Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Companion Animal Clinical Sciences

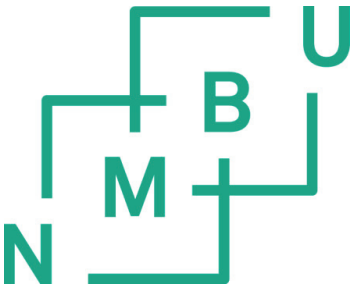
Philosophiae Doctor (PhD)
Thesis 2019:73

The role of the blood supply to growth cartilage in physeal osteochondrosis and septic arthritis/osteomyelitis in foals and pigs

Bjørn Håkon Wormstrand

The role of the blood supply to growth cartilage in physeal osteochondrosis and septic arthritis/osteomyelitis in foals and pigs

Vekstbruskens blodtilførsel i fyseal osteochondrose og septisk artritt/osteomyelitt hos hest og gris



Philosophiae Doctor (PhD) Thesis

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A good plan, violently executed today, is better than a perfect plan executed next week.

George S. Patton, US Army General

Contents

Acknowledgements

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Bjørn Wormstrand, Oslo, July 13th 2019

List of papers

Paper I:

Septic arthritis/osteomyelitis may lead to osteochondrosis-like lesions in foals

Wormstrand B, Østevik L, Ekman S, Olstad K

Vet Pathol. 2018 Sep;55(5):693-702

Paper II:

Osteochondrosis in the distal femoral physis of pigs starts with vascular failure

Olstad K, Wormstrand B, Kongsro J, Grindflek E

Vet Pathol. 2019 May 6:300985819843685. doi: 10.1177/0300985819843685. [Epub ahead of print]

Paper III:

Development of the blood supply to the medial femoral condyle of foals and relevance to osteomyelitis

Wormstrand BH, Fjordbakk CT, Griffiths DJ, Lykkjen S, Olstad K

Submitted, *Equine Veterinary Journal*

Paper IV:

Presence of dye in growth cartilage canals following intraarticular injection, regional intravenous and intraosseous perfusion in an ex vivo porcine model

Wormstrand B, Fjordbakk C, Viscasillas J, Olstad K

Submitted, *Veterinary Surgery*

Summary

Long bone growth takes place by enchondral ossification of growth cartilage, a temporary tissue only present at the articular-epiphyseal cartilage complex (AECC) and the physis in skeletally immature animals and humans. In contrast to articular cartilage, growth cartilage has a blood supply that runs in cartilage canals and consists of an afferent arteriole, a capillary network and one or more efferent venules. Vascular failure of cartilage canals at the point of incorporation into bone may result in focal ischemic chondronecrosis, causing a delay of enchondral ossification. A delay of enchondral ossification is the definition of osteochondrosis, which is a clinical problem in many species including horses and pigs. The presence of cartilage canals relative to age and anatomical location is therefore widely researched in these two species, and there is considerable inter-species similarities. There may be several underlying causes for vascular failure of cartilage canals. For example, in experimental studies in chickens and pigs bacterial binding to growth cartilage extracellular matrix through endothelial discontinuities in cartilage canals was demonstrated. Endothelial discontinuities are present during active cartilage canal ingrowth and regression. Bacterial binding within cartilage canals may result in local infection occluding the cartilage canal, leading to vascular failure. Septic arthritis and osteomyelitis are common clinical entities in foals, the majority of which are of haematogenous origin. Recently, a higher prevalence of osteochondral lesions in horses that survived infection as foals was reported. The aim of this thesis was to investigate the involvement of cartilage canals in the pathogenesis and treatment of septic arthritis/osteomyelitis in foals, and to describe the blood supply with relevance to physeal osteochondrosis.

Bones from foals euthanized due to septic arthritis/osteomyelitis were examined by histology to determine the presence and consequences of bacterial infection in cartilage canals. Bones from healthy pigs and foals were evaluated by histology, computed tomography (CT) and micro-computed tomography (micro-CT) to describe the vascular supply to the physis and potential consequences of vascular failure. Finally, three clinically relevant treatment techniques for local administration of antibiotics were tested in a post mortem porcine model, investigating distribution of dye (used as a histological marker of antibiotics) into cartilage canals, with emphasis on the physis. .

In foals with septic arthritis/osteomyelitis, bacteria were observed in the distal tips of necrotic cartilage canals in both the AECC and the physis. Areas of focal ischemic chondronecrosis centred on one or more necrotic cartilage canals were observed, sometimes with evidence of focally delayed enchondral ossification, i.e. osteochondrosis.

In clinically healthy pigs, physeal lesions observed on CT scans were histologically confirmed to be osteochondrosis. Areas of hypertrophic, retained cartilage were centred on necrotic cartilage canals and eosinophilic streaks in an identical manner to that of necrotic cartilage described for osteochondrosis in the AECC. This observation provides evidence that physeal osteochondrosis may be a consequence of vascular failure.

The configuration and extent of AECC and physeal growth cartilage blood supply in healthy foals changed with age and involved ingrowth and regression of cartilage canals. Both of these processes entail endothelial discontinuities, potentially providing access to extracellular matrix for bacteria. Subchondral blood supply was also dynamic which may entail endothelial discontinuities, providing similar access to extracellular matrix as in cartilage canals.

Of the three investigated treatment techniques, intravenous regional perfusion resulted in significantly wider distribution of dye to cartilage canals and other tissues when compared to intraosseous perfusion and intraarticular injection in pigs. The exception was the joint cavity where only dye injected into the joint was present.

In conclusion and based on the above findings, the blood supply to growth cartilage is essential in the pathogenesis of both physeal osteochondrosis and osteomyelitis. Physeal osteochondrosis may occur due to vascular failure, and physeal osteomyelitis may occur when circulating bacteria gain access to extracellular matrix by endothelial discontinuities in the cartilage canal vessels. These two clinically different diseases are also linked, as osteomyelitis may lead to osteochondrosis as a long term complication by causing septic vascular failure.

Sammendrag

Vekst av rørknokler skjer ved enchondral forbeining i vekstbrusk, et midlertidig vev kun tilstede i epifysen og fysen hos unge, voksende dyr og mennesker. I motsetning til leddbrusk har vekstbrusk en blodforsyning i form av karkanaler, som består av en arterie, et kapillærnøste og en eller flere vener. Svikt av karkanaler når de inkorporeres i underliggende beinvev kan føre til at omkringliggende brusk dør, som igjen kan forårsake fokale forsinkelser i forbeiningen. Dette er definisjonen av osteochondrose, som er en vanlig utviklingssykdom hos blant annet gris og hest, og som ofte karakteriseres av løse beinbiter i ledd (osteochondrosis dissecans, OCD). Distribusjon av karkanaler i forhold til alder og anatomi er derfor godt kartlagt hos disse artene, og viser mange likhetstrekk.

Det kan finnes flere årsaker til at karkanalene svikter. For eksempel har eksperimentelle studier av kylling og gris vist bakteriell binding til ekstracellulær matriks via åpninger i endotelet (det innerste cellelaget i blodkar) i karkanaler. Slike åpninger i endotelet forekommer ved aktiv innvekst eller tilbakedannelse av karkanaler. Bakteriell binding i en karkanal vil kunne resultere i en lokal infeksjon, der karkanalen tilstoppes slik at blodforsyningen svikter.

Infeksjoner i ledd og beinvev er vanlige kliniske problemstillinger hos føll, og skyldes i de fleste tilfeller blodbårne bakterier. Det ble nylig rapportert høyere forekomst av løse beinbiter i ledd hos hester som overlevde infeksjon som føll. Målet med denne avhandlingen var å undersøke karkanalenes rolle i utvikling og behandling ved leddinfeksjoner og osteomyelitt hos føll, samt å beskrive blodtilførsel til fysen og å undersøke om osteochondrose i fysen kan forårsakes av vaskulær svikt.

Knokler fra føll avlivet på grunn av leddinfeksjon og osteomyelitt ble undersøkt histologisk for å kartlegge forekomst og konsekvenser av bakteriell infeksjon i karkanaler. Knokler fra klinisk friske griser og føll ble undersøkt med histologi, datatomografi (CT) og mikro-datatomografi (mikro-CT) for å beskrive blodtilførselen til fysen og eventuelle konsekvenser av en fyseal vaskulær svikt. Deretter ble tre klinisk relevante teknikker for lokalbehandling med antibiotika testet i en post mortem grisemodell for å undersøke distribusjon av fargestoff (brukt som markør for antibiotika) til karkanaler, med hovedfokus på karkanaler i fysen.

Hos føll med leddinfeksjon og osteomyelitt, ble bakterier observert i enden av sviktede karkanaler, lengst ut i brusken, både i epifyseal og fyseal vekstbrusk. Områder med nekrotisk brusk sentrert rundt en eller flere nekrotiske kanaler ble observert, noen ganger med forsinket forbeining, forenlig med osteochondrose.

Lesjoner i fysen observert på CT ble histologisk bekreftet å være osteochondrose hos gris. Områder med forsinket forbeining bestående av hypertrofiske bruskceller var sentrert rundt døde karkanaler og eosinofile streker på samme måte som i død brusk ved tilfeller av osteochondrose i epifysen. Dette bekrefter at osteochondrose i fysen kan skyldes vaskulær svikt på samme måte som i epifysen.

Undersøkelse av knokler fra friske føll viste at blodtilførselen til vekstbrusk både i epifysen og fysen er dynamisk både i antall karkanaler som er tilstede til enhver tid, og, for fysen, hvor disse har sitt opphav. Disse dynamiske prosessene innebærer et potensiale for eventuelle blodbårne bakterier til å binde seg til ekstracellulær matriks i vekstbrusken, siden innvekst og tilbakedannelse av karkanaler medfører åpninger i endotelet. De samme prosessene kan være tilstede i underliggende beinvev, fordi blodtilførselen også her er dynamisk.

Av de tre undersøkte teknikkene for lokalbehandling med antibiotika, gav intravenøs regional perfusjon vesentlig høyere distribusjon av fargestoff til karkanaler og annet vev sammenlignet med intraosseøs regional perfusjon og leddinjeksjon. Derimot var det kun leddinjeksjon som resulterte i fargestoff i leddhulen.

Basert på ovennevnte funn konkluderes det med at blodtilførselen til vekstbrusk spiller en sentral rolle i utviklingen av både fyseal osteochondrose og lokal infeksjon (osteomyelitt). Fyseal osteochondrose kan oppstå ved svikt av fyseale karkanaler, og osteomyelitt kan oppstå ved direkte kontakt mellom blodbårne bakterier og ekstracellulær matriks via åpninger i endotelet, som er tilstede ved innvekst og tilbakedannelse av blodkar i karkanaler. Disse to sykdommene forbindes ved at osteochondrose også kan forekomme som en langtids-komplikasjon av osteomyelitt på grunn av vaskulær svikt med påfølgende ischemisk chondronekrose.

Abbreviations

AECC:	Articular-epiphyseal cartilage complex
CE:	Common era
CT:	Computed tomography
DIRT:	Distal intermediate ridge of the tibia
IgG:	Immunoglobulin G
Micro-CT:	Micro-computed tomography
MRI:	Magnetic resonance imaging
OCD:	Osteochondrosis dissecans
PCR:	Polymerase chain reaction
TEM:	Transmission electron microscopy
WBC:	White blood cell

Introduction

Ex toto non sic pueri ut viri curari debent

[In general, boys should not be treated in the same was as men]

Aulus Cornelius Celsus, *De Medicina,* Book 3, Chapter 7, 1st century CE

A: Brief historical perspective and background for the thesis

To understand pathology, it is first necessary to understand normal anatomy and physiology. The anatomy and physiology of skeletally immature individuals is different from mature individuals. Notably, the immature skeleton contains an additional anatomical structure: the growth cartilage, and is engaged in the physiological process of growth by enchondral (or endochondral) ossification [2]. It is therefore necessary to understand different structures and processes to understand pathology in skeletally immature than mature individuals. In human medicine, paediatrics ("healing of children") was recognised as a speciality by Greek physicians from before Christ, and modern paediatrics were founded by the Swedish physician Nils Rosén von Rosenstein in the 18th century. Veterinary "paediatrics" have a somewhat shorter history, but both neonatology and developmental diseases are recognised as distinct disciplines, in horses and other animal species[290, 293].

In 1986, the term "Developmental Orthopaedic Disease" was introduced in horses to represent a group of clinical entities that were commonly observed in young individuals [1]. The clinical entities were: osteochondrosis dissecans (OCD), subchondral bone cysts, angular limb deformity, physitis, flexural limb deformity, cuboidal bone disease, juvenile arthritis and cervical vertebral myelopathy [1]. In 1887, König applied "osteochondritis dissecans" to fragments in the knee joint of humans with minimal or no history of trauma [149]. "Osteochondritis" was later changed to "osteochondrosis" to reflect the fact that inflammation occurred late, rather than early in the disease [127]. However, physicians continue to use osteochondritis dissecans for reasons of historical, rather than scientific correctness [58]. OCD was first used in horses in 1947, and already then, increased prevalence in the offspring of certain stallions was noted, implying a heritable predisposition [189xxx]. OCD was used in pigs from 1970 [160]. In 1978, OCD was described as a result of the preceding disease process osteochondrosis,

defined as a focal disturbance in enchondral ossification in six different animal species [195]. The extent to which the other developmental orthopaedic diseases occur independently or are also a result of osteochondrosis has been debated [1]. In 1997, osteochondrosis in horses was interpreted to represent lacking/faulty differentiation or dyschondroplasia [116, 242], and this interpretation dominated the literature for the next 10 years. From 2007, an earlier hypothesis of Pool [218, 219] and Carlson [39] was re-examined using a combination of histological and vascular perfusion techniques [199-201]. The results confirmed that articular osteochondrosis in horses was a result of failure of the blood supply to growth cartilage [199, 203], as previously shown in pigs [38]. In 2013, the vascular hypothesis was experimentally reproduced from transection of vessels to OCD [204]. Since then, it has become clear that it is necessary to discover both how vascular failure can be heritably predisposed, and the full range of factors beyond simple, surgical transection that can cause vascular failure and lead to OCD-like fragments [208].

At a thesis defence in Sweden in 2016, nestor of porcine osteochondrosis research Sven Reiland remarked that "someone should really start looking into the pathogenesis of physeal osteochondrosis again" (defined in section B, below). Reiland sadly passed away later the same year, but part of this thesis was completed in honour of his remark [II]. During collaboration from 2004 onwards, porcine researcher Bjørnar Ytrehus alerted his equine colleagues to experimental studies where bacteria injected into pigs [53] and chickens [60] localised to growth cartilage canals shortly after injection, where they caused vascular failure. This initially sparked the equine researchers´ interest in whether bacterial vascular failure could lead to OCD-like lesions, resulting in false-positive diagnosis that could complicate classification, heritability studies and selection [114]. By coincidence, the studies of bacterial vascular failure [I] and physeal osteochondrosis [II] ended up taking place at a similar time. At first, early lesions of physeal osteochondrosis appeared as a spaghetti junction of eosinophilic streaks and were challenging to interpret until knowledge about the pathogenesis of articular osteochondrosis was applied [II]. Lesions of septic vascular failure also became possible to interpret once they were compared and contrasted with aseptic vascular failure in articular and physeal osteochondrosis [I].

Over time, the research into septic vascular failure led to new discoveries about bacterial orthopaedic infections in their own right [I, III-IV]. Some of the developmental orthopaedic diseases can only arise in skeletally immature individuals [1]. Orthopaedic infections are an example of a disease category that can occur at any age, but can have radically different aetiology, pathogenesis, treatment and prognosis between skeletally immature and mature individuals [237]. For example, septic arthritis principally occurs by the haematogenous route and is more often associated with physitis and osteomyelitis [86, 182], so much so that septic arthritis/osteomyelitis are considered a single entity in foals ("joint ill", in layman´s terms) [280]. Septic arthritis can have high incidence and mortality in foals [244, 249], and the importance of early, aggressive treatment was recognised several decades ago [280]. During the 1980ies, Firth first-authored seminal works including the S, P, E and T-type classification described later in this thesis and their likely pathogeneses [70]. Both categorisation and pathogeneses were based on human studies, where establishment of bacterial infection was related to slow flow in subchondral vessels [124, 192, 268]. Since then, this suggestion tends to be repeated whenever pathogenesis is mentioned, seemingly without any drive for further validation. In the studies for this thesis, new discoveries were made which shed an entirely new light on the pathogenesis of septic arthritis/osteomyelitis in immature individuals [I, III-IV].

This thesis is a result of the historical events outlined above: the need to discover causes of vascular failure [I], examine physeal osteochondrosis [II], septic vascular failure and septic arthritis/osteomyelitis [I, III-IV]. Please note that in the thesis summary, more emphasis was deliberately placed on septic arthritis/osteomyelitis than osteochondrosis, because the latter has been extensively reviewed in previous theses, and on equine more than porcine disease, mainly because the thesis was conducted at the Equine Section.

B: Summary of selected relevant normal anatomy and physiology

The synovial joint

The diarthrodial joint provides an articulation between two opposing bones, where the part of the bone constituting the joint surface is covered by articular cartilage (see below). The articular cartilage ensures minimal friction and smooth movement of the opposing bones. The bones are held in place by the outer, fibrous joint capsule, reinforced by ligaments that sometimes contain sesamoid bones. The inner layer of the joint capsule consists of the synovial membrane, which provides a barrier and regulates the contents of the synovial fluid. Joints with high pressure and low motion have a high amount of fibrous tissue in the inner layer of the capsule. In comparison, joints with low pressure and high motion have less fibrous tissue in the inner layer, and may also have villi, increasing the synovial surface area. The synoviocytes have no basement membrane, and are not connected by any cell junctions. This allows for diffusion of water and small solutes between the joint cavity and highly permeable, fenestrated capillaries in the synovial interstitium. The synovial fluid lubricates and protects the joints, and provides nutrition for the articular cartilage [38, 209].

Articular cartilage

The cell constituent of the articular cartilage is the chondrocytes that produce the extracellular matrix, which are organised in four zones [209]. The superficial layer contains the greatest number of chondrocytes per area, which are flattened and oriented tangential with respect to the articular surface [169, 245]. Chondrocytes in the intermediate zone are larger and arranged as single cells or in pairs. The largest chondrocytes are found in the deep layer, where they are arranged in columns that are perpendicular to the articular surface. At the junction with bone, the matrix is calcified and the chondrocytes are organised as single cells or in short columns [245].

At birth, the composition of the articular cartilage matrix is uniform across the joint surface [27, 32, 44]. After birth, physical activity and associated biomechanical forces stimulate the cartilage to adapt to the strain, especially in weight-bearing areas [27, 28]. This includes adaptation of the components of the extracellular matrix [26] and their organisation [32, 44]. The tensile strength of the articular cartilage is provided by a cross-linked framework consisting mainly of collagen type II fibrils, organised in

arcades within the matrix of the four cartilage zones in a configuration known as the Benninghoff structure [169, 194]. The fibrils are anchored in the calcified zone, and extend perpendicular to the joint surface through the deep zone before making a wide, 180°-turn in the intermediate and superficial zones, giving considerable tensile strength to the superficial layer [169]. Compressive strength is provided by negatively charged proteoglycans [194]. The proteoglycans give the cartilage a sponge-like quality, where nutrients are accessed via fluid influx during cyclic, biomechanical decompression, and waste products are removed through fluid egress during compression [90]. In this way, the synovial fluid provides nutrition for the articular cartilage, and it is not supported by any dedicated blood supply [245].

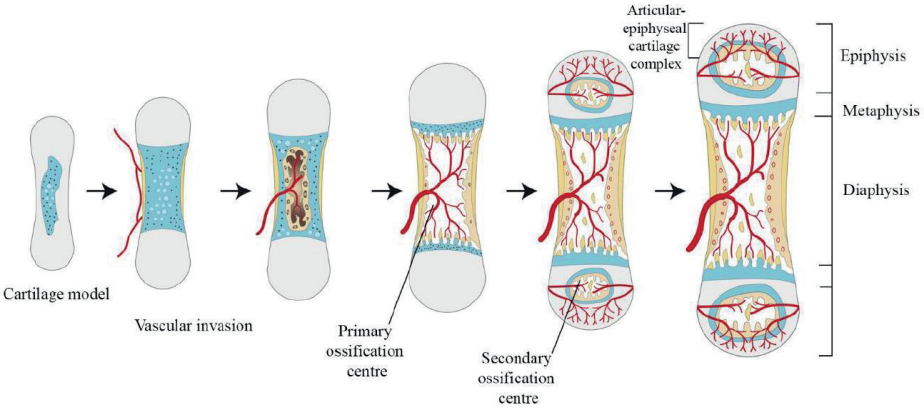

Figure 1: *Schematic diagram of long bone development. From left to right: Mesenchymal cells have differentiated into chondrocytes and formed a cartilage model. Chondrocytes in the centre of the model hypertrophy. Vascular invasion into the diaphysis leads formation of a primary ossification centre (POC). Subsequent vascular invasion into the epiphysis leads to formation of a secondary ossification centre (SOC). As the bone grows and the secondary ossification centre enlarges, blood vessels in the articular epiphyseal cartilage complex become incorporated into the ossification front (far right). NB. The blood vessels of the metaphyseal growth cartilage or physis are not drawn. (Illustrations by Susanna Bateman). Reprinted with permission from Ingunn Risnes Hellings [111].*

Growth cartilage

In addition to articular cartilage, the skeleton of immature individuals contains specialised hyaline growth cartilage [2, 166]. A miniature growth cartilage model of the

mature bone forms in utero, and a primary centre of ossification arises in the diaphysis and secondary centres of ossification arise in the epiphyses of the model (Fig. 1)[2]. Thereafter, longitudinal growth occurs predominantly in the metaphyseal growth plates or physes, located between the primary and secondary centres of ossification [2, 166], whereas shaping of the bone ends takes place in the articular-epiphyseal cartilage complex (AECC) covering the epiphyses [36]. The growth happens by enchondral ossification, where cartilage is continuously produced and replaced with bone [166].

Four distinct zones of chondrocytes are recognised in both the AECC and the physis [166, 305]. Closest to the articular cartilage, the resting zone contains quiescent chondrocytes that rarely divide, in contrast to the subjacent proliferative zone where chondrocytes frequently divide. Deep to the proliferative zone, there is a zone of hypertrophic chondrocytes that produce a specialised matrix that acts as a template for calcification. This happens in the mineralized zone, where chondroclasts remove the transverse septa, allowing bone-forming osteoblasts to advance [155]. The part of the ossochondrous junction where osteoblasts advance into chondrocyte lacunae is referred to as the ossification front. In the physis, the cells of the proliferative, hypertrophic and mineralized zones form distinct columns, whereas in the epiphyseal growth cartilage, chondrocytes are more heterogeneously organised in pairs, short stacks and nests of four or more cells [34].

Hypertrophic zone chondrocytes produce vascular endothelial growth factor (VEGF) that stimulates the ingrowth of blood vessels that is necessary for ossification to proceed [82, 83]. Several other factors regulate the complex process of enchondral ossification, including systemic growth hormone and thyroid hormone, and locally-produced insulin-like growth factors, Indian hedgehog signalling protein, parathyroid hormone-related peptide and bone morphogenetic proteins [46, 166] These factors will not be discussed in further detail, mainly because it is currently unclear how they relate to focal osteochondrosis or orthopaedic infections.

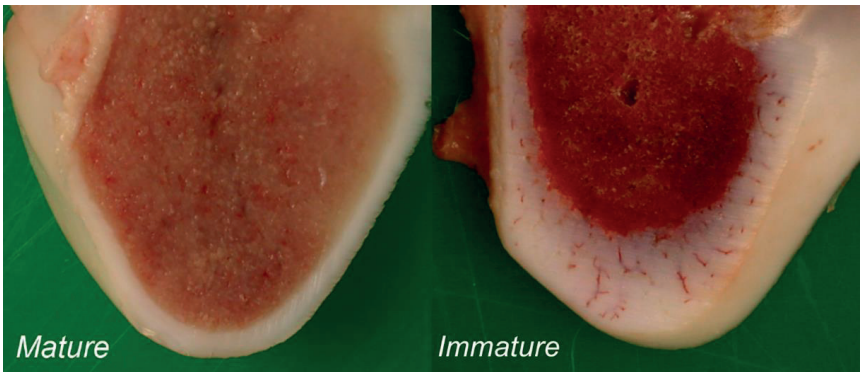

Figure 2: *Sawed slab through the distal end of the lateral trochlear ridge of the talus. The image on the left is from a mature horse and contains only articular cartilage and subchondral bone. The image on the right is from a foal and contains vascularised growth cartilage between the articular cartilage and bone.*

Blood supply to epiphyseal growth cartilage

Growth cartilage is located further away from the synovial fluid, is thicker and has a higher metabolic rate, and is therefore perfused by a dedicated blood supply that is not present in articular cartilage (Fig. 2) [23, 38, 113, 239]. The blood supply courses within channels in the growth cartilage known as cartilage canals. Cartilage canals have been described in mammals [36, 39, 101, 108, 254, 294, 295] and birds [162], but not in marsupials [264]. Within the AECC; the cartilage canals are blind-ending and can therefore be described as having a proximal end near the arterial source and a distal end away from the arterial source [108]. However, the vascular network within the canals is continuous and organised as anatomical end arteries [108]. Each canal contains an afferent arteriole that ends in a glomerular-like capillary network, before the capillaries re-join to form one or a few efferent venules [108, 295]. In addition to the vascular parts, cartilage canals contain perivascular mesenchymal cells [22]; canals have also variably been reported to contain lymphatics and unmyelinated nerve fibres [109, 254, 295]. The function of the cartilage canal blood supply is to provide nutrition and remove waste products from growth cartilage [23]. The cartilage canals originate as invaginations of the perichondrium that invade the cartilaginous epiphyseal model in the foetal or neonatal period and contribute to the formation of the secondary centre of ossification [23, 108, 138]. Finally, perivascular mesenchymal cells within canals contribute cells to growth of the cartilage model [23].

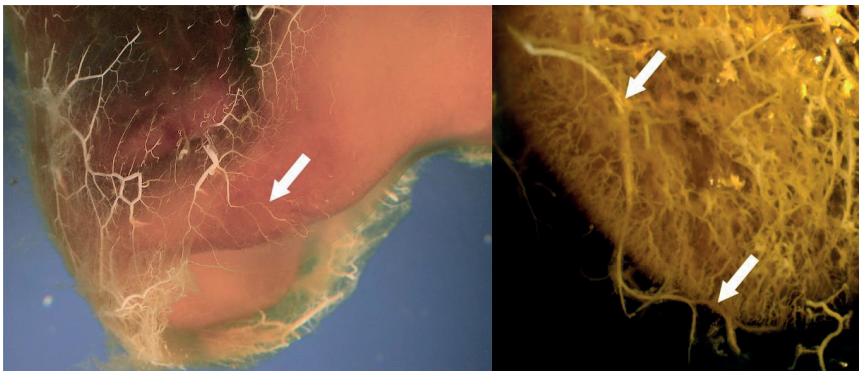

Figure 3: *Arterially barium-perfused and Spalteholz-cleared samples from the cranial distal intermediate ridge of Standardbred foals. The image on the left is of the intact tibia from a 3-week-old foal and shows an artery that is incorporated into bone at a single point (arrow). The image on the right is a sawed, 5-mm-thick sagittal slab from a 4-week-old foal and shows an artery that is incorporated into bone at two points (arrows).*

The presence of cartilage canals can vary considerably between species, joints and epiphyseal regions [208]. In foals, the blood supply is heterogeneously distributed from birth [199-201]. The rate of bone growth changes with age and anatomical location, and gradually declines as the animal approaches mature size [79]. The thickness of growth cartilage also decreases, and the need for a dedicated blood supply declines with it [113]. The blood supply gradually disappears by two processes. Firstly, as the ossification front advances, the cartilage canals are incorporated into bone (Fig. 3) [199, 303]. Incorporation occurs at an earlier stage at the mid-portion than at the proximal and distal portions of the blind-ending canal [199, 303]. During post-natal development, some cartilage canal vessels therefore course from the perichondrium towards the articular cartilage entirely within growth cartilage. However, several vessels course from the perichondrium, into epiphyseal bone and then traverse the ossification front to enter the epiphyseal growth cartilage canals and continue towards the articular cartilage [199, 200]. The cartilage canals also disappear by chondrification, a process that occurs from the distal towards the proximal portion of the blind-ending canal [302]. Chondrification is a physiological process where the canal and its contents are replaced by cartilage through disintegration of the endothelium and transformation of mesenchymal cells into chondrocytes [37, 110, 196]. The regression of the cartilage canal blood supply with age has been most extensively studied in the AECC of pigs and

foals [208, 305]. Several locations have been examined, of which the metatarsophalangeal joint [201] contained cartilage canals for the shortest period of time, the distal tibia contained canals for an intermediate period of time [39, 199] and lastly, the distal femur contained canals for the longest period of time [39, 200, 302].

Blood supply to physeal growth cartilage

The blood supply to physeal growth cartilage has been examined in the distal radius and third metacarpal bone of foals [69, 76]. It was discovered that vessels that entered and traversed the physis originated in epiphyseal bone, and that the number of vessels declined with increasing age. Transphyseal vessels were only present at a young age. The metaphyseal bone contained a dense network of vessels that did not enter the physis. Similar findings have been reported in pigs [122, 123, 286].

The validity of comparing cartilage canal blood supply in foals and pigs

The blood supply to the epiphyseal growth cartilage of the distal femur has been described macroscopically in both foals and pigs (Fig. 4) [38, 200, 302], and in this location, species comparison appears to be valid provided that differences in maturation are accounted for. This is illustrated by the following example: On the medial abaxial aspect of the femur, there is a curved vessel that courses from caudal towards cranial, roughly parallel with the distal contour of the condyle [200]. The curved vessel gives of smaller, radial branches at regular intervals, roughly similar to the spokes of a bicycle wheel. In 7-15 week-old pigs, the radial branches course into the growth cartilage and continue across the distal articular surface of the condyle [302]. With age, the radial branches decrease in number and length, presumably through chondrification, and therefore end up nearer to their original abaxial perichondral source [302]. In 0-7 week-old foals, the same radial branches are observed, but they terminate within the growth cartilage on the medial abaxial aspect of the condyle [200]. The radial vessels observed in the foals are therefore believed to represent a later/more advanced stage of maturation of the radial vessels observed in the pigs. This suggests that the configuration and development of the blood supply is the same [200, 302], but that the femoral growth cartilage is at different stage of maturation in 0-7 week-old foals than in 7-15 week old pigs. Comparison of the blood supply may therefore be valid provided that differences in maturation are taken into consideration.

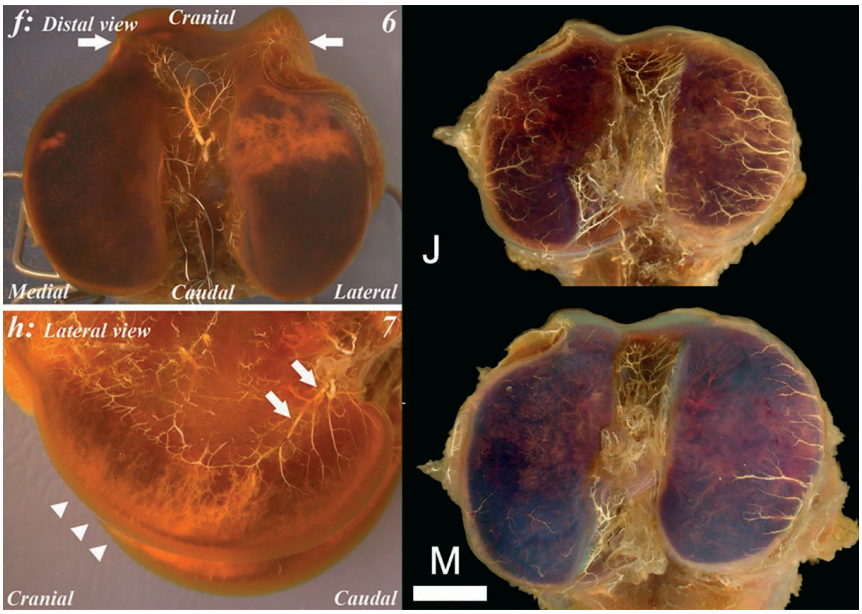

Figure 4: *Comparison of foals and pigs. All images are arterially barium-perfused and Spalteholz distal femurs. The images on the left are from 6-week-old (top) and a 7-week-old (bottom) Standardbred foals. On the medial abaxial aspect of the femur, there is a curved vessel that courses from caudal towards cranial (two arrows in bottom image) that gives off small radial branches that terminate on the abaxial aspect of the femur and do not continue across the distal articular surface (confirmed by top image) [200]. The images on the right are from 13-week-old (top) and a 15-week-old (bottom) Landrace pigs. The radial vessels continue across the distal articular surface of the femur, but the vessels gradually regress towards the abaxial aspect with age [302]. The vessels therefore have the same configuration in foals and pigs, but reflect different stages of maturation.*

C: Documented role of failure of the blood supply in clinical disease in foals: osteochondrosis

Failure of the blood supply to epiphyseal growth cartilage has been associated with osteochondrosis. The following is intended to summarise the aspects relevant to this thesis, rather than provide a complete review of osteochondrosis, available elsewhere [208, 305].

Definitions

Osteochondrosis is defined as a focal disturbance (delay) in enchondral ossification, and can occur in both epiphyseal and physeal growth cartilage, referred to as articular and physeal osteochondrosis, respectively [195, 225, 257]. Articular osteochondrosis can resolve, or progress to OCD or subchondral bone cysts [195, 225, 257]. Physeal osteochondrosis has been associated with angular limb deformity [195, 225, 257].

With the exception of fragments at the sagittal ridge of the third metacarpal/metatarsal bone, fragments in the fetlock joint of young horses tend to be referred to as (developmental) osteochondral fragments, because it has not been definitively determined whether they are a result of osteochondrosis or not [178].

Relevant clinical aspects of articular osteochondrosis in horses

There are five particular aspects of osteochondrosis that set it apart from other orthopaedic diseases:

-Signalement: Osteochondrosis is commonly diagnosed in young horses of large breeds such as Warmblood Horses [56, 136, 253, 278] (up to 69 % prevalence[278]) and Standardbred Horses [93, 94, 163, 234] (up to 50.7 % prevalence[163]). The disease is rare in pony breeds including the Norwegian Fjord Pony, supported by the fact that osteochondral lesions have not been reported when the hocks of Icelandic Horses, a pony-sized breed, are screened for juvenile spavin lesions [21, 256]. A sex predilection as seen in other species is not apparent when representative numbers of horses are examined [95]. Patients may be descended from parents with the disease (see below).

-Clinical signs: Osteochondrosis lesions have been identified in the absence of clinical signs, or associated with joint effusion [31, 176] (ca. 80 % of diagnosed horses) and lameness [31](< 80 % of diagnosed horses). More than one joint is affected in over 50 %

of cases, and multiple affected joints tend to be bilaterally symmetrical [93, 94, 177]. The most commonly affected joints are the fetlock, hock, stifle, shoulder and joints of the vertebral column [177, 178].

-Radiology: Diagnosis of osteochondrosis is usually made based on clinical examination, diagnostic analgesia (if feasible) and radiography. In radiographs, lesions are located at predilection sites that are specific to the affected joint [177, 178]. Multiple predilection sites can be affected simultaneously [93, 94].

-Dynamic development: Longitudinal monitoring has confirmed that lesions arise and may also spontaneously resolve before certain, joint-specific age thresholds [40, 56]. There is some indication that the age thresholds are breed-specific (stifle: up to 18 months in Lusitano Horses [12]), but for Standardbred and Warmblood Horses, the age thresholds from which lesions are considered permanent appear similar at 5 months for the hock and 8-9 months for the stifle joint[40, 56, 95, 213].

-Heritable predisposition: Reported heritability is specific to the studied population, but tends to be moderate to high at around $h^2 = 0.2\text{-}0.3$ for Standardbred Horses [164].

The above five aspects are listed particularly because any hypothesis to explain the pathogenesis of osteochondrosis must be able to explain the clinical characteristics.

Importance of articular osteochondrosis

In some studies, there was little or no impact of osteochondrosis on performance [227, 285], and prognosis following surgical removal of small fragments from the hock and fetlock joints is excellent [31, 179].

However, osteochondrosis remains the most prevalent of the developmental orthopaedic diseases [163, 279], and may also be the cause of some of the entities other than OCD [1], making it the single-most important developmental disease process overall.

Even though morbidity is generally low in the hock and fetlock joint [179, 288], it can be high in other joints. This is especially true for large lesions in the stifle joint [179], which can be associated with more severe lameness and poorer prognosis following surgical removal. In the shoulder, osteochondrosis is associated with rapid progression to irreversible osteoarthritis, and can therefore be a reason for euthanasia on humane

grounds [19, 133, 190]. Osteochondrosis in the spine has been associated with cervical vertebral myelopathy (Wobbler´s syndrome) [251].

The risk and cost of surgical treatment and convalescence can be experienced as high by individual horse owners [284].

The heritable predisposition means that osteochondrosis should be considered in programmes for ethical and sustainable breeding. The two most common breeds in Norway are the Standardbred Horse and the Norwegian-Swedish Coldblooded Trotter. Standardbred stallions with osteochondrosis are included in breeding, whereas Coldblooded Trotter stallions with osteochondrosis are excluded from breeding. The prevalence of osteochondrosis is low in Coldblooded Trotters (ca 4 %) [Arne Holm, personal communication], and lesions are most common in the hock and fetlock, thus it is questionable whether the disease has any major effect on peak racing speed or career longevity in this breed. However, pedigree stallions can be expensive and make good earnings from racing and breeding, thus financial loss from exclusion can actually amount to millions of Norwegian kroner for individual stallion owners.

Articular osteochondrosis therefore remains an important disease.

What about physeal osteochondrosis?

In 1979, Strömberg published a review paper describing how peripheral articular osteochondrosis was associated with OCD, central lesions were associated with subchondral bone cysts and physeal osteochondrosis lesions were associated with disturbed longitudinal growth/angular limb deformity [258]. Physeal osteochondrosis has also been associated with the poorly defined entity “physitis” [1]. Since then, there have been few reports on the concrete relationship between focal osteochondrosis and clinical disease. This may be because angular limb deformity carries a good prognosis, and research tends to be limited to radiographic examination of live foals where there is considerable superimposition, and focal lesions therefore are difficult to identify. When describing physeal osteochondrosis in pigs, Reiland identified a further potential challenge: early lesions were most prevalent in 4 month-old pigs, whereas associated angular limb deformity was most prevalent in 8-9 month-old pigs [224]. There was in other words a considerable lag phase which means that by the time the angular limb deformity is diagnosed, it may no longer be feasible to study the changes that caused it. This means that there is still a lot of information left to be discovered about the

relationship between focal osteochondrosis lesions and angular limb deformities in both species.

Pathogenesis of articular osteochondrosis

Osteochondrosis is often described as a multi-factorial disease, but it is important to distinguish between factors suggested to influence the outcome of osteochondrosis in epidemiological studies, and aetiological hypotheses based on detection of early histological lesions at predilection sites [208, 305]. Factors from epidemiological studies include heritability, biomechanical force, exercise, trauma and diet, comprising both micro- and macronutrients [208, 305].

Histological studies include early work by Carlson et al. [39] suggesting that the blood supply to growth cartilage plays a role, discussed below. In 1996, osteochondral fragments were interpreted as accessory centres of ossification, believed to represent normal anatomic variants with reference to similar centres in humans [96]. In 1997, purported early osteochondrosis lesions were interpreted as primary dyschondroplasia [116, 242], where it has been considered that either the dysplastic chondrocytes or extracellular matrix were responsible for the delay in enchondral ossification [115-117]. It was also proposed that patent blood vessels in areas of delayed ossification allowed exposure of cartilage to imbalances in growth-regulating factors in the circulation [242], or that the vessels represented re-vascularisation of primary dysplastic lesions [116]. The problem with the majority of these hypotheses based on histological studies is that they have thus far failed to explain the multi-focal distribution of osteochondrosis at predilection sites [93, 94, 177].

Lesions at predilection sites for osteochondrosis have long been known to contain areas of growth cartilage necrosis in both pigs [224] and horses [225, 230]. Upon further study, it was discovered that the necrotic cartilage was centred on necrotic cartilage canals AECC [37, 39, 145, 218]. The necrosis was therefore interpreted to represent ischaemic chondronecrosis.

In 1991, Carlson et al. published a study of spontaneous and experimentally induced lesions in pigs, where it was noted that both were multi-lobulated, dubbed stair-step lesions [38]. Ytrehus et al. (2004) later expanded on Carlson´s work, and defined three distinct, consecutive stages of osteochondrosis latens: ischaemic chondronecrosis at intermediate depth of growth cartilage, osteochondrosis manifesta: ischaemic

chondronecrosis and delayed ossification and OCD: pathologic fracture through the area of chondronecrosis [301, 304]. Ytrehus et al. combined arterial perfusion and histological studies, and concluded that vascular failure occurred at the site where the mid-portion of blind-ending cartilage canals was incorporated into the ossification front [303].

When the Carlson/Ytrehus work in pigs was replicated in foals [199, 200] the results indicated that vascular failure occurred at the site of vessel incorporation into bone also in this species. In 2013, Olstad et al. proceeded to perform experimental vascular transection in foals, and succeeded in reproducing all stages from ischaemic chondronecrosis via delayed ossification to OCD (Fig. 5) [204, 205].

The vascular pathogenetic hypothesis explains several of the clinical characteristics of osteochondrosis:

-The blood supply to epiphyseal growth cartilage is heterogeneously distributed from birth, and sites that were the last to lose their blood supply in foals corresponded to predilection sites for osteochondrosis in most bones examined to date [199-201]. Prolonged dependence on a blood supply may therefore explain why certain sites are predilection sites [197].

-During combined computed tomographic (CT) and histological studies in pigs, the only component that matched the distribution of lesions was the blood supply, as opposed to the chondrocytes or matrix [206].

-The multilobulated, stairstep morphology originally observed in pigs [38] and later confirmed in foals [203] was compatible with failure of an end arterial vascular trunk during incorporation into bone, leading to ischaemic chondronecrosis around multiple, smaller vessel branches simultaneously [208].

-Finally, during longitudinal CT monitoring of lesions in pigs, there were two incidence peaks in 4/6 examined joints [207], and comparison to arterial perfusion studies confirmed that most pigs had two vessel trunks left to incorporate during the relevant age interval. Other trigger factors common to multiple pigs were eliminated, and the incidence peaks were therefore compatible with vascular failure during incorporation of cartilage vessels into bone [207].

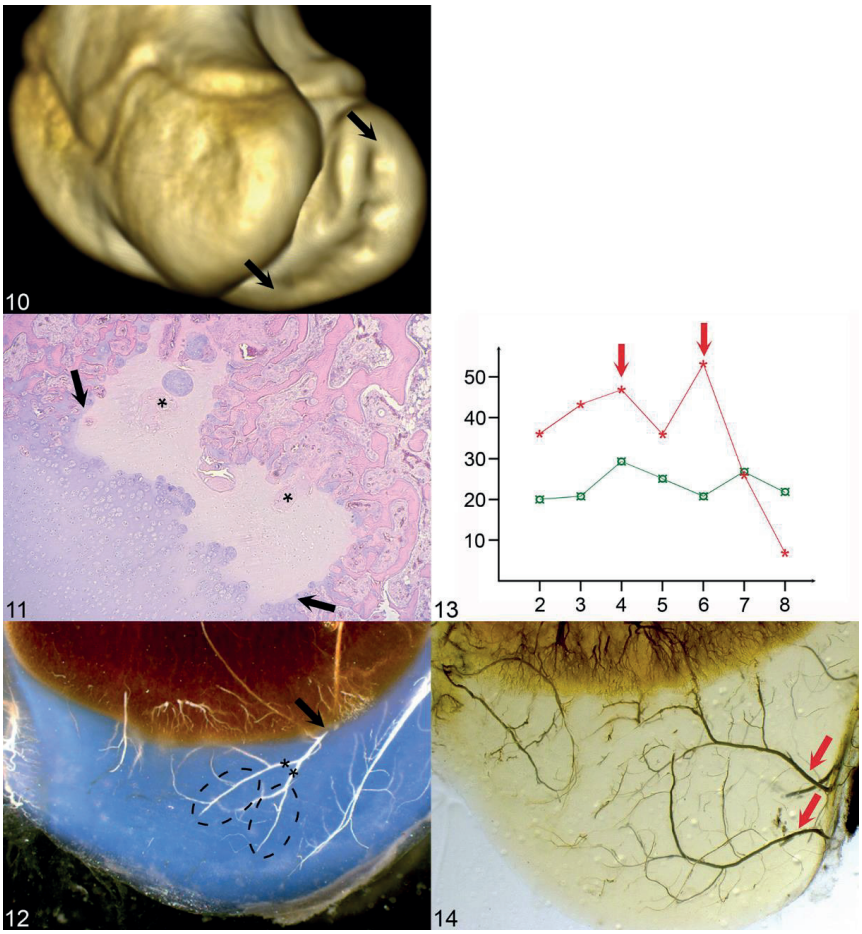

Figure 5: *Vascular pathogenesis of articular osteochondrosis. All figures and data are from the distal femur/medial femoral condyle of pigs [208]. Subfigure 10 shows a multi-lobulated, stair-step lesion in a computed tomography (CT) scan from a 173-day-old Landrace pig. Subfigure 11 shows histology of a multi-lobulated, stair-step lesion from a 174-day-old Landrace pig. Subfigure 12 shows an arterially-barium perfused and cleared, 5-mm-thick slab from a 91-day-old pig. Collectively, subfigures 10-12 show that the only anatomical component that matched the distribution of the stair-step lesions was the cartilage canal blood supply. Subfigure 13 shows that there were two peaks (red arrows) in the incidence of new lesions arising (red asterisks/line) between 70-180 days of age as monitored by longitudinal CT examination (green currency signs/lines: lesions resolving). Subfigure 14 shows that there were two vessels left to incorporate into bone (red arrows) in this 49-day-old Landrace pig.*

In sum, the vascular hypothesis is the pathogenetic hypothesis that explains the most clinical characteristics of articular osteochondrosis to date [208]. Several studies claim to have induced osteochondrosis lesions in horses, but the interventions are often systemic, e.g. manipulation of diet, were performed in foals that were older than the age thresholds for spontaneous lesions and resulted in much more generally distributed lesions [30, 85]. The vascular hypothesis is therefore also the only hypothesis that has been reproduced in a manner that is compatible with the clinical disease [205].

What about the pathogenesis of physeal osteochondrosis?

Physeal osteochondrosis is also defined as a focal delay in enchondral ossification [195, 224], but lesions tend to contain viable, hypertrophic zone chondrocytes and lines of intensely eosinophilic-staining material referred to as eosinophilic streaks [63, 72, 83, 120, 121, 123, 252]. It is believed that the eosinophilic streaks represent remnants of cartilage canal blood vessels [121, 122, 144], but the fact that surrounding chondrocytes are morphologically viable means that it has not been possible to conclude that the lesions are ischaemic. It has been suggested that physeal osteochondrosis is a result of vascular failure, but the suggestion pertains to failure of vessels to penetrate cartilage from bone as required for enchondral ossification to proceed, completely different from failure of vessels already located in cartilage as in articular osteochondrosis [63, 121, 123, 144]. Several studies report on interventions that must have interrupted the blood supply to physeal growth cartilage, but the studies focus more on the effect on longitudinal growth rather than whether that effect occurred via ischaemia [102, 269]. The hypothesis that physeal osteochondrosis is the result of failure of the blood supply to growth cartilage has therefore never been systematically investigated.

Causes of vascular failure

Since the vascular hypothesis was confirmed in spontaneous lesions [39, 196, 199, 203] and experimentally reproduced [204] in foals, it has become clear that it is necessary to discover how vascular failure can be heritably predisposed, and the full range of factors beyond simple, vascular transection that can cause vascular failure and lead to OCD-like lesions.

The status of this on-going work is as follows:

-Spontaneously occurring lesions in foals [199]and pigs [303] indicate that the vascular failure in heritably predisposed osteochondrosis occurs is associated with the process of incorporating vessels into bone during growth [208].

-Epidemiological studies support that biomechanical force influences the outcome of osteochondrosis [283]. Whether biomechanical force is the primary cause of vascular failure in heritably predisposed lesions remains to be determined [208].

-In pigs, it was suggested that vascular failure during incorporation into bone was a result of micro-fractures of trabecular bone at the ossification front [303], but micro-CT

with sufficient resolution to detect micro-fractures revealed only intact trabeculae in the relevant location in heritably predisposed foals [198].

-Lesions from pigs were later examined using advanced, three-dimensional microscopy techniques and there was no evidence of micro-fissures or fractures at the site of vascular failure or in early osteochondrosis latens lesions [66]. However, there was evidence of micro-fissures in some late osteochondrosis manifesta lesions, which can possibly explain why some lesions progress to OCD whereas others resolve [67].

-During the three-dimensional microscopy studies, 3/14 failed vessels in early lesions were traced back to the attachment of the caudal cruciate ligament without being incorporated into bone [66]. It therefore seems that a minority of vascular lesions may occur at sites of ligament attachment, as opposed to at the ossification front [66]. It is possible that both kinds of lesions occur due to biomechanical forces acting at sites of tissue transitions, e.g. the transition between growth cartilage and bone [199, 218, 219, 303] or the transition between growth cartilage and ligaments [66].

Evidence therefore potentially supports that the vascular failure in heritably predisposed osteochondrosis may be a result of micro-, as opposed to macro-mechanical forces [66].

-During the 2000s, it was suggested that weakened collagen type II in dysplastic growth cartilage matrix could predispose for vascular failure [150, 151], but collagen type II is uniformly distributed throughout growth cartilage and this therefore fails to explain focal disease at predilection sites. However, it was later discovered that some parts of cartilage canals were surrounded by fibrillar collagen type I, possibly in preparation for ossification [64, 66, 112]. Cartilage canals are surrounded by collagen type I and II in both breeds with high and low prevalence of osteochondrosis, and the difference in prevalence is therefore unlikely to be due to a difference in collagen type[112]. Instead, the current working hypothesis is that cartilage canals may be surrounded by weaker collagen type I in high prevalence than in low prevalence breeds. It can be speculated whether this is because high prevalence breeds are unable to synthetize strong collagen type I at a fast enough rate, or whether there is a genuine defect on genes coding proteins required for collagen type I synthesis and cross-linking.

-This latter suggestion potentially fits with genome-wide association studies in pigs, where a region was identified on chromosome 13 that remained significantly associated with osteochondrosis after correction for multiple tests [92]. This region contains several genes coding for proteins that sense and respond to biomechanical force, including genes involved in collagen synthesis [92, 111].

-Sensing and responding to micro-mechanical force may of course also be performed by similar protein gene-products at the transition between growth cartilage and bone [199, 218, 219, 303], and growth cartilage and ligaments [66], meaning that the heritable predisposition for vascular failure may be the same in both sites.

This is the current state of knowledge about the cause of vascular failure in spontaneously occurring lesions in heritably predisposed foals and pigs [208].

The fact that surgical incisions can trigger vascular failure means that failure is not limited to heritably predisposed causes, but that acquired, potentially environmental/management triggers must be considered also [208].

As mentioned in part A, an acquired, environmental cause was already known and relatively well-documented in pigs and chickens: bacterial infection [53, 60, 246]. Following experimental injection, bacteria localised to growth cartilage canals, where they caused vascular failure [53, 60, 246]. This means that it is highly necessary to investigate whether bacterial infections can be a cause of acquired vascular failure, leading to ischaemic chondronecrosis, delayed ossification and OCD-like lesions in foals.

C: State of knowledge about septic arthritis and osteomyelitis in foals at the start of the thesis

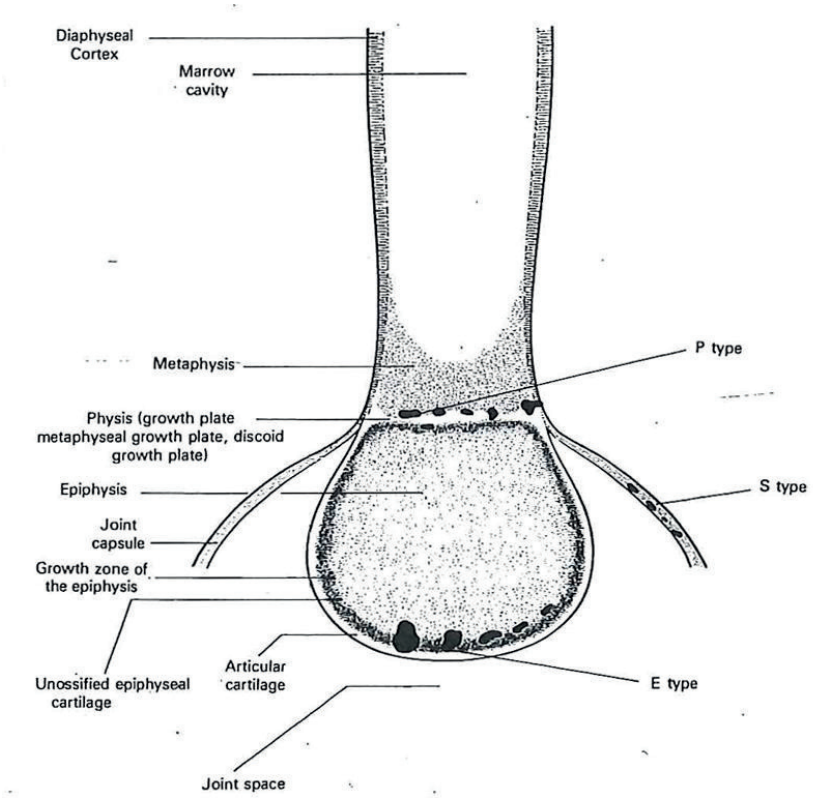

Figure 6: *Schematic representation of the morphology of the end of the long bone and the sites of three types of infection. From Firth 1983 [70].*

Definitions

Bacteraemia is the presence of bacteria in the circulation [3]. Septicaemia is the presence and replication of bacteria in the circulation [3]. Sepsis is the body´s own response to infection, which can be overwhelming and life-threatening [3].

Septic arthritis is defined as infection of a synovial joint cavity by microorganisms, most commonly bacteria [9]. Osteomyelitis is defined as an infection of bone and bone marrow [16, 88], with accumulation of neutrophilic granulocytes, necrosis of bone trabeculae and recruitment of osteoclasts [265]. The distal phalanx and sesamoid bones do not have any medullary cavity and infection in these bones is therefore correctly known as septic osteitis [107, 153, 184].

Historically, septic arthritis in foals was known as "joint ill" [98, 217]. In 1983, Firth introduced more specific classification of infection in foal joints and bones (Fig. 6) [70, 73]:

S-type infection: infection of the joint cavity, including the synovial membrane.

E-type infection: osteomyelitis of epiphyseal bone.

P-type infection: osteomyelitis of metaphyseal bone.

T-type infection: osteomyelitis of cuboidal bones (1985).

Septic arthritis and osteomyelitis can occur independently [70, 185]. However, septic arthritis and osteomyelitis often have a common aetiology, and septic arthritis is so frequently associated with osteomyelitis that the two are recognised as a single entity in foals [70, 185, 217]. Septic arthritis and osteomyelitis are sometimes also associated with septic bursitis and tenosynovitis [91, 118, 152, 185, 281, 300].

It has been described that osteomyelitic foci can extend from bone into adjacent cartilage [74]. Firth's classification pertains to osteomyelitis [70], and does not seem to cover extension into cartilage. Infections in physeal growth cartilage have been referred to as septic physitis, but included changes in metaphyseal bone as well as cartilage [86, 103, 142]. The term "septic chondritis" is used about infection of cartilage elsewhere in the body, and could be used to cover infections in growth and articular cartilage.

Pathology of septic arthritis/osteomyelitis in foals

Bacterial species

In both sepsis and orthopaedic infections in foals, the most frequently isolated microorganisms are Gram-negative bacteria [9, 29, 70, 84, 118, 148, 185, 232, 237, 249]. Gram-negative bacteria still constitute the major portion of isolates from such infections, but in some recent reports, the minor portion of Gram-positive bacteria is increased compared to earlier reports [263, 289, 300]. The most commonly isolated bacterial families in orthopaedic infections are the Enterobacteriaceae (especially *Escherichia coli* and *Klebsiella* spp.), *Actinobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp. [118, 185, 226, 249]. Isolation of *Salmonella* sp. had a negative impact on survival in one study [249]. Less common reported bacterial species include *Chlamydia* spp. [173], *Corynebacterium equi* [33], *Bacteroides* spp. [13, 226], *Clostridium*

spp. [249], *Listeria* ssp. [185], *Actinomyces* spp. [185] and *Rhodococcus equi* [54, 118, 140]. Simultaneous infection with two or more bacterial species is common in sepsis, with a reported incidence of up to 52.6 % [263]. Anaerobic bacteria and yeasts account for a minority of infections [170, 232].

Route of infection

In mature horses, the most common routes of infection in septic arthritis and osteomyelitis are iatrogenic inoculation, inoculation via a penetrating wound or extension from an adjacent focus of infection [20, 161, 182]. In foals, the most common route of infection by far is that bacteria enter the circulation and spread by the haematogenous route [9, 70, 86, 172]. Bacteraemia has been reported in clinically healthy neonatal foals without any obvious route of entry, but equivalent data are not available for older foals [99]. Bacteria may enter the circulation via the navel before this has closed, or via the respiratory system or gastro-intestinal tract [68, 84, 185, 233, 280]. Infections in foals frequently affect multiple organ systems [68, 84, 185, 233, 280]. Septic arthritis/osteomyelitis can arise from septicaemia, but if sepsis develops, the orthopaedic infection merely constitutes a complication to this more life-threatening condition [45, 57, 81, 84, 148]. Orthopaedic infections nevertheless exert a negative influence on sepsis survival [45, 57, 81, 84, 148].

Risk factors

The risk of developing orthopaedic infections is greatest in the first 30 days of life [118, 185, 237]. However, septic arthritis/osteomyelitis have been reported in foals up to 180 days of age [118, 185].

Neonatal foals are at risk for failure of passive transfer of immunoglobulins from colostrum, and if this occurs, it greatly increases the risk of infections, including orthopaedic infections [175, 222, 229, 255, 296]. Neonatal serum immunoglobulin levels below 8 g/l are proportionally associated with mortality [159].

The transition from relying on maternal antibodies to the foal producing own antibodies occurs after 30 days of age, and is associated with reduced levels of immunoglobulin G (IgG) at 1-2 months of age [52, 78]. After this, there is a period of 2-4 months where foals have increased susceptibility to respiratory infections [125], during which time the level of IgG also increases [78].

However, there are two subclasses of IgG, where concentration of IgGa increases from 6 weeks of age and production of IgGb only starts at 12-16 weeks and slowly increases over the first year of life [126, 241]. Class IgGb is critically important for resistance to viral and bacterial pathogens [174, 187, 240]. It has therefore been suggested that, together with low levels of immunoglobulin A, the late onset of production and slow increase in concentration of IgGb is an important factor in the increased susceptibility of foals to infections during the first year of life [126].

Pathophysiology

Bacterial invasion of the joint space and colonization of the synovial membrane leads to a massive immune system response [18, 274]. Inflammatory cells; predominantly neutrophils, localise to the infected area and phagocytose bacteria [168, 210]. They also release a variety of substances, such as collagenases, lysozymes, free radicals and cytokines [210, 247]. This includes interleukin-1 and tumour necrosis factor, which further stimulate the inflammatory cascade [210]. Together with activation of the plasmin, kinin, coagulation and fibrinolytic pathways, the net result is that inflammation is amplified [182]. Inflammation influences joint homeostasis, leading to reduced production of proteoglycans and increased release of matrix metalloproteinases [10, 182, 247]. Matrix metalloproteinases are involved in normal, physiological turnover of cartilage, but are also associated with cartilage degradation in different disease processes and particularly so in septic arthritis [25, 137, 272]. Bacterial toxins also cause direct cartilage damage that contributes to overall tissue destruction and continued stimulation of inflammation [165, 243]. The slow turnover of articular cartilage means that damage quickly exceeds maintenance/repair capacity and becomes irreversible [209]. Septic arthritis is an acute and potentially life-threatening disease, not because it is fatal in itself, but because it is associated with such high levels of destructive factors that cartilage damage quickly becomes so extensive that the horse must be euthanized on humane grounds [182].

Influx of inflammatory cells and mediators and increased permeability of the synovial membrane lead to joint swelling/effusion, increased intraarticular pressure and fibrin deposition [182]. The increase in intraarticular pressure causes pain and reduced blood flow to the synovium and the joint capsule [106]. The joint is slow to remove

accumulated fibrin deposits, and the deposits contain inflammatory cells, necrotic debris and bacteria that contribute to prolonging the infection and inflammation [182].

With respect to osteomyelitis, bone and cartilage have a greater ratio of extracellular matrix to cells than synovial membrane, and this provides a greater surface area for bacteria to adhere to [43, 88]. Often, a biofilm forms that allows bacteria to evade the immune system and continue to proliferate [89, 277]. As in septic arthritis, both the bacterial infection and inflammatory response are associated with physical tissue destruction and necrosis [17, 134, 135, 158]. This can result in formation of isolated areas of necrotic tissue, or sequestra, where the immune system is only capable of accessing the very periphery of the sequestrum and bacteria consequently thrive [193]. Although elimination of osteomyelitis may be challenging and require prolonged treatment, bone has considerably greater potential for healing than articular cartilage [158].

Clinical aspects of septic arthritis/osteomyelitis in foals

Signalement

Infections can affect any age, sex and breed individual, and septic arthritis/osteomyelitis do not show any particular breed or sex predilections [9, 86]. As alluded to above, orthopaedic infections are somewhat more common during particular age windows as follows: orthopaedic infections are most common in foals younger than 30 days [118, 185, 237], i.e. the window when foals are most susceptible to failure of passive transfer and neonatal sepsis. After the age of 1-2 months, there is a window of 2-4 months when foals have increased susceptibility to respiratory infections that may trigger orthopaedic infections [125], and this corresponds more to the transition from maternally derived immunity to the foal starting to produce its own antibodies. Septic arthritis/osteomyelitis are much less common in foals older than 6 months [118, 185, 237, 249].

Clinical signs

Foals with septic arthritis/osteomyelitis may or may not be pyrexic [86]. The more noticeable signs of septic arthritis/osteomyelitis are lameness and joint effusion (Fig. 7).

Septic arthritis tends to be associated with severe lameness [70, 86, 107, 182]. The lameness is often so acute in onset and severe that owners suspect some sort of traumatic injury has occurred [20, 70, 171, 289]. The lameness associated with osteomyelitis is often more insidious in onset and less severe than lameness from septic arthritis, and can increase gradually over days or weeks [103, 185].

Septic arthritis/osteomyelitis are associated with common signs of inflammation associated with any local infection: soft tissue swelling/joint effusion, heat and pain on palpation[9, 86, 107, 182], which may also occur in immune-mediated polysynovitis [167] and therefore are not pathognomonic for bacterial infections. Osteomyelitis can occur without swelling, thus a failure to detect soft tissue swelling does not rule out the possibility that osteomyelitis is present [185]. Thorough palpation of all four limbs is a mandatory part of clinical examination in foals. It may be difficult to detect lameness, joint effusion and soft tissue swelling in foals that are critically ill and recumbent for reasons other than septic arthritis/osteomyelitis [47, 84, 233], where palpation should be repeated daily.

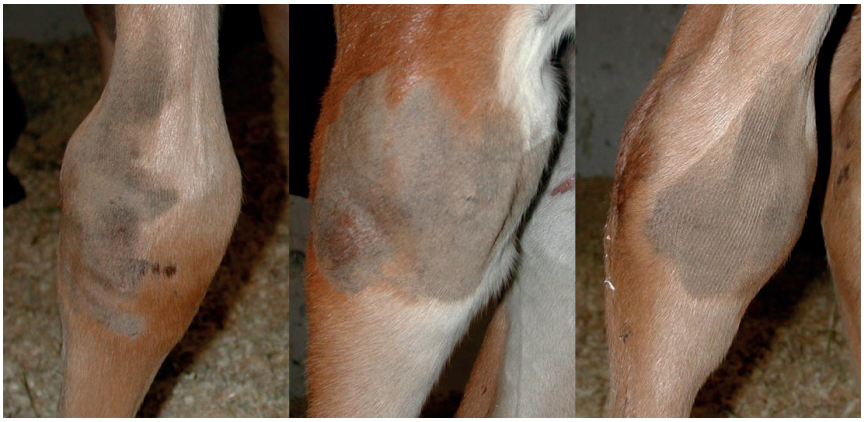

Figure 7: *Simultaneous synovial effusion in the right carpus, right stifle and right hock of case 4 from [I].*

Commonly affected locations

The joints most commonly affected by septic arthritis in foals are the stifle, hock, carpus and fetlock joint [118, 180, 237, 244, 249, 289, 300]. Septic arthritis also occurs somewhat less commonly in the shoulder, elbow hip and interphalangeal joints joint [118, 180, 237, 244, 249, 289, 300]. Septic arthritis has been reported in vertebral

articulations [228]. Infection also occurs in tendon sheaths and bursae, but this is not very common [91, 118, 300].

The most common sites for osteomyelitis in foals are the distal femur, distal tibia, distal radius and distal third metacarpal/metatarsal bones, and for septic osteitis: the distal phalanx [74, 185]. Lesions also occur in or near the hip, shoulder, elbow and interphalangeal joints [74, 185]. Osteomyelitis has furthermore been reported in vertebrae [129, 228, 250], the pelvis [14] ribs [42, 186], the patella [139] and osteitis in the proximal sesamoid bones [153].

Lesions are commonly multiple in foals younger than 30 days at the time of infection, but older foals sometimes also suffer multiple lesions which are then often bilaterally symmetrical [118, 185, 237, 249]. Multiple lesions are most common in foals younger than 30 days of age but occur also in older foals`, sometimes with bilateral lesions in the same location [118, 185].

Diagnosis

A definitive diagnosis of septic arthritis can be made based on positive bacterial culture from a synovial fluid or membrane biopsy, and performing this test/having access to the results can influence prognosis [118, 168, 185, 261]. The bacterial culture, species determination and sensitivity testing that are essential for determining an appropriate antibiotic regime will, however, take some days to complete and bacterial culture can yield a negative result even if infection is present [118, 185].

Polymerase chain reaction (PCR) can be used to detect bacterial DNA, and has sensitivity and specificity equal or superior to bacterial culture [59, 214, 215]. However, PCR does not provide information about antibiotic sensitivity, and has not gained widespread use.

The best and most commonly used method for rapidly confirming a suspicion of bacterial infection is to perform cytology on a sample of synovial fluid to determine total white blood cell (WBC) count and neutrophil percentage [168]. A WBC count of more than 10×10^9 cells/l and <90 % neutrophils are highly suspicious of infection [274, 282]. It is often recommended to treat any increase in WBC count and/or proportion of neutrophils as presence of infection until proven otherwise, and to check adjacent bones and physes for signs of infection [9, 75, 86]. Total protein is also commonly

elevated, but has lower specificity for septic arthritis than cytology [167, 168, 273]. It can, however, be used as a patient-side method for determining if and which samples should undergo cytology and bacterial culture. Other synovial fluid analyses that may be of value include determination of pH, lactate concentration, glucose concentration, and screening for metabolites and markers of cartilage degradation [8, 51, 143, 272, 274, 291].

Criteria are less clear for osteomyelitis than septic arthritis, but the diagnosis is most often made based on a combination of clinical signs, diagnostic imaging and histology or bacterial culture of a lesion biopsy [185]. It is not always possible to obtain a biopsy, but this can readily be done in conjunction with surgical debridement and may help adjust the chosen antibiotic regime. Osteomyelitis in foals has been associated with elevated serum fibrinogen [188].

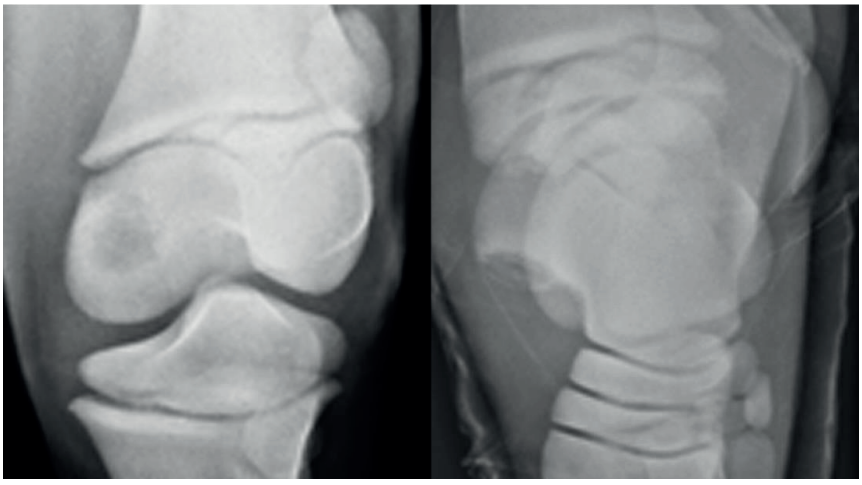

Figure 8: *Radiographic changes in the left stifle and right hock of case 4 from [I].*

Diagnostic imaging

It takes time for bacterial infections to lead to changes that are detectable by diagnostic imaging [80, 154, 185], and as prognosis depends on rapid treatment, imaging should not be relied upon for initial diagnosis.

Radiography is the most commonly used modality in septic arthritis/osteomyelitis in foals [9, 55, 86, 107, 172]. Radiography is especially useful for detecting whether osteomyelitis is present in conjunction with septic arthritis (Fig. 8), but it can also to some extent be used to evaluate soft tissue swelling and cartilage lesions in their own

right. Radiography does, however, have low sensitivity for early osteomyelitis lesions [80, 154]. Superimposition of structures in two-dimensional images makes it challenging to detect small lesions and to determine the full extent of large lesions [15, 154, 212].

Ultrasound scanners are readily available in equine ambulatory and hospital practice, and can be used to examine the echogenicity of synovial fluid (fibrin deposits) and articular cartilage integrity. In one report, ultrasound examination was successful in determining that general neck swelling included septic arthritis of cervical articular process joints [228], and in the accompanying clinical commentary it was suggested that ultrasound examination could be used to detect fluid accumulation adjacent to an irregular bone surface in osteomyelitis[212].

More advanced imaging techniques such as CT and magnetic resonance imaging (MRI) are increasingly used to detect osteomyelitis [5, 14, 80, 154, 183]. There are a number of reports describing the use of CT in cases where osteomyelitis was suspected in the proximal part of a limb, or within the axial skeleton [14, 129]. Computed tomography has superior sensitivity and specificity to radiography for detect of osteomyelitis and allows more accurate assessment of the extent of the lesion [14, 154, 221]. Magnetic resonance imaging is not as commonly used, but osteomyelitis lesions can potentially be detected at an earlier stage in MRI than CT images [80, 216]. Both CT and MRI necessitate general anaesthesia and are therefore more expensive than radiographic examination, but these modalities enable more precise diagnosis and are therefore likely to improve treatment and prognosis. Of the two, CT is often preferred over MRI because large body parts or even a full body scan can be acquired in a short amount of time [86, 154, 212].

Scintigraphy is used to diagnose early osteomyelitis and multifocal lesions in immature and mature humans, and has high sensitivity but low specificity due to the multitude of conditions that can cause increased activity in bone [191, 216]. It has also been recognised in foals that interpretation of scintigraphic images is challenging in osteomyelitis, due to the localisation of lesion adjacent to normal, highly active physes [74, 212, 262, 276].

Treatment

In this section, only the treatment of septic arthritis/osteomyelitis in foal limbs will be addressed. In order to be successful, treatment of septic arthritis/osteomyelitis must address all aspects of morbidity including pain, but a discussion of which analgesics to use is considered to be beyond the scope of this thesis summary.

Early diagnosis and aggressive treatment are key to the successful treatment of septic arthritis in foals, and treatment usually involves antibiotic administration, joint lavage and surgical debridement of lesions [9, 86, 107]. Septic arthritis/osteomyelitis varies in severity from a single affected joint/site, to multiple lesions in critically ill foals with life-threatening systemic disease, but should always be treated as a condition requiring emergency intervention [9, 86, 107].

Systemic antibiotic treatment

Administering systemic antibiotics to foals with septic arthritis has long history in equine medicine [238], and virtually all cases of orthopaedic infections in foals are treated with systemic antibiotics [118, 185, 220, 226, 249, 289, 300]. In general, broad-spectrum antibiotics are started upon examination/admission, as soon as samples for bacterial culture have been acquired. The regime is then adjusted once bacterial culture and sensitivity results become available [9, 86, 107]. There are several differences between skeletally immature and mature horses that must be kept in mind when deciding upon an antibiotic regime, including the fact that some antibiotics are toxic to immature, but not mature individuals [49].

Lavage and debridement

Joint lavage is usually advocated as soon as possible after diagnosis to physically reduce the number of bacteria in the joint.

Accumulation of fibrin and debris in synovial cavities and inflamed, necrotic foci in adjacent tissues promotes further destruction and prolongs disease duration [17, 280]. It is therefore essential to remove material that promotes continued infection and inflammation in order to successfully eliminate infectious agents, remove damaging inflammatory products and restore normal function [171]. This can only be achieved by joint lavage.

Historically, distention-irrigation and open drainage were used, but are now usually only used as adjuncts to other techniques (or as a "last resort") [16, 86, 171, 172, 237]. The technique currently most used in clinical practice is through-and-through lavage using needles or arthroscopic surgery [16, 161]. Arthroscopic surgery offers the possibility of visualising and accessing large parts of the joint [16]. Arthroscopic surgery generally requires more prolonged anaesthesia and is more expensive than needle lavage, but this can be outweighed by the opportunity to physically remove debris, and arthroscopy is superior to needle lavage, especially when large accumulations of fibrin are present [16]. The two techniques are often used alternately, due to the very common requirement for repeated lavage [249].

The need for and feasibility of surgical debridement of osteomyelitis lesions depends on the size, location and accessibility of the lesion, but debridement is generally recommended if feasible due to poor penetration of antibiotics into osteomyelitis lesions [77, 88, 185]. The goal is the same as for septic arthritis: to remove any debris that exacerbates and prolongs the infection [88]. Osteomyelitis lesions are located close to the part of bone where growth occurs, and the concern that surgical debridement may result in inadvertent damage to physeal cartilage has been raised [86]. In one study, outcome after surgical debridement of the physis was good, and the benefits of removing necrotic material were emphasized [103]. On the other hand, there are a number of studies reporting that osteomyelitis resolved after antibiotic treatment alone [139, 185], and the need for surgical debridement must therefore probably be decided on an individual case basis [146].

Local antibiotic treatment

Local antibiotic administration techniques are often used as a complement to systemic antibiotic treatment in order to achieve prolonged high concentration of antibiotics in the infected region in both skeletally immature and mature horses [50, 86, 161]. Notably, with the exception of some studies where the intraosseous route of drug delivery is evaluated [87, 260], all studies of local delivery of medications in equine medicine have been performed in skeletally mature horses [50, 141, 231].

Direct intraarticular injection of antibiotics has been used for a long time and was initially combined with injection of corticosteroids, but that is no longer recommended practice [11, 236, 280].

Regional perfusion techniques using intravenous or intraosseous injection of antibiotics distal to a tourniquet were described in the early 1990ies and are now commonly used [16, 211, 292].

Impregnated poly-methyl-methacrylate beads have been placed inside joints to give continuous release of antibiotics [24, 62], also in foal joints [86]. Antibiotic-impregnated collagen sponges have also been used, as they avoid the need for a second surgery to remove any beads [132, 259]. However, they provide only short duration minimum inhibitory concentration in the joint and are not widely used [50, 86, 132].

Systems are available for continuous infusion of antibiotics, and these provide stable concentration locally [156, 157], but require close monitoring in foals that spend much time in recumbency [86].

Prognosis

The reported overall short-term survival following orthopaedic infection in foals ranges from 45 % to 84 % [118, 180, 185, 237, 244, 249, 289, 300]. Factors documented to reduce short-term survival include: multiple lesions [118, 185, 289], age younger than 30 days [185], concurrent disease in other organs [185, 244, 249], isolation of Gram-negative bacteria [289], isolation of *Salmonella* spp. [249] and presence of both septic arthritis and osteomyelitis [185]. In equine neonates admitted to intensive care units [84], and in neonates diagnosed with bacteraemia [233], presence of orthopaedic infections was associated with reduced survival.

There is some indication that short-term survival rates following orthopaedic infections have improved over the last few decades, with the reservation that the available studies are generally retrospective, include a low number of cases and have different study designs [9, 86]. If the improvement in survival is valid, it may be due to better recognition of the importance of early initiation of treatment, and/or of increased use of combined/multiple treatment techniques [249 668, 289].

In the majority of studies on the longer term effects of surviving orthopaedic infections as a foal, infection reduces the probability of the foal racing once mature [217, 244, 249]. The time to starting in their first race was also longer for foals that survived septic arthritis when compared to the time for unaffected siblings [244]. However, there are examples of studies where surviving septic arthritis as a foal had little effect on the likelihood of racing, and no difference in race earnings between cases and controls [185]. Price at public auction was not different between horses that had received hospital treatment as foals, and controls [48]. Foals that survived bacteraemia competed at a comparable level to controls, but had fewer wins and lower total earnings [233].

As for long-term survival and prognosis: on balance, the majority of studies indicate some form of negative effect on athletic career: may not come to start, may be late to start, may have reduced wins and earnings.

Why is septic arthritis/osteomyelitis important?

It can be difficult to quantify the prevalence or incidence of septic arthritis/osteomyelitis, because these will vary with selection of the study population and time period. In 1977, septic arthritis was found in 1 % of all foals [217]. Increased knowledge about the risk of infectious disease in foals has likely lowered this prevalence; it is also likely to vary with geographic region, farms and management practices [9]. Neonatal sepsis is probably the single-most common and important problem in foals, because of prevalence but also because of morbidity [84].

The main reasons why mortality is so high is an inability to control infection in spite of aggressive treatment, and development of irreversible, irreparable cartilage damage. There is no real reason to believe that septic arthritis/osteomyelitis can be heritably predisposed, and the entity is not monitored or controlled in the same way as osteochondrosis through selection of optimal individuals for breeding. Instead, infections are most likely environmental and must therefore be controlled through hygiene and management from foaling to weaning. This is frustrating because if infections can be prevented through good foaling box hygiene, efficient routines to ensure sufficient colostrum intake and appropriate care of the navel stump then this implies that infection prevalence entails an element of preventable human error.

It is difficult to repair articular cartilage, especially to achieve repair that lasts over time. A major part of the reason why septic arthritis/osteomyelitis has such a high mortality rate is due to destruction of cartilage. This could be different if we had better techniques for more permanent cartilage repair. Also, it would help if we had a better understanding of pathogenesis in terms of when cartilage damage occurs, which mediators are involved and how can we stop or reverse them before the damage is irreparable.

These are some of the reasons why understanding pathogenesis and markers/staging of septic arthritis/osteomyelitis remains an extremely important topic in horses, who are completely reliant on their joints in order to have an acceptable quality of life.

Pathogenesis of septic arthritis/osteomyelitis in foals

The fact that bacteria that enter the circulation of foals show a predilection for establishing infection in joints and bones has been related to characteristics of the vasculature of these structures; a suggestion that was largely extrapolated from human medicine [70, 74] and is repeated whenever pathogenesis is discussed in systematic reviews [86, 107, 172].

Below, aspects of the pathogenesis of the different classifications of septic arthritis and osteomyelitis are discussed in order of how common the different types are in humans, mainly because there is more comparative literature on the pathogenesis of those types that are common in humans as well as in horses.

Metaphyseal osteomyelitis (P-type)

In 1911, Koch reported that bacterial foci were present in metaphyseal bone of rabbits two hours after intravenous injection [147]. Hobo (1921) described vascular sinusoids in metaphyseal bone adjacent to the physis, and suggested that slow blood flow in the sinusoids allowed bacteria to adhere to bone in this region [124]. In an experimental study, Starr (1921) observed fine capillaries in metaphyseal bone and suggested that bacteria could localise to such capillaries [248]. Trueta (1959) also attributed localisation of bacteria to metaphyseal bone to slow flow in vascular sinusoidal loops, and linked different patterns of osteomyelitis to changes in the organisation of vessels in the long bones of children [268]. Ogden (1979) suggested that bacterial binding occurred due to rheologic stasis in sinusoidal veins near the ossification front and led to retrograde thrombosis [193]. The hypotheses presented in these studies are united by the fact that they are based on cross-sectional observation of anatomical features and bacterial localisation in pathologic or experimental samples. This means that the suggestion that flow rate was slow in metaphyseal sinusoids, loops or capillaries represents inference from structural studies, rather than actual measurement of blood flow [60]. Nevertheless, slow blood flow in metaphyseal veins continues to be described as the primary mechanism behind osteomyelitis in children [41, 131].

In electron microscopic studies of chickens [128] and rats [7, 235], gaps were observed in vessels that extended into chondrocyte lacunae at the ossification front. Emslie (1983) combined these data with own observations and suggested that gaps in the

distal tips of vessels (temporarily) advancing into growth cartilage from metaphyseal bone allowed bacteria to come into contact with and bind to extracellular matrix [60].

The fact that vessels could indeed be discontinuous was corroborated by the observation that injected carbon particles or contents of the circulation (erythrocytes, WBC etc.) leaked from it and accumulated in this location [104, 271]. When bacteria were injected intravenously into chickens, this resulted in binding of bacteria to extracellular matrix adjacent to metaphyseal vessels within 12 hours [246]. Bacteria did not bind to either erythrocytes or endothelial cells, but rather adhered to the perivascular matrix by their glycocalyx. Bacterial binding resulted in occlusion of the vessel lumen, due to presence of the bacteria themselves or associated thrombi [246].

Epiphyseal osteomyelitis (E-type)

Epiphyseal osteomyelitis is more commonly recognised in foals than in children [70], and consequently, there is less available comparative literature. In foals, it has been described that vessels traverse the physis from the epiphysis, to the metaphysis [69, 71, 76], and this is the opposite direction of transphyseal vessels in children, which are described to traverse the physis from the metaphysis, towards the epiphysis [193, 267, 268]. The importance of the transphyseal vessels is that it has been suggested that they allow bacterial infection to spread from metaphyseal into epiphyseal bone, leading to E-type osteomyelitis in children [193, 267, 268].

Transphyseal vessels are often mentioned in research studies and reviews of foal orthopaedic infections [86, 107, 300], but they have not been assigned as specific a role as in E-type osteomyelitis in children [193, 267, 268]. Instead, Firth observed accumulations of barium in epiphyseal bone that appeared to correspond to vascular sinusoids[76]. The sinusoids were located in sites where E-type osteomyelitis lesions were also common [76]. E-type osteomyelitis could therefore be caused by the same mechanism as described for P-type osteomyelitis, above: slow flow in sinusoidal vessels permitting bacteria binding to bone [76].

Septic arthritis (S-type)

Synovial membrane has an extensive vascular network for exchange of solutes with synovial fluid [209], and bacteria can colonise the vessels of this network [168]. The network includes a plexus of fenestrated capillaries that allow diffusion between the

circulation and synovial fluid. There is no basement membrane or junctional complexes between cells in the innermost layer of synoviocytes, thus the barrier effect of the synovial membrane is low [209]. The inflammatory response to the bacteria also increases vascular permeability, making it easier for further circulating bacteria to access the joint [223].

Once bacteria are in the joint space, the immune system is inefficient in handling the infection [161]. Accumulation of fibrin and necrotic tissue can lead to formation of a pannus where bacteria thrive, and this acts as a nidus prolonging the infection [299]. S-type infection can occur due to extension from adjacent focus of infection, but this does not appear to be very common [70, 118, 185].

Osteomyelitis in cuboidal bones (T-type)

Osteomyelitis of cuboidal bones was first systematically described in the tarsus in 1985 [73], but had been mentioned in earlier case reports [181, 275]. Three of nine foals described by Firth were one of a set of twins, and the remaining six foals were either premature or had incomplete ossification [73]. Incomplete ossification of cuboidal bones was therefore suggested to predispose for development of T-type osteomyelitis [73]. The third tarsal bone was less ossified than the central tarsal bone, and also had a higher prevalence of infection, supporting this suggestion [73]. The study includes examples of T-type osteomyelitis extending into the overlying cartilage [73].

Role of cartilage canal vessels

The presence and importance of the blood supply to growth cartilage is under-recognised in children, and most experimental studies focus on the vasculature in metaphyseal bone because this is the most common location for osteomyelitis in children. There is therefore virtually no literature on the relationship between cartilage canal vessels and osteomyelitis in children.

In pigs and chickens, there is considerable evidence that experimentally injected bacteria localise to cartilage canal vessels within a short time of injection.

Intraarticular injection of bacteria in to the knee joint of pigs resulted in bacterial colonisation and occlusion of cartilage canal vessels by fibrin in the AECC of the distal femur four days post-injection [53]. Necrosis of cartilage canals occurred from 14 days

post-injection and related partly to damage caused by the immune system, especially neutrophils [53].

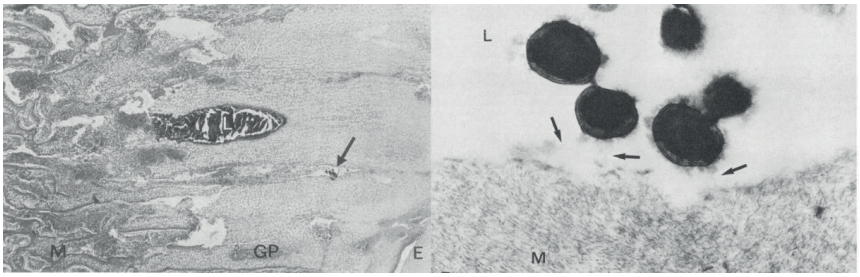

Figure 9: *Bacterial binding to matrix in the distal tips of ingrowing cartilage canals, from Speers, 1985 [246]. Left: Light micrograph of chicken proximal tibia 24 h after bacterial injection. Figure is oriented with the long axis of the bone running from left to right. Within the growth plate (GP), a metaphyseal is totally occluded by bacteria (L). A secondary focus of infection is also present in an adjacent vessel (arrow). E, epiphyseal cartilage; M, metaphysis. Hematoxin and eosin stain. Magnification, x40. Right: Electron micrograph of metaphyseal tunnels in growth plate cartilage 12 h after bacterial injection. Sample treated with antiserum. Arrows indicate the bacterial glycocalyx extending between the bacterial cell wall and the cartilage matrix (M). L, Lumen of metaphyseal tunnel.*

Similarly, bacteria were located in metaphyseal bone vessels within 12 hours, and physeal cartilage canal vessels within 24 hours of intravenous injection in chickens (Fig. 9) [60]. Necrosis of cartilage canals occurred from 24 hours, and abscesses formed from 48 hours post-injection. The focus of infection initially formed at the level of the ossochondrous junction, but resorption of cartilage was impaired superficial to the infection focus. Enchondral ossification advanced as normal in the cartilage adjacent to the focus of infection, which therefore caused a delay in ossification [60, 61]. Upon electron microscopic examination, it was demonstrated that bacteria had bound to extracellular matrix not only in the metaphyseal vessels, but also in cartilage canals [246]. Bacterial colonisation was believed to cause failure and occlusion of cartilage canal vessels, thus limiting access for the heavily mobilised immune system and allowing the infection to progress [6]. Inflammatory cells were only present at the periphery of infected foci and in adjacent, still-functional vessels [6]. Bacteria were not present in synovial fluid until 4 days post-injection, prompting the authors to suggest that septic arthritis was a consequence of osteomyelitis, rather than the other way

around [6]. This is potentially corroborated by an experimental study of osteomyelitis in pigs, where pigs were injected with different, increasing numbers of bacteria [135]. Osteomyelitis was produced at all doses, but septic arthritis only occurred in the pigs that received the highest doses of bacteria [135].

The literature on the relationship between cartilage canals and orthopaedic infections in foals is very limited. At the start of this thesis, there was a case series and a case report where a relationship between an "unknown vascular insult", septic arthritis/osteomyelitis and osteochondral lesions was implied [100, 105, 114]. Mixed aseptic and septic changes were found in 2/9 foals during a histological study of early lesions of ischaemic chondronecrosis/osteochondrosis [203]. Endothelial discontinuities were found in epiphyseal cartilage canal vessels in foals [110]. Higher prevalence of OCD/osteochondral fragments was found in a group of Standardbred horses that survived infection before 6 months of age when compared to prevalence reported in the literature for this breed [114]. The implication was that some of the lesions were caused by acquired septic, rather than heritably predisposed aseptic vascular failure, but this is of course based on inference, rather than actual evidence [114, 208].

The fact that the evidence for bacteria localising to cartilage canal vessels after experimental injection into pigs and chickens is so strong means that it is warranted to investigate whether bacteria also localise to cartilage canal vessels in foals.

Persistent challenges

The vast majority of the literature on orthopaedic infections in horses, pigs and humans focuses on prevalence, diagnosis and treatment. The most well-known hypothesis for how bacteria locate to predilection sites: due to slow flow in metaphyseal blood vessels, lacks experimental corroboration. It has been demonstrated that the blood supply to both growth cartilage and subchondral bone contain endothelial discontinuities, particularly during periods of active in-growth or regression. Discontinuities allow bacteria to come into contact with extracellular matrix. There is some evidence that the prevalence of osteochondral fragments at screening age is higher in horses that survived bacterial infection as foals, than controls. Bacteria colonise cartilage canals in other species, but it has not yet been investigated whether this also occurs in horses.

- It is not known whether bacteria colonise cartilage canals in foals, and if so: what the short-term consequences would be.
- The development of the blood supply to growth cartilage has been studied, but it has not been attempted to systematically determine whether active in-growth occurs in the time period after birth when bacteria typically are encountered.

The importance of the blood supply to the AECC in the pathogenesis of articular osteochondrosis in horses and pigs has been described in a considerable number of publications. Although there is some information available in pigs, the blood supply to the physis has not received the same amount of scientific interest.

- It is currently not known whether focal delay in enchondral ossification occur due to failure of the blood supply to the physis.

Virtually all studies on techniques for local administration of antibiotics have been performed in skeletally mature horses. Mature horses do not have the vascularised growth cartilage that is present in foals during the time when most orthopaedic infections occur.

- There is currently no available information on the distribution of locally administered medications to the cartilage canals of growing individuals.

Aims

Primary aim:

The primary aim of this thesis was to investigate the role of the cartilage canal blood supply to growth cartilage in physeal osteochondrosis and septic arthritis/osteomyelitis in foals and pigs.

Secondary aims:

The secondary aims of this thesis were as follows:

A: To examine whether bacteria were present in cartilage canals of foals with septic arthritis/osteomyelitis, whether this was associated with vascular failure and bacteria therefore caused ischaemic chondronecrosis and osteochondral lesions by the same mechanism as articular osteochondrosis in foals [I].

B: To describe lesions in the distal femoral physis of pigs and determine if these were a result of vascular failure, and therefore represented osteochondrosis with a similar pathogenesis to articular lesions [II].

C: To describe the blood supply to epiphyseal and physeal growth cartilage simultaneously in the medial femoral condyle of foals [III] to identify sites or configurations vulnerable to septic and aseptic vascular failure, which could therefore explain the pathogenesis of lesions identified in secondary aims A and B.

D: To determine the distribution of dye administered by three different regional techniques to growth cartilage canals of the tarsal region, in order to extrapolate results to potential future clinical treatment of lesions detected in secondary aims A and B.

Materials

This section contains a brief summary overview of the materials in papers I-IV. For detailed and complete information, please see the individual papers. This section also contains some additional available information that was cut from the papers due to length or at the request of reviewers.

Sources of material

-The material in paper I comprised two previously described cases [203] and five cases collected for this thesis. The two previously described cases were the inspiration for the funding application for paper I.

-The material in paper II consisted of re-use of a material generated for a previous study [206]; the study concerned articular osteochondrosis, and the material had never previously been examined for physeal osteochondrosis.

-The material in paper III consisted of nine previously used foals [113, 204] and two new foals. None of the foals had previously been used for the purpose of describing the blood supply to the growth cartilage of the medial femoral condyle.

-The material in paper IV consisted of pigs that were available because of other on-going research. The original research studies concerned physiological aspects of the central and peripheral nervous systems [Andreas Lervik, personal communication], and the limbs of these pigs had not previously been used for any research purpose.

Permissions

All permissions for the use of the material in this thesis are listed in the respective papers.

Animals

The thesis population consisted of 18 foals and 30 pigs (Table 1).

Table 1. Material used in the four papers of the thesis

	I	II	III	IV
Number of animals	7	19	11	11
Species	Foals	Pigs	Foals	Pigs
Breed	5 Standardbreds 1 Thoroughbred 1 Warmblood Riding Horse	Landrace	10 Fjord Pony 1 Norwegian-Swedish Coldblooded Trotter	Landrace
Age	9-117 days	82-180 days	228 days gestation - 62 days postpartum	52 – 112 days
Sex	4 males 3 females	All males	7 males 3 females 1 unknown	5 males 6 females
Clinical status	Septic arthritis/ osteomyelitis	Clinically healthy Predisposed for osteochondrosis	Clinically healthy	Clinically healthy
Sampling	Prospective & retrospective	Prospective	Prospective	Prospective
Study type	Cross-sectional	Pseudo-longitudinal	Pseudo-longitudinal	Experimental
Method	Histology	CT & histology	Micro-CT	Histology

Paper I

The study population consisted of seven foals aged 9-177 days collected during routine post mortem examination, and included five Standardbred foals, one Warmblood Riding Horse foal and one Thoroughbred foal. There were four males and three females.

The funding for this study was granted to an application where it was explicitly stated that the research aims were to be answered without the sacrifice of research animals. The study was inspired by detection of mixed aseptic and septic lesions in two cases during a previous study of early lesions of osteochondrosis in the distal femur [203]. Although the lesions in these two foals had been previously described, it had not been examined whether cartilage canals in the lesions contained bacteria or neutrophils and the lesions were therefore re-examined for this purpose.

The study only contains seven cases, but material from a larger number of foals were screened for potential inclusion in the study. In the previous studies of early lesions of osteochondrosis, material was sometimes collected but later excluded due to detection of joint disease other than osteochondrosis; an exclusion criterion in those studies [196, 203]. Material was available from the tibia of 18 such foals and from the femur of a further 13 such foals. Additionally, the patient databases of the Equine Section and the Pathology Section of the Norwegian University of Life Sciences were searched for foals euthanized before the age of 6 months old in the time period from 2006-2015. The search yielded 100 foals, of which 60 foals were instantly excluded because they did not have any infections (28 foals), or because they had infections outside the skeleton and histological cartilage/bone sections were therefore not available (32 foals). Of the remaining 40 foals, eight had non-skeletal *Rhodococcus equi* infection, and 32 had septic arthritis/osteomyelitis (including *Rhodococcus equi* infection). Paraffin blocks and histological sections were available from eight of these foals. Material from 21 foals collected prospectively in conjunction with previous research, and material from eight foals collected retrospectively from the university database was therefore screened in order to arrive at the seven included cases.

Inclusion criteria were treatment for or necropsy diagnosis of septic arthritis/osteomyelitis and availability of histological sections of growth cartilage with or without bone. Both sexes and any breed were included. Cases where necropsy was done more than three days after euthanasia, or where pathologic changes resulted in complete loss of anatomical structure were excluded.

Paper II

The study population consisted of 19 male Landrace pigs that had been purpose-bred from a boar with high breeding value for osteochondrosis among the offspring, and eight different sows. The boar was one of the selected boars that was used for artificial insemination in Norway at the time. The reason for using eight different sows was to generate many half-siblings close together in age/time.

In the original articular osteochondrosis study, a combination of true longitudinal and pseudo-longitudinal design was used [206, 207]. True longitudinal design means the same individual is examined at multiple times, with no possibility for histological

validation, whereas pseudo-longitudinal design means that similar individuals are examined at multiple time points, and one or more of them can be sacrificed for histological validation.

All four limbs of all pigs were CT-scanned eight times at biweekly intervals from age 70-82 at the study start, to 182 days at the study end. One or two pigs were sacrificed at each interval, and the right distal femur was harvested for histological validation.

For this study, ten femurs were available for histological validation because the remainder had already been used for the thesis of Andreas Finnøy in 2017 [65]. All areas of the distal femoral physis containing macroscopically visible changes were processed for histological examination. Longitudinal data from the physis was included in the originally prepared manuscript, but were removed at the request of the reviewers and presented in a separate manuscript currently in review.

Paper III

The study population consisted of 11 foals aged from 228 days of gestation to 62 days postpartum. Ten of the foals originated from a population of Norwegian Fjord Ponies that was purpose-bred for experimental induction of osteochondrosis [204], and for comparison of the blood supply to growth cartilage between horse and pony foals[113]. This included the foetus, which became available when one of the mares put in foal for the experimental study aborted. Six males and four females were included, whereas the sex of the foetus was not recorded. All included foals were clinically healthy and free from signs of joint disease, systemic infections and conditions affecting the circulation.

The foals were euthanized at age intervals that were optimised for histological and immunohistochemical study of the experimentally induced lesions [111, 205].

The 11th foal was a healthy Norwegian-Swedish Coldblooded Trotter foal that was the result of an unplanned mating, and was euthanized at the request of the owner who also granted permission for it to be used for research. The foal was 10 days old and therefore represented an age interval not already covered by the Fjord Pony foals. The Norwegian-Swedish Coldblooded Trotter is a cob-type breed that is similar to the Norwegian Fjord Pony in the sense that it is smaller than the Standardbred Horse and has low OCD prevalence (Fig. 10) [Arne Holm, personal communication].

Figure 10: *Both the Norwegian-Swedish Coldblooded Trotter (left; photograph by Erik Widén) and the Norwegian Fjord Pony (right; photograph by www.fjordhest.no) are cob-type breeds. The stallion on the right was the sire of some of the foals in paper III.*

Paper IV

The study population consisted of 11 pigs originating from other studies unrelated to the skeleton. Nine pigs aged 52-71 days originated from a terminal study at the Anaesthesia Section of the Norwegian University of Life Sciences and in these pigs, the protocol described in paper IV was performed post mortem. Two pigs originated from a terminal study at the Royal Veterinary College in London, and in these pigs, it was possible to perform the protocol described in paper IV in vivo/ante mortem. All pigs were clinically healthy at the time of the original study start.

Bones sampled

A total of 84 bones were sampled in this thesis: 30 foal bones and 54 pig bones (Table 2).

Table 2. Bones sampled in papers I-IV

Paper	Species	Bone	Region	Number of bones sampled	Number of samples
I	Foal	(Please see text for details)		19	49
II	Pig	Right femur	Distal physis	10	28
III	Foal	Left femur	Medial condyle	11	11
IV	Pig	Tibia	DIRT[a]	22	26
		Talus	Distal	22	26
Total				84	140

[a]Distal intermediate ridge of tibia

The 84 bones comprised six different bones, and from these bones, several different epiphyseal regions and whole or part of a physis were examined. In paper I, the

following regions were examined: three medial femoral condyles, two medial and two lateral femoral trochlear ridges, two distal metacarpal and two distal metatarsal bones, one tuber coxa, one distal tibia, and one lateral and one medial trochlear ridge of the talus [I: Supplemental Table 1], totalling 19 examined bones. The bones and regions sampled in papers II-IV are summarised in Table 2. The foetal distal femur in paper III was examined intact, otherwise the bones were examined either as 10 cuboidal tissue samples in paper III and 129 paraffin-embedded tissue blocks in papers I-II and IV (Table 2).

Methods

The methods used in this thesis will be briefly summarised below. For full details, please refer to the respective papers included at the end of the thesis.

Material in papers I, II and IV was evaluated histologically using light microscopy. In paper II, conventional CT was used to examine the ossification front, and changes in CT images were then examined histologically. In paper III, samples from arterially barium-perfused limbs were scanned using high-resolution micro-CT. In paper IV, three techniques for local administration of antibiotics were modelled using histology marking dyes and then evaluated with light microscopy.

Light microscopy (papers I, II and IV)

Bones destined for histologic examination were sawed into slabs in standardised planes of section [II, IV]. The bones processed for paper I were also most often sawed in the same, standardised plane of section, but more lesion-oriented planes of section had also been used [I]. Blocks of tissue for histological evaluation were fixed in 4 % phosphate-buffered formaldehyde for at least 48 hours and then decalcified in 10 % ethylenediaminetetraacetic acid or formic acid. After fixation and decalcification, samples were trimmed to fit into histological cassettes measuring 32 x 25 x 5 mm, embedded in paraffin and sectioned. Sections were stained with haematoxylin and eosin [I, II and IV] and Gram staining [I]. For the different descriptive terminology was used and parameters observed, please refer to the respective papers.

Computed tomography (paper II)

Directly after euthanasia by captive bolt stunning and exsanguination, a hind limb scan was obtained from each pig using a 32-slice helical CT scanner (Fig. 11) (GE Light Speed Pro 32; GE Healthcare, Munich, Germany). The scan extended from the tuber coxa to the claws and was acquired using a fixed kV of 120, a dynamic mA of up to 650 and a slice thickness of 0.625 mm. The result was a scan comprising an average of 960 transverse slices. The acquired images were viewed using a free software package (www.osirix-viewer.com). Each physis was assessed in two-dimensional frontal, sagittal and transverse image planes. Measurements were done with built-in software tools.

Figure 11: *Sedated Duroc boar undergoing routine computed tomography as part of boar testing in Norway (image courtesy of www.norsvin.no).*

Barium-perfusion (paper III)

The terminal barium perfusion procedure is described in full elsewhere [199]. The post-mortem barium perfusion procedure was based on the procedure described by Hertsch & Samy, 1980 [119].

A catheter was placed in the left femoral artery and the limb was flushed with saline until the effluent ran clear. The limb was then perfused with barium micronized to a median particle size of 0.7 µm suspended in saline, followed by barium suspended in formalin. Successful perfusion was confirmed by barium leaking from an incision made in the skin at the level of the coronary band. Other studies have confirmed that this method results in barium filling the arterial side of the circulation [199, 302]. The distal femur was harvested and fixed in 4 % phosphate-buffered formaldehyde for 48 hours. The included foetal femur was so small that the entire distal end could be scanned intact. For the remainder of the included foals, the medial condyle including the AECC and physis were separated from the distal femur by sawing as detailed in paper III, resulting in cuboidal blocks measuring an average of 2.5 x 2.5 x 6.5 cm.

Micro-CT (paper III)

Samples were wrapped in sealing film (Parafilm® M, Merck, Darmstadt, Germany) and scanned using a multi-scale x-ray nano-tomograph (Skyscan 2211; Bruker, Kontich, Belgium) with an open-type x-ray tube working at 110 kV/60µA. The samples rotated at steps of 0.31° per projection around 360°. This generated 1162 transverse images per sample, with an isotropic voxel resolution of 47 µm. The transverse images were imported into a commercial software package (VG Studio Max, version 3.2.4; Volume

Graphics, Heidelberg, Germany) and analysed both in two-dimensional frontal, sagittal and transverse image planes and as three-dimensionally volume-reconstructed models. The origin, course, orientation and branching of vessels were described qualitatively. The size of blocks, physeal area and number of vessels entering growth cartilage were registered quantitatively, including absolute vessel number and vessel number relative to physeal area.

Dye perfusion (Paper IV)

Three methods for local delivery of antibiotics: intraarticular injection, regional intravenous perfusion and intraosseous perfusion, were modelled using dye, in pairs of porcine cadaver hind limbs (n=9) and the hind limbs of anesthetized pigs (n=2). The cadaver limbs were removed immediately after euthanasia by exsanguination during general anaesthesia, flushed with saline and injected with anticoagulant and vasodilator medications. The limbs of the in vivo pigs were not flushed or injected with anticoagulant or vasodilator medications. A tourniquet was placed at mid-height of the tibia of all limbs.

Dyes intended for marking tissues for histological examination (The Davidson Marking System; Bradley Products, Bloomington, MN, USA) were then injected as follows: black dye (diluted with saline to 50 % solution) was injected into both tibiotarsal joints, blue dye (diluted with saline to 10 % solution) was injected intraosseously in the distal third of the tibia in one hind limb and green dye (diluted with saline to 10 % solution) was injected intravenously into the saphenous vein of the contralateral hind limb. The limbs were then kept at room-temperature for 16 hours to allow the dyes time to dry and fix. Samples for histologic examination were then obtained and processed as described above.

Statistical analysis (Paper IV)

Presence of dye in cartilage canals after intravenous and intraosseous perfusion was compared using the chi-square test. Significance level was set at $p \leq 0.05$. The statistical analyses were performed using commercially available software (JMP; Cary, NC, USA).

Results

This section is intended to provide a brief summary of the main results of studies I-IV (Table 3). For full details, please see the respective papers.

Physeal blood supply (papers II and III)

Blood vessels in the physis

The blood supply to the distal femoral physis of foals consisted mainly of anatomical end arteries [III]. The arterial origin of vessels supplying the physis varied from an exclusively metaphyseal origin in the foetus, via both metaphyseal and epiphyseal origin up to 10 days of age, to exclusively epiphyseal origin from 15 days of age [III].

The mid-portion of end arteries with an epiphyseal origin was incorporated into bone on the deep side of the epiphysis [III]. The distal tips of epiphyseal-origin arteries supplying the AECC were located superficially within growth cartilage, near articular cartilage. The distal tips of epiphyseal-origin arteries supplying the physis were located deep within growth cartilage, near metaphyseal bone [III].

The number of observed vessels changed with age in the AECC and the physis, both the absolute number and number relative to physeal area, albeit at slightly different times [III]. From a relatively low number at birth, vessel number increased during the first month of life before decreasing up to 62 days of age [III]. Vessel branching in the cartilage consisted of single branches spread out along the length of vessel trunks, termed monopodial branching [III].

Blood vessels in subchondral bone

A dense network of vessels was observed in metaphyseal bone towards the physis and in epiphyseal subchondral bone towards the AECC [III]. In bone, multiple vessels branched off from a single or a few points, resembling a tree crown and referred to as dichotomous branching [III]. The branching was most extensive in the metaphysis, and became more extensive with increasing age in the period examined [III]. The vasculature was not as dense in areas of monopodial branching as in areas of dichotomous branching, within epiphyseal bone. There was more dichotomous branching within metaphyseal than within epiphyseal bone, and there was also more

dichotomous branching within the metaphyseal bone of old foals than young foals, i.e. the amount of branching varied with site and age.

Vascular failure in the physis

The contents of failed cartilage canal vessels followed a consistent pattern from deep to superficial within the physis [II]. Towards the metaphyseal side of the physis, there were narrow, eosinophilic streaks that were compatible with remnants of failed vessel branches [II]. At mid-depth of the physis, there were remnants of necrotic vessel trunks that contained necrotic endothelium, necrotic perivascular cells and ghost remnant outlines of vascular luminae. Towards the epiphysis, there were chondrifying cartilage canals, and closest to the epiphysis, there was repopulation of necrotic canals by mesenchymal cells that were continuous with cells in the bone marrow [II]. This pattern was compatible with failure of end arteries coursing from the epiphysis, towards the metaphysis [II, III]. Failure could potentially occur at the point of incorporation of the mid-portion of vessels into bone on the deep side of the epiphysis as observed in paper III.

Necrotic vessels were located in the centre of or at the periphery of areas of growth cartilage that contained morphologically viable hypertrophic chondrocytes [II]. These areas protruded into either the metaphyseal or epiphyseal bone, representing areas of focally delayed ossification [II]. Out of 35 lesions, 26 were located on the metaphyseal side and nine were located on the epiphyseal side of the physis. Lesion latero-medial width varied from 1.1 %- 45.6 % (average 12.3 %) of physeal width and lesion cranio-caudal length varied from 1.4 %-38.9 % (average 12.5 %) of physeal length [II]. Repopulation of failed canals with cells from the epiphyseal side, lesion resorption by chondroclast-like cells and different patterns of ossification were observed in association with the physeal lesions and interpreted as reparative processes [II].

Bacterial infection of growth cartilage canals (papers I-III)

Cartilage canals

Bacteria were found in necrotic cartilage canals superficially within the AECC, towards the articular surface and deep in the physis, towards the metaphyseal bone, both intra- and extra-vascularly [I]. By comparison to earlier studies and paper III, it was confirmed that this corresponded to the distal tips of cartilage canals in the AECC [I, III].

Comparison to paper III also confirmed that the location of bacteria corresponded to the distal tips of cartilage canals in the physis [I, III]. This coincides with a location of growth cartilage blood supply that is highly dynamic [III].

In more proximal parts, the cartilage canals contained distinct categories of change that appeared in the same order within canals in the AECC and the physis [I: Figures 1-6]. The septic necrotic canals contained a higher number of degenerated cells, especially neutrophils, than described in earlier reports of aseptic necrotic cartilage canals [I]. Based on patho-morphologic changes in the endothelium, vascular luminae, perivascular cells and adjacent chondrocytes, classification of new types of pathologic cartilage canals associated with bacterial disease was suggested [I: Table 1]. This included necrotic canals with bacteria and/or perivascular neutrophils (acute septic canals) and granulation tissue (chronic septic canals), intact canals with neutrophils (inflamed patent canals) and canals without vascular luminae, but containing fibroblast-like or chondrocyte-like mesenchymal cells (inflamed regressing canals).

Subchondral bone

Bacteria were also found in the epiphyseal and metaphyseal subchondral bone marrow [I], which is also a location of highly dynamic vasculature [III].

Adjacent to septic lesions in growth cartilage, there were sometimes foci of osteomyelitis within subchondral bone either in the epiphysis, the metaphysis or both [I].

Consequences of septic vascular failure

Septic failure of cartilage canal vessels was associated with ischemic chondronecrosis in the AECC of five out of seven cases, and in the physis of two out of seven cases [I]. In five of the seven cases, focal delay in enchondral ossification was also observed [I: Figure 1], and it was proposed that this category of delayed ossification should be termed septic osteochondral lesions. The areas of chondronecrosis in the AECC were more extensive around septic necrotic canals than is usually seen around aseptic necrotic cartilage canals [I, II]. The areas of delayed ossification in the physis consisted of necrotic cartilage and granulation tissue, in contrast to cases of aseptic failure where it consists of viable, hypertrophic chondrocytes [I, II].

The consequences of septic vascular failure were more severe than comparable studies of aseptic vascular failure, with larger areas of ischemic chondronecrosis and, ultimately, destruction of normal anatomy (Fig. 12) [I, II]. Relatively large areas of pathologically altered tissue were observed in young foals, suggesting also a more rapid progression of pathology [I]. There was a marked immune response in septic lesions, not commonly observed in aseptic lesions [I, II].

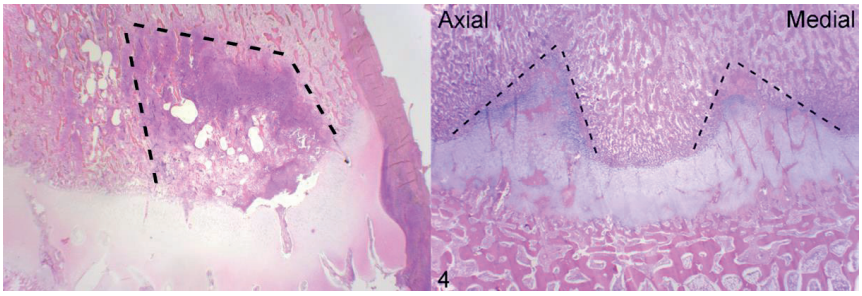

Figure 12: *To the left: Area of delayed ossification (stippled line) in physis of case 1 from paper I consisting of septic granulation tissue. To the right is Figure 4 from paper II showing delayed ossification (stippled lines) in aseptic vascular failure consisting of necrotic cartilage canals/eosinophilic streaks and viable hypertrophic chondrocytes.*

Distribution of injected dye (paper IV)

Intraarticular injection of dye into the tibio-tarsal joint resulted in evenly stained joint surfaces and synovial fluid [IV]. Dye was present in very few cartilage canals in the distal tibia and talus after intraarticular injection, and in very small foci in bone in a few of the samples [IV]. Dye was present in 58.3 % of examined cartilage canals after regional intravenous perfusion, significantly more canals than after intraosseous perfusion [IV; $p \leq 0.05$]. Intravenously perfused dye reached significantly more canals also when different bones and types of growth cartilage were analysed separately [I]. Dye injected by regional intravenous perfusion had better distribution in bone, perichondrium and soft tissues distal to the applied tourniquet than the other examined techniques [IV]. Apart from small, but evenly distributed foci in the tibial metaphysis and talus, very little intraosseously perfused dye was observed in bone. Intravenously or intraosseously injected dye was not found in the joint cavity [IV]. The main part of the study was done in post mortem material, but the inclusion of two pigs where the

techniques were performed ante mortem confirmed that the method is feasible also for use in in vivo studies.

Table 3. Summary of the main results of paper I-IV

Paper	Location of bacteria	Physeal blood supply	Vascular failure	Consequences of vascular failure in the AECC[a]	Consequences of vascular failure in the physis	Treatment of cartilage canals
I	Distal tips of cartilage canals in the AECC and physis. Subchondral bone.	Suggests epiphyseal origin.	Can be caused by bacterial binding in distal tips in cartilage canals both in AECC and physis.	Large areas of ischemic chondronecrosis → delayed ossification → septic osteochondrosis	Small areas of ischemic chondronecrosis → delayed ossification → septic osteochondrosis	Occlusion and failure of septic cartilage canals may prevent distribution of medication to infectious foci.
II	N/A[b]	Suggests epiphyseal origin.	Occurs also in the physis, at the point of vessel incorporation into bone.	N/A	Little ischemic chondronecrosis, mainly hypertrophy of chondrocytes → large areas of delayed ossification → aseptic osteochondrosis	N/A
III	Location of highly dynamic vessels and possibility for endothelial discontinuities [128, 138] coincides with location of bacteria [I].	Confirms mainly epiphyseal origin, age-related, highly dynamic nature.	N/A	N/A	N/A	Retrograde flow from the saphenous vein will reach both AECC and physis.
IV	N/A	Suggests epiphyseal origin.	Possibly prevents local delivery of injected dye to tips of cartilage canals.	Possibly prevents local delivery of injected medications to infectious foci.	Possibly prevents local delivery of injected medications to infectious foci.	Regional intravenous perfusion results in wider distribution of dye compared to intraarticular injection and intraosseous perfusion.

[a]Articular-epiphyseal cartilage complex
[b]Not applicable

Discussion

A: Materials

To answer the thesis aims of investigating anatomy [III], pathology [I, II] and treatment [IV], material was recruited from clinically healthy animals [III, IV], animals that were heritably predisposed for [II] or diagnosed with disease [I].

Material from both foals and pigs was used, due to the similarities in cartilage canal configuration discussed in the Introduction (pages 21-22), and in the disease of interest [86, 208].

A major reason for using the material from pigs was that it was either purpose-bred for investigation of osteochondrosis [II], or constituted a particularly homogeneous group [IV]. To generate similar materials from foals would have been vastly more expensive, and attempting to generate such a material from clinical cases or cases submitted for post mortem examination would have resulted in a much more heterogeneous group [196], potentially increasing the sample size required to answer the aim.

Two of the papers in the thesis [II, III] represents re-use of material originally generated for other studies. The pig population re-used in paper II is special because it contains CT examinations that include both longitudinal scans and histological validation [206, 207]. The Fjord Pony population re-used in paper III is perhaps even more unique because it contains individuals from a breed with low prevalence of OCD that are spaced at very regular intervals during a time that is highly relevant for both normal development and risk of post-natal development [204]. Examining the thesis aims in these populations was considered ethically desirable because it avoided further sacrifice of research animals for this purpose.

Paper I

The aim of paper I was to examine whether bacteria were present within growth cartilage canals and associated with ischaemic chondronecrosis in foals diagnosed with septic arthritis/osteomyelitis. To answer the study aim, the species used had to be horses. The study population included five Standardbred Horses, one Warmblood Riding Horse and one Thoroughbred Horse. In theory, it would have been better to exclude breeds with high prevalence of aseptic ischaemic chondronecrosis to avoid the

risk of classification error. In practice, classification error was easy to avoid because there was no overlap in presence of bacteria and neutrophils within canals between foals with and without septic arthritis/osteomyelitis [196, 203]. The study itself did not include any specific control group, but two of the cases originated from a study of early femoral osteochondrosis [203]. In that study, there were 23/30 lesion-negative foals that acted as historical controls for the septic femoral lesions in paper I. Likewise, in the study of early osteochondrosis in the tibia [196], where macroscopic evidence of joint disease other than OCD was an exclusion criterion, there were 91 lesion negative foals that acted historical controls for the tibial lesions in paper I. Foals older than 6 months were excluded to ensure that there were still blood vessels present within the growth cartilage of included cases. Investigation of the study aim was not dependent on the sex of included foals.

In order to be included, foals had to be diagnosed with septic arthritis/osteomyelitis. It would have been ideal to corroborate the diagnosis by positive bacterial culture in every case. However, bacterial culture results were not available from case 3, but this was not surprising as samples are often taken from the wrong place or at the wrong time, including after start of antibiotic therapy. The fact that bacterial culture results were unavailable from case 3 was therefore representative of the realities of clinical practice.

The principal alternative to the used retrospective design would have been a design where foals were experimentally injected with bacteria. With such a design, it would have been possible to generate a more uniform population with detailed knowledge about the time from bacteraemia to localisation within cartilage canals (and other tissues). In any first attempt to determine if bacteria localise to cartilage canals in foals, histological validation would have been mandatory and the study would therefore have to be terminal. Terminal experimental studies are ethically challenging and should really only be initiated if there is sufficient evidence to support that they will yield results that are important enough to justify the sacrifice of animal lives. Before paper I, the only evidence that bacteria might localise to growth cartilage canals in foals was the observation of mixed aseptic and septic lesions in two foals [203], and this is not considered sufficient evidence to initiate any terminal study. Bacteria in growth cartilage canals are not macroscopically visible, and they do not appear to localise to

predilection sites in the same way as osteochondrosis. Any study to examine early changes following experimental injection of bacteria into foals would therefore have to overcome the challenge of how to decide which pieces of growth cartilage to place in cassettes for histological processing. Of course, "all" tissues could be processed for histology, but this would be prohibitively expensive and labour-intensive.

The study population was successfully used to answer the study aim. There is reason to believe that the results from the study population may be applicable to the general horse population.

Paper II

The aim of paper II was to determine if CT and histological changes in the distal femur of pigs represented vascular failure and had the same pathogenesis as articular osteochondrosis.

According to the study aim, the species used had to be pigs. The Landrace breed was used because this breed is currently routinely CT-scanned during boar testing. If the results were to be implemented in boar testing, they would have to be applicable to this breed.

The material represented re-use from a previous study, where pigs had been purpose-bred from a boar with high breeding value for articular osteochondrosis among the offspring [206, 207]. The original studies by Grøndalen [97] and Reiland [224] indicate that pigs commonly suffer both articular and physeal osteochondrosis, thus a population bred for the study of articular osteochondrosis should also be suitable for the study of physeal osteochondrosis.

It would have been ideal to include pigs older than the maximum age of 180 days, both because the femoral physis appears to be active beyond this age, and to document the relationship between the observed lesions and angular limb deformity, which are more common in 8-9 month old pigs [224]. The study included only male pigs, because female pigs are not allowed to enter the boar testing unit. Results from paper II and future studies in older pigs are probably translatable from male to female pigs, and this is potentially important because more female than male pigs are likely to reach the age when angular limb deformity becomes relevant to health, welfare and longevity.

The prevalence of osteochondrosis in the elbow and stifle joint of contemporary Norwegian Landrace pigs is 90 % [4]. It would therefore have been challenging to recruit a control group that was genuinely osteochondrosis-negative. However, lesions are by definition focal [224], and whenever comparison was needed, the tissue adjacent to focal lesions was used.

The material was successfully used to answer the study aim. The results are likely to be applicable to Landrace pigs, and potentially also to other breeds of pig that suffer physeal osteochondrosis-related angular limb deformities.

Paper III

The aim of study III was to describe the development of the blood supply to the growth cartilage of the medial femoral condyle in foals.

To answer the study aim, the species used had to be foals. The population consisted of the Norwegian Fjord Pony and Norwegian-Swedish Coldblooded Trotter breeds, mainly because it represented re-use from a previous study [204]. Both breeds have low prevalence of articular osteochondrosis, and the parents of the included Fjord Pony foals were radiographically OCD-negative in the stifle [204]. This was considered an advantage, because the growth cartilage of these foals was unlikely to contain lesions of ischaemic chondronecrosis that would have interfered with the study of normal blood supply. It has been documented that Fjord Pony foals have fewer cartilage canal vessels and thinner growth cartilage than Standardbred foals of the same age [113]. However, the configuration of blood vessels in the AECC was the same [113], and the configuration of blood vessels in the physis is therefore also likely to be the same. The qualitative descriptions of configuration in paper III are therefore likely to be directly translatable between different horse/pony breeds. If one should wish to compare quantitative numbers/ratios of cartilage canal vessels, then such comparisons can potentially be made directly between pony breeds, but adjustments would have to be made if comparing horse and pony breeds due to the known difference in canal number/cartilage thickness at the same age [113].

The included age interval was limited by the age interval of the original study [204].

Inclusion of the previously undescribed aborted foetus was considered particularly useful.

In the future, it would be interesting to study more foetuses, and also to include foals older than the maximum age of 62 days to see if further growth spurts dominated by cartilage canal ingrowth or regression occurred before the age of 7 months, around which time femoral AECC cartilage canals probably finally regress [39].

It is not common to distinguish between the sexes in studies of the blood supply to growth cartilage, and the fact that both sexes were included potentially means that the results are applicable to both sexes.

The material was suitable for answering the study aim. The results are likely to be true for Norwegian Fjord Pony foals in the examined age range, and can potentially be translated to other breeds provided that differences in maturation are accounted for.

We wish to use the results in paper III to interpret the results in paper I. The foals are of the same age range, but of different breeds and quantitative results may therefore be different. Qualitative information about the configuration of the blood supply is, however, not likely to be different between breeds [113].

Paper IV

The aim of paper IV was to compare presence of dye within growth cartilage canals following three different techniques for local administration of antibiotics in the tarsal region of pigs. In this study, pigs were used as a model species for foals, and the techniques used are not relevant for clinical practice in pigs.

The choice of pigs as an animal model was partly opportunistic, as a terminal pig study was being conducted within the Anaesthesia Section at a suitable time. Even if this was not so, pigs would have been the model species of choice because the blood supply to growth cartilage has been most extensively studied in pigs and foals. In the Introduction (pages 21-22), it was discussed how it may be valid to compare blood supply between these two species, and comparison of dye presence may be valid provided that percentage/ratio, rather than absolute number of cartilage canals is used. The anaesthesia study meant that it was possible to recruit a large number of closely similar pigs over a short period of time. The pigs in paper IV were being sacrificed for the anaesthesia study, and it was considered ethical to use material from these pigs to answer a second research question because it avoided sacrifice of other pigs (or foals) specifically for that purpose.

Paper IV may be the first study to examine presence of locally injected compounds in growth cartilage canals. As such, histological examination was considered mandatory and the study would have to be terminal, irrespective of species. It might have been possible to conduct such a study in foals. For this, one would either have to breed dedicated research foals[204], which is expensive, or attempt to recruit foals that were being electively euthanized in the hospital. The latter is currently being pursued, and the results will be reported when ready. It can be said that foals recruited in this way generally suffer from some sort of disease or trauma [196]. It could be argued that it is first necessary to generate baseline data about the efficiency of the regional injection techniques in a clinically healthy/normal population, before attempting to draw conclusions about their efficiency in foals at different stages of different disease processes.

The in vivo group of pigs was included to confirm that the injected dyes remained within cartilage canals through processing for histological examination, i.e. that the dyes were not washed out in pigs with a functional circulation. This was considered an important test for potential future terminal studies in foals, and the results indicated that the dye should remain and therefore this methodology can be used.

The age of the included pigs was determined by the original anaesthesia studies. If the population had been recruited specifically for studying dye injection, then younger pigs with more cartilage canals could have been preferable [302]. The fact that the post mortem and in vivo groups of pigs were of slightly different ages was not of major importance because dye presence was reported as a relative ratio, and the in vivo group was included to ensure dye was not washed out by the circulation, rather than for comparing post mortem and in vivo groups.

In sum, it was possible to generate baseline information about the efficiency of the three studied injection techniques in clinically healthy pigs, and the results from the in vivo group indicate that the dye should remain within cartilage canals also in the presence of a functional circulation. As mentioned, study of the injection techniques is currently being pursued in sick foals, and the results will be reported when ready.

B: Methods

Paper I

The aim of paper I was to determine if bacteria were present within cartilage canals, and this can be achieved using light microscopy. Presence of bacteria can be suspected in haematoxylin and eosin-stained sections, but confirmation requires Gram staining which was done in all samples with sufficient tissue left in the stored paraffin blocks. Bacteria are small, and the main challenge with light microscopy is that bacteria can be present, but fail to be included in individual, 4-6 μm-thick sections. This can be alleviated through serial sectioning, but Gram staining was prioritised over serial sectioning in paper I.

In theory, it would have been possible to confirm presence of bacteria (and endothelial discontinuities) using transmission electron microscopy (TEM), as performed by Speers in chickens [246]. The retrospectively recruited sample blocks had not been collected using appropriate fixatives for TEM. The other problem with TEM is that sections are just nanometres thick, and the technique is poorly suited for screening for bacteria. Instead, it is better to use TEM for examination of areas where bacteria have already been confirmed to be present with light microscopy, as appears to have been done by Speers [246].

Paper II

The aim of paper II was to determine if changes detected within the physis in CT scans corresponded to areas with failed cartilage canal vessels in histological sections.

The reason why CT was used was because it is currently routinely used in boar testing in Norway, and it will potentially become necessary to implement the results of paper II in such testing.

In paper II, sawed slabs were evaluated macroscopically for physeal lesions, but as macroscopic evaluation is no longer used, this was done mainly in the interest of comparing sensitivity between macroscopic evaluation and CT, rather than because it was relevant to the pig industry.

In theory, MRI could have been used both in paper II and in boar testing. The pig breeding company that owns the CT scanner, Norsvin SA, considered both methods, but decided on CT because more images can be acquired in a shorter time and CT was

therefore better suited for large-scale, industrial scanning of 3,500 boars annually than MRI.

Paper III

The aim of paper III was to describe the blood supply to the growth cartilage of the medial femoral condyle of foals. The study was limited to using samples that had already been arterially perfused with micronized barium for a previous study [204]. The limitation of the perfusion technique is that the barium lodges in capillaries [302] and therefore does not show the venous side of the circulation. The major advantage is that the barium hardens in the vessels and does not become dislodged during further handling, including sawing of blocks and slabs [302].

In paper III, samples were examined using micro-CT. The advantage of this technique is that it is possible to view three-dimensional models and two-dimensional slice images in any plane of section without destroying the sample. The disadvantage is that vessels that are smaller than the maximal voxel resolution will be lost due to the partial volume effect (structures that do not fill an entire voxel are omitted from the reconstructed image).

In paper III, a deliberate choice was made to scan single, large cuboidal sample blocks containing both the AECC and the physis, as opposed to scanning smaller blocks with the AECC and the physis in different blocks. With smaller blocks, it would have been easier to obtain greater resolution and reduce the partial volume effect. A test scan showed that the resolution obtained in blocks with both the AECC and the physis was similar to that obtained during scans of experimentally induced lesions [202], and in those scans, it was possible to describe the arterial source, orientation and branching of vessels as required for the current study aim, even if the thinnest branches were lost. In paper III, perichondrial vessels coursed towards the secondary centre of ossification, turned and continued in either the superficial or deep direction. We believe that it was easier to appreciate the relationship between such vessels in the chosen large sample blocks containing both the AECC and the physis than it would have been in smaller, separate blocks. The sample blocks remain in storage and can be cut into smaller samples and scanned again if necessary.

The main alternative to micro-CT for describing blood vessels in barium-perfused samples is to use Spalteholz clearing, macroscopic inspection and stereomicroscopy

[302]. Spalteholz clearing is time-consuming (ca 8 weeks), technically challenging and the chemicals used are highly toxic. Entire bones can be examined, but in still-mineralised samples, the mineral component remains opaque and only superficial structures can be examined [302]. Description of the blood supply to the physis would require that bones were sawed into slabs, and description of blood vessels in bone would require decalcification to remove opaque mineral [304], which can take up to 3 months. When all this is done, tissue clearing and stereomicroscopy will be limited to inspection of individual, 3-5 mm thick slabs in a chosen plane of section, rather than the multi-planar reconstruction that is possible with micro-CT. The advantage of stereomicroscopy is that vessels are not lost due to the partial volume effect.

By comparison to Firth [69], it is considered that some extremely thin transphyseal branches were lost in the micro-CT scans that would not have been lost if stereomicroscopy had been used instead. However, in foals above a certain age, the very thin transphyseal branches were no longer present and from this point, branches would therefore not have been lost in the micro-CT scans [69].

The samples used in paper III were taken from the medial condyle of the femur where the blood supply to the lateral trochlear ridge was previously experimentally interrupted [204]. Histological sections are therefore available from the lateral trochlear ridge that confirm that the barium contrast columns in the micro-CT scans correspond to barium within arterioles [204]. The medial femoral condyle blocks remain in storage and can potentially also be decalcified and processed for histological validation, if necessary.

Paper IV

The aim of paper IV was to examine presence of dye within growth cartilage canals following three different local administration techniques.

In order to directly and definitively confirm that dye was present within cartilage canals, it was necessary to use histological validation. It was therefore also necessary to use a dye that was guaranteed to remain within cartilage canals through processing for histological validation. After thorough literature search, a range of coloured dyes developed specifically for marking the different sides of biopsies in humans was identified. The laboratory technician had used similar dyes before, and experienced that even if the dyes had been designed to withstand it, they disappeared during the

embedding and staining procedures. The dyes therefore had to be carefully tested, and fortunately turned out to remain within cartilage canals after processing for histological examination in both post mortem and in vivo examined pigs. Sections are routinely stained with haematoxylin and eosin, and in paper IV, it was easy to see the green and black colours at low power magnification. The blue colour was difficult to see and required scanning of sections at high power magnification, thus in future studies, this colour would not be used.

After paper IV, it should be examined whether antibiotics are present in growth cartilage canals after local injection techniques. It will then be necessary to make the antibiotics detectable by some method. This can potentially be done by mixing the antibiotics with the tissue marking dyes used in paper III. Alternatively, one could attempt to make the drug visible by fluorescence, or by mixing them with some sort of contrast or marker that is detectable by advanced diagnostic imaging. Any such imaging modality would have to have the power to visualise cartilage canals, and this includes MRI at or above 3 tesla magnet strength [266], and contrast-enhanced or positron emission tomography-CT.

C: Results

The relationship between cartilage canals and physeal osteochondrosis [I, II, III]

In CT scans in paper II, the 19 examined femurs contained 31 lesions and all CT lesions matched the definition of osteochondrosis of consisting of focal, sharply demarcated areas of soft tissue density in the ossification fronts of the physis. Further to this, all 31 lesions were multi-lobulated [II], and in previous studies of articular osteochondrosis, the only anatomical component that matched the distribution of multi-lobulated, "stair-step" lesions was the cartilage canal blood supply [206]. Chondrocytes and matrix are generally distributed throughout growth cartilage, and therefore do not match the distribution of focal, multi-lobulated lesions [206]. In articular osteochondrosis, it has been suggested that lesions are the result of failure of the trunk of an end artery during incorporation into bone [199, 303], leading to ischaemic chondronecrosis around multiple, smaller branches simultaneously and the multi-lobulated, stair-step appearance in histological sections and CT scans [206, 208]. The multi-lobulated appearance of physeal lesions in CT scans [II] therefore suggested that they were the result of failure of an end arterial trunk during incorporation into bone. However, in histological sections, lesions contained eosinophilic streaks and morphologically viable, hypertrophic chondrocytes [II]. The width of the eosinophilic streaks varied, they were oriented in many different directions and they were both single and branching and interconnected [II]. On the whole, eosinophilic streaks were initially difficult to interpret. On the one hand, the fact that physeal osteochondrosis lesions contained viable chondrocytes was not surprising, because this was known from the literature [63, 121, 123, 224, 298]. On the other hand, it was somewhat surprising because by comparison to articular osteochondrosis [206], the CT appearance of physeal lesions [II] strongly indicated that they were the result of vascular failure. This raised the questions: could lesions containing viable chondrocytes still be a result of failure of the blood supply to growth cartilage, and if so, why were chondrocytes within lesions viable, rather than necrotic as in the AECC?

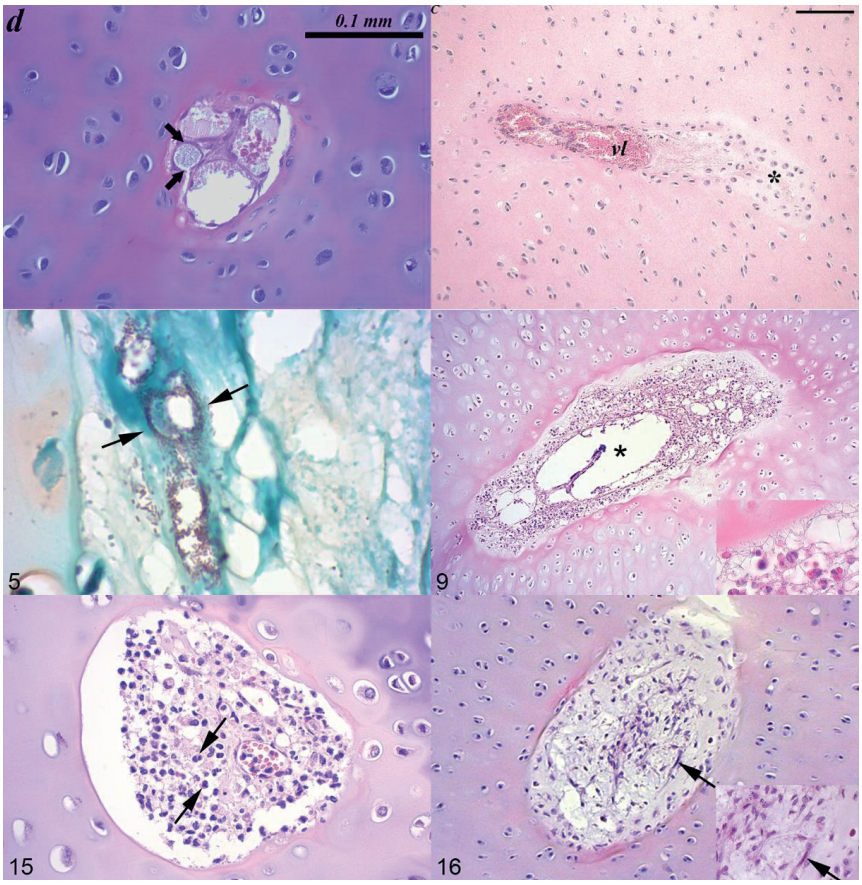

Figure 13: *Top left: Patent canal with arteriole (between arrows) and venules. From [199]. Top right: Chondrifying canal in longitudinal section; towards the left side of the image, the canal contains a still-patent vessel (vl), whereas towards the right side, the canal lumen contains chondrocyte-like cells (asterisk, *). From [196]. 5: Numerous gram-negative bacilli (arrows) are present within the remnant outlines of necrotic vessels Gram fast green (GFG). 9: remnants of degenerated neutrophils (inset) in addition to ghost-like remnant outlines of necrotic vessels (asterisk), referred to as an acute septic canal [I]. 15: A cartilage canal contains neutrophils (arrows), referred to as an inflamed patent canal [I]. 16: A cartilage canal contains no vessels and is surrounded by viable chondrocytes. This was interpreted as regression of an inflamed canal. The canal contains some spindle-shaped, fibroblast-like cells (arrows and inset) and therefore appears to be regressing by fibrosis rather than chondrification [I].*

At the same point in time, the histological sections for paper I were being evaluated. During previous studies of articular osteochondrosis in foals, several different

morphological categories of cartilage canals have been described: patent and chondrifying canals in normal cartilage [38, 196, 199], necrotic and re-populated canals within lesions [38, 196] and reparatively proliferating canals adjacent to lesions [199]. In paper I, new morphological categories were discovered, including inflamed patent and regressing cartilage canals in normal cartilage, and failed acute and chronic septic canals in lesions (Fig. 13). In the AECC, septic canals were always associated with large areas of ischaemic chondronecrosis [I]. However, in the physis, septic canals were either associated with very small areas of ischaemic chondronecrosis, or not associated with necrosis at all [I]. Failed canals are associated with minimal necrosis for the first 5-7 days after experimental transection [205], but notably, septic canals with minimal necrosis in the physis were present at the same time as septic canals with large areas of necrosis in the AECC [I]. This could represent different times of infection, but it could also mean that septic canals genuinely were associated with less ischaemic necrosis in the physis than in the AECC. By this stage, the lesions in paper I confirmed that vascular failure in the physis could be associated with small areas of necrosis. This meant that physeal cartilage was not immune to ischaemia. This reinforced the question from paper II: if septic canals could be associated with ischaemic necrosis in the physis, why were aseptic lesions that appeared to represent vascular failure in CT scans not associated with necrosis in histological sections?

In paper I, it was important to report the exact location of the different categories of canals. Many different kinds of colour-coded maps were drawn, culminating in the map shown in Figure 1 in paper I. Septic canals contained different things at four different depth levels of the AECC [I]. Superficially, there were bacteria; deep to this, there was fibrin and degenerated neutrophils and nearest the ossification front, there was septic granulation tissue that appeared to repopulate canals from epiphyseal subchondral bone [I]. Several of the samples originated from the cranial distal intermediate ridge of the tibia [I], and from this site, we had access to photographs of arterially perfused and cleared slabs from the stereomicroscope [199]. A photograph was superimposed on a map of the different changes at different depth levels (Fig. 14). This confirmed that bacteria were located at the same depth level as the distal tips of the end arteries, corresponding to previous observations [246]. In chickens, bacteria appeared to bind to cartilage matrix through discontinuities in the distal tips of ingrowing vessels [246], and the fact that bacteria were found at the distal tips of vessels could mean that the pathogenesis of bacterial binding was the same in foals.

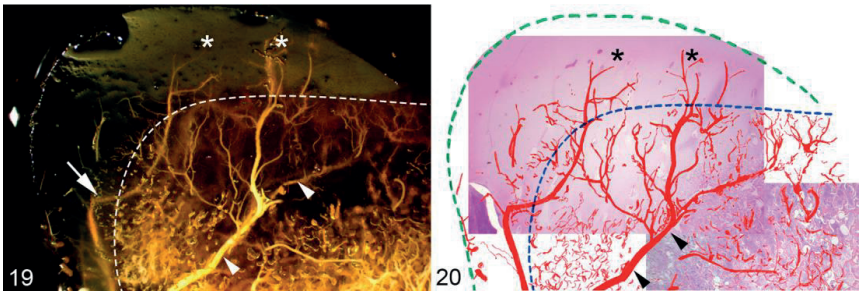

Figure 14: *On the left: The arterial side of the circulation has been perfused with barium; the slab has been decalcified and cleared by the modified Spalteholz technique. Dashed line: Ossochondrous junction. Anatomical end arteries enter epiphyseal growth cartilage canals from the perichondrium. One perichondral arteriole courses within the cartilage (arrow), and an adjacent perichondral arteriole (arrowheads) courses predominantly within subchondral bone, before turning in the distal tip of the canal, located superficially within the growth cartilage (asterisks) [I]. On the right: Tracing of arteries superimposed on histological sections from the AECC and subchondral bone. Dashed green line: Joint cartilage surface. Dashed blue line: Ossochondrous junction. The location of portions of canals containing eosinophilic granular material (see Fig. 2, I)*

in the histological section (between black dashed lines, Fig. 1, I) corresponds to the distal termini of perfused canal arterioles in cleared cartilage (asterisks) [I].

Maps were also drawn of the physeal changes in paper I. Again, changes showed a consistent pattern of bacteria, followed by fibrin, neutrophils and finally, septic granulation tissue. However, in the physis, the pattern was in the opposite direction to the AECC: bacteria were located deep in the physis, and septic granulation tissue was located next to epiphyseal bone. If the pathogenesis of septic vascular failure was the same as in the AECC, the pattern indicated that distal tips were located deep in the physis, proximal ends were located near the secondary centre of ossification, and that end arteries supplying the physis therefore coursed from the epiphysis, towards the metaphysis. The problem was that during the previous perfusion studies [200], samples had been cut through the physis, and we therefore did not have any photographs of arteries to superimpose on the lesions.

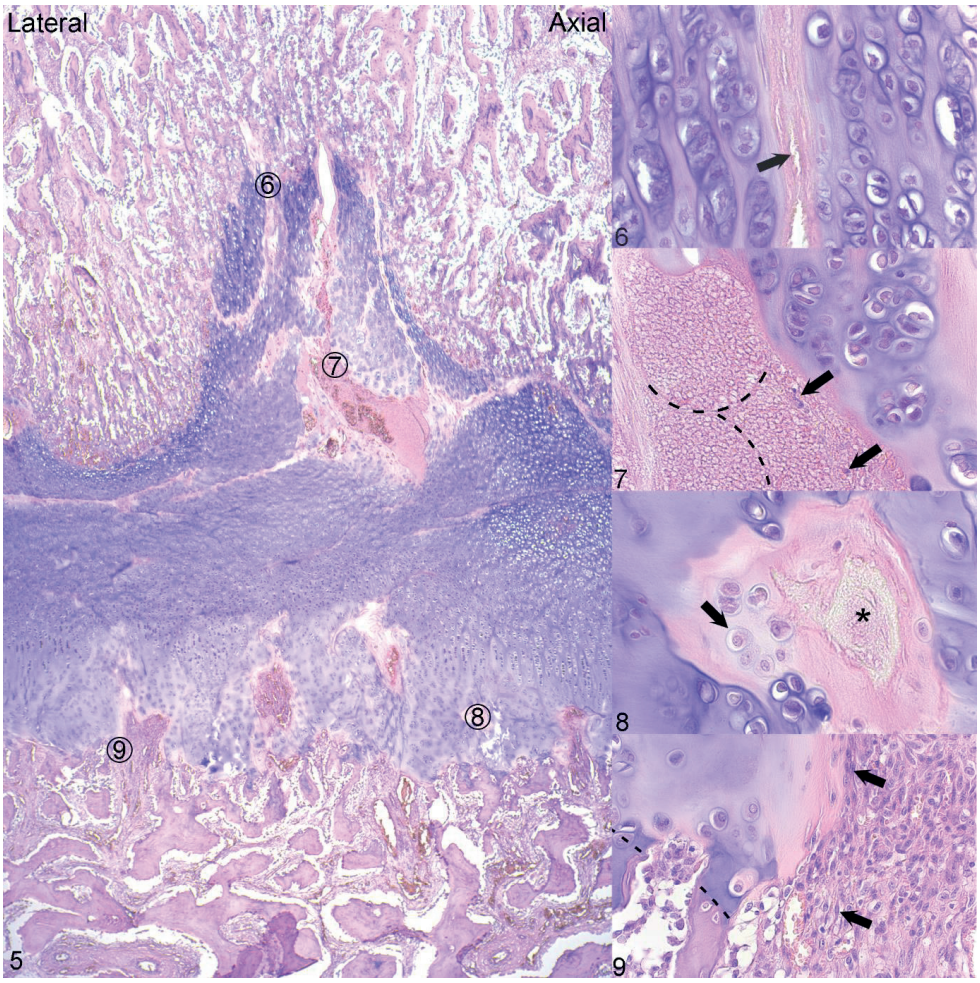

Figure 15: *Figures 5-9 from paper II. Physeal osteochondrosis, distal femur, pig M, lesion 22, central third, lateral half of the physis. Hematoxylin and eosin. 5: At low-power magnification, the distribution of cartilage canal and eosinophilic streak morphological changes within lesions is evident. The locations of Figs. 6 to 9 are indicated. 6: Nearest the metaphysis, there are thin, eosinophilic streaks that are either acellular or contain erythrocytes (arrow), compatible with necrotic vessel branches. 7: Superficial to this, there are wider eosinophilic streaks with remnant outlines of vessels (dashed lines) and necrotic perivascular cells (arrows), compatible with necrotic vessel trunks. 8: Toward the epiphysis, there are canals with remnant vascular luminae (asterisk) and chondrocyte-like perivascular cells (arrow) (i.e., chondrifying canals). 9: Nearest the epiphysis, there are canals with increased perivascular mesenchymal cells that are continuous with cells in bone marrow, interpreted as repopulated canals. Some of the cells are fibroblast-like (arrows). The dashed lines represent the transition between growth cartilage and subchondral bone.*

Inspired by the colour-coded maps in paper I, maps were also drawn of the aseptic physeal lesions in paper II. During the drawing of those maps, we realised that wide eosinophilic streaks contained cells, and upon closer scrutiny, those cells were exactly the same as in (aseptic) vascular failure in the AECC: pyknotic or necrotic endothelial cells, and pyknotic or necrotic perivascular mesenchymal cells [38, 196, 199]. This led to the conclusion that eosinophilic streaks represented necrotic cartilage canals (Fig. 15). The main difference from necrotic canals in the AECC was that necrotic canals in the physis were surrounded by relatively more eosinophilic material, and previous studies from the AECC indicate that this material represents collagen type I produced by perivascular cells within cartilage canals [64, 112].

In the AECC, vessels failed at the point where the mid-portion (trunk) of end arteries was incorporated into bone, resulting in necrosis around small branches and multi-lobulated lesions [199, 208, 303]. Physeal lesions were also multi-lobulated, so could they be the result of failure of arterial trunks during incorporation into bone?

The blood supply to the physis of pigs has been described by several authors [122, 123, 287], but the results tend to focus on transphyseal vessels, and the text contained no concrete information about whether the mid-portion of arteries was incorporated into bone. In contrast to other studies, Fig. 1 in Hills paper from 1985 [122]showed that end arteries supplying the physis were indeed incorporated into bone on the deep side of the secondary centre of ossification, before they continued to course from the epiphysis, towards the metaphysis. When Fig 1 from Hill [122] was superimposed on the lesion maps from paper II, it became possible to interpret eosinophilic streaks within lesions: thin streaks corresponded to failed branches, wide streaks corresponded to failed trunks (Fig. 16). The maps further indicated that changes followed a consistent pattern: deep in the physis, there were failed branches, superficial to this, there were failed trunks, followed by chondrifying and repopulated canals nearest the epiphysis. This pattern was compatible with failure of end arteries coursing from the epiphysis, towards the metaphysis as seen in Hill's Fig 1 [122], and exactly the same as septic physeal vessels in paper I. Historically, the research group has focused on the hind limb, but during paper I, interest in the foal forelimb perfusion papers of Firth was renewed [69, 71, 76]. Firth's studies clearly showed that the physes of the distal radius and third

metacarpal bones were supplied by vessels coursing from the epiphysis, towards the metaphysis in foals [69, 122].

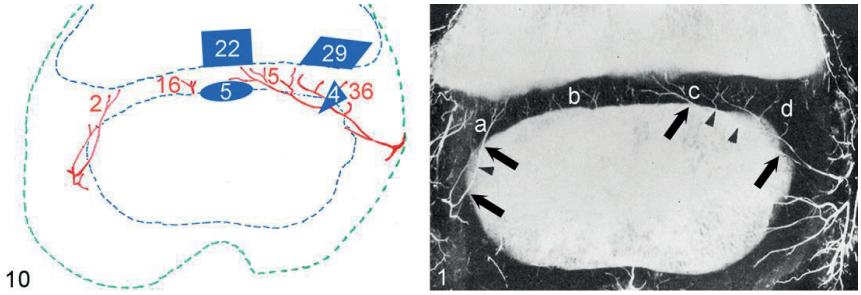

Figure 16: *Location and number of different types of physeal lesions in paper II superimposed on Figure 1 from Hill 1985. Original figure to the right [122].*

At a given point during this thesis, both septic physeal lesions in foals and aseptic physeal lesions in pigs were compatible with failure of end arteries coursing from the epiphysis, towards the metaphysis. Aseptic lesions were compatible with failure at the site of incorporation of vessel trunks into bone. Septic lesions were compatible with bacterial binding in the distal tips of end arteries. In pigs, there was the single perfusion image from Hill [122] to confirm that vessels were indeed incorporated into bone on the deep side of the epiphysis. In foals, there were only the perfusion studies of Firth [69, 71, 76] to compare to; these were from the forelimb and contained no information on vessel incorporation.

With so little information available on the development of the blood supply to hind limb physes in foals, and whether this included incorporation of vessels into bone, we realised we already had the samples to study this. The previously studied femurs of the Standardbred foals had been sawed through the physis [200], but the femurs of the Norwegian Fjord Pony foals used for the experimental study were still intact apart from the operated lateral trochlear ridge [204]. All the pigs in paper II had lesions in the physis deep to the medial femoral condyle, thus when we had to select a smaller region for micro-CT scanning, it appeared especially relevant to study the blood supply to growth cartilage in the medial femoral condyle of the foals. Paper III was therefore a direct result of observations made in papers I and II, and confirmed several interpretations. The femoral physis was supplied by end arteries coursing from the epiphysis, towards the metaphysis, confirming that the location of bacteria in the physis

in paper I corresponded to the distal tips of end arteries; the same as for the AECC and as previously observed in chickens [246]. Vessels supplying the physis in foals [III] were indeed incorporated into bone on the deep side of the epiphysis, supporting that aseptic vascular failure can potentially occur during incorporation into bone in a second species as well as in pigs [II].

Septic vascular failure was associated with small areas of ischaemic chondronecrosis in paper I, whereas aseptic vascular failure was not associated with necrosis in paper II. It is both possible and likely that chondrocytes within the physis have better access to collateral supply than chondrocytes within the AECC. Following experimental interruption of blood supply, chondrocytes adjacent to the ossification front remain viable, presumably due to diffusion from intact vessels in subchondral bone [38]. Chondrocytes in the AECC are located superficial to a single ossification front, whereas chondrocytes in the physis are located between two ossification fronts and therefore have access to diffusion from both sides. Furthermore, there is evidence that the metaphyseal ossification front advances much more rapidly than the ossification fronts of the epiphysis [79], thus devascularised lesions located deep within the physis quickly become surrounded by highly vascularised bone.

The fact that some septic lesions were associated with small areas of necrosis in the physis may be due to these areas being located beyond diffusion distance from both physeal ossification fronts [38]. However, areas of necrosis following septic vascular failure in the AECC were particularly large. The area observed in the 9 day-old case 1 was for example considerably larger than the area of ischaemic chondronecrosis observed 21 days after experimental vascular transection in the Fjord Pony foals [205]. It was considered that areas of ischaemic chondronecrosis in the AECC might become larger in a shorter time following septic than aseptic vascular failure. Bacteria produce toxins, and neutrophils produce inflammatory mediators, both of which may be toxic to chondrocytes and exacerbate necrosis beyond simple ischaemia [165, 209]. This is another potential explanation for why lesions were associated with small areas of necrosis following septic, but not aseptic vascular failure in the physis. As chondrocytes become necrotic following vascular failure in the AECC, it quickly becomes impossible to perform protein or gene expression studies in such cells/lesion [111]. As physeal

chondrocytes mostly remain viable after vascular failure [II], it may be possible to study expression and get further answers about osteochondrosis from these cells.

In sum, the studies in this thesis suggest that the role of cartilage canals in (aseptic) physeal osteochondrosis is as follows:

Eosinophilic streaks in lesions represented necrotic cartilage canals, and physeal osteochondrosis was therefore compatible with vascular failure.

The mid-portion of vessels supplying the physis was incorporated into bone on the deep side of the epiphysis, and vascular failure may therefore occur during incorporation of vessels into bone in the physis, as previously suggested in the AECC.

Vascular failure in the physis was associated with retention of viable hypertrophic chondrocytes rather than necrosis, but nevertheless resulted in focal delay in enchondral ossification/osteochondrosis.

The implication is that both physeal and articular osteochondrosis occur subsequent to failure of the blood supply to growth cartilage. The morphology of vascular failure was the same, and the mechanism and heritable predisposition may be the same in both locations [II].

New working hypothesis:

Aseptic vascular failure occurs mainly in conjunction with the process of incorporating cartilage canal vessels into bone. The distribution of lesions is therefore most likely determined by the distribution of where vessels are incorporated into bone. This may be coupled with individual ability to sense and respond to micromechanical forces at the point of vessel incorporation into bone.

D: Overlap between osteochondrosis and septic arthritis/osteomyelitis

In the AECC, aseptic vascular failure leads to ischaemic chondronecrosis, osteochondrosis and OCD. In paper I, septic vascular failure was associated with ischaemic chondronecrosis, delayed ossification and osteochondral lesions in the AECC.

These lesions can be present during the same age interval. Aseptic lesions in the study of early femoral osteochondrosis lesions were present in foals from 31-336 days old [203]. This overlaps with septic lesions being present in foals from 9-117 days in paper I. Inferred septic lesions in the radiographic prevalence study by Hendrickson were present at the same age as Standardbred Horses are routinely screened for heritably predisposed osteochondral lesions in the hock and fetlock (Fig. 17) [114].

The lesions in the Standardbred Horses examined by Hendrickson are likely to represent a mixture of acquired, septic and aseptic, heritably predisposed lesions [114]. Some of the included horses had osteochondral lesions in both the fetlock and hock joint, and the time when infection was first noticed was after the blood supply had regressed from the fetlock joint [114]. The fetlock lesions in these horses therefore probably arose before infection and were heritably predisposed, whereas infection was noticed while vessels are still present in the hock and lesions therefore could have arisen before infection and been heritably predisposed, or arisen after infection and represented acquired, septic vascular failure [114].

During screening for heritably predisposed OCD at a single time, it was known that false negative diagnosis was possible due to lesions having resolved before screening age. The results of paper I and Hendrickson [114] indicate that false positive diagnosis is also possible, of fragments that are due to acquired, septic vascular failure rather than heritably predisposed OCD.

Before the three-part study, Hance published a series of 20 cases in 1993 (ages: 4-75 weeks-7.5 months) [105] and Haggett published a single case (4 weeks old) report in 2012 [100], describing severe osteochondral lesions in foals with a history of sepsis, or of septic arthritis/osteomyelitis. Hance concluded that lesions most likely were the result of some hitherto unknown vascular insult [105]. We suggest that the pathogenetic mechanism behind the lesions observed by Hance and Haggett [100, 105]

was bacterial binding in growth cartilage canals, followed by septic vascular failure, ischaemic chondronecrosis, delayed ossification and pathologic fracture as described in paper I.

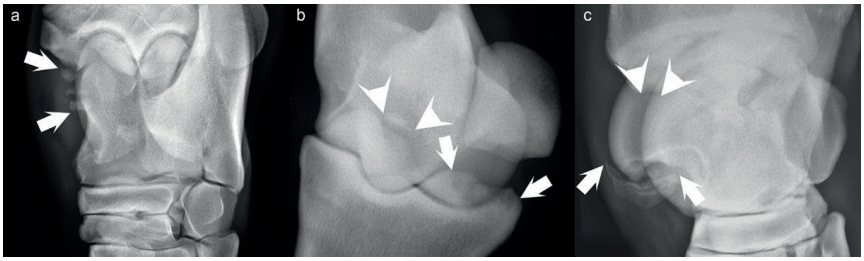

Figure 17: *Figure 2 from Hendrickson 2018 [114]. Complex and large lesions in the sepsis cohort. a: Dorsal-45°-lateral oblique projection of the left hind hock of horse 28. The dorsal-45°-medial oblique projection revealed multiple fragments (between arrows) superimposed on the talus, and this additional projection revealed that the fragments originated from the medial malleolus. b: Dorsal-35°-proximal-45°-lateral oblique projection of the right hind fetlock of horse 8. Axially, there are two mineralised bodies and three radiolucent defects (between arrows) in lateral half of the plantaro-proximal contour of the proximal phalanx. A third mineralised body (between arrowheads) is also visible through the third metatarsal bone, located at the medial half of the plantaro-proximal border of the proximal phalanx. c: Dorsal-45°-medial oblique projection of the right hind hock of horse 8. There is a large mineralised body (between arrows) at the distal end of the lateral trochlear ridge of the talus. There is also a small mineralised body with an associated radiolucent defect (between arrowheads) at the cranial distal intermediate ridge of the tibia[114].*

Prevalence was only 18 % higher in horses that survived bacterial infections as foals than contemporary controls, and septic lesions are therefore likely to represent a minority of lesions detected at screening age [114, 163].

The fact that lesions can be present during the same age interval means that it may become necessary to develop methods to distinguish between septic and aseptic lesions in a clinical setting [I]. It was perfectly possible to distinguish between septic vascular failure in paper I and aseptic vascular failure in historical controls in histological sections [196, 203]. However, owners are unlikely to consent to collection of biopsies for histological examination from prospective racehorses in the clinic. It should be investigated whether (and when) synovial fluid analysis for bacterial DNA is useful for distinguishing between septic and aseptic lesions in the clinic.

In the future, it may become possible to distinguish between horses with acquired, septic and heritably predisposed lesions based on genetic testing, rather than synovial fluid analysis or other, morphological/phenotypic techniques.

E: Role of cartilage canals in septic arthritis/osteomyelitis

Role of cartilage canals in septic arthritis without osteomyelitis

The results of bacterial culture of synovial fluid were available from some, but not all foals included in paper I, and the fluid was not necessarily collected from the joint adjacent to septic lesions. Paper I therefore does not really contain any information about the relationship between bacteria in synovial fluid and in canals, and even if it had done, it would not have been possible to determine if one preceded the other in retrospectively collected samples.

The method in paper IV confirms that an injected dye/compound can get from the synovial cavity, into growth cartilage canals. Importantly, the pigs used in paper IV were clinically healthy, indicating that bacteria can spread by the same route before inflammation is present. The results in paper IV agree with previous studies in pigs [53] where bacteria injected into joints localised to growth cartilage canals within 48 hours of injection. Bacteria injected into the circulation of pigs [135] and chickens [60] can also migrate from the circulation, which includes the cartilage canals, into joints. Following injection of bacteria into the stifle joint of pigs, bacteria were found in distant joints, e.g. the shoulder joint, after 48 hours/days [53], meaning that bacteria moved from the stifle, into the circulation and then out of the circulation and into a joint again.

In sum, bacteria can get from the joint cavity into cartilage canals and from the cartilage canals/circulation into joints. It is not known what happens first, or what is most common in septic arthritis in foals, and the only way to determine this would be by following experimentally injected bacteria over time.

Role of cartilage canals in septic arthritis/osteomyelitis

One of the challenges with histological validation is that one has to decide on which region of a tissue to place into cassettes measuring 32 x 25 x 5 mm for paraffin embedding. The two most common approaches are to use a standardised sampling site [196, 203], or to sample regions based on macroscopically visible changes [II]. Studies support that the earliest cartilage/bone changes following experimental injection of bacteria are not macroscopically visible [53, 134, 246]. Macroscopically visible changes are therefore likely to be chronic, rather than early, and genuinely early changes are difficult to capture in histological sections. Because some of the cases in paper I were

excluded from studies of early osteochondrosis [199, 203], they included macroscopically normal blocks from standardised sampling sites. Lesions detected histologically in macroscopically normal blocks are likely to represent genuinely early changes that would not have been captured if only macroscopically visible changes had been sampled.

The cases in paper I included a number of septic lesions that affected only growth cartilage, not bone. This shows that infection definitively can start in growth cartilage, and is not obliged to start in bone, before it can appear in growth cartilage. The lesions in paper I could be divided into two groups: lesions that affected cartilage only, and lesions that affected cartilage and bone. On the one hand, the common way to interpret this would be that the simpler lesions in one tissue; cartilage represented an earlier stage of the complex changes in two tissues: cartilage and bone [196]. On the other hand, whether lesions initially affect just cartilage, just bone or both may reflect an entirely different factor than disease stage/duration, as discussed further below (pages 96-97).

When Firth studied the site of early osteomyelitis in foals, sampling was limited to tissue with macroscopically visible changes [74]. This is understandable, but the studied lesions may have been chronic simply because they were macroscopically visible. Also in the study by Firth, early lesions of osteomyelitis were located on the bone side of the ossochondrous, just deep to the ossification front [74]. A portion of lesions contained areas of cartilage necrosis [74]. In experimentally induced osteochondrosis, areas of cartilage necrosis became manifest in histological sections from 7 days following vascular transection [205]. As discussed (page 36), bacterial toxins and inflammatory mediators may increase the rate of chondrocyte necrosis in septic, compared to aseptic vascular failure. Even so, lesions that contain areas of cartilage necrosis are likely to be of some days' duration [205]. This is important because in growing foals, the ossification front is constantly advancing. One study suggest that the metaphyseal ossification front may advance by as much as 1-3 mm per day [79], and evidence from chickens suggests that the ossification front advances around septic lesions [60], just as it does in osteochondrosis [205], as opposed to septic lesions advancing with the ossification front. This theoretically means that any lesion with cartilage necrosis of ca. 7 days' duration may have been located as much as 7-21 mm relatively more superficial

within the sample at the time when it was initiated, compared to the time when it is detectable in histological sections [196]. It is therefore perhaps unlikely that the portion of Firth's lesions that contained areas of cartilage necrosis were located just deep to the ossification front at the time when they were initiated [74]. Ultimately, it does not have to be a case of bacterial infection starting either in growth cartilage or in subchondral bone. In some of the (young) cases in study I, bacterial infection appeared to have arisen simultaneously in both growth cartilage and bone.

The aetiology of infection is simple: it starts with bacteria, and is established once those bacteria are actively multiplying in a location/joint cavity or tissue. No one disagrees that the earliest lesions in septic arthritis/osteomyelitis are focal and may arise in several foci simultaneously, i.e. they are multi-focally distributed [9, 70, 86]. As reviewed in the Introduction (pages 47-48), in human studies and the foal studies by Firth the hypothesis for why bacteraemia leads to septic arthritis/osteomyelitis in particular foci is based predominantly on the morphological observation of wide and/or sinusoidal vessels within metaphyseal bone adjacent to the physis. On the one hand, the laws of physics dictate that when diameter increases, flow rate will decrease, but to the best of our knowledge, it has not been attempted to measure actual flow rate within subchondral vessels in metaphyseal bone. Measuring flow rate may be more challenging in bone than in other tissues.

The evidence in this thesis does not disprove any part of the historical hypothesis that localisation of circulating bacteria to the bones and joints of foals may be due to slow flow in subchondral vessels (more below, pages 96-97). However, experimental injection of bacteria in skeletally immature chickens [60, 246] and pigs [53] suggests a slightly different hypothesis, and the evidence in this thesis is more compatible with that alternative hypothesis (endothelial discontinuities), than with the historical hypothesis (slow blood flow). The alternative hypothesis potentially enables more detailed explanation of several of the pathogenetic and clinical aspects of septic arthritis/osteomyelitis in foals than the historical hypothesis. Firstly, the surface glycocalyx of bacteria contains proteins with specific binding affinity for different extracellular matrix components [43]. Secondly, the endothelium of vessels within growth cartilage canals can be discontinuous [7]. Discontinuous endothelium allows bacteria within the circulation to come into direct contact with extracellular matrix

[246]. Binding can then occur between the bacterial glycocalyx and the extracellular matrix, bacteria can start to multiply and infection is established [60]. The reason why bacterial binding occurs in some sites is because the endothelium is only discontinuous in some sites [7, 60, 110, 246]. The reason why bacterial binding only occurs at certain times is because the endothelium is only discontinuous at certain times or at stages of development [7, 60, 110, 246].

Initially, it was suggested that endothelium was discontinuous during active in-growth. Since then, it has also been suggested that endothelium is discontinuous during physiological regression. Endothelial discontinuities have been described in the distal tips of ingrowing cartilage canals in sheep [254], rats [138], guinea pigs [138] and chickens [130]. Endothelial discontinuities have also been described in chondrifying canals in pigs [297] and foals [110]. Chondrification starts in the distal tips of cartilage canals [196, 302], corresponding to the location where bacteria were found in paper I. Following experimental injection, bacteria were found in the distal tips of cartilage canals, and the distal tips correspond to both the site of in-growth and regression/chondrification of vessels [6, 53, 60].

In paper III, barium-perfused vessels were counted in a very detailed manner in samples from the medial femoral condyle of foals. Both absolute counts and ratio relative to physeal area showed the same tendencies, and tendencies were also the same between foals, i.e. there were no outliers. The number of vessels increased up to 17 days of age, and decreased from 40 days of age. In the study, it was suggested that the increase in number could reflect a phase of particularly active enchondral ossification and conversely; that the decrease in number reflected a more quiescent phase, and that the two together therefore reflected that a growth spurt occurred during the studied age interval from 0-62 days of age. Initially, this interpretation was somewhat surprising, because based on earlier studies from foals and pigs, the number of vessels was expected to steadily decrease. When the blood supply to the AECC of the tarsus [199], femur [200] and hind fetlock joint [201] of foals was described qualitatively in 0-7 week-old foals, it steadily declined. Likewise, when the blood supply to the AECC of the femoral condyles was examined in pigs, a vascular index was calculated, representing vessel length per cartilage area [302]. The index was calculated in five groups of 8-10 pigs at 2-week intervals from 7-15 weeks, and during this age

interval, the vascular indices declined linearly in both the medial and lateral condyle. When vessels were counted in paper III, the increase in vessel number up to 17 days was therefore slightly unexpected. The vascular indices in the pigs were calculated in a single section, and may not have been representative for development within the entire condyle [302]. Manual counting of vessels in two planes and a total of 1162 micro-CT slice images per sample was considered to yield numbers that were highly representative for the entire condyle [III]. In the previous foal perfusion studies, vessels were not counted but rather it was noted when they disappeared and any increase in number would have easily been missed [199-201]. However, the most likely explanation for why the number of vessels within the medial femoral condyle increased and decreased in paper III, and decreased linearly in pigs was that the foals and pigs were sampled at different stages of maturation, and that active in-growth only occurs during early post-natal growth.

It was perhaps also slightly surprising that the number of growth cartilage vessels in the distal femur declined as steeply as it did by the age of 62 days, because vessels are known to persist up to at least the age of 7 months in the AECC of the distal femur of foals [39]. If the increase and decrease in vessel number reflect a growth spurt, then it is perhaps likely that further such growth spurts occur between the ages of 62 days and 7 months [39]. This is an attractive proposal, because it could explain why foals would be particularly susceptible to septic arthritis/osteomyelitis at repeated intervals outside the immediate post-natal period when failure of passive transfer may increase the risk of infection. For example, foals have increased susceptibility to respiratory infections at around 2-4 months of age [125], when maternally derived immunity wanes and they start to produce their own antibodies. Pneumonia can be associated with transient bacteraemia. If the growth cartilage in any particular region happened to be undergoing a growth spurt with active in-growth and regression, then vessels could be discontinuous at the time of pneumonia bacteraemia, bacterial binding could occur and this could explain a late wave of septic arthritis/osteomyelitis in older foals.

In several studies, it has been described that sinusoidal vessels are present within subchondral bone [69, 71, 124, 268]. The term “sinusoidal vessels” is not always clearly defined in the text. It could mean: similar to a sinus, or: a particular category of highly fenestrated, filtrating vessels typically found in the liver that can only be identified using

electron microscopy. In the paper published by Firth in 1982, there is a figure of the morphology that is referred to as a sinusoidal vessel [69]. Interestingly, the morphology in Firth's figure appears to correspond to the morphology described as dichotomous vessel branching in paper III. It therefore seems that a similar morphology has been described using different terms.

In paper I, bacterial infection appeared to have arisen simultaneously in growth cartilage and bone in some cases. With respect to the alternative hypothesis that bacterial binding occurs in discontinuous vessels, this raises the question of whether vessels in bone are also discontinuous and permit bacterial binding to matrix. During enchondral ossification, transverse septae are removed and blood vessels and osteoblasts advance into hypertrophic lacunae. Endothelial gaps have been described in advancing metaphyseal vessels in chickens [128], rats [104] and rabbits [270]. Advancement of blood vessels into lacunae is partly stimulated by VEGF produced by hypertrophic chondrocytes in growth cartilage, and vessels have been documented to become discontinuous under the influence of VEGF [35, 83]. However, discontinuities in vessels advancing with the ossification front cannot immediately explain why early osteomyelitis lesions are focal, rather than generalised across the entire ossification front.

According to this, bacterial binding could occur in the same location as observed by Firth (and others) [69, 71, 124, 268] but for a different reason: not due to slow flow, but due to endothelial discontinuities in dichotomously branching vessels, allowing bacteria to bind to matrix. In paper III, dichotomously branching vessels were also observed within epiphyseal bone in the femoral condyle, and this potentially fits with E-type osteomyelitis being common in the distal femur of foals [74, 185].

In earlier studies of osteomyelitis [74, 185], it was described that lesions located at the ossochondrous junction sometimes extended into the overlying cartilage. This could be similar to repopulation of septic cartilage canals with septic, fibro-vascular granulation tissue from subchondral bone described in paper I. The interpretation that this represents repopulation of canals, as opposed to cells that were always present in canals was based on observations from studies of osteochondrosis, especially the pseudo longitudinal study of experimentally induced lesions where canals were repopulated with mesenchymal stem cells from subchondral bone marrow [205]. Cells

involved in disease and repair processes can therefore spread from bone into growth cartilage canals.

As discussed above (pages 93-94), changes that arise within growth cartilage and do not advance with the ossification front [205] will become surrounded by it and relocated to subchondral bone. In contrast to articular cartilage, growth cartilage is destined to be replaced by bone, and it is therefore almost inevitable that changes that arise within growth cartilage will progress to also affect subchondral bone. Bacteria that survive in the distal tips of canals in the AECC could potentially progress to E-type osteomyelitis. Bacteria that survive in the distal tips of canals in the physis could progress to either E- or P-type osteomyelitis. Of these, P-type osteomyelitis is more likely because the distal tips of physeal canals are located closer to, and ossification advances at a much more rapid rate on the metaphyseal [79], than the epiphyseal side of the physis. Again, whether septic vascular failure progresses to become associated with E- or P-type osteomyelitis would then depend on where bacterial binding occurred to begin with: in the distal tips of epiphyseal canals, physeal canals or both.

The work in this thesis has therefore led to a new working hypothesis:

Bacteria will bind anywhere where endothelium is discontinuous during the time when bacteraemia occurs, allowing bacteria to come into contact with the matrix components that they have a binding affinity for. Endothelium can be discontinuous both within growth cartilage canals and within subchondral bone. Endothelium can potentially particularly be discontinuous within dichotomously branching vessels in subchondral bone.

F: Treatment [IV and III]

Experimentally injected bacteria in pigs and chickens localise to growth cartilage canals, and this triggered the idea that it might become necessary to treat cartilage canals for infection. However, in paper I, septic canals did not contain any functional vessels and were plugged with fibrin, and therapeutic agents are therefore unlikely to enter canals unless the fibrin is removed first. In experimental infection of chickens, the infection spread from initially affected canals to neighbouring canals with time [60]. Intact, neighbouring canals have also been documented to be involved in the repair of lesions of aseptic ischaemic chondronecrosis in osteochondrosis [199, 205]. This means that although septic canals may be beyond rescue, it is important to prevent further infection and destruction of neighbouring canals, both to limit the ongoing infection, but also to maximise the chance of repair and limit the risk of progression to osteochondral lesions [114].

In paper IV, regional intravenous perfusion resulted in better distribution of dye to cartilage canals than regional intraosseous perfusion or intraarticular injection. The main reason for this is most likely that the tourniquet more effectively occluded the saphenous vein than the central bone veins that exit in the proximal part of the bone. The result would be higher pressure and more effective retrograde flow with intravenous than intraosseous perfusion. Additionally, the medullar cavity is more voluminous than the saphenous vein, increasing the amount of injected fluid necessary to build up the required pressure.

The results from the medial femoral condyle region of foals in paper III potentially help explain the results of paper IV in that the saphenous vein is directly linked to the perichondral veins. These drain the epiphysis and the physis, and high pressure in the saphenous vein will cause retrograde flow into these areas. Dye injected intraosseously into the diaphysis would have to exit the tibia and enter perichondrial vessels to enter growth cartilage canals, and the results of paper IV indicate that they only did this to a limited extent.

Intravenous regional injection of antibiotics can be recommended in any situation when infection of cartilage canals may be suspected, independent of the age of the foal.

Intraarticular injection of antibiotics remains recommended for all cases of septic arthritis.

Techniques for local injection of antibiotics are currently being investigated in sick foals, and results will require further translation from dye to antibiotic agents in future.

Conclusions

Main conclusion:

In this thesis, a distinct role for septic and aseptic failure of the blood supply to growth cartilage was demonstrated in the pathogenesis of physeal osteochondrosis and septic arthritis/osteomyelitis in foals and pigs.

Subsidiary conclusions:

A: Bacteria were located in cartilage canals of foals with septic arthritis/osteomyelitis, where they were associated with vascular failure, ischaemic chondronecrosis, delayed ossification and pathologic fracture/osteochondral lesions. Septic vascular failure can therefore be a cause of osteochondral lesions in horses.

B: Physeal lesions in pigs were a result of failure of the blood supply to growth cartilage, i.e. the same mechanism as articular osteochondrosis. Vascular failure in the physis led to retention of viable, hypertrophic chondrocytes rather than ischaemic chondronecrosis, and was associated with focal delay in enchondral ossification/osteochondrosis.

C: The blood supply to the growth cartilage of the medial femoral condyle of foals changed over time, including ingrowth/regression and vessels that radiated out from the secondary centre of ossification both towards the superficial articular cartilage and the deep physeal region. This meant that the distal tips of cartilage canals were located superficially in epiphyseal growth cartilage and deep in the physis, corresponding to the location of bacteria/septic cartilage canals in foals with septic arthritis/osteomyelitis [I/A]. It also included incorporation of the mid-portion of cartilage canals on the deep side of the secondary centre of ossification, meaning that vascular failure could occur during the process of incorporating vessels into bone in physeal as well as articular osteochondrosis [II/B].

D: Dye was present in the greatest number of epiphyseal and physeal growth cartilage canals following regional intravenous perfusion, followed by regional intraosseous perfusion and intraarticular injection. This is most likely because intravenously injected dye was distributed retrograde via the veins radiating out from the secondary centre of ossification described in the medial femoral condyle of foals [III/C]. It may not be

possible to rescue failed canals because they are no longer functional, but it may be possible to prevent failure of adjacent, patent canals. Vessels in adjacent canals are involved in lesion repair, and as outcome is a result of the balance between lesions arising and resolving, preserving adjacent vessels can potentially decrease the likelihood of progression to osteochondral lesions [I/A].

New working hypotheses from this thesis:

Aseptic vascular failure occurs mainly in conjunction with the process of incorporating cartilage canal vessels into bone. The distribution of lesions is therefore most likely determined by the distribution of where vessels are incorporated into bone. This may be coupled with individual ability to sense and respond to micromechanical forces at the point of vessel incorporation into bone.

Septic vascular failure appears to be associated with discontinuities in the distal tips of cartilage canal vessels. The initial distribution of lesions is therefore most likely determined by the distribution of constitutional/development-related discontinuities in vessels at the time of bacteraemia. Bacteria and neutrophils can lead to opening up of further, disease-induced discontinuities and subsequent lesions with a different distribution than constitutional/developmental discontinuities.

Future prospects

-One could attempt to confirm or refute discontinuities in ingrowing and regressing cartilage canal vessels, as follows:

- This could be done using transmission electron microscopy, which only allows examination of very small areas of cartilage at a time.
- Another approach would be to use leakage of injected carbon or other particles/solutions, which could allow whole-body examination.
- One could potentially also use contrast-enhanced advanced CT or MRI imaging, included that one could use contrast media with specific bacteria-sized particles.

-The pathogenesis of septic vascular failure could be investigated further in foals:

- One could perform experimental injection of bacteria, with follow-up examination/validation at controlled, regular time intervals.
- In an experimental study, one could attempt to determine whether bacteria localise to the bone, growth cartilage or the synovial cavity first.

-Physeal osteochondrosis could be studied further:

- One could attempt to document the relationship between early physeal lesions and angular limb deformity through longitudinal CT monitoring of older pigs.
- The relationship between angular limb deformity and focal osteochondrosis lesions could be investigated in foals, using CT and histology.

-The blood supply to physeal and epiphyseal growth cartilage could be studied further:

- The blood supply could be studied to more joints and physes in foals, especially in the forelimb.

-The study of local injection techniques should be translated as follows:

- From pigs to foals, from healthy to sick individuals and from injection of dye to injection of different drugs.
- Prognosis/survival could be compared retrospectively before and after the introduction of regional injection techniques in treatment.

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Papers I-IV

I

Domestic Animals–Original Article

Septic Arthritis/Osteomyelitis May Lead to Osteochondrosis-Like Lesions in Foals

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Abstract

Failure of the cartilage canal blood supply leads to ischemic chondronecrosis which causes osteochondrosis, and osteochondral lesions. Osteochondrosis is a disease with a heritable component and usually occurs under aseptic conditions. Because bacteria can bind to growth cartilage and disrupt the blood supply in pigs and chickens, we considered whether this might play a role in development of equine osteochondrosis. The aim of this study was to examine whether bacteria are present in canals in the growth cartilage of foals with septic arthritis/osteomyelitis, and whether this is associated with osteochondrosis. The material consisted of 7 foals aged 9-117 days euthanized because of septic arthritis/osteomyelitis. The 7 cases had 16 lesions in growth cartilage that were evaluated histologically. Bacteria were present in cartilage canals in foals with septic arthritis/osteomyelitis. Portions of necrotic canals adjacent to bacteria frequently contained neutrophils, termed acute septic canals; or granulation tissue with neutrophils, termed chronic septic canals. Acute and chronic septic canals were associated with ischemic chondronecrosis in the articular-epiphyseal cartilage complex (AECC) of 5 cases and in the physis of 2 cases, and ossification was focally delayed in 5 of those 7 cases. Lesions occurred with and without adjacent osteomyelitis. Bacteria were present in cartilage canals and were associated with focal chondronecrosis in both the AECC and the physis. This establishes sepsis as a plausible cause of some osteochondral lesions in horses. It is recommended that horses with sepsis-related osteochondral lesions may be used for breeding without increasing the prevalence of OCD-predisposing genes in the population.

Keywords

bacteria, cartilage canals, foal, growth cartilage, histology, osteochondral lesions, septic arthritis

The long bones of mammals grow by endochondral ossification.[29] The growth cartilage has a temporary blood supply that runs in cartilage canals, which usually contain an arteriole, a capillary network and one or more venules organized as anatomic end arteries, and perivascular mesenchymal cells.[5,26] This renders the blood supply vulnerable in case of damage. Cartilage canals regress by 2 mechanisms; chondrification, that is, becoming filled with cartilage,[4] and incorporation into the advancing ossification front.[37,39] When predilection sites for osteochondrosis in the articular epiphyseal cartilage complex (AECC) were examined in pigs and horses, necrotic cartilage canals surrounded by necrotic chondrocytes were found at intermediate depth.[39,52] Both spontaneous and experimental vascular failure in the AECC result in ischemic chondronecrosis and delayed enchondral ossification, in pigs termed osteochondrosis latens and osteochondrosis manifesta, respectively.[34,48,51,53] Ischemic chondronecrosis and delayed enchondral ossification have the potential to resolve,[7] or develop into osteochondrosis dissecans (OCD).[33] Secondary responses in the growth cartilage including proliferation of morphologically viable chondrocytes and canals have been observed adjacent to areas of ischemic chondronecrosis.[35] After areas of ischemic chondronecrosis are surrounded by the ossification front, repopulation of necrotic canals by cells from subchondral bone marrow has been observed.[35]

Osteochondrosis is most often a multifocal disease and occurs at specific predilection sites in the joints of various species, including pigs,[41] horses,[43] dogs,[31] and humans.[8] A heritable predisposition for osteochondrosis has been documented in horses[16,28,49] and pigs.[1,42] Most lesions of osteochondrosis occur under aseptic conditions.[10,21] Cartilage canals have, however, been experimentally established as a target for bacterial infection in pigs[6] and chickens.[46] On transmission electron microscopy, bacteria were observed in the distal end of ingrowing cartilage

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canals in chickens, where the endothelium is discontinuous.[23,46] Necrosis of cartilage canals followed.[6,9,46] This represents a mechanism where bacteria cause ischemic chondronecrosis, with the potential to develop into OCD.[34] Septic arthritis has been associated with osteochondral lesions in the femoral condyles of foals.[18,20] Higher numbers of osteochondral lesions were also found in the hock and fetlock joints of horses treated for bacterial infections in their first 6 months of life compared to a control group with unknown infection history.(E.H.S. Hendrickson, personal communication)

As osteochondrosis has a heritable component, individuals with the condition are subject to a variety of breeding restrictions. Differentiating individuals with sepsis-related osteochondrosis from the ones with lesions due to heritable predisposition might allow a larger population to be used for breeding. For this to become reality, it is first necessary to investigate whether bacteria colonize cartilage canals in horses. The aim of the current study was to examine whether bacteria are present in canals in the growth cartilage of foals with septic arthritis/osteomyelitis, as previously documented in pig and chickens, and whether this is associated with ischemic chondronecrosis.

Materials and Methods

Cases

The current study contained 7 euthanized foals, 6 from the Equine Hospital of the Norwegian University of Life Sciences and one from the Swedish University of Agricultural Sciences. Inclusion criteria were treatment for septic arthritis/osteomyelitis, or necropsy diagnosis of septic arthritis/osteomyelitis. The upper age limit was 6 months. Both sexes and any breed of horse or pony were included. Histologic sections that included growth cartilage, with or without bone, had to be available. Cases where necropsy was done more than 3 days after euthanasia and where pathologic changes had resulted in complete loss of anatomical structures were excluded. The 7 cases were given ascending numbers by age, summarized in Supplemental Table 1. The femoral lesions in cases 3 and 6 had been previously described.[36]

Sample Collection

The third metacarpal bone (2), ilium (1), femur (6), tibia (5), talus (2), and third metatarsal bone (2) were sampled and sawed into approximately 5 mm thick slabs. The tali and ilium were sawed in a sagittal plane, the tibia was sawed in a slightly oblique sagittal plane parallel to the intermediate ridge and other bones were sawed in a transverse plane. All samples of the AECC included bone, whereas for physes, epiphyseal bone was always included but inclusion of metaphyseal bone varied between samples. All samples were fixed in 4% phosphate-buffered formaldehyde for 24-48 hours and decalcified in 10% ethylenediaminetetraacetic acid (EDTA), except for case 3 which was decalcified in formic acid. Sampled regions were chosen based on macroscopically visible changes or taken from standardized slabs, usually the middle slab. The samples were paraffin-embedded, before at least 2 approximately 5 µm-thick sections were cut from each block and stained with hematoxylin and eosin. Based on initial screening, sections with suspicion of bacteria were subjected to Gram staining (Supplemental Methods 1 and 2), provided that there was tissue left to section in the block.

Parameters Observed

Individual cartilage canals were categorized according to criteria published earlier for patent,[5] chondrifying[3] and necrotic canals.[37] Chondrocytes were interpreted as necrotic when they were shrunken, had cytoplasmic eosinophilia and nuclear pyknosis or karyolysis.[37] When chondrocyte necrosis and matrix change (pallor and relative eosinophilia) were present together, this was referred to as chondronecrosis, and when areas of chondronecrosis were centered on necrotic cartilage canals, they were referred to as areas of ischemic chondronecrosis.[37]

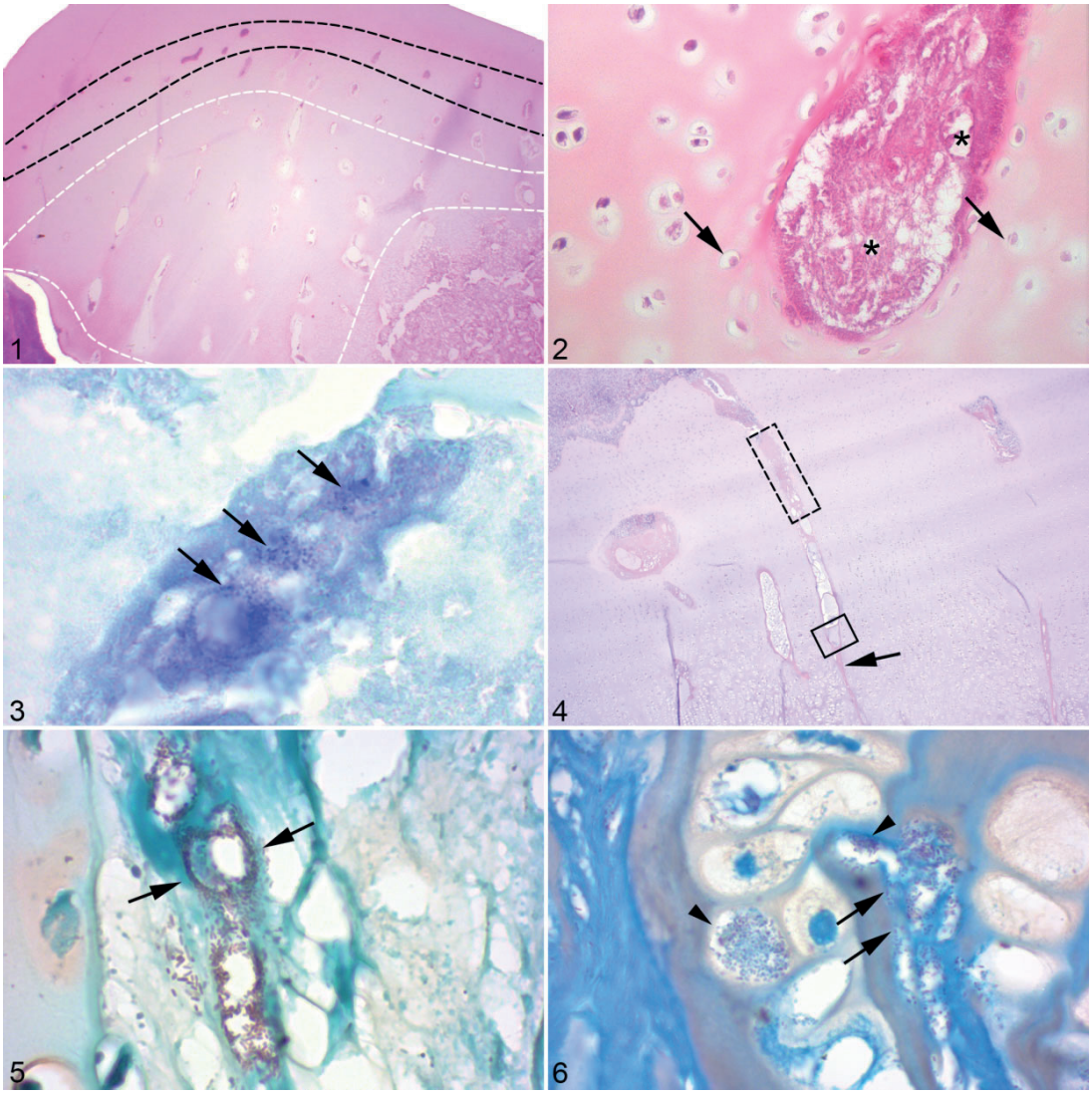
Results

Details of the 7 foals with septic arthritis/osteomyelitis included in the study are presented in Supplemental Table 1.

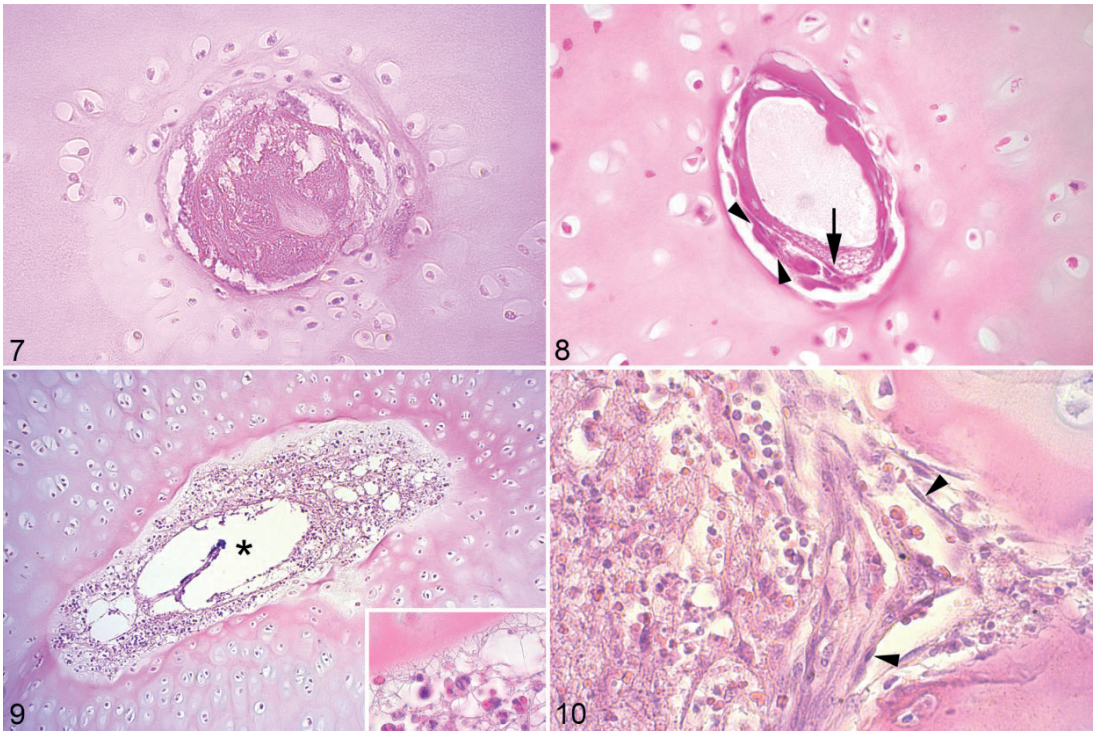
The 7 cases included 30 joints with a diagnosis of septic arthritis/osteomyelitis, and one case had osteomyelitis in the growth cartilage of the tuber coxa of the ilium, that is, there were 31 infected sites (Supplemental Table 1). Histologic material was available from 14 of the 31 infected sites. From these sites, a total of 49 blocks from 19 smaller regions were examined and lesions were found in 41 blocks (16 regions) (Supplemental Table 2).

Bacteria were suspected based on examination of hematoxylin and eosin-stained histologic sections in 6 locations from cases 1, 2, 5, and 7 (Supplemental Table 2). Gram staining confirmed presence of gram-positive (cases 1, 5, and 7) or gram-negative (case 2) bacilli or coccobacilli in the 5 locations with adequate available (Supplemental Table 2).

The most striking feature in the AECC of case 1 was that the most superficial (Fig. 1) portions of the cartilage canals contained only intensely eosinophilic, granular material (Fig. 2) comprising remnants of degenerated erythrocytes, fibrin, canal matrix, and a low number of gram-positive bacilli (Fig. 3). In the physis of cases 1 and 2, large numbers of bacteria and granular material were consistently found within portions of the deep (Fig. 4) cartilage canals (ie, those closer to the metaphyseal than the epiphyseal side of the physis). Between the bacteria-containing portions and the diaphysis, canal lumina were reduced to eosinophilic streaks (Fig. 4). In the physis of case 2, bacteria were located within ghost-like remnants of vascular lumina (Fig. 5), as well as in the perivascular connective tissue where they were apparently bound to cartilage matrix at the canal boundary (Fig. 6) and, occasionally within chondrocyte lacunae (Fig. 6). Bacteria and granular material



Figs. 1–6. Bacterial infection of cartilage canals, foal. Superficial is at the top and deep is at the bottom of each image. **Fig. 1**. Distal tibia, cranial intermediate ridge, articular-epiphyseal cartilage complex (AECC), case 1. Low magnification image to show location of changes. Portions of canals located superficially (between black dashed lines) contain intensely eosinophilic granular material (see Fig. 2). Adjacent canals (between black and white dashed lines), contain fibrin (see Fig. 7). Next to the fibrin there are necrotic canals (between white dashed lines), the majority of which represent acute septic canals (see Fig. 9). Deep to acute septic canals (below white dashed line), there are chronic septic canals (see Fig. 10). The cartilage between the white dashed lines is thickened, representing a focal delay in endochondral ossification, or septic osteochondrosis. Hematoxylin and eosin (HE). **Fig. 2**. Distal tibia, cranial intermediate ridge, AECC, case 1. Canal located superficially contains ghost-like remnant outlines of necrotic vessels (asterisks) and intensely eosinophilic granular material which comprises remnants of degenerated erythrocytes, disintegrated matrix and fibrin. Chondrocytes on the deep side of the canal are necrotic (arrows). HE. **Fig. 3**. Distal tibia, cranial intermediate ridge, AECC, case 1. A low number of gram-positive bacilli (arrows) are present within the granular material in a canal superficially. Gram Twort. **Fig. 4**. Third metacarpal bone, distal physis, case 2. Low magnification image to show location of changes. There are several necrotic canals with suspected bacteria and varying amounts of granular material located deep within the physis (within solid line box, see Figs. 5 and 6). The canals between the 2 boxes (see example, Fig. 7) contain fibrin. Within the dashed box (see example, Fig. 9) are acute septic canals



Figs. 7–10. Lesions of cartilage canals in foals with septic arthritis, distal tibia, cranial intermediate ridge, articular-epiphyseal cartilage complex (AECC). Superficial is at the top and deep is at the bottom of each image. **Fig. 7**. Case 1. Cartilage canal contains fibrillar, eosinophilic material interpreted as fibrin. Hematoxylin and eosin (HE). **Fig. 8**. Aseptic osteochondrosis, Swedish Warmblood horse, 23 days old. Aseptic necrotic cartilage canal contains necrotic endothelial cells (arrow) and necrotic perivascular mesenchymal cells (between arrowheads). HE. **Fig. 9**. Case 1. Cartilage canal contains remnants of degenerated neutrophils (inset) in addition to ghost-like remnant outlines of necrotic vessels (asterisk), referred to as an acute septic canal. HE. **Fig. 10**. Case 1. Cartilage canal contains fibroblast-like cells (arrowheads), interpreted as early repopulation of the canal with fibro-vascular granulation tissue from subchondral bone marrow, referred to as a chronic septic canal. HE.

therefore followed the pattern of being distributed superficially within the AECC (Fig. 1) and deep within the physis (Fig. 4).

Relationship of Bacteria, Cartilage Canal Lesions, and Chondrocyte Necrosis

Adjacent to the bacteria and granular material, there was fibrin (Fig. 7). Next to the fibrin, there were necrotic canals, the minority of which were indistinguishable from necrotic canals earlier described in osteochondral lesions, hereafter called aseptic necrotic canals (Fig. 8). The majority of necrotic canals contained remnants of degenerated neutrophils (Fig. 9) in addition to necrotic endothelial and perivascular mesenchymal cells, and were referred to as acute septic canals (Table 1). Adjacent to the septic canals, and also immediately adjacent to and sometimes visibly bridging the ossification front, there were canal portions that contained morphologically viable cells comprising fibroblast-like cells (Fig. 10), capillaries and neutrophils, interpreted as repopulation of necrotic canals with septic fibrovascular granulation tissue from bone marrow and referred to as chronic septic canals (Table 1). The sequence of changes from canal portions containing bacteria/granular material to the secondary, epiphyseal center of ossification was therefore fibrin (Fig. 7), acute septic canals (Fig. 9) at

Figs. 1–6. (Continued). and above the dashed box (see example, Fig. 10) are chronic septic canals. Portions of a canal (arrow) between the bacteria/granular material and the diaphysis is of reduced diameter and contain eosinophilic-staining material, referred to as eosinophilic streaks. HE. **Fig. 5**. Third metacarpal bone, distal physis, case 2. Numerous gram-negative bacilli (arrows) are present within the remnant outlines of necrotic vessels in canals deep within the physis. Gram fast green (GFG). **Fig. 6**. Third metacarpal bone, distal physis, case 2. Bacteria are closely apposed and apparently bound to cartilage matrix at the canal boundary (arrows), and also present in chondrocyte lacunae immediately adjacent to canals (arrowheads). GFG.

Table 1. Definitions for Lesions of Cartilage Canals in Foals with Septic Arthritis/Osteomyelitis.

Lesion category	Endothelium	Vascular luminae	Perivascular mesenchymal cells	Other cells in canal perivascular space	Pericanalicular chondrocytes
Acute septic canal	Necrotic or lysed	Ghost remnant outlines Bacteria	Necrotic or lysed	Neutrophils	AECC[a]: intervascular necrosis[b] MGP: limited perivascular and intervascular necrosis
Chronic septic canal	Arteriole and venules absent/lysed, capillaries present (angiogenesis)	Capillaries present (angiogenesis)	Absent/lysed	Neutrophils, fibroblasts and capillaries, ie, septic fibro-vascular granulation tissue	AECC: intervascular necrosis MGP: limited perivascular and intervascular necrosis
Inflamed patent canal	Normal	Normal	Normal	Neutrophils	Normal
Inflamed regressing canal	Absent	Absent	Majority: fibroblast-like Minority: chondrocyte-like	Neutrophils	Normal

[a] Articular epiphyseal cartilage complex.
[b] Necrosis: refers to chondrocytes located at intermediate depth of the growth cartilage; in early, intracartilaginous lesions, the most superficial and the deepest chondrocytes may be morphologically viable.

intermediate depth, and chronic septic canals (Fig. 10) immediately adjacent to the ossification front, both for canals in the AECC and in the physis.

Chondrocytes around granular portions superficially within the AECC of case 1 tended to be morphologically viable on the superficial side, and necrotic on the deep side of canals (Fig. 2). All chondrocytes around acute septic canals at mid-depth of the AECC were necrotic, similar smaller areas were detected around mid-depth septic canals in the AECC of cases 3, 4 and 6, respectively (Supplemental Table 2). Chondrocytes immediately adjacent to the secondary center of ossification tended to be viable irrespective of canal status. In the physis of case 1, chondrocyte necrosis was limited to occasional cells or a narrow zone surrounding septic canals. Examples of chondrocyte necrosis being extensive within the AECC and limited within the physis of the same foal were also found in cases 1, 4 and 6 (Supplemental Table 2). The only exception to limited chondrocyte necrosis within the physis was that larger areas of necrosis were observed between canals within the resting zone of the physis in one block from the left metatarsus, and at the AECC/physeal junction in another block from the right distal femur of case 2, also at the level of the resting zone.

The perichondrium of the majority of cases was normal (Supplemental Table 2), but in cases 1 and 5, it contained linear tracts of strongly eosinophilic material (Fig. 11) consisting mainly of degenerated neutrophils, interpreted as inflammation and destruction of perichondral blood vessels.

Delayed Ossification, Septic Osteochondrosis, and Osteomyelitis

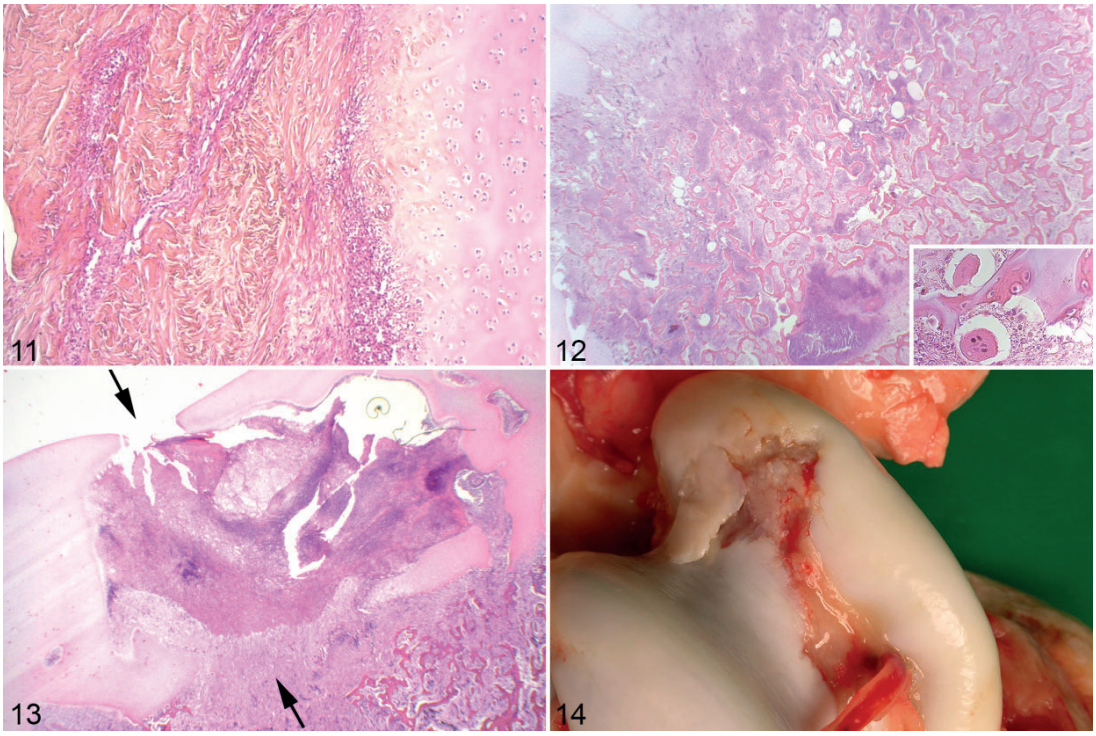
The area of chondronecrosis containing septic necrotic canals within the AECC of case 1 was associated with marked focal delay in endochondral ossification (Fig. 1), hereafter termed septic osteochondrosis. Delayed ossification was also detected in cases 3, 4, 5, and 6, and described macroscopically for case 7 but not represented in the available histological sections (Supplemental Table 2). In 2 sites (the tuber coxa of case 5 and the medial femoral condyle of case 6), the area of delayed ossification consisted predominantly of necrotic vessels and chondronecrosis. In the majority of cases, however, the area of delayed ossification comprised a mixture of necrotic growth cartilage and granulation tissue containing degenerated neutrophils (Supplemental Table 2).

Immediately deep and axially adjacent to the septic osteochondrosis lesion in case 1, there was evidence of neutrophil invasion, necrosis of primary spongiosa and formation of granulation tissue with multinucleated giant cells interpreted as osteoclasts (Fig. 12), that is, suppurative, necrotizing osteomyelitis. Among all cases, some AECC and physeal lesions had no adjacent osteomyelitis, some had adjacent osteomyelitis in some blocks, and some adjacent osteomyelitis in all blocks (Supplemental Table 2). Within the physis, osteomyelitis occurred within the adjacent epiphyseal center of ossification, within the diaphyseal center of ossification, or both (Supplemental Table 2).

Macroscopic Cyst- and OCD-Like Changes

Part of the lesion in case 3 was encapsulated by fibrous tissue, compatible with formation of a Brodie's abscess (Supplemental Table 2). Superficial to this, there were vertical linear areas of septic granulation tissue within the AECC that were compatible with, but considerably larger than, most chronic septic canals. In 2/4 blocks, these extended through the entire thickness of the AECC as nondisplaced pathologic fractures (Fig. 13).

Case 6 had a radiographically visible lesion in the distal femur previously diagnosed as a subchondral bone pseudocyst, in which the chondronecrosis was confirmed to be centered on septic canals (Supplemental Table 2). In the caudal distal



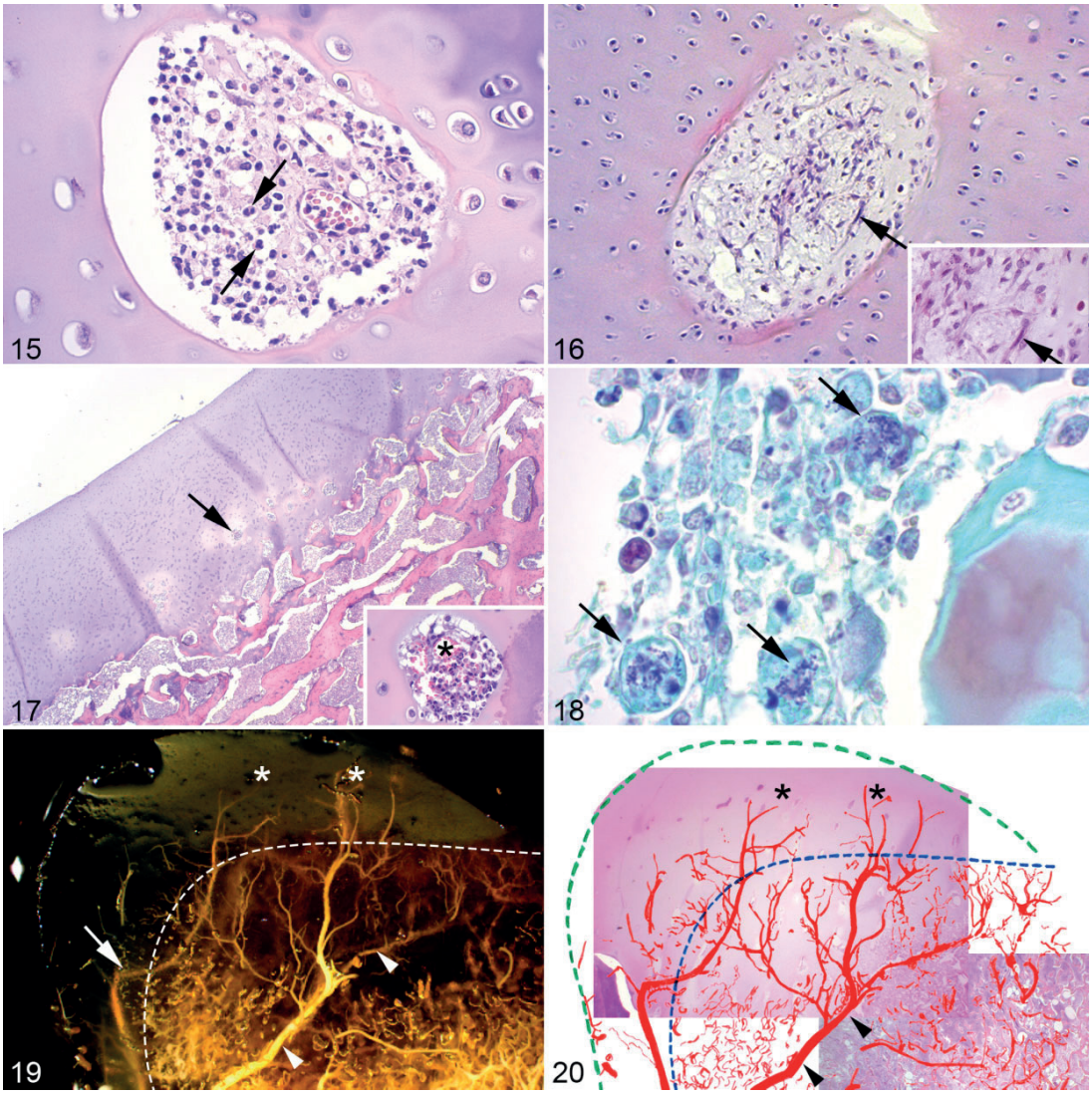
Figs. 11-14. Fig. 11. Inflammation of perichondrium, distal tibia, cranial intermediate ridge, foal, case 1. The perichondrium contains linear tracts of strongly eosinophilic material consisting mostly of degenerated neutrophils, interpreted as inflammation of perichondrial blood vessels. Hematoxylin and eosin (HE). Superficial is at the top and deep is at the bottom in Figs. 11-13. **Fig. 12**. Osteomyelitis with osteochondrosis, distal tibia, cranial intermediate ridge, subchondral bone, foal, case 1. Immediately deep and axially adjacent to the septic osteochondrosis lesion in Fig. 1, within the secondary, epiphyseal center of ossification, there is suppurative osteomyelitis, including necrosis of primary spongiosa and multinucleated giant cells interpreted as osteoclasts (inset). HE. **Fig. 13**. Nondisplaced pathologic fracture, distal femur, medial condyle, AECC, foal, case 3. The lesion extends through the entire thickness of the AECC (between arrows) as a nondisplaced pathologic fracture. HE. **Fig. 14**. Osteomyelitis, talus, medial trochlear ridge, foal, case 6. There is a septic osteochondral lesion with a hinged flap in the proximal half of the medial trochlear ridge and a band of septic fibrous tissue in the distal half.

tibia/hamate process and talus (Fig. 14) of case 6, areas of chondronecrosis were also associated with macroscopically visible wear lines, cartilage loss, protruding bands of fibrous tissue with neutrophils and undermined cartilage margins (tibia) or a hinged cartilage flap (talus). Finally, case 6 had complete pathologic avulsion of the long digital extensor tendon through an area of septic osteochondrosis.

Canal Infection, Inflammation, and Regression Without Necrosis

A low number of patent canals some distance from lesions contained neutrophils outside the circulation, mixed with the perivascular mesenchymal cells, and were surrounded by normal chondrocytes (Fig. 15). Canals with extra-vascular neutrophils were referred to as inflamed patent canals (Table 1). Similarly, in cases 2-7 there were some inflamed canals without evidence of endothelium or vascular luminae that were surrounded by normal chondrocytes and therefore appeared to be regressing (Table 1). Some of the inflamed regressing canals contained chondrocyte-like cells located in lacunar-like structures, and were therefore regressing by chondrification. The majority, however, contained spindle-shaped, fibroblast-like cells (Fig. 16) and were regressing by fibrosis.

In case 7, the physis of the hamate process was virtually completely replaced by granulation tissue containing degenerated neutrophils and there was delayed ossification, osteomyelitis and a subcutaneous closed abscess. The AECC contained only patent or regressing canals located deep in the cartilage (Fig. 17), several of which were inflamed. Numerous gram-positive coccobacilli compatible with the *Rhodococcus equi* cultured from the abscess in case 7 (Supplemental Table 1) were also observed within the osteomyelitic area and the patent or regressing cartilage canals (Fig. 18). The coccobacilli were,



Figs. 15–20. Fig. 15. Inflamed patent canal, third metatarsal bone, distal physis, foal, case 2. A cartilage canal contains neutrophils (arrows), referred to as an inflamed patent canal. The canal and surrounding chondrocytes are morphologically viable. Hematoxylin and eosin (HE). **Fig. 16**. Distal femur, medial condyle, articular-epiphyseal cartilage complex (AECC), foal, case 3. A cartilage canal contains no vessels and is surrounded by viable chondrocytes. This was interpreted as regression of an inflamed canal. The canal contains some spindle-shaped, fibroblast-like cells (arrows and inset) and therefore appears to be regressing by fibrosis rather than chondrification. HE. **Fig. 17**. Distal tibia, caudal intermediate ridge/hamate process, AECC, foal, case 7. Only a low number of regressing or patent canals (arrow and inset) with functional vascular lumina (asterisk) remain deep in the cartilage. HE. **Fig. 18**. Distal tibia, caudal intermediate ridge/hamate process, AECC, foal, case 7. Numerous gram-positive coccobacilli (arrows) compatible with the *Rhodococcus equi* (which was cultured from this case) are present within macrophages within one of the patent cartilage canals shown in Fig. 17. Gram fast green. **Fig. 19**. Standardbred foal, distal tibia, 5 mm thick sagittal slab through cranial intermediate ridge, AECC. The arterial side of the circulation has been perfused with barium, the slab has been decalcified and cleared by the modified Spalteholz technique. Dashed line: Chondro-osseous junction. Anatomical end arteries enter epiphyseal growth cartilage canals from the perichondrium. One perichondrial arteriole courses within the cartilage (arrow), and an adjacent perichondrial arteriole (arrowheads) courses predominantly within subchondral bone, before turning in the distal tip of the canal, located superficially within

however, located within macrophages (Fig. 18) and the surrounding chondrocytes were normal.

Discussion

In this study, bacteria were present in canals in the growth cartilage and bone of the AECC and physis of foals with septic arthritis/osteomyelitis. The typical location of bacteria in cartilage canals was superficially in the AECC and deep in the physis. Anatomical studies have confirmed that a major part of blood supply to the epiphyses in the forelimb of foals originates from perichondral arteries.[13,14] Branches of these vessels enter both the epiphyseal cartilage and the physis.[14,15] A similar arrangement has been identified in the AECC of hind limb joints (Fig. 19), although the physis remains to be investigated.[38,39] Vessels superficial in the AECC and deep in the physis thus are the most distal part of this vasculature (Fig. 20). The location of bacteria here is in accordance with experimental studies in chickens and pigs, where bacteria were first found in the distal tips of metaphyseal vessels.[2,9,24,46]

Discontinuities in the endothelium of epiphyseal chondrifying cartilage canals of foals aged 1-122 days were recently described.[22] Intravenously injected carbon particles accumulated outside growing distal ends of vessels in cartilage canals in noninfected rats.[19] Preexisting fenestrations and gaps in the endothelium could allow small numbers of bacteria to adhere and trigger an inflammation-mediated disruption of endothelium, leading to more widespread colonization and infection.[40] In this cross-sectional study, it was not possible to determine if fenestrations or gaps were present before bacterial adhesion as the endothelium of the cartilage canals was for the most part absent in the lesions examined.

Bacteremia and the accompanying inflammatory response have an impact on the integrity of the endothelium and exacerbate the permeability to bacteria.[40,50] An inflammation-related increase in the permeability of vessel walls has been suggested to allow bacteria to cross where they otherwise would not be able to cross.[6] A more recent study of intravenous inoculation of *Staphylococcus aureus* in 8-week-old pigs found that destruction of endothelium in the vasculature of the physis did not occur until influx of neutrophils at 48 hours.[24] In the same study, micro abscesses in the lungs were resolved within 48 hours, while in the cartilage they persisted and increased in size. The combination of the special vascular anatomy in growth cartilage and a systemic inflammation-induced impact because of bacteremia is a likely reason for the persistence of infections in the physis.[15,38–40]

Neutrophils are not a feature of necrotic canals in studies of osteochondral lesions in heritably predisposed breeds (Fig. 8) or in surgically induced lesions.[3,35,37] Increased numbers of neutrophils in and around cartilage canals were demonstrated in experimental studies 24 hours after inoculation of bacteria in pigs and 12 hours after inoculation in chickens.[9,24] Due to their larger size and more common presence, neutrophils are easier to detect in histologic sections than bacteria.[47] In the absence of bacteria, neutrophils were therefore used as a criterion for identification of septic canals.

Chondronecrosis around septic canals was observed in 6 out of 7 foals in this study, both in the AECC and the physis. The areas of chondronecrosis were consistently more extensive in the AECC than in the physis. Osteochondral lesions have been suggested to be a consequence of septic processes in earlier case reports.[18,20] Hendrickson et al found up to 18% higher frequency of osteochondral lesions in yearlings treated for bacterial infections before 6 months of age than controls with unknown infection history.(E.H.S. Hendrickson, personal communication) Bacterial infection and destruction of cartilage canals may therefore account for a small portion of all developmental osteochondral lesions. Causes of delayed ossification other than failure of incorporation of canals were recently described.[11] It therefore makes sense to add a prefix to osteochondrosis in lesions where the cause of delayed ossification is known. Because bacterial infection can lead to ischemic chondronecrosis and focally delayed ossification—the very definition of osteochondrosis[32,33]—we propose that this condition is termed septic osteochondrosis.

The lesions in cases 3 and 6 (Figs. 13, 14) contained macroscopically visible physical disruptions of the articular cartilage. Whether the disruptions should be termed OCD is debatable. The pathologic fractures observed extended through septic granulation tissue rather than areas of ischemic chondronecrosis, which is the defining criterion for OCD.[33] There is evidence that sepsis-survivors have more OCD lesions than animals with unknown status.(E.H.S. Hendrickson, personal communication) The lesions in these cases were in the caudal part of the distal intermediate of the tibia (hamate process), which is not a recognized predilection site for OCD. None of the sepsis-survivors had lesions at the hamate process, (E.H.S. Hendrickson, personal communication) and this may be because lesions resolved or because they were so extensive that the horses did not survive to OCD-screening age (12 months).[27] Both sepsis-survivors (E.H.S. Hendrickson, personal communication) and many of the present cases had lesions at recognized predilection sites for OCD, making it likely that some lesions do persist to screening age. This could be determined through frequent radiography of survivors from initial sepsis, until screening age.

Cases 1 and 2 had areas in the AECC and physis, or just the physis, respectively, with both early chondronecrosis and early osteomyelitis in the same lesion. Osteomyelitis in foals is

Figs. 15–20. (Continued). the growth cartilage (asterisks). **Fig. 20**. Tracing of arteries in Fig. 19 superimposed on Figs. 1 and 12. Dashed green line: Joint cartilage surface. Dashed blue line: Chondro-osseous junction. The location of portions of canals containing eosinophilic granular material (see Fig. 2) in the histological section (between black dashed lines, Fig. 1) corresponds to the distal termini of perfused canal arterioles in cleared cartilage (asterisks). The location of the osteomyelitis in Fig. 12 corresponds to where one perichondrial arteriole courses predominantly within subchondral bone (arrowheads).

usually found in the junction between bone and cartilage.[12] Various mechanisms for localization of bacteria in this anatomic site have been in different species: sluggish blood flow in sinusoidal loops in the bone marrow,[12] transphyseal vessels allowing migration of bacteria from the diaphysis to the epiphysis,[2,12,30] or endothelial gaps in growing vessels which bacteria may migrate through.[46] In a previous study, it was considered whether infection spread to the growth cartilage from subchondral bone, or the other way around.[36] Given the common observation of chondronecrosis and osteomyelitis in this material harvested from foals, especially in the very early epiphyseal and metaphyseal lesions in the young cases 1 and 2, it is likely that these processes can take place simultaneously. A significant portion of the blood supply to the epiphysis in foals originates from the perichondrium in the form of transverse vessels with perpendicular branches toward the articular surface and the physis.[13,38,39] The branches enter bone, cartilage or both, depending on location in the epiphysis (Fig. 19).[13,38,39] Whether bacterial destruction of these vessels results in chondronecrosis/osteochondrosis or osteomyelitis could be related to the location of the vessel affected at the time of bacteremia (Figs. 19, 20). The distribution of epiphyseal vessels originating in the perichondrium varies depending on bone and age,[13] which could explain the age-related variations of locations of septic arthritis/osteomyelitis in foals.[12] This potentially also explains why osteomyelitis and hematogenous septic arthritis are such rare events in adult horses, as they do not have perichondral or cartilage canal vessels.

The canals categorized as inflamed patent canals (Fig. 15) had morphologically functional vasculature and were surrounded by normal cartilage, and regressed by chondrification. This illustrates that lesser grades of inflammation can be resolved without lasting damage. The majority of inflamed regressing canals (Fig. 16) seemed to regress by fibrosis instead of chondrification. Whether bacteremia results in septic cartilage lesions likely depend on factors like immuno-competence of the foal[44] combined with infective dose[17,25] and pathogenicity[45] of the bacteria involved.

In this study, bacteria were present in cartilage canals in foals and were associated with ischemic chondronecrosis in both the AECC and the physis. Together with evidence from experimental studies of osteochondrosis and higher incidence of OCD in sepsis-survivors, this establishes sepsis as a plausible cause of some osteochondral lesions in horses. It is recommended that horses with sepsis-related osteochondral lesions may be used for breeding without increasing the prevalence of heritable OCD in the population.

Authors' Note

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Supplemental method 1. Protocol for Gram staining, Twort's method
Swedish University of Agricultural Sciences

Chemicals:

- Crystal violet:
  - 12,5 g crystal violet
  - 125 ml ethanol 95%
  - 500 ml ammonium oxalate 1%
- Gram's iodine
- Acetic acid 2 % in absolute alcohol
- Twort's stain:
  - 9 ml neutral red 0,2 %
  - 1 ml fast green 0,2 %
  - 30 ml distilled water

Protocol:

1. Soak sample in distilled water
2. Cover sections in crystal violet for 3 minutes
3. Lavage with water
4. Cover section in Gram's iodine for 3 minutes
5. Lavage with water
6. Decolorize in 2 % acetic acid until no more excess color
7. Lavage with distilled water
8. Counterstain in Twort's stain for 5 minutes
9. Differentiate in 2 % acetic acid until no more stain comes from the section, 2 seconds
10. Clear in xysol

Results:

- Gram positive bacteria: Blue/black
- Gram negative bacteria: Pink
- Nuclei: Red
- Background: Green
- Red blood cells: Green
- Elastic fibers: Black

Protocol for Gram staining, green background
National Veterinary Institute, Norway

- BD Difco™ BBL™ Stain Kit, Gram Stain Kit. Product Number: 212539

Fixation: Formalin

Chemicals:
- Crystal violet
- Iodine
- Decolorizer
- Safranin
- Neutral red 1 %
- Fast green 0,1 %

Procedure:
1. Soak sample in water
2. Cover sections in crystal violet for 1 minute
3. Lavage with water
4. Cover sections in iodine for 1 minute
5. Lavage with water
6. Decolorize with decolorizer for a few seconds (max 30 seconds) until excess fluid is no longer blue
7. Lavage in water
8. Cover sections with 1 % neutral red for 1 minute
9. Lavage with water
10. Cover sections with 0,1 % fast green
11. Lavage with water
12. Dehydrate sections with graded ethanol (15 seconds in each solution)

Results:
- Gram positive bacteria: Purple/blue
- Gram negative bacteria: pink/red
- Nuclei: Red
- Background: Green

Supplemental Table 1. Age, Sex, Breed, Clinical/Necropsy Diagnosis, Duration of Signs, Affected Locations, Histologic Material Available, Isolated Bacteria and Number of Blocks in Seven Foals with Septic Arthritis and/or Osteomyelitis.

Case number	Age (days)	Sex	Breed	Clinical/ necropsy diagnosis	Reported duration of clinical signs (days)	Location affected clinically or at necropsy	Isolated bacteria	Histologic material available	No of blocks
1	9	Male	Warmblood Riding Horse	Septic polyarthritis	4	B carpi B stifles B hocks R hind pastern joint	Joint sample from multiple joints: *Escherichia coli*	L DIRT	4
2	10	Male	Thoroughbred	Septic polyarthritis	4	R elbow L shoulder B carpi B stifles B hocks All fetlock joints	Both femoropatellar: *Eschericia coli*	R MTR L DMC R DMC L DMT R DMT	2 1 1 6 2
3	17	Female	Standardbred	Septic polyarthritis	Unknown	B stifles B hocks	Not available	L LTR L DIRT	2 3
4	30	Female	Standardbred	Septic polyarthritis	20	B stifles R hock	Carpal bursa: *Micrococcus* sp.	L MFC R MFC L MTR R DIRT R LTTa	3 1 2 2 1
5	37	Male	Standardbred	Osteomyelitis	3	L tuber coxa	Left tuber coxa: *Streptococcus dysgalactiae* ssp. Equisimilis, *Eschericia coli*	L TC	4
6	53	Female	Standardbred	Septic polyarthritis Osteomyelitis	53	L shoulder R stifle R hock	Right femoropatellar: *Staphylococcus* sp.	R LTR R MFC R DIRT R MTTa	2 2 4 4
7	117	Male	Standardbred	Septic arthritis	5	R hock	Left shoulder: Right tibiotarsal: *Rhodococcus*	R DT	3

B=both, R=right, L=left, DIRT=distal intermediate ridge of tibia, MTR=medial trochlear ridge of distal femur, DMC=distal metacarpus, DMT=distal metatarsus, LTR=lateral trochlear ridge of distal femur, MFC=medial femoral condyle, LTTa=lateral trochlea tali, TC=tuber coxa. MTTa=medial trochlea tali. DT=distal tibia

Supplemental Table 2. Histopathologic Findings in 16 Lesions in Joints from 7 Foals with Septic Arthritis/Osteomyelitis

Case	Bone, Region Sampled	AECC or Physis	Bacteria	Acute Septic Canals	Chronic Septic Canals	Ischemic Chondronecrosis (ICN)	Cartilage Secondary Responses	Perichondrium and Periosteum	Delayed Ossification; Cause	Changes in Adjacent Bone	Dissecting Lesion; Location	Inflamed Patent Canals	Inflamed Regressing Canals
1	L DIRT	AECC	Some gram-positive bacilli	Many[d]	One	Large intervascular ICN in 3/4 blocks	No	Inflammation	Large; ICN	Epiphyseal OM in 3/4 blocks	No	Single canal	No
1	L DIRT	Physis	Some gram-positive bacilli	Two	Two	Small perivascular and intervascular ICN in 3/4 blocks	No	Normal	No	Diaphyseal OM in 2/4 blocks	No	Single canal	No
2	L DMC[a]	Physis	No	Many	Two	Occasional perivascular chondrocytes	No	Normal	No	No	No	No	No
2	R DMC[b]	Physis	Many gram-positive bacilli	Some[e]	No	No	No	Normal	No	Epiphyseal OM in 1/1 block	No	No	No
2	L DMT[a]	Physis	Suspected; gram not done	Many	No	Perivascular ICN in 1 block, most of resting zone 1 block	No	Normal	No	Diaphyseal OM in 3/4 blocks; epiphyseal OM in 1/4 blocks	No	No	No
2	R DMT[b]	Physis	No	Many	No	Small perivascular ICN	No	Normal	No	OM both sides in 1/1 block	No	No	No
2	R MTR[b]	Physis	No	Some	One	Small perivascular ICN and intervascular ICN 1 block	No	Normal	No	OM diaphysis > epiphysis in 1/2 blocks, epiphyseal OM in 1/2 blocks	No	Some canals	Two canals
3	L LTR[c]	AECC	No	Some	Many	Small perivascular ICN and intervascular ICN 1 block	Proliferating canal	Not included	Medium; vertical line of septic FVGT	Epiphyseal OM with Brodie's abscess in 4/4 blocks	Yes; through septic FVGT in 2/4 blocks	Some	Many
4	R DIRT	AECC	No	Some	Many	Small perivascular ICN and large intervascular ICN 1 block	Chondrocyte proliferation	Normal	Medium; aseptic FVGT	Mix of reparative granulation tissue and epiphyseal OM, 1/2 blocks	No	Some	Some
4	R DIRT	Physis	No	No	No	No	No	Normal	No	No	No	Some	Single canal
4	R LTT	AECC	No	Some	Some	Perivascular and large intervascular ICN	Chondrocyte proliferation	Normal	Large; septic FVGT	Mix of reparative granulation tissue and OM. 1/1 block	No	No	Some
4	R MFC[c]	AECC	No	Many	Many	Perivascular ICN superficially; intervascular ICN deep	Chondrocyte proliferation	Normal	Large; septic FVGT	Mix of reparative granulation tissue and epiphyseal OM in 3/3 blocks	No	No	Some
5	L TC	Tuberosity	Some gram-positive bacilli	Some	Some	Mixed areas, perivascular ICN and large intervascular ICN 1 block	Chondrocyte proliferation, proliferating canal	Inflammation	Medium; ICN and septic FVGT	Diaphyseal OM in 1/2 blocks	No	No	Some
6	R DIRT	AECC	No	Many	Some	Mixed areas, large intervascular ICN and some perivascular ICN, also in flap	Chondrocyte proliferation; proliferating canal	Normal	Large; vertical line of FVGT with pieces of necrotic cartilage	Mix of reparative granulation tissue and epiphyseal OM in 4/4 blocks	Hinged flap; through FVGT in 4/4 blocks	Many	Some
6	R DIRT	Physis	No	No	No	No	No	Normal	No	No	No	Some	No
6	R MTTa	AECC	No	Many	Some	Large intervascular ICN	Chondrocyte proliferation	Normal	Large; septic FVGT with pieces of necrotic cartilage	OM with some hemorrhage at cartilage-bone junction in 4/4 blocks	No	Some	Some
6	R MFC[c]	AECC	No	Many	Some	Large intervascular ICN	Chondrocyte proliferation	Normal	Large; ICN	ICN pseudocyst in 2/2 blocks	No	Some	No
6	R LTR[c]	AECC	No	Some	Some	Large intervascular ICN	Proliferating canals	Normal	Large; septic FVGT with pieces of necrotic cartilage	Mix of reparative granulation tissue and epiphyseal OM in 2/2 blocks	Avulsion through ICN	Many	Many
7	R DIRT	AECC	Many gram-positive cocco-bacilli	No	No	No	No	Normal	No	No	No	Some	Some
7	R DIRT	Physis	No	No	Some	No	No	Normal	Recorded, but not present in sections	OM diaphysis >> epiphysis in 2/3 blocks	No	No	No

R=right, L=left, DIRT=distal intermediate ridge of tibia, MTR=medial trochlear ridge of distal femur, DMC=distal metacarpus, DMT=distal metatarsus, LTR=lateral trochlear ridge of distal femur, MFC=medial femoral condyle, TC=tuber coxa, MTTa=medial trochlea tali, AECC=articular epiphyseal cartilage complex, OM=osteomyelitis, FVGT=fibro vascular granulation tissue

[a]AECC not available
[b]AECC normal
[c]physis not available
[d]Many: >75%
[e]Some: <75%

II

Original Article

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Osteochondrosis in the Distal Femoral Physis of Pigs Starts With Vascular Failure

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Abstract

Articular osteochondrosis (OC) arises due to vascular failure and ischemic chondronecrosis. The aim of the study was to describe the histological and computed tomographic (CT) characteristics of changes in the distal femoral physis of pigs, to determine if they represented OC lesions and if the pathogenesis was the same as for articular OC. The material included 19 male Landrace pigs bred for predisposition to OC. One or 2 pigs were euthanized and CT-scanned at 2-week intervals from 82 to 180 days of age. Material from 10 pigs was available for histological validation. The CT scans revealed 31 lesions confirmed in 3 planes and 1 additional macroscopically visible lesion confirmed in 2 CT planes. Twelve of the lesions were histologically validated. All lesions were compatible with OC. Cartilage canal and eosinophilic streak morphological changes corresponded to failure of end arteries coursing from the epiphysis, toward the metaphysis. The location of lesions was compatible with failure at the point of vessel incorporation into bone. Vascular failure was associated with retention of viable hypertrophic chondrocytes and delayed ossification but not cartilage necrosis. Lesion width ranged from 1.1% to 45.6% of the physis. Several lesions were expected to resolve due to small size and evidence of CT-identifiable, reparative ossification. Angular limb deformity was not detected in any pig. The pathogenesis of physeal OC started with vascular failure that was morphologically identical to articular OC. The heritable predisposition may therefore be the same. The association between lesions and limb deformity should be studied further in older pigs in future.

Keywords

angular limb deformity, bone, growth plate, helical computed tomography, histology, osteochondrosis, swine, vascular failure

Computed tomography (CT) is used in the selection of boars for breeding[2,22] and is an ideal modality for evaluation of skeletal health. Scoring of articular osteochondrosis (OC) lesions in CT scans has previously been histologically validated.[27] Osteochondrosis in the metaphyseal growth plate, or physis, is as important to pig health and welfare as articular OC because it has been associated with angular limb deformity.[3,19,32] During the validation of articular lesions,[27] focal changes were detectable in the physes of pigs, but it is currently not known if the changes represented OC. It is important to determine if this is the case, because it determines whether physeal OC lesions can be scored in the same way as articular OC in CT scans.[27] It is also not known whether physeal OC lesions have the same pathogenesis as articular OC, something that is important because if the pathogenesis is the same, the heritable predisposition[2,33] may also be the same.

Articular OC arises in the epiphyseal growth cartilage that is located between the articular cartilage and the secondary center of ossification.[24,48] Epiphyseal growth cartilage has a temporary blood supply that enters the cartilage from the perichondrium and courses within cartilage canals.[5,49] The circulation is organized as anatomical end arteries,[13] and canals can therefore be said to have a proximal, middle, and distal end. The blood supply becomes incorporated into epiphyseal bone during growth, and this happens earlier at the midportion than at the proximal and distal ends of the canal.[50] In both pigs and foals, early lesions were consistently located axial to the point of incorporation into bone, around the distal portion of the canal.[30,50] The lesions consisted of areas of necrotic cartilage centered on necrotic cartilage canals (ie, ischemic chondronecrosis) that were located at intermediate depth of the growth cartilage outside diffusion distance from alternative sources, termed *OC latens*.[5,30,50] Together, these observations prompted

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the interpretation that lesions were due to failure of vessels during the process of incorporation into bone.[30,50] Endochondral ossification proceeded as normal in the cartilage adjacent to but not within lesions, which therefore caused a focal delay in endochondral ossification, or OC manifesta.[31,50] Articular lesions can resolve[26] or progress to pathologic fracture and loose fragments in joints known as OC dissecans.[25,31]

The hypothesis that articular OC occurred due to failure of vessels during incorporation into bone has since been supported by further evidence. In histological sections, OC manifesta lesions often consisted of multiple areas of ischemic chondronecrosis close together at slightly different depth levels in the ossification front, known as stair-step lesions.[5,27,28] The only anatomical component that matched the geometry of the stair-step lesions was the cartilage canal blood supply.[27] In longitudinal CT scans, multiple lobes appeared at the same time, and the most feasible explanation was that failure of a single vascular trunk during incorporation led to lesions around multiple smaller vessel branches simultaneously.[24,26] The pathogenesis of articular OC has also been experimentally reproduced by transecting the blood supply in pigs,[5,47] foals,[25] and goats.[38] It is known that whereas articular OC lesions consist of areas of ischemic chondronecrosis, physeal OC lesions consist of large numbers of hypertrophic chondrocytes and streaks of intensely eosinophilic-staining material.[7,17,18,31,44] The eosinophilic streaks most likely represent remnants of the cartilage canal blood supply,[16,17,21] and it has been suggested that physeal OC occurs due to vascular failure, but in most cases, this refers to failure of vessels to penetrate growth cartilage from bone rather than failure of cartilage canal vessels.[7,17,18,21] The literature contains some information about the configuration of the blood supply to physeal growth cartilage.[16,18,40] Importantly, the midportion of physeal end arteries is incorporated into bone on the deep side of the secondary center of ossification during growth (Fig. 1).[16] Failure of a vascular trunk during incorporation into bone can therefore potentially lead to lesions around multiple vessel branches simultaneously in exactly the same way in the physis as in articular OC.[30,50]

The aim of the current study was to describe the histological and CT characteristics of changes in the distal femoral physis of pigs, to determine if they represented OC lesions and if the lesions had the same pathogenesis as articular OC.

Materials and Methods

The study was approved by the National Animal Research Authority (FOTS ID: 2010/2630). All pigs were kept according to national legislation (Animal Welfare Act 2009-06-19-97, Regulation for the Keeping of Pigs in Norway 2003-02-18-175).

The material consisted of 19 male Landrace pigs used in previous studies.[26,27] The pigs were purchased with informed consent for research from members of the Norwegian pig breeders' association, Norsvin (www.norsvin.no). The pigs were purpose-bred from a boar with high breeding value for articular OC and were the offspring of 8 different sows whose farrowing dates fitted with the study start date. All pigs were examined at 2-week intervals from 82 to 180 days of age (longitudinal data reported elsewhere). The pigs were visually inspected for angular limb deformity by a veterinary surgeon at each examination interval. One or 2 pigs were selected for euthanasia, CT scanning, and histological validation at each interval. At the eighth and final interval, all remaining pigs were euthanized and examined. Pigs were assigned letters from A to S by ascending age on the study end date and by ascending body weight if they were of the same age (range, 33-128 kg; Suppl. Table S1).

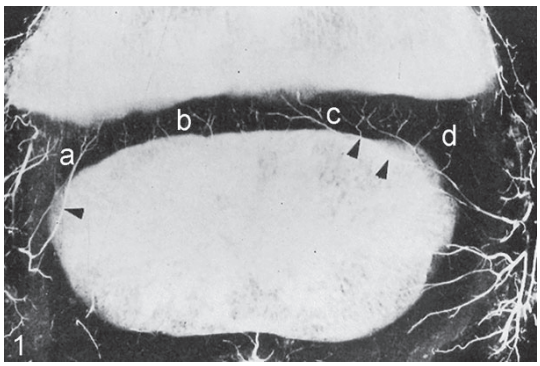
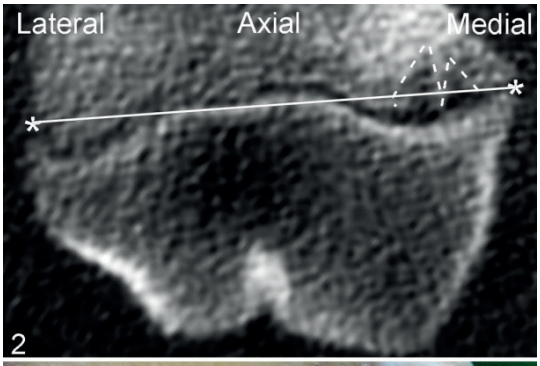


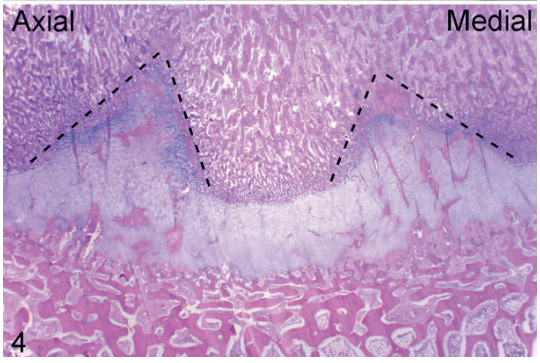
Figure 1. Arterial perfusion, distal femoral physis, 15-day-old pig. Perfused vessels enter the growth cartilage from the perichondrium. The midportion of the end arteries supplying the physis is incorporated into the ossification front on the deep side of the secondary center of ossification (arrowheads). Four main vessel configurations are visible: vessels that enter the physis peripherally and course roughly perpendicular to the epiphyseal-side ossification front, referred to as peripheral-perpendicular vessels (a); vessels that enter the physis centrally and course perpendicular to the epiphyseal-side ossification front, referred to as central-perpendicular vessels (b); vessels that enter the physis centrally and continue in an axially oblique direction, referred to as central-axial vessels (c); and vessels that enter the physis peripherally and continue in an abaxially oblique direction, referred to as peripheral-abaxial vessels (d). Reprinted with permission from Hill et al.[16]

Computed Tomography

Each pig was euthanized by captive bolt stunning and exsanguination and positioned in sternal recumbency with the hind limbs extended on the patient table of a 32-slice helical CT scanner (GE Light Speed Pro 32; GE Healthcare, Munich, Germany). A hind-limb scan comprising an average of 960 transverse slices from the tuber coxae to the claws was acquired using a fixed kV of 120, a dynamic mA of up to 650, and a slice thickness of 0.625 mm.

The right distal femoral physis was selected for evaluation. The maximum mediolateral width of the physis was measured by scrolling to the frontal slice where this dimension subjectively appeared widest (Fig. 2). The software calipers were





Figures 2–4. Methods for evaluating physeal osteochondrosis by computed tomography and histopathology. Physeal osteochondrosis, distal femur, pig N, lesion 23, caudal third, medial half of physis. **Figure 2.** The maximum mediolateral width of the physis (solid line) was measured with software calipers placed midway between the medial extreme of the secondary center of ossification and the metaphysis (asterisk) and midway between the lateral extreme of the secondary center of ossification and the metaphysis (asterisk). Osteochondrosis lesions were defined as focal, sharply demarcated areas of soft tissue hypodensity in or near the epiphyseal-side and metaphyseal-side ossification fronts of the physis. Lesion N23 consists of 2 triangular defects (dashed lines) in the metaphyseal-side ossification front in the medial half of the physis. **Figure 3.** The distal third of each available femur was sawed into approximately 3-mm-thick slabs placed midway between the medial extreme of the secondary center of ossification and the metaphysis and midway between the lateral extreme of the secondary center of ossification and the metaphysis, and the straight line between the 2 points was measured (Fig. 2). The maximum craniocaudal length of the physis was measured by scrolling to the sagittal slice where this dimension subjectively appeared longest. The software calipers were placed midway between the cranial extreme of the secondary center of ossification and the metaphysis and midway between the caudal extreme of the secondary center of ossification and the metaphysis, and the straight line between the 2 points was measured. Proximodistal thickness was not measured because although the physis was open in all pigs, thickness was below the CT slice thickness of 0.625 mm at several locations in the physes of multiple pigs.

Each femur was assessed in the frontal, sagittal, and transverse plane. Lesions of OC were defined as focal, sharply demarcated areas of soft tissue hypodensity in or near the epiphyseal-side and metaphyseal-side ossification fronts of the physis (Fig. 2).[27] Lesions consisted of a single area, referred to as a lobe, or multiple areas close together in the frontal or sagittal plane, referred to as multilobulated lesions. Changes had to be present in all 3 planes and in 2 or more consecutive CT slices to be counted as lesions. Changes that were separated by more than 4 normal CT slices were counted as separate lesions. Recording of the location of lesions was divided into 6 regions: cranial third, central third, or caudal third of the femur and medial or lateral half of the femur. Each lesion was identified by the letter of the pig from A to S and an Arabic numeral that ascended in the same order as the lettering of the pigs (Suppl. Table S1). In pigs with multiple lesions, lesion number ascended in order of decreasing region prevalence (see Results). Secondary responses were recorded, consisting mainly of different patterns of mineral hyperdensity interpreted as ossification.[25,27]

The maximum mediolateral width of lesions was measured by scrolling to the frontal slice where this dimension subjectively appeared widest. The software calipers were placed at the most medially and laterally extreme points of the lesion, and the straight line between the 2 points was measured. The maximum craniocaudal length of lesions was measured by scrolling to the sagittal slice where this dimension subjectively appeared longest. The software calipers were placed at the most cranially and caudally extreme points of the lesion, and the straight line between the 2 points was measured. If the widest or longest part of a lesion was located in a frontal or sagittal slice where the lesion was multilobulated, the normal tissue

Figures 2–4. (Continued). in the frontal plane. All regions of the physis with macroscopically visible changes (for example, focally increased thickness [dashed lines]) were trimmed to fit into cassettes for paraffin embedding. **Figure 4.** A minimum of two 4-μm-thick sections were prepared from each block and stained with hematoxylin and eosin for histological examination. When metaphyseal-side defects were present (dashed lines), the entire thickness of the physis superficial to lesions contained abnormal eosinophilic streaks.

between adjacent lobes was included in the measurement. Measurements therefore represented the maximal span of the lesion, rather than the sum of the width or length of individual lesion lobes. The maximum proximodistal depth of lesions was measured by scrolling to the frontal slice where this dimension subjectively appeared deepest. A line was drawn between the medial and lateral extreme points of the lesion at the level of the ossification front, and the right-angle distance between this line and the deepest point of the lesion was measured.

Histology

Nine femurs were unavailable for histological validation because they had been used in previous studies.[8–10] The 10 available femurs (Suppl. Table S1) were fixed in 4% phosphate-buffered formaldehyde for 48 hours. The distal third of each femur was sawed into approximately 3-mm-thick slabs in the frontal plane (Fig. 3), and the slabs were decalcified in 10% ethylenediaminetetraacetic acid. All regions of the physis with macroscopically visible changes (eg, focally increased thickness) (Fig. 3) were trimmed to fit into cassettes measuring 32 × 25 × 5 mm. After paraffin embedding, a minimum of two 4-µm-thick sections were prepared from each block and stained with hematoxylin and eosin for histological examination (Fig. 4). The blood supply was evaluated according to previously published criteria for identification of patent, chondrifying, and necrotic cartilage canals,[5] whereas growth cartilage and bone were evaluated for primary lesions and secondary responses.[25]

Results

Number, Location, and Definition of Lesions

The 19 examined femurs contained 31 CT lesions confirmed in 3 planes, and all lesions matched the definition of OC given in the Materials and Methods section. In the sawed slabs, an additional lesion (M22) was visible on the cut surface of the slab that was only confirmed in the frontal and sagittal plane in CT scans, and thus the total number of CT and macroscopic lesions was 32 (Suppl. Table S1). The lesions were distributed as follows: 12 caudomedial lesions, 6 combined centro- and caudomedial lesions, 6 centrolateral lesions, 5 centromedial lesions, 2 caudolateral lesions, and 1 lesion that was located caudally in the midline (L20). The 10 available femurs had 15 CT and macroscopic lesions, and 12 of the lesions were visible on the surface of sawed slabs and could be processed for histological validation (Suppl. Table S1).

Lesions were initially defined as defects in the physeal ossification fronts (Figs. 2–4). During histological examination, it was discovered that when metaphyseal-side defects were present, the entire thickness of the physis superficial to lesions contained only abnormal cartilage canals and eosinophilic streaks (Fig. 4; described further below; superficial: toward articular surface). When epiphyseal-side defects were present, the physis deep to lesions contained either normal or abnormal canals and streaks (deep: away from articular surface). The histological definition of lesions was therefore revised to include focal defects in the physeal ossification fronts and all adjacent cartilage containing abnormal canals and streaks.

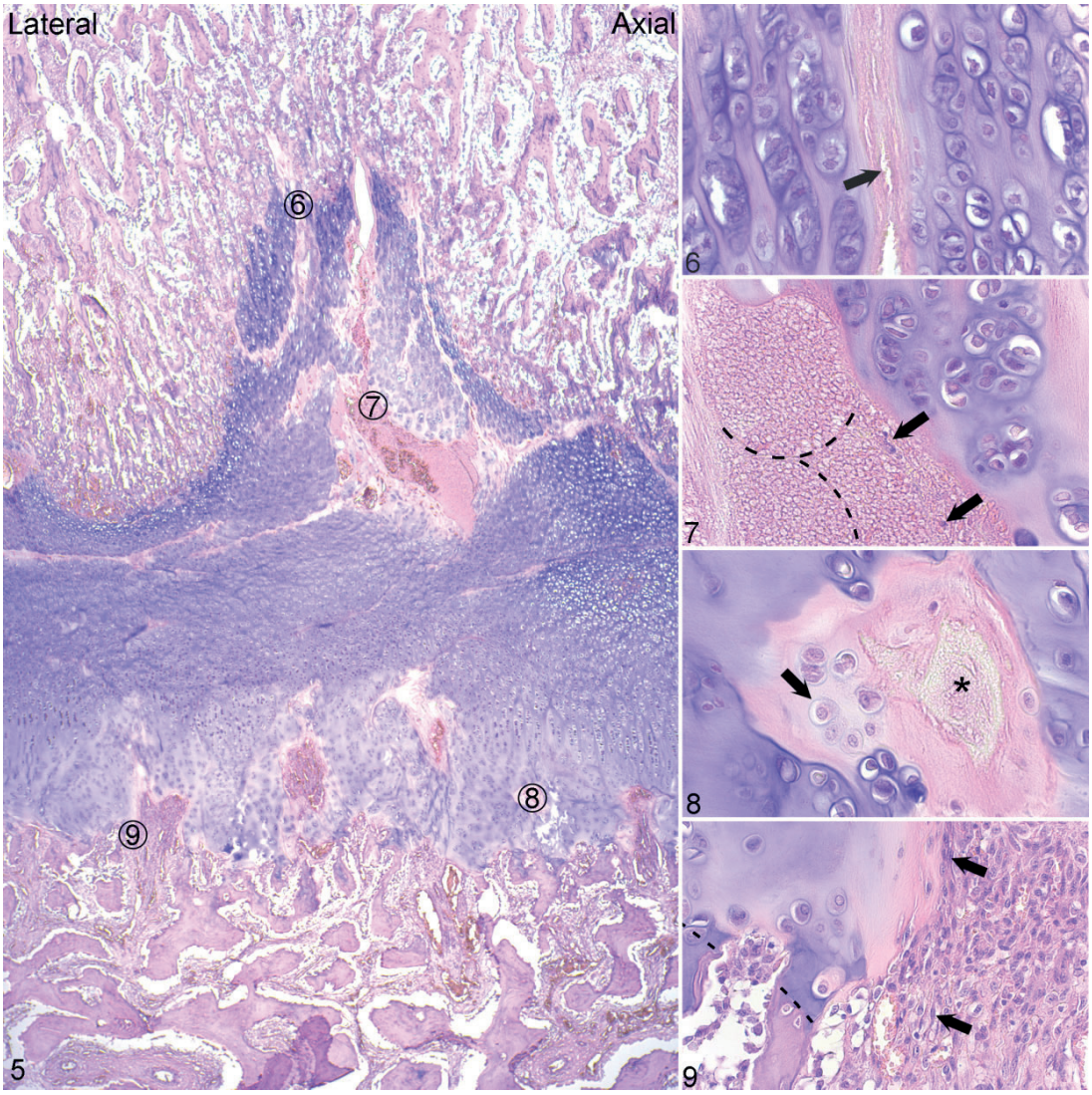
Categories of Cartilage Canal and Eosinophilic Streak Morphological Changes

Categories of cartilage canal and eosinophilic streak morphological changes followed a consistent pattern of distribution from the metaphyseal to the epiphyseal side of the physis in all lesions (Figs. 5–9, Suppl. Table S2). Nearest the metaphysis, there were thin, eosinophilic streaks that were acellular or contained erythrocytes, compatible with necrotic vessel branches (Fig. 6). Superficial to this, there were wider eosinophilic streaks with necrotic or lysed/absent endothelium, ghost remnant outlines of normal or dilated vessels, and necrotic perivascular cells, compatible with necrotic vessel trunks (Fig. 7). Toward the epiphysis, there were chondrifying canals (Fig. 8). Nearest the epiphysis, there were canals with increased perivascular mesenchymal cells that were continuous with cells in bone marrow, interpreted as repopulation of necrotic canals (Fig. 9). The pattern of distribution was therefore compatible with failure of end arteries coursing from the epiphysis toward the metaphysis in all lesions.

Photomicrographs were printed and lines were drawn on the printouts joining adjacent, aligned canal/streak remnants to compare these to the vessel configurations in Fig. 1. The results indicated that the 28 lesion blocks contained remnants of 59 failed vessels (Suppl. Table S3). Two of the remnants corresponded to vessels that entered the physis peripherally and coursed roughly perpendicular to the epiphyseal-side ossification front, referred to as peripheral-perpendicular vessels (Fig. 1). A further 16 remnants corresponded to vessels that entered the physis centrally and coursed perpendicular to the ossification front, referred to as central-perpendicular vessels (Fig. 1). Five remnants corresponded to vessels that entered the physis centrally and continued in an axially oblique direction, referred to as central-axial vessels (Fig. 1), and 36 remnants corresponded to vessels that entered the physis peripherally and continued in an abaxially oblique direction, referred to as peripheral-abaxial vessels (Fig. 1, Suppl. Table S3). The majority of canal/streak remnants were therefore compatible with failure of peripheral-abaxial vessels.

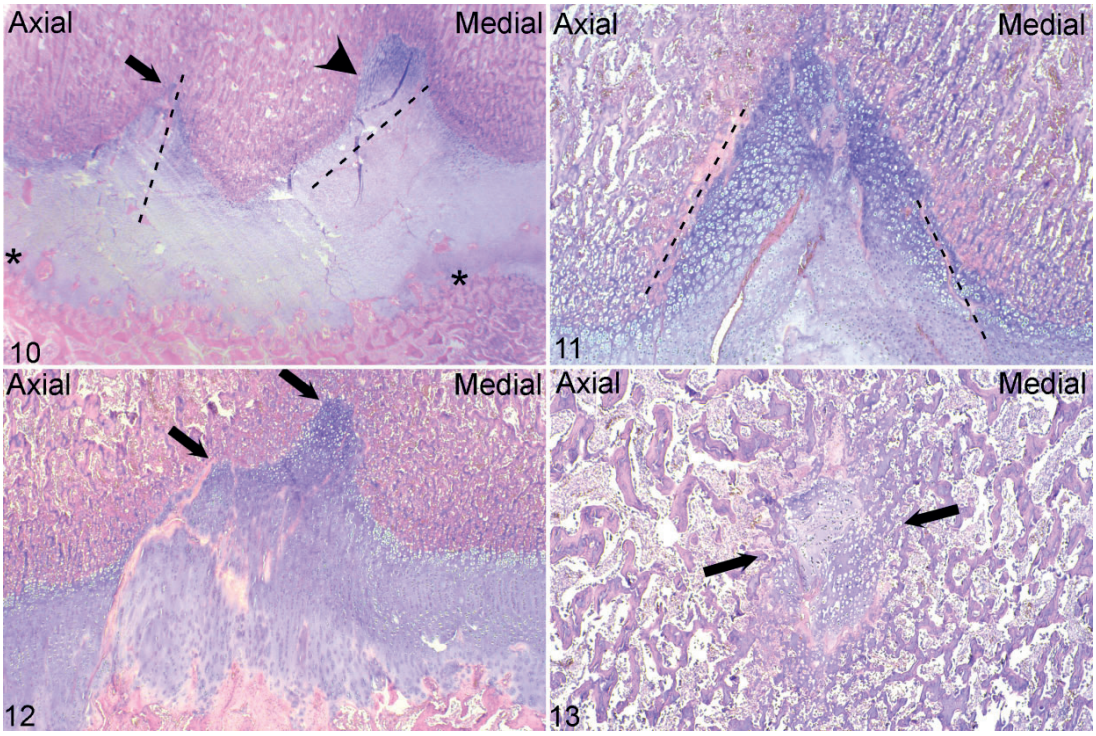
Vascular Failure Was Associated With Delayed Ossification

Nineteen of 28 blocks with lesions contained metaphyseal-side defects, 2 of 28 blocks contained epiphyseal-side defects, and 7 of 28 blocks contained both, and thus there were 26 metaphyseal-side and 9 epiphyseal-side defects (Suppl. Table S3). The 9 epiphyseal-side defects included 4 peripheral defects and 5 central defects. The 4 peripheral epiphyseal-side defects were considered to represent early changes near the point of vessel incorporation into bone and are described further under Causes of Vascular Failure. The 26 metaphyseal-



Figures 5–9. Physeal osteochondrosis, distal femur, pig M, lesion 22, central third, lateral half of the physis. Hematoxylin and eosin. **Figure 5.** At low-power magnification, the distribution of cartilage canal and eosinophilic streak morphological changes within lesions is evident. The locations of Figs. 6 to 9 are indicated. **Figure 6.** Nearest the metaphysis, there are thin, eosinophilic streaks that are either acellular or contain erythrocytes (arrow), compatible with necrotic vessel branches. **Figure 7.** Superficial to this, there are wider eosinophilic streaks with remnant outlines of vessels (dashed lines) and necrotic perivascular cells (arrows), compatible with necrotic vessel trunks. **Figure 8.** Toward the epiphysis, there are canals with remnant vascular luminae (asterisk) and chondrocyte-like perivascular cells (arrow) (ie, chondrifying canals). **Figure 9.** Nearest the epiphysis, there are canals with increased perivascular mesenchymal cells that are continuous with cells in bone marrow, interpreted as repopulated canals. Some of the cells are fibroblast-like (arrows). The dashed lines represent the transition between growth cartilage and subchondral bone.

side defects contained 51 separate lobes of delayed ossification (Suppl. Table S3). Twenty-two defect lobes were central and their proximodistal axes were perpendicular to the metaphyseal-side ossification front, whereas 29 lobes were peripheral and their proximodistal axes were obliquely angled from superficial-axial toward deep-abaxial, mirroring the

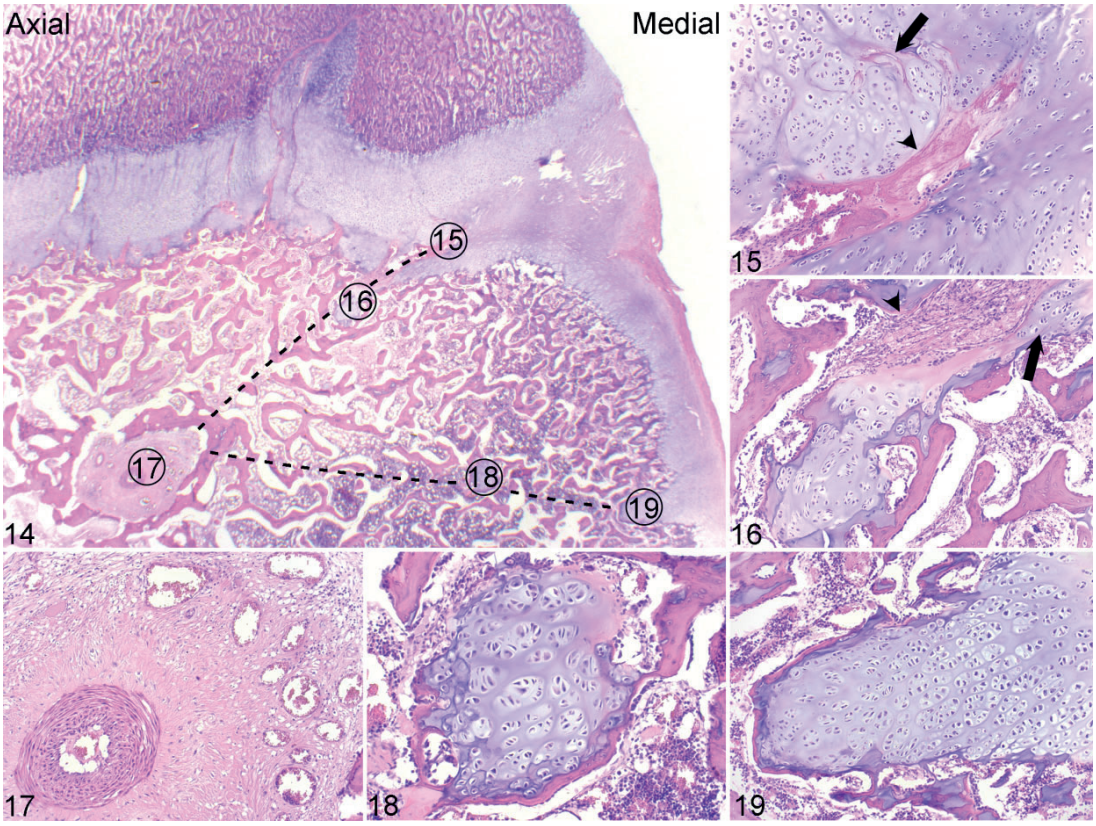


Figures 10–13. Physeal osteochondrosis. Distal femur. Hematoxylin and eosin. **Figure 10.** Pig E, lesion 7, central and caudal thirds, medial half of physis. There is a triangular lobe (arrow) that is centered on a perpendicular, failed vessel (dashed line) and a rectangular lobe (arrowhead) where a failed vessel runs obliquely across the defect (dashed line) in the metaphyseal-side ossification front. There is also a semicircular, wide, shallow, and dish-like defect in the epiphyseal-side ossification front (between asterisks). **Figure 11.** Pig Q, lesion 29, slab 8, central third, medial half of physis. There is a triangular defect in the metaphyseal-side ossification front that is framed by adjacent oblique and converging vessels (dashed lines). **Figure 12.** Pig Q, lesion 29, slab 6, central third, medial half of physis. There is an irregularly rectangular defect in the metaphyseal-side ossification front that appears to represent 2 adjacent, confluent defects (arrows), resulting in a bilobed appearance. **Figure 13.** Pig J, lesion 15, central and caudal thirds, medial half of physis. There is a circular defect (between arrows) that is surrounded by relatively more metaphyseal bone than other defects, which can therefore be described as a pseudocyst-like defect (between arrows).

configuration of peripheral-abaxial vessels. The main reason for the discrepancy between 59 failed vessels and 51 defect lobes was that multiple vessels contributed to single lobes. Both central and peripheral defect lobes had different shapes (Suppl. Table S3). Twenty-five of the 51 defect lobes were triangular (Figs. 10, 11). Triangular lobes were centered on a perpendicular, failed vessel (Fig. 10) or framed by adjacent oblique and converging vessels (Fig. 11). Twenty-four of the 51 defect lobes were rectangular (Fig. 10). Rectangular lobes were centered on a perpendicular vessel that branched toward the deep corners of the rectangle, or failed vessels ran obliquely across the rectangle (Fig. 10). Six of the 24 rectangles were irregular and appeared to represent 2 adjacent, confluent defects, resulting in a bilobed appearance (Fig. 12). Two of the 51 defect lobes were circular and surrounded by relatively more metaphyseal bone than the other defects and could therefore be described as pseudocyst-like defects (Fig. 13). The above observations suggested that there was a close relationship between the configuration of failed vessels and the shape of resultant areas of delayed ossification.

Metaphyseal-side defects contained chondrocytes with the same morphology as the adjacent, normal metaphyseal-side hypertrophic and proliferative growth cartilage zones, except that the number of cells was typically doubled and that columns were sometimes oblique, rather than perpendicular to the ossification front. Chondrocytes tended to be nucleated and morphologically viable.

The 5 central epiphyseal-side defects tended to be semicircular and wider, shallower, and more dish-like than metaphyseal lobes (Fig. 10). In addition to viable hypertrophic and proliferative zone chondrocytes, central epiphyseal defects contained repopulated canals engaged in repair as described below. Metaphyseal-side defects were observed in



Figures 14–19. Physeal osteochondrosis, distal femur, pig G, lesion 10, caudal third, medial half of physis. Hematoxylin and eosin. **Figure 14.** The path of peripheral-abaxial end arteries (dashed line) was followed retrograde from early physeal lesions (Figs. 15, 16) via epiphyseal bone (Figs. 17, 18) toward the site of original perichondrial entry (Fig. 19) to look for evidence of causes of vascular failure. The locations of Figs. 15 to 19 are indicated. **Figure 15.** An early peripheral epiphyseal-side defect contains failed canals/streaks with the same morphology and distribution as metaphyseal-side lesions of necrotic branches (arrow) and necrotic vessel trunks (arrowhead). **Figure 16.** Superficial to the necrotic vessel branches and trunks, the peripheral epiphyseal-side defect contains a chondrifying canal (arrow) and a repopulated canal (arrowhead). **Figure 17.** Within epiphyseal bone, there is a conspicuously large vessel surrounded by fibrous tissue. **Figure 18.** There is also a small island of normal growth cartilage within epiphyseal bone. **Figure 19.** There is a peninsula of normal growth cartilage in the side of the epiphysis facing the perichondrium. Changes along the path of peripheral-abaxial vessels seen in Figs. 15 to 19 were therefore nonspecific for causes of vascular failure.

the absence of central epiphyseal-side defects, whereas the reverse did not occur. Lesions with both central epiphyseal-side and metaphyseal-side defects were therefore interpreted as more chronic than lesions with metaphyseal-side defects only.

It was possible to identify frontal CT slices with defects that corresponded perfectly to the number, shape, and angulation of defects in histological sections (Figs. 2, 4). Lesion F9 contained a single lobe but was located in the same femur as lesion F8, and thus all lesions were either multilobulated or multiple. Detailed descriptions of the CT lesions are available in Supplemental Table S4.

Causes of Vascular Failure

The path of peripheral-abaxial end arteries was followed retrograde from early physeal lesions via epiphyseal bone toward the site of original perichondrial entry to look for evidence of causes of vascular failure (Fig. 14). The aforementioned 4 peripheral epiphyseal-side defects were roughly triangular and sharply angled in the same direction as peripheral-abaxial vessels, and the opposing metaphyseal-side ossification front was normal. Peripheral epiphyseal-side defects contained failed canals/streaks with the same distribution of morphological changes as described for metaphyseal lesions, above, of necrotic branches, necrotic trunks, and chondrifying and repopulated

canals (Figs. 15, 16). The defects contained normal chondrocytes, and lesion N22 contained a small amount of fibrous tissue.

Within epiphyseal bone, there were conspicuously large vessels surrounded by fibrous tissue in 24 of 28 blocks (Fig. 17) and small islands of normal growth cartilage in 7 of 28 blocks (Fig. 18, Suppl. Table S3). The islands sometimes lined up with peninsulas of normal growth cartilage (Fig. 19), present at 1 to 3 depth levels in the side of the epiphysis facing the perichondrium in 8 of 28 blocks. Canal/streak remnants in lesions F8 and F9 contained perivascular neutrophils, compatible with septic vascular failure. All other peninsulas and adjacent perichondrium contained only remnants of chondrifying, fibrosing, necrotic, or repopulated canals (Suppl. Table S3). Changes along the path of peripheral-abaxial vessels were therefore nonspecific for causes of vascular failure. Hematomas, lipid emboli, microfractures, abscesses, or osteomyelitis were not detected.

Secondary Reparative Responses and Computed Tomographic Visibility

The main secondary response within cartilage was repopulation of failed canals with cells from epiphyseal bone marrow. The cells inside repopulated canals were most often fibroblast-like (Fig. 9) but could be undifferentiated, osteoblast-like, or chondrocyte-like (Suppl. Table S2). In the CT scans of lesions E7, J15, M21 (Suppl. Figs. S1, S2), N23, O24, and P27, there were hyperdense lines that matched the location, size, shape, and number of repopulated canals (Suppl. Table S5). This was surprising because although lesions J15, M21, N23, and P27 possibly contained some osteoblast-like cells, lesions E7 and O25 contained only fibroblast-like cells that were expected to have similar attenuation to cartilage. In several lesions, there were larger areas of overt ossification (ie, osteoblasts, osteoid, and trabeculae) in the location of repopulated canals, and such areas corresponded to mineral hyperdense foci in the CT scans of lesions C5 (Suppl. Figs. S3, S4) and J15 (Suppl. Table S5).

The bone adjacent to lesions sometimes contained increased numbers of multinucleated giant cells interpreted as chondroclasts, but the main secondary response within bone was different patterns of ossification. In lesions G10, M21, and O25, there was sclerosis on the margins of lesions in CT scans that corresponded to increased trabecular density in histological sections (Suppl. Table S5). In the metaphyseal defect lobes of lesions E7, J15, M22, P27, Q28, and Q29, there were linear, mineral hyperdensities that advanced horizontally into the lesion at the level of the metaphyseal-side ossification front in CT scans. The lines impinged on rectangular defects, causing them to take on a reverse-C shape and was confirmed to correspond to ossification in histological sections from lesions M22 (Suppl. Figs. S5, S6) and P27. The density of lesions D6, Q28, R30, and S31 was similar to surrounding bone (Suppl. Table S4), and the lesions therefore appeared to be undergoing spontaneous resolution by reparative ossification.

Lesion Size and Outcome

The relative width and length of lesions ranged from 1.1% to 45.6% (average: 12.3%) of physeal width and 1.4% to 38.9% (average: 12.5%) of physeal length (Suppl. Table S6). The absolute smallest lesion was L19 at 166 days, the relative smallest lesion was N23 at 171 days, and the absolute and relative largest lesion was B4 at 96 days, and thus there was no apparent simple, linear relationship between lesion size and age (Suppl. Table S6). Angular limb deformity was not detected in any pig.

Discussion

Physeal Osteochondrosis Starts With Vascular Failure

Cross-sectional studies can only generate limited information about which changes occurred first and which came second. Three current observations nevertheless support the same interpretation. All lesions were focal. Growth cartilage consists of chondrocytes, extracellular matrix, and cartilage canal blood supply, and the only anatomical component that is focally distributed is the cartilage canals.[5,27,49] It is therefore difficult to explain focal growth cartilage lesions in any other way than that they represent primary involvement of the blood supply.[27] All ossification defects were centered on or framed by abnormal vessels/eosinophilic streaks. The vascular hypothesis for the pathogenesis of articular OC originates from cross-sectional studies, where the observation that lesions were centered on necrotic cartilage canals led to the interpretation that they were caused by vascular failure.[4,20,43] This was later confirmed by experimental studies where transection of vessels produced identical lesions.[5,47] With the exception of lesion F9, all lesions were multilobulated. For articular OC, it has been documented that multiple lobes appear at the same time,[26] and the longitudinal data from the current lesions (published elsewhere) indicate that the same is true for physeal OC. The only plausible way to generate multilobulated lesions from a single insult is through failure of 1 vascular trunk, leading to lesions around multiple smaller branches simultaneously.[24,26] Together, these 3 observations support the interpretation that physeal OC starts with vascular failure.

As for articular OC, the vascular hypothesis for the pathogenesis of physeal OC could be tested by experimental transection of vessels. The blood supply to the physis has already been blocked or transected in several studies.[12,39] The problem with these studies is that the blood supply was interrupted to a wider area than 1 vessel trunk and that the studies focus on late effects on ossification, rather than on early changes in growth cartilage. If the vascular pathogenesis of physeal OC was to be tested in future, one should probably make use of recently developed advanced imaging techniques that enable visualization of individual vessel trunks prior to transection, as well as monitoring of resultant lesions.[37]

For articular OC, the hypothesis that vascular failure was associated with incorporation of vessels into bone was based on studies combining arterial contrast perfusion and histology.[30,50] The perfusion showed that the midportion of end arteries was

incorporated into bone at the periphery of the secondary center of ossification, and the histology showed that the lesions were located axial to the point of incorporation.[30,50] The current examined pigs were not perfused, but comparison to Hill et al[16] confirms that all current lesions were located at or axial to the point of incorporation of physeal vessels into bone. Incorporation of physeal vessels should probably be studied in more pigs in future, but until then, the comparison to Hill et al[16] supports that vascular failure in the physis was morphologically identical to vascular failure in epiphyseal growth cartilage, including axial location of lesions.[30,50] Vascular failure can therefore be associated with incorporation of vessels into bone in both physeal and articular OC.

When the morphology of the vascular failure is identical, the heritable predisposition may also be the same.[2,33] This is probably the single-most important implication of the current study because the heritable predisposition is the aspect of OC where there is still the most information left to discover. The current results contribute by indicating that the predisposition may be associated with the same set of genes for both physeal and articular OC, compared to if the pathogenesis had been different and the predisposition therefore was associated with different sets and a greater total number of genes. If discovered, specific genes can potentially be used to refine genomic selection against OC. Also, causes of vascular failure have been easier to discover in acquired than in heritably predisposed lesions (eg, bacteria/perivascular neutrophils in septic vascular failure).[24,45] Following the path of failed vessels has so far only revealed nonspecific changes as described in the Results.[9] Thus, discovering specific genes, researching their functions, and linking these to morphological changes may be the only way to determine the exact cause of vascular failure in heritably predisposed OC.

Physeal Vascular Failure Did Not Lead to Ischemic Chondronecrosis

The vast majority of chondrocytes within the current lesions were morphologically viable. This agrees with Farnum et al,[7] where results were corroborated by transmission electron microscopy, and other studies where necrosis of chondrocytes was observed in only very chronic lesions. [7,15,31] Following vascular failure in epiphyseal growth cartilage, it is chondrocytes at intermediate depth that undergo necrosis.[5,25,47] The proposed explanation is that they are outside the diffusion distance from alternative sources.[5] The physis is responsible for elongation of a cylinder, whereas the epiphyseal growth cartilage is responsible for expansion of a sphere. There may be better access to diffusion from alternative sources following vascular failure within a cylinder, compared to in a sphere. Intermediate depth also corresponds to the resting and proliferative zones of growth cartilage,[29] whereas chondrocytes within the current lesions were hypertrophic. Vascular failure may therefore affect different chondrocyte zones in the physis than in epiphyseal growth cartilage. Growth plate chondrocytes are described as having entered a path of terminal differentiation, and hypertrophic chondrocytes may have reached a stage from which they are able to continue independent of blood supply. Chondrocyte survival following physeal vascular failure may ultimately be due to a combination of factors, and experimental vascular transection could provide more definitive answers. In the meantime, the fact that vascular failure leads to retention of viable chondrocytes in the physis and chondrocyte necrosis in epiphyseal growth cartilage implies that after lesion initiation, diagnostic markers and staging may be different between OC in the 2 sites.

Physeal Vascular Failure Was Nevertheless Associated With Delayed Ossification

Endochondral ossification is a carefully coordinated process that relies on events on the growth cartilage and bone sides of the ossification front.[12] When both physeal and epiphyseal lesions occur due to failure of intra-cartilaginous vessels, this suggests that events on the growth cartilage side may be primarily responsible for the ossification delay in OC. In epiphyseal OC, chondrocytes underwent necrosis before the time they were expected to hypertrophy[5,25,49] and failed to carry out the associated cellular and matrix changes required for ossification to proceed.[12] In physeal OC, chondrocytes underwent hypertrophy, yet ossification was still delayed. The fate of hypertrophic chondrocytes in normal growth cartilage has been much debated.

As others before us,[11,36] we noted that hypertrophic chondrocytes within lesions failed to undergo apoptosis and vacate lacunae. We considered that this might constitute a temporary physical obstacle to osteoblast invasion.[11,12,36] Several studies also document that hypertrophic chondrocytes can transition into osteoblasts.[34,35,46] At present, the relative contribution of apoptosis and osteoblast transition to normal endochondral ossification in pigs is not known. It is therefore not yet possible to speculate which mechanism is responsible for delaying ossification within lesions. More research is needed, and it would be especially interesting to discover the extent to which either mechanism relies upon a vascular signal or gradient, in which case this would explain why the mechanism was upset following vascular failure.

Outcome

Several of the current observed lesions were expected to resolve. Both human[23,41] and animal[28] studies indicate that whether lesions are able to resolve is determined partly by size. In the current study, there was a striking range in terms of lesions affecting from 1.1% to 45.6% of physeal width. Some lesions were expected to resolve based on size alone. Other lesions like D6, Q28, R30, and S31 were expected to resolve due to extensive evidence of secondary, reparative ossification.[3,14] Vessels are spaced at regular intervals within growth cartilage.[42] If the current observed cartilage peninsulas represented points of vessel entry, they were separated by $\sim$0.4 mm of bone (Suppl. Table S4). After vascular failure, the

ossification front could therefore meet with a second, intact vessel in the time it takes to advance 0.4 mm, and this could restore the vascular signals or gradients required for endochondral ossification to resume, as discussed above. This potentially also fits with how ossification was observed to advance horizontally into lesions at the level of the metaphyseal-side ossification front (Suppl. Figs. S5, S6).[14]

Large lesions affecting up to 45.6% of physeal width were expected to persist and potentially cause clinical signs. Angular limb deformity was not observed in any of the current examined 82- to 180-day-old pigs. Histologically, changes represented early lesions, and based on experimental studies of articular OC, a lag phase is expected before clinical signs develop.[29] This is supported by limb deformity becoming manifest from 5 months onward in historical studies.[14,17,18,31] Several studies document limb deformities emanating from the physes around the hock,[1,6] but deformities of the distal femur are difficult to discover in vivo due to the large hams these pigs are bred for. Contemporary finishing pigs also reach slaughter weight by 5 months, but the observation that 23 of the 32 current lesions (72%) were located in the central and caudal thirds of the medial half of the femur agrees with the medial and caudal femoral shortening, angulation, and rotation seen in 8- to 9-month-old Landrace pigs in Figs. 1 and 2 of Reiland.[31] The mechanism for how lesions lead to deformity will have to be studied in older individuals, and with the current histological validation, this can be done by longitudinal CT scanning of sedated pigs.

The pathogenesis of physeal OC started with vascular failure that was morphologically identical to articular OC, and the heritable predisposition may therefore be the same in both sites. Vascular failure in the physis led to retention of viable chondrocytes, compared to chondrocyte necrosis in epiphyseal growth cartilage, and lesion markers and staging may therefore be different between the 2 sites. The mechanism for how lesions lead to angular limb deformity should be studied further in older pigs in future. The authors (would like to) dedicate this article to the memory of Associate Professor Sven Reiland (1935-2016).

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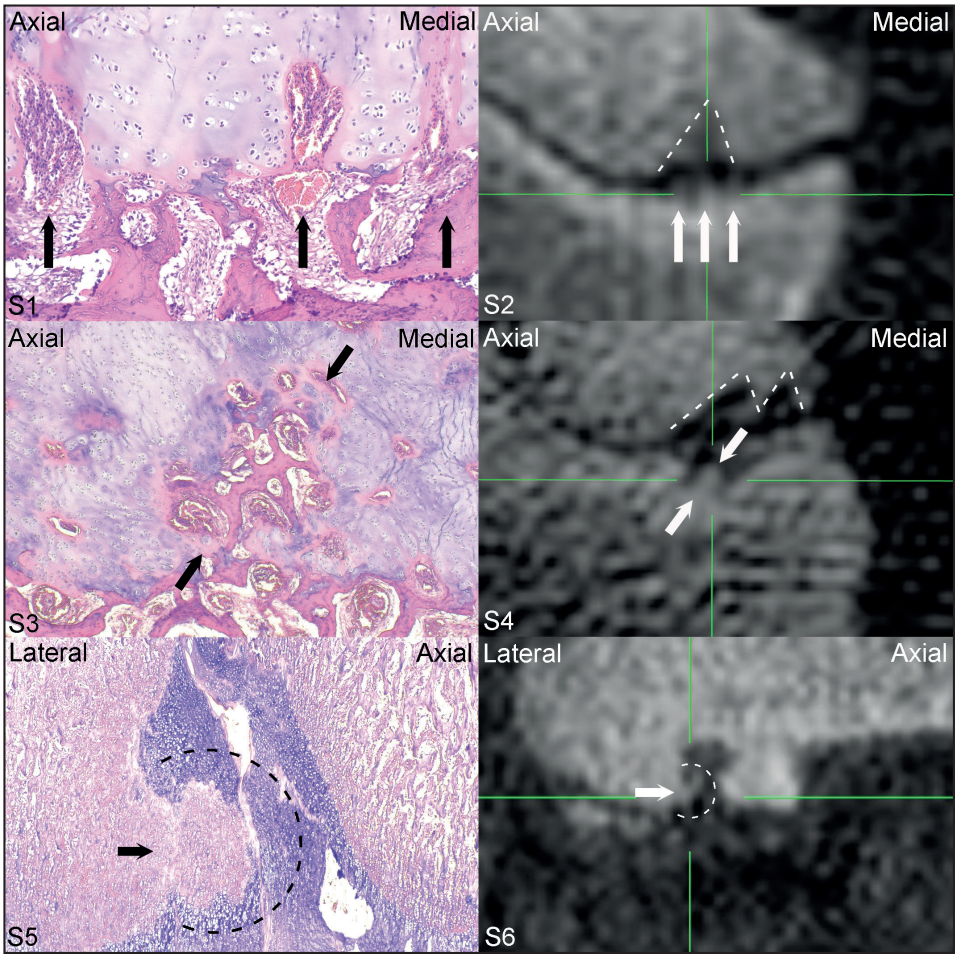

Supplemental Figures S1-S6. Physeal osteochondrosis, distal femur.
Supplemental Figure S1. Pig M, lesion 21, caudal third, medial half of physis. There are three regularly spaced, repopulated canals (arrows) on the epiphyseal side of the lesion. Hematoxylin and eosin (HE).
Supplemental Figure S2. Pig M, lesion 21, caudal third, medial half of physis. There are hyperdense lines (arrows) that match the location, size, shape and number of the repopulated canals in Supplemental Figure S1. Dashed lines: there is a triangular defect in the metaphyseal-side ossification front.
Supplemental Figure S3. Pig C, lesion 5, caudal third, medial half of physis. There is ossification in a location corresponding to the location of canals repopulated from the epiphyseal-side bone marrow (between arrows). HE.
Supplemental Figure S4. Pig C, lesion 5, caudal third, medial half of physis. There is a mineral hyperdense focus (between arrows) in a location corresponding to the ossification in Supplemental Figure S3. Dashed lines: there are two triangular defects in the metaphyseal-side ossification front.
Supplemental Figure S5. Pig M, lesion 22, central third, lateral half of physis. Ossification (arrow) is advancing horizontally into the lesion at the level of the metaphyseal ossification front (arrowheads), causing it to take on a reverse-C-shape (dashed line). HE.
Supplemental Figure S6. Pig M, lesion 22, central third, lateral half of the physis. Horizontal ossification into the lesion (arrow) at the level of the metaphyseal ossification front is causing it to take on a reverse-C-shape (dashed line), matching the shape in Supplemental Figure S5.

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Supplemental Table 1. Clinical Data on the Pigs and Lesion Number and Location Identified by Computed Tomography

Pig	Age	Weight	General Health	Computed Tomography Lesion Number	Location	Number of Available Femur Blocks[a]
A	82 days	33 kg	Normal	1	CdM[b]	-
				2	CeM[c]	-
				3	CeL[d]	-
B	96 days	47 kg	Hernia	4	Ce/CdM[e]	-
C	110 days	42 kg	Diarrhoea	5	CdM	1
D	113 days	57 kg	Normal	6	Ce/CdM	-
E	115 days	58 kg	Normal	7	Ce/CdM	2
F	127 days	68 kg	Coughing	8	Ce/CdM	Not visible on surface
				9	CdL[f]	2
G	132 days	63 kg	Normal	10	CdM	4
H	140 days	80 kg	Normal	11	CdM	-
I	141 days	91 kg	Normal	12	CdM	-
				13	CeM	-
				14	CeL	-
J	159 days	102 kg	Normal	15	Ce/CdM	2
K	162 days	80 kg	Respiratory distress	16	CdM	-
				17	CeM	-
				18	CeL	-
L	166 days	113 kg	Lame left forelimb	19	CdM	-
				20	Mid-caudal	-
M	171 days	96 kg	Normal	21	CdM	2
				22[g]	CeL[g]	2[g]
N	171 days	109 kg	Normal	23	CdM	5
				24	CdL	Not visible on surface
O	171 days	118 kg	Normal	25	CeM	4
				26	CeL	Not visible on surface
P	173 days	115 kg	Normal	27	Ce/CdM	2
Q	174 days	118 kg	Normal	28	CdM	1
				29	CeM	1
R	178 days	128 kg	Normal	30	CdM	-
S	180 days	127 kg	Normal	31	CdM	-
				32	CeL	-

[a]Represents number of slabs with lesions, but not number of lesions. [b]CdM: Caudal third, medial half of physis. [c]CeM: Central third, medial half of physis. [d]CeL: Central third, lateral half of physis. [e]Ce/CdM: Central and caudal third, medial half of physis. [f]CdL: Caudal third, lateral half of physis. [g]Unconfirmed in orthogonal imaging planes, but visible on surface of slab.

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Supplemental Table 2. Categories of Cartilage Canal and Eosinophilic Streak Morphological Changes, and Repopulated Canal Contents Superficial to Metaphyseal Defects in the 12 Histologically Validated Lesions

Lesion	Slab Number from Cranial to Caudal	Nearest the Metaphysis: Thin Eosinophilic Streaks Compatible with Necrotic Vessel Branches				Mid-Depth: Wide Eosinophilic Streaks Compatible with Necrotic Vessel Trunks				Towards Epiphysis	Nearest to and Continuous with the Epiphyseal Bone Marrow, Repopulated Canals Contain[a]		
		Eosinohilic Streak	RBC[b]	Ghost Lumen	Dilated	Eosinophilic Streak	RBC	Ghost Lumen	Dilated	Chondrifying Canals	Fibroblast-Like Cells	Osteoblast-Like Cells	Chondrocyte-Like Cells
C5	1	+	+	+	+	+	+	+	+	+	+	-	-
E7	1	+	+	+	+	+	+	+	+	+	+	-	-
E7	2	+	+	-	-	+	+	-	-	-	+	-	-
F9	1	+	-	-	-	+	+ neutrophils	-	-	+	+	-	-
F9	2	+	+ neutrophils	-	-	+	+	+	+	-	+	-	-
G10	1	+	+	-	-	-	-	-	-	+	+	-	-
G10	2	+	-	-	-	+	+	-	-	+	+	-	-
G10	3	+	+	-	-	-	-	-	-	+	+	-	-
G10[d]	4	+	-	-	-	+	+	+	+/-	+	+	-	-
J15	1	+	+	-	-	-	-	-	-	+	+	+/-	-
J15	2	+	+ remnant PVC[c]	-	-	+	+	+/-	+	+	+	+/-	-
M21	1	+	+	-	-	+	+ remnant PVC	+	-	+	+	+/-	+
M21	2	+	+	-	-	+	+ fibrin	+	+	+	+	+/-	-
M22	1	+	+ fibrin	+	-	-	-	-	-	-	+	+	-
M22	2	+	- hyaline material	+	-	-	-	-	-	-	+	+/-	-
N23[d]	1	+	-	-	-	+	+	+	-	+	+	-	-
N23	2	+	+ fibrin	-	-	-	-	-	-	+	+	-	-
N23	3	+	+	-	-	+	+	-	+/-	+	+	-	-
N23	4	+	+	-	-	+	+ fibrin	+/-	+	+	+	+/-	-
N23	5	+	+	-	-	+	+ fibrin	+	+	+	+	-	-
O25	1	+	+	-	-	+	+	-	-	-	+	-	-
O25	2	+	+	-	-	+	+ fibrin	+	+	+	+	-	-
O25	3	+	+	-	-	+	+	+	+	+	+	-	-
O25	4	+	+	-	-	+	+ fibrin, remnant PVC	-	-	+ fibrin	+	-	-
P27	1	+	+	-	-	+	+ remnant PVC	-	-	+	+	-	-
P27	2	+	+	+	+	+	+ fibrin	+	+	+	+	+/-	+
Q28	1	+	+	-	-	+	+ fibrin, remnant PVC	-	+	+	+	-	-
Q29	1	+	+	-	-	+	+	-	-	+	+	+	-
SUM	28 blocks	28 blocks	23 blocks	5 blocks	3 blocks	22 blocks	22 blocks	11 blocks; 2 blocks +/-	11 blocks; 2 block +/-	23 blocks	28 blocks	2 blocks; 7 blocks +/-	2 blocks

[a]Presence of cell type only; not equivalent to secondary response or ossification recorded in Supplemental Table 1. [b]RBC: Erythrocytes. [c]PVC: Perivascular mesenchymal cells. [d]Lesion G10 slab 4 and lesion N23 slab 1 had epiphyseal-side defects only, but canals followed the same pattern.

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Supplemental Table 3. Configuration of Failed Vessels, Defect Lobes and Changes Between Physeal Lesions and Perichondrium in the 12 Histologically Validated Lesions

Lesion	Slab Number from Cranial to Caudal	Vessels				Metaphyseal-Side Central, Perpendicular Ossification Defect Lobes		Metaphyseal-Side Peripheral, Abaxially Angled Ossification Defect Lobes		Epiphyseal-Side Ossification Defects Towards Physis		Focal Changes Inside Epiphyseal Bone		Focal Changes in Side of Epiphysis Towards Perichondrium Cartilage Peninsulas	Changes in Perichondrium Canals
		Peripheral-Perpendicular	Central-Perpendicular	Central-Axial	Peripheral-Abaxial	Triangular	Rectangular	Triangular	Rectangular	Central, Semi-Circular	Peripheral, Triangular	Conspicuous Large Vessel	Cartilage Islands		
C5	1	-	-	-	1	-	-	-	1	Yes	-	+	2 islands, no canals	-	1 fibrosing canal
E7	1	-	-	1 converge[a]	1 converge	-	1	1	-	Yes	-	+	-	-	-
E7	2	1 converge	-	-	1 converge	-	-	1	-	Yes	Yes	+		1 peninsula, no canal	-
F9	1	-	-	-	1, with neutrophils	-	-	-	1	Yes	-	+	2 islands, no canal	1 peninsula, no canal	-
F9	2	-	-	-	1, with neutrophils	-	-	-	1	No	-	+	-	3 peninsulas, 1 with repopulated canal	-
G10	1	-	-	-	1	-	-	1	-	Undulating	Yes	+	1 island, no canal	1 peninsula, no canal	-
G10	2	-	-	-	2	-	-	3	-	Yes	-	+	1 island, no canal	-	-
G10	3	-	-	-	2	-	-	1	-	No	-	+		3 peninsulas, 1 with chondrifying canal, 1 with acellular streak	-
G10	4	-	-	1	1	-	-	-	-	No	Yes	-	2 islands, no canal	1 peninsula, no canal	-
J15	1	-	2	-	2	1 + 1 circular	-	1	-	Undulating	-	+	-	-	3 chondrifying/fibrosing canals
J15	2	-	-	1 converge	1 converge	-	1	-	-	No	-	+	-	-	-
M21	1	-	-	-	2	-	1 bilobe[b]		1 bilobe	Thinning	-	+	1 island, no canal	-	-
M21	2	-	-	-	1	-	1	-	1	Undulating	-	+	-	-	-
M22	1	-	2	-	-	1	-	1	-	Undulating, possible thinning	-	+	-	-	-
M22	2	-	2	-	-	2	-	-	-	No	-	+	-	-	-
N23	1	-	-	-	1	-	-	-	-	No	Yes	-	-	-	1 fibrosing canal
N23	2	-	-	-	1	-	-	-	1	No	-	+	-	-	-
N23	3	-	-	-	1	-	1	-	-	No	-	+		1 peninsula, 1 necrotic canal	-
N23	4	-	-	1 converge	1 converge	-	2	-	-	Undulating	-	+	-	-	-
N23	5	-	2	-	1	-	1	1	1 bilobe	Undulating, possible thinning	-	+	-	-	-
O25	1	-	3	-	2	2 + 1 circular	-	-	2; 1 of which bilobe	No	-	+	-	-	-
O25	2	-	1	1 converge	1 converge	-	-	1	2; 1 of which bilobe	Undulating	-	+	-	-	2 chondrifying/fibrosing canals
O25	3		1 converge	-	1 converge	-	-	1	1 bilobe	No	-	+	-	-	-
O25	4	1 converge	1	-	1 converge	1	1	-	-	Undulating	-	-		1 peninsula, no canal	1 chondrifying/fibrosing canal
P27	1	-	-	-	2	-	-	1	-	No	-	+	-	-	1 chondrifying/fibrosing canal
P27	2	-	1	-	2	1	-	1	1	Undulating	-	+	-	-	1 chondrifying/fibrosing canal
Q28	1	-	1	-	2	-	1	-	1	Undulating	-	+	-	-	-
Q29	1	-	-	-	3	1	1	-	1	Undulating	-	-	2 islands, no canal	-	1 chondrifying/fibrosing canal
SUM	28 blocks	2 peripheral-perpendicular vessels	16 central perpendicular vessels	5 central axial vessels	36 peripheral abaxial vessels	10 triangular defects and 2 circular defects	10 rectangular defects; 1 of which bilobe	15 triangular defects	14 rectangular defects; 5 of which bilobe	5 blocks with semi-circular defects	4 blocks with triangular defects	24 blocks with conspicuous vessels	11 islands in 7 blocks; no canals	12 peninsulas in 8 blocks; with 1 necrotic, 1 repopulated, 1 chondrifying canal	8 blocks with 11 chondrifying/fibrosing canals

[a]Converge: two adjacent, angled vessels superficial to single defect. [b]Bilobe: shape resulting from two adjacent and confluent defects, any combination of shapes, but overall impression rectangular.

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Supplemental Table 4. Computed Tomographic Observations in 31 Lesions Confirmed in Orthogonal Planes (Excluding Macroscopic Lesion M22)

Lesion	Metaphyseal Ossification Defects	Contents of Defect	Metaphyseal Secondary Response	Epiphyseal Ossification Defect Towards Physis	Epiphyseal Response Opposite Metaphyseal Defect	Focal Changes Inside Epiphyseal Bone	Focal Changes in Side of Epiphysis Towards Perichondrium
A1	1 triangle, abaxially angled 1 triangle, axially angled, becomes rectangle	Uniformly hypodense, with hyperdense lines inside	Partial sclerotic rim	Central, semi-circular defects and possible peripheral triangular defect	Repopulation line at 372 HU	No	Peninsula 0.805 cm from physis
A2	1 small triangle, abaxially angled 1 medium triangle, perpendicular 1 triangle becomes rectangle, axially angled	Uniformly hypodense	Sclerotic foci	Possible peripheral triangle	Repopulation lines at 158, 262 and 280 HU	No	Peninsulas 0.493, 0.915 and 1.382 cm from physis
A3	1 triangle, abaxially angled, with island at tip 1 triangle, perpendicular, becomes rectangle	Uniformly hypodense	Little sclerosis	Central, semi-circular defect	Repopulation line at 221 HU; partial sclerotic rim	No	Peninsulas 0.708 and 1.329 cm from physis
B4	1 cyst-like triangle, axially angledY-fork 1 triangle, perpendicular 1 triangle becomes rectangle, axially angled	Uniformly hypodense, with focus at 275 HU and repopulation line from metaphyseal side at 440 HU	Partial sclerotic rim	Central, semi-circular defect and possible peripheral triangular defect curves back towards perichondrium	Repopulation line at 226 HU	No	Peninsulas 0.327, 0.397 and 0.909 cm from physis
C5	4 small triangles: 2 abaxially angled (one with island), 1 perpendicular, 1 axially angled	Uniformly hypodense at -7, 0, 15 and 100 HU, with hyperdense lines at 171 and 208 HU inside	No	Central, semi-circular defect	Focus in defect at 354 HU	Possible hypodense island	Peninsulas 0.572 and 0.671 cm from physis
D6	1 triangle, abaxially angled 1 bilobe[a], abaxially angled, and more[c]	Mixed density	No	Central, semi-circular defect	Focus at defect edge at 509 HU	No	Peninsulas 0.362, 1.053 and 1.085 cm from physis
E7	1 triangle, perpendicular 1 triangle, axially angled 1 triangle, abaxially angled, island, and more	Uniformly hypodense, with focus inside	Sclerotic foci and horizontal metaphyseal ossification into defect	Central, semi-circular defect	Repopulation line at 223 HU and focus adjacent to defect at 604 HU	Possible small hypodense island	Peninsulas 0.691 and 0.801 cm from physis
F8	1 triangle becomes rectangle, abaxially angled 1 triangle, abaxially angled 1 triangle, axially angled, island at tip, and more	Uniformly hypodense, with focus inside	Sclerotic foci	Central, semi-circular defect and possible peripheral triangular defect curves back towards hypodense island	Repopulation lines at 406 and 435 HU	Hypodense island	Peninsulas 0.503, 0.582 and 0.879 cm from physis
F9	1 small single triangle, perpendicular	Uniformly hypodense	Sclerotic focus	Tiny, central semi-circular defect	No	Possible hypodense island	Peninsula 0.414 cm from physis
G10	1 triangle, abaxially angled 1 large triangle, abaxially angled, connects with rectangle cranially, and more	Uniformly hypodense, with focus at 306 HU and hyperdense line at 412 HU inside	Sclerotic focus	Central, semi-circular defect and peripheral, triangular defect curves back towards island, sclerosis	No	Hypodense islands curve back towards perichondrial peninsula	Peninsula 0.285 and 0.510 cm from physis
H11	1 triangle, abaxially angled, connects with 1 triangle, axially angled 1 triangle, perpendicular, island at tip, and more	Uniformly hypodense, with focus at tip of triangle at 182 HU	Sclerotic foci	Central, semi-circular defect and possible linear defect curves back towards island	Foci in defect at 437 and 445 HU	Hypodense island	Peninsula 0.368 cm from physis
I12	1 triangle, perpendicular 1 triangle, axially angled 1 island	Uniformly hypodense, with focus into defect at 351 HU and hyperdense	Sclerotic foci	Central, semi-circular defect and possible peripheral, triangular	Repopulation lines at 406, 412 and 462		Peninsula 0.615 cm from physis

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		line at 358 HU inside defect		defect curves back towards perichondrial peninsula	HU, and sclerotic margin		
I13	1 triangle, perpendicular	Uniformly hypodense, adjacent focus at 345 HU	Sclerotic foci	No	Sclerosis	No	No
I14	1 triangle, abaxially angled 2 shorter triangles, perpendicular	Uniformly hypodense	Sclerotic foci and ossification into defect from cranial (C-shape)	No	No	No	No
J15	2 connected triangles; 1 axially and 1 abaxially angled 1 rectangle becomes triangle, abaxially angled 1 triangle, axially angled 1 island, and more	Uniformly hypodense at 20, 40 and 69 HU	Sclerotic focus at 398 HU and ossification into defect (C-shape)	Minimal central defect, peripheral, linear hypodense defect; possibly long vessel	Repopulaiton lines at 295, 342 and 426 HU, focus adjacent to lesion at 499 HU, sclerosis	Possible hypodense island curves back towards perichondrial peninsula	Peninsulas at 0.630, 0.949 and 1.012 cm from physis, with foci
K16	1 triangle, abaxially angled, becomes rectangle with island 1 triangle, axially angled 1 triangle, perpendicular 1 rectangle, perpendicular	Uniformly hypodense	Sclerotic focus at 262 HU and ossification into defect (C-shape)	Central, semi-circular defect	Repopulation lines at 342, 366 and 425 HU	Possible hypodense island curves back towards perichondrial peninsula	Peninsula 0.975 cm from physis
K17	1 triangle, perpendicular 1 rectangle, perpendicular	Uniformly hypodense	Ossification into defect	Central, semi-circular defect	Same as above	Same as above	Same as above
K18	2 small triangles, perpendicular	Uniformly hypodense	Sclerotic focus at 534 HU	No	Sclerosis	No	No
L19	2 small triangles, abaxially angled 1 triangle, perpendicular, and more	Uniformly hypodense	Sclerotic foci at 518 and 673 HU	No or very small central, semi-circular defect	Hyperdense line across defect at 478 HU	No	Peninsula 0.974 cm from physis
L20	1 large triangle, becomes rectangle with island, abaxially and caudally angled	Uniformly hypodense	Sclerotic focus at 590 HU	No	Sclerotic focus	No	No
M21	2-3 small triangles, abaxially angled	Uniformly hypodense, with hyperdense line at 534 HU inside	Sclerotic focus at 262 HU	Central, semi-circular defect	Repopulation lines at 431 and 520 HU	Possible hypodense island	Peninsula 0.583 cm from physis
N23	1 large rectangle, perpendicular, with island 3 small triangles; 1 abaxially angled, 1 axially angledand 1 perpendicular, and more	Uniformly hypodense, with hyperdense line at 148 HU inside like peripheral-abaxial vessels	Sclerotic focus at 619 HU	Central, semi-circular defect	Repopulation lines at 323, 386 and 449 HU		Peninsulas 0.560 and 1.286 cm from physis
N24	1 small bilobe, perpendicular or slightly axially angled	Uniformly hypodense	Sclerotic focus at 537 and 580 HU	No	Sclerosis	No	Peninsula 1.597 cm from physis
O25	5-6 triangles, perpendicular and more in several slices	Uniformly hypodense	Sclerotic foci	Tiny central, semi-circular defect	Repopulation line at 309 HU, sclerosis	No	Peninsulas 0.614 and 0.766 cm from physis
O26	2 very small semi-circles, perpendicular	Uniformly hypodense	Sclerotic focus	No	No	No	No
P27	1 triangle/linear defect, abaxially angled 1 rectangle, abaxially angled 1 triangle, perpendicular 1 semi-circle, perpendicular, and more	Uniformly hypodense, with hyperdense, vessel-like lines at 231 and 300 HU inside	Sclerotic foci	Central, semi-circular defect and peripheral, triangular defect, perpendicular and axially angled in same defect	Repopulation lines at 601 and 700 HU, sclerosis	Unconvincing hypodense island	Peninsulas 0.479 and 0.984 cm from physis
Q28	1 bilobe, perpendicular	Mixed density, some hypodense areas with focus at 692 HU and hyperdense line at 421 HU inside	Ossification into defect from edges	No	Sclerotic foci at 687 and 690 HU	No	Peninsula 0.504 cm from physis
Q29	2 small triangles; 1 perpendicular, 1 abaxially angled	Uniformly hypodense	Sclerotic focus at 328 HU and ossification into defect from metaphyseal side	No	Sclerosis	Possible island curves back towards perichondrial peninsula	Peninsulas 0.596 and 1.010 cm from physis
R30	2 triangles, perpendicular, join to form single large triangle, and more	Mixed density, with hyperdense line at 185 HU inside hypodense area like peripheral-abaxial vessel	Sclerotic focus at 469 HU and ossification into defect from edges	Central, semi-circular defect and possible peripheral, linear defect curves back towards perichondrial peninsula	Sclerotic margin including focus at 692 HU	No	Peninsula 0.538 cm from physis
S31	2 triangles; 1 abaxially angledand 1 perpendicular, and more	Mixed density	Ossification into defect from edges	No	No	Possible island curves back towards perichondrial peninsula	Peninsula 0.769 cm from physis
S32	1 small semi-circle, perpendicular becomes 2 small triangles 1 island, and more	Uniformly hypodense	Sclerotic focus at 484 HU	Possible remnant of central, semi-circular defect	Repopulation lines at 287 and 335 HU	No	Peninsula 0.546 cm from physis

[a]HU: Hounsfield units. [b]Bilobe: confluent lobes. [c]The term “and more” used to denote defects that were too small and too many to keep count of individually, e.g. > 5 defects in > 2 CT slices.

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Supplemental Table 5. Secondary Repair Responses and Computed Tomographic Visibility in 11 Lesions (Excluding Macroscopic Lesion M22)

Lesion	Modality	Metaphyseal Side			Epiphyseal Side		
		Inside Defect	Marginal Sclerosis	Horizontal Ossification into Defect[a]	Repopulation Lines[b]	Overt Ossification	Marginal Sclerosis
C5	Histology	Eosinophilic streaks, RBC[c], ghost lumen, dilated	No	No	Fibroblast-like cells	Yes; circular centre	No
	CT	Hyperdense lines at 171 and 208 HU[d]	No	No	No	Focus in defect at 354 HU	No
E7	Histology	Eosinophilic streaks, RBC, ghost lumen, dilated	No	No	Fibroblast-like cells	No	No
	CT	Focus inside	Sclerotic foci	Yes	Repopulation line at 223 HU	No	Focus adjacent to defect at 604 HU
F9	Histology	Eosinophilic streaks, RBC, neutrophils	No	No	Fibroblast-like cells	No	No
	CT	Uniformly hypodense	Sclerotic focus	No	No	No	No
G10	Histology	Eosinophilic streaks, RBC	Possible focus at triangle tip	No	Fibroblast-like cells	Yes; square to mini C-shape	Possible sclerotic rim
	CT	Focus at 306 HU and hyperdense line at 412 HU	Sclerotic foci	No	No	No	Sclerosis
J15	Histology	Eosinophilic streaks, RBC, remnant PVC[e]	No	No	Fibroblast- +/- osteoblast-like cells	No	No
	CT	Uniformly hypodense	Sclerotic focus at 398 HU	Yes; resulting in C-shape	Repopulation lines at 295, 342 and 426 HU	Focus adjacent to lesion at 499 HU	Sclerosis
M21	Histology	Eosinophilic streaks, remnant RBC	Suspicious, but doubtful	No, but in lesion M22; resulting in C-shape	Fibroblast- +/- osteoblast- and chondrocyte-like cells	Yes; short lines	No
	CT	Hyperdense line at 534 HU	Sclerotic focus at 262 HU	No	Repopulation lines at 431 and 520 HU	No	No
N23	Histology	Eosinophilic streaks, RBC, fibrin	No	No	Fibroblast- +/- osteoblast-like cells	No	No
	CT	Hyperdense line at 148 HU	Sclerotic focus at 619 HU	No	Repopulation lines at 323, 386 and 449 HU	No	No
O25	Histology	Eosinophilic streaks, RBC	Possible focus at rectangle tip	No	Fibroblast-like cells	No	No
	CT	Uniformly hypodense	Sclerotic foci	No	Repopulation line at 390 HU	No	Sclerosis
P27	Histology	Eosinophilic streaks, RBC, ghost lumen, dilated Also: streak repopulation from meta side; fibroblast- and chondrocyte-like cells	No	Yes; resulting in C-shape	Fibroblast- +/- osteoblast-like cells	No	No
	CT	Hyperdense lines at 231 and 300 HU	Sclerotic focus	No	Repopulation lines at 601 and 700 HU	No	Sclerosis
Q28	Histology	Eosinophilic streaks, RBC	No	No	Fibroblast-like cells	No	No
	CT	Mixed density, with hyperdense line at 421 HU	Focus at 692 HU	Yes	No	Sclerotic foci at 687 and 690 HU	No
Q29	Histology	Eosinophilic streaks, RBC	No	No	Fibroblast- and osteoblast-like cells	Yes; short lines	No
	CT	Uniformly hypodense	Focus at 328 HU	Yes	No	No	Sclerosis

[a]Defined as lines of osteoblasts, osteoid and trabecular structure in histological sections, and as mineral hyperdense lines in CT scans, advancing horizontally into lesions at the level of the metaphyseal ossification front. [b]Defined as repopulation of failed cartilage canals with cells from epiphyseal subchondral bone marrow in histological sections, and as hyperdense lines in CT scans. [c]RBC: Erythrocytes. [d]HU: Hounsfield units. [e]PVC: Perivascular mesenchymal cells.

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Supplemental Table 6. Size of Physes and 31 Computed Tomographic Lesions[a]

Pig	Physeal medio-lateral width	Physeal cranio-caudal length	Lesion	Absolute lesion medio-lateral width[b]	Absolute lesion cranio-caudal length[b]	Absolute lesion proximo-distal depth	Absolute lesion volume	Relative lesion medio-lateral width[c]	Relative lesion cranio-caudal length[c]
A	43.7 mm	41.3 mm	1	9.84 mm	2.81 mm	0.41 mm	11.34 mm^3	22.5 %	6.8 %
			2	9.85 mm	10.55 mm	0.17 mm	17.67 mm^3	22.5 %	25.5 %
			3	1.40 mm	2.11 mm	0.12 mm	0.35 mm^3	3.2 %	5.1 %
B	46.2 mm	43.7 mm	4	21.09 mm	16.88 mm	0.39 mm	138.84 mm^3	45.6 %	38.9 %
C	48.6 mm	42.3 mm	5	8.44 mm	3.52 mm	0.28 mm	8.32 mm^3	17.4 %	8.3 %
D	50.1 mm	47.9 mm	6	2.11 mm	2.81 mm	0.25 mm	1.48 mm^3	4.2 %	5.9 %
E	47.5 mm	46.1 mm	7	11.95 mm	9.84 mm	0.34 mm	39.98 mm^3	25.2 %	21.3 %
F	49.6 mm	49.6 mm	8	6.33 mm	10.55 mm	0.15 mm	10.02 mm^3	12.8 %	21.3 %
			9	2.11 mm	5.62 mm	0.20 mm	2.37 mm^3	4.3 %	11.3 %
G	48.5 mm	49.7 mm	10	4.12 mm	3.51 mm	0.36 mm	5.21 mm^3	8.5 %	7.1 %
H	55.8 mm	54.5 mm	11	2.82 mm	2.81 mm	0.14 mm	1.11 mm^3	5.1 %	5.2 %
I	48.6 mm	53.8 mm	12	4.53 mm	6.80 mm	0.14 mm	4.31 mm^3	9.3 %	12.6 %
			13	3.02 mm	9.07 mm	0.13 mm	3.56 mm^3	6.3 %	16.9 %
			14	16.63 mm	3.78 mm	0.10 mm	6.29 mm^3	34.2 %	7.0 %
J	54.6 mm	56.8 mm	15	7.08 mm	18.10 mm	0.42 mm	53.82 mm^3	13.0 %	31.9 %
K	53.7 mm	57.4 mm	16	5.94 mm	11.13 mm	0.21 mm	13.88 mm^3	11.1 %	19.4 %
			17	6.68 mm	5.94 mm	0.70 mm	27.78 mm^3	12.4 %	10.3 %
			18	13.36 mm	3.71 mm	0.84 mm	41.64 mm^3	24.9 %	6.5 %
L	57.9 mm	55.2 mm	19	0.83 mm	1.65 mm	0.18 mm	0.25 mm^3	1.4 %	3.0 %
			20	1.60 mm	3.31 mm	0.39 mm	2.07 mm^3	2.8 %	6.0 %
M	57.4 mm	52.3 mm	21	3.22 mm	4.01 mm	1.10 mm	14.20 mm^3	5.6 %	7.7 %
N	59.5 mm	52.9 mm	23	8.22 mm	14.97 mm	0.57 mm	70.14 mm^3	13.8 %	28.3 %
			24	0.63 mm	0.75 mm	0.96 mm	0.45 mm^3	1.1 %	1.4 %
O	56.5 mm	57.2 mm	25	3.36 mm	4.20 mm	0.26 mm	3.67 mm^3	5.9 %	7.3 %
			26	7.56 mm	12.60 mm	0.12 mm	11.43 mm^3	13.4 %	22.0 %
P	58.2 mm	60.0 mm	27	12.20 mm	10.46 mm	0.34 mm	43.39 mm^3	21.0 %	17.4 %
Q	60.6 mm	58.1 mm	28	2.68 mm	2.68 mm	0.16 mm	1.15 mm^3	4.4 %	4.6 %
			29	3.58 mm	4.47 mm	0.96 mm	15.36 mm^3	5.9 %	7.7 %
R	52.3 mm	58.9 mm	30	6.47 mm	7.39 mm	0.54 mm	25.82 mm^3	12.4 %	12.5 %
S	64.4 mm	52.3 mm	31	2.99 mm	3.00 mm	0.35 mm	3.14 mm^3	4.6 %	5.7 %
			32	3.74 mm	1.49 mm	0.21 mm	1.12 mm^3	5.8 %	2.8 %
Range	43.7-64.4 mm	41.3-60.0 mm		0.63-16.63 mm	0.75-18.1 mm	0.1-1.1 mm	0.25-138.84 mm^3	1.1-45.6 %	1.4-38.9 %
Average	53.35 mm	52.10 mm		6.27 mm	6.47 mm	0.37 mm	18.71 mm^3	12.3 %	12.5 %

[a]Excluding macroscopic lesion M22. [b]Absolute lesion width and length refer to the measurements as read off the software calipers. [c]Relative lesion width and length refer to the absolute measurements as read off the software calipers, divided by the absolute measurements for physeal width and length, i.e. the dimension of the lesion expressed as a percentage of the dimension of the physis.

III

Title: Blood supply to femoral growth cartilage in foals and relevance to osteomyelitis

Short title: Blood supply to femoral growth cartilage in foals

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Authorship

All authors contributed to the design of the study, acquiring and interpreting of the data. B.H. Wormstrand drafted the manuscript, and all authors critically revised and approved the final version. B.H. Wormstrand is the responsible author.

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Competing interests

The authors declare no competing interests.

Ethical animal research

The foetus and the nine Fjord Pony foals originated from a previous study, approved by the Norwegian National Research Authority (approval number 2008-783). The Norwegian/Swedish

Coldblooded Trotter foal was euthanized at the owner's request after obtaining informed consent for the foal to be used for research.

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Data accessibility statement

Raw data are available from the corresponding author upon reasonable request.

Keywords

Micro-CT, cartilage canals, foal, physis, stifle

Word count

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Details removed for purposes of masking [with line numbers]

Line 61- 62:	approval number 2008-783
Line 262:	Kristin Olstad

Summary

Background: Osteomyelitis in foals is commonly localized to the ossochondrous junction. Sluggish blood flow in metaphyseal vessels may explain this apparent tissue susceptibility to infections, but fail to explain commonly observed age-related variations in lesion distribution throughout the appendicular skeleton. Vessels within cartilage canals provide temporary blood supply to growth cartilage and regress at different ages in different locations. Bacteria were recently found in cartilage canals of foals euthanized due to septic arthritis/osteomyelitis. Experiments in chickens indicate that vessel endothelium in the distal tips of in-growing cartilage canals is discontinuous and allows bacterial binding to extracellular matrix. Cartilage canals vessel involvement could explain the age-related variation distribution of osteomyelitis lesions, but information is currently missing especially for equine hind limb physes.

Objectives: To describe the blood supply to the growth cartilage of the medial femoral condyle in foals, with emphasis on the physis.

Study design: Pseudo-longitudinal ex vivo arterial perfusion study.

Methods: The left medial femoral condyle of 10 Norwegian Fjord Pony foals and 1 Norwegian-Swedish Coldblooded Trotter foal (228 days of gestation to 62 days of age) was arterially perfused with barium and underwent micro-computed tomography with qualitative and quantitative description of blood vessels.

Results: The blood supply to the femoral physis originated from the metaphysis before formation of the secondary ossification centre, from both the epi- and metaphysis in the neonatal period and from the epiphysis from 15 days of age. The number of vessels in growth cartilage increased until 42/48 days of age before decreasing.

Main limitations: The methodology does not demonstrate vessel endothelial discontinuity.

Conclusions: The blood supply to growth cartilage changed qualitatively and quantitatively with age. These changes are likely to entail endothelial gaps that allow bacteria to adhere to extracellular matrix. Vessels may be particularly susceptible to infection during growth spurts with active in-growth.

Main text (word count 4899)

Introduction

In foals, septic arthritis is often associated with osteomyelitis, and is usually a consequence of bacteraemia or septicaemia [1; 2]. Multiple foci of infection are common, especially in younger animals [2]. Lesions have been reported in most of the appendicular skeleton, but affect some bones more frequently than others including the distal part of the radius, femur, tibia and third metacarpal/metatarsal bones [1; 2]. Young foals are susceptible to lesions in both distal and proximal parts of the limb [2]. In older foals, the proximal extremities, such as the distal tibia and distal femur, are often affected, sometimes with serious long-term, post-infection complications [2-4]. Lesions detectable by radiography or at necropsy are predominantly located immediately deep to the junction between growth cartilage and bone [1]. It has been suggested that this localization is due to sluggish blood flow in subchondral bone vessels [5], but this hypothesis has not been substantiated by experimental studies [6]. It has further been implied that distribution between different regions may be associated with cartilage thickness or growth plate inclination, but there is currently no explanation for why osteomyelitis should affect different bones at different ages [1; 7].

In a recent study of foals euthanized because of septic arthritis/osteomyelitis, bacteria were found in cartilage canals [8]. Cartilage canals carry a temporary blood supply to the growth cartilage of the articular epiphyseal cartilage complex (AECC) and the physis during the early phases of growth [9; 10]. In experimental bacterial inoculation of chickens and pigs, cartilage canals were identified as initial foci of infection [6; 11]. Bacteria were particularly found in the distal tips of actively in-growing canals where there were discontinuities in the vascular endothelium, allowing binding to extracellular matrix [12]. Endothelial discontinuities have since been found in regressing cartilage canals in foals[13]. In the AECC of foals, it is well-recognized that cartilage canals are heterogeneously distributed and regress at different age thresholds[9]. Involvement of cartilage canals can therefore potentially explain why osteomyelitis affects different bones at different ages. However, blood supply has only been studied in a limited number of forelimb physes [10; 14], and the relationship to osteomyelitis is therefore not clear [7].

The aim of the current study was to describe the blood supply to the growth cartilage of the medial femoral condyle of foals, with particular emphasis on the physis. Our hypothesis was that the number of cartilage canal vessels would decrease with increasing age.

Materials and methods

The ex vivo material consisted of the left femur of one Norwegian Fjord Pony foetus and nine foals originally bred for an ethically approved experimental study of osteochondrosis [15] (**removed for peer review**), and the left femur of one Norwegian/Swedish Coldblooded Trotter (NSCT) foal euthanized at the owner's request after obtaining informed consent for the foal to be used for research. Included foals were free of clinical evidence of joint disease, systemic infections or conditions affecting the circulation. Age, breed and sex are detailed in Table 1. The Fjord Pony foals had undergone terminal femoral arterial perfusion with barium as part of the experimental study, whereas the foetus and the NSCT foal were perfused post mortem (see below).

Arterial barium perfusion procedure

The terminal arterial perfusion procedure is described in full elsewhere [16], and the post mortem arterial perfusion procedure was based on Hertsch & Samy, 1980 [17]. A catheter was placed in the left femoral artery, and the limb was flushed with saline until the effluent ran clear. The limb was then perfused with a suspension of micronized barium in saline (25% of total blood volume), followed by barium in formalin (37.5% of blood volume) delivered using a mechanical pump. Previous studies have confirmed histologically that this method results in barium filling of the arterial side of the vasculature, before the barium lodges in the capillaries [16; 18].

Samples

The left femur was harvested and fixed in 4% formaldehyde for 48 hours. A standardised sample containing the medial femoral condyle with physeal and epiphyseal growth cartilage was obtained from all femurs (Fig 1). The samples were cuboidal and measured approximately 2.5 x 2.5 x 6.5 cm. The cubes were obtained by sawing the distal femur into medial and lateral halves using a band saw (Fig 1a). The medial half was then divided into cranial and caudal quarters, and the caudal quarter was finally separated from the femur by a transverse cut immediately proximal to the physis (Fig 1b). In the foetal femur, only the transverse cut proximal to the physis was performed as the distal femur was small enough to be scanned intact.

Micro-CT

The surface of each sample was wrapped in sealing film (Parafilm)[a] to prevent desiccation during scanning. The samples were scanned using a multiscale x-ray nanotomograph (Skyscan 2211) equipped with an open-type X-ray tube working at 110 kV/60μA. The standard wolfram target was used. The samples rotated at steps of 0.31° per projection around 360°, totalling 1162 projections per sample, and images were acquired using a flat panel detector, resulting in an isotropic voxel resolution of 47 μm. The exposure time was 0.370 ms, averaging 4 frames for each projection. A 0.5-mm Cu filter was used to remove low energy x-ray components from the beam. For each sample, a stack of 2D-images was obtained and reconstructed using commercial software (NRecon, Bruker, Kontich, Belgium).

Evaluation of CT-images

The 2D-images were imported into a commercial software package (VGStudio Max version 3.2.4)[c] for evaluation in three orthogonal planes and as 3D volume-rendered models. Larger vessels in the perichondrium and bone were visualised using the software thick-slab option with up to 100 individual 2D-images viewed simultaneously. Smaller vessels in the AECC and physis were visualised using the thick-slab option with a maximum of 30 slices viewed simultaneously. Qualitative description included vessel origin, course, orientation and branching. Additionally, the number of vessels entering the AECC was quantified by manual counting. All vessels were counted in all sagittal plane and all frontal plane images, and the average of these two numbers was defined as the total vessel number of the sample. The difference between the sagittal and frontal plane counts was <5% in all cases. Finally, the maximum medio-lateral width and cranio-caudal length of the physis included in the scanned sample blocks were measured using Vernier callipers. Width was multiplied by length to generate a measure of physeal area. The total sample vessel number was divided by physeal area to produce a relative vessel/physeal area ratio.

Results

Foetus

In the foetal femur, the primary, diaphyseal ossification centre was present, while the secondary, epiphyseal ossification centre had not yet formed (Fig 2a). The foetal femur therefore contained only one ossification front, referred to as the metaphyseal ossification front (Fig 2b). The diaphyseal bone was supplied by arteries which, based on their tapering and orientation, originated from the nutrient artery in the proximal third of the femur. From here, the diaphyseal nutrient artery coursed distally towards the epiphysis and gave off multiple vessel branches along its course, especially towards the metaphyseal ossification front. Most branches terminated on the bone-side of the ossification front, but some branches traversed the ossification front and entered the chondroepiphysis. The location of the future physis was therefore supplied by vessels originating from metaphyseal bone, travelling perpendicular relative to the metaphyseal ossification front, from deep to superficial within the chondroepiphysis and towards the articular surface (Fig 2b).

The distal femoral epiphysis was supplied by four main arterial sources: one midline artery located at the cranio-proximal aspect of the trochlear groove, one midline artery located caudo-distally in the intercondylar fossa, and a medial and lateral abaxial perichondral supply comprising more than one small artery on each side, respectively. Two of these were relevant to the micro-CT-scanned blocks: the caudo-distal midline artery, which constituted the main nutrient artery to the epiphysis, and the perichondral supply on the medial abaxial aspect of the femur (Fig 2b).

The superficial part of the chondroepiphysis, including the site of the future secondary centre of ossification and AECC, was supplied by branches of the epiphyseal nutrient artery and branches of medial perichondral arteries.

1 and 10 day-old foals

The principal difference between the foetus and the 1 and 10 day-old foals was that the secondary centre of ossification had formed (Fig 3a). The foal blocks therefore contained three ossification fronts: the epiphyseal ossification front on the superficial and abaxial periphery of the secondary centre of ossification, and the epiphyseal-side and metaphyseal-side ossification fronts of the physis (Fig 3a). The arterial sources and general configuration of the blood supply to the medial femoral condyle was otherwise similar to that described for the foetus, above. The secondary centre of

ossification and superficial part of the epiphysis were supplied by the epiphyseal nutrient artery centrally, and by perichondral arteries peripherally. Perichondral vessels entered at 2-3 different levels from superficial to deep along the abaxial aspect of the epiphysis. Initially, all vessels coursed towards the centre of the epiphysis. After a short distance, vessels that entered superficially turned and coursed towards the superficial aspect of the epiphysis and the AECC. Likewise, vessels entering deeper on the abaxial side coursed towards the deep aspect of the epiphysis and the physis. As they followed this course, a low number of the superficial vessels coursed entirely within growth cartilage. However, the majority of the perichondral vessels coursed partly within bone as the mid-portion of the vessel was surrounded by the ossification front on both the superficial and deep sides of the secondary centre of ossification.

Vessels underwent two distinct patterns of branching. Dichotomous branching was confined to portions of arteries located within bone. This category was characterised by high numbers of small branches close together from a single or a few points along the trunk of a vessel, diverging outwards with a morphology resembling a tree crown (Fig 3b). Dichotomous branching was frequent towards the metaphyseal-side of the physis, less frequent towards the epiphyseal ossification front and did not occur towards the epiphyseal side of the physis. Within bone and cartilage, vessels also underwent a monopodial branching pattern that was characterised by single branches spread out along the length of vessel trunks (Fig 3c).

Vessels entered growth cartilage via all three ossification fronts. Some vessels entering the AECC or physis from epiphyseal bone turned 90 degrees, before continuing to course perpendicular relative to the underlying ossification front once within growth cartilage. Vessels entering the physis from metaphyseal bone did not turn, but rather continued to course straight and perpendicular to the ossification front within growth cartilage. Vessels entering the physis from the metaphyseal side typically penetrated up to 50% of the total thickness of the physis, whereas vessels entering from the epiphyseal side often extended through the entire physis, without entering metaphyseal bone. Monopodial branching in the cartilage occurred at regular intervals in epiphyseal-side vessels, but was rare in metaphyseal-side vessels. In contrast to the foetus, the physes of the 1 and 10 day-old foals had a dual supply consisting of vessels entering from both the metaphyseal and the epiphyseal sides of the physis (Fig 3d).

15 day-old and older foals

The arterial sources and configuration of the blood supply in the older foals were similar to that described for the foetus and younger foals, above. Subjectively, there was more extensive dichotomous branching within the metaphyseal and epiphyseal bone of foals ≥ 15 days than in the 1 and 10-day old foals (compare Figs 3a and 4a-b). The number and length of vessels within the AECC and physis were also reduced in the older, compared to the younger foals (see below). The frequency of monopodial branching within the physis was lower, markedly within the axial portion of the physis. However, the most pronounced difference was a complete absence of vessels entering the physis from the metaphyseal side in foals from 15 days old onwards. From the age of 15 days, the physis therefore had a single blood supply, originating from the epiphyseal side of the physis and coursing from superficial to deep within the physis only (Fig 4b).

Quantitative results

The absolute and relative numbers of vessels entering the AECC and the physes from different sides are presented in Table 2. The quantitative results supported the qualitative observations, above. In the one-day-old foal, 72 (50.3%) of the vessels entering the physis originated from the epiphysis and 68 (49.7%) vessels originated from the metaphysis (Fig 3d). In the 10-day-old foal, only seven (3.5%) vessels entered the physis from the metaphysis, all in the abaxial region, while 192 (96.5%) entered from the epiphysis.

Both the absolute and relative number of vessels followed the same development over time in the AECC and the physis. The youngest foal had the lowest number of vessels. The numbers increased with increasing age in the next three foals before reaching a plateau stretching from the age of 17 to 48 days in the AECC, and from 17 to 42 days in the physis. The two oldest foals aged 57 and 62 days had a reduced number of vessels compared to the younger animals.

Discussion

The main finding of this study was that the blood supply of the medial femoral condyle growth cartilage changed during the examined age window. In particular, the configuration of the blood supply to the physis changed from metaphyseal-origin vessels in the foetus, via a mixture of metaphyseal- and epiphyseal-origin vessels in the 1 and 10 days old foals, to epiphyseal-origin vessels only in foals ≥ 15 days of age. All physeal vessels appeared to be end arteries, corroborated by previous studies where intravascular AECC contrast columns have corresponded to end arteries when validated by histology [19; 20]. The observed end-points of the intravascular contrast columns could theoretically be due to column diameter dropping below micro-CT voxel resolution (partial volume effect). The obtained resolution was a deliberate choice between observing vessels at sufficient resolution to determine configuration, and being able to detect them within an entire bone region simultaneously. Firth & Poulos [10] described how transphyseal vessels in forelimb physes disappeared with age, leaving only epiphyseal-side end arteries in older foals, thus the interpretation that most physeal contrast columns represented end arteries seems justified. The observation that the blood supply to the medial femoral condylar physis goes through phases with different configuration is corroborated by similar observations from the distal radius and distal metacarpus [10]. Inclusion of a foetus in which the secondary centre of ossification had yet to be formed allowed detection of a phase with arterial supply from the metaphyseal side only, which has not been reported previously [10]. In a recent study of foals euthanized due to septic arthritis/osteomyelitis, bacteria were found in the distal tips of cartilage canals in the AECC [8]. With the current results, it is now possible to confirm that the location of bacteria also corresponds to the distal tips of cartilage canals coursing from the epiphyseal, towards the metaphyseal side of the physis [8]. Data from foals in the current study also support the interpretation that all physeal osteochondrosis lesions in 80-182 day-old pigs were the result of failure of end arteries coursing from the epiphysis, towards the metaphysis [21]. Based on the confirmed progression of osteochondrosis [15], bacterial binding in the distal tips of AECC canals is likely to progress to infected canals becoming surrounded by the advancing ossification front, potentially leading to epiphyseal osteomyelitis lesions [5]. By the same reasoning, bacterial binding in the distal tips of physeal canals could theoretically progress to epiphyseal lesions on the deep side of the epiphysis [5]. However, the distal tips of physeal canals are closer to the metaphyseal side, where ossification advances at a rapid rate compared to the epiphyseal side of the physis [22]; thus,

infection of physeal canals is likely to progress to metaphyseal osteomyelitis [5]. The finding that there appeared to be three distinct phases of physeal blood supply configuration supports the previously observed distribution of aseptic and septic lesions in pigs [21] and foals [8]. The present results indirectly support that vessels in the distal tips of cartilage canals are particularly susceptible to infection [12], since this is where ingrowth and regression takes place, processes which in other studies have been found to involve discontinuities [12; 13].

Two distinct patterns of vascular branching were observed: monopodial branching within growth cartilage and bone, and dichotomous branching within bone. The dichotomous branching was morphologically similar to the pattern described as sinusoidal filling by Firth & Poulos [10]. In foals euthanized due to septic arthritis/osteomyelitis, bacterial binding was sometimes detected simultaneously in vessels in growth cartilage and bone [8]. In the current study, a portion of dichotomous vessels in the primary centre of ossification represented branches of the diaphyseal nutrient artery that appeared to have been located within bone for a long time. Conversely, the secondary centre of ossification was supplied partly by the central nutrient artery, and partly by vessels that were continuously incorporated into bone from growth cartilage on the periphery of the epiphysis. Continuous incorporation of vessels combined with the two distinct branching patterns prompts the suggestion that at least some of the branches within epiphyseal bone represented newly formed dichotomous branches on previously intra-cartilaginous and monopodially branching vessels. Dichotomous branching was also more extensive in ≥ 15 day-old foals than younger foals, further suggesting active branching. It would be interesting to discover the extent to which endothelium is intact during dichotomous branching. Ham et al. [23] described leakage of injected carbon in rats at the cartilage-bone junction, indicating discontinuity, as previously observed in chickens [24]. If vessel endothelium is discontinuous during dichotomous branching, such branching could permit bacterial binding and potentially explain why lesions could occur focally also on the bone side of the ossification front [1; 8].

The number of cartilage canal vessels changed over time. A change in absolute number could have been due to varying sample size as the foals grew older. However, when correcting for sample size by calculating relative vessel numbers per sample, temporal changes in vessels numbers were still observed. The existing literature supports the paradigm that blood supply to growth cartilage should decrease with increasing age and eventually disappear completely [16; 25]. It was therefore

interesting to note that the data from the examined foals support active in-growth up to 42 days in the physis and 48 days in the AECC, after which the number of vessels decreased. Other studies have shown that cartilage canal vessel endothelium is discontinuous during both ingrowth and regression [12; 13]. The variation in number of cartilage canal vessels is therefore likely associated with endothelial discontinuities, which together with bacteraemia episodes may partially explain lesion distribution of osteomyelitis [12].

Cartilage canals are present in the distal femoral AECC until 7 months of age [25] but the number was reduced by nearly 50% from 48 to 62 days of age in the current material. The rate of regression of cartilage canals thus have to slow considerably until the age where they are no longer present. Alternatively, longitudinal studies of pigs confirm that both articular [26] and physeal osteochondral lesions (**removed for peer review**, personal communication) develop in waves, suggesting a fluctuating number of cartilage canals. The waves have been associated with incorporation of vessels into bone [26], but a second factor of cartilage thickness above diffusion distance for nutrients is also required [27], thus growth spurts would increase the likelihood of vascular failure resulting in lesions. The increase and subsequent decrease of number of vessels in the currently examined foals may indicate that these foals went through a phase of accelerated growth, interpreted as a local growth spurt, from 17-48 days. Further to this, it is possible that the foals would have gone through several more growth spurts before vessels disappeared from the AECC at 7 months [25]. As growth cartilage vessels demonstrate a more widespread distribution than where lesions manifest, this apparent discrepancy may be explained by the temporal distribution of vessel endothelial discontinuities coinciding with episodes of bacteraemia or septicaemia [12]. The current results support a growth spurt with active in-growth and regression of vessels from 1-62 days. If later growth spurts occur, these could also be associated with in-growth, regression and discontinuity of vessel endothelium outside the immediate post-natal window, potentially explaining susceptibility to bacterial binding and waves of septic arthritis/osteomyelitis in older foals [2; 5].

In conclusion, this data demonstrates active in-growth and regression of growth cartilage vessels in healthy foals from 1-62 days of age. There was active, dichotomous branching of vessels into bone, and the observed configuration of the blood supply was compatible with the anatomical localization of previously described septic lesions in foals. Although previous studies have shown endothelial discontinuities during active vessel in-growth and regression, the extent to which this may leave distal

tips of the cartilage canal vessels susceptible for bacterial binding and infection should be investigated further.

Manufacturers

[a] Parafilm® M, Merck, Darmstadt, Germany

[b] Bruker Corporation, Kontich, Belgium

[c] Volume Graphics, Heidelberg, Germany

Table 1. Age, sex, breed and weight of nine Fjord foals, one Fjord foetus and one Norwegian-Swedish Coldblooded Trotter foal femorally perfused with barium

Age (days)	Sex	Breed	Weight (kg)
228†	Not recorded	Fjord	Not recorded
1	Female	Fjord	45
10	Male	NSCT‡	73
15	Male	Fjord	63
17	Female	Fjord	75
28	Female	Fjord	94
35	Male	Fjord	81
42	Male	Fjord	102
48	Male	Fjord	114
57	Female	Fjord	111
62	Male	Fjord	113

†Length of gestation

‡Norwegian/Swedish Coldblooded Trotter

Table 2. Number of vessels entering the AECC† and the physis in nine Norwegian Fjord Pony foals and one Norwegian-Swedish Coldblooded Trotter aged 1-62 days.

Age (days)	Vessels entering AECC	Vessels entering physis from epiphysis	Vessels entering physis from metaphysis	Physis area (cm^2)	Vessels AECC/ physis area	Vessels physis/ physis area
1	145	72	68	8.6	16.8	16.2
10‡	196	192	7	10.0	19.6	19.9
15	218	207	0	11.7	18.6	17.6
17	297	284	0	11.0	26.9	25.7
28	313	228	0	8.9	35.2	25.6
35	265	217	0	8.4	31.4	25.7
42	298	288	0	9.6	31.0	30.0
48	321	223	0	9.4	34.2	23.7
57	163	188	0	9.4	17.4	20.0
62	172	188	0	10.9	15.7	17.2

†Articular-epiphyseal cartilage complex

‡Norwegian-Swedish Coldblooded Trotter

Figure legends

Figure 1. Preparation of samples for micro-CT-scanning. **a)** Axial view of the medial half of femur from a 10-day-old foal. Frontal and transverse cuts were made along stippled lines. **b)** Medial view. The cuts in a) resulted in a cuboidal sample of the medial femoral condyle with physeal and epiphyseal growth cartilage for micro-CT-scanning. Barium-perfused vessels are visible caudally on the medial side of the condyle (arrow).

Figure 2. Micro-CT of barium-perfused distal femur of Fjord foetus at 228 days gestation length, voxel size 47 µm. **a)** Disto-proximal view, medial to the right. The origin of a large branch of the central nutrient artery (arrow) entering the medial condyle from the axial aspect is visible. Large vessels (arrowheads) originating in the perichondrium can be seen entering the medial condyle on the medial side. Thick-slab mode, 200 slices. **b)** Frontal view, medial to the right. Branching of vessels from central nutrient artery (asterisk) and perichondral arteries towards the diaphysis and the articular surface are visible. Small vessels (arrows) are also visible entering the chondroepiphysis from the diaphysis where the primary ossification centre subsequently forms. Thick-slab mode, 100 slices.

Figure 3. Micro-CT of barium-perfused medial femoral condyle from one-day-old Fjord foal, voxel sixe 47 µm. **a)** Frontal view, medial to the right. Large vessels from the central nutrient artery (asterisk) are visible branching towards the physis and the articular epiphyseal cartilage complex (AECC). The secondary ossification centre has formed and three ossification fronts can be seen: the metaphyseal side of the physis, the epiphyseal side of the physis and the epiphyseal front. Thick-slab mode, 50 slices. **b)** Frontal view, metaphysis, medial to the right. Dichotomous branching of vessels is visible towards the physis (arrows). Thick-slab mode, 30 slices. **c)** Sagittal view, epiphysis, caudal to the right. Monopodial branching of vessels (arrows) is visible towards the AECC. Thick-slab mode, 50 slices. **d)** Frontal view, medial aspect of physis. Vessels entering the physis are of both metaphyseal (arrow) and epiphyseal (arrowhead) origin. Thick-slab mode, 50 slices.

Figure 4. Micro-CT of barium-perfused medial femoral condyle from Fjord foal aged 62 days, voxel sixe 47 µm. **a)** Frontal view, medial to the right. A large vessel (arrow) from the perichondrium branches towards both physis and articular epiphyseal cartilage complex (AECC). There is dichotomous branching towards the AECC and monopodial branching towards the physis. Thick-slab mode, 200 slices. **b)** Sagittal view, caudal to the right. A vessel (arrow) courses parallel with the

physis in the epiphysis with monopodial branches entering the physis at a perpendicular angle and extending 2/3 of the physeal depth. In the metaphysis, there is extensive dichotomous branching towards, but no vessels enter the physis. Thick-slab mode, 50 slices.

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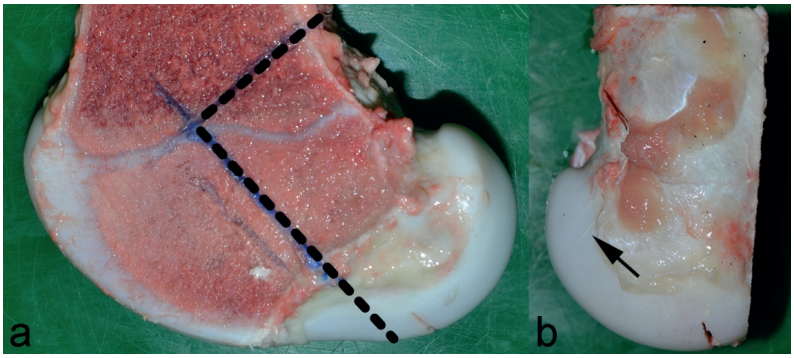
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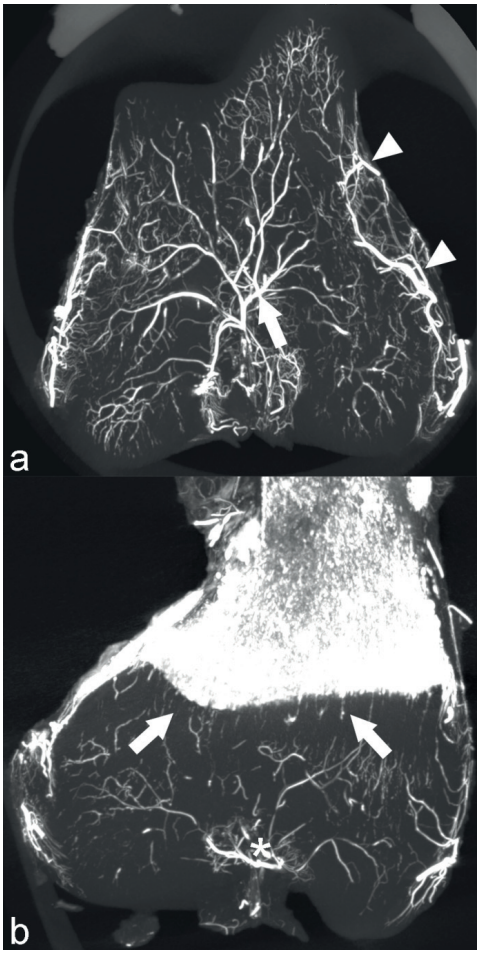
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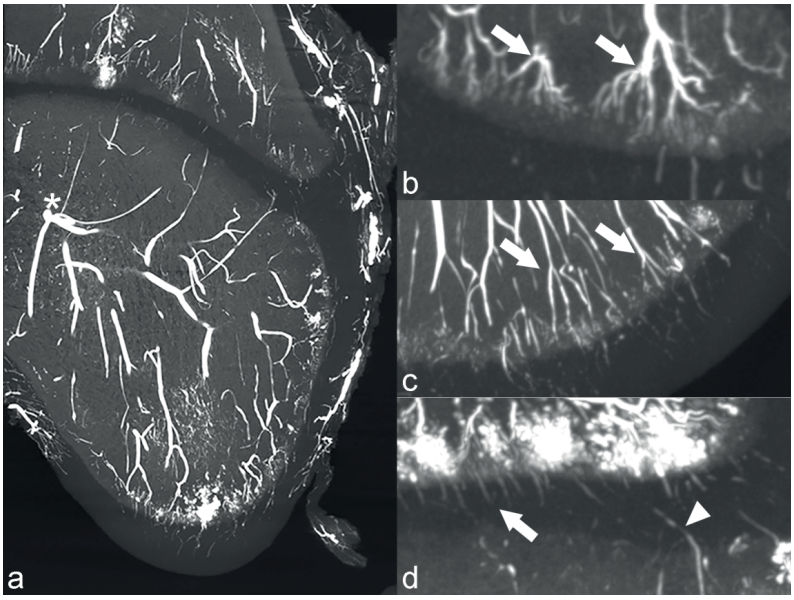
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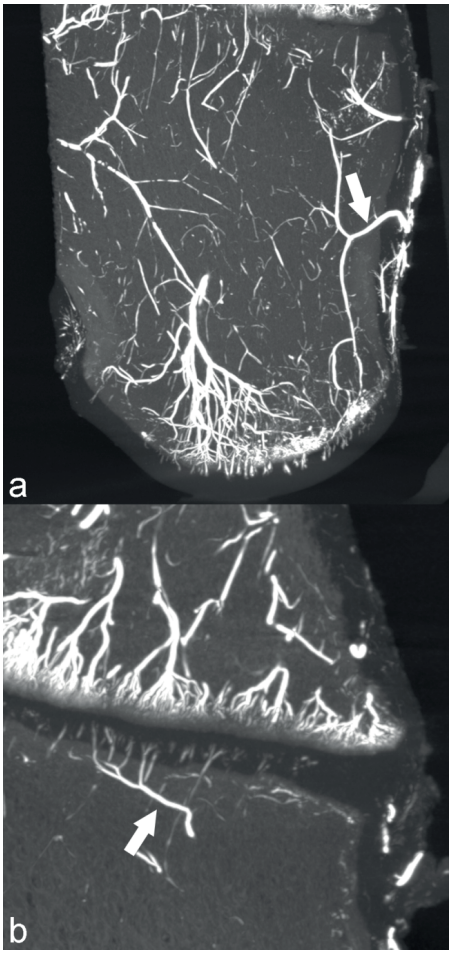
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IV

VETERINARY SURGERY

Presence of dye in growth cartilage canals following intraarticular injection, regional intravenous and intraosseous perfusion in an ex vivo porcine model

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Manuscript ID	VSU-18-306.R1
Manuscript Type:	Original Article - Research
Keywords:	regional perfusion, growth cartilage, osteochondrosis, septic arthritis/osteomyelitis, cartilage canals

1 **Running head: Local delivery of dye to growth cartilage canals**

2 **Article title: Presence of dye in growth cartilage canals following**

3 **intraarticular injection, regional intravenous and intraosseous perfusion in**

4 **an ex vivo porcine model**

5

Abstract

Objective

To compare dye distribution in growth cartilage canals between three techniques of local administration to the tibiotarsal region in pigs.

Study design

Experimental study.

Animals

Nine 52-71 day-old piglets for the post mortem part and two 112 day-old piglets for the in vivo part.

Methods

Different-colored dyes were administered by intraarticular injection into the tibiotarsal joint and by intraosseous and intravenous regional perfusion of the tibia. Presence of dye was evaluated macroscopically in intact and sectioned bones, and histologically in sections from the distal intermediate ridge of the tibia and the talus.

Results

In the post mortem group, dye was found in 134/230 (58.3%) cartilage canals after intravenous perfusion, 20/232 (8.6%) canals after intraosseous perfusion and 6/462 (1.3%) canals after intraarticular injection. Intravenous regional perfusion resulted in the best dye distribution in bone, perichondrium and subcutaneous tissue. In the joint cavity, only intraarticularly injected dye was present. Similar

distribution patterns were found in the in vivo group, supporting feasibility of the method for use in live animals.

Conclusions

Of the examined techniques, regional intravenous perfusion was the best method for delivery of dye to cartilage canals, subchondral bone and soft tissues.

Clinical Impact

Bacteria may infect and occlude growth cartilage canals, leading to vascular failure with the potential for infarction of cartilage and osteochondral lesions. Intravenous regional perfusion of antibiotics could have a benefit when treating septic arthritis/osteomyelitis in skeletally immature animals, potentially reducing the risk of osteochondral lesions as a long term sequela.

Introduction

Growth cartilage is found in the metaphyseal growth plate or physis, and between the articular cartilage and the secondary, epiphyseal centre of ossification.[1] The articular cartilage and epiphyseal growth cartilage are collectively known as the articular-epiphyseal cartilage complex (AECC).[2] Growth cartilage has a temporary blood supply that runs within cartilage canals.[3] The canals are blind-ending, but the circulation within them is continuous and organised as anatomical end arteries.[4] The canals are present during the early phases of growth, and gradually disappear by being incorporated into bone, and by being converted into cartilage/chondrification.[3]

Septic arthritis and osteomyelitis of hematogenous origin are commonly seen in skeletally immature individuals of many domestic species.[5-8] Relapse of clinical signs such as lameness and synovial effusion is frequent despite aggressive treatment, possibly due to multiple bacterial foci not always very accessible to the immune system.[5,9] Bacteria located in growth cartilage canals have been found in both experimental and naturally occurring septic arthritis/osteomyelitis in species such as pigs and horses.[9,10] Bacterial infection of cartilage canals was associated with inflammation, occlusion and, ultimately, necrosis of the infected canal and its contents, resulting in focal failure of the blood supply/circulation.[9,11] Prevalence of osteochondral lesions was higher in a group of horses that survived bacterial infections as foals, when they were compared to controls at screening age approximately 1-2 years, implying that some lesions were due to bacteria rather than heritable predisposition.[12]

Therefore, it seems important that treatment of septic arthritis/osteomyelitis in skeletally immature animals should include methods with therapeutic effect in cartilage canals. In equine practice, treatment of septic arthritis/osteomyelitis commonly involves joint lavage, debridement of infected tissue, systemic antibiotics and anti-inflammatories.[5,13] Additional techniques, such as intra-articular injection of antibiotics, or regional intravenous or intraosseous antibiotic perfusion, are also frequently used to achieve prolonged minimum inhibitory concentration in infected tissues.[14,15] However, there is little evidence regarding the distribution of therapeutic agents into growth cartilage canals.[15] As an initial step in investigating this, the current study was undertaken to evaluate techniques for local administration of dye, comparing dye distribution in growth cartilage canals between techniques. Pigs were used as a model species in this initial study due to similarities in vascular anatomy and developmental pathology.[3,8,13]

The aim of the study was to compare dye distribution in growth cartilage canals between intraarticular injection, regional intraosseous and intravenous perfusion in the distal tibia and talus of piglets. The hypothesis was that more canals would contain dye following regional perfusion than intraarticular injection, and that more canals would contain dye following intravenous than intraosseous perfusion.

Materials and Methods

The study included 11 pigs, with nine pigs in one group and two pigs in a second group. The reason for the number of pigs was that all pigs originated from other, ethically approved studies. The first nine pigs originated from a terminal anesthetic study at the XXX University of XXX approved by the XXX National Animal Research Authority (approval XXX), and was referred to as the post mortem group. The last two pigs originated from an in vivo physiologic study at the XXX (project license: XXX), and was referred to as the in vivo group. The post mortem group comprised five intact male and four female, clinically healthy Landrace pigs with mean age 65 days (range: 52-71), and mean body weight 25.8 kg (range: 20-31). The in vivo group consisted of two clinically healthy, Landrace female pigs that were both 112 days old and weighed 65 and 67.5 kg, respectively. The study aim was predominantly answered in the post mortem group, whereas the purpose of including the in vivo group was to evaluate technical feasibility of the experimental procedure in pigs with a functional circulation (see below).

Both hind limbs were utilized from every included pig. The post mortem group was placed under general anesthesia for the original experiment (XXX, personal communication), which terminated with euthanasia by exsanguination. Both hind limbs were removed by disarticulation of the hip joint immediately after euthanasia. A large-bore catheter was placed in the femoral artery, and 500 IU/kg limb weight of heparin was administered into the catheter, followed by 0.3 mg/kg total bwt of 2 % lidocaine hydrochloride and finally 3 ml/kg of 4.5 % sodium

nitroprusside, for anticoagulation and vasodilation. The femoral artery was then flushed with 5-8 liters of body temperature saline until the venous drainage was free from traces of blood. A latex tourniquet was placed at mid-height of the tibia, and remained in place for the duration of the experiment, ~20 minutes.

The in vivo group was placed under general anesthesia for the original experiment (XXX, personal communication). The pigs were pre-medicated with ketamine (2 mg/kg) and midazolam (0.5 mg/kg) by intramuscular injection, and anesthesia was induced with propofol (2 mg/kg) intravenously and maintained with sevoflurane and continuous rate intravenous infusion of fentanyl (0.2 g/kg/min). Upon completion of the experiment, a tourniquet was placed at mid-height of the tibia. The tourniquet remained in place until the dye procedures were finished and the pig was euthanized by an overdose of barbiturates ~20 minutes later. The in vivo group did not receive any anticoagulant or vasodilatory medications.

The dye procedures were identical for both groups of pigs. For intraarticular injection a volume of 2.5 ml black tissue marking dye (The Davidson Marking System, Bradley Products, Bloomington, MN, USA), diluted to 5 ml total volume with saline, was injected into the tibio-tarsal joint of each left and right hind limb using a 20G needle and a dorsomedial approach. For intraosseous perfusion a custom-made, cannulated 4.5 mm cortical screw was placed in the dorsomedial aspect of one tibia, in the distal part of the diaphysis. Blue tissue marking dye (as before), diluted to 10 % with saline, was injected intraosseously. For intravenous perfusion a 23G butterfly catheter was placed in the saphenous vein in the distal third of the tibia of the contra-lateral hind limb to the intraosseous perfusion, and

10 % green dye (as before) was injected intravenously. A volume of 1 ml/kg bwt dye solution was used for both regional perfusion techniques.

Disarticulated limbs were covered with a plastic drape and kept at room temperature (20-22 °C) for 16 hours after the injection procedures to allow the dyes time to dry and fix. The distal tibia and talus were dissected free from soft tissue and fixed in 4 % phosphate-buffered formaldehyde for 48 hours. The distal tibia was sectioned into two halves that were 10-14 mm thick, in a slightly oblique parasagittal plane parallel with the distal intermediate ridge (Figure 1). The talus was sectioned into 6-8 mm thick slabs in the transverse plane (Figure 2).

Intact bones and slabs were inspected and photographed for macroscopic evaluation of dye distribution. Both halves of the tibia, and the two most distal slabs from the talus were inspected, i.e. four halves/slabs from the left and right limbs were inspected, totaling eight halves/slabs per pig. Presence of dye was recorded separately for epiphyseal, metaphyseal and diaphyseal bone within tibial slabs, and collectively for all bone in talar slabs. Dye presence was recorded as grade 0: no dye, grade 1: small foci, grade 2: large foci and grade 3: generalized distribution (Figure 1).

Slabs were decalcified in 10 % ethylenediaminetetraacetic acid (EDTA). A slab including the intermediate ridge was taken from the distal tibia and the most distal slab from the talus were selected for further processing. The slabs were trimmed to fit into cassettes and paraffin-embedded, and 5 µm-thick sections were prepared and stained with hematoxylin and eosin for histology.

One section was evaluated histologically from the tibia and talus of the left and right limbs of the post mortem group, i.e. four sections times nine pigs, totaling 36 evaluated sections. Because the in vivo pigs were larger, two sections were evaluated histologically from each site, i.e. eight sections times two pigs, totaling 16 evaluated sections. Presence of dye was recorded separately for cartilage canals in the AECC, the physis and the perichondral vasculature, as well as for epiphyseal and metaphyseal subchondral bone (the diaphysis was not included in sections due to cassette size limitations).

Data were analyzed using commercially available software (JMP, Cary, North Carolina, USA). Presence of dye in cartilage canals was compared between intravenous and intraosseous regional perfusion using the chi-square test and a significance level of $p \leq 0.05$.

Results

Intraarticularly administered dye stained all articular and synovial surfaces uniformly black in all pigs, but was not observed macroscopically in bone or perichondrium.

During intraosseous perfusion of the post mortem group, some blue dye leaked out of the femoral vein proximally. Macroscopically, the blue dye was widely and evenly distributed around the injection site in the distal tibial diaphysis (Table 1). Intraosseously injected dye was not observed in the perichondrium, joint cavity, soft tissues or skin of any piglet. Three limbs had small foci of blue dye in the epiphyseal bone (Figures 1A, B), while the epiphyses of the other six limbs contained no observable blue dye.

Intravenous perfusion consistently resulted in widespread distribution of green dye throughout the soft tissues of the limb distal to the tourniquet (Figure 3). Macroscopically, intravenously injected green dye was present within the perichondrium, but not the joint cavity of any limb. Green dye was widely distributed in all parts of the inspected bones, including the talus (Figures 1C, D, Table 1). The metaphyses of the tibia contained subjectively less dye than the other bone areas.

Macroscopic examination yielded similar results in the in vivo group as for the post mortem group.

There were 36 histological sections from the post mortem group and 16 from the in vivo group. The sections from the post mortem group contained 462 cartilage

canals, distributed as 235 canals in the tibial AECC, 122 canals in the tibial physis and 105 canals in the talus (Table 2). The sections from the in vivo group contained 42 cartilage canals, distributed as nine canals in the tibial AECC, 32 canals in the tibial physis and one canal in the talus (Table 3).

Intraarticularly injected black dye was present in 6/462 (1.3%) examined cartilage canals (Figure 4A) of the post mortem group. Black dye was also present in perichondral vessels in 3/36 sections and within bone in 6/36 sections, usually in small, confined foci. Intraarticularly administered dye was not observed in cartilage canals, perichondrium or bone of the in vivo group.

Intraosseously injected blue dye was present in a low number of cartilage canals (Figure 4B and D, Table 2) and in perichondral vessels in 2/18 sections from the post mortem group. Intraosseous dye was not observed in the perichondrium of the post mortem group. Small traces of dye were observed in the tibial metaphysis of all sections and the epiphysis of four of the sections, while blue dye was not observed in any talus. In the in vivo group, a low number of cartilage canals contained intraosseously injected dye (Table 3). Dye was not observed in perichondral vessels or epiphyses in the in vivo group, while all tibial metaphyses contained small traces of dye.

Intravenously injected green dye was present in more than half of cartilage canals in the post mortem group (Figures 4A, C and E, Table 2) and in perichondral vessels in all 18 sections (Figure 5A) and. Green dye was located both in the lumen of vessels and in the extravascular space (Figure 4C). Intravenously injected dye was widely and evenly dispersed within bone in all 18 histological

sections, both in vessels and outside the circulation (Figures 5B and C). Intravenously injected dye was present in more than half the canals in the in vivo group, and was abundant in the perichondrium of all examined sections. Dye was also evenly dispersed within bone similar to, but in smaller amounts than for the post mortem group.

Discussion

The hypothesis was confirmed, regional intravenous perfusion performed better than intraosseous perfusion and intraarticular injection in the observed parameters. The two in vivo subjects supported feasibility of the method for use in live subjects.

Intravenous perfusion resulted in the best distribution of dye to cartilage canals. Based on anatomy, the reason for this is most likely that the dye is contained within the saphenous vein by the tourniquet, and that pressure builds up, forcing retrograde flow of dye into the veins, capillaries and interstitium of the cartilage canals.[15] The proportion of the epiphyseal blood supply that is of perichondral origin has not been quantified, but studies indicate that it is substantial.[16,17] Some of the vessels entering the epiphysis from the perichondrium branch off, enter, and even cross the physis as transphyseal vessels.[17] The perichondral part of the epiphyseal venous drainage appears to constitute the main route for flow of fluid in intravenous perfusion. The intravenously injected dye was most likely distributed from perichondral vessels via the epiphysis where vessels branch off towards the AECC and the physis, terminating inside cartilage canals as described in other studies.[17,18] According to a study in rats, the part of the vasculature nurturing the physis from the epiphyseal side has a greater proportion of veins than the metaphyseal-side vasculature that consists of mainly arterioles.[19] This further explains why intravenous injection may result in wider distribution to cartilage canals also in the physis, as the epiphyseal-side vessels entering the physis are tributaries to the vein where the injection is made.[9,20]

Although intravenous perfusion had wider distribution to cartilage canals, it is important to be aware that in hematogenous septic arthritis/osteomyelitis, some vessels in infected cartilage canals are probably rendered non-functional early in the infectious process.[9] In severe cases, continuous seeding of bacteria may increase the number of affected canals. Nevertheless, the outcome of initiated vascular failure is always a result of the balance between lesions resolving and lesions persisting.[21,22] Resolution of lesions partly depends on proliferation of intact, adjacent vessels, [23,24] and it therefore remains important that administered substances are distributed to intact cartilage canals even if the infected canals themselves are non-functional. By limiting infection of adjacent vessels, intravenous perfusion can potentially encourage repair and possibly limit osteochondral lesions as a long term complication.[9,12]

The perichondral vessels also allow dye to spread into the epiphyseal bone and cross the physis into the metaphysis via transphyseal vessels (Fig. 4E). The close association between septic arthritis and osteomyelitis is due to the hematogenous nature of the disease in foals.[9,25] Injection of lipopolysaccharides into the diaphysis of the third metacarpal bone of foals resulted in septic arthritis of the metacarpophalangeal joint.[26] Septic arthritis can therefore just as well be the result of osteomyelitis as the other way around. Radiography is the most commonly employed diagnostic imaging modality in cases of septic arthritis/osteomyelitis, but has low sensitivity for the early stages of osteomyelitis[27]. Intravenous regional perfusion of antibiotics should therefore be considered even if radiographs are normal.

The main aim of the current study was to determine distribution of dye into cartilage canals, but intravenous perfusion also resulted in the most widespread distribution in soft tissue. Again, this is most likely due to high pressure obtained by occluding the saphenous vein. In an ultrastructural study, the high pressure obtained with intravenous regional perfusion was found to dilate veins and reduce contact between endothelial cells.[28] This resulted in formation of small gaps in the vessel wall, which enhanced filtration and diffusion of molecules into the interstitium.[28] Vascular fenestrations are known from a number of anatomic structures,[29] and both existing gaps and those created because of high pressure will allow more injected fluid to escape the circulation and disperse in the tissue.

Dye administered by intraosseous perfusion was observed in a low proportion of cartilage canals. The anatomy of the medulla implies that injected dye was forced by the achieved pressure into bone veins and then retrograde into cartilage canal veins. The inferior canal distribution is probably partly explained by intraosseous venous outflow in the proximal part of long bones.[30] A tourniquet placed at the mid-level of a long bone would not close off veins in the proximal region of the bone, and injected substances will potentially leak out into the central venous system, as observed from the femoral vein during intraosseous perfusion in the present study. At high pressure, injected contrast entered the medullary venous system in rabbit femurs and exited through veins in the proximal end of the bone.[31] Proximal leakage of fluid potentially also prevents sufficient pressure build-up for retrograde flow to occur into the epiphysis. A more proximal placement of the tourniquet might increase pressure, but the presence of the

282 physis in skeletally immature animals could act as a barrier to distribution to the
283 epiphysis. The situation is different in mature animals, where the lack of a physis
284 presumably allows distribution also to the epiphysis. This is supported by two
285 studies of intraosseous perfusion in mature horses where fluid was detected also
286 around the epiphysis.[32,33] The inferior distribution of intraosseously injected dye
287 within bone and cartilage canals means there may be reason to question the
288 usefulness of this technique for targeted drug delivery in cases of osteomyelitis in
289 bones where a physis is present.

290 Intraarticularly administered dye was found on the cartilage surface and
291 synovium, but was only found in a small proportion of cartilage canals. From the
292 synovial cavity, dye has to cross into capsular veins to enter perichondral veins
293 and cartilage canals. A small proportion of dye entered bone, agreeing with the
294 observation that direct injection of antibiotics into the joint resulted in high
295 concentrations in synovial fluid[34,35] and bone[36] in mature horses. The current
296 results suggest that intraarticularly administered dye does not enter cartilage
297 canals or perichondral vasculature to any great extent, although it obviously will
298 be found in the injected joint.

299 The current method of dye perfusion does not provide any information on the
300 potential tissue concentration of antibiotics when perfused in the same manner.
301 Antibiotics vary in tissue distribution, mechanism and effect on different
302 bacteria.[37] However, this information can likely be extrapolated from studies on
303 adult horses and applied to the treatment of foals. The important thing is to deliver
304 antibiotics as close to the lesions as possible, which was the main aim in this

study. As septic arthritis is common in horses, most studies use maximum concentration of antibiotics in synovial fluid as a measure of success the procedure.[15] The distribution of injected fluid has been evaluated only in a few studies, with contrast studies being part of some them.[38,39] However, perfusion of dye has also been used[38], and was chosen as method in this study because it was necessary to validate presence of dye within cartilage canals by histological examination. None of the other mentioned methods would be suitable for this objective.

Another limitation of this study was the use of clinically healthy pigs post mortem. The results from the in vivo group supported the feasibility of the method for use in live animals, and tentatively supported the results from the post mortem group. An in vivo study would have provided stronger evidence, but because this was an initial study, use of post mortem material was considered more ethical. The impact of septic arthritis/osteomyelitis on the circulation in and close to lesions is not very well described, although some information exists in pigs.[11] This is an area for future research. The research into the pathogenesis of osteochondrosis has demonstrated many similarities in the temporary vasculature in the growth cartilage of pigs and horses.[3,23,40,41] The use of pigs as a model in this study was therefore warranted. Future studies with either foals, live animals, animals with relevant infections, medications instead of dye, or a combination of these, will be necessary to confirm the findings of the current study.

The results of this study indicate that regional intravenous perfusion is the best method for delivery of an injected substance such as dye to cartilage canals,

perichondrium and bone. This suggests that in a clinical setting, where septic arthritis/osteomyelitis often manifests in multiple foci, a combination of intraarticular injection and regional intravenous perfusion will probably provide distribution to all commonly affected tissues.

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Conflict of interest

The authors declare no conflicts of interest.

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447

448

449 **Figure Legends**

450 **FIGURE 1** Distribution of dye was evaluated macroscopically and registered as
451 **A,** grade 0: no dye, **B,** grade 1: small foci, **C,** grade 2: large foci and **D,** grade 3:
452 generalized distribution. All images are from the distal tibia, cranial to the left in
453 all images. Images A-B: intraosseous injected dye. C-D: intravenous injected dye.

454 **FIGURE 2** Transverse sectioning of pig talus along the stippled lines. Lateral to
455 the left.

456 **FIGURE 3** Post mortem group pigs, skin removed, lateral view. **A,** There is
457 widespread distribution of green dye in subcutaneous tissue following intravenous
458 perfusion. **B,** In comparison, there is no blue dye in subcutaneous tissue following
459 intraosseous perfusion.

460 **FIGURE 4** All images from the post mortem group, hematoxylin and eosin. **A,**
461 Presence of black, intraarticularly injected dye and green, intravenously perfused
462 dye within a cartilage canal in the articular-epiphyseal cartilage complex (AECC)
463 of the talus. 400x magnification, 30 µm scale bar. **B,** Presence of blue,
464 intraosseously perfused dye within a cartilage canal in the AECC of the talus.
465 400x magnification, 30 µm scale bar. **C,** Green dye is visible in perivascular
466 mesenchyme (arrowheads) and the lumen of venules in a cartilage canal in the
467 AECC of the talus following intravenous perfusion. **D,** Small amounts of blue
468 dye, intraosseously perfused dye present in a narrow cartilage canal in the distal
469 tibial physis (arrowhead). The neighboring canals is empty. 100x magnification,
470 100 µm scale bar. **E,** Green dye is visible in a narrow cartilage canal in the distal

471 tibial physis following intravenous perfusion. 100x magnification, 100 µm scale
472 bar.

473 **FIGURE 5** All images hematoxylin and eosin. **A,** Green dye is visible within a
474 vessel in the perichondrium of the distal intermediate ridge of the tibia following
475 intravenous perfusion of this post mortem group pig. Black, intraarticularly
476 injected dye is also visible on the articular surface. 100x magnification, 100 µm
477 scale bar. **B,** Green dye (arrowheads) is visible in a cartilage canal and in the
478 subchondral bone of the talus following intravenous perfusion of this post mortem
479 group pig. 100x magnification, 100 µm scale bar. **C,** Green dye is visible in the
480 bone marrow of the distal tibial metaphysis of this in vivo group pig following
481 intravenous perfusion. 400x magnification, 30 µm scale bar.

482

Table 1. Macroscopic distribution of dye in bone after regional intraosseous and intravenous post mortem perfusion in nine piglets.

Bone	Tibia						Talus	
Region	Epiphysis		Metaphysis		Diaphysis		Epiphysis	
Technique	IO	IV	IO	IV	IO	IV	IO	IV
Grade 0* (n)	6	0	2	2	0	0	9	0
Grade 1 (n)	3	0	6	3	1	1	0	1
Grade 2 (n)	0	4	1	4	2	4	0	3
Grade 3 (n)	0	5	0	0	6	4	0	5
Total	9	9	9	9	9	9	9	9

IO, intraosseous; IV, intravenous

* Grading system described in Methods text and Figure 1.

483

484

Table 2. Presence of dye in cartilage canals following regional intravenous and intraosseous perfusion post mortem in nine piglets.

		Cartilage canals containing dye		
Location	Cartilage	Intravenous	Intraosseous	p-value*
Talus	AECC[b]	40/54 (74.1%)	5/51 (9.8%)	<0.001
DIRT[c]	AECC	61/116 (52.6%)	6/119 (5.0%)	<0.001
	Physis	33/60 (55.0%)	9/62 (14.5%)	<0.001
Total		134/230 (58.3%)	20/232 (8.6%)	<0.001

AECC, articular-epiphyseal cartilage complex; DIRT, distal intermediate ridge of the tibia

* Chi-square intravenous vs. intraosseous

485

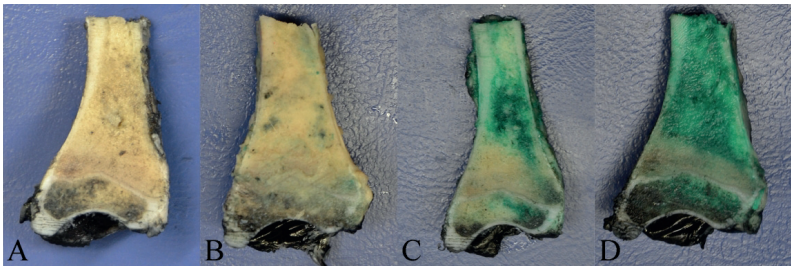
486

Table 3. Presence of dye in cartilage canals following regional intravenous and intraosseous perfusion in vivo in two piglets.

		Cartilage canals containing dye	
Location	Cartilage	Intravenously	Intraosseously
Talus	AECC	1/1 (100%)	0/0
DIRT	AECC	5/8 (62.5%)	1/1 (100%)
	Physis	6/10 (60.0%)	1/22 (4.5%)
Total		12/19 (63.2%)	2/23 (8.7%)

AECC, articular-epiphyseal cartilage complex; DIRT, distal intermediate ridge of the tibia

487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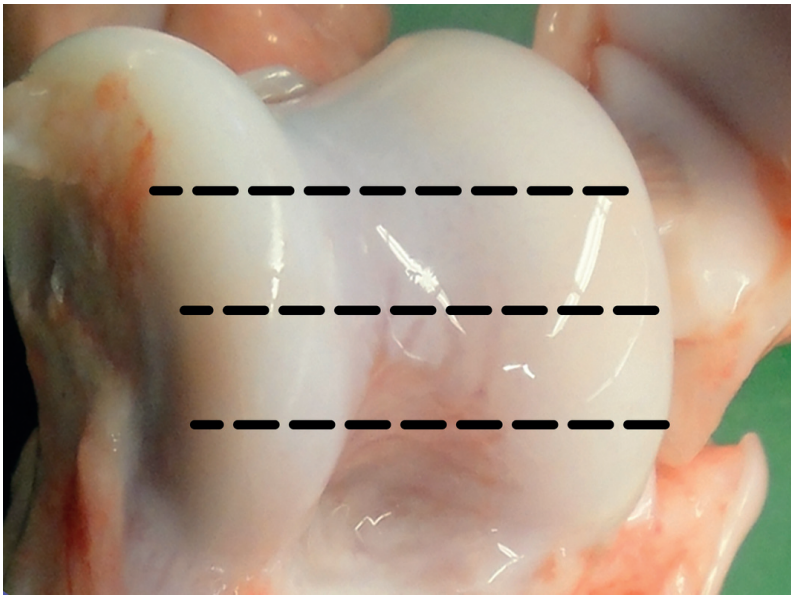

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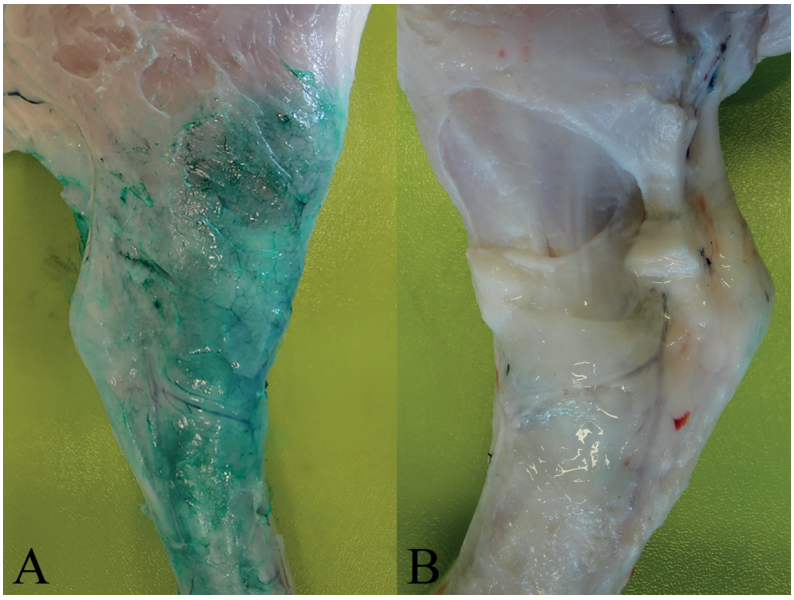

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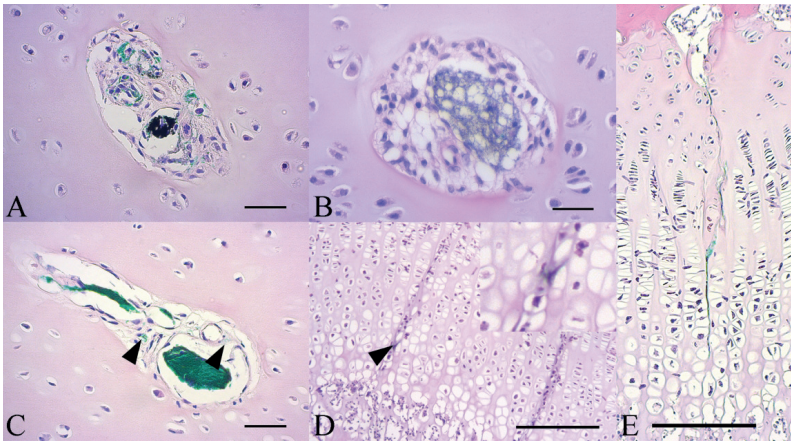
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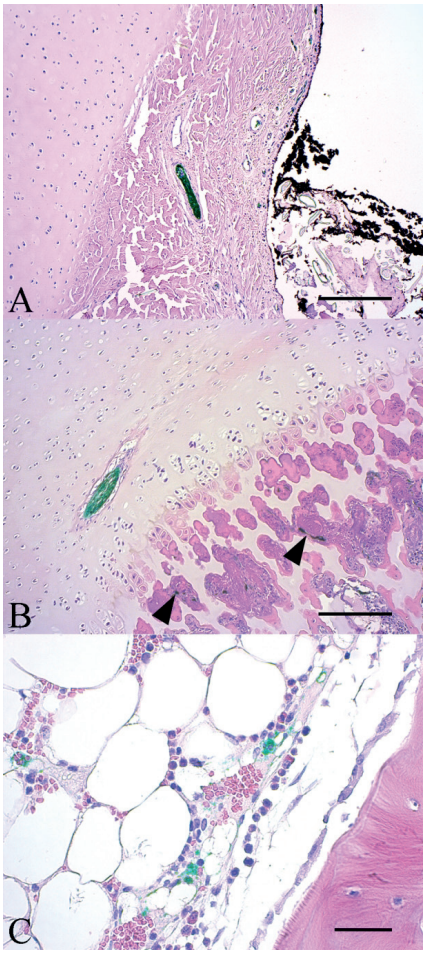

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