Maturation of the equine medial femoral condyle osteochondral unit

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SUMMARY

Objective: The juvenile equine medial femoral condyle (MFC) is frequently affected with radiographic changes (sclerosis and subchondral luencies) that arise at a similar site to juvenile osteochondritis disseicans (JOCD) in children. There is little information on maturation of the MFC. To describe the normal development of the equine MFC osteochondral unit from birth to 2 years.

Methods: Micro CT, histology and immunohistochemistry were performed on healthy equine MFCs (n = 29) at sites where lesions occur. Parameters assessed included: cartilage thickness; the epiphysial growth plate cartilage organization; the osteochondral junction and progression of endochondral ossification.

Results: From 0 to 6 months, chondrocytes near the articular surface are small and flat and have a characteristic hypertrophic appearance near the osteochondral junction but are not arranged in columns like physeal growth plates. The osteochondral junction is also crossed by cartilage canals containing vessels giving a porous appearance on 3D μCT images. At 7 months of age, a subchondral bone plate compact structure emerged histologically coincident with the end of endochondral ossification (absence of type X collagen immunostain and chondrocyte hypertrophy).

Conclusion: New information is provided on MFC osteochondral unit maturation that will improve our understanding of the development of juvenile equine orthopaedic disease. Equine MFC endochondral ossification is complete at 6 months of age. The immature osteochondral junction may be structurally fragile because of its microarchitecture and susceptible to focal traumatic events that induce developmental lesions.

1. Introduction

Understanding the normal maturation of the osteochondral unit of the joint surface is a prerequisite to studying the etiopathogenesis of juvenile orthopaedic developmental disorders at this site in all species. Developmental joint pathology involving the medial femoral condyle (MFC) osteochondral unit includes juvenile osteochondritis disseicans (OCD) in human patients [1] and subchondral cystic-like lesions (or radiolucencies), also linked to osteochondrosis, in horses [2,3]. These pathologies can create joint surface incongruency, subchondral bone inflammation, cartilage degeneration and may lead to debilitating osteoarthritis, if untreated.

In early life the long bones undergo extensive growth and remodeling, particularly in the epiphysis. Longitudinal bone growth of the diaphysis and metaphysis is orchestrated in the epiphyseal plate (physiophyseal cartilage), whose cartilage fully transforms into bone. However the articular surface region of the juvenile femoral epiphysis, termed the articular epiphyseal cartilage complex (AECC) [4], also described by Hunzicker et al. as a surface growth plate in a rabbit study [5], shapes the spheroidal condyle's articular surface. Although there are several histological descriptions of the physseal growth plate structure in many species [6,7] there is sparse information on the structure of the femoral condyle AECC at different stages of postnatal maturation except in a small animal (rabbit) [5]. The deeper epiphyseal growth cartilage expands and transforms, through a sequence of steps of endochondral ossification to become the adult osteochondral unit. The mature osteochondral unit consists of hyaline and calcified cartilage interlinked to the compact cortical subchondral bone plate overlying trabecular bone.

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2. Materials and methods

2.1. Animal and collection procedures

Specimens were collected from the Irish Equine Center’s veterinary post-mortem room. Distal femurs of Thoroughbred foals and horses (\(n = 61\)), presented for post-mortem for reasons unrelated to this study, were harvested within 6 h of death. Femurs (\(n = 122\)) were sectioned proximal to the trochea, dissected of soft tissues and fixed in 10\% formalin until transport to the Comparative Orthopaedic Research laboratory, Université de Montréal.

2.2. Clinical Tomodensitometry (CT) triage for selection of normal specimens

The MFCs of femurs were excised with a band saw to permit rapid CT segmentation. The cartilage was desegmented. The cartilage was decalciﬁed with a parafﬁn acquisition scheme and converted to subchondral bone at the ossiﬁcation front by the process of endochondral ossiﬁcation. At the metaphyseal growth plate, the sequence of events of endochondral ossiﬁcation include proliferation and hypertrophy of the growth cartilage chondrocytes with expression of type X collagen followed by mineralization (calcification) and enzymatic resorption. Osteoid is then synthesized by osteoblasts to form the subchondral bone structure. The organization of epiphyseal growth cartilage has been described as being columnar, similar to the metaphyseal growth plate, but this has been questioned and is not the case in growing rabbits.

Horses are a suitable large animal model for cartilage repair and naturally occurring joint disease investigations due to restricted access to human material and ethical limitations. Consequently, understanding the normal development of the femoral epiphysis, a site of many cartilage repair studies and also OCD and cysts is important in this species. Some aspects of maturation of the equine distal femoral epiphysis from the fetal stages have been previously been investigated by CT, MRI and histology and in 1-day-old foals by 3T MRI, and from birth to 6 months of age by radiography and ultrasonography.

Knowledge of epiphyseal growth plate cartilage maturation may also potentially inform tissue engineering scientists as to strategies to favor neo-tissue integration at the osteochondral junction. Furthermore, endochondral ossiﬁcation cellular and molecular events including both chondrocyte hypertrophy and type X collagen are recapitulated in osteoarthritic and bone marrow lesions, probably due to healing, so the developmental mechanisms are also involved in many diseases.

The earliest histological evidence of osteochondrosis is a focal area of chondronecrosis, due to failure of the cartilage canal blood supply, in the epiphyseal growth cartilage in horses and pigs. The exact cause of the failure is unknown but a focal internal trauma of the epiphyseal growth plate has been postulated (reviewed by Laverty 2013). The objective of the study was to describe the maturation of the equine MFC osteochondral unit by analyzing structural and cellular organization from birth to skeletal maturity in a large animal. The results will be a foundation for future studies of juvenile lesions occurring at this site.

2.4. \(\mu\)-CT analysis

2.4.1. 3D images

The \(\mu\)-CT images were binarized (black and white) using a repeatable optimized threshold of gray scale to distinguish bone from the surrounding marrow cavity. This threshold was defined as a percentage of the gray value of a fixed marker scanned with each specimen. The selected threshold was used for three-dimensional (3D) reconstruction of the entire condyle in order to visualize the ossiﬁcation front. The adult osteochondral unit includes several zones that have been variably deﬁned in prior studies. For the purposes of this investigation imaging juvenile tissues, a non-calciﬁed cartilage zone was deﬁned as incorporating the superﬁcial articular cartilage and underlying non-calciﬁed growth cartilage. It is impossible to differentiate between juvenile calciﬁed cartilage (i.e. growth cartilage that has undergone calcification during endochondral ossiﬁcation at the osteochondral junction) or adult calcified cartilage and the underlying compact subchondral bone on \(\mu\)-CT. Consequently, the mineralized zone (MZ) was deﬁned as either juvenile or mature calciﬁed cartilage with the underlying subchondral cortical bone plate. Qualitative observations were made of the morphological features of the surface and changes with maturation.

2.4.2. MFC region of interest (ROI) analysis

A rectangular prism (1 cm\(^3\) region of interest (ROI)) was manually drawn on the MFC subchondral bone for additional regional investigation (Fig. 1B and Item S2 (video) and Item S3 Supplementary information online). The ROI was located in the craniolateral part of the MFC, oriented craniodistal-caudoproximally in an approximatively sagittal plan (Figure 1C and D). Its position and orientation was selected based on sites where OCD lesions arise in human patients and equine subchondral radiolucenties are identiﬁed in horses.

Supplementary video related to this article can be found at.

2.4.3. Non-calciﬁed cartilage (\(C_{\text{NH}}\)) and mineralised Zone thickness (MZZ)

The non-calciﬁed cartilage (\(C_{\text{NH}}\)) zone and subchondral mineralized zone thicknesses (MZZ) were measured on 2D sagittal images through the center of the ROI. At ﬁrst, the borders of the two zones were manually segmented. The cartilage was deﬁned as the less opaque layer (soft tissue opacity) overlaying the mineralized structure (Fig. 1B). The boundary between the compact subchondral mineralized zone and the trabecular bone was easily identiﬁed as soft tissue opacity (bone marrow) between the bone trabeculae on binarized images. Measurements were taken perpendicular to the articular surface at three sites within the ROI and the average of the three measures was made for each specimen.

2.4.4. Bone volume

The trabecular bone in the ROI was divided into three 1 cm\(^3\) volumes from the boundary between the mineralized zone and trabecular bone to the center of the condyle: BV\(_1\), BV\(_2\) and BV\(_3\) respectively (Fig. 1B). The bone volume fraction (BV/TV) in each region was calculated.

2.5. Histology

The MFCs were sectioned in the sagittal plane through the center of the ROI to obtain 0.5 cm thick osteochondral slabs (Fig. 1D). The slabs were decalcified in 14\% EDTA, ﬁxed in 70\% alcohol and embedded in parafﬁn. Five micrometer thick sections were cut from each slab and stained with hematoxylin, eosin, phloxin and safron (HEPS) and Safranin O Fast micro-computed tomography (\(\mu\)-CT, Xtek HMXST 225, Nexus Metrology, Canada). Projections (\(n = 3142\)) were acquired over 360\(^\circ\). Other imaging parameters were: 65 kV, 64 \(\mu\)A and acquisition time = 2 s. The scan resolution was limited by the specimen size and ranged from 60 to 78 \(\mu\)m per voxel. The scans were reconstructed and viewed using CT Pro 3D software (Nexus Metrology) and Dragonfly 3.0 (Object Research System, Montreal).
Green (SOFG) for evaluation. All the sections were digitalized using a Leica DM4000B microscope attached to an Allied Vision Prosilica GT1920C camera and the software Panoptiq. Calibration was performed using a micrometer slide to permit accurate measurements.

2.5.1. Qualitative assessment

On HEPS stained sections the presence of cartilage canals, the morphology and organization of chondrocytes, the appearance of the ossification front, or the tidemark (junction between calcified and hyaline cartilage), when present [39], and the structure of the subchondral bone were subjectively assessed. An atlas of representative osteochondral units was made for different age groups. A distal femoral physeal growth plate of a 2-month-old foal was also processed for HEPS staining for an illustrative comparison of differences in cellular organization.

2.5.2. Quantitative analysis

The cartilage thickness was measured at 3 sites matched to the μ-CT measurements. To assess endochondral ossification changes with maturation, chondrocyte hypertrophy was assessed on HEPS sections. The diameter of chondrocyte lacunae (n = 6/zone), was measured in a superficial zone closest to the articular surface where the chondrocytes had a comparable appearance to chondrocytes within the resting zone of the physis [40] and a deeper zone directly above the ossification front where chondrocytes appeared hypertrophic. Chondrocyte lacuna dimension is representative of unfixed chondrocyte morphometry [41]. The chondrocyte lacunar volume (V) was derived from the diameter (D) (hypothesizing chondrocytes were spherical) by the following formula: V = (4π/3)(D/2)^3. A ratio of the chondrocyte lacunar volume (V/V_chondr) was also calculated for the superficial and deep hypertrophic zones.

2.6. Type X collagen immunohistochemistry

Type X collagen is expressed in the growth plate in the matrix of hypertrophic chondrocytes and believed to facilitate growth cartilage calcification [42] and considered a marker of endochondral ossification [43]. Mutations in type X collagen are responsible for impaired growth plate function and metaphyseal chondrodysplasias in humans [44]. The sections were immunostained with type X collagen rabbit primary antibody (Abcam inc, Cambridge, MA, USA). The protocol is described in detail in (Item S4, Supplementary information online). Sections were evaluated for the presence or absence of positive type X collagen immunoreactivity. The prevalence (number of specimens with positive immunoreactivity to type X collagen/total number of specimens in the same age group) was calculated for each age group.

2.7. Statistical analysis

For quantitative variables measured on μ-CT images (Cth and MZth), three regression models were tested using the NLIN procedure in SAS 9.4 (SAS Institute., Cary, North Carolina, USA). The first model was a simple linear regression against age. The second was a piecewise regression, which fits two linear segments with different slopes about a breaking point. The third model was a regression involving a power function to capture the non-linear trend in the data. The model retained was the one...
with the lowest Akaike's Information Criterion (AIC) [45]. A mixed linear model with region (BV1, BV2 and BV3) as a fixed factor, specimen as a random factor and age as co-factor was employed to detect differences between bone volumes. This allowed us to test the relationship between volume and age at each position. Tukey's post-hoc tests were used to compare the means in the different age groups. The comparison of cartilage thickness on histologic sections and on μ-CT images for each MFC was performed using a paired t-test. The association between positive type X collagen immunostain and age was compared using Chi-squared test. The difference of the prevalence of positive type X collagen immunostain and age was compared using a paired t-test.

3. Results

3.1. Clinical CT triage - specimen selection

Thirty MFCs were selected after screening CT imaging to obtain healthy specimens to study condyle maturation in different age groups. The age and sex of selected specimens is provided in Table 1. Additional information is provided in the flowchart (Item S5, Supplementary information online). One condyle per animal was included (independent specimens). The specimens were analyzed in six age groups: <2 months (n = 6); 2 - <4 months (n = 4); 4 - <6 months (n = 5); 6 - <8 months (n = 5); 8 - <12 months (n = 3) and 12 - <24 months (n = 6). A maximum of 6 specimens was included in each age group because of financial constraints in respect to uCT imaging.

3.2. Macroscopic evaluation

All the specimens had normal appearing cartilage without lesions on macroscopic examination. Cartilage canal vessels were visible in specimens younger than 2 months old mainly in the cranial part of the condyle (Fig. 2). They decreased between 2 and 6 months and were not visible after 6 months of age.

3.3. μ-CT analysis

3.3.1. 3D images

One specimen (12 - <24 months old) was excluded from further analysis as a focal delay of ossification was identified on μ-CT images but not on clinical CT. Representative 3D reconstructions of the ossification front of the MFCs of each age group are shown in Fig. 3. In the youngest specimens, the mineralized surface had multiple circular small depressions (pores) that were distributed homogenously. The pore number and size decreased with age and disappeared at approximately 6 months (Fig. 3). The pores initially disappeared in the weight-bearing surface, but were still visible in the cranial aspect, the medial third and the caudal half of the MFC (Fig. 3). After 6 months of age, the surface was smooth without pores.

3.3.2. Cartilage thickness (Cth)

The mean non-calcified cartilage thickness ranged from 3.1 mm for specimens less than 2 months old to 1.5 mm in mature specimens (12-<24 months old) (Table 2). Cartilage thickness decreased with age to 213 days but remained constant thereafter (Fig. 4). The best model (AIC) to describe Cth changes with age was the piecewise regression (p < 0.0001) with a breaking point at 213 days.

3.3.3. Mineralized zone thickness - MZth

A compact mineralized zone was present on μ-CT images, in all specimens studied. The mineralized zone varied from 0.5 to 1 mm thickness (Fig. 4, Table 2) and decreased linearly with age up to 80 days and remained constant thereafter (AIC piecewise regression; p = 0.004).

3.3.4. Bone volume

Bone volume data (sites BV1, BV2 and BV3) is presented in Table 2. The mean bone volume was greatest in the superficial zone (BV1), near the articular surface and decreased in BV2 and again in BV3 (Fig. 4). The mixed model revealed a significant effect of the site (p < 0.0001) all ages combined. There was a significant positive linear relation between the age and BV1 (p = 0.0006) but not with BV2 and BV3.

3.4. Histology

No histological lesions were identified in any of the specimens studied.

3.4.1. Cartilage canals

Cartilage canals were detected histologically up to 4 months of age and underwent chondrification (i.e. invasion by chondrocytes) from 2 to 6 months (Fig. 5B). The cartilage canals crossed the ossification front, creating V-shape projections of cartilage into the subchondral bone (Fig. 5A and B) up to 6 months. The V-shapes corresponded with the pores seen on μ-CT images (Fig. 5D). Patent or chondrified canals were no longer observed in these specimens after 7 months of age on histology.

3.4.2. Chondrocyte morphology

Four zones were identified in the epiphyseal growth plate and overlying articular cartilage based on the chondrocyte morphology (Fig. 6). From the articular surface to the osteochondral junction they included: superficial, intermediate, proliferative and hypertrophic zones. In specimens <4 months of age, a thin superficial zone had a dense population of small flattened chondrocytes with the long axis oriented parallel to the articular surface. The next intermediate zone was thicker, with slightly larger and rounder chondrocytes. The proliferative layer had a dense population of slightly larger chondrocytes. The hypertrophic zone adjacent to the ossification front, on the other hand, contained characteristic large hypertrophic chondrocytes that were dispersed relatively evenly throughout the cartilage.

Table 1

| Group       | Age Months | Sex |
|-------------|------------|-----|
| <2 months   | 0.5        | F   |
| n = 6       | 0.5        | F   |
|             | 0.5        | M   |
|             | 0.7        | M   |
|             | 0.7        | M   |
| 2 - <4 months| 2         | F   |
| n = 4       | 2.5        | F   |
|             | 3          | F   |
|             | 3.5        | F   |
| 4 - <6 months| 4         | F   |
| n = 5       | 4          | F   |
|             | 5          | M   |
|             | 4.5        | F   |
| 6 - <8 months| 6         | M   |
| n = 5       | 6          | M   |
|             | 7          | F   |
|             | 7.5        | F   |
| 8 - <12 months| 8        | F   |
| n = 3       | 8.5        | F   |
|             | 9          | M   |
| 12 - <24 months| 12    | M   |
| n = 6       | 14         | M   |
|             | 14.5       | F   |
|             | 17.5       | F   |
|             | 18         | F   |
|             | 19         | F   |

Key: M = male, F = female.
when compared with the characteristic columnar organization identified in physeal cartilage (Fig. 6F). An irregular boundary was present at the osteochondral junction. At 12 months, the superficial and intermediate zones of the articular cartilage were present but only 1 additional zone was present near the osteochondral junction. The chondrocytes in this zone were only slightly larger than in the intermediate zone and a smooth border was present with the underlying bone.

3.4.3. Chondrocyte volume

The mean chondrocyte volume \(V_{\text{chondr}}\) for each age group is presented in (Item S6, Supplementary information online). In the superficial zone, the mean volume remained stable with age whereas in the deeper zone, the mean volume was 11 times larger in the 0-<2 months old group compared to the 12-<24 months old group. The chondrocyte lacunae volume ratios decreased from 23 (0-<2 months) to 3 (8-<12 and 12-<24 months) (Fig. 6B), in the deep zone.

3.4.4. Calcified cartilage

The tidemark (i.e. border between hyaline and calcified cartilage) was first clearly evident as an undulating pale line in some of the specimens over 12 months of age (Fig. 6D) and (Item S7 Figure S7 supplementary information online).

3.4.5. Subchondral bone

In all specimens less than 6 months, the subchondral bone directly underneath the epiphyseal growth cartilage was composed of an uneven margin of trabecular-like bone (Fig. 5A and B). In the youngest specimens, the boundary between cartilage and bone was very irregular and friable when sectioned (Fig. 5A & B). The compact mineralized layer observed on \(\mu\)-CT images in these specimens was interpreted as subarticular epiphyseal growth cartilage undergoing mineralization in the process of endochondral ossification. At about 6 months of age, the subchondral bone structure, immediately below the cartilage, changed from a trabecular to a more compact tissue structure forming a subchondral bone plate (Fig. 5C) on histology and corresponding \(\mu\)-CT.

3.4.6. Quantitative analysis – cartilage thickness

The cartilage thickness measured on HEPS stains ranged from 3.6 mm (0-<2 months) to 1.2 mm (12-<24 months) (Table 2). The cartilage thickness was not significantly different \((p = 0.9)\) from the measurements on \(\mu\)-CT images.

3.5. Type X collagen immunoreactivity

The prevalence of positive type X collagen immunoreactivity, a marker of endochondral ossification, is presented in Table 2. Positive type X collagen immunoreactivity was identified surrounding the
hypertrophic chondrocytes up to 6 months of age (Fig. 7). The positive type X collagen immunoreactive region was wider in specimens less than 2 months old compared to specimens in the 4-<6 months old age group. The exact chi-squared test showed a statistically significant association between the presence of positive type X collagen immunoreactivity and age (p = 0.0002). The Benjamini-Hochberg procedure showed no significant difference of prevalence between the 0-<2 months group and the 2-<4 months (p = 0.40) and 4-<6 months (p = 0.46) groups. The prevalence of positive type X immunoreactivity was lower in the 6-8 months group (p = 0.015), 8-<12 months group (p = 0.012) and 12-<24 months group (p = 0.0022) compared with the 0-<2 months group.

4. Discussion

The results described here represent novel information on the maturation of the osteochondral unit of the equine MFC and provide a foundation for understanding juvenile developmental pathologies that arise at this site. Several interesting findings are documented. First, the 3D ossification front constructed from the μ-CT images is highly porous (portals for blood vessels, confirmed on histology) after birth but matures to a compact smooth interface at 6 months. The subchondral cortical bone plate structure also emerges histologically at this same timepoint but a tidemark was not identified until 12 months of age. Second, we report that the process of epiphyseal growth cartilage endochondral ossification, characterized histologically by chondrocyte hypertrophy and positive type X immunoreactivity, occurs up to 6 months of age in the equine femoral condyle, but is arrested thereafter. The non-calciﬁed cartilage thickness, including both the epiphyseal growth cartilage and overlying articular cartilage, also decreases up to this same timepoint. Interestingly a columnar arrangement of chondrocytes, a characteristic of physisal cartilage, was not detected in the epiphyseal growth cartilage. The chondrocytes were overall more diffusely dispersed throughout the

### Table 2

MFC epiphyseal cartilage growth plate parameters assessed by μ-CT, histology and immunohistochemistry.

| Age (Months) | μ-CT | Histology |
|--------------|------|-----------|
|              | \( C_{th} \) (\( \mu \)m) Mean (\( \pm \)SD) | \( MZ_{th} \) (\( \mu \)m) Mean (\( \pm \)SD) | \( BV_1 \) (%) Mean (\( \pm \)SD) | \( BV_2 \) (%) Mean (\( \pm \)SD) | \( BV_3 \) (%) Mean (\( \pm \)SD) | Chondrocyte volume ratio Mean (\( \pm \)SD) | Prevalence type X Col + |
| <2 (n = 6)   | 3132 (651) | 771 (191) | 37.9 (6.4) | 28.2 (3.9) | 24.7 (3.8) | 2950 (717) | 22.7 (8.6) | 100% |
| 2 - <4 (n = 4) | 2219 (285) | 542 (65) | 50.4 (11.6) | 25.1 (7.4) | 18.4 (7.4) | 2220 (340) | 14.5 (2.8) | 75% |
| 4 - <6 (n = 5) | 2273 (640) | 615 (104) | 43.5 (7.5) | 28.6 (6.8) | 23.3 (6.8) | 2316 (693) | 14.4 (7.0) | 80% |
| 6 - <8 (n = 5) | 1525 (94) | 615 (76) | 50.2 (5.3) | 30.7 (5.9) | 23.3 (5.3) | 1732 (185) | 6.0 (3.2) | 20% |
| 8 - 12 (n = 3) | 1467 (204) | 544 (46) | 50.9 (4.6) | 28.0 (4.1) | 21.2 (5.5) | 1643 (450) | 3.0 (2.1) | 0% |
| 12 - <24 (N = 6) | 1511 (200) | 563 (88) | 53.9 (4.9) | 33.0 (6.9) | 24.4 (7.2) | 1535 (235) | 3.1 (1.7) | 0% |

The prevalence type X Col + was calculated as the number of specimens with positive immunoreactivity to type X collagen/total number of specimens in the same age group.

Key: \( C_{th} \) = non-calciﬁed cartilage thickness; \( MZ_{th} \) = mineralized cartilage zone thickness; \( BV \) = bone volume; Chondrocyte lacunar volume ratio = \( V_{superficial \ zone} / V_{deep \ zone} \).
matrix. Combined, these findings suggest maturation occurs up to 6 months of age in the equine MFC.

The results of our study confirm and extend the findings of others. Micro CT images confirm that the irregular porous ossification front matures into a smoother compact structure similar to the distal tibia in horses and ulna in dogs [33,46]. However, this layer has previously been interpreted as bone in prior studies [33,46,47] without a site-matched histological comparison. The subchondral mineralized zone that we observed in the juvenile specimens (0–6 months) did not correspond to any compact bone structure histologically on site-matched sagittal sections. We believe that the μ-CT subchondral plate-like zone described by others, before 6 months old, corresponds to the juvenile calcified cartilage zone of the epiphyseal growth cartilage, part of the process of endochondral ossification. The calcified cartilage layer of the epiphyseal growth cartilage has been investigated histologically (undecalciified PMMA sections) in rabbits during postnatal development [5]. μ-CT cannot differentiate between calcified cartilage and cortical bone or reveal cellular or matrix details [48] and a comparison with histology is required. The osteochondral unit appears structurally immature on histology up to 6 months of age with an underlying trabecular structure alone on the MFC weight-bearing surface.

Fig. 4. Micro CT determination of cartilage and mineralized zone thickness and bone volume. A. μ-CT sagittal image illustrating the region of interest (ROI). B. Cth decreased from 0 to 213 days of age and remained constant thereafter (piecewise regression with two linear segments; p < 0.0001). C. MZth decreased up to 80 days and remained constant thereafter (piecewise regression with two linear segments; p = 0.004). D. Bone volume at three depths (BV1, BV2 and BV3). BV1 was significantly greater than BV2 and BV3 and BV2 was also significantly larger than BV3. BV1 alone increased significantly with age. Key: μ-CT = micro-computed tomography; Cth = non-calcified cartilage thickness; MZth = mineralized cartilage zone thickness; BV = bone volume.
In the current study, we expanded findings on the osteochondral unit and observed that the porous ossification front on 3D μ-CT images corresponds to cartilage canals on histology crossing the ossification front making V-shape indentations histologically as we observed previously in the equine metacarpus [11]. This is in agreement with a previous μ-CT study of the tarsus of foals [47]. The subchondral cortical bone plate on μ-CT after 6 months is compact with no pores present and corresponds to the histological structure of a compact subchondral bone plate.

Interestingly although the hypertrophic zone of the epiphyseal
growth cartilage contained characteristic large hypertrophic chondrocytes, they were not arranged in the characteristic columnar organization of physeal cartilage. The latter’s growth is in a more unidimensional plane, contributing to longitudinal growth of the bone. The organization we observed is possibly related to a requirement for a more multidimensional expansion in the surface growth plate for the development of the spheroidal shape of the condyle. Currently much of what is known about cartilage growth in long bones relates to longitudinal growth; little is known about the control of features such as the condyles [49]. The 3D μ-CT reconstruction also revealed that endochondral ossification progresses at different phases in different dimensions, but this requires additional quantitative assessments in future studies.

Wolff’s law [50] states that bone adapts to loading and remodeling occurs. We anticipated a change of the bone volume after birth, with maturation, as previously described [46]. However, we did not find any significant change in the bone volume fractions of the condyle ROIs studied with age, except in the region closest to the articular surface (BV1). This may be due to the age group selected in our study and to technical limitations of BV measurement. The aforementioned study detected a difference only between foals of less than 12 h old and a 2-<4 month old group [46]. Moreover, it has been reported that automatic segmentation is more robust to assess bone volume [51]. In our study, this was not easily applicable due to the heterogeneity of the MFC that included variation in size and shape with age.

Our observations on the development of the equine MFC differ from our previous investigations of the equine femoral trochlea where we measured a greater cartilage thickness [13,52]. These findings reveal that within the same bone the osteochondral unit matures at variable rates at different sites (femoral condyle versus trochlea) and could be related to principal biomechanical forces at each site: weight-bearing compressive forces at the MFC versus shear forces at the trochlea.

Some limitations of the current study are acknowledged. For ethical reasons, we did not elect to euthanize large numbers of healthy Thoroughbred foals and yearlings to answer our research questions but rather chose to avail of postmortem material. We also recognize that the exact history of all the specimens is unknown and that perhaps some intercurrent disease could have influenced our findings. However, the included specimens were triaged to include structurally normal appearing condyles to the best of our ability.

The manual selection of the ROIs may not be exactly reproducible because of changes in shape of the condyle with growth. Every effort was made to ensure that the position and orientation of the ROI was comparable among all specimens. Furthermore, the inclusion of non-decalcified histological sections (PMMA) would have made site-matched histological observations of calcified cartilage more robust.

The current study assesses normal development of the equine MFC employing a multimodal approach that included macroscopic assessment, clinical CT triage and subsequently uCT, histological evaluation and collagen X immunoreactivity. The investigation could have been improved by employing additional methodology to assess other pathological parameters such as cell death. This would have permitted us to detect and eliminate potential specimens that we assumed were normal but that had changes compatible with molecular upstream events of early osteochondrosis pathology. Furthermore, the role of type X collagen in endochondral ossification is a subject of debate, but recent in vitro studies employing mesenchymal stem cells have shown that it is essential for endochondral bone formation thereby providing some additional evidence that it may be employed as a marker of this process in juvenile specimens [53].

In addition, the method we employed for the estimation of chondrocyte volume was based on the assumption that chondrocytes are spheres. Although most chondrocytes in the deeper zone of adult human articular cartilage have a spherical shape, when cytoplasmic processes were excluded, 50% of full depth adult articular cartilage chondrocytes are considered spherical on 2 photon laser scanning microscopy [54]. Although the estimation of chondrocyte volume allowed us to detect clear differences between the zones of cartilage, a more exact estimation of chondrocyte volume would require the application of the complex principles of stereology that allow a more accurate assessment of 3D volume from 2D sections [55].

The horse is one of the large animal models recommended for cartilage repair studies by the FDA and also the femoral condyle is the ideal location for these studies [56]. Although it is an endorsed animal model, it could be argued that species differences exist and that the timeline for the development of naturally occurring lesions such as OCD is different from humans. That being said, the study herein also provides important information for those involved in cartilage repair and tissue engineering as it provides new knowledge of the normal structure of equine osteochondral unit during normal development at this key location.

Fig. 7. Type X collagen immunostain: a marker of endochondral ossification. A. At < 2 months of age, hypertrophic chondrocyte lacunae and matrix are strongly stained with type X collagen near the osteochondral junction (blue dotted line). B. At 4–6 months of age, immunostaining decreases indicating a reduction in endochondral ossification with age. C. After 6 months of age, immunostaining is not apparent, indicating endochondral ossification has ceased. Scale bar 200 μm.
In conclusion, the data presented in our study reveals that the osteochondral unit of the equine MFC epiphysis undergoes extensive remodeling and endochondral ossification events up to 6 months of age and the adult mature structure emerges thereafter. Structurally the osteochondral junction could be more susceptible to injury due to the resorption events of endochondral ossification and lack of a subchondral plate structure prior to 6 months, however the biomechanics at this site in relation to developmental disease requires additional study.

Author contributions
Conception and design (TL, SL, CG, ES, UF, GB); Analysis and interpretation of the data (TL, SL, CG, ES, UF, GB, HR, LC); Provision of study materials or patients (UF); Collection and assembly of data (TL, CL, HR); Statistical expertise (GB); Drafting of the article & critical revision of the article for important intellectual content (TL, SL, CG, ES, UF, GB); Final approval of the article (TL, SL, CG, ES, UF, GB, HR, LC); Obtaining of funding (SL); Administrative, technical, or logistic support (CL, HR).

Dr Sheila Laverty (Sheila.laverty@umontreal.ca) takes responsibility for the integrity of the work as a whole, from inception to finished article.

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Declaration of Competing Interest
All authors have no conflicts of interest to report.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2020.100029.

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