RESEARCH ARTICLE

Gene expression analysis of subchondral bone, cartilage, and synovium in naturally occurring equine palmar/plantar osteochondral disease

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Abstract
Osteoarthritis (OA) is a disease of the entire joint but the relationship between pathological events in various joint tissues is poorly understood. We examined concurrent changes in bone, cartilage, and synovium in a naturally occurring equine model of joint degeneration. Joints (n = 64) were grossly assessed for palmar/plantar osteochondral disease (POD) in racehorses that required euthanasia for unrelated reasons and assigned a grade of 0 (n = 34), 1 (n = 17), 2 or 3 (n = 13) using a recognized grading scheme. Synovium, cartilage, and subchondral bone were collected for histological and gene expression analysis. Relations between POD grade, cartilage histological score, and gene expression levels were examined using one-way analysis of variance or Kruskal–Wallis test and Spearman’s correlation coefficient with corrections for multiple comparisons. Cartilage histological score increased in joints with POD grade 1 (p = 0.002) and 2 or 3 (p < 0.001) compared to 0. At grade 1, expression of COL1A1, COL2A1, and MMP1 increased and BGN decreased in subchondral bone while expression of BGN and ACAN decreased in cartilage. These changes further progressed at grades 2 and 3. POD grades 2 and 3 were associated with decreased expression of osteoclast inhibitor OPG and increased markers of cartilage degeneration (MMP13, COL1A1). Expression of the vascular endothelial growth factor decreased with POD grade and negatively correlated with cartilage histological score. Synovium showed no histological or transcriptomic changes related to pathology grade. Cartilage degeneration in POD is likely to be secondary to remodeling of the subchondral bone. Limited activation of proinflammatory and catabolic genes and moderate synovial pathology suggests distinct molecular phenotype of POD compared with OA.

KEYWORDS
horse, joint, markers, osteoarthritis, transcriptomic

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1 | INTRODUCTION

Osteoarthritis (OA) is a chronic disease that can affect all joint tissues. Major pathological changes observed in OA are degradation of the articular cartilage, thickening or sclerosis of the subchondral bone, synovial inflammation, and hypertrophy of the joint capsule.\textsuperscript{1,2} The structural changes in articular cartilage during OA result from disruption of tissue homeostasis at the molecular level. OA is characterized by the shift from extracellular matrix (ECM) synthesis to catabolism, promoted by proinflammatory mediators that are released due to mechanical insult or individual risk factors; such as obesity and ageing.\textsuperscript{3,4} Tissue degradation is followed by inadequate repair response, involving chondrocyte hypertrophy\textsuperscript{5} and changes in bone and cartilage ECM that affect biomechanical properties of the joint tissues.\textsuperscript{6,7} Pathological changes in one joint tissue can affect other tissues due to close biomechanical and biochemical association and so it can be difficult to determine where the primary pathology is initiated.\textsuperscript{8,9} There is growing evidence that subchondral bone alterations precede degeneration of the overlying articular cartilage in some of the OA phenotypes\textsuperscript{10–12} and this sequence of pathological events is thought to result from both biomechanical and biochemical impact of subchondral bone perturbation on articular cartilage.\textsuperscript{11,13} Therefore, better understanding of the crosstalk between bone and cartilage within healthy and diseased joints may help develop strategies to mitigate pathological changes or arrest OA progression.

Animal models that mimic symptoms and pathological features of human OA are essential for studying molecular events underlying joint tissue degeneration and developing interventions that could mitigate disease progression.\textsuperscript{14–16} Various experimental animal models of OA have been shown to be effective in inducing cartilage and subchondral bone lesions characteristic of the late stage joint degeneration in humans. However, induction of OA through surgical procedure or enforced loading can induce rapid extensive changes (affecting subchondral bone and cartilage, simultaneously), which make it difficult to identify the early stages of disease development.\textsuperscript{15,17,18}

Horses can provide a naturally occurring model of OA that resembles human disease on the macroscopic, histological, and molecular level through increased expression of proinflammatory cytokines, and catabolic enzymes in the synovium, synovial fluid, and articular cartilage.\textsuperscript{14,19,20} Joint disease in racehorses, associated with regular, intensive exercise, provides a model for the study of repetitive stress induced microstructural changes in articular cartilage and subchondral bone.\textsuperscript{21,22} A common site of overuse injuries in racehorses are the palmar/plantar distal condyles of the third metacarpal and metatarsal bones (Mc/MtIII). Pathologic lesions commonly referred to as palmar/plantar osteochondral disease (POD), can progressively develop into whole-joint disease, manifested by pain with associated radiographic changes similar to human OA.\textsuperscript{21,23–25} Specifically, the medial McII condyle in the forelimb and the lateral MtIII condyle in the hind limb have been shown to be predisposed to POD.\textsuperscript{24} This can be attributed to the variations in loading of metacarpo- and metatarsalphalangeal joints, due to morphology of the distal condyles of Mc/MtIII.\textsuperscript{24} In early stages of POD, subchondral bone abnormalities occur whilst the articular cartilage remains grossly intact.\textsuperscript{21,24,25} Therefore, POD might provide a plausible model for studying association between subchondral bone injury and development of other OA changes in other tissues such as articular cartilage and synovium.

The main aim of this study was to examine microstructural and molecular changes in different joint tissues associated with different stages of POD in racehorses. Furthermore, we aimed to compare identified changes to those previously reported in OA. We hypothesize that (i) subchondral bone is the predominantly affected tissue in early POD and that development of cartilage and synovial pathologic lesions follow the alterations in bone metabolic equilibrium as POD progresses and (ii) POD could serve as a model for early OA induced by repetitive mechanical stress, due to overlapping structural and molecular phenotype in POD and early OA. The potential impact of this study includes validation of POD as a model for human OA research and improving knowledge of relations between bone, cartilage, and synovial pathologic lesions concurrent with development of OA.

2 | METHODS

2.1 | Sample collection and gross scoring

Metacarpo/metatarsophalangeal (Mc/MtPh) joints were collected from Thoroughbred racehorses that were either in active race training up to the time of death or had previously retired from active race training at the Hong Kong Jockey Club between November 24, 2005 and March 25, 2009. All horses euthanized on welfare grounds for reasons unrelated to the study were eligible for inclusion and samples were collected at postmortem, within 30 min of death. Further details on sample and data collection and the racing population at Hong Kong jockey club were previously described by Pinchbeck et al.\textsuperscript{24} Mc/MtPh joints were examined by gross observation and assigned a POD score using the grading system described by Barr et al.\textsuperscript{26} Briefly, grade 0 represents no gross pathologic lesions, grade 1—discolouration (bruising) of subchondral bone with none or minimal disruption of articular cartilage, grade 2—mild to moderate disruption of articular cartilage, and grade 3—disruption/collapse of articular surface (Figure 1A).

2.2 | Histology

Histological scoring in all samples was performed by two independent observers blinded to the POD grade and the average of the two scores recorded and used for statistical analysis. Sections were obtained from the left medial distal metacarpal condyle and the left lateral distal metatarsal. The condyle was cut with a band saw then two sections each 2-mm-thick were cut with a saline cooled diamond saw. Cartilage was dissected sharply with a scalpel and a section of subchondral bone from the center of the sample at the
anatomical site of POD measuring $15 \times 15$ mm approximately was dissected using a chisel. Examples of affected condyles are shown in Figure 1A–C. Safranin O and Masson’s trichrome staining of the Mc/MtIII condyles were used to score cartilage pathologic lesions using a modified Mankin score. Synovial membranes of the Mc/MtPh joints harvested at postmortem were fixed in cold 4% formaldehyde for at least 30 min before being processed, paraffin-embedded, and cut into 10-µm sections. Hematoxylin and eosin-stained sections of synovial membranes were used to assess the level of synovitis. Three areas of each synovial membrane were evaluated using scoring criteria designed by Krenn et al. and the average score used for statistical analysis. Briefly, the synovitis score is the sum of three features of chronic synovitis (enlargement of lining cell layer, cellular density of synovial stroma, and leukocytic infiltrate), each graded from 0 to 3. The total score is interpreted as follows: 0–1, no synovitis; 2–4, low-grade synovitis; 5–9, high-grade synovitis.

### 2.3 RNA extraction from equine tissue

Cartilage, subchondral bone, and synovial membrane samples were stored in RNAlater (Sigma-Aldrich) at postmortem, snap-frozen in liquid nitrogen, and pulverized using a Mikro-Dismembrator (B. Braun Biotech International) at 2000 rpm for 4 min. Homogenized tissue was reconstituted in 1 ml of Tri-reagent (Ambion) and total RNA was extracted using the phenol-chloroform method. RNA was purified using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions, with on-column DNase treatment (Qiagen). Purified RNA was reconstituted in nuclease-free water, quantified using a Nanodrop ND-100 spectrophotometer (Labtech) and RNA purity was assessed based on the A260/A280 absorbance ratio and samples with ratio above 1.8 were accepted for complementary DNA (cDNA) preparation.
2.4 Reverse transcription and quantitative real-time polymerase chain reaction (PCR)

cDNA was synthesized from 1 µg of total RNA using the M-MLV reverse transcriptase, primed using random primers (Promega). Quantitative real-time PCR (qRT-PCR) was performed on an ABI 7300 system using MESA Blue SYBR Green reagent (Eurogentec) to examine gene expression in equine joint tissues (bone, cartilage, and synovium) as described before.20 Gene expression levels were calculated using the 2^(-ΔΔCt) method with glyceraldehyde 3-phosphate dehydrogenase as the reference gene, using the primers described in Table S1. All primers were provided by Eurogentec. Genes evaluated in all three tissues included marker of inflammation interleukin 1 beta (IL-1β), ECM-degrading enzymes: Matrix metalloproteinases (MMP1, 3 and 13) and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) as well as tissue inhibitor of matrix metalloproteinase 1 (TIMP1). In bone and cartilage only, we evaluated expression of ECM structural proteins: Aggrecan (ACAN), biglycan (BGN), and collagens type I and II (COL1A2, COL2A1), markers of ECM catabolism: ADAMTS12, MMP2, 9, 10, marker of anabolism ADAMTS2, catabolism inhibitors (TIMP3 and 4) and OA markers: vascular endothelial growth factor (VEGF), collagen type X (COL10A1), and caspase 3 (CAS3). Additionally, expression of markers of osteoclast activity (receptor activator of nuclear factor κB [RANK] and its ligand RANKL, tartare-resistant acid phosphatase [TRAP]), osteoblast differentiation (runt-related transcription factor 2 [RUNX2], osteomodulin [OMD], osteoprotegerin [OPG]), bone ECM catabolism (cathepsin K [CATK], MMP16, 23) and non-collagenous ECM component bone sialoprotein (BSP) were evaluated in the subchondral bone.

2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). Due to the low sample number of POD grade 3 samples, samples with POD grades of 3 were combined with those of POD grade 2 for histology and gene expression data analysis. Normalized gene expression data and histological scores were subjected to the Shapiro–Wilk test for normality. Due to the highly skewed distribution, the data were log-transformed (LOG10 2^(-ΔCt) and differences between the POD grades were analyzed with one-way analysis of variance with Tukey posthoc test. Gene datasets that did not fill the criteria of normality (Shapiro–Wilk p < 0.05) despite the log-transformation were analyzed using Kruskal–Wallis test with Dunn’s test for multiple comparisons. Spearman’s correlation coefficient was calculated to investigate relation between gene expression data and the cartilage Mankin score. Benjamini–Hochberg procedure with false discovery rate below 0.05 was performed to correct for multiple comparisons within each tissue type and corrected p < 0.05 considered significant.

3 RESULTS

Sixty-four Mc/MtPh joints from 33 Thoroughbred horses aged 3–10 years (mean 6.3 ± 1.9 y) were used in the study. In 31 horses the left McPh and MtPh joint were included in the study, whereas in two horses only the left MtPh joint was included. Of all the joints examined, 34 were assigned to POD grade 0, 17 to POD grade 1, 9 to POD grade 2, and 4 to POD grade 3.

3.1 POD is associated with degradation of cartilage but not synovitis

With increasing POD grade there was an increase in Mankin score (p < 0.05). This suggests that articular cartilage damage was present even at grade 1 POD (Figure 1D) and became more pronounced at higher grades (p < 0.001). No difference was observed in histological synovitis score across POD grades (Figure 1D).

3.2 Gene expression in subchondral bone is affected in early POD stage and correlates with structural degradation of articular cartilage

Relative expression of ADAMTS12, BGN, and VEGF decreased (p < 0.05), whereas COL1A2, COL2A1, and MMP1 increased (p < 0.05) in subchondral bone from grade 1 POD compared with grade 0 (Figure 2). Further changes in the expression of COL2A1, MMP1, and VEGF were observed in POD grades 2 and 3. OPG expression decreased (p < 0.001) only in the POD grades 2 and 3 (Figure 2). Expression of MMP1, MMP10, and COL2A1 in the subchondral bone was positively correlated with the cartilage Mankin score, whereas BGN, CAS3, IL1β, MMP9, MMP16, OPG, OMD, RANK, TIMP1, TIMP4, and VEGF decreased with articular cartilage degradation (Figure 3). The direction of expression changes relative to POD grade and Mankin score was consistent in the significantly affected genes. No change in expression across POD grades was shown for ACAN, ADAMTS4, and 5, BSP, CAS3, CATK, COL10A1, IL1β, MMP2, MMP3, MMP9, MMP10, MMP13, MMP16, MMP23, OCN, OMD, RANK, RANKL, TIMP1, TIMP3, TIMP4, and TRAP (data not shown).

3.3 Transcriptomic changes in articular cartilage demonstrate altered ECM metabolism in POD grades 2 and 3

There was a significant increase in ADAMTS2, ACAN, and COL1A2 expression and decrease in ACAN, BGN, CAS3, and VEGF expression in articular cartilage in POD-affected joints. Expression of ACAN, BGN, CAS3, and VEGF changed both with increasing POD grade (Figure 4) and Mankin score (Figure 3). Expression levels of ADAMTS2 and
MMP13 increased ($p \leq 0.01$) and superficial zone protein 1 (SZP1) decreased ($p \leq 0.01$) between healthy joints and POD grades 2 and 3. TIMP4 decreased ($p = 0.004$) in POD grades 2 and 3 relative to grade 1 (Figure 4). ADAMTS12, COL2A1, COL10A1, IL1β, MMP1, MMP2, MMP3, MMP9 and MMP10, and TIMP1 and TIMP3 showed no change in expression across different POD grades (data not shown). There were no changes in gene expression relative to POD grade and Mankin score in synovium samples (data not shown).

**DISCUSSION**

When studies are limited to tissues from severely diseased joints with end-stage joint disease, it is difficult to determine whether changes in one tissue preceded changes in others, or if they developed independently in response to the same insult. The main strength of this study was the inclusion of multiple tissues collected from joints in different stages of naturally occurring traumatic joint disease.
disease in which subchondral bone changes are generally considered to be a significant initiating factor. The equine POD model allowed investigation of the sequence of pathological events in different joint tissues and analysis of potential relationships between them. Importantly, we identified 17 joints representing the early phase of POD, characterized by gross subchondral bone pathologic lesions (“bruising”/discolouration) with no or minimal grossly visible disruption of the overlying cartilage. Although macroscopically intact, cartilage in grade 1 POD showed significantly higher Mankin score and decreased gene expression of proteoglycans, ACAN and BGN, VEGF and CAS3 compared to the grossly normal joints. That is contrary to our hypothesis stating that grade 1 POD does not involve significant pathological changes in articular cartilage. Increased Mankin score is in line with the previous studies showing that macroscopically normal cartilage in POD can show extensive levels of microdamage. Decreased proteoglycan content is one of the main characteristics of cartilage matrix loss in OA that results from an imbalance between matrix synthesis and degradation by proteolytic enzymes. Gene expression patterns similar to OA have been reported in other early phase traumatic joint disorders and in the low-load sites of the OA-affected joints, suggesting that molecular changes precede structural changes and may play causative role in their development. Reduced expression of proteoglycans in grade 1 POD correlates with histological signs of cartilage degeneration (Figure 3). CAS3 is one of the effector enzymes driving chondrocyte apoptosis in OA upregulated by proinflammatory cytokines, therefore, lack of change in IL1β expression in POD-affected cartilage in this study may explain the absence of CAS3 activation. Similarly, there was no change in expression of other genes typically associated with OA: COL1A2, COL2A1, MMP13, and COL10.

In the more severe POD stages, where cartilage damage is evident on gross examination, we observed further increases in Mankin score, decreased ACAN expression, and changes in expression of other genes previously associated with spontaneous and experimental OA (increase of ADAMTS2, COL1A2, MMP13, and decrease of S2P1 and TIMP4). ADAMTS-2 is a pro-collagen N-proteinase involved in collagen fibril formation and its upregulation in this study coincided with an increase in COL1A2 expression, suggesting increased type I collagen synthesis. Normal cartilage consists predominantly of type II collagen, although switch to type I collagen production occurs in OA. Increased expression of MMP13 is one of the hallmarks of OA due to its role as a marker of chondrocyte hypertrophy and collagenase targeting mainly type II collagen. Interestingly, increase of MMP13 expression occurred only in POD grades 2 and 3, whereas in OA MMP13 upregulation is present early in the disease onset. Though the underlying cause of late MMP13 expression is unclear, it may be associated with the absence of explicit inflammatory response in the analyzed joints as proinflammatory cytokines are one of the main factors upregulating MMP13 expression. Concurrent downregulation of TIMP4 in severe POD suggests decreased protection of the ECM from MMP-mediated proteolysis. S2P1, also known as proteoglycan 4 or lubricin, is a secreted glycoprotein that provides boundary lubrication between the joint surfaces. S2P1 is secreted mainly in the superficial zone of articular cartilage and, therefore, its reduced expression in POD grades 2 and 3 may be related to significant disruption/collapse, or even loss of the cartilage surface (Figure 1). Altogether, the significant changes in cartilage gene expression in severe POD indicate a disrupted balance between anabolic/catabolic activity and aberrant repair response (fibrillation) that corresponds with gradual destruction of articular cartilage typical in the advanced OA. However, other phenotypic changes typically associated with OA, such as expression of chondrocyte hypertrophy marker COL10 and enzymes degrading other important ECM components—proteoglycans and glycoproteins (ADAMTS4, MMP3, and MMP9) were not observed. It is worth noting that significant downregulation of proteoglycan anabolism occurred before visible gross signs of cartilage compromise, highlighting limitations of the macroscopic methods of joint health assessment (arthroscopy) when assessing trauma-induced disorders.
Gene expression profiles associated with joint degenerative disease have been relatively well characterized in cartilage through human and animal model studies. However, knowledge of the transcriptomic changes affecting the subchondral bone is still limited. In human OA research, subchondral bone sample collection is usually limited to the end-stage disease (joint replacement). Mouse model studies can offer insight into early stages of joint degeneration but they are less suitable for gene expression analysis in specific joint tissues due to their small size. Zhang et al. used experimental rat model of posttraumatic osteoarthritis (PTOA) to analyze the expression profile of the subchondral bone associated with disease progression but without considering corresponding changes in the articular cartilage. In this study, subchondral bone showed more gene expression changes in grade 1 POD than the corresponding cartilage samples (Figures 2 and 4), which fits within the current understanding of POD as predominantly a disease of subchondral bone. Structural integrity of the bone depends on the coordinated response of osteoblasts that produce organic ECM (mainly comprised of type I collagen) and drive its mineralization, and bone-degrading osteoclasts. In grade 1 POD we observed simultaneous increase in expression of COL1A2 and MMP1, the collagen-cleaving protease, suggesting an overall increase in collagen turnover. Expression of ADAMTS2 showed a declining trend in grade 1 POD followed by an increase in more severe POD stages and was accompanied by decreased expression of BGN, a proteoglycan that regulates collagen fibril organization. In POD grades 2 and 3 subchondral bone showed decrease in expression of OPG which inhibits osteoclastogenesis by disturbing the RANK/RANKL pathway, suggesting increased activity of bone-degrading cells. We also observed marked decrease in expression of another ECM protease, MMP16, in POD grades 2 and 3, however, this result may be biased due to there being only a few samples of grade 2 and 3 POD subchondral bone (n = 5, Figure 2). Uncoupled bone remodeling, with bone resorption prevailing over bone formation, leads initially to the loss of bone density, microfractures, and eventually collapse of the subchondral bone and subsequent degradation of the overlying cartilage. Increased expression of COL2A1 observed in POD subchondral bone (Figure 2) was previously reported in human end-stage knee OA and rat experimental PTOA and could reflect attempted repair of microfractures through callus formation and build-up of trabecular bone within bone marrow spaces that can be observed in equine POD. It is possible that the mild cartilage damage identified in grade 1 POD was partially caused by altered biomechanical properties of the subchondral bone undergoing intense remodeling in response to mechanical overuse. Correlations between expression of genes related to tissue remodeling (MMP1, MMP9, TIMP1/4, BGN, OPG, RANK, and OMD) in the subchondral bone and the cartilage Mankin score support our hypothesis that changes in bone metabolic equilibrium are associated with articular cartilage degradation in POD.

Downregulation of VEGF in grade 1 POD was demonstrated simultaneously in cartilage and subchondral bone and negatively correlated with the cartilage Mankin score. VEGF is an established marker of clinical and preclinical OA associated with angiogenesis and procatabolic response in the cartilage and sclerosis of the subchondral bone. VEGF has been also shown to promote osteogenic differentiation of bone marrow-derived mesenchymal stromal cells (BMSCs) yet the evidence on local VEGF expression in the subchondral bone disease is limited. Decreased VEGF expression in POD corresponds with lack of consistent activation of catabolic markers and a decrease in osteoblastic marker of bone formation OPG, which could potentially be attributed to decline in VEGF biological activity in the bone. The study of human femoral joints with signs of cartilage degeneration show unchanged VEGF expression in the subchondral bone despite its upregulation in the overlying cartilage. The experimental induction of PTOA in rodents led to downregulation of VEGF in subchondral bone and pooled joint tissues, similar to our study. It is not clear if increase in VEGF expression in cartilage is specific to the human OA or if animal models of traumatic joint disease fail to replicate that phenotype.

POD has been previously shown to be positively correlated with the presence of gross synovial pathologic lesions. In this study the synovium histological score did not differ significantly between POD groups, however, it did indicate low-grade synovitis in all evaluated joints, even in the absence of POD. Low level of synovial pathologic changes in POD-affected joints was supported by unchanged expression of IL1β and catabolic enzymes between POD grades. Inflammation and hyperplasia of the synovial membrane in Mc/MtPh joints is commonly observed in racehorses and attributed to repetitive mechanical overuse (hyperextension of Mc/MtPh joints during high-speed exercise). Synovitis can accelerate cartilage degradation by release of cytokines, alarmins, and damage-associated molecular pattern molecules (DAMPs), whereas matrix molecules resulting from cartilage damage stimulate synovitis. This cross-talk mediated by the synovial fluid is recognized as part of pathogenic mechanism of OA. Lack of a significant effect of POD grade on synovium histological score and gene expression level in this study suggests that, unlike in OA, synovitis may play limited role in development of POD and observed pathological lesions are more likely to be secondary to subchondral bone and cartilage damage or direct effect of repetitive mechanical loading on the joint capsule.

Some limitations to this study should be considered. Due to the limited number of horses involved in the study, we included Mc/MtPh joints obtained from the same animal. More than a half of the studied horses obtained the same POD grades in the front and hindlimb joint but the difference between POD score in McPh and MtPh in the same horse could also range vastly across the POD grades, suggesting joint reaction to repetitive loading can be highly variable in individual animals or significant variation in loading of the joints between front and hind limbs in the same animal. The inconsistency in the level of disease between animals, or even individual joints in the same animal, is one of the main limitations of using naturally occurring model of joint disease. Although naturally occurring models are more likely to mimic pathological lesions identified in human joint degeneration, the time of disease development is variable across individuals and, thus, the disease...
stage (acute, chronic) in samples collected postmortem can be difficult to determine. Additionally, limitations of the use of gross scoring to identify severity of the lesions (POD grades) should be recognized. It has been previously reported that microstructural damage can be present in equine Mc/MtPh joints despite normal macroscopic appearance.\textsuperscript{21,24} In this study, we aimed to account for that by using two outcome measures to assess joint degeneration, gross joint scoring and histological scoring of articular cartilage, and gene expression changes identified with regard to both measures were most extensively discussed. However, it should be noted that subchondral bone damage can be undetectable from external examination of the joint surface at early POD stage and including histological scoring of the subchondral bone would have been helpful to better define disease severity. The small set of established gene markers of joint degeneration used in this study could not provide exhaustive information about all molecular processes relevant for the disease development, however, it allowed comparison of equine POD to multiple clinical and experimental OA studies in the literature. Despite these limitations, our work provides a rare insight into transcriptomic changes in three different joint tissues at different stages of a naturally occurring degenerative joint disease and their significance in relation to micro- and macrostructural signs of joint pathology.

In conclusion, gene expression analysis in the subchondral bone in POD suggest changes in bone turnover that likely leads to articular cartilage damage, however, changes in cartilage microstructure and gene expression are detectable even at the early stage of disease. Despite altered regulation of several well-established markers of OA (MMP13 and ACAN), limited activation of proinflammatory and catabolic genes suggests that POD has a distinct molecular phenotype compared to PTOA and, therefore, does not constitute an accurate model for studying OA pathogenesis. Transcriptional response of articular cartilage at stages where only microscopic features of cartilage degeneration were detectable supports the putative role of biomechanical and biochemical relationships between subchondral bone and cartilage in development of this degenerative joint disease.

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CONFLICT OF INTERESTS
Chris M. Riggs is an employee of the Hong Kong Jockey Club.

AUTHOR CONTRIBUTIONS
The study was conceived by Chris M. Riggs, Peter D. Clegg, and Alan Boyd. All authors contributed to the design of the study. Chris M. Riggs collected all samples for the study. Laboratory analysis was undertaken by Elizabeth D. Barr and Benjamin T. McDermott. Gina L. Pinchbeck, and Agnieszka J. Turlo supported data analysis. The paper was written by Agnieszka J. Turlo and all authors approved the final manuscript. All authors have read and approved the final submitted manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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