

Evolution of some biochemical markers of growth in relation to osteoarticular status in young horses: results of a longitudinal study in three breeds



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Abstract

Osteocalcin (OC), bone fraction of alkaline phosphatases (BAP) and hydroxyproline (HOP) are markers of bone cell activity. The kinetics of these markers and the analysis of their variations could be related to the osteoarticular status (OAS) of young horses. The growth of Thoroughbreds, French Trotters and Selle Français horses was followed up to 18 months. Blood samples were taken regularly to measure OC, HOP and BAP by standardized techniques. The OAS was evaluated by radiographic examination of the limbs. Based on radiographic findings, two groups of horses were investigated, with no lesions or severely affected. Analysis of variance was used to detect the effects of age and breed, and OAS on parameters. The logarithmic model was used to determine the kinetics of the markers. A rapid decrease in marker concentrations with age and differences between breed was observed. At birth, BAP, OC and HOP concentrations were significantly higher in normal horses (1910 UI l^{-1} , 192 ng ml^{-1} and 35 mg l^{-1} , respectively) than in horses with severe lesions (1620 UI l^{-1} , 149 ng ml^{-1} and 24 mg l^{-1} , respectively). During the first 6 months, OC, HOP and BAP remained lower in severely affected horses.

Keywords: horse; developmental orthopaedic disease; radiographic examination; marker of bone cell activity; longitudinal study

Introduction

Ossification results from the regulation of complex mechanisms by several molecules. Osteocalcin (OC) and bone alkaline phosphatases (BAPs) are produced by osteoblasts and assumed to be direct biomarkers of bone metabolism^{1,2}. Their metabolic activities are related to Somatomedin (IGF-I)³ and controlled by thyroid hormones (thyroxin) and vitamin D_3 ^{2,4}, which are themselves affected by the animal diet; for instance, thyroxin concentration decreases in the case of an excess energy consumption diet⁵.

A perfect equilibrium between bone formation and resorption exists to maintain an appropriate bone mass, but a deregulation of these mechanisms may occur, predisposing to bone lesions called

developmental orthopaedic diseases⁶; DOD include osteochondrosis, physitis and angular limb deformity. Though their pathogeneses are different, these diseases have been classified together due to their similarities⁷. These diseases all appear during the first months of age, a period of particularly rapid growth where equine bones undergo rapid cartilage development as opposed to active enchondral ossification. This process is controlled by the osteoblasts and osteoclasts, and growth regulation factors (sex hormones, vitamin D_3 , thyroxin, Transforming Growth Factor (TGF), etc.). Juvenile osteoarticular lesions or developmental skeletal problems⁸ would then be more appropriate terms. Detection of DOD is based mainly on clinical and radiographic examination, but can also

be documented using ultrasonography or even magnetic resonance imaging where this technique is accessible.

Recent advances^{1,9-11} have been made in the biochemical assays of bone remodelling markers (the ideal marker being specific to the target tissue).

Due to the large physiological variations or the lack of bone specificity of the serum markers, spot samples are of little diagnostic value for the study if not associated with substantial follow-up data collection¹¹. Young horses appear to have higher marker levels than adult horses due to their more rapid bone turnover during growth^{10,12}. Identification of these early predictive markers would be valuable in detecting young horses which are more likely to develop bone abnormalities. A large number of crossover studies have demonstrated that the biochemical marker assays specific to bone metabolism could be used in horses^{3,13-16}.

OC, BAPs and hydroxyproline (HOP) are markers of bone activity during growth. Modifications of these marker concentrations in the equine blood may result in disrupted bone growth, predisposing to orthopaedic lesions.

The aim of this study was to follow up kinetics of OC, HOP and BAP during the first months of life in horses, in order to analyse their variations in relation to the osteoarticular status (OAS) when horses are between 15 and 18 months of age.

Materials and methods

Animals

An epidemiologic, longitudinal study was conducted, in field conditions in Normandy, France, between 1997 and 2003. A total of 398 horses were followed (growth curves, nutrition, locomotion, etc.) from their birth to their departure to the training centre. The subjects studied all belonged to the three main horse breeds used, in France, in horse racing and equine sports: Thoroughbred (Tb), French Trotter (FT) and Selle Français (SF) horses.

Depending on field conditions and economic aspects (death, sales at weanling, etc.), only 262 horses out of the initial 398 had blood samples for the chosen biochemical parameters and radiographic examinations concurrently. There were 86 Tbs, 122 FTs and 54 SF horses.

Blood samples (mean of 6.3 samples by horse, corresponding to a total of 1650 measurements) were taken every 2-3 months. They were done on a very random basis between 08.00 and 15.00, hours, depending on the availability of the breeders, and sent to the reference laboratory (specialized for equine biology and microbiology diagnosis and certified for applying good laboratory practices). Samples were assigned in six classes with respect to the age of the horses (Table 1).

Table 1 Mean values of biochemical markers according to age and breed (average and standard deviation) calculated on 1650 measurements obtained from 262 horses

Period	10 days	<1 month	2nd-3rd months	4th-6th months	7th-12th months	13th-18th months
Mean age (days)	5 (3)	22 (6)	64 (18)	140 (28)	8.9 (1.7)	15.8 (2.4)
Mean age (months)			2.1 (0.6)	4.7 (0.9)	384	245
Measurements	123	196	343	359		
HOP (mg l ⁻¹)	All horses 16.2 (4.4) b Tb 14.6 (4.6) B b SF 18.7 (4.2) A a FT 17.0 (3.2) A a	17.0 (3.6) a 17.0 (3.2) a 17.2 (3.9) b 16.8 (3.9) a	12.8 (3.3) c 12.8 (3.7) c 13.0 (3.7) c 12.5 (2.7) b	8.2 (2.7) d 8.1 (3.0) d 7.8 (2.3) d 8.6 (2.5) c	5.5 (1.2) e 5.5 (1.2) e 6.2 (2.1) e 5.6 (1.2) d	4.4 (1.5) f 4.2 (1.2) f 4.7 (2.2) f 4.4 (1.2) e
BAP (U l ⁻¹)	All horses 1651 (825) a Tb 1988 (890) A a SF 1178 (431) B a FT 1431 (693) B a	696 (279) b 718 (292) b 721 (306) b 657 (244) b	535 (199) c 532 (202) c 537 (228) c 536 (175) c	339 (159) d 340 (175) d 315 (135) d 353 (155) d	227 (103) e 239 (138) e 215 (79) e 224 (82) e	158 (56) f 161 (59) f 157 (53) f 157 (55) f
OC (ng ml ⁻¹)	All horses 130.8 (43.7) a Tb 118.7 (38.3) B a SF 131.7 (29.0) AB a FT 147.7 (52.3) A a	129.3 (38.5) a 127.4 (34.1) a 130.8 (29.0) a 130.1 (45.7) b	101.5 (24.1) b 103.8 (25.1) b 101.6 (23.8) b 99.4 (23.5) c	70.9 (25.1) c 65.9 (25.0) c 63.8 (19.9) c 79.9 (25.9) d	38.5 (12.4) d 37.6 (9.8) B d 33.0 (9.8) C d 43.2 (14.2) A e	32.7 (9.8) e 29.1 (8.1) B e 29.1 (7.8) B e 36.5 (10.1) A e

Results are expressed as mean (SD).

Significance was set at $P < 0.05$.

Letters a-f in a line indicate, for each breed and all horses, differences between ages.

Letters A-B in a column indicate, for each age, differences between breeds.

Radiographic examination

The radiographic evaluation was done at 17.7 ± 2.2 months of age (mean $\pm$ SD) and the median at 16.9 months of age, before the yearlings left the breeding farms to start training.

The radiographic examination included five bilateral views, chosen for their feasibility on the farms, with a mobile radiographic unit: the lateromedial views of the front and hind digits (including the interphalangeal and fetlock joints) and of the hock and the stifle and the dorsopalmar views of the carpus. For economic and radiation safety reasons, and to obtain as much information as possible on the OAS of horses, several joints were examined with one view per joint.

The present radiographic examination was not exhaustive. Other sites (cervical or shoulder) and views could have been obtained to better document the OAS of foals.

Radiographic files were evaluated by a college of three experienced veterinarians. All the radiographic findings (RFs) were categorized by the location and type of change and then graded according to a standardized protocol depending on their severity and clinical relevance¹⁷. Images of little significance on clinical basis were termed suspect RFs and graded 1. Images considered having possible consequences on physical appearance (i.e. joint effusion or local deformation) or locomotion were termed abnormal RFs and graded 2, 4 or 8 if they were considered to have uncertain, probable or certain clinical significances, respectively. Fig. 1 shows RFs of grades 1(a) and 8(b). All observable radiographic changes, including osteochondrosis (i.e. osteochondritis dissecans and subchondral bone cysts), physitis or juvenile degenerative joint disease were categorized. The severity indexes of all the RFs on the ten radiographs were added to calculate a radiographic

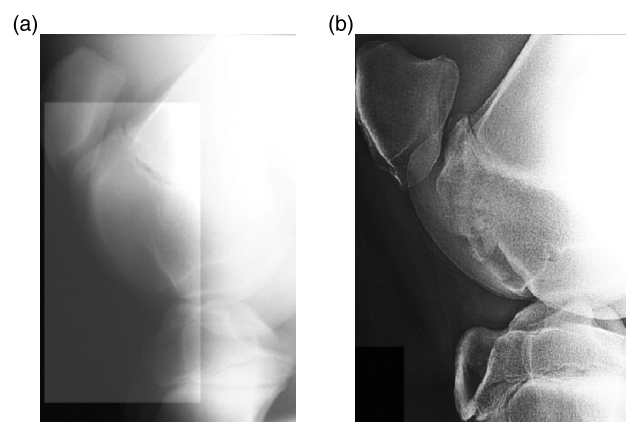


Fig. 1 Example of frequent radiographic findings and their gradation. Osteochondrosis of the lateral trochlear ridge of the femur: (a) graded 1 and (b) graded 8

score (RS) for each yearling. Of the 262 yearlings submitted for radiographic examination, 71 had NRF (no radiographic finding (NRF) group), 45 had SRF (severe radiographic finding (SRF) group) with an RS higher than 5, and 145 had an RS between 1 and 5. The latter represents an intermediate group, with horses that could be considered as moderately or severely affected with osteochondrosis depending on the definition given to osteochondrosis^{8,18,19}. Therefore, they were excluded from the correlation analysis for biochemical parameters and OAS.

In order to compare NRF and SRF groups between birth and 6 months of age, these horses were assigned in six others classes (Table 2).

Biochemical analysis

The following parameters were assayed:

- BAP (UII^{-1}). The Hydrasys SEBIA's semi-automated agarose gel electrophoresis system was used as recommended by the International Federation of Clinical Chemistry (IFCC).
- HOP (mg l^{-1}). A protocol defined by the Frank Duncombe Departmental Laboratory in order to determine the collagen concentration present in meat was adapted to serum. It is based on the extraction of free HOP from the serum with perchloric acid, oxidation of the free HOP by chloramine-T, followed by the formation of a red compound with *p*-dimethylaminobenzaldehyde. Photometric measurement was set at a wavelength of 558 nm²⁰.
- OC (ng ml^{-1}). Concentration of OC was measured in serum samples using a competitive radioimmunoassay (OSTK, CIS-Bio International, Inc., Gif-sur-Yvette, France) that cross-reacts with human OC. This commercial kit had previously been evaluated for humans²¹ and used the same detection strategy as those described or validated for horses¹⁵. In order to ensure that the levels measured fell within the range of the assays ($0\text{--}170 \text{ ng ml}^{-1}$), samples were diluted 1:10 and 1:2 with wash buffer for the foals (1–5 months) and the yearlings (6–18 months), respectively. The limit of sensitivity of the assay was 0.50 ng ml^{-1} and the coefficients of variations of intra- and inter-assays in this study were 3.0 and 5.5%, respectively.

Mathematical model and statistics

The logarithmic model was used to determine the changes of BAP, HOP and OC with age: $Y = a \ln(X) + k$, where X represents the age of the foal in days and k the value at birth (day 1).

Kinetics were linearized by logarithmic transformation of the age and then tested using linear regression. Slopes

Table 2 Mean values of biochemical markers according to age group and osteoarticular status (average and standard deviation) calculated on 395 measurements obtained from the 71 NRF and 45 SRF horses

Period		10 days	10 days–1 month	2nd month	3rd month	4th–6th months
Mean age (days)		5 (3)	22 (6)	46 (8)	77 (9)	140 (28)
Measurements		32	82	61	83	137
HOP (mg l ⁻¹)	SRF	14.3 (3.6) B ab	16.7 (3.2) a	14.9 (1.6) ab	12.4 (2.0) A b	9.3 (3.1) A c
	NRF	16.7 (3.3) A a	16.8 (3.5) a	14.0 (3.4) b	10.9 (2.8) B c	7.6 (2.2) B d
BAP (UI l ⁻¹)	SRF	1171 (677) a	756 (369) b	713 (428) c	524 (138) d	331 (170) e
	NRF	1453 (599) a	690 (299) b	592 (189) c	468 (147) d	319 (132) e
OC (ng ml ⁻¹)	SRF	126.6 (52.1) a	126.4 (32.4) a	97.7 (23.1) b	96.2 (14.6) b	78.3 (21.3) b
	NRF	124.5 (42.8) a	124.7 (26.9) a	106.2 (24.0) b	99.4 (25.7) b	67.2 (25.7) c

Results are expressed as mean (SD).

Significance was set at $P < 0.05$.

Letters a–e in a line indicate, for all horses, differences between ages.

Letters A–B in a column indicate, for each age, differences between osteoarticular status.

and origins were tested using Student's *t*-test. In the aim of amplifying the differences, the comparisons of kinetics were carried out between NRF and SRF horses.

The pertinence of chosen age group was evaluated by the mixed procedure and normal values for age groups were analysed, according to breed and gender, by analysis of variance (General Linear Models (GLM) procedure) with least square means statement to discriminate means.

All statistic tests were performed using the SAS software (SAS Institute).

Results

Mean values

Table 1 indicates mean values, between birth and 18 months of age, established on the 1650 measurements obtained from the 262 horses. The concentrations of bone markers showed a rapid decrease in the first month of age. Between 1 and 6 months, the concentrations of all markers decreased slowly and, after 6 months, there were only small variations. Differences in marker concentrations between breeds appeared clearly during the first 10 days: Tbs had lower values in HOP and OC than FT and SF, but higher values in BAP than the other two breeds. FT had higher values in OC than Tb and SF during the studied period.

There were significant differences between age groups, with large variations from 20 to 40%, or even more, for the BAP within these age groups. The mean values for BAP, HOP and OC between 6 and 12 months were equal to 14, 34 and 29%, respectively, of their values in the first 10 days. There were no significant difference between male and female within each age group.

Table 2 indicates mean values between birth and 6 months of age on 395 measurements obtained from the 71 NRF and 45 SRF horses, according to the breed. For the period of the first 10 days, NRF horses showed a significant higher mean value for HOP (16.7 ± 3.3 vs. 14.3 ± 3.6 mg l⁻¹, $P < 0.05$). For

BAP there was a trend, and NRF horses showed a higher non-significant mean value (1453 ± 599 vs. 1171 ± 677 UI l⁻¹, $P = 0.12$). There was no difference in the mean values between NRF and SRF horses for the other age group.

Bone growth marker kinetics

The predictive average OC value at birth for the 262 horses, was 187 ng ml⁻¹. During the first 6 months, NRF horses had significantly higher OC concentrations than SRF horses (Fig. 2). The values of regression slopes obtained after linearization (-23.95 and -14.85 , respectively) were statistically different when using Student's *t*-test ($t = 2.76$, $P < 0.01$). At birth, the estimated concentrations were 192 and 149 ng ml⁻¹, respectively (significantly different, $t = 3.14$, $P < 0.01$). At the age of 6 months, there was no difference between NRF and SRF horses and the mean OC value was 70 ng ml⁻¹.

The predictive average HOP value at birth for the 262 horses, was 30 mg l⁻¹. During the first 6 months, NRF horses had significantly higher HOP concentrations than SRF horses (Fig. 2). The values of regression slopes obtained after linearization (-5.09 and -2.79 , respectively) were statistically different when using Student's *t*-test ($t = 3.57$, $P < 0.001$). At birth, the estimated concentrations were 35 and 24 ng ml⁻¹, respectively (significantly different, $t = 3.88$, $P < 0.001$). At the age of 6 months, there was no difference between NRF and SRF horses and the mean HOP value was 7 mg l⁻¹.

The predictive average BAP value at birth for the 262 horses was 1895 UI l⁻¹. During the first 6 months, NRF horses had significantly higher BAP concentrations than SRF horses (Fig. 2). The values of regression slopes obtained after linearization (-256.3 and -320.0 , respectively) were slightly but statistically different when using Student's *t*-test ($t = 1.97$, $P < 0.05$). At birth, the estimated concentrations were 1910 and 1620 UI l⁻¹, respectively (significantly different, $t = 2.01$, $P < 0.05$). At the age of 6 months,

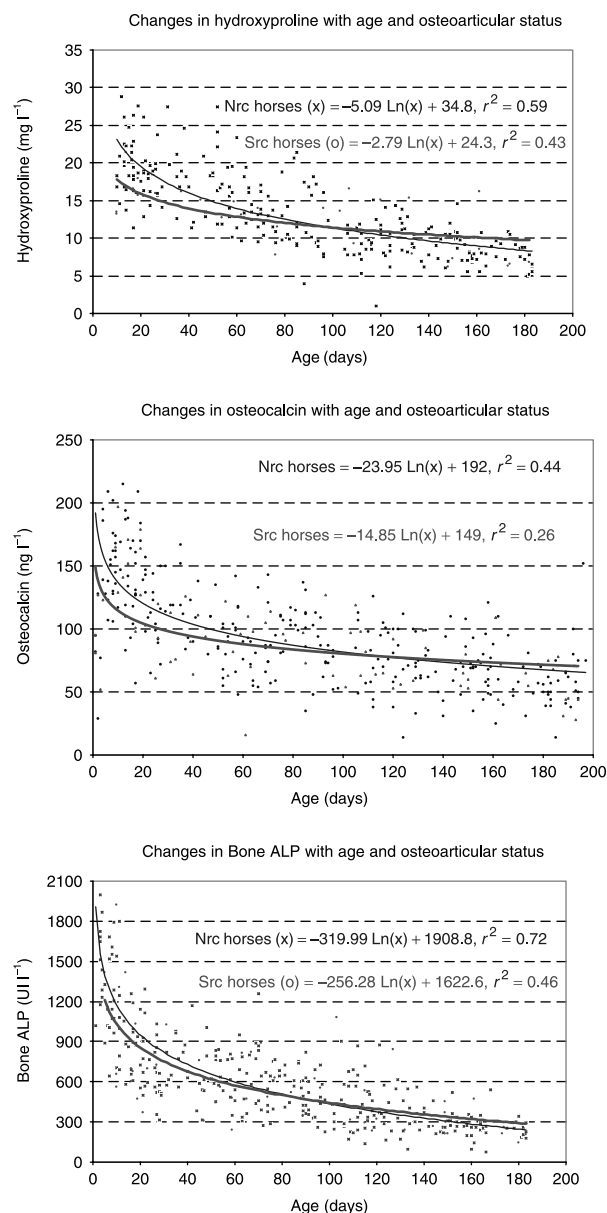


Fig. 2 Changes in biomarkers with age and osteoarticular status (OAS). Fine line represents the regression for the Nrc horses (x) without radiographic changes and bold line represents the regression for Src horses (o) with severe radiographic changes

there was no difference between NRF and SRF horses and the mean BAP value was $260 \text{ U l}^{-1} \text{ mg l}^{-1}$.

There were good correlations between OC and HOP ($r = 0.56$, $P < 0.001$), HOP and BAP ($r = 0.47$, $P < 0.001$) and OC and BAP ($r = 0.41$, $P < 0.001$).

Discussion

Our results showed an inverse relationship among BAP, HOP and OC concentrations and the age of horses as noted in previous works^{3,4,13,14,16,22}. These rapid decreases occurred especially during the first 3 months of life. After 6 months, the values of these

parameters were one-third of or less than the original values.

Slight differences in the serum levels of the markers investigated were found between breeds; during the first 10 days, Tbs had lower values for HOP and OC but higher values for BAP than the other two breeds and, after 1 year of age, FTs had higher values for OC, contrary to the data of Lepage *et al.*¹⁵. On the other hand, we did not find any gender effect, as provided by Carstanjen *et al.*¹² for OC, on the serum levels of these three markers.

Our study was one of the first to examine the relations between the growth biomarker serum levels and the OAS of yearlings at 18 months. Considering the kinetics of the biomarkers, horses with severe radiographic changes had lower concentrations for these parameters than NRF horses during the first months of their life, and not exclusively during the first 10 days of their life when compared by age groups. These observations contradict Billingham *et al.*²³, who found a positive correlation between OC and severity of lesions expressed as macroscopic osteochondrosis severity score and number of osteochondrosis lesions at 5 months of age. Jackson *et al.*²⁴ found that OC concentrations were significantly higher in horses aged 21 months that subsequently developed dorsal metacarpal disease. We also found that the concentration of markers became slightly higher after 3 months in horses with severe radiographic changes but, at this age, marker concentration is not more than one-third of the values at 10–60 days.

These parameters appear to be both good direct biomarkers of bone metabolism: BAP and OC as biomarkers of anabolic processes¹ and HOP as an indicator of catabolic processes⁹. Even if HOP is not a specific bone marker (it is also found in connective tissues), it has been acknowledged that most of the circulating HOP in serum was produced by bone catabolism in growing horses²⁰. Very little is known about the control of bone matrix formation by these markers, but it is thought that OC functions as a negative regulator of osteogenesis. Ducy *et al.*²⁵ demonstrated that non-production of OC was associated with an excessive bone production and a lack of bone resorption in mice. Horses with lower concentration of OC at birth appeared more likely to develop osteoarticular lesions.

Each marker has a different way of being eliminated, which may reflect differences in their specificity with respect to bone. According to that theory, the measurements of several markers are necessary because they all reflect different physiological mechanisms. According to the fact that higher values would be mostly found during the first 3 months, this period seemed to be the optimal one in which to follow these parameters. The variations between the individuals and

age groups indicated that spot sampling is probably of little help in interpreting the information provided, and a longitudinal study involving repeated measurements should be carried out on an appropriate time basis according to Price *et al.*¹⁶.

Our study had the originality to be conducted in field conditions during 4 years with the following of a great number of horses of three breeds from birth to their departure to the training centre. Many biological parameters were followed, including growth, nutrition, locomotion, handling and housing. Despite everything, these field conditions generated a certain number of limits which restrict the range of this study. Sampling was made between 08.00 and 15.00 hours, depending on the availability of the breeders; circadian changes were not taken into account. Jackson *et al.*²⁶ highlighted a significant circadian rhythm for OC with an estimated amplitude of 7.6% and an estimated peak time of 09.00 h. It may have an effect on the sensitivity and ability to identify the changes with age. Meanwhile, we have a great number of measures, and the variations between the groups are c. 20% except for HOP and OC, between group 1 (10 days) and group 2 (< 1 month).

The list of RF presented here is not exhaustive, because some joints were not radiographed and because only one view per joint was obtained. Views chosen in our protocol were not optimal for detection of all forms of osteochondrosis, for which oblique views are usually recommended^{18,27}. Nevertheless, the lateromedial and dorsopalmar views performed here are easier to obtain in a standardized manner in young and seldom-handled animals, which makes later interpretation of the radiographic files easier and quicker to perform. However, this radiographic protocol gave information on all joints that could be radiographed with a mobile unit. Cervical vertebra and shoulders – often affected by osteochondrosis^{8,28} – were not evaluated in our protocol.

In this study, we have considered only healthy horses having no radiographic changes. Inversely, we classified as injured the animals which the clinicians regarded as being eliminated on grounds of having intense sporting exercise. With this restriction, we had a small number of animals classified as injured. It is foreseeable that, with more subjects really injured, it would have been easier to establish normal values for the different categories of horses.

The choice of following the kinetics of bone markers was made after considering the rapid decrease in serum concentrations of these markers and the large variation within age groups. This procedure defined, using all the measurements, the best curve for representing the evolution of a parameter with age. In our study, the logarithmic model best fitted with experimental data for the three parameters. This model had the

advantage of being linearized by logarithmic transformation of the age and then tested using linear regression. Other models such as polynomial of the second or third order, which can fit with data, are not so easy to interpret. Another point of discussion was the choice of these three markers of bone cell activity. Many other markers of bone metabolism are cited that indicated bone formation or bone resorption^{1,10,11}. Among all these markers, we have chosen OC, BAP and HOP because our laboratory was certified for these analyses that are performed routinely for clinicians and breeders.

In conclusion, the measure of these three markers of bone cell activity at birth and the observation of their kinetics during the first months may be a useful tool to identify horses potentially affected by osteochondrosis.

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