonstrated it to have a functional role in tendon organization and strength (Svensson et al 1999, 2000).

We have therefore investigated whether tissue, serum and/or tendon sheath synovial fluid levels of these markers can be used as a biomarker for tendon disease in horses. COMP concentrations in serum were found to vary with age.

Total COMP concentrations in an age-matched group with superficial digital flexor tendinopathy (1.45µg/ml±0.35) were not significantly different from the control group (1.42µg/ml ± 0.31) with no significant effect of severity or duration. Furthermore in a large cross-sectional study of
a population of racehorses, serum COMP levels were not predictive of the presence of superficial digital flexor tendon injury determined ultrasonographically. Exercise, however, appears to influence serum COMP levels. A study on a small group of international event horses demonstrated a significant rise in the mean population serum values between pre-season and after 4 months of training suggesting that COMP levels did reflect training level.

Synovial fluid COMP levels have been shown to be a predictor of intra-thecal tendon pathology (Smith et al 2011). Synovial fluid samples were collected from 77 digital tendon sheaths which were examined either tenoscopically (those with clinically significant pathologic changes; n=37) or at post mortem (to ensure an absence of pathologic change i.e., controls; n=40). Significantly raised levels of COMP occurred in cases of intra-thecal tendon disease compared to controls so this assay may be useful for the prediction of the presence of tendon damage pre-surgery. Further work has been done with a neo-epitope ELISA that only recognizes a specific sequence exposed after enzymatic cleavage of COMP. This COMP fragment is not present in normal digital sheath fluid. An antiserum has been generated to the new N-terminal created by (?? MMP) cleavage in this antiserum labeled a 100kDa fragment on Western Blots. Normal digital sheath synovial fluid contained 5.3 ± 1.5 µg/ml of COMP fragment protein compared to tendon injuries which had 63.2±25.3 µg/ml of the fragment.

A fibromodulin neo-epitope antiserum was evaluated against equine tendon extracts from young and aged tendon and from six injured tendon extracts. This neo-epitope was first identified in cartilage explants stimulated with IL-1 (Heathfield et al 2004). An antiserum, raised against the epitope, recognizes the new N-terminus after MMP-13 cleavage of fibromodulin in bovine cartilage explants and 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 injury.

The development of a serological assay for tendon injury is still the Holy Grail for the tendon clinician and this goal is yet to be realized. However, information above can potentially help understand the disease process as well as potentially be a clinical tool for diagnosis. One essential research tool that is still absent from our armory is the ability to detect the early stages of tendon degeneration (rather than clinical disease) and these tests can potentially enable us to do this.

Current status of imaging biomarkers in horses (Dr. Chris Kawcak)

Diagnostic imaging has been of great importance in equine medicine to characterize a diseased joint. However, diagnostic imaging particularly radiography and ultrasonography often underestimate the amount of damage in the area. In recent years computed tomography (CT) and magnetic resonance imaging (MRI) have allowed for three dimensional characterization of joints. Although this has improved diagnostic capabilities, pathologic tissues must still be present in order for these imaging modalities to be useful. In the past the pathologic changes must have been structural in nature to be detected, although now they may be physiologic. For example, bone marrow lesions on MRI and uptake of contrast agent into diseased tissues on CT examinations are indicative of methods that show the physiologic change when structural changes may not be present (Powell 2011, Vallance et al 2011a, 2011b). Therefore, with the
introduction of three-dimensional imaging techniques, higher resolution and the ability to image physiologic changes has improved early diagnosis of disease. Nuclear scintigraphy is also used and has proven to be a sensitive (but rather non-specific) indicator of early disease. However, one must be cautious that because of higher resolution the potential incidence of over interpretation (and false-positive identification) of images increases.

A correlative study between the various available imaging techniques and the development of experimental OA in the horse has been reported (Kawcak et al 2008). The paper serves as an example of the potential to have multiple imaging biomarkers for clinical OA. At the moment a true imaging biomarker that could be used to predict onset and/or progression of joint disease does not exist in any species including man. Recently researchers have investigated the influence of structural change on disease incidence and there is an emerging field of study in which shape differences in joints are being correlated to disease incidence. Initially these started as simple two-dimensional shape measurements but are now emerging into complex three-dimensional shaped models. The only such published study in horses is by Kawcak et al (2010) where horses with condylar fracture had a narrower lateral condyle in the palmar aspect of the joint compared to those horses without. In addition the contralateral limb in fractured horses showed a narrower lateral condyle in the dorsal aspect of the joint compared to those horses that did not fracture. The measurements in this study were two-dimensional in nature although it lays the groundwork for three-dimensional studies in the future. In addition, some researchers have investigated the influence of subchondral bone density on incidence of osteoarthritis with fracture (Shi et al 2011). The downside of these studies is that nobody has ever been able to predict or determine a density level at which pathologic changes occur. This is compounded in the horse since all athletic horses will adapt to loading by increasing subchondral bone density. The point at which subchondral bone density switches from being adaptive to pathologic is unknown.

**Genetic biomarkers (Dr. Mark Vaudin and Joanna Price)**

Genetic sequence variants hold particular promise for predicting disease risk and guiding appropriate decisions and treatments. Genetic biomarkers are defined as genetic variations (mutations or polymorphisms) that can predict disease susceptibility, disease outcome, or treatment response. The success of the human genome project has facilitated the development of resources to detect altered risks for diseases like cancer, cardiovascular disease and diabetes, as well as chronic episodic conditions like asthma and less common diseases such as inflammatory bowel disease. With a detailed understanding of a patient’s genetic profile and current physiological status, the potential exists for future risk to be accurately predicted and the optimal treatment chosen.

The use of genetic biomarkers to estimate risk is potentially more straightforward than using non-genetic ones because genetic biomarkers can be detected almost without error and do not vary in an individual over time. Also, unlike non-genetic markers, they only need to be determined once, and this can be early in life allowing appropriate decisions on treatments or adjustments to begin earlier, potentially increasing their effects. A possible scenario could be foals routinely having samples collected in the first few weeks of life for genetic screening purposes. Recent developments in next-generation sequencing technologies have resulted in an explosion in the availability of genome-wide polymorphisms in a large number of different
animal and plant species. High throughput genotyping systems using high-density chips containing tens (or hundreds) of thousands of genome-wide single nucleotide polymorphism (SNP) markers, have therefore become widely available to identify genotypes of individuals for a large number of SNPs at relatively low cost. Interpreting this wealth of data and understanding the relationship between genetic variation, environmental influences and epigenetic regulation in determining disease susceptibility is the next great challenge.

This revolution in genomic resources has led to a dramatic impact on the application of genetics and genomics in the horse which can be attributed to the publication of a high quality draft sequence of equine genome (Wade et al 2009). There are approximately 20,000 protein-coding equine genes with a high degree of orthology to the human, mouse and dog gene sets. A SNP map consisting of more than one million markers has been generated by the addition of 100,000 whole genome shotgun reads from each of seven different horse breeds (REFERENCE?). The term haplotype refers to a set of genetic markers that are inherited together as a consequence of their chromosomal co-localization. Haplotype may refer to as few as two genetic variants or to an entire chromosome depending on the number of recombination events that have occurred between a given set of variants. Linkage disequilibrium is the non-random association of alleles at adjacent loci. When a particular allele at one locus is found together on the same chromosome with a specific allele at a second locus more often than expected if the loci were segregating independently in a population the loci are in disequilibrium.

The development of an equine SNP genotyping chip (the Illumina EquineSNP50 BeadChip), and the more recent second generation equine SNP chip (EquineSNP70 BeadChip) with a greater number (approx 74,000) of SNP markers has facilitated a range of genotyping applications including whole genome case-control association studies, commonly used in the study of human disease for many years. These studies include finding a marker associated with foal immunodeficiency syndrome (FIS) at the Animal Health Trust in the UK (Fox-Clipsham et al 2011), as well as a company Equinome Ltd. developing a test for detecting which of three variants (TT, CC, CT) of the myostatin gene (MSTN) a Thoroughbred horse has inherited (Hill et al 2010). The test can apparently reveal whether the horse is best suited to racing over short, middle or middle-to-long distances. The Equinome website (http://www.equinome.com/index.html) claims that “C:C horses are best suited to short distance races, C:T horses are best suited to middle-distance races and T:T horses are best suited to middle- to long-distance races” respectively. There is also preliminary evidence that devastating diseases such as fractures and tendon injury may have some genetic basis.

Post-genomic technologies (Dr. Peter Clegg)
The availability of the equine genome opens up a number of post-genomic technologies to equine researchers. The potential is for the development of novel tests that can be both prognostic and predictive of disease processes in the horse (Ramery et al 2009). For instance, the use of microarray technology may assist us in determining the effects (both positive and negative) of a pharmacological agent such as intraarticular corticosteroids (McIlwraith 2010).

Genomics – While PCR has revolutionized many aspects of biological investigation its major limitation is that each reaction can only identify the expression of a single gene. In order to fully understand the complex process of gene expression in a population of cells, or in tissues,
techniques of global determination of gene expression are required, where it’s possible to determine quantitatively the expression levels of many different genes, in a multiplexed reaction (Clegg unpublished data 2012). However, it is also to be recognized that gene expression data is one dimensional and specific regulation of a particular gene does not always relate to a cellular response of protein transcription (Rousseau et al 2008, Clegg 2011). DNA microarrays used to quantify gene expression in cells or tissues consist of thousands of unique DNA oligonucleotides or probes that are spotted microscopically onto the array. Each spot (which may be present in duplicate or more) on the array consists of a short unique section of a specific gene to which cDNA synthesized from the mRNA of experimental samples is able to hybridize. Successful hybridization is normally detected by fluorophore or chemiluminescence labeled targets to determine relative abundance of the experimental sample (Duggan et al 1999).

Due to the lack of complete equine genome sequences until recently, specific microarray technology for the horse has been relatively slow to develop. The first equine microarray developed contained 3103 specific equine probe sets (Gu and Bertone 2004). Whilst more recently a 12,300 gene whole transcript oligonucleotide microarray (Glaser et al 2009), a 9,367 gene cDNA microarray (Coleman et al 2007, Mienaltowski et al 2008) and a 21,351 gene microarray (Bright et al 2009) have been described but the authors of this review (DFF and CWM) (Eastman 2005) have identified upregulation of certain genes using a 3100 genechip microarray developed in Australia. Microarrays have been used to investigate aspects of cartilage development and repair (Mienaltowski et al 2008, 2009). Commercial equine microarrays are now available from companies such as Affymetrix.

Sequencing the first genomes, for instance the human genome, took many years, but advances in genomic technology have allowed acceleration of sequencing technology. Using technology such as SOLiD and 454 sequencing, which can allow $5 \times 10^9$ base pairs per day to be sequenced, such techniques are beginning to revolutionise the fields of transcriptomics and genomics. Such technology is beginning to be used to define mRNA transcription and has huge potential for use in animals such as the horse where sequence data is currently poorly annotated (Cloonan et al. 2008; Tang et al. 2009).

Proteomics; The study of large-scale protein definition is more widely accessible through the discipline of proteomics and the concept was first proposed by Wilkins (Bayles et al 1996) and is analogous to genomics, the study of the gene. Another challenge has been studying the proteome as the number of proteins that need to be identified as there are a myriad of post-translational modifications that occur following gene expression which results in many times more proteins than the coding potential of the organism. Proteomic techniques rely on techniques to separate proteins, characterization of separated proteins by mass spectrometry (MS) and information mining using bioinformatics tools. Proteomic analysis has been used in muscle biopsies take from horses under different training regimens to identify biomarkers for muscle development in the horse (Bouwman 2010), a pathogenesis of equine recurrent uveitis (Deeg 2009), immunity against R.Equi (Roncada et al 2005), identification of mucins in equine respiratory disease (Rousseau et al 2007) and identification of biological changes in proteins due to anabolic steroid administration in horses (Barton et al 2009).
Metabolomics – in the post-genomic era it has become clear that solely mapping the genes, mRNA and proteins of the living system does not reveal its phenotype. Consequently researchers have turned their interest to the metabolome (or the metabolic compliment to functional genomics) and thus metabolomics is a rapidly expanding post-genomic science that utilizes analytical techniques to measure low molecular weight metabolites in biological samples (Dunn et al 2005, Griffin 2003, Wilson et al 2005). The principal analytical techniques used in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR), spectroscopy. Techniques generate huge amounts of data in complex spectral profiles which must be then analyzed using bioinformatics and statistical methods. Currently there is no published data on metabolomic profiling in the horse and its potential use as a biomarker in musculoskeletal disease, but recent publications in human and animal models OA suggests it may have potential (Blanco et al 2012; Adams et al 2012; Maher et al 2012).

Summary

There are exciting prospects and considerable advances made with biomarkers for equine musculoskeletal disease and injury but there is still considerable work to do before having a clinically available biomarker panel. We will continue to work with colleagues in human medicine to learn from their research but can hopefully contribute back to them with data from the horse which provides a clinically relevant study group with some unique applications of biomarkers in prediction of disease susceptibility, changes with exercise (or over training) and possibly athletic ability.
References


Burton AB, Wagner B, Erb HN, Ainsworth TM. Serum interleukin-6 (IL-6) and IL-10 concentrations in normal and septic foals. Vet Immunolpathol 2009;132:122-128.

Caplan AI. All MSCs are pericytes? Stem Cell Stem 2008;3:229-230.


Clegg PD, Barr ED, Peffers MJ. How can post-genomic science help equine clinical practice? Submitted? (PETE?).


Powell SE. Low-field standing magnetic resonance imaging findings of the metacarpo/metatarsophalangeal joint of racing Thoroughbreds with lameness localised to the region: A retrospective study of 131 horses. Equine Vet J 2011;44:169-177.


Price J. Bone Biomarkers. In, Ross MW and Dyson SJ (eds), Diagnosis and Management of Lameness in the Horse, 2nd edition, Elsevier Saunders, St. Louis 2011;947-952.


