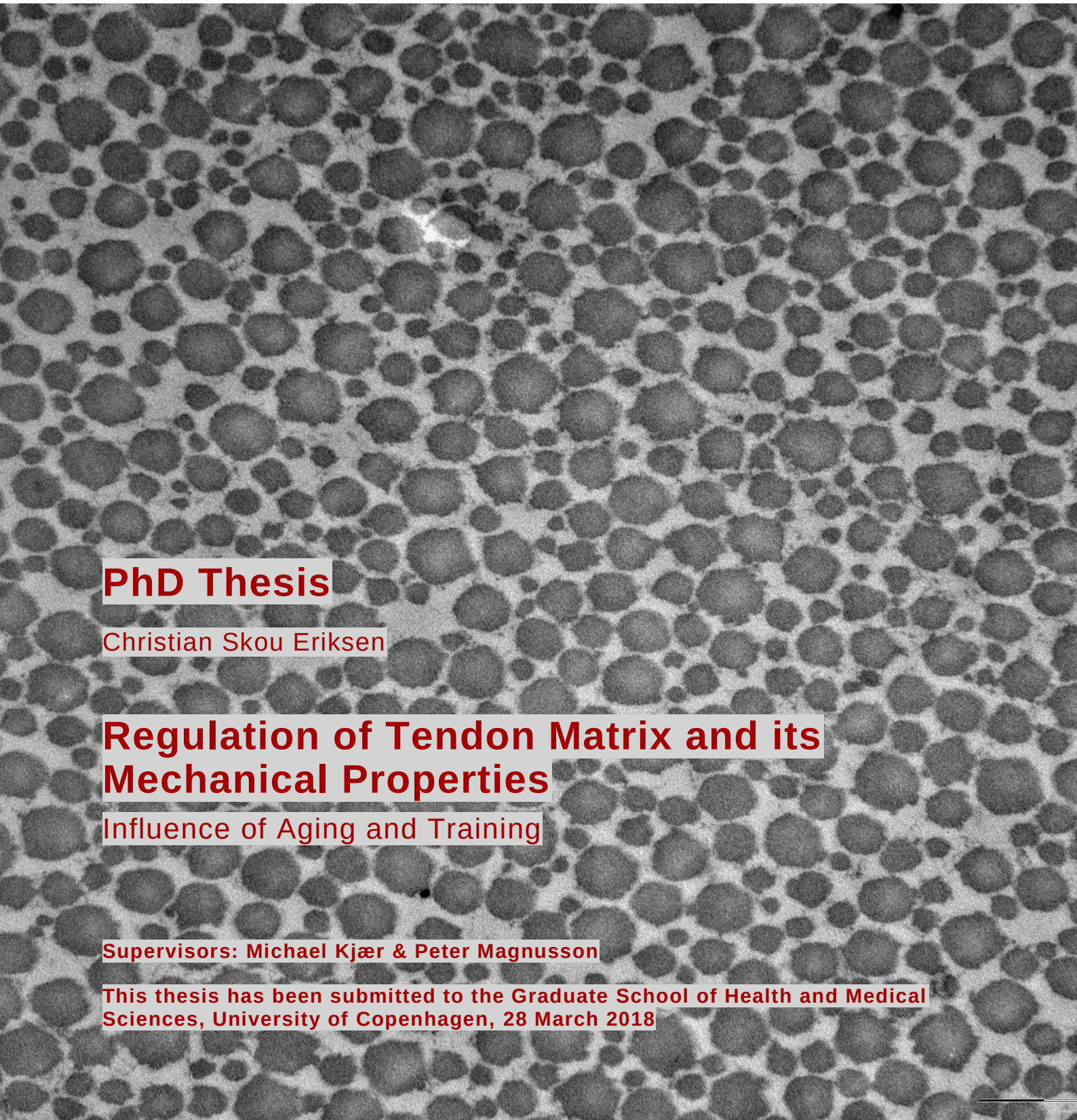
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Institute of Sports Medicine Copenhagen
ISMC Bispebjerg Hospital

Center for Healthy Aging



PhD Thesis

Christian Skou Eriksen

Regulation of Tendon Matrix and its Mechanical Properties

Influence of Aging and Training

Supervisors: Michael Kjær & Peter Magnusson

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, 28 March 2018

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PhD thesis

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1. Preface and acknowledgements

The present PhD thesis is based on three randomized studies conducted at the Institute of Sports Medicine Copenhagen (ISMC) in the years from 2013 to 2017. The conduct and completion of these studies was only possible due to the support of many people to whom I am very grateful.

First of all, I am deeply grateful to my primary supervisor Michael Kjær for giving me the opportunity to conduct the research at ISMC. Thank you for having confidence in me, for letting me work independently, and for always taking responsibility for the research. You demonstrate exemplary leadership and overview.

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A special acknowledgement goes to my colleague Rene Svensson for his friendly, dedicated, and "always available" support with all aspects of the research from technical matters to complicated theoretical and statistical issues. My colleague Christian Couppé taught me the method for assessment of in vivo tendon mechanical properties and has engaged me in many inspiring discussions about tendon mechanics and collagen cross-links. Thank you! I also appreciate the assistance of my colleagues Anne Gylling, Anne-Sofie Agergaard, Cecilie Henkel, Mads Vang Rasmussen, Christian Josefsen, and Camilla Hansen, who put many hours into data acquisition and analysis, and to all the other people organising, training, and testing participants in the three studies. To all my other colleagues and friends at ISMC, I would like to say that you have made every day joyful and meaningful. Thanks for your friendship.

I spend four weeks with the "Protein oxidation group" in their lab at the Biomedical Institute, University of Copenhagen. Thanks Michael Davis, Christine, and Alex for giving me a pleasant and interesting stay in your department and help improve my understanding of advanced glycation end-products and the HPLC method.

I would also like to thank the 96 participants for putting their time and effort into the research. Without you, there is no research.

Last but not least, my family have always backed me up through turbulent periods in the project. Your love and support means everything to me.

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2. List of papers

The thesis is based on the following original papers:

Paper I:

"Lower Tendon Stiffness in Very Old Compared to Old Individuals is Unaffected by Short Term Resistance Training of Skeletal Muscle". J Appl Physiol. Accepted March 2018.

Paper II:

"Counteracting Age-related Changes in Mechanical Properties of Human Tendon: Influence of Strength Training Load". In preparation.

3. List of abbreviations

AGEs	Advanced glycation end-products
ANOVA	Analysis of variance
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
CON	Control
CSA	Cross-sectional area
CV	Coefficient of Variation
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GOLD	Glyoxal-lysine dimer
H&E	Hematoxylin and eosin staining
HP	Hydroxylysyl pyridinoline
HPLC	High pressure liquid chromatography
HRT	Heavy load resistance training
IsoMVC	Isometric quadriceps strength at maximal voluntary contraction
LOX	Lysyl oxidase
LP	Lysyl pyridinoline
LRT	Low load resistance training
MG-H1-3	Methylglyoxal-derived hydroimidazolone 1-3
MOLD	Methylglyoxal-lysine dimer
MPa	Mega Pascal
MRI	Magnetic resonance imaging
MRT	Moderate load resistance training
NIH	National Institute of Health
OLD12	Study 3. Moderately old adults 62-70 years. Interventions: HRT, MRT, CON.
OLD3	Study 2. Moderately old adults >65 years. Interventions: HRT, LRT, CON.
PT	Patellar tendon
Q	Quadriceps muscle
TEM	Transmission electron microscopy
US	Ultrasound
VERY OLD	Study 1. Very old adults age >83 years. Interventions: HRT, CON.
YM	Young's modulus

4. Summary

Aging negatively affects connective tissue structure and biomechanical function throughout the body. Tendons are specialized connective tissue transmitting force from muscle to bone, but this function may be compromised by age-related loss of tensile stiffness. However, in vivo tendon mechanical properties have never been quantified in older adults >80 years. Regular loading seems to have a beneficial effect on tendon tissue in older adults by increasing tensile stiffness, but knowledge about training duration and the magnitude of load needed to improve tendon mechanical properties in older adults is limited. Furthermore, the molecular mechanisms regulating tendon biomechanical adaptation to loading are largely unresolved. The purpose of the PhD thesis was to investigate the effect of aging and training of different durations and load magnitude on patellar tendon (PT) mechanical properties, structure, and biochemical composition.

The thesis comprises sub-studies of three independent randomized trials on muscle adaptations to training. The first study included 30 men and women >83 years (VERY OLD), who were randomized to three months heavy load resistance training (HRT) or no training (CON). The second study included 30 men and women >65 years (OLD3), who were randomized to three months light (LRT) or heavy load resistance training (HRT) or no training (CON). The third study included 36 men and women age 62-70 years (OLD12), who were randomized to 12 months moderate (MRT) or heavy load resistance training (HRT) or no training (CON). Before and after the interventions in vivo PT mechanical properties were determined using the validated ultrasound based ramped contraction method. PT length and CSA was determined with MRI. PT biopsies were obtained in OLD3 and OLD12 to assess collagen fibril morphology, enzymatic cross-links and tendon fluorescence as a measure of advanced glycation end-products (AGEs). Measurements of isometric quadriceps muscle strength (IsoMVC) and cross-sectional area (Q-CSA), physical activity level, as well as blood parameters were also included in the data analysis.

Consistent with our hypothesis, comparison of VERY OLD (~87 years) and OLD3 (~68 years) revealed significantly lower tensile stiffness and Young's modulus in VERY OLD, which was associated with significantly lower physical activity level. Three months HRT did not affect tendon mechanical properties in either age-group, despite significant improvements of IsoMVC (~10%) and Q-CSA (~2.5%). OLD12 displayed a significant effect of training load on muscle strength with greatest improvement after HRT (~21%) compared to MRT (~8%). We found significant group differences in tensile stiffness and Young's modulus after 12 months

intervention with a time-dependent reduction after MRT and CON, which was ameliorated by HRT. Patellar tendon CSA increased after both HRT and MRT, while collagen content, fibril morphology, enzymatic cross-links, and fluorescence were unaffected by training. Comparison of OLD3 and OLD12 showed significantly larger change of PT-CSA following 12 months HRT compared to 3 months HRT, while we were unable to demonstrate significant effects of training duration on tendon mechanical properties, fibril morphology or collagen cross-links.

In conclusion, tendon mechanical and material properties seem to decline through senior life, which may partly be explained by reduced physical activity. Three months heavy resistance training intervention was insufficient to affect tendon size and mechanical properties in both moderately and very old adults despite significant improvements of muscle strength and size. In contrast, twelve months resistance training counteracted a time-dependent reduction of mechanical and material properties and increased tendon size, which suggests that longer training duration may be necessary for tendon adaptation. Furthermore, training with heavy load seemed necessary to improve tendon material properties but not tendon size. The changes in tendon size and mechanical properties could not be related to changes in collagen content, fibril morphology, enzymatic cross-links or AGEs. Further investigations are needed to unravel the molecular mechanisms regulating tendon mechanical adaptation to aging and training. High load training should be incorporated in training programs for older adults to maintain tendon mechanical properties.

5. Dansk resumé

Aldring har en negativ indvirkning på struktur og biomekanisk funktion af bindevæv i alle kroppens organer. Sener er højt specialiseret bindevæv, som overfører kraft fra muskler til knogler. Med alderen mister senen sin trækstivhed, hvilket kompromiterer optimal muskelfunktion, men senens mekaniske egenskaber er aldrig blevet undersøgt hos meget gamle mennesker >80 år. Regelmæssig mekanisk belastning påvirker formentlig senevævet positivt ved at øge stivheden, men der er begrænset viden om størrelsen og varigheden af belastning for at opnå en positiv virkning på senen hos ældre. Ydermere er de molekylære mekanismer, som regulerer senens biomekaniske tilpasning til træning hos ældre stort set ukendte. Formålet med denne Phd afhandling var således at undersøge effekten af alder og træning af forskellig varighed og belastning på knæskalssenens struktur, biokemiske sammensætning og mekaniske funktion.

Afhandlingen var bygget op omkring tre uafhængige randomiserede træningsstudier. Det første studie (studie 1) inkluderede mænd og kvinder >83 år, som blev randomiseret til enten tre måneders tung styrketræning eller ingen træning (kontrol). Det andet studie (studie 2) inkluderede mænd og kvinder >65 år, som blev randomisret til enten let, tung eller ingen styrketræning af tre måneders varighed. Det tredje studie (studie 3) inkluderede mænd og kvinder mellem 62 og70 år, som blev randomiseret til enten moderat, tung eller ingen styrketræning af 12 måneders varighed. Før og efter interventionerne målte vi patellaseenen mekaniske egenskaber in vivo ved hjælp af ultralydsoptagelse synkroniseret med kraftmåling under en gradueret isometrisk kontraktion af knæstrækkermusklen. MR-scanning blev foretaget for at måle senens størrelse og længde. I studie 2+3 blev der yderligere taget vævsprøver fra knæskalssenen til undersøgelse af senens biokemiske sammensætning (kollagenindhold, enzymatiske kollagen krydsbindinger, glykering af kollagen), samt mikroskopiske opbygning (fibril volumen fraktion, fibril diameter, fibril tæthed) med elektronmikroskopi. Endelig fik deltagerne målt m.quadriceps muskelstyrke og tværsnitareal, fysisk aktivitetsniveau samt foretaget blodprøver.

I overensstimmelse med vores forventninger viste sammenligning af studie 1 og 2, at meget gamle mennesker (gns. 87 år) havde signifikant lavere senestivhed og Young's modulus (YM) end moderate ældre (gns. 68 år), og det var associeret med signifikant lavere fysisk aktivitetsniveau hos meget gamle. Tre måneders tung styrketræning havde ingen effekt på senens stivhed eller YM hos hverken moderat eller meget gamle trods signifikant fremgang i m.quadriceps muskelstyrke (~10%) og tværsnitareal(~2.5%). Studie 3 viste en signifikant gruppeforskel i muskelstyrke efter 12 måneders intervention, med størst fremgang efter tung

styrketræning (~21%) sammenlignet med moderat styrketræning (~8%). Ydermere var der signifikant gruppeforskel i senestivhed og Young's modulus efter 12 måneders intervention, med reduktion efter moderat eller ingen styrketræning og bevarede seneegenskaber efter tung styrketræning. Knæskalssenens tværsnitareal var højere efter både moderat og tung styrketræning, mens der ikke var nogen effekt af træning på senens fibril morfologi, kollagenindhold, enzymatiske krydsbindinger eller glykering af kollagen. Sammenligning af studie 2 og 3 viste, at der var en signifikant større ændring i knæskalssenens tværsnitareal efter 12 måneder sammenlignet med 3 måneders træning, mens der ikke kunne vises signifikante forskelle i ændringerne efter 3 eller 12 måneders træning på øvrige variable.

Sammenfattende konkluderes det, at knæskalssenens mekaniske egenskaber reduceres gennem seniorlivet, hvilket til dels kan forklares ved et samtidigt fald i fysisk aktivitetsniveau. Tre måneders styrketræning var for kort tid til at påvirke senevævets mekaniske egenskaber trods fremgang i knæstrækker muskelstyrke og muskelstørrelse, mens tolv måneders styrketræning så ud til at modvirke en alderafhængig nedgang i senemekaniske egenskaber. Ydermere så det ud til, at styrketræning med tung belastning var nødvendig for at forbedre senens mekaniske egenskaber, mens øget senestørrelse ikke var afhængig at tung belastning. Ændringerne i senemekanik og senestørrelse med træning kunne ikke relateres til ændringer i senens kollagenindhold, fibril morfologi, kollagenkrydsbindinger eller glykering af kollagen. Flere undersøgelse er nødvendige for at klarlægge de molekylære mekanismer, som regulerer senens biomekaniske tilpasning til aldring og træning. Træning med høj belastning bør inddrages i træningsprogrammer til ældre for at bevare senemekaniske egenskaber.

6. Introduction

Aging changes structure and mechanical function of collagen-rich tissues like skin, cornea, cartilage, arteries, bone and tendon. The age-associated changes are progressive, may be irreversible, and often impair optimal tissue function. The skin loses elasticity, blood vessels reduce extensibility (6, 20, 140), and tendons seem to lose tensile stiffness (69, 81, 127). The age-related decline of tendon mechanical function may in turn impair muscle force transmission (19) as well as normal and safe locomotion (119). Tendons have historically received far less scientific attention than muscle and bone. This may be due to their relatively slow turnover time, which was previously mistaken for inertness. The past two to three decades have provided evidence that tendons are living tissues which respond to loading with increased metabolic activity (18, 87), at least in younger individuals, and regular loading may even protect tendons from the negative effects of aging (32, 131, 147). However, knowledge about the training duration and magnitude of load required to elicit favorable adaptations in tendons of older adults is limited. Moreover, the molecular changes in tendon matrix which mediate the adaptation of tendon mechanical properties are largely unresolved.

With an increasing population of older adults, it seems more and more urgent to improve our knowledge about tendon composition and mechanical function in older adults. The present PhD thesis investigates tendon mechanical properties and matrix composition of older adults and the potential of physical training to affect the aging tendon. The work contributes with knowledge about connective tissue biology in general and may also have clinical implications because of the possible relation between tendon biomechanics and functional ability. The following sections will outline current knowledge about tendon structure and function relevant to the thesis, as well as the effects of aging and training on tendon matrix and its mechanical properties. Next, I will discuss the applied methods, and finally I will present and discuss the main results.

6.1 Tendon structure

Tendons are highly specialized connective tissues located between muscles and bones. They can take different shapes depending on the specific function of the tendon. The patellar tendon is located between the patellar apex and the tibia tubercle (fig. 6.1A). Is has a relatively short length of approximately 5 cm and a large diameter of approximately 100 mm^2 (variation is large), which makes it suitable for transmission of high forces.

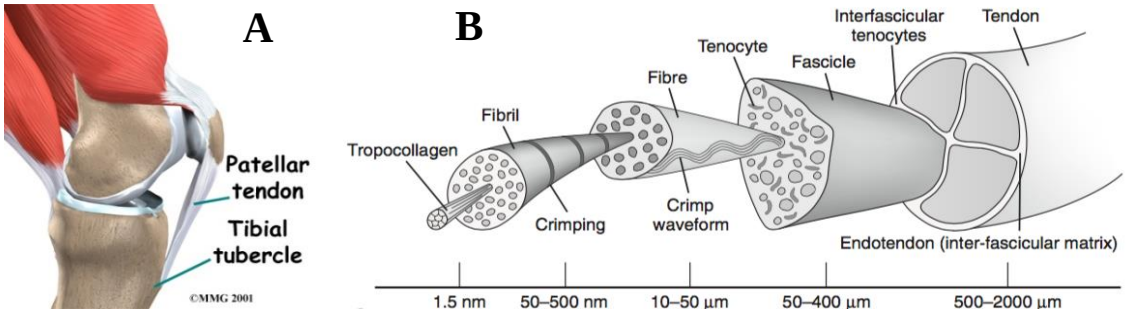

Figure 6.1: A: *The patellar tendon is located between the patella and the tibia tuberosity where it transmits force from the quadriceps muscle to the lower leg* (180). ***B:*** *Transverse section through a tendon illustrating the hierarchical levels* (181).

The tendon has a hierarchical framework, where the tendon proper is divided into continuously smaller subunits called tendon fascicles and fibers, which are supported by loose, irregular connective tissue (fig. 6.1B). Microscopy of H&E stained specimens reveals a densely packed extracellular matrix (ECM) of parallel longitudinal running fibers with relatively few cells (fibroblasts) embedded between the fibers (fig. 6.2A). The smallest functional units in tendon are the collagen fibrils, which can be visualized with transmission electron microscopy (TEM). TEM makes it possible to analyze fibril diameter, fibril density, and fibril volume fraction (fig. 6.2B).

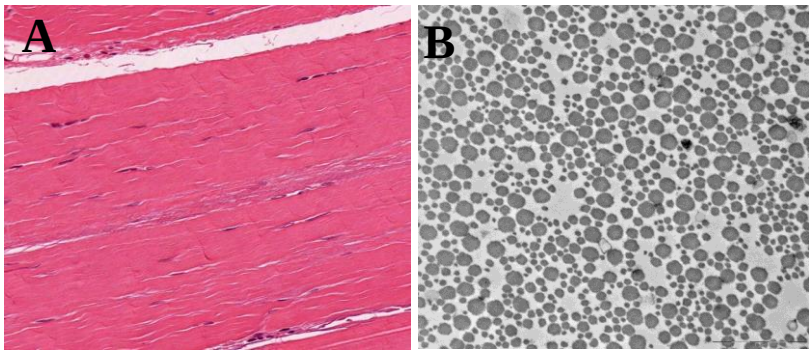

Figure 6.2: A: *Longitudinal section of a tendon seen through the light microscope. H&E staining. The extracellular matrix is densely packed with collagen fibers (pink) with few fibroblasts (purple) embedded between them* (182). ***B:*** *Cross-section of the patellar tendon seen through the transmission electron microscope at 24500 x magnification (4x4µm. Dark-grey elements are collagen fibrils which are surrounded by interfibrillary matrix.*

The collagen fibril is composed of collagen type I molecules arranged longitudinally in a quarter-staggered structure (64) (fig. 6.3A). The collagen molecules are connected by cross-links, which enables transmission of force between individual molecules. There are essentially two types of cross-links. One is enzymatically controlled by the family of lysyl oxidases (LOX) and is essential for normal development and maturation of tendons (42, 63). LOX catalyzes an oxidative deamination of specific lysyl and hydroxylysyl residues, which over a series of reactions mature into the non-reducible covalent cross-links lysyl pyridinoline (LP) and hydroxlysyl pyridinoline (HP) (42). The other type of cross-links form when reducing sugars spontaneously react with protein amino-groups (i.e. lysine or arginine) forming a temporary unstable Schiff-base, which then converts to a stable keto-amine (the Amadori product). The protein-glycation products matures over a series of complex modifications into a large and heterogeneous family of chemical compounds called advanced glycation end-products (AGEs) (7) (fig. 6.3B). Proteins with exposed lysine residues, such as fibrillary collagens, are highly susceptible to glycation and the slow turnover of tendon collagen further aggravates age-related accumulation (8). Some AGEs form cross-links between neighboring molecules affecting the physical properties of the tissue (5) by diminishing collagen fiber sliding (94). Others change conformation of the molecules, which may increase the distance between molecules or change molecular recognition sites (48), gradually impairing normal tissue function.

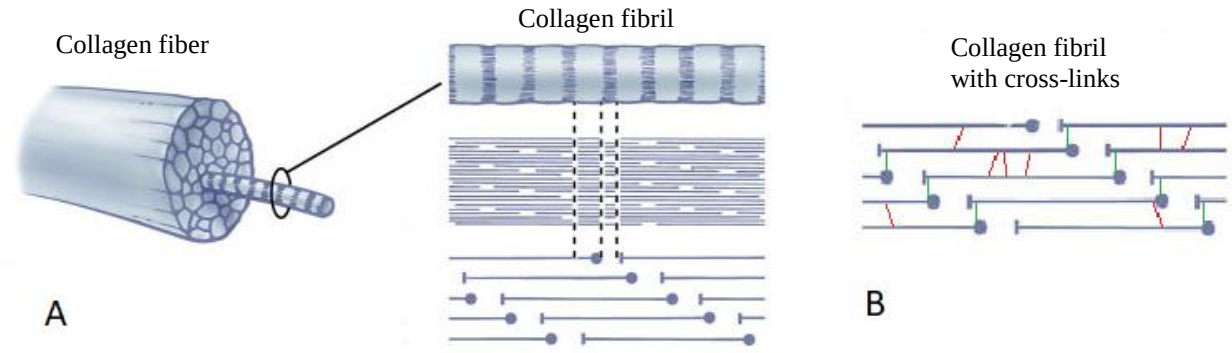

Figure 6.3: A: *Left: Collagen fiber. Right: Collagen fibril and schematic illustration of the arrangement of collagen molecules in the fibril.* ***B:*** *Individual collagen molecules are connected by enzymatic cross-links (green) or non-enzymatic glycation cross-links (red)* (183).

Although collagen makes up 60-80% of the tendon dry-weight, with the majority being type I collagen (~90%) (13, 72, 73), the fibrils and the interfibrillary matrix are composed of many different molecules (table 1). The collagen family alone comprises 28 different fibrillary and non-fibrillary types. In addition, proteoglycans, glycoproteins and several inorganic compounds contribute to the structure, regulation, development, repair, and functional properties of the tendon (table 6.1). A thorough introduction is beyond the scope of this thesis.

Table 6.1: Tendon matrix composition

Fibrillary collagen	*Type I:* Most abundant protein in tendon. ~90% of the collagen content. Forms fibril and is responsible for most of the tensile strength (13, 72). *Type III:* Second most abundant collagen (~10%). Often present in interfascicular matrix. Regulates fibrillogenesis (68). *Type V:* present in the center of type I fibrils. Template for fibrillogenesis (148).
Non-fibrillary collagen	*Type VI, XII, XIV, etc.:* Link collagen to other matrix components and play a role in regulation and development of ECM.
Elastin	Main component in elastic fibers. Contributes to tissue elasticity (50).
Proteoglycans	Protein core with attached glycosaminoglycans (chondroitin sulphate, dermatan sulphate, keratin sulphate). *Large:* aggrecan, versican, lubricin *SLRP's*: small leucine rich proteoglycans: -Type I: decorin (80% of proteoglycans), biglycan. -Type II: lumican, fibromodulin (keratin sulphate). 1-5% of dry weight. Interspersed at all hierarchical levels of the tendon. Plays a role in fibrillogenesis. Attracts water and provides turgor to the ECM. May promote sliding between fascicles and fibrils (13, 159).
Glycoproteins	*COMP:* Cartilage Oligomeric Protein. Binds collagen type I, present in interfibrillary matrix. Uncertain function. *Tenascin-C:* Involved in development and repair of connective tissue. Highly expressed at sites of compression where it has an anti-adhesive role and gives the tendon turgor. *Fibronectin:* Binds ECM components and regulates cell adhesion, migration, and differentiation. (72, 159, 178).
Collagen cross-links	*Enzymatic:* i.e. hydroxylysylpyridinoline (HP), lysylpyridnioline (LP). *Non-enzymatic:* Advanced glycation end-products (AGEs). Connect collagen molecules. See text for details.
Inorganic compounds	i.e. copper, manganese, calcium (67)

Summarized from Ackermann and Hart 2016, Part I: Basic Biology and Anatomy (1).

Though all the biochemical components in table 6.1 may contribute to tendon function, type I collagen because of its abundance, and the cross-linking of collagen play a critical role in tendon mechanical properties in healthy individuals and will be the focus of the present PhD thesis.

6.2 Tendon function

Tendons transmit force from muscles to bones resulting in limb movement. This requires large tensile resistance which is provided by the parallel orientated collagen molecules, but also a certain degree of extensibility to enable energy storing as well as joint flexibility and control. Though the relative contribution of different molecular components to tendon mechanical

properties is not confirmed, the highly specialized composition and organization of the tendon somehow supports all of these qualities (13, 159).

The mechanical properties of tendons can be examined with a force-deformation curve, which resembles a second order polynomial (fig. 6.4). When load is applied, the tendon initially deforms rather fast as the crimps of the fibers straighten out (toe-region). The linear region starts when deformation (mm) increases almost proportionally to the force (N). The slope at this part of the curve expresses tendon stiffness (N/mm), which is a critical property to force transmission. The curve then becomes more flat as damage to the tissue accumulates and stiffness decreases. This yield point marks the transition to the plastic phase, which ends when the tendon ruptures completely (170).

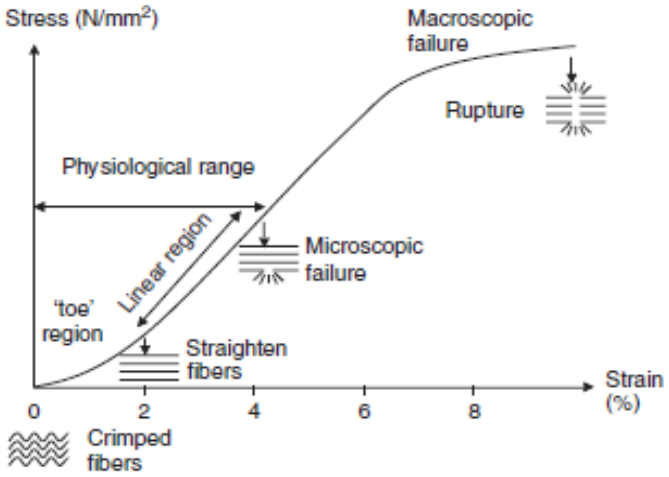

Figure 6.4: *Stress-strain curve of a tendon. Adapted from Wang* (170). *See text for details.*

The tendon operates only on the first part of the linear phase under normal human physical activity, and in vivo testing of tendons obviously only considers this part of the curve. While force-deformation curves describe the *mechanical properties* of individual tendons, normalization to tendon cross-sectional area (CSA) and length gives the *material properties*, which enables comparison between different tendons or time-points. Deformation is normalized to strain (%) by dividing with initial tendon length, force is normalized to stress (MPa) by dividing with tendon CSA and the normalized stiffness (Young's modulus, MPa) is obtained as the stress to strain ratio.

Tendons possess both elastic and viscous qualities which mean that only some of the elastic energy is stored in the tendon during deformation, while the rest is lost as heat (hysteresis). Force thus declines when the tendon is exposed to constant deformation (stress relaxation) and likewise, deformation increase gradually when constant force is applied to the

tendon (creep). The time-dependent viscous quality implies that muscle contraction speed influences tendon mechanical properties.

It is well known that muscle strength is highly dependent on sarcomere length (49, 71). An optimal overlap of the contractile elements requires fine tuning of the series elastic elements, including the tendons. Changes in muscle structure and strength after a training period thus also require adaptation of the tendon mechanical properties to optimize muscle function. However, it remains unresolved what the optimal tendon stiffness is, and this also makes it challenging to determine when an adaptation is beneficial or harmful. Higher tendon stiffness may improve muscle performance (19), electromechanical delay (116) efficiency of human movement (169), and postural balance (119), and is therefore generally considered beneficial to the muscle-tendon unit.

Although the relative contribution of different matrix components to tendon mechanical properties is not confirmed, the collagen fibril is generally believed to be an important functional unit in the tendon (120, 125, 155). Fibrils seem to have the same mechanical properties as the tendon proper (155), and recent research demonstrated that collagen fibrils traverses the entire length of the tendon (157). Molecular changes in the fibril may therefore very well translate into whole tendon mechanical behavior. The next sections introduce current knowledge about the effect of aging and training on tendon mechanical properties, fibril morphology, and collagen cross-links.

6.3 Effect of aging of the tendon

Human tissue develops from fertilization and matures through embryonic life and childhood until adulthood. In the following section, aging generally refers to the time-dependent changes that occur after maturity and through adult life. The possible effects of aging and training on tendons are illustrated in figure 6.5.

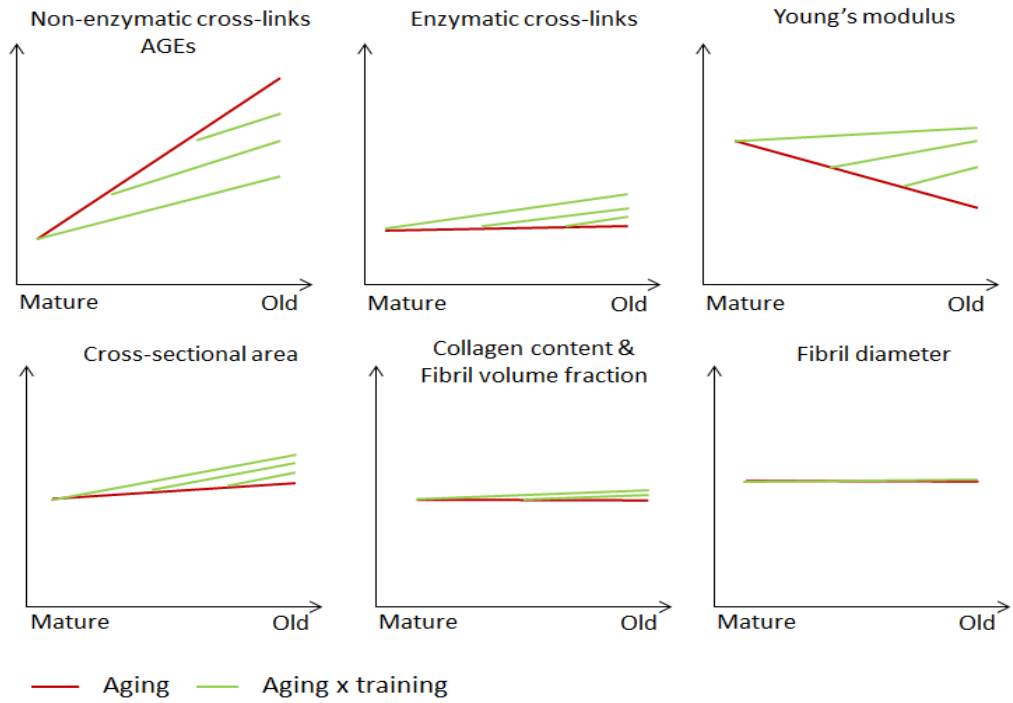

Figure 6.5: *Conceptual illustration of the expected effects of aging and training on the tendon. The axes are arbitrary. AGEs = advanced glycation end-products (non-enzymatic cross-links).*

6.3.1 Effect of aging on tendon mechanical properties

Several studies investigated the influence of aging on tendon stiffness with conflicting results. Animal studies have shown both increased (115, 166, 176), decreased (37, 86, 168), or unchanged (56, 57, 112) stiffness with aging. The discrepancy may be due to differences in the tendons, species, or strains tested. Moreover, rodents mature during most of their lifespan, which make it difficult to separate the effect of maturation from the effect of aging.

Most in vivo human studies have demonstrated lower (69, 81, 106, 127, 152) or unchanged stiffness with age (23, 29, 31), indicating that loss of stiffness may be an age-related deterioration in humans. A limitation to the current available research is however that it only covers aging up to the first part of 8th decade of life. Only one study compared in vitro patellar tendon mechanical properties in young and middle aged (29-50 years) to old and very old adults (64-93 years) and found reduced failure stress in the oldest group but only a tendency toward decreased Young's modulus (66). As people get increasingly older, it becomes more urgent to investigate in vivo tendon mechanics also in very old individuals (>80 years).

An important consideration when investigating the effect of aging on tendon physiology is the confounding effect of physical activity level. While rodents maintain spontaneous physical activity most of their lives, human tend to reduce physical activity level in old age, and this may explain the reduction in tendon stiffness rather than a true aging

phenomenon. Interestingly, young and old men matched for physical activity level do not differ appreciably in tendon mechanical properties (29, 32). The question is if introduction of regular physical activity in old humans can counteract the reduced tensile stiffness.

6.3.2 Effect of aging on collagen content and tendon morphology

Age-related decline of mechanical properties could be related to reduced collagen content since collagen is the most abundant tensile bearing component in tendon. Some studies also found modest age-related reductions of collagen content in both animals (57) and humans (29), but this is not a consistent finding (8, 32). In line with this, there seems to be either no change (176) or possibly a small reduction in average collagen fibril diameter and fibril volume fraction with aging (32, 113, 122, 153) as determined by TEM.

In contrast to fibril size and volume fraction, whole tendon cross-sectional area (CSA) in older individuals are either equal to (23, 29, 32) or higher (152) than in young individuals. No studies have convincingly demonstrated tendon atrophy with aging, which is contrary to the well-established loss of muscle mass with aging (45). The disparity between modest loss of collagen and fibril volume fraction and the possible increased whole tendon dimensions in humans may be due to region specific deposition of collagen (87) or non-collagenous components such as fat, water, and proteoglycans (159, 160), which are not detected in the tendon samples.

Taken together, the collagen content and thereby the bulk mass of tendon ECM does not seem to change appreciably after maturity and throughout life. However, the convincing age-related loss of tendon mechanical properties suggests that changes in other smaller fractions of the ECM components as well as molecular interactions between different components play a key role for the mechanical function of aging tendon. The opposing direction of the aging trends in whole tendon CSA and fibril diameter and fraction further supports the notion that non-collagenous components contribute relatively more to tendon function in old age.

6.3.3 Effect of aging on collagen cross-links

Enzymatic cross-links (i.e LP and HP) are controlled by the LOX enzyme family. Inactivation of the LOX gene results in aortic aneurysms and perinatal death in mice (111) as well as disordered fibrillogenesis and loss of mechanical stiffness in tendon constructs (63). They are therefore considered essential to development of normal force transmission within connective tissue (4, 6, 42). One study reported age-related increase of enzymatic cross-links after maturity in the human

supraspinatus tendon (8) and higher level of HP and LP has been found in the patellar tendon of old compared to young men (29). Age-related increase in enzymatic cross-links is however not a consistent finding (32, 110), and so far no studies have presented a clear relation between enzymatic cross-links and tendon stiffness in old humans (149).

Probably more crucial to aging of connective tissue is the adventitious non-enzymatic collagen cross-linking which is the result of glycation modifications of collagen molecules (4, 7, 14, 140, 165). Accumulation of advanced glycation end-products (AGEs) in skin correlates with the severity of diabetic complications (109, 136), and important for load bearing tissues like tendons, cross-linking AGEs may affect tissue mechanical function (5) by impairing collagen fiber- and intermolecular sliding (94).

Pentosidine is a commonly investigated cross-linking AGE in human tendons. Although there is only about one pentosidine cross-link per 200-300 collagen molecules (6), and pentosidine per se may therefore not be the most important mediator of mechanical properties, it is often used as a biomarker of aging (6) since it increases almost linearly with age in human tendons (8, 29). Pentosidine accumulation has been associated to increased stiffness of blood vessel walls (138, 145) and rabbit Achilles tendon (129), but the relation is not as straight forward in humans, where pentosidine accumulation does not necessarily coincide with increased in vivo tendon stiffness (29, 55).

6.4 Effect of exercise and training on the tendon

Exercise is the performance of a single bout of defined movements with a certain pattern, load size and duration, whereas *training* describes the performance of regular systematic exercises to achieve a certain goal, i.e. to get stronger. *Physical activity* is used more broadly to describe all types of human movement, systematic as well as non-systematic. Exercise and training can be subdivided into endurance- or resistance types. The distinction is not dichotomous and a continuum exists between endurance (i.e. walking, swimming) and resistance types (i.e. lifting heavy weights) depending on load size, time under tension, and number of repetitions. In the following sections, exercise and training are categorized as light, moderate, or heavy load since this distinction may be relevant to tendon adaptation to training. The possible effects of training on the tendon are illustrated in figure 6.5.

6.4.1 Effect of exercise and training on tendon mechanical properties

Increased as well as decreased physical activity has a well-known effect on muscle tissue strength and hypertrophy. Despite the relative inertness of tendon compared to muscle tissue, several studies have shown changes in tendon mechanical properties after only few weeks of immobilization (15, 31, 83), or only few months of increased physical activity (52, 81, 131), indicating a relatively fast adaptation of tendons to loading environment.

Focusing on tendon stiffness, animal studies generally tend to disagree whether training increases or decreases this critical functional property (25, 91, 144, 150, 174, 175). The disagreement may be due to differences in the training loads, testing methods, as well as the species, age-groups, or tendons tested. Use of animal models to investigate the training effects in old age may however be limited by the fact that rodents in contrast to humans maintain spontaneous physical activity most of their lives. Most human studies have found either increased or unchanged tendon stiffness in response to training (17, 173). In fact, a recent meta-analysis showed a moderate to large effect-size of training on Achilles- and patellar tendon stiffness in healthy young and middle-aged adults (17). The positive effect of training on tendon stiffness seems to be maintained in older adults (38, 52, 131), although this is not an entirely consistent finding (24). Until now, there are no reports on the effect of training on tendon mechanical properties in very old age (>80 years).

Although several studies suggest a positive systemic effect of exercise on the tendon (86, 115, 166, 171), direct mechanical loading seems critical to maintenance of tendon homeostasis (28, 73). Surgical release of patellar tendon tension in rabbits induces pro-apoptotic signaling (70). In vitro loading of rat tail tendon inhibits expression of collagen degrading matrix metalloproteinases (3, 89), and this seems to be dependent on load size. Additionally, fibroblasts respond to tension with increased production of growth factors and autocrine induction of ECM protein synthesis (28). The results suggest, that mechanical load is transferred directly from the ECM to the fibroblast cytoskeleton, which further activates gene expression, a phenomenon termed mechano-transduction. Also in humans, heavy load training seems to have a more pronounced effect on tendon stiffness than light load training, at least in young and middle-aged adults (17, 76, 101), and some have argued that there might be a strain threshold below which the mechanical stimulus is too weak to elicit tendon adaptation (2). Only one randomized study investigated the differential effects of light and heavy load resistance training on in vivo tendon mechanical properties in older adults (52), and found superior effect of the heavy load resistance training. However, training volume was much lower in the light load group, and it is likely that training volume was equally important as training load for the adaptations observed in that study.

Another study on 55 year old women found decreased strain of the vastus lateralis aponeurosis after 6 months of 50 daily squats using body weight only (81), suggesting that light load training affect tendon mechanics in older adults, at least if the volume is sufficiently large. It is possible that old age or low habitual physical activity yields a lower tension threshold for adaptation of tendon mechanics, but more studies are needed to determine the effect of load magnitude for tendon adaptation.

Longer training duration may have an additional, protracted effect on tendon mechanical properties due to the relatively slow turnover time of tendons, but few studies have investigated the effect of more than three months training. One recent cross-sectional study showed that four years habitual resistance training was not associated with higher patellar tendon stiffness than three months resistance training intervention (102). Another recent longitudinal study examined the effect of prolonged resistance training on AT mechanical properties in older adults (38) and found increased stiffness and modulus after 14 weeks of training with no further improvements after 1.5 years of training. However, the training volume in that study decreased from three to two weekly sessions after 14 weeks of training, and the blunted tendon response was accompanied by no further improvements in muscle strength from 14 weeks to 1.5 years. Further research is thus warranted to determine the effect of training duration on tendon mechanical properties.

6.4.2 Effect of exercise and training on collagen content and tendon morphology

The collagen transcription machinery in tendons seems to be induced by growth-factors following exercise in rats (60, 117). However, this has not been consistently reproduced in humans (58, 154), and though one study found increased expression of growth factors following low load exercise, this did not coincide with increased collagen synthesis in the patellar tendon (36). Further, chronic exposure to heavy load resistance training was not associated to higher patellar tendon collagen content compared to controls (92). It is possible that human reach maturity relatively early in life compared to rodents, and that this explains the relative inertness of adult human tendons compared to rodents. In line with this, recent research on cadaveric tendon taking advantage of the high levels of carbon-14 formed in the atmosphere during nuclear bomb tests in the 1950's and 60's showed that collagen in the core of human tendons was not renewed after the age of 17 (61). Despite the limited potential for new collagen deposition in the tendon core after maturity, high volume light load exercise seems to increase collagen synthesis in the peripheral tendon region in young individuals (87).

Collagen fibril morphology may or may not be affected by loading, as determined in animal studies (26, 107, 121). Cross-sectional human data have shown no effect of habitual light load exercise on fibril morphology in either young (93) or old adults (32). Twelve weeks heavy load resistance training in young men with patellar tendinopathy did not change fibril fraction but increased fibril density with a concomitant decrease in mean fibril area indicating more small diameter fibrils in response to training (75). The change in fibril diameter distribution may however reflect a restoration of diseased tissue towards more healthy tissue rather than a training effect on healthy tendon. No studies have so far investigated the effect of training on fibril morphology in healthy older adults.

Addition of collagen to the peripheral tendon region following exercise (87) intuitively results in training induced hypertrophy. In contrast to muscle tissue, the extent of training induced tendon hypertrophy is however unclear. Cross-sectional studies on young and middle-aged adults have shown that habitual, light and heavy loading is associated with higher CSA of the patellar (30, 32) and Achilles tendons (99), albeit not a consistent finding (102). In line with the cross-sectional data, a recent meta-analysis of training intervention studies showed a small to moderate effect size on Achilles and patellar tendon CSA in young and middle-aged adults (17). In older adults, most light and heavy load training interventions of short duration (12-14 weeks) have not been able to demonstrate training induced changes in tendon CSA (52, 118, 131, 151), suggesting that longer training periods are required for tendon hypertrophy. However, one recent study found a 6% increase of Achilles tendon CSA in older women following 14 weeks of heavy load isometric plantar flexion training but no further effect when training was continued for 1.5 years (38). The potential training induced tendon hypertrophy thus seems less dependent on load magnitude, but further research is required to determine the effect of training load and duration on tendon dimensions in older adults.

Taken together, it seems unlikely that the apparent training induced changes in tendon mechanical properties are caused by gross changes in collagen content, fibril morphology or whole tendon CSA, and other molecular components may mediate tendon adaptations to training.

6.4.3 Effect of exercise and training on collagen cross-links

Enzymatic collagen cross-links may mediate the increments in mechanical properties observed after training in older adults. Increased contraction induced LOX expression has been demonstrated in rat Achilles tendon (60), whereas 10 weeks of regular low load training did not

change LOX expression in older rats (175). The disparity may relate to differences in the age of the animals, the training load, or the timing of tissue extraction (24h vs. 48h after the last exercise bout). In humans, growth hormone administration attenuated immobilization induced reduction of patellar tendon LOX expression concomitant with attenuated reduction of patellar tendon modulus, suggesting that enzymatic cross-links regulated material properties in response loading environment (16). Few studies have investigated the influence of physical activity on actual HP and LP levels in human tendons. Cross-sectional data have shown no differences in HP or LP between untrained and endurance (32) or resistance trained (92) individuals, and three months heavy resistance training did not affect HP or LP (74) in patients with tendinopathy. Longitudinal training studies are needed to determine the effect of training on enzymatic cross-links in healthy older adults.

Non-enzymatic collagen cross-links have been intensively investigated in relation to aging, whereas the potential of regular training to attenuate the age-related accumulation has received far less attention. A cross-sectional study suggested that life-long light load training attenuated accumulation of the AGE cross-link pentosidine (32), and this is supported by a longitudinal study showing reduced pentosidine in patellar tendon after short-term heavy load resistance training in younger individuals with patellar tendinopathy (74). Tendon AGEs have also shown to be decreased after ten weeks light to moderate load training in old rats (175), but no longitudinal studies have confirmed this in old humans. Training induced reduction in tendon AGEs has been associated with reduced tendon stiffness in old animals (175), but the relation between tendon AGEs and mechanical properties in old humans is not confirmed.

6.5 Summary

Human aging seems be associated with reduced tendon stiffness, but the effect of aging on tendon mechanical properties after the 80th decade of life remains to be determined. Increased habitual physical activity improves tendon mechanical properties in older adults, but the influence of training duration and load size is uncertain. Training induced changes in tendon material properties seems unrelated to gross changes in collagen content or fibril morphology and may instead be regulated by enzymatic collagen cross-linking and attenuated accumulation of AGEs. Although there is a plausible relation between collagen cross-links and tendon mechanical properties, few human studies have reported a clear cut association between the two. More research is thus needed to address the influence of training on collagen cross-links and tendon mechanical properties in older adults.

7. Purpose

The purpose of this PhD thesis was to investigate the regulation of tendon matrix and its mechanical properties in relation to aging and training. Specifically, the investigation compared tendon mechanical properties in moderately old (+65) and very old (+83) adults and the influence of heavy load resistance training. Moreover, the investigation compared the effect of regular resistance training of short (3 months) or long (12 months) durations as well as light/moderate or heavy load on tendon mechanical properties and dimensions, fibril morphology, and collagen cross-links in older adults.

8. Hypotheses

With regards to tendon mechanical properties and dimensions the hypotheses were:

- Compared to moderately old (+65), very old (+83) adults have lower patellar tendon stiffness and Young's modulus, but unchanged dimensions.
- Compared to a control group, resistance training increases patellar tendon stiffness and Young's modulus in both moderately old (+65) and very old (+83) adults.
- Long (12 months) compared to short-term (3 months) heavy resistance training has a more pronounced effect on patellar tendon stiffness, Young's modulus, and dimensions in old adults.
- Moderate load is not inferior to heavy load resistance training with regards to increasing patellar tendon stiffness, Young's modulus and dimensions in old adults.

With regards to fibril morphology and matrix composition the hypotheses were:

- Collagen content and fibril morphology are unaffected by resistance training.
- Compared to a control group, enzymatic collagen cross-links increase in response to 12 months resistance training, and this is related to increased Young's modulus.
- Compared to a control group, accumulation of non-enzymatic glycation products is attenuated by 12 months resistance training.
- Moderate load is not inferior to heavy load resistance training with regards to increasing enzymatic cross-links and attenuating non-enzymatic glycation products.

9. Methodological considerations

The thesis is based on sub-studies of three randomized studies on older adults conducted at the Institute of Sports Medicine between 2013 and 2017 (table 9.1). The studies primarily investigated the effect of training on muscle mass and muscle function. The additional tendon specific tests were in vivo patellar tendon mechanical properties, patellar tendon MRI, and tendon biopsies.

The first study (VERY OLD) primarily investigated the effect of 3 months heavy resistance training and protein supplementation compared to protein supplementation only on muscle hypertrophy and strength gains in very old individuals (+83 years) (10). The 30 participants included in the original study were all recruited for tendon specific tests, except for tendon biopsies.

The second study (OLD3) primarily investigated the effects of 3 and 12 months heavy or light load resistance training and/or protein supplementation on muscle hypertrophy and strength gains in older adults (+65) (11). Thirty out of 206 participants were recruited for the tendon specific tests. They all received whey protein supplementation and were randomly allocated to either light or heavy load resistance training or no training. The data used in the thesis are from baseline and 3 months testing.

The third study (OLD12) primarily investigated the effect of 12 months heavy or moderate load resistance training or no training on muscle power, strength and functional ability in older adults (62-70 years) (40). Thirty-six out of 451 participants were recruited for the tendon specific tests.

Table 9.1: Overview of studies, interventions, and tendon specific measurements

	CON	HRT	MRT / LRT
VERY OLD 0 → 3 months)	Mech. prop. CSA	Mech. prop. CSA	
OLD3 0 → 3 months	Mech. prop. CSA Fibril morphology	Mech. prop. CSA Fibril morphology	Mech. prop. CSA Fibril morphology
OLD12 0 → 12 months	Mech. prop. CSA Fibril morphology Collagen content Enzym. Cross-links AGEs	Mech. prop. CSA Fibril morphology Collagen content Enzym. Cross-links AGEs	Mech. prop. CSA Fibril morphology Collagen content Enzym. Cross-links AGEs

Blue: Paper I. Red: Paper II.
CON = Control (no training), HRT = Heavy load resistance training, MRT = Moderate load resistance training, LRT = Light load resistance training, Mech.prop. = Patellar tendon mechanical properties, CSA = Patellar tendon cross-sectional area.

The three studies are presented in two papers (table 9.1). Paper I compared very old (VERY OLD) and moderately old (OLD3) individuals to investigate the effect of age on tendon mechanical properties and dimensions, and the influence of age on the training response. Paper II investigated the effect of training load on tendon mechanical properties, collagen cross-links and fibril morphology. The thesis additionally presents data on the effect of training duration by comparing the OLD3 and OLD12 studies. In the following sections, I present some general considerations about the study design and methods common to the three studies.

9.1 Study design

Data presented in the manuscripts and thesis were collected in parallel group randomized trials, which have the general advantage of limiting the risk of confounding and strengthen conclusions about causal relationships. The collaboration with larger randomized trials made it possible to collect longitudinal data, not only on tendon physiology, but also blood parameters as well as muscle strength and size, from three different populations, which would have been challenging without the administrative, technical, and practical support from the primary studies. There may also be disadvantages of performing and comparing sub studies (62), since the study populations, interventions, test-protocols, etc. may not be specifically suited for the purpose of the sub-study.

In addition to the longitudinal design, we also used a cross-sectional design to compare VERY OLD to OLD3 and OLD3 to OLD12. The cross-sectional design was of great value when investigating the effect of aging because of the long time perspectives in longitudinal

aging research. It is however important to be aware of confounding factors such as sex, physical activity level, smoking, diet, or other life-style factors, which may be associated with aging. Comparison of OLD3 and OLD12 also added valuable cross-sectional data, which was challenging to obtain in a longitudinal design. While some measurements could have been obtained by adding an extra data acquisition time-point in the same population, tendon biopsies could only be obtained once in each patellar tendon, because of safety issues and the possible influence of the first biopsy on the quality of the second biopsy (59).

9.2 Study populations

Participants were recruited from the greater Copenhagen area via advertisements in local newspapers and on the internet. All three studies included relatively healthy men and women, but also allowed participation of individuals with a certain degree of surgical or stable medical diseases, which did not prevent them from participation in strength training. While this contributes to variation in in the data, it also makes the results more generally applicable to older adults. We specifically paid attention to exclude candidates with previous knee-surgery, history of knee-pain within the past 12 months, or a history of patellar tendon steroid injections, since this may directly affect patellar tendon matrix and mechanical properties. We further excluded candidates taking hormonal or anti-hormonal medications, which may affect muscle and tendon adaptations to resistance training (141). Randomization was stratified for sex to obtain equal sex-distribution in the intervention- groups and avoid statistical interactions. For a more thorough list of in- and exclusion criteria, the reader is kindly referred to previous publications (10, 11, 40).

9.3 Interventions

The interventions are summarized in table 9.2. Heavy load resistance training (HRT) was in all three studies a supervised, whole body, progressive training program. Participants performed 3-5 sets at 70-90% of 1RM three times per week. The leg exercises targeting the patellar tendon were leg press and knee-extension performed in Technogym fitness machines (TechnoGym, Gambettola, Cesena, Italy). The light (LRT) and moderate (MRT) load resistance training was home-based, whole body, progressive training programs performed with TheraBand® elastic bands (Hygenic Corp., Akron, OH, USA) and the participants own body-weight. The exercises were similar to HRT, and each exercise lasted approximately 1-1.5 minutes corresponding to 12-20 repetitions. Training frequency was either 3 (OLD12) or 3-5 (OLD3) times per week, and the

participants were supervised either once a week (MRT) or once a month (LRT). Consequently, LRT and MRT training compliance relies on self-report. CON was encouraged not to change their habitual physical activity level, which was <1 hour of regular strenuous physical activity per week. All participants in VERY OLD and OLD3 received nutritional supplementation.

Table 9.2: Resistance training interventions

	VERY OLD	OLD3		OLD12	
	HRT	**HRT**	**LRT**	**HRT**	**MRT**
Duration	3 months	3 months	3 months	12 months	12 months
Frequency	3/week	3/week	3-5/week	3/week	3/week
Sets	3-5	3-5	3	3	3
Repetitions	6-12	6 - 12	1-1.5 minutes (app. 15-20 reps.)	6-12	12-18
Intensity (% of 1RM)	70-90% [a]	70-90% [a]	40-50% [b]	70-90% [a]	50-60% [b]
Leg-exercises	Leg-press Knee-extension Hamstring-curl	Leg-press Knee-extension Hamstring-curl	Chair-stand/squat Knee-extension	Leg-press Knee-extension Hamstring-curl Hip abduction	Chair-stand/squat Knee-extension Hip abduction Hip extension
Equipment	Fitness Machines	Fitness Machines	Elastic bands and body weight	Fitness Machines	Elastic bands and body weight
Supervision	All sessions	All sessions	Once a month	All sessions	Once a week

[a] *Approximately, according to Brzcyskis formula* (21). [b] *Approximately based on intended number of repetitions to fatigue.*

HRT followed the exact same protocol in OLD3 and VERY OLD, whereas HRT in OLD12 followed a different protocol with slightly lower training volume (only 3 sets of 6 RM compared to 4 or 5 sets in OLD3 and VERY OLD). Overall the relative load was similar between the three studies, but VERY OLD had a lower absolute training load because of lower maximal strength. LRT trained with slightly lower load but higher training frequency than MRT, which makes the overall training volume comparable in these two groups. Training volume was not directly matched between HRT and LRT/MRT, but all participants were instructed that the exercise should feel exhaustive, and the physical trainers paid attention to continuously adjust load to reach the intended number of repetitions

9.4 Assessment of patellar tendon mechanical properties

The first studies to investigate tendon mechanical properties used in vitro testing of animal tissue, and this research was pioneering in the understanding of tendon behavior during loading (132). While in vitro testing is easier to control and gives less variation, it may however not translate directly into in vivo tendon behavior. The first reports on in vivo tendon mechanical

properties during muscle contractions were published in the mid 90'es and used B-mode ultrasonography to visualize tendon deformation (46, 47). Ultrasound (US) imaging quality has since then improved and become more widespread, and is today a validated method for investigation of in vivo tendon mechanical properties, which is used in our own (29, 54) as well as other laboratories (23, 52, 82, 131).

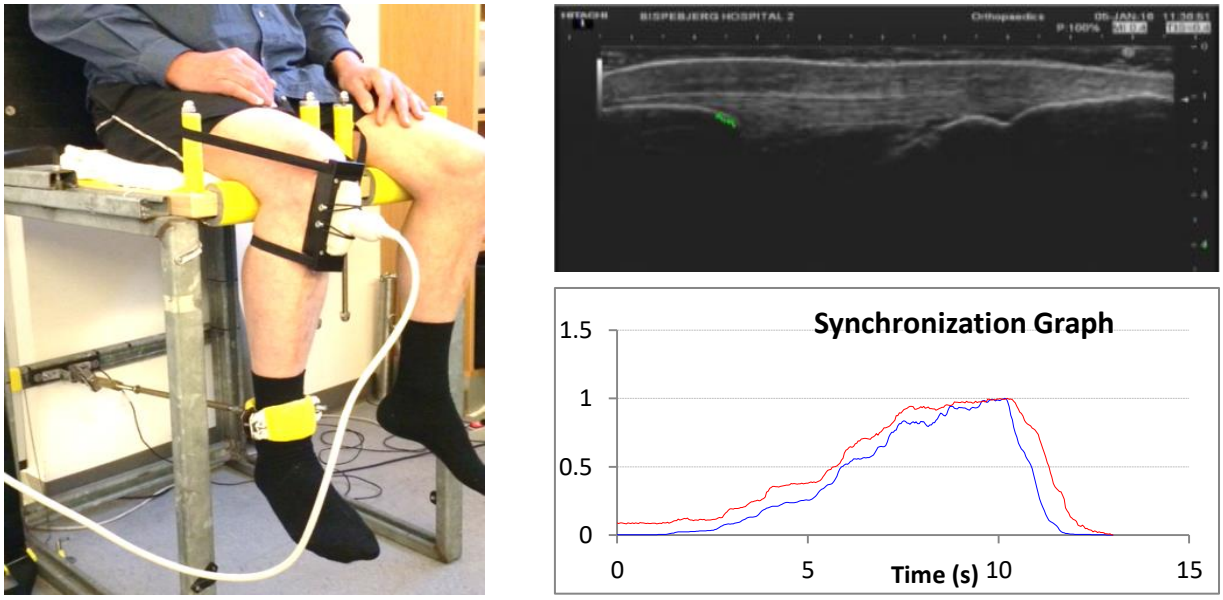

Figure 9.1: *Left: Experimental setup used for the ramped isometric contraction. Top right: US video displayed in the tracking software and marked for tracking (green dots). Bottom right: Example of the relative increase of force (blue) synchronized with the relative increase of deformation (red) based on output from the tracking software and force sampling device.*

The method is based on synchronous measurement of external joint moment and tendon deformation during 8 sec. ramped isometric contractions (fig. 9.1). Although the method is previously validated (54), it is not without limitations. Seynnes and colleagues recently published a critical evaluation of the method (142), where they found up to 30% difference in stiffness between two studies with comparable populations and testing methods (29, 141), and they also noted a large difference between two studies of training induced change in tendon stiffness reported by the same group (80, 82). The large variation is probably due to the complex nature of the method, involving knowledge and presumptions about biomechanics as well as US imaging, digital processing, and physiology (142). The challenges specifically relate to the estimation of tendon force and tendon deformation, as well as preconditioning and rate dependent mechanics, which I will consider in the following.

9.4.1 Estimation of tendon force

Tendon force (N) was calculated by dividing knee joint moment with the estimated internal knee-joint moment arm (167). When calculating the knee joint moment, we chose to leave out the contribution of antagonist (hamstring) co-contraction. Although co-contraction may amount to 10-30% of the resultant knee joint moment estimated by electromyography (EMG) (179), it also adds an additional level of technical variation to tendon force estimation, which in our experience (unpublished data) is even larger in untrained individuals due to EMG cross-talk (overestimated co-contraction due to quadriceps signal).

Internal knee-joint moment arm was estimated as a percentage of femur length according to Visser and colleagues, who found a good correlation between femur length and internal patellar tendon moment arm at different angles in cadavers (167). We measured femur length as the distance from the lateral collateral ligament to the most lateral point of trochanter major on the skin, and used the same estimated internal moment arm in PRE and POST measurements since this factor is not expected to change over time. Other approaches to determine patellar tendon moment arm include different imaging techniques such as X-ray, MRI, and recently also DXA scanning (41). Using these techniques, estimation of patellar tendon moment arm at 90 degrees knee flexion ranges from just above 20 mm to just below 50 mm (162), which emphasizes the impact on accurate assessment of tendon force. With the method used in the present investigation, we found a relatively low average moment arm (28 ±2 mm) compared to the different imaging techniques, which might have resulted in overestimation of tendon force during the ramped contractions. While the absolute values may have been affected, the PRE to POST comparisons would however not be affected.

9.4.2 Estimation of tendon deformation

Tendon deformation was estimated based on movement of the bony insertions relative to one another. With the US-based method it was only possible to visualize movement in the sagittal plane although discrete out of plane movement may contribute significantly to actual tendon deformation. We used a 100 mm linear array probe to keep both insertions in the field of view. Other studies only considered movements of the patella relative to an echo absorptive marker placed on the skin (22, 52, 101), but this approach may under- or overestimate deformation, depending on which way the tibia moves during the isometric contraction (54).

Analysis of the US videos was performed with semi-automated software specifically designed for the purpose (98). A blinded investigator manually selected the

anatomical reference points to be tracked, which were the patella apex and the tibia tuberosity (fig. 6). Besides being faster, automated tracking is generally less biased than manual tracking because the human-computer interaction is minimized (142). One challenge to the method was that the selected area not always maintained the same contrast level throughout the video, making it difficult for the dots to keep track with the actual bone movement. In eight participants where the visual discrepancy was pronounced, we analyzed deformation using a custom implementation of cross-correlation tracking in Matlab (R2015b, MathWorks Inc., USA) developed by one of the co-authors in paper I and II (Rene B Svensson). In these cases, we always made sure to track paired measurements (PRE, POST) with the same software. Another challenge was that even during isometric contraction there is a certain degree of rotation of the bony structures because of soft tissue deformation. Specifically, we noted that the proximal tibia (proximal to the insertion) often rotated posteriorly during the contraction. We therefore always made sure to track the bone movements within the boundaries of tendon insertion on the patella apex and tibia tuberosity respectively.

Participants performed 4-6 trials, and for each trial a blinded investigator performed several trackings of both the patella and tibia. The two best trackings from each bone-insertion were selected based on visual consistency between movement of bone and tracking dots as well as reproducibility of the estimated deformation. We then selected the two most representative trials for further analysis based on the following criteria: good synchronization between force and deformation, a smooth inclining force curve, return to baseline after relaxation, and all other things equal we chose the trials with highest force and/or deformation. We believe that this protocol gave the most reliable results, because it accounted for several aspects of tracking issues compared to only using one or two of these. Blinded investigators performed the analysis to avoid the potential bias introduced by the manual judgment.

The force-deformation data were fitted to second order polynomials because of the non-linear force-deformation behavior of tendons (12) (fig. 9.2). At high forces the tendon response in principal becomes linear, but within the force ranges achieved in the present measurements, a second order polynomial fit provided a good estimate of tendon mechanical behavior. Due to the non-linear force-deformation behavior, stiffness would increase with increasing force, and to account for this we analyzed mechanical and material properties at the highest force level common to the PRE and POST measurement for each individual.

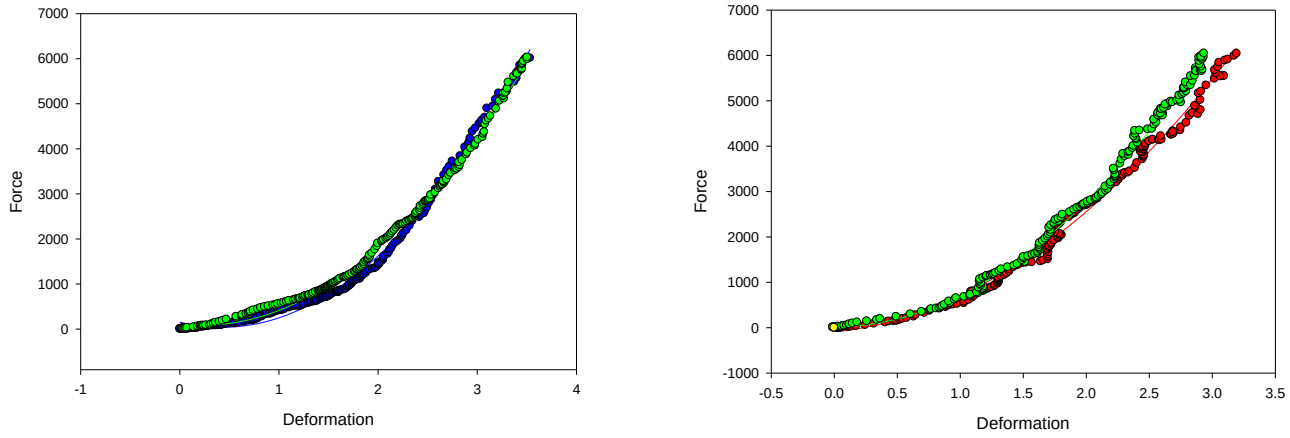

Figure 9.2: *Force-deformation curves with second order polynomial fits before (left) and after (right) the intervention. Note that the curves are cut at common tendon force. Curves are made in Sigma Plot version 10.0 (Systat Software Inc.).*

9.4.3 Preconditioning and rate of ramped contraction

As described in the introduction, tendons exhibit time-dependent mechanical properties because of the viscous component. The term conditioning implies that tendons elongate more when repeatedly loaded, at least in the first approximately 5 trials (95). It is therefore important to precondition the tendon to obtain reproducible measurements. In the present studies, participants performed 5 min warm-up on a cycle ergometer and 1-3 practice trials of the ramped maximal isometric contraction to precondition the tendon before the 4-6 recorded trials. The reason for limiting the number of conditioning trials was that it might compromise the participants in reaching maximal force in the last trials. We did not compare mechanical properties between the trials although another study has reported that stability in mechanical properties is not reached until the 5^{th} or 6^{th} trial (134). In addition to protocol preconditioning, time of day might also influence mechanical properties (123), and we consequently made sure to perform the PRE and POST tests on the same time of day.

Another source of variation caused by the time-dependent nature of tendon mechanical properties is the rate of tendon deformation. Changing deformation rate from 50 Nm/s (~3 sec.) to 110Nm/s (~1.5 sec.) has been shown to increase tendon stiffness by approximately 30% (85), although this is not a consistent finding (79). To help participants perform a smooth and linear increment in force peaking exactly after 8 sec., the investigator counted loudly from 1-8 with increasing loudness and at the same time showed the count with the fingers. A digital feed-back instruction may however provide more precision in future research projects. Other studies have used between 4 and 10 second ramped contractions, which

could give rise to moderate differences in estimation of stiffness between studies, but as long as the same rate was used in repeated measurements, it should not affect the results appreciably in the present investigation.

9.4.4 Summary

Despite the reported variability of in vivo mechanical properties using the ramped contraction and US measurement of tendon deformation (142), it is possible to obtain reliable measurements (54), at least when the tendon has been preconditioned (134). In the present investigation we used the same test protocol in all three studies to limit technical variability. We also made an effort to use the same investigator for data acquisition and analysis. Hence, one investigator performed all data acquisition in VERYOLD and another blinded investigator analyzed the data. In OLD3 and OLD12, one investigator performed all data acquisition except for four tests, and two blinded investigators performed all data analysis.

Other US based methods for determining in vivo tendon mechanical properties are speckle tracking (77) and US elastography (27). Although these methods may be promising, there are very limited data on in vivo patellar tendon mechanical properties, and furthermore we did not have access to this type of equipment. The method presented above therefore was the most feasible and reliable alternative for determining in vivo tendon tensile mechanical properties.

9.5 Measurement of patellar tendon dimensions and signal intensity

The most common in vivo methods for determination of patellar tendon dimensions are ultrasound (US) and magnetic resonance imaging (MRI). Although, both methods have been reported to give reliable estimates of tendon CSA (78, 104), they cannot be used interchangeably since a recent study found a 5.5 % difference in Achilles CSA between the two methods (78). Moreover, the reliability of US based measurement may be more dependent on operator experience than MRI. The literature is more sparse when it comes to assessment of accuracy, but one study verified MRI based measurement of horse patellar tendon with in vitro measurement using optical imaging and found only 2.8% underestimation of the MRI based CSA when measurements was performed using the NIH (National Institute of Health) color scale (33). Although more time-consuming, costly and difficult to access, we therefore chose to use MRI to assess patellar tendon CSA and length in all three studies.

A phantom containing 1.0% $CuSO_4$ was scanned together with each participant and subsequently used to normalize tendon signal intensity before delineation of the tendon borders. A draw-back to this method is that the content of the phantom can change slightly over time giving time-dependent changes of signal intensity. However, the effect on group comparisons would be limited due to the randomized controlled study design. Another possibility would have been to normalize signal intensity to the bone, but the whiteness of bone on T1-weighted MRI can differ slightly between scans, which will give random differences in the signal intensity used to normalize the image. In a few instances however, the phantom was either forgotten by the radiographer or was not in the field of view, in which case tendon signal intensity was normalized to bone before delineation of the tendon borders. We always used the same normalization procedure in PRE and POST measurements on the same participant.

Measurement of patellar tendon dimensions was performed manually in Osirix imaging software (version 2.7.5, Osirix Imaging Medical, Geneva, Switzerland), and we therefore used blinded investigators to avoid bias. One blinded investigator analyzed all OLD3 and VERYOLD, and another blinded investigator analyzed all OLD12 using the same protocol, which makes it reasonable to compare tendon dimensions between studies. In VERY OLD and OLD3, measurements were performed on grey-scale images, but the investigator always verified the selection of grey-scale borders on the NIH color image to provide a more accurate measurement of tendon CSA (33). All measurements in OLD12 were adjusted and measured using NIH color scale (fig. 9.3).

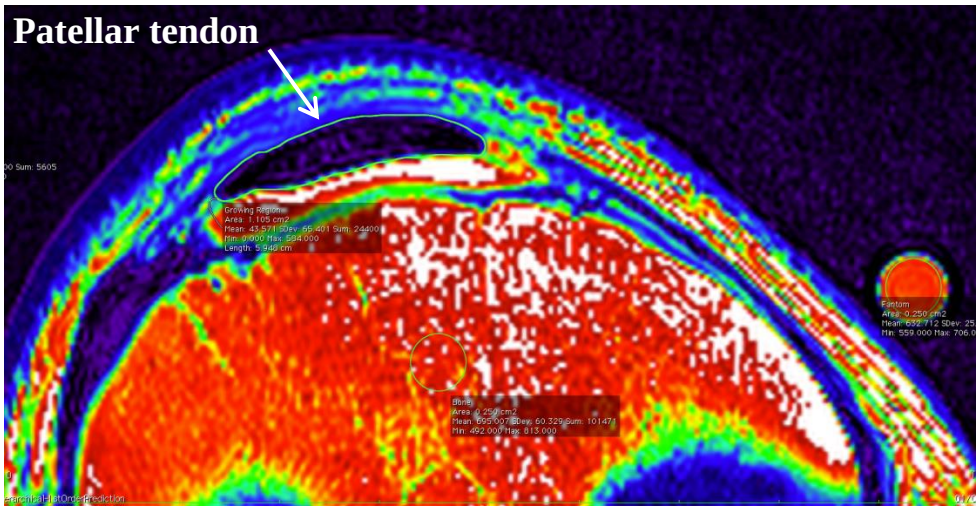

Figure 9.3: *MRI scan of the patellar tendon displayed in Osirix imaging software using the NIH (National Institute of Health) color scale. Note the delineation of the patellar tendon, which was used to calculate tendon cross-sectional area and signal intensity.*

A coefficient of variation corrected for small sample sizes (CV) was calculated for the triplicate measurements. In OLD12, PT length had a mean CV = 1.5% (range: 0.2 – 3.2%), and PT CSA showed an average CV = 2.3% (range: 0.2 – 6.1%). Day to day variation in measurement of CSA showed a CV = 2.4%. This indicates reliable measurements.

A final consideration is that vigorous physical activity may result in water retention in the tendon, and give rise to overestimation of tendon CSA after training (143). We therefore paid attention to perform the MR-scans at the same time of day (15.30-18.00) and carefully instructed participants to avoid strenuous physical activity in the preceding 72 hours to make the PRE and POST intervention conditions as similar as possible.

9.6 Tendon biopsies for structural and biochemical analysis

The patellar tendon is a valuable target of tendon tissue investigation because of its easy accessible location beneath the skin, and its relation the functionally important quadriceps muscle. Biopsies were obtained from all participants in the OLD3 and OLD12 studies, except for one individual, who started anticoagulant therapy during the intervention. No biopsies were obtained in VERY OLD. The patellar tendon biopsy procedure is performed routinely in our lab and has been described in previous publications (29, 75). The biopsies were obtained with a semi-automated biopsy instrument (Bard Magnum, Bard Biopsy Systems, USA) through a skin incision just distal to the patellar apex in a 45º angle relative to the patellar tendon in the proximal to distal direction (fig. 9.4). Using this protocol, we obtained tissue from the mid core region of the tendon.

Figure 9.4: *Left: The sterile patellar tendon biopsy procedure. Right: The semi-automated biopsy instrument (Bard Magnum, Bard Biopsy Systems, USA).*

It is important to be aware that tendons may have region specific variations in composition (34, 160). A patellar tendon biopsy taken from the mid core region might have a different composition and response to training than tissue in the peripheral or proximal/distal regions. To make the biopsies as similar as possible, the same physician obtained or supervised all biopsies

except for four, which were obtained by another experienced physician. We further made an attempt to standardize the biopsies by obtaining them at the same time of day (± 1 hour) before and after the intervention, because of the potential influence of circadian rhythm on tendon tissue (177).

A final consideration of doing repeated biopsies of tendon is that the second biopsy is influenced by a previous biopsy in the same tendon (59). We therefore chose to biopsy the non-dominant leg before and the dominant leg after the intervention, where dominance was determined as "the leg the participant would most likely use to kick a ball". There was a 6% strength difference between the two legs at baseline ($p=0.02$), suggesting a small difference in habitual loading and potentially also in tendon structure and composition, which may influence the results. There was however no correlations between baseline strength and baseline biopsy results, suggesting that muscle strength did not appreciably influence tendon structure and composition. We can however not exclude that the observed changes over time in tendon structure and composition may be due to differences between the dominant and non-dominant leg.

Another investigator prepared the tissue for further analysis under light microscope. The biopsy was dissected free from potential non-tendinous tissue (i.e. fat and subcutaneous tissue). The investigator then cut a small piece with visible regular, longitudinal arrangement of the collagen fibers for TEM. The rest of the sample was immediately frozen in liquid nitrogen stored at -80 ºC until analysis of collagen content and collagen cross-links.

9.6.1 Assessment of fibril morphology

Fibril morphology was assessed using TEM. Although tissue preparation is time-consuming and costly, and may also induce damage to the tissue structure, the method allows reproducible tissue visualization at very high magnification. Preparation and embedment of the tissue is explained in details in previous publications (75, 100).

Images were obtained using a Philips TM 100 transmission electron microscope at 80 kV equipped with Megaview 2 camera. At low magnification, we observed that the samples contained a varying amount of high quality areas with cross-sectioned fibrils in clearly visible fascicles. Obtaining random images from the samples would therefore introduce a risk that a high percentage of the images would not contain cross-sectioned fibrils at all. To avoid bias in the selection of areas of interest, the blinded technician obtaining the images was instructed to zoom to a magnification of 1050 (100x100µm) where the tissue microstructure was not visible,

and to find two different areas with core fibrillary structure (fascicles). The two areas were divided in six fields visually, and the technician selected one area in each field, zoomed to 24500 x magnification (4x4µm), and took a picture. In this way we obtained a total of 12 images per biopsy. We believe that the method provided an unbiased evaluation of fibril morphology within tendon fascicles, because the technician was blinded to group allocation.

Another blinded investigator performed all measurements of fibril diameters in the image analysis software package Image J (NIH, Bethesda, Maryland, USA). One of the co-authors to paper I and II (Rene B. Svensson) developed a macro for semi-automated fibril measurements, which was applied by the investigator on each image initially. The investigator then confirmed the automated measurements within the 90,000 nm^2 (300x300 nm) counting frame, and manually corrected any erroneously measured fibrils (fig. 9.5). We used an elliptic fit because some fibrils were not perfectly round, and chose the smallest diameter perpendicular to the longest axis of each fibril cross-section as the true fibril diameter to eliminate the influence of sectioning angle. The investigator counted on average 335±131 fibrils for each sample (12 images). The data were reduced for each individual and time point to mean fibril diameter, volume fraction (average fibril area x count / counting frame area), and fibril density (number of fibrils/area).

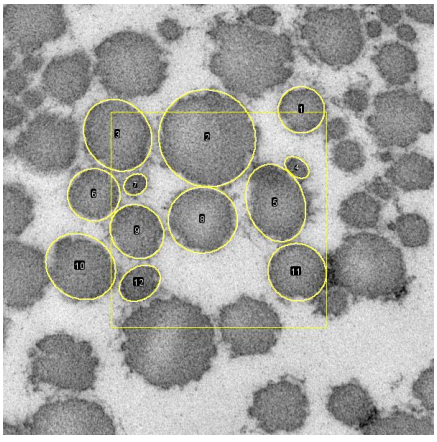

Figure 9.5: *Image of cross-section of patellar tendon obtained through the transmission electron microscope. Dark grey elements are collagen fibrils. The yellow square is the counting frame. Collagen fibril diameter was measured using the smallest diameter perpendicular to the longest axis of the elliptic fits (yellow ellipses).*

9.6.2 Measurement of collagen cross-links

In the present investigation we wished to analyze enzymatic cross-links as a measure of regulated matrix adaptation, as well as pentosidine as a marker of adventitious glycation-induced collagen cross-linking. We also wished to characterize the matrix content of non-cross-linking AGE adducts, because no one has done this in human tendons before. However, it turned out to be significantly more challenging than we had anticipated.

The most commonly measured mature enzymatic cross-links in tendons are LP and HP, of which the latter is present in fairly high amounts in tendon. In humans, the most commonly investigated cross-linking AGE is pentosidine although other cross-linking AGEs are more abundant in collagen-rich tissue (i.e. glucosepane, glyoxal-lysine dimer (GOLD), methylglyoxal-lysine dimer (MOLD)) (5, 135). Pentosidine is often used as a biomarker of aging because it increases almost linearly with age (8, 29) and is relatively easy to measure because of its natural fluorescence. Moreover, it seems to be associated with the degree of organ dysfunction in diabetics (136, 137, 145). Commonly investigated non-cross-linking AGEs are carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), and methylglyoxal-derived hydroimidazolone-1-3 (MG-H1-3). CML probably contributes to arterial stiffening with aging (138, 139). Further, CML, CEL, and MG-H1 were found elevated in tail tendon (39, 133) and Achilles tendon (146) of rodents on an AGE-rich diet, and this was associated with increased tendon stiffness. Moreover, sequestration of non-enzymatic glycation products by addition of their parent amino acids arginine and lysine, reversed glycation induced increase of rat tail tendon stiffness (105). No studies have so far quantified non-cross-linking AGEs in human tendons, leaving their impact on human tendon mechanical properties unresolved.

There are different more or less specific methods of measuring enzymatic cross-links and AGEs including fluorimetry, enzyme-linked immunosorbent assay (ELISA), high pressure liquid chromatography (HPLC), and mass spectrometry (88, 126). We have previously analyzed tendon samples for cross-links and AGEs with the help of collaborators from other laboratories using mass spectrometry (39) and HPLC (29). However, these collaborations were not possible in the present investigation, and we therefore relied on new collaborators with experience in measurement of enzymatic cross-links and AGEs.

One laboratory (The protein oxidation group, Department of Biomedical Sciences, University of Copenhagen) with expertise in measurement of protein modifying AGEs using HPLC and fluorimetry agreed to collaborate, and I went on a research stay in their laboratory to test the method. The HPLC protocol used should be able to detect CML, CEL, and MG-H1-3, but not the cross-linking AGEs pentosidine, GOLD, or MOLD since they elute very late and therefore required long run-time, which could compromise the analysis of the other AGEs. To test the protocol on human tendons, we initially made some test-samples consisting of cadaveric old human patellar tendon, gelatin with different hydrolysis times (a pure collagen sample) and hydroxy-proline (an “AGE-negative” control). Fluorimetry showed higher fluorescence in the old tendon than in any of the other samples, indicating that the method was able to detect accumulation of fluorescent AGEs in tendon samples. Furthermore, longer hydrolysis time

seemed to increase fluorescence. In the first HPLC runs, we found high levels of CML and MG-H2 in all samples except for the hydroxyl-proline sample. Old tendon had higher values of all the AGE products than gelatin, which was also what we expected. To our concern, the results did however not match the expected concentration for at least CML and MG-H2 which was unrealistically high (fig. 9.6).

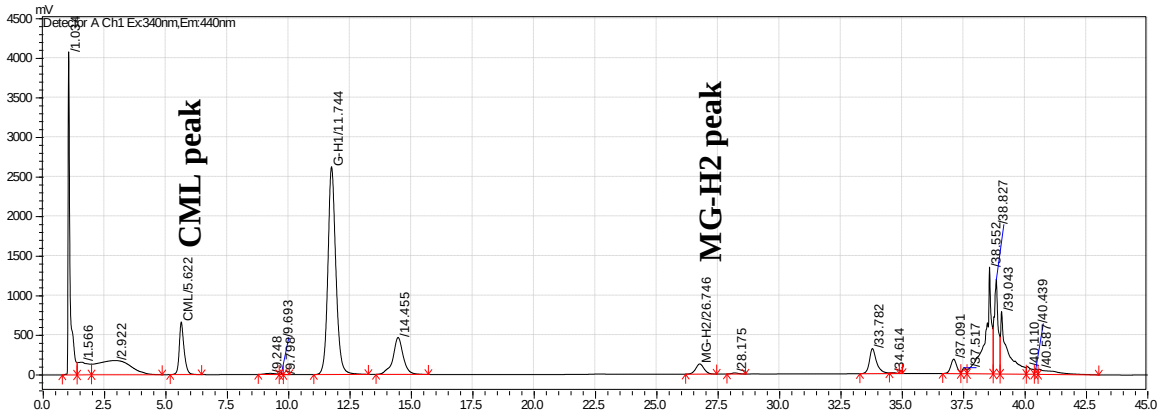

Figure 9.6: *Chromatogram of test samples displaying larger than expected peaks for CML and MG-H2 which may be due to co-elution of amino-acids.*

Additional runs were performed, including spiking with CML and MG-H2 samples and a separate amino acid analysis, and we discovered that the protocol was not able to separate these AGEs from other amino acids, which co-eluted with the expected target AGE peaks. Additional attempts were made to develop at method that could separate the AGE targets of interest from the amino acid peaks, but due to time constraint we did not succeed.

Another laboratory (X-lab, Department of Biomedical Sciences, University of Copenhagen) with expertise in HPLC agreed to collaborate on measurement of HP, LP, and pentosidine. Unfortunately, the HPLC equipment malfunctioned before we could finalize analysis of our samples. Due to time constraint we were again forced to come up with an alternative method. We therefore chose to measure HP and LP with the ELISA technique and estimate AGE content with fluorimetry since this was possible in our own lab.

Although the outcome of ELISA analysis depends on the specificity of the antibody, it is more feasible and less costly than mass spectrometry and HPLC. Two different ELISA kits were used, one made for measuring HP in serum (MicroVue Serum PYD, 8019, Quidel Corp.) and one for measuring LP in urine (MicroVue DPD, 8007, Quidel Corp.). We only had one of each ELISA kit available in our lab, and due to time constraint we therefore only performed single determination of OLD 12 on the ELISA kit, but we plan to make a duplicate analysis. Due to the much higher concentration of HP and LP in the tissue hydrolysates than in

serum and urine, the samples (reconstituted in water at 5 mg/mL) needed to be diluted 2000 fold for the HP analysis and 100 fold for LP analysis, which reduces the risk of interference from compounds that would not normally be present in serum or urine. The assays were both competitive ELISAs, based on a polyclonal rabbit antibody for HP and a monoclonal one for LP. Aside from using a different type of sample the manufacturer's instructions were followed without modification. In addition to the control samples and standards from the kit, a number of test samples were included to help validate the results. A calibrator standard which was used for the initial HPLC measurements (PYD/DPD HPLC Calibrator, 8004, Quidel Corp.) was included as well as a couple of samples that had been successfully analyzed on HPLC prior to the technical problems. Both the calibrator and samples provided results that were fairly consistent with the expected values in relative terms, but the ELISA kits appeared to underestimate the absolute values by approximately 40%. This difference could be due to differences in the standards that came with the kit compared to the HPLC calibrator, but we will investigate this further. Further validation is required, but due to the several prior methodological setbacks, it was not possible within the timeframe of the PhD period. Based on the limited validation we currently believe that the ELISA quantification is suitable for comparison of samples within OLD12.

The fluorimetric assay only provides an overall estimation of AGE accumulation, and we consequently had to give up the quest to quantify specific AGEs in the tendon. As for pentosidine, collagen-linked fluorescence of connective tissue however seems to increase linearly with age and is related to the degree of diabetic complications (108, 109). Given the abundance of different AGEs of which far from all are characterized yet, and the random nature of glycation modifications, it thus seems reasonable to use the total fluorescence as a measure of tissue modifications by AGEs. Fluorescence measurements to quantify AGE accumulation is typically conducted on enzyme digested samples (108). However, the present samples had already been hydrolyzed for HPLC measurements. While several of the AGEs survive hydrolysis, including fluorescent ones like pentosidine, the harsh process is likely to destroy, induce or alter fluorescent compounds, as also evidenced by the increased fluorescence that we observed in gelatin samples with increasing hydrolysis duration. Hydrolysates are therefore suboptimal for AGE quantification by fluorescence. However, since all samples were treated in the same way we have no reason to believe that this would bias our results although it may increase the variance. Fluorescence measurements were made on the hydrolysates reconstituted in water. To ensure a consistent pH and get enough volume for triplicate measurements, samples were diluted 6 fold into 0.12M HCl (0.1M final concentration). We

chose to use HCl rather than a buffer in order to facilitate recovery of the sample for possible use in other measurements, since keeping options open for alternative measurement techniques had proven important previously in this endeavor. The diluted samples were plated onto black 96 well plates and read on a Wallac1420 Victor microplate reader (Perkin Elmer) at 340/5 nm excitation and 460 nm (382-507 nm) emission. Fluorescence was measured in samples containing 0.83 mg tissue/ml. Fluorescence correlated positively to collagen content (r^2=0.52, $p<0.001$), which was also expected given the slow turnover time of collagen relative to other proteins in the samples. We consequently chose to report fluorescence relative to collagen content. The fluorescence values from OLD3 were less reliable than OLD12 since OLD3 samples had been prepared and diluted for HPLC before fluorescence measurements. It was therefore not meaningful to compare fluorescence between OLD3 and OLD12, and only OLD12 data are included in the thesis.

9.7 Muscle strength testing

For the present investigation, we chose to use maximal isometric quadriceps strength (IsoMVC) as a measure of the strength training efficacy. IsoMVC was measured in all studies and is related to the isometric ramped contraction method for determination of PT mechanical properties. A Kinetic Communicator (KinCom) (model 500-11, Kinetic Communicator, Chattex, Chattanooga, TN) was used in VERY OLD and OLD3 and a Good Strength device (V.3.14 Bluetooth; Metitur, Finland) was used in OLD12. The procedures are described in previous publications (10, 11, 40). Since muscle strength was a secondary outcome, we did not make a formal test to compare the two different methods. Although this may limit the comparability of absolute muscle strength in OLD3 and OLD12, the relative changes in repeated measurements should be less affected by the different equipment.

9.8 Assessment of quadriceps muscle size

Data on muscle size were included to evaluate the efficacy of the training protocols. Trained radiographers scanned the participants in VERY OLD and OLD3 in a 1.5 T Philips Ingenia scanner (Philips Healthcare) and blinded investigators measured total quadriceps cross-sectional area (Q-CSA) using Osirix imaging software (version 2.7.5, Osirix Imaging Medical, Geneva, Switzerland). Another trained radiographer scanned all participants in OLD12 with a 3.0 T TX Philips Achieva scanner (Philips Healthcare) and one blinded investigator measured vastus

lateralis cross-sectional area (VL-CSA) using the medical imaging software package Jim version 6.0 (Xynapse Systems, UK). It is thus possible to compare Q-CSA between VERY OLD and OLD3, whereas VL-CSA can only be compared within OLD12.

9.9 Physical activity level

Muscle- and tendon performance may be related to habitual physical activity level. We therefore chose to include data on physical activity level, which was measured in all three studies. A previously validated (35) accelerometer/inclinometer (activPal micro, PAL technologies, Glasgow, Scotland) was worn by the participants for 4 (VERY OLD, OLD3) or 5 (OLD12) consecutive days, always including the weekend. Data were extracted with activPal software (Research edition, V.7.2.32, PAL Technologies, 2013). Here we report daily step count as a measure of habitual physical activity level. Some participants in VERY OLD presented unrealistically low step-counts, and were excluded from the data analysis. We speculated that the activPal device in some instances was not sensitive enough to detect steps when walking speed was too slow. This may have underestimated habitual physical activity level. Slow walking speed would on the other hand also provide less loading on the tendon, and the measured daily step-count in VERY OLD may therefore still represent a realistic estimate of habitual tendon loading although sample size was reduced.

9.10 Statistical analysis

In paper I, we wished to compare the effect of age and also the differential effects of HRT in the two age groups. This gave us three dimensions, which were age-group (OLD vs VERY OLD), intervention group (HRT vs CON), and time-point (0 vs 3 months). Instead of performing a three-way ANOVA, we chose to perform two different statistical tests to simplify interpretation of the statistical outcome. First we analyzed the effect of age on the baseline values using two-way ANOVA with age-group and intervention group as factors. Then we performed another two-way ANOVA on the delta values (change over time) again with age-group and intervention group as factors.

In paper II the setup was simpler since we only wished to analyze the effect of intervention group (HRT, MRT, CON) over time, and we consequently used repeated measures two-way ANOVA with baseline adjustment and Tukey-Kramer post-hoc test.

In the thesis, additional correlational analyses are performed using Pearson's correlation coefficient. Furthermore, the changes in the corresponding intervention-groups in OLD3 and OLD12 (CON3 vs CON12, LRT3 vs MRT12, HRT3 vs HRT12) are compared using Welch's unpaired t-test with Bonferroni correction. Baseline characteristics in all three studies are compared using one-way ANOVA with Bonferroni corrected post-hoc unpaired t-tests.

We purposely chose to exclude individuals from the data analysis, who did not complete the interventions for any reason, medical or personal, because we wanted to investigate the actual training induced adaptations. A specific compliance level was not determined a priori, but we performed secondary statistical analyses post hoc excluding participants with < 80% completed training sessions.

Despite stratified randomization, sex distribution was not entirely equal between groups, and we therefore performed secondary statistical analyses (2-way ANOVA on POST-PRE values) with sex and intervention-group as factors to account for the possible influence of sex on the outcomes.

Normality was confirmed by visual inspection of residual plots. Non-normally distributed variables were log-transformed and analyzed again with the same procedures. Before statistical testing outlier analysis was performed with an online Grubbs test (51).

Sample size was calculated based on expected changes and variation in the AGE marker pentosidine (74). Pentosidine was unfortunately not measured due to the challenges described in section 9.6. We instead performed a post-hoc sample-size calculation using tendon fluorescence as the alternative outcome. Assuming the same effect size in tendon fluorescence as previously observed for pentosidine (~20%), and using the standard deviation of the difference calculated in the present investigation (~200), we needed 10 participants in each group to reach 80% power.

Continuous variables are presented as arithmetic mean ±SE and log-transformed values are presented as geometric mean [upper limit-lower limit]. Baseline data were summarized as mean ±SD. We used SAS statistical Software v. 9.4 (SAS Institute, USA) for ANOVA-tests, and figures and correlational analysis were made in GraphPad Prism (v. 7.0, GraphPad Software Inc., La Jolla, CA).

10. Results & Discussion

This section presents and discusses main findings from the three studies. First, VERY OLD is compared to OLD3 as in paper I to discuss the effect of age and training on tendon mechanical properties. Secondly, results from OLD12 are presented to discuss the effect of training load on tendon adaptations as in paper II. Finally, results from OLD12 and OLD3 are compared, including data not presented in paper I & II, to discuss the influence of training duration. Participant characteristics from all three studies are presented in table 10.1, and the main findings are summarized in table 10.2.

Table 10.1: Participant baseline characteristics

	Study	Total	CON	MRT/LRT	HRT
Participants (men/women)	VERY OLD	26 (16/10)	14 (8/6)		12 (8/4)
	OLD3	28 (15/13)	10 (5/5)	9 (5/4)	9 (5/4)
	OLD12	33 (18/15)	10 (6/4)	13 (5/8)	10 (7/3)
Age (years)	VERY OLD	87 ± 3.2*‡	86 ±2.6		88 ± 3.7
	OLD3	69 ±2.5‡	68 ±1.5	70 ±3.2	69 ±2.2
	OLD12	67 ±2	68 ±1.8	66 ±2.4	67 ±2.3
Height (cm)	VERY OLD	169 ±11*‡	168 ± 12		171 ± 11
	OLD3	172 ±7	171 ± 6	173 ±7	170 ± 10
	OLD12	173 ±8	1.75 ±0.08	1.73 ±0.07	1.71 ±0.08
Weight (kg)	VERY OLD	69.9 ±4.0	69.5 ± 14.8		70.5 ±13.5
	OLD3	73.2 ±10.9	68.5 ± 6.9	74.4 ±8.3	77.2 ±15.2
	OLD12	78.0 ±14	81.6 ±16.9	72.9 ±11.6	79.4 ±13.9
BMI (kg/m^2)	VERY OLD	24.2 ±3.0	24.4 ± 2.9		24.0 ± 3.0
	OLD3	24.8 ±3.2	23.4 ± 2.0	24.8 ±3.2	26.5 ± 3.7
	OLD12	25.8 ±3.7	26.6 ±4.3	24.3 ±3.5	26.9 ±3.1
HbA1c (mmol/l)	VERY OLD	36 ±2.1	36 ± 2.2		36 ± 2.1
	OLD3	35 ±3.0	35 ± 2.7	35 ±3.4	36 ± 3.2
	OLD12	36 ±3.1	37 ±3.5	35.2 ±3.0	35.4 ±2.9
Total-Cholesterol (mmol/l)	VERY OLD	5.2 ±0.9‡	5.3 ± 0.9		5.1 ± 1.0
	OLD3	5.5 ±0.9	5.8 ± 0.5	5.2 ±1.0	5.5 ± 1.0
	OLD12	5.9 ±0.9	5.7 ±0.8	6.0 ±1.0	6.0 ±0.9
Training compliance (%)	VERY OLD	90 ± 8*‡			90 ± 8
	OLD3	79 ±16‡		86 ±12	71 ± 16
	OLD12	86 ±12		86 ±16	86 ±7

Values are mean ±SD. HRT = Heavy load resistance training, MRT = moderate load resistance training, LRT = Light load resistance training, CON = Control, BMI = Body Mass Index. ***ATT:*** *Only HRT and CON are included in the results and discussion of VERY OLD vs OLD3.*Significantly different from OLD3. ‡ Significantly different from OLD12 ($p<0.05$) based on one-way ANOVA and Bonferroni corrected post-hoc t-tests.*

VERY OLD participants were 18 years older than OLD3 ($p<0.05$), and OLD3 were 2 years older than OLD12 ($p<0.05$). OLD3 and OLD12 were 3 and 4 cm taller than VERY OLD ($p<0.001$). TARA12 m had higher total-cholesterol than VERY OLD ($p=0.01$) but it was not different from OLD3. Weight, BMI and HbA1c did not differ significantly between studies. Finally, compliance was different between all three studies. Asides from the impact of different

compliance, which is considered in the respective comparisons of the three studies, the relatively discrete differences in baseline physiological characteristics should not appreciably influence comparisons between studies.

Table 10.2: The effect of aging, training load, and training duration on the patellar tendon

	Aging	Training load	Training duration
Young's modulus	↘	↗	→ ↗ *
Stiffness	↘	↗	→ ↗ *
Cross-sectional area	→	→	↗
Signal intensity	NA	↗	NA
Fibril diameter	NA	→	→
Fibril density	NA	→	→
Volume fraction	NA	→	→
Enzymatic cross-links	NA	→	NA
Non-enzymatic cross links (AGEs)	NA	→	NA

AGEs = advanced glycation end-products. ↘ = reduction. ↗ = increase → = No effect. *NA = Not applicable. Effect of aging: OLD3 vs VERY OLD. Effect of training load: OLD12. Effect of training duration: OLD3 vs OLD12. *Although only OLD12 and not OLD3 increased tendon stiffness and Young's modulus, we could not confirm an effect of training duration. This overall conclusion is discussed in detail on pp. 64-65.*

10.1 Effect of aging on tendon size and mechanical properties (Study 1 & 2)

VERY OLD was to our knowledge the first study to quantify tendon mechanical properties in a group of adults over 80 years of age (87 years in average). Consistent with our hypothesis, we found significantly lower tendon stiffness and Young's modulus in VERY OLD compared to OLD3 (fig. 10.1). Average maximal stiffness was 2187 ± 201 N/mm and average modulus was 856 ±74 MPa in VERY OLD, and this was 43% and 39% lower than OLD3.

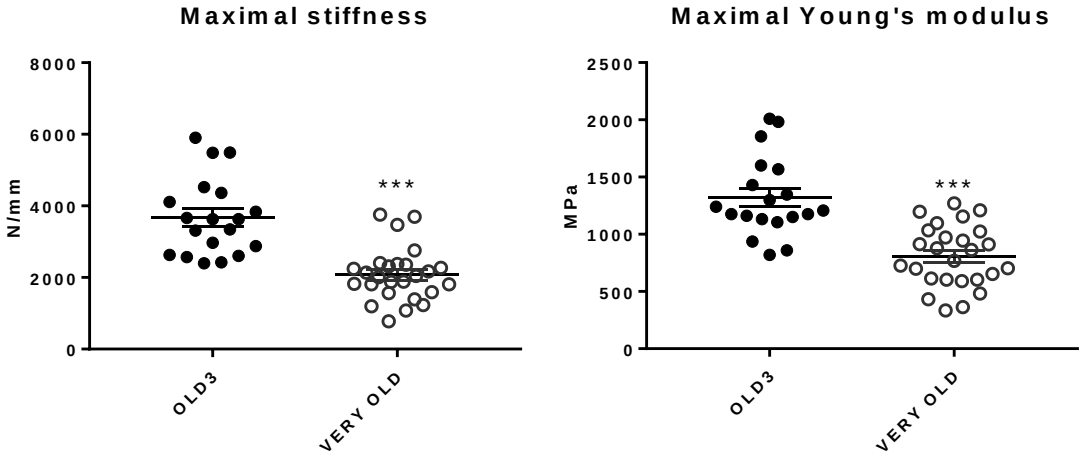

Figure 10.1: *Maximal patellar tendon stiffness and Young's modulus in OLD3 (~68 years in average, n=19) and VERY OLD (~87 years in average, n=26). Lines denote mean ±SE. ***Significant main effect of age (p<0.0001).*

Measurement of in vivo tendon maximal stiffness relies on the subject's ability to produce force. It is therefore important to notice that VERY OLD had 32% lower quadriceps strength and 18% lower maximal tendon stress in the ramped isometric contraction than OLD3. Because of the curvilinear stress-strain relationship, this could explain part of the reduction in tendon stiffness and Young's modulus. However, VERY OLD displayed 37% higher tendon strain and 18% lower stress, suggesting lower tendon modulus even at comparable submaximal stress-levels. This is illustrated in figure 10.2.

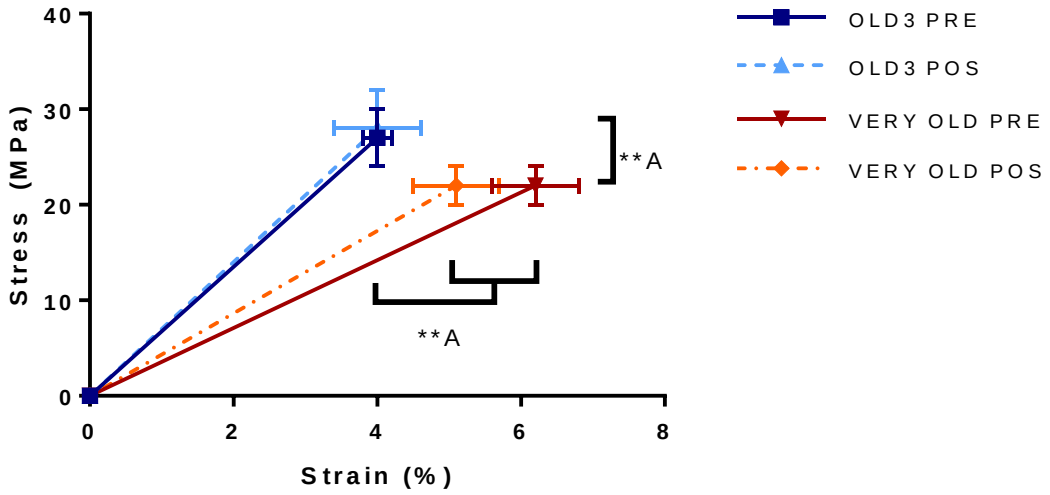

Figure 10.2: *Straight lines drawn on basis of mean ±SE values for maximal stress and strain before (PRE) and after (POST) three months heavy load resistance training in OLD (~68 years, n=9) and VERY OLD (~87 years, n=12). The slope of the lines depicts average Young's modulus. A: main effect of age-group, **p<0.01, based on 2-way ANOVA.*

Age-related reduction of tendon material properties is corroborated by previous studies, which have shown lower in vivo tendon stiffness in older adults ~70 years compared to young (69, 81, 106, 152), and a tendency for lower in vitro tendon modulus in 64-93 year old compared to 29-50 year old (66). Other studies have found no difference in patellar tendon stiffness between young and old individuals (23, 29, 31), which makes it difficult to draw definitive conclusions about the effect of aging on tendon mechanical properties.

Reduced physical activity may have a pronounced influence on tendon mechanical properties (16, 31, 84), and since habitual physical activity tends to fall with aging, this may explain some of the discrepancies. Some studies thus found indistinguishable tendon mechanical properties in young and old with comparable physical activity level (29, 32). In our study, VERY OLD had 43% lower step-count than OLD3 ($p<0.001$), which may partly explain the lower tendon modulus rather than a true effect of aging. Our own data did, however, not display any baseline correlations between maximal modulus and daily step-count when OLD3 and VERY OLD were analyzed separately (fig. 10.3), suggesting that reduced physical activity with aging could not alone explain the observed age differences in maximal modulus.

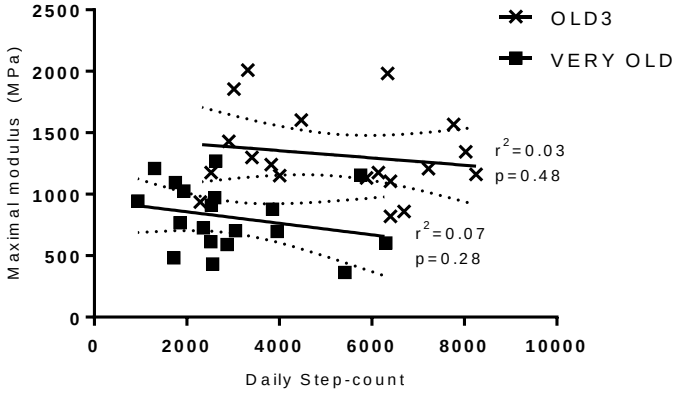

Figure 10.3: *Correlation between daily step-count and maximal modulus in OLD3 (~68 years, n=19) and VERY OLD (~87 years, n=18). Dotted lines are 95% confidence bands of the best linear fit. Eight missing values in VERY OLD due to technical error.*

It is possible that physical activity can partly counteract the effect of aging up to a certain age (~70 years), after which the effect of aging is more pronounced and is not compensated by maintained physical activity. Whether or not the lower tendon modulus in VERY OLD is a true age-phenomenon or mediated by reduced physical activity, it reveals a reduced tendon material quality with aging.

Tendon CSA was not significantly different between the two age-groups, although the mid region tended to be smaller in VERY OLD (p=0.09) (fig. 10.4), and this suggests that the tensile bearing material of the aging (or inactive) tendon is at least partly replaced by other space-filling non-collagenous material like fat, water, proteoglycans, glycoproteins, or AGES (see table 6.1). The unchanged CSA in VERY OLD compared to OLD3 confirms previous comparisons between young and old individuals, that tendons in contrast to muscle do not seem to atrophy with aging (23, 29, 32) but loose tissue quality, but no previous studies exist to confirm our findings in very old individuals.

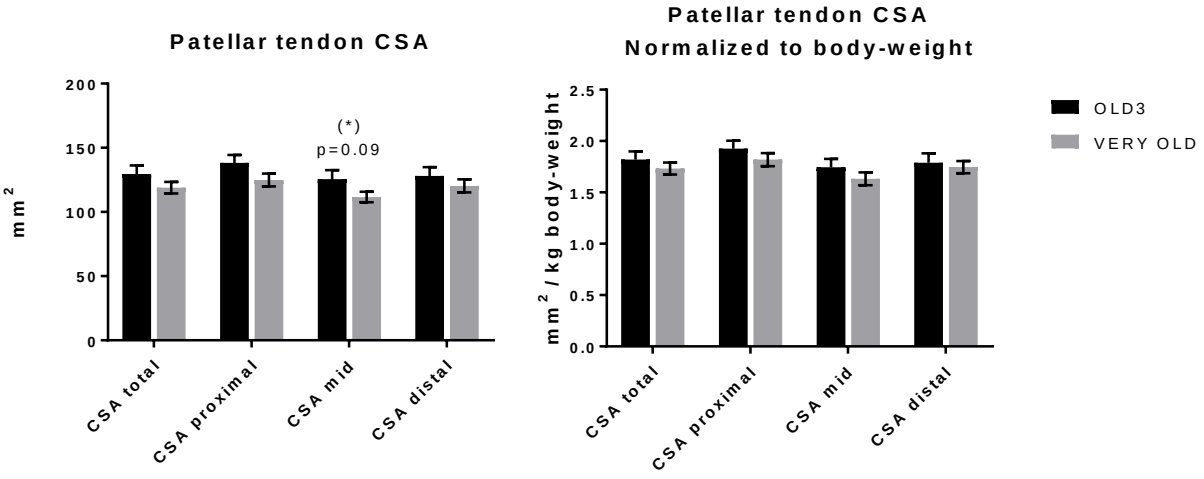

Figure 10.4: *Patellar tendon cross-sectional area (CSA)(left) and normalized CSA (right) in OLD3 (~68 years) and VERY OLD (~87 years). Bars represent mean ±SE. No significant differences.*

The trend towards an age-difference in mid-region CSA could partly be explained by 4% lower body-weight or 2% lower height in VERY OLD. Body-weight displayed the closest correlation to CSA in all regions (r^2 = 0.29-0.37, p<0.0001), and we consequently normalized tendon CSA to body-weight. This removed the trend towards reduced CSA in VERY OLD (fig. 10.4). The consistent absolute differences in the three CSA regions measured was, however, not entirely removed, and although this may be due to biological variation, we cannot exclude a slight loss of tendon material in very old age (or with reduced physical activity), which was not detected due to small sample size. Measurement of tendon CSA in VERY OLD and OLD3 was performed by the same blinded investigator using the exact same protocol, which limits the influence of technical variation on the results.

The consequences of reduced tendon properties with age are uncertain. A more compliant tendon may impair posttural balance (119) and compromise muscle strength and speed of contraction (19, 169) by shifting the muscle length-tension curve to the right (too much

sarcomere overlap) (128). In line with this, one recent study found a significant postitive correlation between rate of muscle force development and tendon stiffness when correlating data from young and old in the same model (127). However, another recent study on young men reported that stiffness of the free patellar tendon did not contribute significantly to quadriceps explosive strength (103), and the functional consequences of age-related decline in tendon mechanics are therefore still uncertain.

Young women tend to have more compliant tendons (97, 172), lower collagen content (92), and maybe also a protracted response to training compared to men (172), which may be due to higher estrogen levels in women (53). Older postmenopausal women do however not seem to differ appreciably from men in tendon mechanical properties (22), and we therefore chose to include both men and women to make the results more generally applicable. Although not statistically significant, slightly more men were included in VERYOLD than in OLD3. We consequently performed a secondary statistical analysis of the sex by age-group interaction. The analysis did not display any significant interaction, and it is therefore unlikely that the slight difference in sex distribution influenced our main findings. Interestingly, the analysis on the other hand showed that women across age-groups had lower tendon stiffness and CSA but unchanged modulus compared to men ($p=0.001$), which is consistent with other studies (22, 118), and confirm equal material properties in men and women in old age.

Total Cholesterol tended to be lower in VERY OLD ($p=0.09$) than OLD3, indicating selection of healthier individuals in VERY OLD. Some (9) but not all (39) studies have associated high serum cholesterol with increased tendon stiffness. In the present investigation, serum cholesterol did not correlate with tendon modulus in OLD3 ($r^2=0.003$) or VERY OLD ($r^2= 0.03$) independently and is therefore unlikely to explain the much lower stiffness found in VERY OLD.

Since VERY OLD was the first study to investigate in vivo tendon physiology of individuals >80 years, comparison with previous studies of tendon aging should be done cautiously. Age-related decline in physiological functions and performance may not be linear (90), and tendon size and mechanical properties may thus change more from moderately to very old age than from youth to moderately old age. This should be pursued in future investigations.

10.1.1 Effect of training dependent on age

According to our expectations both VERY OLD and OLD3 increased maximal strength and quadriceps cross-sectional area in response to HRT (Main effect of HRT, $p<0.05$) (fig. 10.5).

The improvements were not statistically different but VERY OLD displayed larger relative improvements in both muscle strength (OLD3: ~10%, VERY OLD: ~13%) and cross-sectional area (OLD3: ~1%, VERY OLD: ~3.5%), which may be due to the significantly higher training compliance in VERY OLD (90%) compared to OLD3 (71%).

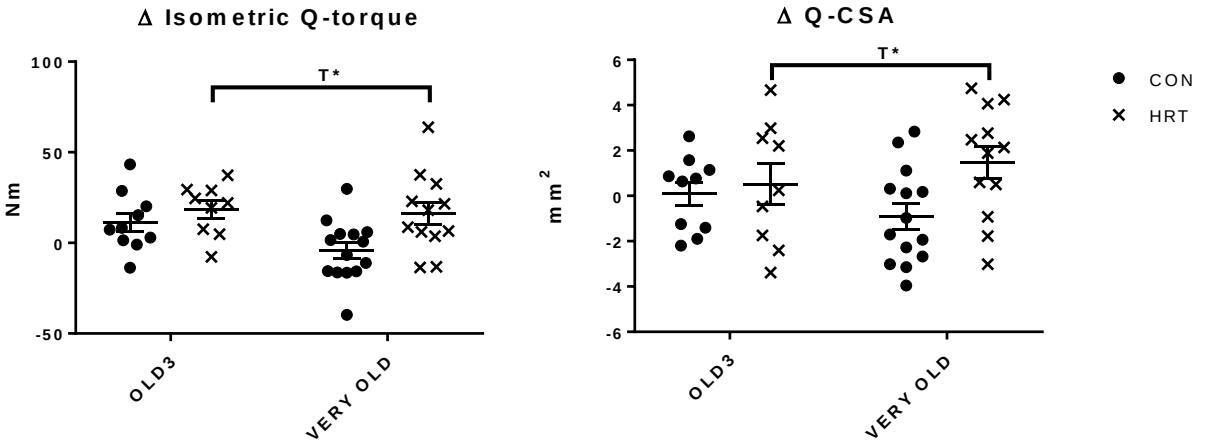

Figure 10.5: *Changes in isometric quadriceps (Q) torque and cross-sectional area (CSA) in OLD3 (~68 years) and VERY OLD (~87 years) after 3 months heavy load resistance training (HRT) or no training (CON). Bars represent mean ±SE. T*: Significant main effect of training (p<0.05).*

In line with the relative improvements in muscle properties, maximal tendon force during the ramped contractions displayed a significant interaction with larger difference between HRT and CON in VERY OLD compared to OLD3 (table 10.3), and the same trend was observed in maximal tendon stress (p=0.053). These results supported a larger quadriceps strength improvement in VERY OLD than OLD3.

Table 10.3: Maximal patellar tendon mechanical properties

		Baseline		Change over time	
	Age group	**CON**	**HRT**	**ΔCON**	**ΔHRT**
Max deformation (mm)	OLD3	2.1 ± 0.2	2.0 ± 0.2	0.1 ± 0.1	0.05 ± 0.2
	VERY OLD A*	2.6 ± 0.4	3.1 ± 0.3	-0.2 ± 0.2	-0.4 ± 0.3
Max force (N) AxT*	OLD3	3940 ± 290	4150 ± 430	280 ± 310	36 ± 340
	VERY OLD A**	3100 ± 310	3130 ± 330	-410 ± 170	330 ± 180
Max stiffness(N/mm)	OLD3	3620 ± 300	3720 ± 420	-190 ± 360	240 ± 500
	VERY OLD A**	2190 ± 200	1950 ± 210	-280 ± 190	280 ± 140
Max strain (%)	OLD3	4.6 ± 0.4	4.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.5
	VERY OLD A**	5.6 ± 0.8	6.7 ± 0.6	-0.4 ± 0.5	-0.8 ± 0.8
Max stress (MPa)	OLD3	32 ± 2	32 ± 3	2 ± 2	1 ± 3
	VERY OLD A*	26 ± 3	26 ± 2	-4 ± 2	3 ± 2
Max modulus (MPa)	OLD3	1330 ± 130	1310 ± 100	-80 ± 120	40 ± 170
	VERY OLD A**	856 ± 74	756 ± 75	-130 ± 80	110 ± 60

*Values are mean ± SE. A: Main effect of age-group at baseline. AxT: Training x age-group interaction. *p<0.05, **p<0.01. HRT = Heavy load resistance training. CON = no training.*

When analyzed at common tendon force before and after training, average stiffness and Young's modulus increased by 9% and 8% respectively in OLD3 HRT but only by 6% and 4% respectively in VERY OLD HRT. In contrast to our hypothesis, we could however not show any significant effect of training on tendon mechanical properties analyzed at common force in either age-group (fig. 10.6).

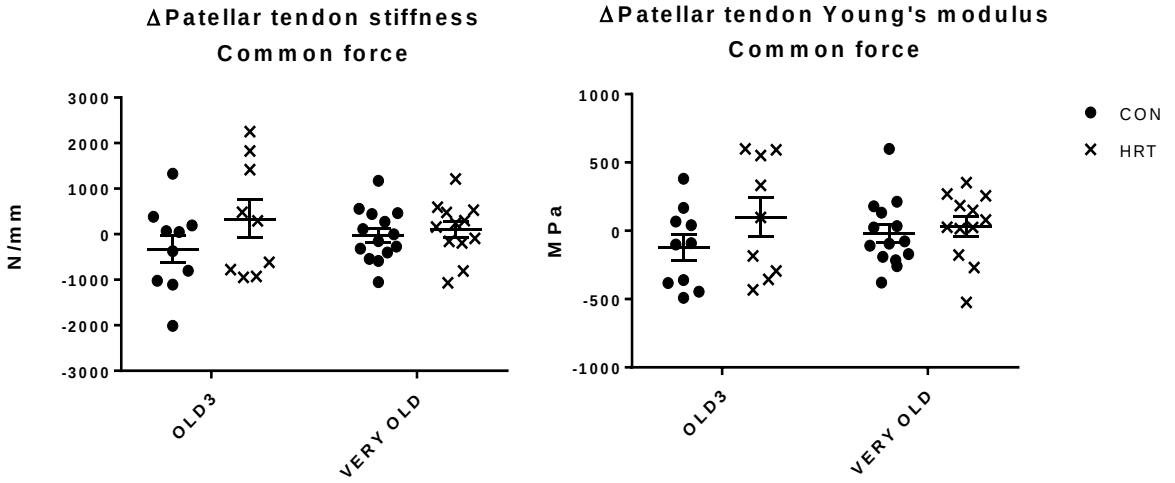

Figure 10.6: *Change in common force patellar tendon stiffness and Young's modulus after 3 months heavy resistance training (HRT) or no training (CON) in OLD3 (~68 years) and VERY OLD (~87 years). No statistically significant differences.*

Moreover, we found no effect of HRT on tendon CSA in either age-group (table 10.4). Our results therefore suggest that muscle adaptation to loading is faster, less variable, and occurs independently of tendon adaptations in both moderately and very old adults.

Table 10.4: Patellar tendon dimensions

		Baseline		Change over time	
	Age group	**CON**	**HRT**	**ΔCON**	**ΔHRT**
CSA total (mm^2)	OLD3	130 ± 10‡	131 ± 9	1 ± 3.2	-2.2 ± 1.7
	VERY OLD	117 ± 5	121 ± 8§	1.4 ± 1.5	0.5 ± 2.3
CSA proximal (mm^2)	OLD3	136 ± 9‡	140 ± 9	1.4 ± 4.9	-2.9 ± 2.9
	VERY OLD	122 ± 5	129 ± 9§	1.9 ± 1.7	-2.9 ± 3.5
CSA mid (mm^2)	OLD3	125 ± 11‡	126 ± 10	0.8 ± 3.5	-1 ± 2.9
	VERY OLD	110 ± 4	114 ± 7§	0.0 ± 1.4	1.5 ± 2.2
CSA distal (mm^2)	OLD3	128 ± 11‡	128 ± 9	0.9 ± 2.8	-2.6 ± 1.4
	VERY OLD	120 ± 5	121 ± 10§	2.3 ± 3	2.8 ± 3.2

Values are mean ±SE. No significant differences. CSA = Cross sectional area. HRT = Heavy load resistance training. CON = no training. §One missing value due to administrative error (n=11). ‡One missing value due to administrative error (n=9).

The lack of training induced tendon hypertrophy after only 3 months training was not surprising since this has been demonstrated before in moderately old adults (52, 118, 131). Given the relative inertness of adult tendons (61), there is no reason to think that very old tendons should

become more responsive to loading. All participants in the OLD3 and VERY OLD studies received whey protein supplementation, which have been shown to augment tendon dimensions in response to resistance training in young adults (43), but the old tendons in our study did not seem to be affected by the different nutritional supplementations.

The lack of improvements in tendon material properties was somewhat surprising since increased tendon modulus after short-term training has been demonstrated in several previous studies on older adults (38, 52, 131). We speculated that the low training compliance in OLD3 (71%, range: 47-89%) might have hampered the training response in this age-group, and consequently performed a secondary analysis including only participants who attended >80% training sessions. This left us with 5 participants in the HRT group with a mean compliance of 83%. The analysis did not change statistical significance of the results but the improvements in quadriceps strength and common force Young's modulus was augmented from 10% to 12% and from 8% to 12% respectively. There was, however, no significant correlation between training compliance and changes in tendon modulus, indicating that the low training compliance only partly explained the lack of significant improvements in tendon mechanical properties. Biological variation in training induced adaptations as well as considerable technical variation in the measurement of tendon mechanical properties may also have prevented us from detecting significant differences. Given the observed variation in the changes over time, and with the number of participants included in the present investigation, Young's modulus should have increased by ~35% to give a power of 80% to detect a significant difference.

Although we could not demonstrate any differences in training induced tendon adaptation between the two age-groups, our own data combined with previous data from our department using the same protocol for testing of tendon mechanical properties, indicated that aging blunted the adaptation to short-term heavy load resistance training in Young's modulus (fig. 10.7). Future research should compare larger samples of different age-groups to give a clearer answer about age-dependent effects on tendon adaptations to training.

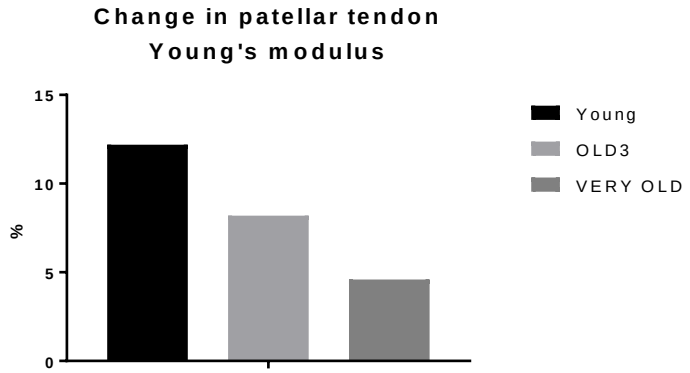

Figure 10.7: *Illustration of relative changes (%) in patellar tendon Young's modulus after 3 months heavy resistance training (HRT) in Young (~25 years), OLD3 (~68 years), and VERY OLD (~87 years) adults. Data on young are adapted from Kongsgaard et al. 2007* (76).

Taken together, our results suggest that aging compromises tendon mechanical function mainly due to a loss of material quality, but three months training is too short to significantly improve tendon function in both moderately and very old individuals. Longer training periods may prove more effective as discussed later in this thesis.

10.2 Effect of training load on tendon adaptation (Study 3)

Study 3 investigated the effect of different load magnitudes on tendon mechanical properties, dimensions and matrix composition. The size and progression of load in the training groups are illustrated in figure 10.8. HRT increased knee extensor load throughout the 12 months intervention, whereas MRT seemed to plateau after week 36.

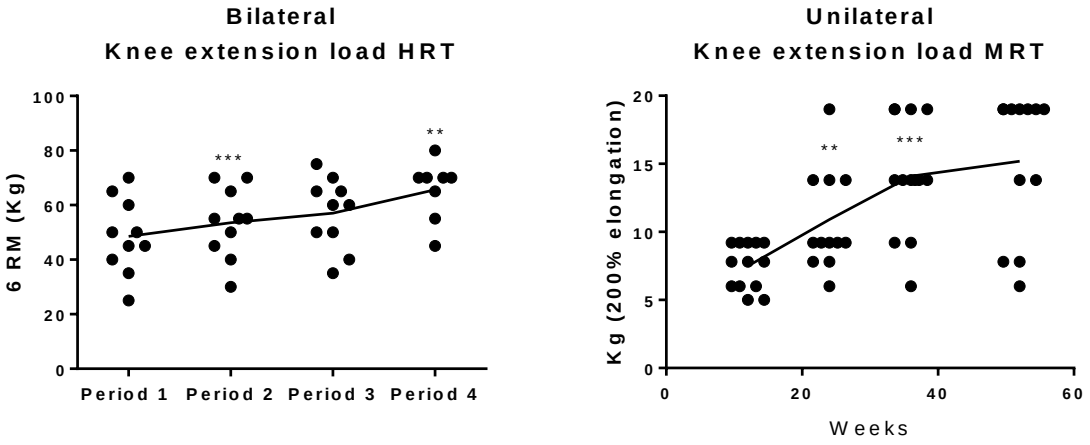

Figure 10.8: *Progression of knee extensor load during 12 months of heavy load resistance training (HRT) or moderate load resistance training (MRT). X-axis for HRT indicates 6 repetition maximum (RM) in kg. X-axis for MRT displays resistance in kg at 200% elastic band elongation (approximate elongation during knee-extension) as indicated by the manufacturer (Theraband): red (5.0 kg), green (6.0 kg), blue (7.8 kg), black (9.2 kg), silver (13.8 kg), gold (19.0 kg). **(p<0.01) or ***(p<0.001). Significant improvement compared to previous assessment.*

It is uncertain what caused the plateau in MRT, but some participants could have improved beyond the gold rubber band, which was the highest resistance available. The large relative improvement in MRT may suggest that training load might have been too low to begin with. It is important to notice that figure 10.8 merely depicts the actual training loads since training progression was not measured in formal maximal or sub-maximal tests.

Maximal isometric quadriceps strength increased significantly after HRT (+21%) and MRT (+8%) but not CON (+7%) (fig. 10.9). The improvement was significantly larger in HRT than MRT, which is consistent with most research regarding the dependency of load size for improvement of muscle strength in older adults (124).

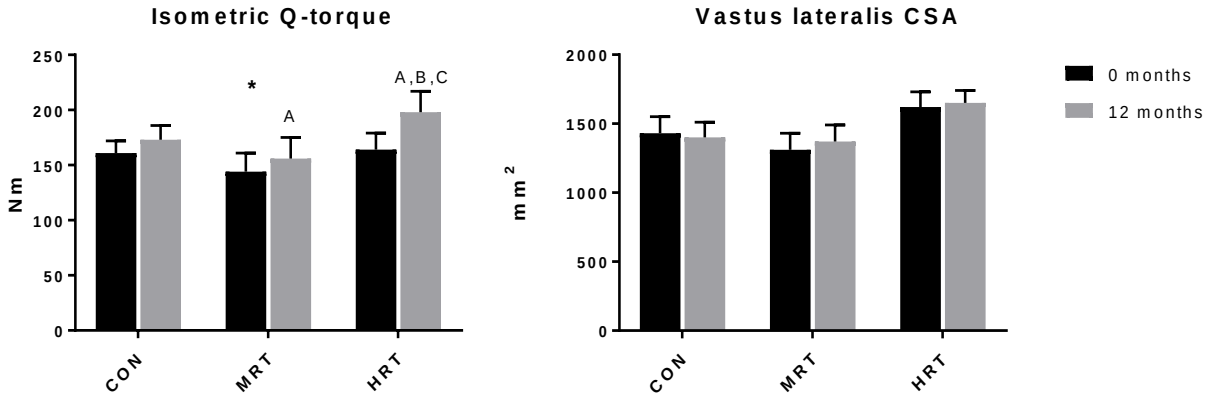

Figure 10.9: *Isometric quadriceps (Q)- torque and vastus lateralis cross-sectional area (CSA) before (0 months) and after 12 months heavy load resistance training (HRT), moderate load resistance training (MRT) or no training (CON). *Significant time x group interaction. Post-hoc tests: A: Significantly different from 0 months, B: Significantly different from CON12, and C: Significantly different from MRT12 ($p<0.05$).*

The augmented adaptation to heavy load compared to moderate load in quadriceps muscle strength seemed to be reflected in maximal patellar tendon mechanical properties (table 10.5). Maximal stiffness and Young's modulus thus displayed significant time by group interactions after 12 months training with reductions in CON and MRT and modest increases in HRT. Maximal force and deformation displayed a main effect of time suggesting better performance in the ramped contraction in all intervention-groups.

Table 10.5: Maximal patellar tendon mechanical properties

	CON (n=10)		MRT (n=13)		HRT (n=10)	
	0 mths	12 mths	0 mths	12 mths	0 mths	12 mths
Max deformation (mm) ‡	2.7 ± 0.3	2.9 ± 0.2	2.3 ± 0.2	2.7 ± 0.2	2.4 ± 0.3	2.5 ± 0.3
Max force (N) ‡‡	4890 ± 390	5020 ± 330	4130 ± 520	4690 ± 490	5020 ± 530	5550 ± 470
Max stiffness (N/mm)**	3530 ± 490	3010 ± 440	3330 ± 290	3170 ± 210	4060 ± 430	4420 ± 340^{B}
Maxs strain (%) ‡	6.0 ± 0.8	6.4 ± 0.5	5.3 ± 0.5	6.1 ± 0.6	5.5 ± 0.6	5.7 ± 0.5
Max stress (MPa)	43 ± 3	43 ± 2	33 ± 3	36 ± 3	43 ± 4	45 ± 3
Max modulus(MPa)*	1430 ± 200	1200 ± 180	1180 ± 70	1090 ± 50	1510 ± 150	1560 ± 140

*Values are means ±SE. Significant Interaction denoted by *(p<0.05) or **(p<0.01). Main effect of time denoted by ‡(p<0.05) or ‡‡(p<0.01). Post hoc tests: B: significant difference from CON12 (p<0.05). CON=Control, MRT = Moderate load resistance training, HRT = Heavy load resistance training.*

More interestingly, when analyzed at common force within subjects, we also found significant time by group interactions in patellar tendon stiffness and Young's modulus, which suggested a time-dependent reduction of mechanical and material properties that was ameliorated by HRT (fig. 10.10).

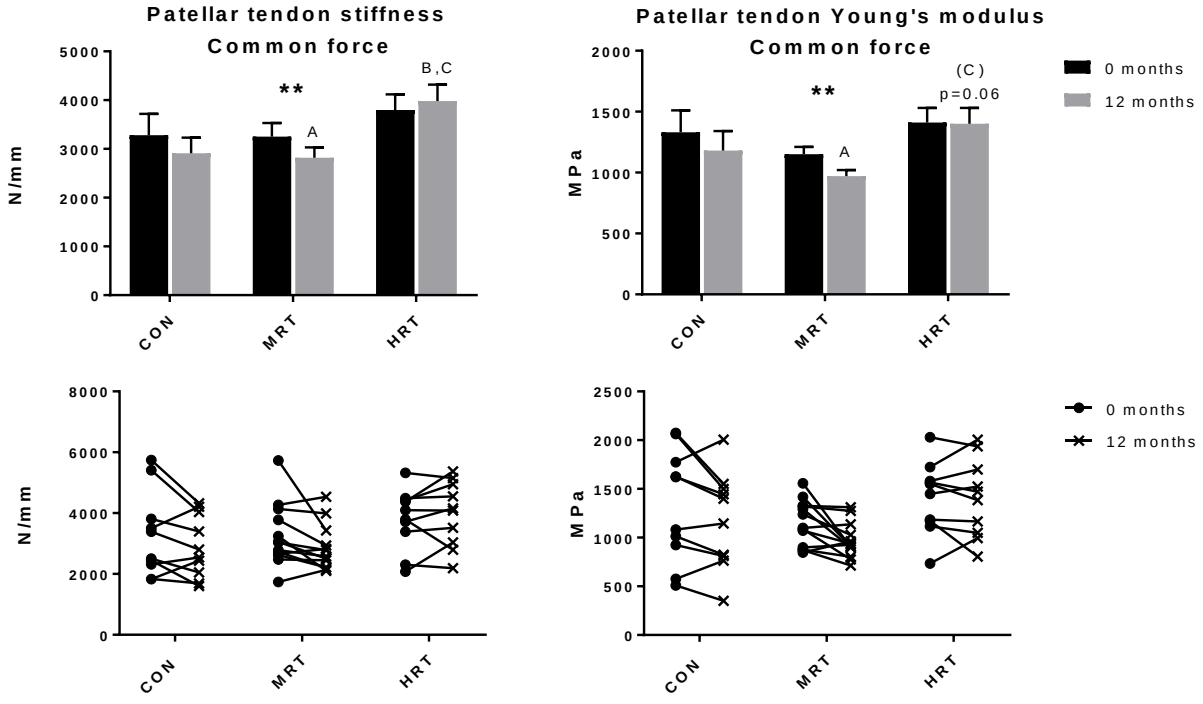

Figure 10.10: *Common force patellar tendon stiffness and Young's modulus before (0 months) and after 12 months heavy load resistance training (HRT), moderate load resistance training (MRT) or no training (CON). Top: Bars represent mean ±SE. Bottom: Spaghetti plot of individual changes over time. Significant time x group interaction denoted by *(p<0.05) or **(p<0.01). Post-hoc tests: A: Different from 0 months, B: Different from CON12, C: Different from MRT12 (p<0.05).*

Although augmented mechanical properties in response to light load training has been suggested previously (81), our results support a recent meta-analysis showing superior effects of heavy load training on tendon stiffness in young and middle-aged adults (17). Furthermore, dependence

of heavy load training for adaptation of patellar tendon mechanical properties has also been demonstrated in older adults (52). In light of the previous research, it was somewhat surprising that tendon mechanical properties were not augmented but rather maintained in response to HRT. One possible explanation is that the relatively long duration of the intervention caused an age-related decline in mechanical properties which was counteracted by heavy but not moderate load resistance training. Decreased stiffness and Young's modulus with aging is consistent with our own data presented above, but we were surprised to see that 12 months was sufficient to show declining mechanical properties.

An alternative previously proposed theory that could explain our results is that moderate load training had a systemic effect on the tendons with increased collagen turnover and decreased accumulation of AGE cross-links, rendering the tendon softer (175), while the local mechanical effect of heavy load training surpassed the systemic softening effect (115, 166). Reduced tendon mechanical properties with increased loading contradicts the evident loss of tendon stiffness seen after reduced loading such as in muscle paralysis (96), microgravity (130), or only 2-3 weeks immobilization (16, 31, 84).

Patellar tendon CSA showed significant time by group interactions in the proximal region, and a trend in total CSA ($p=0.07$) (fig. 10.11). Contrary to our hypothesis, post-hoc analysis revealed improvements of CSA in both HRT and MRT suggesting that both training modalities have a protective effect on the patellar tendon by reducing stress at a given load. Curiously, the increased CSA in MRT was not associated with increased tendon stiffness or modulus, suggesting that tendon hypertrophy is less dependent on training load than tendon material properties. The mid and distal regions showed similar relative increases of CSA as the proximal region after HRT (7% and 6% respectively), but this was not significant (fig. 10.11). In contrast, statistical analysis revealed a main effect of time in the mid and distal regions, which may reflect changes in the phantom used to normalize tendon signal intensity.

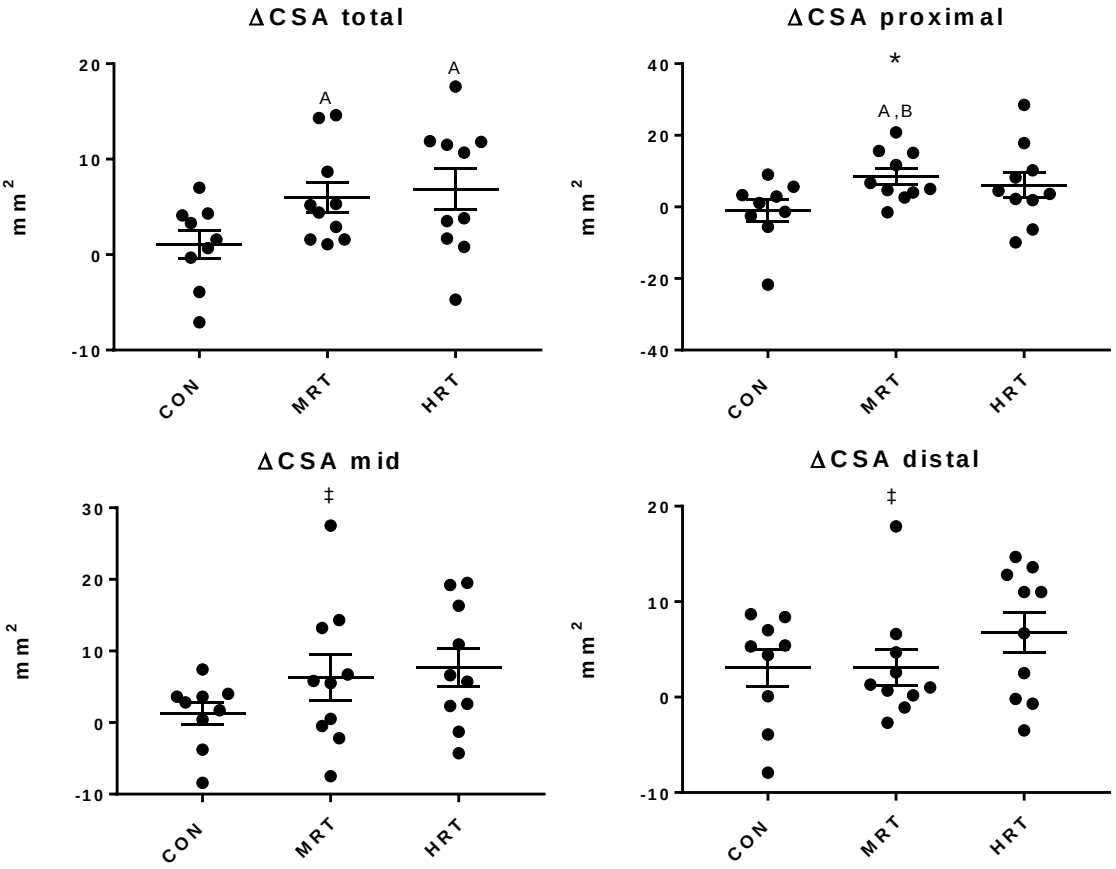

Figure 10.11: *Changes in patellar tendon cross-sectional area (CSA) in total and in the proximal, mid, and distal regions after 12 months heavy load resistance training (HRT), moderate load resistance training (MRT), or no training (CON). Lines denote mean ±SE. Time x group interaction denoted by *(p<0.05). Post hoc tests: A: Significant change from 0 to 12 months, B: Significantly different from CON (p<0.05).*

Although several short-term (3 months) training interventions have found no effect of either light load (151) or heavy load training (52, 118, 131), increased CSA after long-term training with both relatively heavy and light load is corroborated by some (30, 32), albeit not all (102), human cross-sectional data. Consistent with our results, one study also demonstrated increased CSA after 12 weeks light (+7%) and heavy (+6%) load training but only heavy load training increased tendon stiffness (76). Training volume may be more important than load in the long run to induce tendon hypertrophy although the augmented tendon volume only benefits tendon mechanics after heavy load training.

Tendon hypertrophy may be mediated by addition of new collagen to the matrix which would in turn increase fibril diameter or fibril density. Although both heavy and light load training seems capable of activating intramuscular collagen synthesis (65), addition of new collagen to the core tendon matrix does not seem to occur after teenage years (61). In line with

this our results did not demonstrate any changes in tendon collagen content in response to either moderate or heavy load training. Consistent with previous human studies (32), fibril volume fraction, fibril diameter, and fibril density were also unaffected by 12 months training (table 10.6).

Table 10.6: Collagen fibril Morphology

	CON		MRT		HRT	
	0 m (n=10)	12 m (n=9)[a]	0 m (n=13)	12 m (n=12)[a]	0 m (n=10)	12 m (n=9)[a]
Volume Fraction (%)	62 ±2	59 ±1	59 ±2	62 ±1	58 ±2	59 ±2
Fibril diameter (nm)‡	92 ± 5	83 ± 4	86 ± 3	86 ± 5	91 ± 4	78 ± 3
Density (#/μm^2)‡	26 ± 2	31 ± 3	29 ± 2	33 ± 3	26 ± 3	36 ± 3

Values are means ± SE. ‡: main effect of time ($p<0.05$). CON=Control, MRT = moderate load resistance training, HRT = Heavy load resistance training. [a]One value missing due to technical problems.

Another possible mediator of tendon hypertrophy is addition of interfibrillary material to the matrix such as fat, proteoglycans, and glycoproteins, but this seems unlikely since it would have reduced fibril volume fraction. Increased tendon volume measured by MRI immediately following heavy load exercise has previously been proposed to be due to water accumulation (143). Participants in the present study were carefully instructed to avoid strenuous physical activity 72 hours preceding MRI. Although we cannot exclude a water retaining effect of exercise after 72 hours, this was contradicted by increased signal intensity after HRT in the distal region (+29%) and a trend towards increased signal intensity in total (+23%, p=0.09) (table 10.7), whereas increased water content on a T1 weighted MRI would theoretically reduce signal intensity. The specific translation from signal intensity to tendon molecular composition is unknown but given the load dependent increase of signal intensity and the concomitant load dependent effect on tendon Young's modulus, one might speculate that increased signal intensity reflects increased material quality (i.e. higher fraction of load bearing material). We could, however, not demonstrate any correlations between baseline signal intensity and collagen content or fibril volume fraction or between changes in signal intensity and changes in collagen content or fibril volume fraction.

Table 10.7: Patellar tendon signal intensity

	CON		MRT		HRT	
	0 mths (n=10)	12 mths (n=9)[a]	0 mths (n=11)[b]	12 mths (n=12)[a]	0 mths (n=10)	12 mths (n=10)
SI total	13 [11-14]	13 [12-15]	14 [13-15]	14 [13-15]	13 [12-14]	15 [15-16][A]
SI proximal	18 [16-20]	18 [16-20]	21 [19-23]	21 [19-23]	19 [17-22]	21 [20-23]
SI middle‡	10 [9-11]	12 [10-13]	11 [10-11]	11 [10-12]	10 [9-11]	14 [12-15]
SI distal*	10 [9-11]	10 [9-11]	9 [9-10]	9 [8-9]	8 [8-9]	10 [10-11][A,C]

*Values are geometric means [upper limit-lower limit]. *Significant Interaction (p<0.05), ‡: main effect of time (p<0.05). Post hoc tests: A: Different from time-point 0, B: Different from CON12, C: Different from MRT12 (p<0.05). CON = Control, MRT = Moderate load resistance training, HRT = Heavy load resistance training. [a]one value missing due to administrative errors. [b]Two values missing due to administrative errors.*

The apparent disparity between adaptations in tendon signal intensity and CSA on one side and collagen content and fibril morphology on the other side may be attributed to region-specific changes in fibril morphology. It is thus plausible that our core tendon biopsies did not detect changes in the most proximal or distal tendon regions, which was the locations of changes in CSA and signal intensity respectively. Also, changes in the superficial tendon where exercise induced collagen synthesis has been demonstrated (87) might have gone undetected in our core tendon samples.

Enzymatic collagen cross-links are essential to mechanical tissue integrity (5, 42, 63) and could potentially also mediate the increased stiffness and Young's modulus observed after heavy load training. Contrary to our hypothesis, there was however no effect of either moderate or heavy load training on HP and LP concentrations in our tendon biopsies (table 10.8).

Table 10.8: Collagen and collagen cross-links

	CON		MRT		HRT	
	0 mths (n=10)	**12 mths (n=10)**	**0 mths (n=13)**	**12 mths (n=13)**	**0 mths (n=10)**	**12 mths (n=9)[a]**
1Collagen (% dry-weight)	69 ±3	62 ±2	61 ±4	59 ±2	63 ±3	61 ±2
HP (mmol/mol collagen)[b]	289 ±30	316 ±27	294 ±25	263 ±24	254 ±30	259 ±26
LP (mmol/mol collagen)[b]	25.1 ±5.0	27.3 ±3.9	42.5 ±4.5	29.7 ±5.4	37.0 ±6.2	35.2 ±3.3
Fluorescence (AU/mol collagen) ‡	1139 [1096-1185]	1235 [1176-1297]	1155 [1124-1187]	1196 [1161-1232]	1242 [1172-1316]	1328 [1285-1372]

Values are means ±SE or geometric mean [lower limit – upper limit]. No statistical interactions. ‡: Main effect of time (p<0.05). CON = Control, MRT = Moderate load resistance training, HRT = Heavy load resistance training. [a]one missing sample due to anti-coagulant therapy, [b]CON: n=9, MRT: n=11, HRT: n=8, missing samples due to logistics.

Although LOX expression seems to be sensitive to loading (60) and unloading (16) of tendon, our results are in line with previous human studies showing no effect of life-long light load training (32) or short-term heavy load resistance training (74, 92) on patellar tendon HP and LP levels. We suggest that other matrix components mediate the observed training effect on mechanical properties after maturity, or that enzymatic cross-links are only formed in regions with new deposition of collagen, which after maturity would most likely be in the peripheral tendon region (87), and not in the core of the tendon. The latter however contradicts a previous investigation in our department showing higher levels of HP and LP in tendon biopsies from old compared to young men with a similar physical activity level (29), indicating a possibility for core tendon changes in enzymatic cross-links after maturity. Despite the obvious importance of enzymatic cross-links for tissue integrity, we could not demonstrate any correlations between baseline HP or LP and maximal Young's modulus, or between the changes in these variables, in our group of older adults. Others have also failed to correlate enzymatic cross-links to mechanical properties (29, 55, 161). Future research should address region-specific matrix composition in response to aging and training.

The absolute levels of HP as measured with the ELISA technique in the present study (table 10.8) were somewhat lower than previous reports showing HP levels between ~400 mmol/mol (42, 92) and ~600-900 mmol/mol (29, 74) using HPLC, whereas the LP levels (~25-40 mmol/mol collagen) were fairly consistent with previous studies (29, 32, 42, 74). The reason for lower HP measurements in the present study may be due to differences between the ELISA kit and HPLC as mentioned in "methodological considerations".

Patellar tendon fluorescence displayed a significant increase over time across intervention groups (table 10.8 and fig. 10.12), and we also found a significant correlation between age and fluorescence (fig. 10.12), even within this relatively narrow age-span (63-71 years), which is consistent with the existing knowledge about age-related accumulation of AGEs (7). Collagen content was also reduced over time, although not significantly, and this may partly explain the increased fluorescence over time, since fluorescence was normalized to collagen.

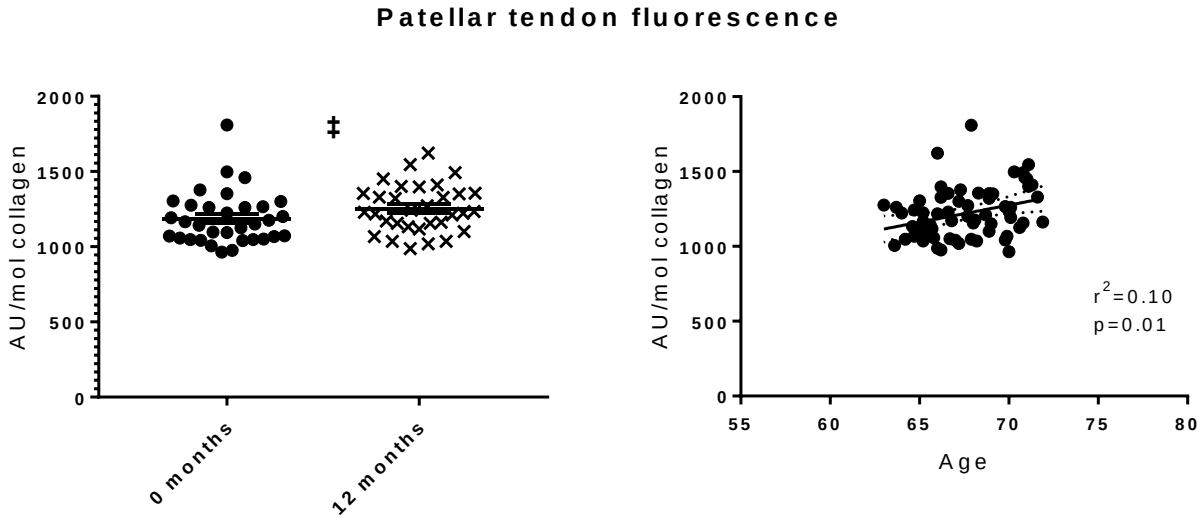

Fig.10.12: *Left: Patellar tendon fluorescence before (0 months) and after 12 months intervention. ‡: significant main effect of time (p<0.05). Right: Correlation between age and patellar tendon fluorescence normalized to collagen. AU=arbitrary units. Dotted lines are 95% confidence bands of the best linear fit.*

Contrary to our hypothesis, fluorescence was however not affected by either moderate or heavy load training. Previous studies have shown reduced AGE content after treadmill running in old mice (175), lower pentosidine in the patellar tendon of endurance trained master athletes (32), and reduced pentosidine after 3 months heavy resistance training in younger men with tendinopathy (74). The fact that the present study failed to support previous findings is difficult to explain. One explanation is that training only prevents accumulation of AGEs over a lifetime but once formed and given years to mature, they are difficult to break down. In younger individuals where the glycation products are less concentrated and more immature compared to older adults, the tissue may on the other hand be easier accessible to matrix degrading matrix metalloproteinases and consequently have a higher training induced matrix turnover.

Some AGEs form intermolecular cross-links which may affect mechanical tissue properties (5, 94, 137), and we consequently expected tendon fluorescence to be correlated to mechanical properties. Since measurement of maximal modulus relies on the maximal strength during the ramped contraction, we correlated fluorescence to maximal modulus as well as the max modulus/max stress ratio. We failed, however, to show any correlations between these variables at baseline or in the changes over time. Our results therefore suggest that total tendon AGE content does not predict in vivo tendon mechanical properties of older adults. It is possible that specific AGE cross-links such as pentosidine, glucosepane, GOLD or MOLD may be differently affected by training in older adults compared to total AGE-content, but this requires further research. As mentioned previously in the thesis, we may also have overlooked changes of AGE content in other regions than the core of the patellar tendon.

In vitro studies on animal tissue indicate that accumulation of both pentosidine (129), CML, CEL and MG-H1 (39, 146), as well as total AGE-adducts (175) are related to increased tendon stiffness. This gives rise to a paradox between the age-related accumulation of AGEs demonstrated in study 3 and previously (8, 29) and the age-related reduction of mechanical properties demonstrated in study 1 compared to study 2 as well as in previous studies (81, 127). The lack of association between AGEs and in vivo patellar tendon mechanical properties however corroborates previous human studies (29, 55). One explanation is that AGE accumulation only affects failure mechanics (39, 158), which in contrast to in vitro studies are far from reached during the voluntary ramped contractions used to measure in vivo tendon mechanics. Another previously proposed explanation is that AGE accumulation reduces collagen packing (114). AGE cross-links would thus alleviate the reduction in collagen concentration to maintain tendon mechanical properties to a certain level (32), whereas AGE adducts would only take up space to make the tissue more compliant. A final consideration is, that accumulation of AGEs may render the tendon more brittle and prone to damage, and damage accumulation would in turn reduce tendon mechanical properties (44, 156, 164). Brittleness is however difficult to define and quantify. Future research should determine more specific AGE cross-links and AGE adducts in tendon and their relation to tendon mechanical function in old age.

Sex distribution was unequal between intervention-groups in OLD12 although not statistically significant. A 2-way ANOVA on PRE-POST values with sex and intervention-group as factors yielded no significant interactions, confirming that the unequal sex-distribution could not explain the effects of training load on tendon mechanical properties, tendon dimensions, fibril morphology, or collagen cross-links.

10.3 Effect of training duration on tendon adaptation (study 2 & 3)

Twelve months training significantly increased quadriceps muscle strength by 21% in HRT and by 8% in MRT, whereas three months training increased strength in HRT by 10% with no changes in LRT (fig. 10.13). It is important to notice that HRT training compliance was significantly lower in OLD3 (71 ±5%) than OLD12 (86 ±2%) ($p<0.05$). Training compliance in OLD3 LRT (86 ±4%) and OLD12 MRT (86 ±4%) was not different, but the protocolled training load was lower in OLD3 LRT (~40-50%) than OLD12 MRT (~50-60%). Given the evidence of load dependence of muscle and tendon adaptation presented above, one might expect augmented training adaptations in OLD12 irrespective of the longer training duration. The strength increase after 12 months was, however, not significantly different from the strength increase in the

corresponding intervention-groups after 3 months (HRT3 vs HRT12: p=0.27, LRT3 vs MRT12: p=0.28) (fig. 10.13). The lack of significance may be due to considerable variation in the training response.

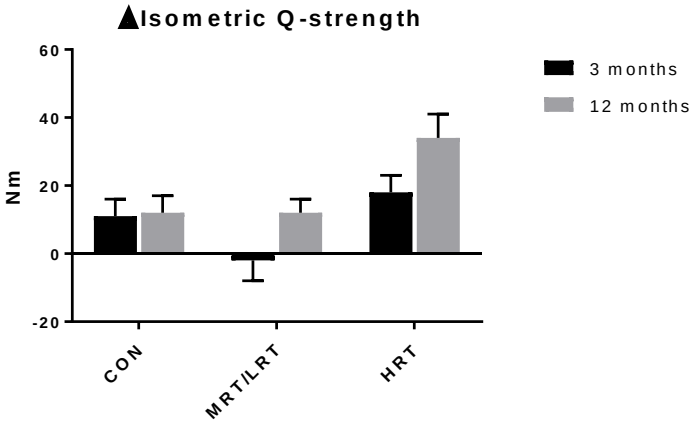

Figure 10.13: *Changes in isometric quadriceps (Q)-strength after 3 or 12 months moderate or light load training (MRT/LRT), heavy load resistance training (HRT) or no training (CON). No significant differences between 3 and 12 months intervention.*

In contrast to three months training, twelve months high load training had a significant effect on tendon mechanical properties. When we compared the changes in mechanical properties between the 3 and 12 months interventions there were however no significant differences between corresponding intervention-groups (CON3 vs CON12, LRT3 vs MRT12, HRT3 vs HRT12) based on unpaired t-tests with Bonferroni correction (fig. 10.14). Looking at Young's modulus, the absolute difference between HRT on one side and MRT/LRT or CON on the other side seemed to be of approximately the same magnitude after 3 and 12 months intervention. However, the variation in OLD3 was somewhat larger than in OLD12 (fig. 10.14). In addition to the lower compliance in OLD3, this may also explain the lack of significant training effects on the tendon after 3 months training. We can thus not confirm that longer training duration yields a more pronounced adaptation of tendon material properties.

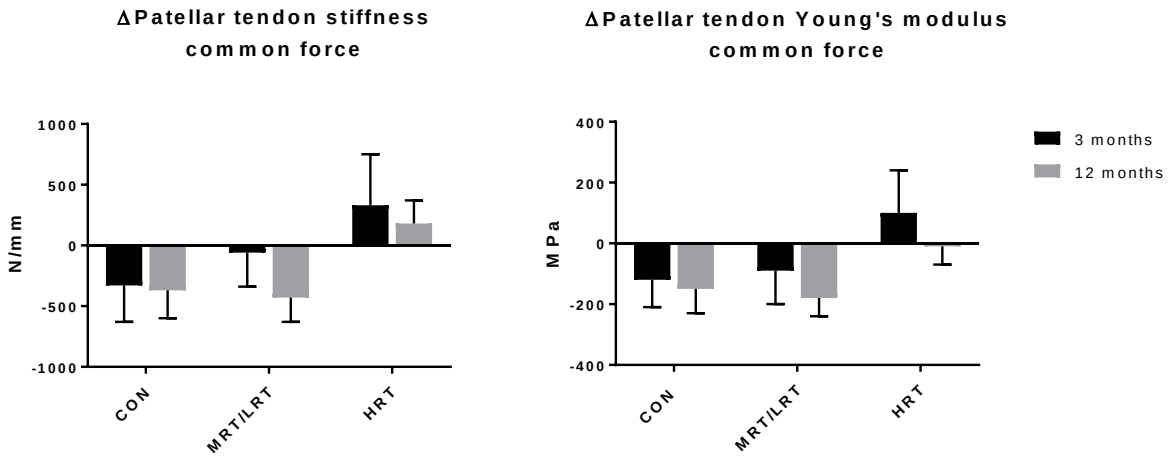

Figure 10.14: *Changes in patellar tendon stiffness and Young's modulus after 3 or 12 months heavy load resistance training (HRT), moderate or light load resistance training (MRT/LRT), or no training (CON). No significant differences between 3 and 12 months intervention.*

OLD12 increased average tendon CSA after both MRT (+5%) and HRT (+6%), and in HRT this was significantly different from OLD3 (p=0.01), where patellar tendon CSA remained unchanged (fig. 10.15). These results indicate that training induced tendon hypertrophy requires longer training duration.

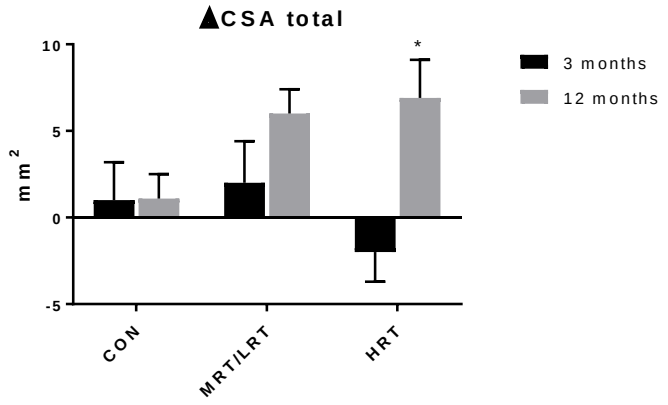

Figure 10.15: *Change in total tendon cross-sectional area (CSA) after 3 or 12 months moderate or light load resistance training (MRT/LRT), heavy load resistance training (HRT) or no training (CON). *Significant difference between 3 and 12 months intervention (p<0.05).*

Increased tendon CSA after long-term heavy loading corroborates some (30) but not all (102) cross-sectional human data. The increased CSA after 12 months MRT but not 3 months LRT further support previous findings that moderate loading can augment tendon CSA if training duration is sufficiently long (32). The difference may however also be attributed to slightly higher training load in MRT compared to LRT. Our results contradict another recent longitudinal study, which demonstrated increased stiffness, modulus, and CSA of the Achilles tendon after

short-term (14 weeks) heavy resistance training with no further improvements after 1.5 years of training (38). It should be mentioned however, that the training volume in that study decreased from three to two weekly sessions after 14 weeks of training, and the blunted adaptation of the tendon was accompanied by no further improvements in muscle strength from 14 weeks to 1.5 years. It is also possible that the time-course of Achilles and patellar tendon adaptations to loading is different. Finally, we cannot exclude that the observed adaptations of tendon CSA and mechanical properties in OLD12 were already present at 3 months, and more longitudinal training studies with repeated measurements of tendon CSA and mechanical properties are warranted to determine the time-course of tendon adaptation to loading in older adults.

Fibril morphology remained unchanged after training in both OLD3 and OLD12, and there was consequently no additional effect of longer training duration (fig. 10.16).

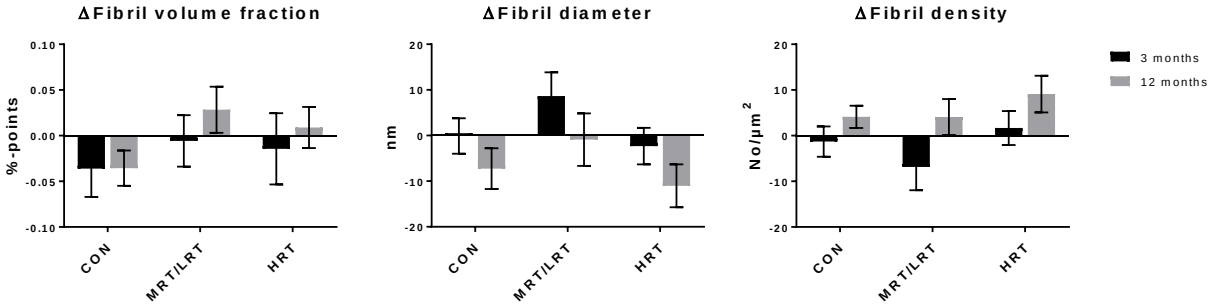

Figure 10.16: *Changes in fibril morphology after 3 or 12 months moderate or light load resistance training (MRT/LRT), heavy load resistance training (HRT) or no training (CON). No significant differences between 3 and 12 months intervention.*

The effect of training duration on collagen fibril morphology has been investigated in young animals with conflicting results (107, 121), but there is limited knowledge about the effect of training duration in humans. One cross-sectional study showed no effect of life-long endurance training compared to sedentary controls on patellar tendon fibril morphology (32), and our results suggest that heavy load training of long duration does not affect fibril morphology either. As suggested previously in the thesis, other molecular matrix components may mediate training induced adaptation of tendon size and mechanical properties.

Aside from the training load in the intervention, daily physical activity also imposes loading on the tendon. OLD12 had an average daily step count at baseline of ~8500 ±2900 steps/day, which is relatively high for the age-group (163), whereas OLD3 had a 40% lower step-count of 5100 ±2000 ($p<0.0001$). Moreover, OLD12 tended to increase physical activity during the intervention across groups ($p=0.07$), whereas OLD3 did not. OLD12 seemed

to differ from OLD3 with regards to absolute changes in daily step-count, but this difference was not significant in the comparable intervention groups (fig. 10.17).

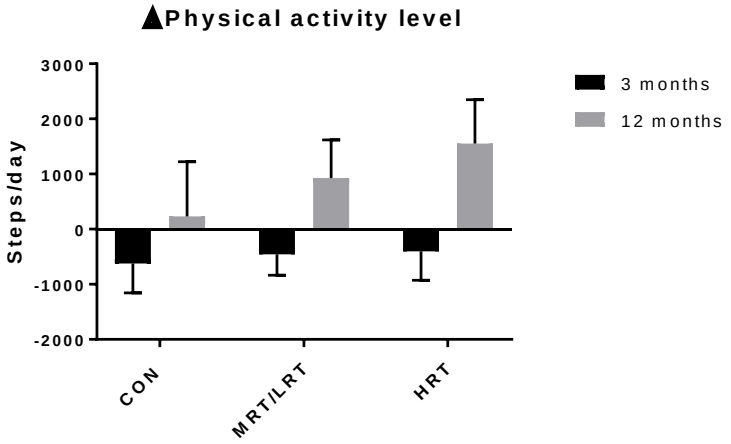

Figure 10.17: *Changes in physical activity level after 3 or 12 months moderate or light load resistance training (MRT/LRT), heavy load resistance training (HRT) or no training (CON). No significant differences between 3 and 12 months intervention.*

One might speculate that these non-significant differences in physical activity could have confounded the changes in tendon mechanical properties and cross-sectional area. Tendon hypertrophy occurred independent of load magnitude in OLD12, and the trend towards increased daily step-count across groups in OLD12 might have contributed to this tendon adaptation (fig. 10.17 + fig. 10.15). Patellar tendon mechanical properties did not seem to be influenced by light or moderate load training in the present investigation, but we cannot exclude that the differences in daily step-count between OLD3 and OLD12 could have contributed to the differences in tendon mechanical adaptation between these two studies. There were, however, no convincing correlations between changes in physical activity and changes in tendon mechanical properties or tendon CSA in either OLD3 or OLD12. Whether or not daily step-count influenced tendon adaptations, the results suggest that longer training interventions may have a more pronounced influence on habitual physical activity level.

11. Conclusions and perspectives

The main findings of the present PhD thesis were that very old age compared to moderately old age was associated with reduced patellar tendon stiffness and Young's modulus. Lower physical activity in very old age may have contributed to the age-related reduction of tendon mechanical

properties. Short-term (3 months) training did not affect tendon mechanical properties, whereas long-term (12 months) training seemed to prevent aging-related reduction of tendon stiffness and Young's modulus. In contrast to our hypothesis, only heavy load training influenced tendon stiffness and Young's modulus, whereas tendon cross sectional area increased after both moderate and heavy load resistance training. This suggests that maintenance of tendon function in old age is dependent on heavy training load, whereas the protective effect of a larger tendon (reduced stress for a given load) is not. We could not identify any changes in load bearing molecular components to explain the changes in tendon material properties and tendon dimensions, since fibril morphology, collagen content, enzymatic cross-links and total AGE content as measured by fluorescence were unaffected by training. This could indicate that other matrix components such as proteoglycans or glycoproteins contribute relatively more to tendon mechanical properties and the adaptations to training in older adults. Another possibility is that tendon matrix adaptation to training occurs in specific tendon regions which were overlooked in the present investigation.

Future research should examine more specific regions of the tendon to explain the apparent training induced changes of mechanical properties and dimensions. It may, however, be difficult to obtain region-specific tendon biopsies in vivo. Without considering ethical limitations, a potential cross-sectional human model could be to examine whole tendon material from cadavers or surgery and retrospectively assess the influence of physical activity and age. Moreover, tendon structure and composition still needs to be investigated in very old adults to determine tendon matrix changes over the entire life-course.

The present investigation could not confirm a causal relation between longer training duration and improved tendon mechanical properties. Future research projects should confirm the temporal adaptations to training in a long-term intervention study with several repeated measurements. Cross-sectional data indicate that several years of habitual light and heavy loading may have a beneficial effect on tendon structure and function (30, 32). It is therefore plausible to think that several years of training is necessary to induce clinically relevant improvements in tendon structure and function. This calls for tendon research of even longer duration, maybe in the form of prospective cohort studies because randomized trials are difficult and perhaps also unethical to control over such long time periods. An interesting cross-sectional model to investigate the effect of long-term habitual loading could be an intra-individual comparison of tendon structure and function in the lower body (loaded) and upper-body (unloaded) tendons of both young and old adults.

Although adaptation of tendon mechanical properties seemed dependent on heavy training load in the present investigation, it is possible that older adults with even lower physical activity level than the ones included in the present studies have a lower loading threshold for tendon adaptations. This would be interesting to pursue in future research projects comparing the effect of different loading magnitudes on tendon adaptations in sedentary and well-trained older adults. Immobilization studies could be another interesting model to determine if the loading threshold for tendon adaptation is reduced when the tendon is "starved" from mechanical loading.

Tendons are only one part of the musculoskeletal system, and more research is needed to encapsulate the complexity of adaptations to aging and training in the entire muscle-tendon-bone unit.

Finally, maintenance of tendon stiffness in old age seems critical to optimal physical function in older adults (19, 119, 169), and our results together with previous findings therefore suggest that heavy load training is incorporated in training programs to maintain tendon function in old age.

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13. Papers

Lower Tendon Stiffness in Very Old Compared to Old Individuals is Unaffected by Short Term Resistance Training of Skeletal Muscle

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Abstract

Aging negatively affects collagen-rich tissue like tendons, but in vivo tendon mechanical properties and the influence of physical activity after the 8th decade of life remains to be determined. This study aimed to compare in vivo patellar tendon mechanical properties in moderately old (OLD) and very old (VERY OLD) adults, and the effect of short term resistance training. Twenty OLD (9 women, 11 men, >65 y) and 30 VERY OLD (11 women, 19 men, >83 years) were randomly allocated to heavy resistance training (HRT) or no training (CON) and underwent testing of in vivo patellar tendon (PT) mechanical properties and PT dimensions before and after three months intervention. Previous measurements of muscle properties, blood parameters, and physical activity level were included in the analysis. Data from 9 OLD HRT, 10 OLD CON, 14 VERY OLD CON, and 12 OLD HRT were analyzed. In addition to lower quadriceps muscle strength and cross-sectional area (CSA), we found lower PT stiffness and Young's modulus ($p<0.001$) and a trend towards lower mid-portion PT-CSA ($p=0.09$) in VERY OLD compared to OLD. Daily step count was also lower in VERY OLD ($p<0.001$). Resistance training improved muscle strength and cross-sectional area equally in OLD and VERY OLD ($p<0.05$), but did not affect PT mechanical properties or dimension. We conclude that PT material properties are reduced in very old age, and this may likely be explained by reduced physical activity. Three months resistance training could, however, not alter PT mechanical properties in very old individuals.

Keywords

Patellar Tendon Mechanical Properties, Aging, Resistance Training

New & Noteworthy

This research is the first to quantify in vivo tendon mechanical properties in a group of very old adults in their eighties. Patellar tendon stiffness was lower in very old (87 years in average) compared to moderately old (68 years in average) individuals. Reduced physical activity with aging may explain some of the loss in tendon stiffness, but regular heavy resistance training for three months was not sufficient to change tendon mechanical properties.

1. Introduction

Aging changes the structure and function of collagen-rich tissues. These changes can be progressive, irreversible and negatively impact tissue function, such as reduced extensibility of blood vessels or thickening of glomerular basement membrane (1, 57). Tendons are collagen-rich tissues that serve to transmit force for locomotion. Yet it remains unclear if tendons become stiffer (65, 70) or more compliant (20, 38, 47, 52) with aging, which would influence optimal muscle contractile properties (9), postural balance, and the risk of fall-related injuries (51). The currently available research in the area is sparse, inconclusive, and limited to comparisons between young, middle aged, and moderately old adults (11, 13, 35, 61, 62). This leaves a gap in the knowledge regarding tendon mechanical properties in very old individuals >80 years.

The effect of aging on tendon mechanical properties has been studied in animal and human models. Animal data show both an increased (65, 70), decreased (20, 39) or unchanged (30, 48) stiffness with age, while studies on humans have most frequently reported lower (15, 33, 35, 47, 52, 61) or unchanged stiffness (11, 13) in old compared to young adults. In vitro mechanical properties in old and very old adults (64-93 years) compared to young and middle-aged adults (29-50 years) suggest that there is no difference in material stiffness (32). However, it remains to be determined if human in vivo tendon mechanical properties decline further after the 8th decade of life.

Physical activity typically declines with age, and therefore separating the effect of aging from that of reduced physical activity can be challenging (41). Thus, studies comparing young and old men with a similar physical activity level found no differences in patellar tendon Young's modulus (13, 16), indicating that aging per se did not alter tendon properties. Moreover, spinal cord injury associated paralysis (42), 90 days of simulated microgravity (54), or 2-3 weeks of immobilization (6, 15, 37) all appear to reduce tendon stiffness. Finally, heavy resistance training of relatively short duration (3 months) seems to increase patellar tendon stiffness in young and middle aged (8, 69) as well as older adults (21, 27, 55), albeit not an entirely consistent finding

(12). However, it is still unknown if regular training affects tendon mechanical properties in very old individuals after the 8th decade of life.

The primary purpose of the present study was therefore to compare patellar tendon mechanical properties in moderately old (+65 years) and very old (+83 years) adults, and to investigate the influence of short term heavy resistance training in the two age groups. We hypothesized that very old individuals would have a lower stiffness of their patellar tendon compared to moderately old. Moreover, we hypothesized that three months of heavy resistance training would increase stiffness in both age-groups, but that this effect would be less pronounced in very old individuals.

2. Materials & Methods

2.1 Study Design

The present investigation was a sub-study of two different randomized training studies conducted at the Institute of Sports Medicine Copenhagen between 2013 and 2017. For details about design and protocols we refer to the original studies (4, 5).

The first study (VERY OLD) primarily investigated the effect of 3 months heavy resistance training (HRT) and protein supplementation compared to protein supplementation alone (CON) on muscle hypertrophy and strength in very old individuals > 83 years (4) . All participants included in the original study underwent the tendon specific tests presented here.

The second study (OLD) primarily investigated the overall effects of both 3 and 12 months light or heavy resistance training (HRT) and/or protein supplementation on muscle hypertrophy and strength in older adults > 65 years (5). The participants in the present sub-study gave consent to undergo additional tendon specific tests before and after 3 months intervention. They were randomly allocated to HRT plus whey protein supplementation or whey protein supplementation

only (CON). The data presented here are from baseline and 3 months in the HRT and CON groups only in order to match the training duration and intensity of VERY OLD.

Combining data from both studies gave us the opportunity to compare the effect of age on tendon biomechanics in a cross sectional design and simultaneously the effect of training in a longitudinal design.

2.2 Ethics and data handling

Both studies complied with the Helsinki Declaration and all participants gave oral and written consent before enrollment. The studies were registered at clinicaltrials.gov and approved by the regional ethical committee and the Danish Data Protection Agency. ID and journal numbers for the respective studies are listed below.

VERY OLD: ClinicalTrials.gov ID: NCT01997320. Danish Regional Ethical Committee (Capital Region): J-no. H-4-2013-068. Danish Data Protection Agency: journal no. 2007-58-0015, local no. BBH-2013-043, I-suite no. 02568.

OLD: ClinicalTrials.gov ID: NCT02034760, registered on January 10th 2014. ClinicalTrials.gov ID: NCT02115698, registered on April 14th 2014. Danish Regional Ethical Committee (Capital Region): J-no. H-4-2013-070. Danish Data Protection Agency: journal no. 2012-58-0004 – BBH-2015-001 I-Suite no. 03432.

2.3 Participants

The VERY OLD study included 11 women and 19 men, age > 83, from local nursery/senior homes. The study excluded candidates with surgical or medical disease preventing them from participating in the intervention or testing-procedures, candidates receiving anticoagulant or hormonal-/antihormonal therapy, and candidates with cognitive impairments. For details, please see the

original study (4). Fifteen (5 women, 10 men) were randomized to HRT, and 15 (6 women, 9 men) to CON.

From the OLD study 9 women and 11 men, age > 65 were included. They were all home dwelling, independent, and untrained. Exclusion criteria were arthrosis, arthritis, or other chronic musculoskeletal pain or impairment affecting the ability to perform resistance training, bilateral knee or hip alloplastic material, severe chronic or unstable medical disease, cognitive impairment, anticoagulant therapy or hormonal-/anti hormonal treatment. The full list of exclusion criteria can be seen in the original study protocol (5). Nine (4 women, 5 men) were randomized to HRT, and 11 (5 women, 6 men) to CON.

2.4 Interventions

The training intervention was a supervised whole body progressive heavy resistance training program (HRT) in both OLD and VERY OLD. Two weeks familiarization, adjustments and a 3-6 repetition maximum (RM) test was performed initially to determine the exercise load. Training volume started at 3 sets of 12 repetitions at 12 RM (~70% of 1RM) and gradually progressed to 5 sets of 6 repetitions at 6 RM (~90% of 1 RM) in week 10 followed by two weeks of tapering that consisted of 3 and 2 sets of 6 repetitions respectively. Load was increased in the following training session if participants could perform more than one repetition after the final set. Load was decreased immediately if participants failed to complete the intended number of repetitions at the pre-specified load. Training frequency was three times weekly, and the leg exercises involving the knee joint were leg press, knee extension, and leg curls performed in Technogym fitness machines (TechnoGym, Gambettola, Cesena, Italy). Although the training principles and relative loads were the same in OLD and VERY OLD, the absolute training load was lower in VERY OLD because of lower maximal muscle strength.

The control groups (CON) were encouraged not to change physical activity level during the intervention. Nutritional supplementation in both groups in VERY OLD was a mixed

nutrient drink at breakfast and lunch (Fresubin® Protein Energy Drink, Fresenius Kabi, Bad Homburg, Germany) containing 20 g of milk protein (whey:casein 20:80), maltodextrin, sucrose, sunflower- and rapeseed oil. Nutritional supplementation in OLD was a drink containing 20 g whey protein and 10 g sucrose in HRT as well as CON (4, 5).

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2.5 Measurements

All measurements were performed before and after three months intervention.

2.5.1 Patellar Tendon Mechanical Properties

The procedure for testing patellar tendon mechanical properties is a validated method, which has been used previously in our own and other laboratories (7, 11, 27, 29, 36). Patellar tendon elongation and knee extensor force were synchronously measured while the participant performed 8 s ramped isometric knee extension with the knee joint fixed in a 90 degree angle. Participants performed 5 min warm-up on a cycle ergometer (Monark, Sweden) at a low resistance before the mechanical testing procedure to precondition the patellar tendon. A couple of practice trials were made before the recordings started. Tendon elongation was visualized with ultrasound (Hitachi Hi Vision, Ascendus, Hitachi Medical Corporation, Tokyo, Japan), using a 10MHz, 100 mm long linear array B-mode transducer to sample videos during the ramped contraction and a common trigger box to synchronize the ultrasound video with the force data. We recorded 4-6 ramps to ensure at least 2-3 satisfactorily completed ramped contractions with a steadily increasing force production and ultrasound videos with a good contrast. One investigator performed all tests in OLD, and another investigator performed all tests in VERY OLD using the same protocol.

Patellar tendon elongation was defined as the change in distance between the patella and tibia insertions of the tendon during the ramped contractions, and analyzed with a previously validated

custom made semi-automated software (45). Patella and tibia movements were always tracked within the area of patellar tendon insertion to reduce error due to rotation of tibia and patella during the isometric contraction. Second order polynomials were fitted to the force-deformation data points in Sigma Plot (Version 10.0, Systat Software, Germany) and used to estimate maximal values for tendon stiffness at the top 10% of the curve.

The investigators performing data analysis selected two curves for further analysis based on the following selection criteria: A smooth inclining force curve, visually reliable tracking, good synchronization between force and deformation, return to baseline after relaxation, and all other things equal, the trials with highest force and/or deformation were chosen. Because the force-deformation relationship of tendon is nonlinear, the raw data in the selected curves was cut off at the highest common force across repeated measurements for each individual. We did not compare age-groups or intervention groups at common force. Stress and strain were calculated using average tendon cross-sectional area (CSA) and length from MRI (see 2.5.2).

The dominant leg was used for mechanical testing at both time-points. Two investigators blinded to group allocation and time point performed all data analysis in OLD, while making sure that the same investigator always performed the PRE and POST analysis for the same individual. Another blinded investigator performed all the analyses in VERY OLD using the same protocol.

2.5.2 Patellar tendon dimensions

Patellar tendon length and CSA were assessed with MRI, which is a reliable and accurate method for determining patellar tendon dimensions (18). Trained radiographers performed the scans on a 1.5-T MRI Philips Ingenia scanner with an axial and sagittal T1-weighted turbo spin echo sequence (TE: 17; TR: 500; matrix: 512×512; FOV: 150 mm; Slice thickness: 3 mm), which has been used in previous human studies in our department (6). The axial slices of the patellar tendon were positioned orthogonal to the length in the sagittal plane covering the distal patellar pole to the tibia insertion. A supportive pillow was placed in the knee coil to ensure slight stretch on the tendon by

bending the knee, which made it easier to measure tendon dimensions. A phantom containing 1.0% $CuSO_4$ was included in the image and subsequently used to normalize tendon signal intensity.

All participants were scanned in their habitual state in the afternoon (15.30 to 17.30), and were instructed to avoid strenuous physical activity in the preceding 48 hours to avoid the possible influence of training on tendon and muscle water content (59).

One experienced, blinded investigator performed all measurements of patellar tendon dimensions using Osirix imaging software (version 2.7.5, Osirix Imaging Medical, Geneva, Switzerland) to manually outline patellar tendon length as well as CSA at three locations (proximal, mid, distal) along the length of the tendon (14, 18). Signal intensity was adjusted on NIH (National Institute of Health) red-green color scale and measurements were performed on grey-scale images. The investigator always verified the selection of grey-scale borders on the red-green color image to obtain more accurate measurements of patellar tendon CSA (18). The proximal CSA was measured just distal to the patellar insertion, the distal CSA was measured just proximal to the tibia insertion, and the mid CSA on the slice midway between the proximal and distal slices. Patellar tendon length was measured as the distance from the most dorsal insertion on the patella apex to the most dorsal insertion on the tibia. All measurements were performed in triplicate with an average CV < 3%.

2.5.3 Quadriceps muscle strength and cross-sectional area

In both studies a Kinetic Communicator (model 500-11, Kinetic Communicator; Isokinetic International, Chattanooga, TN, USA) was used to assess isometric quadriceps strength (IsoMVC) at 70º knee flexion (0º is horizontal). The participants performed a brief cycle ergometer warm-up, two test-sweeps and three verbally encouraged maximal sweeps.

Trained radiographers obtained images for measurements of m. quadriceps cross-sectional area (Q-CSA) with a 1.5 T MRI Philips Ingenia scanner. Image analysis was performed in triplicate

using Osirix imaging software, and we report the mean value. For details about assessment of muscle strength and cross-sectional area we refer to the mother studies (4, 5).

2.5.4 Physical activity level

Physical activity level was measured with a previously validated (19) accelerometer/inclinometer (activPal micro, PAL technologies, Glasgow, Scotland), which was mounted on the thigh of the participants for 4 days (96 hours) always including the weekend. Data were extracted with activPal software (Research edition, V.7.2.32, PAL Technologies, 2013). Here we report daily step count as a measure of physical activity level.

2.5.5 Blood samples

A general health screening was performed at baseline including several analyses on blood samples, which were analyzed with standard assays and kits. We report here values for HbA1c and total cholesterol since they may affect tendon health and biomechanics (3, 17, 28, 67).

2.5.6 Statistical analysis

We combined data from the two studies (age-groups) and reduced all continuous variables to group means. Change over time was also determined (repeated measurements). Drop-outs were excluded completely from the analysis since we were mainly interested in the physiological response to training and not intention to treat. Everyone who completed the intervention was included in the analysis, irrespective of training compliance. We selected a statistical approach that would consider data from both studies in one model, and therefore it differs slightly from the ones used in the mother studies.

To investigate baseline differences, we performed a two-way ANOVA (mixed model) on the baseline values with intervention-group and age-group as factors. In addition, to investigate the effect of exercise two-way ANOVA was performed on the changes over time with intervention-group and age-group as factors. Tukey's post-hoc test was used in all cases to correct for multiple

263 comparisons. Normality was confirmed visually with the residual plots, and all continuous variables
264 were normally distributed. We finally compared satisfactorily completed training sessions between
265 age-groups with Welch's unpaired t-test and differences in sex distribution with chi-square test.
266 SAS 9.4 statistical Software (SAS institute, USA) was used for all statistical analyses.

267 We performed outlier analysis with online Grubbs test (GraphPad Software, 2017) before statistical
268 testing on the change over time of all variables. One outlier was detected in maximal tendon force
269 in OLD and one in maximal tendon deformation in VERY OLD. Statistical testing was performed
270 with and without the outliers and all the variables directly dependent on the outlier, but this did not
271 change the final results. Since we could not detect a specific technical source of the outliers, we
272 chose to include them in the analysis as extreme physiological changes. Missing values due to
273 technical or administrative errors were excluded from the analyses on change over time, whereas
274 they were handled using the software package default imputation technique in the analysis on
275 absolute values. Baseline characteristics are presented as mean ± SD. All other data are
276 presented as mean ± SE unless otherwise stated. A p-value of <0.05 was considered statistically
277 significant.

278

279 **3. Results**

280 *3.1 Participants*

281 One man dropped out of the OLD CON group because he lost interest. One woman and two men
282 dropped out of the VERY OLD HRT group: One due to a hip fracture unrelated to the study, one
283 due to lack of motivation, and one suffered an adverse event (compression fracture of medial
284 femoral condyle). One man dropped out of VERY OLD CON due to surgery of an inguinal hernia.
285 We present data for the remaining 19 OLD and 26 VERY OLD participants, whose baseline
286 characteristics are shown in table 1. OLD were on average 19 years younger than VERY OLD.
287 There were no age differences in height, weight, BMI, HbA1c and total cholesterol although total

cholesterol tended to be lower in VERY OLD than OLD (p=0.09). OLD completed on average 71% (range: 47% to 89%) training sessions satisfactorily, whereas VERY OLD completed 90% (range: 67% to 97%), and this was significantly different (p=0.007). Men and women were not differently distributed between age-groups (p=0.2).

Table 1: Participant baseline characteristics

	Age group	Total	CON	HRT
Participants	OLD	19	10	9
	VERY OLD	26	14	12
Sex (m/f)	OLD	10/9	5/5	5/4
	VERY OLD	16/10	8/6	8/4
Age (y)	OLD	68 ±1.9	68 ±1.5	69 ±2.2
	VERY OLD**	87 ± 3.2	86 ±2.6	88 ± 3.7
Height (cm)	OLD	171 ± 8	171 ± 6	170 ± 10
	VERY OLD	169 ± 11	168 ± 12	171 ± 11
Weight (kg)	OLD	72.6 ± 12.1	68.5 ± 6.9	77.2 ±15.2
	VERY OLD	69.9 ±14.0	69.5 ± 14.8	70.5 ±13.5
BMI	OLD	24.8 ± 3.2	23.4 ± 2.0	26.5 ± 3.7
	VERY OLD	24.2 ± 3.0	24.4 ± 2.9	24.0 ± 3.0
HbA1c (mmol/l)	OLD	36 ± 2.8	35 ± 2.7	36 ± 3.2
	VERY OLD	36 ± 2.1	36 ± 2.2	36 ± 2.1
Total-C (mmol/l)	OLD	5.7 ± 0.8	5.8 ± 0.5	5.5 ± 1.0
	VERY OLD(*)	5.2 ± 0.9	5.3 ± 0.9	5.1 ± 1.0
Compliance (%)	OLD	71 ± 16		71 ± 16
	VERY OLD**	90 ± 8		90 ± 8

Baseline characteristics of OLD (+65 years) and VERYOLD (+83 years) individuals. Values are mean ± SD. **p<0.01 denotes significant difference between age-groups based on two-way ANOVA or Welch's unpaired t-test (compliance). (*) = trend (0.05<p<0.1). No significant differences between HRT and CON at baseline. HRT = Heavy Resistance Training, CON = Control, BMI = Body Mass Index. Total-C = Total Cholesterol. Compliance(%) = Percent satisfactorily completed training sessions.

3.2 Patellar tendon mechanical properties and dimensions

There were no interactions between intervention-group and age-group in any baseline values, which allowed us to look at the main effects of age. Patellar tendon mechanical and material properties displayed a significant main effect of age on all variables (table 2). Maximal deformation and strain were higher (p<0.05), whereas maximal force and stress were lower (p<0.05) in VERY OLD compared to OLD. Hence, maximal stiffness and modulus were also lower in VERY OLD than OLD (p<0.0001).

Table 2: Maximal patellar tendon mechanical properties

		Baseline		Change over time		
	Age group	**CON**	**HRT**	**∆CON**	**∆HRT**	
Max deformation (mm)	OLD	2.1 ± 0.2	2.0 ± 0.2	0.1 ± 0.1	0.05 ± 0.2	
	VERY OLD A*	2.6 ± 0.4	3.1 ± 0.3	-0.2 ± 0.2	-0.4 ± 0.3	
Max force (N)	OLD	3940 ± 290	4150 ± 430	280 ± 310	36 ± 340	AxT*
	VERY OLD A**	3100 ± 310	3130 ± 330	-410 ± 170	330 ± 180	
Max stiffness (N/mm)	OLD	3620 ± 300	3720 ± 420	-190 ± 360	240 ± 500	(AxT)
	VERY OLD A**	2190 ± 200	1950 ± 210	-280 ± 190	280 ± 140	
Max strain (%)	OLD	4.6 ± 0.4	4.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.5	
	VERY OLD A**	5.6 ± 0.8	6.7 ± 0.6	-0.4 ± 0.5	-0.8 ± 0.8	
Max stress (MPa)	OLD	32 ± 2	32 ± 3	2 ± 2	1 ± 3	(AxT)
	VERY OLD A*	26 ± 3	26 ± 2	-4 ± 2	3 ± 2	
Max modulus (MPa)	OLD	1330 ± 130	1310 ± 100	-80 ± 120	40 ± 170	(T)
	VERY OLD A**	856 ± 74	756 ± 75	-130 ± 80	110 ± 60	

Patellar tendon mechanical properties in OLD (+65 years) and VERY OLD (+83 years) individuals, before (baseline) and after three months intervention (Change over time, ∆CON, ∆HRT). Values are mean ± SE. A: Main effect of age-group (VERY OLD different from OLD at baseline). A x T: Training x age-group interaction (training effect different in VERY OLD and OLD), T: Main effect of training (∆HRT different from ∆CON in both age-groups). *p<0.05, **p<0.01. (A), (T), (AxT) = trend (0.05<p<0.1). HRT = Heavy Resistance Training, CON = Control.

The same main effects of age were found when patellar tendon mechanical properties were analyzed at the highest common force level within each participant (table 3).

Table 3: Common force patellar tendon mechanical properties

		Baseline		Change over time	
	Age group	**CON**	**HRT**	**∆CON**	**∆HRT**
Max deformation CF (mm)	OLD	2.0 ± 0.2	1.9 ± 0.2	0.1 ± 0.1	0.0 ± 0.2
	VERY OLD A*	2.2 ± 0.4	2.9 ± 0.3	-0.1 ± 0.2	-0.6 ± 0.2
Max force CF (N)	OLD	3560 ± 340	3620 ± 520	-34 ± 81	1 ± 104
	VERY OLD A**	2310 ± 260	2710 ± 290	9 ± 24	-38 ± 56
Max stiffness CF (N/mm)	OLD	3380 ± 330	3560 ± 510	-330 ± 300	330 ± 420
	VERY OLD A**	1820 ± 160	1800 ± 180	-20 ± 150	100 ± 180
Max strain CF (%)	OLD	4.4 ± 0.4	4.0 ± 0.2	0.1 ± 0.2	-0.1 ± 0.4
	VERY OLD A*	4.9 ± 0.71	6.2 ± 0.55	0.1 ± 0.5	-1.1 ± 0.5
Max stress CF (MPa)	OLD	29 ± 3	27 ± 3	0 ± 1	1 ± 1
	VERY OLD A**	20 ± 2	22 ± 2	0 ± 0	0 ± 0
Max modulus CF (MPa)	OLD	1240 ± 130	1230 ± 130	-120 ± 90	100 ± 140
	VERY OLD A**	706 ± 54	703 ± 73	-20 ± 70	30 ± 70

Within subject common force (CF) patellar tendon mechanical properties and dimensions in OLD (+65 years) and VERY OLD (+83 years) individuals, before (baseline) and after three months intervention (Change over time, ∆CON, ∆HRT). Values are mean ± SE. A: Main effect of age-group (VERY OLD different from OLD at baseline). *p<0.05, **p<0.01. HRT = Heavy Resistance Training, CON = Control.

The analysis of change over time (pre to post) showed a significant interaction in maximal tendon force ($p<0.05$) and stress ($p=0.053$), since the increase in HRT compared to CON tended to be higher in VERY OLD than in OLD (table 2). Further, there was a tendency for a main effect of training on maximal modulus, which increased in HRT but decreased in CON ($p = 0.08$) (table 2).

There were no significant age x training interactions or main effects of training when patellar tendon mechanical properties were analyzed at within-subject common force (table 3).

Patellar tendon CSA tended to be lower in VERY OLD than OLD in the mid region (p=0.09), but not in any other regions (table 4). Training did not change patellar tendon CSA in either age-group.

Table 4: Patellar tendon dimensions

		Baseline		Change over time	
	Age group	**CON**	**HRT**	**∆CON**	**∆HRT**
CSA total (mm2)	OLD	130 ± 10[‡]	131 ± 9	1 ± 3.2	-2.2 ± 1.7
	VERY OLD	117 ± 5	121 ± 8[§]	1.4 ± 1.5	0.5 ± 2.3
CSA proximal (mm2)	OLD	136 ± 9[‡]	140 ± 9	1.4 ± 4.9	-2.9 ± 2.9
	VERY OLD	122 ± 5	129 ± 9[§]	1.9 ± 1.7	-2.9 ± 3.5
CSA mid (mm2)	OLD	125 ± 11[‡]	126 ± 10	0.8 ± 3.5	-1 ± 2.9
	VERY OLD (A)	110 ± 4	114 ± 7[§]	0.0 ± 1.4	1.5 ± 2.2
CSA distal (mm2)	OLD	128 ± 11[‡]	128 ± 9	0.9 ± 2.8	-2.6 ± 1.4
	VERY OLD	120 ± 5	121 ± 10[§]	2.3 ± 3	2.8 ± 3.2

Patellar tendon dimensions in OLD (+65 years) and VERY OLD (+83 years) individuals, before (baseline) and after three months intervention (Change over time, ∆CON, ∆HRT). Values are mean ± SE. (A) = Age main effect trend (0.05<p<0.1). HRT = Heavy Resistance Training, CON = Control, CSA = Cross sectional area. [‡]One missing value due to administrative error (n=9). [§]One missing value due to administrative error (n=11).

3.3 Other physiological characteristics

There was a significant main effect of age on IsoMVC and Q-CSA (p<0.001) which was 32% and 26% lower respectively in VERY OLD compared to OLD (table 5). We also observed a main effect of training on IsoMVC (p<0.01) and Q-CSA (p<0.05), which increased more in HRT than in CON, but the effect was not different between age-groups (no interaction). Daily step count was 43% lower in VERY OLD compared to OLD (p<0.001), and training did not influence this variable in either age-group (table 5). We found a significant age x training interaction of weight and BMI (p<0.05), which increased significantly with training in VERY OLD (p<0.05) but not OLD. The results finally showed a training main effect on total cholesterol (p<0.05), which was more reduced in both HRT groups compared to CON (table 5).

Table 5: Other relevant physiological characteristics

		Baseline		Change over time		
	Age group	**CON**	**HRT**	**∆CON**	**∆HRT**	
Weight (kg)	OLD	68.5 ±2.2	77.2 ±5.1	1.4 ±0.6	0.7 ±0.5	AxT*
	VERY OLD	69.5 ±4.0	70.5 ±3.9	-0.2 ±0.8	2.6 ±0.8*	
BMI	OLD	23.4 ±0.6	26.5 ±1.2	0.47 ±0.19	0.25 ±0.17	AxT*
	VERY OLD	24.4 ±0.8	24.0 ±0.9	-0.03 ±0.27[c]	0.93 ±0.27*	
HbA1c (mmol/l)	OLD	35 ±0.8	36 ±1.1	0.5 ±0.5	0.2 ±0.6	
	VERY OLD	36 ±0.7	36 ±0.6	2.9 ±0.8	1.2 ±0.7	
Total-C (mmol/l)	OLD	5.8 ±0.2	5.5 ±0.3	0.02 ±0.16	-0.59 ±0.16	T*
	VERY OLD (A)	5.3 ±0.2[d]	5.1 ±0.3	-0.52 ±0.17[d]	-0.66 ±0.18	
Steps per day (No.)	OLD	5730 ±599	4630 ±688	-620 ±540	-400 ±520	
	VERY OLD A**	3020 ±420[a]	2820 ±628[b]	-10 ±360[a]	590 ±240[b]	
Isometric MVC (Nm)	OLD	174 ±13	179 ±21	11 ±5	18 ±5	T**
	VERY OLD A**	116 ±14	124 ±11	-4 ±4	16 ±6	
Q-CSA (cm^2)	OLD	54 ±3.6	63 ±5.8	0.1 ±0.5	0.5 ±0.9	T*
	VERY OLD A**	43 ±3.1	43 ±2.8	-0.9 ±0.6	1.5 ±0.7	

Physiological characteristics in OLD (+65 years) and VERY OLD (+83 years) individuals, before (baseline) and after three months intervention (Change over time, ∆CON, ∆HRT). Values are mean ± SE. A: Main effect of age-group (VERY OLD different from OLD at baseline). A x T: Training x age-group interaction (training effect different in VERY OLD and OLD), T: Main effect of training (∆HRT different from ∆CON in both age-groups). *p<0.05, **p<0.01. (A), (T), (AxT) = trend (0.05<p<0.1). HRT = Heavy Resistance Training, CON = Control, MVC = Maximal Voluntary Contraction, Q-CSA = Quadriceps cross sectional area. [a]Two values missing due to technical error (n=12). [b]Five values missing due to technical error (n=7). [c]One participant missing in CON due to peripheral edema (n=13). [d]Two values missing in due to technical error (n=12)

4. Discussion

4.1 The effect of aging

The present investigation was to our knowledge the first to quantify in vivo tendon mechanical properties and dimensions in very old (VERY OLD) compared to old (OLD) adults. Consistent with our hypothesis, patellar tendon mechanical properties were diminished in VERY OLD compared to OLD (Table 2). Specifically, maximal tendon stiffness was 43% lower, maximal deformation was 38% higher, and maximal force was 23% lower in VERY OLD compared to OLD (table 2). Patellar tendon CSA tended to be lower in the mid-region in VERY OLD (p=0.09). We also observed a significant age-related reduction in patellar tendon material properties with a 39% lower maximal modulus, 37% higher maximal strain, and 18% lower maximal stress (table 2). The reduction in mechanical properties with age may partly be attributed to the 32% reduction in maximal quadriceps strength in VERY OLD, which meant that the modulus was not obtained at the same force level in the two age-groups. However, the markedly higher maximal tendon strain coupled

370 with a lower maximal tendon stress in VERY OLD compared to OLD indicated a more compliant
371 patellar tendon in VERY OLD (fig 1).

372 Lower in vivo tendon stiffness in older adults compared to young has previously been
373 reported in some (33, 35, 47, 52, 61) but not all (11, 13, 61) studies. One in vitro study showed a
374 tendency for lower modulus values of the patellar tendon in 64-93 year olds compared to 29-50
375 year olds (32), but no in vivo studies have confirmed this. A few animal studies have even reported
376 an increased stiffness with age (49, 65), which most likely reflects maturation rather than aging per
377 se. Taken together, our data together with previous human studies are, if any change at all, in
378 favor of an age-related reduction in tendon stiffness, and this mechanical deterioration seems to
379 continue throughout life.

380 The participants in the present investigation were generally healthy and comparable
381 across groups with respect to weight, BMI, HbA1c, and cholesterol (table 1). In addition to
382 differences in isometric quadriceps strength, there was an age difference in physical activity level.
383 Daily step count was 43% lower in VERY OLD (table 5), and this may have an influence on tendon
384 mechanical properties, since unloading of tendons such as in muscle paralysis (42), microgravity
385 (54), or even short term immobilization (6, 15) result in a rapid loss of tendon stiffness. Moreover,
386 young and old men with similar activity levels seem to have indistinguishable mechanical
387 properties of the patellar tendon (13, 16). Our own data did not reveal any correlations between
388 daily step count and patellar tendon modulus in either OLD (r^2= 0.003) or VERY OLD (r^2 = 0.03),
389 indicating that the current lower physical activity could not alone explain the observed age
390 difference in tendon modulus.

391 The consequences of reduced tendon stiffness with age are uncertain. A more
392 compliant tendon may impair postural balance (51) and compromise muscle strength and speed of
393 contraction (9, 66) by shifting the muscle length-tension curve to the right (too much sarcomere
394 overlap) (53). In line with this, one recent study found a significant postitive correlation between
395 rate of muscle force development and tendon stiffness when correlating data from young and old in

396 the same model (52). However, another recent study on young men reported that the free patellar
397 tendon did not contribute significantly to quadriceps explosive strength (46), and thus the
398 consequences of reduced tendon stiffness for muscle function still remain to be clarified.
399 Despite significant improvements in ultrasound imaging quality and digital processing
400 of ultrasound videos since the first assessments of in vivo patellar tendon mechanical properties
401 (24, 25), accurate measurement of tendon deformation and hence tendon stiffness remains a
402 challenge (58). Our results for tendon mechanical and material properties in OLD are similar to
403 what we have found previously in the same age group in our own lab (15, 16) and with the same
404 method in other labs (11, 55). However, other studies with slightly different test-protocols have
405 reported lower values for patellar tendon stiffness and modulus and higher values for deformation
406 or strain in older adults (27, 50, 55). The reason for this discrepancy may relate to differences in
407 testing conditions, i.e. speed of contraction, preconditioning, and not least the method for
408 assessment of tendon deformation, which may have a pronounced influence on calculation of
409 tendon stiffness (58). In the present investigation, we used a 100 mm ultrasound probe to keep the
410 patellar and tibia insertions in the field of view simultaneously during the ramped contraction, which
411 is important for accurate assessment of tendon deformation (29).
412 Age-related increases in tendon CSA have been reported in human Achilles tendon
413 (44, 61), but not patellar tendon (11, 13, 15, 16), suggesting that the effect may be tendon specific.
414 Our own data showed a trend ($p=0.09$) towards lower patellar tendon CSA in VERY OLD in the
415 mid-portion of the tendon but not in the average CSA. Reduction of tendon CSA in very old adults
416 would increase stress on the tendon for a given load making it operate closer to maximal
417 properties and thereby increase the risk of tendon injury or even complete rupture. Although the
418 tendency for reduced CSA in the mid-portion of the patellar tendon may be a coincidence, it is
419 possible that tendon size remains unchanged or increases to a certain age, where after there is a
420 gradual loss of tendon material in very old age, but these consideration are beyond the scope of
421 the present investigation. Whether or not tendon CSA is reduced in old age, the lower tendon

422 modulus in VERY OLD suggests that aging reduces material quality and not only the amount of
423 material.

424 The average patellar tendon CSA in the present study (OLD: 131 mm^2, VERY OLD:
425 119 mm^2) was somewhat higher than what many previous studies have found using ultrasound
426 based techniques (10, 27, 50, 55). Although ultrasound may provide reliable estimates, it is
427 important to note that MRI and ultrasound cannot be used interchangeably for evaluation of tendon
428 CSA (34). Previous studies from our own lab have also found lower patellar tendon CSA in the
429 same age-group with a similar 1.5 T MRI based method (15, 16). This is not entirely surprising to
430 us since those studies relied on grey-scale image analysis, which may underestimate actual
431 patellar tendon CSA by as much as 16.5% in 1.5 T MRI scans (18). In the present investigation we
432 made sure to verify the grey-scale measurement on the red-green color image, which may have
433 provided more accurate measurements. A few studies have reported patellar tendon CSA in the
434 same range as us (11, 12) using grey-scale analysis but the participants in those studies also had
435 higher body weight than in our study. We therefore believe that we have reported accurate
436 measurements of patellar tendon CSA.

437 In contrast to young women (43, 68), older postmenopausal women do not seem to
438 differ appreciably from men in tendon mechanical properties (10). Although not statistically
439 significant, slightly more men were included in VERYOLD than in OLD. A secondary statistical
440 analysis of the sex by age interaction was performed, which did not yield any significant
441 interactions, although the difference in tendon stiffness between men and women seemed to be
442 diminished in VERY OLD compared to OLD. It is therefore unlikely that the unequal sex distribution
443 influenced our main findings. The analysis also showed that women across age-groups had lower
444 tendon stiffness and CSA but unchanged modulus compared to men, which is consistent with
445 previous studies (10, 50), and confirms equal material properties in men and women in old age.
446

447 *4.2 The effect of training*

448 HRT increased maximal isometric quadriceps strength by 12% ($p<0.01$) and Q-CSA by 2%
449 ($p<0.05$) (table 5), and the improvements were not different between age-groups despite a lower
450 training attendance in OLD (71%) compared to VERY OLD (90%). VERY OLD showed larger
451 relative improvements of IsoMVC (VERY OLD: +13%, OLD: +10%) and Q-CSA (VERY OLD:
452 +3.5%, OLD: +0.8%) but this was not significant. Further, maximal tendon force ($p<0.05$) and
453 stress ($p=0.053$) calculated on basis of the maximal ramped knee extension increased with training
454 in VERY OLD only (table 2).

455 Patellar tendon CSA did not change significantly after three months resistance
456 training in either age-group, and this corroborates with most (12, 27, 55) but not all (21) previous
457 short-term training studies on older adults. Hence, our training intervention was not successful in
458 reducing tendon stress at a given force level. On the other hand, tendons of younger and middle
459 aged adults generally seem to respond to training with increased CSA (8). Moreover, cross-
460 sectional studies have shown that long-term habitual loading results in higher CSA of both the
461 patellar (14, 16) and Achilles tendons (44), suggesting that younger age or longer training periods
462 are necessary to elicit detectable changes in CSA and reduce tendon stress. Although increased
463 peritendinous collagen synthesis has been observed in response to exercise (40), the lack of
464 changes in patellar tendon dimensions is not surprising since overall collagen turnover in tendon is
465 very slow (2) and new collagen does not seem to be deposited in the tendon to any appreciable
466 extent after teenage years (31). The effect of resistance exercise on non-collagenous components
467 such as proteoglycans, collagen cross-links, fat, and water content remains uncertain.

468 There was a trend towards a main effect of training on maximal patellar tendon
469 modulus with 8% increase in HRT and 10% decrease in CON ($p=0.08$) (table 2). However, the
470 effect was not observed when we analyzed data at common force (table 3), and therefore the
471 results suggest that muscle improvements with training occur faster and independently of tendon
472 adaptations. Long-lasting improvements of muscle function may on the other hand be dependent
473 on proportionate increments in tendon material properties, since maximal muscle performance
474 seems to be correlated to tendon stiffness (9, 52). One recent study investigated the effect of a

longer training period (1.5 years) on calf muscle strength and Achilles tendon stiffness and found no further effect of training after 3 months on either muscle or tendon (21). However, in that study training volume was reduced after three months and muscle strength also failed to improve further from 3 to 18 months.

A 2-way ANOVA (PRE to POST) with sex and intervention-group as factors did not show any interactions or main effects of sex in any muscle or tendon parameters, making it unlikely that sex-distribution could explain the lack of differences in training adaptations between OLD and VERY OLD.

The lack of significant improvements in tendon mechanics in the present study was surprising since several studies (21, 27, 50, 55), although not all (12), on older adults have reported increases of in vivo patellar and Achilles tendon stiffness in response to three months resistance training.

4.3 Limitations:

One limitation in the present investigation was that the lack of comparison between OLD and VERY OLD at a common force level. Common force within subjects is not very restrictive because the variation in strength is small, but between subjects there is a large variation and the weakest person would determine the common force level. Thereby only the toe-region of the mechanical response would be analyzed. However, it appears that VERY OLD had more compliant tendons than OLD (figure 1). Another limitation was that the low training compliance in OLD could have hampered improvements in muscle and tendon adaptation. In fact, the improvement in muscle IsoMVC was only small to moderate (cohen's d = 0.29). Performing an analysis on participants completing >80% of the training sessions (OLD HRT: 2 women, 3 men. VERY OLD HRT: 8 men, 3 women) did not substantially change the conclusions although the training effects were marginally larger. The increase in IsoMVC thus changed from 10 to12% in OLD HRT and the increase in common force modulus changed from 8 to 12%. Training compliance was not statistically different for men (OLD: 74 ±6, VERY OLD: 92 ±4) and women (OLD: 67 ±9, VERY

OLD: 85 ±13). Finally, although training did not affect OLD or VERY OLD differently, we cannot exclude that the different nutritional supplementation in OLD and VERY OLD may have affected the training response, since whey protein seems to augment tendon hypertrophy in young men (23), and diet generally seems to influence tendon composition and mechanics in animals (22, 56, 60). The possible influence of different nutritional supplementations on improvements in muscle strength and cross-sectional area are less relevant to the present investigation, since we compared tendon mechanical properties at the two time-points at common force level.

5. Conclusion

Consistent with our hypothesis, the cross-sectional data suggest lower patellar tendon stiffness and unaltered dimensions in VERY OLD compared to OLD adults. Together with previous data on young vs. old, this indicates an age-related deterioration of tendon function from maturity and throughout life. Reduced material properties may be due to changes in collagen content as well as non-collagenous components such as proteoglycans, collagen cross-links, fat and water (63, 64), but these considerations are beyond the scope of the present investigation. We can also not exclude that a concomitant significant reduction in physical activity with age may have confounded the lower patellar tendon stiffness in VERY OLD. Our longitudinal data showed that three months resistance training augmented maximal quadriceps muscle strength and cross-sectional area, but contrary to our hypothesis resistance training did not affect patellar tendon dimensions or mechanical properties in either age-group.

In conclusion, patellar tendon mechanical and material properties but not dimensions were lower in very old (+83) compared to moderately old (+65) individuals. Three months resistance training did not affect tendon properties or dimensions despite significant improvements in muscle strength.

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8. Disclosures

All authors declare no conflicts of interest.

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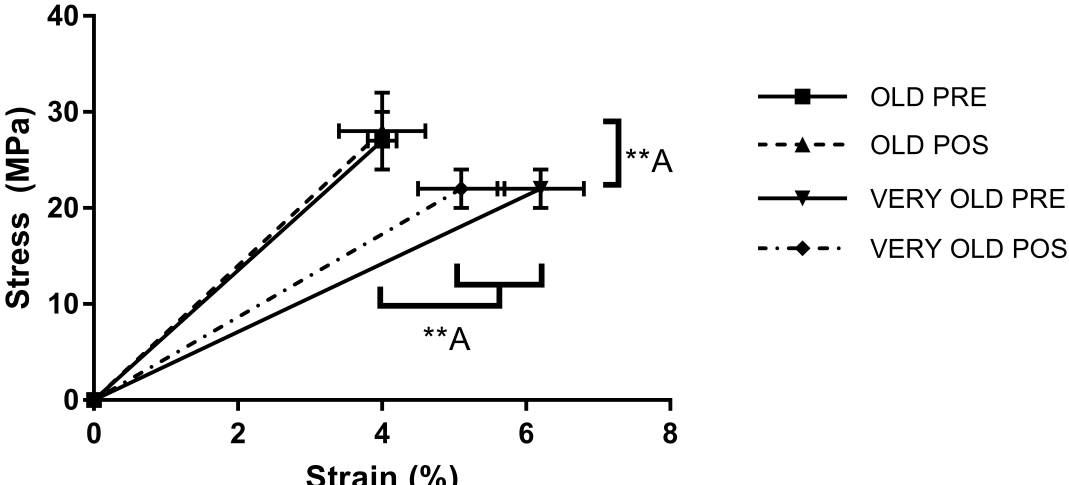
Figure captions

Fig.1: Straight lines drawn on basis of mean ± SE values for maximal stress and strain (common force) before (PRE) and after (POST) three months resistance training in OLD (+65 years, n=9) and VERY OLD (+83 years, n=12) adults. The slope of the lines depicts average Young's modulus. A: main effect of age-group, **p<0.01 based on 2-way ANOVA.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Counteracting Age-related Changes in Mechanical Properties of Human Tendon: Influence of Strength Training Load

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Abstract

Background: Regular loading of connective tissue like tendons may counteract the negative effects of aging. However, the duration of training and magnitude of load required to induce favorable tendon adaptations are uncertain. The purpose of the present study was therefore to compare the effects of moderate or high load long-term resistance training on tendon mechanical properties as well as on matrix structure and composition.

Methods: Thirty-six healthy participants (17 women, 19 men), age 62-70 years, were recruited from a larger training study and randomly allocated to 12 months of heavy load resistance training (HRT: 3 women, 7 men), moderate load resistance training (MRT: 9 women, 6 men) or control (CON: 5 women, 6 men). Pre- and post-intervention testing comprised isometric quadriceps strength test (IsoMVC), ultrasound based testing of in vivo patellar tendon (PT) mechanical properties, MRI for measurement of PT length and cross-sectional area (CSA), PT biopsies for assessment of fibril morphology, collagen content, as well as content of enzymatic collagen cross-links, and tendon fluorescence as a measure of advanced glycation end-products (AGEs).

Results: Data from 33 participants (10 HRT, 13 MRT, 10 CON) were included in the analysis. IsoMVC improved more after HRT (+21%) than MRT (+8%) and CON (+7%) ($p<0.05$). Tendon stiffness and Young's modulus at a common tendon force were also differently affected by training load ($p<0.05$) with a time-dependent reduction in CON and MRT but not in HRT. PT-CSA increased equally after both MRT and HRT whereas signal intensity increased only after HRT. Collagen content, fibril morphology, enzymatic collagen cross-links, and fluorescence were unaffected by training.

Conclusion: Despite equal improvements in tendon size after moderate and heavy load resistance training, only heavy load training counteracted the time-dependent reduction of

tendon material properties in older adults. The changes were unrelated to collagen content, fibril morphology, enzymatic collagen cross-links, or AGEs. This indicates a load dependent effect of strength training upon aging human tendon.

Introduction

Aging negatively affects structure and mechanical function of collagen-rich tissues (3, 5, 12). The skin loses elasticity, blood vessels become stiffer, and tendons may become more compliant (41). The possible age-related decline in tendon mechanical function may in turn impair optimal function of the connected skeletal muscle (11, 53). Regular loading of tendons seems to protect the tissue from the negative effects of aging (17, 58) by increasing tensile stiffness (10), also in older adults (22, 29, 55). However, knowledge about training duration and the magnitude of load needed to induce favorable adaptations in tendons of older adults is limited. Moreover, the molecular changes in matrix composition, which mediate the effect of loading on tendon mechanical properties, are largely unresolved.

Training with heavy load seems to elicit a more pronounced increase in tendon stiffness than training with moderate loads in middle aged (10) as well as older adults (29). However, lower training load promoted collagen synthesis in younger individuals if the training volume was comparable with the high load training (37). Short term (12 weeks) resistance training has effectively increased tendon stiffness in older adults in most (22, 29, 55) but not all (63) studies. Only few studies have investigated the effect of longer training duration and found no additional effect on tendon stiffness after either 18 months (older adults) (22) or 4 years (young adults) (50) compared to only three months of training. However, young athletes engaging in sports where one leg is more loaded than the other over several years display pronounced differences in tendon stiffness and cross-sectional area, suggesting a continued adaptation to loading beyond the first three months (16). Thus, more studies are warranted to investigate the influence

of training load and duration on tendon size and mechanical properties, not least in older adults.

The main contributor to tendon tensile stiffness is type I collagen, which makes up most of the tendon matrix. Acute exercise increases peritendionus collagen synthesis (42), but the overall collagen content and the collagen fibril volume fraction as measured by transmission electron microscopy (TEM) does not seem to be responsive to training (17, 45). Moreover, recent evidence suggest that the vast majority of core tendon collagen molecules are not renewed after late teenage years (35), suggesting that both the aging process and the adaptations to loading are mediated by other molecular components in the matrix.

One important matrix component that can influence tendon mechanical properties are collagen cross-links (25), which are essentially either enzymatic or non-enzymatic. Enzymatic cross-links (i.e. lysylpyrridinoline (LP) and hydroxylysylpyrridinoline (HP)) are tightly regulated in time and space by the family of lysyl oxidases (LOX) and are essential for normal tendon development. Tendon LOX expression is unaffected by regular training (68) but induced by acute exercise (34) in rodents indicating a stimulatory effect of exercise on formation of cross links. Life-long endurance training does not seem to affect HP or LP (17), but it remains unknown how long-term resistance training affects enzymatic cross-links in older adults.

Non-enzymatic cross-links form sporadically when reducing sugars react with amino-groups and mature into advanced glycation end-products (AGEs) (5, 12). AGEs accumulate in collagen rich tissues with age (4), not least in tissues with slow turnover rate like tendon (2, 6), where they may eventually impair material properties (3, 26, 56). Despite the slow turnover rate of tendon, regular training seems to reduce (68) or at least

be associated with an attenuated AGE accumulation in older adults (17), and heavy resistance training reduces patellar tendon pentosidine, which is a cross-linking AGE, in young men (38). However, it is still unknown how regular heavy and light load resistance training in older adults affects AGEs in the human patellar. Intuitively, more collagen cross-links increase tissue stiffness (46, 54, 56) but the contribution of different types of cross-links to tendon mechanical properties in older adults is unknown.

The present investigation aimed to compare the effects of long-term (12 months) heavy or light load resistance training on patellar tendon mechanical properties, morphology and collagen cross-links in older adults. The hypothesis was that compared to a control group, both training loads would increase patellar tendon stiffness. Moreover, we hypothesized that the increased tendon stiffness would be related to higher levels of enzymatic cross-links but unchanged AGE cross-links, collagen content and fibril morphology.

Methods

Study Design

The present investigation was a sub-study of a larger randomized trial started in 2014 at the Institute of Sports Medicine Copenhagen (24). The study primarily investigated the effect of 12 months resistance training on muscle strength and physical function in men and women, 62-70 years old. The 36 participants recruited for the present sub-study gave consent to undergo additional tendon specific tests. They were allocated to 12 months of moderate load resistance training (MRT), heavy load resistance training (HRT), or habitual physical activity level (<1h strenuous exercise/week) (CON).

Ethics and data handling

The study complied with the Helsinki Declaration and all participants gave oral and written consent before enrollment. The study was registered at clinicaltrials.gov (NCT02123641, registered on April 25th 2014) and was approved by the Capital Region ethical committee (J-no. H-3- 2014-017) and the Danish Data Protection Agency (J-no. 2012-58-0004 - BFH-2016-025, I-suite 04545).

Participants

The study included 17 women and 19 men, age 62-70 years. They were all home-dwelling, independent, and untrained. Exclusion criteria were more than 1 hour of systematic strenuous exercise per week, systemic hormonal-/anti-hormonal therapy, anticoagulant therapy, a history of patellar tendinopathy within the past 12 months, chronic knee pain or other musculoskeletal problems impeding participation in resistance training. For the full list of exclusion criteria we refer to the original study protocol (24).

Interventions

The interventions are described in detail in a previous publication (24). Briefly, HRT was a supervised, whole body, progressive, heavy resistance training program. Participants performed 3 sets at 70-90% of 1RM three times per week. The leg exercises targeting the patellar tendon were leg press and knee-extension performed in Technogym fitness machines (TechnoGym, Gambettola, Cesena, Italy).

MRT was a home-based, whole body, progressive, moderate load resistance training program performed with TheraBand® elastic rubber bands (Hygenic Corp., Akron, OH, USA) and own body-weight. The exercises were similar to HRT and were performed

as circuit training with 12-18 repetitions per set. Training frequency was three times per week, and the participants were supervised once a week. Consequently, compliance for MRT relied on self-reports.

CON was encouraged not to change their habitual physical activity level, which was <1 hour of regular strenuous physical activity per week, and were offered participation in social/cultural activities approximately once a month.

Muscle strength assessment

An investigator blinded to group allocation used a Good Strength device (V.3.14 Bluetooth; Metitur, Finland) to assess isometric knee extensor strength (IsoMVC) at 70º knee flexion. The participants performed one test-sweep and at least three verbally encouraged maximal sweeps. Measurements were continued until no further improvement occurred (24).

Muscle size

An experienced radiographer blinded to group allocation acquired MRI of the thighs using a 3.0 T TX Philips Achieva scanner (Philips Healthcare) with a 32-channel body array coil. The scanning was a 2D axial T1-weighted sequence (TR/TE=666/20 ms, FA=90, 672×672 matrix, 3 stacks with 3 slices, gap 1.91 mm, in-plane resolution 0.8 mm, slice thickness 4 mm) acquired at 10, 20 and 30 cm above tibia plateau, with three slices at each level for delineation of vastus lateralis of the quadriceps muscle and calculation of cross-sectional area. Image analysis was conducted by blinded assessors in the medical imaging software package Jim version 6.0 (Xynapse Systems, UK). We report vastus lateralis cross-sectional area (VL-CSA) of the dominant leg only (24).

Blood measurements

A general health screening was performed at baseline including several analyses on blood samples, which were analyzed with standard assays and kits in the clinical biochemistry department at Bispebjerg Hospital (Copenhagen, Denmark). We report here values for HbA1c and total cholesterol since they may affect tendon health and mechanics (7, 18, 30, 66).

Physical activity level

Physical activity level was measured with a previously validated (20) accelerometer and inclinometer (activPal micro, PAL technologies, Glasgow, Scotland), which was mounted on the thigh of the participants for five consecutive days, always including the weekend. Data were extracted with activPal software (Research edition, V.7.2.32, PAL Technologies, 2013). Here we report daily step count as a measure of physical activity level.

Patellar tendon mechanical properties

The procedure for testing patellar tendon mechanical properties is a validated method, which has been used previously in our own and other laboratories (14, 29, 31). The participants initially performed 5 min warm-up on a cycle ergometer (Monark, Sweden) at a low resistance to precondition the patellar tendon. The participants then performed 8 s ramped isometric contractions while the investigator simultaneously recorded knee extensor force (Noraxon Telemyo 2400T G2, USA) and ultrasound videos of the patellar tendon with a 10 MHz, 100 mm linear array transducer (Hitachi Hi Vision, Ascendus,

Tokyo, Japan) to assess tendon elongation. We recorded 4-6 ramps to ensure at least 2-3 satisfactorily completed ramped contractions with a steadily increasing force production and ultrasound videos with a good contrast.

Patellar tendon elongation was defined as the change in distance between the patella and tibia insertions of the tendon during the ramped contractions, and analyzed with a previously validated custom made semi-automated software (48). Patella and tibia movements were always tracked within the area of patellar tendon insertion to reduce error due to rotation of tibia and patella during the isometric contraction. Second order polynomials were fitted to the force-deformation data points in Sigma Plot (Version 10.0, Systat Software, Germany) and used to estimate maximal values for tendon stiffness at the top 10% of the force-deformation relationship.

The investigators performing data analysis selected two trials for further analysis based on the following selection criteria: Visual consistency between bone movement and tracking points, good synchronization between force and deformation, a smooth inclining force curve, return to baseline after relaxation, and all other things equal, the trials with highest force and/or deformation were chosen. Because the force-deformation relationship of tendon is nonlinear, the raw data in the selected curves was cut off at the highest common force across repeated measurements for each individual.

US videos from 8 participants were analyzed using a custom implementation of cross-correlation tracking in Matlab (R2015b, MathWorks Inc., USA) due to technical difficulties with the video analysis in the program otherwise used. In all instances, we made sure to reanalyze both 0 and 12 months measurements so repeated measures on the same subject were analyzed with the same method.

Patellar tendon dimensions

Patellar tendon length and CSA were assessed with a different MR-scanner than that used for muscle size determination. MRI is a reliable and accurate method for determining patellar tendon dimensions (19). Trained radiographers performed the scans on a 1.5-T MRI Philips Ingenia scanner with an axial and sagittal T1-weighted turbo spin echo sequence (TE: 17; TR: 500; matrix: 512×512; FOV: 150 mm; Slice thickness: 3 mm), which has been used in previous human studies in our department (8). The axial slices of the patellar tendon were positioned orthogonal to the length in the sagittal plane covering the distal patellar pole to the tibia insertion. A supportive pillow was placed in the knee coil to ensure slight stretch on the tendon by bending the knee, which made it easier to measure tendon dimensions. A phantom containing 1.0% $CuSO_4$ was included in the image and subsequently used to adjust contrast settings.

All participants were scanned in their habitual state in the afternoon (15.30 to 17.30), and were instructed to avoid strenuous physical activity in the preceding 48 hours to avoid the possible influence of training on tendon and muscle water content (57).

Patellar tendon dimensions and signal intensities were assessed using Osirix imaging software (version 2.7.5, Osirix Imaging Medical, Geneva, Switzerland) to manually outline patellar tendon length as well as CSA at three locations (proximal, mid, distal) along the length of the tendon(16, 19). All images were adjusted according to the phantom and measured using NIH (National Institute of Health) color scale, because this method provides more accurate measurements of patellar tendon CSA (19). The proximal CSA was measured just distal to the patellar insertion, the distal CSA was measured just proximal to the tibia insertion, and the mid CSA on the slice midway between the proximal and distal slices. Patellar tendon length was measured as the distance from the most

dorsal insertion on the patella apex to the most dorsal insertion on the tibia. Signal intensity was expressed relative to bone. Coefficient of variation corrected for small sample size (CV) on the triplicate measurements was on average 1.5% (range: 0.2 – 3.2%) for patellar tendon length, and 2.3% (range: 0.2 - 6.1%) for total patellar tendon CSA. Day to day variation in measurements showed a CV = 2.4%.

Tendon biopsy procedure

The patellar tendon biopsy procedure is a sterile procedure, which has been performed previously in our lab (39). Briefly, the biopsy was obtained in local anesthesia (1 ml lidocaine, Lidokain Mylan 1mg/ml, Mylan, Oslo, Norway) through a medio-lateral skin incision just distal to the patella with a semi-automated biopsy instrument (Bard Magnum, Bard biopsy systems, USA) at an angle of 45º relative to the patellar tendon in the proximal to distal direction.

We obtained all biopsies at the same time of day before and after the intervention (± 1 hour) to avoid the potential influence of circadian rhythm on tendon physiology (69) The non-dominant leg was biopsied before and the dominant leg after the intervention to avoid the influence of the first biopsy on the second (33). The same trained physician obtained or supervised all biopsies except for four biopsies obtained by another experienced physician.

Tissue preparation

Another investigator prepared the tissue for further analysis under light microscope. The tissue was kept moist in isotonic saline during the entire procedure. The biopsy was first dissected free from potential non-tendinous tissue (i.e. fat and subcutaneous tissue).

Then, the investigator cut a small piece for electron microscopy with visible regular, longitudinal arrangement of the collagen fibers, which was immersed in glutaraldehyde and stored at 5 ºC. The rest of the sample was immediately frozen in liquid nitrogen and subsequently stored at -80 ºC until further analysis.

Fibril morphology

Fibril morphology was analyzed with transmission electron microscopy. Preparation of the samples are described in detail in previous publications (9, 39). Images were obtained with a Philips TM 100 transmission electron microscope at 80 kV equipped with Megaview 2 camera. A blinded (to group) technician was instructed to zoom to a magnification of 1050 (100x100µm), where the tissue microstructure was not visible, and identify two different areas with core fibrillary structure (fascicles). The technician then divided the screen visually in six fields, and imaged one area in each field at 24500 x magnification (4x4µm). In this way we obtained a total of 12 unbiased images per biopsy, which all contained core tendon tissue with clearly visible cross-sectioned collagen fibrils.

One blinded investigator performed all measurements of fibril diameters in the image analysis software package FIJI (NIH, Bethesda, Maryland, USA). A macro was initially applied on each image to randomly generate a 300x300 nm^2 unbiased counting frame with guard regions, and semi-automatically delineate fibril contours by best fitting ellipses. The investigator then confirmed the automated measurements within the counting frame, and manually corrected any erroneously measured fibrils. We used an elliptic fit because some fibrils were not perfectly round, and chose the minor axis diameter as the true fibril diameter to alleviate the influence of sectioning angle. The investigator counted

on average 335±131 fibrils for each tendon specimen (12 images). The data were reduced for each individual and time point to mean fibril diameter, volume fraction, and fibril density.

Biochemical analysis

The raw tendon samples were thawed and freeze-dried to give the tendon dry-weight. For biochemical analysis, we used on average 7.2 mg (1.9 to 17.8 mg) tissue with an average water content of 84.2% water, giving 1.1 mg tissue dry weight for biochemical analysis. Afterwards, we performed gas-phase hydrolysis on the samples within sealed and evacuated tubes for 24 hours at 110 ºC followed by freeze-drying the samples again. The freeze-dried samples were dissolved in de-mineralized water to a concentration of 5 mg/ml.

Hydroxy-proline was determined on the hydrolyzed samples as a measure of collagen content as described in detail in a recent publication (61).

HP and LP were determined with two different ELISA kits, one made for measuring HP in serum (MicroVue Serum PYD, 8019, Quidel Corp.), and one for measuring LP in urine (MicroVue DPD, 8007, Quidel Corp.). Due to the much higher concentration of HP and LP in the tissue hydrolysates than in serum and urine, the samples (reconstituted in water at 5 mg/mL) were diluted 2000 fold for the HP analysis and 100 fold for LP, which reduces the risk of interference from compounds that would not normally be present in serum or urine. Both assays were competitive ELISAs based on a polyclonal rabbit antibody for HP and a monoclonal one for LP. Aside from using a different type of sample the manufacturer's instructions were followed without modification.

Total fluorescence was used as a marker of total AGE modification (51). Fluorescence measurements were made on the hydrolysates reconstituted in water. To

ensure a consistent pH and get enough volume for triplicate measurements, samples were diluted 6 fold into 0.12M HCl (0.1M final concentration). The diluted samples containing 83 µg tissue/ml were plated onto black 96 well plates and read on a Wallac1420 Victor microplate reader (Perkin Elmer) at 340/5 nm excitation and 460 nm (382-507 nm) emission. Since fluorescence correlated positively to collagen content (r^2=0.52, p<0.001), we chose to report fluorescence relative to collagen.

Statistical testing

Data were analyzed by repeated measures two-way ANOVA with baseline adjustment using time-point (0 mths vs 12 mths) and intervention-group (CON vs MRT vs HRT) as factors and Tukey-Kramer post-hoc tests. Normality was confirmed by visual inspection of residual plots. Signal intensity and fluorescence were not normally distributed and were log-transformed and analyzed again. All other variables were normally distributed. Outlier analysis was performed with an online Grubb's test (28) before statistical testing (no outliers were detected). SAS statistical Software v. 9.4 (SAS Institute, USA) were used for statistical testing, and figures were made in GraphPad Prism (v. 7.0, GraphPad Software Inc., La Jolla, CA, USA). Continuous variables are presented as arithmetic mean ±SE, and log-transformed values are presented as geometric mean [upper limit-lower limit]. Participant characteristics were summarized as mean ±SD.

Results

Participants

One woman dropped out of CON due to lack of time, and one woman and one man dropped out of MRT due to stroke and meningitis. The remaining 33 participants

completed the study with an average training compliance of 86 ±12%, which was not different between MRT and HRT. Their physiological characteristics are presented in table 1.

Table 1: Participant baseline characteristics

	Total	CON	MRT	HRT
Participants	33	10	13	10
Sex (men/women)	18/15	6/4	5/8	7/3
Age (y)	67 ±2	68 ±1.8	66 ±2.4	67 ±2.3
Height (cm)	173 ±8	175 ±8	173 ±7	171 ±8
Weight (kg)	78 ±14	81.6 ±16.9	72.9 ±11.6	79.4 ±13.9
BMI (kg/m^2)	25.8 ±3.7	26.6 ±4.3	24.3 ±3.5	26.9 ±3.1
HbA1c (mmol/l)	36 ±3.1	37 ±3.5	35.2 ±3.0	35.4 ±2.9
Total-C (mmol/l)	5.9 ±0.9	5.7 ±0.8	6.0 ±1.0	6.0 ±0.9
Training Compliance (%)	86 ±12		86 ±16	86 ±7

Values are mean ± SD. Baseline group-differences within studies evaluated with one-way ANOVA, chi-square test (sex distribution), and Welch's unpaired t-test (compliance). No significant group differences. HRT = Heavy Resistance Training, MRT = moderate load resistance training, CON = Control, BMI = Body Mass Index. Total-C = Total Cholesterol.

Muscle strength and mass

Twelve months intervention significantly improved isometric quadriceps muscle strength in HRT ($p<0.01$) and MRT ($p<0.05$) but not in CON. There was a significant time x group interaction since HRT improved more than both CON and MRT ($p<0.05$) (fig. 1). Vastus lateralis CSA was not significantly affected by training (fig. 1).

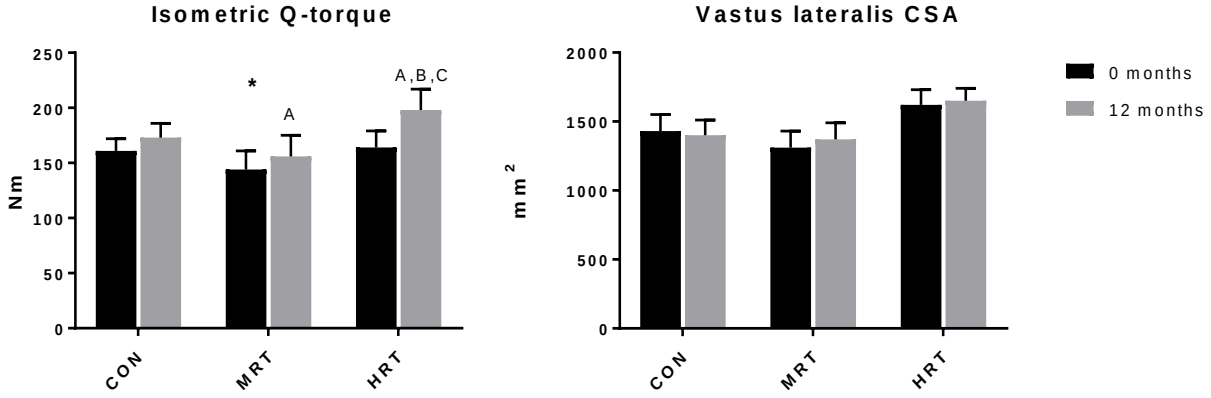

Fig. 1: Isometric quadriceps (Q)-strength and vastus lateralis cross-sectional area (CSA) before (0 months) and after 12 months heavy resistance training (HRT), moderate load resistance training (MRT) or no training (CON). *Significant time x group interaction based on 2-way repeated measures ANOVA ($p<0.05$) A: Significantly different from 0 months ($p<0.05$). B: Significantly different from CON12 ($p<0.05$), and C: Significantly different from MRT12 ($p<0.05$) based on Tukey-Kramer post-hoc test.

Health related variables

Weight, BMI, total cholesterol and HbA1c displayed no time x group interactions. There was however a significant main effect of time ($p<0.001$) on HbA1c which was higher after the intervention in all three groups (table 2). All other health-related variables did not change significantly during the intervention.

Table 2: Health related variables and physical activity level

	CON		MRT		HRT	
	0 mths	**12 mths**	**0 mths**	**12 mths**	**0 mths**	**12 mths**
Weight (kg)	81.6 ± 5.3	79.3 ± 5.2	72.9 ± 3.2	72.8 ± 3.4	79.4 ± 4.4	78.9 ± 4.3
BMI (kg/m^2)	26.6 ± 1.4	25.9 ± 1.4	24.3 ± 1	24.1 ± 1	26.9 ± 1	26.6 ± 0.9
Physical activity (Steps/day)	8200 ± 1100	8500 ± 1100	8900 ± 800	9800 ± 1100	8300 ± 700	9900 ± 700
HbA1c (mmol/mol) ‡‡‡	37.1 ± 1.1	38.3 ± 0.8	35.2 ± 0.8	36.2 ± 0.8	35.4 ± 0.9	36.8 ± 1
Total Cholesterol	5.7 ± 0.3	5.5 ± 0.3	6.0 ± 0.3	5.7 ± 0.2	6.0 ± 0.3	5.7 ± 0.4

Values are means ±SE. Data analyzed by repeated measures two-way ANOVA with baseline adjustment and Tukey-Kramer post hoc test. CON=Control, MRT = Moderate load resistance training, HRT = Heavy load resistance training. ‡‡‡: significant main effect of time ($p<0.001$)

Physical activity level

Physical activity level measured as daily step-count displayed no time x group interactions, but there was a trend towards a main effect of time ($p=0.07$) with increasing step-count from 0 to 12 months across groups (table 2).

Mechanical properties

There were significant time x group interactions in maximal patellar tendon stiffness and Young's modulus. Post hoc tests showed higher maximal stiffness and a tendency for higher maximal modulus (p=0.09) with HRT compared to CON (table 3). Maximal deformation, strain, force, and stress did not display any time x group interactions, but there was a significant main effect of time in all these variables, except for maximal stress (p=0.09).

Table 3: Maximal patellar tendon mechanical properties

	CON (n=10)		MRT (n=13)		HRT (n=10)	
	0 mths	**12 mths**	**0 mths**	**12 mths**	**0 mths**	**12 mths**
Max deformation (mm) ‡	2.7 ± 0.3	2.9 ± 0.2	2.3 ± 0.2	2.7 ± 0.2	2.4 ± 0.3	2.5 ± 0.3
Max force (N) ‡‡	4890 ± 390	5020 ± 330	4130 ± 520	4690 ± 490	5020 ± 530	5550 ± 470
Max stiffness (N/mm)**	3530 ± 490	3010 ± 440	3330 ± 290	3170 ± 210	4060 ± 430	4420 ± 340[B]
Maxs strain (%) ‡	6.0 ± 0.8	6.4 ± 0.5	5.3 ± 0.5	6.1 ± 0.6	5.5 ± 0.6	5.7 ± 0.5
Max stress (MPa)	43 ± 3	43 ± 2	33 ± 3	36 ± 3	43 ± 4	45 ± 3
Max modulus(MPa)*	1430 ± 2000	1200 ± 180	1180 ± 70	1090 ± 50	1510 ± 150	1560 ± 140

Values are means ±SE. Data analyzed by repeated measures two-way ANOVA with baseline adjustment and Tukey-Kramer post hoc tests. CON=Control, MRT = Moderate load Resistance Training, HRT = Heavy Resistance Training. Significant interaction denoted by *(p<0.05) or **(p<0.01). Main effect of time denoted by ‡(p<0.05) or ‡‡(p<0.01). Post hoc tests: B: significant difference from CON12 (p<0.05).

Common force mechanical properties also displayed significant time x group interactions in patellar tendon stiffness and Young's modulus (fig. 2). Common force stiffness was significantly higher after HRT compared to both CON and MRT, which reduced this variable. Common force Young's modulus further tended to be higher after HRT compared to MRT (p=0.06, fig. 2), which reduced the modulus. Common force deformation and strain displayed no time x group interactions or main effects of time.

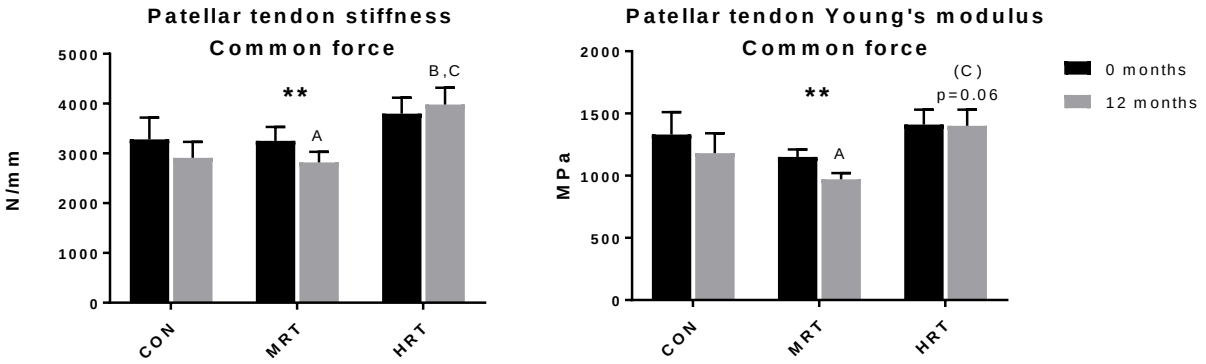

Fig. 2: Common force patellar tendon stiffness and Young's modulus (YM) before (0 months) and after 12 months heavy resistance training (HRT), moderate load resistance training (MRT) or no training (CON). Bars represent mean ±SE. Significant time x group interaction denoted by *(p<0.05) or **(p<0.01) based on repeated measures 2-way ANOVA. A: Significantly different from 0 months (p<0.05). B: Significantly different from CON12 (p<0.05), C: Significantly different from MRT12 (p<0.05) based on Tukey-Kramer post-hoc test.

Patellar tendon MRI

There was a trend towards a time x group interaction of total patellar tendon CSA (p=0.07), which increased significantly over time in HRT and MRT but not in CON (fig. 3). The proximal tendon region (CSA prox) showed a significant time x group interaction, and post-hoc test revealed increase over time in MRT and a similar, although not significant, increase in HRT (p=0.25) (fig. 3). The mid and distal tendon regions did not display any time x group interactions, but there was a main effect of time in both regions where CSA increased from 0 to 12 months across groups.

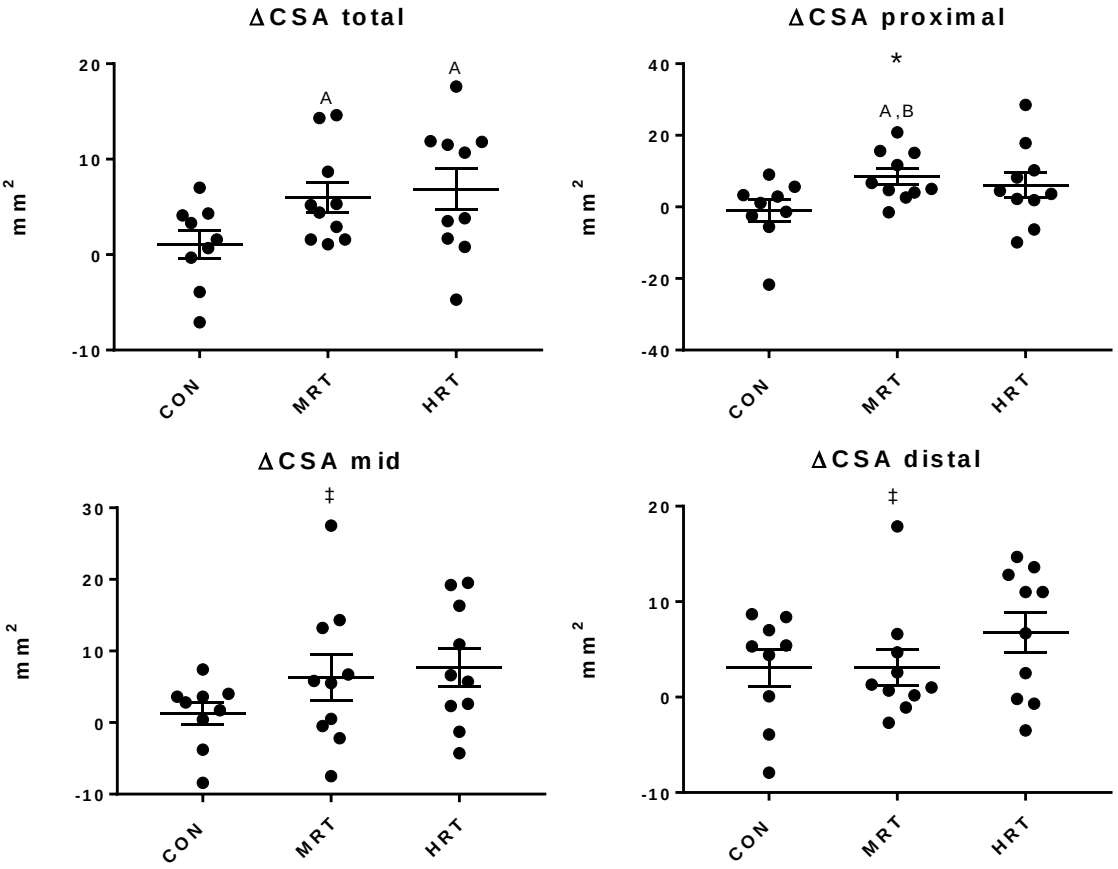

Fig. 3: Changes in patellar tendon cross-sectional area (CSA) in three different regions (proximal, mid, distal) and in total after 12 months heavy resistance training (HRT), moderate load resistance training (MRT) or no training (CON). Bars represent mean ±SE. Significant time x group interaction based on repeated measures ANOVA is denoted by *($p<0.05$) or (*) ($0.05<p<0.1$). Main effect of time is denoted by ‡ ($p<0.05$). A: Significant change from 0 to 12 months ($p<0.05$). B: Significantly different from CON ($p<0.05$), C: Significantly different from MRT ($p<0.05$) based on Tukey-Kramer post-hoc test.

Tendon signal intensity displayed a significant time x group interaction in the distal region and a tendency for a time x group interaction in total signal intensity ($p=0.09$) (table 4). Post-hoc tests revealed significantly increased signal intensity after HRT (total and distal region) but not after CON or MRT. The proximal and mid regions did not display any interactions, but there was a main effect of time in the mid region where signal intensity increased from 0 to 12 months across groups.

Table 4: Patellar tendon signal intensity

	CON		MRT		HRT	
	0 mths (n=10)	12 mths (n=9)[a]	0 mths (n=11)[b]	12 mths (n=12)[a]	0 mths (n=10)	12 mths (n=10)
SI total	13 [11-14]	13 [12-15]	14 [13-15]	14 [13-15]	13 [12-14]	15 [15-16]
SI proximal	18 [16-20]	18 [16-20]	21 [19-23]	21 [19-23]	19 [17-22]	21 [20-23]
SI middle‡	10 [9-11]	12 [10-13]	11 [10-11]	11 [10-12]	10 [9-11]	14 [12-15]
SI distal*	10 [9-11]	10 [9-11]	9 [9-10]	9 [8-9]	8 [8-9]	10 [10-11][A,C]

Values are geometric means [upper limit-lower limit]. Data analyzed by repeated measures 2-way ANOVA with baseline adjustment on log-transformed values Tukey-Kramer post hoc test. CON=Control, MRT = moderate load resistance training, HRT = Heavy load resistance training. SI = signal intensity. *Significant Interaction ($p<0.05$). ‡: Main effect of time ($p<0.05$). Post-hoc tests: A: Different from time-point 0, C: Different from MRT12 ($p<0.05$). [a]one value missing due to administrative errors. [b]Two values missing due to administrative errors.

Collagen fibril morphology

There were no significant time x group interactions in volume fraction, fibril diameter or fibril density (table 5). There was a main effect of time in fibril diameter, which decreased over time, and fibril density which increased over time.

Table 5: Collagen fibril Morphology

	CON		MRT		HRT	
	0 m (n=10)	12 m (n=9)[a]	0 m (n=13)	12 m (n=12)[a]	0 m (n=10)	12 m (n=9)[a]
Volume Fraction (%)	62 ±2	59 ±1	59 ±2	62 ±1	58 ±2	59 ±2
Mean fibril diameter (nm)‡	92 ± 5	83 ± 4	86 ± 3	86 ± 5	91 ± 4	78 ± 3
Density (#/μm^2)‡	26 ± 2	31 ± 3	29 ± 2	33 ± 3	26 ± 3	36 ± 3

Values are means ±SE. Data analyzed by repeated measures 2-way ANOVA with baseline adjustment and Tukey-Kramer post-hoc test. HRT = heavy resistance training, MRT = moderate load resistance training, CON = no training. Main effect of time denoted by ‡($p<0.05$). [a]One value missing due to technical problems.

Collagen and Collagen x-links

There were no significant time x group interactions in tendon collagen content, enzymatic cross-links, or fluorescence (table 6). Tendon fluorescence displayed a significant main effect of time (fig. 4).

Table 6: Collagen and collagen cross-links

	CON		MRT		HRT	
	0 mths (n=10)	12 mths (n=10)	0 mths (n=13)	12 mths (n=13)	0 mths (n=10)	12 mths (n=9)[a]
Collagen (%)	69 ±3	62 ±2	61 ±4	59 ±2	63 ±3	61 ±2
HP (mmol/mol collagen)[b]	289 ±30	316 ±27	294 ±25	263 ±24	254 ±30	259 ±26
LP (mmol/mol collagen)[b]	25.1 ±5.0	27.3 ±3.9	42.5 ±4.5	29.7 ±5.4	37.0 ±6.2	35.2 ±3.3
Fluorescence (AU/mol collagen)‡	1139 [1096-1185]	1235 [1176-1297]	1155 [1124-1187]	1196 [1161-1232]	1242 [1172-1316]	1328 [1285-1372]

Values are means ±SE or geometric mean [lower limit – upper limit]. Data analyzed by repeated measures two-way ANOVA with baseline adjustment and Tukey-Kramer post hoc test. CON = Control, MRT = moderate load resistance training, HRT = Heavy load resistance training. No time x group interactions. ‡: Main effect of time ($p<0.05$). [a]one missing sample due to anti-coagulant therapy. [b]Five missing pairs of samples due to logistics. CON: n=9, MRT: n=11, HRT: n=8.

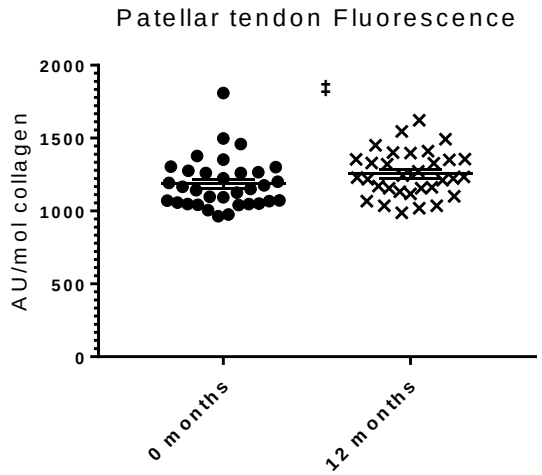

Fig. 4: Fluorescence of tendon biopsies normalized to collagen as a marker of patellar tendon AGE cross-links before (0 months) and after 12 months intervention. ‡: Main effect of time ($p<0.05$) based on repeated measures two-way ANOVA with baseline adjustment. AU = arbitrary units.

Discussion

Primary findings

The present investigation compared the effects of long-term resistance training with high or moderate loads on patellar tendon mechanical properties, macro- and microscopic morphology, and collagen cross-links in older adults. The primary findings were that adaptation of patellar tendon mechanical properties and cross-sectional area were dependent on training load over the 12 month intervention with superior effects of heavy compared to moderate load resistance training. Collagen content, fibril morphology,

enzymatic cross-links, and advanced glycation end-products were not affected by training and could thus not explain the different adaptations between groups.

Muscle properties

Consistent with current knowledge, strength training convincingly improved maximal isometric quadriceps strength (IsoMVC) with a significant effect of training load (fig.1). HRT increased IsoMVC by 21% while MRT and CON increased by 8% and 7% respectively. Vastus lateralis CSA was surprisingly not affected by training, suggesting a quite large neural adaptation to training.

Mechanical properties

Tendon stiffness calculated at common force increased by 6% after HRT, and decreased by 10% and 7.5% after MRT and CON respectively. Young's modulus remained unchanged after HRT but decreased by 14% after MRT and 9% after CON. The different tendon responses in the three groups may suggest a time-dependent decrease of tendon mechanical and material properties, which was ameliorated by HRT (fig. 2).

Although HRT did not increase tendon modulus, our findings support previous studies that have found superior effects of heavy compared to light load training on tendon mechanical properties in young and middle aged (10, 49) as well as older adults (29). The novelty of the present finding is that moderate load training with an approximately comparable volume as heavy load training was also insufficient to induce adaptations of tendon mechanical properties. Our results thus confirm that a certain loading threshold needs to be surpassed in order for tendons to adapt (1, 43). Maintenance of tendon stiffness may be critical to optimal muscle function (11, 65) and postural balance (53) in

old age, and our results together with previous findings therefore highlight the importance of training with a high load magnitude in this age group.

Several previous studies have investigated the effects of short-term (3 months) resistance training on tendon mechanical properties and found either increased (22, 29, 52, 55) or unchanged (14) tendon stiffness. Only few studies have investigated the effect of long-term training and found no additional effects after 1.5 (22) or 4 years (50) compared to three months training. We cannot exclude that the improvements in tendon stiffness and modulus measured after 12 months training were already present after the first three months. In another recent randomized training study in our department (unpublished data) including older adults with an average age similar to the ones in the present study, and using the same test-protocol, we found no significant differences in tendon mechanical properties after 3 months heavy or light load resistance training. It is thus possible that longer training periods are needed to improve tendon mechanical properties in older adults.

Tendon morphology

Patellar tendon CSA (total) tended to be affected by intervention group ($p=0.07$) with increases after both HRT (+6%) and MRT (+5%), but not CON (+1%) (fig. 3). The possible increase in CSA makes the tendon able to support higher loads without imposing more stress on the tendon, and thus protects the tissue from damage. To our surprise, the effect of training on tendon CSA was not dependent on load since MRT in contrast to mechanical properties increased tendon CSA equally much as HRT (fig. 3).

Although several short-term training studies have not been able to show any effect of heavy or light load on tendon CSA in older adults (29, 52, 55, 59), our longitudinal

data supports some (16, 17), albeit not all (50) previous human cross-sectional data, which show that long-term (several years) habitual loading of both relatively low (17) and high load (16) is associated with higher patellar tendon CSA. One recent longitudinal study did, however, not find differences in Achilles tendon CSA in older adults after 1.5 years compared to three months heavy resistance training (22), but in that study training improvements beyond three months was probably hampered by the reduction in training volume from 3 to 2 weekly training sessions after the first three months.

The increments of patellar tendon CSA tended to be region-specific with significant training effects in the proximal region, but not in the mid and distal regions (fig. 3). A previous study in young men found similar region-specific increments of the patellar tendon CSA with 7% higher proximal CSA after light load training and 6% and 4% higher proximal and distal CSA respectively after high load training (40). The increased CSA in that study was only accompanied by increased tendon stiffness in the heavy load group, which corroborates our own data.

Increased CSA with training may be mediated by addition of new collagen to existing fibrils, new fibrils, increased intrafibrillary spacing between collagen molecules, or more interfibrillary material such as fat, water, or proteoglycans. In the present investigation we measured core tendon collagen content, fibril volume fraction, as well as fibril diameter and density but none of these variables were affected by training (table 5). The lack of training effect on collagen content and fibril morphology was not surprising, since it has recently been demonstrated convincingly that no or very little renewal of collagen takes place in the core tendon after teenage years (35). Moreover, it is also consistent with previous human cross-sectional data on master athletes compared to sedentary age-matched controls (17). However, it is possible that adaptations at the

552 fibrillary level have gone undetected in our core tendon biopsies, since new collagen may
553 have been added to the peripheral region of the tendon (42) or to the most proximal
554 region, which would be consistent with the region-specific adaptations in tendon CSA.
555 Region-specificity could thus explain the disparity between adaptations at the fibrillary and
556 whole tendon level.

557 The absolute values of collagen content and fibril morphology were fairly
558 consistent with previous measurements in our own lab (17, 39), and collagen content
559 tended to correlate to fibril volume fraction at baseline (p=0.06, fig. 5), indicating valid
560 measurements of these variables.

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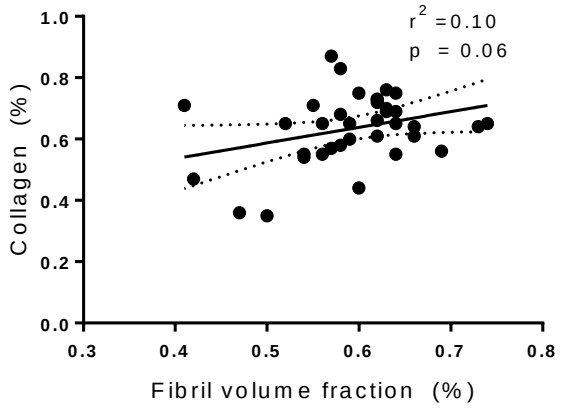

562

563 **Fig. 5:** Correlation between collagen content and fibril volume fraction as determined by Pearson's
564 correlation coefficient. Dotted lines are 95% confidence bands of the best linear fit.

565

566

567 Accumulation of water could also increase tendon CSA, but this explanation is less likely
568 since increased water content would theoretically reduce signal intensity on a T1 weighted
569 scan (more black, less grey). In contrast, our data showed significant increases of signal
570 intensity in the distal region (+29%) and in total signal intensity (+23%) after HRT but not
571 MRT or CON. This indicates a material change in tendon composition in response to HRT
572 but not MRT, which may explain why the mechanical properties were only affected by

HRT. Previous short-term training studies have found no change in patellar tendon signal intensity after either heavy (14) or light load training (59), but cross-sectional data have shown higher signal intensity in the patellar tendon of master endurance athletes compared to both young athletes and old untrained (17) indicating a long-term effect of training on signal intensity. The specific translation from increased signal intensity to tendon molecular composition is unclear but may indicate a difference in tissue quality.

Collagen cross-links

Contrary to our hypothesis, the enzymatic cross-links HP and LP were unaffected by 12 months resistance training (table 6). Previous human data have also shown no difference in enzymatic cross-links between endurance trained and sedentary older adults (17), and no effect of short-term resistance training in young adults (44). Our results thus confirm that enzymatic cross-links do not mediate moderate or high load training induced changes in tendon stiffness or Young's modulus despite long training duration. Since enzymatic cross-links are essential to development of normal force transmission within the tendon (3, 25, 36), we also analyzed correlations between tendon stiffness or modulus and HP or LP, at baseline as well as in the changes over time. The analysis showed no correlations between enzymatic cross-links and tendon mechanical properties in our group of older adults, which corroborate previous human and animal studies (15, 32, 62) and suggests that enzymatic cross-links play a limited role in the adaptation of tendon biomechanics after maturity. It is also plausible that enzymatic cross-links after maturity are only formed in the peripheral regions of the tendon, where there is also evidence of collagen synthesis in response to exercise (42), and future research should investigate region-specific molecular adaptations to training.

Fluorescence which was used as a marker of AGE content increased by ~6% over the 12 months across intervention-groups, but was unaffected by both moderate and heavy load resistance training. The time dependent increase of fluorescence was not surprising given the nature of the glycation process, which results in accumulation of AGEs over the life-course (5). It was however somewhat surprising that 12 months training could not attenuate AGE accumulation since others have found decreased tendon AGE-levels after short-term endurance training in old mice (68), attenuated accumulation of the AGE-marker pentosidine after life-long endurance training (17), and reduced pentosidine after short-term heavy load resistance training in young men with patellar tendinopathy (38). Even longer training duration may be necessary for healthy old humans to significantly attenuate AGE-accumulation.

To support that fluorescence measurement provided a truthful depiction of AGE-accumulation, we performed correlational analysis between age and fluorescence (fig. 6). As expected, fluorescence correlated positively to age even within the relatively small age-range in our population (63-71 years).

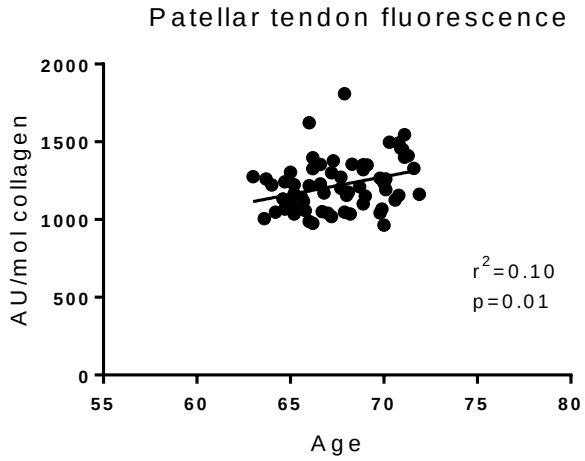

Fig. 6: Correlation between age and patellar tendon fluorescence using bot PRE and POST values as determined by Pearson's correlation coefficient. Dotted lines are 95% confidence bands of the best fit.

Interestingly, HbA1c also increased by ~3% or ~1 mmol/mol ($p<0.0001$) without any difference between groups (table 2). Increased HbA1c confirms the time-dependent accumulation of glycation products, but it may also be due to variation between PRE and POST measurements since the increase was way higher (~1 mmol/mol over 10 years) than what other large-scale studies on healthy individuals have found (21). There was no correlation between tendon fluorescence and HbA1c either at baseline or in the changes over time, indicating different time-patterns of erythrocyte and tendon glycation.

We further correlated fluorescence to Young's modulus since accumulation of AGEs may affect tendon material properties (3, 46), but there was no correlation between these variables either at baseline or in the changes over time. Previous in vitro studies have shown associations between AGEs and tendon mechanical properties (54, 56, 68), but human studies have in line with our results not been able to associate tendon AGEs and in vivo patellar tendon mechanical properties (15, 32), suggesting that other matrix components (i.e. proteoglycans, glycoproteins) contribute more to in vivo mechanical phenotype in old humans. AGEs comprise a large and heterogeneous family of chemical compounds, and it is possible that training induced changes of specific cross-linking AGEs have "drowned" in the measurement of total fluorescence. Further, region-specific changes in AGEs may have occurred in the outer layers or most proximal and distal tendon regions, without being detected in our core tendon samples. Finally, it is possible that AGEs mainly affect tissue mechanics in the failure region of the stress-strain curve (23, 60, 61), whereas in vivo tendon testing only considers mechanical properties up to the first part of the linear phase. It must be noted that recent studies have shown that the deformation mechanism and strength of the tendon are greatly affected by the presence of cross-links (64), and that multiscale mechanical analysis of in vitro glycated tendons strongly suggests that

AGEs reduce tissue viscoelasticity by severely limiting fiber–fiber and fibril–fibril sliding (27).

Limitations

In contrast to young women (47, 67), older postmenopausal women seem to have indistinguishable tendon mechanical properties from men (13). Both men and women were included in the present study to make the results more generally applicable. Although not statistically significant, more men were randomized to HRT and more women were randomized to MRT. To test the potential influence of sex on the training effect, we performed a secondary 2-way ANOVA on the changes over time with sex and intervention-group as factors. The analysis did not show any interactions between sex and intervention-group or main effects of sex in muscle or tendon variables, suggesting that older men and women respond similarly to resistance training. This makes it unlikely that the unequal sex-distribution explained the group-differences in training adaptations.

Despite a high average training compliance of 86% in both MRT and HRT, the range was quite large (51% to 100%). We therefore made a secondary per protocol statistical analysis excluding participants with a compliance < 80% and also excluding one CON who admitted to have initiated strenuous training activities during the intervention. The remaining 7 HRT (compliance = 88 ±5), 9 MRT (compliance = 94 ±5), and 9 CON did not display appreciably different results than the primary statistical analysis and did consequently not affect the reached conclusions.

Daily step-count, which can be considered as light load training, tended to increase over time across intervention-groups ($p=0.07$). Since changes in tendon CSA seemed to be independent of load size, this may explain the main effects of time on mid

and distal tendon CSA. Adaptation of tendon mechanical properties seemed dependent on high load training and may therefore be less influenced by the changes in light load regular physical activity.

Conclusion

Twelve months heavy load resistance training ameliorated the time-dependent reduction of patellar tendon stiffness and Young's modulus, which was observed after moderate load or no resistance training. Tendon cross-sectional area increased with training after both moderate and heavy load resistance training but only heavy load training increased tendon signal intensity. This suggests that high load magnitude is important to tendon performance in old age. The load dependent differences in tendon adaptation could not be related to changes in collagen content, fibril morphology, enzymatic cross-links, or advanced glycation end-products in the tendon core. We suggest that tendon adaptation to training may be mediated by region-specific molecular changes outside the tendon core, or by changes in other non-collagenous matrix components such as proteoglycans or glycoproteins, which were not analyzed in the present investigation. Heavy load resistance training should be included in training programs for older adults to maintain tendon mechanical properties with aging.

Disclosures

All authors declare no conflicts of interest.

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