Relationship between serum biomarkers of cartilage and bone metabolism and joint injury in young Thoroughbred racehorses in training

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OBJECTIVE
To compare serum concentrations of biomarkers of cartilage and bone metabolism between racehorses with a carpal or metacarpophalangeal or metatarsophalangeal (ie, fetlock) joint injury and matched uninjured control horses, determine changes in biomarker concentrations following joint injury, and establish the biomarkers' diagnostic test performance.

ANIMALS
50 Thoroughbred racehorses with a carpal or fetlock joint injury and 50 matched uninjured horses (control horses).

PROCEDURES
Serum concentrations of 2 cartilage synthesis biomarkers (carboxy-terminal propeptide of type II collagen [CPII] and chondroitin sulfate epitope 846 [CS846]), 2 cartilage degradation biomarkers (neoepitope generated by collagenase cleavage of type II collagen [C2C] and cross-linked carboxy-terminal telopeptide fragments of type II collagen [CTX-II]), and serum activity of a bone formation marker (bone-specific alkaline phosphatase [BAP]) were measured around the time of injury diagnosis and monthly thereafter for as long as possible.

RESULTS
Injured horses as a group and horses specifically with fetlock joint injuries had significantly lower serum CPII concentrations and significantly higher serum BAP activities than matched control horses. Concentrations of CTX-II were decreased between 2 and 4 months following joint injury. Measurement of CPII concentration at baseline could distinguish between injured horses and control horses with a sensitivity of 82% and specificity of 50%.

CONCLUSIONS AND CLINICAL RELEVANCE
Although significant differences in specific biomarker concentrations between horses with carpal and fetlock joint injuries and matched control horses were identified, there was no convincing evidence of the suitability of these biomarkers as diagnostic or prognostic tools in a clinical setting. (*Am J Vet Res* 2015;76:679–687)

Carpal and metacarpophalangeal and metatarsophalangeal (ie, fetlock) joint injuries are common in Thoroughbred racehorses but can be difficult to detect clinically during the early stages of disease. As a result, joint disease often progresses to an advanced state before it is detected.[1–7] Identification of more accurate and affordable methods of early diagnosis may limit disease progression prior to detection and allow for earlier and more effective treatment.

A substantial amount of research has focused on determining whether measuring serum or synovial fluid concentrations of biomarkers released during cartilage and bone synthesis and degradation would be useful in identifying animals with joint damage. In the equine field, such studies have involved horses with experimentally induced osteoarthritis,[8,9] horses with osteochondral fragmentation,[10–13] and foals with osteochondrosis.[14–17] Result suggest that synovial fluid and serum concentrations of markers related to syn-

ABBREVIATIONS

AUC	Area under the curve
BAP	Bone-specific alkaline phosphatase
C2C	Neoepitope generated by collagenase cleavage of type II collagen
CI	Confidence interval
CPII	Carboxy-terminal propeptide of type II collagen
CS846	Chondroitin sulphate epitope 846
CTX-II	Cross-linked carboxy-terminal telopeptide fragments of type II collagen
ROC	Receiver operating characteristic

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thesis of type II collagen (CPII) and aggrecan (CS846) are increased in horses with early osteoarthritis[9,13] and that synovial fluid concentrations of markers of type II collagen degradation are increased in horses with osteochondral injury. Synovial fluid concentrations of C2C (the neoepitope present at the carboxy-terminus of the three-quarter length product formed by collagenase cleavage of type II collagen fibrils) have been shown to positively correlate with severity of osteochondral injury in Thoroughbred racehorses,[11] whereas concentrations of another marker of type II collagen degradation, CTX-II (an epitope found in cross-linked carboxy-terminal telopeptide fragments that are formed during type II collagen degradation), have been shown to be increased in both synovial fluid and serum from dogs and in serum from rats with experimentally induced joint injuries.[18,19] A recent study[10] also found significantly higher CTX-II concentrations in synovial fluid from the carpal and fetlock joints of Thoroughbreds with osteochondral injuries.

There is also evidence that changes in bone structure associated with joint disease result in alterations in bone biomarkers, particularly BAP activity. Bone-specific alkaline phosphatase activity has been shown to be higher in the subchondral bone of warmblood horses with osteochondrosis than in the subchondral bone of horses with normal joints,[20] and BAP activity has been found to be significantly higher in synovial fluid from equine joints with gross articular cartilage defects than in healthy joints.[21]

Most equine biomarker studies have involved animals with advanced disease or experimentally induced joint damage or have been performed in a research setting where it has been possible to also collect synovial fluid. However, to evaluate the usefulness of biomarkers as a diagnostic tool in the field, observational studies of horses with naturally occurring joint injury, taking into account clinical signs and severity, are required.

The purpose of the study reported here was to explore possible relationships between serum concentrations of various biomarkers of cartilage and bone metabolism and exercise-induced joint damage in Thoroughbred racehorses in training. Specifically, the objectives were to compare serum concentrations of biomarkers of cartilage and bone metabolism between horses with a carpal or fetlock joint injury and healthy control horses matched for age, sex, and trainer; to evaluate changes in serum biomarker concentrations over time in injured horses; and to determine whether serum biomarker concentrations could be used as a diagnostic test to distinguish between injured and healthy control horses. We hypothesized that injuries not involving fracture or fragmentation would be associated with increased serum concentrations of cartilage synthesis markers; that injuries involving fracture or fragmentation would be associated with increased serum concentrations of markers of cartilage degradation and increased serum activity of a marker of bone formation; that following diagnosis of an injury, serum concentrations of markers of cartilage synthesis and bone formation would increase over time but serum concentrations of markers of cartilage degradation would decrease over time; and that serum biomarker concentrations measured at the time of initial diagnosis could be used to distinguish between injured and uninjured horses.

Materials and Methods

Study design

The study was designed as a nested case-control study. Trainers taking part in a large prospective cohort study[6] of joint injuries in 2- and 3-year-old Thoroughbreds in training in England were invited to participate. In the cohort study,[6] horses were followed up for as long as 2 years after they entered training as yearlings, and information was recorded concerning any joint injuries diagnosed by the trainer's veterinarian.

Horses were eligible for inclusion in the case-control portion of the study if they had a carpal or fetlock joint injury diagnosed by a veterinarian and their trainer agreed to participate. Training yards were visited on a monthly basis to collect data, and blood samples were collected from eligible case horses as soon as possible after the diagnosis of an injury. In liaison with participating trainers and veterinarians, a single randomly selected control horse without a known history of musculoskeletal injury was matched with each case horse on the basis of trainer, sex, and age, and a blood sample was obtained from the control horse at the same time the initial blood sample was obtained from the case horse. Subsequently, blood samples were collected at monthly intervals from each pair of case-control horses until the case or control horse was no longer available or the control horse became injured. The study protocol was approved by the Ethics and Welfare Committee of the Royal Veterinary College.

Sample size calculations

Sample sizes required to detect significant differences in mean serum biomarker concentrations at a single point in time between matched case and control horses were calculated on the basis of assumptions derived from existing literature.[13,18] The largest minimum sample size requirement was for serum CPII concentration, for which it was calculated that 60 case-control pairs would be needed to identify a significant difference in mean concentrations between case and control horses at a single point in time with 80% power and a significance level of $\alpha = 0.05$. Sample size requirements for the other markers were much lower, with only 5 pairs required to detect a significant difference in mean CS846 concentrations between case and control horses and 30 pairs required to detect a significant difference in mean C2C concentrations between case and control horses.

Injury classification

Injured horses were classified as part of the larger cohort study,[6] with 4 injury categories defined: localiza-

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tion to a carpal or fetlock joint on the basis of results of clinical examination or diagnostic analgesia with no diagnostic imaging performed (category 1), localization to a carpal or fetlock joint with no abnormalities detected on diagnostic images (category 2), an abnormality of the subchondral bone or articular margins identified on diagnostic images (category 3), and fracture or fragmentation identified on diagnostic images (category 4).

Blood sample collection

Blood samples were collected by means of jugular venipuncture into plain tubes and allowed to clot. Samples were then centrifuged, and the serum was separated and stored at –80°C until assayed. Samples for each case-control pair were always collected at the same time and analyzed together in the same assay at the end of the study.

Measurement of serum biomarker concentrations and serum BAP activity

Serum concentrations of CS846,[a] CPII,[a] C2C,[a] and CTX-II[b] were measured with commercially available ELISAs validated for use in horses.[16,22–24] Serum BAP activity was determined by use of wheat germ lectin, which selectively binds and precipitates the BAP isoenzyme, according to a method previously validated for use in equine serum in the investigators' laboratory.[25] All samples were analyzed in duplicate at dilutions appropriate for the individual assay.

Statistical analysis

Linear mixed model analysis was used to compare serum C2C, CPII, CS846, and CTX-II concentrations and serum BAP activity between case and matched control horses. Specifically, values for matched control horses were compared with values for all case horses, case horses with carpal joint injury, case horses with fetlock joint injury, case horses with category 1 or 2 injury, case horses with category 3 injury, and case horses with category 4 injury. Additionally, separate analyses were conducted for horses with category 3 and 4 carpal and fetlock joint injuries. Case horses with category 1 and 2 injuries were combined for analyses because of low numbers in each of these categories.

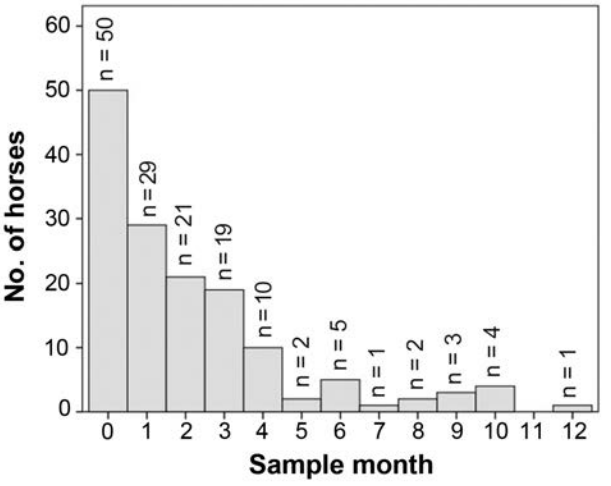

Figure 1—Histogram of sample collection data for a study involving 50 Thoroughbred racehorses with a carpal or metacarpophalangeal or metatarsophalangeal (ie, fetlock) joint injury and 50 control horses without known musculoskeletal injury matched for age, sex, and trainer. Blood samples were collected from injured horses as soon as possible after the diagnosis of an injury (month 0) and from matched control horses at the same time, with subsequent blood samples collected at monthly intervals from each pair of case-control horses until the case or control horse was no longer available or the control horse became injured.

Biomarker measurements were modeled as continuous dependent variables, and case or control status was modeled as a binary fixed variable. The outcome variables CTX-II concentration and BAP activity were log transformed to achieve an approximately normal distribution prior to analysis. To take into account clustering of samples, matched pair and horse were included as random effects and sampling month was specified as the repeated-measures variable (sampling month was based on the sequential order of monthly samples rather than calendar month). The linear mixed model approach allowed inclusion of all available data in a single statistical model to assess differences in mean biomarker concentrations between case and control horses while accounting for repeated measurements and the matched pairs study design.

Analyses were performed with standard statistical software[c] with a first-order autoregressive correlation structure specified to account for correlation between repeated samples taken from the same horses over time. Results from these models are reported as the adjusted means with 95% CIs. Values of $P \leq 0.05$ were considered significant.

To assess the ability of individual biomarkers to distinguish between injured and uninjured horses at the time of first diagnosis, ROC curve analysis was conducted with standard statistical software,[d] with data for the first sample obtained from each horse being used. Separate analyses were conducted for each category of injury and by joint affected. When sufficient data were available, separate analyses were performed for each category of injury by joint affected. Parameters estimated for each biomarker included AUC, which provided an overall measurement of the ability of the biomarker to distinguish between injured and uninjured horses, and sensitivity and specificity. In addition, optimal cutoffs for distinguishing between injured and uninjured horses were determined for each biomarker.

Results

Fifty case-control pairs of horses from 7 training yards were included during the 13-month study **(Figure 1)**. Median follow-up time was 3 months, with number of blood samples collected from each horse ranging from 1 to 7. There were 25 horses with a carpal joint injury, 24 horses with a fetlock joint injury, and 1 horse with both carpal and fetlock joint injuries. Seven horses had category 1 injuries (3 with carpal joint injuries, 3 with

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fetlock joint injuries, and 1 with carpal and fetlock joint injuries), 4 had category 2 injuries (all with fetlock joint injuries), 20 had category 3 injuries (12 with carpal joint injuries and 8 with fetlock joint injuries), and 19 had category 4 injuries (10 with carpal joint injuries and 9 with fetlock joint injuries). Descriptive statistics for biomarker concentrations in the first blood samples obtained from each case and control horse were derived **(Table 1)**.

Differences in biomarker concentrations between case and control horses

Significant differences in biomarker concentrations between case and matched control horses were summarized **(Table 2)**. When results for all blood samples from all case and control horses were considered, serum CPII concentration was significantly lower and serum BAP activity was significantly higher in case horses than in control horses, but no differences in serum CS846, C2C, or CTX-II concentrations were found between case and control horses.

For all 5 biomarkers, no significant differences in biomarker concentrations were found between the 26 case horses with carpal joint injuries and the 26 matched control horses **(Figure 2)** or between the 11 case horses with category 1 or 2 injuries and the 11 matched control horses. However, serum CPII concen-

Table 1—Serum concentrations of biomarkers of cartilage and bone metabolism in samples obtained within 1 month after diagnosis from 50 Thoroughbred racehorses with a carpal or metacarpophalangeal or metatarsophalangeal (ie, fetlock) joint injury and at the same time from 50 control horses without known musculoskeletal injury matched for age, sex, and trainer.

	Injured horses			Control horses		
Biomarker	**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**
CS846 (ng/mL)	224.8	211.5	111.6–447.5	227.5	214.6	89.5–633.0
CPII (ng/mL)	1,236.0	1,250.7*	519.9–2,125.2	1,458.6	1,514.4	557.0–3,181.2
CTX-II (pg/mL)	158.6	139.5	34.5–468.2	143.5	126.0	48.6–395.2
C2C (ng/mL)	200.7	204.4	105.7–310.8	218.6	219.1	100.4–380.2
BAP (U/L)	25.8	25.7	6.3–53.3	24.5	23.6	9.9–54.5

*Median CPII concentration was significantly ($P < 0.001$) lower at baseline in injured horses than in matched control horses.

Table 2—Summary of significant ($P \leq 0.05$) differences in serum biomarker concentrations between injured horses and matched control horses.

	Injured horses		Control horses		
Group and biomarker	**Mean**	**95% CI**	**Mean**	**95% CI**	***P* value**
Carpal and fetlock joint injuries combined (n = 50)					
CPII (ng/mL)	1,586	1,415–1,758	1,771	1,600–1,943	0.002
BAP (U/L)	20.9	18.6–23.4	19.0	17.0–21.4	0.03
Fetlock joint injuries (n = 25)					
CPII (ng/mL)	1,410	1,189–1,630	1,625	1,405–1,846	0.02
BAP (U/L)	22.4	19.5–26.3	19.9	17.4–23.4	0.02
Category 3 injuries (n = 20)					
CPII (ng/mL)	1,690	1,478–1,903	1,902	1,689–2,114	0.005
CS846 (ng/mL)	205.7	175.9–235.6	242.0	212.2–271.8	0.04
Category 3 carpal joint injuries (n = 12)					
CS846 (ng/mL)	203.9	170.2–237.5	241.0	207.4–274.6	0.05
Category 4 injuries (n = 19)					
BAP (U/L)	21.9	18.2–26.9	19.5	15.9–23.4	0.05
CTX-II (pg/mL)	92.0	70.8–109.6	69.2	55.3–85.1	0.01
Category 4 fetlock joint injuries (n = 9)					
BAP (U/L)	24.0	18.2–31.6	20.9	15.5–27.5	0.05
CTX-II (pg/mL)	99.4	71.0–139.2	64.7	46.2–90.6	0.02

Blood samples were collected from injured horses as soon as possible after the diagnosis of an injury (typically, within 1 month) and from matched control horses at the same time. Subsequently, blood samples were collected at monthly intervals from each pair of case-control horses until the case or control horse was no longer available or the control horse became injured. Differences were determined by means of a linear mixed model analysis taking account of repeated measurements and the matched pairs study design.

Injured horses were classified as part of a larger cohort study,[6] with 4 injury categories defined: localization to a carpal or fetlock joint on the basis of results of clinical examination or diagnostic analgesia with no diagnostic imaging performed (category 1), localization to a carpal or fetlock joint with no abnormalities detected on diagnostic images (category 2), an abnormality of the subchondral bone or articular margins identified on diagnostic images (category 3), and fracture or fragmentation identified on diagnostic images (category 4).

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tration was significantly lower and serum BAP activity was significantly higher in the 25 case horses with fetlock injuries than in the 25 matched control horses. Also, serum CPII and CS846 concentrations were significantly lower in the 20 case horses with category 3 injuries than in the 20 matched control horses. Both serum BAP activity and serum CTX-II concentration were significantly higher in the 19 case horses with category 4 injuries than in the 19 matched control horses.

Finally, when horses with category 3 carpal joint injuries and horses with category 3 fetlock joint injuries were analyzed separately, serum CS846 concentration was significantly lower in the 12 case horses with category 3 carpal joint injuries than in the 12 matched control horses, but biomarker concentrations did not differ between the 8 case horses with category 3 fetlock joint injuries and the 8 matched control horses. When horses with category 4 carpal joint injuries and horses with category 4 fetlock joint injuries were analyzed separately, biomarker concentrations did not differ between the 10 case horses with category 4 carpal joint injuries and the 10 matched control horses, but serum BAP activity and serum CTX-II concentration were both significantly higher in the 9 horses with category 4 fetlock joint injuries than in the 9 matched control horses.

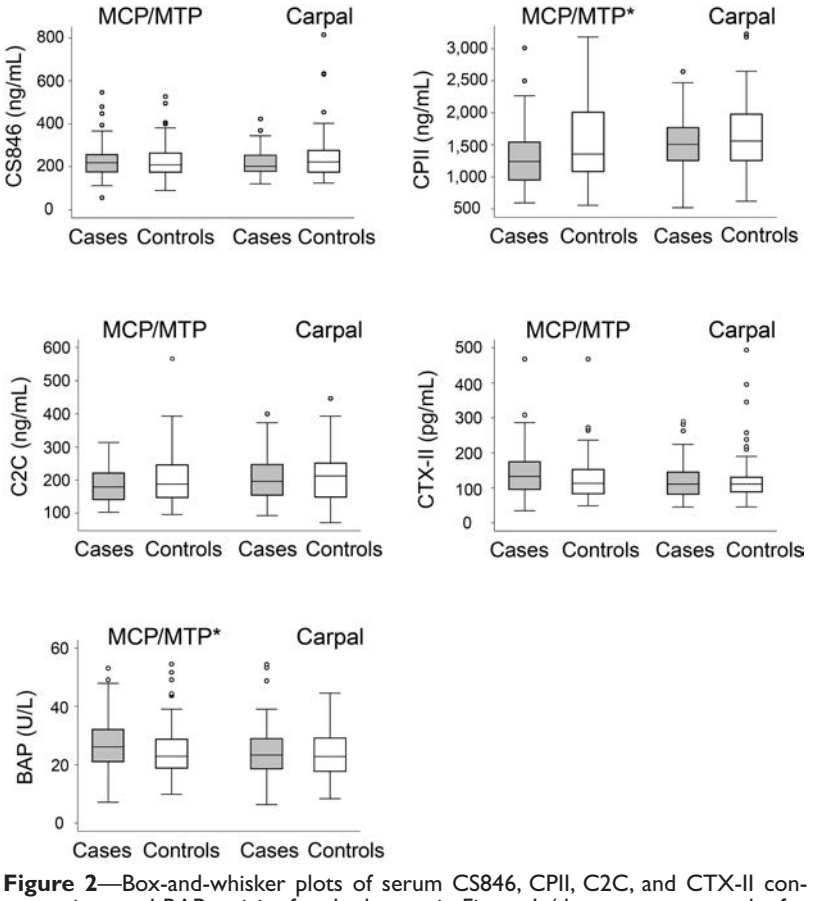

Figure 2—Box-and-whisker plots of serum CS846, CPII, C2C, and CTX-II concentrations and BAP activity for the horses in Figure 1 (data represent results for all samples collected during the study) with a carpal joint injury (n = 26) or fetlock joint injury (MCP/MTP; n = 25). For each plot, the box represents the interquartile range, the horizontal line within the box represents the median, the bars represent the 5th and 95th percentiles, and the circles represent outliers. *Concentration or activity differed significantly ($P = 0.02$ for both biomarkers) between injured horses and matched control horses as determined by use of linear mixed model analysis taking account of repeated measurements and the matched pairs study design.

Changes in biomarker concentrations over time in injured horses

In horses with carpal joint injuries, serum CPII concentration was significantly ($P = 0.02$) lower at the time of the first blood sample collection (mean, 1,431 ng/mL; 95% CI, 1,300 to 1,563 ng/mL; n = 26) than it was 3 months later (mean, 1,847 ng/mL; 95% CI, 1,637 to 2,057 ng/mL; 9). Overall, however, there was no evidence of an increase in concentration over time.

When results for all blood samples from all case horses were considered, serum CTX-II concentrations were highest at the time of the first blood sample collection (mean, 158.6 pg/mL; 95% CI, 132.9 to 184.2 pg/mL; n = 50) and were significantly ($P < 0.001$ for all) lower, compared with initial values, 2 months (mean, 102.3 pg/mL [95% CI, 93.3 to 114.8 pg/mL]; n = 21), 3 months (mean, 100.0 pg/mL [95% CI, 89.1 to 112.2 pg/mL]; 19), and 4 months (mean, 83.2 pg/mL [95% CI, 72.4 to 97.7 pg/mL]; 10) later **(Figure 3)**. Similar results were obtained when data for horses with carpal joint and fetlock joint injuries were analyzed separately.

For horses with category 3 injuries, serum CTX-II concentrations were highest at the time of the first blood sample collection (mean, 143.0 pg/mL; 95% CI, 125.3 to 163.1 pg/mL; n = 20) and were significantly lower, compared with initial values, 4 months (mean, 76.5 pg/mL [95% CI, 58.5 to 99.9 pg/mL]; $P = 0.001$; n = 3) and 5 months (mean, 63.1 pg/mL [95% CI, 40.9 to 97.5 pg/mL]; $P = 0.016$; n = 2) later. Similar results were obtained for horses with category 4 injuries, except that serum CTX-II concentrations were significantly ($P = 0.017$) lower 2 months later (mean, 89.5 pg/mL [95% CI, 75.4 to 106.3 pg/mL]; n = 11), compared with initial values (mean, 122.3 pg/mL [95% CI, 105.2 to 142.2 pg/mL]; 19).

For horses with carpal injuries, serum BAP activity was significantly ($P = 0.047$) lower 4 months later (mean, 17.4 U/L [95% CI, 14.5 to 20.9 U/L]; n = 4), compared with initial values (mean, 22.9 U/L [95% CI, 20.9 to 25.7 U/L]; 26). However, no other significant differences were found.

ROC curve analysis

When all horses were considered, ROC curve analysis indicated that ini-

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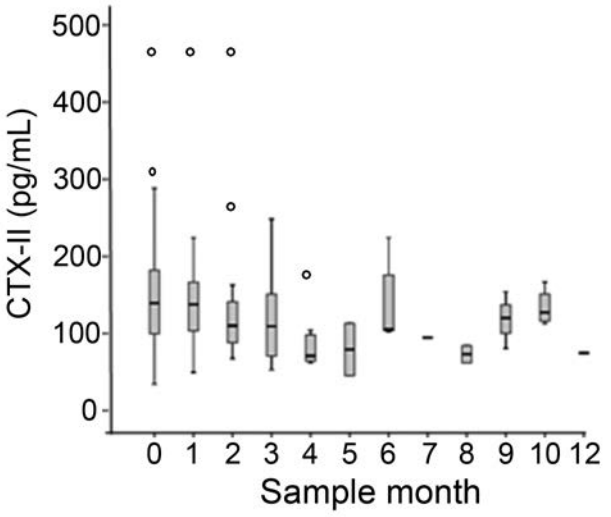

Figure 3—Box-and-whisker plot of serum CTX-II concentrations over time for the case horses in Figure 1. Concentrations were significantly ($P < 0.001$) lower 2, 3, and 4 months after initial samples (month 0) were obtained. ***See*** Figures 1 and 2 for key.

tial serum CPII concentration could be used to distinguish between injured horses and uninjured control horses (AUC, 0.64 [95% CI, 0.53 to 0.72]; $P = 0.017$), with a sensitivity of 82% (95% CI, 68.6% to 91.4%) and specificity of 50% (95% CI, 35.5% to 64.5%) at the optimal cutoff of ≤ 1,509 ng/mL. At the same cutoff, initial serum CPII concentration could also be used to distinguish between horses with carpal injuries and uninjured control horses (AUC, 0.68 [95% CI, 0.53 to 0.80]; $P = 0.024$), with a sensitivity of 81% (95% CI, 60.8% to 93.4%) and specificity of 62% (95% CI, 40.6% to 79.8%).

Receiver operating characteristic curve analysis indicated that initial serum CTX-II concentration could not be used to distinguish horses with category 4 injuries from uninjured matched control horses ($P = 0.094$). However, initial serum CTX-II concentration could be used to distinguish horses with category 4 fetlock joint injuries from uninjured matched control horses (AUC, 0.79 [95% CI, 0.52 to 0.95]; $P = 0.012$), with a sensitivity of 100% (95% CI, 63.1% to 100%) and specificity of 50% (95% CI, 15.7% to 84.3%) at the optimal cutoff of > 73.7 pg/mL.

Discussion

In the present study, we identified significant differences in serum concentrations of some biomarkers of cartilage and bone metabolism between horses with carpal and fetlock joint injuries and uninjured control horses. However, the specific differences depended on the site and severity of injury, and there was no convincing evidence that any of the biomarkers evaluated could be used for diagnostic or prognostic purposes in a clinical setting. Interestingly, serum CS846 and CPII concentrations were both significantly lower in horses with subchondral bone or articular margin lesions (ie, category 3 injuries) than in matched control horses and serum CTX-II concentration and BAP activity were both significantly higher in horses with fracture or fragmentation (ie, category 4 injuries) than in matched control horses, supporting the use of this classification system for joint injuries in a field setting. However, serum biomarker concentrations did not differ between control horses and horses with seemingly mild injuries in which diagnostic imaging was not performed or abnormalities were not detected radiographically (ie, category 1 and 2 injuries). Given that biomarkers would be most beneficial if they could be used to detect joint disease in its early stages, this was somewhat disappointing. It could be that disease in these horses with mild injuries was not sufficiently severe to result in detectable changes. Alternatively, the low number of horses with mild injuries may have resulted in low statistical power to detect significant differences. Further studies including larger numbers of horses with early-stage joint disease from which blood samples were obtained at the time of clinical diagnosis of a joint-related lameness may be warranted.

Our finding that serum concentrations of biomarkers reflective of cartilage synthesis (CS846 and CPII) were decreased in horses with category 3 injuries was contrary to the hypothesis that there would be an increase because of synthesis of type II collagen and aggrecan in horses with these types of injuries as the cartilage attempted to initiate a repair process. It has to be recognized that biomarker concentrations in serum may not reflect disease processes occurring in a single joint.[26] However, concentrations of both CS846 and CPII have been shown to be significantly increased in serum and synovial fluid from horses with early experimentally induced osteoarthritis,[9] whereas another study[13] found that horses classified as having less serious osteochondral fragmentation had significantly increased serum concentrations of CS846 and CPII, compared with horses graded as normal. In contrast, horses in the same study[13] that had more serious osteochondral injuries had CS846 and CPII concentrations that were not significantly different from concentrations in horses graded as normal, possibly suggesting that there is increased synthesis of type II collagen and aggrecan only during the early stages of osteoarthritis as cartilage attempts to initiate a repair process. However, this assumes that less serious injuries in that study[13] reflected the earlier stages of disease progression. In contrast, a recent field study[27] of 2- and 3-year-old racing Thoroughbreds that followed horses in the period leading up to a diagnosis of intra-articular fragmentation found serum CS846 concentrations to be significantly lower than in the control population over the study period as a whole and specifically at 6 months prior to injury. Although the reasons for the differences among these studies are unclear, the most likely explanation is that they reflect differences in the stage of disease progression at the time of sample collection. Thus, it seems possible that for horses in the present study with category 3

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injuries, the joint disease had progressed by the time of blood sample collection to the point that type II collagen and aggrecan synthesis was impaired, but not to the point that cartilage degradation or subchondral bone involvement had become sufficiently severe to cause an obvious change in concentrations of the markers related to cartilage degradation and bone formation. It is notable that serum CTX-II concentrations were significantly decreased, compared with initial concentrations, at 4 and 5 months in horses with category 3 injuries, which may suggest a decrease in cartilage degradation consistent with a repair or attempted repair process in the months following the injury.

The increased serum CTX-II concentration and BAP activity in horses with category 4 injuries, compared with values for control horses, appeared to support the hypothesis that there was substantial cartilage degradation and bone involvement with these injuries. For CTX-II concentration, fetlock joint injuries rather than carpal joint injuries largely accounted for this finding. Granted, few studies have evaluated serum CTX-II concentrations in horses; however, our results are in contrast to those of a recent study[10] that found no difference in serum CTX-II concentrations between horses with osteochondral fetlock joint injuries and healthy adult horses of a similar age. That study[10] included horses from a wider age range than did the present study, possibly reflecting differences in cartilage repair with increasing age. Our results are consistent with results of studies[28,29] of human patients that found significant increases in CTX-II concentration in patients with radiographic evidence of osteoarthritis. Substantially increased serum CTX-II concentrations have also been found in dogs and rats with experimentally induced joint injuries.[18,19] A previous study[26] suggested that circulating CTX-II may originate from calcified cartilage in the tidemark region or even bone. The release of CTX-II would therefore be expected to be greater with increased severity of injury, whereby calcified cartilage or bone was also affected. This may explain why serum CTX-II concentration was significantly increased, compared with the concentration in control horses, for horses with category 4 injuries but not category 3 injuries in the present study. Similarly, higher serum BAP activity in horses with category 4 injuries could have been due to the fact that these injuries involved fracture and fragmentation, resulting in remodelling of subchondral bone in response to the injury or altered mechanical loading. Our results would be consistent with those of a previous study[21] that reported a positive correlation between degree of cartilage damage and synovial fluid BAP activity in Thoroughbred racehorses. In the present study, there were no significant differences in serum CS846 and CPII concentrations between horses with category 4 injuries and uninjured control horses, which is consistent with results of an earlier study[13] in which serum CS846 and CPII concentrations in horses with the most serious osteochondral injuries were not different from concentrations in horses graded as normal. Overall, our data support our original hypothesis that injuries involving fracture and fragmentation would be associated with increased serum concentrations of markers of cartilage degradation and bone formation.

When data were analyzed for horses grouped on the basis of injury location (ie, carpal vs fetlock joint), serum CPII concentration was significantly lower and serum BAP activity was significantly higher in horses with fetlock injuries, compared with values for control horses, but biomarker concentrations in horses with carpal injuries did not differ from concentrations in control horses. It is unclear why significant differences were only found for horses with fetlock joint injuries, although it is possible that disease progression with fetlock joint injuries differs from that for carpal joint injuries. Fetlock joints have a higher range of motion than do carpal joints[30] and are susceptible to joint damage caused by overextension, particularly on the dorsal surface of the distal end of the third metacarpal bone where it articulates with the proximal phalanx.[31] In a recent study[10] of joint-dependent differences in cartilage biomarkers, it was suggested that joint-dependent differences may be attributable to different mechanisms of regulation between joints, which would be consistent with results from a study[32] of humans that found the response to cytokines differed between knee and ankle cartilage.

The second objective of the present study was to identify changes in biomarker concentrations over time, and the most consistent findings in this regard related to serum CTX-II concentration. Concentrations of this marker in horses with joint injuries were highest at the time of initial blood sample collection and were significantly decreased between 2 and 4 months later. This was the case for all injured horses in the present study and for horses with carpal joint injuries, horses with fetlock joint injuries, horses with category 3 injuries, and horses with category 4 injuries. These results may reflect a reduction in degradation of hyaline cartilage following injury diagnosis and institution of treatment (both medical treatment and alterations in exercise patterns). However, treatment information was not included in the present study. Also, these findings were based on small numbers of samples, which may explain why there was no continuing decrease in CTX-II concentration over the entire study period. Although we hypothesized that cartilage synthesis would increase in the period following injury diagnosis, there was no evidence of a clear and consistent increase in relevant biomarker concentrations, other than CPII concentration being significantly higher 3 months after carpal injury diagnosis, compared with the initial concentration.

The third objective of the present study was to use ROC curve analysis to determine the diagnostic test performance of each biomarker in distinguishing between injured and uninjured horses. The only biomarker that could be used to distinguish between horses with any type of joint injury and uninjured control horses was CPII, with an optimal concentra-

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tion cutoff of ≤ 1,509 ng/mL This is consistent with the finding that CPII concentration was significantly lower in horses with any type of joint injury than in uninjured horses over the course of the study as a whole. Although the diagnostic test sensitivity for CPII concentration was good (82%), test specificity was low (50%), which would result in a large number of horses without joint disease being incorrectly identified as injured (ie, false-positive results). At the optimal cutoff of > 73.7 pg/mL, initial serum CTX-II concentration had 100% sensitivity in distinguishing horses with category 4 fetlock joint injury from uninjured horses, but again specificity was low (50%). One would also question the usefulness of a diagnostic biomarker for identifying horses with fracture or fragmentation, given that other diagnostic techniques can easily detect such injuries and provide more information than a biomarker would.

In using ROC curve analysis to evaluate the diagnostic test performance of biomarkers, we acknowledge that the matched nature of the study design may have resulted in biases that could have either under- or overestimated the predictive performance of the biomarker, depending on its strength of association with matching covariates (ie, age, sex, and trainer).[33] Modified statistical methods to correct for potential biases resulting from a matched study design in the evaluation of diagnostic test performance are available,[33] but these were considered beyond the scope of the present study.

On the basis of the findings reported here, our original hypothesis that the biomarkers studied could be used to distinguish between horses with and without joint injury at around the time of diagnosis was only supported for CPII and, in specific cases, for CTX-II. However, the low specificity of both these markers would make their usefulness in a clinical setting questionable.

Potential confounding variables have to be considered when interpreting the findings of this study. Although injured horses and uninjured control horses were matched by age, sex, and trainer, they were not matched for exercise intensity, which has been shown to affect cartilage and bone biomarker measurements[9,24,34,35] as well as injury risk.[36] In addition, articular cartilage constitutes a minority of the cartilage in the body[37] and serum concentrations might not be a good indicator of changes in a single joint.[26,38] In the period following initial injury diagnosis, the changes observed could reflect a response to injury, changes in response to treatment (which may have included medical treatment as well as exercise modification), or, most likely, a combination of these. It also has to be considered that although horses in this study were followed from the time they entered training as yearlings, this did not completely preclude preexisting disease. Also, although control horses did not have musculoskeletal injuries to the best of our knowledge, it is possible that they were not completely free from joint disease, which may have affected concentrations and activities of biomarkers. However, close veterinary involvement and regular data collection (as part of the prospective cohort study[6]) provided some confidence in the clinical information obtained. In addition, the information obtained represented realistic field conditions and the purpose of this study was to evaluate the biomarkers in this context. Finally, although a reasonable number of case-control pairs (n = 50) was studied overall, the number of cases in specific joint or injury categories, particularly mild injuries, was relatively low, which may have resulted in low statistical power to detect significant differences between injured horses and control horses. Similar studies including a larger number of horses in race training, potentially focusing on particular injury types in combination with repeated diagnostic imaging as part of the study design, may be helpful in further clarifying the value of cartilage and bone biomarkers as potential diagnostic or prognostic tools in a clinical setting.

In conclusion, this study demonstrated some differences in serum concentrations of specific biomarkers between horses with carpal and fetlock joint injuries and uninjured control horses, depending on the site and severity of injury. However, the value of biomarkers as diagnostic or prognostic indicators for joint disease in horses, or their potential usefulness for screening subclinically affected horses, remain unclear. Further research, including studies that involve sampling horses prior to injury diagnosis, assessing biomarker concentrations in combination with advanced diagnostic imaging techniques, or correlating findings with synovial fluid biomarker concentrations, is required to further evaluate the role of such markers as diagnostic or prognostic tools for joint disease in horses.

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Footnotes

a. IBEX Technologies Inc, Montreal, QC, Canada.
b. Serum Pre-Clinical CartiLaps, Immunodiagnostics Systems Ltd, Boldon, Tyne and Wear, England.
c. SAS, version 9.2, SAS Institute Inc, Cary, NC.
d. MedCalc, version 12.2.1, MedCalc Software, Mariakerke, Belgium.

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