Intrafascicular chondroid-like bodies in the ageing equine superficial digital flexor tendon comprise glycosaminoglycans and type II collagen

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Abstract
The superficial digital flexor tendon (SDFT) is considered functionally equivalent to the human Achilles tendon. Circular chondroid depositions scattered amongst the fascicles of the equine SDFT are rarely reported. The purpose of this study was the detailed characterization of intrafascicular chondroid-like bodies (ICBs) in the equine SDFT, and the assessment of the effect of ageing on the presence and distribution of these structures. Ultrahigh field magnetic resonance imaging (9.4T) series of SDFT samples of young (1–9 years) and aged (17–25 years) horses were obtained, and three-dimensional reconstruction of ICBs was performed. Morphological evaluation of the ICBs included histology, immunohistochemistry and transmission electron microscopy. The number, size, and position of ICBs was determined and compared between age groups. There was a significant difference \((p = .008)\) in the ICB count between young and old horses with ICBs present in varying number (13–467; median = 47, mean = 132.6), size and distribution in the SDFT of aged horses only. There were significantly more ICBs in the tendon periphery when compared with the tendon core region \((p = .010)\). Histological characterization identified distinctive cells associated with increased glycosaminoglycan and type II collagen extracellular matrix content. Ageing and repetitive strain frequently cause tendon micro-damage before the development of clinical tendinopathy. Documentation of the presence and distribution of ICBs is a first step towards improving our understanding of the impact of these structures on the viscoelastic properties, and ultimately their effect on the risk of age-related tendinopathy in energy-storing tendons.

KEYWORDS
ageing, fascicle, inclusion body, interfascicular matrix, superficial digital flexor tendon

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1 | INTRODUCTION

The equine superficial digital flexor tendon (SDFT) provides positional stability during the stance and swing phase of the stride and acts as the main energy-storing structure of the equine distal limb during locomotion.1 SDFT injury is one of the most common causes of early retirement and wastage in performance horses and has a significant impact on equine welfare and the horse racing industry.2 As the equine SDFT is considered functionally equivalent to the human Achilles tendon it serves as an established model for human tendinopathy.3 The incidence of human Achilles tendon injury continues to increase in the ageing population of developed countries and is associated with chronic pain and restricted mobility.4,5

The SDFT and the human Achilles tendon are both prone to injury as they work close to their functional limit under physiological loading conditions.6,7 These tendons are energy-storing structures that act like springs and are considerably more elastic when compared with positional tendons such as the equine common digital extensor or the human anterior tibialis tendon.8,9 Characteristic features involved in the distinct biomechanics of energy-storing tendons are the interfascicular sliding capacity, as well as the crimp pattern and helical recoil mechanism of the tendon fascicles.8,10

Major risk factors for the development of tendinopathy are increasing age and exercise induced tendon overload.11,14 The specific influence of repetitive load and ageing on the tendon ultrastructure and the mechanisms of energy storage and return have been studied in detail in the equine SDFT model.11,13 Ageing results in the alteration of the tendon matrix composition and homeostasis and cell phenotypic variations have been recognized.13,17 It has additionally been demonstrated that the tendon core region is particularly affected by age-related micro-damage.13–16

The development of chondroid cell differentiation is commonly attributed to chronic overload or injury and has been described in multiple tendons and ligaments including the SDFT and the Achilles tendon.20–24 However, the presence of isolated circular chondroid depositions scattered amongst the tendon fascicular structure as an effect of ageing to date has been rarely reported.29,30 Based on this observation, the aim of this study was to further characterize the location, frequency and age-related appearance of interfascicular chondroid-like bodies (ICBs) as detected histologically and with ultrahigh field magnetic resonance imaging (MRI) in the equine SDFT. The distribution of chondroid depositions was assessed in horses of different age groups with the hypothesis that ICBs are predominantly found in the tendon core region of aged individuals.

2 | METHODS

2.1 | Samples

The study was conducted using SDFT samples harvested from equine cadaver limbs. Randomly selected left or right equine distal forelimbs were collected from a commercial equine abattoir or at a University teaching hospital from horses that were euthanized for reasons other than orthopedic disease or injury. Informed owner consent for tissue retention was obtained and ethical approval for the study was given by the local institutional veterinary research ethics committee (VREC214).

The collected limbs included those from young (1–9 years) and old (17–25 years) Thoroughbred, Thoroughbred Cross and Irish Sport Horses (Supporting information, S1). Horses with evidence of flexor tendon pathology were excluded from the study. Post mortem, the anonymised SDFT samples were dissected free from the surrounding soft tissues with the epitenon left intact. The tendons were excised at the level of the carpo-metacarpal- and the metacarpo-phalangeal joint, wrapped in tissue paper dampened with phosphate-buffered saline and then tin foil. Samples were subsequently stored at −20°C.

2.2 | Histological study

For histological examination, 1 cm tissue specimens were harvested from the proximal, middle, and distal metacarpal region of the SDFT. Specimens were transected in the sagittal plane at the widest part of the tendon to collect samples containing peripheral and core tendon tissue including the dorsal and palmar epitenon. Samples were fixed in 4% phosphate-buffered formaldehyde solution for 48 h, paraffin-embedded longitudinally, and 5 μm sections were mounted on poly-L-lysine coated slides (VWR International Ltd.).

Histological staining included haematoxylin and eosin (H&E) for all tissue sections and the specific stains Toluidine blue, Safranin-O, Alcian blue—Periodic Acid Schiff (PAS), and the modified Von Kossa’s stain for samples containing ICBs (TCS Bioscience Ltd.; Supporting information, S2).31–33 Sections were viewed and imaged (×100 and ×400) using an Eclipse 80i microscope equipped with a Nikon DS 5mc digital camera 1600 × 1200 pixels (Nikon). In the H&E (×400) sections the total width and length (μm) of each ICB, length of the ICB nuclei, width and length of ICB clusters, and number of ICBs in each cluster were determined with the ImageJ Analyze tool (version 1.49, Rasband W.S., National Institute of HealthD) and recorded.34

Immunohistochemical staining for aggrecan, biglycan, decorin, chondroitin-4- and chondroitin-6-sulfate, and collagen II was performed on deparaffinised tissue sections with ICBs.31,35 The immunohistochemistry procedure and antibody details are provided in the Supporting information (S2).

2.3 | Transmission electron microscopy

A large ICB was localized on a fixed, unstained longitudinal section using light microscopy and prepared for transmission electron microscopy. The section was deparaffinised and rehydrated before being fixed in 2.5% buffered glutaraldehyde, post-fixed in 1% osmium tetroxide (Sigma-Aldrich), “en bloc” stained with uranyl acetate (Agar Scientific Ltd.), dehydrated in an ascending series of ethanol and transferred into 100% acetone. The sample was subsequently immersed in an ascending series of epoxy resin (Agar Scientific Ltd.) and acetone solutions to 100% epoxy.
resin. A BEEM capsule (TAAB Laboratories Equipment Ltd.) containing partially polymerized epoxy resin was inverted over the area containing the ICB and the section was fully polymerised at 600°C. To remove the BEEM capsule the section was immersed into liquid nitrogen. Ultrathin sections (60 nm) of the area containing the ICB were cut using a Reichert-Jung Ultratut ultramicrotome (Leica Microsystems Ltd.), placed on copper grids, stained with lead citrate (BDH Laboratory Supplies) and viewed with a Philips EM 208S Transmission Electron Microscope at 80 kV (Philips).31

2.4 | Ultrahigh field MRI study

For 9.4T MR imaging (Bruker Biospec 90/20 USR) tendon samples were thawed at room temperature, attached to a triangular plastic rig and immersed in perfluoropolyether (Fomblin PFPE, Solvay Solexis). The sample tube was subsequently placed in a ¹H quadrature radio-frequency coil (27 mm i.d.; Pulse Tec Ltd.) and the three-dimensional (3D)-FISP (fast imaging with steady-state free precession) gradient echo sequence was used to acquire T2* weighted transverse images with the following scan parameters: field of view = 25 mm × 25 mm × 25 mm, matrix size = 512 × 453, slices = 64, slice thickness = 0.4 mm, echo time (TE) = 4.165 ms, repetition time (TR) = 10.000 ms, average = 7, flip angle = 15°, total scan time = 42 min, and 9 s (Figure 4B). A total of seven overlapping transverse 3D-FISP scans were obtained for each tendon sample, to gather consecutive image data over a minimum tendon length of 14.4 cm (360 slices).36

Following data acquisition overlapping slices were removed and DICOM images were concatenated and registered using the ImageJ StackReg tool (version 1.52a, Rasband W.S., National Institute of Health).36 For segmentation and 3D reconstruction, files were converted into mrc Z-stacks using the IMOD “tif2mrc” command and the Z-scale was set based on image magnification and slice thickness. The 3dmod sculpt, warp and meshing tools (version 4.9, University of Colorado) were utilized to create a 3D model of the ICBs and the SDFT outline. ICBs were reconstructed in different colors to be readily distinguished.

The number, size and position of ICBs was recorded for the proximal, middle and distal tendon samples. ICBs were categorized as small (0.1–1.4 mm), medium (1.5–2.5 mm), or large (>2.5 mm) in size, depending on their width. The width was determined by using the 3dmod measuring tool that was calibrated based on the set size of the triangular sample rig (1 × 1 × 1 cm). In addition, ICB outlines located within 2 mm distance of the SDFT epitenon were categorized as peripheral, whilst the remaining ICBs were classified as being located in the tendon core region.22

2.5 | Data analysis

Data were recorded in Excel (version 16.25, Microsoft Inc.) and analyzed in SPSS (version 25.0, IBM). Descriptive analysis was performed, and data were assessed for normal distribution with the Kolmogorov–Smirnova and the Shapiro–Wilks test. The Mann–Whitney U test was used to analyse the difference in total ICB number between specimens from young and old horses, as detected on Ultrahigh field MRI. The difference in the ICB count between the proximal, middle, and distal metacarpal tendon was assessed using the Kruskal–Wallis test and the difference between the core and peripheral tendon regions was determined with the Wilcoxon signed-rank test. The distribution of small, medium and large ICBs was assessed with Friedman’s Two-Way Analysis of Variance by Ranks. p values less than .05 were considered to be statistically significant.

3 | RESULTS

3.1 | Histological study

Histological examination (H&E) of a proximal, middle and distal SDFT segment was performed in 17 tendon specimens obtained from young horses (1–9 years, median = 7 years) and seven specimens from old horses (17–20 years, median = 18 years). The sections were viewed systematically (×100 and ×400) and ICBs of varying size and distribution were detected in all tendon samples of the aged horse group but not in any young samples (1–9 years of age; Figures 1A,B and S-1). ICBs presented as distinct, round- to oval shaped, pale eosinophilic structures (average width = 23.35 μm; SD ± 7.58/average length = 59.06 μm; SD ± 15.18) with a granulated matrix appearance. The cells within the ICBs contained round- to elliptical basophilic nuclei (average length = 5.77 μm; SD ± 1.34) and were characterized by a round- to oval cell body and the deposition of significant amounts of pale-pink extracellular matrix. Individual as well as clustered ICBs were dispersed between the tendon collagen fibers with their longitudinal axis oriented in the vertical fiber direction (Figures 1A,B and S-1). The shape and size of ICB clusters ranged from 25 × 70 μm to more than 100 × 400 μm and large clusters contained up to 100 round- to cuboid ICBs (average width = 17.81 μm; SD ± 6.24 μm/average length = 22.76 μm; SD ± 7.58; Figures 1B and S-1B). There was no evidence for inflammatory infiltrates or collagen fiber necrosis associated with the presence of the ICBs. However, a number of tenocytes with irregular nuclei of varying size were present at the most proximal and distal extent of the larger ICBs. In addition, the tenocyte nuclei in the collagen fascicles adjacent to the ICBs were round rather than fusiform in appearance.

3.2 | Specialized stains for proteoglycan, glycosaminoglycan, and calcium

Specific staining was performed in sections that contained ICBs which included all samples of the aged horse group (n = 7; 17–20 years, median = 18 years; Figures 1C–F and S3). Toluidine blue staining confirmed the presence of cartilaginous proteoglycans and glycosaminoglycans in the extracellular matrix of the ICBs. The ICB matrix showed basic thiazine stain uptake with increased intensity.
FIGURE 1  Representative hematoxylin and eosin (H&E) and specific stains of intrafascicular chondroid-like bodies (ICBs) in longitudinal equine superficial digital flexor tendon (SDFT) sections of aged horses (17–20 years of age; scale bar = 100 μm). (A) H&E stain of the SDFT section from a 17-year-old Thoroughbred (TB) showing a single, oval-shaped ICB located between SDFT collagen fibers with the long axis of the ICB oriented parallel to the longitudinal fiber direction. Note the granulated appearance of the extracellular matrix, the basophilic nucleus and the scattered tenocytes in the extracellular matrix adjacent to the ICB (black arrow). (B) H&E longitudinal SDFT section of 17-year-old TB with multiple round-shaped ICBs clustered between the SDFT collagen fibers. Note the spherical cell nuclei and the extracellular matrix deposited between the cells. Additionally, there are irregular nuclei of varying size at the proximal extent of the large ICB cluster that are indicative of lesion expansion (black arrow). (C) Longitudinal SDFT sections obtained from a 20-year-old TB, showing a single ICB as well as clustered ICBs (inset) stained with Toluidine blue. There is positive thiazine stain uptake (violet color) in the ICB matrix close to the nucleus as well as in the extracellular matrix at the proximal and distal extent of the ICB indicating the proteoglycan and glycosaminoglycan content of the tissue. The ICB nucleus appears deep blue. Note the positive stain uptake in some of the collagen fibers and tenocytes adjacent to the ICBs (black arrow). (D) Safranin-O staining of a longitudinal SDFT section obtained from a 17-year-old TB. The cellular and the pericellular matrix of the clustered ICBs show red stain uptake indicative of the presence of proteoglycans and type II collagen, with the nuclei appearing black (inset - white arrow). (E) Alcian-blue stained SDFT sections of an 18-year-old TB. The ICB centre displays a bright Alcian blue reaction representing chondroid tissue, with a layered appearance particularly in the area adjacent to the ICB nucleus (inset – black arrow). The proximal and distal aspect of the ICB shows the Periodic Acid Schiff (PAS) magenta color suggestive of glycoprotein and proteoglycan content. (F) Modified Von Kossa’s stained SDFT sections of an 18-year-old TB with bright red stain uptake of a single ICBs and an ICB cluster (inset) showing presence of unmineralized osteoid. Scattered, black mineralized components are evident in the area of the nuclei and the peripheral cellular matrix (black arrow) [Color figure can be viewed at wileyonlinelibrary.com]
evident in the area adjacent to the nuclei. Intensive violet stain uptake was additionally seen in the extracellular matrix lining the ICBs, particularly at the proximal and distal extent. Slightly positive toluidine blue staining was also noted in the collagen fibers and tenocytes in close proximity to the ICBs (Figures 1C and S-2).

Similarly, Safranin-O and Alcian blue-PAS staining identified evidence for proteoglycan and glycosaminoglycan content of the ICBs in the equine SDFT of aged individuals. There was deep orange-to red Safranin-O stain uptake of the ICB matrix. The pericellular matrix close to the ICBs also showed red stain uptake whilst the distant extracellular matrix appeared light blue (Figures 1D and S-3). Alcian blue-PAS staining of ICB sections resulted in bright blue and pink coloring of the ICB matrix and blue staining of the adjacent extracellular matrix proximal and distal to the ICB structures. Larger ICBs showed a layered appearance with a more intense blue color in the core region when compared with the outer margin of the cellular matrix (Figures 1E and S-4).

Modified Von Kossa’s staining was performed for the detection of mineral components (calcium) associated with the ICBs. The red stain uptake of the ICBs indicates the presence of unmineralized components (basic osteoid) with a small amount of black stain highlighting calcium deposition (Figures 1F and S-5). Black stain was mainly distributed close to the nuclei but also in the marginal extracellular matrix in larger ICBs. Evidence for calcium deposition was additionally found in tendon fascicles and the extracellular matrix in close proximity to ICBs.

3.3 Immunohistochemistry and transmission electron microscopy

Immunohistochemistry was performed in sections from aged horse SDFTs that contained ICBs ($n = 7$; 17–20 years, median = 18 years) to further specify the proteins associated with the ICB structure (Figure 2). Positive immunostaining for aggrecan was present with varying intensity. There was more reaction in individual ICBs when compared with the ICB clusters. Positive aggrecan antibody stain uptake was consistently noted in the extracellular matrix and the tendon collagen surrounding the ICBs (Figure 2A). There was little evidence for the presence of significant amounts of biglycan or decorin in the ICBs. An inconsistent intra-cytoplasmatic reaction for biglycan and decorin was noted around the nuclei of some individual ICBs and there was positive immunostaining in the tendon collagen and the IFM, but not in larger ICB clusters (Figure 2B,C). There was no...
positive uptake for chondroitin-4-sulfate (2B6) within ICBs. However, the extracellular matrix adjacent to the ICBs as well as the tendon collagen fibers showed a positive chondroitin-4-sulfate (2B6) antibody reaction (Figure 2D). Individual and clustered ICBs showed a positive immunoreaction to the chondroitin-6-sulfate (3B3) antibody with uptake of varying intensity present in the neighboring extracellular matrix and collagen fibers (Figure 2E). Positive staining for type II collagen was evident with the main stain uptake noted in the tendon IFM and weak staining in the larger clustered ICBs (Figure 2F).

The ICB sample utilized for transmission electron microscopy was obtained from an 18-year-old Thoroughbred (Figure 3). The large ICB showed positive staining for Toluidine blue and Alcian blue-PAS respectively (Figures 1C; E: S-2, and S-4) and the ICB could be localized in the unstained section. The cells contained within the ICB were characterized by a spherical nucleus and a dense lamellar pattern that was occupying the majority of the cytoplasm and is indicative for the presence of a highly developed rough endoplasmatic reticulum (Figure 3A,B). The extracellular components of the ICB appeared as heterogeneous, circular layers interconnected by disorganized micro-fibrils (Figure 3C,D).

3.4 | Ultrahigh field MRI study

Ultrahigh field MRI of the SDFT was performed in specimens obtained from young (n = 5; 1–3 years, median = 3 years) and aged horses (n = 5; 18–25 years, median = 23 years). ICBs were identified as pale circular structures on gross tendon sections and appeared as circular areas of high signal intensity on the MR images (Figure 4). There was a significant difference (p = .008) in the ICB count between the groups of young and old horses with no ICBs or other structures suggestive of chondroid deposition detected in any of the SDFT samples of the young horses examined. In the aged horse group, all SDFT specimens contained multiple distinct circular areas of high signal intensity with the number of ICBs ranging from 13 to 467 (median = 47, mean = 132.6) per tendon (Table 1).

The 3D reconstruction of the ICBs confirmed the spherical- to elliptical shape of the ICBs and allowed for accurate recognition of the ICB position within the tendon (Figure 5). There was no significant difference in the number and size the of ICBs between the proximal, middle and distal aspect of the tendon (p = .50; Figure 6A) but there were significantly more ICBs in the periphery (within 2 mm distance to the epitenon) when compared with the core tendon region (p = .010; Figure 6B). The majority of ICBs were small in diameter and large ICBs were significantly less prevalent (p = .007; Table 1 and Figure 6).

4 | DISCUSSION

In this study intra-fascicular chondroid-like bodies (ICBs) were detected in varying number, size and distribution in the SDFT of aged horses using ultrahigh field MRI. Histological characterization of the
ICBs identified distinctive cells with chondroid composition and highly developed endoplasmatic reticulum, that were interspersed between the tendon collagen fibers either individually or in larger cell clusters.

ICBs were not identified in young horses aged 1–9 years in this study. Earlier reports have described the replacement of the IFM with focal chondroid metaplasia in the equine SDFT starting at the age of 5 years.\textsuperscript{29,30} Chondroid metaplasia in tendons is commonly described to be caused by injury or compressive forces leading to tissue hypoxia and subsequent chondrogenic cell differentiation.\textsuperscript{25,26}

It was therefore hypothesized that there would be more ICBs in the tendon core region where SDFT injuries most commonly occur, or at the level of the fetlock canal where compressive forces act upon the tendon tissue during weight bearing leading to a tendon matrix that

**TABLE 1**  
Spatial distribution of small (S), medium (M), and large (L) intra-fascicular chondroid-like bodies in the equine superficial digital flexor tendon of aged horses

| Sample | SDFT 1 | SDFT 2 | SDFT 3 | SDFT 4 | SDFT 5 |
|--------|--------|--------|--------|--------|--------|
| Horse age | 18 | 20 | 23 | 25 | 25 |
| No of ICBs in the proximal 1/3rd of the tendon – peripheral tendon region | S 64 | 44 | 14 | 8 | 2 |
| | M 31 | 9 | 3 | 2 | 4 |
| | L 4 | 1 | 0 | 0 | 1 |
| No of ICBs in the proximal 1/3rd of the tendon – core tendon region | S 68 | 31 | 1 | 1 | 0 |
| | M 12 | 5 | 1 | 0 | 0 |
| | L 2 | 0 | 0 | 0 | 0 |
| No of ICBs in the middle 1/3rd of the tendon – peripheral tendon region | S 66 | 2 | 11 | 4 | 2 |
| | M 22 | 1 | 2 | 0 | 1 |
| | L 2 | 0 | 1 | 0 | 0 |
| No of ICBs in the middle 1/3rd of the tendon – core tendon region | S 42 | 2 | 6 | 1 | 1 |
| | M 20 | 1 | 0 | 0 | 0 |
| | L 1 | 0 | 0 | 0 | 0 |
| No of ICBs in the distal 1/3rd of the tendon – peripheral tendon region | S 48 | 8 | 6 | 3 | 1 |
| | M 33 | 2 | 1 | 1 | 0 |
| | L 3 | 0 | 0 | 0 | 0 |
| No of ICBs in the distal 1/3rd of the tendon – core tendon region | S 25 | 3 | 1 | 6 | 0 |
| | M 23 | 0 | 0 | 1 | 0 |
| | L 1 | 0 | 0 | 0 | 1 |
| Total number of ICBs | 467 | 109 | 47 | 27 | 13 |

Abbreviations: ICB, intra-fascicular chondroid-like body; SDFT, superficial digital flexor tendon.
FIGURE 5  3D reconstruction of the superficial digital flexor tendon (SDFT) outline and the contained intrafascicular chondroid-like bodies (ICBs) that were detected in aged horses (18–25 years, median = 23 years), displayed as circular shapes in different colors. Transverse (A, C, E, G, I) and sagittal (B, D, F, H, J) views of the SDFTs of an 18-year-old Irish Sport Horse (SDFT 1) (A) and (B), 20-year-old Irish Sport Horse (SDFT 2) (C) and (D), 23-year-old Thoroughbred Cross (SDFT 3) (E) and (F), 25-year-old Thoroughbred (SDFT 4) (G) and (H) and of a 25-year-old Irish Sport Horse (SDFT 5) (I) and (J). Note the proximal distribution of ICBs that was only observed in SDFT 2 (B) [Color figure can be viewed at wileyonlinelibrary.com]
is more chondrogenic in nature. ICBs however appeared to be randomly dispersed throughout the length of the SDFT in the current study with more ICBs present in the proximal one-third in only one tendon (Figure 5D). In addition, a significantly larger number of ICBs were detected in the tendon periphery when compared to the tendon core region. A possible explanation could be that ICBs are a response to continuous inter-fascicular sliding with thinning of the IFM and resulting increase of shear forces rather than chondrogenesis due to compressive forces alone. As the IFM thickness decreases in the tendon core region, the increased strain acting upon the remaining, peripheral IFM may induce the development of ICBs.

The IFM facilitates sliding between tendon fascicles, with an increased IFM stiffness and decreased fatigue life of the IFM and tendon fascicles reported as an effect of ageing in energy-storing tendons. Age-related compositional changes of the tendon

FIGURE 6 Distribution of small, medium and large intra-fascicular chondroid-like bodies (ICBs) in the superficial digital flexor tendon (SDFT) of aged horses (18–25 years, median = 23 years) with the horizontal bars representing the median values. (A) Proximal to distal distribution of ICBs. There was no significant difference in the number and size of ICBs between the proximal, middle and distal aspect of the tendon ($p = .50$). (B) Distribution of ICBs in the tendon periphery (within 2 mm distance of the SDFT epitenon) and the tendon core. Significantly more ICBs were detected in the tendon periphery when compared to the tendon core region ($p = .01$) [Color figure can be viewed at wileyonlinelibrary.com]
extracellular matrix include alterations in the protein profile and turnover rate as well as the increasing disorganization of elastin fibers. Based on the observations of the detailed histological assessment performed in this study, the elevated proteoglycan and glycosaminoglycan content of the ICBs, located mainly intrafascicularly but also between tendon fascicles, may provide an additional explanation for the increased tendon stiffness as detected in the equine SDFT of aged horses. Further studies are required to assess the impact of the ICBs on the fascicular architecture including collagen alignment and structural integrity of collagen fibers as well as the biomechanical properties of energy-storing tendons.

Tenocytes can be located within or in between tendon fascicles and are arranged along the longitudinal axis of the collagen fibers. Up to 11 different tenocyte phenotypes are described in the literature with chondrocyte-like cells generally present close to the tendon’s enthesis or in areas that are exposed to compressive forces. Whilst chondroid-like cell clusters similar to the ICBs have been detected in other species including birds (specifically adult emus), the characterization of the distinct ICB cells in energy-storing tendons like the equine SDFT or the human Achilles tendon appears to be limited to two reports where sections of the equine SDFT were evaluated based mainly on H&E histology. It has been demonstrated that the tenocyte morphology may change with cells becoming more rounded in response to excessive loading. Since the ICB cells as well as the adjacent tenocytes showed a characteristic round appearance in the current study, it is considered likely that the development of ICBs in the IFM of energy-storing tendons may occur in response to micro-trauma caused by nonuniform frictional forces acting between fascicles during repetitive motion. Repetitive loading may induces the aberrant differentiation of resident tendon-derived stem cells. Transmission electron microscopy of an ICB showed a prominent endoplasmatic reticulum with a fingerprint-like appearance. A similar presentation of the endoplasmatic reticulum has been described in chondrocytes of human patients with pseudoachondroplasia and might be indicative of a defect in the extracellular matrix secretion of ICBs.

The histological examination including immunohistochemistry confirmed the presence of cartilaginous extracellular matrix in the majority of the ICBs detected in the equine SDFT samples in this study. However, the Modified Von Kossa's staining showed evidence for calcium deposition in some of the larger ICBs. In poultry species, the development of ICBs is more prominent in the 10th–20th week of life and is considered physiological. In the equine SDFT and the human and murine Achilles tendons, calcification and subsequent ossification has been described as an incidental finding as well as a result of tendon injury. The formation of chondroid metaplasia and calcium deposition in form of ICBs may precede the development of tendon mineralization in energy-storing tendons.

In light of the findings described in this study it would be of interest to perform ultrahigh field MRI studies in a larger sample population, including middle-aged animals with a detailed exercise history to assess the effect of training on the emergence of ICBs in the equine SDFT. In addition, the assessment of middle-aged horses could provide insight into the dynamics of the ICB development and a larger sample number could highlight individual factors associated with their occurrence. In contrast to histological or macroscopic studies, ultrahigh field MRI allows for the non-destructive examination of a consecutive length of tendon and the high contrast resolution of the 3D FISP sequence permits the detection of ICB clusters as well as small ICBs. Consequently, the assessment of other energy-storing tendons including the human Achilles tendon and positional tendons like the equine common digital extensor or human anterior tibialis tendon would be of great interest.

ICBs of varying size were detected in the SDFT of aged equine individuals. Isolated as well as clustered ICBs are characterized by distinct round nuclei surrounded by a cartilaginous extracellular matrix. ICBs were interspersed between tendon fascicles with significantly more ICBs present in the tendon periphery when compared to the tendon core region. Further research is required to understand more about the development of ICBs and their impact on the viscoelastic properties of energy-storing tendons.

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AUTHOR CONTRIBUTIONS
Othman J. Ali and Anna Ehrle contributed to the preparation of the manuscript in equal parts. Anna Ehrle acquired the magnetic resonance imaging data and Othman J. Ali provided the histology. Both authors were involved in the research design, data analysis and drafting of the paper. Eithne J. Comerford and Elizabeth G. Canty-Laird assisted with the data analysis and interpretation of the histological study and Ashleigh Mead performed part of the image data analysis. Peter D. Clegg and Thomas W. Maddox led the design of the study and the preparation of the manuscript. All authors have revised the manuscript and have read and approved the final submitted version.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

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