Biochemical analysis of the articular cartilage and subchondral and trabecular bone of the metacarpophalangeal joint of horses with early osteoarthritis

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Objective—To assess whether site-related changes in biochemical composition are present in the cartilage and subchondral and trabecular bone of the metacarpophalangeal joint of horses with early osteoarthritis.

Sample Population—Right metacarpophalangeal joints from 59 mature warmblood horses.

Procedure—Biochemical data (cross-link, amino acid, DNA, and ash contents; denatured collagen and glycosaminoglycan [GAG] concentrations; bone mineral density; and mineral composition) were obtained from 2 differently loaded sites of phalanx I cartilage and subchondral and trabecular bone samples; data were compared with previously published values from nonosteoarthritic equine joints.

Results—Compared with findings in nonosteoarthritic joints, GAG concentration was lower in cartilage from osteoarthritic joints and there was a loss of site differences in cellularity and lysylpyridinoline (LP) cross-link content. In subchondral bone, LP cross-link content was decreased overall and there was a loss of site differences in osteoarthritic joints; ash content was higher in the osteoarthritic joints. Hydroxyproline content in trabecular bone from osteoarthritic joints was greater than that in nonosteoarthritic trabecular bone. In all 3 layers and at both sites, the linear increase of the pentosidine cross-link content with age had diminished or was not apparent in the horses with osteoarthritic joints.

Conclusions and Clinical Relevance—In equine metacarpophalangeal joints with early osteoarthritis, distinct biochemical changes were detected in the cartilage and subchondral and trabecular bone. The dissimilarity in response of the different tissues and differences between the sites that are affected may be related to differences in biomechanical loading and transmission and dissipation of force. (*Am J Vet Res* 2005;66:1238–1246)

The potential of a horse to perform successfully depends on its inherent athletic ability and capacity to keep free from diseases and conditions that interfere with soundness. Lameness is the most important cause of wastage in various populations of horses.[1,2] Joint diseases are the most important causes of lameness in horses; as in humans, osteoarthritis is by far the most important joint ailment in mature horses.[3–5]

Osteoarthritis is classically considered an inherently noninflammatory disorder of movable joints that is characterized by deterioration of articular cartilage and the formation of new bone at the joint surfaces and margins.[6] Although recent debate has strongly questioned the noninflammatory character of the disease, there is no doubt that the disorder affects both cartilage and underlying bone, at least in the later stages.[7] There is less consensus about the initial phase of the disease. In several reports,[8–13] it is stated that sclerosis of the subchondral bone plate precedes articular cartilage damage. In this scenario, repetitive impulse loading leads to repeated microtrauma and consequently to sclerosis and stiffening of the subchondral bone, followed by cartilage thinning and degeneration.[14] Some support for this was provided by Mansell and Bailey,[15] who reported that bone collagen metabolism was increased in osteoarthritic femoral heads of humans and the greatest changes were identified in the subchondral bone. However, other investigators have detected degradation of articular cartilage that is typical of early osteoarthritis without any signs of pathologic changes in the underlying subchondral bone.[16–18] This observation led to the opposing view that the disease begins in the articular cartilage, possibly promoted by inflammatory mediators that induce primary biochemical changes.[4,19–22] The question of whether subchondral bone change occurs before or subsequent to cartilage deterioration has hence not yet been resolved.[14,23–26] Data that could be used to answer to this question can only be obtained from studies that focus on the very early stages of osteoarthritis.

In recent years, much research has been performed on the molecular composition of the extracellular matrix of articular cartilage in the metacarpophalangeal joint of horses. From such investigations, it emerged that there are distinct site differences with regard to biochemical composition, which parallel the variation in biomechanical loading generated by locomotion.[27,28] In other species, similar assessments of the composition of the subchondral bone plate in relation to loading have revealed comparable site differences.[29–31] In horses, research has been limited so far. Brama et al[32,33] identified site differences in the subchondral bone plate of the proximal articular surface of

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the equine first phalanx with regard to certain matrix constituents (ie, collagen, water, and calcium contents). A relationship between the biochemical compositions of cartilage and subchondral bone and proneness to injury was suggested.[32] Recently, in a comprehensive study,[34] the compositions of articular cartilage and underlying subchondral and trabecular bone at the proximal articular surface of the first phalanx in sound horses were simultaneously analyzed. Results of that study, in which bone and cartilage were considered to be a single functional entity, indicated that there are distinct site-related differences in the distribution of the major biochemical components in the 3 layers and that, to a certain extent, these differences had similar patterns in all 3 layers.

The purpose of the study reported here was to assess whether site-related changes in biochemical composition are present in the cartilage and subchondral and trabecular bone of the metacarpophalangeal joint of horses with early osteoarthritis. It was hypothesized that changes would be identified but that these would not be identical in all 3 layers. An additional objective of this study was to evaluate the character and severity of the pathologic changes in these layers as an indicator of the origin of the primary damage. To this end, comprehensive biochemical analyses were carried out on samples of the cartilage, subchondral bone, and trabecular bone layers from 2 differently loaded sites in a series of mildly osteoarthritic joints from horses; findings were compared with data collected previously from normal nonosteoarthritic joints of horses.

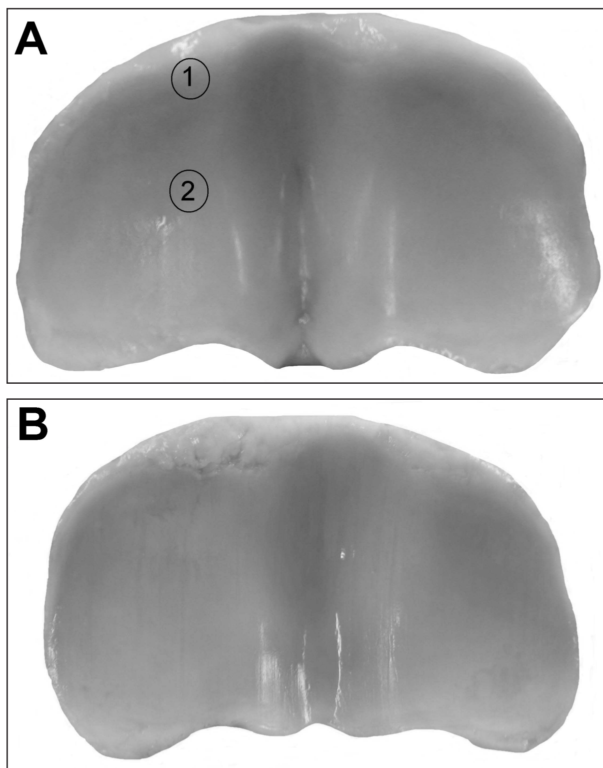

Figure 1—Photographs of the proximal articular surface of the first phalanx of 2 horses. A—Phalangeal articular surface from a metacarpophalangeal joint without osteoarthritis illustrating the sample sites investigated; site 1 was located close to the dorsal articular margin, and site 2 was located at the central fovea. Cartilage degeneration index (CDI) values were calculated for the joint overall (CDI) and at site 1 (CDI_1). In this joint, CDI was 8.8% and CDI_1 was 19.5%. B—Phalangeal articular surface from a metacarpophalangeal joint with early development of osteoarthritis; degenerative changes are principally located at the joint margin (site 1). In this joint, CDI was 18.8% and CDI_1 was 42.5%.

Materials and Methods

Joints—Right metacarpophalangeal joints from 59 adult warmblood horses (mean age, 12.6 years; range, 5 to 23 years) were collected immediately after euthanasia at a slaughterhouse; the joints were stored at –20°C until processing. One day before the analyses were performed, the joints were thawed and opened. The proximal two thirds of the first phalanx was isolated from the rest of the limb, and the surrounding tissue was dissected. Articular cartilage degeneration was quantified by use of the **cartilage degeneration index (CDI)**.[35,36] Briefly, the amount of Indian ink uptake across the entire cartilage surface was quantified via digital imaging of the nonstained and Indian ink–stained articular cartilage surfaces. The increase in mean gray level of the articular surface is the basis for calculation of the CDI (values range from 0% to 100%). This procedure was performed for the entire joint to provide a general CDI value and for 2 specific differently loaded regions of interest: site 1 was located close to the dorsal articular margin (designated as CDI_1), and site 2 was located at the central fovea (designated as CDI_2; **Figure 1**). On the basis of the CDI_1 values, joints were allocated to 1 of 2 groups. Joints in the first group (n = 30) had a $CDI_1 < 25\%$, which is indicative of none to minor degenerative changes. These joints were used to establish the biochemical reference values for clinically normal joints that have been published previously[34]; those data were used in the present study for comparison with findings in osteoarthritic joints. The samples from the second group (n = 29) were analyzed for the present study. Joints in the second group had CDI_1 values > 25%. This meant a much lower CDI for the entire joint and a specific CDI for site 2 that was only slightly higher than zero in most cases; since osteoarthritis in horses is known to start at site 1 and then spread gradually over the joint, site 2 is the last site to be affected.[36] Thus the joints were classified as mildly osteoarthritic.

Sampling procedure—After establishing the CDI values for each joint, a 6-mm-wide slice of phalanx I (containing cartilage and subchondral and trabecular bone) was cut by use of a band saw in the dorsopalmar direction, perpendicular to the articular surface, and through the center of the medial fovea. These slices contained both sites 1 and 2. A slice of 1- to 2-mm thickness was cut off, placed in Burckhardt's fixative for 4 days, and thereafter kept in 100% ethanol until measurement of **bone mineral density (BMD)**. The cartilage was removed by use of a scalpel from the remainder of the sample, and the subchondral and trabecular bones were separated by use of a milling cutter. Each bone and cartilage sample was further divided into 4 pieces and stored at –80°C until further analysis.

Determination of BMD—Bone mineral density was measured by use of a peripheral quantitative computed tomography machine[a] adapted for measuring small bones. Two 360° scans were performed with a thickness of 1 mm and a resolution of 0.148 × 0.148 mm. The peripheral quantitative computed tomography machine was calibrated with a standard of hydroxyapatite embedded in acrylic plastic. The scans were performed in 2 horizontal planes: one at 2 mm under the cartilage layer through the subchondral plate and the other at a depth of 8 mm through the trabecular bone. The BMD value (mg/cm^3) was calculated.

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Mineral analysis—One piece of subchondral bone and 1 piece of trabecular bone from each site was thawed and defatted by placing in ether for 1 week. After the sample was dried for 1 hour at 105°C, dry weight was determined. The drying time of 1 hour proved to be sufficient in a test series in which a variety of samples were dried and subsequently weighed after drying times as long as 24 hours (data not shown). The fat-free, dry samples were processed to ash at 540°C for 6 hours, weighed, and then dissolved in 15 mL of 4M hydrochloric acid. Calcium and magnesium content were determined via atomic absorption spectrophotometry.[b] Phosphorus content was determined according to the method of Quinlan and DeSesa.[37] The amount of each mineral (mmol/g of dry weight of bone) and ash concentration (percentage of dry weight of bone) were recorded.

Cross-link and amino acid analysis—One piece each of subchondral and trabecular bone and cartilage of each site was thawed; bone samples were demineralized for 3 weeks in buffered 0.5M EDTA solution (pH, 7.4). The bone and cartilage samples were hydrolyzed by use of 800 µL of 6M HCl at 110°C for 20 to 24 hours, dried under vacuum,[c] and redissolved in 500 µL (bone) or 200 µL (cartilage) of water containing 10µM pyridoxine (internal standard for the cross-link analysis)[d] and 2.4mM homoarginine (internal standard for the amino acid analysis).[d] Samples were diluted 5-fold with 1% (vol/vol) heptafluorobutyric acid[e] in 10% (vol/vol) acetonitrile[f] for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1M borate buffer (0.1M boric acid adjusted to pH 8.0 by addition of 5M sodium hydroxide) for amino acid analysis.

Reversed-phase **high-performance liquid chromatography (HPLC)** of cross-links was performed on 100-µL aliquots of the 5-fold diluted samples, as described elsewhere.[38,39] **Hydroxylysylpyridinoline (HP)** and **lysylpyridinoline (LP)** purified from adult human bone and calibrated against the pyridinoline-deoxypyridinoline HPLC calibrator and pentosidine (calibrated by mass spectroscopy) were used as standards.[40]

For amino acid analysis, 200 µL of the 250-fold diluted sample was derivatized at room temperature (17°C) with 200 µL of acetone containing 6µM 9-fluorenylmethyl-chloroformate.[e] The termination of the derivatization reaction, removal of excess reagent, and HPLC analysis were performed as described elsewhere.[38] Fluorescence was monitored (excitation, 254 nm; emission, 630 nm). Calibration was performed by use of an amino acid standard for collagen hydrolysates.[38,39] The quantities of cross-links as well as hydroxylysine were expressed as number of residues per collagen molecule, assuming 300 hydroxyproline residues/triple helix of collagen.

Analysis of denatured collagen—The amount of denatured collagen was determined by the assay described by Bank et al,[41] which is based on the observation that α-chymotrypsin digests denatured collagen but not the triple helix form. Briefly, 1 piece each of cartilage and subchondral and trabecular bone from each site were thawed, and bone samples were demineralized for 3 weeks in a buffered 0.5M EDTA solution (pH, 7.4). Bone and cartilage were extracted with 4M guanidinium hydrochloride in 0.1M Tris HCl (pH, 7.3) containing a cocktail of protease inhibitors. The denatured collagen in the bone and cartilage matrix was then digested during 14 hours at 37°C with 0.5 mg of α-chymotrypsin (C-4129)[d] dissolved in 500 µL of PBS solution (pH, 7.4) containing 1mM iodoacetamide and 1mM EDTA. The supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen); both were hydrolyzed with 6M HCl at 110°C for 20 hours. The amount of the collagen-specific amino acid hydroxyproline was measured via reversed-phase HPLC. The amount of denatured collagen was expressed as a percentage of total collagen.

Glycosaminoglycan analysis—Trabecular bone and cartilage samples were digested for approximately 16 hours at 65°C by use of papain (P3125)[d] in 200 µL of a 50mM phosphate buffer (pH, 6.5) containing 2mM Na_2EDTA and 2mM cysteine. Proteoglycan content was determined by measuring the amount of polysulfated **glycosaminoglycans (GAGs)** in the papain digest of the samples by use of a modification of the 1,9-dimethylmethylene blue[d] assay described by Farndale et al.[42] To 10 µL of a diluted sample after papain digestion, 10 µL of 1% (wt/vol) bovine serum albumin,[d] and 200 µL of reagent (46µM 1,9-dimethylmethylene blue, 40mM glycine, and 42mM NaCl adjusted to pH 3.0 with HCl) were added; after 30 minutes, the absorbency at 525 nm was assessed. The assay was standardized with shark chondroitin sulfate.[d] The GAG amount was expressed as micrograms per milligram of dry weight sample of cartilage or bone.

DNA analysis—A 10-µL aliquot of the papain digest was used to determine DNA content as a measure of the amount of cells; the procedure involved the use of a fluorescent dye as described by Kim et al.[43] Briefly, dye solution was added to the papain digest of the bone and cartilage samples; immediately after mixing, fluorescence was measured at excitation of 365 nm and emission of 460 nm. Calf thymus DNA (D-4764)[d] was used as reference. Results were expressed as micrograms of DNA per milligram of dry weight of bone or cartilage.

Statistical analyses—All measured variables were expressed as mean values ± SD. Statistical analyses of the data were performed by use of computer software.[g] Differences between the specific sites in the osteoarthritic and nonosteoarthritic joints were tested by use of a multivariate ANOVA (factors included age, site, and CDI value). When there was an interaction present between osteoarthritis and site, a Tukey multiple group comparison was performed between each combination of sites (ie, 1 and 2) and disease status (ie, osteoarthritis or no osteoarthritis) to identify the nature of this interaction. Correlation was tested by use of a Pearson product moment correlation analysis. The level of significance was set at a value of $P < 0.05$.

Results

Collected data regarding articular cartilage, subchondral bone plate, and trabecular bone samples from joints of horses that were classified as mildly osteoarthritic (ie, osteoarthritic joints) were compared with data obtained from joints of horses that were considered free of osteoarthritis (ie, nonosteoarthritic joints; **Tables 1–3**).

Analyses of the articular cartilage from the osteoarthritic and nonosteoarthritic joints revealed a significant difference only in GAG content, which was considerably less in the osteoarthritic joints than it was in the nonosteoarthritic joints (Table 1). If all horses were taken into account (with and without osteoarthritis), there was a significant negative relationship between the CDI_1 value and GAG content of site 1 (**Figure 2**). There was a significantly greater percentage of denatured collagen at site 1 than site 2 in both nonosteoarthritic and osteoarthritic joints. There was more denatured collagen at site 1 in the osteoarthritic joints, compared with that at site 1 in the nonosteoarthritic joints, but this difference was not significant. All significant differences that were detected

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Table 1—Biochemical variables (mean ± SD) assessed at different sites in cartilage from the proximal articular surface of the first phalanx of the right forelimb of 29 horses with osteoarthritic right metacarpophalangeal joints and 30 horses with nonosteoarthritic right metacarpophalangeal joints.

Variable	Cartilage of nonosteoarthritic joints		Cartilage of osteoarthritic joints		Main statistical analyses		
	Site 1 (n)	Site 2 (n)	Site 1 (n)	Site 2 (n)	Site effect *P* value	Osteoarthritis effect *P* value	Osteoarthritis-site interaction
Hydroxylysine/collagen (mol/mol of collagen)	40.76 ± 13.02 (30)	52.73 ± 5.37 (27)	36.67 ± 11.90 (27)	50.46 ± 7.98 (28)	< 0.001	NS	No
Hydroxyproline/proline (mol/mol)	0.67 ± 0.08 (30)	0.68 ± 0.07 (27)	0.68 ± 0.08 (28)	0.69 ± 0.09 (28)	NS	NS	No
HP/collagen (mmol/mol of collagen)	58 ± 271.57 (30)	853.58 ± 106.08 (27)	641.87 ± 295.99 (29)	815.38 ± 130.13 (27)	< 0.001	NS	No
LP/collagen (mmol/mol of collagen)	26.80 ± 18.64 (30)*	12.39 ± 3.02 (23)	26.63 ± 18.45 (29)	22.22 ± 13.77 (28)	NA	NA	Yes
Denatured collagen (%)	15.28 ± 6.66 (30)	11.73 ± 4.24 (27)	16.94 ± 9.14 (27)	11.60 ± 5.16 (27)	< 0.05	NS	No
GAGs (µg/mg dry weight)	59.27 ± 26.91 (30)	124.05 ± 39.92 (30)	44.30 ± 29.17 (29)	113.32 ± 41.30 (29)	< 0.001	< 0.05	No
DNA (µg/mg dry weight)	2.39 ± 0.90 (30)†‡	1.63 ± 0.49 (29)	1.99 ± 0.83 (29)	1.73 ± 0.37 (29)	NA	NA	Yes
GAG/DNA (µg/µg of DNA)	28.31 ± 15.82 (30)	72.80 ± 17.10 (29)	26.23 ± 20.03 (29)	67.00 ± 22.50 (29)	< 0.001	NS	No
Pentosidine (mmol/mol of collagen)	0.49 ± 0.28 (29)	0.42 ± 0.22 (26)	0.70 ± 0.49 (28)	0.47 ± 0.25 (28)	—	—	—

The *P* values are not given for pentosidine content because this variable is influenced by age and because the age distributions in the osteoarthritic and nonosteoarthritic groups were not identical.

*Value significantly ($P < 0.01$) different from the value for site 2 of the nonosteoarthritic joints. †Value significantly ($P < 0.001$) different from the value for site 2 of the nonosteoarthritic joints. ‡Value significantly ($P < 0.01$) different from the value for site 2 of the osteoarthritic joints.

n = Number of samples. HP = Hydroxylysylpyridinoline. LP = Lysylpyridinoline. GAGs = Glycosaminoglycans. NS = Not significant ($P > 0.05$). NA = Not applicable.

Table 2—Biochemical variables (mean ± SD) assessed at different sites in subchondral bone from the proximal articular surface of the first phalanx of the right forelimb of 29 horses with osteoarthritic right metacarpophalangeal joints and 30 horses with nonosteoarthritic right metacarpophalangeal joints.

Variable	Subchondral bone of nonosteoarthritic joints		Subchondral bone of osteoarthritic joints		Main statistical analyses		
	Site 1 (n)	Site 2 (n)	Site 1 (n)	Site 2 (n)	Site effect *P* value	Osteoarthritis effect *P* value	Osteoarthritis-site interaction
Hydroxylysine/collagen (mol/mol of collagen)	17.74 ± 3.55 (30)	20.08 ± 2.39 (30)	16.20 ± 2.97 (29)	19.07 ± 3.10 (29)	< 0.001	< 0.05	No
Hydroxyproline/proline (mol/mol)	0.64 ± 0.03 (30)	0.67 ± 0.02 (30)	0.64 ± 0.03 (29)	0.67 ± 0.02 (29)	< 0.001	NS	No
HP/collagen (mmol/mol of collagen)	201.61 ± 74.52 (23)	322.06 ± 57.16 (29)	159.79 ± 51.62 (24)	262.44 ± 95.09 (29)	< 0.001	< 0.01	No
LP/collagen (mmol/mol of collagen)	67.31 ± 39.14 (20)*	47.83 ± 13.63 (29)	49.53 ± 28.10 (23)	56.67 ± 24.81 (29)	NA	NA	Yes
Denatured collagen (%)	11.38 ± 2.54 (30)	11.87 ± 2.58 (30)	11.34 ± 2.57 (29)	11.41 ± 1.92 (29)	NS	NS	No
Bone mineral density (mg/cm^3)	990 ± 75 (30)	1,072 ± 59 (29)	992 ± 55 (28)	1,066 ± 63 (28)	< 0.001	NS	No
Ash (%)	58.17 ± 1.42 (28)	60.29 ± 2.42 (29)	59.43 ± 2.62 (29)	61.58 ± 1.83 (28)	< 0.001	< 0.01	No
Calcium (mmol/g dry weight)	5.58 ± 0.20 (29)	5.80 ± 0.21 (29)	5.60 ± 0.29 (29)	5.83 ± 0.22 (29)	< 0.001	NS	No
Phosphorus (mmol/g dry weight)	2.97 ± 0.08 (28)	3.12 ± 0.11 (29)	3.02 ± 0.11 (28)	3.16 ± 0.12 (28)	< 0.001	NS	No
Magnesium (mmol/g dry weight)	0.07 ± 0.005 (29)	0.08 ± 0.006 (29)	0.07 ± 0.005 (29)	0.08 ± 0.007 (29)	< 0.001	NS	No
Pentosidine (mmol/mol of collagen)	0.21 ± 0.10 (19)	0.14 ± 0.05 (29)	0.24 ± 0.09 (21)	0.17 ± 0.07 (29)	—	—	—

The *P* values are not given for pentosidine content because this variable is influenced by age and because the age distributions in the osteoarthritic and nonosteoarthritic groups were not identical.

*Value significantly ($P < 0.05$) different from the value for site 2 of the nonosteoarthritic joints.

See Table 1 for remainder of key.

between sites 1 and 2 in nonosteoarthritic joint cartilage were similarly detected in osteoarthritic joint cartilage, with the exception of LP cross-link and DNA contents. For these 2 variables, there was an interaction with the presence of osteoarthritis, resulting in a loss of site differences. The linear increase of the pentosidine cross-links found in healthy cartilage with age could not be demonstrated in osteoarthritis-affected joints. At both sites 1 and 2, this relationship had lost significance (**Table 4**).

Compared with samples of subchondral bone plate from sites 1 and 2 of nonosteoarthritic joints, subchondral bone from the corresponding site of osteoarthritic joints had a lower hydroxylysine content and appeared to have less HP cross-links per triple helix of collagen (Table 2). In this layer in osteoarthritic joints, the site difference existing in the nonosteoarthritic joints (ie, difference between site 1 and 2) with respect to LP cross-link content had disappeared because of osteoarthritis. In subchondral bone, the change in pentosidine content in relation to age is not as evident as in the cartilage layer, but the increase with age at site 2 appears to be less in the osteoarthritic joints than in the nonosteoarthritic joints (Table 4). There were no

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Table 3—Biochemical variables (mean ± SD) assessed at different sites in trabecular bone from the proximal articular surface of the first phalanx of the right forelimb of 29 horses with osteoarthritic right metacarpophalangeal joints and 30 horses with nonosteoarthritic right metacarpophalangeal joints.

	Trabecular bone of nonosteoarthritic joints		Trabecular bone of osteoarthritic joints		Main statistical analyses		
Variable	Site 1 (n)	Site 2 (n)	Site 1 (n)	Site 2 (n)	Site effect *P* value	Osteoarthritis effect *P* value	Osteoarthritis-site interaction
Hydroxylysine/collagen (mol/mol of collagen)	12.89 ± 2.17 (30)	12.96 ± 1.74 (30)	12.86 ± 1.61 (28)	11.83 ± 1.67 (28)	NS	NS	No
Hydroxyproline/proline (mol/mol)	0.67 ± 0.02 (30)*†	0.69 ± 0.02 (30)	0.66 ± 0.02 (29)†	0.71 ± 0.03 (29)	NA	NA	Yes
HP/collagen (mmol/mol of collagen)	123.62 ± 21.27 (30)	148.59 ± 28.16 (30)	120.88 ± 29.90 (29)	134.70 ± 30.90 (29)	< 0.001	NS	No
LP/collagen (mmol/mol of collagen)	90.44 ± 18.77 (29)	64.65 ± 30.13 (30)	85.20 ± 26.47 (29)	87.26 ± 26.66 (29)	NS	NS	No
Denatured collagen (%)	12.65 ± 3.14 (30)	14.04 ± 4.05 (30)	12.93 ± 3.41 (27)	14.43 ± 2.88 (29)	< 0.01	NS	No
GAGs (µg/mg dry weight)	1.72 ± 0.40 (30)	1.69 ± 0.44 (29)	1.73 ± 0.35 (28)	1.61 ± 0.36 (29)	NS	NS	No
DNA (µg/mg dry weight)	0.95 ± 0.20 (30)	1.03 ± 0.15 (29)	1.03 ± 0.17 (28)	1.07 ± 0.18 (29)	NS	NS	No
GAGs/DNA (µg/µg of DNA)	1.84 ± 0.39 (30)	1.66 ± 0.39 (29)	1.72 ± 0.44 (28)	1.52 ± 0.33 (29)	< 0.05	NS	No
Bone mineral density (mg/cm^3)	875 ± 135 (30)	707 ± 149 (29)	865 ± 164 (28)	703 ± 116 (28)	< 0.001	NS	No
Ash (%)	61.67 ± 1.31 (28)	62.55 ± 1.48 (29)	61.67 ± 1.43 (28)	62.99 ± 1.51 (29)	< 0.001	NS	No
Calcium (mmol/g dry weight)	5.93 ± 0.16 (29)	6.07 ± 0.37 (30)	5.95 ± 0.12 (28)	6.07 ± 0.15 (29)	< 0.01	NS	No
Phosphorus (mmol/g dry weight)	3.17 ± 0.12 (30)	3.29 ± 0.21 (30)	3.19 ± 0.10 (29)	3.27 ± 0.07 (29)	< 0.001	NS	No
Magnesium (mmol/g dry weight)	0.08 ± 0.006 (30)	0.09 ± 0.008 (30)	0.08 ± 0.007 (29)	0.09 ± 0.006 (29)	< 0.001	NS	No
Pentosidine (mmol/mol of collagen)	0.16 ± 0.06 (30)	0.14 ± 0.05 (30)	0.19 ± 0.08 (28)	0.15 ± 0.05 (29)	—	—	—

The *P* values are not given for pentosidine content because this variable is influenced by age and because the age distributions in the osteoarthritic and nonosteoarthritic groups were not identical.

*Value significantly ($P < 0.01$) different from the value for site 2 of the nonosteoarthritic joints. †Value significantly ($P < 0.001$) different from the value for site 2 of the osteoarthritic joints.

See Table 1 for remainder of key.

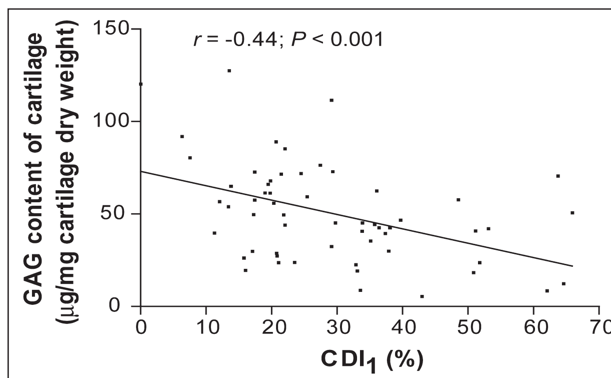

Figure 2—Glycosaminoglycan (GAG) contents of cartilage samples obtained from right metacarpophalangeal joints in relation to the CDI_1 values of the proximal articular surface of the first phalanx of 30 horses without osteoarthritis and 29 horses with osteoarthritis. *r* = Pearson product moment correlation coefficient.

significant differences in any of the mineral-related variables and BMD. However, ash content was significantly higher in the subchondral bone plate underlying cartilage that was affected by osteoarthritis (site 1, 59.43 ± 2.62; site 2, 61.58 ± 1.83) than in the subchondral bone underlying cartilage that was not affected by osteoarthritis (site 1, 58.17 ± 1.42; site 2, 60.29 ± 2.42).

In trabecular bone, an interaction between osteoarthritis and site was detected for hydroxyproline content, which resulted in a significant increase in this variable at site 2 in osteoarthritic joints, compared with the value at site 1 (Table 3). Although no other osteoarthritis-related changes were detected in trabecular bone in either the mineral or the nonmineral variables, the increase of pentosidine cross-links with age had become less strong at site 1 and was not evident at site 2 (Table 4).

Table 4—Pearson correlation coefficients (*r*) for age with pentosidine content at sites 1 and 2 of each of the layers of cartilage, subchondral bone, and trabecular bone from the proximal articular surface of the first phalanx of the right forelimb of 29 horses with osteoarthritic right metacarpophalangeal joints and 30 horses with nonosteoarthritic right metacarpophalangeal joints. There is a significant linear correlation of pentosidine content with age at almost all sites in the joints without osteoarthritis. In the osteoarthritis-affected joints, this correlation has decreased and has lost significance ($P > 0.05$) in most instances, indicating a higher metabolic level.

Tissue type and site	Nonosteoarthritic joints (*r*)	*P* value	Osteoarthritic joints (*r*)	*P* value
Cartilage site 1	0.51	< 0.01	0.07	NS
Cartilage site 2	0.46	< 0.05	0.31	NS
Subchondral bone site 1	0.43	NS	0.42	NS
Subchondral bone site 2	0.73	< 0.001	0.40	< 0.05
Trabecular bone site 1	0.70	< 0.001	0.51	< 0.01
Trabecular bone site 2	0.60	< 0.001	0.19	NS

See Table 1 for key.

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Discussion

Osteoarthritis is typically a chronic and insidious disease that may have affected large parts of the joint before becoming clinically evident. In the ongoing efforts to better understand the pathogenesis of osteoarthritis, these well-established forms of the disease are of little value because secondary processes have obscured or effaced any initial signs. By setting the benchmark at $CDI_1 > 25\%$ in the present study, we made a deliberate selection of joints that could be classified as being at the very early stage of the development of osteoarthritis. Osteoarthritis in horses is known to start at site 1 and then spread gradually over the joint; thus, in joints with $CDI_1 > 25\%$, the general CDI value for the entire joint was low and the specific CDI value for site 2 was only slightly greater than zero in most instances.[36] No clinical data were available from this group of slaughter horses, but it can be assumed that most of them would not have had clinical signs that could have been attributed to the joints examined.

As expected, differences in biochemical variables between osteoarthritic and nonosteoarthritic joints were few because of the very mild form of disease in affected joints. However, differences were identified in all 3 layers. Compared with findings in the cartilage layer of nonosteoarthritic joints, there was a significant decrease in GAG content in the osteoarthritic specimens and a loss of site differences for LP cross-link and DNA contents as a result of the effect of osteoarthritis. Furthermore, compared with findings in the subchondral bone layer of nonosteoarthritic joints, HP cross-link content was considerably and significantly decreased at both sites 1 and 2 in osteoarthritic joints; the difference for LP cross-link content was also lost in this layer. In osteoarthritic joints, the subchondral bone had a lower concentration of hydroxylysine than it did in nonosteoarthritic joints. It is known that as the hydroxylysine content of collagen molecules decreases, the collagen fibrils become thicker and more closely packed.[44–46] In another study,[47] such closer packing of fibrils was related to a higher degree of mineralization. Overall, these changes would result in a denser and probably more brittle tissue. Because 3 hydroxylysine residues are needed for every HP cross-link, a lower hydroxylysine content may be related to a lesser degree of cross-linking,[48] as identified in the present study.

Analysis of samples of trabecular bone revealed a significant osteoarthritis-related increase of the hydroxyproline-to-proline ratio at site 2. Thus, in osteoarthritic joints, there were differences at both sites in the cartilage layer, at both sites in the subchondral bone layer, and only at site 2 in the trabecular bone layer, compared with findings in nonosteoarthritic joints. It can be speculated that this somewhat different reaction pattern of each layer is a consequence of differences in handling of the biomechanical loading that is generated by locomotion. The cartilage layer sustains the initial impact, which is known to be highest at the intermittently loaded site 1.[28] Total cumulative load over time is higher at the continuously loaded site 2; the underlying trabecular layer in this area might be affected to a greater extent because it would receive a larger portion of the dissipating forces than site 1.[34]

The differences in the cartilage layer variables between osteoarthritic and nonosteoarthritic joints that were detected in the present study were minimal, but consistent with other reports concerning the early stage of osteoarthritis. There was a significant correlation between CDI_1 and GAG content. Proteoglycan loss is known to be one of the early signs of osteoarthritis. Indian ink particles are prevented from entering an intact cartilage surface that has an unaffected proteoglycan-rich matrix, but have a high affinity for articular cartilage with surface fibrillation and proteoglycan depletion.[35,49–51] Another early indication of cartilage damage may be the loss of site differences in DNA content. Severe osteoarthritis is known to result in an almost complete loss of site differences of many biochemical variables, and loss of the superficial cartilage layer, where cell density is highest, has been reported as a sign of early osteoarthritis.[52,53] In the osteoarthritis-affected joints examined in the present study, the cell density at site 1 was decreased, compared with that in nonosteoarthritic joints, and became similar to that of site 2 as a result of the disease process.

In the present study, another indication of biochemical changes in association with early development of osteoarthritis was the alteration in the relationship of pentosidine cross-link content with the age of the horses. In healthy animals, there is a linear increase of the nonenzymatic glycation product with age; the accumulation of this advanced glycation end product, which results from the spontaneous reaction of sugars with proteins, is a physiologic process in many tissues. The pentosidine content in any tissue is largely determined by the turnover rate of the extracellular matrix.[54] Therefore, pentosidine content can be used to assess the remodeling rate of the collagen network in a wide range of tissues. In tendons, it has been shown that repeated microdamage, such as that which occurs in the supraspinatus tendon and results in so-called rotator cuff syndrome in humans, will halt the physiologic linear increase of the glycation product through the increase of tissue turnover that leads to removal of the product.[55] In our study, change in the relationship of the pentosidine cross-link content with age was evident; not only in the cartilage layer but in all layers and at all sites (except for site 1 in the subchondral bone at which no such relationship could be found in healthy or diseased joints), there was either a complete loss of the relationship of pentosidine content with age or the increase became diminished.

In the subchondral bone plate, an interesting phenomenon was observed. There was a significant decrease of HP cross-link content at both sites 1 and 2 in the osteoarthritic joints, compared with findings in the nonosteoarthritic joints. This difference was not evident at sites 1 and 2 of the overlying articular cartilage, which is in agreement with earlier findings.[56,57] The HP and LP cross-links are mature cross-links. A decreased amount of these may hence indicate a higher turnover, resulting in what could be called a less

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mature bone matrix. Brama et al[28,32] identified higher cross-link amounts at site 2 in a series of normal specimens and related this finding to the loading pattern determined in another study. They conjectured that the intermittent high peak loading at site 1 would lead to a higher remodeling rate and hence lower amounts of cross-links. The difference in cross-link amounts between sites 1 and 2 in nonosteoarthritic joints that was detected in the present study is similar to that identified by Brama et al.[32] In the osteoarthritic joints, this difference was also detected, but in comparison, the amounts of cross-links at both sites were lower than those in nonosteoarthritic joints. This may indicate that the remodeling rate is higher in osteoarthritic joints than it is in nonosteoarthritic joints. The higher remodeling rate might possibly be interpreted as a very early sign of osteoarthritis in the subchondral bone plate. This is a potentially interesting finding because cross-links influence the structure and mechanical strength of the collagen network.[58,59] Apart from this direct influence on material properties, there is an indirect influence as well because the collagen network appears to regulate the process of fibril mineralization.[60,61] Mineralization has an effect on the packing fraction of collagen fibrils; increased mineralization will result in closer packing of collagen molecules and reduced water content and thus stiffer bone.[47,62] Increasing stiffness of the subchondral bone plate has been incriminated as one of the major initiating factors of osteoarthritis.[8,12] In the present study, there were some indications that probably very early changes in mineral composition were taking place in the osteoarthritic joints. There were small, but significant, differences in ash content at each of the 2 sites between the joints with minimal osteoarthritis and the nonosteoarthritic specimens. However, the changes in the collagen network probably precede these changes in ash content.

In our study, there were no differences in BMD between the joint groups. In reports of studies in which BMD has been measured in osteoarthritic and nonosteoarthritic joints, there are no consistent results. In some investigations,[50,63–65] an increase of BMD in osteoarthritic joints (compared with nonosteoarthritic joints) has been described. However, in several other studies,[8,66–68] no association between BMD and osteoarthritis has been identified. An explanation might be that BMD measured via quantitative computed tomography is a measure of mineral content in a unit volume of bone, which is not a true mineral density but rather an apparent bone mineral density.[69] This may mean that the technique is not sensitive enough to detect early and relatively mild changes in mineral content associated with early developmental stages of osteoarthritis.

Compared with findings in nonosteoarthritic joint specimens, the trabecular bone samples of osteoarthritic joints evaluated in the present study differed only with regard to variables associated with collagen, and these changes were minimal. The increase in hydroxyproline-to-proline ratio indicates a relative increase in collagen content over noncollagenous protein content, which may represent a response to increased loading. The increased collagen amount may be indicative of an increase in tensile strength as adaptation to loading.[58,59]

Our data have led us to conclude that in the very early and mild stages of osteoarthritis in horses that were investigated, changes at the molecular level take place in all 3 layers that comprise the bearing surface of the joint. However, changes take place at different sites in the different layers. In the joints we examined, osteoarthritis-associated changes affected the proteoglycan component of the cartilage layer and characteristics of the collagen network in both bony layers. Changes in any of the mineral-related variables were detected in subchondral bone only and were relatively minimal, suggesting that these develop at a later stage of the disease.

It is acknowledged that the order of events that take place during the early development of osteoarthritis can be definitively determined only on the basis of results of studies in which developments are monitored over time or in which sequential samples are collected for analysis. Furthermore, aspects other than molecular composition alone, such as tissue architecture, should be taken into account. Nevertheless, the fact that most changes were detected in the subchondral bone plate and fewer were detected in cartilage and trabecular bone may perhaps provide an indication of the sequence in which these changes develop.

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a. Stratec XCT 960A, Stratec, Birkenfield, Germany.
b. Perkin Elmer 3300, Norwalk, Conn.
c. Savant SC 110, Hollbrook, NY.
d. Sigma Chemical Co, St Louis, Mo.
e. Fluka, Buchs, Switzerland.
f. Rathburn Chemicals Ltd, Walkerburn, Peebleshire, UK.
g. SPSS, version 10 for Windows, SPSS Inc, Chicago, Ill.

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