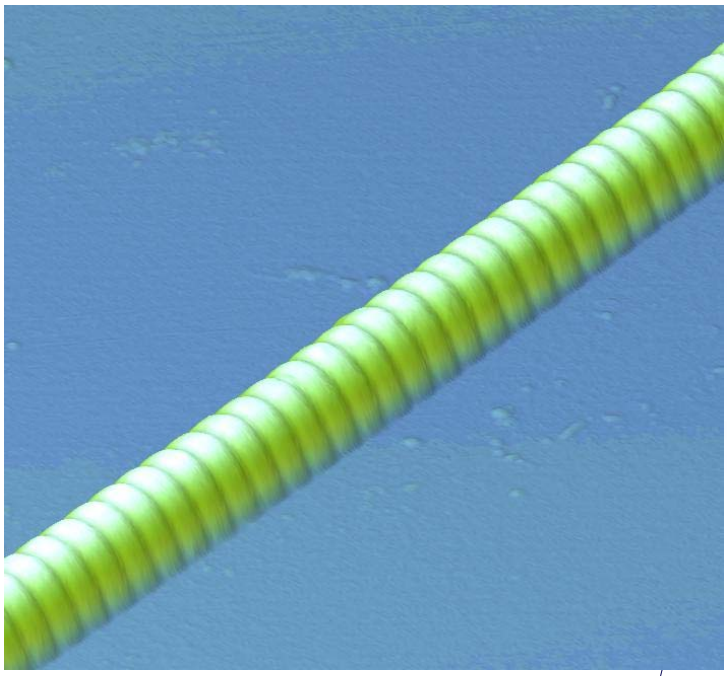
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

# Ph.D. thesis

Philip Hansen

# Human tendon properties: From molecule to man

## A structural, biomechanical and biochemical investigation



Institute of Sports Medicine Copenhagen
Bispebjerg Hospital & Center for Healthy Aging
Copenhagen, Denmark, 2010

Academic supervisors: Peter Magnusson, Michael Kjær, Per Hölmich, Tue Hassenkam

# 1. Preface and acknowledgements

The present PhD-thesis is based on experimental work performed at the *Institute of Sports Medicine Copenhagen (www.ISMC.dk)*, Bispebjerg Hospital & Center for Healthy Aging, University of Copenhagen, Denmark and *Nano-Science Center*, University of Copenhagen, Denmark with the kind assistance form the clinical orthopedic departments at Bispebjerg Hospital and Amager Hospital, Copenhagen, Denmark.

Invaluable additional experimental work was performed at: *Department of Biomedical Sciences* & *Department of Neuroscience and Pharmacology*, The Panum Institute, University of Copenhagen; *School of Physical Education, Sport and Exercise Science, Ball State University, Indiana, USA; Department of Health Sciences*, Biochemistry Laboratory, University of Jyväskylä, Finland and *Department of Clinical Veterinary Science*, Matrix Biology Research Group, University of Bristol, United Kingdom.

My most sincere declaration of gratitude must go to my dear supervisor and friend Professor Peter Magnusson who has provided invaluable guidance. I would not have come far without it. Not many possess the unique combination of in depth scientific knowledge, inspiring enthusiasm, firm empathy and patience with troublesome students. Also sincere thanks to my supervisor Professor Michael Kjær for scientific guidance and for providing such remarkable research facilities for PhD-students. Also, my sincere gratitude to my other supervisors Tue Hassenkam for guiding me in the challenging field of atomic force microscopy and Per Hölmich for clinical guidance and practical assistance in obtaining human tendon tissue samples from Amager Hospital.

Also a great thanks to my collegues in the biomechanical group at ISMC: René Svensson, Mads Kongsgaard, Christian Couppé, Bjarki Thor Haraldsson and Jens Bojsen-Møller. You have all contributed greatly to my work through support and inspiring scientific discussions as well as by providing such enjoyable social company. Also a specific thanks to Professor Per Aagaard for your immense help in terms of data analyses and scientific input. A sincere thanks to Michael Krogsgaard for a great collaboration in obtaining tendon material from Bispebjerg Hospital. Also an unreserved thanks to all co-authors not mentioned above: Nick Avery, Vuokko Kovanen, Todd Trappe, Klaus Qvortrup and Jytte Overgaard Larsen. Without your expertise it would not have been possible to obtain many of the data presented in this thesis. Lastly, pre-graduates Morten Dahl and Martin Hald deserve sincere thanks for much appreciated assistance.

A sincere thanks to all my other colleagues at ISMC. Your positive attitude and helpfulness have been unmistakable throughout my time in the department. It has always been a joyful collaboration and social life with you all.

A word of gratitude to The Danish Rheumatology Association, Bispebjerg Hospital and AMBEHR for funding my work.

Lastly, a special thanks to my dear wife Mette, my lovely daughter Nanna and all of my sweet family. You have supported me and tolerated me in trying phases of my work.

# 2. Content

# 3. Abbreviations

| | |
|---|---|
| ADAM(TS) | A disintegrin and metalloproteinase (with trombospondin motif) |
| AFM | Atomic force microscopy |
| AGE | Advanced glycation endproduct |
| CSA | Cross-sectional area |
| DHLNL | Dehydroxylysinonorleucine |
| ECM | Extracellular matrix |
| GA | Glutaraldehyde |
| HLNL | Hydroxylysinonorleucine |
| HHL | Histidinohydroxylysinonorleucine |
| HPLC | High pressure/performance liquid chromatography |
| HP | Hydroxylysylpyridinoline |
| LP | Lysylpyridinoline |
| LOX | Lysyl oxidase |
| MMP | Matrix metallo-proteinase |
| MRI | Magnetic resonance imaging |
| PICP | Pro-collagen I C-terminal propeptide |
| PIIICP | Pro-collagen III C-terminal propeptide |
| PINP | Pro-collagen I N-terminal propeptide |
| PIIINP | Pro-collagen III N-terminal propeptide |
| PBS | Phosphate buffered saline |
| SEM | Scanning electron microscopy |
| TEM | Transmission electron microscopy |
| TIMP | Tissue inhibitor of metalloproteinases |
| US | Ultrasonography |
| $\varepsilon$ | Strain |
| $\sigma$ | Stress |
| E | Young´s modulus of elasticity |
| ICTP | C-terminal telopeptide of collagen type I |
| IIICTP | C-terminal telopeptide of collagen type III |

# 4. List of included papers

This thesis rests on four studies. The studies are listed below and are referred to in the thesis by their roman numerals.

**I** **Glutaraldehyde Cross-Linking of Te ndon - Mechanical Effects at the Level of the Tendon Fascicle and Fibril.**
Hansen P, Hassenkam T, Svensson RB, Aagaard P, Trappe P**,** Haraldsson BT, Kjaer M, Magnusson SP**.** *Connective Tissue Research* 50: 211-222, 2009

**II** **Hierachical investigation of the human patellar tendon – mechanical properties of whole tendon and collagen fibrils.**
Hansen P, Svensson RB, Hassenkam T, Haraldsson BT, Aagaard P, Krogsgaard M, Kjaer M, Magnusson SP (unpublished, submitted)

**III** **Lower strength of the human posteri or patellar te ndon seems unrelated to mature collagen cross-linking and fibril morphology.**
Hansen P, Haraldsson BT, Aagaard P, Kovanen V, Avery NC, Qvortrup K, Larsen JO, Krogsgaard M, Kjaer M, Magnusson SP.
*Journal of Applied Physiology* Jan;108(1): 47-52, 2010

**IV** **Biomechanical properties and cross-link composition of ruptured human Achilles tendon.**
Hansen P, Kovanen V, Hölmich P, Krogsgaard M, Hansson P, Dahl M, Hall M, Aagaard P, Kjaer M, Magnusson SP (unpublished, submitted)

# 5. Dansk resumé

Det videnskabelige formål med denne afhandling var at belyse strukturelle, biokemiske og biomekaniske faktorer af særlig betydning for seners mekaniske egenskaber.
Selvom sener har været underkastet intensive studier gennem årtier, er der reelt ingen konsensus om, hvordan de betydelige tensile kræfter, som sener udsættes for under bevægelse, overføres imellem senens bestanddele.

Sener er hierarkisk opbyggede og den basale byggesten udgøres af kollagen type I molekylet. Kollagen molekylerne er anordnet i veldefinerede fiberenheder, den såkaldte kollagen fibril, som menes at være den primære kraftoverførende enhed i sene. Bundter af fibriller samles til fasikler, som udgør de største fiberenheder i sene. Fibriller med stort tværsnitsareal antages at besidde relativt større trækstyrke end tyndere fibriller, og styrken formodes betinget af stærke kemiske kovalente tværbindinger (cross-links) mellem kollagen molekylerne i fibrillen. Pyridinium tværbindingerne lysyl-pyridinolin (LP) og hydroxylysyl-pyridinolin (HP) anses for at være essentielle for de mekaniske egenskaber. Disse antagelser er dog aldrig blevet efterprøvet for humane sener. Fravær af tværbindinger har dramatiske negative effekter på mange væv, herunder sener og ligamenter. Disse effekter kan tilbageføres til nedsat mekanisk kvalitet af de kollagene støttevæv, som det ses i sygdommene Ehlers-Danlos syndrom, cutis laxa og Marfans syndrom. Her indgår defekter i kollagen tværbindinger i patofysiologien. Styrken af kollagene væv hos raske øges under opvæksten i takt med at tætheden af enzymatiske tværbindinger øges. Dertil forekommer en alderbetinget dannelse af glucose-deriverede tværbindinger, såkaldte advanced glycation end products (AGE), pga. non-enzymatisk glykosylering af kollagenet. Dette medfører at senestivheden øges, men samtidig reduceres senevævets evne til at absorbere mekanisk energi og dermed dets mekaniske kvalitet.

De nedenfor beskrevne studier havde overordnet til formål at undersøge betydningen af fibril morfologi og sammensætningen af tværbindinger for de basale mekaniske egenskaber i sener samt deres betydning for udvikling af seneskader - herunder den alvorlige totale seneruptur.

**Studie I**

Formålet med dette studie var at undersøge effekten af kunstigt inducerede collagen tværbindinger i senevæv. Ved at behandle sener fra rottehaler med glutaraldehyd, som danner tværbindinger mellem lysin og hydroxylysin sidekæder på kollagenmolekylet, kunne effekten af øget tæthed af tværbindinger måles. De mekaniske egenskaber blev dels målt på rottehalesener vha. mikrotensilt måleudstyr, dels på submikroskopisk niveau ved hjælp af atomic force microscopy (AFM), som tillod at måle den samtidige effekt af en øget koncentration af tværbindinger på hel sene og på kollagen fibriller.

Hovedfundene i studiet var, at både stivhed og brudstyrke af hel sene øgedes efter behandling med glutaraldehyd. På fibril niveau sås en markant øgning af stivheden både i overfladen af fibrillen og i længderetningen som tegn på en stærkere sammenbinding af kollagen molekyler i fibrillen. Samlet var de mekaniske ændringerne mest udtalte for fibrillen.

**Studie II**

Formålet med dette studie var at undersøge de mekaniske egenskaber på flere strukturelle niveauer af human sene. Patienter med overrivning af forreste korsbånd fik inden operation bestemt de mekaniske egenskaber af den store knæsene (patellasenen) ved hjælp af ultralyd, som tillader instantan måling af senens deformation under muskelkontraktion. I forbindelse med operation blev udtaget en seneprøve fra samme sene og kollagen fibriller fra prøven blev efterfølgende undersøgt ved hjælp af AFM. Således var det muligt at bestemme og sammenligne de mekaniske egenskaber på to niveauer af en human sene.

Hovedfundene fra studiet var, at kollagen fibriller umiddelbart synes at udvise større normaliseret stivhed end hel sene (Young´s modulus), men når man tager højde for den fraktion af senens tværsnitsareal, som udgøres af fibriller (ca. 60%) er Young´s modulus sammenlignelig på de to niveauer. Imidlertid er længden af den såkaldte 'tå-region' i de to strukturers kraft-deformations kurver større for hel sene ligesom variationen i Young´s modulus mellem individer syntes mere udtalt. Samlet tydede studiet på, at kollagen fibrillers mekaniske egenskaber er afgørende for de mekaniske egenskaber af hel

sene, særligt i den lineære region af kraftkurven, mens andre faktorer synes at modulere fibrillernes egenskaber i tå-regionen.

**Studie III**

Dette studie havde til formål at belyse betydningen dels af pyridinium og AGE tværbindinger dels af fibril morfologi for de mekaniske egenskaber af den humane patellasene. Desuden blev den betydningen af den nyligt beskrevne enzymatiske tværbinding pyrrol undersøgt. Studiet søgte herudover at afdække mekaniske, biokemiske og strukturelle forskelle mellem den forreste og bagerste del af senen. Fra patienter med overrivning af forreste korsbånd blev der udtaget biopsier fra den forreste og bagerste del af patellasenen. Fasikler fra biopsierne blev testet med mikrotensilt måleudstyr. Desuden blev prøver analyseret for tværbindinger og kollagen indhold ligesom der blev foretaget måling af kollagen fibril diameter ved transmissions elektronmikroskopi (TEM).

Hovedfundende fra studiet var, at styrken af fasikler i den bagerste del af senen var nedsat i sammenligning med den forreste sene. Samtidig var der større tæthed af pyridinium tværbindinger i den bagerste del af senen, mens der var ikke var forskel i indholdet af AGE. Fibriller fra den bagerste sene tenderede samtidig til at have mindre tværsnitsareal. Hverken sammensætning af tværbindinger eller fibril tværsnitsareal var associeret til de mekaniske egenskaber og samlet tyder resultaterne på, at ingen af disse faktorer alene afgør de mekaniske egenskaber herunder trækstyrken af hel sene.

**Studie IV**

Dette studie havde til formål at belyse den patofysiologiske betydning af pyridinium og AGE tværbindinger for overrivning af den humane achillessene. Fra patienter med achillesseneruptur blev udtaget biopsier fra hhv. rupturstedet og et raskt udseende område af den proksimale sene. Desuden blev biopsier udtaget fra aldersmatchede kadaversener. Fra prøverne blev uddissekeret fasikler, som blev mikrotensilt testet og indholdet af pyridinium, AGE og kollagen blev ligeledes bestemt.

Hovedfundende fra studiet var, at der ikke kunne påvises mekaniske eller biokemiske forskelle mellem ruptursted og den proksimale sene. Senefasikler fra kadaver kontrol sener havde højere Young´s modulus og tenderede til at have større trækstyrke end fasikler fra rumperede sener. Kollagenindholdet var ligeledes højest i kadaver sener.

Sammensætningen af tværbindinger var ikke korreleret til de mekaniske egenskaber af senefasikler. For prøver fra rupturstedet korrelerede kollagenindholdet til trækstyrken, mens det ikke var tilfældet for den proksimale sene og kadaver sener. For både rumperede sener og kadaver sener var indholdet af pyridinium tværbindinger påfaldende højt. Samlet set tyder resultaterne på at lavt kollagen indhold har en patofysiologisk betydning for udviklingen af Achillesseneruptur og muligvis skyldes en underliggende ufuldstændig reparationsproces i senen. Det høje indhold af pyridinium tværbindinger i sammenligning med andre sener kan afspejle denne proces eller skyldes adaptation til stor belastning.

# 6. Summary

The purpose of this thesis was to examine structural, biochemical and biomechanical factors of particular importance for tendon mechanical properties.

Although tendons have been studies for decades there is currently no consensus on how force is transferred within the tendon. Tendons are hierarchical structures and the fundamental building block is the collagen type I molecule, which assemble into well-defined collagen fibrils. Fibrils are considered to be the primary force transmitting structures of tendon. Bundles of fibrils coalesce into tendon fascicles, which are the largest well-defined substructures of tendon. The mechanical strength of collagen fibrils is thought to relate to fibril cross-sectional area (CSA). Fibrils with large a large CSA are believed to possess greater relative strength than thin fibrils, however fibril strength is also believed to depend on chemical cross-links between collagen molecules.

In tendon the pyridinium cross-links lysylpyridinoline (HP) and hydroxylysylpyridinoline (LP) are considered important for mechanical strength. Lack of cross-links has dramatic effects on many tissues including tendon. These effects can be traced to inferior quality of collagen supportive tissues. Ehlers-Danlos syndrome, cutis laxa and Marfan's syndrome are examples of such diseases in which cross-link deficiencies are part of the pathophysiology. Tendon strength is increased during maturation as the cross-link concentration increases. An additional detrimental age-dependent cross-linkng process occurs with aging when advanced glycation endproducts (AGE) form additional collagen cross-links, which increase both tendon stiffness and brittleness. It was the purpose of the studies described below to examine the importance of fibril morphology and

cross-link composition for tendon mechanical properties. Additionally, it was the aim to explore these parameters in relation to tendon injury with emphasis on complete tendon rupture.

**Study I**

This study examined the effect of artificial increase in cross-link concentration in rat-tail tendons, which were treated with glutaraldehyde. This reagent forms cross-links between lysine and hydroxylysine sidechains of collagen molecules. Treated and untreated rat-tail tendons were tested both micromechanically and at the sub-microscopic level by atomic force microscopy (AFM), which allowed for evaluation of concomitant mechanical effects of increased cross-link concentration on whole-tendon and collagen fibrils.

Glutaraldehyde treatment increased both stiffness and failure strength at whole-tendon level. The stiffness of the fibril was increased both when the fibril surface was examined and in the axial (tensile) direction of the fibril. These changes indicated a strengthening effect on the collagen lattice within the fibril, which displayed the most pronounced mechanical changes compared to the tendon.

**Study II**

This study examined the mechanical properties at two structural levels of human tendon. The in vivo properties of the patellar tendon were measured by ultrasonography prior to reconstructive surgery of the anterior cruciate ligament (ACL). During surgery a sample was obtained from the same tendon and the properties of collagen fibrils from the sample were measured by AFM. The combined techniques allowed for comparison of mechanical properties at the two structural levels.

The main findings of the study were that fibrils seemed to display larger normalized stiffness (Young´s modulus) than whole-tendon. However, when Young´s modulus was adjusted for fraction of the tendon that is occupied by fibrils (~ 60%) the Young´s moduli at the two levels were comparable. However, the non-linear toe-region of the whole tendon was more pronounced than that for fibrils. In the linear region fibrils displayed only little variation in Young´s moduli between individuals. Collectively, the collagen fibril seems to govern whole tendon properties in the linear region of the stress-strain curve while other factors seem to modulate fibril properties in the toe-region.

**Study III**

This study examined the importance of pyridinium, AGE and the recently discovered pyrrole cross-links in relation to the mechanical properties of the human patellar tendon. Additionally, the importance of fibril morphology was investigated and the study evaluated regional differences for these parameters within the tendon. Biopsies were obtained from patients undergoing ACL reconstructive surgery. Samples were taken from the anterior and posterior aspect of the tendon. Tendon fascicles from the biopsies were procured and tested micromechanically. Remaining sample material underwent biochemical analyses and TEM measurements.

The main findings of the study were that tendon fascicles of the posterior tendon displayed inferior mechanical strength and stiffness compared to the anterior tendon. However, the concentrations of HP and LP cross-links were higher in the posterior tendon while AGE content was similar. Fibrils of the posterior tendon tended to have smaller CSA. Neither cross-link composition nor fibril CSA were associated with fascicle mechanical properties. In conclusion the data indicated that fibril morphology and the measured cross-links are not sole principle determinants of tendon properties.

**Study IV**

The study evaluated whether alterations in pyridinium and AGE cross-links are related to the pathophysiology of Achilles tendon rupture. During surgical reconstruction of the tendon, samples from the rupture site and from healthy appearing tendon proximal to the rupture site was obtained from patients who suffered a complete rupture. Additionally, samples were obtained from age-matched control cadaver tendons during forensic autopsy. Tendon fascicles were procured from the samples and tested micromechanically. Additional sample material was analysed for pyridinium and AGE cross-links.

The main findings of the study were that no regional biomechanical or biochemical differences were apparent between the sample sites within ruptured Achilles tendons. The collagen content was lower in ruptured tendons at both the at both sample sites was reduced compared to cadaver tendon. Fascicles from cadaver tendon displayed larger Young´s moduli and tended to have higher tensile strength. Collagen content was associated with tensile strength of fascicles from the rupture site while no association was

apparent for the proximal tendon and cadaver controls. The content of pyridinium cross-links was remarkably high compared to other human tendons. Collectively these data indicate that decreased collagen content may be reduce the strength of the Achilles tendon and therefore predispose it to rupture as a result of an incomplete repair process. High pyridinium concentration may reflect such a process or, alternatively, may relate to functional adaptation to high loads.

# 7. Introduction

Tendons transmit force generated by muscle contraction to produce joint movement and tendon forces during human locomotion can reach in excess of 4000 N for the patellar tendon while the Achilles tendon is reported to reach peak forces of up to 9000 N during running (45; 46; 102). Tendons tolerate such high loads though countless repetitions with a unique resilience to fatigue wear, however prolonged loading may eventually lead to accumulating damage to the tendon structure, which ultimately may cause tendon rupture (90).

Collagen constitutes the bulk of tendon dry mass (~ 60-85%) (40; 96; 154) of which collagen type I is far most abundant (96; 151). Interestingly, collagen content does not seem to vary greatly between tendon types (12; 18) although different tendons, display different mechanical properties (18; 172). This prompts the notion that tendon material properties are not solely governed by collagen content, albeit collagen content has been demonstrated to be associated with material properties within a certain tendon type (67). Nonetheless, the properties of collagen tissues seems be modulated by further modifications to the basic collagen framework and by variations in the supramolecular organisation (9).

While tendons were previously considered largely inert there is mounting evidence that tendons have the capacity to adapt to variation in loading (96). However, this ability may vary between tendon types and even within the same tendon as some regions along the tendon may respond to loading while others seem not to (33; 107; 172). Furthermore adaptation seems to be prolonged (58) and in some cases insufficient as tendon injuries are common, particularly in relation to sporting activities (43; 80; 110; 114). Some have stated that tendon injuries account for 30-50 % of all sports related injuries (73). Nonetheless, our understanding of injury in mechanistic terms remains unsatisfactory and although the tendon structure has been studied for decades there are many inconsistencies and unresolved questions regarding the transmission of force between the tendon substructures (143; 146; 147). Lack of such basic knowledge impairs chances of improving current tendon injury prevention and treatment. To achieve such goal there is a need to gain further insight into tendon properties by examining its structure and function.

This thesis investigated the importance of both structural and biochemical factors in respect to tendon properties with primary emphasis on collagen cross-link composition and fibril morphology. These factors are believed to be of particular importance for tendon material properties (8; 9; 146), however, currently available information is largely based on animal data, which may not be directly applicable to human tendon. Therefore this thesis strived to investigate the aforementioned variables in human tendon whenever possible. With newly developed techniques it was possible to determine the mechanical properties of human i) whole tendon, *in vivo*, ii) individual human tendon fascicles, *in vitro*, and iii) single collagen molecules, *in vitro*. The included studies utilized ultrasonography, magnetic resonance imaging, transmission electron microscopy, atomic force microscopy in combination with biochemical analyses and micromechanical testing in an attempt to achieve new insight into the structure-function interplay between distinct levels of the tendon hierarchy and to investigate the importance of fibril morphology and cross-link composition in relation to tendon mechanical properties in healthy and injured human tendon.

### 7.1 Tendon composition

The basic structural element is the collagen type I molecule, which consists of three polypeptide strands – two α1 chain and one α2 chain arranged in a helical conformation to form the pro-collagen I molecule. This configuration is allowed by the repetition of a Gly-X-Y aminoacid sequence, where X is usually a proline, and hydroxyproline is typically found at Y-position (166). The initial pro-collagen molecule contains C- and N-terminal propeptides (PINP; PICP) that are enzymatically cleaved by to form the tropocollagen type I molecule denoted 'the collagen molecule' in the following. The collagen molecule measures 300 nm x 1.5 nm. Once propeptides have been cleaved, collagen can self-aggregate into collagen fibrils and it should be noted that other fibril forming collagens exist (types II, III, V, XI). Currently more than 20 distinct collagen types are described in animal tissues, some of which form templates for fibril formation or are associated with the fibril surface (6; 151) (figure 1).

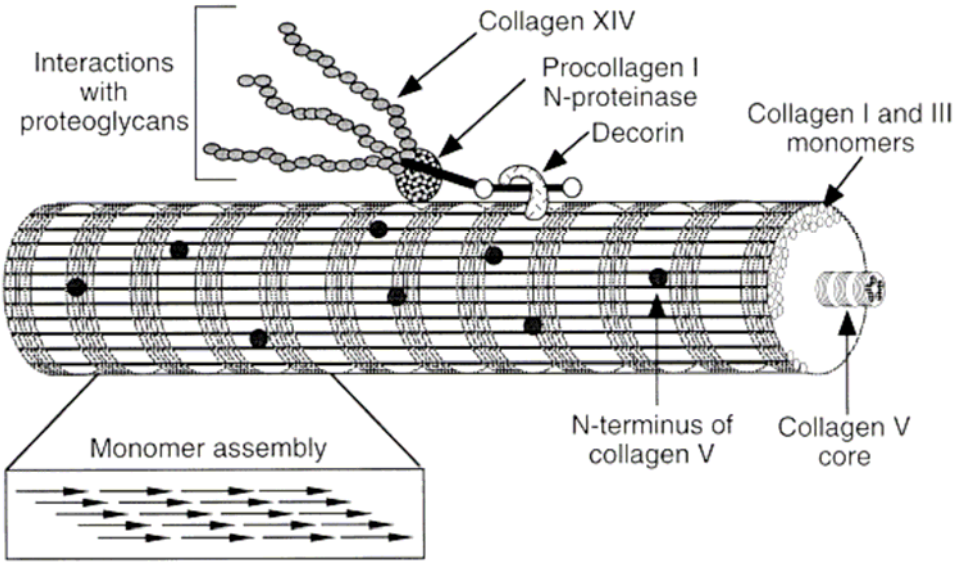



Figure 1. Collagen fibril and associated ECM constituents. Aumailey & Gayraud 1998, ref. (6)

The cells responsible for producing collagen are tenocytes, which are elongated, spindle shaped fibroblasts. In healthy tendon they are aligned along the axis of tendon fibres (95; 169). Tenocytes respond to tendon loading via mechanotransduction which induces the expression of collagens and a wide range of additional ECM constituents (1; 11; 120; 176), of which proteoglycans make up a large proportion (96). Proteoglycans provide a hydrated matrix with a high content of water (~55-70% of wet weight) (96). The collagen fibrils are embedded in the proteoglycans-rich matrix and thus tendon is essentially a fibre-reinforced composite material (82).

.

### 7.2 The collagen fibril

Collagen fibrils in tendon are primarily composed of collagen type I molecules (strictly tropocollagen type I) that aggregate in a highly ordered manner which gives rise to a characteristic cross-banding of the fibril with a periodicity (D-period) of 67-69 nm (figure 1&2). Strictly, collagen molecules are suspected to assemble into *microfibrils* that subsequently aggregate to form the actual fibril, although their existence seems to be subject to debate (131; 132). A so-called quarter stagger of the collagen molecules in the axial direction where an axial displacement of collagen molecules relative to the neighbouring molecules by one D-period explains the fibril banding pattern (70; 141).

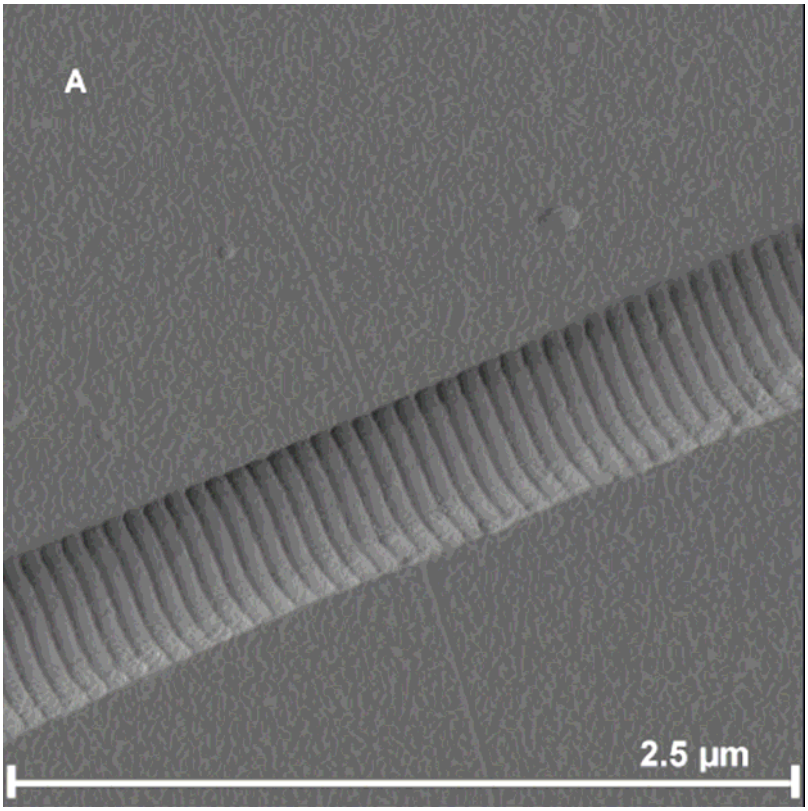



Figure 2. AFM image of a collagen fibril from rat-tail tendon. Hansen et al. 2009, ref. (62).

The integrity of fibrils is critically dependent on intermolecular co-valent cross-links that form gradually with maturation of collagen tissues (8; 9; 167) (see section 7.5). However, polar, hydrophobic and other non-covalent bonds provide the initial structural stability (131). Fibril diameters of various collagenous tissues display a broad range from ~10-500 nm (70; 135).

Although fibrils have the ability to self-assemble (82), proteoglycans seem to be involved in the regulation of fibril morphology (37; 82) and fibril alignment may be cellular in nature (26). Some GAGs are co-assembled with collagen type I during fibrillogenesis (25). Most other GAGs are interfibrillar (6). Small leucine rich proteoglycans (SLRPs) such as decorin, which is abundant in tendon (85), seem to regulate fibril formation and prevent fibril-fibril surface interactions and fusion (98; 180). Knockout mice lacking the SLRP fibromodulin exhibit fewer and abnormal collagen fibrils and the fibril bundles are disorganized (81). Collagen fibrils in tendon display changes in diameter with maturation, aging, loading and tendon type (135; 137; 138; 157; 162). Large diameter fibrils are presumed to possess greater strength than small diameter fibrils. This is due to a potential for higher density of cross-links, since the ratio of fully cross-linked molecules

within the fibril to partially cross-linked peripheral molecules is greater. Small diameter fibrils are assumed more resistant to plastic deformation since the surface area for a given mass is larger, which allows for greater shear forces between the fibril and ECM (134). With maturation there seems to be an increase in fibril diameter and a greater variation in size (broadening of fibril diameter distribution). Depending on tendon type and perhaps species the fibril diameter distribution may be uni- og bimodal (134).

Mature collagen type I fibrils are presumed to be primary load-bearing subunits of tendon (144) albeit their importance relative to the viscous extrafibrillar proteoglycan matrix (ground substance) is subject to debate (146). The fundamental question is whether collagen fibrils are continuous along the entire length of tendons. Provenzano et al. recently investigated fibril morphology in mature tendon and ligament in various animal species by SEM. The authors counted more than 7.000 fibrils and did not observe fibril ends, which lends quite convincing support to the notion that fibrils may span the entire tendon (144). Others, however, have argued that direct interaction between GAG side chains and the fibril surface to provide lateral force transfer between fibril is crucial for force transfer through the tendon structure (49; 147) and addition of GAG to collagen gels have been reported to enhance material properties with little further benefit of cross-linking treatment (130). Conversely, removal of the proteoglycans rich extrafibrillar matrix from tendon fascicles has been shown to have little effect on the mechanical properties of ligament and tendon (44; 115; 159) thus indicating that the extrafibrillar ground substance may not provide a primary pathway for force transmission.

### 7.3 Tendon structure

Tendons display a highly organised and strict hierarchical structure (86). Fibrils assemble into fibre bundles (fibres) and assemblies of fibres give rise to tendon fascicles. The fibre bundles and fascicles are enveloped by the *endotenon*, which is a sheath consisting of reticular connective tissue that carries blood vessels, nerves and lymphatics. Bundles of fascicles group to form whole tendon, which is surrounded by a comparable sheath, the *epitenon*. Many tendons like the Achilles and patellar tendon are further surrounded by a loose areolar connective tissue, the *paratenon,* that provides unhindered movement of the tendon relative to surrounding tissues. The paratenon is contiguous with the epitenon, which in turn connects to endotenon on its inner aspect (83). Fascicles display a

characteristic waveform so-called crimping, which is abolished upon application of tensile loading (38; 57). The microstructural basis for crimps seems to be sharp bends or kinks in the collagen fibre bundles (134; 148).

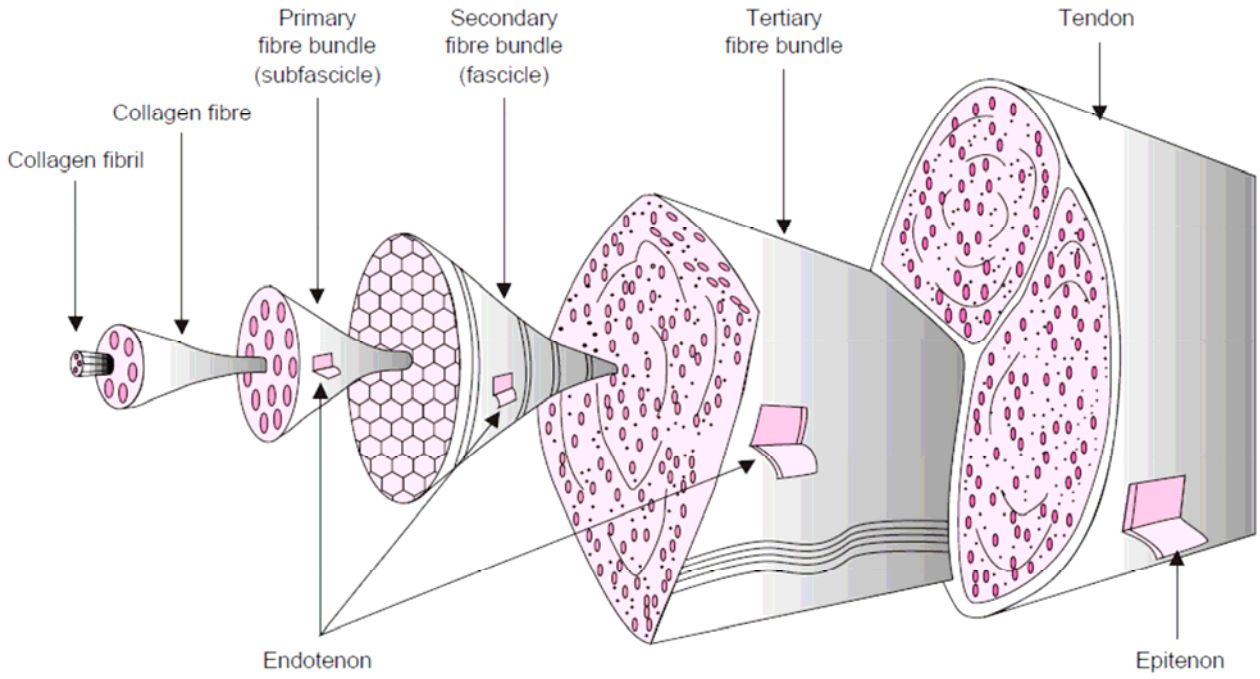



Figure 3. Schematic of the hierarchical tendon structure. Khan 1999, ref. (92).

### 7.4 Tendon biomechanics

The tensile properties of tendons and tendon substructures can be determined by tensile testing that record simultaneous changes in force and elongation of the tendon. Traditionally, whole tendon testing has been performed in vitro (22), however ultrasonographic and MRI methods allow for measurement of in vivo mechanical properties (97). For all measurement modalities some fundamental conventions and parameters are used to describe the mechanical properties.

When tension is imposed on a tendon it will elongate. The ratio of force to elongation ($\Delta F/\Delta L$) defines the tendon *stiffness*. The unit of stiffness is N/m. The shape of the force-elongation curve is curvy-linear with an initial non-linear 'toe-region' and a subsequent 'linear region' (figure 4). Straightening of the aforementioned crimps corresponds with the

toe-region phenomenon (57; 95). Crimping may provide a mechanical 'buffer' against sudden elongation to prevent fibrous damage though shock absorption (128; 134). The linear region likely derives from complex microstructural mechanisms such as elongation of the collagen molecule, increase in the gap region between collagen molecules in the longitudinal direction and relative slippage of laterally neighbouring molecules along the fibril axis (158). Also, non-uniform re-organization and straightening of fibrils may contribute (143).

*Ceteris paribus,* stiffness and elongation will be influenced by changes in tendons dimensions i.e. stiffness and tensile strength will increase with increased tendon thickness, while elongation at failure will remain unaltered (figure 4A). Increased tendon length decreases stiffness and increases elongation at failure while tensile strength is unchanged (figure 4B) (22; 95). Hence, the direct comparison of differences in material properties for tendons of different dimensions is precluded. Therefore, a common approach to circumvent this problem is to normalise ΔL to the initial tendon length ($L_0$), which yields the unit-less parameter *strain*. Strain is most commonly reported as a percentage. Likewise ΔF is normalised to tendon cross-sectional area to provide tendon *stress*. The unit for stress is Pa. The ratio Δstress/Δstrain yields the *Young´s modulus* of elasticity (unit: Pa). Stress, strain and Young´s modulus are utilised to characterise the material properties e.g. of tendon irrespective of material dimensions.

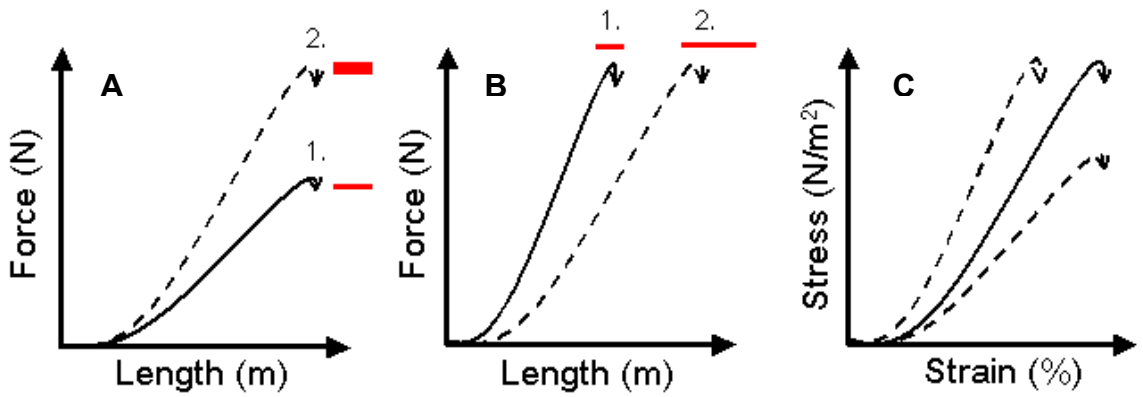



Figure 4. A+B: Graphs illustrating the influence of changes in tendon thickness (A) and length (B) on the force-elongation properties of a tendon (not to scale). C: Normalisation of ΔF and ΔL by tendon dimensions yields a corresponding stress-strain relation, which describes the material properties independent of tendon dimensions. Graphs are not to scale.

Tendons have viscoelastic properties i.e. they exhibit a load dependent (time-*independent*) linear elastic component and a viscous rate dependent (time-*dependent*) component (22). Rheologically, simple spring, dashpot and frictional elements are required to model the viscoelastic behaviour of tendon (22).

*7.4.1 Biomechanical properties of the human patellar and Achilles tendon*

Tendon mechanical properties have been studied extensively, however the majority of studies have utilised in vitro techniques and cadaver material, which may not be directly applicable to human tendons in vivo. By use of such techniques in tail and limb tendons of 10 animal species it has been reported that the Young´s modulus approached a value of ~1.5 GPa at stresses above 30 MPa (15). To circumvent the limited applicability of vitro and animal studies, human *in vivo* techniques have been developed during recent years many of which have exploited the possibilities of real-time US (27; 33; 51; 58; 59; 109; 119; 150; 156; 171). Obviously, the investigation of tendon failure mechanics in vivo is precluded. Nonetheless, the methodology has provided valuable new insight into human tendon biomechanics and importantly has allowed for the investigation of tendon adaptation to various loading regimes. When data from such studies are compiled (see Table 1 below) values of Young´s modulus are quite similar to the value reported by Bennett et al. (15). For the human Achilles tendon (and tibialis anterior tendon) in vivo measurements have yielded comparable result within the range of ~ 0.5-1.2 GPa (partly based on own calculations assuming a tendon length of 60 mm and a CSA of 35 $mm^2$ (58; 105; 118; 156)), so it would seem the modulus of the Achilles tendon is less than average, which may be due to optimization for elastic energy storage during locomotion (160).

Tendons have been reported to tolerate impressive stresses of ~100 MPa ($N/mm^2$) (23; 89; 173). For the human Achilles tendon this is equivalent to a maximal load of 350 kg when a cross-sectional area of 35 $mm^2$ is assumed (19). The male human patellar tendon which has an approximate CSA of 100 $mm^2$ (107) should tolerate a maximal load of ~1000 kg. In general maximal tensile strength of tendon is several times higher than the maximal force of its muscle. This provides an ample tensile safety margin (i.e. the ratio of ultimate tensile stress to peak stresses imposed on the tendon in vivo). Most tendons experience peak stresses below 30 MPa (87; 89). However, since peak loads as high as 9 kN during sprinting and running have been reported for the human

patellar and Achilles tendons (103; 103; 179) these particular high-load tendons may have safety factors below 1 whereas other tendons have been reported to have safety factors of 4 or more (16; 89). Such high in vivo stresses are likely to have implications for the prevalence and incidence of injuries in the human patellar and Achilles tendon.

**Table 1. Patellar tendon mechanical properties, *in vivo***

| **Author, year (reference)** | n | Gender | Method | Stress (MPa) | Strain (%) | Modulus (GPa) |
|---|---|---|---|---|---|---|
| Westh et al., 2007 (171) | 10 | Male | MRI | 46 | 6.1 | 1.5 |
| Kongsgaard et al., 2007 (107) | 12 | Male | MRI | 45 | 6.3 | 1.4 |
| Kongsgaard et al., 2009 (106) | 37 | Male | MRI | 48 | 4.8 | 1.7 |
| Couppé et al., 2009 (young males) (32) | 10 | Male | MRI | 65 | 6.9 | 2.2 |
| Couppé et al., 2009 (old males) (32) | 7 | Male | MRI | 51 | 6.1 | 1.7 |
| Couppé et al., 2008 (lead extremity) (33) | 7 | Male | MRI | 53 | 5.0 | 2.3 |
| Couppé et al., 2008 (non-lead extremity) (33) | 6 | Male | MRI | 66 | 5.9 | 2.2 |
| Carroll et al., 2008, (young males) (27) | 6 | Male | MRI | 30 | 6.0 | 1.0 |
| Carroll et al., 2008, (old males) (27) | 16 | Male | MRI | 25 | 5.4 | 1.0 |
| Onambele et al, 2007 (129) | 10 | Male | US | 29 | 16 | 1.1 |
| Hansen et al., 2006 (59) | 12 | Male | US | 30 | 6.9 | 1.1 |
| **Average** | **(Total: 138)** | - | - | **44** | **6.9** | **1.6** |

## 7.5 Collagen cross-links

The immature tendon possesses little strength. However, as the tendon matures so does tendon strength (8), and there is general agreement that intermolecular co-valent cross-linking are essential for the increase in strength (8; 9) although it should be remembered that other variables increase (collagen content, fibril diameters, water content) also change with maturation (135; 136). Defects in collagen cross-linking (lathyrism) have been associated with devastating adverse effects on various organ systems including the musculoskeletal system (121), which trace back to inferior quality of supporting connective tissues (108). For some types of Ehlers-Danlos and Marfan's syndrome secondary cross-link defects appear to be part of the pathophysiology (42), the former condition may be associated with rare types of tendon rupture (123; 125).

Cross-linking of collagen type I occurs extracellularly through posttranslational modification to the collagen molecule by the copper dependent enzyme LOX (166). LOX catalyses oxidative deamination of the ε-amino group of a lysine or a hydroxylysine

residue located on the collagen type I molecule at two specific telopeptide sites (one C- and one N-terminal), which results in the formation of two corresponding aldehydes, allysine or hydroxyallysine of which the latter is prevalent in tendon (155; 166). Subsequently, the aldehydes can condense with a helical lysine or hydroxylysine to form a so-called 'immature' cross-link. Immature cross-links are divalent in nature, however they spontaneously convert to trivalent 'mature' forms. Immature cross-links bind two adjacent molecules in a head-to-tail fashion between molecules in axial alignment. Trivalent mature cross-links are believed to provide an additional transverse cross-link to a third collagen molecule in parallel alignment (possibly located on another microfibril i.e. interfibrillar) (9; 13). The trivalent bond should provide additional strength by resisting molecular slippage (Figure 5)(8).

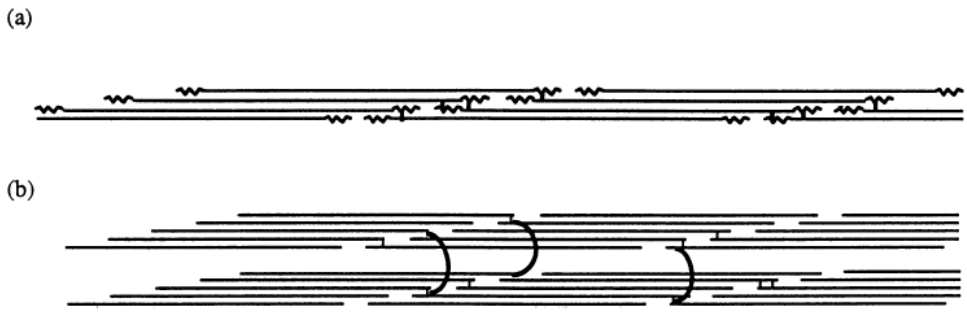



Figure 5. (a) Immature cross-links provide head-to-tail bonds between two collagen molecules. (b) Mature cross-links are trifunctional and provide an additional transverse interfibrillar bond
Bailey et al. 1998, ref. (9)

Lysine residues in the helix are often hydroxylated in tendon, which gives rise to the immature *keto-imine* cross-link hydroxylysino-5-ketonorleucine (HLKNL), which is unstable. Before analyses it can be stabilized by borohydride reduction to form DHLNL. Remaining non-hydroxylated lysine gives rise to the much less abundant HLNL i.e. the stabilized product of lysino-5-ketonorleucine (LKNL) (9; 166). Other cross-link types may also occur depending on tendon type (8). HLNL and DHLNL (strictly HLKNL and LKNL) spontaneously transform into the mature pyridinium (or pyridinoline) cross-links LP and HP, respectively. It should be noted that some propose HP and LP to be divalent rather than trivalent and would therefore they will not provide additional strength compared to immature cross-links (9; 13). However this seems to be a matter of debate (68). HP has been shown to be related to material behavior of rabbit medial collateral ligament when the

results for healing and control tissue were pooled (48). In healing rat patellar tendon pyridinoline was found to be a better indicator of ultimate tensile stress than hydroxyproline (29). Others have shown a positive relationship between HP content and the stiffness (modulus) of goat bone-tendon-bone ACL graft and ACL (127). However, despite similar HP concentrations the modulus of the graft and ACL differed considerably, which suggested that factors other than HP co-determine tendon mechanics. Hence, pyridinium cross-links seem related to mechanical function in healing ligament and tendon tissue in animals. However, the importance of mature cross-links in relation to the mechanical properties of healthy human tendon remains unexplored. Pyrrole (Ehrlich´s chromogen) is a third mature collagen crosslink, which forms by reaction with a non-hydroxylated telopeptidyl lysine-aldehyde and HLKNL (7; 9)

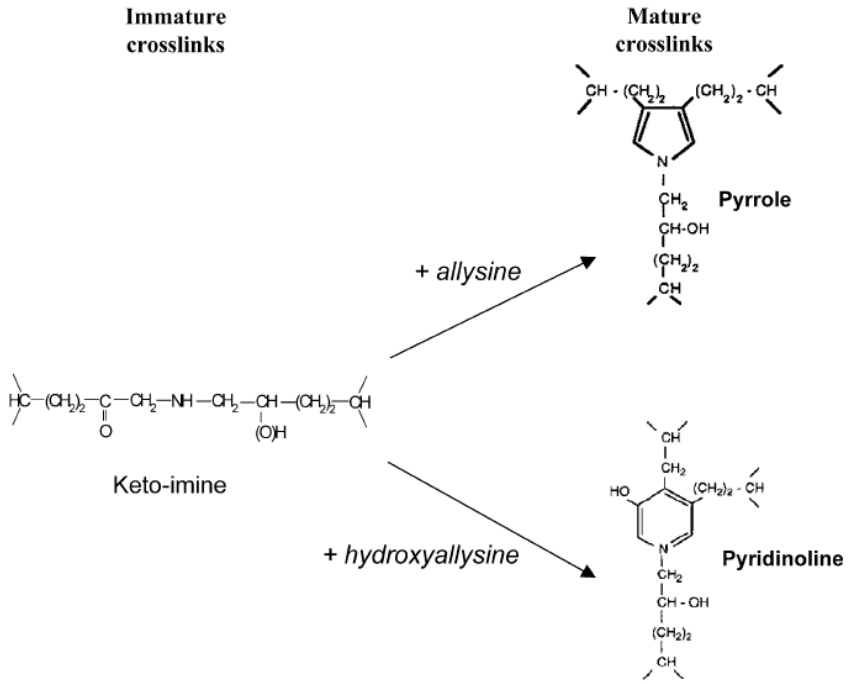



Figure 6. Immature ketoimine cross-links can spontaneously transform into mature pyrrole or pyridinoline depending on the degree of telopeptide hydroxylation. From Viguet-Carrin et al. 2006, ref. (166).

If the telopeptides are highly hydroxylated pyridinolines will preferentially form, if not pyrrole is favoured (Figure 6). The isolation of pyrrole has posed a challenge since the

compound is very instable during isolation procedures (9). Additionally, the pyrrole assay is somewhat unspecific compared to HPLC, which is the most widely applied method for cross-link analyses (7; 9). Pyrrole is suspected to be trivalent and has been shown to correlate with bending strength of cortical bone whereas no association was found for pyridinoline (100). It should be noted that a third mature trivalent cross-link HHL derived from aldimine precursors is reported to be present in some tendons (8; 18). It was beyond the scope of the present thesis to examine its presence and significance in human tendon.

### 7.6 Tendon degenerative disease

Although tendons posses impressive strength, tendon injury is common. Some have proposed tendon injury to account for 30-50 % of all sports related injuries (73). Such injury can be subdivided into acute injuries such as tendon rupture and chronic painful conditions so-called *tendinopathy*. This generic term compiles the clinical triad of pain, swelling and impaired performance (117) but also underlines the frequently reported absence of inflammation in the affected tendons (92; 93). However, both conditions may be interrelated as tendon degenerative changes are associated with both tendon degeneration and rupture (164). It should be noted that tendon injury is seen in conjunction with various systemic diseases such as autoimmune, infectious, neurological, renal, calcium metabolic, hypercholesterolemic and heritable collagen diseases (31; 72; 116; 122; 142) as well as iatrogenic causes such as local or systemic corticosteroid injection (66; 116) and treatment with quinolones (116).

Among the frequently injured tendons in humans are the Achilles, patellar and supraspinatus tendons (151). These tendons are subjected to high loads and generally tendon injury is suspected to result from repeated microinjury and gradual fatigue wear (88; 90). An X-ray diffraction study has revealed that fibrillar damage occurs with excessive loading immediately prior to failure of larger collagen fibres (99).

At the microscopic and submicroscopic level there is histopathologic evidence for at multi-facetted degenerative process that involves both cellular and structural changes. Adverse changes such as disorganization of collagen fibrils and fibre bundles, increased amounts of glycosaminoglycans (GAGs) and type III collagen, deposition of lipids and calcification of collagen fibres, vascular abnormalities, signs of cellular hypoxia

and variations in cellular density and morphology (77; 79; 84; 84; 164). Such changes are embraced by the term *tendinosis* (116; 145).

The underlying mechanisms for tendon degeneration are not fully understood, however, there is mounting evidence that the biochemical composition of degenerated tendon is altered and especially the members of the MMP, TIMP, ADAM and ADAMTS families have received attention. The interplay between these factors which regulate the composition of the ECM seems to be disturbed in both tendinopathy and tendon rupture which may cause degradation of the ECM (36; 76; 85; 85; 152; 153). On the basis of such studies it has been proposed that tendinopathy and tendon rupture are distinct entities since their gene expression profiles are dissimilar (76). In Achilles tendon ruptures increased gelanolytic activity has been demonstrated at the rupture site compared to a healthy appearing proximal area of the tendon (85). Such changes are suspected to cause mechanical weakening yet there are few attempts to link tendon degenerative disease and tendon mechanical properties. However, recently Kongsgaard et al. investigated mechanical properties and biochemical changes in human patellar tendinopathy. Interestingly, no change in whole tendon mechanical properties in vivo seemed to affect the injured tendon (106).

### 7.7 Achilles tendon rupture

The human Achilles tendon is particularly prone to complete rupture in comparison to other tendons and seem more prevalent in westernised countries (69; 74; 113; 116; 124). There is a male predominance (78) and interestingly, the incidence of lower extremity tendon rupture seems higher in black than white American U.S. soldiers (133). The aforementioned tendon degenerative changes have been convincingly associated with Achilles tendon rupture (4; 28; 79; 84; 164). Such changes are likely to cause mechanical weakening of the tendon albeit this assumption has been challenged (71). It seems likely that etiology is multifactorial as numerous factors have been associated with the condition (116). However, from a structural viewpoint insufficient dimensions of the tendon may an important risk factor. The so-called *safety factor* (i.e. the ratio of ultimate tendon stress to peak forces during in vivo loading) of the Achilles is low and especially so in Achilles tendon rupture patients (105). As such the tendon seems to operate close to its limit in

vivo and minor perturbations in tendon strength and excessive loading are not unlikely to cause rupture. (For a more detailed discussion the reader is referred to paper IV).

# 8. Methodological considerations

### 8.1. Atomic force microscopy

The microscope was first developed in the 1980s (17) and offers tremendous advantages in terms of resolution compared to optical techniques. In principle resolution is atomic under ideal conditions. The microscope offers the additional advantage of allowing imaging and force measurement in both dry and liquid conditions. AFM is a scanning probe technique, which consists of a precisely controlled piezoelectric element onto which a sample can be mounted and a deflectable cantilever with a very sharp tip. By controlled movement of the piezo the tip can be brought into contact with the sample (Figure 7).

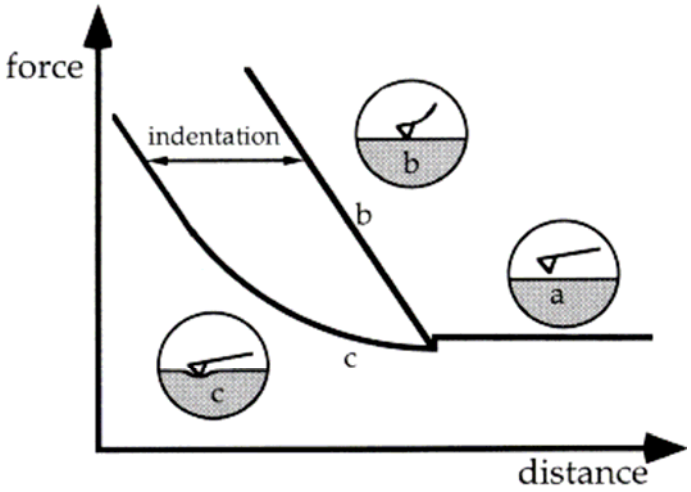



Figure 7. Illustration of the basic principle of AFM. From . Vinckier&Semenza, 1998, ref. (168).

Minute tip-sample forces such as electrostatic and Van der Waal´s forces cause deflection of the cantilever. The deflection causes alterations in the reflection of a laser beamed onto the cantilever surface. The sample surface is scanned in the X and Y directions while height changes (Z-direction) are optically detected (47; 168). By detection of changes in the laser position as a function of the piezo movement, the sample surface topography can be imaged at very high resolution. Imaging can be performed either in contact mode in which the tip-sample force is maintained constant. Since the tip is essentially “dragged”

across the surface, damage to the tip or surface can occur. In tapping mode the tip is oscillated and brought in very close proximity of the surface. Tip-sample forces change the amplitude of oscillation, which can be registered and utilised to construct an image without the risk of surface and tip damage.

Uniquely, the AFM also allows for mechanical measurements performed on a sample of interest by measuring the cantilever deflection as a function of the piezo movement in the Z-direction as the cantilever is alternately moved towards (often indented into) and retracted from the surface which allows for constructing force-distance curves (Figure 7).

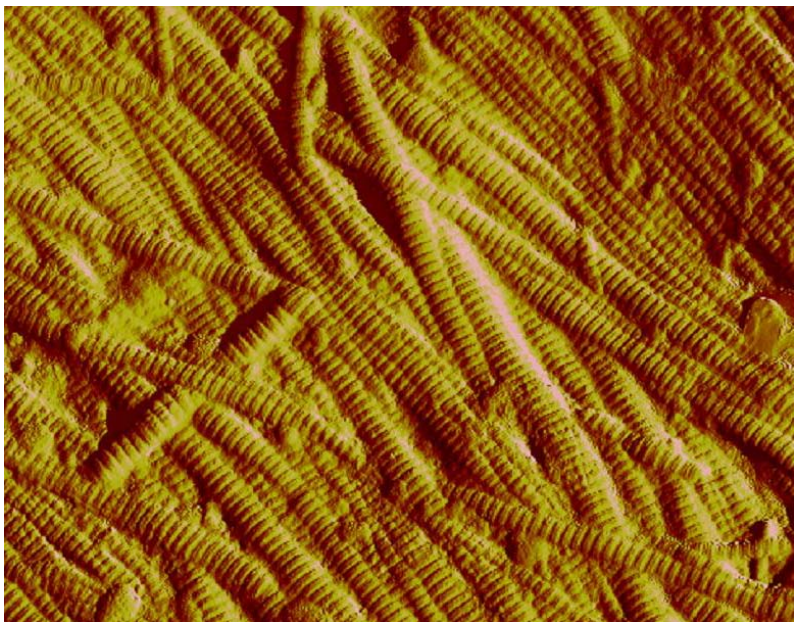


Figure 8. AFM image of substrate surface covered by collagen fibrils . The image was obtained during pilot investigations on human semitendinosus tendon. Hansen P (unpublished).

This measuring mode is termed *force spectroscopy*. The technique allows for measurement of minute forces e.g. from breakage of inter- and intra molecular bonds. AFM has been widely utilised in the investigation of various biological materials and macromolecules (47) and specifically force spectroscopy has been applied to examine the mechanical properties of collagen fibrils (50; 54; 55; 56; 170). Gutsmann et al. investigated the mechanical properties collagen fibrils covering a substrate surface (Figure 8) and reported that the fibril surface was comparable to a so-called “polymer brush” with a

combination of collagen and other filamentous fibril material protruding from the surface, which could not be distinguished (56). The authors proposed that the surface behaved as a “nano-scale polymer Velcro” with successive release of interchain bonds that had the capacity to reform. Such studies can provide knowledge about the fibril molecular structure. However, since fibrils are loaded along their axis it is essential to investigate the properties of collagen fibrils in the axial direction in order to relate nano-scale fibril mechanical properties to higher order structures of the tendon hierarchy. Recently, attempts have been made to measure the “true” tensile properties of single fibrils by AFM, which has provided new insight into fibril mechanical properties (163; 165). In these studies the collagen fibril was glued to the cantilever and the underlying substrate, which allowed for actual tensile testing of the fibril. Since the dimensions of the tested fibril were measured, the stress, strain and Young´s modulus could be determined to allow for direct comparison of fibril properties to higher order tendon structures (see section 8.1.1.)

*8.1.2. Study I and II – the application of AFM.*

The AFM technique was utilized in study I and II. Study I aimed to investigate the effect of artificially increased concentrations of collagen cross-links on the mechanical properties of rat-tail tendon fascicles, while study two aimed to compare the mechanical properties of collagen fibrils from the human patellar tendon to its in vivo properties as evaluated by US. It is beyond the scope of the present thesis to provide at detailed overview of the pit-falls and inherent limitations of the AFM technique.

However, some important issues of relevance to the AFM work performed in this thesis must be mentioned. Both for AFM imaging and force spectroscopy contamination of the cantilever tip can occur. This potentially changes the geometry of the tip as well as the tip-sample interaction, which can lead to imaging artifacts and unreliable force-distance measurements. This problem is hard to circumvent and often necessitates changing the cantilever. Also, insufficient adsorption of the sample material to the underlying substrate may distort images and force measurements (177). One obvious caveat when probing a multi-composite biological structure like the collagen fibril is that it cannot be determined with certainty which surface structure is being probed (56).

In the present thesis we attempted to circumvent the methodological issues of conventional force spectroscopy. Since the mechanical tensile properties along the fibril

axis were the main interest of the thesis a novel single fibril force spectroscopy technique was applied in study I and II. The technique poses the challenge of anchoring a collagen fibril between the AFM cantilever and the substrate underlying the substrate (Figure 9) to perform true tensile testing of the collagen fibril. It was developed only recently (165) and has subsequently been refined (163).

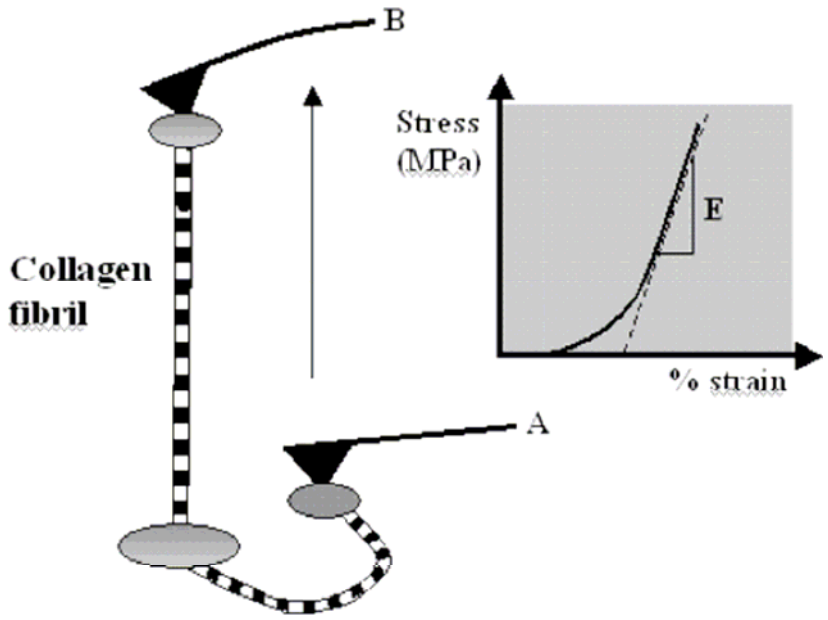



Figure 9. Schematic illustration of tensile testing of a single collagen fibril by means of AFM. Hansen et al., 2009, ref. (62).

This method avoids the uncertainty as to which structure is being measured and it allowed for measurement of the direct effect of GA cross-linking in study I. Study II also applied single fibril force spectroscopy to investigate the tensile fibril properties in comparison to whole tendon using a *within-subject* design. A few concerns regarding this methodology should be noted. Although all measurements on collagen fibrils were performed in a physiologically hydrated state the sample preparation requires the fibril to be dry while the glue is curing, and the effect of drying at the fibril level is unknown but could potentially alter the mechanical properties of the fibril relative to its native properties. Furthermore, it is not possible to monitor the integrity of the glue-fibril interface during testing, and some deformation of glue could occur, which would influence mechanical measurements. However, for the methodology applied the contribution from deformation of the glue was estimated to be <0.01% and therefore negligible. Finally, the geometry of the fibril attachment forces it to bend at the ends, inducing internal shear that can affect the

measured forces. We expect this to only affect the low stress mechanics since the shear modulus has been found to be less than 2% of the tensile modulus of native fibrils (178). The methodology is further discussed in study I and II.

*8.1.3. Micromechanical testing – study I, III, IV*

Micromechanical testing procedures have been applied during recent year to gain insight into the mechanical properties of tendon substructures. Some studies have examined split tendon specimens (23; 24; 30) while others have examined properties of tendon fascicles (63-65; 175). The methodology is comparable to in vitro testing of whole tendon. Hence, it offers the possibility to precisely control tensile load, elongation and strain rate. Therefore both linear and viscous properties of the mounted tendon structure can be investigated. Depending on the testing apparatus it is possible to perform mechanical tests in buffer solution. Thus drying of the sample is prevented and different buffer solutions can easily be applied. Another advantage to whole-tendon testing is that specimen dimensions can be more accurately evaluated due to smaller and more defined “insertion” sites (24).

An important methodological concern is clamping of the tendon sample. It has long been known that strain values obtained by measurement of the displacement of mounting clamps (grip-grip strain) is greater than locally measured strain, which can be measured by tracking of ink markers on the tendon surface (23). This phenomenon has also been observed for tendon fascicles at our facility (64) and it is believed to result from stress-concentrations and sliding at the clamping sites, which may produce excessive strain values and reduced Youngs´s moduli (23). Importantly, however, the discrepancy between grip-grip and local strain values does not seem to be abolished when bone-tendon-bone specimens are tested (23), which is puzzling since this gripping technique mimics the conditions for tendon in situ. Recently a variety of gripping techniques were examined and the majority of gripping techniques seemed to yield similar strain values and there were no differences in strength, elastic modulus and strain between samples that failed at the clamping sites compared to mid-substance failures (126). In our experience sliding of fascicle specimens at the clamping sites was rare with careful drying and adequate time for the cyanoacrylate glue to cure. Additionally, specimens rarely failed at the clamps, rather failure more often occurred along the mid substance of the fascicle (unpublished observation). A recent study compared tendon grip-grip and local strains.

This study found a stress-hardening effect with measurement of local strains by which modulus increased dramatically at stress levels above 20 MPa (174). To this author's knowledge such a phenomenon is not described in studies that have applied in vivo techniques to measure whole-tendon properties. Additionally, it must be considered that during elongation of a tendon specimen the borders of ink markings may become increasingly blurred, likely due to non-uniform elongation of tendon fascicles or fibers (depending on specimen size). Additionally, ink markings merely reflect the surface strains, which may differ from the more central parts of the specimen. This may hamper accurate measurement of marker displacement (Figure 10). Recently, Baker et al. found that repeated measurements of elastic modulus of canine digital tendons varied 10-15%, which was primarily ascribed to inconsistencies in optical strain measurement (Figure 10) (10). Thus, an open question remains whether local strain measurements can be considered of greater physiological relevance than grip-grip measurements. Study I, III and IV report strain values based on grip-grip measurements. It was beyond the scope of the studies to apply additional optical surface marker measurements. However, the optimal choice of methodology currently does not seem to be clarified.

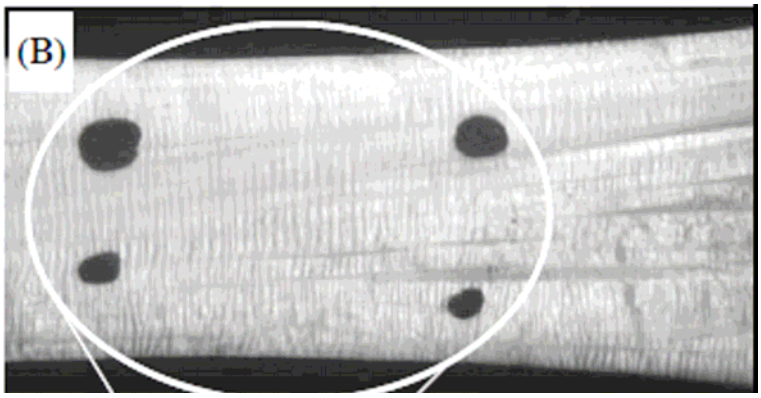



Figure 10. Photo illustrating ink markings on surface of a canine digital tendon. Note the numerous fascicles that form the tendon surface. Fascicles have been shown to be mechanically independent with no lateral force transfer (65). Image from Baker et al. 2004, ref. (10)

The choice of buffer medium during microtensile testing should be considered. For all micromechanical experiments (study I, II, III, IV) an isotonic PBS solution (0.15 M) was used. Screen et al. have proposed that PBS markedly reduces failure strength and

elastic modulus in rat-tail tendons. This effect was ascribed to swelling of the proteoglycans-rich matrix, which supposedly lead to an increased distance between interfibrillar GAG chains and consequently decreased fibril-fibril resistance to shear. The mechanical changes were accompanied by increased fibril diameter and larger interfibrillar spaces alongside decreased fibril circularity, which were assumed to cause impaired fibril properties (159). However, the fascicles were incubated for an extended period (overnight) compared to the trials of the present thesis (~15-20 min), which may have produced more pronounced effects from swelling. Trials have been conducted at our lab to manipulate buffer salt concentration to ascertain any corresponding mechanical effects. We did not observe such effects over a wide range of PBS concentrations (Figure 11). Yamamoto et al. have performed micromechanical testing on rabbit patellar tendon fascicles. Importantly, these authors reported slightly lower elastic moduli ~180-250 MPa) than those reported in the present thesis (~260-625 MPa). These authors used a physiological saline solution for hydration of fascicles, which indicates that PBS per se does not cause adverse mechanical effect. PBS was chosen as buffer medium all studies in the present thesis to maintain avoid pH changes that could potentially affect mechanical properties to some unknown extent and e.g. non-buffered isotonic saline may suffer from variations in pH (4.5-7.5) (ref.: www.lmk.dk). Low pH has been reported to affect aldimine cross-links and cause pronounced creep in rat-tails (35).

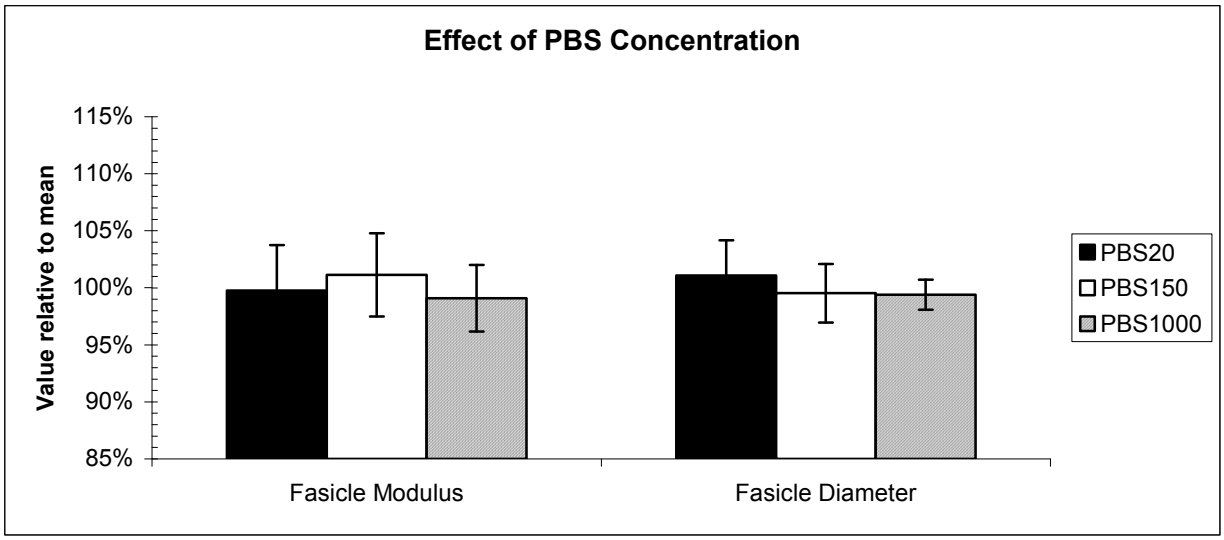



Figure 11. The effect of PBS concentration on mechanics and structure of collagen fascicles (n=6). Values for each parameter are normalized. No differences between 20mM (PBS20), 150mM (PBS150) and 1000mM (PBS1000) PBS solutions were observed. Courtesy of René Brüggebusch Svensson (unpublished).

Lastly, it should be mentioned that tensile testing of tendon substructures such as fascicles or split-tendon specimens seem to yield lower values of Young´s modulus (< 1 GPa) and tensile strength along with increased strain at failure (5; 30; 175) compared to whole tendon (15; 160). The reason for such phenomenon is presently unclear but may relate to frictional forces between fascicles and anatomical orientation (175).

### 8.2 Measurement of tendon mechanical properties *in vivo*

The US-based method applied in study II has previously been applied in several studies that examined the mechanical properties of the human patellar tendon (27; 32; 33; 60; 129; 139; 149; 171) (Table 1). An exhaustive review of the methodology was recently provided by Kongsgaard (104). However, it should be emphasized that to obtain reliable measurements by this method the examined subject must be positioned in a rigid setup and particular attention must be paid to external fixation of the US probe, since slight rotation of the transducer on the sagittal axis or medio-lateral translation may introduce significant errors in the measurement of tendon elongation since the insertions on the patella and tibial tuberosity are not horizontal (frontal plane). Also, tibial co-movement during isometric knee extension should be accounted for to avoid significant underestimation of patellar tendon elongation (60). Additionally, since MRI is required to determine structural dimensions of the patellar tendon adequate image resolution and meticulous measurements of tendon length and CSA by an experienced examiner are a prerequisite for accurate determination of stress-strain properties. It should be noted that while US images are obtained at a 90˚ knee joint angle MRI measurements do not allow for this position during scanning, which may introduce some systematic bias in the absolute magnitude of stress values obtained by the method (27).

## 8.3 Statistics

All data are reported as group mean ± SD. All parametric and non-parametric statistical comparisons were performed with the GraphPad Prism® statistical software package version 4.01. For linear regression analyses a SPSS® Standard Version 10.0.5 statistical software was used. Prior to analyses all data were investigated by D'Agostino Omnibus normality test in GrapPad Prism® to ascertain Gaussian distribution or non-Gaussian distribution. For all studies an alpha level of $p<0.05$ was chosen.

**Study I**

The D'Agostino and Pearson omnibus normality test revealed non-Gaussian distribution of the pooled data from force spectroscopy and Hertz indentation trials. A Mann-Whitney test was used to detect differences between treated and non-treated specimens. For fascicle mechanical testing data a two-tailed paired Student's *t*-test was applied to compare the effect of GA treatment on tail tendon fascicles.

**Study II**

To evaluate the variation in Young´s modulus of fibril and whole tendon the coefficient of variation (CV) i.e. the ratio of SD to the mean was determined. A student´s paired t-test was used to determine differences between fibril and whole tendon mechanical parameters (stress, strain, Young´s modulus and length of the toe-region).

**Study III**

Differences in mechanical (stress, stain, Young´s modulus, failure energy), biochemical (HP, LP, pentosidine, pyrrole and collagen concentrations) and structural properties (fibril CSA, fibril density, fibril volume fraction) between the anterior and posterior fascicles were investigated using a two-tailed paired t-test. To determine associations between structural and biochemical vs. biomechanical parameters linear regression analysis (Pearson product-moment method) was performed.

**Study IV**

Mechanical properties (stress, stain, Young´s modulus, failure energy) and biochemical composition (HP, LP, pentosidine, and collagen concentrations) were compared. Differences between from the rupture site compared to the proximal uninjured tendon were investigated by a two-tailed paired t-test for normally distributed data or a Wilcoxon matched pairs test for non-Guassian data. Differences between external controls and rupture tendons were examined by a two-tailed unpaired t-test for normally distributed data. For non-parametric unpaired data a Mann-Whitney test was applied. To determine associations between biochemical and biomechanical parameters linear regression

analysis was performed using Pearson correlation analysis. In case of non-Gaussian distribution of data a Spearman's rho test was used.

# 9. Results and discussion

## 9.1 Study I

This study exploited the cross-linking effect of GA to evaluate the effect of increased collagen cross-link density on mechanical properties. GA has the ability to cross-link collagen tissues by through GA aldehyde residues reacting with ε-amine groups on lysine and hydroxylysine sidechains (34). GA can form covalent inter- and intramolecular bonds that stabilize the overall structure of the collagen tissue (29, 30, 31) and GA has been widely applied to augment collagen biomaterials (20; 94; 161). However, GA cross-linking in collagenous tissues have produced contrasting results. In bovine pericardial tissue GA has been reported to yield decreased stiffness values and unchanged ultimate tensile stress (112; 140). In porcine pericardial tissue ultimate tensile stress was even found to decrease (111). A clear strengthening effect of GA was found in rat-tail tendon fascicles (Table 1).

Table 1: Effect of glutaraldehyde on fascicle mechanical properties

| | Untreated (n=3) | Treated (n=3) | *p* | *% change* |
|---|---|---|---|---|
| Yield stress (MPa) | 8.1±1.9 | 39.3±16.0 | <0.01 | +385 |
| Yield strain (%) | 3.7±0.4 | 10.6±2.5 | <0.01 | +187 |
| Cumulated E (mJ) | 0.4±0.2 | 3.9±1.9 | <0.05 | +875 |
| Young´s modulus (MPa) | 329.2±62 | 489±137 | <0.01 | +49 |

Values are means ± SD. From Hansen et al., 2009, ref. (62)

Inconsistencies as to the effect of GA are difficult to reconcile but may be related to differenced in tissue type, species and testing procedures. Whether GA cross-links can span between individual microfibrils as proposed for mature enzymatic cross-links is unknown (Figure 5). Damink and co-authors found that GA strengthened dermal collagen only when the tissue was pre-strained during GA treatment. The authors

suggested that the effect of GA depended on whether GA could breach the steric gap between collagen fibre bundles (34). It follows that structural differences may contribute to variation in the mechanical effects of GA cross-linking.

Our data clearly indicate that increased cross-link density augmented the strength of rat-tail tendon fascicles. Interestingly, we observed a marked plateau in force elongation curves, which was abolished by GA treatment. The plateau may reflect low levels of mature cross-links and pronounced creep behaviour, since a similar phenomenon is seen in β-APN fed cross-link deficient rats (146). GA abolished the plateau, which supports the general notion that cross-links provide augmented strength in tendinous tissue.

Study I also aimed to investigated the sub-microscopic alterations induced by GA at the level of the collagen fibril by AFM measurements. Using force spectroscopy we observed higher peak forces, higher cumulated energy (i.e. area under the force-length curve) and decreased pulling length. Small tendon samples from the same fascicles that underwent micromechanical testing were examined. Also the retraction length at which peak force was reached decreased with cross-linking, which resulted in an increased peak-force-to-length ratio. The ratio was considered a surrogate measure of “stiffness” of the probed sample surface. GA treatment also yielded a clear difference in force curve morphology (Fig. 2). Treated samples presented much simpler curves with only few drops in force and very little variation between consecutive extension-retraction cycles.

As discussed in section 8.1.2 conventional force spectroscopy may not be ideal to evaluate changes from cross-link density for the tensile properties of collagen fibrils. Therefore, single fibril measurements were performed. Due to the novelty of the technique only one fibril was tested which precludes any firm conclusion. However, since measurements of the GA cross-linking were performed within the same trial and on the same fibril while other parameters were kept constant, the observed changes in terms of increased stiffness and Young´s modulus are likely directly attributable to increased cross-link density within the fibril. This assumption is supported by the fact that the single fibril is secluded from other ECM components and could not have been affected by concomitant changes to the fibril-ECM interface due to some unknown effect of GA treatment.

It should be noted that presently the single fibril method does not allow for failure testing. The fibril was tested to a stress level of ~60 MPa for the control and the

treated fibril, respectively. Importantly, no indications of early failure of the fibril were observed. Since untreated fascicles showed signs of failure (yield) at much lower stress (Table 1) is seems that the strength of the fibril was greater that its whole-tendon counterpart.

### 9.2 Study II

The aim of study II was to investigate the mechanical properties at two distinct structural levels of the human patellar tendon. Currently, how tensile force is distributed through the tendon during loading is debated. Some authors have proposed that the elastic modulus of the collagen molecule > fibril > tendon (158) while others have proposed that pro-collagen molecules < collagen fiber < collagen fascicle < tendon (3). Such basic knowledge is crucial to fully understand injury mechanisms in tendon. Study I compared the collagen fibril to whole patellar tendon based on the hypothesis that the two structures would display comparable properties. The underlying rationale for the hypothesis is the notion that collagen fibrils are thought to be continuous along the length of a mature tendon (see section 7.2). To investigate this hypothesis a *within* subject design was chosen to avoid intersubject variation.

The overall findings of the study were that fibrils displayed higher Young´s moduli than the whole tendon. The average elastic fibril modulus was 2.83±0.35 GPa compared to 1.91±0.50 GPa for the whole tendon. The latter value is well within the range reported for the patellar tendon albeit slightly higher than the average value (1.6 GPa) reported in table 1 (section 7.4.1). The inherent between day variation for Young´s modulus for the applied method is ~10% in our laboratory (60). Additionally, slight methodological differences may cause further variation for the reported values of Young´s modulus between studies e.g. the determination of tendon CSA by US may yield greater tendon dimensions than MRI (21), which in turn will lead to lower values of tendon stress. Other factors are such as training status and subject age may contribute further to variation (33). An important observation from study I was the large inter-subject variability for Young´s modulus that was not apparent for collagen fibrils (Figure 10). Likely, the in vivo variation represents a combination of measurement and inter-subject variability. Possible sources of variation for fibril measurements are described in section 8.1.2.

Currently, the magnitude of variation between measurements is not known partly due to the novelty of the technique. When various nano-scale techniques to measure single fibril properties are compared the reported range for Young´s modulus of collagen fibrils is very large (32 MPa to 6 GPa) (41; 53; 165). Such variation may be ascribed to methodological differences and possibly to variation in collagen composition of the examined fibrils.

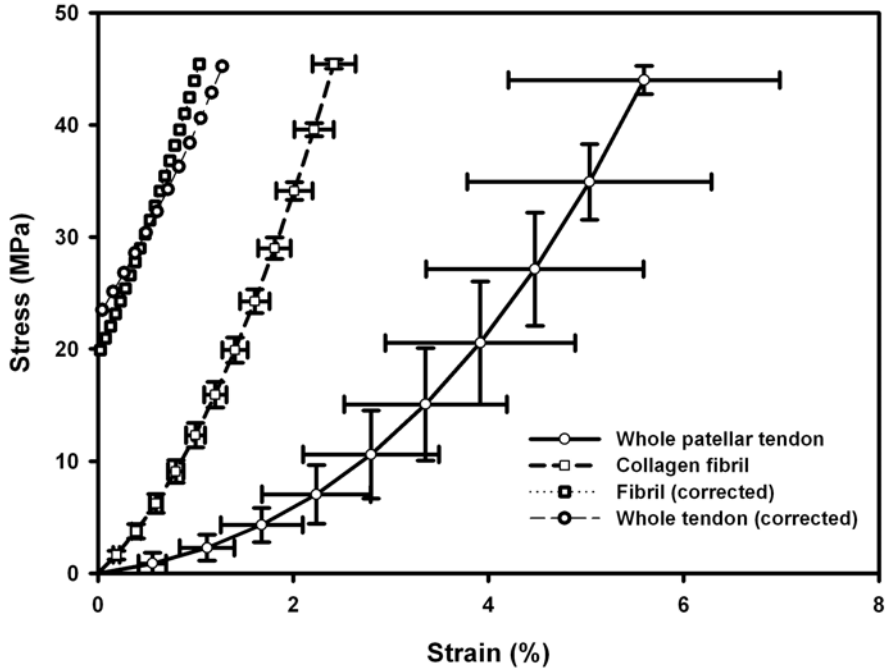



Figure 11. Graph illustrating averaged stress-strain properties of whole patellar tendon, in vivo and collagen fibrils, in vitro obtained from human subjects (n=5). From study II.

Collectively, by direct comparison the collagen fibrils appeared stiffer than whole tendon. However, when tendon Young´s modulus was adjusted for the fibril volume fraction of the tendon CSA the moduli at the two levels were comparable (Figure 11). A volume fraction of 0.6 was assumed based on previous TEM measurements on human patellar tendon (Kongsgaard et al. 2010 (*in press*); Hansen et al., 2010 (61)). These data lend support to the notion of fibrils acting as primary load-bearing units. The toe-region of whole tendon was markedly less pronounced for the collagen fibril. Bearing in mind that the toe-region may to some degree derive from the testing geometry this finding lends support to the notion that low-stress properties of whole tendon are governed primarily by the non-fibrillar ECM.

There are inherent limitations in study II. Firstly, the fibril CSA was measured from dry fibrils while the whole tendon CSA was measured in vivo As discussed in detail in study II such differences could influence the absolute magnitude of the obtained stress and modulus values and the drying in itself may alter the native fibril properties to some unknown degree. Also it should be noted, that only two fibrils were measured per subject while the absolute number of fibrils constituting the whole tendon is immense. However, it was beyond the scope of this thesis to evaluate the inter-individual variation in fibril properties.

### 9.3 Study III

This study aimed to investigate the importance of collagen fibril morphology and cross-link composition of the human patellar tendon in relation to tendon mechanical properties. Based on a previous study of Haraldsson et al. (64) is it was hypothesized that regional differences in mechanical strength would affect tendon fascicles of the anterior and posterior patellar tendon and that such differences would be accompanied by detectable differences in collagen composition and fibril morphology. The rationale for this hypothesis was the general notion that both fibril morphology and collagen cross-links are essential determinants of tendon mechanical properties (Figure 12).

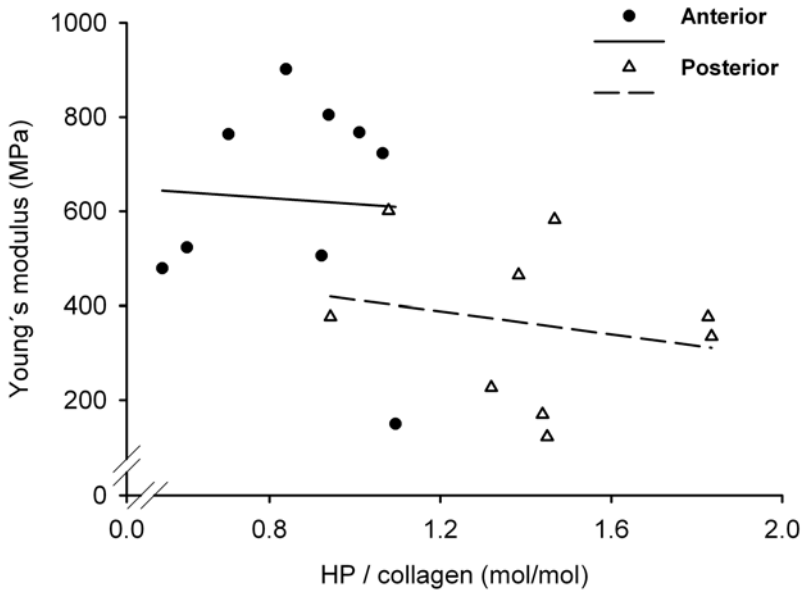



Figure 12. HP content of the anterior (filled circles) and posterior (open triangles) patellar tendon plotted against Young´s modulus (n=9). No association was found between the two variables ($r^2$=0.04). Hansen et al. 2009, ref (61) (unpublished graph).

The main findings of the study were, that fascicles located in the posterior tendon displayed lower elastic moduli and tensile strength. Concomitantly, the content of pyridinium cross-links were markedly higher compared to the anterior tendon while the pe content was similar. Fibril diameters of the posterior tendon tended to be smaller. Importantly, neither collagen content nor cross-link composition displayed any association with mechanical parameters (Figure 12). Pyridinoline is believed to confer strength to collagenous tissue and have been shown to correlate with elastic modulus in horse tendon (8; 9; 52), however this does not seem to be the case for the human patellar tendon. It has been demonstrated that pyrrole correlates with bending strength in avian cortical bone (101). To the best of this author's knowledge study III is the first to investigate the pyrrole content of human patellar tendon. We did not find any correlation between pyrrole and mechanical parameters.

Collectively, study III demonstrated unequivocal differences in pyridinoline levels between the anterior and posterior tendon. The combination of high levels of HP, low strength and a tendency towards smaller fibril CSA in the posterior tendon could relate to a different functional demands in the posterior and anterior tendon. Several authors have reported that differential intratendinous strain occurs within the human patellar tendon (2; 14; 39) albeit with contrasting results, which may in part be due to technical differences in the applied measurements. The present data do not exclude this possibility. Interestingly the posterior patellar tendon (denoted patellar ligament in some species) bears some resemblance to ligament biochemically and structurally (see discussion in study III). Alternatively, the observed differences could relate to tendon degeneration and it should be noted that there seems to be a predilection of the posterior tendon for the overuse injury jumper's knee (75; 91), which could relate to tendon degeneration. However, pentosidine concentration was similar in both tendon regions, which did not point to any ongoing renewal of the collagen matrix speaking against an injury process in posterior tendon (12).

It was beyond the scope of study II to investigate whether other cross-link parameters. Measurement of immature cross-links, MMPs and collagen type III would have aided the interpretation of the results and it remains to be established whether

alternative mature cross-links are associated with mechanical properties of the human patellar tendon. Additionally, the sample size of the study was limited and needless to say data must be interpreted with caution. However, the study demonstrated unequivocal regional differences in cross-link composition between the anterior and posterior tendon.

### 9.4 Study IV

This study attempted to investigate the significance of mechanical properties and collagen composition in tendon rupture, which represents the most severe human tendon injury. The mentioned parameters were measured in tendon samples obtained from patients that had suffered a complete Achilles tendon rupture. It was hypothesized that a pre-existing weakening of the tendon at the site of rupture would be present alongside biochemical changes when compared to a non-injured proximal area of the same tendon. Additionally, non-injured age-matched cadaver Achilles tendons were examined to provide data on non-ruptured tendons for comparison.

The main findings of the study were that no regional mechanical or biochemical differences were present within ruptured tendons when the rupture site and proximal tendon were compared. Compared to non-ruptured cadaver tendons collagen content was lower at both locations in injured tendons while HP content tended to be greater in cadaver tendons (Figure 13).

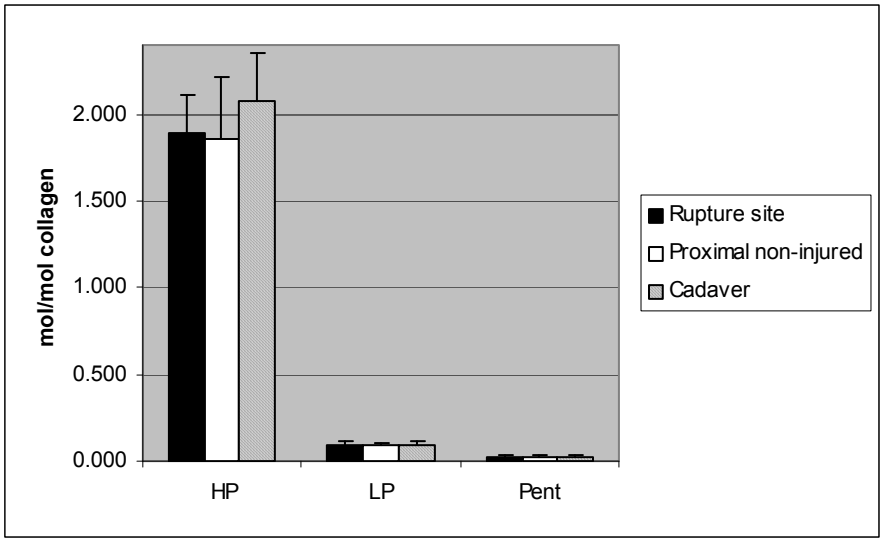



Figure 13. Comparison of cross-link composition. HP content tended to be greater in cadaver tendon compared to the rupture site (p=0.07) and internal control site (p=0.12) (n=16). Data from study IV.

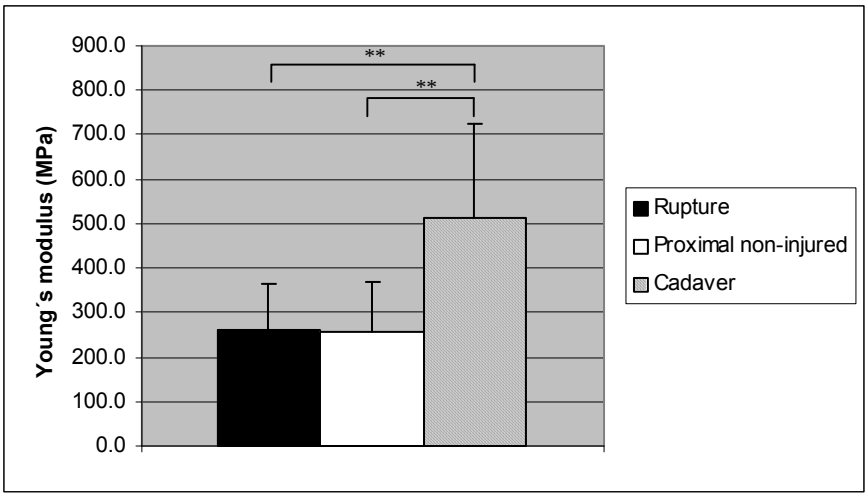



Figure 14. Graphs illustrating differences in fascicle Young´s modulus. Fascicles from cadaver tendons displayed the higher modulus values compared to fascicles from the rupture ($p<0.01$) and non-injured proximal tendon ($p<0.01$) (n=16). Data from study IV.

Micromechanical testing of fascicles from cadaver tendons displayed higher Young´s moduli and a tendency to larger tensile strength (Figure 14). Similar to study III there was no association between pyridinium cross-links and mechanical parameters. Albeit HP has been shown to predict scar tissue weakness in healing rabbit ligament (48) and to be associated with modulus in animal tendon (52) the present data and findings in study III indicate that HP is not a principle determinant of tensile strength and elastic modulus in the human patellar tendon. This is supported by recent in vivo data from our lab that demonstrated no association between modulus and HP (104) and a pilot study performed in connection with the present thesis (Figure 14).

Interestingly, collagen content at the site of rupture was positively correlated with tensile fascicle strength. In the aforementioned study on healing rabbit ligament collagen content was associated with failure strength only in the early phases of healing (48). Early healing and chronic fibrosis has been associated with high HP levels (12). Study IV demonstrated high concentration of HP at the site of rupture, the proximal uninjured tendon and in control cadaver tissue. A previous study that applied a similar study design to evaluate collagen composition in tendinopathic tendons found a similar pattern for HP and collagen content (Figure 13), however lysine hydroxylation was increased at the rupture site which suggested early fibrotic repair and the pattern of MMP expression alongside decreased pentosidine content pointed to increased turnover with deposition of a young collagenous matrix (36).

However, changes in tendinopathy seem be slightly different from tendon rupture since no reduction of pentosidine was observed in study IV. It has been suggested that painful tendinopathy and rupture are distinct disease entities (76). The present data and previous reports seem to indicate impaired capacity for collagen type I synthesis and reduced collagen content, possibly due an impaired repair process. Such changes may have caused the observed mechanical changes and weakening of the Achilles tendon.

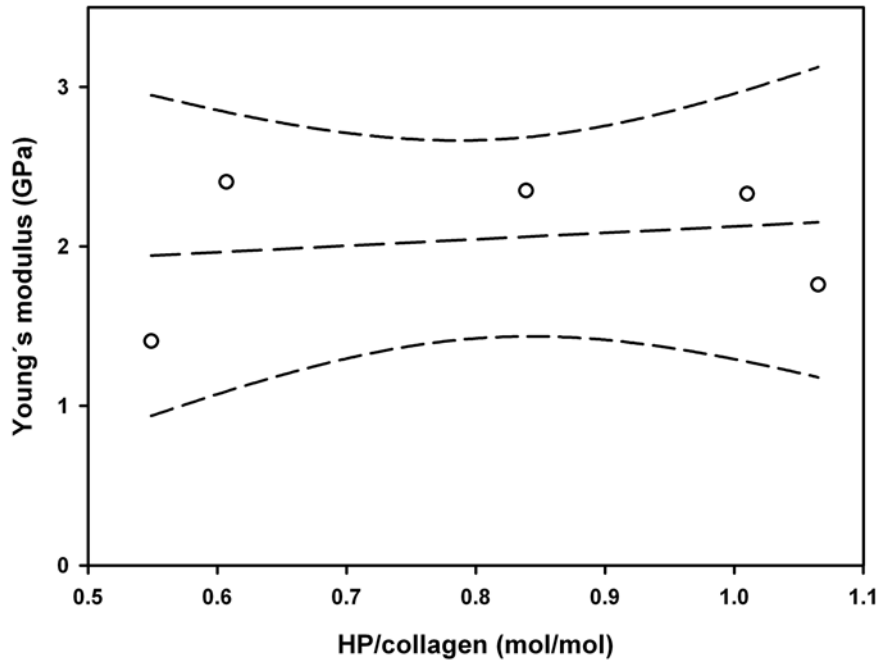



Figure 14. HP content (anterior tendon) plotted against in vivo Young´s modulus of the human patellar tendon. No association was found between the variables. Hansen et al. 2009 (unpublished data).

This study is the first to relate collagen composition and mechanical properties in ruptured Achilles tendon. However, future studies should include the evaluation of parameters such as collagen type III, immature cross-links, collagen hydroxylation, MMP activity and other measures of collagen age and turnover to provide a more complete picture of the pathophysiology underlying Achilles tendon rupture.

# 10. Conclusions

This thesis aimed to investigate the importance of cross-link composition and fibril morphology in relation to tendon mechanical properties. The data presented in this thesis demonstrate that artificial cross-linking can confer strength to tendon likely through an augmenting effect on collagen fibrils. Collagen fibrils are considered essential for tensile force transfer in tendon, indeed collagen fibrils seem to govern human patellar tendon properties in the high-stress region of the stress-strain curve while other factors are responsible for the low-stress behaviour in the toe-region. Fibril morphology does not seem to correlate closely with mechanical characteristics in patellar tendon and similarly mature pyridinium cross-links appear unrelated to both tensile strength and Young´s modulus in both the human patellar and Achilles tendons. This supports the notion that HP may not be tri-functional. Pyrrole cross-links are trivalent and detectable in the patellar tendon, however similar to pyridinoline pyrrole did not correlate with mechanical parameters. Marked regional differences in HP and LP content exist between the anterior and posterior patellar tendon. Interestingly, the posterior and weaker posterior tendon contains the higher amounts of HP. Such findings may relate to the etiology of patellar tendinopathy. In severe tendon injury namely Achilles tendon rupture low collagen content and high concentrations of HP cross-links may relate to the injury mechanism possibly reflection impaired capacity for tendon repair.

# 11. Perspectives

It was the overall aim of the present thesis to provide new insight into the interplay between the hierarchical structural elements of tendon and how collagen cross-link composition and fibril morphology affect tendon strength. To date only limited data on these parameters in human tendon is available. This thesis describes the combined application of various measurement techniques in the attempt to link biochemical, structural and biomechanical factors and to put them into a clinical context relating to tendon injury. However, many questions are prompted from the data presented herein. For instance the lack of association between pyridinium cross-links and fibril morphology in relation to tendon properties was unexpected. Hence, in future studies cross-link analyses should aim to examine alternative and perhaps novel cross-links that were not measured in the present thesis. Additionally, to allow for better evaluation of tendon remodelling and

turnover cross-link analyses should be combined with measurement of other factors involved in regulating the tendon matrix composition and a combined biochemical, stuctural and biomechanical (preferably *in vivo*) approach is encouraged.

Nano-scale techniques such as AFM are extremely promising in the field of tendon research. With little doubt failure testing of single collagen fibrils will soon provide new and important knowledge about this fundamental tensile structure in tendon. Knowing the nano-scale failure mechanisms of fibrils will hopefully shed new light on whole tendon injury mechanisms. Further development of nano-scale techniques should focus on mimicking the natural tendon environment, as should micro-tensile techniques to optimize the physiological relevance of the data.

The direct comparison of mechanical properties at distinct levels of the tendon hierarchy is challenging yet necessary to fully understand the structural basis for tendon viscoelastic behaviour. Current limitations are largely technical in nature. For instance the normalization of force-deformation data obtained at distinct levels of the tendon hierarchy can still be optimized to allow for a more direct comparison of mechanical properties of different tendon substructures and the force transfer between them.

Reference List


1. **Almekinders LC, Banes AJ and Ballenger CA**. Effects of repetitive motion on human fibroblasts. *Med Sci Sports Exerc* 25: 603-607, 1993.

2. **Almekinders LC, Vellema JH and Weinhold PS**. Strain patterns in the patellar tendon and the implications for patellar tendinopathy. *Knee Surg Sports Traumatol Arthrosc* 10: 2-5, 2002.

3. **An KN, Sun YL and Luo ZP**. Flexibility of type I collagen and mechanical property of connective tissue. *Biorheology* 41: 239-246, 2004.

4. **ARNER O and LINDHOLM A**. Subcutaneous rupture of the Achilles tendon; a study of 92 cases. *Acta Chir Scand Suppl* 116: 1-51, 1959.

5. **Atkinson TS, Ewers BJ and Haut RC**. The tensile and stress relaxation responses of human patellar tendon varies with specimen cross-sectional area. *J Biomech* 32: 907-914, 1999.

6. **Aumailley M and Gayraud B**. Structure and biological activity of the extracellular matrix. *J Mol Med* 76: 253-265, 1998.

7. **Avery NC**. Quantitative determination of collagen cross-links. In: Methods in Molecular Biology vol. 522. Extracellular Matrix Protocols, Humana Press, 2009, p. 103-121.

8. **Avery NC and Bailey AJ**. Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. *Scand J Med Sci Sports* 15: 231-240, 2005.

9. **Bailey AJ, Paul RG and Knott L**. Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* 106: 1-56, 1998.

10. **Baker AR, Abreu EL, Mascha E and Derwin KA**. Homotypic variation of canine flexor tendons: implications for the design of experimental studies in animal models. *J Biomech* 37: 959-968, 2004.

11. **Banes AJ, Horesovsky G, Larson C, Tsuzaki M, Judex S, Archambault J, Zernicke R, Herzog W, Kelley S and Miller L**. Mechanical load stimulates expression of novel genes in vivo and in vitro in avian flexor tendon cells. *Osteoarthritis Cartilage* 7: 141-153, 1999.

12. **Bank RA, TeKoppele JM, Oostingh G, Hazleman BL and Riley GP**. Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon

collagen: changes with age and in chronic rotator cuff tendinitis. *Ann Rheum Dis* 58: 35-41, 1999.

13. **Barnard K, Light ND, Sims TJ and Bailey AJ**. Chemistry of the collagen cross-links. Origin and partial characterization of a putative mature cross-link of collagen. *Biochem J* 244: 303-309, 1987.

14. **Basso O, Amis AA, Race A and Johnson DP**. Patellar tendon fiber strains: their differential responses to quadriceps tension. *Clin Orthop* 246-253, 2002.

15. **Bennett MB, Ker RF, Dimery NJ and Alexander McN**. Mechanical properties of various mammalian tendons. *J Zool Lond(A)* 209: 537-548, 1986.

16. **Biewener AA and Roberts TJ**. Muscle and tendon contributions to force, work, and elastic energy savings: a comparative perspective. *Exerc Sport Sci Rev* 28: 99-107, 2000.

17. **Binnig G, Quate CF and Gerber C**. Atomic force microscope. *Phys Rev Lett* 56: 930-933, 1986.

18. **Birch HL**. Tendon matrix composition and turnover in relation to functional requirements. *Int J Exp Pathol* 88: 241-248, 2007.

19. **Bojsen-Moller J, Hansen P, Aagaard P, Kjaer M and Magnusson SP**. Measuring mechanical properties of the vastus lateralis tendon-aponeurosis complex in vivo by ultrasound imaging. *Scand J Med Sci Sports* 13: 259-265, 2003.

20. **Bowes JH and Cater CW**. The interaction of aldehydes with collagen. *Biochim Biophys Acta* 168: 341-352, 1968.

21. **Brushoj C, Henriksen BM, brecht-Beste E, Holmich P, Larsen K and Bachmann NM**. Reproducibility of ultrasound and magnetic resonance imaging measurements of tendon size. *Acta Radiol* 47: 954-959, 2006.

22. **Butler DL, Grood ES, Noyes FR and Zernicke RF**. Biomechanics of ligaments and tendons. *Exerc Sport Sci Rev* 6: 125-181, 1978.

23. **Butler DL, Grood ES, Noyes FR, Zernicke RF and Brackett K**. Effects of structure and strain measurement technique on the material properties of young human tendons and fascia. *J Biomech* 17: 579-596, 1984.

24. **Butler DL, Kay MD and Stouffer DC**. Comparison of material properties in fascicle-bone units from human patellar tendon and knee ligaments. *J Biomech* 19: 425-432, 1986.

25. **Canty EG and Kadler KE**. Collagen fibril biosynthesis in tendon: a review and recent insights. *Comp Biochem Physiol A Mol Integr Physiol* 133: 979-985, 2002.

26. **Canty EG, Starborg T, Lu Y, Humphries SM, Holmes DF, Meadows RS, Huffman A, O'Toole ET and Kadler KE**. Actin filaments are required for

fibripositor-mediated collagen fibril alignment in tendon. *J Biol Chem* 281: 38592-38598, 2006.

27. **Carroll CC, Dickinson JM, Haus JM, Lee GA, Hollon CJ, Aagaard P, Magnusson SP and Trappe TA**. Influence of aging on the in vivo properties of human patellar tendon. *J Appl Physiol* 105: 1907-1915, 2008.

28. **Cetti R, Junge J and Vyberg M**. Spontaneous rupture of the Achilles tendon is preceded by widespread and bilateral tendon damage and ipsilateral inflammation: a clinical and histopathologic study of 60 patients. *Acta Orthop Scand* 74: 78-84, 2003.

29. **Chan BP, Fu SC, Qin L, Rolf C and Chan KM**. Pyridinoline in relation to ultimate stress of the patellar tendon during healing: an animal study. *J Orthop Res* 16: 597-603, 1998.

30. **Chun KJ and Butler DL**. Spatial variation in material properties in fascicle-bone units from human patellar tendon. *Key Engineering Materials* 326-328: 797-802, 2006.

31. **Cirincione RJ and Baker BE**. Tendon ruptures with secondary hyperparathyroidism. A case report. *J Bone Joint Surg Am* 57: 852-853, 1975.

32. **Couppe C, Hansen P, Kongsgaard M, Kovanen V, Suetta C, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J Appl Physiol* 107: 880-886, 2009.

33. **Couppe C, Kongsgaard M, Aagaard P, Hansen P, Bojsen-Moller J, Kjaer M and Magnusson SP**. Habitual loading results in tendon hypertrophy and increased stiffness of the human patellar tendon. *J Appl Physiol* 105: 805-810, 2008.

34. Damink, L. O. Gluataraldehyde as a crosslinking agent for collagen based biomaterials. Dijkstra, P. J., van luyn, J. A, Van Wachem, P. B., Nieuwenhuis, P., and Feijen, J. J Mater Sci Mat Med 6, 460-472. 1995.
Ref Type: Generic

35. **Davison PF**. The contribution of labile crosslinks to the tensile behavior of tendons. *Connect Tissue Res* 18: 293-305, 1989.

36. **de Mos M., van EB, Degroot J, Jahr H, van Schie HT, van Arkel ER, Tol H, Heijboer R, van Osch GJ and Verhaar JA**. Achilles Tendinosis: Changes in Biochemical Composition and Collagen Turnover Rate. *Am J Sports Med* 35: 1549-1556, 2007.

37. **Derwin KA, Soslowsky LJ, Kimura JH and Plaas AH**. Proteoglycans and glycosaminoglycan fine structure in the mouse tail tendon fascicle. *J Orthop Res* 19: 269-277, 2001.

38. **Diamant J, Keller A, Baer E, Litt M and Arridge RG**. Collagen; ultrastructure and its relation to mechanical properties as a function of ageing. *Proc R Soc Lond B Biol Sci* 180: 293-315, 1972.

39. **Dillon EM, Erasmus PJ, Muller JH, Scheffer C and de Villiers RV**. Differential Forces Within the Proximal Patellar Tendon as an Explanation for the Characteristic Lesion of Patellar Tendinopathy: An In Vivo Descriptive Experimental Study. *Am J Sports Med* 2008.

40. **ELLIOTT DH**. Structure and Function of Mammalian Tendon. *Biol Rev Camb Philos Soc* 40: 392-421, 1965.

41. **Eppell SJ, Smith BN, Kahn H and Ballarini R**. Nano measurements with micro-devices: mechanical properties of hydrated collagen fibrils. *J R Soc Interface* 3: 117-121, 2006.

42. **Eyre DR, Paz MA and Gallop PM**. Cross-linking in collagen and elastin. *Annu Rev Biochem* 53: 717-748, 1984.

43. **Ferretti A**. Epidemiology of jumper's knee. *Sports Med* 3: 289-295, 1986.

44. **Fessel G and Snedeker JG**. Evidence against proteoglycan mediated collagen fibril load transmission and dynamic viscoelasticity in tendon. *Matrix Biol* 28: 503-510, 2009.

45. **Finni T, Komi PV and Lepola V**. In vivo human triceps surae and quadriceps femoris muscle function in a squat jump and counter movement jump. *Eur J Appl Physiol* 83: 416-426, 2000.

46. **Finni T, Komi PV and Lepola V**. In vivo muscle mechanics during locomotion depend on movement amplitude and contraction intensity. *Eur J Appl Physiol* 85: 170-176, 2001.

47. **Fotiadis D, Scheuring S, Muller SA, Engel A and Muller DJ**. Imaging and manipulation of biological structures with the AFM. *Micron* 33: 385-397, 2002.

48. **Frank C, McDonald D, Wilson J, Eyre D and Shrive N**. Rabbit medial collateral ligament scar weakness is associated with decreased collagen pyridinoline crosslink density. *J Orthop Res* 13: 157-165, 1995.

49. **Fratzl P, Misof K, Zizak I, Rapp G, Amenitsch H and Bernstorff S**. Fibrillar structure and mechanical properties of collagen. *J Struct Biol* 122: 119-122, 1998.

50. **Friedrichs J, Taubenberger A, Franz CM and Muller DJ**. Cellular remodelling of individual collagen fibrils visualized by time-lapse AFM. *J Mol Biol* 372: 594-607, 2007.

51. **Fukunaga T, Kubo K, Kawakami Y, Fukashiro S, Kanehisa H and Maganaris CN**. In vivo behaviour of human muscle tendon during walking. *Proc Biol Sci* 268: 229-233, 2001.

52. **Gillis C, Pool RR, Meagher DM, Stover SM, Reiser K and Willits N**. Effect of maturation and aging on the histomorphometric and biochemical characteristics of equine superficial digital flexor tendon. *Am J Vet Res* 58: 425-430, 1997.

53. **Graham JS, Vomund AN, Phillips CL and Grandbois M**. Structural changes in human type I collagen fibrils investigated by force spectroscopy. *Exp Cell Res* 299: 335-342, 2004.

54. **Gutsmann T, Fantner GE, Kindt JH, Venturoni M, Danielsen S and Hansma PK**. Force spectroscopy of collagen fibers to investigate their mechanical properties and structural organization. *Biophys J* 86: 3186-3193, 2004.

55. **Gutsmann T, Fantner GE, Venturoni M, Ekani-Nkodo A, Thompson JB, Kindt JH, Morse DE, Fygenson DK and Hansma PK**. Evidence that collagen fibrils in tendons are inhomogeneously structured in a tubelike manner. *Biophys J* 84: 2593-2598, 2003.

56. **Gutsmann T, Hassenkam T, Cutroni JA and Hansma PK**. Sacrificial bonds in polymer brushes from rat tail tendon functioning as nanoscale velcro. *Biophys J* 89: 536-542, 2005.

57. **Hansen KA, Weiss JA and Barton JK**. Recruitment of tendon crimp with applied tensile strain. *J Biomech Eng* 124: 72-77, 2002.

58. **Hansen P, Aagaard P, Kjaer M, Larsson B and Magnusson SP**. Effect of habitual running on human Achilles tendon load-deformation properties and cross-sectional area. *J Appl Physiol* 95: 2375-2380, 2003.

59. **Hansen P, Bojsen-Moller J, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties of the human patellar tendon, in vivo. *Clin Biomech* 21: 54-58, 2006.

60. **Hansen P, Bojsen-Moller J, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties of the human patellar tendon, in vivo. *Clin Biomech (Bristol , Avon )* 21: 54-58, 2006.

61. **Hansen P, Haraldsson BT, Aagaard P, Kovanen V, Avery N, Qvortrup K, Larsen JO, Krogsgaard M, Kjaer M and Magnusson SP**. Lower strength of the human posterior patellar tendon seems unrelated to mature collagen cross-linking and fibril morphology. *J Appl Physiol* 2009.

62. **Hansen P, Hassenkam T, Svensson RB, Aagaard P, Trappe T, Haraldsson BT, Kjaer M and Magnusson P**. Glutaraldehyde cross-linking of tendon--mechanical effects at the level of the tendon fascicle and fibril. *Connect Tissue Res* 50: 211-222, 2009.

63. **Haraldsson BT, Aagaard P, Crafoord-Larsen D, Kjaer M and Magnusson SP**. Corticosteroid administration alters the mechanical properties of isolated collagen fascicles in rat-tail tendon. *Scand J Med Sci Sports* 2008.

64. **Haraldsson BT, Aagaard P, Krogsgaard M, Alkjaer T, Kjaer M and Magnusson SP**. Region-specific mechanical properties of the human patella tendon. *J Appl Physiol* 98: 1006-1012, 2005.

65. **Haraldsson BT, Aagaard P, Qvortrup K, Bojsen-Moller J, Krogsgaard M, Koskinen S, Kjaer M and Magnusson SP**. Lateral force transmission between human tendon fascicles. *Matrix Biol* 27: 86-95, 2008.

66. **Haraldsson BT, Langberg H, Aagaard P, Zuurmond AM, van EB, Degroot J, Kjaer M and Magnusson SP**. Corticosteroids reduce the tensile strength of isolated collagen fascicles. *Am J Sports Med* 34: 1992-1997, 2006.

67. **Haut RC, Lancaster RL and DeCamp CE**. Mechanical properties of the canine patellar tendon: some correlations with age and the content of collagen. *J Biomech* 25: 163-173, 1992.

68. **Henkel W, Glanville RW and Greifendorf D**. Characterisation of a type-I collagen trimeric cross-linked peptide from calf aorta and its cross-linked structure. Detection of pyridinoline by time-of-flight secondary ion-mass spectroscopy and evidence for a new cross-link. *Eur J Biochem* 165: 427-436, 1987.

69. **Houshian S, Tscherning T and Riegels-Nielsen P**. The epidemiology of Achilles tendon rupture in a Danish county. *Injury* 29: 651-654, 1998.

70. **Hulmes DJ, Wess TJ, Prockop DJ and Fratzl P**. Radial packing, order, and disorder in collagen fibrils. *Biophys J* 68: 1661-1670, 1995.

71. **Inglis AE and Sculco TP**. Surgical repair of ruptures of the tendo Achillis. *Clin Orthop Relat Res* 160-169, 1981.

72. **Jakobsen LP, Knudsen TB and Bloch T**. [Spontaneous infrapatellar tendon rupture in a patient with systemic lupus erythematosus]. *Ugeskr Laeger* 162: 5088-5089, 2000.

73. **Jarvinen TA, Kannus P, Maffulli N and Khan KM**. Achilles tendon disorders: etiology and epidemiology. *Foot Ankle Clin* 10: 255-266, 2005.

74. **Jarvinen TA, Kannus P, Paavola M, Jarvinen TL, Jozsa L and Jarvinen M**. Achilles tendon injuries. *Curr Opin Rheumatol* 13: 150-155, 2001.

75. **Johnson DP, Wakeley CJ and Watt I**. Magnetic resonance imaging of patellar tendonitis. *J Bone Joint Surg Br* 78: 452-457, 1996.

76. **Jones GC, Corps AN, Pennington CJ, Clark IM, Edwards DR, Bradley MM, Hazleman BL and Riley GP**. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. *Arthritis Rheum* 54: 832-842, 2006.

77. **Jozsa L, Balint BJ, Reffy A and Demel Z**. Hypoxic alterations of tenocytes in degenerative tendinopathy. *Arch Orthop Trauma Surg* 99: 243-246, 1982.

78. **Jozsa L, Balint JB, Kannus P, Reffy A and Barzo M**. Distribution of blood groups in patients with tendon rupture. An analysis of 832 cases. *J Bone Joint Surg Br* 71: 272-274, 1989.

79. **Jozsa L and Kannus P**. Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7: 113-118, 1997.

80. **Jozsa L, Kvist M, Balint BJ, Reffy A, Jarvinen M, Lehto M and Barzo M**. The role of recreational sport activity in Achilles tendon rupture. A clinical, pathoanatomical, and sociological study of 292 cases. *Am J Sports Med* 17: 338-343, 1989.

81. **Kadler KE, Holmes DF, Graham H and Starborg T**. Tip-mediated fusion involving unipolar collagen fibrils accounts for rapid fibril elongation, the occurrence of fibrillar branched networks in skin and the paucity of collagen fibril ends in vertebrates. *Matrix Biol* 19: 359-365, 2000.

82. **Kadler KE, Holmes DF, Trotter JA and Chapman JA**. Collagen fibril formation. *Biochem J* 316 ( Pt 1): 1-11, 1996.

83. **Kannus P**. Structure of the tendon connective tissue. *Scand J Med Sci Sports* 10: 312-320, 2000.

84. **Kannus P and Jozsa L**. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J Bone Joint Surg Am* 73: 1507-1525, 1991.

85. **Karousou E, Ronga M, Vigetti D, Passi A and Maffulli N**. Collagens, Proteoglycans, MMP-2, MMP-9 and TIMPs in Human Achilles Tendon Rupture. *Clin Orthop Relat Res* 2008.

86. **Kastelic J, Galeski A and Baer E**. The multicomposite structure of tendon. *Connect Tissue Res* 11-23, 1978.

87. **Ker RF**. The design of soft collagenous load-bearing tissues. *J Exp Biol* 202: 3315-3324, 1999.

88. **Ker RF**. The implications of the adaptable fatigue quality of tendons for their construction, repair and function. *Comp Biochem Physiol A Mol Integr Physiol* 133: 987-1000, 2002.

89. **Ker RF, Alexander RM and Bennett MB**. Why are mammalian tendons so thick? *J Zool, Lond* 216: 309-324, 1988.

90. **Ker RF, Wang XT and Pike AV**. Fatigue quality of mammalian tendons. *J Exp Biol* 203: 1317-1327, 2000.

91. **Khan KM, Bonar F, Desmond PM, Cook JL, Young DA, Visentini PJ, Fehrmann MW, Kiss ZS, O'Brien PA, Harcourt PR, Dowling RJ, O'Sullivan RM, Crichton KJ, Tress BM and Wark JD**. Patellar tendinosis (jumper's knee): findings at

histopathologic examination, US, and MR imaging. Victorian Institute of Sport Tendon Study Group. *Radiology* 200: 821-827, 1996.

92. **Khan KM, Cook JL, Bonar F, Harcourt P and Astrom M**. Histopathology of common tendinopathies. Update and implications for clinical management. *Sports Med* 27: 393-408, 1999.

93. **Khan KM, Cook JL, Kannus P, Maffulli N and Bonar SF**. Time to abandon the "tendinitis" myth. *BMJ* 324: 626-627, 2002.

94. **Khor E**. Methods for the treatment of collagenous tissues for bioprostheses. *Biomaterials* 18: 95-105, 1997.

95. **Kirkendall DT and Garrett WE**. Function and biomechanics of tendons. *Scand J Med Sci Sports* 7: 62-66, 1997.

96. **Kjaer M**. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 84: 649-698, 2004.

97. **Kjaer M, Langberg H, Bojsen-Moller J, Koskinen SO, Mackey A, Heinemeier K, Holm L, Skovgaard D, Dossing S, Hansen M, Hansen P, Haraldsson B, Caroe I and Magnusson SP**. Novel methods for tendon investigations. *Disabil Rehabil* 30: 1514-1522, 2008.

98. **Kjaer M, Langberg H and Magnusson P**. [Overuse injuries in tendon tissue: insight into adaptation mechanisms]. *Ugeskr Laeger* 165: 1438-1443, 2003.

99. **Knorzer E, Folkhard W, Geercken W, Boschert C, Koch MH, Hilbert B, Krahl H, Mosler E, Nemetschek-Gansler H and Nemetschek T**. New aspects of the etiology of tendon rupture. An analysis of time-resolved dynamic-mechanical measurements using synchrotron radiation. *Arch Orthop Trauma Surg* 105: 113-120, 1986.

100. **Knott L, Tarlton JF and Bailey AJ**. Chemistry of collagen cross-linking: biochemical changes in collagen during the partial mineralization of turkey leg tendon. *Biochem J* 322 ( Pt 2): 535-542, 1997.

101. **Knott L, Whitehead CC, Fleming RH and Bailey AJ**. Biochemical changes in the collagenous matrix of osteoporotic avian bone. *Biochem J* 310 ( Pt 3): 1045-1051, 1995.

102. **Komi PV**. Relevance of in vivo force measurements to human biomechanics. *J Biomech* 23 Suppl 1: 23-34, 1990.

103. **Komi PV, Fukashiro S and Jarvinen M**. Biomechanical loading of Achilles tendon during normal locomotion. *Clin Sports Med* 11: 521-531, 1992.

104. **Kongsgaard M**. *Structural and mechanical adaptations of tendinous tissue. Effects of exercise, loading and injury. Ph.D thesis* (Dissertation). Copenhagen: University of Copenhagen, 2008.

105. **Kongsgaard M, Aagaard P, Kjaer M and Magnusson SP**. Structural Achilles tendon properties in athletes subjected to different exercise modes and in Achilles tendon rupture patients. *J Appl Physiol* 99: 1965-1971, 2005.

106. **Kongsgaard M, Kovanen V, Aagaard P, Doessing S, Hansen P, Laursen AH, Kaldau NC, Kjaer M and Magnusson SP**. Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand J Med Sci Sports* 2009.

107. **Kongsgaard M, Reitelseder S, Pedersen TG, Holm L, Aagaard P, Kjaer M and Magnusson SP**. Region specific patellar tendon hypertrophy in humans following resistance training. *Acta Physiol (Oxf)* 191: 111-121, 2007.

108. **Kovanen V and Suominen H**. Effects of age and life-long endurance training on the passive mechanical properties of rat skeletal muscle. *Compr Gerontol [A]* 2: 18-23, 1988.

109. **Kubo K, Kanehisa H, Ito M and Fukunaga T**. Effects of isometric training on the elasticity of human tendon structures in vivo. *J Appl Physiol* 91: 26-32, 2001.

110. **Kujala UM, Sarna S and Kaprio J**. Cumulative incidence of achilles tendon rupture and tendinopathy in male former elite athletes. *Clin J Sport Med* 15: 133-135, 2005.

111. **Lee JM, Boughner DR and Courtman DW**. The glutaraldehyde-stabilized porcine aortic valve xenograft. II. Effect of fixation with or without pressure on the tensile viscoelastic properties of the leaflet material. *J Biomed Mater Res* 18: 79-98, 1984.

112. **Lee JM, Haberer SA and Boughner DR**. The bovine pericardial xenograft: I. Effect of fixation in aldehydes without constraint on the tensile viscoelastic properties of bovine pericardium. *J Biomed Mater Res* 23: 457-475, 1989.

113. **Levi N**. The incidence of Achilles tendon rupture in Copenhagen. *Injury* 28: 311-313, 1997.

114. **Lian OB, Engebretsen L and Bahr R**. Prevalence of jumper's knee among elite athletes from different sports: a cross-sectional study. *Am J Sports Med* 33: 561-567, 2005.

115. **Lujan TJ, Underwood CJ, Jacobs NT and Weiss JA**. Contribution of glycosaminoglycans to viscoelastic tensile behavior of human ligament. *J Appl Physiol* 106: 423-431, 2009.

116. **Maffulli N**. Rupture of the Achilles tendon. *J Bone Joint Surg Am* 81: 1019-1036, 1999.

117. **Maffulli N, Khan KM and Puddu G**. Overuse tendon conditions: time to change a confusing terminology. *Arthroscopy* 14: 840-843, 1998.

118. **Maganaris CN, Narici MV and Maffulli N**. Biomechanics of the Achilles tendon. *Disabil Rehabil* 30: 1542-1547, 2008.

119. **Maganaris CN and Paul JP**. In vivo human tendon mechanical properties. *J Physiol* 521 Pt 1: 307-313, 1999.

120. **Majima T, Marchuk LL, Sciore P, Shrive NG, Frank CB and Hart DA**. Compressive compared with tensile loading of medial collateral ligament scar in vitro uniquely influences mRNA levels for aggrecan, collagen type II, and collagenase. *J Orthop Res* 18: 524-531, 2000.

121. **Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R and Myllyharju J**. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. *Am J Pathol* 167: 927-936, 2005.

122. **Mathiak G, Wening JV, Mathiak M, Neville LF and Jungbluth K**. Serum cholesterol is elevated in patients with Achilles tendon ruptures. *Arch Orthop Trauma Surg* 119: 280-284, 1999.

123. **Matziolis G, Drahn T and Perka C**. [Spontaneous patellar tendon rupture in a patient with Ehlers-Danlos syndrome]. *Unfallchirurg* 106: 1051-1053, 2003.

124. **Moller A, Astron M and Westlin N**. Increasing incidence of Achilles tendon rupture. *Acta Orthop Scand* 67: 479-481, 1996.

125. **Moretti B, Notarnicola A, Moretti L, Garofalo R and Patella V**. Spontaneous bilateral patellar tendon rupture: a case report and review of the literature. *Chir Organi Mov* 91: 51-55, 2008.

126. **Ng BH, Chou SM and Krishna V**. The influence of gripping techniques on the tensile properties of tendons. *Proc Inst Mech Eng H* 219: 349-354, 2005.

127. **Ng GY, Oakes BW, Deacon OW, McLean ID and Eyre DR**. Long-term study of the biochemistry and biomechanics of anterior cruciate ligament-patellar tendon autografts in goats. *J Orthop Res* 14: 851-856, 1996.

128. **O'Brien M**. Structure and metabolism of tendons. *Scand J Med Sci Sports* 7: 55-61, 1997.

129. **Onambele GN, Burgess K and Pearson SJ**. Gender-specific in vivo measurement of the structural and mechanical properties of the human patellar tendon. *J Orthop Res* 25: 1635-1642, 2007.

130. **Osborne CS, Barbenel JC, Smith D, Savakis M and Grant MH**. Investigation into the tensile properties of collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines. *Med Biol Eng Comput* 36: 129-134, 1998.

131. **Ottani V, Martini D, Franchi M, Ruggeri A and Raspanti M**. Hierarchical structures in fibrillar collagens. *Micron* 33: 587-596, 2002.

132. **Ottani V, Raspanti M and Ruggeri A**. Collagen structure and functional implications. *Micron* 32: 251-260, 2001.

133. **Owens B, Mountcastle S and White D**. Racial differences in tendon rupture incidence. *Int J Sports Med* 28: 617-620, 2007.

134. **Parry DA**. The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. *Biophys Chem* 29: 195-209, 1988.

135. **Parry DA, Barnes GR and Craig AS**. A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proc R Soc Lond B Biol Sci* 203: 305-321, 1978.

136. **Parry DA, Craig AS and Barnes GR**. Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibres as a function of age. *Proc R Soc Lond B Biol Sci* 203: 293-303, 1978.

137. **Patterson-Kane JC, Firth EC, Parry DA, Wilson AM and Goodship AE**. Effects of training on collagen fibril populations in the suspensory ligament and deep digital flexor tendon of young thoroughbreds. *Am J Vet Res* 59: 64-68, 1998.

138. **Patterson-Kane JC, Parry DA, Birch HL, Goodship AE and Firth EC**. An age-related study of morphology and cross-link composition of collagen fibrils in the digital flexor tendons of young thoroughbred horses. *Connect Tissue Res* 36: 253-260, 1997.

139. **Pearson SJ, Burgess K and Onambele GN**. Creep and the in vivo assessment of human patellar tendon mechanical properties. *Clin Biomech (Bristol , Avon )* 22: 712-717, 2007.

140. **Pereira CA, Lee JM and Haberer SA**. Effect of alternative crosslinking methods on the low strain rate viscoelastic properties of bovine pericardial bioprosthetic material. *J Biomed Mater Res* 24: 345-361, 1990.

141. **Pestruska JA and HODGE AJ**. A subunit model for the tropocollagen macromolecule. *Proc Natl Acad Sci U S A* 51: 871-876, 1964.

142. **Pritchard CH and Berney S**. Patellar tendon rupture in systemic lupus erythematosus. *J Rheumatol* 16: 786-788, 1989.

143. **Provenzano PP, Hurschler C and Vanderby R, Jr.** Microstructural morphology in the transition region between scar and intact residual segments of a healing rat medial collateral ligament. *Connect Tissue Res* 42: 123-133, 2001.

144. **Provenzano PP and Vanderby R, Jr.** Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. *Matrix Biol* 25: 71-84, 2006.

145. **Puddu G, Ippolito E and Postacchini F**. A classification of Achilles tendon disease. *Am J Sports Med* 4: 145-150, 1976.

146. **Puxkandl R, Zizak I, Paris O, Keckes J, Tesch W, Bernstorff S, Purslow P and Fratzl P**. Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos Trans R Soc Lond B Biol Sci* 357: 191-197, 2002.

147. **Raspanti M, Congiu T and Guizzardi S**. Structural aspects of the extracellular matrix of the tendon: an atomic force and scanning electron microscopy study. *Arch Histol Cytol* 65: 37-43, 2002.

148. **Raspanti M, Manelli A, Franchi M and Ruggeri A**. The 3D structure of crimps in the rat Achilles tendon. *Matrix Biol* 24: 503-507, 2005.

149. **Reeves ND, Maganaris CN and Narici MV**. Effect of strength training on human patella tendon mechanical properties of older individuals. *J Physiol* 548: 971-981, 2003.

150. **Reeves ND, Narici MV and Maganaris CN**. Strength training alters the viscoelastic properties of tendons in elderly humans. *Muscle Nerve* 28: 74-81, 2003.

151. **Riley G**. Chronic tendon pathology: molecular basis and therapeutic implications. *Expert Rev Mol Med* 7: 1-25, 2005.

152. **Riley GP**. Gene expression and matrix turnover in overused and damaged tendons. *Scand J Med Sci Sports* 15: 241-251, 2005.

153. **Riley GP, Curry V, DeGroot J, van EB, Verzijl N, Hazleman BL and Bank RA**. Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. *Matrix Biol* 21: 185-195, 2002.

154. **Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE and Hazleman BL**. Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. *Ann Rheum Dis* 53: 359-366, 1994.

155. **Robins SP and Bailey AJ**. The chemistry of the collagen cross-links. The mechanism of stabilization of the reducible intermediate cross-links. *Biochem J* 149: 381-385, 1975.

156. **Rosager S, Aagaard P, Dyhre-Poulsen P, Neergaard K, Kjaer M and Magnusson SP**. Load-displacement properties of the human triceps surae aponeurosis and tendon in runners and non-runners. *Scand J Med Sci Sports* 12: 90-98, 2002.

157. **Sargon MF, Ozlu K and Oken F**. Age-related changes in human tendo calcaneus collagen fibrils. *Saudi Med J* 26: 425-428, 2005.

158. **Sasaki N and Odajima S**. Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy. *J Biomech* 29: 1131-1136, 1996.

159. **Screen HR, Chhaya VH, Greenwald SE, Bader DL, Lee DA and Shelton JC**. The influence of swelling and matrix degradation on the microstructural integrity of tendon. *Acta Biomater* 2: 505-513, 2006.

160. **Shadwick RE**. Elastic energy storage in tendons: mechanical differences related to function and age. *J Appl Physiol* 68: 1033-1040, 1990.

161. **Simionescu A, Simionescu D and Deac R**. Lysine-enhanced glutaraldehyde crosslinking of collagenous biomaterials. *J Biomed Mater Res* 25: 1495-1505, 1991.

162. **Strocchi R, De P, V, Guizzardi S, Govoni P, Facchini A, Raspanti M, Girolami M and Giannini S**. Human Achilles tendon: morphological and morphometric variations as a function of age. *Foot Ankle* 12: 100-104, 1991.

163. **Svensson RB, Hassenkam T, Hansen P and Magnusson SP**. Viscoelastic behavior of discrete human collagen fibrils. *J Mech Behav Biomed Mater* 2009.

164. **Tallon C, Maffulli N and Ewen SW**. Ruptured Achilles tendons are significantly more degenerated than tendinopathic tendons. *Med Sci Sports Exerc* 33: 1983-1990, 2001.

165. **van der Rijt JA, van der Werf KO, Bennink ML, Dijkstra PJ and Feijen J**. Micromechanical testing of individual collagen fibrils. *Macromol Biosci* 6: 697-702, 2006.

166. **Viguet-Carrin S, Garnero P and Delmas PD**. The role of collagen in bone strength. *Osteoporos Int* 17: 319-336, 2006.

167. **Viguet-Carrin S, Roux JP, Arlot ME, Merabet Z, Leeming DJ, Byrjalsen I, Delmas PD and Bouxsein ML**. Contribution of the advanced glycation end product pentosidine and of maturation of type I collagen to compressive biomechanical properties of human lumbar vertebrae. *Bone* 39: 1073-1079, 2006.

168. **Vinckier A and Semenza G**. Measuring elasticity of biological materials by atomic force microscopy. *FEBS Lett* 430: 12-16, 1998.

169. **Wang JH**. Mechanobiology of tendon. *J Biomech* 39: 1563-1582, 2006.

170. Wenger, P. E., Bozec, L., Horton, M., and Mesquida, P. Mechanical Properties of Collagen Fibrils. Biophys.J 93, 1255-1263. 2007.
Ref Type: Generic

171. **Westh E, Kongsgaard M, Bojsen-Moller J, Aagaard P, Hansen M, Kjaer M and Magnusson SP**. Effect of habitual exercise on the structural and mechanical properties of human tendon, in vivo, in men and women. *Scand J Med Sci Sports* 2007.

172. **Woo SL, Gomez MA, Woo YK and Akeson WH**. Mechanical properties of tendons and ligaments. II. The relationships of immobilization and exercise on tissue remodeling. *Biorheology* 19: 397-408, 1982.

173. **Wren TA, Yerby SA, Beaupre GS and Carter DR**. Mechanical properties of the human achilles tendon. *Clin Biomech (Bristol , Avon )* 16: 245-251, 2001.

174. **Wu JZ, Brumfield A, Miller GR, Metheny R and Cutlip RG**. Comparison of mechanical properties of rat tibialis anterior tendon evaluated using two different approaches. *Biomed Mater Eng* 14: 13-22, 2004.

175. **Yamamoto E, Hayashi K and Yamamoto N**. Mechanical properties of collagen fascicles from the rabbit patellar tendon. *J Biomech Eng* 121: 124-131, 1999.

176. **Yang G, Crawford RC and Wang JH**. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. *J Biomech* 37: 1543-1550, 2004.

177. **Yang J and Shao Z**. Recent advances in biological atomic force microscopy. *Micron* 26: 35-49, 1995.

178. **Yang L, van der Werf KO, Koopman BF, Subramaniam V, Bennink ML, Dijkstra PJ and Feijen J**. Micromechanical bending of single collagen fibrils using atomic force microscopy. *J Biomed Mater Res A* 82: 160-168, 2007.

179. **Zernicke RF, Garhammer J and Jobe FW**. Human patellar-tendon rupture. *J Bone Joint Surg Am* 59: 179-183, 1977.

180. **Zhang G, Ezura Y, Chervoneva I, Robinson PS, Beason DP, Carine ET, Soslowsky LJ, Iozzo RV and Birk DE**. Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development. *J Cell Biochem* 98: 1436-1449, 2006.

# 13. Appendix Study I

*Connective Tissue Research*, 50:211–222, 2009

ISSN: 0300-8207 print / 1607-8438 online
DOI: 10.1080/03008200802610040

informa
healthcare

# Glutaraldehyde Cross-Linking of Tendon—Mechanical Effects at the Level of the Tendon Fascicle and Fibril

**Philip Hansen**

*Institute of Sports Medicine Copenhagen, Bispebjerg Hospital & Centre for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark*

**Tue Hassenkam and René Brüggebusch Svensson**

*Nano-Science Center, University of Copenhagen, Copenhagen, Denmark*

**Per Aagaard**

*Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Copenhagen, Denmark*

**Todd Trappe**

*Human Performance Laboratory, Ball State University, Muncie, Indiana, USA*

**Bjarki Thor Haraldsson, Michael Kjaer, and Peter Magnusson**

*Institute of Sports Medicine Copenhagen, Bispebjerg Hospital & Centre for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark*



**Conclusive insight into the microscopic principles that govern the strength of tendon and related connective tissues is lacking and the importance of collagen cross-linking has not been firmly established. The combined application of whole-tissue mechanical testing and atomic force spectroscopy allowed for a detailed characterization of the effect of cross-linking in rat-tail tendon. The cross-link inducing agent glutaraldehyde augmented the tensile strength of tendon fascicles. Stress at failure increased from ∼8 MPa to ∼39 MPa. The mechanical effects of glutaraldehyde at the tendon fibril level were examined by atomic force microscopy. Peak forces increased from ∼1379 to ∼2622 pN while an extended Hertz fit of force-indentation data showed a ∼24 fold increase in Young's modulus on indentation. The effect of glutaraldehyde cross-linking on the tensile properties of a single collagen fibril was investigated by a novel methodology based on atomic force spectroscopy. The Young's modulus of a secluded fibril increased from ∼407 MPa to ∼1.1 GPa with glutaraldehyde treatment. Collectively, the findings indicate that cross-linking at the level of the collagen fibril is of key importance for the mechanical strength of tendon tissue. However, when comparing the effects at the level of the tendon fascicle and fibril, respectively, further questions are prompted regarding the pathways of force through the tendon microstructure as fibril strength seems to surpass that of the tendon fascicle.**

**Keywords** Tendon, Connective Tissue, Collagen, Cross-Linking, Mechanical Properties, Atomic Force Microscopy

## INTRODUCTION

Understanding the structure and function of tendons remains an intricate scientific challenge with many yet unanswered questions. A primary characteristic of tendons is the great tensile strength, which is apparent as some tendons can tolerate peak loads several times the body weight (BW) of an individual during physical activity [1, 2]. The multi-composite structure of tendon allows it to withstand tremendous forces and whole-tendon biomechanics have been studied extensively. However, the specific pathways for force transmission are only incompletely understood and clarification of tendon properties at the nanometer level is required to successfully mimic the remarkable load-bearing properties of tendon and related connective tissues. This study directly compared concomitant mechanical effects of glutaraldehyde (GA) cross-linking at two structural levels of the tendon hierarchy.

Received 1 July 2008; Revised 19 October 2008; Accepted 7 November 2008.

Address correspondence to S. Peter Magnusson, Institute of Sports Medicine Copenhagen, Bispebjerg Bakke 23, DK-2400 Copenhagen, Denmark. E-mail: p.magnusson@mfi.ku.dk, www.ismc.dk

Tendons are hierarchical structures primarily composed of collagen molecules arranged in a highly ordered quarter stagger array, which gives rise to a distinct 67 nm banding periodicity (D-period) of the collagen fibril. In vivo, the collagen molecules assemble into microfibrils, fibrils, and fiber bundles [3, 4], which are embedded in a proteoglycan-rich ground substance [5]. Fiber bundles coalesce into fascicles that constitute the largest subcomponent of whole tendon, which was recently shown to be mechanically independent structures [6]. Therefore, tendon fascicles would be expected to display biomechanical behavior comparable to the whole-tendon level.

During loading tendons display an initial non linear region in the load-deformation curve (the so-called "toe region") followed by a linear region preceding tissue failure (yield) [7]. In the latter region relative slippage between neighboring collagen molecules and perhaps between neighboring tendon fibrils has been suggested [8–12]. Such slippage would imply considerable tensile forces acting on intermolecular cross-links [11] and cross-links that interconnect collagen molecules and fibrils are likely to provide additional strength to the tendon [13]. Indeed it has been shown that the absence or abnormal formation of cross-links is associated with decreased tendon strength and reduced mechanical properties in collagenous tissues [14, 15].

Nonetheless, there have been conflicting reports on the exact influence of cross-links on collagen material properties. Increased density of cross-links has been reported to confer additional tensile strength to collagen tissues [16–19] whereas other authors have found unaltered [20, 21] or reduced ability of the tissue to withstand tensile loading [22].

These findings do not provide a coherent picture of the importance of cross-linking in collagen materials. Factors other than cross-links, such as collagen concentration, fibril diameter, and additional components of the extracellular matrix, have been implicated in contributing to the mechanical properties of tendon [5, 12, 23]. Therefore, it is necessary to independently vary the density of cross-links to ascertain the relative effect on the mechanical properties of the tissue.

Glutaraldehyde (GA) is known to cause extensive cross-linking of collagen lysine and hydroxylysine sidechains thereby stabilizing the overall collagen structure [21]. Therefore, GA has been widely applied to augment collagen biomaterials [24–26]. In the present study we utilized the cross-linking ability of GA to examine the changes with increased cross-linking in rat-tail tendon at two structural levels of its hierarchy. By combining micromechanical testing and atomic force spectroscopy measurements we were able to directly compare the relative mechanical changes resulting from increased cross-linking at the collagen fibril level and at the whole-tissue fascicle level.

## MATERIALS AND METHODS

### Ethical Approval

All experiments complied with the European Convention for the Protection of Vertebrate Animals Used for Experiments or Other Scientific Purposes (ETS No. 123, Strasbourg, France, 1985).

### Preparation and Glutaraldehyde Treatment of Tendon Samples

Rat-tail tendon fascicles obtained from male Wistar rats ∼10 weeks old weighing ∼250 g were used for all mechanical experiments described below. The tails were stored at −20°C until testing. Tendon fascicles were gently teased out from the middle one-third of the tail and divided in two equal segments. Each segment pair consisted of a proximal and distal segment that were alternately assigned to 'treated' or 'control' to remove any variation secondary to heterogeneous mechanical properties along the fascicle. The control segment was immediately wrapped in gauze soaked in PBS buffer solution (0.15M, pH 7.4) and frozen at −20°C. The treated segment was incubated for 24 hr in glutaraldehyde (25% in aqueous solution for electron microscopy, Merck KGaA, Darmstadt, Germany) diluted with distilled water to a concentration of 0.5% at 4°C without any constraints [27]. Subsequently, the treated segments were washed in copious amounts of PBS (0.15 M, pH 7.4), wrapped in PBS soaked gauze (0.15 M, pH 7.4) and frozen at −20°C. The treatment was considered to induce an adequate number of cross-links to sufficiently evaluate the effect of increased cross-linking [27, 28].

### Mechanical Testing and Imaging Experiments

*Mechanical Testing*

The four mechanical methodologies used are illustrated in Figure 1.

Atomic force microscopy (AFM) investigations were performed using a Veeco MultiMode microscope (Santa Barbara, CA, USA) with a Nanoscope IIIa controller whereas data were collected using Veeco Nanoscope v612r1 software.

*Experiment 1: Force Spectroscopy*

From tail tendon fascicles of 1 Wistar rat 3 treated and 3 control sample pairs were prepared for force spectroscopy. Each pair derived from the same fascicle. The treated sample underwent the GA treatment described above. Subsequently, small fascicle segments (∼3–5 mm) were prepared on a metal disc that was cleaned several times in washing detergent and thoroughly rinsed in milli-Q water (18MΩ). While the fascicle segment was maintained moist, the middle portion was gently spread out on the metal disk by sharp tweezers to yield a central thinned rhomboid area extensively covered by collagen fibrils, which was subsequently subjected to force spectroscopy measurements. The tissue surrounding the thinned area was glued onto the disk by a commercial two-part slow hardening epoxy glue (Danalim, Epoxy Universal 335, Koege, Denmark). The sample was then mounted in the AFM using a liquid cell setup and allowed to equilibrate for at least 1 hr in a buffer

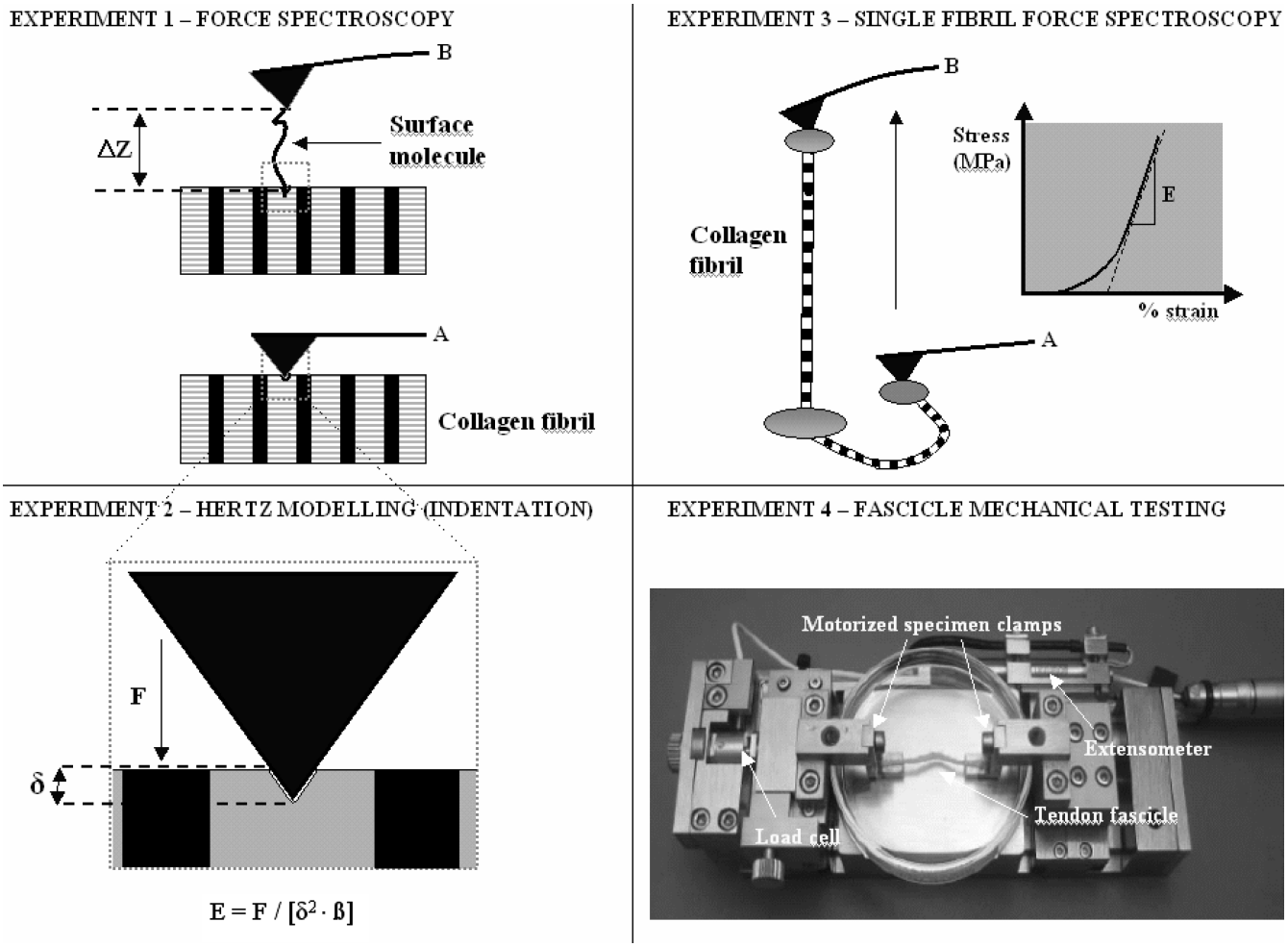



FIG. 1. Illustration of the mechanical experiments (sizes of the cantilever and fibril are not to scale). *Experiment 1*: Force spectroscopy of collagen fibrils. The cantilever is indented into the fibril (position A) and subsequently retracted (position B). On retraction one or more molecules of the collagen fibril surface can randomly adhere to the tip. The adhering molecule is pulled to a given pulling length while force is recorded. The size of the molecule is exaggerated for clarity. *Experiment 2*: During force spectroscopy the tip is indented into the fibrillar surface (position A, experiment 1) at a given indentation depth $\delta$. The Young's modulus E on indentation can then be deduced. *Experiment 3*: Single fibril force spectroscopy. An isolated collagen fibril secluded from other fibrils is selected. The fibril is glued to the sample and to the cantilever tip. In position A the fibril is relaxed. In position B the cantilever has extended and elongated the fibril, which leads to cantilever deflection. By combining force-extension data and dimensional data obtained from tapping mode images of the fibril, the Young's modulus 'E' is obtained. *Experiment 4*: Tensile stage for mechanical testing of fascicles. The fascicle is glued to motorized clamps, which extend the fascicle. Force-elongation data are recorded real-time during elongation. The fascicle dimensions are obtained from video images of the mounted fascicle to allow for stress-strain calculations.



containing 150 mM NaCl, 2 mM Tris adjusted to a pH of 7.4 at room temperature (26°C) [29].

The force spectroscopy measurements were performed with a Veeco MultiMode microscope mounted with a PicoForce scanner using a liquid cell setup. Commercially available cantilevers were used (Biolever BL-RC150VB), Olympus, Tokyo, Japan). Thermal calibration of the cantilevers used was performed and yielded spring constants between 0.030–0.036 N/m (manufacturer specification: 0.030 N/m). Once centred above the central fibril covered area the cantilever was ramped continuously up and down with a ramp size of 1 $\mu$m and a pulling rate of 2 $\mu$m·s$^{-1}$ (200%·s$^{-1}$). The pressure by which the cantilever tip was inserted into the sample could not be maintained exactly equal between each ramp cycle due to a slight thermal drift inherent in the AFM system. Therefore, the pressure was manually adjusted to maintain and indentation force of ~500–1000 pN by use a force-feedback 'pico-angler' controller. The indentation into the sample was maintained for 10 s before retraction of the cantilever. For each sample 30 force spectroscopy curves were obtained at three separate sample locations which were randomly chosen. In total 90 force curves were obtained per sample.

*Experiment 2: Extended Hertz Modelling*

To aid the interpretation of retraction data, the elastic (indentation) properties of the samples were evaluated by

applying an extended Hertz fit to force curve (approach curves) obtained during the force spectroscopy trials in experiment 1. The Hertz model estimates the elastic properties (Young's modulus) when pushing a spherical or cone-shaped object into a flat sample which is equivalent to indenting the AFM tip into the sample surface [30]. A cone shape of the AFM tip was assumed with a tip radius of 30 nm (manufacturer specifications). Collagen samples were expected to behave in an elastic manner. Other researches have made similar assumptions [31, 32], but some caution is called for since the native structure of a fibril is not likely to resemble the structure of an ideally elastic material. As such, some error may be introduced to the Young's moduli obtained by this method. Nonetheless, the applicability of the Hertzian model to study the indentation characteristics of collagen type I fibrils of rat tail tendon and sea cucumber has previously been demonstrated [31, 32].

The extended Hertz fit was performed using a custom made Labview ® routine with a graphical interface that superimposes the Hertzian fit onto the data of each individual approach curve. The program is semi automated since the user defines the baseline force value whereupon the routine calculates the Young's modulus on indentation. The underlying algorithm is based on the equation:

$$E = F/[\delta^2 \cdot ß]$$

where E = Young's modulus (MPa); F = indentation force (N); $\delta$ = indentation depth (m).

The factor ß equals:

$$2 \cdot \tan\alpha/[\pi(1 - \nu^2)]$$

where $\alpha$ is the half opening angle of the indenting cone; $\nu$ is the Poisson's ratio [30].

We chose a Poissons's ratio of 0.3 for the analysis [32].

*Experiment 3: Single Fibril Force Spectroscopy*

To examine cross-linking effects on a single collagen fibril an experimental approach comparable to the method presented by van der Rijt et al. [33] was applied. An Olympus OMCL-TR800-PSA cantilever (nominal spring constant 0.57 N/m) was used for force spectroscopy. A sample was prepared in milli-Q water (18 MΩ) on a $SiO_2$ substrate by cutting ~1 mm of a rat tail tendon fascicle and gently sheering it with pincers across the substrate. The sample was then dried under ambient conditions, rinsed in milli-Q water, and left to dry again. A fibril easily accessible with the AFM cantilever was selected with no specific attention to size. The dry secluded fibril was imaged by AFM in tapping mode to determine the cross-sectional area and ensure the integrity of the fibril (see imaging section below). The cross-sectional area was defined as the integrated area of the fibril cross-section obtained from tapping mode images.

Using a two-component epoxy glue (Danalim, Epoxy Universal 335, Koege, Denmark) a small droplet was transferred to the substrate in the vicinity of the secluded fibril. The AFM cantilever was dipped into the glue and used to deposit a minute amount onto the substrate ~ 25 $\mu$m from the fibril end. Additional glue was adsorbed to the cantilever and set down covering ~ 10 $\mu$m of the fibril end. The glue was allowed to cure for ~20 hours. Subsequently, a PBS buffer solution (0.15 M) was introduced and left for 45 min to hydrate the fibril. The glued cantilever-fibril complex was then released by moving the cantilever a few $\mu$m horizontally toward the first glue droplet. To bring the fibril into a vertical position and center the tip of the cantilever above the anchoring point on the sample surface, the tip-to-surface distance was increased using the piezo until a slight force was detectable. The tip was then moved in a certain direction that reduced the cantilever deflection. This procedure was repeated until movement of the tip in any direction would increase the force.

After positioning the tip, fibril length was determined by retraction of the cantilever from substrate surface until a 5 nm downward deflection was detected. Force measurements were done by slowly increasing the tip-to-surface distance to ~130% of the fibril length, and then running cyclic ramps from this position to the surface at 1 Hz. Due to the magnitude of forces measured, the deflection would fall outside the optical detector window with continued cantilever retraction. Therefore, it was necessary to stitch together the entire force-extension curve from several curve segments obtained by adjusting the detector window between measurements. To ensure that curve segments were consecutive, the fibril was preconditioned by ramping the cantilever continuously at 1 Hz until no stress-relaxation phenomena were observed. After ~10 ramp cycles force curves remained stabile.

After force-extension measurements on the untreated fibril the PBS solution was removed with a small piece of filter paper and a solution of 0.5% GA in demineralized water was introduced for 1 hr. The liquid cell was rinsed three times with PBS (0.15 M) by alternate renewal and removal of the solution with filter paper. The measurements described above were repeated to obtain force-extension data on the GA-treated fibril. Following the experimental procedure the cantilever spring constant was determined with a thermal tune, using a Lorentzian curve fit.

*Experiment 4: Mechanical Testing of Fascicles*

A total of 6 ($n = 6$) treated and 6 control ($n = 6$) segments were used for fascicle mechanical testing. The method used for determination of mechanical properties of the fascicles has been described in detail elsewhere [34]. Briefly, approximately 5 mm of each fascicle segment end was allowed to air dry at room temperature while the mid substance of the specimen was kept moistened in PBS-soaked gauze. The dried ends were then glued to aluminium plates of a mechanical tensile testing device with cyanoacrylate glue. The glue was allowed to cure for ~15 min.

Subsequently, the fascicle was submerged in PBS buffer and allowed to equilibrate for 15 min before testing to failure

at a fixed extension rate of 0.07 mm·s$^{-1}$ which corresponded to a strain rate of ~0.40%·s$^{-1}$. Force-displacement data were sampled at 20 Hz and synchronized video imaging of the fascicle sampled at 15 Hz was obtained during testing for subsequent analysis. Video images obtained at the onset of force were used to determine fascicle diameter, which allowed fascicle cross-sectional area to be calculated.

## Imaging

*Single Fibril Tapping Mode Imaging*

For all tapping mode imaging a J-type scanner was used. For tapping mode imaging in the dry state Olympus OMCL-AC160TS cantilevers (nominal resonance frequency 300 kHz; spring constant 42 N/m) were used. For wet tapping mode imaging, an OMCL-TR800-PSA chip housing 2 cantilevers with nominal spring constants of 0.15 N/m and 0.57 N/m, respectively, was used running a liquid cell setup. The former cantilever was used for soft tapping mode imaging in liquid while the latter was used for hard tapping mode imaging in liquid.

Secluded fibrils, which were easily identifiable by characteristic landmarks e.g., crossing of two single fibrils, were chosen to examine fibril morphology in both dry and hydrated conditions. By switching to a liquid cell setup the same fibril was identified and imaged in the wet state. The sample was hydrated in PBS (0.15 M) for a minimum of 30 min before imaging. Tapping mode images of these fibrils were used for measurements of D-band periodicity and fibril dimensions.

## Data Reduction and Analysis

*Fascicle Mechanical Testing*

The average of three measures along the length of the fascicle was used to calculate the cross-section, assuming the fascicle was circular. For the mechanical testing of fascicles, the cross-sectional area was used to calculate stress ($\sigma = \mathrm{N/m^2}$), and plate-to-plate displacement was used to calculate strain ($\varepsilon = (l_1\text{-}l_0)/l_0$). As described previously [34], least-squares linear regression analysis was performed on the curve portion with a tangential slope (i.e., point-to-point slope) of 85.5–100% of the maximum tangential slope, which yielded a correlation coefficient that exceeded $r = 0.99$ ($p < 0.001$) in all instances.

*Yield stress* was identified at the instant when the difference between recorded force and the extrapolated linear regression line exceeded 0.5 MPa. This point was considered to be the first indication of structural failure of the fascicle. The linear curve phase never overlapped the yield phase, i.e. in all cases the yield point occurred outside (rightward to) the linear regression interval. The tangent modulus (E, $\sigma/\varepsilon$) was calculated as the mean of three separate measurements in the linear portion of the stress-strain curve. *Cumulated energy* was defined as the area under the force-elongation curve ($\int F \cdot dl$; F = strain force; l = elongation).

*Force spectroscopy and extended Hertz modelling*

Pooled results for the 3 treated and 3 control samples prepared from tail tendon fascicles of 1 Wistar rat are reported. Each sample was probed 90 times, i.e., a total of 270 pulling curves for the treated and control samples, respectively. *Peak force* was the greatest force recorded during the entire pull. Baseline force was considered to correspond to the horizontal portion of the force-extension curve. In some ramp cycles pull-off of the probed structure for the tip did not occur within the chosen ramp size. In cases where tip-sample pull-off did not occur—i.e., the force never returned to baseline—the pulling length was set to ~1 $\mu$m (corresponding to the maximum pulling length that could be recorded within the predefined travel length of the microscope).

Force spectroscopy trials on control samples resulted in nonhomogeneous complex force curves. The complexity of the curves precluded reasonable application of a worm-like chain (WLC) model or similar curve fit. The *ratio of peak force divided by pulling length at peak force* (pN · nm$^{-1}$) served as a 'surrogate' measure of stiffness of the probed molecular structures of the sample. This ratio is expected to be rather insensitive to variations in measurements e.g., due to variations in tip-sample adhesion forces resulting from tip contamination. Such variations could influence the magnitude of peak forces and pulling length. However, variations in tip-sample adhesion are not expected to influence the force-to-length relation (stiffness) to the same extent. Therefore we consider this ratio more robust than the isolated force and pulling length values. A D'Agostino and Pearson omnibus normality test revealed no normality for any of the pooled data from force spectroscopy and Hertz modelling. Therefore, a Mann-Whitney test was applied for all force spectroscopy data.

For fascicle mechanical testing data, two-way paired Student's *t*-tests were applied to examine the effect of the glutaraldehyde treatment. No outliers were removed for any of the above experiments. All results are reported as mean ± SD.

# RESULTS

## Force Spectroscopy

Data are presented in Table 1. Treated fascicles displayed greater peak force and decreased pulling length. Also the length at which peak force was reached decreased with cross-linking which resulted in an increased peak-force-to-length ratio. The cumulated energy for the entire pulling length was increased by treatment. In 1 sample peak forces were slightly lower in the treated sample (1072 pN vs. 1340 pN; $p < 0.01$). Thus, GA treatment did not seem to influence tip-sample adhesive forces per se. Importantly, GA treatment yielded a striking difference in force curve morphology (Figure 2). Typically control samples yielded complex curves that resembled those reported by other groups [29, 35] with considerable variation in

TABLE 1
Force spectroscopy

| | Untreated | Treated | $p$ | Percent change |
|---|---|---|---|---|
| Peak force (pN) | $1379 \pm 596$ | $2622 \pm 1441$ | $< 0.0001$ | $+90$ |
| Pulling length (nm) | $758 \pm 221$ | $264 \pm 179$ | $< 0.0001$ | $-65$ |
| Cumulated energy (pJ $\cdot$ $10^{-6}$) | $503 \pm 618$ | $556.3 \pm 430$ | $< 0.0001$ | $+11$ |
| Peak force:length ratio (pN $\cdot$ nm$^{-1}$) | $6.3 \pm 2.7$ | $14.9 \pm 4.2$ | $< 0.0001$ | $+137$ |

Values are means $\pm$ *SD* ($n = 270$).

morphology between individual pulls and multiple force drops, possibly corresponding to sequential breakage of inter- and intramolecular bonds. Treated samples presented much simpler curves with only few drops in force and very little variation between consecutive pulls.

## Extended Hertz Modelling

In GA-treated samples the Young's modulus extracted from the indentation data was markedly greater compared to untreated samples (Table 2). In some force curves in the treated samples the small magnitude of indentation approached the limit of detection with very small indentation depths (2–3 nm) corresponding to a Young's modulus of roughly 5 GPa. Lesser indentation depths were masked by the inherent background noise of the AFM system. The upper Young's modulus that could be extracted from the indentation data was therefore ~5 GPa.

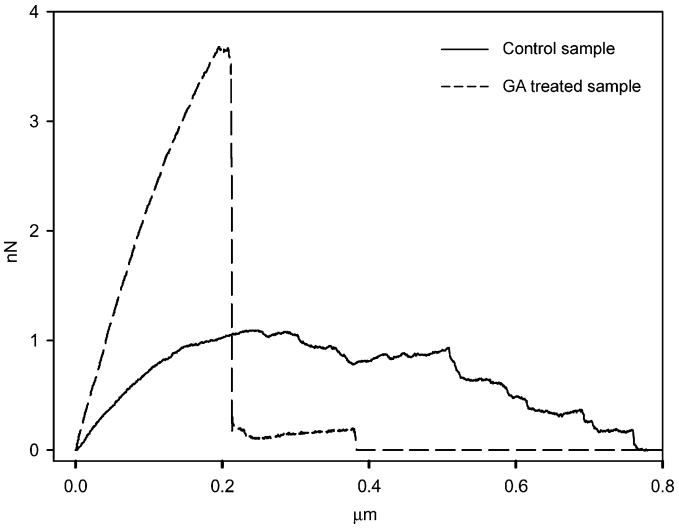



FIG. 2. Force-extension curves from a single force spectroscopy pull illustrate the typical curve morphology observed during force spectroscopy trials. The micromechanical differences between GA cross-linked (dashed line) and untreated samples were apparent with treated samples displaying only few bond ruptures. Peak forces were increased with GA treatment from ~1379 pN to ~2622 pN and total pulling length decreased from ~758 nm to ~264 nm.

## Single Fibril Force Spectroscopy

By single fibril force spectroscopy GA treatment increased Young's modulus considerably (Table 3). The fibril chosen for force spectroscopy measured 13 $\mu$m with a cross-section of 14700 nm$^2$. The greatest common stress was ~60 MPa for the control and the treated fibril, respectively. At this level of stress the corresponding strain was decreased in the treated fibril (~16% vs. ~29%) (Figure 3).

## Fascicle Tensile Testing

Table 4 displays data for the mechanical testing of glutaraldehyde-treated fascicles. Treated fascicles showed increased Young's modulus, yield strain, and yield stress. The cumulated energy (area under the curve) to yield was also increased in treated fascicles. A difference in curve morphology was noted. All control samples presented a classical force-elongation relation in the "toe" and "linear" region. However, a marked "plateau" in force between yield and rupture points was noticed, where force remained fairly constant despite a considerable increases in strain (Figure 4). Following the plateau region tissue failure occurred with gradually decreasing force. In GA treated samples no plateauing was observed. Failure was preceded by a normal toe and linear region.

## Tapping Mode Imaging

Interestingly, the hydrated fibril did not reveal its D-banding in *soft* tapping mode images. *Hard* tapping mode images revealed no apparent differences in D-periodicity (~69–70 nm) between treated and control samples regardless of hydration state. Hydration increased fibril height and width dimensions comparably in both untreated and GA cross-linked fibrils (Figure 5 A–D).

TABLE 2
Extended Hertz modelling

| | Untreated | Treated | $p$ | Percent change |
|---|---|---|---|---|
| Young's modulus (MPa) | $0.1 \pm 0.1$ | $2.4 \pm 3.3$ | $< 0.0001$ | $+2300$ |

Values are means $\pm$ *SD* ($n = 270$).

TABLE 3
Single fibril force spectroscopy

| | Untreated | Treated | $p$ | Percent change |
|---|---|---|---|---|
| Young's modulus (GPa) | 0.4 | 1.1 | - | +170 |

Values are absolute ($n = 1$).

TABLE 4
Fascicle mechanical testing

| | Untreated | Treated | $p$ | Percent change |
|---|---|---|---|---|
| Yield stress (MPa) | $8.1 \pm 1.9$ | $39.3 \pm 16.0$ | $< 0.01$ | +385 |
| Yield strain (%) | $3.7 \pm 0.4$ | $10.6 \pm 2.5$ | $< 0.01$ | +187 |
| Cumulated E (mJ) | $0.4 \pm 0.2$ | $3.9 \pm 1.9$ | $< 0.05$ | +875 |
| Young's modulus (MPa) | $329.2 \pm 62$ | $489 \pm 137$ | $< 0.01$ | +49 |

Values are means $\pm$ *SD* ($n = 6$).

## DISCUSSION

We have found that GA cross-linking of rat-tail tendon can induce considerable increases in the tensile strength of tendon fascicles. AFM measurements revealed concomitant micromechanical changes occurring at the level of the tendon fibril, which suggests that whole-tissue strength in tendon depends on cross-linking of collagen fibrils. In the following the findings of the present study are discussed in further detail.

To augment the integrity of collagen biomaterials, GA cross-linking has been widely applied [21, 36]. The biochemical reaction with collagen is diverse, but the primary reaction is between GA and free lysine and hydroxylysine $\varepsilon$-amino groups to form both intra- and intermolecular covalent cross-links [24, 25]. Head-to-side cross-linking that span between neighboring microfibrils in parallel alignment have been expected to increase tissue strength as opposed to immature native head-to-tail cross-links [13]. GA cross-links can span between collagen molecules on separate microfibrils and perhaps between fibrils [21, 37]. Indeed, GA treatment of collagenous heart valve biomaterial has been associated with augmented mechanical properties such as increased strain at failure, decreased stress relaxation, increased toughness (area under the stress-strain curve), and increased tensile strength [37]. Curiously, tensile strength was not augmented by GA in all reports that examined the effect on heart pericardial tissue [20, 38, 39], and one study even reported a decrease in strength with GA treatment [22].

Similarly, in dermal sheep collagen Damink and co-workers [21] found no strengthening effect of GA per se. In the latter study the authors proposed that the distance between unstrained collagen fibres exceeded the sterical cross-bridging ability of GA, which allowed for slippage between collagen fibres. However, an increase in tensile strength was observed when strain was applied to the tissue combined with GA. The authors speculated that pre straining the tissue brought collagen fibres in closer approximation, which allowed for the formation of *inter fiber* GA cross-links that resisted fiber slippage. Thus, subtle ultrastructural differences between various collagen tissues

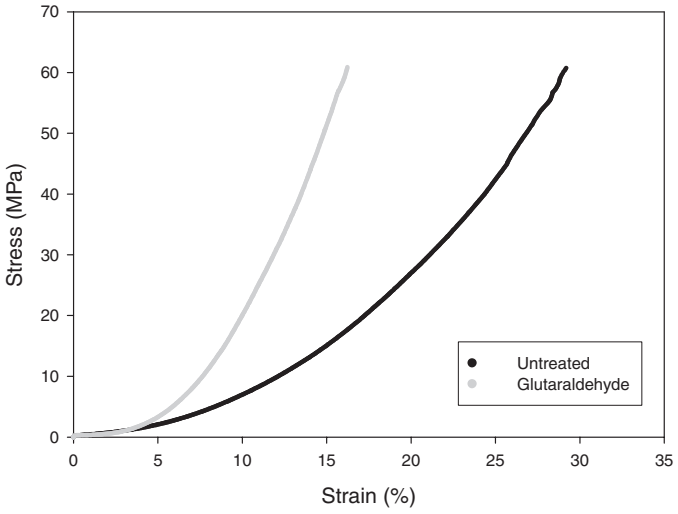



FIG. 3. Force-elongation curves obtained from micromechanical testing of a single isolated collagen fibril by atomic force spectroscopy before and after GA treatment. GA cross-linking increased the Young's modulus of the fibril. Within the applied force range no indications of structural failure were noted from the force-extension curves obtained.

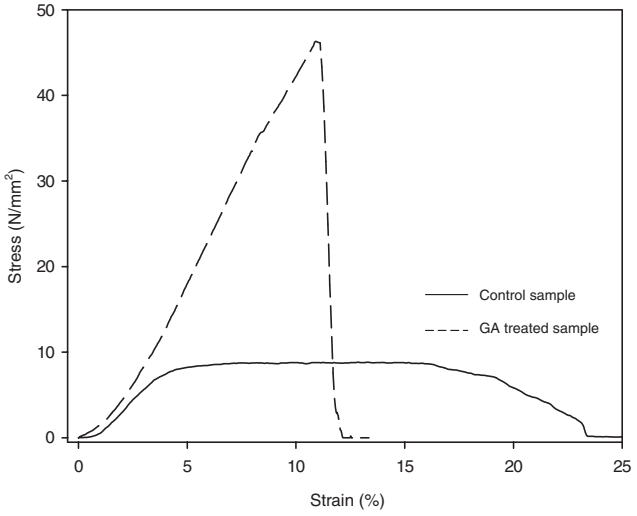



FIG. 4. Comparison of typical stress-strain curves from whole-tissue tensile testing of an untreated and a GA treated (dashed line) tendon fascicle. Note the markedly increased stress at failure resulting from GA treatment illustrating the importance of collagen cross-linking in providing increased tensile strength in tendon tissue.

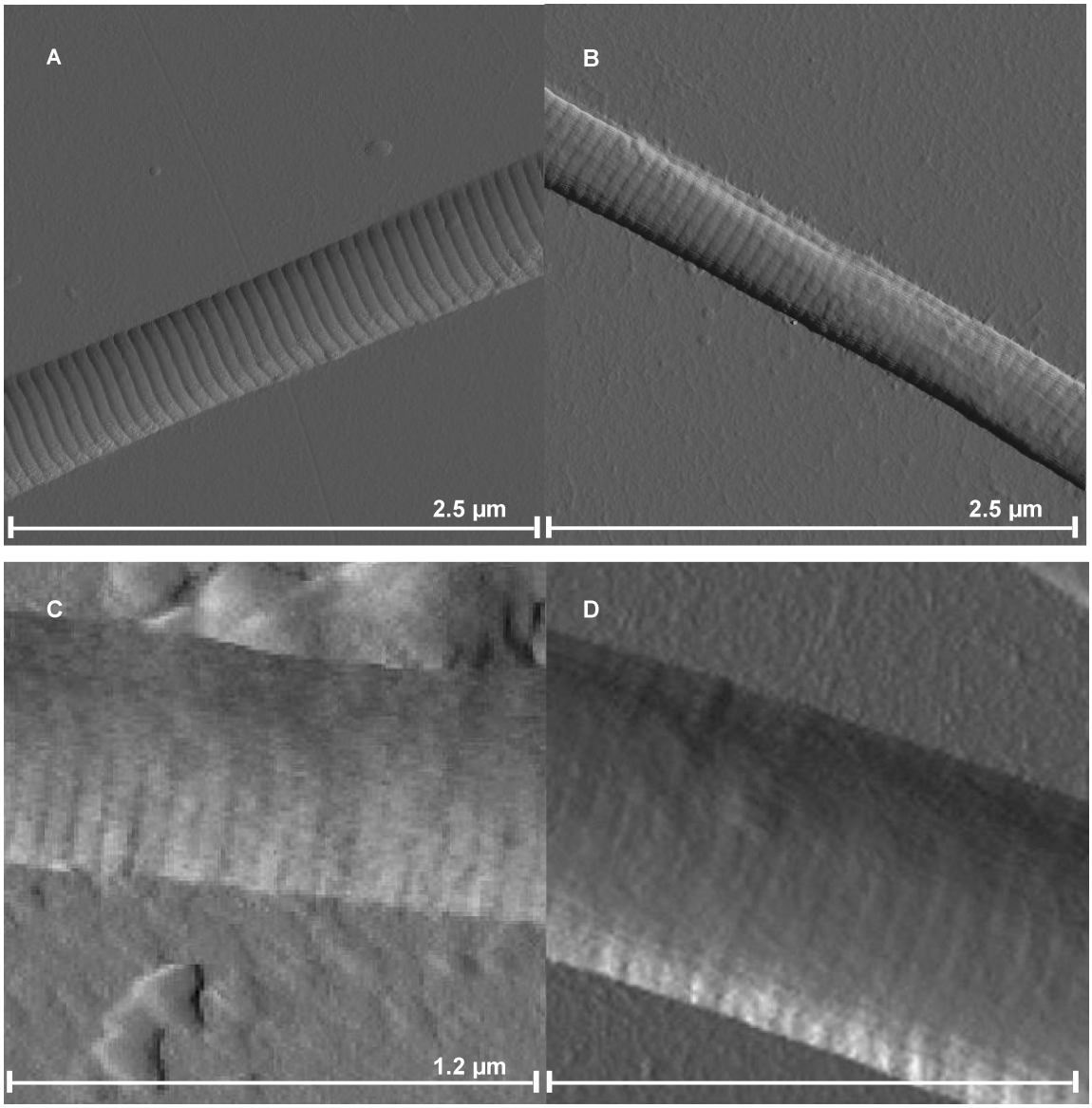



FIG. 5. Tapping mode images of isolated fibrils in dry ambient conditions (A: untreated; B: treated) and in a hydrated state (PBS, 0.15M) (C: untreated; D: treated). No apparent structural changes occurred with GA treatment and D-periodicity was unchanged with treatment under both dry and hydrated conditions. In the hydrated state D-periodicity was only apparent at high cantilever pressures. Both untreated and treated fibril dimensions increased with hydration in PBS solution.



could evoke very different mechanical effects of cross-linking as could different cross-linking agents due to variations in the spanning width of the agent [40].

The effect of exogenous cross-linking in tendon tissue has been scarcely studied. Cross-linking by means of non enzymatic glycation has been reported to increase stiffness and tensile strength in rat-tail and rabbit Achilles tendon [17, 41]. The effect of GA treatment on bovine whole-tendon mechanical properties has been reported to increase stiffness slightly while tendon strength was considerably increased [42], which is in agrees with our findings lending support to our belief that the properties of tendon fascicles closely reflect whole-tendon properties. To the best of our knowledge, a comparative study on the mechanical effects of cross-linking different levels of the tendon hierarchy has not been conducted previously.

In the present study, GA treatment did not result in apparent changes in the fibril ultrastructure as evaluated by AFM tapping mode imaging (Fig. 5A–D). Thus, the basic collagen quarter stagger arrangement seemed unaffected by cross-linking, yet there was a considerable augmenting effect of GA treatment on the tensile strength of the tendon fascicles paralleled by marked mechanical alterations at the level of the fibril. AFM measurements revealed large relative changes in peak force, pulling length and peak-force-to-length ratio.

These changes are likely to relate closely to the strengthening effect observed at the whole-tissue level. Since the applied methodologies for mechanical testing did not allow for similar strain rates for the two structural levels, a direct comparison of mechanical parameters in absolute terms may be problematic since strain rate-dependent viscoelastic properties may come into play. Therefore, in the following we primarily focus on the relative changes resulting from cross-linking for each structural level.

The effect of cross-linking was obvious in force-extension curves obtained by conventional force spectroscopy. In untreated samples, highly heterogeneous force curves with multiple rupture events with no reproducible periodicity between ruptures and quite slow decrements in force were observed. GA treatment produced much simpler curve morphology with only few ruptures before sudden 'pull-off' occurred (Figure 2). Thus, GA seemed to 'strengthen' surface collagen lattice of the fibrils covering the sample surface. Cross-linking seemed to prevent fibril surface disruption and breakage of molecular bonds. This difference was well reflected by the ratio of peak force to length, which can be considered a 'surrogate' measure of stiffness for the probed molecular structures. This parameter was increased in all treated samples, i.e., for any given force the molecular structures of the fibril surface could not be extended to same degree as in untreated. A 65% reduction in pulling length was observed following GA treatment.

Albeit speculative, these phenomenona could relate to the fascicle level since similarities between force-extension curves at the two different structural levels were apparent—i.e., the larger pulling length and gradual force decrements in untreated samples could relate to the force plateau and gradual failure observed in untreated fascicles (Figures 2 and 4). The observed force plateau for untreated fascicles is likely related to the relatively young age of the rats used for experiments. As the rats were juvenile they are likely to have had an abundance of head-to-tail immature cross-links of inferior mechanical strength [13]. Interestingly, the force plateau was abolished by GA treatment indicating the introduction cross-links spanning between neighboring microfibrils in parallel alignment.

Similarly, increases in peak forces with GA treatment observed by AFM could be closely coupled to the strengthening of the fascicle, although the relative increase in fascicle yield stress seemed to exceed increases in peak forces. However, it must be kept in mind that 'pull-off' in treated samples does not necessarily reflect rupture of the molecular structures of the fibril surface, but could result from tip-sample detachment. Therefore, the observed increase in peak forces with GA treatment is likely to underestimate a strengthening effect on the fibril surface. This assumption is supported by results obtained from extended Hertz fitting of indentation data since GA treatment increased Young's modulus on indentation markedly (Table 2). These data indicate that GA treatment reduced the degrees of freedom for collagen molecules to displace inside the fibril as a likely effect of extensive intermolecular cross-linking.

A recent study points to the fibril as the primary load-bearing unit of tendon. The study demonstrated that fibrils ran seemingly uninterrupted across the entire length of the examined tendons and ligaments. Hence, the integrity of the fibril becomes crucial for whole-tissue tensile strength [43]. Since conventional force spectroscopy only indirectly describes the tensile properties of the molecular structures of the fibril surface with cantilever probing forces acting in a perpendicular direction to the fibril, this methodology does not necessarily reflect the longitudinal tensile-loading behavior of the fibril. Therefore, we applied a novel AFM methodology that allows for true tensile testing of individual fibrils [33]. By this technique we were able to assess the mechanical properties of a single isolated collagen fibril and the effect of GA cross-linking.

It is noteworthy that the shape of the stress-strain curves obtained by this method closely resembles that of the whole fascicle, i.e., with an initial toe and a subsequent linear region (Figure 3). If uninterrupted tendon fibrils are primary transmitters of tensile force, the mechanical behavior of the untreated fibril, as well as changes occurring with GA cross-linking of the fibril, should resemble the changes observed by mechanical testing of the fascicle. Interestingly, the Young's modulus of the untreated fibril determined from single fibril force spectroscopy was comparable to the average Young's moduli of untreated fascicles (407 MPa vs. 329 MPa) suggesting a close link between the two structural levels. However, GA treatment increased the fibril Young's modulus by $\sim$170%, which exceeded the relative increase at the fascicle level ($\sim$50%). We also attained strain values in both the untreated ($\sim$29%) and the treated fibril ($\sim$16%), which were in excess of yield strain values observed at the fascicle level ($\sim$4% and $\sim$11%, respectively).

These discrepancies are not readily reconciled. However, we may have overestimated the magnitude of fibril strain since part of the apparent toe region is likely derived from bending of the fibril. Since the single fibril was glued to the substrate surface in horizontal position, we speculate that the transition to a vertical position would require some amount of force to straighten the fibril tightly around the edge of the glue with a corresponding deflection of the cantilever but only negligible elongation of the fibril. This would result in overestimation of tensile fibril strain to an uncertain extent. Such a phenomenon would also have influenced the data of van der Rijt et al. [33] However, these authors examined much longer fibrils (100–200 $\mu$m) and therefore overestimation of relative fibril strain would be greater in the present study. Also, we attained a greater level of fibril stress in the hydrated state ($\sim$60 MPa) than van der Rijt et al. [33] ($\sim$15 MPa), which would explain the greater strain values.

Interestingly we found the fibril to behave in a reversible manner (i.e., without signs of permanent deformation) at stress levels exceeding 20 MPa, which is contrary to van der Rijt et al., [33] who reported permanent deformation of the fibril at stress levels above 20 MPa. At stresses below 15 MPa van der

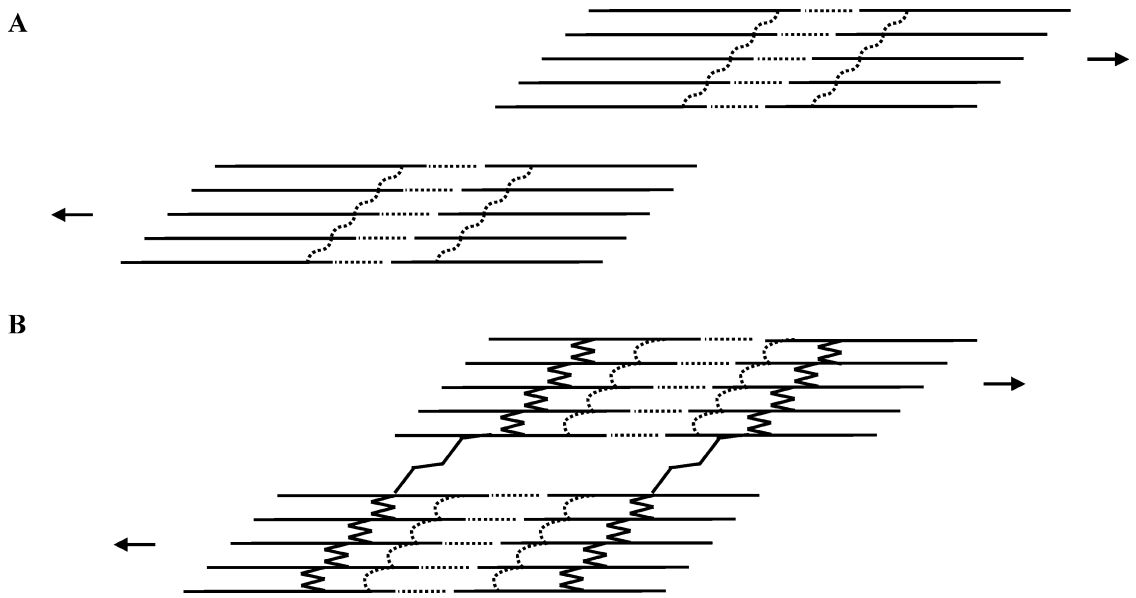



FIG. 6. Illustration of possible gliding mechanisms between two neighboring microfibrils. Tensile loading leads to marked slippage between the microfibrils (A). GA induces cross-links between neighboring microfibrils to resist intermolecular slippage (B).



Rijt et al. observed no rupture events, which agrees with our findings. Importantly, by comparing the average failure stress of the fascicle it would seem that the single fibril possesses the greater relative strength since no failure was observed at ∼60 MPa, while untreated fascicles failed at ∼8 MPa. Additionally, it would seem GA treatment had a larger relative impact on elastic properties of the fibril compared to the fascicle. The reason for such differences is unclear, but one possibility is that GA induced a greater density or a more homogenous distribution of cross-links within the single fibril as compared to the fascicle. It has previously been demonstrated that GA may polymerize on the surface of a collagen fiber and hinder further penetration of GA into the fiber [44]. Such a mechanism could surely contribute to the observed discrepancies and lead to a lesser effect of treatment for the fascicle due to its greater size. The degree of polymerization may be affected by variations in pH. Since pH was not strictly controlled for during the cross-linking procedure, differences in polymerization may have introduced variations in mechanical effects of GA since the steric reach of GA cross-links will increase with polymerization.

Differences in the relative tensile strength at the two structural levels were pronounced in the untreated fascicle. The discrepancy was reduced but not abolished by GA treatment, which increased the stress at failure to ∼39 MPa in treated fascicles. Failure of the fascicle may occur through slippage between fibrils in the untreated fascicle with interfibrillar GA cross-links resisting such slippage as proposed by Damink et al. (Figure 6 A and B) [21]. Slippage as a failure mechanism would imply that fibrils do not span the entire length of the tendon as proposed by Provenzano et al. [43]. Interruption of fibrils along the fascicle would also have implications for lateral force transmission occurring between fibrils, likely through proteoglycans intercalated between fibrils [12]. We cannot exclude a potential albeit unknown effect of GA on the interaction between ground substance and fibrils, which may have added to the observed changes with GA treatment. Also, we cannot not exclude that some solubilization of the tendon polysaccharide matrix may have resulted from the cross-linking procedure. This could have affected the strain rate-dependent viscous properties to some degree.

Alternatively, the overall physical properties of the fascicle could be influenced by the presence of "weak spots" along its length due to subtle regional alterations in the molecular structure of load-bearing fibrils. Assuming there is a given chance of such "weak spots" along a given continuous fibril, and that this chance scales with length, then the longer fibril is more likely to be weakened from such a phenomenon. Conversely, the shorter isolated fibril used for single fibril force spectroscopy is less likely to have weak spots since it represents a minute fraction of the length of the entire fascicle. Hence, the secluded fibril would appear stronger than its fascicle.

Collectively, the data from the AFM experiments described above indicate that much of the strengthening effect of GA treatment on the tendon fascicle level is derived from micromechanical changes occurring at the level of individual fibrils. Also cross-linking seems to be of crucial importance in providing additional strength to tendon tissue. However, when comparing the effects at the level of the tendon fascicle and fibril, respectively, further questions are prompted regarding the pathways of force through the tendon microstructure as fibril strength seems to surpass that of the tendon fascicle. Future studies should seek to clarify such discrepancies.

## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

## REFERENCES


1. Fukashiro, S., Komi, P.V., Jarvinen, M., and Miyashita, M. (1995). In vivo Achilles tendon loading during jumping in humans. *Eur. J. Appl. Physiol. Occup. Physiol.*, 71, 453–8.
2. Finni, T., Komi, P.V., and Lepola, V. (2000). In vivo human triceps surae and quadriceps femoris muscle function in a squat jump and counter movement jump. *Eur. J. Appl. Physiol.*, 83, 416–26.
3. Kastelic, J., Galeski, A., and Baer, E. (1978). The multicomposite structure of tendon. *Connect. Tissue Res.*, 6, 11–23.
4. Kannus, P. (2000). Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sports*, 10, 312–20.
5. Scott, J.E. (1991). Proteoglycan collagen interactions in connective tissues. Ultrastructural, biochemical, functional and evolutionary aspects. *Int. J. Biol. Macromol.*, 13, 157–61.
6. Haraldsson, B.T., Aagaard, P., Qvortrup, K., Bojsen-Moller, J., Krogsgaard, M., Koskinen, S., et al. (2008). Lateral force transmission between human tendon fascicles. *Matrix Biol.*, 27, 86–95.
7. Butler, D.L., Grood, E.S., Noyes, F.R., and Zernicke, R.F. (1978). Biomechanics of ligaments and tendons. *Exerc. Sport Sci. Rev.*, 6, 125–81.
8. Mosler, E., Folkhard, W., Knorzer, E., Nemetschek-Gansler, H., Nemetschek, T., and Koch, M.H. (1985). Stress-induced molecular rearrangement in tendon collagen. *J. Mol. Biol.*, 182, 589–96.
9. Sasaki, N., and Odajima, S. (1996). Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy. *J. Biomech.*, 29, 1131–6.
10. Sasaki, N., and Odajima, S. (1996). Stress-strain curve and Young's modulus of a collagen molecule as determined by the X-ray diffraction technique. *J. Biomech.*, 29, 655–8.
11. Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H., and Bernstorff, S. (1998). Fibrillar structure and mechanical properties of collagen. *J. Struct. Biol.*, 122, 119–22.
12. Puxkandl, R., Zizak, I., Paris, O., Keckes, J., Tesch, W., Bernstorff, S., et al. (2002). Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos. Trans. R Soc. Lond. B Biol. Sci.*, 357, 191–7.
13. Bailey, A.J., Paul, R.G., and Knott, L. (1998). Mechanisms of maturation and ageing of collagen. *Mech. Ageing Dev.*, 106, 1–56.
14. Misof, K., Landis, W.J., and Klaushofer, K. (1997). Collagen from the Osteogenesis Imperfect Mouse Model (OIM) Shows Reduced Resistance Against Tensile Stress. *J. Clin. Invest.*, 100, 40–5.
15. Haut, R.C. (1985). The effect of a lathyritic diet on the sensitivity of tendon to strain rate. *J. Biomech. Eng.*, 107, 166–74.
16. Andreassen, T.T., Seyer-Hansen, K., and Bailey, A.J. (1981). Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. *Biochim. Biophys. Acta.*, 677, 313–7.
17. Reddy, G.K. (2004). Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit achilles tendon. *Exp. Diabesity Res.*, 5, 143–53.
18. Chuang, S.Y., Odono, R.M., and Hedman, T.P. (2007). Effects of exogenous crosslinking on in vitro tensile and compressive moduli of lumbar intervertebral discs. *Clin. Biomech. (Bristol, Avon).*, 22, 14–20.
19. Cornwell, K.G., Lei, P., Andreadis, S.T., and Pins, G.D. (2007). Crosslinking of discrete self-assembled collagen threads: effects on mechanical strength and cell-matrix interactions. *J. Biomed. Mater. Res. A.*, 80, 362–71.
20. Lee, J.M., Haberer, S.A., and Boughner, D.R. (1989). The bovine pericardial xenograft: I. Effect of fixation in aldehydes without constraint on the tensile viscoelastic properties of bovine pericardium. *J. Biomed. Mater. Res.*, 23, 457–75.
21. Damink, L.O., Dijkstra, P.J., van luyn, J.A., Van Wachem, P.B., Nieuwenhuis, P., and Feijen, J. (1995). Gluataraldehyde as a crosslinking agent for collagen based biomaterials. *J. Mater. Sci. Mat. Med.,* 6, 460–472.
22. Lee, J.M., Boughner, D.R., and Courtman, D.W. (1984). The glutaraldehyde-stabilized porcine aortic valve xenograft. Effect of fixation with or without pressure on the tensile viscoelastic properties of the leaflet material. *J. Biomed. Mater. Res.,* 18, 79–98.
23. Parry, D.A., Craig, A.S., and Barnes, G.R. (1978). Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibres as a function of age. *Proc. R Soc. Lond. B Biol. Sci.,* 203, 293–303.
24. Bowes, J.H., and Cater, C.W. (1968). The interaction of aldehydes with collagen. *Biochim. Biophys. Acta.*, 168, 341–52.
25. Simionescu, A., Simionescu, D., and Deac, R. (1991). Lysine-enhanced glutaraldehyde crosslinking of collagenous biomaterials. *J. Biomed. Mater. Res.*, 25, 1495–505.
26. Khor, E. (1997). Methods for the treatment of collagenous tissues for bioprostheses. *Biomaterials.*, 18, 95–105.
27. Miles, C.A., Avery, N.C., Rodin, V.V., and Bailey, A.J. (2005). The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. *J. Mol. Biol.*, 346, 551–6.
28. Jorge-Herrero, E., Fernandez, P., Turnay, J., Olmo, N., Calero, P., Garcia, R., et al. (1999). Influence of different chemical cross-linking treatments on the properties of bovine pericardium and collagen. *Biomaterials.*, 20, 539–45.
29. Gutsmann, T., Fantner, G.E., Kindt, J.H., Venturoni, M., Danielsen, S., and Hansma, P.K. (2004). Force spectroscopy of collagen fibers to investigate their mechanical properties and structural organization. *Biophys. J.*, 86, 3186–93.
30. Domke, J., and Radmacher, M. (1998). Investigation of the mechanical properties of thin polymeric films. *Langmuir.*, 14, 3320–3325.
31. Wenger, P.E., Bozec, L., Horton, M., and Mesquida, P. (2007). Mechanical properties of collagen fibrils. *Biophys. J.*, 93, 1255–1263.
32. Heim, A.J., Koob, T.J., and Matthews, W.G. (2007). Low strain nanomechanics of collagen fibrils. *Biomacromolecules.*, 8, 3298–3301.
33. Van Der Rijt, J.A., Van Der Werf, K.O., Bennink, M.L., Dijkstra, P.J., and Feijen, J. (2006). Micromechanical testing of individual collagen fibrils. *Macromol. Biosci.*, 6, 697–702.
34. Haraldsson, B.T., Aagaard, P., Krogsgaard, M., Alkjaer, T., Kjaer, M., and Magnusson, S.P. (2005). Region-specific mechanical properties of the human patella tendon. *J. Appl. Physiol.*, 98, 1006–12.
35. Bozec, L., and Horton, M. (2005). Topography and mechanical properties of single molecules of type I collagen using atomic force microscopy. *Biophys. J.*, 88, 4223–31.
36. Nimni, M.E., Cheung, D., Strates, B., Kodama, M., and Sheikh, K. (1987). Chemically modified collagen: a natural biomaterial for tissue replacement. *J. Biomed. Mater. Res.*, 21, 741–71.
37. Sung, H.W., Chang, Y., Chiu, C.T., Chen, C.N., and Liang, H.C. (1999). Crosslinking characteristics and mechanical properties of a bovine pericardium fixed with a naturally occurring crosslinking agent. *J. Biomed. Mater. Res.*, 47, 116–26.
38. Bedran-Russo, A.K., Pereira, P.N., Duarte, W.R., Drummond, J.L., and Yamauchi, M. (2007). Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. *J. Biomed. Mater. Res. B Appl. Biomater.*, 80, 268–72.
39. Pereira, C.A., Lee, J.M., and Haberer, S.A. (1990). Effect of alternative crosslinking methods on the low strain rate viscoelastic properties of bovine pericardial bioprosthetic material. *J. Biomed. Mater. Res.*, 24, 345–61.
40. Sung, H.W., Chang, W.H., Ma, C.Y., and Lee, M.H. (2003). Crosslinking of biological tissues using genipin and/or carbodiimide. *J. Biomed. Mater. Res. A.*, 64, 427–38.

41. Andreassen, T.T., Oxlund, H., and Danielsen, C.C. (1988). The influence of non-enzymatic glycosylation and formation of fluorescent reaction products on the mechanical properties of rat tail tendons. *Connect Tissue Res.*, 17, 1–9.
42. McMaster, W.C. (1986). Mechanical properties and early clinical experience with xenograft biomaterials. *Bull. Hosp. Jt. Dis. Orthop Inst.*, 46, 174–84.
43. Provenzano, P.P., and Vanderby, R. Jr. (2006). Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. *Matrix Biol.*, 25, 71–84.
44. Cheung, D.T., Perelman, N., Ko, E.C., and Nimni, M.E. (1985). Mechanism of crosslinking of proteins by glutaraldehyde III. Reaction with collagen in tissues. *Connect Tissue Res.*, 13, 109–15.

# Study II

# Human patellar whole tendon, in vivo, and isolated collagen fibrils have comparable mechanical properties

**Philip Hansen[1], René Brüggebusch Svensson[1,2], Tue Hassenkam[2], Bjarki Thor Haraldsson[1], Per Aagaard[3], Michael Krogsgaard[4], Michael Kjaer[1], S. Peter Magnusson[1]**

*[1]Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery, Bispebjerg Hospital & Center for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, Denmark*
*[2]Nano-Science Center, University of Copenhagen, Denmark*
*[3]Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark*
*[4]Department of Orthopedic Surgery, Bispebjerg Hospital, Copenhagen, Denmark*

**Abstract**
Tendons are strong hierarchical structures, but knowledge about the mechanical properties of individual structural levels and how tensile forces transmit between them remains incompletely understood. Collagen fibrils are thought to be primary determinants of whole tendon properties, although this is a matter of debate. We hypothesized that the human patellar tendon and its collagen fibrils would display similar mechanical properties for strain and Young´s modulus. Human patellar tendon biopsies (n=5) were obtained and individual collagen fibrils were dissected from the biopsies and tested mechanically by atomic force microscopy. The mechanical properties of the patellar tendon from which the fibrils derived were examined by ultrasonography, in vivo. The Young´s modulus of the patellar tendon was 1.91±0.50 GPa. From the obtained force-deformation data the length of the toe region was calculated (3.1±1.6%). The average diameter of the fibrils tested by AFM was 131±18 nm. The measured Young´s modulus was 2.83±0.35 GPa and the calculated length of the toe region was 0.8±0.1%. The mechanical properties of collagen fibrils displayed more homogenous mechanical behavior than whole tendon with only small inter-individual differences. When adjustment of whole tendon stress-strain data for a fibril volume fraction of 60% was performed, Young´s modulus (~2.7 GPa) compared well with that of the fibril. These data imply that collagen fibrils act as the primary load bearing units of tendon. Collagen fibrils may govern the stress-strain properties of tendon in the linear region, while other components of the extracellular matrix contribute more in the toe region.

**Keywords:** Patellar tendon, collagen fibril, mechanical properties, atomic force microscopy

## Introduction

Tendon is regarded a biological composite with collagen fibrils embedded in a ground substance of primarily proteoglycans and water (41). The fibril is considered the basic force transmitting unit of tendon, although its importance in comparison to other matrix components such as proteoglycans is yet to be firmly established (37; 38; 40; 41). Fibrils are made up of tropocollagen molecules arranged in a quarter-staggered manner as described by the Hodge-Petruska model (39). However, exact knowledge of how forces transfer through the tendon structure remains to be determined. It has been suggested that fibrils are continuous throughout the length of the tendon (12; 40), which would imply the that the mechanical strain of the fibril and the whole tendon are similar. On the other hand, it has been suggested that fibrils are discontinuous in which case force transfer must occur between neighbouring fibrils, e.g. via interfibrillar proteoglycans (17; 34; 41). Discontinuous fibrils would mean that strain of the whole tendon would exceed that of the lower levels of the hierarchy (17).

Few available studies have attempted to examine the mechanical properties at different hierarchical levels, and none have done so in a human model. Sasaki et al (48) reported the tangent modulus of the collagen molecule > fibril > tendon. In contrast Kai-Nan An (1) summarized mechanical data from the existing literature and showed the modulus of pro-collagen molecules < collagen fiber < collagen fascicle < tendon. These conflicting observations leave questions regarding the mechanical properties of individual structural levels of tendon and the force transfer between them unanswered. Previous attempts to compare different hierarchical levels of tendon may have been hampered due to distinctions between phenotype, species, age, gender and loading history of the examined tendons (2) in addition to methodological issues of mechanical testing (7).

Ultrasonography-based measurement of tendon properties has proven an attractive non-invasive technique of determining whole-tendon behaviour *in vivo* (5; 25; 31). This methodology has allowed for characterisation of the human patellar tendon mechanical properties (20) and the technique has been widely applied to examine tendon adaptation to e.g. aging and habitual loading (11; 19; 28; 29; 35; 45; 46). For *ex vivo* testing of human tendon substructures, micro-tensile devices have been applied to describe the behaviour of tendon fascicles (22; 54). However, testing of individual collagen fibrils has not been possible until very recently, but with the advent of atomic force microscopy (AFM) it has become possible to measure the mechanical properties of individual collagen fibrils by force spectroscopy using various approaches (18; 50; 51).

To date no studies have attempted to compare *in vivo* whole-tendon properties with properties of lower-level hierarchical structures within the same individual. In the present study we applied the AFM and ultrasonography methodologies to investigate and compare the hierarchical mechanical properties of 1) whole patellar tendon, *in vivo* and 2) collagen fibrils, *in vitro*, in healthy human subjects. We hypothesized that the two distinct structural levels would display similar mechanical properties.

## Materials & methods

Five healthy males (age: 32±7 years; weight: 90±11 kg; height: 183±6 cm; BMI: 26.7 kg/m$^2$) participated in the study. Subjects were scheduled for elective reconstructive surgery of the anterior cruciate ligament (ACL). At the time of elective surgery the patients had normal range of knee joint motion. Patients were not impaired during normal activities of daily living, but they were unable to participate in sports due to knee instability. All subjects gave informed consent prior to inclusion in the study. Approval of the study was obtained from the local ethics committee prior to initiation of the study.

*In vivo whole tendon mechanical testing:*

The details of the measurement, including the reliability of the method in our laboratory, has been reported elsewhere (20). All measurements were performed <1 month prior to the ACL-reconstruction.

The subjects were seated in a custom made rigid chair with both hips and knees flexed to an angle of 90°. A leg cuff, which was connected to a strain gauge (Bofors KRG-4, Bofors, Sweden) through a rigid steel rod perpendicular to the lower leg, was mounted on the leg just above the medial malleolus. An ultrasound probe (7.5 MHz, linear array B-mode, Sonoline Sienna, Siemens, Erlangen, Germany) was fitted into a custom made rigid cast that was secured to the skin above the patellar tendon in the sagital plane. The ultrasound probe and cast was positioned so that the patella, the patellar tendon and the tibia were all visible within the viewing field throughout the ramp contractions (Figure 1). The ultrasound S-VHS video images obtained during the ramp trials were sampled at 50 Hz on a PC using frame-by-frame capturing (Matrox Marvel G400-TV, Dorval, Canada). Force was sampled on two separate PCs at 50 Hz via a 12-bit A/D converter (dt 2810A, Data Translation, MA, USA). The two computers were inter-connected to permit synchronous sampling of all data using a custom-built trigger device (5). The subjects performed 4-5 slow isometric knee extensions ramps by applying gradually increasing force until maximum over a 10 s. period during which patellar tendon displacement and knee extension force were measured simultaneously. All measurements were performed on the injured side knee. During the ramp contractions, force was sampled

at 50 Hz and low pass filtered at a 1.0 Hz cutoff frequency using a 4th order zero-lag Butterworth filter.

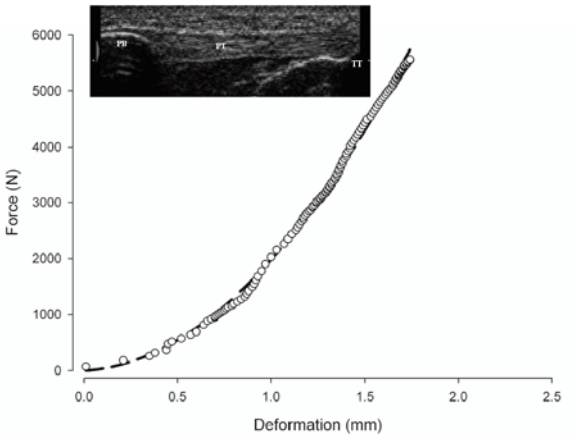



*Figure 1: Representative graph illustrating in vivo force-deformation data for patellar tendon elongation obtained by ultrasonography. The force-deformation data was fitted to a 2. or 3. order polynomial (black dotted line). The corresponding stress-strain relation was calculated by normalization for tendon dimensions and the Young´s modulus was subsequently determined in the final 20% of the force range.*

*MRI measure:*
Patellar tendon cross-sectional area (CSA) and length were determined by MRI (General Electric, Sigma Horizon LX 1.5 Tesla, T1 weighted SE) (Figure 2). Tendon CSA was determined by axial plane MR using the following parameters: TR/TE 400/14 ms, FOV 20, matrix 256x256, slice thickness 5.0 mm and spacing 0 mm. The axial scans were performed perpendicular to the patellar tendon. The tendon CSA was measured at the *proximal* (just distal to the patellar bone), *mid* and *distal* (just proximal to the tibial tuberosity) parts of the tendon. The patellar tendon length was determined from sagittal plane MRI using the following parameters: TR 500, ET: 3 x (TE:12.4 ms), FOV 16, matrix 256x192, slice thickness 4.0 mm and no spacing. The patellar tendon length was obtained by measuring the distance from the dorsal insertion at the patella apex to the dorsal insertion on the tibia. The PT CSA and PT length were manually outlined using the software program Osiris 4.19 (http://www.sim.hcuge.ch/osiris/). The color intensity of each image was adjusted using the NIH color scale mode of the software. Tendon CSA and length was measured using the gray scale image display. The mean value of three measurements of the same image was used for analysis. The reproducibility data showed that the typical error percent of repeated measures of tendon CSA was 2.5 % for repeated measurements at the proximal tendon level, 2.5 % for repeated measurements at the mid-tendon tendon level and 2.0 % for repeated measures at the most distal tendon level.

*Tendon biopsies:*
During ACL reconstructive surgery a small tendon sample was teased from patellar tendon ACL-graft of each patient. The sample was obtained from the anterior aspect and mid one-third of the tendon. A thin tendon fiber bundle containing ~10-15 fascicles was obtained from the anterior region of the tendon. The biopsies were then wrapped in sterile gauze moistened by 0.15 M phosphate buffered saline (PBS) and stored at -20°C. On the day of single fibril mechanical testing the sample was allowed to thaw in PBS (0.15M) moistened gauze.

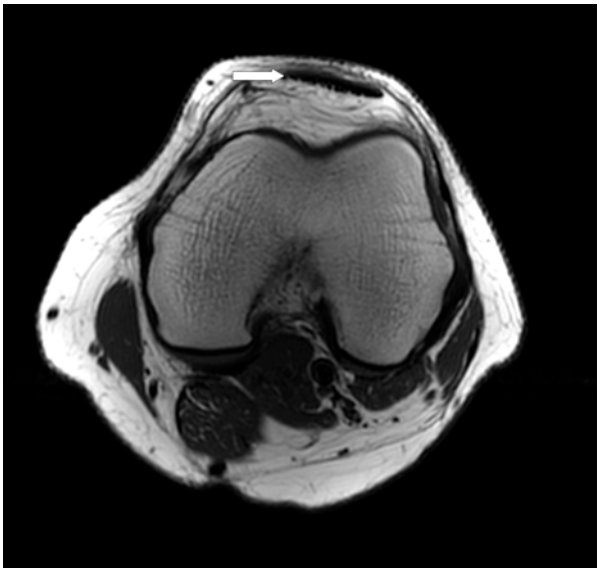


*Figure 2: Axial MRI image of the human patellar tendon (arrow). Patellar tendon cross-sectional area was determined from axial images while tendon length was measured from similar images obtained in by sagittal plane scans.*

*Single collagen fibril mechanical testing*:
Mechanical testing of collagen fibrils was performed using a recently described method (50). Briefly, two fibrils were tested for each subject. A small piece of a tendon fascicle was transferred to a silicon substrate and spread on the substrate surface with a pair of tweezers. The sample was subsequently allowed to dry. Next, a collagen fibril positioned in a secluded manner on the substrate was identified with an optical microscope. Using an atomic force microscope (AFM) (DI-Veeco MultiMode), small droplets of glue were deposited with an AFM cantilever on either end of the identified fibril ~ 200µm apart (Figure 3). After the glue had cured the fibril dry CSA was determined by AFM imaging. One of the droplets was then detached from the substrate surface using a sharp AFM tip mounted in the AFM. The freed glue deposit (secured to one end of the fibril) was then attached with fresh

glue to another AFM cantilever ($k_{spring}$ ~ 0.42 N/m). When the glue had cured, a PBS buffer solution (0.15M) was introduced and the fibril was mechanically tested using a picoforce AFM scanner (Figure 4). The fibril length was determined in two different ways. Firstly the length of fibril between the two glue droplets was measured by optical microscopy after the end-glue had been cut free of the surface. Secondly the distance moved with the stepping motor in the z direction to stretch the fibril was determined. The two measures were in good agreement and the average of the two were used ($r^2$=0.94; $p < 0.01$; mean absolute difference=3.25 µm). During the experiment the onset of force was set at a cantilever deflection of 5 nm. The ramp size was 18 µm and the frequency was 0.272 Hz, which corresponded to a strain rate of 9.81µm/s (~ 5%/s).

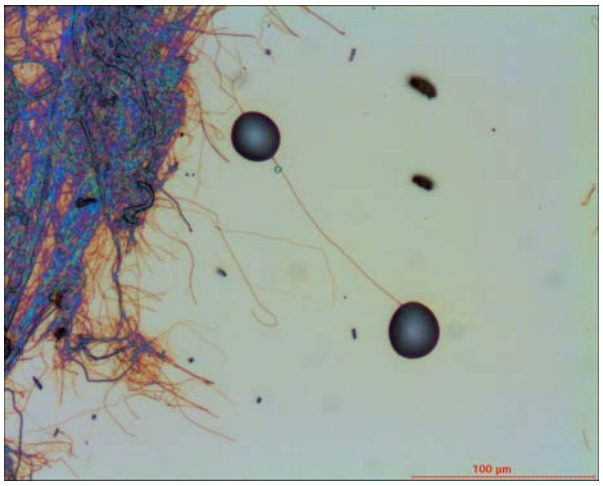



*Figure 3: Optical image of a secluded fibril from a human patellar tendon. The fibril has been glued to the surface by two glue deposits placed ~ 200 µm apart.*

Using the stepping motor, the fibril was stretched until the distance between the onset point and the peak of the ramp was equal to 4% of the fibril length plus an additional 1µm to approximately account for the cantilever bending. The use of a different force onsets in the final data treatment and cantilever-bending deviating from 1µm caused peak strains to differ slightly from 4%. The optical detector used to determine the cantilever deflection had a limited linear range, therefore, data was acquired in a piecewise manner by physically moving the detector (Figure 4) (50). By retaining an overlap between the data recorded at two subsequent detector positions, it was possible to stitch together the data. Each curve was stitched together by 20-30 pieces. It is necessary for the stitching to work that a mechanical equilibrium is reached, which was achieved by continuously cycling for at least 1 min before starting data acquisition. Visible changes in the mechanical response disappear after ~ 20 sec.

**Data reduction and statistics**

Ultrasound tendon force-deformation data were analyzed to a greatest common force for each subject. Each force–deformation curve was fitted to a 2nd or 3rd order polynomial with an $R^2 > 0.97$ in all cases (Figure 1). In vivo whole tendon tangent (Young´s) modulus was calculated in the upper 20 % stress of the fitted stress–strain curve. For calculation of tendon stress, the tendon force was divided by an average tendon cross-sectional area based on individual cross-sections at the proximal, mid- and distal portion of the tendon. Tendon force was calculated by dividing the estimated total knee extension moment by the internal moment arm, which was estimated from individually measured femur lengths (52). Tendon stress was calculated by dividing tendon force with the average CSA at 3 levels (proximal, mid and distal) of the patellar tendon determined from MRI. Tendon length was determined on sagittal MRI images and defined as the distance between the inferior margin of the patellar apex and superior margin of the tibial tuberosity. Tendon deformation was defined as the change in distance between the patellar apex and the tibia. The deformation was measured by automated tracking software that analyses sequential ultrasonographic images (20; 32).

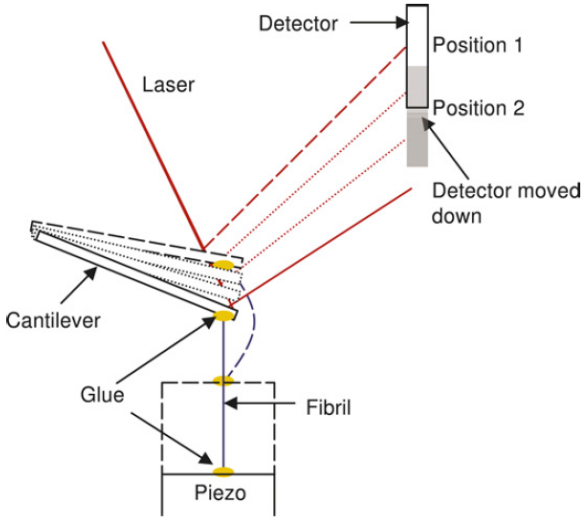



*Figure 4: Schematic of the single fibril AFM setup for mechanical testing, which was described in detail previously. The single fibril is attached at one end to the AFM cantilever and to the sample surface at the other.*

For fibrils no fitting was performed and Young´s modulus was determined by linear regression to the final 20% stress of the stress-strain data. The dry fibril area was used for calculation of fibril stress. The fibril area was calculated as the average of (1) the integrated area under the fibril contour and (2) the cross-sectional area assuming an elliptical shape of the fibril with one diameter equal to the fibril height and the other equal to the fibril width at half the height. For comparison of fibril mechanical

behavior compared to whole tendon an average stress-strain curve for all tested fibrils and whole tendon measurements, respectively, was calculated based on individual fitted curve data for both fibril and whole tendon. To construct the fitted average curve for all subjects (Figure 5), the lowest common stress across all subjects was determined and used as cut-off value.

To evaluate the non-linear properties of fibrils compared to whole tendon the length of the toe-region of the two structures was determined by extrapolating the linear region onto the strain axis:

$$\text{Toe length}=\varepsilon-(\sigma/E)$$

Where $\varepsilon$ and $\sigma$ are the maximal strain and stress and E is the modulus.

To evaluate the variation of Young´s modulus the coefficient of variation (CV) i.e. the ratio of SD to mean was determined. Toe length was expressed as a finite percent strain. An average of all fitted stress-strain curves for whole tendon was plotted and compared to an averaged stress-strain curve for all fibrils (based on data fitted by a second order polynomial fit). For comparison of the elastic properties of fibril compared to whole tendon the toe region was subtracted from both of the averaged stress-strain curves (Figure 5). Additionally, we adjusted the average whole tendon stress-strain data by a typical volume fraction (i.e. the fraction of the total tendon cross-sectional area occupied by fibrils) of 60% based on own observations (21). The stress-strain data were plotted to a common stress level. A student´s paired t-test was used to determine differences between fibril and whole tendon mechanical parameters. A 0.05 level of significance was used. Results are reported as group means ± SD.

**Results**

*Whole tendon measurements, in vivo:*

The average tendon length was 45.1±5.7 mm and the average tendon CSA was 97.3±10.1 $mm^2$. The common force for all subjects was 5442±471 N, which corresponded to a common stress of 55.6±9.8 MPa and a strain of 6.0±1.6%. The Young´s modulus was 1.91±0.50 GPa. The coefficient of variation (CV) for the Young´s modulus was 0.23. The calculated length of the 'toe' region was 3.1±1.6% (i.e. ~ 50% of total strain).

*Single fibril force spectroscopy:*

Fibril diameter was 131±18 nm (range: 107-160 nm) and fibril length was 200±15 µm (range: 167-225 µm). The Young´s modulus was 2.83±0.35 GPa, and the associated CV was 0.12. The average maximum stress was 79.7±12.0 MPa and the corresponding strain was 3.6±0.3%. Calculated length of the 'toe' region was 0.8±0.1%.

*Comparison between fibril and whole tendon mechanical properties:*

The Young´s modulus of collagen fibrils exceeded that of whole tendon ($p < 0.01$), while the toe length was shorter for fibrils ($p < 0.05$). Correction of the average whole tendon stress-strain data for fibril volume fraction (60%) yielded a Young´s modulus of 2.71 GPa.

**Discussion**

To the best of our knowledge, this study is the first to compare the mechanical properties at two structural levels of the human patellar tendon for the same individual, namely the whole tendon and the tendon fibril. The results show that collagen fibrils appear stiffer than the whole tendon, however, the Young´s moduli may in fact converge when methodological limitations are appropriately corrected for. The absolute modulus was ~ 3 GPa for fibrils and ~ 2 GPa for whole tendon in vivo, while the non-linear toe region of the stress-strain curves was less for the fibril (~ 1%) than the whole tendon (~ 3%). Interestingly, the inter-subject coefficient of variation for Young´s modulus was greater for the whole tendon (0.23) compared to collagen fibril (0.12).

The collagen fibril is thought of as the principle load bearing unit of tendon (37; 40), although its relative importance in comparison to other constituents of the matrix has not been firmly established. Few studies have addressed the mechanical properties at different hierarchical levels of tendon, and none in human tissue (1; 48). We show that the fibril modulus > modulus of whole tendon, which is in agreement with Sasaki et al (48). However, they reported a modulus of ~ 430 MPa for the fibril and ~ 400 MPa for tendon, which is far below that of the present study. Sasaki et al measured strain values following complete stress-relaxation of the fibril, which may in part explain the discrepancy. We have previously shown that collagen fibril mechanics are strain rate dependent and that stress-relaxation clearly lowers the modulus (50). Also, Sasaki et al. tested sub-units of 'whole tendon', which may have underestimated the modulus since testing of macroscopic substructures such as isolated fascicles or fascicle bundles often yields lower Young´s modulus values (< 1 GPa) compared to whole tendon testing (typically ≥ 1 GPa) (2; 7-9; 24). It is well known that testing of tendon substructures can be confounded by issues related to clamping technique (7). Human patellar tendon fascicles yielded modulus values of ~ 1.2 GPa when strain was obtained from optical markers, while the corresponding modulus using grip-to-grip strain was a mere 510 MPa (22), and similar differences has been shown by others (8). Excessive strain, slippage, or stress concentration near the clamping sites are circumvented by the in vivo ultrasound methodology applied in the present study,

and have typically yielded modulus values in the range of 0.8-2.2 GPa (4; 10; 11; 20; 27; 36; 45; 53). Notably, this range of moduli of human in vivo studies compare well with the summarized data of a multitude of mammalian whole tendons (2; 24).

A human *within* subject study design to compare mechanical properties at different hierarchical levels of tendon has never been applied previously. The absolute data show that fibrils are stiffer than whole tendon. Importantly, it should be noted that fibril stress was based on dry fibril cross-sectional area. Pilot studies from our lab have shown that the dry fibril diameter may increase ~ 100 % when hydrated in isotonic PBS solution (0.15 M), and others have reported a swelling of 73% upon hydration (51). However, some of this swelling may be that of the proteoglycan layer covering the fibril surface (42; 43), which is unlikely to contribute to the tensile mechanical properties of an isolated fibril and should therefore not be included in the calculation of fibril stress. Furthermore adhesion of the fibril to the surface may cause a flattening effect that could increase the apparent fibril diameter (51), which we attempted to circumvent by deriving the diameter from both the integrated area under the fibril contour and by calculation of fibril cross-sectional area assuming an elliptical shape of the fibril. We found a mean fibril diameter of ~ 130 nm, which is quite large compared to previous transmission electron microscopy studies where there is a dominance of fibrils < 100 nm (21; 49). However, the calculations based on dry fibril area likely did not lead to gross overestimation of 'true' fibril modulus since fibril diameter measured by transmission electron microscopy may be underestimated due to shrinkage from chemical fixation and embedding (33).

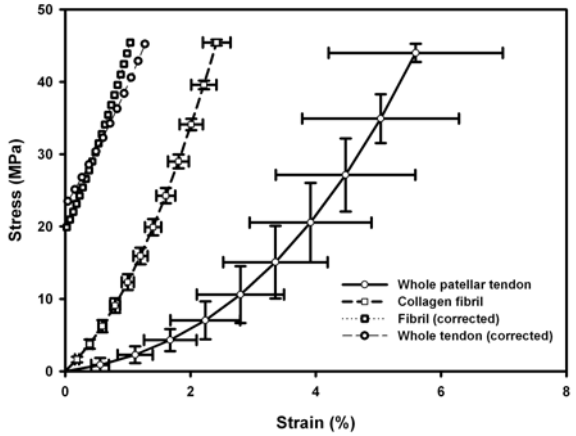



*Figure 5: Plot showing the average of all stress-strain curves for whole tendon (line) and collagen fibrils (dashed line). Averaged data were corrected by subtraction of the calculated toe region and whole tendon data were further corrected for volume fraction of collagen fibrils (fibril: bold squares; tendon: bold circles).*

The difference in toe length indicates that the non-linear properties of whole tendon and fibril are dissimilar. The toe region of the fibril may represent shear due to bending of the fibril ends at the glue-fibril interface since the test is conducted in a vertical orientation, and this would result in a small force before the fibril is fully stretched. Deformation of the glue could have contributed to the toe region, although this contribution is likely negligible (50; 51). The structural basis for the pronounced toe phenomenon of whole tendon is likely at the level of higher order tendon structures. The general notion is that the toe region is unfolding of macroscopic waviness (crimping) of tendon fiber bundles (23), and there is a structural basis of crimping localized as microscopic kinks in collagen fibrils (16; 44). We were unable to observe such kinks in the tested fibrils, and this lack of kinking may have contributed to the observed small fibril toe region.

Human collagen fibrils have not previously been examined, and the existing studies on various types of collagen fibrils have produced dissimilar modulus values. A modulus of 32 MPa and 450 MPa have been reported for fibrils derived from cultured human dermal fibroblasts and bovine Achilles tendon, respectively (18; 51). Fibrils of sea cucumber appear quite soft (modulus ~ 400 MPa) at low strains, but very stiff (modulus ~ 6 GPa) at very large strains (~ 30%) (13). Van der Rijt et al. (51) used an AFM setup similar to the present study to test fibrils from bovine Achilles tendon and reported permanent deformation of the fibril at stresses above 20 MPa with a corresponding strain of ~ 4 %. We tested human fibrils to a similar magnitude of strain (3.6 %) with an accompanying stress of ~ 80 MPa, but without any signs of permanent deformation. The reason for the discrepancy in fibril strength could in part be due to differences in fibril cross-link density (6; 37). It is also possible that that the extensive biochemical and mechanical treatment of the fibril prior to mechanical testing by Van der Rijt et al. (51) may have altered the fibril properties. In the present study we only examined fibrils from the anterior aspect of the tendon because it has previously been shown that tendon fascicles of the posterior region are mechanically weaker (22). Since the applied AFM methodology does not allow for testing the fibrils to failure (the single fibrils were strong enough to cause the applied AFM cantilevers to bend beyond the measurement range) we can only speculate whether such mechanical weakness could be explained by inferior strength of collagen fibrils.

Interestingly, we observed relatively little variation in the modulus of fibrils from different individuals (Figure 5), and this variation was 2-fold greater on the whole tendon level, in vivo. This divergence may suggest that the fibril is the fundamental tensile bearing unit of tendon, but that the extracellular matrix components can exert some

influence on the overall properties in vivo. To what extent interfibrillar proteoglycans may play a role in force transmission remains a debate (40). Human tendon contains only small amounts of proteoglycans while type I collagen accounts for the major portion of the tendon dry matter (24), and therefore it is relevant to consider the volume fraction of collagen. We have previously shown that the fibril volume fraction is ~ 60% in human patellar tendon (21), and moreover, the volume fraction appears to be a better predictor of the modulus than collagen content or fibril diameter (30; 47). Therefore, we adjusted the average whole tendon stress-strain data for volume fraction. This manoeuvre produced quite similar modulus values for whole tendon and fibrils (Figure 5), which lends support to the notion that fibrils are the load bearing units of tendon. It must be noted that the correction does not account for the *between* subject variation, which was greater in vivo than for fibrils. Additionally, is does not explain the large differences for the magnitude of the toe region that was more pronounced for the whole tendon. These findings indicate that the fibril properties may be modulated by other factors such interfibrillar proteoglycan ground substance. Also, inter-fibrillar sliding processes, which imply discontinuity of fibrils, have previously been suggested by some authors (17; 41) and such a mechanism could in part explain the larger variation for whole tendon. On the other hand, if continuity of fibrils is assumed, it is possible that the variation for modulus values of whole tendon and the pronounced toe region may be due to different initial resting lengths of fibrils. Sequential tightening of subgroups of fibrils during tensile loading could produce an initial non-linear increase in stiffness, as seen in the toe region of whole tendon force-elongation behavior. With continued increases in tensile strain all fibrils are eventually taught and beyond this point one would expect a more linear region as seen in typical tendon stress-strain curves. Indeed, non-uniform reorganization of tendon during loading has previously been suggested (40). Additionally, one would expect a quite abrupt shift (biphasic appearance) from the toe to the linear region if all fibril were of similar resting lengths.

During daily walking activity, the human patellar tendon seems to operate in the toe region of the stress-strain curve. Previous studies describe the human knee extensor forces during walking and small amplitude hopping (3; 14), and from these studies a patellar tendon stress during walking should be in the range of 5-15 MPa assuming a tendon cross-sectional area of 100 $mm^2$, which is a reasonable estimate for an average human male (26; 36; 53). During high intensity jumping, the tendon can reach ~ 40 MPa (15). If these assumptions hold true perhaps loading of the tendon in the toe region during walking allows for some protection from fatigue damage to fibrils because the viscous component dominates and fibrils are relatively unloaded.

In conclusion the findings of the present study provide some support for the notion that fibrils are primary load bearing units of human tendon. Fibrils appear stiffer than whole tendon, however, when fibril volume fraction is accounted for the modulus values of the two structural levels approximate each other, which is in accordance with our hypothesis. However, the mechanical properties of collagen fibrils from different individuals display more homogenous mechanical behavior than whole tendon. This in addition to the larger toe region in tendon indicate that fibril properties may govern the linear region while other components of the extracellular matrix contribute more to the toe region.

Reference List


1. **An KN, Sun YL and Luo ZP**. Flexibility of type I collagen and mechanical property of connective tissue. *Biorheology* 41: 239-246, 2004.

2. **Bennett MB, Ker RF, Dimery NJ and Alexander McN**. Mechanical properties of various mammalian tendons. *J Zool Lond(A)* 209: 537-548, 1986.

3. **Besier TF, Fredericson M, Gold GE, Beaupre GS and Delp SL**. Knee muscle forces during walking and running in patellofemoral pain patients and pain-free controls. *J Biomech* 42: 898-905, 2009.

4. **Bojsen-Moller J, Brogaard K, Have MJ, Stryger HP, Kjaer M, Aagaard P and Magnusson SP**. Passive knee joint range of motion is unrelated to the mechanical properties of the patellar tendon. *Scand J Med Sci Sports* 17: 415-421, 2007.

5. **Bojsen-Moller J, Hansen P, Aagaard P, Kjaer M and Magnusson SP**. Measuring mechanical properties of the vastus lateralis tendon-aponeurosis complex in vivo by ultrasound imaging. *Scand J Med Sci Sports* 13: 259-265, 2003.

6. **Buehler MJ**. Nanomechanics of collagen fibrils under varying cross-link densities: atomistic and continuum studies. *J Mech Behav Biomed Mater* 1: 59-67, 2008.

7. **Butler DL, Grood ES, Noyes FR, Zernicke RF and Brackett K**. Effects of structure and strain measurement technique on the material

properties of young human tendons and fascia. *J Biomech* 17: 579-596, 1984.

8. **Butler DL, Kay MD and Stouffer DC**. Comparison of material properties in fascicle-bone units from human patellar tendon and knee ligaments. *J Biomech* 19: 425-432, 1986.

9. **Chun KJ and Butler DL**. Spatial variation in material properties in fascicle-bone units from human patellar tendon. *Key Engineering Materials* 326-328: 797-802, 2006.

10. **Couppe C, Hansen P, Kongsgaard M, Kovanen V, Suetta C, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J Appl Physiol* 107: 880-886, 2009.

11. **Couppe C, Kongsgaard M, Aagaard P, Hansen P, Bojsen-Moller J, Kjaer M and Magnusson SP**. Habitual loading results in tendon hypertrophy and increased stiffness of the human patellar tendon. *J Appl Physiol* 105: 805-810, 2008.

12. **Craig AS, Birtles MJ, Conway JF and Parry DA**. An estimate of the mean length of collagen fibrils in rat tail-tendon as a function of age. *Connect Tissue Res* 19: 51-62, 1989.

13. **Eppell SJ, Smith BN, Kahn H and Ballarini R**. Nano measurements with micro-devices: mechanical properties of hydrated collagen fibrils. *J R Soc Interface* 3: 117-121, 2006.

14. **Finni T, Komi PV and Lepola V**. In vivo muscle mechanics during locomotion depend on movement amplitude and contraction intensity. *Eur J Appl Physiol* 85: 170-176, 2001.

15. **Finni T, Komi PV and Lepola V**. In vivo human triceps surae and quadriceps femoris muscle function in a squat jump and counter movement jump. *Eur J Appl Physiol* 83: 416-426, 2000.

16. **Franchi M, Fini M, Quaranta M, De P, V, Raspanti M, Giavaresi G, Ottani V and Ruggeri A**. Crimp morphology in relaxed and stretched rat Achilles tendon. *J Anat* 210: 1-7, 2007.

17. **Fratzl P, Misof K, Zizak I, Rapp G, Amenitsch H and Bernstorff S**. Fibrillar structure and mechanical properties of collagen. *J Struct Biol* 122: 119-122, 1998.

18. **Graham JS, Vomund AN, Phillips CL and Grandbois M**. Structural changes in human type I collagen fibrils investigated by force spectroscopy. *Exp Cell Res 2004 Oct 1* 335-342, 299.

19. **Hansen P, Aagaard P, Kjaer M, Larsson B and Magnusson SP**. Effect of habitual running on human Achilles tendon load-deformation properties and cross-sectional area. *J Appl Physiol* 95: 2375-2380, 2003.

20. **Hansen P, Bojsen-Moller J, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties of the human patellar tendon, in vivo. *Clin Biomech (Bristol , Avon )* 21: 54-58, 2006.

21. **Hansen P, Haraldsson BT, Aagaard P, Kovanen V, Avery N, Qvortrup K, Larsen JO, Krogsgaard M, Kjaer M and Magnusson SP**. Lower strength of the human posterior patellar tendon seems unrelated to mature collagen cross-linking and fibril morphology. *J Appl Physiol* 2009.

22. **Haraldsson BT, Aagaard P, Krogsgaard M, Alkjaer T, Kjaer M and Magnusson SP**. Region-specific mechanical properties of the human patella tendon. *J Appl Physiol* 98: 1006-1012, 2005.

23. **Jarvinen TA, Jarvinen TL, Kannus P, Jozsa L and Jarvinen M**. Collagen fibres of the spontaneously ruptured human tendons display decreased thickness and crimp angle. *J Orthop Res* 22: 1303-1309, 2004.

24. **Ker RF**. The implications of the adaptable fatigue quality of tendons for their construction, repair and function. *Comp Biochem Physiol A Mol Integr Physiol* 133: 987-1000, 2002.

25. **Kjaer M, Langberg H, Bojsen-Moller J, Koskinen SO, Mackey A, Heinemeier K, Holm L, Skovgaard D, Dossing S, Hansen M, Hansen P, Haraldsson B, Caroe I and Magnusson SP**. Novel methods for tendon investigations. *Disabil Rehabil* 30: 1514-1522, 2008.

26. **Kongsgaard M, Kovanen V, Aagaard P, Doessing S, Hansen P, Laursen AH, Kaldau NC, Kjaer M and Magnusson SP**. Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand J Med Sci Sports* 2009.

27. **Kongsgaard M, Kovanen V, Aagaard P, Doessing S, Hansen P, Laursen AH, Kaldau NC, Kjaer M and Magnusson SP**. Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand J Med Sci Sports* 2009.

28. **Kongsgaard M, Reitelseder S, Pedersen TG, Holm L, Aagaard P, Kjaer M and Magnusson SP**. Region specific patellar tendon hypertrophy in humans following resistance training. *Acta Physiol (Oxf)* 191: 111-121, 2007.

29. **Kubo K, Kanehisa H, Ito M and Fukunaga T**. Effects of isometric training on the elasticity of human tendon structures in vivo. *J Appl Physiol* 91: 26-32, 2001.

30. **Lavagnino M, Arnoczky SP, Frank K and Tian T**. Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons. *J Biomech* 38: 69-75, 2005.

31. **Maganaris CN and Paul JP**. In vivo human tendon mechanical properties. *J Physiol* 521 Pt 1: 307-313, 1999.

32. **Magnusson SP, Hansen P, Aagaard P, Brond J, Dyhre-Poulsen P, Bojsen-Moller J and Kjaer M**. Differential strain patterns of the human gastrocnemius aponeurosis and free tendon, in vivo. *Acta Physiol Scand* 177: 185-195, 2003.

33. **Meller D, Peters K and Meller K**. Human cornea and sclera studied by atomic force microscopy. *Cell Tissue Res* 288: 111-118, 1997.

34. **Mosler E, Folkhard W, Knorzer E, Nemetschek-Gansler H, Nemetschek T and Koch MH**. Stress-induced molecular rearrangement in tendon collagen. *J Mol Biol* 182: 589-596, 1985.

35. **Narici MV and Maganaris CN**. Adaptability of elderly human muscles and tendons to increased loading. *J Anat* 208: 433-443, 2006.

36. **Onambele GN, Burgess K and Pearson SJ**. Gender-specific in vivo measurement of the structural and mechanical properties of the human patellar tendon. *J Orthop Res* 25: 1635-1642, 2007.

37. **Parry DA**. The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. *Biophys Chem* 29: 195-209, 1988.

38. **Parry DA, Barnes GR and Craig AS**. A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proc R Soc Lond B Biol Sci* 203: 305-321, 1978.

39. **PETRUSKA JA and HODGE AJ**. A subunit model for the tropocollagen macromolecule. *Proc Natl Acad Sci U S A* 51: 871-876, 1964.

40. **Provenzano PP and Vanderby R, Jr.** Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. *Matrix Biol* 25: 71-84, 2006.

41. **Puxkandl R, Zizak I, Paris O, Keckes J, Tesch W, Bernstorff S, Purslow P and Fratzl P**. Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos Trans R Soc Lond B Biol Sci* 357: 191-197, 2002.

42. **Raspanti M, Alessandrini A, Ottani V and Ruggeri A**. Direct visualization of collagen-bound proteoglycans by tapping-mode atomic force microscopy. *J Struct Biol* 119: 118-122, 1997.

43. **Raspanti M, Congiu T, Alessandrini A, Gobbi P and Ruggeri A**. Different patterns of collagen-proteoglycan interaction: a scanning electron microscopy and atomic force microscopy study. *Eur J Histochem* 44: 335-343, 2000.

44. **Raspanti M, Manelli A, Franchi M and Ruggeri A**. The 3D structure of crimps in the rat Achilles tendon. *Matrix Biol* 24: 503-507, 2005.

45. **Reeves ND, Maganaris CN and Narici MV**. Effect of strength training on human patella tendon mechanical properties of older individuals. *J Physiol* 548: 971-981, 2003.

46. **Reeves ND, Narici MV and Maganaris CN**. Strength training alters the viscoelastic properties of tendons in elderly humans. *Muscle Nerve* 28: 74-81, 2003.

47. **Robinson PS, Lin TW, Jawad AF, Iozzo RV and Soslowsky LJ**. Investigating tendon fascicle structure-function relationships in a

transgenic-age mouse model using multiple regression models. *Ann Biomed Eng* 32: 924-931, 2004.

48. **Sasaki N and Odajima S**. Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy. *J Biomech* 29: 1131-1136, 1996.

49. **Svensson M, Movin T, Rostgard-Christensen L, Blomen E, Hultenby K and Kartus J**. Ultrastructural collagen fibril alterations in the patellar tendon 6 years after harvesting its central third. *Am J Sports Med* 35: 301-306, 2007.

50. **Svensson RB, Hassenkam T, Hansen P and Magnusson SP**. Viscoelastic behavior of discrete human collagen fibrils. *J Mech Behav Biomed Mater* 2009.

51. **van der Rijt JA, van der Werf KO, Bennink ML, Dijkstra PJ and Feijen J**. Micromechanical testing of individual collagen fibrils. *Macromol Biosci* 6: 697-702, 2006.

52. **Visser JJ, Hoogkamer JE, Bobbert MF and Huijing PA**. Length and moment arm of human leg muscles as a function of knee and hip-joint angles. *Eur J Appl Physiol Occup Physiol* 61: 453-460, 1990.

53. **Westh E, Kongsgaard M, Bojsen-Moller J, Aagaard P, Hansen M, Kjaer M and Magnusson SP**. Effect of habitual exercise on the structural and mechanical properties of human tendon, in vivo, in men and women. *Scand J Med Sci Sports* 18: 23-30, 2008.

54. **Yamamoto E, Hayashi K and Yamamoto N**. Mechanical properties of collagen fascicles from stress-shielded patellar tendons in the rabbit. *Clin Biomech (Bristol , Avon )* 14: 418-425, 1999.

# Study III

*J Appl Physiol* 108: 47–52, 2010.
First published November 5, 2009; doi:10.1152/japplphysiol.00944.2009.

# Lower strength of the human posterior patellar tendon seems unrelated to mature collagen cross-linking and fibril morphology

**Philip Hansen,[1] Bjarki Thor Haraldsson,[1] Per Aagaard,[2] Vuokko Kovanen,[3] Nicholas C. Avery,[4] Klaus Qvortrup,[5] Jytte Overgaard Larsen,[6] Michael Krogsgaard,[7] Michael Kjaer,[1] and S. Peter Magnusson[1]**

[1]*Institute of Sports Medicine Copenhagen, Bispebjerg Hospital & Center for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark;* [2]*Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark;* [3]*Department of Health Sciences, Biochemistry Laboratory, University of Jyväskylä, Jyväskylä, Finland;* [4]*Department of Clinical Veterinary Science, Matrix Biology Research Group, University of Bristol, Langford, North Somerset, United Kingdom;* [5]*Department of Biomedical Sciences, The Panum Institute, University of Copenhagen, Copenhagen, Denmark;* [6]*Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark;* [7]*Department of Orthopedic Surgery, Bispebjerg Hospital, Copenhagen, Denmark*

Submitted 21 August 2009; accepted in final form 2 November 2009

**Hansen P, Haraldsson BT, Aagaard P, Kovanen V, Avery NC, Qvortrup K, Larsen JO, Krogsgaard M, Kjaer M, Magnusson SP.** Lower strength of the human posterior patellar tendon seems unrelated to mature collagen cross-linking and fibril morphology. *J Appl Physiol* 108: 47–52, 2010. First published November 5, 2009; doi:10.1152/japplphysiol.00944.2009.—The human patellar tendon is frequently affected by tendinopathy, but the etiology of the condition is not established, although differential loading of the anterior and posterior tendon may be associated with the condition. We hypothesized that changes in fibril morphology and collagen cross-linking would parallel differences in material strength between the anterior and posterior tendon. Tendon fascicles were obtained from elective ACL surgery patients and tested micromechanically. Transmission electron microscopy was used to assess fibril morphology, and collagen cross-linking was determined by HPLC and calorimetry. Anterior fascicles were markedly stronger (peak stress: 54.3 ± 21.2 vs. 39.7 ± 21.3 MPa; $P < 0.05$) and stiffer (624 ± 232 vs. 362 ± 170 MPa; $P < 0.01$) than posterior fascicles. Notably, mature pyridinium type cross-links were less abundant in anterior fascicles (hydroxylysylpyridinoline: 0.859 ± 0.197 vs. 1.416 ± 0.250 mol/mol, $P = 0.001$; lysylpyridinoline: 0.023 ± 0.006 vs. 0.035 ± 0.006 mol/mol, $P < 0.01$), whereas pentosidine and pyrrole concentrations showed no regional differences. Fibril diameters tended to be larger in anterior fascicles (7.819 ± 2.168 vs. 4.897 ± 1.434 nm$^2$; $P = 0.10$). Material properties did not appear closely related to cross-linking or fibril morphology. These findings suggest region-specific differences in mechanical, structural, and biochemical properties of the human patellar tendon.

patellar tendon; jumper's knee; cross-links; mechanical properties; fibril morphology

IN PATELLAR TENDINOPATHY the pathological tendon changes are typically located in the posterior proximal tendon at the insertion on the deep aspect of the patellar bone (17). The reason for this is not established. Khan et al. demonstrated marked histopathological changes in the proximal patellar tendon accompanied by clear abnormalities in the same location demonstrated by magnetic resonance imaging and ultrasonography (19). Variations in intratendinous loading patterns may be implicated since studies have demonstrated that the anterior and posterior aspects of human patellar tendon are exposed to different magnitudes of tensile strain during knee flexion, (1, 7, 13). A study on isolated tendon fascicles showed decreased material strength of fascicles of the posterior patellar tendon (16). It is possible that such variations in loading patterns may correlate to variations in mechanical properties within the patellar tendon. In support of this notion, a recent study by Lavagnino et al. elegantly demonstrated increased localized tendon strain at the classic lesion site of "jumper's knee" (22). Collectively, there seems to be evidence that the susceptibility to tendinopathy of the patellar tendon is related to differential intratendinous loading patterns and regional variation in tendon strength.

Tendon strength is related to both ultrastructure and biochemical composition of the tendon. Type I collagen molecules constitute the fundamental structural components of tendon. Collagen molecules assemble into fibrils (18), which are thought to be major load-bearing units of the tendon, although their importance compared with other matrix components such as proteoglycans has yet to be firmly established (29, 30, 32, 33). The mechanical integrity of fibrils is thought to be augmented by so-called "mature" cross-links. Mature cross-links are trivalent chemical bonds that link neighboring collagen molecules together and strengthen the collagen lattice. Immature enzymatically derived cross-links spontaneously convert into the trivalent form with maturation (5). In human tendon, the pyridinium type cross-links lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) constitute such mature cross-links (4, 5). Additionally, nonenzymatic glycation of collagen occurs with aging, leading to the accumulation of advanced glycation end-products (AGEs), which further cross-link the collagen molecules and lead to a "stiffening" of the tissue. One such AGE is pentosidine (PENT), which is commonly used as a biomarker of nonenzymatic glycation (4, 5).

Collagen fibril morphology has implications for the mechanical tissue properties. Small-diameter fibrils are expected to be weaker than large-diameter fibrils, which in turn are associated with increased tensile strength of dense collagenous tissues (29). In animal studies, the fibril diameter distribution has been shown to differ within individual tendon regions (31) and between individual tendon types (12). Recently, a site-specific loss of large-diameter fibrils in the central part of ruptured human Achilles tendons was suggested to have reduced failure strength and contributed to overt tendon rupture (25).

Address for reprint requests and other correspondence: S. Peter Magnusson, Institute of Sports Medicine Copenhagen, Bispebjerg Bakke 23, Bldg. 8, 1st Floor, DK-2400 Copenhagen NV, Denmark (e-mail: p.magnusson@mfi.ku.dk).

We hypothesized that region-dependant differences in mechanical properties exist between the anterior and posterior regions of the patellar tendon and that such differences are associated with alterations in collagen fibril morphology and the biochemical cross-link composition of the tendon fascicles.

## MATERIAL AND METHODS

*Preparation of tendon fascicles.* Before initiation of the study, approval was obtained from the local Ethics Committee. Informed consent was acquired from all patients included in the study. During elective anterior cruciate ligament (ACL) reconstructive surgery, tissue samples were obtained from healthy young men (mean ± SD, age 32 ± 9 yr; body mass 85.6 ± 11.6 kg; $n = 9$). At the time of elective surgery, the patients had full range of knee motion and were able to perform normal activities of daily living without disabling symptoms but were unable to participate in sports due to knee instability. The middle third of the patellar tendon was harvested for the ACL graft. From the graft, a thin bundle of tendon (~35 mm in length and ~3.5 mm in diameter) was obtained from the anterior and posterior tendon regions, respectively. From the tendon bundle, three fascicles (length ~35 mm; diameter ~300–400 μm) were dissected under stereomicroscopic guidance. The fascicles were transferred to PBS-moistened (0.15 M) sterile gauze and stored at −20°C. Immediately before mechanical testing of a fascicle, the fascicle was allowed to thaw while moistened in PBS (0.15 M)-soaked gauze for ~30 min at room temperature.

*Instrumentation.* A micro tensile testing stage was used for mechanical testing of tendon fascicles. The stage (200-N tensile stage, petridish version, Deben, Suffolk, UK) consists of a load cell (1% accuracy), a specimen liquid chamber, and two specimen mounting plates driven by a computer-controlled motor fitted with a linear variable differential transducer (LVDT) that registers changes in mounting plate displacement. The stage with the sample mounted is placed directly under a stereomicroscope (SMZ1000, Nikon, Tokyo, Japan) with a C-mount lens (×0.38). The microscope is equipped with a 15-Hz digital camera (DFWX700, Sony, Tokyo, Japan) with a 1,024 × 768 output signal format that allowed acquisition of detailed image data of the sample.

*Mechanical testing procedures.* Fascicles of ~25 mm were prepared for mechanical testing as described previously (16). Briefly, each 5-mm end of the collagen fascicle was allowed to air dry at room temperature, whereas the central region of the fascicle was wrapped in PBS-soaked (0.15 M) gauze. Next, the dried fascicle ends were glued to the uncoated aluminum specimen mounting plates of the mechanical rig with cyanoacrylate. The fascicle and mounting plates were then immersed in PBS solution (0.15 M) in a Petri dish fitted in the mechanical rig. The fascicle was hydrated for 20 min before the failure tests were conducted at a strain rate of 2.0 mm/s. The initial specimen testing length (mounting plate-to-mounting plate distance) was ~15 mm, except in four fascicles where the testing length needed to be reduced to ~10 mm due to the shorter length of the biopsy in four patients. For all samples, the original length ($L_0$) was determined once the fascicle was mounted in the testing apparatus. $L_0$ was measured at force onset by slowly straining the fascicle until a slight rise in force (~0.03 N) was detected. A high-resolution stereomicroscopic image of the mounted fascicle was obtained in this position, and the length was measured from the acquired image. The measured $L_0$ was subsequently used for strain calculations. Since the exact $L_0$ was accounted for in all measurements, we do not expect the inclusion of the ~10-mm biopsies to have influenced the measurements.

*Biochemical analysis.* The analysis of collagen, pyridinium cross-links, and PENT are described in detail elsewhere (21). Collagen concentration was measured spectrophotometrically as described by Creemers et al. (11). Cross-link analysis was performed by HPLC. The calculation of collagen cross-link concentration was based on concentrations of pure compounds of HP, LP, and PENT as external standards in each HPLC run. The collagen content was expressed as milligram per milligram dry weight. The quantities of cross-links were expressed as mol/mol collagen. Pyrrole was assayed colorimetrically as described by Avery et al. (3) and expressed in the same fashion (mol/mol collagen).

*Transmission electron microscopy.* Biopsy specimens for transmission electron microscopy (TEM) were collected from six (means ± SD; 31 ± 12 yr; body mass 82 ± 7 kg) of the nine patients. Following dissection of the biopsy, fascicle specimens were fixed in a 2% glutaraldehyde solution in 0.05 M sodium phosphate buffer (pH 7.2) and stored at 4°C until subsequent analysis. The procedure for the TEM and the following measurements of collagen fibril diameter is described in detail previously (25). Following fixation, specimens were dehydrated in graded series of ethanol, transferred to propylene oxide, and embedded in Epon (Hexion, Houston, TX) according to standard procedures. Ultra-thin sections were cut with a Reichert-Jung Ultracut E microtome, collected on one-hole copper grids with Formvar supporting membranes, and stained with uranyl acetate and lead citrate. The sections were examined with a Philips CM 100 TEM operated at an accelerating voltage of 80 kV. Images were recorded with a Megaview 2 camera and processed with the AnalySis software package.

*Stereology.* Stereological analysis of collagen fibrils were performed on a random sample of 10 TEM images from each biopsy cross section with the following parameters: the volume fraction ($V_V$) of collagen fibrils; the numerical concentration of collagen fibrils per cross sectional area ($N_A$); and the individual diameters ($d$) of the collagen fibers. Each randomly sampled EM image was examined with 16 uniformly positioned, unbiased counting frames, each of area 0.0426 $mm^2$, in a fixed position relative to the image (which was sampled randomly within the ultra thin cross section of the biopsy, ensuring all profiles in all locations have an equal probability of being sampled within the biopsy cross section). The unbiased counting frame ensures that all profiles, regardless of shape, size, or orientation, have an equal probability of being sampled within an area probe. The stereological analysis of collagen fibrils was carried out on a computer monitor onto which the digitized EM image was merged with a graphic representation of the stereological test systems (C.A.S.T.-grid software, The International Stereology Center at Olympus). On average 199 ± 25 (SE) fibrils (range: 137–287) were analyzed per biopsy cross section in the specimens from the anterior aspect and 278 ± 19 (SE) from each of the posterior specimens (range: 217–327). The fibril density was expressed as the absolute number of fibrils per square micrometer. The fibril volume fraction represents the area occupied by fibrils within the sample area. A single experienced investigator performed all stereological analyses. All measurements were performed in a blinded fashion. The investigator was unaware of the study hypothesis, subject characteristics, and whether the sample was obtained from the anterior or posterior aspect of the tendon.

*Data reduction and analysis.* The cross-sectional area of the fascicles was calculated based on the diameters of the fascicles measured from stereomicroscopic images obtained while the fascicle was mounted in the experimental setup. Images were obtained while the specimen was immersed in the PBS solution (0.15 M), and diameters were measured at three locations along the length of the specimen, and an average of the three measures was used. Fascicle stress was calculated as the tensile force (N) divided by the cross-sectional area ($m^2$) of the fascicle and reported in Pascal (MPa). Peak stress was defined as the highest level of stress (MPa) measured during failure testing of the fascicle. Fascicle strain was defined as $\Delta L$ divided by $L_0$ and was expressed as a percentage [$(\Delta L/L_0) \times 100$]. Fascicle strain was calculated from the instantaneous distance between specimen mounting plates. Young's tangent modulus was calculated in the linear portion of the stress-strain curve. Energy to failure was calculated as the area under the force-deformation curve. Fifteen fascicle specimens ruptured between the end plates of the mechanical rig,

approximately in the middle of the specimen. On three occasions, rupture occurred near the specimen plates 2–3 mm from the plate.

Since the amount of tissue in each biopsy was limited, there was insufficient tissue to perform biochemical analysis for pyrrole in two subjects, i.e., only sample pairs from seven patients could be analysed. For TEM, three sample pairs could not be analyzed. Therefore, only six sample pairs were examined.

Differences in mechanical, biochemical, and structural properties between the anterior (AF) and posterior (PF) fascicles were investigated using a two-tailed paired *t*-test. Since excessive strain due to insufficient clamping and stress concentrations near clamping sites have been known to introduce variation in tendon mechanical measurements (8), the fascicle that yielded the highest peak stress was used for analysis. To determine associations between structural and biochemical vs. biomechanical parameters, linear regression analysis (Pearson's product-moment method) was performed. A 0.05 level of significance was used. Results are reported as group means ± SD.

## RESULTS

*Mechanical properties (n = 9).* Peak stress levels were higher for AF (54.3 ± 21.2 vs. 39.7 ± 21.3 MPa; $P < 0.05$), strain at failure was larger for PF (12.7 ± 3.4 vs.16.2 ± 2.8%; $P < 0.01$), and tangent modulus was higher for AF (624 ± 232 vs. 362 ± 170 MPa; $P < 0.01$) (Fig. 1). Absorbed energy at failure did not differ between AF and PF (63.8 ± 41.0 vs. 63.5 ± 41.4 mJ/mm$^2$; $P = 0.98$).

*Biochemical analyses (n = 9).* Collagen content was similar in AF compared with PF (0.63 ± 0.05 vs. 0.62 ± 0.05 mg/mg; $P = 0.90$). HP and LP content were higher for PF than for AF (HP: 0.859 ± 0.197 vs. 1.416 ± 0.250 mol/mol, $P = 0.001$; LP: 0.023 ± 0.006 vs. 0.035 ± 0.006 mol/mol, $P < 0.01$) (Fig. 2). There was no difference for PENT (0.024 ± 0.015 vs. 0.024 ± 0.013 mol/mol; $P = 0.92$). Likewise, pyrrole ($n = 7$) concentration was similar in both regions of the tendon (0.202 ± 0.151 vs. 0.170 ± 0.136 mol/mol; $P = 0.76$).

*TEM (n = 6).* Fibril diameter tended to be smaller in PF (7,819 ± 2,168 vs. 4,897 ± 1,434 nm$^2$; $P = 0.10$) (Fig. 3). Fibril density was higher for PF than AF (71 ± 17 vs. 96.6 ± 15 μm$^{-2}$; $P = 0.05$), whereas volume fraction was similar for the two regions (61.0 ± 0.7 vs. 59.3 ± 0.1%; $P = 0.82$).

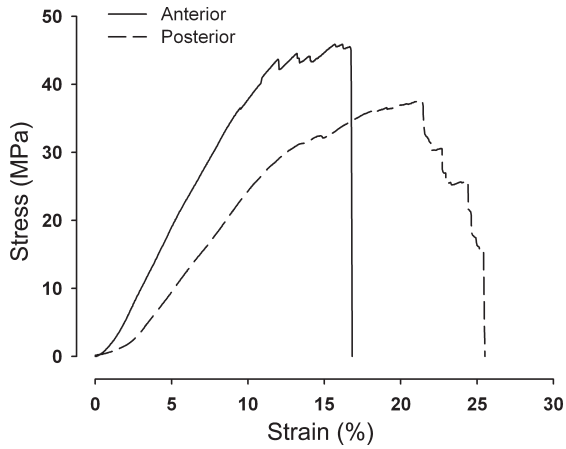



Fig. 1. Graph showing typical stress-strain properties of one anterior compared with one posterior tendon fascicle from the patellar tendon of one patient. On average, anterior fascicles were stronger and stiffer than posterior fascicles (ultimate tensile stress ± SD: 54.3 ± 21.2 vs. 39.7 ± 21.3 MPa, $P < 0.05$; Young's modulus ± SD: 624 ± 232 vs. 362 ± 170 MPa, $P < 0.01$).

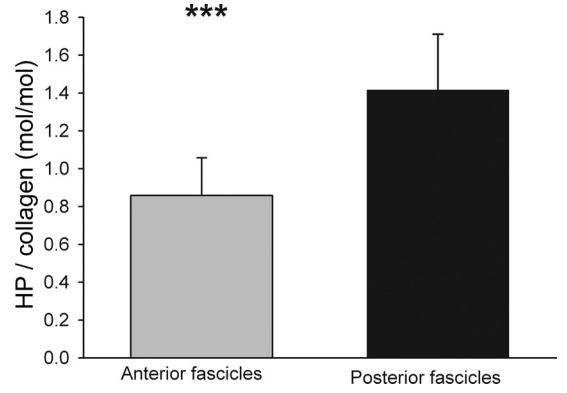



Fig. 2. Graph showing differences for in hydroxylysylpyridinoline (HP) concentration (±SD) between anterior and posterior fascicles. HP concentration was considerably higher for posterior fascicles (HP: 0.859 ± 0.197 vs. 1.416 ± 0.250 mol/mol; $P < 0.01$).

*Regression analysis.* No statistically significant associations were observed between any of the mechanical parameters measured for AF and PF (Young's modulus and ultimate tensile stress) and any of the measured cross-link parameters (HP, LP, pyrrole, and PENT), respectively (Fig. 4). However, PENT concentration and subject age were strongly related

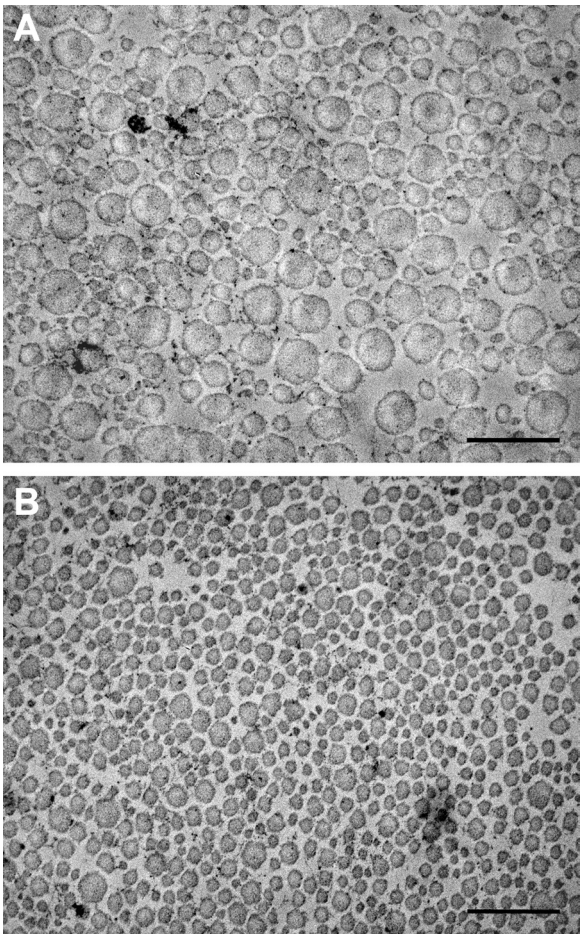



Fig. 3. Representative transmission electron microscopy images of transverse sections of collagen fascicles from the anterior (*A*) and the posterior (*B*) aspect of the patellar tendon. Posterior fascicles tended to have smaller fibril cross-sectional areas (7,819 ± 2,168 vs. 4,897 ± 1,434 nm$^2$; $P = 0.10$) (scale bar = 500 nm).

(Fig. 5) for both AF ($r^2 = 0.903$; $P < 0.01$) and PF ($r^2 = 0.902$; $P < 0.01$). No correlations were observed for any of the measured cross-link types and fibril morphology. Likewise, fibril mean diameter, fibril concentration, and fibril volume fraction were unrelated to Young's modulus and peak stress (Fig. 6).

## DISCUSSION

The findings of the study were that PF from human patellar tendon reached lower peak stress and modulus values before failure compared with the AF. PF also demonstrated greater fibril density and a tendency toward smaller fibril cross-sectional area. There were no differences in fibril volume fraction. Biochemically, PF had greater concentrations of enzymatically derived HP and LP cross-links but similar collagen, PENT, and pyrrole concentrations. Finally, measures of cross-linking and fibril morphology seemed unrelated to the mechanical properties of the fascicles. Notably, PENT cross-linking correlated very strongly with subject age.

Collagen cross-links are considered essential for providing strength to dense collagen tissues (4, 5); however, few studies have directly assessed the importance of HP and LP in relation to mechanical tissue properties. HP has been shown to be related to material behavior of rabbit medial collateral ligament when the results for healing and control tissue were pooled (15). Others have shown a positive relationship between HP content and the stiffness (modulus) of goat bone-tendon-bone ACL graft and ACL (28). However, the modulus of the graft and ACL differed considerably despite comparable HP concentrations, which suggests that factors other than HP likely play an important role in tendon mechanics. It has been reported that pyridinoline may be a better indicator of ultimate tensile stress than hydroxyproline (9). In summary, pyridinium cross-links seem related to mechanical function in healing ligament and tendon tissue, but we are unaware of reports that address the importance of mature cross-links in relation to the mechanical properties of healthy human tendon.

In the present study, we were unable to show any association between the mechanical properties of the tested fascicles and the concentration of the pyridinium type cross-links HP and LP, suggesting that other factors are also important for tendon strength. One such factor could be the putative inter-fibrillar pyrrole cross-link, which has been positively associated with strength of cortical bone (20) but so far has not been explored in relation to tendon mechanics. We observed no association between pyrrole concentrations and mechanical properties of the isolated tendon fascicles. Accordingly, the present data do not support the notion that pyridinium, pyrrole cross-links, or the AGE marker PENT are sole principle determinants of tendon mechanical function. However, it must be kept in mind that the sample size of the present study was limited, which precludes firm conclusions.

Importantly, we did observe marked differences in the concentration of HP and LP between AF and PF. In humans, it has been shown that the supraspinatus tendon displays elevated HP density compared with the biceps tendon, which may reflect different loading patterns and perhaps morphology between the two tendons (6). In the present study, the observed differences in pyridinium type cross-links may relate to different loading patterns of the anterior and posterior patellar tendon regions.

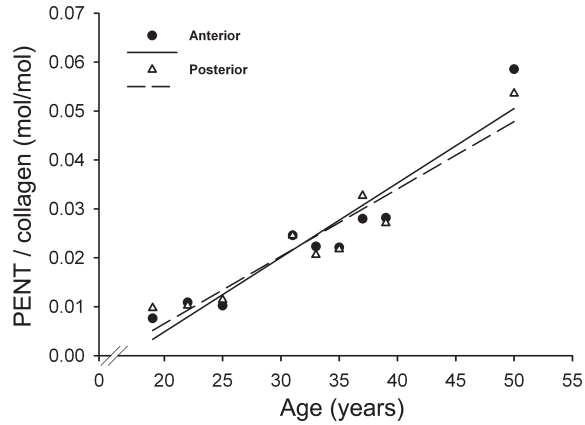



Fig. 5. First-order linear regression plot comparing pentosidine concentration against patient age. There was a remarkably linear association between the variables for both anterior (●) and posterior fascicles (△) (AF: $r^2 = 0.903$, $P < 0.01$; PF: $r^2 = 0.902$, $P < 0.01$).

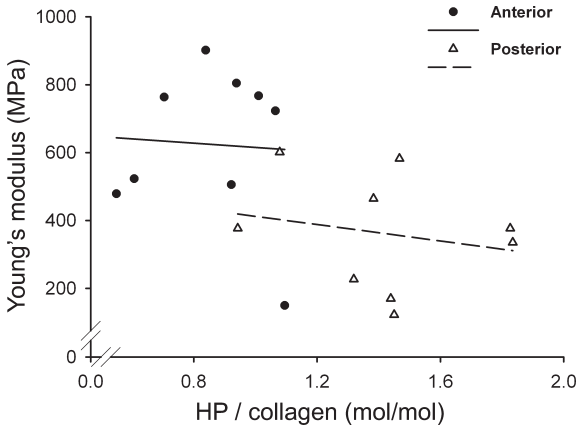



Fig. 4. First-order linear regression plot of HP concentration against Young's modulus of anterior (AF; ●) and posterior fascicles (PF; △). There was no association between HP and modulus (AF: $r^2 = -0.003$, $P = 0.9$; PF: $r^2 = -0.044$, $P = 0.6$).

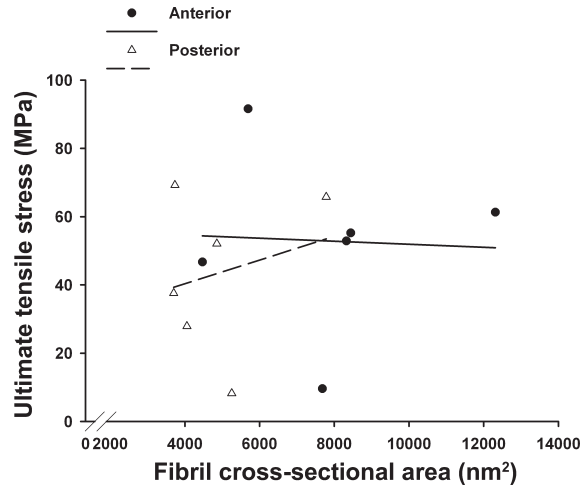



Fig. 6. First-order linear regression plot of fibril cross-sectional area against peak stress of anterior (●) and posterior fascicles (△). There was no association between fibril cross-sectional area and fascicle strength (AF: $r^2 = -0.002$, $P = 0.9$; PF: $r^2 = 0.05$, $P = 0.7$).

Adventitious, nonenzymatic cross-linking by advanced glycation end products (AGEs) has previously been shown to markedly affect mechanical properties of collagen tissues. AGE accumulation appears to increase stiffness and breaking strength in tendon tissue (2, 34), and the AGE concentration as represented by PENT is known to increase in a remarkably linear fashion with aging of various collagen tissues (6, 14, 36). Interestingly, a "leveling-off" of PENT has been reported in normal supraspinatus tendon in subjects >50 yr of age, whereas HP and collagen content did not show aberrant values (6). This pattern of cross-linking was believed to represent a history of repetitive injury and repair with deposition of newly synthesized but functionally organized tendon tissue containing low levels of PENT. In degenerate supraspinatus tendon affected by tendinitis, the authors reported abnormally high HP concentration as a likely result of either an early healing process with deposition of scar tissue or chronic fibrosis (6). The present study demonstrated a strong positive relationship between subject age and PENT (Fig. 5) in both AF and PF and, furthermore, that PENT concentration was unrelated to tendon mechanics. PENT is generally considered a suitable marker of collagen biological age (4–6). PENT has also been utilized to evaluate the degree of remodeling in tendon tissue (6). The similar levels of PENT between PF and AF do not indicate recent remodeling in the posterior tendon since newly deposited collagen from tendon repair would contain reduced amounts of PENT due to insufficient time for the constitutive process of AGE accumulation. Although we did not observe any signs of pathology, we cannot exclude the possibility that the patellar tendon grafts in the study could be affected by tendinosis to some degree since as much as one-third of jumping athletes suffer from subclinical tendinosis (24). The presence of tendinosis could have influenced mechanical, structural, and biochemical properties of the tendon samples. However, we suspect that significant degeneration of the examined grafts would have influenced the PENT concentration in the affected samples, which does not seem to be the case. However, assay of the matrix metalloproteinases, their tissue inhibitors, and collagen intermediate cross-linking would provide unequivocal evidence of this possibility and should be included in further studies.

Fibril morphology has previously been associated with mechanical properties of collagen tissues, where a positive relationship was suggested between the "mass average diameter" of fibrils and ultimate tensile strength of the tissue (30). Large-diameter fibrils are thought to possess augmented tensile strength due to greater potential for intra-fibrillar cross-links, whereas smaller fibrils are weaker but more creep resistant due to a higher surface area per unit mass, which allows for increased electrostatic interactions between fibrils and glycosaminoglycans (30). However, increased levels of glycosaminoglycans and possible resultant increased hydration have also been shown to influence the structural integrity and strength of the anterior cruciate ligament in dogs (10). Consequently, further studies must address these questions.

The present study also showed that the stronger AF tended to have a larger mean fibril area; yet, there was no relationship between the fibril morphology and mechanical properties in individual patients. Our findings corroborate those of Lavagnino et al. who could not demonstrate a relationship between fibril diameter distribution and mechanical properties in stress deprived rat tail tendons (23). Few studies have addressed the impact of altered loading on fibril morphology. A tendon lesion animal model showed a transient decrease followed by an increase in fibril diameters in noninjured tissue surrounding the lesion, which was considered to be an adaptation to increased tendon stress (due to reduced functional cross-sectional area of the noninjured tendon) with subsequent remodeling of the tendon (26). Others have observed a positive correlation between fibril diameters and ultimate strength in collagen tissues (29, 30). The present data show a tendency toward decreased fibril diameters in the posterior fascicles compared with the AF (Fig. 3), which suggests that the fibrils of the posterior tendon region may be relatively stress shielded (27). Since the similar concentration of PENT in the two regions indicates equivalent remodeling, the tendency toward smaller posterior fibril diameter likely reflects a congenitally moderated functional adaptation. Perhaps the human patellar tendon should be regarded as two distinct functional units; an anterior tendon unit and a posterior ligament unit. Anatomically, the posterior portion of patellar tendon connects the patellar sesamoid bone to the tibial tuberosity, whereas the anterior portion appears as a continuity of the quadriceps tendon. Furthermore, compared with tendon, ligaments have increased concentration of the immature cross-link dihydroxylysinonorleucine (DHLNL) [which is a precursor of HP (37)], a higher concentration of pyridinoline (36), and a larger number of small fibrils (35), which corresponds well with the present data, although obviously further studies are warranted.

We hypothesized that region-dependent differences in mechanical properties would exist between the anterior and posterior regions of the patellar tendon and that such differences would be associated with alterations in collagen fibril morphology and the biochemical cross-link composition of the tendon fascicles. We observed inferior mechanical strength of the posterior tendon. The reduced strength of the posterior tendon was accompanied by more densely packed fibrils that displayed a tendency toward smaller cross-sectional areas. Fibril morphology appeared unrelated to mechanical properties, although it should be noted that the sample size was limited. Biochemically, PENT concentration was similar in the anterior and posterior tendon. Surprisingly, the concentration of mature HP and LP cross-links was greater in the posterior and weaker part of the patellar tendon. The lower levels of HP and LP in the anterior tendon were not accounted for by the putative interfibrillar pyrrole cross-link. These findings suggest region-specific differences in mechanical, structural, and biochemical properties of the human patellar tendon.

## REFERENCES


1. **Almekinders LC, Vellema JH, Weinhold PS.** Strain patterns in the patellar tendon and the implications for patellar tendinopathy. *Knee Surg Sports Traumatol Arthrosc* 10: 2–5, 2002.
2. **Andreassen TT, Oxlund H, Danielsen CC.** The influence of non-enzymatic glycosylation and formation of fluorescent reaction products on the mechanical properties of rat tail tendons. *Connect Tissue Res* 17: 1–9, 1988.
3. **Avery NC.** Quantitative determination of collagen cross-links. In: *Methods in Molecular Biology. Vol. 522. Extracellular Matrix Protocols*. New York: Humana Press, 2009, p. 103–121.
4. **Avery NC, Bailey AJ.** Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. *Scand J Med Sci Sports* 15: 231–240, 2005.

5. **Bailey AJ, Paul RG, Knott L.** Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* 106: 1–56, 1998.
6. **Bank RA, TeKoppele JM, Oostingh G, Hazleman BL, Riley GP.** Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon collagen: changes with age and in chronic rotator cuff tendinitis. *Ann Rheum Dis* 58: 35–41, 1999.
7. **Basso O, Amis AA, Race A, Johnson DP.** Patellar tendon fiber strains: their differential responses to quadriceps tension. *Clin Orthop* 246–253, 2002.
8. **Butler DL, Grood ES, Noyes FR, Zernicke RF.** Biomechanics of ligaments and tendons. *Exerc Sport Sci Rev* 6: 125–181, 1978.
9. **Chan BP, Fu SC, Qin L, Rolf C, Chan KM.** Pyridinoline in relation to ultimate stress of the patellar tendon during healing: an animal study. *J Orthop Res* 16: 597–603, 1998.
10. **Comerford EJ, Tarlton JF, Avery NC, Bailey AJ, Innes JF.** Distal femoral intercondylar notch dimensions and their relationship to composition and metabolism of the canine anterior cruciate ligament. *Osteoarthritis Cartilage* 14: 273–278, 2006.
11. **Creemers LB, Jansen DC, van Veen-Reurings A, van den BT, Everts V.** Microassay for the assessment of low levels of hydroxyproline. *Biotechniques* 22: 656–658, 1997.
12. **Davankar SP, Deane NJ, Davies AS, Firth EC, Hodge H, Parry DA.** Collagen fibril diameter distributions in ligaments and tendons of the carpal region of the horse. *Connect Tissue Res* 34: 11–21, 1996.
13. **Dillon EM, Erasmus PJ, Muller JH, Scheffer C, de Villiers RV.** Differential forces within the proximal patellar tendon as an explanation for the characteristic lesion of patellar tendinopathy: an in vivo descriptive experimental study. *Am J Sports Med* 36: 2119–2127, 2008.
14. **Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW.** Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 91: 2463–2469, 1993.
15. **Frank C, McDonald D, Wilson J, Eyre D, Shrive N.** Rabbit medial collateral ligament scar weakness is associated with decreased collagen pyridinoline crosslink density. *J Orthop Res* 13: 157–165, 1995.
16. **Haraldsson BT, Aagaard P, Krogsgaard M, Alkjaer T, Kjaer M, Magnusson SP.** Region-specific mechanical properties of the human patella tendon. *J Appl Physiol* 98: 1006–1012, 2005.
17. **Johnson DP, Wakeley CJ, Watt I.** Magnetic resonance imaging of patellar tendonitis. *J Bone Joint Surg Br* 78: 452–457, 1996.
18. **Kadler KE, Holmes DF, Trotter JA, Chapman JA.** Collagen fibril formation. *Biochem J* 316: 1–11, 1996.
19. **Khan KM, Bonar F, Desmond PM, Cook JL, Young DA, Visentini PJ, Fehrmann MW, Kiss ZS, O'Brien PA, Harcourt PR, Dowling RJ, O'Sullivan RM, Crichton KJ, Tress BM, Wark JD.** Patellar tendinosis (jumper's knee): findings at histopathologic examination, US, and MR imaging. Victorian Institute of Sport Tendon Study Group. *Radiology* 200: 821–827, 1996.
20. **Knott L, Whitehead CC, Fleming RH, Bailey AJ.** Biochemical changes in the collagenous matrix of osteoporotic avian bone. *Biochem J* 310: 1045–1051, 1995.
21. **Kongsgaard M, Kovanen V, Aagaard P, Doessing S, Hansen P, Laursen AH, Kaldau NC, Kjaer M, Magnusson SP.** Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand J Med Sci Sports*. In press. DOI:10.1111/1.1600-0838.2009.00949.X.
22. **Lavagnino M, Arnoczky SP, Elvin N, Dodds J.** Patellar tendon strain is increased at the site of the jumper's knee lesion during knee flexion and tendon loading: results and cadaveric testing of a computational model. *Am J Sports Med* 36: 2110–2118, 2008.
23. **Lavagnino M, Arnoczky SP, Frank K, Tian T.** Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons. *J Biomech* 38: 69–75, 2005.
24. **Lian O, Scott A, Engebretsen L, Bahr R, Duronio V, Khan K.** Excessive apoptosis in patellar tendinopathy in athletes. *Am J Sports Med* 35: 605–611, 2007.
25. **Magnusson SP, Qvortrup K, Larsen JO, Rosager S, Hanson P, Aagaard P, Krogsgaard M, Kjaer M.** Collagen fibril size and crimp morphology in ruptured and intact Achilles tendons. *Matrix Biol* 21: 369–377, 2002.
26. **Matthew C, Moore MJ, Campbell L.** A quantitative ultrastructural study of collagen fibril formation in the healing extensor digitorum longus tendon of the rat. *J Hand Surg [Br]* 12: 313–320, 1987.
27. **Muellner T, Kwasny O, Loehnert V, Mallinger R, Unfried G, Schabus R, Plenk H Jr.** Light and electron microscopic study of stress-shielding effects on rat patellar tendon. *Arch Orthop Trauma Surg* 121: 561–565, 2001.
28. **Ng GY, Oakes BW, Deacon OW, McLean ID, Eyre DR.** Long-term study of the biochemistry and biomechanics of anterior cruciate ligament-patellar tendon autografts in goats. *J Orthop Res* 14: 851–856, 1996.
29. **Parry DA.** The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. *Biophys Chem* 29: 195–209, 1988.
30. **Parry DA, Barnes GR, Craig AS.** A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proc R Soc Lond B Biol Sci* 203: 305–321, 1978.
31. **Patterson-Kane JC, Wilson AM, Firth EC, Parry DA, Goodship AE.** Comparison of collagen fibril populations in the superficial digital flexor tendons of exercised and nonexercised thoroughbreds. *Equine Vet J* 29: 121–125, 1997.
32. **Provenzano PP, Vanderby R Jr.** Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. *Matrix Biol* 25: 71–84, 2006.
33. **Puxkandl R, Zizak I, Paris O, Keckes J, Tesch W, Bernstorff S, Purslow P, Fratzl P.** Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos Trans R Soc Lond B Biol Sci* 357: 191–197, 2002.
34. **Reddy GK.** Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit achilles tendon. *Exp Diabesity Res* 5: 143–153, 2004.
35. **Rumian AP, Wallace AL, Birch HL.** Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features: a comparative study in an ovine model. *J Orthop Res* 25: 458–464, 2007.
36. **Suzuki D, Takahashi M, Abe M, Nagano A.** Biochemical study of collagen and its crosslinks in the anterior cruciate ligament and the tissues used as a graft for reconstruction of the anterior cruciate ligament. *Connect Tissue Res* 49: 42–47, 2008.
37. **Viguet-Carrin S, Garnero P, Delmas PD.** The role of collagen in bone strength. *Osteoporos Int* 17: 319–336, 2006.

# Study IV

# Biomechanical properties and cross-link composition of ruptured human Achilles tendon

**Hansen P[1], Kovanen V[2], Hölmich P[3], Krogsgaard M[4], Hansson P[1], Dahl M[1], Hald M[1], Aagaard P[5], Kjaer M[1], Magnusson SP[1]**

[1] *Institute of Sports Medicine Copenhagen, Bispebjerg Hospital & Center for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, Denmark*
[2] *Department of Health Sciences, Biochemistry Laboratory, University of Jyväskylä, Finland*
[3] *Department of Orthopedic Surgery, Amager Hospital, Copenhagen, Denmark*
[4] *Department of Orthopedic Surgery, Bispebjerg Hospital, Copenhagen, Denmark,*
[5] *Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark*

**Abstract**

**Introduction:** The human Achilles tendon is the most commonly ruptured Achilles tendon in humans. Tendon degeneration is suspected to cause weakening of the tendon, however no attempts have been made to evaluate the mechanical properties in ruptured and healthy Achilles tendon. We hypothesized that tendon fascicles from acute Achilles tendon ruptures would be weaker compared to both non-injured tissue proximal to the rupture site and to cadaver Achilles tendon. Mechanical weakening was expected to be accompanied by changes in biochemical cross-link composition. **Materials & Methods:** Biopsies were obtained during surgical repair in patients with acute Achilles tendon ruptures. Additional samples were collected from age and weight matched cadaver Achilles tendon. Tendon fascicles were dissected from the biopsies and tested micromechanically. Additional tendon material underwent biochemical analyses for collagen content and cross-link composition. **Results:** Young´s modulus was greater in external cadaver controls than in fascicles from ruptured tendons ($p<0.01$) and internal control fascicles ($p<0.01$). Failure stress in external controls did not differ from rupture fascicles ($p=0.16$) but tended to be different from internal control fascicles ($p=0.06$). Energy absorption and fascicle strain to failure did not differ between rupture fascicles, internal controls and external (cadaver) control fascicles. Collagen content was greater in external cadaver controls compared to the rupture patients ($p<0.001$). Collagen content, lysylpyridinoline (LP), hydroxylysylpyridinoline (HP) and pentosidine (PENT) did not display regional differences in rupture patients. PENT was similar in cadaver tendon compared to both the rupture site ($p=0.9$) and internal control site ($p=0.9$) while HP content tended to be greater in cadaver tendons. PENT was positively related to age in rupture site ($r^2=0.70$; $p<0.01$), internal control site ($r^2=0.72$; $p<0.01$) and cadaver ($r^2=0.44$;$p<0.05$) samples. Collagen content was positively related to failure stress but only in rupture fascicles ($r^2=0.36$; $p<0.05$). In all subject groups HP, LP and pentosidine content were unrelated both to failure stress and Young´s modulus, respectively. **Conclusion:** the present data imply a mechanical weakening of the ruptured Achilles tendon, which extends beyond the rupture site. This indicates a generalized involvement of the entire tendon. The weakening may relate to reduced type I collagen content. Notably, the measured cross-links do not appear to be associated with impaired mechanical properties of individual tendon fascicles in ruptured and uninjured tendons. In fact, elevated pyridinoline levels in both ruptured and control cadaver Achilles tendons may reflect an impaired repair process that relates to mechanical weakness of the Achilles tendon.

## Introduction

The human Achilles tendon is particularly prone to complete rupture in comparison to other tendons and especially so in western populations (30; 33; 50; 51; 57). Not surprisingly, the Achilles tendon has been studied extensively to reveal the underlying injury

mechanisms. Although there is convincing histopathological evidence of numerous adverse changes in the tendon tissue such as disorganization of collagen fibre bundles, increased amounts of glycosaminoglycans (GAGs) and type III collagen, deposition of lipids, calcification of collagen fibres, vascular abnormalities with signs of cellular hypoxia and variations in cellular morphology (35; 36; 38; 51; 68), there is insufficient understanding of the underlying disease processes. A recent comprehensive study by de Mos et al. demonstrated that Achilles tendinosis was accompanied increased MMP activity and changes in collagen hydroxylation and cross-link composition which suggestive of an early repair process due to microruptures (15). Jones et al found distinct changes in the expression of extracellular proteinases in painful compared to ruptured Achilles tendon and suggested that the conditions were different phenotypes (34). However, to which extent mechanical weakening results from such changes is currently not known. Some authors have proposed that a mere mismatch between tendon strength and excessive mechanical force can lead to rupture of an otherwise healthy Achilles tendon (31).

However, a mechanical weakening as a predisposing contributor to rupture must be suspected since degeneration of the tendon seems to be more the rule than the exception albeit a causal relation has not been established (2; 11; 37; 38).

Studies of ruptured Achilles tendons at the sub-microscopic level have demonstrated a decreased cross-sectional area and other abnormalities of the important load-bearing collagen fibrils that (36; 38; 55). Reduced fibril CSA is expected to reduce the strength of the tendon (60). However, studies have failed to demonstrate a clear association between fibril size and mechanical properties within a distinct tissue type including tendon (16; 25; 49; 61). Strength of collagen fibrils is not solely dependent on fibril diameter since it also depends on enzymatic intermolecular cross-links that covalently link neighbouring collagen molecules (4; 5). Strength of collagen fibrils is thought to derive from intermolecular cross-links that are enzymatically introduced by lysyl oxidase. Cross-links are strong chemical bonds that link specific telopeptide and helical regions of neighbouring collagen molecules. The pyridinium type cross-links lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) are considered to be important for the mechanical strength of collagen type I fibrils (5; 60). Non-enzymatic cross-linking by advanced glycation end products (AGEs) physically stabilize collagen tissues further with aging and pentosidine (PENT) is a biomarker of AGE accumulation. In human tendon, changes in the cross-link composition have been linked to tendon degeneration and injury (7; 15; 17). However, to our knowledge no studies have attempted to compare concomitant changes in the cross-link composition and mechanical properties in ruptured human Achilles tendons.

We hypothesized that tendon fascicles from acute Achilles tendon ruptures would be weaker compared to both non-injured tissue proximal to the rupture site and to control tissue from cadaver Achilles tendons. It was also hypothesized that the weakening of injured tendons would be accompanied by changes in biochemical cross-link composition at the site of injury.

**Materials & methods**

*Ethical considerations*

Achilles tendon rupture patients were recruited from two local hospitals in Copenhagen, Denmark. The study was approved by the local ethics committee. All patients gave written informed consent before inclusion in the study. Harvesting of cadaver material was performed in the state of Arkansas, USA. Tendon material was obtained during forensic autopsy. Approval was obtained by the local ethics committee.

*Patient inclusion:*

15 males and 2 females (39±8 years; 28-53 years; 85±18 kg; BMI: 26±5 kg/m$^2$) were included in the study. Additionally, Cadaver Achilles tendon material was obtained from 10 males without known Achilles tendon pathology (40±years; 30-55 years; 78±9 kg; BMI: 24.2±2.2 kg/m$^2$). Inclusion criteria for rupture patients were: (1) Acute Achilles tendon rupture, (2) open surgical repair performed within ≤ 48 hours from trauma. Exclusion criteria were: (1) age > 65, (2) previous ipsilateral rupture, (3) sharp or blunt trauma to the Achilles tendon, (4) systemic disease: rheumatoid arthritis and other connective tissue diseases including Marfan´s/Ehlers Danlos syndrome, diabetes, vascular disease (ischemic/atherosclerotic), (4) previous systemic corticosteroid treatment, (6) peritendinous corticosteroid injection within 2 months before trauma, (7) other than Caucasian ethnicity.

*Biopsy procedure*

Achilles tendon biopsies were obtained during open surgical repair. Average time form tendon rupture to surgical repair was ~26 hours (range: 14-48 hours). One thin collagen bundle ~ 20-25 mm in length, 3-5 mm in width was gently isolated from the rupture site (denoted rupture biopsy). Care was taken not to apply any tensile loading that could damage the biopsy or alter its mechanical properties. A similar sized bundle was then obtained as far proximally as possible from an area where the tendon appeared macroscopically healthy. The biopsy was obtained within the limits of a conventional skin incision. The internal mechanical control biopsy (internal control) was always obtained at least 15-20 mm proximal to the proximal end of the rupture biopsy. The biopsy was wrapped in 0.15M phosphate buffered saline (PBS) moistened gauze and frozen at –80º C. Subsequently, two well-defined

fascicles were dissected from the biopsies. The fascicles underwent subsequent micromechanical testing. Prior to dissection the biopsies were allowed to thaw while maintained moist by PBS (0.15M). The fascicles were then re-frozen within 1-2 hours until subsequent micromechanical testing. For biochemical analysis, one small rupture biopsy ~ 5 x 5 mm was obtained from the central (core) area of one of the ruptured tendon ends and one similar sized internal control biopsy was harvested from the proximal tendon approximately 50-60 mm proximal to the rupture site. The biopsies were gently washed in PBS (0.15M) to remove blood residues. Subsequently, the biopsies were snap frozen in liquid nitrogen in the operating theatre in cryotubes and subsequently stored at at –80º C until biochemical analysis.

*Cadaver material*
The cadaver tissue served as external control for both mechanical tests (denoted external control) and cross-link measurements. Biopsies were obtained within ≤ 24 hours post mortem. Biopsies ~ 30 mm were obtained from the distal part of the tendon. The biopsy was wrapped in sterile gauze and stored at –80º C until biomechanical measurements, at which time the biopsy was allowed to thaw at room temperature while kept moist by PBS (0.15M) to allow for dissection of two well-defined fascicles from the biopsy. The fascicles were then re-frozen until micromechanical testing. Additionally, ~20 mg (dryweight) was harvested from the biopsy for biochemical analyses.

*Mechanical testing procedures*
Fourteen rupture, 14 internal control and six external control fascicles were tested micromechanically.
Measurements were performed using a micro-tensile testing apparatus (200 N tensile stage, petridish version, Deben Ltd., Suffolk, UK). The dissected fascicles were prepared for mechanical testing by reducing the length to ~ 20 mm. The methodology was described in detail previously (27). Briefly, to strongly fix the fascicle to the mounting plates of the micromechanical rig each 5 mm end of the fascicle was allowed to air dry at room temperature until this section had a hard pellucid appearance, while the central region of the fascicle remained wrapped in PBS-soaked (0.15M) gauze. The dry fascicle ends were glued with cyanoacrylate to uncoated, rifled aluminum specimen mounting plates of the mechanical rig. The fascicle was then immersed and hydrated for 20 min in PBS solution (0.15M) in a Petri dish where it remained during mechanical testing. Strain rate was 2.0 mm/min. The initial specimen testing length (mounting plate-to-mounting plate distance) was ~10 mm.

*Micromechanical testing*
The cross-sectional area of each fascicle was calculated based on the three diameters measured from stereomicroscopic images at three locations (right, mid and left one-third) along the length of the specimen. Images were obtained while the fascicle was mounted and immersed in solution (PBS 0.15M) in the experimental setup and diameters were measured.

*Biochemical analysis*
Sixteen rupture, 16 internal control and 10 external control samples were analyzed.
Prior to analysis tendon samples were freeze-dried. The samples underwent hydrolyzation in HCL (+108° C, 24h). They were evaporated into dryness and then dissolved in $H_2O$. Collagen specific hydroxyproline was measured by spectrophometry as described by Creemers et al. (13). Collagen content was expressed as mg/mg dry weight. The analyses of pyridinium cross-links (HP, LP) and PENT are described in detail elsewhere (6; 46). The calculation of collagen crosslink concentrations was based on the use of pure compounds of HP, LP and PENT as external standards in each HPLC run. The HPLC system included: Quaternary Gradient Pump unit, PU-2089 Plus, Intelligent Autosampler AS-2057 Plus, Intelligent Fluorescence Detector, FP-2020. The applied column was a LiChroCART® 125-4 column (Merck KGaA, Darmstadt, Germany). Data processing software was Jasco Chrompass (Jasco Scandinavia AB, Möllndal, Sweden). The quantities of cross-links were expressed as mol/mol collagen.

**Data reduction and analysis**
*Exclusion from analyses*
One subject was excluded from all analyses since surgery revealed that the rupture was a complete avulsion of the Achilles tendon insertion on the calcaneus, whereas all other patients suffered a rupture of the tendon proper. Fascicles from two patients could not be analyzed micromechanically due to technical problems. Still, biochemical analyses were performed on the two patients.

*Micromechanical testing:*
The smallest cross-sectional area of the three measurements was used for calculation of stress. Fascicle stress was calculated as the tensile force (N) divided by the cross-sectional area ($mm^2$) of the fascicle and reported in Pascal (MPa; $10^6$ $N/m^2$). Failure stress was defined as the highest stress measured during failure testing of the fascicle. The resting length ($L_0$) of the fascicle was defined as the length at force onset, which was obtained by slowly straining the fascicle (0.5 mm/min) until the delta change in instantaneous passive fascicle force exceeded the mean value of the preceding 100 datapoints by 0.075% of peak passive force (~ 0.03N). Fascicle strain, calculated from the instantaneous distance between specimen mounting plates, was defined as $\Delta L$ divided by $L_0$ and was expressed as a percentage [($\Delta L/L_0$) x 100]. Young´s tangent modulus was calculated in the linear part of the stress-strain

curve (for typical stress-strain plots, see (25; 27)). The analysis of fascicle stiffness and energy absorption was performed at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 percent of peak failure force. Young´s modulus was reported for the 10% interval that yielded the highest value. Energy absorption at failure was calculated by integrating the area below the force-deformation curve from force onset ($L_0$) until peak force and reported as $mJ/mm^2$.

*Statistical analysis*

All data were examined for normality by a D'agostino Omnibus normality test. Differences between rupture tissue vs. internal control tissue were investigated by a two-tailed paired t-test for normally distributed data or a Wilcoxon matched pairs test for non-Guassian data. Differences between external controls and rupture tendons were examined by a two-tailed unpaired t-test or a Mann-Whitney test for non-parametric data. The fascicle that yielded the highest failure stress was used for analysis. To determine associations between biochemical and biomechanical parameters linear regression analysis was performed using Pearson correlation analysis. In case of non-Gaussian distribution of data a Spearman's rho test was used. For all statistical analyses a 0.05 level of significance was applied. Results are reported as group mean ± SD.

**Results**

*Micromechanical testing:*

Mechanical data are summarized in Table 1. Young´s modulus was greater in external cadaver controls than in fascicles from ruptured tendons ($p<0.01$) and internal control fascicles ($p<0.01$). Failure stress in external controls did not differ from rupture fascicles ($p=0.16$) but tended to be different from internal control fascicles ($p=0.06$). Energy absorption and fascicle strain to failure did not differ between rupture fascicles, internal controls and external (cadaver) control fascicles (failure energy: p=0.27-0.67; strain: p=0.18-0.26).

*Biochemical measurements:*

Data are summarized in Table 2. Collagen content was greater in external cadaver controls compared to the rupture site ($p<0.001$) and proximal internal control site ($p<0.001$) (Figure 1). Collagen content, HP, LP and PENT did not display any differences between the rupture site and the proximal tendon (internal control) (Collagen content: p=0.46; HP: p=0.65; LP: p=0.81; PENT: p=0.76).

**Table 1**: Fascicle mechanical properties.

| Achilles tendon | Rupture (n=14) | Internal control (n=14) | External control (n=6) |
|---|---|---|---|
| Failure stress (MPa) | 33.7±19.2 | 30.0±14.9 | 44.1±13.1[*#] |
| Strain (%) | 21.0±9.1 | 18.9±4.4 | 15.9±4.6 |
| Young´s modulus (MPa) | 256.7±100.8 | 262.4±111.5 | 512.9±209.6[a,b] |
| Failure energy ($mJ \cdot mm^2$) | 33.8±21 | 25.4±16.3 | 30.0±6.2 |

Values are mean±SD. [a]Larger than rupture ($p<0.01$) Larger than internal control ($p<0.01$).
[*] Higher than rupture ($p=0.16$). [#]Higher than internal control ($p=0.06$)

**Table 2**: Achilles tendon collagen content and cross-link composition.

| Achilles tendon | Rupture (n=16) | Internal control (n=16) | External control (n=10) |
|---|---|---|---|
| LP (mol/mol) | 0.090±0.024 | 0.091±0.014 | 0.096±0.016 |
| HP (mol/mol) | 1.891±0.215 | 1.855±0.359 | 2.074±0.280 |
| PENT (mol/mol) | 0.024±0.009 | 0.024±0.009 | 0.024±0.013 |

Values are mean±SD.

PENT was similar in cadaver tendon compared to both the rupture site ($p=0.9$) and internal control site ($p=0.9$) while HP content tended to be greater (2.074±0.280 mol/mol collagen) than the rupture site (1.891±0.215 mol/mol collagen; $p=0.07$) and internal control site (1.855±0.359 mol/mol collagen; $p=0.12$). LP content did not differ between samples from the rupture site, internal control site and external controls (p=0.46-0.80).

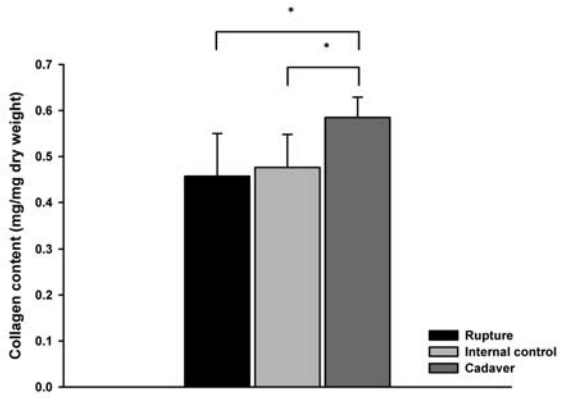



**Figure 1.** *Total collagen content in biopsies of ruptured Achilles tendon (n=16) compared to non-injured material in the same tendon (n=16) and cadaver control Achilles tendons (n=10). (* p≤0.05).*

*Regression analyses:*

PENT was positively related to age in rupture site ($r^2=0.70$; $p<0.01$), internal control site ($r^2=0.72$; $p<0.01$) and cadaver ($r^2=0.44$;$p<0.05$) samples (Figure 2). PENT content at the rupture site was positively related to PENT content at the internal control site ($r^2=0.81$; $p<0.01$) (Figure 3). Collagen content was positively related to failure stress but only in rupture fascicles ($r^2=0.36$; $p<0.05$) (Figure 4). In all subject groups (rupture, internal control and external control fascicles) HP, LP and pentosidine content were unrelated both to failure stress and Young´s modulus, respectively. HP displayed a negative relation to age in rupture site samples ($r^2=0.32$; $p<0.05$) (Figure 5), a similar slight trend in internal controls ($r^2=0.11$;

p=0.20) and no association in external control samples ($r^2$=0.19; p=0.60).

**Discussion**

To our best knowledge, this is the first study on ruptured human Achilles tendons that attempts to examine both tendon mechanical properties and biochemical composition in the same individual. We hypothesized that the ruptured tissue would be weaker and contain reduced cross-link density and collagen content. The main findings of the study were that tendon fascicles obtained from the rupture site in patients with a spontaneous Achilles tendon rupture showed similar mechanical strength, cross-link density and collagen content compared to an internal control sample from a healthy appearing area of the same tendon proximally to the rupture site (Figure 3). In contrast, control tissue from human cadaver Achilles tendon contained significantly greater amounts of collagen, displayed increased Young´s modulus and tended to have greater tensile strength than tendon material from rupture patients.

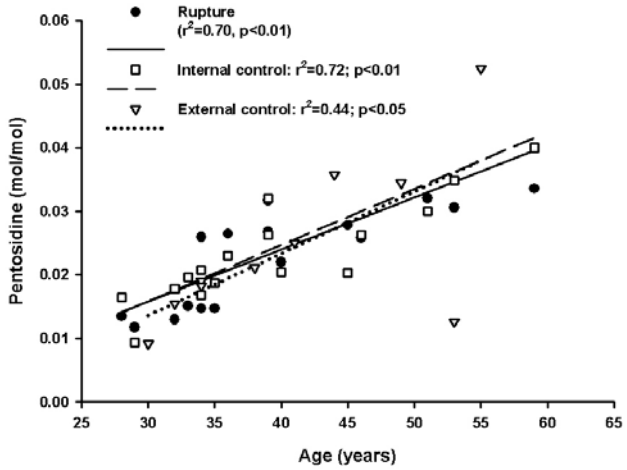



**Figure 2.** *Pentosidine content plotted agains subject age. For all subject groups pentosidine was positively correlated with age. Pentosidine content is considered a marker of collagen remodelling.*

Degenerative changes in the Achilles tendon seem strongly associated with rupture (2; 32; 38; 52; 68). However, a direct causal relation between degeneration and rupture has never been demonstrated in ruptured Achilles tendon and some authors have suggested that an excessive mechanical load may cause rupture in an otherwise healthy Achilles tendon (31). Since the injury most frequently occurs at a relatively young age when significant degeneration is otherwise not expected (30-40 years) and since incidence seems to decline with further aging this standpoint cannot be entirely dismissed. From a structural point of view, the typical rupture site of 3-6 cm above the calcaneus coincides with the narrowest area of the tendon (45), which results in greater tendon stress (force divided by CSA) for a given force, and in turn reduces the safety factor (i.e. the ratio of ultimate tensile strength to peak forces, in vivo) The fact that persons with an acute rupture seem to have smaller tendon CSA than non-injured runners (45) give some support to the notion that there may be a mismatch between tendon strength and the imposed load. Peak tendon forces can reach in excess of 2-3 kN for the Achilles tendon during jumping (19; 20) which equals a stress of ~70 MPa. This value is in accordance with other studies on Achilles whole tendon (40; 71). Tendons have been reported to fail at a stress of ~100 MPa (9).

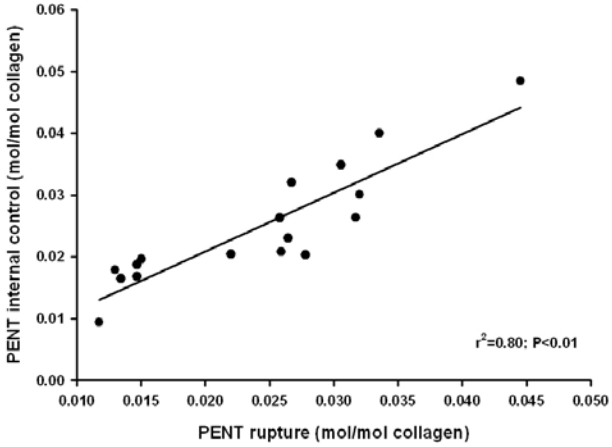



***Figure 3.*** *Pentosidine content at the site of rupture compared to a non-injured region of the same tendon. Pentosidine content was positively associated between the two sites, which indicates similar degrees of remodelling.*

Hence the human Achilles tendon possesses a safety factor of ~1.5 (45) while the more rarely ruptured human patellar tendon has a safety factor of ~2.5, assuming a peak load of 4000 kN (20) and a CSA of 100 $mm^2$ (12; 46; 59; 70). As such the Achilles tendon seems to operate close to its failure limit in vivo, and therefore even small perturbations in Achilles tendon strength may predispose to rupture (54).

We found reduced Young´s modulus (Table 1) in Achilles tendon fascicles from rupture patients both at the site of rupture as well as at non-rupture site when compared to external control fascicles obtained from matched (age, weight) non-injured individuals. Similarly, peak stress of the rupture fascicles and internal controls were of a lower magnitude compared to external control samples, albeit not reaching statistically significance (Table 1), which however may be due to the limited sample size of external controls (n=6). These data indicate for the first time that the Achilles tendons from persons with an acute rupture have altered mechanical properties and potentially reduced mechanical strength, which may predispose the tendon to rupture.

Our biochemical analyses revealed that collagen content of external controls exceeded that of rupture patients (Figure 1). Tendon is composed

primarily of collagen type I structurally organized into collagen fibrils. A previous study has demonstrated a reduced fibril area in the core of ruptured Achilles tendons (55). Since the fibril is the smallest functional tensile bearing unit in tendon and principally made of type I collagen, it is possible that the smaller collagen content in rupture tissue represents a reduction of the tensile load bearing material, which may have contributed to tendon rupture. Interestingly, we found a positive relation between collagen content and strength of fascicles from the rupture site (Figure 4), however the association was not apparent for internal control and cadaver fascicles. It is noteworthy that both reduced fibril area and collagen content have been described in human tendon degeneration (7; 46; 65). Moreover, a previous study on human Achilles tendon found reduced amounts of collagen in ruptured tendons compared to healthy cadaver material (17) while de Mos et al. observed a similar trend in tendinopathic Achilles tendons (15). It is tempting to speculate that bulk loss of collagen content may be an important etiologic factor for Achilles tendon rupture (Figure 4). Such loss may be exacerbated by increased proteolytic activity through MMPs and aggrecanases acting on collagen and proteoglycans (15; 34; 39).

Enzymatically induced cross-links are believed to confer strength to collagen fibrils (5) and abnormalities in collagen cross-linking have dramatic deleterious effects on various organ systems such as in the heritable collagen diseases Ehlers-Danlos Syndrome and Marfan's Syndrome (5; 18; 56) and a dramatic decrease in tensile strength has been demonstrated in rat-tail tendons from rats fed with the lathyric agent β-APN (63). Conversely, artificially increased amounts of mature cross-links have been associated with increased tensile strength in rat tail tendon (14). Therefore, we did not expect to find equal amounts of HP and LP cross-links in rupture, internal control and cadaver control material. Bank et al. investigated the biochemical composition of intact and degenerated human supraspinatus tendons (rotator-cuff tear) compared to biceps tendons (7). High amounts of HP and LP were found in severely degenerated supraspinatus tendons compared to biceps tendon. In Achilles tendon de Mos et al. reported equal amounts of HP and LP in tendinopathic and cadaver tendons while collagen hydroxylation was greater in tendinopathic tendons (15). Both groups proposed that an early fibrotic repair process could explain the biochemical observations. Interestingly, when we compare our present data with previous measurements of cross-links in the anterior region of uninjured human patellar tendon (12; 25) and patellar tendinopathy patients (46), the Achilles tendon seems to contain greater amounts of HP and LP (Table 1). In the posterior and more frequently injured part of the patellar tendon (the classical region of the jumper´s knee lesion) high HP levels were noted which we interpreted as likely congenital or functional in nature since PENT was unaltered. Moreover, HP and LP did not appear to correlate to Young´s modulus or failure stress of individual fascicles (25). Although it seems contradictory to the notion that mature cross-links confer strength to collagen tissues, it is in agreement with previous findings on the patellar tendon (44). The present data support the notion that pyridinoline may not be tri-functional but rather divalent as previously suggested (5; 8), although this has been subject to debate (29). Thus, pyridinoline may not confer appreciable strength compared to 'immature' cross-links. However, HP has been positively associated with Young´s modulus in horse tendon (23).

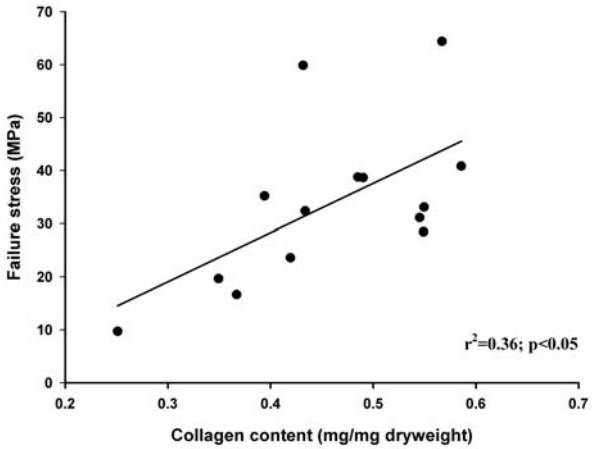



**Figure 4.** *Collagen content plotted against failure stress (n=14). Collagen content was positively associated failure stress of fascicles from the rupture site.*

Eriksen et al. investigated the biochemical composition of ruptured human Achilles tendons and cadaver controls and reported an age-related reduction of the pyridinoline cross-linked collagen type 1 carboxy-terminal telopeptide (ICTP), which suggested a change in the cross-link type to non-pyridinoline compounds (17). The authors speculated that a novel cross-link found in skin could perhaps explain decline in ICTP. Moriguchi et al. previously described a similar decrease in pyridinoline content of the human Achilles tendon decreases after the age of 30 years and suggested that pyridinoline was transformed into other compounds with aging (58). We observed small age related decline ($r^2$=0.3; p=0.05) in HP content in rupture samples ($r^2$=0.1; p=0.2) (Figure 5). However, we are hesitant to speculate whether these subtle regional differences have implications for Achilles tendon rupture since HP levels seem similar for the age range of 30-40 years when Achilles tendon rupture is most prevalent HP levels seem similar (Figure 5). Birch et al. recently described the presence of a mature cross-link histidinohydroxylysinonorleucine (HHL) abundant in skin to be present in some types of horse tendon. However, the authors but did not examine its mechanical importance (10). We recently assessed

whether the tri-valent enzymatic cross-link pyrrole was related to fascicle strength of the patellar tendon (25). Pyrrole derives from the same precursor as HP (3; 69) and has been positively associated with bone strength, perhaps owing to the formation of inter-microfibrillar (as opposed to intrafibrillar) cross-links (43). However, we found no association between pyrrole and mechanical properties for the patellar tendon (25). Future studies should seek to clarify whether HHL or other types of cross-link are of mechanical importance in the human tendon.

Collagen type III is known to posses less tensile strength than type I collagen and increased amounts of collagen type III may accumulate in ruptured Achilles tendons over a long period of time, while collagen III content appeared to be lower in uninjured cadaver tendons at a site equivalent to the rupture site (17). Collagen type III contains pyridinoline, however the molecule only has a single telopeptide cross-linking site compared to two sites in collagen I and it contains only half as many hydroxylation residues (7; 26; 28). In the present study HP tended to be higher in uninjured cadaver tendons compared to both rupture (p=0.07) and internal control samples (p=0.12). Hence, it is possible that ruptured tendons contained significant amounts of type III collagen (7).

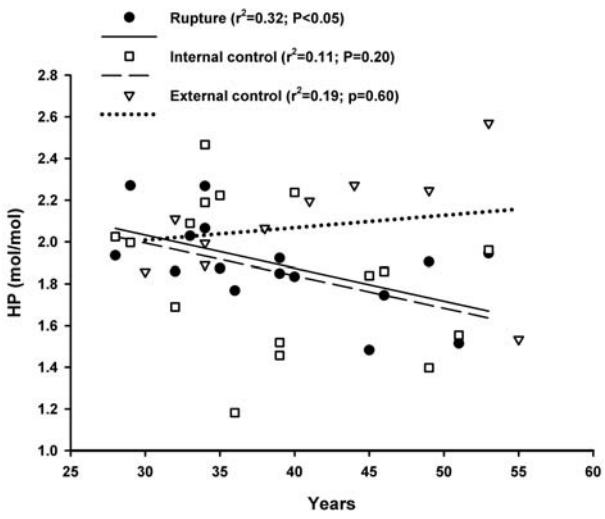



**Figure 5.** *Hydroxylysylpyridinoline plotted against subject age in injured tendons (n=16) and cadaver control tendons (n=10). In biopsies from the rupture site, that HP content was negatively associated with age.*

Lastly, in the present study the AGE marker pentosidine was strongly associated with age (Figure 2). Such an relationship is expected in collagen tissue (4) and has been demonstrated previously in human tendon (7; 12; 67). Bank et al. proposed PENT as a marker of tendon remodelling and interestingly, the authors found a levelling off of the accumulation of PENT in human supraspinatus tendons > 50 years, while the accumulation seemed uninterrupted in the biceps tendons (7). Newly deposited collagen lacks PENT and the authors concluded that repetitive injury had led to a replacement of some 50 % of the collagen in supraspinatus tendons. We found no differences in PENT levels between rupture, internal control and cadaver samples. Also, we observed no sudden slowing of the accumulation of PENT with age (Figure 2), however only few patients and cadavers were > 50 years and none > 60 year which precludes the evaluation of changes during seniti. However, our data on PENT indicate that there is no marked deposition of newly made collagen even in rupture patients, which would speak against the notion of an ongoing repair process prior to rupture. Alternatively, if a degenerative or remodelling process progresses slowly a noticeable levelling off phenomenon may not occur. Under those circumstances PENT may not be ideal to evaluate remodelling. Also, since PENT accumulation is influenced by tissue glucose concentration and oxidative stress (66), Riley et al. recently suggested the percentage D-aspartic acid (%D-asp) as a more reliable marker for collagen age (64).

Indications exist that the Achilles tendon may possess limited potential for de novo synthesis of collagen type I. When Eriksen et al. compared ruptured Achilles tendons to cadaver material they found no differences in the concentration of carboxyterminal pro-peptide of type I collagen PICP (carboxyterminal pro-peptide of type I collagen), which is an indirect marker of collagen type I synthesis (17). Again, no ongoing renewal of collagen type I appeared to occur in ruptured tendons. Curiously, at the level of gene expression collagen mRNA seems to be upregulated in Achilles tendinopathy and rupture. Such upregulation could, however, occur secondary to rupture in the attempt to restore the collagen composition (34; 39). This prompts the question whether the human Achilles tendon has an impaired capacity for remodelling? Decreased cellularity has been reported in ruptured tendon including the Achilles tendon (34; 38), although others have reported increased cellularity in degenerate Achilles tendons (68). Importantly, tenocytes harvested from ruptured Achilles tendons have been shown to stain more strongly for collagen type III compared to type I than normal tenocytes (53). Perhaps abnormal and smaller sized collagen fibrils in ruptured Achilles tendons (37; 38; 55) represent partly degraded nascent type I collagen fibrils and some type III collagen fibrils which in combination weaken the tendon. In contrast to the notion of impaired collagen type I synthesis Langberg and co-workers demonstrated increased PICP after both acute and chronic loading of healthy Achilles tendon (running and military training, respectively) (48). It is noteworthy that degradation (ICTP) was increased 4 weeks into the chronic training regime while returning to baseline at 11 weeks. Kongsgaard et al. found increased cross-sectional area in runners compared to kayakers and Achilles tendon rupture

patients (45) and regional hypertrophy of the patellar tendon following strength training (47), while Arampatzis et al. found region-specific hypertrophy in human Achilles tendon following controlled cyclical loading (1). However, no increase in the Achilles tendon cross-sectional area was found after 9 months of running in previously untrained subjects (24). Hence, net collagen type I synthesis may potentially cause hypertrophy of the Achilles tendon, however the response seems slow. Moreover, the initial response to unaccustomed loading may favour collagen degradation. Indeed, local loss of strain e.g. from microtrauma may lead to increased proteolytic activity (34). Ruptures frequently occur in subjects who resume sports after a longer period of absence (55) or participate only occasionally in sports (51). Hence, insufficient adaptation to a sudden increase in tendon loading and transient inadvertent weakening from degradation may lead to a catastrophic failure of the tendon. We found that collagen content was positively related to failure stress of fascicles from the rupture site (Figure 4), but not for internal and external control fascicles. A threshold may exist below which collagen is a key determining factor of tendon strength. Above the threshold other factors modulate the strength of the tendon Such a phenomenon has been observed for healing rabbit ligament (21). Moreover, since tendon is poorly innervated bulk tissue failure may have been preceded by a partial rupture that went unnoticed yet weakened the tendon (22; 42).

The present study has certain limitations. The sample size for external controls was limited (mechanical measurements: n=6; biochemical measurements: n=10) which precludes firm conclusions. Additionally, we tested the tendon fascicles at a strain rate below the in vivo strain rate during sporting activities such as jumping when most rupture occur. Low strain rate has previously been shown to reduce failure stress and strain in the human Achilles tendon while Young´s modulus remained unaltered (71). However, Yamamoto et al. have reported that the strain rate dependence of isolated tendon fascicles is less than for whole tendon (72). Also, since all mechanical testing conditions were maintained constant during all trials we expect that the noted mechanical differences from micromechanical measurements are valid and expect them to be of relevance to the in vivo situation. Since our cadaver material was not evaluated histologically, we cannot be certain that the specimens were not afflicted by degeneration, which has been reported to occur in 34% of cadaver tendons (38). Jones et al. found some cell and matrix abnormalities in 27% of human cadaver Achilles tendon. Since the age span of our cadaver tendons was narrower and the tendons were younger we do not expect a greater prevalence of degeneration in our samples. It was beyond the scope of the study to explore the role of cross-links other than PENT and pyridinoline but future studies should seek to do so. Additionally, the measurement of collagen type III, immature cross-links, MMP activity and %D-asp would have allowed more precise evaluation of the degree of tendon remodelling (3; 64) and would have provided aid for the overall interpretation of biochemical data. Lastly, it should be mentioned that proteoglycan content is known to influence tendon mechanical properties (41; 62) and should considered in future studies.

In conclusion, the present data provide support for our hypothesis of preexisting mechanical weakening of the ruptured Achilles tendon since cadaver tendons displayed higher elastic modulus and tended to be stronger. However, contrary to our hypothesis there were no regional mechanical or biochemical differences within the rupture tendons when the rupture site was compared to non-injured tissue proximal to the rupture. Collagen content was lower in ruptured tendons and correlated positively with strength of fascicles obtained from the rupture site. Collectively, adverse structural and biochemical changes may extend beyond the rupture site. Hence a preexisting, generalized weakening of the ruptured Achilles tendon may relate to reduced type I collagen content. Notably, the cross-linking pattern that pertains to pyridinium type cross-links and pentosidine does not appear to be associated with mechanical properties of tendon fascicles in ruptured and uninjured tendons. Elevated pyridinoline levels may reflect functional adaptation or an impaired repair process that relates to mechanical weakness of the Achilles tendon.

Reference List


1. **Arampatzis A, Karamanidis K and Albracht K**. Adaptational responses of the human Achilles tendon by modulation of the applied cyclic strain magnitude. *J Exp Biol* 210: 2743-2753, 2007.
2. **ARNER O and LINDHOLM A**. Subcutaneous rupture of the Achilles tendon; a study of 92 cases. *Acta Chir Scand Suppl* 116: 1-51, 1959.
3. **Avery NC**. Quantitative determination of collagen cross-links. In: Methods in Molecular Biology vol. 522. Extracellular Matrix Protocols, Humana Press, 2009, p. 103-121.
4. **Avery NC and Bailey AJ**. Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. *Scand J Med Sci Sports* 15: 231-240, 2005.
5. **Bailey AJ, Paul RG and Knott L**. Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* 106: 1-56, 1998.
6. **Bank RA, Beekman B, Verzijl N, de Roos JA, Sakkee AN and TeKoppele JM**. Sensitive fluorimetric quantitation of

pyridinium and pentosidine crosslinks in biological samples in a single high-performance liquid chromatographic run. *J Chromatogr B Biomed Sci Appl* 703: 37-44, 1997.

7. **Bank RA, TeKoppele JM, Oostingh G, Hazleman BL and Riley GP**. Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon collagen: changes with age and in chronic rotator cuff tendinitis. *Ann Rheum Dis* 58: 35-41, 1999.
8. **Barnard K, Light ND, Sims TJ and Bailey AJ**. Chemistry of the collagen cross-links. Origin and partial characterization of a putative mature cross-link of collagen. *Biochem J* 244: 303-309, 1987.
9. **Bennett MB, Ker RF, Dimery NJ and Alexander McN**. Mechanical properties of various mammalian tendons. *J Zool Lond(A)* 209: 537-548, 1986.
10. **Birch HL**. Tendon matrix composition and turnover in relation to functional requirements. *Int J Exp Pathol* 88: 241-248, 2007.
11. **Cetti R, Junge J and Vyberg M**. Spontaneous rupture of the Achilles tendon is preceded by widespread and bilateral tendon damage and ipsilateral inflammation: a clinical and histopathologic study of 60 patients. *Acta Orthop Scand* 74: 78-84, 2003.
12. **Couppe C, Hansen P, Kongsgaard M, Kovanen V, Suetta C, Aagaard P, Kjaer M and Magnusson SP**. Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J Appl Physiol* 107: 880-886, 2009.
13. **Creemers LB, Jansen DC, van Veen-Reurings A, van den BT and Everts V**. Microassay for the assessment of low levels of hydroxyproline. *Biotechniques* 22: 656-658, 1997.
14. **Davison PF**. The contribution of labile crosslinks to the tensile behavior of tendons. *Connect Tissue Res* 18: 293-305, 1989.
15. **de Mos M., van EB, Degroot J, Jahr H, van Schie HT, van Arkel ER, Tol H, Heijboer R, van Osch GJ and Verhaar JA**. Achilles Tendinosis: Changes in Biochemical Composition and Collagen Turnover Rate. *Am J Sports Med* 35: 1549-1556, 2007.
16. **Derwin KA and Soslowsky LJ**. A quantitative investigation of structure-function relationships in a tendon fascicle model. *J Biomech Eng* 121: 598-604, 1999.
17. **Eriksen HA, Pajala A, Leppilahti J and Risteli J**. Increased content of type III collagen at the rupture site of human Achilles tendon. *J Orthop Res* 20: 1352-1357, 2002.
18. **Eyre DR, Paz MA and Gallop PM**. Cross-linking in collagen and elastin. *Annu Rev Biochem* 53: 717-748, 1984.
19. **Finni T, Komi PV and Lepola V**. In vivo muscle mechanics during locomotion depend on movement amplitude and contraction intensity. *Eur J Appl Physiol* 85: 170-176, 2001.
20. **Finni T, Komi PV and Lepola V**. In vivo human triceps surae and quadriceps femoris muscle function in a squat jump and counter movement jump. *Eur J Appl Physiol* 83: 416-426, 2000.
21. **Frank C, McDonald D, Wilson J, Eyre D and Shrive N**. Rabbit medial collateral ligament scar weakness is associated with decreased collagen pyridinoline crosslink density. *J Orthop Res* 13: 157-165, 1995.
22. **Gibbon WW, Cooper JR and Radcliffe GS**. Sonographic incidence of tendon microtears in athletes with chronic Achilles tendinosis. *Br J Sports Med* 33: 129-130, 1999.
23. **Gillis C, Pool RR, Meagher DM, Stover SM, Reiser K and Willits N**. Effect of maturation and aging on the histomorphometric and biochemical characteristics of equine superficial digital flexor tendon. *Am J Vet Res* 58: 425-430, 1997.
24. **Hansen P, Aagaard P, Kjaer M, Larsson B and Magnusson SP**. Effect of habitual running on human Achilles tendon load-deformation properties and cross-sectional area. *J Appl Physiol* 95: 2375-2380, 2003.
25. **Hansen P, Haraldsson BT, Aagaard P, Kovanen V, Avery N, Qvortrup K, Larsen JO, Krogsgaard M, Kjaer M and Magnusson SP**. Lower strength of the human posterior patellar tendon seems unrelated to mature collagen cross-linking and fibril morphology. *J Appl Physiol* 2009.
26. **Hanson DA and Eyre DR**. Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. *J Biol Chem* 271: 26508-26516, 1996.
27. **Haraldsson BT, Aagaard P, Krogsgaard M, Alkjaer T, Kjaer M and Magnusson SP**. Region-specific mechanical properties of the human patella tendon. *J Appl Physiol* 98: 1006-1012, 2005.
28. **Henkel W**. Cross-link analysis of the C-telopeptide domain from type III collagen. *Biochem J* 318 ( Pt 2): 497-503, 1996.
29. **Henkel W, Glanville RW and Greifendorf D**. Characterisation of a type-I collagen trimeric cross-linked peptide from calf aorta and its cross-linked structure. Detection of pyridinoline by time-of-flight secondary ion-mass spectroscopy and evidence for a new

cross-link. *Eur J Biochem* 165: 427-436, 1987.
30. **Houshian S, Tscherning T and Riegels-Nielsen P**. The epidemiology of Achilles tendon rupture in a Danish county. *Injury* 29: 651-654, 1998.
31. **Inglis AE and Sculco TP**. Surgical repair of ruptures of the tendo Achillis. *Clin Orthop Relat Res* 160-169, 1981.
32. **Jarvinen M, Jozsa L, Kannus P, Jarvinen TL, Kvist M and Leadbetter W**. Histopathological findings in chronic tendon disorders. *Scand J Med Sci Sports* 7: 86-95, 1997.
33. **Jarvinen TA, Kannus P, Paavola M, Jarvinen TL, Jozsa L and Jarvinen M**. Achilles tendon injuries. *Curr Opin Rheumatol* 13: 150-155, 2001.
34. **Jones GC, Corps AN, Pennington CJ, Clark IM, Edwards DR, Bradley MM, Hazleman BL and Riley GP**. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. *Arthritis Rheum* 54: 832-842, 2006.
35. **Jozsa L, Balint BJ, Reffy A and Demel Z**. Hypoxic alterations of tenocytes in degenerative tendinopathy. *Arch Orthop Trauma Surg* 99: 243-246, 1982.
36. **Jozsa L and Kannus P**. Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7: 113-118, 1997.
37. **Jozsa L and Kannus P**. Histopathological findings in spontaneous tendon ruptures. *Scand J Med Sci Sports* 7: 113-118, 1997.
38. **Kannus P and Jozsa L**. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J Bone Joint Surg Am* 73: 1507-1525, 1991.
39. **Karousou E, Ronga M, Vigetti D, Passi A and Maffulli N**. Collagens, Proteoglycans, MMP-2, MMP-9 and TIMPs in Human Achilles Tendon Rupture. *Clin Orthop Relat Res* 2008.
40. **Ker RF, Alexander RM and Bennett MB**. Why are mammalian tendons so thick? *J Zool, Lond* 216: 309-324, 1988.
41. **Kjaer M**. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 84: 649-698, 2004.
42. **Kjaer M, Langberg H and Magnusson P**. [Overuse injuries in tendon tissue: insight into adaptation mechanisms]. *Ugeskr Laeger* 165: 1438-1443, 2003.
43. **Knott L, Whitehead CC, Fleming RH and Bailey AJ**. Biochemical changes in the collagenous matrix of osteoporotic avian bone. *Biochem J* 310 ( Pt 3): 1045-1051, 1995.
44. **Kongsgaard M**. *Structural and mechanical adaptations of tendinous tissue. Effects of exercise, loading and injury. Ph.D thesis* (Dissertation). 2008.
45. **Kongsgaard M, Aagaard P, Kjaer M and Magnusson SP**. Structural Achilles tendon properties in athletes subjected to different exercise modes and in Achilles tendon rupture patients. *J Appl Physiol* 99: 1965-1971, 2005.
46. **Kongsgaard M, Kovanen V, Aagaard P, Doessing S, Hansen P, Laursen AH, Kaldau NC, Kjaer M and Magnusson SP**. Corticosteroid injections, eccentric decline squat training and heavy slow resistance training in patellar tendinopathy. *Scand J Med Sci Sports* 2009.
47. **Kongsgaard M, Reitelseder S, Pedersen TG, Holm L, Aagaard P, Kjaer M and Magnusson SP**. Region specific patellar tendon hypertrophy in humans following resistance training. *Acta Physiol* 191: 111-121, 2007.
48. **Langberg H, Skovgaard D, Petersen LJ, Bulow J and Kjaer M**. Type I collagen synthesis and degradation in peritendinous tissue after exercise determined by microdialysis in humans. *J Physiol* 521: 299-306, 1999.
49. **Lavagnino M, Arnoczky SP, Frank K and Tian T**. Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons. *J Biomech* 38: 69-75, 2005.
50. **Levi N**. The incidence of Achilles tendon rupture in Copenhagen. *Injury* 28: 311-313, 1997.
51. **Maffulli N**. Rupture of the Achilles tendon. *J Bone Joint Surg Am* 81: 1019-1036, 1999.
52. **Maffulli N, Barrass V and Ewen SW**. Light microscopic histology of achilles tendon ruptures. A comparison with unruptured tendons. *Am J Sports Med* 28: 857-863, 2000.
53. **Maffulli N, Ewen SW, Waterston SW, Reaper J and Barrass V**. Tenocytes from ruptured and tendinopathic achilles tendons produce greater quantities of type III collagen than tenocytes from normal achilles tendons. An in vitro model of human tendon healing. *Am J Sports Med* 28: 499-505, 2000.
54. **Maganaris CN, Narici MV and Maffulli N**. Biomechanics of the Achilles tendon. *Disabil Rehabil* 30: 1542-1547, 2008.
55. **Magnusson SP, Qvortrup K, Larsen JO, Rosager S, Hanson P, Aagaard P, Krogsgaard M and Kjaer M**. Collagen fibril size and crimp morphology in ruptured and

intact Achilles tendons. *Matrix Biol* 21: 369-377, 2002.
56. **Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R and Myllyharju J**. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. *Am J Pathol* 167: 927-936, 2005.
57. **Moller A, Astron M and Westlin N**. Increasing incidence of Achilles tendon rupture. *Acta Orthop Scand* 67: 479-481, 1996.
58. **Moriguchi T and Fujimoto D**. Age-related changes in the content of the collagen crosslink, pyridinoline. *J Biochem* 84: 933-935, 1978.
59. **Onambele GN, Burgess K and Pearson SJ**. Gender-specific in vivo measurement of the structural and mechanical properties of the human patellar tendon. *J Orthop Res* 25: 1635-1642, 2007.
60. **Parry DA**. The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. *Biophys Chem* 29: 195-209, 1988.
61. **Parry DA, Barnes GR and Craig AS**. A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. *Proc R Soc Lond B Biol Sci* 203: 305-321, 1978.
62. **PARTINGTON FR and WOOD GC**. The role of non-collagen components in the mechanical behaviour of tendon fibres. *Biochim Biophys Acta* 69: 485-495, 1963.
63. **Puxkandl R, Zizak I, Paris O, Keckes J, Tesch W, Bernstorff S, Purslow P and Fratzl P**. Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. *Philos Trans R Soc Lond B Biol Sci* 357: 191-197, 2002.
64. **Riley GP, Curry V, DeGroot J, van EB, Verzijl N, Hazleman BL and Bank RA**. Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. *Matrix Biol* 21: 185-195, 2002.
65. **Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE and Hazleman BL**. Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. *Ann Rheum Dis* 53: 359-366, 1994.
66. **Sell DR and Monnier VM**. Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem* 264: 21597-21602, 1989.
67. **Suzuki D, Takahashi M, Abe M and Nagano A**. Biochemical study of collagen and its crosslinks in the anterior cruciate ligament and the tissues used as a graft for reconstruction of the anterior cruciate ligament. *Connect Tissue Res* 49: 42-47, 2008.
68. **Tallon C, Maffulli N and Ewen SW**. Ruptured Achilles tendons are significantly more degenerated than tendinopathic tendons. *Med Sci Sports Exerc* 33: 1983-1990, 2001.
69. **Viguet-Carrin S, Garnero P and Delmas PD**. The role of collagen in bone strength. *Osteoporos Int* 17: 319-336, 2006.
70. **Westh E, Kongsgaard M, Bojsen-Moller J, Aagaard P, Hansen M, Kjaer M and Magnusson SP**. Effect of habitual exercise on the structural and mechanical properties of human tendon, in vivo, in men and women. *Scand J Med Sci Sports* 18: 23-30, 2008.
71. **Wren TA, Yerby SA, Beaupre GS and Carter DR**. Mechanical properties of the human achilles tendon. Clin Biomech (Bristol , Avon ) 16: 245-251, 2001.
72. Yamamoto E, Hayashi K and Yamamoto N. Mechanical properties of collagen fascicles from the rabbit patellar tendon. J Biomech Eng 121: 124-131, 1999.