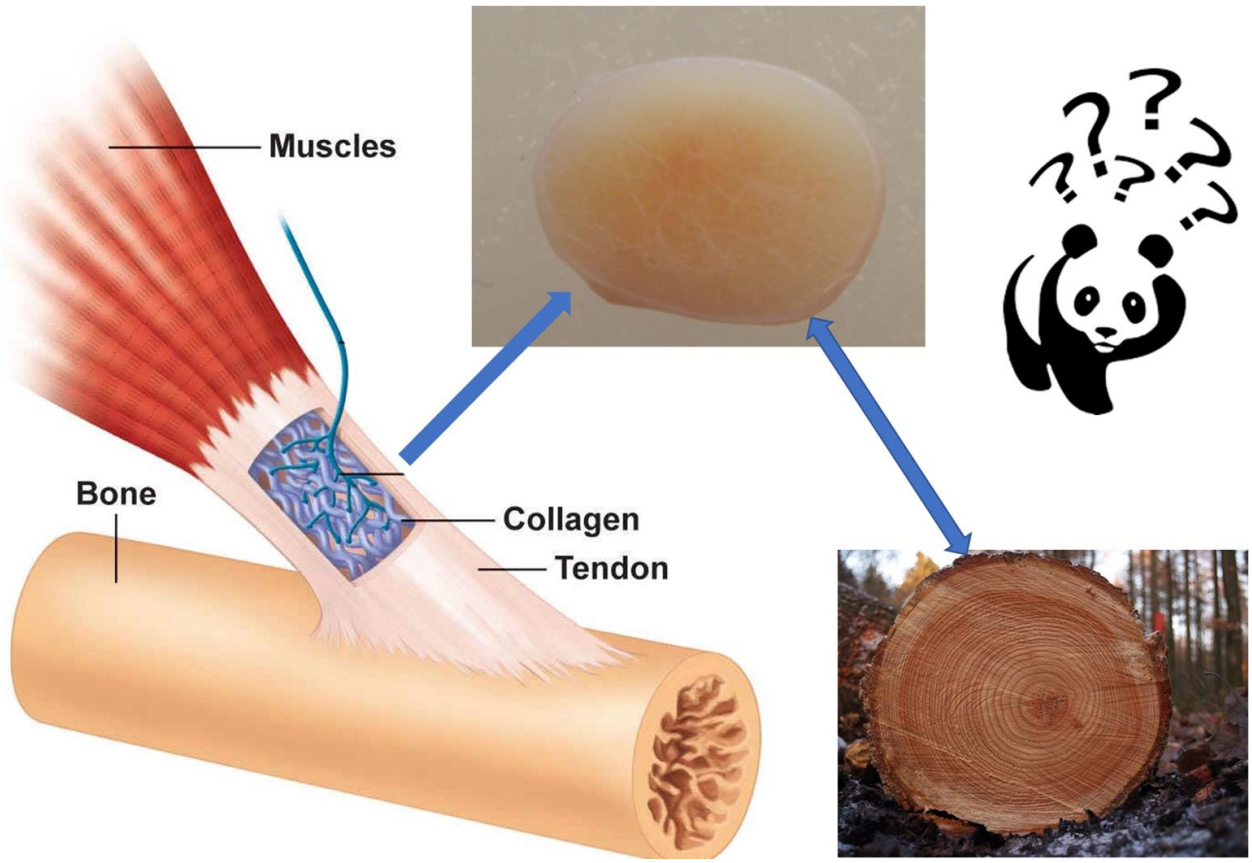
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Tendon composition and turnover
-Is it uniform throughout the tissue?



PhD thesis 2020

Cheng Zhang

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This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen

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Cover image:	Tendon structure image was referred from Karin Lienhard published on Research Gate on 2014. The tree rings image was referred from Wikimedia Commons and photographed by Arnoldius. The panda question mark image was from google image, failed to find the original resource. All images were organized by CZ.

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Preface and Acknowledgements

The last 3 years was like only one moment in my life, I cannot believe I am going to complete my PhD life. Even the moment I am sitting here writing the acknowledgements, it is too unreal.

Back to the autumn in 2016, that time I was bored of the repetitive life in clinic, and wanted to go further and deeper of my career. Only after a short thought, I decided to start a PhD life, even though, at that moment I had no plan, I just knew I wanted to have a different life.

In the very beginning, I wanted to look for a PhD position in Germany, because of its nice reputation and personally I had my intern there. Things were going well then, I sent some emails to some cooperated labs there, and got some nice response. But one day, by chance, a friend who I respected Shifu Peng told me I could also try to come to Denmark, where he was studying in. That time, about Denmark, the only thing I knew could be H.C. Anderson and his little mermaid. But finally, I am here.

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Sincerely yours,
Cheng Zhang

Resumé

Omsætningen i senevæv menes at være meget begrænset, men alligevel kan senen vokse ved fysisk belastning og i et vist omfang hele efter skader. Denne øjensynlige uoverensstemmelse mangler endnu at blive belyst. Desuden forekommer seneskader normalt i specifikke regioner af senen, hvilket kan være relateret til disse regioners mekaniske egenskaber, biokemiske sammensætning, cellepopulationer eller seneomsætning, men den nøjagtige årsag kendes ikke.

Sener består primært af kollagen samt en mindre mængde glykosaminoglykan (GAG) og relativt få celler. Seneomsætning er vanskelig at måle in vivo, derfor udførte vi in vitro forsøg på dyremodeller og humant væv. Seneomsætningen kan blandt andet måles ved ^{14}C bombe-puls metoden, som har høj præcision men kræver at personen er født omkring 1950erne hvor atomprøvesprængninger blev udført. Dyremodeller er oftest for unge til ^{14}C metoden men tidligere studier har vist at såkaldte *advanced glycation end-products* (AGEs) akkumuleres lineært med alderen og derfor kan anvendes som en indirekte markør. AGEs kan måles indirekte ved hjælp af autofluorescens eller direkte ved massespektrometri.

Formålet i dette ph.d.-studie er at undersøge de regionale forskelle i seneomsætning og de potentielt relaterede mekanismer. I vores første studie undersøgte vi den dybe digitale bøje-sene fra slanke (raske) og diabetiske Göttingen-minigrise. Biokemiske (AGE-akkumulering, kollagenindhold, DNA-indhold, GAG-indhold) og mekaniske tests blev udført for at undersøge egenskaberne i forskellige dele af senen (opdelt efter langsgående og radial position). Resultatet af AGE-akkumuleringen viste, at autofluorescensen var større i den proksimale end den distale del af senen i både de slanke og diabetiske dyr. Derudover var AGE-akkumulering i den slanke kontrolgruppe også større i kernen end overfladen af den distale del af senen. Disse fund indikerer, at senesomsætningen varierer i forskellige regioner.

Det andet studie blev udført på humane patellar-sener, hvilket er mere klinisk relevant og også gør det muligt at måle seneomsætningen mere direkte ved hjælp af ^{14}C-metoden. Ligesom i det foregående studie blev senen opdelt i forskellige regioner, og den samme type biokemiske målinger (AGE-akkumulering, kollagenindhold, DNA-indhold og GAG-indhold) blev udført. Der ud over blev et antal specifikke AGE produkter også målt og ^{14}C-metoden blev anvendt til direkte at bestemme kollagenomsætningen i senen. ^{14}C-analyserne viste, at forskellige regioner i den humane patellar-sene havde en ensartet kollagenomsætning, som fandt sted i løbet af de første 15 år af personens liv. Fluorescens og de specifikke AGE målinger viste heller ingen forskelle mellem regionerne. Samlet set understøttede alle resultaterne, at alle dele af den humane patellar-sene havde en lignende kollagenomsætning.

Det første studie havde indikeret et højere indhold af celler og større seneomsætning i overfladen af senen end i kernen. Det tredje studie undersøgte derfor cellekulturer fra disse regioner. I dette studie blev den overfladiske digitale bøjesene fra heste anvendt. Tenocytter blev isoleret fra henholdsvis kernen og overfladen af senen, proliferationsevnen blev bestemt ved hjælp af vækstkurver og produktionen af matrix proteiner blev karakteriseret ved histologiske farvninger og proteom-analyser. Cellekulturerne udviste lignende proliferationshastigheder og meget ensartede proteom-profiler mellem de to regioner, men udviste biologisk variation i deponeringen af type I kollagen.Samlet set fandt vi at de regionale forskelle i seneomsætning varierede mellem de forskellige modeller, hvilket indikerer, at der ikke er noget fastlagt mønster for vækst eller fornyelse. Særligt i den humane patellar-sene så det ud til, at ingen regioner blev omsat efter kroppen er fuldt udvokset.

Summary

The turnover of tendon tissue is thought to be very limited, but tendon can still grow in response to loading and to some extent renew after injuries, a conflict that remains to be investigated. In addition, tendon injuries normally occur in specific regions, which may relate to the mechanical properties, tendon composition, cell populations or tendon turnover, but the exact reason remains unknown.

Tendon consists mainly of collagen with a smaller amount of glycosaminoglycan (GAG) and contains relatively few cells. It is difficult to measure tendon turnover in vivo, therefore animal models and human tissue were used to do the research in vitro. One method for measuring turnover is ^{14}C bomb-pulse dating, which can be considered a gold standard but requires that subjects were born around the time of nuclear tests in the 1950s. Animal models are typically too young for the ^{14}C method but previous studies have shown that advanced glycation end-products (AGEs) accumulate linearly with age, which can therefore be used as an indirect marker. AGEs can be measured indirectly by autofluorescence or directly by mass-spectrometry.

The aim of this PhD study was to investigate the regional differences of tendon turnover, and the potentially related mechanisms. In our first study, the deep digital flexor tendon of lean and diabetic Göttingen minipigs was investigated. Biochemical tests (such as AGE accumulation, collagen contents, DNA content and GAG content) and mechanical tests were performed to investigate the properties in different parts (longitudinal and radial) of the tendon. The result of the AGE measurements showed that autofluorescence was greater in the proximal than the distal part in both the lean control and diabetic groups. Moreover, in the lean control group AGE accumulation was also greater in the core than the periphery of the tendon. These findings indicate that tendon turnover varies between different regions.

The second study was performed on human patellar tendon, which is more clinically relevant and enables a more direct measurement of turnover with the ^{14}C method. Similar to the previous study, the tendon was divided into different regions and biochemical measurements (AGE accumulation, collagen content, DNA contents and GAG content) were performed. In addition, a number of specific AGEs were measured and the ^{14}C method was used to directly determine the collagen turnover. The ^{14}C results showed that all regions of human patellar tendon had similar collagen turnover, and this turnover occurred during the first 15 years of the person's life. Fluorescence results and specific AGEs also did not differ between regions. All in all, the results showed that different regions of human patellar tendon had similar collagen turnover.

The first study had indicated differences in cellularity and turnover between the tendon core and periphery. The third study was therefore a cell culture study of regional differences. In this study, equine superficial digital flexor tendons (SDFT) were used. Tenocytes were derived from the tendon core and periphery, the proliferation capacity was determined by growth curve assay and matrix protein production was characterized by histological staining and proteomics. Cell cultures from the two regions exhibited similar proliferation rates and very similar proteome profiles, but showed biological variation in collagen type I deposition.

To conclude, the regional differences in turnover of different species varied, indicating that there is no consistent pattern of growth or renewal. In particular, no region of human patellar tendon appeared to turn over after skeletal maturity.

Abbreviations

MSD: Musculoskeletal disorder
CSA: Cross-sectional area
RC: Rotator cuff
STIR: Short TI inversion recovery
MR: Magnetic Resonance
RCT: Randomized controlled trial
NSAIDs: Nonsteroidal anti-inflammatory drugs
PRP: Platelet-rich plasma
CT: Computed tomography
HDCT: Heritable disorders of connective tissue
OI: Osteogenesis imperfecta
IFM: Interfascicular matrix
ECM: Extracellular matrix
EDS: Ehlers–Danlos syndromes
LOX: Lysyl oxidase
HP: Hydroxylysyl pyridinoline
ACL: Anterior cruciate ligaments
PCL: Posterior cruciate ligaments
AGEs: Advanced glycation end products
GAGs: Glycosaminoglycans
CS: Chondroitin sulfate
DS: Dermatan sulfate
KS: Keratan sulfate
HS: Heparin sulfate
HA: Hyaluronic acid
SDFT: Superficial digital flexor tendon
MS: Mass spectrometry
D-Asp: D-aspartate
L-Asp: L-aspartate
pH: Acidity
CML: Carboxymethyllysine
CEL: Carboxyethyllysine
STZ: Streptozotocin
HPLC: High Performance Liquid Chromatography
DMB: 1,9-Dimethylmethylene blue
ANOVA: Analysis of variance
Ins: Insertion
PS: Proximal Side
PCA: Proximal Central Anterior
PCP: Proximal Central Posterior
MS: Middle Side
MCA: Middle Central Anterior
MCP: Middle Central Posterior
Dis: Distal
AMS: Accelerator mass spectrometry
pMC: Percentage modern carbon

C/N: Carbon/nitrogen
UPLC MS/MS: Ultra-performance liquid chromatography tandem mass spectrometry
AF: Atrial fibrillation
TBS: Tris-buffered saline
HCD: Higher-energy collisional dissociation
LFQ: Label-free quantitation
PC: Principle components
FBLN5: Fibulin 5
PGE_2: Prostaglandin
TXB_2: Thromboxane

Introduction

Tendon injuries

Tendons are dense connective tissues attaching muscles to bones, and they exhibit mechanical properties that effectively transmit force from the muscles to the bone [1]. Tendon and ligament injuries are some of the most common musculoskeletal problems for which patients come to the clinic [2, 3]. Based on the musculoskeletal disorders (MSD) report, in the United States of America, 33 million musculoskeletal injuries are reported per year, and 50 % of these involve tendon and ligament injuries [4]. In the European Union Member states around 40 million workers are estimated to have a musculoskeletal problem, and the number is increasing constantly [5]. Tendon injuries are increasingly becoming a very common clinical problem, due to age-related degeneration and overuse and represents 30% of consultations for musculoskeletal pain [6]. When tendons and ligaments are damaged they heal slowly and normally are involved with damage of structural integrity [7].

Despite the fact that tendon injures are common, research in the area has been relatively sparse until recently. It is very hard or even near impossible to get healthy tendon tissue biopsies from patients or participants. Almost all existing data of basic mechanisms of tissue physiology, or investigations of tendon damage and repair are from non-primate animal studies [8]. In our studies, we used different tendons from different species, porcine, equine and human tendons.

In general, tendon injuries are common medical conditions, the causes of tendon injuries are intrinsic and extrinsic. Acute tendon injuries were more often caused by extrinsic factors, while chronic tendon injuries were normally caused by multiple factors [9]. Extrinsic factors include overloads on the body, extreme environmental circumstance, also training errors or ineffective rules in sports. Intrinsic factors include malalignments of the tendons, different leg length, muscle weakness, loss of flexibility and laxity of joints, female sex, diabetes etc. [9] (Fig. 1). Meanwhile, both chronic and acute injuries occur in specific regions of the tendons, but the reasons behind this are not clear.

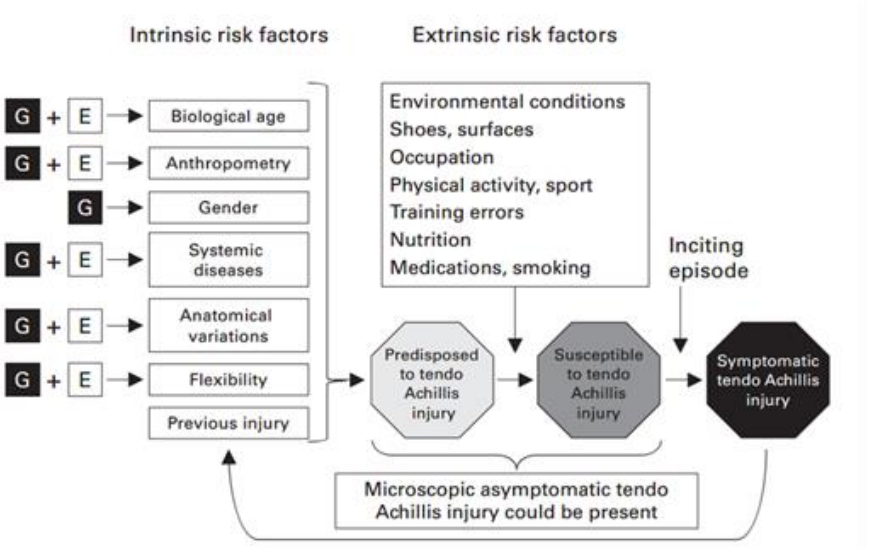

Figure 1: The flow chart showed the relationship between the intrinsic and extrinsic risk factors of Achilles tendon injuries.[10]

Chronic injuries – tendinopathy

The tendon conditions that contain chronic pain are referred to ‘tendinopathies’ instead of ‘tendinosis’ or ‘tendinitis’ [3], ‘itis’ means inflammation and some studies suggest that there are no signs of inflammation. However, recent studies suggest that there may be some inflammation in the early stage [11, 12], so the underlying pathology is still not clearly known. Tendinopathy normally relates to overuse and degeneration, and it is usually painful and it can severely reduce the quality of life. Therefore, it also becomes a very sever burden to both the individual person and the entire society [13].

Research have showed that tendon overuse is normally caused by two type of sport activities, endurance activities (such as middle to long-distance running), and sports with repetitive motion (such as volleyball and tennis) [14]. But tendon overuse injury is not related to some specific sports activities but rather relates to increased use, frequency, and intensity.

Tendinopathies can occur in different tendons [15, 16, 17], and some of the common ones include Achilles, patellar posterior tibialis, and rotator cuff tendinopathies [18, 19].

Achilles tendon is the largest and strongest tendon, and it is involved in almost half of all sports-related injuries [20]. Achilles tendinopathy has the highest occurrence in middle and long distance running, tennis, badminton and football among sports amateurs [21]. It occurs frequently at the insertion site (the enthesis) and mid-portion of the tendon [22, 23].

Patellar tendinopathy is also a very common painful disorder that normally affects athletes engaged in jumping sports, such as basketball and volleyball [24]. The healthy patellar tendon is heterogeneous along its length with the cross-sectional area (CSA) increasing from the proximal to the distal region [25]. In the tendinopathic tendon, there is a thickening of the posterior proximal region [26] in addition to neovascularization [27, 28].

Shoulder pain is the third most prevalent musculoskeletal disorder [29, 30, 31], and almost two thirds of patients are diagnosed with rotator cuff (RC) tendinopathy [31]. Rotator cuff tendinopathy is a broad diagnosis, which also includes shoulder impingement syndrome and subacromial bursitis [32]. The life quality of patients diagnosed with rotator cuff tendinopathy is severely affected due to decreased function [33], poor sleep quality [34], and work absenteeism [35].

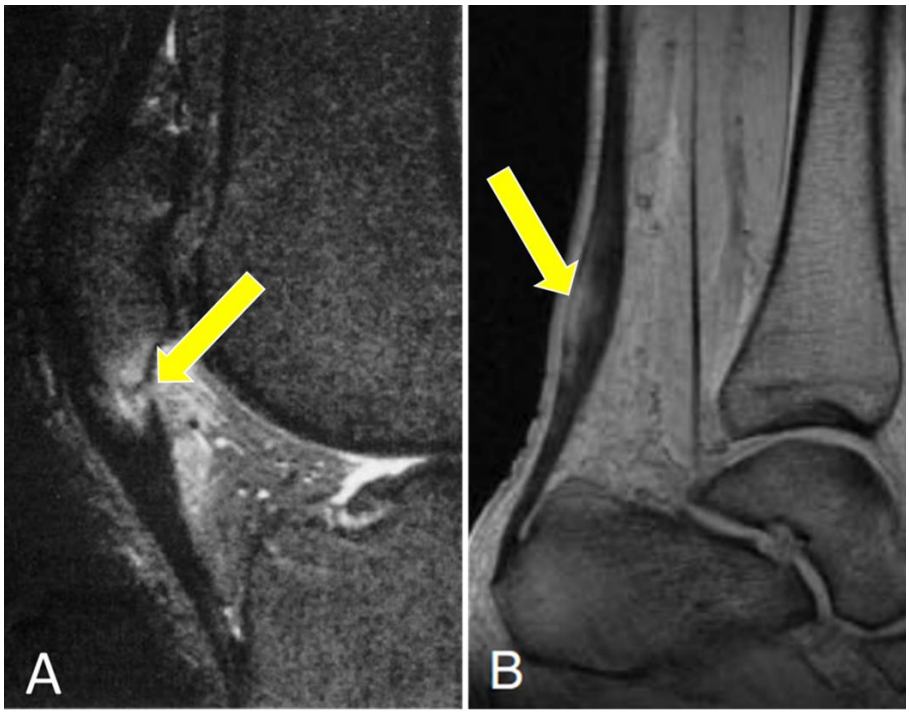

Figure 2. Tendinopathies occur in specific regions as shown by the arrows show. A) Patellar tendinopathy shown with a fast short TI inversion recovery (STIR) sagittal Magnetic Resonance (MR) scan [26]. B) Achilles tendinopathy shown with thickened and increased intratendinous signal in the mid-potion in MR [36].

Although tendinopathies can occur in many different tendons, it often occurs in some specific regions of a tendon. For example, Achilles tendinopathy frequently occurs at the insertional region and in the mid portion (Fig 2B) [22, 23]. In the patellar tendon, tendinopathy more frequently (about 65%) involves the proximal and posterior portion [24, 26, 37, 38, 39], and this has been verified by both imaging and histological investigations [26, 39, 40]. The proximal tendinopathy is also structurally located in the posterior region (Fig. 2A).

Tendinopathy blood flow

Previous studies have shown that tendinopathies are often companied by neovascularization and increased intratendinous blood flow [41, 42, 43]. It has been shown that that there are more cells in tendinopathic region; both the volume fraction and the number per unit volume [44]. The collagen turnover is also increased in the tendinopathic region with both collagen synthesis and degradation being up-regulated [45].

Treatment for tendinopathy

A review based on 36 randomized controlled trials (RCTs) showed that there does not exist a standard form of treatment that is appropriate for all types of tendinopathy. Normally when patients with tendinopathies come to the physician, they cannot name a specific injury incident or activity, but rather they described a process of worsening pain over time. So the treating physician should consider the history and the physical examination of the individual.

Nowadays, there are quite a few interventions for tendinopathies, including surgery, nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, shockwave therapy, platelet-

rich plasma (PRP) injection, sclerosing injection, intratendinous hyperosmolar dextrose injection (prolotherapy), high-volume injections of 10 mL 0.5% bupivacaine and 40 mL normal saline into the paratenon, therapeutic ultrasound and Kinesio Tape [46, 47, 48]. Even though there are a number of treatment options for tendinopathy, loading based rehabilitation is currently considered the gold standard [46, 49]. Studies have demonstrated that the loading based rehabilitation can result in beneficial clinical [50, 51, 52], structural [44] and biomechanical outcomes [53].

20 years ago a treatment regime that isolated eccentric contraction without any accompanying concentric component was suggested, and this regime showed a good clinical outcome of tendinopathic patients [54]. It was though as the gold standard that time, even though there is no convincing evidence that this regime is the most effective one, it remains very popular [49]. In recent years, other loading based such as heavy slow resistance training [53, 55], isolated concentric training [51], concentric/eccentric progressing to eccentric training [52, 56] showed up, some people think the loading based training is the gold standard, while others may not agree.

Acute injuries - tendon rupture

Achilles ruptures typically (up to 90% of all cases) occur in the low vascularised region, 2 cm to 6 cm to the osteotendinous junction [57], Typically the patients presents to the clinic with a spontaneous rupture without any prior symptom, pain, discomfort, edema, stiffness or any other diagnosed disease in the ruptured region [23].

Different research showed that from 60% up to 75% of Achilles tendon ruptures were associated with sports activities [58, 59], and the specific sports involved in Achilles tendon rupture varies from country to country, as football, tennis, downhill skiing in Europe and American football, basketball, baseball in United States [60, 61].

Patellar tendon rupture is a relatively uncommon injury (the incidence rate is less than 0.5-6%), although it is a severe injury [62]. Patellar tendon rupture is caused by sudden trauma, but the histories of patients showed that they could be the result of patellar tendinopathy [62]. To diagnose patellar tendon rupture, many risk factors of history need to be considered, as corticosteroids local injections [63], diabetes [64], and fluoroquinolone systemic use [65]. Even the gold standard for patellar rupture is an MRI scan, but due to its higher cost and lower availability [66], computed tomography (CT) scan and ultrasound are used in the clinic.

Since the strain is greater at the osteotendinous insertion than the other regions in patellar tendon, the avulsion occurs in this site more than other regions [62]. Conservative treatment with extension immobilization is recommended for incomplete ruptures with intact function [67]. For the other operative cases, there are two settings, the acute setting and the chronic setting. In the acute setting, the transpatellar bone tunnel technique with an augmentation is the gold standard treatment. On the other hand, patellar tendon reconstruction is recommended for chronic setting.

Genetic disease

Tendon are not just affected by overuse or degeneration, but genetic factors could also play a role. Genetic diseases normally not specifically affect tendon injuries, but some common genetic connective tissue disorders are affecting bone, muscle, skin, cornea or tendon and

ligament. These genetic connective tissue diseases are often called heritable disorders of connective tissue (HDCTs), which are the result of changes in certain genes.

Marfan syndrome is a genetic disease, which affects the ligaments, bones, eyes, heart, and blood vessels. Marfan syndrome is seen more frequently in basketball players, because people with Marfan syndrome tend to become tall and have very long bones, typically called 'spider-like' fingers. Marfan syndrome is caused by mutations in the gene that regulates the structure of fibrillin-1. With Marfan syndrome can cause a number of negative consequences such as scoliosis, kyphosis, sunken or protruding chest, joint hypermobility, flat feet, crowded teeth and aortic aneurisms.

Osteogenesis imperfecta (OI), also known as brittle bone disease, is a group of genetic disorders that have brittle bones, lax joints and ligaments, and also a low muscle mass. The severity may be mild to severe. OI is caused by the mutation in two genes responsible for type 1 collagen, which is important for building the structure of bones.

Tendon structure & function

Tendon structure

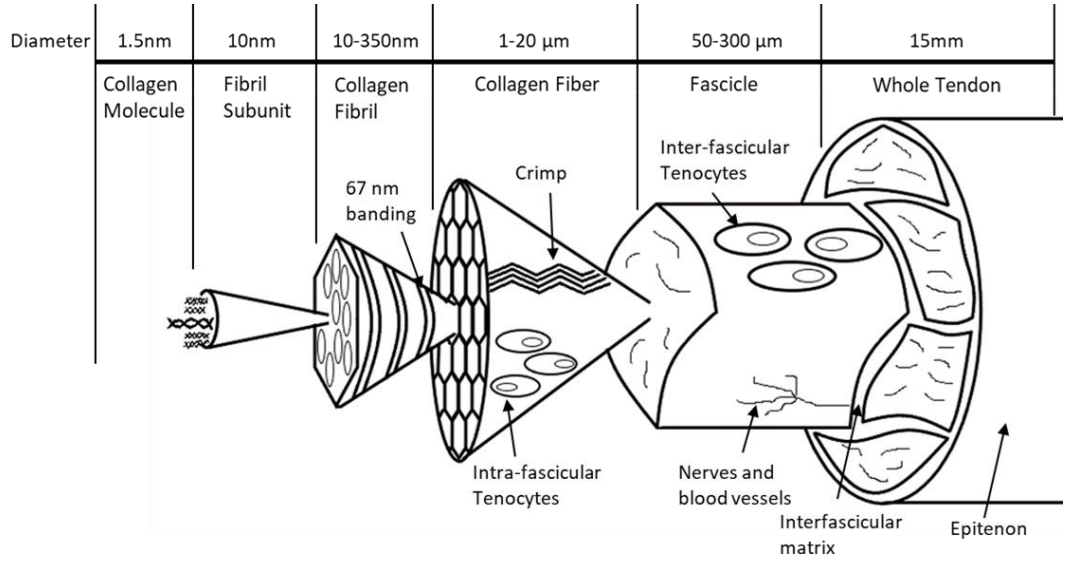

Figure 3. A schematic of the tendon hierarchical structure

Tendon is a typical connective tissue, with a water content of 55-70% of total weight, while collagens make up the majority of tendon dry weight (60-85%) [68].

The collagen is composed of a triple helix structure, which consists of two identical chains (α1) and an additional chain (α2). Collagen molecules in tendons are arranged in a hierarchical manner (Fig. 3), and in each layer of the hierarchy the collagen is assembled with fibers and ground substance [69, 70, 71]. Tendon is a good example of an aligned fiber composite with a hierarchical structure. The size of tendon composite involves the scale from nano to macro.

In general, tendons are comprised by two compartments, the intrinsic compartment and the extrinsic compartment [72]. Collagen molecules are the smallest building blocks. Five of collagen molecules are cross-linked together to form pentafibrils or microfibrils, and then these microfibrils are assembled together becoming fibrils. Collagen fibrils are the minimal functional

mechanical unit with a scale ranging from 10 to 500 nm in diameter [8, 73, 74]. Fibrils combined together forming the next level structure fibers, then fibers were combined together again to form the next level structure fascicles, which are identified as the basic functional unit of the tendon and the largest subunit of tendon [69, 75]. The fascicles range from 150 to 500 μm, and they can be seen with naked eyes. All the structures above form the intrinsic compartment or tendon core, they are surrounded by endotenon which is the lowest level extrinsic compartment and also called interfascicular matrix (IFM). The tendon is surrounded by the epitenon, which is a connective tissue sheath continuous with IFM [76, 77]. Finally, at the tissue level tendon may or may not be surrounded by paratenon, which is a loose connective tissue layer [75].

Besides the structural components, there also exist many types of cells in the hierarchical-structure tendons, such as tenocytes, chondrocytes, vascular cells and synovial cell. Tenocytes take account most of the cells in tendons. Tenocytes intersperse between collagen fiber bundles and align longitudinally along the tendon. Tenocytes could produce the extracellular matrix (ECM), including collagen, fibronectin and proteoglycans, which are involved in maintaining tendon homeostasis and repairing tendon juries [78].

Composition

Collagen type I is the primary component of healthy tendon, and it makes up about 80% of dry mass, which means collagen I makes up to 90% of total collagen content [79, 80]. Besides collagen I, other collagens also have important function even if they only make up a small amount, like collagen III, collagen V, collagen VI, collagen XI and XII.

Collagen type III is the second most abundant fibrillar collagen matrix in tendon with studies showing that it accounts for up to 10% of the total collagen content [81]. Even though there is not much collagen III in tendons, it has an important function in collagen fibrillogenesis. Collagen III can regulate the size of collagen I fibrils [82], and the synthesis is involved in early stages of wound repair, following on fibronectin matrix templating by tendon fibroblasts [83]. However, increased presence of collagen III is a sign of degeneration of tendons and has been associated with increased tissue disorder and reduced mechanical properties [8, 84].

Collagen type V was found in the center of collagen I fibrils, it has a low abundance in tendon, but it also plays an important role in providing template for fibrillogenesis [85], ordering and stabilizing collagen I structure during self-assembly [86]. Abnormalities in collagen V can cause the genetic connective tissue disorder called classical Ehlers–Danlos syndromes (EDS) [87]. The classical EDS is a heritable disorder, and the symptoms includes hyperflexible joints, tearing of tendons, skin hyperextensibility, poor wound healing and abnormal scar formation [88].

Collagen type VI is one of the non-fibrillar collagens in tendon, it is normally attached to pericellular matrix. Proteomic studies showed that collagen VI also plays an important role in collagen fibrillogenesis [89], and it is a very important component of tendon ECM [90].

Collagen type XII and XIV have important function during tendon development by being a molecular bridge between collagen I and other matrix molecules [91].

Elastin is one of components of elastic fibers, and it is an important protein of ECM. Because of its elasticity, elastin allows connective tissues to keep their original position or shape after stretching or contracting. Elastin plays important role in many tissues, such as blood vessels, the lung, the bladder and so on. More flexible tissues contain more elastin, while less flexible tissue does not have so much, such as tendons [92]. Elastin plays a very important role

in tendon tissue in recoiling of the matrix after repetitive mechanical loading [93] while it only takes 1-2% of tendon dry mass.

Cross-linking

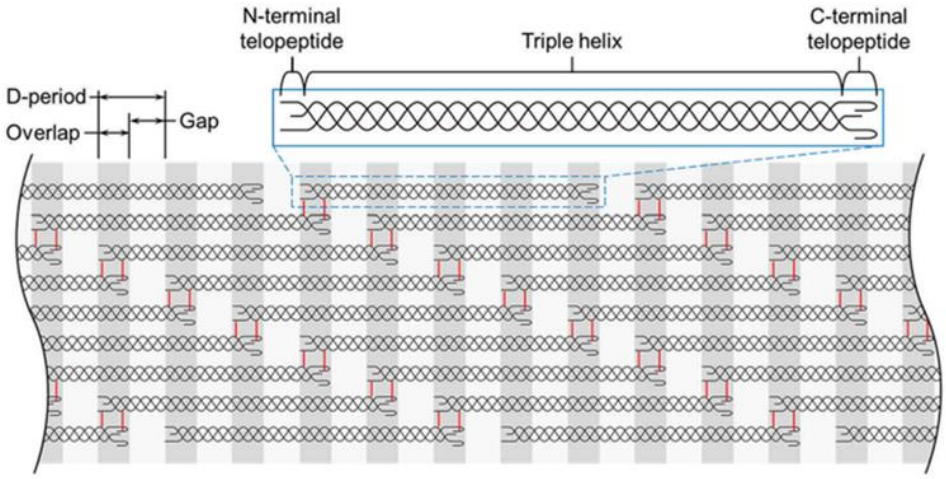

Figure 4. Molecular structure of collagen. A triple helix structure with N- and C-terminal telopeptides [94]. The collagen molecules are aligned in a quarter-staggered arrangement with distinctive overlap and gap regions at each end giving rise to a D-period of around 67 nm. The collagen fibrils are stabilized by intermolecular cross-links in the overlap regions (shown in red) [95].

Collagen molecules are formed with a triple helix structure, which generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2), and short nonhelical telopeptide regions are at the N- and C-terminals (Fig. 4) [95]. The fibrillar molecules are quarter-staggered to adjacent molecules, forming a characteristic D-period of about 67nm. In each repetition of D-period, there are two distinct regions called overlap and gap [96]. These overlap and gap regions are retained as microfibrils assemble into fibrils. There are also some covalent cross-linking within the triple helices, and between tropocollagen helices to form the well-organized aggregates [97]. Cross-links are formed between the collagen molecules, to stiffen the fibrils and fibers. There are two kinds of cross-links added into the molecular network after the formation of fibrils to bond collagen molecules together, which are enzymatic and nonenzymatic cross-links [95].

Enzymatic cross-links are considered to be beneficial to tendon function and will briefly be introduced in this section. Lysyl oxidase (LOX) should be mentioned here, since it is the enzyme directly involved in formation of collagen cross-links. The major trivalent cross-links in tendon tissue are pyridinolines and pyrrole formed between hydroxylysine and lysine [98, 99, 100]. The most abundant cross-link in tendons is hydroxylysyl pyridinoline (HP) [101, 102]. Previous studies showed that HP accumulates most in anterior cruciate ligaments (ACL) and posterior cruciate ligaments (PCL) [103, 104, 105]. Aside from differences between ligaments, within the ACL HP and is reported to accumulate more in the posterior fascicles than anterior fascicles [106]. Some studies have reported a relation between mechanical properties and cross-link accumulation and in addition, increased mature cross-linking has been found in tendons with endurance exercise [107, 108].

Besides the enzymatic cross-links, both intermolecular and intramolecular non-enzymatic cross-links are formed in collagen through glycation via the Maillard reaction, which in the end results in the formation of advanced glycation end products (AGEs) [109, 110, 111].

Since glycation is a non-enzymatic reaction, AGEs take considerable time to accumulate in tissue. However, the turnover of tendon collagen is remarkably slow, which provides a possibility for this long process of AGEs accumulation [112, 113, 114]. Several studies have shown that non-enzymatic cross-links accumulate linearly with age in tendon tissue [102, 104, 106, 112, 115], but some research showed that AGE accumulation decreased in tendinopathy indicating a higher collagen turnover than in healthy tissue [116, 117, 118]. In addition, AGE accumulation in tissue is expected to proceed more quickly in diabetics and hyperglycemia, since there is more sugar to be involved in glycation [113], however, there are inconsistent experimental evidence for increased AGEs accumulation in diabetes with studies showing either that AGE accumulation is increased [119, 120] or similar [121, 122] compared to control groups.

Pentosidine is one of the most studied non-enzymatic cross-linking, since it is easy to quantify due to natural fluorescence [123, 124], but it only constitutes a minor quantity of the non-enzymatic cross-links in extracellular matrix (ECM). Other AGEs such as glucosepane have been reported to constitute a magnitude greater quantity than other minor AGEs, including pentosidine, and it has been suggested that glucosepane contributes the most mechanical effects of glycation [119, 125, 126].

GAGs

Even though Glycosaminoglycans (GAGs) only constitute a very small proportion of the tendon ECM, they play a significant role in fibrillogenesis by controlling fibril size and mechanical integrity [127, 128]. GAGs are long mucopolysaccharides that are consisting of repeating disaccharide (double sugar) units of uronic acid and an amino sugar [129, 130]. The most common classification of GAGs is based on the degree of sulfation, as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparin sulfate (HS), and the only non-sulfated GAG is hyaluronic acid (HA). GAGs are usually covalently attached to glycoproteins to form proteoglycans, which are the structure that surround collagen fibrils [131, 132]. They are also associated with modifying collagen organization [133], tissue repair and regeneration [134] and improving mechanical and degradation properties [135, 136]. GAGs play an important role in maintaining the elastic and viscoelastic properties of tendon [137, 138], however, GAGs do not appear to play an essential role in mediating the tensile force transmission in human patellar tendon [139]. GAGs also play a very significant role in resisting compression forces. Due to their negative charge, osmotically active cations are attracted by the GAGs, which in turn causes retention of a large amount of water. This enables tissue with high GAG contents such as cartilage to resist compression forces from outside [140]. Research has shown that GAG content decreases with aging while exercise can counteract the process [141, 142].

Mechanical properties

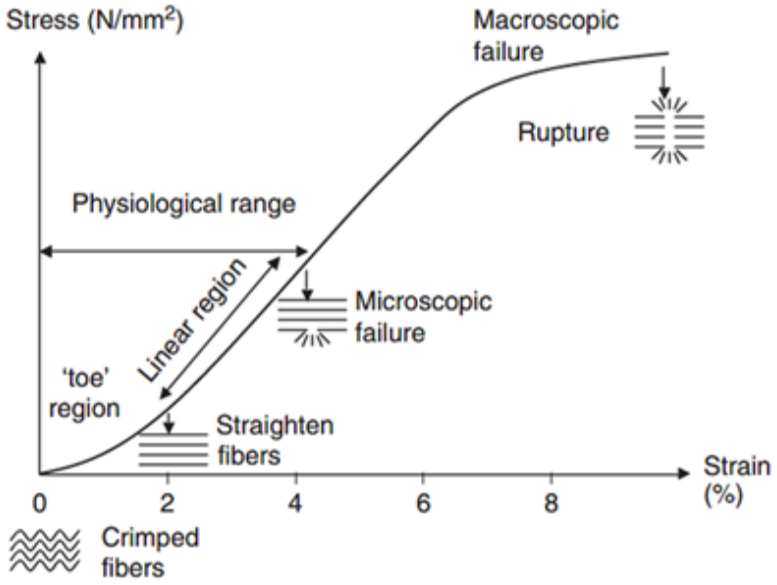

Figure 5. A schematic for strain-stress and tendon injuries. When the repetitive tendon strain is under 3%, it is considered the physiologically relevant range for daily activities. When the strain reaches >3% microtrauma of tendon fiber may occur. If tendon strain is over 8%, acute tendon rupture will ensure [23]. This curve also presents the development of tendon injuries. When the repetitive stain is less than 3%, it is the physiological coiling. When the stain is from 3-5%, microtrauma in tendon fiber my occur. When the stain is over 8%, it exceeds the reparative capacity of a tendon, an acute rupture will happen [143].

As mentioned previously, tendon has an important role in transmitting contractile forces from muscle to bones. Tendons are designed to resist large loads (up to 8 times body weight) and bear dynamic mechanical force during human locomotion [101, 144, 145].

The stress-strain curve of tendon has a characteristic of three distinct phases (Fig. 5). In the beginning of a mechanical test, collagen fibers in the tendons are crimped into a wavy pattern, with increasing force, fibers begin to be aligned along the force direction. Depending on the type of tendon, the strain required to align the fibers is commonly up to 2% and defines the so-called 'toe region'. Next is the 'linear region'. In this region, tendons are stretched up 6%-8%, and the slope of the stress-strain curve in the linear region defines the Young's modules. The linear response result from stretching of the collagen molecules helix, if the tendon continues being stretched over approximately 4%, micro damage can occur. As stretching continues, it reaches the third phase 'the plastic region'. This phase begins at the end of the 'linear region', micro and macro tears occur, until the tendon is ruptured [146].

Tendon mechanical properties have different levels, from macro scale to micro scale. At the organ level, plenty of literature demonstrate a very wide range of tendon mechanical properties [147]. This wide rage may depend on the species [148], anatomical site [149] and methodological challenges related to mechanical testing, in particular the clamping technique [150]. At the level of tendon fascicles, there exists a large body of research and experiments on mechanical properties with mouse and rat tail tendons being particularly popular [8]. This may be due to the ease of isolating mouse tail tendon fascicles with minimal damage. On the other hand, to research the physiology of tendon, rodent tail tendon models are very reproducible

[151, 152, 153, 154], but may not be very comparable to human physiology and therefore studies have also been made on human fascicles [101, 155, 156]. This was also a reason why we did not use rodent models in our studies. Mechanical properties at this level highly depend on the structural organization of the collagen fibers and the cross-linking of fibers [8, 157, 158]. At the level of fibers, the mechanical properties cross over the cell level, since tendon cells directly interact with the structural unit.

The exact mechanism of how tendons transfer forces remains unknown. There are two different hypotheses of the mechanisms, some research showed that tendon fibrils are continuous along the tendon length [159, 160], which means at fibril level and organ level, tissue has similar strains. Some other research supported that the fibrils are discontinuous, which means when fibrils transfer force, there would be forces transmission between neighboring fibrils [161, 162]. In this case, the strain of tendon could be higher than the strain of fibrils and the fibril stiffness could be higher than tendon stiffness.

Tendons can be classified in many different ways. According to the function, they can be classified as energy storage or positional tendons [163]. Positional tendons are those tendons that transmit muscle force to a bone to position a joint and should be relatively inextensible under physiological loads in this process, such as anterior tibial tendon [164]. While energy storing tendons are those tendons that need to store and release elastic strain energy to increase the efficiency of locomotion, such as Achilles tendon and equine superficial digital flexor tendon (SDFT) (Fig. 6) [164, 165, 166]. Compared to positional tendons, energy storing tendons are subjected to higher strain during physiological activity, an in vivo measurement in human Achilles tendon showed an up to 10.3% strain during one-legged hopping, while another in vivo study showed that the maximum stain of positional tendon was only 3.1% [167, 168].

Tendon mechanical properties may relate to the tendon injuries, it is possible that when tendon transfers forces throughout the length, the cross-links at some structural level could be weaker [169]. Research showed that energy storing tendons such as the Achilles, which need to respond to muscle contraction with low energy dissipation are highly cross-linked due the requirement of minimal gliding. But other tendons that are required to perform precise movements at relatively lower forces, are less cross-linked, these tendons are defined as positional tendons such as the digital extensor tendons [170].

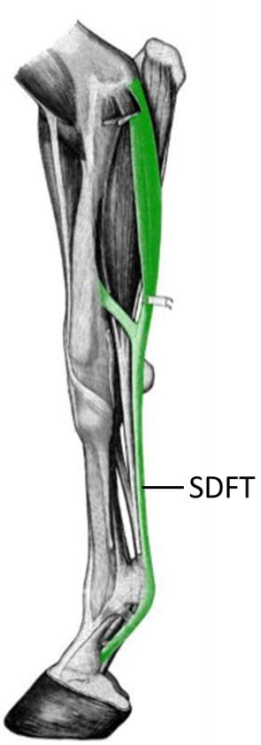

Figure 6. A schematic of equine superficial digital flexor tendon (SDFT).

Region-specific injuries of tendons

As previously mentioned, patellar tendinopathy normally occurs in the proximal and posterior region of the tendon, supported by imaging [26, 40]. This may relate to region-specific strain during loading [155, 171, 172] or regional differences in composition. A study from our group showed that fascicles taken from the anterior region of patellar tendon showed a greater peak and yield stress and also tangent modulus compared to the posterior region [106, 155]. A possible reason could be the differences in lever arm [173], since the lever arm on the anterior regions have greater advantage compared to the posterior regions, the proximal posterior region of the tendon may be stress shielded [171]. This finding showed that human patellar tendon displays region-specific mechanical properties, but whether this relates to the tendon turnover remains unknown.

Tendon turnover

Different tissues have different turnover rate, studies showed that the skin has a half-life of 14.7 years, muscle has a few months half-life, [114, 174, 175, 176], while other tissues have slower turnover, the cartilage collagen has a half-life of 117 years, and dentin is more than 500 years, so do the eye lens and tooth enamel [177, 178]. The turnover varies from tissue to tissue. Tendons display very limited regenerative capacity [114], which may relate to a slow tissue turnover. A previous study investigated the metabolic activity of healing tendon, and showed that it could be enhanced for more than 1 year after an Achilles tendon rupture, meanwhile, the vascularization was up-regulated for more than 6 months [179], the finding showed that the healing process takes considerable time. The same study also reported that the metabolic activity was higher in the core than the periphery at 3 and 6 months, but at 12

months this region-specific response was interchanged to be higher in the periphery. These regional differences in metabolic activity suggest that the overall tendon turnover may also vary with region.

Tenocytes

As we have mentioned above, there are many types of cells in tendon, such as tenocytes, chondrocytes, vascular cells and synovial cell. Tenocytes take account most of the cells in tendons. In the ECM network, tenoblasts and tenocytes constitute about 90% to 95% of the cellular elements of tendons, while chondrocytes, synovial cells and vascular cells constitute the other 5% to 10%. Tenoblasts are immature tendon cells, and they are highly proliferative. When they mature, they are elongated and give rise to tenocytes that occupy the core of the tendon. Tenoblasts and tenocytes are responsible for the synthesis and turnover of collagen and other components of the extracellular matrix network [180, 181, 182].

Generally, tendons have few cells compared to other organs, and the internal part is thought to contain no nerve fibers. But the sheaths of the epitenon and paratenon have some nerve endings [60].

Proteomics

The proteome is the entire set of proteins that are expressed by a given organism or system at a certain time, under a defined condition. While proteomics deals with characterizing biological processes, and enables the identification of increasing numbers of proteins [183, 184]. It represents a large-scale research of proteins, and covers all-level research of proteomes from protein composition and structure to the activity. Proteomic analysis is contributing in understanding the pathogenesis and developing new treatments in many diseases [185].

Usually, proteomics makes use of mass spectrometry (MS), which is an analytical technique that measures the mass-to-charge ratio of ions. In the MS procedure, the sample is ionized by bombarding with electrons. Molecules from sample are broken into charged fragments, and these ions are separated according to their mass-to-charge ratio by the mass-spectrometer. Results would be presented as spectra of the signal intensity of detected ions [186]. The molecules in sample could be identified by the matching to the known masses of the proteins they came from. The mass spectrometry-based proteomics are for example being used in assembling the map of human tissues [187, 188]. MS-based proteomics can identify and quantify hundreds of proteins in one single sample, and does not need antibodies [189]. This technology has successfully been used to identify disease biomarkers and reveal the hidden mechanisms of different diseases in many fields, such as cancer research [190] and osteoarthritic cartilage research [191, 192]. But the research of tendons is limited, so it would be interesting to investigate tendons with proteomic analysis.

Hypotheses

As mentioned in the beginning, tendon plays a very important role in the musculoskeletal system, however, it is still an under researched tissue. One of the important outstanding questions is tendon could recover to some extent after got injured, even though research showed that tendon has a very limited turnover [112, 114]. As mentioned in the preceding introduction it has been shown that sample with limited turnover was taken from the tendon core [114]. Therefore the three manuscripts of the present PhD study investigated the three following hypotheses.

1. AGE accumulation can be used as an indirect measure of collagen turnover and previous work have shown that the tendon core in humans has very limited turnover after 17 years of age. In the first study we therefore hypothesized that the AGEs accumulate to a greater extent in the core compared to the periphery of the tendon, which would indicate that the tendon growth and collagen turnover occurs at the peripheral region of the tendon in a pig model.

2. The first study showed that AGE accumulation is greater in the proximal than the distal part of the porcine flexor tendon, indicating that collagen turnover could be higher in the distal part. The AGE accumulation was also found to be higher in the core than the periphery. With these new findings, we wanted to investigate if there were regional differences in the collagen turnover and biochemistry of healthy human patellar tendon, which could perhaps relate to the region specificity of patellar tendinopathy. Aside from being more clinically relevant, the use of human tissue also enables a more specific measure of turnover by the ^{14}C bomb-pulse method. We hypothesized that the collagen turnover of patellar tendon would be greater in the proximal region, where tendinopathy typically occurs.

3. The first study showed that DNA content was higher in the surface of tendon, and previous studies have successfully used label-free mass spectrometry for protein identification and quantification of ECM components in tendon tissues. So in the third study, we derived tenocyte populations from the core and periphery of horse SDFTs and characterized their proliferation and ECM deposition. We hypothesized that the regional biological variation of a tendon would come from the difference of cell amount, not from the different properties of single cell from different regions.

Methods

The primary purpose of this PhD project was to study regional differences in tendon turnover and therefore the methods to detect tendon turnover are important in the project.

Methods to measure tendon turnover

There are several ways to estimate the turnover of tendon tissue. Microdialysis and stable isotopes are possible methods. Microdialysis is a microinvasive sampling technique that allows for in vivo monitoring of biochemical substances in tissue during various forms of intervention [193]. This method can be applied to the peritendinous space around the Achilles' tendon to measure low molecular mass substances, such as glucose, lactate, glycerol, prostaglandin (PGE_2) and thromboxane (TXB_2) but also peptides generated during formation or breakdown of collagen. A previous study using microdialysis of procollagen peptides taken from the peritendinous region suggested that collagen protein synthesis is increased after exercise in the human Achilles tendon [193]. This was supported by another study using stable isotope labelled amino acid incorporation into patellar tendon [194].

Aspartic acid racemization method

Measuring the rate of amino acid racemization is one of the widely accepted methods in assessing tissue age. Homochirality is a common property of amino acids, and they are incorporated into protein in the L-form, but over time, spontaneous racemization occurs and they convert to the D-form. Aspartic acid is amongst the most rapidly racemizing amino acid [195] and therefore measurement of the ratio of D-aspartate (D-Asp) to L-aspartate (L-Asp) is used to assess the protein half-life. With this method, a 200 years half-life of horse high stress tendon was reported [112]. This method however has some limitations, it depends on environment, such as the average temperature, humidity, acidity (pH), and other characteristics.

Gold standard: ^{14}C bomb pulse method

As the result of nuclear bomb testing during 1955-1963, ^{14}C level in the atmosphere dramatically increased, referred to as the ^{14}C bomb pulse. The $^{14}CO_2$ was incorporated into plants, and then were eaten by animals and humans, resulting in tissue ^{14}C levels that are close to the atmospheric concentration when the tissue was formed.

It should be noted that the ^{14}C method is both a stable and a non-stable (radio-) isotope method. When this method is used to date very ancient things, such as fossils from thousands of years ago, it is the radioactive decay that is measured. However, when the ^{14}C method is used as the bomb pulse method to measure on shorter time scales (<100 year) it is used as a stable isotope, because it only decays a little. The ^{14}C method is considered as a gold standard for measuring long-term turnover, as their usage can result in exceptionally clear records. This ^{14}C method can be utilized to measure the turnover of different tissues. The method measures the percentage of ^{14}C carbon isotope in tissues. Through the difference relative to modern carbon percentage, we can tell the age of the tested tissue because the ^{14}C concentration in the atmosphere following the bomb pulse is well recorded.

AGEs measurement

As described previously, advanced glycation end-products (AGEs) are proteins that have been glycated as a result of exposure to sugars, resulting in a number of different compounds [196, 197]. AGEs could also be used as an indicating factor in measuring tissue turnover. Studies have reported that the accumulation of AGEs in cartilage and skin collagen increases linearly with age [123, 178], other studies have shown that the carboxymethyllysine (CML), carboxyethyllysine (CEL) and pentosidine products accumulate linearly with age, and that it does so to a greater extent in cartilage, which has a slow turnover than in skin with a faster turnover [176]. This suggests that AGE accumulation may be used as an indirect measure of collagen turnover. Moreover, in tissues with hyperglycemia like in diabetes the accumulation is even higher [176, 198, 199].

Study 1

Animal model

The first study made use of legs from Göttingen minipigs collected in relation to a larger prospective intervention study investigating atherosclerosis, diabetes, and obesity [200]. With this animal model we were able to use the entire length of relatively large tendons, which facilitated the regional analyses. The study was approved by the Animal Experiment Inspectorate, Ministry of Justice, Denmark [200]. Previous research showed that mice fed with food containing high AGE levels lead to higher accumulation of AGEs in their tissue, such as heart, Achilles tendon and tail tendon [201, 202, 203]. As a negative consequence, stiffness was increased in these tissues. In the intervention study that the pig tendons were collected from, animals were fed with different diets to investigate the potential effects in different tissues and disease. However, in our study we did not plan to compare the effect of different diets and therefore we used tissue from the diabetic animals and lean control animals with relative normal diet.

All animals were acquired at 6-7 months of age, at which point the treatments started, lasting until they were sacrificed at 20 months. For the diabetic pigs in this study, diabetes was chemically induced by IV injection of streptozotocin (STZ) (Sigma-Aldrich A/S), then insulin was used to maintain the fasted morning blood glucose between 14 and 16 mM. Another point of the treatment was the different diets; in the lean control group, four minipigs were fed with lean standard minipig (SDS) diet for the entire period, which is considered as a normal diet (Special Diet Service, UK). In the diabetic group, there were two different diets, two minipigs were fed with a high fat diet (5B4L 1% cholesterol, TestDiets) and four pigs were fed with the same diet with extra salt (5B4L 1% cholesterol + 2.5% salt, TestDiets) for the entire period. In our tendon study, the two diet groups for the diabetic animals were combined into a single diabetic group, since no differences were observed in any of the measured parameters. Furthermore, since our study looked at regional differences within the same animal this alleviates issues related to differences between animals. Even though the diabetic animals were combined, the sample size in the study was still limited, but several significant differences could still be detected.

One hind limb (Fig. 7A) from each of ten pigs were used. Animals were 20 months old and ~38 kg in the lean control group when they are sacrificed. For reference, Göttingen minipigs are considered mature at ~35 kg and ~24 months of age [204].

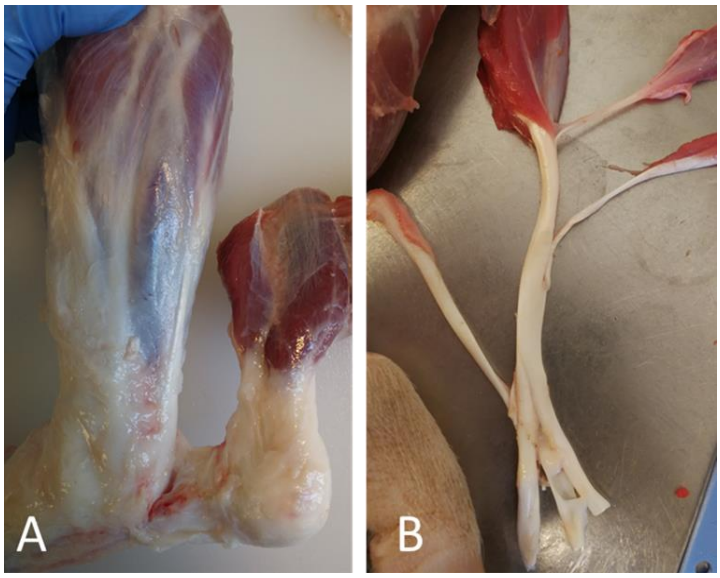

Figure 7. Schematic diagram of a dissected hind limb. A) Skinned hind limb showing the porcine Achilles tendon and deep digital flexor tendon. B) Dissected deep digital flexor tendon.

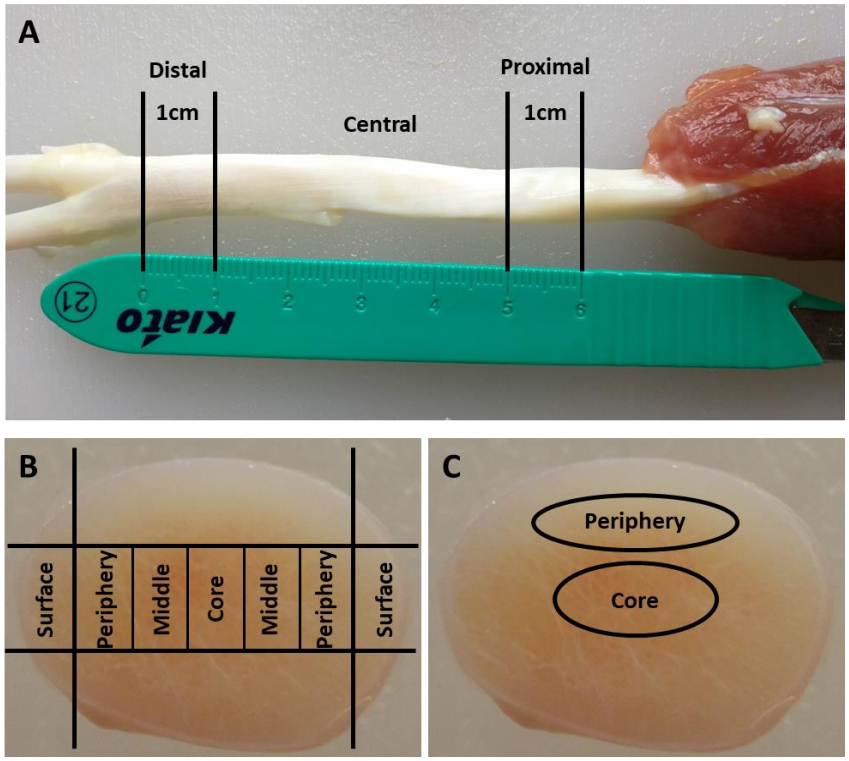

Figure 8. Schematic diagram of deep digital flexor tendon. A) 1 cm long sample taken from proximal and distal parts were used for biochemical measurements, while the central part was used for the mechanical test. B) Cross section of sample taken from the proximal and distal part. Four regions were separated: surface, periphery, middle, and core. C) Fascicles for mechanical test were taken from the core and the periphery of the central tendon segment. (Figure comes from Paper 1 [205] with permission from Taylor & Francis)

Sample preparation

As we have emphasized, the initial purpose of the study was to investigate the turnover differences in different regions and consequently the sample preparation and how regions were defined was very important.

In this study, the deep digital flexor tendon was used instead of the Achilles tendon, because of its large diameter and circular shape. From the clinical perspective, the Achilles tendon would perhaps be more relevant [16], but it is very short in pigs, and is also a fusion of three tendons coming from the soleus and two heads of gastrocnemius [206], which would have precluded the detailed regional analysis possible with the flexor tendon.

All surrounding tissues such as skin, fat, and muscle were removed. The tendon was cut into three parts, proximal, central, and distal, respectively. The proximal part was 1 cm long and taken approximately 0.5 cm distal to the muscle insertion. The distal part was also 1 cm long and taken approximately 0.5 cm proximal to the bifurcation of the toes. The central part was approximately 4 cm long depending on the total tendon length.

In this study, AGE measurement was used as a method for investigating tendon turnover, with a potentially added effect in the diabetic group since hyperglycemia may increase AGE accumulation in the tissue. To perform the AGE measurements, biochemical test were performed on proximal and distal parts (Fig. 8A, 8B). In addition, the turnover may relate to the composition of tendon, so we also measured other components. Finally, the mechanical properties were also assessed by mechanical testing of fascicles in the central part (Fig. 8A). It was impossible for us to measure biochemical and mechanical properties on the exact same regions of tissue, which prevents a direct relation of mechanics and biochemistry and this is a limitation of this study.

As mentioned, biochemical tests were performed on the proximal and distal parts (Fig. 8A), and the cross section of tendon is shown in figure 9B. We separated the cross sections into 4 different regions (Fig. 8B), the detailed information about how we define the regions was described in the paper.

Biochemical test

Biochemical analyses were performed based on papain digested tissue according to previously published protocols [207]. The papain digest was used for analyses of AGEs by autofluorescence, DNA content, and glycosaminoglycan (GAG) content. The samples were kept wet to avoid changes in fluorescence due to drying and consequently values are normalized to wet rather than dry weight. Furthermore, a separate piece of tissue was taken for measuring collagen content, which was freeze dried first and then weighed. Secondly, the collagen sample was delipidated in acetone, the acetone was removed and samples were freeze dried and weighed again. Finally, samples were hydrolyzed in hydrochloric acid (6 M, 110°C).

Papain digestion was performed using papain (P3125, Sigma, 0.125 mg/ml) in PBE buffer (100 mM sodium phosphate, 10 mM EDTA, pH 6.5) with L-cysteine [207]. Samples were digested in an oven at 60 °C overnight. The next day tubes were removed from the oven, vortexed and spun briefly.

AGE content

As we have mentioned above, there are many methods to investigate the tendon turnover. The ‘gold standard’, ^{14}C method cannot be applied here, since it requires tissues with

a long life, while the minipigs were only 20 months old, which is too short to perform the ^{14}C method. The aspartic acid racemization method normally requires a stable temperature during measuring, however, the porcine samples had been frozen for a while and we do not have the setup for doing the measurement in our lab , we could not use this method either.

In this study we therefore estimated turnover from AGE accumulation. High Performance Liquid Chromatography (HPLC) is a method often used to measure the AGE called pentosidine, which can be used as an indirect factor indicating the turnover, however, when the study was carried out, the lab that we have previously cooperated with could not perform this measurement for us. Instead AGE accumulation was determined from total tissue autofluorescence, which has the downside that it is an indirect method that can be affected by other sources of fluorescence [208]. In addition, the autofluorescence should only be considered a marker of AGE accumulation since several AGEs, including the major AGE cross-link glucosepane, are not fluorescent. However, autofluorescence measured in a similar way has previously been shown to correlate well with turnover measured by aspartic acid racemization [112].

AGE accumulation was assessed in papain digests by autofluorescence [209, 210, 211]. Triplicate sample were pipetted into wells of a 96-well black microwell plate (237107, Thermo scientific) and the plate was read on a fluorometer (Wallac1420 Victor, Perkin Elmer).

DNA content

DNA content was also measured in this study, as it may relate to the tendon turnover. It was measured by binding of Hoechst [207]. Papain digests and DNA standards made from salmon DNA were plated in triplicate into a black 96-well microwell plate. To each well, TEN buffer (100 mM NaCl, 10 mM Tris-HCl, 1mM EDTA) was added and the autofluorescence was measured. Subsequently freshly prepared 0.8 µg/mL Hoechst 33258 (H3569, Invitrogen) in TEN was added to all wells and fluorescence was measured immediately. DNA content was calculated from the standard curve based on the difference between fluorescence before and after addition of Hoechst. This was modified from the original reference where they added the Hoechst from the start and used the absolute fluorescence value, rather than the difference between before and after addition of Hoechst. In our study, we calculated the difference in fluorescence, because our samples have a high level of autofluorescence to begin with. The presence of dust or other small particles, can be a problem for fluorescence measurements, since it may reflect the light. We therefore made triplicate measures and used the median to avoid strange high values possibly caused by a dust particle.

There are other methods that could be used to determine cellularity, such as cell culture many of which require live cells, but in this study, the pig legs had already been collected and frozen, so those methods would not work here.

GAG content

For GAG quantification, papain digest was mixed with 1,9-dimethylmethylene blue (DMB) (341088, Sigma-Aldrich) solution (38 µM DMB in 40 mM NaCl, 40 mM glycine, pH 3.0), and absorbance was read at 540 and 595 nm (the reading at 595 nm was subtracted from the reading at 540 nm). Sample values were compared to a standard curve made with 12.5-150 µg/mL of chondroitin sulfate B (C3788, Sigma-Aldrich).

Collagen content

Hydrolysates were dried on a heating block at 95°C, rehydrated with distilled water, and dried again. The hydrolysates were resuspended in 0.2 M HCl and diluted 100 fold in the buffer used for the hydroxyproline assay (acetate-citrate). Hydroxyproline was measured by a colorimetric assay based on 4-dimethylaminobenzaldehyde, the detailed procedure used in our lab has previously been published [212]. Hydroxyproline concentrations were converted to collagen concentration by multiplying with 7.5 (13.3% hydroxyproline in collagen) [213]. This factor of HYP percentage in collagen is different in different species [214] and we did not determine it for the present samples specifically, but for comparability we used a factor of 7.5, which we have also used in previous studies. Another potential problem is the method of drying, in this study, the hydrolysates were dried in a heating block in air, this may have reduced the amount of HYP, because some of the HYP could react with oxygen. This was noted in study 2 where we improved the method by drying the hydrolysates under nitrogen flux, which reduced the HYP loss.

Mechanical test

Mechanical measurements were a secondary objective of this study. In the very beginning of the study, we mistakenly thought that there were only 2 instead of 4 animals in the lean control group. So we decided to focus on the diabetic group. After submitting the manuscript and based on comments from the reviewers, we added the lean control group, which we now realized had 4 animals, thereby making it more suitable for statistical analysis. However, due to time limitations and other practical reasons, we only did the biochemical measurements on these samples but did not do the mechanical tests on the lean control group.

In this study, fascicles were dissected from each of the core and periphery of the central 4 cm piece of the tendon (Fig. 8A and 8C). When we were dissecting the fascicles, it was only possible to isolate fascicles from two areas (core and periphery) (Fig. 8C) rather than the four regions used for the biochemical measurements, because we had to ensure the integrity and not cut too many fascicles. Fascicles did not run straight in the tissue and several fascicles were bound together making it difficult to dissect them without a tear or cut. Twelve individual intact fascicles around 20 mm long were obtained from each region, discarding shorter fascicles. A total of 144 fascicles from 6 tendons were obtained, and mechanical tests were conducted on these fascicles.

Mechanical measurements were performed using a microtensile testing apparatus (20N tensile stage, Petri dish version, Deben Ltd) at a sampling rate of 10 Hz (Fig. 9A and 9C). The micromechanical testing procedure has been described in detail previously [155]. In this study, 2 cm long fascicles were set on the clamps with the middle 10 mm wrapped in PBS (0.15 M, pH 7.4) moistened gauze and the ends allowed to air dry at room temperature. The dried ends were glued with cyanoacrylate to the aluminum specimen mounting plates of the device. Fascicles were then immersed in PBS solution during the testing. The nominal testing length of each specimen was 10 mm and the testing rate was set at 6.0 mm/min. The start point was defined when the stress value first reached 0.5 MPa. Peak modulus (slope at the steepest point of the curve), peak stress (considered to be the ultimate failure point), and the strain corresponding to the peak stress were determined. We did not perform preconditioning prior to the mechanical test, because preconditioning should be performed far from the failure point, which is typically ~2-3% strain, but in this study the pig tendon fascicles broke at ~4-5%, so 2-3% preconditioning would be too close to failure. Microscopy images were taken while the

fascicle was mounted and immersed in PBS solution, when tension just started rising. Fascicle diameter was measured from the microscopy images and used to calculate fascicle cross-sectional area assuming circularity.

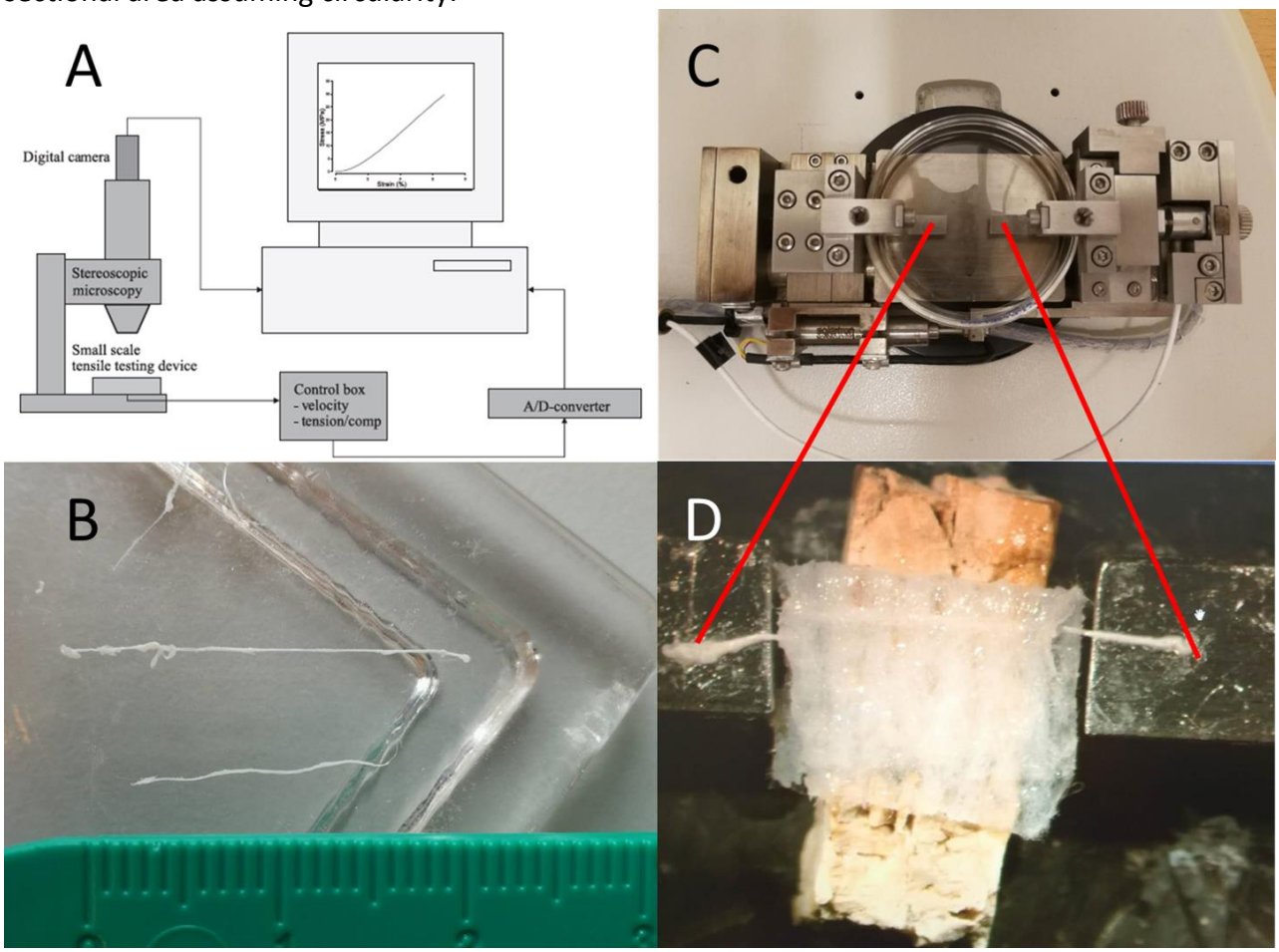

Figure 9. Schematic diagram of mechanical testing. A) Setup with the stereoscopic microscope mounted with a 10-Hz digital camera [155]. B) Fascicle dissected from the central tendon, with a 2 cm length and 0.5 mm diameter. C) Small-scale tensile testing device placed under the microscope as shown in A. D) Large-scale of the plated tendon fascicle with the central part moistened in wet gauze.

Data analysis and statistics

All data are presented as means ± SE. Paired students t-tests were used for comparing the mechanical differences between the core and periphery.

To compare regional differences in the biochemical tests on samples from the proximal and distal parts, two-way ANOVA was used with longitudinal (proximal, distal) and radial (core, mid, periphery, surface) position as the two factors. Analysis of variance (ANOVA) is a statistical model and the associated estimation procedures, which is used to analyze the differences among group means. The two-way ANOVA not only aims at assessing the main effect of each independent variable, but also interaction between them. For a main effect of longitudinal position, comparisons were made between the proximal and distal region at each radial position with a Sidak correction for multiple comparisons. For a main effect of radial position, each radial position was compared to the core region at each of the longitudinal positions, with

Dunett's correction for multiple comparisons. Differences were considered significant when $P < 0.05$. All statistical tests were performed in GraphPad Prism v.7.0 (GraphPad Software, La Jolla).

Study 2

Study design

The first study showed that the AGE accumulation was found to be higher in the core than the periphery, and it was also greater in the proximal than the distal part of the porcine flexor tendon, indicating that collagen turnover could be higher in the distal part. Having seen this effect in the young pig tendon, we wanted to investigate more clinically relevant adult human tissue, which also allowed us to apply the ^{14}C method as a more direct measure of turnover. Regional differences in turnover could perhaps relate to the regional specificity of patellar tendinopathy. In the second study, to investigate collagen turnover, three independent methods were applied; 1) biochemical measurement of autofluorescence as a general marker of AGE accumulation (just like we had done in study 1), 2) direct measurement of tissue age by the ^{14}C bomb pulse method (the gold standard), 3) mass spectrometry based measurement of specific non-fluorescent AGEs. To support these measurements, basic composition (content of collagen, glycosaminoglycans (GAGs) and DNA) was also measured on the same samples used for fluorescence measurements, similar to what was done in study 1.

Tendon samples

Tissues were collected from different regions of patellar tendon (n=5) from five cadavers between 68 to 86 years of age (mean 76.2 years ± 7.3 (SD); 4 males and 1 female). All subjects were born from 1931 to 1950, before the nuclear bomb test, as we mentioned above, the ^{14}C concentration in the atmosphere dramatically increased during 1955-1963. If we had included subjects born after 1955, the same atmospheric $^{14}C/^{13}C$ value present at their birth would have occurred again at a later year (see Fig. 18), which could have made interpretation of the results more difficult. Tissues from the anonymous Danish body donation program were collected and their use do not require separate ethical approval.

Samples were taken from 10 different regions of each patellar tendon (Fig. 10). The insertion was taken from the anterior tendon above the distal patella pole. The proximal tendon was divided into side and central parts with the central part divided into an anterior and a posterior. A similar scheme was used for the mid-tendon, which was divided into side and central parts with an anterior and posterior layer. Finally, a sample was taken from the distal, central tendon just proximal to the insertion. In this study, we did not divide the patellar tendon into a core and periphery region because patellar tendon is quite flat compared to Achilles tendon and pig's flexor tendon, and it was therefore technically not possible to separate the tendon into core and periphery.

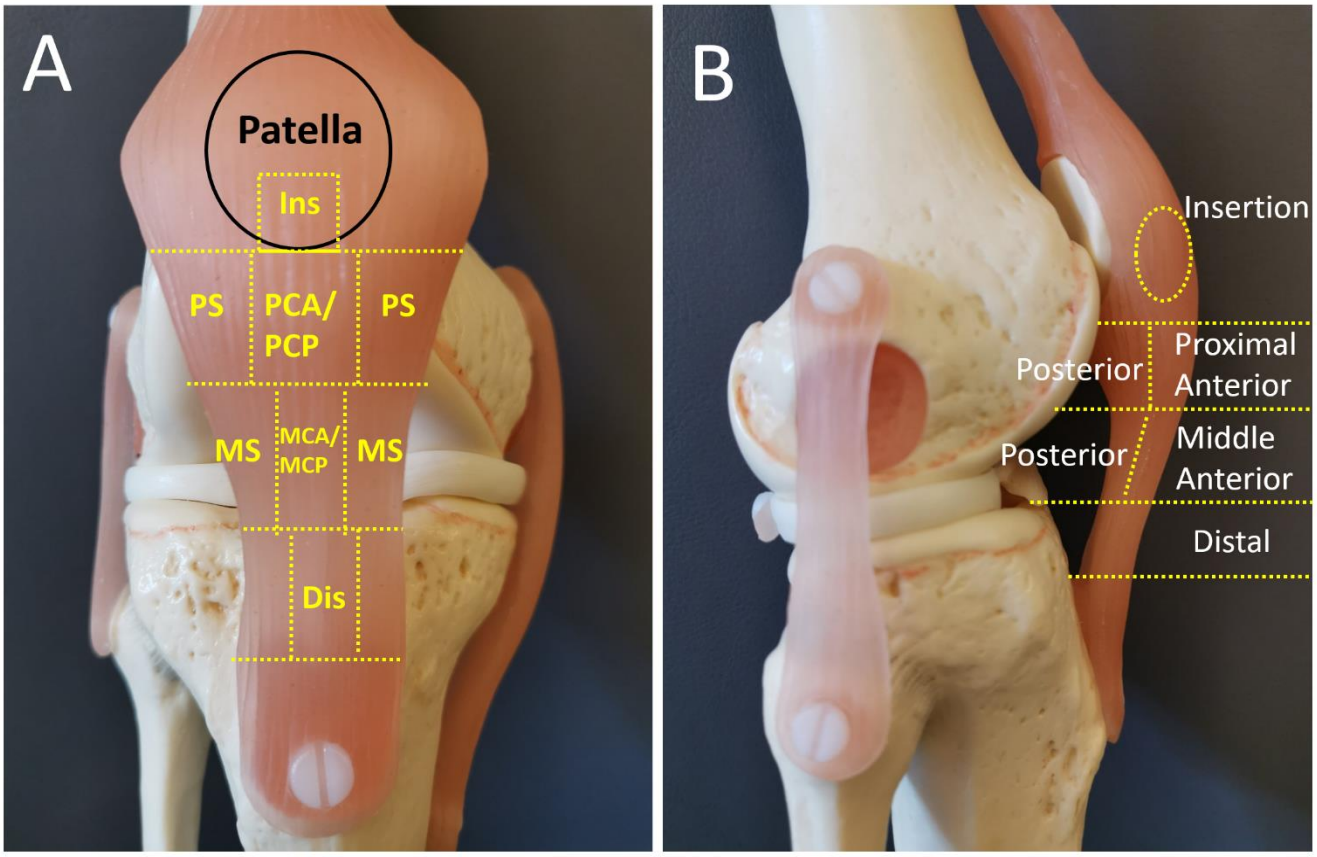

Figure 10. Schematic diagram of patellar tendon. A) Front view of patellar tendon. Samples were taken from 10 different regions. The insertion on patella (Ins). The Proximal Side (PS), the Proximal Central Anterior (PCA) and Proximal Central Posterior (PCP) layers. A similar scheme was used for the mid-tendon, divided into Middle Side (MS), Middle Central Anterior (MCA) and Middle Central Posterior (MCP) layers. Finally distal central tendon (Dis). B) Side view of patellar tendon. In proximal and middle parts of tendon, a sample was taken from anterior and posterior regions. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

Sample preparation

In this study, sample preparation was a bit complicated because we used 3 different methods to detect collagen turnover and remaining tissue was used to do the biochemical measurements. We therefore included a flow chart of sample analyses (Fig. 11).

This section briefly illustrates how we prepared the samples and the detailed information can be found in paper 2. A big piece was taken from each of the 10 different regions. These samples were further divided for biochemical measurements, ^{14}C bomb-pulse dating and measurement of specific AGEs. To ensure homogeneity the samples were initially cut into smaller pieces, which were then scrambled before dividing between the analyses. All samples were freeze dried overnight before further measurements.

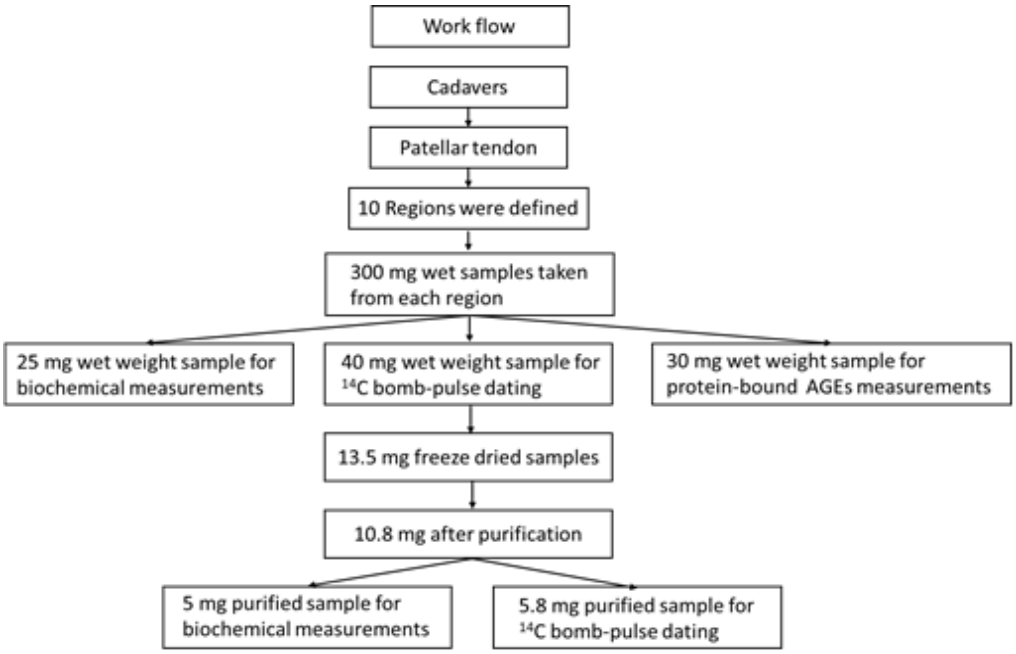

Figure 11. Flow chart of sample analyses. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

Biochemical measurements

To remove lipids, samples were washed 3 times in acetone. Subsequently, samples were freeze dried overnight and weighed. The biochemical analyses were similar to study 1 [205], which was based on published protocols [207]. Samples were digested in papain then the papain digest was used for analyses of fluorescent AGEs (autofluorescence), DNA content, glycosaminoglycan content and collagen content.

As described later, the same biochemical measurements were also performed on samples after purification for ^{14}C bomb-pulse dating.

Fluorescent AGE measurement

As described in study 1, AGE accumulation was measured by autofluorescence [209, 210, 211]. Papain digest (25 µL) was mixed with 75 µL distilled water for a 4-fold dilution. The subsequent steps were similar to study 1. In study 1 we had avoided freeze drying samples prior to this measurement because we were unsure if drying would alter the fluorescence. However, wet weight is generally less accurate than dry weight due to difficulty ensuring a fixed level of hydration and we therefore wanted to be able to dry the samples if possible. We conducted a small test with triplicate measures on three different samples which had been homogenized and divided into a wet and a freeze-dried portion. The fluorescence normalized to wet weight was 2524 ±254 AU/mg (mean ±SD) for the wet sample and 2486 ±146 AU/mg for the freeze dried, showing that freeze drying did not affect the measurement.

DNA content and GAG content

DNA content and GAG content measurements were similar to study 1, and detailed information was in the paper 1 and 2.

Collagen content

For collagen content measurement, it was a little different from study 1. As in study 1 collagen content was assessed by a hydroxyproline assay [205, 212], however, in study 2 it was measured in the papain digest rather than a separate sample by hydrolyzing 5 µL in 195µL 6M HCl. After hydrolysis the next step is to dry the sample, but in study 2 samples were dried under nitrogen flux rather than in air. This was different from study 1, and it helped to protect the sample hydroxyproline, likely from getting oxidized. After obtaining the dry hydrolysates, the next steps were similar to study 1.

^{14}C bomb-pulse method

Sample purification

As we have mentioned above, the preparation of samples in this study is very important. To avoid modern carbon from GAGs, fat and other non-collagenous substances, a collagen purification procedure was performed as described in a previous paper [118]. This is because due to the higher turnover of these tissues (as we mentioned in the introduction), they may contain more modern carbon. For ^{14}C measurement, on average 13.5 mg freeze dried sample was used (Fig. 11). To remove GAGs, fresh hyaluronidase solution was added and incubated to break down the hyaluronate. Then samples were washed with sterile saline and trypsin solution was added to samples to remove cellular and other non-collagenous proteins. Subsequently samples were washed with PBS buffer, KCl solution and distilled water. Samples were freeze dried, and lipids were removed by acetone washes. Finally samples were washed 3 times in ethanol, followed by 3 times distilled water wash. This last step was to remove carbon originating from the acetone itself. Sample dry weight was around 10.8 mg after purification. To assess the influence of the purification, part of the sample was analyzed for fluorescence, GAG and collagen in the same manner as described in biochemical measurements.

^{14}C analysis

From the biochemical tests, we did not see any differences in collagen turnover (fluorescence) among the different regions and because of the high cost of the analyses (3500 DKK per sample for ^{14}C dating), we decided to focus the ^{14}C and specific AGE measurements on four regions: the insertion, the distal, Middle Central Anterior, and Middle Central Posterior region. The results of biochemical tests showed that the insertion region and distal region contained more GAGs compared to other regions, and tended to higher DNA (see Fig. 17B and C). So that these two regions were picked, then one sample from each part was picked in the central patellar tendon.

We did not do the ^{14}C measurements by ourselves, instead we prepared the purified samples and sent them to the AMS ^{14}C Dating Centre, Aarhus University to do the measurements. Samples for accelerator mass spectrometry (AMS) were combusted with CuO in sealed combustion tubes at 950°C, and converted to graphite prior to ^{14}C analysis at the 1 MV Tandetron accelerator [216]. The radiocarbon dating results are reported according to international convention [217], and ^{14}C content are given as percentage modern carbon (pMC) based on the measured ^{14}C/^{13}C ratio. Stable isotope values of δ 13C, δ 15N carbon and nitrogen

fraction (by weight) and carbon/nitrogen (C/N) atomic ratios were measured by continuous-flow isotope-ratio mass spectrometry.

Specific AGE content

As described for ^{14}C analyses, only four of the regions were included in this analysis. Specific AGEs were measured as previously described [218, 219, 220]. Again, we did not perform the actual measurements ourselves, but we prepared samples and sent them to our collaborators at the Maastricht University. In brief, high-performance liquid chromatography (HPLC) was used to quantify pentosidine with fluorescence, as described in a previous paper [220]. The specific AGEs chosen for analysis in this study, to a large extent, are the ones that the lab is able to measure. However, pentosidine is among the most commonly measured AGEs in the past due to being fluorescent and carboxymethyl- and carboxyethyl-lysine (CML and CEL) have also been commonly reported. As for methylglyoxal it is present in fairly large quantities compared to the other AGEs and may therefore be more functionally relevant and not just be used as a marker for AGE accumulation. Ultra-performance liquid chromatography tandem mass spectrometry (UPLC MS/MS) was used to quantify CML, CEL, MG-H1 (methylglyoxal-derived hydroimidazolone) and lysine [218, 219]. Concentrations of pentosidine, CML, CEL and MG-H1 were adjusted to levels of lysine and expressed as nanomoles per millimole lysine. Hydroxyproline could not be measured directly in these samples, but correlated well with lysine in previously analyzed tendon samples. A ratio (Hydroxyproline/Lysine) of 3.29 was used to estimate the hydroxyproline concentration (Fig. 12).

Correlation between hydroxyproline and lysine

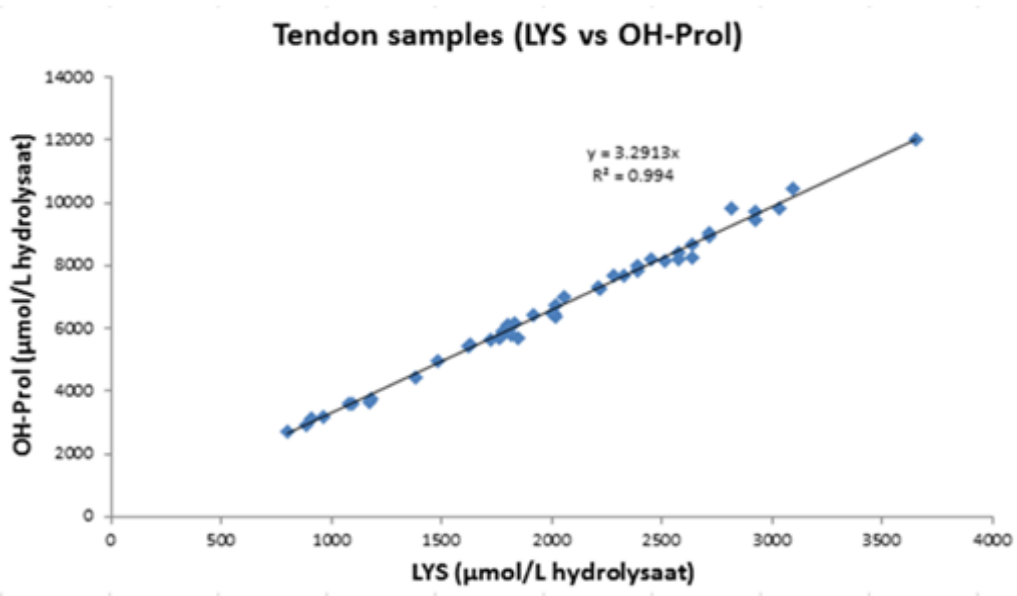

Figure 12. The data are based on previous (unpublished) measurements of hydroxyproline and lysine on tendon. A ratio (Hydroxyproline/Lysine) of 3.29 was used to estimate the hydroxyproline concentration. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

Data analysis and statistics

All data are presented as means ± SE. For comparing different regions, repeated measures one-way ANOVA was used, with Tukey's correction for multiple comparisons. Differences were considered significant when $P < 0.05$. All statistical tests were performed in GraphPad Prism v.8.0 (GraphPad Software, La Jolla).

Study 3

As we mentioned above, the first study showed that DNA content was higher in the surface of tendon, and previous studies have successfully used label-free mass spectrometry for protein identification and quantification of ECM components in tendon tissues. So we designed the third study to investigate tenocyte populations from the core and periphery of horse SDFTs and characterized their proliferation and ECM deposition.

Animals

Tendon tissue samples used in this study were from a larger prospective intervention study. All horses were standardbreds, mares or geldings between 4 and 10 years of age. For this current study, five horses between 4 to 10 years of age (mean 6.4 years ± 2.3 (SD); three mares and two geldings were selected. All five horses were untrained. Before inclusion in the study an experienced equine surgeon performed a lameness examination of all included horses and only those with lameness grade <3 were included.

Tendon cells

To simplify the text below, cell media used in this study are listed here:
Pen-Strep: Final concentrations of 50 U/ml penicillin and 50 µg/ml streptomycin in the solution.
Culture-medium: DMEM/F-12 supplemented with 10% fetal calf serum (FCS) and Pen-Strep.
Matrix-medium: Culture-medium supplemented with 200 µM ascorbate-2-phosphate (ascorbate is included to promote ECM deposition).

Superficial digital flexor tendons (SDFT) were dissected from the left forelimb. All surrounding tissues were removed. Three 1-cm sections were made from the mid-portion of the tendon 8-10 cm proximal to the sesamoid bone (Fig. 13A and B). From each 1-cm section the lateral and medial parts of the SDFT were removed (Fig. 13C). The remaining SDFT was divided into three parts: the anterior and posterior were considered as the periphery, the inner part was considered as the core of the tendon (Fig. 13C). Tendon cells were released from the tendon tissue as previously described [221]. In brief, tendon tissues were washed in phosphate buffered saline (PBS) with Pen-Strep. Afterwards, tendon tissue was cut into smaller pieces and incubated overnight in collagenase type 2 prepared in DMEM/F-12. Cells were then pelleted by centrifugation. The cell pellets were resuspended in PBS, pelleted again and resuspended in culture-medium. Medium was changed every 3-5 days. After approximately 10 days of culture, tendon cells derived from the core and the periphery of horse SDFT were frozen at passage 1 in liquid nitrogen and then stored at -80°C.

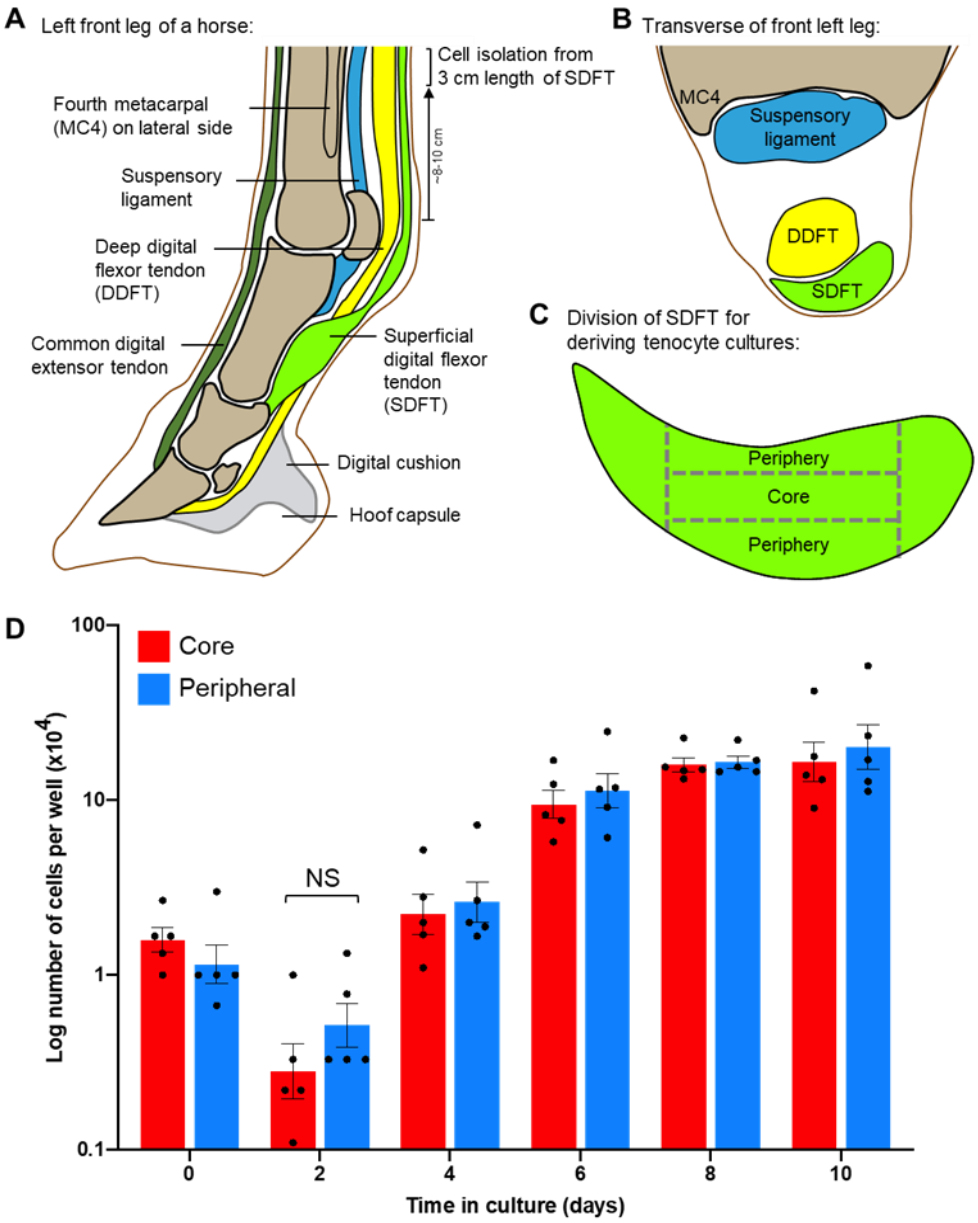

Figure 13. Proliferation of tendon cells isolated from the core and periphery of horse SDFTs. The SDFT was dissected from the left forelimb (AB). The SDFT was separated into core and periphery tissues and the tendon fibroblasts within were released and cultured (C). Mean (log) number of tendon cells from the core or periphery of horse SDFTs were counted every 2 days during 10 days of culture (n=5) (D). (Figure comes from Paper 3)

Growth curve assay

Experiments were performed based on a previous published protocol [222]. To detect the proliferative capacity of tenocytes derived from the core and periphery of horse SDFT in vitro, cells between the passage of 1 and 3 were plated in a 24-well plate at a density of 1×10^4 cells/well and cultured for 10 days in culture-medium. For each cell line, trypsinized cells from three individual wells were counted every 2 days (at day 0, 2, 4, 6, 8, 10) and from each well, the mean number of cells was calculated from three measurements. Cells were counted

manually with haemocytometer (a counting-chamber). The gridded area of the haemocytometer consists of nine squares, and in our study, numbers were counted from 5 of those squares then used for calculating the total number.

Staining of ECM deposition

Immunofluorescence staining and Alcian blue staining were performed in this study. For each cell line, cells were seeded onto glass coverslips in a plate and cultured for 4 days in matrix-medium. Afterwards, cells were fixed with 0.5 ml Histofix. The fixed cells were then washed with PBS, permeabilized, and finally coverslips were washed and incubated as follows.

To perform the immunofluorescence staining, coverslips were blocked with BSA and primary antibodies specific to type I collagen were added to the coverslips and incubated overnight. Subsequently, coverslips were washed and incubated with Alexa-568-conjugated secondary antibodies while protected from light. Finally, coverslips were washed and mounted with DAPI.

Alcian blue was used to stain acidic polysaccharides, such as GAGs. The coverslips were stained with 1% Alcian blue prepared in 3% glacial acetic acid. Afterwards, coverslips were washed, air dried and mounted without DAPI. For both type I collagen and Alcian blue, images were captured by an Olympus BX51 microscope with CellSens software, using a 10x objective.

Label-free quantitative LC-MS-based proteomics analysis

For isolation of proteins to be used in proteomics analyses, cells from each cell line (3×10^4 cells/cm^2) were seeded into two wells of a 6-well plate and cultured for 4 days in matrix-medium. Afterwards, cells were washed in PBS and lysed for mass spectrometry (MS) analysis. Protein concentrations were determined by Quick Start Bradford Protein Assay according to manufacturer's instructions. After protein isolation, the samples were handed over to our collaborators at DTU for proteomics analyses and we did therefore not perform the following steps ourselves.

The isolated proteins were digested with Lys-C protease and trypsin. Subsequently samples were acidified with trifluoroacetic acid and vortexed. Acidified samples were then filtered through equilibrated stage tips containing Empore C18 filters. Peptides were eluted in acetonitrile and formic acid, dehydrated and resuspended together with indexed retention time peptides. Peptide concentrations were measured using a spectrophotometer.

For each sample, peptides were loaded onto a trap column, connected in-line to a C18 analytical column, using the Easy-nLC 1200 high-performance liquid chromatography system. Peptides were eluted and measured on the Orbitrap Fusion instrument using a data-dependent-MS/MS (DD-MS2) top speed method. Full MS spectra were collected with a scan range of 400–1500 m/z. Ions with a charge state <2, >7 or unknown were excluded. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility.

Analysis of MS data

The raw files were analyzed using Proteome Discoverer 2.4. Label-free quantitation (LFQ) was enabled in the processing and consensus steps, and spectra were matched against the Equus caballus (horse) validated and unvalidated databases obtained from Uniprot. Dynamic modifications were set as oxidation (M), deamidation (N, Q) and acetyl on protein N-

termini. Cysteine carbamidomethyl was set as a static modification. All results were filtered to a 1% FDR, and protein quantitation done using the built-in Minora Feature Detector.

The normalized protein intensities generated by LC-MS were analysed using the R-based integrated web application Differential Expression and Pathway version 0.90 (iDEP) [223]. Only proteins for which two peptides were detected were analyzed.

Statistics

For statistical analysis comparing cell growth curves, repeated measures two-way ANOVA was used, with Sidak's correction for multiple comparisons using GraphPad Prism v.8.0 (GraphPad Software, La Jolla).

Results and discussion

Study 1

In the first study we aimed to investigate the regional differences in tendon turnover. We hypothesized the AGE accumulation would be higher in the core in the cross-section of tendon, but we did not suspect that the AGE accumulation could be different along the length of the tendon. It was also unexpected that the AGE accumulation differed in the diabetic and lean control groups.

Mechanical properties from the central tendon

The mechanical results are shown in figure 14. The peak modulus and peak stress in the core were higher compared to the periphery (Fig. 14A and 14B), and the strains are similar in the core and periphery (Fig. 14C). These data show that the core had higher mechanical properties than the periphery of porcine flexor tendon, which corresponds to previous studies on human tendon [155, 224]. Studies on different tendons from different animal species have yielded very dissimilar mechanical results [155, 224, 225]. Studies on human tissue have shown that anterior fascicles of the patellar tendon have higher mechanical properties than the posterior fascicles [155, 224]. In contrast, research on rabbit patellar tendon have shown a lack of regional differences in mechanics [225], although structural differences have been reported [226].

After the mechanical tests were performed, biochemical tests were conducted on the tissue left over. The fluorescence was similar in both regions, but the collagen content was greater in the core compared to the periphery. This difference in collagen content could be a possible explanation for the mechanical differences. However, it should be noted that only the wet weight of samples was recorded, which is a limitation.

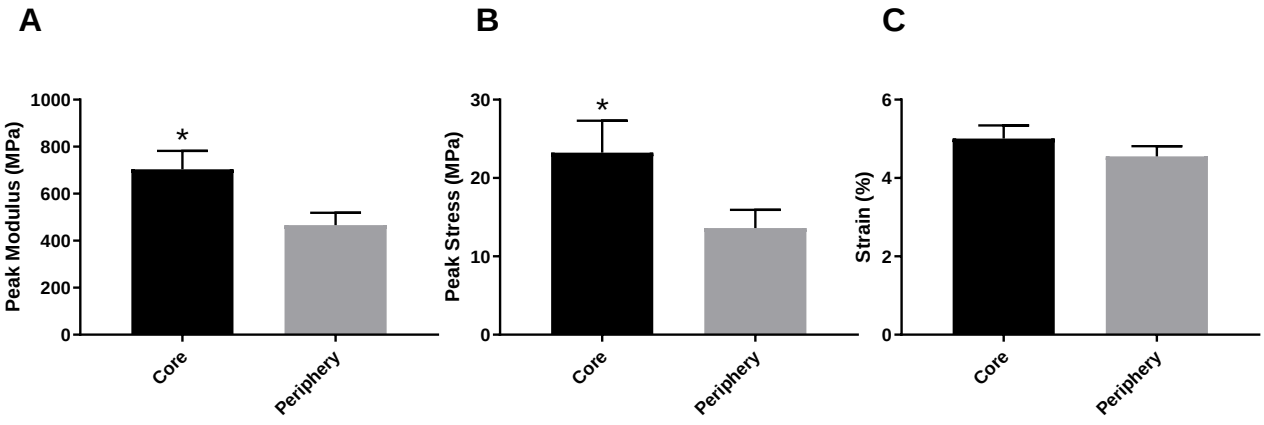

Figure 14. Mechanical test results of fascicles taken from the core and periphery of the central tendon segment. A) Peak modulus. B) Peak stress. C) Strain at peak stress. Mean±SE, * $p<0.05$, N=6. (Figure comes from Paper 1 [205] with permission from Taylor & Francis)

Biochemical measures from the proximal and distal tendon

In general, the pattern of biochemical measurements were similar in diabetic and lean control groups (Fig. 15 and 16). From previous studies we know that AGE accumulation is dependent on collagen turnover rates; tissues with higher collagen turnover have less AGE accumulation [114, 116, 227, 228]. An important finding in this study was that AGE accumulation was higher in the proximal part than that in the distal part in both groups (Fig. 15A and 16A), indicating a higher turnover in the distal part. Within the proximal and distal part, AGE accumulation was only found to be higher in the core region in the distal part of lean control pigs (Fig. 15A), while the tendon turnover was similar in all regions of the cross section of the proximal parts.

To the best of our knowledge, this was the first study investigating differences in collagen turnover radially and longitudinally throughout a tendon. Previous studies have shown that the periphery of a tendon have higher collagen turnover or growth in different models [107, 229], so we expected similar results in the this study, However, similar result was only shown in the periphery of lean control pigs in the distal part. This difference indicated that the tendon growth in the cross section may occur at outer regions in the distal part but not the proximal part.

Besides the AGE content; DNA content, GAG content and collagen content were also measured. In both lean control and diabetic groups, DNA content was higher in the surface region compared to that in the core region (Fig. 15B and 16B). Previous research has shown that the sheath of the tendon contained more cells [229], and in the current study the surface region was a combination of tendon sheath and some of the peripheral tendon proper, and thus the increased cellularity may be due to the sheaths. We hypothesized that collagen turnover was higher in the periphery of a tendon, and that tendon growth may also occur in this region, which could be related to the cell density and activity. Therefore we expected the cell density to be higher in the periphery.

In general, the pattern of GAG content was similar to AGE content. GAG content was higher in the proximal part than that in the distal part (Fig. 15C and 16C). Higher GAG content is normally in regions of higher compression in tendon tissue [230], but in the current study the proximal part of the tendon was just below the muscle-tendon junction and should therefore not be subjected to higher compression. It is also known that GAGs play a role in inhibiting collagen fibril growth [231],so it may be that the proximal part has lower fibril growth since it contained higher GAGs. This was consistent with the AGE accumulation observed in the same study, although it is speculative and the real reason remains unclear.

The collagen content showed different characterization in lean control and diabetic group (Fig. 15D and 16D). In the lean control group, collagen content was slightly higher in the proximal than the distal part of core, middle and surface regions, while the different radial regions had similar collagen content (Fig. 15D). In contrast, the diabetic group showed similar collagen content in all regions and across both proximal and distal parts (Fig. 16D). The percentage of collagen content in the diabetic group was higher than that in the lean control group, which corroborates previous animal data showing that streptozotocin-induced diabetic rats had higher collagen content [232].

This study of porcine tendons suggested that in both the healthy and the diabetic group, DNA content was higher in the surface region of the tendon compared to the core [205], which indicates that the outer regions have more cells. With this finding, it is possible that different tenocyte populations reside in different regions of the tendon, which could be the source of dissimilarity between core and periphery tendon turnover. We also observed that the AGE accumulation was greater in the proximal part, which indicates that the collagen turnover in

the distal part was higher. This observation was not associated with differences in cells, but could be related to GAGs instead since GAG content followed a similar pattern.

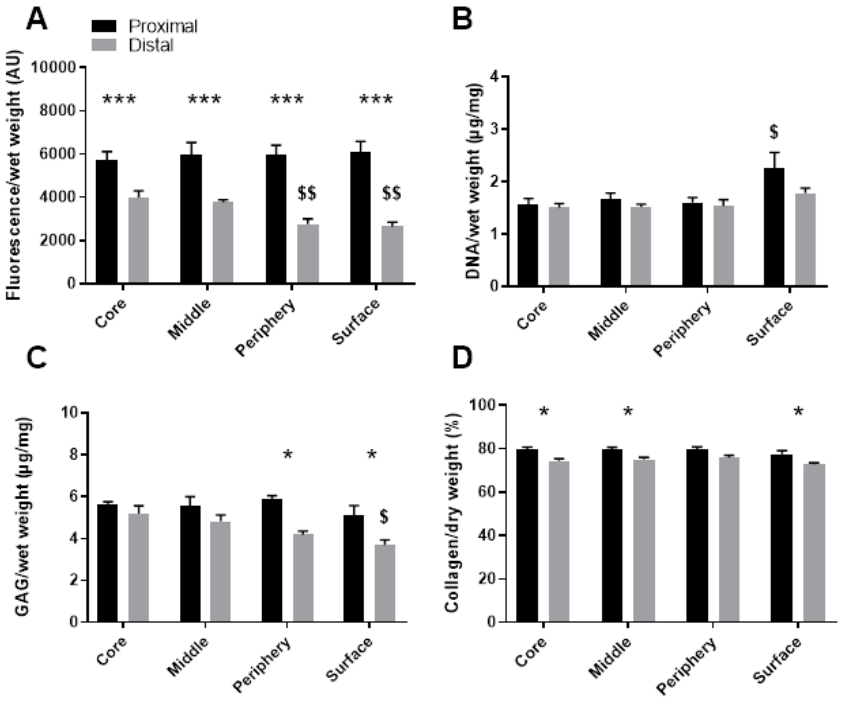

Figure 15. Biochemical test results of lean control pigs. Samples were taken from different radial (core, middle, periphery, and surface) and longitudinal (proximal and distal) positions. A) Autofluorescence (AGEs) normalized to wet weight. B) DNA content normalized to wet weight. C) GAG content normalized to wet weight. D) Collagen content in percent of dry weight. Mean±SE, * Significantly different between proximal and distal part, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. $ Significantly different from the core region, $ $p<0.05$, $$ $p<0.01$. N=4. (Figure comes from Paper 1 [205] with permission from Taylor & Francis)

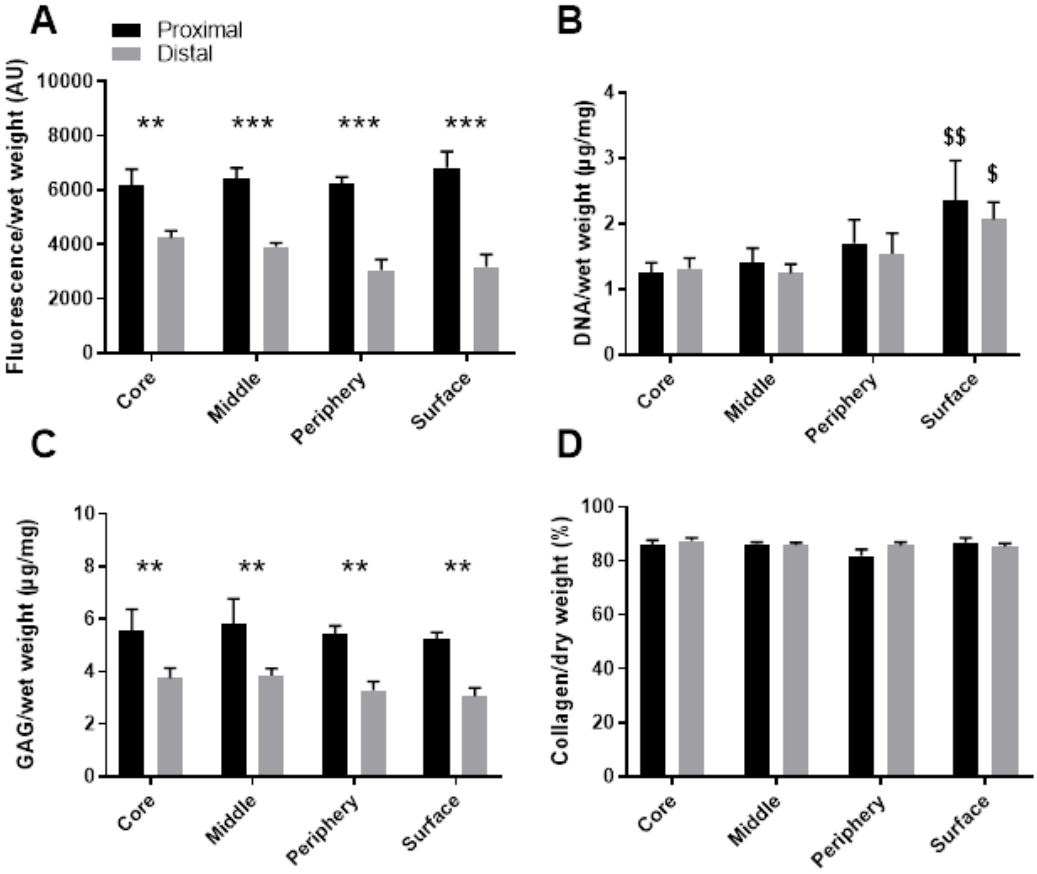

Figure 16. Biochemical test results of diabetic pigs. Samples were taken from different radial (core, middle, periphery, and surface) and longitudinal (proximal and distal) positions. A) Autofluorescence (AGEs) normalized to wet weight. B) DNA content normalized to wet weight. C) GAG content normalized to wet weight. D) Collagen content in percent of dry weight. Mean±SE, * Significantly different between proximal and distal part, ** $p<0.01$, *** $p<0.001$. $ Significantly different from the core region, $ $p<0.05$, $$ $p<0.01$. N=6. (Figure comes from Paper 1 [205] with permission from Taylor & Francis)

Study 2

From our first study, AGE accumulation was greater in the proximal site, and comparing the core and periphery, it accumulated more in the core of the lean control group, which indicated a reginal difference of tendon turnover. In study 2, the human patellar tendon was used to perform all measurements. As we have mentioned above, patellar tendinopathy normally occurs in a specific region, i.e. the proximal posterior region of the tendon [26, 39]. As a consequence, this region contains more cells and has higher blood flow, and thus we hypothesized that the collagen turnover might be higher in this region already in the healthy tendon, but this experiment showed that all regions had similar turnover in human patellar tendon.

Biochemical measurements

Similar to study 1, biochemical measurements were also performed in this study with AGE accumulation as an important indirect marker of collagen turnover [176, 201, 205, 233]. DNA, GAG and collagen content were also measured (Fig. 17).

AGE accumulation measured by fluorescence was similar in all regions (Fig. 17A), indicating that collagen turnover was equal in all regions of the patellar tendon. This was consistent with the ^{14}C results shown below (Fig. 18). The finding was unexpected because our first study showed that AGE accumulated to a greater extent in the core region in pigs tendon [205]. The reason of this difference could be structural dissimilarity since the porcine flexor tendon was rounder whereas the human patellar tendon is flatter, or it could be a species differences, but it remains unknown.

In the current study, the DNA content did not display any regional differences (Fig. 17B). However, even thought it was not statistically different, the insertion seemed to numerically contain more cells than other regions. This may relate to the location; the insertion sample was taken above the patella bone, which could have higher blood flow, although we are not aware any evidence for higher blood flow in this region.

In general, the insertion and distal regions contained a higher GAG content than other regions (Fig. 17C). This finding makes sense since both regions are subjected to compression (Fig. 10), and it is known that GAG contents relate to compression [230]

Collagen content was very similar in all regions of the human patellar tendon (Fig. 17D), around 80% of dry weight.

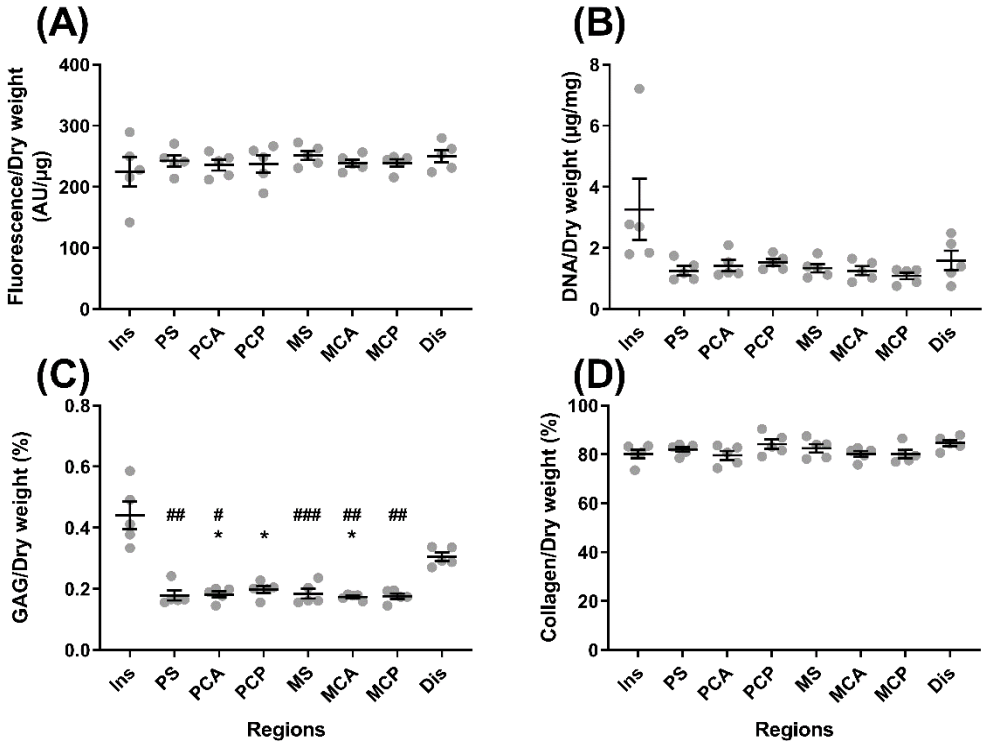

Figure 17. Biochemical results. Samples were taken from different regions, Insertion (Ins), Proximal Side (PS), Proximal Central Anterior (PCA), Proximal Central Posterior (PCP), Middle Side (MS), Middle Central Anterior (MCA), Middle Central Posterior (MCP), Distal (Dis). A) Autofluorescence (AGEs) normalized to dry weight. B) DNA content normalized to dry weight.

C) GAG content in percent of dry weight. D) Collagen content in percent of dry weight. * Significantly different from Ins, * p<0.05. # Significantly different from Dis, # p<0.05, ## p<0.01, ### p<0.001. Mean ±SE, N=5. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

^{14}C bomb-pulse

^{14}C measurement is considered the gold standard of measuring the age of tendon tissue and is therefore the most important method employed in this study. The results are shown in Fig. 18 where the X axis is the birth year of each individual subject, and the Y axis is the ^{14}C concentration of samples taken from each region. The main purpose of this study was to investigate the regional difference of collagen turnover in human patellar tendon, and when we examine each individual subject it becomes evident that there is no differences in the ^{14}C level between regions, indicating that all regions had similar collagen turnover. A single sample from the insertion region of the oldest subject appeared to have an increased ^{14}C level (Fig. 18), which was consistent with this sample also having lower AGE content (Fig. 17A) and more DNA content (Fig. 17B). Collectively these results reflect a higher collagen turnover in this sample. While we unfortunately did not have medical information on the subjects, we speculate that there could have been a prior injury that may explain these observations.

When we look at the ^{14}C levels across the subjects, they appear to be shifted compared to the average level in atmosphere, which fitted well to a 15-year moving average of the atmospheric ^{14}C level (Fig. 18). This finding suggests that the collagen turnover mainly occurred during the first 15 years, and thereafter the collagen turnover became extremely low for the remaining lifetime. This finding was supported by a previous study on human Achilles tendon, which showed that the core of Achilles tendon stopped renewing after the age of 17 [114, 118]. The current study extended on the previous findings by showing that all regions had similar collagen turnover rather than high turnover occurring in specific regions of the tendon.

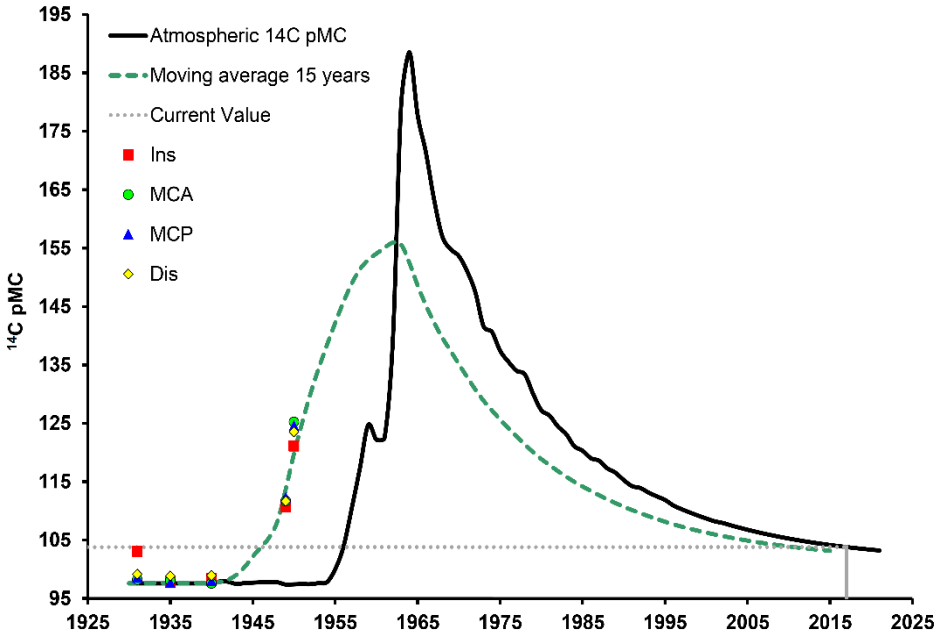

Figure 18. ^{14}C in different regions of patellar tendon. Solid black line shows the ^{14}C level in the atmosphere [234, 235] up to 2012 (extrapolated to 2020). Levels of ^{14}C in purified collagen from

different regions of patellar tendon are plotted according to the birth year of the donors (n=5). The green dashed line shows a moving average of the atmospheric ^{14}C concentration over the following 15 years. The ^{14}C levels of patellar tendon fit well with the dashed curve, suggesting that the patellar tendon was gradually formed during the first 15 years with little to no turnover thereafter. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

Biochemical measurements after purification

Before undergoing ^{14}C measurement, the samples were purified to remove modern carbon. Afterwards, the biochemical measurements were performed on the purified samples and the results are shown in figure 19. Similar to non-purified samples, all regions contained similar AGE content and the particular sample mentioned above still had the lowest value of AGE accumulation (Fig. 19A). The purpose of purification was to remove modern carbon including GAGs and it can be seen that most GAGs were removed by the purification before ^{14}C measurement (Fig. 19B).

Collagen content was still similar in all regions, but the content decreased from 80% to 60% after purification (Fig. 19C). This loss of collagen content was counterintuitive and we believe that it is due to a technical error rather than an actual relative loss of collagen. After noticing this apparent loss of collagen, we subsequently did a small test and found that tendon tissue hydrolysed and dried without nitrogen flow has approximately 30% less hydroxyproline content compared to samples dried with nitrogen. In the current study, hydrolysates from the purified samples were dried without nitrogen flow, and oxidation could be the reason for the decreased value.

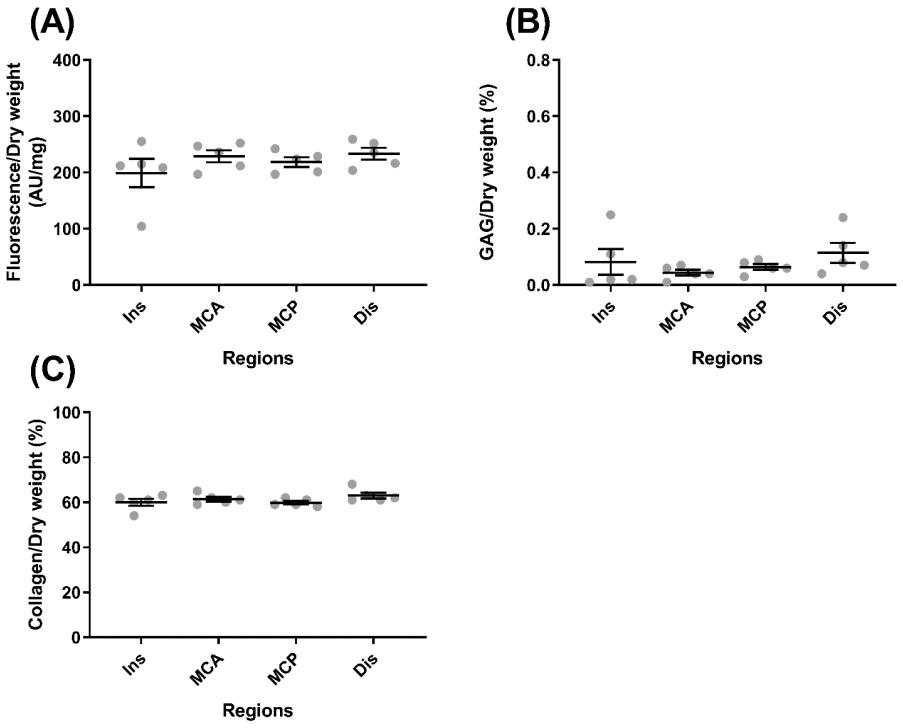

Figure 19. Biochemical test results after purification. Samples were taken from 4 different regions, Insertion (Ins), Middle Central Anterior (MCA), Middle Central Posterior (MCP) and

Distal (Dis). A) Autofluorescence (AGEs) normalized to dry weight. B) GAG content in percent of dry weight. C) Collagen content in percent of dry weight. Mean ±SE, N=5. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)

Specific AGEs and lysine measurement

To make all of our data comparable, the same four regions analyzed for ^{14}C were also used to analyse specific AGEs. In study 1 and this study, the fluorescence measurements of all fluorescent AGEs was used as an indirect measure of collagen turnover, but not all AGEs are fluorescent, and therefore protein-bound AGEs were also measured. This method was based on mass-spectrometry quantification of CML, CEL, MG-H1 and pentosidine, which were shown to correlate well with turnover [176]. But no regional differences were observed of all four protein-bound AGEs (Fig. 20), which was consistent with the fluorescence results. So we concluded that AGE measurements revealed that collagen turnover is equal in all regions of patellar tendon, in agreement with the ^{14}C measurements.

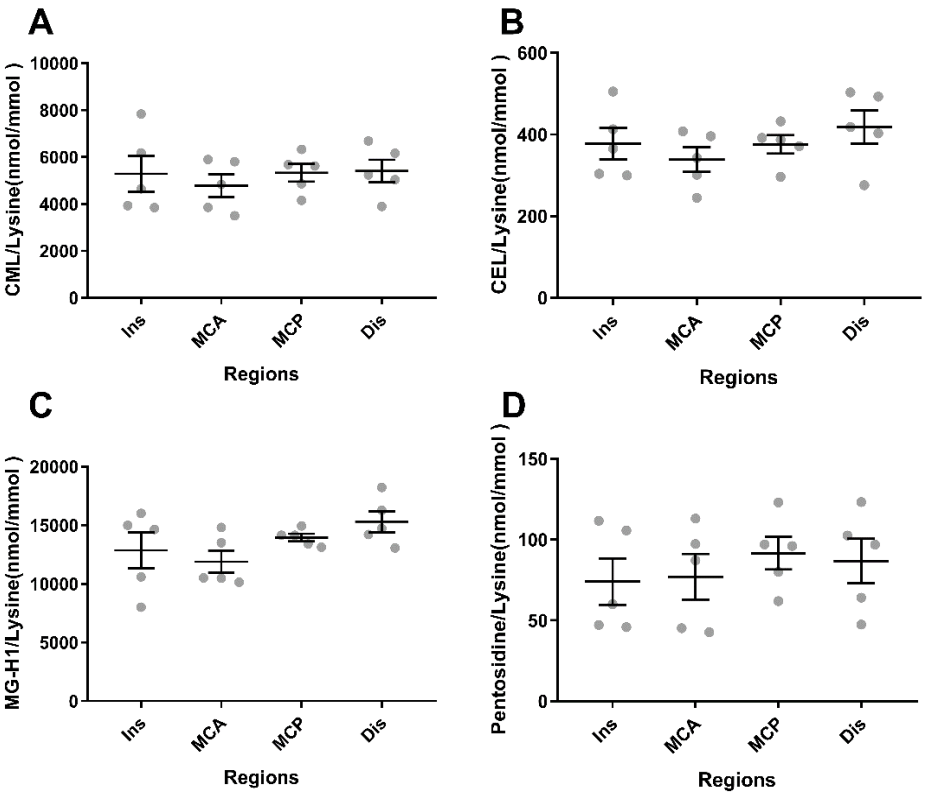

Figure 20. Protein-bound AGEs. Samples were taken from 4 different regions, Insertion (Ins), Middle Central Anterior (MCA), Middle Central Posterior (MCP) and Distal (Dis). A) CML normalized to lysine. B) CEL normalized to lysine. C) MG-H1 normalized to lysine D) Pentosidine normalized to lysine. Mean ±SE, N=5. (Figure comes from Paper 2 [215] with permission from The American Physiological Society)
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Study 3

In this study, cell proliferation assays, immunofluorescence staining and label-free quantitative LC-MS-based proteomics analysis were performed to characterize any potential differences between primary cultures of cells derived from the core and periphery of horse SDFTs.

Cell proliferation

Previous work have shown that tendon turnover is higher in the periphery [114, 193], and tendon growth could occur in the very same region [229]. Our first study also showed that DNA content was higher in the outer region, which is the same region of higher tendon turnover. A difference in turnover could be mediated either by a difference in the matrix synthesizing ability of cells from different regions, or differences in the cell densities between the regions. In this study, cells cultured from the core and periphery of porcine tendon displayed a decrease in cell number from day 0 to day 2, and afterwards the cell number exponential increased up to day 6. Subsequently it slowed down with a density almost 10 times that at day 0 (Fig. 13D). There was no significant difference in the proliferation rate between cells from the periphery and the core of the tendon (Fig. 13D and 21).

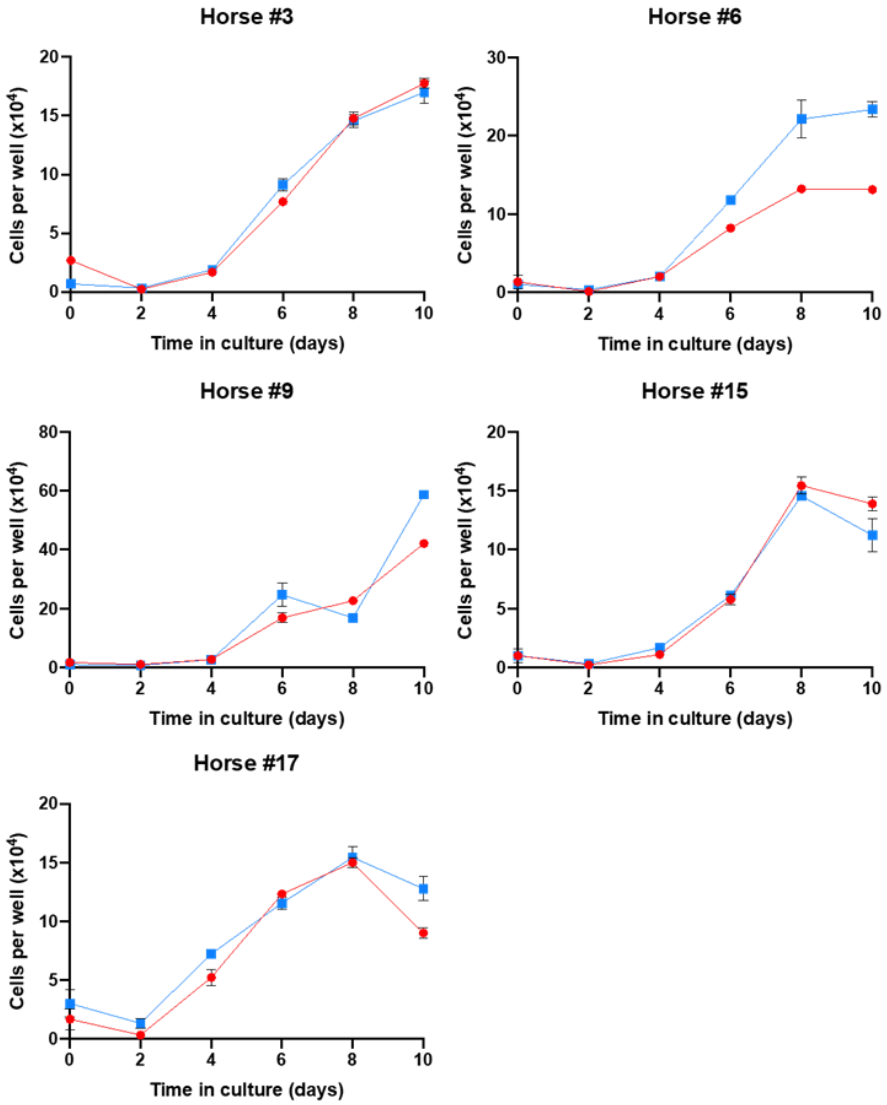

Figure 21. Individual growth curves for tendon cells of each horse. (Figure comes from Paper 3)

A previous study on equine tendons showed a different result from the current study. In that study, cells derived from the outer loose peritenon had a higher cell proliferation rate than those from the core [76]. The peritenon is mainly comprised of type III and type V collagen, and may contain other cell types than tenocytes, for example, pericytes. In our study, the peripheral cells were derived from the outer parts of the tendon proper rather than the peritenon, and thus represented a more well-defined fibroblast population responsible for synthesizing, maintaining and degrading the collagen-rich ECM. However, a previous study has shown that cells from peritenon could secrete stimulatory factors to cells in the tendon proper [236], thus it is possible that in vivo tenocytes from the tendon periphery are more active than cells from the core since they are closer to the peritenon. This could be the explanation why previous measurement of ECM turnover at the tendon surface is high in vivo. We initially hypothesized that cell activity and proliferation would not be different between the cell populations from core and periphery, when the cells were stripped of their native microenvironment, and this is supported by the present results. This data suggests that tenocytes from the tendon core and periphery have the same intrinsic capacity to proliferate.

Matrix deposition and proteomics

In the current study, ECM was deposited after 4 days of culture, as established by immunofluorescence analysis for type I collagen (Fig. 22) and Alcian blue staining for glycosaminoglycans (Fig. 23). Type I collagen deposition varied largely between horses, and did not correlate with age or sex (Fig. 22). Tenocytes derived from the core and periphery behaved similarly in terms of morphology, and appeared fibroblastic in culture (Fig. 23). Very little glycosaminoglycans were seen in all five horses through Alcian blue staining (Fig. 23).

Proteomics analyses were performed with proteins isolated after 4 days of culture. 4082 unique proteins were detected by LC-MS in samples of core and periphery SDFT cell cultures. The distribution of protein abundances was similar in all samples (Fig. 24A and B). Pearson's correlation coefficients of all samples showed strong correlation (Fig. 24C). A heatmap showing hierarchical clustering of samples based on the protein abundances ranked by standard deviation did not show any differences between samples from core and periphery (Fig. 24D). Proteomics analysis of the ECM deposition showed that the proteome profiles of core and periphery cultures were very similar. However, there were still a few ECM proteins that showed differential abundance. The matrix formed by cells from the tendon core contained 27 proteins with significantly greater abundance than those from the periphery, including collagen alpha-2(IV) (COL4A2) (Fig. 24E). Matrix formed by cells from the periphery contained 32 proteins that were significantly more abundant than those from the core, including lysyl oxidase-like 2 (LOXL2), fibulin 5 and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (Fig. 24E).

Collagen IV is the major structural component of basement membranes and is important in the formation of complex covalently linked structural scaffolds [237, 238]. The proteomics data also identified ADAM10 as significantly more abundant in periphery than core SDFT cultures. The ADAM family of proteins are called sheddases and their primary function is to cleave membrane proteins at the cellular surface [239]. Catalytically active ADAM10 mediates the shedding of meprinβ [240], which when released from the plasma membrane can cleave ECM molecules, including collagen IV, nidogen and fibronectin [241]. The high expression of ADAM10 by periphery SDFT cells may therefore explain why core SDFT cultures exhibited a significantly higher abundance of collagen IV.

The levels of fibulin 5 and LOXL2 were found to be higher in the periphery SDFT than core SDFT cultures. Fibulin 5 (FBLN5) is a short glycoprotein that interacts with integrin receptors. The family of fibulins plays an important role in the assembly of elastic fibers and in regulating transforming growth factor β during fibrosis [242, 243, 244]. LOXL2 is involved in the formation of cross-links in collagens, elastin, and other extracellular matrix proteins [245]. Together these data suggest that cells from the periphery of horse SDFT may have more potential for high ECM turnover than cells from the core SDFT. However, it must be stressed that the proteomics data showed that there were largely no differences (>98% proteins) between core and periphery SDFT cells in vitro.

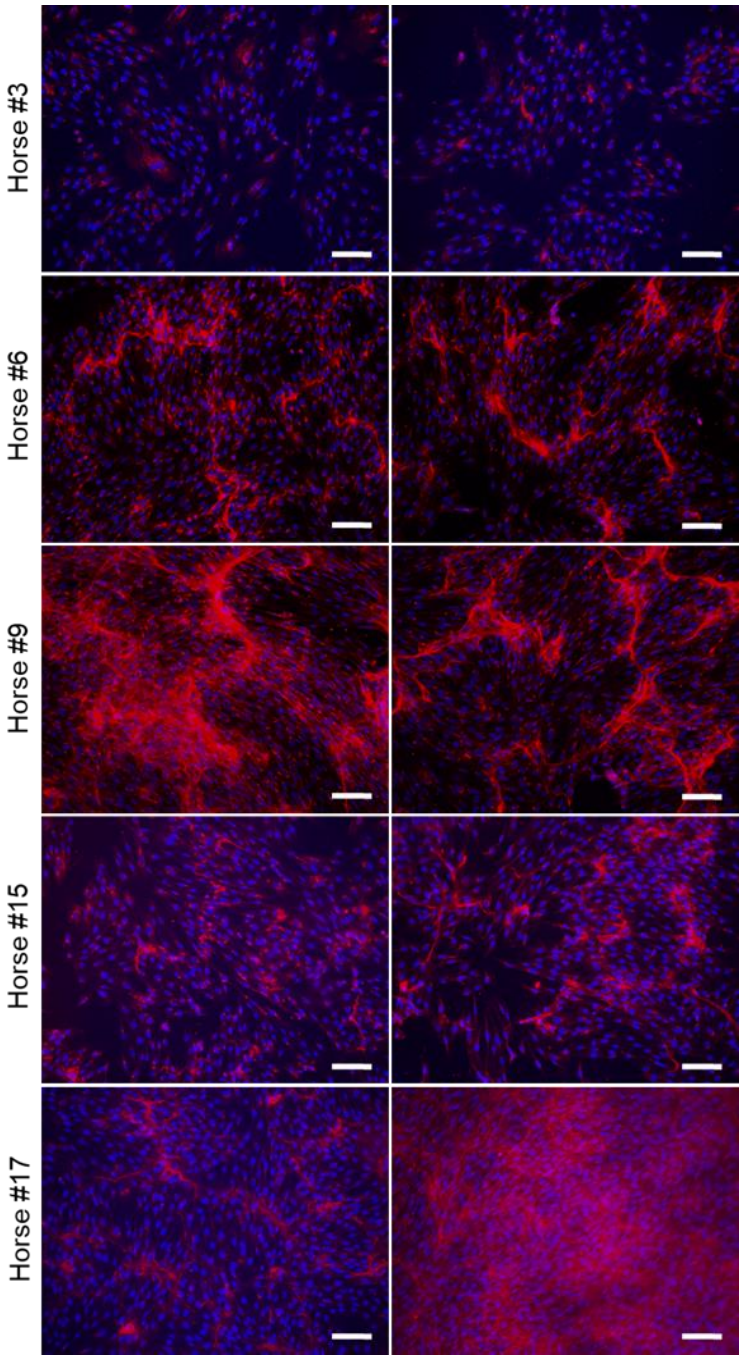

Figure 22. Collagen I deposition by core and periphery SDFT cells in culture. Representative images of immunofluorescence staining for collagen I in 4-day old cultures of core and periphery SDFT cells from five horses. Red, collagen I. Blue, DAPI. Bars, 100 µm. (Figure comes from Paper 3)

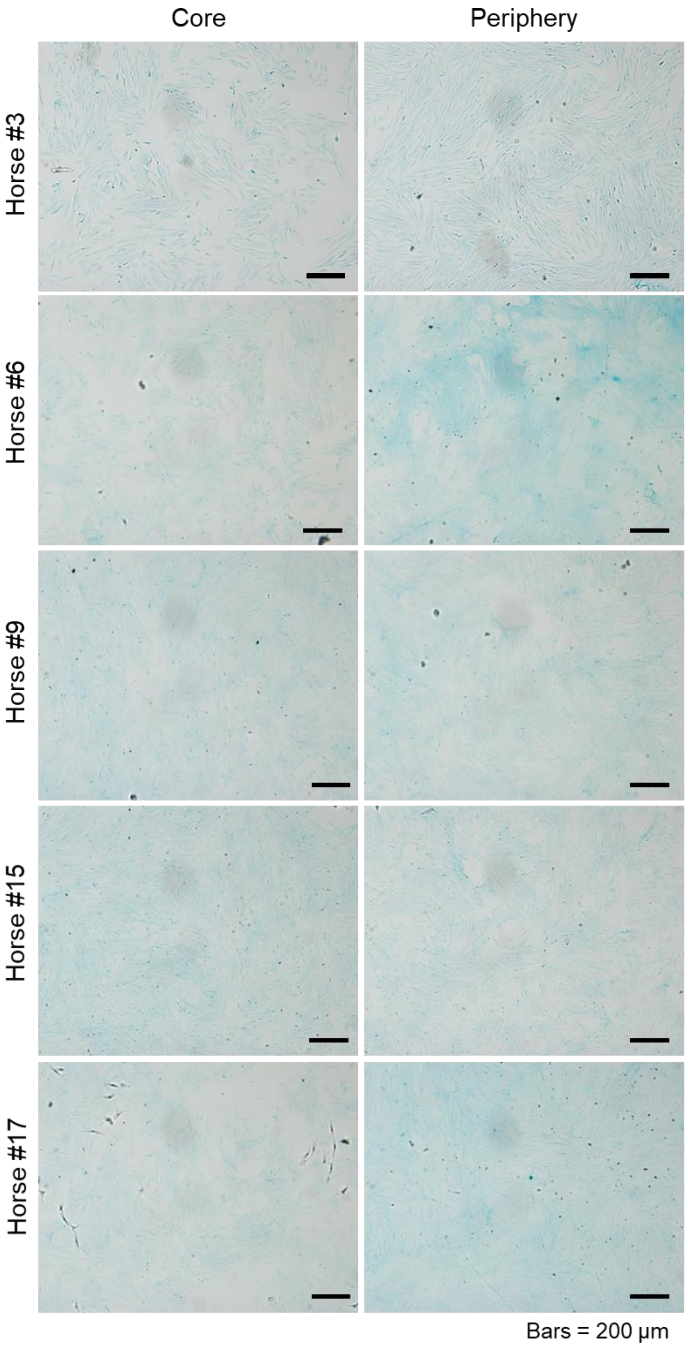

Figure 23. Alcian blue staining by core and periphery SDFT cells. (Figure comes from Paper 3)

Principal component analysis did not show a clear separation of core and periphery samples (Fig. 24F). T-distributed stochastic neighbour embedding showed that the proteome profile of cultures of cells taken from the same horse are more similar than cultures of cells taken from the same region (Fig. 24G).

In conclusion, tenocyte cultures derived from the core and periphery of horse SDFTs behave similarly in proliferation rates and ECM proteome profiles, which suggests that other factors may play a role in regulating ECM turnover than the intrinsic properties of tendon fibroblasts.

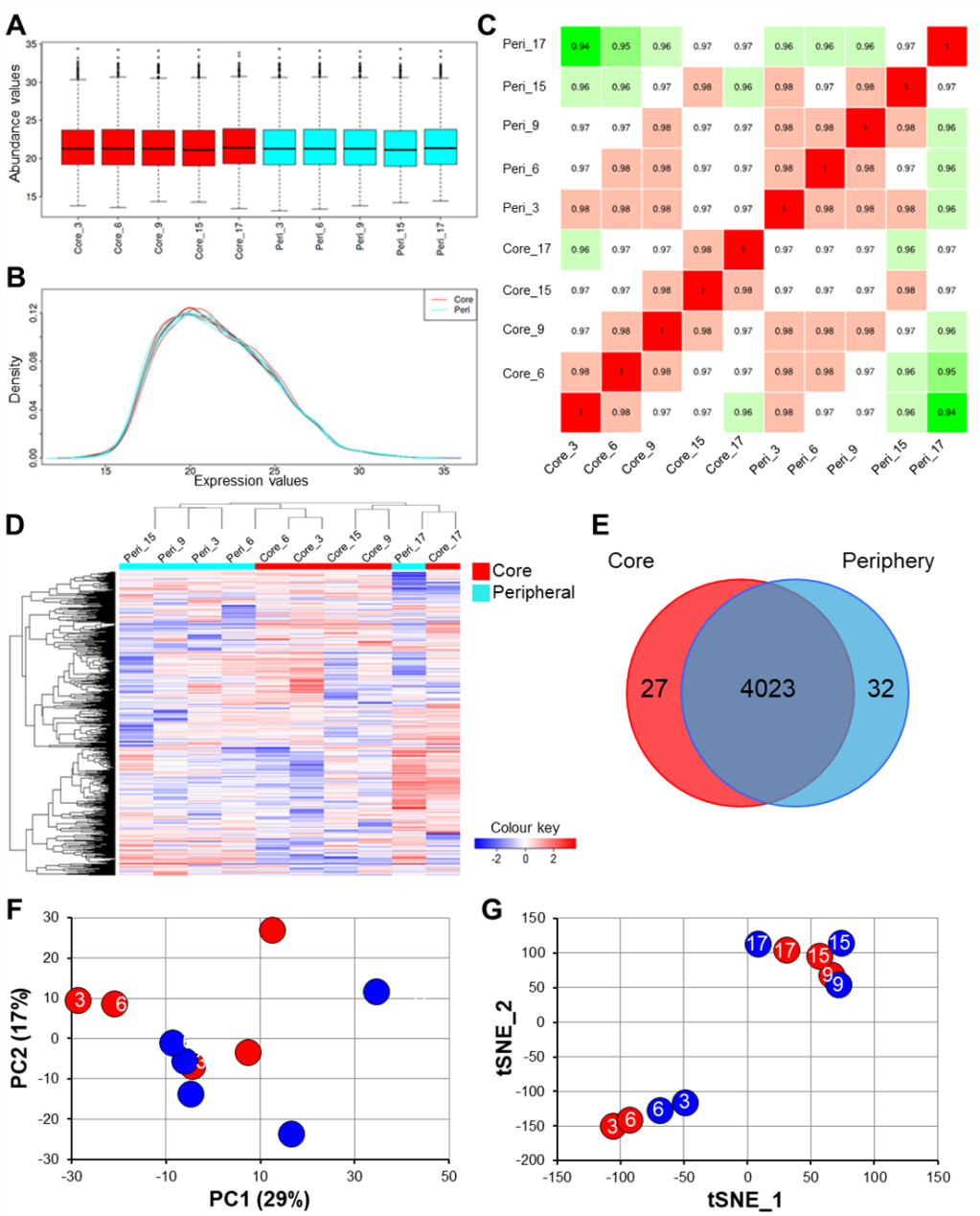

Figure 24. Similarities of proteome profiles of core and periphery SDFT cells in culture. Distribution of protein abundances from the core and periphery as detected by LC-MS shown in box plot and density plot (AB). Heatmap of Pearson correlation coefficient (r) for pairwise comparison (C). High r values (red squares) denote high correlation between samples. Heatmap showing hierarchical clustering of samples based on their protein abundances ranked by standard deviation (D). Venn diagram showing the majority of proteins (4023) was not significantly different in abundance between core and periphery SDFT cells (E). There were 27 proteins that were significantly more abundant in core SDFT cell cultures and 32 proteins that were significantly more abundant in periphery SDFT cell cultures (false discovery rate $p<0.1$, >1.5 fold change). Principal component analysis (PCA) was used to provide a statistical summary of proteins. The first two principle components (PC1 and PC2) are shown (F). tSNE (t-distributed stochastic neighbour embedding) plot showed the similarity between proteins from different regions (G). (Figure comes from Paper 3)

Conclusions

In the first study, AGEs accumulated more in the proximal than the distal part in both lean and diabetic animals, indicating that collagen turnover could be higher in the distal part. Comparing the core and periphery, lean pigs had higher AGE accumulation in the core compared to the periphery in the distal part, but this was not the case in the proximal part or in the diabetic pigs. Overall, the study found evidence of regional differences in turnover of uninjured tendons, which has not previously been investigated.

In the second study, collagen turnover was assessed by the bomb-pulse dating technique and by measurement of AGE accumulation. There was no difference between any regions of the patellar tendon. Meanwhile, the insertion and distal regions contained more GAG content. Overall, the results did not support that tendon regions commonly afflicted in tendinopathy would display an increased turnover.

In the third study, tenocyte cultures derived from the core and periphery of equine SDFTs behave similarly in proliferation rates and ECM proteome profiles. The largest source of variation was between biological replicates; cells from the same horse were more similar than cells derived from the same region of the SDFT from different horses.

The overall conclusion of the thesis is that collagen turnover does not appear to differ between regions in adults. Even though we saw some differences of collagen turnover in study one, that could be because the pigs were relatively young compared to the subjects in study two and three. In addition, study two and three dealt with more clinically relevant populations, thus we believe that the inherent collagen turnover is even throughout a tendon.

Perspectives

In this PhD project, three different species were used to detect the tendon turnover in different regions of a tendon. AGE accumulation was used as an indirect marker of tendon turnover, and the results differed in porcine tendons and human patellar tendons, so in the future species specificity needs to be considered. If possible, I would like to do more experiments with different tendons from human, the results would be more comparable to the human patellar tendon results. Even though Achilles tendon is perhaps be the most researched tendon tissue in human, I still want to apply our methods in Achilles tendon, because I believe that there could be regional differences in human Achilles tendon based on our porcine data. Achilles tendon would in some ways be more comparable to DDF tendon since it its longer and more round size relative to the patellar tendon. In addition, the Achilles tendon is also an energy storing tendon and therefore the mechanical demand is more similar to the DDF tendon of porcine. It would be difficult to get enough amount of Achilles tendons because it requires the entire tendon from muscle junction to the calcaneus insertion.

To perform ^{14}C method in future experiments, donors born before 1950s would be preferred, and as a limitation in the second project, number of subjects is important.

More methods could be used to detect the tendon turnover besides AGE accumulation and ^{14}C methods. As we have mentioned above, different methods focus on different factors, isotopes could be used to measure the synthesis of new collagen, which is a dynamic process.

Cell culture of human tendon is also a direction, in our research horse tendon cells were used, which is comparable to human tissue.

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Study 1

CONNECTIVE TISSUE RESEARCH
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Regional differences in turnover, composition, and mechanics of the porcine flexor tendon

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ABSTRACT

Purpose: Recent data suggest that there is a lack of turnover in the core of human tendon, but it remains unknown whether there are regional differences between core and periphery of the cross section. The purpose of this project was to investigate regional differences in turnover as estimated by the accumulation of fluorescent Advanced Glycation End-products (AGEs) and regional differences in mechanical properties.

Materials and methods: Tendons were obtained from lean control ($n = 4$) and diabetic Göttingen minipigs (streptozotocin-induced, $n = 6$). The deep digital flexor tendon of one hind limb was separated into a proximal, central and distal part. Autofluorescence was measured in the core and periphery of the proximal and distal parts of the tendon, and mechanical properties were tested on fascicles taken from the core and periphery of the central tendon (only diabetic animals).

Results: Autofluorescence was greater in the proximal than the distal part. In the distal part of the lean control animals, autofluorescent AGE accumulation was also greater in the core than the periphery. Peak modulus in the core region (704 ± 79 MPa) was higher than the periphery (466 ± 53 MPa, $p < 0.05$) in diabetic tendons.

Conclusion: Taken together, autofluorescence varied both along the length and across the tendon cross section, indicating higher turnover in the distal and peripheral regions. In addition, mechanical properties differed across the tendon cross-section.

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Collagen turnover; regional differences; advanced glycation end-products; autofluorescence; collagen content

Introduction

Tendon is a dense connective tissue, which links muscle to bone, and has particular mechanical properties enabling it to respond to load transmitted by muscles.[1]. To what extent and exactly how it adapts to habitual loading and injury remains largely unknown. Tendon hypertrophy has been shown to occur with long-term exercise in humans,[2] and in a mouse model, this exercise-induced hypertrophy has been shown to occur in the tendon periphery.[3] Furthermore, the metabolic activity of healing tendon can be enhanced for more than 1 year after an Achilles tendon rupture, and vascularization can be elevated for more than 6 months,[4] which indicates that the healing process takes considerable time. The same study also showed that metabolic activity was higher in the core than in the periphery at 3 and 6 months, but at 12 months this region-specific response was interchanged.[4] Chronic tendinopathy represents 30% of consultations for musculoskeletal pain,[5] and can occur in many different tendons.[6–8] In the patellar tendon, tendinopathy happens more frequently (65%) in the proximal and posterior portion,[9–11] and in the Achilles tendon, it occurs frequently at the insertional region or in the mid portion.[12] The exact reason why tendinopathy appears to be region specific remains an enigma.

Prior research has suggested that there is limited regenerative capacity of tendons,[13] which may be due to a slow tissue turnover. A half-life of tendon collagen protein of around 200 years has been suggested based on determination of aspartic acid racemization in horses.[14] Nuclear testing in the fifties and sixties led to a dramatic time-limited spike in atmospheric ^{14}C, which effectively isotope labeled all organic matter formed during that period. Using this "bomb pulse" label, it was revealed that the renewal of human Achilles tendon tissue taken from the core is extremely limited following maturation. The lack of tissue turnover could explain the relatively poor regenerative capacity of tendon tissue, although turnover in the periphery of the tendon remains unknown.[13]

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The ^{14}C method can be used to explore collagen turnover of human tendon, but cannot be used in animal models with relatively short lifespan. Aspartic acid racemization is somewhat temperature dependent, which may be more difficult to control in superficial tendons. Advanced glycation end-products (AGEs) are proteins or lipids that have been glycated as a result of exposure to sugars. Previous studies have shown that the accumulation of AGEs in cartilage and skin collagen increases linearly with age,[15,16] and that the rate of accumulation is higher in cartilage than in skin.[17] Moreover, in tissues exposed to hyperglycemia like in diabetes, the accumulation is even higher.[17–19] These findings suggest that AGE accumulation may be used as an indirect measure of collagen turnover. In this experiment, we used the deep flexor tendon from lean control and diabetic pigs to examine regional differences. We hypothesized that tendon growth and turnover occur at the peripheral region of the tendon, and consequently that there would be a greater accumulation of AGEs in the core compared to the peripheral region. We did not expect to see any difference between the proximal and distal parts.

Materials and methods

Animal model

This study used tissue from a larger prospective intervention study in male Göttingen minipigs investigating atherosclerosis, diabetes, and obesity.[20] Due to hyperglycemia, we expected that AGEs would accumulate at a greater rate in the diabetic model, which makes it easier to detect, and therefore both the diabetic and lean control animals were used in this experiment. The animals were acquired at 6–7 months of age. For diabetic animals, diabetes was chemically induced after approximately 2 months by IV injection of 60 mg/kg streptozotocin (STZ) (Sigma-Aldrich A/S) once per day over 3 consecutive days. Afterward, insulin was used and dose was individually adjusted to maintain fasted morning blood glucose between 14 and 16 mM. In the lean control group, pigs were fed a lean standard minipig (SDS) diet for the entire period (Special Diet Service, UK), according to breeder's recommendations. In the diabetic group, two pigs were fed a high-fat diet 9G4U (5B4L 1% cholesterol, TestDiets) and four pigs were fed a similar diet with extra salt 5BTJ (5B4L 1% cholesterol + 2.5% salt, TestDiets) for 13 months before being sacrificed. Diabetic animals on both the normal and high salt diet were combined into a single diabetic group (no differences were observed in any of the presently measured parameters). One hind limb from each of six diabetic and four lean control pigs were used in this study. When sacrificed the animals were 20 months old and had an average weight of 38 kg in the lean control group. Göttingen minipigs are considered mature at ~35 kg and ~24 months of age.[21] The study was approved by the Animal Experiment Inspectorate, Ministry of Justice, Denmark.[20]

Sample preparation

Because of its relatively large diameter and circular shape, the deep digital flexor tendon was used in this study. All surrounding tissues were removed, including skin, fat, and muscle. The tendon was cut into three parts, proximal, central, and distal, respectively. The proximal part was 10 mm long and taken approximately 5 mm distal to the muscle insertion, taking care to avoid any muscle. The distal part was 10 mm long and taken approximately 5 mm proximal to the bifurcation of the toes. The remaining central part was approximately 40 mm long depending on the total tendon length.

Biochemical tests were performed on the proximal and distal parts (see Figure 1(a)). The cross section of tendon is shown in Figure 1(b). This tendon does not have a distinct paratenon, but still has a thin sheath of loose connective tissue on the surface. To distinguish this layer from the tendon proper the outermost part of the tendon, which contains the sheath and some of the underlying tendon, was removed initially and combined into one sample per tendon per distal/proximal part. This tissue is referred to as the surface and since it is a mixture of sheath and peripheral tendon, the results from this region may be ambiguous. Samples from each of the five central regions (two peripheral, two middle, and one core) were approximately 5 × 2.5x10 mm and were analyzed separately. Because no difference was observed between the medial and lateral pairs of periphery and middle samples the average value is reported here.

Biochemical test

A number of biochemical analyses were made based on papain-digested tissue according to previously published protocols.[22] This papain digest was used for analyses of AGEs (autofluorescence), DNA content, and glycosaminoglycan (GAG) content. The samples were kept wet to avoid changes in fluorescence due to drying and consequently values are normalized to wet rather than dry weight. In addition, a piece of tissue was taken for measuring collagen content, which was freeze-dried first, and then weighed. Secondly, the

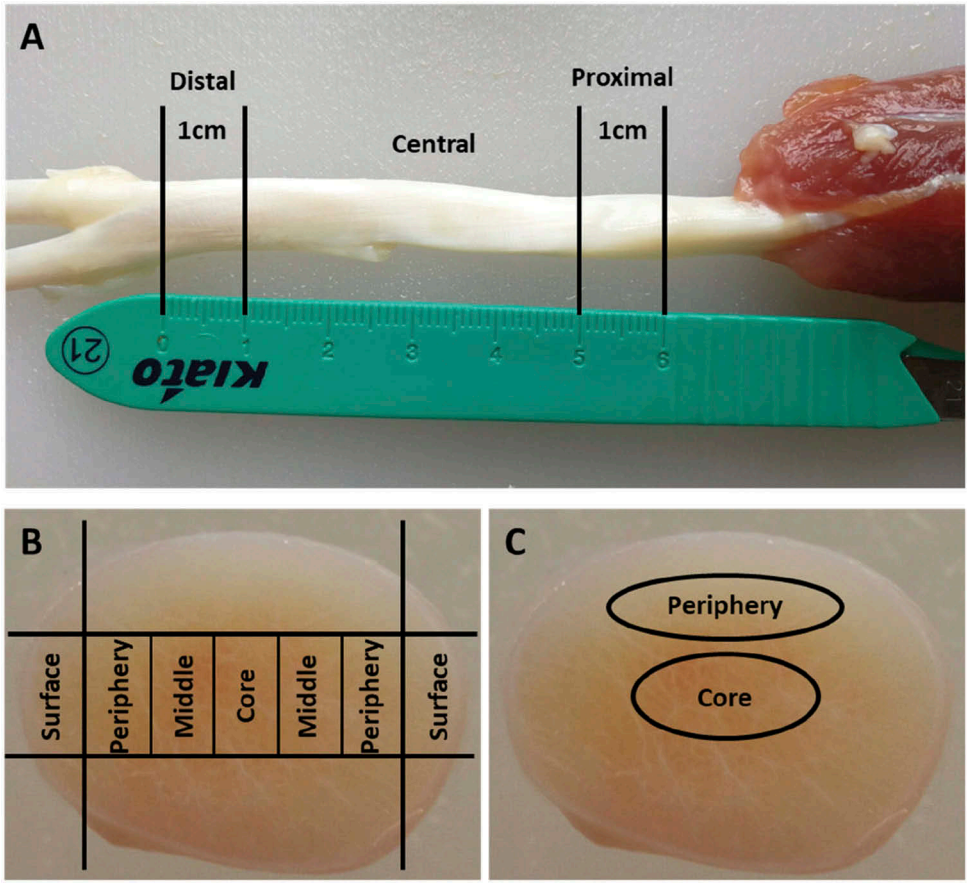

Figure 1. Schematic diagram of deep digital flexor tendon. (A) A 1 cm long sample was taken from proximal and distal parts, respectively, and used for biochemical measurements. The central part was used for the mechanical test. (B) Cross section of sample taken from the proximal and distal part. Four regions were separated: surface, periphery, middle, and core. (C) Fascicles for mechanical test were taken from the core and the periphery of the central tendon segment.

sample was delipidated in two overnight 0.5 ml acetone washes. Third, acetone was removed, and samples were freeze dried and weighed again. Finally, samples were hydrolyzed in 0.5 mL hydrochloric acid (6 M, 110 °C) for 24 h.

Papain digestion was performed using papain (P3125, Sigma, 0.125 mg/ml) in PBE buffer (100 mM sodium phosphate, 10 mM EDTA, pH 6.5) with 10 mM L-cysteine.[22] To each sample, 0.5 mL was added, and two more tubes with just papain solution were made as blanks. Samples were digested in an oven at 60 °C overnight. The next day tubes were removed from the oven, vortexed and spun briefly.

AGE content

AGE accumulation was assessed in papain digests by autofluorescence.[23–25] Triplicates of 100 µL sample were pipetted into wells of a 96-well black microwell plate (237,107, Thermo scientific). The plate was read at (338–343 nm)ex/(382–507 nm)em on a fluorometer (Wallac1420 Victor, Perkin Elmer).

DNA content

DNA content was measured by binding of Hoechst.[22] Papain digests and DNA standards made from salmon DNA (2–100 µg/mL) (10 µL) were plated in triplicate into a 96-well black microwell plate. To each well, 150 µL of TEN buffer (100 mM NaCl, 10 mM Tris-HCl, 1mM EDTA) was added and the autofluorescence was measured. Subsequently, 50 µL of freshly prepared 0.8 µg/mL Hoechst 33,258 (H3569, Invitrogen) in TEN was added to all wells and fluorescence was measured immediately at (338–343 nm)ex/(450–476 nm)em. DNA content was calculated from the standard curve based on the difference between fluorescence before and after addition of Hoechst.

GAG content

For GAG quantification, 10 µl of papain digest was mixed with 190 µl 1,9-dimethylmethylene blue (DMB) (341,088, Sigma-Aldrich) solution (38 µM DMB in 40 mM NaCl, 40 mM glycine, pH 3.0), and absorbance was read at 540 and 595 nm (the reading at 595 nm was

subtracted from the reading at 540 nm). Sample values were compared to a standard curve made with 12.5–150 μg/mL of chondroitin sulfate B (C3788, Sigma-Aldrich).

Collagen content

Hydrolysates were dried on a heating block at 95 °C, rehydrated with 0.5 mL of distilled water, and dried again. The hydrolysates were resuspended in 0.5 mL of 0.2 M HCl and diluted 100-fold in the buffer used for the hydroxyproline assay (acetate-citrate). Hydroxyproline was measured by a colorimetric assay based on 4-dimethylaminobenzaldehyde, the detailed procedure used in our lab has previously been published.[26] Hydroxyproline concentrations were converted to collagen concentration by multiplying with 7.5 (13.3% hydroxyproline in collagen).[27]

Mechanical test

Mechanical measurements were a secondary objective of this study and for practical reasons was only performed on tissue from diabetic animals. Fascicles were dissected from each of the core and periphery of the central 40 mm piece of the tendon (Figure 1(a) and 1(c)). It was only possible to isolate fascicles from two areas (Figure 1(c)). Twelve individual intact fascicles around 20 mm long were taken from each region, discarding shorter fascicles.

Mechanical tests were conducted on 144 fascicles from six tendons. Measurements were performed using a microtensile testing apparatus (20 N tensile stage, Petri dish version, Deben Ltd) at a sampling rate of 10 Hz. The micromechanical testing procedure has been described in detail previously.[28] In brief, approximately 20 mm long fascicles were set on the clamps with the middle 10 mm wrapped in PBS (0.15 M, pH 7.4) moistened gauze and the ends allowed to air dry at room temperature. The dried ends were glued with cyanoacrylate to the aluminum specimen mounting plates of the device. Fascicles were then immersed in PBS solution during the testing. The nominal testing length of each specimen was 10 mm and the testing rate was set at 6.0 mm/min. The start point was defined when the stress value first reached 0.5 MPa. Peak modulus (slope at the steepest point of the curve), peak stress (considered to be the ultimate failure point), and the strain corresponding to the peak stress were determined. Microscopy images were taken while the fascicle was mounted and immersed in PBS solution, when tension just started rising. Fascicle diameter was measured from the microscopy images and used to calculate fascicle cross-sectional area assuming circularity. Preconditioning was not performed.

Data analysis and statistics

All data are presented as means ± SE. Paired students t-tests were used for comparing the mechanical differences between the core and periphery. For comparing regional differences in the biochemical tests on samples from the proximal and distal parts, two-way ANOVA was used with longitudinal (proximal, distal) and radial (core, mid, periphery, surface) position as the two factors. For a main effect of longitudinal position, comparisons were made between the proximal and distal region at each radial position with a Sidak correction for multiple comparisons. For a main effect of radial position, each radial position was compared to the core region at each of the longitudinal positions, with Dunett's correction for multiple comparisons. Differences were considered significant when $p < 0.05$. All statistical tests were performed in GraphPad Prism v.7.0 (GraphPad Software, La Jolla).

Results

Mechanical properties from the central tendon

The mechanical data is shown in Figure 2. The average diameter of fascicles was 161 ± 53 μm. Note that there was some level of selection when dissecting out the fascicles because very thin or thick fascicles may have been discarded due to uncertainty of whether they were damaged (for the thin ones) or they were in fact a bundle rather than a single fascicle (for the thick ones). Therefore, these diameters are not necessarily representative of the entire sample and consequently group-comparisons were not made. The peak modulus in the core (704 ± 79 MPa) was significantly higher than that of the periphery (466 ± 53 MPa), $p < 0.05$ (Figure 2(a)). Peak stress in the core (23.2 ± 4.1 MPa) was significantly higher than that of the periphery (13.6 ± 2.3 MPa), $p < 0.05$ (Figure 2(b)). Strain at peak stress did not differ between core (5.0 ± 0.34%) and the periphery (4.6 ± 0.26%) (Figure 2(c)).

Biochemical measures from the proximal and distal tendon

The data are shown in Figures 3 and 4. Sample wet weight was similar in all groups (~5 mg) and there was no difference in water content between regions (average 43% water, data not shown).

The fluorescence data showed that the proximal part had significantly greater AGE content than distal part, which was consistent in both the lean control group ($p < 0.01$) (Figure 3(a)) and the diabetic group ($p < 0.005$) (Figure 4(a)). In the proximal part, there was no

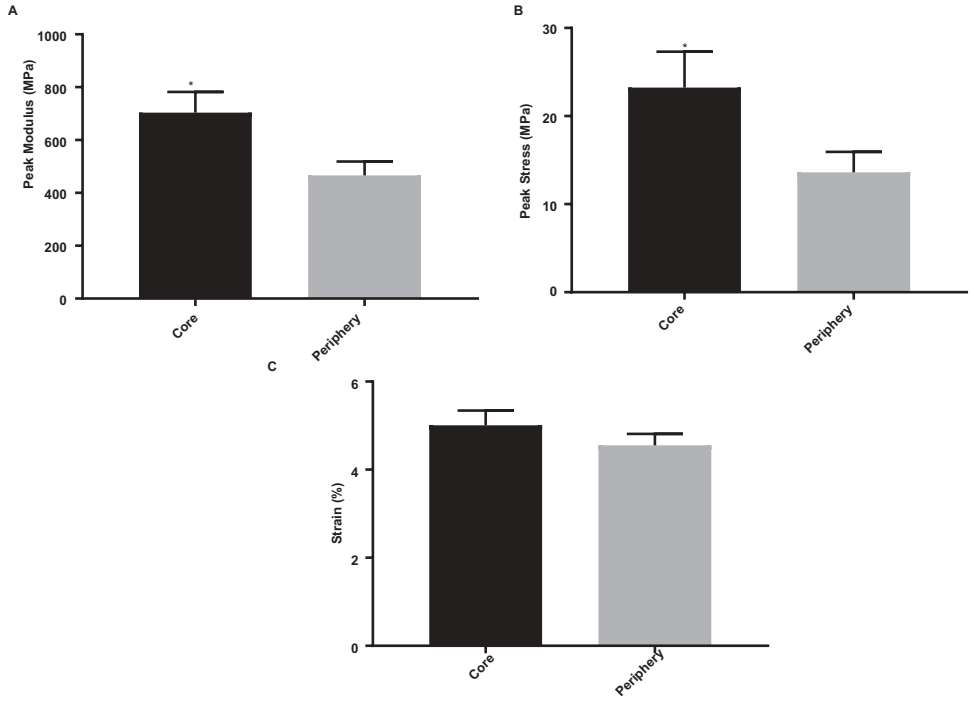

Figure 2. Mechanical test results of fascicles taken from the core and periphery of the central tendon segment. (A) Peak modulus. (B) Peak stress. (C) Strain at peak stress. Mean ± SE, * p < 0.05, N = 6.

difference between the core and any of the radial regions in both lean control and diabetic pigs. In the distal part, AGE content was greater in the core compared with the periphery ($p < 0.01$) and surface ($p < 0.01$) for the lean control animals, and there was a tendency in the same direction (core vs. periphery $p = 0.07$) in the diabetics.

With respect to DNA content, the only difference was that the surface had more cells than the core region in both lean control and diabetic groups (Figures 3(b) and 4(b)). In the diabetic group, the post-test was significant for both the proximal ($p < 0.01$) and distal ($p < 0.05$) surface, while it was only significant in the proximal part for the lean controls ($p < 0.05$).

Similar to the AGE content, GAG content was significantly greater in the proximal than distal part ($p < 0.01$). This was significant in all regions of the diabetic group (Figure 4(c)), but in the lean control group, the difference was only significant in the periphery ($p < 0.05$) and surface ($p < 0.05$) (Figure 3(c)). In the lean control group, the distal part also showed a significantly lower GAG content in the surface compared with the core ($p < 0.05$) (Figure 3(c)).

There were no significant differences in collagen content between regions or between the proximal and distal parts for the diabetic animals (Figure 4(d)). For the lean controls, there was also no difference between regions, but collagen content was greater in the proximal than distal part in all regions except for the periphery where there was only a trend ($p = 0.07$) (Figure 3(d)).

Discussion

To the best of our knowledge regional differences in tendon turnover across and along the tendon have not been reported previously. Somewhat contrary to our hypotheses the main finding of this study was that AGE accumulation was greater in the proximal than the distal part of the tendon in both lean control and diabetic animals. In the distal part, the AGE accumulation was greater in the core than in the periphery for the lean controls and tended to be so for the diabetics. This indicates that turnover varies more along the tendon length than across the cross-section. The GAG content followed a similar pattern to the AGEs and was surprisingly not increased in the surface. In the central tendon, the core displayed higher mechanical modulus and stress.

Biochemical properties

From previous studies, we know that AGE accumulation is dependent on collagen turnover rates,[29] such that a greater accumulation suggests a lower collagen turnover, e.g., tendons and cartilage accumulate more

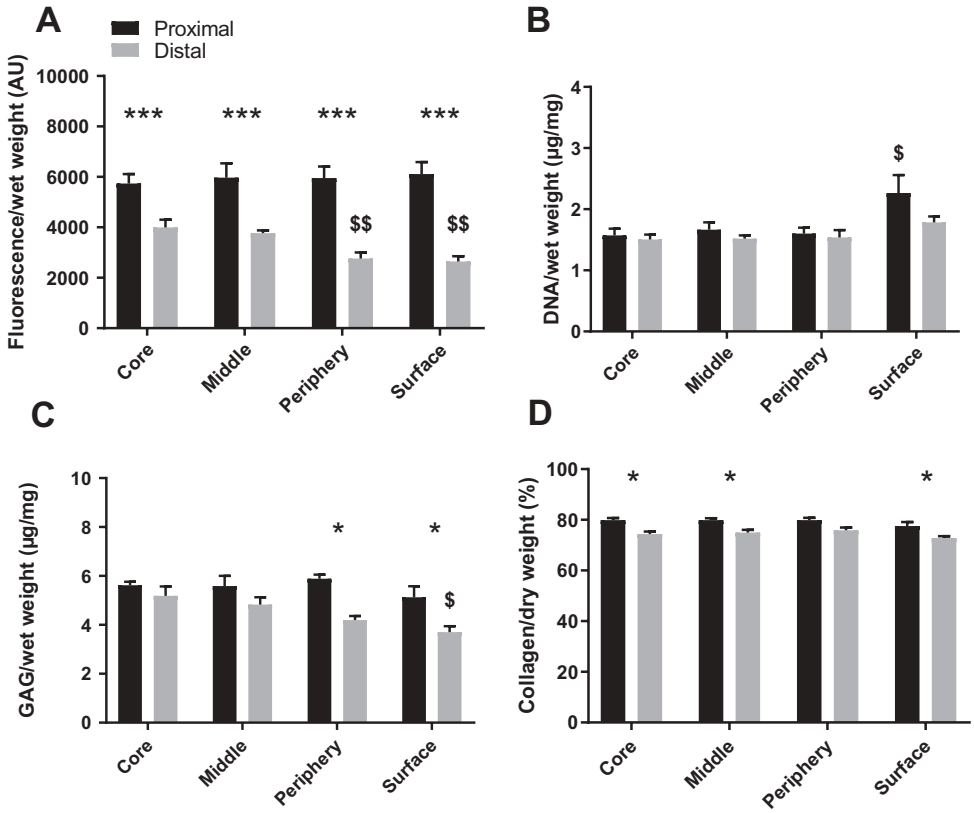

Figure 3. Biochemical test results of lean control pigs. Samples were taken from different radial (core, middle, periphery, and surface) and longitudinal (proximal and distal) positions. (A) Autofluorescence (AGEs) normalized to wet weight. (B) DNA content normalized to wet weight. (C) GAG content normalized to wet weight. (D) Collagen content in percent of dry weight. Mean ± SE, * Significantly different between proximal and distal part, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $ Significantly different from the core region, $ $p < 0.05$, $$ $p < 0.01$. N = 4.

AGEs than skin and muscle.[13,30,31] At the same time, previous studies have reported substantial growth in the periphery but not the core of the Achilles in an overload mouse model,[3] and it has also been reported that exercise can induce tendon growth in adult and even old human patellar tendon.[2] Tissue growth with the apparent lack of turnover in the core suggests that turnover would take place at the periphery. We therefore expected that AGE accumulation would also be greater in the core than the periphery. The present study found no indication of regional difference in the proximal part of the tendon. However, in the distal part, the lean controls had greater AGE accumulation in the core compared to the periphery and surface, and the diabetic animals displayed a similar tendency. Furthermore, there was a consistently greater AGE accumulation in the proximal compared with the distal part across all samples, which indicates that tendon turnover may be greater in the distal part. To our knowledge, this is the first study demonstrating different collagen turnover along the length of a tendon. Contrary to our expectations, there was no distinct increase in AGE autofluorescence in the diabetic animals. We speculate that this may be due to the limited duration of diabetes and the unspecific nature of the fluorescence measurement, since different products of glycation have been reported to respond differently to diabetes and age.[32]

Overall, a higher turnover in the distal part could indicate that longitudinal growth of the tendon may be occurring at a greater rate at the distal than proximal end. During embryogenesis, tendon growth has been shown to occur from both the muscle and bone/cartilage insertions in mouse front limbs[33]. However, to our knowledge, no studies have investigated the origins of tendon growth during postnatal maturation.

Similar to the AGEs, GAG content was greater in the proximal compared with the distal part. For lean control pigs, GAG content also tended to continually decrease from the core toward the surface, although it was only significant in the surface compared to the core. Increased GAG content has been associated with compressed regions in tendon[34]; however, we would not expect the proximal region just below the muscle

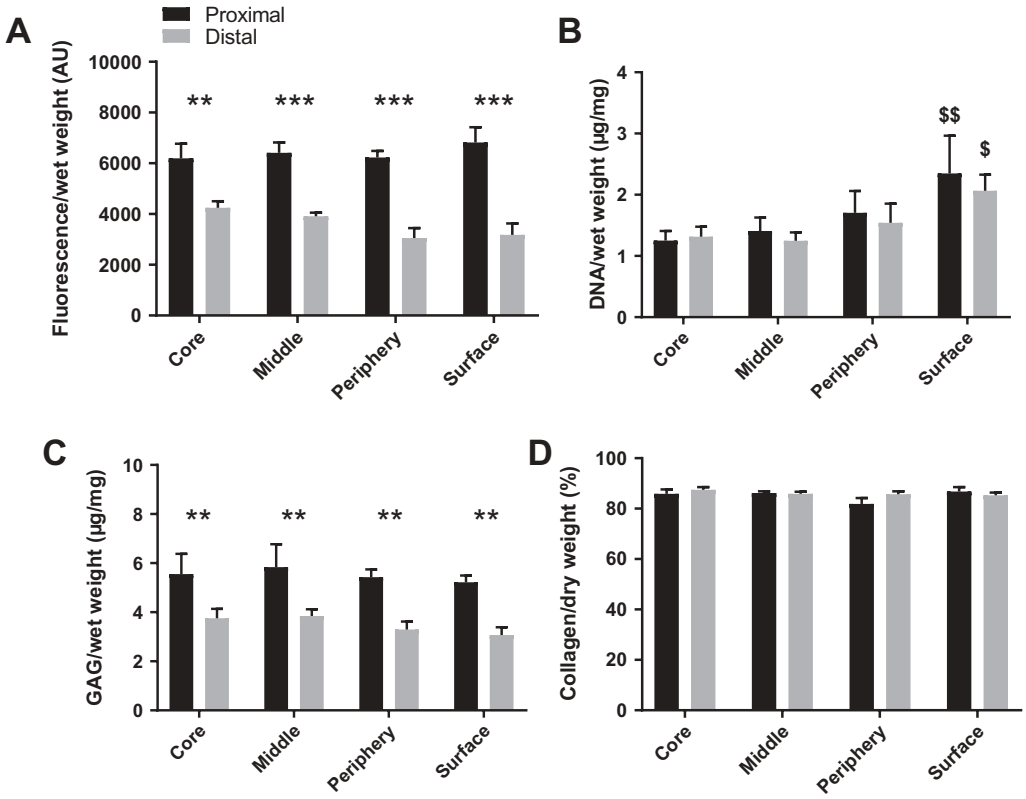

Figure 4. Biochemical test results of diabetic pigs. Samples were taken from different radial (core, middle, periphery, and surface) and longitudinal (proximal and distal) positions. (A) Autofluorescence (AGEs) normalized to wet weight. (B) DNA content normalized to wet weight. (C) GAG content normalized to wet weight. (D) Collagen content in percent of dry weight. Mean±SE, * Significantly different between proximal and distal part, ** $p < 0.01$, *** $p < 0.001$. $ Significantly different from the core region, $ $p < 0.05$, $$ $p < 0.01$. $N = 6$.

insertion to experience significant compression. If anything the distal region could be expected to be under more compression.[34] One of the roles of GAGs is to inhibit collagen fibril growth,[35] so a lower GAG concentration in regions with higher turnover might promote growth of newly formed fibrils in those regions. However, this is speculative, and the reason for the observed difference in GAG content is therefore not entirely clear.

With respect to collagen content, there were no regional differences across the tendon section, but in the lean control group, collagen content was higher in the proximal than the distal part. Interestingly the diabetic group had higher collagen content in all regions than the lean control group, and this is consistent with a previous animal study, which showed that streptozotocin-induced diabetic rats had an increase in collagen content.[36]

There was also no difference in cell density in the core, mid, and peripheral part (as indicated by DNA content), although the surface had a greater cell density than the core. While interpretation of the results from the surface can be ambiguous due to being a mixture of sheath and tendon proper, greater cellularity was expected from the sheath.[3] However, we had expected that the peripheral part of the proper tendon would also have a higher cellularity. Previous research has shown that cell density drops dramatically in tendon with maturity,[37–39] and since we hypothesized that tendon growth occurs in the periphery, we expected the peripheral tissue to be more immature and consequently have higher cell density, but that was not the case.

Mechanical properties

Previous human studies have shown that peak stress, yield stress, and tangent modulus of young patellar tendon were higher for anterior fascicles than posterior, indicating that tendons have different regional material properties.[28,38] Other studies on rabbit patellar tendon have reported regional differences in structure,[40] but not mechanics.[41] It is plausible that the mechanics of the knee joint influences the anterior and posterior portions

of the patellar tendon. However, the present study shows that pig flexor tendon exhibit greater peak modulus and peak stress in the core than in the periphery, which corroborates earlier studies in human tendon.[28,38] To have a more direct comparator to the mechanical findings, the remaining tissue after mechanical testing was assessed for collagen content and fluorescence, and collagen content normalized to wet weight was greater in the core (11.6%) than the periphery (9.6%), while fluorescence was not significantly different. It is therefore possible that the greater mechanical properties in the core region were related to greater collagen content. However, for these samples, we only had the wet weight, and total sample weight was small, which makes the measurements considerably less reliable. Therefore, these measurements were not included in the study results.

Limitations

There are inherent limitations associated with the study. The sample size was limited, although significant differences could be detected. Diabetic animals on normal and high salt diet were combined, but the small sample size precluded any analysis of the effect of dietary salt. From a clinical perspective, it would have been relevant to examine the Achilles tendon,[7] but this is quite short in pigs, and is also a fusion of three tendons coming from the soleus and two heads of gastrocnemius,[42] which would have precluded the detailed regional analysis possible with the flexor tendon. It was not possible to measure biochemical and mechanical properties on the exact same regions of tissue, which prevents a direct relation of mechanics and biochemistry. It is known that physical activity may influence tendon properties,[43] but this was not possible to control for in this study. Also, the pigs were euthanized at 20 months of age, which is just around the point of maturity but still relatively young since their lifespan is around 15 years old.

Turnover was estimated from AGE accumulation based on total tissue autofluorescence, which is an indirect method that can be affected by other sources of fluorescence.[44] In addition, it is unknown to what extent other factors such as the level of perfusion could influence the site-specific AGE accumulation. Finally, fluorescence should only be considered a marker of AGE accumulation since several AGEs including the major AGE cross-link glucosepane is not fluorescent. However, autofluorescence measured in a similar way has previously been shown to correlate well with turnover measured by aspartic acid racemization.[14]

Perspective

Substantially more work is required to elucidate the growth and turnover of tendon tissue. Future work is planned on human tendon tissue, which will have greater clinical relevance. In addition, using tissue from humans will allow a sufficient age range to utilize more accurate measures of tissue turnover such as carbon-14 bomb-pulse dating. Further work to determine regions of postnatal tendon growth in animal models would also be valuable to understand mechanisms of growth and possibly regeneration in mature tissue, which could have implications for healing of injured tendon.

Conclusion

To our surprise, AGEs and GAG accumulated more in the proximal than the distal part in both lean and diabetic animals, indicating that collagen turnover could be higher in the distal part. In cross section, lean pigs had higher AGE accumulation in the core compared to the periphery of the distal part as expected, but this was not the case in the proximal part or in the diabetic pigs. Similar to AGEs, GAG content was also greater in the core compared with the surface. For the mechanical test, the core displayed higher mechanical modulus and sustained higher mechanical stress compared with the peripheral region.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Study 2

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RESEARCH ARTICLE

Regional collagen turnover and composition of the human patellar tendon

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[1]*Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery, Bispebjerg Hospital, Copenhagen, Denmark;* [2]*Center for Healthy Aging, University of Copenhagen, Copenhagen, Denmark;* [3]*Department of Physical and Occupational Therapy, Bispebjerg Hospital, Copenhagen, Denmark;* [4]*CARIM School for Cardiovascular Diseases, Maastricht University Medical Centre, Maastricht, The Netherlands; and* [5]*Department of Internal Medicine, Maastricht University Medical Centre, Maastricht, The Netherlands*

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Zhang C, Couppé C, Scheijen JLJM, Schalkwijk CG, Kjaer M, Magnusson SP, Svensson RB. Regional collagen turnover and composition of the human patellar tendon. *J Appl Physiol* 128: 884–891, 2020. First published March 12, 2020; doi:10.1152/japplphysiol.00030.2020.—Tendon pathology (tendinopathy) typically occurs in specific regions of a tendon, and growth in response to exercise also appears to be more pronounced in specific regions. In a previous study in animals we found evidence of regional differences in tendon turnover, but whether the turnover of human patellar tendon differs in different regions still remains unknown. Patellar tendons were obtained from cadavers of healthy men and women (body donation program, $n = 5$ donors, >60 yr of age). Samples were taken from 10 different regions along the length, width, and thickness of the tendon. Turnover was measured by ^{14}C bomb pulse dating and also estimated from the accumulation of advanced glycation end products (AGEs) by fluorescence (340/460 nm) in addition to measurement of specific AGEs by mass spectrometry. Composition in terms of collagen, glycosaminoglycans (GAGs), and DNA was also assessed in each region. ^{14}C results showed that all tendon regions had a similar ^{14}C concentration, which was equal to the average atmospheric ^{14}C concentration during the first 15 yr of the person's life. Fluorescence normalized to dry weight did not differ between regions, nor did specific AGEs. Higher GAG content was observed in the proximal and near the distal insertion of the tendon. In conclusion, healthy human patellar tendon displays no regional differences in collagen turnover throughout life.

NEW & NOTEWORTHY Tendon injuries and tendinopathies typically occur in specific regions of the tendon, but the reason for this specificity is not well understood. A potential factor in injury susceptibility is tissue turnover, and previous work suggests that the tendon core has practically no turnover during adult life; however, it is not known whether this is true for other regions of the tendon. Our present results on healthy human patellar tendon clearly demonstrate that turnover does not differ between regions and thereby cannot explain differences in injury susceptibility. The findings also indicate that all regions of the tendon are formed simultaneously during skeletal maturation and do not turn over appreciably during adulthood. This is an important finding because little is known about tendon growth during maturation in humans.

advanced glycation end products; autofluorescence; ^{14}C bomb pulse; collagen turnover; regional differences

INTRODUCTION

Tendons are dense connective tissues that attach muscles to bones, and they exhibit mechanical properties that enable them to effectively transmit load generated by muscles to the bone (34). The patellar tendon connects the patella to the tibial tuberosity and transmits quadriceps muscle forces that extend the leg. The patellar tendon structure has some inherent complexity to it because the anterior layer runs over the surface of the patella to the muscle, whereas the posterior part acts more like a ligament by attaching the patella bone to the tibia.

Patellar tendinopathy is a painful disorder that commonly affects athletes engaged in jumping sports, such as basketball and volleyball (28), and it most frequently involves the proximal posterior portion of the tendon (65%; 20, 21, 28), which has been verified by both imaging and histological investigations (20, 21, 40). The healthy patellar tendon is heterogeneous along its length with the cross-sectional area (CSA) increasing from the proximal to the distal region (23). In the tendinopathic tendon there is a thickening of the posterior proximal region (20) in addition to neovascularization (1, 7). From these observations it is clear that structural changes with patellar tendinopathy are commonly focused in the proximal posterior region; however, the exact reason why tendinopathy appears to be region specific remains an enigma.

It is possible that the region-specific tendinopathy is related to differences in tensile strain during loading (2, 6, 15). In fact, isolated tendon fascicles in the posterior regions appear to be mechanically considerably weaker than those of the anterior part of the tendon (13, 15). Differences in lever arm could be a possible reason for the regional variation in mechanical properties in patellar tendon (50). Because the lever arm on the anterior region has a greater advantage compared with the posterior region, the proximal posterior region of the tendon may be stress shielded (2). It is also possible that differences in collagen cross-linking (9), fibril morphology (38), and composition of the extracellular matrix (22) contribute to the region-specific tendinopathy.

Studies have shown that the regenerative capacity of tendons is limited, which may be due to slow tissue turnover (16). However, the rate of tendon tissue turnover is not fully understood. Data based on aspartic acid racemization in high-stress tendon in horses suggest that the half-life of collagen protein is ~200 yr (46), and data using the ^{14}C bomb pulse method in human tendon samples indicate that renewal of adult core

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tendon tissue is extremely limited past 17 yr of age (16). Yet, using microdialysis of procollagen peptides taken from peritendinous tissue and using stable isotope-labeled amino acid incorporation into patellar tendon tissue suggested that tendons have a measurable increase in collagen protein synthesis after exercise (25, 30). These conflicting results underline that many details regarding tendon growth and turnover still remain unknown. Regional differences could contribute to the inconsistent findings since microdialysis was applied in the tendon periphery, whereas ^{14}C measurements were made in the tendon core. Tendinopathy is often associated with neovascularization, and an increased intratendinous blood flow (37, 41, 49), which may bring more cells (36) to the affected region and thereby affect tendon turnover.

Advanced glycation end products (AGEs) are proteins or lipids that have been glycated as a result of exposure to sugars, forming a number of different compounds (3, 4). Previous studies have shown that at least the carboxymethyllysine (CML), carboxyethyllysine (CEL), and pentosidine products accumulate linearly with age and that they do so to a greater extent in cartilage than in skin (48). This age-associated AGE accumulation may therefore be used as an indirect measure of collagen turnover, and we have recently reported that tendon turnover is higher in the distal part of pig flexor tendons (52) by measuring accumulation of advanced glycation end products.

In the present study, we investigated whether there were any regional differences in the biochemistry and turnover of collagen in healthy human patellar tendon, which may help explain the region specificity of patellar tendinopathy. The turnover was investigated by employing both the ^{14}C bomb pulse method and the AGE accumulation technique. We hypothesized that patellar tendon turnover would be greater in the proximal region, where tendinopathy typically occurs.

MATERIALS AND METHODS

Study Design

To investigate collagen turnover, three independent methods were applied: *1*) biochemical measurement of autofluorescence as a general marker of AGE accumulation, *2*) direct measurement of tissue age by the ^{14}C bomb pulse method, and *3*) mass spectrometry-based measurement of specific nonfluorescent AGEs. To support these measurements, basic composition [content of collagen, glycosaminoglycans (GAGs), and DNA] was measured on the same sample used for fluorescence measurements (see Supplemental Fig. S1; all Supplemental Material is available at https://doi.org/10.6084/m9.figshare.11591436).

Tendon Samples

Tissues were collected from different regions of patellar tendon from five cadavers between 68 and 86 yr of age [mean 76.2 ± 7.3 (SD) yr; 4 men and 1 woman]. Tissues from the anonymous Danish body donation program were collected, and their use does not require separate ethical approval. Samples were taken from 10 different regions of each patellar tendon (Fig. 1). The insertion [insertion (Ins)] was taken from the anterior tendon above the distal patella pole. The proximal tendon was divided into side [proximal side (PS)] and central parts with the central part divided into an anterior [proximal central anterior (PCA)] and a posterior [proximal central posterior (PCP)] layer. A similar scheme was used for the midtendon, which was divided into side [middle side (MS)] and central parts with an anterior [middle central anterior (MCA)] and posterior [middle central posterior (MCP)] layer. Finally, a sample was taken from the distal, central tendon [distal (Dis)] just proximal to the insertion.

Sample Preparation

About 300 mg wet wt were taken from each of the 10 different regions. These samples were further divided for biochemical measurements (~25 mg), ^{14}C bomb pulse dating (~40 mg), and measurement of specific AGEs (~30 mg). The remaining tissue was stored at −80°C. To ensure homogeneity, the samples were initially cut into smaller pieces, which were then scrambled before dividing between the analyses. All samples were freeze-dried overnight before further measurements (water content ~65%).

Biochemical Measurements

To remove lipids, samples were washed three times in 0.5 mL acetone for 1 h, overnight, and 1 h, respectively. Subsequently, samples were freeze-dried overnight and weighed.

The biochemical analyses were similar to our previous study (52), which was based on published protocols (19). Samples (~8 mg dry wt)

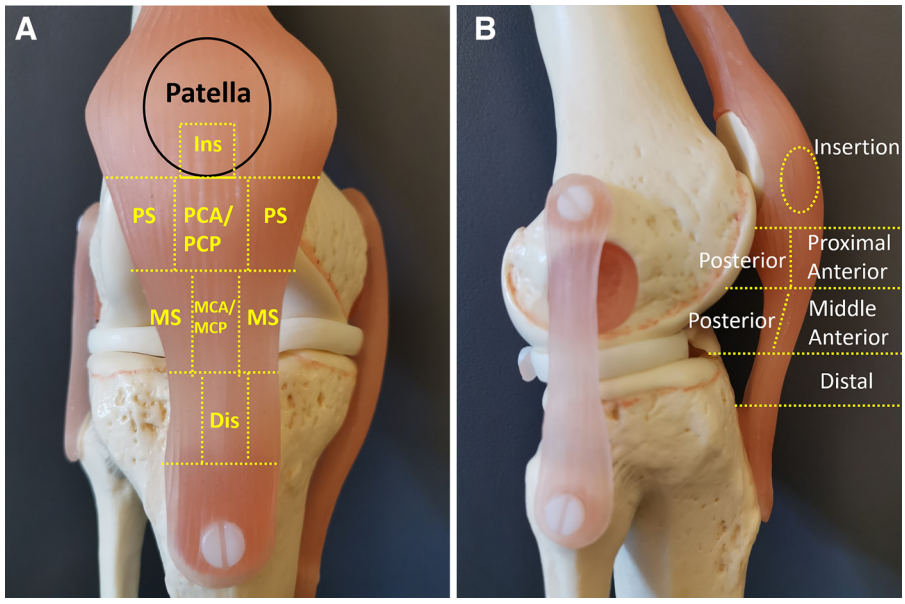

Fig. 1. Schematic diagram of patellar tendon. *A*: front view of patellar tendon. Samples were taken from 10 different regions. The insertion on patella (Ins). The proximal side (PS), the proximal central anterior (PCA), and proximal central posterior (PCP) layers. A similar scheme was used for the midtendon, divided into middle side (MS), middle central anterior (MCA), and middle central posterior (MCP) layers. Finally, distal central tendon (Dis). *B*: side view of patellar tendon. In proximal and middle parts of tendon, a sample was taken from anterior and posterior regions each.

and blanks (empty tubes) were digested in 0.5 mL papain overnight at 60°C. Papain digestion was made by using papain (P3125; Sigma, 0.125 mg/mL) in phosphate-buffered EDTA (PBE) buffer (100 mM sodium phosphate and 10 mM EDTA, pH 6.5) with 10 mM L-cysteine (52). This papain digest was used for analyses of fluorescent AGEs (autofluorescence), DNA content, glycosaminoglycan content, and collagen content.

Fluorescent AGE content. As previously described, AGE accumulation was measured by autofluorescence (12, 31, 32). Papain digest (25 μL) was mixed with 75 μL distilled water for a fourfold dilution. Triplicate samples were pipetted into wells of a 96-well black microwell plate (237107; Thermo Scientific). The plate was read at 338–343-nm excitation and 382–507-nm emission wavelengths, respectively, on a fluorometer (Wallac1420 Victor; PerkinElmer).

DNA content. DNA content was measured by binding of Hoechst stain as described in a previous paper (52). Papain digests (10 μL) were plated in triplicate, 150 μL of Tris-EDTA-NaCl (TEN) buffer (100 mM NaCl, 10 mM Tris·HCl, and 1 mM EDTA) were added, and the autofluorescence was measured at 338–343-nm excitation and 450–476-nm emission wavelengths, respectively. Subsequently, 50 μL of freshly prepared Hoechst stain (0.8 μg/mL in TEN) were added, and then fluorescence was immediately measured again. The difference in fluorescence before and after addition of Hoechst stain was calculated, and DNA content was determined from a standard curve with known concentrations of salmon DNA.

GAG content. As previously described (52), 10 μL of papain digest were mixed with 190 μL of 1,9-dimethylmethylene blue (DMB, 341088; Sigma-Aldrich) solution (38 μM DMB in 40 mM NaCl and 40 mM glycine, pH 3.0), and absorbance was read at 540 and 595 nm. Sample values were compared with a standard curve made with 12.5–150 μg/mL of chondroitin sulfate B (C3788; Sigma-Aldrich).

Collagen content. Collagen content was assessed by a hydroxyproline assay (45, 52). From the papain digest, 5 μL were hydrolyzed in 195 μL of 6 M HCl. Samples were dried under nitrogen flux for ~1.5 h and reconstituted in 1 mL acetate citrate buffer. Samples were diluted 100-fold into acetate citrate. Hydroxyproline was measured by a colorimetric assay based on 4-dimethylaminobenzaldehyde. Hydroxyproline concentrations were converted to collagen concentration by multiplying by 7.5 (assuming 13.3% hydroxyproline by mass in collagen; 33).

^{14}C Bomb Pulse Method

Sample purification. To avoid modern carbon from GAGs, fat, and other noncollagenous substances, a collagen purification procedure was performed as described in a previous paper (18). For ^{14}C measurement, on average 13.5 mg of freeze-dried sample were used. To each sample, 1 mL of fresh hyaluronidase solution (5 units/mL) was added and incubated on a shaker overnight in an oven at 37°C. Samples were washed with sterile saline (0.9%), and 1 mL of trypsin solution (1 mg/mL in PBS buffer) was added to all samples, with incubation overnight at 37°C. Subsequently, samples were washed with PBS buffer for 10 min, 0.7 M KCl solution for 1 h, and three times 10 min with distilled water. Samples were freeze-dried, and lipids were removed by three acetone washes of 0.5 mL for 1 h, overnight, and 1 h, respectively. Finally, samples were washed three times in 0.5 mL 96% ethanol for 2 h each, followed by three times distilled water wash for 1 h each. Samples were freeze-dried overnight and weighed immediately. Sample dry weight was ~10.8 mg after purification. To assess the influence of the purification, part of the sample (~5 mg) was analyzed for fluorescence, GAG, and collagen in the same manner as described in *Biochemical Measurements*.

^{14}C analysis. Nuclear bomb testing during 1955–1963 dramatically increased the amount of ^{14}C in the atmosphere, leaving a spike in the atmospheric ^{14}C level referred to as the ^{14}C bomb pulse. The $^{14}CO_2$ is incorporated into plants and then passed to animals and humans leading to levels of ^{14}C in tissue that are close to the atmospheric concentration when the tissue was formed. For economical reasons, ^{14}C and specific AGEs were only measured in four regions: the insertion (Ins), distal (Dis), middle central anterior (MCA), and middle central posterior (MCP) regions.

About 5-mg purified samples were used for analysis at the AMS ^{14}C Dating Centre, Aarhus University. Samples for accelerator mass spectrometry (AMS) were combusted with CuO in sealed combustion tubes at 950°C and converted to graphite before ^{14}C analysis at the 1-MV Tandetron accelerator (35). The radiocarbon dating results are reported according to international convention (43), and ^{14}C content is given as a percentage of modern carbon (pMC) based on the measured ^{14}C/^{13}C ratio corrected for the natural isotopic fractionation by normalizing the result to the standard δ^{13}C value of −25‰ Vienna Pee Dee Belemnite (VPDB; δ^{13}C calibration standard). Stable isotope values of δ^{13}C, δ^{15}N carbon and nitrogen fraction (by weight) and carbon-nitrogen (C/N) atomic ratios were measured at the Aarhus AMS Centre by continuous-flow isotope ratio mass spectrometry.

Specific AGE Content

As mentioned in MATERIALS AND METHODS, *^{14}C Bomb Pulse Method*, *^{14}C analysis*, only four of the regions were included in this analysis. Specific AGEs were measured as previously described (14, 29, 39). In brief, high-performance liquid chromatography (HPLC) was used to quantify pentosidine with fluorescence, as described in a previous paper (39). Ultraperformance liquid chromatography-tandem mass spectrometry (UPLC MS/MS) was used to quantify CML, CEL, methylglyoxal-derived hydroimidazolone (MG-H1), and lysine (14, 29). Concentrations of pentosidine, CML, CEL, and MG-H1 were adjusted to levels of lysine and expressed as nanomoles per millimole lysine. Hydroxyproline could not be measured directly in these samples but correlated well with lysine in previously analyzed tendon samples (see Supplemental Fig. S2).

Data Analysis and Statistics

All data are presented as means ± SE. For comparing different regions, repeated measures one-way ANOVA was used, with Tukey's correction for multiple comparisons. Differences were considered significant when $P < 0.05$. All statistical tests were performed in GraphPad Prism v.8.0 (GraphPad Software, La Jolla).

RESULTS

Biochemical Measurements

The biochemical data are shown in Fig. 2. The fluorescence data revealed no regional differences (Fig. 2*A*). DNA content also did not display any regional differences (Fig. 2*B*). With respect to GAGs, the insertion (Ins) had a greater content compared with the proximal central anterior (PCA; $P < 0.05$), proximal central posterior (PCP; $P < 0.05$), and middle central anterior (MCA; $P < 0.05$) regions. Distal (Dis) also had greater GAG content compared with proximal side (PS; $P < 0.01$), proximal central anterior (PCA; $P < 0.05$), middle side (MS; $P < 0.001$), middle central anterior (MCA; $P < 0.01$), and middle central proximal (MCP; $P < 0.01$; Fig. 2*C*). There were no significant differences in collagen content between regions (Fig. 2*D*).

^{14}C Bomb Pulse

Results of the ^{14}C measurements are shown in Fig. 3. Concentrations of ^{14}C from each region are shown at the birth year of each individual. The ^{14}C levels did not differ between

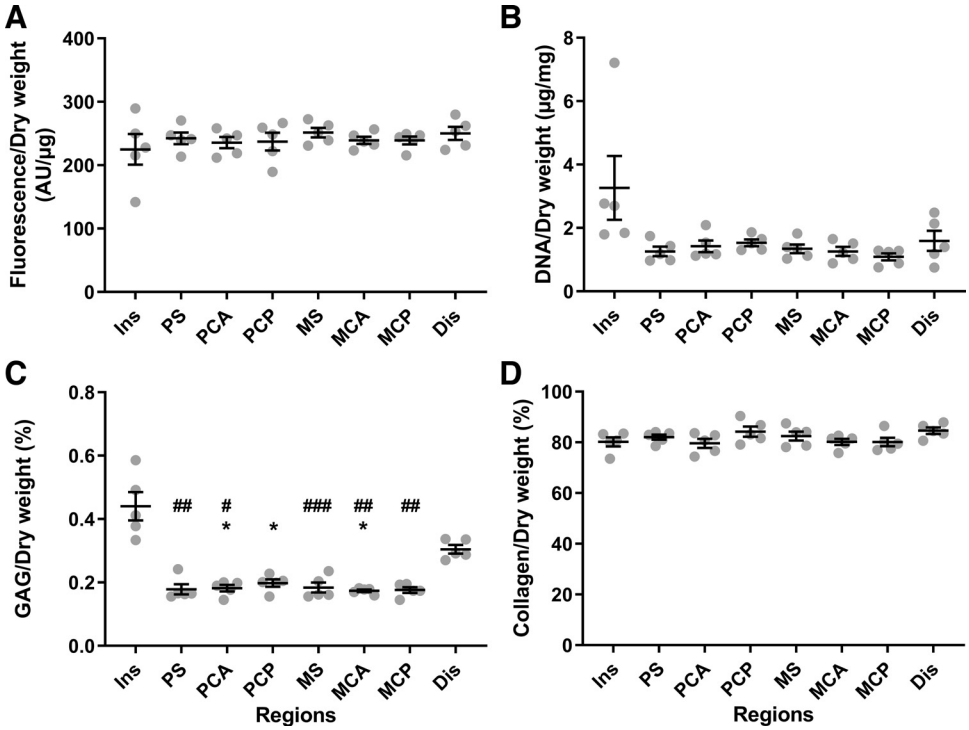

Fig. 2. Biochemical results. Samples were taken from different regions: insertion (Ins), proximal side (PS), proximal central anterior (PCA), proximal central posterior (PCP), middle side (MS), middle central anterior (MCA), middle central posterior (MCP), and distal (Dis). *A*: autofluorescence (advanced glycation end products) normalized to dry weight. *B*: DNA content normalized to dry weight. *C*: glycosaminoglycan (GAG) content in %dry weight. *D*: collagen content in %dry weight. Means ± SE; $n = 5$ donors. AU, arbitrary units. $^{*}P < 0.05$ significantly different from Ins. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ significantly different from Dis.

the regions within each individual. Three donors born before 1940 had ^{14}C levels that equaled the prebomb levels. The remaining two donors had elevated ^{14}C levels, which were found to fit well with a moving average of atmospheric ^{14}C covering the first 15 yr after birth.

Samples used for ^{14}C measurements underwent a collagen purification procedure, and biochemical measurements performed after purification are shown in Supplemental Fig. S3. Fluorescence did not change much with purification and was still not different between regions (Supplemental Fig. S3*A*). GAG content decreased from around 0.27% to 0.075% and no longer differed between regions (Supplemental Fig. S3*B*). Collagen content showed no differences between regions (Supplemental Fig. S3*C*) but appeared to be reduced after purification, most likely because of a technical error (see Supplemental Material).

Specific AGEs and Lysine Measurement

The same four regions used in the ^{14}C measurements were used in the analysis of protein-bound AGEs. CML concentration normalized to lysine was ~5,500 (nmol/mmol) and did not

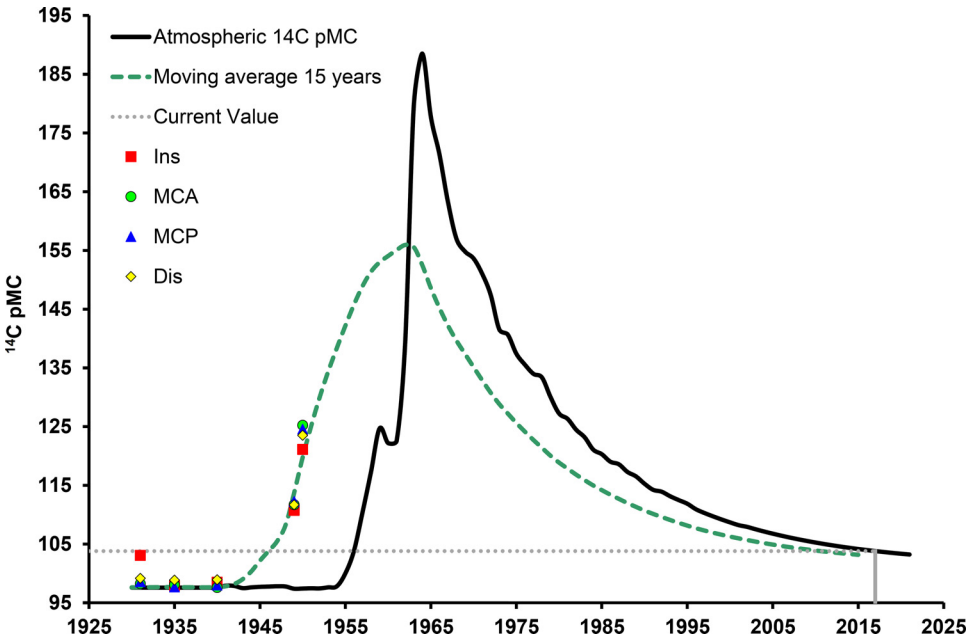

Fig. 3. ^{14}C in different regions of patellar tendon. The solid black curve shows the ^{14}C level in the atmosphere (24, 27) up to 2012 (extrapolated to 2020). Levels of ^{14}C in purified collagen from different regions of patellar tendon are plotted according to the birth year of the donors ($n = 5$ donors). The green dashed curve shows a moving average of the atmospheric ^{14}C concentration over the following 15 yr. The ^{14}C levels of patellar tendon fit well with the dashed curve, suggesting that the patellar tendon was gradually formed during the first 15 yr with little to no turnover thereafter. Dis, distal; Ins, insertion; MCA, middle central anterior; MCP, middle central posterior; pMC, percentage of modern carbon.

differ between regions (Fig. 4*A*). CEL normalized to lysine was ~400 (nmol/mmol) and did not differ between regions (Fig. 4*B*). Similar to these two AGEs, MG-H1 normalized to lysine was ~13,000 (nmol/mmol) and did not differ between regions (Fig. 4*C*). Pentosidine normalized to lysine was ~80 (nmol/mmol), without any regional difference (Fig. 4*D*).

DISCUSSION

To the best of our knowledge, this is the first paper to investigate collagen turnover of the patellar tendon in different regions along the length, width, and thickness. Previous research has shown that patellar tendinopathy most frequently involves the tissue of the proximal posterior region (20, 21), which results in greater blood flow and a higher number of cells in the tendon. Therefore, we hypothesized that the turnover would differ by region in patellar tendon, but contrary to our hypothesis all of our data convincingly show that there was no difference in any region of patellar tendon with respect to turnover.

^{14}C Bomb Pulse

We consider the ^{14}C bomb pulse method to be the gold standard for measuring turnover in tendon. The four regions had very similar ^{14}C levels within subjects, which indicates similar collagen turnover in all regions. Furthermore, the turnover throughout adult life appears to be extremely low with collagen formation chiefly occurring during the first 15 yr of life. The present result is in accordance with previous research on the Achilles tendon, which showed that the tendon core is developed during the first 13 or 17 yr of life and is not renewed during adulthood (16, 18). However, we extend on the prior findings by showing that it is not a region-specific event, but rather that growth occurs evenly throughout the tendon. The reason for the difference between 13 and 17 yr may be attributed to purification since the 17-yr-formation data (16) were based on unpurified samples, whereas the 13-yr-formation data (17) were on samples purified to reduce the amount of modern carbon. A single sample from the insertion region on the patella of the oldest individual appeared to display a higher content of modern carbon (Fig. 3). This particular sample also displayed lower AGEs by autofluorescence (Fig. 2*A*) and higher DNA content (Fig. 2*B*), all consistent with greater collagen turnover. It therefore seems likely that this region in this particular individual indeed had greater turnover. Although we do not have any medical records, it could potentially be related to an injury at some point in the life of that specific person.

Biochemical Measures

The average composition of the tendon (82% collagen, 0.23% GAG, and 1.6 μg/mg DNA) was comparable to previous studies having found collagen content in the range of 65–90% (8, 10, 26) and GAG content of 0.37% (44). We are not aware of DNA measurements in human patellar tendon, but a study in pigs reported ~1.6 μg/mg (51).

AGE accumulation has been shown to depend on collagen turnover rates (5), with a greater AGE accumulation indicating a lower collagen turnover. Thus, AGEs are useful as a surrogate to indicate tendon turnover (42, 47, 48, 52). The present data showed that the fluorescent AGE accumulation was very similar in all tendon regions, suggesting that the collagen turnover is equal in all regions, which is in agreement with the ^{14}C results.

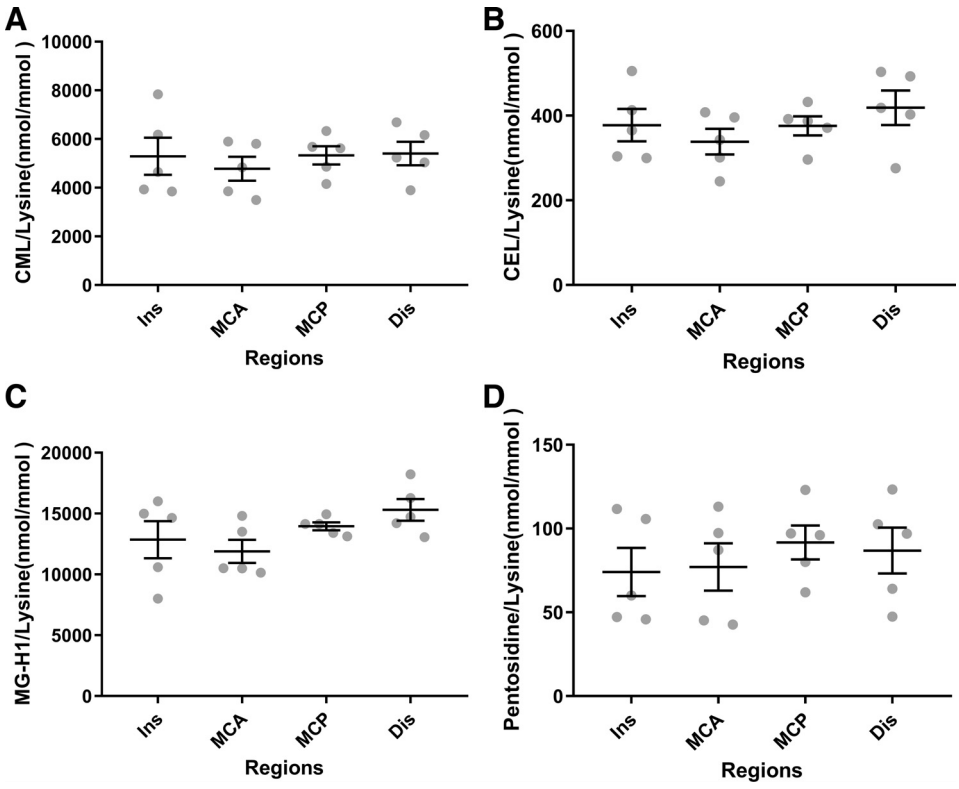

Fig. 4. Protein-bound advanced glycation end products. Samples were taken from four different regions: insertion (Ins), middle central anterior (MCA), middle central posterior (MCP), and distal (Dis). *A*: carboxymethyllysine (CML) normalized to lysine. *B*: carboxyethyllysine (CEL) normalized to lysine. *C*: methylglyoxal-derived hydroimidazolone (MG-H1) normalized to lysine. *D*: pentosidine normalized to lysine. Means ± SE; $n = 5$ donors.

The AGE results differed from our expectation since we have shown that the core of the tendon had greater AGE accumulation than the periphery in porcine flexor tendon (52). This difference may relate not only to species differences but also to the structure since the patellar tendon does not have a distinct core and periphery because of its more flattened morphology.

GAG content was greater in the insertion and distal regions compared with the rest. The insertion region lies on the surface of the patella, and the distal region makes contact with the tibial plateau when the knee joint is flexed (Fig. 1); therefore these two regions may be exposed to higher mechanical compression, which could explain the greater GAG content (11).

Although there were no significant differences, the insertion and distal regions appeared to have numerically higher DNA content compared with the other regions, whereas the other six regions displayed very similar DNA content. This pattern is somewhat similar to that of the GAG content, but not the collagen content or turnover, suggesting that cells may be involved in turning over GAGs, but not collagen, in the adult tendon. We have previously observed more cells in the proximal part of the porcine digital flexor tendon than the distal part (52), which may be related to its closer proximity to the muscle with greater available blood flow. In the present work, the insertion was also obtained from the proximal site of the patellar tendon in close proximity to the patella bone, but to our knowledge the blood flow is not expected to be greater in this region.

Specific AGEs and Lysine Measurement

The fluorescence measurements in the present study represent an indirect measure of all fluorescent AGEs; however, not all AGEs are fluorescent. Therefore, mass spectrometry-based quantification of specific AGEs was assessed to get a more direct measure, including some important nonfluorescent AGEs such as CML, CEL, and MG-H1. Previous work has shown that pentosidine, CML, and CEL correlate well with collagen turnover (48). Similar to the fluorescence, there were no differences between regions with any of the specific AGEs measured. Collectively, the AGE measurements further support the notion that collagen turnover is similar in all regions of human patellar tendon.

Limitations

In the present study, the sample size was rather limited, and therefore smaller regional differences could have been present that the study was underpowered to detect. However, the AGE and ^{14}C results obtained were very consistent and generally displayed little variance between regions, making us fairly confident that the collagen turnover did in fact not differ between regions.

Conclusions

In conclusion, collagen turnover assessed by the bomb pulse dating technique and by measurement of AGE accumulation consistently indicated no differences between any regions of the patellar tendon. Meanwhile, the insertion and distal regions displayed higher GAG content. Overall, the results did not support our hypothesis that tendon regions commonly afflicted in tendinopathy would display an increased turnover.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.K., S.P.M., and R.B.S. conceived and designed research; C.Z., J.L.J.M.S., C.G.S., and R.B.S. performed experiments; C.Z., J.L.J.M.S., C.G.S., and R.B.S. analyzed data; C.Z., C.C., M.K., S.P.M., and R.B.S. interpreted results of experiments; C.Z. prepared figures; C.Z. drafted manuscript; C.Z., C.C., J.L.J.M.S., C.G.S., M.K., S.P.M., and R.B.S. edited and revised manuscript; C.Z., C.C., J.L.J.M.S., C.G.S., M.K., S.P.M., and R.B.S. approved final version of manuscript.

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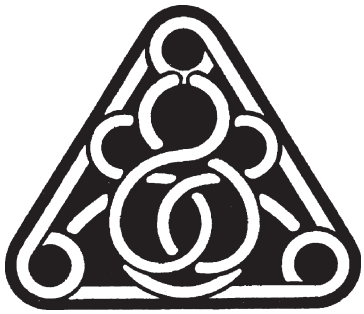

Study 3

For submission to Journal of Proteome Research. Article written in American English.

A Comparison of Tenocyte Populations from the Core and Periphery of Equine Tendons

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RUNNING TITLE: Proteomics analysis of horse tendon cell cultures

ABSTRACT

Tendon is a highly organized, dense connective tissue that has been demonstrated to have very little turnover. In spite of the low turnover, tendon can grow in response to loading, which may take place primarily at the periphery. Tendon injuries and recurrence of injuries are common in both human and animal in sports. It is unclear why some areas of the tendon are more susceptible to such injury and whether this is due to intrinsic regional differences in extracellular matrix (ECM) production or tissue turnover. This study aimed to compare populations of tenocytes derived from the tendon core and periphery. Tenocytes were isolated from equine superficial digital flexor tendons (SDFT), and the proliferation capacity was determined. ECM production was characterized by immuno- and histological staining and by liquid chromatograph-mass spectrometry-based proteomics. Core and periphery SDFT cultures exhibited comparable proliferation rates and had very similar proteome profiles, but showed biological variation in collagen type I deposition. In conclusion, the intrinsic properties of tenocytes from different regions of the tendon are very similar and other factors in the tissue may contribute to how specific areas respond to loading or injury.

KEYWORDS: proteomics, fibroblasts, cell proliferation, extracellular matrix

INTRODUCTION

Tendons are dense connective tissues connecting muscle to bone, and their principle function is force transmission in both humans and animals, such as horses (1, 2). Tendon injuries are common in sports among both athletes and the general public, and they account for up to 50% of all sports injuries (3). In humans, Achilles tendinopathy has the highest occurrence in middle and long distance running, tennis, badminton and football among sports amateurs (4) and frequently occurs at the insertion site (the enthesis) and mid-portion of the tendon (3, 5). Patellar tendinopathy is a very common disorder in jumping sports, such as volleyball and basketball (6). Imaging and histological investigations showed that patellar tendinopathy normally occurs in the proximal and posterior regions of the patellar tendon (65%) (7-9). Why some areas of tendons are more susceptible to injuries and tendinopathies is unclear, but differences in collagen cross-linking (10), composition of extracellular matrix (ECM) (11), fibril morphology (12) or metabolic activities of cells residing in the tissue are key contributing factors to be considered.

Tendons are able to grow in response to long-term exercise (13, 14), and a mouse model of severe overload indicates that growth may primarily take place at the tendon periphery (15). A study using microdialysis on the surface of tendon found a measurable increase in collagen protein synthesis after exercise (16), however, a pioneering study that measured tissue incorporation of atmospheric radioactive isotope carbon-14 (^{14}C) resulting from the bomb-pulse between 1955 and 1963 found that the core of healthy human Achilles tendons have very limited renewal after 17 years of age (17). Our previous study of porcine flexor tendons showed that DNA content was higher in the tendon periphery compared to the core of the tendon (18), which indicates that the outer regions of tendons have more cells than the core of the tendon. Therefore we hypothesized that the apparent differences observed in turnover of different tendon regions may be a result of differences in cell number.

Recent advances in single cell RNA sequencing technologies and bioinformatics have identified more than one cell type in tendons. One study showed that the pericytes are a progenitor cell population for adult tendon fibroblasts that can produce distinct components of tendon extracellular matrix (ECM) (19). Another study combined surface proteomics with gene expression analysis of single cells to show that human tendon consists of at least eight sub-populations of cells (20). Therefore it is probable that different tenocyte populations residing in specific regions of the tendon and possessing distinct ECM regulation properties are a source of variation between core and periphery tendon turnover.

Most animals are not prone to develop tendon injuries, but horses are an exception. In horses, forelimb tendons are much more prone to injury (97-99%) than any other tendons, and the superficial digital flexor tendon (SDFT) accounts in 75-93% of these cases (21, 22). Similar to humans, the majority of these injuries are tendinopathies with less than 10% of them being tendon ruptures. The equine SDFT is therefore a

good model to study whether cellular properties play a role in regulating regional differences to tendon turnover.

In the current study, we derived tenocyte populations from the core and periphery of horse SDFTs and characterized their proliferation and ECM deposition. To determine whether there are differences in ECM production and the expression of ECM-regulating enzymes, we employed mass-spectrometry based proteomics (23). Label-free mass spectrometry has previously been successful for protein identification and quantification of ECM components in tendon tissues (24, 25). Elucidating the tissue components responsible for the variation in turnover between the core and periphery of tendons will improve the understanding of why some tendons or areas of tendons are more susceptible to injuries and tendinopathies.

EXPERIMENTAL PROCEDURES

Animals

This study utilized tendon tissue samples from a larger prospective intervention study in trained and untrained horses, which investigated the changes in the equine atria after high intensity training. All horses were standardbreds, mares or geldings between 4 and 10 years of age. For this current study, five horses between 4 to 10 years of age (mean 6.4 years ± 2.3 (SD); three mares and two geldings were selected. All five horses belonged to the control group consisting of animals that were either untrained or had been out of training for a minimum of 1 year. Before inclusion in the study an experienced equine surgeon performed a lameness examination of all included horses. The horses were graded according to the Equine Practitioners lameness grading scale (score 0-5) and only horses scoring <3 were included. The study was approved by the local ethical committee at the Department of Veterinary Clinical Sciences, University of Copenhagen and the Danish Animal Experiments Inspectorate (license number 2016-15-0201-01128) and was performed in accordance with the European Commission Directive 86/609/EEC.

Cell media

Pen-Strep: Final concentrations of 50 U/ml penicillin and 50 µg/ml streptomycin (Thermo Fisher Scientific) in the solution.

Culture-medium: DMEM/F-12 (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS) (BioWest) and Pen-Strep.

Matrix-medium: Culture-medium supplemented with 200 µM ascorbate-2-phosphate (Sigma).

Tendon cells

Superficial digital flexor tendons (SDFT) were dissected from the left forelimb (Fig. 1A and 1B). All surrounding tissues were removed, including skin, muscle, and any fat and loose connective tissues. Three 1-cm sections

were made from the mid-portion of the tendon 8-10 cm proximal to the sesamoid bone (Fig. 1A and B). From each 1-cm section the lateral and medial parts of the SDFT were removed (Fig. 1C). The remaining SDFT was divided into three parts: the anterior and posterior were considered as the periphery, the inner part was considered as the core of the tendon (Fig. 1C). Tendon cells were released from the tendon tissue as previously described (26). In brief, tendon tissues were washed in phosphate buffered saline (PBS) with Pen-Strep. After, tendon tissue was cut into smaller pieces and incubated overnight in 400 U/ml collagenase type 2 (Worthington Biochemical Corporation) prepared in DMEM/F-12 supplemented with 20% FCS and Pen-Strep at 37°C in 5% CO_2. Cells were then pelleted by centrifugation at 600 × g for 6 min. The cell pellets were resuspended in PBS, pelleted again and resuspended in culture-medium at 37°C in 5% CO_2. Medium was changed every 3-5 days. After approximately 10 days of culture, tendon cells derived from the core and the periphery of horse SDFT were frozen at passage 1 in liquid nitrogen and then stored at -80°C.

Growth curve assay

Experiments were performed based on a previously published protocol (27). To detect the proliferative capacity of tenocytes derived from the core and periphery of horse SDFT *in vitro*, cells between the passage of 1 and 3 were plated in a 24-well plate at a density of 1×10^4 cells/well and cultured for 10 days in culture-medium at 37°C in 5% CO_2. For each cell line, trypsinized cells from three individual wells were counted every 2 days (at day 0, 2, 4, 6, 8, 10) and from each well, the mean number of cells was calculated from three measurements.

Immunofluorescence staining

For each cell line, 3×10^4 cells/cm^2 were seeded onto glass coverslips in a 12-well plate and cultured for 4 days in matrix-medium at 37°C in 5% CO_2. After, cells were fixed with 0.5 ml Histofix (Histolab) for 10 minutes at RT. Cells were then washed with PBS for 5 minutes three times. Fixed cells were permeabilized with 0.5% Triton X-100 in Tris-buffered saline (TBS) for 10 minutes at RT. Coverslips were then washed with TBS three times and incubated with 5% bovine serum albumin (BSA), 0.1% Tween 20 in TBS for 30 minutes at RT. After blocking, primary antibodies specific to type I collagen (clone COL-1, Sigma) diluted 1:500 in 1% BSA, 0.1% Tween 20 in TBS were added to the coverslips and incubated overnight at 4°C. After, coverslips were washed with TBS for 5 minutes three times and incubated for 45 min at RT, protected from light with Alexa-568-conjugated goat anti-mouse antibodies (A11031, Thermo Fisher Scientific) diluted 1:200 in 1% BSA, 0.1% Tween 20 in TBS. Coverslips were washed with TBS for 5 minutes three times. The coverslips were mounted with Prolong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were captured on an Olympus BX51 microscope (Olympus, Japan) with CellSens software, using a 10x objective.

Alcian blue staining

Cells were cultured on coverslips, fixed and washed in PBS as described above for immunofluorescence. The coverslips were then washed with distilled water three times, and stained with 1% Alcian blue (Sigma) prepared in 3% glacial acetic acid, pH 2.5 for 30 minutes. After, coverslips were washed with distilled water, air dried and mounted with Prolong Gold Antifade Mountant without DAPI (Thermo Fisher Scientific). Images were captured by an Olympus BX51 microscope with CellSens software, using a 10x objective.

Protein isolation and measurement

For each cell line, 3×10^4 cells/cm^2 were seeded into two wells of a 6-well plate and cultured for 4 days in matrix-medium at 37°C in 5% CO_2. After, cells were washed in PBS three times and lysed with 6 M guanidinium hydrochloride, 10 mM Tris (2-carboxyethyl) phosphine hydrochloride, 40 mM 2-chloroacetamide, 100 mM Tris pH 8.5 for mass spectrometry (MS) analysis. Protein concentrations were determined by Quick Start Bradford Protein Assay (Bio-Rad) according to manufacturer's instructions.

Tryptic digestion of proteins

Proteins were digested with Lys-C protease (20 ng/µg protein) in 10% acetonitrile with 50 mM HEPES pH 8.5 at 37°C for 3 h and with additional trypsin (10 ng/µg protein) for 16 h. After, samples were acidified with an equal volume of 2% trifluoroacetic acid and vortexed. Acidified samples were then run through equilibrated stage tips containing Empore C18 filters (3M). Peptides were eluted in 40% acetonitrile and 0.1% formic acid. Dehydrated samples were then resuspended in 2% acetonitrile and 1% trifluoroacetic acid containing indexed retention time peptides (iRT peptides at 1:500, Biognosys AG). Peptide concentrations were measured using a DS-11 FX+ spectrophotometer (DeNovix).

Liquid chromatography-mass spectrometry (LC-MS)

For each sample, 1 µg peptides were loaded onto a 2-cm C18 trap column (Thermo Fisher Scientific), connected in-line to a 50-cm C18 reverse-phase analytical column (Easy-Spray LC column, Thermo Fisher Scientific) using 0.1% formic acid in water at 750 bar, using the Easy-nLC 1200 high-performance liquid chromatography system (Thermo Fisher Scientific), and the column oven operating at 45°C. Peptides were eluted over a 140-minute gradient ranging from 6% to 60% of 80% acetonitrile, 0.1% formic acid at 250 nl/min, and the Orbitrap Fusion instrument (Thermo Fisher Scientific) was run in a data-dependent-MS/MS (DD-MS2) top speed method. Full MS spectra were collected at a resolution of 120 000, with an AGC target of $4x10^5$ or maximum injection time of 50 ms and a scan range of 400–1500 m/z. The MS2 spectra were obtained in the ion trap operating at rapid speed, with an AGC target value of 1×10^4 or maximum injection time of 35 ms, a normalized higher-energy collisional dissociation (HCD) collision energy of 30 and an intensity threshold of $1.7e^4$. Dynamic exclusion was

set to 60 s, and ions with a charge state <2, >7 or unknown were excluded. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility.

Label-free quantitative proteomics analysis

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride/archive/) with the data set identifier. The raw files were analyzed using Proteome Discoverer 2.4. Label-free quantitation (LFQ) was enabled in the processing and consensus steps, and spectra were matched against the *Equus caballus* validated and unvalidated databases obtained from Uniprot. Dynamic modifications were set as oxidation (M), deamidation (N, Q) and acetyl on protein N-termini. Cysteine carbamidomethyl was set as a static modification. All results were filtered to a 1% FDR, and protein quantitation done using the built-in Minora Feature Detector.

Statistical analysis of MS data

The normalized protein intensities generated by LC-MS were analyzed using the R-based integrated web application Differential Expression and Pathway version 0.90 (iDEP) (28). Only proteins for which two peptides were detected were analyzed (Data S1). Data S2 contains the customized R code for the iDEP workflow. Data S3 contains the transformed protein intensities. iDEP-generated values for heatmaps can be found in Data S4. Data S5 contains the results from the DESeq2 (an iDEP package), using a threshold of false discovery rate (FDR) $p<0.1$ and fold-change $>\pm1.5$.

Statistics

For statistical analysis comparing cell growth curves, repeated measures two-way ANOVA was used, with Sidak's correction for multiple comparisons using GraphPad Prism v.8.0 (GraphPad Software, La Jolla).

RESULTS

Tenocytes derived from the tendon core and periphery have similar capacity of proliferation

Tenocytes derived from horse SDFTs appeared fibroblastic in culture and there were no apparent differences in cell morphology between cells derived from the core and the periphery of the tendons (Fig. S2). Growth curves of core and periphery tenocytes from each of the five horses analyzed are shown in Fig. S1. No significant differences in cell numbers were detected between the core and periphery SDFT-derived tenocytes at each time point (Fig. 1D). Cell number decreased from day 0 to day 2, followed by exponential growth up to day 6, at which point growth slowed down with the density reaching ~10 times the original seeding density (Fig. 1D).

ECM deposition varied between individual horses

ECM deposition was examined by immunofluorescence for type I collagen and Alcian blue staining for glycosaminoglcycans. Positive staining for collagen type I was localized to fibrillar networks in the ECM (Fig. 2). We observed a large variation in type I collagen deposition between individual horses but no clear differences in type I collagen deposition was observed between tenocytes derived from the core SDFT and those from the peripheral SDFT (Fig. 2). Very little collagen I staining was observed in cell cultures from horses #3 and #15 compared to cells from horses #6 and #9. Only tenocytes from horse #17 appeared to show a difference in the amount of type I collagen ECM deposited (Fig. 2). Very little Alcian blue staining of glycosaminoglycans was observed in all five horses (Fig. S2).

Similar proteome profiles of core and periphery tenocyte cultures

LC-MS detected 4082 unique proteins in samples of core and periphery SDFT cell cultures (Data S1). Distribution of the protein abundances was similar in all ten samples analyzed (Fig. 3A-B). Pearson's correlation coefficients of individual core and periphery replicates showed strong correlation in all sample comparisons despite coming from different horses, with the lowest r = 0.94 (Fig. 3C). Heatmap showing hierarchical clustering of samples based on their protein abundances ranked by standard deviation did not reveal any expression pattern similarities within core or periphery samples (Fig. 3D, Data S4). Differential analysis was applied to identify any significant differences in protein abundance between core and periphery SDFT cultures. Only 27 proteins were identified as significantly more abundant in core SDFT cultures, including collagen alpha-2(IV) (COL4A2) (Fig. 3E, Data S5). Thirty-two proteins were significantly more abundant in periphery SDFT cultures, including lysyl oxidase-like 2 (LOXL2), fibulin 5 and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (Fig. 3E, Data S5). Principal component analysis of the LC-MS data did not show a clear separation of core and periphery samples (Fig. 3F). T-distributed stochastic neighbor embedding, which looks at how similar samples are, showed quite clearly that the proteome profile of cultures of cells taken from the same horse look more similar than cultures of cells taken from the same regions (Fig. 3G).

DISCUSSION

In this study, we performed cell proliferation assays, immunofluorescence staining and label-free quantitative LC-MS-based proteomics analysis to characterize potential differences between primary cultures of cells derived from the core and periphery of horse SDFTs. The results did not display any well-defined differences between the two regions, instead, the largest source of variation was between biological replicates; cells from the same horse were more similar than cells derived from the same region of the SDFT from different horses.

As it is, tendon turnover appears to be greater at the periphery (16, 17), and tendon growth may also occur at the surface (15), which suggests a difference in the ability of cells from the two regions to generate

new ECM. Such a difference could either be due to differences in the synthetic activity of cells or due to the amount of cells in different regions. Tenocytes derived from the SDFT core and the periphery did not show any significant difference in proliferation rate. We hypothesized that there would be no difference in cell activity, including proliferation, but it was surprising that in standard cell culture conditions, where cells are stripped off their native microenvironment, periphery SDFT cells did not have a greater capacity of proliferation than core SDFT cells when more cells are found in the periphery of porcine tendons (18) . This data suggest that given the right conditions, such as the low cell density, high serum, low ECM ligand microenvironment provided by cell culture, tenocytes from the tendon core and periphery have the same intrinsic capacity to proliferate.

In a similar study cells derived from the periphery (loose peritenon) and the core of the tendon were compared and it was found that cells derived from the periphery have a higher proliferation rate than cells from the core of the tendon (29). The peritenon tissue is a thin gliding membrane of loose connective tissue comprised of mainly type III and type V collagen (30). It has been proposed that cells from the peritenon may secrete stimulatory factors to cells in the tendon proper (30). We suspect that the peritenon may contain cell types other than tenocytes, e.g. pericytes, whereas the present work studied the cells from the tendon proper, which likely represents a more well-defined fibroblast population responsible for synthesizing, maintaining and degrading the collagen-rich ECM. It is possible that *in vivo*, tenocytes from the tendon periphery could be more active than core tendon cells due to their proximity to the peritenon, potentially as a result of regulation by non-tenocytes, which could explain why previous measurement of ECM turnover at the tendon surface *in vivo* is high.

In this study, cells were cultured for 4 days before the proteomics analysis. A previous study showed that the culture of equine SDFT can generate reasonable numbers of cells within one week and is a bit more rapid than the deep digital flexor tendon (31). The doubling time (from day 2 to 6) of our cultures was ~1 day and immunofluorescence analysis established that ECM was deposited after 4 days. We observed a large variation in collagen type I deposition between biological replicates, which had no correlation with age or sex of the horse. Heterogeneity in collagen synthesis has also been observed in human skin fibroblasts (32) and these individual differences may relate to susceptibility to injuries. Proteomic analysis of the deposited ECM showed a few ECM proteins (COL4A2, ADAM10, FBLN5, LOXL2) were differential abundance between core and periphery cultures, but there were largely no differences (>98% proteins) between core and periphery SDFT cells *in vitro*.

Overall, through cell proliferation, immunofluorescence staining and LC-MS analyses, we observed that tenocytes derived from the core and periphery of horse SDFT behave very similarly in culture. Our previous studies have also demonstrated that there are no regional differences in turnover of human patellar tendons, while the turnover and DNA content of porcine flexor tendons is higher in the periphery compared to the core, but only in the distal part of the tendon (18, 33). However, in all these studies, we have looked at healthy

tendons. It remains unknown whether introducing a challenge to the cells specifically in the periphery or core *in vivo*, such as an injury from overloading, might show stark differences in ECM turnover, where circulation and the peritenon may play crucial regulatory roles.

In conclusion, tenocyte cultures derived from the core and periphery of equine SDFTs have similar proliferation rates and ECM proteome profiles, which suggests that factors other than the intrinsic properties of tendon fibroblasts may play a role in regulating ECM turnover.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

CZ performed the experiments and analyzed the data, prepared the data, and drafted the manuscript. RBS designed and supervised the study and reviewed the manuscript. CM prepared the primary cell cultures. HC and RB prepared horse materials. EMS performed the LC-MS and curated the mass spec data. MK and SPM acquired funding, supervised the study and reviewed the manuscript. CY designed and supervised the study, analyzed the data, wrote and revised the manuscript. All authors approved the manuscript.

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FIGURE LEGENDS

Figure 1. Proliferation of tendon cells isolated from the core and periphery of horse SDFTs.

The SDFT was dissected from the left forelimb **(AB)**. The SDFT was separated into core and periphery tissues and the tendon fibroblasts within were released and cultured **(C)**. Mean (log) number of tendon cells from the core or periphery of horse SDFTs were counted every 2 days during 10 days of culture (n=5) **(D)**.

Figure 2. Collagen I deposition by core and periphery SDFT cells in culture.

Representative images of immunofluorescence staining for collagen I in 4-day old cultures of core and periphery SDFT cells from five horses. Red, collagen I. Blue, DAPI. Bars, 100 µm.

Figure 3. Similarities of proteome profiles of core and periphery SDFT cells in culture.

Distribution of protein abundances from the core and periphery as detected by LC-MS shown in box plot and density plot **(AB)**. Heatmap of Pearson correlation coefficient (r) for pairwise comparison **(C)**. High r values (red squares) denote high correlation between samples. Heatmap showing hierarchical clustering of samples based on their protein abundances ranked by standard deviation **(D)**. Venn diagram showing the majority of proteins (4023) was not significantly different in adbundance between core and periphery SDFT cells **(E)**. There were 27 proteins that were significantly more abundant in core SDFT cell cultures and 32 proteins that were significantly more abundant in periphery SDFT cell cultures (false discovery rate $p<0.1$, >1.5 fold change). Principal component analysis (PCA) was used to provide a statistical summary of proteins. The first two principle components (PC1 and PC2) are shown **(F)**. tSNE (t-distributed stochastic neighbour embedding) plot showed the similarity between proteins from different regions **(G)**.

LIST OF SUPPLEMENTARY FILES

Supplementary Figure S1. Individual growth curves for tendon cells of each horse.

Supplementary Figure S2. Alcian blue staining by core and periphery SDFT cells.

Supplementary Data 1. Mass spectrometry data of list of proteins and normalized intensities in all samples.

Supplementary Data 2. Customized R code for iDEP analyses.

Supplementary Data 3. Log transformed intensity list with missing values filled in by imputation.

Supplementary Data 4. Heatmap values for proteins ranked by SD.

Supplementary Data 5. Lists of proteins, fold changes and FDR values from DESeq2 analysis.

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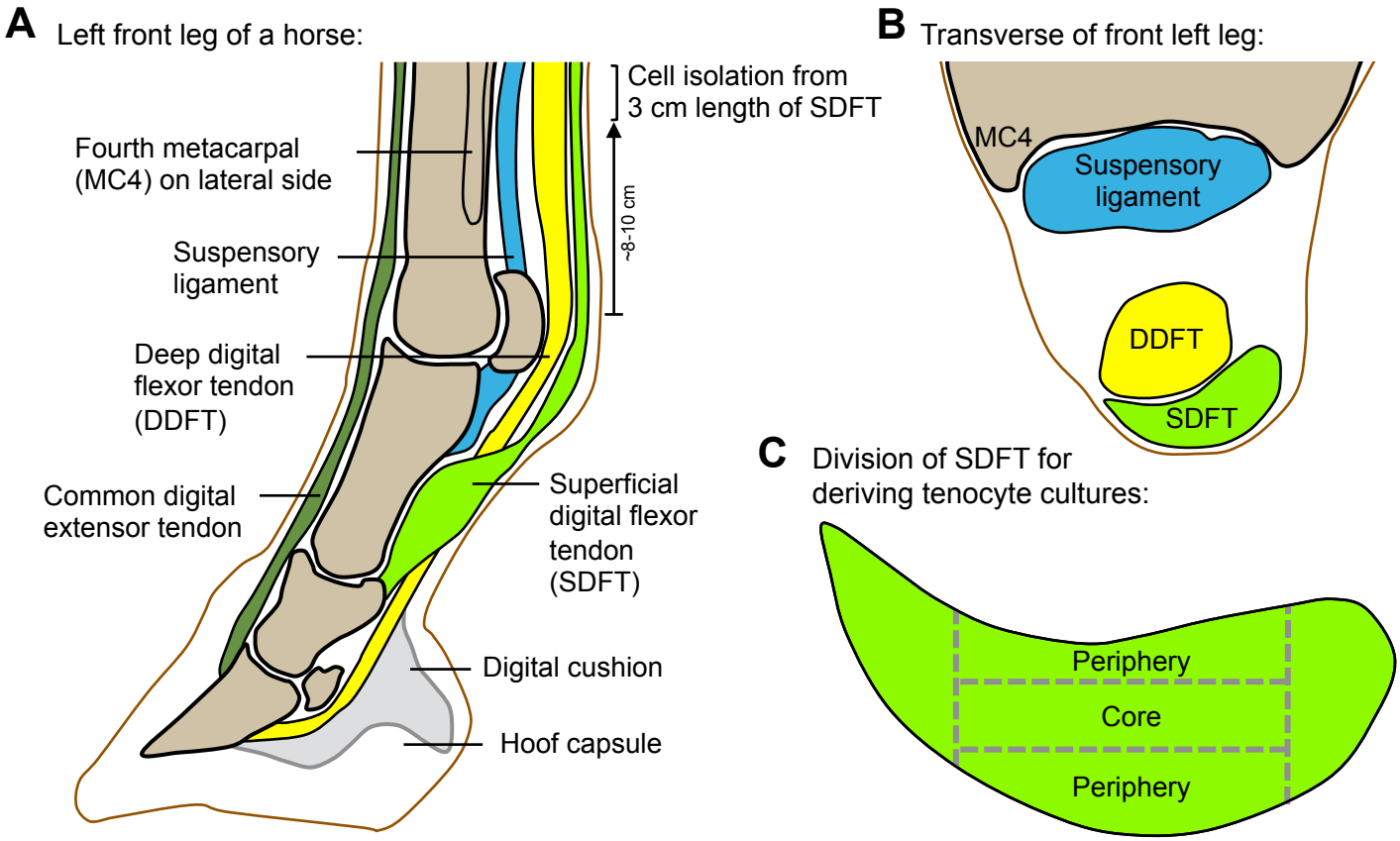
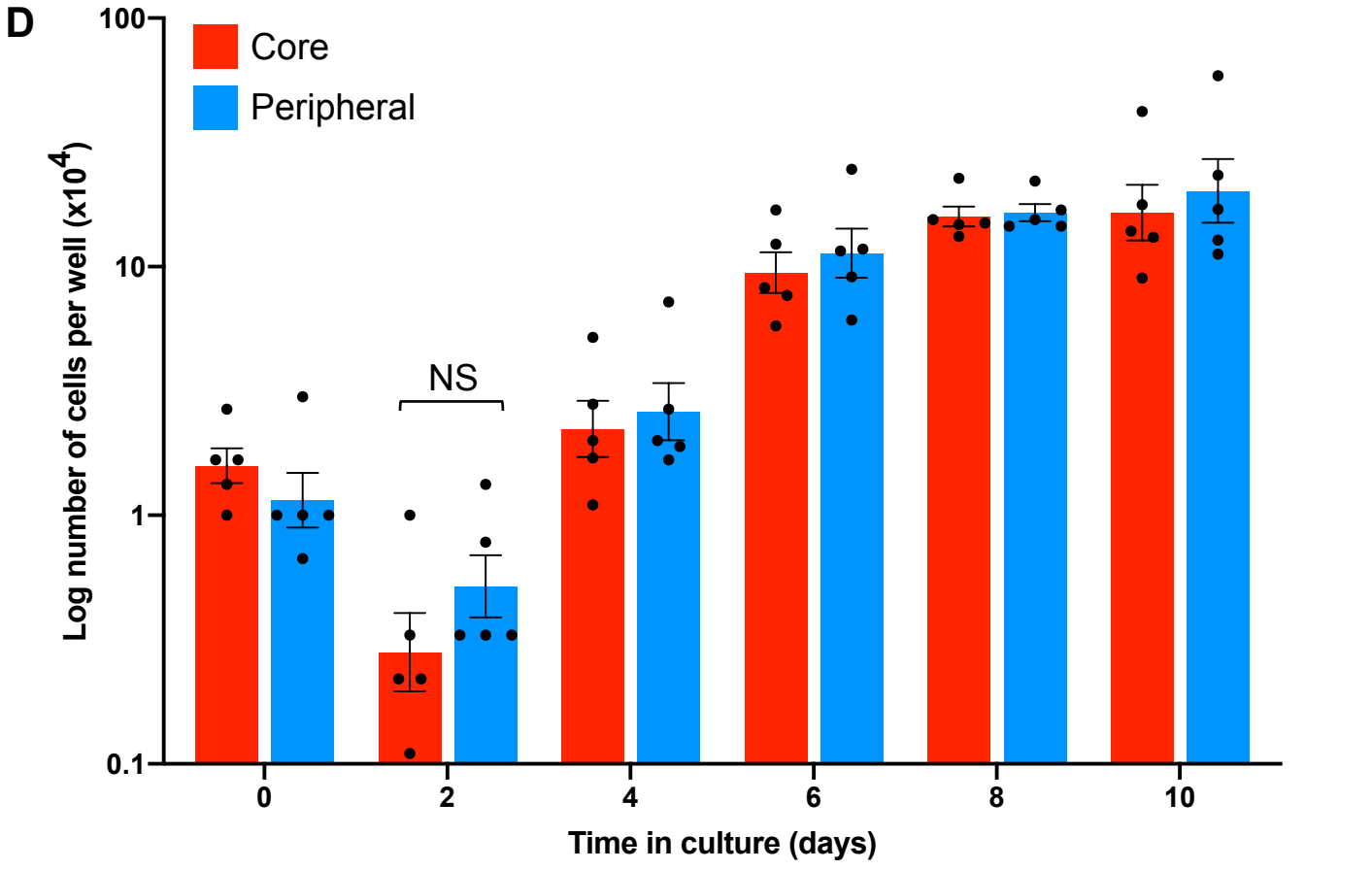
Zhang – Figure 1





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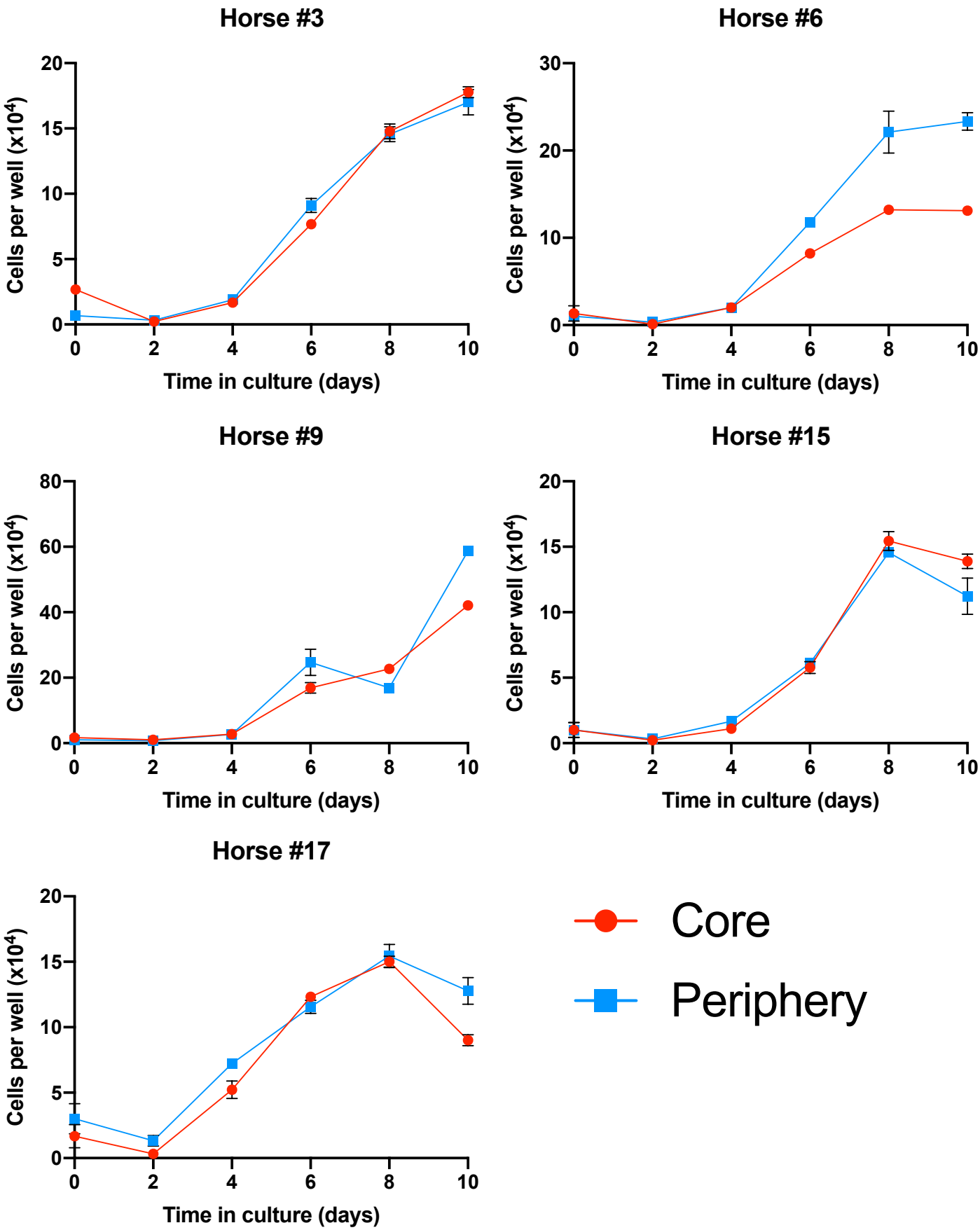

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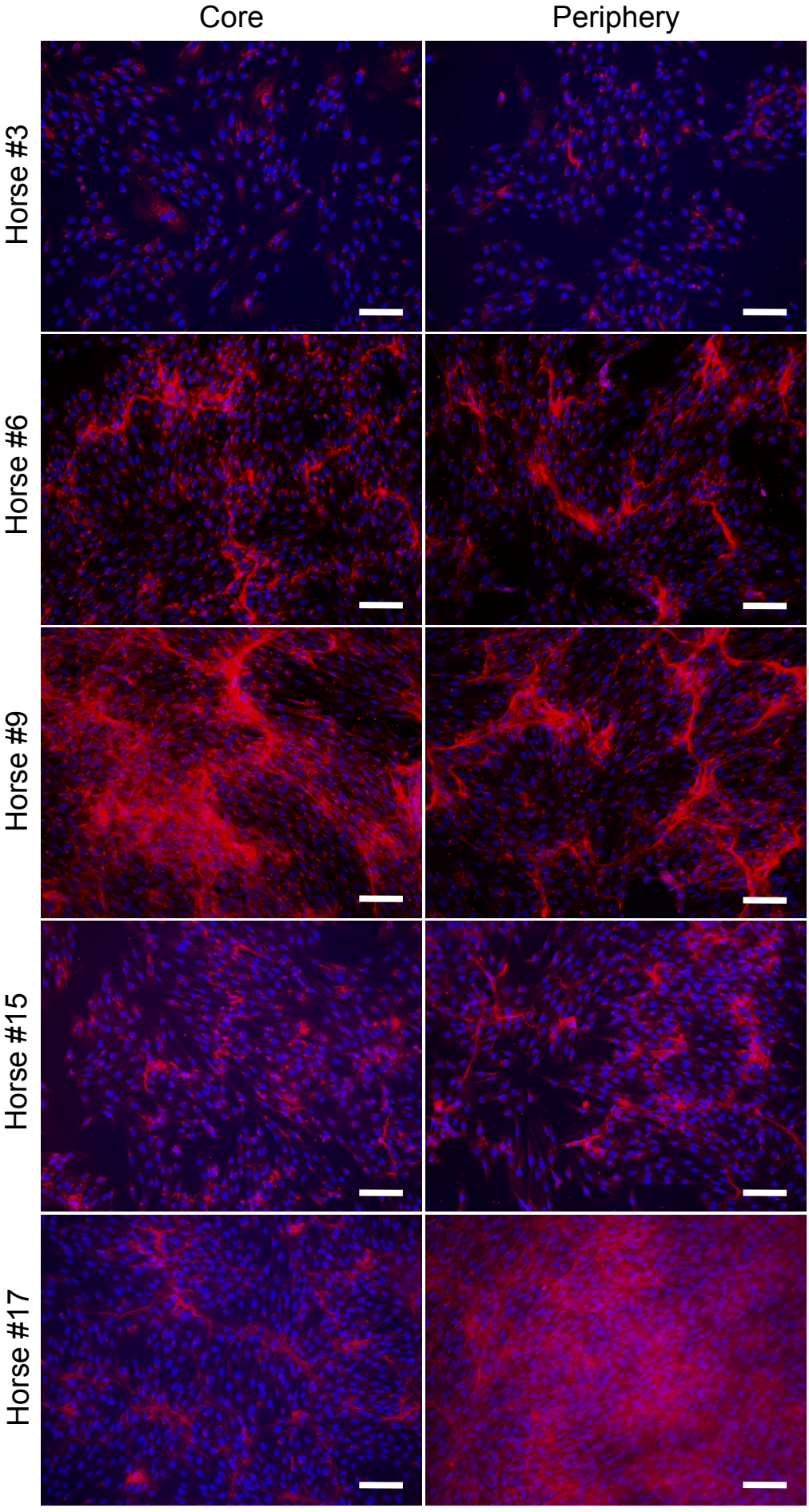

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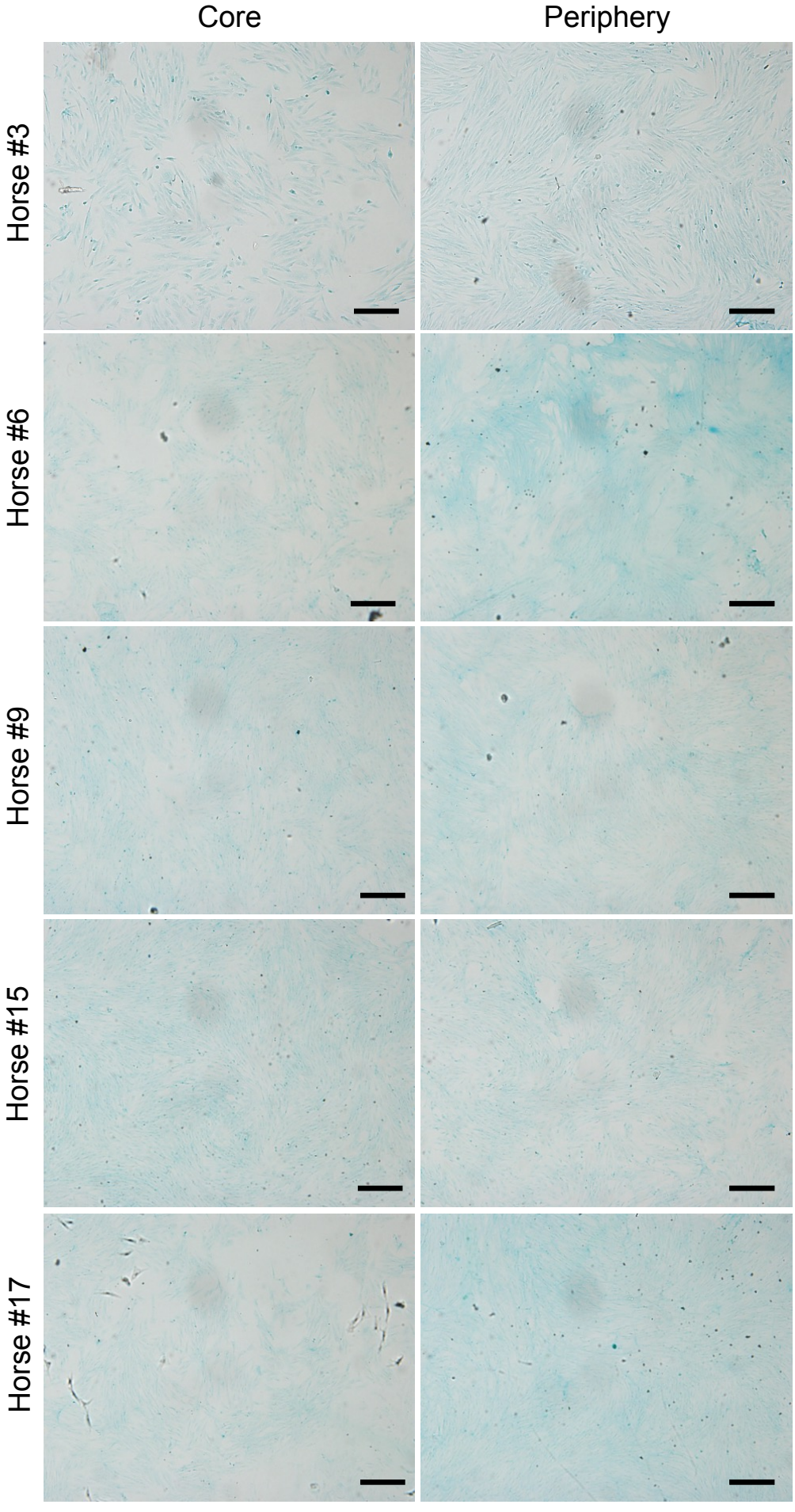
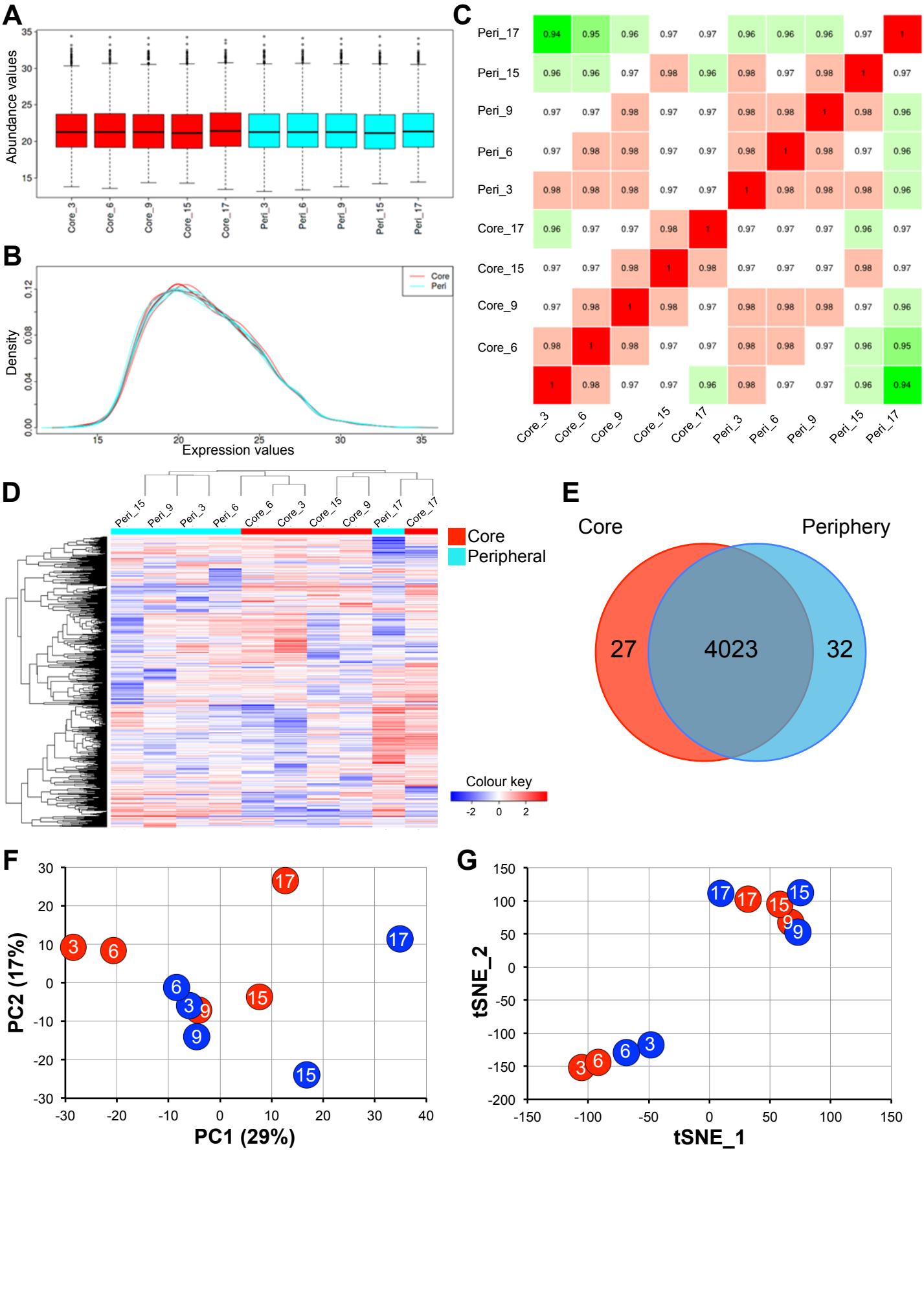


Zhang – Figure 3



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Name of PhD student	CHENG ZHANG
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Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Tendon composition and turnover - Is it uniform throughout the tissue?

2. The declaration applies to the following article	
Title of article	Regional differences in turnover, composition, and mechanics of the porcine flexor tendon
Article status	
Published ☒ Date: 2019/05/28	Accepted for publication ☐ Date:
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If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	Connective Tissue Research. 2019 May 28;1-10. doi: 10.1080/03008207.2019.1620222.

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5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B
Provide a short description of the PhD student´s specific contribution to the article.[i] The PhD student contributed in the animal and lab work, analysis of data and writing the manuscript.	

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	23/5/20	S. Peter Magnusson	Prof	
2.	25/5-2020	Michael Kjær	Prof	
3.	25/5/2020	Rene B. Svensson	PhD	
4.	28/5-20	Peter Schjerling	PhD	
5.	23/5/20	Christian Couppé	Associated professor	
6.	25/5/20	Dorthe Skovgaard	MD, PhD	
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 23/5/20
Principal supervisor:

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 27/5/20
PhD student:

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.
[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):
"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."
[iii] If more signatures are needed please add an extra sheet.

GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	CHENG ZHANG
E-mail	zhangchengmed@163.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Tendon composition and turnover - Is it uniform throughout the tissue?

2. The declaration applies to the following article	
Title of article	Regional Collagen Turnover and Composition of the Human Patellar Tendon
Article status	
Published ☒ Date: 2020/04/01	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	J Appl Physiol (1985). 2020 Apr 1;128(4):884-891. doi: 10.1152/japplphysiol.00030.2020.

3. The PhD student's contribution to the article *(please use the scale A-F as benchmark)* Benchmark scale of the PhD-student's contribution to the article **A.** Has essentially done all the work (> 90 %) **B.** Has done most of the work (60-90 %) **C.** Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	B
2. Development of the key methods	C
3. Planning of the experiments and methodology design and development	B
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	A
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B
Provide a short description of the PhD student´s specific contribution to the article.[1] The PhD student contributed in the human tissue and lab work, analysis of data and writing the manuscript.	

Latest update of the declaration: December 2018

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Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
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5.	25/5/20	Casper G. Schalkwijk	Prof	
6.	27/5/20	Jean L. J. M. Scheijen	PhD	
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1. Declaration by	
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E-mail	zhangchengmed@163.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Tendon composition and turnover - Is it uniform throughout the tissue?

2. The declaration applies to the following article	
Title of article	A Comparison of Tenocyte Populations from the Core and Periphery of Equine Tendons
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

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5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B
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4.	23/5/20	Costanza Montagna	Postdoc	
5.	25/5/20	Ching-Yan Chloé Yeung	Dr.	
6.	25/5/20	Erwin M. Schoof	Dr.	
7.	28/05/20	Rikke Buhl	Professor	
8.	17/06/20	Helena Carstensen	DVM	
9.				
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6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 25/5 2020
Principal supervisor:

7. Signature of the PhD student

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