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Original Research

Differential Protein Expression of the Marginal Transitional Zone in Foals with Osteochondrosis

Elizabeth A Marchant, Stacy A. Semevolos*

Department of Clinical Sciences, Carlson College of Veterinary Medicine, Oregon State University, Corvallis, OR

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ABSTRACT

The marginal transitional zone is peripherally located within the diarthrodial joint, and represents the interface of articular cartilage, periosteum, and the fibrous joint capsule. The purpose of this study is to characterize the protein expression of matrix and molecular regulators in the marginal transitional zone of foals having osteochondrosis (OC) compared to normal foals. Several families of proteins with known roles in cartilage and bone development are investigated, including matrix molecules, Wnt signaling, apoptotic factors and paracrine cell signaling molecules. Our results demonstrate differential protein expression in the marginal transitional zone from the lateral femoral trochlear ridge of foals affected by osteochondrosis. Alterations in protein expression of OC-affected foals mainly involve components of extracellular matrix homeostasis and canonical Wnt signaling. Matrix expression of collagen type IIB and lubricin are decreased and matrix metalloproteinase-3 expression is increased in OC-affected marginal transitional zone samples. Canonical Wnt signaling is inhibited in OC-affected marginal transitional zone samples, based on increased Dickkopf-1 and decreased β-catenin protein expression. Most apoptotic and paracrine signaling proteins are not altered in OC-affected marginal transitional zone samples.

1. Introduction

The marginal transitional zone (MTZ) is peripherally located within the diarthrodial joint, and represents the interface of articular cartilage, periosteum, and the fibrous joint capsule [1]. The MTZ is distinct from more centrally located articular cartilage which possesses a more homogenous tissue profile [2,3]. Electron micrograph-based descriptions of the MTZ of juvenile rabbits show a wide tissue heterogeneity. In this study, the MTZ consists of a fibrous joint capsule supplied by capillary networks, with cells consisting of several synovial cell subtypes of differing orientation and morphology. The chondrocytes of this region have a homogeneous morphology in young animals, and there is a distinct demarcation when meeting the synovium. At the edge of the MTZ, collagen fibrils are oriented in a transverse direction around the perimeter of the articular cartilage, referred to as the “cartilage edge belt” [1].

Descriptions of the MTZ are rare in the equine literature and are limited to osteophyte formation at the junction between synovium, periosteum, and articular cartilage secondary to osteoarthritis [4]. Another study examines the blood supply in this region of the lateral femoral trochlear ridge through perfusion and imaging techniques [5]. Further work shows that transection of vessels within this region results in chondronecrosis and subsequent osteochondrosis lesions [6].

Other studies of equine osteochondrosis (OC) characterize gene and protein expression patterns of articular cartilage along the central portion of the lateral femoral trochlear ridge [7–9]. These studies show disruption of multiple cellular pathways regulating endochondral ossification in OC-affected samples along the cartilage canals and osteochondral junction [7–9]. However, the marginal transitional zone was not evaluated in these studies. The unique tissue composition of the MTZ represents an opportunity to further define microscopic and immunohistochemical features of articular cartilage which may aid our understanding of protein expression differences in OC-affected cartilage in horses.

Therefore, **the purpose of this study was to characterize the protein expression of matrix and molecular regulators in the marginal transitional zone of foals having OC compared to**

Conflict of Interest Statement: The authors declare no conflicts of interest.

Animal Welfare/ethical statement: Osteochondral samples were harvested in a previous study following humane euthanasia [see reference below]. The study was approved by the institutional animal care and use committee. Foals were euthanized for reasons unrelated to joint sepsis using an overdose of sodium pentobarbital(105 to 187 mg/kg IV).

* Corresponding author at: Stacy A. Semevolos, Department of Clinical Sciences, Carlson College of Veterinary Medicine, Oregon State University, 2014 Magruder Hall, Corvallis, OR 97331.

E-mail address: stacy.semevolos@oregonstate.edu (S.A. Semevolos).

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normal foals. For this research, several families of proteins with known roles in cartilage and bone development were investigated, including matrix molecules (Collagen type IIB, Sox9, Osteocalcin, Lubricin, MMP3, MMP13), Wnt signaling (β-catenin, Wnt-4, Wnt-11, Dkk-1), apoptotic factors (Caspase-10, Fas, Cytochrome-C, Thymosin β10) and paracrine cell signaling molecules (Ihh, PTHrP, VEGF, PDGF). **We hypothesized that the marginal transitional zone in OC-affected samples would have increased protein expression of apoptotic factors, Wnt signaling members, matrix degradative enzymes and paracrine signaling molecules and decreased protein expression of extracellular matrix components, when compared to the MTZ of normal control samples.**

2. Materials and Methods

2.1. Sample collection and processing

Osteochondral samples were harvested from the lateral femoral trochlear ridge of 15 foals aged 1 – 6 months (7 OC-affected, 8 normal) from a previous study, including 5 intact males and 10 females [7]. The study was approved by the institutional animal care and use committee. Foals were euthanized for reasons unrelated to joint sepsis using sodium pentobarbital (105 to 187 mg/kg IV). All osteochondral samples (n = 2 per trochlear ridge, 3 – 4 mm thickness, ~1 – 1.5 cm in depth, ~2 cm in length) were sharply dissected from mid-lateral trochlear ridges of both distal femurs and included the MTZ (See Appendix 1). Cartilage was sharply cut with a scalpel down to bone, followed by sectioning of the underlying bone using a sharp osteotome. Osteochondral samples were fixed in 4% paraformaldehyde for 48 hours and transferred to 10% EDTA solution for decalcification (2 – 4 weeks). Decalcified samples were embedded in paraffin and sectioned in 6 μm sections in preparation for immunohistochemistry, Toluidine Blue, and H&E staining.

2.2. Gross and histological assessment

Samples were evaluated grossly at the time of collection and histologically following H&E staining to classify them as early OC, advanced OC, or normal as described in a previous study [7] (See Appendix 2). Normal cartilage was defined as having no gross or histologic abnormalities. Early OC was defined as samples having altered endochondral ossification (locally thickened cartilage [class 1], loss of normal columnar arrangement of chondrocytes, chondrones) (class 2) or separation (fissures, necrosis) along the osteochondral junction without concurrent superficial cartilage lesions (class 3). Advanced OC was defined as having separation (fissures, necrosis) along the osteochondral junction with concurrent superficial lesions (OCD flaps, osteochondral fragments, fibrocartilage formation) (class 4) [7,10]. Based on these criteria, 7 foals were determined to have early OC, and 8 were classified as normal. In OC samples, 5 foals had separation along the osteochondral junction and 4 foals had locally thickened cartilage (2 with concurrent osteochondral separation). None had concurrent superficial lesions. Areas of chondronecrosis along the osteochondral junction were apparent in 2 foals, while focal areas of chondronecrosis in the epiphyseal cartilage were apparent in 2 foals.

2.3. Toluidine blue staining

Samples were sequentially deparaffinized in xylene, brought to water, rinsed in phosphate buffered saline (PBS) and then stained with Toluidine Blue for 45 seconds.

2.4. Immunohistochemistry

Immunohistochemistry was performed, as described previously [7–9], using the Supersensitive Link-Label Multilink Immunohistochemistry System (Biogenex, Fremont, CA). Briefly, samples were sequentially deparaffinized in xylene, brought to water, and rinsed in phosphate buffered saline (PBS). Antigen retrieval was performed with testicular hyaluronidase (cartilage samples) at 37◦C for 60 minutes, or with pepsin (connective tissue controls) at 37◦C for 5 minutes. Endogenous peroxidases were quenched with hydrogen peroxide and methanol. Protein block was only used for polyclonal antibodies. Incubation with primary antibodies was performed using 1:20 dilution of rabbit α-human polyclonal (Ihh, PTH-rP, VEGF, PDGF, β-catenin, Wnt 11, Lubricin, Caspase-10, Fas, Thymosin β10, Sox9), mouse α- human monoclonal (MMP-3, MMP-13, Dkk-1, Collagen type IIB, Osteocalcin, Cytochrome C), or rat α- human monoclonal (Wnt-4) (Biogenex, Fremont, CA; Research Diagnostics, Inc., Flanders, NJ; Abcam, Cambridge, MA, USA; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Thermo Fisher Scientific, Hampton, NH). Negative procedural controls were performed using normal IgG non-immune serum (Super Sensitive Mouse, Super Sensitive Rabbit, or Super Sensitive Rat, Biogenex, Fremont, CA) instead of primary antibodies (using the same species as primary antibodies). Secondary biotinylated multilink antibodies were applied, followed by labeling with streptavidin conjugated peroxidase, and then applying diaminobenzidine tetrachloride (DAB) as a chromogen for production of color product (brown). The sections were counterstained with Harris hematoxylin and mounted for microscopy.

Positive tissue controls were used to confirm specificity in equine tissue, including equine samples of liver (Ihh, Caspase-10, β-catenin, Wnt-4, Wnt-11, Dkk-1), skin (PTH-rP, MMP-13), hemangioma (VEGF, Fas), lung (thymosin-β10, cytochrome C), squamous cell carcinoma (PDGF), and cartilage (MMP-3, Collagen IIB, Sox9, Osteocalcin, Lubricin).

2.5. Immunohistochemistry and toluidine blue scoring

Slides were scored by 2 investigators (EAM and SAS), blinded to group allocation. Matrix and cellular protein expression were scored separately in each of the following layers of the MTZ: superficial synovium, deep synovium, fibrocartilage, superficial cartilage, middle cartilage, deep cartilage, and bone layers (Fig. 1). Protein expression from immunohistochemistry and toluidine blue staining was scored from 0 to 3 using a semiquantitative system: 0 (no staining/expression), 1 (mild staining/expression), 2 (moderate staining/expression), or 3 (strong staining/expression) for each layer of the MTZ. The percentage of cells or matrix staining positive was then estimated for each layer. Scores of both investigators were averaged at each location. For immunohistochemistry scoring only, the HSCORE was then calculated for each MTZ layer by taking the sum of the percentage of positive staining cells or matrix (Pi) at each intensity and multiplying this value by its respective intensity score (i). HSCORE = Σi i $*$ Pi [11].

When scoring cytochrome C immunohistochemistry samples, mitochondrial expression was also scored. Mitochondral expression was noted as a stippled pattern within the cytoplasm of chondrocytes [12]. Diffuse cellular immunostaining without distinct stippling was considered a cytoplasmic/cellular pattern [12].

2.6. Statistical analysis

Comparison of HSCOREs and toluidine blue scoring from OC-affected versus normal samples for each protein was conducted using a Mann Whitney U test ($p \leq 0.05$).

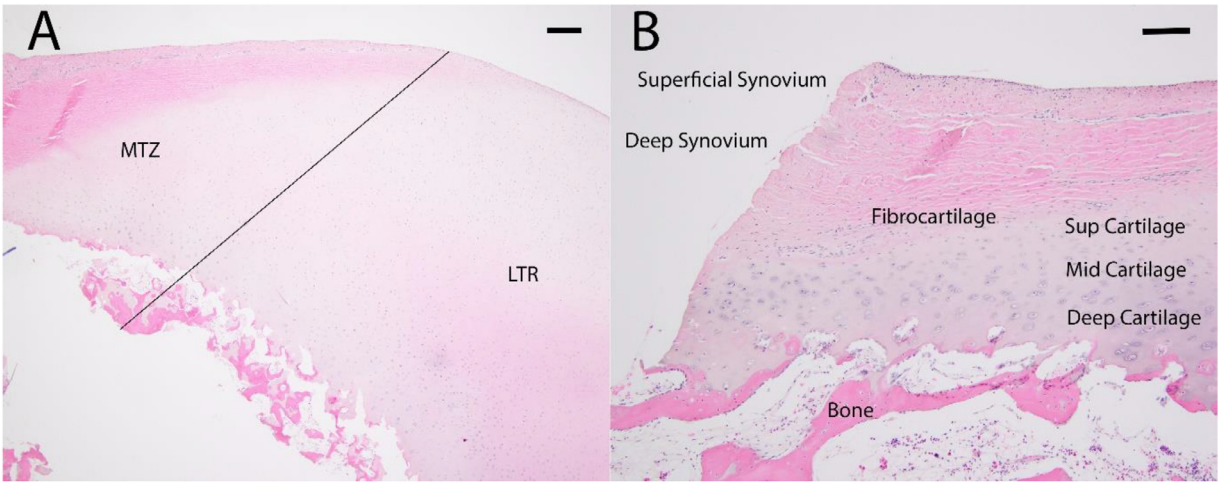

Fig. 1. Photomicrographs of the marginal transitional zone (MTZ) of the lateral trochlear ridge (LTR) of the distal femur from (A) a 5-month old foal and (B) a 4-month old foal. (H&E staining; Bar = 100μm; A = 4x objective, B = 10x objective).

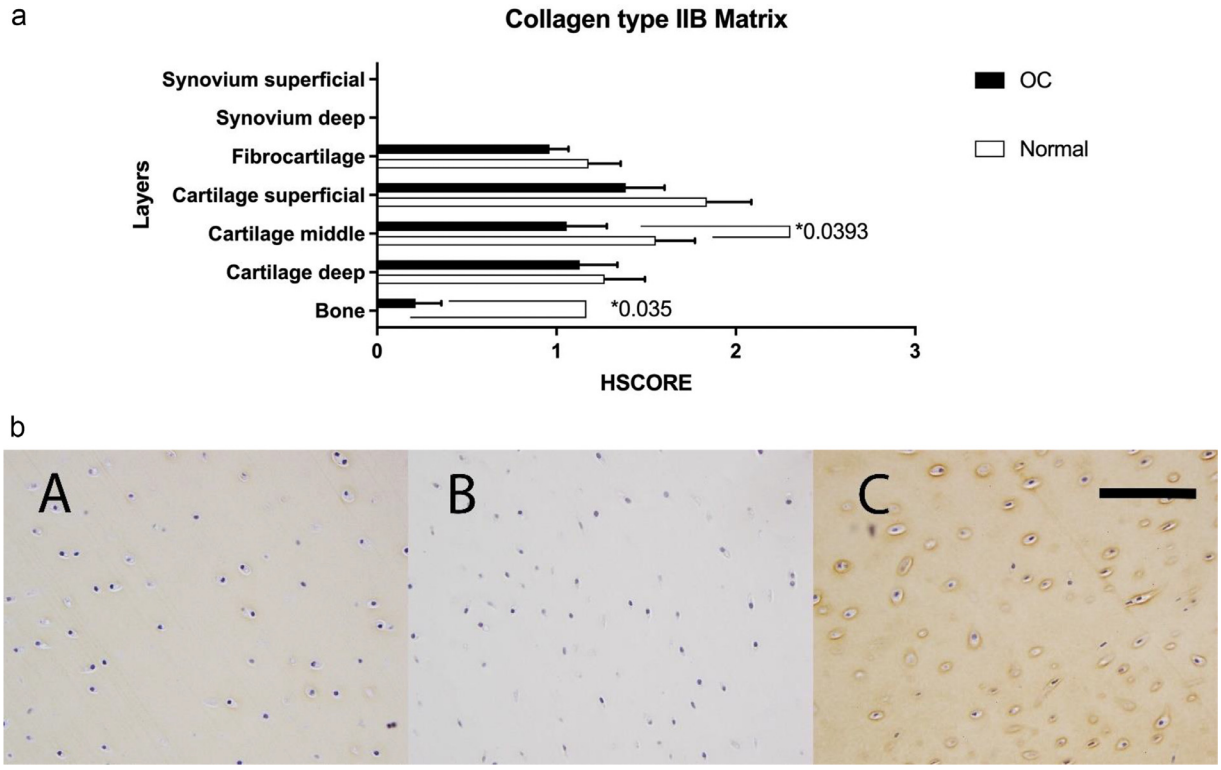

Fig. 2. a Mean immunohistochemistry HSCOREs +/- SEM for matrix protein expression of Collagen type IIB in the MTZ. Collagen type IIB expression was decreased in the middle cartilage layer and increased in bone layer compared to normal matrix bone *P values ≤.05 displayed above error bars. b: Photomicrographs of the middle cartilage layer of MTZ sections following immunohistochemical localization using antibodies directed against collagen type IIB. (A) OC-affected 5-month old foal showing mild matrix expression of collagen type IIB (HSCORE = 1.1). (B) Negative control of (A). (C) Normal 4-month old foal having moderate matrix expression of collagen type IIB (HSCORE = 2.0). (Hematoxylin counterstaining; DAB chromogen; Bar = 100μm; 40x objective).

3. Results

3.1. Matrix molecules

In OC samples, matrix expression of Collagen type IIB was significantly decreased in the middle cartilage layer (P = .0393) and increased in bone (P = .035) when compared to normal samples (Figs. 2A and 2B). Lubricin matrix expression was also significantly decreased in the deep (P value = .0196) cartilage layer in OC samples (Figs. 3A and 3B). Conversely, MMP-3 matrix expression was significantly increased in the deep cartilage layer (P = .0295) and superficial synovium (P = .0095) in OC samples compared to normal controls (Figs. 4a and 4b). There were no significant differences in mean matrix or cellular expression between OC-affected and normal samples for Sox9, Osteocalcin, and MMP-13 (See Appendix 3). Similarly, no significant differences were found in tolu-

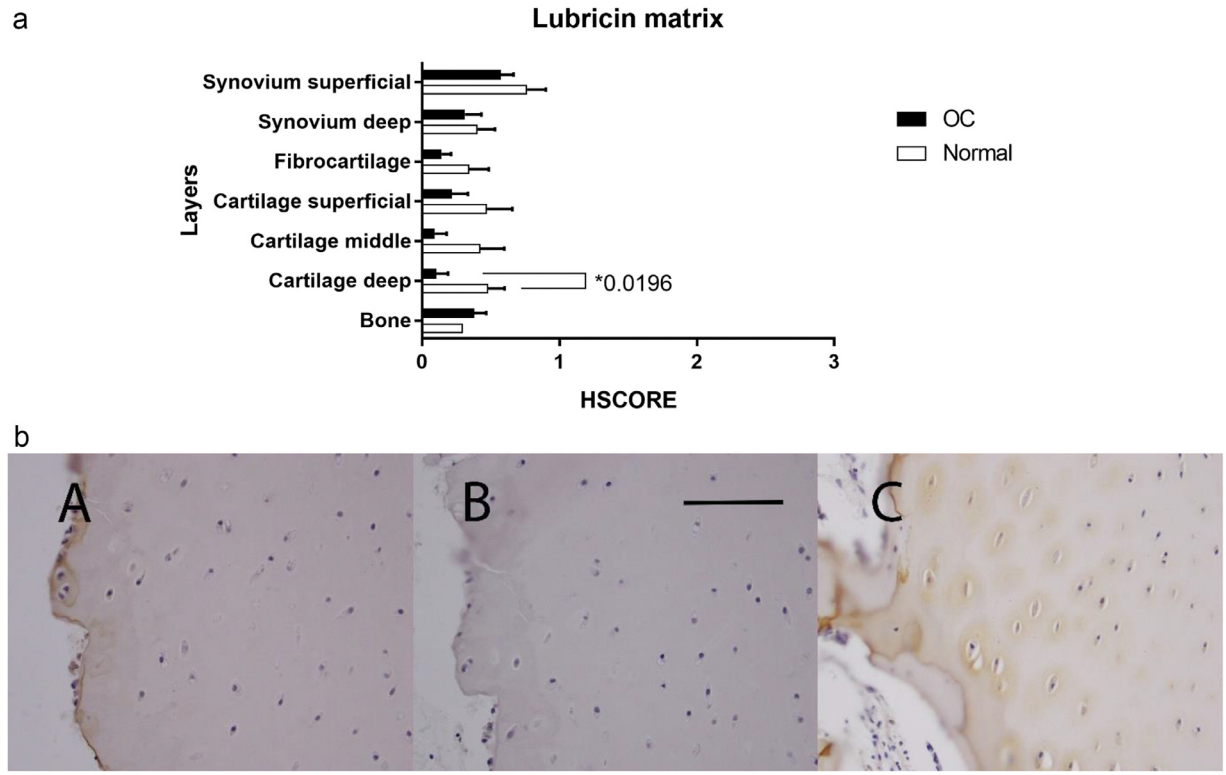

Fig. 3. a: Mean immunohistochemistry HSCOREs +/- SEM for matrix protein expression of lubricin in the MTZ. Matrix expression in the deep cartilage layer was significantly lower in OC-affected versus normal samples $^{*}P$ values $\leq$.05 displayed above error bars. b: Photomicrographs of deep cartilage layer of MTZ sections following immunohistochemical localization using antibodies directed against lubricin. (A) OC-affected 5-month old foal showing minimal matrix expression of lubricin (HSCORE = 0). (B) Negative control of (A). (C) Normal 4-month old foal having mild territorial matrix expression of lubricin (HSCORE = 1.0). (Hematoxylin counterstaining; DAB chromagen; Bar = 100µm; 40x objective).

idine blue staining (matrix metachromasia) of the MTZ layers between OC and normal foals (Figs. 5a and 5b).

3.2. Wnt signaling

In OC samples, β-catenin cellular expression was significantly decreased in the superficial (P = .0355) and middle (P = .0256) cartilage layers compared to normal controls (Figs. 6a and 6b). In contrast, Dickkopf-1 (Dkk-1), a Wnt inhibitor, had significantly increased matrix expression in the superficial (P = .0067) and deep (P = .015) cartilage layers and in bone (P = 0.0057) of OC samples versus normal controls (Figs. 7a and 7b). No significant difference in mean protein expression was found for Wnt-4 and Wnt-11 between OC and normal controls (See Appendix 4).

3.3. Apoptotic factors

In OC-affected samples, mitochondrial expression of Cytochrome C was significantly decreased in the middle cartilage layer compared to normal controls (P-value = .0448) (Figs. 8a and 8b). No significant differences in protein expression were found between OC and normal samples for Caspase-10, Fas, and Thymosin β10 (See Appendix 5).

3.4. Paracrine signaling

No significant differences in protein expression were found in the MTZ between OC-affected and normal foals for Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTH-rP), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (See Appendix 6).

4. Discussion

This is the first known investigation of protein expression in the MTZ of OC-affected and normal foals. We hypothesized there would be increased protein expression of apoptotic factors, Wnt signaling, matrix degradative enzymes and paracrine signaling molecules and decreased protein expression of extracellular matrix (ECM) components in OC-affected MTZ layers. Our results partially support these hypotheses, particularly in regards to extracellular matrix components and degradative enzymes. Decreased matrix expression of collagen type IIB and lubricin and increased expression of MMP-3 indicate potentially abnormal cartilage matrix in OC-affected MTZ samples. However, contrary to our hypotheses, apoptotic and paracrine signaling proteins were not increased in OC-affected MTZ samples. Additionally, canonical Wnt signaling appears to be inhibited in OC-affected MTZ samples, based on increased Dkk-1 and decreased β-catenin protein expression.

Our collagen type IIB findings support previous research showing decreased immunofluorescence of type II collagen in porcine OC-affected whole cartilage samples [13]. Another study found differential type II collagen and aggrecan turnover in the cartilage matrix of young animals having osteochondrosis [14,15]. Based on our findings, the observed decrease in collagen type IIB expression in the MTZ may contribute to changes in cartilage matrix structure, but it is difficult to conclude whether this change is a cause or consequence of OC.

The role of lubricin in OC pathogenesis is unclear. Lubricin is a glycoprotein secreted by chondrocytes and synovial fibroblasts that has boundary lubricant effects in articular cartilage. Lubricin has

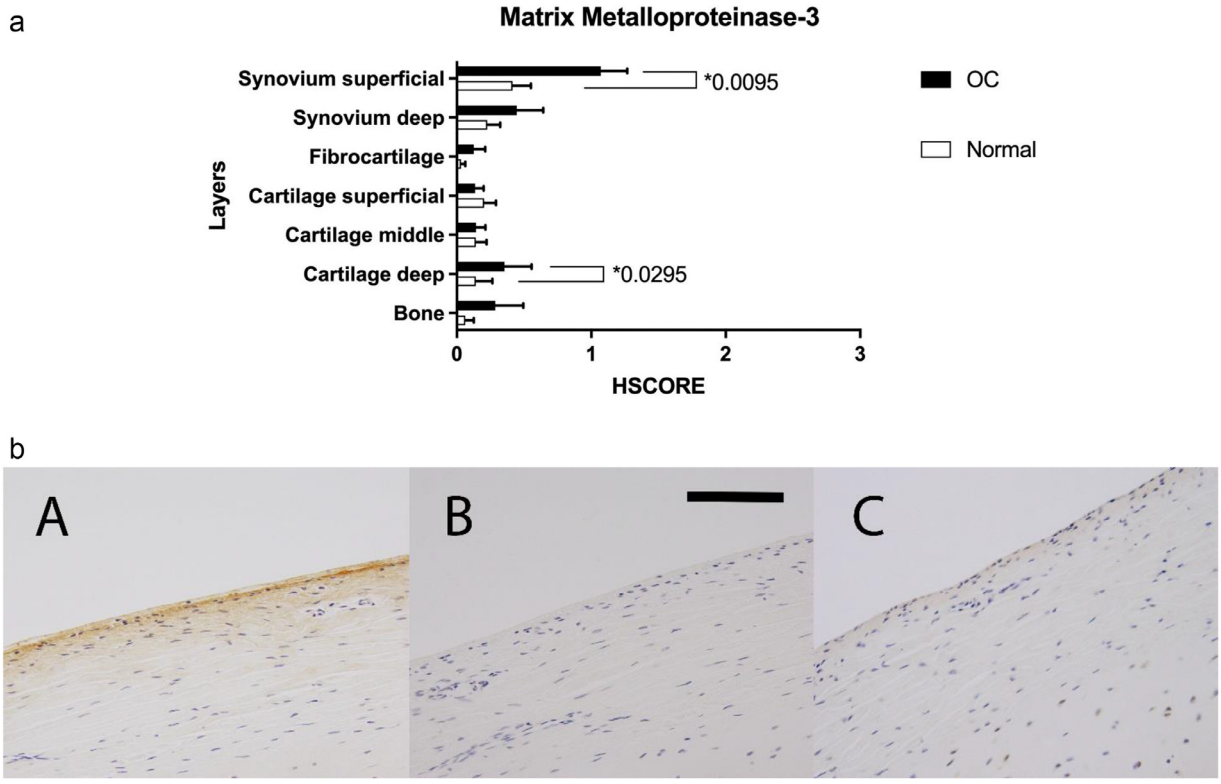

Fig. 4. a: Mean immunohistochemistry HSCOREs +/- SEM for matrix protein expression of MMP-3 of MTZ. Matrix expression in the deep cartilage layer and superficial synovium were significantly higher in OC samples than normal controls. *P values ≤.05 displayed above error bars. b: Photomicrographs of superficial synovium of MTZ sections following immunohistochemical localization using antibodies directed against MMP-3. (A) OC-affected 5-month old foal showing moderate matrix expression of MMP-3 (HSCORE = 2.0). (B) Negative control of (A). (C) Normal 4-month old foal having minimal matrix expression of MMP-3 (HSCORE = 0.31). (Hematoxylin counterstaining; DAB chromogen; Bar = 100μm; 40x objective).

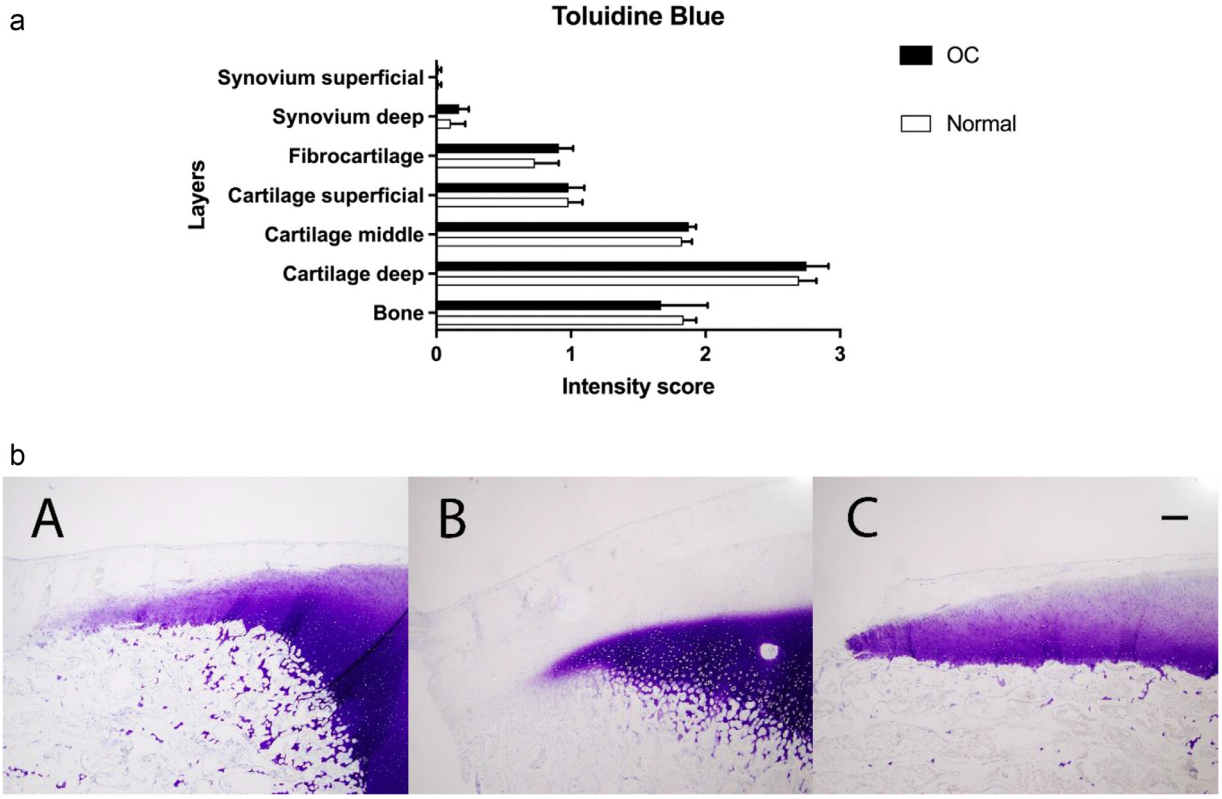

Fig. 5. a: Toluidine blue staining intensity scoring of matrix metachromasia in the MTZ. No significant differences were found between OC and normal samples for any of the layers evaluated. b: Matrix metachromasia of lateral trochlear ridge MTZ from (A) OC-affected 4-month old filly, (B) OC-affected 4-month old colt and (C) normal 4-month old filly. (Toluidine Blue staining; Bar = 100μm; 4x objective).

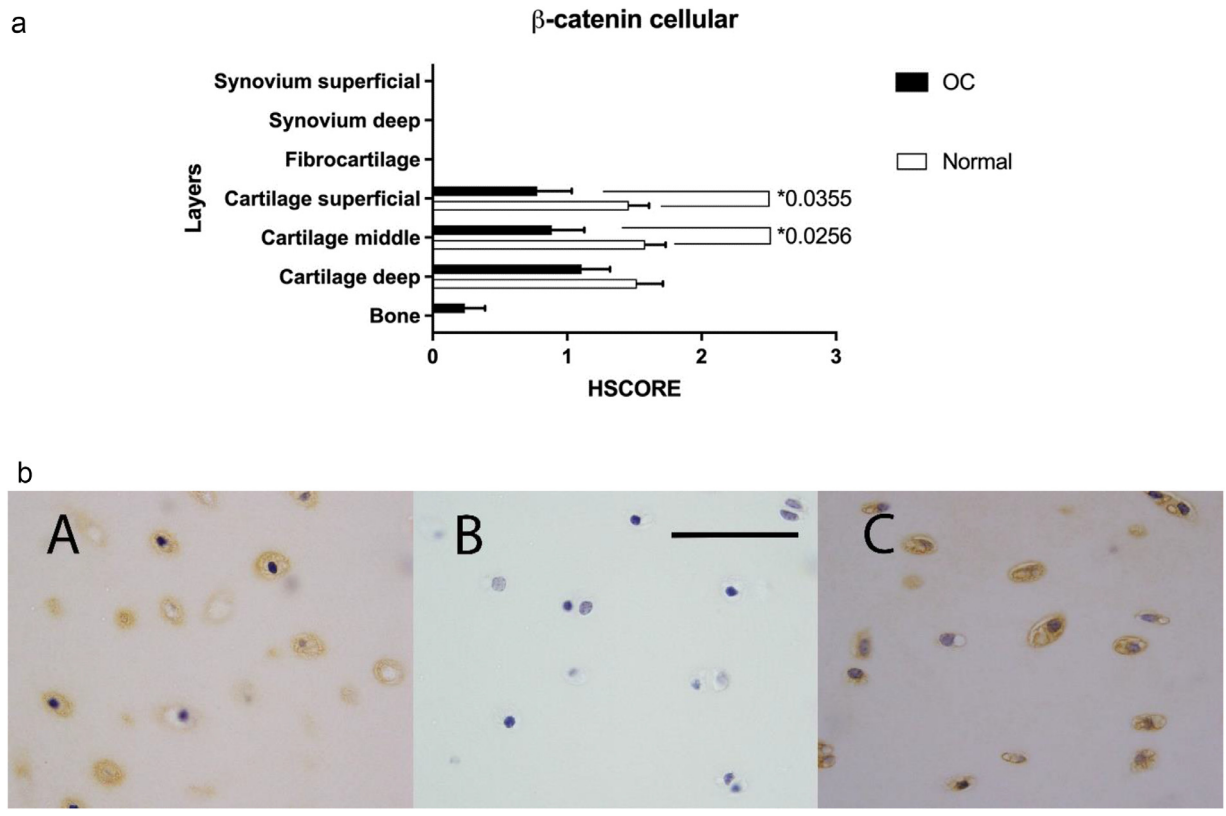

Fig. 6. a: Mean immunohistochemistry HSCOREs +/- SEM for cellular protein expression of β-catenin in MTZ. OC samples had significantly lower cellular expression in the superficial and middle cartilage layer of the MTZ than normal samples. $^{*}P$ values $\leq$.05 displayed above error bars. b: Photomicrographs of middle cartilage layer of MTZ sections following immunohistochemical localization using antibodies directed against β-catenin. (A) OC-affected 5-month old foal showing mild cellular expression of β-catenin (HSCORE = 0.59). (B) Negative control of (A). (C) Normal 4-month old foal having moderate cellular expression of β-catenin (HSCORE = 2.1). (Hematoxylin counterstaining; DAB chromogen; Bar = 50μm; 100x objective).

been most studied in horses for its potential role in osteoarthritis [16,17]. This glycoprotein occupies the outermost superficial layer of the cartilage matrix (lamina splendins) and upper middle cartilage zone [18], providing a chondroprotective effect to articular cartilage. However, it has also been evaluated in foals with osteochondrosis, where no difference was found in lubricin expression between normal and OC cartilage in the central portion of the lateral femoral trochlear ridge [19]. In contrast, our results show decreased lubricin expression in OC middle and deep cartilage layers of the MTZ. More work is needed to define what role lubricin may play in OC at this location.

Interestingly, our findings of increased MMP-3 protein expression in the MTZ deep cartilage and superficial fibrous layer support a potential loss of matrix integrity in OC samples. MMP-3 is part of a metalloproteinase superfamily that governs matrix assembly and degradation [20], digesting cartilage ECM, and activating other degradative enzymes responsible for matrix remodeling [21]. Previous work evaluating MMP-3 immunostaining found a trend for increased protein expression in the superficial and deep layers of OC-affected articular cartilage, with minimal expression along the osteochondral junction in the central portion of the lateral femoral trochlear ridge [7]. Another study evaluating cartilage distribution of MMPs in normal and osteoarthritic cartilage found the highest MMP-3 expression in the superficial zone of articular cartilage in osteoarthritic cartilage [20–22]. Our results partially support the above-mentioned findings, indicating a possible role for dysregulated matrix remodeling in OC. However, further studies evaluating MMP activity in this region are needed to corroborate these findings.

Despite alterations in collagen type IIB, lubricin, and MMP-3 expression in OC samples, no difference was found in toluidine blue intensity scores. Toluidine blue staining enhances visualization of proteoglycans by matrix metachromasia, with the degree of metachromasia approximating the proteoglycan amount. Previous work using toluidine blue found decreased matrix metachromasia in OC affected cartilage compared with normal cartilage from age matched foals [15]. Although the toluidine blue scores for each layer in the MTZ were not significantly different between OC and normal samples in the current study, we did see varying patterns of toluidine blue staining in several OC samples. These differing patterns may indicate a degree of matrix proteoglycan disruption in the MTZ of select animals; however, it does not appear to be a widespread finding.

Contrary to our original hypothesis, canonical Wnt signaling appears to be inhibited in OC-affected MTZ samples. β-Catenin is a transcriptional regulator in the canonical Wnt pathway. In the absence of Wnt signal (Off-state), β-catenin is targeted for degradation. In the presence of Wnt ligand (On-state), β-catenin is translocated to the nucleus via intracellular signaling cascades to initiate transcription of Wnt target genes through various co-factors [23]. Previous work evaluating Wnt/B-catenin signaling in osteochondrosis found no significant differences in cartilage canal, osteochondral junction, or total cartilage immunohistochemistry scores between OC and normal samples for β-catenin [9] despite having significant increased gene expression. Our immunohistochemistry findings, at least in the superficial and middle layer of MTZ cartilage, show decreased β-catenin cellular expression in OC samples. This decrease may indicate lower canonical Wnt

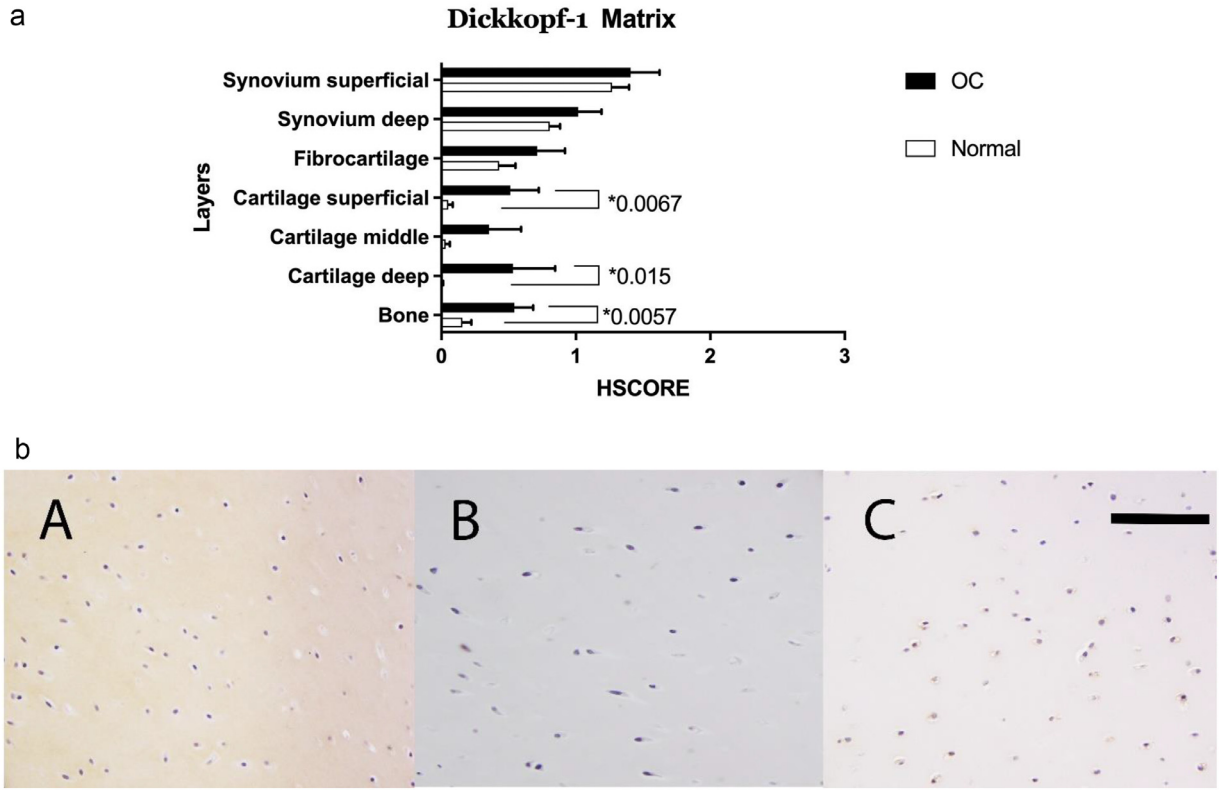

Fig. 7. a: Mean immunohistochemistry HSCOREs +/- SEM for matrix protein expression of Dickkopf-1 in MTZ. Significantly higher matrix expression of Dkk-1 was found in the superficial cartilage, deep cartilage and bone layers in OC samples. $^{*}P$ values $\leq$.05 displayed above error bars. b: Photomicrographs of superficial cartilage layer of MTZ sections following immunohistochemical localization using antibodies directed against Dkk-1. (A) OC-affected 5-month old foal showing mild matrix expression of Dkk-1 (HSCORE = 0.5). (B) Negative control of (A). (C) Normal 4-month old foal having minimal matrix expression of Dkk-1 (HSCORE = 0). (Hematoxylin counterstaining; DAB chromogen; Bar= 100μm; 40x objective).

activity in the MTZ but further work is needed to determine its effect in OC.

Supporting this supposition is the concurrent finding of increased inhibitor (Dkk-1) expression in the superficial and deep layers of MTZ cartilage and bone of OC samples. Dickkopf proteins consist of 4 members (Dkk-[1-4]), with Dkk-1 having a primary role in canonical Wnt signal inhibition [24]. Studies on canonical/non-canonical Wnt pathway in foals with osteochondrosis found no significant difference in immunohistochemistry scores for Dkk-1 between OC and normal articular cartilage in the central portion of the lateral femoral trochlear ridge. However, Dkk-1 immunostaining was noted to be the strongest along the osteochondral junction, with mild to moderate expression along the superficial, middle, and deep cartilage, indicating zonal differences in cartilage expression [9]. In our current study, significantly higher Dkk-1 expression and lower β-catenin expression in the MTZ of OC samples support possible canonical signaling inhibition in this location.

Contrary to our hypotheses, apoptotic and paracrine signaling proteins are not increased in OC-affected MTZ samples. However, our current study does support previous findings of decreased mitochondrial expression of cytochrome C in OC samples [8]. In that previous study, mitochondrial cytochrome C was lower in OC samples of the middle and deep cartilage layers compared to normal controls [8,9]. We similarly find decreased mitochondrial protein expression in OC samples in the middle cartilage layer of the MTZ. Cytochrome C, fundamental to ATP synthesis, is a mitochondrial protein released into the cytosol and subsequently into the extracellular matrix when a cell undergoes apoptosis [9,25]. However, in our current study, we do not concurrently find increased cytochrome C in the matrix. Therefore, it is unclear if translocation of cytochrome C is occurring from the cell to the extracellular environment in the MTZ of OC cartilage or what role it may it may play in OC.

In this study, OC samples were classified as they were originally assessed in a previous study [7]. This classification was based on an earlier published grading scheme [10] that was then grouped into early OC and late OC [7]. Terminology has evolved over the years as the understanding of OC pathogenesis has progressed [26,27]. Some authors have recommended the term, Juvenile Osteochondral Conditions (JOCC) to describe developmental disorders related to immature joints or growth plates, including osteochondrosis [26]. JOCC may be further subdivided into: 1) osteochondral fragmentation of the articular surface (AS-OCF), 2) periarticular osteochondral fragmentation (PA-OCF), or 3) juvenile subchondral bone cyst-like lesions (JSBC) [26]. Based on this terminology, OC samples in our study may be classified as osteochondral fragmentation of the articular surface, as shearing of the osteochondral junction was evident in most cases. OC may be further characterized using the terms latens, manifesta, and dissecans to describe the stage of disease [26,27]. Denoix et al. [26] defines OC latens as not detectable by routine diagnostic techniques, OC manifesta as detectable on standard radiographs, and OC dissecans as featuring a loose fragment. Olstad et al. [27] further refines the definition of OC latens to be areas of ischemic necrosis within growth cartilage, while OC manifesta is chondronecrosis within the ossification front. Based on these criteria, 2 of our foals may be classified as latens and 5 as manifesta lesions.

Limitations to this study include a limited sample size, issues of correlating protein expression to protein function or activity,

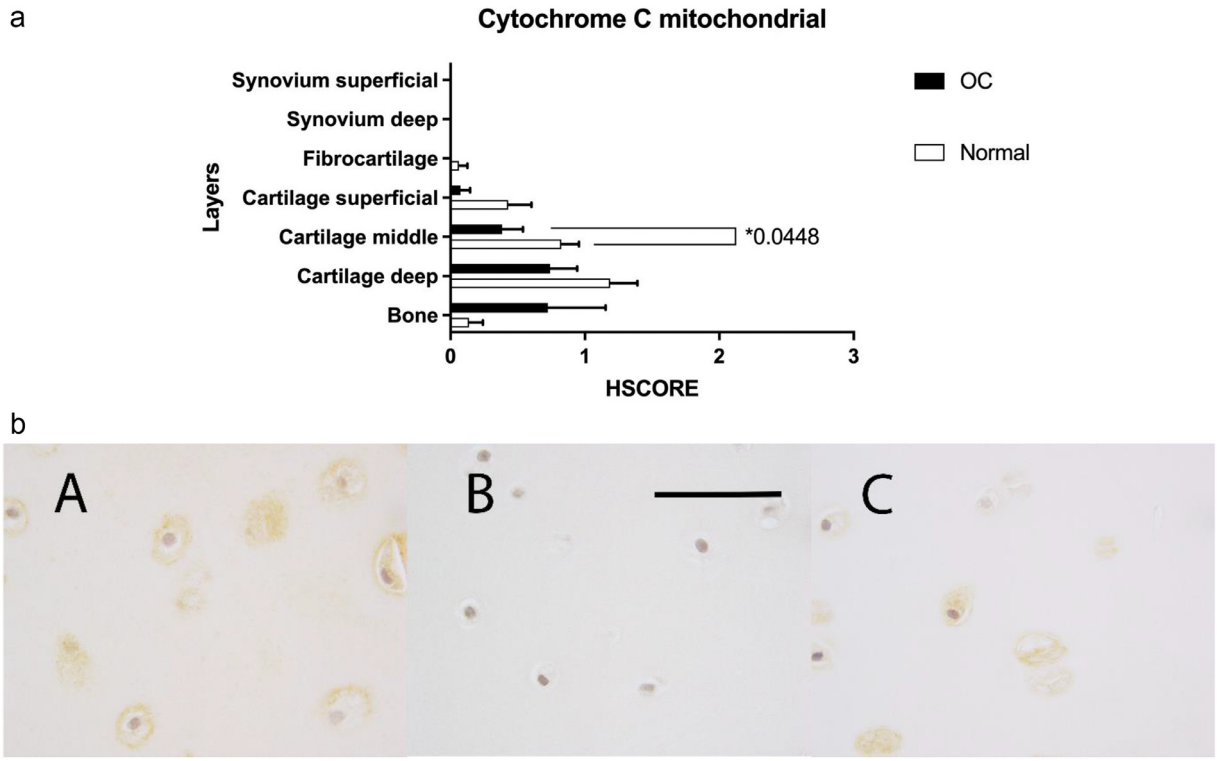

Fig. 8. a: Mean immunohistochemistry HSCOREs +/- SEM for mitochondrial protein expression of cytochrome C in MTZ. Mitochondrial expression in middle cartilage layer of OC affected samples was significantly decreased compared to normal controls. $^{*}P$ values $\leq$.05 displayed above error bars. b: Photomicrographs of middle cartilage layer of MTZ sections following immunohistochemical localization using antibodies directed against cytochrome C. (A) OC-affected 5-month old foal showing mild cellular expression of cytochrome C (HSCORE = 0.75). (B) Negative control of (A). (C) Normal 4-month old foal having mild mitochondrial expression of cytochrome C (HSCORE = 0.78). (Hematoxylin counterstaining; DAB chromogen; Bar = 50μm; 100x objective).

and the ongoing challenge of determining cause and effect when evaluating only one timepoint in a dynamic disease. In addition, semi-quantitative assessments of immunohistochemistry protein expression were performed using manual scoring methods rather than software-based techniques, which increases the subjectivity of analysis.

5. Conclusion

In conclusion, our results demonstrate differential protein expression in the marginal transitional zone from the lateral femoral trochlear ridge of foals affected by osteochondrosis. Alterations in protein expression of OC-affected foals mainly involve components of extracellular matrix homeostasis and canonical Wnt signaling. Decreased expression of collagen type IIB and lubricin and increased expression of MMP-3 indicate potentially abnormal cartilage matrix in OC-affected MTZ samples. Additionally, canonical Wnt signaling appears to be inhibited in OC-affected MTZ samples, based on increased Dkk-1 and decreased β-catenin protein expression. These results differ from previously reported expression changes in the central portion of LTR articular cartilage of OC affected foals, thus supporting zonal differences in protein expression. Future work should further examine the role of MTZ in the pathogenesis of OC, focusing on predilection joints and sites for osteochondrosis. In addition, temporal expression patterns of the MTZ in normal foals are needed to determine zonal differences related to age.

Author Credit statement

Dr. Semevolos is responsible for conceptualization and design of the study. Both authors were responsible for data acquisition, analysis and interpretation. Dr. Marchant drafted the original article and both authors revised it critically and gave final approval.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2022.104055.

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